# GENETICALLY-ENGINEERED BONE MARROW STROMAL CELLS AND COLLAGEN MIMETIC SCAFFOLD MODIFICATION FOR HEALING CRITICALLY-SIZED BONE DEFECTS

A Dissertation
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Engineering
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Georgia Institute of Technology
Emory University
August 2009

# GENETICALLY-ENGINEERED BONE MARROW STROMAL CELLS AND COLLAGEN MIMETIC SCAFFOLD MODIFICATION FOR HEALING CRITICALLY-SIZED BONE DEFECTS

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To my parents, who have always believed in me

# ACKNOWLEDGEMENTS

There are many people who guided me in the completion of this dissertation either by scientific contribution or by emotional support. All of their contributions have been invaluable, and I am lucky to have such a long list of people to thank.

First and foremost, I would like to thank my advisor, Dr. Andrés García, without whom this work would not have been possible. Andrés has been an outstanding mentor to me, and I have matured not only as a researcher but also as a student, employee and person under his guidance. I thank him for persistently challenging while simultaneously supporting and encouraging me. No matter how many meetings were scheduled, grants due, papers to review, conferences to chair, classes to teach, seminars to host or basketball games to coach, I always felt I could knock on Andrés' door any day and he would make time to meet with me. And there are 9 other grad students in the lab! To feel like this much of a priority kept me motivated to stick with it, even when research was a struggle. I have also learned a lot from Andrés, including how to write a killer paper or abstract (by including words or phrases like "simple and elegant," "robust," and "parsimonious"), and speaking up and being assertive when it comes to science ("confidence bordering on arrogance"). I have really enjoyed working for Andrés for these past 5 years, and I am looking forward to maintaining ties with him and the García lab for many years to come.

My thesis committee members, Drs. Bob Guldberg, Todd McDevitt, Johnna Temenoff, Athanassios Sambanis, and Ken Gall, have provided many fruitful discussions about my work, which have helped shape this dissertation and improve the quality of the

research. After leaving a committee meeting, I always felt excited about their enthusiasm for my work. Scientific discussion is my favorite part about science research, and I thank them for providing their insight through rigorous discussion of my data and how it fits into the bigger picture.

A particular note of thanks goes to Dr. Bob Guldberg, who not only provided many insightful discussions about the animal model used in this research, which was developed in his lab, but also trained me in the subtleties of the surgical technique. Bob played an integral role in the completion of this research because he actually scrubbed in and served as one of the primary surgeons in all of my segmental bone defect studies. I thank him for his dedication to the project and the long hours spent in the PRL with the García Lab.

A number of other people were also critical to the success of the animal work in this dissertation. Dr. Laura O'Farrell, the research veterinarian, provided insightful advice about the surgical technique and ensured that the well-being of the animals was always a priority. Kim Benjamin, the PRL lab manager, placed animal orders and organized the lab space. Andrea Gibson, John Duncan and Autumn provided excellent daily care of the animals and made sure the lab was always clean. Finally, Dr. Megan Oest, Ken Dupont, and Joel Boerckel served as primary surgeons. Dr. Andrés García, Asha Shekaran and Brian Dierckman were sterile surgeons' assistants. Kellie Burns did animal prep, and almost everyone else in the García lab served as non-sterile surgical assistants at some point or another. Thank you to everyone for making these surgeries successful.

My next acknowledgement is to the members of the García Lab, who are extremely fun and energetic and make the everyday grind of research more enjoyable. The first shout out goes to Kellie Burns, an amazing lab manager, who keeps the lab running smoothly and will bend over backwards to help you out. Kellie is a fun person, who is loyal to her friends, and I will miss our 11:30 am trips to Einstein's or Tech Square for lunch! Kellie is always good for a laugh and makes sure none of us crazy science geeks starts taking ourselves too seriously. I will miss Kellie a lot, and I wish her the best in all she does in the future.

Sean Coyer, Dave Dumbauld and Tim Petrie are closest to me in "lab age," and we have shared a lot of fun experiences both in and out of the lab. I'm disappointed that Sean has still not figured out that Danville is not in *southern* Illinois, but he did figure out the right phrasing to encourage me to have a little fun. And he also let me copy his idea of the deli-style lunch, which saved me both time and money! Because of that I can forgive him for his geographical ignorance of his home state, as long as we can still get together for a drink every once in a while after grad school. Dave is my fellow soul-less ginger in the lab, who once changed a burnt out light bulb on a high ceiling for me without even standing on a chair! An amazing feat, and one that demonstrates Dave's earnest willingness to lend a helping hand without expecting anything in return. I'm sure he has literally spent more time fixing broken lab equipment than all of the rest of us combined, for which all of us owe him big. On top of that, Dave always knows where the party is, and we have had a lot of fun around Atlanta. Defending his thesis within one day of me, Tim Petrie is the end of an era in the García lab. He was there before I started, and will likely stay on a few months after I leave, making it difficult to imagine life in the

García lab without him. My guess is that the lab computers will have significantly fewer trojans, viruses, music downloads, gambling software, and jelly on them after Tim's departure from the lab, which will certainly lighten everyone else's computer repair load. But Tim really is a brilliant scientist and will never hesitate to discuss experiments, protocols and data to help you out with your work. Tim helped guide the GFOGER portion of this work, and I thank him tremendously for that.

The next generation of García Lab members includes some pretty amazing people, who I'm glad I've gotten the chance to know. Rachel Whitmire kept me having fun by hosting several girls nights and made sure to get me out swing dancing at least once before I left Atlanta. Ed Phelps keeps us all entertained with the latest everyday science news, like the giant sea worm discovered killing the coral in an aquarium in Great Britain or the fact that you can't make your foot go clockwise while your hand goes counterclockwise. Nduka Enemchukwu is an extremely motivated individual, who has carried on the age old "tradition" of using silicon grease to design custom-made lab parts. Asha Shekaran is a small person with a huge smile and an even huger laugh, who I believe can finish a whole ghetto burger in one sitting if she puts her mind to it. Kadija McAnuff has done an outstanding job working as my undergraduate research assistant, and I have enjoyed mentoring her over the past year. It has also been a pleasure sharing an office with Dr. Ram Selvam over the past few months. Finally, Chi-Chi Esimai and Stacie Gutowski are the newest addition to the García Lab, and I'm sure they will keep the good times rolling.

Several former lab members have helped shape this work giving both scientific advice and personal encouragement. Entering the lab at the same time as I did, former

García Lab member, Dr. Amanda Wall Bridges, was always there to help keep things in perspective and make sure I still allowed myself to have some fun despite hard work or failed experiments in the lab. My original mentor in the lab, Dr. Jenn Phillips, initially showed me the "Runx2 ropes" and encouraged me to focus on the science not on the stress when things didn't work out as expected. Jenn is a very motivated and successful scientist with a keen sense for what to do next. She gave me something to look up to when I first joined the lab, and she continues to impress me with her hard work and dedication. I'm also glad to have gotten to know her better on a personal level by sharing an office with her during her last few months in the lab. Former García Lab member, Dr. Charlie Gersbach, also helped me get started on the Runx2 project by answering my endless questions. At first I was intimidated by Charlie, a 5$^{th}$ year student who seemed to have it all figured out, and I worried that I would ask the wrong questions – incriminating myself so that it was obvious I didn't know what I was doing! But even for a seemingly simple question, Charlie would pause to think about it (leaving me more time for self-doubt), then head to the white board and draw up a 15 minute mini-lecture (indicating that I had actually asked the right question). I thank him for always taking an interest in helping me get started. Finally, Drs. Catherine Reyes and Ben Byers, completed the initial work with GFOGER and Runx2, respectively, on which this dissertation is founded. I thank them for paving the way for my work.

Outside of the García Lab, there are many other people at Georgia Tech who have helped me make it through grad school. First, I'd like to extend a thank you to everyone in Wing 2D, and particularly those who sit or have ever sat in the Lair of Inefficiency. The Lair truly is an inefficient place to work, but being there reminds you to laugh and

not stress out so much about work. In particular, several members of the Guldberg Lab deserve special mention. Ever so long ago, Yash Kolambkar, Mela Johnson and I used to sit in the same cubicle in the Lair, bonding over our common agonies of working with bone marrow stromal cells. They have been great friends to have around. Angela Lin is the microCT lab manager, who is a whiz at all things CT. She takes an active interest in helping people figure out the best way to scan samples and analyze data, and I thank her for her direct contributions to this work. It has also been a pleasure getting to know Dr. Tamim Diab as we shared an office together during the last several months of my Ph.D. There are also former members of the Guldberg lab who helped me get started. I did a research rotation under the guidance of Dr. Craig Duvall, who was very good at getting quality work done efficiently so that we didn't have to work late at night. Dr. Megan Oest originally developed the animal model used for this research, and she scrubbed in with me for my first segmental defect surgery to give me a rigorous hands-on surgery training. Dr. Blaise Porter showed me the art of putting collagen meshes into PCL scaffolds and imaging them on the confocal. And finally, Dr. Srin Nagaraja stole a lot of Goldfish from me and never let me leave the Lair without a good laugh.

I have really enjoyed working in such a collaborative environment as IBB. The personnel who run the core lab facilities around IBB are top notch and have helped me with several of my projects. Aqua Asberry, the histologist, Allen Echols, the building manager, Johnafel Crowe, the confocal lab manager and, Steve Woodard, the core lab facilities manager, have all helped benefit this work.

In completing a PhD, there are also many administrative duties that must be taken care of, and there are several people that helped me figure it all out. Shannon Sullivan,

Beth Bullock and Sally Garrish, all from BME, made sure I had all the proper forms filled out and turned in on time for my degree requirements. Vivian Johnson took care of my travel reimbursements.

Outside of the lab, many of my Georgia Tech classmates have helped round out the social side of grad school. My first acknowledgement in this category goes to Laveeta Joseph, Rekha Nair, Priya Santhanam, and Swathi Ravi, who have shared more memories with me than can be recounted here. A brief word about each of them… Laveeta and I were roommates for almost the entire time in grad school, and we had fun hosting parties and dinners at our cute little Midtown apartment. Towards the end of grad school, I had the pleasure of being a bridesmaid in Laveeta's wedding, and I traveled to India with her and the other girls for the occasion, which was an experience of a lifetime. Rekha and I have been running buddies ever since our first half marathon during our second year. There's nothing better than staying in shape while catching up with a good friend, because as everyone knows, chatting is an essential part of running! Rekha has one of the kindest hearts of anyone I know and will bend over backwards to make everyone happy. Priya was actually the first person I met at Georgia Tech. We were roommates during the prospective student weekend before our first semester, and together we missed the bus to Emory on the first day of the visit! Ever since sharing a cab over to Emory that day, Priya and I have become fast friends. Priya is a great person to relax and share a drink with, and her sense of humor keeps me laughing, even during hard times. Swathi was my first roommate at the Graduate Living Center our first year, and together we explored Midtown from Joe's on Juniper to Cosmo/Lava. She never fails to keep you in check with her sarcastic comments and she was a great travel

companion in Goa. I am so lucky to have gotten to know these amazing women over the past 5 years, and I thank them for the tremendous support and friendship they have given me throughout my time in grad school.

Several other friends deserve special mention. Victoria Stahl and Adele Doyle, who along with Rekha and I, formed the bio study group turned volunteer group. Julia Henkels, an honorary García Lab member, is as passionate about dancing and dance shoes as I am! Dr. Claire Honeycutt was my church chat/coffee talk buddy. Laura-Lee Farrell and I shared many movie watching nights together. Frances Brady, my college roommate, was always encouraging and supportive, even if we hadn't talked in a while. Dr. James Wang, my college bio study buddy, could relate to my grad school struggles as he struggled in parallel through pharmacy school. Sheena Burbridge was my roommate during the final 6 months of grad school. And Kelly Erby Dumbauld shared a common interest in attending the Emory Mixers! I thank all of them for being great friends.

A special note of thanks goes to Dr. Joe Charest. Joe's continued support, advice and encouragement kept me motivated during my final two years in grad school. I thank him for the patience it took to believe in me.

Finally, I am so lucky to have a tremendous family, who never doubts that I can accomplish anything, even though I don't always believe it myself. My grandparents, Ed and Irene Wojtowicz and Betty Shinn, and my brother, Ben Wojtowicz, and sister-in-law, Louriz Calderon Wojtowicz, have all been supportive over the past 5 years. And there is not enough space here to write about all the encouragement my parents, Jeff and Patty Wojtowicz, have given me over the years. I hope that the dedication of this work to them points to how much they mean to me.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ALP | alkaline phosphatase |
| αMEM | alpha modified eagle's medium |
| BCA | bicinchoninic acid |
| BMP | bone morphogenetic protein |
| BMSCs | bone marrow stromal cells |
| BSA | bovine serum albumin |
| $CO_2$ | carbon dioxide |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| ECM | extracellular matrix |
| EDC | 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride |
| EDTA | ethylenediaminetetraacetic acid |
| eGFP | enhanced green fluorescent protein |
| ELISA | enzyme-linked immunosorbent assay |
| ΦNX | phoenix helper cells |
| FBS | fetal bovine serum |
| FTIR | fourier transform infrared |
| GFOGER | amino acid abbreviation for the synthetic peptide $GGYGGGPC(GPP)_5GFOGER(GPP)_5GPC$ |
| HA | hydroxyapatite |
| IACUC | Institute Animal Care and Use Committee |
| IRES | internal ribosomal entry site |

| | |
|---|---|
| kVp | kilovolt potential |
| μA | microamperes |
| MES | (2-*N*-morpholino) ethanesulfonic acid |
| MicroCT | micro-computed tomography |
| MMA | methyl methacrylate |
| MUP | 4-methylumbelliferyl phosphate |
| $NaN_3$ | sodium azide |
| PBS | phosphate buffered saline |
| PCL | poly caprolactone |
| PEG | polyethylene glycol |
| P/S | penicillin/streptomycin |
| TFA | trifluoroacetic acid |

# SUMMARY

Non-healing bone defects have a significant socioeconomic impact in the U.S. with approximately 600,000 bone grafting procedures performed annually. Autografts and allografts are clinically the most common treatments; however, autologous donor bone is in limited supply, and allografts often have poor mechanical properties. Therefore, tissue engineering and regenerative medicine strategies, which employ the use of cells or growth factors to heal bone, are being developed to address issues with clinical bone grafting. However, the need for an abundant mineralizing cell source limits the progress of these therapies. The overall objective of this work was to develop bone tissue engineering strategies that enhance healing of orthotopic defects by targeting specific osteogenic cell signaling pathways. The general approach included the investigation of two different tissue engineering strategies, which both focused on directed osteoblastic differentiation to promote bone formation.

In the first cell-based strategy, we hypothesized that constitutive overexpression of the osteoblast-specific transcription factor, Runx2, in bone marrow stromal cells (BMSCs) would promote orthotopic bone formation in vivo. We tested this hypothesis by delivering Runx2-modified BMSCs on synthetic scaffolds to critically-sized defects in rats. We found that Runx2-modified BMSCs significantly increased orthotopic bone formation compared to empty defects, cell-free scaffolds and unmodified BMSCs. This gene therapy approach to bone regeneration provides a mineralizing cell source which has clinical relevance.

In the second biomaterial-based strategy, we hypothesized that incorporation of the collagen-mimetic peptide, GFOGER, into synthetic bone scaffolds would promote orthotopic bone formation in vivo without the use of cells or growth factors. We tested this hypothesis by passively adsorbing GFOGER onto poly-caprolactone (PCL) scaffolds and implanting them into critically-sized orthotopic defects in rats. We found that GFOGER-coated scaffolds significantly increased bone formation compared to uncoated scaffolds in a dose dependent manner. Development of this cell-free strategy for bone tissue engineering provides an inexpensive therapeutic alternative to clinical bone defect healing, which avoids issues of immune response from implanted materials and could be implemented in a point of care application.

Both of the strategies developed in this work take advantage of specific osteoblastic signaling pathways involved in bone healing. Further development of these tissue engineering strategies for bone regeneration will provide clinically-relevant treatment options for healing large bone defects in humans by employing well-controlled signals to promote bone formation and eliminating the need for donor bone.

# CHAPTER 1

# INTRODUCTION

## Specific Aims

Non-healing bone defects have a significant socioeconomic impact in the U.S. with approximately 600,000 bone grafting procedures performed annually (Bucholz 2002). Current clinical treatment of large bone defects employs the use of autografts and allografts; however, autografts can cause donor site morbidity and pain and must be taken from a limited supply of donor bone, while allografts may have poor mechanical properties and present a risk of disease transmission (Meyer *et al.* 2004; Hutmacher and García 2005). As an alternative approach for bone repair, tissue engineering and regenerative medicine strategies are being developed to address the problems associated with current bone grafting procedures. Many of these strategies make use of a mineralizing cell source that is delivered to a defect site to promote bone formation. However, the identification of the ideal cell source for bone tissue engineering remains to be uncovered. The overall ***objective*** of this work was to develop bone tissue engineering strategies that enhance healing of orthotopic defects by targeting specific osteogenic cell signaling pathways. These specific targeting strategies, which exert control over donor and host cell signaling pathways, address the current limitations of cell sourcing for bone tissue engineering by increasing the osteogenic capacity of donor cells or by promoting host osteogenesis in the absence of donor cells. These strategies improve upon existing bone tissue engineering strategies and present valuable alternatives for clinical healing of large bone defects.

Bone marrow stromal cells (BMSCs) are a heterogeneous population of multipotent cells, easily obtained from the bone marrow, that have the ability to differentiate towards the osteogenic, chondrogenic or adipogenic lineages (Pittenger *et al.* 1999). However, the growth rate and osteoblastic differentiation potential of BMSCs is negatively affected by ex vivo manipulation and expansion of the cells (Derubeis and Cancedda 2004). Runx2 is an osteoblast-specific transcription factor that upregulates osteoblast-specific genes and promotes osteoblastic differentiation of BMSCs both in vitro and in vivo (Byers and García 2004). For this work, we ***hypothesized*** that the delivery of BMSCs, genetically modified to overexpress Runx2, into critically-sized orthotopic defects would facilitate increased healing of the defects compared to delivery of unmodified BMSCs.

Synthetic polymeric scaffolds have been used to deliver cells and growth factors to critically-sized orthotopic defects to facilitate defect healing (Rohner *et al.* 2003; Schantz *et al.* 2003; Oest *et al.* 2007). However, polymeric scaffolds alone are not sufficient for repair of critically-sized defects. Recently, the use of biomimetic strategies which attempt to recapitulate the environment of the bone ECM have been used to promote osseointegration and bone formation in vivo (Hubbell 2003). In particular, surface modification of titanium implants with the synthetic peptide GFOGER, derived from collagen I, has been shown to significantly increase osseointegration and implant fixation in vivo to levels greater than that of unmodified titanium implants or implants coated with full length collagen I (Reyes *et al.* 2007). Furthermore, GFOGER signaling occurs via binding of the $\alpha_2\beta_1$ integrin receptor and upregulation of Runx2 (Xiao *et al.* 1998). Therefore, we further ***hypothesized*** that surface modification of synthetic polymer

scaffolds with the peptide, GFOGER, would significantly improve healing of critically-sized orthotopic defects compared to unmodified scaffolds without the use of cells or growth factors. These hypotheses were tested via the following three specific aims:

1. *To examine the Runx2-induced osteogenic potential of BMSCs from an inbred rat strain compared to that of BMSCs from a previously established outbred rat strain.*

Genetic modification of BMSCs with Runx2 upregulates the expression of osteoblast-specific genes and subsequently increases mineralization in BMSCs isolated from Wistar rats, an outbred rat strain (Byers and García 2004). To proceed with in vivo studies, we were interested in using inbred rats to address concerns with immune rejection of implanted cells. However, strain differences in rats affect a number of physiological processes, such as skeletal development and ectopic bone formation (DeMoss and Wright 1998; Li *et al.* 2003). Our hypothesis for this aim was that the strain of rat from which BMSCs were isolated would affect the in vitro osteogenic potential of BMSCs but that Runx2 modification would remain effective in promoting osteoblastic differentiation. We tested this hypothesis by harvesting BMSCs from both Lewis and Wistar rats, an inbred and outbred strain, respectively. Osteogenic potential of all cells was quantified via alkaline phosphatase activity and in vitro mineralization of 2D surfaces. Furthermore, the effect of Runx2 modification and passage number on all cell types was assessed.

2. *To evaluate bone regeneration and mechanical strength induced by Runx2-engineered BMSCs in critically-sized segmental defects.*

The implantation of BMSCs, engineered to overexpress Runx2, causes significant healing of critically-sized rat calvarial defects compared to treatment with unmodified BMSCs (Byers *et al.* 2006). Although these results are promising, segmental defects provide a more rigorous test bed than calvarial defects for bone tissue engineering by eliminating the host response from the cranial periosteum and underlying dura mater (Aalami *et al.* 2003). For this aim, we hypothesized that delivery of Runx2 modified BMSCs to critically-sized segmental defects would significantly increase bone formation and mechanical strength of defects compared to treatment with unmodified BMSCs. To test this hypothesis, we seeded BMSCs, retrovirally-transduced to overexpress Runx2, onto PCL scaffolds and implanted the cell/scaffold constructs into critically-sized defects in rat femurs. Control groups included empty defects, cell-free scaffolds, and scaffolds seeded with unmodified BMSCs or empty-vector-modified BMSCs. Bone volume in defects was quantified at 4, 8 and 12 weeks via microCT. Postmortem analysis of explanted samples included histology, FTIR, and mechanical testing.

3. *To evaluate the extent of bone regeneration and mechanical strength induced by GFOGER-modified PCL scaffolds in critically-sized orthotopic defects.*

Although the synthetic polymer, PCL, can be used as a biomaterial for bone tissue engineering scaffolds (Zein *et al.* 2002; Byers *et al.* 2006), the surface hydrophobicity of

these scaffolds causes non-specific protein adsorption, resulting in a non-specific cell signaling response. For this aim, we hypothesized that incorporation of the bioadhesive molecule, GFOGER, into synthetic bone scaffolds would promote healing of critically-sized bone defects without the use of cells or growth factors. We tested this hypothesis by passively adsorbing GFOGER onto PCL scaffolds and implanting GFOGER-coated scaffolds into critically-sized defects in rat femurs. Control groups included empty defects and uncoated PCL scaffolds. Bone volume in defects was quantified at 4, 8 and 12 weeks via microCT. Postmortem analysis of explanted samples included histology and mechanical testing.

This work is innovative because it uses novel genetic and extracellular cues for osteoblastic differentiation and mineralization to address the issue of cell sourcing, a common problem for tissue engineered constructs. By engineering BMSCs to overexpress Runx2, issues with donor variation and ex vivo manipulation of BMSCs are abrogated. Furthermore, surface modification of synthetic scaffolds with GFOGER, confers biofunctionality to otherwise non-bioactive substrates and promotes specific signaling to host cells, thereby eliminating the need for donor cells. We expect that development of these tissue engineering approaches to bone regeneration will provide a clinically-relevant strategy for the treatment of bone defects by eliminating the need for donor bone and employing well-controlled signals for bone formation.

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# CHAPTER 2

# BACKGROUND

## Bone Structure and Repair

### *Structure*

Bone is a highly organized tissue that can be categorized into several structural types. In particular, long bones, the focus of this dissertation, are composed of a cortical shaft with trabecular bone on either end. Cortical bone is dense and comprises about 80% of the human skeleton, while trabecular bone consists of an array of plates and rods that form a lattice-like structure (Liebschner 2004). In the present work, where bone tissue engineering strategies are evaluated in segmental defects created in long bones, structural and functional recapitulation of cortical bone is the goal.

At the microstructural level, normal adult human cortical bone is arranged into osteons and lamellar sheets. Osteons are formed when planar sheets of mineralized collagen fibers called lamellae form concentric rings around an open channel. Lamellae can also form parallel arrays called circumferential lamellar bone (Rho *et al.* 1998). Although lamellar bone is common to all vertebrate animals, generally only large animals have osteonal lamellar organization, whereas the skeletons of small animals are primarily arranged in lamellar plates. The particular arrangement of lamellae within cortical bone lends structural support to the tissue, creating a niche for the characterization of structure function relationships in bone. However, the complexity of bone tissue and the difficulty in measuring the mechanical properties of bone on a molecular level make this task

challenging. To date, the structure function relationship in lamellar bone has not been fully elucidated (Weiner *et al.* 1999).

*Bone repair*

Bone is one of the few tissues with the unique ability to regenerate after injury with full restoration of function to the injured site. The process of bone repair is highly complex and involves the coordination of many different cell and tissue types to produce the desired response. There are four main tissue types that contribute to fracture healing in bone (Figure 2.1), which participate in both endochondral and intramembranous bone formation (Einhorn 1998). The general process involves hematoma formation and inflammation, leading to development of a soft tissue callus surrounding the defect site, which is eventually mineralized and remodeled into mature lamellar bone (Einhorn 1998; Duvall *et al.* 2007). This process occurs naturally after injury to bone tissue; however, successful healing is only realized in fractures that do not result in substantial bone loss. Defects resulting in non-union of the injured tissue require clinical therapy for healing.

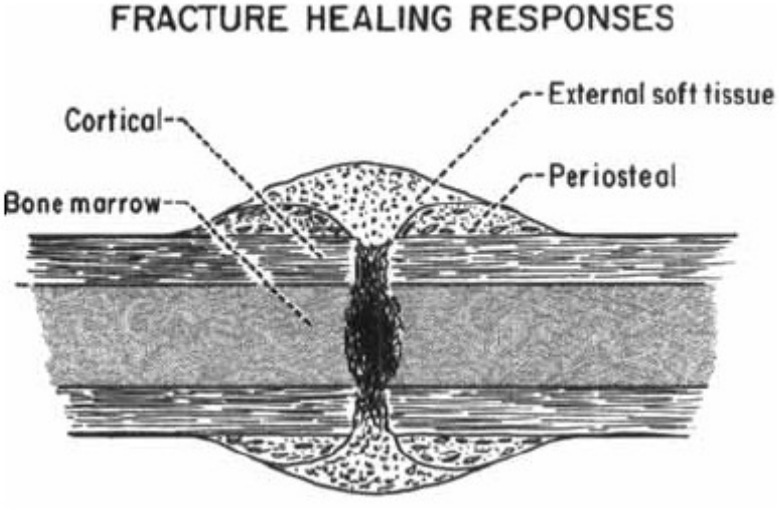


**Figure 2.1.** The tissue types that contribute to fracture healing in bone. (adapted from Einhorn 1998)

## Clinical Repair of Bone Defects

Although fracture repair of long bones occurs successfully with little to no surgical intervention, non-unions, in which a large portion of the bone is injured or must be resected, will remain as open defects until treated. The gold standard for clinical treatment of non-unions remains as the autograft, followed closely in number of treatments by allograft. However, recent use of bone morphogenetic proteins (BMPs) to treat large bone defects in humans is a newly emerging standard of care despite its high cost. These therapies, along with other current clinical treatments, will be discussed.

### *Autografts and Allografts*

In 2002, it was estimated that 600,000 bone grafting procedures were performed annually in the U.S., consisting of spinal fusions, general orthopaedic procedures, and synthetic bone grafts (Bucholz 2002). In 2004, approximately $5 billion was invested in more than 1,000,000 procedures involving bone grafting, bone excision and fracture repair (Kretlow and Mikos 2007). Autografts remain the gold standard for orthopaedic replacement procedures, including cases of non-union in long bones or craniomaxillofacial procedures. The graft material is most commonly taken from the iliac crest providing both an osteoconductive matrix for bone cell adhesion and an osteoinductive growth factor environment promoting osteoblastic differentiation (De Long *et al.* 2007). Furthermore, because bone is transferred from one site in the patient to another site in the same patient, autografts elicit no immunogenic host response. However, the repair of large bone defects in humans remains a significant clinical problem despite successful healing of defects treated with autografts. Although

autografts contain the appropriate cues for osteogenesis and elicit no immunogenicity, donor site morbidity and pain affect as many as 30% of patients who have undergone a bone harvest from the iliac crest (Gottfried and Dailey 2008; Rawashdeh and Telfah 2008).

Approximately 1/3 of all bone grafts in North America are allografts, where cadaveric bone is taken from an unrelated donor and frozen until use (De Long *et al.* 2007). Allografts are widely used to address the issue of donor tissue availability for autografts; however, they present a different set of problems. Because bone is transferred from donor to patient, graft material may be infected or cause a significant immunological host response. The process of freezing allografts has reduced immune issues, but major infections causing graft failure are still reported to occur in about 8% of patients. Furthermore, extensive processing of allografts causes poor mechanical properties and increased rates of resorption and fracture, leading to graft failure rates as high as 50% (Sorger *et al.* 2001; Mankin *et al.* 2005) and leading many researchers to investigate alternative strategies.

*Bone Morphogenetic Proteins*

The first well documented study of bone formation by osteoinduction was described by Marshall Urist in 1965 by the implantation of demineralized bone matrices into ectopic sites in rabbits and rodents (Urist 1965). From this experiment came the discovery and characterization of bone morphogenetic proteins (BMPs), soluble growth factors that work through Smad-dependent signaling pathways to promote osteoblastic diffentiation (Derynck and Zhang 2003). More than 15 individual BMPs have now been identified and several studies have shown that BMP-2 and BMP-7 promote healing of

critically-sized bone defects in both small and large animals (Yasko *et al.* 1992; Cook *et al.* 1994; Cook *et al.* 1994; Cook *et al.* 1995) and in humans (Govender *et al.* 2002). Additionally, BMP-2 and BMP-7 are currently approved for human use in the United States and Europe (Boden 2005; Bishop and Einhorn 2007; Vaibhav *et al.* 2007), making treatment with BMPs a newly emerging standard of care that may soon replace autografts as the gold standard of treatment.

Although the use of BMPs in clinical repair of bone defects has demonstrated success, several factors contribute to the continued search for bone tissue engineering alternatives. First, the residence time of BMP in a defect site following localized delivery directly affects its osteogenic potential. As a soluble factor, diffusion of BMP away from the delivery site decreases the amount of bone formed in an implant (Uludag *et al.* 2000). Efforts are being made to increase BMP residence time at the defect site (Yamachika *et al.* 2009). Second, the dosage of BMPs required to stimulate bone formation in large bone defects in humans far exceeds the successful working dosage described in preclinical trials. In fact, one vial of BMP-7 contains as much BMP-7 as is found in two entire human skeletons. Thus, production of enough recombinant protein for one vial of BMP for human use is a very costly procedure (Alt and Heissel 2006; Bishop and Einhorn 2007; Cancedda *et al.* 2007).

*Synthetic Bone Grafts: Calcium phosphates*

Despite the increasing need for more suitable bone replacement therapies, synthetic bone graft substitutes make up only about 10% of all clinical bone grafting procedures (Bucholz 2002). Most of FDA approved synthetic grafts are composed of hydroxyapatite or other ceramic composites which possess inherent osteoconductive

properties. Pro-Osteon, the first bone graft substitute to gain FDA approval, is an interporous hydroxyapatite material derived from coral with a natural architecture similar to that of native human cancellous bone. Blocks of the material can be milled to fit the shape and size of a defect, and bone forms readily within and around the implant, making it an effective filler. Due to the brittle nature of hydroxyapatite, Pro-Osteon is restricted to use in non-loaded defects, or defects in which internal rigid fixation is applied. For the FDA-approved product, Vitoss, small beta-tricalcium phosphate particles are formed into a 90% porous matrix. The resulting scaffold has both microscale and macroscale porosity, aiding in nutrient diffusion through the scaffold. Furthermore, this material has a resorption rate similar to that of native bone, which is conducive to eventual remodeling of treated defects. Collagraft is a composite scaffold consisting of porous calcium phosphate granules contained with a matrix of bovine-derived collagen fibrils. Before implantation, autogenous bone marrow is added to the scaffold. Collagraft has demonstrated similar results to autograft in clinical trials. These products join an ever growing list of commercially available calcium phosphate grafts (De Long *et al.* 2007).

*Autologous bone marrow progenitor cells*

Although not yet common practice, the use of autogenous bone marrow derived cells for clinical treatment of non-unions has recently gained attention. The first clinical report of the use autologous bone marrow progenitor cells for treatment of large bone defects in humans was in 2001 by Quarto and colleagues (Quarto *et al.* 2001). Three patients with substantial defects in their long bones (4 cm defect in the tibia, 4 cm ulna, and 7 cm humerus) underwent bone marrow harvests from the iliac crest, and the cells were cultured ex vivo according to previous methods (Martin *et al.* 1997).

Hydroxyapatite scaffolds were modified to fit the size and shape of each defect, and cells were seeded onto scaffolds prior to implantation. Six months to one year after surgery, external fixation was removed, and all patients had recovered normal limb function. In a 6-7 year follow-up study, which included a fourth patient with a 6cm defect in the ulna, all patients demonstrated complete bone implant integration as determined by radiography and CT analysis (Marcacci *et al.* 2007). The success of these studies warrants further investigation of the use of autologous BMSCs in treating clinical non-unions. However, no subsequent reports have been made.

**Bone Tissue Engineering Strategies**

As an alternative approach to current clinical bone repair, tissue engineering and regenerative medicine strategies are being developed to address the problems associated with current bone grafting procedures (Hutmacher and García 2005; Awad *et al.* 2007; Cancedda *et al.* 2007; Kimelman *et al.* 2007). These strategies involve the development of tissue-engineered cell/scaffold constructs, whereby cells on synthetic scaffolds are implanted into a defect site, directly associate with native host tissue, and eventually restore natural tissue structure and function (Langer and Vacanti 1993). For bone regeneration, tissue engineering strategies focus on the isolation and control of a mineralizing cell source as well as the development of scaffolds with relevant mechanical properties and appropriate pore volumes (Guillot *et al.* 2007; Kretlow and Mikos 2007).

*Cell types*

An appropriate cell source for bone tissue engineering must address several criteria. First, cells must be available in abundant quantities and non-invasively obtained from the patient. Further, transplantation of the cells should be safe and pose no risk of

disease transmission to the patient (Gimble *et al.* 2007). Many cell types for bone tissue engineering are currently being investigated, the most common of which is bone marrow stromal cells.

Bone marrow stromal cells (BMSCs) are easily obtained from bone marrow aspirates and have the ability to differentiate down several mesodermal tissue lineages (Pittenger *et al.* 1999). BMSCs provide an appropriate autologous cell source for human bone tissue regeneration (Bruder *et al.* 1994; Quarto *et al.* 2001; Derubeis and Cancedda 2004). They can be extracted in large quanties from the iliac crest with minimal donor site morbidity (De Long *et al.* 2007), can be expanded in culture, and respond to intra- and extracellular cues for differentiation (Majors *et al.* 1997). BMSCs have the capability of differentiating into osteoblasts, among other cell types of the mesodermal lineage, and they mineralize constructs both in vitro and in vivo (Goshima *et al.* 1991; Ishaug *et al.* 1997; Krebsbach *et al.* 1997; Cartmell *et al.* 2004). BMSCs also enhance bone healing in critically-sized orthotopic defects in rodents and large animals compared to treatment with a scaffold alone (Werntz *et al.* 1996; Bruder *et al.* 1998; Kon *et al.* 2000; Petite *et al.* 2000). Recent evidence suggests that BMSCs may also be used as an allogeneic cell source due to the secretion of immunosuppressive trophic factors (Caplan 2007), making these cells a good source for "off the shelf" tissue engineering strategies.

Although BMSCs are an appropriate cell source for bone tissue engineering, extensive in vitro culture causes morphology changes, reduction in proliferation rate, and reduced osteogenic differentiation ability (Banfi *et al.* 2000; Derubeis and Cancedda 2004; Yeon Lim *et al.* 2006). Furthermore, the mineralization potential and proliferation rate of BMSCs varies widely with individual donors and is significantly affected by

donor age (Phinney *et al.* 1999; Mendes *et al.* 2002; Kretlow *et al.* 2008). These variations in BMSC function imply that the treatment of large bone defects with autologous BMSCs may not produce effective results for all patients. Alternative cell types with osteogenic potential for bone tissue engineering are currently being explored. Initial work with adipose-derived stem cells and amniotic fluid stem cells have shown promising potential for the ability of these cells to differentiate down an osteoblastic lineage (Muschler *et al.* 2004; Gimble *et al.* 2007; Ilancheran *et al.* 2007). However, the search for the ideal cell source for bone tissue engineering continues.

*Gene therapy*

To improve the osteogenic potential of cells used for bone tissue engineering, gene therapy strategies, which force overexpression or silencing of a target gene in a given cell population have been developed. Genetic modification of cells can be achieved through a variety of carriers, may promote stable or transient gene expression, and can be applied in vivo or ex vivo (Gersbach *et al.* 2007). Extensive work has focused on the delivery of BMPs to promote osteoblastic differentiation of cells delivered to bone defect sites (Hanada *et al.* 1997; Gazit *et al.* 1999; Huang *et al.* 2005; Edgar *et al.* 2007; Hsu *et al.* 2007). Ex vivo gene transfer of BMP-2 to BMSCs accelerates the time course of bone defect healing compared to treatment with unmodified BMSCs (Lieberman *et al.* 1999; Baltzer *et al.* 2000; Blum *et al.* 2003).

In addition to BMP, gene therapy strategies targeting osteoblast-specific transcription factors, such as Runx2 and, more recently, osterix, have been used to increase the bone forming potential of the target cell population. Runx2/Cbfa1 and osterix are transcription factors that upregulate the expression of many osteoblast-specific

genes, such as osteopontin, osteocalcin, bone sialoprotein and collagen I (Ducy *et al.* 1997; Nakashima *et al.* 2002). Osterix acts downstream of Runx2, such that forced overexpression of osterix in BMSCs does not upregulate Runx2, and Runx2 null mice do not express osterix (Nakashima *et al.* 2002; Tu *et al.* 2006). The role of Runx2 in osteoblastic differentiation has been demonstrated both in vitro and in vivo, in human and rodent systems (Otto *et al.* 1997; Shui *et al.* 2003). Furthermore, retroviral delivery of Runx2 to rodent BMSCs promotes osteoblastic differentiation of these cells even after multiple passages in vitro (Byers and García 2004; Byers *et al.* 2006).

Skeletal abnormalities have been observed in the absence of Runx2 function. For example, transgenic mice expressing a dominant negative form of Runx2 develop abnormally even after normal embryonic development (Ducy *et al.* 1999). Homozygous deletion of Runx2 in mice results in embryonic lethality and a complete lack of bone formation (Komori *et al.* 1997; Otto *et al.* 1997), while heterozygous Runx2 mutant mice show impaired mineralization and bone formation compared to wild-types (Otto *et al.* 1997). Finally, the human disease, cleidocranial dysplasia, occurs as a result of genetic mutations in Runx2 (Lee *et al.* 1997; Mundlos *et al.* 1997; Zhang *et al.* 2000).

Forced overexpression of Runx2, therefore, has been used to promote osteogenesis in vitro and in vivo in BMSCs (Byers and García 2004), and recent use of osterix has produced similar results (Tu *et al.* 2006). Interestingly, Runx2 overexpression also causes osteoblastic differentiation in non-osteoblastic cell lineages, such as myoblasts and dermal fibroblasts (Gersbach *et al.* 2004; Gersbach *et al.* 2004; Phillips *et al.* 2007), demonstrating the nature of Runx2 as a molecular switch in osteoblast biology (Ducy 2000). Recently, several studies have shown that Runx2-modified or osterix-

modified BMSCs delivered to cranial defects in rodents significantly increase defect healing compared to unmodified BMSCs (Zheng *et al.* 2004; Zhao *et al.* 2005; Byers *et al.* 2006; Tu *et al.* 2007), pointing to gene therapy strategies which incorporate transcription factors as a viable alternative to BMP gene delivery.

*Synthetic and Biomimetic Scaffolds*

Delivery of a therapeutic load of genetically-modified or unmodified BMSCs to a critically-sized bone defect requires a support scaffold that is well-suited for bone regeneration. Ideal bone tissue engineering scaffolds have mechanical properties that match those of the host tissue, allow new tissue ingrowth and removal of waste products through an interconnected pore volume, exhibit biocompatibility in the implant site, and support cell adhesion, proliferation and differentiation (Hutmacher *et al.* 2001). The most commonly used synthetic polymers for bone tissue engineering are poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers of poly(lactic-*co*-glycolic acid) (PLGA) (Liu and Ma 2004; Rezwan *et al.* 2006). PLGA foams support osteoblastic differentiation and bone formation both in vitro (Ishaug *et al.* 1997) and in vivo (Fialkov *et al.* 2003; Karp *et al.* 2003). Poly(ε-caprolactone) (PCL) is another synthetic polymer suitable for bone tissue engineering applications, that can be formed into scaffolds by fused deposition modeling to create unique architectures and variable pore sizes (Zein *et al.* 2002). PCL promotes cell-based mineralization both in vitro (Cao *et al.* 2003; Schantz *et al.* 2003; Byers *et al.* 2004; Phillips *et al.* 2006; Zhou *et al.* 2007) and in vivo (Rohner *et al.* 2003; Schantz *et al.* 2003; Byers *et al.* 2006; Rai *et al.* 2007) and is well tolerated in vivo. Poly(L-lactide-*co*-D,L-lactide) (PLDL) is a synthetic polymer with degradation and mechanical properties appropriate for bone tissue (Cartmell *et al.* 2004).

PLDL promotes cellular mineralization in vitro (Cartmell *et al.* 2004) and in vivo (Oest *et al.* 2007), and it can be fabricated with longitudinally oriented pores as well as randomly dispersed, interconnected pores within the same scaffold (Lin *et al.* 2003).

While synthetic polymeric scaffolds support bone ingrowth and mineralization, composite structures and surface modifications improve the osteogenic response. For example, various PCL/ceramic composites, such as PCL/tricalcium phosphate (TCP) and PCL/hydroxyapatite increase cell proliferation and matrix deposition rate (Zhou *et al.* 2007), alkaline phosphatase activity (Venugopal *et al.* 2007) and mineralization (Causa *et al.* 2006; Wutticharoenmongkol *et al.* 2007) compared to PCL scaffolds alone. Furthermore, chemical surface modification, such as ion irradiation, enhances cell adhesion and osteoblastic differentiation (Amato *et al.* 2007; Marletta *et al.* 2007).

To more specifically modulate the osteoblastic response to synthetic materials, surface biofunctionalization strategies which mimic the extracellular matrix allow precise control over cell signaling and differentiation (García and Reyes 2005). These strategies attempt to recapitulate the signaling environment of the bone ECM and have been used to promote osseointegration and bone formation in vivo (Hubbell 2003). For example, short immobilized peptide sequences derived from ECM proteins, such as the RGD sequence from fibronectin, have been used to promote cell attachment to titanium implants as an improved method of implant fixation and osseointegration (Ferris *et al.* 1999; Bernhardt *et al.* 2005; Elmengaard *et al.* 2005). These strategies take advantage of the specific interactions between ECM protein ligands and integrin cell surface receptors.

The integrin receptors play a crucial role in cell attachment and ECM-mediated cell signaling. Integrin dimers, consisting of one $\alpha$ and one $\beta$ subunit, bind specifically

to active sites contained within ECM proteins, thereby promoting cell attachment, migration, mechanotransduction, and numerous other cell functions (Clark and Brugge 1995; Hynes 2002). In particular, the specific interactions of fibronectin with the $\alpha_5\beta_1$ integrin and of type I collagen with the $\alpha_2\beta_1$ integrin have been shown to mediate osteoblast differentiation and subsequent mineralization of osteoblastic and pre-osteoblastic cells (Moursi *et al.* 1997; Takeuchi *et al.* 1997; Xiao *et al.* 1998; Jikko *et al.* 1999; Mizuno *et al.* 2000). The $\alpha_2\beta_1$ receptor is highly expressed on the surface of osteoblastic cells (Gronthos *et al.* 1997), and $\alpha_2\beta_1$ activation is presently known to occur via binding of distinct adhesive sites contained within collagen I, namely DGEA and GFOGER (Xiao *et al.* 1998; Knight *et al.* 2000). Isolation of the active binding sequence, GFOGER, via synthetic fabrication of a triple helical GFOGER peptide, allows engineered control over osteoblastic differentiation of cells on GFOGER coated 2D surfaces (Reyes and García 2003; Reyes and García 2004). Interestingly, the use of GFOGER as an implant coating for titanium tibial plugs in rats enhances osseointegration in vivo to levels greater than that of uncoated implants or implants coated with full length collagen I (Reyes *et al.* 2007). This collagen mimetic strategy for osseointegration specifically targets osteoblastic cells through $\alpha_2\beta_1$-mediated signaling by eliminating extraneous binding sites contained in the full length protein. Furthermore, the synthetically derived peptide is cheap and easy to fabricate and poses no risk of disease transmission.

Another collagen mimetic peptide, termed P-15, exhibits collagen-mimetic cell signaling when used as a surface coating and promotes osteoblastic differentiation and mineralization on anorganic bone matrices in vitro (Qian and Bhatnagar 1996; Bhatnagar

*et al.* 1999; Yang *et al.* 2004). Preclinical studies using P-15/ABM implants in orthotopic defects have demonstrated successful defect healing, and numerous case reports have demonstrated successful healing in humans due to P-15/ABM implants (Scarano *et al.* 2003; Cakmak *et al.* 2006; Gomar *et al.* 2007; Trombelli and Farina 2008).

*Animal models*

Presently, no standard procedure for evaluating bone tissue engineering strategies in large and small animal models exists (Reichert *et al.* 2009). Researchers often test subcutaneous implantations as a first level evaluation of the tissue engineered construct. If substantial mineralization is formed subcutaneously, intramuscular implantation may be studied at an alternative ectopic site. However, while these strategies provide initial insight into implant-host interaction and can verify survival and function of implanted cells, no information regarding successful healing of a large bone defect can be obtained. For functional assessment of the implant, an orthotopic model must be used.

To test the advantage of an implantation strategy in bone, a critically-sized defect must be established for each animal model and each anatomic location. A critically-sized defect is defined by the inability of the defect to heal without surgical intervention and is the appropriate test bed for bone tissue engineering strategies. For many models, the critical size of the defect is approximately a length 2 to 2.5 times the diameter of the bone (Reichert *et al.* 2009). For example, a rat femur is approximately 4 mm in diameter, and a defect of 8 mm has shown consistent non-union (Oest *et al.* 2007; Rai *et al.* 2007). On the other hand, 3-5 mm defects have been tested in rat femurs with varying results.

In addition to size of the defect, many other factors must be considered when developing an animal model for bone tissue engineering strategies. Most importantly, the model should mimic the human setting for which the strategy is being developed as closely as possible. For example, the cranial defect model provides relevant information regarding the healing of craniofacial defects. However, conflicting evidence regarding the role of periosteum, dura mater, and surrounding healthy bone tissue in cranial defect healing makes separation of host response from treatment effect difficult in this model (Aalami *et al.* 2003). In particular, dura mater has been shown to have a significant effect on cranial defect healing (Ozerdem *et al.* 2003). Therefore, a cranial model may not be an appropriate test bed for strategies aimed at healing defects in long bones, where contributions from the dura mater are not present.

Another aspect for consideration in animal models for bone tissue engineering is the methods used to evaluate healing. Many studies that report successful healing of bone defects rely solely on a combination of X-ray analysis and histological evaluation as a measure of defect healing. However, these methods do not provide functional information, which is necessary to fully evaluate the success of a given treatment strategy for bone healing (Liebschner 2004). Bone quality is a description of all skeletal aspects of bone, except bone mass, that affect bone strength, such as shape, size, and trabecular connectivity. (Hernandez and Keaveny 2006). Recent evidence suggests that patients with a high level of bone turnover may be at higher risk for bone fracture regardless of their bone mineral density (Hernandez 2008). Therefore, models which only evaluate bone volume or percent area of the defect healed via radiographic and histological methods are not enough to fully characterize the extent of bone defect healing.

Functional mechanical evaluation of bone defects should be used in conjunction with other methods for complete analysis of both bone mass and bone quality.

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# CHAPTER 3

# EFFECTS OF RAT STRAIN AND CULTURE METHODS ON RUNX2-ENHANCED OSTEOBLASTIC DIFFERENTIATION AND MINERALIZATION IN BONE MARROW STROMAL CELLS*

## Introduction

Bone marrow stromal cells (BMSCs) are a heterogeneous population of progenitor cells, easily obtained from the bone marrow, that have the potential to differentiate along several tissue lineages, including adipogenic, chondrogenic, and osteogenic (Pittenger *et al.* 1999). Because of their stem cell-like nature, BMSCs have been explored as a possible cell source for many tissue engineering applications, including bone tissue engineering (Haynesworth *et al.* 1992; Bruder *et al.* 1994; Quarto *et al.* 2001; Derubeis and Cancedda 2004). However, in vitro expansion of BMSCs, a necessary step in obtaining sufficient cell numbers for therapeutic applications, results in a significant decrease in BMSC proliferation rate and osteoblastic differentiation and mineralization (Banfi *et al.* 2000; Lim *et al.* 2006). To overcome these limitations, Runx2 genetic modification has been employed as a method of maintaining the osteogenic potential of these cells even after multiple passages in vitro (Byers and García 2004; Byers *et al.* 2006).

Runx2/Cbfa1 is an osteoblast-specific transcription factor that upregulates the expression of many osteoblast-specific genes, such as osteopontin, osteocalcin, bone sialoprotein and collagen I (Ducy *et al.* 1997). The role of Runx2 in osteoblastic

*Adapted from Wojtowicz AM and García AJ. JTERM, in revision.*

differentiation has been demonstrated both in vitro and in vivo, and in both human (Shui *et al.* 2003) and rodent systems (Otto *et al.* 1997). Furthermore, skeletal abnormalities have been observed in the absence of Runx2 function. For example, transgenic mice expressing a dominant negative form of Runx2 develop abnormally even after normal embryonic development (Ducy *et al.* 1999). Homozygous deletion of Runx2 in mice results in embryonic lethality and a complete lack of bone formation (Komori *et al.* 1997; Otto *et al.* 1997), while heterozygous Runx2 mutant mice show impaired mineralization and bone formation compared to wild-types (Otto *et al.* 1997). Finally, the human disease, cleidocranial dysplasia, occurs as a result of genetic mutations in Runx2 (Lee *et al.* 1997; Mundlos *et al.* 1997; Zhang *et al.* 2000). Forced overexpression of Runx2, therefore, has been used to promote osteogenesis in vitro and in vivo in bone marrow stromal cells (Byers and García 2004; Zheng *et al.* 2004; Zhao *et al.* 2005; Byers *et al.* 2006). Interestingly, this phenomenon has also been demonstrated in non-osteoblastic cell lineages, such as myoblasts and dermal fibroblasts (Gersbach *et al.* 2004; Gersbach *et al.* 2004; Phillips *et al.* 2007), demonstrating the role of Runx2 as a molecular switch in osteoblast biology (Ducy 2000).

Although Runx2 genetic engineering provides a way to overcome the reduced osteogenic potential of in vitro expanded-BMSCs, it should be noted that in vitro expansion is not the only variable that affects BMSC function. Isolation and culture procedures strongly influence BMSC growth rate and osteogenic differentiation potential (Phinney *et al.* 1999) as well as alkaline phosphatase-positive colony forming units (CFUs) (Muschler *et al.* 1997). Culture conditions, such as serum source, media composition and even media volume, modulate osteogenic differentiation (Jaiswal *et al.*

1997; Anselme *et al.* 2002; Mendes *et al.* 2002; Abdallah *et al.* 2006). Finally, donor age has been shown to affect the growth rate of human BMSCs older than 40 years of age (Mendes *et al.* 2002), but not that of BMSCs when donors were younger than 40 years of age (Phinney *et al.* 1999). Taken together, these studies illustrate that cell source (i.e. donor) and culture conditions affect the function of BMSC populations.

In the present work, we examined the osteogenic differentiation and mineralization capacity of unmodified and Runx2-modified BMSC populations isolated from commonly used strains of rats. We compared cells isolated from Lewis rats, an inbred strain, to those isolated from Wistar rats, an outbred strain. We demonstrate that the BMSC population derived from Wistar rats has a greater propensity for retroviral Runx2 transduction than the population derived from Lewis rats, but that Lewis BMSC populations have a greater capacity for subsequent osteoblastic differentiation than Wistar BMSC populations.

## Materials and Methods

### *Cell harvest and isolation*

Rat BMSCs were isolated from the hind legs of young Lewis or Wistar rats (Charles River Labs) by commonly used methods (Javazon *et al.* 2001; Byers and García 2004). Briefly, the rats were euthanized by $CO_2$ asphyxiation (protocol approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology), and the hind-limbs removed, taking care to minimize bleeding and subsequent clotting. Surrounding soft tissue was removed from each bone, and the separated femurs and tibias were soaked in cell culture media consisting of αMEM (Invitrogen) + 1% penicillin-streptomycin (Invitrogen) + 10 or 20% fetal bovine serum (FBS – HyClone). The

epiphyses of each bone were removed and the marrow was flushed from the diaphysis of the bone into a sterile tube. The marrow pellet was centrifuged, resuspended, and then transferred to tissue culture-grade polystyrene (Corning).

Two different methods of stromal cell isolation were tested. Both methods begin as described above but differ after plating. Method 1 involves a 30 minute incubation step, whereby plated cells are maintained at 37$^{o}$C for 30 minutes, after which time, non-adherent cells are removed and replated, while those that have adhered are discarded. This procedure is designed to remove monocytes/macrophages from the heterogeneous BMSC population. Method 2 does not include this adhesion-selection step, and cells are plated onto tissue culture-grade polystyrene immediately following centrifugation. The following three strain/culture condition groups were examined:

1. Lewis BMSC harvested without the 30-min adhesion-selection step, isolated in 20% FBS, then switched to 10% FBS after one week. This group is referred to as Lewis (serum).
2. Lewis BMSC harvested without the 30-min adhesion-selection step, isolated and cultured in 10% FBS throughout culture. This group is referred to as Lewis.
3. Wistar BMSC harvested with the 30-min adhesion-selection step, isolated and cultured in 10% FBS throughout culture. This group is referred to as Wistar.

*Runx2 retroviral transduction*

Following isolation, cells were expanded to passage 3 or passage 5 in αMEM + 1% penicillin-streptomycin + 10% FBS (Invitrogen), and then plated at 5 000 cells/cm$^2$ in 6 well plates (Corning) coated with type I collagen (MP Biomedicals). For each strain/culture condition, four treatment groups were examined: (i) Runx2-engineered

cells, (ii) Runx2-engineered cells + dexamethasone (dex, 10 nM), (iii) unmodified cells, and (iv) unmodified cells + dex. Dex is a synthetic glucocorticoid that induces osteoblastic differentiation of BMSCs in vitro (Maniatopoulos *et al.* 1988; Cheng *et al.* 1994). Genetically engineered cells were transduced with Runx2 retrovirus as previously described (Byers *et al.* 2002). Two days after transduction, culture media was supplemented with 50 μg/mL ascorbic acid and 3 mM Na β-glycerophosphate. All media supplements were purchased from Sigma. Media was changed every 2-4 days.

*Flow cytometry for transduction efficiency*

Our Runx2 retrovirus has an internal ribosomal entry site that encodes for enhanced green fluorescent protein (eGFP) as a marker of Runx2 transduction (Byers *et al.* 2002). To measure Runx2 transduction efficiency, eGFP expression was measured by flow cytometry. Three days after Runx2 transduction, cells were trypsinized (0.05% trypsin/EDTA – Invitrogen) and resuspended in phosphate buffered saline (PBS – Invitrogen) with 10% FBS. Cell suspensions were then centrifuged for 5 minutes at 200-300g, resuspended in PBS and then passed through a 40 μm filter (BD Falcon). eGFP fluorescence was measured on a flow cytometer (Becton Dickinson, BD LSR II), and 10 000 events were measured for each sample. Data analysis was performed using WinMDI v.2.8, and graphs were made in FlowJo v.7.2.1.

*Alkaline phosphatase activity*

Alkaline phosphatase (ALP) activity was evaluated as an early marker of osteoblastic differentiation at 7 days after Runx2 transduction (Byers *et al.* 2002). Cultures were rinsed with PBS, then lysed with 50 mM ice cold Tris-HCl. Lysates were

sonicated 2 times each for 10 seconds at 5 Watts and then centrifuged at 10 000g for 5 minutes. The supernatant, containing the soluble protein component, was then frozen at -20$^{o}$C.

For each sample, total protein concentration was determined by averaging the results of a bicinchoninic acid (BCA) reaction (Pierce Biotechnology, Micro BCA Protein Assay kit #23235) with those obtained from a Nanodrop (ND-1000 Spectrophotometer). To measure ALP activity, 2.5 μg of total protein was added to 60 μg/mL 4-methyl-umbelliferyl-phosphate, a fluorescent substrate for the ALP reaction, in diethanolamine buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$, 0.2 M $NaHCO_3$, pH=9.5). Samples were incubated at 37$^{o}$C for 40 minutes in the dark to allow the reaction to occur. Activity was measured as relative fluorescence units on a Bio Assay Reader (Perkin Elmer, HTS 7000 Plus) and standardized using purified calf intestinal ALP (Sigma).

*von Kossa staining for mineralization*

Twenty one days after Runx2 transduction, cultures were stained for phosphate deposits, indicative of mineral formation, by von Kossa staining (Byers *et al.* 2002). Briefly, plates were rinsed with PBS and then fixed in 70% ethanol at 4$^{o}$C overnight. After fixation, plates were rinsed with $ddH_2O$, stained with 5% $AgNO_3$ for 30 minutes on a light table, rinsed again with $ddH_2O$, fixed with 5% $NaS_2O_3$ for 2 minutes, and rinsed again with $ddH_2O$. Plates were stored in the dark until completely dry. Images were captured with a digital camera (Kodak Digital Science DC120 Zoom Digital Camera). Mineral area was quantified using an inverted microscope (Nikon Eclipse TE300) with ImagePro Plus v.4.5.1.22.

*Statistics*

All statistics were performed using Systat v.11.00.01. For flow cytometry data, ANOVA analysis was performed using the post-hoc Tukey method to determine differences between groups. A p-value $< 0.05$ was considered significant. For ALP activity and mineralization, data was divided into three groups: treatment (4 levels: Control, Control+dex, Runx2, Runx2+dex), strain/harvest (3 levels: Lewis (serum), Lewis, Wistar), and passage (2 levels: passage 3 and passage 5). These three groups were analyzed by a general linear model ANOVA to determine overall differences between the levels in each group.

**Results**

*Runx2 transduction efficiency*

To quantify the transduction efficiency of the Runx2 retrovirus, flow cytometry analysis was used to determine the percentage of cells expressing eGFP after transduction. Unmodified BMSCs (no Runx2 transduction) were used as a negative control, and the top 2% of cells in each control population were used to set the lower limits for transduction. Figure 3.1 shows flow cytometry histograms of both control and Runx2 transduced cells for all strain/harvest groups.

For all groups, passage 3 cells demonstrated significantly different transduction efficiencies when compared to passage 5 cells within the same strain/harvest group. Interestingly, Wistar cultures at passage 5 showed greater transduction efficiency compared to Lewis and Lewis (serum) cultures at the same passage. This result could be due to differences in proliferation rate, which affects retroviral transduction, or

differences in donor strain or culture/isolation procedures. Finally, passage 3 Lewis (serum) cultures showed significantly less transduction efficiency than the other two

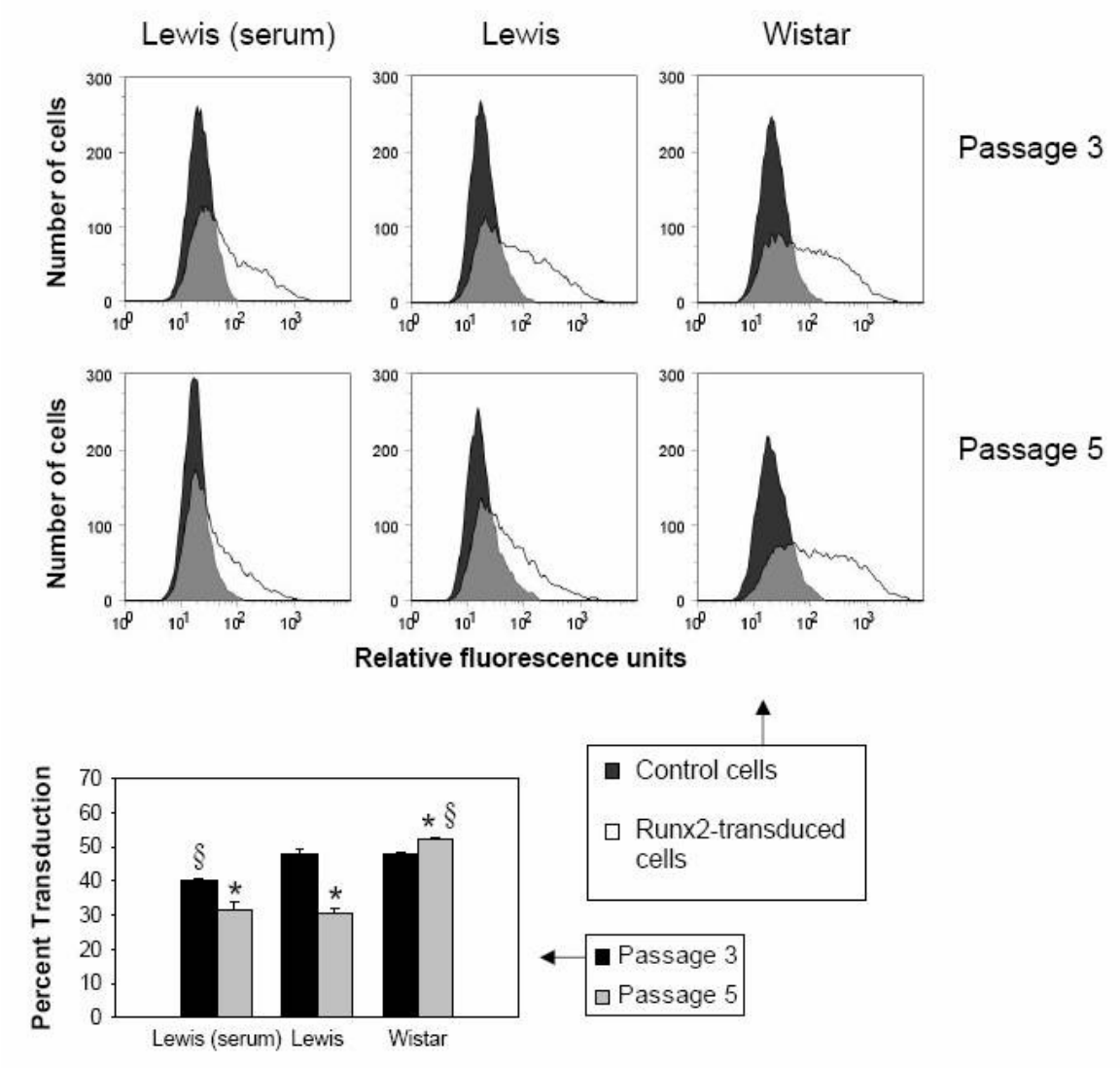


**Figure 3.1.** Runx2 retroviral transduction efficiency is differentially modulated by cell passage and strain/harvest procedures. § Different from other harvest groups within the same passage. Furthermore, transduction efficiency is significantly different at P3 than at P5 for all groups. * Different from P3 within the same harvest group. $p<0.05$, n=3. Error bars represent standard deviation.

groups at the same passage. These results indicate that a change in media components (i.e. 20% serum switched to 10%) significantly impacts transduction efficiency.

*Alkaline phosphatase activity*

Alkaline phosphatase (ALP) activity was examined at 7 days after Runx2 transduction as a marker of early osteoblastic differentiation. Runx2 treatment significantly increased ALP activity both with and without dexamethasone (Figure 3.2). Further analysis by general linear model ANOVA showed that ALP activity was also significantly modulated by strain/harvest group as shown in the inset in Figure 3.2A. This statistical analysis allowed the simultaneous comparison of several experimental groups containing multiple levels, and the variance of each group was taken into account when calculating the significance. Finally, passage did not have a significant effect on ALP activity under these conditions.

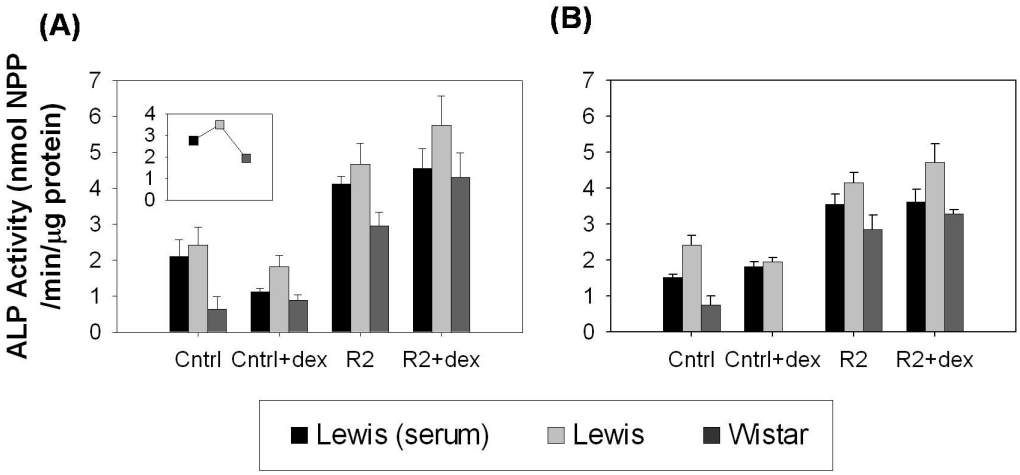


**Figure 3.2.** Treatment with Runx2 significantly increases alkaline phosphatase (ALP) activity after 7 days for (A) passage 3 cultures and (B) passage 5 cultures. Furthermore, as shown in the inset, strain/harvest group has an overall significant effect on ALP activity. $p<0.05$, $n=3$. Error bars represent standard deviation.

*von Kossa staining for mineralization*

Cultures were maintained for 3 weeks under osteogenic conditions to examine mineral deposition, which was assessed by von Kossa staining and image analysis. von Kossa analysis revealed differences in mineralization among experimental groups (Figure

3.3A). The percentage of mineralized area on each plate was quantified for passage 3 (Figure 3.3B) and passage 5 (Figure 3.3C) cultures. Runx2 treatment, strain/harvest conditions, and passage all had a significant effect on mineralization. Overall differences for strain/harvest group are illustrated in the inset in Figure 3.3B.

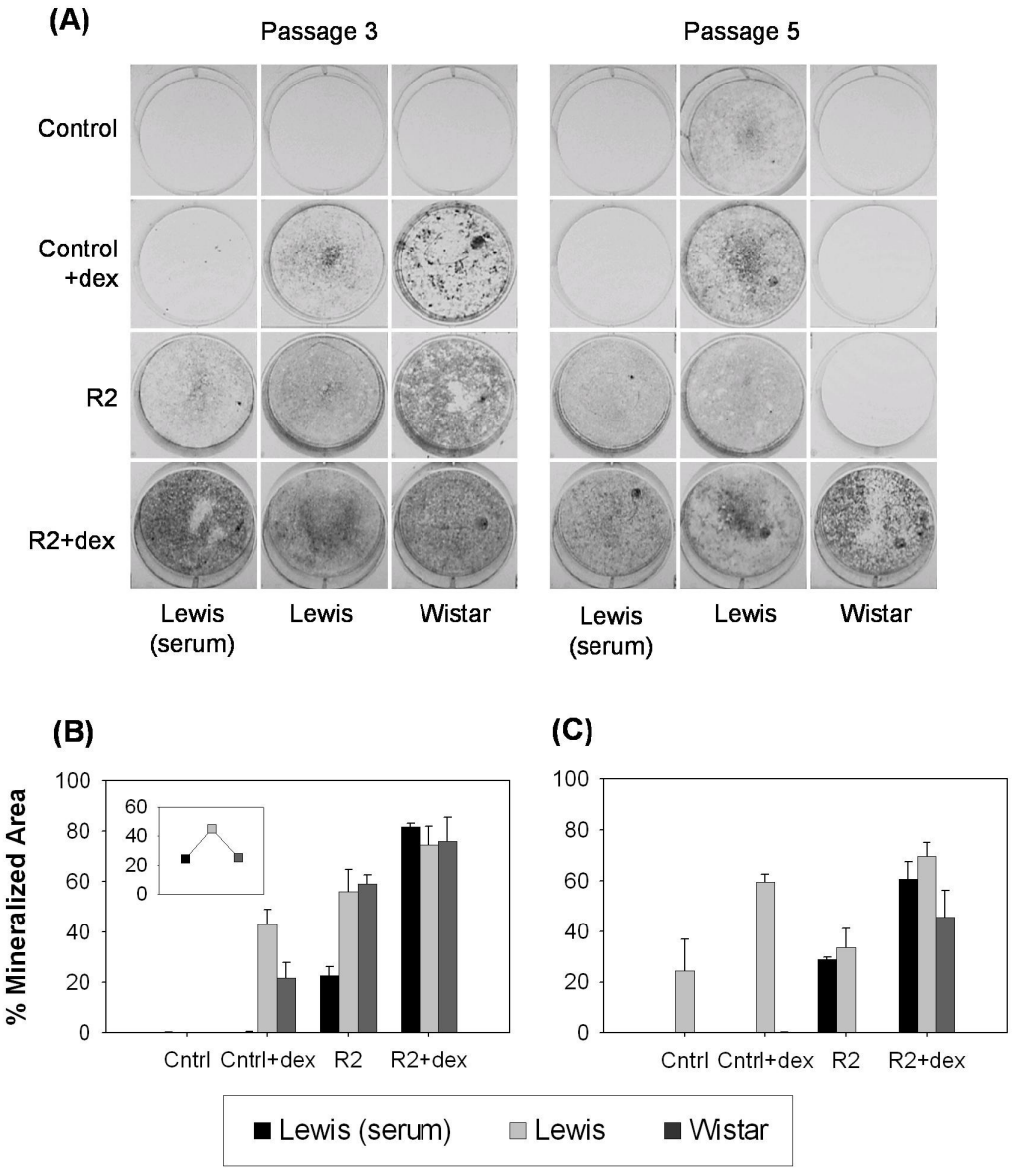


**Figure 3.3.** Unmodified Lewis cultures (control and control+dex) display the greatest amount of mineralization compared to unmodified Lewis (serum) and Wistar cultures. Treatment with Runx2 + dex increases mineralization for all groups. von Kossa staining is shown in (A). Mineralization was quantified by image analysis and results are shown for (B) P3 cells and (C) P5 cells. Using a general linear model for ANOVA analysis, strain, passage and treatment all showed overall significant differences. Overall strain differences are illustrated in the inset. $p<0.05$, $n=3$. Error bars represent standard deviation.

The trends observed for mineralization match those for ALP activity, namely that Runx2 treatment with and without dexamethasone significantly increased both ALP activity and mineralization. Furthermore, Lewis cultures displayed significantly greater ALP activity and mineralization compared to Lewis (serum) and Wistar cultures. These results demonstrate that both early (ALP) and late (mineral) markers of osteogenic differentiation are affected in the same manner by Runx2 treatment and strain/harvest procedures in this study. Finally, Lewis BMSCs in combination with Runx2 genetic engineering produced the most mineral.

**Discussion**

In this study we demonstrate differences in the osteogenic differentiation ability of bone marrow stromal cells harvested from different strains of rat using different methods of isolation. Although it has already been established that cell source has a significant effect on the function of a BMSC population (Phinney *et al.* 1999; Anselme *et al.* 2002), cell populations derived from different animal strains have not yet been compared. Our work suggests that donor strain differences may have a significant effect on the function of BMSC populations.

The current work shows that overall mineralization of Lewis BMSC cultures is significantly greater than that of Wistar BMSCs, suggesting a difference between BMSC populations harvested from these two strains of rats. These differences are most noticeable for unmodified passage 5 cells (control and control + dex conditions), whereas Runx2 treatment with and without dex essentially eliminated the differences between the two strains. The mechanistic reasons for these apparent strain differences in unmodified rat BMSCs are not yet clear; however, strain differences in rats cause a number of

physiological differences, including behavioral differences (Brimberg *et al.* 2007; Kosten *et al.* 2007), differences in platelet aggregration and thrombus formation (Sudo *et al.* 2007), skeletal development (DeMoss and Wright 1998), and ectopic bone formation in response to BMP9 (Li *et al.* 2003), among others. These results taken together with this study suggest that strain differences play an important role in many biological systems of the rat. Therefore, regarding human tissue engineering therapies, results validated in pre-clinical studies may be largely dependent on the strain of the animal used.

It is well-established that serum concentration and differences in serum source, such as the specific lot of serum, influence mammalian cell culture. Here we report that changing the serum concentration from 20% to 10% after one week had a significant effect on BMSC retroviral transduction, alkaline phosphatase activity and matrix mineralization. The switch in serum concentration from 20% to 10% had a negative effect when compared to sustained culture in 10% serum. Although these two cultures were initially expanded for one week in different serum concentrations, they were both cultured in 10% serum after one week. This indicates that a change in serum concentration may have a greater effect on cell function than the concentration alone.

Although there may be differences in the osteogenic capacity of BMSCs derived from Lewis versus Wistar rats, a limitation of this study is that our isolation methods differed slightly between Lewis and Wistar cells. Our harvest from Wistar rats included a 30 minute adhesion selection step whereby cells that were readily adherent to the plates were discarded. In contrast, our harvest of Lewis cells did not include this step. The inclusion of this adhesion selection step is meant to remove monocytes/macrophages from the heterogeneous BMSC population; however, it is possible that in discarding these

readily adherent cells, many of the desired osteogenic cells of the population may have been discarded as well. Therefore, the differences observed in this study could have been due to either strain or isolation methods or a combination of both. Further investigation is required to fully elucidate the cause of the observed differences.

In conclusion, we have demonstrated significant differences in osteoblastic differentiation and mineralization of BMSC populations due to differences in donor rat strain and culture conditions. Although BMSCs are a robust potential cell source for orthopaedic tissue engineering applications due to their ability to differentiate into functional osteoblastic cells, differences between donors and differences in culture and isolation procedures have a significant impact on cellular differentiation and mineralization. Therefore, donor strain and isolation procedures should be carefully considered when choosing a cell source for pre-clinical tissue engineering research. The ultimate goal in tissue engineering is the development of therapies for human disease states; therefore, the significance of the current work emphasizes the importance of choosing an appropriate animal model for a particular human application. With regard to bone tissue engineering, certain rat strains may be more conducive to in vitro BMSC mineralization than others. The current study demonstrates that cell type (i.e. BMSC) is not the only criterion necessary for defining a cell source for orthopaedic tissue engineering applications and that donor strain and isolation procedures also have a significant impact on BMSC differentiation and mineralization.

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# CHAPTER 4

# RUNX2 OVEREXPRESSION IN BONE MARROW STROMAL CELLS ACCELERATES BONE FORMATION IN CRITICALLY-SIZED RODENT FEMORAL DEFECTS

## Introduction

The repair of large bone defects in humans remains a significant clinical problem despite successful healing of defects treated with auto- and allografts. Although autografts contain the appropriate cues for osteogenesis and elicit no immunogenicity, donor site morbidity and pain affect as many as 30% of patients who have undergone a bone harvest from the iliac crest (Gottfried and Dailey 2008; Rawashdeh and Telfah 2008). Allografts address these sourcing issues but are further complicated by poor mechanical properties, increased rates of resorption, and increased risk of infection (Sorger *et al.* 2001; Mankin *et al.* 2005). As an alternative approach for bone repair, tissue engineering and regenerative medicine strategies are being developed to address the problems associated with current bone grafting procedures (Hutmacher and García 2005; Awad *et al.* 2007; Cancedda *et al.* 2007; Kimelman *et al.* 2007).

For successful repair and remodeling of large bone defects, a cell population capable of producing and remodeling bone must be present in the defect site. These cells can either be recruited from host tissues or implanted via surgical intervention. Bone marrow stromal cells (BMSCs) offer an attractive solution to cell sourcing for bone tissue engineering because they are part of a multipotential cell population that contributes to

the early stages of fracture healing in bone (Einhorn 1998), and human BMSCs can be isolated from bone marrow and delivered directly to a defect site to induce healing (Muschler *et al.* 1997; Smiler and Soltan 2006). BMSCs have the capability of differentiating into osteoblasts, among other cell types of the mesodermal lineage, and they mineralize constructs both in vitro and in vivo (Goshima *et al.* 1991; Ishaug *et al.* 1997; Krebsbach *et al.* 1997; Pittenger *et al.* 1999; Cartmell *et al.* 2004). BMSCs also enhance bone healing in critically-sized orthotopic defects in rodents and large animals compared to treatment with a scaffold alone (Werntz *et al.* 1996; Bruder *et al.* 1998; Kon *et al.* 2000; Petite *et al.* 2000). Recently, Marcacci et al. have reported successful healing of large bone defects in humans treated with autologous BMSCs seeded on bioceramic scaffolds. These implantations were initially reported in 2001, and a 6-7 year follow-up of each patient demonstrated that complete bone implant integration was achieved in all patients as determined by radiography and CT analysis (Quarto *et al.* 2001; Marcacci *et al.* 2007).

The success of these implants makes a strong case for the use of autologous BMSCs in treating large bone defects in humans. However, multiple factors affect the inherent ability of BMSCs to differentiate into osteoblasts. For example, in vitro expansion of BMSCs, a necessary step to obtain sufficient numbers for implantation, causes dedifferentiation and subsequent loss of mineralization capacity (Banfi *et al.* 2000; Derubeis and Cancedda 2004; Yeon Lim *et al.* 2006). Furthermore, the mineralization potential and proliferation rate of BMSCs varies widely with individual donors and is significantly affected by donor age (Phinney *et al.* 1999; Mendes *et al.* 2002; Kretlow *et al.* 2008). These variations in BMSC function imply that the treatment of large bone

defects with autologous BMSCs may not produce effective results for all patients. A method which overcomes these difficulties with BMSCs would provide great benefit to clinical bone healing.

To address these limitations with BMSCs, extensive work has focused on BMP delivery to BMSCs for upregulation of osteoblastic differentiation (Hanada *et al.* 1997; Gazit *et al.* 1999; Huang *et al.* 2005; Edgar *et al.* 2007; Hsu *et al.* 2007). However, the diffusion of these soluble factors away from a defect site makes the effective dosage for human BMP treatment very high, which in turn makes treatment with BMPs an expensive therapy (Bishop and Einhorn 2007). Furthermore, as a soluble factor, diffusion of BMP away from the delivery site decreases the amount of bone formed in an implant and may lead to unregulated signaling in remote sites (Uludag *et al.* 2000). Alternatively, the type 2 runt-related Cbfa1 gene, Runx2, encodes an osteoblast-specific transcription factor, which works *intracellularly* to upregulate a host of bone specific genes, including osteocalcin and collagen I (Ducy *et al.* 1999). Runx2 plays an important role in both bone development and bone remodeling/repair (Ducy 2000). For example, homozygous deletion of Runx2 in mice causes the formation of a completely non-mineralized, cartilaginous skeleton and results in immediate postpartum death (Komori *et al.* 1997; Otto *et al.* 1997). Meanwhile, mice heterozygous for Runx2 display a pathology similar to that observed in the sketetal disease cleidocranial displasia (Mundlos *et al.* 1997; Otto *et al.* 1997). In fact, the human disease, cleidocranial dysplasia, occurs as a result of genetic mutations in Runx2 (Lee *et al.* 1997; Mundlos *et al.* 1997; Zhang *et al.* 2000). The role of Runx2 as an osteoblastic transcription factor in human BMSCs has been demonstrated in vitro via DNA binding assays (Shui *et al.* 2003). Furthermore,

dominant negative expression of Runx2 after osteoblast differentiation causes skeletal abnormalities (Ducy *et al.* 1999), while ex vivo overexpression of Runx2 in both osteoblastic and non-osteoblastic cells promotes upregulation of bone-specific genes and subsequent mineralization (Xiao *et al.* 1999; Byers *et al.* 2002; Gersbach *et al.* 2004; Phillips *et al.* 2007). Finally, overexpression of Runx2 in BMSCs accelerates osteoblastic differentiation and subsequent mineralization both in vitro and in vivo when compared to unmodified BMSCs (Byers *et al.* 2004; Zhao *et al.* 2005), making Runx2 overexpression an attractive option for regulating BMSC function.

Recently, our group and others have reported significant healing of critically-sized defects in a calvarial defect model treated with BMSCs engineered to overexpress Runx2 (Zheng *et al.* 2004; Byers *et al.* 2006; Zhao *et al.* 2007). However, results obtained from calvarial defect studies do not necessarily translate to healing in long bones due to the involvement of the dura mater in healing cranial defects (Aalami *et al.* 2003). In this study, we examine the effect of constitutive overexpression of Runx2 in BMSCs implanted in critically-sized segmental defects in rat femurs. This model provides a more rigorous test bed than cranial defects by eliminating contributions to healing from the dura mater. This segmental defect study of Runx2-modified BMCSs for the treatment of large bone defects provides better insight into this alternative cell-based gene therapy method for clinical bone defect healing.

## Materials and Methods

### *Cell harvest and isolation*

Rat BMSCs were isolated from the hind legs of young Lewis rats (Charles River Labs) by commonly used methods (Javazon *et al.* 2001; Byers and García 2004). Briefly,

the rats were euthanized by $CO_2$ inhalation (Georgia Tech IACUC-approved protocol), and the hind limbs removed, taking care to minimize bleeding and subsequent clotting. Surrounding soft tissue was removed from each bone, and the separated femurs and tibias were soaked in cell culture media consisting of αMEM (Invitrogen) + 10% fetal bovine serum (FBS – HyClone) + 1% penicillin-streptomycin (Invitrogen) + 0.3 μg/mL fungizone. The distal end of each bone was removed and the marrow was flushed from the diaphysis via centrifugation into a sterile tube. The marrow pellet was then resuspended, and transferred to tissue culture-grade polystyrene (Corning). Three days after harvest, plates were rinsed twice in PBS to remove non-adherent hematopoietic cells, and fresh media was added. Media was changed every 3 days. When plates were 80-90% confluent, passage 0 cells were cryopreserved in 10% DMSO in FBS at -80$^{o}$C overnight, then transferred to liquid nitrogen for long term storage.

*Scaffold fabrication*

Polycaprolactone (PCL) scaffolds were produced in 9 mm thick sheets by fused deposition modeling as described previously (Zein *et al.* 2002). Sheets were cut into scaffolds using a 4 mm diameter dermal biopsy punch (Miltex). MicroCT analysis was used to characterize the structural parameters of the scaffolds (Figure 4.2C). For in vivo studies, scaffolds were 81-85% porous, average pore size was 890 μm and average rod thickness was 310 μm.

For all studies, scaffolds were cleaned in 70% ethanol for 30 minutes, rinsed 3 times in sterile water to remove ethanol, then soaked in PBS for 10-30 minutes prior to cell seeding. For in vivo studies, an additional step was added prior to scaffold

sterilization for endotoxin removal. For this step, scaffolds were rinsed in 70% ethanol for 4 days on a shaker plate, and ethanol was replaced every day.

To produce collagen meshes within each PCL scaffold, a collagen solution was polymerized inside the scaffolds, then lyophilized as previously described (Porter *et al.* 2007). Briefly, a sterile solution of 1.5 mg/mL type I collagen (MP Biomedicals) was polymerized inside sterile PCL scaffolds at 37$^{o}$C for 30 minutes. Constructs were then frozen for 1 hour at -80$^{o}$C and lyophilized overnight to produce a mesh of collagen within the scaffolds.

*Retroviral transduction*

The Runx2 type II MASNSLF isoform was expressed via the pTJ66 vector as a single bicistronic mRNA sequence encoded by murine cDNA (Figure 4.2A). An internal ribosomal entry site, located downstream from and adjacent to the Runx2 insert, allowed co-expression of a fusion protein of zeocin resistance and enhanced green fluorescent protein (eGFP). eGFP expression was quantified via flow cytometry and used as a measure of Runx2 transduction efficiency (Byers *et al.* 2002; Gersbach *et al.* 2004). Empty pTJ66 vector, which was missing the Runx2 insert but still encoded the fusion protein, was used as an empty vector control (Phillips *et al.* 2007).

To package Runx2 retrovirus, ΦNX helper cells, stably transfected with the Runx2 plasmid, were grown to sub-confluency in DMEM + 10% FBS + 1% P/S. Twenty four hours before the first viral harvest, media was changed and cells were transferred to a 32$^{o}$C incubator to minimize heat-induced viral degradation. To harvest virus, media supernatant was collected every 12 hours, filtered at 0.45 μm, snap frozen and stored at -80$^{o}$C.

To transduce primary BMSCs with Runx2 or empty-vector virus, bone marrow stromal cells were expanded to passage 3, then trypsinized and seeded into T-75 flasks at a density of 5,000 cells/$cm^2$. One day after seeding into flasks, media was replaced with retrovirus supplemented with 0.4 μg/mL polybrene, as previously described (Byers *et al.* 2002). Briefly, flasks were incubated with viral media at 32°C for 15 minutes, then centrifuged at 2500 RPM for 30 minutes. Viral media was then replaced with fresh αMEM + 10% FBS + 1% P/S + 0.3 μg/mL fungizone/amphotericin B, and cells were incubated at 37°C. After 12 hours, a second transduction was performed. Two days after transduction, cells were trypsinized from flasks and seeded onto scaffolds or analyzed for eGFP expression by flow cytometry.

*Cell seeding on scaffolds*

Immediately prior to cell seeding, PCL-collagen scaffolds were pre-wet in PBS, then wicked on Kimwipes to remove excess fluid. Unmodified, Runx2-modified and empty-vector-modified cells (passage 4) were trypsinized and seeded at 500,000 cells per scaffold in 50 μL of media (25 μL per side). Scaffolds were placed in scaffold holders inside 24 well plates and incubated at 37°C to allow cell attachment to the scaffolds. After 30 minutes of incubation, 2 mL of media were added to each well for complete submersion of each scaffold. Media was changed every 3 days.

To ensure equal cell numbers were seeded onto all scaffolds, unmodified BMSCs were seeded onto PCL-collagen scaffolds, as described above, in three separate batches, n=4 scaffolds for each batch. Three days after cell seeding, DNA content on each batch of scaffolds was analyzed via Picogreen staining (Quant-iT™ Picogreen® dsDNA Assay Kit – Molecular Probes) as previously described (Gersbach *et al.* 2004; Phillips *et al.*

2006). Briefly, constructs were rinsed in PBS, then frozen at -80°C. Thawed samples were dried in a Savant DNA120 SpeedVac Concentrator (Thermo Electron Corporation), then digested in 700 μg/mL Proteinase K (Promega) for 48 hours in a 45°C water bath. Picogreen staining was used to quantify average DNA content/cell numbers on each batch of scaffolds. No differences in cell number were observed among groups (data not shown), confirming that cell seeding was reproducible across multiple batches.

*Flow cytometry for transduction efficiency*

Three days after Runx2 or empty-vector transduction, cells were trypsinized (0.05% trypsin/EDTA – Invitrogen) and resuspended in PBS with 10% FBS. Cell suspensions were centrifuged for 5 minutes at 200-300g, resuspended in PBS and then passed through a 40 μm filter (BD Falcon). eGFP fluorescence was measured on a flow cytometer (Becton Dickinson, BD LSR II), and 10,000 events were measured for each sample. Data analysis was performed using WinMDI v.2.8.

*Live/Dead staining*

Cell viability on PCL scaffolds was assessed using the Live/Dead kit (Invitrogen). Unmodified and Runx2 modified cells were seeded onto PCL scaffolds with and without collagen meshes. Three days after seeding, constructs were rinsed 3 times in PBS, then stained with 4 μM calcein and 4 μM ethidium homodimer in PBS for 45 minutes at room temperature. After staining, constructs were rinsed 3 times in PBS, then imaged on a confocal microscope (Zeiss LSM 510 NLO). LSM 5 Image Browser was used to stack groups of 2-dimensional image slices, and image brightness was increased to reduce background.

*Segmental Defect surgery*

Femoral defects were created bilaterally as previously described (Oest *et al.* 2007). Briefly, 13- to 15-week old female Lewis rats were anesthetized using isoflurane, and the hind limbs were shaved and swabbed with cycloheximide and alcohol to prepare the skin for incision. An anterior incision was made from the hip to the knee to allow blunt separation of the quadriceps muscles, exposing the femur (Figure 4.1A). Before the defect was created, a modular fixation device was attached to the bone for mechanical support. The device consisted of two stainless steel plates affixed directly to the bone via screws and one polysulfone plate, which spanned the defect and was attached to the stainless steel plates (Figure 4.1B). Use of this modular system was advantageous for postmortem mechanical testing because the polysulfone plate could be removed before defect testing without removing the screws or stainless steel plates from the bone, thus avoiding any incidental damage to the repair tissue prior to testing. Furthermore, use of a polysulfone plate for support allowed non-invasive in vivo X-ray analysis of defects due to the low X-ray attenuation of polysulfone. After the fixation device was attached, an 8.0-mm segment of bone was removed via bone saw, and a scaffold was press fit into the defect (Figure 4.1A). Notches in the polysulfone plate, spaced 8.0-mm apart, ensured each defect was consistently created the same length (Figure 4.1B). Muscle was closed around the plate and defect using Vicryl sutures, and the skin was closed using sutures and wound clips.

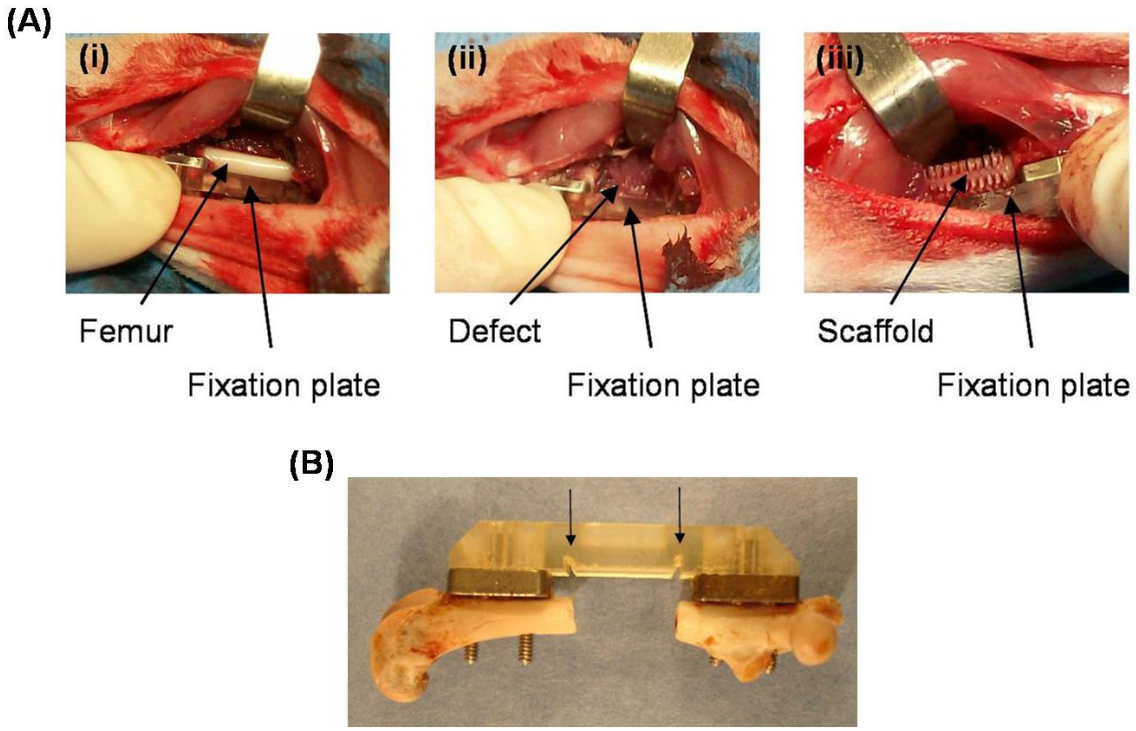


**Figure 4.1.** Surgical procedure for critically-sized segmental defects in rat femurs. (A) Each defect is stabilized by a fixation plate. (i) Blunt dissection of the quadriceps exposes the femur and enables placement of the fixation plate. (ii) An 8.0 mm segment is removed from the femur via bone saw, and (iii) scaffolds are press fit into the defect. (B) An explanted femur shows the modular fixation plate attached to the bone via stainless steel screws. Notches in the polysulfone plate (marked with arrows) are spaced 8.0 mm apart ensuring each defect is created at the same size.

For in vivo studies, five groups were tested, consisting of 4 PCL-collagen scaffold groups each with a different cell condition, and one empty defect control group. For the empty defect group, the surgical procedure remained the same, but no scaffold or cells were placed in the defect. The groups tested were PCL-scaffolds seeded with (i) unmodified BMSCs, (ii) Runx2-modified BMSCs, (iii) empty vector-modified BMSCs or (iv) no cells and (v) empty defect control. These 5 groups are outlined in Table 4.1.

**Table 4.1.** Outline of experimental groups for in vivo segmental defects.

| Group Name | Defect contains: |
|---|---|
| Empty Defect | No scaffold and no cells |
| *The following groups all contain a PCL-collagen scaffold seeded with:* | |
| PCL-col | No cells |
| Empty Vector | Empty vector-modified BMSCs |
| BMSC | Unmodified BMSCs |
| Runx2 | Runx2-modified BMSCs |

Following surgery, animals were given 3 daily doses of buprenorphine at 0.03 mg/kg for 2 consecutive days and 3 doses of 0.01 mg/kg on the third day to control pain. Animals were monitored daily for signs of pain and distress, progress of wound closure, regular eating habits and normal ambulation. A small percentage (< 8%) of rats developed infections in or around the surgery site, or experienced mechanical failure of the fixation device. These animals were removed from the study and euthanatized, and any data collected from these animals was excluded. Two weeks post surgery, when skin wounds had completely healed, animals were anesthetized with isoflurane and wound clips were removed. At 4, 8, and 12 weeks post-surgery, animals were anesthetized with isoflurane and the hind legs were scanned via radiography and microCT as described below. Twelve weeks post-surgery, animals were euthanized by $CO_2$ inhalation, and the femurs, along with surrounding muscle tissue, were harvested for postmortem microCT evaluation, histology, FTIR analysis and mechanical testing.

*Radiography and MicroCT Analysis*

Every 4 weeks following surgery, 2-dimensional X-ray images of each sample were non-invasively obtained using an MX-20 Specimen Radiography System (Faxitron X-ray Corporation) to make gross morphological observations of bone formation in each defect site. For X-ray analysis, animals were anesthetized in a gas chamber filled with 5% isoflurane and maintained under anesthesia using 2% isoflurane flow into a face mask. Each hind leg of anesthetized animals was scanned for 15 s with an X-ray beam energy of 23 kV.

In addition to radiographic imaging, samples were non-invasively analyzed every 4 weeks post-surgery by microCT using a vivaCT 40 (Scanco Medical) to quantify bone volume in each defect site. For microCT, animals were anesthetized in the same manner as for radiography and placed in a rodent holder with one leg outstretched for scanning. The defect area in between the stainless steel plates of the fixation device was imaged with an X-ray beam energy of 55 kVp and intensity of 109 μA, and the integration time was 200 ms. Scanning resolution was 38 μm. After imaging was complete, noise was reduced from 3-dimensional reconstructions of each scan by applying a Gaussian filter (sigma=1.2, support=2) using the Scanco Medical μCT Evaluation Program. Images were thresholded at 270 mg HA/ccm to isolate mature bone from soft tissue and the polymer scaffold and polysulfone plate. Bone volume was quantified using directly computed values.

*Histological Analysis*

Following euthanasia, samples for histological analysis were fixed in 10% neutral buffered formalin immediately after harvest. One day after fixation, soft tissue was removed, and specimens were placed in fresh formalin. Prior to embedding, fixed tissues were scanned ex vivo in formalin via microCT as described above to allow matching of histological sections with microCT slices. After scanning, specimens were dehydrated in a series of alcohols, cleared in xylene, and embedded in methyl methacrylate (MMA). Ground sections, 50-80 μm thick, were prepared by Wasatch Histo Consultants, Inc. (Winnemucca, NV) and stained using Sanderson's Rapid Bone Stain™ and a van Gieson counterstain (Reyes *et al.* 2007). Stained histological sections were then matched to thresholded microCT scans to confirm that microCT analysis was representative of mature bone.

*FTIR spectroscopy*

Explanted samples for FTIR analysis were wrapped in PBS-soaked gauze and frozen at -20$^{o}$C until use. Upon thawing, chips of mineralized tissue were removed from the defect area of each sample using a bone cutter. Care was taken to remove only newly formed mineralized tissue in the defect area and no native host bone. Mineral chips were fixed in ethanol, then dried overnight at 50$^{o}$C, ground with a mortar and pestle, pressed into KBr pellets, and read on a Nexus 470 FT-IR (Thermo Nicolet) using 64 scans at 4 cm$^{-1}$ resolution. Native bone from age-matched Lewis rat femurs were used as positive controls for FTIR bone spectra. Mineral recovered from the defect region of empty defect samples was used a negative control.

*Mechanical testing*

Samples for mechanical testing were explanted and wrapped in PBS-soaked gauze and frozen at -20$^{o}$C until use. Mechanical testing was performed as previously described (Oest *et al.* 2007). Briefly, samples were thawed in room temperature PBS and most of the soft tissue was removed, taking care not to mechanically disrupt tissue in the defect site. The ends of each bone were potted in Wood's metal up to the polysulfone plate and secured with pins into potting blocks. Blocks were loaded onto an ElectroForce® mechanical testing machine (Elf 3200 by Bose) and the polysulfone plate was removed just before testing. Samples were loaded in torsion at a displacement rate of 3$^{o}$/s up to 360$^{o}$. Maximum torque before failure was recorded for each sample.

*Statistical Analysis*

Data from two independent studies was pooled, and a mixed model ANOVA was used to define experiment and individual animal as sources of error. This analysis was performed using the Hierarchical Linear Mixed Models function in Systat v12, which uses a Satterthwaite approximation to account for differences in sample sizes. A p-value $< 0.05$ was considered significant.

**Results**

*Runx2 expression and cell viability*

To measure transduction efficiency of the Runx2 and empty-vector retroviruses, eGFP expression was measured via flow cytometry. Unmodified cells were used as a control population, and transduction efficiency of retrovirus-transduced cells was determined using the 2% of background method (Overton 1988). Runx2-modified cells

showed 40% transduction efficiency as measured by eGFP expression compared to control cells (Figure 4.2B). Empty vector-transduced cells showed 60% transduction efficiency (data not shown).

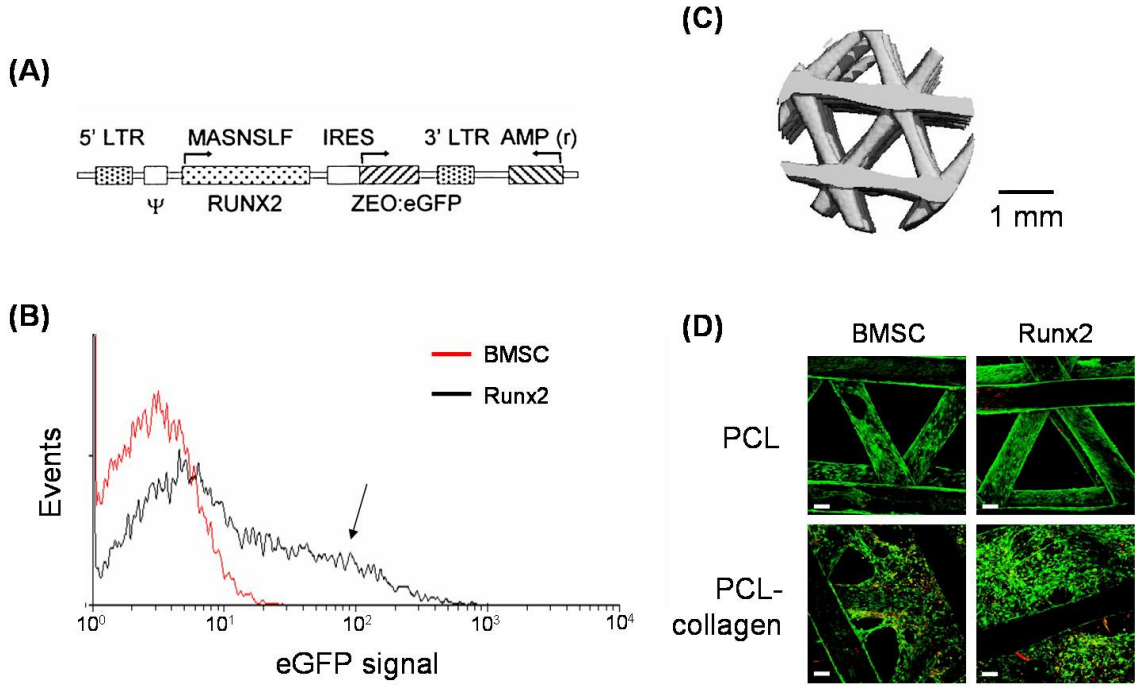


**Figure 4.2.** Runx2-modified BMSCs show high eGFP expression at 3 days post-transduction and are viable on PCL scaffolds containing lyophilized collagen mesh. (A) Diagram of Runx2 plasmid showing internal ribosomal entry site (IRES) for eGFP expression. (B) Flow cytometric detection of eGFP expression in unmodified and Runx2-modified BMSCs. Transduction efficiency of Runx2-modified cells (arrow) is 40% compared to unmodified controls. (C) Cross-sectional microCT image of a PCL scaffold. (D) Confocal live/dead images of unmodified and Runx2-modified BMSCs on PCL scaffolds with and without collagen meshes. Live cells are shown in green and dead cells in red. Scale bar is 200 μm. Cells populate the pore volume of the PCL in (C) by adhering to collagen meshes lyophilized inside the scaffold.

PCL scaffolds without collagen meshes were analyzed by microCT to determine average pore volume and pore size (Figure 4.2C). Live/Dead staining of cells on scaffolds 3 days post-seeding shows unmodified and Runx2-modified BMSCs are viable on PCL scaffolds both with and without collagen meshes. Scaffolds with collagen meshes promote an even distribution of cells throughout the pore volume of the PCL

while scaffolds without meshes do not retain cells in the pore volume (Figure 4.2D). PCL scaffolds with collagen meshes were subsequently used for all in vivo studies.

*Radiography and MicroCT Analysis*

Immediately following surgery, animals were monitored several times daily for signs of pain or stress, regular eating habits, and normal ambulation. Within one week following surgery, signs of stress were minimal, regular eating had returned and normal ambulation using both hind limbs was restored.

To monitor bone formation in critically-sized defects, animals were anesthetized every 4 weeks and defects were scanned via X-ray and microCT. X-ray images show gross morphological changes in bone growth at the defect site over time. While empty defects, PCL and BMSC groups showed minimal bone formation, Runx2 showed substantial increases in bone growth over time (Figure 4.3). Representative X-ray images from each group are shown in Figure 4.3A, and the corresponding three-dimensional microCT reconstructions at 12 weeks are shown in Figure 4.3B. No differences were observed between BMSC and empty vector groups (data not shown).

At 4, 8 and 12 weeks post-surgery, bone volume in all defects was quantified via microCT. Negligible bone formation occurred at the ends of host bone in empty defects and PCL defects. However, bone formation in BMSC and Runx2 defects increased over time. At 4 and 8 weeks post-surgery, Runx2 defects contained significantly more bone than unmodified BMSC defects ($p < 0.05$, $n=8$). At 12 weeks, bone volume in Runx2 defects was not significantly different compared to unmodified BMSC defects ($p = 0.059$) (Figure 4.3C). This differential time course of bone formation in BMSC and Runx2 defects indicates that Runx2-modified BMSCs initially accelerate bone formation

in critically-sized defects but that the inherent osteogenic capacity of unmodified BMSCs eventually produces a similar level of bone as Runx2-modified BMSCs.

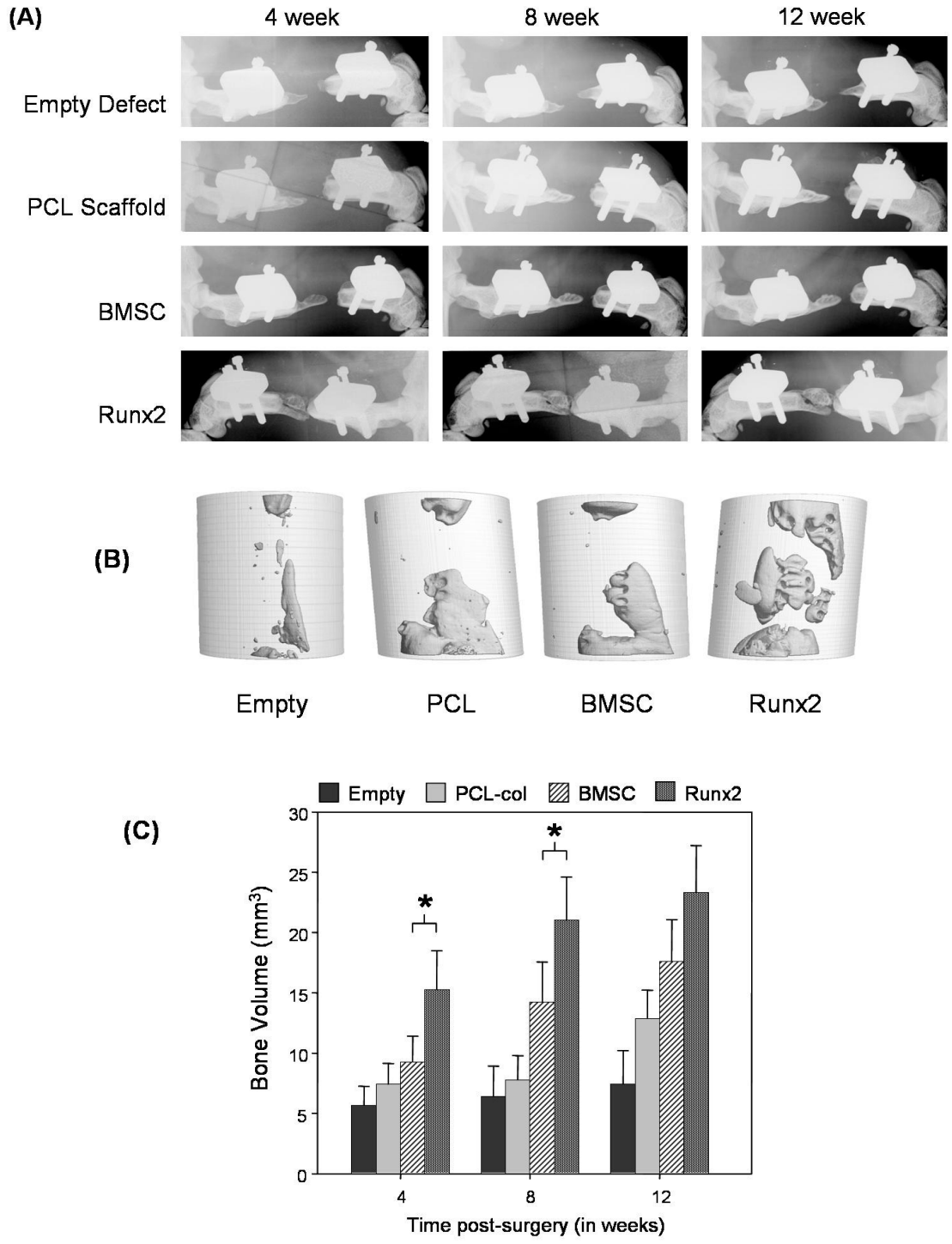


**Figure 4.3.** Runx2-modified BMSCs accelerate bone formation in critically-sized defects compared to unmodified BMSCs. (A) X-ray images showing representative images for each group. (B) MicroCT images showing the same samples from (A). (C) Bone volume is significantly greater in Runx2-treated defects compared to BMSC-treated defects at 4 and 8 weeks (* $p < 0.05$). At 12 weeks, bone formation due to unmodified BMSCs is not significantly different from Runx2-modified cells ($p=0.059$), indicating that Runx2-modified cells initially accelerate healing but unmodified cells produce similar levels of bone at late time points.

*Histological Analysis*

To more fully characterize areas of high attenuation imaged by microCT, one sample from the Runx2 defect group was subjected to histological analysis at 12 weeks. Prior to embedding, the sample was scanned via microCT and thresholded in the same manner as all other samples. Then, Sanderson's Rapid Bone Stain™, which distinguishes areas of mineralized bone from demineralized connective tissue and osteoid, was applied to the sample revealing mineralized bone tissue in a red/pink color and demineralized osteoid in blue/green (Sanderson and Bachus 1997). Because this sample underwent both Sanderson's stain and microCT scanning, matching slices from histology and microCT were compared. This analysis shows that areas of high attenuation that were thresholded in microCT and used as a measure of bone volume in Figure 4.3, directly match areas of red/pink staining defined as mineralized bone tissue in the Sanderson's stain (Figure 4.4).

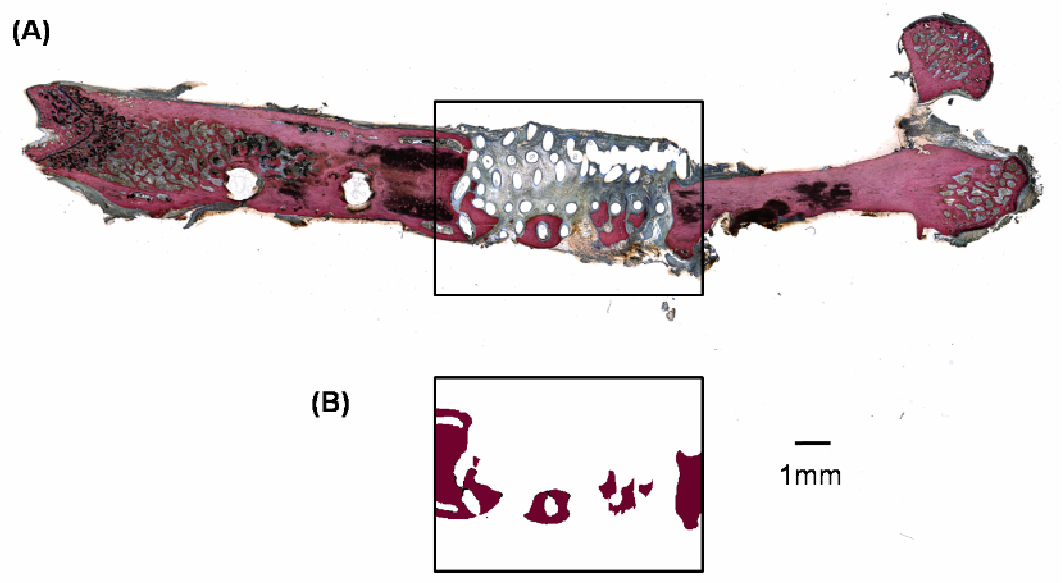


**Figure 4.4.** Histological analysis confirms that areas of high attenuation revealed by microCT are bone. (A) Sanderson's rapid bone stain for a Runx2-modified BMSC sample showing bone in red/orange and soft tissue in blue/green. (B) A corresponding 2D microCT slice from the same sample, thresholded to isolate areas of high attenuation within the sample. MicroCT analysis shows high correlation with Sanderson's bone stain.

*FTIR Spectroscopy*

To determine the composition of bone formed in all defects, samples of bone taken from the defect area in all groups were analyzed via FTIR analysis. Spectra from native bone contains all peaks expected for biologic apatite, including amide peaks for protein at 1700 and 1550 $cm^{-1}$, a small carbonate peak at 900 $cm^{-1}$, a broad phosphate peak for stretching vibrations at 900 – 1200 $cm^{-1}$, and a phosphate doublet for bending vibrations at 525 – 625 $cm^{-1}$ (Paschalis *et al.* 1997; Bonewald *et al.* 2003; Byers and García 2004). Cell-loaded samples having either unmodified BMSCs or Runx2-modified BMSCs displayed all of these expected peaks (Figure 4.5), indicating that the mineralized tissue in these defects was a biological, poorly crystalline hydroxyapatite. Cell-free PCL scaffolds containing lyophilized collagen also displayed all expected peaks for native bone; however, $CO_3$ and $PO_4$ (bending) peaks were less prominent in these cell-free samples as compared to native control bone or cell-loaded samples. Finally, empty defect negative controls showed amide peaks and some phosphate deposits; however, $CO_3$ peaks and prominent $PO_4$ (stretching) peaks were not present, indicating that the mineral deposited in these samples did not have the chemical composition of native bone.

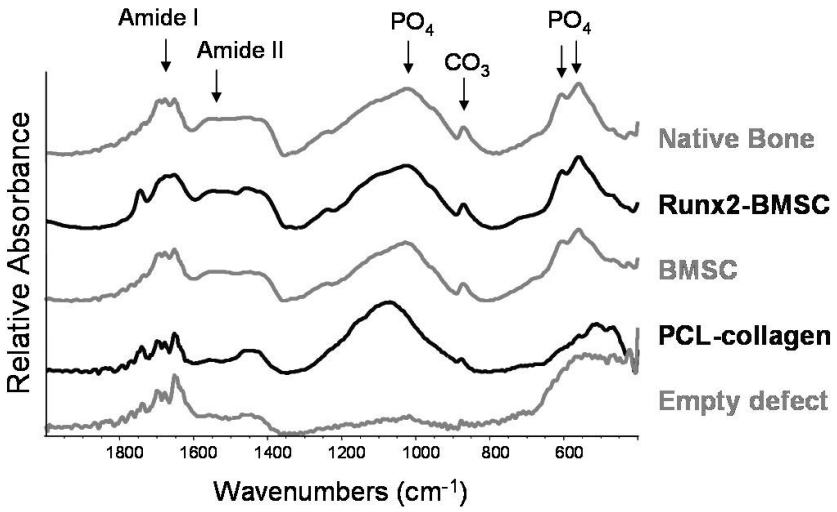


**Figure 4.5.** FTIR spectra demonstrate that the structural composition of cell-mediated bone formation is similar to that of native bone. Bands characteristic of biologic hydroxyapatite, namely a small carbonate peak at 855 – 890 $cm^{-1}$, a broad phosphate peak at 900 – 1200 $cm^{-1}$, and a phosphate doublet at 525 – 625 $cm^{-1}$, are present in Runx2-modified and unmodified BMSC-treated defects, as well as native bone. Cell-free scaffold spectra contain peaks that are shifted compared to native bone, and empty defects do not contain characteristic peaks.

*Mechanical strength of repaired defects is dependent on bridging*

To assess mechanical functionality of new bone present in critically-sized defects, femurs were harvested 12 weeks post-surgery and subjected to postmortem torsional testing. Although microCT revealed differences in bone volume between BMSC and Runx2 defects at 4 and 8 weeks, maximum torque sustained at 12 weeks was not significantly different between these two groups (Figure 4.6A). Stiffness and work to failure were also evaluated and no significant differences among experimental groups were observed (data not shown). We hypothesized that mechanical strength is dependent on defect bridging. Whereas some samples contain a large amount of bone in the defect site, high levels of mechanical strength are only present when this bone is firmly attached

to both the proximal and distal end of the host bone. Without full attachment (i.e. complete bridging) samples that have large bone volumes sustain low torque loads.

To demonstrate that mechanical strength is dependent on defect bridging, X-ray images for all samples were blindly assessed for their extent of bridging and assigned a bridging score from 0-5 based on the criteria outlined in Figure 4.6B, where 0 is no bone in the defect and 5 is a fully bridged defect. Representative X-ray images for each score are shown in Figure 4.6C. By looking at bone volume versus maximum torque for each sample grouped by briding scores, a trend is observed where samples with higher bridging scores generally have greater max torque (Figure 4.6D). Although there are no significant differences in torsional strength between BMSC and Runx2 defects, a distribution of bridging scores from each group shows that Runx2-modified defect scores are shifted towards fully bridged or nearly fully bridged (scores 4 and 5) compared to unmodified BMSC scores (Figure 4.6E). A Kruskall Wallis one way ANOVA by ranks shows that this shift is significant ($p < 0.05$).

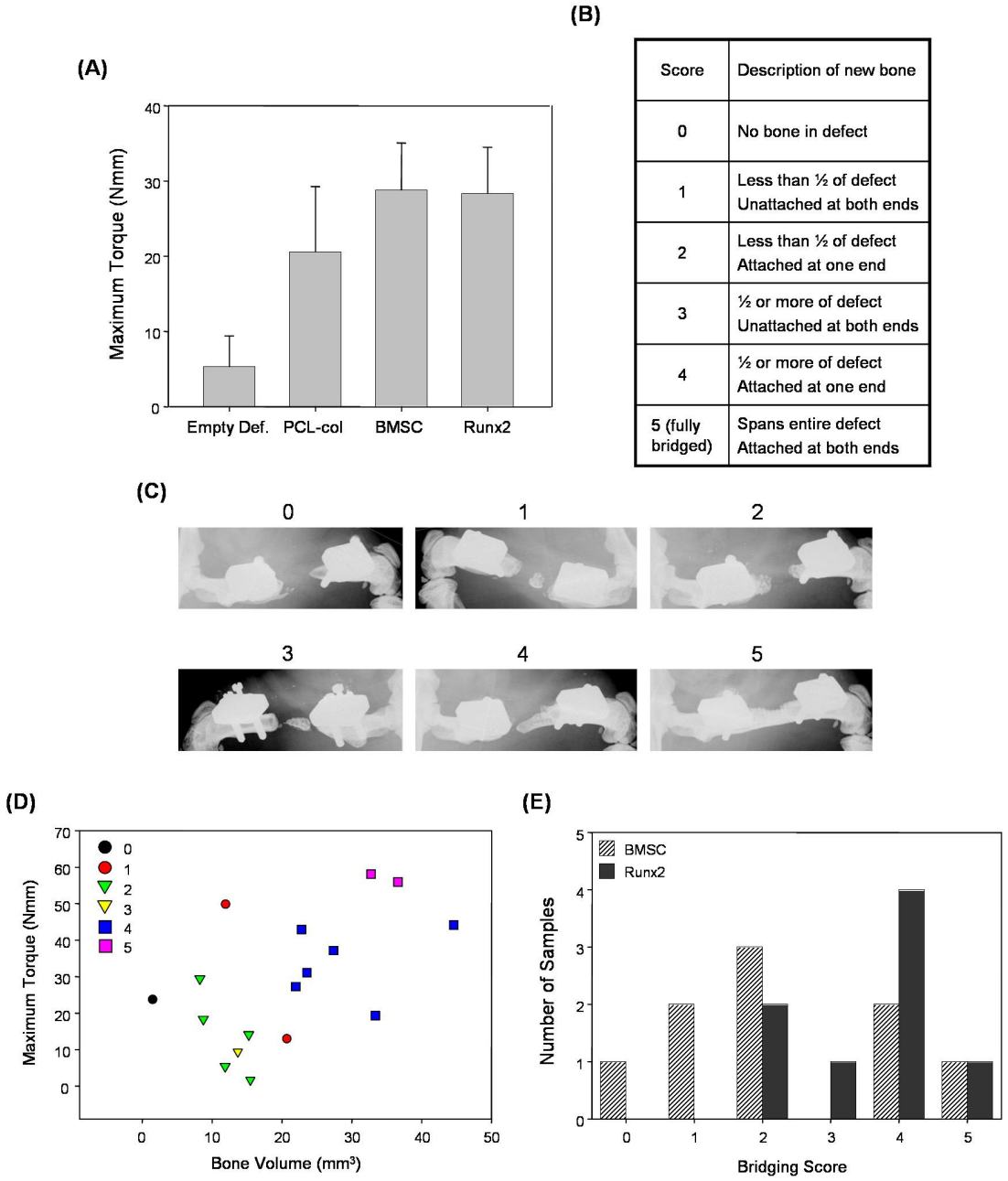


**Figure 4.6.** Mechanical properties of repaired segmental defects. (A) Despite differences in bone volume between Runx2-treated and BMSC-treated defects, maximum torque is not significantly different. (B) Criteria for bridging scores assigned to each sample. (C) Representative faxitron images of scores described in (B). (D) Samples with higher bridging scores are shifted to the upper right of a bone volume versus maximum torque correlation graph. (E) The distribution of bridging scores for Runx2-treated defects is shifted righted compared to that for BMSC-treated defects ($p < 0.05$).

**Discussion**

This study examined the effects of treating critically-sized segmental defects in rat femurs with cell/scaffold constructs containing Runx2-modified BMSCs. In comparison to unmodified BMSCs on scaffolds, scaffolds alone, or empty defects, we demonstrate that bone healing in rat femurs is accelerated by treatment with Runx2-modified BMSCs. At both 4 and 8 weeks post-surgery, quantitative microCT analysis showed significantly more bone formation in defects treated with Runx2-modified BMSCs versus unmodified BMSCs, indicating accelerated healing due to Runx2 treatment at early time points. However, at 12 weeks post-surgery, no statistical differences in bone formation between unmodified and Runx2-modified BMSCs were present, highlighting the inherent osteogenic capabilities of this cell type. Taken together, this study presents an accelerated method of tissue engineering for healing large bone defects in vivo.

Runx2 has been described as a molecular switch for osteoblast biology (Ducy 2000), yet few studies have examined the potential use of Runx2 in healing critically-sized bone defects. Three previous orthotopic analyses of Runx2-modified BMSCs have been performed and each one focused on healing critically-sized calvarial defects (Zheng *et al.* 2004; Byers *et al.* 2006; Zhao *et al.* 2007). Although the cranial defect model provides relevant information regarding the healing of craniofacial defects, conflicting evidence regarding the role of periosteum, dura mater, and surrounding healthy bone tissue in cranial defect healing makes separation of host response from treatment effect difficult in this model (Aalami *et al.* 2003). In particular, dura mater has been shown to have a significant effect on cranial defect healing (Ozerdem *et al.* 2003). To our

knowledge, no other study to date has examined the use of Runx2 as a gene therapy strategy for the healing of critically-sized segmental defects in long bones. The present study examined Runx2 treatment in a segmental defect model, where contributions from the dura mater, which are irrelevant to anatomical locations other than the calvaria, are not present, making this a more rigorous test bed for the healing of large bone defects.

In addition to differences in anatomical location between this study and other orthotopic studies of Runx2, factors such as species, time points, and scaffold types also varied between the studies. Zheng *et al.* implanted adenovirally transduced Runx2-BMSCs on collagen sponges for 4 weeks in BALB/c mice. Using manual segmentation of radiographic images, they found significantly more bone in defects treated with Runx2 transduced cells over unmodified cells, cell-free scaffolds and empty defects (Zheng *et al.* 2004). Zhao *et al.* used adenoviral transduction to deliver Runx2 and LacZ (control) to BMSCs. Cells were implanted on gelatin sponges for 7 weeks in C57BL6 mice, and CT quantification showed more bone in defects treated with Runx2 over LacZ. However, no cell-free or empty defect controls were included in this study (Zhao *et al.* 2007). Our group has also previously reported on Runx2 treatment of cranial defects. We have investigated BMSCs retrovirally tranduced with Runx2 and implanted on PCL scaffolds for 4 weeks in the rat calvarium. MicroCT analysis showed more bone in Runx2 treated defects, compared to unmodified controls and empty defects, following a 21 day pre-culture period. However, due to the open pore structure of our PCL scaffolds, cell-free scaffolds performed as well as Runx2-engineered cell-loaded scaffolds in this study (Byers *et al.* 2006). Given these differences, all 3 previous orthotopic studies comparing Runx2 transduced BMSCs to unmodified or LacZ transduced BMSCs showed a

significant increase in bone formation due to Runx2 treatment, in agreement with the present study.

Although Runx2-modified BMSCs accelerated bone repair in critically-sized defects in this study, unmodified BMSCs eventually produced equivalent amounts of bone, pointing to the inherent ability of BMSCs to mineralize bone defects. Many studies have demonstrated successful healing of bone defects using unmodified BMSCs in pre-clinical trials (Bruder *et al.* 1998; Kon *et al.* 2000; Petite *et al.* 2000), and recently, a pilot study of the implantation of autologous human BMSCs for repair of large bone defects in humans was reported by Marcacci *et al.* 2007. In this study, 4 patients presented with large bone diaphysis defects, for which previous treatment with conventional surgical therapies had failed. Each patient underwent a BMSC harvest from the iliac crest, and the isolated cells were then cultured and seeded onto hydroxyapatite scaffolds custom made to fit the size and shape of the defects. For patients 1, 2, and 4, complete consolidation between the implant and host bone was radiographically evident between 5-7 months post-surgery, at which time external fixation devices were removed. For these patients, limb function was gradually regained within 8 months post-surgery. For patient 3, whose injury was more complex and involved the elbow joint, a custom made cast was fitted over the defect after removal of the Ilizarov apparatus at 8 months post-surgery. This patient recovered limb function after 16-24 months post-surgery. For all patients, a 6-7 year follow-up revealed that stable bone-implant integration was maintained (Marcacci *et al.* 2007). The success of this study makes treatment with autologous BMSCs an attractive option for patients with large bone defects. However, a

reduction in the time needed for external fixation is desirable, making the accelerated healing strategy presented in the current study relevant for clinical application.

The success of unmodified BMSCs for healing critically-sized defects in other pre-clinical studies may largely depend on the scaffold type used to deliver the cells. For example, all of the aforementioned studies which demonstrate successful healing (i.e. complete bridging) of segmental defects when treated with unmodified BMSCs employed the use of a hydroxyapatite scaffold to deliver the cells to the defect site. The osteoconductive properties of hydroxyapatite have been reviewed elsewhere (LeGeros 2002). In brief, calcium phosphate ceramics demonstrate bioactivity and osteoconductivity leading to rapid and strong osseointegration with host bone tissue when implanted into an orthotopic site. In fact, implantation of HA alone (without cells) has been shown to heal osteotomy defects in human patients (Meyer *et al.* 2007). In the current study, the use of a polymer scaffold, which is not osteoconductive by itself, in combination with the rigorous 8mm segmental defect test bed, may explain why defects treated with unmodified BMSCs resulted in non-union after 12 weeks. This result is in agreement with other studies that also show non-union of segmental defects treated with unmodified BMSCs when a non-ceramic scaffold is the delivery vehicle (Turgeman *et al.* 2001; Fialkov *et al.* 2003). Recent evidence suggests that the modification of synthetic PCL scaffolds with hydroxyapatite nanoparticles increases the bone forming response of cells seeded on the scaffolds (Wutticharoenmongkol *et al.* 2007). In this study, the rationale behind including a collagen matrix within PCL scaffolds was to increase the therapeutic load of cells delivered the defect site. It is possible that incorporation of an osteoconductive material into our scaffolds would further enhance the cellular response.

However, in this study, the use of a collagen-PCL scaffold, which did not heal defects in the cell-free condition, allowed us to demonstrate the effect of Runx2 treatment without contributions from the scaffold.

A distinct advantage of this study over many reports of segmental defect healing is the use of mechanical testing to assess functionality of the defects. A large majority of studies that report successful healing of bone defects rely solely on a combination of X-ray analysis and histological evaluation as a measure of defect healing. However, this approach does not provide functional information, which is necessary to fully evaluate the success of a given treatment strategy for bone healing (Liebschner 2004). Few groups report mechanical testing analysis, but of those that do, the most common test methods are torsion (Cook *et al.* 1994; Cook *et al.* 1994; Cook *et al.* 1995; Hsu *et al.* 2007; Oest *et al.* 2007; Rai *et al.* 2007). The current study demonstrates the importance of including mechanical testing analysis in any bone healing study because, in this case, the significant differences in bone volume measured by microCT did not translate to significant differences in torsional strength between Runx2 and BMSC treated defects. However, we did observe differences in torsional strength between fully bridged samples compared to non-unions. This result is in agreement with a mounting body of work suggesting that greater bone mass will not necessarily result in greater bone strength. For example, recent evidence suggests that patients with a high level of bone turnover may be at higher risk for bone fracture regardless of bone mineral density (Hernandez 2008). This concept is known as bone quality, a term used to describe any skeletal aspect of bone, excluding bone mass (i.e. bone shape, size, trabecular connectivity, etc) that affects its strength (Hernandez and Keaveny 2006). In the present study, although significant

increases in bone mass were observed due to Runx2 treatment, bone quality was not affected. The implication of these results is that radiographic and histological methods of evaluating bone tissue are not sufficient to fully characterize the extent of bone defect healing. Functional mechanical evaluation of bone defects should be used in conjunction with other methods for complete analysis of both bone mass and bone quality.

In summary, we have demonstrated accelerated bone healing in critically-sized defects in rat femurs due to treatment with Runx2-modified BMSCs delivered on synthetic polymer scaffolds. This strategy targets a specific osteoblastic signaling pathway to upregulate osteoblastic differentiation of BMSCs to promote bone formation, and may shorten the long time scale required for complete healing of defects with unmodified BMSCs. Further investigation into increasing functional strength is warranted. With further development, this gene therapy technique could be a useful strategy for healing large bone defects in humans.

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# CHAPTER 5

# SIMPLE COLLAGEN-MIMETIC SURFACE MODIFICATION INCREASES BONE FORMATION IN CRITICALLY-SIZED RODENT FEMORAL DEFECTS

## Introduction

Limitations with current clinical bone grafting procedures, namely autografts and allografts, have led to the development of alternative methods of bone defect healing via regenerative medicine strategies (Hutmacher and García 2005; Awad et al. 2007; Cancedda et al. 2007). Most tissue engineering strategies can be broadly categorized as osteoinductive or osteoconductive, whereby a tissue engineering construct *stimulates differentiation* of host cells to subsequently form bone or *promotes attachment* of bone forming cells, respectively (De Long et al. 2007). The first well documented study of bone formation by osteoinduction was described by Marshall Urist in 1965 by the implantation of demineralized bone matrices into ectopic sites in rabbits and rodents (Urist 1965). From this experiment came the discovery and characterization of bone morphogenetic proteins (BMPs), soluble growth factors that work through Smad-dependent signaling pathways to promote osteoblastic differentiation (Derynck and Zhang 2003). More than 15 individual BMPs have now been identified and several studies have shown that BMP-2 and BMP-7 promote healing of critically-sized bone defects in both small and large animals (Yasko *et al.* 1992; Cook *et al.* 1994; Cook *et al.* 1994; Cook *et al.* 1995) and in humans (Govender *et al.* 2002). Additionally, BMP-7 is

currently FDA-approved for human use in long bone non-unions where autograft treatment is not feasible or has failed, and BMP-2 is available for clinical use in the treatment of open tibial fractures and for lumbar spinal fusion (Boden 2005; Bishop and Einhorn 2007).

Although the use of BMPs in clinical repair of bone defects has demonstrated success, several factors contribute to the continued search for regenerative medicine alternatives. First, the residence time of BMP in a defect site following localized delivery directly affects its osteogenic potential. As a soluble factor, diffusion of BMP away from the delivery site decreases the amount of bone formed in an implant (Uludag *et al.* 2000). Efforts are being made to increase BMP residence time at the defect site, by the immobilization of BMP onto implants (Yamachika *et al.* 2009). Second, the dosage of BMPs required to stimulate bone formation in large bone defects in humans far exceeds the successful working dosage described in preclinical trials. Thus, production of enough recombinant protein for one vial of BMP for human use is a very costly procedure (Alt and Heissel 2006; Bishop and Einhorn 2007; Cancedda *et al.* 2007). Clinical bone healing would benefit from a more targeted and cost effective solution.

Recently, the use of biomimetic strategies which attempt to recapitulate the environment of the bone ECM have been used to promote osseointegration and bone formation in vivo (Hubbell 2003). Short immobilized peptide sequences and fragments derived from ECM proteins, such as the RGD sequence from fibronectin, have been used to promote cell attachment to titanium implants as an improved method of implant fixation and osseointegration (Ferris *et al.* 1999; Bernhardt *et al.* 2005; Elmengaard *et al.*

2005; Reyes *et al.* 2007; Petrie *et al.* 2008). These strategies take advantage of the specific interactions between ECM protein ligands and integrin cell surface receptors.

The integrin receptors play a crucial role in cell attachment and ECM-mediated cell signaling. Integrin dimers, consisting of one α and one β subunit, bind to specific sites contained within ECM proteins, thereby promoting cell attachment, migration, mechanotransduction, differentiation, and numerous other cell functions (Clark and Brugge 1995; Hynes 2002). In particular, type I collagen-mediated activation of the $\alpha_2\beta_1$ integrin receptor promotes osteoblastic differentiation of bone marrow stromal cells (BMSCs) and pre-osteoblastic cells in vitro leading to the production of mineralized matrices on 2-dimensional surfaces (Jikko *et al.* 1999; Mizuno *et al.* 2000). The $\alpha_2\beta_1$ receptor is highly expressed on the surface of osteoblastic cells (Gronthos *et al.* 1997), and it binds specifically to the triple helical hexapeptide domain, GFOGER, contained within collagen I (Knight *et al.* 2000). We have previously demonstrated that isolation of this active binding sequence, GFOGER, via synthetic fabrication of a triple helical GFOGER peptide, allows engineered control over osteoblastic differentiation of cells on GFOGER coated 2D surfaces (Reyes and García 2003; Reyes and García 2004). Furthermore, use of GFOGER as an implant coating for titanium tibial plugs enhances osseointegration in vivo to levels greater than that of unmodified titanium or titanium coated with full length collagen I (Reyes *et al.* 2007). This collagen mimetic strategy for osseointegration specifically targets osteoblastic cells through $\alpha_2\beta_1$-mediated signaling by eliminating extraneous binding sites contained in the full length protein. Furthermore, the synthetically derived peptide is inexpensive, easy to fabricate and poses no risk of disease transmission.

In this study, we examined the effects of GFOGER surface modification on the healing of critically-sized bone defects in rats. We hypothesized that the specific targeting of $\alpha_2\beta_1$ receptors in orthotopic segmental bone defects via GFOGER-coated polymer scaffolds would increase bone formation in critically-sized defects compared to uncoated scaffolds. The fabrication of synthetic peptide sequences provides an inexpensive alternative to costly methods of bone tissue engineering, such as those employing BMPs or using cells. This simple surface modification strategy, which imparts specific biologic functionality to synthetic implants represents an elegant yet facile procedure for future clinical bone healing.

## Materials and Methods

### *GFOGER synthesis*

The synthetic peptide GGYGGGPC$(GPP)_5$GFOGER$(GPP)_5$GPC, where O is hydroxyproline, was fabricated by the Emory University Microchemical Facility using stepwise solid-phase procedures, as previously described (Reyes and García 2003). This peptide has a triple helical conformation (Figure 5.1A), which mimics the structure of collagen I and is essential for peptide bioactivity (Knight *et al.* 2000). The purified peptide was stored as a TFA salt at -20$^{o}$C. For short term storage, the peptide was reconstituted to 10 mg/mL in 0.1% TFA and 0.01% sodium azide and stored at 4$^{o}$C. For all surface coating experiments, the reconstituted peptide solution was further diluted in PBS.

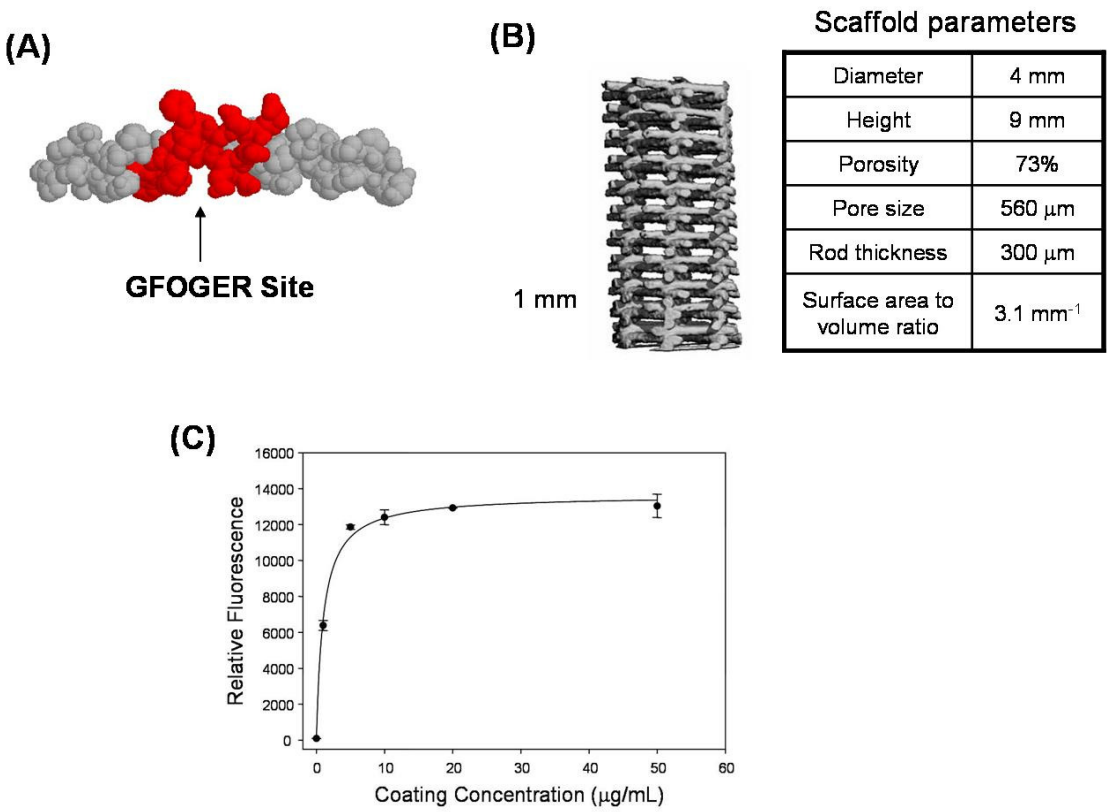


**Figure 5.1.** The synthetic peptide fragment, GFOGER, is passively adsorbed to PCL scaffolds and saturates the surface. (A) Space filling model of the GFOGER molecule shows its triple helical, collagen mimetic conformation. (B) MicroCT is used to characterize the structure of PCL scaffolds. Scaffold parameters for the first in vivo study are listed. (C) GFOGER surface saturation on PCL scaffolds occurs at a coating concentration of 20 μg/mL. Biotinylated GFOGER was adsorbed onto PCL scaffolds and detected via ELISA. Error bars represent standard deviation, n=3.

*PCL scaffold fabrication and coating with GFOGER*

Polycaprolactone (PCL) scaffolds were produced in sheets 9 mm thick by fused deposition modeling as previously described (Zein et al. 2002). Sheets were cut into scaffolds using a 4 mm diameter dermal biopsy punch (Miltex), and microCT analysis was used to characterize the structural parameters of the scaffolds (Figure 5.1B). For the first in vivo study, scaffold pore volume was 73% of the total volume, and pore size was 560 μm.

For passive adsorption of GFOGER onto PCL scaffold surfaces, scaffolds were cleaned in 70% ethanol for 30 minutes, rinsed in sterile dd$H_2O$ 3 times, then soaked in PBS for 10 minutes prior to peptide coating. For in vivo studies, an additional step was added for endotoxin removal, which involving rinsing scaffolds in 70% ethanol for 4 days on a shaker plate with daily ethanol replacement. To coat with GFOGER, scaffolds were removed from PBS, wicked on Kimwipes, and then added to a solution of 50 μg/mL of GFOGER in PBS for 2 hours at room temperature. Uncoated scaffolds received the same treatment, but were kept in PBS without GFOGER. Prior to implantation, scaffolds were rinsed briefly in PBS to remove any unbound peptide.

*Biotinylation and detection of GFOGER peptide*

The carboxyl end of the GFOGER peptide was biotinylated using an EZ-Link® Amine-$PEG_3$-Biotin kit (Pierce Biotechnology) to allow detection of the peptide after adsorption onto PCL scaffolds. Briefly, reconstituted GFOGER peptide was added to MES buffer, mixed with Amine-PEG-Biotin solution and EDC according to manufacturer's instructions, then incubated with shaking for 3.5 hours at room temperature. Unreacted biotin was removed via dialysis overnight into PBS using a Slide-A-Lyzer Dialysis Cassette with a molecular weight cut off of 3500 (Thermo Scientific). After dialysis, protein concentration was measured using a BCA Protein Assay kit (Pierce Biotechnology).

For in vitro detection of biotinylated GFOGER on PCL scaffolds, a modified ELISA against biotin was performed. Briefly, scaffolds were coated with biotinylated GFOGER, rinsed thoroughly in PBS, wicked on Kimwipes, then blocked in 0.25% heat denatured BSA with 0.0005% Tween 20, 1 mM EDTA, and 0.025% $NaN_3$ in PBS for 1

hour at 37°C. After blocking, scaffolds were rinsed rigorously in PBS, incubated in a 1:2000 dilution of anti-biotin antibody conjugated to alkaline phosphatase (clone BN-34, Sigma) for 1 hour at 37°C, rinsed again rigorously, then incubated with 60μg/mL of 4-methylumbelliferyl phosphate (MUP) substrate in diethanolamine buffer (pH 9.5) for 1 hour at 37°C. Fluorescent signal was measured by transferring 100 μL from each scaffold to the well of a u-bottom black plate and reading excitation/emission spectra at 360/465 nm on a HTS 7000 Plus Bio Assay Reader (Perkin Elmer). Uncoated PCL scaffolds served as negative controls and were incubated in PBS without GFOGER, then rinsed and blocked following the same procedure as GFOGER-coated scaffolds. Substrate-only negative controls were coated with GFOGER but excluded use of the anti-biotin antibody.

*Segmental Defect Surgery*

Femoral defects were created bilaterally as previously described (Oest et al. 2007). Briefly, 13 to 15 week old female Lewis rats were anesthetized using isoflurane, and the hind limbs were shaved and swabbed with cycloheximide and alcohol to prepare the skin for incision. An anterolateral incision was made from the hip to the knee to allow blunt separation of the quadriceps muscles, exposing the femur (Figure 5.2A). Before the defect was created, a modular fixation device was attached to the bone for mechanical support. The device consisted of two stainless steel plates affixed directly to the bone via screws and one polysulfone plate, which spanned the defect and was attached to the stainless steel plates (Figure 5.2B). Use of this modular system was advantageous for postmortem mechanical testing and non-invasive in vivo X-ray and microCT analysis. After attachment of the fixation device, an 8.0 mm segment of bone

was removed via bone saw with irrigation, and a scaffold was press fit into the defect (Figure 5.2A). Notches in the polysulfone plate, spaced 8.0 mm apart, ensured each defect was consistently created the same length (Figure 5.2B). Muscle was closed around the plate and defect using Vicryl sutures, and the skin was closed using sutures and wound clips.

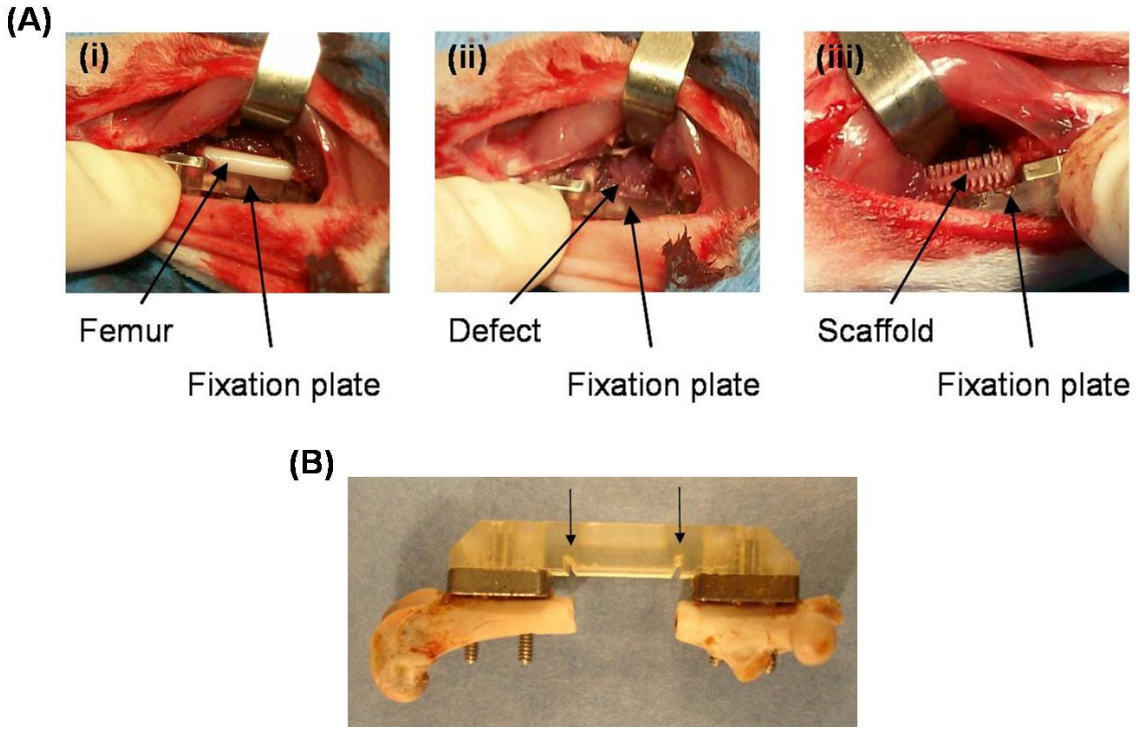


**Figure 5.2.** Surgical procedure for critically-sized segmental defects in rat femurs. (A) Each defect is stabilized by a fixation plate. (i) Blunt dissection of the quadriceps exposes the femur and enables placement of the fixation plate. (ii) An 8.0 mm segment is removed from the femur via bone saw, and (iii) scaffolds are press fit into the defect. (B) An explanted femur shows the modular fixation plate attached to the bone via stainless steel screws. Notches in the polysulfone plate (marked with arrows) are spaced 8mm apart ensuring each defect is created at the same size.

For in vivo studies, the following 3 groups were tested: (i) uncoated PCL scaffolds, (ii) GFOGER-coated PCL scaffolds and (iii) empty defect control. For the empty defect group, the surgical procedure remained the same, but no scaffold was

placed in the defect. A coating concentration of 50 μg/mL was used for all implanted GFOGER-coated scaffolds.

Following surgery, animals were given 3 daily doses of buprenorphine at 0.03 mg/kg for 2 consecutive days and 3 doses of 0.01 mg/kg on the third day to control pain. Animals were monitored daily for signs of pain and distress, progress of wound closure, regular eating habits and normal ambulation. A small percentage (< 8%) of rats developed infections in or around the surgery site, or experienced mechanical failure of the fixation device. These animals were removed from the study and euthanatized, and any data collected from these animals was excluded. Two weeks post surgery, skin wounds were completely healed, and animals were anesthetized with isoflurane for removal of wound clips. At 4, 8 and 12 weeks post-surgery, animals were anesthetized with isoflurane and the hind legs were scanned via radiography and microCT as described below. Twelve weeks post-surgery, animals were euthanized by $CO_2$ inhalation, and the femurs, along with surrounding muscle tissue, were harvested for postmortem microCT evaluation, histology and mechanical testing.

*Radiography and MicroCT Analysis*

Every 4 weeks following surgery, 2-dimensional X-ray images of each femur were non-invasively obtained using an MX-20 Specimen Radiography System (Faxitron X-ray Corporation) to make gross morphological observations of bone formation in each defect site. To obtain X-ray images, animals were anesthetized in a gas chamber filled with 5% isoflurane and maintained under anesthesia using 2% isoflurane flow into a face mask. Each hind leg of anesthetized animals was scanned for 15 s with a X-ray beam energy of 23 kV.

In addition to radiographic imaging, samples were non-invasively analyzed every 4 weeks post-surgery by microCT using a vivaCT 40 (Scanco Medical) to quantify bone volume in each defect site. For microCT, animals were anesthetized in the same manner as described above for radiography and placed in a rodent holder with one leg outstretched for scanning. The defect area in between the stainless steel plates of the fixation device was imaged with a X-ray beam energy of 55 kVp and intensity of 109 μA, and the integration time was 200 ms. Scanning resolution was 38 μm. After imaging was complete, noise was reduced from 3-dimensional reconstructions of each scan by applying a Gaussian filter (sigma=1.2, support=2) using the Scanco Medical μCT Evaluation Program. Images were thresholded at 270 mg HA/ccm to isolate mature bone from soft tissue and the polymer scaffold and fixation plate. Bone volume was quantified using directly computed values.

*Histological Analysis*

Immediately following euthanasia, samples for histological analysis were fixed in 10% neutral buffered formalin. One day after fixation, soft tissue was removed, and specimens were placed in fresh formalin. Prior to embedding, fixed tissues were scanned ex vivo via microCT as described above to allow matching of histological sections with microCT slices. After scanning, specimens were dehydrated in a series of alcohols, cleared in xylene, and embedded in methyl methacrylate (MMA). Ground sections, 50-80 μm thick, were prepared by Wasatch Histo Consultants, Inc. (Winnemucca, NV) and stained using Sanderson's Rapid Bone Stain™ and a van Gieson counterstain (Reyes et al. 2007). Stained histological sections were then matched to thresholded microCT scans to confirm that microCT analysis was representative of mature bone.

Mechanical Testing

Explanted samples for mechanical testing were wrapped in PBS-soaked gauze and frozen at -20$^{o}$C until use. Mechanical testing was performed as previously described (Oest et al. 2007). Briefly, samples were thawed in room temperature PBS and most of the soft tissue was removed, leaving some soft tissue surrounding each defect and taking care not to mechanically disrupt tissue in the defect site. The ends of each bone were potted in Wood's metal up to the polysulfone plate and secured with pins into potting blocks. Blocks were loaded onto an ElectroForce® mechanical testing machine (Elf 3200, Bose) and the polysulfone plate was removed just before testing. Samples were loaded in torsion at a displacement rate of 3$^{o}$/s up to 360$^{o}$. Maximum torque before failure was recorded for each sample. Stiffness and work to failure were calculated.

*Statistical Analysis*

Data was analyzed using ANOVA in Systat v11. Samples identified as statistical outliers that also met additional criteria for removal, such as improper plate placement, were removed from analysis. A p-value $< 0.05$ was considered significant.

**Results**

*Saturation of GFOGER on PCL scaffolds*

To determine a coating concentration for GFOGER saturation on PCL scaffolds, varying concentrations of biotinylated GFOGER were adsorbed onto PCL scaffolds in vitro and a saturation curve was generated via ELISA for anti-biotin. Figure 5.1C shows that GFOGER saturates the surface of PCL scaffolds at a coating concentration of 20

μg/mL. For all in vivo studies, GFOGER was used at a coating concentration of 50 μg/mL to ensure saturation of GFOGER on PCL scaffold surfaces.

*Bone formation in critically-sized defects*

Immediately following surgery, animals were monitored several times daily for signs of pain or stress, regular eating habits, and normal ambulation. Within one week following surgery, signs of stress were minimal, regular eating had returned and normal ambulation using both hind limbs was restored.

To monitor bone formation in critically sized defects, x-ray and microCT scans were performed every 4 weeks post surgery. X-ray images show gross morphological changes in bone growth at the defect site over time. While empty defects and uncoated PCL-treated defects showed minimal bone formation, GFOGER-treated defects showed substantial increases in bone growth over time. Representative X-ray images from each group are shown in Figure 5.3A, and the corresponding three-dimensional microCT reconstructions at 12 weeks are shown in Figure 5.3B.

At 4 and 12 weeks post-surgery, animals were anesthetized and bone volume in all defects was quantified via microCT. Negligible bone formation occurred at the ends of host bone in empty defects and uncoated PCL defects. However, bone formation in GFOGER defects was significantly greater compared to PCL and empty defects (Figure 5.3C). These indicate that specific targeting of the $\alpha_2\beta_1$ integrin via GFOGER surface modification promotes osteoblastic differentiation and bone formation in vivo.

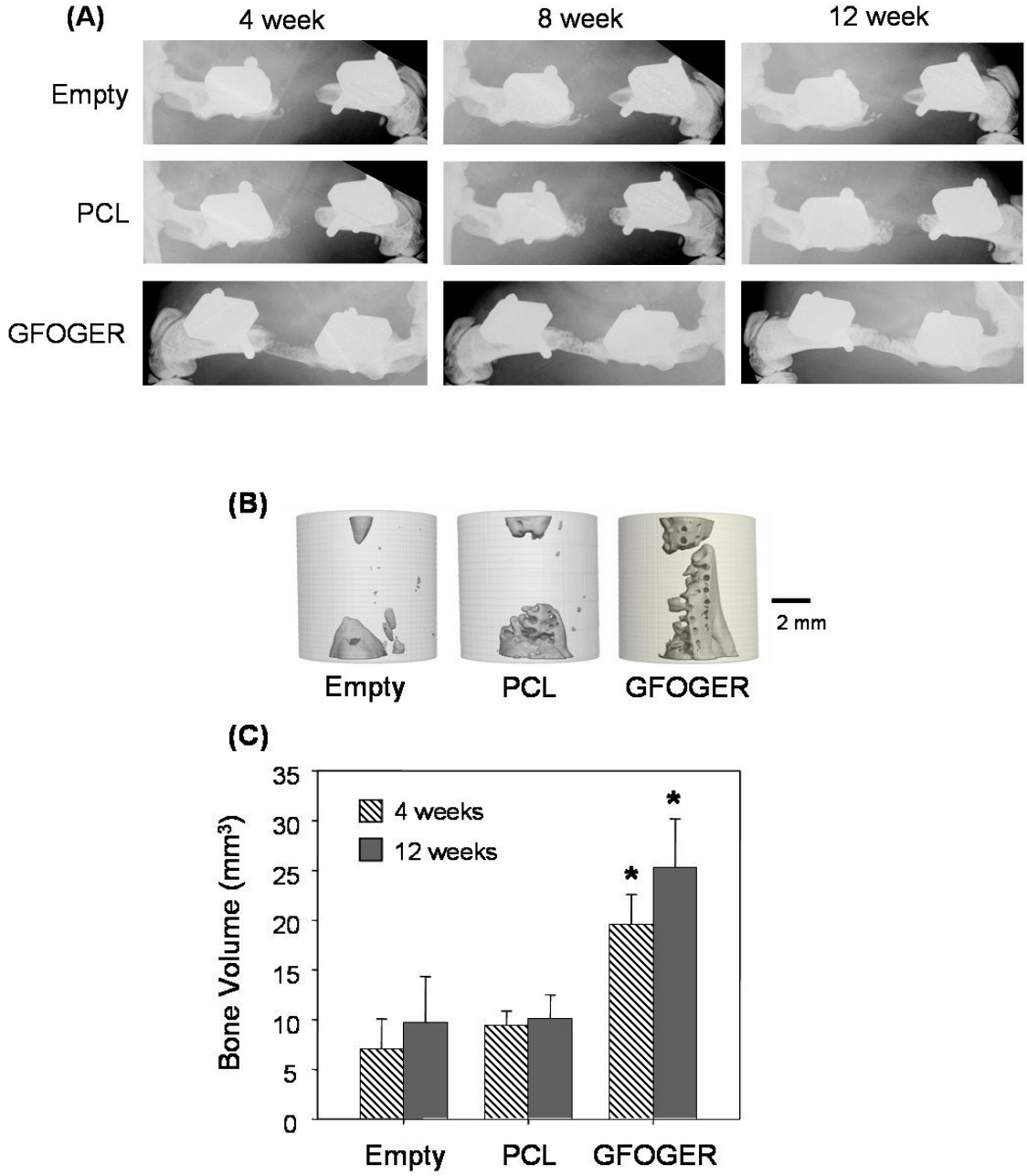


**Figure 5.3**. GFOGER-coated scaffolds significantly enhance bone formation in critically-sized defects compared to uncoated scaffolds and empty defect controls. (A) Faxitron images show that empty defects do not heal after 12 weeks, and negligible bone formation is present in uncoated PCL-treated defects. However, GFOGER-treated defects promote bone formation as early as 4 weeks. Representative samples are shown for each group. (B) MicroCT images show the same samples from (A) at 12 weeks. (C) Bone volume is significantly greater in GFOGER-treated samples at both 4 and 12 weeks compared to empty defects and uncoated PCL-treated samples. Error bars represent standard error of the mean. n=8 and n=9 for PCL and GFOGER at 4 weeks, respectively. n=7 and n=8 for PCL and GFOGER at 12 weeks, respectively. * Different from empty defect and PCL ($p<0.05$).

*Histological Analysis*

To more fully characterize areas of high attenuation imaged by microCT, one sample from the GFOGER group was subjected to histological analysis at 12 weeks. Prior to embedding, the sample was scanned via microCT and thresholded in the same manner as all other samples. Then, Sanderson's Rapid Bone Stain™, which distinguishes areas of mineralized bone from demineralized connective tissue and osteoid, was applied to the sample revealing mineralized bone tissue in a red/pink color and demineralized osteoid in blue/green (Sanderson and Bachus 1997). Because this sample underwent both Sanderson's stain and microCT scanning, matching slices from histology and microCT were compared. This analysis shows that areas of high attenuation that were thresholded in microCT and used as a measure of bone volume in Figure 5.3, directly match areas of red/pink staining defined as mineralized bone tissue in the Sanderson's stain (Figure 5.4).

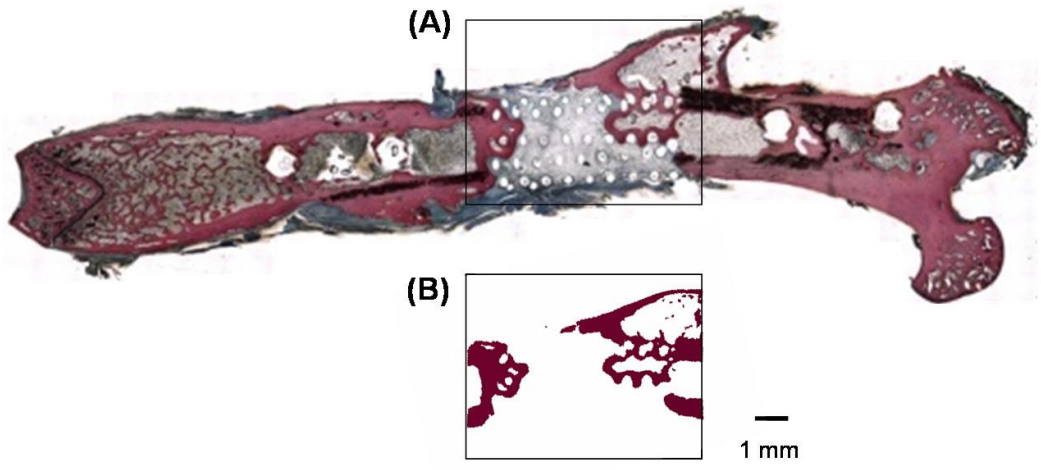


**Figure 5.4.** Histological analysis confirms that areas of high attenuation revealed by microCT are bone. (A) Sanderson's rapid bone stain for a GFOGER-coated sample showing bone in red/orange and soft tissue in blue/green. (B) Two-dimensional microCT image from the same sample as (A) revealing bone formation in the same location.

*Mechanical strength of repaired defects is dependent on bridging*

To assess functionality of new bone present in critically-sized defects, femurs were harvested 12 weeks post-surgery and subjected to postmortem torsional testing. Although microCT reveals differences in bone volume between GFOGER-treated and PCL-treated defects, maximum torque sustained was not significantly different between these two groups (Figure 5.5A). Stiffness and work to failure were also evaluated and no significant differences were observed (data not shown). We hypothesized that mechanical strength is dependent on defect bridging. Whereas some samples contain a large amount of bone in the defect site, high levels of mechanical strength are only present when this bone is firmly attached to both the proximal and distal end of the host bone. Without full attachment, or bridging of the defect, samples that have large bone volumes sustain low torque loads.

To demonstrate that mechanical strength is dependent on defect bridging, Faxitron images for all samples were blindly assessed for their extent of bridging and assigned a bridging score from 0-5 based on the criteria outlined in Figure 5.5B, where 0 is no bone in the defect and 5 is a fully bridged defect. Representative Faxitron images for each score are shown in Figure 5.5C. By looking at bone volume versus maximum torque for each sample grouped by briding scores, a trend is observed where samples with higher bridging scores generally have greater max torque (Figure 5.5D). Although there are no significant differences in torsional strength between uncoated PCL-treated defects and GFOGER-treated defects, a distribution of bridging scores from each group shows that GFOGER scores are shifted towards fully bridged or nearly fully bridged

(scores 4 and 5) compared to PCL scores (Figure 5.5E). This shift found to be significant by a Kruskall Wallis one way ANOVA by ranks ($p < 0.05$).

*Effects of GFOGER on bone formation are surface area dependent*

We hypothesized that the effects of GFOGER on bone formation are dependent on the area of scaffold in direct contact with host bone because the surface area of the scaffold directly affect the dose of GFOGER delivered to the defect site. Uncoated and GFOGER-coated PCL scaffolds with varying surface area to volume ratios were implanted in critically-sized segmental defects to test for surface area dependent effects of GFOGER. MicroCT analysis shows that GFOGER significantly enhances bone formation compared to uncoated PCL scaffolds only when the surface area to volume ratio of the scaffolds is above 3.1 $mm^{-1}$ (Figure 5.6). In vitro ELISA detection of biotinylated GFOGER adsorbed at a saturating concentration of 50 μg/mL onto PCL scaffolds with various surface area to volume ratios shows significantly more total GFOGER on scaffolds with more surface area (Figure 5.6B).

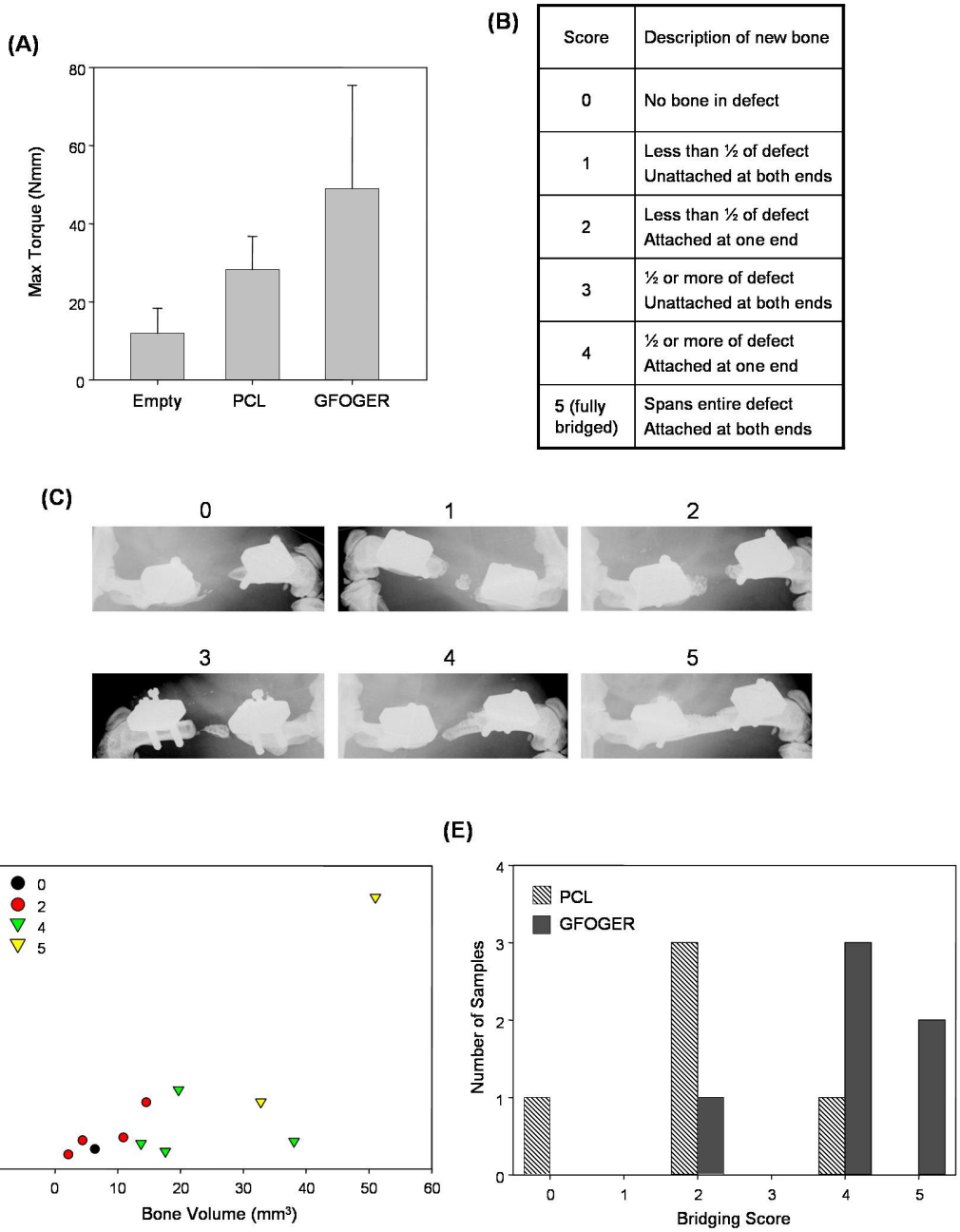


**Figure 5.5.** Mechanical properties of repaired segmental defects. (A) Despite differences in bone volume between GFOGER-treated defects and PCL-treated or empty defects, maximum torque is not significantly different. (B) Criteria for bridging scores assigned to each sample. (C) Representative faxitron images of scores described in (B). (D) Samples with higher bridging scores are shifted to the upper right of a bone volume versus maximum torque correlation graph. (E) GFOGER-coated samples result in a greater number of fully bridged samples than uncoated samples ($p < 0.05$).

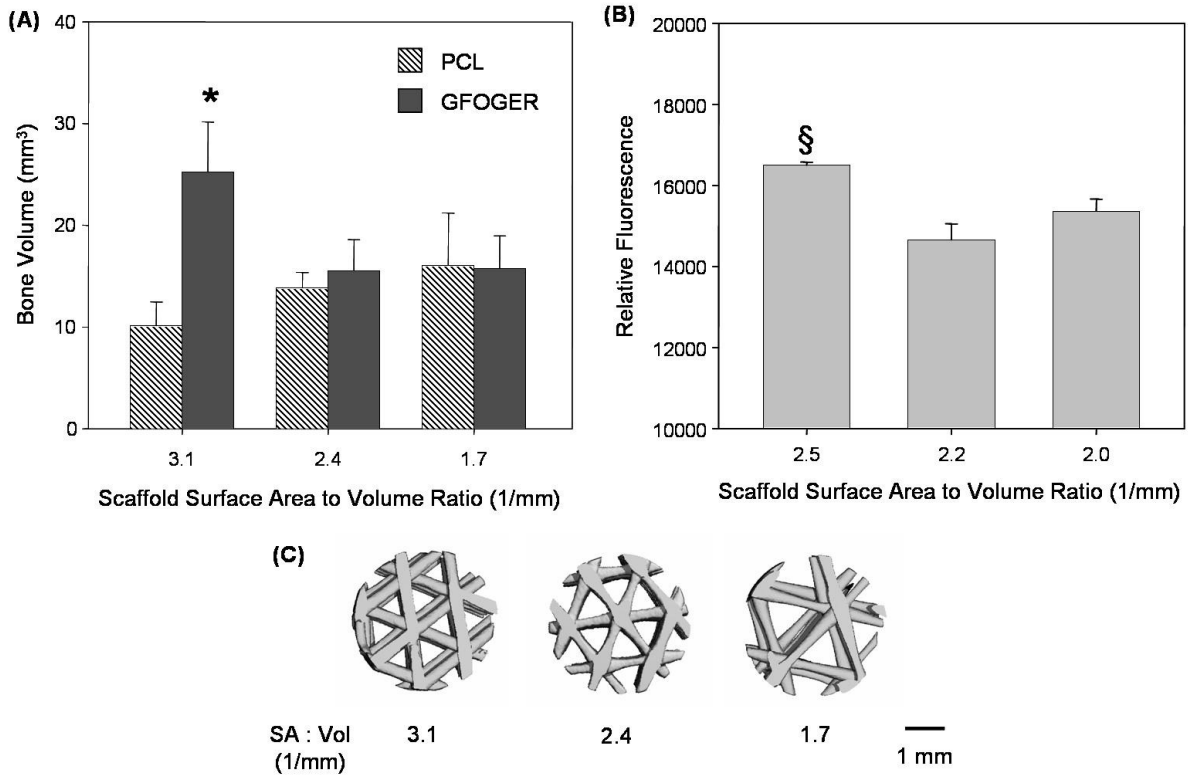


**Figure 5.6.** Effects of GFOGER are dependent on scaffold surface area to volume ratio. (A) In vivo results show greater bone volume in GFOGER-treated defects compared to uncoated PCL only when the scaffold surface area to volume ratio is high. Bone volume is quantified by microCT at 12 weeks post-op (*Different from uncoated scaffolds in the same SA:Vol group, $p < 0.05$). (B) Biotinylated GFOGER detected on the surface of PCL scaffolds with varying surface area to volumes ratios via ELISA. Scaffolds with the highest surface area to volume ratio have more GFOGER than the others (§ Different from all other groups, $p < 0.05$). (C) Cross-sectional microCT images of scaffolds with different surface area to volume ratios.

**Discussion**

This study examined the effect of coating synthetic polymer scaffolds with a biomimetic peptide sequence from collagen I on the in vivo bone healing of critically-sized defects in rats. We found that simple surface modification by adsorption of the peptide, GFOGER, to the scaffold accelerated and promoted significantly more bone formation in segmental femoral defects treated with coated scaffolds compared to uncoated scaffolds. We further demonstrated that GFOGER-mediated bone formation is dependent on scaffold surface area, an indication that the dose of GFOGER delivered to the site or the area of GFOGER in direct contact with host cells directs the host reparative response. The implications of this study are significant for clinical healing of large bone defects because this cell and growth factor-free method for bone regeneration addresses many issues with current regenerative medicine technologies. Namely, the fabrication of the synthetic peptide sequence, GFOGER, is inexpensive and provides a more cost effective method treatment method than strategies employing BMPs. Furthermore, the use of synthetic materials avoids the issue of immune response to these biologically-derived biomolecules. This simple surface modification strategy which uses passive adsorption to coat biomaterials for bone regenerating scaffolds provides a simple method for imparting biofunctionality to synthetic surfaces, which could easily be translated to other materials.

The critical role of integrin activation in cell adhesion and signaling has been well established (Clark and Brugge 1995; Hynes 2002). In particular, the specific interactions of fibronectin with the $\alpha_5\beta_1$ integrin and of type I collagen with the $\alpha_2\beta_1$ integrin have been shown to mediate osteoblast differentiation and subsequent mineralization of

osteoblastic and pre-osteoblastic cells (Moursi *et al.* 1997; Takeuchi *et al.* 1997; Xiao *et al.* 1998; Jikko *et al.* 1999; Mizuno *et al.* 2000). Over the past decade, targeting of these specific ECM-integrin interactions to exert engineered control over osteoblastic function has been accomplished via isolation of specific binding sequences, such as RGD, from ECM proteins. Successful osseointegration and implant fixation has been demonstrated in vivo using RGD as a biomaterial coating (Ferris *et al.* 1999; Bernhardt *et al.* 2005; Elmengaard *et al.* 2005), and these strategies often enhance osteoblastic activity to a greater extent than the native ECM molecules (Kurihara and Nagamune 2005). Recently, work in our group has demonstrated a significant improvement in osseointegration and implant fixation over RGD using the fibronectin fragment, $FNIII_{7-10}$, which contains a synergy site in addition to RGD (Petrie *et al.* 2008). Although these strategies are promising, little work has focused on the presentation of non-RGD peptide motifs that target the collagen-$\alpha_2\beta_1$ interaction (García and Reyes 2005).

Activation of the $\alpha_2\beta_1$ integrin is presently known to occur via binding of distinct adhesive sites contained within collagen I, namely DGEA and GFOGER (Xiao *et al.* 1998; Knight *et al.* 2000). In studies comparing RGD to DGEA, cell adhesion strength was greater on surfaces presenting RGD over DGEA (Harbers and Healy 2005). Although several studies have characterized the effect of DGEA on other cell types, the role of DGEA in osteoblast signaling and differentiation is presently unclear (Marquis *et al.* 2008). On the other hand, our group has previously reported increased osteoblastic differentiation on GFOGER coated surfaces in vitro, and this effect translated to increased osseointegration and implant fixation on GFOGER-coated titanium implants in vivo (Reyes and García 2003; Reyes and García 2004; Reyes *et al.* 2007). In those

studies, the effects of GFOGER on osteoblastic differentiation and mineralization were greater than that for native collagen I, indicating that the GFOGER peptide has greater targeting specificity than full length collagen I, most likely due to the elimination of other binding sites (such as DGEA) present on collagen I. Many studies have explored the use of collagen sponges as carriers for cells and growth factors to heal segmental orthotopic defects. While collagen sponges have demonstrated good use as carriers, no significant effect on orthotopic bone formation has been demonstrated with empty collagen carriers alone (Pekkarinen *et al.* 2006; Chen *et al.* 2007; Azad *et al.* 2009). To our knowledge, the present study is the first to examine the use of the collagen-mimetic peptide, GFOGER, in a critically-sized orthotopic segmental defect. While collagen alone does not successfully heal critically-sized bone defects, we demonstrate a significant enhancement in bone formation, with complete defect bridging occurring in some cases, due to GFOGER coating alone, without the use of cells or growth factors. It is likely that the suboptimal results obtained from uncoated scaffolds is due to non-specific adsorption of serum proteins to the scaffold surface, leading to non-specific signaling and an unregulated host cell response. Meanwhile, GFOGER-coated scaffolds promote specific binding of $\alpha_2\beta_1$ integrin, thereby upregulating osteogenesis in surrounding host cells. This signaling response may via preferential binding and recruitment of osteoprogenitor cells present in or around the defect site to GFOGER, or via increased differentiation of uncommitted cells bound to GFOGER on the scaffold or some combination thereof.

In the late 1990s, a 15 amino acid peptide sequence, termed P-15, was isolated from collagen I, and shown to exhibit collagen-mimetic cell signaling when used as a surface coating (Qian and Bhatnagar 1996; Bhatnagar *et al.* 1999). Although P-15

signaling has not been specifically linked to the $\alpha_2\beta_1$ integrin, use of P-15 as a coating on anorganic bone matrix (ABM) promotes osteoblastic differentiation and mineralization in vitro (Yang *et al.* 2004). Preclinical studies using P-15/ABM implants in orthotopic defects have demonstrated successful defect healing in two different models. Scarano *et al.* report significant healing of a monocortical tibial defect in rabbits using P-15/ABM (Scarano *et al.* 2003). Similarly, Cakmak *et al.* report significant healing due to P-15/ABM in a segmental rat radial defect (Cakmak *et al.* 2006). However, in both of these studies, P-15/ABM was compared to empty defect controls, but uncoated ABM controls were not included. Furthermore, no quantitative CT analysis of bone volume was performed in either of these studies, making direct comparison to the present study difficult. A third pre-clinical study employing P-15/ABM did not show a significant enhancement in healing of a rabbit segmental femoral defect. However, in this study, empty defects demonstrated complete bridging as often as P-15/ABM treated defects after 12 weeks, indicating that the 5 mm rabbit radial defect used in this study was not of a critical size (Sarahrudi *et al.* 2008). Despite few reports on successful preclinical testing of P-15/ABM in orthotopic sites, recent clinical data has demonstrated successful healing in humans due to P-15/ABM implants in several case reports (Gomar *et al.* 2007; Trombelli and Farina 2008). Although these results are promising, little effort has been made to test uncoated ABM compared to P-15 complexed ABM. ABM is porous particulate bone mineral derived from bovine sources, which is likely to be a naturally osteoconductive material. In the present study, uncoated synthetic polymeric scaffolds did not produce significant healing in orthotopic defects when compared to GFOGER-coated scaffolds. This choice of scaffold, which does not heal the defect alone, allowed

us to evaluate the effects of GFOGER on defect healing without contributions from the scaffold. Interestingly, a recent study comparing DGEA, P-15, GFOGER, and RGD, showed that GFOGER produced no cell adhesion when used as an HA coating (Hennessy *et al.* 2009). The authors attribute this surprising result to the HA substrate employed. Taken together, these results indicate that the effects of GFOGER may be substrate dependent.

It is well known that ligand density and clustering have a direct effect on integrin activation and signaling (Massia and Hubbell 1991; Maheshwari *et al.* 2000). Recent work in our group has demonstrated that adhesion of HT1080 human fibrosarcoma cells to GFOGER coated surfaces is directly dependent on the surface density of GFOGER (Reyes *et al.* 2008). In the present study, we determined the coating concentration necessary to produce a saturating surface density of GFOGER on PCL scaffolds (Figure 5.3C). This saturating concentration was used to coat all GFOGER-coated PCL scaffolds used for in vivo implantations. Because coating was performed via passive adsorption of GFOGER onto PCL scaffold surfaces, and ligand density and clustering were not precisely controlled, we, therefore, assume that ligand density and clustering were constant between different batches of PCL. However, we observed that PCL scaffolds with varying surface area to volume ratios produced differential effects on bone formation in vivo (Figure 5.6A). Because ligand density and clustering were not specifically varied in this experiment, this difference in host response to GFOGER on different PCL scaffolds was most likely due to a dose dependent effect of GFOGER at the tissue level. With relatively few studies that characterize the 3 dimensional in vivo binding environment of short synthetic integrin ligands used as biomaterial coatings,

future work should focus on the optimization of GFOGER dosage for in vivo applications. Exploring scaffold materials, architectures, and surface areas for GFOGER therapy is warranted.

Finally, a distinct advantage of this study over many reports of segmental defect healing is the use of mechanical testing to assess functionality of the defects. A large majority of studies that report successful healing of bone defects rely solely on a combination of X-ray analysis and histological evaluation as a measure of defect healing. However, this approach does not provide functional information, which is necessary to fully evaluate the success of a given treatment strategy for bone healing (Liebschner 2004). Few groups report mechanical testing analysis, but of those that do, the most common test methods are torsion (Cook *et al.* 1994; Cook *et al.* 1994; Cook *et al.* 1995; Hsu *et al.* 2007; Oest *et al.* 2007; Rai *et al.* 2007). In this study, we used torsional testing to evaluate the functional strength of the defects. Despite differences in bone formation, the average torsional strength of GFOGER treated defects was not significantly different from that of PCL treated defects, pointing to the need for careful mechanical evaluation in bone tissue engineering to assess function of tissue engineered defects. However, we did observe differences in torsional strength between fully bridged samples compared to non-unions. This result is in agreement with a mounting body of work suggesting that greater bone mass will not necessarily result in greater bone strength. For example, recent evidence suggests that patients with a high level of bone turnover may be at higher risk for bone fracture regardless of areal bone mineral density (Hernandez 2008). This concept is known as bone quality, a term used to describe any skeletal aspect of bone, excluding bone mass (i.e. bone shape, size, trabecular connectivity, etc) that affects its

strength (Hernandez and Keaveny 2006). In the present study, although significant increases in bone mass were observed due to GFOGER treatment, bone quality was not affected. The implication of these results is that radiographic and histological methods of evaluating bone tissue are not sufficient to fully characterize the extent of bone defect healing. Functional mechanical evaluation of bone defects should be used in conjunction with other methods for complete analysis of both bone mass and bone quality.

In conclusion, this simple surface modification of synthetic bone implants imparts biologic functionality without the use of cells or growth factors. The advantages of this method include reduced cost compared to purification of natural proteins, reduced risk of disease transmission and reduced reliance on variability in biologics, making GFOGER therapy a viable treatment for future clinical bone healing.

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# CHAPTER 6

# SUMMARY AND FUTURE CONSIDERATIONS

Clinical healing of large non-unions in bone is a significant socioeconomic problem in the United States. As many as 30% of patients undergoing autografts have been reported to suffer from donor site morbidity and pain, while approximately 30% of allografts fail due to fracture. To address these issues, bone tissue engineering strategies, which eliminate the need for autogenous or cadaveric donor bone, present valuable alternatives to healing large bone defects. In this work, two tissue engineering strategies, one cell-based and one biomaterials-based, were developed to promote directed osteoblastic differentiation for healing critically-sized defects in bone. Although the approaches differed, both strategies focused on exerting control over specific signaling pathways involved in osteogenesis to promote bone formation. The two strategies are summarized below, along with future considerations for further development of this research.

**Runx2 Genetic Engineering for Bone Regeneration**

In this work, we presented a cell-based strategy for bone tissue engineering, in which BMSCs were genetically modified to constitutively overexpress the osteoblast-specific transcription factor, Runx2. We demonstrated that delivery of Runx2-modified BMSCs to critically-sized orthotopic defects in rats accelerated the bone formation rate in defects at early time points compared to treatment with unmodified cells or negative controls. At late time points, unmodified BMSCs eventually produced similar levels of bone volume, highlighting the inherent osteogenic ability of this cell type. Recent

clinical work suggests that successful treatment of large bone defects in humans with unmodified BMSCs requires at least 5 months of external fixation of the injured limb post-surgery (Marcacci *et al.* 2007). Therefore, a reduction in recovery time after surgery would add significant benefit to this treatment, making the gene therapy strategy for accelerated bone formation presented in the current work a valuable alternative to current treatments.

Although Runx2-modified BMSCs accelerated bone formation in critically-sized defects compared to unmodified BMSCs, the mechanical strength of the defects after 12 weeks was not significantly different among unmodified BMSC and Runx2-modified BMSC treatments. Runx2-modified cells did result in a population shift towards fully bridged or nearly fully bridged defects; however, for successful healing of large bone defects in humans complete bridging and functional restoration of mechanical strength should be achieved. To improve mechanical functionality in defects treated with Runx2-modified cells, several parameters may be considered. These include the therapeutic load of cells delivered to the defect site, scaffold type, scaffold architecture, transduction efficiency of the target gene, and cell type. These parameters are briefly discussed below in the context of future directions for this work.

Few studies have directly examined the effect of cell concentration on the in vivo osteogenic potential of bone tissue engineering constructs. However, evidence suggests that an increase in the number of cells present on constructs upon implantation significantly increases osteogenesis (Connolly *et al.* 1989). Recently, Yoshii *et al*. reported the use of a simple pressurized seeding strategy to increase cell retention and improve cell distribution on β-TCP scaffolds, which resulted in greater and more uniform

bone formation on scaffolds implanted ectopically (Yoshii *et al.* 2009). Furthermore, previous work in our group demonstrated that Runx2-expressing dermal fibroblasts had significantly greater mineralization capacity in vitro on fibrous collagen sponges, which retained more cells upon initial seeding than PCL scaffolds with an open pore structure or PLGA foams (Phillips *et al.* 2006). This study points to an intricate interplay between cell number, scaffold architecture and scaffold material. The high surface area to volume ratio of a fibrous mesh in combination with the active binding sites present within collagen I are most likely more conducive to cell adhesion and subsequent differentiation than synthetic scaffolds with more open pore structures that lack adhesive sequences. In the current work, a collagen mesh was incorporated into PCL scaffolds to increase cell retention in the void volume of the scaffolds compared to PCL scaffolds alone. Perhaps the use of a fibrous collagen sponge without PCL in this model would allow greater cell attachment, further increasing the therapeutic load of cells delivered to the defect site, thereby enhancing bone healing beyond levels reported in the current work. Another scaffold option is the use of naturally osteoconductive materials, such as hydroxyapatite or β-TCP, which promote attachment of host bone cells that will mineralize the construct (LeGeros 2002). These materials have been used for bone regeneration; however, radiographic quantification of bone formed in the defect is difficult because these calcium phosphate ceramics have X-ray attenuation values very similar to bone, making image segmentation to isolate bone from scaffold difficult. Alternatively, synthetic scaffolds presenting specific adhesive sequences, such as those investigated in Chapter 5 of this work, may provide an extracellular signaling environment more conducive to osteoblastic differentiation than unmodified synthetic scaffolds. Because retroviral transduction of

primary cells is not 100% efficient, a second level of signaling presented on the scaffold, in addition to genetic modification of the cells, may encourage differentiation of the untransduced cell population delivered to the defect site. All in all, optimization of the interplay between cell and scaffold parameters may enhance the bone formation and subsequent mechanical strength of bone defects treated with cell/scaffold constructs containing Runx2-modified BMSCs.

A major contributing factor to the success of any gene therapy strategy is the type of gene delivery vehicle used to efficiently transduce the target cell population with the gene of interest. Several gene delivery vehicles, including both viral and non-viral vectors and naked plasmid DNA, have been used for bone tissue engineering (Gersbach *et al.* 2007). In the current work, Runx2 overexpression in BMSCs was achieved by retroviral transduction, promoting stable integration of the Runx2 transgene in 40% of the recipient cells. It is possible that an increase in the transduction efficiency of Runx2 to donor cells would enhance the bone formation rate in critically-sized defects treated with Runx2-modified cells beyond levels achieved in this work. As discussed previously in this dissertation, the proliferation rate and differentiation ability of BMSCs gradually decrease as time in culture time increases. Because retroviral transduction only occurs in dividing cells, perhaps the propensity for decreased proliferation of this cell type would be better suited to a different vector system. For example, lentiviral vectors promote stable, long-term integration of the gene of interest and effectively target non-dividing cells (Naldini *et al.* 1996). On the other hand, alternative donor cell types or the elimination of the need for donor cells could be explored. Recent work in our lab has demonstrated that dermal fibroblasts, a cell type less susceptible to loss of proliferation

than BMSCs, can be retrovirally transduced to express Runx2 with 65% transduction efficiency and mineralize collagen scaffolds implanted ectopically (Phillips *et al.* 2007). Perhaps the use of dermal fibroblasts in this model would allow greater Runx2 transduction efficiency, leading to further enhancement of bone formation in critically-sized defects. Finally, our lab has also recently demonstrated that biomaterial-mediated delivery of a retroviral vector can efficiently promote transduction of target cells when biomaterial surface chemistry is well-controlled (Gersbach *et al.* 2007). Therefore, implantation of a biomaterial carrier for the Runx2 retrovirus directly into a defect site could promote in situ transduction of host cells, thereby promoting bone formation without the need for donor cells. Implementation of these gene therapy strategies may enhance Runx2-mediated healing of critically-sized defects in bone.

**GFOGER Surface Modification for Bone Regeneration**

In this work, we developed a biomaterials-based strategy for bone tissue engineering, in which synthetic PCL scaffolds were biofunctionalized with the collagen-mimetic peptide, GFOGER. We demonstrated that passive adsorption of GFOGER onto synthetic PCL scaffolds significantly increased bone formation in critically-sized orthotopic defects treated with GFOGER-coated scaffolds compared to defects treated with uncoated PCL scaffolds or empty defects. We further demonstrated that this effect was dependent on scaffold surface area to volume ratio, indicating a dose dependency or threshold effect of GFOGER in critically-sized bone defects. This simple surface modification strategy imparts specific biologic functionality to synthetic surfaces, promoting enhanced bone healing without the use of donor cells or growth factors. The implications of this study are significant for clinical healing of large bone defects because

this cell and growth factor-free strategy is cost-effective, evokes no immune response, avoids regulatory issues involving the implantation of biologic materials, and could be made readily available as a point of care clinical application.

Despite differences in bone formation between GFOGER-coated and uncoated PCL scaffolds at 12 weeks, no significant differences in the average mechanical properties of defects treated with GFOGER-coated or uncoated PCL scaffolds were observed. As with our cell-based approach, GFOGER-coated scaffolds resulted in a population shift towards fully bridged or nearly fully bridged defects compared to uncoated PCL scaffolds. However, for successful healing of large bone defects in humans complete bridging and functional restoration of mechanical strength should be achieved. To improve mechanical functionality in defects treated with GFOGER-coated scaffolds, several parameters may be considered. These include the surface area to volume ratio of the scaffold used, scaffold architecture, scaffold material, method of ligand tethering, and ligand clustering. These parameters are briefly discussed below in the context of future directions for this work.

The structure of synthetic scaffolds for bone tissue engineering must meet several criteria in order to support bone tissue ingrowth, vascularization, and tissue remodeling. Importantly, scaffolds must contain a high degree of porosity, an interconnected pore volume, and an average pore size of at least 100 μm (Liu and Ma 2004). In the current work, PCL scaffolds were created with well-controlled architectures, which provided an open interconnected pore volume to support bone ingrowth. We demonstrated that the effects of GFOGER on bone formation in critically-sized defects depended on the surface area to volume ratio of the scaffold. This surface area dependency most likely occurs

either because the amount of GFOGER delivered to the defect site is directly related to the surface area of the scaffold (i.e. scaffolds with greater surface area contain a greater amount of GFOGER) or because scaffolds with greater surface area have more opportunity for direct contact with host cells. Thus, it is possible that the implantation of GFOGER-coated scaffolds with a greater surface area to volume ratio than the scaffolds examined in this work could result in greater bone formation than observed here. However, the caveat to this design change is that scaffold porosity must remain open and interconnected to continue to allow tissue infiltration and bone formation. Taking scaffold surface area and porosity into account, synthetic scaffolds may be specifically designed to optimize GFOGER efficacy in vivo, thereby enhancing bone healing to levels beyond that of the current work.

Finally, it is well established that ligand surface density and clustering have a direct effect on integrin signaling and activation (Massia and Hubbell 1991; Maheshwari *et al.* 2000). Indeed, our group has demonstrated that cell adhesion to GFOGER coated surfaces is directly dependent on GFOGER surface density (Reyes and García 2003; Reyes *et al.* 2008). In the current work, we used passive adsorption of GFOGER to saturate PCL scaffold surfaces. The application of this strategy is simple, making it attractive for use in the clinic. However, it is possible that well controlled deposition or tethering of GFOGER to specifically modulate ligand density and clustering may produce surfaces that are better suited for bone regeneration than those prepared by passive adsorption of the ligand. Recent work in our lab has demonstrated that surface modification of titanium implants with non-fouling brushes that present $\alpha_5\beta_1$ specific ligands at well-defined densities increase osseointegration and implant fixation in vivo

compared to unmodified titanium implants (Petrie *et al.* 2008). This tethering strategy has also been demonstrated for GFOGER, where titanium implants, modified with well-controlled densities of GFOGER on a non-fouling background, support cell adhesion in vitro (Raynor *et al.* 2009). Perhaps the implementation of this strategy to generate bone tissue engineering scaffolds could improve the host response to GFOGER-modified surfaces. Furthermore, we have observed widely varying adsorption profiles for GFOGER on different materials, including tissue culture polystyrene, PCL, titanium and glass (unpublished data), indicating that the base material used for GFOGER adsorption may have a significant effect on cell response to the construct. Recently, *Hennessy et al.* found that GFOGER did not promote cell adhesion on hydroxyapatite scaffolds despite their own verification of GFOGER-induced cell adhesion on tissue culture polystyrene (Hennessy *et al.* 2009). It is possible that choosing a different scaffold material or tethering GFOGER to materials that do not naturally adsorb GFOGER would expand the available options for delivery of GFOGER to critically-sized bone defects. All in all, well-controlled methods for surface modification with GFOGER could more specifically modulate the osteogenic response of host cells to GFOGER-modified scaffolds, further enhancing GFOGER-mediated bone formation in critically-sized bone defects.

**Conclusions**

The work presented in this dissertation describes two strategies for bone tissue engineering which both target specific osteoblastic signaling pathways to promote enhanced healing of critically-sized bone defects. This work is innovative because genetic and extracellular cues for osteoblastic differentiation are used to exert control over cell signaling and promote bone healing in a robust segmental defect model. By

engineering BMSCs to overexpress Runx2, issues with BMSC dedifferentiation in culture and donor cell variability are addressed, providing a strategy for accelerated bone formation in large bone defects. Furthermore, development of a cell and growth factor free strategy for bone regeneration via surface modification of PCL scaffolds with GFOGER confers biofunctionality to synthetic substrates and promotes specific signaling to host cells, eliminating the need for donor cells and providing a simple and elegant point of care strategy. Further development of these tissue engineering strategies for bone regeneration will provide clinically-relevant treatment options for healing large bone defects in humans by eliminating the need for donor bone and employing well-controlled signals to promote bone formation.

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