TOP DOWN AND BOTTOM UP APPROACHES TO ELUCIDATING MULTISCALE PERIOSTEAL MECHANOBIOLOGY: TISSUE LEVEL AND CELL SCALE STUDIES

by

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Submitted in partial fulfillment of the requirements

For the degree of Master of Science

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ACKNOWLEDGEMENTS

I would like to thank a number of people who have contributed to my experiences as a graduate researcher. First, I would like to thank Dr. Knothe Tate for her outstanding mentorship during my tenure in her lab. She has helped me to develop both as a scientist and professionally. Dr. Knothe Tate continually challenged me to reach for the impossible, think outside of the box and challenge the limits of science. Furthermore, she provided me with the opportunity to participate in transdisciplinary as well as transcontinental research as a member of her lab group. My international research experiences helped me to grow both as a person and scientist and these experiences will prove valuable for the remainder of my career regardless of if I am working in industry or academia.

In addition, I would like to thank all the members of the MechBio lab group here at Case, both past and present. Special thanks to Sara McBride for mentoring me as an undergraduate student and sparking my interest in biomedical materials research. I would also like to thank Hana Chang for being a part of my international research experience. Thanks is also due to Dr. Ralf Richter and the members of his lab group at CICbiomaGUNE in San Sebastian, Spain for teaching me everything I know about biochemistry and the nanoscale world of cell and molecular interactions.

Finally, I would like to thank my family and friends for providing constant support and encouragement throughout my graduate career.
Top Down and Bottom Up Approaches to Elucidating Multiscale Periosteal Mechanobiology: Tissue Level and Cell Scale Studies

Abstract

by

SARAH FRANCES EVANS

The periosteum serves as a stabilizing boundary membrane to the bone it envelopes and contains mechanosensitive progenitor cells capable of promoting new bone formation. Knowledge of the structure-function relationships underlying the dynamic mechanical and regenerative properties of the periosteum is lacking. The following work investigates the structure-function relationships present in periosteum at both the tissue and cellular level. At the tissue level, periosteum permeability is characterized and found to exhibit barrier membrane properties. Subsequently, cell scale studies investigate the molecular characteristics of periosteum cells responsible for enabling tissue-level barrier membrane properties. Lastly, a biomimetic membrane system for investigating cell-cell adhesions of periosteum cells is developed. Culture of periosteum cells on the model membrane system results in signs of early osteogenic lineage commitment. Characterization of structure-function relationships in periosteum across multiple lengths scales provides knowledge valuable for the development of predictive computational models and tissue engineered periosteal replacement membranes.
Chapter 1

General Introduction and Literature Review

*Portions of this chapter are intended for submission as a review paper to Journal of Biomechanics, March 2012

1. Introduction

As the population continues to age, trauma is expected to rise from the seventh to the third leading cause of disability in adults within the next decade\(^1\). Bone fractures are a common result of trauma and accounted for 26\% of total reported musculoskeletal injuries in 2004\(^2\). Fractures or conditions resulting in critical size bone defects are especially debilitating, as such defects are incapable of healing without surgical intervention. Defects of this severity arise from trauma, tumor, infection or congenital malformations\(^3\).

The healing of critical size bone defect is of great interest as they present one of the most challenging problems in orthopedic surgery. Current methods to heal critical size bone defect utilize surgical procedures such as the Ilizarov technique of distraction osteogenesis and typically involve use of bone graft and/or graft substitutes. In the Ilizarov technique, the distal and proximal ends of the injured bone are stabilized using an external cylindrical frame that is affixed to the stable bone ends as well as osteotomized bone segments via fixation wires. The transport segment is then distracted a millimeter or so per day (via a screw or motor on the external frame), which stimulates bone regeneration in the widening gap, until it reaches the proximal or distal segment on the other side of the defect zone\(^4\). Hence, new bone regenerate emanates from the osteotomy gap, which is placed under tension through distraction of the fragment; cells
and factors related to this new bone formation likely derive from the bone itself as well as the medullary niche, if an external fixator is used. Adaptations of the technique include stabilization and bone transport over an intramedullary nail\textsuperscript{5-11}, which would significantly reduce the involvement of intramedullary factors on the regeneration of bone. Distraction osteogenesis has several disadvantages, including long and labor intensive treatment times, a high risk of complications, patient discomfort, and scarring. Furthermore, distraction osteogenesis requires significant technical expertise, limiting the number of surgeons qualified to perform the procedure and hence access for patients.

Recent research in the field of orthopedics has drawn attention to the power of periosteal tissue and periosteal derived cells to heal bone defects\textsuperscript{3,5,12-15}. Specifically, the periosteum has been found to be able to regenerate woven bone within a critical size defect within as little as two weeks from time of treatment\textsuperscript{3,12-13}. The biology of the periosteum has been well studied\textsuperscript{16,17} but until recently little research has focused on the mechanical properties or on the mechanobiology of the periosteum. Yet, recent research suggests periosteal tissue and periosteum cell mechanobiology play an important role in defect healing. Since the periosteum serves as the bounding envelope of bone, anisotropy in periosteal properties may play a key role in modulating bone growth, healing and adaptation, in health, disease and trauma. The following reviews the current state of periosteal mechanobiology from the cell to tissue length scale, in bone health and during healing of bone. Furthermore, open questions and barriers to understanding are addressed, both from the perspective of defining important future directions for the field and also from the perspective of identifying the technologies that are missing to
understand the multiscale (length and time scales) of the highly (visco)elastic biomaterial, periosteum.

2. Periosteum Tissue Mechanobiology

The periosteum is a bi-layered, soft tissue sleeve that envelopes bone (Figure 1). Periosteum is highly vascularized and provides at least 1/3 of the blood supply to cortical bone, with the remaining supply coming from the intramedullary niche\textsuperscript{18}. The tissue exhibits composite structure comprised of an outer fibrous layer and an inner cambium layer. The outer fibrous layer contains fibroblasts, collagen and elastin fibers, and microvessels\textsuperscript{17}. Histological data reveals axially aligned collagen fibers\textsuperscript{19} and a high density of elastin\textsuperscript{20,21} throughout mid-diaphyseal periosteum, likely providing mechanical (structural) strength to the tissue\textsuperscript{22}. The cambium or cellular layer contains adult mesenchymal stem cells known as periosteal derived cells (PDCs), differentiated osteogenic precursor cells, and osteoblasts\textsuperscript{23}. PDCs have been shown to play a major role in bone fracture healing by

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{diagram.png}
\caption{Schematic of the structure of the bi-layered periosteum.}
\end{figure}
constantly building\textsuperscript{20} and repairing bone\textsuperscript{12,15,24-26}. Adjacent PDCs are joined together by tight junctions\textsuperscript{27} a hallmark of epithelial membrane morphology\textsuperscript{28}. The progenitor cells proliferate and then differentiate into osteoblastic cells, driving the process of bone repair.

The periosteum is anchored to the bone by Sharpey’s fibers, strong fibers with high collagen content. Sharpey’s fibers serve as a link between the exterior musculature and the interior skeleton\textsuperscript{16} and allow the periosteum to remain intact and attached to the bone, even after severe trauma occurs. In certain bones, Sharpey’s fibers anchor tendons and ligaments to the bone. Periosteum is absent at sites of tendon attachments. As tendon and ligament attachments vary by bone, periosteum morphology is highly variable between bones and even within bones\textsuperscript{21}.

The periosteum is often thought to behave as a mechanically stabilizing boundary membrane which functions in skeletal growth and musculoskeletal biomechanics\textsuperscript{16,18}. Damage to or absence of the periosteum is known to cause developmental abnormalities. For example, in bones without periosteum, the development of a substantial layer of regenerated bone adjacent to intact cortical bone is unlikely\textsuperscript{29}. The anatomy of the periosteum, as well as its osteoinductive and nutrient transport and inductive capabilities\textsuperscript{30}, are well characterized. Characterization of the mechanics of the periosteum will allow for a better understanding not only of the periosteum’s mechanical role as the bounding element of bones as organs, but also of the mechanical environment in which PDCs reside. Characterization of the mechanical environment of PDCs will provide knowledge valuable for the fabrication of biomimetic tissue engineered periosteum. Ultimately, knowledge of periosteal mechanobiology will allow the use of
native and tissue engineered periosteum to become common place in regenerative medicine.

3. Periosteum Derived Cells

PDCs are a subpopulation of mesenchymal stem cells (MSCs) that can be isolated from the periosteum. PDCs are commonly used for bone and cartilage tissue engineering applications due to their ability to differentiate into tissues of mesodermal origin, specifically bone and cartilage. Current methods for isolating PDCs from periosteum include enzymatic digestion or explant culture. In enzymatic digestion, the tissue is digested by an enzyme such as collagenase, leaving behind progenitor cells from the cambium layer as well as fibroblasts from the fibrous layer. In explant culture, minced pieces of periosteal tissue are placed cambium side down on tissue culture plastic and covered with cell culture media. Seven to ten days are allowed to pass during which PDCs migrate out of the cambium layer and attach to the tissue culture plastic. After this period of time, cells are trypsinized and passaged to obtain the desired PDCs.

Few studies have characterized surface antigens unique to PDCs. Two recent studies characterized surface antigens of PDCs isolated via enzymatic digestion from human proximal tibial periosteum. One study classifies PDCs as CD90 PE, CD105 PE, CD29 FITC, CD73, CD34, CD45 and HLA-DR FITC positive while the second classifies PDCs as Stro-1, CD34, and ALP positive. Like other stem cells, the number of PDCs residing in the cambium layer is known to decline with age. PDCs play an important role in modulating bone and cartilage formation during growth and healing. Recent research has focused on identifying the mechanobiological properties of both
PDCs and the tissue in which they reside in order to understand their role throughout growth and development.

4. Periosteal Cell Mechanics

Extensive research has been performed to classify the structural adaptations of MSCs in response to fluid shear stress, substrate stiffness and seeding density\(^{40}\). Substrate stiffness and cell shape are known to control MSC fate decisions including self renewal and lineage commitment. Topographic changes also influence MSC fate and exogenous dilatational and deviatoric stresses modulate changes in MSC gene expression indicative of early fate decisions\(^{41-48}\). Furthermore, mechanical signals are known to affect growth and differentiation of MSCs (Figure 2). Mechanical stimuli are increasingly recognized as key regulators of cell structure and function\(^{40,43,49}\). The ability of a cell to sense mechanical forces, transmit them to the interior or to other cells, and transduce mechanical signals into biochemical signals forms the concept of cellular mechanobiology\(^{40,49}\).

\[\text{Figure 1.2: Mechanical signals known to regulate cell volume, shape, proliferation rate and differentiation.}\]
As PDCs are a sub-population of MSCs, it is often assumed that PDCs will respond in a similar manner as MSCs in the same mechanical environment. However, PDCs are currently only broadly characterized and little is known about their morphology. Research indicates that the phenotypical and morphological properties of PDCs differ from bone marrow derived mesenchymal stem cells (BMSCs) in response to similar environments\textsuperscript{50}. PDCs and BMSCs cultured in the same osteo-inductive medium have shown different growth patterns, with PDCs reaching confluence 5-7 days earlier than BMSCs\textsuperscript{33}. Furthermore, PDCs and BMSCs have been shown to differentiate along different lineages when cultured on the same roughened titanium surface\textsuperscript{52,53}. It is therefore important to investigate periosteal cell mechanics independently of MSC mechanics.

The native environment of PDCs is mechanically regulated by a combination of tension and shear (given that the periosteum itself exhibits different moduli of elasticity in the longitudinal and circumferential directions, discussed below). The intracellular tension PDCs experience is suggested to regulate long bone growth\textsuperscript{34,36}. Surgical release of the inherent tension within the periosteum alters the local mechanical environment of PDCs. Periosteal release has been shown to increase cambium cell proliferation as early as one hour after periosteal release as measured by mRNA expression for BMP-2\textsuperscript{36}, a growth factor commonly expressed during bone and cartilage development. Histological analysis indicates that four-sided periosteal release results in greater bone formation compared to two-sided periosteal release, indicating that cells are sensitive to the magnitude, and potentially to the direction, of mechanical stimulus\textsuperscript{36}. 
PDCs' capacity to carry intracellular tension through their active microfilament network has been postulated to regulate a signaling cascade which in turn is responsible for the expression of soluble factors that modulate cartilage growth. The stiffness of the culture surface determines the magnitude of intracellular tension placed on the actin microfilament network. PDCs were cultured on substrates of varying stiffnesses which allowed cells to generate various magnitudes of intracellular tension. Conditioned medium from these cell cultures was added to cultures of intact or periosteum stripped embryonic chick tibiotarsi to evaluate the growth response. Culture medium from cells grown on low stiffness substrates (3kPa) resulted in greater cartilage growth of periosteum stripped bones compared to medium from stiffer substrates (80 kPa). Furthermore, culture of stripped tibiotarsi in medium taken from cells cultured on 80 kPa substrates and treated with cytochalasin D to disrupt the actin microfilament network showed significantly increased cartilage growth. This indicates a mechanobiological feedback mechanism between growing cartilage and tension in the periosteum in which expression of soluble growth inhibitors is regulated by the intracellular tension of PDCs.

In addition to being regulated by tension in the local microenvironment, PDCs respond to the topographical surface on which they are cultured, culture density, shear fluid flow, dynamic fluid pressure (DFP), and mechanical strain. PDCs cultured on machined and acid etched titanium surfaces proliferate at significantly different rates, with cells cultured on the machined surface exhibiting increased proliferation. The acid etched surface was shown to promote chondroblastic differentiation of PDCs while suppressing osteoblastic differentiation. Culture density of PDCs has been found to affect the level of type II collagen mRNA expression, a marker for chondrogenesis. The
level of type II collagen mRNA was significantly higher in PDCs cultured in a high
density, micromass culture system compared to cells cultured in a monolayer\textsuperscript{38}. Furthermore, PDCs cultured in a micromass culture system in the absence of TGF-\(\beta\)3, a chondrogenic growth factor also exhibited type II collagen expression, indicating mechanical signaling alone can cause PDCs to differentiate\textsuperscript{38}. Culture of periosteal explants in spinner flask bioreactors resulted in a fourfold increase in cartilage yield compared to static culture conditions\textsuperscript{31}. The majority of tissue growth occurred in the longitudinal direction, indicating PDCs are sensitive to the direction of applied shear stress. Results of shear flow studies on stem cells must be interpreted with caution as consistency of flow and minimization of turbulent flow are often of concern\textsuperscript{31}.

DFP has been shown to induce PDC proliferation in vitro\textsuperscript{35}. Periosteal explants are subjected to cyclic hydrostatic pressure during culture using a pneumatically driven membrane chamber which creates dynamic pressurization in the gas phase. Upon comparison to control explants, samples cultured under DFP expressed 60\% higher DNA content after three days of culture, representative of an increase in cell proliferation. Proliferating cells were found to be localized in the fibrous layer on day two of culture and progressed to the cambium layer by day three. Immunostaining with proliferating cell nuclear antigen confirmed that, on day four of culture, explants exposed to DFP showed a significantly greater number of proliferating cells localized in the cambium layer of the periosteum\textsuperscript{35}. Thus, PDC proliferation during periosteal chondrogenesis can be stimulated by DFP. In addition, small magnitude strains comparable to those experienced by the periosteum in vivo have been found to stimulate PDC proliferation\textsuperscript{54}. Small magnitude mechanical strains have also been found to upregulate Runx2 and
ColIa1, transcription factors critical for osteoblast differentiation\textsuperscript{55}. One hypothesis to explain this increase in cell proliferation is that a paracrine signaling mechanism exists in which cells in the fibrous layer release soluble factors such as Runx2 in response to mechanical stimuli, stimulating the PDCs in the cambium layer to divide and differentiate\textsuperscript{35}.

PDCs reside in a mechanobiologically dynamic environment. Model embryonic mesenchymal stem cells (C3H3t1/2) have been shown to be more than 1000x more sensitive to exogenous mechanical stimuli than terminally differentiated cells, changing their baseline gene expression significantly after exposure to short term (30 min) and small magnitude shear stresses (0.1 dyn/cm\textsuperscript{2})\textsuperscript{42}. Pluripotent cells\textsuperscript{49}, including PDCs have been shown to exhibit mechanical sensitivity as well\textsuperscript{54,55}. Mechanical stimulation of PDCs is thought to stimulate paracrine signaling pathways\textsuperscript{34-36}, leading to the absence of production of soluble factors which stimulate cell proliferation and differentiation. The specific cells stimulated (cambium vs. fibrous layer PDCs) remain to be elucidated. Further research is necessary to identify specific soluble factors released via different means of mechanical stimulation of PDCs and how these stimuli and soluble factors interact to influence cell proliferation, cell migration (e.g. egression from the periosteum), and lineage commitment.

5. Periosteum Tissue Mechanics

Understanding the mechanical properties of the periosteum at the tissue level is necessary to predict the environment of the pluripotent and osteochondroprogenitor cells known to reside within the periosteum\textsuperscript{14}. Furthermore, knowledge of periosteum
mechanical properties may be key to development of novel materials designed to replace periosteal function or to mimic periosteum’s smart properties. Since periosteum exhibits composite structure at multiple length scales and viscoelastic behavior over a range of time scales, the measurement and description of a given property should always reference the length and time scale of interest.

It is understood that PDCs taken from a variety of animals and bones possess the ability to generate bone \textit{de novo} \textsuperscript{3,5,56-58}. Research has shown that net changes in periosteal strain during stance shift loading after surgery correlate to rapid \textit{de novo} bone generation in critically sized defects\textsuperscript{13}. Therefore, mechanical signaling at the tissue level may be responsible for the initiation of bone regeneration at the cellular level. Determination of the mechanobiological environment in which PDCs reside will give insight into how the regenerative capacities of these cells are turned on to form new bone.

\textit{Ex vivo} periosteal tissue mechanics have been tested primarily using one of two testing methods. In the first method, periosteal mechanics are investigated \textit{in situ}, while the tissue is still anchored to the bone. The second, more commonly used method involves harvesting periosteum from intact bones and investigating solely the tissue mechanics.

Periosteum stabilizes bones mechanically during failure. To investigate the biomechanical capacity of the periosteum in intact long bones, femora and tibiae of Wistar rats were subjected to a destructive three-point-bending test protocol. The ultimate strength, stiffness, energy absorption, and deflection were measured in bones stripped of periosteum and bones with intact periosteum. For the femora, all parameters measured exhibited a statistically significant difference, with periosteum-covered femora displaying
higher values compared to periosteum-stripped femora. In the tibia, only energy absorption and deflection were significantly higher, indicating that the mechanical role of periosteum varies between long bones\textsuperscript{22}. In bones with preserved periosteum, fractured bone ends remained in close apposition and the periosteum remained intact at the conclusion of the bending test. In contrast, periosteum-stripped bones fractured completely, indicating that periosteum plays a mechanical role in stabilizing bone fractures\textsuperscript{22}.

Mechanical loading of periosteal tissue stimulates changes in behavior of the cells within the tissue. The mechanical response of the periosteum to cyclic loading and the effects of loading frequency were studied in the ulnae of adult female rats subjected to dynamic loading at frequencies of 1, 5, and 10 Hz for 360 cycles/day with peak loads ranging from 4.3 to 18 N. After two weeks of loading bone formation on the periosteal and endocortical surfaces was measured. Periosteal bone formation increased in a dose-dependent manner with peak load and compressive strain. No significant bone formation was seen on the endocortical surface for any loading frequency. To determine the effect of loading frequency on periosteal bone formation, a mathematical model was developed in which osteogenesis was assumed to be proportional to the bone tissue strain rate, which in turn was proportional to the extracellular fluid shear stress divided by the extracellular stiffness. This model effectively predicted periosteal bone formation rate at all loading frequencies, showing that mechanical loading at the tissue level modulates the cellular level via fluid shear stresses\textsuperscript{59}. In a separate study using the same rat ulna end loading model to induce fatigue fracture, rapid proliferative woven bone from the
periosteum was evident at one week after fracture and had consolidated completely to heal the fracture within one month\textsuperscript{60}.

Further studies demonstrate the effects of tissue level mechanical loading on cell behavior. Cyclic bending loads have been applied to stimulate bone regeneration from the periosteum\textsuperscript{56}. In one study, a vascularized flap of periosteum was resected from the rabbit tibia and subsequently sutured to the medial side of the knee. The knee underwent mechanical loading as rabbits were allowed to move spontaneously outside their cages for one hour per day. Four days after surgery chondrogenic differentiation of mesenchymal precursor cells was observed. The initial cartilage generated was replaced by fibrous tissue and bone, 15 to 30 days post surgery. At 30 days a segment of long bone comparable in size to the implanted periosteal flap had formed along the medial side of the knee\textsuperscript{56}. The results of this study once again show mechanical loading at the tissue level is transduced to the cellular level, resulting in the formation of new bone from periosteal tissue.

Recently new methods were developed to measure the pre-stress of healthy periosteum \textit{in situ}. Studying fresh ovine femoral periosteum resected from the mid-diaphysis of the femur, pre-stress was measured as shrinkage of the periosteal tissue upon release from the underlying bone. While it has been known for some time that the periosteum retracts upon release from the underlying bone\textsuperscript{36}, this study quantified the amount of retraction for the first time. By measuring shrinkage after release of the periosteum from the Sharpey’s fibers that anchor the tissue to the bone, it was possible to estimate the amount of endogenous tension (prestress) present in native periosteum. Periosteum from all aspects of the bone experienced an approximately 50% decrease in
area immediately upon release from the underlying bone. Significantly more shrinkage occurred in the axial direction, parallel to the axis of the bone, compared to the circumferential or transverse direction for all aspects\textsuperscript{14}. Hence, not only is the periosteum anisotropic (shows different mechanical behavior dependent on direction of loading) but it also resides in a mechanically loaded state \textit{in vivo} (pre-stress).

The long bones of vertebrates are subjected to substantial mechanical loads on a daily basis during activities such as walking, running, and jumping. Bones and the attached periosteum experience increased mechanical stresses during fracture and limb lengthening. The mechanical properties of resected periosteum under different loading conditions have been characterized using a number of experimental protocols. Experimentally determined stress-strain behavior of resected periosteum from ovine femora, avian tibiotarsus, bovine tibia and swine metacarpus has been reported in the literature\textsuperscript{14,18,61,62} (Table 1).

\textbf{Table 1.1:} Elastic moduli of resected periosteum from different bones and animals. Confidence intervals for the elastic moduli are listed in parentheses.

<table>
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<th>Region</th>
<th>E Toe (MPa)</th>
<th>E Axial (MPa)</th>
<th>E Circ (MPa)</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine Femur</td>
<td>1.93 (0.79, 3.06)</td>
<td>25.67 (18.8, 32.5)</td>
<td>4.41 (3.19, 5.62)</td>
<td>\textsuperscript{14}McBride et al., 2011</td>
</tr>
<tr>
<td>Pig Metacarpus</td>
<td>N/A</td>
<td>79 (42, 116)</td>
<td>96 (64,128)</td>
<td>\textsuperscript{18}Popowics et al., 2002</td>
</tr>
<tr>
<td>Chick Tibiotarsus</td>
<td>3.4 (1.5, 5.2)</td>
<td>230 (140, 320)</td>
<td>N/A</td>
<td>\textsuperscript{61}Bertram et al., 1998</td>
</tr>
<tr>
<td>Bovine Tibia</td>
<td>0.43 (0.16, 0.7)</td>
<td>51 (15, 87)</td>
<td>72 (22,122)</td>
<td>\textsuperscript{62}Uchiyama et al., 1998</td>
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In all studies listed in the above table, the bones from which periosteum was to be obtained were resected immediately upon death of the animal. Overlying muscle and tendon insertions were cleaned from the bone prior to periosteal removal. In certain
instances, bones with intact periosteum\textsuperscript{14} or resected periosteum sheets\textsuperscript{18,62} were cryopreserved until the time of experiment. In some instances of mechanical testing, periosteum was removed using a template that allowed for the tissue to be kept at its precise \textit{in vivo} length\textsuperscript{14,61}. In other studies, observable tissue shrinkage occurred once tissue was removed from the bone prior to insertion in the test fixture\textsuperscript{18,62}. Samples resected from the axial\textsuperscript{14,18,61,62} and circumferential\textsuperscript{14,18,62} orientations of long bones have been tested. In all cases, samples were secured in a set of grips designed to prevent the tissue from slipping during testing. A controlled loading rate ranging from a high speed of 0.42 mm/s\textsuperscript{62} to a low speed of 0.004 mm/s\textsuperscript{14} was applied until samples reached failure. In one instance, samples were preconditioned for 3-4 cycles before loading to failure\textsuperscript{18}. The force experienced by the tissue due to loading was measured by a load cell in all instances. Collected data was used to generate periosteal stress-strain curves. For all animals and bones, periosteal stress-strain curves obtained for axial samples generally exhibit a compliant toe region followed by a much stiffer elastic region (Table 1). The elastic modulus for axial samples varies depending on the animal and bone from which the sample was obtained. Circumferential samples exhibit highly variable behavior dependent upon the bone and animal of origin (Table 1).

To date, the only report of anisotropic mechanical properties in periosteum are from measurements of the ovine femur. Interestingly, bone denuded of periosteum shows robust regeneration of periosteum over time, but the thickness of regenerated periosteum does not correlate to the loading history to which the tissue is exposed\textsuperscript{63}. Unpublished measurements show that periosteal thickness varies significantly depending on the bone, site on the surface of a particular bone, within and between animals of experimental
cohorts (Evans, unpublished data). Similar to the structural properties, the mechanical properties of the periosteum are site specific, depending on the bone, location on the surface of the bone, and the species and age of animal of origin. Given structure-function relationships in biomaterials, the varying mechanical properties of long bone periosteum likely indicate varying tissue composition and collagen fiber alignment\textsuperscript{14,19,61}.

In addition to the long bone periosteum, the mechanical properties of swine zygomatic arch and mandible\textsuperscript{18} and human nasal\textsuperscript{64} periosteum have been characterized. The mechanical properties of periosteum from swine zygomatic arch and mandible showed no directional differences as compared to the swim metacarpal in which directional differences were noted\textsuperscript{18}. Furthermore, shrinkage of the tissue along the long axis varied greatly between the two bones while shrinkage in the circumferential direction did not vary. Human nasal periosteum has been tested and found to be less extensible than fascia. Furthermore, nasal periosteum exhibited a higher tensile strength than fascia, indicating it may be suitable for implant fixation\textsuperscript{64}. These results further indicate the variability of the mechanical properties of periosteum amongst bones. A limitation to consider is that the above mentioned studies characterized periosteum obtained from bones that do not experience a significant amount of mechanical loading, although it may be argued that these bones, especially the mandible, experience loading of varied direction, on a daily basis.

The degree to which periosteal tissue serves as a functional interface between muscle and bone is reflected by greatly varying elastic moduli and failure properties of periosteum in varying animals and anatomical locations. This evidence as a whole suggests that the periosteum is a highly specialized, mechanosensitive tissue. The degree
to which periosteum serves as a functional barrier between the muscle and bone is an area of intense current interest.65,66 Whereas periosteum is sometimes assumed to exhibit complete barrier function, acting as an outer “seal” to bone,67 it has been postulated that the robust regenerative capacity of the periosteum to infill defects derives from pluripotent cells that ingress, via the periosteum, to the defect site.3,12,15 Recent studies indicate that the periosteum may exhibit directionally dependent permeability and that permeability is highly dependent on the stress-state of the tissue.65,66

The mechanical response of the periosteum to injury may greatly vary dependent upon the location and severity of injury. For example, a mild to moderate tibial fracture may result in fracture of the bone but allow the periosteum to remain intact, thus keeping the two ends of fractured bone in close apposition. In this example, the periosteum would operate in the linear elastic region of the stress-strain curve14 and only small stress signals would be sent to PDCs, initiating proliferation and differentiation. In a more severe case, such as a fractured femur, the periosteum as well as the bone may fracture. The high stiffness incurred by the periosteum just prior to fracture would correlate to the linear region of the stress-strain curve14 (Figure 3). The large strains experienced would be transduced to PDCs residing in the inner cambium layer and stimulate rapid proliferation and differentiation of all periosteal progenitor cells from local as well as surrounding areas.
Figure 1.3: Stress-strain properties of ovine femoral periosteum removed from the A) axial and B) circumferential orientations of the bone. Axial periosteum exhibits a high stiffness (E = 25.67 MPa) in the linear region of the stress-strain curve. Used with permission from [14].

6. Bridging the Cell-Tissue Level: Tissue Engineered Periosteum

Although a number of studies have used native periosteum to heal critical size bone defects\(^5,6,56-58\), alternatives are sought for the same when periosteum availability is limited. In many cases native periosteum contains too few PDCs or has been damaged therefore limiting the tissue available for use. It is therefore desirable to create a tissue engineered periosteum (TEP) for use in these instances.

Many attempts to create a successful TEP have occurred over the last two decades. Early attempts at designing TEP focused on creating a confluent sheet of cells mechanically robust enough to be removed from the tissue culture plastic without tearing\(^68\). Few studies have characterized the mechanical properties of TEP. Current TEP is often intended for use in oral applications, where it would experience significantly
less mechanical stress and strain than in a dynamically loaded environment such as the femur. It is imperative to consider the mechanical properties of the native tissue in order to design TEP to be used in mechanically loaded environments such as the long bones of the leg.

TEP has been created using a variety of natural and synthetic biomaterials in combination with BMSCs and PDCs (Table 2). Porcine small intestinal submucosa (SIS) seeded with BMSCs is one popular combination for creating TEP\(^{68,69}\). Use of a natural scaffold such as SIS eliminates concerns related to biodegradability and toxicity that are often associated with synthetic polymer biomaterials. SIS periosteum membranes were 100-200 μm thick after seven days of growth. The membranes were somewhat permeable and allowed for the diffusion of nutrients from interstitial fluid to the cells. Membrane mechanics were characterized qualitatively and found to be of a flexible texture similar to natural bone membrane\(^{68,69}\).

**Table 1.2:** Published work designing TEP \(^{30,68-84}\).

<table>
<thead>
<tr>
<th>Publication</th>
<th>Animal</th>
<th>Source</th>
<th>Cell Type</th>
<th>Scaffold</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhao et al., 2008</td>
<td>Rabbit</td>
<td>Femoral Medullary Cavity</td>
<td>BMSC</td>
<td>SIS</td>
<td>Radial defects</td>
</tr>
<tr>
<td>Zhao et al., 2011</td>
<td>Rabbit</td>
<td>Femoral Medullary Cavity</td>
<td>BMSC</td>
<td>SIS</td>
<td>N/A</td>
</tr>
<tr>
<td>Ma et al., 2011</td>
<td>Rabbit</td>
<td>Iliac Crest</td>
<td>BMSC</td>
<td>N/A</td>
<td>Mandibular fracture</td>
</tr>
<tr>
<td>Fan et al., 2010</td>
<td>Human</td>
<td>N/A</td>
<td>BMSC</td>
<td>Collagen membranes</td>
<td>Skull defects</td>
</tr>
<tr>
<td>Schönmer et al., 2009</td>
<td>Rat</td>
<td>Femoral and tibial medullary cavity</td>
<td>BMSC</td>
<td>Acellular human dermis</td>
<td>Mandibular defects</td>
</tr>
<tr>
<td>Warnke et al., 2009</td>
<td>Human</td>
<td>Mandible</td>
<td>PDC</td>
<td>Collagen Membrane</td>
<td>N/A</td>
</tr>
<tr>
<td>Puelacher et al., 1996</td>
<td>Calf</td>
<td>Humerus</td>
<td>PDC</td>
<td>PGA</td>
<td>Femoral defects in rats</td>
</tr>
<tr>
<td>Keskin et al., 2008</td>
<td>Rat</td>
<td>Tibia</td>
<td>PDC</td>
<td>SIS</td>
<td>Critical size calvarial defects</td>
</tr>
<tr>
<td>Gassling et al., 2010</td>
<td>Human</td>
<td>Mandible</td>
<td>PDC</td>
<td>Collagen membranes with platelet rich fibrin</td>
<td>N/A</td>
</tr>
<tr>
<td>Perka et al., 2000</td>
<td>Rabbit</td>
<td>Tibia</td>
<td>PDC</td>
<td>Fibrin gel or Ethisorb-fleece cylinders</td>
<td>Ulna defects</td>
</tr>
<tr>
<td>Arnold et al., 2002</td>
<td>Human</td>
<td>Tibia</td>
<td>PDC</td>
<td>Ethisorb polymer scaffolds</td>
<td>N/A</td>
</tr>
<tr>
<td>Ribeiro et al., 2010</td>
<td>Dog</td>
<td>Mandible</td>
<td>PDC</td>
<td>Either no scaffold or PTFE</td>
<td>Mandibular defects</td>
</tr>
<tr>
<td>Kawase et al., 2010</td>
<td>Human</td>
<td>Mandible</td>
<td>PDC</td>
<td>Hydroxyapatite (HA) blocks</td>
<td>Periodontal defects</td>
</tr>
<tr>
<td>Yamamiya et al., 2008</td>
<td>Human</td>
<td>Mandible</td>
<td>PDC</td>
<td>N/A</td>
<td>Periodontal defects</td>
</tr>
<tr>
<td>Knothe Tate et al., 2011</td>
<td>Ovine</td>
<td>Femora</td>
<td>PDC</td>
<td>Elastomeric membrane</td>
<td>Critical size femora defects</td>
</tr>
<tr>
<td>Kawase et al., 2011</td>
<td>Human</td>
<td>Mandible</td>
<td>PDC</td>
<td>Porous poly(L-lactic acid) membranes</td>
<td>N/A</td>
</tr>
<tr>
<td>Lee et al., 2011</td>
<td>Human</td>
<td>Mandible</td>
<td>PDC</td>
<td>Polydioxanone (PDO)/Pluronic F127</td>
<td>Swine mandibular defects</td>
</tr>
</tbody>
</table>

Scaffold free TEP has been grown from BMSCs cultured in osteogenic media. At 10 days of culture a thin membrane with poor resistance to external forces had formed. Further culture until day 17 resulted in a mechanically robust sheet with a tissue structure similar to the cambium layer of native periosteum. In the above studies, the mechanical properties of the TEP were not characterized quantitatively.

In addition to BMSCs, many varieties of PDCs have been used to engineer TEP. PDCs have been used to construct TEP with and without the use of scaffolds. Rat
femoral shaft defects were successfully healed using TEP constructed from PDCs seeded on a polyglycolic acid (PGA) scaffold\textsuperscript{74}. TEP constructs constructed from porcine SIS and PDCs were not as effective as autologous bone grafts in healing rat calvarial critical size defects\textsuperscript{75}. A TEP construct composed of an elastomer surgical membrane filled with autologous periosteum strips was found to promote healing of critically sized ovine femoral defects\textsuperscript{82}. The membrane allowed for the directional delivery of periosteal factors to the defect. Studies are currently in progress to determine the material and mechanical properties of the elastomer membrane.

Scaffolds used to create TEP are designed to influence cell differentiation via cell-matrix interactions. It is therefore important to classify the mechanical properties of TEP in order to match those of the native tissue. Furthermore, creating TEP with variable stiffness and elastic moduli is desirable as the local mechanical environment in which the tissue is implanted varies from bone to bone. In a load bearing bone such as the femora or tibia, TEP with a high elastic modulus would be desired whereas a TEP with a lower elastic modulus could be used in a non-mechanically loaded environment such as the mandible.

7. Summary

As the behavior of PDCs in response to tissue level mechanical forces is further characterized, it will become possible to create a biomimetic TEP. In addition to the tensile mechanical properties of the tissue, it is important to characterize other mechanical and material properties such as the fatigue life of the tissue and the permeability, pore size, and transport gradients. Together with what is already known
about the periosteum, knowledge of these properties will allow for an understanding of the mechanobiological environment in which PDCs reside.

Studies described in this paper serve to further identify both the mechanical and biological environment in which the pluripotent and osteochondroprogenitor cells of the periosteum reside in order to better understand the role of periosteum in both health and disease. The mechanobiological properties of the periosteum are classified at both the tissue and cellular level in order to understand the structure-function relationship of the periosteum at multiple length scales. The results of these studies are meant to be incorporated as design criteria for the development of periosteal replacement membranes as well as incorporated into predictive computational models.

Chapter 2 begins at the tissue level of the periosteum and investigates the permeability and fluid transport properties of the tissue. A custom-designed permeability tester is used to apply a controlled volumetric rate flow rate to tissue samples. The pressure drop across the periosteum specimen is measured using a mechanical tester and Darcy’s Law is used to calculate the hydraulic permeability coefficient, $k$, of the periosteum samples. Femoral and tibial periosteum samples are tested under a variety of physiological as well as physiologically relevant conditions. Periosteum permeability exhibits loading rate dependent behavior; an increase in the flow rate by one order of magnitude results in a permeability increase by one order of magnitude. In addition, femoral periosteum samples exhibit bidirectional permeability properties at a high flow rate, with the tissue being most permeable in the bone→muscle direction. Lastly, the permeability properties of periosteum are dependent upon the stress state of the tissue and the inherent pre-stress of the periosteum serves to decrease fluid permeability.
Knowledge of the permeability properties of the periosteum is important for incorporation into predictive computational models and design of periosteal replacement membranes.

Chapter 3 studies the properties of the periosteum at the cellular level. The junctional adhesion markers of periosteum derived cells are classified in order to understand the molecular mechanisms by which the individual cells of the tissue interact. Periosteum derived cells are cultured on a solid-supported lipid bilayers (SLBs) functionalized with N-cadherin to mimic the cell-cell interactions experienced by *in vivo* periosteum progenitor cells (PDCs). Culture of PDCs on the functionalized SLBs results in an upregulation of the pre-mesenchymal condensation transcription factor *Runx2*. The upregulation of *Runx2* provides early evidence that N-cadherin functionalized SLBs promote differentiation of PDCs into osteoblasts via intramembranous ossification. The development of a SLB functionalized with cell-cell adhesions markers provides a model membrane system for investigate the molecular mechanisms by which cells interact and provides a culture platform with the ability to guide cell fate and lineage commitment.

Finally in Chapter 4 the conclusions of this body of research are presented. The importance of this work is discussed in context to understanding the mechanobiological properties of the periosteum as well as the role periosteum plays as bone’s bounding membrane. Lastly, possible future directions for this topic of research are discussed.

References


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Chapter 2

Periosteum Permeability Exhibits Mechanically Responsive Behavior

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Intended for submission to Bone, March 2012

Abstract

The periosteum serves a bone’s epithelial bounding membrane and contains mechanically sensitive progenitor cells capable initiating bone regeneration. Critical size bone defect healing has been shown to be maximal when cells egress from periosteum left in situ around the defect. Recent studies have characterized the dynamic mechanical environment of periosteum progenitor cells but to date no studies have investigated the permeability and bidirectional transport properties of the periosteum. To better understand the extent to which the periosteum serves a bone’s bounding membrane permeability testing was performed on ovine periosteum. A custom designed permeability tester was used to provide a controlled, volumetric flow of phosphate buffered saline through resected periosteum samples. Darcy’s Law was used to calculate the hydraulic permeability coefficient, k, of the periosteum. In response to convective fluid transport, periosteum exhibits loading rate and directionally dependent behavior. Furthermore, the stress state of the tissue plays an important role in regulating fluid transport through the tissue. Knowledge of the permeability properties of the periosteum

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will allow for incorporation of the periosteum into computation models that can predict the cellular environment of periosteal progenitor cells as well as for the development of biomimetic tissue engineered periosteum.

1. Introduction

The permeability properties of the periosteum are currently uncharacterized. A characterization of these properties is necessary to understand the dynamic mechanical environment in which the pluripotent cells of the periosteum reside as well as the role periosteum plays in controlling fluid flow into and out of the bone as bone is the largest reservoir of calcium ions in the body and thus a “living electrophoresis column”\(^1,2\). Furthermore, knowledge of periosteum permeability is necessary to optimize the flow directing properties of periosteal replacement membranes\(^3\).

The periosteum serves as a stabilizing boundary membrane to the bone it envelopes and is known to contain pluripotent and osteochondroprogenitor cells\(^4-6\). Structurally, the periosteum is a bi-layered membrane consisting of an outer fibrous layer and an inner cellular, or cambium, layer which provides a habitat for adult mesenchymal stem cells known as periosteal derived cells (PDCs)\(^4\). The periosteum is highly vascularized and contains a blood supply in order to provide nourishment to bone\(^7\). Histological studies of periosteal architecture reveal an axially aligned collagen structure in the mid-diaphysis\(^8\) and a high density of elastin\(^9,10\). Furthermore, recent work has found PDCs to express \textit{zona occludens} proteins\(^11\), hallmarks of tight junctions between cells, which confer barrier membranes their directional transport properties. Therefore, it is expected that the periosteum exhibits anisotropic fluid transport properties, but this theory has not been tested to date.
A recent study characterized the material and mechanical properties of ovine femoral periosteum and showed periosteum to be highly pre-stressed and to have an anisotropic elastic modulus, with the axial elastic modulus of the tissue more than five times greater than that of the circumferential. Furthermore, a series of previously published studies have characterized the dynamic mechanical environment of periosteal progenitor cells using an ovine femora critical sized defect model. Periosteal cells have been shown to have the capacity to rapidly infill critical size defects with proliferative woven bone in as little as two weeks and complete bridging of the defect a 16 weeks post-surgery. Critical sized defect healing has been shown to be maximal when cells egress from periosteum left in situ around critical sized defects, followed by cells egressing from autologous periosteum transplants, or periosteum derived cells seeded on a collagen membrane, when encased by a flow directing periosteal replacement implant.

The permeability properties of bone have been studied extensively using both experimental and computational approaches. When small molecular weight tracers are subjected to convective transport via load induced fluid flow transport proceeds from the vasculature of the intramedullary canal into the pericellular spaces of the bone cortex and then tends toward the periosteum. The pericellular permeability of bone has been modeled computationally and a healthy cellular network devoid of cellular structures exhibits permeability on the order of $2.78 \times 10^{-16}$ m$^2$. Inclusion of cellular structures reduces permeability to the order of $10^{-17}$-10$^{-18}$ m$^2$. The permeability of a porous material such as bone or the pericellular network may be modeled using Darcy’s Law which is the scientific basis of fluid flow through a homogenous porous medium.
Darcy’s Law has been found valid for any Newtonian fluid and is a proportional relationship between \( v \), the bulk flow velocity through a specimen (m/s); \( \mu \), the viscosity of the fluid (Pa·s); \( L_s \), the thickness of the specimen (m); \( (P_d - P_u) \), the pressure drop over a given distance (Pa); and \( k \), the hydraulic permeability of the specimen (m\(^2\)). A high value of \( k \) indicates a material is highly permeable while a low value of \( k \) indicates a material through which it is difficult to transmit fluids. The periosteum consists of a cellular network and can therefore be modeled as a porous material using Darcy’s Law.

In addition to bone, the permeability properties of bidirectional permeable biomembranes such as the peritoneum\(^{24} \), nuclear envelope\(^{25} \), and blood-brain barrier (BBB)\(^{26} \) have been characterized. To our knowledge, no work to date has characterized the permeability properties of periosteum. Thus in order to further characterize the dynamic mechanical environment in which periosteum cells reside we sought to determine the permeability properties of ovine femoral and tibial periosteum. Specifically we hypothesize (i) periosteal permeability depends on the volumetric flow rate applied across the tissue, (ii) periosteal permeability is significantly different in prestressed versus non-prestressed samples and (iii) permeability is anisotropic with respect to the direction of flow through the tissue.

Our approach was to design a custom chamber allowing for the application of a controlled volumetric flow rate fluid to periosteum samples resected from ovine femora and tibiae. The hydraulic permeability, \( k \), of the periosteum samples was then calculated using Darcy’s Law.
2. Methods

2.1 Specimen Preparation

Periosteum samples were harvested from the femora or tibia of skeletally mature female sheep (n = 71, 36 mm diameter). Two cohorts of animals were used throughout the permeability studies. Cohort number 1 consisted of six 2 year old ewes, ¾ Hampshire and ¼ Dorset, 62.2-79 kg (Ohio Agricultural Research and Development Center, Wooster, OH). Cohort number 2 consisted of six 3 year old ewes, dorset, 52-60 kg (Archer Farms, Darlington, MD). Animals were cared for and euthanized in accordance with Case Western Reserve University Animal Resource Center guidelines under IACUC approved protocols (2010-0014).

Samples were either tested within four hours of animal euthanasia or cryopreserved until the time the time of testing. Previous research has indicated that the mechanical and material properties of ovine femoral periosteum are maintained during cryopreservation, (Zhen and Evans, unpublished studies). Samples tested the same day as euthanasia were treated using the following protocol. After animal euthanasia the hind limbs of the sheep were disarticulated from the hip. The skin and overlying muscle were removed to expose the femora or tibiae. The periosteum was carefully cleaned of all muscle and tendon insertions using tweezers and a #10 surgical blade. Periosteum samples were harvested from the mid-diaphyseal region of the anterior of the femora or tibiae (n = 2-3 per bone). For pre-stressed samples, samples were affixed to a circular, flexible plastic template 36 mm in diameter using super glue to allow for removal of the tissue at its in situ length and width (Figure 1). A periosteal lifter was then used to gently separate the Sharpey’s fibers from the underlying bone, resecting the periosteum. For
non-pre-stressed samples, 20 mm by 20 mm samples were removed by resection with a periosteum lifter. These samples experienced an approximately 54% reduction in area immediately upon removal from the underlying bone\textsuperscript{12}.

Samples subjected to cryopreservation were treated to the following protocol. Within one hour of euthanasia, the femora and tibiae were disarticulated and overlying skin was removed while preserving all overlying muscle. The femora and tibiae were then sealed in a Ziploc bag and stored in a -80\textdegree C cryofreezer. The sealed femora were left to thaw at room temperature for 24 hours prior to specimen testing. After thawing for 24 hours, the thick muscle tissue was trimmed back preserving a thickness of one inch of tissue from the bone surface. Then femora were resealed in a Ziploc bag and placed in an incubator (5\% CO\textsubscript{2}, 37\textdegree C) for one hour in order to raise the samples’ temperature to ovine body temperature while maintaining tissue hydration. Periosteum samples were then resected according to the protocol detailed above.

Figure 2.1: Pre-stressed periosteum sample attached to flexible, plastic template post-resection from underlying bone.

2.2 Permeability Tester Design
A custom designed permeability tester (Figure 2) allowed for the application of a controlled, constant (volumetric flow rate) flow of phosphate-buffered saline (PBS) through periosteum samples. The permeability tester consisted of a 5 mL syringe plunger (BD Safety-Lok Syringe #30558, polycarbonate) inserted into a 7.62 cm tall opening syringe tube (7.62 cm outside diameter, 12.06 mm inside diameter, PVC) with a 1.27 cm thick plate and four symmetrical thread holes (#10-32), a 1.27 cm thick specimen holding plate with a hole (12.06 mm diameter), four screws with washers (#10-32, 1 ½ inches), and a 29 mL prescription vial (polypropylene). Two O-ring seals located on the bottom of the syringe plunger and an additional O-ring on the bottom of the opening syringe created a fluid-tight seal and prevented lateral leakage of PBS from the tester.

Figure 2.2: Schematic of permeability tester design and experimental set-up.
2.3 Permeability Study

The permeability coefficient of periosteum was determined for 8 sample groups (n = 5-6 samples per group) (Table 1). The permeability coefficient of pre-stressed femoral periosteum was determined at two flow rates, a low flow rate of 0.5 μl/s and a high flow rate of 6 μl/s and for two directions of flow, from the bone $\rightarrow$ muscle (B $\rightarrow$ M) side of periosteum and the muscle $\rightarrow$ bone (M $\rightarrow$ B) side of periosteum (Figure 3A,B). The permeability coefficient of tibial periosteum was determined at a constant flow rate of 6 μl/s for pre-stressed and non-pre-stressed samples for both sample orientations. To maximize physiological relevance, the low flow rate corresponded to fluid flow induced via strain during normal gait$^{15,27}$ and the high flow rate is an estimated high threshold for interstitial flow through periosteum, based on blood flow rates measured in situ in ovine tibial periosteum$^{28}$.

Figure 2.3: Schematic of periosteum permeability test set up. A) Permeability was tested to mimic transport in the bone $\rightarrow$ muscle and muscle $\rightarrow$ bone directions. B) Periosteum samples were placed into the permeability tester either bone- or muscle-side upwards.
Table 2.1: Periosteum permeability sample groups. The independent variables in the experiment were bone, flow direction, flow rate and application of pre-stress to the periosteum during sample removal.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bone</th>
<th>Flow Direction</th>
<th>Flow Rate (μL/s)</th>
<th>Pre-stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Femur</td>
<td>Muscle→Bone</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Femur</td>
<td>Bone→Muscle</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>Femur</td>
<td>Muscle→Bone</td>
<td>0.5</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>Femur</td>
<td>Bone→Muscle</td>
<td>0.5</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Tibia</td>
<td>Muscle→Bone</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>Tibia</td>
<td>Bone→Muscle</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>Tibia</td>
<td>Muscle→Bone</td>
<td>6</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>Tibia</td>
<td>Bone→Muscle</td>
<td>6</td>
<td>No</td>
</tr>
</tbody>
</table>

For the permeability experiment, the opening syringe of the previously described permeability tester was inverted and filled with 5 mL of PBS (Gibco, Invitrogen, Carlsbad, CA) without creating air bubbles. The periosteum sample was positioned across the hole of the opening syringe with the desired tissue orientation (Figure 3B) and secured by the specimen holding plate with the washers and screws. A constant displacement was applied to the plunger using a mechanical tester (Enduratec ELF 3200, Bose Co, Eden Prairie, MN), pressing PBS through the specimen at room temperature and constant bulk flow rate for 200 or 2500 seconds depending on the flow rate. The hydraulic permeability, \( k \), was derived from the equation for Darcy’s Law (1) using MATLAB (Mathworks, Natick, MA)

\[
v = \frac{k(P_u - P_d)}{\mu L_s} \quad (1)
\]

where \( v \) is the bulk flow velocity through the specimen (m/s); \( \mu \) is the viscosity of PBS (1.05 x 10^3 Pa·s); \( L_s \) is the thickness of the specimen (m); \( P_u - P_d \) is the pressure drop across the specimen (Pa) where \( P_u \) is the upstream pressure at the start point and \( P_d \) is the downstream pressure at the end point; and \( k \) is the hydraulic permeability of the
specimen (m$^2$). In this experiment, $P_u$ was normalized to 0 Pa as the experiment was conducted under steady state conditions. $P_d$ was the average steady-state pressure as recorded by the mechanical tester.

### 2.4 Statistics

Permeability, $k$, was calculated for the specimens of each group. Groups were compared using one-way ANOVA with tukey pair-wise comparisons (S-PLUS, TIBCO Software Inc, Somerville, MA) between groups where $p < 0.05$ was defined as a statistically significant difference. In addition, a non-parametric, multivariate correlation analysis (Spearman’s $\rho$, S-PLUS, TIBCO Software Inc, Somerville, MA) was performed between all independent variables and permeability in order to determine the variables with significant influence ($p < 0.05$) on permeability.

### 3. Results

Femoral periosteum samples exhibit direction and loading rate dependent behavior. For the test conditions used in this experiment, the periosteal surface closest to the bone exhibits significantly higher permeability ($p < 0.05$, Figure 4) than the periosteal surface closest to overlying muscle at a high flow rate. In contrast, no significant difference can be attributed to the direction of flow at the low flow rate. Permeability is also significantly increased ($p < 0.001$, Figure 4) when flow rates are increased, proportional to the increases in flow rate. An increase in flow by one order of magnitude led to an increase in permeability by one order of magnitude. Table 2 lists the average hydraulic permeability, $k$ for each sample group.
Figure 2.4: Femoral periosteum permeability coefficients. Permeability was measured at two different volumetric flow rates (0.5 and 6 \( \mu \text{L/s} \)), as well as in two flow directions (muscle \( \rightarrow \) bone, bone \( \rightarrow \) muscle) for pre-stressed femoral periosteum samples. Error bars show 95\% standard error; *\( p<0.05 \), **\( p<0.001 \).

Table 2.2: Average hydraulic permeability coefficients, \( k \), for each sample group as calculated by Darcy’s law.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hydraulic permeability coefficient, ( k ) (x10(^{-5} ) ( \mu \text{m}^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>36.83</td>
</tr>
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<td>2.02</td>
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<td>27.57</td>
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<td>8</td>
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Tibial periosteum samples exhibit significantly lower permeability for pre-stressed samples ($p < 0.05$, Figure 5). Although not significant, tibial permeability tends to be greater in the bone→muscle direction for pre-stressed samples, the same direction in which femoral periosteum is significantly more permeable. Interestingly, the removal of pre-stress leads to tendency for the periosteum to be more permeable in the muscle→bone direction, although the difference is non-significantly (Figure 5).

**Figure 2.5:** Periosoteum permeability coefficients for tibial periosteum samples at a constant volumetric flow rate of $6 \, \mu$L/s. Application of pre-stress during periosteum sample removal significantly decreases the permeability of the tissue ($p<0.05$) for two tissue orientations (muscle→bone, bone→muscle). Error bars show 95% standard error; *$p<0.050$, **$p<0.001$. 
Correlation analysis of pooled data for all groups indicates flow rate and pre-stress to show significant ($p < 0.05$, Table 3) positive correlation with permeability. The independent variables bone and flow direction do not significantly influence periosteum permeability.

**Table 2.3:** Correlation analysis to determine relationships between independent variables and periosteum permeability. Spearman’s $\rho$ correlation coefficients and $p$-values between independent variables and permeability. Statistically significant correlations ($p < 0.05$) are highlighted in green and lack of significance is indicated by pink highlighting.

<table>
<thead>
<tr>
<th>Correlation Coefficients</th>
<th>Bone</th>
<th>Flow Direction</th>
<th>Flow Rate</th>
<th>Pre-stress</th>
<th>Permeability</th>
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<tr>
<td>Bone</td>
<td>X</td>
<td>-0.0217</td>
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<td>Permeability</td>
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<td>-0.0847</td>
<td>0.7335</td>
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</table>

<table>
<thead>
<tr>
<th>P-values</th>
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<th>Flow Rate</th>
<th>Pre-stress</th>
<th>Permeability</th>
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<td>0.5653</td>
<td>0</td>
<td>0.0008</td>
<td>X</td>
</tr>
</tbody>
</table>

**4. Discussion**

Experimental permeability studies show the periosteum to exhibit significant flow rate and stress state dependence. A higher flow rate correlates to higher periosteal permeability while a low flow rate corresponds to decreased periosteal permeability. Release of the inherent tension of periosteum results in increased tissue permeability. Furthermore, femoral periosteum shows significant anisotropy in flow direction at high volumetric flow rate while tibial periosteum does not show significant anisotropy in
response to flow direction. At a high flow rate of 6 μL/s, the periosteum shows significantly higher permeability in the bone→muscle direction than vice versa, which challenges current paradigms with regard to bone physiology and healing.

In the standard context, Darcy’s Law gives a linear relationship between the flow rate of fluid through a membrane and the driving pressure across the membrane. Therefore, the hydraulic permeability of the membrane depends only on pore size and is expected to be independent of the flow rate. The current study found significant positive correlation between periosteum permeability and flow rate. We hypothesize that this increase in permeability is attributable to the flow rate acting as a mechanical lever on the tissue and opening more pores within the tissue creating an increase in tissue permeability.

Periosteum and periosteum cells have been previously shown to be mechanically responsive to fluid flow when cultured in bioreactors that deliver a controlled shear stress to the tissue. Periosteum explants cultured in recirculating flow-perfusion bioreactors for a six week period showed approximately a fivefold increase in cartilage yield over explants cultured in static conditions. During this time period, the periosteal cells undergo proliferation and differentiation to initiate tissue growth. The processes of proliferation and differentiation require the reorganization of cells and their extracellular matrix components. This reorganization of cells and extracellular matrix components at the molecular level may lead to changes in the porosity and thus the permeability at the tissue level of the periosteum.

Tight junctions are an integral component of the extracellular matrix and are responsible for enabling the barrier membrane properties of epithelial tissues. Barrier
properties at the tissue level depend on the level of interaction between the proteins making up the tight junction and the mean number of strands comprising the tight junction\footnote{1}. Studies characterizing the permeability of the blood brain barrier, an epithelial membrane consisting of many tight junctions, have found that edema of the brain is associated with decreased tight junction organization and increased BBB permeability, indicating mechanical forces (ie. pressure) play a role in the regulation of epithelial tight junctions\footnote{2}. In addition, vascular endothelial cell monolayers exposed to high shear stress gradients have shown increases in transendothelial transport of dextran tracers, indicating endothelial barrier function as governed by tight junctions is regulated by shear stress\footnote{3}. Therefore, as the periosteum exhibits an epithelial morphology increased permeability proportional to the increase in flow rate is can be attributed to the degradation of tight junctions resulting in increased pore size.

The application of pre-stress during periosteum resection is necessary in order to maintain the tissue’s native properties as the tissue is in tension when attached to the bone by Sharpey’s fibers\footnote{4,5}. A 54% reduction in periosteum surface area occurs immediately upon removal of the tissue from the underlying bone when pre-stress is not maintained\footnote{6}. Allowing the tissue to freely shrink during removal significantly alters the permeability properties of the membrane with pre-stressed periosteum being significantly less permeable than non-pre-stressed periosteum. \textit{In vivo} the fibrous layer of the periosteum contains highly aligned collagen fibers\footnote{7} and a high density of elastin\footnote{8,9} providing mechanical strength and structural integrity to the tissue. In cortical bone, digestion of collagen from the extracellular matrix results in a significant increase in permeability\footnote{10}, demonstrating the role of ECM components in regulating fluid transport. When the
periosteum is allowed to shrink during removal from the bone, the highly oriented ECM structure of the fibrous layer is disrupted, likely resulting in an increase in pore size which is responsible for a subsequent increase in permeability. To picture this phenomenon, the periosteum can be thought to contain many rubber bands stretched axially along the length of the tissue and anchored by pins at various points along the bone. If these pins are removed, the rubber bands contract to a relaxed state and no longer possess any orientation. The resulting orientation may consist of large areas no longer encompassed by the rubber bands, allowing an area through which fluid may freely flow.

Femoral periosteum subjected to a physiologically relevant high flow rate is significantly more permeable in the bone→muscle direction than vice versa and thus exhibits anisotropy. This directional permeability indicates that signaling molecules from within bone may be transported into the surrounding muscle during when interstitial fluid flow within bone is high. Evidence for muscle and bone communicating at a molecular level via biochemical factors is supported by recent research which has shown conditioned media from MLO-Y4 osteocytes accelerates the differentiation of C2C12 myotubes. Pre-stressed tibial periosteum shows a non-statistically significant tendency to more permeable in the bone→muscle direction. The difference in permeability between the femur and tibia may be explained by the fact that periosteum experiences site-specific differences between and within bones. Specifically, femoral periosteum is surrounded by a thick layer of musculature whereas tibial periosteum is surrounded by a thinner layer of muscle which is easily separated from the underlying periosteum. Epithelial barrier membranes such as the peritoneum, nuclear envelope, and blood-
brain barrier (BBB)\textsuperscript{26,32} have also been shown to exhibit anisotropy during transport of fluids as well as small molecules and as thus bidirectional transport is a distinguishing feature of epithelial boundary membranes.

The present study is limited by the fact it only investigated the ability of physiological fluid to be transported across the periosteum. The permeability of the periosteum to small biological molecules known to reside in bone such as Ca\textsuperscript{2+} was not investigated in the present study but is of relevant interest. Furthermore, in order to maximize the number of samples per animal, periosteum samples were collected from both the femora and tibia of each animal; correlation analysis shows the bone from which samples were collected to have no significant influence on permeability. Lastly, the study is limited due to inherent differences in local periosteum microstructure.

The results of this study show that ovine periosteum demonstrates mechanically responsive and directionally dependent permeability properties. The inherent tension within native periosteum serves to decrease periosteum permeability in both the muscle\textendash\textrightarrow\text{bone} and bone\textendash\textrightarrow\text{muscle} directions, further indicating the structure of the periosteum plays an important role as bone’s bounding membrane. The permeability properties are important in understanding periosteal mechanobiology and demonstrate the extent to which periosteum exhibits properties of a smart biomaterial by allowing bidirectional and mechanically responsive fluid transport. These studies may have important implications with regard to the development of predictive computational models and periosteal replacement implants for use when healthy periosteum is absent. Recent studies have developed flow directing\textsuperscript{3} and microporous\textsuperscript{37} periosteal membranes for tissue engineering applications. Knowledge of periosteal permeability will allow for
structural optimization of tissue engineered periosteum in order to achieve biomimicry and ultimately a structure-function relationship capable of supporting bone regeneration.

Acknowledgements

We would like to thank the AO Research Fund (F-07-99k) for their financial support. We would also like to thank the staff of the Case Western Reserve Animal Research Center for their assistance in sample collection.

References


Chapter 3

Solid-Supported Lipid Bilayers as a Surface for Periosteal Cell Culture

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In preparation for journal submission, Spring 2012

Abstract

Solid-supported lipid bilayers (SLBs) provide a dynamic surface to study cell-cell and cell-matrix adhesions. Cell-cell and cell-matrix adhesions play an important role in skeletal condensation and regulate cell fate and lineage commitment. SLBs can be functionalized with cell-cell and cell-matrix proteins in order to mimic the environment cells encounter in vivo. The present studied elucidated the cell-cell adhesion proteins expressed by periosteum derived cells in order identify a cell adhesion protein optimal for SLB functionalization. The morphology and gene expression of periosteum derived cells seeded on SLBs functionalized with N-cadherin, a cell-cell adhesion protein expressed by mesenchymal stem cells, was investigated using phase contrast microscopy and real-time PCR. PDCs grown on N-cadherin functionalized SLBs show an upregulation of Runx2, a transcription factor expressed in pre-mesenchymal condensations as well as early osteogenic lineage commitment. Functionalized SLBs have the ability to act as a tissue patterning template by directing cell fate and lineage commitment.
1. Introduction

The periosteum is known to contain multipotent progenitor cells shown to have the capacity to infill critical sized defects with proliferative woven bone in as few as two weeks post-injury\textsuperscript{1,2}. In instances where native periosteum is insufficient, periosteal substitutes or biomimetic engineered periosteum tissue is desirable. Progenitor cells receive a combination of biochemical and biomechanical signals \textit{in vivo} which are responsible for guiding cellular proliferation and differentiation. A current challenge in engineering \textit{de novo} tissue formation is achieving the targeted differentiation of cells that will secrete extracellular matrix appropriate for the desired tissue function\textsuperscript{3}.

The periosteum serves as a stabilizing boundary membrane to the bone it envelopes and consists of an outer fibrous layer and inner cellular layer which provides a habitat for adult mesenchymal stem cells known as periosteal derived cells (PDCs)\textsuperscript{4-7}. PDCs are commonly used for bone and cartilage tissue engineering applications as they possess the ability to differentiate into osteoblasts and chondrocytes\textsuperscript{8}. Mechanical stimuli are increasingly recognized as key regulators of cell structure and function and are essential for a variety of cellular responses including motility of cells, differentiation, and proliferation\textsuperscript{9}. PDCs subjected to a physiological strain magnitude of 3000 μstrain for one cycle per day for eight days exhibit increased cell proliferation\textsuperscript{10}. Furthermore, uni-axial tensile strain has been found to up-regulate \textit{Runx2} and \textit{ColIa1} expression in PDCs\textsuperscript{11}. In additional cell, morphology is notably different after eight hours of strain with strained cells being elongated and flattened compared to non-strained cells\textsuperscript{11}.

Substrate microtopography and stiffness also play a key role in determining cell shape and lineage commitment. PDCs cultured on a smooth titanium surface differentiate
into osteoblasts whereas PDCs cultured on a roughened titanium surface differentiate into chondrocytes\textsuperscript{12}. With respect to substrate stiffness, cells that inhabit more compliant matrices deform the matrix more than cells that inhabit less compliant matrices\textsuperscript{13}. Thus, more compliant matrices are unable to resist the forces generated by the cell’s extracellular matrix (ECM) and cell spreading and adhesion results in local deformation of the matrix. Additionally, a very compliant matrix allows for the extension of filopodia\textsuperscript{13} which allow for cellular migration, and promote the formation of cellular networks between cells and focal adhesions between the cell and its substrate\textsuperscript{14}. Stiffer matrices effectively resist cell forces, allowing for increased cell spreading, which in turn promotes commitment to osteogenic and endothelial lineages\textsuperscript{13}.

Glass coverslips are very stiff substrates and have an elastic modulus approximately 50 times greater than that of a mesenchymal stem cell\textsuperscript{15}. As glass coverslips provide a local microenvironment very different than that experienced by PDCs \textit{in vivo} we sought to investigate the phenotype and genotype of PDCs cultured on solid-supported lipid bilayers (SLBs) with the ultimate goal of controlling cell differentiation. SLBs provide \textit{in vitro} models of the biological membrane systems that play key roles in cell life and can be used to investigate the dynamic molecular process occurring at the interface of biological membranes\textsuperscript{16}. The majority of studies involving SLBs are used to characterize interactions between cell membrane proteins and the membrane surface as represented by the lipid bilayer\textsuperscript{17-20}. A small number of studies have used SLBs to create semi-3D, biomimetic surfaces for cell culture\textsuperscript{21,22}. For optimal functionalization, SLBs should be decorated with junctional adhesion or ECM proteins to which native cells are known to attach. Previous studies creating SLBs for use in cell
culture have functionalized the surface with E-cadherin\textsuperscript{21}, a cell-cell adhesion protein, and type I collagen\textsuperscript{22}, a key component of the ECM.

Other proteins of interest for SLB functionalization include N-cadherin, a cell-cell adhesion protein and ZO-1, a tight junctional protein. N-cadherin is a classic type I cadherin responsible for the formation of junctional cell-cell adhesions\textsuperscript{23} and joining cells together in the appropriate configuration to form tissues. N-cadherin is one of a limited number of cell-cell adhesion molecules expressed by skeletal cells\textsuperscript{23}. Inhibition of the extracellular domain of N-cadherin in human trabecular bone-derived cells leads to loss of cell-cell adhesions and a reduction in size or even absence of cell pellet\textsuperscript{24}. Therefore, N-cadherin plays an important role in pre-mesenchymal condensation and expression is necessary to initiate mesenchymal condensation. ZO-1 (\textit{zona occludens}) is responsible for the formation of tight junctions between cells and confers barrier properties to epithelial membranes\textsuperscript{25}.

To date no studies have characterized the junctional adhesion proteins by which PDCs interact or the behavior (phenotype and genotype) of PDCs seed on a representative cell membrane. Therefore, in order to functionalize the SLB with an appropriate protein, it was first necessary to determine the cell-cell junctional adhesion proteins expressed by human PDCs. As the periosteum is a bilayered tissue and exhibits directional transport characteristics consistent with an epithelial barrier membrane\textsuperscript{26}, we hypothesize one of these proteins to be ZO-1. In addition, we have a specific interest in N-cadherin as the expression of N-cadherin is a hallmark of mesenchymal cells. We then sought to create a model membrane system functionalized with recombinant N-cadherin, creating a functionalized surface for cell culture with the ability to mimic cell-cell
interactions. Finally, we investigated the short term phenotypic and genotypic behavior of PDCs in response to the dynamic mechanical environment of the SLB.

2. Materials and Methods

2.1 Periosteum Cell Isolation

Human femoral neck periosteum was obtained from an 81-year-old female who suffered a pertrochanteric fracture of the right femur and underwent subsequent hip replacement. The periosteum sample was obtained under Ludwig Maximilians University IRB protocol 311-04. Sections of periosteum were placed in 25 ml of low glucose Minimum Essential Medium alpha (MEM-α) (Invitrogen, Carlsbad, CA) with 1% Penicillin-Streptomycin (PAA, Pasching, Austria) and Patricin with 3 mg/ml collagenase II (Worthington, Lakewood, NJ) in T-75 tissue culture flasks (Nunc, Wiesbaden, Germany) and incubated overnight at 37°C. The tissue suspension was passed through a 100 μm cell strainer to obtain periosteum derived cells (PDCs). Primary PDCs (P0) were plated in a T-75 tissue culture flask in MEM-α medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Munich, Germany) and 1% Penicillin-Streptomycin and cultivated at 37°C and 5% CO2 in a humidified incubator until passage 1 (P1). At P1, PDCs were stored in MEM-α with 40% FBS and 10% DMSO at -80°C until needed.

2.2 Western Blot

ECM markers expressed by periosteum cells were investigated using qualitatively by western blot analysis. Passage 5 (P5) PDCs were seeded on 100 mm diameter polystyrene tissue culture dishes (BD Biosciences, Heidelberg, Germany) and grown to a
density of 1.0x10^4 cells/cm^2. Cells were washed with ice-cold phosphate buffered saline (PBS), harvested with a cell scraper and total protein was extracted by sonication in RIPA extraction buffer (0.1% SDS, 1% NaDOC, 1% Triton X-100, 50 mM Tris-HCl (pH 8.2), 150 mM NaCl, 10 mM EDTA, and 20 mM NaF) with protease inhibitor (Roche, Mannheim, Germany). Cellular debris was pelleted at 10,000 g (10 minutes, 4°C). Protein was diluted 1:1 with loading buffer consisting of a 3:1 ratio of 5x Laemmli and 1M dithiothreitol and heated at 99°C for 5 minutes prior to gel loading. Thirty microliters of protein extract was then separated on a 8% SDS-PAGE gel and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Roche, Mannheim, Germany) overnight using a Bio-Rad transferring unit (Bio-Rad, Munich, Germany). Membranes were blocked in 5% (w/v) skimmed milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 90 minutes at room temperature. Membranes were incubated overnight at 4°C with primary antibodies to tight junction protein ZO-1 (Invitrogen, Carlsbad, CA) and N-cadherin (R&D Systems, Abingdon, United Kingdom) in 5% (w/v) skimmed milk in TBST at concentrations of 2 μg/ml and 0.5 μg/ml respectively. Membranes were then washed extensively in TBST and incubated in horseradish peroxidase-conjugated (HRP) secondary antibodies diluted 1:1000 in 5% (w/v) skimmed milk in TBST for one hour at room temperature. HRP was detected using ECL Plus Detection Reagent and detected on ECL-Hyperfilm (GE Healthcare, Munich, Germany).

2.3 Solid-Supported Lipid Bilayer Experiments

2.3.1 Buffers

A Hapes buffer solution of 10 mM Hapes, 150 mM NaCl, pH 7.4 in ultrapure water was used to prepare lipid vesicles and in all experiments reported. For the
formation of the supported lipid bilayers 10 mM NiCl₂ was added to the Hepes buffer in the initial incubation step with the tris-NTA functionalized lipids. In the cell culture experiments, the Hepes buffer was exchanged with MEM-α without the addition of FBS as the FBS was found to attach to the lipid bilayer.

2.3.2 Preparation of Lipids and Vesicles

Lyophilized 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Tris-NTA functionalized steroyl-oleoyl-amine (SOA-tris-NTA) was generously provided by Jacob Piehler (Osnabrück University, Germany) and prepared as previously described 27. The trivalent presentation of Ni²⁺-loaded NTA lipids allows increasing binding stability of histidine-tagged proteins to solid-supported membranes 28. A 19:1 molar ratio of DOPC:SOA-tris-NTA was dissolved in chloroform, dried, resuspended in Hepes buffer, and homogenized as previously described by Richter et al 29. Small unilamellar vesicles (SUVs) were obtained by sonication 30. SUV suspensions were stored at 4°C under nitrogen and diluted to desired concentrations before use.

2.3.3 Substrate Preparation

Silica-coated QCM-D sensors (Q-Sense AB, Västra Frölunda, Sweden) were immersed in an aqueous solution of 3% (w/v) SDS overnight, rinsed abundantly with ultrapure water, blow-dried in N₂, and treated with UV/ozone (Bioforce Nanoscience, Ames, IA) for 30 minutes and stored in air. For use in cell culture experiments, glass coverslips 20 mm in diameter were immersed into Piranha solution (H₂O₂/H₂SO₄) for 1 hour, rinsed extensively in ultrapure water, blow-dried with N₂ and stored under nitrogen
in sterile containers. Cleaned coverslips were exposed to UV/ozone for 30 minutes prior to use.

2.3.4 Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D) Measurements

QCM-D measures changes in resonance frequency, $\Delta f$, and dissipation, $\Delta D$, of a sensor crystal upon interaction with soft matter. The QCM-D response is sensitive to the mass (including coupled water) and the mechanical properties of the surface-bound layer. For sufficiently rigid biomolecular layers at high surface coverage, the (acoustic) film thickness can be estimated by the Sauerbrey equation\textsuperscript{31} (1)

$$d \approx \frac{\Delta m}{\rho_{film}} = -\frac{C}{\rho_{film}} \times \frac{\Delta f_n}{n} \quad (1)$$

where $\rho_{film} = 1.1$ g·cm$^{-3}$ has been assumed for the density of the protein film including coupled water. $\Delta m$ and $C = 18.0$ ng·cm$^{-2}$·Hz$^{-1}$ are the absorbed areal mass density including coupled water, and the sensor’s mass sensitivity constant, respectively.

Adsorption and interfacial processes were monitored \textit{in situ} with sub-second time resolution. Measurements were performed on silica-coated 4.95 MHz QCM-D sensors with a Q-Sense E4 system (Q-Sense AB, Västra Frölunda, Sweden) in flow mode at a temperature of 23ºC. Changes in dissipation and normalized frequency, $\Delta f_n/n$, of the fifth overtone ($n=5$) are presented.

2.3.5 Model Membrane Assembly

For QCM-D experiments, SUVs consisting of a 19:1 molar ratio of DOPC:SOA-tris-NTA were incubated at a concentration of 50 μg/ml in the presence of 10 mM NiCl$_2$. The SLB was rinsed and recombinant human N-cadherin with a 6-Histidine tag on the C-
terminus (R&D Systems, Abingdon, United Kingdom) was attached to the SLB at a concentration of 10μg/ml. To ensure N-cadherin was binding to the nickel loaded NTA lipids, the SLB was rinsed with 500 mM imidazole, a compound with the ability to elute histidine tagged proteins bound to nickel\textsuperscript{30}.

For cell culture experiments, sterilized 20 mm diameter glass coverslips were attached to the bottom of 12 well polystyrene tissue culture plates using an inert, two component dental glue (Picodent, Munich, Germany). SUVs consisting of a 19:1 molar ratio of DOPC:SOA-tris-NTA were incubated at a concentration of 100 μg/ml in the presence of 10 mM NiCl\textsubscript{2} in a total buffer volume of 200 μl for 90 minutes at room temperature. The SLB was gently washed with buffer 10× to remove any excess SUVs and recombinant human N-cadherin was added to the buffer at a concentration of 20 μg/ml in a total buffer volume of 500 μl and allowed to attach for 90 minutes at room temperature. The SLB was then washed with buffer 10× to remove non-bound proteins.

2.4 Cell Culture Experiments

2.4.1 Cell Seeding and Culture

Prior to cell seeding, the buffer in which SLBs were formed was exchanged 10× with MEM-α so that each well contained 500 μl of media. PDCs were seeded on three types of surfaces: 1) plain glass coverslips (control), 2) unfunctionalized SLBs on glass coverslips (SLB), and 3) N-cadherin functionalized SLBs on glass coverslips (SLB + N-cad). All three groups underwent the same incubation and buffer exchange steps where incubations for the control group were done in the absence of SUVs and N-cadherin and incubations for the SLB group were done in the absence of N-cadherin. Passage 3 (P3) PDCs were seeded at a low density of 5,000 cells/cm\textsuperscript{2} (LD5000) or a high density of
35,000 cells/cm$^2$ (HD35000) with N=3 samples per density for each type of surface. Seeding densities were chosen to achieve developmental contexts previously utilized for stem cells$^{32-35}$. After 24 hours in culture PDC morphology was investigated using phase contrast microscopy (Axio Observer D1; Zeiss, Oberkochen, Germany) and cells were harvested for real-time PCR analysis.

2.4.2 RNA Isolation and Real-Time PCR

Transcriptional profiles for early markers of mesenchyme condensation, and cell commitment to osteogenic, chondrogenic, and adipogenic lineages were measured using quantitative real-time PCR$^{36}$. In addition, makers for the development of extracellular matrix and junctional adhesion proteins were probed. For the control group, cell culture media was aspirated, lysing buffer was added directly to the cells and a cell scraper used to collect cell lysate. For the SLB and SLB + N-cad groups, the majority of cells grew in suspension on the fluid lipid bilayer and thus the cells were collected and pelleted by centrifuging for 5 minutes at 300$\times$ g. Supernatant was removed by aspiration and lysis buffer was added to the cell pellet. In addition, to ensure collection of all cells, lysis buffer was added directly to the glass coverslips and a cell scraper was used to collect any attached cells. Cell lysate from the suspended and attached cells was then combined prior to homogenization. Cell lysate was then placed in a Qiashredder (Qiagen, Valencia, CA) and centrifuged to homogenize the cells. The RNeasy Mini Kit (Qiagen) was used to isolate total RNA from the samples.

Quantitative real-time PCR analysis (including cDNA preparation) was performed at the Gene Expression and Genotyping Facility (shared resource of the Comprehensive Cancer Center at Case Western Reserve University, Cleveland, OH) using TaqMan Gene
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Expression Assays (Applied Biosystems, Foster City, CA) and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Twelve genetic markers (in addition to \textit{HPRT1} used as the endogenous control) were examined in each of the samples (Table 1). The markers chosen are key to mesenchymal condensation and subsequent differentiation\textsuperscript{36}. Prior to condensation, cells produce primarily a mesenchymal matrix comprising type I and III collagens\textsuperscript{36,37}. The initiation of the condensation step is associated with an upregulation of N-cadherin, a cell-cell junction adhesion marker expressed by mesenchymal cells\textsuperscript{23} as well as an upregulation of \textit{CD44}, a membrane receptor for hyaluronan extracellular matrix proteins and a downregulation of hyaluronan\textsuperscript{38}. During and after condensation the cells produce a cartilaginous matrix comprising types II, IX and XI collagens\textsuperscript{36,37} as well as chondroitin sulfate\textsuperscript{38}. Upregulation of \textit{Sox9} controls the production of \textit{ColIIa1}\textsuperscript{36,37}. Hence, activity of markers including \textit{HAS3, CD44, CDH2, Colla1, Sox9}, and \textit{ColII} were assessed to determine the relative stage of mesenchymal condensation for each of the groups studied. Furthermore, to assess differentiation state, early and late markers of osteogenic (\textit{Runx2, Postn, Osx}, and \textit{Msx2}), chondrogenic (\textit{ACAN}), and adipogenic (\textit{Pparg}) lineage commitment were assessed\textsuperscript{37}. We normalized the results of three independent trials for each sample to the \textit{HPRT1} gene expression. Analysis was completed with with Excel (Microsoft, Redmond, WA) using the $\Delta\Delta$Ct method and the LD5000 and HD35000 control groups as a baseline for comparison.
Table 3.1: Genetic markers investigated in PDCs seeded on SLBs. Genes investigated are markers for mesenchyme condensation, cell lineage commitment and junctional and cellular adhesions.

<table>
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<tr>
<th>Common Gene Name</th>
<th>Gene Abbreviation</th>
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<td>Runt-related transcription factor 2</td>
<td>Runx2</td>
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<td>Pparg</td>
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<td>Acan</td>
<td>Hs00153936_m1</td>
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<td>Hs00983056_m1</td>
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<td>Postn</td>
<td>Hs00170815_m1</td>
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<tr>
<td>CD 44 molecule</td>
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2.5 Statistics

For statistical analysis of real time PCR data, two-way ANOVA with tukey pairwise comparisons (S-PLUS, TIBCO Software Inc, Somerville, MA) was used to determine differences in gene expression between cell seeding surfaces and densities with $p < 0.05$ defined as a statistically significant difference. In addition, a non-parametric, multivariate correlation analysis (Spearman’s $\rho$, S-PLUS) was performed to correlate changes in gene expression with changes in seeding conditions (cell seeding surface, density) with $p < 0.05$ defined as statistically significant.
3. Results

3.1 Western Blot

Human PDCs express proteins for the tight junction protein ZO-1 and the cell adhesion and transmembrane protein N-cadherin (Figure 1). ZO-1 is a 225 kDa protein and appears as a band at 250 kDa. N-cadherin is 135 kDa protein and appears as a band at approximately 140 kDa.

![Western blot of human PDCs showing expression of ZO-1 and N-cadherin. The unspecific binding at 72 kDa was not characterized.](image)

Figure 3.1: Western blot of human PDCs showing expression of ZO-1 and N-cadherin. The unspecific binding at 72 kDa was not characterized.
3.2 Solid-Supported Lipid Bilayer Formation

Exposure of silica surfaces to SUVs of 19:1 DOPC/SOA-tris-NTA in Hepes buffer with 10 mM NiCl₂ resulted in the characteristic response for SLB formation\(^{16}\) (Figure 2). Incubation of the SLB with N-cadherin at a concentration of 10 μg/ml resulted in an additional frequency shift of 85 Hz. This frequency shift corresponds to an acoustical thickness of 13.9 nm (Equation 1). The thickness of the N-cadherin protein layer is in agreement with the size of N-cadherin extracellular domain which is approximately 18.7 nm\(^{39}\). Rinsing of the SLB with 500 mM imidazole in Hepes buffer successfully removed the N-cadherin, proving that binding of the protein to the lipid bilayer occurred via specific interactions between the NTA groups on the lipids and the 6-Histidine tag on the protein.

**Figure 3.2:** A: Schematic presentation of the SLB functionalized with N-cadherin extracellular domains. B: Construction of lipid bilayer as followed by QCM-D. Changes in frequency, Δf, and dissipation, ΔD, are shown for each incubation step. Arrows indicated start and length of incubations.
3.3 Cell Morphology

Phase contrast images (10× magnification) of cell morphology show PDCs seeded on glass coverslips are well spread (Figure 3A) with cells seeded at low density having very few cell-cell contacts while cells seeded at high density contain areas of cell-cell contact. PDCs seeded on the unfunctionalized SLB (Figure 3B) are grown in suspension and can be seen floating in the media as scrolling through the plane of focus brought cells into and out of focus. No noticeable difference is seen between low and high seeding densities. PDCs seeded on the N-cadherin functionalized SLB (Figure 3C) attach to SLB and grow in suspension as seen by the round morphology of cells in the focal plane of the SLB surface. At high density, many cells aggregate together presumably around a portion of the SLB that is functionalized with N-cadherin. At low density, cells adhere to the functionalized SLB but aggregation is less evident. Both low density and high density seeded cells on functionalized SLBs exhibit less spreading than on glass coverslips.
Figure 3.3: PDCs seeded on A) glass coverslips, B) unfunctionalized SLBs, C) N-cadherin functionalized SLBs at low density (5,000 cells/cm²) and high density (35,000 cells/cm²). A) PDCs exhibit a spread morphology on glass coverslips. B) PDCs are in suspension and unattached to the SLB. C) PDCs are in suspension and attached to the N-cadherin functionalized SLB.

3.4 Gene Expression

Real-time PCR did not detect expression of ColIIα1 for any sample; therefore, no data is presented for this gene. Human PDCs seeded on the unfunctionalized SLB show increases in gene expression for Runx2, Msx2, Osx, CDH2, and HAS3 after 24 hours of culture on the SLB (Figure 4). In addition PDCs seeded on the unfunctionalized SLB
show a 15% decrease in Postn expression over the same time period. PDCs cultured on the SLB functionalized with N-cadherin showed significant upregulation of Runx2 and a 40%, 30% and 45% decrease from baseline for Colla1, ACAN, and Postn after 24 hours of culture on the dynamic surface (Figure 5). In addition, two-way ANOVA revealed that Pparg, Sox9, HAS3, and CD44 show density dependent effects (Figure 6). When low density samples are separated from high density samples, Pparg expression decreases and Sox9 expression increases for PDCs cultured on both SLB and SLB + N-cad surfaces. CD44 expression increases for PDCs grown at high density on the unfunctionalized SLB and decreases by approximately 20% for PDCs grown at high density on the N-cadherin functionalized SLB. Additionally, PDCs cultured on the unfunctionalized SLB as well as the N-cadherin functionalized SLB show a significant increase in the expression of HAS3 which is a regulator of the ECM protein hyaluronan.
Figure 3.4: Expression of gene markers associated with events prior to and during mesenchyme condensation and early markers of cell lineage commitment and extracellular and junctional adhesions. Data is presented for PDCs seeded on unfunctionalized SLBs at LD5000 and HD35000. The baseline comparison for each group was cells seeded on glass coverslips at the respective density. Chart shows pooled data for high and low density SLB samples. Error bars show 95% standard error.
Figure 3.5: Expression of gene markers associated with events prior to and during mesenchyme condensation and early markers of cell lineage commitment and extracellular and junctional adhesions. Data is presented for PDCs seeded on N-cadherin functionalized SLBs at LD5000 and HD35000. The baseline comparison for each group was cells seeded on glass coverslips at the respective density. Chart shows pooled data for high and low density SLB + N-cadherin samples. Error bars show 95% standard error.
Figure 3.6: Genes that exhibit a significant density dependence as detected by two-way ANOVA. *Pparg*, *Sox9*, and *CD44* to not show any significant increases or decreases when data was pooled for LD5000 and HD35000 but do show significant differences for the HD35000 samples. Error bars show 95% standard error.
4. Discussion

PDCs express proteins for both ZO-1 and N-cadherin, providing insight into the molecular mechanisms by which the cells of the cambium layer of the periosteum interact. Tight junction proteins such as ZO-1 play an important role in enabling tissue barrier function. Tight junctions are characterized by a network of strands within the plane of the plasma membrane between neighboring cells. Tight junctions are a key characteristic of epithelial tissues and form boundaries between distinct biological environments and regulate paracrine signaling and fluid and solute exchange between environments. The identification of ZO-1 in PDCs implicates that tight junctions are the molecular basis for the selective, multiscale barrier membrane properties of the periosteum. In addition, the expression of N-cadherin suggests that PDCs express characteristics of mesenchymal cells as well as the epithelial characteristics discussed above. N-cadherin is a part of the cadherin superfamily of molecules and is responsible for the formation of junctional cell-cell adhesion such as adherens junctions. The mesenchyme is generally a loosely organized tissue but it is known to express N-cadherin, which is implicated in the condensation of chondrocytes in the limb bud as well as in the early stages of membranous osteogenesis. Furthermore, an increase in N-cadherin expression is a major criteria for a cell undergoing an epithelial to mesenchymal transition (EMT). The tendency of PDCs to express both epithelial and mesenchymal junctional adhesion proteins may be due to the fact that the periosteum is a composite structure with an outer fibrous layer and inner cambium layer. The inner cambium layer is known to contain a mixed population of cells including adult mesenchymal progenitor cells, differentiated osteogenic progenitor cells and fibroblasts. The expression of both
epithelial and mesenchymal cellular adhesion proteins in PDCs illustrates the complex cellular dynamics of the periosteum.

PDCs seeded on the N-cadherin functionalized SLB display a remarkably different morphology than those cultured on glass coverslips (Figure 3A,C). N-cadherin is traditionally expressed during development in cells of the mesenchyme and upregulation of N-cadherin expression is associated with mesenchymal condensations which are the leading step in the differentiation of skeletal tissues such as bone, cartilage, muscle, tendon and ligament\textsuperscript{36}. between cell seeding conditions and genes expressed during pre-, peri- and post- mesenchymal condensation and cells shows cells seeded on N-cadherin functionalized SLBs to have no significant change in \textit{CDH2} mRNA expression, evidence that the PDCs are in a pre-mesenchymal condensation state. PDCs seeded on the N-cadherin functionalized SLB at high density exhibit a downregulation of \textit{Colla1, ACAN, Postn, Pparg}, and \textit{CD44} (Figure 5, 6). The collective downregulation of these genes indicates PDCs seeded on the N-cadherin functionalized SLB have yet to undergo mesenchymal condensation as all of the down regulated genes are post-mesenchymal condensation markers (Figure 7). Specifically, upregulation of both \textit{CD44} and \textit{Colla1} have been shown to occur prior to mesenchymal condensation\textsuperscript{37,38}. On the N-cadherin functionalized surface, these PDCs show a nearly 40\% decrease in \textit{Colla1} expression, preventing mesenchymal condensation from occurring and thus the formation of permanent chondrocytes as well as osteoblasts via endochondral ossification.

In addition to the downregulated genes, PDCs seeded at high density on the N-cadherin functionalized surface show significant upregulation of \textit{Runx2} (pre-condensation, osteogenic lineage commitment), \textit{Sox9} (peri-condensation), and \textit{HAS3}
(pre-condensation) expression. Expression of Runx2 is indicative of cells that do not undergo mesenchymal condensation but instead differentiate into osteoblasts directly via intramembranous ossification\textsuperscript{37,38}. Expression of HAS3, a hyaluronan sythnase, indicates increased hyaluronan production, blocking chondrogenesis\textsuperscript{38}. The collective changes in gene expression indicate the N-cadherin functionalized SLB to inhibit mesenchymal condensation of PDCs.

**Figure 3.7:** Patterns of gene expression indicative of relative stage in lineage commitment over time for mesenchymal multipotent progenitor cells such as PDCs. Genetic markers for pre-, peri- and post-mesenchymal condensation are shown in red text while genetic markers for the formation of cell-cell and cell-matrix adhesions are shown in blue. The mesenchymal condensation event is shown in the blue dotted square. Mesenchymal stem cells can differentiate along chondrogenic (orange), osteogenic (blue), and adipogenic (green) paths toward lineage commitment. Used with permission and modification from [36]. Cell-cell and cell-matrix adhesion data plotted after [23, 38, 42, 43].
The gene expression pattern of PDCs seeded on N-cadherin functionalized SLBs provides early evidence for PDCs differentiating along an osteogenic lineage via intramembranous ossification (Figure 7). Previous research has shown PDCs to induce repair via endochondral ossification after injury to the periosteum\textsuperscript{44}. The present study indicates PDCs may be able to induce bone repair via intramembranous ossification as well. The present study investigated only short term behavior (24 hours in culture) of the PDCs on the N-cadherin functionalized SLB. Longer times in culture may allow PDCs to undergo mesenchymal condensation and differentiate along the respective osteogenic and chondrogenic lineages.

The development of a SLB decorated with N-cadherin provides a novel substrate for the culture of PDCs that mimics the \textit{in vivo} environment of the cells more closely than glass or tissue culture plastic. In an \textit{in vivo} environment cells grow on top of one another or the ECM secreted by an adjacent cell. In these environments cell attach to one another via cell-cell and cell-matrix adhesions. Substrate stiffness has been found to influence the fate decisions of multipotent progenitor cells\textsuperscript{13}; therefore, model systems such as SLBs may more accurately instruct and guide cell fate as well as provide a means for characterizing the interaction between neighboring cells and their adhesion proteins\textsuperscript{21,22}. To our knowledge this is the first study to investigate the fate of multipotent progenitor cells seeded on a functionalized SLB. Based upon the results of this study, SLBs have the potential to provide a tissue-patterning template and direct cell fate and lineage commitment based upon the molecular dynamics of the functionalized surface.
Acknowledgements

We would like to thank the Alexander von Humboldt Foundation and National Science Foundation (#CNNI-0826435) for the funding support necessary to carry out the international collaboration presented in this work. Thanks to Jacob Piehler (University of Osnabrück, Germany) for providing the SOA-tris-NTA lipids. Special thanks to Hana Chang (CWRU), Cvetan Popov (LMU), and Nico Eisele (CICbiomaGUNE) for assistance and guidance. Lastly, we would like to thank the Case Western Reserve University Gene Expression and Genotyping Facility for assistance in collecting the real-time PCR data.

References


Chapter 4

Concluding Remarks

The periosteum exhibits a remarkable, composite structure which enables its selective, multiscale barrier properties. In addition, the periosteum contains multipotent progenitor cells which express tight junction and cadherin proteins, hallmarks of epithelial as well as mesenchymal tissue morphology. These multipotent progenitor cells have the ability to sense their surrounding environment and differentiate along different lineages based upon their surrounding environment. This research elucidates how structure at the cellular level enables function at the tissue level. This scientific knowledge allows us to better understand the mechanobiological properties of the periosteum as well as the environment of the multipotent progenitor cells residing within the tissue. These insights can be used to create predictive computational models of bone healing in procedures such as the one-stage bone-transport procedure, design biomimetic periosteal replacement membranes and modulate cell seeding conditions for the design of tissue engineered periosteum. The over-arching goal of this research is to better understand the role of periosteum in bone regeneration both in health and disease.

Beginning at the tissue level, Chapter 2 characterizes the permeability properties of ovine femoral and tibial periosteum. Ovine femoral periosteum demonstrates mechanically responsive as well as bidirectional permeability properties. Periosteum is capable of modulating its permeability based upon the applied fluid flow rate. An increase in fluid flow rate leads to a corresponding increase in tissue permeability. Furthermore, the pre-stress experienced by *in vivo* periosteum samples serves to regulate
tissue permeability. Removal of periosteal pre-stress markedly changes periosteum tissue morphology and results in an increase in tissue permeability. Thus, the periosteum acts a bone’s bounding membrane and possesses the ability to regulate fluid transport into and out of the bone.

At the cellular level, Chapter 3 characterizes the junctional adhesion proteins expressed by periosteum progenitor cells and discovers PDCs express proteins for both tight junctions and cadherins. PDCs seeded on a model cell membrane functionalized with N-cadherin respond to their extracellular environment by upregulation and downregulation of proteins and transcription factors associated with mesenchymal condensation. PDCs grown on the functionalized membrane show early signs of differentiation into osteoblasts by intramembranous ossification. Thus PDCs have the potential to generate bone de novo via endochondral ossification as well as intramembranous ossification. The functionalized model membrane system can be used to further investigate and cell-cell and cell-matrix adhesions by which PDCs interact as well as a template for creating tissue engineered periosteum.

OUTLOOK

The periosteum has great potential to advance the field regenerative medicine. The unraveling of the periosteum’s mechanical and well as biological properties presented in this work provide insight into the structure-function relationship of the periosteum. One of the most important aspects of this work is the characterization of the structure-function properties at the tissue and cellular level, and thus providing insight into the multiscale mechanobiology of the periosteum. The work presented in this thesis
demonstrates how the structure of the periosteum at the cellular level is responsible for controlling the function of at the tissue level. This innate control of the structure-function relationship allows for classification of the periosteum as a “smart” (bio)material.

The work presented in this thesis only begins to unravel the structure-function relationship exhibited by the periosteum. Further research is necessary in order to fully classify and understand the material as well as mechanical properties of the periosteum. The discovery of the important role pre-stress plays in periosteum mechanics has yet to be studied at a structural level. It would be very interesting to investigate how the alignment of the collagen fibers within the periosteum is altered when the tissue is removed from the bone. Furthermore, in order to fully understand the role of periosteum as bone’s bounding membrane it is important to classify the molecular species to which periosteum is permeable. In addition, classification of the pore size in both the fibrous and cambium layer of the periosteum will provide a structural explanation for the tissue level directionally permeability exhibited by ovine femoral periosteum.

This research also highlights the lack of information available about periosteum cell mechanobiology. The periosteum contains many types of cells and a standard, homogeneous population of periosteum cells has yet to be characterized. Mechanical stimulation of intact periosteum surrounding a bone defect leads to new bone formation. It has yet to be determined if periosteum cells respond directly to these mechanical signals, if mechanical signals lead to the release of paracrine signaling molecules which in turn stimulate periosteum cells or if a combination of these two mechanisms stimulates the regenerative capacity of periosteum cells. Furthermore, knowledge is lacking
regarding the specific types of mechanical signals which promote periosteum cell
egression to begin the process bone regeneration.

Knowledge of periosteum and periosteum cell mechanobiology is important for
incorporation in multi-scale models. The ability to predict stresses, strains, and fluid flow
at the tissue and cellular level will allow for determination of the effects of these
variables on the organ scale structure of bone. Multi-scale models will prove valuable for
understanding perisoteum’s structure-function properties and the development of tissue
engineered strategies in healing bone as well as physical therapy protocols to improve
outcomes post-surgery. The mechanobiological properties of the periosteum are of
increasing importance to understand bone’s micromechanical milieu and fully harness the
regenerative potential of the periosteum in critical size bone defect healing. Finally, an
understanding of the periosteum’s smart properties provides inspiration for a new class of
advanced (bio)materials that harness and change their behavior based on the mechanical
cues inherent to their environment.


