METHODS TO QUANTITATIVELY ASSESS THE PERFORMANCE
OF CONNECTIVE TISSUE PROGENITOR CELLS IN RESPONSE
TO SURFACE MODIFIED BIOMATERIALS

BY
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*We also certify that written approval has been obtained for any
proprietary material contained therein.
To my parents and my wife Puja, for their unconditional support, encouragement, and sacrifices
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The field of orthopaedic tissue engineering includes a broad range of treatment options that seek to regenerate or accelerate the repair of bone tissue. Formation of new bone by the activity of connective tissue progenitor cells (CTPs) is a central feature of each of these treatment options. CTPs are defined as the heterogeneous population of stem and progenitor cells that are resident in native tissues and are capable of proliferation to give rise to progeny that will differentiate to express one or more connective tissue phenotypes. The strategy of using a cell-based therapy to achieve bone regeneration may include: targeting CTPs, and guiding their downstream progression towards the osteogenic pathway using a combination of scaffolds, bioactive factors, and mechanical and biophysical methods. Recent advancements in biomaterials science include promising technologies for modifying biomaterial surfaces with pro-survival or pro-osteogenic bioactive ligands. Before testing the pre-clinical efficacy of these surface modified biomaterials in animal models, it is important to test and optimize the influence of these materials on cells in an in vitro setting.

The purpose of this dissertation is to introduce and demonstrate a new method to assess the response of CTPs to surface-modified biomaterials using a standardized automated colony forming unit (CFU) assay. Individual components of the method are
verified for their compliance with the technical specifications. The method is validated using β-TriCalcium Phosphate (TCP), a bone scaffold material that was modified with Epidermal Growth Factor (EGF), a pro-survival and proliferation bioactive ligand, using a novel TCP-binding peptide. The amount of tethered EGF on the TCP surface was quantified, and its stability was demonstrated. Surfaces modified with tethered EGF were shown to enhance the colony forming efficiency of CTPs obtained from human bone marrow and the trabecular surface of bone. These data support the potential of tethered EGF presentation on the surface of implantable TCP biomaterials as a means of enhancing the performance of local and transplanted CTPs in a setting of bone repair or other tissue engineering applications.
CHAPTER 1: PREFACE

The purpose of this dissertation is to introduce and demonstrate a new quantitative method to assess the response of connective tissue progenitor (CTP) cells to surface-modified biomaterials by applying an automated colony forming unit (CFU) assay that has been recently adopted by the American Society for Testing and Materials (ASTM) (ASTM F2944-12) [1] (referred here as “Colonyze™”). This new method uses engineering design principles to combine the following components: (1) a novel method to prepare two dimensional surfaces of a bone scaffold material, with or without surface modification using a bioactive ligand; (2) a method to verify and optimize these surfaces with respect to their repeatability, stability, uniformity in coverage, and smoothness; and (3) a method to assess the material and surface modification effects on CTPs using Colonyze™.

The combined method was validated using β-TriCalcium Phosphate (TCP), a clinically used bone scaffold material with, and without surface modification with Epidermal Growth Factor (EGF), a pro-survival and proliferation bioactive ligand, using a novel TCP-binding peptide that has been developed by researchers at MIT [2]. The unique contribution of this work is the development, verification, and validation of the method; and the results demonstrating that the colony forming efficiency of CTPs is enhanced in response to the surface modification of TCP with EGF.

This dissertation should be of interest to the scientific community performing basic and translational research in the fields of tissue engineering, stem cell therapies, and biomaterial science. The work reported in this dissertation was published, in part, in reports and publications of the Armed Forces Institute of Regenerative Medicine.
(AFIRM) [3], and in several national and international conference proceedings. This research may lead to accelerating rational development of new biomaterials and their surface modifications that improve the rate and reliability of regeneration of large traumatic bone defects. This research is relevant to both injured warriors and to those suffering traumatic injuries in the civilian population.

Chapter 2 of this dissertation introduces the underlying components of the method, and principles of their use. These components include the use or targeting of CTPs in bone tissue engineering applications; assessment of CTP performance using Colonyze™ [1]; techniques of surface modification of biomaterials; interactions between cells and biomaterials; and their role in the efficacy of the biomaterials in orthopaedic tissue engineering applications. The purpose of this chapter is to provide an adequate summary of these components and it is not intended to be a comprehensive review.

Chapter 3 introduces the design of a method to quantitatively assess the response of CTPs to surface modified biomaterials using Colonyze™. Engineering design principles were applied to design this method. The scope of the design was defined, and constraints were identified based on the available knowledge and the unmet need. The technical specifications were defined, forming the basis for verification of the compliance of individual components.

A novel method to prepare two-dimensional surfaces of biomaterials was developed. Three designs were tested. The design most effective in meeting technical specifications was chosen, and optimized for uniformity in coverage and smoothness. The feasibility of using Colonyze™ to quantify the CTP response was verified. The surface
density of tethered EGF and the stability of the presentation of tethered EGF on TCP surfaces were verified.

Chapter 4 integrates these components, and describes the validation of this method using a real-world example. TCP surfaces were modified with tethered EGF using a novel TCP-binding peptide that has been developed in Dr. Linda Griffith’s laboratory at MIT [2]. CTPs were harvested from bone marrow and the trabecular surface of bone. The response of CTPs to TCP surfaces modified with tethered EGF was quantified using Colonyze™. The results discussed in this chapter not only validated the method, they also demonstrated that tethered EGF increases CTP colony forming efficiency. These data and methods can be used to develop a combination product consisting of TCP scaffolds modified with tethered EGF and bone marrow derived cells, which could be transplanted into large orthopaedic defects. This strategy may lead to new clinical methods that significantly improve the rate and reliability of regeneration of large traumatic bone defects.

Chapter 5 provides the conclusions and the summary of the method discussed in Chapters 3 and 4. This summary will help researchers apply this method to other types of synthetic biomaterials, bioactive factors, and stem cells that can be used for orthopaedic tissue engineering and other tissue engineering applications. It also provides guidance to the proposed future studies in Chapter 6.

Chapter 6 proposes future studies to progress the work discussed in this dissertation. These proposed studies will advance understanding of the effects of tethered EGF in influencing the response of CTPs. Each study is introduced with a significance, hypothesis and rationale, followed by the experimental design. The following proposed
studies are discussed: quantification of CTP response to tethered EGF under normoxia, and cellular stress induced by hypoxia and inflammatory cytokines; characterizing the mechanism of CTP response to tEGF under the cellular stress induced by hypoxia and inflammatory cytokines; assessment of the effect of tethered EGF on bone regeneration; improving the surface modification of TCP scaffolds; and assessment of CTP response to surface modifications of other bone graft materials. These contributions enable exploration of additional scientific questions that are significant for the field of orthopaedic tissue engineering in general, and for the efficacy of surface modified TCP materials in bone grafting in particular. This work is intended to advance the current standard of orthopaedic trauma care.

**Works Cited:**


CHAPTER 2: INTRODUCTION TO THE COMPONENTS OF THE METHOD

Current orthopaedic tissue-engineering strategies recognize the critical importance of osteoprogenitor cells, biomaterials, and bioactive stimuli, among other factors, as orthopaedic graft components. Generation of new bone is assisted by transplanting osteogenic cells along with the scaffold material, particularly when large defects are involved [1-12]. The local and transplanted cells can be targeted, and their performance can be modified using a scaffold matrix that facilitates the distribution of osteogenic cells through attachment and migration. In particular, it is desired to use a biomaterial that is osteoconductive, i.e., graft material that facilitates the attachment and migration of cells, and thereby the distribution of a bone healing response throughout the grafted volume [13-15]. The performance of osteogenic cells may be further influenced by osteoinductive stimuli, mass transport and revascularization of the defect, and by controlling the mechanical environment [13-15]. Osteogenesis occurs when osteoprogenitor cells respond to osteoconductive and/or inductive stimuli by differentiating along the osteoblastic lineage, and contributing to new bone formation.

This chapter provides an overview of the principles of the use of connective tissue osteo-progenitor (CTP) cells in bone tissue engineering, and methods to assess CTP performance using an automated assay. In addition, principles of surface modification of biomaterials are introduced, and the interactions between cells and biomaterials are discussed, including how these interactions play a critical role in the efficacy of the biomaterials in orthopaedic tissue engineering applications. This introduction forms a foundation for Chapters 3 and 4, which elaborate a novel method to assess CTP response
to surface modified biomaterials designed for bone tissue engineering applications in vitro.

2.1. **Role of Connective Tissue Progenitor Cells (CTPs) in Bone Repair:** Stem cells and progenitor cells are present in all adult tissues and are critical to tissue health, maintenance, and response to injury or disease throughout life. Stem cells are the source of all new tissues arising from repair and remodeling, and are modulated by chemical and physical signals that control their activation, proliferation, migration, differentiation, and survival. Stem cells give rise to progenitor cells and are distinguished from progenitor cells by their capacity for self-renewal, or self regeneration by asymmetric cell division [2]. In contrast, progenitor cells (also called transit cells) proliferate and expand in number. Progenitor cells have a limited capacity for self-renewal and are committed to progress toward a more differentiated phenotype. In bone, stem cells give rise to progenitor cells, which advance to become pre-osteoblasts and then osteoblasts.

Various stem and progenitor cells from mammalian postnatal bone marrow have been extensively used in tissue engineering applications because of their potential to differentiate into specialized connective tissue cells, including bone, cartilage, muscle, fat, tendon, ligament, fibrous tissue, perivascular adventitia, as well as blood cells [12, 16-20]. This has important implications with regard to the design of tissue engineering strategies, in that cells derived from one tissue might be useful in forming a different tissue.

During skeletal development and repair, bone formation can occur via the processes of endochondral or intramembranous ossification. Endochondral ossification
occurs primarily in long bones while in the flat bones of the skull and also the mandible, bone formation occurs via the process of intramembranous ossification. During endochondral ossification, undifferentiated mesenchymal stem cells condense at the site of future bone formation. Cells within the condensation center differentiate into chondrocytes to produce cartilage while those at the periphery differentiate into fibroblast like perichondrial cells to produce perichondrium. During this process, cartilage becomes mineralized as the chondrocytes become hypertrophic, followed by vascular invasion of the mineralized cartilage. Subsequently, chondroclasts degrade the mineralized cartilage allowing osteoblast migration and osteoid deposition onto the cartilaginous matrix with replacement of the cartilage precursor with mineralized bone [21, 22].

In contrast to endochondral ossification, no cartilage precursor is formed during intramembranous ossification. Cell differentiation occurs within a membranous, condensed plate of mesenchymal cells present in fibrous connective tissue with differentiation of these cells directly into those of an osteoblastic lineage. These cells continue to differentiate and proliferate into osteoblasts that deposit bone matrix resulting in the formation of woven bone [21, 23].

The number of stem and progenitor cells in various tissues can be assayed in vitro by harvesting cells from the tissues and growing them in tissue culture under conditions that promote their activation, proliferation and phenotypic differentiation. The number of stem cells and progenitors can be estimated on the basis of the number of colony-forming units [3, 24]. The underlying assumption of this approach is that one progenitor cell leads to the formation of one colony containing the progeny of that progenitor cell.
2.1.1. Definition of CTPs: CTPs denote a combined heterogeneous population of stem cells and progenitor cells that is capable of both proliferation and differentiation into one or more connective tissue phenotypes. The term CTP was introduced to enable exploration of the concentration, prevalence, diversity, and spectrum of biological potential among cells that are natively present in different tissues, and the changes in these parameters that can be related to aging, gender, disease and responses to chemical conditions (e.g. hypoxia) or pharmaceuticals (e.g. steroids and nicotine) [1, 2, 24, 25]. It is important to recognize that a CTP is a tissue resident cell that is assayed in vitro and detected by the fact that it gives rise to a colony of progeny. However, only the colony founding cell is a CTP. The true number of CTPs in various tissues is most often estimated with use of the Colony-Forming Unit (CFU) assays, that is, cells that give rise to a colony of proliferating progenitor cells in vitro under conditions that are selected to promote activation and proliferation of one or more fractions of the connective tissue progenitor population. In these assays, each colony represents the progeny of a founding stem or progenitor cell. Functional biological differences between the colony-founding cells are revealed by differences in the proliferation rate, the morphology, the migration, and the differentiation of their progeny in each colony.

The cells that make up a proliferating colony are the progeny of the founding CTP, but they change rapidly under in vitro conditions and quickly adopt a phenotype that is distinctly different than the tissue resident CTP that gave rise to the colony. It is also important to recognize that even bone forming CTPs (CTPs capable of giving rise to progeny that can differentiate into bone –which we identify by the nomenclature CTP-O) in a given tissue are a heterogeneous population. This is evidenced by the fact that the
progeny of some CTP-Os divide rapidly and others divide slowly, and that some colonies express bone markers quickly in vitro (often without osteogenic stimuli) while others require exogenous osteogenic stimuli. Thus, it should be evident that there is no one phenotypic marker or set of markers that defines a CTP, because CTPs include many types of progenitors. However, it is possible to characterize the population of CTPs from a given tissue by applying methods that assay their concentration, prevalence, and variation in biological potential.

2.1.2. Role of CTPs in Bone Regeneration: Bone repair requires the activity of osteoblasts, which are derived from cells of the mesenchymal lineage, such as CTP-Os. Since bone defects are, by definition, deficient in CTP-Os, their treatment cannot be optimized without CTP-O transplantation from another source, such as bone marrow. Bone marrow has been widely used clinically as a source of autogenous CTP-Os. CTP-Os can be harvested from a bone marrow aspirate with very low morbidity and minimal pre-processing compared to other sources of CTP-Os such as fat and muscle. The established clinical procedures to increase CTP-O concentration and/or reduce number of competing non-CTP-O cell population from bone marrow are relatively uncomplicated. These procedures involve centrifugation of the marrow to remove majority of red blood cells and plasma, and concentrate the CTPs, white blood cells, platelets and other nucleated cells. The CTP population increases 4-6 fold using this approach [26]. Alternatively, bone scaffold materials can be used to selectively retain the CTPs on the scaffold surface, by transforming the bone marrow through a column of scaffold material at a low speed, which enables retention of 40-70 % CTPs on certain scaffolds [27]. Bone
marrow derived CTP-Os can be transplanted to the defect site in a single intra-operative procedure, and is a more viable alternative than transplantation of culture expanded population such as MSCs, whose therapeutic usage requires at least two operative procedures (cell harvest and implantation), and a complex laboratory treatment that is time intensive and expensive. The strategy of CTP-O transplantation has led to improved bone formation compared to scaffolds without CTP-Os [7-9, 28-34].

2.1.3. **Difference between CTPs and MSCs:** CTPs differ from *in vitro* culture expanded cells, such as bone marrow stromal cells (BMSCs), mesenchymal stem cells (MSCs), and adult multipotential progenitor cells (MAPCs), which are more purified and homogeneous in nature. Mesenchymal stem cells (MSC) are defined as a population of “purified, homogeneous culture expanded cells that retain the capacity for differentiation in to multiple mesenchymal lineages, where mesenchymal lineages include bone, cartilage, fat, muscle, tendon and bone marrow stroma” [19, 35]. A rigorous protocol for demonstrating multilineage potential was defined and used in the pioneering work of Dr. Arnold Caplan [19, 35]. This work demonstrated that not all batches of culture expanded cells would satisfy the full set of requirements to call them MSCs. Further, culture expansion favors cells that divide quickly. Therefore, the process of producing a batch of MSCs removes from consideration stem cells that grow slowly. Analysis of CTPs, which by definition does not include culture expansion, allows for information to be obtained from all of the potential founding cells, including those that grow slowly.

By definition, to be counted as a CTP, a bone marrow derived cell needs to be capable of giving rise to progeny that can form one of the connective tissue phenotypes.
In contrast, to be counted as an MSC a cell must have the capacity to differentiate into all “mesenchymal phenotypes (bone, cartilage, tendon, muscle, fat, marrow stroma). However, the heterogeneity presented by CTPs more closely mimics the multifunctional nature of cells within the bone marrow environment and this heterogeneity can be viewed as a source of valuable information that can be dissected experimentally to understand the prevalence and kinetics of various connective tissue stem-cell populations associated with tissue engineering strategies.

2.1.4. The Colony Forming Units: Friedenstein et al. (1970) demonstrated that bone marrow cells explanted and grown in monolayer cultures form colonies [36]. The number of CFUs formed was a linear, increasing function of the number of plated explanted cells. Both time lapse photography and mixed cultures of male/female cells indicated that each colony was derived from a single progenitor cell. Additionally, colonies derived from different sources exhibited different morphologies [37-39]. Bone marrow cultures contained colonies with dense centers at day ten, while pleural and peritoneal cultures contained looser and smaller colonies [40, 41].

In vivo and in vitro studies demonstrated the pluripotent nature of the CFU progeny [42]. Passaged progeny of bone marrow derived CFUs placed in diffusion chambers formed osteogenic tissue [41]. In vitro colony assays methods applied to hematopoietic progenitors have been used to demonstrate the heterogeneity of the progenitor population and the capacity for osteogenic differentiation [37, 39, 43, 44]. Individual colonies express alkaline phosphatase (an early osteogenic marker) and vary widely in size, morphology and level of alkaline phosphatase activity.
2.2. The Colony Forming Unit Assay: Cells and tissues commonly used in tissue engineering and cellular therapy are routinely assayed and analyzed to define the number, prevalence, biological features and biological potential of the original stem cell and progenitor population(s). The number of stem cells and progenitor cells in various tissues can be assayed in vitro by harvesting cells from the tissues using methods that preserve their viability and biological potential, and culturing in vitro that leads to activation and proliferation of stem and progenitor cells into the formation of colonies. A colony is defined as a cluster of a predetermined number of cells present within predefined dimensional parameters. The true number of stem cells and progenitors in a population can be estimated on the basis of the number of colony-forming units (CFUs) observed. The prevalence of stem cells and progenitors can be estimated on the basis of the number of observed CFUs detected, divided by the number of total cells assayed.

2.2.1. Limitations of Manual Observer-Dependent Assay: Current standards utilize user input for defining the presence and location of colonies based on visualization of an entire culture surface at low magnification using a microscope. The cell culture surface sample may be viewed in transmission light mode (unstained or with a histochemical marker) or fluorescently with a dye or antibody. For this method, colony count is the only measurable output parameter. The manual quantification of cell and CFU cultures based on an observer-dependent judgment is an extremely tedious and time-consuming task, and can be significantly impacted by user bias. In addition to being highly time and labor intensive and subjective, manual enumeration has been shown to have a significant
degree of intra- and inter-observer variability, with coefficients of variation (CV) ranging from 8.1% to 40.0% and 22.7% to 80%, respectively [45]. When multiple observers are employed, observer fatigue can be reduced, but the accuracy and reproducibility of cell and colony identification can be significantly compromised due to significant intra- and inter-observer variability. Standard CVs for cell viability assessment and progenitor (colony) type enumeration range from 19.4% to 42.9% and 46.6% to 100%, respectively [45]. In contrast, studies focusing on bacteria, bone marrow-derived stem cells and osteogenic progenitor cells have collectively concluded that automated enumeration provided significantly greater accuracy, precision, and/or speed for counting and sizing cells and colonies, relative to conventional manual methodologies. Automated methods for enumerating cells and colonies are less biased, less time consuming, less laborious, and provide greater qualitative and quantitative data for intrinsic characteristics of cell and colony type and morphology.

2.2.2. ASTM F2944-12-Automated CFU Assay: A fully automated, accurate, and standardized method for characterizing the biological potential and functional potency of the cultured cells can be achieved by utilizing an automated, microscope-based imaging system that is able to stitch together sequentially scanned, high resolution images to form a single mosaic image of the entire culture surface, and subsequently employing image processing algorithms to identify cells and assign them to individual colonies. In addition to the calculation of prevalence, extracted parameters include colony level statistics such as cell/nuclear density and count, colony morphology (shape and size parameters), secondary marker coverage and effective proliferation rates.
Colonyze™ is an integrated software suite for image acquisition and cell and colony analysis. This method utilizes standardized protocols for image capture of cells and colonies derived from in vitro processing of a defined population of starting cells in a defined field of view (FOV), and standardized protocols for image processing and analysis. The primary outcome measure is the number of colonies present in the FOV. In addition, the characteristics and sub-classification of individual colonies and cells within the FOV also provides useful information, based on extant morphological features, distributional properties, or properties elicited using secondary markers (e.g., staining or labeling methods). The procedure, imaging-set up, hardware and software requirements, image acquisition and processing methods and available options, data acquisition and analysis methods, and limitations are discussed in the recently published ASTM Standard Method, (ASTM F2944-12) [46]: “Standard Test Method for Automated Colony Forming Unit (CFU) Assays- Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture”.

2.3. Surface Modification of Biomaterials: Recently developed biomaterials are designed to be not only biocompatible, and/or osteoconductive, but also to promote tissue specific behaviors that can assist regeneration of functional target tissue. These materials can broadly be categorized as bioactive biomaterials. The surface properties of the biomaterial are primarily responsible for dictating cell behavior in response to and interactions with the biomaterial. Modification of biomaterial surfaces with bioactive ligands such as cell adhesion molecules, bioactive factors that promote cell survival, proliferation, migration or differentiation can be used to induce selective cell interaction
with the specific adhesion and bioactive factor receptors expressed by autologous or
exogenous cells at the defect site.

Ligands used for surface modified biomaterials include adhesion molecules such
as RGD peptides, growth factors such as EGF, PDGF, FGF and BMPs. However, growth
factors in the physiological environment can be rapidly degraded or rendered inactive
prior to reaching their target, and biomaterial systems designed to deliver soluble growth
factors to sites of tissue repair are therefore often ineffective and costly. Large amounts
of soluble growth factors are frequently needed to effect cellular outcomes, although the
delivery of such large quantities has the potential to damage cells and tissues.

Imobilization of growth factors to biomaterial substrates has emerged as a
method for improving the stability and persistence of growth factor delivered to cells.
These goals cannot be achieved via simple growth factor adsorption, which does not
allow control over delivery, retention, orientation, or desorption rate [47, 48].
Immobilization strategies can prolong growth factor availability, allow spatial control,
and reduce the amount of growth factor required, thereby potentially reducing the cost
and increasing the efficacy of various bioactive ligands.

The process of covalently or preferentially adhering these molecules to the
biomaterial using a high affinity binding is referred to as tethering. Tethering ensures that
the signaling molecule does not diffuse away from the cells rapidly, and enables control
over the concentration and 3 dimensional conformation of the molecule that preserves its
ability to interact with receptors. The presentation of growth factors in an immobilized
form also has physiological relevance, as both soluble and matrix-bound growth factors
perform distinct functions in the in vivo environment [49, 50].
There are several approaches to modify the biomaterial surface with tethered growth factors. Table 1 summarizes conjugation techniques to tether growth factors to biomaterials [51]. Figures 1-3 depict the generalized mechanisms of tethering growth factor to a substrate [51]. The choice of tethering approach depends upon the chemistry of the substrate and the reactive groups on the growth factors, structure, and the location of reactive groups relative to the receptor-binding area of the growth factor structure. Several studies have used these conjugation chemistries to tether a variety of growth factors in bioactive form to biomaterial substrates, and are summarized in Table 2.

2.3.1. Mechanism of Cell-Surface Interaction: When a biomaterial comes into the contact with the human body, the proteins, lipids, saccharides and other extra-cellular molecules present in physiological fluid get adsorbed on the biomaterial surface [52-54]. The adsorption of extracellular molecule occurs through complex and poorly understood mechanisms. Cells arrive on the implant surface at later times. Therefore, cell-biomaterial interaction is mediated via the adsorbed protein layer [54, 55]. Biomaterials surface properties influence cell response in terms of the kinetics and thermodynamics of the protein adsorption. The nature of the conditioning biomolecules and their orientation on the surface will have direct consequences on the protein adsorption and thereby the recruitment, attachment, proliferation and differentiation of cells [54-66]. Further, the efficacy of a biomaterial depends on the ability of the biomaterial to provide environmental cues to the recruitment and stability of multiple cell phenotypes in culture, proliferative and/or differentiative signals, and cell migration.
2.3.2. **Mechanism of Adhesion**: The extracellular matrix of bone is composed of 90% collagen (type I collagen 97% and type V collagen 3%) and of 10% other proteins (osteocalcin 20%, osteonectin 20%, bone sialoproteins 12%, proteoglycans 10%, osteopontin, fibronectin, growth factors, bone morphogenetic proteins, etc.) [67]. All these proteins are synthesized by osteoblasts and most are involved in adhesion [67]. *In vitro*, other proteins such as fibronectin or vitronectin have been shown to be involved in osteoblast adhesion. Some of the bone proteins, including collagen and fibronectin, have chemotactic or adhesive properties, notably because they contain an Arg–Gly–Asp (RGD) sequence which is specific to the fixation of cell membrane receptors like integrins [60, 68-72].

The sites of adhesion between tissue cultured cells and substrate surfaces are called focal contacts or adhesion plaques [73-75]. Focal contacts are closed junctions where the distance between the substrate surface and the cell membrane is between 10–15 nm. The focal contacts include specialized microstructures anchored within the cell to cytoskeletal microfilaments which underlie the cell membrane. The external faces of focal contacts present specific cell receptor proteins that facilitate cell adhesion. There are more than 20 distinct cell-matrix receptors in the integrin family alone [76, 77]. The type of focal adhesion and its geometry can influence the shape the cell assumes, ultimately influencing phenotypic expression.

2.3.3. **Mechanism of Migration**: Cell migration is facilitated by an interaction between the cell, its substrate and its cytoskeleton. Firstly, cells develop a projection of their leading edge to form a lamellipodium. Cells subsequently use adhesive interactions (via
integrins) to generate the traction and energies required for cell movement. The last step of the migratory cycle is the release of adhesions at the lagging end followed by cell detachment and retraction [78, 79]. In general, cells with a low motility form strong focal adhesions while motile cells form less adhesive structures [78, 79], and an intermediate level of attachment force induces a maximal migration rate.

2.3.4. Mechanism of Osteoblastic Differentiation: After the attachment phase, extracellular collagenous and non-collagenous matrix osteoblastic proteins (e.g. alkaline phosphatase, thrombospondin, osteopontin, osteocalcin, osteonectin, type I collagen and bone sialoprotein) are synthesized and released by cells. These proteins regulate the fate of cells that produce them (autocrine regulation) or of other cells in their immediate environment (paracrine regulation). A subset of these factors are called as morphogens; which are the factors that have a capability of initiating a cascade of events leading to differentiation of a progenitor cell into a committed end line cell, like an osteoblast or a chondrocyte. Members of the Transforming growth factor beta superfamily play a special role in the morphogenesis of bone and cartilage. A member of the family, the bone morphogenetic proteins (BMPs), is particularly important in inducing Osteogenesis [80-82]. When BMPs are implanted in a non-osseous mesenchymal tissue, they may induce chondrogenesis [83-87]. If this cartilage is able to fully differentiate along the endochondral lineage, it becomes calcified and supports subsequent vascularization and bone formation. The type of protein and their amounts depend on the cell type and nature of the surface.
2.3.5. **Influence of Surface properties on Cell-Biomaterial Interactions:** Surfaces are different from the corresponding bulk of the material. They contain unsaturated bonds which lead to the formation of surface reactive layers and adsorbed contamination layers. Preparation technique effects such as the type of material (metal, ceramics, and polymers), type of chemicals used for preparation, type of sterilization techniques, even though plays a key role in determining the bulk properties, the surface topography, chemistry or surface energy predominantly govern the surface properties. As previously discussed, cells in contact with a surface will firstly adhere and migrate. This first phase depends on previously described adhesion proteins. Thereafter, the quality of this adhesion and influences their morphology, capacity for proliferation and differentiation.

2.3.5.1. **Effect of Surface Morphology and Roughness on Cell Behavior:** Cells are also sensitive to the surface roughness and the fluctuations in surface structure. Variations in surface texture, or microtopography, can also affect the cellular response to an implant [88]. In case of osteoblasts, a higher percentage of cells adhere to the rougher surface [88]. In contrast, fibroblasts prefer smoother surfaces, and epithelial cells attach only to the smoothest surfaces [88]. The overall roughness of the surface influences the nature of the host response. *In vivo*, the surface characteristics of an implant, particularly roughness, may control tissue healing and therefore subsequent implant success [62, 89, 90]. Logically, the distribution of focal contacts dominates the mode of adhesion of osteoblasts on the different roughnesses. On smooth surfaces, focal contacts are distributed uniformly on the entire membrane surface which was in contact with the
surface. On rough surfaces, focal contacts are visible only at the extremities of cell extensions where cell membranes were in contact with the substrate [91-93].

2.3.5.2. Effect of Surface Energy and Chemistry on Cell Behavior: The hydrophilic and hydrophobic characteristics and the net positive or negative charge on the surface are also of great importance for cell response. Cell adhesion is generally better on hydrophilic surfaces [94-96]. Cell migration can be altered using the degree of hydrophilicity of the surface. It has been shown that human skin fibroblast migration increased with increasing hydrophilicity of the surface [97, 98]. The energy at the surface of a biomaterial is defined by its general charge density and the net polarity of the charge. A surface with a net positive or negative charge may be hydrophilic in character, whereas a surface with a neutral charge may be more hydrophobic. The net effect of the surface charge is to create a local environment with a specific surface tension, surface free energy and energy of adhesion [54, 55]. The net surface charge also plays a key role in cell attachment and spreading. This may be conceptualized as the interfacial free energy. As the initial events that led to protein adsorption are altered with time, a series of interfaces is established, each with an independent interfacial free energy. Differences in chemistry of the functional groups of a surface also influence the cell attachment and proliferation, although the exact mechanism is not clearly established. Presentation of ions or specific molecules (e.g., poly-ethylene glycol) that alter the hydrophobicity of the surface can change the orientation of binding proteins, and ultimately, the binding of cells to the material [99, 100].
2.3.5.3. Effect of Scaffold Osmolarity and pH on Cell Behavior: A scaffold must provide and maintain an environment with physiological pH (7.3 to 7.4) and osmolarity (280 to 310 mOsmol/Litre). A deviation of pH and osmolarity from physiological conditions may lead to cell injury [101-104]. For most scaffolds, simple hydration with normal saline solution prior to exposing them to cell can avoid cell injury. However, some matrices do not allow an isotonic condition to be created for cell delivery. Many bone matrix materials that are prepared with use of solutions containing high concentrations of low-molecular weight materials to improve handling (e.g., glycerol) and materials that dissolve rapidly in water, releasing hyper-osmolar concentrations of local ions (e.g., calcium sulfate) present this risk. If such materials are mixed with cells, they can induce osmotic injury, reducing or precluding cell viability.
<table>
<thead>
<tr>
<th>Immobilization molecule/strategy</th>
<th>Reactive GF group(s) needed</th>
<th>Reactive substrate group(s) needed</th>
<th>Immobilization mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-soluble carbodiimide (e.g., EDC)</td>
<td>Amine or carboxyl</td>
<td>Amine or carboxyl</td>
<td>Coupling reaction</td>
</tr>
<tr>
<td>Succinimidyl ester-phenyl azide (e.g., sulfo-SANPAH)</td>
<td>Amine</td>
<td>Double bonds or C–H bonds, or N–H bonds, or Nucleophiles (e.g., amines)</td>
<td>UV light</td>
</tr>
<tr>
<td>Monoacylated PEG-succinimidyl ester</td>
<td>Amine</td>
<td>Acrylate</td>
<td>UV light or free radical polymerization</td>
</tr>
<tr>
<td>Sulfo succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC)</td>
<td>heterobifunctional crosslinker with an amine-reactive NHS group and a sulphydryl-reactive maleimide</td>
<td></td>
<td>Heterobifunctional PEG</td>
</tr>
</tbody>
</table>

Table 1. Summary of common immobilization chemistries.
<table>
<thead>
<tr>
<th>Immobilization molecule/strategy</th>
<th>Growth Factor</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-soluble carbodiimide (e.g., EDC)</td>
<td>VEGF</td>
<td>Collagen</td>
<td>[105-107]</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>PCL</td>
<td>[108, 109]</td>
</tr>
<tr>
<td></td>
<td>BMP-2</td>
<td>Collagen</td>
<td>[110]</td>
</tr>
<tr>
<td>Succinimidy l ester-phenyl azide</td>
<td>VEGF</td>
<td>SEM</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>Glass</td>
<td>[112, 113]</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>Polystyrene</td>
<td>[114-116]</td>
</tr>
<tr>
<td>Monoacrylated PEG-succinimidy l ester</td>
<td>RGD</td>
<td>PEG-based polymers</td>
<td>[117, 118]</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>PEG-based polymers</td>
<td>[119, 120]</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>PEG-based polymers</td>
<td>[121-126]</td>
</tr>
<tr>
<td></td>
<td>PDGF-BB</td>
<td>PEG-based polymers</td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>FGF-2</td>
<td>PEG-based polymers</td>
<td>[128]</td>
</tr>
<tr>
<td></td>
<td>TGF-beta</td>
<td>PEG-based polymers</td>
<td>[129]</td>
</tr>
<tr>
<td>Sulfo-SMCC</td>
<td>VEGF</td>
<td>Agarose</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>Collagen</td>
<td>[131]</td>
</tr>
<tr>
<td></td>
<td>PDGF-BB</td>
<td>DBM</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>BMP-2</td>
<td>PCL</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>BMP-2</td>
<td>Chitosan</td>
<td>[134]</td>
</tr>
</tbody>
</table>

**Table 2.** Summary of common methods of growth factor tethering to biomaterial substrates
Figure 1: General scheme for a growth factor immobilization to a substrate with Water-soluble carbodiimide

Figure 2: General scheme for modifying a growth factor with a photo-reactive phenyl azide group and subsequent immobilization on a biomaterial substrate
Figure 3: General scheme for modifying a growth factor with a photo-reactive acrylate group and subsequent immobilization on an acrylated biomaterial substrate.

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CHAPTER 3: DESIGN AND VERIFICATION OF METHODS TO ASSESS THE RESPONSE OF CONNECTIVE TISSUE PROGENITOR CELLS TO SURFACE MODIFIED BIOMATERIALS

3.1. Unmet Need: The field of tissue engineering and regenerative medicine has generated a broad range of biomaterials that can be applied in many different ways as treatment options that seek to regenerate or accelerate the repair of various tissues. Bone regeneration is one of the most intensely studied approaches. Over 50 biomaterial products are currently available for bone regeneration. Formation of new bone by the progeny of native connective tissue progenitor cells (CTPs) is a central feature of each of these treatment options. The strategy of using a cell-based therapy to achieve bone regeneration inevitably includes the potential to use biomaterials to “target” CTPs and to use their interaction with a biomaterial surface to enhance their attachment, survival, migration, proliferation, or differentiation. Many of these same cellular responses can be targeted using other means such as mechanical or biophysical environment or soluble bioactive factors. However, biomaterials surfaces represent a unique opportunity due to the potential that they offer to control the special orientation of the cells and signals in a tissue region as well as the concentration and presentation of specific signals.

Recent advancements in biomaterials science include promising technologies for modifying biomaterial surfaces with pro-survival or pro-osteogenic bioactive ligands. These surface modifications use biomaterial-specific chemistry to present bioactive ligands in a controlled, stable and biologically active form.

Before testing the pre-clinical efficacy of these surface modified biomaterials in animal models, it is important to test the influence of these materials on cells in an in
**In vitro** setting. *In vitro* assays using cell culture preparations can be used to: characterize the response of specific cell types to biomaterials and possible degradation products, confirm the bioactivity and stability of a tethered molecule, characterize the dose responsiveness of a tethered ligand, characterize the differences between substrate surfaces and tethering chemistries for presentation of individual tethered ligands, *In vitro* assays can also be used to advance the understanding of fundamental biological outcomes in response to biological signals. However, in order to provide the stem cell and regenerative medicine community with the greatest possible and generalizable benefit, the methods available for assessment should be standardized, systematic, repeatable, quantifiable, user-independent, automated, cost effective and applicable to a vast array of biomaterials, surface modification strategies and cell sources. A platform offering such methods is not yet available, and represents the unmet need in both the biomedical research and the clinical community.

3.2. **Proposed Solution:** In this dissertation, I have addressed this gap in standardized methodology and advance the field of biomedical engineering by designing and applying a quantitative method to assess the response of CTPs (or other cell types) to a biomaterial surface or a surface-modified biomaterial by applying the recently adopted ASTM standard automated colony forming unit (CFU) assay (ASTM F2944-12). This new method uses engineering design principles to combine the following components:

1. a novel method to prepare two dimensional surfaces of a particulate of any bone scaffold material, with or without surface modification using a bioactive ligand;
(2) a method to verify and optimize these surfaces with respect to their repeatability, stability, uniformity in coverage, and smoothness; and

(3) a method to assess the material and surface modification effects on CTPs using a standardized method for the automated CFU assay.

Figure 1 describes the flowchart of the design process. Constraints were identified based on the available knowledge and the unmet need. The technical specifications were defined, forming the basis for verification of the compliance of individual components. The combined method was validated using β-TriCalcium Phosphate (TCP), a widely available and clinically useful bone scaffold material. TCP surfaces were further evaluated with and without surface modification with Epidermal Growth Factor (EGF), a pro-survival and proliferation bioactive ligand. Technology for tethering EGF to a TCP-containing surface using fusion protein containing both EGF and a novel TCP-binding peptide was developed by the Griffith Lab at MIT, as described in Chapter 4.

The methods described for surface fabrication and testing are applicable to a wide variety of cell types, biomaterials, and bioactive ligands. Some examples include: ceramics (e.g., β-TriCalcium Phosphates (TCPs), hydroxyapatites, nanocrystalline calcium phosphates), biologics (e.g., demineralized bone matrix, chitosan, lyophilized collagen) or synthetic polymers (e.g. Poly(methyl methacrylate) (PMMA), Polycaprolactone (PCL), poly(propylene fumarate) PPF) are some examples of such biomaterials. While this presentation is focused on materials and applications in bone tissue regeneration, these methods can apply to materials and cell types well beyond bone regeneration, including cells and materials relevant to: skin and wound healing, nerve
recovery, muscle regeneration, tendon/ligament, vascular reconstruction, as well as hollow and solid organs.

3.3. **Components of the Design Relevant to Bone Tissue Regeneration:** Osteogenic Connective Tissue Progenitors (CTPs): The recent developments in stem cell and progenitor cell biology has highlighted the fact that bone marrow, trabecular surface bone, muscle, periosteum, perivascular tissue, adipose tissue and blood can each provide a potential niche and source for a heterogeneous population tissue derived stem cells and progenitor cells capable of contributing to the forming bone tissues. Bone repair requires osteogenic Connective Tissue Progenitors (CTPs), defined as a tissue resident population of cells that are capable of proliferation and osteoblastic differentiation. No new bone can be formed without a source of cells capable of generating progeny that ultimately secrete osteoid, the hallmark of bone formation. Many preclinical studies have demonstrated improved graft performance when CTPs are added, even to small graft sites in young healthy animals, supporting the premise that the CTP population is suboptimal in virtually all clinical settings and that optimal performance from any osteoconductive or osteoinductive biomaterial may require augmentation with local CTPs [1-10].

3.3.1. **Osteoconductive Materials:** An osteoconductive surface is one that permits the attachment, migration, proliferation and differentiation of CTPs and other contributing cells throughout a grafted region, and thereby facilitates the distribution of bone formation and bridging or connection to adjacent bone structures. An ideal osteoconductive scaffold is biocompatible, reproducible, resorbable, porous, and cost
effective. A variety of synthetic materials are used clinically as bone grafts, including ceramics, collagen, non-collagenous proteins, bioactive glasses, and biodegradable polymers.

3.3.2. **Osteoinductive Stimuli:** Osteoinductive stimuli are defined as soluble factors that modify (induce a change in) cell fate in a definitive direction towards bone differentiation. Bone Morphogenetic Proteins (BMPs) are the prototypical inductive signaling molecule in the musculoskeletal system. However, many other factors have important osteotropic effects. Platelet Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF) and EGF are each identified as key effectors in improving the performance of stem and progenitor cells in orthopaedic tissue engineering applications.

EGF is a potent mitogen for osteoblastic progenitors, and is both necessary and sufficient to induce colony growth in bone marrow-derived cells [11, 12]. EGF signaling has been shown to prevent TNF-induced apoptosis in a cell line derived from MSCs [13, 14]. The EGF receptor (EGFR) is expressed by virtually every cell type, including stem and progenitor cells. EGFR is a receptor tyrosine kinase that activates intracellular signaling cascades that influence cell proliferation, migration, and differentiation. EGF-EGFR interaction induces EGFR dimerization and autophosphorylation which activates downstream intracellular pathways. Once bound to the receptor, EGF is rapidly internalized by receptor-mediated endocytosis leading to receptor desensitization, thereby down-regulating the effects of exogenous EGF [15].

Osteoinductive stimuli in the form of growth factors and bioactive peptides can also be selectively concentrated and presented by either preferential adsorption or
tethering- i.e. covalently linking them to a surface. There are several potential advantages in presenting growth factors as a matrix-tethered molecule for applications in tissue engineering. Tethering ensures that the signaling molecule does not diffuse away from the cells rapidly, and enables control over the concentration and three dimensional conformation of the molecule that preserves its ability to interact with receptors. In case of epidermal growth factor (EGF), tethering it to the scaffold has shown to be competent to bind and activate the EGF receptor, but not internalized and degraded. This mode mimics features of the physiological presentation of EGF receptor ligands that act in juxtacrine fashion or are matrix bound.

EGF is a candidate for relatively simple chemical modifications, compared to other growth factors. EGF is a relatively small protein with a structure that is characterized by a single amine group at its N-terminus. EGF has been shown to be bioactive when tethered to biomaterials, in contrast to many other factors, which have limited applications when presented in a tethered form [16, 17]. Currently, most growth factors (e.g., BMPs) are implanted in very high local concentrations and released by diffusion, making it extremely difficult to control their local concentration and rate of clearance. In case of epidermal growth factor (EGF), tethering it to the scaffold has shown to be competent to bind and activate the EGF receptor, but not internalized and degraded. This mode mimics features of the physiological presentation of EGF receptor ligands that act in juxtacrine fashion or are matrix bound.

3.3.3. CFU assay for CTPs: When placed in the in vitro culture system, under appropriate conditions, CTPs adhere to the surface on which they are plated, survive, and
go on to proliferate to form a colony of cells that represent the progeny of the original founding CTP. Colony forming unit (CFU) assays have been the gold standard for the quantitative assessment of CTPs since the 1980s. Several methods have been used to define colonies for the purposes of quantification of the number, concentration or prevalence of colony founding cells in a starting population. Colonies are identified based on gross size, morphology, number of cells and expression of specific markers or enzyme activity [18-21]. However, the vast majority of these methods have been based on subjective and qualitative assessments based on skilled observers, which have been shown to be prone to large variation within and between observers [22]. In this work we have chosen to adopt automated image-based methods of colony assessment consistent with the recently adopted ASTM Standard Method, (ASTM F2944-12) [23]: “Standard Test Method for Automated Colony Forming Unit (CFU) Assays- Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture”. An image capture and analysis system platform developed in the Muschler Lab and named Colonyze™ is used for this purpose. In the form that was available at the beginning of this project, Colonyze™ was designed and partly optimized for the assessment of colonies on glass or tissue culture plastic surfaces, or with surface modifications applied to glass or tissue culture plastic [24-27]. These assays are not optimized for a three-dimensional scaffold or the use of an optically opaque material.

3.4. **Constraints of the design:**

3.4.1. **Use of Beta-tricalcium phosphate as a biomaterial that is modified with tethered EGF:** Beta-tricalcium phosphate (TCP) is a clinically used bone graft material
and is widely used as bone void filler [28-34]. These materials have been widely used as bone graft materials due to their biocompatibility and similarity in composition to bone mineral [35, 36]. TriCalcium Phosphate [Ca₃(PO₄)₂] is not a natural component of bone but has chemical proportions of calcium and phosphate similar to bone mineral [37]. These materials provide a biocompatible osteoconductive scaffold for new bone formation.

The continued development and use of TCP as a bone graft material is limited by its material properties, and the disintegration of TCP in aqueous solutions at physiological pH which do not permit stable direct covalent chemical surface modification using covalent binding methods. As a means of overcoming the surface constraints of TCP related to covalent binding, the surface of TCP scaffolds was modified in this project using tethered EGF via a high-affinity, non-covalent binding novel TCP binding peptide TCPBP [38]. We examined the effect of tethered TCPBP-EGF on the in vitro performance of bone marrow-derived CTPs. Verification of TCPBP surfaces required measurement of TCPBP-EGF concentration, biological activity, and stability. The tethering concentration of TCPBP is dependent on the concentration in tethering solution and incubation time. The specific receptor mediated biological TCPBP-EGF could be confirmed based on the activation of ERK in passage 3 MSCs. The stability of tethered EGF on TCP scaffolds could be characterized using the standardized BCA assay.

3.4.2. Use of CTPs and osteogenic conditions to screen for pro-osteogenic biological response: Cell sourcing for these methods was designed to utilize human bone marrow or discarded bone collected from patients undergoing invasive arthroscopic surgeries
under an IRB approved protocol. CTPs show a large patient to patient variation in both the concentration of cells and the prevalence of colony founding CTPs among the cells isolated. The number of patients recruited for the study will be determined using statistical methods employing power analyses. As the intended usage of the surface modified TCP biomaterials being tested for use as bone void fillers that promote osteogenic potential of local or transplanted CTPs, the CTP response was evaluated in osteogenic culture conditions.

3.4.3. Use of Colonyze™, a standardized automated CFU assay to assess CTP response: The traditional CFU assay determines the observed CTP prevalence manually by counting the number of colonies on the adherent surface. However, this method is highly user dependant and the assessment may be influenced by user bias in determination of a colony, and inaccuracy in colony identification. The user-to-user variability has been shown to be as high as 40%. The method developed in this dissertation will use an automated CFU assay that uses an algorithm that is accurate, consistent, automated and independent of user bias, and is compliant with the ASTM Standard Method (ASTM F2944-12) [23]: “Standard Test Method for Automated Colony Forming Unit (CFU) Assays- Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture”.

While verification and validation of this method is demonstrated using one class of biomaterial, one class of bioactive stimulus, and one cell type, this method can easily be generalized to any combination of biomaterials, bioactive stimuli and cells. Potential applications of this method are discussed in Chapter 6.
3.5. **Technical Specifications of the Design:**

3.5.1. **Design of biomaterial surfaces:** Biomaterials can be prepared in a variety of shapes, dimensions and sizes. However, the following surface properties are desired:

1. **Ease of preparation and repeatability:** the method of biomaterial surface preparation must be consistent, repeatable, and relatively easy to execute.

2. **Uniformity:** Surfaces must be uniform in terms of the surface properties (coverage, smoothness, porosity, hydrophilicity, or charges where applicable), and interaction between the cell and surface,

3. **Stability:** the surface must be stable for the duration of culture, and may not degrade or disintegrate.

4. **Detection of colonies:** the form of the biomaterial (shape and dimensions) must be such that the cells which comprise the colonies formed by the progeny of CTPs can be detected in an accurate and consistent manner enabling the primary outcomes of colony formation and performance to be quantified.

In order to choose the optimal surface design, a scoring system will be implemented for the verification step. Surfaces will be scored for: (1) Ease of Preparation (2) Repeatability (3) Surface Uniformity (4) Stability During Culture Period (5) and Identification and Detection of Colonies. A scoring system is defined where the biomaterials will be scored on the scale of 0 to 5 (5 for Excellent, 4 Above Average, 3 Average, 2 Acceptable, 1 Poor, And 0 Unacceptable).
Four samples from each surface condition will be evaluated. In addition to the visual qualitative assessment, brightfield microscopy, and scanning electron microscopy will be used to confirm the surface properties, and cell culture will be used to determine the cell response properties. The biomaterial with the highest overall score, AND no score below 3 in any of the criteria will be advanced to the validation step.

3.5.2. **Surface density and stability of bioactive ligands:** Surface density of the bioactive ligand on the test material is measured in units of number of ligands per µm². On theoretical grounds, the surface area of a spread cell in contact with the biomaterial surface is ~ 500 µm². A typical MSC expresses 50-100,000 EGF receptors (which gives the estimated surface density as 100-200 receptors per µm²). A significant portion of these cells can interact with tethered EGF molecule over the course of time. Therefore a minimum surface density that is at least 100 times more than the surface density of the receptor on the cell surface is like to be sufficient to stimulate the EGFRs on the cell surface to a maximum level [26, 38, 39]. For the TCPBP-EGF protein (molecular weight 77 kDa), that has been tethered to a 18 mm diameter coverslip (surface area 254 x 10⁶ µm²), a desired surface density of 20000 molecules per µm² translates to 650 nanograms per coverslip. Including a 40% excess, the minimum expected amount of tethered TCPBP-EGF is set at at least 1000 nanograms per coverslip. The repeatability and stability of surface modification will be verified for at least 5 days.

3.5.3. **Primary and secondary outcomes of the CFU assay:** Primary outcomes of the automated CFU assays will be:
(1) Number of colonies on the biomaterial surface,

(2) Number of cells in each colony,

(3) Colony density, a ratio of the number of cells in colony and the colony area

(4) Alkaline phosphatase (AP) positive area in the cell cytoplasm.

A colony is defined as a cluster of 8 or more cells separated by less than 142 µm (an empiric metric based providing clustering of cells into colony objects matching determinations made by skilled observers). Cells are identified using a means of counting individual cells. In this project the means of cell identification is based on staining of fixed cells using 4',6-diamidino-2-phenylindole (DAPI), a nuclear fluorescent stain. DAPI stained nuclei can be identified under fluorescence with 340-380 nm excitation and >425 nm emission wavelengths. Alkaline phosphatase (AP) is a marker for early osteogenesis. AP expression will be determined using a alkaline phosphatase Vector Red stain kit (Vector Labs), that produces a precipitate which has a red color in the visual spectrum and under fluorescence with 566 nm excitation and 575-670 nm emission wavelengths.

(1) Relative Colony Forming Efficiency (CFE): CFE of the test material will be calculated as a ratio of the mean number of colonies on the surface of a test material divided by the mean number of colonies on the control material. CFE will be used to determine the influence of the test material on the colony forming ability of CTPs. Further, all the colony data will be pooled together for each test condition for all patient samples. A median will be calculated for each colony metric. Due to the known wide variation in CTP prevalence and performance between individual subjects, the
outcome corresponding to the test surface will be divided by the outcome corresponding
to the control surface.

(2) Colony Size (metric of effective proliferation): determined as the median
number of cells per colony on the test surface standardized to the median number of cells
per colony on the control surface, (3) Relative Migration: determined as the median
number of colony density on the test surface standardized to the median number of
colony density on the control surface, (4) Osteoblastic differentiation: determined as the
median number of AP positive cell area divided by cell number on the test surface
standardized to that on the control surface. Table 1 summaries the primary and
secondary outcomes and technical specifications:

3.5.4. CFU Analysis - Cell culture conditions, inclusion/ exclusion criteria and
sample size determination: Bone marrow derived cells are cultured for 9 days in
osteogenic conditions containing 10% fetal bovine serum, 50 μM ascorbate, 10 nM
dexamethasone. The initial plating density per surface will be 500,000 for bone marrow
or marrow space-derived cells and 200,000 for cells derived from trabecular surface.
Based on previous data, the expected range of colony prevalence at these plating densities
is 4 to 50 per surface. A prevalence of more than 50 leads to a state of confluence in
which the progeny of individual colonies often overgrow into one another compromising
the ability to identify discrete colonies. A prevalence of less than 4 colonies limits the
accuracy of the assessment - as one error in positive or negative colony identification can
cause a 25% or more difference in overall assessment. Therefore, confluent samples
(more than 50 colonies) or samples with less than 4 colonies are excluded from the analysis.

3.5.5. **Criteria for success:** Based on historical data correlating CTP prevalence (observed colony formation) and in *in vivo* biological response [25, 40], a minimum of 50% improvement in CFE or cell number per colony, a function of effective proliferation rate (EPR) represents an appropriate (albeit as yet unvalidated) threshold for demonstrating a biological response to a test condition (e.g. a surface modified biomaterial) compared to the control (material with no surface modification) that is of potential clinical value.

Metrics of colony density (associated with migration) and osteoblastic differentiation, are also potentially relevant from a clinical perspective. However, these metrics, taken by themselves may or may not measure positive aspects of biological response from a clinical perspective, and must be interpreted in the overall context of performance, starting with CFE and effective proliferation rate (EPR). An increased level of osteoblastic differentiation (i.e. expression of markers or activities associated with osteoblastic differentiation, such as alkaline phosphatase activity or PTH receptors) indicates more CTPs committed to osteogenesis (i.e. demonstrate performance as osteogenic CTPs). However, because differentiation is often associated with a shift away from proliferation, osteogenesis alone, and particularly premature osteogenesis may have the adverse effect of reducing the overall number of mature osteoblasts generated and the resultant amount of new bone tissue. Similarly a decreased level of colony density indicates more migration of CTP-progeny, which may be biologically desirable in
distributing progenitors throughout the grafted volume. However, an increased colony density, suggesting less migration of CTP-progeny, may also be biologically desirable, since high density may be associated with both an increased EPR and increase osteogenic activity.

Patient-to-patient variability and the known heterogeneity of CTP in cells harvested from bone marrow and bone tissue samples must be considered in determining sample size when making generalizable comparisons between test conditions. A power analysis is performed to determine the necessary sample size to yield a significant difference with $\alpha = 0.05$ and power $= 0.80$. In general, assessment in samples from at least 5 and up to 20 human subjects is necessary to document the generalizability of a given finding across subjects. Within subject samples, analysis of at least 20 and up to 200 colonies (progeny samples of individual CTPs) is necessary to document differences between test conditions (i.e. biomaterials and surface modifications).

3.6. Design and Verification:

3.6.1. Assessment of existing TCP scaffolds: TCP TheriLok™ scaffolds (“crosses”), a gift from Integra® Lifesciences (Plainsboro, NJ), were used as a generic substrate for assessment of CTP-derived colony formation on a 3D surface and as a substrate for tether in of TCPBP-EGF to a TCP surface. Crosses are fabricated using a 3D printing methodology (3DP) followed by sintering to shape TCP granules into a 3D TCP scaffold of defined shape. The nominal size of the TCP TheriLok™ scaffolds is 3 mm from tip to tip of each cross and 1.8 mm in height. The “cross” design was intended to facilitate
interlocking between crosses when placed into a defect site as a means of enhancing stability. TheriLok™ scaffolds are 60% porous with a pore size of 250 µm.

Scaffolds were first scored for compliance with technical specifications (i.e. broken or misshaped scaffolds were discarded). Scaffolds were sterilized using a steam autoclave (20 minutes at 121°C and 15 psi) and hydrated in 1X sterile PBS under vacuum. Individual scaffolds were transferred to a sterile well in a 96 well polypropylene plate. 150 uL of bone marrow aspirate containing was gently loaded into each well. Crosses loaded with marrow-derived cells were cultured in osteogenic conditions for 6 days. Culture media was replaced every 72 hours. Cells adherent to each cross were fixed with 1:1 acetone/methanol (Fisher Scientific, Silver Spring, MD) for ten minutes at room temperature and air dried. Prior to staining, cells were hydrated with deionized water for 10 minutes. VECTASHIELD™ (Vector Labs, Burlingame, CA) mounting medium containing DAPI (0.75 µg/µL) was applied in the dark overnight, followed by a rinse using deionized water. Alkaline phosphatase activity was assayed using SK5100 Vector Red kit (Vector Labs, Burlingame, CA) according to the manufacturer’s instructions.

The crosses were scanned using a fluorescent microscope using a 2048 x 3072 Quantix K6303E 12 bit digital camera (Roper Scientific) attached to a Leica DMXRA motorized microscope controlled by ImagePro imaging software. 48 individual images were collected in a total of 40 focal planes starting at the top of the scaffold at 461 x 344 pixels (1641.16 µm x 1224.64 µm), 8-bit gray level, using a 10x objective (pixel size = 3.56 µm). Each plane was separated by 50 µm. Individual images were stitched together to form one montaged image per plane. This allowed capture and reconstruction of 3D
images from the top 50 μm of the 1800 μm thick scaffold. DAPI fluorescence localizing individual cell nuclei were obtained with UV light (340-380 nm) excitation and fluorescence was read at >425 nm. Alkalike phosphatase expression was imaged with 566 nm excitation and 575-670 nm emission wavelengths.

3.6.2. Verification: Images associated with the assessment of colony formation on TheriLok™ scaffolds are shown in Figures 1 and 2. A movie moving through each of the focal planes can be provided as supplemental material. Overall, evaluation of CTP-derived colony formation was found to be challenged by several factors. TCP TheriLok™ scaffolds are brittle and subject to fracture and deformation during processing and handling. This required hand selection of intact scaffold. The scaffolds are optically opaque and also geometrically complex and porous. As a result, cells and colonies were distributed into the pores of the material and along the vertical walls of the scaffolds, making many of the cells present unobservable in the center and along the sides of the scaffold below a depth of 50 μm. These limitations were judged to make the overall utility of the method of colony assessment unacceptable, as outlined below.

<table>
<thead>
<tr>
<th></th>
<th>Ease of Preparation</th>
<th>Repeatability</th>
<th>Surface Uniformity</th>
<th>Stability During Culture Period</th>
<th>Identification and Detection of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Score</td>
<td>5</td>
<td>2.5</td>
<td>2.5</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

3.6.3. Revised Design: To overcome the limitations of the TCP scaffolds, methods were explored to develop a two dimensional presentation of the TCP scaffold surface was designed in which TCP could be presented on the surface of a substrate, reproducing the surface texture of the TheriLok™ scaffolds, but limiting the depth of the porosity to less
than 30-40 µm. This exploration followed two primary phases in design. In Phase I, three basic designs strategies were explored using TCP particles 46-106 µm diameter:

2. Design 2 - Spin coating PDMS with addition of TCP particles before curing.
3. Design 3 - Spin coating preparation of PDMS with TCP particles followed by mechanically pressing particles into the PDMS surface before curing of the PDMS.

In Phase II, following the selection of the design that satisfied the criteria of success, was chosen as a preferred strategy, the design was further explored for the optimization with respect to two primary variables: a) PDMS thickness (15 vs 35µm) and b) TCP particle size (46-106 vs 26-45 vs less than 26 µm), as illustrated in Figure 6. Ultimately, Design 2 using PDMS thickness of 35µm and TCP particle size of less than or equal to 25 µm was selected as optimal, based on the stability of particles on the surface, superior uniformity of coverage and smoothness.

The following detailed discussion as well as the images presented in Figure 6, summarizes this process.

3.6.3.1. Phase I: Design 1: (manual preparation of PDMS coating with TCP particles 46-106 µm diameter and removal of non-attached particles by manual tapping): 18 mm diameter circular glass coverslips (thickness- 0.13-0.17 mm) (VWR, Batavia, IL) were used as a base surface. Coverslips were cleaned by swirling them for four hours in 2% ChemSolv glass cleaner solution (Mallinckrodt Baker, Phillipsburg, NJ) at room temperature, followed by swirling three times for 5 minutes in deionized water. All swirling steps were performed at 100 rpm using a MaxQ 2000 Rotary shaker (Thermo
Coverslips were then silanized by swirling in filtered 2% Siliclad (Gelest, Morrisville, PA) for 20 seconds, followed by swirling three times for 5 minutes in deionized water. Silanized coverslips were cured at 100°C for 20 minutes, left at room temperature overnight, and then stored in a vacuum oven at room temperature.

PDMS (polydimethylsiloxane) was used to provide an adherent surface for TCP particles. PDMS mixture was prepared using SYLGARD 184 Silicone Elastomer kit (Dow Corning, Midland, MI) by mixing the Elastomer Base and curing agent at 10:1 ratio. A 100 µl droplet of PDMS was placed in a parafilm-lined plastic tray. A silanized coverslip was placed on top of the droplet, and pressed gently using a separate larger glass coverslip (2 cm x 6 cm). TCP powder (particle size < 106 µm) was provided as a gift from Integra Lifesciences (Plainsboro, NJ). Six PDMS-coated coverslips were placed in a TCP powder-PDMS side down, and pressed gently for 30 seconds. Excess unattached TCP particles were removed by manually holding the slide at a 90 degree angle to the table top and tapping gently against a clean surface. Coverslips were cured at room temperature overnight, washed twice with deionized water and stored till further use.

3.6.3.2. Design 2: (Spin coated PDMS with TCP particles 46-106 µm diameter layered on the surface, removal of non-adherent particles using sonication and air blowing): In this design, a PDMS layer was applied on silanized glass coverslips by the spin coating process. SYLGARD 184 Silicone Elastomer Base and the curing agent were mixed in a 10:1 ratio at room temperature. 1 mL PDMS mixture was prepared for a batch of 12 coverslips. The mixture was placed in a vacuum oven at room temperature for 20
minutes to remove air bubbles. Silanized glass coverslips were placed on a spin coater controller head (Headway Research, Garland, TX). A 50µL PDMS mixture was deposited on each coverslip, and was spun at 2500 rpm for 30 seconds. Each coverslip was manually examined to ensure that there was no lint, debris or irregularities.

TCP powder (particle size < 106 µm) was sieved on the PDMS layer immediately after spin-coating was completed. 1 gm TCP powder was poured into the sieve (VWR Scientific), and TCP particles were gently pushed through the sieve using a spatula. TCP particles were allowed to deposit onto the PDMS coat. TCP coated coverslips were gently tapped to remove unattached TCP particles, and cured in a vacuum oven at 60°C for 2 hours at atmospheric pressure. This curing step hardens the PDMS layer, thereby providing a stable TCP coating. TCP surfaces were prepared in batches of 12. TCP coated coverslips were sonicated upside down for 2 minutes in deionized water to remove non-adherent TCP particles using the Aquasonic sonicator (VWR Scientific) at 50 Hertz. A gentle air stream was used to blow any remaining loose TCP from each coverslip. Coverslips were then dried overnight and examined under the microscope to determine coverage and artifacts. Coverslips were then dried overnight and stored at room temperature till further use.

3.6.3.3. Design 3: (Spin coated PDMS with TCP particles 46-106 µm diameter layered on the surface and secondarily pressed into the PDMS): In this approach, design 2 was modified to include a step in which the TCP layer was pressed into the PDMS layer using a clean 250 gm glass block for 10 seconds prior to curing. Pressing the TCP layer was expected to provide a more uniform surface. The procedure developed in
design 2 was repeated, except the surfaces were pressed. The resulting surfaces were scored for compliance with technical specifications.

To test these surfaces for effectiveness in detection of colony formation, surfaces were sterilized under UV for 45 minutes each side, and then washing twice in 70% ethanol followed by sterile 1X PBS. Coverslips were placed in a sterile 2cm x 2 cm LabTek chamber (Nunc, Naperville, IL) and 500,000 nucleated marrow derived cells were plated in 2mL osteogenic culture medium. Culture media was replaced after 72 hours. Scaffolds were fixed after 9 days with 1:1 acetone methanol and stained with DAPI and AP, and colony formation was verified qualitatively under a fluorescent microscope using appropriate filters for DAPI and AP wavelengths.

3.6.3.4. Verification: In Phase I, all three surface designs scored better than the TCP scaffolds on repeatability, uniformity, stability during the culture time. As shown below, Design 2 as scored higher than Designs 1 and 3 in ease of preparation, repeatability, and surface uniformity. Based on this assessment and overall scores, Design 2 was chosen for further verification experiments and validation.

<table>
<thead>
<tr>
<th>Average Score</th>
<th>Ease of Preparation</th>
<th>Repeatability</th>
<th>Surface Uniformity</th>
<th>Stability During Culture Period</th>
<th>Identification and Detection of Colonies</th>
<th>Overall Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Design 1</td>
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<td>5</td>
<td>4.5</td>
<td>22.5</td>
</tr>
<tr>
<td>Design 3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>4.5</td>
<td>19.5</td>
</tr>
</tbody>
</table>
3.6.4. **Phase II - Optimization of Design 2 (with respect to PDMS thickness and TCP particle size):** The thickness of the PDMS coating depends on the viscosity of the PDMS solution and the spin coat speed. A thin PDMS layer with larger particle size will make the surface non-uniform and rough, and on the other side a thick PDMS layer with smaller particle size may compromise the exposure of TCP particles on the surface. To optimize the surface properties we used different combinations of the PDMS thicknesses and the TCP particle sizes.

Two spin coat speeds (2000, 5000 rpm) were used to vary the thickness of the PDMS coating. The expected coating thickness was 35 and 15 µm, respectively [41, 42]. Sintered TCP granules, were sieved onto the PDMS coated coverslip to obtain particles that were ≤25 µm, 26 - 45 µm, and 46-106 µm in size. To confirm that the TCP was exposed on the surface (not covered by PDMS) and that the TCP was intimately entrapped by the PDMS and not resting directly on the glass coverslip, a subset of TCP coated coverslips were demineralized by rocking coated side down in 3 mL of 1M HCL overnight and rinsed 3X for 5 min in deionized water, and left again overnight at 4C in 3 mL of 1X PBS.

A Scanning Electron Microscope (SEM) was used to obtain high magnification images of the surfaces. SEM images were used to confirm the removal of TCP granules from the surface and to screen for patches of exposed glass coverslip that would imply a non-uniform thickness of PDMS.
3.6.5. Verification: A scoring system introduced about was used again to assess the surface uniformity and smoothness. Surfaces were scored on the scale of 0 to 5 (5 for Excellent, 4 Above Average, 3 Average, 2 Acceptable, 1 Poor, And 0 Unacceptable). See Figure 6 for visual illustration of the findings for each thickness and particle size combination.

PDMS Thickness of 15µm and Particle size: 46-106 µm: (Figure 6. Images A-C): SEM images before and after demineralization demonstrated non-uniformity of the PDMS surface, as evidenced by patches of exposed glass in Figure 6B. The larger particle size lead to a rough pattern on the surface, with crevices with a depth more than 50 µm. The 15µm thickness of PDMS layer was inadequate to hold TCP particles in many areas. Overall surface was scored as 1.

PDMS Thickness of 15µm and Particle size: 25-45 µm: (Figure 6 D-F): The uniformity and smoothness of the TCP layer improved considerably. Further, SEM images of demineralized surfaces verified that a PMDS thickness of 15µm provided a stable base for 25-45 µm TCP particles. These surfaces were scored as 3.

PDMS Thickness of 15 µm and Particle size: <=25 µm: (Figure 6 G-I): Surfaces prepared with 15µm thick PDMS also provided a stable base for TCP particles <=25 µm in size. However, some irregularities were observed. (Figure 6G). These surfaces were scored as 3.
**PDMS Thickness of 35µm and Particle size: 46-106 µm:** (Figure 6. Images J-L): The increase in the thickness of PDMS To 35 µm improved the binding of TCP particles. The PDMS thickness was adequate to hold the TCP particles. However, the roughness of the surface was observed to be similar to figure (A). These surfaces were also scored as 3.

**PDMS Thickness of 35µm and Particle size: 25-45 µm:** (Figure 6 Images M-O): the PDMS thickness was adequate to provide a binding surface for TCP particles. The smoothness and the uniformity in coverage improved compared to previous combinations. These surfaces were also scored as 4.

**PDMS Thickness of 35µm and Particle size: < =25 µm:** (Figure 6 Images P-R): Surfaces prepared with 35 µm thick PDMS and <=25um particle size provided the most uniform and the smoothest surface. The average score for these surfaces was 5. This composition was used in subsequent experiments to evaluate *in vitro* performance with and without TCPBP-EGF. These scored are summarized below:

<table>
<thead>
<tr>
<th>PDMS Thickness (µm)</th>
<th>15</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP Particle Size (µm)</td>
<td>46-106</td>
<td>25-45</td>
</tr>
<tr>
<td>Average Score</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

**3.6.6. Colonyze™ algorithm:** The existing Colonyze™ image processing algorithm has been described in detail in prior publications [22, 25, 43]. As previously defined, each imaging application (cell source and surface often produce colonies with a different spectrum of features. As a result, the Colonyze™ method used in an individual setting was refined to create a defined “profile” which sets specific variables used in identifying
cells and clustering cells into defined colony objects (e.g. thresholds for nuclear size, staining intensity). The Colonyze™ profile refined for these applications was verified to accurately detect at least 95% of the nuclei that were segmented against the auto-fluorescent TCP background, as shown in Figure 7.

3.6.7. Verification of the binding and stability of tethered EGF: Preparation of tethered EGF modified TCP surfaces: Eight TCP coverslips were tethered in one parafilm-layered plastic tray. Plastic trays and parafilms were first cleaned with 70% ethanol. Parafilms were then treated with sterile 1% BSA prepared in sterile 1X PBS (Calbiochem) overnight at 4°C in a sterile chamber, rinsed twice with sterile 1X PBS, and allowed to dry. BSA coated parafilms were kept at 4°C till further use. TCP surfaces were sterilized under UV for 45 minutes each side, and then washed twice in 70% ethanol followed by sterile 1X PBS. TCPBP-EGF protein was a provided by Dr. Linda Griffith’s laboratory at MIT (Cambridge, MA). TCPBP-EGF solution was prepared at desired concentrations on ice using 1X PBS as a diluting agent. BSA-coated parafilms were placed inside plastic trays. 150 µL TCPBP-EGF droplets were placed on the parafilm, and TCP coverslips was gently placed on the droplet with the TCP side interacting with the protein solution. The entire assembly was sealed, and incubated at 4°C for 36 hours in a humidifying chamber.

3.6.8. Quantification of the surface density of tethered EGF and stability analysis: TCPBP-EGF was tethered to TCP coverslips as described above. EGF-tethered coverslips were rinsed twice with 1X PBS, placed in fresh PBS solution, and then stored
at 4°C for 0, 24 hours or 5 days (N=4). To measure stability, surfaces were removed and rinsed twice with 1X PBS. The amount of EGF associated with each coverslip was assessed by the BCA assay (Piercenet, Rockford, IL ) [44, 45].

3.6.9. Verification: At time = 0 the mass of tethered EGF per TCP coverslip was in the range of 6000-7000 nanograms (mean= 6187, std. dev. 779) (Figure 8A), which met the criteria for success (at least 1000 nanograms). We demonstrated the repeatability of the tethering process by quantifying the amount of tethered EGF using two separate batches of coverslips using the same reagents in a repeated experiment. The P value was calculated as 0.16 using the standardized ANOVA methods for a 95% confidence interval; it verified that the binding was repeatable. The coefficient of variation for this comparison was 12.5%.

The stability of the tethered EGF surface (tethering concentration = 2.7 µM) to TCP coverslips was assessed in triplicate after storage at 0, 1, and 5 days in 1X PBS at 4°C. No significant degradation was detected over this interval. The P value was calculated as >0.15 using the standardized ANOVA methods for a 95% confidence interval. For the Day 0 vs. Day 1 comparison, the P value was 0.22, where for Day 0 vs Day 5 comparison, the P value was 0.15. The overall coefficient of variation was 7.98%. These data verify the stability of the presentation of tethered TCPBP-EGF. Tethered EGF presentation met the defined criteria of success.

3.7. Discussion: In this chapter, we have introduced a method to assess the CTP response to the surface modification of a biomaterial using an automated colony forming-unit
assay. This method overcomes several limitations of existing methods, enabling colony detection on an opaque surface or a 3-dimensional form of scaffold material, and the application of automated image capture and analysis to extract colony level assessment of the CTP response to the surface modified biomaterials.

We first determined the scope and components of the design. This design is applicable to biomaterials that are in a solid form (such as amorphous or crystalline ceramics, hydroxyapatites, demineralized tissue powder, or micro and nanoscale solid polymers), and can be used to prepare a stable surface (at least for the duration of the assay -10 days). In the application demonstrated here, we used TCP, a synthetic bone void filler, as a biomaterial, and then modified the surface of the TCP with EGF, using a specific binding protein. We used bone marrow aspirate and trabecular surface-derived cells as sources of connective tissue progenitors (CTPs). The CTP response was quantitatively assessed using an automated CFU assay system, Colonyze™ that meets the standards in ASTM Standard Method (F2944-12) [23].

We defined the technical specifications associated with this method. These included primary and secondary measurable outcomes of the CFU assay, and the criteria for success. We proposed to assess CTP performance colony forming efficiency, proliferation, migration and osteoblastic differentiation. We further established the inclusion/ exclusion criteria for colonies, and established criteria of success to assess whether a surface modification is sufficient to significantly influence the CTP response.

A scoring system was designed to assess biomaterial surfaces, and surface modification. Surfaces were scored for ease of preparation, repeatability, uniformity and consistency in coverage, stability and colony formation. Surfaces that did not meet the
criteria for success were revised until the criteria were met. The best performing surface design was optimized for particle size and PDMS thickness. Surfaces with the maximum score were advanced to analysis using Colonyze™ to assess the CTP response.

We first used existing scaffolds (TheriLok™ TCP scaffolds) to verify whether they satisfy the technical specifications. Existing scaffolds had several limitations, including a lack of stability during the culture period, and an inability to quantify colony level parameters in 3-dimensional structure due to its architecture and optical opacity. These limitations warranted a two-dimensional representation of a scaffold material. A novel approach to surface preparation was introduced to prepare two-dimensional TCP surfaces. Three surface designs were prepared, and scored for the compliance to the technical specifications defined for biomaterial surfaces. The design with the highest score (Design 2- Spin coated PDMS with TCP particles 46-106 µm diameter layered on the surface, removal of non-adherent particles using sonication) was advanced for optimization. This method can be used for any type of particulate biomaterial, ceramic or polymeric to create a two-dimensional surface on a glass coverslip using a PDMS layer as a binder to hold the particulate surface in place on the spin-coated on the glass.

Surface topography plays a key role in the cell response. Topography has effects on cell-surface interactions, surface charges and hydrophobicity. The cell-surface interactions and their effect on cell response have been discussed in detail in Chapter 2. Considering the importance of surface topography on cell response, we verified means to measure the surface uniformity, and smoothness based on the SEM images. A scoring system was defined, and three particle sizes and two PDMS thickness layers were verified for compliance. The surface with <=25 µm particles size and 2000 rpm rotation
speed provided the best score. These surfaces were advanced to the surface modification and colony formation tests.

We used TCP surfaces with tethered EGF as a prototype of a surface modified biomaterial. We quantified the amount of tethered EGF in separate a series of 4 experiments, and at different time points up to 5 days. We verified that the amount of tethered EGF was sufficient to induce a ligand-receptor interaction. We verified that EGF can be tethered to TCP surface in a repeatable, consistent manner. We also verified that the EGF remains stable for at least 5 days in 1X PBS.

We further verified that these surfaces prepared using these methods can be used to assess CTP colony level response using an automated CFU assay, Colonyze™ platform. The benefits of using Colonyze™ are discussed in Chapter 2. Expanding this range of Colonyze™ analysis capabilities from translucent two dimensional surfaces to non-translucent textured surfaces using PDMS as a binder rather than the surface itself opens the potential for exploration of broad range of biomaterials and surface modification methods. Application of this method required the development of an application-specific profile in Colonyze™ to enable accurate cell segmentation and identification on the auto-fluorescent surface background. Further, we verified at least 90% accuracy in cell segmentation. In Chapter 4, we will use this unified approach to validate this method by quantifying the CTP response to TCP coverslips with tethered EGF.
3.8. **Tables and Figures:**

Figure 1: The engineering design method: Use of engineering design principles to design a method to assess the connective tissue progenitor cell-response to surface modified biomaterials
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Outcomes (Directly Measured)</strong></td>
<td></td>
</tr>
<tr>
<td>Colony</td>
<td>A cluster of 8 or more cells separated by less than 142 µm from each other</td>
</tr>
<tr>
<td>Colony Size</td>
<td><strong>Number of cells in each colony</strong> (counted based on DAPI stained nuclei)</td>
</tr>
<tr>
<td>Colony density</td>
<td>Ratio of the number of cells and colony area</td>
</tr>
<tr>
<td>Area of Alkaline phosphatase (AP) (A&lt;sub&gt;AP&lt;/sub&gt;)</td>
<td>Area of the colony that is stained positively for AP expression</td>
</tr>
<tr>
<td><strong>Secondary Outcomes (Calculated)</strong></td>
<td></td>
</tr>
<tr>
<td>Relative Colony Forming Efficiency (CFE)</td>
<td>Ratio of the mean observed prevalence of colony forming units on the surface of a test material divided by the mean number of colonies on the control material</td>
</tr>
<tr>
<td>Colony Size (metric of effective proliferation)</td>
<td>Median number of cells per colony on the test surface standardized to the median number of cells per colony on the control surface</td>
</tr>
<tr>
<td>Relative Migration (metric related to Colony density)</td>
<td>Ratio of colony density on the test surface divided by colony density on the control surface.</td>
</tr>
<tr>
<td>Area Fraction of Alkaline phosphatase (AP) (metric of differentiation)</td>
<td>Area of the colony that is stained positively for AP expression divided by total number of cells in each colony</td>
</tr>
</tbody>
</table>

Table 1: Primary and secondary outcomes of the method

Figure 2: Assessment of TheriLok™ TCP scaffolds. (A) Macroscopic dimensions, (B) Disintegration of scaffolds after hydration in 1X PBS.
Figure 3: Imaging approach to quantify colony-parameters in TheriLok™ scaffolds. (A) A schematic of Z-stacking method, (B-E) Segmentation of nuclei within a plane below 100 µm. (B,C) Note the limitation of the opacity of the scaffold in identifying the nuclei in the middle part of the scaffold (Only the cells on the edge are segmented). (D) Composite image of the DAPI-stained nuclei on the scaffold combining all 48 planes. (E) Composite image of the DAPI-stained nuclei that positively expressed alkaline phosphatase.
### Design 1

Manual preparation of PDMS coating with TCP particles 46-106µm diameter

#### Silanized Coverslip is pressed on PDMS
- 100 µL PDMS Droplet
- Parafilm

#### TCP Particles
- PDMS
- Silanized Coverslip

### Design 2

Spin coated PDMS with TCP particles 46-106µm diameter layered on the surface

#### 50uL PDMS Droplet is spin coated
- Silanized Coverslip
- Spin Coater

#### TCP Particles
- PDMS
- Silanized Coverslip

### Design 3

Modified Design 3: TCP particles are pressed into the PDMS

#### 50uL PDMS Droplet is spin coated
- Silanized Coverslip
- Spin Coater

#### TCP Particles are pressed using glass
- TCP Particles
- PDMS
- Silanized Coverslip

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**Figure 4: Initial proof of design of 2D TCP coverslips:** Three designs, as described in section 5.3.2 are shown here. Second row provides the design description. Third row depicts the preparation of PDMS layer. Fourth row depicts the deposition of PDMS particles. Fifth row provide snapshots of these surfaces. Sixth (bottom) shows the formation of CTP-derived colonies. Cells were fixed after 9 days of culture and stained for alkaline phosphatase (in red).
Figure 5: Expected thickness of the PDMS layer as a function of spin coater speed:
(A) The expected thickness of Slygard-184 PDMS layer is between 15-35 µm for the speeds of 2000-5000 rpm [42]. (B) The thickness was confirmed to be 35 µm at 2000 rpm using SEM image analysis. (C) an SEM image at the scale of 100 µm to demonstrate the uniformity in coverage of TCP coating on PDMS.
Figure 6: Optimization of the TCP surface design, effect of particle size and PDMS thickness on the uniformity of TCP surfaces: (A-C) Particle size 46-106 μm, PDMS thickness 15 μm. Images were obtained at 100X magnification using a Scanning Electron Microscope (SEM). (A) SEM image at the edge of a coverslip. Note the roughness of the surface. (B) SEM image of the coverslip that was demineralized with HCl treatment. TCP particles were removed and PDMS layer was maintained. Note that the PDMS thickness was inadequate to provide a binding surface for TCP particles of 46-106 μm size. (C) SEM image at the center of the coverslip. Note the overall consistency of the coverage.
X100, Angle 45°
PDMS spin coat speed = 5000 rpm
PDMS Thickness = 15 µm
TCP Particle size 26-45 µm

Figure 6: Optimization of the TCP surface design, effect of particle size and PDMS thickness on the uniformity of TCP surfaces: (D-F) Particle size- 26-45 µm, PDMS thickness- 15 µm. Images were obtained at 100X magnification using a Scanning Electron Microscope (SEM). (D) SEM image at the edge of a coverslip. Smoothness of the surface is improved compared to image (A) (E) SEM image of the coverslip that was demineralized with HCl treatment. TCP particles were removed and PDMS layer was maintained. Note that, unlike image (B), the PDMS thickness was sufficient to provide a binding surface for TCP particles of 26-45 µm size. (F) SEM image at the center of the coverslip. Note the overall consistency of the coverage.
Figure 6: Optimization of the TCP surface design, effect of particle size and PDMS thickness on the uniformity of TCP surfaces: (G-I) Particle size ≤ 25 µm, PDMS thickness 15 µm. Images were obtained at 100X magnification using a Scanning Electron Microscope (SEM). (G) SEM image at the edge of a coverslip. Smoothness of the surface was inconsistent compared to image (D) (H) SEM image of the coverslip that was demineralized with HCl treatment. TCP particles were removed and PDMS layer was maintained. Note that, unlike image (B), the PDMS thickness was sufficient to provide a binding surface for TCP particles of ≤ 25 µm size. (I) SEM image at the center of the coverslip. Note that the overall consistency of the coverage is improved compared to (C) and (F).
Figure 6: Optimization of the TCP surface design, effect of particle size and PDMS thickness on the uniformity of TCP surfaces: (J-L) Particle size- 46-106 µm, PDMS thickness- 35 µm. Images were obtained at 100X magnification using a Scanning Electron Microscope (SEM). (J) SEM image at the edge of a coverslip. Note that the roughness of the surface was similar to (A). (K) SEM image of the coverslip that was demineralized with HCl treatment. TCP particles were removed and PDMS layer was maintained. Note that the PDMS thickness was adequate to provide a binding surface for TCP particles of 46-106 µm size. (L) SEM image at the center of the coverslip. Note the overall consistency of the coverage.
Figure 6: Optimization of the TCP surface design, effect of particle size and PDMS thickness on the uniformity of TCP surfaces: (M-O) Particle size 25-45 µm, PDMS thickness 35 µm. Images were obtained at 100X magnification using a Scanning Electron Microscope (SEM). (M) SEM image at the edge of a coverslip. Note that the roughness of the surface was similar to (D). (N) SEM image of the coverslip that was demineralized with HCl treatment. TCP particles were removed and PDMS layer was maintained. Note that the PDMS thickness was adequate to provide a binding surface for TCP particles of 46-106 µm size. (O) SEM image at the center of the coverslip. Note the overall consistency of the coverage.
X100, Angle 45°
PDMS spin coat speed 2000 rpm
PDMS Thickness = 36 µm
TCP Particle size ≤ 25 µm

Figure 6: Optimization of the TCP surface design, effect of particle size and PDMS thickness on the uniformity of TCP surfaces: (P-R) Particle size- <= 25 µm, PDMS thickness- 35 µm. Images were obtained at 100X magnification using a Scanning Electron Microscope (SEM). (P) SEM image at the edge of a coverslip. Note that the roughness of the surface reduced compared to that in (M). (Q) SEM image of the coverslip that was demineralized with HCl treatment. TCP particles were removed and PDMS layer was maintained. Note that the PDMS thickness was adequate to provide a binding surface for TCP particles of <= 25 µm size. (O) SEM image at the center of the coverslip. Note the overall consistency of the coverage. These surfaces provided best smoothness, consistency in coverage and TCP exposure, and hence were chosen for the final design.
Figure 7: Verification of nuclei segmentation on opaque TCP surfaces using a Colonyze™ algorithm: (A) A montaged image of a colony on the TCP surface. Cells were stained with DAPI, and the image was obtained using a fluorescence microscope using DAPI excitation and emission wavelengths. (B) The Colonyze™ algorithm segments nuclei against the auto-fluorescent TCP background. (C) A colony was identified from (B); Nuclei were colored blue. (D) Image (C) was superimposed on image (A) to verify that at least 95% nuclei are accurately identified.
Figure 8: Quantification of the surface density of tethered TCPBP-EGF. (A) Amount of tethered EGF is consistent in repeated experiments. Two repeated experiments (Batch 1 and Batch 2) were conducted to quantify the amount of tethered EGF on TCP coverslips at Day 0. Coefficient of variation: 12.5%, P=0.16. The results show consistency within each experiment, and between experiments (B) Tethered EGF presentation is stable for at least 5 days at 4°C. Tethered EGF was quantified at three time points (Days 0, 1 and 5). Day 0 vs Day1: P=0.22; Day 0 vs Day 5: P=0.15. The results show the stability of tethered EGF presentation.
3.9. Works Cited:


33. Szabo, G., et al., A prospective multicenter randomized clinical trial of autogenous bone versus beta-tricalcium phosphate graft alone for bilateral sinus


CHAPTER 4: ASSESSMENT OF HUMAN CONNECTIVE TISSUE PROGENITOR CELL RESPONSE TO B-TRICALCIUM PHOSPHATE SURFACES WITH TETHERED EPIDERMAL GROWTH FACTOR

4.1. Abstract: Connective Tissue Progenitors (CTPs) are defined as the heterogeneous population of stem and progenitor cells that are resident in native tissues and are capable of proliferation to give rise to progeny that will differentiate to express one or more connective tissue phenotypes. Preclinical studies in both small and large animal models have suggested that the concentration and prevalence of CTPs in many, if not all bone defect sites is suboptimal. This is supported by the evidence that the transplantation of sources of CTPs into bone defects improve bone regeneration. A variety of methods have been used to harvest, process and transplant CTPs from bone or bone marrow. However, the survival of transplanted cells is threatened both by profound hypoxia and by the presence of pro-apoptotic cytokines. This work seeks to develop practical methods to improve CTP performance using epidermal growth factor (EGF) as a signaling molecule. EGF increases proliferation of culture expanded stem cells without compromising their osteogenic potential, and thus has a potential to play a critical role in osteogenesis. EGF was tethered to a β-tricalcium phosphate (TCP) scaffold via a novel TCP binding peptide (TCPBP). TCPBP was identified using a phage display library, a gene fusion was created to fuse EGF to TCPBP into a single TCPBP-EGF protein, which was then expressed in E. coli, and purified. TCPBP-EGF was tethered to a TCP surface. Tethering reduces the EGF degradation rate, improves control over its concentration and presentation, and enables prolonged signaling. We evaluated the effect of tethered TCPBP-EGF (tethered EGF) on CTP performance in vitro. The amount of tethered EGF
on the TCP surface was quantified, and its stability was demonstrated. CTPs were obtained from trabecular surface or bone marrow samples. Tethered EGF enhanced colony forming efficiency of CTPs in all eight patient samples (p< 0.03), with an average increase of 62%. These data support the potential of tethered EGF presentation on the surface of implantable TCP biomaterials as a means of enhancing the performance of local and transplanted CTPs in a setting of bone repair or other tissue engineering applications.

4.2. Introduction: Each year, roughly 500,000 bone grafting procedures are performed in the United States to treat various forms of orthopedic fixations [1-3], including critical segmental bone defects (defined as a defect that cannot heal without external intervention) [4]. Bone grafts and associated devices represent a $1.5 billion industry in the United States [1-3]. Autogenous cancellous bone (ACB) is currently the gold standard for the graft material, and is used in ~50% of these procedures [5-7]. However, the harvest of ACB is associated with significant morbidity, including surgical scars, blood loss, pain, prolonged surgical time and rehabilitation, and infection risk [7]. These co-morbidities emphasize the need for effective alternatives to autografts that could significantly improve the outcome, reduce the morbidity and/or cost of these procedures.

Bone repair requires the activity of osteoblasts, which are derived from osteogenic CTPs. Connective Tissue Progenitors (CTPs) are defined as the heterogeneous population of stem and progenitor cells that are resident in native tissues and are capable of proliferation to give rise to progeny that will differentiate to express one or more
connective tissue phenotypes [8-10]. Bone marrow has been widely used as a clinical source of autogenous CTPs [11-14]. Bone marrow can be harvested from a bone marrow aspirate with very low morbidity and minimal pre-processing compared to other sources of CTPs such as fat and muscle [10, 15]. Several procedures have been explored to increase CTPs concentration and/or reduce number of competing non-CTP cell population from bone marrow [16-18]. In large bone defects, which are often associated with local tissue trauma such as loss of muscle, periosteum and supporting vasculature, the performance of transplanted CTPs may be significantly hindered by a harsh wound environment, characterized by hypoxia and pro-inflammatory signaling molecules that may apoptosis or necrosis [8, 10, 16, 19, 20].

EGF is one of many potential modulators of stem and progenitor cells in orthopaedic tissue engineering applications, along with Bone Morphogenetic Proteins (BMPs), Platelet Derived Growth Factor (PDGF), and basic Fibroblast Growth Factor (bFGF). EGF is a potent mitogen for osteoblastic progenitors, and is both necessary and sufficient to induce colony growth in bone marrow-derived cells [21, 22]. EGF signaling prevents TNF-induced apoptosis in culture expanded marrow stromal cells [23-28]. Receptors for EGF ligand (EGFR) are expressed by virtually every cell type, including stem and progenitor cells. EGFR is a receptor tyrosine kinase that activates intracellular signaling cascades that influence cell proliferation, migration, and differentiation. EGF-EGFR interaction induces EGFR dimerization and autophosphorylation, which activates to downstream intracellular pathways. Once bound to the receptor, EGF is rapidly internalized by receptor-mediated endocytosis leading to receptor desensitization, thereby down-regulating the effects of exogenous EGF [29]. Compared to other growth factors,
EGF is a relatively small protein with a structure that is suitable for chemical modification because of its singular amine group [30, 31].

Molecular Surface Design can be used to tether growth factors such as EGF to the surface of a biomaterial as a means of improving control over the cellular and tissue response to the implant material. Tethering ensures that the signaling molecule does not diffuse away from the cells rapidly, and enables control over the concentration and 3-dimensional conformation of the molecule, which preserves its ability to interact with receptors [22, 27, 32, 33]. In contrast, most current clinical therapies deliver growth factors by diffusion release (e.g., BMPs). When released by diffusion, it is difficult to control their local concentration and rate of clearance.

Beta-tricalcium phosphate (TCP) is clinically used osteoconductive bone void filler [34-40]. The ability to modify the surface of TCP with growth factors could extend its biofunctionality and improve its clinical performance. To explore this potential, novel methods have been developed to tether bioactive EGF molecule to the surface of a TCP coverslip using a high-affinity, non-covalent binding of a novel TCP binding peptide (TCPBP) (Figure 1). This chapter tests the hypothesis that tethered EGF will significantly improve the in vitro performance of CTP when cultured on a TCP surface.

4.3. **Methods:** In Chapter 3, a new method was introduced to prepare reproducible textured 2D surfaces suitable for the assessment of cellular response to a biomaterial surface or a surface-modified biomaterial using any relevant adherent cell type. In this Chapter, this method was validated using TCP that was modified with Epidermal Growth
Factor (EGF), a pro-survival and proliferation bioactive ligand, using a novel TCP-binding peptide (TCPBP-EGF).

This method several limitations of existing methods, enabling colony detection on an opaque surface or a highly textured presentation of a scaffold material that is intended to be used in a 3-dimensional form. This method is based on the preparation of the biomaterial of interest in a particulate form (generally less than 25 µm in diameter, but 50-100 µm may be acceptable for some materials), and adhering these particles to the substrate surface using polydimethylsiloxane (PDMS). The PDMS surface is first prepared in a uniform thickness using spin coating. A thickness of 35 µm was selected for TCP, but may require further optimization for larger particle sizes or materials with different wetting properties. Using this method of preparation, the biological response to a variety of biomaterials can be compared in a setting where the effect of texture and larger scale structural properties is controlled or limited.

4.3.1. Fabrication of TCP surfaces: 18 mm diameter circular glass coverslips (thickness- 0.13-0.17 mm) (VWR, Batavia, IL) were used as a base surface. Coverslips were cleaned by swirling them for four hours in 2% ChemSolv glass cleaner solution (Mallinckrodt Baker, Phillipsburg, NJ) at room temperature, followed by swirling three times for 5 minutes in deionized water. All swirling steps were performed at 100 rpm using a MaxQ 2000 Rotary shaker (Thermo Scientific, Brookfield, WI). Coverslips were then silanized by swirling in filtered 2% Siliclads (Gelest, Morrisville, PA) for 20 seconds, followed by swirling three times for 5 minutes in deionized water. Silanized coverslips
were cured at 100°C for 20 minutes, left at room temperature overnight, and then stored in a vacuum oven at room temperature.

A polydimethylosiloxane (PDMS) coating was used to provide a uniform adherent surface for TCP particles (PDMS is a silicon-based organic polymer that is inert, non-toxic, non-flammable and optically clear). A PDMS layer was applied on silanized glass coverslips by the spincoating process. SYLGARD 184 Silicone Elastomer Base and the curing agent were mixed in a 10:1 ratio at room temperature. 1 mL PDMS mixture was prepared for a batch of 12 coverslips. The mixture was placed in a vacuum oven at room temperature for 20 minutes to remove air bubbles. Silanized glass coverslips were placed on a spin coater controller head (Headway Research, Garland, TX). A 50µL PDMS mixture was deposited on each coverslip, and was spun at 2000 rpm for 30 seconds. Each coverslip was manually examined to ensure that there was no lint, debris or irregularities.

Thickness of the PDMS layer depends upon the speed of rotation and viscosity of the mixture. Based on the values reported in the literature [41, 42], the thickness of the PDMS layer is expected to be 30-40 µm. The thickness was later confirmed using scanning electron microscope (SEM) images. Each coverslip coated with non-cured PDMS was manually examined to ensure that there was no lint, debris or irregularities.

TCP powder (particle size < 106 µm) was a gift from Integra Orthobiologics (Plainsboro, NJ). TCP was sieved on the PDMS layer immediately after spin-coating was completed. 1 gm TCP powder was poured into the 25 µm sieve (VWR Scientific), and TCP particles were gently pushed through the sieve using a spatula. TCP particles were allowed to deposit onto the PDMS coat. TCP coated coverslips were gently tapped to remove unattached TCP particles, and cured in a vacuum oven at 60°C for 2 hours at
atmospheric pressure. This curing step hardens the PDMS layer, thereby providing a stable TCP coating. TCP surfaces were prepared in batches of 12. TCP coated coverslips were sonicated upside down for 2 minutes in deionized water to remove non-adherent TCP particles using the Aquasonic sonicator (VWR Scientific) at 50Hertz. A gentle air stream was used to blow any remaining loose TCP from each coverslip. Coverslips were then dried overnight and examined under the microscope to determine coverage and artifacts. Coverslips were then dried overnight and stored at room temperature till further use.

4.3.2. Synthesis and purification of TCPBP-EGF protein: The TCPBP-EGF protein was a gift from Dr. Linda Griffith’s laboratory at MIT (Cambridge, MA). A TCP-binding peptide (TCPBP), discovered through phage display, was fused to EGF (TCPBP-EGF) and produced in E. coli [43]. An amylose resin was used to purify the final fusion protein and an on-column method was used for endotoxin removal. This on column method was modified from a previously published protocol [44]. Briefly, the column-bound protein was washed with 25 column volumes of a 1% Triton X-114 in 20mM Tris-buffer pH 7.4 and then washed with 25 column volumes of the same buffer without Triton X-114. The purification was carried out at 4°C.

Purified TCPBP-EGF protein was tested for endotoxin using a standardized Limulus Amebocyte Lysate (LAL) assay. Charles River Endosafe (CRE) system, a standardized apparatus for LAL assay, was used (LAL is a lysate from horseshoe crab (Limulus polyphemus) amoebocytes. LAL reacts with bacterial endotoxin or lipopolysaccharide, which is a membrane component of gram negative bacteria. This
reaction is the basis of the LAL assay, which is used for the quantification of bacterial endotoxins. The sensitivity of this assay kit was 0.05-5 Endotoxin units (EU)/mL.

All reagents, including the protein sample to be tested, were warmed to room temperature prior to use. The TCPBP-EGF protein was diluted to the desired concentration in endotoxin-free certified water (Gibco, Billings, MT) under UV sterilization to avoid any contamination. 25 µL of TCPBP-EGF protein sample was loaded in each of 4 wells (n=4) of the CRE reader. The peak endotoxin reading, spike recovery and coefficient of variation of TCPBP-EGF protein were compared with that of the standard curve to determine the amount of endotoxin present in the protein.

The endotoxin level in TCPBP-EGF was found to be less than 0.05 EU/mL. The endotoxin removal step reduced endotoxin levels in untreated TCPBP-EGF more than 100-fold over treated TCPBP-EGF.

4.3.3. Tethering TCPBP-EGF to TCP coverslips: A 4 cm x 6 cm plastic tray, lined with parafilm (Sigma-Aldrich) was used as an apparatus for the tethering process. The parafilm provides a uniform, chemically inert, hydrophobic surface for tethering. Eight TCP coverslips were tethered in one parafilm-layered plastic tray. Plastic lids and parafilms were first cleaned with ethanol and sterilized under UV (60-75 J/m²) for 45 minutes on each side. Parafilms were treated with sterile 1% BSA (Calbiochem) overnight at 4°C in a sterile chamber, rinsed twice with sterile 1X PBS, and allowed to dry in a sterile hood. BSA-coated parafilms were kept in a sterile container at 4°C till further use. The BSA treatment is used to minimize the non-specific adsorption of TCPBP-EGF on parafilms. TCP coverslips were sterilized using UV for 45 minutes on
each side, and subsequently with EtOH prior to tethering. TCPBP-EGF protein solution was steriley prepared at 2.7 µM on ice using sterile 1X PBS as a diluting agent. Sterile BSA-coated parafilms were placed inside sterile plastic trays. 150 µL TCPBP-EGF droplets were placed on the parafilm, and sterile TCP coverslips was gently placed on the droplet with the TCP side interacting with the protein solution. The entire assembly was sealed to maintain sterility, and was incubated at 4°C for 36 hours in a humidifying chamber. Coverslips were washed once with sterile 1X PBS immediately before cell culture.

4.3.4. **Quantification of the amount of tethered EGF and stability analysis:** TCPBP-EGF was tethered to TCP coverslips as described above. EGF-tethered coverslips were rinsed twice with 1X PBS, placed in fresh PBS solution, and then stored at 4°C for 0, 24 hours or 5 days (N=4). To measure stability, surfaces were removed and rinsed twice with 1X PBS. The amount of EGF associated with each coverslip was assessed by the BCA assay (Piercenet, Rockford, IL) [45, 46].

4.3.5. **Isolation of cells from bone marrow and trabecular space:** Bone marrow aspirates from the iliac crest, and discard bone cores from the proximal femur of human subjects undergoing elective hip arthroplasty procedures were obtained under in an IRB approved protocol with informed consent. Cells were harvested from either bone marrow aspirate (BMA), or from trabecular surface (TS) of bone. Each marrow aspirate was limited to a two ml volume from a given needle site, to limit dilution with peripheral blood. Each sample was aspirated into one ml of normal saline containing 1000 units of
Na-Heparin. Ten aspirates were harvested from both the left and the right iliac crest (20 total). Each marrow sample was suspended in 20 mL α-minimal essential medium (α-MEM) (Gibco, Billings, MT) containing 2 unit/mL Na-heparin to obtain a 23 mL suspension for each aspirate.

Each heparinized bone marrow suspension was centrifuged at 400 x g for 10 minutes at room temperature. Nucleated cells were selected using a buffy coat isolation [47, 48] (approximate volume 1.8mL) and re-suspended in α-MEM containing 0.3% Bovine Serum Albumin (BSA) (Calbiochem). Suspension with buffy-coated cells was re-centrifuged at 400 x g for 10 min. The second buffy coat was isolated and re-suspended in α-MEM containing 0.3% BSA. The double-buffy-coated cells were then pooled together and nucleated cells in each aspirate were counted using a hemocytometer. This cell suspension was used as a source of bone marrow aspirate (BMA) derived CTPs (Cohort 1- three patients).

To harvest cells from the trabecular surface, the cancellous bone was washed in α-MEM with antibiotic/antimycotic (Life Technologies, Carlsbad, CA), cut into small pieces (1-2 mm in diameter) with an osteotome, and washed thoroughly to remove cells from the marrow space. Cells that remained adherent to the trabecular surface were then recovered by collagenase type I (100U/ml, Sigma, Inc.) digestion [49]. This cell suspension was used as a source of trabecular surface (TS) derived CTPs (Cohort 2- 5 patients).

Cells from the BMA were plated at a density of 500,000 nucleated cells per coverslip, and cells from TS were plated at a density of 200,000 cells per coverslip, respectively (N=3). These plating densities were based on variations in the prevalence of
CTPs in BMA and TS samples (Data not Shown). The plating densities were chosen to optimize colony formation with overgrowth (confluence) of colonies. Coverslips were placed in 2.05 x 2.05 cm² Lab-Tek™ (Nunc, Logan, UT) glass chamber slides. An osteogenic medium (α-MEM, 10% fetal bovine serum, 50 µM ascorbate, 10 nM dexamethasone and penicillin/streptomycin) was used for all cell culture conditions, and was changed every 3 days. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ at 20% oxygen.

4.3.6. Staining, imaging and data analysis: Cells were harvested after nine days of culture. At the time of cell harvest, the growth medium was removed by aspiration, and culture chambers were vigorously rinsed with 1X PBS to remove non-adherent cells and debris. The remaining cells were fixed with 1:1 acetone/methanol (Fisher Scientific, Silver Spring, MD) for ten minutes at room temperature and air dried. Prior to staining, cells were hydrated with deionized water for 10 minutes. VECTASHIELD™ (Vector Labs, Burlingame, CA) mounting medium containing DAPI (0.75 µg/µL) was applied in the dark overnight, followed by a rinse using deionized water. Alkaline phosphatase activity was assayed using SK5100 Vector Red kit (Vector Labs, Burlingame, CA) according to the manufacturer’s instructions. Dry coverlips were stored in the dark at room temperature until image acquisition.

Coverslips were imaged using a 2048 x 3072 Quantix K6303E 12 bit digital camera (Roper Scientific) attached to a Leica DMXRA motorized microscope controlled by ImagePro imaging software. 240 individual images were collected at 461 x 344 pixels (1641.16 µm x 1224.64 µm), 8-bit gray level, using a 10x objective (pixel size = 3.56
µm). Images of DAPI fluorescence localizing individual cell nuclei were obtained with UV light (340-380 nm) excitation and fluorescence was read at >425 nm. Images of Alkalike Phosphatase stained cells were obtained with 566 nm excitation and 575-670 nm emission wavelengths.

Image processing and analysis were performed using a software algorithm developed in the Muschler laboratory using algorithms written in the C/C++ programming language and Motif X-Windows TM environment [50]. Algorithms were designed for background correction, montaging, nuclei segmentation, and quantitative characterization of CTP-derived colonies. Briefly, individual images of one coverslip were background-corrected by subtracting a smoothed (15 x 15 square average filter) background image and montaged together to create one image of the entire coverslip. Nuclear segmentation was accomplished using a gray value threshold and area calculation. Colony clusters were identified in an automated fashion using Colonyze™. Euclidian Distance Map was used to group nuclei falling within 142.4 µm of at least one other nucleus into colony clusters of at least eight nuclei. Identified colonies on each slide were reviewed using Colonyze™ to minimize any errors. Reviewed chambers were quantified and analyzed for different colony parameters as described below.

4.3.7. Data validation and analysis: The effect of tethered EGF on CTP metrics (number of colonies, number of cells per colony, expression of alkaline phosphatase, cell density) was evaluated. A mean was calculated for the number of colonies for all patients under each condition. A median was also calculated for each colony metric for each patient sample: number of cells (nuclei) within the colony, cell density (nuclei per mm²),
and percentage of alkaline phosphatase positive area divided by cell number for all colonies from each patient sample under each condition. Due to the known wide variation in CTP prevalence and performance between individual subjects, the outcome of CTP response to TCP surfaces with tethered EGF were standardized by dividing the outcome with the corresponding outcome for TCP surfaces with no tethered EGF for each patient sample. This ratio provided a relative measure of various parameters for each condition compared to control.

4.3.8. **Statistical analysis:** Statistical analysis was performed using standard ANOVA methods. Given the historical patient-to-patient variability and heterogeneity of CTPs in terms of colony forming efficiency (CFE) and proliferation, power analyses indicated that a sample size of at least 8 patient samples was sufficient to yield a significant difference with $\alpha = 0.05$ and power > 0.80.

4.4. **Results and Discussion:**

4.4.1. **Characterization of 2D TCP coverslips:** The average mass of a PDMS-coated glass coverslip was 152 mg (ST DEV = 1.9, n = 5). The average mass of a TCP coated coverslip was 172 mg (ST DEV = 4.3, n=5). Based on these calculations, the average mass of TCP per coverslip was expected to be 20 mg. The presence of TCP particles, consistency of the thickness of the TCP layer, and TCP particle size (less than 25 µm) was later confirmed using SEM images (Figure 2B). SEM also confirmed the uniformity of TCP surfaces within a range of 50 µm.
4.4.2. **Quantification of the concentration and stability of tethered EGF on TCP coverslips:** The concentration and purity of TCPBP-EGF protein (molecular weight: 77 kDa, 70% purity) was determined by J. Rivera, MIT. We demonstrated the reproducibility of the tethering process within and between batches by quantifying the amount of tethered EGF in two separate experiments. At time = 0 the mean amount of tethered EGF per TCP coverslip was in the range of 6000-7000 ng (Figure 3A) (P=0.16, coefficient of variation: 12.5%).

4.4.3. **Quantification of the stability of tethered EGF on TCP coverslips:** Quantification of tethered EGF (tethering concentration = 2.7 µM) to TCP coverslips after storage up to 5 days in 1X PBS at 4°C demonstrated that there was no statistically significant difference in the amount of tethered EGF remaining on the surface after different storage times (p-value>0.15, N = 3) (Figure 3B).

4.4.4. **CTP response to tethered TCPBP-EGF:**

4.4.4.1. **Tethered EGF enhances CFE in bone marrow as well as trabecular surface:** Formation of osteogenic colonies in culture requires cell adhesion, survival, proliferation, and expression of differentiation markers. Osteogenic colony formation is typically assessed by plating cells in serum-containing medium onto glass or plastic substrates so that adhesion is mediated by adsorbed serum proteins. In the CFU assay, CTPs were plated on surfaces at the cell loading densities determined using historical data on CTP prevalence from bone marrow aspirates and cells isolated from the trabecular surface (Figure 4). The number of colonies (8 or more cells in a cluster)
formed, number of cells per colony, colony area, along with determination of
differentiation status, were counted after 9 days of culture.

Tethered EGF increased CFE on TCP surfaces by more than 62% when
compared to TCP surfaces without tethered EGF, including both BMA- and TS-derived
cells (P<0.02) (Figure 4). Differences in the final colony number may arise from
differences in the number of CTPs adhering initially, activation of CTPs for form
colonies or differences in proliferation. These processes are both influenced by cues from
the culture substrate and cues from soluble medium components. Because the prevalence
of CTPs within a BMA sample differs from the prevalence of CTPs from the TS, we
asked if the ability of tethered EGF to affect colony forming efficiency might be
influenced by the source of CTPs. Tethered EGF improved CFE in both BMA and TS
derived CTPs, with an average increase of 57% in BMA derived cells and 72% in TS
derived cells (P<0.02) (Figure 4). The increase in CFE was consistent across all patient
samples.

These data emphasize the robustness of the observed CFE increase associated
with tethered EGF. Tethered EGF could cause an increase in CFE by increasing
adhesion, survival, proliferation, or a combination of these processes. While an initial
enhancement of CTP activation and proliferation would likely lead to an increase in
colony number, induction of proliferation at a later point would lead to an increase in the
number of cells per colony, and possibly the colony area. Therefore, we used quantitative
image analysis to further study CTP response by examining colony number and size.
4.4.4.2. **Tethered EGF does not influence the number of cells per colony or cell density:** The median number of cells per colony remained the same in the presence of tethered EGF, in both BMA- and TS-derived samples (Figure 5). Median colony area was similar as well across all patients. Because an increase in colony area could be a result of increased cell number resulting from proliferation, or increased migration, we also assessed colony density by dividing the number of cells per colony by median colony area. Our analysis revealed that colonies formed in the presence of tethered EGF had a similar density to colonies without tethered EGF (Figure 6). This suggests the possibility that an increase in CTP CFE is predominantly governed by the events of increased adhesion and survival, and less so by proliferation or migration. It is possible that the effect of tethered EGF could be in combination with other synergistic biological cues. Further studies are needed to elucidate these interactions.

4.4.4.3. **Tethered EGF maintains osteogenic potential of CTPs:** With Colonyze™, it is possible to examine the number of cells expressing alkaline phosphatase within a colony by measuring the area of a colony expressing alkaline phosphatase, and normalizing it to the number of cells within that colony. Figure 7 shows that alkaline phosphatase expression within a colony was similar across all conditions (p>.05 for all comparisons). These results are similar to the previously reported influence of tethered EGF on the alkaline phosphatase expression in culture expanded MSCs as well as CTPs [22, 43].

4.5. **Discussion:** In this study, we examined the effect of tethered EGF on CTP colony formation from cells obtained from freshly aspirated human bone marrow as well as cells
isolated from the surface of trabecular bone for potential tissue engineering applications. We demonstrated that the amount of tethered EGF was adequate to induce a biological cell response. However, we have not yet explored the possibility of a dose response. TCPBP-EGF construct was previously found to be biologically active on passage 3 human MSCs [J Rivera and L Griffith, MIT- unpublished results]. We also demonstrated the stability of the tethered EGF constructs for at least 5 days of incubation. We also demonstrated that the endotoxin levels in the protein were 10-fold below commercial standards of protein production.

This work demonstrated an increase in the CTP colony forming efficiency. This observation could be due to an increase in the activation of CTPs in the sample. It is also possible that an increase in the attachment or survival of CTPs could contribute towards this effect. This data cannot distinguish between these mechanisms. Many prior studies have demonstrated that EGF-induced signaling is a key effector of cell survival and proliferation [25, 51-53]. Due to the inherent limitations in working with bone marrow as well as trabecular bone, we could not perform the CFU assay using inhibitors to verify the dependence of colony formation on EGF-induced signaling. Therefore, we cannot definitively rule out other possible underlying mechanisms that are not induced by EGFR-signaling. One possibility would be that increased colony formation was solely a result of an increase in the mechanical attachment of cells resulting from a large number of EGF-EGFR binding events. However, this is unlikely due to the fact that the density of tethered EGF is significantly lower than the density of other cell-surface interaction moieties. Another possibility is that the tethered EGF is promoting the selective adsorption of serum factors that can then promote differential cell signaling. However,
previous results with culture expanded CTPs using a similar experimental system suggest that our observed effects are EGFR-mediated [22, 32].

4.6. Conclusion:

In conclusion, this study has successfully demonstrated tethering EGF to TCP surfaces via a novel TCP binding peptide. TCPBP-EGF was tethered to a TCP surface at 6000-7000 ng per coverslip, and was stable for at least five days of incubation. Human bone marrow and trabecular surface-derived cells demonstrated an increase in CFE on the TCP surfaces with tethered EGF when compared to TCP surfaces without tethered EGF, while maintaining the osteogenic potential. There was no change due to tethered EGF in the number of cell per colony or colony density. The CTP response to tethered EGF was seen in both bone marrow (three out of three) and trabecular surface (five out of five) derived CTPs. In contrast, addition of soluble EGF did not improve CFE [data not shown]. These findings support a possible value of tethered TCPBP-EGF as a modulator of the clinically applicable biomaterials.

In future studies, the effect of tethered EGF on CTP response could be further explored in terms of the effect of tethered EGF on long-term differentiation and mineralization of CTPs. The dose response relationship between concentrations of tethered EGF and CTP response could be evaluated. Further, TCP scaffolds with tethered EGF could be tested for their efficacy in bone regeneration in an animal model, such as the canine femoral multidefect model that has been used previously by the investigators to assess the efficacy of bone graft materials [17, 54, 55]. The efficacy of tethered EGF
in augmenting CTP response can further be tested in presence of pro-inflammatory cytokines or hypoxia, where the survival of cells could be a challenge.

The presentation strategy of tethered EGF can be further advanced to co-tether other relevant bioactive factors when TCP-containing scaffolds are used. A similar strategy might be implemented to identify binding peptides to other forms of biomaterials, and tether bioactive factors to those biomaterials via a fusion of corresponding binding peptides and growth factors.

4.7. Figures and Tables:

![Diagram](image.png)

**Figure 1:** A schematic of presentation of tethered TCPBP-EGF on TCP surfaces. The TCPBP is the binding domain that tethers the protein. The Coil domain provides flexibility to the EGF molecule which is important for its biological activity. The coiled domain also provides additional stability due to the coil-coil interactions between two TCPBP-EGF proteins. The EGF domain is linked to the TCPBP at its C-terminus.
50uL PDMS Droplet is spin coated

Silanized Coverslip

Spin Coater

(A)

TCP Particles

PDMS

Silanized Coverslip

(B)

Figure 2: Preparation and characterization of 2D TCP surfaces. (A, B) A schematic of the process that involves coating a PDMS layer on a glass coverslip using a spin-coater, and deposition of TCP particles on the PDMS coat, followed by a curing process for surface hardening. (C) SEM image of a TCP surface, demonstrating the uniformity and smoothness of the surface with 50 um range, (D) Colony formation on the TCP coverslip. CTPs were plated on a TCP surface, fixed after 9 days of culture, and stained for alkaline phosphatase (in red), a marker of early osteogenesis.
Figure 3: Quantification of the surface density of tethered TCPBP-EGF. (A) Amount of tethered EGF is consistent in repeated experiments. Two repeated experiments (Batch 1 and Batch 2) were conducted to quantify the amount of tethered EGF on TCP coverslips at Day 0. Coefficient of variation: 12.5%, P=0.16. The results show consistency within each experiment, and between experiments (B) Tethered EGF presentation is stable for at least 5 days at 4\(^\circ\) C. Tethered EGF was quantified at three time points (Days 0, 1 and 5). Day 0 vs Day1: P=0.22; Day 0 vs Day 5: P=0.15. Data was obtained using ANOVA. The results show the stability of tethered EGF presentation.
Figure 4: Tethered EGF improves Colony Forming Efficiency (CFE) at Day 9. (A) CFE of TCP surfaces with tethered EGF standardized to control (TCP coverslips with no tethered EGF) for all 8 patients combined. (B) Tethered EGF improves CFE of CTPs derived from bone marrow aspirates (3 patients) (C) Tethered EGF improves CFE of CTPs derived from trabecular surface of bone (5 patients)
Figure 5: tethered EGF does not change number of cells per colony at Day 9. (A) Number of cells per colony on TCP surfaces with tethered EGF standardized to control (TCP coverslips with no tethered EGF) for all 8 patients combined. (B) CTPs derived from bone marrow aspirates (3 patients) (C) CTPs derived from trabecular surface of bone (5 patients).
Figure 6: Tethered EGF does not change relative colony density at Day 9. (A) Number of cells per colony on TCP surfaces with tethered EGF standardized to control (TCP coverslips with no tethered EGF) for all 8 patients combined. (B) CTPs derived from bone marrow aspirates (3 patients) (C) CTPs derived from trabecular surface of bone (5 patients).
Figure 7: tethered EGF does not change % AP expression in colony cells at Day 9.  
(A) Percentage Alkaline phosphatase (AP) expression is quantified as AP-positive area of the colony divided by colony cell number. % AP expression on TCP surfaces with tethered EGF standardized to control (TCP coverslips with no tethered EGF) for all 8 patients combined. (B) CTPs derived from bone marrow aspirates (3 patients) (C) CTPs derived from trabecular surface of bone (5 patients).
4.8. Works Cited:


49. Siclari, V.A., et al., *Mesenchymal progenitors residing close to the bone surface are functionally distinct from those in the central bone marrow*. Bone, 2012.


CHAPTER 5: CONCLUSIONS

The overall goal of this dissertation was to contribute to the development of tissue engineering strategies that could be used in the evaluation of cell interactions with biomaterials or modified biomaterial surfaces. The specific design was validated using β-TriCalcium Phosphate (TCP), a common biomaterial used in bone regeneration, and the assessment of the targeted modification of the surface of TCP was performed with respect to the in vitro performance of human bone and marrow-derived connective tissue progenitors (CTPs) and their progeny. The assessment of cellular response to biomaterials utilizes the recently adopted standard method for automated colony forming unit (CFU) assay (ASTM F2944-12) [1], and is enabled by a customized software platform developed in the Muschler Lab within the Department of Biomedical Engineering (referred to as “Colonyze™”).

In Chapter 3, a new method was introduced to prepare reproducible textured 2D surfaces suitable for the assessment of cellular response to a biomaterial surface or a surface-modified biomaterial using any relevant adherent cell type. In Chapter 4, the method was validated using TCP that was modified with Epidermal Growth Factor (EGF), a pro-survival and proliferation bioactive ligand, using a novel TCP-binding peptide (TCPBP-EGF). This Chapter (Chapter 5) summarizes the conclusions of Chapter 3 and 4 that may be helpful to future investigators.

5.1. The Need – Quantitative Assessment of the Effect of Scaffold Biomaterials and Biomaterial surface Modifications on Relevant Cell Populations: Three dimensional scaffolds that are relevant to biomedical applications are valuable as potential surfaces to
assess cellular interactions (attachment, survival, migration, proliferation, differentiation). However, the vast majority of three dimensional biomaterial scaffolds are optically opaque and also geometrically complex and porous. As a result, cells and colonies which may adhere and respond to these materials are often distributed into the deep pores of the material and along the vertical walls or overhangs in the scaffold structures, making many of the cells present on the scaffolds unobservable below a depth of 30-40 µm. Moreover, even if the deeper regions of a scaffold were accessible to direct measurement, the thickness of these structures creates large variation in the local environment around cells (due to diffusion gradients in standard media conditions).

The TCP TheriLok™ scaffolds used in the validation of this approach had these limitations. In addition, the TCP TheriLok™ scaffolds are brittle and subject to fracture and deformation during processing and handling.

A new method was introduced to overcome several limitations of existing methods, enabling colony detection on an opaque surface or a highly textured presentation of a scaffold material that is intended to be used in a 3-dimensional form. This method is based on the preparation of the biomaterial of interest in a particulate form (generally less than 25 µm in diameter, but 50-100 µm may be acceptable for some materials), and adhering these particles to the substrate surface using Polydimethylsiloxane (PDMS). The PDMS surface is first prepared in a uniform thickness using spin coating. A thickness of 35 µm was selected for TCP, but may require further optimization for larger particle sizes or materials with different wetting properties and densities. Using this method of preparation, the biological response to a variety of biomaterials can be compared in a setting where the effect of texture and larger...
scale structural properties is controlled or limited. Once a physically and chemically stable 2D particulate surface is prepared, it is then possible to secondarily modify the chemical surface of the particular bulk material systematically to assess the biological effect of surface modification.

While these methods were applied and validated specifically for TCP and CTPs and their progeny, with a focus on applications in bone tissue regeneration, these methods can readily be applied to a variety of materials and cell types which have relevance well beyond bone regeneration, including cells and materials relevant to: skin and wound healing, nerve regeneration, muscle regeneration, tendon/ligament, vascular reconstruction, as well as hollow and solid organs. The only intrinsic requirements in this method is that the bulk biomaterial utilized in this process be relatively chemically stable in a aqueous (culture media) environment at physiological pH.

A scoring system was designed to qualitatively evaluate and compare biomaterial surface preparations. Surfaces were scored for ease of preparation and repeatability, uniformity and consistency in coverage, stability and detection of colonies. This scoring system can be further modified according to the requirements of the surface design, or the available skillset of the user.

5.2. Quantitative Assessment using Colonyze™ and Analytical Principles in the ASTM Standard Method (F2944-12): The feasibility of using Colonyze™ to facilitate automated colony forming unit assay based on the recently adopted ASTM Standard Method (F2944-12) was demonstrated. Colonyze™ was used to identify cells on the material surface and to group the identified cells into colony objects, each representing
the progeny of a colony founding connective tissue progenitor present in the initial tissue sample. An increase in the number of colonies formed (observed prevalence of CTPs) when the TCP surface was modified using the TCPBP-EGF peptide provided confirmation that this surface modification resulted in an increase in colony forming efficiency (CFE) by tissue derived CTPs. These methods also enable the detection of relative differences in effective proliferation rate (EPR), cell density within colonies (a metric of migration, expression of differentiation markers, as well as metrics of cell survival.

5.3. Qualitative Scoring System for Surface Preparation: A qualitative scoring system was used to evaluate and rank the value of various biomaterial surface preparations for their compliance to technical specifications. The table below summarizes the results of the verification step. The method was verified for six technical specifications. In all cases, the criterion for success was met.

<table>
<thead>
<tr>
<th>Technical Specification</th>
<th>Criteria for Success</th>
<th>Measured Performance</th>
<th>Criteria met?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Repeatability</td>
<td>No score below 3, and the best overall score</td>
<td>4</td>
<td>Yes</td>
</tr>
<tr>
<td>2 Uniformity in coverage</td>
<td>No score below 3, and the best overall score</td>
<td>4.5</td>
<td>Yes</td>
</tr>
<tr>
<td>3 Stability during culture period</td>
<td>No score below 3, and the best overall score</td>
<td>5</td>
<td>Yes</td>
</tr>
<tr>
<td>4 Ability to use Colonyze for CFU assay</td>
<td>No score below 3, and the best overall score</td>
<td>4.5</td>
<td>Yes</td>
</tr>
<tr>
<td>5 Surface density of bioactive ligand</td>
<td>At least 1000 ng per coverslip</td>
<td>6000-7000 ng per coverslip</td>
<td>Yes</td>
</tr>
<tr>
<td>6 Stability of surface modification</td>
<td>At least 5 days</td>
<td>5 days</td>
<td>Yes</td>
</tr>
</tbody>
</table>
5.4. Characterization of Surface Preparation of TCP with and without Surface Modification using TCPBP-EGF: In Chapter 4, the method was validated in its entirety using β-TriCalcium Phosphate (TCP), a bone void filler material that was modified with Epidermal Growth Factor (EGF), a pro-survival and proliferation bioactive ligand, using a novel TCP-binding peptide. The amount of tethered EGF on the TCP surface was quantified, and its stability was demonstrated. The colony forming efficiency was measured as a ratio of the mean observed prevalence of colony forming units on the surface of TCP surfaces with tethered Effective Proliferation Rate was measured as median number of cells per colony on the TCP surface with tethered EGF standardized to the median number of cells per colony on the TCP surface without tethered EGF. Migration was estimated using the relative colony density, which as measured as a ratio of colony density on TCP surface with tethered EGF divided by colony density on TCP surface without tethered EGF. Osteoblastic differentiation was measured in terms of the area fraction of Alkaline phosphatase (AP) which was measured as the area of the colony that is stained positively for AP expression divided by total number of cells in each colony.

This work has successfully demonstrated tethering EGF to TCP surfaces via a novel TCP binding peptide. TCPBP-EGF was tethered to a TCP surface 6000-7000 molecules per µm², and was stable for at least five days of incubation. Human bone marrow and trabecular surface-derived cells demonstrated an increase in CFE on the TCP surfaces with tethered EGF when compared to TCP surfaces without tethered EGF, while maintaining the osteogenic potential. There was no change due to tethered EGF in the number of cell per colony or colony density. The CTP response to tethered EGF was
seen in both bone marrow (three out of three) and trabecular surface (five out of five) derived CTPs. In contrast, addition of soluble EGF did not improve CFE [data not shown].

The efficacy of the TCP surfaces with tethered EGF was verified for four technical specifications. In two cases, the criterion for success was met. The results of validation are summarized below:

<table>
<thead>
<tr>
<th></th>
<th>CFU assay</th>
<th>At least 50% Improvement</th>
<th>Improvement</th>
<th>Yes/No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Colony Forming Efficiency</td>
<td>62%</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Proliferation</td>
<td>No change</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Migration</td>
<td>No change</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Osteoblastic differentiation</td>
<td>No change</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

These results demonstrated that modifying bone scaffold materials with EGF may have a value in improving the performance of CTPs in bone repair. These data support the potential of tethered EGF presentation on the surface of implantable TCP biomaterials as a means of enhancing the performance of local and transplanted CTPs in a setting of bone repair or other tissue engineering applications.

5.5. Works Cited:

CHAPTER 6: FUTURE DIRECTIONS

The long-term goal of this research is to advance the field of orthopaedic tissue engineering by improving the performance of osteogenic connective tissue progenitor cells (CTPs) transplanted in a bone graft using surface modification of the graft material. The purpose of this dissertation is to introduce and demonstrate a new method to assess the response of CTPs to biomaterials or surface-modified biomaterials using an automated colony forming unit (CFU) assay that has been recently adopted by the American Society for Testing and Materials (ASTM) [1] (referred here as “Colonyze™”). This method is particularly applicable to biomaterials that are solid, opaque, and water insoluble or minimally degradable. Bone graft materials such as ceramics (e.g., β-TriCalcium Phosphates (TCPs), hydroxyapatites, nanocrystalline calcium phosphates), biologics (e.g., demineralized bone matrix, chitosan, lyophilized collagen) or synthetic polymers (e.g. Poly(methyl methacrylate) (PMMA), Polycaprolactone (PCL), poly(propylene fumarate) PPF) are some examples of such biomaterials.

The method described here is validated in its entirety using β-TriCalcium Phosphate (TCP), a common bone void filler material. TCP was also modified with Epidermal Growth Factor (EGF), a pro-survival and proliferation bioactive ligand, using a novel TCP-binding peptide. The amount of tethered EGF on the TCP surface was quantified, and its stability was demonstrated. TCP surfaces with tethered EGF were shown to enhance colony forming efficiency (CFE) of CTPs obtained from trabecular surface or bone marrow samples. These data support the potential value of tethered EGF presentation on the surface of implantable TCP biomaterials as a means of enhancing the
performance of local and transplanted CTPs in a setting of bone repair or other tissue engineering applications.

The work discussed in this dissertation could be continued in several possible ways: to advance the understanding of the role of tethered EGF in influencing the osteogenic progenitor cell response; to optimize the surface modification of TCP; or to assess CTP response to other types of biomaterials and surface modifications that are relevant to tissue engineering. In this chapter, these proposed studies are presented with their significance, hypothesis and rationale, followed by the design of experiments. These proposed studies include: assessment of CTP response to tethered EGF under normoxia, and the stress induced by hypoxia and pro-death cytokines; understanding the mechanism of CTP response to tethered EGF under hypoxia; assessment of the effect of tethered EGF on bone regeneration in a large animal model; optimization of the surfaced modification of TCP scaffolds; and assessment of CTP response to other bone graft materials with or without surface modifications.

6.1. Assessment of CTP response to tethered EGF under normoxia, and the cellular stress induced by hypoxia and pro-death cytokines: transplantation of osteogenic connective tissue progenitors (CTPs) into large bone defects is a clinical therapeutic strategy that is based on the premise that the concentration and prevalence of osteogenic cells in these defects sites is suboptimal. The performance of transplanted cells is threatened both by the profound local hypoxia and the presence and pro-death chemical signals that tend to drive cells to die by apoptosis or necrosis. Reduced oxygen tension limits energy production and triggers various signaling pathways
regulating proliferation, apoptosis and differentiation [2-5], and therefore can influence the performance of CTPs and other progenitor populations. The inflammation of bone tissue elevates hypoxic stress, by increasing the density of metabolically active cells in a wound site. Inflammation also increased the local release and activity of multiple pro-death ligands including Fas ligand (FasL), tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), interferon γ (IFN-γ), and interleukin 1 (IL-1) [6-9]. In many cell types, including culture expanded MSCs, FasL induces apoptosis by activation of caspases [10-14]. However, the response of bone marrow derived to tethered EGF in the presence of pro-death ligands such as FasL, particularly under conditions of hypoxia is as yet poorly understood. Also, the CTP response to tethered EGF under normal physiological oxygen tension (normoxia) is unknown. In this context, it is relevant to test the efficacy of TCP modified with tethered EGF in improving CTP response under these conditions.

The goal of this study is to test the influence of tethered EGF on the CFE, proliferation, migration and osteoblastic differentiation of CTPs under conditions of normoxia, hypoxia, and presence of FasL. Even though the mechanism of the response can be assessed using a hypothesis based study that is described in Section 6.2, it is relevant to first quantify the CTP response under these conditions using the method described in this dissertation.

Many cell types, including culture expanded MSCs, respond to hyoxia or FasL by activating canonical signal transduction pathways including, but not limited to, the Mitogen-activated protein kinase (MAPK)- extracellular-regulated kinase (ERK)
pathway [5]. The MAPK-ERK signaling can be modulated through the EGF receptor (EGFR) [15-19]. EGFR is expressed by virtually every cell type, including stem and progenitor cells. EGFR is a receptor tyrosine kinase that activates intracellular signaling cascades that influence cell proliferation, migration, and differentiation. The presence of soluble EGF reduces hypoxia-induced apoptosis via EGFR-mediated MAPK activation [20-22]. In the presence of FasL, the presence of tethered EGF improved the initial attachment and survival of culture-expanded MSCs by sustained MAPKK-ERK signaling [10, 11, 14]. The mechanism of tethered EGF-mediated cell response also involves activation of MAPK-ERK pathways [12, 23]. Therefore, TCP surfaces with tethered EGF are expected to improve the response of CTPs, compared to TCP surfaces without EGF.

All experiments will use bone marrow as a source of CTPs. The CTP response will be evaluated at 0.1% oxygen (to mimic the hypoxic environment that develops after closure of the surgical wound due to lack of vascular mass transport) [24], 3% oxygen (to mimic normoxia- the physiological conditions in bone marrow) [24], and in the presence of 100 ng/ml FasL (a dose that is sufficient to induce a 50% cell death in culture expanded MSCs) [10]. CTP response at 20% will be used as a positive control. The outcome measures will be colony forming efficiency CFE, proliferation, migration, and osteoblastic differentiation at Days 6 and 9 using Colonyze™. These results will also form a basis for the proposed study described in Section 6.2.

6.2. Understanding the mechanism of CTP response to tethered EGF under the cellular stress induced by hypoxia and pro-death cytokines: The mechanism by which tethered EGF mediates effects on CFE and proliferation under hypoxia or in
the presence of pro-death cytokines is uncertain. The objective of this proposed follow-up study is to advance our understanding of the mechanism(s) by which tethered EGF influences CTP performance under these conditions. The central hypothesis of this study is that tethered EGF-dependent improvement in the *in vitro* performance of CTPs is mediated, at least in part, by the activation of the Akt, MAPK (Mitogen Activated Protein Kinase) and/or HIF-1α (Hypoxia Inducible Factor-1α) signaling pathways, alone or in combination. This hypothesis is based on the following rationale: in many cells types, including culture-expanded mesenchymal stem cells, hypoxia stimulates cell death via apoptosis [25-27] and an increase in caspase-3 activation (an intracellular shift from inactive to active caspase-3) [22, 28]. Akt activation is necessary for cell survival in hypoxia [5]. Activation of the Akt and MAPK pathways (phosphorylation of Akt and MAPK) inhibits apoptosis by inhibiting caspase-3 activation and stabilization of HIF-1α. In addition to survival, activation of the MAPK pathway can improve cell proliferation and attachment, in many cell types [19, 29]. EGF is known to increase phosphorylation of Akt and MAPK in normoxia in many cell types [19, 29]; however, its effect on osteogenic progenitor cells under conditions of hypoxia is unknown. This study will define the extent to which the biological effects of a biomaterial surface on osteogenic progenitor cells under conditions of hypoxia are mediated through one or more of these mechanisms.

This study can be advanced by first testing the hypothesis that tethered EGF increases the phosphorylation of Akt and MAPK, increases HIF-1α stabilization and decreases caspase-3 activation in osteogenic progenitor cells, human dermal fibroblasts
and endothelial cells when cultured in hypoxia. Further, the relative effect of tethered EGF on the survival, proliferation and attachment of osteogenic progenitor cells, dermal fibroblasts and endothelial cells under conditions of hypoxia can be quantified. This mechanism can further be explored by testing the hypothesis that selective inhibition of Akt, MAPK, and/or HIF-1α pathways will reduce survival, proliferation and/or attachment of osteogenic progenitor cells, dermal fibroblasts and endothelial cells under conditions of hypoxia.

Passage-1 cells obtained from adult human bone marrow derived connective tissue progenitor cells (CTPs) can be used as a clinically relevant source of CTPs for this study. Passage-1 human dermal fibroblasts and vascular endothelial cells derived from the same patient subject can be isolated [30-32], and used as an in-patient control to determine the extent to which tethered EGF effects may be specific for osteogenic progenitor cells or generalized to other clinically relevant human cell types.

The response of cells will be evaluated at 0.1% oxygen, and in presence of 100 ng/ml FasL. The cell EGF receptor (EGFR), pEGFR, Akt, pAkt, MAPK, pMAPK, HIF-1α, stabilized HIF-1α (sHIF-1α), caspase-3, and cleaved caspase-3 (cCaspase-3) levels will be measured using standard protein immunoblotting assays at early and late cell culture periods to assess the durability of tethered EGF effects on cells. These levels will be standardized to the levels of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene that is often stably and constitutively expressed at high levels in most tissues and cells. The standardized pEGFR can confirm that tethered EGF-mediated changes in downstream signaling are due to EGFR activation. Standardized levels of
pAkt, pMAPK, sHIF-1α, and cCaspase-3 can be used to determine the effect of tethered EGF on the activation of these pathways.

The effect of tethered EGF on cell survival will be determined using a standardized live-dead cell assay. The effect of tethered EGF on proliferation can be determined in terms of the total DNA content of cells and a standardized Bromodeoxyuridine (BrdU)-cell proliferation assay [33]. An imaging-based cell quantification assay can be used to quantify the number of adherent cells.

Osteogenic progenitor cells can be treated with the selective inhibitors of Akt, MAPK, HIF-1α and caspase-3 pathways respectively prior to seeding. The relative roles of these pathways on tethered EGF-mediated cell survival, proliferation and attachment as well as interaction between these pathways can be characterized. Cell response evaluated at early and late time points will determine the effect of selective inhibition as a function of time.

The characterization of the mechanism and pathways responsible for mediating tethered EGF effects on the survival, proliferation and/or attachment and retention of osteogenic progenitor cells on a biomaterial surface, as well as characterization of the dose dependent effects of tethered EGF presentation, will enable rational development of biomaterials that are modified with tethered EGF. Characterization of the effects on dermal fibroblasts and endothelial cells will contribute to an understanding of the likely safety profile of biomaterials presenting tethered EGF, as well as the potential application of tethered EGF biomaterials to other tissue engineering applications.
6.3. Assessment of the effect of tethered EGF on bone regeneration \textit{in vivo}: The efficacy of tethered EGF in improving bone regeneration can be tested in a large animal bone defect model. The canine femoral multidefect (CFMD) model and the chronic caprine tibial defect (CCTD) model are relevant animal models for this study. The rationale for choosing an animal model for the \textit{in vivo} testing of biomaterials has been discussed in [34].

The CFMD model has been used previously to assess the efficacy of bone graft materials with transplanted bone marrow derived cells [35-37]. This model consists of four equally sized and equally spaced cylindrical defects measuring 15 mm in depth and 10 mm in diameter created in the femur of a single dog. Bone regeneration is assessed at four weeks. The defects are of sufficient size such that the interior of the defect is characterized by profound hypoxia [24], a key feature of large clinical defects that is not modeled in small animal defects. Bone formation and revascularization within the defect occur through a process of ingrowth that has a radially oriented outside-in pattern, which can be readily measured and characterized using microcomputed tomography (MicroCT) and histological methods. The CFMD model system is a robust and sensitive system with which to make objective comparisons between materials. The CFMD model allows these comparisons to be made in a rapid and efficient manner (only 4 weeks) using a large animal system, in a defect of clinically relevant size. By collecting four data points from each animal, the CFMD model maximizes the value of each subject and minimizes the number of subjects that are needed to provide sufficient power for comparisons. Moreover, in addition to total bone formation and histology, the CFMD model provides
an opportunity in one model to assess the pattern, distribution, and remodeling features associated with a material in varying biological settings.

The CFMD model will be used for initial screening and the dose response study. TheriLok™ (Integra Orthobiologics) TCP scaffolds will be used for in vivo studies, as their biocompatibility, and clinical efficacy as a bone graft has been tested [38-42]. TheriLok™ scaffolds with tethered TCPBP-EGF will be prepared using methods described in Chapters 3 and 4. The selective retention method that has been described in detail in [43] will be used for loading bone marrow-derived CTPs to the scaffolds. Previous studies have demonstrated the efficacy of selective retention to load freshly aspirated bone marrow-derived cells to graft materials with an increase in CTP loading efficiency by 40-70% [37, 43]. TheriLok™ scaffolds with tethered TCPBP-EGF will be implanted in two defect sites and TheriLok™ scaffolds without TCPBP-EGF will be implanted in the other two defect sites. Implants will be fixed with a metal plate to prevent scaffold movement and loss. The implant sites will be harvested four weeks post implantation and the defect sites will be fixed and embedded till further use. Defect sites will be isolated from femurs and will be imaged using microCT to measure the density of newly mineralized tissue in the defect. We will also use histomorphologic methods to further evaluate the extent of bone formation using Giemsa, Hematoxylin & Eosin (H&E), and Goldner’s Trichrome stained 5 μm cross sections of the tissue from the defect site. Giemsa and H&E will be used to differentially detect infectious and inflammatory cells, while Goldner’s Trichrome will be used to identify mineralized bone as well as calculate a percent of bone area using thresholding of microscopy images of the mineralized bone stain. Analysis will be performed using ImagePro software for
reconstruction of microCT images and calculation of newly mineralized tissue volume. A dose response study can be conducted to determine the best possible tethered EGF dose that leads to maximum bone regeneration.

The Muschler lab, in collaboration with the University of Minnesota and the Institute for Surgical Research, has developed the CCTD model, which creates a 5 cm segmental bone defect, and incorporates the loss of regional soft tissues and periosteum, local tissue scarring, and a fibrous induced membrane that forms around a PMMA spacer placed at the site of the defect. It is intended to better represent the complexity and severity of the actual biological environment in which clinical bone grafts currently fail. In this model, each animal undergoes two surgical procedures, a “Pre-procedure” in which a 5 cm tibial defect is created and preserved with the use of a polymethylmethacrylate (PMMA) spacer, followed by the “Treatment Procedure” in which various clinically relevant bone grafting treatment scenarios can be implemented. During the Pre-procedure, the 5 cm defect is created and the tibia stabilized with an intramedullary interlocking nail. The newly created defect is filled with a PMMA spacer to induce a membrane to form at the defect site. At the treatment procedure, the PMMA spacer is removed and the defect is filled with the test material used in the study; the induced membrane serves to contain the test material.

The best performing tethered EGF dose from the CFMD study can be tested for its efficacy using this model. Preparation of graft materials will be similar to the CFMD study. Grafts will be implanted during the Treatment Procedure, 4 weeks after the Pre-procedure. The tibia will be harvested for further assessment of defect healing using
microCT and histological analysis 12 weeks after implantation, and fixed for analysis. The analysis methods will be same as the ones used for the CFMD model.

6.4. **Optimization of the surface-modification of TCP scaffolds:** The presentation strategy of tethered EGF can be further optimized in terms of the dose of tethered EGF, co-presentation of other relevant bioactive factors along with EGF, and identification of binding peptides for other forms of biomaterials.

In this dissertation, the efficacy of tethered EGF was demonstrated using only one dose of tethered EGF, but its optimization was not assessed. In future experiments, a dose response of tethered EGF could be assessed. The surface density of tethered EGF on the TCP surface can be controlled using the concentration of the protein solution used for the tethering step [44]. A dose response study can include 4 concentrations of tethered EGF (50, 10, 0.1, 0.05-fold the concentration of tethered EGF used in this dissertation, respectively). A tethered EGF dose that was used in Chapter 2 (incubation of scaffolds in 2.7 uM TCPBP-EGF solution) will be used as a positive control.

Optimization of TCPBP-EGF protein is an active area of research. Researchers at MIT are modifying the TCPBP protein construct to improve the stability and binding. These modifications include addition of repeated binding domains, and modification of TCPBP-EGF protein to improve binding and biofunctionality. In addition, novel proteins constructs are being fabricated to co-tether other bioactive ligands, such as Heregulin, with EGF using a single binding domain.

The efficacy of tethered EGF can be further assessed in terms of its effect on the long-term differentiation and mineralization of CTPs. As shown in chapter 4, there was
no tethered EGF-induced effect on osteoblastic differentiation of CTPs after 9 days of culture. However, it is conceivable that tethered EGF could affect the differentiation process at later time points. A positive tethered EGF-induced effect on long term differentiation and mineralization would indicate that the improved colony formation of CTPs translates to improved long term osteogenesis.

6.5. Assessment of CTP response to surface modifications of other bone graft materials:

In chapter 3, we introduced a novel method to prepare 2-dimensional surfaces of TCP material. These surfaces were characterized for their presentation and surface uniformity. TCP is a class of ceramics used as bone void filler. A similar approach could be used to prepare 2 dimensional surfaces of other amorphous and crystalline materials used in tissue engineering applications. These materials include ceramics (e.g. hydroxyapatites, nanocrystalline calcium phosphates), biologics (e.g. demineralized bone matrix, chitosan, lyophilized collagen) or synthetic polymers (e.g. Poly(methyl methacrylate) (PMMA), Polycaprolactone (PCL), poly(propylene fumarate) PPF). The response of CTPs to these materials can be assessed using Colonyze™, using the method described in Chapters 3 and 4. This method could be used for a comparative assessment of these graft materials for their effect on CTP performance. In addition, the efficacy of novel peptide binding domains that are specific to these materials can be tested using this method.

6.6. Optimization of the assessment of the two-dimensional TCP surfaces: A novel method to prepare two dimensional surfaces of a particulate of any bone scaffold material
was introduced in Chapter 3. This method was verified using the TCP material. These surfaces were assessed with respect to their repeatability, stability, uniformity in coverage, and smoothness using a semi-quantitative scoring system. High resolution Scanning Electron Miscoscope (SEM) images were used to assess the smoothness and the uniformity in coverage. A more quantitative method could be implemented for the assessment in future studies. Atomic Force Microscopy (AFM) is a method to measure the surface roughness. However, the AFM method is not well suited for the TCP surfaces as AFM can measure the surface roughness only upto 20 micron range, whereas the roughness of TCP surfaces was around 30-50 microns. Further, the use of AFM is limited due to the fact that the AFM uses a very fragile sensor tip, which has a risk of damage when used with a hard ceramic material such as TCP. Replacement of the tip can be very expensive. To circumvent these limitations, a more suitable method to measure the surface roughness in this range could be using non-contact, optical profilometers. There are several optical profilometers that use non-contact mode to measure surface roughness in the low to high micrometer range. The non-contact mode uses a light beam that is emitted from a stationary tip. The light beam is reflected from the surface whose roughness is being measured. The reflected beam is received at the same stationary tip where the light beam is is originated. The time between the emitted and received light beams is measured. The roughness of the surface is estimated using the variations in the measured time, and is displayed in microns. In addition to the sensitivity range, the non-contact profilometer also eliminates the risk of damaging the sensor tip due to the surface material that is being tested.
The uniformity of coverage of the TCP material can be assessed quantitatively using an imaging-based approach. The low resolution (20-50 X magnification) SEM images can be analyzed using advanced image processing software such as ImageJ or ImagePro. The thresholds can be defined for the gray-levels that represent the TCP particles (higher gray-level) and the PDMS layer (lower gray-level), and the image can be analyzed to filter the relative values of the corresponding gray-levels. This method will facilitate the quantitative assessment of the uniformity in terms of % area that is occupied by TCP relative to the total area of the surface.

The scoring system that is introduced in Section 3.6 does not differentiate between the relative significance of the parameters that were assessed, namely ease of preparation, repeatability, surface uniformity, stability during culture period, and identification and detection of colonies. Consequently, the parameters are valued at the same level of significance (or importance). For example, a score of 3 in ease of preparation is valued same as the score of 3 in the stability during culture period, even though the stability during culture period is far more important in the response of cells to the surface than the ease of preparation. This limitation of the proposed scoring system can be circumvented by assigning a multiplier for each assessment parameter. The value of the multiplier can be assigned based on the relative significance of the parameters. In case of the scoring system discussed in Section 3.6, ease of preparation, repeatability, surface uniformity, stability during culture period, and identification and detection of colonies can be assigned the multiplier values of 1, 3, 5, 5 and 5 respectively. The individual scores for the coverslip design will be multiplied by these values.
Consequently, the relative significance of the parameters will be reflected in the overall score of the design.

6.7. **Concluding Remarks:** The work in this dissertation has developed and documented a new and novel method for quantitative assessment of the effects of biomaterials and modified biomaterial surfaces on the performance of relevant cell populations. Specifically relevant to bone tissue engineering, these data provided clear evidence that the modification of a TCP biomaterial surface using a TCP binding peptide fusion protein with EGF (TCPBP-EGF) will increase colony forming efficiency by bone marrow-derived human connective tissue progenitors. Together with the proposed future studies, this work can provide an important contribution towards advancing the current standard of care of orthopaedic trauma in a clinical setting.

6.8. **Works Cited:**


20. Liu, L.Z., et al., *Reactive oxygen species regulate epidermal growth factor-induced vascular endothelial growth factor and hypoxia-inducible factor-1alpha*


