Specific Aims

Clinical strategies to restore lost bone are ineffective when used to treat composite fractures, bone defects presenting with significant muscle loss, suggesting a critical role for neighboring muscle tissue in bone repair. Adipose-derived stromal cells (ASCs) have multi-lineage capabilities including myogenic phenotypes, exhibiting greater myogenic capacity than bone marrow-derived mesenchymal stem cells (MSCs). The transplantation of dissociated ASCs into damaged muscle increases muscle regeneration but is limited by insufficient retention. Alternatively, the aggregation of progenitor cells into spheroids results in improved cell survival, increased secretion of endogenous trophic factors, and accelerated tissue formation, providing a simple approach for enhancing the efficacy of cell-based therapies.

Overall hypothesis: Composite fracture healing will be enhanced by promoting muscle regeneration *via* transplanting myogenically-induced ASC spheroids in engineered biomaterials.

Aim 1. Evaluate the survival and capacity of myogenically-induced ASCs to stimulate differentiation of myoblasts and osteoblastic cells *in vitro* when entrapped in insulin-like growth factor (IGF-1)-eluting alginate cryogels.

Hypothesis: Myogenically-induced ASC survival is improved with spheroid geometry and will enhance differentiation of cells from the myogenic and osteoblastic lineage.

Aim 2. Test the ability of ASC spheroids transplanted on IGF-eluting cryogels to concurrently stimulate repair of a rat bone and muscle composite injury.

Hypothesis: ASC spheroids transplanted in an IGF-1-eluting cryogels will speed neovascularization and muscle regeneration of a volumetric muscle defect, while restoring the potency of a BMP-2-loaded implant for bone repair in a rat model of composite tissue injury.

Background and Significance

The role of soft tissue injury and its relationship to bone regeneration has become a recent focus of study in improving fracture healing. Clinical studies have observed that composite fractures involving both osseous and muscular injury result in an increased incidence of complications including delayed healing, nonunion, infection, re-hospitalization, and revision surgeries.[1-5] Furthermore, the musculoskeletal system has been largely characterized by its mechanical nature. However, the muscle as a secretory source of "myokines" which affect the musculoskeletal environment has been demonstrated in the literature including the secretion of numerous osteogenic factors such as insulin-like growth factor [IGF]-1, fibroblast growth factor [FGF]-2, and transforming growth factor [TGF]-beta under basal conditions, with increased secretion in regenerating muscle.[6-9] The periosteum has receptors for these secreted factors suggesting a pathway for tissue cross-talk.[8] Additionally, the role of bone as an endocrine organ has garnered appreciation.[10,11,12] A shift in research and focus on the musculoskeletal system as a complex network of molecular and biochemical interactions will yield an increased understanding of the factors involved in osseous and muscle healing.

Muscle tissue may contribute to bone formation by secreting soluble factors that promote tissue vascularization, progenitor cells, osteogenic cytokines, and providing biomechanical stimuli.[13,14] Myoprogenitor cells have the potential to differentiate into osteogenic cell lineages and incorporate into regenerated bone in an open fracture model.[15-17] Clinically, coverage of an open fracture with a muscle flap is the gold standard for treatment with proven results of improved fracture healing, but this usually involves autograft harvest with donor site morbidity.[18-20] Hence, a cell-based synthetic construct may provide the therapeutic advantage of contributing to muscle regeneration/repair and fracture healing without the need for additional surgery and harvest of autologous muscle. The proposed project aims to elucidate the biochemical nature of muscle regeneration and provide a foundation for the development of a clinically applicable device to promote muscle and bone healing in an open fracture with significant muscle damage.

Research Design and Method

Aim 1. The objective is to determine the survival and role of myogenically-induced ASC spheroids on muscle and bone cell response *in vitro* when entrapped in IGF-1 eluting materials. RGD is the functional peptide sequence in extracellular matrix proteins such as fibronectin and collagen that enables cell adhesion.[21] Ongoing studies with our collaborator demonstrate RGD ligand density guides spheroid function for osteogenic differentiation, but no studies have been performed to evaluate myogenic potential.

Study 1. Determine the contribution of RGD ligand density on ASC spheroid survival and function in alginate cryogels. RGD-modified alginate cryogels will be synthesized as described[22,23] with increasing ligand density to examine the role of adhesion sites for ASCs (**Table 1**). Human ASCs will be purchased from commercial suppliers. ASC spheroids containing 15,000 cells per spheroid will be formed and undergo myogenic induction as previously described.[24,25] Spheroids will be seeded in alginate cryogels of varied RGD density and IGF concentration using dosages previously reported[22] and maintained in complete media (n=5 per group per time point). At designated time points, conditioned media will be collected and frozen until analyzed. Cell number will be quantified from a DNA assay, and the survival of ASCs in cryogels will be analyzed by a live/dead assay.

Table 1. Design for Study 1A.

Ligand density (μM RGD)	IGF dosage (μg/mL)	Endpoints t=0,1, 7, 14 days
0 150 1500	0 60	• ASC survival • VEGF secretion • Bone and muscle cell differentiation

The bioactivity of conditioned media on bone and muscle cells will be determined upon exposure to cultured human ASCs, primary osteoblasts, and primary skeletal myoblasts. The effect of IGF-1 and conditioned media on bone cells will be measured by assessing metabolic activity by MTT assay, apoptosis *via* caspase 3/7 activity, and biochemical markers of proangiogenic potential and osteogenesis. The osteogenic response of stimulated bone cells will be quantified by measuring alkaline phosphatase activity, osteocalcin secretion by ELISA, and qPCR detection of genes involved in osteogenesis (*RUNX2*, *SP7*, *IBSP*), myogenesis (*MYOD*, *MYOG*, *MYHC*), and angiogenesis (*VEGFA, FGF2*). Proangiogenic cues in ASC conditioned media will be quantified with protein arrays. All studies will be performed in parallel with conditioned media from dissociated myogenically-induced ASCs entrapped at the same initial density, and data will be normalized to DNA content to account for proliferation differences. The results of this study will reveal the role of cell adhesion from ASC spheroids on their proangiogenic, myogenic, and osteogenic potential.

Aim 2. The objective of Aim 2 is to examine the capacity of myogenically-induced ASC spheroids transplanted in engineered alginate hydrogels, optimized to potentiate cell function, to accelerate muscle repair and concurrent formation in a rat composite injury model.

Study 2A. RGD-modified alginate cryogels will be prepared as described. The formulation identified from Aim 1 that maximizes survival, proangiogenic, and regenerative potential of ASC spheroids will be deployed *in vivo*. Human ASCs transduced to express luciferase will be used to follow cell survival over time, and cells will be suspended at $20x10^6$ cells/mL in alginate cryogels for subsequent implantation.

We will study the capacity of ASC spheroids transplanted in engineered alginate cryogels to promote repair of

Table 2. Experimental groups for *in vivo* study.

Treatment Group	Purpose
Bone defect only (BMP-2 eluting gel)	Bone healing without muscle injury; positive control
Bone defect with untreated muscle defect	Rate of bone healing without intervention for muscle repair
Bone defect with treated muscle defect	Rate of bone healing when stimulating muscle repair
Time points of tissue collection: 12 weeks	
Animals per group (n): 7	
Total animals: 24	

composite injuries of critical-sized segmental femur defects and volumetric muscle loss in the quadriceps muscle.[26] We will create 6 mm segmental defects in the femur of skeletally mature immunocompromised rats (male and female, 12 weeks old) as described.[27] Defects will be immediately treated with 2 μg BMP-2 eluting alginate gels. Animals will be placed in one of three injury groups (**Table 2**). Negative controls will not be included, as this model confirms no union without intervention. Muscle defects will be created through the full thickness of the quadriceps down to the femur using a 6 mm-diameter biopsy punch. The bone defect will be made first, then, once the incised muscles have been closed with suture, the muscle defect will be created and treated with cryogels carrying myogenically-induced ASC spheroids.

ASC persistence in the muscle defects will be monitored noninvasively weekly using optical imaging. Bone formation will be followed longitudinally by plain film radiography at 2, 6, and 12 weeks. Femurs, both treated and native, will be recovered at 12 weeks. Osteogenesis will be quantified using microCT to obtain bone mineral density and bone volume fraction of the scanned region of interest. Representative femora (n=3/group) will be processed for H&E for tissue morphology, von Kossa stain for mineralized tissue, and immunohistochemistry (IHC) for vessel count. Remaining femora will undergo torsional testing to evaluate the mechanical properties of repair tissue when compared to native bone.[28] Muscle repair will be evaluated longitudinally at 2, 6, and 12 weeks by measuring contractile force as an indicator of extent of repair.[26]

Statistical analysis. Statistical comparisons will be made on the basis of Student's *t*-test or ANOVA followed by *post hoc* comparisons when statistically appropriate. The number of necessary animals (α=0.05; power level=80%) was determined by power analysis based on published data comparing bone volume fraction in repair bone in the presence of large muscle injuries.

Role of the Resident

The resident serving as the principal investigator will lead this project while on their research year with full release from surgical training. The resident has completed literature review and been integral in the project development. The resident will be responsible for mammalian cell culture, *in vitro* studies to evaluate the interplay between muscle and bone cells, and leading *in vivo* studies, including performing technical procedures, to evaluate the therapeutic potential of this approach. The resident will lead data analysis. The resident will be primary lead on manuscript formulation and writing. The resident will meet with the co-PIs on a biweekly basis to discuss research progress. Established milestones for the research year include at least one full-length research paper and conference presentation from the work.

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Names of specific individuals and names of institutions should NOT be in the body of the budget.

BUDGET

SALARIES AND WAGES (List all personnel for whom money is requested)	% effort on project	Requested from OTA (round to $)
None	%	$
	%	
	%	
	%	
Fringe Benefits _____% of Salaries and Wages Salaries and Wages plus Fringe Benefits	TOTAL	$ 0

PERMANENT EQUIPMENT (append justification)	
None	
Subtotal	$ 0

CONSUMABLE SUPPLIES (exclude animals and animal care)	
Human ASCs (Lonza)	$ 2,190
Human myoblasts and osteoblasts	$ 1,000
Cell culture consumables, materials for hydrogel synthesis	$ 2,000
Assays for measuring cell response (i.e ELISA, PCR, protein arrays, antibodies)	$ 4,000
Subtotal	$ 9,190

ANIMALS AND ANIMAL CARE	
Athymic rats	$ 2,520
Per diem for rats	$ 790
Instrumentation for segmental defect in rats	$ 3,500
In vivo imaging and mechanical characterization of rat tissue	$ 4,000
Subtotal	$ 10,810

ALL OTHER EXPENSES	
None	
Subtotal	$ 0

TOTAL DIRECT COSTS $ 20,000