FINAL SCIENTIFIC / TECHNICAL REPORT

Project Title:	Regeneration of tissues and organs using autologous cells
Award Number:	DE-FG02-06ER64240
Reporting Period:	August 1, 2006 – July 31, 2008
Recipient Organization:	Wake Forest University Health Sciences
Project Location:	Winston-Salem, NC
Project Contact:	Anthony Atala (Principal Investigator)

Executive Summary:

There are three major challenges to the field of regenerative medicine: 1) the growth and expansion of regenerative cells outside the body in controlled *in vitro* environments, 2) supportive vascular supply for large tissue engineered constructs, and 3) interactive biomaterials that can orchestrate tissue development *in vivo*. The Wake Forest Institute for Regenerative Medicine (WFIRM) proposes to engage in multidisciplinary projects to address all three. The WFIRM is home to a team of scientists with expertise in cell and molecular biology, physiology, biomaterials, controlled release, nanomaterials, tissue engineering, bioengineering, and clinical medicine. This combination of resources, combined with the vast infrastructure of the WFIRM, will be brought to bear on projects to discover and test new sources of autologous cells that can be used therapeutically, novel methods to improve vascular support for engineered tissues *in vivo*, and to develop intelligent biomaterials and bioreactor systems that interact favorably with stem and progenitor cells to drive tissue maturation.

The Institute's current programs are aimed at developing technologies using regenerative methods that employ a patient's own cells to help restore or replace tissue and organ function. This DOE program will serve to solve some of the vexing problems that are germane to many tissue engineering applications, regardless of tissue type or target disease. By providing new methods that are the underpinning of tissue engineering, this program will provide advances that can be applied to conditions including heart disease, diabetes, renal failure, nerve damage, vascular disease, and cancer, to name a few. These types of conditions affect millions of Americans at a cost of more than $400 billion annually. Regenerative medicine holds the promise of harnessing the body's own power to heal itself. By addressing the fundamental challenges of this field in a comprehensive and focused fashion, this DOE program will open new opportunities to treat conditions where other approaches have failed.

Project Summary:

Introduction

The Joint Commission for Health Care Organizations recently declared the shortage of transplantable organs and tissues a public health crisis. As such, there is about one death every 30 seconds due to organ failure. Complications and rejection are still significant albeit underappreciated problems. It is often overlooked that organ transplantation results in the patient being placed on an immune suppression regimen that will ultimate shorten their life span. Patients facing reconstruction often find that surgery is difficult or impossible due to the shortage of healthy autologous tissue. In many cases, autografting is a compromise between the condition and the cure that can result in substantial diminution of quality of life. The national cost of caring for persons who might benefit from engineered tissues or organs has reached $600 billion annually.

Autologous tissue technologies have been developed as an alternative to transplantation or reconstructive surgery. Autologous tissues derived from the patient's own cells are capable of correcting numerous pathologies and injuries. The use of autologous cells eliminates the risks of rejection and immunological reactions, drastically reduces the time that patients must wait for lifesaving surgery, and negates the need for autologous tissue harvest, thereby eliminating the associated morbidities. In fact, the use of autologous tissues to create functional organs is one of the most important and groundbreaking steps ever taken in medicine. Although the basic premise of creating tissues in the laboratory has progressed dramatically, only a limited number of tissue developments have reached the patients to date. This is due, in part, to the several major technological challenges that require solutions. To that end, we have been in pursuit of more efficient ways to expand cells *in vitro*, methods to improve vascular support so that relevant volumes of engineered tissues can be grown, and constructs that can mimic the native tissue environment to ensure tissue integration, maturation, and survival. Other long-term benefits of this research are likely to be cell-based drug delivery mechanisms, intelligent biomaterials, bio-nano technologies, as well as controlled delivery using advances in materials science.

Project Goals and Objectives

The major challenges to the goal of producing tissues and organs for transplantation and reconstruction are three-fold and will form the basis for the goals of this research. These include 1) Identifying sources of autologous cells and developing methods to expand them in large number *in vitro*, 2) Providing vascular support for growing constructs, and 3) Developing biomaterials and bioreactor systems that mimic the native tissue environment.

<u>Project 1: Cell Sources</u>

It has become widely accepted that almost every tissue in the body contains some type of stem or progenitor cell, including brain, liver, circulating blood, and heart, as well as skin, and fat. These stem and progenitor sources provide ample opportunity to address the need for autologous cells for transplantation or reconstruction. Research that addresses the technologies necessary to procure, purify, expand, differentiate, and mature these cells into functional tissues and organs is the first objective of the present research. Many such methods have been

developed for cells derived from animal tissues, and some techniques have been used with human cells as well. However, the clinical applicability of these approaches has not been convincingly demonstrated as the relationship between donor age and stem cell growth potential has not been established. As such, it is critical that the growth and tissue formation potential of autologous cells must be evaluated and characterized for their utility in regenerative therapies.

Project 2: Providing Vascular Support

Adequate vascularization is also essential for the engineering of large tissues. Neovascularization via the formation of new capillaries may be from existing vessels (angiogenesis) induced by vascular endothelial growth factor (VEGF), or by the formation of new vessels (vasculogenesis) from endothelial cells (EC). We have previously combined the use of VEGF gene delivery and implantation of vascular EC to enhance the neovascularization of engineered muscle tissues with successful results. Currently we propose to use VEGF and circulating endothelial progenitor cells (cEPC, progenitors of EC) to derived EC and enhance the neovascularization of engineered tissues. cEPC are regenerative cells that are mobilized into the circulation and differ from sloughed mature, circulating endothelial cells that randomly enter the circulation as a result of blunt vascular injury. cEPC proliferate and/or migrate in response to angiogenic growth factors and differentiate into mature EC *in situ* for neovascularization during tissue and organ regeneration. We propose to use cEPC to enhance neovascularization in our engineered tissues by co-culturing cEPC with other cell types, thereby incorporating them into our implantable constructs. We will also employ a genetic modification technique similar to one previously developed in our labs to enhance angiogenesis through a cell-mediated delivery of VEGF.

Project 3: Mimicking the Native Tissue Environment

Engineering clinically useful tissues will require that each component of the construct is optimized for tissue growth and maturation on a larger scale. This optimization will require the development of new, “intelligent” biomaterial scaffolds that facilitate neovascularization, innervation, and tissue maturation through controlled release of growth factors, that provide the spatial and orientational cues necessary to facilitate formation of functional tissues *in vivo*, and that will recruit additional host progenitor and stem cells necessary for complete maturation of the tissue. Simple biomaterials cannot accomplish this feat, so a scaffold system comprised of a natural matrix with the desired morphology, as well as controlled release of angiogenic and neurogenic factors must be developed. In the current project we will develop new biomaterials that possess biomolecular compositions capable of mediating cell attachment, growth, and differentiation. We will utilize advanced bioreactor systems that transfer the appropriate environmental cues to the developing cells such that accelerated tissue maturation can be achieved.

Results:

Project 1: Cell Sources:

Identification and expansion of sources of autologous cells are critical in the successful translation of tissue engineering technologies, as cells for the basis for tissue reconstruction. In this project, we have focused in developing reliable cell culture systems for muscle and endothelial cells. Muscle cells are an essential component of soft tissue that permits coordinated locomotion through contraction and relaxation. To build a functioning muscle tissue for reconstruction, a reliable cell culture system that allows for expansion of cell quantity must be achieved. In this project we have successfully developed a culture system from skeletal muscle tissue biopsy and expanded in vitro while maintaining cellular phenotypic and functional characteristics. Another important cell type is endothelial cells. These cells are known to promote angiogenesis and neovascularization. This is a critical component in tissue building process, as implanted tissues must establish vascular supply for cell survival and tissue formation. During the award period, we have developed culture systems for these two types of cells and have made substantial progress toward building tissues.

Muscle cell culture system.

To obtain autologous muscle precursor cells (MPCs), a 1 cm^3 muscle tissue biopsy was placed in sterile Dulbecco's Modified Eagles Medium (DMEM, Gibco, Grand Island, NY) on ice. The muscle was minced into small pieces (≤1 mm^3). The isolated muscle fibers were incubated for 2 hours at 37 ^{O}C in 35 mm dishes containing filter-sterilized 0.2 % (wt/vol) type I collagenase (Worthington Biochemical, Lakewood, NJ) in DMEM. After digestion, the enzymatic reaction was terminated through repetitive washes with PBS. The muscle fibers were then transferred into 35 mm dishes containing 3 ml of myogenic medium consisting of 20 % fetal bovine serum, 10 % horse serum (Gibco, Grand Island, NY), 0.5 % chick embryo extract, and 1 % Penicillin/ Streptomycin (Gibco, Grand Island, NY) in DMEM. Single muscle fibers were liberated by repeated pipetting and confirmed by microscopy. The separated muscle fibers were plated on 35 mm dishes precoated with Matrigel (1 mg/ml, BD Matrigel Basament Membrane Matrix HC, Becton Dickinson) and incubated at 37 ^{O}C and 5 % CO_2. MPCs migrating from the plated myofibers were trypsinized and plated in 10 cm culture dishes with complete medium containing 10 % fetal bovine serum and 1 % Penicillin/Streptomycin in DMEM. This culture system showed that MPCs were migrated from individual muscle fibers and grew in culture and fused to form muscle fibers in vitro (Fig 1). The muscle cells grown in culture were expanded over multiple passages and retained their phenotypic and functional characteristics.

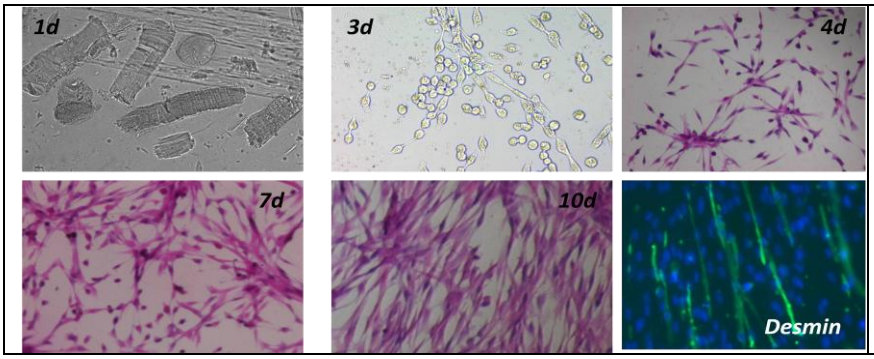

Figure 1. Muscle precursor cells (MPC) are isolated from individual muscle fibers. MPCs migrated from satellite cells of individual muscle fibers are grown under culture conditions and fuse to form into mature muscle fibers *in vitro*.

Use of muscle cells derived from donors of different ages.

To use expanded muscle cells clinically, it is essential to demonstrate that muscle cells can be grown from different age groups. However, it was unclear whether adequate muscle could be engineered equally from all age groups. Would the engineered tissue from muscle cells from a patient be the same as the engineered tissue formed from older or younger cells? To that end, we investigated the age-related effects on muscle cells with respect to cell growth and recombinant protein expression. We used our established methods to culture muscle precursor cells (MPCs) from young (Y, 2 weeks), mature (M, 3 months) and old (O, 18 months) Balb/C mice. Cell expansion was successfully performed with muscle precursor cells derived from all 3 age groups. We analyzed the cells in vitro for fiber formation, proliferation and recombinant protein expression. It was evident that the young cells had a better performance and growth rate than the cells obtained from mature and the older animal tissues (Figure 2A). The doubling time was 41.2 ± 6.7 h of MPC from young donor age, 67.3 ± 10.3 h for cells grown from mature age and 88.1 ± 18.9 h from old donors. The slower growth of old cells made it necessary to extend the time of culture to reach sufficient cell number for tissue engineering (80 million cells) by an average of 10 days (Figure 2B).

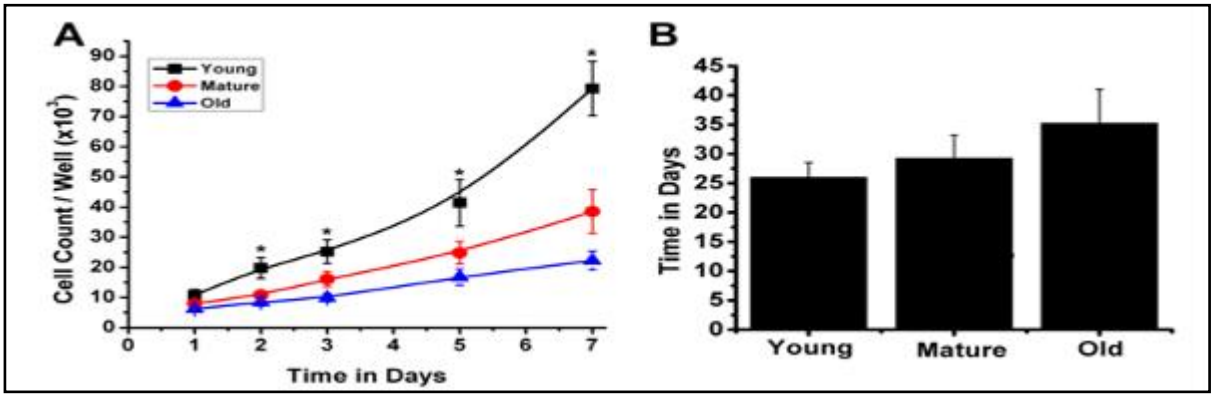

Figure 2. Proliferation Assay of MPCs. The proliferation analysis was performed at passage 3. Cells were plated in a 48 well plate. After 1, 2, 3, 5, and 7 days, the cells were harvested and counted. A. The proliferation assay demonstrated that there was a significant increased growth in the young MPCs. B. The slower growth rate of old cells increased the time required to culture sufficient cell numbers for tissue engineering (80 million).

The evaluation of myofiber formation in vitro showed significant differences between the age groups (Figure 3A-C). MPCs from young animals demonstrated a high regenerative potential with significantly higher number of cells participating in the new myotubes (281.3 ± 30.9) when compared to cells from mature and old age (Y vs. M: $p=0.001$, Y vs. O: $p<0.001$) (Figure 3D). Cells from mature age formed shorter myofibers with a lower number of participating cells (136.5 ± 18.3, M vs. O: $p=0.32$). Cells from old age formed even fewer myofibers with a lower number of cells (59 ± 10.5) participating (Figure 3E).

The evaluation of myofiber density revealed a similar pattern with young cells forming the highest density of myofibers (29.3 ± 1.7, Y vs. M: $p=0.004$, Y vs. O: $p<0.001$) followed by the mature cells with 19.0 ± 1.1 (M vs. O: $p=0.067$) and the old cells with 13.5 ± 2.5. These results showed that the ability of MPCs to fuse to mature myofibers decreases with age.

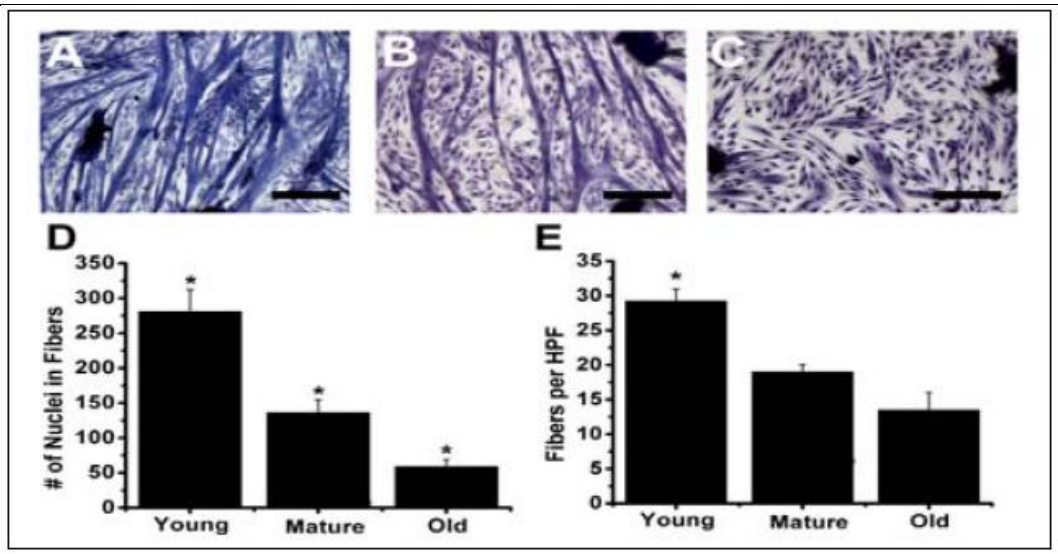

Figure 3. Fiber Formation and Fusion Rates. The ability of MPCs from different donor ages to form myofibers was assessed at passage 2. A Giemsa stain was used to identify formed myofibers. A. young, B. mature, C. old donor age. (Scale bar represents 100 µm). D. Histomorphological assessment demonstrated a significant age-related decline in the number of nuclei incorporated in myofibers/high power field. E. Evaluation of fiber density showed a similar trend. However, only young cells were significantly different from the other groups. Overall, histomorphometry showed a clear age-related decline in myofiber formation with fewer and shorter myofibers in older cells.

Endothelial Cell Culture System: peripheral blood

Endothelial cells (EC) are an important cell type in tissue engineering. These cells are known to promote angiogenesis and facilitate neovascularization when implanted in vivo. We have developed an endothelial cell culture system from two different cell sources: peripheral blood and placental cells. Peripheral blood (20mL per sample) was collected from the jugular vein of sheep. The mononuclear cell fraction, which contains EPCs, was isolated using Ficoll-Histopaque-1083 (Sigma, St. Louis, MO) density-gradient centrifugation. The cells were washed with phosphate buffered saline (PBS) to remove contaminating platelets. An aliquot of mononuclear cells was initially cultured in a 35mm culture dish with Endothelial Cell Growth Medium (EGM-2) supplemented with Single-Quot (Lonza, Walkersville, MD), ascorbic acid, heparin, gentamicin, VEGF, IGF, FGF, EGF, and 2% fetal bovine serum as supplied by the manufacturer for 24 h. Cells were then transferred to fibronectin-coated 35mm culture plates (Corning, Corning, NY). EC differentiation was initiated with EGM-2. Mononuclear cells were cultured until cells with endothelial morphology were visible and began to proliferate. Cells were further expanded and differentiated into mature ECs. At passage five, the differentiated ECs were characterized by immunohistochemistry using mature EC-specific markers. Further, cells were cultured on Matrigel to confirm their potential to form small capillaries. The cells were successfully grown from every sample collected.

EPC-derived ECs were plated onto six-well plates for 24 h, fixed with ice-cold methanol, and incubated with primary antibodies, including CD31 (Santa Cruz, Santa Cruz, CA), Flk-1 (Santa Cruz), Ulex-1 (Sigma), von Wilbrand factor (vWf; Dako, Carpinteria, CA), and CD146 (BD Bioscience, San Jose, CA) diluted at 1:50. This was followed by incubation with FITC-

conjugated or Texas red–conjugated secondary antibody diluted at 1:300. Samples of cell-seeded valve tissue were embedded in Optimal Cutting Temperature solution (Sakura Finetek, Tokyo, Japan). The whole leaflet with commissure and part of the cusp were included in every block. The frozen tissue block was sectioned at a thickness of 5 mm using a cryotome. H&E staining was performed. For immunohistochemistry, sections of cell-seeded valve tissue were incubated with anti-human vWF (Dako) at room temperature for 2 h, followed by fluorescent-labeled secondary antibodies at room temperature for 30 min. The tissue slide was rinsed, counterstained with DAPI, and mounted using Vectashield fluorescent mounting medium (Vector, Burlingame, CA).

Mononuclear cells were successfully isolated from 20mL of sheep blood and plated on fibronectin-coated plates. Formation of colonies was observed a few days later (Fig. 4A). Cells grown in differentiation medium showed the typical cobblestone appearance, indicative of ECs (Fig. 4B). At passage 5, which was chosen for all of our experiments based on cell numbers and proper cell characteristics, cells were characterized by immunocytochemistry to demonstrate differentiation into ECs. These cells strongly expressed the endothelial-specific cell markers, including CD31, CD146, Ulex-1, vWF, and Flk-1 (Fig. 4C, D, E, F, and G, respectively). In addition, these cells formed capillary structures typical of ECs when seeded onto Matrigel (Fig. 4H). These data indicate that EPCs from circulating peripheral blood had differentiated into mature ECs in vitro.

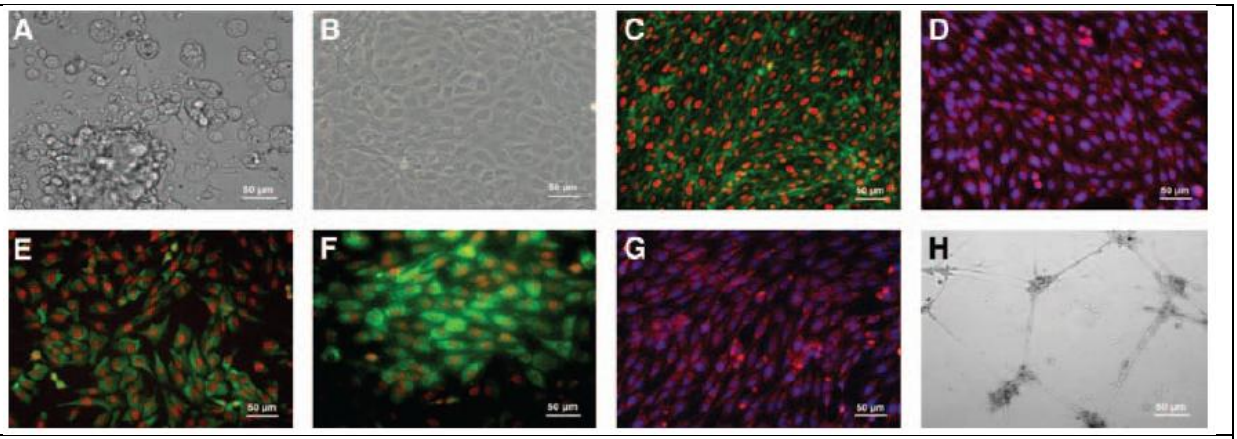

Figure 4. Characterization of EPC-derived ECs. (A) A colony derived from the mononuclear cell fraction. Differentiated ECs show the typical cobblestone morphology. Cells also stained positive for the following EC-specific markers: (B) CD31, counterstained with PI; (C) Flk-1, counterstained with DAPI; (D) Ulex Europa Agglutinin-1, counterstained with PI; (E) vWf, counterstained with PI; (F) CD146, counterstained with DAPI. (G) Capillary formation after dispersion and culture of EPC-derived ECs on Matrigel.

Endothelial progenitor cells (EPC) from human placental tissue

Placenta is a highly vascularized tissue and characterized by routine regeneration. This property suggests that placental tissue contains an abundant resource of vascular progenitors. Thus, the placenta may be the ideal source to obtain vascular progenitors for tissue engineering. Examples include endothelial progenitor cells (EPC) to coat the vasculature lumen (endothelial cells) and mesenchymal stem cells (MSC) as a source of pericytes to support the vascular structures. The use of full term placental tissue to obtain vascular progenitors offers a safer

approach than placenta villi biopsy that poses a risk to the fetus and the mother. In this study, we examined methods to isolate and culture EPC and MSC from termed placental tissue and tested their potential for neovascularization of bioengineered tissues. In this study we have focused to achieve the following goals: 1) Isolation of EPC and MSC from placental tissue, 2) Purification EPCs population by magnetic cell sorting and Fluorescence-activated cell sorting (FACS), 3) Expansion of EPC, 4) Characterize populations of EPCs.

In this study we discovered that endothelial cells like population of the cord blood and umbilical vein lumen cells cultured and isolated by immune-selection with anti CD31 antibodies, comprises endothelial progenitor cells with robust proliferation ability. To date we have successfully implemented this approach to obtain fetus-derived EPC from multiple human pregnancies. Analyses by flow cytometry have shown that human EPC express cell surface markers characteristic of endothelial progenitor cells, including CD31, CD34, ULEX, CD146, CD105 and c-kit. The EPCs are negative for markers of the hematopoietic lineage (CD45). Immunohistochemistry have shown placenta derived EPCs expressing makers for functional endothelial cells, namely eNOs and vWF, and they are also positive with Dil-AC-LDL endocytosis assay (Fig 5).

Placental MSC have a high proliferation rate (typical doubling time ca. 36 hours) and can be propagated easily in culture. Analyses by flow cytometry have shown that human PMSCs express cell surface markers characteristic of mesenchymal stem cells, including CD44 (hyaluronan receptor), CD146 and CD105 (endoglin). The MSC are negative for markers of the hematopoietic lineage (CD45) and of hematopoietic stem cells (CD34, CD133). We have shown that placental MSC can be induced, under specific growth conditions in vitro, to multilinage. In osteogenesis condition, the MSC have shown calcium deposition, and lipid droplets formation in adipogenesis condition.

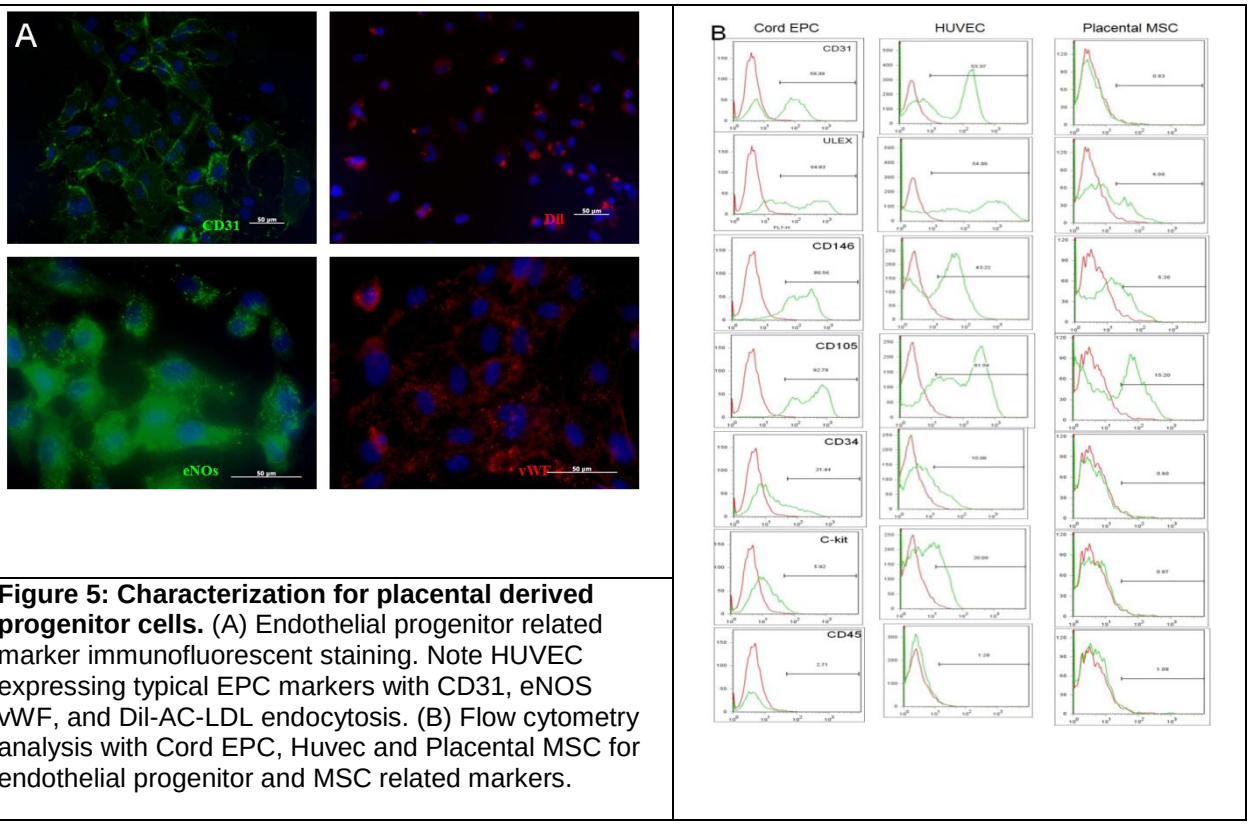

Figure 5: Characterization for placental derived progenitor cells. (A) Endothelial progenitor related marker immunofluorescent staining. Note HUVEC expressing typical EPC markers with CD31, eNOS vWF, and Dil-AC-LDL endocytosis. (B) Flow cytometry analysis with Cord EPC, Huvec and Placental MSC for endothelial progenitor and MSC related markers.

Project 2: Providing Vascular Support

Achieving adequate blood supply is one of the major hurdles for clinical translation of tissue engineering technology. To facilitate neovascularization of engineered tissues upon transplantation, we proposed to combine the use of angiogenic growth factors and vascular cells, together with the bioengineered tissue and cells. As such, our work was focused on identification, isolation and growth of vascular cells and provision of vascular growth factors to support neovascularization. In this project, combination of muscle progenitors, endothelial progenitor cells and angiogenic factors was performed to achieve clinically relevant muscle tissue mass for muscle tissue engineering. Toward this goal, delivery of vascular endothelial growth factor (VEGF) was investigated. The effects of VEGF and endothelial progenitor cells (EPC) of engineered muscle tissue volumes were evaluated.

Release kinetics of VEGF embedded in collagen gel and secreted from cells.

We examined VEGF release kinetics from muscle progenitor cells that were transiently expressing VEGF. VEGF concentrations in the culture media steadily increased in the first 10 days, reaching 1 µg VEGF secreted from 5 x 106 cells. After 10 days, the amount of secreted VEGF decreased sharply until day 20, when only 0.1 µg VEGF was secreted from 5 x 10^6 cells. Lower VEGF amounts were detected until day 35. Taken together, we have documented that we can control VEGF release from a gel suspension and from cells that transiently secrete VEGF. The release kinetics show a gradual decrease in VEGF amounts with time and that VEGF could be detected after more than 2 weeks. These results are significant because they will enable us to control tissue neovascularization by controlling secretion of angiogenic factors such as VEGF (Fig 6).

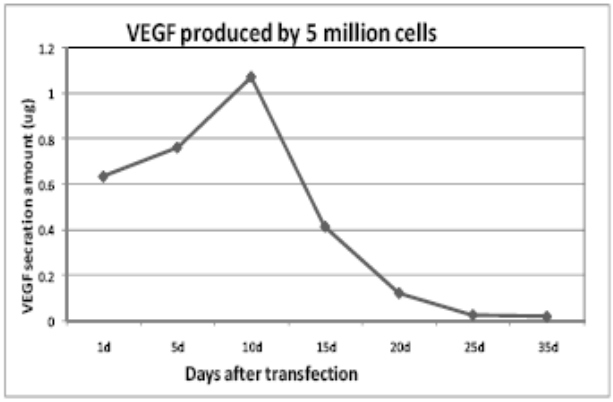

Figure 6. VEGF secretion. Muscle progenitor cells were infected with a recombinant adenovirus that expresses VEGF. VEGF concentrations in the medium were measured every 5 days using ELISA. The amounts of VEGF secreted from 5 x 106 cells were calculated. VEGF secretion peaked after 10 days and then decreased sharply by day 20th. VEGF could be detected until day 35.

The effects of VEGF and EPC of engineered muscle tissue volumes.

We have previously shown that VEGF supplementation helps to preserve the volumes of regenerated muscle tissue upon injection of muscle progenitor cells (MPC) to mice. The goal of the current experiments was to test the combine effects of VEGF and human EPC of the muscle volumes. We obtained human EPC that were differentiated from human embryonic stem cells. We injected different combinations of myoblasts, myoblasts expressing VEGF and EPC into the

subcutaneous space of immune-deficient mice. Volume measurements of the regenerated muscle tissues showed a significant advantage to VEGF-secreting myoblasts. Furthermore, addition of human EPC provided a significant improvement of muscle volume preservation over VEGF-secreting myoblasts only. Explantation of the regenerated muscle tissue confirmed these results and showed generous vascularization around the tissue. Importantly, immunostaining for human cells in the regenerated muscle tissue showed many human cells integrated in the mouse capillaries (Fig 7). These results confirm our previous results that angiogenic factors such as VEGF have essential role in inducing neovascularization wich, in turn, aids in tissue volume preservation. These results also demonstrate that human EPC participate in blood vessel formation and suggest their role in the neovascularization process.

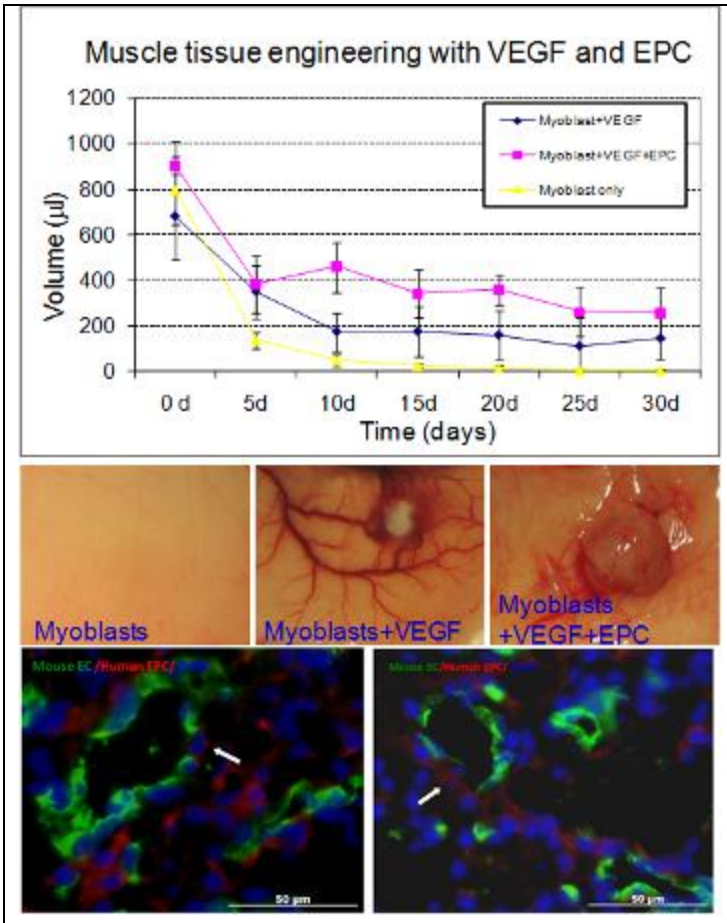

Figure 7: VEGF and EPC help to preserve engineered muscle tissue volumes. For these experiments we used 3 cell combinations: 1. Myoblasts infected with an adenovirus expressing VEGF, 2. Myoblasts infected with an adenovirus expressing VEGF mixed with human EPC and 3. Unmodified myoblasts. The cell combinations were mixed with collagen and injected subcutaneously into nude mice. Regenerated muscle volumes were recorded for 30 day. Subsequently, the skin was opened and images of the regenerated muscle tissues from the different groups were obtained. The regenerated muscle tissues from mice injected with Myoblasts infected with an adenovirus expressing VEGF mixed with human EPC (group 2) was processed for immunohistology using antibodies against human specific mitochondrial antigen (red) and against mouse CD31 (green) to detect human and mouse cells, respectively. The images at the bottom show integration of human EPC (red) within capillaries of mouse origin (green).

Project 3: Mimicking the Native Tissue Environment

To engineer clinically useful tissues, the tissue constructs need to be optimized for tissue growth and maturation. This optimization will require the development of new biomaterial scaffolds that facilitate adequate tissue maturation and provide the spatial and orientational cues necessary to form functional tissues *in vivo.* In this project we have focused on fabricating scaffolds that provide adequate microenvironment mimicking native tissue. We have also utilize bioreactor systems that transfer the appropriate environmental cues to the developing cells such that accelerated tissue maturation can be achieved.

Scaffolds, human muscle progenitor cells (MPCs) and bioreactor protocols for tissue engineered skeletal muscle.

As part of Project 3 we proposed to develop bioreactor systems capable of recreating critical aspects of the *in vivo* environment for the purposes of accelerated tissue maturation (both *in vitro* and *in vivo*), with parallel development of human cell sources and scaffolds that can be used for regenerative medicine. Figure 8 shows our initial strategy and results in this regard, using a modified decellularized bladder acellular matrix (BAM), human MPCs and a cyclic stretch protocol in a custom designed bioreactor. Full details can be found in a subsequent publication (Moon et al., 2008). Briefly, conditions were optimized so that human MPCS could be cultured and differentiated into myoblasts that stained with characteristic muscle markers (Fig 8). Cells were then seeded on a modified BAM scaffold to ensure maximal surface area for cellular integration and maturation. As illustrated, we developed, in parallel, a tissue culture container with a motor controller system to provide cyclic strain to tissue engineered constructs. Furthermore, this system employed computer control of the preconditioning protocol; which initially consisted of 10% stretch at the rate of 3X/minute for the first 5 minutes of every hour. Use of this system provided evidence for formation of organized human skeletal muscle tissue *in vitro* that can be subsequently implanted *in vivo*. Further details can be found in a recent publication (Moon et al., 2008). In short this initial work has laid the foundation for future studies and further research and development of the optimal cell source(s), scaffold(s), and bioreactor protocol for tissue engineered skeletal muscle.

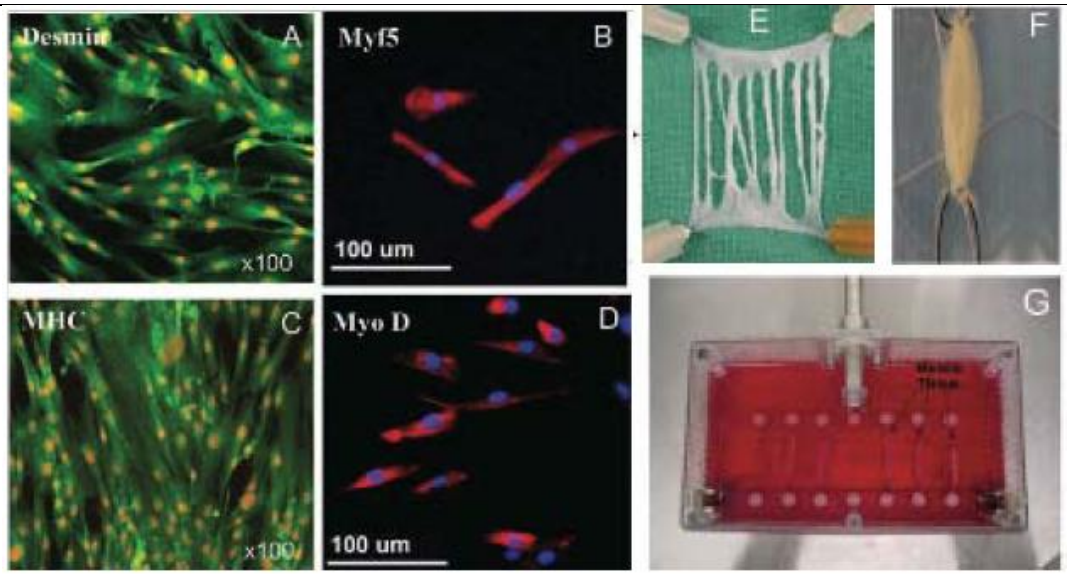

Figure 8. Cell culture and bioreactor system. Panels A–D depict the homogeneity of the muscle cell phenotype observed after explant cell culture of human skeletal muscle biopsies (see Methods). Panels E and F illustrate the preparation of the decellularized bladder submucosa scaffold for cell seeding and bioreactor placement. Panel G illustrates the bioreactor chamber with the engineered skeletal muscle constructs in place.

Scaffolds, vascular smooth muscle cells and bioreactor protocols for tissue engineered blood vessels (TEBV).

In our continuing effort to identify the optimal scaffold for TEBV, we have further extended our decellularization efforts on porcine arteries. One of the current limitations of TEBV for vascular replacement is that they do not possess a functional medial smooth muscle cell layer. Without question, the presence of a functional medial smooth muscle cell layer has important implications to vessel function throughout the vascular tree. During this funding cycle we extended this line of investigation to include development of TEBV with a more physiologically relevant medial smooth muscle layer. Specifically, our strategy was to use a specially tailored bioreactor system and preconditioning protocol in combination with surface modification of decellularized vascular scaffolds to increase smooth muscle cellular seeding efficiency and proliferation. Our rationale was that this approach would promote a more uniform deposition and density of mature VSMCs in TEBV. The overall goal of these experiments was to continue development and optimization of *in vitro* scaffold, cell seeding and bioreactor preconditioning techniques for enhanced fabrication of TEBV with a more native vessel phenotype. A schematic depiction of the system we used is shown in Figure 9, and the salient features of our findings are described below and in more detail in a recently published manuscript based in part on this study.

In short, decellularized vessels were prepared in the absence and presence of the adventitial layer, and statically seeded with a pipette containing a suspension of rat aortic VSMCs (primary cultures prior to passage 4 in all cases). After cell seeding, recellularized engineered vessel segments (≈5 mm) were placed in a custom bioreactor system for 1–2 weeks to evaluate cellular attachment, proliferation, alignment, and maturation. Of note, removing the adventitial layer of the decellularized porcine artery dramatically enhanced the initial attachment of VSMCs. Moreover, cyclic bioreactor conditioning (i.e., changes in flow and pressure) produced increased VSMC proliferation, and moreover, accelerated formation of a muscularized medial layer in the absence of the adventitial layer during the first week of preconditioning. Again, the major finding is that bioreactor preconditioning accelerates the formation of a significant muscular layer on decellularized scaffolds, in particular on adventitia-denuded scaffolds. These initial findings represent an important step toward the development of TEBV that more closely mimic native vasculature. Because the vessel segments used in this approach can be removed at various time points during the same bioreactor preconditioning cycle, without affecting the remaining vessel segments, we anticipate that this customized bioreactor approach will provide a relatively “high content” and potentially “high throughput” screening tool to evaluate and optimize a variety of cell source and biomaterials for TEBV.

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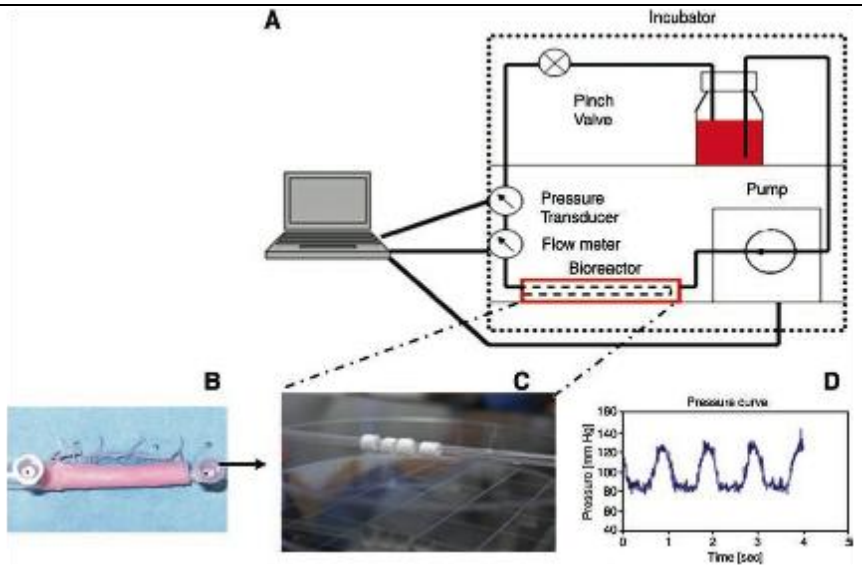


Figure 9. Schematic depiction of the bioreactor system used for preconditioning of VSMC-seeded rings of engineered vessel. (A) Illustration of the flow system setup to induce cyclic strain onto the engineered vessels. (B) Removal of adventitial layer. (C) Snap image demonstrating the position of the vascular rings on the custom sylgard tube. (D) Corresponding pressure measurements of the bioreactor system.

Despite this encouraging success, there was still little penetration of the smooth muscle cells into the vessel wall (Figure 10). Thus, we evaluated scaffold etching using various peracetic acid concentrations (1-10%), but were unable to establish a protocol that increased porosity without compromising biomechanical integrity (i.e.,the scaffolds fell apart in the bioreactor; data not shown). However, our initial findings with decellularized vein indicate significantly greater smooth muscle cell penetration following 1 week of bioreactor preconditioning. We are continuing to pursue this line of investigation.

Figure 10. Left Panel: H&E staining of decellularized porcine carotid artery following removal of the adventitia 24 hours after cell seeding. **Middle Panel:** H&E staining of decellularized porcine carotid artery following removal of the adventitia and 4 weeks of bioreactor preconditioning. **Right Panel:** DAPI staining of decellularized porcine vein after 1 week of bioreactor preconditioning.

Development of Extracellular Matrix for Bone Tissue Regeneration

Large craniofacial defects create a significant obstacle in reconstructive surgery. Current treatments for these defects are inadequate and fail to address the clinical need. Tissue engineering of bone substitutes is a potential avenue to address the problem of large defects; however, very few successful bone substitutes have been engineered. Progress in development of these bone substitutes has been slow due to limitations in scaffold properties, which include little to no osteoinductive activity, resulting in restricted stem and progenitor cell migration as well as restricted calcified matrix deposition in the scaffold. Osteoinductive factors are especially important when defects are of critical size (i.e. sufficient size to prevent spontaneous healing). One such factor, bone morphogenetic protein 2 or BMP-2, has been approved by the Food and Drug Administration FDA for spinal fusion applications. However, off-label usage and reporting of adverse outcomes has lead to some safety concerns related to the biomaterial delivery system. As a potential solution to the need for new biomaterials for bone regeneration, we are investigating the matrix secreted by differentiating stem cells as a source for new osteogenic biomaterials.

Our preliminary investigations suggest that potent osteoinductive factors are produced by differentiating stem cells during the process of osteogenesis. Key among these are extracellular matrix (ECM) molecules that provide a solid or semi-solid microenvironment (i.e. the stem cell niche) essential to the maturation of osteogenic cells into mature osteoblasts. *We hypothesize that ECM proteins are instructive to stem cells and play a critical role in driving the differentiation process.* Moreover, we postulate that certain ECM proteins may serve as the basis for biomaterials development and that these important compounds can be used as or incorporated into bone scaffolds once their identity and role in osteogenesis is elucidated. We propose to test our hypothesis by using differentiating stem cell cultures as a model system. The overall goal of this study is to identify salient osteoinductive ECM components produced during adipose derived stem cell- (ADSC)-mediated osteogenesis that may be responsible for the increase in osteoinductive activity of the ECM. The following specific aims are being performed to meet this goal:

Aim 1: To identify genes expressed by ADSC during osteogenesis *in vitro* that produce extracellular proteins which could play a role in building the stem cell niche and guiding differentiation.

Adult stem cells provide a readily available, autologous source of multipotent cells. We chose adipose-derived stem cells (ADSC) because they are relatively easy to obtain and differentiate into bone through the addition of soluble factors *in vitro*. ADSCs will be differentiated to osteoblasts *in vitro*. Gene arrays will be performed at specific time points along the osteogenic differentiation pathway. Genes of interest will be those that encode ECM proteins or could be associated with ECM and are significantly up-regulated during osteogenesis in relation to ADSC controls. Array data will be analyzed by computer software such as the Database for Annotation and Integrated Discovery (DAVID). The candidate genes will be categorized into functional groupings and network analysis of gene products will be performed in order to identify predicted signaling pathways and to visualize the molecular interaction networks.

Aim 2: To investigate the composition of osteogenic ECM *in vitro* by proteomic analysis.

ADSCs will be differentiated to osteoblasts *in vitro*. At predetermined time points, the cell secreted ECM will be harvested from culture dishes. Resulting ECM preps will be analyzed using 2D gel electrophoresis and differential analysis will be employed to identify proteins that

vary between differentiated cells and undifferentiated controls. Proteins that are differentially expressed will be identified by mass spectrometry. These data will be correlated with the gene expression data from Aim 1 to determine if gene expression results in the presence and accumulation of gene products in the stem cell niche. The presence of select proteins of interest will be confirmed by western blot analysis.

Preliminary data suggests that stem cells undergoing differentiation modify their microenvironment. Several findings suggest that dynamic changes occur within the extracellular matrix of differentiating ADSC which can alter or guide the stem cell differentiation process. One study in particular focused on defining osteogenic activity of the ECM produced by differentiating ADSC *in vitro* (Fig 11). ADSC were grown in 2D culture using osteoinductive media. At progressive time points in differentiation, the monolayer of cells was decellularized leaving a culture dish coated with residual ECM produced at a specific time in osteogenesis. To assess the osteogenic activity of these matrices, fresh ADSC were reseeded onto the dishes with residual cell secreted ECM and induced to differentiate to bone. The resulting differentiation was then characterized to assess whether the presence of the cell-secreted ECM could enhance or guide the differentiation of the re-seeded cells.

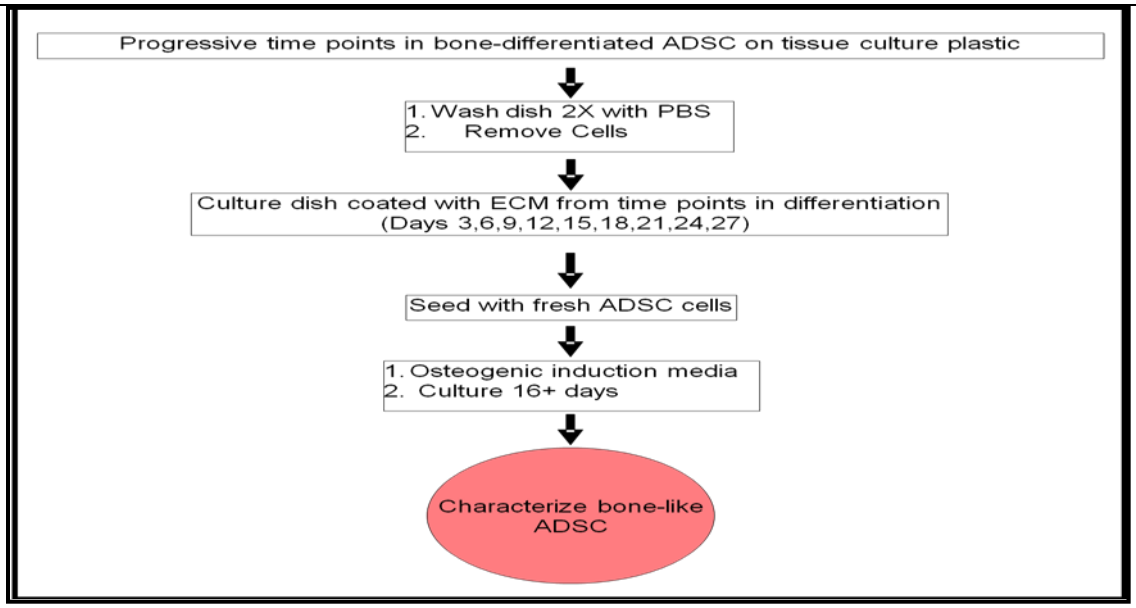


Figure 11. Experimental Design.

Cells seeded onto day 3, 6, 9, 12, 15, 18, 21, 24, and 27 ECM showed differential alizarin red staining, and indication of formation of a calcified matrix. Robust calcium deposition was clearly evident by day 15, but not before. Matrices from later time points appear to display similar or lesser calcium levels, but cell detachment was prevalent (Fig 12). These findings suggest that the matrices from time points prior to day 12 display less osteogenic activity when compared to matrices from time points following day 15. Further characterization of the reseeded cells on day 15 matrices revealed that day 15 matrices also induced high expression of osteogenic markers. qPCR results showed earlier and increased expression of bone markers such as

osteocalcin and RUNX2 in cells grown on day 15 ECM compared to cells grown on plain tissue culture plastic (Fig 13). These experiments demonstrated that ADSC differentiated on day15 ECM were osteoblast-like and had produced a robust calcified matrix in half the time as ADSC differentiated on uncoated dishes, thereby suggesting the presence of strong osteogenic factors in the day 15 matrix that had remained on the dish.

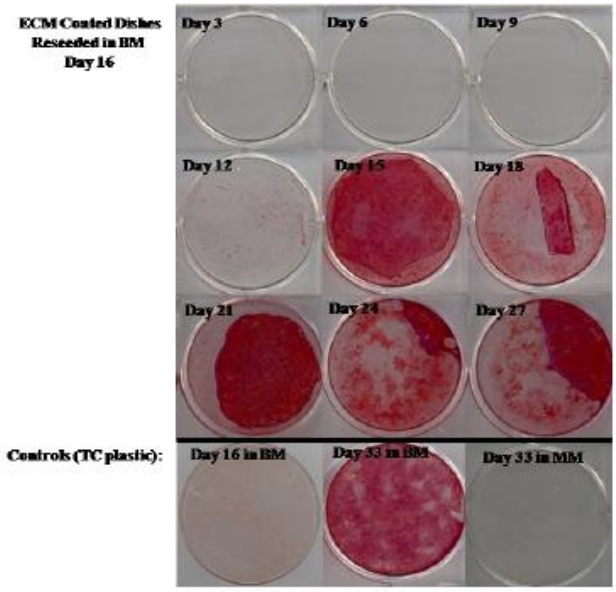


Figure 12. Calcium deposition visualized with alizarin red. Top squares (above the black line) show calcium deposition by ADSC on ECM from progressive time points in differentiation. Day 15 ECM appears to have a significant effect on calcium deposition compared to earlier time points. Similar staining in cultures grown on TCP does not occur until day 33 (bottom panel).

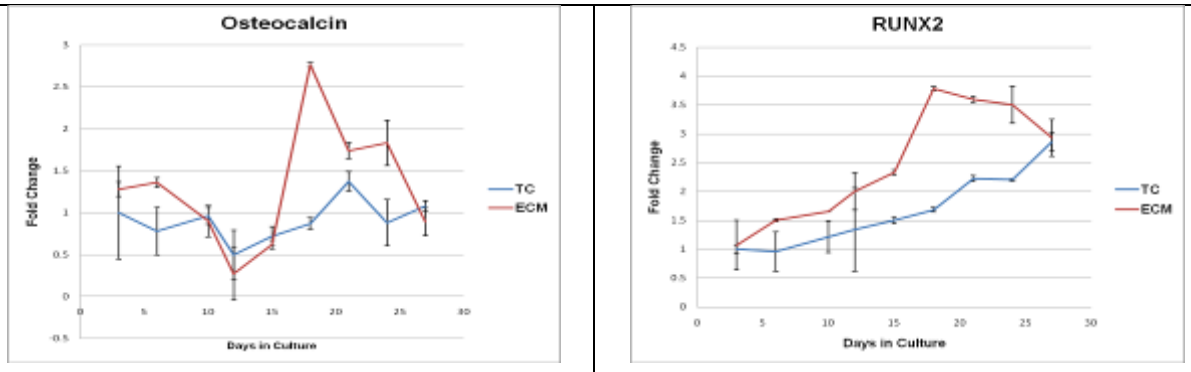


Figure 13. Bone marker mRNA expression levels across differentiation on tissue culture plastic (TC) compared to day 15 ECM in bone media. n=6; *p≤0.05

To further investigate this phenomenon, we prepared ECM isolated from day 11 and day 16 in the osteogenic differentiation process for isoelectric focusing and subsequent 2D SDS PAGE electrophoresis. Time points were extended by a day to provide a safety net for possible differences in the differentiation process which might occur under separate differentiation cycles. Spots for tryptic digestion were chosen on the basis of absence in the contrasting gel. Subsequent LC/MS/MS analyses, followed by Mascot search analysis were performed in order

to identify selected spots. Analysis revealed a large number of spots at each time point, suggesting that numerous ECM proteins are present on the dishes. In addition, highly abundant intracellular proteins are present (data not shown; from previous gel experiments) particularly proteins present in high amounts within the cytoplasm. Of particular interest was the presence of Histone (H4) on gels from day 11 ECM and absent on gels from day 16 ECM. H4 mRNA is commonly used as a marker of cell proliferation. Increased mRNA levels of H4 indicate that cells are actively proliferating. Increased mRNA levels would lead to high levels of H4 in the cytoplasm prior to protein transport into the nucleus (Fig 14). This finding suggests that matrices from day 11 in the differentiation process are secreted while the cells are still undergoing proliferation and have not yet become fully committed to the osteogenic lineage. The absence of H4 on gels from day 16 ECM suggests that by this time point, cytoplasm levels of H4 have decreased leading to the absence of H4 on gels from day 16. Therefore, day 16 ECM, which possesses high osteogenic activity, is likely being secreted by cells which have committed down the osteogenic lineage and are no longer undergoing proliferation.

Additional analysis revealed high amounts of collagen present in gels from day 16 ECM. It is well documented that collagen provides a template for accessory ECM proteins to bind. Additionally, collagen is a major component of the osteoid or organic matrix deposited prior to mineralization. The high level of collagen present on day 16 ECM when compared to day 11 provides more evidence to suggest that the cells which secreted the day 16 matrices are committed down the osteogenic lineage. In depth analysis of differentially expressed proteins will provide more insight into the increase in osteogenic activity from day 16 ECM.

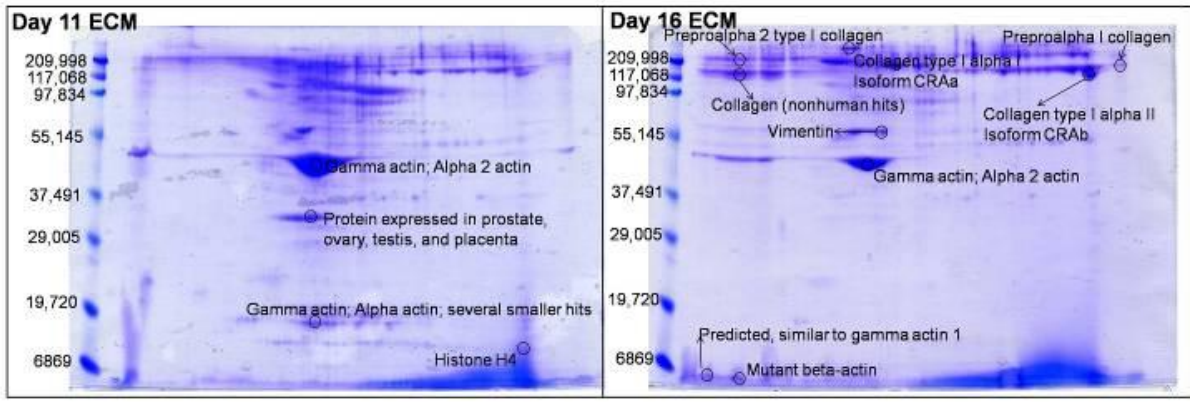


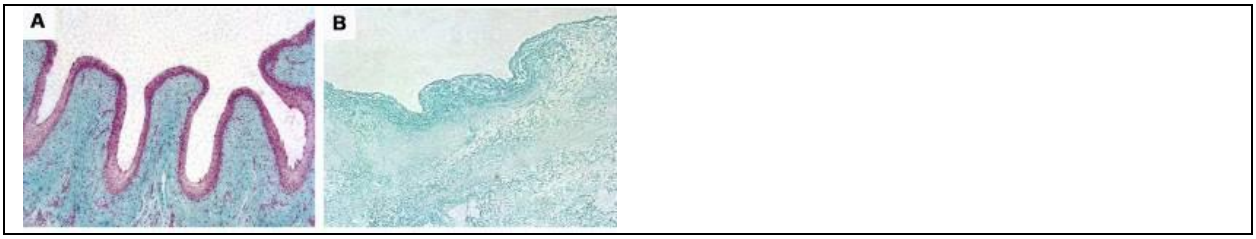
Figure 14. 2D gel electrophoresis of day 11 and day 16 ECM. Spots were chosen for analysis based on absence in the contrasting gel. pH 3-10;n=3

To understand the underlying mechanisms and pathways involved in ECM enhanced osteogenesis, gene array analyses will be performed at specific time points along the osteoblast differentiation pathway with cells on tissue culture plastic controls as well as on day 16 matrices. RNA has been collected for each of these conditions and whole transcript Affymetrix arrays will be performed in collaboration with the David H. Murdock Research Institute. This data will be correlated with proteomic data in order to further ascertain the affect of ECM on the process of stem cell mediated osteogenesis. Findings from this project can be further translated into an *in vivo* setting. Osteogenic ECM components discovered from this process can be immobilized onto scaffold material, or be used to create novel bone scaffolds, in order to create more biologically relevant bone substitute materials which address limitations of synthetic substitute materials.

Development of Intelligent Bioscaffolds for Bone Tissue Engineering

Numerous materials have been proposed for bone tissue regeneration. However, none has been shown to be entirely satisfactory. In this study we fabricated a hybrid composite scaffold composed of poly(D,L-lactide-*co*-glycolide) (PLGA) and a naturally derived collagen matrix derived from porcine bladder submucosa matrix (BSM), and evaluated the biological activities and physical properties of the scaffold for use in bone tissue regeneration. Naturally derived collagen-based matrix derived from the bladder has been used as scaffolds for multiple applications, including bladder and urethral tissue reconstruction. BSM has been shown to be biocompatible and promote cell adhesion and proliferation. Therefore, BSM was selected for its biocompatibility, hydrophilic nature and ability to induce cell proliferation. BSM consists mainly of type I and type III collagen, elastin fibers and various proteins such as fibronectin and vitronectin that contain the arginine-glycine-aspartate (RGD) peptide binding motif. The presence of these extracellular proteins may enhance cell adhesion, survival and proliferation. However, BSM alone is not suitable for bone graft applications due to its small pore size, poor interconnectivity and inability to maintain structural integrity. To overcome these limitations, we designed a natural-synthetic hybrid scaffold that would possess an interconnected network of pores and sufficient mechanical and physicochemical properties that would maintain structural integrity, thus preventing collapse during the handling and implantation process. The microarchitecture of the composite scaffolds did not differ from the control PLGA scaffold, indicating that the incorporation of BSM did not alter structural changes during the fabrication process. Further, the composite scaffolds provided desirable surface properties necessary for cell attachment and proliferation.

In this study we fabricated composite bone scaffolds using PLGA and BSM. The composite scaffolds were configured to accommodate cells and designed to provide adequate structural support. We evaluated the biological activity, physical and structural properties of the scaffolds for their use in bone tissue regeneration using two different cell types. Porcine bladders, obtained from donor animals, were decellularized using a multiple step process. Briefly, the bladders were rinsed thoroughly with phosphate-buffered saline (PBS). The bladder submucosa was microdissected and isolated from the muscular and serosal layers. Under continuous agitation, the bladder submucosa was rinsed with deionized water for 24 hr and placed in a solution containing 0.2% Triton X-100 and 0.03% ammonium hydroxide for 14 days to remove all cellular components. The bladder submucosa was washed with deionized water containing 10% cefazolin (APOTHECON®, G. C. Hanford Mfg. Co., Syracuse, NY, USA) for an additional 24 hr. The BSM was freeze-dried at -50°C for 48 hr using a lyophilizer (FreeZone® 12, Model 775410, LABCONCO, Kansas City, MO, USA), followed by pulverization in a freezer mill (SPEX 6700, Mutchen, USA) at -198°C (**Fig 15**).



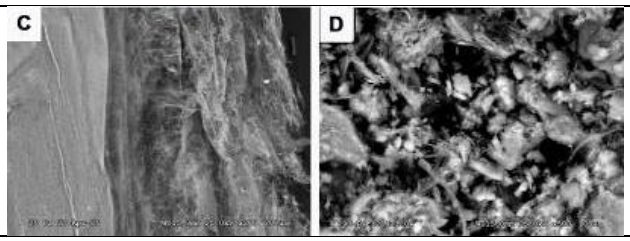


Figure 15. Decelullarization process of porcine bladders: Histological evaluations of (A) native and (B) decellularized bladder submucosa matrix (BSM). SEM images of (C) decellularized BSM and (D) pulverized BSM.

The BSM-PLGA composite scaffolds, fabricated by the solvent casting/particulate leaching process, exhibited highly porous and uniform interconnected structures. BSM particles with a size ranging from 6~32 μm were homogeneously embedded in the composite scaffolds as demonstrated by SEM (**Fig 16**). There were distinct morphological differences between the BSM-PLGA composite scaffold and Collagraft®, while the BSM-PLGA composite scaffold and PLGA scaffold were similar in microstructure (**Fig 16**), pore size and porosity. The exterior surface, serial cross-section and side wall morphologies of the BSM-PLGA composite scaffolds and PLGA scaffolds exhibited a highly porous structure with interconnectivity that would support adequate cell seeding, adhesion and proliferation. The average pore size of the BSM-PLGA composite scaffolds was 121.84 ± 23.44 μm. The average pore size and porosity of the BSM-PLGA composite scaffolds were significantly different compared to Collagraft® ($P < 0.05$). The BSM-PLGA composite scaffold exhibited uniformly distributed-interconnecting pores in its inner microstructures which were successfully achieved by a particulate leaching process.

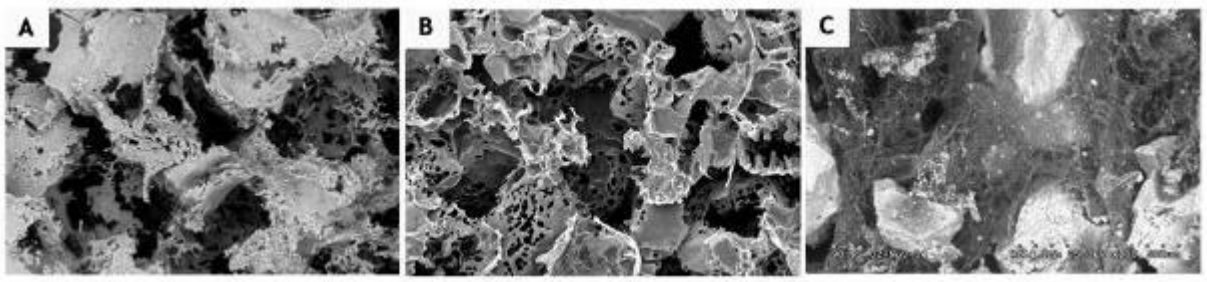


Figure 16. SEM images of cross-sectional (A) BSM-PLGA composite scaffold (B) PLGA scaffold and (C) Collagraft®. The BSM-PLGA composite scaffold and PLGA scaffold were similar in microstructure. Collagraft® had separated hydroxyapatite particles which did not embed in the scaffold (C). Original magnification: ×100.

The fluid uptake ability of the BSM-PLGA composite scaffolds was superior when compared with PLGA scaffold and Collagraft® ($P < 0.01$) (**Figure 17A**). The fluid uptake ability of BSM-PLGA composite scaffolds increased with the elevation of the BSM to PLGA ratio due to the hydrophilic characteristics of the BSM).

Cytotoxicity assay using Neutral Red indicated that the BSM-PLGA composite scaffold, PLGA scaffold and Collagraft® did not demonstrate significant differences in cell viability, when compared to cells grown on tissue culture plates as a negative control (**Figure 17B**). Meanwhile, the latex used as a positive control, showed a cell viability of 7.3% of dissociated cells from *h*EBs and 10.5% of *b*OBs when compared to the negative control, indicating a high cytotoxicity. The mitochondrial metabolic activity of the BSM-PLGA composite scaffold, PLGA scaffold and

Collagraft® was also determined by the MTT assay using the direct contact method (**Figure 17C**). Direct contact with latex significantly decreased mitochondrial activity by 92.1% in *h*EBs cultures and 96% in *b*OBs cultures after 7 days.

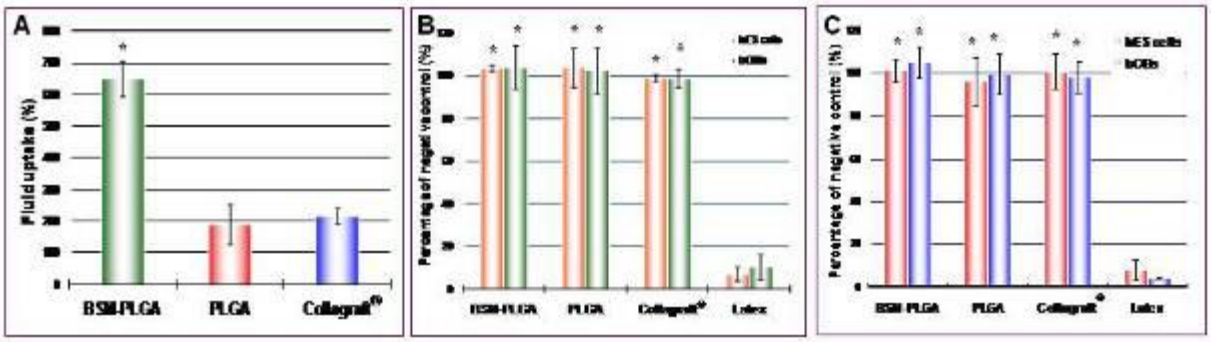


Figure 17. (A) Fluid uptake ability of the BSM-PLGA composite scaffold *P<0.01 compared to PLGA and Collagraft®. Fluid uptake (%) was calculated from the weight difference between the wet and dry condition of the samples. (B) The viability and (C) mitochondrial metabolic activity of *h*ES cells and *b*OBs cultured in direct contact with bone scaffolds at 7 days, as determined by Neutral Red assay and MTT assay, respectively. *P < 0.01

Histological evaluations of the BSM-PLGA composite and PLGA scaffolds at 1 day after seeding with *h*ES are shown in Figure 18 (Left). In the BSM-PLGA composite scaffold, the seeded cells were uniformly distributed and adhered to the walls of the pores (Figure 18 (Left) A,B). However, there were only a few cells found in the pores of the PLGA scaffolds (Figure 18 (Left) C,D). At 8 days of culture, both cell types proliferated on the BSM-PLGA composite scaffold, indicating that the scaffold was conducive to cell adhesion and proliferation. A uniform cell distribution was observed throughout the entire BSM-PLGA scaffold. MTT staining of the BSM-PLGA composite scaffold showed the uniform penetration of formazan crystals on the surface and vertical cross-sections, indicating adequate cell adhesion and distribution throughout the inner structure (Figure 18 (Right) A-D). Cell adhesion on the BSM-PLGA composite scaffold was significantly higher than on Collagraft® (Figure 18 (Right) E-H).

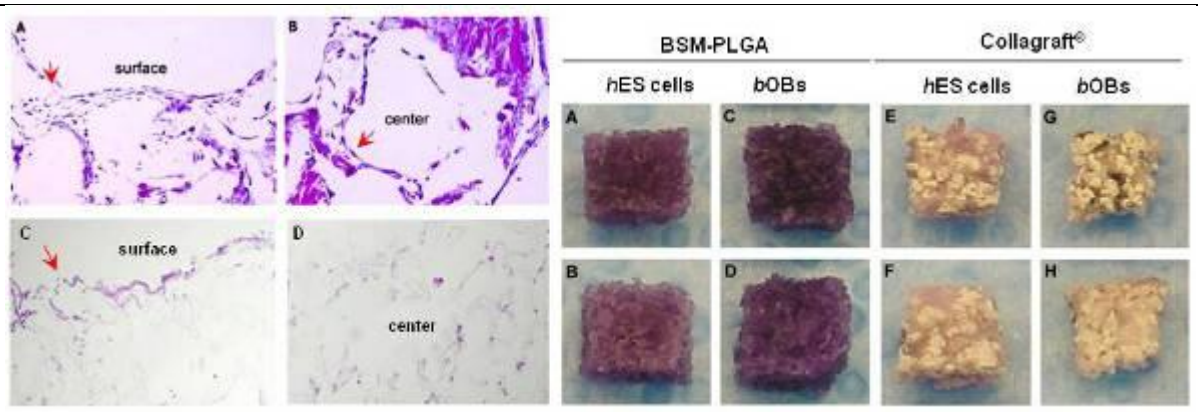


Figure 18. (Left) Histological evaluation of *h*ES cells seeded (A, B) BSM-PLGA composite scaffold and (C, D) PLGA scaffold at 1 day culture as stained by H&E staining. (Right) Gross observation of cells attached bone scaffolds at 1 day culture as stained by MTT. (A, C, E, G) Top and (B, D, F, H) bottom of the scaffolds.

The percentage of the seeded *h*ES cells and *b*OBs adhered to the BSM-PLGA composite scaffold was 87.70 ± 5.46% and 82.23 ± 8.77% of the initial cell number, respectively. The levels of cell adhesion showed a significant difference ($P < 0.01$) between the BSM-PLGA composite scaffold and Collagraft®(**Figure 19A**). Both the *h*ES cells and *b*OBs were cultured on the BSM-PLGA composite scaffold, PLGA scaffold and Collagraft® for up to 8 days. The rate of the cell proliferation on the BSM-PLGA composite scaffold was significantly higher than that of the PLGA scaffold and Collagraft® ($P < 0.05$) (**Figure 19**), and did not show significant differences between the cell types at different culture time points.

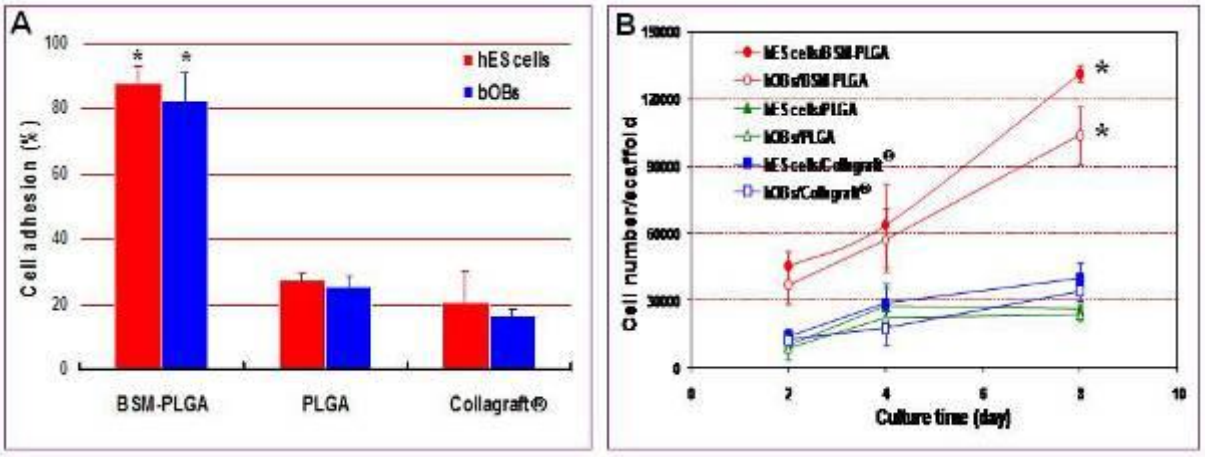


Figure 19. (A) Cell adhesion (%) of the BSM-PLGA scaffold at 1 day culture as determined by MTT assay. (B) Cell Proliferation of the BSM-PLGA scaffold at 2, 4 and 8 days culture as determined by MTT assay. *$P < 0.01$ compared to PLGA scaffold and Collagraft®.

We successfully fabricated a bio-hybrid bone scaffold composed of BSM and PLGA that possess necessary characteristics for bone tissue regeneration. The BSM-PLGA composite scaffolds possessed uniform porous structures with a consistent interconnectivity throughout the entire scaffold. The hydrophilicity of the BSM-PLGA composite scaffolds was significantly enhanced, when compared to the hydrophilicity of each material separately, resulting in uniform cell seeding and distribution. Introducing of the BSM to PLGA synthetic polymeric scaffold results in improved cell adhesion and proliferation compared to PLGA and Collagraft® due to containing of natural source, such as collagen, elastin and bioactive molecules. The BSM-PLGA composite scaffolds are non-toxic, easily fabricated and provide structural features, consisting of abundant pores that are homogenously distributed throughout the inner structure allowing cell adhesion and proliferation. The use of the composite scaffolding system with cells may enhance the formation of bone tissue for therapeutic regeneration.