Final Scientific/Technical Report

Project title: Regeneration of Tissues and Organs Using Autologous Cells

Award Number: DE-FG02-08ER64711

Reporting Period: September 15, 2008 – September 14, 2010

Recipient Organization: Wake Forest University School of Medicine

Project Location: Winston Salem, NC

Project Contact: Anthony Atala, M.D. (Principal Investigator)

Executive Summary:

The proposed work aims to address 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*. Toward this goal, we have engaged a team of scientists with expertise in cell and molecular biology, physiology, biomaterials, controlled release, nanomaterials, tissue engineering, bioengineering, and clinical medicine to address all three challenges. This combination of resources, combined with the vast infrastructure of the WFIRM, have 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 ongoing programs are aimed at developing regenerative medicine technologies that employ a patient's own cells to help restore or replace tissue and organ function. This DOE program has provided a means 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 facilitated 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 has opened new opportunities to treat conditions where other approaches have failed.

Project Summary:

Introduction

The shortage of transplantable organs and tissues has become 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.

Project 1: Cell Sources

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

Autologous Urine-Derived Stem Cells Expressing Vascular Endothelial Growth Factor for Potential Use in Reconstruction
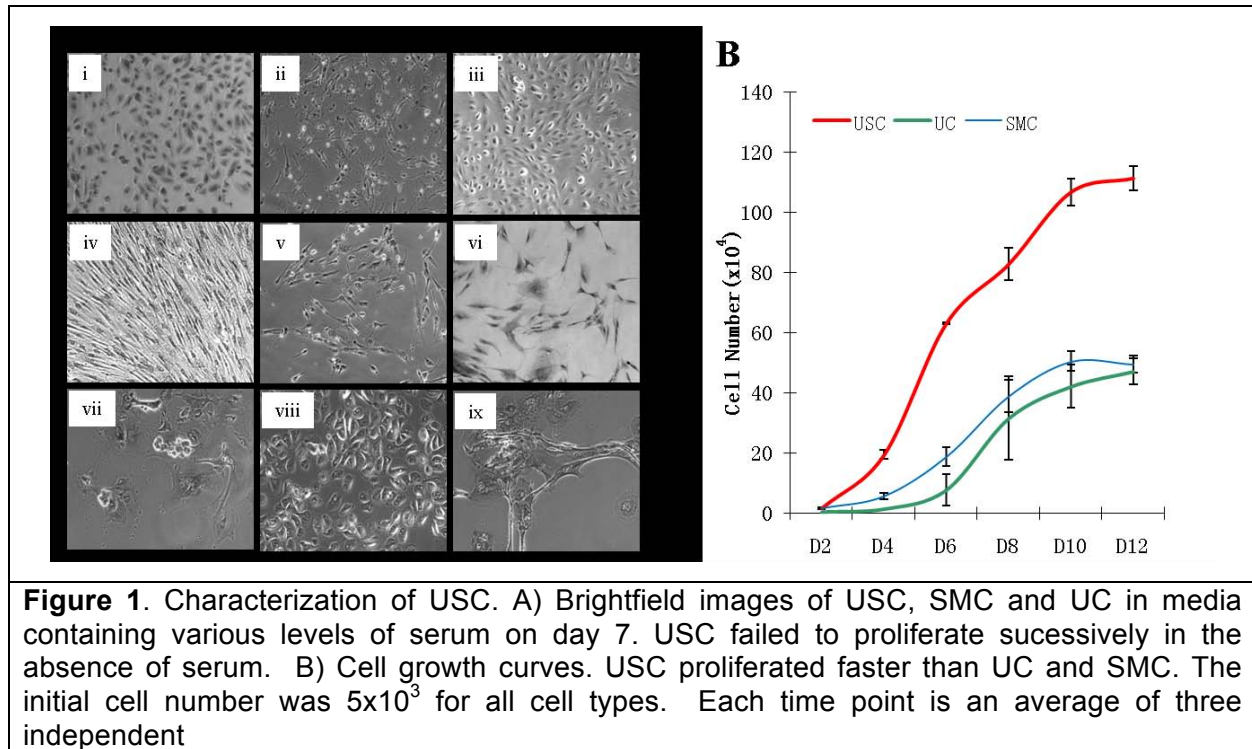
We have developed a stem cell source derived from urine. The goal of this study was to evaluate effects of vascular endothelial growth factor (VEGF) over-expression on urine-derived stem cell (USC) survival and myogenic differentiation, and to determine whether these cells can be used as a novel cell source for genitourinary reconstruction.

USC were isolated from 31 urine samples (6 healthy individuals; ages 3-27 years). USC were infected with an adenoviral vector containing the mouse VEGF gene (USC/Ad-VEGF). USC/Ad-VEGF was mixed with human umbilical vein endothelial cells (HUVEC) (total, $5x10^6$ cells) in a collagen-I gel. These cell-containing gels were subcutaneously implanted along with six other controls into 18 athymic mice. The grafts were assessed up to 28 days post-injection for gross appearance and immunocytochemistry.

VEGF levels in the media from infected USC cultures reached a peak value on day 10 after infection. Grafts composed of USC/AdVEGF and HUVEC were larger and better vascularized compared to uninfected USC control grafts. Additionally, more implanted cells expressed human nuclear markers in the VEGF-expressing grafts. VEGF-expressing grafts also contained more cells expressing the endothelial markers CD 31 and von Willebrand factor (vWF) and smooth muscle markers (α-smooth muscle actin, desmin and myosin). More nerve fibers were present in USC/AdVEGF plus HUVEC grafts than in controls.

Optimization of Culture Medium for USC

USC, smooth muscle cells (SMC), and urothelial cells (UC) were cultured in three different culture media to optimize conditions for USC growth. USC proliferated robustly in KSFM and EFM (1:1) (**Fig. 1A iii** and **Fig. 1B**) containing 5% FBS, maintaining their expansion potential for several passages (p10-p14). However, USC failed to grow in KSFM containing low levels of calcium/ magnesium (UC-specific medium) (**Fig. 1A ii**). These cells differentiated and lost their expansion potential (**Fig. 1A i**) when cultured in high-glucose DMEM containing 10% FBS (SMC-specific medium). Similarly, UC and SMC only grew well in their favorite media (**Fig 1A iv, viii** and **Fig. 1B**) but not in others (**Fig 1A v, vi, vii and ix**). The growth curves for USC, SMC and UC are shown in **Fig. 1B**. Compared to SMC and UC, USC proliferation was at least two fold higher from day 4 onwards. USC have a distinct growth pattern *in vitro* significantly different from SMC and UC for up to 12 days of culture (P<0.01).

Figure 1. Characterization of USC. A) Brightfield images of USC, SMC and UC in media containing various levels of serum on day 7. USC failed to proliferate sucessively in the absence of serum. B) Cell growth curves. USC proliferated faster than UC and SMC. The initial cell number was 5×10^3 for all cell types. Each time point is an average of three independent
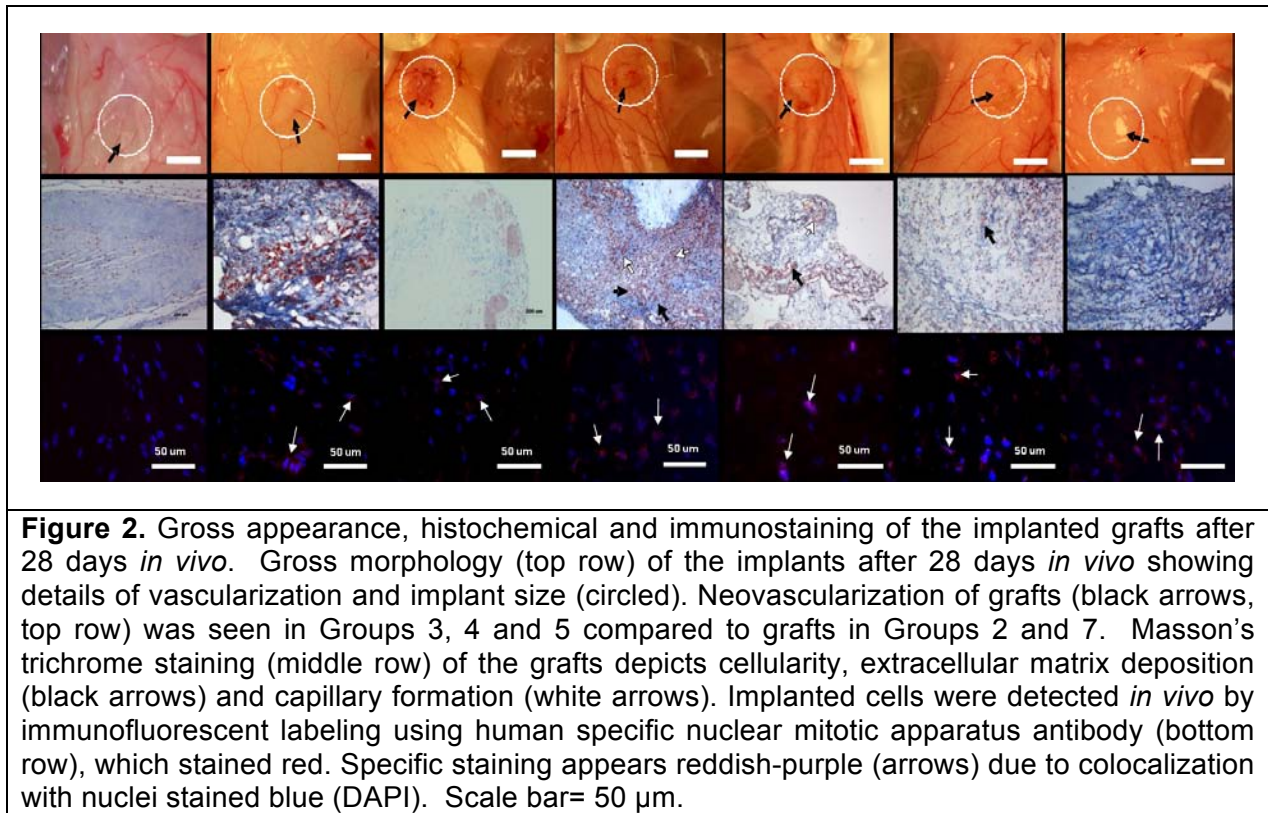
VEGF Release Kinetics in Culture Medium

USC infected with AdVEGF showed maximum infection efficiency at an MOI of 250 with transduction efficiency around 90-95%. Increasing the MOI above 250 led to cellular toxicity. VEGF secretion peaked on day 10 post-infection, after which it rapidly declined and returned to pre-induction levels by day 28. This reflects the acute and transient nature of adenoviral infections, which causes the transfected gene to be lost with successive cell division.

Gross Appearance and Analysis of Implanted Grafts

Graft volume size were larger in most groups on day 14 after implantation, particularly in VEGF transfection groups, and decreased as tissue edema lessened by day 28. In animals receiving cell-free collagen gel injections (Group 1), graft size was significantly reduced by day 14, and at day 28, cell-free grafts were barely visible. However, in all animals receiving cell-containing injections, a larger tissue mass was observed at day 28 after implantation compared to the cell-free implant. Importantly, grafts consisting of USC expressing VEGF with HUVEC (Group 4) and without HUVEC (Group 5) were larger than those consisting of uninfected USC alone (Group 3) or uninfected USC with the VEGF-containing gel (Group 6 and Group 7) (**Fig. 2**). However, graft sizes were similar among those with VEGF-expressing USC and HUVEC (Group 4) and those without HUVEC (Group 5). Similarly, graft sizes were similar among groups implanted with USC that did not express VEGF (G3, G6 and G7), even if VEGF was provided by the collagen matrix. Notable angiogenesis was observed in implants from Groups 4 and 5 compared to other groups on days 14 and 28. Little difference in capillary formation between gels containing VEGF (G6 and G7) and gels that did not release VEGF (G2) was seen (**Fig. 2**).

6

Figure 2. Gross appearance, histochemical and immunostaining of the implanted grafts after 28 days *in vivo*. Gross morphology (top row) of the implants after 28 days *in vivo* showing details of vascularization and implant size (circled). Neovascularization of grafts (black arrows, top row) was seen in Groups 3, 4 and 5 compared to grafts in Groups 2 and 7. Masson's trichrome staining (middle row) of the grafts depicts cellularity, extracellular matrix deposition (black arrows) and capillary formation (white arrows). Implanted cells were detected *in vivo* by immunofluorescent labeling using human specific nuclear mitotic apparatus antibody (bottom row), which stained red. Specific staining appears reddish-purple (arrows) due to colocalization with nuclei stained blue (DAPI). Scale bar= 50 µm.

Histology and Immunocytochemistry

The total cell density increased between days 14 and 28 in each group receiving a cell-containing implant. Furthermore, total numbers of cells (stained red in **Fig. 2**, middle row) within grafts and the cells expressing human nuclear-specific antibody significantly increased in implants from Groups 4 and 5 (USC/AdVEGF groups) compared to other groups on day 28 (**Fig. 2**, bottom row). A higher density of micro-vessels (**Fig. 2** middle, white arrow) appeared within the USC/Ad-VEGF (Group 4) grafts compared to the other groups 28 days after subcutaneous implantation. More cells expressed smooth muscle markers (α-smooth muscle actin, desmin, and myosin, **Fig. 3**) and endothelial cell markers (vWF and CD31, **Fig. 4**), which increased in the groups with VEGF (Groups 4, 5, and 6). Similarly, more cells expressed SMC (**Fig. 3**) and HUVEC markers (Fig. 4) in groups with implanted HUVEC (Groups 3, 4 and 6) compared to the groups without HUVEC implantation (Groups 2, 5 and 7). Interestingly, more nerve fibers (**Fig. 5**) were identified using antibodies to the peripheral nerve cell markers NF, S-100 and GFAP both 14 and 28 days after implantation in Group 4 (USC/AdVEGF plus endothelial cells) compared to other groups. Moreover, in this group, more new nerve fibers were seen at 28 days than at 14 days after implantation. However, they did not display any human nuclear staining, indicating they were derived from host tissue.
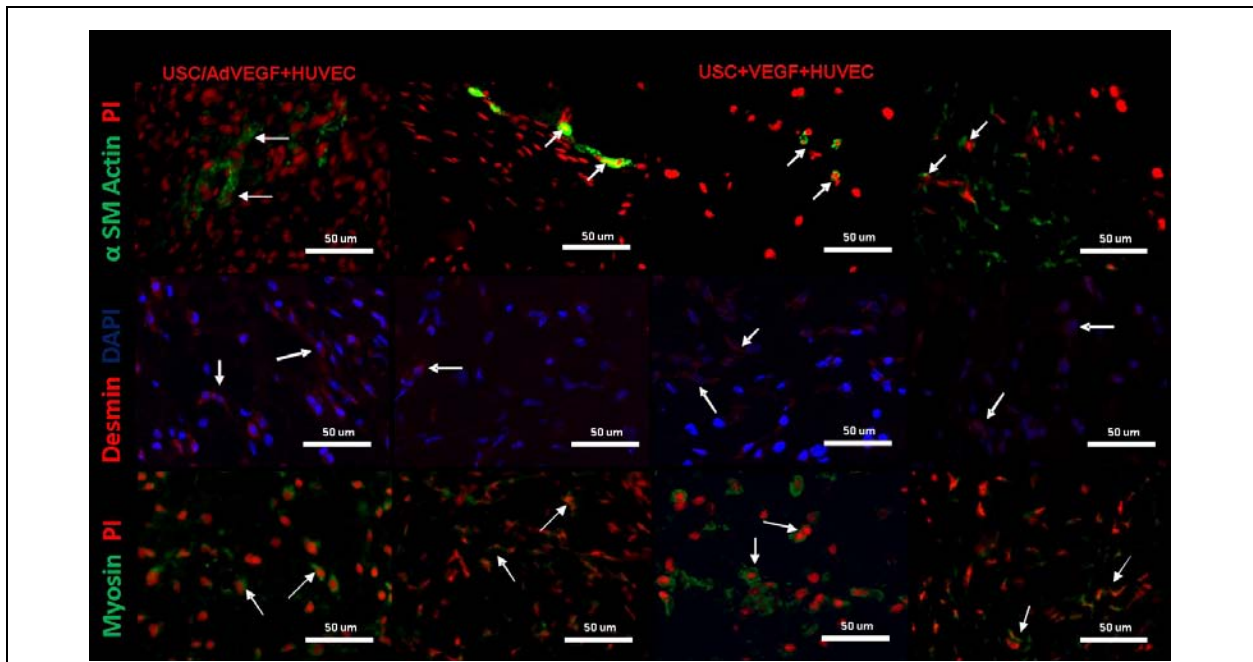
Figure 3. Immunofluorescent staining of implanted cell-collagen gel with myogenic-specific markers *in vivo*. Implants of different treatment groups of USC (p4) were harvested after 28 days *in vivo* and subjected to immunofluorescent staining using myogenic markers (ASMA-top row, desmin- middle row and myosin- bottom row). Specific staining (depicted by white arrows) appear green (ASMA and myosin) or red (desmin). The nuclei were counterstained with PI (ASMA and myosin) or DAPI (desmin). Scale bar= 50 µm.
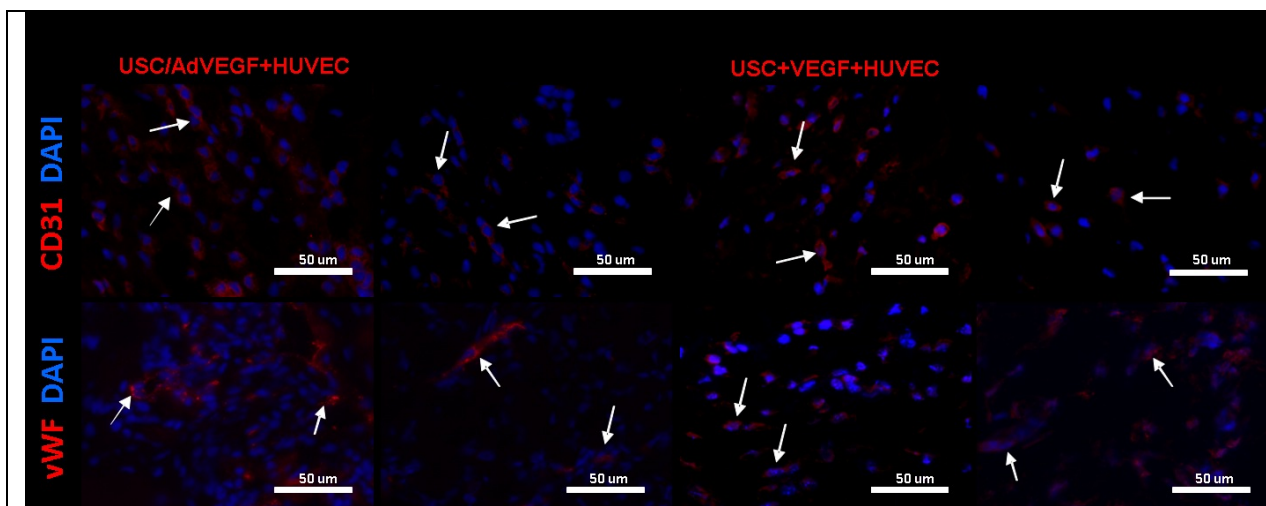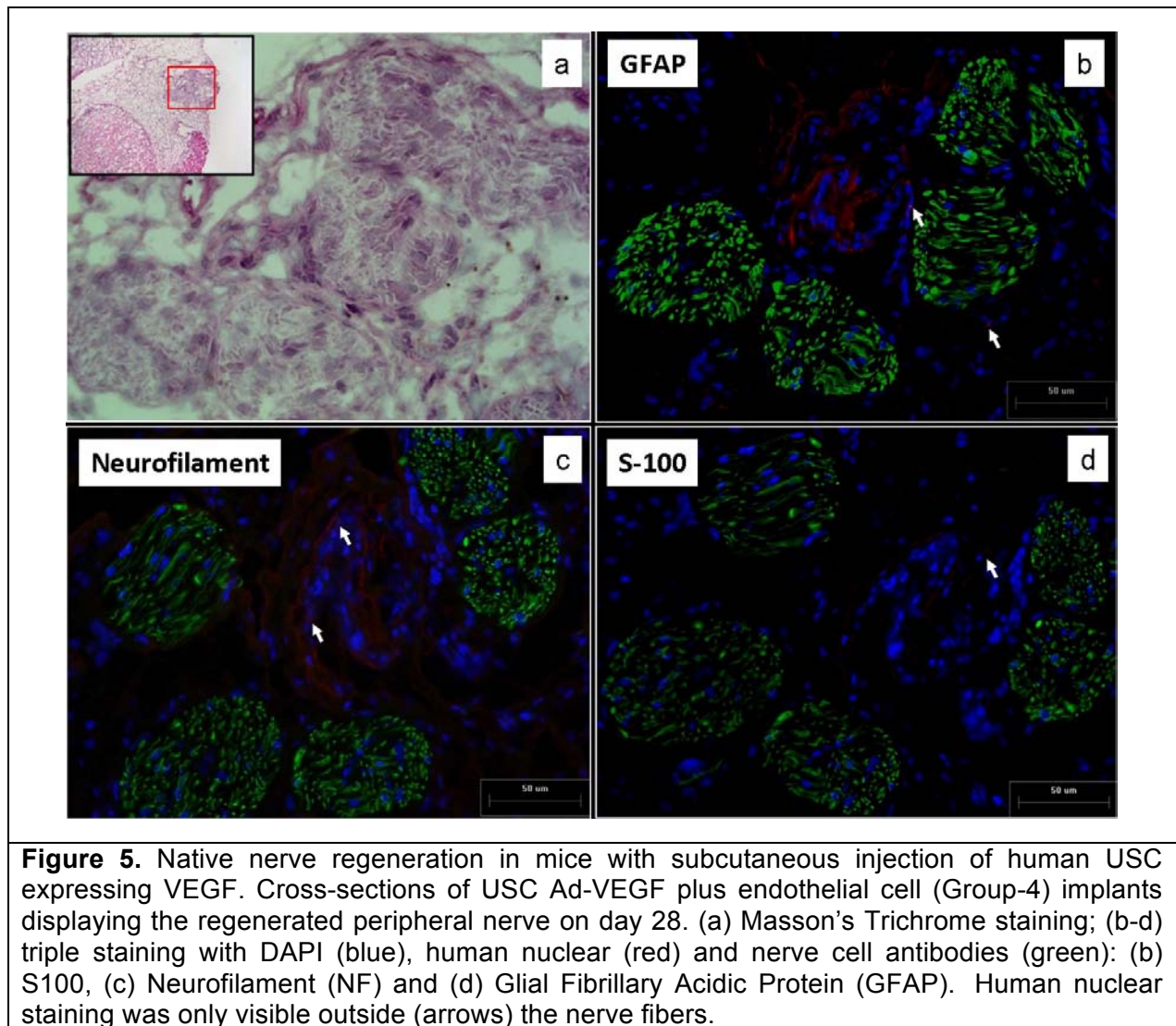

Figure 4. Immunofluorescent staining for endothelial-specific markers *in vivo*. Implants of the different USC (p4) groups were harvested after 28 days *in vivo* and subjected to immunofluorescent staining using epithelial markers (CD31-top row and von Willebrand factor (vWF) - bottom row). Specific staining (shown by arrows) appears red with nuclear staining in blue (DAPI). Most cells stained positive at 28 days compared to 14 days. Scale bar= 50 µm.

Figure 5. Native nerve regeneration in mice with subcutaneous injection of human USC expressing VEGF. Cross-sections of USC Ad-VEGF plus endothelial cell (Group-4) implants displaying the regenerated peripheral nerve on day 28. (a) Masson's Trichrome staining; (b-d) triple staining with DAPI (blue), human nuclear (red) and nerve cell antibodies (green): (b) S100, (c) Neurofilament (NF) and (d) Glial Fibrillary Acidic Protein (GFAP). Human nuclear staining was only visible outside (arrows) the nerve fibers.

CONCLUSIONS:

VEGF expression by USC, along with concurrent endothelial cell implantation, promoted angiogenesis, significantly improved *in vivo* cell survival and myogenic differentiation of USC, and enhanced nerve regeneration within the graft, which maintained graft size. The safety of using cells that over-express VEGF remains a concern, because VEGF is associated with urothelial cancer. The optimal dose of VEGF and long-term follow-up after implantation of cells expressing VEGF needs further investigation. Using autologous VEGF-expressing USC combined with HUVEC as an alternative cell source for urological cell therapy appears feasible and may be useful in genitourinary reconstruction, such as treating VUR, SUI with cell therapy or even in repairing urethral stricture and neuropathic bladder with tissue engineering technology.

Project 2: Providing Vascular Support

Neovascularization of engineered or regenerated tissues is essential for successful translation of regenerative medicine technologies to clinical practice. Blood supply is one of the major obstacles for the reconstruction of large tissues, applicable for clinical use. 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 is focused on the optimal combination of these components, *in vitro*, in order to facilitate their use *in vivo*. 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. We were further able to isolate and grow vascular cells from peripheral blood and placenta and tested their angiogenic potential. The early results show that the cells, in combination with an angiogenic growth factor, VEGF, are able to support neovascularization. The focus of the experiments in the past year was to combine muscle cells, endothelial cells and pericytes in order to create a vascularized tissue *in vitro*.

1. Pericyte progenitors stabilize in vitro capillary formation.

Our in vivo results indicated that transplanted EC can participate in the neovascularization process. However, the exact conditions for the combinations of cells and angiogenic growth factors are not well defined. We developed an in vitro capillary formation assay in order to optimize these conditions. We first tested if and how the presence of pericyte progenitors affects capillary formation. We labeled HUVEC and 10T½ mouse embryonic pericyte progenitors with 2 different fluorescent dyes and performed a series of co-culture experiments. Although HUVEC formed capillaries in the absence of 10T½ cells, these disappeared after about a week. In contrast, the 10T½ stabilized the capillaries that were evident for at least 21 days (**Fig. 6**). Fluorescence microscopy revealed several large capillaries that have both cell types integrated in.

We next tested if such capillaries can be formed when HUVEC and 10T½ cells are co-cultured together with mouse myoblatst (**Fig. 7**). Several capillaries, stained red, were observed within the muscle fibers. Although the 10T½ cells could not be observed under the fluorescent microscope, we were able to detect non-EC cells associated with the HUVEC capillaries. Taken together, these results indicate the essential role of pericyte progenitors in stable capillary formation within a regenerated muscle tissue.
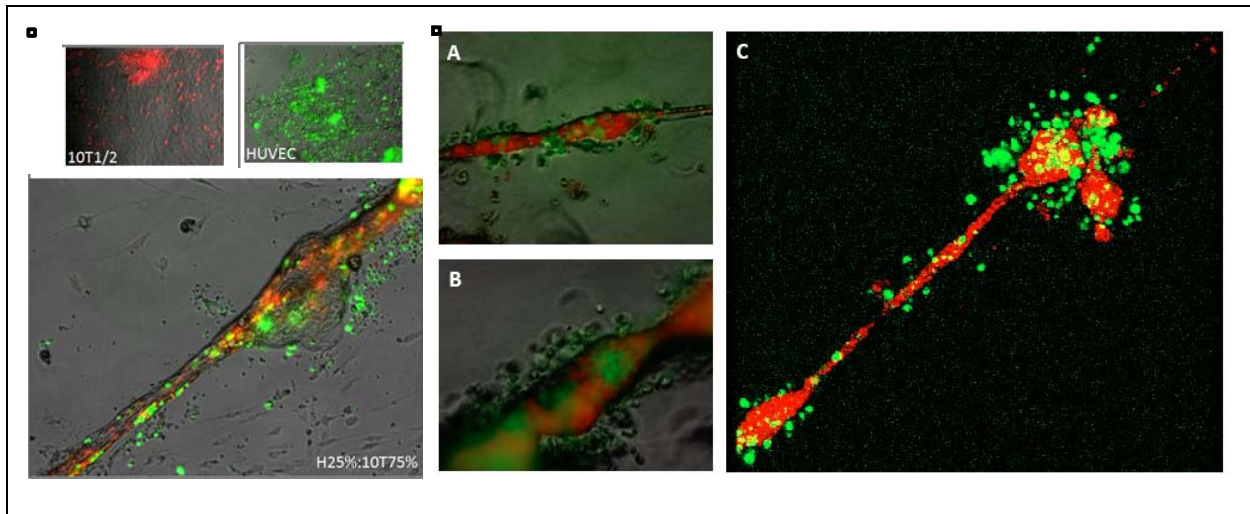
Figure 6: In vitro capillary formation of EC co-cultured with pericyte progenitors. Left panels. HUVEC and 10T½ co-culture. HUVEC were labeled green with Vybrant® CFDA SE Cell Tracer and 10T½ mouse embryonic pericyte progenitors were labeled red with red fluorescent CM-Dil. The labeled cells were mixed with 1:3 matrigel:collagen suspension and plated in culture dishes in DMEM + 10% FBS. Capillaries containing EC and pericytes were observed in cultures of HUVEC alone and in co-cultures. In HUVEC alone, capillaries were dissolves after about a week whereas in co-cultures, capillaries remained stable for at least 3 weeks. **Right Panels.** Pericytes (green) grow along side and surround human umbilical vein endothelial cells (HUVECs -red) when grown together in matrigel. CD146 high expressing adipose-derived mesenchymalstem cells were used as pericytes. Pericytes were labeled with VybrantCFDA SE (Invitrogen) and HUVECs were labeled with VybrantCM-Dil(Invitrogen). Cells were grown in matrigelfor 5 days before being imaged with a ZeissAxiophot inverted fluorescent microscope at either 10X magnification (A) or 20X magnification (B). In (C), a Z-stack (10X) was obtained with a ZeissLSM 510 confocalmicroscope and a 3-dimensional image was constructed using the LSM Image Browser software.

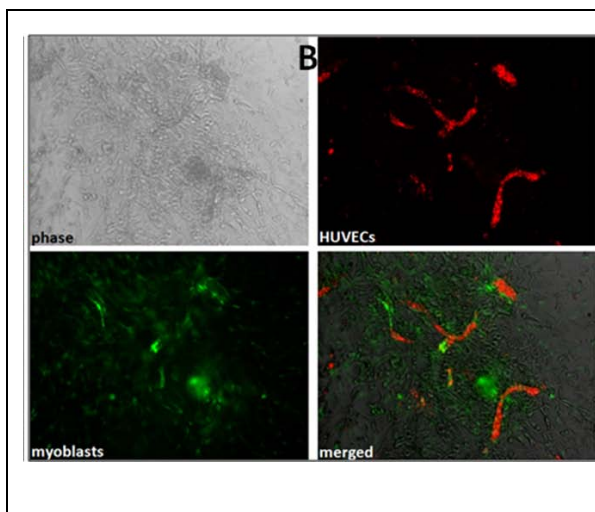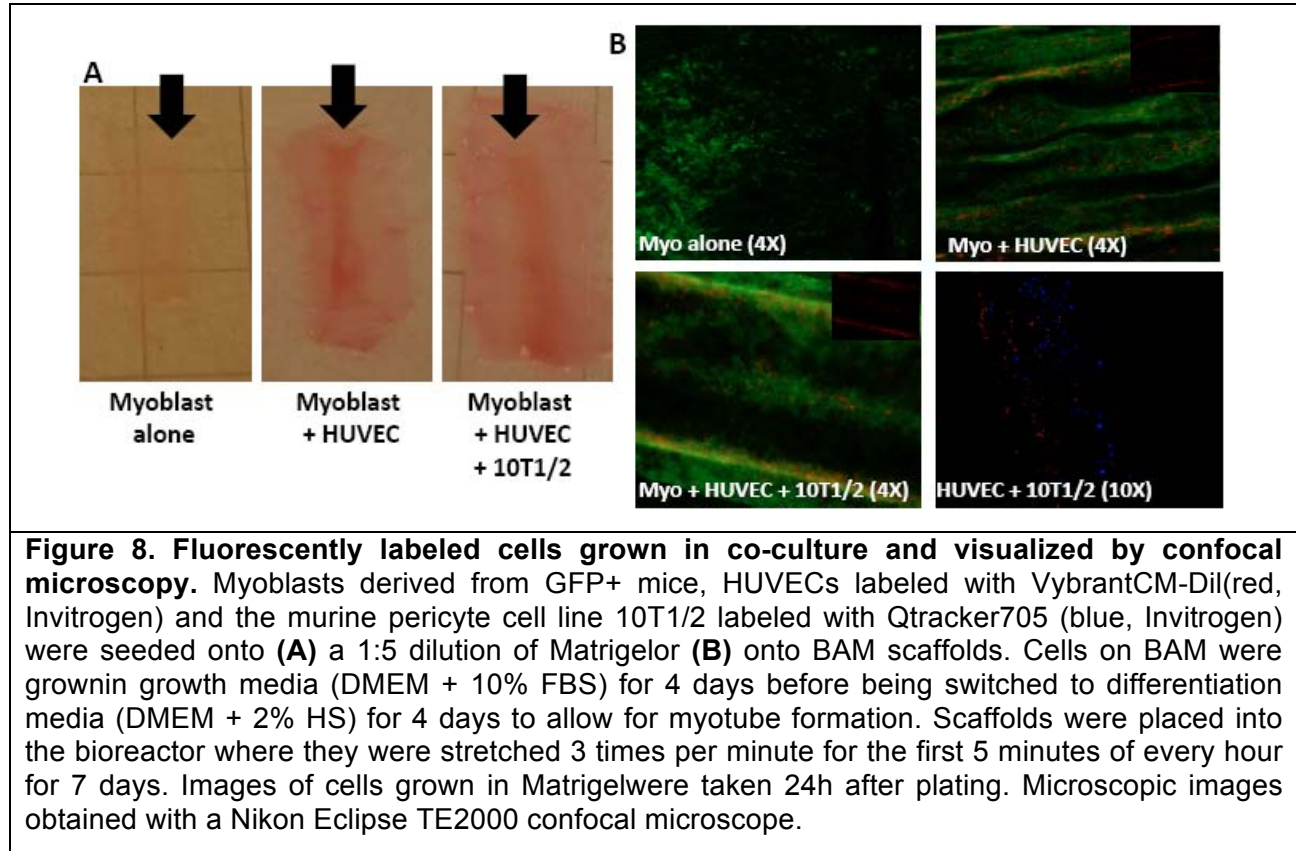**Figure 7: Co-culture of EC, pericytes and myoblasts.** HUVEC, mouse myoblasts and 10T½ co-culture. HUVEC were labeled green wih red fluorescent CM-Dil and mouse myoblasts were labeled green with Vybrant® CFDA SE Cell Tracer. 10T½ cells were unlabeled. Co-culture of the 3 cell types show capillaries embedded within the muscle fibers.

11

2. Incorporation of endothelial cells and pericytes into bioengineered muscles.

We next combined myoblasts endothelial cells (HUVEC) and pericytes and grew them on a collagen scaffold. We could see alignment of the EC and the myoblasts and association between perycites and EC. Interestingly, scaffolds that had been seeded with a combination of the 3 cell types had greater tissue volume than the scaffold with myoblasts alone (**Fig. 8**).

Figure 8. Fluorescently labeled cells grown in co-culture and visualized by confocal microscopy. Myoblasts derived from GFP+ mice, HUVECs labeled with VybrantCM-Dil(red, Invitrogen) and the murine pericyte cell line 10T1/2 labeled with Qtracker705 (blue, Invitrogen) were seeded onto **(A)** a 1:5 dilution of Matrigelor **(B)** onto BAM scaffolds. Cells on BAM were grownin growth media (DMEM + 10% FBS) for 4 days before being switched to differentiation media (DMEM + 2% HS) for 4 days to allow for myotube formation. Scaffolds were placed into the bioreactor where they were stretched 3 times per minute for the first 5 minutes of every hour for 7 days. Images of cells grown in Matrigelwere taken 24h after plating. Microscopic images obtained with a Nikon Eclipse TE2000 confocal microscope.
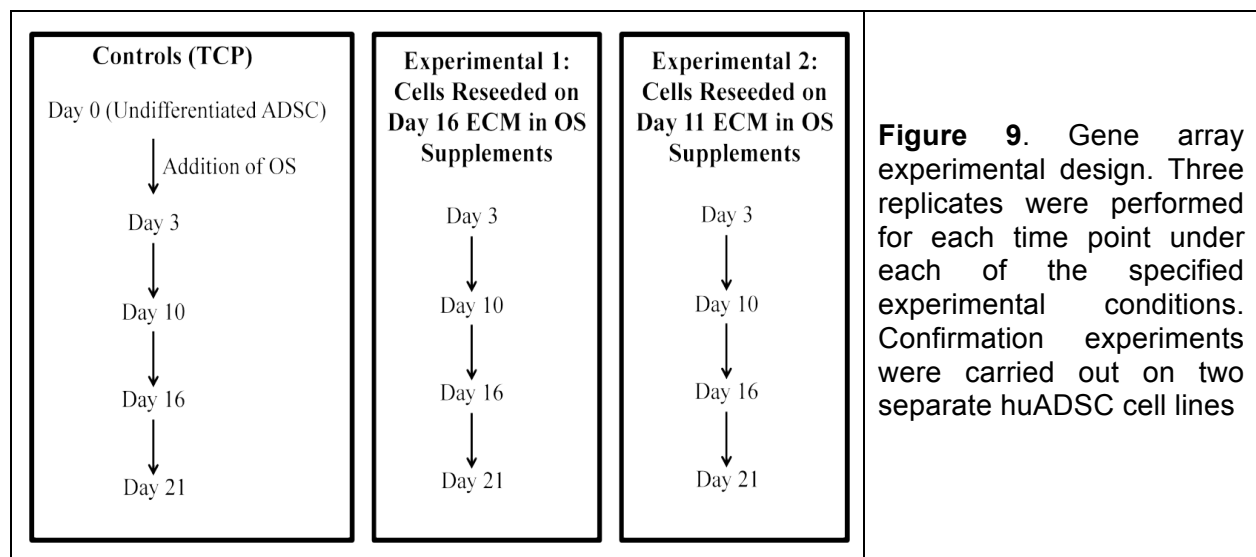
The results of our experiments in the last year are in line with achieving the goals of our research. We used a combination of vascular cells, EC and pericytes, together with muscle progenitor cells, myoblasts. This approach resulted in the formation of vascularized muscle tissue in vitro. We performed fluorescence imaging to confirm these findings. In the future we will use this approach to enhance neovascularization in our engineered tissues by co-culturing EC 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**

Development of extracellular matrix for bone tissue regeneration

Mesenchymal stem cells (MSC) are an important component of many tissue engineering and regenerative medicine (TE/RM) strategies. Their multipotential characteristics make them extremely useful as a source of cells for TE/RM applications. Adipose-derived stem cells (ADSC) represent one type of MSC and have many characteristics that make them a clinically relevant source of MSC. Specifically, in the field of bone tissue engineering (BTE), ADSC are commonly used for *in vitro* osteogenesis studies. Although these cells display similar characteristics as bone marrow-derived stem cells (BMSC), the mechanisms and pathways that these cells utilize to differentiate down the osteogenic pathway are still unclear. Specific differences exist between BMSC and ADSC osteogenesis. However, more detailed analysis of how the two MSC types differ will be important for enhanced clinical relevance of ADSC. The goal of this work was to identify important genes in ADSC osteogenesis, investigate those genes related to building the cell niche of ADSC undergoing osteogenesis, and to determine if secreted extracellular matrix (ECM) proteins could strongly influence cell fate.
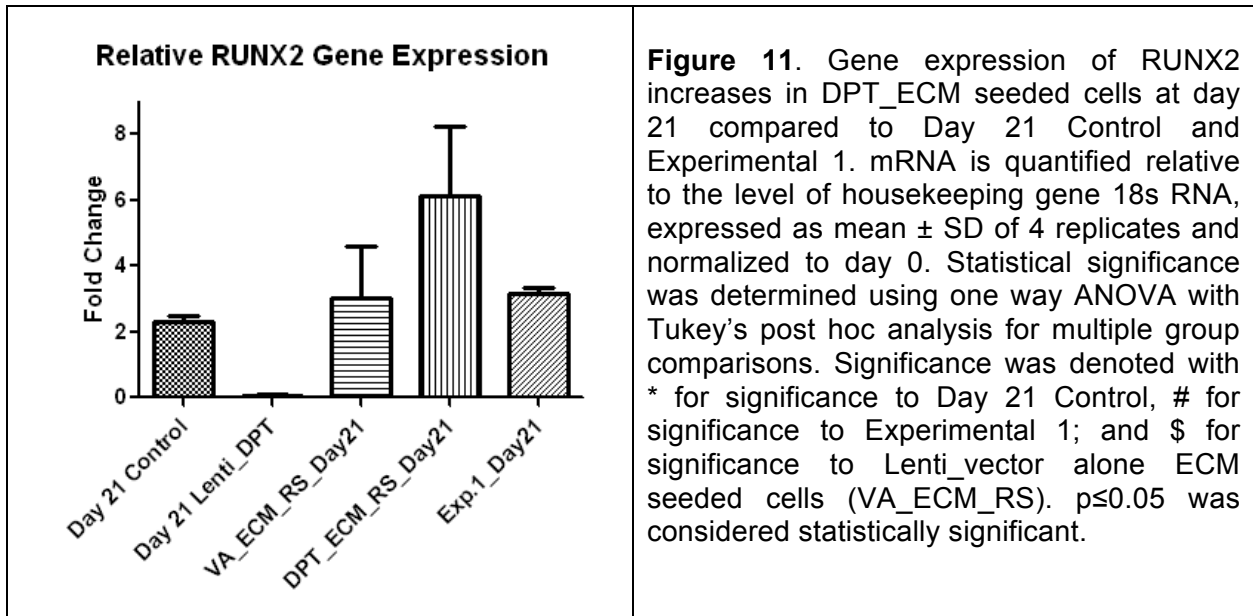
METHODS: Genes of interest were identified by conducting a simple experiment in which human ADSC were differentiated to osteoblasts using commonly employed media supplements (dexamethasone, β -glycerol phosphate, ascorbic acid). At various time points during differentiation, cells were removed and the cell secreted ECM left behind on the culture dish. Quantitative PCR was conducted to determine which ECM had the greatest influence on differentiation as determined by high and/or early gene expression. Fresh ADSC were seeded and grown with osteogenic supplements on day 10 (low osteoinductive potential) and ay 16 (high osteoinductive potential) ECM, as well as uncoated tissue culture plastic (TCP), and full gene array was conducted to identify up-regulated genes. This experimental design is shown in **Fig. 9**.

Figure 9. Gene array experimental design. Three replicates were performed for each time point under each of the specified experimental conditions. Confirmation experiments were carried out on two separate huADSC cell lines
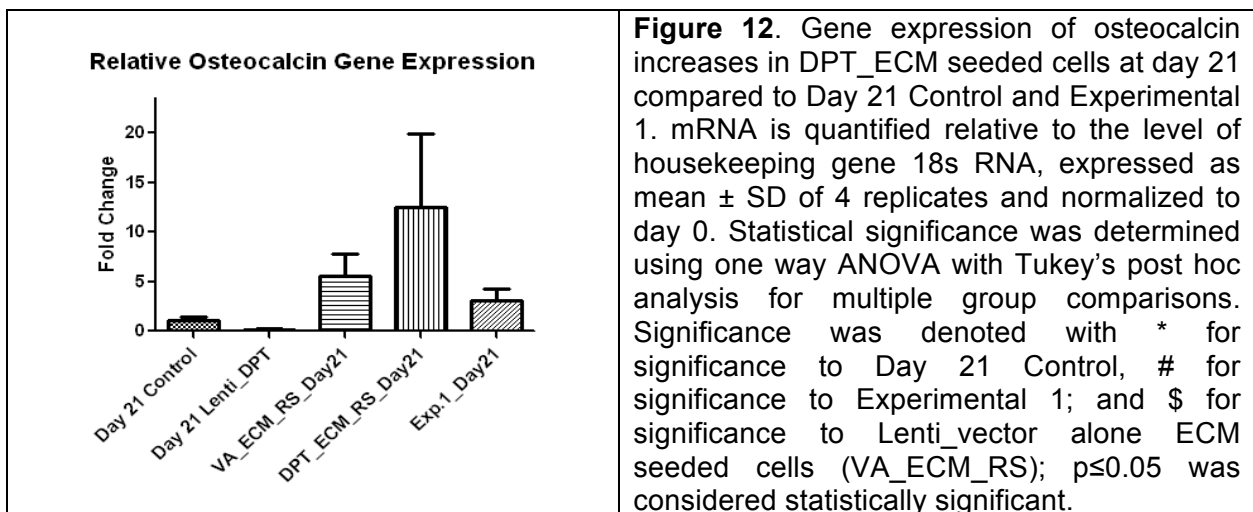
Array analysis was performed using an assortment of software and programs (**Fig. 10**). Raw data files (CEL files) were imported into Affymetrix Expression Console (AEC). AEC was used to generate CHP files. CHP files were normalized to median CHP intensity. Background correction, normalization, and probe summarization were performed using Robust Multichip Averaging RMA [96]. Data was analyzed for quality control (QC) (Figure 3). Normalized intensity values were mapped to gene summarization and annotation using AEC and uploaded into Excel based add-on, BRB array tools. Analyses were performed using BRB-ArrayTools. Differentially expressed genes were detected between groups and time points using *t*-test adjusted with *Benjamini and Hochburg* False Discovery Rate (FDR) method.

Figure 10. Gene array analysis workflow.
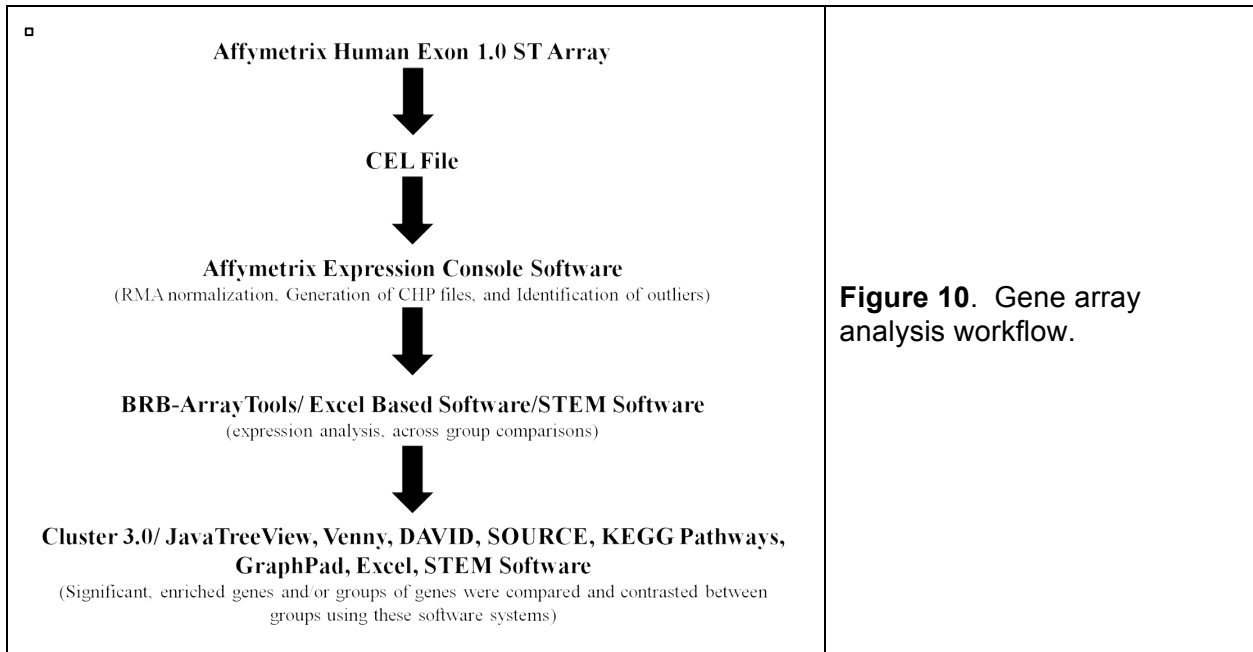
This analysis resulted in the identification of several genes that were significantly up-regulated as a result of the cells being exposed to the ECM microenvironment provided by the day 16 secreted matrix. We hypothesized that this overexpression resulted in secretion of the coded proteins, and that accumulation of these components in the ECM would be responsible for outside-in signaling mechanisms that would enhance osteogenesis of ADSC. To test this hypothesis, one such gene, dermatopontin (DPT), an ECM protein abundant in bone ECM that was expressed at significantly higher levels in day 16 compared to day 10 of osteogenesis, was selected as a proof-of-concept ECM component. Stable overexpression of DPT was achieved through lentiviral-mediated overexpression. ADSC overexpressing DPT were induced to differentiate on TCP and the cells removed at day 16. This ECM was seeded with normal ADSC (noted as DPT_ECM in **Figs. 11 and 12**) as well as ADSC overexpressing DPT (noted as Day 21 Lenti_DPT in Figures 3 and 4); cells cultured on TCP were used as a control. Osteogenic genes were assessed by q-PCR to determine if the presence of DPT-enhanced ECM would have an effect on osteogenesis.

Figure 11. Gene expression of RUNX2 increases in DPT_ECM seeded cells at day 21 compared to Day 21 Control and Experimental 1. mRNA is quantified relative to the level of housekeeping gene 18s RNA, expressed as mean ± SD of 4 replicates and normalized to day 0. Statistical significance was determined using one way ANOVA with Tukey's post hoc analysis for multiple group comparisons. Significance was denoted with * for significance to Day 21 Control, # for significance to Experimental 1; and $ for significance to Lenti_vector alone ECM seeded cells (VA_ECM_RS). p≤0.05 was considered statistically significant.

Expression of osteogenic related genes was upregulated in cells seeded onto DPT_ECM at day 21 compared to Controls and Experimental 1 cells (cells seeded onto day 16 ECM as described previously). Specifically, RUNX2 expression was significantly higher than both Control cells on TCP and Experimental 1 cells on day 16 ECM (**Fig. 11**). Osteocalcin gene expression was also significantly higher than both Controls at day 21 on TCP and Experimental 1 cells on day 16 ECM (**Fig. 12**). ECM was also isolated from ADSC infected with the Lenti_vector alone. ADSC were seeded onto this ECM and induced to differentiate as another control. Expression of osteogenic markers for cells on Lenti_vector alone ECM would be expected to show similar patterns to Experimental 1 cells. Assuming lentiviral infection did not result in insertion of viral components into genomic areas important in osteogenesis, this ECM should be essentially equal to ECM used in Experimental 1 cells. Expression of RUNX2 in these cells was similar to Experimental 1 expression. However, in the case of osteocalcin, expression was not statistically significant to DPT_ECM seeded cells.

Figure 12. Gene expression of osteocalcin increases in DPT_ECM seeded cells at day 21 compared to Day 21 Control and Experimental 1. mRNA is quantified relative to the level of housekeeping gene 18s RNA, expressed as mean ± SD of 4 replicates and normalized to day 0. Statistical significance was determined using one way ANOVA with Tukey's post hoc analysis for multiple group comparisons. Significance was denoted with * for significance to Day 21 Control, # for significance to Experimental 1; and $ for significance to Lenti_vector alone ECM seeded cells (VA_ECM_RS); p≤0.05 was considered statistically significant.

Osteogenic differentiation of MSC is an important concept for the field of BTE. The identification of biological factors which can influence MSC osteogenic lineage specification is important in developing a broader understanding of how complex microenvironments play a role in differentiation. In this work we demonstrate that ADSC osteogenesis is enhanced through interaction with ECM secreted from the midpoint in osteogenesis. We show that not only are osteogenic markers highly enriched compared to ADSC induced on tissue culture plastic, but that osteogenic maturational phases occur more rapidly in the presence of this ECM. Finally, we demonstrate that a direct correlation may exist between ECM-related genes expressed at a particular time point and the corresponding activity of the secreted and subsequently entrained proteins from that same time point. We show that DPT, highly expressed at day 16 in osteogenesis, has a profound effect on differentiation. Specifically, abundant DPT in the ECM (obtained through lentiviral infection), induces high expression of osteogenic genes; higher than cells seeded onto ECM as shown in other published studies. This approach, as well as the biomolecules such as DPT identified in our experiments, can serve as a foundational discovery tool to identify potential biomaterials that can be used to actively direct cell differentiation and tissue formation, in bone as well as other important tissues.

Publications

Original Article
Ju YM, Choi JS, Atala A, Yoo JJ, and Lee SJ. Bilayered scaffold for engineering cellularized blood vessels. Biomaterials 2010;31:4313-4321

Lang R, Stern MM, Smith L, Liu Y, Bharadwaj S, Liu G, Baptista P, Bergman CR, Soker S, Yoo J, Atala A and Zhang Y. Three-dimensional Culture of Hepatocytes on Porcine Liver Tissue-Derived Extracellular Matrix. Biomaterials, *in press*.

Book Chapter
Ladd MR, Hill TK, Yoo JJ, and Lee SJ. Electrospun nanofibers in tissue engineering, *In Nonofibers,* Edited by Lin T, ISBN 978-953-307-420-7, InTech Open Access Publisher, *in press*