Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** University of Pittsburgh – Commonwealth System of Higher Education

2. **Reporting Period (start and end date of grant award period):** 1/1/2010 – 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Margaret C. McDonald, PhD

4. **Grant Contact Person's Telephone Number:** 412-383-7474

5. **Grant SAP Number:** 4100050913

6. **Project Number and Title of Research Project:** 01- Application of Adult Stem Cells for Tissue Regeneration

7. **Start and End Date of Research Project:** 1/1/2010 – 12/31/2013

8. **Name of Principal Investigator for the Research Project:** Rocky S. Tuan, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 3,588,983.76

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Tuan, Rocky	Principal Investigator	10%	$95,125.38
Baker, Natasha	Post-doctoral Fellow	100%	251,596.99
Statman, Lauren	Pre-doctoral Fellow	100% Yr 1-3 only	107,716.49
Diederichs, Solvig	Post-doctoral Fellow	100% Yr 2-3 only	56,278.75
Ulici, Veronica	Post-doctoral Fellow	76% Yr 2-3 only	84,820.99
Clark, Karen	Lab Manager	69%	282,741.42
McCann, Timothy	Grants Administrator	20%	58,317.68
Tan, Jian	Research Specialist	68%	222,575.65
Taboas, Juan	Co-Investigator	87.5% Yr 1 only	85,587.67
Boyette, Lisa	Post-doctoral Fellow	100% Yr 1-2 only	93,209.93
Cheng, Wai Ming	Post-doctoral Fellow	25% Yr 2, 48% Yr 3	23,683.36
Jiang, Yangzi	Post-doctoral Fellow	100% Yr 3-4 only	72,021.67
Lin, Hang	Post-doctoral Fellow	11% Yr 3 only	3,678.01
Hofer, Heidi	Graduate Stud. Researcher	100%	119,716.72
Bean, Allison	Graduate Stud. Researcher	100% Yr 1-2 only	36,336.25
Yang, Guang	Graduate Stud. Researcher	100% Yr 2 only	16,000.01
Langhans, Mark	Graduate Stud. Researcher	100% Yr 2 only	10,625.00
Brick, Rachel	Graduate Stud. Researcher	100% Yr 3 only	13,005.00

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
ABI PCR Machine	Used for all research projects.	$75,924.32
GloMax Luminometer	Used for all research projects.	7,046.10
HeraCell 150I Dual Stack Copper Chamber	Used for all research projects.	10,776.44
Deconvolution Module	Used for all research projects.	7,560.00
High Speed Mandrel Assembly	Used for all research projects.	5,850.00
CellKraft Humidifier	Used for all research projects.	31,300.00
Incubator	Used for all research projects.	5,803.65
Syringe Pump	Used for all research projects.	6,003.40
Heating Chamber module	Used for all research projects.	14,580.00

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No___X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
"3-D Osteochondral Micro-tissue to Model Pathogenesis of Osteoarthritis"	☒NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	January 2012	$721,450	$711,565
"Regenerative Repair of Traumatic Articular Cartilage Injuries: Point-of-Care Application of Mesenchymal Stem Cells and Chondrocytes"	☐NIH ☒ Other federal (specify: Dept of Defense__) ☐ Nonfederal source (specify:_)	January 2012	$299,218	$294,276
"Regenerative Repair of Traumatic Articular Cartilage Injuries: Point-	☐NIH ☒ Other federal (specify:_Dept of	July 2012	$1,000,000	$1,000,000

of-Care Application of Mesenchymal Stem Cells and Chondrocytes”	Defense__) ☐ Nonfederal source (specify:_)			

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

We plan to submit the following grant applications to continue the research:

1. NIH – “Cholesterol Sensitivity and Mechanisms of MSC Responses to 3D Substrate Rigidity.” To be submitted in February 2014.

2. Department of Defense – “Polyphenolic Compounds for the Prevention and Treatment of Osteoarthritis.” To be submitted in February 2014.

3. NIH – “Cartilage Stem Cells: Inflammatory Target in Pathogenesis of Osteoarthritis.” To be submitted in February 2014.

12. Future of Research Project. What are the future plans for this research project?

Activities related to this research will be continued in the lab and additional grant funding is being sought to support these activities. We will continue to explore the use of adult stem cells, with their trophic and differentiation activities, as a means of developing therapeutic solutions for degenerative joint diseases.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male			2	2
Female			4	5
Unknown				
Total			**6**	**7**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			6	7
Unknown				
Total			**6**	**7**

	Undergraduate	Masters	Pre-doc	Post-doc
White			4	4
Black				
Asian			2	3
Other				
Unknown				
Total			**6**	**7**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes____X____ No_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

Predoctoral:

Lauren Statman	BS	Columbia University, NY
Heidi Hofer	BS	University of Arizona, AZ
Allison Bean	BS	Rice University, TX
Guang Yang	BS	University of Science and Technology, China
Rachel Brick	BS	Pennsylvania State University, PA

Postdoctoral:

Natasha Baker	PhD	East Anglia University, United Kingdom
Solvig Diederichs	PhD	Heidelberg University, Germany
Veronica Ulici	PhD	University of Western Ontario, Canada
Lisa Boyette	MD	University of Virginia, VA
Wai Ming Cheng	PhD	Duke University, NC
Yangzi Jiang	PhD	Zhejiang University, China
Hang Lin	PhD	Chinese Academy of Sciences, China

Staff:

Jian Tan	MD	Southeast University Medical School, Nanjing, China

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

This research project has enhanced the strength of the stem cell and regenerative medicine programs at the University of Pittsburgh and provided opportunities for predoctoral and postdoctoral fellows to explore stem cell and regenerative medicine research, particularly related to musculoskeletal applications.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No___X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures

of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project Title and Purpose

Application of Adult Stem Cells for Tissue Regeneration - Degenerative skeletal diseases like osteoarthritis are the primary cause of disability, particularly in the elderly. The purpose of this research project is to develop novel tissue regenerative approaches for the treatment of such diseases. The included studies will investigate the regenerative activities of human adult stem cells, specifically the nature of the optimal physical and biological microenvironment that supports their activities. This knowledge will guide the design of biomaterial scaffolds and the selection of bioactive factors for effective stem cell-based therapy for the engineering, regeneration, and functional restoration of diseased or injured skeletal tissues.

Project Overview

Degenerative skeletal diseases are the primary cause of disability in the elderly. Cell-based therapy is a promising treatment approach for the engineering and regeneration of the diseased or injured tissues. Adult stem cells, known as mesenchymal stem cells (MSCs), possess the ability to differentiate into musculoskeletal cells and represent a promising cell type for skeletal tissue engineering. Successful application of MSCs in cell-based therapy requires a full understanding of the mechanisms that regulate their biological activities, specifically proliferation and differentiation. Three interconnected projects are described here. The first project focuses on elucidating the mechanism of action of selected genes previously identified as potential

"stemness genes" (i.e., genes that encode proteins that act to maintain the potency of the MSCs). These genes include both secreted factors and extracellular matrix components and will be analyzed in terms of their control of MSC proliferation and differentiation and their cellular signaling pathways. Functional molecules identified in this study will be considered as ligands for incorporation into a three-dimensional (3D) matrix (e.g., using nanostructured biodegradable polymer fibers to construct a bioactive scaffold for MSCs). Using bioreactor technology, culture conditions will be optimized to induce the cell-seeded constructs to differentiate into a hyaline cartilaginous tissue with biological and mechanical characteristics approximating those of native articular cartilage. The utility of the engineered constructs for cartilage repair will be tested in animal models of traumatically induced lesions in the femoral condylar articular cartilage. In summary, these studies will identify the key molecules important for MSC activity and exploit the identified pathways to produce optimal 3D scaffolds for cartilage engineering that will be tested for cartilage repair in clinically relevant animal models. Positive results from these proof-of-concept studies will yield a rational basis for the design of clinical trials using this technology.

The project will have the following specific aims:

Aim 1 – To analyze the molecular mechanism of action of a specific "stemness gene" previously identified and associated with the maintenance of proliferative and differentiation-readiness of MSCs (specific attention will be paid to identifying molecular domains or moieties that may be applied as reagents to generate active MSCs)

Aim 2 – To apply the reagents generated in Aim 1 as ligands for incorporation into and/or conjugated onto biodegradable polymeric nanofibers (produced via electrospinning) to construct bioactivated scaffolds that will be seeded with MSCs; the efficacy of such MSC-seeded constructs to produce improved engineered cartilage will be tested and optimized *in vitro.*

Aim 3 – To test the ability of the engineered cartilage construct to repair cartilage lesions produced via supraphysiological impact in medium (rabbit) and large (goat) animal models.

Background and Significance

Osteoarthritis (OA) is the most common joint disease and is characterized by degeneration of the articular cartilage, ultimately leading to joint destruction. Currently OA is a major cause of disability in the elderly, and the prevalence of OA is expected to increase dramatically during the next 20 years as the population ages. The burden of OA is exacerbated by the inadequacies of current treatments. Early to moderate OA is typically addressed with several nonpharmacologic and pharmacologic measures, but no drug used in the treatment of OA has been convincingly shown to protect articular cartilage to date. When the medical approaches fail to control the symptoms and progression of OA, surgical interventions may be indicated. Arthroscopic lavage and debridement can provide symptomatic relief, although a recent major study has cast doubt on this practice. Also, various methods for the repair of articular cartilage lesions have been developed. These methods include the transplantation of osteochondral grafts, microfracturing, and autologous chondrocyte implantation (ACI) with or without the assistance of a scaffold matrix for cell delivery. A common feature of these techniques is that their use is limited to the

repair of focal lesions. As a consequence, patients with OA are currently excluded. An osteotomy is sometimes performed in the case of joint malalignments. This measure can provide pain relief for several years until the newly weight-bearing articular cartilage erodes and is only considered a delaying tactic that buys time until a total knee replacement (TKR) becomes necessary. Thus, the challenge for researchers to develop disease-modifying OA treatments becomes an issue of paramount importance.

Adult MSCs that have the ability to differentiate into cells of the chondrogenic lineage have recently emerged as a candidate cell type with great potential for future cell-based articular cartilage repair technologies. These MSCs can be isolated from a variety of adult tissues, be readily culture-expanded without losing their multilineage differentiation potential, and have been shown to undergo chondrogenic differentiation *in vitro* and *in vivo*. Unlike chondrocytes, the use of MSCs is not hindered by the limited availability of healthy articular cartilage and the intrinsic tendency to lose their phenotype during expansion. Also, the use of MSCs obviates the need for a cartilage biopsy that causes considerable donor site morbidity to the articular surface.

Etiopathophysiology of OA

Because of the late stage at which OA is diagnosed, difficulties in studying the disease in humans, and inadequacies with animal models of OA, the disease is poorly understood. Much of the research into the pathophysiolology of OA has focused on loss of articular cartilage caused by mechanical and oxidative stresses, aging, or apoptotic chondrocytes. Articular chondrocytes within the diseased cartilage synthesize and secrete proteolytic enzymes, like matrix metalloproteinases (MMPs) and aggrecanases, that are responsible for degrading the cartilaginous matrix. The pro-inflammatory cytokine, interleukin-1β (IL-1β), has been shown to be the most potent inducer of these enzymes and other mediators in OA (like nitric oxide [NO] and prostaglandin E_2) by articular chondrocytes, leading to matrix depletion through a combination of accelerated breakdown and reduced synthesis. Other pro-inflammatory cytokines, like tumor necrosis factor-α (TNF-α) have also been shown to be involved in cartilage breakdown and contribute to biomechanical factors implicated in the etiopathophysiology of OA. Although the pharmaceutical industry has devoted considerable effort to the development of inhibitors of these targets as novel anti-OA drugs, clinical success with respect to prevention of further cartilage matrix breakdown or cartilage restoration in OA remains elusive.

Potential of MSCs to aid cartilage restoration in OA

Given the various disease pathologies in OA, it is conceivable that some of these may be obviated by the application of cell-based treatments. The use of MSCs provides an attractive alternative to chondrocytes, as MSCs have been shown to be multilineage progenitors that can be stimulated to differentiate along specific pathways, including chondrogenesis. Unlike mature chondrocytes, which must be surgically harvested from a very limited supply of non-weight-bearing articular cartilage, MSCs can be readily harvested from bone marrow or other tissues of mesenchymal origin and will maintain their multilineage potential with extended passage, enabling considerable expansion in culture. MSCs are commonly isolated by adherence to cell culture plastic or density-gradient fractionation and, therefore, represent a heterogeneous population of cells. Although no definitive MSC marker has been identified, an immunophenotype positive for STRO-1, CD73, CD146, CD105, CD106, and CD166, and

negative for CD11b, CD45, CD34, CD31, and CD117 has been shown to most reliably characterize the MSC population. In cartilage regeneration, extensive analyses of the appropriate microenvironment to stimulate MSCs toward chondrogenesis *in vitro* have been performed. Positive selection for chondroprogenitor cells has been achieved by conditioning the culture medium with growth factors, such as fibroblast growth factor-2 (FGF-2) or transforming growth factor-β (TGF-β), during monolayer expansion. Additional critical steps in using MSCs for articular cartilage regeneration include (1) the development of effective methods to maintain MSCs in an active state and, thus, highly responsive to chondrogenic stimulation, (2) the retention of a hyaline articular cartilage phenotype without hypertrophy, ossification or fibrinogenesis, and (3) a delivery system to localize the cells within a lesion without compromising chondrogenic differentiation or the integrity of the repair tissue as well as promotion of integration with surrounding host tissues.

Despite these promising features of MSCs and their potential to reverse some of the pathologies of OA, the fact that cartilage defects arising from an underlying disease process like OA are distinct from focal cartilage lesions that result from acute injury or osteochondrosis dissecans (OCD) must be taken into consideration. Specifically, acute cartilage injury and OCD often take place in an otherwise healthy joint; the patient may be young, and the focal defect will likely require a localized treatment. In contrast, in late-stage OA, the patient is likely to be older and to require treatment of the entire articulating surface. Consequently, repair of the lesion may provide symptomatic relief and delay symptom progression, but unless the underlying disease is also treated effectively, any improvement is unlikely to be long-term.

Outstanding challenges in MSC-based cartilage repair
This project aims to address some of the major challenges that must be resolved to apply MSC-based therapy to repair articular cartilage: (1) identify specific mechanisms to maintain MSCs in an active stem cell state, ready for guided induction to differentiate along chondrogenic lineage; (2) design three-dimensional scaffold materials that will promote the activity of MSCs, permit their guided chondrogenic differentiation, provide physical support for the engineered cartilage, and allow integration of engineered cartilage with surrounding host tissue; and (3) understand the trophic activities of MSCs, particularly their interaction with endogenous cells of the host tissue, in order to achieve successful articular cartilage repair in clinically relevant animal models. The projects build on previous and recent findings and the demonstrated expertise of our laboratory, in particular investigations of the basic regulatory mechanisms governing MSC proliferation and differentiation, the development of a biomimetic tissue engineering scaffold, and experience with animal models of cartilage disease and repair.

Summary of Research Completed

AIM 1: Maintenance of Proliferative and Differentiation-Readiness of MSCs

Project (1) *Characterization of Molecular Control of Stemness and Proliferative/Differentiative State of Adult Stem Cells*

A. *Role of Caveolin-1 (Cav-1), Cholesterol, PI3K/Akt Signaling, and Age in MSC Biology*
Introduction Caveolin-1 (Cav-1) is the main protein component of cell membrane cholesterol-

rich caveolae lipid rafts. As Cav-1 is an important scaffolding protein and cell signaling regulator, we hypothesized that this protein could have a strong influence on some aspects of adult MSC biology. We set out to test this hypothesis to gain knowledge that may benefit the application of stem cells in regenerative medicine. We were particularly interested in the effect of Cav-1 on adult MSC osteogenesis, because the Cav-1 null mouse has a greater rate of postnatal bone formation. Furthermore, as the activity of Cav-1 is closely related to cholesterol levels, and Cav-1 expression is induced by high cholesterol, we examined the role of both Cav-1 and cholesterol in MSC biology, as well as possible cell signaling pathways that are dually regulated by both. One signaling pathway of interest became the PI3K/Akt pathway. The relationship of this pathway with integrin adhesion has led us in a new direction to investigate the potential interplay between Cav-1, cholesterol, and substrate rigidity-driven MSC responses. Because cell adhesion plays a major role in this, and Cav-1 also affects cell aging, we also sought to determine the affects of MSC donor age on MSC adhesion dynamics.

Methods

(1) *MSC Culture and Cav-1 Expression*

MSCs were isolated from the bone marrow of femoral heads obtained following total hip replacement surgery with IRB approval, and cultured in growth medium (GM) with 10% fetal bovine serum (FBS). Gene expression was assessed using RT^2 Profiler PCR Arrays (Qiagen/SABiosciences) or standard RT (real time)-PCR using an ABI PCR Machine - 7900HT. MSC proliferation was measured by cell counting on days 1, 5, and 10 of culture in GM. The pattern of Cav-1 expression in MSCs was examined by immunofluorescent staining visualized by standard fluorescence and confocal microscopy. In addition, the caveolar localization of Cav-1 was probed by detergent-free cell lysis, followed by lysate fractionation through sucrose gradient ultra-centrifugation, and then western blotting of the resultant fractions.

(2) *Osteogenic Differentiation*

MSCs were cultured in GM supplemented with 5 mM β-glycerophosphate, 50 µg/ml ascorbate, 10 nM 1,25-dihydroxyvitamin D_3, and 10 nM dexamethasone (referred to as osteogenic medium, OM). MSCs cultured in standard GM were used as a control in all osteogenesis studies. Medium was changed on every third day of culture. Osteogenic differentiation was assessed by measuring alkaline phosphatase activity after 4 days, osteogenic gene expression by real time (RT)-PCR after 10 days, and matrix mineralization by Alizarin Red staining after 21 days, respectively. The expression of Cav-1 was examined at various time points during osteogenic differentiation by RT-PCR and western blotting.

(3) *Perturbation of Cav-1 and Cholesterol*

Cav-1 expression was disrupted using small interfering RNA (siRNA). For controls, untransfected cells and cells transfected with a non-targeting control siRNA were used. In some experiments, Cav-1 expression was stably knocked-down by transduction with lentiviral siRNA. The lentiviral construct conferred resistance to puromycin, and cells infected with lentivirus were selected using puromycin-containing GM. Parental untransfected cells were used as a control for experiments with these cells. In all cases, Cav-1 knockdown was confirmed by RT-PCR and western blotting. The cholesterol content of the MSC cell membrane was depleted by treatment with methyl-β-cyclodextrin, which is known to deplete cell membrane cholesterol. Conversely, MSCs were also cultured in the presence of excess cholesterol.

(4) *Cell Signaling Studies*
Lysates were harvested from siRNA-transfected cells at various time points following stimulation with OM or GM and probed for the phosphorylation (and hence activation) of various cell signaling proteins by western blotting. To complement these studies, RNA was isolated from the cells at a later time point and analyzed for the expression of various gene targets known to be immediately downstream of different cell signaling pathways. We accomplished this using an RT^2 Profiler PCR Array for Human Signaling (Qiagen/ SABiosciences), which was run on an ABI PCR Machine - 7900HT. To investigate the localization of signaling proteins in caveolar and non-caveolar lipid rafts, buoyant lipid raft-containing cell fractions (generated through sucrose-gradient cell fractionation as described above) were probed for the presence of Cav-1 and for various cell signaling molecules by western blotting. The role of PI3K/Akt signaling was probed using the PI3-Kinase inhibitor LY294002. Integrin signaling was activated using divalent cations and β-catenin signaling activated using Wnt3a or lithium chloride. For co-immunoprecipitation experiments, cultures were treated with the chemical crosslinker dithiobis[succinimidylpropionate] (DSP) before harvesting and immunoprecipitation of Cav-1 protein using a polyclonal Cav-1 antibody (BD Biosciences) and immunopreciptation kit (Pierce).

(5) *3D and Adhesion Dynamics Studies*
For 3D culture studies MSCs were pre-cultured in GM, then detached and re-suspended in gelatin containing a light-activatable photocrosslinker. MSC/gelatin suspensions were then exposed to light for varied lengths of time to generate 3D cultures with soft and stiff gelatin substrate rigidity. In addition, subsets of MSCs were either: (1) treated with LY294002 after photocrosslinking, (2) pre-treated for 1 hour with methyl-β-cyclodextrin before dilution in gelatin, (3) pre-incubated in cholesterol-containing medium for 24 hours before dilution in gelatin, or (4) treated with a short peptide mimetic of part of the Cav-1 protein (Cavtratin) after photo-crosslinking. Medium was changed 24 hours after photo-crosslinking (with LY294002 or Cavtratin where necessary) and cells viewed by calcien-am staining after a further 24 hours. Meanwhile, to study the possible influence of donor source age on MSC adhesion, we isolated MSCs from six young (20 yrs–45 yrs) and six old (75 yrs–84 yrs) donor sources, and observed their adhesion to tissue culture plastic in real time using an ACEA RTCA-DP xCELLigence analyzer and culture plates with integrated microelectrodes.

Results and Conclusions

As reported in our recent paper (Baker et al., 2012), we determined that Cav-1 protein is expressed in human bone-marrow derived MSCs and in caveolae raft fractions, and that Cav-1 mRNA and protein expression increases upon MSC osteogenic differentiation. By knocking down Cav-1 expression, we determined that Cav-1 is a negative regulator of MSC proliferation as measured by cell counting. However, this result occurred only in female MSCs, and gene expression studies suggested that POU5F1/Oct4 expression is reduced in male, but not female, Cav-1 knockdown MSCs. These results highlighted that there may be gender-dependent differences in Cav-1 effects on MSC growth. The possibility of gender-related differences in Cav-1 effects were also highlighted in signaling studies, in which genes downstream of estrogen and androgen signaling pathways showed slight gender-dependent changes.

Other effects of Cav-1 knockdown were not gender dependent. Cav-1 knockdown enhanced

MSC osteogenesis, as measured by alkaline phosphatase activity, Alizarin Red staining and osteogenic gene expression. As a result of this work, which was published in 2012, we postulated that Cav-1 expression may increase during MSC osteogenesis as part of a negative feedback loop. Meanwhile, signaling protein studies revealed that Cav-1 may suppress osteogenesis by partial inhibition of Akt phosphorylation and β-catenin-dependent signaling. We also found that Cav-1 physically interacts with Akt in MSCs, though this interaction was only detectable after chemical crosslinking. Further studies using pharmacological agents revealed that PI3K/Akt signaling was essential for MSC osteogenesis in our system, and this result may be at least partially through the activation of β-catenin signaling. This outcome suggests a possible osteogenic pathway inhibited by Cav-1, with PI3K/Akt signaling upstream of β-catenin signaling. Cav-1 inhibition of Akt, and separately, Akt activation of β-catenin signaling, have certainly been observed by other researchers in other cell types in other contexts.

Activation of integrin signaling enhanced β-catenin signaling and PI3K/Akt signaling induced by osteogenic supplements. Osteogenic PI3K/Akt signaling may therefore act in concert with or downstream of integrin signaling. Additionally, we found that osteogenic PI3K/Akt signaling and MSC osteogenesis were sensitive to cholesterol perturbations. We determined that the role of cholesterol in MSC osteogenesis and PI3K/Akt signaling is complex. This complexity is most likely due to the division of Akt between caveolar and non-caveolar cholesterol-rich lipid rafts. Our data suggest that non-caveolar cholesterol may be required for MSC osteogenesis and PI3K/Akt signaling. In connection with data from studies in three-dimensional (3D) cultures (see below), we intend to publish this mechanistic data in a second manuscript.

From our published findings (Baker et al., 2012) and other reports in the literature (cited in our review paper, Baker and Tuan, 2013), we predict that Cav-1 forms the connection between the caveolar and non-caveolar cholesterol signaling compartments, the latter of which is most likely positively associated with integrins. Outside of caveolae, Cav-1 may introduce cholesterol to integrin focal adhesions and promote integrin outside-in signaling, downstream of which PI3K/Akt signaling is activated. Conversely, Cav-1 is well known for inducing the internalization of cholesterol and focal adhesion proteins upon cell-substrate detachment, thus leading to the down-regulation of integrin outside-in signaling. We thus concluded our study with the hypothesis that the cholesterol/Cav-1/caveolae homeostatic system has an important influence on the regulation of adhesive signaling and downstream regulation of MSC differentiation pathways.

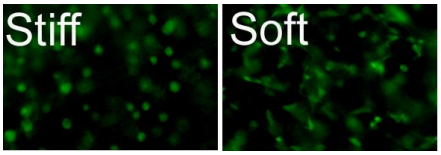

Figure 1: *Calcein-AM staining showing P3 human MSC morphology in 3D scaffolds with relatively stiff (25 kPa left) and soft (4 kPa right) elasticity.*

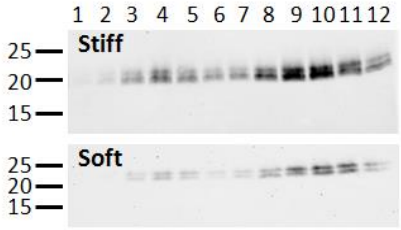

Figure 2: *Western blots for Cav-1 protein (22 KDa) in sucrose gradient fractions of cell lysate from MSCs cultured in a stiff 3D gel (top) or a soft 3D gel (bottom). Cav-1 protein is abundant in the expected fractions containing caveolae (7-11) and appears to be stronger in cells seeded on stiff gels (gradients were loaded with equal total protein).*

In the last phase of our study we began to test this hypothesis in a 3D culture system, with measures of MSC responses to substrate rigidity. Preliminary results (see Figures 1, 2 & 3) suggest that both cholesterol and Cav-1 expression, as well as cholesterol level, have some role to play in the adhesion responses of cells in this context. Therefore, these recent data, together with our published findings, have formed the basis of our recent grant application to NIH for further funding of this research, which may benefit the rational development of substrate-controlled, MSC-based tissue engineering.

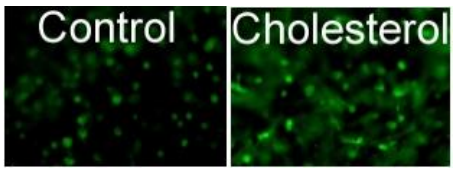

Figure 3: *Calcein-AM staining of human MSCs cultured in standard growth medium (control, top panel) or cultured in growth medium supplemented with cholesterol for 18 hours (cholesterol, bottom panel) before growth in stiff 3D scaffolds for 48 hours. MSCs pretreated with cholesterol appear to become less rounded and more elongated in stiff gels than control MSCs.*

Finally, preliminary results from our final months of the study suggest that MSC age does not significantly affect MSC adhesive characteristics (unpublished) . Although further work is required, this finding strengthens our hypothesis that MSC membrane cholesterol status may be more important than MSC age in determining adhesion responses. This hypothesis could also be the subject of further study and of interest to those considering the application of MSCs for regenerative medicine.

B. *Derivation and Characterization of MSCs from Induced Pluripotent Stem Cells Produced via Reprogramming Using Stemness Genes*

MSCs have a high potential for therapeutic efficacy in treating diverse musculoskeletal injuries and cardiovascular diseases and ameliorating the severity of graft-versus-host and autoimmune diseases. While most of these clinical applications require substantial cell quantities, the number of MSCs that can be obtained initially from a single donor is limited. Reports on the derivation of MSC-like cells from pluripotent stem cells (PSCs) are thus of interest, since the infinite proliferative capacity of PSCs opens the possibility of generating large numbers of uniform batches of MSCs. However, characterization of such MSC-like cells is currently inadequate, especially as to whether these cells are equivalent or identical to MSCs.

We have recently derived MSC-like cells, induced multipotent progenitor cells (iMPCs) using three different methodologies from a newly established induced PSC line reprogrammed from human bone marrow stromal cells (BMSCs): (1) embryoid body formation, (2) indirect co-culture with MSCs, and (3) forced differentiation of colonies. We have compared the iMPCs directly to the originating parental BMSCs. The iMPCs exhibited typical MSC/fibroblastic morphology (Figure 4) and an MSC-typical surface marker profile (Table 1), and were capable of differentiation *in vitro* along the osteogenic, chondrogenic, and adipogenic lineages (Figure 5), as measured by Alizarin Red staining and alkaline phosphatase activity (osteogenesis),

glycosaminoglycans (GAG) assay (chondrogenesis), oil red-O staining (adipogenesis), and lineage specific gene expression. In particular, when implanted into intramuscular sites, the iMPCs, upon induction with bone morphogenetic protein-2, are able to form bone nodules (Figure 6), demonstrating their mesenchymal characteristics. However, compared to the parental BMSCs, iMPCs displayed a unique expression pattern of mesenchymal and pluripotency genes and were less responsive to traditional BMSC differentiation induction protocols. We conclude that iMPCs generated from PSCs via spontaneous differentiation represent a distinct cell population that resemble MSCs but are at a more primitive developmental stage. This work has been accepted for publication (Diederichs S Tuan R. Functional comparison of human induced pluripotent stem cell-derived mesenchymal cells and bone marrow-derived mesenchymal stromal cells from the same donor. **Stem Cells Develop.** 2014. In press).

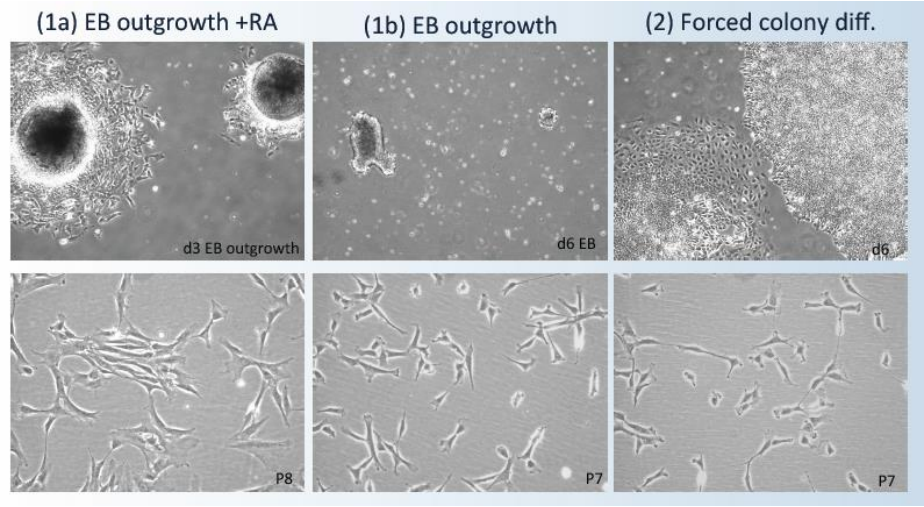

Figure 4. *Derivation of induced multipotent progenitor cells (iMPCs) from iPSCs using embryoid body outgrowth (1a & 1b) and forced colony differentiation (2). Cells assume a fibroblastic morphology and are able to propagate on standard MSC culture medium. Bottom panels show cells at passage 7-8.*

Table 1
Surface Epitope of iMPCs versus iPSCs and MSCs

	CD73	CD90	CD105	CD44	CD146
MSCs	+	+	+	+	+
iPS	-	+	-	-	-
iMP	+	+	+	+	+

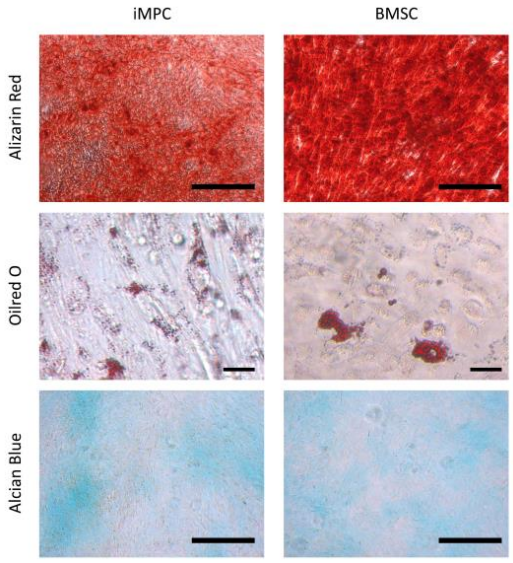

Figure 5. *Trilineage differentiation potential of iMPCs compared to bone marrow derived MSCs (BMSCs). Top: osteogenesis (Alizarin Red staining); Middle: adipogenesis (Oil Red O staining); bottom: chondrogenesis (Alcian Blue staining).*

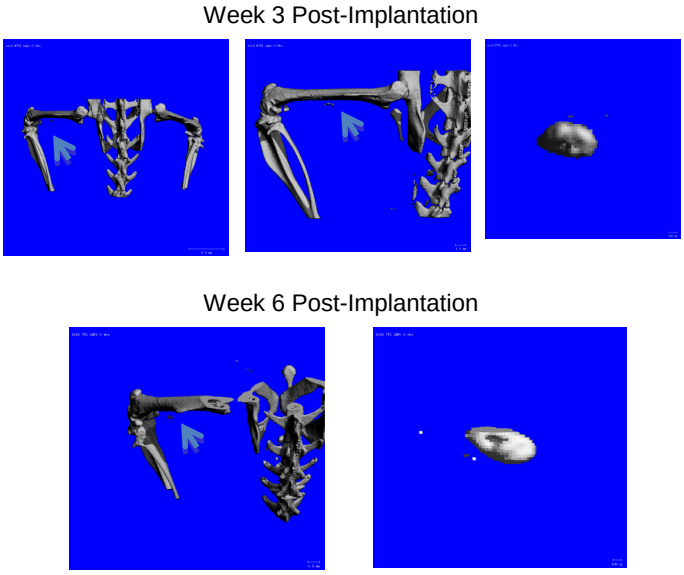

Figure 6. *iMPCs are capable of BMP-2 induced osteogenesis in vivo. iMPCs were implanted intramuscularly in the presence of BMP-2 and bone formation is monitored by micro-CT at weeks 3 and 6. Formation of a bone nodule is visible by week 3 and appears to be stabilized by week 6.*

Until now, there have been no standard protocols for the derivation of mesenchymal progenitors from pluripotent cells or verification of their mesenchymal nature. Our use of iPSCs reprogrammed from human MSCs allows us to compare key characteristics of iMPCs with original MSCs from the same donor and draw more specific conclusions about their similarities and differences than would be possible with iMPs and MSCs from different sources and donors.

<u>AIM 2. Tissue Engineering Applications of MSCs</u>

Project (2) *Application of Human Mesenchymal Stem Cells To Enhance Innervation, Mobility, and Joint Function*
Musculoskeletal injuries and trauma, the ultimate underlying justification for the entire project,

are often accompanied by peripheral tissue damage, which severely compromises functional repair. Tendon/ligament and peripheral nerves injuries are the most common. Below we present our studies using both MSCs and biodegradable scaffolds to address these issues. We have been studying MSCs as a potential therapeutic cell type to repair these injuries and reported on their role in bone and tendon interaction (also below). In addition, innervation is critical to effective musculoskeletal regeneration. In studying adult stem cells for their potential regenerative application, we are constantly examining all cellular activities that are potentially therapeutic, particularly activities that may influence both the stem cells and other cells in the injured/diseased tissue site. In a similar manner, in developing MSCs as an agent for functional tissue engineering, we pay attention to applications that will address multiple cell types that together contribute to functional tissue engineering.

A. *Neural Differentiation of MSCs for Peripheral Nerve Repair*

Studies estimate that there are approximately 800,000 peripheral nerve injuries (PNI) every year, with over half of these PNI involving partial or complete laceration of the nerve. Because PNIs heal slowly, with significantly impaired function, they are good candidates for regenerative or tissue engineering approaches for repair. Tissue engineering seeks to optimize a combination of cells and their environmental stimulants, both physical and chemical (classically termed scaffold and growth factors), to create functional, robust tissue replacements. Current understanding of nerve repair after traumatic injury supports the use of an isolating conduit that incorporates physical and biochemical cues, such as neurotrophic factors (NFs) to encourage nerve growth cone migration through the conduit. We have recently reported that autologous multipotent progenitor cells (MPC) isolated from a wound site (e.g., debrided tissue from blast-damaged muscle) produce complex mixtures of neurotrophic factors when both the neurons and the MPC are on tissue culture plastic. NFs are known to be secreted by endothelial cells (EC); gene expression and factor production of many cells changes upon three dimensional culture, often enhancing the differentiation of stem cells. We propose that MPCs, acting alone and in concert with other support cells, alter the innate healing ability of native cells in a wounded environment. We hypothesize that MPCs interact dynamically, both affecting the proliferation, migration, and function of neighboring cells and modulating their mesenchymal function according to the inflammatory environment. We tested this hypothesis by examining the interaction of MPCs with endothelial and nerve cells, two cell types whose activities are critical for functional restoration after injury. To understand the activity of MPCs and the role of inflammation on tissue function, we investigated MPC- and EC- interaction in three scenarios.

We have developed a simplified nerve conduit of aligned nanofibers that allows the *in vitro* manipulation of neuron clusters as well as the neural support cells (MPC and EC). We hypothesize that this construct could enhance the neural activity of fiber-seeded MPC and EC. Our preliminary results as described below support this hypothesis, as (1) seeded MPC/EC act as sources of neurotrophic factors when cultured on nanofiber scaffolds; and (2) the presence of either or both cell types supported neurite outgrowth from co-cultured chick dorsal root ganglion (DRG). These results suggest that a construct consisting of electrospun naonofibers, combined with MPC and EC, may be further developed as a conduit- and cell-based therapy to promote peripheral nerve regeneration.

We also investigated MPC and endothelial cell (EC) interaction. First, MPC promotion and/or inhibition of angiogenesis, specifically vessel recruitment, was assessed using an avian chorioallantoic membrane (CAM) model (Figure 7). MPCs and their conditioned media were encapsulated in methacrylated gelatin and incubated for two days on the CAM. The results showed that MPCs serve to recruit blood vessels from the surrounding vasculature. Although vascular endothelial growth factor (VEGF) is present in MPC-conditioned media at a concentration of ~ 1 ng/ml and is a known pro-angiogenic factor, 1x- and 10x-conditioned media did not initiate vascular invasion of the gelatin plug as strongly as live MPCs, suggesting that it is the dynamic response of the living MPCs to the invading vessels that contributes to successful vessel recruitment.

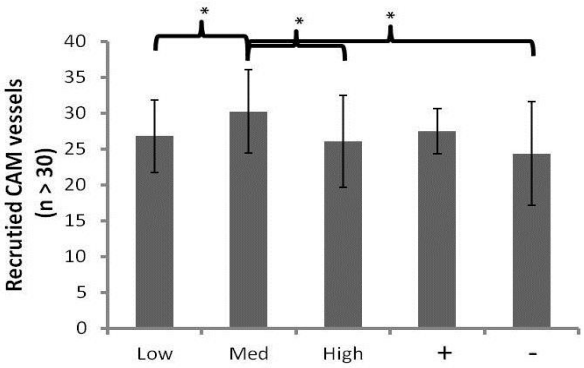

***Figure 7**. MPC-mediated CAM vessel recruitment. CAM small vessel recruitment (angiogenesis) was tracked 48 h after gel placement and compared to positive (10 ng/ml FGF) and negative (blank) controls. Viable MPCs at the medium density (0.5 million) recruited the greatest number of vessels.*
** = $p < 0.05$.*

Next, we investigated the attenuation of mesenchymal differentiation of MPCs under inflammatory conditions and the role of ECs in rescuing those phenotypes. Preliminary data show that EC-conditioned media, regardless of the presence of inflammatory factors TNF-α and IL-1β increased the early but not the late osteogenic differentiation capacity of bone marrow-derived MSCs and MPCs (alkaline phosphatase expression). Previous gene expression research in our laboratory identified a number of "stemness" factors—molecules with decreased expression when cells were differentiated and increased expression when cells were maintained in an undifferentiated state (Song et al. Identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency. Stem Cells. 2006. 24(7):1707-18.).

Among those recognized factors were two secreted agents: interleukin-6 (IL-6) and chemokine (C-C motif) ligand-2 (CCL-2). Our laboratory previously demonstrated the ability of IL-6 to affect the proliferation, survival, and differentiation capacity of MSCs. We therefore sought to examine the role of CCL-2 in regulating bone marrow-derived MSC differentiation. We confirmed a reduction in CCL-2 secretion with MSC differentiation but found little difference in the ability of MSCs to differentiate along adipo- and osteogenic pathways in the presence of exogenous CCL-2. While there CCL-2 appeared to affect MSC proliferation and promoted IL-6 secretion, high patient-to-patient variability made identifying a clear role for this chemokine difficult.

Finally, we assessed the combined MPC and EC support of nerve growth. We had previously reported that MPCs were a source of NFs that encourage nerve growth cone migration through the conduit. NFs are also known to be secreted by ECs. Gene expression and factor production of many cells changes upon three-dimensional culture, often enhancing the differentiation of stem cells, so we investigated the effect of polymer fiber culture on MPC, EC, and MPC-EC combinations. MPC/EC survival and proliferation were determined by fluorescent live-dead staining (Figure 8).

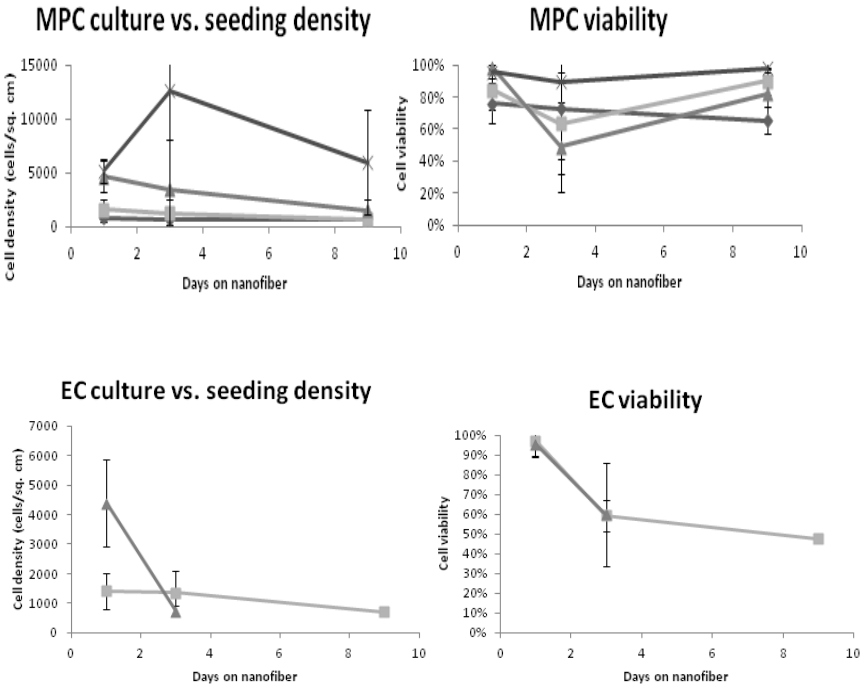

Figure 8. *Cell viability and proliferation were measured 1, 3, and 9 days after seeding using Live-Dead stain and ImageJ software. >10 images covering representative areas of each scaffold were counted (mean±SD, no significant differences between groups). Although ECs (p.6-10) were maintained at approximately the same density as MPCs, EC viability was compromised, perhaps due to their crowding on the small scaffold. Only cells seeded at 1,000 cells/sq. cm maintained viability long enough to measure at day 9. Where possible to measure, all groups were initially significantly different from one another but not significantly different when viable at later time points. Further experiments were performed using MPCs and ECs seeded at 1,000 cells/sq cm.*

NF gene expression and protein production were assessed via RT-PCR and confirmed with ELISA. VEGF and brain-derived neurotrophic factor (BDNF) were produced in high (~1-2 ng/ml and 1-200 pg/ml, respectively) amounts in both EC and MPC-conditioned media was collected from cells grown on tissue culture plastic (TCP) and poly-ε-caprolactone (PCL) nanofibers (Figure 9), but factor production dropped precipitously when those cells were seeded on polymer fibers. TCP-conditioned media and an embryonic chicken dorsal root ganglia (DRG) culture system were used to analyze MPC effects on neurite outgrowth by live-dead staining (Figure 10). While neither cell type caused extreme increases in neurite extension and survival, the presence of either or both cell types supported neurite outgrowth upon custom fabricated conduit-mimics of longitudinally aligned fibers. A PCL fiber, MPC, and EC combination construct may be further developed as a conduit- and cell-based therapy to promote peripheral nerve regeneration.

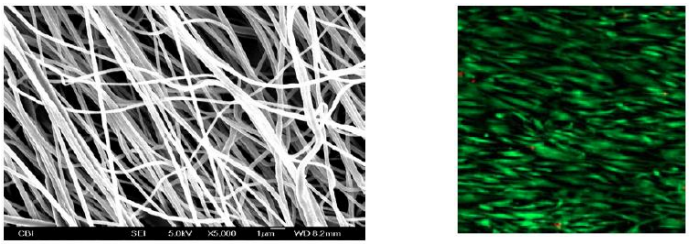

***Figure 9**. Aligned poly-ε-caprolactone nanofibers generated by electrospinning (left), and seeded with MPCs (stained with Live/Dead dye showing abundant live cells (green) on culture day 9 (right).*

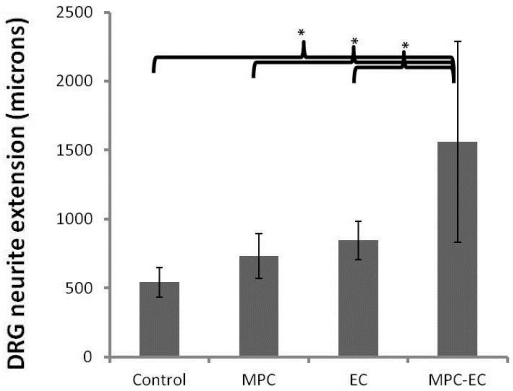

***Figure 10**. MPC, EC, and combination MPC-EC induced neurite extension. DRG neurite extensions were labeled with fluorescent dye and traced after 4 days of culture on aligned PCL nanofibers. The MPC-EC cell combination induced the longest neurite extensions. * = $p < 0.05$.*

In conclusion, with the goal of enhanced understanding of tissue repair, we have generated model systems that allow a more thorough investigation of physiologically relevant post-trauma cell interactions that may lead to the development of more nuanced regenerative medicine approaches.

B. <u>*Human Mesenchymal Stem Cells in Bone and Tendon Interaction*</u>

This project focuses on the biological interactions between bone and tendon/ligament tissue and the role adult MSCs may play in the osteointegration of graft to bone during anterior cruciate ligament (ACL) reconstruction surgery. Primary tenocytes and primary calvarial osteocytes were isolated from White Leghorn chicks at incubation day 14 and co-cultured in the following ratio (1:1 and 1:3) for a period of seven days. Samples were collected for RNA isolation, and the expression of fibrocartilaginous interface-associated genes including collagen type II, aggrecan, and cartilage oligomeric matrix protein (COMP) was examined using quantitative real-time polymerase chain reaction (qPCR). As shown in Figure 11, cartilage marker gene expression

peaks at day seven. Co-cultures grown for 10 days were then analyzed to see if longer time in culture can induce interface markers. Samples were collected for RNA isolation, and interface-specific genes including collagen type II, aggrecan, and COMP were examined using qPCR. After 10 days of co-culture, there was no increase in collagen type II or COMP gene expression but there was an increase in aggrecan expression in the 1:1 tenocyte to osteocyte cultures. Next, tenocytes, osteocytes, and adult bone marrow-derived MSCs were co-cultured in a 1:1:1 ratio for seven days. However, the presence of MSCs in the cultures did not cause an increase in cartilage marker gene expression (Figure 11). In conclusion, these findings failed to demonstrate a beneficial effect of MSCs on tendon-bone interface formation.

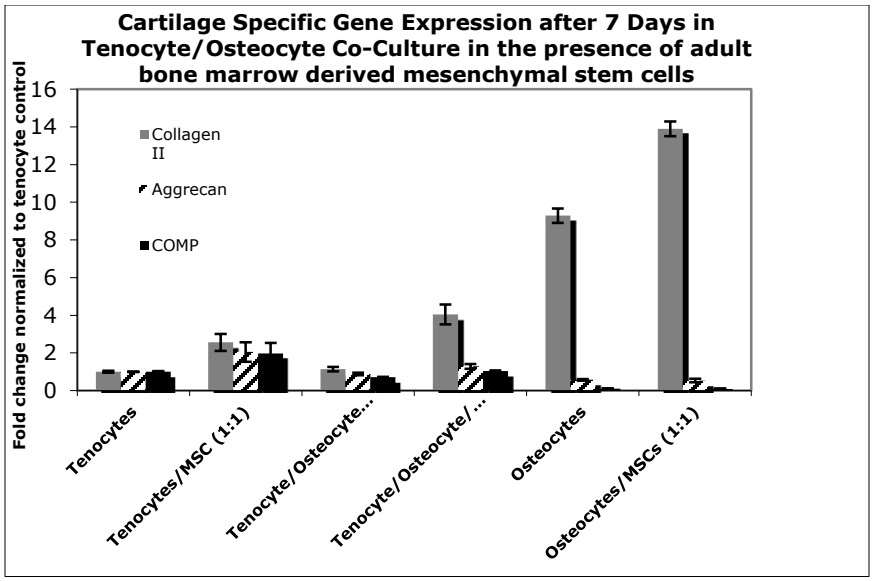

Figure 11: *Co-culture of primary tenocytes and primary calvarial osteocytes in the presence of adult bone marrow MSCs does not induce the expression of cartilage specific genes. Primary chick embryonic tenocytes and primary calvarial osteocytes were isolated at incubation day 14 and co-cultured with adult human MSCs in a 1:1:1 ratio for 7 days. RNA samples were isolated and gene expression of the cartilage (interface) specific genes- collagen type II, aggrecan, and cartilage oligomeric matrix protein (COMP), were analyzed by qRT-PCR.*

AIM 3. Cartilage Degeneration and Regeneration: *In vitro Mechanisms and In vivo Repair*

Project (3) Pathogenic Mechanisms of Cartilage Degeneration and Application of Adult Stem *Cells for Cartilage Tissue Regeneration: In vitro and In vivo Studies*

A. Epigenetic Changes during Mechano- and Inflammation-Mediated Injury of Chondrocytes
Introduction We are exploring molecular mechanisms responsible for the effects of injurious mechanical strain and inflammatory environment on chondrocytes, which may lead to post-traumatic cartilage degeneration. Specifically, we are targeting the group of enzymes, histone deacetylases (HDACs), which modify chromatin structure and subsequent gene expression activities. HDACs are known to be involved in epigenetic mechanisms and have been observed to change in levels of expression in osteoarthritic chondrocytes. In this study, we have used both 2-dimensional (2D) culture and 3D cartilage tissue explant systems to analyze the potential functional involvement of HDACs in degenerative changes in chondrocytes. Specifically, we investigated the potential of HDAC inhibitors (HDIs) to ameliorate the effects associated with cartilage mechanical and inflammatory insults. The relationship between mechanical injury and cartilage degeneration is of particular importance since >12% of all cases of osteoarthritis (OA) may be traced to a post-traumatic origin.

There are as yet no effective therapeutic interventions that ameliorate the progression of the disease. Conventional treatment for OA utilizes non-steroidal anti-inflammatory drugs (NSAIDS) which act mainly to reduce pain, and ultimately replacement of the joint is required. In addition, NSAIDS have well-known and severe side-effects. Intra-articular hyaluronan injection represents another palliative treatment, while the effectiveness of the neutraceuticals, such as glucosamine/chondroitin, remains unclear. Therefore, new, safe and more efficacious therapeutic agents are needed in the treatment of this debilitating disease. The search for candidate disease modifying osteoarthritis drugs (DMOADs) is one of the major goals of current OA research.

Epigenetic changes in chromatin structure represent a principal means of gene regulation, and their role in OA has attracted attention recently. Among these, histone modification through reversible acetylation is a crucial event in gene expression. Histone acetylation state is regulated by the activity of the enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, the acetylation of histones promotes increased access to the DNA for transcription factors – thus generally HDACs act as transcriptional suppressors while HATs promote gene expression.

Pharmacological inhibition of HDAC activity has been shown to be beneficial in various malignancies and a wide variety of immunological and inflammatory disorders. Many of these studies suggest HDAC involvement in mediating degradative responses in these diseases. It is reported that HDAC inhibitors (HDIs) can reduce the expression of inflammatory mediators, such as tumor necrosis factor-α (TNFα), interleukin (IL)1β, IL6, IL8, transforming growth factor-β (TGFβ), and nitric oxide (NO) that are involved in the pathogenesis of inflammatory diseases including arthritis.

The mechanism of HDAC stimulation and its exact contribution to cartilage degeneration is not clearly understood, but likely involves signaling via NF-kB, mitogen activated protein kinase (MAPK), and STAT, and mediated by NO and PGE2 production, both prevalent mediators of early OA-like degradation following traumatic injury.

Recent findings have suggested an important role for HDAC-mediated epigenetic changes in OA: (1) chondrocytes from OA patients showed higher levels of HDAC1, 2 and 7 and associated down-regulation of cartilage anabolic genes; (2) chondrocytes transfected with HDAC1 and 2 down-regulate cartilage matrix gene expression, including collagen type II α1 (COL2A1) and aggrecan (ACAN); (3) pharmacologic inhibition of HDAC increased SOX9-regulated cartilage matrix genes and antagonized FGF2 and IL1β induction of MMP expression in human articular chondrocytes in vitro; and (4) administration of trichostatin-A (TSA), a pan-HDI, in animal models of OA resulted in reduced cartilage degradation.

Overall findings from these previous studies suggest that classical HDACs have a negative role in cartilage homeostasis and are associated with OA, and therefore represent a potential target for OA therapy. However more studies are needed to understand the mechanism of HDAC/HAT regulation of genes involved in OA

Methods Different culture systems such as bovine and human chondrocytes in monolayer cultures and bovine cartilage explants were used in this study. Second passage bovine chondrocytes, isolated from knees of adult cows were plated as high density monolayer cultures on regular 6-well tissue culture plates or Bioflex (Flexcell) culture plates for mechanical loading experiments. The Bioflex plates were previously coated with fibronectin (5 μg/ml) for 2 h at room temperature. The cells were cultured for 2 days until they reached confluence and then serum-starved in 1% serum-containing medium for 24 hours before the start of the loading experiment or the IL1β treatment. The Bioflex plates were loaded using a custom-made mechanical loading system using the following parameters: cyclic tensile strain (CTS) at 16% tension and 0.5 Hz for 3, 24 or 48 h, conditions we previously established to create a chondrocyte gene expression pattern similar to that of OA chondrocytes. Initially the cells were loaded for 48h and after loading was stopped they were treated for additional 24h with a number of HDIs, such as trichostatin A (TSA), valproic acid (VPA), MS-275 (MS), PCI 34015 (PCI), at different concentrations. Therefore the subsequent analysis was done 24h following loading. We believe that it is possible that a number of the changes seen at this point are not directly caused by mechanical injury, but could be the effect of compensatory mechanisms. In order to eliminate this potential problem chondrocytes exposed to 48 h of loading were treated with HDIs after 24 h of loading and continued for 24 more hours while being mechanically loaded. In addition, the bovine chondrocytes were incubated with HDI in the presence of IL1β for 24 hours.

Human OA chondrocytes were isolated from cartilage obtained as surgical waste from patients undergoing joint replacement surgery and normal chondrocytes were obtained from cartilage procured at autopsy by NDRI. Human passage 2 chondrocytes were incubated with IL1β and HDI for 24 hours, following a similar protocol as for bovine chondrocytes.

RNA was isolated after treatment and cartilage gene expression was analyzed by SYBR Green real-time PCR with ΔΔ Ct method. In addition, melting curve analysis was performed for all the primer-pairs investigated. Cellular protein was also isolated after 3, 24 and 48 h of loading and after HDI and IL1b treatments. Western blot analysis was performed for acetyl-histone 3 (A-H3), total H3, p-NF-kB p65, HDAC2, HDAC8 and β-actin. Culture medium was also collected after loading or IL1β treatment and MMP3 secretion was analyzed in these samples by western blotting.

We also used an explant culture model, in which adult bovine articular cartilage plugs harvested from the patellofemoral groove were either unimpacted (CTRL) or impacted with loads of 17 or 36 MPa, representing sub-injurious and injurious loads, respectively. Cartilage explants were cultured in media and then collected at several time points (1, 3 and 7 days). Total RNA was isolated from 3 independent experiments. Global gene expression changes were determined by microarray analysis using bovine Gene Chip v1 Affymetrix arrays and the J5 algorithm within the web application caGEDA developed at University of Pittsburgh Genomics and Proteomics Core. The resulting gene lists, ranked by their J5 score, were then uploaded into the Ingenuity Pathway Analysis (IPA) for identification of relevant biological groups.

In addition 5 mm bovine cartilage explants were collected and equilibrated in medium for 2 days before exposure to IL1β and HDI (TSA, VPA, MS, PCI, tubastatin A). The samples were harvested after 72 hours of treatment, while culture medium was collected after 24 and 72 hours.

MMP3 secretion was analyzed in the sampled medium at both time points.

Results

Bovine chondrocytes treated with different HDIs demonstrated increased acetylated-histone 3 (A-H3) levels, which is consistent with suppression of HDAC activity. VPA proved to be the least potent, as a high concentration (10 mM) was needed to obtain increased levels of A-H3 (Figure 12).

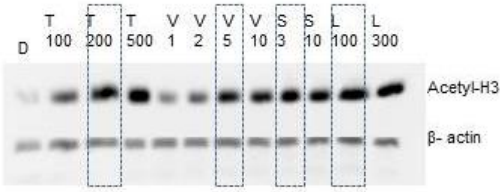

Figure 12: *Western blot of acetylated-H3 in bovine chondrocytes treated with HDAC inhibitors. Boxed doses were chosen for subsequent experiments – TSA-T, VPA-V, SAHA-S, LAQ-L*

One of the most significant and consistent gene expression changes under loading conditions was represented by increased iNOS expression at all the time points investigated (Figure 13). The factors responsible for this increase in iNOS expression appeared to be secreted in the medium, as treatment of chondrocytes with medium obtained from cultures after 48 h of loading also increased iNOS expression, although to a lower extent than immediately after loading. The HDIs produced a generalized decrease in cartilage gene expression in both the anabolic category and as well as in the catabolic group. Among these genes we report here: *COL2A1*, *ACAN*, *iNOS* and *MMP13*. These effects were noticed at different inhibitor concentrations, usually in a dose-dependent manner (Figure 14). These changes did not seem to be caused by a general toxic effect of the inhibitors as both Live/Dead staining and LDH production were not significantly changed between inhibitors and control conditions. The changes in gene expression were noticed in both non-loading and loading conditions, with some exceptions: *MMP13* was decreased by HDAC inhibitors under control conditions, but it was not significantly changed under CTS.

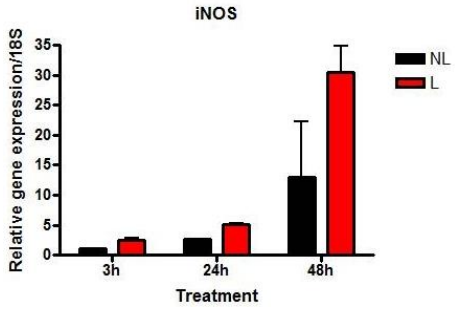

Figure 13: *iNOS mRNA expression in bovine chondrocytes subjected to 3, 24 and 48h CTS - loading (L) compared to non-loading (NL).*

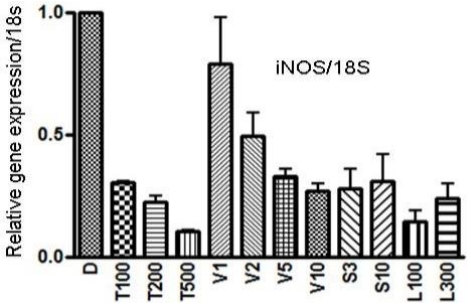

Figure 14: *iNOS expression in bovine chondrocytes treated with four HDAC inhibitors or DMSO (D) control for 24 hours, TSA (T) at 100, 200 and 500 nM, VPA (V) at 1,2,5 and 10 mM, SAHA (S) at 3 and 10 µM and LAQ824 (L) at 100 and 300 nM.*

While broad spectrum HDIs, e.g., TSA (trichostatin A), decreased MMP13 (matrix metallopeptidase 13 gene) and iNOS (inducible nitrous oxide synthase gene) expression in bovine chondrocytes, they also upregulated MMP3 and COX-2 (cyclooxygenase-2) levels, which are involved in cartilage degeneration. IL1β treatment further enhanced the effects of TSA (Figure 15). Because these inhibitors could influence a number of HDACs with either protective or degenerative activities in chondrocytes, we investigated the effects of more specific HDIs. An HDAC8-specific inhibitor, PCI, did not show the additive effects of TSA and IL1β on the MMP3 and COX-2 expression. A slight increase in HDAC8 protein levels was seen after 24 hours of loading. PCI is thus a promising candidate for reducing the levels of inflammatory and catabolic markers in chondrocytes exposed to pro-inflammatory cytokines.

The additive effect of broad HDIs (e.g., TSA) and IL1β seems to be specific to bovine chondrocytes in monolayer cultures as experiments with human chondrocytes and bovine cartilage explants showed either a beneficial effect of HDI (bovine cartilage plugs - Figure 16A) or no change (human chondrocytes) in the case of MMP3 secretion (Figure 16B).

One possible explanation for this difference in response between bovine and human chondrocytes is the presence of a dimer product of phospho-NF-kB p65 in the presence of IL1β in the human chondrocytes treated with L1β and IL1β + TSA. This product was only present (or produced at much higher levels) in bovine chondrocytes treated with both IL1β and TSA but not IL1β alone.

Surprisingly, explant culture treatment with PCI was not as efficient as in monolayer cultures, as the levels of MMP3 were not affected in the presence of IL1β (Figure 16A). Additional work is thus warranted.

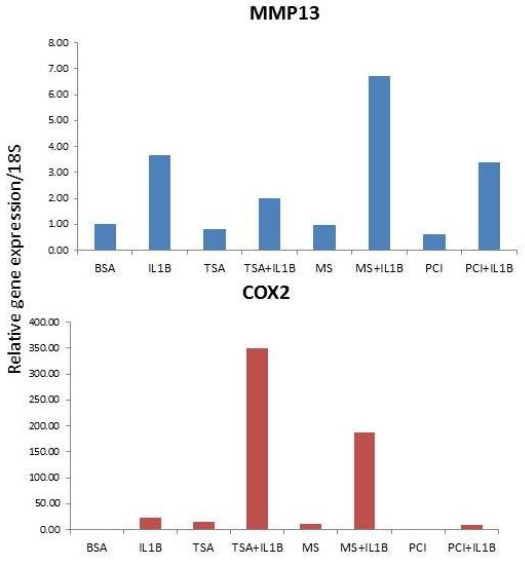

Figure 15: *Treatment with IL1β and HDI (TSA, MS275) results in higher COX-2 expression than IL1β alone. Treatment with PCI decreased IL1β-induced COX-2 expression. IL1β induced -MMP13 expression is decreased by TSA, but not by MS275.*

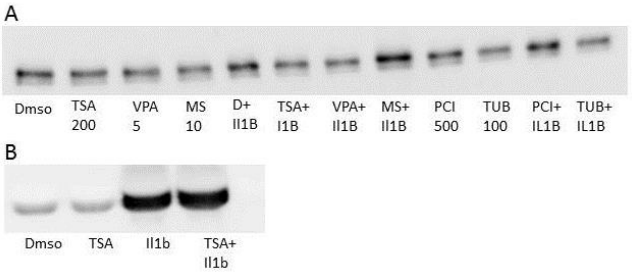

Figure 16. (A) *MMP3 secretion in media from bovine cartilage plugs treated with IL1β and HDIs for 72h. Decreased IL1β-induced MMP3 secretion with TSA, VPA and tubastatin A (TUB).* ***(B)*** *Normal human chondrocytes treated with IL1β & TSA for 24h.*

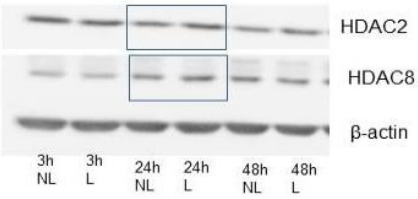

Figure 17: *Increased HDAC2 and HDAC8 after 24 h of loading*

Another possible target for OA therapy could be HDAC6, as treatment with an HDAC6-specific inhibitor, tubastatin A, decreased MMP3 secretion under both basal and IL1β stimulated-conditions in the explant culture model (Figure 16A). In addition, injurious mechanical loading in healthy bovine chondrocytes resulted in decreased levels of acetylated histone 3 (A-H3), accompanied by increased levels of HDAC2 and HDAC8 (Figure 17). Given that the main activity of HDACs is to de-acetylate histones, generally thought to inhibit gene transcription, increased HDAC activity is probably responsible for reduction in A-H3 with mechanical insult.

In the last part of our study we aimed to analyze the genes differentially expressed within the first week after applying mechanical trauma to articular cartilage. Investigating the global gene expression profiles would allow us to identify functional gene groups affected by mechanical injury that could be overlooked by candidate gene analysis. These gene expression changes are likely to provide insight into the early etiological mechanisms of physical trauma and osteoarthritis (PT-OA) that may lead to identification of early therapeutic targets. We will then

investigate the effects of HDIs on gene expression changes following mechanical (impact) injury.

Our results showed that, common among all affected groups in comparison to controls at every time point was the increased gene expression associated with the Ingenuity Pathway Analysis (IPA) canonical pathway/functional category "role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis," containing genes such as *BMP2*, *BMP4*, *MMP13*, *FRZB*, *TNSF11*, and *ITGP3* . The biological networks associated differed with impact magnitude and time. After 24 hours, gene networks associated with "skeletal and muscular system development and function, tissue development, connective tissue disorders" (*MATN3*, *MMP13*, *FGF18*, *DMP1*, *BMP2*, *BMP4*, *COL9A1*, *TGFB3*, etc.) were active after a 17 MPa mechanical impact as compared to the biological network "cellular development, growth, and proliferation, skeletal and muscular system development and function" (*COL10A1*, *VCAN*, *WISP1*, *TGFB3*, etc.), which was differentially expressed after tissues were subjected to a 36 MPa mechanical force. This force also increased expression of genes in the category "granulocyte adhesion and diapedesis" (*CCL20*, *CXCL14*, *CXCR2*, *PECAM1*, etc.), indicating a greater inflammatory response. Three days after mechanical trauma, both the 17 Mpa and 36MPa groups showed increased expression of genes in the "cell cycle, cellular assembly and organization, DNA replication, recombination and repair" group represented by genes such as *CDK1*, *CENPE*, *CENPF*, *MATN3*, among others. Similar biological groups were also identified seven days after impact in the 17 MPa group with representative genes such as *BMP4*, *MMP13*, *OMD*, *COL10A1*, *COL1A1*, *and DMP1*. In the seven day, 36 MPa group, we observed markers such as *IBSP*, *BAMBI*, *FGF7*, *SP7*, and *POSTN*.

While we observed differential expression of catabolic genes such as *MMP13* and *MMP3* and anabolic genes like *COL2A1*, *COL1A1*, and *MATN3* in most force-exposed groups and at most time points, we found that each condition was also characterized by the expression of specific sets of genes. Twenty-four hours after high impact we noticed the expression of pro-inflammatory genes (e.g. *IL6*). Three days following impact, the top functional pathway identified by IPA in both the 17 MPa and the 36 MPa groups contained genes involved in cell cycle and DNA repair, suggesting the activation of a common reparative mechanism. At seven days, gene expression of cells in the 17MPa and 36 MPa samples diverged between chondrogenic/hypertrophic and osteogenic functional groups. This difference may reflect a more advanced disease state in the high impact group or a different response altogether.

A number of these genes were validated by qRT-PCR and demonstrated results similar to the microarray analysis (e.g., IL6 and TGFB3 increased 24 hours following 36MPa impact while DMP1 was increased by the 17MPa force). Many of the genes identified in this study were previously known markers of OA, indicating that further validation and functional analyses of novel loci identified here could lead to the development of new PT-OA therapeutic candidates.

Taken together, our findings shown above suggest that a number of HDACs may be important players in regulating chondrocytes, and we are therefore investigating their possible role as therapeutic targets in the treatment of OA and the optimization of cartilage repair.

B. *Mechanosensitive β-Catenin Signaling Modulates MSC Chondrogenesis*

While MSCs are considered a promising candidate as chondroprogenitor cells, the current

differentiation protocols result in the development of additional phenotypic features common to hypertrophic and osteoarthritic chondrocytes, which are undesirable in the context of articular cartilage repair. We have investigated the roles of cyclic hydrostatic pressure and canonical Wnt/β-catenin signaling in regulating the phenotype of chondrogenic MSCs. We used a standard chemically-defined medium containing TGF-β to induce chondrogenic differentiation in pellets of human MSCs. Hydrostatic pressure was applied using a custom-built loading chamber. β-catenin signaling was modified using the small molecules XAV939 (for inhibition) and CHIR99021 (for stimulation). The chondrogenic MSCs in this culture system resembled osteoarthritic chondrocytes, co-expressing features of both articular and hypertrophic chondrocytes. Cyclic hydrostatic pressure favored a modest shift in the phenotypic balance away from hypertrophy and toward chondrogenesis. In addition, cyclic hydrostatic pressure inhibited intracellular signaling via the ERK1/2 and canonical Wnt/β-catenin pathways. Inhibition of β-catenin signaling with XAV939 significantly augmented chondrogenesis (Figure 18) and inhibited hypertrophy, suggesting that this may be a potential mechanism by which hydrostatic pressure is transduced in MSCs. Stimulation of β-catenin with CHIR99021, inhibited both types of chondrogenic differentiation. These results strongly suggest that β-catenin is an attractive target for phenotypic modulation of chondrogenic MSCs, and has potential applications in cartilage regeneration therapies from tissue engineering to targeted drug delivery to gene therapy.

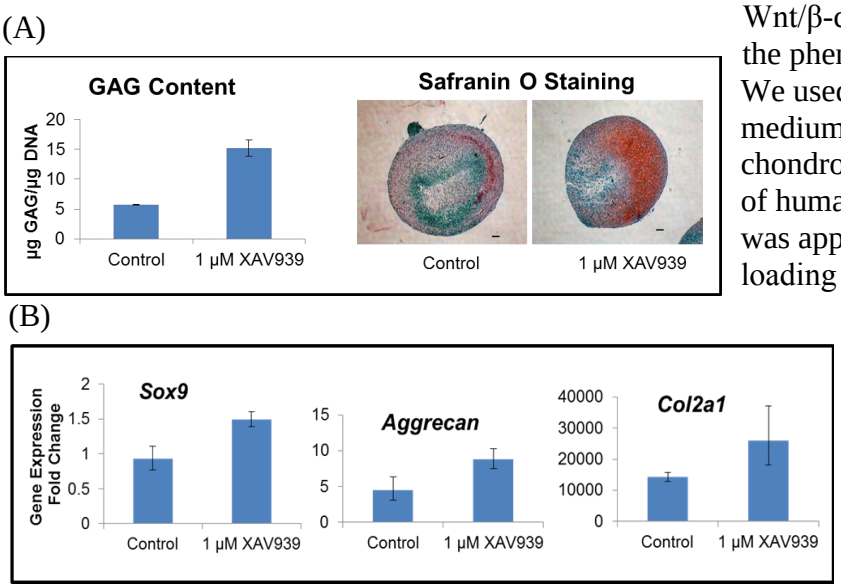

Figure 18. *Effect of XAV939, a β-catenin inhibitor, on MSC chondrogenesis. (A) Cartilage matrix deposition (GAG content and Safranin O staining); (B) Expression of chondrocyte-specific genes, Sox9, aggrecan, and Col2a1.*

C. *Repair of Articular Cartilage Defect In Vivo*

We have initiated articular cartilage repair studies using two animal models. In a rabbit study, we have recently established a mechanical impact-mediated model of articular cartilage degeneration. A spring-loaded device was used to impact the femoral condyle, creating cartilage fissure that results in frank degeneration in 12 weeks. This model, as well as a surgically produced defect model of the femoral condyle, are currently being used to examine the efficacy of tissue-engineered osteochondral constructs.

In a goat study, which was started in April, 2013, we began by surgically creating a seven millimeter-diameter defect to optimize placement of an engineered osteochondral construct-plug to repair the defect. Initial surgery proceeded in a satisfactory manner, to be followed in the near future with the repair study. For the production of 3-dimensional cell-based tissue constructs, we plan to use our recently developed method of 3-D printing (projection stereolithography),

using MSCs seeded within a photocrosslinked polymeric scaffold, to fabricate the osteochondral unit for implantation (Figures 19 and 20).

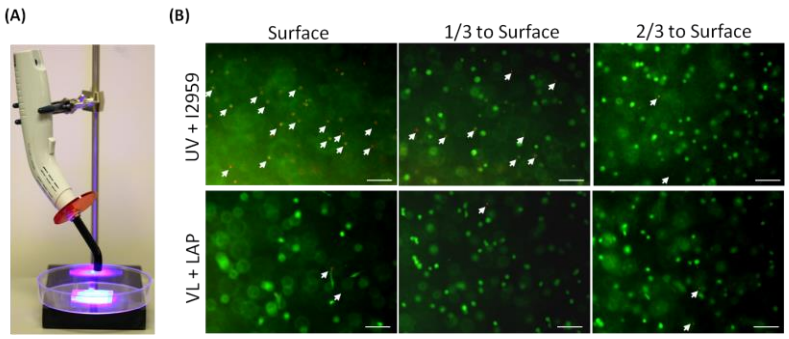

***Figure 19**. (A) Visible light activated-gelation using illumination from a dental lamp (wavelength 430~490nm, power 1,400 mw/cm^2). (B) Live/Dead analysis of hBMSCs encapsulated into the methacrylated gelatin hydrogel at different depth, using traditional ultraviolet (UV)/I2959 or visible light(VL)/LAP. Green = Live cells, Red or orange = Dead cells as indicated by arrows. Bar= 100 µm. Improved, high viability of cells is seen with the visible light (LAP) photocrosslinked hydrogel.*

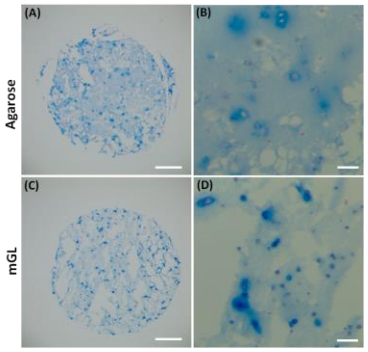

***Figure 20**. Alcian blue staining of sGAG in histological sections of the hBMSC-laden mGL constructs compared to agarose constructs after 90 days of chondrogenic culture with Fast Red as nuclear counterstaining. Bar= 1 mm (A, C) or 100 µm(B, D).*

<u>Posters and Presentations at Meetings</u>:

Author(s)	Title	Meeting	Abstract
Boyette L, Creasey O, Tuan RS	β-catenin/FoxO signaling in adult stem cells at low oxygen tension (poster)	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2011, Farmington, PA (poster)	
Boyette L, Xu W, Creasey O, Tuan RS	Inducing resistance to senescence in adult stem cells using transcriptional reprogramming (oral presentation)	Orthopaedic Research Society Annual Meeting, Long Beach, CA 2011 (poster)	
Boyette L, Tuan RS	Reprogramming transcription factors induce differential gene expression in pathways regulating stemness versus differentiation	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2010, Farmington, PA (poster)	

Jiang Y, Lin H, Tuan RS	The tropism of cartilage stem/progenitor cells from tissue and the genotype signature during in vitro culture	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2013, Farmington, PA (poster)	
Jiang Y, Hu CC, Zhang W, Zhang SF, Lu P, Yang D, Yin Z, Wang LL, Tuan RS, Ouyang H-W	Cartilage stem/progenitor cells: characterization and role in osteoarthritis	11th ISSCR (International Society for Stem Cell Research), June 12-15, 2013 Boston, MA (poster)	
Jiang Y, Chen, L, Zhang S, Tong T, Zhang W, Liu W, Tuan RS, Xiao Y, Ouyang H-W	Iodine promotion of osteogenic differentiation enhances stem cell-bioscaffold based subchondral bone regeneration	11th ISSCR (International Society for Stem Cell Research), June 12-15, 2013 Boston, MA (poster)	
Statman LY, Taboas JM, Aliston T, Tuan RS	Mechanobiology of cyclic hydrostatic pressure in chondrogenic MSCs	Orthopaedic Research Society Annual Meeting, January 2011 Long Beach, CA (poster)	
Statman LY, Taboas JM, Alliston T, Tuan RS	Inhibition of β–catenin enhances mesenchymal stem cell chondrogenesis	Orthopaedic Research Society Annual Meeting, February 2012 San Francisco, CA (poster)	
Ulici V, Taboas JM, Alexander PG, Tuan RS	HDAC inhibitors decrease gene expression in chondrocytes cultured under high tensile strain	Orthopaedic Research Society Annual Meeting February 2012 San Francisco, CA (poster)	
Ulici V, Taboas JM, Alexander PG, Tuan RS	HDAC inhibitors decrease gene expression in chondrocytes cultured under high tensile strain	Keystone Symposia – Regenerative Tissue Engineering and Transplantation, Breckenridge, CO 2012 (poster)	
Ulici V, Taboas JM, Alexander PG, Tuan	HDAC inhibitors decrease gene	University of Pittsburgh 7th Annual	

RS	expression in chondrocytes cultured under high tensile strain	Data & Dine, Pittsburgh, PA 2012 (poster)	
Ulici V, Zavadak E, Tuan R	Effect of inhibitors of specific histone deacetylases (HDAC) on chondrocytes	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2013, Farmington, PA (poster)	
Ulici V, Zavadak E, Tuan R	Effect of inhibitors of specific histone deacetylases (HDAC) on chondrocytes	2013 OARSI (Osteoathritis Research Society International) World Congress, April 18-21, Philadelphia, PA (poster)	Abstract published:Osteoarthritis and Cartilage 21: S171-172, 2013
Hofer HR, Alexander PG, Gorman S, Tuan RS	Co-cultures for enhanced conduit-based peripheral nerve repair	Military Health Sciences Research Symposium, August 2013, Ft. Lauderdale, FL (poster)	
Hofer HR, Alexander PG, Gorman S, Tuan RS	Neurotrophic activity of multipotent progenitor cells cultured on nanofiber scaffolds	University of Pittsburgh Pathology Retreat, May 2013, Pittsburgh, PA (poster)	
Hofer HR, Alexander PG, Tuan RS	Neurotrophic activity of multipotent progenitor cells cultured on nanofiber scaffolds	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2013, Farmington, PA (poster)	
Hofer HR, Alexander PG, Tuan RS	Nerve support: differentiated mesenchymal stem cells and substrate guidance	Military Health Sciences Research Symposium, August 2012, Ft. Lauderdale, FL (poster)	

Hofer HR, Alexander PG, Tuan RS	Nerve support: differentiated mesenchymal stem cells and substrate guidance	University of Pittsburgh Pathology Retreat, May 2012, Pittsburgh, PA (poster)	
Hofer HR, Alexander PG, Tuan RS	Mesenchymal stem cells as neural support cells	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2012, Farmington, PA (poster)	
Hofer HR, Tuan RS	Mesenchymal stem cells and the functional analysis of stemness factor CCL-2	University of Pittsburgh Pathology Retreat, May 2011, Pittsburgh, PA (poster)	
Hofer HR, Tuan RS	Mesenchymal stem cells and the functional analysis of stemness factor CCL-2	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2011, Farmington, PA (poster)	
Lin H, Tang Y, Oyster N, Wang B, Tuan RS	Projection stereolithographic fabrication of bone scaffold encapsulated with Lenti-BMP-2 transduced human bone marrow stem cells (hBMSCs)	Tissue Engineering and Regenerative Medicine International Society (TERMIS) Americans Annual Meeting, 2013, Atlanta, GA (poster)	
Lin H, Tang Y, Tan J, Wang B, Tuan RS	Single-step projection stereolithographic fabrication of gene-activated bone scaffold encapsulating human bone marrow stem cels and Lenti-BMP-2 viral vector	Orthopaedic Research Society (ORS) Annual Meeting, 2013, San Antonio, TX (NIRA finalist presentation, poster)	

Lin H, Cheng AWM, Alexander PG, Beck AM, Tan J, Tuan RS	Tissue engineering application of visible light-activated gelatin hydrogel with in situ gelation capability in both air and aqueous solution	Orthopaedic Research Society (ORS) Annual Meeting, 2013, San Antonio, TX (poster)	
Lin H, Zhang DN, Yang G, Tan J, Alexander P, Tuan RS	Fabrication of 3-dimensional live cell biomaterial constructs using a visible light-based stereolithographic printer	Orthopaedic Research Society Annual Meeting, February 2012 San Francisco, CA (podium presentation)	
Lin H, Ma ZW, Tan J, Alexander P, Tuan RS, Taboas JM	Alginate methacrylation in dimethyl sulfoxide for photopatterning tissue engineering scaffolds	Tissue Engineering and Regenerative Medicine International Society (TERMIS) – North America 2011, Houston, TX (poster)	
Lin H, Taboas JM, Tan J, Tuan RS	Decellularized autologous mesenchmal stem cell (MSC0 extacellular matrix enhances MSC migration and differentiation potential	Orthopaedic Research Society Annual Meeting, January 2011 Long Beach, CA (poster)	
Yang G, Rothrauff BB, Lin H, Alexander PG, Tuan RS	Tendon-derived extracellular matrix enhances tenogenic differentiation of human adipose derived stem cells in 3D collagen scaffold under tension	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2013, Farmington, PA (poster)	

Yang G, Rothrauff BB, Lin H, Gottardi R, Alexander PG, Tuan RS	Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix	Tissue Engineering and Regenerative Medicine International Society (TERMIS) Americans Annual Meeting, 2013, Atlanta, GA (oral presentation)	

Lin H, **Cheng AWM**, Alexander PG, Beck AM, Tan J, Tuan RS	Tissue engineering application of visible light-activated gelatin hydrogel with in situ gelation capability in both air and aqueous solution	Orthopaedic Research Society (ORS) Annual Meeting, 2013, San Antonio, TX (poster)	
Cheng AW, Alexander PG, Tuan RS	Co-culturing with MSCs in vitro alters early post-traumatic	Gordon Research Conference: Musculoskeletal	
Baker N, Tuan R	Promotion of human MSC osteogenesis by P13-kinase/Akt signaling and potential influence of cholesterol	2013 OARSI (Osteoathritis Research Society International) World Congress, April 18-21, Philadelphia, PA (poster)	Abstract published:Osteoarthritis and Cartilage 21: S121-122, 2013
Baker N, Tuan R	Promotion of human MSC osteogenesis by P13-kinase/Akt signaling and potential influence of cholesterol	University of Pittsburgh 8th Annual Data & Dine, Pittsburgh, PA 2013 (poster)	

Baker N, Malagise C, Tuan R	Caveolin-1 in human mesenchymal stem cells: involvement in cell proliferation, osteogenic differentiation, and differential effects on Wnt/β-catenin signaling in male and female cells	Orthopaedic Research Society Annual Meeting, January 2011 Long Beach, CA (poster)	
Baker N, Malagise C, Tuan R	Caveolin-1 in human mesenchymal stem cells: involvement in cell proliferation, osteogenic differentiation, and differential effects on Wnt/β-catenin signaling in male and female cells	Gordon Research Conference in Musculoskeletal Biology & Bioengineering, New Hampshire, August,2010 (poster)	
Baker N, Tuan R	Caveolin-1 in human mesenchymal stem cells: involvement in cell proliferation, osteogenic differentiation, and differential effects on Wnt/β-catenin signaling in male and female cells	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2010, Farmington, PA (poster)	
Diederichs S, Tuan R	Mesenchymal progenitors derived from human induced pluripotent stem cells	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2012, Farmington, PA (poster)	

Diederichs S, Tuan R	Mesenchymal progenitors derived from human induced pluripotent stem cells	Wurzbug Initiative Tissue Engineering (WITE): Strategies in Tissue Engineering, May 23-25, 2012, Wurzburg, Germany (oral presentation)	
Diederichs S, Tuan R	Mesenchymal progenitors derived from human induced pluripotent stem cells	International Society for Stem Cell Research (ISSCR), June 13-16, 2012, Yokohama, Japan (poster)	
Diederichs S, Tuan R	Mesenchymal progenitors derived from human induced pluripotent stem cells	Tissue Engineering International and Regenerative Medicine Society (TERMIS), September 5-8, 2012, Vienna, Austria (poster)	
Bean AC, Tuan RS	Influence of scaffold fiber diameter and cell source on in vitro stem cell-based skeletal tissue engineering	MSC 2013, Adult Stem Cell Therapy & Regenerative Medicine, Cleveland, OH, August 19, 2013 (poster)	
Bean AC, Koch RG, Tuan RS	Effects of seeding density and fiber diameter on chondrogenesis of mesenchymal stem cells in vitro	Tissue Engineering and Regenerative Medicine International Society Annual Meeting, Houston, TX, December 19, 2011 (poster)	

Bean AC, Alexander PG, Tuan RS	Comparison of chondrogenic differentiation of bone marrow and adipose-derived stem cells seeded on electospun fibers of different diameters	Tissue Engineering and Regenerative Medicine International Society Annual Meeting, Houston, TX, December 19, 2011 (poster)	
Bean AC, Tuan RS	Effects of fiber diameter and surface area on protein adsorption onto poly (ε−caprolactone) scaffolds	7th Annual Meeting of the American Physician Scientists Association, Chicago, IL, April 2011 (poster)	
Brick R, Tuan R	Induced mesenchymal progenitor cells produce neurotrophic factors to id nerve regeneration	BSGA Symposium, October 23, 2013, University of Pittsburgh, Pittsburgh, PA (poster)	
Langhans MT, Cui C, Lo CW, Tuan RS	Exploring the effects of defective ciliogenesis in the Mks1 mutant mouse	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2012, Farmington, PA (poster)	
Langhans MT, Alexander PG, Tuan RS	Wdpcp affects skeletal tissue differentiation and patterning	McGowan Institute for Regenerative Medicine Annual Retreat, March, 2013, Farmington, PA (poster)	

Brick R, Tuan R	Induced mesenchymal progenitor cells produce neurotrophic factors to id nerve regeneration	Cellular and Molecular Pathology Retreat, May 22, 2013, University of Pittsburgh, Pittsburgh, PA (poster)	

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one

publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.Influence of decellularized matrix derived from human mesenchymal stem cells on their proliferation, migration and multi-lineage differentiation potential	Hang Lin, Guang Yang, Jian Tan, Rocky S. Tuan	Biomaterials	January 2012	☐Submitted ☐Accepted ☒Published
2.Caveolin-1 regulates proliferation and osteogenic differentiation of human mesenchymal stem cells	Natasha Baker, Guofeng Zhang, Yang You, Rocky S. Tuan	Journal of Cellular Bochemistry	June 2012	☐Submitted ☐Accepted ☒Published
3.The promise and challenges of stem cell-based therapies for skeletal diseases	Solvig Diederichs, Kristy M. Shine Rocky S. Tuan	Bioessays	2012	☐Submitted ☐Accepted ☒Published
4. Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture	Hang Lin, Dongning Zhang, Peter G. Alexander, Guang Yang, Jian Tan, Anthony Wai-Ming Cheng, Rocky S. Tuan	Biomaterials	August 2012	☐Submitted ☐Accepted ☒Published
5. Incorporation of bioactive polyvinylpyrrolidone-iodine within bilayered	Yangzi Jiang, Longkun Chen, Shufang Zhang, Tong Tong,	Acta Biomaterialia	February 2013	☐Submitted ☐Accepted ☒Published

collagen scaffolds enhances the differentiation and subchonral osteogenesis of mesenchymal stem cells	Wei Zhang, Wanlu Liu, Gurwei Xu, Rocky S. Tuan, Boon Chin Heng, Ross Crawford, Yin Xiao, Hong Wei Ouyang			
6.The less-often-traveled surface of stem cells: caveolin-1 and caveolae in stem cells, tissue repair and regeneration	Natasha Baker, Rocky S. Tuan	Stem Cell Research & Therapy	2013	☐Submitted ☐Accepted ☒Published
7. Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix	Guang Yang, Benjamin B. Rothrauff, Hang Lin, Riccardo Gottardi, Peter G. Alexander, Rocky S. Tuan	Biomaterials	August 2013	☐Submitted ☐Accepted ☒Published
8.Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm	Guang Yang, Benjamin B. Rothrauff, Rocky S. Tuan	Birth Defects Research (Part C)	2013	☐Submitted ☐Accepted ☒Published
9. Human bone marrow-derived mesenchymal stem cells display enhanced clonogenicity but impaired differentiation with hypoxic preconditioning	Lisa B. Boyette, Olivia A. Creasey,Lynda Guzik, Thomas Lozito, Rocky S. Tuan	Stem Cells Trans Med	April 2013	☐Submitted ☐Accepted ☒Published
10. Maintaining the adult stem cell to combat diseases of aging	Lisa B. Boyette, Rocky S. Tuan	J Clin Med	November 2013	☐Submitted ☒Accepted ☐Published
11. Cartilage tissue engineering application of injectable gelatin hydrogel with in situ visible light-activated gelatin capabilitly in both air and aqueous solution	Hang Lin, Anthony Cheng, Peter G. Alexander, Angela M. Beck, Rocky S. Tuan	Tissue Engineering, Part A	October 2013	☐Submitted ☒Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____X____ No_____

If yes, please describe your plans:

We will continue to prepare manuscripts using data and findings obtained during the funding period of the grant. These publications will acknowledge the support of the Commonwealth of PA, Department of Health.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

Tuan , Rocky S., Professor and Executive Vice Chair for Orthopaedic Research, Department of Orthopaedic Surgery; Professor, Department of Bioengineering

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YR CONFERRED	FIELD OF STUDY
Swarthmore College, Swarthmore, PA			Chemistry
Berea College, Berea, KY	B.A.	1972	Chemistry
Rockefeller University, New York, NY Biochem./Cell Biol.	PhD	1977	
Harvard Medical School, Boston, MA	Postdoc	1977-80	Develop. Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE:

A. PERSONAL STATEMENT

My longstanding research activities in musculoskeletal biology and tissue regeneration cover basic science and engineering, as well as translational and clinical applications. In basic research, my interests range from skeletal patterning and embryonic cartilage development, to mechanistic aspects of cell signaling and matrix biology, to the biology of adult stem cells. My translational and engineering investigations include nanomaterials, bioscaffold design, bioreactor technology, the biology of cell-material interactions, and tissue engineering and regeneration. My clinical research topics of interest include periprosthetic osteolysis, molecular diagnosis of orthopaedic infection, development of biodegradable drug delivery systems, osteoarthritis and articular cartilage repair, and developmental skeletal defects. Our overall experimental approach has been to elucidate the cellular and molecular mechanisms of tissue growth and regeneration and to apply this knowledge in combination with biomaterials and mechanobiological manipulations to restore function and structure to damaged, injured or diseased tissue. Specifically, in the last decade, we have made significant progress in studying the fundamental mechanisms regulating adult stem cell proliferation, activation, and commitment and differentiation, which is now a major focus in the laboratory. In addition, I have actively mentored young scientists and taken part in the management of education and research programs in multiple roles – mentor of PhD and MD-PhD students; Academic Director of the MD-PhD (Thomas Jefferson University); founder and director of the Cell and Tissue Engineering PhD Program at Jefferson; training clinical residents and fellows in basic science research; and Co-Director of the Armed Forces Institute of Regenerative Medicine, a national initiative devoted to the development of regenerative therapies for battlefield injuries. I am the founder and Editor of two journals relevant to developmental biology and stem cells - ***Birth Defects Research. Part C: Embryo Today,*** and ***Stem Cell Research and Therapy***. The funding support by the Commonwealth of Pennsylvania Department of Health has enabled us to continue our productive investigation on the application of adult stem cells for the development of regenerative approaches to musculoskeletal diseases, particularly those involving tissue degeneration and dysfunction. By working with a group of young and talented investigators, ranging from students, to postdoctoral fellows and staff members, we have put together a dynamic research team that has made significant progress during the funding period. The Commonwealth support has been critical in enabling this success. I envision the continuation of these activities and the leveraging of our research efforts for additional, future funding support from federal agencies and private foundations.

B. POSITIONS AND HONORS

1974-1977 Laboratory of Cellular Physiology and Immunology, Rockefeller University
Ca^{2+}-binding protein of chick chorioallantoic membrane (PhD Thesis), Sponsor: Dr. Zanvil A. Cohn

1977-1978 Research Fellow, Dept Orthopaedic Surgery, Harvard Med Sch, Children's Hospital, Boston
Embryonic vitamin K metabolism and calcium transport (Sponsor: Dr. Melvin J. Glimcher)

1978-1980	Research Fellow, Dept Medicine, Harvard Med Sch, Developmental Biol. Lab, Mass. General Hospital Embryonic osteogenesis and calcium metabolism (Sponsor: Dr. Jerome Gross)
1980	Instructor in Medicine, Harvard Medical School, Boston
1980-1993	Assistant/Associate Professor, Adjunct Professor, Dept Biology, University of Pennsylvania
1992-1995	Academic Director, M.D.-Ph.D. Program, Thomas Jefferson University
1996-2001	Vice Chairman, Department of Orthopaedic Surgery, Thomas Jefferson University
1997-2001	Director, Ph.D. Graduate Program in Cell & Tissue Engineering, Thomas Jefferson University
1988-2004	Professor, Department of Orthopaedic Surgery, Thomas Jefferson University
2001-2009	Chief, Cartilage Biology and Orthopaedics Branch, NIAMS, NIH, Bethesda
2009-present	Professor & Executive Vice Chairman, Department of Orthopaedic Surgery, and Director, Center for Cellular and Molecular Engineering, University of Pittsburgh School of Medicine
2010	Arthur J. Rooney, Sr. Chair in Sports Medicine, University of Pittsburgh School of Medicine
2010-present	Co-Director, Armed Forces Institute of Regenerative Medicine
2012-present	Director, Center for Military Medicine Research, University of Pittsburgh School of Medicine
2012-present	Associate Director, McGowan Institute for Regenerative Medicine, Pitt/UPMC

MEMBERSHIPS AND EDITORIAL BOARDS

1992-present	Advisory Editor, *Journal of Arthroplasty*
1993/94-present	Editorial Boards, *Osteoarthritis and Cartilage, Cell Communication and Adhesion*
2003-present	Editor-In-Chief, *Birth Defects Research. Part C: Embryo Today (Wiley)*
2009-present	Co-Editor-in-Chief, *Stem Cell Research and Therapy*
2010-present	Council Member, *Society for Matrix Biology, Tissue Engineer & Regenerative Medicine Intl Society*
2011/2013	Vice Chair/Chair, Gordon Research Conf *"Cartilage Biology and Pathology"*
2011-present	Editorial Board, *Biomaterials, Tissue Engineering, Stem Cells Translational Medicine*
2012	Co-Organizer: Keystone Symposium "*Regenerative Tissue Engineering & Transplantation"*

C. SELECTED RELEVANT PEER-REVIEWED PUBLICATIONS (Total: over 400)

1. Jackson WM, Lozito TP, Djouad F, Kuhn N, Nesti LJ, **Tuan RS**. Differentiation and regeneration potential of mesenchymal progenitor cells derived from traumatized muscle tissue. J Cell Mol Med. 2011. 15(11):2377-88.
2. Jackson WM, Aragon AB, Onodera J, Koehler SM, Ji Y, Bulken-Hoover J, Vogler JA, **Tuan RS**, Nesti LJ. Cytokine expression in muscle following traumatic injury. J Orthop Res. 2011. 29(10):1613-20.
3. Mueller MB, **Tuan RS.** Anabolic/catabolic balance in pathogenesis of osteoarthritis. 2011. Phys Med Rehab. 3:S3-11
4. Jackson WM, Nesti LJ, **Tuan RS**. Clinical translation of wound healing therapies based on mesenchymal stem cells. Stem Cells Transl Med. 2012 Jan;1(1):44-50
5. Bulken-Hoover JD, Jackson WM, Ji Y, Volger JA, **Tuan RS**, Nesti LJ. Inducible expression of neurotrophic factors by mesenchymal progenitor cells derived from traumatically injured human muscle. Mol Biotechnol. 2012. 51(2):128-36.
6. Steinert AF, Rackwitz L, Gilbert F, Nöth U, **Tuan RS**. Clinical application of mesenchymal stem cells for musculoskeletal regeneration: Current status and perspectives. Stem Cells Transl Med. 2012. 1(3):237-47.
7. Jackson WM, Nesti LJ, **Tuan RS**. Mesenchymal stem cell therapy for attenuation of scar formation during wound healing. Stem Cells Res Ther. 2012. 3(3): 20.

8. Rackwitz L, Eden L, Reppenhagen S, Reichert JC, Walles H, **Tuan RS**, Rudert M, Nöth U. Stem cell and growth factor-based regenerative therapies for avascular necrosis of the femoral head. Stem Cell Res Ther. 2012. 3(1): 7.
9. Alexander PG, McCarron JA, Levine MJ, Melvin GM, Murray P, Manner PA, **Tuan RS**. An *in vivo* lapine model for impact-induced injury and osteoarthritic degeneration of articular cartilage. Cartilage. 2012. 3: 323-333.
10. Lin H, Taboas JM, Tan J, Yang G, **Tuan RS.** Decellularized matrix derived from human mesenchymal stem cells enhances their proliferation, migration and multi-lineage differentiation potential. Biomaterials. 2012. 33(18):4480-4489.
11. Xie X, Wang Y, Zhao C, Guo S, Liu S, Jia W, **Tuan RS**, Zhang C. Comparative evaluation of MSCs from bone marrow and adipose tissue seeded in PRP-derived scaffold for cartilage regeneration. Biomaterials. 2012. 33:7008-18.
12. Baker N, Zhang G, You Y, **Tuan RS**. Caveolin-1 regulates proliferation and osteogenic differentiation of human mesenchymal stem cells. J. Cell Biochem. 2012.113(12):3773-3787.
13. Jackson WM, Alexander PG, Bulken-Hoover JD, Vogler JA, Ji Y, McKay P, Nesti LJ, **Tuan RS**. Mesenchymal progenitor cells derived from traumatized muscle enhance neurite growth. J Tissue Eng Regen Med. 2013 Jun;7(6):443-51
14. Yagi H, Tan J, **Tuan RS**. Polyphenols suppress hydrogen peroxide-induced oxidative stress in human bone-marrow derived mesenchymal stem cells. J. Cell. Biochem. 2013. 114(5):1163-1173.
15. Diederichs S, Shine KM, **Tuan RS**. The promise and challenges of stem cell-based therapies for skeletal diseases: Stem cell applications in skeletal medicine: Potential, cell sources and characteristics, and challenges of clinical translation. BioEssays. 2013. 35(3):220-230.
16. Lin H, Zhang D, Alexander PG, Yang G, Tan J, Cheng AW, **Tuan RS**. Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture. Biomaterials. 2013. Jan;34(2):331-339.
17. **Tuan RS**, Chen AF, Klatt BA. Cartilage regeneration. J Am Acad Orthop Surg. 2013 May;21(5):303-11.
18. Alexander PG, Wang X, Song Y, Taboas J, Chen FH, Levin MJ, McCarron JA, Melvin GM, Manner PA, **Tuan RS.** Development of a spring-loaded impact device to deliver injurious mechanical impact to articular cartilage surface.
19. Cartilage. 2013. 4:(1): 52-62.
20. **Tuan RS**, Chen AF, Klatt BA. Cartilage regeneration. J Am Acad Orthop Surg. 2013. 21(5): 303-311
21. Baker N, **Tuan RS**. The less-often-travelled surface of stem cells: Caveolin-1 and caveolae in stem cells, tissue repair and regeneration. Stem Cell Res Ther. 2013. Jul 30; 4(4):90.
22. Yang G, Rothrauff BB, Lin H, Gottardi R, Alexander PG, **Tuan RS.** Enhancement of tenogenic differentiation of human adipose stem cells by tendon-drived extracellular matrix. Biomaterials. 2013. Dec;34(37):9295-306.
23. Yang G, Rothrauff BB, **Tuan RS**. Tendon and ligament regeneration and repair: Clinical relevance and developmental paradigm. Birth Defects Research C: Embryo Today. 2013. Sep;99(3):203-22
24. Lozito T, Alexander PG, Lin H, Gottardi R, Chen AWM, **Tuan R**S. Osteochondral microtissue to model pathogenesis of osteoarthritis. Stem Cell Res Ther. 2013. Dec. 4 (Suppl 1).S6.
25. Boyette LB, Creasey OA, Guzik L, Lozito T, **Tuan RS**. Human bone marrow-derived mesenchymal stem cells display enhanced clonogenicity but impaired differentiation with hypoxic preconditioning. Stem Cells Transl Med. 2014. Feb;3(2):241-54
26. Xie X, Zhang C, **Tuan RS.** Biology of platelet-rich plasma and its clinical application in cartilage repair. Arthr Res Ther. 2014. *In press.*
27. Lin H, Cheng AWM, Alexander PG, Beck AM, **Tuan RS**. Cartilage tissue engineering application of injectable gelatin hydrogel with *in situ* visible light-activated gelation capability in both air and aqueous solution. Tissue Engin. Part A. 2014. *In press.*