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AWARD NUMBER: W81XWH-14-1-0217

TITLE: "Customized Fabrication of Osteochondral Tissue for Articular Joint Surface Repair"

PRINCIPAL INVESTIGATOR: Rocky S. Tuan, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213

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Fort Detrick, Maryland 21702-5012

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University of Pittsburgh,
450 Technology Drive,
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Osteoarthritis (OA) is a chronic degenerative disease of the articular joint that involves more than just the destruction of the articular cartilage – it is a disease of the cartilage, bone and surrounding soft tissue that disables 9-10% of the US population. In the US military, combat and non-combat injuries experienced during deployments, training and generally active life-style can cause traumatic mechanical destruction or progressive biological degeneration of the articular cartilage. It has been reported that OA is the most frequent cause of disability in the armed forces. There are currently no effective therapies for OA. A regenerative surgical approach that aims for biological resurfacing of the joint is thus needed.

In this study, we will construct adipose stem cell-laden scaffolds loaded with microparticles, designed to provide temporospatially specific differentiation cues for chondrogenesis and osteogenesis, by the 3D printing method of projection stereolithography (PSL). We further test the applicability of these novel osteochondral tissues for articular cartilage repair in rabbit model, using medical imaging-guided PSL. Such an approach may be adapted for the development of a single-step, point-of-care procedure for clinical cartilage repair. If successful, the technology being developed here could accelerate the rate of recovery and therefore shorten the time away from active duty status and the duration of rehabilitation, while allowing full recovery of joint function and minimizing the impact on military medical care costs.

15. SUBJECT TERMS
18 months

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TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Osteoarthritis (OA) is a chronic degenerative disease of the articular joint that involves more than just the destruction of the articular cartilage – it is a disease of the cartilage, bone and surrounding soft tissue that disables 9-10% of the US population. In the US military, combat and non-combat injuries experienced during deployments, training and generally active life-style can cause traumatic mechanical destruction or progressive biological degeneration of the articular cartilage. It has been reported that OA is the most frequent cause of disability in the armed forces. There are currently no effective therapies for OA. A regenerative surgical approach that aims for biological resurfacing of the joint is thus needed.
In this study, we will construct adipose stem cell-laden scaffolds loaded with microparticles, designed to provide temporospatially specific differentiation cues for chondrogenesis and osteogenesis, by the 3D printing method of projection stereolithography (PSL). We further test the applicability of these novel osteochondral tissues for articular cartilage repair in rabbit model, using medical imaging-guided PSL. Such an approach may be adapted for the development of a single-step, point-of-care procedure for clinical cartilage repair. If successful, the technology being developed here could accelerate the rate of recovery and therefore shorten the time away from active duty status and the duration of rehabilitation, while allowing full recovery of joint function and minimizing the impact on military medical care costs.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Post Traumatic Osteoarthritis, Injury, Articular Cartilage, tissue-engineered, 3D printing, Osteochondral constructs.

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Goals:
1. Convert micro-computed tomography (micro-CT) images of bone and cartilage of the joint into 3D .stl files to yield a virtual, structural replica of the articular joint to be used for projection stereolithography (PSL).
2. Fabricate bony and cartilage tissues using different biomaterials using PSL.
3. Implant pre-differentiated or undifferentiated osteochondral constructs for focal cartilage lesion repair and assess their reparative efficacy.
4. Test the applicability of tissue engineered osteochondral construct for repair of post-traumatic OA articular cartilage, using micro-CT imaging guided PSL fabrication.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

All the results are presented in the end of the report.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

1. Micro-CT scan the knee of rabbits with or without surgically created defect. 2. Converting micro-computed tomography (micro-CT) images of bone and cartilage of the joint into 3D .stl files to yield a virtual, structural replica of the articular joint to be used for projection stereolithography. 3. Apply PSL to fabricate and assemble osteochondral tissues, in which cartilage side will be composed with hASCs and TGF-β3/BMP-6 loaded PDLLA-PEG hydrogel and bone side will be composed with BMP-2 loaded porous PCL-Heparin scaffolds. 4. Implant pre-differentiated or undifferentiated osteochondral constructs for focal cartilage lesion repair and assess their reparative efficacy. 5. Testing efficacy of tissue engineered osteochondral construct for repair of post-traumatic OA articular cartilage by repairing focal defects in animals, *in vivo*.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

In the original proposal, we intended to use microparticles to delivery growth factors in order to induce chondrogenesis or osteogenesis in situ without the additional supplement of growth factors. This is critical for the success of this project because for point of care application it is impossible to predifferentiate cells in vitro or administrate TGF-β3 regularly in vivo. However, in our preliminary tests, microparticle fabrication was time consuming and impacted the bioactivity of growth factors as well. Therefore, on the cartilage side, we will directly incorporate TGFβ3/BMP-6 into a gelatin hydrogel since gelatin has been reported to have the capacity for controlled release. As shown below, such a method did result in the controlled release of TGFβ3/BMP-6 and sufficient GAG deposition thus meeting the requirements for making cartilage and offering an efficacious alternative to microparticles. For the bone side, we have grafted heparin-DOPA onto PCL, which is able to specifically bind with BMP-2 and release it in a controlled manner. As shown below, BMP-2 was slowly released from DOPA-heparin/PCL scaffolds up to 1 month. In summary, our changes in the way of delivering growth factors have no adverse impact on completing this project. Indeed, new methods used here suggest a new way for growth factor delivery and could be applied in other tissue engineering.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We have achieved IACUC approval on June 2, 2015 and submitted application for ACURO approval. However, we did not get confirmation yet. This will delay the project significantly. Consequently, we may file a no-cost extension for this project. However, we will still work hard in order to keep the schedule.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Sun AX, Lin H, Beck AM, Kilroy EJ, Tuan RS. (2015) Projection Stereolithographic Fabrication of Human Adipose Stem Cell-incorporated Biodegradable Scaffolds for Cartilage Tissue Engineering. Frontiers in Bioengineering and Biotechnology, In press.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

(1) Live Cell-scaffold Printing using Biodegradable PDLLA-PEG/ Hyaluronic Acid Copolymer for Cartilage Tissue Engineering H. Lin, A. Sun, A. Beck, E. Kilroy, R. Tuan; 2015 4th TERMIS World Congress Boston, Massachusetts.
(2) Medical Imaging-guided Additive Manufacturing of Human Osteochondral Tissues H. Lin, E. Kilroy, P. Alexander, R. Tuan; 2015 4th TERMIS World Congress Boston, Massachusetts
(3) Medical Imaging-guided Additive Manufacturing of Human Osteochondral Tissues H. Lin, E. Kilroy, P. Alexander, R. Tuan; 2015 Military Health System Research symposium, Ft. Lauderdale, FL

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	*Rocky S. Tuan, PhD*
Project Role:	*Principal Investigator (PI)*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1.50 Person Months*
Contribution to Project:	*He will have direct responsibility for the overall design and conduct of the study, oversight of data analysis, and preparation of research manuscripts and research reports. Dr. Tuan will supervise the day-to-day research activities of all personnel.*
Funding Support:	*Supported by this Award (W81XWH-14-1-0217)*

Name:	*Peter G. Alexander, PhD*
Project Role:	*Data Analyst Scientist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4.80 Person Months*
Contribution to Project:	*In addition to contributing to the overall study design, he will be directly responsible for day-to-day organization of the bench-top research associated with all proposed tasks. He will also play a leading role in the implantation, in vivo imaging and end-point assessment of the constructs in Task 3A & B. He will also be responsible for training the Postdoctoral Associates on all assays proposed, and working with them to complete theses assays.*
Funding Support:	*Supported by this Award (W81XWH-14-1-0217)*

Name:	*Hang Lin, Phd.*
Project Role:	*Postdoctoral Associate*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*6.00 Person Months*
Contribution to Project:	*He will participate in all activities that require cell entrapment in 3-D matrices, scaffold culture and testing. Thus, he will be involved in optimization of 3D scaffold preparation by PSL, culture of scaffolds, and mechanical and biological testing of the scaffolds.*
Funding Support:	*Supported by this Award (W81XWH-14-1-0217***)**

Name:	*Kanika Rautji*
Project Role:	*Graduate Student Researcher*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*12.00 Person Months*
Contribution to Project:	*Ms. Rautji will be testing the biomechanical aspects of the different materials used for the osteochondral construct and also the biocompatibility and regenerative capacity of the printed tissue with the stem cells used. She will also be assisting with the 3D printing of the material.*
Funding Support:	*Supported by this Award (W81XWH-14-1-0217***)**

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Rocky Tuan	**Closed** Pittsburgh Foundation grant MR2013-66653 "Investigating the Role of WDPCP and Primary Cilia in Articular Cartilage Homeostasis" Role: PI Effort: 2% Date: 7/1/2013 - 6/30/2014 No impact on this project (W81XWH-14-1-0217)
Rocky Tuan	**Closed** DoD grant W81XWH-10-2-0084 "Stem Cell-Based Neurotrophic Enhancement of an Aligned Nanofiber Scaffold for Nerve Repair" Role: PI

	Effort: 5% Date: 9/1/2010 - 8/31/2014 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Closed** DoD grant W81XWH-10-1-0850 "Development of Novel Point-of-Care Treatment for Articular Cartilage Injury" Role: Partnering PI Effort: 5% Date: 9/30/2010 - 3/29/2015 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Closed** NIH grant 5U18 TR000532-02 "3-D Osteochondral Micro-tissue to Model Pathogenesis of Osteoarthritis" Role: PI Effort: 5% Date: 7/1/2013 - 6/30/2015 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Closed** PADoH, SAP 4100050913 "Application of Adult Stem Cells for Tissue Regeneration" Role: PI Effort: 10% Date: 1/1/2010 - 12/31/2013 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** DOD grant W81XWH-15-1-0104 "Cell-Based Meniscal Repair Using an Aligned Bioactive Nanofibrous Sheath" Role: PI Effort: 6% Date: 6/15/2015 - 12/14/2016 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** Vanderbilt U. Subaward from their EPA Award# 83573601 "Vanderbilt-Pittsburgh Resource for Organotypic Models for Predictive Toxicology (VPROMPT)" Role: Co-PI Effort: 7.5% Date: 12/1/2014 - 11/30/2018 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** NIH Grant 5R01 EB019430-A11 "Cholesterol Sensitivity and Mechanisms of MSC Responses to 3D Substrate Rigidity" Role: PI Effort: 10% Date: 4/1/2015 - 3/31/2019 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** DOD Grant W81XWH-15-2-0011 "Treatment of Orthopaedic Infections using Activated Adult Mesenchymal Stem Cells" Role: PI Effort: 12.5% Date: 4/10/2015 - 4/9/2018 No impact on this project (*W81XWH-14-1-0217)*
Rocky Tuan	**Received** Amniox Medical Inc., No Award#/Identifier "Determination of the role of human amniotic membrane and umbilical cord membrane materials in musculoskeletal tissue repair and regeneration" Role: Co-Investigator Effort: 3.5% (Cost Shared) Date: 7/1/2015 -3/31/2016 No impact on this project (*W81XWH-14-1-0217)*

Rocky Tuan	**Received** DOD, Grant W81XWH-14-2-0003 "AFIRM II Admin Core" Role: Director Effort: 10% Date: 1/1/2014 -12/31/2018 No impact on this project (W81XWH-14-1-0217)
Rocky Tuan	**Received** DOD, Grant W81XWH-14-2-0003 "Regenerative Repair of Traumatic Articular Cartilage Injuries: Point-of-Care Application of Mesenchymal Stem Cells and Chondrocytes" Role: Project Leader Effort: 5% Date: 1/1/2014 -12/31/2018 No impact on this project (W81XWH-14-1-0217)

Peter G. Alexander	**Closed** DoD grant W81XWH-10-2-0084 "Stem Cell-Based Neurotrophic Enhancement of an Aligned Nanofiber Scaffold for Nerve Repair" Role: Co-Investigator Effort: 8% Date: 9/1/2010-8/31/2014 No impact on this project (*W81XWH-14-1-0217)*
Peter G. Alexander	**Closed** DoD grant W81XWH-10-1-0850 "Development of Novel Point-of-Care Treatment for Articular Cartilage Injury" Role: Data Analyst Scientist Effort: 40% Date: 9/30/2010 - 3/29/2015 No impact on this project (*W81XWH-14-1-0217)*
Peter G. Alexander	**Closed** NIH grant 5U18 TR000532-02 "3-D Osteochondral Micro-tissue to Model Pathogenesis of Osteoarthritis" Role: Data Analyst Scientist Effort: 9.5% Date: 7/1/2013 - 6/30/2015 No impact on this project (*W81XWH-14-1-0217)*
Peter G. Alexander	**Received** DOD, Grant W81XWH-14-2-0003 "Regenerative Repair of Traumatic Articular Cartilage Injuries: Point-of-Care Application of Mesenchymal Stem Cells and Chondrocytes" Role: Data Analyst Scientist Effort: 10% Date: 1/1/2014 -12/31/2018 No impact on this project (W81XWH-14-1-0217)
Peter G. Alexander	**Received** DOD grant W81XWH-15-1-0104 "Cell-Based Meniscal Repair Using an Aligned Bioactive Nanofibrous Sheath" Role: Co-Investigator Effort: 10% Date: 6/15/2015 - 12/14/2016 No impact on this project (*W81XWH-14-1-0217)*
Peter G. Alexander	**Received** Vanderbilt U. Subaward from their EPA Award# 83573601 "Vanderbilt-Pittsburgh Resource for Organotypic Models for Predictive Toxicology (VPROMPT)" Role: Data Analyst Scientist Effort: 20% Date: 12/1/2014 - 11/30/2018 No impact on this project (*W81XWH-14-1-0217)*

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

What was accomplished under these goals?

1. Fabricate a-cellular molds and test the mechanical propertyes and degradation charactiristics of scaffolds

We have successfully fabricated acelluar PDLLA-PEG and PCL scaffolds using PSL (Figure 1,2). Solid PDLLA-PEG scaffold (30%) possesses a compressive modulus up to 800kPa and is biodegradable in aqueous solution (Figure 3). Pure PCL scaffolds displayed 120Mpa compressive modulus.

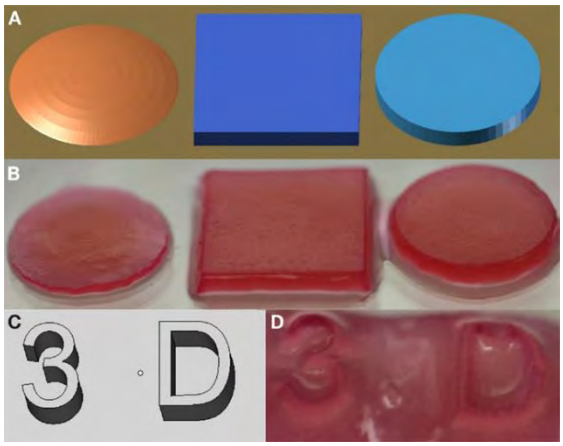

Figure 1. mPDLLA-PEG constructs generated using VL-PSL. (A,B) PDLLA-PEG hydrogels with spherical, cuboidal, and cylindrical architecture (B) based on CAD models (A). (C,D) Alphanumeric mPDLLA-PEG hydrogels (D) based on CAD models (C). Formulation used includes mPDLLA-PEG (30% w/v), mHA (0.5% w/v), LAP (0.6% w/v), and phenol red (0.025% w/v).

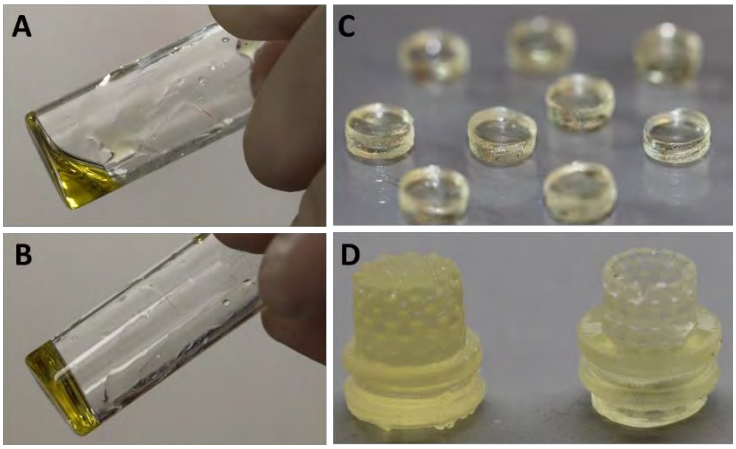

Figure 2. PCL scaffolds. A,B. PCL before and after curing. C.D PCL scaffolds generated using VL-PSL. Formulation used includes 100% PCL with the inclusion of 1% Diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide

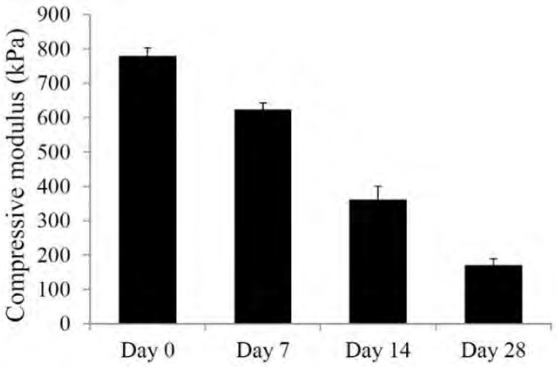

Figure 3. Mechanical property of mPDLLA-PEG/HA scaffolds incubated in HBSS at 37°C for different times up to 28 days. Difference between every two groups is statistically significant.

2. Cell preparation.

We have isolated human adipose-derived stem cells (hASCs) and characterized surface marker profile (Figure 4). Their potential was also tested (Figure 5).

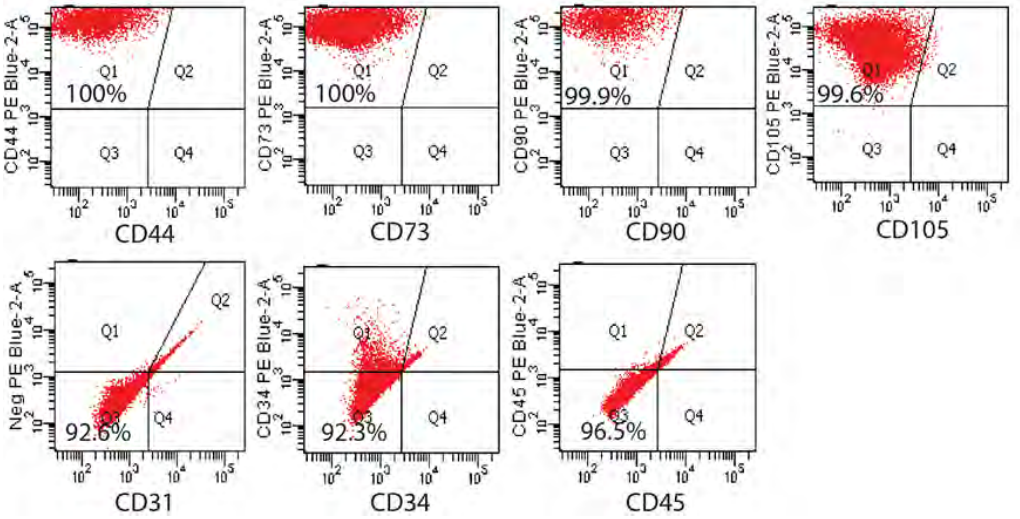

Figure 4. Flow cytometry analysis of the expression profile of cell surface markers related to mesenchymal stem cells, hematopoietic and endothelial cells. The results suggested a homogeneous population that expressed mesenchymal cell markers (CD44, CD73, CD90 and CD105) but was free of hematopoietic and endothelial markers (CD31, CD34, CD45).

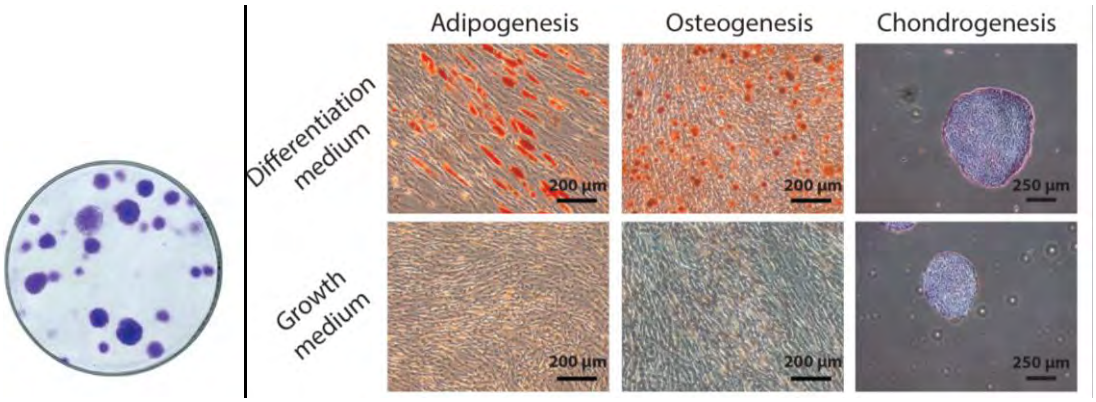

Figure 5. Left: Crystal violet staining visualizes the colonies formed by proliferating cells. In our study, after 14 days of culture, 28.83±3.31% of the adherent cells were found proliferating by CFU assay, indicating a self-renewal capability within the cell population. Right: Histological staining to test ASCs multipotency. When treated with corresponding differentiation media, cells in culture were able to undergo differentiation toward multiple mesenchymal lineages, including adipogenesis, osteogenesis, and chondrogenesis Adipogenesis was identified by Oil Red staining (red), osteogenesis by Alizarin Red staining (red), and chondrogenesis by Safranin-O staining (red).

3. hASCs ASC differentiation in completed PSL-fabricated scaffold

Solutions of polymer, LAP, and phenol red dye were prepared in 50 ml tubes. Thirty percent PDLLA-PEG was chosen because it not only maintained the fabrication fidelity of VL-PSL but also formed a scaffold with mechanical properties more similar to that of cartilage. HA was used at 0.5% concentration because it yielded sufficient specific gravity to suspend cells but did not compromise routine cell mixing by trituration, for example, by repeated pipetting. The solution was titrated to pH 7.4 with 10 N NaOH and adjusted to 40 ml using HBSS. P3 hASCs were pelleted by centrifugation, and the supernatant was completely removed. The polymer solution prepared above was added on top of the pellets and mixed with cells thoroughly by gently pipetting up and down 20 times. The final hASC density was 4×10^6 cells/ml. The cell-polymer solution was immediately poured into the basement plate of the PSL device for printing with different 3D models as the template.

As shown in Figure 6, uniform distribution of single cells throughout the construct was clearly seen, suggesting that cells remained suspended and separated from each other in the fabrication solution for at least 30 min. Cell viability was determined to be high at 81% after fabrication.

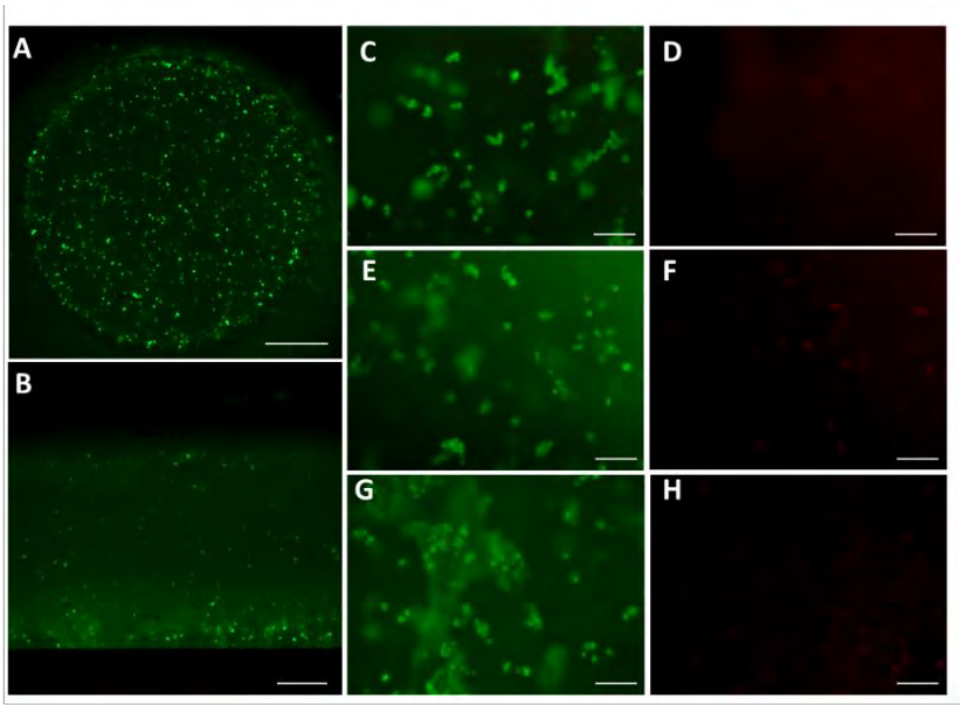

Figure 6. Cell viability in VL-PSL built PDLLA-PEG-PDLLA scaffolds at 0 days and 28 days after fabrication. (A, B, C, E, G) Calcein-AM staining (green, live cells) and (D, F, H) EthD-1 staining (red, dead cells) in scaffold show the cell viability following fabrication throughout VL-PSL method. (A) shows a surface view of a Calcein-AM stained construct, and (B) shows a cross sectional view of the construct at day 0. Uniform distribution of single cells throughout the construct was clearly seen. Cell viability was determined to be high at 81% right after fabrication (C&D) and 77% at 28 days culture in control (E&F)or chondrogenic medium (G&H).

hMSCs also show high viability (>95%)after 3 days culture on PCL scaffolds.

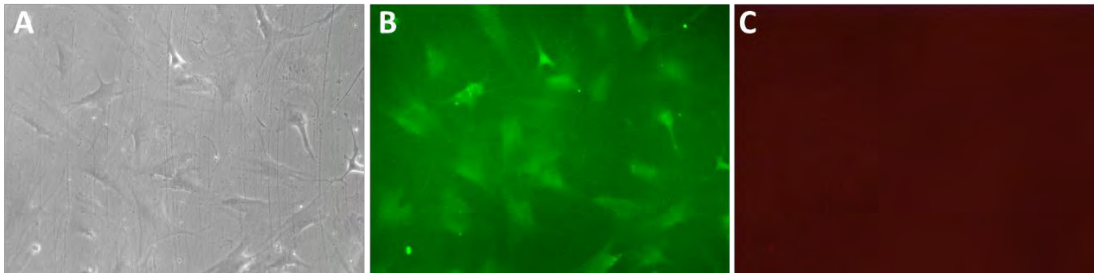

Figure 7. Cell viability PCL scaffolds at 0 days and 28 days 3 days after seeding. A, Phase contrast image of cells. B, Calcein-AM staining (green, live cells). C, EthD-1 staining (red, dead cells).

Chondrogenic differentiation of the hASCs seeded within the dense mPDLLA-PEG/HA scaffolds and exposed to TGF-β3-containing chondrogenic medium was analyzed by real time RT-PCR for the expression of genes associated with chondrogenesis. Figures 8A–D show the relative levels of gene expression for Sox 9, aggrecan, collagen type II, and Runx2. All three chondrogenic genes, Sox 9, aggrecan, and collagen II, were also

considerably higher in the TGF-β3 group. In contrast, Runx2, an osteogenesis marker, expression was found to be higher in the control group. Thus, TGF-β3 chondrogenic medium effectively induced chondrogenesis and concurrently inhibited osteogenesis in hASCs encapsulated within the PSL-fabricated scaffolds.

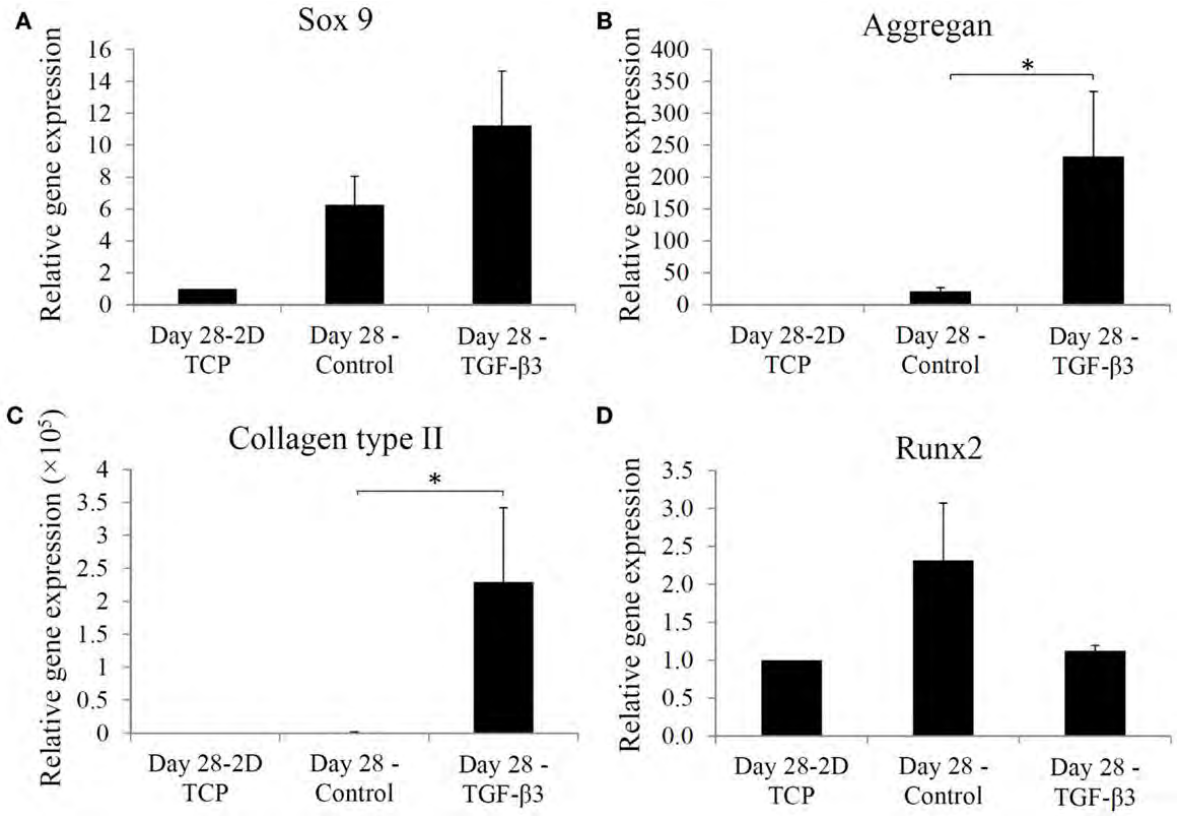

*Figure 8. Real time-PCR analysis of gene expression in hASCs at day 28 in 2D tissue culture plate, control group, and TGF-β3 group. Relative gene expression levels of (A) Sox 9, (B) aggregan, (C) collagen type II, and (D) Runx2 at day 28, are normalized to gene expression in 2D culture without chondrogenic induction. *p < 0.05.*

The chondrogenic activity of the cells within the constructs cultured in the chondrogenic medium was next assessed based on hydroxyproline assay to estimate the content of newly synthesized collagen. The TGF-β3 group showed a hydroxyproline level of 21.16 ± 7.15 μg/construct versus an undetectable level in the control group (Figure 9). Since this assay did not differentiate between the different collagen types, we further performed immunohistochemistry (IHC) to examine the presence of collage type II. IHC revealed positive staining for collagen type II, a major ECM component in cartilage, only in the TGF-β3 group (Figure 10). GAG and proteoglycan content were also estimated by histological staining with Alcian Blue and Safranin O, respectively.

Weak staining was seen in the control group after 28 days culture (Figures 11A,C), while dense, strong staining was seen in the TGF-β3 group (Figures 11B,D). Taken together, these results clearly demonstrate that with TGF-β3 induction, robust chondrogenic differentiation of the hASCs took place within the PSL-fabricated constructs. Lastly, the compressive moduli for the constructs containing the mPDLLA-PEG polymer were measured. At day 0, the compressive modulus was 780 ± 23 kPa, which fell to 240 ± 20 kPa in the control group and 238 ± 25 kPa in the TGF-β3 group by day 28 (Figure 12), principally due to the degradation of the scaffold material.

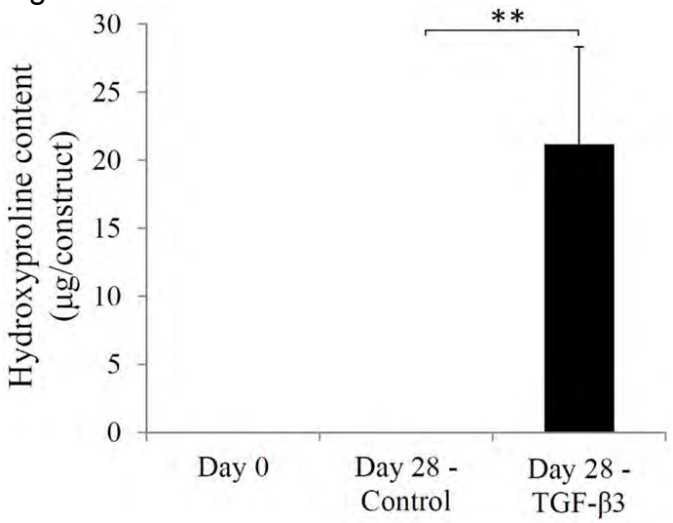

*Figure 9. Hydroxyproline levels in hASC-encapsulated VL-PSL-fabricated constructs in the control and TGF-β3 groups at days 0 and 28. Levels of hydroxyproline in days 0 and 28 control groups were negligible, and levels in the day 28 TGF-β3 group measured at 21.16 ± 7.15 µg/construct. **p < 0.01.*

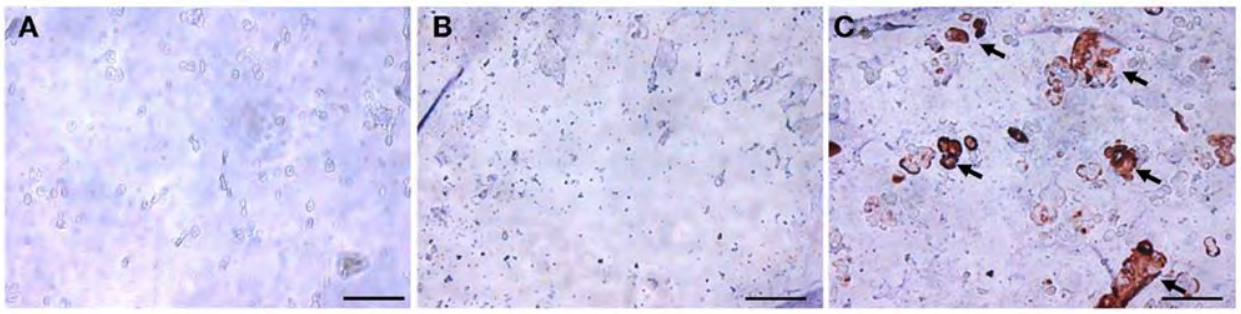

Figure 10. Immunohistochemical staining for collagen type II in hASC-encapsulated VL-PSL-fabricated constructs. (A) Day 0 immediately after fabrication; (B) day 28 control group; and (C) day 28 TGF-β3 group. Positive collage type II staining (brown) was only seen in the day 28 TGF-β3 treated group as indicated by arrows. Scale bar: 50 µm.

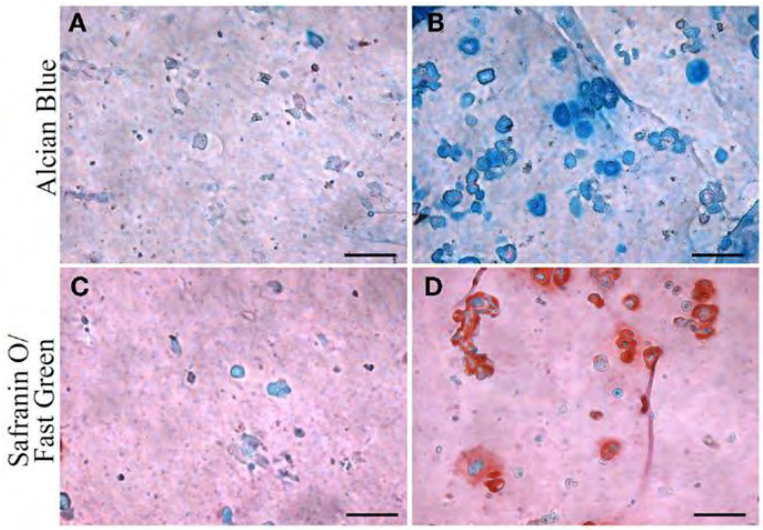

Figure 11. Glycosaminoglycan (GAG) and proteoglycan content in hASC-encapsulated VL-PSL-fabricated constructs visualized by Alcian blue and safranin O/fast green staining at day 28. (A,C) Alcian blue and safranin O staining, respectively, of control group. Negligible amounts of GAG and proteoglycan are detected. (B,D) Alcian blue and safranin O staining, respectively, of TGF-β3 group at day 28, showing strong staining. Scale bar: 50 μm.

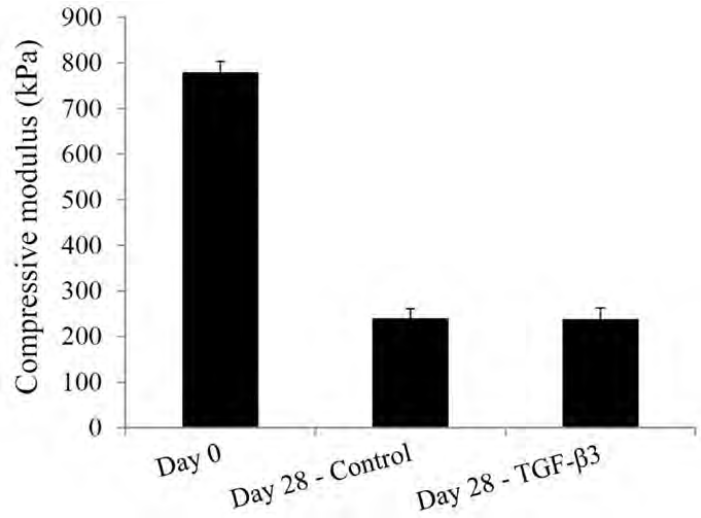

Figure 12. Compressive moduli of hASC-encapsulated VL-PSL-fabricated constructs measured at culture days 0 and 28 in the control and TGF-β3 groups.

4. Controlled release of TGFβ3 from gelatin incorporated PDLLA-PEG and its effect on chondrogenesis.

In the original proposal, we intended to use microparticles to delivery growth factors in order to in situ induce chondrogenesis or osteogenesis without the additional supplement of growth factors. This is critical for the success of this project because for point of care purpose it is impossible to predifferentiate cells *in vitro* or administrate TGF-β3 regularly *in vivo*. However, in our preliminary test, microparticle fabrication is time consuming and

impacts the bioactivity of growth factors as well. Therefore, in cartilage side, we will directly incorporated TGFβ3/BMP-6 into gelatin hydrogel since gelatin was reported to have controlled release capacity. For bone side, we will incorporate heparin-DOPA into PCL, which is able to specifically bind with BMP-2 and release BMP-2 in a controlled manner.

We made 10%PDLLA-PEG/5% gelatin as the monomer and encapsulated 10, 50, 200, 1000, 2000, 3300 ng/ml TGFβ3 (final concentration). hMSCs were also encapsulated with a cell density 20×10^6/ml. After curing with visible light, the constructs were cultured in basal chondrogenic medium without the supplement of TGFβ3 up to 28 days. The constructs without TGFβ3 cultured in chondrogenic medium with or without TGFβ3 were served as positive and negative control. We have performed this experiment for 3 times with cells from 3 patients.

As shown in Figure 13, chondrogenic genes expression increased with incorporated TGFβ3 amount until reaching a peak at 2 μg/ml. Excess of TGF β3 (3.3 μg/ml) seemed to impair the chondrogenesis. Interestingly, 2 μg/ml TGF β3 within scaffolds is able to induce higher SOX 9 and Aggregan expression than that cultured in TGF β3 supplemented medium.

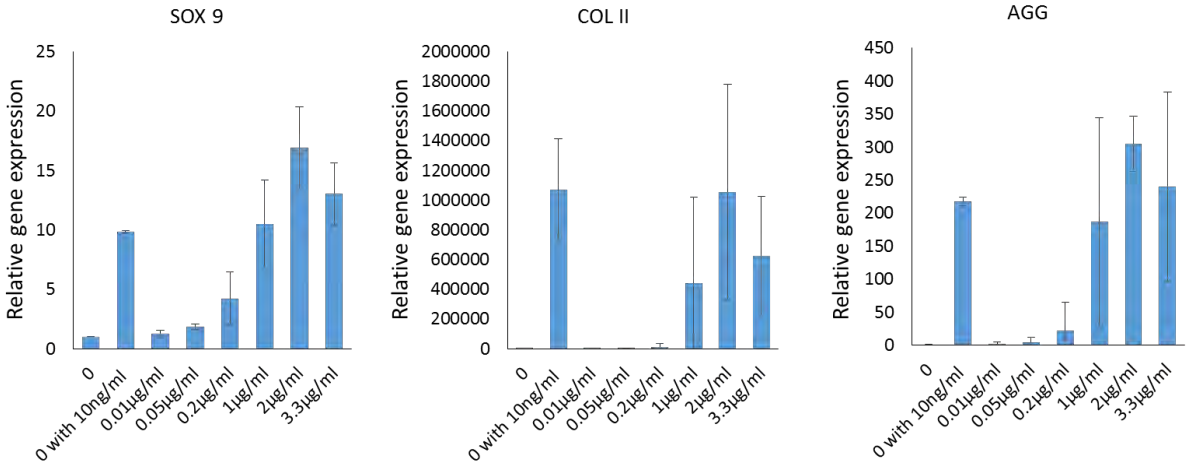

Figure 13. Real time-PCR analysis of gene expression at day 28. Relative gene expression levels of Sox 9, collagen type II (COL II) and aggregan (AGG) are normalized to gene expression in 0 group.

0: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs cultured in medium without TGFβ3.

0 with 10ng/ml: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs cultured in medium with 10ng/ml TGFβ3.

0.01, 0.05, 0.2, 1, 2 and 3.3μg/ml: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs containing 1μg/ml, 2μg/ml and 3.3μg/ml, respectively, and cultured in medium without TGFβ3.

2 μg/ml TGF-β3 incorporation was also able to promote cell-laden construct produce GAG with highest efficiency without TGF-β3 supplement. More importantly, the total GAG amount is comparable to those from constructs cultured in TGF-β3-containing medium (Figure 14, 15). Therefore, we will use 2 μg/ml TGF-β3 in future application.

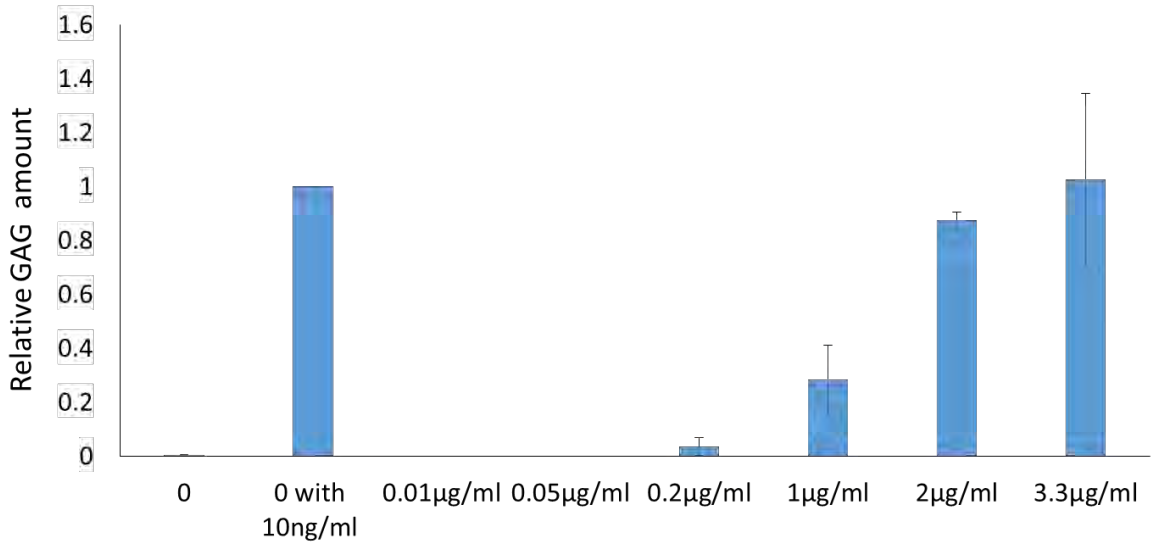

Figure 14: GAG deposition in constructs after normalized to positive group (0 with 10 ng/ml).

0: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs cultured in medium without TGFβ3.

0 with 10ng/ml: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs cultured in medium with 10ng/ml TGFβ3.

0.01, 0.05, 0.2, 1, 2 and 3.3µg/ml: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs containing 1µg/ml, 2µg/ml and 3.3µg/ml, respectively, and cultured in medium without TGFβ3.

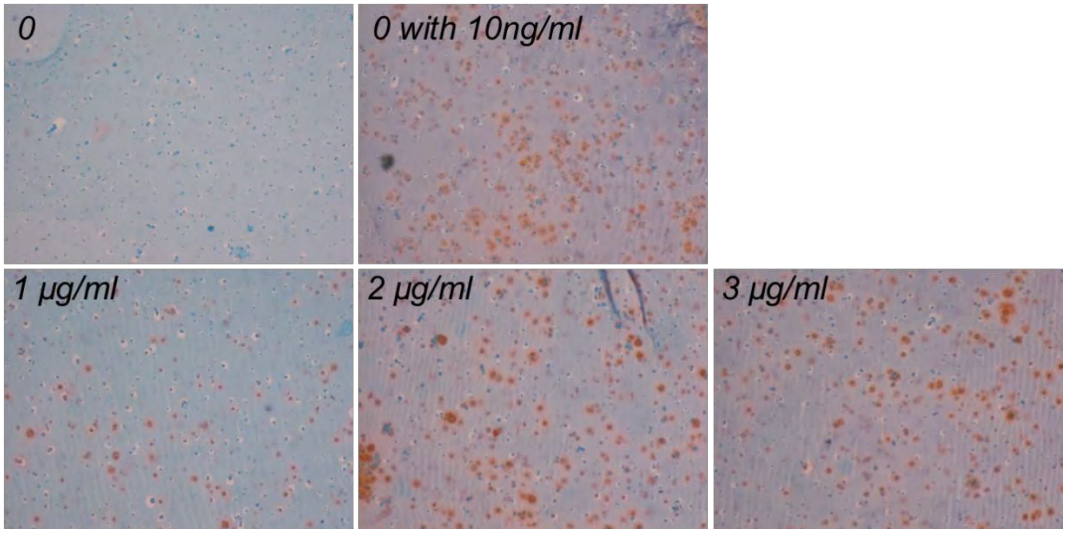

Figure 15: Safranin O staining of slides from different groups after 28 days culture.

0: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs cultured in medium without TGFβ3.

0 with 10ng/ml: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs cultured in medium with 10ng/ml TGFβ3.

0.01, 0.05, 0.2, 1, 2 and 3.3μg/ml: cell seeded PDLLA-PEG-PDLLA/Gelatin constructs containing 1μg/ml, 2μg/ml and 3.3μg/ml, respectively, and cultured in medium without TGFβ3.

We then test the release profile of TGF-β3 from PDLLA-PEG/Gelatin constructs. 2 μg/ml TGF-β3 was encapsulated into PDLLA-PEG/Gelatin and soak in PBS. PBS were changed every 3 days up to 28 days. TGF-β3 released to PBS were quantitated using an ELISA kit. The data is under analyzing.

4. We are currently working on the controlled release of BMP-2 from PCL scaffolds.