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TITLE: Optimizing Skin-Implant Interface of Osseointegrated Device

PRINCIPAL INVESTIGATOR: Jonathan Forsberg, MD, PhD

RECIPIENT: Henry M Jackson Foundation for the Advancement of Military Medicine
Bethesda, MD 20817

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This study focuses on an OI prosthetic implant anchored in the long bone of a residual limb and exiting through the skin. Implant and soft tissue infections and implant loosening are common complications for both upper and lower extremity bone-anchored implants, resulting in revision surgeries and increased morbidity. We explore the possibility of creating a tight, durable skin-implant interface for OI implants using mesenchymal stem cells (MSCs) derived from naturally occurring porcine integumentary tissues or human induced pluripotent stem (iPS) cells, which have the intrinsic potential to form an impervious seal at hard and soft tissue junctions. Initial porcine cell characterization is ongong.

15. SUBJECT TERMS

Osseointegration, implants, titanium, scaffold, MSCs, iPS cell

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

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

The study addresses two focus areas of research with pressing clinical need: **1.** Optimization of the skin-implant interface for osseointegrated (OI) implants, and **2.** Prevention of infection at the skin-implant interface. The goal is to address both these issues while maintaining residual limb skin integrity and durability. We address the issue of skin-to-implant healing and attachment for osseointegrated (OI) prostheses by focusing on integration and durability of their microbially, mechanically and biologically challenging skin-to-implant interface. This study focuses on an OI prosthetic implant anchored in the long bone of a residual limb and exiting through the skin. Implant and soft tissue infections (29-38%) and implant loosening (13-29%) are common complications for both upper and lower extremity bone-anchored implants, resulting in revision surgeries and increased morbidity. These complications develop due to lack of a tight, impervious seal at the skin-percutaneous implant interface, resulting in exposure of soft tissue and vasculature, thereby increasing chances of infection as well as implant loosening. For both focus areas, we explore the possibility of creating a tight, durable skin-implant interface for OI implants using mesenchymal stem cells (MSCs) derived from naturally occurring porcine integumentary tissues or human induced pluripotent stem (iPS) cells, which have the intrinsic potential to form an impervious seal at hard and soft tissue junctions. We hypothesize that comparative analysis of the differentiation and adhesion properties of naturally occurring cells of the integumentary system, present at hard and soft tissue junctions at the dermis, nails or hoof, periodontal ligament as well as iPS cells could enable us to engineer durable and impervious cell-based scaffolds for placement at the skin-implant interface.

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

Osseointegration, implants, titanium, scaffold, MSCs, iPS cells, differentiation, adhesion, tissues, bone, cartilage, adipose, muscle, ligament, tendon, dermis

3.ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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.

Research-Specific Aims and Tasks	Mos.	Percent completed
Administrative Aims and Tasks: 1. Establish subaward agreement between HJF and MMRF 2. Develop and sign USU-MMRF CRADA 3. Recruit and hire support personnel a. Stem Cell Biologist (USU) b. Research Associate (USU) c. Stem Cell Biologist (MMRF-UMN)	1-4	100% 100% 100% a. 100% b: 100% c: 100%
Specific Aim 1 Specific Aim 1: in ex vivo culture (a) steer differentiation for human and swine MSC, iPS cells and mature site-specific (gingival and hoof/nail bed) cells to adhesive/epithelial phenotypes, (b) Characterize and rate the ingrowth of these cells into scaffold and their adhesive potential to metal substrate.	**1-24**	30%
Major Task 1: Develop cell culture	1-16	50%
Subtask 1.1: In vitro isolation and characterization of *porcine* cells.	1-16	50%
Subtask 1.2: In vitro development and characterization of *human* cells.	1-16	0%
Major Task 2: *In vitro*: evaluate cell adhesion to metal substrate	7-16	30%
Subtask 2.1a: Test porcine cells for adhesion	7-16	30%
Subtask 2.1b: Test human cells for adhesion	7-16	0%
Major Task 3: Scaffold development for cell growth and anchorage to underlying tissue.	5-24	0%
Subtask 3.1: Complete scaffold design (constructs) for "sleeve" & "transition designs	5-16	0%
Subtask 3.2: Seed and grow porcine cells on flat collagen sheets, assemble scaffolds	17-24	0%
Subtask 3.3: Seed and grow human cells on flat collagen sheets, assemble scaffolds	17-24	0%
Major Task 4: Complete full statistical analysis, complete/submit 2-4 manuscripts.	18-24	0%
Milestone(s) Achieved: Characterization of 3-4 cell choices for optimal adhesivity - in vitro; submission of 2-4 manuscripts.	*1-24*	0%
Specific Aim 2 Specific Aim 2: In vivo large animal (swine) testing of transdermal implants with and without subdermal cellular augmentation (SA2a), +/- septal /strain limiting scaffold (SA2b), and topical bacterial challenge (SA2c).	**25-60**	0%
Major task 5: (SA2a) Implant 8 implants/animal with "best of" cells from Specific Aims 1 and 2 x 10 animals at USU-Surgery	25-35	0%
Subtask 5.1: Implant initial 5 animals with 3-4 types of cell augmentation per animal	25-27	0%
Subtask 5.2: Initial evaluation of skin integration - assessment of initial results; experiment modification as necessary.	28-29	0%

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.

Administrative tasks:

- Sub award to Minnesota Medical Research Foundation (MMRF) – complete 14Jan2018
- Recruited and hired Stem Cell Biologist (USU) – complete 02Jan2018
- Initiated the development of a USU-MMRF data agreement - pending
- Recruited and hired Research Associate (USU) – complete 12March2018
- Recruited Stem Cell Biologist (MMRF-UMN) – complete 21April2018
- Hire Stem Cell Biologist (MMRF-UMN) - complete 25July2018

Subtask 1.1: In vitro isolation and characterization of *porcine* cells.

Methods:

Porcine MSCs were isolated from female Gottingen, Yucatan or Yorkshire minipigs under USUHS Surgery and Veterinary Medicine tissue sharing protocol (LAM-17-540 entitled "Education and Training Protocol for Techniques in Animal Care and Use"). All tissues were collected in sterile collection media (1:1 DMEM-F12, containing 2% FBS and 2X antibiotics; namely penicillin, streptomycin and fungizone). Bone marrow (BM) cells were isolated after multiple washes in antibiotic-containing PBS and post RBC lysis. For all other tissues, standard mechanical and enzymatic digestion protocols were followed, as shown in **Table 1**. The nine different tissues from which MSCs were derived include bone marrow, muscle, abdominal and hind limb adipose, abdominal and hind limb dermis, tendon, hoof, and molar-associated periodontal ligament. Isolation was carried out from 4-6 animals.

	Tissue	Enzyme cocktail	Duration of digestion
1	**Bone marrow**	NA; Obtained by aspiration from iliac crest or flushing ribs, immediately followed by resuspension in ice-cold collection media*	Not Applicable
2	**Muscle**	Col I (3 mg/ml) + Col II (1.42 mg/ml) + Dispase (3 mg/ml)	2 hours
3	**Abdominal adipose**	Col I (1 mg/ml)	2 hours
4	**Hind Limb adipose**		
5	**Achilles Tendon**	Col I (3 mg/ml)+Col II (3 mg/ml)+ Dispase (4 mg/ml)	1.5 hours
6	**Hoof**		
7	**Abdominal dermis**	Col I (0.25%) in DMEM-F12 (1:1) containing 10% FBS	10-12 hours
8	**Hind Limb dermis**		
9	**Molar-associated Periodontal ligament**	Col I (3 mg/ml)+ Dispase II (4 mg/ml)	1 hour

**Collection media: 1:1 DMEM-F12 + 2% FBS + 2x Pen-Strep-Fungizone*

Table I. Details of swine tissue collection and the respective digestion protocols.

Cells at Passage 1 and 2 were frozen at a concentration of 1-2 x 10^6 cells/vial. Passage 2 cells were used for all experiments.

Differentiation studies

Comparative analysis of multi-lineage differentiation potential was carried out for the cells isolated from all the nine tissues. We focused on osteogenic, chondrogenic and adipogenic differentiation, which are the hallmark of mesenchymal stem cells (MSCs).

Differentiation assays and gene expression was used to assess lineage-specific differentiation. For induction of osteogenic and adipogenic differentiation, equal number of cells were seeded in tissue culture plates and cultured for either 21 days for long-term differentiation assay, or 7 days for analysis of lineage-specific gene expression. For chondrogenic differentiation, we tested both 2-dimensional micromass culture and 3-dimensional pellet culture.

Osteogenic differentiation was induced by replacing growth media with osteogenic media (OM; StemPro Osteogenesis kit) 24 hours post seeding. Early osteogenic differentiation was assessed by pNpp based alkaline phosphatase activity assay (Sigma Aldrich) after 1 week of osteogenic differentiation. Briefly, cells were washed with PBS, followed by lysis in 50 µl of 1% Triton X-100 for 2-3 minutes. Equal volume of pNpp substrate was added, and absorbance was recorded immediately at 405 nm in kinetic mode for 20 cycles (Tecan). Terminal osteogenic differentiation was assessed by measuring Ca^{2+} deposition after 3 weeks in differentiation media. Briefly, at the end of 3 weeks, cells were washed in PBS, fixed in 0.5% glutaraldehyde and stained with 2% alizarin red solution for 20-30 minutes. Quantification of alizarin red staining was done in 10% formic acid and absorbance read at 414 nm. Both early and terminal osteogenic differentiation was carried out in a 96-well plate, with 100 cells/well. Cells were harvested after 5 days of osteogenic differentiation for osteogenic gene expression studies. Trizol was used for homogenization, followed by RNA isolation (Qiagen mRNAeasy kit), cDNA synthesis (ABI) and Syber Green (Bio-Rad) based quantitative Real-Time PCR run on Quant-Studio.

Results:

Assessment of early osteogenic differentiation demonstrated significantly higher osteogenic potential in molar-associated periodontal ligament tissue among all tissues, followed by hind limb dermis **(Fig. 1A).**

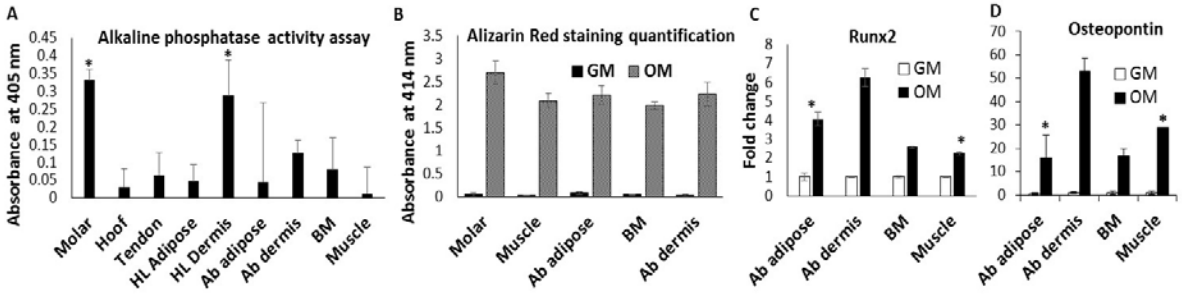


Figure 1. **Characterization of osteogenic differentiation potential of porcine integumentary and connective tissues. A.** Quantification of alkaline phosphatase activity measured after culturing cells in osteogenic differentiation media for 1 week. **B.** Quantification of alizarin red staining of cells cultured in growth media (GM) or osteogenic media (OM) for 3 weeks. Fold change in expression of **C.** *Runx2* and **D.** *Osteopontin* gene in cells cultured in OM with respect to levels in GM

After 3 weeks of differentiation, molar ligament derived cells were once again the cell type with highest calcium deposition among the five tissues tested, as measured by alizarin red staining **(Fig. 1B)**. Expression levels of three osteogenic genes, *Runx2, Osteopontin* and *Osterix* was assessed in four tissues; namely abdominal adipose, dermis, bone marrow and muscle. Out of these, abdominal dermis showed the highest fold change in expression of both *Runx2* **(**6.25-fold; **Fig. 1C)**, a key transcription factor in osteoblast differentiation and the secreted phosphoprotein 1, *Osteopontin* **(**>50-fold; **Fig. 1D**), which is known to be important for attachment of osteoclasts to mineralized bone matrix. There was no increase in expression of *Osterix* in any of these tissues with respect to their undifferentiated controls (data not shown). Analysis of these genes in the other tissues, and analysis of additional osteogenic genes, such as (*BSP, Osteocalcin*) is currently underway.

Chondrogenic differentiation was induced by replacing growth media with chondrogenic media (CM) prepared in-house (1:1 DMEM-F12 containing 10% FBS, 2x Pen-Strep-Fungizone, 40 µg/ml L-Proline, 10 ng/ml TGF-β1, 1 µM Dexamethasone, 50 µg/ml L-Ascorbic Acid, 1 mM sodium pyruvate and 1% ITS Supplement) after 4-5 hours of micromass or pellet seeding. For both 2D and 3D cultures, seeding density was 5 x 10^5 cells. For micromass cultures, this cell number was resuspended in 5 µl GM at the center of a 12-well plate; CM was added to it after 3-4 hours. Pellet cultures were carried out in 15 ml conical Falcon tubes, in which 5 x 10^5 cells resuspended in 1 ml of GM was centrifuged at 1200 rpm for 10 minutes, and the pellet was left undisturbed by incubating the tubes upright at 37°C. Media was replaced to CM after 4-5 hours after centrifuging the cells at 1200 rpm for 10 minutes. Fresh media was replenished every 3 days after centrifugation, followed by discarding spent media without disturbing the pellet. For gene expression analysis, micromass or pellet was subjected to lysis and homogenization in Trizol, followed by RNA isolation (Qiagen mRNAeasy kit), cDNA synthesis (ABI) and Syber Green (Bio-Rad) based quantitative Real-Time PCR run on Quant-Studio. Deposition of proteoglycans was assessed only for micromass cultures so far, by staining with 1% Alcian Blue (prepared in 3% acetic acid).

Results:

Chondrogenic lineage-specific genes (*Sox9, Aggrecan and Col II*) genes were assessed for four tissues, namely hoof connective tissue, hind limb adipose, tendon and hind limb dermis. Hoof connective tissue demonstrated significantly higher expression of Sox9, as compared to the other tissues **(Fig. 2A)**. No significant change was seen for the other genes. We are currently testing expression of these genes in the remaining tissues. We also carried out a 3-week differentiation assay of abdominal dermis and hindlimb adipose cells in micromass cultures, followed by assessment of proteoglycan deposition by alcian blue staining. Since we did not observe a strong staining for preoteoglycans in the micromass cultures, we are currently culturing the cells as pellet cultures for proteoglycan analysis after 21 days.

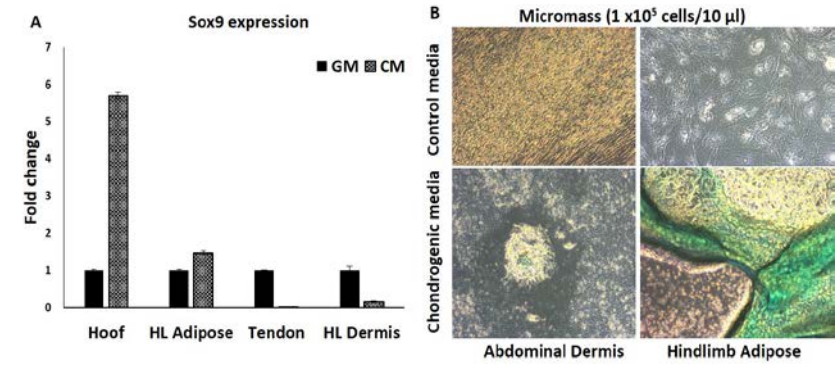


Fig. 2. Chondrogenic differentiation studies. **A.** Comparative analysis of Sox9 gene expression in 4 tissues after 1 week in pellet cultures, either in growth media (GM) or chondrogenic media (CM); * p-value<0.05. **B.** Staining of micromass cultures of abdominal dermis and hind limb adipose with Alcian Blue after 21 days in either control media or chondrogenic media

Adipogenic differentiation was induced by replacing growth media with commercially available adipogenic media (AM; LaCell) when cells reached ~80% confluency at 5-7 days post seeding. For gene expression studies, 1 x10^5 cells was seeded per well of a 6-well plate, and for 21-day differentiation assay, 500 cells/well was seeded in a 96-well plate. Gene expression studies are currently underway and was carried out the same way as described for osteogenic and chondgrogenic differentiation. Induction of adipogenic differentiation was visualized by formation of lipid droplets, which are stained with Oil Red O (ORO). Briefly, at the end of 3 weeks cells were washed in PBS, fixed in 0.5% glutaraldehyde and washed with 60% isopropanol 30 minutes, followed by staining with working solution of ORO, prepared in isopropanol. This was followed by 3-4 washes in tap water.

Results:

Human adipose derived MSCs (hASCs) were used as positive control to standardize ORO staining. After 21 days in culture, distinct lipid droplets were seen in hASCs cultured in AM (**Fig. 3B**), while no lipids were formed in hASCs in control media (**Fig. 3A**). The same conditions were used for differentiation assessment of four porcine cell types (**Fig. 3C-F**). We are currently assessing the adipogenic differentiation potential of the remaining cell types.

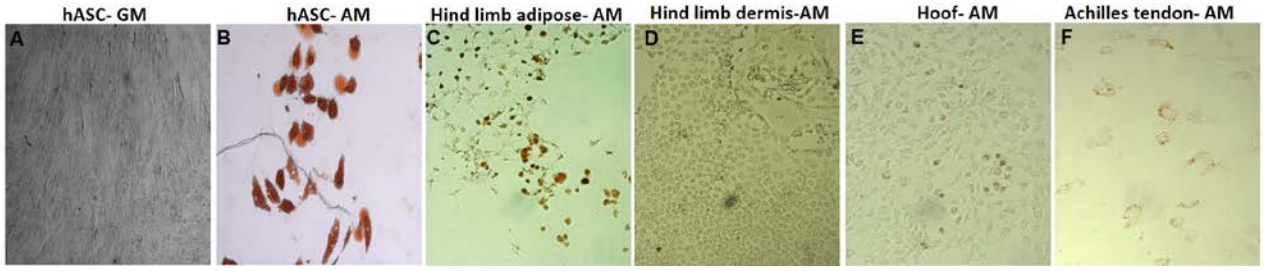


Fig. 3. Adipogenic differentiation studies. ORO staining of human adipose derived MSCs (hASCs) cultured in **A.** control media, **B.** adipogenic media (AM). **C-F.** Staining of porcine cells cultured in AM for 3 weeks.

Subtask 2.1a: Test porcine cells for adhesion

Purpose:
The purpose of this study was to carry out a preliminary screening of all MSCs vis-à-vis the positive control fibroblasts for their ability to adhere to medical-grade Ti alloy and control glass surfaces by tracking expression of focal adhesion proteins and cell spreading using immunofluoresence.

Methods:
The following mesenchymal cells were identically tested (Gottingen Pig 3; isolated as previously described):
1. Bone marrow harvested from the iliac crest (Bone Marrow)
2. Muscle from hind limb (Muscle)
3. Achilles tendon (Tendon)
4. Subcutaneous connective tissue underlying the hoof of hind limb (Hoof)
5. Adipose tissue from the flank (HL Adipose)
6. Abdominal Adipose tissue (AB Adipose)
7. Dermal tissue from hind limb (HL Dermis)
8. Abdominal dermal tissue (AB Dermis)
9. Periodontal ligament associated with molars (Molar)

NIH-3T3 (ATCC, Manassas, VA) murine embryonic fibroblasts were tested as positive controls due to our previous expertise with these cells in the lab.

Ti-6Al-4V extra low interstitial (ø=7mm, President Titanium) disks were polished to a 20-60 nm colloidal silica finish. Glass (ø=7mm, Harvard Apparatus) was used as a positive control substrate. Cells were seeded on titanium and glass disks at 1,000 cells/disk for 4, 24, and 72 hours. At each time point, cell spreading and focal adhesions characteristics were determined with immunofluorescence. Seeded disks (n=10; both Ti and glass) were washed in phosphate buffered saline (PBS) to remove weakly adhered cells and fixed in 4% paraformaldehyde. Then, adhered cells were permeabilized with Triton X-100 in PBS and blocked in 5% bovine serum albumin (BSA). After PBS washing, cells were stained with an anti-vinculin antibody (MilliPore Sigma) overnight. Following washing, secondary antibody (Cy5, Abcam) was added for 1 hour. Seeded disks were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 300 nM in PBS) for 10 minutes. Following extensive washing, the seeded disks were mounted with ProLong (Fisher) and imaged (Olympus FV1000 - 40X). Other disks (n=5) were stained with rhodamine-phalloidin (Fisher) and processed similarly with DAPI and mounted. ImageJ (NIH) was used for image analysis with 5 fields of views (FOVs) per sample.

Proliferation (number of DAPI-positive nuclei), cell surface area per field of view, average individual cell surface area, focal adhesion integrated intensity per FOV, average focal adhesion integrated intensity per cell, and average focal adhesion integrated intensity per cell surface area (FApSA) were calculated. A t-test with False Discovery Rate (FDR) correction, an analysis of variance (ANOVA) with Dunnett's multiple

comparison (against NIH-3T3 cells), and ANOVA with a Tukey's post hoc analysis were used for statistical analysis (GraphPad Prism 7.0d). A threshold value of p = 0.05 was chosen. Values are presented as mean ± standard deviation.

Results:
Proliferation, cell surface area per FOV, and average individual cell surface area results showed a significant increase through time (from 4 hours to 72 hours) for all cell types, with no significant differences between fibroblasts and each of the MSCs. Although MSCs showed significantly lower focal adhesion intensity than fibroblasts at 4 and 24 hours, no differences were seen at 72 hours. FApSA results showed no significant differences between each MSC and fibroblasts. There were no notable differences in cellular behavior between titanium and glass. Differences in focal adhesion and cell surface area among MSCs were minimal, but proliferation of MSCs from hindlimb dermis, hoof, and abdominal dermis was higher than other tissue-derived MSCs. Proliferation of muscle-derived MSCs was the lowest among all MSCs. Representative results from abdominal dermis derived MSCs are shown in **Figure 4**.

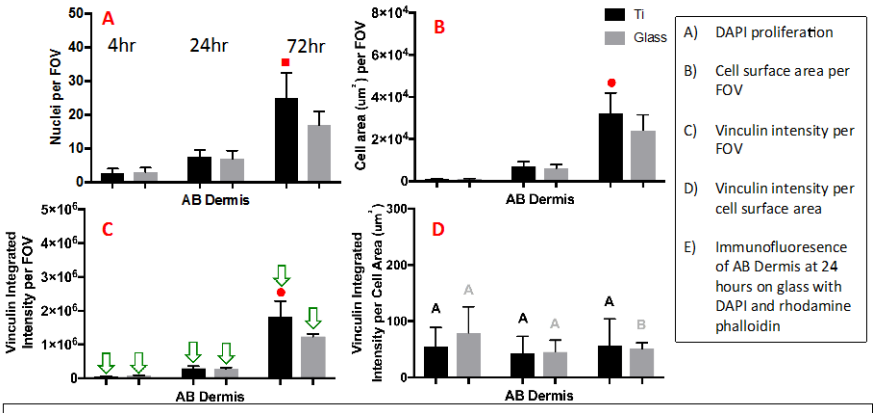
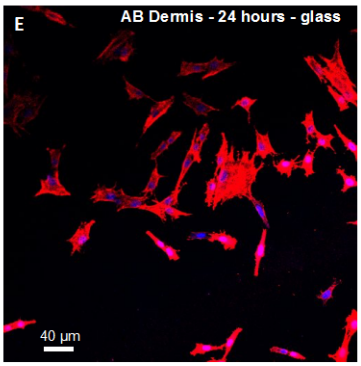


Figure 4: Proliferation and adhesion measurements from porcine-derived abdominal dermis MSCs
• shows significant differences between Ti and glass under the same conditions (*t*-test with FDR)
ABC shows significant differences through time for each cell/substrate combination (ANOVA/Tukey)
↓ shows significantly *lower* factor for each condition as compared to equivalent conditions for NIH-3T3 fibroblasts (Dunnett's)



DAPI / Phalloidin

Conclusions:
Our results demonstrate that all porcine MSCs have the ability to adhere and proliferate on polished medical-grade Ti alloy and control glass surfaces. Preliminary studies indicate minimal differences in adhesion and proliferation. Future work will include gene expression and other adhesion markers on a subset of highest performing MSCs based on differentiation and adhesion results.

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.

Nick Fischer: Attended 2018 Institute for Engineering in Medicine Annual Retreat at the University of Minnesota, Minneapolis, MN on September 24, 2018.

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.

USUHS

- Completion of the comparative multilineage differentiation potential of all derived porcine cells.
- Increasing sample size for molar-derived periodontal ligament tissue, abdominal and hind limb dermis
- Completion of gene expression analysis of osteo-, chondro- and adipogenic differentiation markers across the nine tissues
- Phenotypic analysis of MSC markers across the nine tissues by qRT-PCR based gene expression for CD45, CD90, CD105, Sca1
- Manuscript preparation to report the comparative profiling of nine porcine tissue-derived MSCs

MMRF-UMN

- Optimizing the protocol for MSC and keratinocyte differentiation of iPS cells, which will then be confirmed by gene and/or protein expression, and immunofluorescence.
- Assessing the adhesion and proliferation of iPS-derived MSCs and keratinocytes on titanium alloy and glass substrates.
- A new postdoc will be hired at MMRF who will be helping with the scaffold design and in the *in vivo* studies as part of Specific Aim 2.
- Develop initial parameters for the design of the scaffold which will then be used for cell adhesion and growth experiments (both porcine derived cells and iPS derived MSCs and/or keratinocytes).

Both sites

- Complete the submission of a review article on the general topic of 'Osseointegration'.

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 PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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:

The signing of the subcontract with MMRF and to all third party subawards through MMRF, was delayed because of required budget adjustments, statement of work updates, and further DoD approvals routed through the Prime awardee. A final subaward was signed in January, 2018 with the further UMN subaward executed in February, 2018. This delayed the initiation of tasks associated with the work to be supported by UMN-MMRF

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.

Nothing to Report

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.

Hiring of a postdoctoral associate with stem cell expertise to complete tasks at MMRF-UMN was delayed as the recruited associate had to get a J1 visa to join the project and she was not able to secure an interview in the US embassy for this purpose until 6July2018. This postdoctoral associate started her appointment at UMN on 25July2018.

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

Not applicable

Significant changes in use or care of vertebrate animals

Significant changes in use of biohazards and/or select agents

Not applicable

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).*

Nothing to Report

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.*

Nicholas G. Fischer, Devaveena Dey, Mehdi Belkhodja, Thomas A. Davis, Joan E. Bechtold, Jonathan A. Forsberg, Conrado Aparicio. *In vitro Adhesion of Porcine Mesenchymal Stem Cells to Percutaneous Osseointegrated Prostheses for Improved Skin/Implant Therapies. 2018 IEM Annual Review, University of Minnesota, Minneapolis, MN. Poster presentation.* (**Won the first place in poster session in Regenerative Medicine**)

Nicholas G. Fischer, Devaveena Dey, Mehdi Belkhodja, Thomas A. Davis, Joan E. Bechtold, Jonathan A. Forsberg, Conrado Aparicio. *In vitro Adhesion of Porcine Mesenchymal Stem Cells to Percutaneous Osseointegrated Prostheses for Improved Skin/Implant Therapies*. 2019 Orthopedic Research Society Annual Meeting. Austin, TX. Submitted.

Devaveena Dey, Mehdi Belkhodja, Jonathan A. Forsberg, Thomas A. Davis. *In vitro Characterization of Osteogenic Differentiation Potential of Porcine Mesenchymal Stem Cells Derived from Multiple Integumentary Tissues for Improved Durability of the Skin/Implant Interface. 2019 Orthopedic Research Society Annual Meeting. Austin, TX. Submitted.*

- **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. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**

•**Other Products**

Identify any other reportable outcomes that were developed under this project.

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".

Example:

Name: Jonathan A Forsberg, MD, PhD.
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: CDR Forsberg responsible for overall project coordination.

Name: Joanie Bechtold, PhD
Project Role: Site PI (MMRF)
Researcher Identifier (e.g. ORCID ID): 0000-0002-7090-4270
Nearest person month worked: 1
Contribution to Project: Responsible for overall project coordination at subaward site(s).

Name: Thomas A. Davis, PhD
Project Role: Associate Investigator (USUHS)
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Oversight of project conducted at USUHS.

Name: Conrado Aparicio, PhD.
Project Role: Associate Investigator (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0003-2969-6067
Nearest person month worked: 1
Contribution to Project: Oversight of iPSC in vitro studies and cell adhesion studies conducted at UMN.

Name: Devaveena Dey, PhD
Project Role: Associate Investigator (USUHS)
Researcher Identifier (e.g. ORCID ID): 0000-0002-0507-5701
Nearest person month worked: 1
Contribution to Project: Oversight of in vitro porcine cells studies conducted at USUHS.

Name: Isha Mutreja, PhD
Project Role: Postdoctoral Associate (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0002-8998-7563
Nearest person month worked: 1
Contribution to Project: Oversight of in vitro iPS cells studies conducted at UMN.

Name: Isha Mutreja, PhD
Project Role: Postdoctoral Associate (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0002-8998-7563
Nearest person month worked: 1

Name: Nicholas Fischer, B.S.
Project Role: Graduate Student (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0003-2230-5158
Nearest person month worked: 1
Contribution to Project: Performance of porcine cell adhesion experiments conducted at UMN.

Name: Mehdi Belkhodja, B.S.
Project Role: Research Associate (USUHS)
Researcher Identifier (e.g. ORCID ID): 0000-0002-7796-4807
Nearest person month worked: 1
Contribution to Project: Performance of porcine cell based experiments conducted at USUHS.

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."

Nothing to report

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);*

- *Other.*

Organization Name: Stem Cell Institute – Professor Jakub Tolar's lab
Location of Organization: University of Minnesota, Minneapolis, MN
Partner's contribution to the project: Facilities, collaboration, personnel exchange (Isha Mutreja-Aparicio's lab, Kirk Twaroski-Tolar's lab).

8.SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (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.

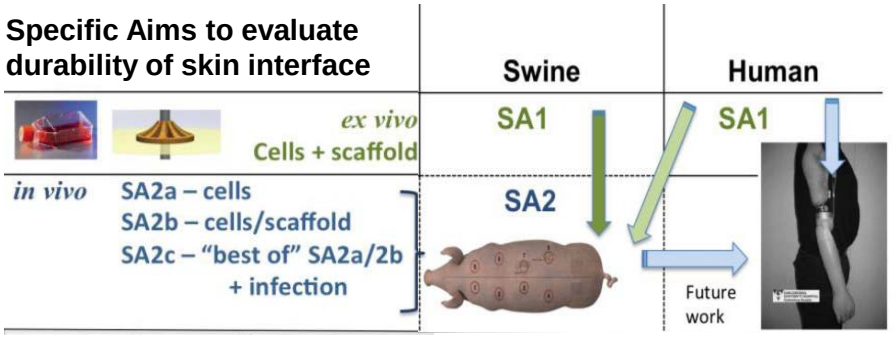
9.APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Optimizing Skin-Implant Interface of Osseointegrated Device, W81XWH-17-2-0059; Log# BA150755

PI: Forsberg, Jonathan Org: Henry M Jackson Foundation/ USU Award Amount: $2,499,220

Study/Product Aim(s): Our goals to optimize Osseointegration implant-interface with transdermal coupling based on examining three hypotheses: Hypothesis 1, septal scaffolds designed to limit tissue strain by providing gradual mechanical transition between hard implants and soft tissues will provide superior durability and sealing compared to sleeve scaffolds (with or without cells). Hypothesis 2, cells with the normal physiologic function to adhere soft tissues to hard tissues will provide superior ex vivo and in vivo attachment to metal implant substrates and superior resistance to infection compared to normal epithelial cells. Hypothesis 3, antimicrobial treatment of implant surfaces will improve sealing, durability, and resistance to skin infection

Approach: We based our approach on existing clinical work with OI implants; our strategy is to identify specific problems with currently available systems and to use basic science and our swine model to address these problems in a logical, step-wise approach. Our use of human cell types (including iPSC) has precedent in clinical trials. Swine skin models are highly relevant to represent structure and response of human skin. To maximize translational potential, we will use both swine and human cells to identify those that perform the best. Risk will be minimized by using candidate swine cells in swine model, and then using corresponding "best of best" human cells in the final cohort to provide data needed to use these human cells for human clinical trials in the US.



Accomplishments: We are continuing with studies to isolate, ex vivo expand, biobank and functionally characterize swine tissue-derived stem/progenitor cells from healthy swine tissues (bone marrow, adipose, muscle, Achilles tendon, nail/hoof bed, gingival and periodontal ligament). We have started studies evaluating the capacity of these cells to adhere and proliferate on a meta substrate-titanium.

Timeline and Cost

Activities / Yr	1	2	3	4	5	Total
Evaluate human and porcine MSCs/iPSCs; cell characterization						
Initial scaffold development						
Scaffold / cell adherence optimization						
Porcine modelling						
Estimated Budget ($)	$528k	$539K	$508k	$457k	$467k	$2.499M

Goals/Milestones

Y1 Goals

- ✓ Establish Subaward with MMRF……**COMPLETE**
- ❑ Evaluate differentiation of nail stem cells (NSC) in iPSCs – in progress
- ✓ Characterize cell system

Y2-3 Goals

- ☐ Scaffold development and adhesion testing – in progress
- ☐ Optimum scaffold/cell combination determined

Y4 Goals

- ☐ Initial Porcine model and cell integration testing
- ☐ Present/ publish initial results

Y5 Goals

- ☐ Final Porcine infection challenge model testing
- ☐ Present/ publish final results