**AWARD NUMBER:** W81XWH-17-2-0059

**TITLE:** Optimizing Skin-Implant Interface of Osseointegrated Device

**PRINCIPAL INVESTIGATOR:** Jonathan Forsberg, MD, PhD

**RECIPIENT:** Henry M Jackson Foundation, Bethesda, MD

**REPORT DATE:** October 2022

**TYPE OF REPORT:** Annual

**PREPARED FOR:** U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

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| **1. REPORT DATE** | **2. REPORT TYPE** | **3. DATES COVERED** |
|---|---|---|
| October 2022 | Annual | 15Sep2021-14Sep2022 |

**4. TITLE AND SUBTITLE**

**Optimizing Skin-Implant Interface of Osseointegrated Device**

**5a. CONTRACT NUMBER**
**W81XWH-17-2-0059**

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Dr. Jonathan Forsberg, MD, PhD

E-Mail:

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
HENRY M. JACKSON FOUNDATION FOR THE ADVA
6720-A ROCKLEDGE DR STE 100
BETHESDA MD 20817-1891

**8. PERFORMING ORGANIZATION REPORT**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

The proposed scope work addresses bio-engineering strategies to enhance healing and functionality of osseointegrated devices for the person with an upper extremity amputation. Recognizing the role of the septal compartments at the hard tissue-soft tissue interface at the tips of appendages, the investigators are investigating methodologies to create a more durable interface. The approach is to apply a scaffold to mimic septal compartments, select and propagate stem cells with enhanced adhesive properties to promote healing and osseointegration and to prevent infection with antimicrobial coated metal device material. We have isolated, characterized and selected highly adhesive stem cells (MSCs) with plasticity for multilineage differentiation that have good scaffold ingrowth, intending them to be a population of cells that can be seeded into the scaffold and transplanted to the interface of the metal device. Animal studies are being designed and the IACUC protocol developed to measure outcome to test depth of tissue growth, preventing or limiting infection, metal-skin interface strength, and histopathology.

**15. SUBJECT TERMS**
None listed

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| | | | | | USAMRDC |
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | **19b. TELEPHONE NUMBER** ***(include area code)*** |
| Unclassified | Unclassified | Unclassified | Unclassified | 59 | |

**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

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## 1. INTRODUCTION:

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 and connective 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, adipose tissues, 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:

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

## 3. ACCOMPLISHMENTS:

**What were the major goals of the project?**

| Research-Specific Aims and Tasks | Mos. | Percent complete | Sub- |
|---|---|---|---|
| **Administrative Aims and Tasks**:<br>1. Establish subaward agreement between HJF and MMRF<br>2. Develop and sign USU-MMRF CRADA<br>3. Recruit and hire support personnel<br>a. Stem Cell Biologist (USU)<br>b. Research Associate (USU)<br>c. Stem Cell Biologist (MMRF-UMN) | 1-4 | 100%<br>100%<br>100%<br>a. 100%<br>b: 100%<br>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** | 100% | X |
| **Major Task 1:** Develop cell culture | 1-16 | 100% | |
| **Subtask 1.1:** In vitro isolation and characterization of *porcine* cells. | 1-16 | 100% | |
| **Subtask 1.2:** In vitro development and characterization of *human* cells. | 1-16 | 100% | X |
| **Major Task 2:** *In vitro*: evaluate cell adhesion to metal substrate | 7-16 | 100% | X |
| **Subtask 2.1a**: Test porcine cells for adhesion | 7-16 | 100% | X |
| **Subtask 2.1b:** Test human cells for adhesion | 7-16 | 100% | X |
| **Major Task 3**: Scaffold development for cell growth and anchorage to underlying tissue. | 5-24 | 100% | X |
| **Subtask 3.1:** Complete scaffold design (constructs) for "sleeve" & "transition designs | 5-16 | 100% | X |
| **Subtask 3.2:** Seed and grow porcine cells on flat collagen sheets, assemble scaffolds | 17-24 | 100% | |
| **Subtask 3.3:** Seed and grow human cells on flat collagen sheets, assemble scaffolds | 17-24 | 100% | X |
| **Major Task 4:** Complete full statistical analysis, complete/submit 2-4 manuscripts. | 18-24 | 80% | X |
| *Milestone(s) Achieved: Characterization of 3-4 cell choices for optimal adhesivity - in vitro; submission of 2-4 manuscripts.* | *1-24* | 90% | *X* |
| **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** | 15% | X |
| **Major task 5**: (SA2a) Implant 8 implants/animal with "best of" cells from Specific Aims 1 and 2 x 10 animals at University of Minnesota | 25-35 | 5% | X |
| **Subtask 5.1:** Implant initial 5 animals with 3-4 types of cell augmentation per animal | 25-27 | 0% | X |
| **Subtask 5.2**: Initial evaluation of skin integration - assessment of initial results; experiment modification as necessary. | 28-29 | 0% | X |

**What was accomplished under these goals?**

**Specific Aim 1: *Ex vivo* culture (a) steer differentiation for human and swine MSC**

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

**Isolation of Hoof and Tendon MSC cells**

**Objective:** Development of biobank of isolated, expanded and characterized swine multipotent mesenchymal stem cells in order to complete the large animal study.

**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), as described in previous reports.

**Results:** In this past year, we increased the size of our swine cell biobank by isolating and freezing mainly the two most pertinent Swine MSCs cell types (Hoof- and Tendon-derived pMSCs). We updated our inventory shown **in Table 1.**

**Conclusion:** We successfully isolated enough Hoof- and Tendon-derived MSCs to accomplish the entire large animal study.

***Table 1: Inventory of Hoof- and Tendon-derived MSC cells localized at USU***

| Tendon MSC | Number of vials | Total of frozen cells |
|---|---|---|
| Passage 0 | 5 | 2,300,000 |
| Passage 1 | 31 | 25,000,000 |
| Passage 2 | 10 | 5,940,000 |
| Passage 3 | 35 | 189,000,000 |
| Passage 4 | 1 | 450,000 |
| Passage 5 | 4 | 3,100,000 |

| Hoof MSC | Number of vials | Total of frozen cells |
|---|---|---|
| Passage 0 | 3 | 1,200,000 |
| Passage 1 | 15 | 20,000,000 |
| Passage 2 | 12 | 11,000,000 |
| Passage 3 | 22 | 11,000,000 |
| Passage 5 | 6 | 6,800,000 |

**Large scale ex vivo expansion tendon-derived swine MSCs for in vivo application**

**Objective:** We tested the efficiency of Human Platelet Lysates and heparin (HPL+H) on cellular proliferation rate of multipotent MSCs derived Achilles tendon in order determine the maximum cell density that could be obtained after 10 and 15 days of culture.

**Methods:** The Tendon-derived MSCs (passage 1) were thaw and seeded at 350,000 cells in a 10 cm culture dish in standard MSC growth media (GM) (1:1 DMEM-F12, supplemented with 2% FBS and antibiotics (2x) consisting of penicillin, streptomycin and fungizone) After 24h the GM was exchanged with fresh GM supplemented with 2% of HPL (StemCell) plus 2 IU/ml of sodium heparin (StemCell) every 72h for 10 days at which point adherent confluency was ~ 90%. Cells were trypsinized, collected washed and counted. Cells were expanded for another 5 days by passaging at 1:3 ratio (**Figure 1**).

**Results:** The addition of Human Platelet Lysates and heparin promoted robust Tendon-derived MSC growth. The number of MSC cells increased 28-fold (1x107 total cells) and 200 fold (7 x107) after 10 and 15 days of ex vivo culture, respectively (**Figure 2**). Culturing swine MSCs rendered significant large number of undifferentiated adherent fibroblastic cell-type in only 15 days of culture, giving us enough material to pursue the in vivo experiment.

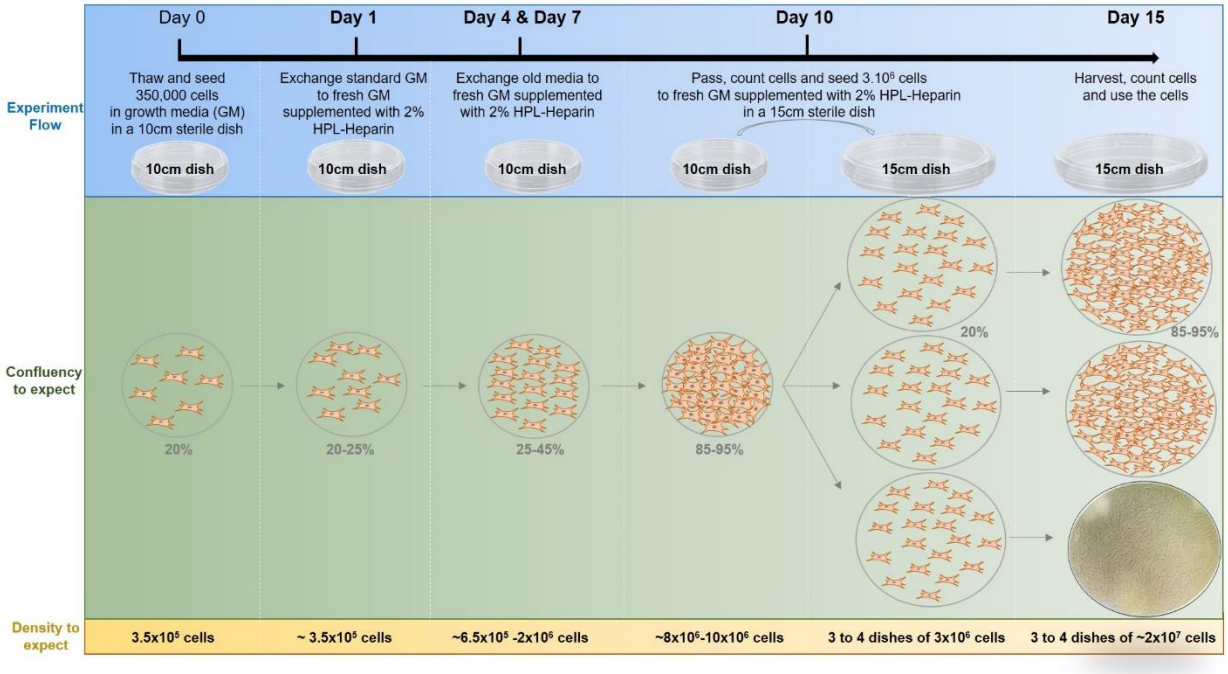



***Figure 1:*** *Cell expansion diagram showing the experiment flow, the confluency and cell density to expect after 1, 4, 7, 10 and 15 days of culture.*

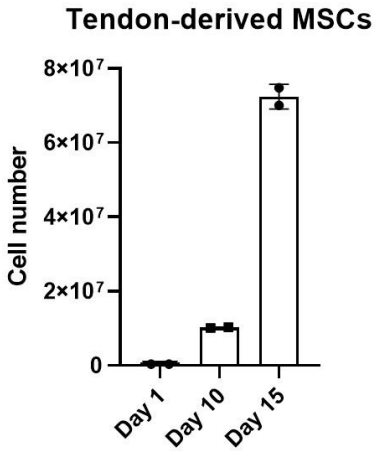



***Figure 2:*** *Tendon-derived MSC expansion characterization in sterile growth media supplemented with 2% of Human Platelets lysate and 2 IU/ml Sodium Heparin. Average of cell numbers at Day 1, Day 10 and Day 15 of Tendon-derived MSCs cultivated in cMSC-GM+HPL+Heparin.*

**Evaluation of platelet lysate and heparin as media additives on porcine MSCs marker transcription level.**

**Objective:** We tested the effect of Human Platelet Lysate and Heparin (HPL+H) on both Hoof and Tendon-derived MSC marker transcription using real time quantitative Polymerase Chain Reaction (RT q-PCR).

**Methods:** Hoof-associated superficial flexor tendon and Achilles tendon-derived MSCs were cultivated in parallel with commonly used growth media DMEM-F12+10% FBS and antibiotics (cMSC-GM) and in cMSC-GM supplemented with 2% Human Platelet Lysate and Heparin (2 IU/ml as final concentration) (cMSC-GM+HPL+H). We assessed comparative gene expression profiles for 2 housekeeping genes (B-actin and B2M) and 2 MSCs marker (CD90 and CD29) using quantitative Polymerase Chain Reaction (qPCR).

**Results:** Remarkably, in addition of HPL+H promoted robust swine MSC growth (**Figure 2**), we wanted to validate that HPL+H does not affect gene expression of porcine MSCs markers. We compared the transcript expression of two housekeeping genes (B-actin, B2M) and two putative MSCs markers (CD90 and CD29) after 11 days in culture either in cMSC-GM or in cMSC-GM+HPL+H (**Figure 3**). All transcripts were highly expressed independent of the culture conditions, and no marked changes in gene transcript expression were observed.

A

| | Primer sequences |
|---|---|
| B-actin-FW | ATCCACGAGACCACCTTCAA |
| B-actin-RV | TGATCTCCTTCTGCATCCTG |
| B2M-FW | CCATCCGCCCCAGATTGAAA |
| B2M-RV | CACGCGGCAGCTATACTGAT |
| CD90-FW | ACAGTCTTGCAGGTGGCCCG |
| CD90-RV | TCAGGGACCCCCACAGTGCC |
| CD29-FW | CGAATGTGAATGCCAGAGCG |
| CD29-RV | AATGTCTCCCAACACGTCCC |

B

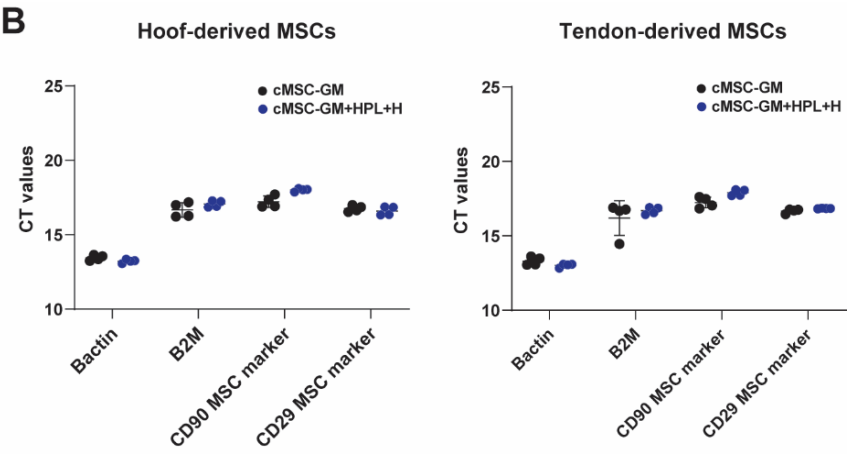



***Figure 3:*** *Hoof and Tendon-derived MSC gene expression study. A. Forward (FW) and reverse (RV) primer sequences used for the qPCR study. B. Average of CT values obtained from quantitative Polymerase Chain Reaction (qPCR) using of B-actin, B2M, CD90 and CD29 primers and comparing Hoof and Tendon-derived MSCs cultured 12 days in cMSC-GM or cMSC-GM+HPL+H. Data presented as mean +/- SD.*

**Evaluation of platelet lysate and heparin as media additives on Tendon-derived MSCs cytoskeleton structure and morphology**

**Objective:** The objective of this study was to evaluate the effect of platelet lysate and heparin as media additive on cytoskeleton of tendon-derived MSCs selected for in vivo implantation. To do so, we optimized a cytoskeleton-staining protocol to stain filamentous actin (F-actin) on MSCs cultured in standard (DMEM) and platelet lysate and heparin supplemented (DMEM+HPL+H) MSC growth medium. The cytoskeleton of cells plays important roles in cell morphology, adhesion, growth, and signaling. The actin network has been shown to play a major role in the mechanical properties of cells.

**Methods:** The Tendon-derived MSCs were seeded on a 0.2% gelatin-coated glass coverslip in either a standard MSC growth media (1:1 DMEM-F12, containing 2% FBS and 2X antibiotics) or MSC growth media supplemented of HPL+Heparin for 48h. Cells were fixed using cold 4% paraformaldehyde (Thermofisher) and permeabilized using 0.25% Triton-X100 (Thermofisher)-Phosphate Buffered Saline (PBS, Gibco). Cells were stained with ActinGreen 488 Readyprobes reagent (Thermofisher) which has a high-affinity for filamentous actin, main components of the cytoskeleton. The coverslip was mounted in a microscope slide using EverBrite™ Hardset Mounting Medium (Biotium) containing Dapi dye. The microcopy slide was imaged on an epifluorescence microscope (Zeiss) using an oil-immersion 40X objectives.

**Conclusion:** The ActinGreen staining highlights the structure and morphology of well-spread Tendon-derived MSC cytoskeleton by binding directly to actin filaments (grey) (**Figure 4**). Since implants will be coated with cells cultured in the presence of HPL, we assessed if this method of culture results in any cytoskeletal structural changes in MSCs that may be impact cell adhesion and differentiation. No significant difference was observed on cell cytoskeleton structure and

morphology using standard (DMEM) or platelet lysate and heparin supplemented (DMEM+HPL+H) MSC growth medium. In both cases, the actin filaments appear long, thin and well organized (**Figure 4**).

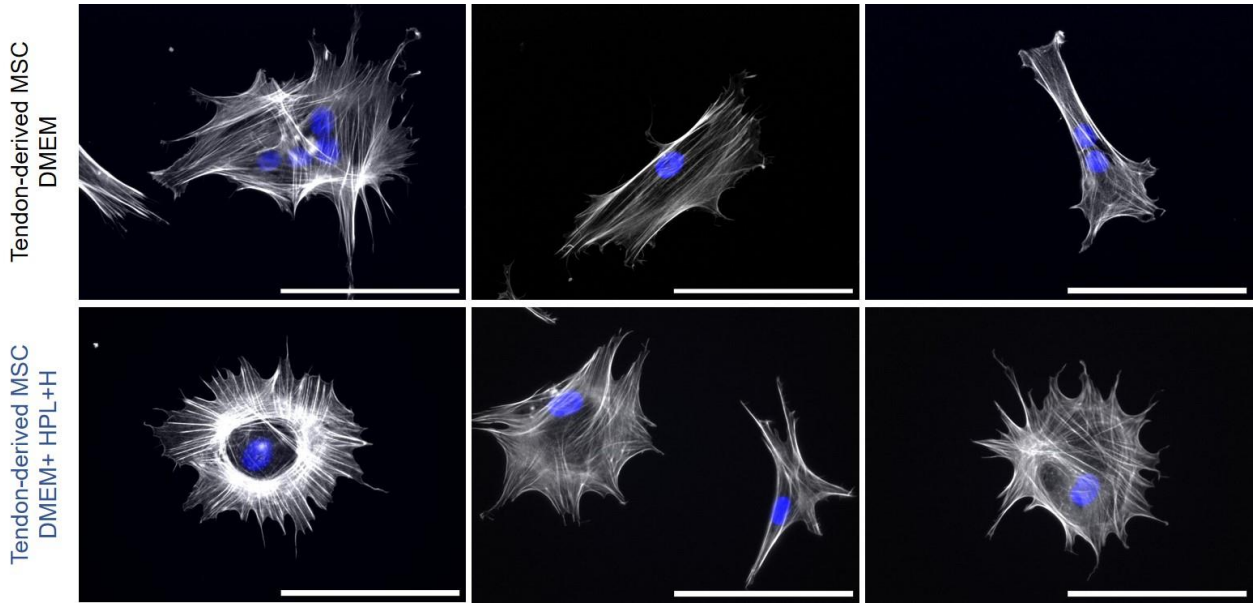



***Figure 4:*** *Tendon-derived MSCs cytoskeleton image using ActinGreen 488nm (grey) and Dapi (blue) staining. Scale bars equal 100 μm.*

One of the microscopy image obtained was submitted to "USU Research Day 2022- Art of Science Competition" and was awarded first place (see Appendix 1).

**Conclusion on using platelet lysate and heparin as media additives:** The use of HPL+H has a highly stimulatory effect on swine MSC proliferation while maintains cytoskeleton morphology, and the expression of B-actin, B2M even CD90 and CD29 (MSC markers) and consequently can be used to rapidly accelerate the ex vivo growth of hoof-associated superficial flexor tendon and Achilles tendon-derived MSCs.

**Shipment of frozen cells from USU to the University of Minnesota**

Eleven vials were successfully shipped from USU to The University of Minnesota on July 26$^{th}$ 2022 (see Table 2). A cell expansion standard operational procedure have been validated at USU and forwarded to the University of Minnesota.

***Table 2: Inventory of Tendon-derived MSC cells which have been shipped to the UMN***

| Tendon-derived MSCs | | | | | |
|---|---|---|---|---|---|
| **Passage** | **Pig #** | **Freeze date** | **Cell numbers in the shipped vial** | **Expected cell numbers after expansion** | **Purpose** |
| P1 | 11 | 06/05/2020 | 500,000 | 100,000,000 | Cell expansion protocol testing |
| P1 | 19 | 11/25/2019 | 690,000 | 138,000,000 | Implant coating for Large animal study |
| P1 | 19 | 11/25/2019 | 690,000 | 138,000,000 | Implant coating for Large animal study |
| P1 | 19 | 11/25/2019 | 690,000 | 138,000,000 | Implant coating for Large animal study |
| P1 | 19 | 11/25/2019 | 690,000 | 138,000,000 | Implant coating for Large animal study |
| P1 | 20 | 11/20/2019 | 700,000 | 140,000,000 | Implant coating for Large animal study |
| P1 | 20 | 11/20/2019 | 700,000 | 140,000,000 | Implant coating for Large animal study |
| P1 | 20 | 11/20/2019 | 700,000 | 140,000,000 | Implant coating for Large animal study |
| P1 | 20 | 11/20/2019 | 700,000 | 140,000,000 | Implant coating for Large animal study |
| P1 | 20 | 11/20/2019 | 540,000 | 108,000,000 | Implant coating for Large animal study |

### Training and education

During the performance period, our team mentored H2M Shruti Kamath, an EMPD2- upcoming medical student, in a variety of molecular and cell culture methodologies and techniques pertinent to studies in this proposal. Dr. Ronzier served as her mentor.

## <u>Specific Aim 2</u>: *In vivo* large animal (swine) testing of transdermal implants

- A Material Transfer Agreement (MTA) was executed on March 17th 2020 between Zimmer, Inc., and the Henry M. Jackson Foundation to provide 150 OsseoTi Transdermal Collars to support projected in vivo studies.

- Transdermal implants were fabricated by Zimmer Biomet and received by the University of Minnesota.

- The ex-vivo study to assess skin attachment onto titanium rods in the presence of cell-laden hydrogel was completed and the samples have been provided to the University of Minnesota imaging center for tissue clearance and imaging to analyze cellular responses. The center is optimizing the staining protocols to minimize strong background fluorescence which should be completed soon after which the samples will be imaged.

- Two swine cadaver pilot studies were performed to optimize surgical techniques and to refine the IACUC protocol and address potential IACUC questions and concerns. These studies determined the optimal spacing of the implants, incision placement, sizing of the transdermal hole and the optimal procedure for applying scaffold treatments during surgery (**Figure 5**). The scaffold treatment was stained to allow visual inspection of the placement and ensure even distribution on the implant.

- The large animal protocol for the in vivo study was approved by IACUC on May 06th 2022 (see Appendix 2) and approved by ACURO on July 05th 2022 (see Appendix 3). The approved protocol is presented in Appendix 4.

- An ex-vivo cell culture study to assess skin attachment onto titanium rods in the presence of cell-laden hydrogel was completed and the samples have been provided to University Imaging Center at UMN for tissue clearance and imaging to analyze cellular responses.

**Conclusion:** Significant progress has been made in regards to developing the experimental design and surgical procedures in order to conduct the in vivo-large animal study. All materials have been procured. Protocol optimization and mock surgical implantation procedures have been developed and optimized using pig cadavers at UM. Personal, facilities and supplies are in place to commence with the proposed in vivo studies once IACUC/ACURO approval is obtained and the custom-made titanium implants from Zimmer Biomet are received.

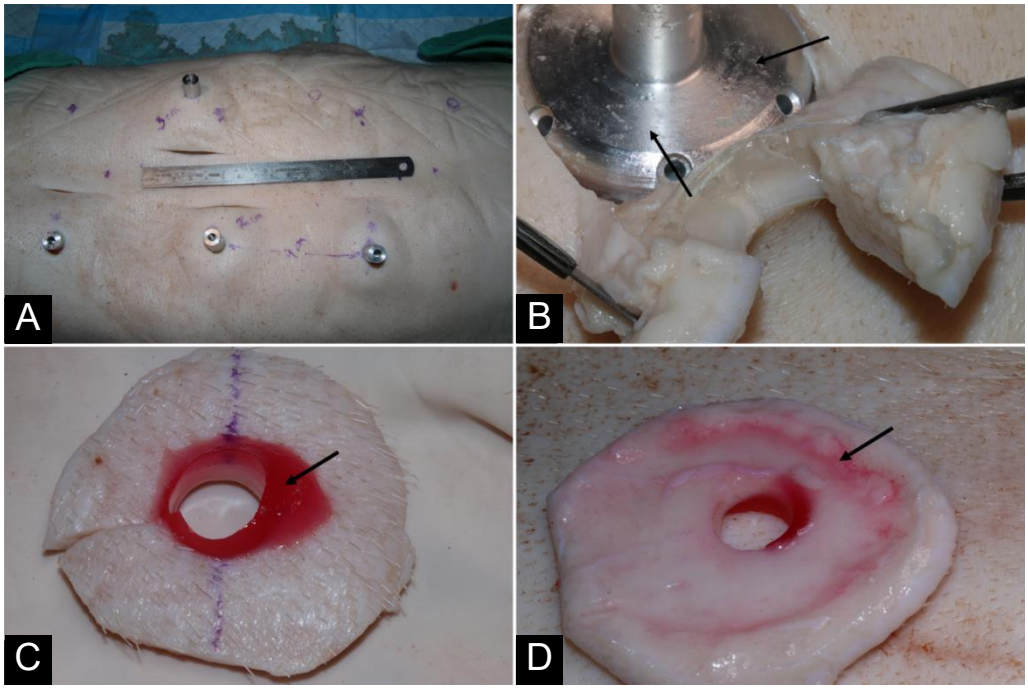



***Figure 5:*** *A. Picture shows the placements of implants on the cadaver along with the incisions made for inserting the implant, post-insertion ~17mm of the implant post protrudes out of the skin whereas. B. A picture of the base of the implant with scaffold residue (black arrows) after the scaffold was injected through the space between the post and the skin and cross-linked using visible light ($\lambda$ = 405 nm). C-D shows spatial distribution of colored scaffold (carmine was incorporated within scaffold for better visualization) demonstrating that there is a layer of cross-linked scaffold around the central hole (where the metal post was) and underneath the skin between the tissue and the base plate of the implant (as marked by black arrow).*

**<u>Major Task 4:</u> Complete full statistical analysis, complete/submit 2-4 manuscripts**

A peer-reviewed manuscript entitled "Culture and characterization of various porcine integumentary-connective tissue-derived mesenchymal stromal cells to facilitate tissue adhesion to percutaneous metal implants" was accepted for publication in an International peer-reviewed journal "Stem Cell Research and Therapy' on November 19th 2021 and is available on-line as of December 19, 2021 (see Appendix 5).

**What opportunities for training and professional development has the project provided?**

During the past year, Dr. Ronzier from the USU team mentored H2M Shruti Kamath, an EMPD2- upcoming medical student, in a variety of molecular and cell culture methodologies and techniques pertinent to studies in this proposal.

**How were the results disseminated to communities of interest?**

Nothing to report

**What do you plan to do during the next reporting period to accomplish the goals?**

- Finalize the second manuscript entitled *"Use of iMSCs-laden scaffolds for epithelial attachment onto metal abutment for improved performance of osseointegrated devices for amputees: in vitro and ex vivo analysis"*.
- Complete ~ 25% of the large animal study and submit 6-week tissue integrated implants for histological evaluation (uncoated implants, scaffold coated implants and scaffold-coated/plus cell-coated implants).

## 4. IMPACT:

**What was the impact on the development of the principal discipline(s) of the project?**

Nothing to Report

**What was the impact on other disciplines?**

Nothing to Report

**What was the impact on technology transfer?**

Nothing to Report

**What was the impact on society beyond science and technology?**

## 5. CHANGES/PROBLEMS:

Nothing to Report

**Changes that had a significant impact on expenditures**

Nothing to Report

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**

Not applicable

**Significant changes in use or care of vertebrate animals**

Not applicable

**Significant changes in use of biohazards and/or select agents**

Not applicable

## 6. PRODUCTS:

- **Publications, conference papers, and presentations**

  **Journal publications.**

**Publication:**
Dey D, Fischer NG, Dragon AH, Ronzier E, Mutreja I, Danielson DT, Homer CJ, Forsberg JA, Bechtold JE, Aparicio C, Davis TA. Culture and characterization of various porcine integumentary-connective tissue-derived mesenchymal stromal cells to facilitate tissue adhesion to percutaneous metal implants. Stem Cell Res Ther. 2021 Dec 18;12(1):604. doi: 10.1186/s13287-021-02666-2. PMID: 34922628; PMCID: PMC8684200.

**Poster presentation:**
Mutreja1 I. et al. Human iMSC-laden photocurable hydrogel for improved durability of the skin/implant interface – An in vitro and ex vivo analysis. MHSRS 2022 poster presentation.

**Books or other non-periodical, one-time publications.**

Nothing to Report

**Other publications, conference papers and presentations**.

Nothing to Report

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

Nothing to Report

- **Technologies or techniques**

Nothing to Report

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

- **Other Products**

Nothing to report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

| | |
|---|---|
| *Funding Support:* | *The Ford Foundation (Complete only if the funding support is provided from other than this award.)* |

*Name:* *Jonathan A Forsberg, MD, PhD.*
*Project Role:* *Principal Investigator*
*Nearest person month worked:* *1*
*Contribution to Project:* CAPT Forsberg responsible for overall project coordination.

*Name:* *Joan Bechtold, PhD*
*Project Role:* *Site PI (MMRF)*
*Researcher Identifier (e.g. ORCID ID):* 0000-0002-7090-4270
*Nearest person month worked:* *3*
*Contribution to Project:* Responsible for overall project coordination at sub award site(s).

*Name:* *Thomas A. Davis, PhD*
*Project Role:* *Associate Investigator (USUHS)*
*Nearest person month worked:* *3*
*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:* *3*
*9*
*Contribution to Project: Oversight of iPSC in vitro studies and cell adhesion 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:* *6*
*Contribution to Project: Oversight of in vitro iPS cells studies conducted at UMN.*

*Name:* *Nicholas Fischer, B.S.*
*Project Role:* *Graduate Student (UMN)*
*Researcher Identifier (e.g. ORCID ID):* 0000-0003-2230-5158
*Nearest person month worked:* *6*
*Contribution to Project: Performance of porcine cell adhesion experiments conducted at UMN.*

*Name:* *Elsa Ronzier, PhD*
*Project Role:* *Associate Investigator (USUHS))*
*Researcher Identifier (e.g. ORCID ID):* 0000-0003-1008-4271
*Nearest person month worked:* *12*
*Contribution to Project: Extraction of porcine tissues, cell isolation and characterization conducted at USUHS.*

*Name:* *Andrea Dragon, B.S.*
*Project Role:* *Research Associate (USUHS)*
*Researcher Identifier (e.g. ORCID ID):* 0000-0002-3257-1567
*Nearest person month worked:* *3*
*Contribution to Project: Extraction of porcine tissues, cell isolation and characterization 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?**

Nothing to report

**What other organizations were involved as partners?**

*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:**

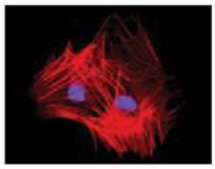
**QUAD CHARTS:**

## 9. APPENDICES:

**Appendix 1: USU Research Day 17th-19th May 2022 – Art of Science Competition Winner announcement**

Research Days 2022

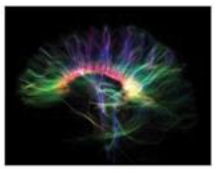
## Art of Science Competition Winners



### 1st Place Award

**HM2 Shruti Kamath**
US Navy, Enlisted to Medical Degree Preparatory Program

*Pig Cell Actin' Nuclear!*



### 2nd Place Award

**Herman Morris PhD**
MRI physicist in the USU Department of Radiology and Radiological Science

*Structural Brain Connectivity visualized using the USU MAGNUS MRI scanner at WRNMMC Brain*

### 3rd Place Award

**Rohini Manickham**
PhD candidate Molecular and Cell Biology PhD Program, GEO, School of Medicine-USU Department of Biochemistry and Molecular Biology

*Stress Fibers in Color*

50TH ANNIVERSARY 1972 2022 USU Uniformed Services University

## Appendix 2: IACUC protocol approved letter

**From:** iacuc@umn.edu
**Sent Date:** Friday, May 06, 2022 16:39:20 PM
**To:** sugorr@umn.edu, bechtold@umn.edu, carne115@umn.edu, pluha006@umn.edu
**Cc:**
**Bcc:**
**Subject:** IACUC Protocol Approved: 2112-39687A, Gorr, Sven-Ulrik
**Message:**

Dear Dr. Gorr, Sven-Ulrik,

The Institutional Animal Care and Use Committee (IACUC) reviewed your recent IACUC submission. We are happy to inform you that the information provided satisfies the requirements set by the IACUC and as such, you have received approval for the following project.

Please note that while the IACUC has approved your proposal, initiation of the project cannot begin without adherence to any additional restrictions that have been implemented by federal and local government and/or the University of Minnesota in response to the COVID-19 pandemic. IACUC approval does not supersede mandates made by these agencies and their requirements must be followed before initiating any research at this time.

Ongoing guidance about University of Minnesota research activities during the COVID-19 pandemic can be found at the OVPR page, COVID-19 Guidance for the Research Community: https://research.umn.edu/covid-19

IACUC approved protocol:

Protocol ID: 2112-39687A
Principal Investigator: Gorr, Sven-Ulrik
Department:
Protocol Title: The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Approval Date: May 06, 2022

For your records and for grant certification purposes, the approval date for the referenced project is May 06, 2022. The date of our last AAALAC accreditation was October 2021. The University's NIH Animal Welfare Assurance number is D16-00288 (A3456-01). The code number and the title of your study should be used in all communication with the IACUC office.

Approval for this protocol will expire three years from this date and a new application will be required in order to work past that time. If annual Continuing Review is required by your funding source, please contact the IACUC to arrange for a Continuing Review of your protocol. You are required to participate in the post-approval monitoring process and IACUC inspections during the life of your protocol, if applicable.

As Principal Investigator of this project, you are required by federal regulations to inform the IACUC of any proposed changes in your research involving the use of animals. Changes should not be initiated until written IACUC approval is received. It is also the responsibility of the Investigator to notify the appropriate funding agency of any changes to your proposed animal care and use that have occurred as a result of IACUC review and approval. Finally, the Investigator is responsible for maintaining copies of this application within the laboratory for reference by individuals conducting the work described in this protocol.

If you would like certification of approval sent to your funding agency or a paper copy of this approval letter, please email your request and the relevant information to iacuc@umn.edu.

## Appendix 3: ACURO protocol approved letter

**DEPARTMENT OF THE ARMY**
HEADQUARTERS, U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000
July 05, 2022

Director, Office of Research Protections
Animal Care and Use Review Office (ACURO)

Subject: Approval of Proposal Number BA150755, Award Number W81XWH-17-2-0059 entitled, "Optimizing Skin-Implant Interface of Osseointegrated Device"

Dr. Jonathan Forsberg, MD, PhD
Naval Medical Research Center
Silver Spring, MD, US

Dear Dr. Jonathan Forsberg, MD, PhD:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Conducted and Supported Research and Training"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"

In accordance with the above references, ACURO protocol BA150755.e001 entitled, "The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration," IACUC protocol number 2112-39687A, Protocol Principal Investigator Dr. Sven-Ulrik Gor, is approved by ACURO as of 06/30/2022 for the use of pigs and will remain so until modification, expiration or cancellation. This protocol was approved by the University of Minnesota Twin Cities IACUC on 05/06/2022; IACUC approval expires 05/06/2025.

**Required Actions:**

**A. Submit to ACURO for review and approval prior to implementing:**

- IACUC-approved de novo reviews of the protocol
- IACUC-approved significant changes to this protocol (see guidance document)

**B. Notify ACURO within 5 business days of any of the following:**

- Any noncompliance, suspensions or adverse events (see guidance document)
- Receipt of notification that the institution is under investigation by USDA
- AAALAC, International accreditation status change

For further assistance, please contact ACURO at (301) 619-6694, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

***NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.***

Sincerely,

Krinon Moccia, DVM, MPH, DACLAM
LTC, VC, USA
Director, Animal Care and Use
Review Office

Copies Furnished:
University of Minnesota Twin Cities IACUC
Patrick Struzky
Dr. Sven-Ulrik Gor
Dr. Lynn Impelluso
Mr. D. Jason Ghannadian
Lisa Straker

# Appendix 4: IACUC protocol approved



Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

## * * * Personnel Information * * *

### Principal Investigator (PI)

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**Department Name**
Dent Basic Sciences, Div of

**Dept ID**
11456

**Job Title**
Associate Dean

**Job Code Group**
Faculty - Regular

**Job Code**
9401

**Physical Address for PI Lab**

**Works with Animals*** No
**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?***
**Indicate with which procedures or species this person is not experienced and how they will be trained.***
**Click here for additional information about training.**
**Click to check Medgate Status**
**Training Details**
No training data is available.
**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**

### Alternate Submitter





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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**Dept ID**
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**Job Title**
Researcher 5

**Job Code Group**
Civil Service

**Job Code**
8351

**Works with Animals*** Yes
**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?*** Y
**Indicate with which procedures or species this person is not experienced and how they will be trained.***
**Click here for additional information about training.**
**Click to check Medgate Status**
**Training Details**
No training data is available.
**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**
**This individual will be copied on emails from IACUC.**

### Secondary Investigator





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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ORSU Orthopaedic Admin

**Dept ID**
11824

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Non-Exempt Temporary or Casual

**Job Code Group**
Temp Casual

**Job Code**
0001

**Works with Animals*** No
**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?***
**Indicate with which procedures or species this person is not experienced and how they will be trained.***
**Copied on email from IACUC?** Y
**Click here for additional information about training.**
**Click to check Medgate Status**
**Training Details**
No training data is available.
**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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11652

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Professor

**Job Code Group**
Faculty - Regular

**Job Code**
9401

**Works with Animals*** Yes
**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?*** Y
**Indicate with which procedures or species this person is not experienced and how they will be trained.***
**Copied on email from IACUC?** Y
**Click here for additional information about training.**
**Click to check Medgate Status**
**Training Details**
No training data is available.
**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**

### Additional Staff

Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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**Dept ID**
11884

**Job Title**
Researcher 5 No New Entry

**Job Code Group**
Civil Service

**Job Code**
8352ZF

**Works with Animals*** Yes

**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?*** Y

**Indicate with which procedures or species this person is not experienced and how they will be trained.***

**Copied on email from IACUC?** N

**Click here for additional information about training.**

**Click to check Medgate Status**

**Training Details**

**No training data is available.**

**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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**Dept ID**
11884

**Job Title**
Admin Assoc 3 Supv No Entry

**Job Code Group**
Academic Administrative

**Job Code**
9341ZA

**Works with Animals*** No

**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?***

**Indicate with which procedures or species this person is not experienced and how they will be trained.***

**Copied on email from IACUC?** N

**Click here for additional information about training.**

**Click to check Medgate Status**

**Training Details**

**No training data is available.**

**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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**Department Name**
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**Dept ID**
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**Job Title**
Researcher 6 No New Entry

**Job Code Group**
Civil Service

**Job Code**
7353

**Works with Animals*** Yes

**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?*** Y

**Indicate with which procedures or species this person is not experienced and how they will be trained.***

**Copied on email from IACUC?** N

**Click here for additional information about training.**

**Click to check Medgate Status**

**Training Details**

**No training data is available.**

**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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**Department Name**
Research Animal Resources Mpls

**Dept ID**
11342

**Job Title**
Researcher 2

**Job Code Group**
Civil Service

**Job Code**
8352R2

**Works with Animals*** Yes

**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?*** Y

**Indicate with which procedures or species this person is not experienced and how they will be trained.***

**Copied on email from IACUC?** N

**Click here for additional information about training.**

**Click to check Medgate Status**

**Training Details**

**No training data is available.**

**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**

Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

**Name***
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**Dept ID**
11342

**Job Title**
Researcher 3

**Job Code Group**
Civil Service

**Job Code**
8352R2

**Works with Animals*** Yes
**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?*** Y
**Indicate with which procedures or species this person is not experienced and how they will be trained.***
**Copied on email from IACUC?** N
**Click here for additional information about training.**
**Click to check Medgate Status**
**Training Details**
No training data is available.
**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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**Department Name**
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**Dept ID**
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**Job Title**
Researcher 2

**Job Code Group**
Civil Service

**Job Code**
8352R2

**Works with Animals*** Yes
**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?*** Y
**Indicate with which procedures or species this person is not experienced and how they will be trained.***
**Copied on email from IACUC?** N
**Click here for additional information about training.**
**Click to check Medgate Status**
**Training Details**
No training data is available.
**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

**Name***
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**Pager**

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**Mailing Address**

**City / State / Zip**

**Department Name**
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**Dept ID**
11884

**Job Title**
Researcher 2

**Job Code Group**
Student Employee

**Job Code**
2226

**Works with Animals*** Yes
**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?*** Y
**Indicate with which procedures or species this person is not experienced and how they will be trained.***
**Copied on email from IACUC?** N
**Click here for additional information about training.**
**Click to check Medgate Status**
**Training Details**
No training data is available.
**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

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**Dept ID**
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**Job Title**
Research Professional 3

**Job Code Group**
LR - Clerical

**Job Code**
1815

**Works with Animals*** No
**Is this person experienced with all the species that he/she will use and all procedures he/she will be performing on this protocol?***
**Indicate with which procedures or species this person is not experienced and how they will be trained.***
**Copied on email from IACUC?** Y
**Click here for additional information about training.**
**Click to check Medgate Status**
**Training Details**
No training data is available.
**If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):**

* * * Funding * * *

Funding

**How will the work covered in this protocol/agreement be funded?**
**(Please include All funding sources)***

☐ **No Funding.**

Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

### Sponsored Projects Funding

| | Federal sponsor | Non-federal sponsor |
|---|---|---|
| Funding expected to come from future proposal(s) intended to be submitted to Sponsor | Y | Y |

### Internal University Of Minnesota Funding

| Internal Funding Source(s) |
|---|
| Internal Grant Program |

### Fund Source(s) Outside The University

---

## * * * Rationale * * *

**Project Title**

The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration

**Is this a teaching and/or wildlife study?** X No Teaching Wildlife

**Rationale**

**Note: Use language understandable to a layperson as you answer questions in this section. Avoid overly technical terms and define abbreviations. The readability should be similar to a newspaper article.**

**Study Objectives:**

**Address the following five points:**

a. **Background and significance (1-2 sentences):**

Osseointegration is a method of attaching a prosthesis to the residual limb of an amputee. Surgically, a threaded titanium implant (rod) is inserted into the medullary canal of the residual limb. Over time the titanium implant becomes infused with bone becoming a solid-stable structure (osseointegration). A titanium metal extension (abutment) is surgically attached to the implant and brought through the soft tissue and skin at the amputation site (transdermal interface). A prosthesis can then be attached to the exposed titanium abutment. Superficial and deep infection remains unsolved. The bone/implant/skin interface is critical in the maintenance of skin barrier function and defense against infection
(permanent skin seal at the implant-skin interface). Porous titanium discs will be coated with biodegradable vehicle material in the presence or absence of ex vivo cultured cells derived from swine and human stem cell populations and then implanted transdermally in the dorsal lumber region of pigs.

b. **What is the question the research addresses (1-2 sentences):**

Porous titanium discs will be coated with biodegradable vehicle material (GelMA) in the presence or absence of ex vivo cultured cells derived from swine stem cell populations and then implanted transdermally in the dorsal lumber region of domestic pigs. We will investigate new approaches to develop ex vivo engineered living tissues which can be transplanted and integrate with soft tissue, bone and metal to form a seal at the transdermal interface to prevent bacterial leakage. The ultimate goal of this study is optimization of the skin-implant interface, including skin-to-implant healing and attachment, remodeling of connective tissue during wound-healing processes, prevention of infection and control strategies.

c. **How will the results of this study be used? (1-2 sentences):**

We will investigate new approaches to develop ex vivo engineered living tissues which can be transplanted and integrate with





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
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Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

soft tissue, bone and metal to form a seal at the transdermal interface to prevent bacterial leakage. Furthermore, using novel treatment strategies we address recognized complications associated with the transdermal interface such as: wound dehiscence, delayed wound healing, bacterial leakage and colonization (infection), destructive microenvironmental pH levels surrounding implanted metal surfaces, chronic inflammation and long-term in vivo survival, growth and differentiation of bioengineered tissue allografts.

d. **Summarize the specific aims (derived from the grant proposal):**

NA

e. **For 3 year renewals, please provide a brief summary of the research results from animals obtained during the prior approval period.**

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## * * * Species * * *

**Species**

**Common Name *** Pig (Biomedical)
**Housing Location *** RAR

1. **Pain Class Categorization (Check all that apply then add the number of animals by acquisition method. List all animals produced in conjunction with the study even those that are the wrong genotype and are euthanized.)**

**Class A: No pain, distress or use of pain-relieving drugs.**
**Class B: Potential pain/distress WITH appropriate analgesia/anesthesia/tranquilizers.**
**Class C: Pain/distress WITHOUT analgesia/anesthesia/tranquilizers.**

| | Purchase (or transfer from external source) | Internal Transfer (from another IACUC protocol) | Produced In-House | Misc. | Total by Pain Class |
|---|---|---|---|---|---|
| ☐ Pain Class A | | | | | |
| ☒ Pain Class B | 25 | | | | 25 |
| ☐ Pain Class C | | | | | |
| Total by Acquisition method | 25 | 0 | 0 | 0 | 25 |

2. **For animals that are acquired by transfer from another IACUC protocol, please provide IACUC protocol Number(s):**

3. **Rationale for Species Selection**

a. **Why are the species you have selected the most appropriate for these studies?**

There are advantages of the swine model over other animal models. Swine share with humans similar anatomic and physiologic characteristics with respect to clinical, histological and immunohistological features associated with tissue regeneration and cutaneous skin healing. Pig skin and underlying fascia/muscle tissue has been shown to be the most similar to human and have made the pig a standard model of wound healing and reconstructive surgical treatments. The utilization of another species other than swine would require use of





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animals that would allow for a less than optimal comparison to the human tissue ingrowth and regeneration.

b. **Will you be conducting the same experiment in multiple species?** N

**If Yes, please justify.**

c. **Check all of the statements below that apply:**

X **This model has previously been used.**

**Provide citations, specify and explain why the citation is applicable to your study:**

The investigators have been involved with previous use of the model under a grant from the Advanced Medical Development Program and US Department of Defense DRMDP Clinical Rehabilitative Medicine Research Program at the Department of Regenerative Medicine at the Naval Medical Research Center.

**The results will be directly applicable to the health, care, or study of the species used.**

**Describe how results will benefit the species used.**

X **This is a new model. Describe the features of the species (e.g., anatomic, physiologic, genetic, etc.) that make it desirable for this model. Contrast with other available models, if any. (RAR veterinarians are available for consultation on new model development)**

Osseointegration research has been done in relation to dental implants and other implant models, but the utilization of an amputation swine model has not been created according to the literature search performed

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## * * * Experimental Design * * *

**Experimental Design**

1. **Explain the experimental design for this IACUC Protocol.**

**Please provide a brief overview of your study and be sure to include a clear description of the design, treatments, procedures and animal numbers. A complete, sequential, and accurate description of what procedures will be performed on/with the animals must be provided.**

**Within each species and for each group (treatments within a study, etc.) of animals, be sure to describe all procedures performed on/with the animals and indicate how often and when these procedures will be performed within a study. Be certain to identify each animal group and make sure this corresponds to the information you provided in the Species Table.**

**YOU ARE STRONGLY ENCOURAGED TO PROVIDE TABLES AND FLOW CHARTS TO EXPLAIN EXPERIMENTAL GROUPS, DESIGNS, AND SEQUENTIAL PROCEDURES.**

**PLEASE NOTE THAT DETAILS FOR EACH SEPARATE PROCEDURE ANIMALS WILL UNDERGO SHOULD BE LISTED UNDER PROCEDURES RATHER THAN IN THIS EXPERIMENTAL DESIGN SECTION.**

**For animals used in facilities with an IACUC approved Standard Operating Procedure for animal care (primarily agricultural facilities), you do not need to describe specific details of animal care and**





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**(primarily agricultural facilities), you do not need to describe specific details of animal care and management if they do not differ from those described in the approved Standard Operating Procedure for the facility being used. In this situation, simply report the IACUC protocol number for the Standard Operating Procedure and state that your study animals will be managed as described in the Standard Operating Procedure. Be sure to identify any exceptions and to address all associated questions.**

**Background Protocol Information:**
The ESS personnel that will be performing work on this protocol are accounted for in the ESS Roster Protocol 2101-38784A.

**Experimental Design:**
Epidermal down growth and marsupialization at the skin-implant interface has been identified as one of the prominent factors leading to infection of percutaneous devices as the sinus tract developed provides an ideal environment for bacterial growth. Therefore, methods that promote or accelerate soft tissue integration and sealing of the skin interface could eliminate the risk of infection at percutaneous sites.
Gelatin methacryloyl (GelMA) is a versatile material for a wide range of bioapplications. There is an intense interest in the use of GelMA in orthopedic use as an independent therapy or vehicle for infection prevention applications. In the proposed experimental groups, cells are placed in GelMA that has been optimized for adhesivity and other attributes.The objective of this study is to determine if the addition of porcine or human stem cells to a GelMA vehicle improves fixation over control 1 (no cells, no GelMA) or control 2 (no cells, with GelMA). The two controls are necessary due to the potential for the biomaterial GelMA to assert an effect.
The study design is based on findings from the Investigator's previous work which provided data on which to base cell choice, for porcine cells and for human cells.
•Porcine cell = pMSC selected candidate/Tom Davis

•Human cells = (a) Gelma + human BM-MSC and (b) human iPSC-derived MSCs/Mutreja

*Experimental Group 1: Porcine Test Therapy (3 Treatment Groups x 9 animals)*
Porcine cells will be evaluated in pigs and human cells will be evaluated in immunocompromised pigs.
•Ti implant/no cells/no GelMA (control 1)

•Ti implant/no cells/with GelMA (control 2)

•Ti implant / GelMA + pMSC selected candidate/Tom Davis

*Experimental Group 2: Human Test Therapy (4 Treatment Groups x 12 animals)*
•Ti implant/no cells/no GelMA (control 1)

•Ti implant/no cells/with GelMA (control 2)

•Ti implant / GelMA + human BM-MSC

•Ti implant / GelMA + human iPSC-derived MSCs/Mutreja

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•Ti implant / GelMA + human iPSC-derived MSCs/Mutreja

Group sizes were determined based on the following assumptions: (a) 6 implants will be placed per animal (3 rows of bilateral implants), (b) this results in 3 implants for histology and 3 implants for mechanical evaluation per animal.Sample size of 9 implants per treatment group requires 9 animals for the porcine cell evaluation and 12 animals for the human cell evaluation. Visual representation of study groups is provided in the attached document "Proposed Porcine Study Design_BTS."
21 animals have been determined to be sufficient to fulfill the study design. At 80% power and an accepted error of 0.05,if the variable has a standard deviation of 25% of the mean (assumption), then a 9 sample size would allow us to statistically find a significant difference >33% between the two compared groups. If the sample size would be of 12, then the significant difference detectable at this power would be >29%. We are requesting 4 additional animals for use as replacements in the case of unforeseen adverse events, so as to complete the study with the number of statistically relevant number of animals needed to fulfill the study design. The number of replacement animals was determined for an anticipated study fallout rate of 20% for swine studies which represents the maximum observed fallout rate for swine studies requiring complex cardiac and vascular procedures performed by the ESS laboratory and clinician partners. Should adverse events require the use of replacement animals, the Investigator will work closely with RAR veterinarians in determining the root cause of the events or possible amendments / refinements to the surgical procedure or postoperative care to improve outcomes for the animals used as replacements. We are requesting 25 animals in total to be approved for this protocol.

**Experimental Procedure:**

All animals may have a control blood sample drawn by venipuncture preoperatively during anesthesia induction and preparation for surgery.
Animals will be anesthetized, intubated, and prepared for aseptic surgery. Once transported into the operating room, the animal will be placed in ventral recumbency, connected to mechanical ventilation and vital monitoring leads, and the dorsal aspect of the animal prepared for aseptic surgery for chronic implant and evaluation.
Six areas on each animal's dorsal aspect will be selected for subcutaneous implant of the titanium disc. Small incisions will be made in each selected area to facilitate insertion of the titanium disc. Six additional incisions may be made with a skin biopsy punch or blade to facilitate externalization of the titanium disc plug, so that there is communication between the disc and the external aspect of the dorsum.
Upon completion of the implant insertions, all skin incisions will be closed in a standard manner with absorbable suture. Concurrently, the test / control therapies will be applied to the externalized disc sections in a manner outlined by the approved study group and dosing strategy.
Once therapies have been applied and incisions closed, the animals will be weaned from mechanical ventilation and anesthesia, and recovered to sternal / ambulatory prior to transfer to RAR postoperative housing. During recovery from anesthesia, jackets or wraps may be placed on animals to facilitate protection of the surgical sites from mechanical damage associated with a recovering large animal. Wrapping will be





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performed in line with best practices as recommended by the attending RAR postoperative veterinarian.
Following surgery and insertion of discs and the test/control therapy, animals will be recovered and monitored for a maximum duration of 12 weeks post-op, however the investigator is targeting a term of 8 weeks based on previous work. Any extension of the recovery period beyond 12 weeks will be requested via amendment to the IACUC and approved prior to extending study duration past 12 weeks.
At the end of the study term, animals will be humanely euthanized prior to gross examination and tissue collection during a comprehensive necropsy.

**2. Justification for Number of Animals**

**How did you determine how many animals were needed for the activities described in this protocol?**
**A statistical analysis should be used to justify animal numbers whenever possible. Statements such as "The study requires 50 animals or 50 experiments," are not sufficient.**

**Check all that apply and provide specific answers to the associated questions:**

x **Pilot study or preliminary project, group variances unknown at present. Describe the information used to estimate how many animals are needed.**

21 animals have been determined to be sufficient to fulfill the study design. At 80% power and an accepted error of 0.05,if the variable has a standard deviation of 25% of the mean (assumption), then a 9 sample size would allow us to statistically find a significant difference >33% between the two compared groups. If the sample size would be of 12, then the significant difference detectable at this power would be >29%. We are requesting 4 additional animals for use as replacements in the case of unforeseen adverse events, so as to complete the study with the number of statistically relevant number of animals needed to fulfill the study design. The number of replacement animals was determined for an anticipated study fallout rate of 20% for swine studies which represents the maximum observed fallout rate for swine studies requiring complex cardiac and vascular procedures performed by the ESS laboratory and clinician partners. Should adverse events require the use of replacement animals, the Investigator will work closely with RAR veterinarians in determining the root cause of the events or possible amendments / refinements to the surgical procedure or postoperative care to improve outcomes for the animals used as replacements. We are requesting 25 animals in total to be approved for this protocol.

**Group sizes determined statistically. Describe the statistical analysis used to estimate the number (N) of animals needed. A common approach is to estimate N from a simple power analysis for the most important measurement in the study. This is usually based on the expected size of the treatment effect, the standard error associated with the measurement, and the desired statistical power. Data analysis methods should not be submitted unless directly applicable to the estimate of N.**

**Group sizes based on quantity of harvested cells or amount of tissue required. Explain how much tissue is needed based on the number of experiments you will conduct and how much tissue you expect to obtain from each animal. Example: Need 10 g tissue. Can get 2 g tissue per animal = Need 5 animals.**

**Product Testing. If the number of animals needed is based on FDA guidelines, provide the citation from the regulations, the IND tracking number, or relevant FDA correspondence:**

**Other - Elaborate, indicating method used to determine group size:**





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*** * * Procedures * * ***

| | |
|---|---|
| **Procedure Type: *** | Blood Collection |
| **Procedure Title: *** | Scheduled Blood Collections |
| **Species: *** | Pig (Biomedical) |
| **Pain/Distress Category: *** | B |
| **Copy procedure location details** | |
| **Procedure Location:** | RAR |
| **Other:** | |
| **Room Number:** | RAR housing, Post op, or basement of PWB, or mezzanine KE |
| **Will you be moving Animals from Primary Housing for this Procedure?** | N |

**Information on Animal Transport**

| | |
|---|---|
| **From where to where will animals be transported?** | |
| **Via what route will the animals be transported?** | |
| **Who will transport the animals?** | |
| **What equipment will be used to transport them?** | |
| **At what time(s) of the day will transport occur?** | |

*** * * Procedure Description * * ***

**Blood Collection**

**Please check this box if this blood collection is done immediately prior to euthanasia. Provide details regarding anesthetics given prior to euthanasia below.**

**This section does not need to be completed if blood collection is immediately prior to euthanasia.**

**Blood Collection Guidelines**

**Blood Collection Details**





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| | |
|---|---|
| **Frequency : * (How many collections per unit of time)** | once |
| **Duration : * (Once, weekly through study, etc.)** | a baseline sample for clinical chemistry and hematology, 7 days, monthly, not to exceed 5 collections |
| **Volume Collected : * (Per collection instance)** | 12 ml |
| **Method and Anatomical Site of Collection : *** | venipuncture, jugular or other peripheral vein |
| **Do you need an exception to the blood collection limits ?** | **N** |

**If yes, then provide details including the scientific justifcation:**

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## * * * Anesthetic Regimen * * *

**Anesthetic Regimen**

**Will you be performing this procedure with anesthesia?** Y

**If yes, please add at least one Anesthetic/Sedative, Analgesic and/or Paralytic Agent.**

### Anesthetic Agent(s) and Sedatives(s)

**Agent Name *** Tiletemine/zolazepam

**Dosage (in mg/kg if possible) *** 1-8 mg/kg

**Route of Administration *** Intramuscular( IM )

**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? *** N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

**Agent Name *** Xylazine

**Dosage (in mg/kg if possible) *** 1-3 mg/kg

**Route of Administration *** Intramuscular( IM )

**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? *** N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

**Agent Name *** Ketamine Hydrochloride

**Dosage (in mg/kg if possible) *** 250mg

**Route of Administration *** Intramuscular( IM )





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**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? *** N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

### Analgesic agents (Prophylactic and Intra-procedural)

### Paralytic Agent(s)

**Please describe intended use if agents will be used in combination.**

Sedation drugs are included in this procedure so that blood samples by venipuncture can be collected minimizing undue stress to the animals.
Tiletaminexzolazepam and xylazine may be mixed in the same syringe and administered in a single IM injection.

In the case of difficult sedation recovery, another option is to use all 3 in a pre-mix (Dose: 0.02-0.04 mls/kg of mixture, Route: IM, Preparation: Reconstitute an unused vial of Telazol with 2.5 mL ketamine (100 mg/mL) and 2.5 mL xylazine (100 mg/mL). This results in 100 mg/mL dissociative (tiletamine and ketamine), and 50 mg/mL each of xylazine and zolazepam))

**Other support agents used during procedure:**

Yohimbine 1-2 mg/kg, IM or IV OR

1 mg atipmaezole for every 10 mg of xylazine given.

**Parameters monitored during procedure:**

**Please indicate methodology and physiological parameters used to assess depth of anesthetics, paralytics and analgesics. Include the acceptable ranges for these procedures, (e.g., respiratory rate range of 60-80 per minute, or heart rate of 100-150 bpm).**

We will used the lowest dose possible to achieve sedation, generally we only monitor respiratory rate (6-20 breaths per min) and heart rate (60-140 beats per min).

**What specific steps will be taken in case any of the measured values are outside acceptable ranges?**

Still our goal is light sedation, animals should not become too deeply anesthetized. We will have endotracheal tubes, a scope and an ambu bag to intubate and ventilate the animal to provide respiratory support. We can use the partial reversal agent as this time as well.





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**Anesthesia and Intra-operative Surgery Records. Please describe your recordkeeping plans and forms:**

ESS personnel will record the drugs administered, along with the dose, time, and route of administration.

**All animals experiencing anesthesia or surgical procedures must have a concurrently recorded and readily available record detailing anesthesia data, intra-operative monitoring, and post-procedural care. Continuous documentation is expected to be recorded at a minimum of 15 minute intervals.**

**Sample Anesthesia, Surgery, and Post-Procedural Recordkeeping Forms**

**Do you need a recording frequency different than the guidelines?** N

**If yes, then provide details including the scientific justification:**

---

**Procedure Type: *** Surgery

**Procedure Title: *** Titanium Disc Insertion and Application of Experimental Therapies

**Species: *** Pig (Biomedical)

**Pain/Distress Category: *** B

**Copy procedure location details**

**Procedure Location:** KE / DVCCRC

**Other:**

**Room Number:** ESS Basement ORs

**Will you be moving Animals from Primary Housing for this Procedure?** Y

**Information on Animal Transport**

**From where to where will animals be transported?** Animals will be moved from RAR housing to ESS induction room for prep for surgery

**Via what route will the animals be transported?** Animals will be moved from RAR housing, be transported from PWB to blue tube hallway to passenger elevators, to KE basement.

**Who will transport the animals?** ESS personnel

**What equipment will be used to transport them?** Transport cages and gurneys

**At what time(s) of the day will transport occur?** 6 AM to 6 PM





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## * * * Procedure Description * * *

**Please select one:** S

**Description of Surgical Procedure**

**Provide sufficient detail, including closure, use of eye lubricant and other support drugs used. Helpful guidelines and tips can be found at the link below:**
**General Surgery Tips and Requirements**

Three days prior to the surgical implantation of transdermal titanium discs, pigs will be fitted with jackets as part of their acclimation prior to surgery. This will allow comfort in wearing the jackets post-surgery and protection of surgical site.

Animals will be anesthetized, intubated, and prepared for aseptic surgery. Once transported into the operating room, the animal will be placed in ventral recumbency, connected to mechanical ventilation and vital monitoring leads, and the dorsal aspect of the animal prepared for aseptic surgery for chronic implant and evaluation.

Six areas on each animal's dorsal aspect will be selected for subcutaneous implant of the titanium disc. Small incisions will be made in each selected area to facilitate insertion of the titanium disc. Six additional incisions may be made with a skin biopsy punch or blade to facilitate externalization of the titanium disc plug, so that there is communication between the disc and the external aspect of the dorsum.

Upon completion of the implant insertions, all skin incisions will be closed in a standard manner with absorbable suture. Concurrently, the test / control therapies will be applied to the externalized disc sections in a manner outlined by the approved study group and dosing strategy.

Once therapies have been applied and incisions closed, the animals will be weaned from mechanical ventilation and anesthesia, and recovered to sternal / ambulatory prior to transfer to RAR postoperative housing. During recovery from anesthesia, jackets or wraps may be placed on animals to facilitate protection of the surgical sites from mechanical damage associated with a recovering large animal. Wrapping will be performed in line with best practices as recommended by the attending RAR postoperative veterinarian.

**Please describe aseptic technique (e.g. instrument sterilization methods, surgical incision/site preparation, etc.)**
**http://www.ahc.umn.edu/rar/surgery.html#Preparation**

This is an chronic procedure, the surgical sites will be shaved and cleaned for this procedure. Aseptic preparation is necessary with a betadine alcohol solution applied to the surgical site. The entire animal (except the surgical sites) will then be draped with sterile towels.

Sterilization:
All equipment will be sterile for this procedure. This includes surgical instruments, drapes, supplies, instruments, and supplies.

**Will any animal undergo more than one survival surgical procedure?** N
**(This includes surgeries the animals may have had on another protocol or at another institution.)**

**Indicate the estimated time between each survival surgery.**

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**If yes, please provide justification for exception request.**
**Survival procedures on the same animal are not permitted, unless the procedures are essential components of the same project. Include in your justification any pain, distress, or functional deficit that may result and describe how such distress will be minimized.**

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## * * * Surgeon Details * * *

**Surgeon Details**

**Only personnel listed below will be approved to perform surgery on this protocol.**

### Surgeon Details

**Surgeon Name ***: Lahti, Matthew Theodore

**Please provide relevant training and experience with this species and surgical procedure. E.G., "In (Month/Year) (Name of the individual performing surgery) was trained to perform the (name of surgery) by (Name of the trainer) who has (X-number) years of experience with the technique.":***
Matt has over 25 years of experience performing complex cardiac and vascular surgery in swine.

**Surgeon Name ***: Carney, John

**Please provide relevant training and experience with this species and surgical procedure. E.G., "In (Month/Year) (Name of the individual performing surgery) was trained to perform the (name of surgery) by (Name of the trainer) who has (X-number) years of experience with the technique.":***
John has over 12 years of experience performing complex cardiac and vascular surgeries in large and small animal models, with an extensive publication record in the use of animal models of human disease.

**Surgeon Name ***: Pluhar, Liz

**Please provide relevant training and experience with this species and surgical procedure. E.G., "In (Month/Year) (Name of the individual performing surgery) was trained to perform the (name of surgery) by (Name of the trainer) who has (X-number) years of experience with the technique.":***
Dr. Pluhar is a practicing clinical veterinary surgeon at the UMN Department of Clinical Veterinary Sciences. Her research interests include improving total joint longevity, development of bone graft substitutes, the study of osteoclasts and bone graft incorporation, and the investigation of novel gene and immunotherapies for brain tumors.

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## * * * Anesthetic Regimen * * *

**Anesthetic Regimen**

**Will you be performing this procedure with anesthesia?** Y

**If yes, please add at least one Anesthetic/Sedative, Analgesic and/or Paralytic Agent.**

### Anesthetic Agent(s) and Sedatives(s)

**Agent Name ***: Propofol
**Dosage (in mg/kg if possible) ***: 1-2 mg/kg
**Route of Administration ***: Intravenous (IV)

**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? ***: N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

**Agent Name ***: Isoflurane
**Dosage (in mg/kg if possible) ***: 1-4%
**Route of Administration ***: Inhalation (IN)

**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? ***: N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

**Agent Name ***: Tiletemine/zolazepam
**Dosage (in mg/kg if possible) ***: 1-8 mg/kg
**Route of Administration ***: Intramuscular( IM )





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**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? ***: N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

**Agent Name ***: Xylazine
**Dosage (in mg/kg if possible) ***: 1-3 mg/kg
**Route of Administration ***: Intramuscular( IM )

**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? ***: N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

**Agent Name ***: Ketamine Hydrochloride
**Dosage (in mg/kg if possible) ***: 2-4mg/kg
**Route of Administration ***: Intramuscular( IM )

**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? ***: N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

### Analgesic agents (Prophylactic and Intra-procedural)





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**Agent Name ***: Buprenorphine SR
**Dosage (in mg/kg if possible) ***: 0.1-0.3mg/kg
**Route of Administration ***: Subcutaneous (SC)
**Duration and Frequency ***: once, 24hours to 30 minutes prior to surgery, last up to 72 hours

**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? ***: N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

**Agent Name ***: Carprofen (Rimadyl)
**Dosage (in mg/kg if possible) ***: 1-4mg/kg
**Route of Administration ***: Subcutaneous (SC)
**Duration and Frequency ***: SID or BID per veterinarian in the early postoperative period

**It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? ***: N

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

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| | |
|---|---|
| **Agent Name *** | Lidocaine |
| **Dosage (in mg/kg if possible) *** | 1-2mg/kg |
| **Route of Administration *** | Intramuscular( IM ) |
| **Duration and Frequency *** | may be injected IM at the site of disc insertion at the time of closure |
| **It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? *** | N |

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

| | |
|---|---|
| **Agent Name *** | Bupivicaine |
| **Dosage (in mg/kg if possible) *** | 1.0-2.0 mg/kg |
| **Route of Administration *** | Intramuscular( IM ) |
| **Duration and Frequency *** | may be administered at sites of disc insertion prior to closure |
| **It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline ? *** | N |

**If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. ***

Paralytic Agent(s)

**Please describe intended use if agents will be used in combination.**

Tiltemine/Zolazepam and xylazine will be administered as described in the table above to sedate the animal. Propofol will be administered to anesthetize the animal for placement of an endotracheal tube and surgical preparation. Once in the OR, isoflurane will be administered IN to maintain anesthesia throughout the case. Ketamine will be used only if difficult recoveries are observed, and was requested in previous reviews. It is unlikely that this cocktail will be necessary, but we will consider it, at the instruction of the reviewers and RAR attending veterinarians.

**Other support agents used during procedure:**





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atropine 0.04 mg/kg, IM
ceftiofur, 3 mg/kg, IM; alternatively Cefazolin may be administered 50mg/kg IV during anesthesia induction
normal saline (1-15 ml/kg/hour, IV)

In the case of difficult postoperative recovery, another option is to use all 3 in a pre-mix (Dose: 0.02-0.04 mls/kg of mixture, Route: IM, Preparation: Reconstitute an unused vial of Telazol with 2.5 mL ketamine (100 mg/mL) and 2.5 mL xylazine (100 mg/mL). This results in 100 mg/mL dissociative (tiletamine and ketamine), and 50 mg/mL each of xylazine and zolazepam))

**Parameters monitored during procedure:**

**Please indicate methodology and physiological parameters used to assess depth of anesthetics, paralytics and analgesics. Include the acceptable ranges for these procedures, (e.g., respiratory rate range of 60-80 per minute, or heart rate of 100-150 bpm).**

We monitor:ECG, heart rate, respiratory rate, temperature, end tidal CO2, & oxygen saturation.
HR 60-140 beats per min
blood pressure; mean >60 mmHg, systolic > 90 mmHg
oxygen saturation 90-100% is preferred
end tidal CO2 35-40 mmHg is preferred, 30-45 mmHg is acceptable

**What specific steps will be taken in case any of the measured values are outside acceptable ranges?**

-Changes in individual parameters may not be a good indication of anesthetic depth. Changes in two or more parameters are indications of anesthetic depth.
-If the change in the animals intrinsic values are significant (approximately 20% increase or decrease from baseline) the following steps will be taken:
-If parameters are below the expected ranges, the animal may be too deeply anesthetized. Anesthesia (inhalation or injectable) will be reduced and IV fluid volume will be increased.
-If parameters are above the expected ranges, the animal may be to lightly anesthetized. Anesthesia will be increased; IV anesthesia may be given if needed.
-If the pupils become too dilated, the animal may be too deeply anesthetized. Anesthesia will be reduced.
-If pupils become reactive, this is an indication that anesthesia is too light. Anesthesia will be increased, IV anesthesia maybe given if needed.
-If a palpebral reflex is present, this is an indication that anesthesia is too light. Anesthesia will be increased, and IV anesthesia maybe given if needed.

**Anesthesia and Intra-operative Surgery Records. Please describe your recordkeeping plans and forms:**

Records will document personnel, procedures, and drugs used (dose, time, and route of administration). Vital parameters will be recorded every 15 min.

**All animals experiencing anesthesia or surgical procedures must have a concurrently recorded and readily available record detailing anesthesia data, intra-operative monitoring, and post-procedural care. Continuous documentation is expected to be recorded at a minimum of 15 minute intervals.**
**Sample Anesthesia, Surgery, and Post-Procedural Recordkeeping Forms**

**Do you need a recording frequency different than the guidelines?** N

**If yes, then provide details including the scientific justification:**





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*** * * Post-operative Care * * ***

**Post-operative Care**

**1. How long will animals be maintained after surgery?**

Maximally 12 weeks following surgery. The current anticipated duration of the study is 8 weeks following surgery, but will be guided by procedural and in-life outcomes following surgery and initial study groups.

**2. Post-Operative Care Provider (Choose One):**

Y **A. Post-operative care will be provided by RAR or under RAR direct supervision for dogs, cats, non human primates or farm animals housed on the Minneapolis campus.**

**1) Does this study have requirements for specific analgesic, antibiotic or other post-operative practices that RAR Post-op staff will need to follow?** Y

**a. List the specific study requirements in the text box below. You must also list these requirements on the RAR Intake forms when animals are brought to RAR post-op.**

We will induce immunosuppression by treating with cyclosporine (15-30 mg/kg SID PO starting 1 day prior to xenotransplantation on the Ti implants for animals enrolled in Experiment 2, receiving humans MSC based therapy.

Clavamox- Antibiotic 13-20mg/kg PO BID for 10-14 days; Alternatively Cephalexin 500mg PO BID on post-operative day 1 (if Cefazolin is administered 50mg/kg IV on DOS)

Carprofen- Analgesic 1-4mg/kg PO/IM q 12-24 hours, PRN

**B. Post-Operative Care will not be provided by RAR.**

**1) Describe post-operative care: State the physiological parameters assessed (e.g. hydration, body temperature, parameters used to determine evidence of pain), frequency of monitoring, and any other post-procedural care details.**

**2) Post-operative Care and Monitoring Records. Please describe your recordkeeping plans and forms.**

**3) Click on "Add" to add Analgesic Agent(s).**
**NOTE: Adding several analgesic options in your protocol will provide flexibility as the study proceeds.**

Post-Operative Analgesics

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**3. Please describe intended use if agents will be used in combination.**

We will administer an opioid, may administer local blocks, and nsaids in combination to provide multi modal pain management. SR Buprenorphine will be administered prior to surgery as indicated in the table above. Carprofen will be administered as described in the table above. Lidocaine and bupivacaine may be administered during the surgical procedure as described in the table above. In cases of breakthrough pain, we will defer to RAR veterinarian's guidance.

**4. All animals experiencing surgical procedures must have a concurrently recorded and readily available record detailing anesthesia data, intra-operative monitoring, and post-procedural care. Post-operative/procedural records are expected to be recorded daily for a minimum of 3 days..**

**Do you need a recording frequency different than the guidelines ?** N
**If yes, then provide details including the scientific justification.**

**5. IACUC guidelines indicate that analgesics must be administered for 72 hours post-operatively.**

**Do you need an exception to this guidelines ?** N
**If yes, then provide details including the scientific justification.**

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* * * Health and Monitoring * * *

**Health & Monitoring**

**Please provide Species - Specific responsees to the questions below**

You must add at least one species on the Species tab to answer questions 1, 2, and 3.

**1. List the anticipated specific study-induced or related adverse health conditions that the animals might experience (i.e. pain, distress, complications, etc.) or any health problems that will or might result from the genotype or phenotype of the animal. Note that momentary pain or distress does not need to be described (e.g. venipuncture or injections). If applicable, consider and include any repeated handling and/or confinement stress associated with maintenance on a scientific study.**

| Species | Description |
|---|---|





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| Species | Description |
|---|---|
| Pig (Biomedical) | There is always a risk of complications with anesthesia.<br><br>There could be illness and/or death due to a non-efficacious infection prevention therapy administered during disc insertion, or infection occurring during the postoperative in-life period that does not respond to antibiotic therapies as suggested by RAR veterinarians.<br><br>To minimize the possibility of infection, animals will be singly housed with other pigs, surgical sites will be wrapped in lormir jackets with the addition of clean bandaging and Mepilex Ag foam dressing --that incorporates the rapid and sustained antimicrobial action of ionic silver with the benefits of Safetac soft silicone adhesive technology. The combined attributes of each component of this dressing allow both the control of pain and infection to be achieved simultaneously.<br><br>Fresh dressings will be applied daily until circumferential healing is observed in relation to the portion of the externalized implant, anticipated to be within the first 7-10 days of postoperative recovery. Once observed, jackets and dressings will be removed and animals will be socially housed (multiple pigs in a pen). The investigators will defer to best practices suggested by the attending RAR veterinarians in all cases. |

**2. How will pain and/or distress be monitored? Provide the specific clinical signs which will be monitored as well as the frequency of monitoring by personnel on this protocol and indicate if this monitoring will include weekends and holidays.**

| Species | Description |
|---|---|





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| Species | Description |
|---|---|
| Pig (Biomedical) | After surgery animals are monitored by RARs post operative care unit. After three days, if animals are recovering normally, the veterinarian will release the animal back into ESS care where they will continue to receive antibiotics and any other medications that have been prescribed. RAR will discuss continuation of care with ESS.<br><br>Animals are monitored, minimally, once a day by ESS which includes weekends and holidays.<br><br>We observe animals for (including but not limited to the following): normal recovery from surgery, eating, drinking, voiding, ambulation, respiratory rate, respiratory effort, heart rate and characterization, willingness to stand when approached, and willingness to ambulate.<br><br>We will monitor the subcutaneous pockets and implant sites made for the titanium discs and test/control therapies. The pocket will be inspected for temperature (heat from the pocket), redness, and fluid accumulation.<br><br>If clinical signs are noted it is our priority to have animals seen by an RAR vet. A veterinarian will be alerted to examine the animal as soon as possible. The veterinarian, in combination with the PI, will develop a treatment plan that will be specific to the animal and the clinical symptoms.<br><br>We will induce immunosuppression by treating with cyclosporine (15-30 mg/kg SID PO starting 1 day prior to xenotransplantation on the Ti implants for animals enrolled in Experiment 2, receiving humans MSC based therapy. Based on feedback from attending veterinarians and citing the attached Dean and Zhang publications, there are no anticipated adverse events from administration of immunosuppression therapies. As in all cases, we will follow the guidance of the RAR attending veterinarians. |

**3. Explain what steps will be taken to alleviate any pain, distress or discomfort the animals may experience. Provide the dose (in mg/kg), route of administration, frequency, and type of analgesic or tranquilizers to be administered. Consider warming pads, fluids, soft bedding, etc. You may provide a range of doses, frequencies, and drug classes for committee consideration. For animals undergoing multiple class B or C procedures, include environmental modifications that will be incorporated to offset experimental and/or confinement demands, and improve animals' coping abilities.**

| Species | Description |
|---|---|





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| Species | Description |
|---|---|
| Pig (Biomedical) | Animals are administered SR Buprenorphine the day prior to the surgical event.<br><br>Animals are given a combination of Telazol and Xylazine as a sedative prior to administration of IV anesthesia.<br><br>eye lubrication is applied during induction.<br><br>Local blocking agents may be administered prior to the closure of the disc insertion sites(bupivicaine 1-2 mg/kg, IM at incision sites) (lidocaine, 1-2 mg/kg, IM at incision sites).<br><br>Surgery is performed on a heated surgical table OR a warming pad will be placed under the animal during the surgical procedure.<br><br>An NSAID will be administered by Post Op when animals arrive in the post op care unit area after surgery.<br><br>To minimize the possibility of infection, animals will be singly housed with other pigs, surgical sites will be wrapped in lormir jackets with the addition of clean bandaging and Mepilex Ag foam dressing --that incorporates the rapid and sustained antimicrobial action of ionic silver with the benefits of Safetac soft silicone adhesive technology. The combined attributes of each component of this dressing allow both the control of pain and infection to be achieved simultaneously.<br><br>Fresh dressings will be applied daily until circumferential healing is observed in relation to the portion of the externalized implant, anticipated to be within the first 7-10 days of postoperative recovery. Once observed, jackets and dressings will be removed and animals will be socially housed (multiple pigs in a pen). The investigators will defer to best practices suggested by the attending RAR veterinarians in all cases. |

**4. Animals housed in RAR will be provided with species specific environmental enrichment. Do you need a modification or exception to this guideline ?** N

If yes, please select at least one checkbox below.

**a. Requesting a modification, please explain proposed modification:**

**b. Requesting an exception, please explain and provide scientific justification for withholding enrichment:**

**5. Social animals housed in RAR will be pair or group housed. Do you need an exception to this guideline ?** Y

**If requesting an exception, please explain and provide scientific justification:**

To minimize the possibility of infection, animals will be singly housed with other pigs in the same area, surgical sites will be wrapped in lormir jackets with the addition of clean bandaging and Mepilex Ag foam dressing --that incorporates the rapid and sustained antimicrobial action of ionic silver with the benefits of Safetac soft silicone adhesive technology. The combined attributes of each component of this dressing allow both the control of pain and infection to be achieved simultaneously.

Fresh dressings will be applied daily until circumferential healing is observed in relation to the portion of the externalized implant, anticipated to be within the first 7-10 days of postoperative recovery. Once observed, jackets and dressings will be removed and animals will be socially housed (multiple pigs in a pen). The investigators will defer to best practices suggested by the attending RAR veterinarians in all cases.

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## * * * Experimental Endpoints * * *

### Experimental Endpoints

1. **The endpoint is when an animal or a group of animals will no longer be used in the protocol. To the extent possible, endpoints should be defined using measurable elements such as time after administration of a compound or surgical procedure, number of successful trials required for statistical significance, or the relative change in a measured parameter (e.g. a 50%decrease in tumor size). If an endpoint is not time-dependent (such as the latter two examples above), the maximum amount of time that an animal will be kept on protocol (whether or not the endpoint is achieved) should be indicated. Study endpoint(s) should be both humane and scientifically sound. Identify and explain the rationale for the specific endpoint(s) for each animal or group of animals used in this protocol. Make sure the endpoints match those identified in your experimental design. You may provide a range of endpoints for committee consideration.**

We estimate that it will take 12 weeks (84 days) on this protocol to complete the work. The pilot study is tentatively schedule to have a postoperative in-life period of 8 weeks (+/- 7 days) This experimental endpoint may be shortened or extended to no more than 84 days based on the scientific needs of the sponsor.

Animals may be euthanized earlier if there are complications that are not responsive to treatment.

The clinical symptoms that would prompt examination are already listed in the Health and Monitoring section.

2. **Indicate the disposition of each species. For approval of multiple euthanasia methods per species, re-enter the species for each requested method. "Not euthanized" and "Other Euthanasia Method" are options.**

**Euthanasia Standard Methods and CO2 Instructions**

#### Euthanasia Method

| | |
|---|---|
| **Species*** | Pig (Biomedical) |
| **Method of Euthanasia Primary *** | Other Euthanasia |
| **Provide agent name, route of administration and dosage when applicable. You may provide a range of options for committee consideration.*** | Beuthanasia, Euthasol, Sleepaway, or similar euthanizing agent<br>87-90 mg/kg, IV |
| **Method to Ensure Euthanasia (optional)** | |
| **It is expected that investigators use pharmaceutical-grade compounds whenever they are available in non-experimental research. Do you need an exception to this guideline?*** | N |
| **If yes, then justify exception to guideline, and detail preparation and storage of non-pharmaceutical-grade compound. *** | |

3. **Animals that are experiencing unrelieved pain or distress prior to the defined experimental endpoint must be humanely euthanized, unless doing so would interfere with, or compromise the scientific goals of the experiment. List the frequency of observation of animals who may be experiencing unrelieved pain/distress, the training of personnel on what to look for (this should also be documented in the personnel training records), and the specific actions to be taken depending on what is observed.**

For three days post operatively, animals will be under RAR post operative care. For the duration of study, ESS will check on animals minimally once a day.

Once an animal has been identified as having clinical symptoms, we request an examine by a veterinarian. Together with the veterinarian, we develop a treatment and monitoring plan based on the severity of the symptoms.

We have an Animal Observations SOP that describes what to look for when observing animals, such as normal versus abnormal





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behavior in multiple species. All ESS personnel are trained to document any abnormal observations and contact the veterinarian so that the animal can be assessed.

**Euthanasia Criteria for Animals Used in Research, Teaching, & Display**

**These criteria apply to all animals on experimental protocols, whether manipulated or not. Additional criteria may be specified on the Animal Usage Form. Fulfillment of one criterion can constitute grounds for euthanasia. Exceptions are permitted only if approved by the Committee as part of the protocol review process (i.e. the clinical signs listed below are expected as part of the experiment and appropriate measures are taken to minimize pain or discomfort in the animals):**

a. **Weight loss: loss of 20-25% (depending on attitude, weight recorded at time of arrival, and age: growing animals may not lose weight, but may not gain normally) or if not measured, characterized by cachexia and muscle wasting.**
b. **Inappetance: complete anorexia for 24 hours in small rodents, up to 5 days in large animals; partial anorexia (less than 50% of caloric requirement) for 3 days in small rodents, 7 days in large animals.**
c. **Weakness/inability to obtain feed or water: inability or extreme reluctance to stand, which persists for 24 hours, assuming that the animal has recovered from anesthesia.**
d. **Moribund state: in rodents, measured by a lack of sustained purposeful response to gentle stimuli (example of purposeful response- weak attempt to get up; if animal is on its side, attempts should be asymmetrical in nature); in larger animals, measured by depression coupled with body temperature below 99F (assuming in either case that the animal has recovered from anesthesia).**
e. **Infection: Infection involving any organ system (either overt, or indicated by increased body temperature or WBC parameters), which fails to respond to antibiotic therapy within an appropriate time and is accompanied by systemic signs of illness.**
f. **Signs of severe organ system dysfunction non-responsive to treatment, or with a poor prognosis as determined by an RAR veterinarian:**

**Do the IACUC Criteria for Euthanasia interfere with your experimental objectives?**

**Yes. Provide the criteria to be used by the PI to determine that euthanasia would be required prior to the approved endpoint AND provide scientific justification for why earlier IACUC guideline-based euthanasia criteria cannot be used:**

Y **No. All of the personnel on this study have read and will comply with the IACUC Guidelines for early euthanasia.**

**N/A. Client owned animals - the decision to euthanize a client-owned animal is between the owner and their veterinarian and cannot be required as an endpoint for clinical studies.**

4. **Will death or moribund condition be used as the experimental endpoint?**

**Humane Endpoints in Animal Care and Use Protocols**

**Yes (Select "Death" or "Moribundity" below and provide justification for exception request.)**

**Death**

**Moribundity will be used as the experimental endpoint. These animals are classified as pain class C.**

**Provide justification for experimental endpoint exception:**

Y **No. IACUC Criteria for Euthanasia guidelines will be followed.**

5. **Food Chain Approval** N

**Describe the treatment to be administered.**

**Note that separate sections are provided for chemicals, biologics, and devices. Chemicals (part A) include drugs and hormones. Biologics (part B) include innoculants (i.e. bacterial or viral suspension, vaccine) and antibodies. Devices (part C) include transponders or other electrical or mechanical devices that might be implanted, retained in the digestive tract, etc. In some instances, more than one section may need to be filled out. For example, use of implantable items, such as the Alzet minipump, would require completion of part A (chemicals) or B (biologics) depending upon the item being administered and part C for the device.**





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#### Chemicals / Biologics

#### Devices (transponders, pumps, etc.)

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## * * * Alternative Search * * *

### Alternative Search

**This protocol is using pain class B and/or C.**

**Pain Classes (Instructional Text)**

#### Pain Class B and/or C

**Briefly describe how you have considered each of the following alternatives or how they are not applicable.**

**Replacement of vertebrate animals (e.g. with in vitro models, computer models or less sentient animals)**

The animal model must be of sufficient size to accommodate the materials utilized for this surgery. Less sentient animal models have already been utilized in early evaluation of this technology and in the testing of various infection prevention therapies. This investigator has moved on to a large animal model for testing, a large animal who has been used previously in this type of research.

Computer modeling, in this case, is not appropriate method as we are evaluating the test article in a complex, dynamic biological system.

**Refinement of experimental procedures to minimize pain or distress (e.g. early endpoints; use of analgesics, anesthetics, or sedatives; techniques that reduces stress in the animals):**

We administer a sedative reduce stress of handling and as a best practice to facilitate surgical induction.

Our analgesic methods are mutli modal, and include an opiod, local blocks at the incision site, and an NSAID.

If animals present with clinical signs, and examination and monitoring demonstrate that the animals is in distress, we will opt to euthanize animals early.

ESS personnel are trained in the proper handling of pigs.

**Reduction in the number of animals (e.g. using appropriate statistical methods in the design and analysis of the study; reduction in experimental variability by using animals of defined genetic or microbiological status; sharing tissue among investigators):**

Replacement animals requested on this protocol will not be used if they are not needed.

When possible, ESS participates in tissue sharing with other investigators.

All animals will be utilized as their own control in this study.

**Methods used to search for alternatives (indicate all that apply):**

**Per USDA request, the alternative search must include a search strategy for the use of USDA Class B or C animals. Please include this search strategy as an attachment to the protocol. The search strategy should include a summary of the combinations of search terms used for each search along with results and a narrative addressing whether the results were applicable and if not applicable, why.**

**For help, see links below.**

**Resources on Alternative Search and Search Strategy:**

- **Worksheet: Alternative Literature Searching https://www.nal.usda.gov/sites/default/files/altwkaht_march15.pdf**
- **Alternative Search Summary (Example) https://www.nal.usda.gov/awic/alternative-search-summary-example**
- **Sample Searches on the USDA Website https://www.nal.usda.gov/awic/sample-searches**

X **Literature search conducted -**





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**Please note that while the keywords section is auto-populated with procedural titles, these fields should be expanded upon to ensure that the keywords and general search are completed to identify potential options for not only replacement but also refinement of procedures and reduction of animals used without sacrificing statistical validity.**

**NOTE: At least one database must be used.**

**(See http://www.ahc.umn.edu/rar/ethics.html#Alternatives or http://www.nal.usda.gov/awic for resources to assist in the search)**

#### Search Data

| | |
|---|---|
| **Search Date** | 01/19/2022 (MM/DD/YYYY) |
| **Keywords** | |
| **Scheduled Blood Collections (Pig (Biomedical))** | **Alternative to "Scheduled Blood Collections (Pig (Biomedical))"** |
| **Titanium Disc Insertion and Application of Experimental Therapies (Pig (Biomedical))** | **Alternative to "Titanium Disc Insertion and Application of Experimental Therapies (Pig (Biomedical))"** |

**Name of the database(s)**

- ☒ **Medline / Pub Med**
- ☐ **NIH website on Model Organisms for Biomedical Research**
- ☐ **The Animal Welfare Information Center**
- ☐ **The Johns Hopkins Center for Animal Alternatives**
- ☐ **The University of California-Davis Center for Animal Alternatives**
- ☐ **Other**

**The above searches were completed from the following date range.**

**Search Range From** 2000 (YYYY)
**To** 2021 (YYYY)

**Consultation with colleagues. Please note that this consult should be related to alternatives to the use of animals, not simply the science behind the research.**

**Name(s) of those with whom you consulted:**

**Provide their qualifications:**

**Date of Consult:**

**Content of Consult:**

**Other information/service utilized. Elaborate, providing specific information:**

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## * * * Controlled Substances * * *

### Controlled Substances

**Are you using Controlled Substances in this protocol?** Y

**Use of Controlled Substances requires DEHS Approval. Please submit a separate controlled substances protocol in eProtocol if you haven't already done so or if your current one needs updating (over three years old, safe moved, registrant changed, added controlled substances).**

**Select Controlled Substance(s) being used on this protocol:**

Controlled Substances
Name of Controlled Substance* Pentobarbital and noncontrolled active ingred.

Controlled Substances
Name of Controlled Substance* Tiletamine and Zolazepam Combination Product

Controlled Substances
Name of Controlled Substance* Buprenorphine SR

Controlled Substances
Name of Controlled Substance* Ketamine

**Guidance for using controlled substances for research can be found on the DEHS website at: http://www.dehs.umn.edu/conub.htm**

**Questions: Contact the Department of Environmental Health and Safety (DEHS) at cahelp@umn.edu for more information on controlled substances used for research.**

## * * * DEHS & OHS * * *

### DEHS & OHS

**Are you using any of the following potentially hazardous materials: Anesthetic Gases, Chemicals, Hazardous Waste, Radiation, Animal Pathogens, or Human blood, body fluids, normal or neoplastic tissues?** Y

**Human blood, body fluids, normal or neoplastic tissue (including human cell lines):**
X **Anesthetic Gases**
X **Chemicals**
**Radioactive Material and/or Radiation Producing Equipment**
**Animal Pathogens**

**To request information on appropriate safety procedures on any of the sections below call DEHS at 615-626-6002, or visit the web at http://www.dehs.umn.edu. For occupational health questions, call OHS at 612-626-5006, or visit the web at http://www.ohs.umn.edu.**

**Anesthetic Gases**

**Please verify that all halogenated anesthetic gas use is in accordance with DEHS Policy. Please review the training module on the safe use of anesthetic gases. All personnel that use anesthetic gases must receive training and a record of the training must be available or accessible in your laboratory.**





Protocol Title : The development and characterization of a soft-tissue attachment to a transdermal skin-implant interface in a swine model of osseointegration
Protocol Type : IACUC-R1S1
Approval Period : 05/06/2022-05/05/2025
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

### Anesthetic Gases

**Anesthetic Gas** Isoflurane
**Delivery Method(s)** Intubation
**Scavenging Method(s)** **vacuum**
**Location (Bldg, Room #)** KE basement ORs.

X **This laboratory has verified that all anesthetic gas use complies with DEHS policy and all relevant personnel have been trained on the safe use of anesthetic gases.**

**Chemicals**

**In the table below list any toxic or poisonous chemicals, carcinogens, chemotherapeutic agents and other hazardous drugs, reproductive hazards or nanomaterials used in this protocol. Also list compounds where the hazards have not been fully studied. Contact DEHS, 612-626-6002, labsafe@umn.edu, if you have questions regarding what to include.**

**You must attach a Toxic Hazard Class Standard Operating Procedure (SOP) that covers all the chemicals listed in this section. SOPs on file with DEHS may be used as long as they have been updated within the last 3 years.**

**A collection of resources, including step-by-step instructions, form templates, sample SOPs, and lists of chemicals that require SOPs can be found at: www.z.umn.edu/iacucresources**

### Chemicals

**Chemical** Cyclosporine
**Dose Regimen (dose, frequency of dosing, total number of doses)** cyclosporine (15-30 mg/kg SID PO starting 1 day prior to xenotransplantation on the Ti implants for animals enrolled in Experiment 2, receiving humans MSC based therapy.
**Chemical hazard SOP** attached I have attached in this protocol
I have previously submitted (within past 3 years)
Other

**Sample SOP's : Toxic Hazard Class SOP Template**

**It is the responsibility of the PI to ensure that RAR staff (or other animal care staff if animals are not housed in RAR) are notified two weeks in advance of administering a hazardous agent to an animal.**

## * * * IBC * * *

### IBC

**Does your protocol requires IBC approval?** N

**The university of Minnesota's Institutional Biosafety Committee (IBC) reviews research involving rDNA, infectious agents and biological toxins. The committee also approves practices and procedures that aim to prevent biosafety hazards across campus, in the environment and for the general public. Use of any of the items identified in IBC requires an application to, and approval from, the institutional biosafety committee (IBC). If you are unsure or have questions, please contact IBC at 612-626-5654.**





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## * * * Attachments * * *

| Document Type | Attachment Name | Attached Date | Attached By | Submitted Date |
|---|---|---|---|---|
| Other | Alternatives Literature Search_BTS study | 01/19/2022 | Carney, John | 02/24/2022 |
| Other | Proposed Porcine Study Design_BTS_updated | 03/17/2022 | Carney, John | 03/17/2022 |
| Procedures Additional Description | Disc insertion and experimental therapy application_BTS_updated | 03/17/2022 | Carney, John | 03/17/2022 |
| Other | [American Journal of Veterinary Research] Effect of immunosuppressive doses_of cyclosporine on pancreatic beta cell_function in pigs | 04/29/2022 | Carney, John | 04/29/2022 |
| Other | cyclosporine CIRCULATIONAHA.106.659730 | 04/29/2022 | Carney, John | 04/29/2022 |
| Chemical Hazard SOP | sop_toxic_hazard_class_2019_11_21 (2)_ESS lab cyclosporine | 04/29/2022 | Carney, John | 04/29/2022 |
| Chemical Hazard SOP | product_safety-data-sheets_ah-sds_Cyclosporine Formulation_AH_AR_EN | 04/29/2022 | Carney, John | 04/29/2022 |

## * * * Helpful Links * * *

### HELPFUL LINKS

**NIH,USDA, AAALAC**

**The UMN's USDA Registration Number is 41-R-005**
**The UMN's NIH Animal Welfare Assurance Number is A3456-01**
**NIH Vertebrate Animal Section Template**





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**REGULATORY LINKS**

**Public Health Service Policy on Humane Care and Use of Laboratory Animals**
**U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training**
**Guide for the Care and Use of Laboratory Animals (The Guide)**
**Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Ag Guide)**
**NIH Office of Laboratory Animal Welfare (OLAW)**
**USDA Animal Welfare Act Regulations (AWAR) - Federal Law**

**UNIVERSITY LINKS**

**Board of Regents Policy on Animal Care and Usage**
**Research Animal Resources**
**Office of Occupational Health and Safety (The Guide)**
**Office of the Vice-President for Research (OVPR)**
**Institutional Animal Care and Use Committee (IACUC)**

All personnel assigned to your protocol must have user ID. You may request an an eProtocol user id for external collaborators by completing the eProtocol Access Request Form Clicking this link will take you directly to the form. For more information about requesting users please see the job aid - "Adding Personnel - Non University Access Request" eProtocol Information and Training Page
Rights and Privileges associated with eProtocol roles

Principal Investigator (PI) - may create, edit, view, print and submit the initial application and all subsequent submissions including changes in protocol, incident reports and continuing review forms. ONLY the PI may check the PI assurance statement found on the Assurances tab

Alternate Submitter - the PI may appoint an alternate submitter who may take action on behalf of the PI. The Alternate Submitter may create, edit, view, print and submit the initial application and all subsequent submissions. Secondary Investigators may initiate, edit and submit amendments,

Secondary Investigator - may create, edit, view, and print the initial application and all subsequent submission.

Staff/Lab - Individuals assigned as staff lab may view or print the protocol. This role does not allow individuals to edit, submit or initiate changes to the protocol.

For assistance, please contact IACUC staff at 612-626-2126 or, via email, at iacuc@umn.edu. Additional information can be found on the IACUC website
eProtocol job aids and instructional videos are available on the eProtocol training page.

## * * * Assurance * * *

### Assurance

**Principal Investigator**

**As Principal Investigator, I agree to execute all activities as described; request approval from the IACUC for changes; comply with the guidelines set forth by the IACUC, Research Animal Resources, Environmental Health & Safety; and be responsible for the supervision, training, documentation of training, and work of staff listed on this protocol. I affirm that the activities described in this study do not unnecessarily duplicate previous experiments and that this form accurately and completely reflects the animal use in the full grant application, if applicable. I will notify the funding agency of any subsequent protocol amendments that represent a change in the scope of the grant.**

**Conflict Of Interest**

**Please remember that you were required to file a Report of External Professional Activities (REPA) in February. If you have not completed that process, do so as soon as possible.**

**Please also remember that you are required to file a new REPA within 30 days of a "substantial change" in a financial or business interest that relates to your University expertise or responsibilities, or a change in your University responsibilities that relates to an existing business or financial interest. Should you have questions, the FAQ on the Conflict of Interest Program website may be helpful to you.**
**http://www.compliance.umn.edu/conflictHome.htm**

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**I certify that I understand the University of Minnesota Code of Conduct policy and agree to abide by the rights and responsibilities as identified therein.**

X **The Principal Investigator has read and agrees to abide by the above assurances.**

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## * * * Event History * * *

### Event History

| Date | Status | View Attachments | Letters |
|---|---|---|---|
| 12/21/2021 | NEW FORM PROTOCOL CLONED (1902-36741A) | | |
| 02/24/2022 | NEW FORM SUBMITTED | Y | |
| 02/24/2022 | NEW FORM PANEL ASSIGNED | | |
| 02/24/2022 | NEW FORM REVIEWER(S) ASSIGNED | | |
| 03/17/2022 | NEW FORM SUBMITTED (CYCLE 1) | Y | |
| 03/29/2022 | NEW FORM SUBMITTED (CYCLE 2) | Y | |
| 04/01/2022 | NEW FORM SUBMITTED (CYCLE 3) | Y | |
| 04/05/2022 | NEW FORM PANEL REASSIGNED | | |
| 04/07/2022 | NEW FORM PANEL REASSIGNED | | |
| 04/12/2022 | NEW FORM REVIEWER(S) ASSIGNED | | |
| 04/12/2022 | NEW FORM REVIEWER(S) ASSIGNED | | |
| 04/19/2022 | NEW FORM REVIEWER(S) ASSIGNED | | |
| 04/19/2022 | NEW FORM CONTINGENT | | |
| 04/29/2022 | NEW FORM SUBMITTED (CYCLE 4) | Y | |
| 05/06/2022 | NEW FORM APPROVED | Y | N |

**Appendix 5: Dr. Dey's published manuscript.**

Dey et al. Stem Cell Research & Therapy (2021) 12:604
https://doi.org/10.1186/s13287-021-02666-2

Stem Cell Research & Therapy

RESEARCH Open Access

# Culture and characterization of various porcine integumentary-connective tissue-derived mesenchymal stromal cells to facilitate tissue adhesion to percutaneous metal implants

Devaveena Dey[1,2†], Nicholas G. Fischer[3†], Andrea H. Dragon[1,2], Elsa Ronzier[1,2], Isha Mutreja[3], David T. Danielson[1], Cole J. Homer[3,5], Jonathan A. Forsberg[1], Joan E. Bechtold[4,5,6], Conrado Aparicio[3] and Thomas A. Davis[1*]

## Abstract

**Background:** Transdermal osseointegrated prosthesis have relatively high infection rates leading to implant revision or failure. A principle cause for this complication is the absence of a durable impervious biomechanical seal at the interface of the hard structure (implant) and adjacent soft tissues. This study explores the possibility of recapitulating an analogous cellular musculoskeletal-connective tissue interface, which is present at naturally occurring integumentary tissues where a hard structure exits the skin, such as the nail bed, hoof, and tooth.

**Methods:** Porcine mesenchymal stromal cells (pMSCs) were derived from nine different porcine integumentary and connective tissues: hoof-associated superficial flexor tendon, molar-associated periodontal ligament, Achilles tendon, adipose tissue and skin dermis from the hind limb and abdominal regions, bone marrow and muscle. For all nine pMSCs, the phenotype, multi-lineage differentiation potential and their adhesiveness to clinical grade titanium was characterized. Transcriptomic analysis of 11 common genes encoding cytoskeletal proteins *VIM* (*Vimentin*), cell–cell and cell–matrix adhesion genes (*Vinculin*, *Integrin β1*, *Integrin β2*, *CD9*, *CD151*), and for ECM genes (*Collagen-1a1*, *Collagen-4a1*, *Fibronectin*, *Laminin-α5*, *Contactin-3*) in early passaged cells was performed using qRT-PCR.

**Results:** All tissue-derived pMSCs were characterized as mesenchymal origin by adherence to plastic, expression of cell surface markers including *CD29*, *CD44*, *CD90*, and CD105, and lack of hematopoietic (*CD11b*) and endothelial (*CD31*) markers. All pMSCs differentiated into osteoblasts, adipocytes and chondrocytes, albeit at varying degrees, under specific culture conditions. Among the eleven adhesion genes evaluated, the cytoskeletal intermediate filament vimentin was found highly expressed in pMSC isolated from all tissues, followed by genes for the extracellular matrix proteins *Fibronectin* and *Collagen-1a1*. Expression of *Vimentin* was the highest in Achilles tendon, while

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Full list of author information is available at the end of the article

*Fibronectin* and *Col1agen-1a1* were highest in molar and hoof-associated superficial flexor tendon bone marrow, respectively. Achilles tendon ranked the highest in both multilineage differentiation and adhesion assessments to titanium metal.

**Conclusions:** These findings support further preclinical research of these tissue specific-derived MSCs in vivo in a transdermal osseointegration implant model.

**Keywords:** Transdermal osseointegrated implant, Mesenchymal stromal cells, Integumentary tissues, Cell adhesion, Titanium surfaces

## Introduction

Osseointegration is the direct apposition of bone onto a metallic implant, ideally, without fibrous tissue interposition [1]. Transdermal systems have been recently popularized for the use in patients with limb amputations. In amputees, bone anchored devices traverse the skin-bone-implant interface in order to form a direct mechanical connection between the bone and the external prosthesis [2]. Indeed, transdermal systems have revolutionized the quality of life for patients living with major limb amputations, particularly for military service members and veterans. In a national survey of wounded service members and veterans with unilateral upper limb amputations, using traditional (non-osseointegrated) socket prosthesis, it was found that nearly a third completely abandoned their prosthetic device due to skin irritation, discomfort, prosthesis weight and associated morbidities [3]. Unlike socket-based prosthesis, osseointegration results in direct load transfer to the skeleton, elimination of the need for prosthetic revisions due to residual limb shape changes, optimum control of the prosthetic movement, and minimal risk of nerve compression or skin irritation. Anchoring the prosthetic directly to bone also affords the same restitution of sensory and tactile function, osseoperception, as well as a sense of proprioception, a sensory function thought to be impossible to achieve with conventional socket prostheses [4]. All these advantages culminate in overall improved quality of life, increased functional independence, and prompt return to an active lifestyle [5, 6].

While the transdermal nature of osseointegrated implants enables the attachment of a variety of prosthetic devices, the skin-implant interface, known as the aperture, is a unique and ill-defined microenvironment that is integral to prolonged implant stability and homeostasis. The possibility of deep and superficial infection complications remains a major clinical concern for both patients and surgeons, posing a substantial barrier to widespread use. Further, excessive soft tissue motion at the aperture result in progressive exposure of adjacent soft tissues thereby increasing chances of infection, osteomyelitis, implant loosening, surgical revisions and implant failure [7].

Techniques to augment adhesion and durability of the skin and underlying connective tissue to metal at the aperture may improve the durability and long-term viability of the skin-implant 'seal,' resulting in reduction of infection and implant loosening. Natural transdermal structures such as nail bed, hoof and tooth, generate an effective mechanical barrier to prevent infections and create a strong, durable hard-soft tissue interface. Recapitulating an analogous cellular transdermal musculoskeletal-connective tissue interface for osseointegrated implants may be a potentially powerful strategy to reduce infection and enhance long-term clinical outcomes [8].

Mesenchymal stromal cell (MSC) therapies have become a widely explored method for treating diseases and potentiating regeneration [9]. Integration with tissue engineering approaches have successfully incorporated MSCs into biomaterial carriers to reduce immediate clearance of transplanted MSC, prolong survival and localization of these cells, thereby enhancing the long-term paracrine effector function of MSCs [10]. However, predicting clinically meaningful outcomes has been difficult for many reasons, such as MSC heterogeneity based on tissue and isolation methods, thus necessitating robust characterization prior to delivery [11–13]. Given the multi-lineage differentiation potential of MSCs into bone, cartilage and fat tissues, our approach for recapitulating cellular transdermal barriers is to deliver MSCs in a biomaterial construct to form a flexible and durable impervious seal.

Here, as a necessary first step toward translation, we characterized the phenotype, multi-lineage differentiation potential and adhesiveness of various tissue-derived porcine MSCs (pMSCs) to clinical grade titanium. The tissues selected for this study can be categorized into two broad groups: 1) those present at the junction of a hard and soft tissue (such as periodontal ligament), present at the interface of the tooth cementum and the supporting bone, and the superficial flexor tendon (anchoring the hoof bone to the soft tissue) and 2) connective tissues such from dermis, adipose tissue and Achilles tendon tissue. We hypothesized that multi-lineage differentiation and metal adhesion properties of porcine integumentary and associated connective tissues would be different than

muscle and bone marrow-derived MSCs commonly used for MSC therapy. Porcine musculoskeletal tissues are similar to humans with respect to clinical, histological and immunohistological features associated with tissue regeneration and cutaneous skin healing [14, 15]. Findings from this study will subsequently be tested in vivo using a porcine transdermal implant model developed by our team for studying wound healing and infections at percutaneous titanium (Ti) implant sites. Overall, our detailed characterization and comparative analysis of a broad panel of integumentary and connective tissue-derived MSCs will enable selection of best cell candidates and contribute to developing therapies to enhance transdermal system outcomes.

## Materials and methods

### Animals and tissue collection

Tissues were aseptically collected under institutional tissue sharing protocols from 6-month-old female Gottingen, Yucatan or Yorkshire minipigs, *Sus scrofa domesticus,* immediately after humane euthanization, per approved IACUC guidelines. Bone marrow cells were harvested and isolated from ribs or iliac crest by marrow aspiration using a heparin pre-coated syringe fitted with a Jamshidi bone marrow biopsy aspiration needle. For all other tissues the collection sites were aseptically cleansed prior to incision with ethanol and iodine wipes, then irrigated with phosphate-buffered saline (PBS; Gibco, Gaithersburg MD) supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin and 250 µg/ml amphotericin B (Gibco, Gaithersburg MD). Details regarding tissue description and the sites of tissue collection are listed in Additional file 1: Table S1.

### Cell culture

Heparinized bone marrow was washed twice, treated with ACK lysing buffer to deplete RBCs, and resuspended in complete MSC stromal growth medium (cMSC-GM; DMEM-F12 (1:1); Sigma Aldrich, St. Louis, MO), supplemented with 2% FBS and antibiotics (200 units/ml penicillin, 200 µg/ml streptomycin and 250 µg/ml amphotericin B; Gibco, Gaithersburg MD). Periodontal ligament tissue was scraped off the outer surface of the molars. Harvested tissues were placed in a 10 cm tissue culture dish (Fisher Scientific, Waltham MA) containing 5–7 ml of cMSC-GM, then finely minced into a slurry of small pieces ($<1mm^3$) using fine sterile scissors. Refer to Additional file 1: Table S1 for enzyme concentrations and tissue digestion conditions. Digested tissue cell suspensions were washed, resuspended in 15 ml of cMSC-GM and filtered through 100, 70 and 40 µm cell strainers sequentially (Fisher Scientific) to obtain a single-cell suspension. Cells were placed into a 10-cm sterile tissue culture dish. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$ until 70–80% confluent.

Dermal tissue was processed differently to preserve viability [16]. Briefly, the epidermal skin layer was gently scraped using a scalpel blade to remove hair prior to overnight enzymatic digestion. Digested skin was vigorously vortexed to mechanically remove the epidermal layer. Dermal tissue explants (~4 cm × 2 cm) were cut into small pieces, and evenly placed on a 10-cm tissue culture dish (Fisher Scientific). A minimum volume of cMSC-GM was added and replenished every 3–5 days near the explants dropwise, ensuring no tissue movement.

### Osteogenic differentiation

For induction of osteogenic differentiation, Passage-2 pMSCs resuspended in cMSC-GM were seeded in either in a 96-well plate (100 cells/well) for assessing early osteogenic differentiation via a pNPP based alkaline phosphatase assay, in a 6-well plate ($1 \times 10^5$ cells/well) for osteogenic gene expression studies or at $2.5 \times 10^5$ cells / well in a 6-well plate for assessing long term osteogenic differentiation potential, as previously described [17–19]. Media was changed from growth media to StemPro® Osteogenic Differentiation media ('OM'; Life Technologies, Carlsbad CA) for half of the wells 24 h post seeding, with the other half (control wells) maintained in growth media (GM). Both OM and GM were replaced every 3 days. For pNPP-based assay, media was aspirated after 7 days, washed with PBS, followed by cell lysis in 1% TritonX-100 and addition of the pNPP substrate (Sigma Aldrich). Absorbance was measured immediately at 405 nm on a kinetic mode for 20 cycles on a Tecan Infinite 200 PRO fluorometer (Tecan; Morrisville NC). Cultured pMSCs were harvested in QIAzol Lysis Reagent (Qiagen, Germantown, MD) for gene expression studies. For long term osteogenic differentiation studies, adherent cell cultures after 14–28 days of culture were fixed in 0.5% glutaraldehyde for 20 min, followed by multiple PBS washes, and mineralization (presence of calcium-rich hydroxyapatite of the extracellular matrix) was assessed by staining with 2% alizarin red solution (prepared in distilled water; pH = 4.2) for 3–5 h at room temperature. Post staining, adherent cells were washed multiple times with tap water, followed by deionized water, and imaged microscopically (Axio Observer Z1, Zeiss). For quantification, alizarin red stained calcium crystals were solubilized using 10% formic acid, incubated for 45–60 min with shaking at room temperature, and absorbance measured at 414 nm (Tecan Infinite 200 PRO fluorometer; Tecan, Morrisville NC). Osteogenic differentiation

experiments were carried out for all nine tissues in triplicates; i.e., isolated from 3 different animals.

#### Chondrogenic differentiation

Chondrogenic differentiation of Passage-3 pMSCs was performed using pellet cultures. Briefly, cells were expanded in GM until attaining 80% confluency, after which cells were dissociated with trypsin and re-suspended in chondrogenic differentiation medium in V-well polypropylene plates at a density of $5.0 \times 10^5$ cells /well. Cells were centrifuged at 200 g for 4 min and maintained in the media for 21 days with media changes every three days. The standard chondrogenic differentiation media consisted of DMEM (high glucose) media supplemented with 1% ITS+(Sigma, USA; St. Louis, MO), 100 nM dexamethasone (Sigma, USA; St. Louis, MO), 1.25 mg/ml bovine serum albumin (Sigma, USA; St. Louis, MO), 10 ng/ml TGF-β1 (R&D systems, USA), and 0.1 mM ascorbic acid 2 phosphate (Sigma, USA; St. Louis, MO). Pellets (in triplicates) were collected either at 7 days for gene expression analysis (in QIAzol Lysis Reagent), or after 21 days for histological and quantitative assessments of differentiation. Synthesis of glycosaminoglycans (GAG) was measured to quantify chondrogenic differentiation. For GAG analysis, collected pellets (in triplicates) were digested at 56 °C in 300 µL of 1 mg/mL proteinase-K solution to digest the matrix, followed by addition of dimethyl-methylene blue (DMMB) dye. Total GAG content was determined by measuring the absorption of the molecular complex at 492 nm (Synergy TM 2, Biotek multi-mode microplate reader). Chondroitin sulphate B was used to prepare a standard curve. GAG content was normalized against the total DNA content, measured using the cell lysate following the manufacturer's protocol (CyQuant cell proliferation assay). Briefly, diluted cell lysate was mixed with 2X GR-dye and incubated for 1 h at room temperature following which the fluorescence was measured at $\lambda_{ex}$ of 480 nm and $\lambda_{em}$ of 520 nm (Synergy TM 2, Biotek multi-mode microplate reader). Chondrogenic differentiation was quantified as the ratio of GAG to the DNA content (GAG/DNA). For histological analysis, collected pellets (in triplicates) were fixed in 4% paraformaldehyde for 30 min at room temperature, cryo-sectioned (30 µm thick sections) and stained with Alcian blue to assess levels of sulphated glycosaminoglycans and counterstained with Nuclear Fast Red. The set of pellets collected at day 7 post chondrogenic differentiation was used to assess the expression of three chondrogenic genes: *SOX9, Collagen-2a1* and *Aggrecan*.

#### Adipogenic differentiation

Adipogenic differentiation of Passage-2 pMSCs resuspended in cMSC-GM seeded in either in a 6-well plate ($1 \times 10^5$ cells/well) for adipogenic gene expression studies, or at $2.5 \times 10^4$ cells/well in a 6-well plate for assessing long term adipogenic differentiation potential. Media was changed from GM to AdipoQual adipogenic media ('AM'; Obatala Sciences; New Orleans, LA) for half of the wells when cells reached 40–60% confluency, with the other half (control wells) continued in GM. Both AM and GM were replaced every 3 days. For gene expression studies, cells were harvested after 7 days in differentiation or growth media, via cell scraping, and stored at − 80 °C in 1 ml of QIAzol Lysis Reagent until further processing. For long term adipogenic differentiation studies, cultured pMSCs were harvested between 14–28 days; fixed in 0.5% glutaraldehyde for 20 min, followed by multiple PBS washes, and stained with Oil Red O Staining Solution (Obatala Sciences; New Orleans LA) for 30 min to an hour. Post staining, wells were washed multiple times with DI water and imaged microscopically (Axio Observer Z1, Zeiss). For quantification, Oil Red O-stained lipid droplets were solubilized using 100% isopropanol, incubated for 10 min with shaking at room temperature, and fluorescence measured at $\lambda_{ex}$ of 500 and $\lambda_{em}$ of 595 nm (Tecan Infinite 200 PRO fluorometer; Tecan, Morrisville NC). All adipogenic differentiation experiments were carried out for nine tissues in triplicates; i.e. each of the nine tissues isolated from 3 different animals.

#### Gene transcripts associated with cell adhesion

Adhesion gene profiling was conducted using Passage-5 pMSCs ($1 \times 10^5$ cells/well) grown in cMSC-GM, OM, CM and AM for 7-days. Adherent cell cultures were washed twice with PBS, scraped from the plate, and pelleted by centrifugation, stored at − 80 °C in 1 ml of QIAzol Lysis Reagent (Qiagen, Germantown MD). Total RNA was isolated using the miRNAeasy Mini Kits (Qiagen). Quantitative and qualitative evaluation measurements of RNA samples were conducted using NanoDrop and Agilent 2100 Bioanalyzer instruments (Thermo Scientific, Waltham, MA). cDNA was synthesized using a high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA) and qPCR was carried out using a Syber Green based detection (BioRad, Hercules, CA) on the Quantstudio (QuantStudio 7 Flex Real-Time System; Applied Biosystems, Waltham, MA). The list of primers and sequences used for this study have been listed in Additional file 1: Table S2 with β-actin as a housekeeping gene.

### Immunophenotype

Passages 3–5 pMSCs were harvested for flow cytometric analysis. pMSCs were washed once with room temperature PBS, incubated with 15 mM EDTA in PBS solution for 12 min at 37 °C and detached off the plate. Detached cells were washed in DMEM media supplemented with 10% FBS, centrifuged and counted. Aliquots of $2 \times 10^5$ cells were suspended in 2 ml of Flow Cytometry Staining buffer (eBioscience™, Invitrogen, Waltham, MA), washed (centrifugation for 5 min at 1400 rpm). Cell pellets were resuspended in 100 µl of FACS buffer then incubated on ice for 30 min with FITC-conjugated anti-CD44 (#MA1-10228; Invitrogen, Waltham, MA), anti-CD90 (#A15761; Invitrogen), and anti-CD29 (#MA1-19566; Invitrogen, Waltham, MA), and anti-CD105 (#NB11081749; Novus Biologicals, Centennial, CO). FITC-conjugated isotype matched IgG1 (#50-204-9474; Cell Signaling Technology, Danvers, MA) and IgG2 (#PA5-33239, Invitrogen) were used as controls. After 30 min of incubation in the dark, cells were washed twice with 2 ml of Flow Cytometry Staining buffer and then resuspended 300 µl of cold FACS buffer. To detect dead cells, 1ul of SYTOX™ Blue Dead Cell Stain (Invitrogen, #S34857) was added to the cells 5 min prior to being analyzed using a BD LSRII Flow Cytometry System (BD BioSciences, Rockville, MD). Viable cells were gated in a dot plot of forward versus side scatter signals wherein 10,000 gated events were acquired over a log fluorescence scale. Flow cytometric data were analyzed, and histograms generated using FlowJo software version 10 (TreeStar Inc, Ashland, OR).

### Cell adhesion experiments on titanium alloy

#### *Intracellular focal adhesion quantification*

Passages 2–3 cells were seeded on Ti-6Al-4 V (Ti) polished to a 20–60 nm colloidal silica finish (President Titanium, Hanson, MA) and glass disks (Harvard Apparatus, Holliston, MA) at $1 \times 10^3$ cells/disk for 4 h, 1 day, and 3 days. NIH-3T3 (CRL-1658, ATCC, Manassas, VA) embryonic murine fibroblasts (hereafter, NIH-3T3 fibroblasts) which adhere, proliferate and migrate on metal and glass surfaces served as controls Overmann [20]. Cell spreading and focal adhesion characteristics were determined with immunofluorescence. Seeded disks ($n=10$) were washed in PBS (Gibco), fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in PBS, blocked in 5% bovine serum albumin (BSA), followed by overnight anti-vinculin primary antibody staining (MAB3574; MilliPore Sigma, Burlington, MA), incubation with secondary antibody (ab97037; Abcam, Cambridge, UK) in PBS for 1 h at room temperature, followed by incubation with 4',6-diamidino-2-phenylindole (DAPI; 300 nM in PBS; D9542; Fisher Scientific, Waltham, MA) for 10 min. After each step, the cells were rinsed in PBS. Disks were mounted with ProLong (Fisher) and imaged (Olympus FV1000, Tokyo Japan or Leica DM 6B, Wetzlar Germany). Cytoskeletal architecture was assessed by rhodamine-phalloidin (R415, ThermoFisher Scientific, Waltham, MA) based actin staining on the remaining disks ($n=5$). These slides were processed using the same protocol as described above, counterstained with DAPI and mounted. ImageJ (NIH) was used for image analysis of all fluorescent images, with 5 fields of views (FOVs) per sample. Proliferation (number of DAPI-positive nuclei), cell surface area per FOV, individual cell area, vinculin (focal adhesion) intensity per FOV, average vinculin (focal adhesion) intensity per cell, and average vinculin (focal adhesion) intensity per cell area were calculated and normalized to NIH-3T3 fibroblasts on Ti at 4 h for each measure to account for differences in microscope usage. All experiments were repeated on pMSCs derived from at least 3 animals.

#### *Cell proliferation and viability*

A colorimetric assay (CCK-8) was used to compare the proliferation and viability of pMSCs on both Ti and control glass. At 4 h, 1 day and 3 days, seeded disks ($n=7$; both Ti and glass) were washed thrice in PBS to remove weakly adherent cells, transferred to a virgin 48 well-plate, and incubated for 2.5 h in a 1:10 dilution of CCK-8 solution (Dojindo Laboratories, Japan) in culture media. Afterward, 100 µL of the CCK-8/media solution was transferred to a 96-well plate for absorbance reading (Synergy, BioTek—450 nm) expressed as optical density (O.D.). A blank, comprising of CCK-8/media solution, without cells was used to subtract background absorbance. All experiments were repeated on pMSCs derived from at least 3 animals.

(See figure on next page.)

**Fig. 1** Isolation and phenotypic characterization of nine porcine tissue-derived mesenchymal stromal cells (pMSCs). **a** Representative phase contrast images (50 × magnification) of Passage-0 cells, 10 days after isolation from the different porcine integumentary and connective tissues as specified. For all figures, *Ab* Abdominal, *HL* Hind limb, *Hoof* Hoof-associated superficial flexor tendon, *Molar* Molar-associated periodontal ligament, *Muscle* Gastrocnemius muscle **b** Phenotypic characterization of the pMSCs by gene transcripts of MSC (*CD90, CD44, CD29, CD105*), hematopoietic (*CD11b*) and endothelial (*CD31*) markers in Passage-2 cells cultured in growth media. β-Actin was used for normalization of gene expression. Data presented as mean ± SEM ($n=3$; $^*p<0.05$)

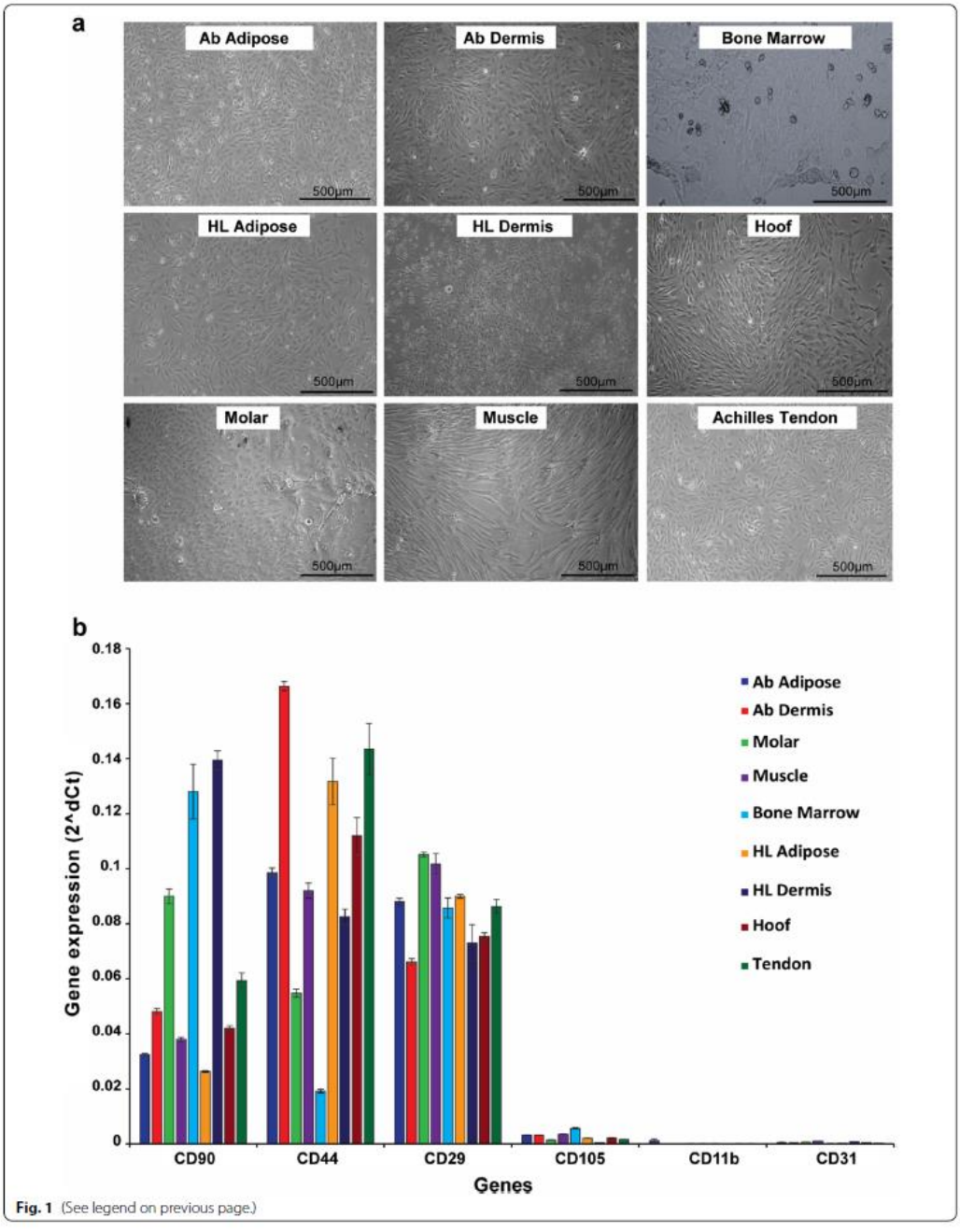


**Fig. 1** (See legend on previous page.)

#### *Centrifugal assessment of cell adhesion*

A centrifuge-based assay was used to apply shear to pMSCs to quantify their physical adhesion to titanium [21]. pMSCs ($5 \times 10^3$) were seeded onto polished titanium disk surfaces in 48-well plates for 48 h then gently washed thrice with PBS to remove non-adherent cells. Oral keratinocytes (OKF6/TERT-2; BWH Cell Culture and Microscopy Core, Boston, MA) were used as a positive control given their strong adhesion to titanium [22]. One set of disks ($n=3$) were immediately removed and fixed in 4% paraformaldehyde (hereafter, control-pre). The second set ($n=3$) were inserted into a three-dimensionally (3D) printed (Dental Resin SG, Formlabs, Somerville, MA) disk-holder (Additional file 1: Fig. S1). This holder fits inside a 48-well such that disks (one disk per holder per well) are oriented perpendicular to the ground when centrifuged. Wells were filled with culture media and then centrifuged (3 min) at 350 g or 500 g ($n=3$ per sample). Control disks ($n=3$ sample; hereafter, control-post) were treated identically, but placed next to the centrifuge (5810R; Eppendorf, Hamburg Germany) while the other two groups were centrifuged. Disks were then subsequently stained with DAPI for 15 min at room temperature and imaged (Lecia DM6 B). ImageJ (NIH) was used for quantification of nuclei in three random field of views per disk.

#### Statistical analysis

GraphPad Prism (v8; GraphPad Software, San Diego, CA) was used for statistical analyses and graphic drawings. Experimental data were evaluated by one-way ANOVA, followed by the Tukey HSD multiple comparison test. A *t*-test with False Discovery Rate (FDR) correction ($Q=1\%$) was used to compare titanium vs. glass for each measure. An ANOVA with Dunnett's multiple comparison was used to compare the various pMSCs cell types to NIH-3T3 fibroblasts (control group) for each measure. Inter-cell population differences between control-post, 350 g, and 500 g were detected with a two-way analysis of variance with a Dunnett correction where the control mean (null) was set as keratinocytes. A value of $p<0.05$ was considered as significant difference.

## Results

#### Isolation and culture of porcine mesenchymal stromal cells (pMSCs) derived from integumentary and connective tissues

As shown in Fig. 1a, a population of "fibroblast-like" cells were successfully isolated from nine distinct porcine tissues, comprising either integumentary (periodontal ligament, hoof-associated flexor tendon) or connective (Achilles tendon, dermis, adipose) tissues, in addition to bone marrow and muscle, using enzymatic and mechanical dissociation as detailed in Methods and Additional file 1: Table S1. Except for dermal tissue, single cell suspensions obtained from post-tissue digestion were seeded on tissue-culture treated polystyrene (TCPS). Dermis-resident cells were derived from epithelial layer-depleted skin explants, seeded on TCPS. By day five, distinct foci (clusters of 5–10 cells) of plastic adherent cells were evident in all single-cell suspension derived cultures and retained high viability and proliferation potential after extended in vitro expansion. Outgrowth of pMSCs was greatest in adipose tissue and least from dermal tissue; however, by Passage-2 growth across all cell type were comparable through Passage-5. In contrast, pMSCs derived from periodontal ligament exhibited a mixed population of fibroblast like and spherical cells, while pMSCs derived from hind limb dermis often contained a mixture of fibroblast and cobblestone shaped cells. pMSCs isolated and expanded from all three pig strains (Gottingen, Yucatan and Yorkshire) revealed similar characteristics.

#### Gene expression of pMSC-surface markers

We used RT-PCR to profile the mRNA expression patterns of MSC-specific cell surface markers in Passage-2 cells (Fig. 1b). All the nine tissue-derived cells expressed high levels of mRNA transcripts for porcine cell surface markers whose expression can be used to functionally characterize isolated pMSCs, such as *CD29*, *CD44* and *CD90*, while negative for hematopoietic (*CD11b*) and endothelial (*CD31*) gene transcripts [23]. The expression of the *CD105* transcript was not strongly detected in any of the nine pMSCs comparing to others MSC markers studied but still significantly higher than the expression of hematopoietic and endothelial markers (Fig. 1b). The expression of *CD105* as a pMSC marker in pigs is still controversial [24, 25]. The pattern of gene transcript

(See figure on next page.)

**Fig. 2** Cell surface marker immunophenotype of porcine tissue-derived mesenchymal stromal cell (pMSCs) using flow cytometric analysis. Flow cytometric analysis of cell surface protein expression on pMSCs using FITC-conjugated anti CD29, CD44, CD90, and CD105 antibodies. Cell surface log fluorescent measurements were obtained on 10,000 viable cells using forward versus side scatter (FSC vs SSC) gating. Representative fluorescent histograms of cells stained with isotype matched (control) and MSC surface marker antibodies are shown

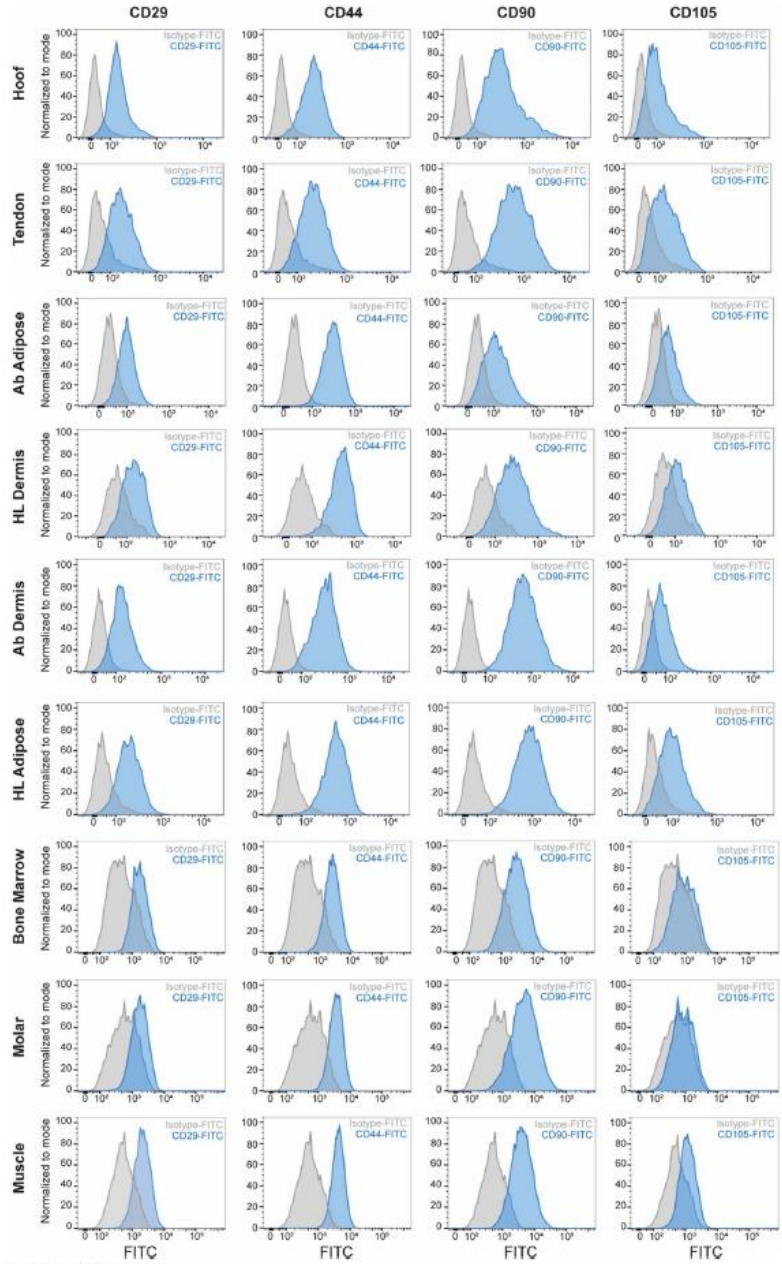


**Fig. 2** (See legend on previous page.)

expression for the pMSC-specific markers varied across the nine tissues; molar-derived periodontal ligament (Molar), bone marrow and hind limb (HL) dermis-derived cells expressed significantly higher levels of the *CD90* transcript, compared to the other cell types; while abdominal (Ab) dermis, hind limb adipose and tendon-derived cells expressed significantly high levels of the *CD44* transcript. Most of the cell types expressed comparable levels of *CD29* gene transcript expression, although the expression levels in molar and muscle-derived cells were significantly higher than in all other cell types.

#### Cell surface immunophenotype of pMSCs

In order to confirm the gene expression data, cell surface protein expression was assessed using flow cytometric analysis. In comparison to median fluorescent signals on isotype control stained pMSCs, we observed that all of the pMSC-derived cell types strongly expressed CD29 (3–6 fold increase), CD44 (5–14 fold increase) and CD90 (4–26 fold increase), albeit the expression of CD105 (1.5–4 fold increase) was significantly less (Fig. 2). These data and the mRNA transcript results demonstrate that pMSCs isolated from different pig tissues share a similar mesenchymal cell surface marker phenotype ($CD29^{low}$, $CD44^{high}$, $CD90^{high}$ and $CD105^{dim}$) immunophenotype.

#### pMSCs exhibit trilineage differentiation potency

Further functional characterization of the pMSCs was carried out by assessing the multilineage potential of these cells during expansion. On induction of differentiation conditions, all pMSCs lines differentiated into osteoblasts, adipocytes and chondrocytes, albeit at varying degrees, under specific culture conditions.

##### *Osteogenic differentiation*

pMSCs derived from the adipose tissue of the hind limb (HL) adipose, bone marrow and molar-associated periodontal ligament demonstrated the highest potential of early osteogenic lineage commitment, as assessed by the alkaline phosphatase activity assay (Fig. 3a). In long term culture under extended osteogenic differentiation conditions, calcium deposition as a late indicator of osteogenic differentiation, was markedly increased in HL adipose and bone marrow-derived pMSCs (Fig. 3b–c). Analysis of osteoblast-specific genes in these cells after one week in osteogenic differentiation media demonstrated relatively high expression level of *BGLAP* (*Osteocalcin)* and *COL1A1 (Collagen-1a1)* genes in HL adipose-derived pMSCs (Fig. 3d, e), when compared to cells cultured in growth media. The expression level of these genes in HL adipose-derived pMSCs was comparable to expression in pMSCs derived from other tissues, such as Achilles tendon, muscle, hoof-associated tendon, abdominal adipose and dermal tissues; indicating potent osteoblastic differentiation potential of all these cell types. In addition, the same osteogenic induction signals resulted in induction of other pro-osteogenic genes in the different tissue-derived pMSCs. For example, there was pre-dominance of expression of *BGLAP* (Fig. 3d) and *RUNX2 (Runt-related transcription factor 2*) (Additional file 1: Fig. S2a) in abdominal dermis and abdominal adipose; *COL1A1* (Fig. 3e) and *BSP (Bone sialoprotein)* (Additional file 1: Fig. S2b) in muscle, tendon and hoof-associated superficial flexor tendon pMSCs, and *OPN* (*Osteopontin)* in bone marrow and molar-derived pMSCs (Additional file 1: Fig. S2c).

##### *Chondrogenic differentiation*

As shown in Fig. 4, Achilles tendon-derived pMSCs had the greatest chondrogenic differentiation capacity, as assessed by Alcian blue staining (Fig. 4a) and measurement of normalized glycosaminoglycans (GAG) (Fig. 4b). This was confirmed by gene expression analysis of chondrocyte-specific genes, such as *ACAN* (*Aggrecan), COL2a1 (Collagen-2a1) and SOX9 (SRY-box transcription factor 9)* in pMSCs harvested after 7 days in pellet cultures (Fig. 4c–e). Achilles tendon-derived pMSCs demonstrated the highest expression of these pro-chondrogenic genes compared to the corresponding cells cultured in growth media.

##### *Adipogenic differentiation*

All the tissue-derived pMSCs populations demonstrated varied low-to-modest levels of intracellular lipid droplets accumulation (Fig. 5a). Isopropanol-based solubilization of the lipid droplets, followed by spectrophotometric quantification, indicated that hoof-associated superficial flexor tendon-derived pMSCs had the highest level of adipogenic differentiation, followed by HL adipose

(See figure on next page.)

**Fig. 3** Assessment of in vitro osteogenic differentiation of pMSCs. **a** Quantification of early osteogenic differentiation potential of pMSCs cultured in osteogenic induction media for 7 days, assessed by pNPP-based alkaline phosphatase activity assay. **b** Quantification of alizarin red stain-based assessment of late osteogenic differentiation of cells cultured in osteogenic induction media for 14–28 days. **c** Representative phase contrast images (50 × magnification) of cells stained with alizarin Red. **d**–**e** Fold change in gene expression of osteogenic transcription factors, *Osteocalcin (BGLAP)* (**d**) and *Collagen-1a1* (**e**) in cells cultured in osteogenic media (OM) normalized to their expression under growth media (GM) for 7 days. β-Actin was used for normalization of gene expression. Data presented as mean ± SEM ($n = 3$; $^{*}p < 0.05$)

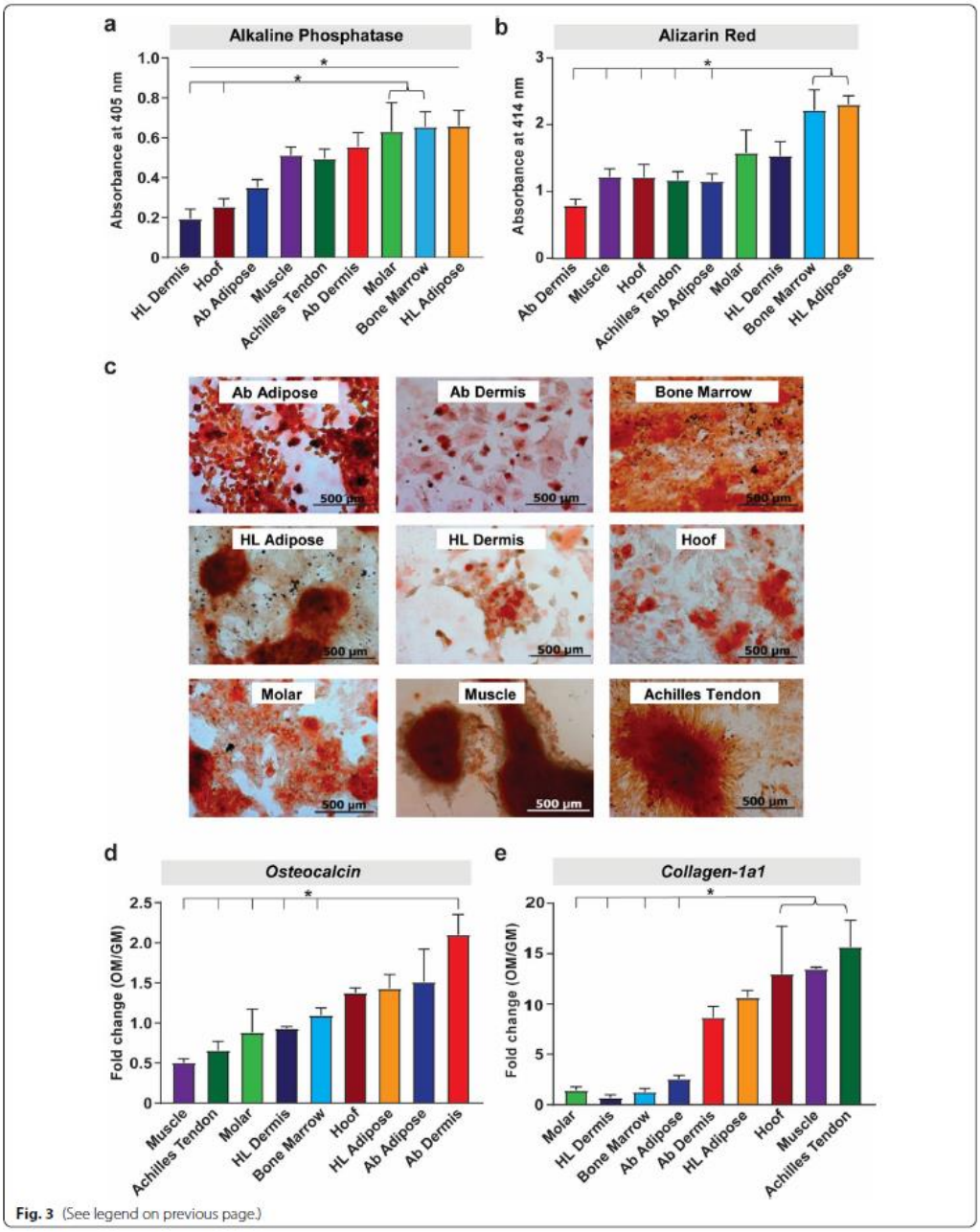


**Fig. 3** (See legend on previous page.)

tissue-derived pMSCs (Fig. 5b). This was corroborated by the highest upregulation of the adipogenic transporter, *AP2 (Adipocyte protein 2),* in hoof-associated superficial flexor tendon pMSCs observed after a week in adipogenic induction medium (Fig. 5c). While hoof-associated superficial flexor tendon pMSCs also had high expression of another well-known adipogenic transcription factor, *PPARγ (Peroxisome proliferator activated receptor gamma)* (Fig. 5d), muscle and tendon-derived pMSCs also demonstrated significantly high upregulation of *PPARγ* in early stages of adipogenic induction.

#### Profiling of cell adhesion-related genes in pMSCs

Others have shown that surface modifications (porosity, surface roughness, scaffold coatings) of the titanium (Ti) metal can improve cell adhesion and osseointegration [20]. One of the critical parameters for selection of a promising cell type to reinforce the aperture is its adhesion strength to metal, scaffold and surrounding tissue. Such adhesion may also result in MSC tissue retention. Thus, non-stimulated pMSCs were profiled for inherent differences in expression of structural and adhesion-related genes. Using qRT-PCR based expression, an assessment of the expression of 11 common genes encoding cytoskeletal proteins *VIM* (*Vimentin*), cell–cell and cell–matrix adhesion genes (*Vinculin, Integrin β1, Integrin β2, CD9, CD151*), and for ECM genes (*Collagen-1a1, Collagen-4a1, Fibronectin, Laminin-α5, Contactin-3*) in early (Passage 2) and late (Passage 5) cells was performed. At Passage 5, all pMSCs showed high levels of expression of *Vimentin,* followed by *Collagen-1a1, Fibronectin, Vinculin, Integrin β1, Collagen-4a1* (Fig. 6) and *CD9, CD15, Laminin-α5, Contactin-3* and *Integrin β2* (Additional file 1: Fig. S3). Achilles tendon-derived pMSCs had the highest expression level of vimentin. Among all the cell types, hoof-associated superficial flexor tendon pMSCs expressed high levels of the majority of adhesion-related genes tested (6/11; *Collagen-1a1, Integrin β1, CD9, CD151, Laminin-α5, Contactin-3),* followed by muscle-derived pMSCs (5/11).

#### pMSCs adhesion to titanium alloy surface

To further characterize the potential of pMSCs strengthening the skin-implant (*i.e.*, Ti) interface, a CCK-8 based metabolic activity assay, measuring cell adhesion, proliferation and viability on titanium or glass control surfaces, was carried out. Here, we compared pMSCs to control NIH-3T3, whose proliferation on Ti is well described [26], and typical bone marrow pMSCs. These results for the top two performing pMSCs demonstrated significantly higher proliferation and viability of hind limb dermis-derived pMSCs on titanium as early as 4 h (Additional file 1: Fig. 7a), followed by hind limb adipose and bone marrow-derived cells, with significantly higher values on titanium at 72 h and 24 h with respect to glass, respectively. Molar-derived pMSCs also had high cell proliferation and viability on titanium versus glass surface at 72 h (Additional file 1: Fig. S4). As shown in Fig. 7b, abdominal dermis-derived pMSCs cultured on titanium demonstrated significantly high levels of intracellular focal adhesion marker vinculin levels when compared to glass at 72 h. Focal adhesions are known to mediate cell-Ti adhesion and proliferation [27]. This was followed by hoof-associated superficial flexor tendon pMSCs cultured on titanium, with significantly higher vinculin levels compared to cells cultured on glass at 24 h (Fig. 7b). All the other cell types generally demonstrated higher vinculin intensity on glass surface (Additional file 1: Fig. S5). Finally, a stringent functional test of the pMSCs' adhesion to titanium was carried out by subjecting the nine pMSCs seeded on titanium disks to centrifugal forces of 350 g and 500 g, followed by assessing the percentage of cells that remained adhered to titanium post centrifugation. The positive control, keratinocytes with their well described adhesion to Ti [22], had no significant change in percentage of adhered cells on titanium pre- and post-centrifugation (Fig. 7c). However, post centrifugation titanium surfaces showed significantly fewer attached cells for all tissue-derived pMSCs (Fig. 7c and Additional file 1: Fig. S6). Bone marrow-derived pMSCs had the lowest decrease in percentage of cells adhered to titanium post centrifugation at 350 g. HL dermis and Achilles tendon-derived pMSCs had the highest remaining percent amongst the other pMSCs at 500 g (Fig. 7c).

(See figure on next page.)

**Fig. 4** Assessment of in vitro chondrogenic differentiation of pMSCs. **a** Histological staining (Alcian blue/Nuclear Fast Red) of pellets derived from pMSCs of different tissue sources cultured in chondrogenic media after 21 days in culture (50 × magnification). **b** Quantification of chondrogenic differentiation by calculating the levels of glycosaminoglycans (GAG) secreted by cells under chondrogenic differentiation conditions, normalized to the cell density in the pellets (DNA) after 21 days in culture. Data presented as mean ± SD ($n = 3$ biological replicate with three pellets per replicate). *$p < 0.05$ and **$p < 0.01$ **c–e** Fold change in gene expression profiling of chondrogenic genes, *SOX9* (**c**) *Aggrecan* (**d**) and *collagen II* (**E**) in pMSCs cultured in chondrogenic media (CM) vs growth media (GM) for 7 days. β-Actin was used for normalization of gene expression. Data presented as mean ± SEM ($n = 3$; *$p < 0.05$)

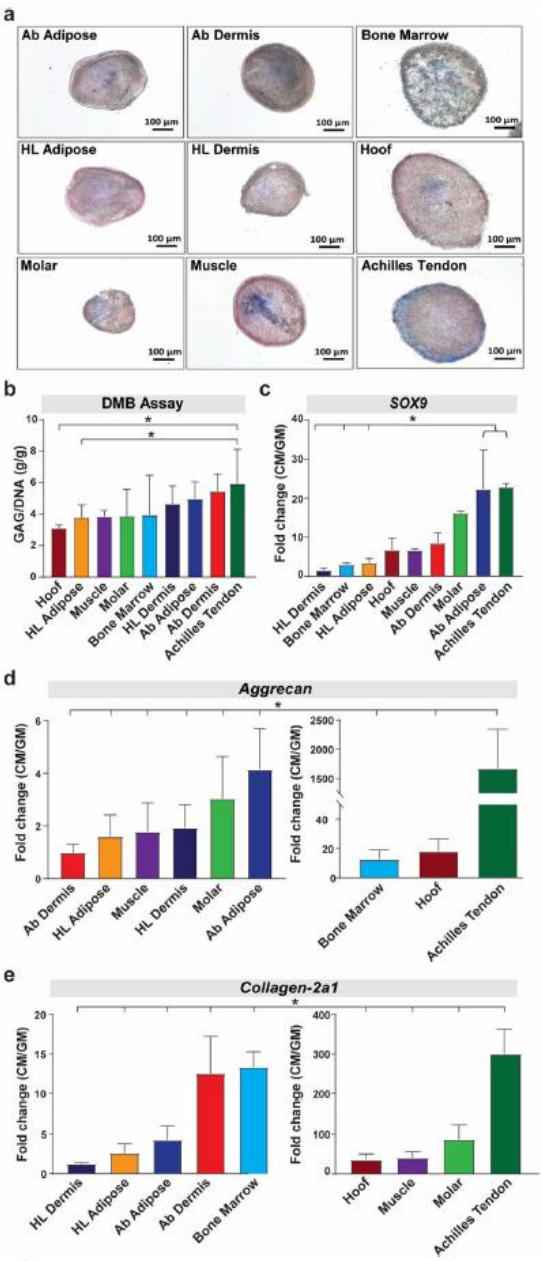


**Fig. 4** (See legend on previous page.)

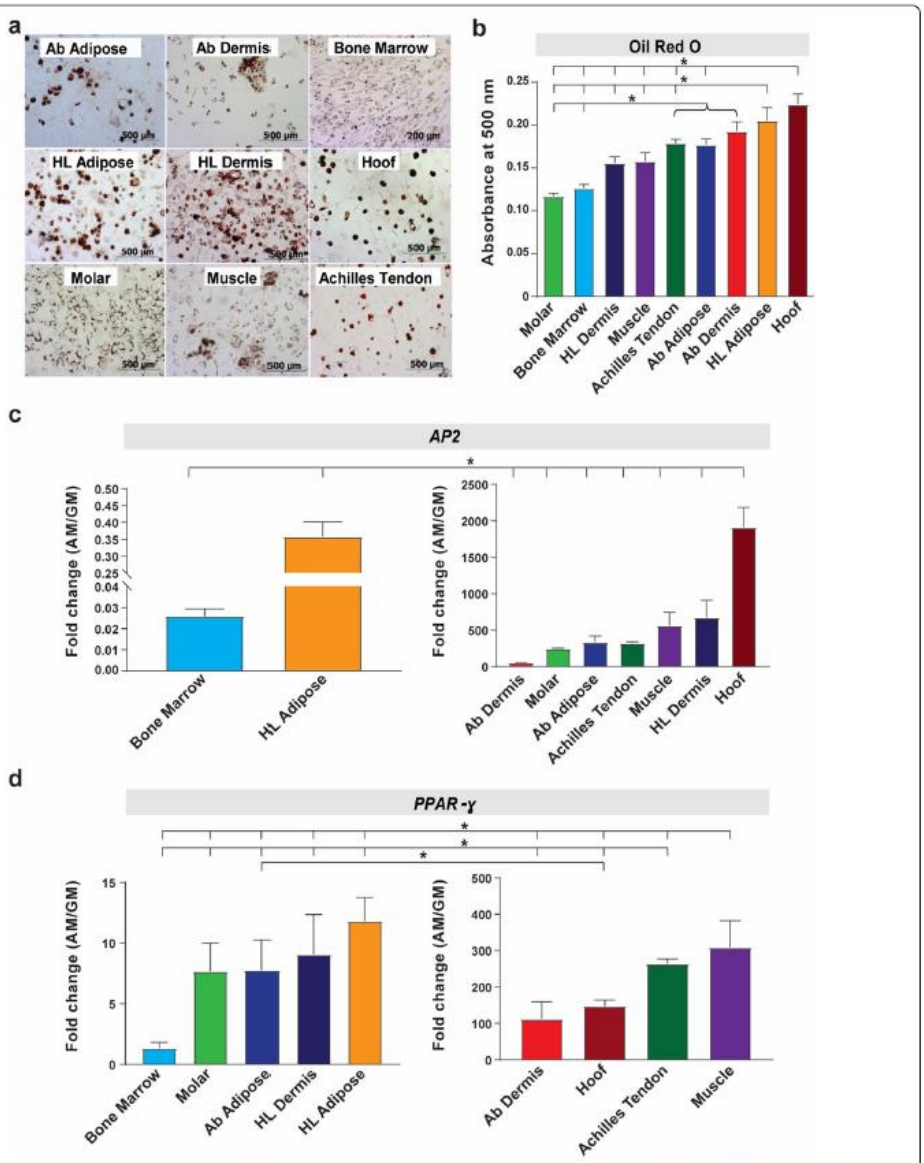


**Fig. 5** Assessment of in vitro adipogenic differentiation of pMSCs. **a** Histological staining (Oil Red O) of the nine pMSCs cultured in adipogenic media after 21–28 days in culture (50 × magnification). **b** Quantification of intracellular lipid droplet formation by measuring absorbance of solubilized Oil Red O at 500 nm. **c**–**d** Fold change in gene expression of the adipogenic transporter *AP2* (**c**) and receptor *PPAR-γ* (**d**) in pMSCs cultured in adipogenic media (AM) versus growth media (GM). β-Actin was used for normalization of gene expression. Data presented as mean ± SEM ($n = 3$; $^*p < 0.05$)

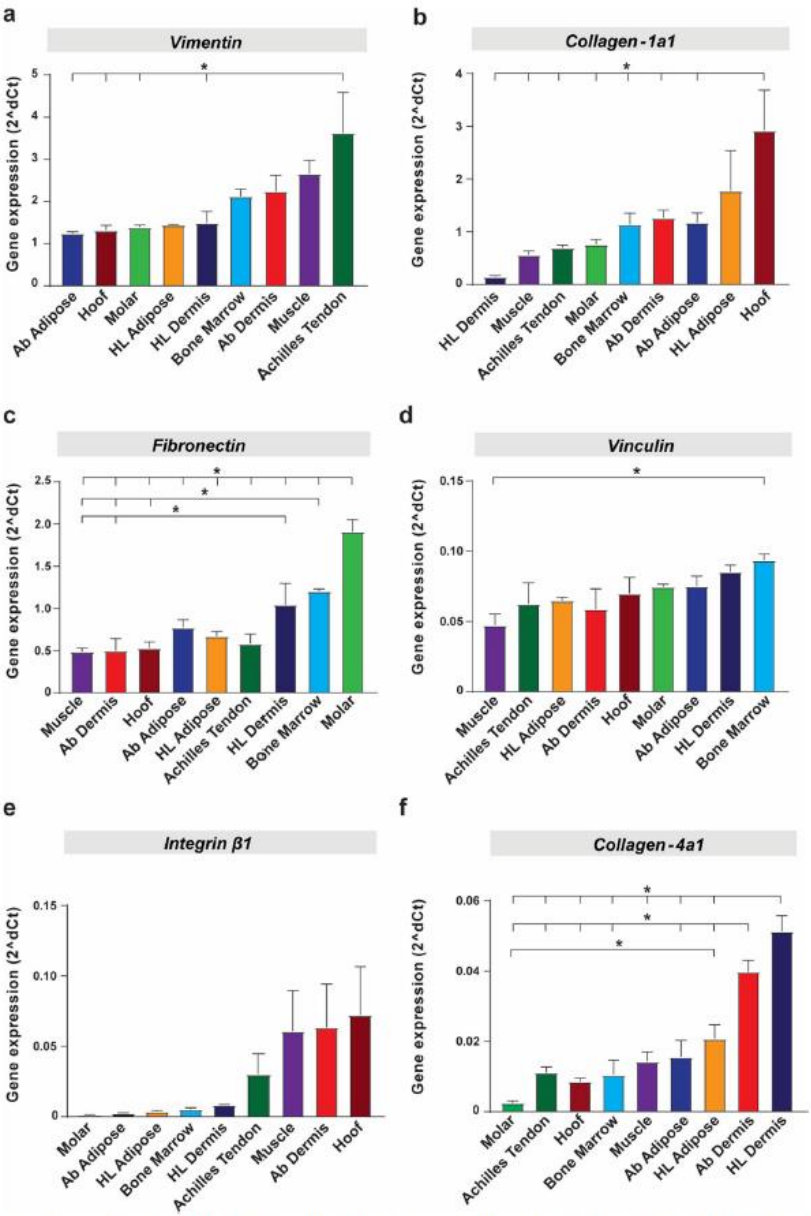


**Fig. 6** Expression profiling for a panel of adhesion genes in nine pMSCs. Syber Green based qRT-PCR for a panel of 6 adhesion genes in Passage 5 pMSCs cultured in growth media. **a** *Vimentin* **b** *Col1agen-1a1* **c** *Fibronectin* **d** *Vinculin* **e** *Integrin β1*, and **f** *Collagen-4a1*. β-Actin was used for normalization of gene expression. Data presented as mean ± SEM ($n = 3$; $^*p < 0.05$)

### Summary of key functions tested

A summary table (Table 1) was created to rank the top 3 tissues for each assessment (tri-lineage differentiation, adhesion gene expression, focal adhesion to Ti, proliferation on Ti, and adherence to Ti after centrifugation). The number of times a pMSCs appeared in the list was summed to create an overall ranking, where hoof-associated superficial flexor tendon pMSCs appeared the most.

## Discussion

This is the first comprehensive study, in our knowledge, which reports detailed characterization and comparative analysis of a broad panel of porcine integumentary and connective tissue-derived mesenchymal stromal cells. Indeed, the bulk of literature on pMSCs and their preclinical uses in the porcine model are restricted to bone marrow and adipose-derived cells [28–32], with very few reports on the isolation of multipotent pMSCs from porcine skeletal muscle [33–35], dermis [36, 37], and periodontal ligament [38]. While several reports used decellularized porcine tendon as a biological scaffold to seed human tissue-derived stromal cells [39, 40], there exist limited reports on the isolation and characterization of pMSCs derived from porcine tendon tissues. These findings indicate that these tissue-derived regenerative cells can have potential applications in bioengineering a durable skin-implant interface.

Successful isolation, ex vivo expansion, and in vitro multi-lineage differentiation of plastic adherent cells with self-renewal capacity from all nine porcine tissues demonstrated the presence functionally competent MSCs within these tissues. High expression of vimentin in all these cell types corroborated the mesenchymal origin of these cells [41]. Our results indicated that these cells can be steered to any of the three lineages studied here (osteogenic, chondrogenic and adipogenic), based on the combination of i*n vitro* induction signals the cells are exposed to. However, the varying level of expression of the different cell surface marker transcripts across the pMSCs indicated tissue-selectivity of these surface proteins or their compensatory roles in regulating pMSC function. Tissue-specificity and selectivity of the nine pMSCs was also reflected in the differentiation studies, with distinct transcription factors upregulated in the different pMSCs during the early stages of differentiation. It is important to consider species-specific differences between porcine and human MSCs that came up in this study, exemplified by the dim expression of CD105 on pMSCs, which on the other hand, is a well-recognized marker for human MSCs [23, 25]. Others have reported CD105 is [42] and is not [25] expressed by pMSCs, further supporting tissue-specificity. Here, we were able to detect low levels of mRNA gene transcript and cell surface protein expression of CD105.

Cell survival and proliferation assays demonstrated the ability of the different pMSCs to withstand forces on clinical grade titanium alloy, further supporting preclinical research of these cells in vivo. Centrifugation-based functional adhesion test of the pMSCs on titanium indicated that some cell types are more sensitive to handling than the others. For example, muscle-derived pMSCs seeded on titanium disks detached more than others at 500 g centrifugation test indicating that once seeded on titanium, these cells may not be amenable to additional manipulations and handling; thereby making them unfit for clinical purposes. Practical considerations associated with the ease of manipulation of cells are highly relevant as future steps are taken towards translation of this approach into a clinical therapy.

We ranked the nine sources of pMSC-derived with regard to osteogenic, chondrogenic and adipogenic differentiation capacity, expression of genes involved in cell adhesion, and their ability to adhere to and proliferate on titanium metal (Table 1). The results showed that hoof-associated superficial flexor tendon and Achilles tendon ranked the highest in both differentiation and adhesion assessments. Anatomical study of the porcine hoof region provides insights into the different tissue types, whose synchronous function is critical in imparting the strength, flexibility and mechanical stability of this region. Located right below the keratinized hoof is the modified dermis, corium, which generates cells that gradually keratinize to form the outer 'horn' of the hoof [43]. Superficial and deep flexor tendons attach the pedal bone inside the hoof to the muscles at the back of the leg, allowing for movement and flexing of the limb [44], providing a graded tensile strength along the tendon-hoof bone insertion junction, ranging from 200 MPa tensile modulus at the tendon end to 20 GPa tensile modulus

(See figure on next page.)

**Fig. 7** Adhesion potential of pMSCs to titanium. **a** CCK8-based assessment of pMSCs proliferation and viability demonstrating significantly higher values on titanium versus glass surface at 4, 24 and 72 h. NIH-3T3 has been used as a positive control. (Ti: Titanium; $n=3$; $^{*}p<0.05$ between Ti and Glass) **b** Measurement of intracellular levels of the focal adhesion protein vinculin in pMSCs on titanium versus glass surface at 4, 24 and 72 h. NIH-3T3 has been used as a positive control. (Ti: Titanium; $n=3$; $^{*}p<0.05$ between Ti and Glass) **c** Centrifugation based functional test of pMSCs adhesion to titanium, where control-post is the sham group; 350 g and 500 g are the two centrifugal forces tested. Keratinocytes have been used as a positive control. ($n=3$; $^{*}p<0.05$)

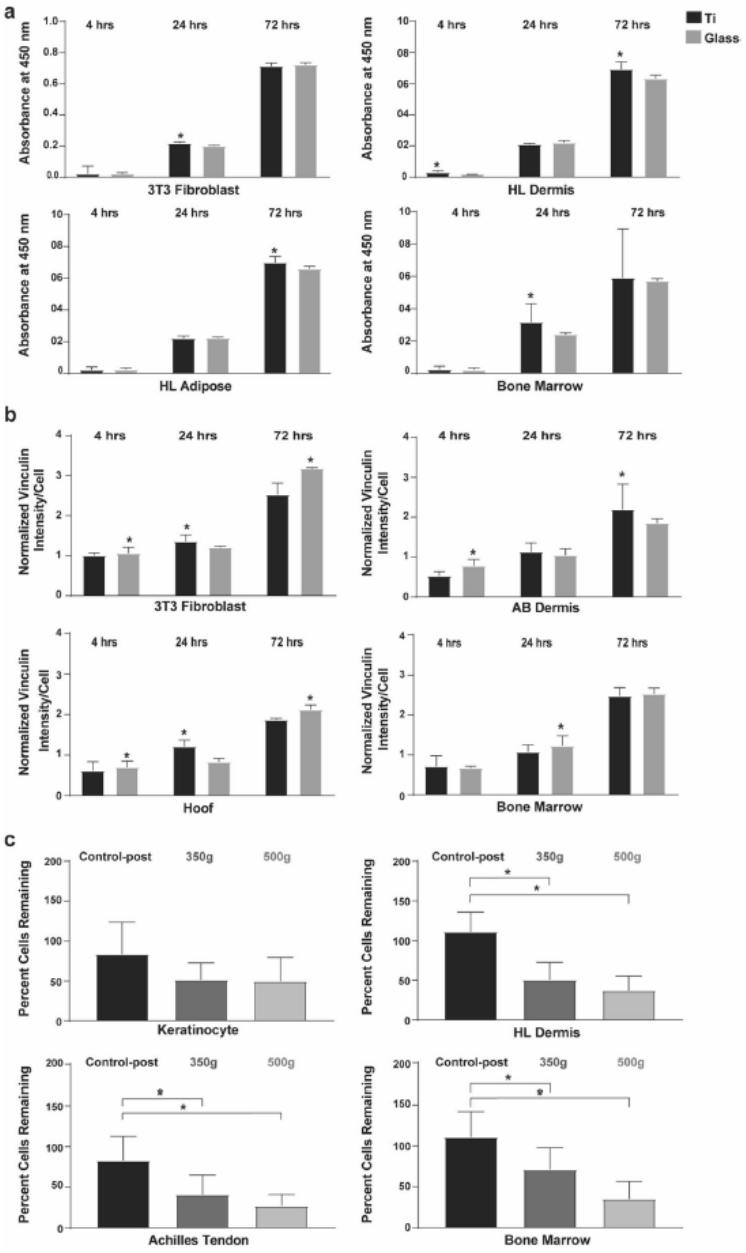


**Fig. 7** (See legend on previous page.)

**Table 1** Summary and ranking of pMSCs potential for application to transdermal device therapies.

| Function tested | Top 3 tissues for each assessment | | |
|---|---|---|---|
| Osteogenic differentiation | Hoof* | Bone Marrow | HL Adipose |
| Chondrogenic differentiation | Ab Dermis | Molar | Achilles Tendon |
| Adipogenic differentiation | Muscle | Achilles Tendon* | Hoof |
| Adhesion gene expression | Ab Adipose | HL Dermis | Hoof |
| Focal adhesion density on titanium | Bone Marrow | Hoof | Ab Dermis |
| Proliferation and viability on titanium | HL Adipose | HL Dermis | Ab Adipose |
| Adherence to titanium post centrifugation | Bone Marrow | HL Dermis | Achilles Tendon |

| Hoof | Achilles Tendon | Bone Marrow | HL Dermis | Ab Adipose | HL Adipose | Ab Dermis | Muscle | Molar |
|---|---|---|---|---|---|---|---|---|
| 4 | 3 | 3 | 3 | 2 | 2 | 2 | 1 | 1 |

(Top) Each row represents the cellular characteristic/function studied, and the columns indicate the top 3 pMSCs (in no particular order) with highest values for each function tested. * Based on gene expression results. (Bottom) Summarized table indicating the number of times a given pMSCs appears in the top 3 list

at the bone end [45]. Right below the keratinized portion of the hoof, the fatty, digital cushion, composed of adipose tissue provides a shock absorbing and sensory function to the hoof [46]. pMSCs from Achilles tendon showed robust chondrogenic differentiation potential and relatively high resistance to high shear forces when cultured on titanium, while the cells from the hoof-associated flexor tendon demonstrated high adipogenic and osteogenic differentiation, expression of multiple adhesion-related genes, and high expression levels of the focal adhesion protein, vinculin, when cultured on titanium. These observations corroborated with the intrinsic function of tendons as the tissue connecting a hard tissue (bone) to a soft tissue (muscle), and thus the capability to withstand mechanical loads [47] such as those present at the aperture created by transdermal devices.

Despite the underlying similarities in hoof and Achilles tendon tissues, there is a possibility of the distinct anatomical locations (microenvironment) of the two tendons dictating their structure and function, which reflects in the function of the progenitor cells derived from these tissues. There is limited literature comparing the cellular and acellular features of these two tendon tissues, and what distinguishes them. Interestingly, there were substantial differences in the adhesion gene expression profiles between pMSCs derived from these two tendons, with cells from the hoof-associated superficial flexor tendon expressing significantly more cell–cell and cell–matrix adhesion genes than Achilles tendon derived progenitor cells.

Going forward, the validity of these in vitro findings needs to be tested in vivo before any decision can be taken on the ideal cell type or combination to stabilize the unique aperture created by transdermal bone anchored devices. Cell survival, proliferation and lineage-specific differentiation of transplanted donor MSCs in vivo is highly dependent on the microenvironment and timing of injection/persistence within the receipient tissue [48, 49]. While there are reports of MSCs mediating direct tissue repair in vivo [50–52], multiple studies have reported trophic factors such as growth factors, morphogens, chemokines, cytokines, extracellular vesicles and extracellular matrix proteins triggering activation and homing of recipient's stem cells to the injury site, followed by tissue repair [49, 53–57]. Initial survival and adhesion of donor MSCs to the transplantation site is a critical parameter; however, the fate and contribution of the adhered MSCs to tissue repair and homeostasis would largely depend on their response to the local inflammatory milieu that exists at the site of injury, which in this context is the site of osseointegration [58]. A recent study indicated that ex vivo 'priming' of MSCs in an inflammatory microenvironment, before their transplantation to such an environment, significantly

enhanced MSC survival and tissue reparative functions when transplanted in vivo y [59]. For our current study, it would be interesting to observe the fate of these tissue-specific pMSCs after priming them in 'conditioned media' of cultured tissue explants from the OI abutment site. More broadly, trophic factor expression and pMSCs delivery methods must be defined in our future work.

## Conclusions

We characterized nine pMSCs derived from different porcine integumentary and connective tissues, adipose and dermal tissues from the hind limb and abdominal regions, bone marrow and muscle. Hoof-associated superficial flexor tendon and Achilles tendon ranked the highest in both differentiation and adhesion assessments. Tissue-specific differences between MSCs may be exploited toward bioengineering a durable skin-implant interface to reduce failure of transdermal osseointegrated implants.

### Abbreviations

Ab: Abdominal; ACAN: Aggrecan; ACK: Ammonium-chloride-potassium; ALP: Alkaline phosphatase; AM: Adipogenic medium; AP2: Adipocyte protein 2; BGLAP: Bone gamma-carboxyglutamate protein; BM: Bone marrow; BSA: Bovine serum albumin; BSP: Bone sialoprotein; CCK8: Cell counting kit 8; CM: Chondrogenic media; cMSC-GM: Complete MSC stromal growth medium; COL1A1: Collagen Type I Alpha 1 Chain; COL2A1: Collagen Type 2 Alpha 1 Chain; DAPI: 4′,6-Diamidino-2-phenylindole; DMEM: Dulbecco's modified eagle's medium; DMMB: Dimethyl-methylene blue; DNA: Deoxyribonucleic acid; ECM: Extracellular matrix; EDTA: Ethylenediaminetetraacetic acid; FACS: Fluorescence-activated single cell sorting; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate; FOV: Fields of views; GAG: Glycosaminoglycans; GPa: Gigapascal; GM: Growth medium; HL: Hind limb; HSD: Honestly significant difference; mRNA: Messenger RNA; NIH: National Institutes of Health; OI: Osteointegration implant; OM: Osteogenic media; OPN: Osteopontin; PBS: Phosphate-buffered saline; pMSCs: Porcine mesenchymal stromal cells; pNPP: P-Nitrophenyl phosphate; RBC: Red blood cell; RNA: Ribonucleic acid; RT-PCR: Reverse transcription- polymerase chain reaction; SEM: Standard error of the mean; SOX9: *SRY-box transcription factor 9*; Ti: Titanium; TCPS: Tissue-culture treated polystyrene; VIM: Vimentin.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-021-02666-2.

**Additional file 1:** Supplementary Tables and Figures.

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### Disclaimer

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### Author' contributions

DD: conception, design, performed experiments, assembly, analysis, interpretation of the data and wrote the manuscript, final approval of manuscript; NGF: cell adhesion, differentiation studies, and data collection, analysis, assembly and interpretation, and manuscript writing, final approval of manuscript; AHD: samples collection, cell culture, cell differentiation and gene expression studies, and data analysis and assembly, final approval of manuscript; ER: cell culture, flow cytometry experiment and data collection, final approval of manuscript. IM: cell adhesion, differentiation studies, and data collection, analysis, assembly and interpretation, final approval of manuscript; DTD: design, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; CJH: cell adhesion studies and data collection, final approval of manuscript; JAF, JEB: conception, data interpretation, and final approval of the manuscript; CA: design, analysis and interpretation of data, financial support; TAD: conception, design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information file.

## Declarations

### Ethics approval and consent to participate

Tissues used in this study were collected under an approved IACUC institutional tissue sharing protocol at Uniformed Services University of the Health Sciences.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Publisher's Note

## Appendix 6: Dr. Mutreja's MHSRS 2022 poster

# Human iMSC-laden photocurable hydrogel for improved durability of the skin/implant interface – An in vitro and ex vivo analysis


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## Introduction

- Implant loosening (13-29%) and surrounding soft tissue infection (29-38%) are common complications of upper and lower extremity bone-anchored implants.[1] These failures are due to absence of a tight, impervious seal at the soft tissue skin - percutaneous implant interface.
- Exploiting the ability of cells to interact with the dynamic nature of the soft tissue to increase tissue attachment on the implant has gained notable interest.[2]
- ***Our goal is to use MSC's differentiated from human induced pluripotent stem cells (iPSCs) and combine them with gelatin-based hydrogel for engineering a cell-based scaffold that can stimulate the formation of a tight seal at the skin-implant interface.***

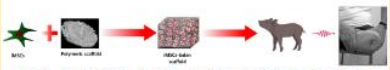


Figure 1. Biological strategy to form impervious seal at skin-percutaneous implant interface

## Aims

- Differentiation of human iPSCs into MSC-like cells (iMSCs) and perform detailed characterization of differentiated iMSCs in terms of the surface marker expression and their ability to undergo tri-lineage differentiation.
- Fabrication of methacrylated gelatin hydrogels with varying stiffness and assessing their effect on survival and migration of encapsulated iMSCs.

## iPSCs - derived MSCs

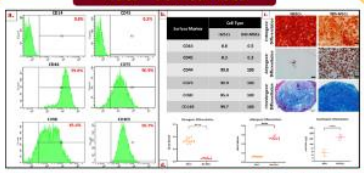


Figure 3: (a) Flow cytometry analysis to confirm presence of MSC-specific cell surface (b) Quantitative comparison of surface marker expression between iMSCs and BM-MSCs, (c) qualitative and (d) quantitative assessment of functional capability of iMSCs and BM-MSCs to undergo tri-lineage differentiation. Scale: 50 µm

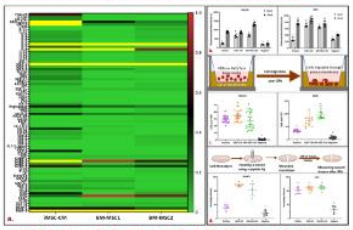


Figure 4. (a) Heatmap of cell secretome collected in CM released by iMSCs and BM-MSCs cultured in 2D measured by cytokine analysis, yellow color represents cytokine expression higher than 1 (b) Effect of CM on cell proliferation (c) cell migration and (d) wound closure capability of HaCaTs and HDFs cultured in presence of CM collected from iMSCs and BM-MSCs. Positive is serum supplemented DMEM and negative is serum starved DMEM.

## Methods

- **Derivitizing MSC-like cells from iPSCs[3]**

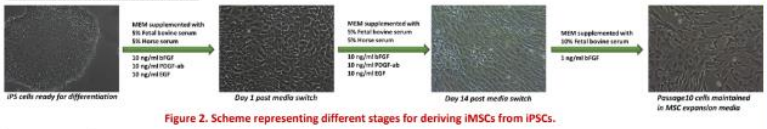
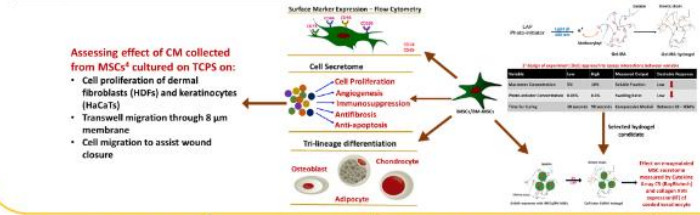



Figure 2. Scheme representing different stages for deriving iMSCs from iPSCs.

- **Assessing iMSC function in 2D and within optimized GelMA scaffold**




## Photo-labile GelMA hydrogel

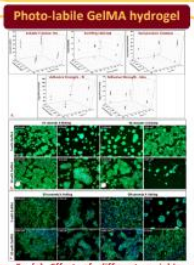


Figure 5: (a) Effect of different variables on physico-mechanical properties of fabricated GelMA hydrogel and attachment of (b) keratinocytes and (c) fibroblasts seeded on different hydrogels as measured by expression of Hoechst and Igβ4 for HaCaTs and Hoechst and Collagen I for HDFs. Scale bar is 100 µm

## Stem cell-laden GelMA hydrogels

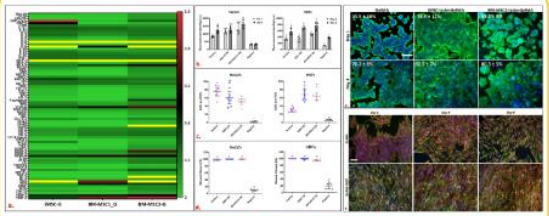


Figure 6: (a) Heatmap of cell secretome collected in CM released by iMSCs and BM-MSCs encapsulated within selected GelMA hydrogel (10 wt% macromer, 0.05% LAP, X-linked for 90 seconds), yellow color represents cytokine expression higher than 1. Effect of CM released from cells encapsulated within the selected GelMA candidate on (b) cell proliferation (c) cell migration and (d) wound closure capability of HaCaTs and HDFs. (e) Immunofluorescent (Hoechst and Igβ4) micrographs of keratinocytes (f) Immunofluorescent (Hoechst, Collagen I and Igαvβ1) micrographs of fibroblasts cultured on control and iMSC-laden GelMA. Scale bar is 100 µm

## Summary

- iPSCs derived MSCs demonstrated all the characteristic properties comparable with BM-MSCs in their morphology, cell surface antigen profile, their capacity for tri-lineage differentiation and cell secretome profile.
- DoE based approach confirmed that *the key variable which significantly affects the measured response for GelMA scaffold is time; suggesting as we increase time, there is significant reduction in soluble fraction and swelling ratio and increase in material stiffness.*
- HaCaTs and HDFs cultured on different GelMA hydrogel scaffolds showed no differences in proliferative capabilities, however, scaffold '10 wt% macromer, 0.05% LAP and X-linked for 90 seconds showed presence of HaCaTs with highest expression of Integrin β4, key marker for hemidesmosome formation and Collagen I expression for HDFs confirming its suitability for stem cell encapsulation.
- The selected scaffold when combined with stem cells (iMSCs and BM-MSCs) supported proliferation, migration and wound closure capability of HaCaTs and HDFs. Additionally, iMSC-laden hydrogel upregulated the expression of Igβ4 in HaCaTs and collagen I and Igαvβ1 expression in HDFs.

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