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

The study addresses two focus areas of research with pressing clinical need: **1.** Optimization of the skin-implant interface for osseointegrated (OI) implants, and **2.** Prevention of infection at the skin-implant interface. The goal is to address both these issues while maintaining residual limb skin integrity and durability. We address the issue of skin-to-implant healing and attachment for osseointegrated (OI) prostheses by focusing on integration and durability of their microbially, mechanically and biologically challenging skin-to-implant interface. This study focuses on an OI prosthetic implant anchored in the long bone of a residual limb and exiting through the skin. Implant and soft tissue infections (29-38%) and implant loosening (13-29%) are common complications for both upper and lower extremity bone-anchored implants, resulting in revision surgeries and increased morbidity. These complications develop due to lack of a tight, impervious seal at the skin-percutaneous implant interface, resulting in exposure of soft tissue and vasculature, thereby increasing chances of infection as well as implant loosening. For both focus areas, we explore the possibility of creating a tight, durable skin-implant interface for OI implants using mesenchymal stem cells (MSCs) derived from naturally occurring porcine integumentary 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: Provide a brief list of keywords (limit to 20 words).

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

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

What were the major goals of the project?

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

Research-Specific Aims and Tasks	Mos.	Percent completed
Administrative Aims and Tasks: 1. Establish subaward agreement between HJF and MMRF 2. Develop and sign USU-MMRF CRADA 3. Recruit and hire support personnel a. Stem Cell Biologist (USU) b. Research Associate (USU) c. Stem Cell Biologist (MMRF-UMN)	1-4	100% 100% 100% a. 100% b. 100% c. 100%
Specific Aim 1 <u>Specific Aim 1: in ex vivo culture</u> (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	50%
Major Task 1: Develop cell culture	1-16	75%
Subtask 1.1: In vitro isolation and characterization of <u>porcine</u> cells.	1-16	80%
Subtask 1.2: In vitro development and characterization of <u>human</u> cells.	1-16	75%
Major Task 2: <u>In vitro</u> : evaluate cell adhesion to metal substrate	7-16	50%
Subtask 2.1a: Test porcine cells for adhesion	7-16	75%
Subtask 2.1b: Test human cells for adhesion	7-16	25%
Major Task 3: Scaffold development for cell growth and anchorage to underlying tissue.	5-24	10%
Subtask 3.1: Complete scaffold design (constructs) for “sleeve” & “transition designs	5-16	15%
Subtask 3.2: Seed and grow porcine cells on flat collagen sheets, assemble scaffolds	17-24	0%
Subtask 3.3: Seed and grow human cells on flat collagen sheets, assemble scaffolds	17-24	5%
Major Task 4: Complete full statistical analysis, complete/submit 2-4 manuscripts.	18-24	15%
<i>Milestone(s) Achieved: Characterization of 3-4 cell choices for optimal adhesivity - in vitro; submission of 2-4 manuscripts.</i>	1-24	20%
Specific Aim 2 <u>Specific Aim 2: In vivo large animal (swine) testing of</u> transdermal implants with and without subdermal cellular augmentation (SA2a), +/- septal /strain limiting scaffold (SA2b), and topical bacterial challenge (SA2c).	25-60	0%
Major task 5: (SA2a) Implant 8 implants/animal with “best of” cells from Specific Aims 1 and 2 x 10 animals at USU-Surgery	25-35	0%
Subtask 5.1: Implant initial 5 animals with 3-4 types of cell augmentation per animal	25-27	0%
Subtask 5.2: Initial evaluation of skin integration - assessment of initial results; experiment modification as necessary.	28-29	0%

What was accomplished under these goals?

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

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

A. Isolation of porcine MSC and expression of MSC markers:

Porcine MSCs were isolated from female Gottingen, Yucatan or Yorkshire minipigs under USUHS Surgery and Laboratory Animal Medicine tissue sharing protocol (LAM-17-540 entitled "Education and Training Protocol for Techniques in Animal Care and Use"), as described in the last annual report. Briefly, all tissues were collected in sterile collection media (1:1 DMEM-F12, containing 2% FBS and 2X antibiotics; namely penicillin, streptomycin and fungizone). Cells were harvested after mechanical and enzymatic digestion, followed by RBC lysis. The nine different tissues from which MSCs were derived include bone marrow, muscle, abdominal and hind limb adipose, abdominal and hind limb dermis, tendon, hoof, and molar-associated periodontal ligament. Expanded cells from the nine tissues from 4-5 animals have been biobanked at different passages from P0 to P5 at a concentration of $1-2 \times 10^6$ cells/vial.

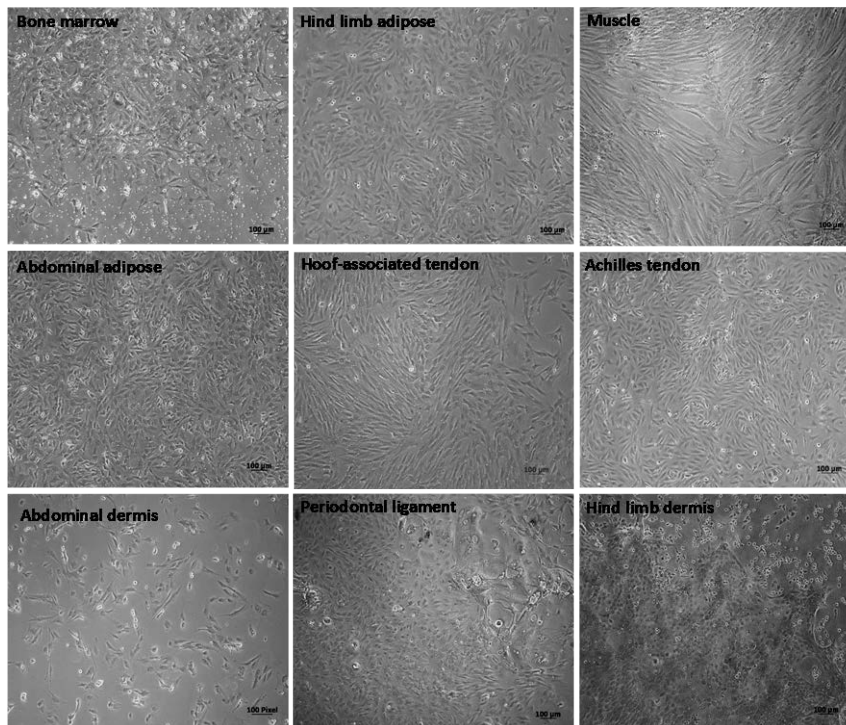


Figure 1. Phase contrast images (50x) of Passage 0 cells, 10 days after isolation from the different porcine integumentary and connective tissues.

Characterization of early passage tissue-derived cells for mesenchymal stem cell markers

Cells derived by mechanical and enzymatic digestion of all the nine tissues were cultured and expanded in regular growth media (GM), comprising of 1:1 DMEM-F12, supplemented with 10% FBS and antibiotics. On reaching 80-90% confluency, cells were passaged, and 1×10^5 cells were seeded per well in a 6-well plate in GM for gene expression studies. At the end of 1 week, cells were harvested and homogenized in 1 ml of Qiazol

which was carried out using the RNA isolation mRNeasy kit (Qiagen). cDNA synthesis (ABI) and Syber Green (Bio-Rad) based quantitative Real-Time PCR was run on Quant-Studio. We assessed the expression of four MSC-associated markers, CD90, CD44, CD105 and CD29. In addition, we tested for expression levels of CD11b, a hematopoietic cell-specific marker and CD31, an endothelial cell marker. β -Actin was used as the housekeeping gene. All primers for this study were designed in-house.

Results: All the nine cell types expressed high levels of the MSC markers, CD90, CD44 and CD29 as shown in **Figure 2**. Expression of CD105 was however low across all cell types. CD11b and CD31 were expressed at very low levels in all cell types, indicating minimal contamination of hematopoietic and endothelial cells. For the genes with low expression, the PCR was repeated with new primer sets, and we did not observe any changes with the new set.

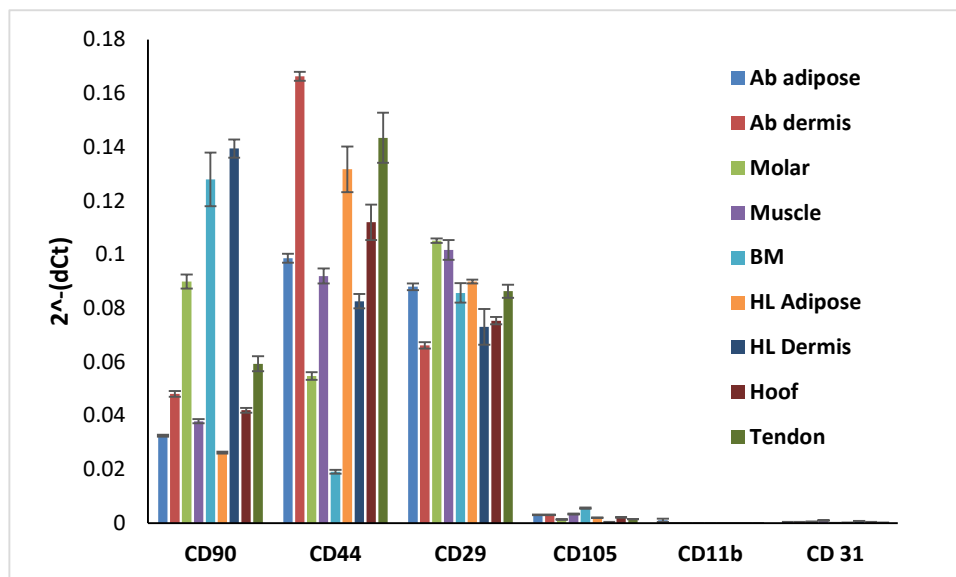


Figure 2. Characterization of MSC, hematopoietic and endothelial marker expression in porcine integumentary and connective tissue cells. Expression of MSC markers (CD90, CD44, CD29 and CD105), hematopoietic (CD11b) and endothelial marker (CD31) in the nine different cell types cultured in GM as indicated.

B. Osteogenic, adipogenic and chondrogenic differentiation capacity of isolated MSCs

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

Long term differentiation assays and gene expression was used to assess lineage-specific differentiation. For induction of osteogenic and adipogenic differentiation, equal number of cells were seeded in tissue culture plates and cultured for either 21 days for long-term differentiation assay, or 7 days for analysis of lineage-specific gene expression.

Osteogenic differentiation was induced by replacing growth media with osteogenic media (OM; StemPro Osteogenesis kit) 24 hours post seeding. Early osteogenic differentiation was assessed by pNpp based alkaline phosphatase activity assay (Sigma Aldrich) carried out after one week of initiation of osteogenic differentiation.

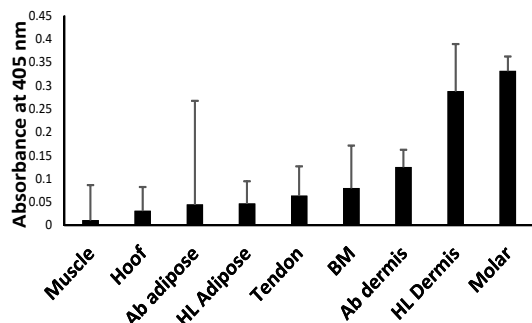


Figure 3. Characterization of early osteogenic differentiation potential of porcine integumentary and connective tissues by alkaline phosphatase activity measured after culturing cells in osteogenic differentiation media for 1 week

Briefly, cells were washed with PBS, followed by lysis in 50 μ l of 1% Triton X-100 for 2-3 minutes. Equal volume of pNpp substrate was added, and absorbance was recorded immediately at 405 nm in kinetic mode for 20 cycles. (Tecan).

Terminal osteogenic differentiation was assessed by measuring Ca^{2+} deposition after 3 weeks in differentiation media, and the analysis has been completed in all the nine tissues. Alizarin Red staining was used to assess Ca^{2+} deposition as described in previous reports and staining was quantified by formic acid based solubilization of the stain, followed by absorbance reading at 414 nm. Both early and terminal osteogenic differentiation was carried out in a 96-well plate, with 100 cells/well. We have also completed the osteogenic gene expression profiling for all nine cell types after 5 days of osteogenic differentiation for *Runx2*, *Osterix*, *Osteopontin*, bone-specific protein (BSP) and *Osteocalcin*.

Results:

Assessment of early osteogenic differentiation demonstrated the highest alkaline phosphatase activity in molar-associated periodontal ligament cells among all tissues, as shown in **Fig 3**, followed by hind limb dermis.

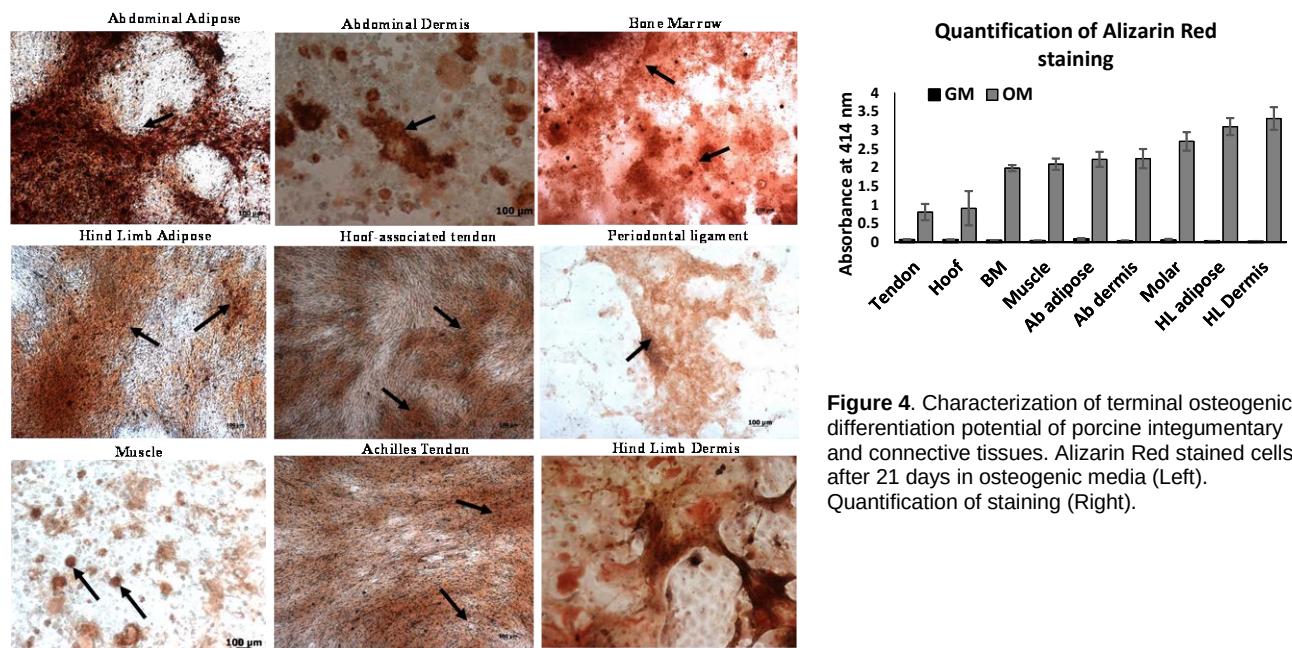


Figure 4. Characterization of terminal osteogenic differentiation potential of porcine integumentary and connective tissues. Alizarin Red stained cells after 21 days in osteogenic media (Left). Quantification of staining (Right).

After 3 weeks of differentiation, hind limb-derived dermal cells displayed the highest Ca^{2+} deposition, followed by hind limb adipose and molar associated periodontal ligament derived cells (**Figure 4**). Gene expression analysis of five osteogenic genes, namely *Runx2*, *Osterix*, *Osteopontin*, *BSP* and *Osteocalcin* in the nine tissues, carried out 7 days after osteogenic differentiation demonstrated differential upregulation of the genesets in the different tissues. While abdominal dermis-derived cells showed highest expression of *Runx2* and *Osteopontin*, molar-derived cells showed highest expression of *Osterix*. Tendon-derived cells showed highest expression of *BSP*, while hind limb adipose cells had high expression of *BSP* as well as *Osteocalcin* (**Figure 5**).

Adipogenic differentiation was induced by replacing growth media with commercially available adipogenic media (AM; LaCell) when cells reached ~80% confluency at 5-7 days post seeding. For gene expression studies, 1×10^5 cells was seeded per well of a 6-well plate, and for 21-day differentiation assay, 500 cells/well was seeded in a 96-well plate. Induction of adipogenic differentiation was visualized by formation of lipid droplets, which are stained with Oil Red O (ORO), as described in previous reports. Gene expression analysis of the adipogenic-specific gene, *PPAR-γ* has been completed for all the cell types.

Results:

Human adipose derived stem cells (hASCs) were used as positive control for adipogenic differentiation studies. After 21 days in culture, distinct lipid droplets were seen in hASCs cultured in AM (**Figure 6A**), while the different porcine cell types showed differential formation of lipids droplets. Among them, abdominal adipose and muscle cells showed highest adipogenic differentiation (**Figure 6A**).

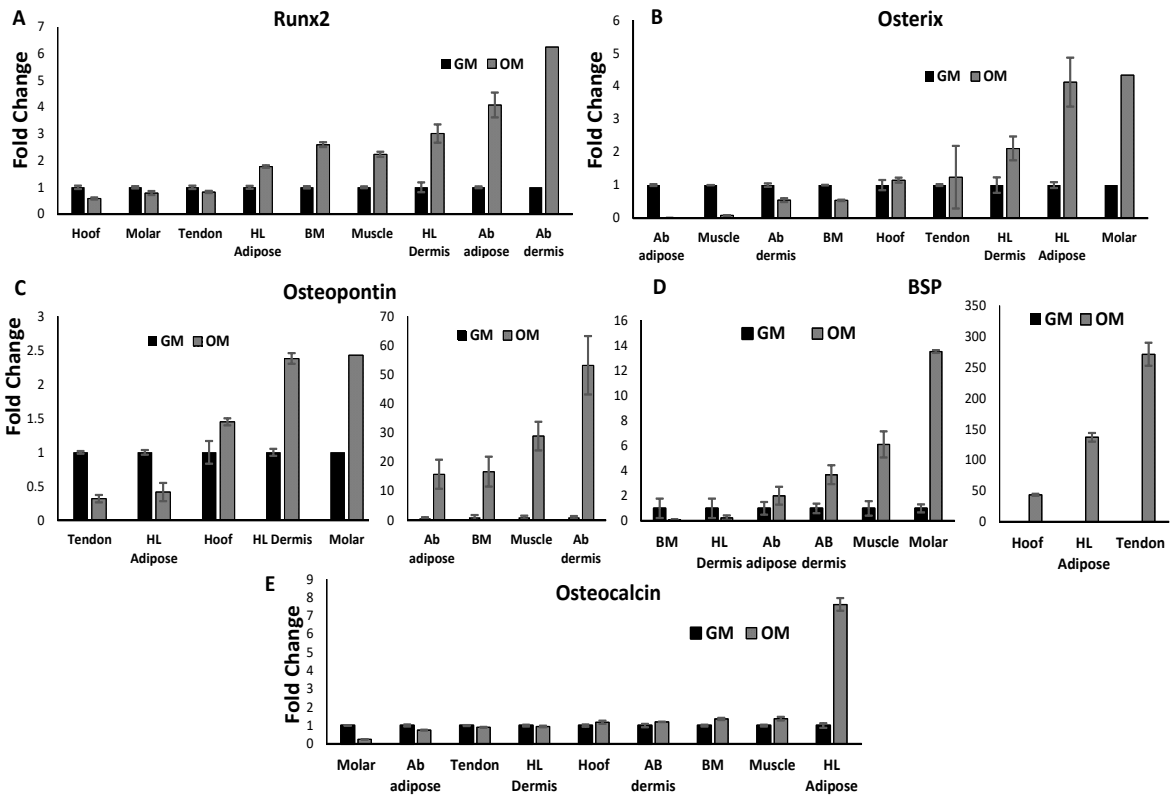


Figure 5. Gene expression analysis of five osteogenic-specific genes in the nine tissues. A. *Runx2*, B. *Osterix*, C. *Osteopontin*, D. *Bone-Specific Protein (BSP)*, E. *Osteocalcin*, assessed in osteogenic media (OM) with respect to growth media (GM) as baseline.

Analysis of PPAR- γ expression after 7 days in adipogenic media however demonstrated highest expression in bone marrow cells (**Figure. 6B**). These studies would have to be repeated in additional biological samples.

Chondrogenic differentiation was induced by seeding Passage 3 cells (P3) in v-well plates at two different densities (2.5×10^5 or 5×10^5 cells) in chondrogenic media (CM). Plated cells were centrifuged at 200g or 4 minutes. Pellets were cultured in CM for 21 days, with media changes done twice a week. After 35 days in culture, pellets were either fixed in 4% paraformaldehyde for sectioning or washed with PBS and frozen for quantitative analysis of proteoglycan deposition by GAG/DNA quantification or qRT-PCR based gene expression studies. Pellets were cryosectioned at 30 μ m thickness and stained with alcian blue (glycosaminoglycan (GAG) staining) and counterstained with neutral red (nuclear stain for DNA). Images were taken at 10x and 20x magnification. GAG was quantified using DMMB assay and CyQuant was used for measurement of chondroitin sulphate and DNA content.

Results:

Pellets were successfully formed under the culture conditions, and cryosections of the pellet were obtained. Sections were successfully stained with Alcian blue, as observed in **Figure 7A**. Pellets formed by the BM-MSCs and hoof-associated tendon cells demonstrated the highest chondrogenic differentiation, comparable to the positive control (human MSCs), as shown in **Figure 7A**. GAG/DNA quantification of this staining was done, as shown in **Figure 7B**. Gene expression analysis of the pellets is currently underway.

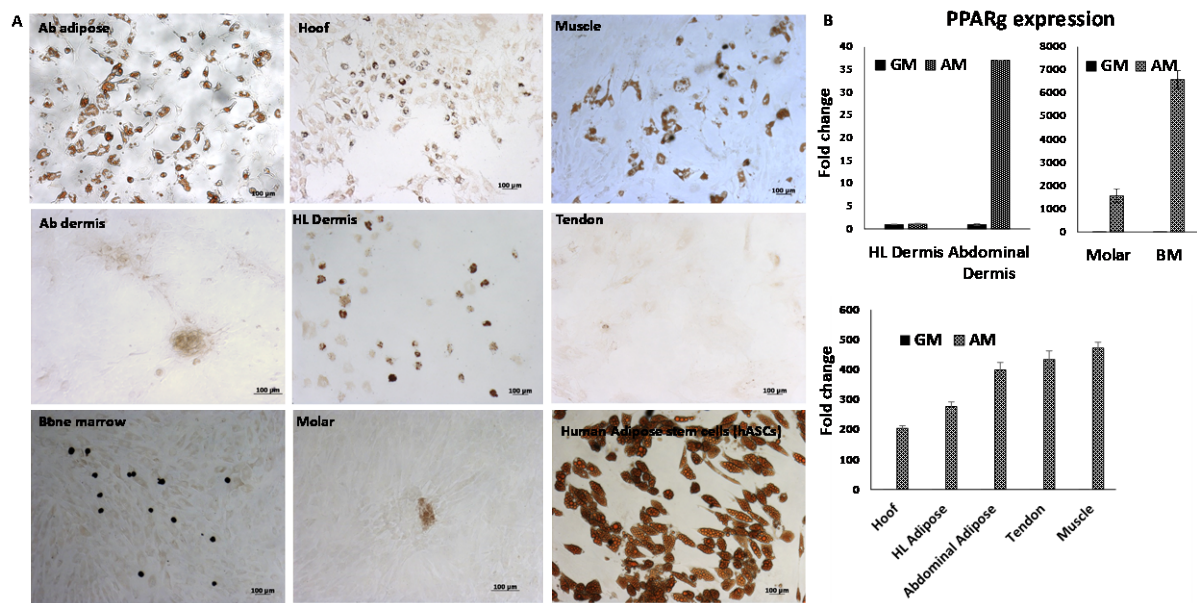


Figure 6. Characterization of adipogenic differentiation potential of porcine integumentary and connective tissues. A. Oil Red O staining of cells cultured in adipogenic differentiation media for 21 days B. Gene expression analysis of PPAR γ after 7 days in adipogenic media (AM). Fold change is calculated based on expression of the gene in growth media (GM)

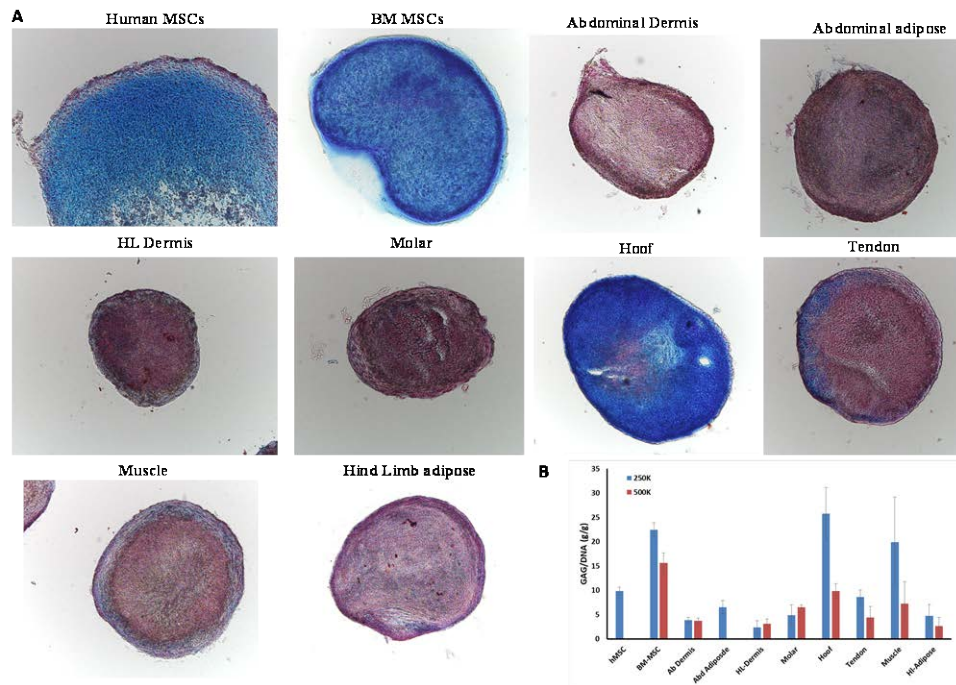


Figure 7. Chondrogenic differentiation studies. A. Alcian blue staining of pellets formed for the nine cell types cultured in chondrogenic media for 35 days. B. Quantitative analysis of proteoglycan staining by DMMB assay, and normalized to DNA content (GAG/DNA).

C. Characterization of early passage tissue-derived MSCs for expression of cell adhesion genes

In order to determine the cell type with high expression of cell adhesion genes in their native state, passage 2 (P2) cells from all the nine tissues were cultured in regular growth media (GM) at a density of 1×10^5 cells per well in a 6-well plate, and were harvested after 1 week in Trizol Reagent for RNA isolation. Post RNA isolation and cDNA synthesis, qPCR was carried out for a panel of cell adhesion genes; namely vimentin, fibronectin, integrin B1, vinculin, CD151, laminin, collagens 1A1, 2A1 and 4A1.

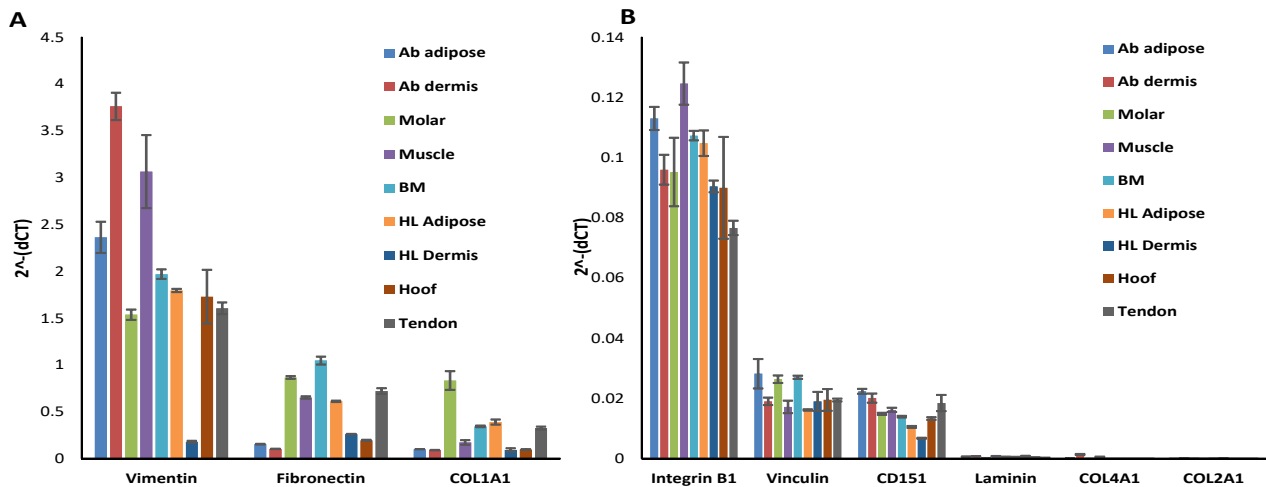


Figure 8. Gene expression analysis of cell-adhesion specific genes. A. High expressing; B. Low expressing genes

Results

Among both high and low expressing adhesion genes, molar-associated periodontal ligament cells demonstrated comparatively high expression pattern of all the genes (**Figure 8**), indicating to a potential active synthesis of the adhesion proteins, vimentin fibronectin, collagen 1A1, integrin B1, vinculin, and CD151. Expression of additional adhesion markers are currently underway, and new primers are being tested for the low expressing genes.

Subtask 1.2: In vitro isolation and characterization of human cells.

The main focus of this subtask was to optimize differentiation of human iPSCs into MSC-like cells and perform detailed characterization of the differentiated cells via surface marker analysis and tri-lineage differentiation capability.

A.iPS cell maintenance and culture

Fully characterized human iPS cells derived from wild type fibroblasts have been used for this study. These cells are part of Dr. Jakub Tolar's lab protocol entitled 'Comparison of normal skin biology to the biology of diseased skin' (STUDY00001939, University of Minnesota), exempt for IRB approval ("WORKSHEET: Exemption (HRP-312)." as 'Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked of the subjects.'

iPS cells at passage P36 were cultured in 6-well Matrigel®-coated plates with mTeSR1 Plus media (Stem Cell Technology) containing 5 μ M Rock Inhibitor (RI). mTeSR1 Plus media without the RI was used for cell culture and once the cell colonies were matured, they were passaged using 500 μ M EDTA in PBS. This was done every 4 days to prevent the colonies from overgrowing and preventing cells from losing their pluripotency. iPS cell maintenance training has been successfully completed, so that the iPS cells can be maintained, cultured and frozen without losing their potency.

B.iPS cell differentiation to iMSC cells

To differentiate human iPS cells into MSC-like cells (iMSC), MSC differentiation media was used when the cells were 50% confluent. The composition of the differentiation media is Minimum Essential Media (MEM) containing 5% horse serum, 5% fetal bovine serum, pen-strep, glutamax, 10 ng/ml recombinant epidermal growth factor (EGF), 10 ng/ml human recombinant basic fibroblast growth factor (bFGF) and 10 ng/ml human recombinant platelet derived growth factor (PDGFab). The iPS cells were cultured in differentiation media for 14 days, followed by passaging from one well of 6-well plate to a Matrigel®-coated 10-cm plate.

Cells were maintained in the differentiation media until confluence, following which they were passaged and seeded on 0.1% gelatin coated plate in complete media up to P4.

After P4, cells were cultured and maintained in MEM media, which is serum-supplemented medium with 1 ng/ml bFGF. This was done to assess optimal media conditions required for maintaining MSC phenotype.

C. Characterization of iMSC phenotype

To confirm MSC phenotype of iMSCs, flow cytometry was performed on cells at P10 for a panel of surface markers. PerCP-Cy5.5 labelled antibodies specific for CD14, CD45, CD44, CD73, CD90 and CD105 were used. iMSC were prepared following manufacturers instruction. Briefly, 90% confluent cells were trypsinized, filtered to obtain single cell suspension, counted and then stained with fluorescently-labelled antibody along with respective isotype control. Flow cytometry was performed on a BD LSR II H1010.

D. Tri-lineage assessment of iMSCs

iMSCs were also tested for tri-lineage differentiation, namely, osteogenic, chondrogenic and adipogenic differentiation. For osteogenic and adipogenic differentiation, cells were seeded in 12-well plate at a seeding density of 20,000 cells/cm². For osteogenic differentiation cells were cultured in MEM media supplemented with FBS (10% v/v), 0.2 mM ascorbic acid 2 phosphate, 10 mM β -glycerophosphate and 100 nM dexamethasone. Cells were fixed after 21 days and stained for calcium mineralization using alizarin red. For adipogenic differentiation commercial adipogenic differentiation medium from Stem Cell technologies was used. Oil red O stain was used to assess the presence of lipid vesicles. Chondrogenic differentiation was performed via pellet culture method. Expanded iMSC were passaged, counted and re-suspended in chondrogenic differentiation medium and plated in v-well polypropylene plate at a density of 250,000 cells/well in 0.290 ml medium. Cells were centrifuged at 200x g for 4 minutes. Cells were maintained in the media for 21 days with media changes every three days. The chondrogenic differentiation media consists of DMEM high glucose media supplemented with 1% ITS, 100 nM dexamethasone, 1.25 mg/ml bovine serum albumin, 10 ng/ml TGF- β 1, 0.1 mM ascorbic acid 2 phosphate. Pellets were collected after 21 days, fixed, cryo-sectioned and stained with alcian blue and counterstained with nuclear fast red.

Results:

Induced pluripotent stem cells were successfully differentiated into MSC-like cells. Following two weeks in the differentiation medium, changes in cellular morphology were observed, with appearance of cells showing fibroblast-like morphology.

Flow cytometry of iMSCs at P10 confirmed the presence of CD44, CD73, CD90 and CD105 (MSC positive markers) and were negative for CD14 and CD45 (**Figure 9**). The cells were 99.8% CD44+, 90.9% CD73+, 85.4% CD90+ and 99.7% CD105+ Cells were also assessed for surface markers at higher passages and no significant changes in surface expression was observed, which confirmed that these cells can be used up to P14.

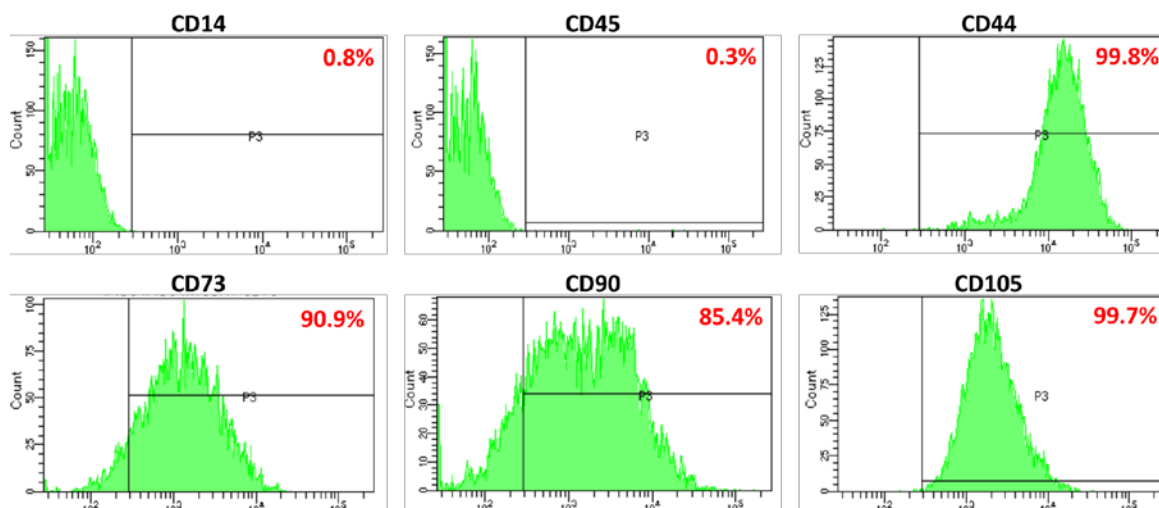
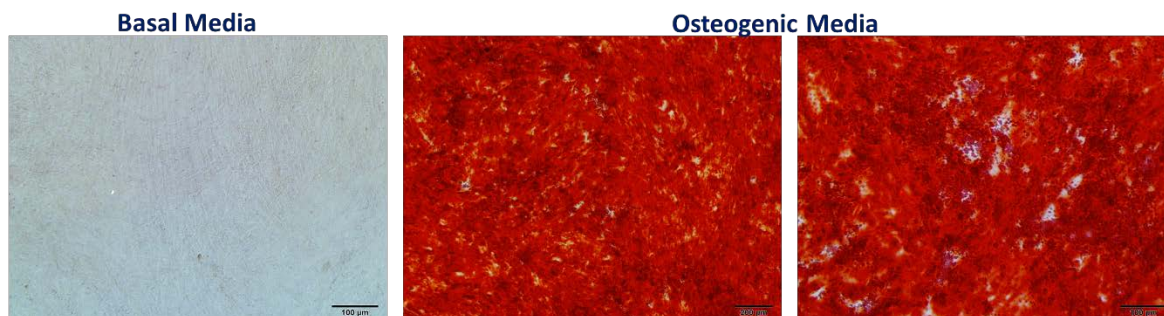


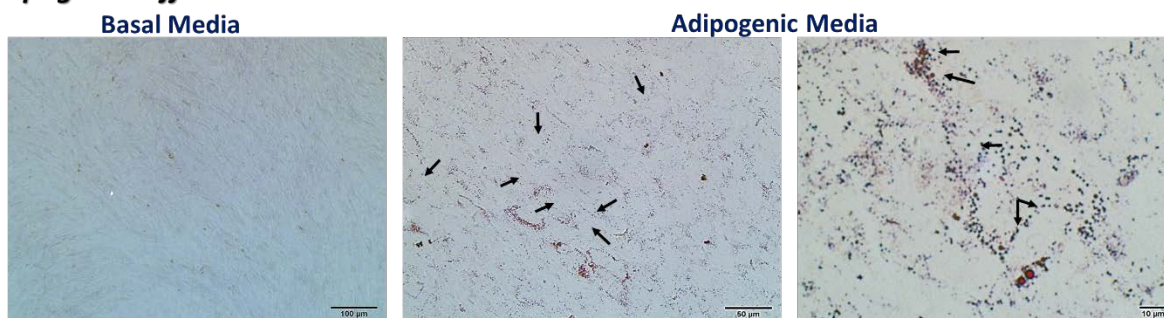
Figure 9: Flow scatter plots for iMSC (passage 10) cultured in MEM media

For osteogenic differentiation, cells were cultured in osteogenic differentiation medium for 21 days. iMSC showed high levels of mineralization as evident with alizarin red staining performed at day 21 (**Figure 10, top**). Calcium deposition started as early as after 12 days in culture. For adipogenic differentiation, oil droplets were observed as stained by oil red O stain after 21 days of culture (**Figure 10, middle**). Chondrogenic pellets positively stained for alcian blue confirming the presence of sulphated glycosaminoglycans in the extracellular matrix as shown in the image blue (**Figure 10, bottom**). The surface marker analysis and tri-lineage differentiation confirmed the iMSCs derived from iPSCs had all characteristic properties associated with primary human MSCs.

Osteogenic Differentiation



Adipogenic Differentiation



Chondrogenic Differentiation

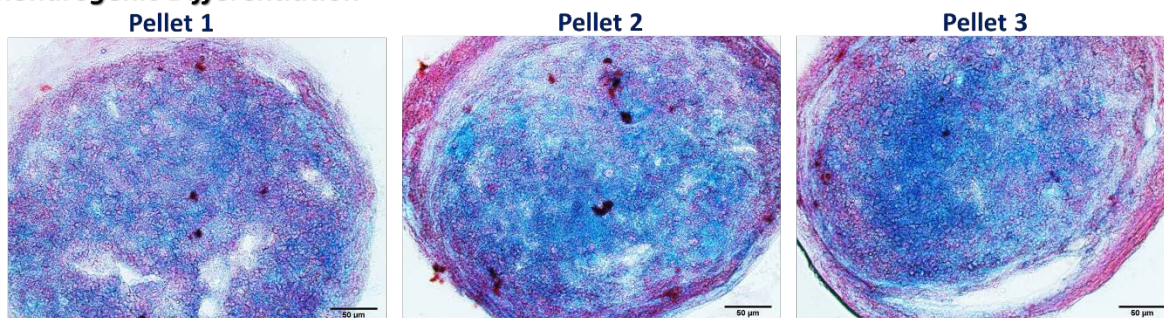


Figure 10: Alizarin red (top), oil red o staining (middle) and alcian blue staining for iMSCs cultured for 21 days in osteogenic, adipogenic and chondrogenic differentiation conditions, respectively

Conclusion:

We successfully differentiated iPSCs to MSCs. iPSCs derived MSCs demonstrated all the characteristic properties associated with primary human MSCs in their morphology, cell surface antigen profile, and their capacity for tri-lineage differentiation.

Subtask 2.1: Test porcine cells for adhesion to metal substrate.

Purpose:

The purpose of this task was to test the mechanical adhesion of porcine MSCs to medical-grade titanium alloy using a centrifuge to apply forces and track cell attachment with immunofluorescence. We completed a full analysis of cells from one animal.

Methods:

A centrifugation method was used to mechanically detach cells based on work from Reyes and Garcia (JBMR Part A, 2003) and Wu *et al.* (Biomaterials, 2012) and as previously detailed in a previous progress report. This method applies controlled and uniform detachment forces to a large population of adherent cells. In order to perform this, nine MSC populations from integumentary tissues 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") as detailed earlier and cultured on titanium disks. Orally-derived keratinocytes (Dickson, MCB, 2000), which are known to have high resistance to mechanical detachment, were used as controls. Disks were centrifuged at 350g (corresponding to a cell adhesion force of approximately 2 pN based on Wu *et al.*) and 500 (approximately 3pN). Control groups included "control-pre," which were disks that were not inserted into disk-holders, and "control-post," which were disks that were inserted into disk-holders but were not centrifuged.

Statistical Analysis:

Mean and standard deviation values are presented of the percentage of the mean number of cells of control-pre disks (n=3). Means within each cell population were compared with a one-way analysis of variance followed by Tukey's honest significant difference post hoc test [GraphPad Prism 8.1.2 (GraphPad Software, San Diego, CA, USA)]. Different letter labels (A, B, and C) indicate statistically significantly different groups. Inter-cell population differences between 350g and 500g were detected with a one-way analysis of variance with a Dunnett correction where the control mean (null) was set as keratinocytes. Green arrows label a statistically significant difference compared to keratinocytes. Statistical significance was determined with a nominal α equal to 0.05.

Results:

Results are shown in **Figure 11**. No statistically significant differences were found between control-pre and control-post suggesting that handling necessary to prepare disks for centrifugation did not affect cellular adhesion. All groups except keratinocytes and molar-derived MSCs (which decreased but not significantly) showed a significant decrease in percent cells adhered following centrifugation at 350g and 500g. Abdominal adipose and dermis MSCs showed a significant decrease in percent of cells adhered following 500g compared to 350g. All other groups except keratinocytes showed a non-significant decrease following 500g compared to 350g. There were no significant differences in MSC adhesion at 350g compared to keratinocytes. However, hindlimb adipose, molar, muscle, abdominal adipose, abdominal dermis, hoof, and tendon-derived MSCs showed a significantly smaller percent of cells adhered following 500g centrifugation than keratinocytes did.

Conclusion:

We successfully implemented a method to test the mechanical attachment of cells to medical-grade titanium. All cells showed suitability for attachment to medical-grade titanium but adhesion of hindlimb adipose, molar, muscle, abdominal adipose, abdominal dermis, hoof, and tendon-derived MSCs was lower than keratinocytes at 500g.

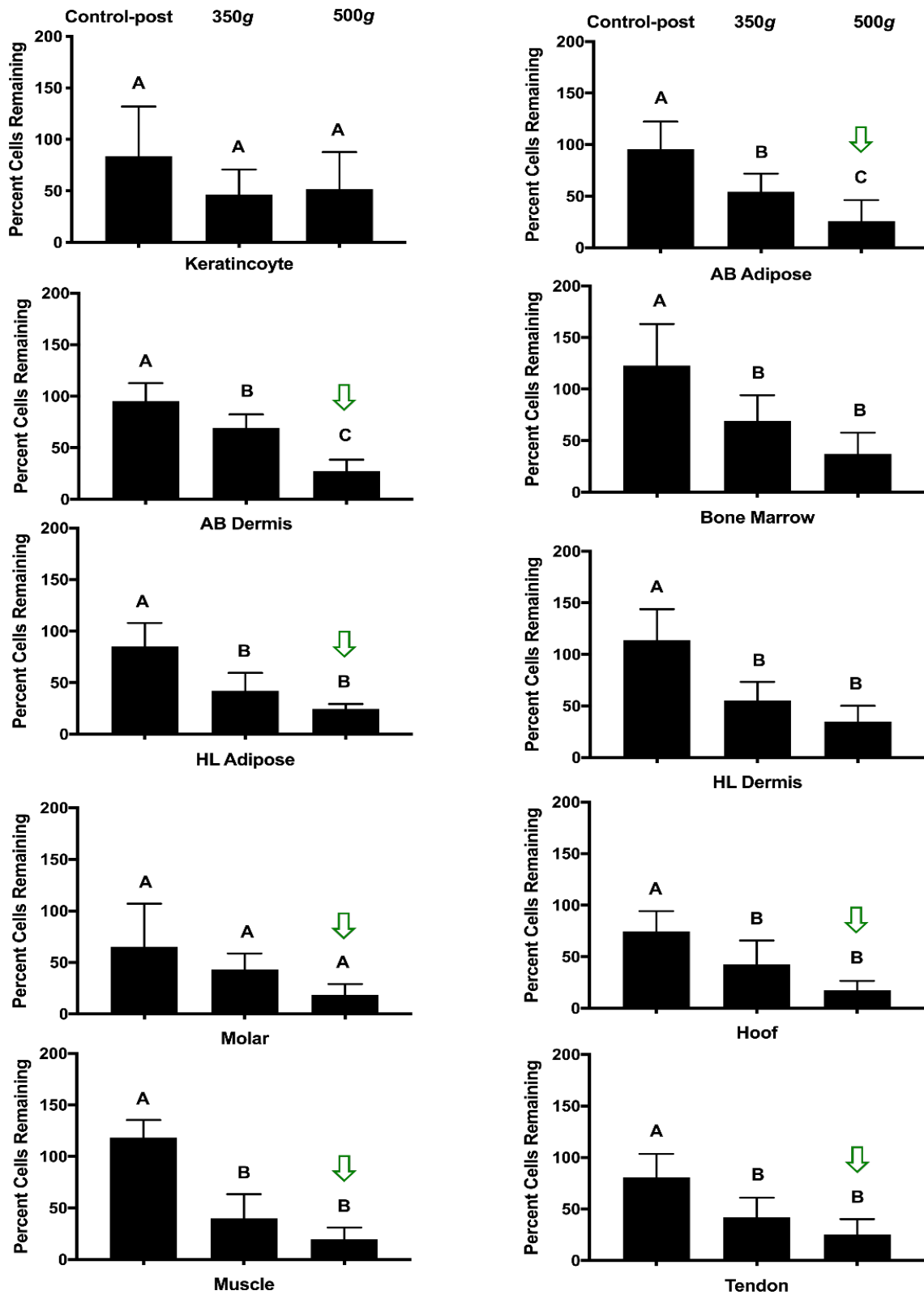


Figure 11: Percent of candidate MSCs and control keratinocytes remaining on titanium disks following centrifugation at 350g and 500g.

Subtask 2.2: Test human cells for adhesion to metal substrate.

The experiments for this task aims to assess the adhesion and proliferation of iPS derived MSCs at different passages along with relevant bone marrow derived MSCs as internal control. Following adhesion and proliferation, cells will be subjected to mechanical shear forces to assess post-force cell attachment. This study will further confirm the ideal passage that can then be used for future studies. This subtask has been initiated and the methods used are a combination of the methods used in Subtask 1.2 for cell culturing and Subtask 2.1 for assessment of cell adhesion in Year 1 and Year 2.

Major Task 3: Scaffold development for cell growth and anchorage to underlying tissue.

Subtask 3.1: Complete scaffold design (constructs) for “sleeve” and “transition designs.

We have selected functionalized gelatin to develop a new type of scaffold, as a potential alternative to the use of collagen membranes/scaffolds. Functionalized gelatin will enable the type of multifunctionality need for the ideal scaffolds: biocompatibility, easy to handle in the OR, guidance for cell adhesion and growth, and biodegradability. Specifically, methacryloyl-gelatin (GelMA) has been well recognized as a cell-carrier, which not only facilitates light-activated in-situ cross-linking but also supports cell proliferation and function. We are designing and developing different strategies to improve adhesion of cross-linked GelMA to both the metal substrate and to cells that form soft tissue. The next set of experiments will tests these materials for adhesion to substrates and cells of interest as well as for their ability to support biological function of encapsulated cells.

Subtask 3.2: Seed and grow porcine cells on flat collagen sheets, assemble scaffolds

RCF resorbable collagen foam (ACESurgicals) have been selected as an ideal candidate for this study, following previous experience of veterinarian surgeons in the project for their proper handling characteristics and good in vivo outcomes. The next experimental step will include comparing adhesion and proliferation of selected pMSC, based on results of Subtasks 1.1 and 2.1a. This will be the focus for the next quarter.

Major Task 4:

We have submitted a review article on the general topic of ‘Osseointegration’ to the Journal of Orthopedic Research. First round of revisions have been submitted to the journal.

We have also started the assembly of a research manuscript on the comparative profiling of nine porcine tissue-derived MSCs with results from Subtasks 1.1 and 2.1a.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

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

- Nick Fisher, Conrado Aparicio and Joan Bechtold (UMN) and Devaveena Dey (USUHS): Attended 2019 Orthopedic Research Society meeting held in Austin, Texas (February 2-5, 2019)
- Isha Mutreja and Joan Bechtold (UMN): Attended International Combined Orthopedic Research Society (ICORS) meeting held in Montreal, Canada (June 19-22, 2019)
- Devaveena Dey (USUHS): Attended the ‘Military Health System Research Symposium’ (MHSRS) held in Kissimmee, Florida (Aug 18-22, 2019)
- Isha Mutreja and Nick Fischer (UMN): Attended 2019 Institute for Engineering in Medicine Annual Retreat at the University of Minnesota, Minneapolis, MN on September 23, 2018.
- Conrado Aparicio and Joan Bechtold (UMN): Attended DoD Osseointegration Annual Meeting in San Diego, CA (September 24, 2019)
- David Danielson (2LT, USA, MS), a second year medical student at USUHS is currently involved in studies of comparative expression profiling of MSC and adhesion markers in the porcine cells derived from multiple integumentary and connective tissues. This grant serves as a training opportunity for medical students in the Department of Surgery at USUHS.

How were the results disseminated to communities of interest?

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

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

Nothing to Report

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

If this is the final report, state “Nothing to Report.”

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

USUHS

- Repetition of the multilineage differentiation experiments with cells from two additional animals.
- Completion of gene expression analysis of chondrogenic differentiation markers across the cell pellets collected.
- Analysis of adhesion markers across the nine tissues harvested at a later passage (P5).
- Increasing biobanked sample size for hind limb dermis and molar-derived periodontal ligament tissue.

MMRF-UMN

- Optimize the protocol for MSC and keratinocyte differentiation of iPS cells, which will then be confirmed by gene and/or protein expression, and immunofluorescence.
- Assess the adhesion and proliferation of iPS-derived MSCs and keratinocytes on titanium alloy and glass substrates.
- Develop a GelMA-based biocompatible scaffold with enhanced adhesion to titanium and soft tissue (both porcine derived cells and iPS derived MSCs and/or keratinocytes).
- Assess the cell seeding culture conditions and its impact on the cell proliferation and differentiation potential of cells in collagen and a new GelMA-based scaffold using iPS derived MSCs and/or chondrocytes
- Select the best-of-breed scaffold and MSC cell type and culturing conditions for *in vivo* animal assessment.

Both sites

- Complete an in-progress manuscript describing the phenotype, differentiation potential and metal adherence capacity of tissue specific derived MSCs from nine defined tissue types.

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

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

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

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

Nothing to Report

What was the impact on other disciplines?

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

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

Nothing to Report

What was the impact on technology transfer?

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

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

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

Nothing to Report

What was the impact on society beyond science and technology?

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

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

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

Nothing to Report

5.CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change

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

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

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

Nothing to Report

Changes that had a significant impact on expenditures

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

Nothing to Report

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

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

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Significant changes in use of biohazards and/or select agents

Not applicable

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

•**Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

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

Nothing to Report

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

Nothing to Report

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

- Isha Mutreja, Kirk Twaroski, Joan E Bechtold, Jakub Tolar, Jonathan A. Forsberg, Conrado Aparicio. *Human iMSC-laden hydrogel scaffold for improving the durability of skin/implant interface*. 2020 Orthopedic Research Society Annual Meeting. Phoenix, AZ. Submitted.
- Isha Mutreja, Devaveena Dey, Kirk Twaroski, Mehdi Belkhodja, Joan E. Bechtold, Jakub Tolar, Jonathan A. Forsberg, Thomas A. Davis, Conrado Aparicio. *Stem cell sources for regenerative strategies to improve durability of skin/implant interfaces*. 2019 International Combined Orthopedic Research Society (ICORS) meeting. Montreal, Canada. (Poster presentation).
- Nicholas G Fischer, Devaveena Dey, Mehdi Belkhodja, Thomas A. Davis, Joan E. Bechtold, Jonathan A. Forsberg, Conrado Aparicio. *In vitro adhesion of porcine mesenchymal stem cells to percutaneous osseointegrated prosthesis for improved skin/implant therapies*. 2019 Orthopedic Research Society Annual Meeting. Austin, TX. (Poster presentation).

- Devaveena Dey, Mehdi Belkhodja, Jonathan A. Forsberg, Thomas A. Davis. *In vitro Characterization of Osteogenic Differentiation Potential of Porcine Mesenchymal Stem Cells Derived from Multiple Integumentary Tissues for Improved Durability of the Skin/Implant Interface*. 2019 Orthopedic Research Society Annual Meeting. Austin, TX (Poster presentation).
- Devaveena Dey, Mehdi Belkhodja, Alisha Rhodes, David Danielson, Jonathan A. Forsberg, Thomas A. Davis. *In vitro Characterization of Osteogenic Differentiation Potential of Porcine Mesenchymal Stem Cells Derived from Multiple Integumentary Tissues for Improved Durability of the Skin/Implant Interface*. 2019 Military Health System Research Symposium, Kissimmee, FL (Poster presentation).

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

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

Nothing to Report

•**Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

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

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

•**Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- data or databases;
- physical collections;
- audio or video products;
- software;
- models;

- educational aids or curricula;
- instruments or equipment;
- research material (e.g., Germplasm; cell lines, DNA probes, animal models);
- clinical interventions;
- new business creation; and
- other.

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

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

Name: Jonathan A Forsberg, MD, PhD.

Project Role: Principal Investigator

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 3

Contribution to Project: CDR Forsberg responsible for overall project coordination.

Name: Joanie Bechtold, PhD

Project Role: Site PI (MMRF)

Researcher Identifier (e.g. ORCID ID): 0000-0002-7090-4270

Nearest person month worked: 3

Contribution to Project: Responsible for overall project coordination at subaward site(s).

Name: Thomas A. Davis, PhD

Project Role: Associate Investigator (USUHS)

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 3

Contribution to Project: Oversight of project conducted at USUHS.

Name: Dan Kaufman, MD, PhD

Project Role: Associate Investigator (UCSD)

Researcher Identifier (e.g. ORCID ID): 0000-0002-2003-2494

Nearest person month worked: 1

Contribution to Project: Analysis of iPSC in vitro studies conducted at UMN.

Name: Conrado Aparicio, PhD.
Project Role: Associate Investigator (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0003-2969-6067
Nearest person month worked: 3
Contribution to Project: Oversight of iPSC in vitro studies and cell adhesion studies conducted at UMN.

Name: Devaveena Dey, PhD
Project Role: Associate Investigator (USUHS)
Researcher Identifier (e.g. ORCID ID): 0000-0002-0507-5701
Nearest person month worked: 3
Contribution to Project: Oversight of in vitro porcine cells studies conducted at USUHS.

Name: Isha Mutreja, PhD
Project Role: Postdoctoral Associate (UMN)
Researcher Identifier (e.g. ORCID ID): 0000-0002-8998-7563
Nearest person month worked: 3
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: 2
Contribution to Project: Performance of porcine cell adhesion experiments conducted at UMN.

Name: Alisha Rhodes, B.S.
Project Role: Research Associate (USUHS)
Researcher Identifier (e.g. ORCID ID): 0000-0002-6139-0036
Nearest person month worked: 1
Contribution to Project: Extraction of porcine molar tissues 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: 1
Contribution to Project: Performance of porcine cell based experiments conducted at USUHS.

Name: Cole Homer
Project Role: Undergraduate Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Performance of porcine cell adhesion experiments conducted at UMN.

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

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

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

Nothing to report

What other organizations were involved as partners?

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

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

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

Organization Name: Stem Cell Institute – Professor Jakub Tolar’s lab

Location of Organization: University of Minnesota, Minneapolis, MN

Partner’s contribution to the project: Facilities, collaboration, personnel exchange (Isha Mutreja-Aparicio’s lab, Kirk Twaroski-Tolar’s lab).

8.SPECIAL REPORTING REQUIREMENTS

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

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

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



Optimizing Skin-Implant Interface of Osseointegrated Device,

W81XWH-17-2-0059; Log# BA150755

PI: Forsberg, Jonathan

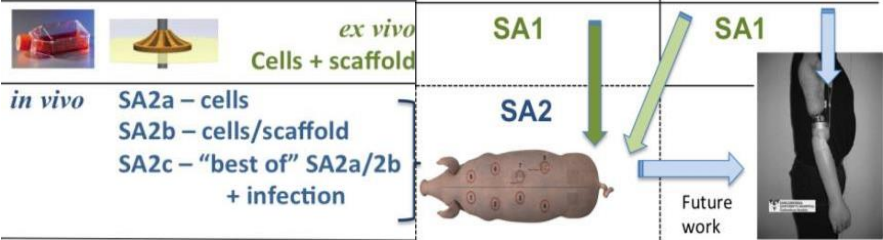
Org: Henry M Jackson Foundation/ USU

Award Amount: \$2,499,220

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

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

Specific Aims to evaluate durability of skin interface



Accomplishments: We have completed the isolation, ex vivo expansion, biobanking and functional characterization of swine tissue-derived stem/progenitor cells from healthy swine tissues (bone marrow, adipose, muscle, Achilles tendon, nail/hof bed, gingival and periodontal ligament) and have successfully differentiated and characterized human iPSC cells to MSCs. We have completed adhesion studies of the porcine cells to titanium.

Timeline and Cost

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

Goals/Milestones

Y1 Goals

- ✓ Establish Subaward with MMRF.....**COMPLETE**
- ✓ Evaluate lineage differentiation and metal adhesiveness of swine MSCs and human iPSCs.....**IN PROGRESS.**
- ✓ Characterize cell system..... **COMPLETE.**

Y2-3 Goals

- ✓ Scaffold development and adhesion testing....**IN PROGRESS.**
- ✓ Optimum scaffold/cell combination determined....**IN PROGRESS**

Y4 Goals

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

Y5 Goals

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

In vitro Adhesion of Porcine Mesenchymal Stem Cells to Percutaneous Osseointegrated Prostheses for Improved Skin/Implant Therapies

Nicholas G. Fischer¹, Devaveena Dey², Mehdi Belkhdja², Thomas A. Davis², Joan E. Bechtold^{1,3}, Jonathan A. Forsberg², Conrado Aparicio¹

¹University of Minnesota, Minneapolis, MN, ²Uniformed Services University of the Health Sciences - Walter Reed National Military Medical Center, Bethesda, MD, and ³Hennepin County Medical Center, Minneapolis, MN

Email of Presenting Author: NGF, Fisc0456@umn.edu

Disclosures: Nicholas G. Fischer (N), Devaveena Dey (N), Mehdi Belkhdja (N), Thomas A. Davis (N), Joan E. Bechtold (3C-SI Bone, 5-Medtronic, 7B and 8-JBJS, 9-ASB), Jonathan A. Forsberg (3B, 3C, and 5) and Conrado Aparicio (5- 3M Oral Care and Mozo Grau Dental Implants)

INTRODUCTION: The overall goal of this work is to develop therapies that induce durable skin/implant interface healing for osseointegrated (OI) prostheses. Transhumeral OI prostheses have a 5 year post-implantation 38% infection rate and 17% failure rate.¹ This problem is not limited to orthopedics but extends rather to any percutaneous device, such as catheters or dental implants for which infection rates are similar. A key contributor to this persistent problem is a lack of durable mechanical sealing between the skin and implant surface, where the skin ideally serves as a barrier against the external environment. Currently, we are developing therapies that will deliver porcine-derived mesenchymal stem cells (MSCs) to the implant site to promote skin attachment. Here, we aimed to identify porcine-derived MSCs isolated from multiple tissues with robust adhesion to titanium alloy. The best candidate cells will be used in a tissue-engineered construct to induce strong and durable skin/implant interfacial sealing.

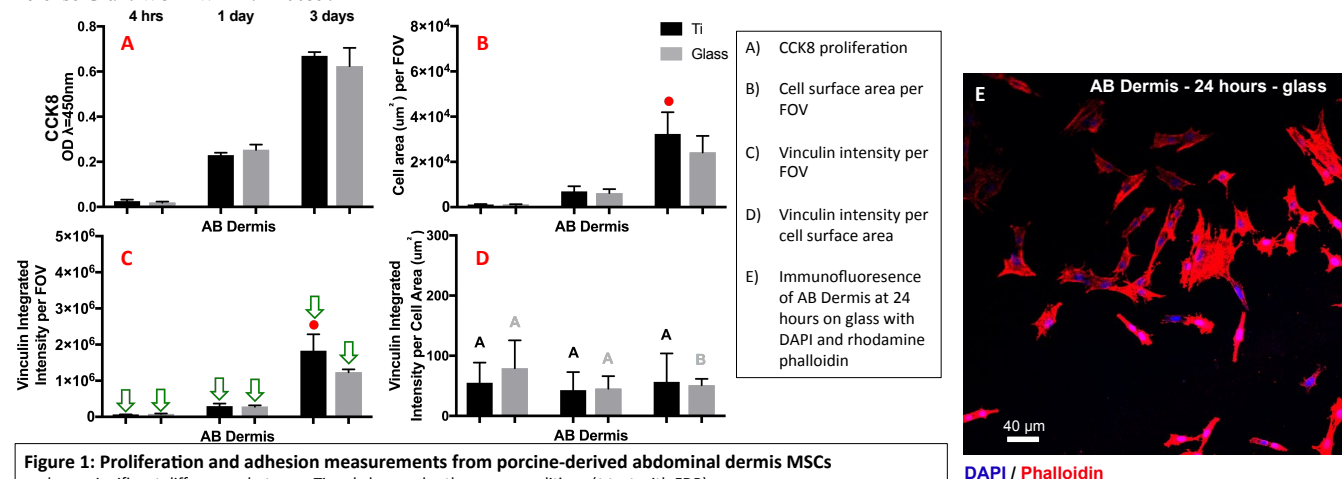
METHODS: Porcine MSCs were isolated from female Gottingen minipigs under the USUHS Surgery and Veterinary Medicine tissue sharing protocol (LAM-17-540 entitled "Education and Training Protocol for Techniques in Animal Care and Use"). Standard mechanical and enzymatic digestion protocols were used to isolate MSCs from nine different tissues; namely bone marrow, muscle, abdominal adipose, hind limb dermis, hind limb adipose, tendon, hoof, abdominal dermis, and molar-associated periodontal ligament. Mouse fibroblast (NIH-3T3, ATCC) cells were used as a gold standard due to extensive knowledge of their adhesion response to metallic devices. Passage 2 MSCs were evaluated for their tri-lineage differentiation potential to osteocytes, chondrocytes, and adipocytes. Ti-6Al-4V extra low interstitial ($\sigma=7\text{mm}$, President Titanium) disks were polished to a 20-60 nm colloidal silica finish. Glass ($\sigma=7\text{mm}$, Harvard Apparatus) was used as a positive control substrate. Cells were seeded on titanium and glass disks at 1,000 cells/disk ($n=17$ disks per cell for both titanium and glass) for 4, 24, and 72 hours. At each time point, proliferation was measured with Cell Counting Kit 8 (CCK8, Dojindo Laboratories; $n=7$). Absorbance was read on a plate reader (450 nm, Synergy) and expressed as optical density. Additionally, cell spreading and focal adhesions characteristics were determined with immunofluorescence. Seeded disks ($n=10$; both Ti and glass) were washed in phosphate buffered saline (PBS) to remove weakly adhered cells and fixed in 4% paraformaldehyde. Then, adhered cells were permeabilized with Triton X-100 in PBS and blocked in 5% bovine serum albumin (BSA). After PBS washing, cells were stained with an anti-vinculin antibody (MilliPore Sigma) overnight. Following washing, secondary antibody (Cy5, Abcam) was added for 1 hour. Seeded disks were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 300 nM in PBS) for 10 minutes. Following extensive washing, the seeded disks were mounted with ProLong (Fisher) and imaged (Olympus FV1000 - 40X). Other disks ($n=5$) were stained with rhodamine-phalloidin (Fisher) and processed similarly with DAPI and mounted. ImageJ (NIH) was used for image analysis with 5 fields of views (FOVs) per sample. Proliferation (number of DAPI-positive nuclei), cell surface area per field of view, average individual cell surface area, focal adhesion integrated intensity per FOV, average focal adhesion integrated intensity per cell, and average focal adhesion integrated intensity per cell surface area (FAPSA) were calculated. A t -test with False Discovery Rate (FDR) correction, an analysis of variance (ANOVA) with Dunnett's multiple comparison (against NIH-3T3 cells), and ANOVA with a Tukey's post hoc analysis were used for statistical analysis (GraphPad Prism 7.0d). A threshold value of $p = 0.05$ was chosen. Values are presented as mean $\pm$ standard deviation.

RESULTS: Proliferation (both CCK8 and DAPI counting), cell surface area per FOV, and average individual cell surface area results showed a significant increase through time (from 4 hours to 72 hours) for all cell types, with no significant differences between fibroblasts and each of the MSCs. Although MSCs showed significantly lower focal adhesion intensity than fibroblasts at 4 and 24 hours, no differences were seen at 72 hours. FAPSA results showed no significant differences between each MSC and fibroblasts. There were no notable differences in cellular behavior between titanium and glass. Differences in focal adhesion and cell surface area among MSCs were minimal, but proliferation of MSCs from hindlimb dermis, hood, and abdominal dermis was higher than other tissue-derived MSCs. Proliferation of muscle-derived MSCs was the lowest among all MSCs. Representative results from abdominal dermis-derived MSCs are shown below in **Figure 1**.

SIGNIFICANCE: Understanding intrinsic differences in MSC adhesion allows for the best candidate MSCs to be further studied to enhance durability at the skin/material interfaces in percutaneous OI devices. This may contribute to longer lifespan of OI devices and improved patient outcomes. Preliminary studies indicate minimal differences in adhesion and proliferation of MSCs derived from the different porcine tissues on medical-grade titanium alloy. Follow up differentiation and adhesion gene expression studies on titanium alloy will be needed to determine the optimum tissue-derived MSC for improving the skin/implant interface of OI.

REFERENCE: Tsikandylakis *et al. Clin. Orthop. Relat. Res.* **2014**, 472 (10), 2947–2956 DOI: 10.1007/s11999-014-3695-6.

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Stem cell sources for regenerative strategies to improve durability of skin/implant interfaces

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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 implants¹. These failures occur due to absence of an impervious seal at the skin-percutaneous implant interface. Utilizing cells derived from naturally occurring integumentary tissues, which form impervious seal at hard-soft tissue junctions is a promising approach to address the complications associated with transdermal implants. Our goal is to use porcine mesenchymal stem cells (pMSCs) derived from multiple integumentary tissues and/or MSCs differentiated from human induced pluripotent stem cells (iPSCs) for engineering a cell-based scaffold that enables formation of a tight seal of skin around the implant. Here, we carried out a comparative assessment of downstream osteogenic differentiation of pMSCs from multiple porcine integumentary tissues and human iPSCs.

Methods: pMSCs were isolated from female Gottingen minipigs under the USUHS and Veterinary Medicine tissue sharing protocol (LAM-17-540 entitled "Education and training Protocol for Techniques in Animal Care and Use"). pMSCs from different tissue sources were isolated using standard mechanical and enzymatic digestion and isolated cells were maintained in DMEM-F12 media supplemented with 10% fetal bovine serum (FBS). Fully characterized human iPSCs (IRB exempt) were maintained on Geltrex-coated tissue culture plastic in complete Essential E8 medium (Gibco).² To differentiate iPSCs into MSCs, media was switched to complete differentiation media (CDM) consisting of MEM supplemented with 5% FBS, 5% horse serum (HS), 10 ng/ml of EGF, bFGF and PDGF-AB once cells were 50% confluent. Cells were cultured in CDM for two weeks, followed by passage after which they were maintained in CDM. To confirm cell phenotype, flow cytometry was performed on cells at passage 5. For assessing osteogenic differentiation capability of different MSCs, cells were cultured in osteogenic differentiation media for 21 days. Alkaline phosphatase activity (ALP) was measured at day 7, and terminal osteogenic differentiation was determined by measuring Ca²⁺ deposition after 3 weeks using Alizarin Red S staining.

Results: pMSCs derived from different tissue sources expanded successfully. pMSCs derived from molar associated periodontal ligament showed the highest expression of ALP and calcium deposition compared to cells derived from other porcine tissues. Uniform cultures of hMSCs derived from human iPSCs were obtained between passages 5-10 and flow cytometric analysis

demonstrated the expression of MSC markers CD73 (99.5%), CD90 (94.2%) and CD105 (97.4%). iPSCs induced hMSC demonstrated high a mineralization capacity upon osteogenic induction by day 21.

Conclusion: Preliminary studies indicate that mesenchymal stem cells derived from naturally occurring integumentary tissues have promising application in creating a durable, impervious skin-implant interface, in part via their osteogenic differentiation potential. Follow up analysis of gene expression of these cells is currently underway to confirm the differences observed in their differentiation profile. Human iPSC derived MSCs are an alternative, promising cell type for this application as well, based on their robust osteogenic differentiation. Together these findings suggest that human iPSCs, vis-à-vis the insights from the porcine system, may offer therapeutic value in assessing cell based therapy(ies) required to support a durable and impervious coating on transdermal implants.

Acknowledgement: This study was supported by the DoD Grant titled “Optimizing Skin-Implant Interface of Osseointegrated Device” (W81XWH-17-2-0059).

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References

1. Atallah, R.; Leijendekkers, R. A.; Hoogeboom, T. J.; Frölke, J. P., Complications of bone-anchored prostheses for individuals with an extremity amputation: A systematic review. *PloS one* **2018**, *13* (8), e0201821, doi:
2. Rousseaux, M. W.; Tschumperlin, T.; Lu, H.-C.; Lackey, E. P.; Bondar, V. V.; Wan, Y.-W.; Tan, Q.; Adamski, C. J.; Friedrich, J.; Twaroski, K., ATXN1-CIC complex is the primary driver of cerebellar pathology in spinocerebellar ataxia type 1 through a gain-of-function mechanism. *Neuron* **2018**, *97* (6), 1235-1243. e5, doi:

In vitro Characterization of Osteogenic Differentiation Potential of Porcine Mesenchymal Stem Cells Derived from Multiple Integumentary Tissues for Improved Durability of the Skin/Implant Interface

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INTRODUCTION: This study explores the possibility of creating a tight, durable skin-implant interface for osseointegrated (OI) implants using MSCs derived from naturally occurring integumentary tissues, which have the intrinsic potential to form an impervious seal at hard and soft tissue junctions. Implant and soft tissue infections (29-38%) and implant loosening (13-29%) are common complications for both upper and lower extremity bone-anchored implants [1], 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. We hypothesize that identifying and characterizing naturally occurring cells of the integumentary system, present at the junction of hard and soft tissues, such as dermis, nails or hoof, periodontal ligament etc. could enable us to engineer durable and impervious cell-based scaffolds for placement at the skin-implant interface. We report here the isolation of porcine mesenchymal stem cells from multiple integumentary and connective tissues, namely dermis, hoof, periodontal ligament, Achilles tendon, adipose tissue, muscle and bone marrow, followed by comparative characterization of osteogenic potential of these cell types. The cell type with optimum osteogenic differentiation potential could be expected to form a durable mineralized matrix at the skin-implant interface, thereby forming a tight seal.

METHODS: Porcine MSCs were isolated from female Gottingen, Yucatan or Yorkshire minipigs under our institution's Surgery and Veterinary Medicine tissue sharing protocol (LAM-17-540 entitled "Education and Training Protocol for Techniques in Animal Care and Use"). All tissues were collected in sterile collection media (1:1 DMEM-F12, containing 2% FBS and 2X antibiotics; namely penicillin, streptomycin and fungizone). Bone marrow (BM) cells were isolated after multiple washes in antibiotic-containing PBS and post RBC lysis. For all other tissues, standard mechanical and enzymatic digestion protocols were followed. The nine different tissues from which MSCs were derived include bone marrow, muscle, abdominal and hind limb adipose, abdominal and hind limb dermis, tendon, hoof, and molar-associated periodontal ligament. Post digestion, cells were seeded in growth media (GM), comprising of 1:1 DMEM-F12, containing 10% FBS and 2X antibiotics. Osteogenic differentiation was induced by replacing growth media with osteogenic media (OM; StemPro Osteogenesis kit) 24 hours post seeding. Passage 2 cells were used for all experiments. Early osteogenic differentiation was assessed by pNpp based alkaline phosphatase activity assay (Sigma Aldrich) after 1 week of osteogenic differentiation. Briefly, cells were lysed in 50 µl of 1% Triton X-100 for 2-3 minutes. Equal volume of pNpp substrate was added, and absorbance was recorded immediately at 405 nm in kinetic mode for 20 cycles (Tecan). Terminal osteogenic differentiation was assessed by measuring Ca²⁺ deposition after 3 weeks in differentiation media. Briefly, at the end of 3 weeks cells were fixed in 0.5% glutaraldehyde and stained with 2% alizarin red solution for 20-30 minutes. Quantification of alizarin red staining was done in 10% formic acid, and absorbance read at 414 nm. Both early and terminal osteogenic differentiation was carried out in a 96-well plate, with 100 cells/well. Cells were harvested after 5 days of osteogenic differentiation for osteogenic gene expression studies. Trizol was used for homogenization, followed by RNA isolation (Qiagen mRNAeasy kit), cDNA synthesis (ABI) and Syber Green (Biorad) based quantitative Real-Time PCR run on Quant-Studio.

RESULTS: All cell types expanded successfully, and a cell bank of all the tissues collected from 4-6 animals has been frozen. Assessment of early osteogenic differentiation demonstrated significantly higher osteogenic potential in molar-associated periodontal ligament tissue among all tissues, followed by hind limb dermis (**Fig. 1A**). After 3 weeks of differentiation, molar ligament derived cells was once again the cell type with highest calcium deposition among the five tissues tested, as measured by alizarin red staining (**Fig. 1B**). Expression levels of three osteogenic genes, *Runx2*, *Osteopontin* and *Osterix* was assessed in four tissues; namely abdominal adipose, dermis, bone marrow and muscle. Out of these, abdominal dermis showed the highest fold change in expression of both *Runx2* (6.25-fold; **Fig. 1C**), a key transcription factor in osteoblast differentiation and the secreted phosphoprotein 1, *Osteopontin* (>50-fold; **Fig. 1D**), which is known to be important for attachment of osteoclasts to mineralized bone matrix. There was no increase in expression of *Osterix* in any of these tissues with respect to their undifferentiated controls (data not shown). Analysis of these genes in the other tissues, and analysis of additional osteogenic genes (*BSP*, *Osteocalcin*) is currently underway.

SIGNIFICANCE: Understanding the cellular and molecular mechanisms of cells within naturally occurring tissues at the junction of hard structures and soft tissues will provide critical insights on the engineering of a durable, tight seal at the skin-implant interface of OI devices. Our preliminary studies indicate superior mineralizing/osteogenic potential of periodontal ligament and dermal tissues. Further differentiation and gene expression studies are underway to confirm these results and to dissect the mechanism underlying this difference.

REFERENCES

1. Atallah, R., et al., *Complications of bone-anchored prostheses for individuals with an extremity amputation: A systematic review.* PLoS One, 2018. 13(8): p. e0201821.

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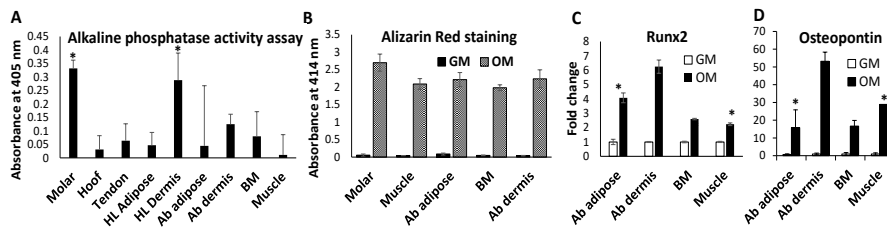


Figure 1. Characterization of osteogenic differentiation potential of porcine integumentary and connective tissues. **A.** Quantification of alkaline phosphatase activity measured after culturing cells in osteogenic differentiation media for 1 week. **B.** Quantification of alizarin red staining of cells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

of **C. Runx2** and **D. Osteopontin** genes in cells cultured in OM with respect to levels in GM. Significance was analyzed using Student's t-test; *p<0.05.

Title: In vitro Characterization of Osteogenic Differentiation Potential of Porcine Mesenchymal Stem Cells Derived from Multiple Integumentary Tissues for Improved Durability of the Skin/Implant Interface

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INTRODUCTION: This study explores the possibility of creating a tight, durable skin-implant interface for osseointegrated (OI) implants using MSCs derived from naturally occurring integumentary tissues, which have the intrinsic potential to form an impervious seal at hard and soft tissue junctions. 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, resulting in exposure of soft tissue and vasculature, thereby increasing chances of infection as well as implant loosening. We hypothesize that identifying and characterizing naturally occurring cells of the integumentary system, present at the junction of hard and soft tissues, such as dermis, nails or hoof, adipose tissues, periodontal ligament etc. could enable us to engineer durable and impervious cell-based scaffolds for placement at the skin-implant interface. We report here the isolation of porcine mesenchymal stem cells from multiple integumentary and connective tissues, namely dermis, hoof, periodontal ligament, Achilles tendon, adipose tissue, muscle and bone marrow, followed by comparative characterization of osteogenic potential of these cell types. The cell type with optimum osteogenic differentiation potential could be expected to form a durable cellular and extracellular interface matrix at the skin-implant interface, thereby forming a tight seal.

METHODS: Porcine MSCs were isolated from female Gottingen, Yucatan or Yorkshire miniature pigs under our institution's 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 media (1:1 DMEM-F12, containing 2% FBS and 2X antibiotics; namely penicillin, streptomycin and fungizone). Unfractionated bone marrow (BM) cells were isolated after multiple washes in antibiotic-containing PBS and post RBC lysis. Standard mechanical and enzymatic digestion protocols were followed for all other tissues. Tissue-MSCs (n = 4-6 animals) were isolated from skeletal muscle, abdominal and hind limb adipose tissue, abdominal and hind limb dermis, Achilles tendon, hoof-associated superficial flexor tendon, and molar-associated periodontal ligament using tissue-specific mechanical and enzymatic digestion protocols. Post digestion, cells were seeded in growth media (GM), comprising of 1:1 DMEM-F12, containing 10% FBS and 2X antibiotics. Osteogenic differentiation was induced by replacing growth media with osteogenic media (OM; StemPro Osteogenesis kit) 24 hours post seeding. Passage 2 cells were used for all experiments. Early osteogenic differentiation was assessed by pNpp based alkaline phosphatase activity assay (Sigma Aldrich) after 1 week of osteogenic differentiation. Briefly, cells were washed with PBS, followed by lysis in 50 μ l of 1% Triton X-100 for 2-3 minutes. Equal volume of pNpp substrate was added, and absorbance was recorded immediately at 405 nm in kinetic mode for 20 cycles (Tecan). Terminal osteogenic differentiation was assessed by measuring Ca^{2+} deposition after 3 weeks in differentiation media. Briefly, at the end of 3 weeks cells were washed in PBS, fixed in 0.5% glutaraldehyde and stained with 2% alizarin red solution for 20-30 minutes. Quantification of alizarin red staining was done in 10% formic acid, and absorbance read at 414 nm. Both early and terminal osteogenic differentiation was carried out in a 96-well plate, with 100 cells/well. Cells were harvested after 5 days of osteogenic differentiation for osteogenic gene expression studies. Trizol was used for homogenization, followed by RNA isolation (Qiagen mRNAeasy kit), cDNA synthesis (ABI) and Syber Green (Biorad) based quantitative Real-Time PCR run on Quant-Studio.

RESULTS: Assessment of early osteogenic differentiation demonstrated significantly higher osteogenic potential in molar-associated periodontal ligament tissue among all tissues, followed by hind limb dermis. After 3 weeks of differentiation, molar ligament derived cells had demonstrated the highest calcium phosphate deposition (mineralization), as measured by alizarin red staining. Transcript expression levels of three osteogenic genes, *Runx2*, *Osteopontin* and *Osterix* was assessed in four tissues; namely abdominal adipose, dermis, bone marrow and muscle. Out of these, abdominal dermis showed the highest fold change (6.25-fold) in expression of both *Runx2*, a key transcription factor in osteoblast differentiation and the secreted phosphoprotein 1, *Osteopontin* (>50-fold), which is known to be important for attachment of osteoclasts to mineralized bone matrix. There was no increase in expression of *Osterix* in any of these tissues with respect to their undifferentiated controls. Analysis of these genes in the other tissues, and analysis of additional osteogenic genes, such as (*BSP*, *Osteocalcin*) is currently underway.

Significance: Understanding the cellular and molecular mechanisms of cells within naturally occurring tissues at the junction of hard structures and soft tissues will provide critical insights on the engineering of a durable, tight seal at the skin-implant interface of OI devices. Our preliminary studies indicate superior mineralizing/osteogenic potential of periodontal ligament and dermal tissues. Further differentiation and gene expression studies are underway to confirm these results and to dissect the mechanism underlying this difference.

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Orthopaedic Osseointegration: Implantology and Future Directions

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Keywords:	Gait, Rehabilitation, Trauma, Reconstruction, Osteoblasts
Areas of Expertise:	Amputation, Osseointegration, Military



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Orthopaedic Osseointegration: Implantology and Future Directions

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Keywords: Osseointegration, Amputation, Trauma, Military

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27 **Author Contributions Statement**

28 **Archie Overmann:** primary author, contributed to project conceptualization, writing the original
29 draft, review, and editing of the final manuscript

30 **Conrado Aparicio:** contributed to original draft, figure creation, editing the revised manuscript.

31 **John Richards:** contributed to original draft, figure creation, review and editing of the final
32 manuscript.

33 **Isha Mutreja:** contributed to original draft and figure creation.

34 **Nicholas Fischer:** contributed to original draft and figure creation.

35 **Sean Wade:** contributed to original draft.

36 **Benjamin Kyle Potter:** contributed to project supervision, original draft creation, revision and
37 editing of the final manuscript, and project conceptualization

38 **Thomas Davis:** contributed to project conceptualization, supervision, editing and revising the
39 final manuscript.

40 **Joan Bechtold:** contributed to supervision, revision and editing of the final manuscript.

41 **Jonathan Forsberg:** contributed to project conceptualization, supervision, original manuscript
42 draft, review and editing the final manuscript, contributed clinical photographs and created
43 figures.

44 **Devaveena Dey:** Senior author, contributed to project conceptualization, supervision, original
45 draft, review and editing of the final manuscript.

46 **All Authors have read and approved the final submitted manuscript.**

47

48

Abstract

Osseointegration (OI) is the direct anchorage of a metal implant into bone, allowing for the connection of an external prosthesis to the skeleton. Osseointegration was first discovered in the 1960's based on the microscopic analysis of titanium implant placed into host bone. New bone was observed to attach directly to the metal surface. Following clinical investigations into dentistry applications, OI was adapted to treat extremity amputations. These bone anchored implants, which penetrate the skin and soft tissues, eliminate many of the challenges of conventional prosthetic sockets, such as poor fit and suspension, skin breakdown and pain. Osseointegrated implants show promise to improve prosthesis use, pain, and function for amputees.

The successful process of transcutaneous metal integration into host bone requires three synergistic systems: the host bone, the metal implant, and the skin-implant interface. All three systems must be optimized for successful incorporation and longevity of the implant. Osseointegration begins during surgical implantation of the metal components through a complex interplay of cellular mechanisms. While implants can vary in design – including the original screw, press fit implants, and compressive osseointegration - they face common challenges to successful integration and maintenance of fixation within the host bone. Overcoming these challenges requires the understanding of the complex interactions between each element of OI. This review outlines 1) the basic components of OI, 2) the science behind both the bone-implant and the skin-implant interfaces, 3) the current challenges of OI, and 4) future opportunities within the field.

71 **Introduction**

72 Osseointegration (OI) refers to the direct integration of bone to metal, resulting in
73 structural and functional integration between the living bone and the surface of the implant.¹
74 Per-Ingvar Brånemark first coined the term “OI” in the early 1950’s. He performed macroscopic
75 and microscopic analysis of the rabbit bone marrow and cortex following titanium screw
76 placement. Brånemark noted that compact cortical bone grew directly onto the implant, without
77 any intervening soft tissue, following a period of immobilization.¹ Microscopically, the bone
78 tissue grew into very thin spaces in the titanium. Since then, OI and its applications have been
79 extensively studied in both preclinical and clinical settings.

80 One of the most notable applications of OI is in amputee care. Prosthesis use for the
81 treatment of limb amputation has been documented throughout medical history.² For centuries,
82 prosthesis attachment has been socket-based and prone to soft tissue pain, skin irritation and
83 ulcerations, as well as difficult fit and suspension.¹ While the material, dimensions, and socket
84 structure have undergone significant refinements, the principles guiding socket design have
85 remained largely unchanged for over a century.^{4,5} For this reason, and despite significant
86 advances in material science, the constant friction between the ‘socket’ and skin of residual limb
87 remains the most significant barrier to functional mobility and quality of life.^{2 3}

88 Osseointegration-based prostheses overcomes the challenges of traditional sockets by
89 eliminating the constant friction between the device and skin, as well as difficulties related to fit
90 and suspension. Additionally, direct bone anchorage enables physiological weight bearing,
91 increased flexibility and control over range of motion, through mechanoreception-based sensory
92 feedback (AKA osseoproprioception or osseoperception).⁴ Osseointegration holds promise for
93 improving the lives of amputees and their family members. This review outlines 1) the basic

components of OI, 2) the science behind both the bone-implant and the skin-implant interfaces, 3) the current challenges of OI, and 4) future opportunities within the field.

The Elements of OI for Limb Prosthesis

The Implant

In general, OI implants consist of a fixture or stem that is implanted into the residual bone and an abutment that percutaneously connects the external prosthesis to the fixture (Figure 1). Long term endosseous implant stability directly depends on implant design.^{5 6} The key design features that alter load-bearing conditions at the endosteal interface include the length, diameter and the body/thread shape or porous coating. Notably, the relationship between implant design and long term stability also depends on the quality of the bone.⁷ Large diameter implants have been shown to be more successful in poor quality bone because the stress distribution becomes more homogenous across the implant-bone interface.^{8 9} Baggi et al. assessed the influence of length and diameter on stress distribution in five implants using linearly elastic 3-dimensional finite element analysis. An increase in implant diameter induced a significant reduction in stress peaks at the cortical bone, whereas changes in length altered stress patterns at the cancellous bone-implant interface.¹⁰ Kruyt et al. confirmed these findings clinically in a randomized control trial of bone-anchored hearing aids. Significantly higher implant stability quotient was determined for 4.5 mm than for 3.75 mm wide implants.¹¹ Overall, implant diameter can be considered a more effective design parameter determining the load transfer pattern at the interface compared to implant length.

Another key design parameter that dictates local stress patterns is the thread shape and geometry or surface topography of the implant. Thread depth, the distance between major and

minor diameter of the thread, has a significant effect on implant stability of screw-based implants (Figure 2) in both trabecular bone and cortical bone, with greater thread depth both increasing initial stability as well as further increasing the surface area for osseointegration to occur.¹² Chung et al. showed that a decrease in thread pitch increased surface area, which thereby resulted in a more uniform stress distribution.¹³ Decreasing thread pitch could improve mechanical anchorage and result in early endosseous integration, especially in low-density cancellous bone.¹⁴ The resulting increased surface area distributes newly deposited bone more uniformly at the endosteal interface.

For screw-based fixtures, thread shape also determines load transfer to the surrounding bone. Amongst different thread shapes available, including v-shaped, square, buttress, reverse-buttress, and spiral-shaped, the square thread profile provides the best primary stability under immediate loading conditions with highest bone implant contact. Both Misch et al. and Yadav et al. suggested that shear forces in V-thread and reverse buttress threads are 10-fold higher than in a square thread.^{15, 16} This reduction in shear loading at the square thread-bone interface induces more compressive load transfer, which is important in conditions where there is compromised bone density. However, there is still no consensus that square threads are the optimum design. Chien et al. reported that implants with a trapezoidal thread design achieved more rapid OI in comparison to implants with a square thread design.¹⁵

Porosity

Press fit or compressive OI implant surface geometry can improve OI by applying a porous coating or by designing a porous implant.¹⁶ Porous implants undergo bone ingrowth within the interconnected channels and neovascularization, ensuring faster OI when compared to solid materials.¹⁷ Pore morphology and the interconnection pattern dictate cell distribution and

liquid flow. Recent studies suggest that 20-50% porosity¹⁸ is an optimal porosity range to stimulate bone ingrowth.¹⁹ Pore size also affects fixation. Taniguchi et al. demonstrated that a 600 μm pore size, in comparison to 300 and 900 μm , had superior fixation at the same level of porosity.²⁰ It is important to note that there is a tradeoff between an increase in porosity and with a decrease in material strength and fatigue resistance.²¹ Compressive osseointegration achieves terminal bone ingrowth via application of a controlled compressive load between proximal transfixion pins and the distal spindle to create a traction bow, but also ultimately relies on terminal surface porosity at the bone-implant interface.

Material properties

The bulk properties of OI implants, such as strength, toughness, and elastic modulus, are determined by the chemical composition and structure of the materials used to manufacture the implant. Metals and metal alloys, most commonly commercially pure titanium CP1-Grade 4 and titanium Grade 5- titanium 6Al-4V alloy, are primarily used to manufacture OI implants due to their strength and toughness.^{22,23 24} Titanium has a high specific strength (strength per density) and high corrosion resistance (due to the formation of a passive titanium oxide layer) compared to other metals. It is also biocompatible with bone.^{25,26 27,28}

Titanium alloys do have some drawbacks.²⁹ Titanium can undergo severe wear due to its high coefficient of friction. This can be overcome through manufacturing strategies, such as using ultrafine-grained materials, thermal oxidation, or laser texturing.³⁰ Furthermore, titanium has a much higher elastic modulus than the surrounding bone. This mismatch can lead to bone resorption, stress shielding, and OI failure. However, titanium and titanium alloys are generally more flexible and thus superior to cobalt chrome-based implants with regard to the latter.

The Living Bone and the endosteal-Implant Interface

Osseointegration begins by injuring the endosteal surface during surgical implantation (Figure 3) and inducing a healing response to cover the implant in bone. The characteristics and quality of the living bone is critical for both initial and long-term success of OI. Endosteal injury rapidly activates the immune response to tissue injury resulting in a highly coordinated cascade of cellular responses involving soluble mediators, blood cells, extracellular matrix, and the mobilization-migration of parenchymal cells that promote cell proliferation-differentiation, extracellular matrix (ECM) deposition, stimulate growth factor secretion, promote neoangiogenesis and tissue regeneration. Hemostasis and coagulation occurs as early as one-hour post implantation and onset of granulation tissue development at the injury site seen within two hours.^{31,32} Nguyen et al. observed the expression of coagulation factors and extrinsic coagulation initiator tissue factor by osteoblasts. This study further demonstrated the previously unknown regulatory role of such extravascular coagulation factors on the endosteal stem cell niche and the activation of their egress to areas of injury.³³ Secreted extracellular matrix glycoproteins initiate replacement of the granulation tissue starting as early as 24 hours post implantation.³⁴ Ultrastructural studies have observed bone-specific extracellular matrix proteins, such as osteonectin and fibronectin, at the bone-biomaterial interface during this period.³⁵ Macrophages are thought to be the primary cell type mediating the gradual replacement of granulation tissue with extracellular matrix proteins, thereby stimulating wound vascularization, migration of mesenchymal stem cells, and clearing dead cells.³⁶

Woven bone is observed within the empty spaces at the bone-implant interface within one week following surgery.³⁷ The woven bone is replaced by trabecular bone within 2-3 weeks post insertion. Bone fragments (35-220 μm) from surgical damage are also enveloped within the

new bone tissue. It has been speculated that these fragments enhance peri-implant osteogenesis.³⁸ The same study observed the longitudinal progression of this *de novo* ossification. Trabecular bone was replaced within 12 weeks with mature lamellar bone containing marrow, suggestive of endochondral ossification remodeling. However, no osteogenesis was observed in regions where implant threads were in tight contact with the native bone surface.³⁹

Histological analysis demonstrates the formation of *de novo* bone along the surface of the native bone, in addition to deposition of bone on the implant surface. This indicates that the presence and activity of osteoconductive factors within the 'gap' between the native bone and implant.⁴⁰ Bridging of gaps (1-2 mm) between the bone and implant by *de novo* bone is dependent on the surface coating of the implant. In this regard, hydroxyapatite (HA) coatings have been shown to be superior to titanium alloy coating for stimulating implant bone ingrowth from the native bone.⁴¹ However, the highly osteophilic surface created by HA can result in stronger bone-HA coating at the expense of bonding of HA to the metal implant. This can result in a separation of the bone coating interface. It is important to note that despite their routine use in external fixator pins, HA coatings are not a routine in OI because the layer separates from the titanium surface over time.⁴² Hence, the long-term physical implications of HA coatings are unclear and require further study.^{43,44}

Proteoglycan appears as early as twelve weeks post implantation. Early electron microscopic analysis of the bone-titanium interface in a rabbit long bone model revealed the presence of a 20 nm thick layer of proteoglycans, with HA-crystals in direct contact with the titanium surface.⁴⁵ This proteoglycan layer separated the implant surface from the collagen and cellular layers. The collagenous matrix has been shown to be mostly comprised of Type I

collagen.⁴⁶ These observations present a target for enhancing integration through implant surface coatings. Over the years, several different coatings have been compared vis-à-vis HA. A long term *in vivo* study demonstrated that bone ingrowth within tri-calcium phosphate (TCP) scaffold over a 12-month period was significantly higher than in HA scaffolds.⁴⁷

Transdermal interface

The skin-implant interface, or skin penetration site (SPS) is where the implant exits the skin and attaches to an external prosthetic device (Figure 4). The use of half-pins in external fixators is exemplary of the challenges inherent to percutaneous implants. Lessons learned from their management can be applied to OI.⁴⁸ Pin tract infections (PTI) are a common complication of half-pins given their percutaneous nature.^{49,50} Changes in surgical techniques and post-operative care are still being tested to address this issue.⁵¹ In principle, an ideal scenario for half-pins involves a setting with minimal to no motion at the skin-pin interface, thus producing less local irritation, inflammation, and pain, and potentially less risk of infection. Indeed, applications that involve placing half-pins through deeper tissue, such as in the case of femoral or pelvic external fixation, where there is more motion at the skin-implant interface, produce the highest rates of pin tract infections.⁵²

There are several issues that remain unaddressed regarding the extent of soft tissue coverage. These include the role of the pre-surgical health of surrounding soft tissue, ideal surgical manipulation of this tissue, the significance and feasibility of a firmly attached soft tissue interface, and the long-term follow up of soft tissue healing. While some surgeons and OI devices emphasize ample soft tissue coverage, others prefer minimal coverage so as to minimize motion at the skin-implant interface. Preferences for soft tissue layer composition of also vary. Some implants are designed to accommodate ample soft tissue coverage with a substantial layer

of muscle and fascia.⁵³ Others prefer cutaneous fat and skin layers for coverage or skin coverage alone (Figure 5).^{1, 54}

In clinical practice, the SPS of all commonly utilized OI implants include a smooth, polished abutment, seeking to minimize irritation and inflammation at the SPS with no attempt a skin or dermal adhesion to the device itself. Early attempts to apply a porous coating for soft tissue attachment to a cobalt chrome abutment led to a high infection and failure rate, which improved dramatically with a change to the more common, smooth and polished design.⁵⁵ Further, of two custom implants placed with a porous titanium subdermal cap attempting to create a biologic dermal seal, at least one has failed due to recurrent soft tissue infections within two years of placement (unpublished data).

Current investigational efforts aim to create just such an impervious seal and develop bacterial-resistant implant surfaces. One such effort is the ‘Skin and Bone Integrated Pylon’ (SBIP) technology, which aims to promote skin ingrowth within the abutment at the skin-implant interface. The skin and bone become a continuum by integrating to the implant. Pitkin et al. reported that a porous titanium abutment, instead of the typical smooth surface, significantly promotes the ingrowth of dermal fibroblasts and mesenchymal cells at the interface.⁵⁶ A more recent study using the SBIP technology demonstrated that coating porous titanium pylons with TiO₂ nanotubes followed by seeding the surface with fibroblasts resulted in higher cellular density on implant surface and increased contact between the implant and soft tissue. In contrast, the control samples of this study (without nanotube coating and/or cell coating) were observed to have patchy fibrovascular ingrowth.⁵⁷ The authors concluded that the combination of a nanotextured surface of implant and pre-seeding the implant with cells at the skin-implant interface could potentially create a stable integration of the skin and implant.

In addition to efforts to create an integrated skin-implant system, there have been parallel efforts to prevent infection at the interface by altering surface characteristics. Such efforts include special coating of implant surfaces and improved diagnostics for early detection. A recent study exploited the change in tissue pH that occurs during an infection to develop a non-invasive system for monitoring infection in OI devices.⁵⁸ This system integrates a pH sensitive thin film coated on the prosthesis surface with an electrical capacitance tomography (ECT) algorithm. The algorithm is capable of detecting, localizing and representing the permittivity variations, which correlates with alterations in pH. This system can be a robust early diagnostic tool to monitor, detect, and treat superficial bacterial infections at the skin-implant interface.

Use of cathodic voltage-controlled electrical stimulation (CVCES) is another recently reported powerful modality to inhibit the growth of typical implant-associated microbes [methicillin resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii*]⁵⁹ Canty et al. demonstrated that CVCES for any duration between 4-8 hours was effective in attaining this inhibition for both Gram positive (MRSA) and Gram negative (*A. baumannii*) microbes present on commercially pure titanium. This technique reduced bacterial formation by 99.9%, which is very difficult to attain by conventional antimicrobial agents owing to issues with biofilm penetration.

Current Challenges:

Infection

In general, the most common complication surrounding OI prosthetic implants is superficial infection. The most common organisms implicated in these infections are *Staphylococcus aureus* and coagulase-negative staphylococci species. While deep infection

leading to implant failure is infrequent, the risk of superficial infection with OI amputation implants may exceed 50%. Superficial infections are often managed effectively with oral antibiotics alone and do not require surgical debridement. Superficial infections most commonly present with mild pain, erythema, swelling, or purulent discharge at the skin/abutment interface (Figure 6).⁶⁰ In their series of transfemoral implants, Brånemark et al.⁶¹ reported a superficial infection rate of 54.9%. Superficial infection occurred 41 times in 28 patients. The majority of these were successfully treated with a ten-day course of oral antibiotics and only 4 patients required prolonged antibiotic treatment. More importantly, none the patients who were treated for superficial infection developed a deep infection and none of these patients required implant removal.

Bacterial infection of OI percutaneous devices most commonly occurs through direct inoculation.⁶² This has led to the paradigm described by Gristina et al.⁶³ known as the “race to the surface,” based on the idea of a competition between bacterial adhesion and tissue integration. Ideally, bone and epithelial tissue colonize the surface of OI percutaneous devices and outcompete bacteria. However, if bacteria successfully colonize the surface of the device to a critical mass, tissue cells may not be able to displace bacterial colonies resulting in decreased tissue integration and infection. The first few hours following device placement are most critical to preventing initial bacterial colonization.⁶⁴

Bacterial colonization of a surface is a multistep process and is often preceded by initial conditioning of surfaces with biomolecules, such as proteins from blood, through adsorption in nanoseconds.⁶⁵ The first phase of bacterial attachment to a surface is dominated by nonspecific associations between bacteria and surface through long (e.g., van der Waals, and electrostatic interactions) and short range (e.g., hydrogen bonding, dipole–dipole, and hydrophobic

interactions) forces.⁶⁶ Next, bacterial cell proliferation, intercellular adhesion, and eventual biofilm formation take place. Biofilm formation takes place at a critical local density (i.e., quorum). Microbes multiply and form multi-layered cell clusters with complex architecture and significant internal heterogeneity. Eventually a polysaccharide matrix is formed. Extra polysaccharide substances (EPS) are produced and are responsible for binding and cell adhesion of the microbial cell clusters to OI percutaneous device surfaces.⁶⁷ The EPS matrix acts as a barrier and protects the microbes during adverse conditions such as shear force, contains channels for nutrient flow, and acts as an ion exchange resin critical to biofilm-based antimicrobial resistance.⁶⁸ Eventually, the biofilm reaches a critical mass and planktonic bacterial cells are generated. This generation is not a passive process but rather activated through similar signals used for quorum sensing.

Osteomyelitis

Osteomyelitis is inflammation of bone caused an infecting organism resulting in bone destruction. Traumatic injury, surgery, the presence of foreign bodies, or the placement of prostheses may disrupt bony integrity and lead to the onset of bone infection. Microorganisms often reach bone by traveling through the bloodstream or spreading from nearby infected tissue,⁶⁹ but in OI implants, there is obviously further concern of migration down the abutment through the SPS with deep infection developing. *Staphylococcus aureus* is classically associated with osteomyelitis, accounting for 45% of infections, but the appearance of this microorganism has reportedly dropped off in the past few decades.^{70,71} Osteomyelitis is characterized by tissue necrosis and destruction of bone matrix and trabeculae.⁷² Vascular channels are collapsed and destroyed by inflammation and the resultant lack of functional vascularization contributes to

bone necrosis.⁷³ Bone segments without blood supply can become isolated and difficult to treat.

⁷⁴

The cumulative risk of osteomyelitis in a group of patients receiving femoral implants was recently placed at 20% at 10 years Kaplan-Meier survivorship.⁷⁵ Treatment is typified by a combination of surgical debridement and antimicrobial therapies, with implant removal being required due to loosening or for infection eradication being required in over ½ (10 of 16) affected patients (9% risk at 10 years in Kaplan-Meier survivorship).⁷⁰ Of note, seven of the 16 affected patients were implanted and treated before the current surgical protocol for that implant was developed. A more recent prospective series of 51 patients with 55 transfemoral amputations reported a deep infection rate of 10% (11 of 55 limbs), with only one implant being removed for deep infection at five-year follow-up.⁷⁶

Skin Complications

In the absence of infection, other complications can occur at the SPS (“stoma”) and skin/abutment interface. The skin around the implant can change color, becoming erythematous or purple. There can be serous, non-purulent drainage, and formation of granulation tissue surrounding the abutment. These skin changes vary in the degree of severity of symptoms experienced by the patient. The majority of these issues can be managed conservatively, including observation, local wound care and hygiene, chemical cauterization. Rarely they may require surgical debridement or revision of the skin/implant interface in refractory cases. Tsikandylakis et al. reported skin reactions accounted for 19% of all adverse events and occurred in eight of eighteen patients in their series.⁶⁰

The skin implant interface can be complicated by partial or complete necrosis of the distal skin flap (Figure 7). This necrosis is most commonly related to thinning of the distal skin, resulting in insufficient viability of the skin. When the necrosis is mild, it is often managed with wound care and antibiotics. Revision surgery is occasionally necessary.^{60,77}

Loosening/Failure of Fixation

Loss of integration and implant fixations can have several etiologies. Fortunately, for transcutaneous OI in amputees, inflammation secondary implant loosening due to wear of metal, ceramic, or polyethylene bearing surfaces is not a concern, as there is no internal motion or bearing surface. Thus, the chief concerns for loosening of OI implants relate to failure of primary ingrowth, loosening due to infection, and stress shielding.

Fixture apposition to the endosteal surface during implantation helps produce initial mechanical stability. Mobile or loosely fitting implants lead to fibrous tissue deposition between the bone and metal surface, resulting in instability of the implant.⁸¹ Macromotion may lead to implant failure, while micromotion is beneficial to bone-implant integration. Implant micromotion is equally important for stable OI,⁸² whereby micromotions up to 30 μm enhances OI, and micromotions $>150 \mu\text{m}$ inhibits it.⁸³

One consequence of OI is marginal bone loss (MBL), which occurs due to inflammation around an implant with a roughened surface.^{84 85} Additionally, the shape and placement timing of the abutment may contribute to this inflammation (termed 'peri-implantitis') and subsequent MBL.⁸⁶ Canullo et al. observed that placement of a conical abutment in a one-step surgery resulted in healthier peri-implant soft tissue.⁸⁷ Peri-implantitis and MBL are common in

osseointegrated dental implants.^{88,89} However, the clinical importance of peri-implantitis in extremity OI is unclear.

Stress shielding may also affect the long-term stability of an OI implant. Over time, well fixed metal implants decrease the physiologic load on the surrounding bone. This results in reduced bone density (osteopenia), and cortical bone thickness of the implant-associated bone.^{90,91} Altering implant design,⁹² stiffness,⁹³ biomaterial coating,⁹⁴ and manufacturing⁹⁵ can address stress shielding to some extent.

In the future, real time quantitative monitoring of the status of implant integration could extend the life of the implant. This was not possible until the recent work by Wang et al., which uses a sensing strategy integrated at the percutaneous end of the prosthesis.⁹⁶ Piezoelectric elements mounted at the percutaneous end of the prosthetic device generate guided waves that are sensitive to both the degree of bone healing at the prosthetic surface, and the movement of the prosthetic device over time. The guided waves travel along the length of the prosthetic. The healing process, indicating positive OI, decreases the energy of the longitudinal waves, while increase in this wave energy corresponds with implant loosening. This is a very elegant sensing system to interrogate the quality of OI and its long-term success in individual patients.

Peri-implant Fracture

As with all intramedullary implants, fractures can occur proximal to the implant or at the level of the implant, disrupting the bone-implant interface. Three periprosthetic fractures reported by Brånemark et al.⁹⁷ were ipsilateral hip fractures, proximal to the implant. As with all implants, failure/fracture can also occur through the implant itself secondary to fatigue or intense stress. In the same series by Brånemark et al.⁹⁸ nine implant failures were reported in 4

patients and included bending or fracture of the abutment or abutment screw. All were treated successfully with revision surgery and implant replacement. Six of the nine implant failures occurred in the same patient, indicating likely individual patient specific factors that overestimate the rate of overall implant failure in this study. In a more recent prospective series of 51 patients/55 limbs and five-year clinical follow-up, mechanical complications with bent abutments or abutment screw failures remained common, including 16 bent abutments in nine patients.⁷⁶ In both series, no implant failures occurred at the fixture itself.⁷⁶

Future Opportunities

The field of OI continues to see advancements in multiple areas of implant design. One particular area receiving attention is the integration of electronic sensors and the neuromuscular-implant connection. The Enhanced Osseointegrated Prostheses for the Rehabilitation of Amputees(e-OPRA) system allows for bidirectional communication between implanted neuromuscular electrodes and the external prosthesis. This system uses advanced algorithms and neural stimulation to provide sensory feedback to the patient and allow for volitional motor control of the prosthesis.⁹⁹ Additionally, integrated sensors could provide real-time information to clinicians including implant stability and early signals of infection. Recent studies demonstrate that applying a cathodic electrical stimulation can eradicate bacteria, including antibiotic resistant strains, *in vivo* without negatively affecting the surrounding host bone.¹⁰⁰ Finally, no early detection method exists for the loss of OI that occurs in infection or aseptic loosening. Guided wave strategy could be a novel method to quantify OI. A recent study successfully measured the change in energy absorption of guided waves in piezoelectric wafers installed on an OI implant surface.¹⁰¹ Real time monitoring of fixation status could not only aid clinicians in detecting early failure, but also accelerate rehabilitation and weight bearing.

Despite its five-decade history, extremity OI is still in its infancy and the long-term outcomes are unknown. This treatment has the potential to benefit tens of thousands of amputee patients and, if the costs of implants decrease as utilization of OI techniques increase, potentially save medical costs related to the complications of socket-based prosthesis. In the future, the outcomes of amputees with osseointegrated limbs can be collated and analyzed using an international patient registry.

Figure Legends

Figure 1: Schematic image of the Osseointegrated Prostheses for the Rehabilitation of Amputees (OPRA) Device system illustrating (a) the screw and the abutment, (b) the position of the implant system within the residual limb and (c) a depiction of the post-implantation coagulum initiating the osseointegration process. Created with BioRender

Figure 2: From left to right, the (a) pre-operative, (b) Stage I post-operative, and (c) Stage II post-operative radiographs of the OPRA device implanted in a Transhumeral Amputee. The OPRA device is implanted in two stages to allow for bone graft incorporation and soft tissue healing. In the first stage, the fixture is placed into the distal end of the residual bone and in the second stage, a stoma is created for the external abutment to connect to the fixture.

Figure 3: Surgical implantation of the OPRA device after reaming and tapping to create both endosteal injury necessary for osseointegration as well as to create grooves for the controlled

purchase of implant threads into the bone for initial stability and controlled fixture placement and orientation.

Figure 4: Clinical photographs of a transhumeral amputee after completing the two-stage OPRA device implantation.

Figure 5: Clinical photograph of a transfemoral amputee following two-stage OPRA device implantation. The skin overlying the residual bone has been defatted and thinned to minimize motion of the soft tissue.

Figure 6. Clinical photograph of a superficial infection at the stoma of an OPRA device.

Figure 7. Clinical photograph of skin necrosis at the stoma around an OPRA device.

References

1. Brånemark R. Osseointegrated prostheses for rehabilitation following amputation. *Der Unfallchirurg*. March 2017;1-8. doi:10.1007/s00113-017-0331-4.
2. Gailey R, McFarland LV, Cooper RA, et al. Unilateral lower-limb loss: Prosthetic device use and functional outcomes in servicemembers from Vietnam war and OIF/OEF conflicts. *JRRD*. 2010;47(4):317–16. doi:10.1682/JRRD.2009.04.0039.
3. Van de Meent MD PhD H, PhD MTH, PhD JPFM. Walking Ability and Quality of Life in Subjects With Transfemoral Amputation: A Comparison of Osseointegration With Socket Prostheses. October 2013:1-5. doi:10.1016/j.apmr.2013.05.020.
4. Häggström E, CPO, Hagberg K, et al. Vibrotactile evaluation: Osseointegrated versus socket-suspended transfemoral prostheses. *JRRD*. 2013;50(10):1423-1434. doi:10.1682/JRRD.2012.08.0135.
5. DDS OEO. Implant Surface Material, Design, and Osseointegration. *Dental Clinics of*

- 454 NA. 2015;59(2):505-520. doi:10.1016/j.cden.2014.12.003.
- 455 6. Vairo G, Sannino G. Comparative Evaluation of Osseointegrated Dental Implants Based
456 on Platform-Switching Concept: Influence of Diameter, Length, Thread Shape, and In-
457 Bone Positioning Depth on Stress-Based Performance. *Computational and*
458 *Mathematical Methods in Medicine*. 2013;2013(1):1-15. doi:10.1155/2013/250929.
- 459 7. The effect of implant length and diameter on the primary stability in different bone
460 types. 2013;10(5):449-455. /pmc/articles/PMC4025419/?report=abstract.
- 461 8. The effect of implant length and diameter on the primary stability in different bone
462 types. 2013;10(5):449-455. /pmc/articles/PMC4025419/?report=abstract.
- 463 9. Three-year Outcomes of a Randomized Controlled Trial Comparing a 4.5-mm-Wide to a
464 3.75-mm-Wide Titanium Implant for Bone Conduction Hearing. 2018;39(5):609-615.
465 doi:10.1097/MAO.0000000000001761.
- 466 10. The influence of implant diameter and length on stress distribution of osseointegrated
467 implants related to crestal bone geometry: a three-dimensional finite element analysis.
468 2008;100(6):422-431. doi:10.1016/S0022-3913(08)60259-0.
- 469 11. Three-year Outcomes of a Randomized Controlled Trial Comparing a 4.5-mm-Wide to a
470 3.75-mm-Wide Titanium Implant for Bone Conduction Hearing. 2018;39(5):609-615.
471 doi:10.1097/MAO.0000000000001761.
- 472 12. Effects of thread features in osseointegrated titanium implants using a statistics-based
473 finite element method. 2012;28(8):919-927. doi:10.1016/j.dental.2012.04.035.
- 474 13. Effects of implant geometry and surface treatment on osseointegration after functional
475 loading: a dog study. 2008;35(3):229-236. doi:10.1111/j.1365-2842.2006.01653.x.
- 476 14. Dental implant thread pitch and its influence on the osseointegration process: an in vivo
477 comparison study. 2012;27(2):383-392.
478 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=22442779&retmode=ref&cmd=prlinks>.
479
- 480 15. Influence of Thread Design on Dental Implant Osseointegration Assayed Using the Lan-
481 Yu Mini-Pig Model. 2017;37(5):627-638. doi:10.1007/s40846-017-0240-6.
- 482 16. Engineering biocompatible implant surfaces: Part I: Materials and surfaces.
483 2013;58(3):261-326. doi:10.1016/j.pmatsci.2012.09.001.
- 484 17. Porous Titanium Implants: A Review. 2018;20(5):1700648.
485 doi:10.1002/adem.201700648.
- 486 18. Evaluation of bone ingrowth into porous titanium implant: histomorphometric analysis
487 in rabbits. 2010;24(4):399-405. doi:10.1590/S1806-83242010000400005.

- 488 19. Scaffold Design and Manufacturing: From Concept to Clinic. 2009;21(32-33):3330-
489 3342. doi:10.1002/adma.200802977.
- 490 20. Effect of pore size on bone ingrowth into porous titanium implants fabricated by
491 additive manufacturing: An in vivo experiment. 2016;59:690-701.
492 doi:10.1016/j.msec.2015.10.069.
- 493 21. Fatigue characterization of Titanium Ti-6Al-4V samples produced by Additive
494 Manufacturing. 2016;2:3168-3176. doi:10.1016/j.prostr.2016.06.395.
- 495 22. Bone and metal: an orthopaedic perspective on osseointegration of metals.
496 2014;10(10):4043-4057. doi:10.1016/j.actbio.2014.06.004.
- 497 23. *Osseointegration: a Review of the Fundamentals for Assuring Cementless Skeletal*
498 *Fixation*. 300AD. [http://scholar.google.com/javascript:void\(0\)](http://scholar.google.com/javascript:void(0)).
- 499 24. Thesleff A, Brånemark R, Håkansson B, Ortiz-Catalan M. Biomechanical
500 Characterisation of Bone-anchored Implant Systems for Amputation Limb Prostheses: A
501 Systematic Review. *Annals of Biomedical Engineering*. 2018;46(3):377-391.
502 doi:10.1007/s10439-017-1976-4.
- 503 25. Bone and metal: an orthopaedic perspective on osseointegration of metals.
504 2014;10(10):4043-4057. doi:10.1016/j.actbio.2014.06.004.
- 505 26. *Osseointegration: a Review of the Fundamentals for Assuring Cementless Skeletal*
506 *Fixation*. 300AD. [http://scholar.google.com/javascript:void\(0\)](http://scholar.google.com/javascript:void(0)).
- 507 27. An overview of recent advances in designing orthopedic and craniofacial implants.
508 2013;101(11):3349-3364. doi:10.1002/jbm.a.34605.
- 509 28. Biological strategies for improved osseointegration and osteoinduction of porous metal
510 orthopedic implants. 2015;21(2):218-230. doi:10.1089/ten.TEB.2014.0333.
- 511 29. Biocompatibility of Ti-alloys for long-term implantation. 2013;20:407-415.
512 doi:10.1016/j.jmbbm.2012.11.014.
- 513 30. Current advances in enhancement of wear and corrosion resistance of titanium alloys - a
514 review. 2016;31(12):696-704. doi:10.1080/10667857.2016.1212780.
- 515 31. Biological fixation of endosseous implants. 2005;36(7-8):665-671.
516 doi:10.1016/j.micron.2005.05.010.
- 517 32. De novo alveolar bone formation adjacent to endosseous implants. 2003;14(3):251-262.
518 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=12755774&retmode=ref&cmd=prlinks>.
519
- 520 33. Extravascular coagulation in hematopoietic stem and progenitor cell regulation.
521 2018;132(2):123-131. doi:10.1182/blood-2017-12-768986.

- 522 34. De novo alveolar bone formation adjacent to endosseous implants. 2003;14(3):251-262.
523 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=12755774&retmode=ref&cmd=prlinks>.
524
- 525 35. Ultrastructural characterization of the implant/bone interface of immediately loaded
526 dental implants. 2004;25(10):1959-1967.
527 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=14738860&retmode=ref&cmd=prlinks>.
528
- 529 36. Hesketh M, Sahin KB, West ZE, Murray RZ. Macrophage Phenotypes Regulate Scar
530 Formation and Chronic Wound Healing. *IJMS*. 2017;18(7):1545–10.
531 doi:10.3390/ijms18071545.
- 532 37. De novo alveolar bone formation adjacent to endosseous implants. 2003;14(3):251-262.
533 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=12755774&retmode=ref&cmd=prlinks>.
534
- 535 38. Biological fixation of endosseous implants. 2005;36(7-8):665-671.
536 doi:10.1016/j.micron.2005.05.010.
- 537 39. Biological fixation of endosseous implants. 2005;36(7-8):665-671.
538 doi:10.1016/j.micron.2005.05.010.
- 539 40. De novo alveolar bone formation adjacent to endosseous implants. 2003;14(3):251-262.
540 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=12755774&retmode=ref&cmd=prlinks>.
541
- 542 41. Hydroxyapatite ceramic coating for bone implant fixation. Mechanical and histological
543 studies in dogs. 1993;255:1-58.
544 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=8237337&retmode=ref&cmd=prlinks>.
545
- 546 42. Kim J-S, Kang S-M, Seo K-W, et al. Nanoscale Bonding between Human Bone and
547 Titanium Surfaces: Osseohybridization. *BioMed Research International*.
548 2015;2015(1):1-10. doi:10.1155/2015/960410.
- 549 43. The contributions and limitations of hydroxyapatite coatings to implant fixation: A
550 histomorphometric study of load bearing implants in dogs. 1996;20(1):1-6.
551 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=8881881&retmode=ref&cmd=prlinks>.
552
- 553 44. Van Hamersveld KT, Marang-Van De Mheen PJ, Nelissen RGHH, Toksvig-Larsen S.
554 Peri-apatite coating decreases uncemented tibial component migration: long-term RSA
555 results of a randomized controlled trial and limitations of short-term results. *Acta*
556 *Orthopaedica*. 2018;89(4):425-430. doi:10.1080/17453674.2018.1469223.
- 557 45. Electron microscopic analysis of the bone-titanium interface. 1983;54(1):45-52.
558 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=6829281&retmode=ref&cmd=prlinks>.

- mode=ref&cmd=prlinks.
46. The concept of osseointegration and bone matrix expression. 1991;2(1):83-101.
<http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=1912145&retmode=ref&cmd=prlinks>.
47. Bone ingrowth into two porous ceramics with different pore sizes: an experimental study. 2004;70(6):598-603.
<http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=15669463&retmode=ref&cmd=prlinks>.
48. Ferreira N, Marais LC. Prevention and management of external fixator pin track sepsis. *Strategies in Trauma and Limb Reconstruction*. 2012;7(2):67-72. doi:10.1007/s11751-012-0139-2.
49. Ceroni D, Grumetz C, Desvachez O, Pusateri S, Dunand P, Samara E. From prevention of pin-tract infection to treatment of osteomyelitis during paediatric external fixation. *Journal of Children's Orthopaedics*. 2016;10(6):605-612. doi:10.1007/s11832-016-0787-8.
50. Kazmers NH, Fragomen AT, Rozbruch SR. Prevention of pin site infection in external fixation: a review of the literature. *Strategies in Trauma and Limb Reconstruction*. 2016;11(2):75-85. doi:10.1007/s11751-016-0256-4.
51. Pin-site care: can we reduce the incidence of infections? 2015;46 Suppl 3:S35-S39. doi:10.1016/S0020-1383(15)30009-7.
52. Complications of temporary and definitive external fixation of pelvic ring injuries. 2005;36(5):599-604. doi:10.1016/j.injury.2004.11.016.
53. McGough RL, Goodman MA, Randall RL, Forsberg JA, Potter BK, Lindsey B. Transkutanes Compress®-Implantat zur Rehabilitation nach Extremitätenamputation. *Der Unfallchirurg*. 2017;120(4):300-305. doi:10.1007/s00113-017-0339-9.
54. Osseointegrated Prosthetic Limb for the treatment of lower limb amputations : Experience and outcomes. 2017;120(4):306-311. doi:10.1007/s00113-016-0296-8.
55. Juhnke D-L, Beck JP, Jeyapalina S, Aschoff HH. Fifteen years of experience with Integral-Leg-Prosthesis: Cohort study of artificial limb attachment system. *JRRD*. 2015;52(4):407-420. doi:10.1682/JRRD.2014.11.0280.
56. Skin and bone integrated prosthetic pylon: a pilot animal study. 2006;43(4):573-580. /pmc/articles/PMC4859765/?report=abstract.
57. Shevtsov MA, Yudintceva N, Blinova M, et al. Application of the skin and bone integrated pylon with titanium oxide nanotubes and seeded with dermal fibroblasts. *Prosthet Orthot Int*. 2015;39(6):477-486. doi:10.1177/0309364614550261.

58. Noncontact Electrical Permittivity Mapping and pH-Sensitive Films for Osseointegrated Prosthesis and Infection Monitoring. 2017;36(11):2193-2203. doi:10.1109/TMI.2017.2707390.
59. Cathodic voltage-controlled electrical stimulation of titanium for prevention of methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii* biofilm infections. 2017;48:451-460. doi:10.1016/j.actbio.2016.11.056.
60. Tsikandylakis G, Berlin Ö, Brånemark R. Implant Survival, Adverse Events, and Bone Remodeling of Osseointegrated Percutaneous Implants for Transhumeral Amputees. *Clinical Orthopaedics and Related Research*®. 2014;472(10):2947-2956. doi:10.1007/s11999-014-3695-6.
61. A novel osseointegrated percutaneous prosthetic system for the treatment of patients with transfemoral amputation: A prospective study of 51 patients. 2014;96-B(1):106-113. doi:10.1302/0301-620X.96B1.31905.
62. Does endotoxin contribute to aseptic loosening of orthopedic implants? 2005;72(1):179-185. doi:10.1002/jbm.b.30150.
63. Biomaterial-centered infection: microbial adhesion versus tissue integration. 1987;237(4822):1588-1595. <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=3629258&retmode=ref&cmd=prlinks>.
64. Reducing implant-related infections: active release strategies. 2006;35(9):780-789. doi:10.1039/b515219b.
65. Reducing implant-related infections: active release strategies. 2006;35(9):780-789. doi:10.1039/b515219b.
66. Bacterial adherence and biofilm formation on medical implants: a review. 2014;228(10):1083-1099. doi:10.1177/0954411914556137.
67. The biofilm matrix. 2003;19(2):139-150. doi:10.1080/0892701031000072190.
68. The EPS matrix: the "house of biofilm cells". 2007;189(22):7945-7947. doi:10.1128/JB.00858-07.
69. Infection and musculoskeletal conditions: Osteomyelitis. 2006;20(6):1065-1081. doi:10.1016/j.berh.2006.08.014.
70. Ribeiro M, Monteiro FJ, Ferraz MP. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. *Biomatter*. 2014;2(4):176-194. doi:10.4161/biom.22905.
71. Osteomyelitis: Clinical overview and mechanisms of infection persistence. 2006;28(9):65-72. doi:10.1016/j.clinmicnews.2006.04.001.

- 629 72. Infection and musculoskeletal conditions: Osteomyelitis. 2006;20(6):1065-1081.
630 doi:10.1016/j.berh.2006.08.014.
- 631 73. Osteomyelitis: the past decade. 1980;303(7):360-370.
632 doi:10.1056/NEJM198008143030703.
- 633 74. Osteomyelitis. 2004;364(9431):369-379. doi:10.1016/S0140-6736(04)16727-5.
- 634 75. Tillander J, Hagberg K, Berlin Ö, Hagberg L, Brånemark R. Osteomyelitis Risk in
635 Patients With Transfemoral Amputations Treated With Osseointegration Prostheses.
636 *Clinical Orthopaedics and Related Research*®. 2017;475(12):3100-3108.
637 doi:10.1007/s11999-017-5507-2.
- 638 76. Brånemark RP, Hagberg K, Kulbacka-Ortiz K, Berlin Ö, Rydevik B. Osseointegrated
639 Percutaneous Prosthetic System for the Treatment of Patients With Transfemoral
640 Amputation: A Prospective Five-year Follow-up of Patient-reported Outcomes and
641 Complications. *J Am Acad Orthop Surg*. 2019;27(16):e743-e751. doi:10.5435/JAAOS-
642 D-17-00621.
- 643 77. Hebert JS, Rehani M, Stiegelmar R. Osseointegration for Lower-Limb Amputation.
644 *JBJS Reviews*. 2017;5(10):e10-e12. doi:10.2106/JBJS.RVW.17.00037.
- 645 78. Osseointegration in Irradiated Cancer Patients: An Analysis With Respect to Implant
646 Failures. 2005;63(5):579-585. doi:10.1016/j.joms.2005.01.008.
- 647 79. Soft tissue reactions around percutaneous implants: a clinical study on skin-penetrating
648 titanium implants used for bone-anchored auricular prostheses. 1987;2(1):35-39.
649 [http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=3471713&ret](http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=3471713&retmode=ref&cmd=prlinks)
650 [mode=ref&cmd=prlinks](http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=3471713&retmode=ref&cmd=prlinks).
- 651 80. Reconstruction of nasal defects with implant-retained nasal prostheses. 1998;36(5):341-
652 345.
653 [http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=9831053&ret](http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=9831053&retmode=ref&cmd=prlinks)
654 [mode=ref&cmd=prlinks](http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=9831053&retmode=ref&cmd=prlinks).
- 655 81. Immediate post-implantation skin immobilization decreases skin regression around
656 percutaneous osseointegrated prosthetic implant systems. 2013;101(7):2075-2082.
657 doi:10.1002/jbm.a.34510.
- 658 82. Validation of value of actual micromotion as a direct measure of implant micromobility
659 after healing (secondary implant stability). An in vivo histologic and biomechanical
660 study. 2016;27(11):1423-1430. doi:10.1111/clr.12756.
- 661 83. Immediate post-implantation skin immobilization decreases skin regression around
662 percutaneous osseointegrated prosthetic implant systems. 2013;101(7):2075-2082.
663 doi:10.1002/jbm.a.34510.
- 664 84. Peri-implantitis. 1998;102(3-4):74-79.

- 665 <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10756848&retmode=ref&cmd=prlinks>.
- 666
- 667 85. Marginal bone loss as success criterion in implant dentistry: beyond 2 mm.
- 668 2015;26(4):e28-e34. doi:10.1111/clr.12324.
- 669 86. The effect of one-time abutment placement on interproximal bone levels and peri-
- 670 implant soft tissues: a prospective randomized clinical trial. 2017;28(4):443-452.
- 671 doi:10.1111/clr.12818.
- 672 87. Marginal soft tissue stability around conical abutments inserted with the one abutment-
- 673 one time protocol after 5 years of prosthetic loading. 2018;20(6):976-982.
- 674 doi:10.1111/cid.12683.
- 675 88. Influence of the abutment height and connection timing in early peri-implant marginal
- 676 bone changes: A prospective randomized clinical trial. 2018;29(9):907-914.
- 677 doi:10.1111/clr.13343.
- 678 89. An Evaluation of the Relationship Between Peri-implant Soft Tissue Biotype and the
- 679 Severity of Peri-implantitis: A Cross-Sectional Study. 2019;34(1):187-196.
- 680 doi:10.11607/jomi.6958.
- 681 90. On stress/strain shielding and the material stiffness paradigm for dental implants.
- 682 2017;19(5):935-943. doi:10.1111/cid.12509.
- 683 91. Stress shielding of the humerus in press-fit anatomic shoulder arthroplasty: review and
- 684 recommendations for evaluation. 2018;27(6):1139-1147. doi:10.1016/j.jse.2017.12.020.
- 685 92. Stress shielding of the humerus in press-fit anatomic shoulder arthroplasty: review and
- 686 recommendations for evaluation. 2018;27(6):1139-1147. doi:10.1016/j.jse.2017.12.020.
- 687 93. On stress/strain shielding and the material stiffness paradigm for dental implants.
- 688 2017;19(5):935-943. doi:10.1111/cid.12509.
- 689 94. To reduce the maximum stress and the stress shielding effect around a dental implant-
- 690 bone interface using radial functionally graded biomaterials. 2017;20(7):750-759.
- 691 doi:10.1080/10255842.2017.1299142.
- 692 95. Open-cellular metal implant design and fabrication for biomechanical compatibility with
- 693 bone using electron beam melting. 2017;76:164-177. doi:10.1016/j.jmbbm.2017.02.019.
- 694 96. IWSHM 2017: Application of guided wave methods to quantitatively assess healing in
- 695 osseointegrated prostheses. 2018;17(6):1377-1392. doi:10.1177/1475921718782399.
- 696 97. A novel osseointegrated percutaneous prosthetic system for the treatment of patients
- 697 with transfemoral amputation: A prospective study of 51 patients. 2014;96-B(1):106-
- 698 113. doi:10.1302/0301-620X.96B1.31905.

- 699 98. A novel osseointegrated percutaneous prosthetic system for the treatment of patients
700 with transfemoral amputation: A prospective study of 51 patients. 2014;96-B(1):106-
701 113. doi:10.1302/0301-620X.96B1.31905.
- 702 99. Myoelectric signals and pattern recognition from implanted electrodes in two TMR
703 subjects with an osseointegrated communication interface. 2018;2018:5174-5177.
704 doi:10.1109/EMBC.2018.8513466.
- 705 100. Nodzo S, Tobias M, Hansen L, et al. Cathodic Electrical Stimulation Combined With
706 Vancomycin Enhances Treatment of Methicillin-resistant *Staphylococcus aureus*
707 Implant-associated Infections. *Clinical Orthopaedics and Related Research*®. April
708 2015:1-9. doi:10.1007/s11999-015-4280-3.
- 709 101. IWSHM 2017: Application of guided wave methods to quantitatively assess healing in
710 osseointegrated prostheses:. 2018;17(6):1377-1392. doi:10.1177/1475921718782399.

711

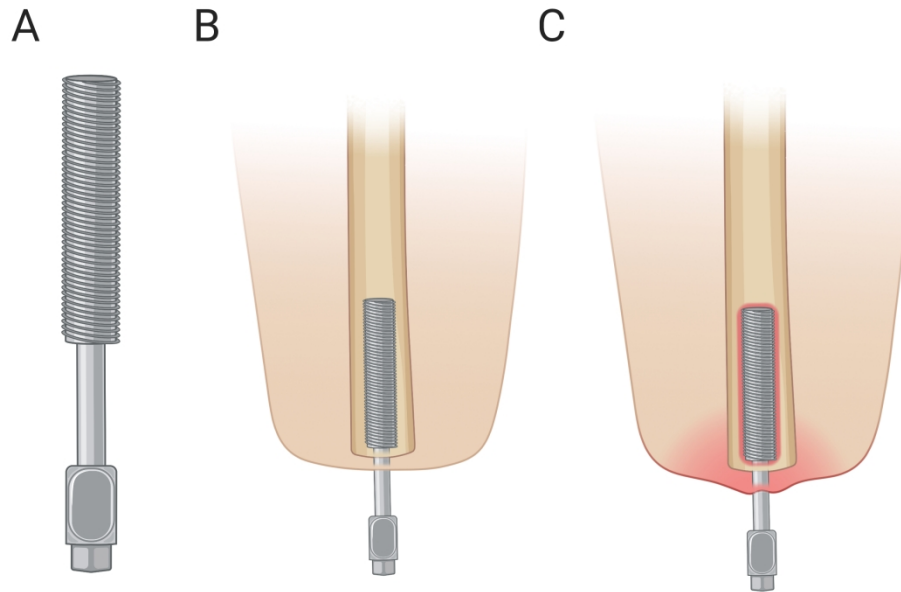
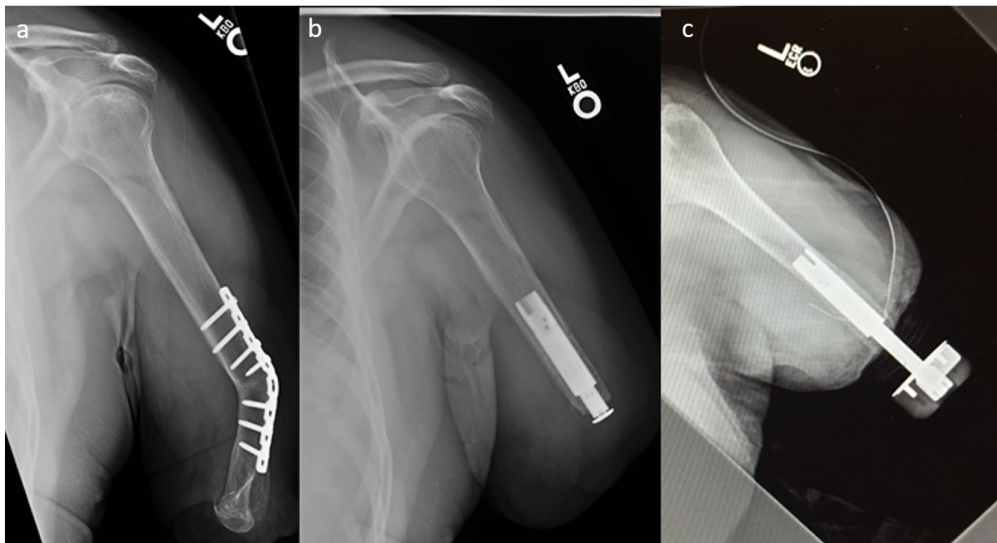


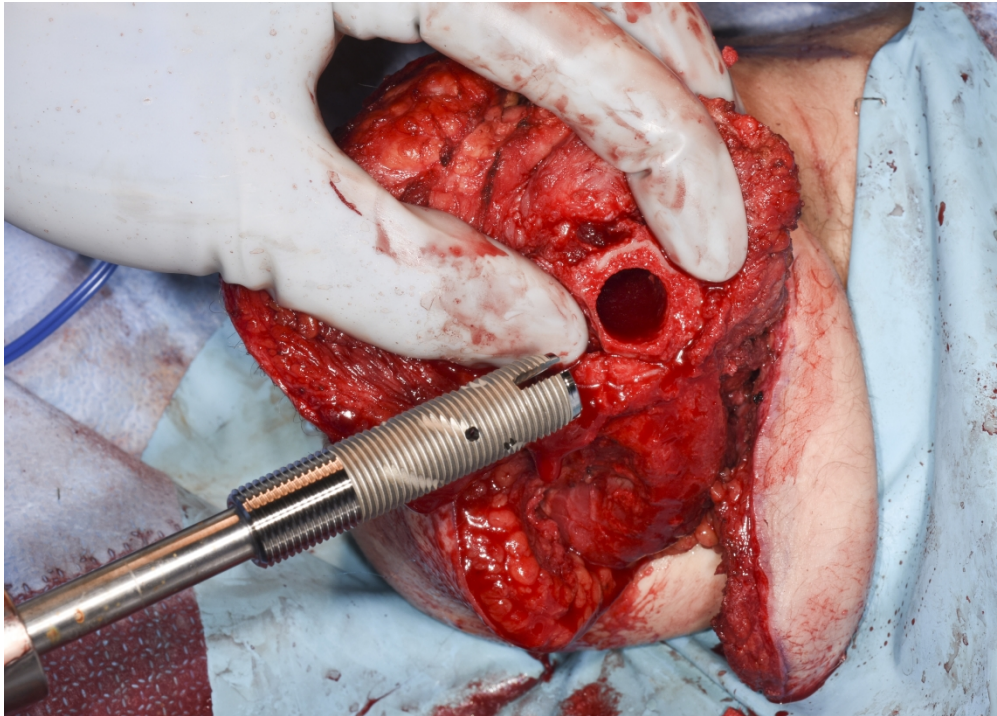
Figure 1: Schematic image of the Osseointegrated Prostheses for the Rehabilitation of Amputees (OPRA) Device system illustrating (a) the screw and the abutment, (b) the position of the implant system within the residual limb and (c) a depiction of the post-implantation coagulum initiating the osseointegration process.
Created with BioRender

381x304mm (200 x 200 DPI)



From left to right, the (a) Preoperative, (b) Stage-One Post-Operative, and (c) Stage-Two Post-Operative Radiographs of the OPRA Device Implantation in a Transhumeral Amputee. The OPRA Device is implanted in two stages to allow for bone graft incorporation and soft tissue healing. In the first stage, the fixture is placed into the distal end of the residual bone and in the second stage, a stoma is created for the external abutment to connect to the fixture.

227x123mm (150 x 150 DPI)



Surgical implantation of the OPRA Device.

391x279mm (300 x 300 DPI)



Clinical photographs of a transhumeral amputee after completing the two-stage OPRA device implantation.

332x168mm (150 x 150 DPI)



Clinical photograph of a transfemoral amputee following two-stage OPRA device implantation. The skin overlying the residual bone has been defatted and thinned to minimize motion of the soft tissue.

1066x1066mm (72 x 72 DPI)



Clinical photograph of a superficial infection at the stoma of an OPRA device.

355x266mm (72 x 72 DPI)



Clinical Photograph of skin necrosis at the stoma around an OPRA device.

1066x1422mm (72 x 72 DPI)