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14. ABSTRACT

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

15. SUBJECT TERMS

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

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1. INTRODUCTION:

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

2. KEYWORDS:

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

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

What were the major goals of the project?

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

Research-Specific Aims and Tasks	Mos.	Percent completed
Administrative Aims and Tasks: 1. Establish subaward agreement between HJF and MMRF 2. Develop and sign USU-MMRF CRADA 3. Recruit and hire support personnel a. Stem Cell Biologist (USU) b. Research Associate (USU) c. Stem Cell Biologist (MMRF-UMN)	1-4	100% 100% 100% a. 100% b: 100% c: 100%
Specific Aim 1 Specific Aim 1: in ex vivo culture (a) steer differentiation for human and swine MSC, iPS cells and mature site-specific (gingival and hoof/nail bed) cells to adhesive/epithelial phenotypes, (b) Characterize and rate the ingrowth of these cells into scaffold and their adhesive potential to metal substrate.	**1-24**	50%
Major Task 1: Develop cell culture	1-16	90%
Subtask 1.1: In vitro isolation and characterization of *porcine* cells.	1-16	100%
Subtask 1.2: In vitro development and characterization of *human* cells.	1-16	90%
Major Task 2: *In vitro*: evaluate cell adhesion to metal substrate	7-16	55%
Subtask 2.1a: Test porcine cells for adhesion	7-16	100%
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	40%
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	40%
Major Task 4: Complete full statistical analysis, complete/submit 2-4 manuscripts.	18-24	30%
Milestone(s) Achieved: Characterization of 3-4 cell choices for optimal adhesivity - in vitro; submission of 2-4 manuscripts.	*1-24*	40%
Specific Aim 2 Specific Aim 2: In vivo large animal (swine) testing of transdermal implants with and without subdermal cellular augmentation (SA2a), +/- septal /strain limiting scaffold (SA2b), and topical bacterial challenge (SA2c).	**25-60**	5%
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 and bio-banking of nine porcine tissue-derived MSCs:

Porcine mesenchymal stem cells (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 previous reports, MSCs have been derived from nine different integumentary tissues, namely bone marrow, muscle, abdominal and hind limb adipose, abdominal and hind limb dermis, tendon, hoof, and molar-associated periodontal ligament from 4-6 animals for each tissue. Briefly, 1-2 gm of each tissue was collected in sterile collection media (1:1 DMEM-F12, containing 2% FBS and 2X antibiotics; namely penicillin, streptomycin and fungizone), followed by RBC lysis, and isolation of cells after mechanical and enzymatic digestion, as detailed in **Table 1**. The cell pellet obtained at the end of digestion from each tissue was resuspended in growth media (GM; same as collection media), and seeded in 10-cm tissue-culture dishes, with media change every 3 days. Within the first 7-10 days, while the majority of cells underwent cell death, MSCs were seen to attach to the plate, and expand in distinct colonies. Expanded MSCs from the nine tissues from 4-6 animals have been bio-banked at multiple passages from P0 to P5 at a concentration of 1-2 x 10^6 cells/vial. Cells at P2 or P3 have been used for all downstream characterization studies. The hallmark characteristics of MSCs are **1.** adherence to plastic surface, the basis of our first enrichment step, and **2.** Ability to differentiate into osteo-, chondro- and adipogenic lineages (multi-lineage differentiation). Qualitative and quantitative assays have been used to analyze the multi-lineage differentiation potential of these nine tissue-derived MSCs. **Over this past year, we have completed repeats for all characterization studies in at least 3 animals per tissue as shown in the heat map (Figure 1).**

S. No.	Tissue	Enzyme cocktail	Duration of digestion
1	**Bone marrow**	NA; Obtained by aspiration from iliac crest or flushing ribs, immediately followed by resuspension in ice-cold collection media*	Not Applicable
2	**Muscle**	Col I (3 mg/ml) + Col II (1.42 mg/ml) + Dispase (3 mg/ml)	2 hours
3	**Abdominal adipose**	Col I (1 mg/ml)	2 hours
4	**Hind Limb adipose**		
5	**Achilles Tendon**	Col I (3 mg/ml)+Col II (3 mg/ml)+ Dispase (4 mg/ml)	1.5 hours
6	**Hoof**		
7	**Abdominal dermis**	Col I (0.25%) in DMEM-F12 (1:1) containing 10% FBS	10-12 hours
8	**Hind limb dermis**		
9	**Molar-associated Periodontal ligament**	Col I (3 mg/ml)+ Dispase II (4 mg/ml)	1 hour

Table 1. Protocol for digestion and processing of the nine porcine tissues. Col: Collagenase;
** Collection media: 1:1 DMEM-F12 + 2% FBS + 2X Pen-Strep-Fungizone*

	Osteo-ALP	Osteo-AR	Chondro-AB	Adipo-ORO	AM Genes	OM Genes	CM Genes	Adhesion P1	Adhesion P5
Ab Adipose	5	4	3	5	2	2	2	2	2
Ab Dermis	3	3	3	3	2	2	2	2	2
Bone Marrow	3	4	3	3	2	2	2	2	2
HL Adipose	3	4	3	3	2	2	2	2	2
HL Dermis	3	3	3	4	2	2	2	2	2
Hoof	4	3	3	4	2	2	2	2	2
Molar	3	3	3	3	2	2	2	2	2
Muscle	4	3	3	3	2	2	2	2	2
Tendon	3	3	2	2	2	2	2	1	2

Figure 1. Heat map representing the status of completion of characterization studies. The tissues are listed along the rows, and each column corresponds to the assay. The first two columns comprise early (Osteo-ALP) and late (Osteo-AR) osteogenic differentiation respectively. The third column represents chondrogenic differentiation (Chondro-AB), and fourth column is for adipogenic differentiation (Adipo-ORO). The last three columns detail the status of differentiation gene expression profiling.

Methods: For long term osteogenic and adipogenic differentiation assays, cells were seeded in two sets at a density of 25,000 cells/well of a 6-well plate, or at a density of 100,000 cells/well of a 6-well plate for gene expression assays. Early osteogenic lineage commitment, as assessed by alkaline phosphatase assay, was carried out by seeding 100 cells per well in 12 wells of a 96-well plate. Osteogenic differentiation was induced in the second set of cells by replacing the growth media with osteogenic media (OM) after 24 hours of seeding. The control set continued in growth media. Plates for gene expression and alkaline phosphatase assays were harvested after 1 week in OM, while for long term differentiation studies, plates were harvested after 21 days in differentiation media. For gene expression, cells were harvested in 1 ml Qiazol (Qiagen). Seeding densities and cell harvest time points for adipogenic differentiation are identical to that of osteogenic differentiation. However, induction of adipogenic differentiation is started when cells reach ~80 confluency. Chondrogenic differentiation is carried out via the pellet culture, at a cell seeding density of $5x10^5$ cells per pellet, and pellets either harvested at day 28 for long term differentiation analysis, or on days 7 and 21 for gene expression analysis. Osteogenic and adipogenic differentiation were carried out at USU, while chondrogenic differentiation was carried out at UMN. Alkaline phosphatase (ALP) assay to measure early osteogenic differentiation has been repeated in 3 or more tissues as shown in the heatmap **(Figure 1)**. Alizarin Red (AR) staining to quantify calcium deposits at the end of 3 weeks of terminal osteogenic differentiation has been completed for at least 3 replicates for all tissues. Chondrogenic differentiation assays, by alcian blue (AB) staining and DMMB assay have been completed for 3 biological replicates for all nine tissues, as detailed below. Adipogenic differentiation, assessed by oil red O (ORO) staining has been repeated for two additional

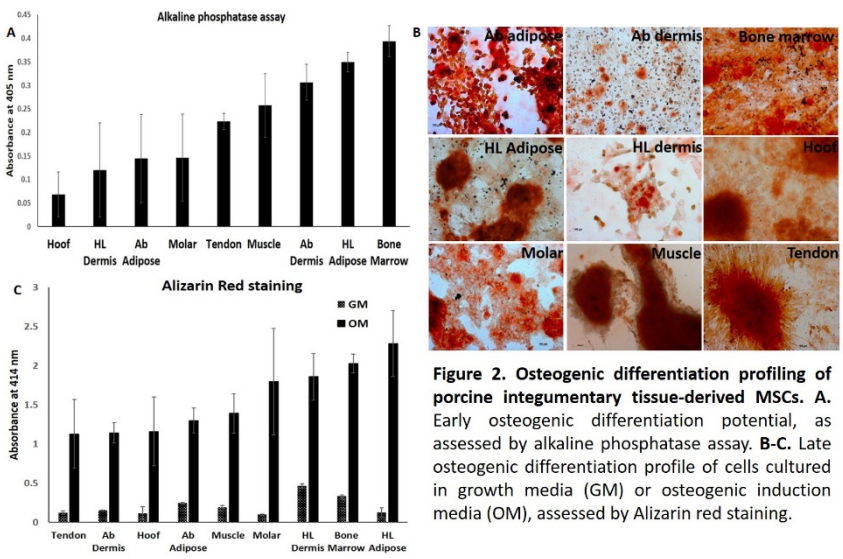

Figure 2. Osteogenic differentiation profiling of porcine integumentary tissue-derived MSCs. A. Early osteogenic differentiation potential, as assessed by alkaline phosphatase assay. **B-C.** Late osteogenic differentiation profile of cells cultured in growth media (GM) or osteogenic induction media (OM), assessed by Alizarin red staining.

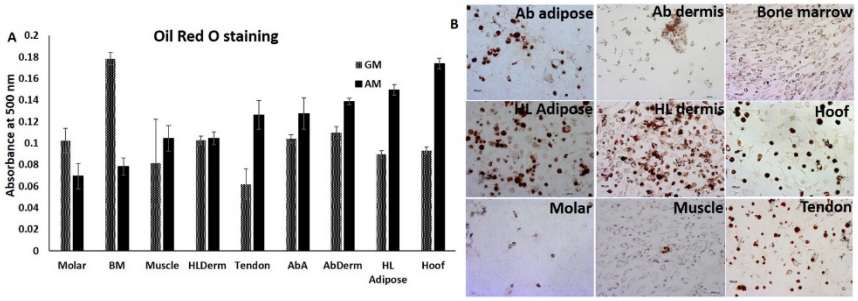

Figure 3. Adipogenic differentiation profiling of porcine integumentary tissue-derived MSCs. A. Quantification of adipogenic differentiation potential of porcine integumentary tissues cultured in either growth media (GM) or adipogenic media (AM) as assessed by Oil Red O staining **B.** Representative bright field images of cells cultured in AM

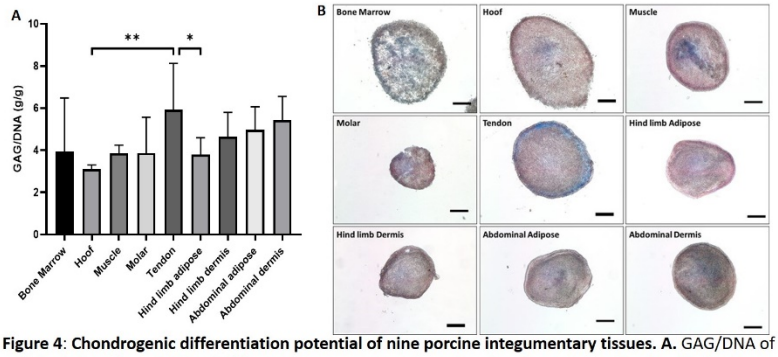

Figure 4: **Chondrogenic differentiation potential of nine porcine integumentary tissues. A.** GAG/DNA of pellets derived from MSCs of different tissue sources cultured in chondrogenic media after 21 days in culture. Data presented as mean ± SD (n=3 biological replicate with three pellets per replicate). * $p<0.05$ and ** $p<0.01$ **B.** Histological staining (alcian blue/nuclear fast red) of pellets derived from MSCs of different tissue sources cultured in chondrogenic media after 21 days in culture. Scale is 100 µm.

animals. Chondrogenic differentiation for three biological replicates for all nine tissues was completed at UMN in the past year. Passage 2-3 cells were used for all experiments. While Alcian Blue stain was used to qualitatively assess deposition of proteoglycans, DMMB assay was used to quantify differentiation by measuring levels of glycoaminoglycans.

Results: Multi-lineage differentiation assays have been completed for all tissues from at least 3 animals and gene expression studies have been completed for all tissues from at least 2 animals, in triplicates. HL adipose and bone marrow-derived cells demonstrate the highest early and late osteogenic differentiation potential (**Figure 2**), while HL adipose and hoof-derived cells demonstrated the highest adipogenic potential (**Figure 3**). Quantitative (GAG/DNA) and qualitative (histology) analysis of micromass cultures confirmed that the cells derived from the abdominal dermis and tendon showed the highest chondrogenic differentiation potential (**Figure 4**).

B. Characterization of early and late passage tissue-derived MSCs for expression of cell adhesion genes:

In order to identify progenitor cells with strong adhesion potential to metal and/or scaffold, cells were assessed for the expression levels of a panel of well characterized adhesion genes, namely vimentin, fibronectin, integrin B1, vinculin, CD9, CD151, integrin B2, CNTN3, laminin, collagens 1A1, 2A1 and 4A1. In order to determine the stage at which progenitor cells have highest level of expression of the adhesion genes, cells at both early (P1) and late passage (P5) were assessed for these genes. Expression analysis for 12 adhesion genes has been completed in all nine tissues in at least 2 animals in duplicates. All gene expression studies were carried out on cells seeded in a 6-well plate (1 x10^5 cells per well in GM), and harvested after 1 week in Qiazol. RNA isolation was carried out using the miRNeasy mini kit (Qiagen). cDNA synthesis (ABI) and Syber Green (Bio-Rad) based quantitative Real-Time PCR was run on Quant-Studio (Applied Biosystems). β-Actin was used as the housekeeping gene.

Results: Out of the 12 genes tested, vimentin showed the highest level of expression in abdominal dermis, muscle and tendon, followed by vinculin, Col1A1, integrin B1, fibronectin, integrin B2, as shown in **Table 2**. Late passage cells for all four tissues had significantly higher expression of adhesion genes, compared to the early passage cells. Muscle and hoof derived cells had relatively high expression of the majority of adhesion genes (6 genes), followed by HL dermis with high expression of 5 adhesion genes. Based on these results, **A.** late passage tissue-derived cells appear to be a better candidate for seeding on scaffolds, and **B.** Muscle, hoof and abdominal dermis-derived MSCs have the highest expression of multiple genes encoding ECM proteins and regulating cell-cell and cell-matrix adhesion.

Adhesion gene	Top 3 tissues with highest expression		
Vimentin	Ab dermis	Muscle	Tendon
Vinculin	Ab adipose	HL Dermis	Bone Marrow
Col1A1	Ab adipose	HL Adipose	Hoof
Integrin B1	Ab dermis	Hoof	Muscle
Fibronectin	HL Dermis	Bone Marrow	Molar
Integrin B2	Ab adipose	HL Dermis	Bone Marrow
CD9	HL Dermis	Hoof	Muscle
CD151	HL Adipose	Hoof	Muscle
Col4A1	Ab adipose	Ab Dermis	HL Dermis
Laminin	Hoof	Muscle	HL Adipose
CNTN3	Muscle	Tendon	Hoof

Table 2. List of adhesion genes in order of decreasing expression. qRT-PCR based expression analysis of adhesion genes in nine integumentary tissue-derived Passage 5 MSCs cultured in growth media. Vimentin showed the highest expression among all tissues. The top 3 tissues with highest expression for each gene has been shown.

Result summary of Subtask 1.1

Tri-lineage differentiation profiling of MSCs derived from nine integumentary tissues from 3 or more animals demonstrate that **MSCs derived from the hind limb adipose possess the highest osteo- and adipogenic differentiation potential, while cells of the tendon and abdominal dermis have highest chondrogenic differentiation potential.** Expression profiling of adhesion genes at early and late passages indicates highest expression of multiple ECM, cell-cell and cell-matrix adhesion genes in muscle, hoof and abdominal dermis vimentin in late passage (P5) cells. Put together, MSCs derived from hind limb adipose and abdominal dermis appear to be the ideal cell populations for adherence to scaffold-coated titanium implants and to the surrounding matrix, in addition to undergoing multi-lineage differentiation, thereby providing a strong, but flexible attachment of the implant to the surrounding dermal interface tissue. This will be tested in the next phase of *in vivo* preclinical studies in a porcine model.

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

The main focus of this subtask was to compare the performance of iPSCs (induced pluripotent stem cells) derived MSCs (iMSCs) relative to bone marrow derived MSCs. This was done by comparing the surface marker expression of different cells (iMSCs, BM-MSC1 and BM-MSC2; BM-MSC1 being commercially available bone marrow derived MSCs from Lonza, USA and BM-MSC2 was kindly provided by Tolars lab at the University of Minnesota). Cell function was assessed by comparing the capability of cells to undergo tri-lineage differentiation. We also assessed the capability of conditioned media collected from different MSCs in supporting proliferation, migration and wound closure capabilities of human dermal fibroblasts (HDFs) and epithelial keratinocytes (HaCaTs, AddexBio, USA).

Method: Different MSCs were cultured according to methods mentioned in previous reports. Briefly, cells were cultured in FBS supplemented MEM media with 1ng/ml b-FGF. Comparative analysis of surface marker expression was assessed using flow cytometry. Tri-lineage differentiation of different MSC sources was also performed where cells were cultured either in 2D (osteogenic and adipogenic differentiation) or micromass cultures (chondrogenic differentiation) for 3 weeks in defined culture conditions to support cell differentiation. Following three weeks of culture, cells were fixed and stained with alizarin red (matrix mineraliztion) or Oil Red O stain (oil droplets formation) and stain was quantified by eluting in 10% acetic acid or 100% isopropyl alcohol. For assessing chondrogenic differentiation, micromass pellets were digested in 1mg/ml proteinase K after which secreted glycolsaminoglycans was quantified using DMMB assay which was then normalized to total DNA content.

a.

Surface Marker	Cell Type		
	iMSC	BM-MSC1 (Lonza)	BM-MSC2 (Patient derived)
CD14	0.8	5	0.5
CD45	0.3	0	0.3
CD44	99.8	100	100
CD73	90.9	99	100
CD90	85.4	100	100
CD105	99.7	100	100

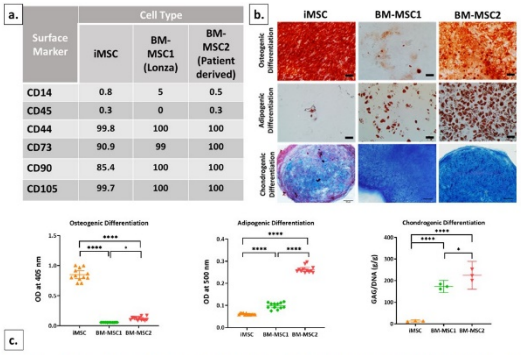

Figure 5: In vitro characterization of human cells. Summary of surface marker expression (a), characterization of terminal osteogenic, adipogenic and chondrogenic differentiation of different MSCs (b) and quantification of alizarin red, Oil Red O and DMMB assay based GAG content, normalized to CyQuant based total DNA content (c). One way ANOVA with post hoc Tukey test was used for statistical analysis. * p<0.05

Results: Surface marker analysis by flow cytometry confirmed that 85% of cell population of iPSCs derived MSCs were positive for markers associated with MSCs and did not express CD14 and CD45 **(Figure 5a)**. Whereas BM-MSC1 and BM-MSC2 had 99% cell population positive for MSC-associated markers. Tri-lineage differentiation confirmed that different MSC sources showed tri-lineage differentiation, with iMSCs being more committed towards the osteogenic lineage than bone marrow derived cells **(Figure 5b,c)**. On the contrary, BM-MSCs (BM-MSC1 and BM-MSC2) favored differentiation into chondrogenic and adipogenic lineages.

Effect of conditioned media (CM) from different MSC on keratinocytes and dermal fibroblasts

MSCs are known to secrete a wide array of growth factors and cytokines that show positive implication on cells associated with wound healing. This part of the study was to assess the effect of MSCs secretome (by means of using MSCs conditioned media) on proliferation, migration and wound healing capability of keratinocytes and dermal fibroblasts.

Method: Different MSCs were expanded in expansion media (MEM supplemented with FBS and b-FGF) until cells were 90% confluent. Once the desired confluency was achieved, cells were washed with PBS and were cultured in serum starved media (half the normal culture volume) and cells were cultured for another 72h followed by collection of the 'conditioned media' (CM). To prepare the CM for different analysis, the media was centrifuged to remove cell debris and filtered using 0.22 µm filter and stored in -20°C until use. HaCaTs (AddexBio) and HDF (ATCC) were used in this study as model cells associated with wound healing. Cells were cultured in serum supplemented DMEM media and were passaged when the cells were 80% confluent. For assessing the effect of CM collected from different MSCs, HaCaTs and HDFs were plated in a 48 well plate at a density of 7200 cells/well in presence of CM. Cells were cultured in MEM media (used for collecting CM) as negative control and serum supplemented DMEM as positive control.

Metabolic activity of the cells at day 2 and day 4 was determined using Alamar Blue following manufacturers instructions. The fluorescence (Synergy HT, Biotek, USA) was measured at 540/590 excitation/emission wavelength and was used as a measure of cell proliferation. For cell migration, transwells (Corning, 8 µm, PET) were used. For assessing the effect of CM to support cell migration through transwell membrane, HaCaTs ($5x10^4$ cells/membrane) and HDFs ($1x10^4$ cells/membrane) were seeded on the membrane. The bottom chamber of the well included either CM from different MSCs or MEM media (negative control) or serum supplemented DMEM (for HDFs) or 50ng/ml TGF-β1 added to serum supplemented DMEM (for HaCaTs) as positive control. Cells were cultured for 24h after which the number of cells that migrated through the membrane was determined using crystal violet stain. Briefly, cells in the upper chamber of the transwell were removed using cotton swab after which the cells were washed and stained with 0.1% crystal violet for 10 minutes followed by washing the membranes in PBS. Optical micrographs from different fields of view were taken to quantify number of cells that migrated through the transwell membrane.

To determine the effect of CM on wound healing capability of HaCaTs and HDFs, cells were cultured in 48 well plate at a density of $0.25x10^6$ cells (HaCaTs) and $0.12x10^6$ (HDFs) per well in 24 well plate. Cells were cultured for 24h until a confluent monolayer was formed. A 'wound' was created in this monolayer using 200 µl pipette tip and washed several times with PBS to remove cell debris. Cells were then exposed to different culture conditions (CM from MSCs, DMEM+10%FBS (positive control for HDFs) or 50ng/ml TGF- β1 supplemented DMEM+10%FBS (positive control for HaCaTs) or MEM media (negative control)). Micrographs were taken at t-0 to determine area of initial wound after which cells were cultured for another 48h. Micrographs were taken at t-24h and t-48h to determine area of wound closure.

Results: Assessing the effect of CM on proliferation of HaCaTs and HDFs using Alamar Blue confirmed significantly higher levels of cell proliferation relative to the positive control and negative control groups (**Figure 6**). The results confirmed that the CM derived from iMSCs performed on-par and in some cases outperformed CM collected from BM-MSCs, thereby supporting the utilization of iMSCs as a replacement for primary MSCs for our future in vivo experiments.

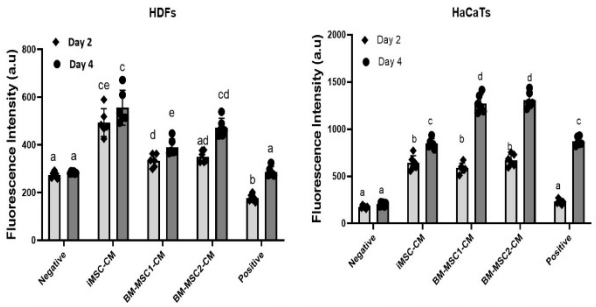

Figure 6: HDF and HaCaT proliferation under different treatment conditions. One way ANOVA with post hoc Tukey test was used for statistical analysis; p<0.05 is considered significant. The same alphabet represents non-significant differences.

Transwell migration assay confirmed the presence of bioactive cues in CM that support migration of both keratinocytes and fibroblasts through the transwell membrane **(Figure 7)**. For HDFs, significantly high levels of migration were observed in presence of MSC-derived CM relative to the positive control whereas for HaCaTs, similar levels of migration were observed in CM-treatment groups relative to positive control.

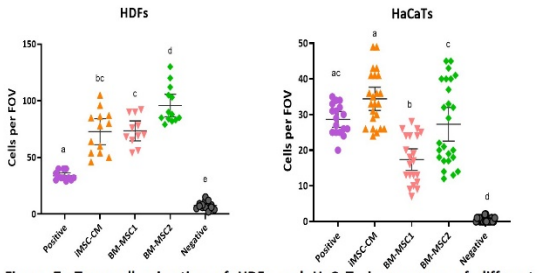

Figure 7: Transwell migration of HDFs and HaCaTs in presence of different treatment conditions. One way ANOVA with post hoc Tukey test was used for statistical analysis p<0.05 is considered significant. The same alphabet represents non-significant differences.

For wound healing assay, keratinocytes showed a higher rate of wound closure in presence of CM whereas complete wound closure was observed after 48h under different treatment conditions, except for the negative control **(Figure 8A-B)**. On the contrary, HDFs showed faster wound closure at 24h time point for all the treatments except the negative control. Complete wound closure was also observed for HDFs after 48h for different conditions barring negative control **(Figure 8C-D)**.

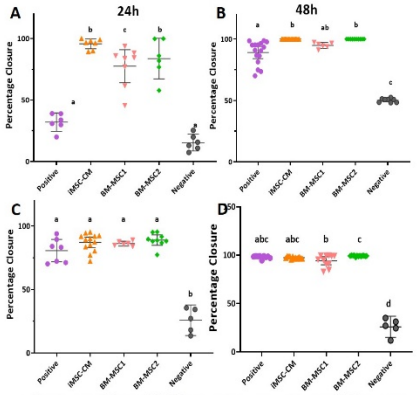

Figure 8: Effect of different treatment conditions on wound closure after 24h and 48h in HaCaTs **(A-B)** and HDFs **(C-D)**. One way ANOVA with post hoc Tukey test was used for statistical analysis p<0.05 is considered significant. The same alphabet represents non-significant differences.

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

The experiments are being planned while titanium substrates are being polished and will start over in the next quarter. The objective of the experiment is 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.

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

Subtask 3.1: Subtask 3.1: Complete scaffold design (constructs) for "sleeve" and "transition designs. Decision on collagen scaffold

The collagen sponge procured from Integra didn't support cell infiltration and acted more like a 2D substrate instead of 3D porous scaffold. This is not ideal for a cell delivery system which is why alternative cell-delivery system was investigated. For this purpose, we have chosen methacryloyl-functionalized gelatin as a potential alternative to the use of collagen membranes/scaffolds. Functionalized gelatin enables the type of multi-functionality needed 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 does not only facilitate light-activated in-situ cross-linking but also supports cell proliferation and function. The scaffold properties especially the viscosity and stiffness can be altered by changing the macromer (pre-polymer) concentration. How this alters the viability and proliferation of porcine and human cells is still being investigated under our experimental conditions. Visible-light mediated crosslinking using Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Sigma, USA) photo-initiator has been selected **(Figure 9)**.

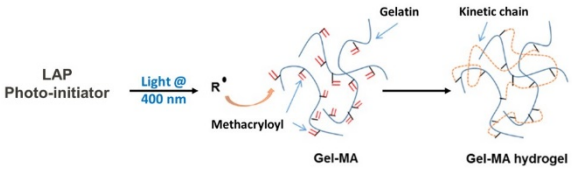

Figure 9: Schematic representation of LAP-mediated photo-crosslinking of GelMA

Purpose: To select optimal physical chemical properties of GelMA scaffold using design of experiments.

Method: A 2^3 design of experiments (DoE) was used to determine main effects and interactions of three variables on physico-mechanical properties of different hydrogel formulations. The low and high levels of macromer concentration, photo-initiator (LAP) concentration, and time of crosslinking were set to 5 and 10 weight%, 0.05 and 0.1 weight% and 30 and 90 seconds, respectively. Based on DoE, 8 different combinations were achieved which were then used to assess the outcomes. The outcomes that were assessed include soluble fraction (measure of uncrosslinked macromer), swelling ratio (measure of water retention capability) and compressive moduli (measure of stiffness).

Briefly, different macromer concentrations (5 wt%, or 10 wt%) prepared by dissolving lyophilized GelMA in PBS were mixed with 0.05% or 0.1% LAP (prepared in PBS) and cross-linked in silicon molds (6 mm diameter, 1 mm thickness) using light with maximum at 400 nm for 30 or 90 seconds. Prepared hydrogel discs were used to determine soluble fraction (macromer crosslinking efficiency) swelling ratio and compressive moduli (determined using Mach1, Biomomentum). To calculate soluble fraction and swelling studies, six samples were weighed immediately after cross-linking to obtain initial wet mass ($m_{initial}$, t0), and three samples were lyophilized to obtain their dry weights (m_{dry}, t=0). The actual macromer fraction was then calculated using equation (1).

actual macromer fraction = $\frac{m_{dry;\,t0}}{m_{initial;t0}}$ (1)

The remaining samples were incubated in PBS at 37 °C to allow swelling and the soluble fraction to leach out of the hydrogel network. After 24 h, the samples were weighed again ($m_{swollen}$). The samples were subsequently lyophilized and weighed a final time (m_{dry}). The sol fraction was defined as the mass loss after 24 h and was calculated using equations (2) and (3). Mass swelling ratio (q) was calculated using equation (4).

$m_{initial;\ dry} = m_{initial}$ (actual macromer fraction) (2)

$$\text{sol fraction} = \frac{m_{initial,dry} - m_{dry}}{m_{initial,dry}} \times 100\% \quad (3)$$

$$q = \frac{m_{swollen}}{m_{dry}} \quad (4)$$

Results: Based on DoE, 8 different combinations were used and the effect of different variables on each measured outcome is represented below. The results suggest that for the tested variables, time is a predominant factor that positively influences physico-mechanical properties of the hydrogels resulting in reduced soluble fraction and swelling ratio along with increased compressive modulus (**Figure 10**). The effect of the tested variables on hydrogel adhesivity to skin and metal still need to be investigated which will be the primary focus for the next quarter along with assessing the handleability as a measure of viscosity. Along with the material characterization, biological performance of keratinocytes on different scaffold system will also be investigated on cell-free and MSC-containing hydrogel scaffolds.

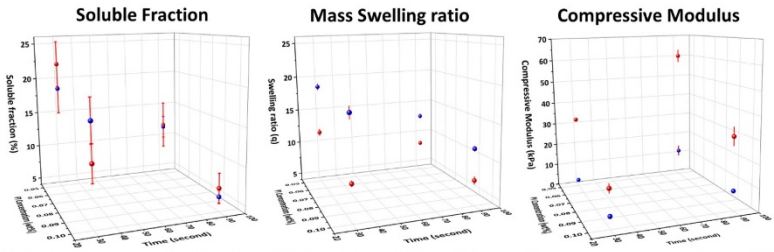

Figure 10: Effect of different tested variables on the soluble fraction, swelling ratio and compressive modulus of the hydrogel. The red dots correspond to macromer concentration of 10 weight% and blue for 5weight%.

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

Purpose: To study the cell survival and proliferation of human iMSCs encapsulated within GelMA hydrogels of different stiffnesses

Method: Expanded human iMSCs cultured in MEM media supplemented with 1% Pen-Strep, 10% FBS and 1 ng/ml bFGF were used for the experiment. Passaged cells were incorporated within sterile GelMA (5, 7.5 and 10 wt%) and photo-initiator (0.05 wt% LAP) at a density of 5 x 10^6 cells/ml and cell-laden discs were prepared in silicon molds (6 mm diameter, 1 mm thickness) and crosslinked for 30 seconds. Prepared cell-laden hydrogel discs were cultured in FBS supplemented MEM media for up to 7 days. Cell metabolic activity was assessed using Alamar Blue cell viability reagent (Thermo Fisher Scientific) assay and live-dead staining (calcein (live) and propidium iodide (dead) stain) (Sigma, USA) used at day 1. For alamar blue assay, at each time point, cell culture media was replaced with media containing 10% CCK8 reagent and cells incubated for 3 h. After this 150 µL of the alamar blue/media solution was transferred to a 96-well plate for fluorescence reading (Synergy, BioTek – excitation @ 560 nm and emission @ 590 nm) expressed as fluorescence intensity. For live-dead staining, samples were transferred to fresh plate, washed with PBS twice and incubated with working solution of stains prepared in PBS (1 µg/ml of calcein

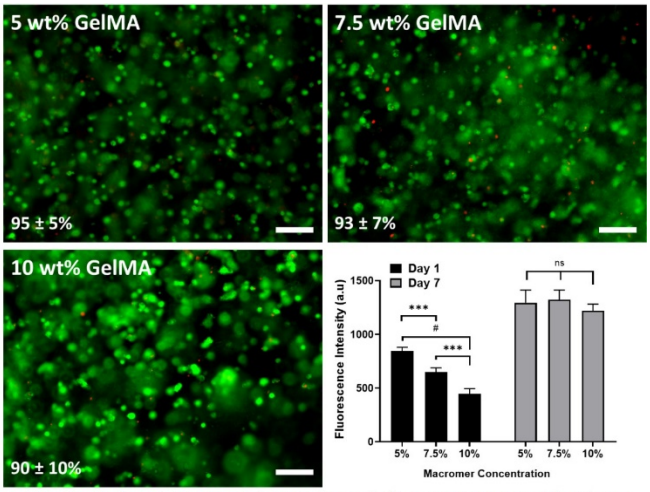

Figure 11: Live/Dead staining at day1 and metabolic activity measured by Alamar Blue at day 1 and 7. Scale - 100µm.

and 1 µg/ml of PI) for 10 minutes after which samples were washed twice with PBS. Samples were then imaged on Leica DM6 B upright microscope and cell viability measured by calculating percentage number of green-labelled cells to the total number of cells (green and red – labelled) in a given field of view (FOV).

Results: Alamar Blue assay confirmed cell survival and proliferation for the cells encapsulated within different GelMA hydrogels. Day 1 showed significant differences in metabolic activity, with lower metabolic activity for stiffer hydrogels. However, by day 7, significant increase in metabolic activity relative to day 1 was observed with no significant differences observed within different sample groups (**Figure 11**). Live/dead imaging confirmed cell viability higher than 90% or cells encapsulated within different hydrogel systems suggesting that the cross-linking mechanism was not detrimental to the cells (**Figure 11**).

Purpose: To assess cell migration from cell laden hydrogels of different stiffnesses (5, 7.5 and 10 wt%) onto underlying G5 titanium substrate or surrounding cell free hydrogel.

Method: Cell laden macromer of different macromer concentrations was prepared as discussed previously, was cross-linked onto titanium substrate and cultured for 7 days, following which the cells were stained with calcein/PI and Z-stack images collected to determine cell migration (**Figure 12A**). For migration onto hydrogel, 5 wt% GelMA was used to mimic skin as the stiffness of 5% GelMA is comparable to skin and using GelMA allows flexibility to chemically crosslink cell-free, skin mimicking hydrogel to the cell-laden hydrogel (**Figure 13A**)

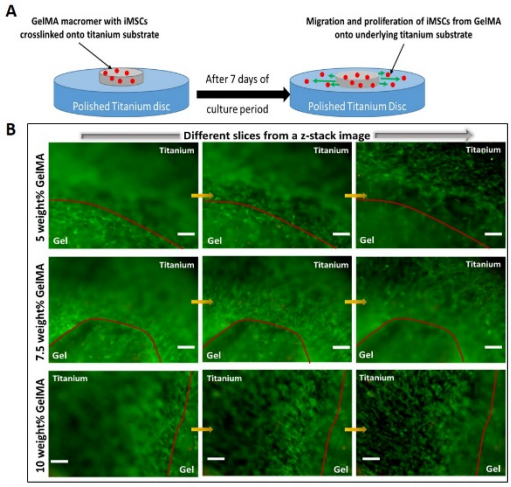

Figure 12: Migration of encapsulated iMSCs from GelMA hydrogels of varying stiffnesses onto underlying Grade 5 titanium substrate. Red line indicates the interface between hydrogel and titanium substrate. Scale - 100µm

The experiments are being repeated and their effect on adhesivity will be determined using lap-shear test. The adhesivity will be measured with and without cells and the ideal candidate will be tested with selected porcine MSCs.

Results: The results showed no significant differences in cell survival and migration onto titanium (**Figure 12B**) and cell-free (CF) soft GelMA (**Figure 13B**) from different GelMA hydrogels of varying stiffnesses prepared by using different macromer concentrations.

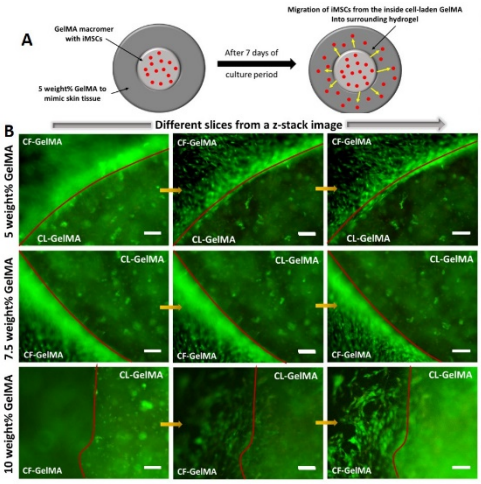

Figure 13: **Scaffold design rationale. A.** Diagrammatic representation of scaffold-cell seeding, and projected outgrowth of cells after a week of seeding. **B.** Migration of encapsulated iMSCs from GelMA hydrogels of varying stiffnesses into surrounding soft GelMA (5 wt%) hydrogel. The red line indicates the interface between cell-laden (CL) hydrogel and surrounding cell free (CF) GelMA. Scale - 100µm

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

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training"

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

Nothing to Report

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

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

Nothing to Report

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

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

USUHS

- Submit animal use protocol for IACUC review and approval for the next phase of *in vivo* studies
- Completion and submission of the manuscript on the comparative analysis and characterization of multiple porcine integumentary tissue-derived MSCs for use in optimizing the osseointegrated skin-implant interface.
- Continue expansion and biobanking of selected tissue-derived MSCs (HL adipose, abdominal dermis, tendon) for *in vivo* studies

MMRF-UMN

- Assessing the adhesion and proliferation of iPS-derived MSCs and keratinocytes on titanium alloy and glass substrates.
- Cytokine analysis of CM collected from different MSC sources (iMSCs and BM-MSCs)
- Detailed characterization of the adhesiveness of the scaffolds to metal (titanium alloy-grade 5) and skin-mimicking tissue using lap-shear tests based on ISO-standards.
- Optimize the scaffold physico-mechanical properties to enhance scaffold toughness and adhesivity without interfering with its biocompatibility.
- Perform similar characterization with the selected porcine MSC candidate to ensure cell survival and proliferation within GelMA – based hydrogel system.
- Assess adhesion and proliferation of pMSCs and iMSCs on collagen sponges and/or clinically used sponge/scaffold for wound healing application with different scaffold parameters by DoE.

- Manuscript preparation on the use of iMSCs-laden scaffold for epithelial attachment onto metal abutment for improved performance of osseointegrated devices: in vitro study.

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.

Since March 9th, 2020 a modest amount of new studies and experimentation covered under this proposal have been initiated due to the covid-19 situation. Only work on critical/essential in-progress studies has been permitted within USUHS and UMN under strict social distancing guidelines. Phase-I reopening at USUHS (25% of personnel) commenced on June 22, 2020. Animal studies have been restricted to only mission critical projects, and animal housing significantly reduced. This might impact the next phase of in vivo studies at USU. We have completed drafting of the animal protocol, which we plan to submit to IACUC within the next quarter.

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

Not applicable

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).*

  Orthopedic Osseointegration: Implantology and future directions. Overmann AL, Aparicio C, Richards JT, Mutreja I, Fischer NG, Wade SM, Potter BK, Davis TA, Bechtold JE, Forsberg JA, **Dey D.** J Orthop Res. 2020 Jul;38(7):1445-1454

  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.*

- I. Mutreja, K. Twaroski, J.E. Bechtold, J. Tolar, J.A. Forsberg, C. Aparicio. Human iMSC- laden hydrogel scaffold for improving the durability of skin/implant interface. ORS 2020 Annual Meeting. Orthopedic Research Society. Phoenix, AZ, 2020. Poster presentation.

- Mutreja, K. Twaroski, J.E. Bechtold, J. Tolar, J.A. Forsberg, C. Aparicio. Human iMSC encapsulated Gelatin-based hydrogel for improving the durability of skin/implant interface. Military Health System Research Symposium 2020 (Meeting cancelled due to COVID-19 outbreak).

- I. Mutreja, K. Twaroski, J.E. Bechtold, J Tolar, J.A. Forsberg, C. Aparicio. Optimization, Feasibility and Cellular Interactions of a Human iMSCs-laden photocurable hydrogel for improved durability of the skin/implant interface. Submitted to ORS 2021 Annual Meeting. Orthopedic Research Society. Virual Conference, 2021.

- **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*
Nearest person month worked: *3*
Contribution to Project: CAPT Forsberg responsible for overall project coordination.

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

Name: *Thomas A. Davis, PhD*
Project Role: *Associate Investigator (USUHS)*
Nearest person month worked: *3*
Contribution to Project: Oversight of project conducted at USUHS.

Name: *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.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

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

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

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc. available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *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.