AWARD NUMBER: W81XWH-17-2-0059

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

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REPORT DATE: OCTOBER 2021

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
OCTOBER 2021	Annual Progress report	09/15/20-9/14/21

4. TITLE AND SUBTITLE

Optimizing Skin-Implant Interface of Osseointegrated Device

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

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry M Jackson Foundation, 6720a Rockledge Dr, Suite 100, Bethesda, MD 20817

8. PERFORMING ORGANIZATION REPORT

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

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

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

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

15. SUBJECT TERMS
NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	**U**	**U**	UU	50	**19b. TELEPHONE NUMBER** *(include area code)*

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

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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 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**	90%
Major Task 1: Develop cell culture	1-16	100%
Subtask 1.1: In vitro isolation and characterization of *porcine* cells.	1-16	100%
Subtask 1.2: In vitro development and characterization of *human* cells.	1-16	100%
Major Task 2: *In vitro*: evaluate cell adhesion to metal substrate	7-16	100%
Subtask 2.1a: Test porcine cells for adhesion	7-16	100%
Subtask 2.1b: Test human cells for adhesion	7-16	30%
Major Task 3: Scaffold development for cell growth and anchorage to underlying tissue.	5-24	50%
Subtask 3.1: Complete scaffold design (constructs) for "sleeve" & "transition designs	5-16	90%
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	90%
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.

Characterization of MSC cell surface marker expressions of nine porcine tissue-derived MSCs

Objective: We had completed the subtask 1.1 but based on a reviewer comment after a manuscript submission, we evaluated the immunophenotypic cell surface expression of MCS-derived cells by Fluorescence-Activated Cell Sorting (FACS).

Methods: Cells were washed once with Phosphate-Buffered Saline (PBS) and detached with 15mM EDTA in PBS solution for 12min at 37°C. Cells were harvested after addition of DMEM media complemented with 10% FBS, centrifuged and counted. A minimum of 200,000 cells by condition was transfer into a FACS tube. Cells were washed twice by adding 2ml of FACS buffer and following by a centrifugation for 5min at 1400 rpm. The supernatant was removed and the cell pellet was stained with 0.5 µg of antibody in 100 µl of FACS buffer for 30min on ice. The antibodies used were Mouse IgG1 isotype control FITC conjugated (Cell Signaling technology, #50-204-9474), CD44 Monoclonal Antibody FITC conjugated (Invitrogen, #MA1-10228), CD90 Monoclonal Antibody FITC conjugated (Invitrogen, #A15761), ITGB1 Monoclonal Antibody FITC conjugated (CD29) (Invitrogen, #MA1-19566), Mouse IgG2a Isotype Control FITC conjugated (Invitrogen, #PA5-33239) and CD105 Monoclonal Antibody FITC conjugated (Novus Biologicals, #NB11081749). Cells were washed twice with 2ml of FACS buffer and following by a centrifugation for 5min at 1400 rpm. After the last wash, 300 µl of FACS buffer was added to the cell pellet and cells were kept on ice until the FACS analysis. Five minutes before the fluorescence reading using BD LSRII Flow Cytometer, 1ul of SYTOX™ Blue Dead Cell Stain (Invitrogen, #S34857) was added to the cells.

Results: The immunophenotypic cell surface expression of MSCs cell surface markers CD29, CD44, CD90 and CD105 was determined using FACS on Hoof-derived pMSCs, tendon-derived pMSCs, abdominal dermis-derived pMSCs and hind limb adipose cells (**Figure 1**). We found that all of the MSC-derived cell types tested strongly expressed CD29, CD44 and CD90. The CD105 marker is less expressed comparing to CD29, CD44 or CD90 but still detectable in all of the MSC lines comparing to its isotype control. We currently, expanding the other five MSC-derived cell types. Once this work is completed within the next 2-weeks, we will include these findings along with other manuscript revisions and then resubmit for peer-reviewed publication.

Conclusion: MSCs isolation from different pig tissues share a similar mesenchymal cell surface marker ($CD29^+$, $CD44^+$, $CD90^+$ and $CD105^+$) immunophenotype.

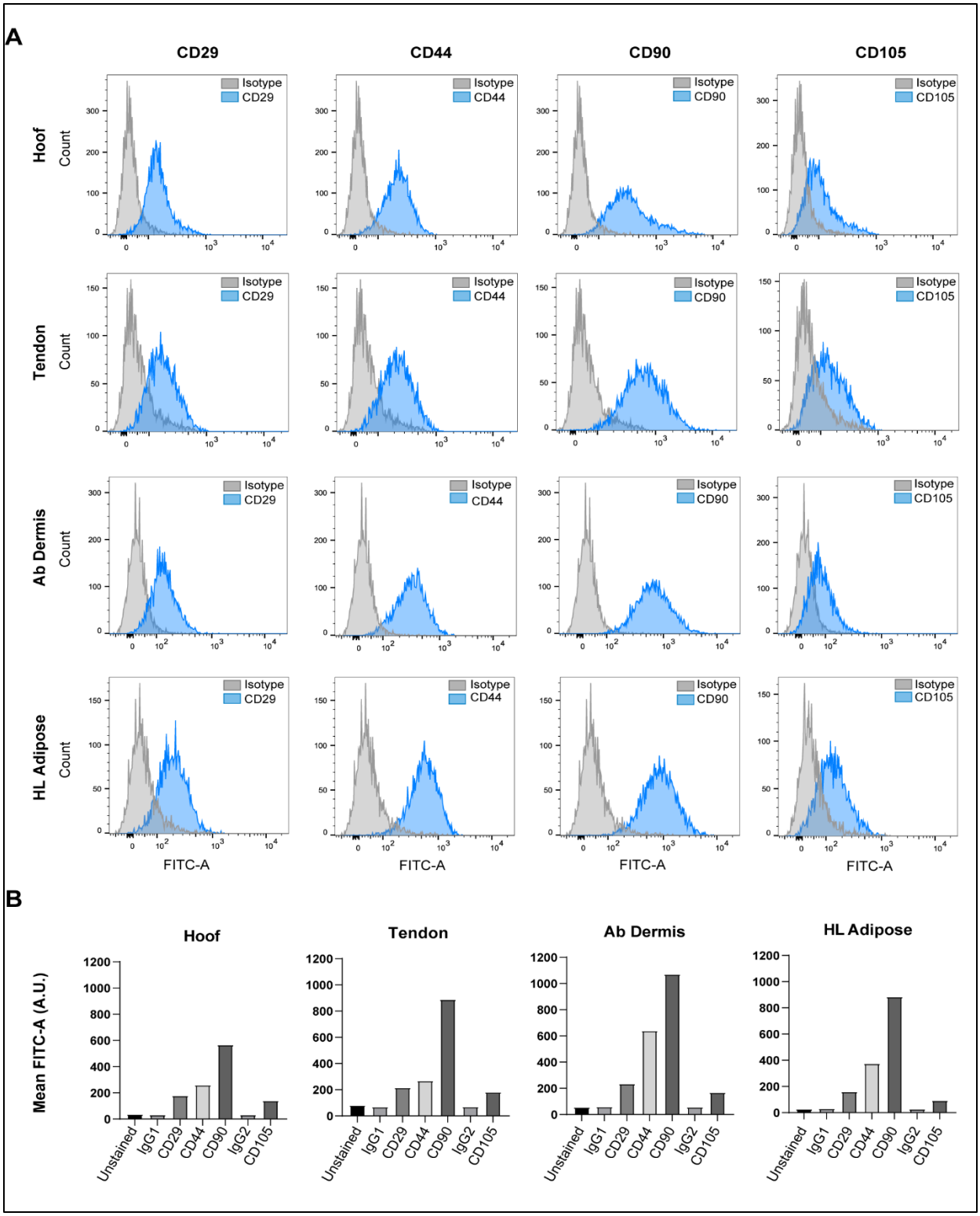


Figure 1: A. Representative FACS histograms showing positive expression of MSCs markers (CD29, CD44, CD90 and CD105) (Blue histogram) comparing to their isotype controls (light grey histogram). B. Mean of fluorescence intensity (FITC-A) for unstained and stained cells, n=1 experiment

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

Objective: The purpose of this task was to further characterize the human iPSC derived MSCs (iMSCs) bydetermining the trophic factors released by iMSCs and compare it with bone marrow derived MSCS (BM-MSCs). Ray Biotech Human Cytokine Array C5 kit was used to assess an array of 80 cytokines released to identify factors released by MSCs which can play an important role in wound healing.

Methods: Human iMSCs and BM-MSCs (commercial cells purchased from Lonza (BM-MSC1) and other cells kindly provided by Tolar's lab, University of Minnesota (BM-MSC2)) were expanded in expansion media (MEM media with 10% FBS and 1ng/ml bFGF) until the cells were 90% confluent. Cells were washed with PBS and cultured in MEM media without FBS for another 72 h to collect conditioned media (CM). After 72 h, the CM was collected, centrifuged to remove cell debris, filtered and used to perform cytokine array following manufacturers protocol. The membrane was read on BioRad under chemiluminescence settings. The intensity of each of the blot on the membrane was measured and normalized against the positive control spots on each membrane. The collected data for all the 80 trophic factors for the CM collected from the three MSC sources is presented as heat map. Also, factors comparison of some of the key factors that play an important role in wound healing were quantified and compared to assess differences, if any between the CM collected from different MSCs.

Statistical Analysis: The data analysis for statistical significance was performed using the GraphPad Prism 9.0.0 software. Data is represented as scatter plot as mean ± standard deviation of n = 3 replicates. After validating normality of sample distribution and homogeneity of variance, one-way analysis of variance (ANOVA) with Tukey test was used to assess the statistical significance between groups at p-value <0.05.

Results: The results from the cytokine array confirms that between the three different MSC sources, there was not notable differences in their released cytokine profile, data presented as heat map in **Figure 2**. However, a closer look on some of the cytokines that play an important role in wound healing revealed that they were significantly higher in CM collected from iMSCs relative to the BM-MSCs. This included IL-8, Angiogenin and IGF; which positively influence cell migration and proliferation and in some cases influence vascularization. Some other cytokines that showed higher expression in CM collected from iMSCs and play an indirect role in wound healing included Gro-α (helps in re-epithelialization) and IL-7 (assists in keratinocyte migration). High expression of TIMP1, TIMP2 and IGFBP-2 was observed in the CM collected from all three MSCs (**Figure 3 and 4**).

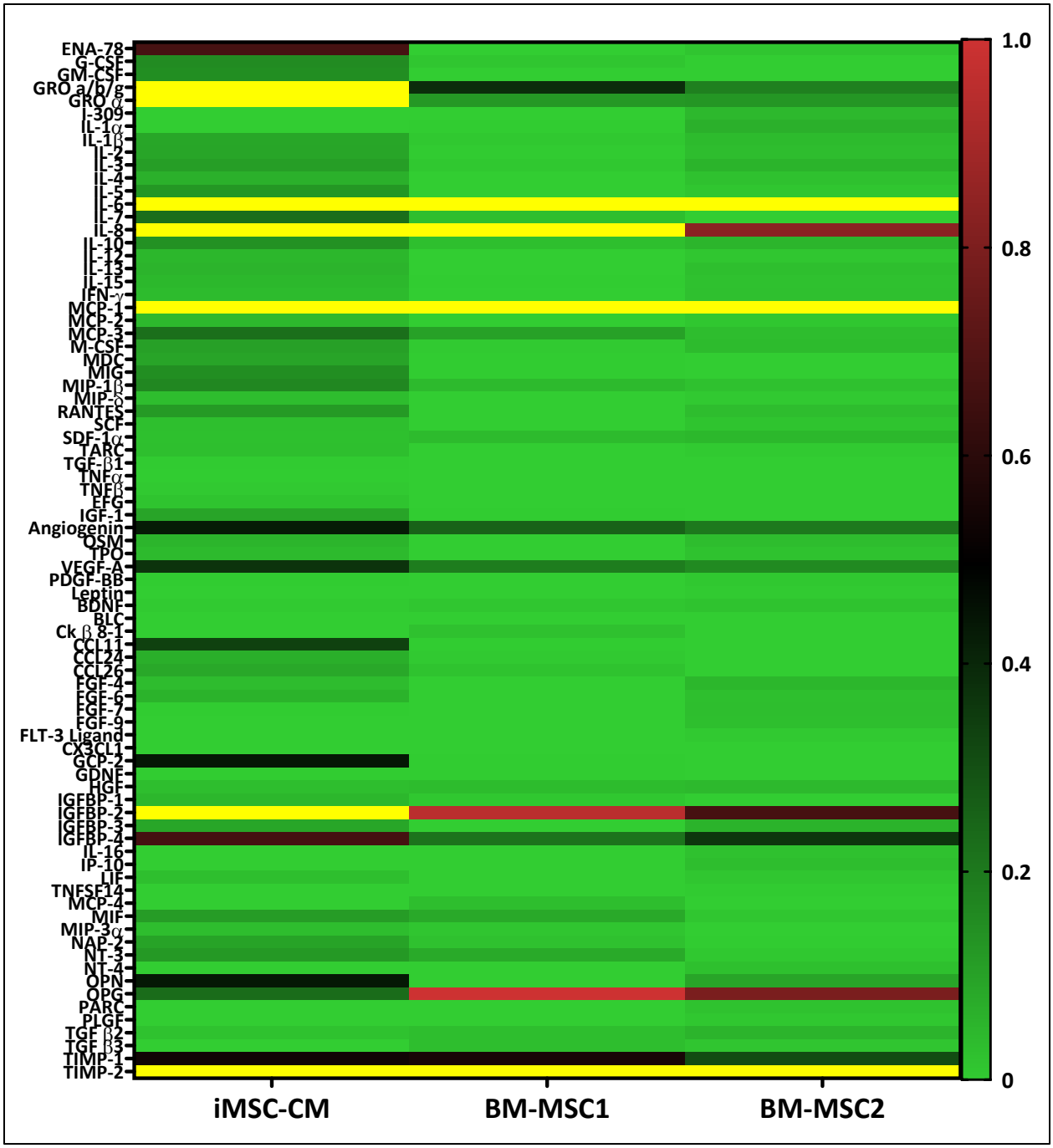


Figure 2: Heatmap of the cytokines present in CM collected from iMSC, BM-MSC1 and BM-MSC2. Bands colored yellow showed expression of those cytokines higher than 1.

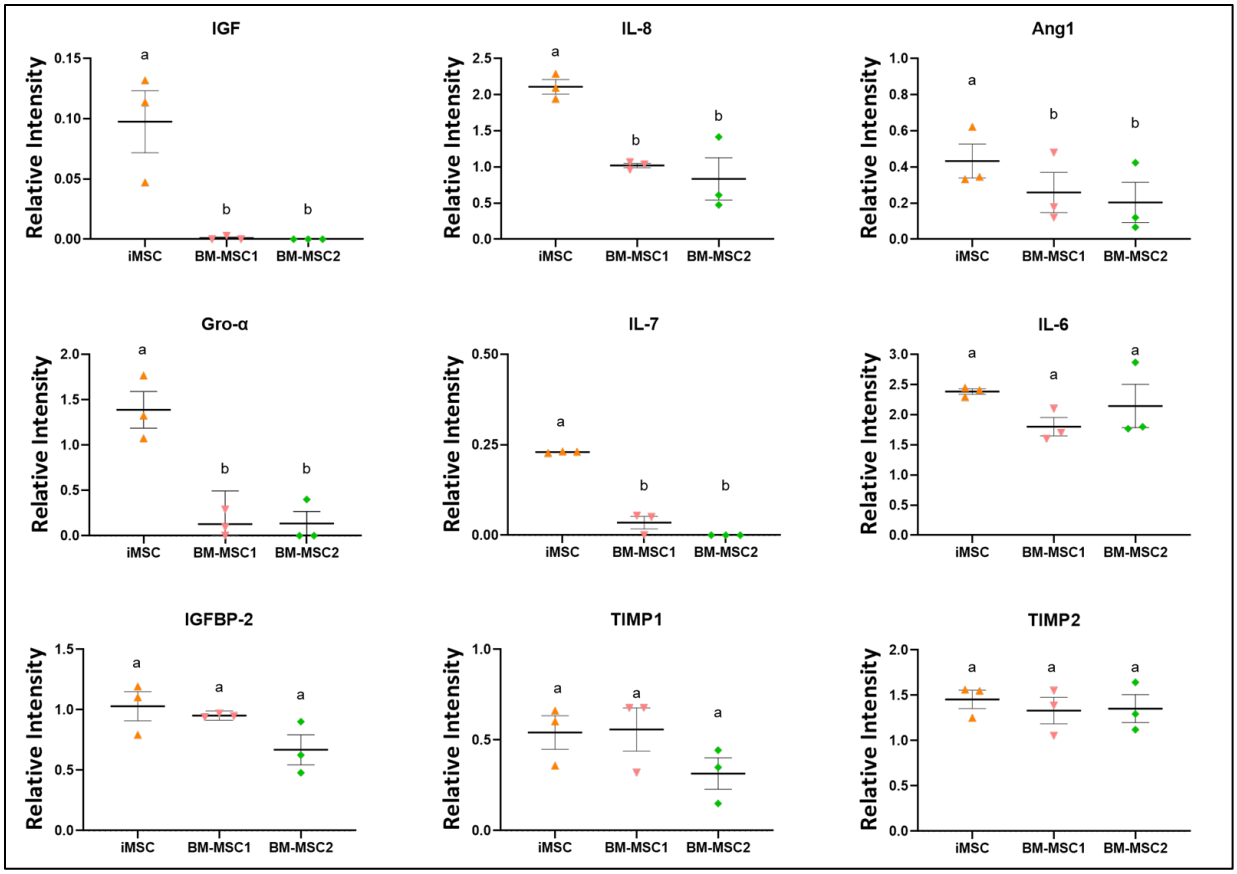


Figure 3: Quantitative analysis of the cytokines present in the CM collected from different stem cell sources which play an important role in wound healing by controlling cell migration and proliferation, cell apoptosis and pro-angiogenesis.

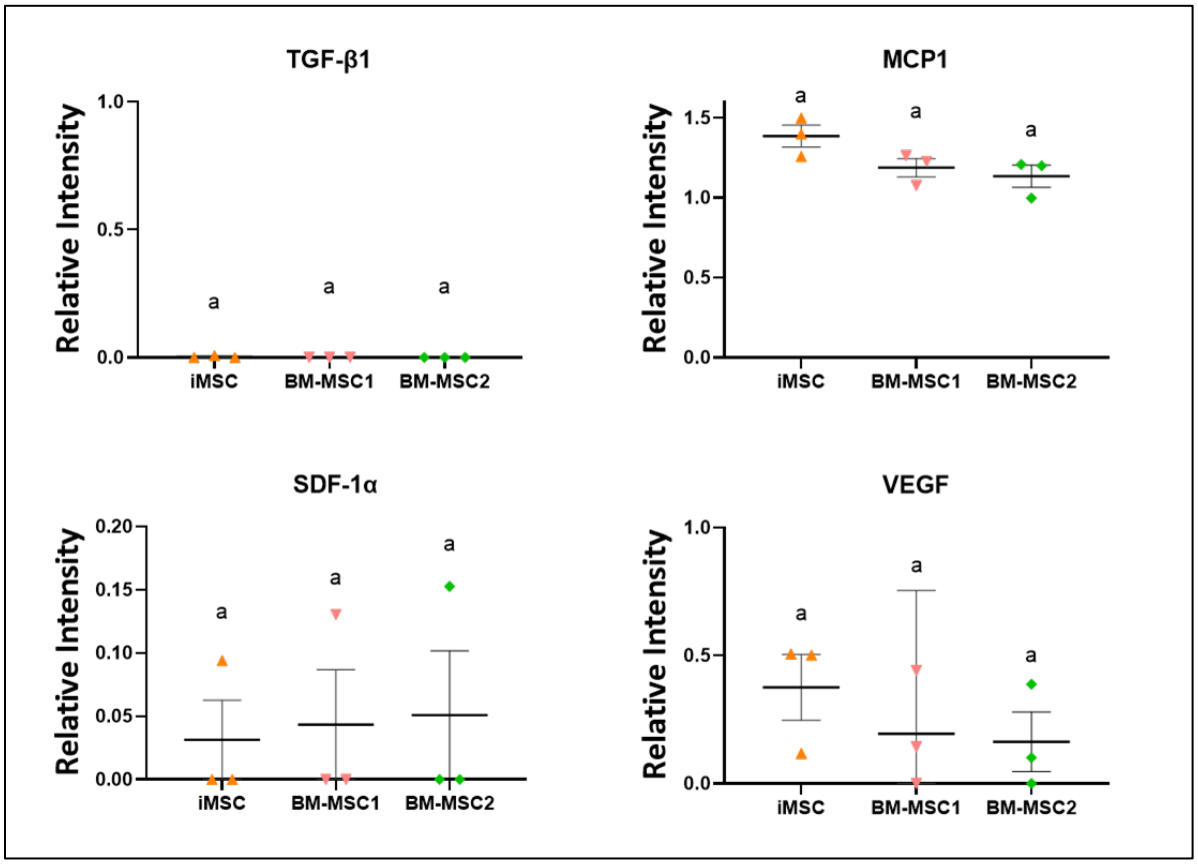


Figure 4: Quantitative analysis of additional cytokines present in the CM collected from different stem cell sources which play an important role in wound healing by controlling cell migration and proliferation, cell apoptosis and pro-angiogenesis.

Conclusion: These results confirm that the nature and amount of different cytokines present in the CM collected from different MSCs (iMSCs and BM-MSCs) was comparable demonstrating that the iMSCs can be potentially used as a cell source. By combining these cells with an adhesive scaffold and applied as cell-laden glue, the system will have the capability to create a reparative environment for supporting soft tissue attachment around the metal implant.

Subtask 2.1a: Test porcine cells for adhesion

Objective: The purpose of this task was to screen all porcined-dervied MSCs and positive control fibroblasts and keratinocytes for their ability to adhere to medical-grade Ti alloy and control glass surfaces by measuring proliferation, cell spreading, focal adhesion expression, and mechanical adhesion *via* resistance to centrifugation. We previously reported these results with one replicate. Here, we show pooled, final results from three different Gottingen pigs, i.e. in triplicate.

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

Immunofluorescence analyses of adhesion to titanium and control glass: Ti-6Al-4V extra low interstitial (Ti, President Titanium) disks were polished to a 20-60 nm colloidal silica finish. Glass (Harvard Apparatus) was used as a positive control substrate. Cells were seeded on titanium and glass disks at 1,000 cells/disk for 4 hours, 1 day, and 3 days. NIH-3T3 (CRL-1658, ATCC, Manassas, VA) embryonic murine fibroblasts (hereafter, 3T3 Fibroblasts) were tested as positive controls due to their well-documented adhesion to titanium and long history of studies on their adhesome. At each time point, cell spreading and focal adhesion characteristics were determined with immunofluorescence. Seeded disks (n=10; both Ti and glass) were washed in phosphate buffered saline (PBS) to remove weakly adherent 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 (as a marker of focal adhesions; MAB3574, MilliPore Sigma) overnight. Following washing, a secondary antibody (ab97037, Abcam) was added for 1 hour. Seeded disks were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 300 nM in PBS; D9542, Fisher) for 10 minutes. Following extensive washing in PBS, the seeded disks were mounted with ProLong (Fisher) and imaged (Olympus FV1000). In some cases, seeded disks were imaged on a different microscopy (Leica DM 6B). Other disks (n=5) were stained with rhodamine-phalloidin (R415, Fisher) and processed similarly with DAPI and mounted. ImageJ (NIH) was used for image analysis with 5 fields of views (FOVs) per sample. Proliferation (**Figure 5)**; number of DAPI-positive nuclei (**Figure 6)**, cell surface area per FOV (**Figure 7)**, individual cell area (**Figure 8**), vinculin (focal adhesion) intensity per FOV (**Figure 9)**, average vinculin (**Figure 10**; focal adhesion) intensity per cell, and average vinculin (focal adhesion) intensity per cell area (**Figure 11**) were calculated and normalized to 3T3 fibroblasts on Ti at 4 hours for each measure to account for differences in microscope usage.

Metabolic activity on titanium and control glass: At 4 hours, 1 day, and 3 days, seeded disks (n=7; both Ti and glass) were washed thrice in PBS to remove weakly adherent cells, transferred to a virgin 48 well-plate, and incubated for 2.5 hours in a 1:10 dilution of CCK-8 solution (Dojindo Laboratories, Japan) in culture media. Afterward, 100 µL of the CCK-8/media solution was transferred to a 96-well plate for absorbance reading (Synergy, BioTek - 450 nm) expressed as optical density (O.D.). A blank was used to subtract background absorbance. Results are shown in **Figure 4.**

Centrifugation adhesion measurements on titanium: After cell seeding for 2 days (5,000 cells per 48-well) on polished Ti, disks were gently washed thrice with phosphate buffered saline (PBS) to remove nonadherent cells. Oral keratinocytes[5] were used as a positive control given their strong adhesion to titanium.[6] Some disks (n=3) were immediately removed and fixed in 4% paraformaldehyde (hereafter, control-pre). Remaining disks were inserted into a three-dimensionally (3D) printed (Dental Resin SG, Formlabs) disk-holder. This holder was designed to fit inside a 48-well such that disks (one disk per holder per well) were perpendicular to the ground when centrifuged. Wells were filled with culture media and then centrifuged (3 minutes) at 350 (n=3) or 500 (n=3) gravitational forces (*g*). Control disks (n=3; hereafter, control-post) were treated identically but placed next to the centrifuge (5810R, Eppendorf) while the other two groups were centrifuged. Disks were then subsequently stained with DAPI for 15 minutes at room temperature and imaged (Lecia DM6 B). ImageJ (NIH) was used for image analysis with 3 random fields of views (FOVs) per sample. Results are shown in **Figure 12**.

Statistical analysis: Values are presented as mean ± standard deviation. A *t*-test with False Discovery Rate (FDR) correction was used to compare Ti vs. glass for each measure, an analysis of variance (ANOVA) with Dunnett's multiple comparison was used to compare each MSC vs 3T3 fibroblasts (control group) for each measure, and an ANOVA with a Tukey's post hoc analysis was used to compare each cell on each substrate for each measurement through time (4 hours vs. 1 day vs. 3 days). For the centrifugation adhesion test, values are presented as mean ± standard deviation of the mean percentage of cells of control-pre. An ANOVA followed by a Tukey's honest significant difference post hoc test was used to compare control-post vs. 350*g* vs. 500*g* for each cell. Inter-cell population differences between control-post, 350*g*, and 500*g* were detected with a two-way analysis of variance with a Dunnett correction where the control mean (null) was set as keratinocytes. Statistical significance was determined with a nominal α equal to 0.05 (GraphPad Prism 9.0.0).

Results:

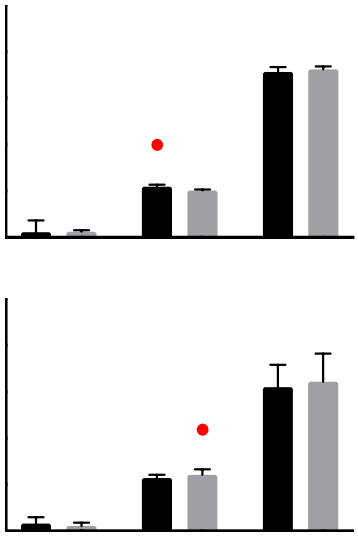
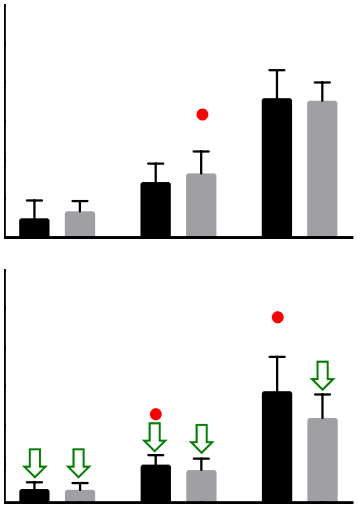


Figure 5:MSC proliferation (metabolic activity) when cultured on Ti and glass compared to positive control 3T3 fibroblasts at 4 hrs, 1 day, and 3 days. Red dots indicate a statistically significant difference between Ti and glass, where the dot is above the statistically larger group. Each cell on each substrate showed a statistically significant increase from 4 hrs to 1 day to 3 days.



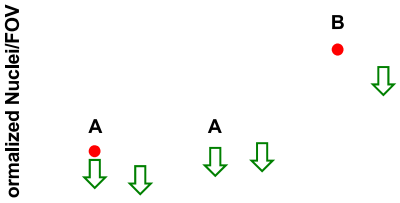


Figure 6: MSC proliferation [numbers of cell per field of view (FOV)] when cultured on Ti and glass compared to positive control 3T3 fibroblasts at 4 hrs, 1 day, and 3 days. Red dots indicate a statistically significant difference between Ti and glass, where the dot is above the statistically larger group. Green arrows indicate a statistically significant decrease compared to the positive control 3T3 fibroblasts at the equivalent time point and substrate. Letters indicate a trend other than a significant increase from 4 hrs to 1 day to 3 days for each cell on each substrate, where different letters indicate a statistically significant difference.

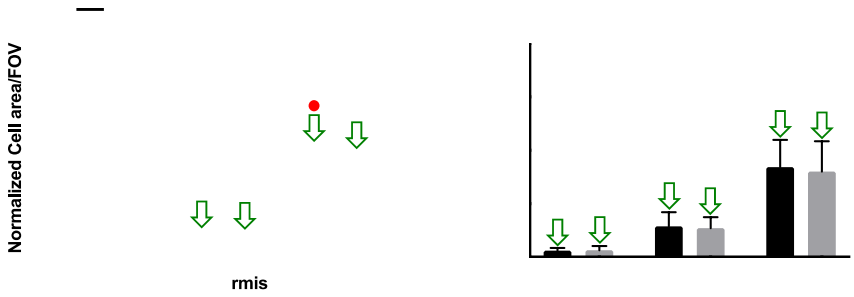


Figure 7: MSC spreading per FOV when cultured on Ti and glass compared to positive control 3T3 fibroblasts at 4 hrs, 1 day, and 3 days. Red dots indicate a statistically significant difference between Ti and glass, where the dot is above the statistically larger group. Green arrows indicate a statistically significant decrease compared to the positive control 3T3 fibroblasts at the equivalent time point and substrate. Each cell on each substrate showed a statistically significant increase from 4 hrs to 1 day to 3 days.

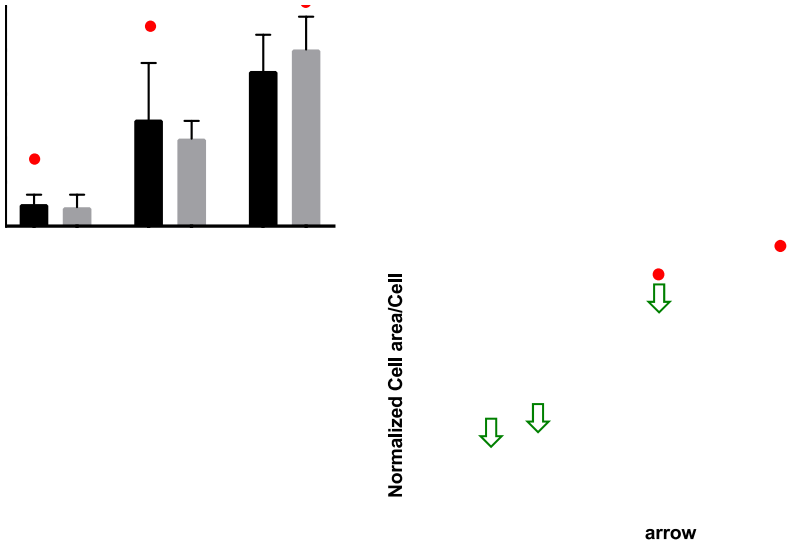


Figure 8: Individual MSC spreading when cultured on Ti and glass compared to positive control 3T3 fibroblasts at 4 hrs, 1 day, and 3 days. Red dots indicate a statistically significant difference between Ti and glass, where the dot is above the statistically larger group. Green arrows indicate a statistically significant decrease compared to the positive control 3T3 fibroblasts at the equivalent time point and substrate. Letters indicate a trend other than a significant increase from 4 hrs to 1 day to 3 days for each cell on each substrate, where different letters indicate a statistically significant difference.

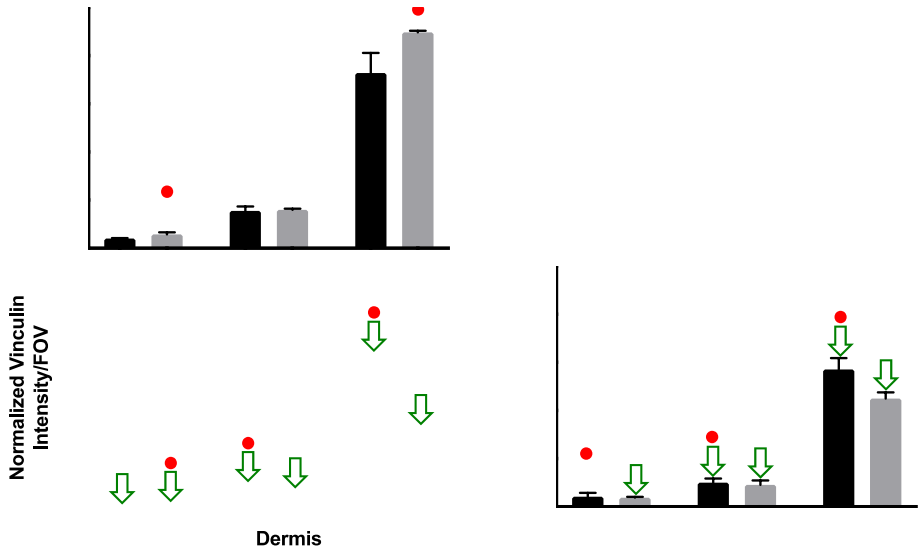


Figure 9: MSC vinculin expression per field of view determined using immunofluorescence when cultured on Ti and glass compared to positive control 3T3 fibroblasts at 4 hrs, 1 day, and 3 days. Red dots indicate a statistically significant difference between Ti and glass, where the dot is above the statistically larger group. Green arrows indicate a statistically significant decrease compared to the positive control 3T3 fibroblasts at the equivalent time point and substrate. Letters indicate a trend other than a significant increase from 4 hrs to 1 day to 3 days for each cell on each substrate, where different letters indicate a statistically significant difference.

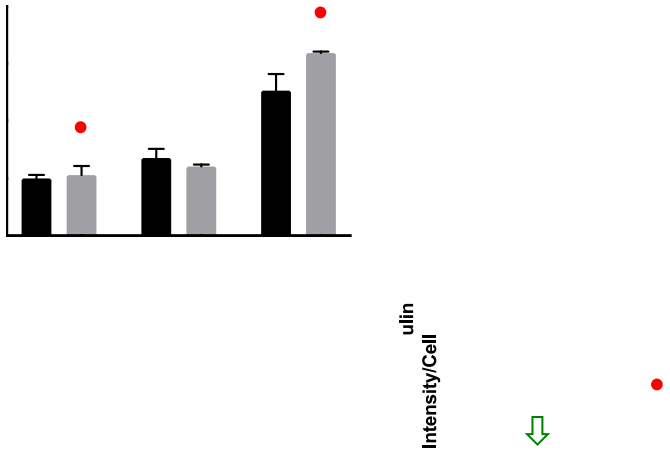


Figure 10: Individual MSC vinculin expression determined using immunofluorescence when cultured on Ti and glass compared to positive control 3T3 fibroblasts at 4 hrs, 1 day, and 3 days. Red dots indicate a statistically significant difference between Ti and glass, where the dot is above the statistically larger group. Green arrows indicate a statistically significant decrease compared to the positive control 3T3 fibroblasts at the equivalent time point and substrate. Each cell on each substrate showed a statistically significant increase from 4 hrs to 1 day to 3 days.

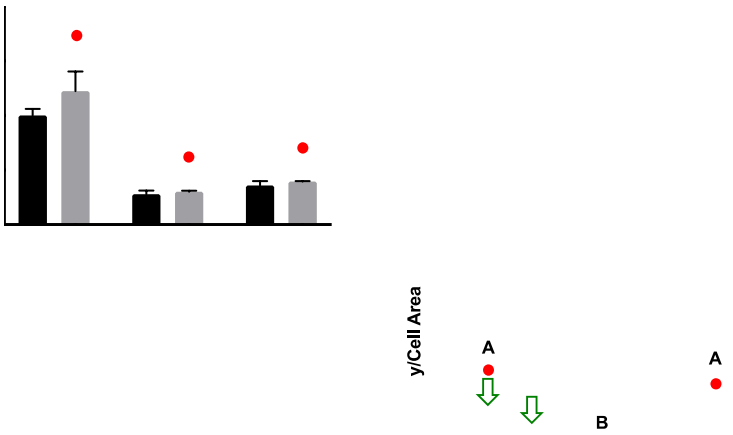


Figure 11: Individual MSC vinculin expression determined using immunofluorescence per individual cell area determined when cultured on Ti and glass compared to positive control 3T3 fibroblasts at 4 hrs, 1 day, and 3 days. Red dots indicate a statistically significant difference between Ti and glass, where the dot is above the statistically larger group. Green arrows indicate a statistically significant decrease compared to the positive control 3T3 fibroblasts at the equivalent time point and substrate. Letters indicate a trend other than a significant increase from 4 hrs to 1 day to 3 days for each cell on each substrate, where different letters indicate a statistically significant difference.

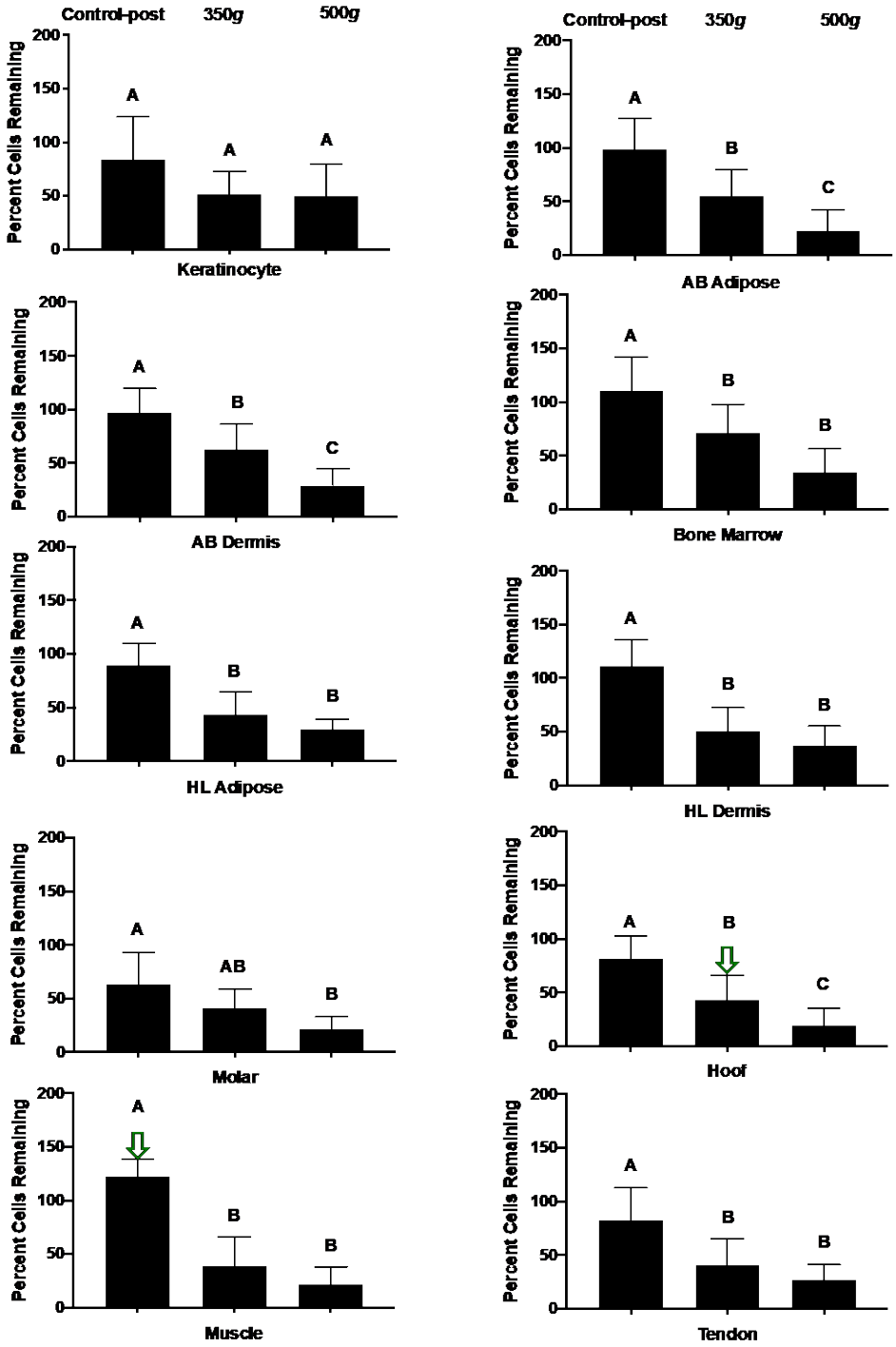


Figure 12: Percent cells remaining after control-post, 350g, and 500g centrifugation when compared to control-pre. Green arrows indicate a statistically significant decrease compared to the positive control keratinocytes at the equivalent time point. There were no statistically significant differences between control-pre and control-post for any of the cells. Different letters indicate statistically significant differences between control-post, 350g, and 500g for each cell.

Results (continued):

Proliferation (metabolic activity) on titanium and control glass

All porcine derived MSCs proliferated on both Ti and glass over three days. No statistically significant differences were seen between any of the MSCs compared to positive control 3T3 fibroblasts. Overall, no notable differences were seen in proliferation between Ti and positive control glass.

Proliferation (number of cells per field view) on titanium and control glass

All porcine derived MSCs proliferated on both Ti and glass over three days. Fewer cells per field of view were found for most MSCs at most timepoints, with the notable exception of hoof-derived MSCs that showed no statistically significant differences, compared to control 3T3 fibroblasts. Overall, no notable differences were detected between Ti and positive control glass.

MSC spreading per field of view on titanium and control glass

All porcine derived MSCs continued to spread within the field of view on both Ti and glass over three days. Lower cell spreading per field of view was found for almost all timepoints for all MSCs compared to control 3T3 fibroblasts. Overall, no notable differences were detected between Ti and positive control glass.

Individual MSC spreading on titanium and control glass

Almost porcine derived MSCs continued to spread on both Ti and glass over three days except for hoof-derived MSCs cultured on both Ti and glass and AB adipose cultured on Ti. Lower cell spreading was found for almost all timepoints for all MSCs compared to 3T3 fibroblasts except for HL Adipose-derived MSCs. Overall, no notable differences were detected between Ti and positive control glass.

MSC vinculin expression determined using immunofluorescence per field of view on titanium and control glass

All porcine derived MSCs expressed vinculin on both Ti and glass over three days. Total vinculin expression per field of view was lower for all MSCs compared to control 3T3 fibroblasts for almost all timepoints on both substrates for all MSCs. All MSCs exhibited increased expression per field of view from 3 hrs to 1 day to 3 days except for AB Adipose, which plateaued at 1 day. Overall, no notable differences were detected between Ti and positive control glass.

Individual MSC vinculin expression determined using immunofluorescence on titanium and control glass

Vinculin expression per cell was similar to control 3T3 fibroblasts for almost all timepoints on both substrates for all MSCs. All MSCs exhibited increased expression per cell from 3 hrs to 1 day to 3 days. Overall, no notable differences were detected between Ti and positive control glass.

Individual MSC vinculin expression determined using immunofluorescence per individual cell area on titanium and control glass

Vinculin expression, normalized to cell surface area, peaked at 4 hrs for both Ti and glass for 3T3 fibroblasts and most MSCs. Few differences were seen comparing each MSC to 3T3 fibroblasts. Overall, no notable differences were detected between Ti and positive control glass.

Percent cells remaining after control-post, 350g, and 500g centrifugation when compared to control-pre on titanium

Most MSCs showed fewer cell remaining when comparing 500*g* centrifugation to 350*g* centrifugation, except for bone marrow, muscle, tendon HL Adipose, HL Dermis -derived MSCs, and control keratinocytes, which showed no differences. Nearly no differences were seen when comparing each MSC to control keratinocytes for either 350g or 500*g*.

Conclusions: Our results demonstrate that all porcine MSCs have the ability to adhere and proliferate on polished medical-grade Ti alloy and control glass surfaces. Minimal differences in proliferation, cell spreading, focal adhesion expression, and mechanical adhesion *via* resistance to centrifugation were seen between different MSCs.

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''

As mentioned in our last annual report, we have focused on using photo-curable GelMA hydrogel as a biologic-glue between skin and titanium abutment. In our last report, we highlighted the effect of different input variables on physico-mechanical characterization (soluble fraction, swelling ration and compressive modulus) of fabricated hydrogels. In this report, we highlight adhesive and biological effects of different hydrogels and select our candidate hydrogel for stem cell delivery.

Objective 1: Using the 'Design of Experiments' (DoE) statistical tool to systematically assess the effect of different variables on the adhesive properties of different hydrogel formulations to grade 5 titanium and porcine skin

Method: A 2^3 DoE was used to determine main effects and interactions of three variables on the adhesive properties of GelMA hydrogels. 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 tested; which were then used to assess the outcomes. The assessed outcomes included hydrogel adhesivity to titanium via the lap shear test. The setup used is presented in **Figure 13 a and c**.

Briefly, macromer concentration (5 wt%, or 10 wt%) were prepared by dissolving lyophilized GelMA in PBS, mixed with 0.05% or 0.1% LAP (prepared in PBS) and then, cross-linked between titanium coupon/porcine skin and gelatin coated glass slide using light with maximum wavelength at 400 nm for 30 or 90 seconds. Post cross-linking, the adhered glass-titanium or glass-skin sample was mounted between custom-made fixtures and strained at a speed of 1.5 mm/min until failure using an MTS Criterion 41 mechanical testing machine equipped with a 100 N load cell.

Results: Based on DoE, 8 different combinations were tested and the effect of different variables on metal and skin adhesivity is shown in **Figure 13 b and d**, respectively. Among tested variables, macromer concentration and time of cross-linking were the predominant factors positively influencing adhesivity of the hydrogels to the metal and skin. This result validated our preferred conditions for hydrogel manufacturing as results from our previous report showed that an increase

in macromer concentration and time for crosslinking (except for soluble fraction) resulted in reduced soluble fraction, reduced swelling ratio, and increased compressive modulus.

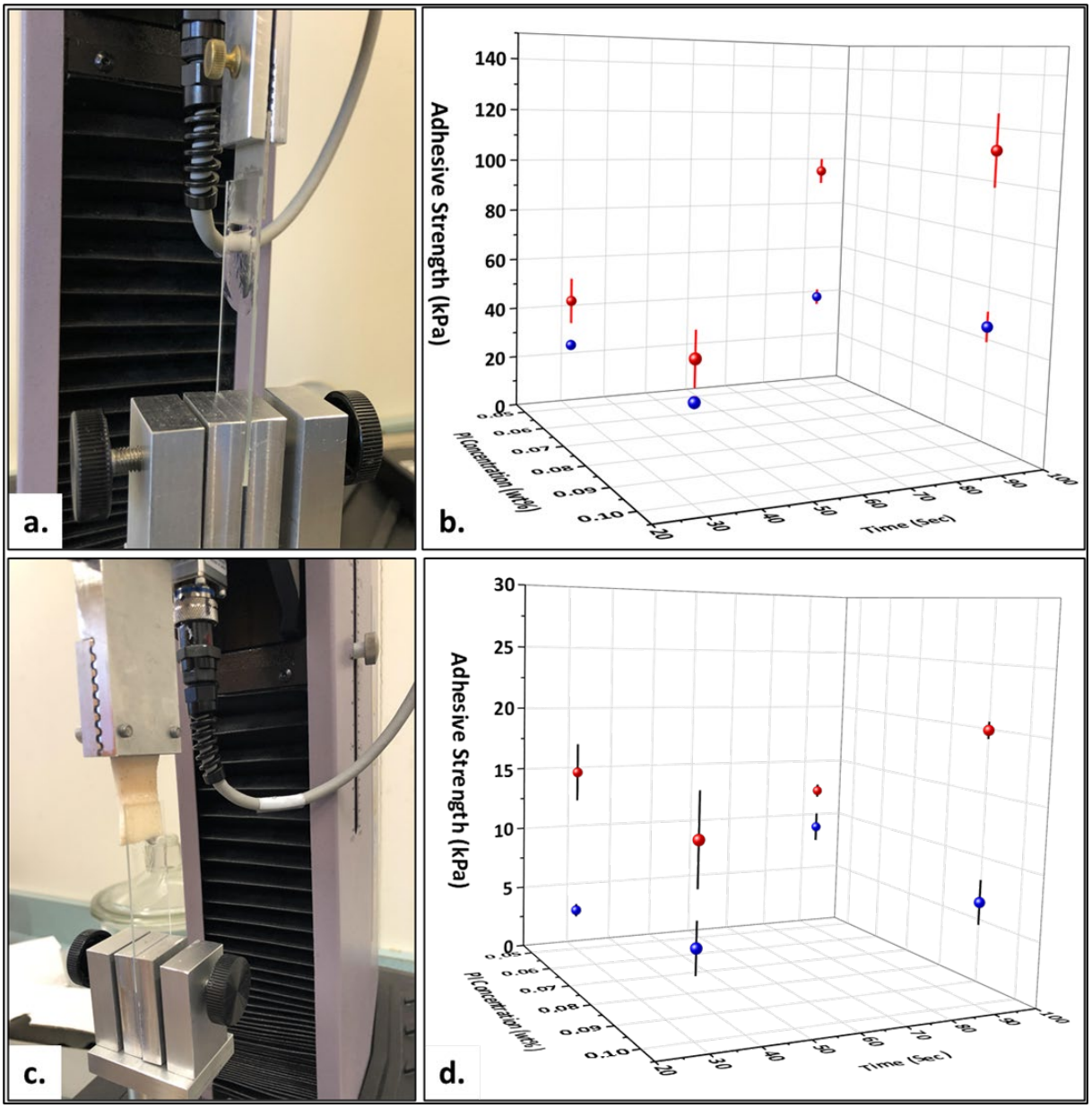


Figure 13: Setup used for performing lap shear test to assess effect of different tested variables on hydrogel adhesivity to (a) grade 5 titanium substrate and (c) procine skin; summary of the effect of different tested variables on the adhesive strength to (b) grade 5 titanium substrate and (d) porcine skin. Blue dots are for 5 wt% GelMA and red dots are for 10 wt% GelMA.

Objective: To perform statistical analysis to assess which input variable significantly influenced the measured output properties of different hydrogel formulations using Design Expert.

Method: Design Expert software was used to perform statistical analysis to determine what factors (independently or in combination with other factors) influenced measured output responses. The output values corresponding to each measured response for different combinations of input variables was provided. The analysis function was performed and based on the ratio of maximum to minimum, transformation or no-transformation was applied. The ratio of maximum value of a response to minimum value of the same measured response determines if response transformation is required or not. If the ratio is greater than 5 then transformation is applied (nature of transformation depends on the value of λ) or else the data is analyzed as is. The dependence of a response on input variables and/or their interaction with other variables is presented here as half-normal plot and the Pareto chart which highlights what factors significantly influence the measured response.

Results: **Figure 14** is a table with the measured output responses for different combinations of input variables used in Design Expert software for analysis. The half-normal plot and the Pareto chart for each outcome highlighted which of the input variables (either alone or in combination with other variables) significantly influenced the output property positively (if variable value increases then outcome value increases) or negatively with adhesive strength to titanium being an exception. The response for the metal adhesivity was transformed (inverse square root transformation was applied as $\lambda = -0.5$). This means that the input variables highlighted as negative (blue bars) positively influence the outcome. (**Figure 15 and Figure 16**).

Run	Factor 1 A:Macromer co...	Factor 2 B:PI concentration	Factor 3 C:Time of crossl...	Response 1 Soluble fra...	Response 2 Swelling rat...	Response 3 Compressi...	Response 4 Adhesive streng... kPa	Response 5 Adhesive Streng... kPa
1	5	0.1	30	9.06489	16.375	2.29	32.1057	7.01205
2	10	0.1	90	10.1871	9.10345	28.08	98.9896	19.5077
3	10	0.05	30	16.5685	9.6087	15.11	62.5195	10.9235
4	5	0.05	30	18.4398	16.1429	2.3	26.2568	3.83857
5	10	0.1	30	11.0045	9.37037	21.6	40.8931	14.0747
6	10	0.1	30	7.70836	8.89286	14.43	59.7234	18.3258
7	10	0.05	90	21.7539	8.63636	21.68	84.1732	13.7932
8	5	0.05	30	17.7144	17	2.88	31.4764	1.90345
9	5	0.05	90	26.7253	15.0909	4.33	46.3581	9.12184
10	5	0.1	90	15.8745	18.5833	3.61	50.8451	9.72299
11	10	0.1	30	12.3632	9.44444	15.98	42.4359	22.8096
12	10	0.05	90	16.8463	8.26923	26.76	85.4365	12.1494
13	5	0.1	30	13.7719	18.9286	2.78	31.476	9.27854
14	5	0.1	30	8.01794	18.4667	1.87	37.8431	2.86792
15	5	0.05	90	19.4783	14.6	4.28	35.5623	11.4964
16	10	0.05	30	15.1301	8.69565	17.72	53.2904	11.571
17	5	0.05	30	25.7986	17.7692	1.87	29.4232	2.78016
18	10	0.1	90	7.73086	9.18519	34.83	101.896	21.24
19	10	0.05	30	16.2135	9.56522	16.09	35.1527	16.0769
20	5	0.05	90	21.2751	16	4.1	38.2176	7.26364
21	10	0.05	90	17.8284	7.95833	20.84	92.6795	13.1941
22	5	0.1	90	11.3723	17.0769	3.95	45.338	7.2067
23	5	0.1	90	10.3788	16.4167	5.2	56.3522	6.33416
24	10	0.1	90	7.45162	7.53333	24.43	139.288	20.1439

Figure 14: The summary of the table used for analysis in Design Expert software for understanding the effect of different input variables on output responses

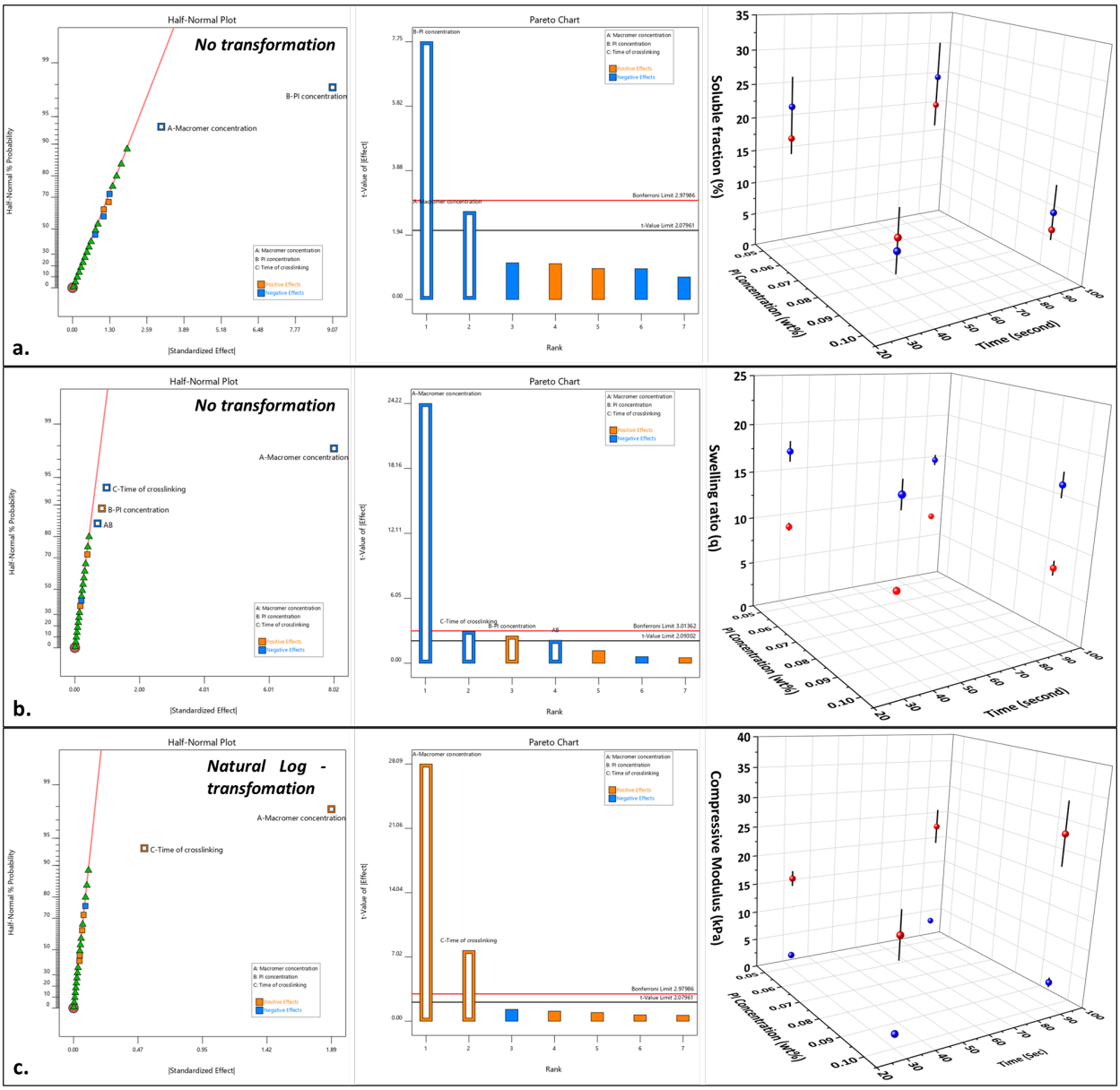


Figure 1: Half-normal plot and Pareto chart along with summary of effect of different variables on (a) soluble fraction, (b) swelling ratio and (c) compressive modulus of fabricated hydrogels. Blue dots are for 5 wt% GelMA and red dots are for 10 wt% GelMA. The orange bars in the pareto chart represent the variable negatively influences the response and the blue bar is for the input variable or combination of variables positively influencing the output response.

Based on the half-normal plot and Pareto chart, it is confirmed that one input variable that positively influences the output variables (low soluble fraction and swelling ratio and high compressive modulus) is the macromer concentration. Thus, as the macromer concentration is increased from 5 wt% to 10 wt%, the fabricated hydrogels are stiffer with lower soluble fraction and lower swelling ratio.

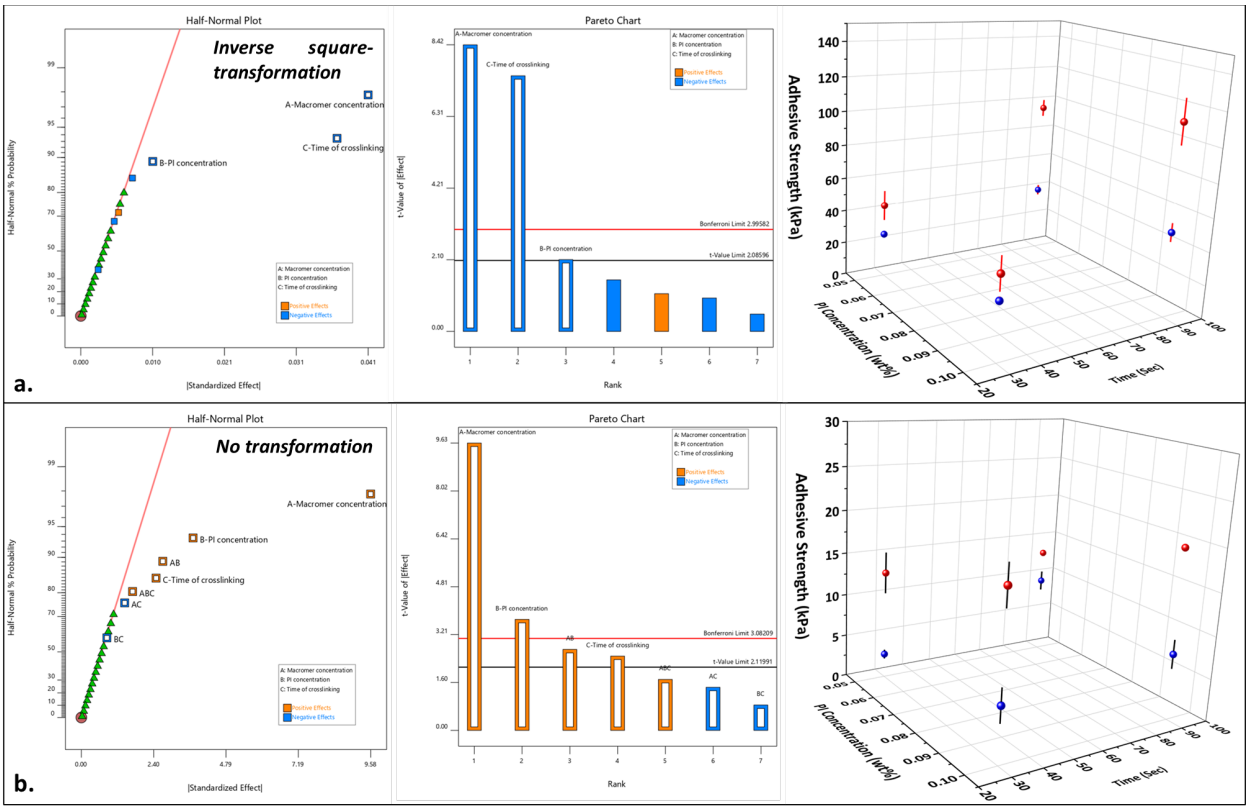


Figure 16: Half-normal plot and Pareto chart along with summary of effect of different variables on adhesive strength to (a) grade 5 titanium and (b) porcine skin of different hydrogels acting as a glue. Blue dots are for 5 wt% GelMA and red dots are for 10 wt% GelMA. The orange bars in the pareto chart represent the variable negatively influences the response and the blue bar is for the input variable or combination of variables positively influencing the output response.

The half-normal plot and pareto chart confirmed that, for the adhesive strength to both metal and skin tissue, the macromer concentration had a positive effect, as shown in **Figure 16**. This indicates that as the macromer concentration is increased from 5 wt% to 10 wt%, the adhesive strength increased from 26.2 kPa for 5 wt% GelMA crosslinked for 30 seconds in presence of 0.05% PI to 113.4 kPa for 10 wt% GelMA crosslinked for 90 seconds in presence of 0.01% PI. Similar trend was observed for hydrogel adhesivity to skin where increase in the macromer concentration improved its adhesivity to porcine skin.

Objective 2: The purpose of this task was to assess the effect of different hydrogel formulations on the expression of key markers associated with hemidesmosome formation (for keratinocytes) and collagen I (for fibroblasts). Assessing the biological performance in combination with the physico-mechanical and adhesive profile will determine the final hydrogel candidate that can be used as stem cell carrier applied as a transient interface between the metal abutment and skin.

Methods: Different hydrogel formulations were fabricated using DoE, as mentioned previously and keratinocytes (HaCaTs, addexbio) and human dermal fibroblasts (HDF) (ATCC) were seeded separately on fabricated hydrogels at a density of 25000 cells/gel and 10000 cells/gel, respectively. Cells were cultured for 72h after which they were fixed for ten minutes in 4% paraformaldehyde. Cells were immersed in 5% bovine serum albumin (BSA) in PBS and probed with a primary rabbit polyclonal antibody for collagen XVII [(critical later marker for hemidesmosome assembly which

is important for cell-matrix adhesion) ab28440; Abcam; 1:300] for HaCaTs. HDF-seeded samples were probed with collagen I [(extracellular matrix marker) ab34710; Abcam; 1:200] for 2 h at room temperature. After 2h of incubation in primary antibody, samples were washed with PBST (0.05% Tween-20 in 1X PBS). Samples were immersed in an anti-rabbit secondary (ab97037; abcam; 1:500) for 1 hour. Samples were counterstained with nuclear stain Hoechst (Sigma-Aldrich). Micrographs (×10) were obtained on an upright fluorescent Leica DM6 fluorescence microscope as previously described. Representative micrographs have been presented here.

Results: The results from the collagen XVII expression (**Figure 17**) of seeded keratinocytes shows no direct correlation between independent variables and collagen XVII expression. However, there was some effect of hydrogel stiffness and collagen XVII expression, a small correlation was observed. The hemidesmosome intensity lower for very soft (2.7 kPa; 5% GelMA 0.1% LAP and 30 seconds cross-linking) and very stiff (60.2 kPa; 10% GelMA, 0.1% LAP and 90 seconds crosslinking) hydrogels.
For HDFs on the other hand, 5% hydrogel fabricated using 0.1% LAP didn't support cell spreading (cells grew in small clusters) especially for the hydrogels prepared at 90 second crosslinking which was not observed on other formulations (**Figure 18**). All the other formulations supported cell attachment, spreading and collagen I expression.

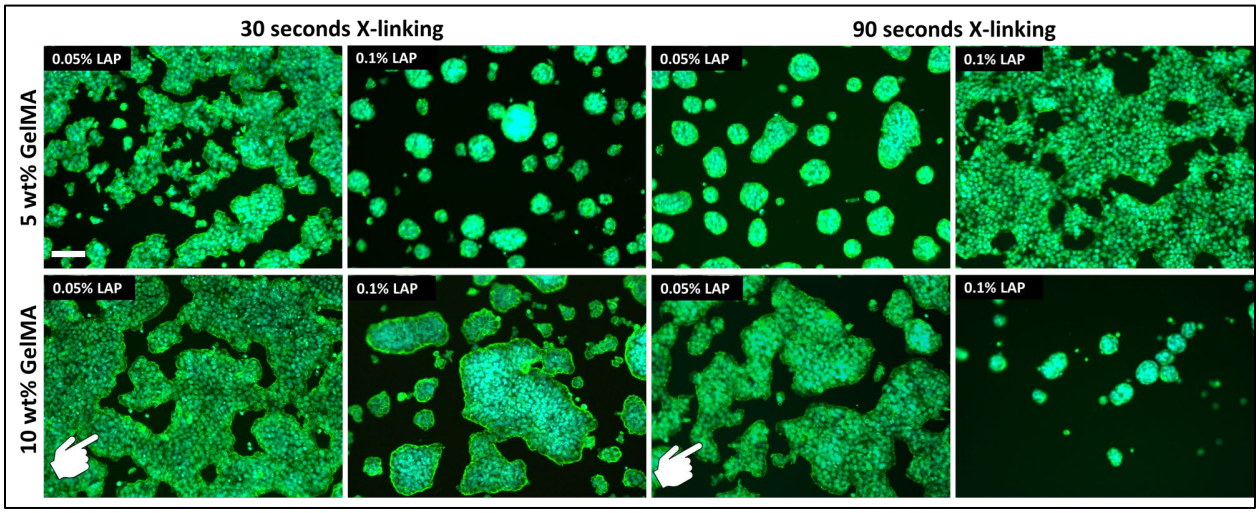
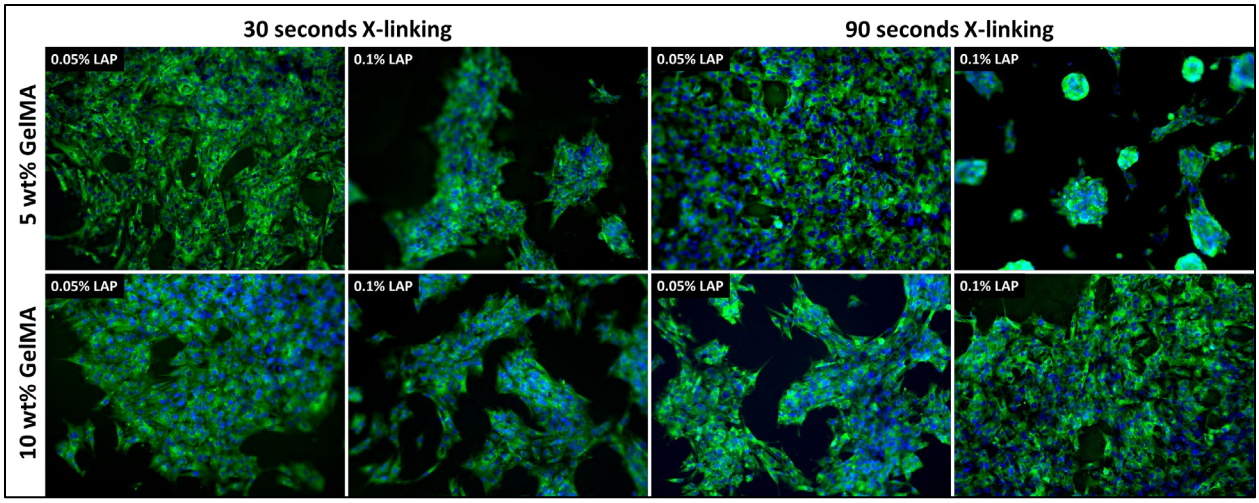


Figure 17: Immunofluorescent micrographs (Hoechst and Collagen XVII-stained) of keratinocytes at 3 days. Scale bar is 100 µm



Conclusion: Taken together, these results confirm that by carefully choosing our input variables, we can carefully tune the output properties of our fabricated hydrogels. Based on the physico-mechanical properties, hydrogel adhesivity to both metal and skin and the biological performance of seeded fiborblasts and keratinocytes, the hydrogel formulation **10 wt% GelMA, 0.05% PI and cross-linked for 90 seconds** was selected for MSC encapsulation. This formulation permits low soluble fraction (highly cross-linked matrix), low swelling ratio (dimensional changes over time) and high adhesive strength to both titanium and porcine skin. Cellular functionality in terms of expression of key markers associated with wound healing was also supported by the selected formulation

Subtask 3.3: Seed and grow human cells on flat collagen sheets, assemble scaffolds

Objective 1: The purpose of this task was to assess if the hydrogel scaffold used as a carrier will influence the profile of trophic factors released by MSCs. For this, iMSCs and the two BM-MSCs were encapsulated within the scaffold and the cytokines released by the cells.

Methods: Human iMSCs and the two BM-MSCs used in Subtask 1.2 were expanded and once cells were confluent, cells were passaged and encapsulated with GelMa hydrogel (10 weight % GelMA, 0.05% PI) and the hydrogels were crosslinked for 90 seconds. After encapsulation, cells were cultured in expansion media for 48 h after which they were cultured in FBS-free MEM media for 72 h to collect CM. The CM was analyzed the same way as done previously for 2D cultures (subtask 1.2) and differences in the profile was quantified.

Statistical Analysis: The data analysis for statistical significance was performed using the GraphPad Prism 9.0.0 software. Data is represented as scatter plot as mean ± standard deviation of n = 3 replicates. After validating normality of sample distribution and homogeneity of variance, one-way analysis of variance (ANOVA) with Tukey test was used to assess the statistical significance between groups at p-value <0.05.

Results: The results from the cytokine array (**Figure 19**) confirmed that post-encapsulation within the hydrogel, the nature of cytokines secreted by the cells were similar to those secreted in 2D. However, the levels of some of the cytokines like that of IL-6, IGFBP2 and TIMP2 were lower than the levels determined in the CM collected from 2D cultures (**Figure 3 for 2D culture and Figure 20 for 3D culture**). However, some of the cytokines like TGF-β1 which was not secreted by cells in 2D **(Figure 4)** was present in the CM collected from cells encapsulated in the hydrogel **(Figure 21)**.

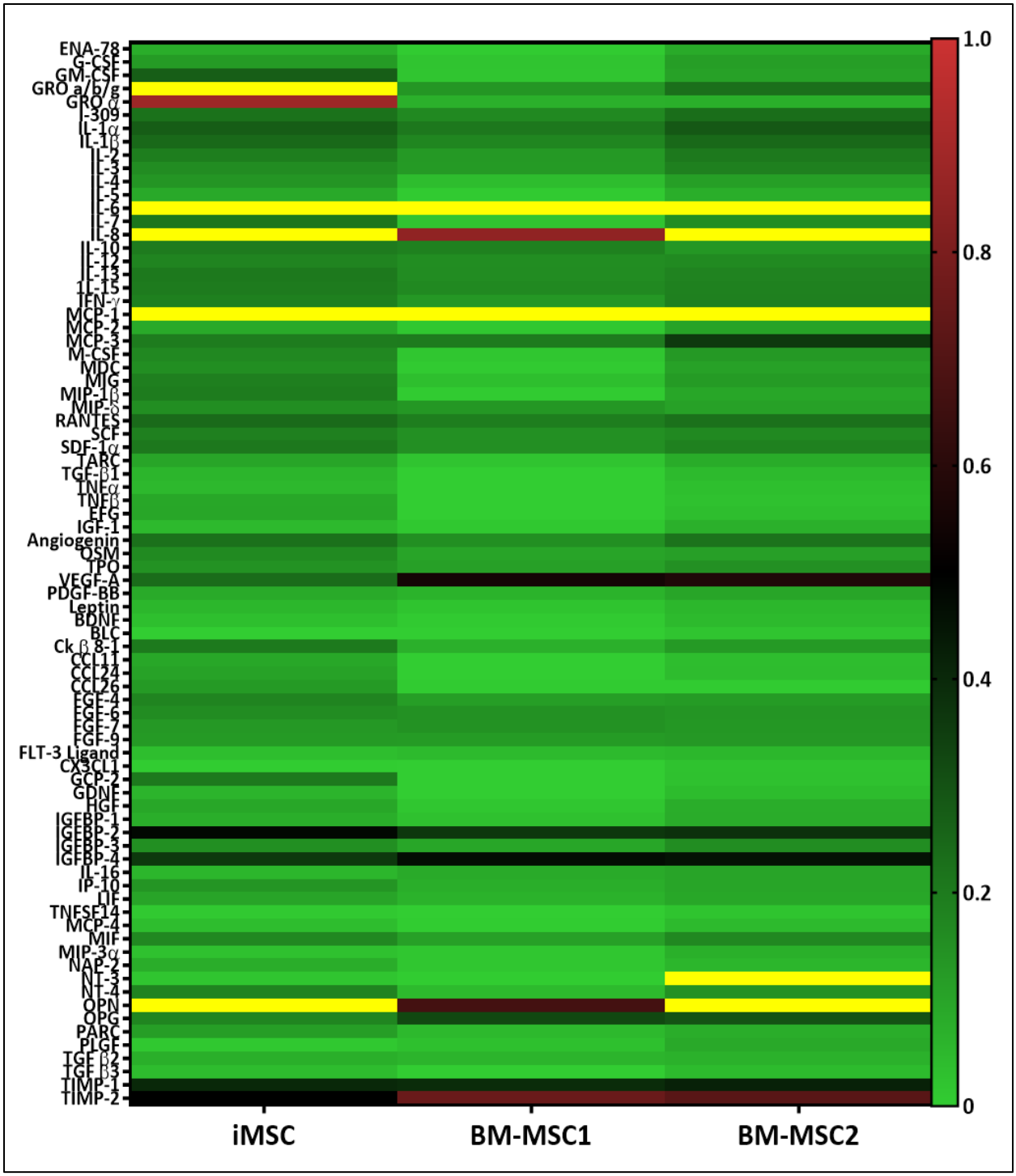


Figure 192: Heat map of the cytokines secreted by different MSCs encapsulated withing GelMA hydrogel. Bands colored yellow showed expression of those cytokines higher than 1.

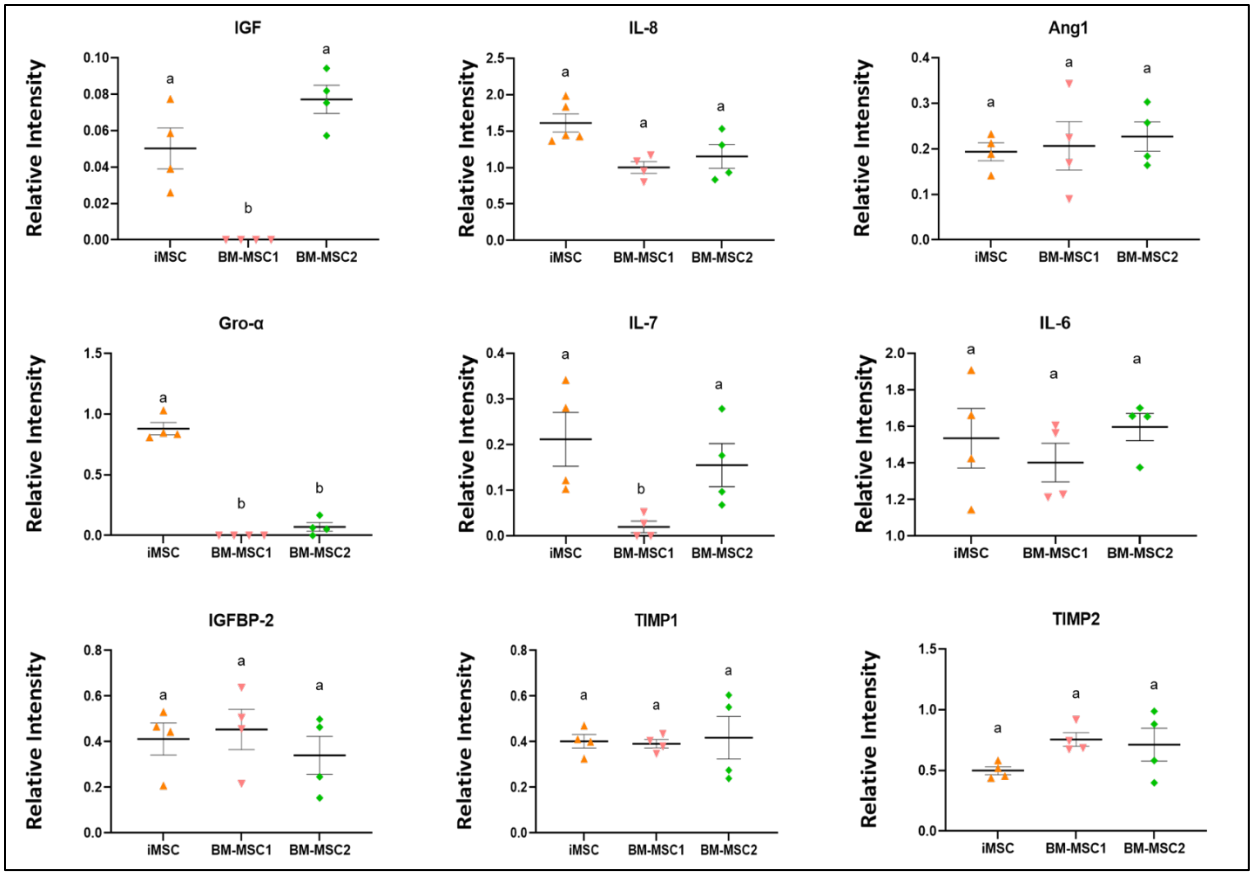


Figure 20: Quantitative analysis of the cytokines present in the CM collected from different stem cell sources encapsulated with the carrier hydrogel, GelMA which play an important role in wound healing by controlling cell migration and proliferation, cell apoptosis and pro-angiogenesis.

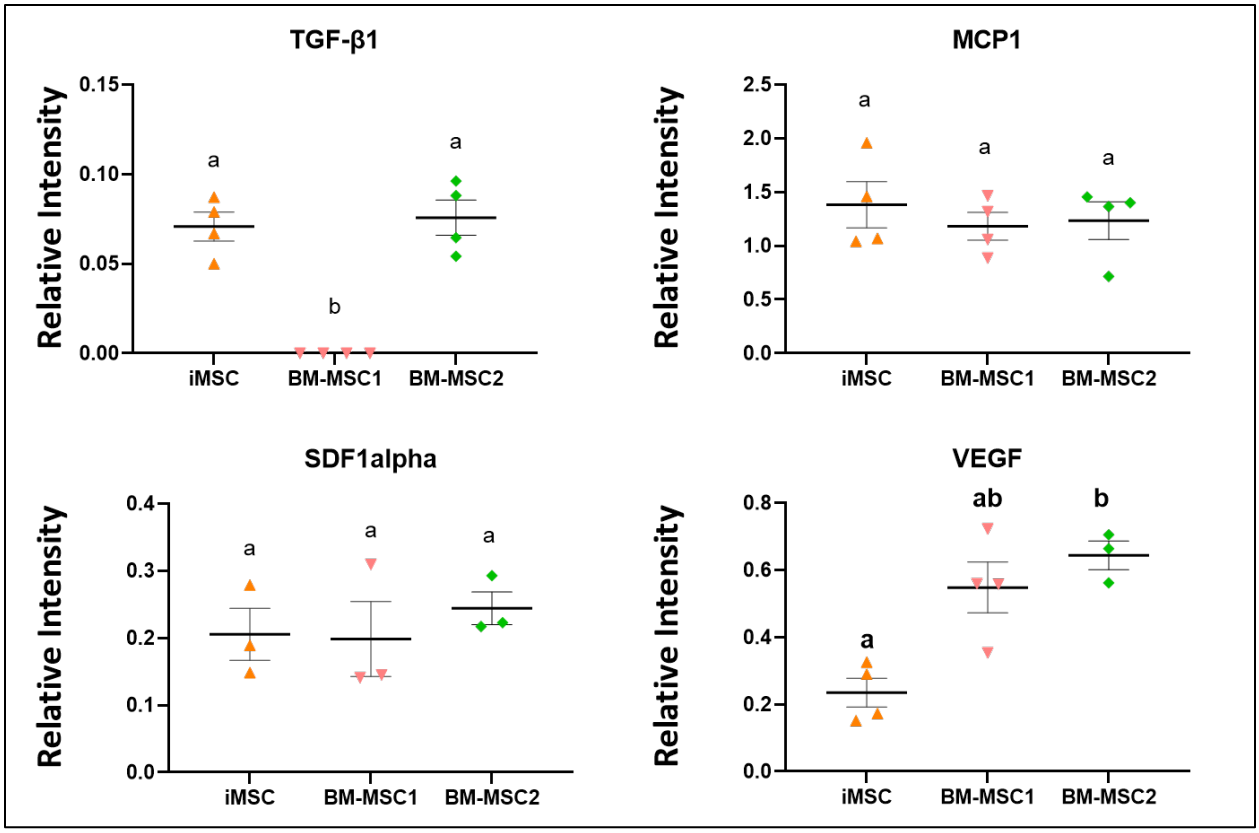


Figure 21: Quantitative analysis of additional cytokines present in the CM collected from different stem cell sources encapsulated with the carrier hydrogel, GelMA which play an important role in wound healing by controlling cell migration and proliferation, cell apoptosis.

Objective 2: To confirm functionality of CM collected from iMSCs and BM-MSCs encapsulated within GelMA hydrogel.

Method:

Cell culture and conditioned media: iMSCs and BM-MSCs were expanded in expansion media (MEM supplemented with FBS and b-FGF) until cells were 90% confluent. Once cells were 90% confluent, cells were passaged, counted and encapsulated within GelMA hydrogel at a density of 5 x 10^6 cells/ml. The hydrogel formulation that was selected for encapsulation was 10 wt% GelMA, 0.05% PI and cross-linked for 90 seconds. Post-encapsulation, cells were cultured in expansion media for 72h after which the media was switched to basal media (MEM media without FBS) and the cell-encapsulated hydrogels were cultured for another 72h. Post-culture, the media was collected, 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.

Cell proliferation: 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 the manufacturer's instruction. The fluorescence was measured at

540/590 excitation/emission wavelength (Synergy HT, Biotek, USA) and was used as a measure of cell proliferation.

Cell migration: For cell migration, transwells (Corning, 8 µm, polycarbonate) 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 includes 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 after which the membranes were washed in PBS. Optical micrographs from different fields of view were taken to quantify number of cells that migrated through the transwell membrane.

Wound healing: To determine the effect of CM on wound healing capability, HaCaTs cells were seeded at a density of $0.25x10^6$ cells/well while HDF cells were cultured at a density of $0.12x10^6$ cells/well in a 24 well plate. Cells were cultured for 24h until a confluent monolayer was formed after which a wound was created 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), 50ng/ml TGF- β1 supplemented DMEM+10%FBS (positive control for HaCaTs) or MEM media (negative control)). Micrographs were taken at 0h to determine the area of the initial wound after which cells were cultured for another 48h. Micrographs were taken at 48h to determine the extent of wound closure.

Results: Assessing the effect of CM on proliferation of HaCaTs and HDFs using alamarBlue™ confirmed significantly higher levels of cell proliferation relative to the negative control. CM from iMSCs and BM-MSCs performed similar to positive control for HDFs whereas the CM from BM-MSCs outperformed positive control for HaCaTs (**Figure 22**).

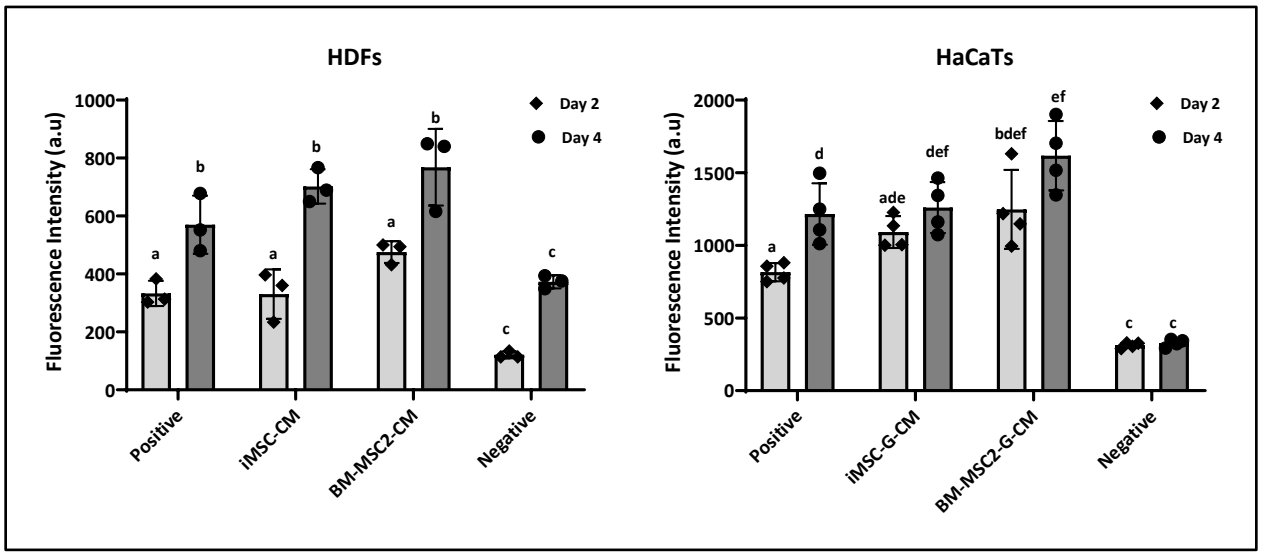


Figure 22: HDF and HaCaT proliferation under different treatment conditions. Statistical analysis: one way ANOVA with post hoc Tukey test $p<0.05$ is considered significant. Dissimilar letters represent significant differences between different groups.

Transwell migration assay confirmed the presence of bioactive cues in CM that support migration of both keratinocytes and fibroblasts through the transwell membrane. For HDFs, significantly higher levels of migration were observed in presence of iMSC-derived CM relative to the positive control whereas for HaCaTs, similar levels of migration were observed in CM collected from iMSCs relative to positive control (**Figure 23**).

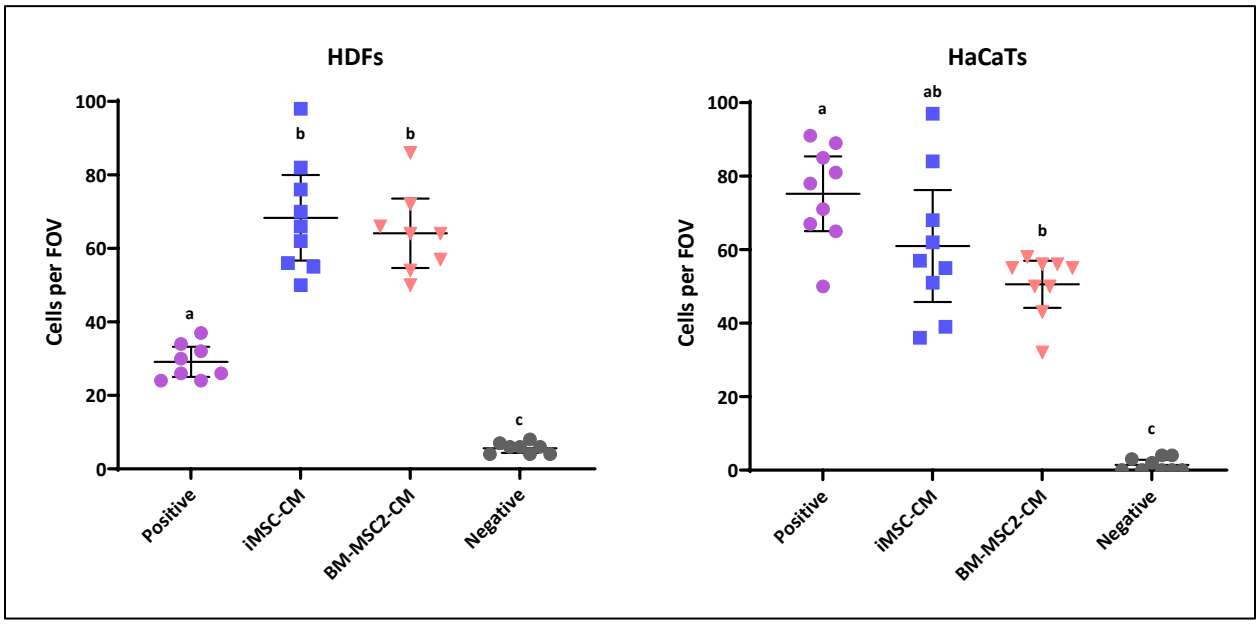


Figure 23: Transwell migration of HDFs and HaCaTs in presence of different treatment conditions. Statistical analysis: one way ANOVA with post hoc Tukey test $p<0.05$ is considered significant. Dissimilar letters represent significant differences between different groups.

For wound healing assay, complete wound closure was observed for all the treatment groups relative to positive control in both HaCaTs and HDFs (**Figure 24**).

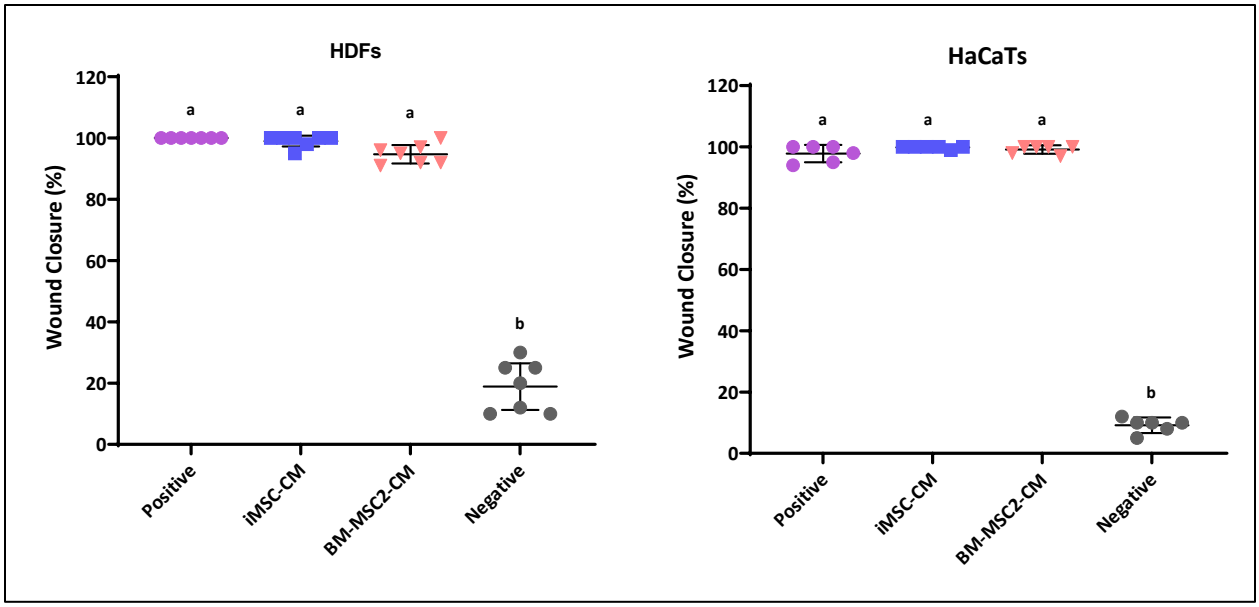


Figure 24: Effect of different treatment conditions on HDFs and HaCaTs wound closure after 48h. Statistical analysis: one way ANOVA with post hoc Tukey test $p<0.05$ is considered significant Dissimilar letters represent significant differences between different groups.

Objective 3: Effect of encapsulated iMSCs on hydrogel physico-mechanical properties and macromer handleability

Method: Based on the physico-mechanical and adhesive properties along with biological response of HaCaTs, GelMA hydrogels fabricated using 10 wt% GelMA, 0.05% LAP and cross-linked for 90 seconds was chosen for further evaluation. Confluent iMSCs (passage 12) were trypsinized, counted and encapsulated at a density of $5X10^6$ cells/ml. Fabricated hydrogels were compared with cell-free hydrogels prepared at the same time in terms of soluble fraction, swelling ratio and compressive modulus. This was done in accordance to previously mentioned methods.

Results: The results confirm that the presence of encapsulated iMSCs didn't influence the physico-mechanical properties of fabricated hydrogel with no significant differences between cell-free hydrogels and cell-encapsulated hydrogels (**Figure 25**).

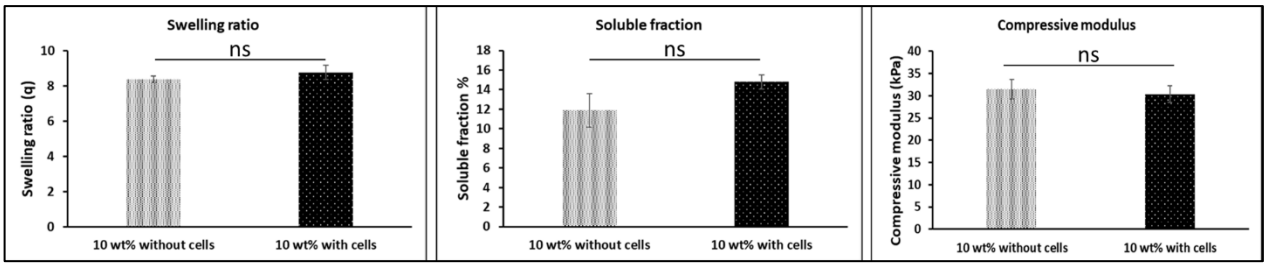


Figure 25: Effect of encapsulated iMSCs on swelling ratio, soluble fraction and hydrogel stiffness of hydrogels fabricated using 10 wt% GelMA, 0.05% LAP and cross-linked for 90 seconds.

Objective 4: To assess the effect of cell encapsulated hydrogel formulations on the hemidesmosome expression of seeded keratinocytes and Collagen I and Igαvβ1 expression for seeded fibroblasts. Collagen XVII (a late marker for hemidesmosome assembly) was used for DoE groups assessed in Section 3.1 (Objective 2) but for these experiments Igβ4 was used. Both Collagen XVII and Igβ4 can be used for assessing hemidesmosome assembly and previous published reports indicate the direct correlation between the expressions of these two markers: if one is high the other marker expression is expressed at higher levels as well. Collagen I and $Ig\alpha_v\beta_1$ marker expression was determined for fibroblasts. Collagen I is one of the key components of the newly synthesized extracellular matrix by fibroblasts during wound healing whereas $Ig\alpha_v\beta_1$ is expressed in fibroblast and is important for $TGF\beta_1$ activation which is critical for tissue healing.

Methods: The chosen hydrogel platform (10 wt% GelMA, 0.05% PI and cross-linked for 90 seconds) was mixed with iMSCs to have a final encapsulation of 5 x 10^6 cells/ml. Cell-free hydrogels were used as a control. Post fabrication, HaCaTs (25,000 cells/gel) were seeded on fabricated hydrogels and cultured for 72h. Samples were collected after 24h or 72h and fixed for ten minutes in 4% paraformaldehyde. Samples were then immersed in 5% bovine serum albumin (BSA) in PBS and probed with a primary rabbit polyclonal antibody for Igβ4 [(critical early marker for hemidesmosome assembly) NB10065599; Novus Biologicals; 1:500] followed by an FITC-anti-rabbit secondary (ab97037; abcam; 1:500). Samples were counterstained with nuclear stain Hoechst (Sigma-Aldrich).
For HDFs, 10000 cells/gel was seeded on cell-free or iMSC-containing GelMA hydrogel and cultured upto 8 days. The samples were collected at different time points (day 1, 4 and 8) and fixed using 4% paraformaldehyde for 10 minutes. Samples were stained with Cy3 conjugated-$Ig\alpha_v\beta_1$ [(BS-2016R-CY3), Bioss Antibodies; 1:200] and Collagen I [(extracellular matrix marker) ab34710; Abcam; 1:200] for 2 h at room temperature. Samples were immersed in an anti-rabbit secondary (ab97037; abcam; 1:500) after extensive washing for 1 hour. Samples were counterstained with nuclear stain Hoechst (Sigma-Aldrich). Micrographs (×10) were obtained on an upright fluorescent Leica DM6 fluorescence microscope as previously described. Representative micrographs have been presented here.

Results: The HaCaTs seeded on both cell-free and iMSC-encapsulated hydrogels showed Igβ4 expression (**Figure 26**) but the presence of iMSCs supported higher levels of HaCaT proliferation (evident from surface coverage) and Igβ4 expression relative to cell free control. Similar observation was made for HDFs (**Figure 27**) where presence of iMSCs significantly upregulated the expression of Collagen I and $Ig\alpha_v\beta_1$ relative to GelMA control.

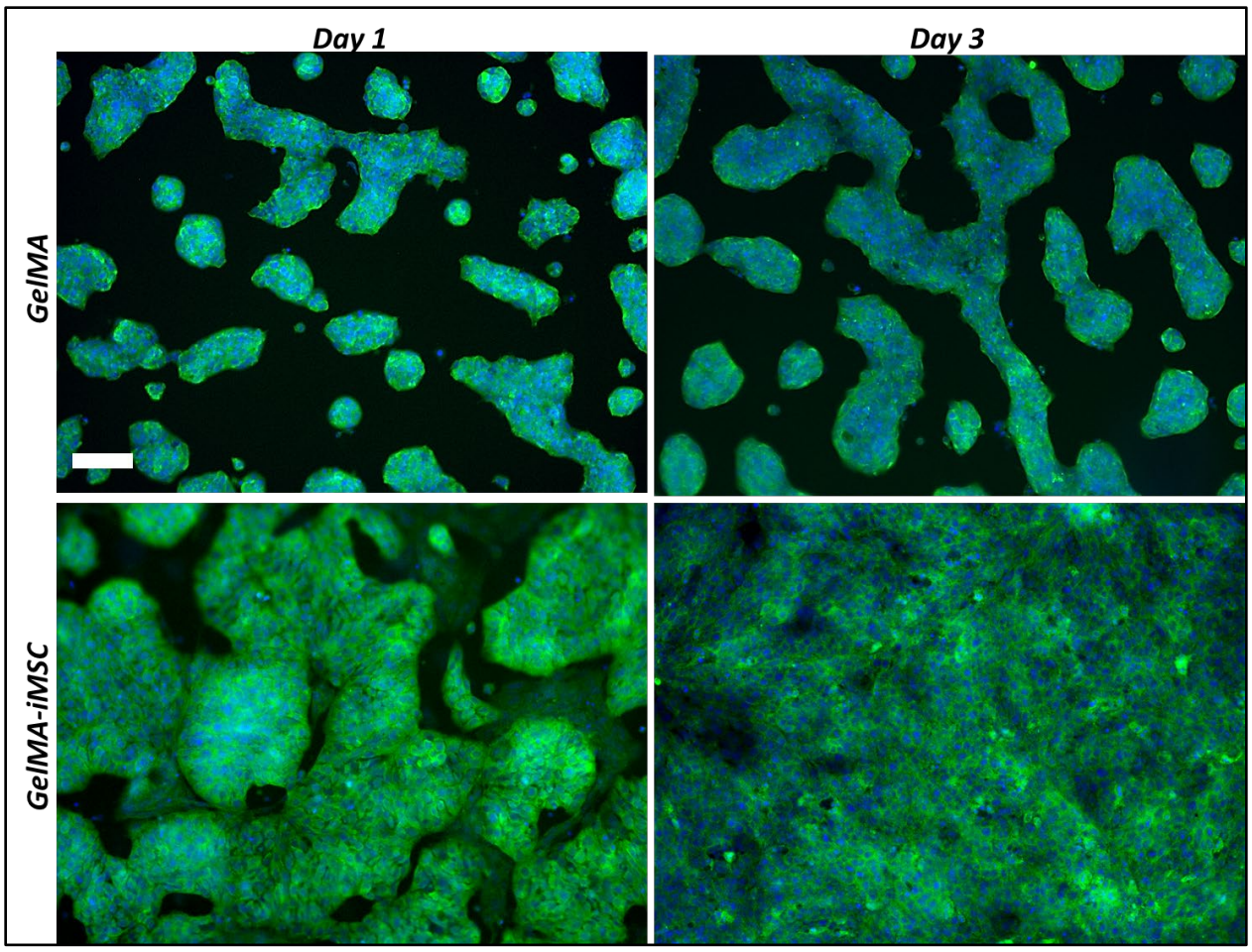


Figure 26: Immunofluorescent (Hoechst and Igβ4) micrographs of keratinocytes at day 1 and 3. Scale bar is 100 µm

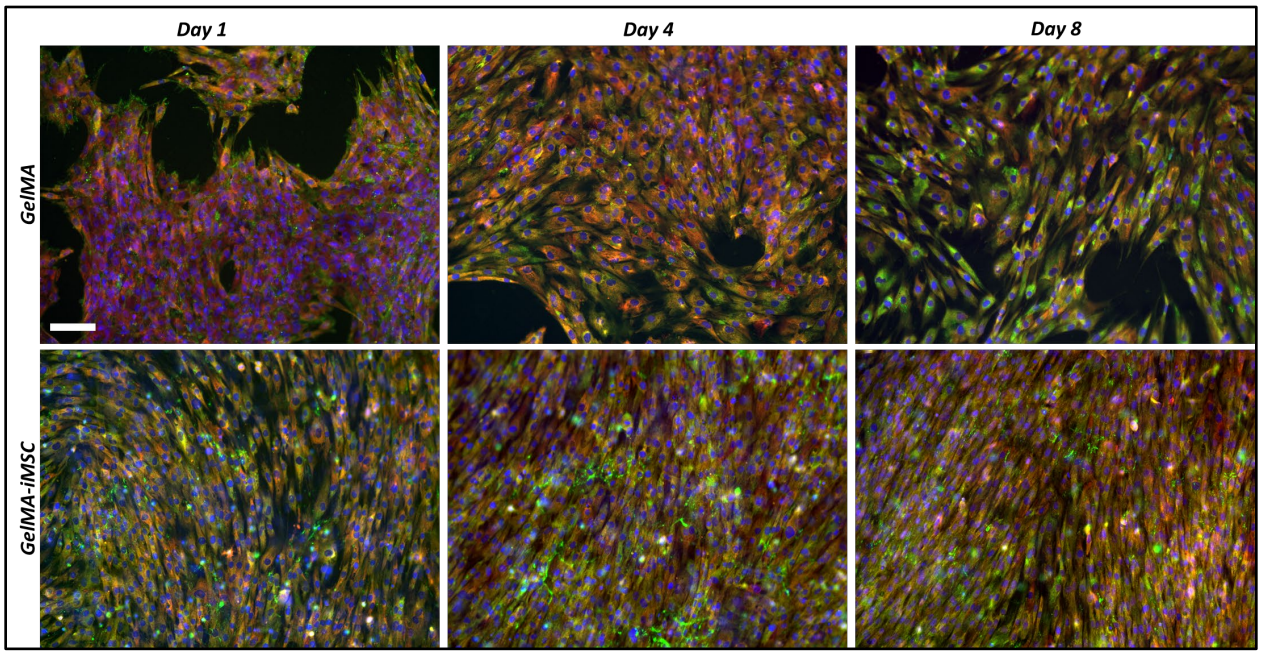


Figure 27: Immunofluorescent (Hoechst, Collagen I and Igαvβ1) micrographs of fibroblasts at day 1 and 3. Scale bar is 100 µm

Conclusion:
These results confirm that the selected GelMA scaffold to be used as a carrier for the stem cells to help create an environment that can recruit the cells and help in healing of the soft tissue around the metal implant not only maintains the viability (data shown in previous report) but also maintains the capability of cells secreting trophic factors. Also, the CM collected from iMSCs and BM-MSCs encapsulated GelMA supported growth and migration of cells associated with wound healing, which is comparable to the CM collected from stem cells cultured on tissue culture polystyrene. Also, inclusion of stem cells within the hydrogel did not influence the physico-mechanical properties of the hydrogel. However, the presence of iMSCs within the hydrogel upregulated the expression of key markers; hemidesmosomes (Igβ4) in HaCaTs and collagen I and Igαvβ1 expression in HDFs.

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.

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- 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.
- Perform similar characterization with the selected porcine MSC candidate to ensure cell survival and proliferation within GelMA – based hydrogel system.
- An ex-vivo skin model will be used to assess the efficacy of cell-laden hydrogel scaffold to improve epithelial attachment on titanium rods.
- Manuscript preparation: *'Use of iMSCs-laden scaffolds for epithelial attachment onto metal abutment for improved performance of osseointegrated devices for amputees: in vitro and ex vivo analysis'.*

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:

Exploration of potential for in vivo experiments to be conducted at University of Minnesota:

Given the reduced capacity for animal work at USUHS following 2020 pandemic restrictions and 2021 queue, the team has begun exploring conducting the swine experiments at the University of Minnesota.

- Experimental Surgical Services (ESS) has been consulted, given their experience in conducting large animal studies for internal and external customers. https://med.umn.edu/surgery/research/experimental-surgical-services
- An initial meeting was held to describe the animal model, observation period. Surgical procedure, implants, team requirements, IACUC process, tissue and fluid collection, histopathologic and imaging requirements and timing for completion.
- ESS states they do have capacity to meet scientific aims within the time constraints of the funding period and agency.
- ESS generated a budget estimate, which is in the process of being adjusted to correctly represent needed services. Additional costs include:
    - implants (to be fabricated through Zimmer Biomet),
    - Histopathology services (two vendors are candidates),
    - HHRI and the University of Minnesota are discussing potential for F&A waiver or reduction for this additional work.

- Our team has fine-tuned the budget to correspond to experimental plan, and are in final stages of updating the Statement of Work.

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. 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' was presented virtually at ORS 2021, where the poster was selected as finalist in Implant Section.

II. I. Mutreja, K. Twaroski, J.E. Bechtold, J. Tolar, J.A. Forsberg, C. Aparicio. 'iMSCs-laden adhesive polymer matrix for improving soft tissue attachment at skin/implant interface' was accepted for poster presentation at MHSRS 2021. Military Health System Research Symposium 2020 (Meeting cancelled due to COVID-19 outbreak).

III. I. Mutreja, K. Twaroski, J.E. Bechtold, J. Tolar, J.A. Forsberg, C. Aparicio. Human iMSCs-laden photocurable hydrogel for improved durability of the skin/implant interface – An in vitro and ex vivo analysis. Submitted to ORS 2022 Annual Meeting

- **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".

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

Name: *Jonathan A Forsberg, MD, PhD.*
Project Role: *Principal Investigator*
Nearest person month worked: *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: *Elsa Ronzier, PhD*
(Replacement of Dr. Devaveena Dey whom was spearheading the project at USUHS resigned to taken-on an administrative position with the FDA on December 24, 2020)
Project Role: *Associate Investigator (USUHS)*
Researcher Identifier (e.g. ORCID ID): 0000-0003-1008-4271
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: 1
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.