Diabetic Complications Consortium

Application Title: Development of an off-the-shelf injectable MSC Microcarrier for Diabetic Wound Healing
Principal Investigator: James Ankrum, PhD

1. Project Accomplishments:

This project pursued 3 main goals: i.)Understand the impact of cryopreservation on MSC's ability to function in vivo, ii.) Refine a cell carrier system to enable efficient delivery and localization of transplanted MSC to a local site of injury, iii.) Examine MSC's ability to promote wound resolution in a mouse model of diabetic wound healing.

As the wound model was new to our lab, we initially pursued the first goal using a retinal ischemia reperfusion model made available to us through a collaborator. There have been several reports in recent years of cryopreservation affecting MSC potency in *in vitro* assays, but little investigation of the impact of cryopreservation on MSCs within in vivo disease environemnts. We sought to determine if using standardized freezing protocols with commercially available freezing reagents would yield MSCs that upon thaw would behave similarly to freshly cultured MSCs allowing for banking of MSCs for use in any hospital facility. We found that while cryopreserved MSCs showed a slight deficiency in viability, immunomodulatory function, and suppression of T lymphocytes compared to fresh MSCs *in vitro*, there was no difference in MSC performance in regards to their ability to rescue retinal ganglion cells following I/R injury. Therefore, it appears that in the case of ischemic conditions, cryopreserved MSCs can be used with the same effectiveness as fresh MSCs using cryopreservation methods optimized for MSCs. This works proved the concept that MSC's can be used off-the-shelf out of cryostorage without needing a recovery period in culture

The second goal of refining a cell carrier system was carried out through both in vitro and in vivo testing. We were also able to explore different microcarrier scaffolds for their suitability for MSC transplant into an *in vivo* model. Our goal was to find a system that would allow for dense packing of MSCs, would be imaging friendly, and would increase MSC retention in a wound site after transplant. We started with a simple gelatin scaffold crosslinked with genipin and found that a complex leaching protocol was required to remove unreacted genipin. Additionally, we found that even when we were able leach all free genipin and have high MSC viability, the microcarrier material became autofluorescent and interfered with fluorescent imaging making assessment of cell function via optical based assays impractical. To overcome the autofluorescence problem, we changed the crosslinker to glutaraldehyde which reduced autofluorescence, but still required a significant processing protocol to remove excess glutaraldehyde which would decrease MSC viability. We found that an additional treatment of the glutaraldehyde microcarriers with sodium borohydride greatly improved the optical properties of the microcarriers and additionally allowed for dense packing of MSCs onto the microcarriers with high viability. We were able to find optimal parameters for loading MSCs efficiently onto these microcarriers without significant loss. We also optimized and streamlined microcarrier production by making large quantities of scaffold, milling the scaffold, and using a sieve to select the microcarrier size range which was most advantageous for MSC loading and application. MSC's on the scaffold maintained high expression of IDO and growth factors. Prior to in vivo testing, MSCs were transduced with mCherry lentivirus to allow for in vivo tracking. We found that the carriers enhanced MSC retention in the wound when applied topically. However, the scaffold did not protect MSCs from detection by the immune system after transplantation. A marked decline in MSC numbers over time was observed by measuring fluorescence signal from the wound throughout the wound recovery phase. Based on the observed immunogenicity of our cells, we added a new design criteria to our microcarrier development: protection from immune recognition. Based on this criteria, we have begun developing an alginate based microencapsulation protocol for MSCs to allow for protection of MSCs from immune system detection. As this technique has been performed extensively in the past for islet transplantation, we are confident we

will be able to find encapsulation parameters that support MSC function while protecting transplanted cells from immune detection.

For the third goal, we sought to test our MSC loaded microcarriers in a mouse model of diabetic wound healing. While a number of mouse models of diabetic wound healing have been used to evaluate wound closure and angiogenesis, many of them rely on single genetic mutations or STZ based induction of diabetes and the animals maintain relatively normal wound healing. Thus, we sought to establish a wound healing model in a polygenic model of type 2 diabetes that has been reported to more closely mimic diabetes in humans. Initially we sought to use, NONcNZO10/LtJ mice, but due to colony limitations with the supplier, this was not a feasible option. We then turned to another polygenic model of diabetes, the TallyHo mouse from Jax. The male TallyHo mice spontaneously develop Type 2 Diabetes between 12 and 14 weeks of age and display impaired wound healing to a much greater degree than db/db or ob/ob mice. We used a full thickness excisional wound model on the dorsum of the mice. As mouse wound heal primarily through contraction instead of reepithelialization, the wounds were stented with stainless steel washers and sutures after wounding, forcing wound to heal via epithelialization. We found that within the TallyHo model, the wounds took >2 weeks to heal demonstrating a vastly impaired healing rate compared to other models. In this process, we found that use of metal stents were required over silicone stents, as the long healing time led to increased wear of the silicone stents and a high rate of stent failure. We also sought to develop a method to use optical coherence tomography (OCT) to serially image healing and revascularization within the same animal. While OCT has been set up for monitoring tumor growth using implanted windows, no protocol for wound imaging was available and thus we developed the method ourselves with collaboration with Dr. Bret Bouma's group at MGH. We developed a animal stabilization system to hold animals in place during imaging and are in the process of identifying the optimal acquisition settings and topical wound dressing to allow for repeat imaging of neovascularization within the wound bed. Upon completion of protocol development, we plan on publishing this protocol as it will enable histology quality imaging of wound resolution within a single animal over time. Overall, we were not able to achieve improved wound healing in our MSC treated mice compared to controls. We have several hypothesis for why this may have occurred. Topical administration of the microcarriers to the wound appear to have retarded the progression of wound healing and contributed to a large scab formation. Future iterations of this technique are focusing on subcutaneous delivery of MSCs and improved topical dressing of the wound to prevent dessication and scab formation. Additionally, to our surpise, mCherry transduction of MSCs appears to dramatically reduce their ability to modulate inflammation. In follow-up examinations in in vitro assays, mCherry MSCs lost their ability to suppress PBMC inflammation. Thus, in future assays, chemical based dyes instead of viral vectors will be used for tracking MSC location.

2. Specific Aims:

Specific Aim 1. Tune biodegradable injectable microcarriers to optimize cell loading, viability, function, and recovery after cryopreservation.

Results: MSCs were loaded onto two different gelatin microcarrier systems. Both gelatin crosslinked with genipin or glutaraldehyde showed significant autofluorescence when imaged on a fluorescent microscope. This autofluorescence interferes with with the standard FITC, TR, and Cy5 channels making many standard assays incompatible with the microcarriers. However, when glutaraldehyde crosslinked gelatin was neutralized with sodium borohydride, these microcarriers became significantly less autofluorescent making assays such as live dead staining possible.

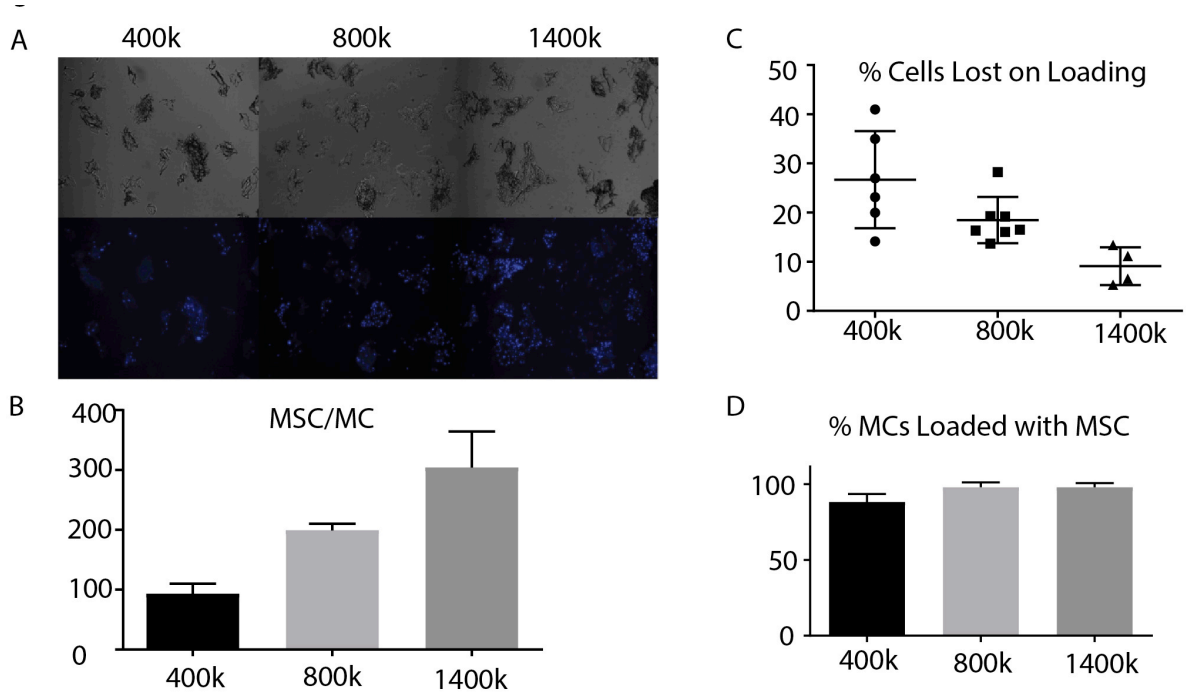

Figure 1. As MSC dose is increased, MSC density on microcarriers increases with minimal loss. (A) Bright field and DAPI images of cells on microcarriers with increasing initial cell dose. (B) Quantification of MSC density on microcarriers. (C) Flow cytometry counts of cells lost post-microcarrier loading. (D) Percentage of microcarriers with at least one MSC for each loading condition.

For these microcarriers we found that as we increased the total MSC number per microcarriers, the MSCs packed more densely (Fig. 1 A,B) and there was a only a minor loss from MSCs not loaded as determined by flow cytometry on free cells post-loading (Fig. 1C). We also saw that the number of carriers loaded with at least 1 MSC changed depending on the initial loading conditions (Fig. 1D). MSC viability on microcarriers was slightly lower than MSCs cultured on tissue culture plastic (Fig. 2A). Additionally, we found that the cumulative VEGF and kynurenine production per MSC in response to IFN-γ stimulation were not significantly different between free and microcarrier bound MSCs (Fig. 2B,C).

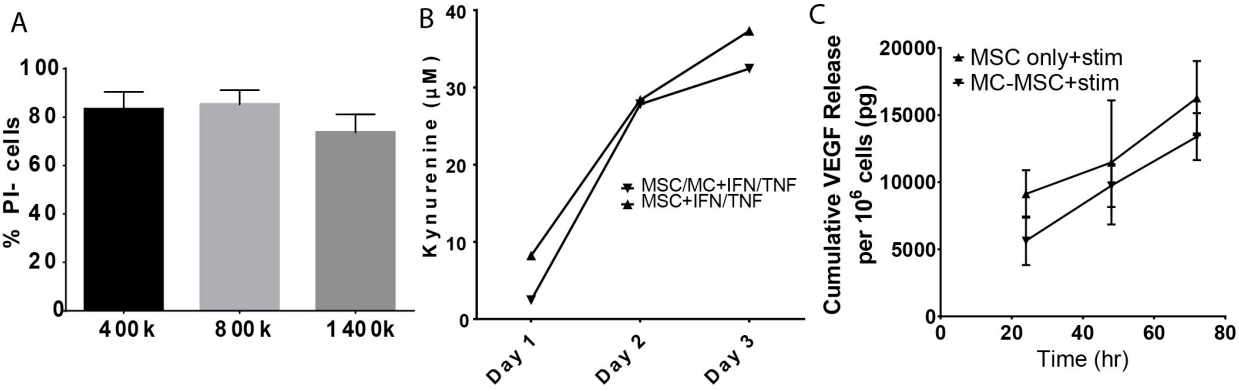

Figure 2. MSCs are viable and produce a secretome similar to that of free MSCs. (A) Viability of MSCs on microcarriers for increasing loading conditions measured by PI/Hoechst live/dead staining. (B) Cumulative kynurenine production by free and microcarrier adherent MSCs. (C) Cumulative VEGF production by free and microcarrier adherent MSCs.

In parallel, to MSC scaffold creation, we examined MSC function after cryopreservation both in vitro and in a retinal ischemia/reperfusion disease model with a collaborator. We found that cropreserved MSCs

showed minor decreased viability out to 72 hours Figure 3A, but there was no significant difference in cryopreserved MSCs' ability to proliferate Figure 3B. We observed that functionally, cryopreserved MSCs did not show any significant difference in kynurenine production, an immunomodulatory factor produced by the conversion of tryptophan by IDO (Fig. 4A), but cryo-MSCs had a marginal decline in their ability to suppress T-cells (Fig. 4B). We used a growth factor array to determine if there was any major differences in the repetoir of growth factors secreted by MSCs after cryopreservation. We found that while there were some differences in production levels among the growth factors, to a large extent the growth factor profiles were largely similar between fresh and cryo-MSCs (Fig. 4C,D). Finally in our retinal ischemia reperfusion model, we saw that there was no difference between fresh and cryo MSCs in their ability to rescue retinal ganglion cells Figure 5.

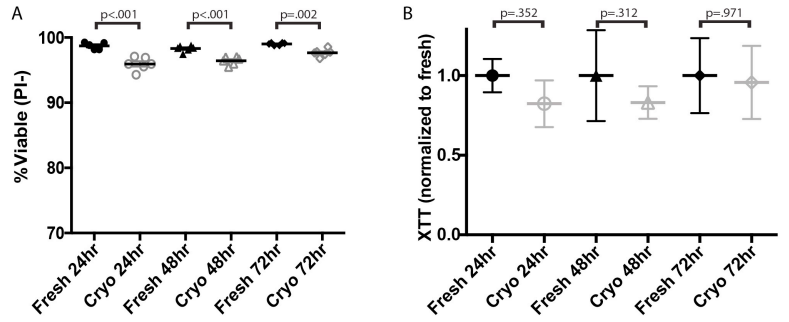

Figure 3. Cryopreservation minimally affects MSC viability and metabolic function. (A) Viability of MSC plated after thawing was compared to donor and passage matched MSC from fresh cultures 24, 48, and 72 hours after thawing. Cells were stained with Hoechst 33342 and PI and imaged with a fluorescence microscope. Cells double positive for Hoechst 33342 and PI were considered dead. (One-way ANOVA with Sidak correction for multiple comparisons, $p<0.05$ considered significant, n=5). (B) The metabolic activity of MSC after cryopreservation was compared to donor and passage matched MSC from continuous cultures using XTT (mean±SD, Oneway ANOVA with Sidak correction for multiple comparisons, $p<0.05$ considered significant, n=6). All experiments performed with MSC from donors 8002L and 7083 at passages P3–P5.

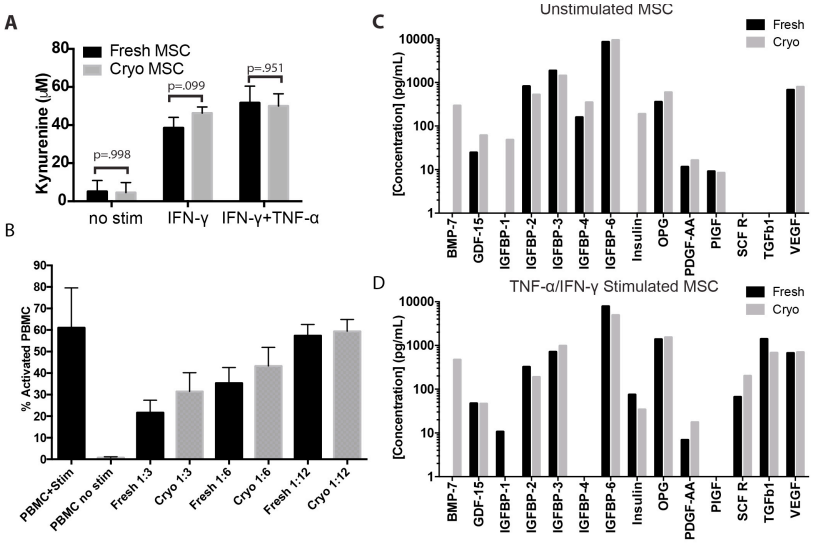

Figure 4. Cryopreserved MSC maintain immunosuppressive and secretory potential. (A) IDO activity as measured by the concentration of kynurenine in the conditioned media collected from fresh or cryo-MSC exposed to IFN-γ or TNF-α/IFN-γ for 48hours (mean±SD, One-way ANOVA with Sidak correction for multiple comparisons, $p<0.05$ considered significant, n=6). (B) Quantification of the percent of activated PBMCs in each co-culture condition compared to unstimulated and stimulated controls. No statistical differences between fresh MSC and cryo-MSC at each ratio. (mean±SD, One-way ANOVA with Sidak correction for multiple comparisons, $p<0.05$ considered significant, 1:3 and 1:6 n=4, 1:12 n=3). All experiments performed with MSC from donors 8002L and 7083 at passages P3–P5. (C) The concentration of each growth factor produced by fresh and cryo-MSC over 48hours in unstimulated conditions. (D) The concentration of each growth factor produced by fresh and cryo-MSC over 48hours in stimulated IFN-γ/TNF-α conditions.

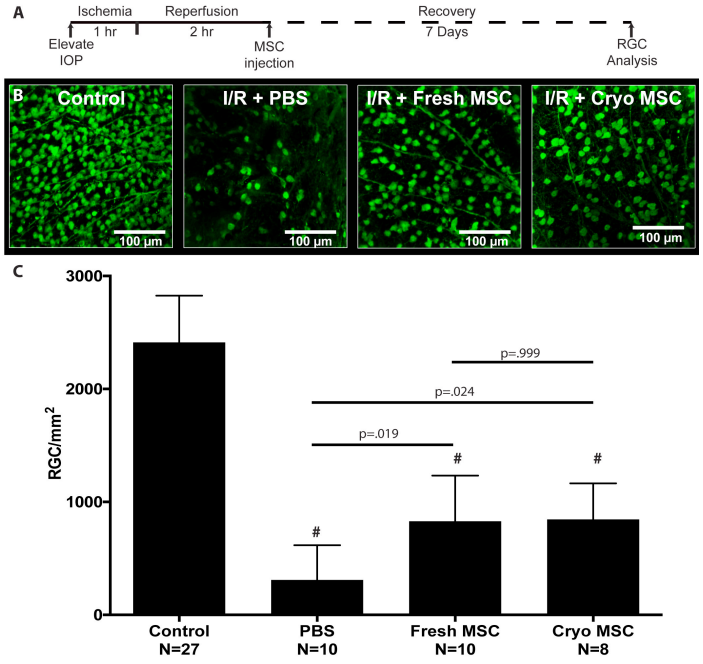

Figure 5. Cryopreserved MSC prevent RGC loss after ischemia/reperfusion injury in vivo. (A) Injury and treatment timeline for all retinal ischemia/reperfusion model. Intraocular pressure (IOP) was elevated to blanch the fundus for 1hour, after which perfusion was restored. 2hours after reperfusion, eyes were injected with one of the MSC groups or PBS as a vehicle control. 7 days later, animals were sacrificed and eyes were analyzed for RGC counts. (B) Representative images of γ-synuclein immunostaining of whole-mounted retina from non-ischemic retinas (control) and retinas after I/R treated with vehicle (I/R+PBS), fresh MSC (I/R+Fresh MSC) or cryo-MSC (I/R+Cryo-MSC). (C) Quantitative analysis of RGC survival in eyes after I/R revealed a significant rescue effect after transplantation of both fresh MSC and cryo-MSC (mean±SD, One-way ANOVA with Tukey honest significant difference post hoc test to correct for multiple comparisons, $p<0.05$ considered significant). #denotes $p<0.001$ in comparison to healthy control. Both cryo-MSC and fresh MSC were from donor 7083.

Specific Aim 2. Pilot study to determine the ability of cryopreserved microcarriers to accelerate healing in an excisional diabetic wound model.

Results: We used a multigenic type 2 diabetic TallyHo mouse model which has been shown previously to have impaired wound healing ability. We used a stented excisional wound to model a diabetic wound after surgical debridement (Fig. 6A). By analyzing brightfield wound images, we found that there was no difference in wound closure rates between our untreated control groups and our MSC treated groups (Fig. 6B).

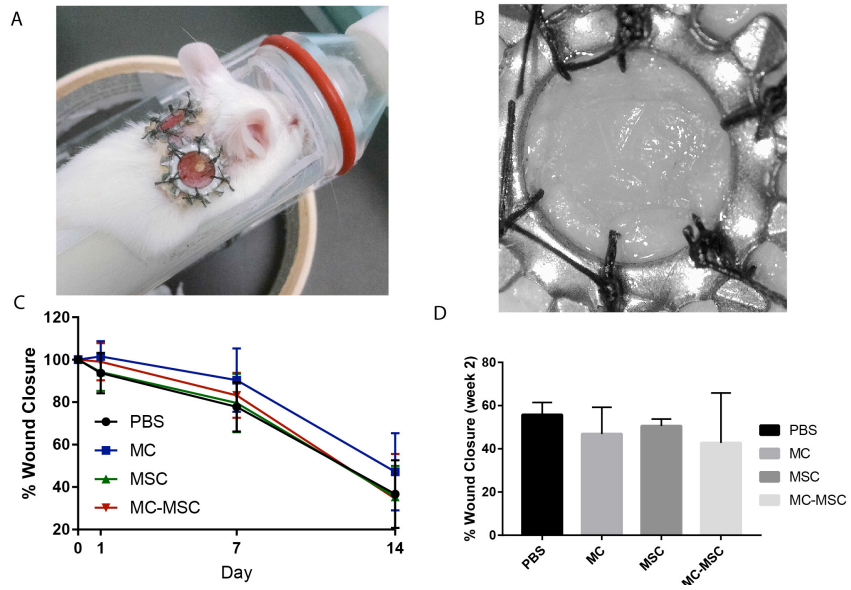

Figure 6. TallyHo diabetic wound model shows no difference in healing rate between groups. (A) TallyHo mouse with two excisional wounds on back. (B) Close up optical of stented wound. (C) Percentage wound closure after 1, 7, and 14 days. (D) Wound closure percentage from initial wounding at end of week 2.

This was surprising, as previous publications had demonstrated improved healing upon addition of MSCs in other wound models. We have several hypotheses that could explain the lack of improved healing in our model. MSCs infected with a mCherry expressing adenovirus decreased their immunomodulatory function and increased their immunogenicity when exposed to allogeneic T-cells. We ran a western blot looking at IDO production in MSCs modified with and without mCherry and found a large decrease in IDO production (Fig. 7A). We also co-cultured MSCs with and without mCherry modification with T-cells and found that mCherry modified cells were not able to suppress T cell proliferation (Fig. 7B,C).

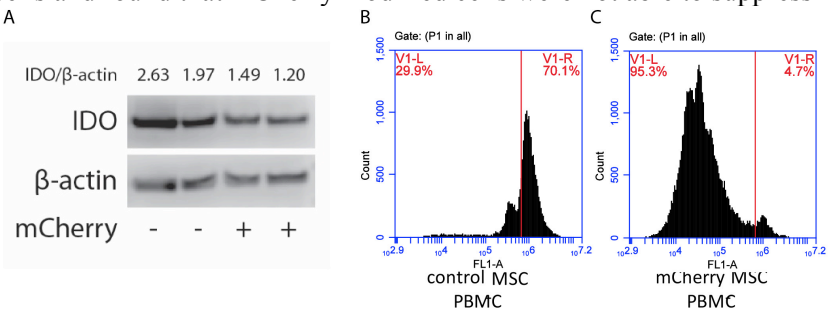

Figure 7. mCherry adenovirus modified MSCs demonstrate increased immunogenicity. (A) Western blot of IDO protein from MSCs modified with and without mCherry. (B) Control MSCs suppress 70% of PBMCs while (C) mCherry modified MSCs only suppress 4.7% of PBMCs indicating a complete loss of immunosuppressive ability.

Future studies will be preceeded by pilot studies focused on examining the immunogenicity of the labeling strategy. We also believe that stent failure during the second week of the study significantly affected the final wound closure measurements as once the stents no longer held the wounds open, we saw significant contraction of the wound. We believe that using a silicone glue which is more flexible along with a continuous running suture will better secure the stent to the animal. Another option being explored is a cranial skin wound model because cranial skin doesn't contract as readily as skin on the rest of the body. A cranial skin wound would also be more stable for OCT imaging allowing us to image angiogenesis *in situ*. We tracked mCherry tagged MSCs with a dinolite fluorescent microscope and found that there was better long term retention of the cells over time on MCs compared to free MSCs, although both free and MC adherent MSCs failed to persist long-term in the wound bed (Fig. 8). Shift in macrophage polarization from M1 inflammatory macrophages to M2 anti-inflammatory macrophages is an important aspect of diabetic wound healing. We looked at macrophage polarization markers iNOS (M1 macrophage) Arginase 1 (M2 macrophage) in wound tissues via PCR. We found that there was no statically significant shift towards M2 phenotype in the MSC treated groups during the first week (Figure 9A), but there appears to be a trending, but non-significant, increase in M2/M1 gene expression after the second week in the MSC treated groups (Figure 9B). We have made significant progress on developing a mouse diabetic wound model that can evaluate a cryopreserved off the shelf therapy and with the proposed modifications, we believe that we will be able to more thoroughly evaluate and develop a cryopreservable microcarrier systems for MSC therapy.

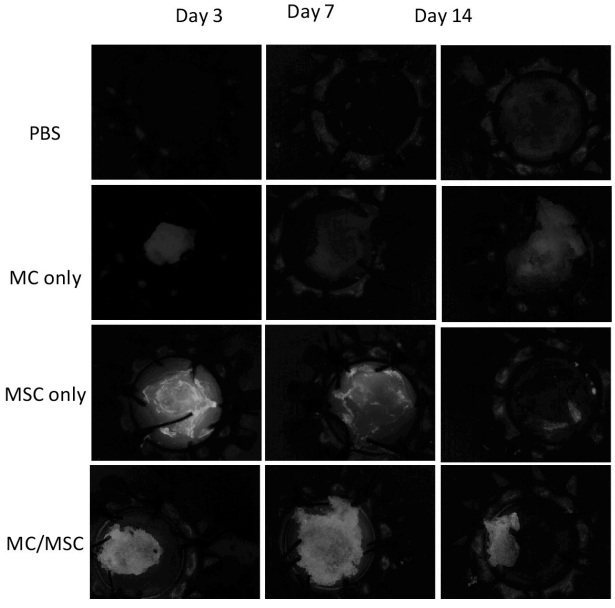

Figure 8. Microcarriers improve MSC retention in the wound site. Fluorescent images of TallyHo wound beds at day 1, 7, and 14. Despite receiving a second dose of MSCs on day 7, MSCs fail to persist in the wound bed throughout the 14 day test period.

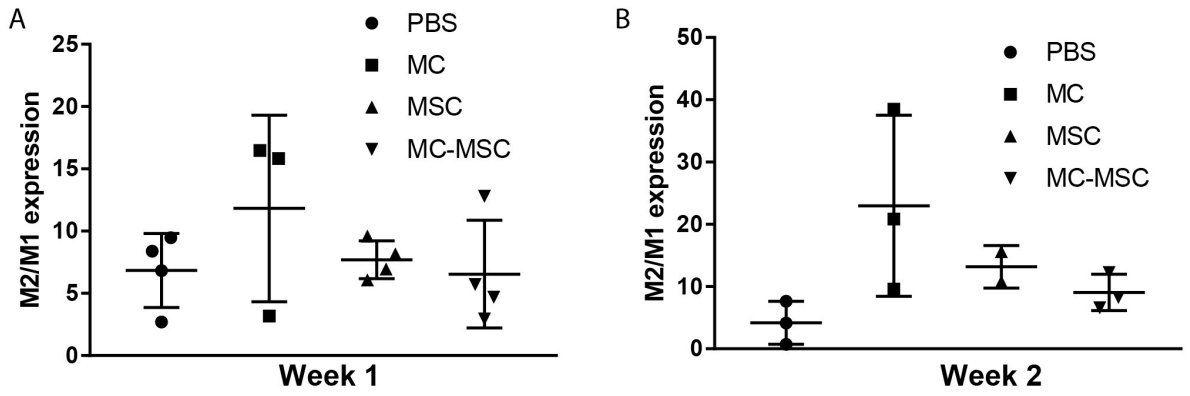

Figure 9. MSC treated wounds showed trending, but non-significant, shifts towards M2 macrophage phenotype. (A) RT-PCR of Arg1 mRNA expression to iNOS mRNA expression at week 1. (B) RT-PCR of Arg1 mRNA expression to iNOS mRNA expression at week 2.

3. Publications:

Peer-Reviewed Publications

1. Burand, A. J., Gramlich, O. W., Brown, A. J. & Ankrum, J. Function of Cryopreserved Mesenchymal Stromal Cells With and Without Interferon-γ Prelicensing is Context Dependent. *Stem Cell* (2016). doi:10.1002/stem.2528

2. Gramlich, O. W. *et al.* Cryopreserved Mesenchymal Stromal Cells Maintain Potency in a Retinal Ischemia/Reperfusion Injury Model: Toward an off-the-shelf Therapy. *Sci. Rep.* **6,** 26463 (2016).

Published Conference Abstracts

1. OW Gramlich, W Zhu, A Burand, JA Ankrum, MH Kuehn. Transplantation of cryopreserved MSC rescues retinal ganglion cells following I/R injury. Investigative Ophthalmology & Visual Science (2016). 57 (12), 4402-4402

2. AJ Burand Jr, AJ Brown, JA Ankrum. Development of an Injectable Cell-laden Microniche for Mesenchymal Stem Cell Therapy. TISSUE ENGINEERING PART A (2015) 21, S275-S275