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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this study is to test the hypothesis that hypertrophic burn scars can be remodeled by fractional laser treatment and administration of stem cells. Finding the best ways to combine these approaches is a goal of this proposal. During the period of this report we have been completing an examination of the effect of administering autologous and allogeneic bone marrow derived mesechymal stem cells (BM-MSCs) and adipose derived stem cells (ADSC) to hypertrophic third degree burn scars in Red Duroc pigs using ablative fractional CO_2 or Erbium:YAG lasers. Epidermal and superficial dermal remodeling was noted to varying degrees in specimens treated with BM-MSCs and ADSCs by both lasers. Further Western blot, cell culture and Real Time PCR analysis along with additional studies performed on uninjured skin have indicated that: i) CO_2 laser can have a more tissue damaging effect than Erbium:YAG laser, ii) autologous stem cells appear to generate a less fibrotic response, iii) cells derived from autologous stem cell treated burns have a more immature phenotype, iv) collagen indicative of dermal remodeling may be more persistent when burns are treated with autologous BM-MSCs, and v) cells derived from stem cell treated burns appear to peak 14 Days after treatment and taper significantly beyond 21 Days after treatment. Overall, these findings indicate that Erbium:YAG and perhaps autologous stem cells may have and advantage in treating hypertrophic burn scars. They also suggest that repeat administration is likely to be beneficial, again favoring the use of autologous cells.

15. SUBJECT TERMS
fractional laser, stem cells, scarring, dermal remodeling

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INTRODUCTION: The purpose of this study is to develop an optimal delivery system for stem cells that can reduce burn scars. In this unique approach both the delivery system and delivered agents have been shown to have an effect in scar reduction. Combining these technologies could have a greater than additive effect on skin regeneration with normalization of function. New technologies that reduce burn scars would have a significant impact not only for wounded warrior, but also civilians who suffer from burn injuries. This proposal aims to evaluate the effectiveness of these novel delivery systems and cell-based therapies for third degree burns in a porcine model. We will test the hypothesis that hypertrophic burn scars can be remodeled by fractional laser treatment and administration of stem cells. Stem cells will be administered alone or incorporated into a chitosan fibrin matrix. Finding the best ways to combine these approaches is a goal of this proposal.

BODY:

Over the past year we have completed animal work where burn scars were administered directly applied stem cells or stem cells incorporated in PEGylated fibrin gels after ablative fractional laser treatment. This last year has focused predominately on burn scars treated with stem cells incorporated in PEGylated fibrin gels and treated with Erbium:YAG laser at the high and low settings.
An overall improved scar scale scoring was observed in wounds treated with autologous ADSC in PEGylated gels when Erbium:YAG laser was used at the high setting. These changes were noted at Days 14, 21 and 35, with the greatest change occurring at Day 21. These findings are consistent with previous observations that there may be a prolonged effect afforded by the incorporation of stem cells in a gel matrix. There was however very little change noted when Erbium:YAG was used at the low setting. In the previous reports we have noted a more prominent tissue destructive effect associated with laser treatment at higher settings (including Erbium:YAG). The PEGylated gel matrices could be providing several benefits (over direct application of stem cells) by allowing cells more access to deeper tissues (possibly by serving as a moist occlusive like dressing) and by supporting the stem cells in a less hostile environment.
In evaluating scar scale scores burn wounds treated with allogeneic ADSC directly applied to burn scars, there appeared to be better improvement when scars were treated with Erbium:YAG laser at the high setting at Day 21. There was however an increase in scar score over baseline (Day 0) for all burn scars (untreated control and ADSC treated) at Day 14. Increases over baseline were noted in both control and ADSC/Erbium low treated burn scars at Day 21. We have observed similar effects in animals treated with directly applied allogeneic stem cells (particularly ADSC) which could represent a local and/or systemic response to allogeneic cells. We have compared these findings to data on burns treated with autologous and allogeneic ADSC delivered directly or incorporated into PEGylated gels. In comparing scar scores in burns treated allogeneic ADSC administered in PEGylated gels to those treated with directly applied ADSC to there is a similar but much less dramatic change in (increased) scores for ADSC/PEGylated gel treated burns at Days 14 and 21. As with directly applied allogeneic ADSC, improved scores were noted when burn wounds were first treated with Erbium:YAG at the high setting. These findings confirm several previous observations that allogeneic stem cells produce a different response than autologous cells. Among the differences observed are a shorter lived response (e.g. here we see tapering of better scoring by Day 35) than autologous cells and possibly a greater host response (e.g. by increased scar scoring at Days 14 and 21) to allogeneic cells (more apparent with ADSC). We have hypothesized that these findings could be due to limiting direct access of cells to tissues. We believe paracrine effects likely mediate the beneficial effects of stem cells incorporated into PEGylated gels. This is supported by our molecular studies illustrating a prolonged effect when allogeneic BM-MSC were incorporated in PEGylated gels.
While the scar scoring system has been a useful tool in integrating molecular, cell culture and histologic findings we have found that there can be shortcomings in relying solely on scar score assessment. We have been evaluating other means of clinical assessment to better characterize changes that may be missed. In the last year we have begun a closer examination of burn scars by photographic evidence, which can be enlarged. Photographic analysis of burn wounds treated with Autologous BM-MSC in PEGylated gels, Allogeneic BM-MSC in PEGylated gels or PEGylated gels were reviewed after standardizing photographs for size and adjusting orientation. Photographic analysis of burn wounds treated with Autologous BM-MSC in PEGylated gels, Allogeneic BM-MSC in PEGylated gels or PEGylated gels was reviewed after standardizing photographs for size and adjusting orientation. Burn wounds treated with Autologous M-MSC in PEGylated gels after Erbium:YAG laser appeared overall improved at Days 15 and 21. This effect was noted when Erbium:YAG was used at both the high and low setting, but seemed to have slightly better outcome when the high setting was used. This was similar to some previous observations that indicated stem cells delivered without gels may have produced a better outcome when lasers were used at the higher settings. Observations made previously in normal skin also suggest that higher laser energies may be associated with a debridement effect that might facilitate cells to enter tissues or deliver materials to the burn scar. This effect is noted more prominently with higher energies than those used for cell delivery in this proposal. This illustrates the need to examine even higher laser energies for cell delivery, whether in matrices or not. While photographs of burn wounds treated with Allogeneic BM-MSC in PEGylated gels showed some improvement (at both high and low laser energy settings) this was less noticeable than burns treated with Autologous BM-MSC in PEGylated gels. Photographs of wounds treated with PEGylated gels alone did not show much change with perhaps some improvement when Erbium:YAG laser was used at the higher energy setting. These findings support that while there may be some improvement with the use of PEGylated gels alone, there is an additional benefit of incorporating stem cells. Similar to several other previous findings, this effect appears to be better when autologous stem cells are used. In addition, these findings illustrate the transient nature of these benefits and support the need to examine repeat administration to maximize this approach towards an effective therapy.
Molecular analysis of burn scars treated with PEGlyated fibrin gels alone and stem cells incorporated in PEGylated fibrin gels were underway in the last year. As we have previously reported, these studies have focused on Collagen I, Collagen III, TGFβ I, TGFβ III, α-SMA and decorin in tissues and cells derived from treated burn scars. Western blot analysis of wounds PEGylated fibrin gels without cells have helped to examine effects that may have been contributory only to the gels themselves. TGFβ-1 expression (commonly associated with increased scarring) was elevated in untreated control scars and scars treated with Erbium:YAG laser at the

high setting and PEGylated fibrin gels (Figure 1). Elevated TGFβ-1 levels noted in burn scars treated with Erbium:YAG laser at the high setting and PEGylated fibrin gels over untreated controls are likely due to the greater tissue destructive effects of fractional lasers at higher settings. While we cannot exclude that the PEGylated gels could have somewhat ameliorated this effect, their influence was not enough to reduce TGFβ-1 levels at or below control levels. Analysis of burn scars treated with Erbium:YAG laser at the low setting and PEGylated gels however revealed a reduction of TGFβ-1 levels over controls. This finding is supportive our initial observations in Specific Aim 1 where we noted that Erbium:YAG laser alone at the low setting produced superior results. There however appeared to be at least a limited short term effect of applying PEGylated gels following Erbium:YAG laser at a low setting. These findings again support that repeat administration (of laser, stem cells and/or gels) is likely to provide a greater benefit.

Real Time PCR analysis of cells derived from burn scars treated with PEGylated gels alone or containing either autologous or allogeneic BM-MSC were also examined. Analysis of αSMA levels in these cells (Figure 2) reveal an increased level of expression in burn scars treated with allogeneic BM-MSC in PEGylated fibrin gels at Day 14 (and to a lesser extent at Day 21 for laser at the low setting). These finding correlated with increased scar scoring at these time points and could illustrate an initial inflammatory host response to being administered allogeneic cells.

In burn wounds treated with PEGylated gels alone, Real Time PCR indicated an elevation in α-SMA at Day 35 including when Erbium:YAG was administered at the lower setting. As this analysis is based on isolated cells in culture, this would seem to indicate a more myofibroblast phenotype.

Real time analysis of cells derived from wounds treated with Allogeneic BM-MSC PEGylated gels and Autologous BM-MSC in PEGylated gels revealed low levels of α-SMA on all days. There were however slightly higher levels in cells derived from scars treated with Erbium:YAG laser at the high setting compared to those treated at the lower setting. In Western Blots protein analysis of treated tissues there was however elevated tissue αSMA protein expression (relative to untreated controls) in scars treated with Allogeneic BM-MSC in PEGylated gels at the lower setting at Day14 possibly indicating a greater inflammatory response to allogeneic cells. Increased levels of Decorin expression were noted in Real Time PCR analysis of cell derived from wounds treated with Allogeneic BM-MSC PEGylated gels and Autologous BM-MSC in PEGylated gels at Day 14. This was not observed in cells derived from burn wounds treated with PEGylated gels alone. Again, this indicates a benefit of incorporating stem cells in PEGylated fibrin gels.

Real Time PCR was examined in cells derived from scars treated with Autologous and Allogeneic ADSC in PEGylated fibrin gels following Erbium:YAG fractional laser at both the high and low settings. Increased levels of Decorin, TGFβ3, Collagen IIIα1, and Collagen IIIα1/ Collagen Iα2 ratio were noted at Days 21 and 35 for Allogeneic ADSC in PEGylated fibrin gels at the higher laser setting. These values were all significantly higher than those noted for Autologous ADSC in PEGylated fibrin gels (both laser settings) and those for Allogeneic ADSC in PEGylated gels when laser was used at the lower setting. TGFβ1 was however at a sustained low level (compared to controls) for Allogeneic ADSC in PEGylated gels as the lower energy setting.

Real Time PCR expression of TGFβ-1 was elevated at Day 35 in burn wounds treated with PEGylated fibrin gels alone. TGFβ-1 expression remained low in all days for wounds treated with Autologous BM-MSC in PEGylated fibrin gels or Allogeneic BM-MSC in PEGylated gels. This suggests a limited effect of PEGlyated fibrin gels alone to diminish TGFβ-1 over a prolonged time.

Real time PCR expression of Collagen αI was low at Days 14 and 21 in cells derived from wounds treated with Autologous BM-MSC in PEGylated fibrin gels at when Erbium:YAG laser was used at the low setting. The findings at Days 14 and 21 for Autologous BM-MSC in PEGylated fibrin gels support a limited effect within a time range we have repeatedly noted. This again reinforces the need to examine repeat administration.

TGFβ-3 expression by Real Time PCR was elevated at Day 35 in cells derived from wounds treated with Allogeneic BM-MSC and Erbium laser at the lower setting. Smaller elevations in TGFβ-1 were noted at Day 35 in cells derived from wounds treated with PEGylated gels alone but overall total levels of expression numbers were relatively small. This might suggest an additional benefit of adding stem cells.

In Real time analysis of Collagen αIII expression appeared elevated at Day 21 in cultured cells derived from wounds treated with Autologous BM-MSC in PEGylated gels when Erbium:YAG laser was used at the high setting. This level of Collagen αIII expression was increasing from Day 14. There was also an increase in Collagen αIII expression at Day 35 noted in cultured cells derived from wounds treated with PEGylated gels alone. It is possible that the stem cells stimulated an earlier response. Again, the peak effect for stem cells is within the 14 -21 day post treatment period, supporting the need for repeat administration.

Protein expression in tissues treated with Allogeneic ADSC in PEGylated gels we have not show a comparable increase in Collagen IIIα1 protein to that seen for BM-MSC. Changes in Collagen IIIα1 protein expression for scars treated with Autologous BM-MSC also appear to better correlated to prior increases in Real Time PCR expression of Collagen IIIα1. These findings may support a potentially greater influence of other cells, importantly inflammatory cells, in scars treated with allogeneic cells. It will be increasingly important to investigate this as it could lead to safety concerns for Allogeneic stem cells in the multiple dosing schedules that will likely be needed to achieve an optimal outcome.

In comparing burn wounds treated with autologous ADSC (delivered directly by injections) and autologous ADSC in PEGylated gels, various clinical measurements including scar size and clinical scoring were evaluated. In both cases there seemed to be more improvement when Erbium:YAG laser was used at higher energies. These findings, although preliminary, were noted predominately in the scar scoring for autologous ADSC in PEGylated gels and in overall size differences in scars for autologous ADSC delivered by injection. These improvements appeared to peak at Day 21 post treatment and again confirm an apparent maximum benefit of stem cell delivery within the 14 to 21 day post treatment period we have observed in several other experiments. These findings again strongly support the need to examine repeat administrations.

In this year we have gone through a more thorough analysis of previous animal experiments. This has included completing molecular analyses, some of which has required repeating and/or re-analyzing samples lost during processing and those thought to subject to

processing error. As we will complete analysis of more than 350 tissue samples requiring more than 1,000 molecular assays, this has proven to be an arduous task. We are continuing in this effort.
Focusing on a more thorough cumulative analysis of animal experiments has allowed us to begin a better detailed comparative analysis between treatment groups. In this quartile we have begun on a more concentrated analysis of clinically derived data.
In examining the changes in burn wound size observed clinically for control wounds in all animals treated to date we have found that there is change in size that occurs after treatment is applied to other wounds. We have speculated that some of this change may be due to growth in these animals as they are kept for long periods. Growth however would not explain why the wound sizes decrease from Day 7 after burn injury to Day 62 as the animal is growing significantly during that time. At Day 69 (the day of treatment to other wounds) however, control wounds begin to get larger (Figure 3). This change in size may have to do with a systemic effect of treating with laser and/or delivery of stem cells. Duroc pigs were chosen for their ability to form hypertrophic scars and therefore may be particularly sensitive to systemic changes. These findings have required the use of an additional animal that would remain untreated to better determine the natural course of scar growth in a similar timeframe and would be an invaluable source as a comparative tool.
We have used the cumulative control wound size measurement as a comparison to treated burn scars beginning at the day of treatment. We have noted that in most cases, the wound size increases above the cumulative control measurements after treatment and remains higher for 14 to 21 days after treatment. While an initial/immediate increase in size is expected as laser treatment tends to relax the tight collagen network of scars, it was surprising to see this effect last in during the 14 to 21 day time period. This finding is again consistent molecular and clinical photographic findings that indicate a maximum benefit 14 to 21 days after treatment.
In general, wound sizes fell below the cumulative control wound size measurement when Erbium:YAG laser is used at the high setting. A similar effect can be seen with CO_2 laser. This is most likely is due to greater tissue damage we have seen with fractional lasers (particularly CO_2) which may result in a greater degree of contraction in these burn scars over time. These findings are supported by histologic changes noted previously.
When CO_2 laser was used at the higher setting, there was a greater variability in wound size measurements. Again, this is probably due to variability in the greater degree of tissue effect/damage caused by CO_2 administered at higher energies.
When examining treated wound sizes (regardless of autologous/allogeneic or BM-MSC/ADSC) the effect compared to time matched cumulative control measurements was prolonged when stem cells were placed in PEGylated fibrin gels. This effect had been noted previously when allogeneic cells were incorporated into gels but was now also evident with autologous cells. This could be due to greater survival of stem cells being placed in gels. It should be noted that when cells were injected into burn scars, delivered cells did not persist long locally. Perhaps placing cells in gels also served to immobilize cells at the site and allow greater amounts of secreted materials to reach the burn scar (via laser conduits).
When stem cells are placed in PEGylated gels, the effect in changing wound size (as compared to cumulative control measurements) appears to be biphasic. There appears to be an early effect noted from Days 7 to 21post treatment with a second change noted after Day 25. This later effect did not appear to be different when Erbium:YAG laser was used at either high or low settings and was noted to be somewhat greater when BM-MSC were incorporated into PEGylated fibrin gels. Burn scars treated with Allogeneic ADSC in PEGylated gels however were noted to have larger wound size deviations from cumulative control measurements at Days 21 and 35, indicating a greater physiologic response at that those time points. This deviation is consistent with the second portion of a biphasic response we have noted with stem cells. PEGylated fibrin gels also appear to prolong (or even delay) the response to stem cell treatment. Taken as a whole, these observations point to the need to examine multiple dosing schemes in the future to maximize the response to these potential treatments.
Having he cumulative data we have derived up to now has allowed us to make several new important observations. Among the more recent has been the need to isolate certain variables such as the systemic effect of delivering stem cells and/or ablative fractional laser. In previous preclinical (porcine) work on wounds and intact skin we have observed that cells delivered by ablative fractional laser can traffic to distant sites including the bone marrow and wounds that had not been treated with stem cells. Similar findings were observed in experiments performed in this proposal as well. While it is not certain that these “mobile” stem cells exert a physiologic effect, there is indirect evidence that they do. We have observed in a previously performed clinical trial that patients with recalcitrant chronic wounds treated with bone marrow stem cells show improvement in both the chronic wounds treated as well as other chronic wounds they may have that were not treated. In this proposal, the changes observed in the cumulative data on control wounds indicates changes after treatment has been applied to other wounds. This has been observed in all groups including control wounds from animals treated only with ablative fractional laser, animals treated with ablative fractional laser and directly applied stem cells and animals treated with ablative fractional laser and stem cells incorporated in PEGylated fibrin gels.
To better characterize any systemic effects, we determined that an additional completely untreated animal be examined. At the end of this year an additional animal underwent burn injury (with the same number of wounds) and supportive care identical to previous animals provided - but this animal had no treatment was applied. This work is currently in progress. All wounds will be harvested and analyzed as was done for all other animals in this proposal. Once all clinical, photographic and molecular data has been obtained from this last experiment, it will be compared to previous data to better examine the potential systemic and local effects of the treatment we have applied. In addition, this last animal will allow us to far better define the burn scar model developed in this proposal.

KEY RESEARCH ACCOMPLISHMENTS:

REPORTABLE OUTCOMES:

Rodriguez-Menocal L, Salgado M, Christy RJ, Becerra S, Gil J, Candanedo A, Guzman W, Natesan S, Valdes J, Solis M, Higa A, Schulman CI, Waibel JS, Davis SC, Badiavas EV: Fractional Laser and Stem cell treatment As A Tool In Burn Scar Modulation. 5th Congress of the World Union of Wound Healing Societies, Florence, Italy September 25 – 29 2016.

Rodriguez-Menocal L, Salgado M, Christy R, Becerra S, Gil J, Candanedo A, Guzman W, Natesan S, Valdez J, Solis M, Higa A, Schulman C, Waibel J, Davis S, Badiavas E: Stem Cells to Prevent Contraction and Enhance Healing In A Third Degree Burn Model. Military Health System Research Symposium, Orlando, FL, August 15 – 18, 2016.

Rodriguez-Menocal L, Salgado M, Christy R, Becerra S, Gil J, Candanedo A, Guzman W, Natesan S, Valdez J, Solis M, Higa A, Schulman C, Waibel J, Davis S, Badiavas E: Fractional laser and stem cell treatment as tools in burn scar modulation. Wound Healing Society and Symposium on Advanced Wound Care, Spring Meeting, Atlanta GA, April, 2016.

Invited Speaker: "Stem Cells: Bench To Bedside", University of Miami Department of Dermatology and Cutaneous Surgery 60th Anniversary, Miami Beach FL, Jan 14-17 2016.

Invited Speaker: "Innovative Strategies For Wound Healing", Wound Healing Society and Symposium on Advanced Wound Care, Spring Meeting, April, 2016

Maranda EL, Rodriguez-Menocal L, Badiavas**,** EV, "Role of Mesenchymal Stem Cells in Dermal Repair in Burns and Diabetic Wounds" Curr Stem Cell Res Ther. 2016 Jul 14.

CONCLUSION:

As this proposal nears completion of the Specific Aims outlined, we provide a broader list of overall observations made over the last year. More specific details of the progress made in the last year have been described above.

- In several instances, stem cells incorporated in PEGylated gels appear to have a greater reparative effect than when they are applied directly.
- Incorporating stem cells in PEGylated gels seems to extend the beneficial effects for allogeneic stem cells. This was most apparent for ADSC.
- Autologous stem cells (particularly BM-MSC) incorporated in PEGylated gels appear to have greater beneficial effects than allogeneic cells incorporated in PEGylated gels.
- Applying PEGylated gels alone after fractional ablative laser appears to have an effect but is shorter lived than when stem cells (particularly BM-MSC) are incorporated into these gels.
- There appears to be an additional separate effect when stem cells are incorporated into PEGylated gels.
- The findings listed above are supported by clinical, histologic and molecular analyses.
- Numerous lines of evidence indicate that the effect of fractional lasers and/or stem cells (whether delivered alone or incorporated in PEGylated gels) is limited and diminishes after 21 Days.
- The limited effect of single dosing in these experiments strongly argues to examine multiple dosing in future work in order to maximize the potential benefits in scar remodeling and tissue regeneration.
- Variability in response to treatments appears to be related to factors including inflammatory response to cells delivered (particularly allogeneic) and tissue destruction produced by ablative fractional lasers administered at higher energies.
- A longitudinal analysis of control wounds for all treatment groups supports that there is a systemic effect when treating other wounds with ablative fractional laser with or without stem cells (directly applied or incorporated in PEGylated gels).
- An additional untreated animal is currently being processed to better characterize any systemic effects due to treatment and to better define the burn scar model developed in this proposal.

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1. Rodriguez-Menocal L, Salgado M, Davis S, et al. Percutaneous bone marrow transplantation using fractional ablative Erbium:YAG laser. PLoS One 2014;9:e93004.
2. Badiavas AR, Badiavas EV. Potential benefits of allogeneic bone marrow mesenchymal stem cells for wound healing. Expert Opin Biol Ther 2011;11:1447-54.
3. Badiavas EV, Abedi M, Butmarc J, Falanga V, Quesenberry P. Participation of bone marrow derived cells in cutaneous wound healing. J Cell Physiol 2003;196:245-50.
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APPENDICES:

Fractional laser and stem cells treatment as tool in burn scars modulation

Rodriguez-Menocal L[1], Salgado M[1], Christy RJ[2], Becerra S[2], Gil J[1], Candanedo A.[1], Natesan S[2], Valdes J[1], Solis M[1], Waibel JS[1], Davis SC[1], Schulman CI[3], Badiavas EV[1]

[1] University of Miami, Department of Dermatology & Cutaneous Surgery/ ISCI, Miami, FL, 33136, USA, [2]United States Army Institute of Surgical Research, Department of Extremity Trauma Research and Regenerative Medicine, Fort Sam Houston, TX 78234-6315, USA, [3] University of Miami, Department of Surgery, Miami, FL, 33136, USA.

ABSTRACT

Background: Burn scars represent a significant cause of morbidity in the injured warrior. Therapies designed to mitigate the effects of hypertrophic scarring including scar massage, topical treatments, steroid injections, and compression garments have limited success. Finding the best ways to remodel hypertrophic burn scars through fractional laser and stem cells (SCs) treatment is the goal of this study.

Methods: An in vivo Red Duroc model was used to evaluate the effect of ablative fractional lasers on hypertrophic third degree burn scars. Third degree burn wounds were created on the backs of Red Duroc pigs and burn scars were allowed to develop for 70 days prior to treatment. Scars received treatment with either CO2 or Erbium: YAG ablative fractional lasers at various settings with or without AD-SCs or BM-MSCs (allogeneic or autologous). Biopsy samples were taken from burn scars at various days post treatment for histologic evaluation, protein expression and cell culture analysis.

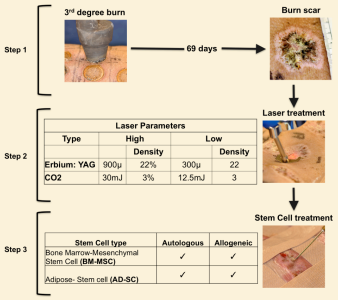
Results: Histology, Western blot, cell culture and qPCR analysis have indicated that CO_2 laser can have increased tissue damaging effect than Erbium:YAG laser, autologous SCs appear to generate a less fibrotic response, iii) cells derived from autologous SCs treated burns have more immature phenotype, iv) collagen indicative of dermal remodeling, may be more persistent when burns are treated with autologous BM-MSCs, and v) cells derived from SC treated burns appear to peak 14 Days after treatment.

Conclusion: These findings indicate that Erbium:YAG and perhaps autologous SCs may have benefit in treating hypertrophic burn scars. They also suggest that repeat administration is likely to be beneficial, again favoring the use of autologous cells. This study supports that hypertrophic third degree burn scars can be modified by fractional laser and SCs treatment with favorable clinical, histological and molecular changes.

MATERIALS AND METHODS

Animal Model:

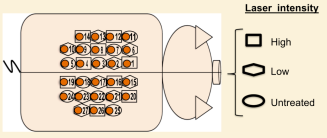
Swine Red Duroc were used as our experimental animal due to the morphological, physiological, and biochemical similarities between porcine skin and human skin. The gross appearance of scar in Red Duroc is thick, hairless, firm and hypo or hyperpigmented.

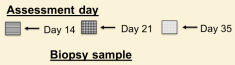


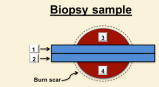
Laser Parameters				
Type	High		Low	
		Density		Density
Erbium: YAG	900µ	22%	300µ	22
CO2	30mJ	3%	12.5mJ	3

Stem Cell type	Autologous	Allogeneic
Bone Marrow-Mesenchymal Stem Cell (**BM-MSC**)	✓	✓
Adipose- Stem cell (**AD-SC**)	✓	✓

MATERIALS AND METHODS (cont.)





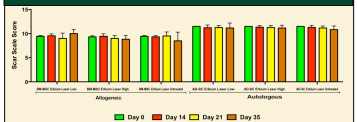


1. **Excisional Biopsy: Formalin H&E**
2. **Excisional Biopsy: Liquid N_2 frozen**
3. **Punch Biopsy 8mm: Liquid N_2 for Western blot**
4. **Punch Biopsy 8mm: Fibroblast culture for qPCR**

Protein	Scar Tissue	Hypertrophic Scar	Function
TGFβ1	Low	High	➢ One of the most important growth factors that regulate tissue regeneration, cell differentiation, embryonic development and regulation of the immune system.
α SMA	Low	High	➢ Myofibroblasts phenotype
Collagen I α 1	Low	High	➢ Most abundant protein produced by mammals and it is fundamental in contiguous formation of the interstitum trough epidermis.
Collagen III α1	Low	High	

RESULTS

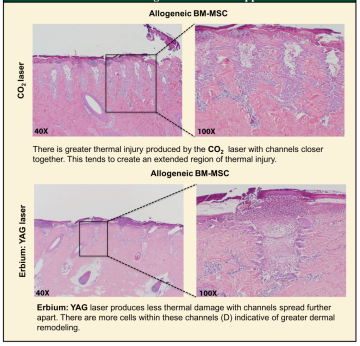
Clinical scoring of burn wounds at 0, 14, 21 and 35 days.



The treated burn wounds were then covered with an occlusive dressing. Clinical evaluation of these wounds was performed by a specialist who was blinded to the treatment groups. The scores were processed by the grading system we had previously established which includes vascularity, pliability, color and texture.
Improvement from Day 0 was noted in all groups treated with Erbium:YAG laser at the high setting. In particular there is an improvement in at Day 21 in all groups which may be occurring following the peak appearance of the Fibrocytes like cells noted in cultures.
Autologous ADSC did perform somewhat better by clinical scoring in this experiment, possibly due to the better preparation of these cells.

RESULTS

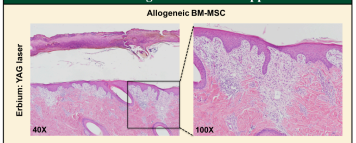
H&E stained section injured skin at Day 3 post high dose laser treatment and allogeneic BM-MSC application.



There is greater thermal injury produced by the CO_2 laser with channels closer together. This tends to create an extended region of thermal injury.

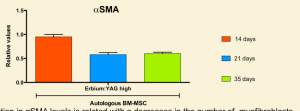
Erbium: YAG laser produces less thermal damage with channels spread further apart. There are more cells within these channels (D) indicative of greater dermal remodeling.

H&E stained sections of uninjured skin at Day 7 post high dose laser treatment allogeneic BM-MSC application



At Day 7 there was greater evidence of dermal remodeling in the Erbium:YAG treated skin. Dermal remodeling cells have an immature "fibrocyte" like morphology.
With doses at the high level (30mJ for CO2 and 900microns for Erbium:YAG) the epidermis and occasionally portions of the superficial dermis were disrupted for both lasers.
High energy settings could therefore provide greater access of applied cells into the dermis. This could explain why we tended to see greater improvement in burn scar remodeling when delivering cells using the high dose laser settings.

Western blot densitometry analysis of burn scar treated with laser plus Stem Cell



This reduction in αSMA levels is related with a decreases in the number of myofibroblasts able to express this protein in scar tissue after treatment with autologous BM-MSC.

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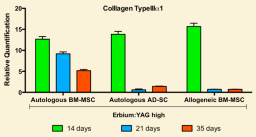
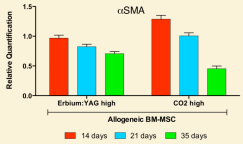
Cellular mRNA expression for ECM-related biomarkers in hypertrophic scar evaluated by qPCR.



Elevated Collagen IIIα1 expression (at times more than 10 fold) was sustained Day 14 through Day 35 for Autologous BM-MSC whereas Allogeneic BM-MSC showed elevated Collagen IIIα1 expression only at Day 14. Expression of Collagen IIIα1 in burn scars treated with Autologous ADSC was similar to Allogeneic BM-MSC in being elevated at Day 14 but not sustained.
This could indicate the persistence of an immature phenotype in the fibroblast population for wounds treated with autologous BM-MSCs.
This greater expression of Collagen Type IIIα 1 would indicate the presence of a more immature phenotype of cells that might be capable of more elastic/pliable dermal remodeling less characteristic of a scar.



These results for αSMA reveal a reduction on expression in burn scar treated with BM-MSC, which is consistent with a reduced transformation of fibroblasts into myofibroblasts similar to the results obtained by western blot

CONCLUSION

- Molecular changes noted in the areas of dermal remodeling propose that biomarkers **αSMA** and **Col3α1** play significant roles in tissue regeneration induced by fractional laser and stem cell therapy.
- These results for **αSMA** and **Col3α1** are consistent with histological observations of less scarring when BM-MSC are delivered
- Higher doses of laser energy could also serve as a type of superficial debridement that might be stimulatory to stem cell engraftment.

REFERENCES

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ACKNOWLEDGMENTS

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Fractional laser and stem cell treatment as tools in burn scar modulation

Rodriguez-Menocal L[1], Salgado M[1], Christy RJ[2], Becerra S[2], Gil J· Candanedo A.[1], Natesan S[2], Valdes J[1], Solis M[1], Waibel JS[1], Davis SC[1], Schulman CI[3], Badiavas EV[1]

[1] University of Miami, Department of Dermatology & Cutaneous Surgery/ ISCI, Miami, FL, 33136, USA, [2]United States Army Institute of Surgical Research, Department of Extremity Trauma Research and Regenerative Medicine, Fort Sam Houston, TX 78234-6315, USA, [3] University of Miami, Department of Surgery, Miami, FL, 33136, USA.

ABSTRACT

Background: Burn scars represent a significant cause of morbidity in the injured warrior. Therapies designed to mitigate the effects of hypertrophic scarring including scar massage, topical treatments, steroid injections, and compression garments have limited success. Finding the best ways to remodel hypertrophic burn scars through fractional laser and stem cells (SCs) treatment is the goal of this study.

Methods: An in vivo Red Duroc model was used to evaluate the effect of ablative fractional lasers on hypertrophic third degree burn scars. Third degree burn wounds were created on the backs of Red Duroc pigs and burn scars were allowed to develop for 70 days prior to treatment. Scars received treatment with either CO2 or Erbium: YAG ablative fractional lasers at various settings with or without AD-SCs or BM-MSCs (allogeneic or autologous). Biopsy samples were taken from burn scars at various days post treatment for histologic evaluation, protein expression and cell culture analysis.

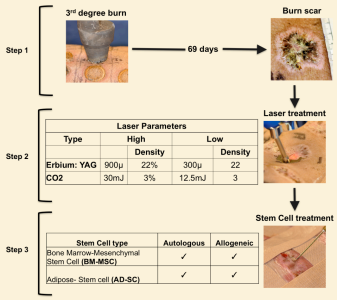
Results: Histology, Western blot, cell culture and qPCR analysis have indicated that CO_2 laser can have increased tissue damaging effect than Erbium:YAG laser, autologous SCs appear to generate a less fibrotic response, iii) cells derived from autologous SCs treated burns have more immature phenotype, iv) collagen indicative of dermal remodeling, may be more persistent when burns are treated with autologous BM-MSCs, and v) cells derived from SC treated burns appear to peak 14 Days after treatment.

Conclusion: These findings indicate that Erbium:YAG and perhaps autologous SCs may have benefit in treating hypertrophic burn scars. They also suggest that repeat administration is likely to be beneficial, again favoring the use of autologous cells. This study supports that hypertrophic third degree burn scars can be modified by fractional laser and SCs treatment with favorable clinical, histological and molecular changes.

MATERIALS AND METHODS

Animal Model:

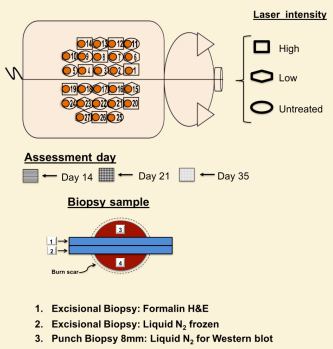
Swine Red Duroc were used as our experimental animal due to the morphological, physiological, and biochemical similarities between porcine skin and human skin. The gross appearance of scar in Red Duroc is thick, hairless, firm and hypo or hyperpigmented.



Laser Parameters				
Type	High		Low	
	Density		Density	
Erbium: YAG	900µ	22%	300µ	22
CO2	30mJ	3%	12.5mJ	3

Stem Cell type	Autologous	Allogenic
Bone Marrow-Mesenchymal Stem Cell (**BM-MSC**)	✓	✓
Adipose- Stem cell (**AD-SC**)	✓	✓

MATERIALS AND METHODS (cont.)

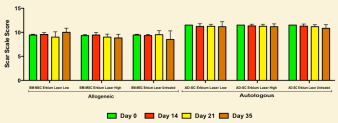


1. **Excisional Biopsy: Formalin H&E**
2. **Excisional Biopsy: Liquid N_2 frozen**
3. **Punch Biopsy 8mm: Liquid N_2 for Western blot**
4. **Punch Biopsy 8mm: Fibroblast culture for qPCR**

Protein	Scar Tissue	Hypertrophic Scar	Function
TGFβ1	Low	High	➢ One of the most important growth factors that regulate tissue regeneration, cell differentiation, embryonic development and regulation of the immune system.
α SMA	Low	High	➢ Myofibroblasts phenotype
Collagen I α 1	Low	High	➢ Most abundant protein produced by mammals and it is fundamental in contiguous formation of the interstitum trough epidermis.
Collagen III α1	Low	High	

RESULTS

Clinical scoring of burn wounds at 0, 14, 21 and 35 days.



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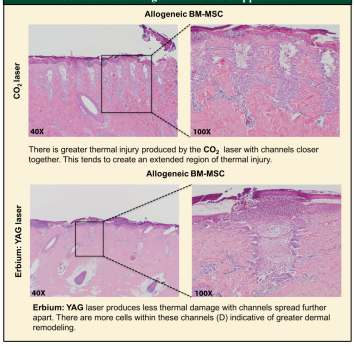
Autologous ADSC did perform somewhat better by clinical scoring in this experiment, possibly due to the better preparation of these cells.

RESULTS

H&E stained section injured skin at Day 3 post high dose laser treatment and allogeneic BM-MSC application.



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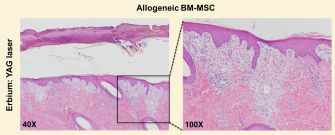

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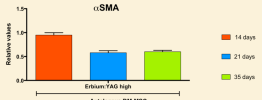
Western blot densitometry analysis of burn scar treated with laser plus Stem Cell



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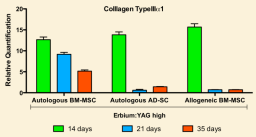

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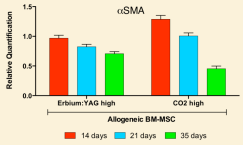

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CONCLUSION

- Molecular changes noted in the areas of dermal remodeling propose that biomarkers **αSMA** and **Col3α1** play significant roles in tissue regeneration induced by fractional laser and stem cell therapy.
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[1] University of Miami, Department of Dermatology, Miami, FL, USA

[2]United States Army Institute of Surgical Research, Fort Sam Houston, TX, USA [3] University of Miami, Department of Surgery, Miami, FL, USA

ABSTRACT

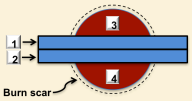
Background & Aims: Burn scars represent a significant cause of morbidity for which there are few therapeutic options. We have studied the ability of fractional lasers and stem cells (SCs) to remodel hypertrophic burn scars.

MATERIALS AND METHODS (cont.)

Laser intensity — High, Low, Untreated

Assessment day — Day 14 ← Day 21 ← Day 35

Biopsy sample



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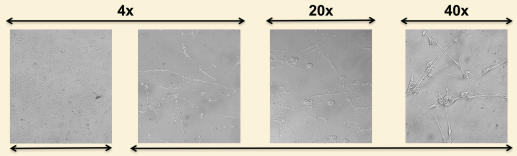
2. **Excisional Biopsy: Liquid N_2 frozen**
3. **Punch Biopsy 8mm: Liquid N_2 for Western blot**
4. **Punch Biopsy 8mm: Fibroblast culture for qPCR**

Cultured fibroblasts from punch biopsy 8mm

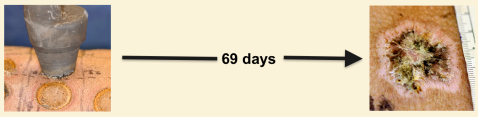


MATERIALS AND METHODS

Animal Model:

Swine Red Duroc were used as our experimental animal due to the morphological, physiological, and biochemical similarities between porcine skin and human skin. The gross appearance of scar in Red Duroc is thick, hairless, firm and hypo or hyperpigmented.

Step 1



Step 2

Laser Parameters				
Type	High		Low	
		Density		Density
Erbium: YAG	900µ	22%	300µ	22
CO2	30mJ	3%	12.5mJ	3

Step 3

Stem Cell type	Autologous	Allogeneic
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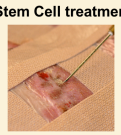
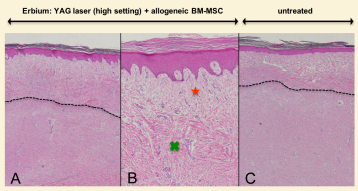
Adipose- Stem cell **(AD-SC)**	✓	✓



RESULTS

H&E of biopsies taken at Day 14 from an animal treated with Erbium:YAG laser high setting plus allogeneic BM-MSC



A: There is epidermal thickening and superficial dermal remodeling. Deep dermal scar is present below the dotted line (40X).

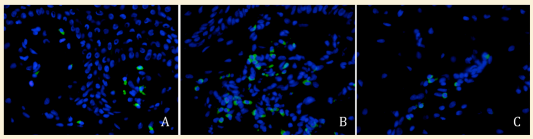
B: Higher magnification (100X) that better illustrates the epidermal thickness and dermal remodeling.

Red star is in an area of papillary dermal remodeling. There are more stellate fibroblasts and ground substance in this area, indicative of dermal regeneration.

Green arrow is in an area where there appears to be some reticular dermal regeneration. The more random orientation of the collagen fibers is more consistent with a normal appearing dermis than scar.

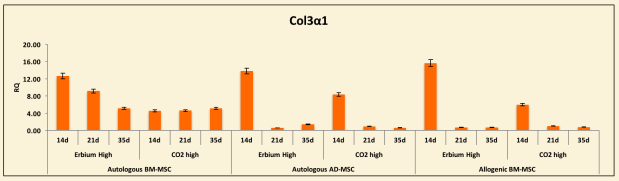
C: Untreated control has a larger area of dermal scar (below the dotted line) that appears more dense than that seen in the laser plus allogeneic BM-MSC treated wound. There is minimal increase in epithelial thickness with the absence of rete ridge formation. Little if any papillary dermal regeneration is noted. There is minimal reticular dermal regeneration.

DAPI stained slides of burn wounds at Day 14 (400X)



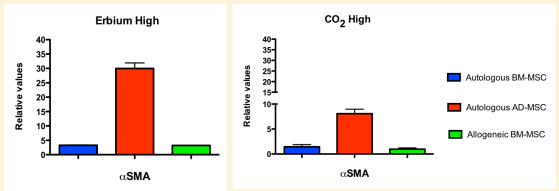
A: Erbium:YAG at low setting plus allogeneic BM-MSCs labeled (YFP).
B: Erbium:YAG at high setting plus allogeneic BM-MSCs labeled (YFP).
C: Untreated burn wound (control).

Quantitative PCR of burn scar fibroblasts for Col3α1



This reduction in collagen Type-III after treatment could indicate the presence of a more immature phenotype of cells that might be capable of more elastic/ pliable dermal remodeling, which is less characteristic of a scar.

Western blot densitometry analysis of burn scar treated with laser plus stem cell



The presence of αSMA is greater in animals treated with autologous AD-SC regardless of the laser used to deliver them.

CONCLUSION

- **Molecular changes noted in the areas of dermal remodeling propose that αSMA and Col3α1 play significant roles as molecular marker in tissue regeneration induced by fractional laser and stem cell treatment.**
- **Using this burn scar model we were able to examine the effects of ablative fractional laser and stem cell combined on hypertrophic scar remodeling.**

REFERENCES

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Modulation of Burn Scars Through Laser Assisted Delivery of Stem Cells

Proposal Number: 12340037

W81XWH-12-2-0078

PI: Evangelos Badiavas **Org:** University of Miami School of Medicine **Award Amount:** $2,246,074.80

Study/Product Aim(s)

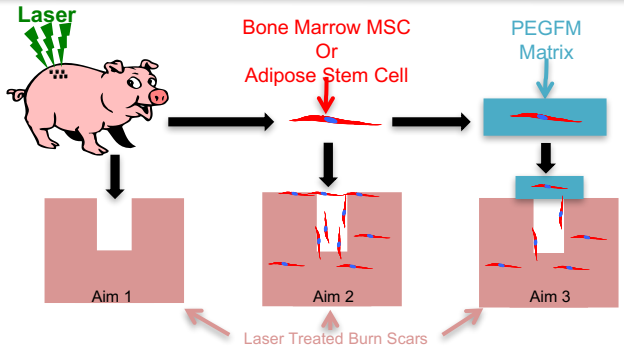
•**Objective 1**: To determine if fractional laser alone can remodel hypertrophic burn scars.

•**Objective 2:** To determine if fractional laser plus stem cells is more effective than fractional laser alone in treating hypertrophic burn scars.

•**Objective 3**:To determine if applying stem cells in a PEGylated Fibrin Matrix (PEGFM) following fractional laser treatment would provide additional benefits in reducing hypertrophic scarring associated with burns.

Approach

The approach is directed at evaluating the above treatment groups in a third degree burn wound model developed by our laboratory and collaborators involved in this proposal



Stem cells delivered in PEGylated fibrin gels can deliver similar effects to burn scars after treatment with laser. PEGylated fibrin gels may prolong and/or enhance some of the effects of allogeneic stem cells. Repeat administration of stem cells will be important.

Timeline and Cost

Activities	FY1	FY2	FY3	FY4	FY5
Determine optimal laser dosimetries which reduce burn scars. Maximize physical properties and stem cell loading of matrices for laser delivery					
Evaluate topical application of stem cells following fractional laser					
Assess PEGFM as a delivery mechanism for stem cells to burn scars					
Estimated Total Budget ($K)	$519	$500	$500		

Updated: (04-Nov-2016)

Goals/Milestones

☑**FY1 Goal** – Determine effect of fractional laser alone on 3rd degree burn scars and compare the results of fractional CO_2 versus Erbium-YAG laser treatment.

☑ **FY2 Goal** – Measure the effect of topical application of adipose stem cells and bone marrow derived mesenchymal stem cells over laser treated 3rd degree burn wounds.

FY3 Goal – Determine if PEGylated matrices with or without chitosan can improve on the delivery of stem cells to fractional laser treated 3rd degree burn scars and measure their effect on scar reduction.

Comments/Challenges/Issues/Concerns

- None at present

Budget Expenditure to Date

Projected Expenditure: $2,246,074.80

Actual Expenditure $ 2,119,091.96

SUPPORTING DATA: Figures are below:

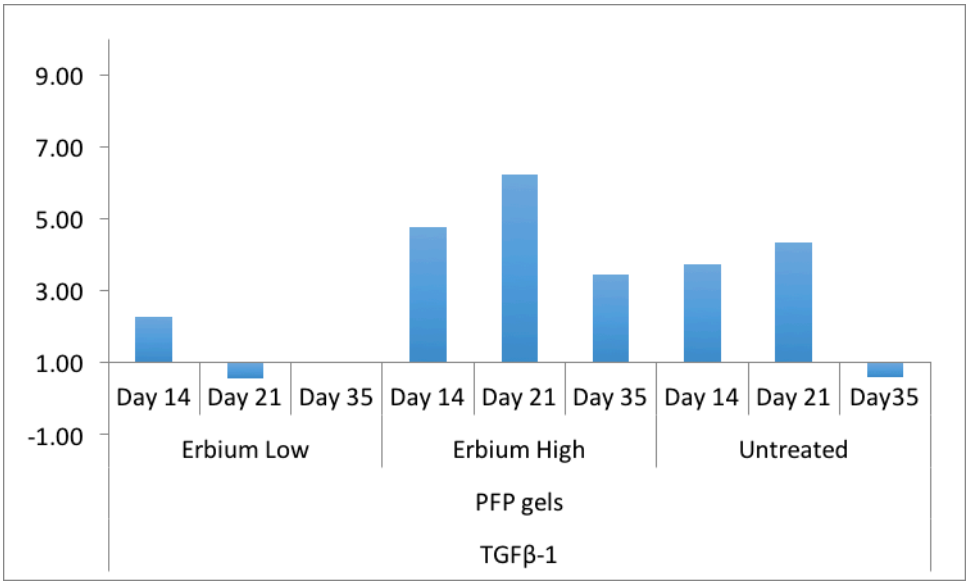
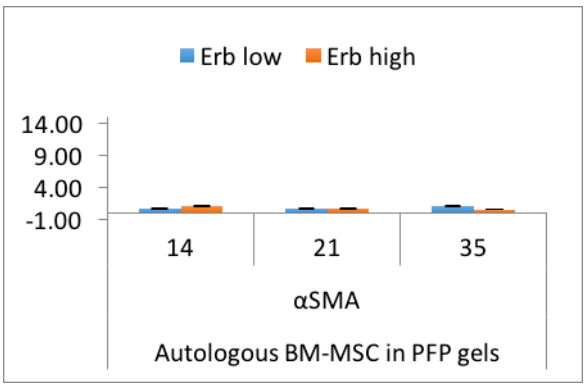
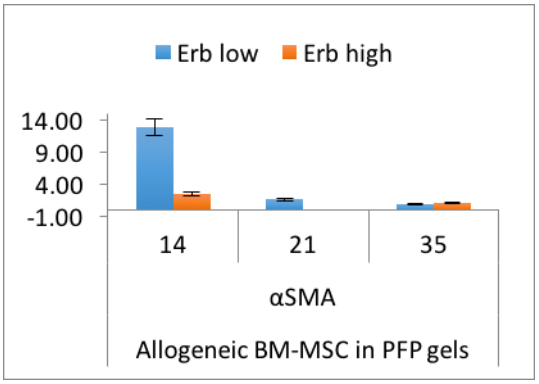


Figure 1: Western blots of tissues derived from pigs treated with PEGylated fibrin (PFP) gels without incorporated cells. All burn scars administered PFP were first treated with Erbium:YAG laser at high or low settings. All values have been normalized to a housekeeping gene (GAPDH) and are expressed relative to untreated control values. All analyses have been performed at Days 14, 21 and 35 post treatment. There is an increased level of TGFβ-1 most proximately when laser is first administered at the high setting and in untreated controls. Laser treatment at the lower setting appeared to have a beneficial effect.





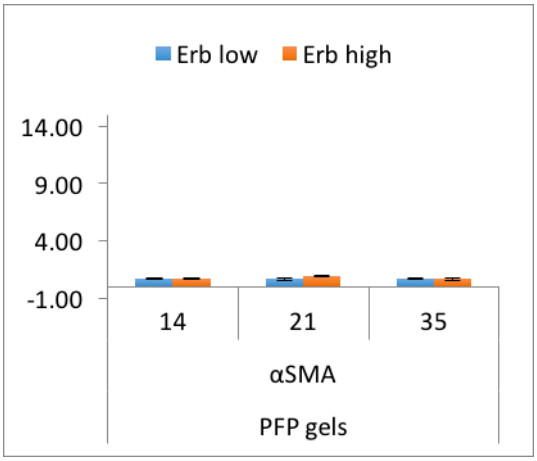
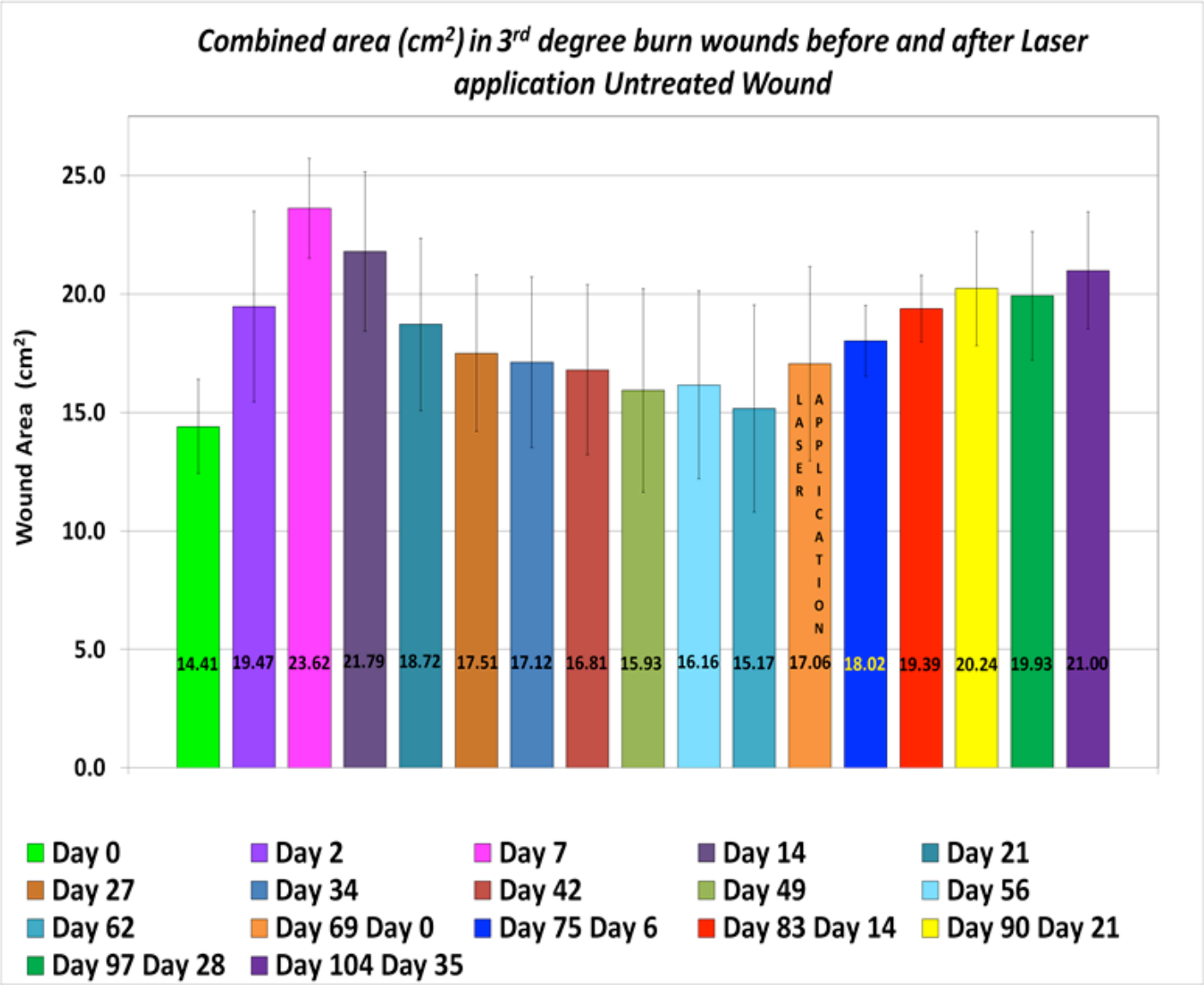


Figure 2: Real Time PCR of cultured cells derived from pigs treated with PEGylated fibrin gels (PFP) with our without incorporated cells. All burn scars administered PFP were first treated with Erbium:YAG laser at high or low settings. All values have been normalized to a housekeeping gene (GAPDH) and are expressed relative to untreated control values. All analyses have been performed at Days 14, 21 and 35 post treatment. A: Pig treated autologous BM-MSC in PFP. B: Pig treated with allogeneic BM-MSC in PFP. C: Pig treated with PFP without cells. There is an increased level of αSMA noted at Day 14 that could indicate a reaction to allogeneic cells, possibly reflected in increased scar scoring at Day 14. This was not observed for autologous cells or with PFP alone.

Figure 3: Combined wound areas for all untreated wound, showing the decrease in wound size leading to day 69 (day of treatment) and then the wound area increase day 69.



Modulation of Burn Scars Through Laser Assisted Delivery of Stem Cells

Proposal Number: 12340037
W81XWH-12-2-0078

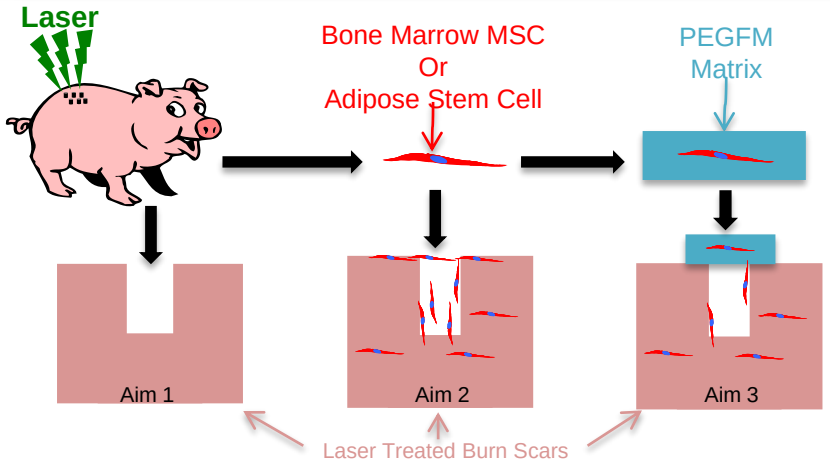
PI: Evangelos Badiavas **Org:** University of Miami School of Medicine **Award Amount:** $2,246,074.80

Study/Product Aim(s)

- **Objective 1**: To determine if fractional laser alone can remodel hypertrophic burn scars.
- **Objective 2:** To determine if fractional laser plus stem cells is more effective than fractional laser alone in treating hypertrophic burn scars.
- **Objective 3**:To determine if applying stem cells in a PEGylated Fibrin Matrix (PEGFM) following fractional laser treatment would provide additional benefits in reducing hypertrophic scarring associated with burns.

Approach

The approach is directed at evaluating the above treatment groups in a third degree burn wound model developed by our laboratory and collaborators involved in this proposal



Stem cells delivered in PEGylated fibrin gels can deliver similar effects to burn scars after treatment with laser. PEGylated fibrin gels may prolong and/or enhance some of the effects of allogeneic stem cells. Repeat administration of stem cells will be important.

Timeline and Cost

Activities	FY1	FY2	FY3	FY4	FY5
Determine optimal laser dosimetries which reduce burn scars. Maximize physical properties and stem cell loading of matrices for laser delivery					
Evaluate topical application of stem cells following fractional laser					
Assess PEGFM as a delivery mechanism for stem cells to burn scars					
Estimated Total Budget ($K)	$519	$500	$500		

Updated: (04-Nov-2016)

Goals/Milestones

☑**FY1 Goal** – Determine effect of fractional laser alone on 3rd degree burn scars and compare the results of fractional CO_2 versus Erbium-YAG laser treatment.

☑ **FY2 Goal** – Measure the effect of topical application of adipose stem cells and bone marrow derived mesenchymal stem cells over laser treated 3rd degree burn wounds.

FY3 Goal – Determine if PEGylated matrices with or without chitosan can improve on the delivery of stem cells to fractional laser treated 3rd degree burn scars and measure their effect on scar reduction.

Comments/Challenges/Issues/Concerns

- None at present

Budget Expenditure to Date

Projected Expenditure: $2,246,074.80
Actual Expenditure $ 2,119,091.96

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