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14. ABSTRACT

Neuromusculoskeletal injuries sustained in recent military conflicts have been notable for their number and complexity. Post-traumatic heterotopic ossification (HO) is the development of bone in the soft tissues and it is a significant sequela of these traumatic wounds occurring in approximately 60-70% of the war wounded. HO is the end product of a deranged fibroproliferative healing response and can render the extremity disfigured, painful and nonfunctional. Our group has studied this condition in combat related injuries at the cell and molecular level for the greater part of the last decade. In addition to identifying a progenitor cell population involved in this healing response, we have also identified TGF-b1 mediated tissue fibrosis to be one of the key initial steps in the pathogenesis of HO and that dysregulation of the SMAD3 intracellular signaling protein in conjunction with a fibrotic microenvironment to be a central feature of the bone forming process

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Introduction

Current combat operations yield large numbers of traumatic neuromuscular injuries. As a consequence of these battlefield injuries traumatized muscle is intimately associated with the development of post-traumatic heterotopic ossification (HO). To identify a treatment strategy for HO it is necessary to understand the details of the process by which bone forms in muscle tissue. Despite extensive study, much of the cellular and molecular mechanisms underlying HO are still unknown. What is known is that following combat trauma, wound closure is often delayed, leading to sub-optimal healing. While modulation of the local inflammatory response following injury is essential for wound healing and repair, severe traumatic injuries appear to initiate an over-exuberant response, thus compromising efficient wound healing & tissue regeneration. As a result of the chronic inflammation in the wound, endogenous tissue regeneration mechanisms are overshadowed by a generalized healing response that leads to formation of scar tissue. Fibrosis therefore limits the functional regeneration of the musculoskeletal tissues. Further, tissue fibrosis appears to be a salient feature in the HO lesion. Repeated anecdotal surgical observations have linked areas of abundant fibrotic scarring within the wound to an increased chance of HO formation. Fibrosis therefore appears to be an intermediate step in the onset of HO in a way that is not understood. It is possible that regions of fibrosis convert directly to bone. Additionally, it is possible that regions of fibrosis contribute osteogenic signals that drive neighboring cells to proceed down a bone-forming pathway. It can be predicted that any reduction in fibrosis should lead to a reduction in HO severity. Therefore, an appropriate treatment strategy for HO would therefore be to suppress fibrosis and promote the endogenous mechanisms of tissue repair. Ultimately, a timely administration of an effective HO prophylaxis to wounded soldiers in theater or soon thereafter could eliminate ectopic bone-related complications in blast amputation stumps and expedite rehabilitation, prosthetic limb use, and a return to productive life. Depending upon the efficacy-safety balance of the specific HO prophylaxis, it may be administered to all injured soldiers or only those at high risk for HO.

Over the past several years our lab has examined the cells, the molecular signals and the cellular scaffolds that are present in the wound-healing environment. We have identified a population of primary mesenchymal progenitor cells (MPCs) harvested from debrided human muscle tissue. These cells which are capable of differentiating down multiple pathways including bone and cartilage. MPCs have the potential to modulate differentiation due to their expression of multiple cytokines and growth factors. For example, they express TGFβ1, TGF 3, IL-10, GDF10 and BDNF at elevated levels compared to mesenchymal stem cells derived from human bone marrow. Consistent with this data, tissue samples from traumatized muscle also display elevated TGFβ1, TGF 3, IL-10, and BDNF, compared to normal muscle. Given the capacity of MPCs to undergo osteogenesis and to express cytokines and growth factors, it is likely that these MPCs play a critical role in both fibrosis and HO formation. We have therefore used these MPCs as a platform onto which we hope to rationally generate a treatment strategy for HO. In addition, we have made use of a recently generated rat model for post-traumatic HO, which very closely mimics that seen in wounded veterans. We have combined these *in vitro* and *in vivo* approaches to identify a potential novel target for trauma-mediated muscle fibrosis that could block the formation of HO and potentially aid in muscle regeneration.

Keywords

Heterotopic ossification (HO), mesenchymal progenitor cells (MPCs), fibrosis, trauma, TGF-beta.

Accomplishments

Previously, we showed that inhibition of the TGF-beta pathway with targeted pharmacological inhibitors reduced the mRNA expression of fibrotic markers compared to TGF-beta alone treated Mesenchymal Progenitor Cells (MPCs). Moreover, upon the pharmacological inhibition of the TGF-beta pathway, the mRNA expression levels of the osteogenic regulator *CBFA1* was moderated reversed compared to TGF-beta alone treated cells. At the protein level, a dose response effect (inhibition) was observed on the phosphorylation of SMAD2 and SMAD3 upon treatment with the ALK5 inhibitors (SB431542 and LY2157299), while the total levels of SMAD2 were not significantly changed and the total levels of SMAD3 were moderately modulated in some conditions. As expected, the SMAD inhibitors (Halofuginone and SIS3) demonstrated no effects in the phosphorylation of SMAD2 and total SMAD2, and a dose-dependent response on the phosphorylation of SMAD3, while some decrease was also observed on the levels of total SMAD3. More recently, we focused our efforts to investigate the potential for the TGF-beta targeted therapies to inhibit the formation of fibrotic nodules. We showed that SB431542, LY2157299 and SIS3 significantly inhibited nodule formation induced by the TGF-beta treatment. Overall, these results confirm the effectiveness of the all the inhibitors used in this study to block the SMAD2 and/or SMAD3 pathway in MPCs *in vitro* and demonstrate clinically-relevant effects on the inhibition of fibrotic markers and fibrotic nodules formation. Following these findings, we focused our efforts to (i) induce osteogenic differentiation following fibrotic nodule formation and (ii) investigate the gene expression profile of fibrotic markers following the formation of fibrotic nodules *in vitro*. Since TGF-beta is known as a major regulator of the initial inflammatory and wound healing response in the traumatized muscle bed, and fibrosis appears to play a central role in heterotopic ossification (HO) development and progression, we continued to pursuit the *in vitro* model of fibrotic nodule formation using human primary MPCs derived from high- and low-energy traumatized muscle tissue (J Tissue Eng Regen Med. 2009;3(2):129-38) treated with TGF-beta. An important aspect of this model is that it is mediated by TGF-beta, an inflammatory factor understood to drive the fibrotic response. This model relies on the plating of cells on poly-L-lysine coated dishes, which are then subsequently treated with TGF-beta (Am J Physiol Renal Physiol. 2007;293(2):F631-40). In the absence of TGF-beta, the cells remain in a monolayer and when treated with TGF-beta, the cells migrate into spherical nodules within 48-96 hours. The cells strongly adhere to each other and form a multicellular 3-dimensional nodule which is quite stable over days in culture. In addition, these nodules are remarkably similar to those observed in traumatized muscle tissue.

Our initial efforts focused on inducing osteogenic differentiation following the fibrotic nodule formation to asses if the fibrotic nodules have the ability to undergo osteogenic induction (similar to MPCs when cultured in monolayer conditions), and if treatment with the TGF-beta inhibitors would have any effect on the osteogenic induction. Osteogenic differentiation

was performed as previously described (J Bone Joint Surg Am. 2008;90(11):2390-8, PLoS One. 2014;9(12):e114318), followed by 2% Alizarin Red S at pH 4.2 for evidence of a mineralized matrix. Briefly, monolayer cultures of MPCs were seeded at a density of 5,000 cells/cm^2 and treated for 2-, 4- or 5-weeks with osteogenic medium, consisting of Dulbecco's Modified Eagle Medium with 10% fetal bovine serum supplemented with10 mM β-Glycerol phosphate, 50 μg/mL ascorbic acid, 10 nM 1,25-di-hydroxyvitamin D3 and 0.01 μM dexamethasone.

As shown on two independent donors (Figures 1-2), after 5-weeks in osteoinductive medium, we observed histological evidence of increased matrix mineralization by Alizarin red staining. However, no differences were observed between the treatments. Interestingly, shorter times in osteoinductive medium (2- and 4-weeks, Figures 3 and 4, respectively) did not demonstrate any differences in the increase of matrix mineralization by Alizarin red staining.

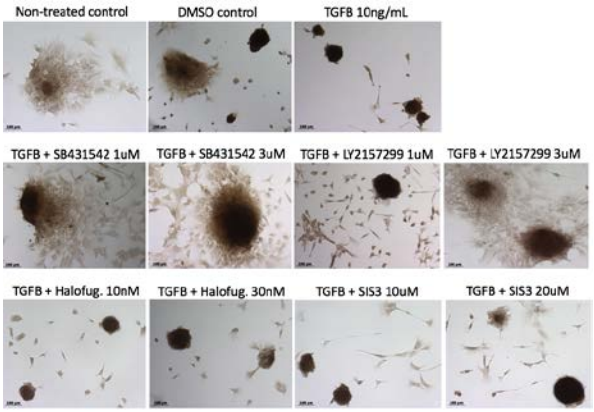


Figure 1 – Osteogenic differentiation (5-weeks, Donor 1) following the fibrotic nodule formation assay. MPCs (Donor 1) cultured on poly-L lysine coated 96-well plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [1uM and 3uM], TGF-beta [10 ng/mL] + LY2157299 [1uM and 3uM], TGF-beta [10 ng/mL] + Halofuginone [10nM and 30nM] and TGF-beta [10 ng/mL] + SIS3 [10uM and 20uM] for 4-days followed by osteogenic induction for 5-weeks. Non-treated cells and DMSO treated cells were used as treatment controls. TGFB = TGF-beta, Halofug. = Halofuginone. 10X magnification.

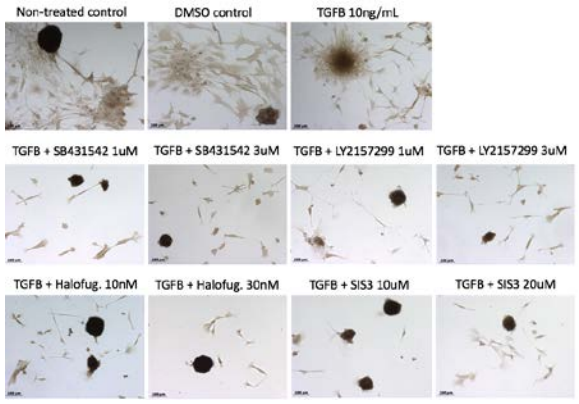


Figure 2 – Osteogenic differentiation (5-weeks, Donor 2) following the fibrotic nodule formation assay. MPCs cultured on poly-L lysine coated 96-well plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [1uM and 3uM], TGF-beta [10 ng/mL] + LY2157299 [1uM and 3uM], TGF-beta [10 ng/mL] + Halofuginone [10nM and 30nM] and TGF-beta [10 ng/mL] + SIS3 [10uM and 20uM] for 4-days followed by osteogenic induction for 5-weeks. Non-treated cells and DMSO treated cells were used as treatment controls. TGFB = TGF-beta, Halofug. = Halofuginone. 10X magnification.

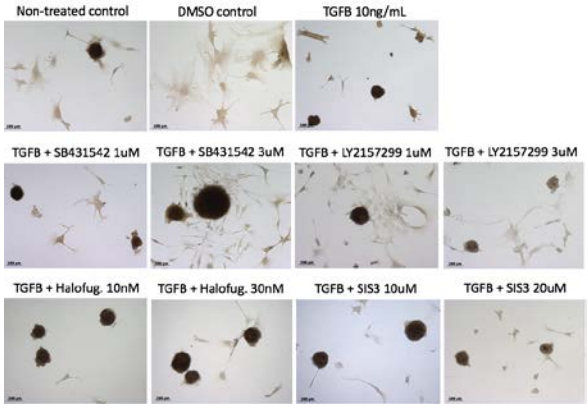


Figure 3 – Osteogenic differentiation (2-weeks) following the fibrotic nodule formation assay. MPCs cultured on poly-L lysine coated 96-well plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [1uM and 3uM], TGF-beta [10 ng/mL] + LY2157299 [1uM and 3uM], TGF-beta [10 ng/mL] + Halofuginone [10nM and 30nM] and TGF-beta [10 ng/mL] + SIS3 [10uM and 20uM] for 4-days followed by osteogenic induction for 2-weeks. Non-treated cells and DMSO treated cells were used as treatment controls. TGFB = TGF-beta, Halofug. = Halofuginone. 10X magnification.

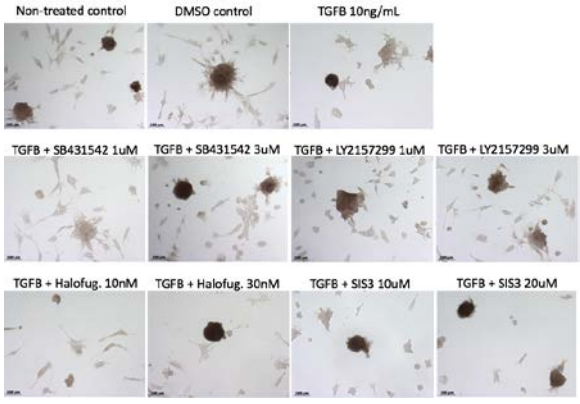


Figure 4 – Osteogenic differentiation (4-weeks) following the fibrotic nodule formation assay. MPCs cultured on poly-L lysine coated 96-well plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [1uM and 3uM], TGF-beta [10 ng/mL] + LY2157299 [1uM and 3uM], TGF-beta [10 ng/mL] + Halofuginone [10nM and 30nM] and TGF-beta [10 ng/mL] + SIS3 [10uM and 20uM] for 4-days followed by osteogenic induction for 4-weeks. Non-treated

cells and DMSO treated cells were used as treatment controls. TGFB = TGF-beta, Halofug. = Halofuginone. 10X magnification.

In addition to the osteogenic induction, we are focusing on the investigation of the gene expression profile of fibrotic markers following the formation of fibrotic nodules *in vitro*. To investigate that we collected RNA and performed quantitative PCR analyses for target genes following the 4-days of the fibrotic nodules' formation assay. In accordance to our previously shown data on the total number of fibrotic nodules formed with this assay, the expression of the fibrotic markers *COL1A1*, *FN1* and *SERPINE1* are strongly down-regulated (Figures 5-6) upon treatment with the ALK5 inhibitors (SB431542 and LY2157299) and up-regulated or equivalent to the TGF-beta control upon treatment with the SMAD inhibitors (Halofuginone and SIS3). Interestingly, the fibrotic marker *ACTA2* is also down-regulated upon treatment with the ALK5 inhibitors and mostly equivalent or decreased (Halofuginone [30nM]) to the TGF-beta control upon treatment with the SMAD inhibitors (Figures 5-6). Additional qPCRs are currently ongoing to investigate the expression of osteogenic markers on these samples.

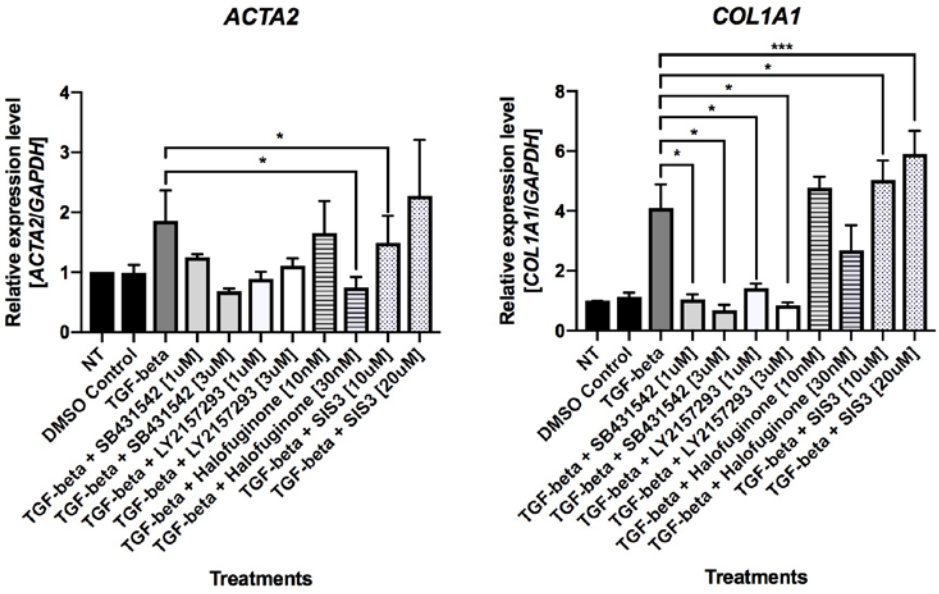


Figure 5 – Quantitative PCR analysis of the fibrotic markers *ACTA2* and *COL1A1* following the fibrotic nodule formation assay. MPCs were cultured on poly-L lysine coated 96-well plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [1uM and 3uM], TGF-beta [10 ng/mL] + LY2157299 [1uM and 3uM], TGF-beta [10 ng/mL] + Halofuginone [10nM and 30nM] and TGF-beta [10 ng/mL] + SIS3 [10uM and 20uM] for 4-days followed by RNA extraction and qPCR analysis. *$p<0.05$, *** $p\leq0.001$, T-test 1-tail.

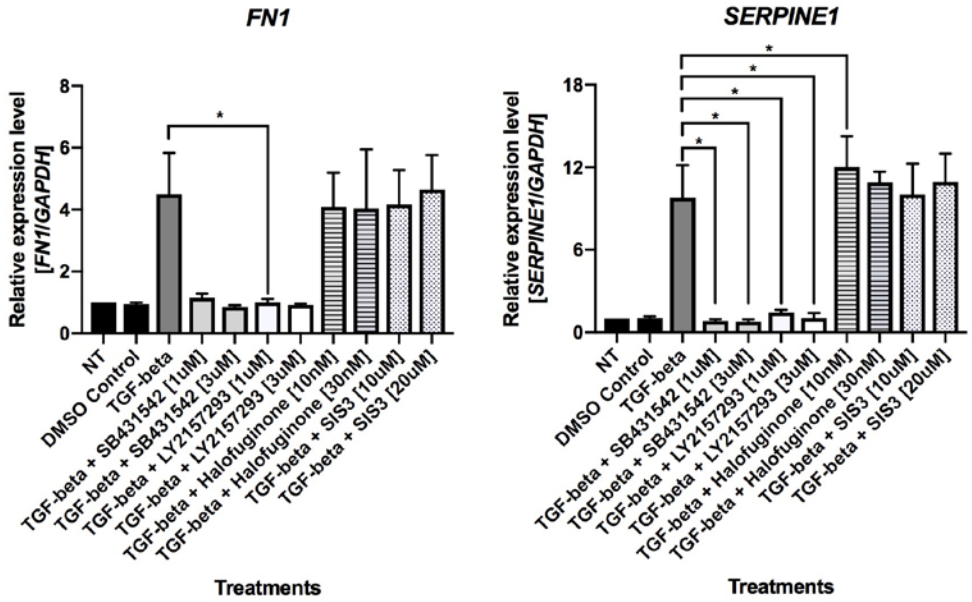


Figure 6 – Quantitative PCR analysis of the fibrotic markers *FN1* and *SERPINE1* following the fibrotic nodule formation assay. MPCs were cultured on poly-L lysine coated 96-well plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [1uM and 3uM], TGF-beta [10 ng/mL] + LY2157299 [1uM and 3uM], TGF-beta [10 ng/mL] + Halofuginone [10nM and 30nM] and TGF-beta [10 ng/mL] + SIS3 [10uM and 20uM] for 4-days followed by RNA extraction and qPCR analysis. *p<0.05, T-test 1-tail.

Following these findings, we investigated the expression of the osteogenic markers *CBFA1*, *ALP* and *Osteocalcin* on these samples (following the formation of fibrotic nodules *in vitro*). As shown on Figure 7, *CBFA1* was significantly down-regulated upon treatment with the ALK5 inhibitors (SB431542 and LY2157299) and moderately up-regulated - but not statistically significant - upon treatment with the SMAD inhibitors (Halofuginone and SIS3). Interestingly, *ALP* was decreased upon treatment with TGF-beta alone, up-regulated upon treatment with the ALK5 inhibitors (compared to TGF-beta alone) and down-regulated or unchanged upon treatment with the SMAD inhibitors (compared to TGF-beta alone). Finally, *Osteocalcin* remained without statistically significant changes.

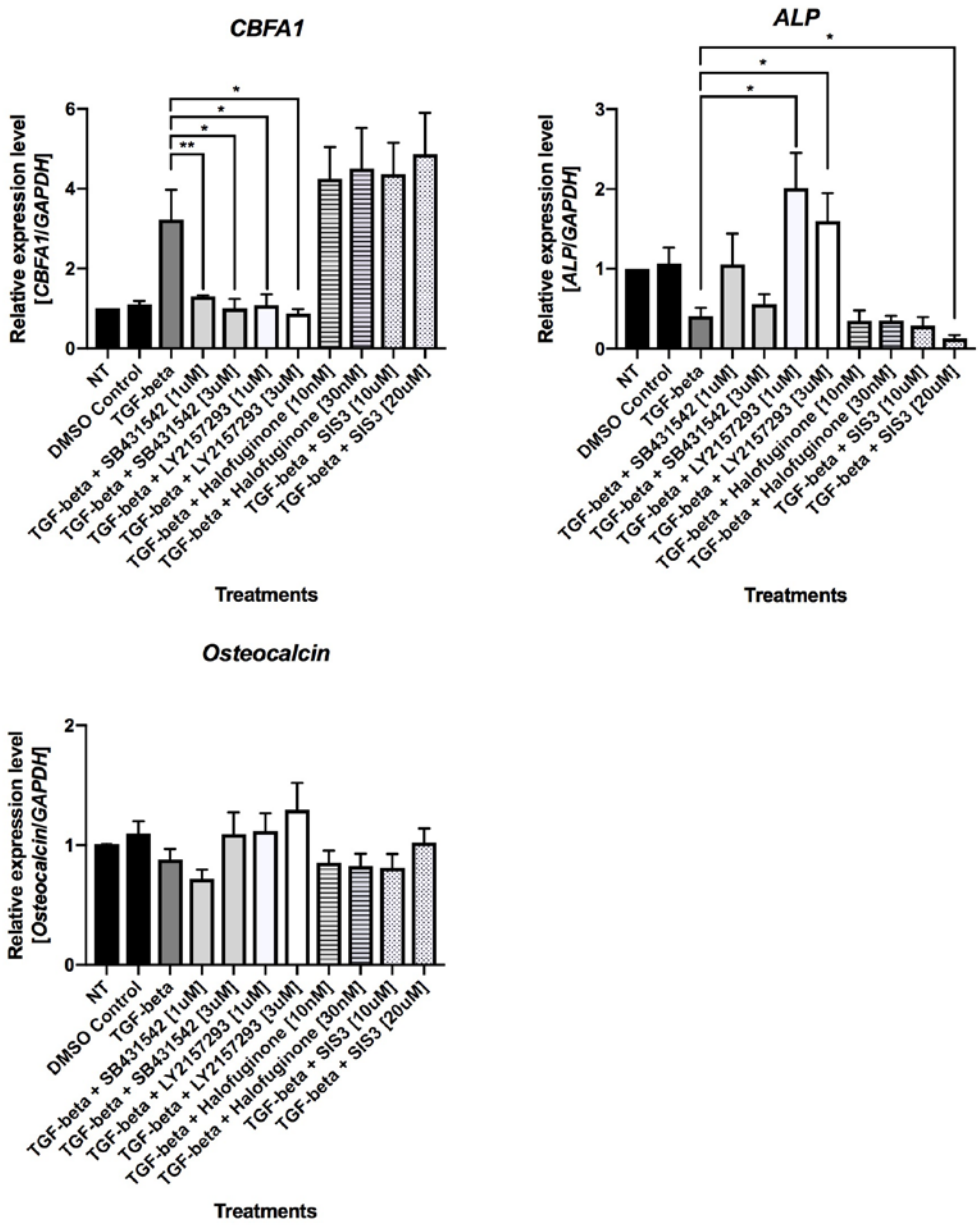


Figure 7 – Quantitative PCR analysis of the osteogenic markers *CBFA1*, *ALP* and *Osteocalcin* following the fibrotic nodule formation assay. MPCs were cultured on Poly-L-Lysine coated 96-well plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [1uM and 3uM], TGF-beta [10 ng/mL] + LY2157299 [1uM and 3uM], TGF-beta [10 ng/mL] + Halofuginone [10nM and 30nM] and TGF-beta [10 ng/mL] + SIS3 [10uM and 20uM] for 4-days followed by RNA extraction and qPCR analysis. DMSO alone was used as vehicle control. *p<0.05, **p□0.01, *** p□0.001, T-test 1-tail.

In addition, we chose to perform a comparison between the gene expression profile of the fibrotic and osteogenic markers following the formation of fibrotic nodules *in vitro* and following MPCs cultured and treated with TGF-beta inhibitors in a 3-dimensional nanofiber plate. The aim of this comparison was to investigate if the type of 3-dimensional system where the

cells are cultured has an impact on the response to the TGF-beta inhibitors as assessed by the mRNA expression of fibrotic and osteogenic markers. To investigate that we collected RNA and performed quantitative PCR analyses for fibrotic and osteogenic target genes following 4-days of treatment with the TGF-beta inhibitors when the MPCs where cultured in commercially available nanofiber plates. As shown on Figure 8, the commercial nanofiber plate used on these experiments has fibers on a random orientation, which we believe mimics the fibers formed on the fibrotic/scar tissue following a traumatic injury *in vivo*.

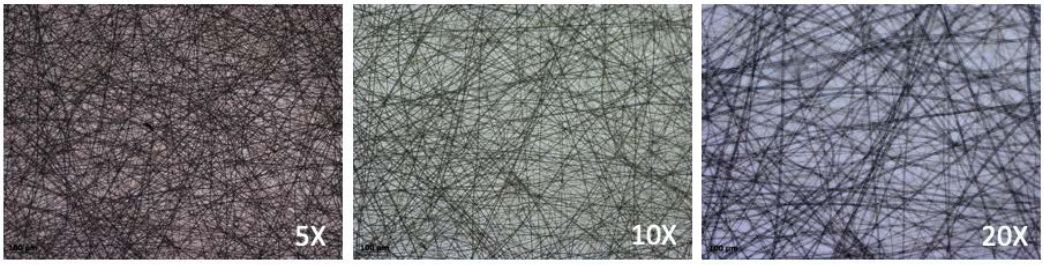


Figure 8 – Commercial nanofiber plate displays randomly orientated fibers. Different magnifications are indicated in each panel.

Following treatment with the TGF-beta inhibitors, the MPCs cultured on the nanofiber plates demonstrated mostly a down-regulated expression of the fibrotic markers *ACTA2*, *COL1A1*, *FN1* and *CDH2*, with the exception of the cells treated with Halofuginone [30nM], where the levels of *CDH2* were up-regulated (Figures 9-10). In addition, *SERPINE1*, *VIMENTIN* and *MMP2* were mostly unchanged, again with the exception of the cells treated with Halofuginone [30nM], where the levels of *SERPINE1* were robustly up-regulated and the levels of *MMP2* were robustly down-regulated. Finally, *MMP9* levels were unchanged on cells treated with SB431542 [3uM] and down-regulated on cells treated with LY2157299 [3uM], Halofuginone [30nM] and SIS3 [20uM], with the less robust effect observed upon the SIS3 [20uM] treatment.

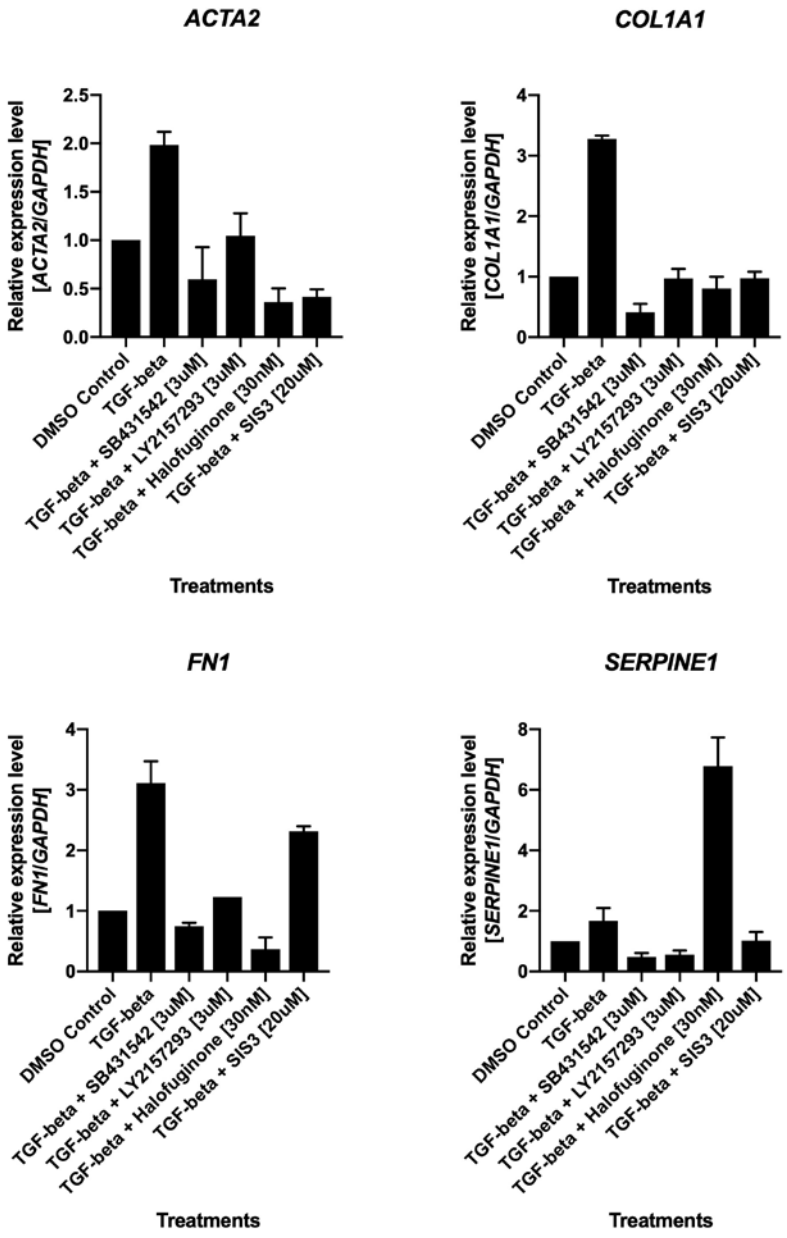


Figure 9 – Quantitative PCR analysis of the fibrotic markers *ACTA2*, *COL1A1*, *FN1* and *SERPINE1*. MPCs were cultured on randomly oriented nanofiber plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [3uM], TGF-beta [10 ng/mL] + LY2157299 [3uM], TGF-beta [10 ng/mL] + Halofuginone [30nM] and TGF-beta [10 ng/mL] + SIS3 [20uM] for 4-days followed by RNA extraction and qPCR analysis. DMSO alone was used as vehicle control. Results represent the average of two independent donors.

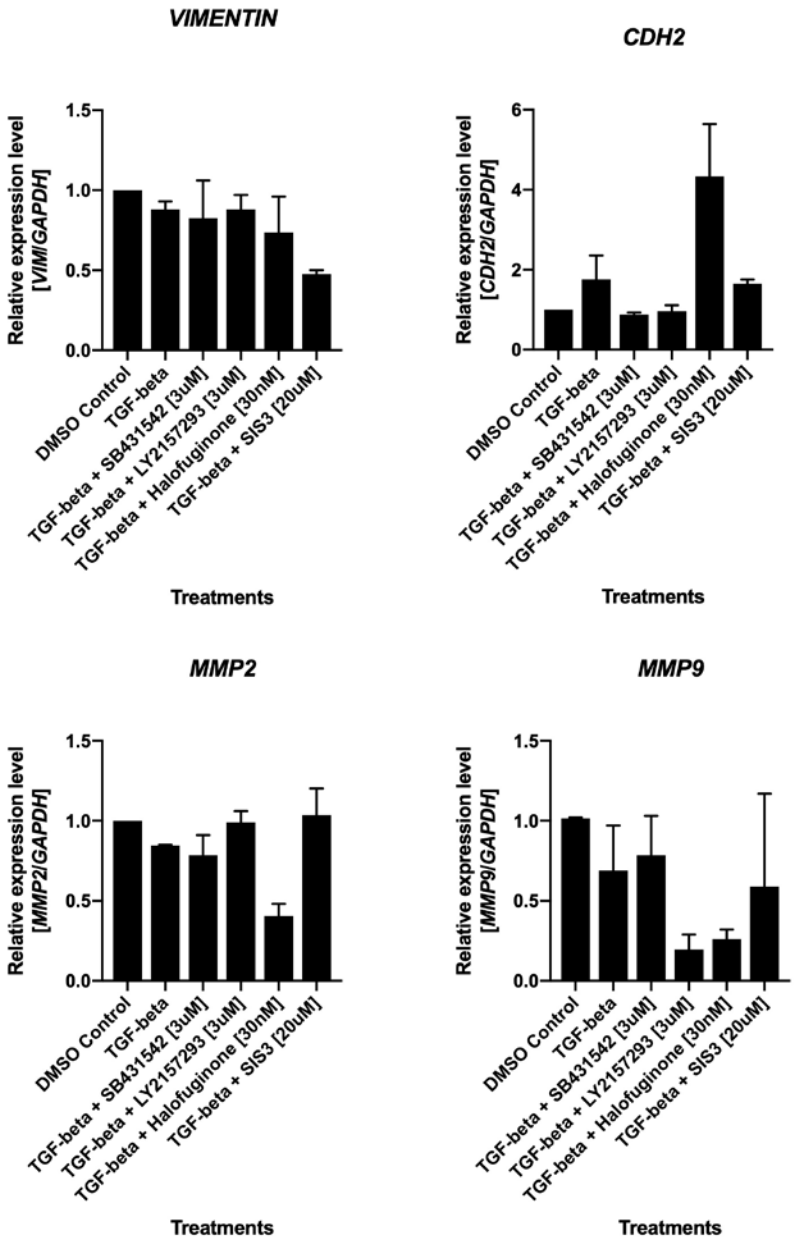


Figure 10 – Quantitative PCR analysis of the fibrotic markers *VIMENTIN*, *CDH2*, *MMP2* and *MMP9*. MPCs were cultured on randomly oriented nanofiber plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [3uM], TGF-beta [10 ng/mL] + LY2157299 [3uM], TGF-beta [10 ng/mL] + Halofuginone [30nM] and TGF-beta [10 ng/mL] + SIS3 [20uM] for 4-days followed by RNA extraction and qPCR analysis. DMSO alone was used as vehicle control. Results represent the average of two independent donors.

Interestingly, following treatment with the TGF-beta inhibitors, the MPCs cultured on the nanofiber plates demonstrated no significant changes on the expression levels of *CBFA1*, while treatment with the TGF-beta inhibitors consistently down-regulated the expression levels of *ALP* (Figure 11). Lastly, the levels of *Osteocalcin* were mostly unchanged, with the

exception of the cells treated with SB431542 [3uM], which demonstrated increased expression of *Osteocalcin* mRNA levels (Figure 11).

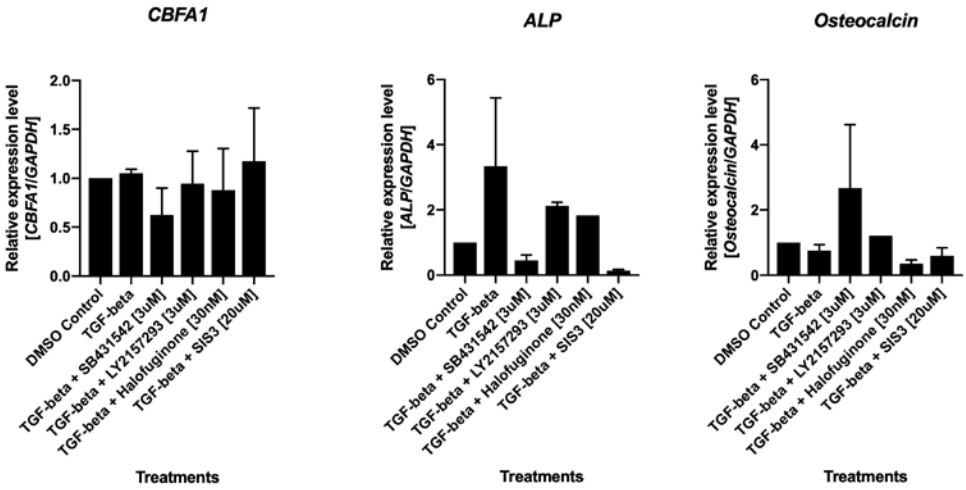


Figure 11 – Quantitative PCR analysis of the osteogenic markers *CBFA1*, *ALP* and *Osteocalcin*. MPCs were cultured on randomly oriented nanofiber plates and treated with TGF-beta [10 ng/mL] alone (control), TGF-beta [10 ng/mL] + SB431542 [3uM], TGF-beta [10 ng/mL] + LY2157299 [3uM], TGF-beta [10 ng/mL] + Halofuginone [30nM] and TGF-beta [10 ng/mL] + SIS3 [20uM] for 4-days followed by RNA extraction and qPCR analysis. DMSO alone was used as vehicle control. Results represent the average of two independent donors.

Finally, we focused our efforts to investigate the ability of the fibrotic nodules to undergo osteogenic differentiation following the formation of fibrotic nodules *in vitro*. Following the fibrotic nodules formation *in vitro*, cells (nodules) were induced to osteogenic differentiation for 4-weeks with an *in-house* previously reported osteogenic media (J Bone Joint Surg Am. 2008;90(11):2390-8) and commercially available osteogenic media. A control plate were cells grew in a monolayer condition was cultured at the same time with the same two osteogenic media and general media (non-osteogenic condition). Osteogenic differentiation was confirmed by Alizarin Red staining following a previously published protocol (J Bone Joint Surg Am. 2008;90(11):2390-8). As shown on Figure 12 A-B, both osteogenic media conditions induced cells to osteogenic differentiation on the control plate, however, the commercial media did appear to be more efficient to induce cells into the osteogenic lineage compared to the *in-house* media (compare intensity of the Alizarin Red staining on Figure 12A *vs*. Figure 12B). As expected, the cells cultured in general media (DMEM + 10% Fetal Bovine Serum + 1% Penicillin/Streptomycin + 1% Fungizone) did not stain (negative control) for Alizarin Red (Figure 12C).

Interestingly, as shown on Figures 13-14, both osteogenic medias (*in-house vs*. commercial) successfully induced the fibrotic nodules towards the osteogenic pathway. However, the nodules induced with the commercial media (Figure 14) stained stronger for Alizarin Red compared to the nodules induced with the *in-house* media (Figure 13), which suggests that osteogenic differentiation for 4-weeks was more robustly induced when the commercial media was used on our culture

conditions. Also of importance, no significant differences were observed between the treatments with different TGF-beta inhibitors and the ability of the fibrotic nodules to undergo osteogenic differentiation, independent of the type of osteogenic media used in the induction (Figures 13 and 14).

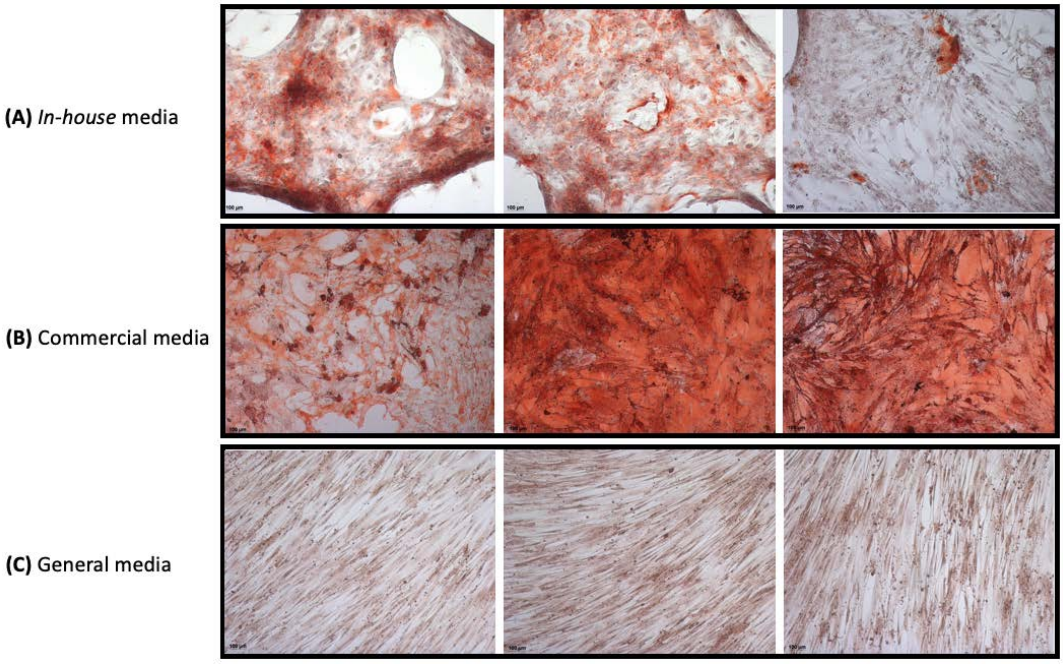


Figure 12 – Mesenchymal progenitor cells cultured in a conventional (monolayer) plate induced to osteogenic differentiation for 4-weeks with **(A)** *in-house* osteogenic media (J Bone Joint Surg Am. 2008;90(11):2390-8) and **(B)** commercially available osteogenic media. **(C)** Cells were cultured in general media (DMEM + 10% Fetal Bovine Serum + 1% Penicillin/Streptomycin + 1% Fungizone) as control. Following differentiation, cells were stained with 1.5% Alizarin Red.

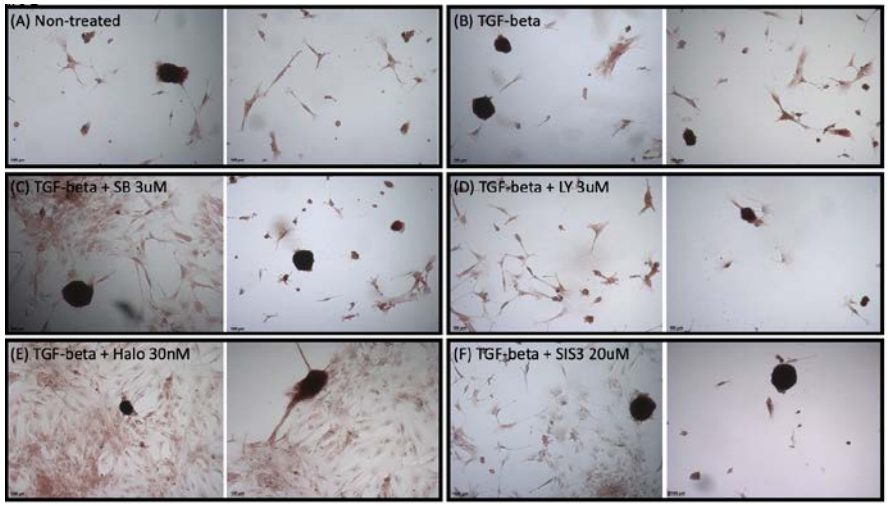


Figure 13 – Mesenchymal progenitor cells treated with TGF-beta for fibrotic nodules formation for 4-days followed by osteogenic differentiation for 4-weeks with *in-house* osteogenic media (J Bone Joint Surg Am. 2008;90(11):2390-8). **(A)** Non-treated control, **(B)** TGF-beta treatment control [10 ng/mL], **(C)** TGF-beta [10 ng/mL] + SB431542 [3uM], **(D)** TGF-

beta [10 ng/mL] + LY2157299 [3uM], **(E)** TGF-beta [10 ng/mL] + Halofuginone [30nM] and **(F)** TGF-beta [10 ng/mL] + SIS3 [20uM]. Treatments were performed for 4-days during the fibrotic nodule formation phase. Following differentiation, cells were stained with 1.5% Alizarin Red. SB = SB431542, LY = LY2157299 and Halo = Halofuginone.

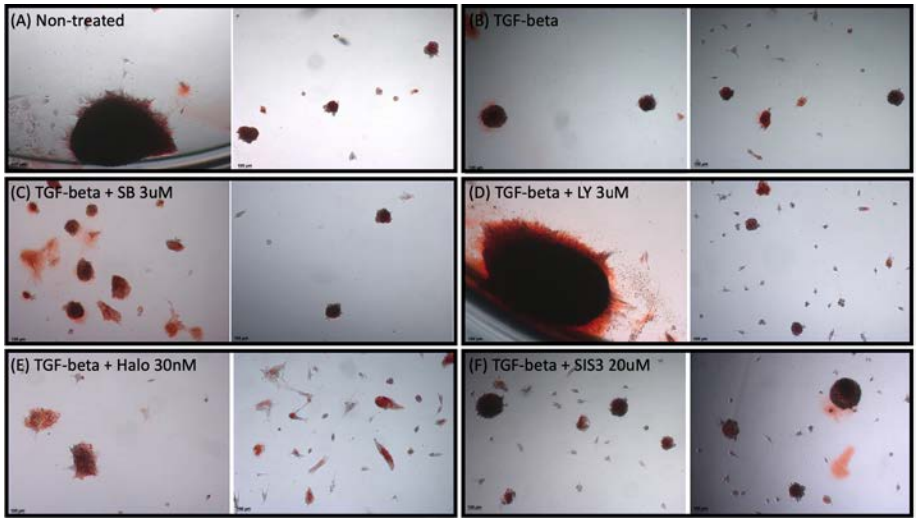


Figure 14 – Mesenchymal progenitor cells treated with TGF-beta for fibrotic nodules formation for 4-days followed by osteogenic differentiation for 4-weeks with *commercial* osteogenic media. **(A)** Non-treated control, **(B)** TGF-beta treatment control [10 ng/mL], **(C)** TGF-beta [10 ng/mL] + SB431542 [3uM], **(D)** TGF-beta [10 ng/mL] + LY2157299 [3uM], **(E)** TGF-beta [10 ng/mL] + Halofuginone [30nM] and **(F)** TGF-beta [10 ng/mL] + SIS3 [20uM]. Treatments were performed for 4-days during the fibrotic nodule formation phase. Following differentiation, cells were stained with 1.5% Alizarin Red. SB = SB431542, LY = LY2157299 and Halo = Halofuginone.

We also chose to perform a comparison between the gene expression profile of 2 fibrotic markers (*FN1* and *COL1A1*) and the osteogenic marker *CBFA1* following the formation of fibrotic nodules *in vitro* and following mesenchymal progenitor cells (MPCs) cultured and treated with TGF-beta in a monolayer plate (conventional culture conditions), a 3-dimensional commercial polycaprolactone (PCL) nanofiber plate and a 3-dimensional collagen nanofiber plate electrospun by Dr. Frank Alexander Jr. and Dr. Lee Johnson from 4D Bio^3, a collaboration that we recently established to test this collagen electrospun plates with the MPCs. The aim of this comparison was to investigate if the type of 3-dimensional system where the cells are cultured has an impact on the response to TGF-beta as assessed by the mRNA expression of fibrotic and osteogenic markers. To investigate that we collected RNA and performed quantitative PCR analyses for fibrotic and osteogenic target genes following 4-days of treatment with TGF-beta. Both the commercial PCL nanofiber plate and the collagen nanofiber plate used on these experiments have fibers on a random orientation, which we believe best mimics the fibers formed on the fibrotic/scar tissue following a traumatic injury *in vivo*.

As shown on Figure 15, both *FN1* and *COL1A1* were up-regulated in all culture conditions following treatment with TGF-beta [10 ng/mL]. Remarkably, the fold-change was consistently higher on cells cultured on the fibrotic nodule formation assay, followed by PCL nanofiber plate, collagen nanofiber plate and regular (monolayer) plates, respectively.

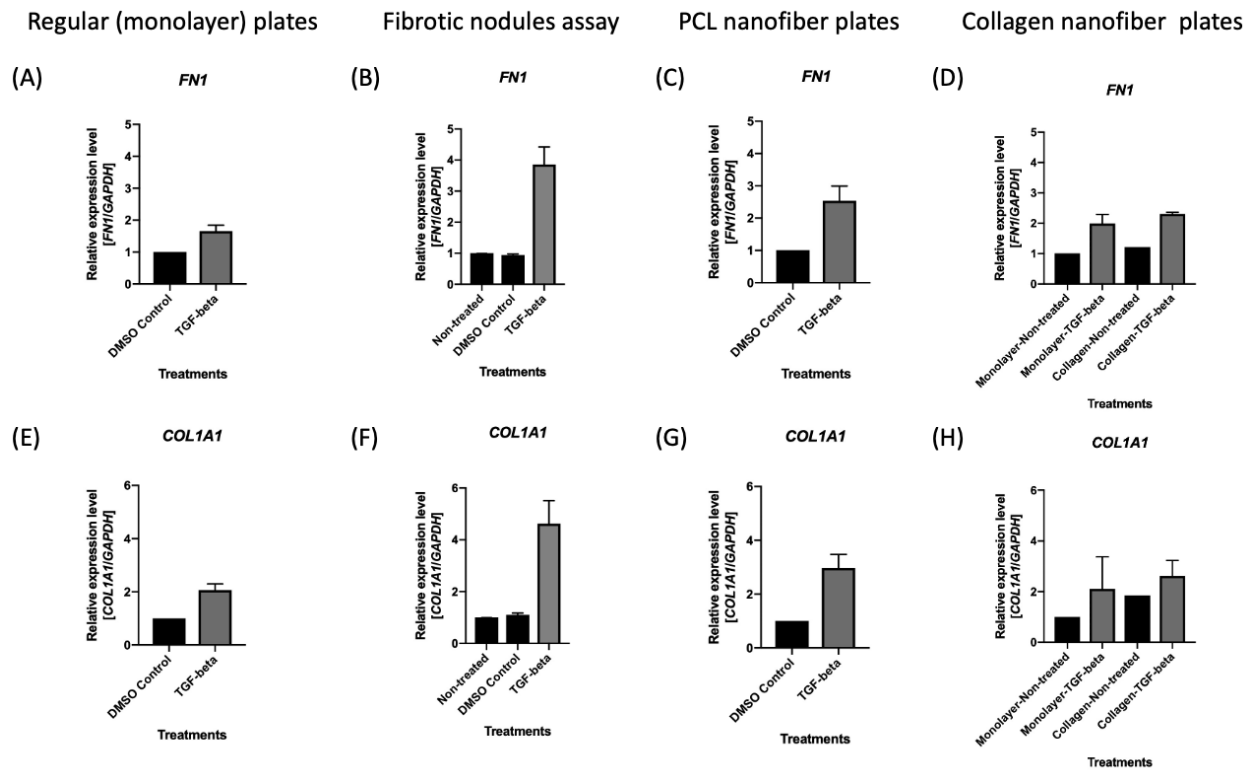


Figure 15 – Quantitative PCR analysis of the fibrotic markers **(A-D)** *FN1* and **(E-H)** *COL1A1*. MPCs were cultured on a regular (monolayer) plate (A and E), fibrotic nodules assay (B and F), commercial PCL nanofiber plate (C and G) or collagen nanofiber plate (D and H) treated with TGF-beta [10 ng/mL] for 4-days followed by RNA extraction and qPCR analysis. Non-treated cells and/or DMSO alone were used as controls. Results represent the average of at least three independent donors.

Furthermore, as shown on **Figure 16**, the osteogenic marker *CBFA1* mRNA levels were higher following treatment with TGF-beta [10 ng/mL] on the fibrotic nodule formation assay followed by collagen nanofiber plate, PCL nanofiber plate and regular (monolayer) plates, respectively. Altogether, these results demonstrate that the fibrotic nodule formation assay induces the highest (up-regulation) changes on the fibrotic and osteogenic markers analyzed.

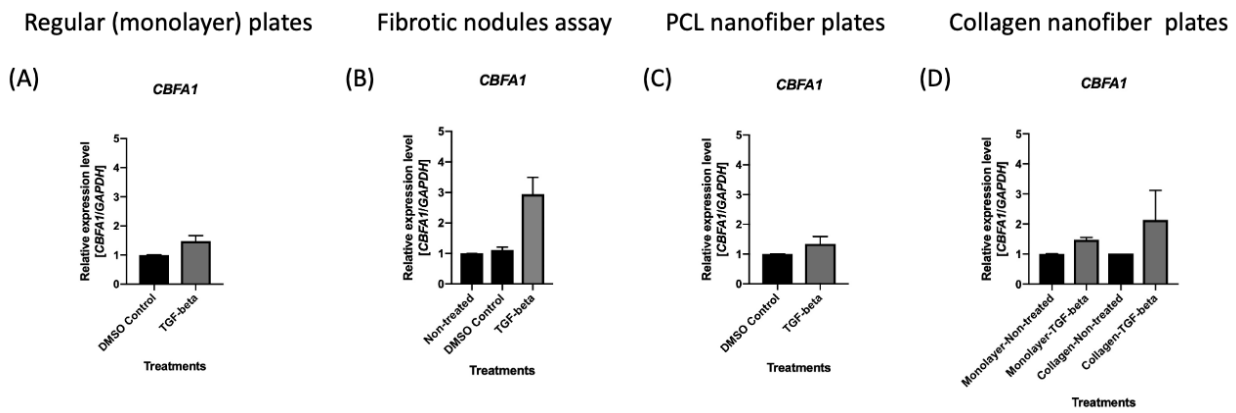


Figure 16 – Quantitative PCR analysis of the osteogenic marker **(A-D)** *CBFA1*. MPCs were cultured on a regular (monolayer) plate (A), fibrotic nodules assay (B), commercial PCL nanofiber plate (C) or collagen nanofiber plate (D) treated with TGF-beta [10 ng/mL] for 4-days followed by RNA extraction and qPCR analysis. Non-treated cells and/or DMSO alone were used as controls. Results represent the average of at least three independent donors.

Impact

Musculoskeletal injuries sustained in recent military conflicts have been notable for their number and complexity. Our group has studied this condition in combat related injuries for the greater part of the last decade and we have focused on the earliest and most basic parts of the process to gain insight into the key cell and molecular events leading to heterotopic ossification (HO) formation which is disfiguring, painful and functionally debilitating. Our efforts have enabled us to identify a population of cells that we feel are integral to the process of HO formation, a set of growth factors that are dysregulated in the healing response and a structural microenvironment which may lead stem cells to an undesirable cell fate. Together these components of wound healing identified in combat-related extremity injuries suggest that over stimulation of a specific fibrotic pathway may be one of the key triggers that initiates these undesirable healing events. This study capitalizes on the years of research devoted to studying these traumatic war wounds and uses a pre-clinical model to help translate the basic science findings into a new clinical treatment. The result will be improved functional recovery after traumatic musculoskeletal injuries for our injured service members.

Products

Manuscript published: de Vasconcellos JF, Zicari S, Fernicola SD, Griffin DW, Ji Y, Shin EH, Jones P, Christopherson GT, Bharmal H, Cirino C, Nguyen T, Robertson A, Pellegrini V, Nesti LJ. In vivo model of human post-traumatic heterotopic ossification demonstrates early fibroproliferative signature. *J Transl Med*. 2019;17(1):248.

Manuscript submitted (under review):

1. de Vasconcellos JF, Jackson WM, Dimtchev A, Nesti LJ. A microRNA signature for post-traumatic human heterotopic ossification. Submitted (under review).

Abstracts presented:

1. Piscoya A, Desraj C, Dingle M, de Vasconcellos JF, Dimtchev A, Nesti LJ. Inhibition of TGF-beta signaling modulates fibrosis in a model of human post-traumatic heterotopic ossification. In: *Walter Reed National Military Center Research Symposium*, Bethesda, 2020. Finalist for the Robert A. Phillips Award Competition. Oral and poster presentation.
2. de Vasconcellos JF, Dingle M, Desraj C, Piscoya A, Dimtchev A, Nesti LJ. TGF-beta signaling inhibition modulates fibrosis in a model of human post-traumatic heterotopic ossification. In: *2020 Orthopaedic Research Society Annual Meeting*, Phoenix, 2020. Poster presentation.
3. de Vasconcellos JF, Dingle M, Dimtchev A, Desraj C, Piscoya A, Nesti LJ. Effects of a targeted-therapy approach for post-traumatic heterotopic ossification. In: *61th Society of Military Orthopaedic Surgeons (SOMOS) Annual Meeting*, Palm Spring, 2019. Oral presentation.
4. de Vasconcellos JF, Dingle M, Dimtchev A, Desraj C, Piscoya A, Nesti LJ. Inhibition of TGF-beta signaling in a cellular model of human post-traumatic heterotopic ossification. In: *American Society for Cell Biology Annual Meeting*, Washington DC, 2019. Poster presentation.
5. de Vasconcellos JF, Dingle M, Dimtchev A, Nesti LJ. Effects of TGF-beta targeted-therapies in primary traumatized muscle derived multipotent progenitor cells as a model of human post-traumatic heterotopic ossification. In: *5th Stem Cell Biology Meeting from Cold Spring Harbor Laboratory*, Cold Spring Harbor, 2019. Poster presentation.
6. de Vasconcellos JF, Dingle M, Dimtchev A, Nesti LJ. Effects of TGF-beta targeted-therapies in an *in vitro* model of post-traumatic heterotopic ossification. In: *Military Health System Research Symposium*, Kissimmee - FL, 2019. Poster presentation.

Participants & Other Collaborating Organizations

Name: Leon Nesti
Project Role: PI
Nearest person month worked: .25
Contribution to Project: no change

Name: Jaira Vasconcelos
Project Role: Scientist
Nearest person month worked: 12
Contribution to Project: Tasks 1 and 2; scientific oversight

Name: Noreen Gervasi

Project Role: Scientist
Nearest person month worked: 12
Contribution to Project: Task 2 and 3; scientific oversight

Name: Vincent Pellegrini, Jr.
Project Role: Sub award PI
Nearest person month worked: 12
Contribution to Project: Sub award performance

Special Reporting and requirements

None

Appendices

Appendix 1
Post-Operative Blast Protocol –11.16.2018

Protocol #: IACUC #-2018-00344

Post-Operative Environment:

- Ensure ambient room temperature is at or above 70F.
- Prepare a clean housing unit with alpha-dri bedding, crushed wet food, water source, and cotton padding (layers of Webril)
- Provide supplemental heating with heat lamps above the unit and warm packs below the unit o Heat warming pack for 1 minute on each side before placing under ½ of the unit. Provide a buffer layer between warming pack and bottom of housing unit with paper towels, layers of Webril, etc. o Maintain appropriate distance (approximately 1-1.5ft) distance from heat lamp to top of housing unit
    - o Allow for half of the housing unit to be free of any supplemental heat source

Post-Operative Assessment:

- Measure and record rectal temperatures at scheduled post-operative intervals to ensure appropriate thermal resuscitation o Measure at 0 mins, 30 mins, 1 hr and thereafter as clinically indicated o Target Temperature Range 35.0 C to 37.5 C
- Maintain thermal support until rectal temperature reaches 35 C to 37.5 C, then remove housing unit from supplemental heat exposure.
- Monitor activity level, respiratory pattern, and general appearance

Treatment Algorithm:

- For temperatures < 35 C, continue to provide supplemental heating and monitor activity and respiratory pattern
- For temperatures within the target temperature range (35 C to 37.5 C), the supplemental heating may be removed from housing unit

- For temperatures > 37.5 C to 38.5 C, remove animal from supplemental heating source and monitor closely. Recheck temperature in 2 minutes
- For temperatures > 38.5 C, administer intraperitoneal injection of 10 cc sterile saline warmed to approximately 37 C and place under cool running water for 30 seconds - 1 minute. Monitor closely and recheck temperature in 2 minutes.

Indications for Veterinarian Consultation:

- Animal body temperature > 40 C
- Body temperature not responsive to specified interventions.
- Unexplained mortality associated with elevated body temperature.

References:

- Cowles, R.B., & Bogert, C.M. (1944) A preliminary study of the thermal requirements of desert reptiles. *Ball. Am. Mus. nat. Hist.* 83, 265-296. Czeisler, C.A., Weitzman, E.
- Erskine, D.J., & Hutchinson, V. H. (1982) Critical thermal maxima in small mammals. *J. Mammal*. 63, 267-273.
- Lillie, L.E., Temple, N.J., & Florence, L.Z. (1996, August). Reference values for young normal Sprague-Dawley rat: Weight gain, hematology and clinical chemistry. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8863053
- Stoner, H.B. (n.d.). Energy metabolism after injury. Retrieved November 24, 2018, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1519986/

Novel anti-fibrotic strategies in the targeted treatment and prevention of post-traumatic Heterotopic Ossification and enhancement of post-traumatic tissue regeneration.

Opportunity: W81XWH-17-2-0009 **Log:** BA150280

PI: LTC Leon Nesti M.D. Ph.D. **Org:** USU **Proposal Amount:** $1,992,386

Summary: We hypothesize that interrupting this TGF-b1 mediated signaling process at the level of Smad protein activation will suppress the fibroproliferative response and reduce or eliminate the formation of HO. We propose a preclinical trial to investigate the effect of specifically blocking the activation of the TGF-b1 intracellular signaling proteins, SMAD2 and SMAD3, on the development of posttraumatic HO using the inhibitors SB431542, Galunisertib, SIS3, and Halofuginone.

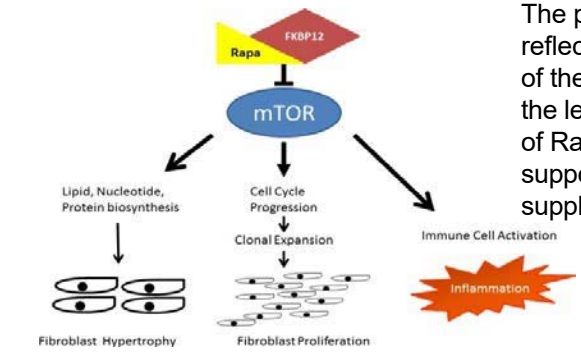
Aim 1 To assess the efficacy of SB431542, Galunisertib, SIS3, and Halofuginone treatment in preventing fibrosis in a cell culture model.

Aim 2 To assess the efficacy of SB431542, Galunisertib, SIS3, and Halofuginone based therapy in preventing fibrosis and ectopic bone formation in an animal model.

Aim 3 To assess the effectiveness of SB431542, Galunisertib, SIS3, and Halofuginone in promoting muscle regeneration.

Muscle Fibrosis & HO Mediattion

Muscle Trauma → *MPCs* → *Myofibroblasts* → *Myofibroblast Proliferation* → ***Fibrosis*** → ***HO***



The progression along the top reflects the working hypothesis of the project while the figure to the left shows the known impact of Rapamyacin on mTOR and supports our choice of supplementing with Proleukin

Accomplishment: Animal studies completed; pathology analyses in progress

Timeline and Cost

Activities CY	17	18	19	20
Dosing and in-vitro studies				
Small animal studies				
Data Analysis				
Publication & Closeout				
Estimated Budget ($1,992K)	**$498K**	**$664**	**$664**	**$166**

Updated 06/11/2020

Goals/Milestones

- ☐ **CY17 Goal** – IACUC Submission, dosing and in vitro studies
- ☐ **CY18 Goals** – Complete In vitro studies and start small animal study
- ☐ **CY19 Goals** - Data Analysis, Publication
- ☐ CY'20-Goals – Data Analysis, Publications and Closeout

Endpoint: To reduce fibrosis and subsequent osteogenesis in a cell culture model and HO formation in a blast injury rat model.

Comments/Challenges/Issues/Concerns: Sub-awardee Dr. Pellegrini moved to Dartmouth College; will resume upon approval of sponsor. Dr. Nesti is supportive of continuation of his move.

Budget Expenditure to Date:
Projected Expenditure: $1.91M
Actual Expenditure: $1.70M.