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TITLE: A Novel M2 Lipogenic Macrophages Associated with Fracture Callus Are Altered in Type 2 Diabetes

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CONTRACTING ORGANIZATION: Methodist Hospital Research Institute, Houston, TX

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14. ABSTRACT
Type II diabetes (T2DM) is usually viewed as a systemic disease where there is insulin resistance accompanied by increased glucogenesis by the liver leading to increased systemic glucose levels. However, much evidence suggests that in T2DM there is also chronic proinflammation with an increase in M1 proinflammatory macrophages. Little is known about how these changes in macrophage phenotype contribute to the disease phenotype. The findings from the research presented in this proposal will have a large impact in the PRMRP topic areas of diabetes and prevention of complications. Specifically, this proposal aims to understand the mechanisms surrounding the diabetic complication of impaired wound healing. Ideally, this work will result in a therapeutic to treat this complication. In addition, the findings from these studies may also allow for the identification of novel and unique biomarkers. It is hypothesized that the novel macrophages described in this application may be altered in diabetic wound healing and these cellular and metabolic responses may serve as novel and unique biomarkers for the disorder. These metabolic or functional differences may also allow for the characterization of disease progression as these cellular populations are uncharacterized in pre-diabetic and diabetic molecular environments. Key differences in these macrophages may also reveal potential molecular changes eliciting the heterogeneity in the disease complications.

15. SUBJECT TERMS
Type 2 diabetes, Macrophages, wound repair

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1. INTRODUCTION:

Based on preliminary data, we hypothesize that alteration in expansion, recruitment, and/or function of ADRbeta 3+ macrophages, directly result in impaired tissue regeneration, fracture repair observed in T2DM. Further, suppression of metabolic pathways by delivery of systemic medications to treat T2DM may further suppress the M2 macrophage recruitment and function. We propose to test our hypothesis through 2 aims. The first is to determine if the recruitment and expansion of the specialized ADRbeta 3+ macrophages are altered in diabetes, leading to impaired fracture healing. The second aim is to determine if typical anti-T2DM medications, such as metformin, glibenclamide (glyburide), and pioglitazone (glitazone), suppress the production or function of the ADRβ3+ macrophages, thereby compromising bone repair.

2. KEYWORDS:

Type 2 diabetes mellitus, M2 macrophages, beta adrenergic receptor positive macrophages.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1:
Task 1: Comparison of macrophages
- Subtask 1- Gain ACURO approval – Completed July 02, 2021
- Subtask 2- Compare ADRB3+ macrophages in diabetic animals to wild type mouse models of fracture repair. Completed
- Subtask 3 – Characterization of macrophage populations present in fracture callus – Completed

Task 2: Functional testing of macrophages
- Subtask 1 – Functional analysis of wild type ADRβ3+ macrophages to rescue fracture repair in T2DM mouse models- 50% Completed
- Subtask 2 – Functional analysis of ADRβ3+ macrophages isolated from T2DM mice to determine if they lead to impaired fracture repair. – 50% Completed

Aim 2:
- Subtask 1 – Gain ACURO approval - Completed
- Subtask 2 – Establish model –Completed
- Subtask 3 – Delivery of drug treatment or vehicle and glucose monitoring -30% Completed

What was accomplished under these goals?

Many factors contributed to slowing down the progress of this project. The move of the PI to a new institute and gaining approvals to transfer the grant and to then have a subcontract back to the co-investigator took a significant amount of time (Approximately 6 months). Gaining DOD approval took several months. Then it took some time for my colleague to gain the subcontract and access the funds to initiate the experiments. As mentioned below, we also had issues with Baylor College of Medicine closing during the initial COVID pandemic. The institute was not able to have enough personnel working to maintain the animal colonies, and any that we had purchased and bred to use for experiments all had to be terminated. It was approximately 1-1.5 years before we could purchase and breed mice. In the meantime, we initiated some different experiments to look at the origin of the cells. While these experiments were not in our original application, a colleague that shares lab space had mice including bone marrow transplanted animals that they were told to euthanize. We chose to do a quick experiment, so as not to waste the animals. Therefore, we did not perform the transplantations, but took advantage of a difficult situation, to obtain publishable data from this award, and build a basis for future studies that we could work on during the closure.

The major activities performed under this award have been to purchase the mice, and allow the diet induced obese mice to age, where they will have noticeable elevated blood glucose levels, and to breed the lepOB mice to obtain sufficient numbers for experiments. We finished the characterization of the ADRB3$^+$ macrophages in fracture callus and published this work (see appendix for results).

Our objectives were to introduce bone fractures in mice and isolate macrophages as well as determine if they are altered between in fracture repair. In our original pilot data, we observed a reduction in ADRB3+ macrophages in the diabetic lep OB mice that appeared to correlate with blood sugars.

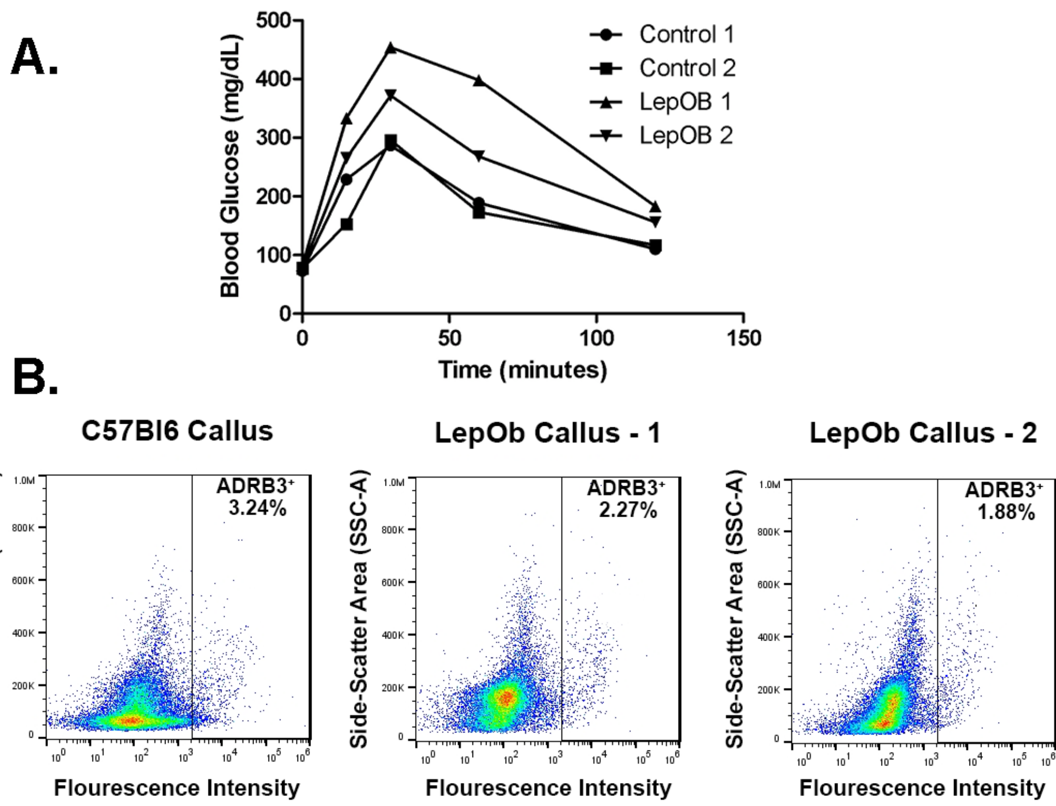


Figure 1: Correlation of blood glucose levels and percentage of ADRB3+ macrophages from fracture callus in lep OB mice.

We next looked at whether there was a change in the diet induced obese mice. In these mice we set up heterotopic ossification (HO) rather than fracture, to determine if there was a change in macrophages. Again, there is a trend in ADRB3+ macrophages to be reduced in the diabetic animals, but to our surprise we also saw a decrease in CD11b macrophages which would be a more total population. We are repeating these experiments and will again correlate them with blood sugar. Further, upon repeat we will introduce additional M1 (CD38) and M2 (Egr2) macrophage markers to attempt to sort whether M2 macrophages are decreasing or all macrophages. Finally, we are in the process of analyzing the microCT data from fracture repair of a larger cohort of mice from (Control, DIO, and LepOb mice) to determine if bone healing is reduced due to the loss of specific macrophage populations. Thus, in conjunction with the fracture repair, we have also isolated the mice, and performed flow cytometry on the tissues to isolate M1, M2 and ADRB3 macrophages to look for changes. This data is still being analyzed.

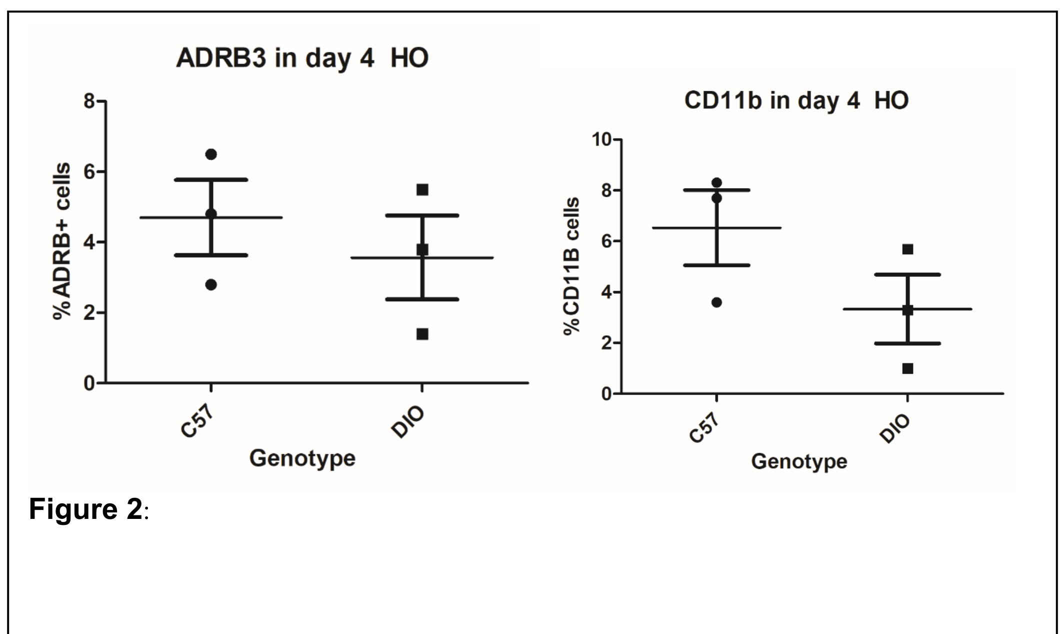


Figure 2:

In this set of experiments, the mice were fasted for 12 hrs and a glucose tolerance test was

performed as shown in figure 1A prior to fracture. Macrophages were isolated from the fracture and quantified using flow cytometry. While the mice ADRB3+ CD11- lipogenic macrophages were decreased, the difference was not statistically different (Fig 2). Further, analysis of bone fracture healing in these mice and the leptin mice, as quantified using microCT did not appear to vary statistically. Thus, while the models appear to have a decrease in these macrophage numbers, again this change may not have been sufficient to alter the bone fracture repair. Alternatively, the bone fracture repair could be independent of these macrophages, but not be sufficient in these mouse models to be readily detectable by microCT.

These studies were initiated during Covid. At the time we were told that the Baylor College of Medicine did not have enough staff to handle their animal colony during the closure due to COVID, so we were instructed to eliminate our colonies of mice. This meant we had to rebreed all the animals, after the institute fully opened again, almost 1 year later. Thus, we did not have a lot of time to be able to trouble shoot this model, due to lack of mice.

Additionally, we have started experiments using metformin. Initially we looked at blood glucose in the mice to ensure that delivery of metformin to wild type mice did not drive them into a hyperglycemic state. We next set up a pilot using metformin and vehicle to determine maximal effects on macrophages that may be observed. In these studies, we used all three groups (Dio, LepOb and wild type mice) to determine the effects on macrophages isolated 4 days later. While we were able to start these experiments, we were not able to use the other models than wild type, due to COVID closures. We saw a statistically significant decrease in ADBR3+ Cd11b- cells in fractures from animals treated with Metformin as compared to those that receive vehicle (**Fig 3).**

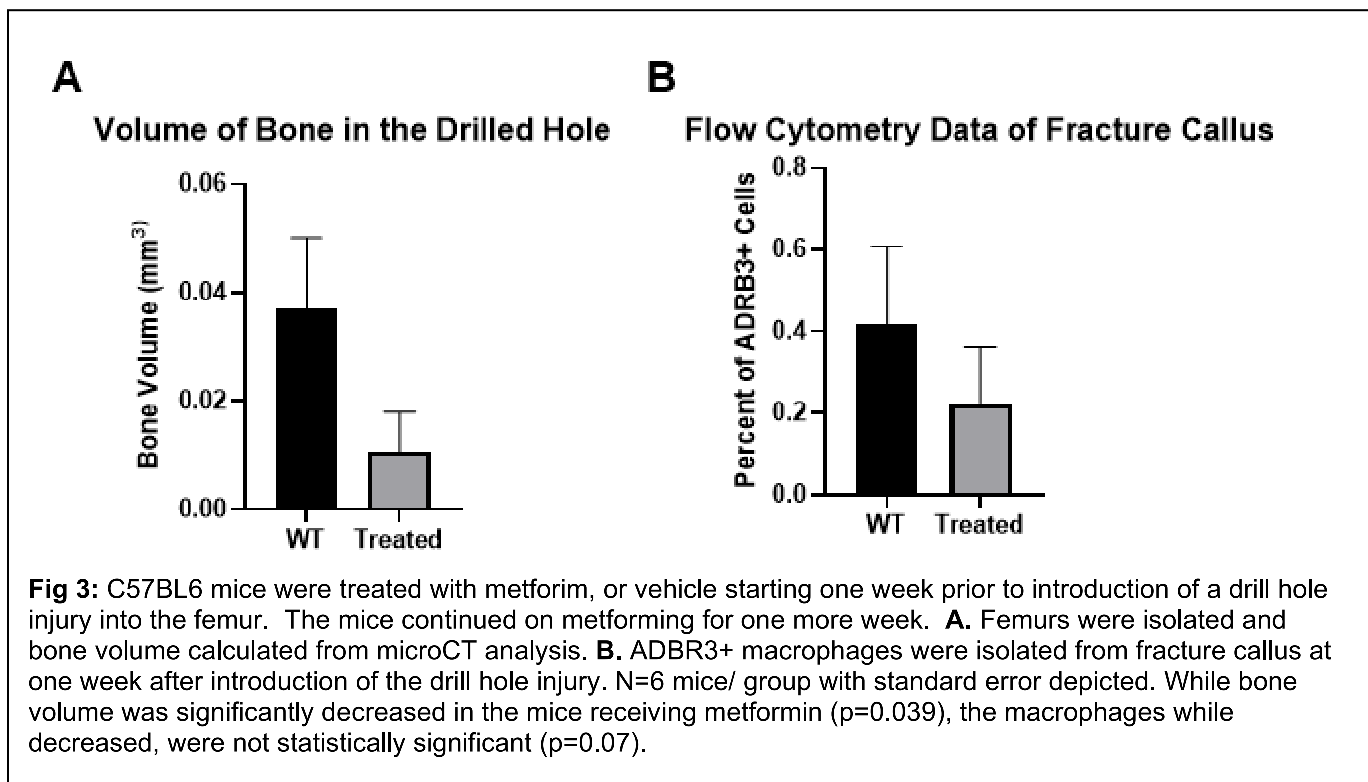


Fig 3: C57BL6 mice were treated with metforim, or vehicle starting one week prior to introduction of a drill hole injury into the femur. The mice continued on metforming for one more week. **A.** Femurs were isolated and bone volume calculated from microCT analysis. **B.** ADBR3+ macrophages were isolated from fracture callus at one week after introduction of the drill hole injury. N=6 mice/ group with standard error depicted. While bone volume was significantly decreased in the mice receiving metformin (p=0.039), the macrophages while decreased, were not statistically significant (p=0.07).

The Lipogenic Macrophages are derived from a common progenitor as the osteoblasts during bone formation:

The ADRB3+ lipogenic macrophages/monocytes were subjected to single cell RNAseq. The resultant populations yielded a population that appeared to be identical to the early GLAST + osteoprogenitor population[1] with the top 30 genes in each cluster having 20 overlap or approximately 97% similarity in gene expression. The major genes that separated the two populations are a set of hematopoietic markers such as CSFR1, CD45, and CD68, in the macrophages, whereas the osteoprogenitor expressed collagen Col1A1, col2a1, col5a2, or myofibroblast markers (**Fig 4**).

To determine if these cells could be from a common progenitor, we performed bone marrow transplantation in mice. First CD45.1 mice were transplanted with CD45.2 whole bone marrow, which can easily be distinguished using a selective CD45 antibody. Briefly, the mice were bone marrow ablated, and repopulated with whole bone marrow isolated from the donor mouse (CD45.2) (**Fig 5**). The ADRB3+ cells were then isolated using antibodies against CD45.1 and CD45.2 to determine if these cells are arising from whole bone marrow. Results of flow cytometry analysis suggested that the majority of the ADRB3+ macrophages were derived from bone marrow (**Fig 6**).

We next decided to determine if the osteoprogenitor could be derived from the same population. Hematopoietic stem cells were isolated from mice possessing a GLAST-Cre$^{ERT:}$:TdTomato red transgene that were also CD45.2. The HSCs were then transplanted into lethally irradiated CD45.1 mice. The donor tagged HSCs were allowed to repopulate bone marrow of recipient wild type mice and then ADRB3+ cells were isolated and approximately 80% were shown to possess CD45.2. We next set up a uni-cortical femur drill hole or bone microfracture repair in the transplanted mice. We also looked at BMP2 induced heterotopic ossification in the mouse muscle to see if those cells were derived from the common progenitor. Tamoxifen was delivered to the mice and then 3 days later either the fracture or HO were introduced into the mouse. At approximately 4 days, blood was isolated and analyzed for the presence of CD45.2. The mice showed approximately 80% efficiency in engraftment of these cells. The mice were then given tamoxifen and several days later cells were isolated from fracture callus and heterotopic bone, and sorted for expression of the GLAST-Cre^{ERT}:TdTomato red reporter. Approximately 1-5% of all cells isolated from these tissues were positive for the tomato red donor cell tag. While this was a minor population, it was distinct osteoprogenitor cells. We have previously shown in GLAST-Cre^{ERT}-TdTomato red mice that the only cells labeling with tamoxifen are the osteogenic cells [1] These numbers were even higher in heterotopic bone, with approximately 10% of the cells positive for the reporter. ***The data suggests that these cells are derived from a hematopoietic stem cell (HSC).***

Much evidence in the literature suggests that endothelial cells upon activation can activate a pathway leading to epigenetic reprogramming to a myofibroblast, and vice versa [2, 3]. These endothelial cells have been shown to undergo de-differentiation and eventually an EMT to form other cells [1, 4]. This model has been shown to occur during vascular ischemia [5] and suggests that the cells within the wound may have multiple regenerative capacity including creation of the lipogenic macrophages.

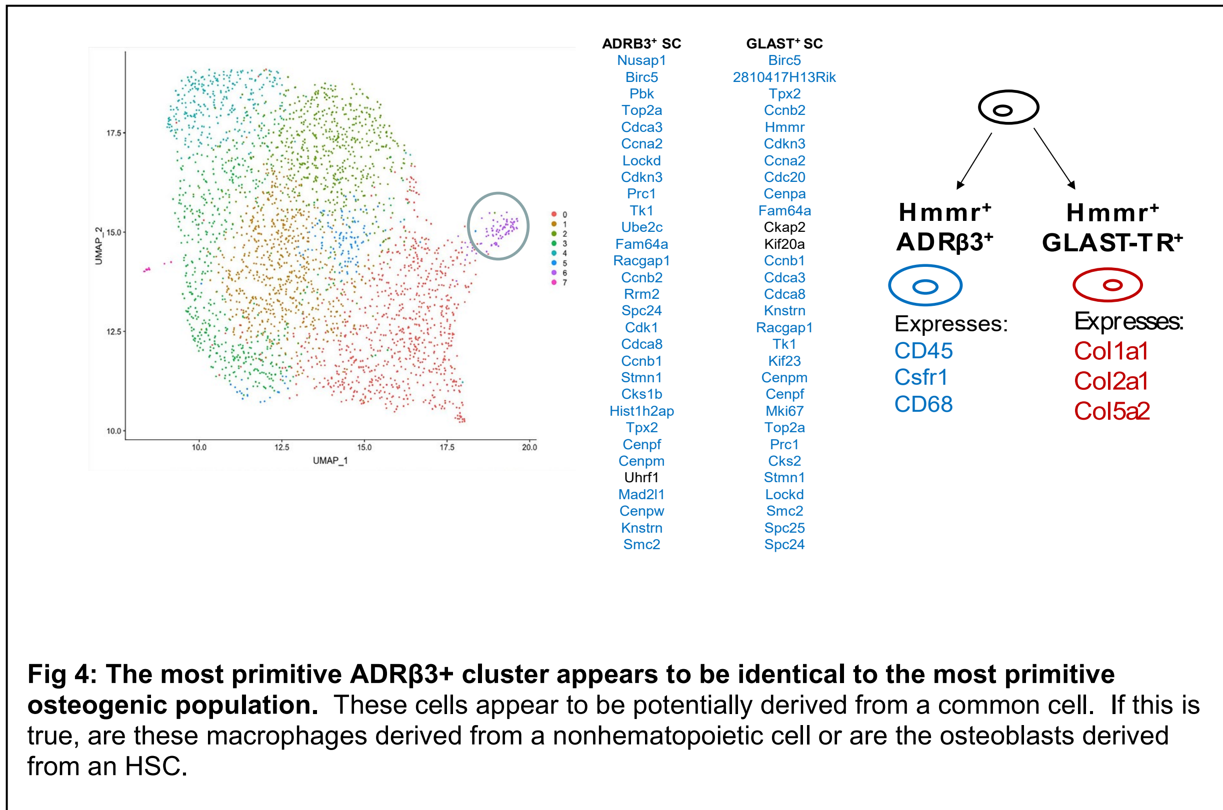
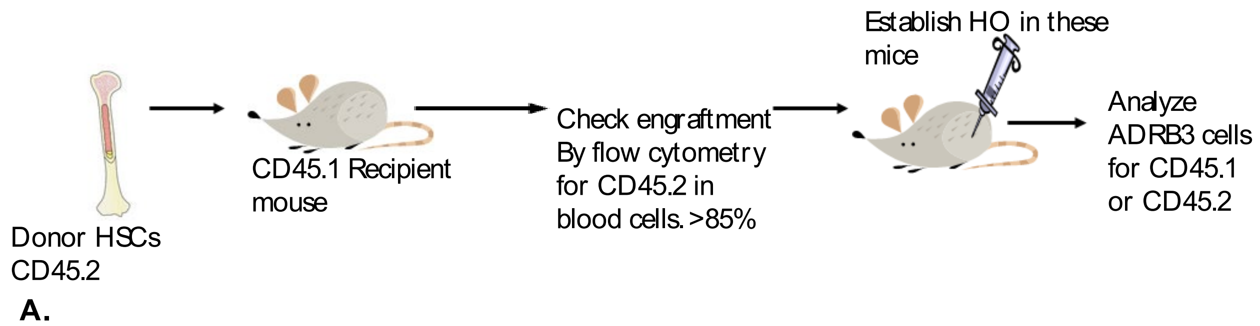


Fig 4: The most primitive ADRβ3+ cluster appears to be identical to the most primitive osteogenic population. These cells appear to be potentially derived from a common cell. If this is true, are these macrophages derived from a nonhematopoietic cell or are the osteoblasts derived from an HSC.

However, it is still unclear how the transplanted HSCs are able to repopulation the osteoprogenitors. Further analysis of the progenitor population that appears to overlap in both macrophages and osteoblasts suggests that they express approximately 50 genes associated with epigenetic reprogramming again suggesting that they may be derived from a reprogramming event. Both cell populations also express histone acetylase 1 (HAT1) known to be responsible for the acetylation of Lysine 27 (K27) on histone 3 and expression of H2zfa gene, which encodes a histone 2A variant, H2A.Z. Studies showed that incorporation of H2A.Z in nucleosomes, when it co-occurs with H3.3, makes their DNA interactions weaker. Moreover, the presence of H2A.Z containing nucleosomes around transcription start sites has now been shown to enhance gene expression.

Of further note, a small population of cells appeared to be a hemangioblast-like cell based on their transcriptome. Hemangioblasts are multipotent precursors that can differentiate into either hematopoietic or endothelial cells. They appear early in development and form “blood islands”, which is the start of hematopoiesis and vasculature. However, evidence suggests that these cells may exist in adults to assist in giving rise to blood and endothelial cells during injury. These cells express Notch 1 and Hmmr, but also

Ace/Cd143, vascular-endothelial cadherin, Cd31/Pecam, Endoglin, Sox 17, EphB4, Kdr, and Lmo1all genes associated with endothelial progenitors. We hypothesize that this hemangioblast-like cell may be an endothelial-blood progenitor cell derived potentially derived from the HSC. The data also suggests that the HSC was not the only cell contributing to the new bone formation, and perhaps other types of cells are also undergoing epigenetic reprogramming.



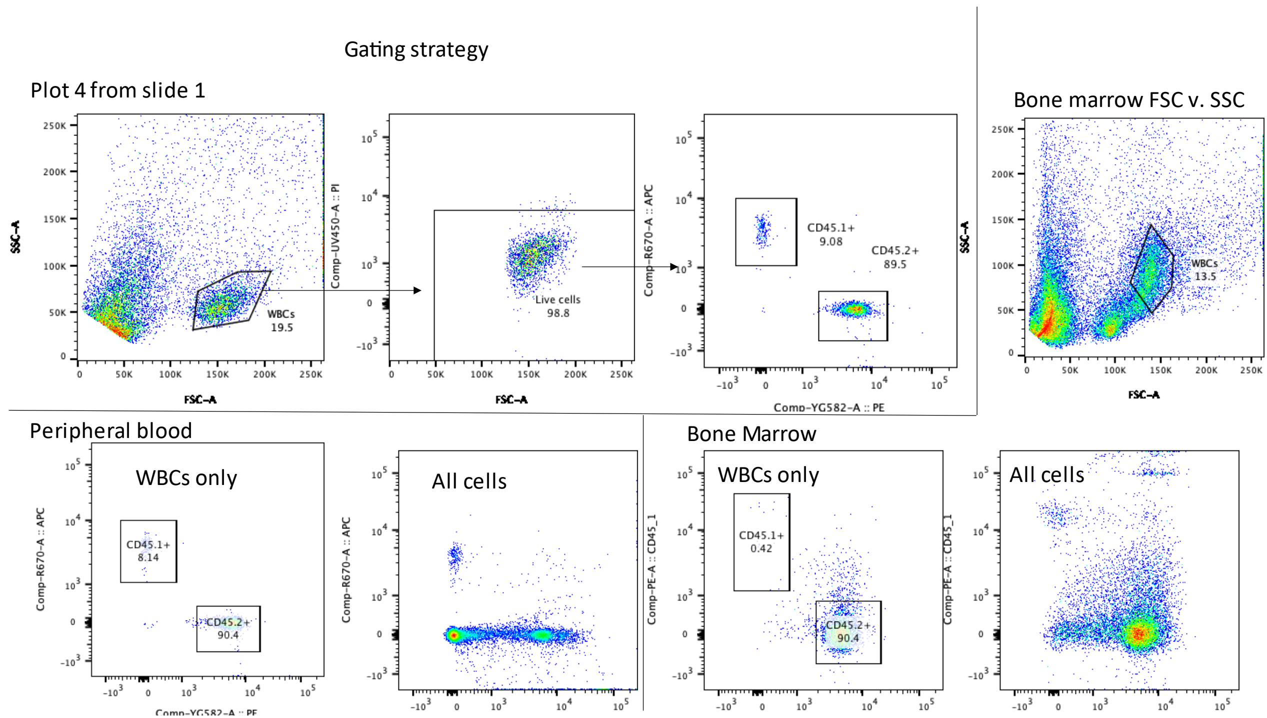


Fig 5: Mice underwent bone marrow transplantation with hematopoietic stem cells isolated from a CD45.2 donor and were engrafted into a CD45.1 recipient. One year later transplanted mice or 3 month old C57BL/6 mice (n=6 per group) were induced to form heterotopic bone, and ADRB3+ macrophages isolated 4 days later. **A)** ADRβ3+ cells were analyzed for their expression of CD45.1 and CD45.2.

Lipogenic Nature of the Macrophages: We have reported the lipogenic nature of this macrophage[6]. These cells appear to be undergoing both gluconeogenesis and uncoupled oxidative phosphorylation (**Fig 3**). Thus, these cells appear to be expending energy to make glucose, while they are also burning oxygen. Our initial thoughts were that this could drive chondrogenesis, but new data from the vascular biology field suggests that epigenetic reprogramming of cells requires a glycolytic switch, where endothelial cells undergoing reprogramming must switch to glycolysis in order to make acetyl coA required for histone acytelation [2]. Thus, dropping the oxygen levels at the site of tissue regeneration may be a way to induce the switch to glycolysis and downstream epigenetic reprogramming [2]. Further the robust oxidative phosphorylation has been associated with a regenerative phenotype of macrophage. Since the diabetic drugs suppress gluconeogenesis, we have found that they appear to suppress this lipogenic phenotype and thus, cannot induce contribution of epigenetic reprogramming to wound repair. This includes vascular, bone and skin according to our preliminary data.

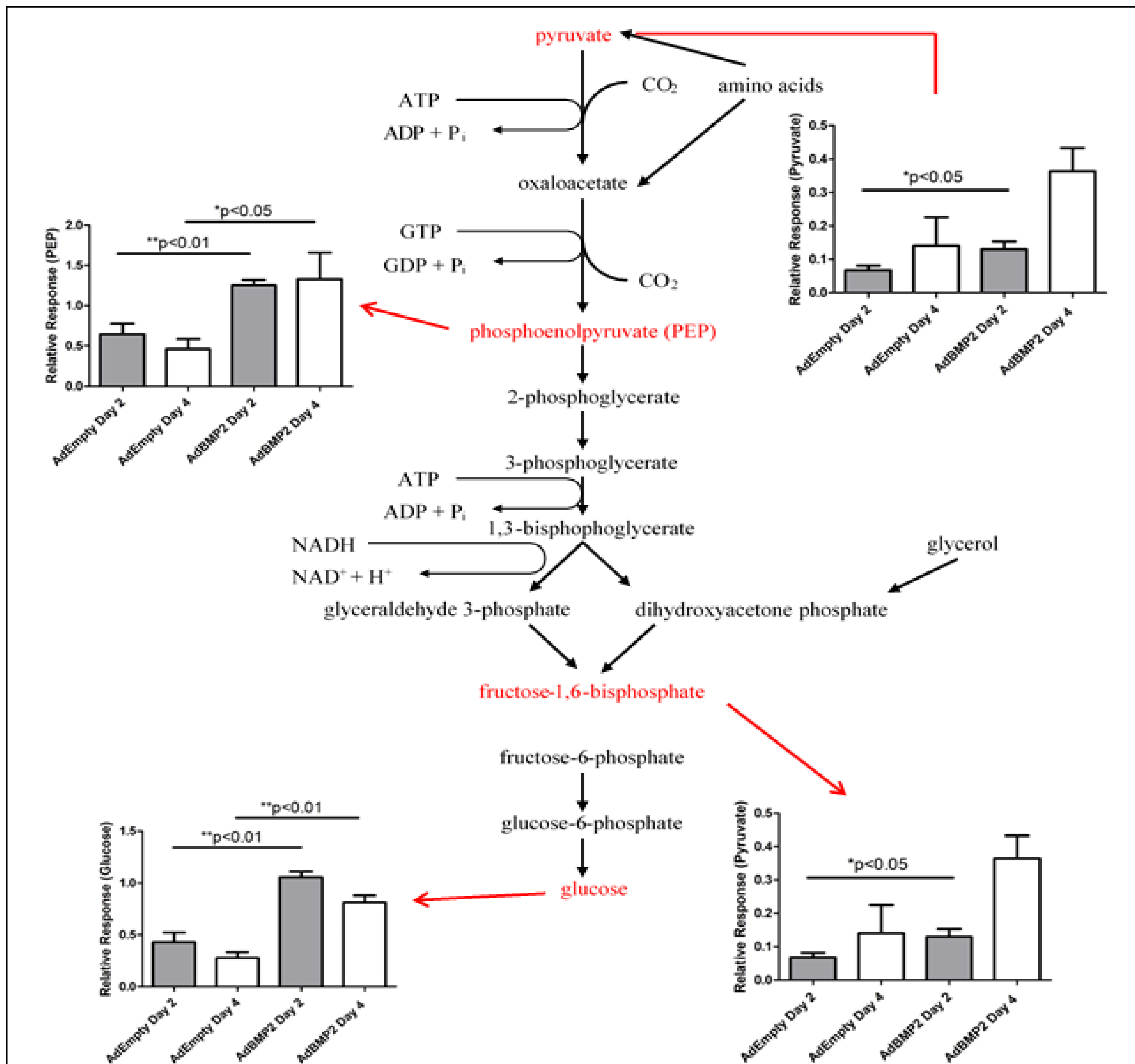


Fig 6: ADRB3+ macrophages undergo gluconeogenesis, to generate glucose. ADRB3+ macrophages were isolated and subject to unbiased metabolomics screen. The factors in red were significantly elevated in these macrophages as compared to bone marrow derived macrophages. This pathway is the target of metformin. Surprisingly, these cells are also undergoing robust uncoupled oxidative phosphorylation. In this latter pathway, oxidative phosphorylation is uncoupled from the ATPase, and thus the high energy is lost as heat rather than converted to ATP.

Clinical Significance:
We are currently working on assembling this manuscript as a follow up to our previous manuscript on the two cell types discussed in this review. The clinical significance of these studies is with the finding that metformin will inhibit the ability of the macrophages to differentiate correctly into a lipogenic macrophage that can regulate the oxygen environment and potentially cause the cells to undergo a glycolytic shift, that will allow them to produce acetyl coA for histone acetylation and downstream epigenetic reprogramming. Thus, the cells may fail to actually reprogram and shunt to form scar tissue, rather than to regenerate the damaged wound. Additionally, the ability of multiple cell types including an HSC to differentiate into a mesenchymal cell that can contribute to multi-tissue repairs shows how the entire wound and vasculature may be essential for healing and potentially should be allowed to remain in place as much as possible for tissue regeneration. Finally, the ability to induce or direct epigenetic reprogramming in the wound would be a novel approach to tissue repair particularly in T2DM.

1. Mejia, J., et al., *A replicating stem-like cell that contributes to bone morphogenetic protein 2-induced heterotopic bone formation.* Stem Cells Transl Med, 2021. **10**(4): p. 623-635.
2. Lai, L., et al., *Glycolytic Switch Is Required for Transdifferentiation to Endothelial Lineage.* Circulation, 2019. **139**(1): p. 119-133.
3. Meng, S., et al., *Reservoir of Fibroblasts Promotes Recovery From Limb Ischemia.* Circulation, 2020. **142**(17): p. 1647-1662.
4. Medici, D., et al., *Conversion of vascular endothelial cells into multipotent stem-like cells.* Nat Med, 2010. **16**(12): p. 1400-6.
5. Meng, S., et al., *Transflammation: Innate immune signaling in nuclear reprogramming.* Adv Drug Deliv Rev, 2017. **120**: p. 133-141.
6. Olmsted-Davis, E., et al., *A Population of M2 Macrophages Associated With Bone Formation.* Front Immunol, 2021. **12**: p. 686769.

What opportunities for training and professional development has the project provided?

The project has allowed me to collaborate with Dr. Li Lai at Houston Methodist Hospital, where I have been learning about endothelial cells, and vascular biology. Understanding the vasculature is critical for wound repair and is assisting me in being able to further make progress in this area.

How were the results disseminated to communities of interest?

Due to covid restrictions there has been very limited opportunities to disseminate the research. We have made some of the data public through publication and recently through oral presentation at the Gulf Coast Consortium meeting on immunology, and poster presentations at the American Society for Bone and Mineral Research.

What do you plan to do during the next reporting period to accomplish the goals?

We are currently working on assembling a grant proposal using this preliminary data. This is a final report so there will be no other reports generated.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have obtained preliminary data that suggests that cells within a wound are able to transform to other cells through reprogramming their DNA. This reprogramming is done, by adjusting their metabolism. In order to accomplish this process during injury, the cells must react to their environment, and switch metabolism to then reprogram their DNA and contribute to tissue repair. We have identified an accessory blood cell that contributes to this process by possessing unique metabolism that can assist providing the essential environment for the cells to make a metabolic switch to become reprogrammed regenerative cells. Thus, patients with T2DM, particularly those who take metformin a drug that suppresses the essential metabolism that these cells utilize to provide the overall healing environment in the wound, are not able to launch an appropriate healing response and either take much longer to heal or do not heal correctly.

What was the impact on other disciplines?

The findings also have significant impact on the tissue regeneration and traumatic injury repair fields, where blast injury wounds heal differently than other types of traumatic injury wounds etc. The idea that cells utilize their metabolic environment to reprogram their cell fate to contribute to the populations of cells needed for regeneration of the tissues is a novel finding and will impact the field of traumatic injury. If the cells are damaged or unable to reprogram correctly due to an altered environment, they may be unable to heal the tissues as well, and even generate scar tissue in place of the original.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Due to COVID and other circumstances, we were not able to purchase and breed mice, therefore we used mice from a colleague that had been transplanted with tagged bone marrow. While this was a deviation from the original proposal, due to circumstances we attempted to continue the work, and gain publications and a foundation for future funding. We were also able to utilize mice that would otherwise be euthanized due to BCM closure for COVIDWhen working on the proposed studies, we observed the common progenitor, which led us to perform a couple of additional experiments. These experiments allowed us to see that several cell types respond and reprogram to contribute to wound repair and that these cells all require the lipogenic macrophage.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Olmsted-Davis E, Mejia J, Salisbury E, Gugala Z, Davis AR. A Population of M2 Macrophages Associated With Bone Formation. Front Immunol. 2021 Oct 12;12:686769. doi: 10.3389/fimmu.2021.686769. eCollection 2021.PMID: 34712222.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Oral presentation: A Population of M2 Macrophages Associated With Bone Formation. Gulf Coast Consortium, Immunobiology research conference, 3/2022.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Elizabeth Olmsted-Davis
Project Role: Principal Investigator
Research Identifier: 0000-0001-9457-1848
Nearest person month worked: 3 months
Contribution to Project: Dr. Olmsted-Davis, has been training the technician on all the technologies described in the grant. She has been working with Dr. Davis to interpret all data and was instrumental for publishing the first paper on the work.

Name: Alan R. Davis
Project Role: Principal Investigator
Research Identifier:
Nearest person month worked: 3 months
Contribution to Project: Dr. Davis, has also focused on training the technician on all the technologies described in the grant. He has been working with the PI to interpret all data and was instrumental for publishing the first paper on the work.

Name: Ashwin Vivekananthan
Project Role: Research Technician
Research Identifier:
Nearest person month worked: 3 months
Contribution to Project: Ashwin has been responsible for conducting the experimental procedures outlined in the proposal.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Baylor College of Medicine:
One Baylor Plaza
Houston, TX77030
Co-Investigator is performing all the animal studies. He receives a subcontract from Houston Methodist Hospital.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: