Metabolically labeling an iP-hMSC derived modified bone matrix to a hydrogel scaffold and its effects on osteogenic advancement

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Mesenchymal stem cells (MSCs) have very promising regeneration potential and have been explored in cancer treatment for several years. Because of their stellar differentiation capabilities, they have started to be considered as strong candidates for tissue engineering and the like; however, MSCs have been found to have limited proliferation capabilities. By deriving MSCs from induced pluripotent stem cells (iPSCs), we can work with cells that can be standardized across a large sample as well as express increased proliferation, differentiation and expansion. We then culture these iP-hMSCs and evaluate and synthesize the resulting anabolic bone extracellular matrix (ECM) containing the non-canonical amino acid (methionine analog), L-azidohomoalanine (AHA). Furthermore, we aim to metabolically label this AHA-modified anabolic bone ECM to a hydrogel scaffold through click-chemistry applications in order to visualize ECM deposition and observe the hydrogel scaffold's ability to covalently bind with the AHA modified anabolic bone ECM.

tissue regeneration | biomaterials | mesenchymal stem cells | click-chemistry | modified bone ECM | hydrogel scaffold

INTRODUCTION

Bone is a vascularized tissue that must be able to provide firm structural support, withstand load bearing, and rapidly respond to metabolic demand. Over the past decade, there has been an increased need for bone replacement due to bone diseases, osteoporosis-related fractures, trauma, bone malformations, and tumor resections. Worldwide, an estimated 2.2 million bone graft procedures are performed annually, with autografts and allografts being a standard treatment method[1]. Autografts are the most common treatment for bone defects; however, the limited supply and potential complications at the donor site remains a significant problem. Allografts are a widely used alternative, but these are expensive and pose a serious risk of disease transmission[1]. In addition, synthetic implants can be used to temporarily stabilize fracture injuries, however it can lead to an inadequate cellular response and in turn, delay healing. Failed implants are very hard to fix and take a large toll on the health of the patient[1]. In addition, bone substitutes taking the form of synthetic material or decellularized bone are abundant but come with their own disadvantages. They support poor osteoconductivity, poor cell adhesion properties, and immune rejection. They provide a well-defined region of containment but are rather insufficient when it comes to supporting proliferation, nutrition and adherence. Bone tissue engineering has been spurred to develop treatment alternatives for an aging population and the steady growth of conditions that negatively impact bone healing.

The ultimate goal for bone tissue engineering would be to fabricate scaffolds with the healing potential of autografts. Newer approaches to tissue engineering focus heavily on the combination of cells and other biological molecules that are seeded onto a three-dimensional biomaterial scaffold to create an osteogenic implant. This technique is used not to replace damaged tissue but to promote normal deposition of tissue with eventual degradation for the restoration of original structure and function[2]. Human mesenchymal stem cells (hMSCs) have promising regeneration potential after being originally explored in cancer treatment research for repairing cancer damaged tissue[3]. hMSCs are traditionally isolated from the bone marrow and have the potential to differentiate into multiple connective tissues -- mainly bone, fat and cartilage. In addition, there is emerging evidence that the immunosuppressive nature of hMSCs, along with their differentiation abilities, makes them highly attractive for regenerative techniques due to transplant rejections being a huge limiting factor for stem cell-based therapies[1].

Although hMSCs have been observed to have outstanding differentiation potential, they have limited abilities in proliferation, losing a few important biological features as they begin expanding[4]. Hence, they can be difficult to prepare in large quantities due to discrepancies in biological capacities and expressions from cell to cell. Being unable to standardize these cells can become detrimental when trying to compare data from various publications[5]. In order to bypass this obstacle and address the complications associated with limited proliferation and standardization of these cells, we further derived hMSCs from induced pluripotent stem cells (iPSCs) to allow them to be better expanded to accommodate large cell banks originating from a single cell. These iPSC-derived hMSCs (iP-hMSCs) express the traditional surface level characteristics of hMSCs; moreover, they are capable of multilineage differentiation and can be thoroughly expanded, but they do not retain the pluripotency of iPSCs[3].

Furthermore, these iP-hMSCs can be stimulated with GW9662 (GW) (Sigma Aldrich) to deposit an anabolic bone extracellular matrix (ECM), which is comprised of high levels of collagen VI and XII. These two proteins play an important role in endowing the osteoconductive properties of the anabolic bone ECM. However, currently the anabolic bone ECM has been prepared by either 1) culturing the stem cells on established scaffolds which are then decellularized[6], or 2) by passively coating scaffolds through adsorption. A technique that covalently binds the ECM to scaffolds without interfering with their osteoinductive properties would be ideal.

L-azidohomoalanine (AHA) is a non-canonical amino acid (methionine analog) containing an azide group that has been used for the detection of nascent proteins through a metabolic labeling process[9]. In short, the cultured cells recognize AHA as a normal methionine amino acid, begin to metabolize it, and then incorporate it into proteins during active protein synthesis. After it has been incorporated into protein synthesis, the azide group can then be utilized in a chemoselective click-chemistry reaction. While this technique has been used to visualize ECM deposition by providing alkynes that are paired with a fluorescent tag, we propose fabricating a hydrogel scaffold with DBCO alkynes that can covalently bind with the AHA containing anabolic bone ECM. In this proof of concept work, we investigate whether the anabolic bone ECM deposited by iP-hMSCs contains the azide functional groups.

METHODS

Tissue Culture

To generate the anabolic bone ECM, we cultured iP-hMSCs as described in Zeitouni 2012[8]. In short, iP-hMSCs were grown in 150 mm dishes for 10 days in the presence of 10 uM of GW with and without 300 uM of AHA. We also cultured iP-hMSCs treated with an equal volume of DMSO (a vehicle for GW), with and without 300 uM of AHA. Protein samples were then stored in the freezer until needed.

Protein Extraction

Within a sterile biosafety cabinet environment, cultured media was aspirated out of the well plates containing the iP-hMSCs. Then cells were washed by adding ice-cold PBS to each well. PBS was then aspirated and ice-cold RIPA buffer was added to each well. Cells were lysed leaving only the ECM, and samples were vortexed. The samples were then centrifuged for five minutes at 500g, and then stored in the freezer until needed.

BCA Assay and Protein Quantification

Multiple samples of DMSO, DMSO + AHA, GW, and GW + AHA were organized in a 96 well plate, performed at both five-fold and ten-fold dilutions for each sample. The Pierce™ Micro BCA™ Protein Assay Kit, Thermofisher was used to perform the assay. A cell plate reader, calibrated at 562 nm, was used to obtain the absorbances of each sample.

Gel Electrophoresis and Western Blotting

Electrophoresis was performed using hand casted 8% polyacrylamide gels (Bio-Rad). We then probed our membrane for Collagen VI presence by adding our protein-specific primary antibody, Collagen VI Alpha 1 (Rabbit anti-Human, Novus Biological) and our species-specific secondary antibody (Goat anti-Rabbit, Protein Tech), at a dilution of 1-1000. We then imaged our membrane on a Licor gel reader. We repeated this process on the same membrane

probing for Collagen XII using our primary (Rabbit anti-Human, Novus Biological) and the same secondary described above. Finally, we probed for our housekeeping protein, GAPDH, using GAPDH Mouse anti-Human, Protein Tech to obtain GAPDH bands. After verifying the molecular weights of Collagen VI and XII using the molecular ladder against those reported by the manufacturers (Precision Plus Protein™, Bio-Rad), we normalized the Collagen VI and Collagen XII bands to our GAPDH bands.

Imaging and Quantification
Membrane was imaged on a Licor Gel Reader and signal intensity of the bands were quantified using Licor Studio, Image Studio Lite.

Protein Precipitation
488-DBCO dye was added to each protein sample at 1:200 dilution. Samples were then vortexed and stored in the dark for an hour. Chloroform and methanol were added, and the samples were centrifuged for one minute at 12,000g. The resulting top aqueous layer was removed, and the samples were centrifuged again for five minutes at 20,000g. Samples were wrapped in aluminum foil to prevent light exposure and then placed in an orbital overnight. Methanol was removed and the samples were dried under vacuum. Then samples were resuspended in warmed RIPA buffer and stored in the freezer until needed.

In-Gel Fluorescence
The samples were loaded into the appropriate lanes in equal amounts and the protein ladder (Precision Plus Protein™, Bio-Rad) was loaded in the first lane. The stained gels were then imaged by a gel reader.

Coomassie Blue Stain
Using the imperial Protein Stain (224615), the gel was stained for an hour and washed overnight.

RESULTS AND DISCUSSION

BCA Assay analysis reveals GW treatment to be more effective for ECM production compared to DMSO
We conducted a BCA Assay to quantify the total protein in a given mixed sample.

We organized multiple samples of DMSO, DMSO + AHA, GW, and GW + AHA in a 96 well plate and simply followed the manufacturer's instructions. Using a plate reader we collected the absorbances at 562 nm for all the samples in our well plate. A standard linear curve was then used to calculate the sample concentration. As shown in Figure 1, we see that the iP-hMSCs treated with GW produced higher levels of extracellular matrix compared to DMSO treatment. In addition, our data shows that there seems to be no difference in the effects of AHA in terms of the production of anabolic bone ECM. The iP-hMSCs given AHA produced a similar amount of ECM when compared to their DMSO and GW counterparts. Furthermore, iP-hMSCs given GW had nearly three times the ECM protein production frequency of cells given DMSO, making them more suitable for mass production of ECM.

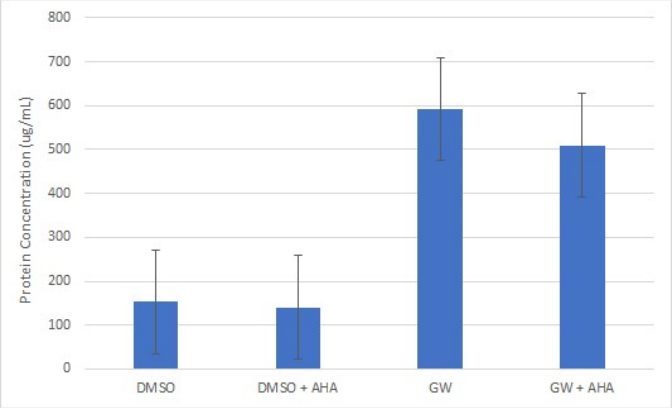

Figure 1: *Standardized BCA assay measured at 562 nm, quantifying the total amount of DMSO, DMSO + AHA, GW, and GW + AHA concentration at a five-fold dilution.*

Gel Electrophoresis and Western Blot confirms Collagen VI and XII production within ECM
We then investigated whether the composition of the ECM was affected by the addition of AHA. After separating the proteins through electrophoresis and transferring them from the gel to the membrane, we probed the membrane three different times for Collagen VI, Collagen XII, and GAPDH, whose bands can be seen in Figure 2 A-C, respectively. The normalized band signals are shown in Figure 2 D-E. With our Collagen VI bands, we saw a molecular weight of approximately 100 kD, close to the reported weight of 108 kD for the alpha 1 chain. Our Collagen XII bands measured at approximately

150-250 kD, compared to its reported weight of 220 kD. When analyzing the normalized signals, we observed similar levels of Collagen VI for our DMSO (lanes 1-2) and GW (lanes 5-6) samples, but a lot more of Collagen XII in the GW samples compared to the DMSO samples. In addition the lanes that were given AHA alongside DMSO (lanes 3-4) or GW (7-8) had similar normalized signal compared to the samples that were not given AHA.

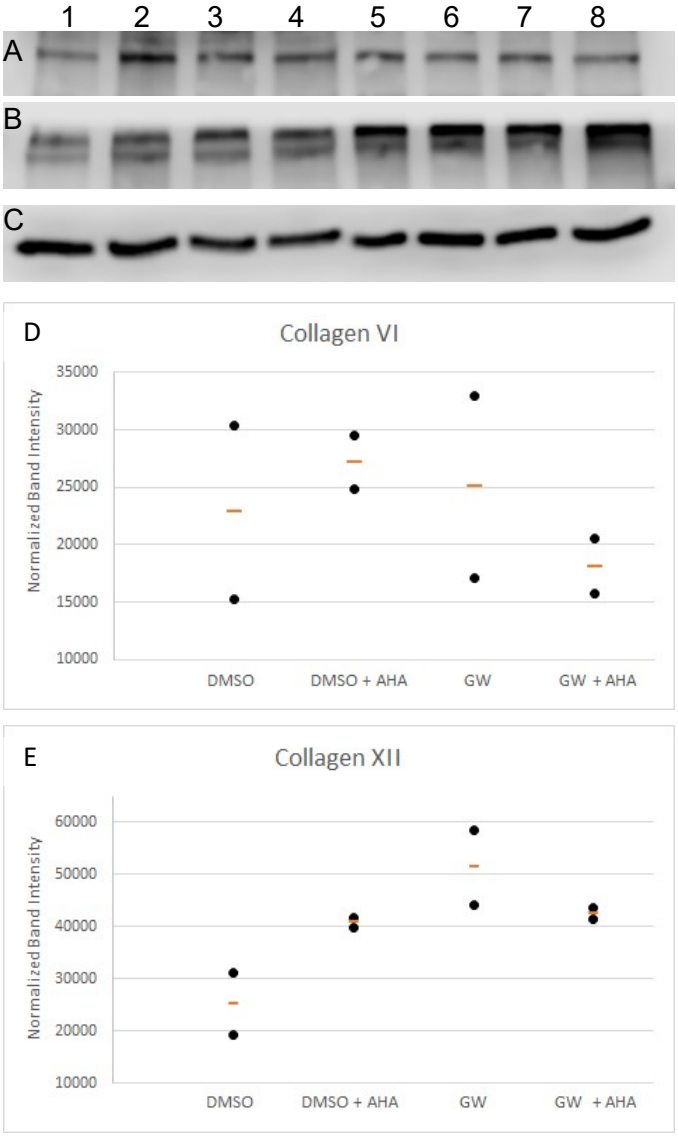

Figure 2: *Western blot protein quantification bands for proteins of interest: Collagen VI* ***(A)*** *and Collagen XII* ***(B)****, and for our housekeeping protein: GAPDH* ***(C)****. Our normalized signal intensity is shown for Collagen VI* ***(D)*** *and Collagen XII* ***(E)*** *with the horizontal line depicting the mean value for each protein.*

Our data shows that GW treatment increased the expression of Collagen XII, agreeing with previous publications[6] with hMSCs treated with GW. Interestingly, Collagen VI expression did not seem to improve with GW treatment. However, it should be mentioned that when we ran the electrophoresis, the same amount of protein was added to each lane. In contrast, our BCA assay results showed that GW treatment resulted in a three-fold increase in the protein content of the ECM. Thus, we hypothesize that the total amount of Collagen VI in the ECM would still be higher in the GW treated samples.

AHA (methionine analog) enables the fluorescent labeling of extracellular nascent proteins

For the present work, we opted to label our anabolic bone ECM with a bio-orthogonal methionine analog: L-azidohomoalanine (AHA). This methionine analog is structurally and functionally similar to the original methionine amino acid, however it includes an azide functional group. Following the AHA culture preparation, we extracted the proteins and identified the incorporated AHA by clicking the azides with an alkyne linked to a fluorescent tag (Alexa 488-DBCO, Click Chemistry Tool) through a sterically hindered azide-alkyne click chemistry reaction. The samples were then resolved on an SDS-PAGE gel and imaged at the appropriate wavelength. This allowed us to detect whether the AHA was incorporated into the ECM. After imaging, we were able to see the level of fluorescence in each lane (Figure 3A).

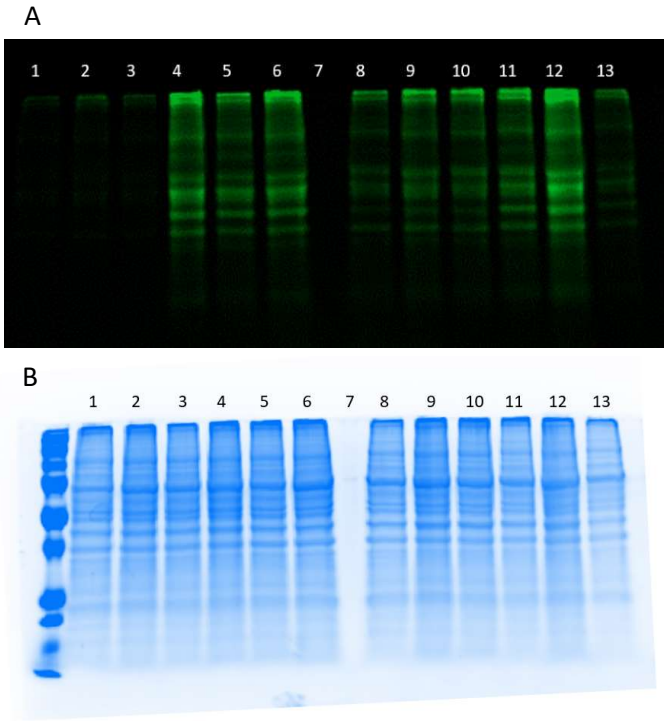

***Figure 3**: The proteins were imaged for fluorescence to determine if AHA was able to covalently label nascent proteins in order to visualize ECM deposition **(A).** After proteins underwent immunoblotting, the gel was then stained with Coomassie Blue to detect the protein bands and confirm all lanes were loaded equally **(B)**.*

We confirmed fluorescence due to AHA click chemistry reactions in the DMSO + AHA (lanes 4-6) and the GW + AHA lanes (lanes 11-13). However, we also observed similar fluorescence intensity in the lanes containing proteins from iP-hMSCs given GW without AHA (lanes 8-10). When cross-referencing our in- gel fluorescence with Coomassie blue staining, it became clear that lack of fluorescence in the DMSO lanes was not due an unequal protein loading. However, it does seem our washing protocol was not sufficient in removing unconjugated fluorescent 488-DBCO dye from the GW samples. A possible explanation could be that the GW proteins are better at retaining the 488-DBCO dye. This can only be confirmed with more trials which were not able to occur due to time shortage. However, the DMSO + AHA and the GW + AHA lanes retained the dye very well.

CONLUSION AND FUTURE DIRECTION

Typical designs for tissue regeneration biomaterials are focused on providing the appropriate ECM analog to mimic the native tissue within the human body compositionally, structurally, and mechanically. By using an iP-hMSC derived anabolic bone ECM, we are able to work towards mimicking an environment of regenerating bone. The ultimate goal would be to covalently tether the anabolic bone ECM to a hydrogel scaffold without negatively affecting its osteoinductive properties. In this work, we have begun to apply metabolic labeling, a technique that has been primarily used to visualize proteins, to develop a novel biomaterial. A simple interpretation of these results leads to the conclusion that GW treatment provides an avenue to stimulate iP-hMSCs to deposit higher levels of ECM than DMSO. More importantly, our AHA methionine analog can be effectively incorporated into the anabolic bone ECM.

Although we have confirmed that AHA does not affect the amount of proteins our iP-hMSCs produce or its composition (Collagen VI and Collagen XII), we still have to verify that the AHA does not interfere with normal anabolic bone ECM function. We must make sure that hMSCs seeded onto the decellularized, purified AHA-containing anabolic bone ECM experience the same osteoinductive effects observed from a standard anabolic bone ECM. This can be achieved by analyzing the expression of ALP and OPG, both of which are important bone markers that are upregulated when cultured on this ECM.

Future work could also focus on using an alanine substitute instead of methionine substitute. The alanine amino acid was found to be present at a much higher percentage in the anabolic bone ECM[10] and therefore might be a more suitable candidate to use in click chemistry applications.

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