OPTIMIZATION OF MBV ISOLATION THROUGH MATRIX DISSOCIATION

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INTRODUCTION
Decellularized extracellular matrix (ECM) has been shown to promote a regenerative response when administered at the site of a wound [1]. ECM is known to recruit perivascular stem cells, polarize macrophages in an anti-inflammatory manner, and increase collagen deposition at the site of implantation. However, little is known about the mechanisms or specific components of ECM that facilitate this response. Recently, a new bioactive component of ECM was discovered, termed membrane-bound vesicles (MBVs) [2]. MBVs are nanovesicles that are embedded within the collagen fibers of the matrix. Interestingly, they have only been able to be isolated following digestion of ECM by a protease enzyme, followed by ultracentrifugation. MBVs have exhibited the ability to mimic the regenerative response produced by unaltered ECM. If MBVs are indeed the mechanism by which ECM acquires its renowned regenerative properties, isolating MBVs in their purest form could be of great use therapeutically and for the advancement of basic concepts of regenerative medicine.

OBJECTIVE
This study aims to devise a method of isolating MBVs in their purest, most undamaged form, while maintaining high vesicle yields. Previously, a procedure for isolating MBVs has been described [] that is effective for isolating MBVs, but introduces large amounts of proteolytic enzymes and protein molecules that are not associated with MBVs to the isolation solution. If MBVs are to be utilized as a therapeutic regenerative treatment, it is not ideal for large amounts of a protease to be administered along with an MBV dose to a patient. The MBVs isolated by this procedure are difficult to characterize, because the exposure to a proteolytic enzyme, while cleaving collagen fibers in order to release MBVs into solution, likely cleaves surface proteins that can be used to characterize these vesicles. As little is known about the origins of these ECM components, it is of great importance to determine whether these vesicles are a distinct type of exosome intended deposited into ECM or another class of vesicle entirely.

HYPOTHESIS/SUCCESS CRITERIA
The method mentioned previously for MBV isolation called for the use of collagenase for use as the proteolytic enzyme in order to cleave only the collagen fibers to which the MBVs are bound. This enzyme, while effective in cleaving the fibers, contains unspecific proteases in its enzyme mixture as well as a significant amount of background RNA. We reason that if a “dissociating agent” can be utilized in place of an enzymatic digestion, or in conjunction with very small amounts of enzyme, there is a better chance that surface markers of MBVs will be maintained, and high yields of MBVs are possible with a higher purity of sample. We also hypothesize that since MBVs are similar in many ways to exosomes, isolation techniques that improve exosome yields may also lead to higher MBV yields. We will quantify yield of MBVs by direct correlation with amount of RNA present in the sample that is resistant to RNase. If RNA is able to be isolated from a sample exposed to RNase, it must be protected from contact in some manner, in this case the vesicle lipid bilayer. A high concentration of RNA correlates with higher yields of MBVs.

METHODS

A. Ionic Salts + Enzymatic Digestion
Urinary bladders were collected from market weight adult pigs and decellularized by mechanical scraping, leaving the lamina propria and basal membrane intact. The bladders were lyophilized as sheets and ground into a fine powder.
125 mg of urinary bladder matrix (UBM) powder was suspended in 10 mL of phosphate buffered saline (PBS) and exposed to the enzyme AOF collagenase C, a type of collagenase with very few unspecific proteases in its enzyme mixture. Also, AOF collagenase type C was derived from a non-animal source, thereby reducing the amount of background RNA it added to the sample. After investigating promising exosome isolation techniques [3], we decided that we would add varying molarities of potassium chloride, an ionic salt, to the digest. KCl was added for molarities of 0.2M, 0.4M, 1M, and 2M. The digest with added KCL was incubated for 30 minutes at 37 °C with agitation. The digest was then incubated for 30 minutes at 4°C with agitation in order to stop the collagenase reaction. The digest was subjected to a centrifugation spin of 12000 x g for 45 minutes. The pellet was discarded, and the supernatant was subjected to ultracentrifugation at 100000 x g for 130 minutes. The barely visible pellet from ultracentrifugation contains the isolated MBVs. The MBV samples were exposed to 5uL of RNase and incubated at 37° C for 10 minutes.

B. Ionic Salt Only
This experiment was conducted in a very similar manner as the experimental setup outlined above, with the difference between the two being the omission of enzymatic digestion from the sample. 0.2M KCl was added to pure UBM powder, incubated at 37°C for 30 minutes with agitation, and then incubated at 4°C for 30 minutes with agitation. The same centrifugation procedure was followed, with a spin at 12000 x g for 45 minutes and an ultracentrifugation at 100000 x g for 130 minutes.

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minutes. The MBV samples were exposed to 5uL of RNase and incubated at 37°C for 10 minutes.

C. RNA Isolation
RNA isolation was conducted by following the guidelines of the EXIQON miRCURY kit. RNA concentration was quantified using NanoDrop.

RESULTS
Potassium chloride showed the ability to increase yield of MBVs harvested from a single isolation. As seen in Figure 1, addition of KCl for a molarity of 0.2M doubled the yield of MBVs that a digest with no KCl produced (0M KCl denotes standard protocol). Although the trial was only an n of 1, we saw increases in MBV yield across all molarities of KCl. The AOF collagenase, introduced very low levels of background RNA, conclusively showing the presence of isolated RNA resistant to RNase.

![Graph](image)

Figure 1. Yield of MBVs, quantified by RNA concentration. All molarities of KCl increased yield, with the largest increase at a concentration of 0.2M. n=1

These results were encouraging, so an experiment in which only the “dissociating agent,” KCl, was introduced to the UBM suspension. The experiment had an n of 3 and used only 0.2M as the concentration of KCl. As shown in Figure 2, KCl exhibited the ability to liberate MBVs from the matrix without digestion by a proteolytic enzyme. Furthermore, it did so while producing a higher MBV yield than the standard protocol produced.

![Graph](image)

Figure 2. Yield of MBVs, quantified by RNA concentration. No enzymatic digest was conducted. Addition of solely KCl resulted in liberation of MBVs from UBM powder. n=3.

DISCUSSION
These results mark the first time that MBVs have been successfully isolated from ECM without digestion of the ECM by a proteolytic enzyme. This is very exciting, because these results open the door for MBVs to be characterized as a specific type of exosome or something else entirely. Since no proteolytic enzyme was introduced into the isolation solution, it is reasonable to assume the surface markers of the MBVs remain intact. Also, these MBVs are very likely suitable for injection in an in vivo model because no protease will be injected into the subject. We believe that these isolated MBVs are in the purest form in which they are able to be isolated. Moving forward, these MBVs must be subjected to Western Blot analysis for common exosome markers, as well as utilized in in vivo animal models to confirm the recapitulation of the regenerative response seen with the implantation of a UBM sheet or injection of a hydrogel. It is not currently known how MBVs bind to the matrix collagen fibers, but from these results we may speculate that the binding complex contains very charge-sensitive binding sites. The ionic salt dissociation method of isolating MBVs produces MBVs in high yields that have the potential to be characterized for further knowledge in the field of regenerative medicine, as well as the potential for implementation as an in vivo therapeutic regenerative treatment.

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REFERENCES
