

ABSTRACT

Title of Document: EFFECTS OF METABOLISM ON
MESENCHYMAL STEM CELL-
DERIVED EXTRACELLULAR
VESICLES
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Mesenchymal stem cells (MSCs) are under investigation for a wide variety of therapeutic applications. It has been determined that paracrine secretions are responsible for a significant portion of MSC bioactivity. Among these secretions, extracellular vesicles (EVs) have been discovered to have therapeutic potential. EVs have many applications, such as reduction of myocardial injury, wound repair, and promotion of angiogenesis. A recent spark in MSC- derived EV interest stems from the potential advantages they have over MSC transplantation. EVs are considered more stable, have a well-defined clearance pathway *in vivo*, and pose less safety risks due to their inability to differentiate. There is increasing interest in MSC-derived EVs and their potential clinical application, however, there are many barriers to realistic, widespread EV-based therapy. One of the more prevalent issues is a lack of knowledge behind the impact of various cell culture parameters, which have been shown to affect both MSCs and the EVs they produce. Specifically, evidence shows that cell culture conditions impact metabolic pathways, which provide important signals that contribute to MSC behavior and function. However, there have been no specific studies on the potential impact of MSC metabolism on EVs. My overarching hypothesis is that MSC EV production and function are dependent on the metabolic state of the parent MSCs. To test this hypothesis, two key metabolic pathways, glycolysis and oxidative phosphorylation, were inhibited to examine their effects on EV production. Metabolic effects on MSC EV bioactivity were also assessed using gap closure assays. Experiments were performed in both 2D and spheroid culture to assess continuity of results between platforms. In 2D culture, metabolic pathway inhibition did not impact MSC EV production capacity but did decrease EV bioactivity. In microcavity-well culture plates, metabolic pathway inhibition decreased both MSC EV production capacity and EV bioactivity. These results indicate it is very likely that metabolism plays a mechanistic role in MSC EV production and bioactivity. Further studies are required to conclusively determine if metabolism impacts MSC EV production capacity, and they are also needed to better understand what causes metabolism to affect MSC EV bioactivity.

EFFECTS OF METABOLISM ON MESENCHYMAL STEM CELL-DERIVED
EXTRACELLULAR VESICLES

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of a
Master of Science
2020

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Dedication

To Tyler, you are my rock. Thank you for always being there for me. I could truly never repay you.

To my Dad, you taught me the value of passion and hard work. Your guidance and support has helped me both in this process and in life.

To my Mom, whose optimism and grace is unparalleled. You taught me to never give up, and always see the bright side of life. I will always be thankful all you have given me.

Acknowledgements

Thank you to...

My advisor, Dr. Steven Jay. Your guidance helped me to develop a project that I was incredibly interested in, and it broadened my horizons by introducing me to many new concepts and ideas. I appreciate your counsel not only with my research but also with my plans to find work in the industry. Thank you so much for all your help!

My lab mates: Stephanie, Louis, Eli, Dan, and Dipankar. I appreciate your input during lab meeting, willingness to answer any of my questions, and flexibility toward the end of my time in lab. You all made me experience as a Masters so positive, and I wish everyone the best as you continue your research.

Lab alumna, Divya Patel: Your previous work is what led to the development of my project, and I will forever be grateful to you for paving the way. I appreciate your willingness to train and help me learn when I first joined the lab, as well as the kindness you showed me. Thank you so much!

Corning: Thank you for providing the microcavity-culture plates used in this project. They allowed me to investigate metabolism effects in a different culture system, which proved to be incredibly valuable.

My committee members: Dr. Steven Jay, Dr. Alisa Clyne, and Dr. Katharina Maisel. Thank you very much for accepting a position on my committee, and thank you for agreeing to evaluate my research and results. I truly appreciate your contributions and insight, and I will use your input to improve this body of work.

My mother. Your lifelong battle with cancer is what motivated me to enter this field and try to make a difference. You will never know how much your experiences have given me direction and purpose. I will forever be thankful.

My family and friends. Thank you so much to those that helped me throughout this process. Your support and encouragement is what enabled me to complete this degree; I will remember it always.

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Chapter 1: Introduction

1.1 Motivation

Mesenchymal stem cells (MSCs) are under investigation for a wide variety of therapeutic applications as evidenced by hundreds of current clinical trials. Recently, a consensus has merged suggesting that paracrine secretions are responsible for a significant portion of MSC bioactivity.¹ Among these secretions, which include chemokines and cytokines,² extracellular vesicles (EVs) have more recently been discovered to have therapeutic potential. EVs are bioactive components secreted by MSCs,³ and they have been shown to have many applications, such as reduction of myocardial injury,⁴ wound repair, promotion of angiogenesis,⁵ and so on. In addition, the therapeutic use of MSC EVs has potential advantages over MSC transplantation. EVs are considered more stable than MSCs, while also having a well-defined clearance pathway *in vivo*. EVs also pose less safety risks because of their inability to differentiate or divide. Because of these factors, there is increasing interest in MSC-derived EVs and their potential clinical application.

Despite the potential of EVs, there are many barriers to realistic, widespread EV-based therapy development and use. One of the more prevalent issues is the lack of an established, standardized, and scalable biomanufacturing process. Currently, the common cell culture parameters used for EV biomanufacturing follow conventional methods, which were designed prior to the knowledge of EVs. It would be hugely beneficial to tailor cell culture toward optimal EV production conditions, which would enable higher yield and greater manufacturing efficiency. However, this is not currently possible due to lack of knowledge behind the cell culture parameters and mechanistic factors that drive

MSC EV production. To make therapeutic EV use a reality, we must better understand EVs and the factors that drive their production.

1.2 Mesenchymal Stem Cells

Stem cells have various abilities such as self-renewal, capacity to differentiate into various lineages, and proliferation potential. There are two main types of pluripotent stem cells: embryonic (ESCs) and induced-pluripotent stem cells (iPSCs). ESCs are pluripotent stem cells isolated from the inner cell mass of human or mouse fibroblasts, and they have the potential to differentiate into three germ lines.¹⁵ ESCs originated prior to iPSCs and generated a lot of interest due to their vast therapeutic potential, but they have restrictions use due to ethical conflicts. This leads to the generation and use of iPSCs. iPSCs are produced from human fibroblasts through the overexpression of the transcription factors Oct4/3 (octamer binding transcription factor 4/3), Sox2 (sex determining region Y), Klf4 (kruppel-like factor 4), and c-Myc (Avian Myelocytomatosis virus oncogene cellular homologue),¹⁶ and, at the cellular level, they are almost completely like ESCs in terms of function. iPSCs avoid the ethical dilemma present with ESC use but may lack in function due to less genomic stability.

Stem cells, as stated, can differentiate into many lineages and have therapeutic potential. Specifically, MSCs, which can be produced from either ESCs or iPSCs, have been of recent interest due to their regenerative abilities. For instance, MSCs have been shown to facilitate the recovery of tissue injury in organs such as the heart, liver, and pancreas.¹⁷ MSCs are multipotent stromal cells, likely originating from the mesoderm, with the ability to differentiate into specialized cell lines such as osteoblasts and chondrocytes. A significant portion of MSC bioactivity stems from the cell's paracrine

secretions, which are thought to be a major source of tissue repair. This mode of healing can enhance cell viability and proliferation, reduce apoptosis, and regulate immune response. Among these paracrine secretions are growth factors, hormones, cytokines, and extracellular vesicles.⁴¹ MSCs are self-renewable, easily accessible, and able to be expanded culturally *in vitro*.¹⁸ It is important to note that MSCs have high genomic stability and face very few ethical conflicts, making them good candidates for clinical translation and application.

1.3 Mesenchymal Stem Cell Metabolism Effects

Many different mechanisms contribute to the self-renewal and differentiation potency of MSCs. Current evidence has demonstrated that, in addition to growth factors and extracellular matrix, metabolic pathways provide important signals that contribute to the optimization of MSC behavior and function.¹⁹ It has been shown that metabolic profile distinguishes the differentiated and undifferentiated state, with a changing mitochondrial morphology. There is a shift between glycolysis and oxidative phosphorylation that is linked to the cell's pluripotent state²⁰ (Figure 1). Glycolysis utilizes glucose to produce pyruvate and adenosine triphosphate (ATP), and oxidative phosphorylation functions under the TCA cycle. Pyruvate enters the TCA cycle initiating oxidative phosphorylation, which produces a higher ATP yield than glycolysis.²¹

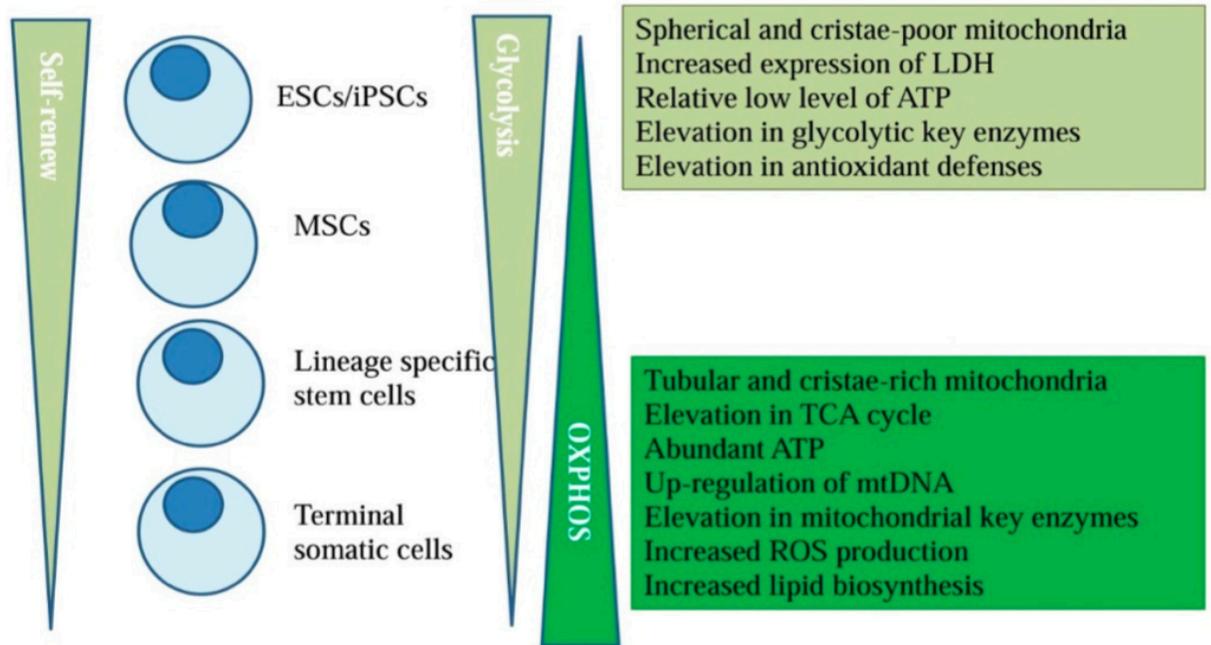


Figure 1. Metabolic pathways may provide crucial signals that contribute to the direction of pluripotency and self-renewal of stem cells. Glucose and oxidative phosphorylation have been shown to play a role in this process. Image adapted from Hu et al. 2015.²⁴

In undifferentiated MSCs, mitochondrial activity is low and glycolytic activity is high.²²

During the early stages of differentiation, MSC fate is directed through the downregulation of genes relating to pluripotency, upregulation of terminal-specific genes, and alteration of relevant metabolic enzymes.²³ This information shows that metabolism is likely to play a role in MSC self-renewal, differentiation, and overall function.

1.4 Extracellular Vesicles

All cells are capable of secreting different types of membrane vesicles, known as EVs. This process was conserved throughout evolution, and it occurs in bacteria, plants, and humans.⁶ The secretion of EVs was initially thought to be a means of eliminating waste, but it is now known that EVs have the capacity to exchange components between cells. Components exchanged vary from nucleic acids, lipids, proteins, and much more.

EVs are also able to act as signaling vehicles in homeostatic processes or pathological development.⁷

The term EVs is a blanketed description for the secreted materials, but the membrane vesicles produced are not that straightforward. The population is highly heterogeneous (Figure 2), with each different vesicle having its own characterization, function, and array of properties. This discovery allows for EVs to be classified into two types: exosomes and microvesicles.⁸

| | Exosomes | Microvesicles |
|--|--|---|
| Origin | Endosome | Plasma membrane |
| Size | 50–150 nm | 50–500 nm (up to 1 μm) |
| Other names (according to their origin, size and morphology) | <ul style="list-style-type: none"> • Protasomes • Tolerosomes • Dexosomes • Nanovesicles • Exosome-like vesicles and others | <ul style="list-style-type: none"> • Microparticles • Blebbing vesicles • Shedding vesicles • Oncosomes • ARRs • Migrasomes • Neurospheres • Apoptotic bodies |

Figure 2. Extracellular vesicles encompass a heterogeneous population produced by the cell. The different vesicles have varying origins, sizes, and morphology. This allowed for two classes of EVs to be identified. Image adapted from Niel et al. 2018.⁸

Exosomes are intraluminal vesicles (ILVs) formed through the inward budding of the membrane of multivesicular endosomes (MVEs) then release when there is fusion between MVEs and the plasma membrane (Figure 3). Their average diameter is reported to range from 50 to 150 nm. Exosomes are secreted from a variety of cell types, such as B lymphocytes⁹ and dendritic cells,¹⁰ and they have been shown to have potential involvement in immune regulation and intracellular communication.¹¹ Microvesicles are subcellular materials generated by the outward budding of the plasma membrane and release to the extracellular space¹² (Figure 3). They are known for their involvement with blood coagulation¹³ but also have a role in cell-cell communication in many cell types,

such as oncosomes.¹⁴ It is important to note the understanding of these processes is still questionable as EV biogenesis pathways may differ across cell types. Also, EV characterization is constantly changing and evolving as more knowledge is gained on the topic.

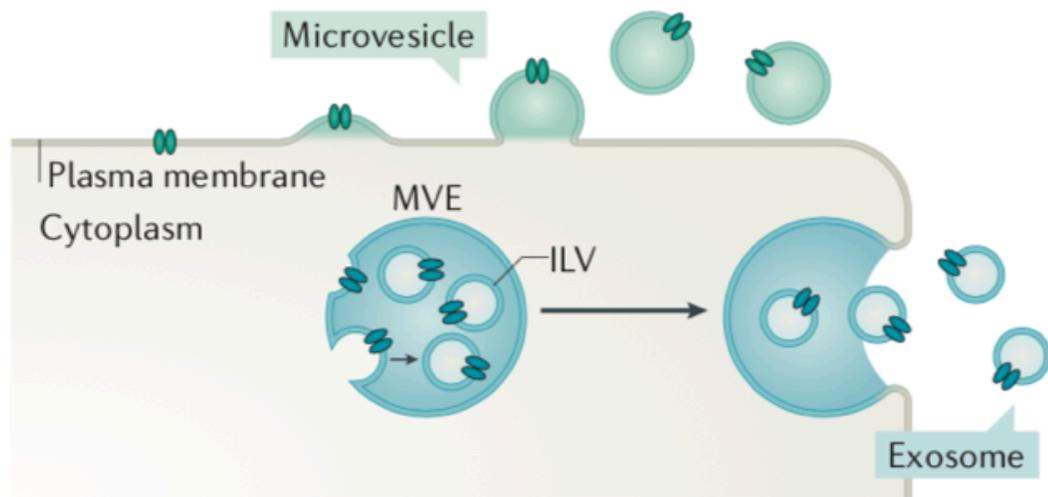


Figure 3. Extracellular vesicles form in two ways. First, they are formed through budding within the lumen of MVEs. MVEs then fuse with the plasma membrane to release the ILVs called exosomes. Second, there is outward budding of the plasma membrane, which leads to the formation of microvesicles. Image adapted from Niel et al. 2018.⁸

Mechanistic details of EV biogenesis have only recently been specified.

Compared to microvesicles, mechanisms of exosome biogenesis are well understood.

This allows them to be more easily manipulated and studied for therapeutic potential.

Aside from exosomes and microvesicles, many EVs do not support therapeutic interests, such as shedding vesicles and apoptotic bodies. For these reasons, exosomes are of most interest and are often used in clinical studies and applications over other EVs.

Although some EV biogenesis is well-defined, the mechanisms contributing to the efficiency of their release is still vague. When culturing MSCs for EV production, there is no optimal cell culture standard currently in place. This is mainly due to lack of

knowledge surrounding the specific mechanisms and cell culture parameters that contribute to MSC processes. For example, initial seeding density is a parameter of recent interest because it has been shown to effect MSC behavior, including EV production abilities. Studies have shown that when using a lower MSC seeding density (1E2 cells/cm²), there is higher EV production.²⁵ This behavior is not limited to MSCs. Human dermal microvascular endothelial cells (HDMECs), human embryonic kidney 293T (HEK) cells, and human umbilical vein endothelial cells (HUVECs) have also followed the trend of lower seeding density leading to greater EV production.²⁵ Although there is a clear relationship between seeding density and MSC EV production, the mechanisms behind this are not currently understood. It is hypothesized that MSC metabolism is the cause of this trend, as studies have shown lower seeding densities are associated with increased metabolic rates in MSCs.²⁷ Metabolism may be the mechanism behind many MSC behaviors, particularly pertaining to EV production and function. However, there is currently no literature confirming this. This lack of understanding prevents the optimization of MSCs in culture for EV biomanufacturing purposes, which is crucial for the potential production and clinical translation of EVs.

1.5 Three-Dimensional Cell Culture

Two-dimensional (2D) cell culture has been normal practice for many years. However, three-dimensional (3D) culture is growing in popularity, as it allows a realistic microenvironment for the cells to grow. 3D cultures allow cells to grow in all directions, surround one another, and simulate *in vivo* conditions. The addition of a dimension creates more realistic spatial organization and physical constraints, which can affect both intracellular and extracellular signaling.³⁸ Many groups have found that cells cultured in a

3D environment differ in morphology and physiology than those cultured in a 2D environment.^{33,34} Because of the *in vivo* like microenvironment, 3D culture provides more physiologically accurate and predictive cell behavior and data than that of 2D culture, which has been shown in various studies.^{35,36}

3D culture plates and scaffolds can mimic the extracellular matrix of 3D tissues and have controlled pore size and interconnectivity, making the environment more realistic.²⁸ For the current development of 3D culture plates, the hanging drop method is the most common in manufacturing. This method uses spontaneous aggregation of cells on a drop of media and gravity to create 3D spheroids.²⁹ However, this method is labor intensive and not realistically scalable, so other 3D cell culture methods are being explored. Microwell-containing well plates have been shown to be cost-effective, high throughput, and flexible in terms of size. Multiple groups have created sufficient microwells on culture plates and have found success using them for 3D culture.^{30,31} For example, 3D spheroids of MSCs have been successfully formed using a microwell-containing culture plate as well. MSCs aggregated and formed spheroids in 24 hours; they maintained this morphology at 48 hours (Figure 4).³²

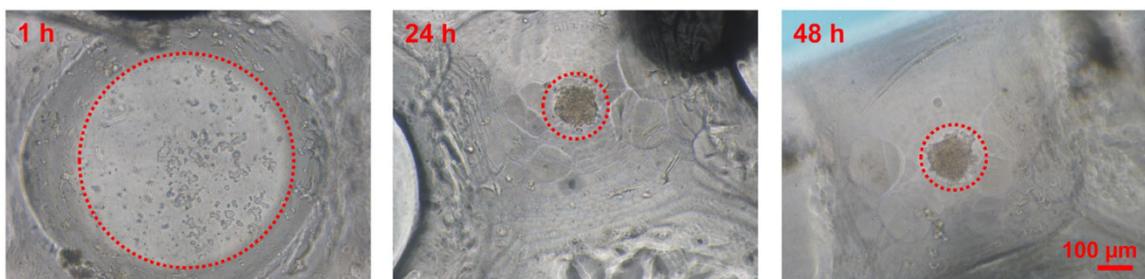


Figure 4. Mesenchymal stem cells form spheroids in a 3-dimensional microwell culture plate. At initial seeding, the cells sat at the bottom of the microwell in a monolayer. After 24 hours, the cells migrated to form a spheroid. This shape was maintained after 48 hours. Image adapted from Lee et al. 2019.³²

Mesenchymal stem cells cultured in a 3D space have been studied by various groups, and this culture medium has been shown to have both effects on cell behavior and benefits. Compared to 2D culture, 3D culture conditions promote MSC self-renewal, differentiation, and upregulation of paracrine signaling.³⁹ Ong SM, et al. demonstrated this when culturing MSCs three-dimensionally using a microfluidic system consisting of polymeric linkers and micro-fabricated pillar arrays. They were able to achieve total cell viability and 3D morphology using this culture method. Interestingly, they also saw improved cell functionality over those in 2D culture, and the MSCs exhibited the capability to differentiate.³⁷ In addition to improved cell behavior and functionality, 3D-cultured MSCs have improved therapeutic potential. It is thought MSC spheroids have increased survival after transplantation due to the enhanced angiogenic and anti-inflammatory properties that come with the 3D culture approach, as well as increased stemness.⁴⁰ The 3D culture of MSCs has a multitude of effects, as evidenced, but there is very little literature examining its impact on MSC EV production and behavior. The microenvironment of MSC culture is incredibly variable based on the purpose of culture, whether it be for small-scale culture and study or large-scale production and manufacturing. The likelihood of variations in MSC culture technique is high when the potential for a scalable culture format is considered. Therefore, it is important to understand the differences between MSC and MSC spheroid-produced EVs. For MSC EV therapeutic translation and use to become a reality, there must be a greater awareness of how MSC culture can affect EV production and function.

1.6 Objectives

Metabolism affects various aspects of MSC activity and behavior, such as pluripotency and self-renewal. However, there is no literature examining the relationship between metabolism and MSC EVs. My overarching hypothesis is that metabolism plays a role in MSC EV production and bioactivity. I will examine this relationship by focusing on two key metabolic pathways, glycolysis and oxidative phosphorylation (OXPHOS), as these have been shown to impact MSC function and behavior.²⁴ These pathways will be inhibited in MSCs, and the effects of this in EVs will be observed. Previous studies have shown that MSC proliferation increases when glycolysis is favored over oxidative phosphorylation.²² Therefore, we also hypothesize one pathway will have more of an impact on MSCs than the other, namely, glycolysis inhibition will more greatly affect EVs than OXPHOS inhibition.

To assess the effects of glycolysis and OXPHOS, I will inhibit these pathways chemically *in vitro*. MSC EV production capacity will be assessed using nanoparticle tracking analysis (NTA). EV bioactivity will be evaluated using a gap closure assay. Experiments will be performed in both 2D and spheroid culture to determine the similarities and differences between results from the two microenvironments.

Chapter 2: Materials and Methods

2.1 Two-Dimensional Cell Culture

Bone marrow derived MSCs were obtained from ATTC (PCS-500-012). Tissue culture polystyrene flasks were used. Cells were cultured in Dulbecco's Modification of Eagles Medium (DMEM) with 10% fetal bovine serum (FBS), 1% Penn Strep (P/S), and 1% non-essential amino acids (NEAA) added. The passage number used for experiments was 3 and 4, as it is shown that bioactivity does not vary between these passage numbers.²⁵ When preparing for EV collection, EV-Depleted medium was used. Here, FBS was EV-Depleted by ultracentrifugation for 16 hours, then added to DMEM along with 1% P/S and 1% NEAA. This is referred to as "EV-Depleted medium."

Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (C-12203). Tissue culture polystyrene flasks were coated with 0.1% gelatin at 37°C for 1 hour. Once the flask was coated, the HUVECs, at passage number 3, were seeded. Cells were cultured using the Endothelial Cell Growth Medium GM MV 2 kit (PromoCell: C-39221), where all supplements added are provided and amounts are specified.

2.2 Microcavity-Well Plate Cell Culture

Bone marrow derived MSCs were obtained from ATTC (PCS-500-012). MSCs were cultured in Corning Prototype Ultra Low Attachment (ULA) microcavity 6-well plate inserts. Cells were cultured in DMEM with 10% FBS, 1% P/S, and 1% NEAA. All MSCs used for spheroid formation were at passage number 4.

To obtain spheroid formation, 500 MSCs were seeded in each microcavity (Figure 5a). Because the culture flasks and well plate-inserts were ULA, extreme care was taken

during handling prevent spheroid detachment and loss. The angle of the vessel during media collection and exchange was kept to a minimum ($\sim 10^\circ$), and all actions were gentle. The timeline for MSC seeding and media collection and replacement can be seen in Figure 5b.

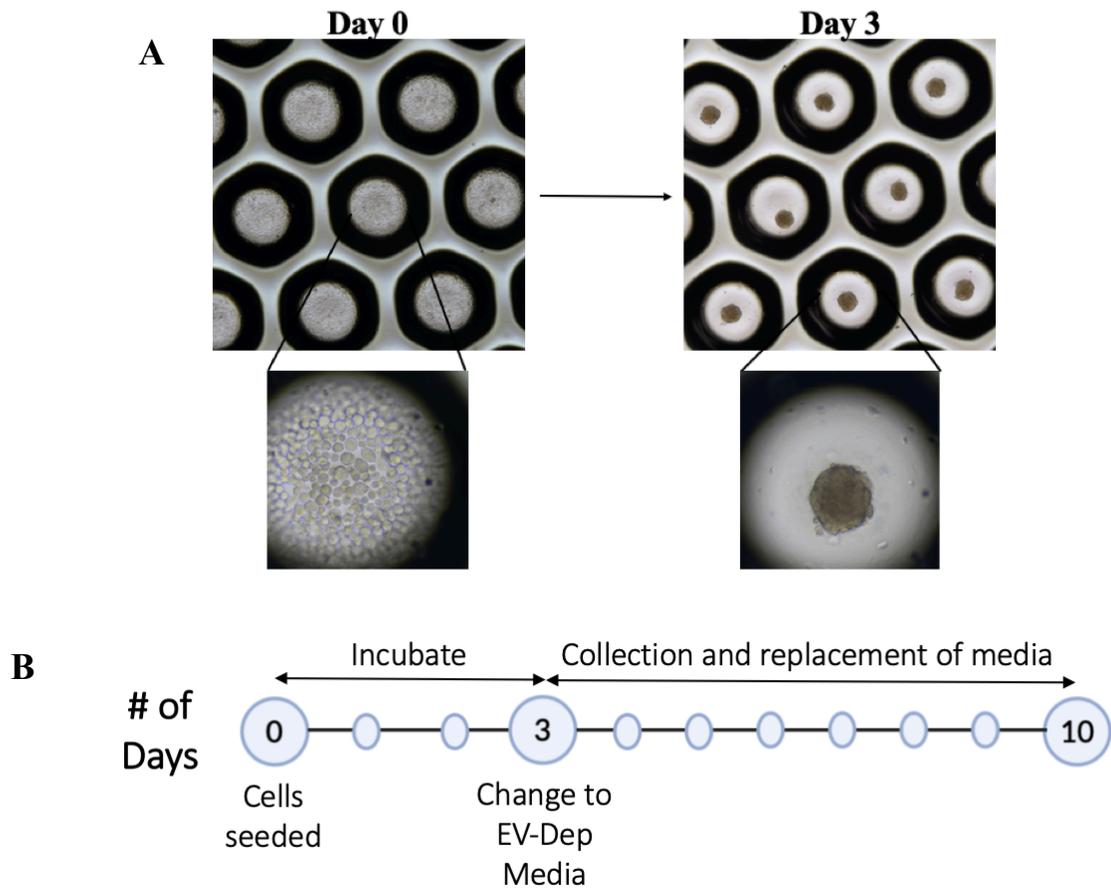


Figure 5. Timeline used during microcavity-well MSC culture. MSCs were seeded, then allowed to incubate, undisturbed for 3 days to allow spheroid formation. On day 3, the medium is changed to EV-depleted medium. After 24 hours, the medium is collected for EV isolation and replaced with EV-depleted medium. This process continues until day 10, where the spheroids are then discarded.

2.3 Metabolic Pathway Inhibition

During pathway inhibition, MSCs were cultured in Glucose-Free DMEM obtained from Sigma-Aldrich (D5030-10X1L) in the form of a powder. The powder was resuspended in one liter of deionized water, and 3.7 grams of Sodium Bicarbonate (NaHCO_3) was added for each liter of media. The solution was then filtered for sterility. Prior to use in culture, 10% EV-Depleted FBS (obtained via 16 hours ultracentrifugation of FBS), 1% P/S, and 1% NEAA were added to the media. This formulation was used for all experiments where metabolism was inhibited.

Two metabolic pathways of MSCs were inhibited: glycolysis and oxidative phosphorylation (OXPHOS). Glycolysis was inhibited using 2-Deoxy-D-Glucose obtained from TCI Chemicals (D0051-1G). Deionized water was used for resuspension. OXPHOS was inhibited using Antimycin-A obtained from BioVision (2247-50). 95% Ethanol was used for resuspension.

24 hours prior to EV isolation, MSC culture medium was changed with Glucose-Free, EV-Depleted DMEM with either 5mM 2-Deoxy-D-Glucose or 1 μ M Antimycin-A added. These concentrations were chosen based on preliminary results, which can be found in Chapter 3. MSCs incubated at 37°C, 5% CO₂ for 24 hours, when medium was collected for EV isolation.

2.4 EV Collection

For the 2D EV production capacity experiments, MSCs were seeded at 100cells/cm² into T-25 tissue culture flasks using EV-rich medium. 24 hours later, the medium was aspirated and disposed. MSCs were washed twice with 1X PBS to ensure complete removal of EVs from the previous medium. 5mL of EV-Depleted medium was

then added. When either metabolic pathway was inhibited, 5mL of Glucose-Free, EV-Depleted medium was added with either 5mM 2-Deoxy-D-Glucose or 1 μ M Antimycin-A included. After 24 hours, the medium was collected for EV isolation. The flasks were then trypsinized and cells were counted to develop an EV/cell measure. 4mL of trypsin was added to completely cover all cells, and the flasks were incubated for 5 minutes at 37°C. Cells were collected and counted using a Hemocytometer.

For the MSC spheroid EV production capacity experiments, MSCs were seeded into microcavity 6-well plate inserts at 500 cells per microcavity. MSCs incubated at 37°C, 5% CO₂ for 48 hours to allow for spheroid formation. Once spheroids formed, MSCs were washed twice with 1X PBS and 2mL of EV-Depleted medium was added to each well. Like in the 2D experiment, Glucose-Free, EV-Depleted medium with either 5mM 2-Deoxy-D-Glucose or 1 μ M Antimycin-A was used for metabolic pathway inhibition. After 24 hours, the medium was collected for EV isolation. The well plates were trypsinized to determine cell viability and count for an EV/spheroid measure. 1mL of trypsin was added to each well, and the plates were incubated for 5 minutes at 37°C. Cells were collected and counted using a Hemocytometer.

For 2D EV bioactivity experiments, MSCs were seeded into a T-175 tissue culture flasks with EV-rich medium for a cell count of 250,000. 24 hours later, the medium was aspirated, cells were washed twice with 1X PBS, and 25mL of EV-Depleted medium was added. When a metabolic pathway was inhibited, 25mL of Glucose-Free, EV-Depleted medium with either 5mM 2-Deoxy-D-Glucose or 1 μ M Antimycin-A was added. After 24 hours, the medium was collected for EV isolation. Again, the cells were washed twice and the respective EV-Depleted medium was added. This cycle continues

until the MSCs reached confluency. This enabled enough EVs to be obtained for each bioactivity assay. Once confluent, flasks were then trypsinized and cells were counted to develop an EV/cell measure.

For MSC spheroid-derived EV bioactivity experiments, MSCs were seeded into microcavity 6-well plate inserts at 500 cells per microcavity and incubated for 48 hours to allow spheroid formation. After 48 hours, the MSCs were washed twice with 1X PBS and 2mL EV-Depleted medium was added per well. Glucose-Free, EV-Depleted medium with either 5mM 2-Deoxy-D-Glucose or 1 μ M Antimycin-A was added when a metabolic pathway was inhibited. After 24 hours, the medium was collected for EV isolation. Again, the cells were washed twice and the respective EV-Depleted medium was added. This cycle continued for 12 days to allow for an adequate amount of EVs to be obtained for each bioactivity assay. After 12 days, the well plates were trypsinized to determine cell viability and count for an EV/spheroid measure.

2.5 EV Isolation

EVs were isolated using differential centrifugation and multiple steps. To start, the collected medium was centrifuged at 1,000xg for 10 minutes. The supernatant was then centrifuged at 2,000xg for 20 minutes, then 10,000xg for 30 minutes. The supernatant was then ultracentrifuged at 40,000xg for 2 hours to pellet the EVs. Optiseal tubes and a T70i ultracentrifuge rotor (both from Beckman Coulter) were used, and all centrifugation was at 4°C. Pelleted EVs were resuspended in 1X PBS and washed twice with 1X PBS buffer using Nanosep 300kDa molecular weight cut-off (MWCO) spin columns (Pall; OC300C35). EVs were then resuspended with 1X PBS for collection.

2.6 BCA Assay

EVs were resuspended with 1X PBS so total protein could be measured using a bicinchroninic acid (BCA) assay kit (G-Biosciences; 786-571). The kit included two reagents, Reagent A and Reagent B. This assay requires the preparation of standards, so diluted albumin (BSA) standards were created based on a dilution scheme provided by Thermo Fisher.²⁶ 96-well plates were used: two wells for each standard and two wells for each sample to achieve duplicates. Each cuvette contained 200 μ L of a working reagent, which was created using 50 parts of BCA Reagent A and 1 part Reagent B, and 25 μ L of standard or sample. 96-well plates were then covered from light and incubated at 37°C for 30 minutes. After incubation, the absorbance of each well was measured at 562nm using a Tecan Spark multimode plate reader provided by the University of Maryland Bioworkshop.

2.7 EV Quantification by NTA

EV size and concentration was assessed by nanoparticle tracking analysis (NTA) using a NanoSight LM10. Samples were manually injected into the sample chamber using a syringe, and each sample was measured in triplicate. The camera setting varied from 10-14 based on the sample, and the detection threshold was set to 3. An acquisition time of 30 seconds was used. NTA analytical software version 2.3 was used to obtain data.

2.8 Extracellular Oxygen Consumption Assay

As a measure of cellular metabolism, an extracellular consumption assay (Abcam; ab197243) was used. The assay was performed using the protocol provided by the

manufacturer. Briefly, BDMSCs were seeded in a 96-well plate in 200 μ L of culture medium. For the initial seeding density experiment, cells were seeded at 100, 1,000, and 10,000 cell/cm² and allowed to incubate overnight at 37°C, 5% CO₂. For the chemical inhibition experiment, cells were seeded at 4x10⁴ cells/well as specified in the provided protocol. The cells incubated overnight, then the medium was changed with 200 μ L of Glucose-Free culture medium, and the cells were allowed to incubate overnight at 37°C, 5% CO₂. After the final incubation period, the medium was replaced with 150 μ L fresh medium, and empty wells (without cells) were filled with 150 μ L fresh medium to act as blanks. 10 μ L of Extracellular O₂ Consumption Reagent was added to each sample well; 10 μ L of fresh medium was added to blank wells to maintain consistent volumes. 1 μ L of the test compound (2-Deoxy-D-Glucose or Antimycin-A) was added to the necessary wells. Each well was sealed using 100 μ L of warmed high sensitivity mineral oil, which is provided by the manufacturer and is designed to prevent back diffusion of ambient oxygen. The plate was then inserted into a Tecan Spark multimode plate reader warmed to 37°C, which was provided by the University of Maryland Bioworkshop. A measurement was taken at 2 min intervals for 90 min at Ex/Em=380/650nm.

For data analysis, intensity was plotted against time, each sample intensity value corrected with that of the blank. The linear portion of the plot was selected, and linear regression was used to determine the slope and correlation coefficient.

2.9 Gap Closure Assay

HUVECs were seeded at 20,000 cells/well into a 96-well plate. The cells were allowed to incubate for 24 hours at 37°C, 5% CO₂. BioTek's AutoScratch Wound Making Tool (Part Number: 1750012) was used to disrupt the cell monolayer and create a

gap. The wells were washed once with 1X PBS to remove any cell debris. The treatments were then applied, which are detailed in Table 1. EGM2 was made by adding a supplement pack provided by the manufacturer to EBM2, with the purpose being to promote HUVEC functionality. An image of the gap was taken immediately (0 hr) using a Nikon Eclipse Ti2 inverted microscope. The cells were allowed to incubate for 20 hours at 37°C, 5% CO₂. An image of the gap was taken after the incubation period (18-20hr). ImageJ was used to determine gap closure by tracing the gaps at 0 and 18 or 20 hr to determine the gap area.

| Treatment | Medium and Chemicals Used |
|--|--|
| Negative Control | Endothelial Cell Basal Medium MV 2 (EBM2) (PromoCell; C-22221) |
| Positive Control | Endothelial Cell Growth Medium MV 2 (EGM2) |
| BDMSC-EV Treatment | EBM2 + 100 ug/mL EVs |
| EV Treatment derived from 5mM 2-Deoxy-D-Glucose treated BDMSCs | EBM2 + 100 ug/mL EVs |
| EV Treatment derived from 1uM Antimycin-A treated BDMSCs | EBM2 + 100 ug/mL EVs |

Table 1. Description of the different treatments used in the gap closure assay. There are positive and negative controls included. There are three treatments, where 100 ug/mL of EVs are used for each. The treatments use EVs derived from normal BDMSCs, 2-Deoxy-D-Glucose treated BDMSCs, and Antimycin-A treated BDMSCs.

2.10 Statistics

Data are presented as mean±SEM. Differences between groups were analyzed by unpaired t-test. To compare data sets of three, one-way ANOVA was used. Notation for significance in figures are: ns p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Chapter 3: Influence of Metabolic Pathways

Inhibition on MSC EVs

3.1 Preliminary Experiments and Results

3.1.1 Confirmation of Initial Seeding Density Effects on MSC EV Production Capacity

An in-house study was performed to evaluate cell culture parameter effects on MSC EVs. This study's goal is to corroborate the results obtained by Patel, D. et al²⁵, who determined that MSC EV production capacity was impacted by initial MSC seeding density. The group found that a lower seeding density lead to higher EV production per cell.

MSCs were seeded in a T-25 at varying densities (100, 1,000, and 10,000 cells/cm²), and the EVs were collected, isolated, and quantified using NTA. As seen in previous studies²⁵, seeding density impacts EV production capacity. The number of EVs produced per cell decreased between densities 100 and 10,000 cell/cm² by ~100 fold (p<0.0001) (Figure 6). This finding supports the notion that cell culture parameters, such as initial seeding density, have a significant effect on MSC EV production capacity. The mechanisms behind this, however, are unknown.

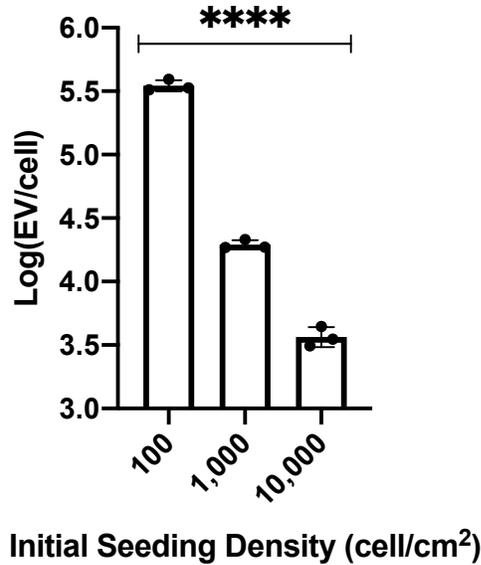


Figure 6. Initial seeding density impacts MSC EV production capacity. MSCs were seeded at 100, 1,000, and 10,000 cell/cm². EVs were collected and isolated. Quantification was performed using NTA, and the data was normalized to determine the number of EVs produced per cell. Three T-25 flasks were used for each condition (n=3). Significant difference was calculated using one-way ANOVA (**p<0.0001).**

3.1.2 Initial Seeding Density Affects MSC Extracellular Oxygen Consumption

To explore the potential relationship between cell culture parameter effects and metabolism, an extracellular consumption assay was used to determine metabolic changes experienced by MSCs are varying seeding densities. As previously mentioned, oxygen consumption is an established means of measuring cellular metabolism, as it is a direct measure of mitochondrial function. Metabolic activity can drive favorable or unfavorable reactions that directly impact the mitochondria, making the two directly dependent on either other.

MSCs were seeded into a 96-well plate at 100, 1,000, and 10,000 cells/cm², and the assay was performed using the protocol provided by the manufacturer. The results of the assay show that oxygen consumption rate is impacted by initial seeding density, with

the highest oxygen consumption rate occurring at the lowest seeding density (n=1) (Figure 7). No statistical analysis could be performed on this data set.

These results provide evidence that there could be a direct relationship between cell culture parameters and cellular metabolism, which could potentially impact MSC and EV function. To explore this relationship, further experimentation will be performed.

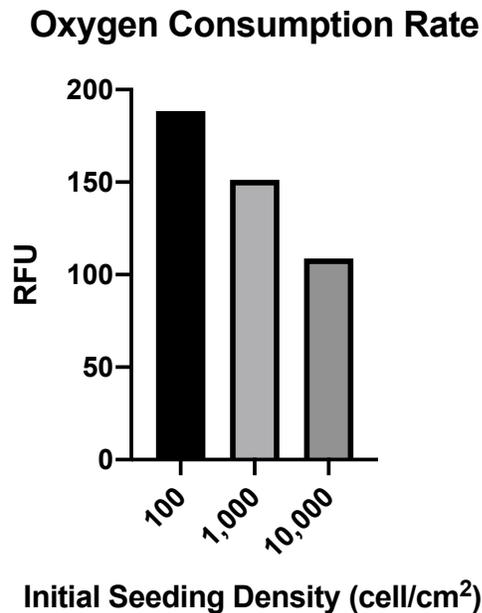


Figure 7. Metabolic activity is affected by MSC initial seeding density. MSCs were seeded at 100, 1,000, and 10,000 cell/cm² in a 96-well plate. An extracellular consumption assay (Abcam) was performed using the protocol provided by the manufacturer. Fluorescent signal was measured using Tecan Spark multimode plate reader (n=1).

3.1.3 Successful Chemical Metabolic Pathway Inhibition in MSCs

To evaluate the effect of metabolism on MSC and EV activity, glycolysis and OXPHOS were inhibited chemically using 2-Deoxy-D-Glucose and Antimycin-A, respectively. These chemicals were added to Glucose-Free DMEM, and they were introduced to the cells *in vitro*. The MSCs incubated with the chemicals 24 hours. To ensure this process would effectively inhibit metabolism, an extracellular consumption

assay was performed. In using this assay, a drop of metabolic activity can be seen, and proper inhibition can be verified. Untreated MSCs were used as a negative control, where regular, glucose-rich medium was used. Two concentrations of each chemical were used to test for potential superior dosing.

In most cases, metabolism was successfully inhibited using the two chemicals (Figure 8). The untreated MSCs (negative control) acted as expected, maintaining high metabolic activity compared to those chemically treated. MSCs treated with 1mM 2-Deoxy-D-Glucose experienced slight metabolic inhibition, but greater inhibition was experienced when 5mM 2-Deoxy-D-Glucose was used. MSCs treated with 1 μ M Antimycin-A experience similar metabolic inhibition to those treated with 5 μ M Antimycin-A. No statistical analysis could be performed on this data.

Proper metabolic inhibition was achieved when chemically treating the MSCs during culture. Based on this data, 5mM 2-Deoxy-D-Glucose will be used when inhibiting glycolysis, and 1 μ M Antimycin-A was be used when inhibiting OXPHOS.

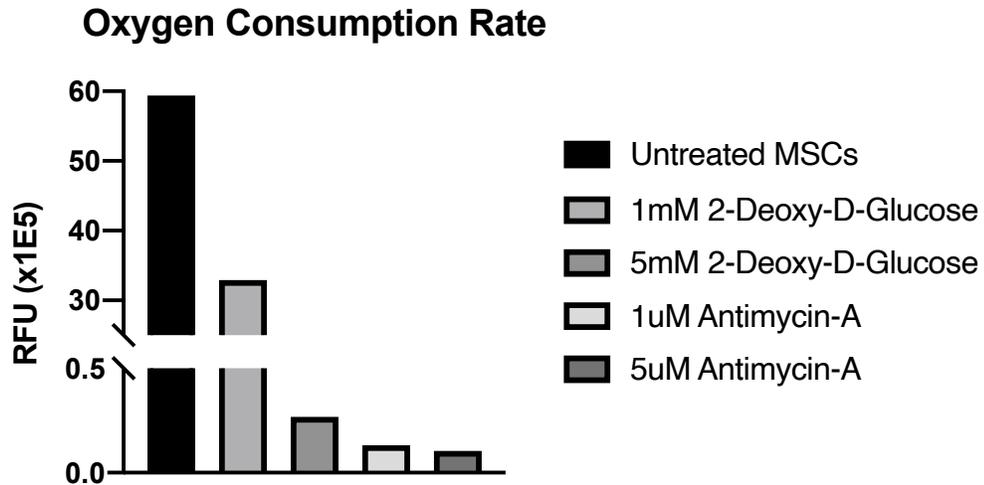


Figure 8. MSC metabolism can be inhibited chemically using 2-Deoxy-D-Glucose and Antimycin-A. MSCs were seeded in a 96-well plate, and an extracellular consumption assay (Abcam) was performed using the protocol provided by the manufacturer. Fluorescent signal was measured using Tecan Spark multimode plate reader (n=1).

3.2 Results From 2D-Cultured MSCs

3.2.1 Metabolic Inhibition Does Not Affect 2D-Cultured MSC EV Production

MSC EV production capacity was assessed to better understand if metabolic pathway inhibition affected MSC behavior when the cells were cultured in a 2D-culture system. EV production rate is a critical parameter in biomanufacturing and better understanding what affects it is valuable. MSCs were seeded in a T-25 culture flask at 100 cells/cm². The MSCs receiving no treatment were cultured in regular, glucose-rich medium, and the chemically-treated MSCs were cultured in Glucose-Free DMEM. EVs were collected, isolated, then characterized and quantified using NTA. The average mode size of the particles for all experimental groups was within the sizes used to characterize EVs (n=3) (Table 2). There was no significant difference of the particle's mode sizes

between the experimental groups, indicating physical EV characteristics were unchanged during chemical inhibition (n=3) (Figure 9).

| | Untreated MSC EVs | 5mM 2-Deoxy-D-Glucose Treated-MSC EVs | 1 μ M Antimycin-A Treated-MSC EVs |
|----------------|-------------------|---------------------------------------|---------------------------------------|
| Mode Size (nm) | 147.1 \pm 20.63 | 185.5 \pm 27.43 | 120.5 \pm 12.63 |

Table 2. Average mode size of the imaged particles from 2D-cultured MSCs. The average mode size (diameter) was below 200nm for all groups. Based on size, the particles imaged can safely be characterized as EVs. Three T-25 flasks were used for each condition (n=3).

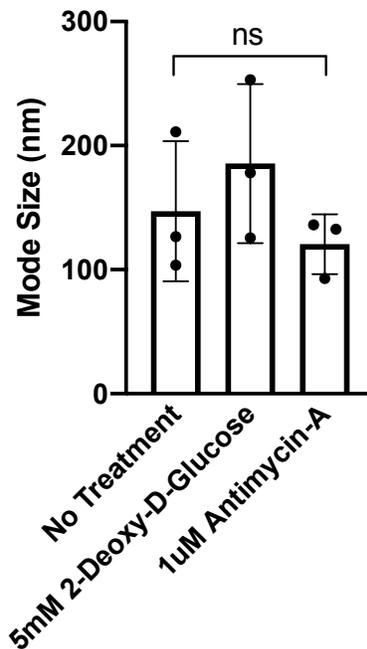


Figure 9. There is no significant difference of the MSC-derived particle's mode sizes when using a 2D-culture system. MSCs were seeded at 100 cell/cm², and EVs were collected and isolated. Size characterization was performed using NTA. Three T-25 flasks were used for each condition (n=3). No statistical difference found between groups using one-way ANOVA (p>0.05).

There was no significant change in MSC EV production capacity when glycolysis and OXPHOS were inhibited (n=3) (Figure 10). This indicates there is no relationship between metabolism and MSC EV production when a 2D-culture system is used.

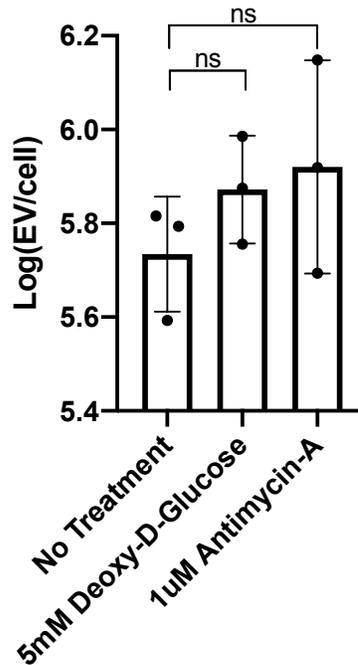


Figure 10. There is no significant difference in 2D-cultured MSC EV production capacity when glycolysis and OXPHOS are inhibited. MSCs were seeded at 100 cell/cm², and EVs were collected and isolated. EV quantification was performed using NTA. Three T-25 flasks were used for each condition (n=3). No statistical difference found between groups using unpaired t-test (p>0.05).

3.2.2 OXPHOS Inhibition Affects 2D-Cultured MSC EV Bioactivity

MSC EV bioactivity was tested to determine the effect of metabolic pathway inhibition when the parent MSCs were cultured in a 2D medium. EV bioactivity has a direct correlation to their therapeutic efficacy so understanding how it can be impacted is critical. MSCs were seeded in a T-175 culture flask at a cell count of 250,000 cells. The MSCs receiving no treatment were cultured in regular, glucose-rich medium, and the chemically-treated MSCs were cultured in Glucose-Free DMEM. The MSC-derived EVs

were collected and isolated. EV concentration was determined using a BCA assay. The effect of metabolic pathway inhibition on these EV's bioactivity was evaluated using an *in vitro* gap closure assay. A 20 hour wait time after the initial scratch was adequate for sufficient gap closure. A significant difference in gap closure was seen when comparing the EGM2 (positive control) to the EBM2 (negative control) (n=4); this is to be expected, meaning to assay was performed properly. For specific gap closure values, reference Table 3.

| | EGM2 | EBM2 | Untreated MSC EVs | 5mM 2-Deoxy-D-Glucose Treated-MSC EVs | 1 μ M Antimycin-A Treated-MSC EVs |
|-----------------|------------------|------------------|-------------------|---------------------------------------|---------------------------------------|
| Gap Closure (%) | 92.38 \pm 1.67 | 36.71 \pm 2.96 | 53.78 \pm 1.39 | 40.88 \pm 5.53 | 43.12 \pm 1.58 |

Table 3. Specific mean gap closure values for 2D-cultured MSC EVs. The results indicate that basal medium (negative control) has a much lower closure value when compared to growth medium (positive control). This is to be expected, showing the assay functioned properly. The specific gap closures from all EV treatments can be seen as well. Data are representative of four independent experiments (n=4).

When a 100 μ g/mL dose of EVs were applied, all EV populations increased HUVEC gap closure compared to basal medium (Figure 11a). However, a significant decrease in gap closure was observed when comparing the EVs isolated from regular MSCs (no chemical treatment, no metabolic pathway inhibition) to those isolated from OXPHOS-inhibited MSCs (n=4) (Figure 11b). This indicates a potential link between MSC EV bioactivity and the OXPHOS metabolic pathway.

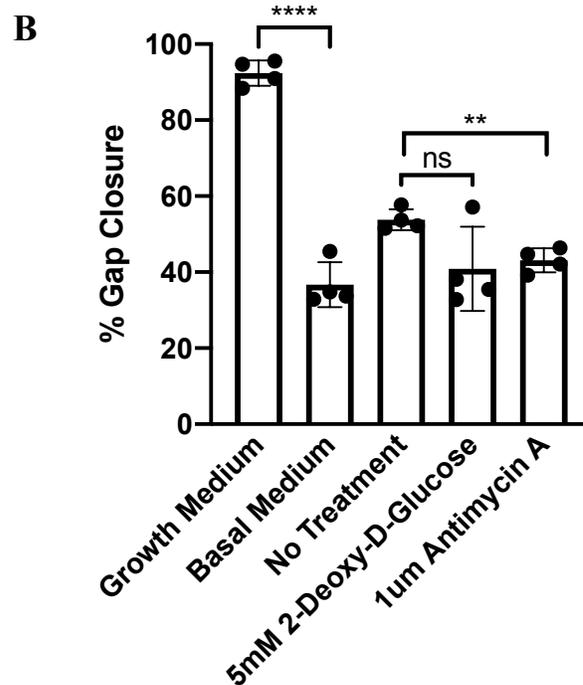
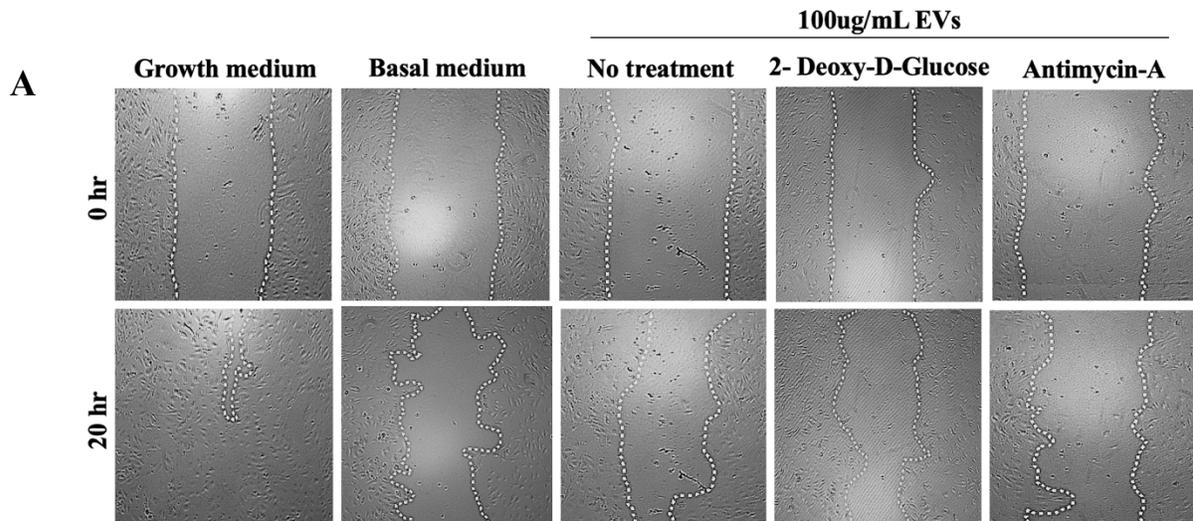


Figure 11. Impact of metabolic pathway inhibition on MSC EV bioactivity using a 2D-culture system. (A) HUVECs were stimulated with growth medium (positive control), basal medium (negative control), or 100 μ g/mL EVs isolated from MSCs with the indicated treatments. Representative images captured at 0 and 20hr are shown. (B) ImageJ analysis comparing of gap closure at 20hr to that at 0hr. The gap area is indicated by the white dotted lines. There is a significant decrease in gap closure when OXPHOS is inhibited in MSCs, indicating a decrease in EV bioactivity. Data are representative of four independent experiments (n=4). Statistical comparisons determined by unpaired t-test (ns p>0.05, **p<0.01, **p<0.001).**

3.3 Results from Microcavity-well Cultured MSCs

3.3.1 Metabolic Inhibition Affects MSC Spheroid EV Production

MSC EV production capacity was also assessed in a microcavity-culture system to test consistency between culture platforms. MSCs were seeded in a 6-well microcavity plate, with 500 MSCs seeded into each microcavity for proper spheroid formation. The MSCs receiving no treatment were cultured in regular, glucose-rich medium, and the chemically-treated MSCs were cultured in Glucose-Free DMEM. The MSC-derived EVs were collected, isolated, then characterized and quantified using NTA. The average mode size of the particles for all experimental groups was within the sizes used to characterize EVs (n=3) (Table 4). There was no significant difference of the particle's mode sizes between the experimental groups, indicating physical EV characteristics were unchanged during chemical inhibition (n=3) (Figure 12).

| | Untreated MSC EVs | 5mM 2-Deoxy-D-Glucose Treated-MSC EVs | 1 μ M Antimycin-A Treated-MSC EVs |
|----------------|-------------------|---------------------------------------|---------------------------------------|
| Mode Size (nm) | 176.33 \pm 9.63 | 189.53 \pm 16.93 | 164.00 \pm 11.93 |

Table 4. Average mode size of the imaged particles from microcavity-well cultured MSCs. The average mode size (diameter) was below 200nm for all groups. Based on size, the particles imaged can safely be characterized as EVs. Three T-25 flasks were used for each condition (n=3).

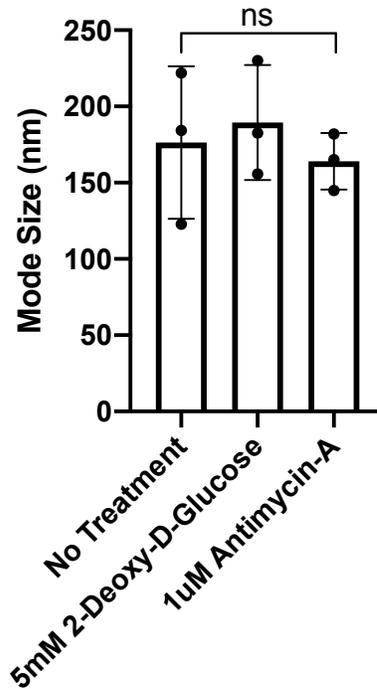


Figure 12. There is no significant difference of the MSC-derived particle's mode sizes when using a microcavity-well culture system. MSCs were seeded at 500 cells/microcavity, and EVs were collected and isolated. Size characterization was performed using NTA. Three microcavity-well plates were used for each condition (n=3). No statistical difference found between groups using one-way ANOVA ($p>0.05$).

There was a significant decrease in MSC EV production capacity when glycolysis and was inhibited, as well as when OXPHOS was inhibited (n=3) (Figure 13). This indicates that metabolism impacts and MSC EV production when a microcavity-well culture system is used. This result does not reflect those seen when MSCs were cultured in a 2D medium (Figure 10), which implies there could be a mechanistic difference in MSC EV production between 2D and microcavity-well culture systems.

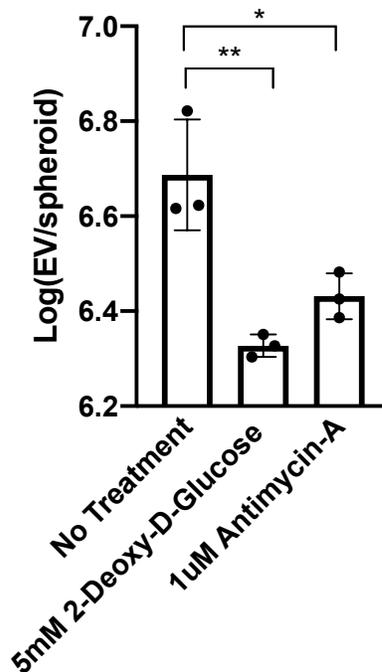


Figure 13. Inhibition of glycolysis and OXPHOS cause a significant decrease in MSC EV production capacity when using a microcavity-well culture system. MSCs were seeded at 500 cells/microcavity, and EVs were collected and isolated. EV quantification was performed using NTA. Three microcavity-well plates were used for each condition (n=3). Statistical comparisons determined by unpaired t-test (*p<0.1, **p<0.01).

3.3.2 OXPHOS Inhibition Affects Microcavity-Cultured MSC EV Bioactivity

MSC EV bioactivity was also assessed in a microcavity-culture system to determine if there is consistency between culture mediums. MSCs were seeded in a 6-well microcavity plate, with 500 MSCs seeded into each microcavity for proper spheroid formation. The MSCs receiving no treatment were cultured in regular, glucose-rich medium, and the chemically-treated MSCs were cultured in Glucose-Free DMEM. EVs were collected and isolated, and their concentration was determined using a BCA assay. As before, EV bioactivity was evaluated using an *in vitro* gap closure assay. An 18 hour wait time after the initial scratch was adequate for sufficient gap closure. There was significant decrease in gap closure when comparing basal medium to growth medium,

which acted as the negative and positive controls, respectively (n=6). This behavior is to be expected, indicating the assay is functioning properly. For specific gap closure values, reference Table 5.

| | Growth Medium | Basal Medium | Untreated MSC EVs | 5mM 2-Deoxy-D-Glucose Treated-MSC EVs | 1 μ M Antimycin-A Treated-MSC EVs |
|-----------------|------------------|------------------|-------------------|---------------------------------------|---------------------------------------|
| Gap Closure (%) | 96.46 \pm 0.39 | 36.82 \pm 4.66 | 53.21 \pm 3.52 | 46.53 \pm 3.57 | 35.53 \pm 1.93 |

Table 5. Specific mean gap closure values for microcavity-well cultured MSC EVs. The results indicate that basal medium (negative control) has a much lower closure value when compared to growth medium (positive control). This is to be expected, showing the assay functioned properly. The specific gap closures from all EV treatments can be seen. Data are representative of six independent experiments (n=6).

When a 100 μ g/mL dose of EVs were applied, the untreated and 5mM Deoxy-D-Glucose treated MSC-derived EV populations increased HUVEC gap closure compared to basal medium (Figure 14a). A significant decrease in gap closure was observed when comparing the EVs isolated from untreated MSCs to those isolated from OXPPOS-inhibited MSCs (n=6) (Figure 14b). These results implicate a potential relationship between OXPPOS and EV bioactivity. The same decrease in EV bioactivity was also seen when MSCs were cultured in a 2D-system (Figure 11b), which shows that the bioactivity of EVs remains constant despite the culture system used for the parent cells.

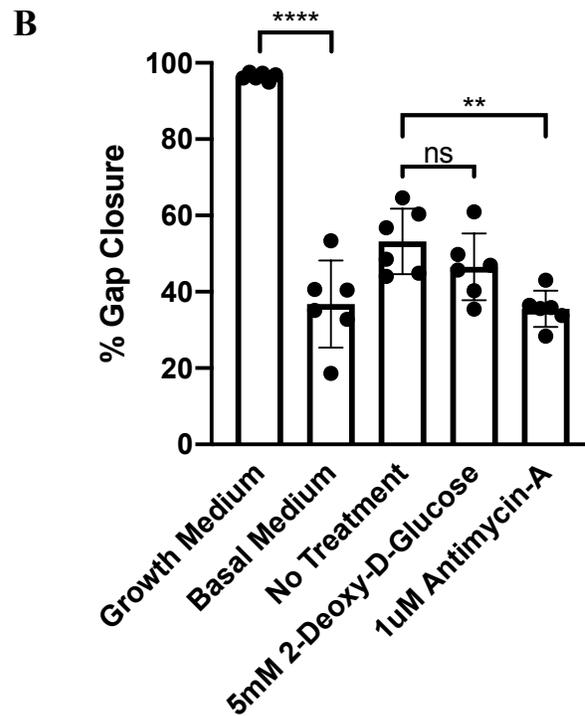
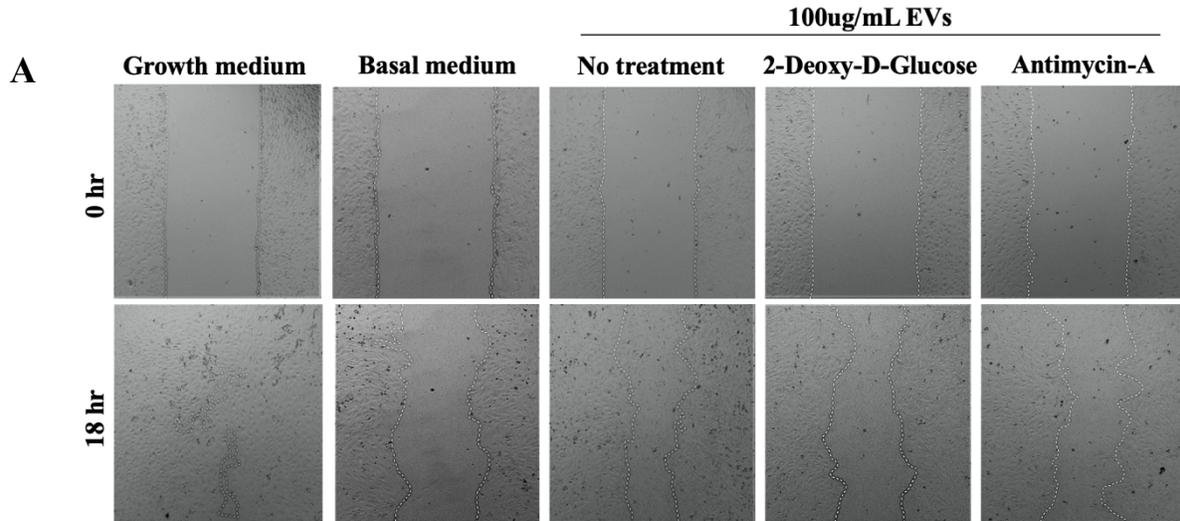


Figure 14. Impact of metabolic pathway inhibition on MSC EV bioactivity using a microcavity-well culture system. (A) HUVECs were stimulated with growth medium (positive control), basal medium (negative control), or 100 μ g/mL EVs isolated from MSCs with the indicated treatments. Representative images captured at 0 and 18hr are shown. (B) ImageJ analysis comparing of gap closure at 18hr to that at 0hr. The gap area is indicated by the white dotted lines. There is a significant decrease in gap closure when OXPHOS is inhibited in MSCs, indicating a decrease in EV bioactivity. Data are representative of six independent experiments (n=6). Statistical comparisons determined by unpaired t-test (ns p>0.05, **p<0.01, **p<0.001).**

3.4 Discussion

Many different factors impact MSC behavior, including as proliferation, differentiation, and migration. Various factors can contribute to altering MSC function, such as growth factors and extracellular matrix.¹⁹ Here, we investigated how cellular changes caused by metabolic pathway inhibition may affect the production and bioactivity of MSC EVs. Mechanical parameters such as a 2D and 3D culture system have been shown to impact MSC activity^{33,34}, and both platforms are commonly used in both experimentation and commercial production. Because of this, experiments were performed in both 2D and microcavity-well culture to investigate the translation of results between the two. Our results suggest a relationship between MSC metabolism, EV production capacity, and EV bioactivity.

3.4.1 Metabolic Inhibition Effects on MSC EV Production

The results of this study show that inhibition of both glycolysis and OXPHOS significantly decrease MSC EV production capacity when a microcavity-well culture system is used, with an inhibition of glycolysis having a greater effect (Figure 13). However, in a 2D culture system, there is no significant difference in production capacity between untreated and chemically inhibited MSCs (Figure 10). Based on these results, the effect of metabolic pathway inhibition on MSCs does not translate between a 2D and microcavity-well culture system. This difference in results supports various studies detailing the immense change in cellular morphology, physiology, and behavior experienced by cells when cultured in a 3D medium.^{33,34} It is thought that, because of this, a 3D-culture system provides more predictive information than that of a 2D-culture

system.^{35,36} This ideology indicates that the decreased EV production capacity resulting from the MSC spheroid culture may be more indicative of *in vivo* phenomena.

The differing results between culture systems supports previous work suggesting cell culture parameters, including mechanical factors, can impact MSC EV production.²⁵ The use of 3D culture has been shown to effect extracellular signaling in various cell types.³⁸ Specifically, it can enhance paracrine signaling and secretions in MSCs³⁹, and amongst these paracrine secretions are extracellular vesicles⁴¹. Glycolysis and OXPHOS may have an impact on MSC paracrine signaling, which could be intensified when paired with the enhanced properties of MSC spheroids. Further study is required to identify the mechanistic link between metabolic pathway inhibition and paracrine signaling. In doing this, there can be a better understanding as to why different dimensional culture systems yield different MSC EV production results when glycolysis and OXPHOS are inhibited.

3.4.2 Metabolic Inhibition Effects on MSC EV Bioactivity

The results of this study show that the inhibition of OXPHOS in MSCs decreases EV bioactivity, and the inhibition of glycolysis does not have a statistically significant effect. This result is translatable across 2D and microcavity-well culture systems (Figures 11 and 14). Previous studies have shown that metabolic pathways contribute to both the optimization and detriment of MSC function.¹⁹ This same effect could be translated to the EVs they produce; however, this fact has not previously been proven. These results are a good first step in uncovering the potential link between metabolism and EV functionality.

This result goes against the initial hypothesis, which predicted that glycolysis inhibition would have a more significant effect on MSC EVs than the inhibition of OXPHOS. The effect of OXPHOS on MSC EVs could be due to the pathway's

relationship to ATP production. Because OXPHOS operates on the TCA cycle, it produces more ATP than glycolysis. Varying levels of interaction between ATP and EVs have been shown to modify the EV proteome in other cell types⁴², and the same may occur in MSCs. It is possible that the decreased ATP level associated with OXPHOS production altered the common ATP-EV interaction, resulting in a changed EV proteome. Further study is required to analyze the potential effects of OXPHOS inhibition on the MSC EV proteome and identify it as a potential mechanism behind the decrease in EV bioactivity. In doing this, there can be a better understanding of the factors that contribute to and impact EV biogenesis.

3.5 Significance

MSC EVs are responsible for a large portion of MSC bioactivity, and they contribute to the many regenerative and healing properties experienced by their parent cells. MSC EVs have great therapeutic potential, which has been evidenced in various studies and disease models.⁴⁻⁵ However, the lack of knowledge behind the culture parameters and mechanisms that drive MSC EV biogenesis and bioactivity acts as a barrier to their widespread clinical use. Metabolism has been shown to greatly contribute to MSC behavior and functionality¹⁹⁻²⁰, and it is hypothesized to be the mechanism behind the many variations in MSC EV production and bioactivity experienced in cell culture.²⁵ Here, we take the first steps toward revealing a mechanistic relationship between metabolism and MSC EVs. By better understanding how the two are related, there can be a greater insight into the factors that drive MSC EV production and bioactivity, improving the knowledge behind the mechanisms that drive EV biogenesis.

A greater understanding increases the possibility for realistic clinical translation and application of EVs.

When considering the results obtained when using a microcavity-based culture system, the inhibition of glycolysis and OXHPOS significantly decreases MSC EV production capacity. This result is valuable, as it reveals a previously unknown potential mechanism driving EV biogenesis in MSCs. It also allows for the possibility to optimize MSC EV production. Once EVs are cleared for clinical use, glycolysis and OXPHOS can be utilized as a vehicle to enhance MSC EV production in the biomanufacturing process. Future studies can explore the potential for metabolic pathway promotion, rather than inhibition, to see if MSC EV production is increased. If this is the case, this mechanism can be used to optimize the biomanufacturing and production of MSC EVs, which is a crucial component to the realistic use of EVs as a therapeutic.

While the inhibition of glycolysis seems to have no effect, the inhibition of OXPHOS significantly decreases MSC EV bioactivity. This result reveals that not only can metabolic pathways play a role in MSC EV production capacity, but it may also be a mechanism impacting EV behavior and functionality. This is a fact that can, again, be used to optimize MSC EV biomanufacturing. Future studies can observe the effects of metabolic pathway promotion on EVs, and it can be determined if EV bioactivity can potentially be improved. If this is observed, it is a mechanism that has incredible value. The therapeutic potential of MSC EVs can be enhanced, and the regenerative effects seen in previous studies⁴⁻⁵ can be improved. The manipulation of MSC metabolic pathways *in vitro* can positively impact the efficacy of MSC EV use in various medical treatments.

Chapter 4: Conclusions

4.1 Summary

MSC EVs are responsible for a large amount of MSC function and bioactivity. These EVs have great therapeutic potential that has been proven in many different applications, such as reduction of myocardial injury and wound healing.⁴ EV treatment has potential advantages over MSC transplantation in that they are considered more stable, have a well-defined clearance pathway, and are unable to proliferate or differentiate. Despite the rising interest and advantages of MSC EVs, there are many barriers to realistic clinical use, the most prevalent of which being lack of knowledge detailing the driving forces of their biogenesis. It is unknown how different cell culture parameters impact MSC EVs on a mechanistic level, and this leads to great variation in both MSC EV production and bioactivity across different culture systems. It has been previously hypothesized that cellular metabolism is the mechanism behind the variations in MSC EVs experienced between cell cultures²⁵, but this has never been proven.

The enclosed findings provide insights into the effects of cellular metabolism on MSC EV production capacity and bioactivity. These results provide a greater understanding of metabolism as a mechanistic link between cell culture effects of MSCs and the EVs they produce. The results also provide potential ways to better improve biomanufacturing processes in the future, which is critical to the efficient clinical translation of EVs. Key findings of this study include:

- **Metabolic pathway inhibition may impact MSC EV production.** The inhibition of glycolysis and OXPHOS did not significantly decrease MSC EV production when a 2D-culture system was used. However, when using a

microcavity-well culture system, the inhibition of glycolysis and OXPHOS did significantly decrease MSC EV production capacity. The lack of translation between culture systems can be attributed to the changes in behavior experienced by MSC spheroids. Glycolysis and OXPHOS may have an impact on MSC paracrine signaling, which could be enhanced with the improved properties associated with MSC spheroids. Further study is needed to identify if there is truly a mechanistic relationship between metabolism and MSC EV production capacity.

- **Metabolic pathway inhibition significantly decreases MSC EV bioactivity.** In both a 2D and microcavity-well culture system, the inhibition of OXPHOS significantly decreases MSC EV bioactivity. The inhibition of glycolysis does not have a statistically significant effect. These results defy the initial hypothesis that glycolysis would have a more significant effect than OXPHOS. Further studies are required to better understand this mechanistic relationship and the details behind it.

4.2 Future Directions

This work was completed to better understand what mechanisms effect MSC EV biogenesis. More specifically, we investigated if metabolism plays a significant role in MSC EV production capacity and bioactivity. Conclusions from this research indicate that this is very much a possibility. The next steps will be to further study the relationship between metabolic pathways and MSC EVs. In doing this, a greater understanding of MSC EV biogenesis can be gained, and there can be improved efficiency in potential strategies for MSC EV biomanufacturing, making the clinical translation of EVs more realistic.

To determine if metabolism truly impacts MSC EV production capacity, further study is necessary. Because of the inconsistency in results between 2D and microcavity-well culture systems, the results received here are indicative of a potential relationship, but they are not conclusive. The experiments could be repeated using a different quantitative method than NTA to determine EV count. Tetraspanin CD63 is a common biomarker associated with EVs, and it has been deemed by the International Society of Extracellular Vesicles as an acceptable protein to characterize EVs by.⁴³ Therefore, a CD63 ELISA can be performed to quantify the MSC EVs. In using this method, more decisive results may be achieved.

To better understand the role of metabolism in MSC EV bioactivity, further study is needed. The results obtained here indicate that the inhibition of OXPHOS significantly decreases MSC EV bioactivity. Although this relationship is established, the mechanism behind it is still unknown. To potentially gain insight into this mechanism, future studies can investigate if changes in MSC EV proteome are experienced during OXPHOS inhibition. OXPHOS is responsible for a large amount of ATP production. Previous studies have shown that interactions between ATP and EVs can modify the EV proteome. This may be the mechanism behind the changes in EV bioactivity seen here.

It would be beneficial to perform the experiments described with metabolism promoters instead of inhibitors. Whether it by the addition of nutrients (e.g. glucose) or metabolites (e.g. lactate), the aim would be to increase metabolic activity. Here, MSC EV production and bioactivity would be tested in the interest of seeing if these parameters are increased when metabolism is enhanced. If this is the case, metabolism is a cellular parameter that can be utilized in MSC EV biomanufacturing to make processes more

fruitful and efficient. By providing a means to improve MSC EV yield during biomanufacturing, as well as enhance EVs therapeutic potential by improving its bioactivity, the clinical translation of EVs is significantly more achievable.

If future experiments are performed to better understand the effects of metabolism on MSCs and their EVs, it may be worthwhile to explore a different culture system than the microcavity-well plate. Although the microcavity-well plate was able to successfully form MSC spheroids (Figure 5), it seems improved cell-cell interactions is the only altered cell culture condition. Aside from this, the culture system is like that of a standard, 2D 6-well plate. Other multi-dimensional culture systems allow for more realistic cell conditions, such as complex surface shapes, altered mechanical properties, and the application of shear. The creation of a more realistic cell culture environment could more significantly impact cell behavior, which could translate to the EVs they produce. For example, in a study using a scaffold-perfusion bioreactor system to culture endothelial cells, both EV production and bioactivity was enhanced compared that of a 2D-culture system⁴⁴. To achieve more realistic cell culture conditions and changes in MSC behavior, any future studies should consider using a bioreactor system instead of the microcavity-well plates.

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