HUMAN MEGAKARYOCYTIC MICROPARTICLES TARGET MURINE HEMATOPOIETIC STEM CELLS TO STIMULATE *IN VIVO* PLATELET BIOGENESIS

by

Christian Escobar

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Winter 2018

HUMAN MEGAKARYOCYTIC MICROPARTICLES

TARGET MURINE HEMATOPOIETIC STEM CELLS

TO STIMULATE *IN VIVO* PLATELET BIOGENESIS

by

Christian Escobar

Approved: _____

Eleftherios T. Papoutsakis, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

E. Fidelma Boyd, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____

George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____

Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I would like to thank Professor Papoutsakis for his guidance and support that has been critical in the completion of this project and the development of my own way of thinking about science and for that I am indebted and grateful. In addition, I would like to thank my committee members for my project: Dr. Donna Woulfe and Dr. Anja Nohe, who went above and beyond to ensure the success of my projects to the extent where I felt I was a part of their research groups and I am equally grateful for their time. I would like to thank all the members of the Papoutsakis' lab and extend my deepest thanks to Stephanie Luff, Chen-Yuan Kao and Jinlin Jiang who were my mentors and assisted me through the completion of my thesis. I would also like to thank Dr. Erica Selva and Dr. Matthew Butchbach who have bestowed upon me the confidence I was lacking and have grown to be lifelong mentors who have demonstrated their commitment to my success.

I would like to thank everyone at the OLAM facility where all of my animals were housed for being so kind and welcoming me as one of their own. In addition, I thank all of the animals whose sacrifice allowed me to collect these data and their sacrifices were integral in the advancement of science. Additionally, I was a recipient of personal funding for two years through the University of Delaware Graduate Scholars award for which I am grateful.

In addition, I would like to thank all of my friends that I have made and made my time at the University worthwhile; specifically, the Dr. Xinqiao Jia group who were nothing short of welcoming and treating me as one of their own specifically

Aidan Zerdoum, Alex Stuffer, Eric Fowler, Dakota Kelly, and Anitha Ravikrishnan who were always accommodating. I would like to thank all of my friends I have made specifically; Becca Scott, Ishnoor Shidu, Conroy Field, Hannah Street, Erica Winter, Vrathasha, Sambee Kanda, Katie Robbins, Effie Halakos, Francyne Kubaski and Moni Phay who were nothing short of inviting and always believed in my ability as a scientist. I would also like to thank my old friends in New York specifically, Joseph Quinones, Annette Demir, Jessica Drenzyk, Abigail Paul, Rosalie Melo, Jormary Melo and Ashley Merentis who have been nothing but unwavering support despite our long distance apart.

At this time, I would like to acknowledge two key people who without the help of I would not have ever attended the University of Delaware. Dr. Melinda Duncan and Betty Cowgill. Dr. Melinda Duncan did everything in her capacity as graduate director to ensure I entered the University of Delaware and had belief in me as a scientist when I had lost that belief. Without her guidance, and that fateful phone call on her time. I would have never attended and for that you have my sincerest gratitude. I would also like to thank Betty Cowgill who is the gear that keeps our program running in shape. Without her, I would be lost in navigating the unfamiliar landscape and has been nothing short of being the most kind-hearted woman I have met. Once again, I extend my deepest gratitude.

Finally, I would like to thank every one of my family members whose unwavering support allowed me to attend the University of Delaware and succeed as a scientist and I am forever grateful that all of you made sacrifices on my behalf.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

One of the greatest challenges in the production of platelets *ex vivo* is implementing a system that does not require expensive media and time-consuming laboratory practices that rival the price of a platelet transfusion. Platelet transfusions are required for people who suffer from thrombocytopenia, a condition characterized by low platelet counts. Recently, new evidence has emerged suggesting that megakaryocyte derived microparticles (MkMPs) can be utilized to induce differentiation of the hematopoietic stem cell to megakaryocytes, the precursor of platelets through *an ex vivo* co-culture system without the cytokines traditionally used. Through endocytotic or membrane fusion events, MkMPs have been shown to deliver their miRNA rich cargo to the target cell and induce the target cell to commit to megakaryopoiesis (the differentiation of hematopoietic stem cell to megakaryocyte) and ultimately generate platelets.

To determine the effectiveness of MkMPs in inducing platelet biogenesis *in vivo*, this project utilizes a thrombocytopenic mouse model, whereby thrombocytopenia is induced by an antibody targeting the important CD41 complex on the surface of platelets and megakaryocytes. Before the *in vivo* murine experiments, it was important to examine if human MkMPs can recognize and target murine hematopoietic stem and progenitor cells (HSPCs). By performing *ex vivo* co-cultures of the murine HSPCs with the human MkMPs, we confirmed that there was uptake of the human MkMP by the murine HSPCs, thus confirming that there is cross reactivity

between the human MkMPs and the murine species and that the miRNA cargo of human MkMPs can likely be delivered to murine HSPCs.

The next step was to determine if the human MkMPs can induce platelet biogenesis in non-thrombocytopenic mice. Mice were injected human MkMPs to examine if they could increase to platelet concentration in the mice. After examining three increasing dosages of human MkMPs, there was an observed dose dependent increase in platelet concentration in the mice. Further studies with thrombocytopenic mice showed human MkMPs can partially ameliorate the low platelet concentration resulting from the antibody-induced thrombocytopenia. Confirmation of this increase was performed by observing reticulated (newly synthesized) platelet levels. As demonstrated and expected, there was an increase in the percentage of circulating reticulated platelets confirming that human MkMPs are, in fact, causing *de novo* megakaryopoiesis and platelet biogenesis.

Taken together, these data suggest that, *in vivo*, human MkMPs can target murine hematopoietic stem cells and induce them into *de novo* thrombopoiesis, thus increasing the platelet levels in non-thrombocytopenic mice and ameliorating the induced thrombocytopenia in thrombocytopenic mice.

Chapter 1

INTRODUCTION

The study of megakaryocytes has uncovered many different biological roles these cells perform. Megakaryocytes are the precursors of platelets; the cells responsible for blood clots in the event of an injury or opening in the blood vessels. Platelets and megakaryocytes originate from the hematopoietic stem cell. Megakaryopoiesis is defined as the differentiation of hematopoietic stem cells to megakaryocytes. Recently, it has been observed that along with the formation of platelets, small membrane bound vesicles around the size of 0.5 microns in diameter have been observed along with the formation of platelets referred to as microparticles (MPs) (Jiang, Woulfe et al. 2014). The MPs produced are not specific to only being produced when platelets are being formed as MPs can be formed from any cell, however, the biological role of megakaryocyte-derived microparticles (MkMPs) have not yet been determined (Jiang et al., 2014). That was the case before a study by the Papoutsakis group showed the effect of MkMPs on hematopoietic stem and progenitor cells (HSPCs). Before the study, the role of MkMPs biological role were not characterized however, the Papoutsakis group has shown that MkMPs influence HSPCs to differentiate along the megakaryopoietic lineage *ex vivo* (Jiang et al., 2014). To show the effectiveness of MkMPs in vivo as observed ex vivo, I propose to utilize a murine model with induced thrombocytopenia (low platelet counts) to examine if introduction of MPs can influence platelet levels via *de novo, in vivo* thrombopoiesis.

1.1 The Importance of Platelets

The human body has many different fundamental cell types circulating through the system of blood vessels. These blood types have a multitude of different capabilities from the transportation of oxygen to the ability to recognize foreign bodies to the repair of injured or broken vasculature. One of the cell types circulating in the blood are platelets. Under normal conditions, platelets circulate through the blood, in a quiescent state (McFadyen and Kaplan 2015). Upon recognition of damage to the endothelial walls of the circulatory system, the platelets become activated through a series of intracellular and extracellular signaling cascades (Jones 2016). The breakage in the endothelial walls of the tunica intima exposes proteins that permit the adherence of platelets, such as Von Willebrand Factor (Rhodes and Simons 2007, Jones 2016). Over time, more platelets are recruited to the site through extracellular signaling thus leading the aggregation of platelets referred to the platelet plug. Once an initial platelet plug forms, intracellular and extracellular signaling will recruit fibrinogen and stabilize the platelet plug at the site of vascular injury and tether the platelets to the site of injury forming a platelet clot that consists of the fibrinogen sticking the platelets together in a tight aggregate (Y.-Q. Ma 2007, Italiano, Richardson et al. 2008). Once all the responses have been coordinated effectively, the clot will effectively seal off the injured site preventing more loss of blood.

1.2 The Formation of Platelets from HSPC

In the human circulatory system, all blood cells arise from a common cell, the hematopoietic stem progenitor cell (HSPC) (Palis 2016). Located in the bone marrow, HSPCs are regulated by the microenvironment through a variety of different

regulatory factors such as different cytokines, relative oxygen composition and sheer stress.(Kovtonyuk, Fritsch et al. 2016, Luff and Papoutsakis 2016).

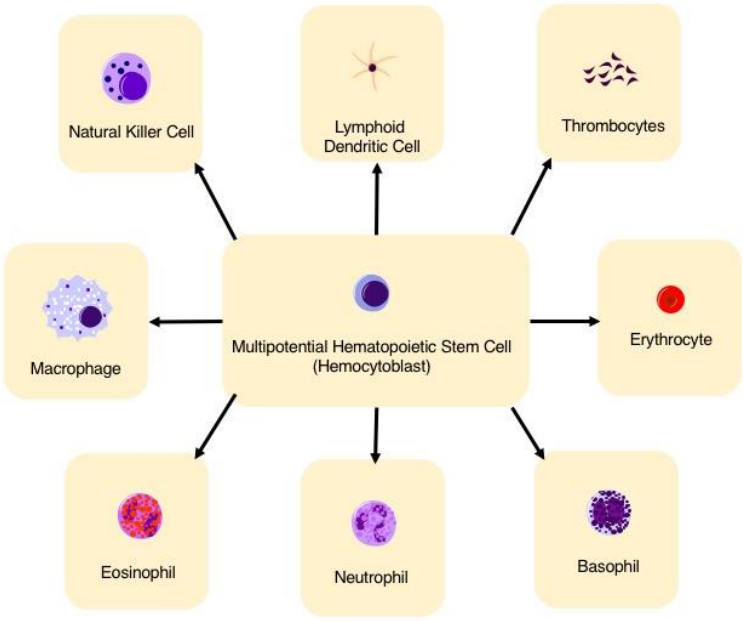


Figure 1. **The multiple fates of the Multipotential Hematopoietic Stem cell**.

Depiction of Hematopoietic stem cell undergoing multiple routes of differentiation. The fate is determined through the environment as well as chemical signals from surrounding resident bone marrow cells

Because the microenvironment of the bone marrow is rich in different cytokines and chemical signals, the HSPC has the ability to commit to a particular cell linage to differentiate terminally to one of the many blood cells within the human body such a erythrocytes, monocytes or leukocytes (Seita and Weissman 2010) (Figure 1) . Focusing on megakaryopoiesis, or the commitment of a HSPC to become

a megakaryocyte which will ultimately produce platelets, HSPCs will lose their ability to divide and become multipotent progenitors (MPPs) (Seita and Weissman 2010). From there, the MPPs will further become specialized by becoming a Megakaryocyte/Erythrocyte progenitor and eventually form into a megakaryocyte (MEP) (Seita and Weissman 2010).

1.2.1 Maturity of Megakaryocytes

An essential differentiation factor for megakaryopoiesis is thrombopoietin (Kaushansky 2008). For *in vivo* megakaryopoiesis, the MEP begins to increase in cell diameter and increase the amount of DNA within the nucleus. This is caused by a special process in megakaryocytes referred to as endomitosis (Guo, Wang et al. 2015). Comparing the megakaryocytes to its progenitor cell, the cell cycle is followed through until cytokinesis, where there is a failure to produce 2 daughter cells and there is an increase in cytoplasm volume and DNA content (Guo, Wang et al. 2015). Early Mks express CD41 on their cell surface which allow them to be identified from other cells, have typically 2N-4N the amount of DNA and are around 10 microns in diameter (Kaushansky 2008, Ru, Dong et al. 2016) (Figure 2).

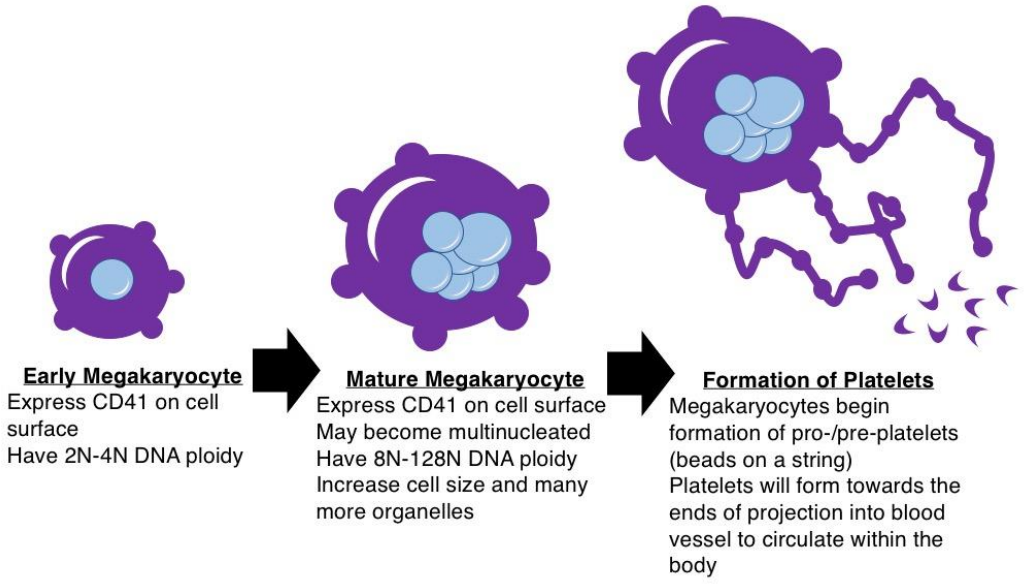


Figure 2. **The cycle of a megakaryocyte from early to the formation of platelets**.

As megakaryocytes mature, the cytoplasm and cell size increase, more nuclei begin to form and make the cell multinucleated, have DNA content anywhere from 8N-128N, have an increase in organelle number, and begin migration toward the sinusoid vessels of the bone marrow (Guo, Wang et al. 2015, Ru, Dong et al. 2016). Throughout the Mk maturation process, Mks begin to express CD61 which can be used to select between immature and mature megakaryocytes (Kaushansky 2008). Once mature, megakaryocytes migrate toward of the endothelial lining where they penetrate gaps within the sinusoids to extend their protrusions to form proplatelets (Jiang, Woulfe et al. 2014, Luff and Papoutsakis 2016). Once extended, the proplatelets are subjected to different factors such as intracellular signaling as well as shear stress from the blood vessel (Machlus, Thon et al. 2014, Guo, Wang et al. 2015, Luff and Papoutsakis 2016). These intracellular signals include increased amounts of

pro apoptotic signals such as caspase 3 and 9. As these signals intensify, the Mk extrudes its entire body into the sinusoid vessel where it is transformed to proplatelets and the remaining nucleus is degraded. (Machlus, Thon et al. 2014, Luff and Papoutsakis 2016). Typically, 1 Mk can produce anywhere from 1,000- 5,000 platelets (Ru, Dong et al. 2016).

1.2.2 *Ex Vivo* Production of Platelets

Understanding of the environment the bone marrow provides is crucial for the development of techniques capable of a viable production of *ex vivo* platelets. As demand for platelet based transfusions continue to grow, *ex vivo* platelet production is viewed as a possible solution for meeting the platelet demand (Whitaker, Henry et al. 2011). Several groups have examined the possibility of *ex vivo* platelet production from donor or universal (e.g., from iPSC [induced pluripotent stem cells] or HSPCs [hematopoietic stem and progenitor cells]),(Nakagawa, Nakamura et al. 2013, Panuganti, Schlinker et al. 2013, Luff and Papoutsakis 2016, Nurhayati, Ojima et al. 2016). This will circumvent many problems regarding the supply of platelets in Transfusion Medicine, including the need for blood donations, the risk of allo-immunization and the contamination of platelet preparations with blood-borne pathogens (Avanzi, Chen et al. 2012, Nakagawa, Nakamura et al. 2013, Panuganti, Schlinker et al. 2013, Luff and Papoutsakis 2016, Nurhayati, Ojima et al. 2016). The main issue with *ex vivo* platelet production is the cost of production (Nakagawa, Nakamura et al. 2013, Luff and Papoutsakis 2016, Nurhayati, Ojima et al. 2016). As stated previously, the bone marrow environment has evolved to provide an environment suitable for hematopoietic differentiation in terms of specific cytokine availability, oxygen tension, cell-to-cell interactions, ECM (extracellular matrix) to

cell interactions and exposure to shear forces as maturing blood cells cross the wall of the BM sinusoids to release more mature or generally more differentiated blood cells into the systemic blood circulation. To emulate, to the extent possible, this complex BM environment, HSCs grown *ex vivo* must be grown in media supplemented with different cytokines depending on the desirable differentiation lineage, but also provide suitable levels of oxygen tension and pH (Walasek, van Os et al. 2012, Avanzi and Mitchell 2014, Luff and Papoutsakis 2016, Nurhayati, Ojima et al. 2016). These are fairly complex and expensive cultures to carry out, require extensive care, do not produce many platelets *ex vivo*, and may not be functional as their counterparts produced *in vivo* (Mattia, Vulcano et al. 2002, Pallotta, Lovett et al. 2011, Avanzi and Mitchell 2014, Nurhayati, Ojima et al. 2016) Thus, *ex vivo* platelet production is not currently not a feasible way to produce mass quantities of platelets and other methods of *ex vivo* platelet production should be explored. One such alternative is the use of megakaryocytic microparticles (MkMPs). Dr. Jiang recently reported that Mks producing platelets also produce small membrane bound vesicles called Mk microparticles (MkMPs)(Jiang, Woulfe et al. 2014).

1.3 Defining Microparticles

Virtually all eukaryotic cells have the ability to produce MPs (Mause and Weber 2010). MPs are defined as a heterogeneous population of membrane bound spherical particles typically between 0.1-1 micron in diameter (Mause and Weber 2010, Loyer, Vion et al. 2014) Processes that lead to MP formation include but not limited to, pro-inflammatory signals, apoptotic signals, general stress signals such as sheer stress, and stimulatory or activation signals (Mause and Weber 2010, Jiang, Woulfe et al. 2014). Upon formation and shedding, the MP is loaded with different

proteins, nuclear material, organelles and RNAs enriched in microRNAs (miRNAs) (Mause and Weber 2010, Loyer, Vion et al. 2014, Zhao, Zhou et al. 2017). Different MPs derived from different cell types utilize different cytokines that target cells emit and lure the MPs to an area of interest where further cellular cascades such as coagulation in the case of Platelet MPs can occur (Piccin, Murphy et al. 2007, Mause and Weber 2010). At this time, a review paper on how MkMPs may interact with target cells has yet to be determined however, it has been shown that MkMPs utilize the circulatory system and are predominately the MP that can be found in blood (Italiano, Mairuhu et al. 2010). One interest factor that has been shown is the ability for the MPs to be engulfed in a endocytotic manner to their target cell (Mause and Weber 2010, Jiang, Woulfe et al. 2014). This allows the cargo the MPs is carrying to become integrated into the cytosol of the target cell, thus delivering the cargo the microparticles carry and allowing the cell to utilize the cargo (Mause and Weber 2010, Jiang, Woulfe et al. 2014). MPs also carry the same cell surface markers that are expressed from the parent cell. In circulation, MkMPs express CD41 on their surface (Italiano, Mairuhu et al. 2010). The CD41 marker as defined earlier is representative of both megakaryocytes and platelets however upon closer examination, the MPs in circulation lack CD62p on their surface (Italiano, Mairuhu et al. 2010). The CD62p marker is indicative of platelets and not megakaryocytes, and, as defined by the Italiano group, the majority of MPs circulating in the blood are $CD41^{+}$ and $CD62^{-}$ (Italiano, Mairuhu et al. 2010).

1.3.1 Distinction between Microparticles and Exosomes

At first glance, the distinction between a MP and an exosome based off their relative sizes toward one another are small however there are some key differences

that allow one to distinguish between the two microvesicles. Per the reviews of Mause and Weber, MPs range from sizes anywhere between 0.1-1 microns in diameter as opposed to exosomes whose sizes are reported between 0.05-0.1 microns in diameter (Mause and Weber 2010, Loyer, Vion et al. 2014). Another key difference is the formation of the two particles. MPs are formed from membrane blebbing carrying components of the parent cell such as proteins, RNAs and lipids while exosomes are created through the fusion of vesicular bodies to the plasma membrane of parent cells (Booth, Fang et al. 2006) (Loyer, Vion et al. 2014). However, there are many key differences in determining what are microparticles and what are exosomes. One of these differences is based off size. Microparticles range from 100 nm- 1000 nm in size while exosomes fall below the 100 nm (Pols and Klumperman 2009, Mause and Weber 2010, Azevedo 2012, Raposo and Stoorvogel 2013). In addition, MP generation comes from blebbing of the plasma membrane while exosomes are exocytosed out of the cell into the extracellular matrix (Simons and Raposo 2009, Azevedo 2012). In addition, exosomes are rich in tetraspanning proteins such as CD63 while MPs display proteins abundant on the surface of the cell which they were generated from (Simons and Raposo 2009, Azevedo 2012). Shifting toward the generation of MkMPs, flow cytometry is the preferred method of determining MP populations due to its strict discrimination on size and ability to use immunohistochemistry to isolate specific MP populations. Through immuno-staining, the MPs can be stained with the appropriate antibody to isolate a specfic MP population (Chou, Mackman et al. 2004, Jiang, Woulfe et al. 2014, Garraud, Khacef et al. 2016).

1.3.2 Utilization of Microparticles for Cell Therapy

As it stands currently, platelet production *ex vivo* is extremely costly and does not yield anywhere near the number of platelets as the functioning human body does (Luff and Papoutsakis 2016). As previously reported, the role of MPs was poorly understood for quite some time before research was conducted that elucidated their roles when it came to endothelial cell derived MPs (Mause and Weber 2010). Although the endothelial cell derived MPs were better understood, the biological purposes of MkMPs remain unknown. A recent study published by the Papoutsakis group showed that MkMPs can localize with HSPCs in a highly targeted matter to influence megakaryopoiesis in an environment not conducive for HSPCs to undergo megakaryopoiesis (Jiang, Woulfe et al. 2014). Their study showed that only MkMPs can be taken up by HSPCs and allowed the cargo to influence the fate of the targeted cells as opposed to platelet MPs (PMPs) that showed no localization with these cells; furthermore, this study elucidated the biological role of MPs *ex vivo* (Jiang, Woulfe et al. 2014). In addition, their study showed different mechanisms canonical to previous research which suggested MPs have multiple routes of delivering their cargo to target cells (Raposo and Stoorvogel 2013, Jiang, Kao et al. 2016). Through the combination of flow cytometry and microscopy, and utilizing media that didn't have the traditional cytokines conducive to megakaryopoiesis, they showed the visual localization of the MkMPs with the HSPCs, and quantified the amount of megakaryocytes depending on their maturity level. (Jiang, Woulfe et al. 2014). One of the most impactful results was the rate of maturation the MkMPs had with the HSPC. Compared to their control HSPC that was plated in media that did not have media or cytokines that allowed for megakaryopoiesis, not only did the MkMPs cause a significant rate of cells committing to megakaryopoiesis, but those cells were showing signs of maturity as

their DNA content was in the 8N-16N ploidy class (Jiang, Woulfe et al. 2014). This data suggests that MkMPs play a role in megakaryopoesis by delivering their cargo to target cells and this cargo is conducive to HSPCs to differentiate into Mks.

1.4 Research Proposal: Use of Megakaryocytic Microparticles to Increase Platelet Concentrations in Thrombocytopenic Mice

Theoretically, as megakaryocytes become platelets, they produce MPs. The study of the Papoutsakis group has shown that, *in vitro*, MkMPs interact with HSPCs to induce them to differentiate into megakaryopoiesis. If this takes place *ex vivo*, then MkMPs can be used to induce *in vivo* thrombopoiesis and thus be used in lieu of platelet transfusions.

Utilization of MkMPs mitigate a couple of the challenges that *ex vivo* platelet production faces. First, the MkMPs will be produced from stem cells donated from people that have platelet deficiency issues such as thrombocytopenia meaning the platelets produced will be classified in terms of donor as self, and upon transfusion, the risk of blood borne illness greatly decreases (Luff and Papoutsakis 2016). In addition, those MkMPs can be used further to differentiate HSPCs *ex vivo* to produce more MkMPs mitigating the cost of expensive cytokines (Luff and Papoutsakis 2016). Finally, using MkMPs themselves as a targeted vesicle that can deliver their megakaryocytic inducing cargo to the target HSPCs to differentiate them into Mks and produce platelets which should reverse the phenotype and effectively cure them of their ailment.

This project aims to take the observed work of MkMPs from an *ex vivo* model to an *in vivo* model to effectively show that MkMPs do indeed interact with HSPCs to produce platelets. The first aim is to visualize that MkMPs from human donors can

interact with target murine stem cells *ex vivo*. The purpose of the study is to see if there is an interaction that can be visualized where the MkMPs can deliver dye and observe how the cells uptake the dye. In addition, as we are proposing human MkMPs for the purposes of treating thrombocytopenia, we are interested in the developing of an experimental model to show the effectiveness of MkMPs in thrombocytopenic treatment.

The second aim will utilize the thrombocytopenic mouse model and human MkMPs to show that human MkMPs can reverse the phenotype produced by thrombocytopenia. To do so, we must inject mice with an agent that has been known to sharply reduce platelet levels to a condition known as thrombocytopenia (Apostolidis, Woulfe et al. 2012). Once platelet levels have stabilized and significantly decreased, we will perform a rescue injection into the mice to observe the effectiveness human MkMPs have on the platelet levels. These two aims coupled together will give us a better understanding in how MkMPs, specifically human MkMPs, can change the microenvironment of the body in times of low platelets and effectively elucidate the role of MkMPs in an *in vivo* system. Overall, MPs can be used as an answer for the high cost of platelet production *ex vivo* as well as prevention of allo-immunization and the need for constant platelet donors (Luff and Papoutsakis 2016). By utilizing MPs, purchasing of costly cytokines can be effectively reduced and as HSCs are collected from donors, the MPs will express all the necessary proteins to allow the donor to recognize the MPs as self. In conclusion, I plan to show that MPs can be used as a novel treatment for the condition known as thrombocytopenia by showing the MPs effectiveness *in vivo*. First, I would like to show that MPs can react with hematopoietic stem cells *ex vivo*. Once the interaction has been established, I will

then introduce the MPs into mice that have been depleted of platelets through an injection of antibodies to determine the effectiveness of a MP treatment and to see if the phenotype can be rescued.

Chapter 2

MATERIALS AND METHODS

2.1 Mice

All procedures involving mice were approved by the University of Delaware Institutional Animal Care and Use Committee and are in agreement with the guide for the care and use of laboratory animals published by the National Research Council of the National Academies, 8th ed., Washington, D.C (publication 85-23, revised 2011). Female 4-6 weeks old Balb/c mice were purchased from Jackson Laboratories and housed at the University of Delaware Animal Facility with access to free food and water.

2.2 Extraction of Bone Marrow from Mice

Extraction of bone marrow was performed based on the procedure published by the Abdel-Wahab group (Chung, Kim et al. 2014). Mice were collected and are sacrificed via CO_2 asphyxiation followed by cervical dislocation to confirm death. Mice were then prepared by spraying them with 70% ethanol. Mice were cut open above the hip and the femurs are extracted by cutting the areas where the femur meet the tibia and the hip. Collected femurs were placed in RPMI 1640X (Fisher Scientific Cat # 11875085) with 10% FBS and 1% Antibiotic-Antimycotic (Gibco 15240062) to prevent neutrophil activation. Femurs were then decontaminated by triple washes of Phosphate Saline Buffer (PBS) with 1% Antibiotic-Antimycotic. Once decontaminated, the epiphyses of the femur were cut and a 20G needle attached to a

12cc syringe filled with media was passed into the femur. The effluent was collected and passed through a pre-separation filter (30 microns, Miltenyi Biotech, 130-041-407). This process continues until the bone appeared white.

2.3 Linage Depletion and Harvesting of Murine Hematopoietic Stem Cells

Cells collected as described above were spun at 300 *g* for 10 minutes. After, the spin, the supernatant was discarded. Red blood cells were depleted following the protocol depleted in Fuhrken et. al (Fuhrken, Apostolidis et al. 2008). The cell pellet was treated with Ammonium- Chloride- Potassium (ACK) buffer for 5 minutes. After that, the cells were spun at 300 *g* for 10 mins. The cell pellet was collected and the supernatant was discarded. The pellet was washed twice with PBS +1% Antibiotic-Antimycotic.

The remaining cells were selected through MACS cell sorting (Miltenyi Biotech). The cells were stained with an antibody cocktail that targets blood cell line committed to any blood lineage. Following the manufacturer's instructions of the lineage depletion kit (Miltenyi Biotech, 130-090-858) 10 µL of the antibody cocktail was added for every 10^7 cells. In addition, 40 µL of PEB (PBS+EDTA+BSA) were added to the cell pellet for every 10^7 cells. After 10 mins, the cells were spun at 300 *g* for 10 minutes, the supernatant was discarded and 20 µL of Anti-Biotin microbeads and 30 µL of PEB were added for every 10^7 cells. The cells were then passed through a magnetic column, where the column was washed with PEB 4 times to ensure collection of the cells. The effluent is the hematopoietic stem cell population. Finally, the effluent collected from the column was centrifuged at 300 x *g* for 10 minutes to remove the supernatant from the MACS cell sorting system.

2.4 Culture of Murine Hematopoietic Stem Cells to Megakaryocytes

Cells were cultured in Iscove's modified Dulbecco's medium +20% Bovine Serum Albumin, Insulin and Transferrin (BIT, StemCell Technologies, Canada 09500) + 1% Anti-Anti with 2.5 ng/mL recombinant human interleukin (rhIL)-3 (Perprotech Inc. 200-03), 10 ng/mL of rhIL-6, rh IL-11 (Perprotech Inc., 200-06, 200-11), 100 ng/mL recombinant human Thrombopoietin (rhTPO) (Perprotech Inc., 300-18), 100 ng/mL recombinant human stem cell factor (rhSCF) (Perprotech Inc. 300-07) and 1 µg/mL of lipoprotein, low density from human plasma (hLDL) (Sigma-Aldrich L7914). Cells were incubated at 5% CO_2, 5% O_2, 95% relative-humidity, 37ºC for 3 days at 37 ºC afterward the cells were transferred to 20 % O_2 and 5% CO_2 for 3 days at 37ºC. At Day 6, culture media was collected and the MPs were analyzed for expression of megakaryocytic cell surface markers.

2.5 Culture of Human Hematopoietic Stem Cells to Megakaryocytes

Frozen human G-SCF-mobilized peripheral blood $CD34^+$ cells (purchased from Fred Hutchinson Cancer Research Center) were cultured as described previously (Panuganti, Schlinker et al. 2013). Cells were thawed and plated for 5 days in IMDM medium supplemented with 20% BIT+ 1% Anti-Anti+ 100 ng/mL rhTPO+ rhSCF+ 2.5 ng/mL of rhIL-3, 10 ng/mL of rhIL-6, rhIL-11 and 1 µg/ml of hLDL in 5% CO_2, 5% O_2, 95% relative-humidity, 37ºC. On Day 5, the cell-culture suspension was collected and centrifuged at 300 *g* for 10 minutes to collect the cells for re-suspension in new media. For Days 5-7 the culture medium of cells comprised of IMDM medium+ 20% BIT+ 1% Anti-Anti, 100 ng/mL rhTPO+ rhSCF, 10ng/mL rhIL-3, rhIL-9, rhIL-11, 1 µg/mL of hLDL, and the culture was incubated in 5% CO_2, 20%

O_2, 95% relative-humidity, 37ºC. On Day 7, dead cell removal and $CD41^+$ enrichment was performed.

First, the cell culture medium was spun at 300 *g* for 10 minutes to remove the supernatant. After that, the cells were re-suspended specific microbeads (Miltenyi 130-090-101) to remove dead cells. 100 µL of such microbeads was added for every 10^7 cells. The cell-microbead suspension was incubated for 15 minutes at room temperature. Afterward, 500 µL of 1X binding buffer provided with the microbeads was added to the suspension. Afterward, the cell suspension was passed through a MS column (Miltenyi 130-042-201) setup utilizing the MACS system and the manufacturer's protocol. The live cells that passed were collected in a 15mL conical tube. The column was washed 4 times with 500 µL of 1X binding buffer. After the wash, 7 mL of PEB buffer (PBS+ 5% BSA+ 2mM of EDTA) was added to the cell suspension and then the suspension was centrifuged at 300 *g* for 10 minutes.

Afterward, the supernatant was discarded and CD61 microbeads (Miltenyi 130-051-101) were added to the cell pellet. 20 µL of microbeads and 80 µL of PEB were added for every 10^7 cells. The suspension was then incubated at 4ºC for 15 minutes. Afterward, the cells were centrifuged at 300 *g* to remove excess microbeads and the cells were re-suspended in 500 µL of PEB. The cell suspension was then passed through an LD column (Miltenyi 130-042-901). The labeled cells were then captured by the column and the cells were released through forcefully passing 3 mL of PEB through the column in the absence of the MACS setup following the manufacturer's protocol for removing cells trapped in the magnetic column. Afterwards, 7 mL of IMDM were added to the cells and the suspension was spun at 300 *g* for 10 min. After the centrifuge, the supernatant was discarded.

After enrichment $CD41^+$ cells are plated in IMDM medium+ 20% BIT+ 1% Anti-Anti, 100 ng/mL hypo+ risk, 6.25 mm nicotinamide, 1µg/mL hid incubated in 5% CO_2, 20% O_2, 95% relative-humidity, 37ºC. At Day 12, the medium was collected to separate and collect the Moms.

2.6 Preparation of Megakaryocytic Micro particles, their Collection, and Characterization

For all cultures described above, MPs were collected as previously described (Jiang, Woulfe et al. 2014). Cell medium was spun at 1000 *g* for 10 mins and the MP rich supernatant was collected. MPs were enriched through ultra-centrifugation (25000 *g*, for 30 minutes at 4ºC Beckman Coulter Optima Max Centrifuge). After collection, MPs were washed three times with fresh IMDM medium. Characterization of MPs were performed through flow cytometry as described previously (Jiang, Woulfe et al. 2014). Namely, Moms were characterized by staining with FITC-rat anti mouse CD41 (BD Biosciences 561849) for 15 minutes. The concentrations of the MPs were standardized using 1.34 µm microbeads (Spherules).

2.7 Preparation of Platelet Micro particles, their Collection, and Characterization

Blood for isolation of human platelets was collected by venipuncture from adult human volunteers after providing written informed consent as approved by the Institutional Review Board at the University of Delaware (IRB protocol # 622751-1). Blood was collected from healthy donors and PMPs were prepared as follows. First, 50 mL of blood was collected into syringe with ACD buffer (disodium citrate, 65 mm; citric acid, 70 mm; dextrose, 100 mm; pH 4.4) at a volume ratio of 1:6 (Audibled). 6.7 mL of ACD (Acid-Citrate-Dextrose) buffer was prepared for each platelet donor.

After collection at the blood bank, whole blood was centrifuged for 200 x *g* for 10 minutes at room temperature (without brake). Once the blood was separated, the platelet rich plasma was collected and 1 µM of prostaglandin E1 (PGE 1) (Sigma P5515) was added to inhibit platelet activation. The platelet rich plasma was spun at 800 x *g* for 10 minutes at room temperature with no brake to pellet down the platelets. The platelet poor plasma was discarded and the platelets were washed with 5 mL of Tyrode's buffer. After the wash, the platelet pellet was suspended in 10 mL of Tyrode's buffer and 1 mm of $CaCl_2$ was added to the suspension before platelet activation. Platelets were activated by adding 2U/mL of human thrombin (T4393) and incubated at 37ºC for 30 minutes. The activated platelets were then centrifuged at 800 x *g* for 10 minutes. The supernatant was collected and micro particles were collected as previously described.

2.8 Staining of Micro particles with PKH 26 and CFDA-SE Dye

MPs were pelleted and suspended in 200 µL Diluent C (Sigma Cat# G8278). Simultaneously, a 2X dye solution of PKH 26 ethanolic dye solution (Sigma Cat# P9691) was prepared by adding 0.8 µL of PKH 26 to 200 µL of diluent C. After both suspensions were made, the 200 µL of dye solution was added to the 200 µL of the MP suspension. The MPs incubated for 1-5 minutes with occasional mixing. After the incubation period, 400 µL of 1% BSA was added to the MPs suspension and incubated for 1 minute. After the MPs were centrifuged at 25000 rpm for 30 mins. Following the PKH 26 staining MPs were then stained with CFDA-SE dye (life technologies Cat# C1157). Following the centrifugation of the MPs, 1 mL of pre-warmed IMDM was added to the MP pellet and mixed. After mixing, 4 µL of CFDA-SE was added and incubated at 37ºC for 20 minutes. After the incubation period, the

MPs were washed three times with fresh IMDM. After the staining, the MPs were used for co-culture with murine hematopoietic stem cells.

2.9 Co-culture of Human Microparticles with Murine Hematopoietic Stem Cells

Murine HSPCs were collected following the linage depletion protocol as explained previously. In a 24 well plate, 750 µL of growth media (consisting of IMDM+ 20% BIT+ 1% Anti-Anti+ 50 ng/ µL of hSCF) was added to a well. A transwell membrane insert (Costar Cat#3470) was inserted into the well. Following the insertion, cells were added to the membrane. For certain conditions, MPs were added to the membrane as well. For reference, cells and MPs were added at a ratio of 1:30. The cells and MPs incubated for approximately 1.5-5 hours or 5 days depending on experimental procedure. For the 1.5-5-hour incubation period, the cell and MP suspension was plated onto a µ-slide 8 chamber poly-L-lysine (ibidi cat # 80826). Images of co-cultured cells were collected through confocal microscopy (Zeiss LSM880 multiphoton confocal microscope). For studies involving a 5-day co-culture of murine HSPCS to human MkMPs, the cells were incubated with either 50 ng/mL of hSCF, a 1:30 ratio of murine HSPCs to human MkMPs in 50 ng/mL of hSCF, or 50 ng/mL of hTPO with 50 ng/mL of hSCF. After the 5-day incubation, the cells were imaged utilizing the Nikon Eclipse Ti-E microscope.

2.10 Ploidy Assay of 5-day Co-Culture of Murine Hematopoietic Stem Cells and Human MkMPs

Cells were collected after 5 days of culture of murine HSPCs with or without human MkMPs. Cells were washed in triplicate with PEB at 400 x *g* for 5 minutes and the supernatant discarded. Following the washes, cells were incubated with 10 µL of Rat Anti Mouse CD41 (BD biosciences 561849) for 15 minutes at room temperature

in the absence of light. Afterward cells were washed in triplicate. Following the washes, 500 µL of 0.5 % formaldehyde was added to each cell sample for 10 minutes at room temperature. The cells were washed in triplicate after the addition of formaldehyde. After the washes, 500 µL of 70% methanol was added to the cells and the suspension were incubated at 4ºC for 1 hour. The cells were then washed in triplicate. Next, 10 µL of RNase was added the cells were incubated at 37ºC for 20 minutes. After the incubation, 100 µL of propidium iodine (1 mg/mL) was added to the cell suspension. The suspension incubated at room temperature for 20 minutes in the absence of light. After the propidium iodine stain, the cells were analyzed for DNA content and maturity using flow cytometry.

2.11 Induction of Thrombocytopenia

Balb/c mice 4-6 week were weighed before any handling and use in these experiments. Up to 1 µg of LEAF rat anti-mouse CD41 (MWReg 30, BioLegend, 133910) was injected once intraperitoneally per gram of body weight of the mouse depending on the assay. Bleeding was performed at 48 or 72 hours retro-orbitally per the approved protocol by the University of Delaware.

2.12 Platelet Counting Blood Extracted from Mice

Mice were first anesthetized through isoflurane inhalation. Mice were confirmed anesthetized by squeezing toe pad on foot with a pair of tweezers. The squeeze was done to ensure the mice were fully anesthetized. Blood was collected through one eyeball using a non-heparinized glass tube. From the blood collected, 10 µL of blood was collected using capillary tubes provided by the Thrombo-TIC kit (Bioanalytic GmbH 004015-0006). Following the manufacturer's instructions, the

capillary was placed in a tube with 990 µL of 1% oxalate buffer and shaken until the blood was expelled from the tube and dissolved within the solution. Platelet counts were performed by taking 100 µL of the platelet-oxalate solution and analyzed through flow cytometry BDFACS Aria II (Alugupalli, Michelson et al. 2001). Platelets were gated by using the forward-scatter and side-scatter gates and standardized using microbeads of various sizes ranging from 0.2 µm to 1.34 µm. The gating was also confirmed utilizing FITC rat anti-mouse CD41(BD 553848) to distinguish between noise and platelet events.

2.13 Human Megakaryocytic Microparticle Transfusion into Wild type or Thrombocytopenic Mice

Mice were separated into distinct groups based off which treatment they are going to receive. MPs were injected through tail vein injection as previously approved by the University of Delaware. MPs from human cells were collected by differentiating the donated stem cells in media as described above. MPs were counted using flow cytometry and then were then dissolved in 100 µL of saline and the MPs solution was injected into the mice through an intravenous injection into the tail vein once. Platelet counts were performed depending on the experiment after 48 hours or 72 hours post injection of the MPs.

2.14 Analysis of Reticulated Platelets from Murine Blood

Murine blood was collected via retro-orbital bleed as approved by the University of Delaware. Blood was collected through one eyeball using a non-heparinized glass tube. From the blood collected, 10 µL of blood was collected using capillary tubes provided by the Thrombo-TIC kit (Bioanalytic GmbH 004015-0006). Following the manufacturer's instructions, the capillary was placed in a tube with 990

µL of 1% oxalate buffer and shaken until the blood was expelled from the tube and dissolved within the solution. 500 µL of the platelet suspension was mixed with 1 mL of BD Retic-Count (BD 349204) which contained a concentration of thiazole orange at 0.01 mg/mL as described in previous assays (Apostolidis, Woulfe et al. 2012). The platelet suspension was incubated at room temperature in the dark for at least 30 minutes followed by analysis via flow cytometry.

2.15 Statistical Analysis

Statistical analysis was performed using a two- tailed Student's *t*-test or means ± standard error of means (SEM) using Microsoft Excel software. Differences were considered when $P < 0.05$.

Chapter 3

RESULTS

3.1 Human Microparticles Interact with Murine Stem Cells *Ex Vivo* and Induce Stem Cells to Undergo Megakaryocytic Differentiation in the Absence of Thrombopoietin

Before utilizing human MkMPs as a novel agent to ameliorate thrombocytopenia in murine experiments, we must first ask whether there is an interaction between human MkMP and murine HSPCs. Instead of utilizing murine MkMPs, which are hard to generate in the needed large quantities, human MkMPs offer not only an established protocol to produce them in large batches but ultimately will be the agent that will potentially be ameliorating thrombocytopenia in humans (Jiang, Woulfe et al. 2014). With that in mind, we utilized a similar co-culture approach to carry out the study to examine if human MkMPs can interact with murine HSPCs (Jiang, Woulfe et al. 2014, Jiang, Kao et al. 2016). The co-culture design utilized 2 dyes to study the uptake of human MkMPs by murine HSPCs: the membrane-staining red dye PKH26 and the green cytosolic dye CFDA SE (CFSE) dye. Application of these two dyes makes it possible to microscopically visualize the process of human MkMP uptake by murine HSPCs.

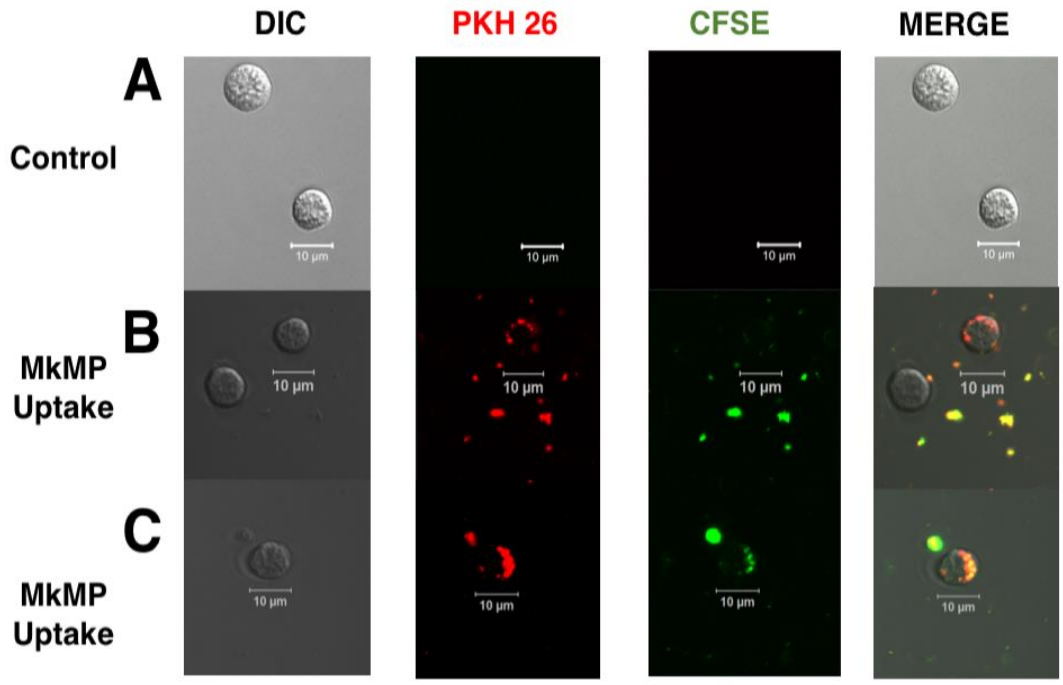
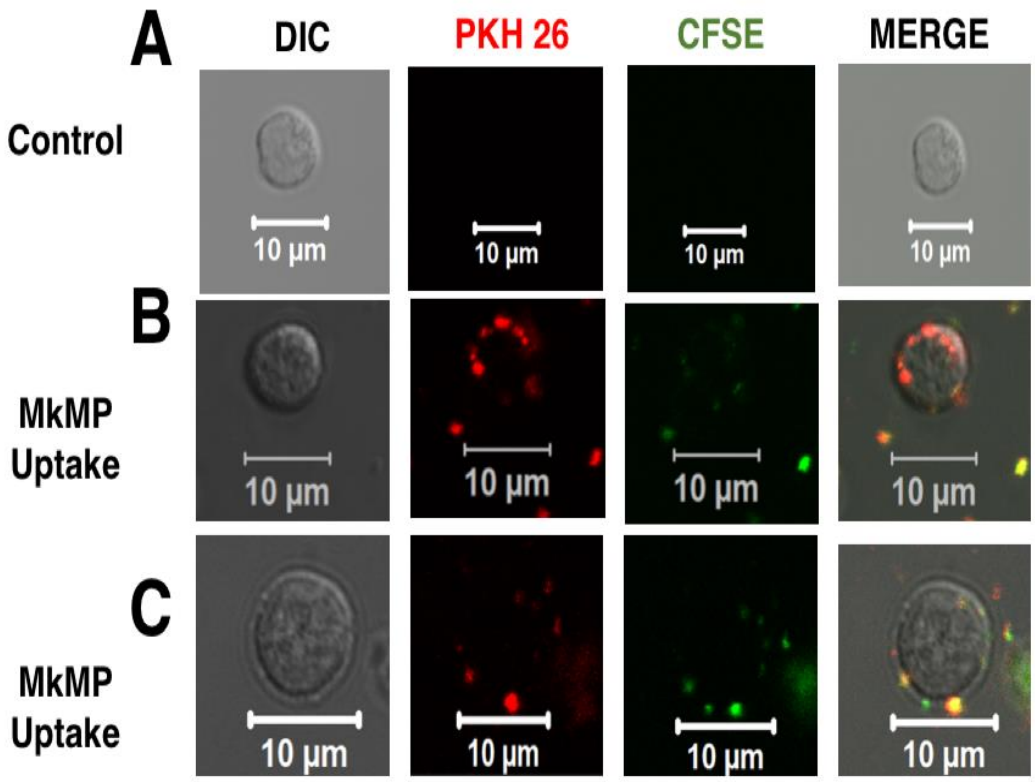
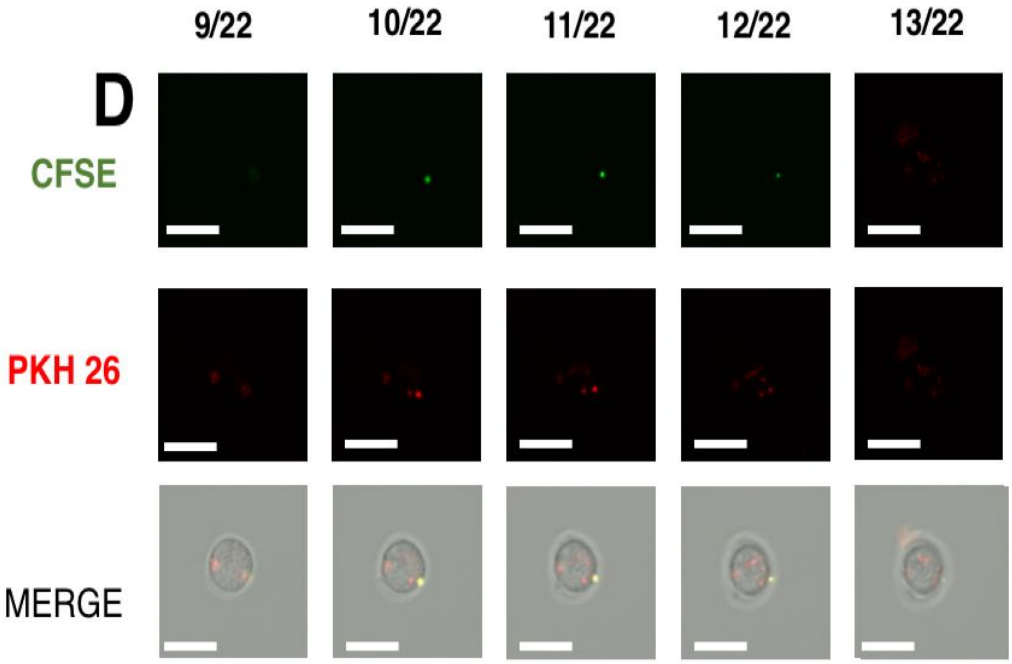


Figure 3. **Human MkMPs interacting with Murine HSPCs after 1.5 hours of co-culture.**

Human MkMPs interact with murine HSPCs after being co-cultured for 1.5 hours. Human MkMPs were stained with PKH 26 dye followed by CFDA-SE (CFSE) (green) dye which were co-cultured with murine HSPCs after a 12-hour culture following lineage depletion. Fluorescent and Differential Interference Contrast (DIC) images were collected via confocal microscopy. (A) Vehicle control cultures without human MkMPs display no background red or green signal. (B - C) Human MkMP co-cultures (~ 1.5 hr.) contain cells with intact fluorescence appearing on the surface of the cells indicating the possibility of human Mumps uptake by murine HSPCs.


A
DIC
PKH 26
CFSE
MERGE
Control
B
MkMP
Uptake
C
MkMP
Uptake
10 µm


9/22
10/22
11/22
12/22
13/22
D
CFSE
PKH 26
MERGE

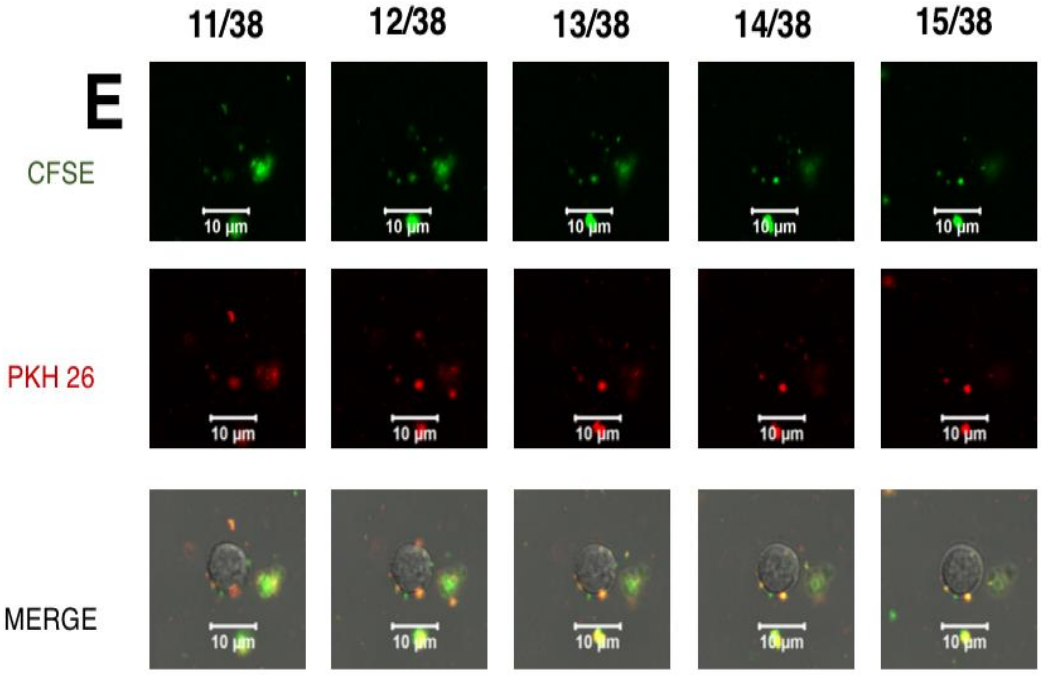

11/38
12/38
13/38
14/38
15/38
E
CFSE
PKH 26
MERGE
10 µm

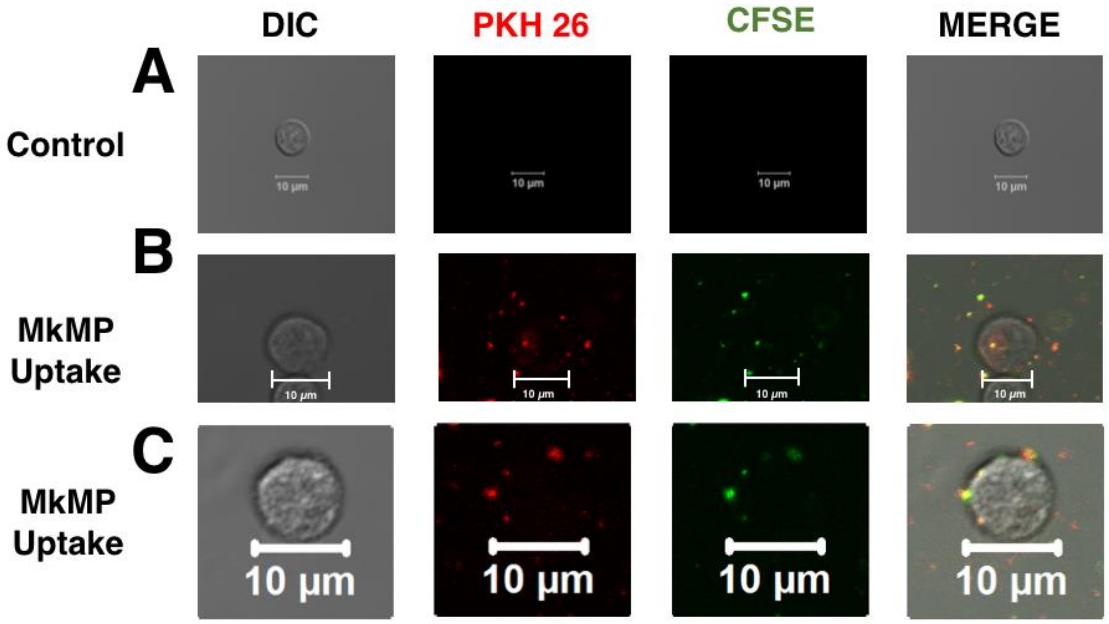
Figure 4. **Human MkMPs interacting with Murine HSPCs after 3 hours of co-culture.**

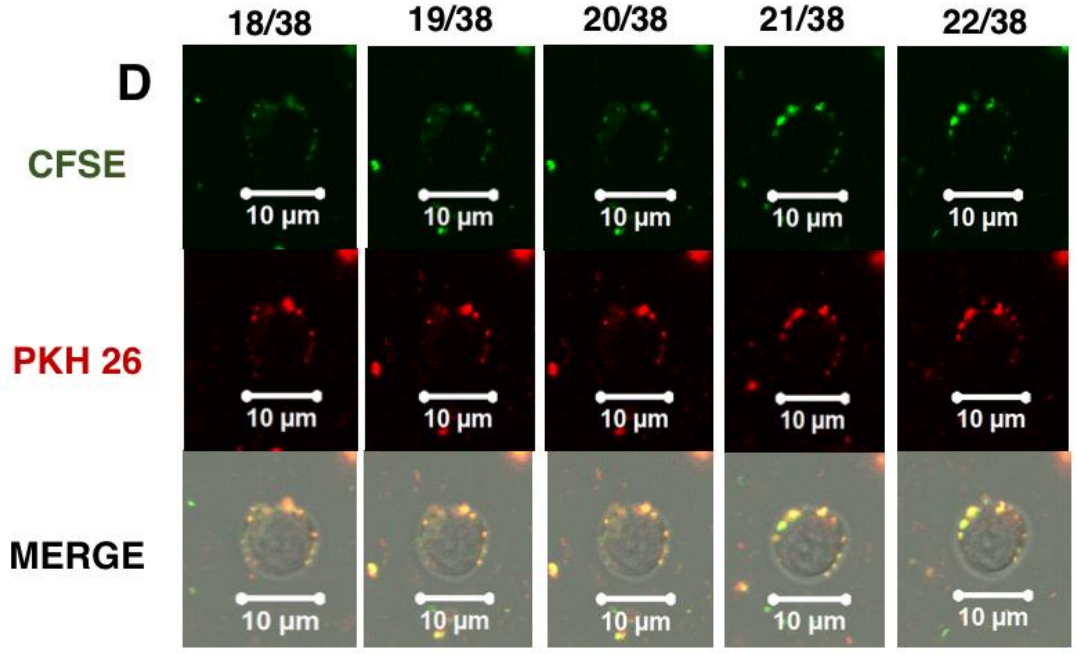
Human MkMPs show an interaction with murine HSPCs after being co-cultured for 3 hours. Human MkMPs were stained with PKH 26 dye followed by CFDA-SE (CFSE) (green) dye and then co-cultured with murine HSPCs after a 12-hour culture following lineage depletion. Fluorescent and Differential Interference Contrast (DIC) images were collected via confocal microscopy. (A) Control cultures without human MkMPs display no background red or green signal. (B–C) Human MkMP co-cultures (~ 1.5 hr.) contain cells with intact fluorescence appearing on the surface of the cells indicating a possibility of an uptake occurring.

As shown in Figure 3, the co-culture of stained human Moms with PKH 26 and CFSE dye shows co-localization of the human MkMPs on the cellular membrane of the HSPCs after 1.5-hours of co-culture. We note that the PKH 26 is a cellular membrane dye while the CFSE dye stains all cellular content of the MkMPs (Gray, Mitchell et al. 2015). Thus, upon uptake of the human MkMPs by murine HSPCs, the PKH 26 dye will remain as a signal on the MkMP surface, while the CFSE dye will spread throughout the cytosol of the cell as the human MkMP are engulfed by the HSPC in the event of a membrane fusion. In Figure 3, we can observe an interaction consistent with a membrane fusion of the human MkMPs and the murine HSPCs (Raposo and Stoorvogel 2013). Confirmation of this interaction is the spreading of the green CFSE dye throughout the target cell while the PKH 26 dye remains on the MkMP membrane. This interaction is consistent with one of the ways MPs interact with target cells (Raposo and Stoorvogel 2013).

The next question was to determine if a longer co-culture incubation period can lead to more co-localization events of human MkMPs to murine HSPCs. When the HSPCs are co-cultured for 3 hours, the dye loaded MkMPs adhere to the surface of the cells. As shown in Figure 4, the PKH26 red color from the human MkMPs are localizing strongly around the cellular membrane, however the CFSE dye hasn't spread throughout the cytosol of the cell. Upon closer observation, Figure 4B and 4C both depict strong localization of human MkMPs dyed with PKH 26 and CFSE dye around the outer edges of murine HSPCs. In addition, the Z-stack represented in Figure 4E also shows this strong localization of human MkMPs around the membrane of the murine HSPCs and in addition, demonstrates that these human MkMPs are in the same plane as the cell and not false results above or below the cell. These data

indicate that there appears to be interaction as the human MkMPs are localizing in the area of the cell however, the CFSE dye has not spread throughout the cytosol as a true fusion has not occurred. An explanation may be that the human MkMPs haven't been completely digested in the cells.


DIC
PKH 26
CFSE
MERGE
A
Control
B
MkMP
Uptake
C
MkMP
Uptake
10 µm


18/38
19/38
20/38
21/38
22/38
D
CFSE
PKH 26
MERGE
10 µm

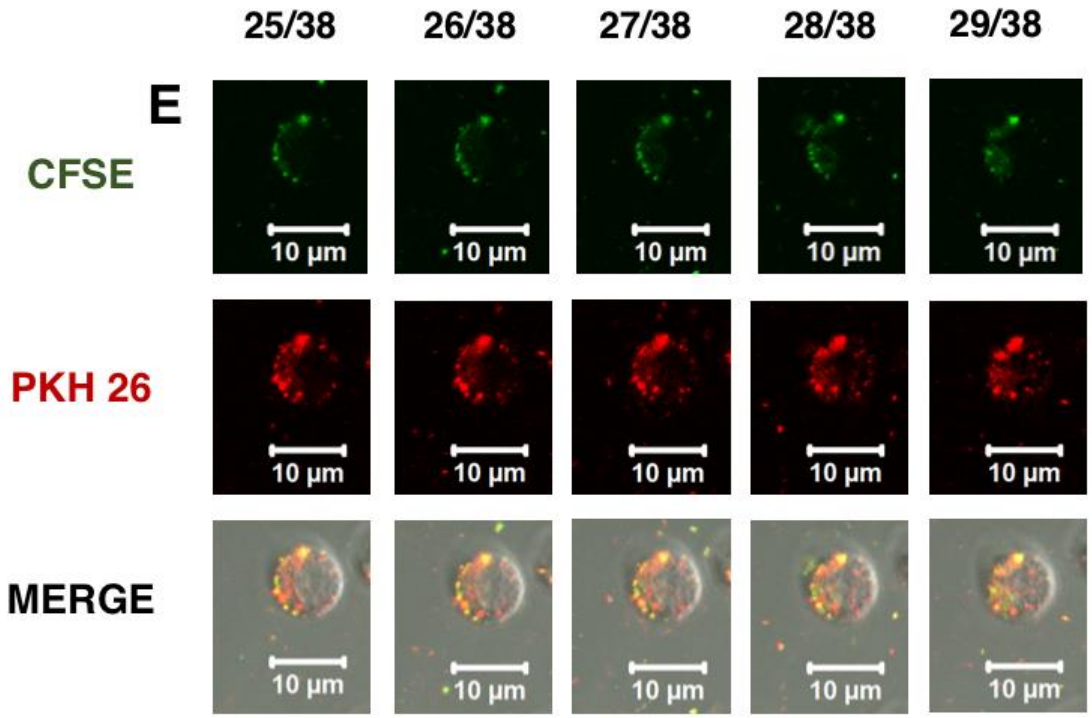
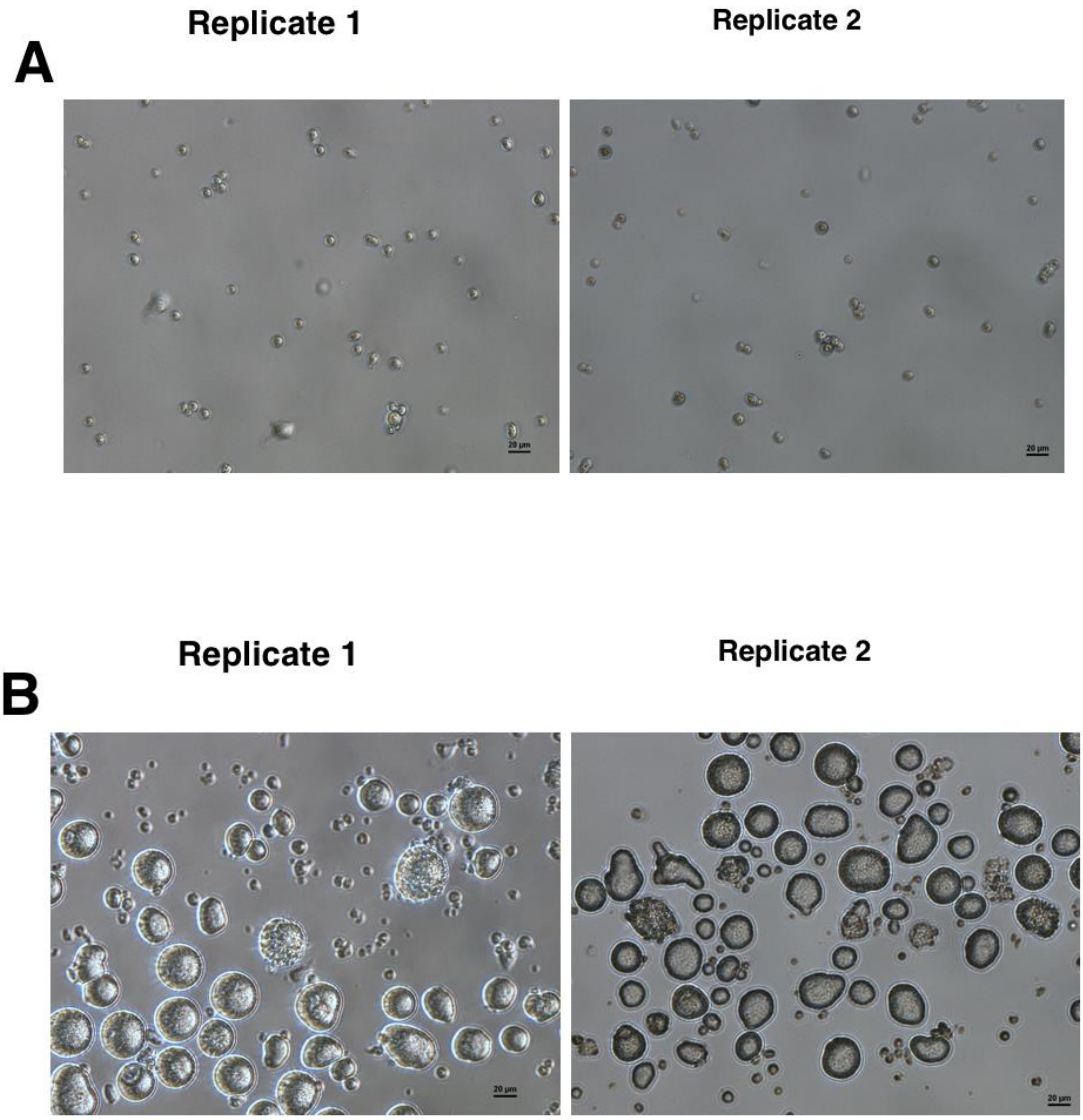


Figure 5. **Images of human MkMPs interacting with murine HSPCs after 5 hours of co-culture.**

Human MkMPs show an interaction and uptake through membrane fusion with murine HSPCs after being co-cultured for 5 hours. Human MkMPs were stained with PKH 26 dye followed by CFDA-SE (CFSE) (green) dye and then co-cultured with murine HSPCs after a 12-hour culture following lineage depletion. Fluorescent and Differential Interference Contrast (DIC) images were collected via confocal microscopy. (A) Vehicle control cultures without human MkMPs display no background red or green signal. (A) Human MkMP co-cultures (~ 5 hr.) contain cells with intact fluorescence appearing on the surface of the cells indicating a possibility of an uptake occurring with intact signals appearing around the membrane. This represents a possibility of membrane fusion of human Moms with murine HSPCs. (B) Human MkMPs are depositing their CFSE dye to the target HSPC. The CFSE spreads throughout the cytosol of the cell as the membranes of the human MkMP and the HSPC fuse. Strong signals of PKH 26 appear also on the outer membranes of the murine HSPCs indicating the strength and quantity of the human MkMPs.

Although data supported the interaction of human MkMPs with murine HSPCs, the cytosolic CFSE dye has not spread through the cytosol as previously reported (Jiang, Kao et al. 2016). The possibility exists that the incubation period was not sufficient to observe the uptake of human MkMPs by the murine HSPCs. As such, a longer co-culture incubation period from 3 hours to 5 hours was performed.

Figure 5 shows that after 5 hours of co-culture an interaction of human MkMPs and murine HSPCs were occurring. As seen in Figure 5C the membrane of the murine HSPCs were stained indicating that human MkMPs have been involved in membrane fusion with murine HSPCs. Figure 5D and Figure 5E shows a Z-stack of the human MkMPs at different slices showing this strong localization of human MkMPs around the membrane of the murine HSPCs and in addition, demonstrates that these human MkMPs are in the same plane as the cell and not false results above or below the cell. In addition, both Figures 5D and Figure 5E show the CFSE dye spreading throughout the cytosol of the cell, confirming the human MkMPs have fused membranes with the murine HSPCs and deposited their CFSE dye thus confirming an interaction and an uptake.


A
Replicate 1
Replicate 2
B
Replicate 1
Replicate 2

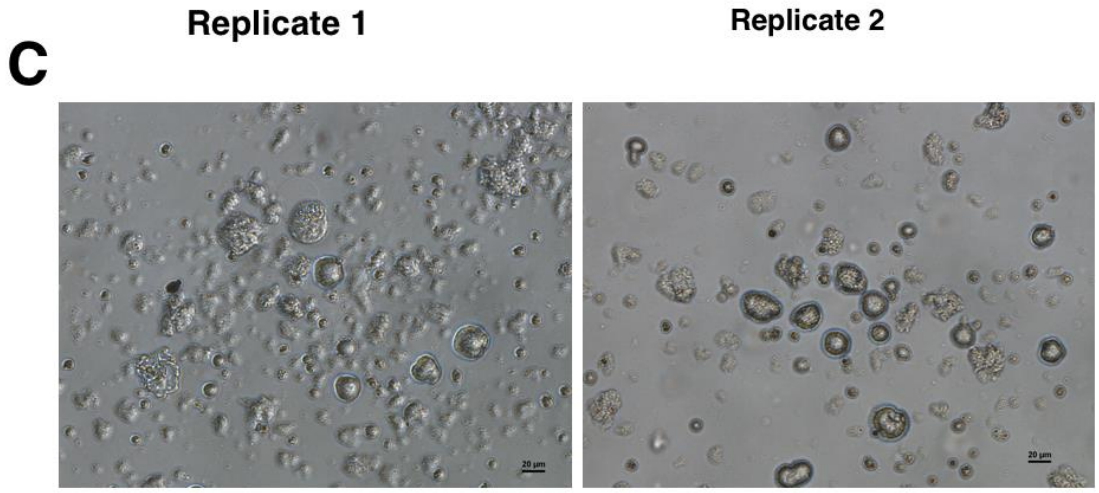


Figure 6 **Co-culture of Human MkMPs and murine HSPCs show an increase in *ex vivo* megakaryopoiesis**

Human MkMPs were co-cultured with murine HSPCs for 5 days and bright field images were obtained. (A) Control Images of murine HSPCs cultured in media with human SCF (50 ng/mL). (B) Murine HSPCs cultured with rhSCF (50 ng/mL) with rhTPO (50 ng/mL), show larger cells indicative of megakaryopoiesis. (C) Human MkMP co-cultured with murine HSPCs in media only containing rhSCF (50ng/mL) show an increase in the amount of larger cells indicative of megakaryopoiesis compared to the control Scale bar = 20 µm. N=4 for biological replicates. N=3 technical replicates of human MkMP/ murine HSPC co-cultures.

We demonstrated above that human MkMPs interact with and are taken up by murine HSPCs. The goal of these experiments was to address the question as to whether or not human MkMPs can induce the megakaryocytic differentiation of murine HSPCs as they do for human HSPCs (Jiang, Woulfe et al. 2014). To answer, the question, we cultured freshly harvested into media without any cytokines that can cause the murine HSPCs to undergo megakaryopoiesis such as TPO as a negative control (Control), media containing TPO as a positive control (TPO) and media containing human MkMPs (huMkMPs).

We first examined the cultured cells at day 5 of the three types of cultures (Control, TPO, MkMPs) for observable differences in the number and size of cells at day 5. As seen in Figure 6A, in the control culture with only risk added, there appears to be no signs of megakaryocytes as most of the cultured cells remained small and well below the scale of 20 microns in diameter. In Figure 6B, in the presence of TPO, there is an observable increase in the number of large cells in both replicates, indicating that the murine HSPCs have undergone megakaryopoiesis. In the co-culture group (Figure 6C), there is an observable increase in the number of larger cells compared to that of the control, indicating the possibility that co-culture with the human MkMPs is driving the murine HSPCs to undergo megakaryopoietic differentiation.

To further show that indeed the human MkMPs are causing the murine HSPCs to differentiate, we quantified the cultured cells for the mekagaryocytes ($CD41^+$ cells) and their ploidy classes using flow cytometric analysis (Figure 7).

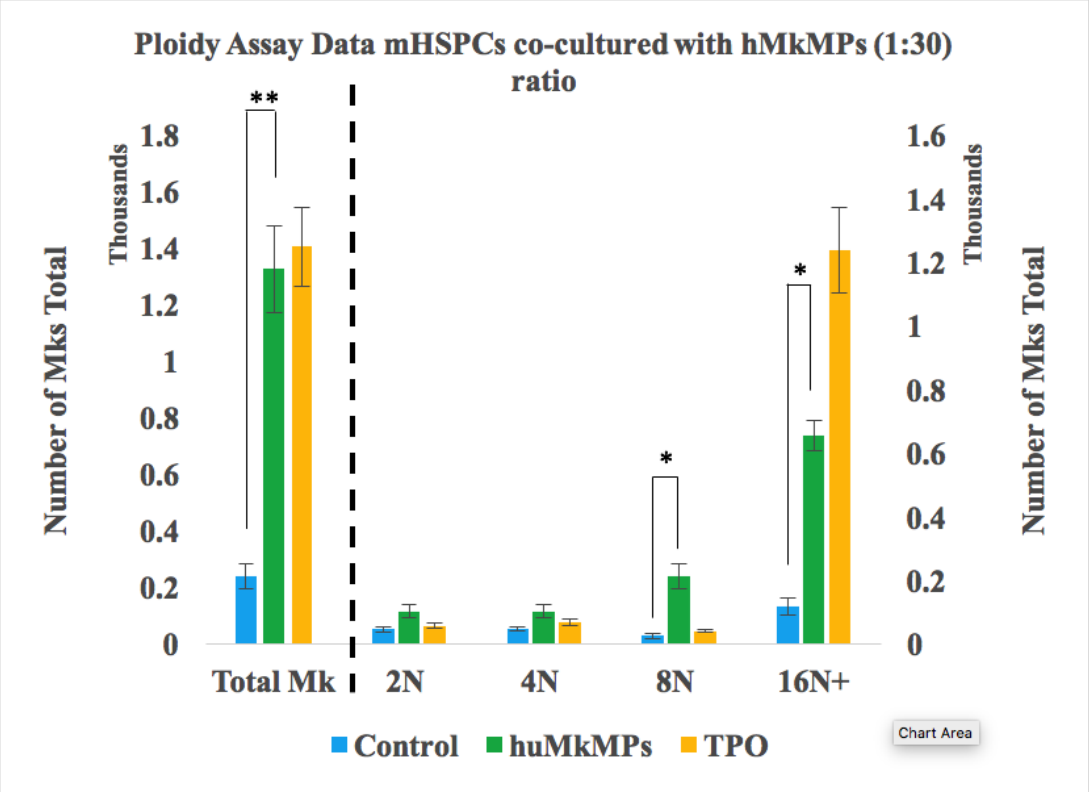


Figure 7 **Human MkMPs promote the megakarocytic differentiation of murine HSPCs.**

Graph shows the total number of megakaryocytes after 5 days of culture. Murine HSPCs were cultured either without any TPO/human MkMPs (Control), with human MkMPs (huMkMPs) or with TPO (TPO). After 5 days, cells were counted, stained with propidium iodine, and rat anti mouse CD41 antibody and quantified based off total number of megakaryocytes as well as ploidy class. N=4 for all groups. Error bars ± 1 S. E. *= P<0.05, **= P<0.01.

As seen in Figure 7, there is an increase in the number of megakaryocytes in the murine HSPCs culture containing human MkMPs compared to the culture which only contained SCF, indicating that the human MkMPs can drive the murine HSPCs to undergo megakaryopoiesis (Figure 7). Observing the different ploidy classes, the data indicates that there is an increased number of megakaryocytes in the mature ploidy classes (8N and above) in murine HSPCs co-cultured with human MkMPs compared to the "control" showing that the human MkMPs can increase the number of mature megakaryocytes rather than simply increasing the number of megakaryocytes in the lower ploidy classes, thus demonstrating that the human MkMPs can deliver their cargo and drive megakaryopoiesis in murine HSPCs.

These data support that hypothesis there is an interaction of the human MkMPs with murine HSPCs and that interaction is similar to the interaction seen with the co-culture of human MkMP and human HSPCs indicating that the mechanism of uptake is conserved (Jiang, Woulfe et al. 2014). In addition, these results show that there is cross reactivity between human MkMPs and murine HSPCs in that human MkMPs can be taken up by and induce the megakaryocytic differentiation of murine HSPCs. This interaction is crucial for the design of experiments to treat mice with human MkMPs aiming to examine if human MkMPs can induce *in vivo* platelet biogenesis in mice.

3.2 Transfusion of Human Megakaryocyte-Derived Microparticles in Wild Type Mice Alters the Murine Platelet Concentrations

After determining that, *ex vivo,* human MkMPs can interact with murine HSPCs isolated from the bone marrow, we next examined if the interaction of human MkMP and murine HSPCs is conserved and if this interaction can lead to *de novo*

platelet biogenesis and would result in increases in platelet concentration in the murine circulation. As mentioned previously, megakaryocytes are the precursor to platelets, and if human MkMPs can induce murine HSPCs to differentiate into megakaryocytes, then newly synthesized platelets might result in increased platelet concentrations. As observed previously (Figure 3, Figure 4, Figure 5, Figure 6), human MkMPs can interact with murine HSPCs. If this cross reactivity observed *ex vivo* is conserved in the *in vivo* setting, then transfused human MkMPs might result in an increase in biogenesis of murine platelets.

An important decision we sought to make was to decide the number of MkMPs to be injected into the mice. Literature searches revealed a broad range in the number of MP used and injection/delivery routes (Table 1).

MP Type	Strain of Mice Used	Dosage of MPs	Route of Administration	Journal Reference
Platelet/Leukocyte MPs	C57BL/6	80,000-160,000 MPs/dose	Intraverebroventricular injection	(Ramacciotti, Hawley et al. 2009)
Endothelial MPs	C57BL/6	10,000,000 MPs/dose	Intravenously	(Jansen, Yang et al. 2013)
Endothelial Exosomes	C57BL/6	10,000,000 exosome/dose	Intranasally	(Kim, Haney et al. 2016)
Tumor Exosomes	Balb/c	10,000,000 exosomes/dose	Intravenous	(Takahashi, Nishikawa et al. 2013)
Tumor Exosomes	C57BL/6	260,000,000 exosome/dose	Intravenously	(Plebanek, Angeloni et al. 2017)
Endothelial MPs	C57BL/6	2,000 MPs/dose/mL of mouse blood	Intravenously	(Buesing, Densmore et al. 2011)
Cancer MPs	C57BL/6	18,000 MPs/dose/g of mouse	Jugular veins/intravenously	(Thomas, Panicot-Dubois et al. 2009)

Table 1. **Reference table of MP usage in research.**

Reference table indicating the broad range of extracellular vesicle numbers and administration routes used in murine experiments.

Based on this literature survey, a consensus was reached that the human MKMPs should be injected through an intravenous route (i.e., be infused into the mice) to maximize the probability that the infused human MkMPs will reach their target murine HSPCs.

The first study we carried out was to examine if human MkMP infusion can alter the steady state of murine platelet concentration. For these studies, we used wild type mice for two reasons. First, there were chosen due to their availability and low cost compared to NOD/SCID mice. In addition, other research groups have used MPs without utilizing an immunocompromised strain or depleting the macrophages in the mice (Jansen, Yang et al. 2013, Jansen, Yang et al. 2015). Secondly, we determined that if human MkMPs are fast to reach and program murine HSPCs, these human MkMPs will not stay in circulation in the mouse long enough to be cleared by macrophages or induce an immune response. Even if macrophage clearance is at play, we thought that we might still be able to observe a phenotype through changes in platelet concentration.

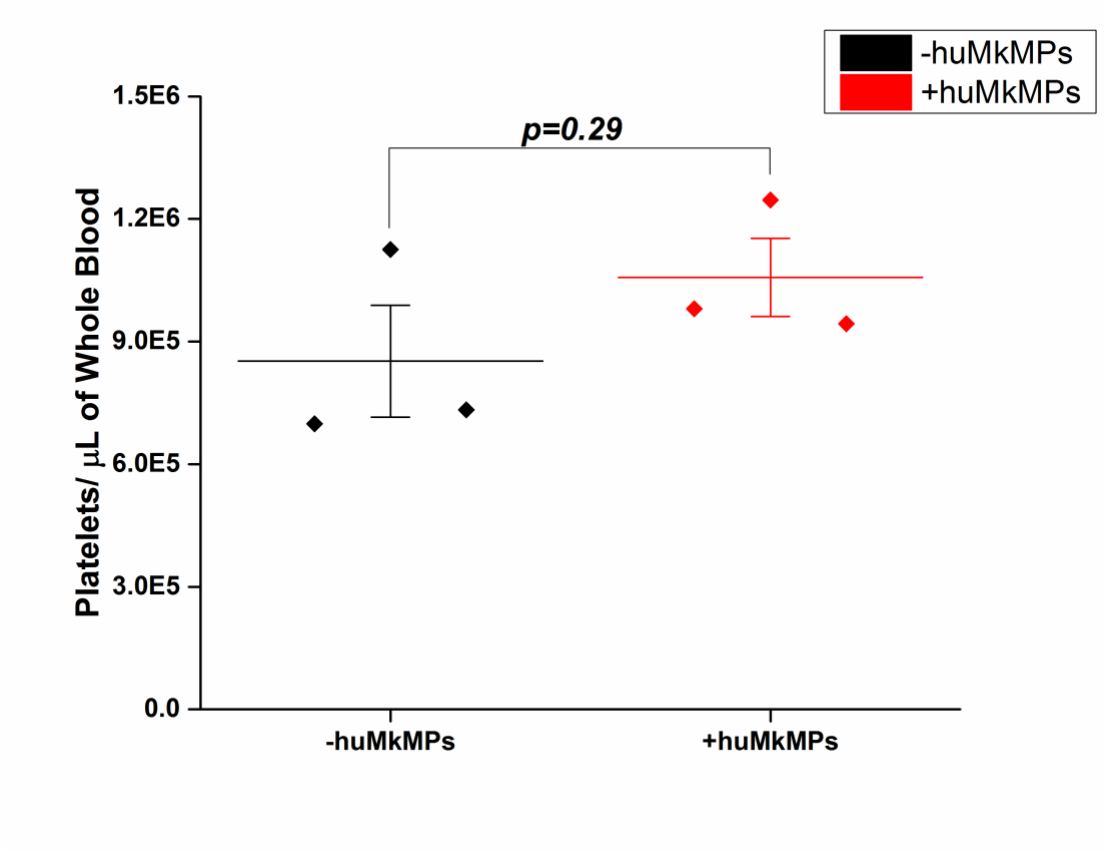


Figure 8. **Infusion of 2,000,000 human MkMPs into non-thrombocytopenic mice.**

Platelet concentration in mice 72 hours after an injection of MkMPs intravenously. Mice in the "+HuMkMPs" group received an infusion of 2,000,000 human MkMPs. -HuMkMPs mice received a dose of saline instead of MkMPs. Blood was collected through retro-orbital bleed and platelet counts were analyzed via flow cytometry. For both groups: N=3. Error bars = ± 1 S.E.

The first study was infusion of 2,000,000 human MkMPs into each mouse in the "+HuMkMPs" group. As shown in Figure 8, the concentration of platelets in mice that received human MkMPs was higher compared to the saline control (the "-HuMkMPs"), but the effect was not statistically significant. This indicates that

transfusion of human MkMPs may be affecting platelet levels in mice. However, it is possible that the human MkMP dose used did not provide a significant effect that can be attributed to the number of human MkMPs being transfused, or that the sampling time was not optimal for observing an effect. To remedy the effect, we decided to increase the dose of human MkMPs from 2,000,000 to 4,000,000 human MkMPs (Figure 8, Figure 9).

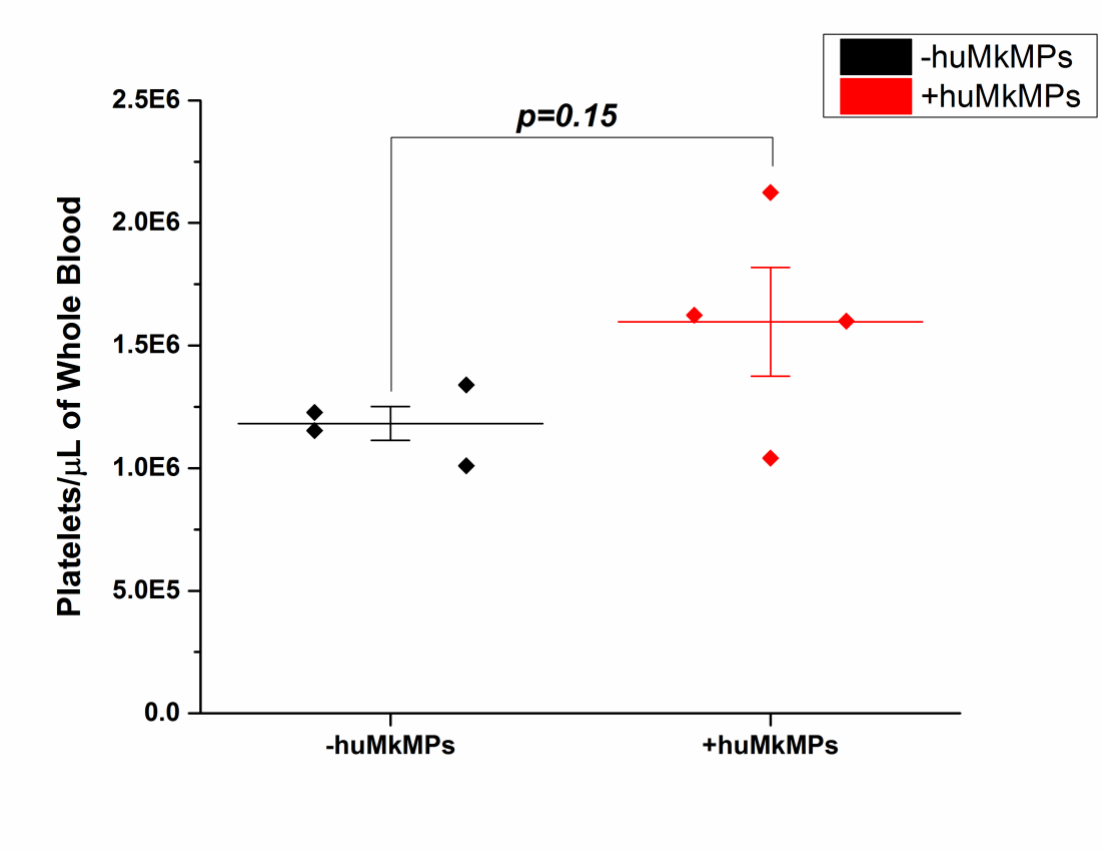


Figure 9. **Infusion of 4,000,000 human MkMPs into non-thrombocytopenic mice.**

4,000,000 human MkMPs ("+HuMkMPs") were infused into wild type mice at 0 hrs. through intravenous injection. Control mice ("-HuMkMPs") received a dose of saline in place of the human MkMP injection. 72 hours post infusion, blood was drawn retro-orbitally and platelets were analyzed through flow cytometry. For both Control and human MkMP groups: N=4. Error bars = ± 1 S.E.

As seen in Figure 9, when human MkMPs were introduced at a dose of 4,000,000 human MkMPs / mouse and blood was sampled at 72 hours, there was an increase in platelet concentrations in circulating blood but again, the increase was not statistically significant ($p=0.15$). We thus decided to increase the dosage from to 6,000,000 human MkMPs.

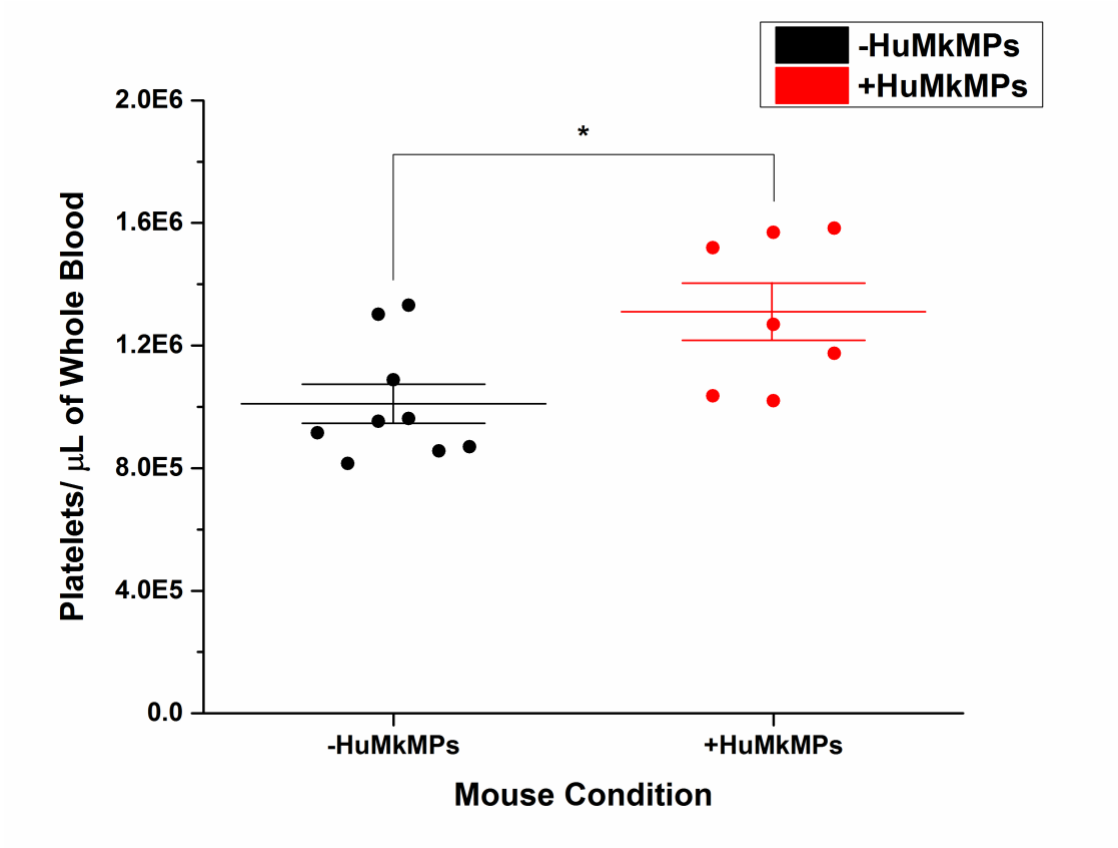


Figure 10. **Infusion of 6,000,000 human MkMPs into non-thrombocytopenic mice.**

6,000,000 human MkMPs ("+HuMkMPs) were infused into wild type mice at 0 hrs. through intravenous injection. Control mice (-HuMkMPs) received a dose of saline in place of the human MkMP injection. 72 hours post injection, blood was drawn retro-orbitally and platelets were counted through flow cytometry. N=9 for Control group, N=7 for human MkMP (Microparticles) group. Error bars = ± 1 S.E. * indicates $P<0.05$.

Upon increasing the dosage of the MPs from 4,000,000 to 6,000,000 human MkMPs per injection, there was an observed significant change in platelet

concentration compared to the control mice (Figure 10). Taken together, these data suggest that platelet concentrations increase in a dose dependent manner upon infusion of human MkMPs. We next wanted to examine if the infusion of human MkMPs can be used to ameliorate induced thrombocytopenia in mice.

3.3 Infusion of human MkMPs ameliorates antibody-induced thrombocytopenia in mice

Thrombocytopenia may be caused by several mechanisms and agents. For our studies, we will utilized a murine thrombocytopenic model whereby thrombocytopenia was induced by injecting an anti-CD41 antibody to deplete the circulating platelets (Apostolidis, Woulfe et al. 2012). To establish a thrombocytopenic murine model, a common method is to inject an antibody against a major surface antigen of platelets. The antibody results in an thrombocytopenic response by recruiting phagocytic macrophages that destroy cells that express the CD41 antigen, namely platelets and Mks (Neschadim and Branch 2015). Here, we utilized an Anti-CD41 antibody (LEAF [Low endotoxin Azide Free] Anti-CD41 antibody (Biolegend MWReg30 Cat # 133910), which has been shown to decrease platelet concentration significantly compared to controls (Marjon, Marnell et al. 2009, Sullivan, Wang et al. 2010, Apostolidis, Woulfe et al. 2012).

Once thrombocytopenia was induced, we infused human MkMPs to the thrombocytopenic mice to examine if they would ameliorate the low platelet concentration in the murine blood.

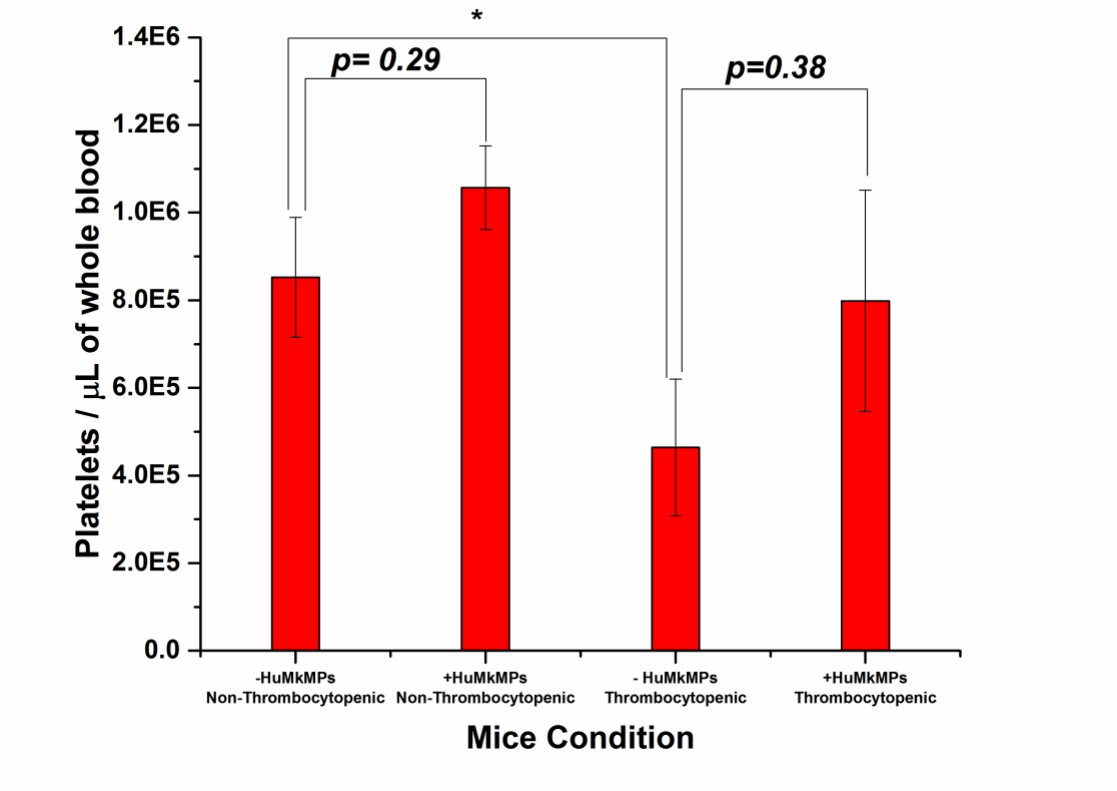


Figure 11. **Infusion of human MkMPs in mice following induced thrombocytopenia**.

At time 0, mice received a dose of 200 µL of saline (non-thrombocytopenic groups) or 0.5 mg of anti-CD41 antibody per kg of mouse body weight dissolved in 200 µL of saline ("thrombocytopenic groups"). 8 hours post antibody injection, mice were transfused with 200 µL of saline ("-HuMkMPs) or 2,000,000 human MkMPs (+HuMkMPs) suspended in 200 µL of saline. Blood was collected via retro-orbital bleed 72 hours post antibody injection and analyzed via flow cytometry. N=3 for all groups. Error bars are ± 1 S.E. * = $P < 0.05$.

The first set of experiments was carried out using 2,000,000 human MkMPs for infusion into both non-thrombocytopenic mice and thrombocytopenic mice. The results (Figure 11) demonstrated an increase (albeit not statistically significant) in

platelet concentration in both the non-thrombocytopenic groups and the thrombocytopenic groups when treated with human MkMPs respectively (P=0.29 and P=0.38, respectively). A concern was that the anti-CD41 antibody might deplete the infused human MkMPs, and thus suppress the observable effect of human MkMP infusion. However, this should not be a concern as the antibody is specific to cells expressing the murine CD41, which is different from the human CD41 expressed on human MkMPs (Teeling, Jensen-Hendriks et al. 2001). There is still of course the concern that the anti-CD41 antibody might continue to deplete newly synthesized murine Mks and platelets. This led us to re-examine the experimental design for these experiments.

In a previous study from the Papoutsakis lab, Anti-CD41 antibody injection resulted in a sharp decrease in platelet concentrations within 24 hours and a quick recovery in platelet concentration starting 48 hours post antibody injection (Figure 12) (Apostolidis, Woulfe et al. 2012). In view of these data, we decide to sample the murine blood for platelet concentration at 24 hours post antibody injection instead of 72 hours.

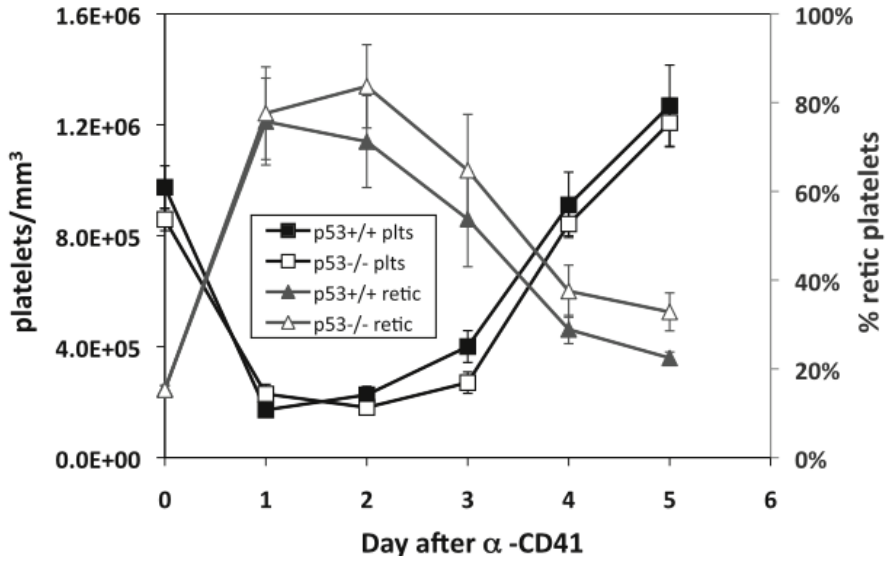


Figure 12. **Induced thrombocytopenia following injection of Anti-CD 41 antibody.**

Platelet levels after the addition of 0.5 mg of anti-CD41 antibody/kg of mouse body weight. "Plts" indicate platelets while "retic" indicate reticulated (newly synthesized) platelets. Image adapted from (Apostolidis, Woulfe et al. 2012). The wild-type mice correspond to the $p53^{+/+}$ mice.

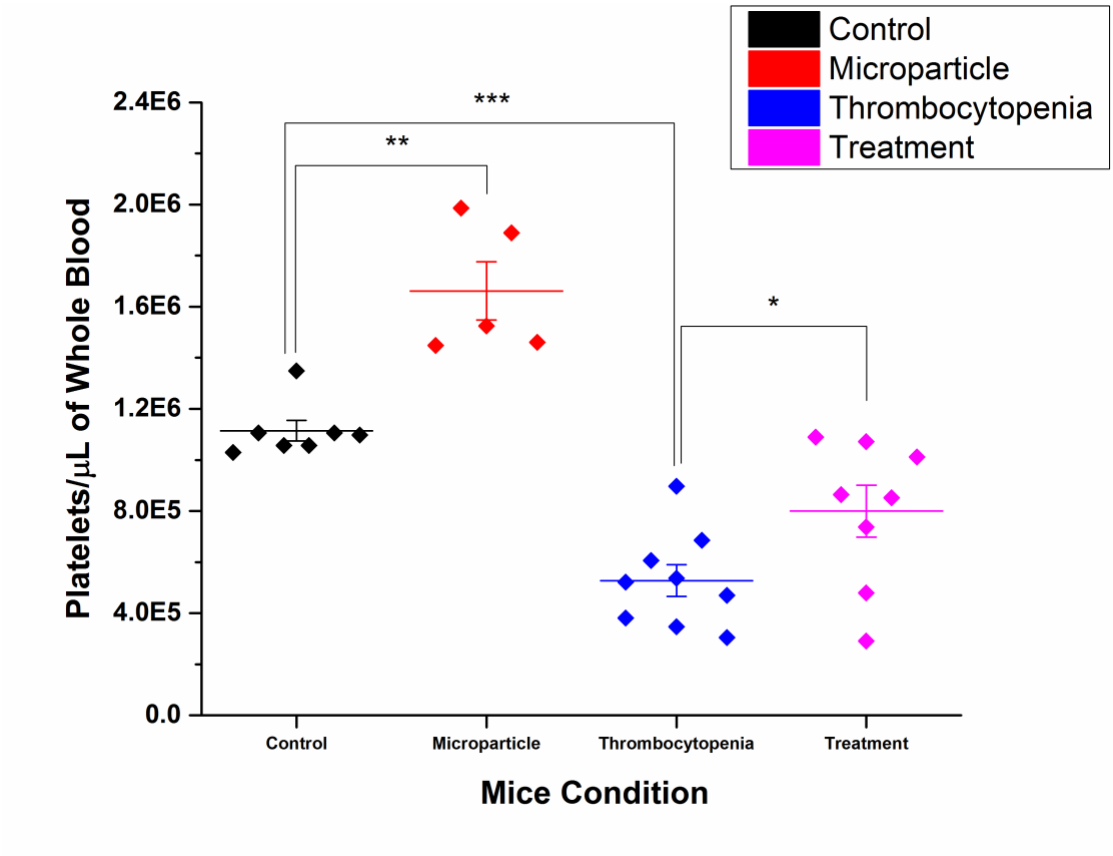


Figure 13. **Transfusion of human MkMPs in wild type mice following induction of antibody induced thrombocytopenia**.

At time 0, mice received a dose of 200 µL of saline or 1 mg of anti-CD41 antibody per kg of mouse body weight dissolved in 200 µL of saline. 8 hours post antibody injection, mice were transfused with 200 µL of saline or 2,000,000 human MkMPs suspended in 200 µL of saline. Blood was collected 24 hours post antibody injection via retro-orbital bleeding and analyzed via flow cytometry for platelet concentration. N=8 for the Control group and Treatment group. N=5 for the Microparticle group, N=9 for Thrombocytopenia group. Error bars are ± 1 S.E. * = $P < 0.05$. **=$P<0.01$, ***= $P<0.001$.

As seen in Figure 13, there is an increase in the concentration of platelets between “Control” mice and “Microparticle” mice. This shows that human MkMPs at low dosages can influence platelet concentrations at 24 hours compared to 72 hours as performed in Figure 9. In addition, we observed that human MkMPs can ameliorate the effects of antibody induced thrombocytopenia. As seen in Figure 13, comparing the “Thrombocytopenia” group and the “Treatment” group (where the treatment group received both antibody and human MkMPs), there was a statistically significant increase in platelet concentrations. The only explanation of this increase is an increased in megakaryopoiesis resulting from the *in vivo* interaction of human MkMPs with murine HSPCs. These data show that infusion of human MkMPs results in increased platelet levels in both a non-thrombocytopenic and a thrombocytopenic mouse.

3.4 Transfusion of human MkMPs shows synthesis in reticulated platelets in non-thrombocytopenic mice and thrombocytopenic mice

Finally, to confirm that murine platelets are newly synthesized as the result of human MkMP infusion, we wanted to examine if there is an increase in the level of reticulated (newly synthesized) platelets circulating in murine blood. Reticulated platelets are considered a marker of *de novo* megakaryopoiesis due to their retention of RNAs early in their lifespan (Monteagudo, Amengual et al. 2008). Utilizing a dye that stains RNA allows us to differentiate the populations of newly synthesized platelets versus older mature platelets circulating in murine blood (Monteagudo, Amengual et al. 2008). Our data (Figure 14) shows that compared to the control group, there was an increase in the number of reticulated platelets in all 3 groups. The increase was expected in the thrombocytopenic mice as shown in Figure 9 reproduced from

(Apostolidis, Woulfe et al. 2012). Interestingly, comparing the "Control" group with the "+HuMkMPs" group (the non-thrombocytopenic mice group that received the human MkMP infusion), there was an expected, statistically significant increase in the percentage of reticulated platelets (Figure 14). This trend of increased reticulated platelet percentage was also seen in the "Thrombocytopenic" group and "Thrombocytopenic+HuMkMPs" group compared to the "Control" group. These data confirm that an infusion of human MkMPs to mice increases *de novo* platelet biogenesis.

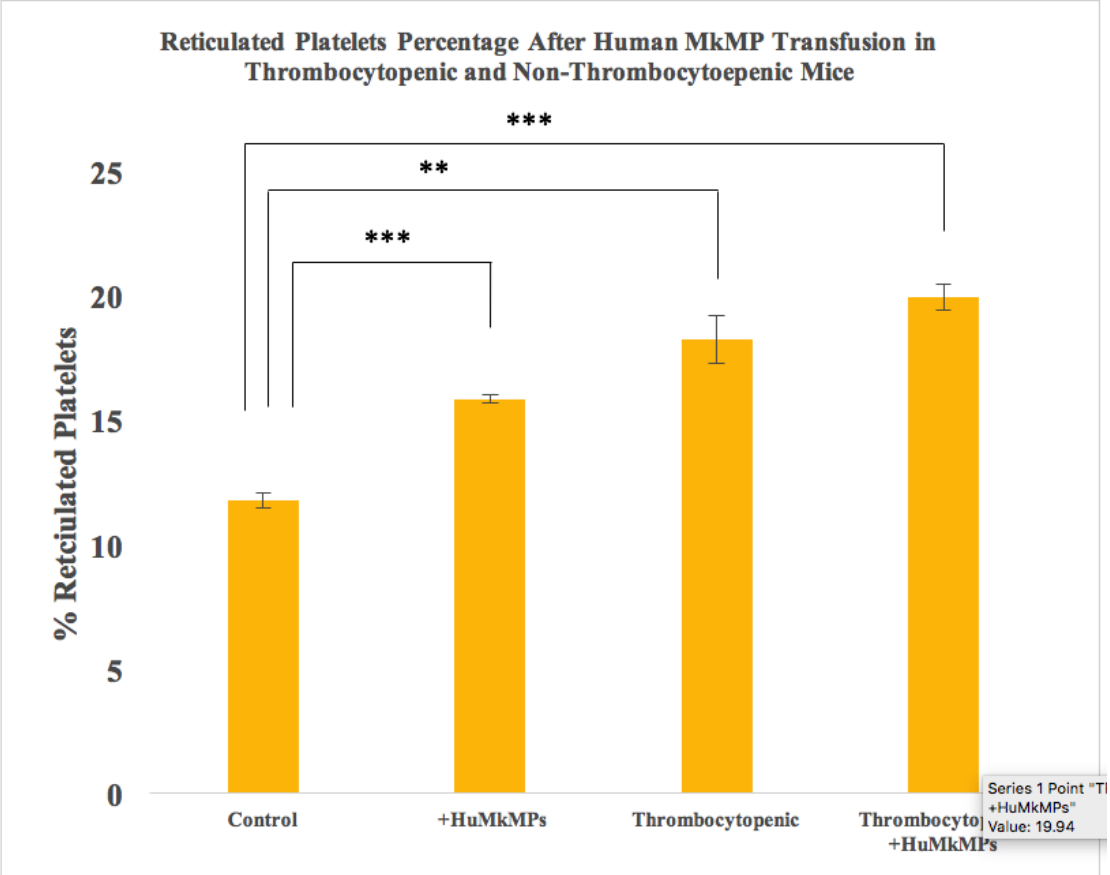


Figure 14. **Determining the percentage of reticulated platelets following transfusion of human MkMPs in wild type following induction of thrombocytopenia**.

At the beginning of the assay, mice received a dose of 200 µL of saline 1 mg of anti-CD41 antibody per kg of mouse body weight dissolved in 200 µL of saline. 8 hours post antibody injection, mice were transfused with 200 µL of saline or 2,000,000 human MkMPs suspended in 200 µL of saline. Blood was collected 24 hours via retro-orbtial bleed post injection with respect to the antibody, stained with thiazole orange and analyzed via flow cytometry. N=5 for all groups Error bars are ± 1 S.E. **=P<0.01, ***= P<0.001.

3.5 Transfusion of Platelet Microparticles does not Result in *de novo* Platelet Generation in Normal or Thrombocytopenic Mice

In order to validate the effectiveness of human MkMPs in an *in vivo* model, we sought to utilize control MPs that also expresses CD41. For these studies, we utilized human PMPs as they express CD41 as well as CD62p and have been shown to be involved in different physiological processes such as enhanced platelet activity, and increased angiogenesis, advanced progression of diseases such as cancer, and increased atherosclerosis (Burnouf, Goubran et al. 2014, Melki, Tessandier et al. 2017). In addition, previous research has also shown that, *in vitro*, PMPs are not taken up by hematopoietic stem cells, but rather they appear to promote aggregation of human HSPCs, and, significantly, it was shown that upon co-culture with HSPCs, unlike MkMPs, they do not promote mekagaryopoietic differentiation (Jiang, Woulfe et al. 2014, Jiang, Kao et al. 2016). Based on this information, we hypothesized that, *in vivo*, in the murine model, PMPs should not promote *de novo* platelet biogenesis and thus should not affect platelet concentrations and that only MkMPs are involved in platelet generation. For these reasons, utilizing human PMPs was essential in validating the results shown previously (Figures 10-14).

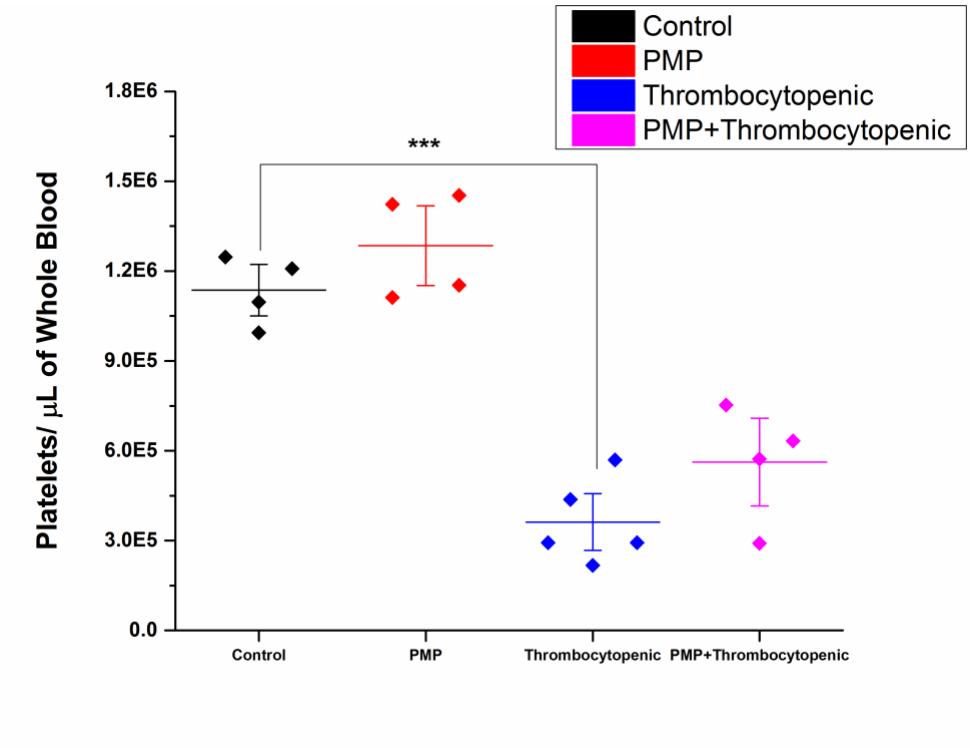


Figure 15. **Transfusion of human PMPs in wild type mice without or with induction of antibody-induced thrombocytopenia**.

At time 0, mice received a dose of 200 µL of saline or 1 mg of anti-CD41 antibody per kg of mouse body weight dissolved in 200 µL of saline. 8 hours post antibody injection, mice were transfused with 200 µL of saline or 2,000,000 human PMPs suspended in 200 µL of saline. Blood was collected 24 hours post antibody injection via retro-orbital bleeding and analyzed via flow cytometry for platelet concentration. N=4 for the Control group, Microparticle group and PMP+Thrombocytopenic group. N=5 for the Thrombocytopenic group. Error bars are ± 1 S.E. ***= P<0.001. P-value between Control and PMP groups (P=0.21). P-value between Thrombocytopenic and PMP+Thrombocytopenic (P=0.12).

As shown in Figure 13, there is not a statistically significant increase in platelet concentrations between "Control" mice and "+PMP" treated mice. This shows that human PMPs do not influence platelet concentration at 24 hours compared to the effect seen between the "Control" and "+PMP" group in mice treated with human MkMPs (Figure 13). In addition, we observed that human PMPs cannot ameliorate the effects of antibody induced thrombocytopenia. As seen in Figure 15, comparing the "Thrombocytopenic" group and the "PMP+Thrombocytopenic" group, there also was not a statistically significant increase in platelet concentrations further validating that only human MkMPs influence an increase in platelet concentrations.

Although the increase in platelet concentration was not statistically significant, there existed the possibility that *de novo* platelet synthesis was occurring but possibly at a lower rate compared to that of human MkMPs. As seen in Figure 16, staining for reticulated (newly synthesized) platelets show no statistical increase in new platelet biogenesis between the "Control" and "PMP" groups compared to the statistically significant increase between the "Control" and "MPs" group when mice were injected with human MkMPs (Figure 14) thus indicating no *de novo* platelet biogenesis. In fact, the comparison between the "Control" group and the "PMP" group showed a trend of decreasing in the percentage of newly synthesized platelets indicating that a PMP infusion does not promote new platelet biogenesis (Figure 16). In addition, the comparison between the thrombocytopenic groups ("Thrombocytopenic" and "Thrombocytopenic+PMP") shows a decrease in the percentage of *de novo* platelet synthesis indicating that a transfusion of PMPs shows a trend of decreasing newly synthesized platelets in thrombocytopenic mice (Figure 16). These data indicate that

only human MkMPs and not human PMPs can influence platelet concentrations through increased *de novo* platelet synthesis.

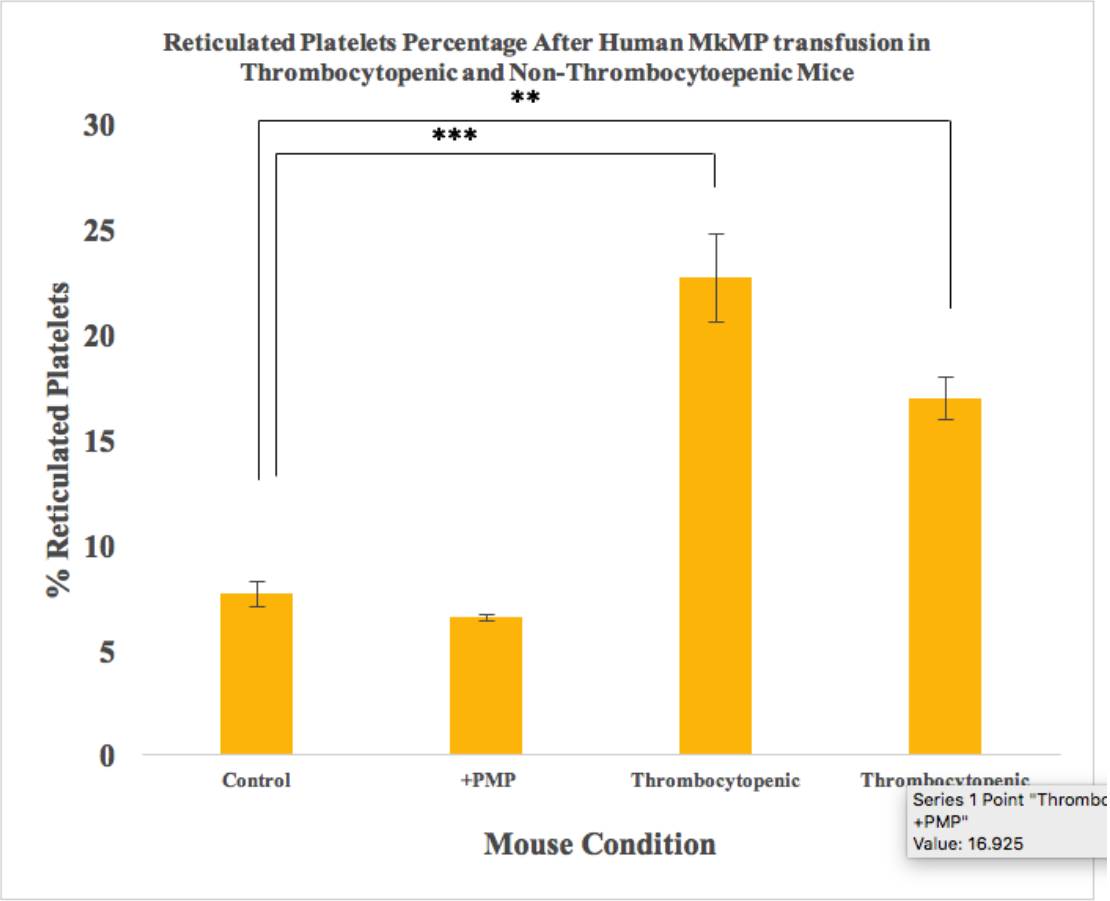


Figure 16. **Determining the percentage of reticulated platelets following transfusion of human PMPs in wild type mice without or with antibody-induced thrombocytopenia.**

At the beginning of the assay, mice received a dose of 200 µL of saline 1 mg of anti-CD41 antibody per kg of mouse body weight dissolved in 200 µL of saline. 8 hours post antibody injection, mice were transfused with 200 µL of saline or 2,000,000 human MkMPs suspended in 200 µL of saline. Blood was collected 24 hours via retro-orbital bleed post injection with respect to the antibody, stained with thiazole orange and analyzed via flow cytometry. N=4 for all groups except Thrombocytopenic (N=5) Error bars are ± 1 S.E. **=P<0.01, ***= P<0.001.

Chapter 4

DISCUSSION

Based on the presented data, we have shown that human MkMPs have a considerable potential as an application in transfusion medicine to increase platelet concentration and reverse the effects of thrombocytopenia. First, we have shown that the human MkMPs interact with murine HSPC (Figure 4, Figure 5, and Figure 6). This supports the hypothesis that human MkMPs can deliver RNA cargo to promote the megakaryocytic differentiation of murine HSPCs. In addition, our data shows that human MkMPs cross react with murine HSPCs with respect to targeting/uptake as well the ability to differentiate them into Mks as seen in the bright field images and confirmed utilizing a ploidy assay (Figure 7 and Figure 8).

For the *in vivo* studies, we showed that an infusion of human MkMPs into both normal and thrombocytopenic mice results in an increase in the platelet concentration. We also showed that the increase in platelet concentration results from *de novo* platelet biosynthesis as demonstrated by the increase in the concentration of reticulated platelets (Figure 13). When utilizing PMPs as an appropriate control for the thrombocytopenic studies, we determined no statistically significant increase in platelet concentrations in normal and thrombocytopenic mice infused with PMPs and no increase in the amount of *de novo* platelet biosynthesis as well (Figure 15, Figure 16 respectively).

To further examine the interaction of human MkMPs to murine HSPC, an *in vivo* co-localization of human MkMPs and murine HSPCs should be performed to

visually identify that the increase in platelet concentrations can be attributed directly to human MkMPs localizing in areas rich in murine HSPCs. These studies typically involve staining the human MkMPs with a dye (such as the PKH26 dye used for membrane staining in our *ex vivo* studies) (Figures 4, Figures 5, and Figure 6) and *in vivo* florescent detection using a suitable instrument such as an IVIS (*in vivo* imaging system) as reported, for example, in (Zhuang, Xiang et al. 2011, Tang, Zhang et al. 2012). Utilizing this technology, we can hone in on an area or organ of the animal of interest where HSPCs may reside such as the bone marrow, the liver or the lungs (Lefrançais, Ortiz-Muñoz et al. 2017). Another way of investigating where human MkMPs may localize is employing immunohistochemistry of tissues of interest where HSPCs reside such as the bone marrow, liver or lungs. Previous studies utilizing platelet MPs have utilized immunohistochemistry to isolate areas of ischemia in heart tissue and incubated these tissues with antibodies against a receptor of the platelet MP to examine the effect that platelet MPs have on ischemia (Brill, Dashevsky et al. 2005, Sprague, Elzey et al. 2008).

More assays may be employed to confirm the effectiveness of human MkMPs in inducing *de novo* megakaryopoiesis and platelet biogenesis. Additional assays could employ immunocompromised mice such as NOD/SCID mice. The NOD/SCID mouse provides a great insight in greatly decreasing the possible clearance of human MkMPs (Belizario 2009, Zitvogel, Pitt et al. 2016). In addition, a humanized mouse strain (such as NSG mice) can also be used (Shultz, Ishikawa et al. 2007, Hu and Yang 2012). Finally, another method to reduce the possible clearance of human MkMPs is using macrophage depletion. This is done by using liposomes containing clodronate (a chemical agent that promotes “suicide” of macrophages and dendritic cells) and

injecting into immunocompetent mice to decrease their macrophage number (Weisser, van Rooijen et al. 2012). Reducing the macrophage number will reduce the likelihood of clearance of human MkMPs caused by immunity.

Microparticles from different cells such as endothelial cells, platelets and tumor cells have been and are actively investigated for different applications. For example, regarding platelet MPs, some of their effects observed were repair of damaged infarcted tissue in the heart, interaction with immune cells to elicit a pro-inflammatory response, and enhancement of platelet aggregation by different pro-thrombotic factors such the Tissue Factor 2 (Badimon, Suades et al. 2016). Endothelial MPs have been shown to have similar effects to those of platelet MPs, and in addition may protect endothelial cells from apoptosis (Dignat-George and Boulanger 2011). In the case of cancer MPs, it has been shown that they may affect other cancer cells by increasing resistance to chemotherapeutic drugs and transfer pro-metastatic components to downstream targets (Gong, Jaiswal et al. 2015). Consistently, the similarities between these MPs show that their cargo is extremely potent in affect downstream target cells and not specific only to MkMPs.

Chapter 5

FUTURE WORK

As many of these experiments are preliminary trials, there still are areas of interest that require further investigating that will be performed to continue determining the efficiency of the human MkMP. Continuing with the *ex vivo* studies, a more comprehensive study with utilizing human MkMPs to differentiate murine HSPCs should be further investigated and quantified.

For the *in vivo* studies utilizing the thrombocytopenic mice. More studies with higher numbers of human MkMPs transfused into mice should be performed. These numbers should be consistent with the numbers of human MkMPs transfused into wild type mice to further validate the effectiveness of human MkMPs in a dose dependent manner. In a thrombocytopenic murine model.

An important study that requires consideration is the inclusion of an immuno-deficient mouse model. As immunity and platelets are interrelated with one another, it's important to remove a variable factor moving forward as we would be able to confirm the effect we are observing with the increased number of platelets in circulation can be attributed solely on the human MkMPs and not an effect associated with immunity (Semple, Italiano et al. 2011).

Finally, another important experiment to consider is the determination of the effectiveness of the newly synthesized platelets and notably tail-bleeding assays and other in vivo platelet function assays to determine if platelets made through human MkMP infusion are functional (Apostolidis, Woulfe et al. 2012, Wei, Gao et al. 2016).

REFERENCES

Alugupalli, K. R., A. D. Michelson, M. R. Barnard and J. M. Leong (2001). "Serial Determinations of Platelet Counts in Mice by Flow Cytometry." Thrombosis and Haemostasis **86**(2): 668-671.

Apostolidis, P. A., D. S. Woulfe, M. Chavez, W. M. Miller and E. T. Papoutsakis (2012). "Role of tumor suppressor p53 in megakaryopoiesis and platelet function." Exp Hematol **40**(2): 131-142 e134.

Avanzi, M. P., A. Chen, W. He and W. B. Mitchell (2012). "Optimizing megakaryocyte polyploidization by targeting multiple pathways of cytokinesis." Transfusion **52**(11): 2406-2413.

Avanzi, M. P. and W. B. Mitchell (2014). "Ex vivo production of platelets from stem cells." Br J Haematol **165**(2): 237-247.

Azevedo, L. C. P. (2012). "Microparticles and Exosomes: Are they part of important pathways in sepsis pathophysiology." Severe Sepsis and Septic Shock-Understanding a Serious Killer.

Badimon, L., R. Suades, E. Fuentes, I. Palomo and T. Padró (2016). "Role of Platelet-Derived Microvesicles as Crosstalk Mediators in Atherothrombosis and Future Pharmacology Targets: A Link between Inflammation, Atherosclerosis, and Thrombosis." Frontiers in Pharmacology **7**.

Belizario, J. E. (2009). "Immunodeficient Mouse Models: An Overview." The Open Immunology Journal **2**: 79-85.

Booth, A. M., Y. Fang, J. K. Fallon, J. Yang, J. E. K. Hildreth and S. J. Gould (2006). "Exosomes and HIV Gag Bud from Endosome-like Domains of the T Cell Plasma Membrane." The Journal of Cell Biology **172**(6): 923-935.

Brill, A., O. Dashevsky, J. Rivo, Y. Gozal and D. Varon (2005). "Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization." Cardiovasc Res **67**(1): 30-38.

Buesing, K. L., J. C. Densmore, S. Kaul, K. A. Pritchard, Jr., J. A. Jarzembowski, D. M. Gourlay and K. T. Oldham (2011). "Endothelial microparticles induce inflammation in acute lung injury." J Surg Res **166**(1): 32-39.

Burnouf, T., H. A. Goubran, M. L. Chou, D. Devos and M. Radosevic (2014). "Platelet microparticles: detection and assessment of their paradoxical functional roles in disease and regenerative medicine." Blood Rev **28**(4): 155-166.

Chou, J., N. Mackman, G. Merrill-Skoloff, B. Pedersen, B. C. Furie and B. Furie (2004). "Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation." Blood **104**(10): 3190-3197.

Chung, Y. R., E. Kim and O. Abdel-Wahab (2014). "Femoral bone marrow aspiration in live mice." J Vis Exp(89).

Dignat-George, F. and C. M. Boulanger (2011). "The many faces of endothelial microparticles." Arterioscler Thromb Vasc Biol **31**(1): 27-33.

Fuhrken, P. G., P. A. Apostolidis, S. Lindsey, W. M. Miller and E. T. Papoutsakis (2008). "Tumor suppressor protein p53 regulates megakaryocytic polyploidization and apoptosis." J Biol Chem **283**(23): 15589-15600.

Garraud, M., K. Khacef, A. C. Vion, C. Leconte, M. Yin, J. M. Renard, C. Marchand-Leroux, C. M. Boulanger, I. Margaill and V. Beray-Berthat (2016). "Recombinant tissue plasminogen activator enhances microparticle release from mouse brain-derived endothelial cells through plasmin." J Neurol Sci **370**: 187-195.

Gong, J., R. Jaiswal, P. Dalla, F. Luk and M. Bebawy (2015). "Microparticles in cancer: A review of recent developments and the potential for clinical application." Semin Cell Dev Biol **40**: 35-40.

Gray, W. D., A. J. Mitchell and C. D. Searles (2015). "An accurate, precise method for general labeling of extracellular vesicles." MethodsX **2**: 360-367.

Guo, T., X. Wang, Y. Qu, Y. Yin, T. Jing and Q. Zhang (2015). "Megakaryopoiesis and platelet production: insight into hematopoietic stem cell proliferation and differentiation." Stem Cell Investig **2**: 3.

Hu, Z. and Y. G. Yang (2012). "Full reconstitution of human platelets in humanized mice after macrophage depletion." Blood **120**(8): 1713-1716.

Italiano, J. E., Jr., A. T. Mairuhu and R. Flaumenhaft (2010). "Clinical relevance of microparticles from platelets and megakaryocytes." Curr Opin Hematol **17**(6): 578-584.

Italiano, J. E., Jr., J. L. Richardson, S. Patel-Hett, E. Battinelli, A. Zaslavsky, S. Short, S. Ryeom, J. Folkman and G. L. Klement (2008). "Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released." Blood **111**(3): 1227-1233.

Jansen, F., X. Yang, K. Baumann, D. Przybilla, T. Schmitz, A. Flender, K. Paul, A. Alhusseiny, G. Nickenig and N. Werner (2015). "Endothelial microparticles reduce ICAM-1 expression in a microRNA-222-dependent mechanism." J Cell Mol Med **19**(9): 2202-2214.

Jansen, F., X. Yang, M. Hoelscher, A. Cattelan, T. Schmitz, S. Proebsting, D. Wenzel, S. Vosen, B. S. Franklin, B. K. Fleischmann, G. Nickenig and N. Werner (2013). "Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles." Circulation **128**(18): 2026-2038.

Jiang, J., C. Y. Kao and E. T. Papoutsakis (2016). "How do megakaryocytic microparticles target and deliver cargo to alter the fate of hematopoietic stem cells?" J Control Release **247**: 1-18.

Jiang, J., D. S. Woulfe and E. T. Papoutsakis (2014). "Shear enhances thrombopoiesis and formation of microparticles that induce megakaryocytic differentiation of stem cells." Blood **124**(13).

Jones, C. I. (2016). "Platelet function and ageing." Mamm Genome **27**(7-8): 358-366.

Kaushansky, K. (2008). "Historical review: megakaryopoiesis and thrombopoiesis." Blood **111**(3): 981-986.

Kim, M. S., M. J. Haney, Y. Zhao, V. Mahajan, I. Deygen, N. L. Klyachko, E. Inskoe, A. Piroyan, M. Sokolsky, O. Okolie, S. D. Hingtgen, A. V. Kabanov and E. V. Batrakova (2016). "Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells." Nanomedicine **12**(3): 655-664.

Kovtonyuk, L. V., K. Fritsch, X. Feng, M. G. Manz and H. Takizawa (2016). "in amm-Aging of Hematopoiesis, Hematopoietic Stem Cells, and the Bone Marrow Microenvironment." Frontiers in Immunology **7**.

Lefrançais, E., G. Ortiz-Muñoz, A. Caudrillier, B. Mallavia, F. Liu, D. M. Sayah, E. E. Thornton, M. B. Headley, T. David, S. R. Coughlin, M. F. Krummel, A. D. Leavitt, E. Passegué and M. R. Loonet (2017). "The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors." Letters to Nature **544**.

Loyer, X., A. C. Vion, A. Tedgui and C. M. Boulanger (2014). "Microvesicles as cell-cell messengers in cardiovascular diseases." Circ Res **114**(2): 345-353.

Luff, S. A. and E. T. Papoutsakis (2016). "Megakaryocytic Maturation in Response to Shear Flow Is Mediated by the Activator Protein 1 (AP-1) Transcription Factor via Mitogen-activated Protein Kinase (MAPK) Mechanotransduction." J Biol Chem **291**(15): 7831-7843.

Machlus, K. R., J. N. Thon and J. E. Italiano, Jr. (2014). "Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation." Br J Haematol **165**(2): 227-236.

Marjon, K. D., L. L. Marnell, C. Mold and T. W. Du Clos (2009). "Macrophages Activated by C-Reactive Protein through Fc RI Transfer Suppression of Immune Thrombocytopenia." The Journal of Immunology **182**(3): 1397-1403.

Mattia, G., F. Vulcano, L. Milazzo, A. Barca, G. Macioce and H. J. Hassan (2002). "Different ploidy levels of megakaryocytes generated from peripheral or cord blood CD34+ cells are correlated with different levels of platelet release." Blood **99**(3): 888-897.

Mause, S. F. and C. Weber (2010). "Microparticles: protagonists of a novel communication network for intercellular information exchange." Circ Res **107**(9): 1047-1057.

McFadyen, J. D. and Z. S. Kaplan (2015). "Platelets are not just for clots." Transfus Med Rev **29**(2): 110-119.

Melki, I., N. Tessandier, A. Zufferey and E. Boilard (2017). "Platelet microvesicles in health and disease." Platelets **28**(3): 214-221.

Monteagudo, M., M. J. Amengual, L. Munoz, J. A. Soler, I. Roig and C. Tolosa (2008). "Reticulated platelets as a screening test to identify thrombocytopenia aetiology." QJM **101**(7): 549-555.

Nakagawa, Y., S. Nakamura, M. Nakajima, H. Endo, T. Dohda, N. Takayama, H. Nakauchi, F. Arai, T. Fukuda and K. Eto (2013). "Two differential flows in a bioreactor promoted platelet generation from human pluripotent stem cell-derived megakaryocytes." Exp Hematol **41**(8): 742-748.

Neschadim, A. and D. Branch (2015). "Mouse Models of Autoimmune Diseases: Immune Thrombocytopenia." Current Pharmaceutical Design **21**(18): 2487-2497.

Nurhayati, R. W., Y. Ojima and M. Taya (2016). "Recent developments in ex vivo platelet production." Cytotechnology **68**(6): 2211-2221.

Palis, J. (2016). "Hematopoietic stem cell-independent hematopoiesis: emergence of erythroid, megakaryocyte, and myeloid potential in the mammalian embryo." FEBS Lett **590**(22): 3965-3974.

Pallotta, I., M. Lovett, D. L. Kaplan and A. Balduini (2011). "Three-dimensional system for the in vitro study of megakaryocytes and functional platelet production using silk-based vascular tubes." Tissue Eng Part C Methods **17**(12): 1223-1232.

Panuganti, S., A. C. Schlinker, P. F. Lindholm, E. T. Papoutsakis and W. M. Miller (2013). "Three-stage ex vivo expansion of high-ploidy megakaryocytic cells: toward large-scale platelet production." Tissue Eng Part A **19**(7-8): 998-1014.

Piccin, A., W. G. Murphy and O. P. Smith (2007). "Circulating microparticles: pathophysiology and clinical implications." Blood Rev **21**(3): 157-171.

Plebanek, M. P., N. L. Angeloni, E. Vinokour, J. Li, A. Henkin, D. Martinez-Marin, S. Filleur, R. Bhowmick, J. Henkin, S. D. Miller, I. Ifergan, Y. Lee, I. Osman, C. S. Thaxton and O. V. Volpert (2017). "Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche." Nat Commun **8**(1): 1319.

Pols, M. S. and J. Klumperman (2009). "Trafficking and function of the tetraspanin CD63." Exp Cell Res **315**(9): 1584-1592.

Ramacciotti, E., A. E. Hawley, D. M. Farris, N. E. Ballard, S. K. Wrobleski, D. D. Myers Jr., P. K. Henke and T. W. Wakefield (2009). "Leukocyte- and platelet-derived microparticles correlate with thrombus weight and tissue factor activity in an experimental mouse model of venous thrombosis." Thromb Heamost. **101**(4): 748-754.

Raposo, G. and W. Stoorvogel (2013). "Extracellular vesicles: exosomes, microvesicles, and friends." J Cell Biol **200**(4): 373-383.

Rhodes, J. M. and M. Simons (2007). "The extracellular matrix and blood vessel formation: not just a scaffold." J Cell Mol Med **11**(2): 176-205.

Ru, Y. X., S. X. Dong, H. Y. Liang and S. X. Zhao (2016). "Platelet production of megakaryocyte: A review with original observations on human in vivo cells and bone marrow." Ultrastruct Pathol **40**(4): 163-170.

Seita, J. and I. L. Weissman (2010). "Hematopoietic stem cell: self-renewal versus differentiation." Wiley Interdiscip Rev Syst Biol Med **2**(6): 640-653.

Semple, J. W., J. E. Italiano, Jr. and J. Freedman (2011). "Platelets and the immune continuum." Nat Rev Immunol **11**(4): 264-274.

Shultz, L. D., F. Ishikawa and D. L. Greiner (2007). "Humanized mice in translational biomedical research." Nat Rev Immunol **7**(2): 118-130.

Simons, M. and G. Raposo (2009). "Exosomes--vesicular carriers for intercellular communication." Curr Opin Cell Biol **21**(4): 575-581.

Sprague, D. L., B. D. Elzey, S. A. Crist, T. J. Waldschmidt, R. J. Jensen and T. L. Ratliff (2008). "Platelet-mediated modulation of adaptive immunity: unique delivery of CD154 signal by platelet-dervied membrane vesicles." Blood **111**(10).

Sullivan, B. P., R. Wang, O. Tawfik and J. P. Luyendyk (2010). "Protective and damaging effects of platelets in acute cholestatic liver injury revealed by depletion and inhibition strategies." Toxicol Sci **115**(1): 286-294.

Takahashi, Y., M. Nishikawa, H. Shinotsuka, Y. Matsui, S. Ohara, T. Imai and Y. Takakura (2013). "Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection." J Biotechnol **165**(2): 77-84.

Tang, K., Y. Zhang, H. Zhang, P. Xu, J. Liu, J. Ma, M. Lv, D. Li, F. Katirai, G. X. Shen, G. Zhang, Z. H. Feng, D. Ye and B. Huang (2012). "Delivery of chemotherapeutic drugs in tumour cell-derived microparticles." Nat Commun **3**: 1282.

Teeling, J. L., T. Jensen-Hendriks, T. W. Kujipers, M. de Haas, J. G. J. van de Winkel, C. Erik Hack and W. K. Bleeker (2001). "Therapeutic efficacy of intravenous immunoglobulin preparations depends on the immunoglobulin G dimers: studies in experimental immune thrombocytopenia." Blood **98**(4).

Thomas, G. M., L. Panicot-Dubois, R. Lacroix, F. Dignat-George, D. Lombardo and C. Dubois (2009). "Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo." J Exp Med **206**(9): 1913-1927.

Walasek, M. A., R. van Os and G. de Haan (2012). "Hematopoietic stem cell expansion: challenges and opportunities." Ann N Y Acad Sci **1266**: 138-150.

Wei, X., J. Gao, R. H. Fang, B. T. Luk, A. V. Kroll, D. Dehaini, J. Zhou, H. W. Kim, W. Gao, W. Lu and L. Zhang (2016). "Nanoparticles camouflaged in platelet membrane coating as an antibody decoy for the treatment of immune thrombocytopenia." Biomaterials **111**: 116-123.

Weisser, S. B., N. van Rooijen and L. M. Sly (2012). "Depletion and reconstitution of macrophages in mice." J Vis Exp(66): 4105.

Whitaker, B. I., R. A. Henry and S. Hinkins (2011). "Report of the United States Department of Health and Human Services: the 2011 national blood collection and utilization survey report, US Department of Health and Human Services, Office of the Assistant Secretary for Health, Wash- ington, D.C." US Department of Health and Human Services, Office of the Assistant Secretary for Health.

Y.-Q. Ma, J. Q. a. E. F. P. (2007). "Platelet integrin aIIbb3: activation mechanisms." Journal of Thrombosis and Haemostasis(5): 1345-1352.

Zhao, Z., Y. Zhou, Y. Tian, M. Li, J. F. Dong and J. Zhang (2017). "Cellular microparticles and pathophysiology of traumatic brain injury." Protein Cell **8**(11): 801-810.

Zhuang, X., X. Xiang, W. Grizzle, D. Sun, S. Zhang, R. C. Axtell, S. Ju, J. Mu, L. Zhang, L. Steinman, D. Miller and H. G. Zhang (2011). "Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain." Mol Ther **19**(10): 1769-1779.

Zitvogel, L., J. M. Pitt, R. Daillere, M. J. Smyth and G. Kroemer (2016). "Mouse models in oncoimmunology." Nat Rev Cancer **16**(12): 759-773.

Appendix A

HUMAN CONSENT FORM

HUMAN SUBJECTS PROTOCOL
University of Delaware

Protocol Title: Utilizing Primary Human Cells in the Papoutsakis Lab

Principal Investigator
Name: Eleftherios T. Papoutsakis
Department/Center: Chemical and Biomolecular Engineering
Contact Phone Number: 302-831-8376
Email Address: epaps@udel.edu

Advisor (if student PI): N/A
Name:
Contact Phone Number:
Email Address:

Other Investigators: N/A

Investigator Assurance:

By submitting this protocol, I acknowledge that this project will be conducted in strict accordance with the procedures described. I will not make any modifications to this protocol without prior approval by the IRB. Should any unanticipated problems involving risk to subjects occur during this project, including breaches of guaranteed confidentiality or departures from any procedures specified in approved study documents, I will report such events to the Chair, Institutional Review Board immediately.

Rev. 05/2017

HUMAN SUBJECTS PROTOCOL
University of Delaware

Protocol Title: Generation of Platelet-derived Microparticles

Principal Investigator
Name: Eleftherios T. Papoutsakis
Department/Center: Chemical and Biomolecular Engineering
Contact Phone Number: 302-831-8376
Email Address: epaps@udel.edu

Advisor (if student PI): N/A
Name:
Contact Phone Number:
Email Address:

Other Investigators: N/A

Investigator Assurance:

By submitting this protocol, I acknowledge that this project will be conducted in strict accordance with the procedures described. I will not make any modifications to this protocol without prior approval by the IRB. Should any unanticipated problems involving risk to subjects occur during this project, including breaches of guaranteed confidentiality or departures from any procedures specified in approved study documents, I will report such events to the Chair, Institutional Review Board immediately.

Appendix B

ANIMAL CONSENT FORM

RECEIVED
DEC 18 2015
IACUC

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research and Teaching

Title of Protocol: Collection of Microparticles for Thrombocytopenic Recovery	
AUP Number: 1290-2016-0	**← (4 digits only — if new, leave blank)**
Principal Investigator: E. Terry Papoutsakis	
Common Name: Mouse **Genus Species:** Mus Muculus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ **B**	Breeding or holding where NO research is conducted
☒ **C**	Procedure involving momentary or no pain or distress
☐ **D**	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ **E**	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: Jean Tallon, DVM

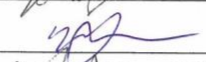
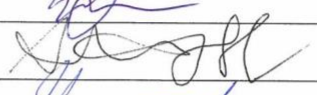
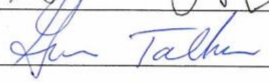
Date of Approval: 2/5/16

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to by the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. ***(Teaching Protocols Exempt)***
12. I understand that by signing, I agree to these assurances.  _____ Signature of Principal Investigator ___12-18-15_____ Date

NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

Name	Signature
1. Christian Escobar	
2. Chen-Yuan Kao	
3. Stephanie Luff	
4. Gwen Talham	
5. Click here to enter text.	
6. Click here to enter text.	
7. Click here to enter text.	
8. Click here to enter text.	
9. Click here to enter text.	
10. Click here to enter text.	
11. Click here to enter text.	
12. Click here to enter text.	
13. Click here to enter text.	
14. Click here to enter text.	

Appendix C

RIGHTS PERMISSION POLICIES

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 27, 2017

This Agreement between Univeristy of Delaware -- Christian Escobar ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4237120544730
License date	Nov 27, 2017
Licensed Content Publisher	Elsevier
Licensed Content Publication	Experimental Hematology
Licensed Content Title	Role of tumor suppressor p53 in megakaryopoiesis and platelet function
Licensed Content Author	Pani A. Apostolidis,Donna S. Woulfe,Massiel Chavez,William M. Miller,Eleftherios T. Papoutsakis
Licensed Content Date	Feb 1, 2012
Licensed Content Volume	40
Licensed Content Issue	2
Licensed Content Pages	16
Start Page	131
End Page	142.e4
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Supplemental Figure E.1
Title of your thesis/dissertation	THE UTLIZATION OF HUMAN MEGAKARYOCYTIC MICROPARTICLESTO AFFECT MURINE HEMATOPOIETIC STEM CELLS AND STIMULATE PLATELET PRODUCTION IN NON THROMBCYTOPENIC AND THROMBOCYTOPENIC MICE
Expected completion date	Dec 2017
Estimated size (number of pages)	60
Requestor Location	Univeristy of Delaware 15 Innovation Way Room 243 NEWARK, DE 19711