

The Characteristics of rabbit and rat mesenchymal stromal cell
growth and attachment to mesh used in hernia repair

by

Melissa Lydic

Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in the Biological Sciences Program

YOUNGSTOWN STATE UNIVERSITY

MAY, 2010

The characteristics of rabbit and rat mesenchymal stromal cell
growth and attachment to mesh used in hernia repair

by

Melissa Lydic

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

Melissa Lydic, Student

Date

Approvals:

Diana L. Fagan, Thesis Advisor

Date

Gary R. Walker, Committee Member

Date

Johanna Krontiris-Litowitz, Committee Member

Date

Peter J. Kasvinsky, Dean of School of Graduate Studies and Research

Date

ABSTRACT

The goal of this project is to create an optimal environment for healing at an incision site in the abdominal fascia. Long cycles of recurring hernias are one of the major complications that patients can endure following surgery. This research project will compare various mesh material embedded with mesenchymal stromal cells (MSC's) to find the optimal environment to enhance hernia repair. The four mesh materials that will be used in this study are Bard Colla Mend, Vicryl Knitted Mesh, Gore-Tex Soft Tissue Patch, and Bard Mesh. Mesh supports have been used in the treatment of hernia repair and MSC's have been used to treat cartilage and cutaneous tissue repair. Since MSC's attach to a collagen matrix, we predict that the MSC's will adhere to the mesh materials. We also predict that MSC's once attached to the mesh materials, will increase the amount of collagen and elastin production at the incision site. The Cell Titer 96 Cell Proliferation Assay will be used to measure the number of MSC's that adhere to the mesh and stains will be used to measure collagen and elastin production. Future studies will examine how MSC's along with mesh supports can be used to strengthen hernia repair sites. Hopefully, this will diminish or eliminate the vicious cycle of hernia reappearance.

Friday, January 29, 2010

Dr. Diana Fagan
Biology Department
UNIVERSITY

Re: IACUC Protocol # 02-09
Title: Autologous Mesenchymal Stem Cell Transplantation in Rats to Improve Fascial Repair

Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration titled "**Autologous Mesenchymal Stem Cell Transplantation in Rats to Improve Fascial Repair**" and determined it should be unconditionally approved for the period of **July 2, 2009** through its expiration date of **July 2, 2012**.

This protocol is approved for a period of three years; however, it must be updated yearly via the submission of an Annual Review-Request to Use Animals form. These Annual Review forms must be submitted to the IACUC at least thirty days **prior** to the protocol's yearly anniversary dates of July 2, 2010 and July 2, 2011. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Sincerely,

Dr. Peter J. Kasvinsky
Associate Provost for Research
Dean School of Graduate Studies

PJK:dka

C: Dr. Walter Home, Consulting Veterinarian, NEOUCOM
Dr. Robert Leipheimer, Chair IACUC, Chair Biological Sciences
Dawn Amolsch, Animal Tech., Biological Sciences

TO: Jeremy J. Heffner, M.D. – Surgical Education
St. Elizabeth Health Center

FROM: Donna King, Ph.D.
Chairperson, Institutional Animal Care and Use Committee

SUBJECT: Protocol Approval by the Northeastern Ohio Universities College of Medicine
(NEOUCOM) Institutional Animal Care and Use Committee (IACUC)

DATE: May 29, 2007

The following NEOUCOM protocol was reviewed and approved by this Institution's Animal Care and Use Committee (IACUC) on May 29, 2007. This protocol is approved for a three (3) year period of time; however you must submit an annual renewal to the IACUC each year for review. Protocols involving the use of human tissues require Institutional Review Board (IRB) approval. Any serious or adverse events regarding the use of animals approved in this study must be reported immediately to the IACUC Chairperson or the Attending Veterinarian.

NEOUCOM Protocol No.:	#07-012
Title of Protocol:	Autologous Mesenchymal Stem cell Transplantation to Improve Fascial Repair
Type of Vertebrate:	Rabbits
Funding Agency:	St. Elizabeth Surgery Education and Research Fund
Protocol Expiration Date:	May 29, 2010

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance number is A3474-01. This institution is also registered with the United States Department of Agriculture (USDA). The USDA registration number is 31-R-0092.

The Comparative Medicine Unit (CMU) at the Northeastern Ohio Universities College of Medicine (NEOUCOM) has been accredited with the Association for Assessment for Accreditation of Laboratory Animal Care (AAALAC) International since June 8, 1982. Full accreditation was last renewed on June 29, 2005.

Thank you.

DK:lkn

Cc: Walter E. Horton, Jr., Ph.D.
Vice President for Research
NEOUCOM Institutional Official

File

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Diana L. Fagan for allowing me to work on this research project. She was an excellent thesis advisor. I would also like to thank my committee members, Dr. Gary Walker and Dr. Jodie Krontiris-Litowitz. I also want to thank my family and friends for their guidance and support during my graduate experience.

LIST OF FIGURES

	Page #
Figure 1: Thickness of Collagen Deposition following Rabbit Surgeries	36
Figure 2: Thickness of Collagen Deposition following Rabbit Surgeries	37
Figure 3: Standard Curve using Cell Titer 96 Aqueous One Solution and Rabbit MSC's	40
Figure 4: Standard Curve using Rabbit MSC's	42
Figure 5: Standard Curve using Rabbit MSC's	43
Figure 6: Standard Curve for the Adherence assay using Rabbit MSC's	44
Figure 7: Cell Adhesion Assay: Cell Titer 96 AQ One Solution Cell Proliferation Assay	46
Figure 8: Cell Adhesion Assay: Cell Titer 96 AQ One Solution Cell Proliferation Assay	48
Figure 9: Cell Adhesion Assay: Cell Titer 96 AQ One Solution Cell Proliferation Assay	49
Figure 10: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	51
Figure 11: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	52
Figure 12: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	56
Figure 13: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	57

Figure 14: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	58
Figure 15: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	59
Figure 16: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	60
Figure 17: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	61
Figure 18: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	62
Figure 19: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	63
Figure 20: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	64
Figure 21: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	65
Figure 22: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	66
Figure 23: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	67
Figure 24: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	68
Figure 25: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	69

Figure 26: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	70
Figure 27: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	71
Figure 28: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	72
Figure 29: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count	73
Figure 30: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	74
Figure 31: Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's	75
Figure 32: Standard Curve for the Adherence assay using Rat MSC's	76
Figure 33: Cell Adhesion Assay: Cell Titer 96 AQ One Solution Cell Proliferation Assay using Rat MSC's	77
Figure 34: Cell Proliferation Assay comparing different types of media using Rat MSC's_Cell Count	79
Figure 35: Cell Proliferation Assay comparing different types of media using Rat MSC's_Cell Count	80
Figure 36: Cell Proliferation Assay comparing different types of media using Rat MSC's_Cell Count	81
Figure 37: Cell Proliferation Assay comparing different types of media using Rat MSC's	82

Figure 38: Cell Proliferation Assay comparing different types of media using Rat MSC's	83
Figure 39: Cell Proliferation Assay comparing different types of media using Rat MSC's	84
Figure 40: Percent highest for Cell Proliferation assays using Rabbit MSC's at 1000 MSC's	85
Figure 41: Percent highest for Cell Proliferation assays using Rabbit MSC's at 2700 MSC's	86
Figure 42: Percent highest for Cell Proliferation assays using Rabbit MSC's at 5400 MSC's	87
Figure 43: Percent highest Cell Count using Rabbit MSC's at 1000 cells/well	88
Figure 44: Percent highest Cell Count using Rabbit MSC's at 2700 cells/well	89
Figure 45: Percent highest Cell Count using Rabbit MSC's at 5400 cells/well	90

LIST OF TABLES

	Page #
Table 1: Rabbit Experimental Protocol	25
Table 2: Cell Culture Media	33

LIST OF ABBREVIATIONS

IL	Interleukin
MSC	Mesenchymal Stromal Cell
CD	Cluster of Differentiation
FGF	Fibroblast growth factor
DMEM	Dulbecco's Modified Eagle's Medium
DMEM HG	Dulbecco's Modified Eagle's Medium with high glucose
MEM	Modified Eagles Medium
MDM	Modified Dulbecco's Medium

TABLE OF CONTENTS

I.	Introduction	1-22
II.	Materials	23
III.	Methods	24-32
	A. Animal Procedures	24-25
	B. Bone Marrow Stromal Cell Culture Protocol	25-26
	C. Trypsinization of MSC's	26
	D. Cryopreservation of cells	27
	E. Preparing the cells when frozen	27
	F. Histology	28
	G. Hematoxylin and Eosin Stain: Progressive Method	28
	H. Masson's Trichrome Staining Protocol	29
	I. Measuring Collagen Thickness	29-30
	J. Cell Adhesion Assay using Cell Titer 96 AQ One Solution Cell Proliferation Assay	30-31
	K. Cell Proliferation Assay	31-32
IV.	Results	34-91
V.	Discussion	92-100
VI.	References	101-111

Introduction:

One major area of concern for surgeons involves fascia healing. Wound sites can heal improperly following surgical repair, leading to the surgical site reopening and exposing abdominal contents, incisional hernia formation, or abdominal contents protruding through the surgical site. Long cycles of recurring hernias are one of the major complications that patients can endure if these hernias are not healed properly, as well as disfigurement of the body and bowel problems. Even though fascial healing has been a problem for many years, the progression towards finding a procedure that will alleviate this problem is extremely slow. Studies have focused on many mechanical factors including the technique used to close the incision site, incision orientation, suture material, and wound physiology. However, none of these factors seem to make a difference in the strength of healing, or in decreased incidence of herniation (Tyrone et al., 2000).

In order to improve facial repair, the process of wound healing needs to be understood. If researchers can provide an optimal environment for a surgical site to heal properly, this may reduce hernia formation. The process of wound healing is a natural event that can be divided into four distinct phases: homeostasis, inflammation, proliferation, and remodeling with scar maturation (Enoch and Leaper, 2007). The cells involved with wound healing include neutrophils, a phagocyte and the first cell to arrive at the site of injury. Platelets are the main source of growth factors involved in the wound healing process. Monocytes (or macrophages) are a phagocytic cell

responsible for clearing away dead tissue, stimulating fibroblast division and collagen synthesis, as well as angiogenesis. Lymphocytes are thought to produce cytokines at the wound site. Fibroblasts are responsible for producing molecules of the extracellular matrix, including fibronectin, hyaluronic acid, collagen, and proteoglycans, as well as granulation tissue.

Wound healing involves a variety of growth factors, proinflammatory cytokines and chemokines (Barrientos et al., 2008). The first proinflammatory cytokine that is released into damaged tissue is IL-1, which is secreted from injured keratinocytes, the most common cell found in the epidermis. Some of the proinflammatory cytokines involved with wound healing include IL-1 and IL-6. Both of these cytokines are largely involved with inflammation. IL-1 and IL-6 are both produced by neutrophils, and monocytes, and only IL-1 is produced by macrophages and keratinocytes. Epidermal growth factor is secreted by platelets, macrophages and fibroblasts. It is involved with epithelialization, and acts upon keratinocytes. Fibroblast growth factor is produced by keratinocytes, fibroblasts, endothelial cells, smooth muscle cells, chondrocytes and mast cells. It is involved with the formation of granulation tissue, epithelialization, and tissue remodeling. Fibroblast growth factor stimulates fibroblasts to produce the components of the extracellular matrix, increasing the motility of keratinocytes during epithelialization. Fibroblast growth factor also regulates fibroblast activity. Transforming growth factor beta is produced by macrophages, fibroblasts, keratinocytes and platelets. It is involved with inflammation, epithelialization, and the production of the extracellular matrix. It also plays an important role in the production of collagen.

Platelet-derived growth factor is produced by platelets, macrophages, cells found in the endothelium, fibroblasts, and keratinocytes. It is primarily involved in stimulating the chemotaxis (movement to the site) of neutrophils and macrophages. It is also involved with the formation of granulation tissue, epithelialization, the production of the extracellular matrix, and remodeling. Vascular endothelial growth factor is produced by endothelial cells, keratinocytes, platelets, neutrophils and macrophages. It stimulates endothelial cell migration and proliferation, which is important during angiogenesis. It is also involved with the formation of granulation tissue. Chemokines (chemotactic cytokines) are important in the wound healing process because they attract multiple cells, especially inflammatory cells, to the injured tissue. It is thought that chemokine receptors found on host tissue cells may play a part in epithelialization, remodeling, and angiogenesis.

The first stage of wound healing involves homeostasis, bringing the site of injury back to normal. As soon as the blood vessel wall is damaged, vasoconstriction occurs, as well as the activation of the coagulation cascade (Enoch et al., 2007).

Vasoconstriction and the coagulation cascade activation result in clot formation and platelet aggregation preventing further blood loss. Platelets are also responsible for increasing vascular permeability via the release of vasoactive amines, such as serotonin, which leads to the production of exudates in the extravascular space. The intrinsic and the extrinsic coagulation pathways are both involved in the coagulation cascade, and both result in the activation of thrombin, which is responsible for converting plasma fibrinogen to fibrin following tissue injury (Romo et al., 2008). The intrinsic pathway is

also known as the contact activation pathway, plays a minor role in the initiation of clot formation. The extrinsic pathway is also known as the tissue factor pathway, and is the primary pathway for the initiation of blood coagulation, and its role is to create a thrombin burst (thrombin is released instantaneously). Thrombin's role in the process of wound healing is to promote vascular permeability, making it easier for white blood cells and platelets to get into the injured tissue. The activated platelets aggregate at the site of injury, stimulating the plasma coagulation factors. This leads to the production of a fibrin clot. Fibrin is an important component of wound healing, and is responsible for providing the wound matrix, to which inflammatory cells, platelets, and plasma proteins can migrate. The clot is also made up of fibronectin, vitronectin, von Willibrand factor, and thrombospondin. Clot formation depends upon the magnitude of the various stimuli, as well as the site of injury. The site is important because the collection of platelets can be inhibited by chemicals such as prostacyclin produced by nearby uninjured endothelial cells. Prostacyclin can also act as a potent vasodilator (Romo et al., 2008). Once the process of wound healing begins, the platelets and fibrin clot are broken down.

The clot initiates homeostasis, and drives inflammatory cells to the site of injury. The platelets undergo degranulation resulting in the release of many growth factors, such as epidermal growth factor, platelet-derived growth factor, vascular endothelial growth factor, insulin-like growth factor-1, platelet factor-IV and transforming growth factor-beta (Enoch and Leaper, 2007). These proteins initiate wound healing by attracting fibroblasts, endothelial cells, and macrophages.

After clot formation, the inflammatory phase of wound healing begins.

Inflammation can be further broken down into the early and late phases (Enoch and Leaper, 2007). Inflammation begins with the activation of the classical and alternative complement cascades. Complement, plays an important role during an innate immune response. Complement is responsible for removing bacteria and viruses from an organism. Complement is a series of proteins in the blood that once activated have the ability to activate an inflammatory response (C3a, C4a, C5a), chemotaxis (C5a, C567), degranulation of mast cells (C3a, C4a, C5a), and cytolysis (C5b6789 or MAC). Some of the signs of inflammation include redness, heat, swelling and pain. The reddish appearance of a wound site results from the process of vasodilation, which increases the amount of blood flow to the injured tissue. Other inflammatory mediators include histamine, prostaglandins, kinins, and leukotrienes. The increase in blood flow resulting from an inflammatory response allows white blood cells to reach the site, and allows them to do their job fighting off an infection and removing dead cells from the wound.

Chemotactic compounds call inflammatory cells to the site of injury within hours.

The first and most common inflammatory cell to migrate to the site of injury is the neutrophil. Neutrophils are attracted to various substances, such as fragments of protein that make up the extracellular matrix, IL-1, transforming growth factor-beta, complement components (C3a, and C5a), as well as products from bacteria (Barrientos et al., 2008). Neutrophils enter the wound site via diapedesis, which involves the neutrophil first attaching to the endothelial cells, and then pushing through the vessel.

The main function of leukocytes, such as the neutrophil, is to phagocytose (eat) bacteria, preventing bacterial contamination of the wound.

During the late phase of inflammation, monocytes are attracted to the wound via various chemicals. The monocytes then differentiate into macrophages. The macrophage, also a phagocyte, is the most important cell during the late stage of inflammation. They secrete various cytokines which attract other cells, and are responsible for tissue repair to the damaged site. They also secrete various enzymes such as collagenases and elastases that help break down the injured tissue. Macrophages also secrete platelet derived growth factor, a cytokine that stimulates the proliferation and the production of collagen by fibroblasts, the proliferation of smooth muscle cells, and the proliferation of endothelial cells, which are responsible for angiogenesis (Romo et al., 2008). Angiogenesis is the formation of new capillaries which bud off of the preexisting capillaries. Macrophages also produce angiogenic factors that attract endothelial cells in response to low oxygen levels. Consequently, if macrophage numbers decline, this would result in the depletion of many important growth factors and the wound healing process would become severely altered. This could result in decreased fibroblast proliferation, and inadequate angiogenesis. After the inflammatory response, the proliferation stage begins, the second phase of wound healing.

Proliferation involves fibroblast migration to the site, formation of the extracellular matrix, the formation of granulation tissue, and epithelialization (Rosch et

al., 2003). There are many substances that attract fibroblasts to the wound site, including platelet-derived growth factor and transforming growth factor-beta. Fibroblasts are responsible for the production of collagen, fibronectin, hyaluronan, and proteoglycans, which all are components of the extracellular matrix.

The extracellular matrix is extremely important, and has many functions. It serves as an adhesion site for a variety of cells involved with the wound healing process, such as fibroblasts (Enoch and Leaper, 2007). The extracellular matrix (the structural support for connective tissue) allows the attached cells to differentiate, and is involved with their movement and growth. The extracellular matrix, which consists of the basement membrane (sheet that epithelial cells rest) and the interstitial matrix (the space between animal cells), is composed of collagen and elastin fibers, creating a strong yet flexible matrix. The interstitial matrix is composed of adhesive glycoproteins, and a gel-like material that is composed of proteoglycans and glycosaminoglycans. Epithelial cells, endothelial cells, and smooth muscle cells are then embedded in the interstitial matrix. Each component of the interstitial and extracellular matrix has a unique role in the maintenance of the extracellular matrix. Collagen fibers give connective tissue its strength and structure, and are extremely important in the proliferative phase of the wound healing process. Collagen is secreted in the form of procollagen in the extra cellular matrix. The cleavage of the procollagen molecule at the terminal segment produces tropocollagen. Tropocollagen can aggregate together to form collagen filaments, the filaments aggregate to form fibrils, and the fibrils aggregate to form the collagen fiber.

Intermolecular cross-links form between the fibrils, producing the collagen protein (Zimmerman et al., 1973). These intermolecular cross-links give collagen its mechanical strength, producing an extremely strong and supportive substance, which is resistant to damage. The most common types of collagen found in connective tissue are I, III, and V. Adhesive glycoproteins, such as fibronectin, laminin, and thrombospondin, connect all the protein fibers and cells found in the extracellular matrix. Fibronectin is especially important in the attachment and migration of cells in the extracellular matrix. Proteoglycans (a glycoprotein with a protein core connected to glycosaminoglycan chains) are involved with the structure and permeability of the extracellular matrix.

Granulation tissue is formed 3-5 days after the initial injury (Enoch and Leaper, 2007). This is a sign that the wound healing process is moving along without any complications. Granulation tissue can appear as pink, moist and shiny, which would indicate successful healing. The tissue can also appear as red and soft, which would indicate poor healing. Specifically, granulation tissue consists of proliferating fibroblasts within a matrix of capillaries and loose connective tissue. Angiogenesis is a prime component in the formation of granulation tissue. Some of the growth factors that stimulate angiogenesis include vascular endothelial growth factor, platelet-derived growth factor, basic fibroblast growth factor, and transforming growth factor beta. The first step of angiogenesis involves new vessels beginning to sprout off of the original vessel. The second and third step of angiogenesis involves the migration and proliferation of endothelial cells towards the angiogenic stimulus. Finally, the

endothelial cells mature and the newly formed blood vessels are complete.

Angiogenesis is important because it increases blood flow to the wound site. The next step in the wound healing process is epithelialization.

Epithelialization, involves the migration of epidermal cells to the wound site, which provides protection from the outside environment (Romo et al., 2008). This process occurs within a few hours after injury (Enoch and Leaper, 2007). The basal epidermal cells proliferate rapidly, and migrate over the wound site by the help of extremely small fibers called intracellular actin microfilaments. Keratinocyte growth factor, epidermal growth factor, and basic fibroblast growth factor stimulate epithelialization. After epithelialization is complete, the final stages of wound healing take place, including remodeling and scar maturation.

The formation of granulation tissue, and the synthesis and remodeling of the extracellular matrix are initiated simultaneously (Enoch and Leaper, 2007). The remodeling of the extracellular matrix involves the production and breakdown of collagen fibers, and this process lasts approximately 21 days after the initial injury. The fibroblasts adhere to the nearby molecules found in the extracellular matrix, leading to contraction of the wound. This process of contraction is stimulated by transforming growth factor beta, platelet-derived growth factor, and basic fibroblast growth factor.

Fibroblasts, neutrophils and macrophages at the site of injury produce various enzymes called metalloproteinases that break down collagen (Enoch and Leaper, 2007). Some examples of metalloproteinases include interstitial collagenases, gelatinases, and

stromelysins. Interstitial collagenases cleave collagen types I, II, and III. Gelatinases degrade unstructured collagen and fibronectin. Stromelysins break down components of the extracellular matrix such as the proteoglycans, laminin and fibronectin. The synthesis and secretion of all of these enzymes are regulated by growth factors, cytokines and phagocytic debris. The regulation of these enzymes is vital to wound healing because structured collagen fibers can also be degraded. After a while, further remodeling leads to a decrease in the breakdown of collagen, the number of macrophages and fibroblasts at the site of injury, the rate of angiogenesis, and the rate of contraction. At this time the granulation tissue is converted into an avascular scar, consisting of inactive fibroblasts, and a dense matrix of collagen and elastic fibers. During scar maturation, fibronectin and hyaluronan are broken down (Enoch and Leaper, 2007). The collagen bundles increase in diameter, which explains the increase in tensile strength of the scar tissue. However, the increase in strength is only 80% of the original level, before the tissue was injured.

Fibroblasts are responsible for the production of collagen, glycosaminoglycans (an important part of connective tissues), and fibronectin (an extracellular matrix glycoprotein that binds to integrins). Fibronectin binds to extracellular matrix components like collagen, along with other extracellular components, such as elastin and proteases, which play an important role in blood-clotting. Fibroblasts begin to increase rapidly in number as the inflammatory cells decrease in number. This process, called fibroplasia, can last up to 14 days, and starts about three to five days after injury. Fibroblast cells are brought to the site of injury and proliferate when

fibronectin, platelet derived growth factor, fibroblast growth factor, transforming growth factor, and C5a are present (Romo et al., 2008). Fibroblasts provide the structural framework for many tissues, and play a primary role in wound healing. They are derived from mesenchymal tissue, like other cells that make up the connective tissue. Fibroblasts are diverse, and their location and activity will determine their appearance. However, fibroblasts are not the only cell that contributes to wound healing, cells from the bone marrow stroma are also involved.

The bone marrow stroma contains a heterogeneous population of cells, including cells with stem cell characteristics such as hematopoietic, mesenchymal and endothelial stem cells. These bone marrow cells also secrete cytokines; however the exact role of these cytokines in wound healing is still unclear. Many studies have shown that bone marrow-derived stem cells are found in the wound and contribute to tissue repair as well as to the regeneration of various tissues such as damaged bone, blood vessels, skin, and cartilage. Recent studies have found that the cells found in the skin are derived from cells found in the bone marrow, and bone marrow derived cells found in the skin are involved in wound healing. These bone marrow stem cells may home to injured tissue, such as the skin.

The type of stem cell that this project will focus on is the mesenchymal stem cell (MSC). Bone marrow MSC's, are multipotent, spindle-shaped cells that are able to differentiate into various cell types (Markowicz et al., 2005). These stem cells are undifferentiated, with the ability to divide and replicate themselves longer than other

cells. They have the ability to become specialized cells to replace dead or lost cells (Han et al., 2005). Bone marrow stromal cells are the group of cells that MSC's belong to, and were first discovered by Friedenstein in the late 1900's. He was able to see that these stem cells were able to differentiate into many different types of cells such as fibroblasts, osteoblasts, chondroblasts, and adipocytes *in vitro* (Friedenstein et al., 1968). Stem cells are either categorized as multipotent or pluripotent, depending upon whether the cell can differentiate into more than one cell type, or differentiate into every cell type of the organism, respectively. A stem cell is also capable of self renewal. When it divides it produces identical cells that are still multipotent (Oreffo et al., 2005). MSC's adhere to plastic, and this is the method used to separate MSC's from the other cells found in bone marrow (Horwitz, 2002). Even though MSC's are primarily found in the bone marrow, it has been understood that MSC's also exist in various tissue types, such as circulating blood, cord blood, placenta, amniotic fluid, heart, skeletal muscle, adipose tissue, synovial tissue and the pancreas. Basically, if an organ contains connective tissue, MSC's can be found. In 2005, the International Society for Cellular Therapy stated that mesenchymal stem cells should be referred to as mesenchymal stromal cells (Dominici et al., 2006). The difference between a stem cell and a stromal cell is the ability to divide indefinitely, and many laboratories have not been able to accomplish this with MSC's consistently. In order for progress in this field of study, a universally accepted definition of MSC's was necessary. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy decided to take action, and develop a set a standards to

define MSC's that are either used in a laboratory setting or in pre-clinical studies. These standards are solely for research purposes using human MSC's, and should be updated as soon as new information about MSC's is discovered. The standards are as follows: MSC's are able to adhere to plastic, when cultured in tissue cultured treated flasks. They must express a specific cell surface antigen. They must be positive for CD105, also known as endoglin, CD73, and CD 90, also known as Thy-1. They must be negative for CD 45, a pan-leukocyte marker, CD34, a hematopoietic progenitor marker, CD14 or CD11b, a monocyte and macrophage marker, CD79 α or CD19, a B cell marker, and HLA class II. They must be capable of multipotent differentiation, meaning they must be able to differentiate into osteoblasts, adipocytes, and chondroblasts under differentiating culturing conditions, *in vitro*. Osteoblast differentiation can be confirmed by Alizarin red staining. Adipocyte differentiation can be confirmed by Oil Red O staining. Chondroblasts differentiation can be confirmed by Alcian blue staining. These standards are not universally accepted for every species, and each species will possess different surface antigens.

There are many unknowns about MSC's, because their use in research studies is fairly new. One problem is the low number of MSC's compared to other cells in the bone marrow. It is estimated that there are 10 MSC's for every one million bone marrow cells (Vaananen, 2005). Another problem that involves MSC research is the lack of knowledge about surface markers that will identify these cells (Horwitz, 2002) (Vaananen, 2005). Even following Friedenstein's developments, such as plastic adherence, medium modification, and single cell cloning, a complete understanding of

specific surface markers or antigenic determinants that can distinguish MSC's from every other cell in the bone marrow is still lacking. Another talked about problem is whether or not MSC's can turn malignant due to their uncontrolled proliferation and growth, and if a tumor should develop, was it derived from local or bone marrow derived stem cell. In 2005, Han and coworkers established a tumor cell line from mutated human embryonic bone marrow MSC's (Han et al., 2005). It was also demonstrated by Rubio and coworkers that MSC's could spontaneously transform when cultured *in vitro* for long periods of time, such as 4-5 months instead of 6-8 weeks (Vaananen, 2005) (Rubio et al., 2005).

The intra and extra cellular pathways that determine a cell's lineage still remains a major challenge for many researchers. These signaling pathways and transcriptional regulation are both necessary to determine cellular fate. This needs to be known in order to grow cells *in vitro* and to allow for proper cell expansion. The importance of the microenvironment, including soluble factors as well as cell-cell and cell-matrix contacts, has been demonstrated in various animal experiments. The effects of hormones, vitamins, growth factors, and cytokines on MSC proliferation and differentiation have been tested, looking at primarily osteogenic, chondrogenic or adipogenic differentiation. It is important to use growth factors that contribute to their self renewing capabilities, and to maintain their multilineage potential, such as the fibroblast growth factor family. FGF-2 plays a huge role in self renewal, as does vascular endothelial growth factor, a potent substance with mitogenic qualities involved in endothelial proliferation (Markowicz et al., 2005)(Vaananen, 2005). More examples

include transforming growth factor β , which aids in cartilage differentiation, and bone morphogenetic proteins, which enhance osteoblast differentiation, and adipogenic complete medium containing dexamethasone which induces adipogenic differentiation. There are also many transcription factors that will direct MSC's towards a specific lineage and these factors can be extremely helpful in creating the optimal microenvironment to generate specific tissues needed in clinical studies (Vaananen, 2005).

Previous work from our laboratory examined tensile strength of the wound, collagen deposition, and collagen remodeling following the addition of MSC's to a repaired incision, using a rabbit model. The purpose of this project was to find a way to improve fascial healing using autologous MSC's. This study showed a promising use of MSC's in combination with platelet rich plasma-enhanced fibrin glue on bovine type 1 collagen. It was known that collagen type I can act as a vehicle for MSC's and helps organize tissue remodeling (Juncosa-Melvin et al., 2006). It is also known that platelet enriched plasma provides increased levels of vascular endothelial growth factor and platelet-derived growth factor- β , which can induce MSC growth and collagen remodeling (Eppley et al., 2004). Our previous studies applied MSC's to the wound with a vehicle containing collagen, platelet rich plasma and fibrin which is an important component of the wound, allowing MSC movement through the matrix (Greling and Clark, 1997).

Hernia repair may be more difficult than the repair of cutaneous tissues as the

inflammatory cells and fibroblasts that migrate from the local blood vessels must travel through the surrounding tissue to get to the healing fascia. In the abdominal region the fascia is located just below the skin and just above the peritoneum. There are three types of fascia, which include the subcutaneous, deep and subserous fascia, and these layers are found in this order moving from superficial to deep. The subcutaneous fascia covers the entire body and is located between the skin and other structures such as muscle, and it blends with the reticular layer of the dermis. It is primarily adipose tissue and loose areolar connective tissue, and serves as a storage medium for fat and water. The blood vessels are located between a fatty and elastic layer, along with nerves and lymph vessels. The deep fascia is primarily dense fibrous connective tissue, with densely packed collagen and elastic fibers, covering muscles, bones, nerves and blood vessels. It makes up tendons, ligaments, aponeuroses, and joint capsules found in the body. The deep fascia also makes up many envelopes of bone, cartilage, and muscles, known as the periosteum and endosteum, the perichondrium, and the epimysium, perimysium and the endomysium, respectively. The subserous fascia is between the deep fascia and the serous membranes that line all the body cavities. Because blood vessels are only found in the subcutaneous fascia, the fibroblasts must travel through many layers of tissue during fascial repair. In a cutaneous wound, fibroblasts and inflammatory cells are able to migrate from the blood vessels directly to the wound site, and begin the healing process immediately (Tyrone et al., 2000). Just below the skin, the subcutaneous layer or the hypodermis is highly vascularized. The fibroblasts and inflammatory cells leave the hypodermis, and travel

through the dermis, and epidermis. Therefore, there is a significant difference in the healing time, and strength, when comparing the cutaneous layer and the fascia.

This research project will investigate whether MSC's, embedded in mesh supports, will enhance hernia repair. The mesh supports will be attached to the wound site and help support the healing environment, which is standard procedure for hernia repair. There have been some previous studies in which researchers used various mesh matrices to support the growth of stem cells, and then used those matrices to aid in wound healing, or tissue engineering. One research project used collagen matrices made of collage type I isolated from bovine skin, which were then freeze dried to a non-directed structure with various pore sizes. The collagen matrices were then cut into small cubes, and used in the experiment. Some of these small cubes were then heparinized and cross-linked with 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide and N-hydroxysuccinimide, which basically activated the carboxylic acid groups of heparin. These modified scaffolds increased the proliferation of endothelial cells, and demonstrated that these endothelial cells can form organized structures within the scaffolds (Markowicz et al., 2005). Another group performed a ceramic cube assay to test the ability of MSC's to produce bone as well as cartilage. They cultured the MSC's in 11 different types of serum, changing the percent of horse, chick, or fetal calf serum. They used ceramic cubes coated in fibronectin, and loaded them with MSC's. These cubes were then implanted subcutaneously into syngeneic rats, and the cubes were harvested 3-6 weeks after the implantation. The ceramic cubes were processed for histological evaluation, to analyze the presence of bone formation. This

study showed that MSC's produced mineralized tissue in almost all of the cubes after 6 weeks of implantation, and were also able to produce cartilage but to a lesser extent (Lennon et al., 1996).

In this study we will use four different types of mesh materials that are normally used in hernia repair, to determine if the MSC's adhere to the mesh. This will allow surgeons to then implant the mesh into the surgical site, providing added support to the incision site, as well as MSC's to enhance the wound healing process. This will test the hypothesis that adding MSC's may aid in the reconstruction of hernia repair or soft tissue deficiencies, decreasing the risk of recurrence.

The first type of mesh material that will be used in this project is called Bard CollaMend Implant. The Bard CollaMend is a lyophilized acellular porcine dermal collagen matrix, which was chemically cross-linked to increase strength and endurance, and is used in soft tissue repair (LeBlanc, 2008). The final product is composed of a matrix or framework of natural biological components, which allow the body to undergo its own tissue regeneration process. Colla Mend is chemically modified to reduce antigenicity, stimulate acellularity, and to inhibit enzymatic degradation, due to cross-linking the mesh with 1-ethyl-carbodiimide hydrochloride (Scott et al., 2007). This mesh material was developed to eliminate the need for long term use of synthetic material in patients that need hernia repair or soft tissue reconstruction. CollaMend is considered to be a minimally processed material that is similar in structure to basement membrane and can be used in cases where a synthetic prosthesis is not appropriate. It

works by containing fibers that organize into various load-bearing structures, which then strengthens tissues between tendons, skin, and organs. It controls cell shape and differentiation, and provides the blueprint for how to remodel the tissue in its anatomical environment.

The second mesh material that will be used is called Gore-Tex soft tissue patch. This is a synthetic prosthetic patch used in soft tissue repair (Gore Tex Soft Tissue Patch, 2007). It is composed of polytetrafluoroethylene, and it features a microporous structure allowing for host tissue incorporation. It is promising in laparoscopic hernia repair as well as for use in temporary bridging of fascial defects. The material used in Gore-Tex is stronger than most commonly used meshes. The biological inertness and softness of the prosthesis minimizes a foreign-body response, erosion in the bowel, and adhesions to surrounding viscera.

The third type of material used is called VICRYL, a synthetic knitted mesh made of an absorbent material called polyglactin 910 (Vicryl (polyglactin 910) woven mesh, 2009). It is a knitted mesh prepared from a synthetic absorbable copolymer of glycolide and lactide, derived respectively from glycolic and lactic acids. VICRYL's knitted mesh structure in vivo reinforces wound strength and improves the rate of absorption. It can be used wherever temporary wound or organ support is required, especially when a stretchable support material is desired. VICRYL is only intended for temporary support during the healing process because it is absorbable.

The fourth type of material used is called Bard Mesh, a synthetic mesh made of

monofilament knitted polypropylene fibers, which cannot support the growth of bacteria (Bard Mesh Flat Sheets, 2010). This mesh was first discovered by Lichtenstein when he developed the “tension-free” repair system, about 45 years ago. The tension free healing environment allows the fascia to adjust to the patient’s position, and its use results in fewer complications. This type of mesh stays in the body permanently, is immunologically inert and well tolerated by the body (Amid et al., 1992). Bard Mesh is able to restore the strength and elasticity of damaged tissue by providing an environment for fibroblast ingrowth, and by withstanding more than normal levels of pressure. Bard Mesh also has a low recurrence rate, which averages less than 1%.

In general, any type of mesh material needs to meet four requirements: The mesh must be pliable and able to adjust to the patient’s anatomy, the mesh should not be able to stimulate a severe inflammatory response, the mesh should be porous for drainage to occur and for fibroblast ingrowth, and the mesh should also be able to maintain its integrity.

The primary purpose of these mesh materials is to maintain the viscera of the abdominal cavity, allowing the wound to fully develop, and to prevent any defects. It has been shown that the weight of the mesh material can influence the degree of inflammation and cell proliferation. In a study by Rosch and coworkers, the researchers found that the polypropylene + polyglactin mesh (55g/m²) produced a lower inflammatory response. The researchers looked at the macrophage index (number of ED3-positive macrophages per total number of cells), T-cell index (number

of CD 3-positive cells per total number of cells), and mast cell index (mast cells per total number of cells) and cell proliferation during long-term and short-term implant durations (Rosch et al., 2003). Rosch and coworkers found that large pore and low weight mesh materials had a reduced inflammatory response. In 1996, Beets and coworkers showed that the pore size of the mesh material influences the foreign body response. They found that the mesh with small pores initiated a larger immune response (Beets et al., 1996). In the future when our research group implants these mesh materials into a rat model we would expect the lightest mesh material with the largest pore size to stimulate a decreased inflammatory response and increased cellular proliferation.

The main focus of this project is to see how well synthetic mesh, and other mesh-like supports act as a scaffold for the attachment and growth of MSC's. This will allow surgeons to apply these mesh supports containing MSC's at surgical sites in an attempt to decrease the rate of recurrence of hernia's. Hopefully this will diminish, or in some cases eliminate hernia reappearance. In the past, mesh supports have been used in the treatment of hernia repair. MSC's have been used to treat cartilage repair and cutaneous tissue repair. Future studies are designed to see how MSC's along with mesh supports can be used to strengthen hernia repair sites. This study will look at various types of biosynthetic mesh materials as well as synthetic mesh to determine which mesh allows the MSC's to bind effectively. Studies have found that collagen is an effective scaffold for these stromal cells. The goal in this project is to see how the stromal cells attach to other types of mesh materials. Hopefully, this project will aid

surgeons with their struggle to eliminate hernia problems associated with laparotomies.

In order for MSC's to be used therapeutically, the culture conditions must be optimal to allow for high levels of cell proliferation, and ultimately high numbers of MSC's. Various factors that are relevant to optimization include cell plating density, the concentration of fetal calf serum, passaging density, the effect of cryopreservation, and the addition of growth and other supplementary factors (Sotiropoulou et al., 2009). Currently, most research facilities use a different recipe for culturing MSC's, and there is very little information about the optimal culture conditions. Most importantly, culture conditions need to be sterile, with an abundant supply of nutrients. The pH level and temperature are also vital factors. This research project compares various media recipes, and cell plating densities to determine the optimal culture conditions for rabbit and rat MSC's.

Materials:

Bard Colla Mend and Bard Mesh were purchased from C.R.Bard Inc. (Madison, GA). Vicryl was purchased from Ethicon (Cornelia, GA). Gore-Tex was purchased from W.L.Gore & Associates (Newark, DE). Cell Titer 96 AQ One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI). Bovine calf serum was purchased from Hyclone Laboratories Inc. (Logan, UT). Trypsin-ethylene diamine tetraacetic acid, L-glutamine, penicillin/streptomycin solution, HEPES, α MEM media, Ham's F-12 media, amphotericin, Dulbecco's modified Eagle's medium low glucose media, Dulbecco's modified Eagle's medium high glucose, Glutamax, Modified Eagle's medium with Earle's

salt were all purchased from Gibco (Carlsbad, CA). Gentamycin was purchased from One Cell Systems (Cambridge, MA). Basic fibroblast growth factor was purchased from PeproTech (Rocky Hill, NJ). ITS Premix, and non tissue cultured treated nonsterile polyvinyl chloride 96 well plate was purchased from BD BioSciences (Durham, NC). Dexamethasone was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Eosin Y, Phloxine B, Hematoxylin, mercuric acid, ammonium aluminum sulfate, absolute ethyl alcohol, 95% Alcohol, glacial acetic acid, xylene, ammonia, 29% aqueous solution of ferric chloride, 1% aqueous solution of Biebrich Scarlet, 1 % aqueous solution of acid fuchsin, aniline blue, phosphomolybdic acid, phosphotungstic acid were purchased from Fisher Chemical Company (Fair Lawn, NJ). Xylene was purchased from VWR Scientific (San Francisco, CA)

Methods:

Animal Procedures:

In order to obtain Mesenchymal Stromal Cells (MSC's) from rats, femurs and tibias were removed from a euthanized rat. Bone marrow was collected by flushing the bone with DMEM. Rabbit bone marrow was collected from an isofluorane anesthetized rabbit by

making a small incision and exposing the tibia. A 10mL syringe with 1mL of heparin solution was used to aspirate 4-5mL of bone marrow from the proximal tibia. Bone marrow culture to produce mesenchymal stromal cells is described below (Bone Marrow Stromal Cell Culture).

The next phase of the research involved the rabbit surgeries, where autologous MSC's were applied to fascial defects. Each rabbit was its own control, as described by Tyrone and coworkers (Tyrone et al., 2000). A midline abdominal wall skin incision was created in anesthetized rabbits, using isoflurane. This was followed by bilateral fascial incisions, approximately 6cm in length and 1cm lateral to the midline. All defects were closed with 6-0 running nylon sutures. The controls were on the right side of the rabbit, and the test group was on the left side of the rabbit. The collagen vehicle containing the PRPFG with MSC's was placed on the fascial defect. The control side contained the same treatment as the test group, except for the addition of the MSC's. The three experimental groups are described in Table 1.

Table 1. Rabbit Experimental Protocol

Group	Control	Test
1	Type 1 collagen tape	Type 1 collagen tape + MSC's
2	Platelet rich plasma with fibrin glue	Platelet rich plasma with fibrin glue + MSC's
3	Platelet rich plasma with fibrin glue on collagen tape	Platelet rich plasma with fibrin glue on collagen tape + MSC's

The skin was then closed with running 4-0 cutaneous sutures and the animals were closely monitored by the veterinary staff for 8 weeks. After the 8 weeks, the entire abdominal fascia was harvested, as outlined by DuBay and coworkers (DuBay et al., 2005). The tissue was paraffin embedded for histologic analysis or transferred to PBS for analysis of tensile strength (data not shown in this thesis) by the engineering department.

Bone Marrow Stromal Cell Culture Protocol:

The culturing of rat and rabbit MSC's was performed by the method of Im and coworkers (Im et al., 2001). The bone marrow was centrifuged and washed with PBS once. The supernatant was removed and the cells were resuspended in 10mL of high-glucose Dulbecco's Modified Eagle's Medium containing 100U/ml penicillin, 100ug/ml streptomycin, 2.5ug/ml amphotericin B, 1mM of L-glutamine and 10% fetal calf serum (MSC media). The cells were counted with a hemocytometer and 10^6 cells were added to a T-75 cell culture flask. The cells were incubated at 37°C in the presence

of 5% CO₂ for 4 days, the non-adherent MSC's were removed, and new medium was added to the flask. The cells were fed every 3-4 days, and when the cells were 80% confluent they were passed and a new flask was started. MSC's from 21 different rabbits were used in this study, keeping all cells labeled to identify the rabbit identification. MSC's were removed from the culture flasks and added to platelet rich plasma enhanced fibrin glue and embedded in collagen. Fibrin glue was acquired from Baxter (Deerfield, IL) in the form of TISSEEL VH (fibrin sealant). The fibrin glue, isolated MSC's and the platelet gel were mixed together according to Ito and coworkers (Ito et al., 2006).

Trypsinization of MSC's

To transfer cells, trypsinization of MSC's was performed by the method of Morgan and coworkers (Morgan, 1993). The stem cell media was removed and the cells were washed with 1 ml of phosphate buffer saline. Next 2mL/T25 or 5mL/T75 of 0.25% trypsin in 1.0 mM ethylene diamine tetraacetic acid was added to the flask of MSC's. When the MSC's started to round up, the flask was tapped to dislodge the MSC's from the flask. Enough stem cell media with 10% fetal calf serum was added to the flask to inactivate the trypsin. For every 1ml of trypsin, 10ml of media needs to be added to inactive the trypsin effectively.

Cryopreservation of cells

Cryopreservation of cells was performed in order to store the MSC's until they are

needed for an experiment. The cryopreservation of cells was performed by the method of Morgan and coworkers (Morgan, 1993). A concentration of 10^6 MSC's were centrifuged at 400 *xg* for 8 minutes. The MSC's were resuspended in media containing 10% DMSO, and the MSC's were placed in a labeled cryogenic vial. The MSC's were exposed to a final temperature of -80°C, decreasing the temperature slowly. The MSC's were then transferred to liquid nitrogen.

Preparing the cells when frozen

The preparation of cells when frozen was performed by the method of Morgan and coworkers (Morgan, 1993). Approximately 5 ml of MSC media was added to a sterile centrifuge tube. Immediately after taking the vial of MSC's out of the liquid nitrogen tank, they were thawed out slightly in a water bath set at 37°C. The cap was rinsed with methanol and the MSC's were quickly decanted into the media and mixed gently to thaw the MSC's completely. The MSC's in media were centrifuged at 400 *xg* for 8 minutes. The media was aspirated off and the MSC's were resuspended in 4 ml of MSC media. The MSC's were plated in a 24 well plate, putting 1 ml in each well. The MSC's were stored in an incubator set at 37 °C in the presence of 5% CO₂. The MSC's were gradually moved into larger plates until the desired number of cells was reached.

Histology

The slides examined were prepared by paraffin embedding and sectioning. This was performed by the Histology department at St. Elizabeth.

Hematoxylin and Eosin Stain: Progressive Method

This stain was used to view basic tissue morphology. The Hematoxylin and Eosin stain was performed by the method of Carson (Carson, F). The slides were washed with xylene 3 times for 2 minutes each. To hydrate the slides, they were dipped 10 times in absolute alcohol, and then dipped 10 times in 95% alcohol. The slides were then washed with tap water. The slides were saturated with the Harris Hematoxylin dye (5g Hematoxylin, 50ml of absolute ethyl alcohol, 100g of ammonium aluminum sulfate, 1,000ml of distilled water, 2.5g of mercuric oxide) for 1-3 minutes. The slides were dipped in tap water 10 times each, making sure to change the water 2 times. The slides were then placed in 0.25% ammonia water for 10 to 30 seconds. The slides were rinsed in tap water 10 times each, making sure to change the water frequently. The slides were saturated with the eosin-phyloxine dye (100ml of a 1% Eosin Y solution, 10ml of a 1% Phloxine B solution, 780ml of 95% alcohol, and 5ml of glacial acetic acid) for 1-3 minutes. The slides were dipped in 95% alcohol 10-15 times each, making sure to change the alcohol 2 times, and then dipped in absolute alcohol 10-15 times each, making sure to change the absolute alcohol 3 times. Finally the slides were dipped 10-15 times in xylene, making sure to change the xylene 3 times, keeping the slides in the xylene until the coverslip is ready to be applied.

Masson's Trichrome Staining Protocol

This stain was used to differentiate between collagen and smooth muscle. The Masson's Trichrome stain was performed by the method of Carson (Carson, F). The

slides were deparaffinized, and hydrated in distilled water. The slides were placed in the Weigert Hematoxylin (Stain consisted of equal part of Solution A and B. Solution A: 10g Hematoxylin, and 1,000ml of 95% alcohol. Solution B: 20ml of a 29% Ferric chloride aqueous solution, 475ml of distilled water, and 5ml of glacial acetic acid) for 10 minutes. The slides were washed in running water for 10 minutes. Slides were rinsed in distilled water. The slides were placed in the Biebrich scarlet-acid fuchsin solution (360 ml of a 1% Biebrich scarlet aqueous solution, 40ml of a 1% acid fuchsin aqueous solution, and 4ml of glacial acetic acid) for 2 minutes. The slides were rinsed in distilled water. The slides were placed in the phosphomolybdic/phosphotungstic acid solution (25g of phosphomolybdic acid, 25g of phosphotungstic acid, and 1,000ml of distilled water) for 10-15 minutes. The slides were placed in the aniline blue solution (25g of aniline blue, 20ml of glacial acetic acid, and 1,000ml of distilled water) for 5 minutes. The slides were rinsed with distilled water. The slides were placed in a 1% acetic acid solution for 3-5 minutes. The slides were dehydrated with 95% alcohol and absolute alcohol, making sure to change the solution 2 times. The slides were cleared with 2 or 3 changes of xylene and mounted with synthetic resin.

Measuring Collagen Thickness

The scar tissue was sectioned, stained with Masson's Trichrome stain and Hematoxylin and Eosin stains, and the collagen thickness was measured. Masson's Trichrome stained allowed the identification of collagen as a blue band next to the fascia.

Collagen thickness on the prepared slides were measured using the computer program

Moticlimage Plus 2.0. The two blue dots that were made by the histologists were located, and this is the area of scar tissue. Images of scar tissue were captured with a digital microscope. Scar thickness was measured as the distance from the inferior to superior border and reported as the average of 10-15 observations. The camera was calibrated every time the images were taken. A Motic calibration slide spot was used to calibrate the camera at the 4x objective magnification.

Comparison of control versus test samples was performed using a Student's *t*-test.

Cell Adhesion Assay using Cell Titer 96 AQ One Solution Cell Proliferation Assay

The cell adhesion assay was performed to determine which mesh material allowed the MSC's to adhere. The number of cells bound to mesh materials was determined using the colorimetric assay Cell Titer 96 AQ One Solutions Proliferation Assay, produced by Promega Corporation (The cell adhesion assay was performed by the method of Zhu and coworkers (Zhu et al., 2006). The matrix materials used in this procedure included Bard Colla Mend, Gore-Tex, vicryl and Bard Mesh. These matrix materials were cut into circles that measured approximately 3mm in diameter, and placed into the wells of a sterile tissue culture treated or non-tissue cultured treated, nonsterile polyvinyl chloride 96 well plate. The Bard Colla Mend was rehydrated with sterile saline (.85 % NaCl) for 3 minutes at 37°C in the presence of 5% CO₂. The matrix materials were washed 3 times with MSC media (DMEM-High glucose with 10% FCS, 100U/ml penicillin,

100ug/ml streptomycin, 2.5 ug/ml amphotericin B, 1mM of L-glutamine). Viable MSC's were counted using trypan blue exclusion on a hemocytometer. The MSC's were centrifuged at 400 *xg* for 8 minutes and resuspended at 4×10^4 MSC's/100ul and 100ul of MSC's were added to the appropriate wells containing mesh. The stem cell media remained in the wells that did not receive MSC's. To interpret the absorbance values from the adhesion assay, a standard curve was performed. MSC's in 200ul of stem cell media was pipetted into two wells, and 100ul of stem cell media was added to 4 additional wells. Serial 2-fold dilutions were performed down the wells. The MSC's were allowed to incubate with the matrix materials for 30-60 minutes at 37°C in the presence of 5% CO₂. All wells containing mesh and cells or only mesh was washed gently with PBS 3 times, and 100ul of PBS was added back to each well. To account for the reactivity between the reagent and PBS, 4 wells contained only PBS. To account for the reactivity between the mesh and the reagent 3 wells/mesh contained only mesh. To account for the reactivity between the MSC media and the reagent in the standard curve, 4 wells contained only MSC media. The number of adherent MSC's was indicated by adding 20μl of Cell Titer 96 AQ One solution reagent to each well. The plate was incubated for 0-4 hours at 37°C in the presence of 5% CO₂. The absorbance at 490 nm was determined before and after taking out the mesh, using a 96-well plate reader.

Cell Proliferation Assay

This method was performed to see which type of MSC media provided the optimal

environment for the proliferation of MSC's. This will allow future research to be accomplished in a timely manner. MSC's were counted on a hemocytometer using trypan blue exclusion. Cells were centrifuged at 400 *xg* for 8 minutes and resuspended in MSC media #5 (our normal media). Cells were plated at 3 concentrations, 1000 MSC's/well, 2700 MSC's /well, and 5400 MSC's well, in triplicate in a tissue culture treated 96-well plate. The plate was incubated for 12 hours. The MSC media was replaced with the designated media (1-10) (Table 2), and the MSC's were fed twice a week. When one well reached 80% confluent, all wells were washed 3x with PBS, then 100ul of PBS was added back to all wells. The cells were counted using an inverted microscope, counting 1 field of vision/well at the highest magnification (total magnification=200x). The cell number was also determined by adding 20ul of Cell Titer 96 AQ One solution reagent to each well and the plate was incubated for 2 hours in 37°C in the presence of 5% CO₂. The absorbance was measured at 490nm using a standard plate reader.

Table 2. Cell Culture Media

Media	FCS^k	Additives^l	Species
Alpha MEM ^a	20%	1mM L-glutamine	Human rat
Alpha MEM ^b	10%	2mM L-glutamine	human
Alpha MEM ^c	10%	1mM L-glutamine 25 mM HEPES	human
Hams F12 ^d	10%	0.25ug/ml amphotericinB	rabbit

DMEM HG ^e	10%	1mM L-glutamine 0.25ug/ml amphotericinB	rabbit
DMEM HG ^f	20%	10mM HEPES	rabbit
DMEM ^g	10%	25 ug/ml gentamycin	human
DMEM ^h	10%	1mM L-glutamine 1mM glutamax	rat
MEM Earles ⁱ	10%	1mM L-glutamine 1mM glutamax	rat
Iscove's MDM ^j	10%	2mM L-glutamine 10ng/ml bFGF	human

^a Gregory, CA, *et.al.*, Stem Cells. 24 (2006): 2232-2243.

^b Bartmann, C, *et.al.*, Transfusion. 47 (2007): 1426-1435.

^c Schallmoser, K, *et.al.*, Transfusion. 47 (2007): 1436-1446.

^d Im, G, *et.al.*, Journal of Bone and Joint Surgery. 83(2001): 289-94.

^e Solchaga, LA., *et.al.*, Cell Transplantation. 8 (1999): 511-519.

^f Yan, H and Yu, C. Arthroscopy: The Journal of Arthroscopic and Related Surgery. 23 (2007): 178-187.

^g Han, Seung-Kyu, *et.al.*, Annals of Plastic Surgery. 55 (2005): 414-419

^h Farrell, E, *et.al.*, Tissue Engineering. 12 (2006): 459-469.

ⁱ Kuo, TK, *et.al.*, Gastroenterology. 134(2008): 2111-2121.

^k Fetal calf sera was heat-inactivated for 45 min at 55-60°C

^l All media contained 100U/ml penicillin and 100ug/ml streptomycin

Results:

The goal of this study was to determine if the addition of mesenchymal stromal cells (MSC's) to an incision site will improve wound healing in a rabbit model. Twenty-one rabbits were used in this study. This study involved 3 phases. The first phase was the harvesting of MSC's from the rabbits tibia/iliac crest. The cells were grown in culture, and frozen for later use. In the second phase, the MSC's were transplanted onto rabbit fascia at the site of a repaired incision. A midline abdominal wall skin incision was performed in anesthetized rabbits, along with bilateral fascial incisions. Four incisions were performed on each animal with two control (no MSC) and two test (with MSC) incisions. The control group was on the right and the test group was on the left, therefore each rabbit was its own control. The incisions were closed with nylon sutures. The rabbits were divided into three groups, with seven rabbits in each group. Group one control incisions, had no additions while test incisions received MSC's added to type 1 collagen tape. Group two control incisions received platelet rich plasma mixed with fibrin glue with test incisions receiving MSC's in the same vehicle. In group three the vehicle was platelet rich plasma, fibrin glue and collagen tape with the test side receiving MSC's in the same vehicle. The MSC's were added to platelet rich plasma enhanced fibrin glue, embedded in a collagen vehicle, and placed on the fascial defect. The skin was closed with cutaneous sutures and the rabbits were monitored for 8 weeks. In the third phase the abdominal fascia was harvested, embedded in paraffin, sectioned and stained. The Masson Trichrome stain

was used to measure collagen production at the site of the incision. In Masson Trichrome stained tissue, the muscle tissue is stained pink and the collagen is stained blue. An example of a stained tissue section is shown in **Figure 1**. Each line represents a measurement taken of the collagen band. Approximately 10-15 measurements were taken for each slide. The two blue dots were added by the Histology department, and the area between the blue dots is thought to be scar tissue.

The Histology department at St. Elizabeth sectioned and stained the slides for this study. A blue dot was placed on either side of the scar tissue when the site of the incision could be identified on the slide. An incision site could not be found on all of the tissue sections following paraffin embedding, so the collagen was only measured on slides in which the incision site was identified with blue dots. To remedy this problem, in future studies the area of the scar will be marked with india ink dots prior to paraffin embedding. The thickness of the collagen deposited at the test incision site shown in **Figure 1** was 523.36 μm . This was calculated by taking the average of the 13 measurements. The control incision site for this rabbit was 299.55 μm , indicating that the MSC's increased the amount of collagen deposited at the test incision site. The thickness of collagen deposition between control and test groups could only be compared for 6 out of the 21 rabbits. Measurements of collagen deposition at the incision site for these rabbits are shown in **Figure 2**. Rabbit #1 in this figure was from group 1 (collagen tape +/- MSC). No obvious difference was seen in the thickness of collagen deposition in these samples, with the control measuring 647 μm and the sample which received MSCs being 574 μm . Rabbits 2-4 were from group #2 which

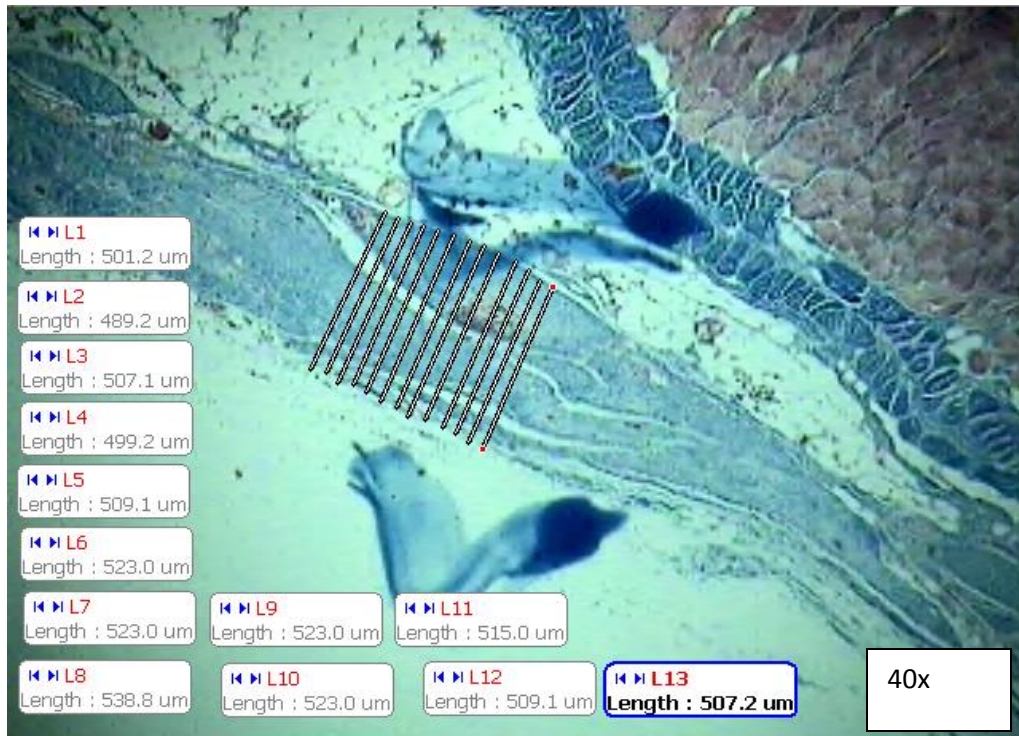


Figure 1. Thickness of Collagen Deposition following Rabbit Surgeries: Tissue section from the abdominal wall of a New Zealand white rabbit, stained with the Masson Trichrome stain. The staining was performed by the Histology department at St. Elizabeth Hospital. The computer program used to measure the collagen thickness was MoticImage Plus 2.0. The two blue dots that were made by the histologists were located, and the collagen band in this area was measured. Images of scar tissue were captured with a digital microscope. Scar thickness was measured as the distance from the inferior to superior border and reported as the average of 10-15 observations. The camera was calibrated every time the images were taken. A Motic calibration slide spot was used to calibrate the camera at the 4x objective magnification. Each slide was analyzed and the values were averaged together to get one value for the collagen thickness. This figure is from rabbit #21 which was in group #1.

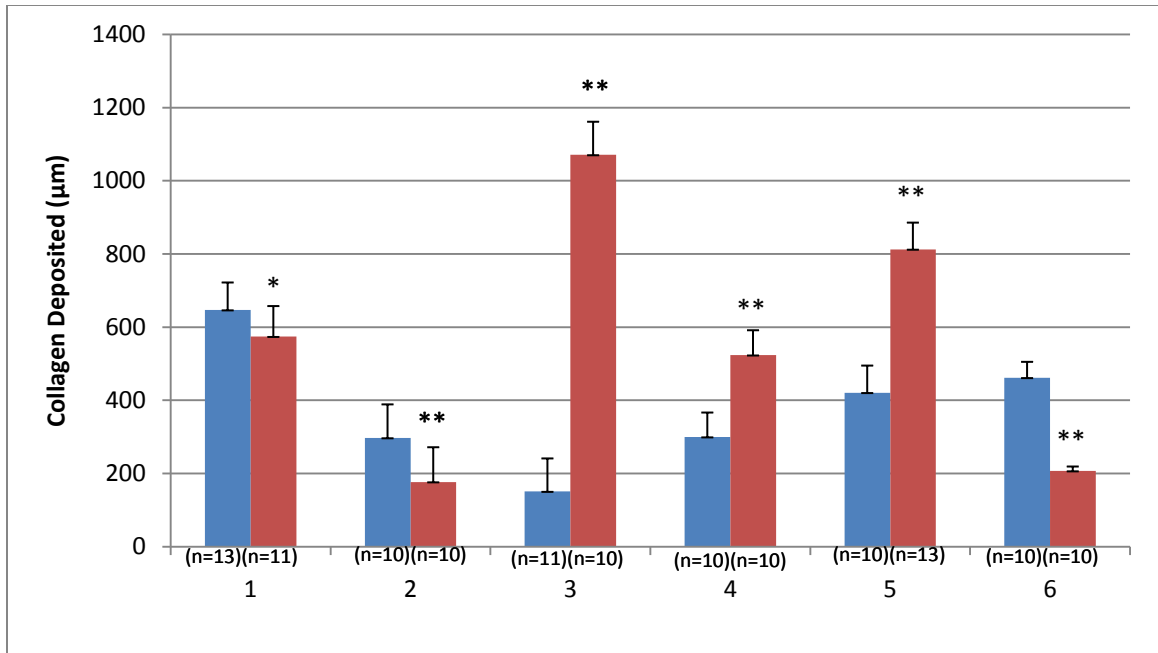


Figure 2. Thickness of Collagen Deposition following Rabbit Surgeries:

Measurements of collagen thickness from six rabbits. Measurements were performed as described in Figure 1. Rabbit 1 was from Group 1 (collagen +/- MSC). Rabbits 2-4 were from Group 2 (platelet rich plasma/fibrin glue +/- MSC). Rabbits 5 and 6 were from Group 3 (collagen/ platelet rich plasma and fibrin glue +/- MSC). The control group (no MSC) is represented by the blue bar, and the test group (+MSC) is represented by the red bar. Statistical analysis was performed using a Student's *t*-test (* = $p < 0.005$, ** = $p < 0.001$).

received platelet-rich plasma and fibrin glue +/- MSC. In this group two of the three rabbits demonstrated increased collagen deposition in the wound containing MSCs (#2: 297 μm in the control, 177 μm with MSC; #3: 151 μm in the control, 1071 μm with MSC; #4: 300 μm in the control, 523 μm with MSC). In group #3, which received platelet-rich plasma, fibrin glue and collagen tape with or without MSC, one rabbit showed increased collagen deposition (#5: 421 μm in the control, 812 μm with MSC) while one rabbit showed decreased collagen deposition in the MSC treated tissue (#6: 462 μm in the control, 207 μm with MSC). These results are not conclusive and statistical analysis could not be performed due to the low number of samples tested. However, there is an indication that the addition of MSCs to the wound may have increased collagen deposition in the tissue, which could strengthen the scar formed following surgery.

Hernia surgery usually involves the addition of mesh materials to the surgical site, in an attempt to strengthen the site and prevent re-herniation. The next part of the research project investigated the ability of MSC's to adhere to various types of mesh material currently used in hernia repair. This experiment involved adding the mesh materials to a 96 well plate, and then adding the cells to the appropriate wells. Rabbit MSC's have not been used in this type of cell adhesion assay previously. Adhesion could be measured by directly counting the cells that attach to the mesh. However, visual counts are time consuming, susceptible to investigator bias, and could possibly be difficult to perform with the mesh under the cells. An alternative method of determining cell number, the Promega Cell Titer Cell Titer 96 AQ One Solution Cell

Proliferation Assay was investigated as an alternative to manual microscopic cell counts (Zhu et al., 2006). This assay involves the addition of a solution containing an MTS tetrazolium compound (Owen's reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt). This compound is reduced (presumably by NADPH or NADH produced by dehydrogenase enzymes) in metabolically active cells to a colored formazan product. The amount of colored product produced indicates the number of cells present (Promega product insert).

As this product has not been used to measure rabbit MSCs previously, a standard curve was performed to determine the feasibility of using this product. The standard curve shown in **Figure 3** was performed using rabbit MSC's, adding 4×10^4 cells to the first well of a 96 well plate and performing serial 2-fold dilutions of the cells in the remaining wells. The absorbance readings were performed immediately (—), and at 1 (— —), 2 (— — —), 3 (— — — —), and 4 (— — — — —) hours after the addition of the reagent. The standard curves at 1 and 2 have the steepest slope, with the largest differences between samples. At the point where the steepest part of the curve levels off, the minimum amount of cells detectable in this experiment was 2×10^4 MSCs. Higher concentrations of cells were tested in **Figure 4** using the same method. The curve appears to still be linear at the highest number of cells tested (1.75×10^5 MSCs). Based upon these results, the solution could not be used to measure cells at numbers lower than 2×10^4 MSCs. However, additional tests, shown in **Figure 5 and 6**, were able to detect MSCs at concentrations as low as 5×10^3 . We concluded from this data that the

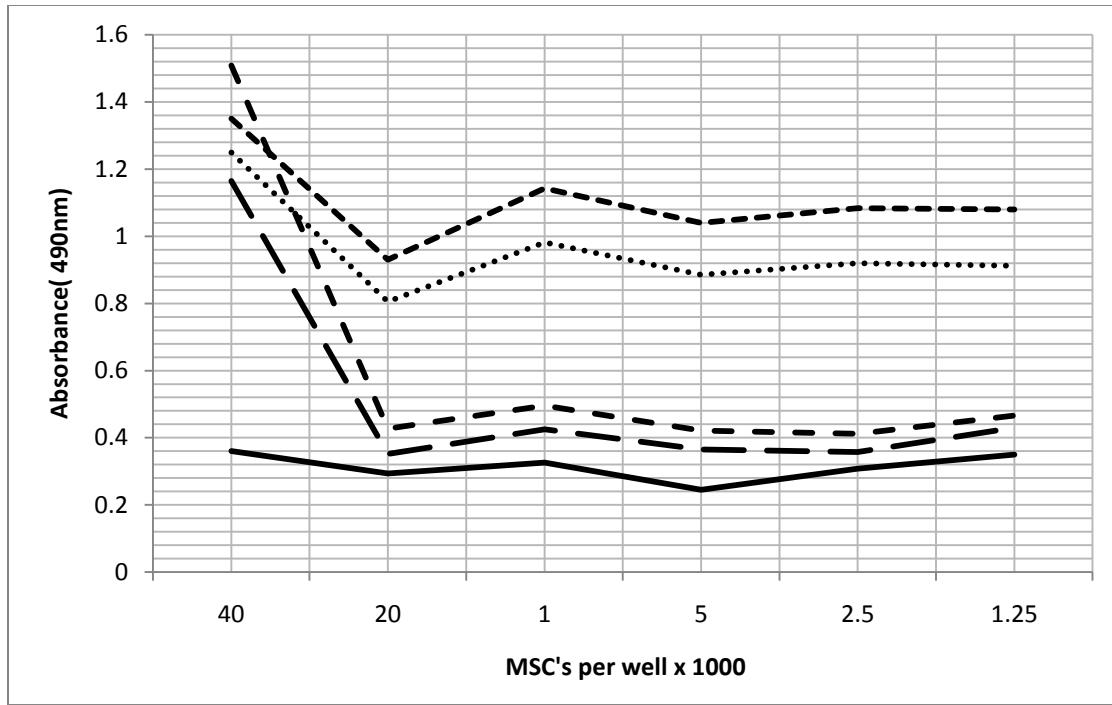


Figure 3. Standard Curve using Cell Titer 96 Aqueous One Solution and Rabbit MSC's:

The MSC's were trypsinized and resuspended at 4.0×10^5 MSC's/ml in MSC media (DMEM-High glucose with 10% FCS, 100U/ml penicillin, 100ug/ml streptomycin, 2.5 ug/ml amphotericin B, 1mM of L-glutamine). MSC's (200ul) were added to 4 wells of a 96 well tissue cultured treated plate, and 100ul of MSC media was added to five wells below each well. Serial 2-fold dilutions were performed down the wells. As a control, 4 wells contained only MSC media. To each well, 20ul of Cell Titer 96 Aqueous One Solution reagent was added and the absorbance was immediately recorded at 490 nm using a 96 well plate reader. The plate was incubated for 1-4 hours, recording the absorbance at every hour. The MSC's used in this experiment are from rabbit #14. The y-axis indicates the absorbance reading, and the x-axis indicates the number of MSC's in each well. The first absorbance reading (____) was taken as soon as the

reagent was added. The second absorbance reading (_ _) was taken one hour after the reagent was added. The third absorbance reading (_ _ _) was taken two hours after the reagent was added. The fourth absorbance reading (.....) was taken three hours after the reagent was added. The fifth absorbance reading (_ _ _ _) was taken four hours after the reagent was added.

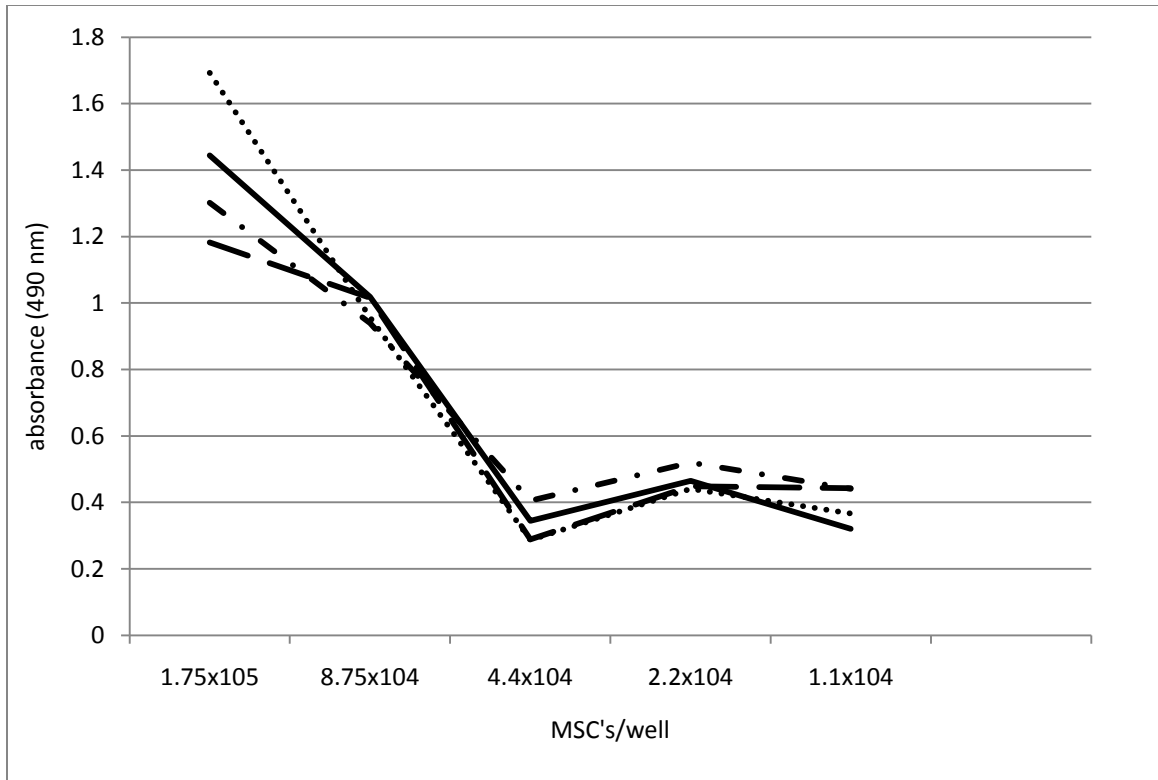


Figure 4. Standard Curve using Rabbit MSC's: The procedure used for this standard curve is identical to the method described in Figure 3. However, the cell number was increased. In this standard curve 3.5×10^5 MSC's were added to one well, and 2-fold dilutions were performed as previously described. As a control MSC media was added to four wells. The MSC's used in this experiment were from rabbit #14. The y-axis indicates the absorbance reading, and the x-axis indicates the number of MSC's in each well. The first reading (.....) was taken as soon as the reagent was added. The second reading (___ ___) was taken 1 hour after the reagent was added. The third reading (_____) was taken 2 hours after the reagent was added. The fourth reading (. _ . _) was taken 3 hours after the reagent was added.

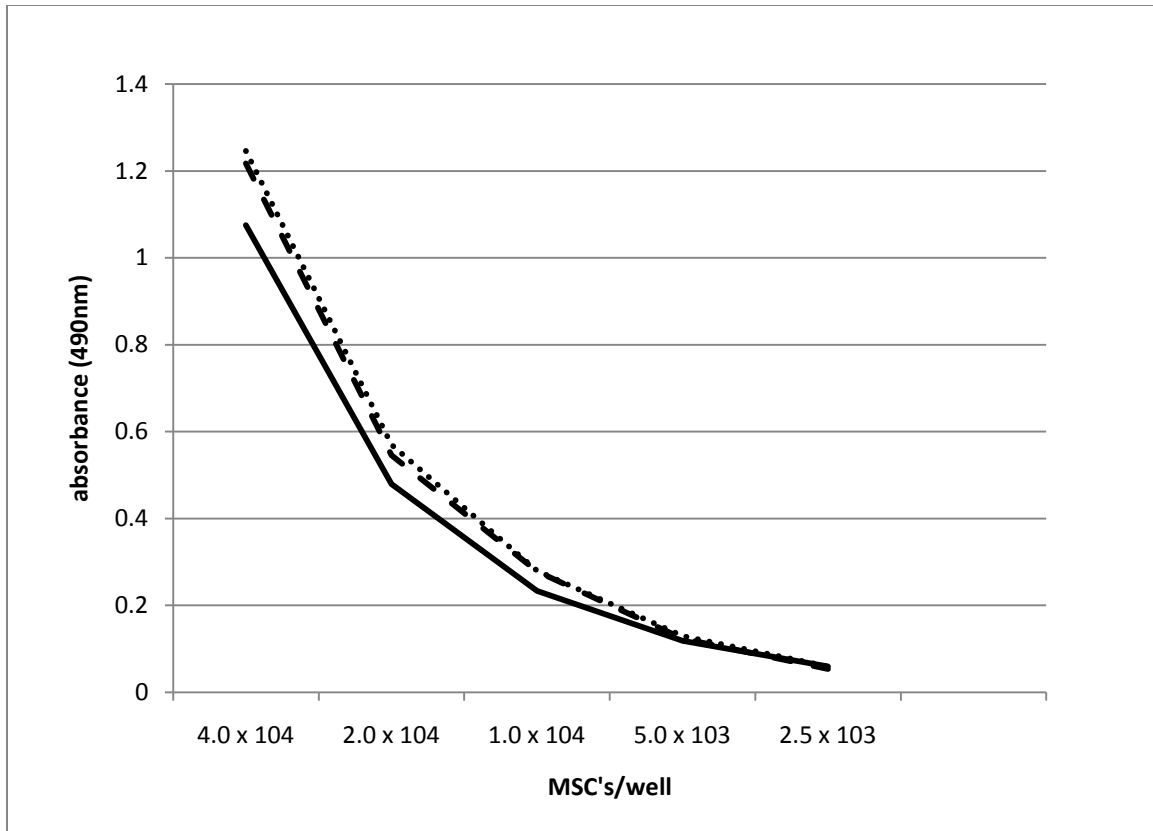


Figure 5. Standard Curve with Rabbit MSC's: This standard curve was performed using the same procedure as Figure 3. In this standard curve 8.0×10^4 MSC's were added to the first two wells, and serial 2-fold dilutions were performed as previously described. The MSC's used in this experiment were from rabbit #14. The y-axis indicates the absorbance reading, and the x-axis indicates the number of MSC's in each well. The first reading (____) was taken 1 hour after the reagent was added. The third reading (___) was taken 2 hours after the reagent was added. The fourth reading (.....) was taken 3 hours after the reagent was added.

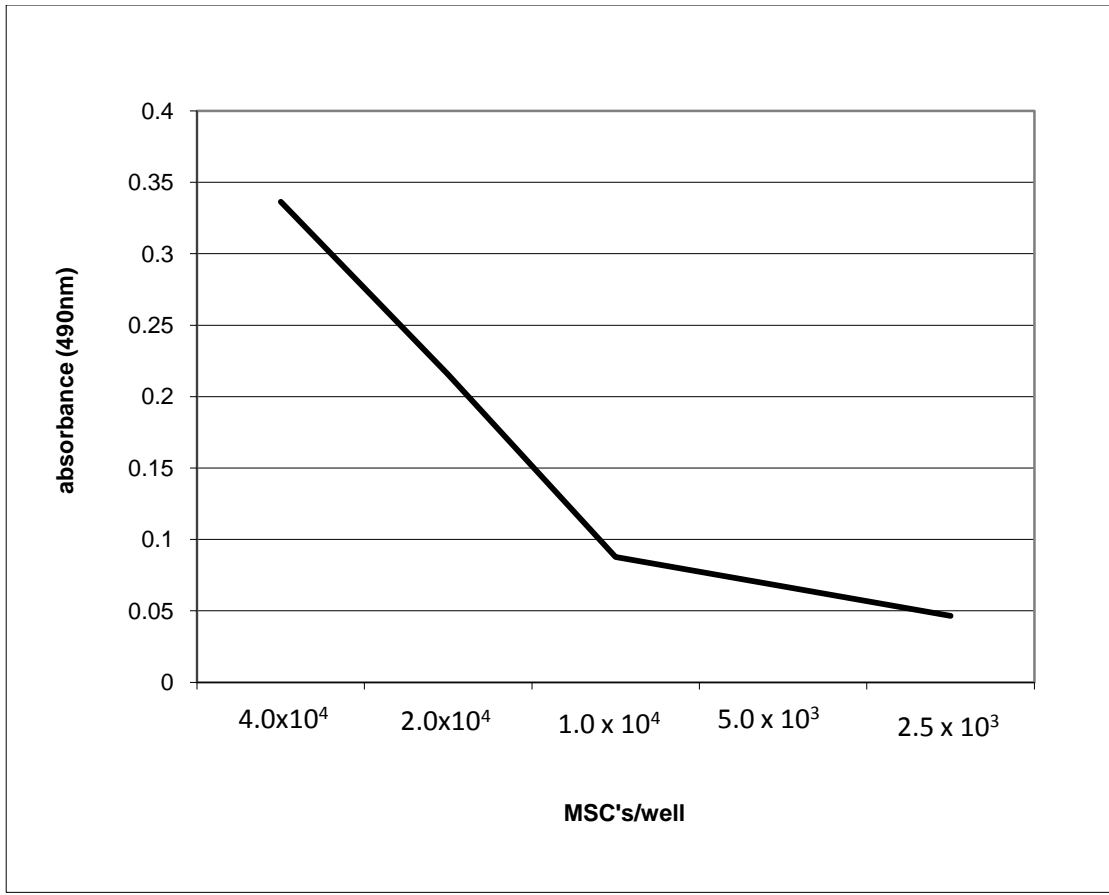


Figure 6. Standard Curve for the Adherence assay using Rabbit MSC's:

This standard curve was performed by using the same procedure as Figure 3. In this standard curve 8.0×10^4 MSC's were added to the first two wells, and serial 2-fold dilutions were performed as previously described. The absorbance reading was taken two hours after the reagent was added. The MSC's used in this experiment were from rabbit #19. The y-axis indicates the absorbance reading, and the x-axis indicates the number of MSC's in each well.

results varied with the cell preparation used and, when possible, a standard curve would be necessary for each experiment using this method.

Adherence assays examining whether MSCs could adhere to mesh materials were performed using the colorimetric assay to determine number of cells bound. The first adherence assay using rabbit MSC's is shown in **Figures 7 and 8**. MSCs (4×10^4 cells) were incubated with the mesh materials for one hour at 37°C . Unbound cells were removed and the colorimetric reagent incubated with the cells for 2 hours. The absorbance values were first taken while the mesh was still in the wells (**Figure 7**). The mesh materials were removed from the wells and the absorbance reading was performed again (**Figure 8**). When comparing **Figure 7** to the standard curve in **Figure 6**, more than 4.0×10^4 MSC's were bound to the Bard Colla Mend. Less than 2.5×10^3 MSC's were bound to the Gore-Tex, and approximately 2.0×10^4 MSC's were bound to Bard Mesh and/or vicryl mesh. When the absorbance readings were repeated without the mesh (**Figure 8**), approximately 3×10^4 MSC's were able to adhere to the Bard Colla Mend. Approximately 2.5×10^3 MSC's were able to adhere to the Gore-Tex which is higher than when the mesh was still in the well. The other absorbance readings were slightly lower with the mesh removed (1.5×10^4 MSC's with Bard Mesh and the vicryl mesh). The mesh materials most likely inhibit the plate reader from accurately measuring the absorbance. Therefore, in future adherence assays the mesh will be removed before the absorbance is measured.

The second adherence assay using rabbit MSC's is shown in **Figure 9**. In this experiment, untreated polypropylene plates were used instead of tissue culture plates

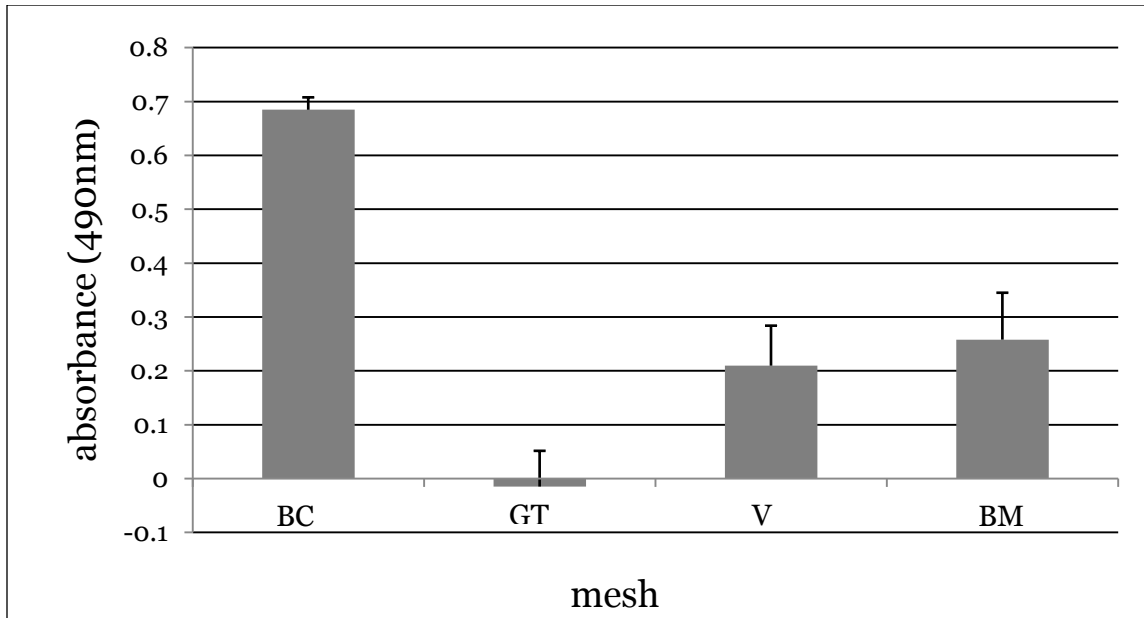


Figure 7. Cell Adhesion Assay: Cell Titer 96 AQ One Solution Cell Proliferation Assay:

Mesh materials were cut with sterile surgical scissors into small circles with a 3mm diameter. Using sterile technique the mesh materials were placed into the appropriate wells of a 96 well tissue cultured treated plate. The four mesh materials used were Bard Colla Mend (BC), Gore-Tex (GT), Bard Mesh (BM), and vicryl (V). For this experiment, each test was performed in triplicate. The Bard Colla Mend was rehydrated for three minutes with sterile saline. The mesh materials were washed three times with MSC media (DMEM-High glucose with 10% FCS, 100U/ml penicillin, 100ug/ml streptomycin, 2.5 ug/ml amphotericin B, 1mM of L-glutamine). MSC's were resuspended in MSC media to a final concentration was 4.0×10^4 MSC's/100ul. The MSC's were added to the appropriate wells, and the MSC's were allowed to incubate with the mesh materials for 30 minutes. The wells were washed three times with PBS to remove the non-adherent MSC's. To each well, 100ul of PBS was added back, and 20ul of the Cell Titer 96 reagent was added to each well. As a control, four wells

contained PBS + 20ul of the Cell Titer reagent. The plate was allowed to incubate for two hours, and the absorbance was recorded at 490nm. Each bar represents the three absorbance values averaged together (y-axis). The MSC's used in this experiment were from rabbit #19. Standard error bars are included in the graph.

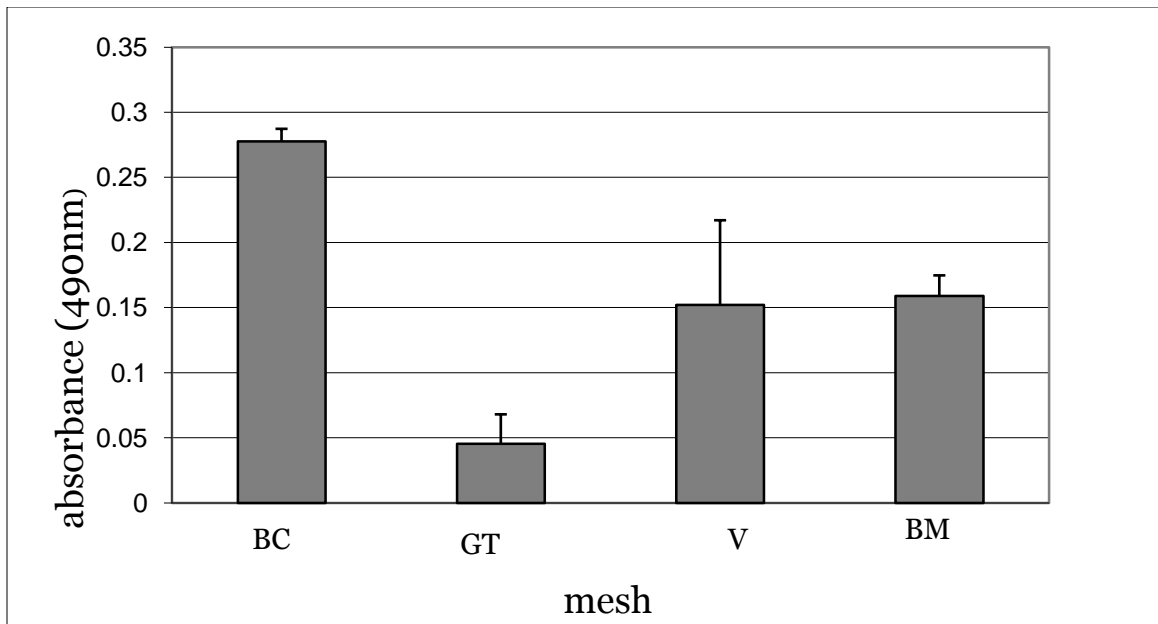


Figure 8. Cell Adhesion Assay: Cell Titer 96 AQ One Solution Cell Proliferation Assay:

This figure is a continuation of Figure 7. Following the procedure described in Figure 7, the mesh was removed from the wells, and the absorbance was recorded again at 490nm.

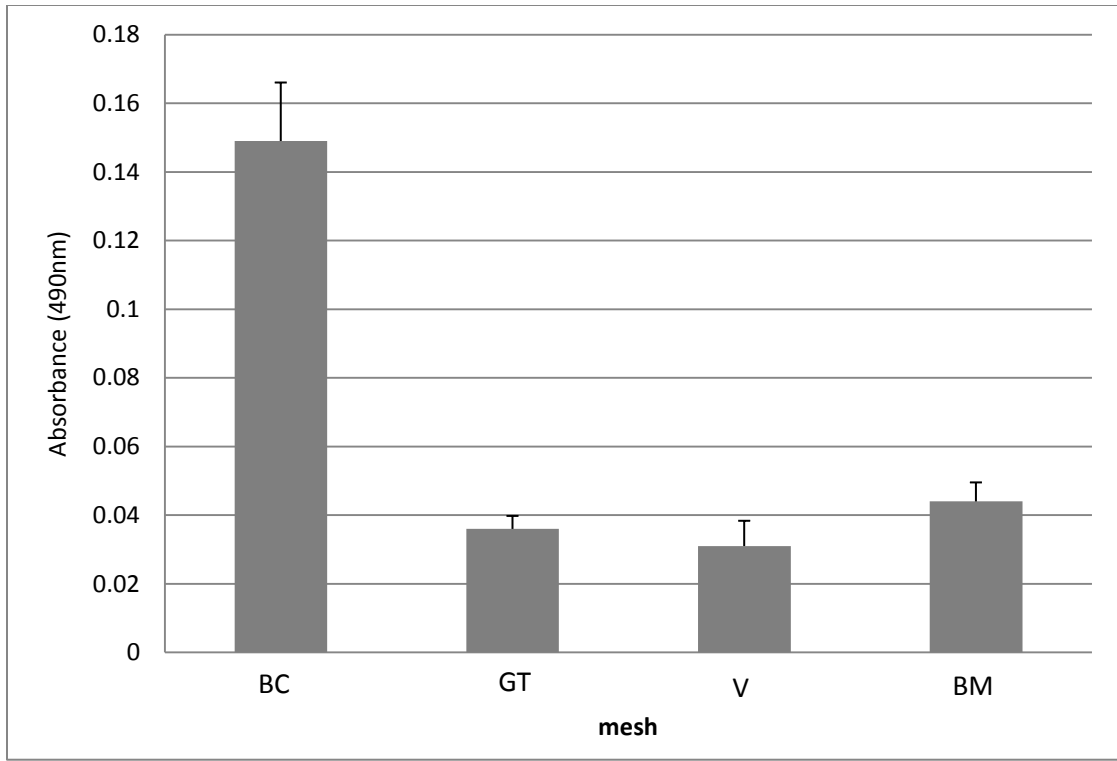


Figure 9. Cell Adhesion Assay: Cell Titer 96 AQ One Solution Cell Proliferation Assay:

The procedure used in this experiment is identical to Figure 7, except a non-tissue cultured treated nonsterile polyvinyl chloride 96 well plate was used. To make sure the MSC's were not adhering to the plate, four wells with only MSC's were used as a control. Because the plates were non-tissue cultured treated, the media used in the standard curve could not be replaced with PBS; therefore four wells with only media were used as a control. For this experiment, each mesh was performed in triplicate. The absorbance was only recorded after the mesh was taken out of the wells. The 1st mesh was the Bard Colla Mend, the 2nd mesh was the Gore-Tex, the 3rd mesh was the Bard Mesh, and the 4th mesh was the vicryl. Each bar represents the three absorbance values averaged together (y-axis). The MSC's used in this experiment were from rabbit #19. Standard error bars are included in the graph.

to make sure the MSC's were sticking to the mesh and not the plates. This was a concern primarily with the Bard Mesh and vicryl because of gaps in the mesh material. When comparing **Figure 9** to the standard curve in **Figure 6**, less cell adherence was seen in all conditions (1.5×10^4 MSC's with Bard Colla Mend and less than 2.5×10^3 with Gore-Tex, Bard Mesh, and vicryl). However, the results were consistent with the data from the previous figure, as the MSC's were able to adhere to Bard Colla Mend the best. The values for Bard Mesh and vicryl decreased when the untreated plate was used; therefore, some of the MSC's were most likely attaching to the plate instead of the mesh materials. All remaining and future adherence assays will be performed in non-tissue culture treated plates.

In order to improve future proposed studies using rabbit or rat MSCs, we felt it was necessary to investigate the best cell culture conditions for MSC growth and differentiation. We performed a review of the literature to determine the culture conditions used by various laboratories (Table 1) and tested the various cell culture media for their effect on MSC proliferation. The first cell proliferation assay is shown in **Figures 10 and 11**.

Many different types of media were used in this study. Some of the basal media's used were α MEM and DMEM, and both support a wide range of cell lines (Stacey, 2010). DMEM is α MEM modified containing more amino acids and vitamins. The complex media used was Iscoves DMEM, which is a modified version of DMEM and supports high density growth. The serum free media used was Hams F12, and this

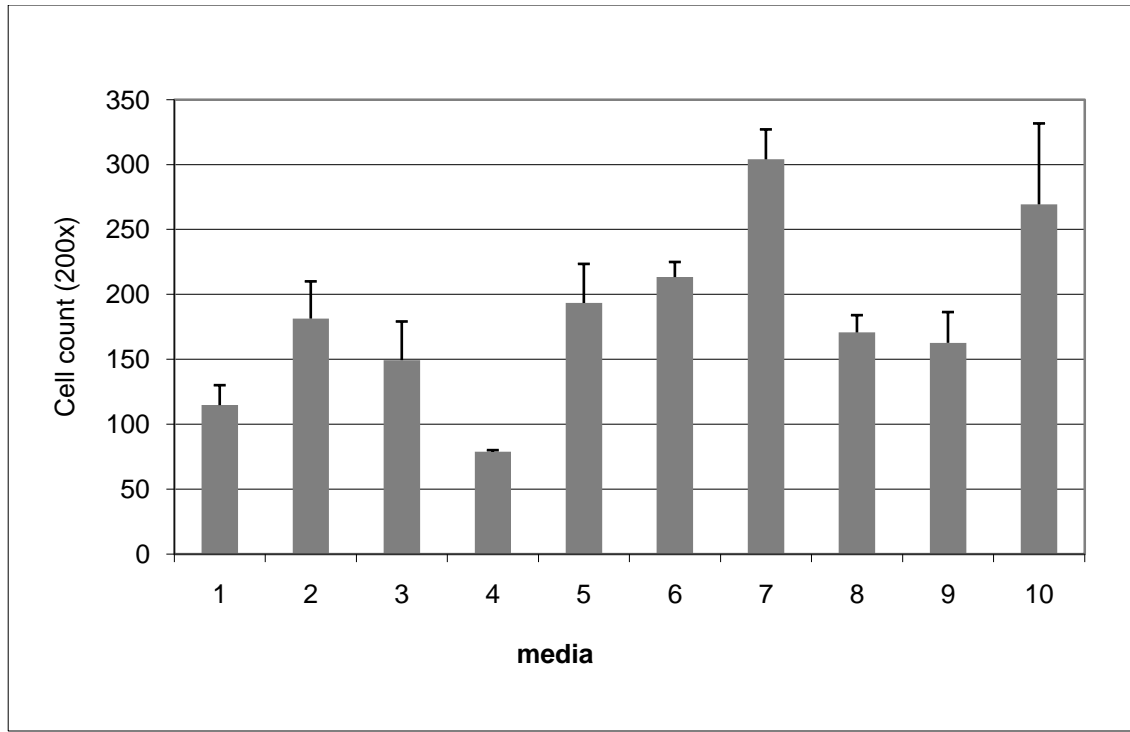


Figure 10. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's _Cell Count: MSC's were thawed and diluted to 9000 MSC's/100ul of MSC media (DMEM-High glucose with 10% FCS, 100U/ml penicillin, 100ug/ml streptomycin, 2.5 ug/ml amphotericin B, 1mM of L-glutamine). MSC's (100ul) were added to a 96 well tissue cultured treated plate, coated with poly-L lysine. The plate was incubated for 12 hours. The media was aspirated off, and the appropriate media was added to each well. The cells were incubated with 10 different cell culture media, as described in Table 2. Cells were fed twice a week until one well was 80% confluent. At that time, the wells were washed three times with PBS, adding back 100ul of PBS to each well. A cell count was performed on 1 well was used and 3 fields of vision using the highest magnification (200x). Each bar represents the averaged values +/- SEM. The MSC's used in this experiment were from rabbit #3.

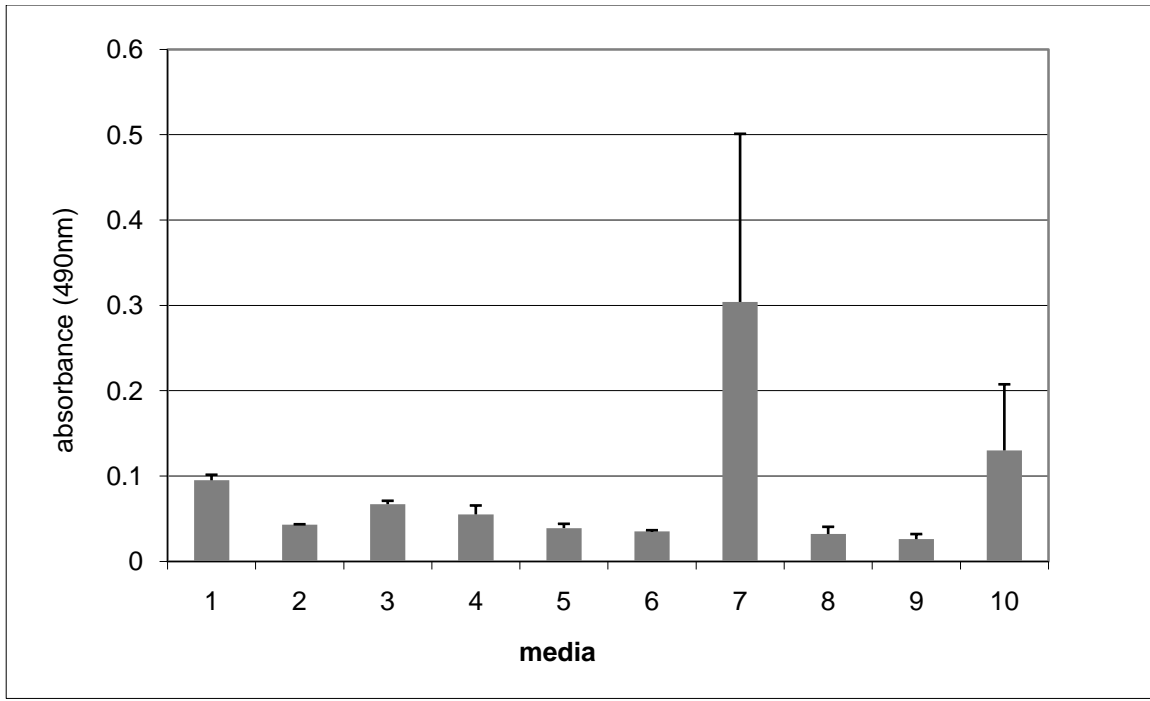


Figure 11. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: After the cell count was complete (as described in Figure 10) 20ul of the Cell Titer 96 reagent was added to the 100ul of PBS in each well. As a control, 100ul of PBS was added to two wells, along with 20ul of the Cell Titer 96 reagent. The absorbance assays were performed in triplicate. The plate was allowed to incubate for two hours. The absorbance was measured by using a 96 well plate reader at 490nm. Each bar represents the two absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard deviation bars are included in this graph.

media is designed for serum free culture conditions. Usually a serum free media needs to be supplemented with insulin or growth factors.

Maintaining the pH around 7.2-7.4 is important for optimal growth (Stacey, 2010). Normally a natural buffering system is used, where gaseous CO₂ is injected into the incubator. A chemical method is also available, and this involves using HEPES, which is a zwitterion. HEPES can be toxic to some cell types, and is rather expensive. Most media's contain phenol red which turns the media yellow, if acidic, and purple, if alkali.

Carbohydrates are the main source of energy for the cells (Stacey, 2010). The most common sugars used in media are glucose and galactose. Usually complex media contains 4x more sugar than basal media.

Vitamins, proteins, peptides, fatty acids, and lipids are usually added in the form of serum (Stacey, 2010). Vitamins are involved with cell growth and proliferation, and are precursors for co-factors which are important in metabolism. Common proteins found in serum include albumin, transferrin and fibronectin. Some fatty acids are necessary for specialized cells.

Serum is the most important component of cell culture media, and the most commonly used serum is fetal bovine serum. It is vital to low density cultures or slow growing cells. Serum has the ability to neutralize toxins, and protect the cell against mechanical damage. Every batch of serum is different, and this may prevent

consistency within a protocol. Some batches of serum may be contaminated, and heat inactivation can reduce the risk of contamination.

In these experiments, 9000 MSCs were added to each well and the cells were incubated with the ten different cell culture medias listed in **Table 1**. The cells were maintained and fed three times a week until the cells in one of the conditions reached 80% confluence. At that point, the experiment was stopped. The cells in one well for each condition were counted manually. Counts from 3 fields of view at 200X magnification were averaged and the data is shown in **Figure 10**. After the cell count was complete 20ul of the Cell Titer 96 reagent was added to the 100ul of PBS in each well. As a control, 100ul of PBS was added to 2 wells, along with 20ul of the Cell Titer 96 reagent. The plate was allowed to incubate for 2 hours. The absorbance was measured by using a 96 well plate reader at 490nm (**Figure 11**). Each bar represents the 2 absorbance values for each media averaged together. Cell counts appeared to be more sensitive, as they detected growth at lower cell numbers than were detected with the colorimetric assay. However, the two methods gave identical results when looking at wells containing higher cell numbers. With both methods, optimal cell proliferation was seen in DMEM containing 10% FCS (condition 7) and in Iscoves MDM containing 10% FCS (condition 10).

The growth of large numbers of MSCs for future studies would be simplified if the cells could be plated at low densities and allowed to proliferate longer before it became necessary to split the cells and dilute them into new plates. This would also

decrease the chance of contamination of the cells during trypsinization and passage to new flasks. In order to determine the proliferation capacity of the cells in the various media, the cells were plated at three different concentrations; 1000, 2700, and 5400 cells per well. The cells were grown in the various media. As before, the experiment was stopped when cells grown in one condition reached 80% confluence. The cells were then counted visually and quantitated using the Cell Titer method. The results for cells plated at 1000 cells/well and counted visually are shown in **Figures 15, 21, and 29**. The data for the same cells, but quantitated using the colorimetric assay are shown in **Figures 14, 18, and 25**. The results for cells plated at 2700 cells/well and counted visually are shown in **Figures 16, 22, 23, and 30**. The data for the same cells, but quantitated using the colorimetric assay are shown in **Figures 12, 19, 26, and 27**. The results for cells plated at 5400 cells/well and counted visually are shown in **Figures 17, 24, and 31**. The data for the same cells, but quantitated using the colorimetric assay are shown in **Figures 13, 20, and 28**. All of the data was normalized by recalculation as percent of the highest value for that experiment to allow the data from the 3 to 4 replicate experiments to be averaged. The averaged data for visual counts is found in **Figures 44** (1000 cells/well), **45** (2700 cells/well), and **46** (5400 cells/well). The averaged data for colorimetric quantitation is found in **Figures 41** (1000 cells/well), **42** (2700 cells/well), and **43** (5400 cells/well). The aggregate data show that alpha MEM with 20% FCS (media #1) and Hams F12 with 10% FCS (media #4) appear to be the best media for the proliferation of rabbit MSCs. Future studies will use alpha MEM for the culture of rabbit MSCs.

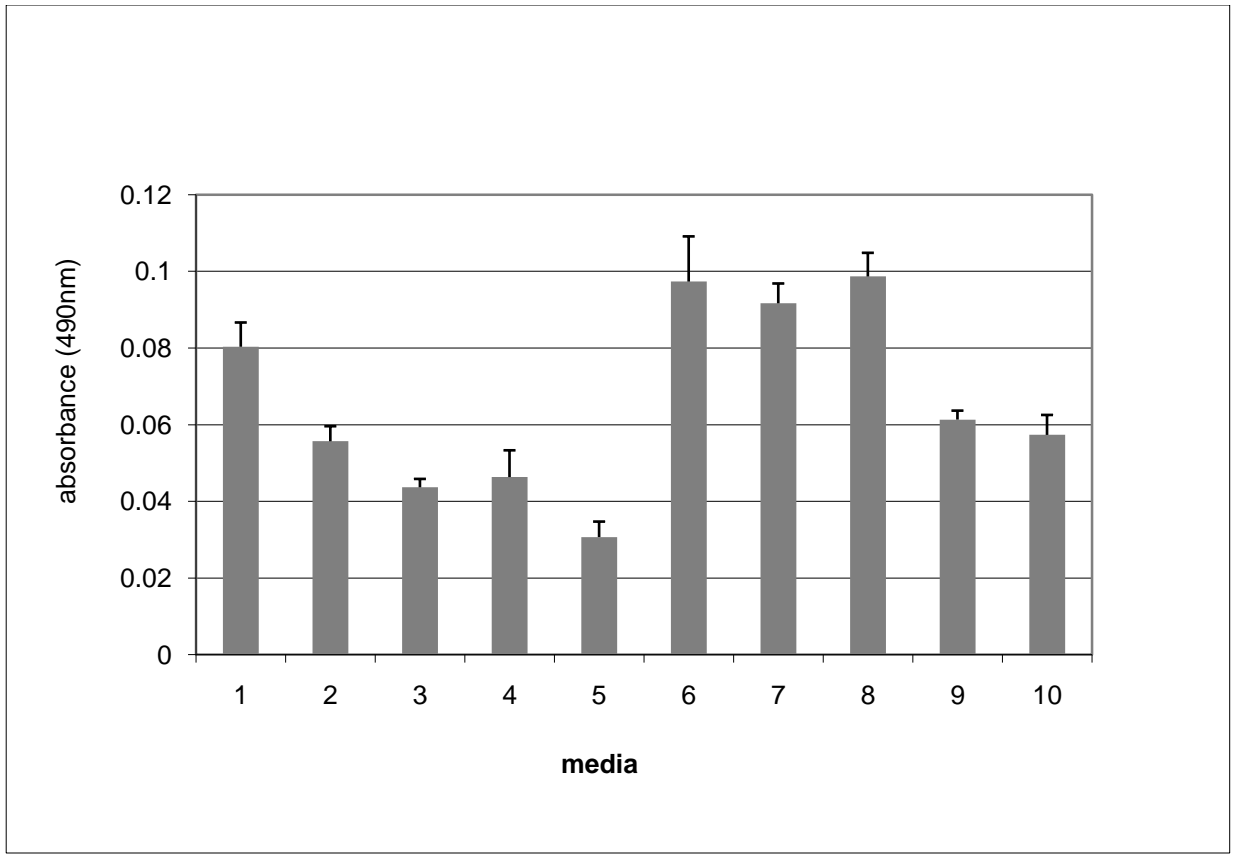


Figure 12. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's: The procedure is identical to that in Figures 10 and 11. In each well 2700 MSC's were added. This cell proliferation assay was performed at the same time as the assay in Figure 13. Each incubation with media was performed in triplicate. As a control, four wells contained only PBS + 20ul of the Cell Titer 96 reagent. No cell count was performed in this experiment. The bars represent the three absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. The MSC's used in this experiment were from rabbit #1. Standard error bars are included in the graphs.

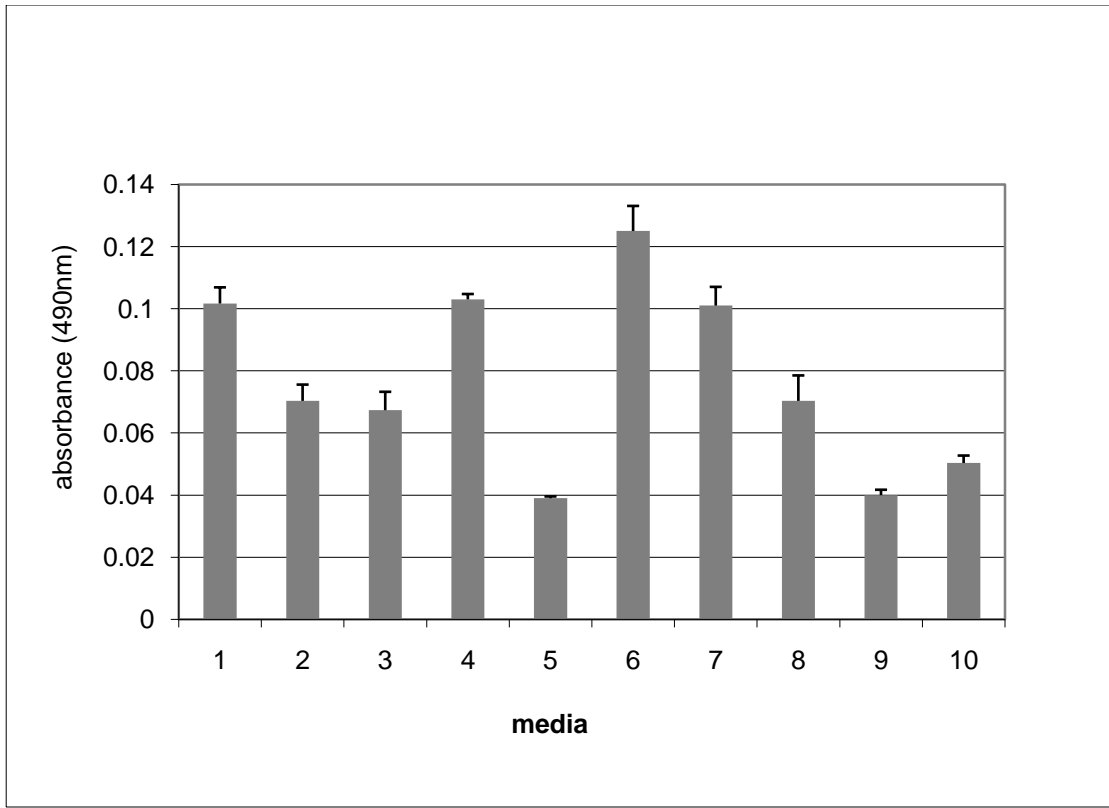


Figure 13. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's: The procedure used in Figure 13 is the same as in Figures 10 and 11,

except the concentration of MSC's added to each well was different. In each well 5400

MSC's were added. This cell proliferation assay was performed at the same time as the

assay in Figure 12. Each media was performed in triplicate. As a control four wells

contained only PBS + 20ul of the Cell Titer 96 reagent. No cell count was performed in

this experiment. The bars represent the three absorbance values for each media

averaged together. The y-axis indicates the absorbance values, and the media used

(x-axis) in each condition can be referenced in Table 2. The MSC's used in this

experiment were from rabbit #1. Standard error bars are included in the graphs.

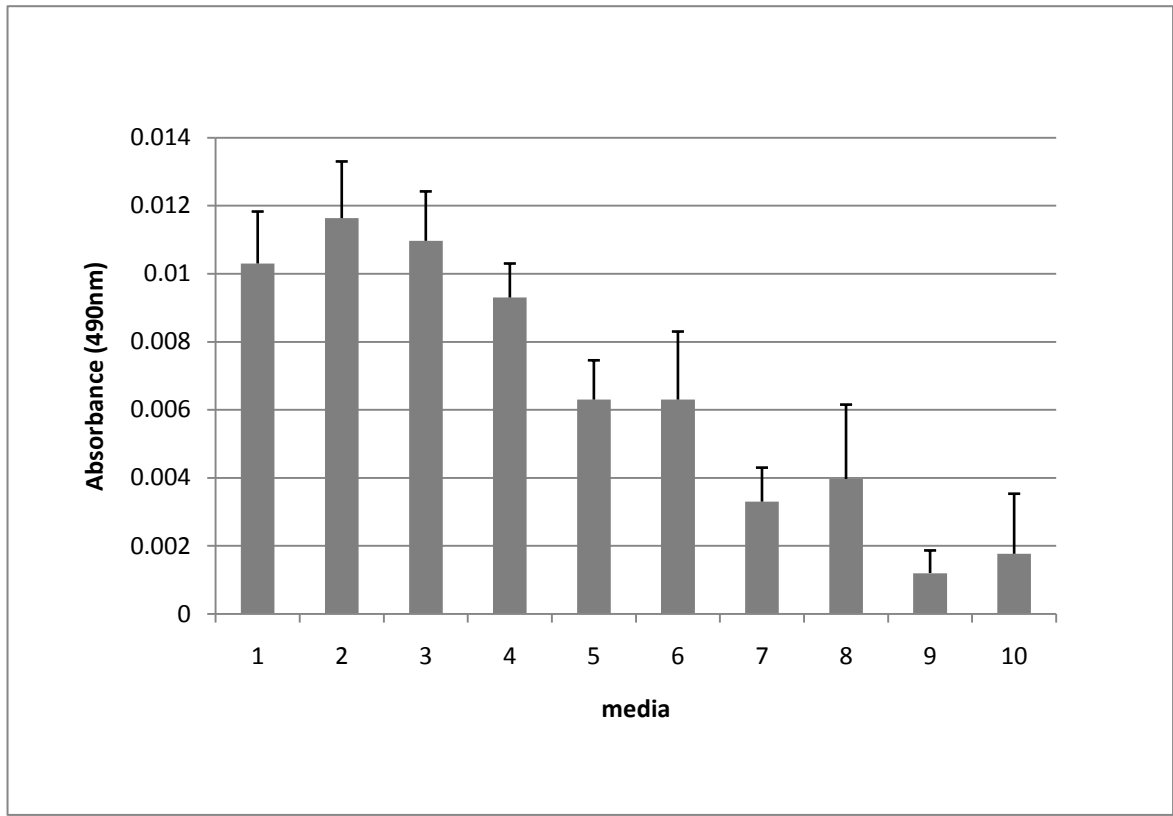


Figure 14. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: The procedure used in this experiment is the same as in Figures 10 and 11, except the concentration of MSC's added to each well was different. In this experiment 1000 MSC's were added to each well. Each media was performed in triplicate, and as a control four wells contained only PBS + 20ul of the Cell Titer 96 reagent. A cell count was not performed. Each bar represents the three absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. The MSC's used in this experiment were from rabbit #15. Standard error bars are included in the graph.

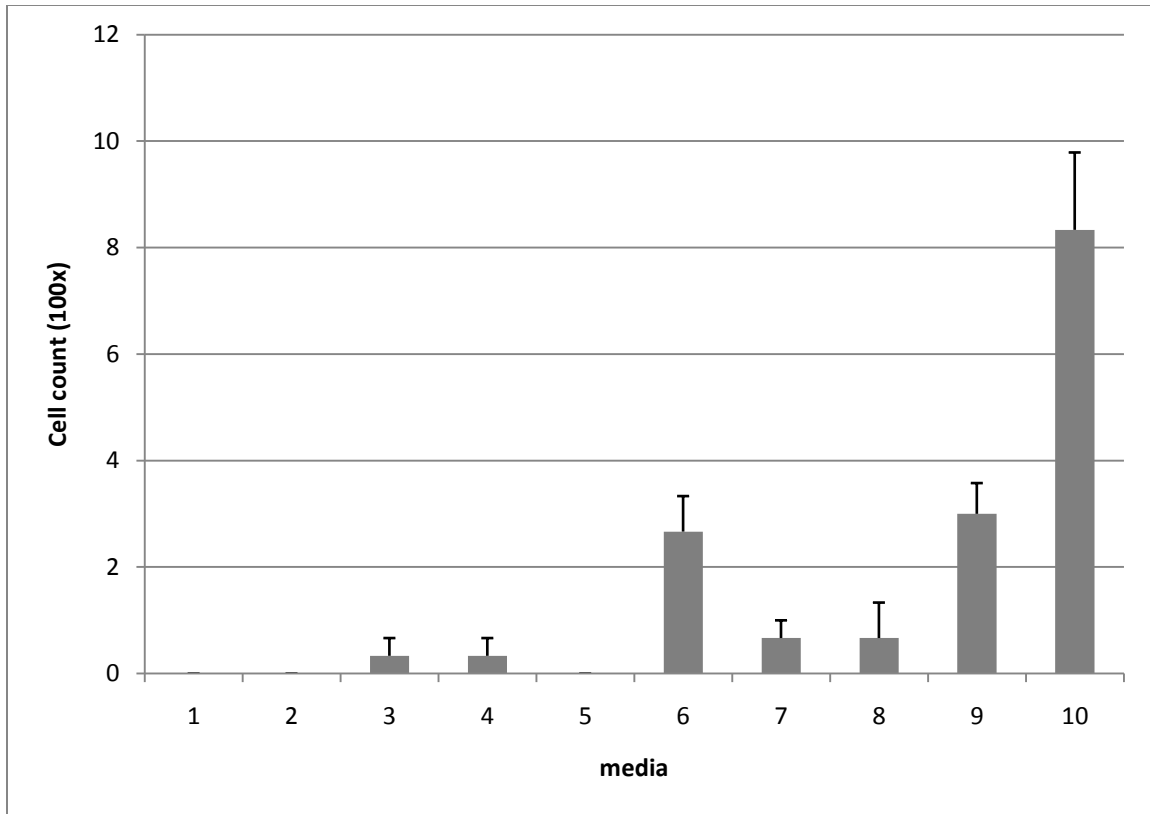


Figure 15. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's_Cell Count: The procedure used in this experiment is the same as in Figure

10, except the concentrations of MSC's added to each well was different. A

concentration of 1000 MSC's was added to each well. Each media was performed in

triplicate. The magnification used in these experiments was 100x, because there were

so few cells in each well. For each well, three fields of vision were counted and

averaged together. Each bar represents the averaged values (y-axis). The media

used (x-axis) in each condition can be referenced in Table 2. The MSC's used in this

experiment were from rabbit #15. Standard error bars are included in the graphs.

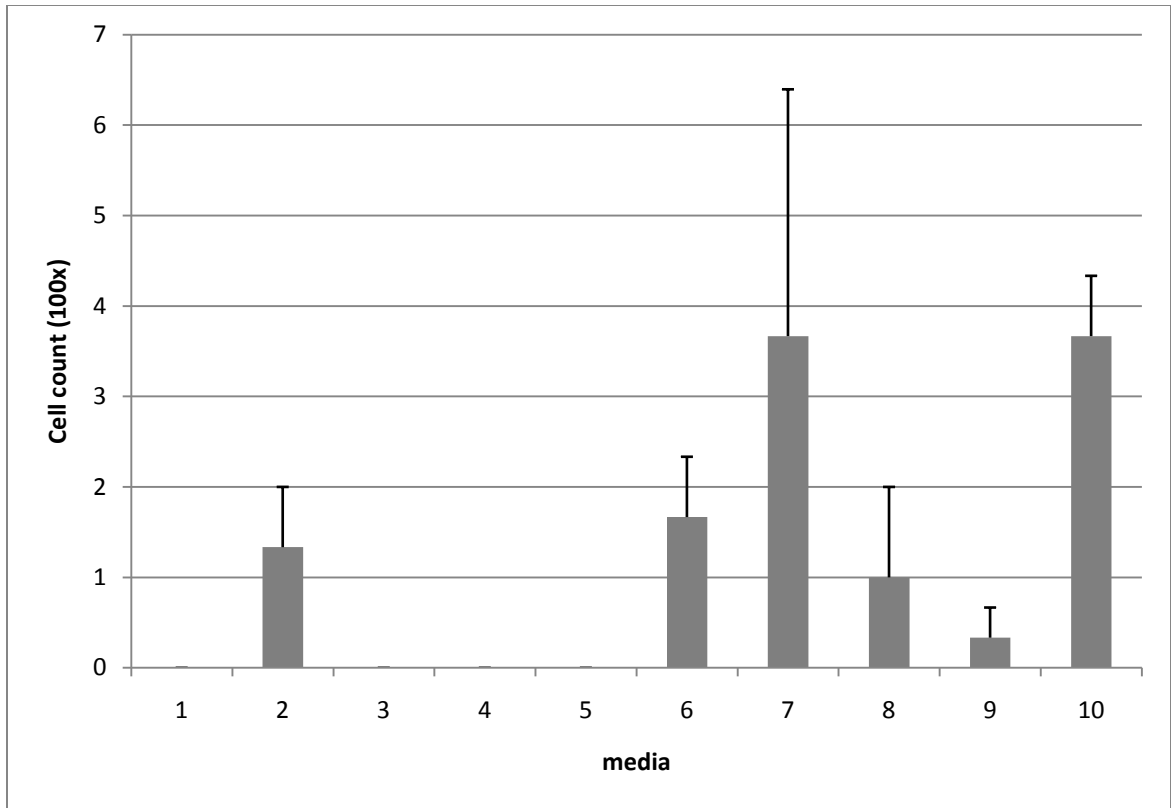


Figure 16. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except. A concentration of 2700 MSC's was added to each well. Each incubation with media was performed in triplicate. The magnification used in these experiments was 100x, only because there were so few cells in each well. For each well, three fields of vision were counted and averaged together. Each bar represents the averaged values (y-axis). The media used (x-axis) in this each condition can be referenced in Table 2. The MSC's used in this experiment were from rabbit #15. Standard error bars are included in the graphs.

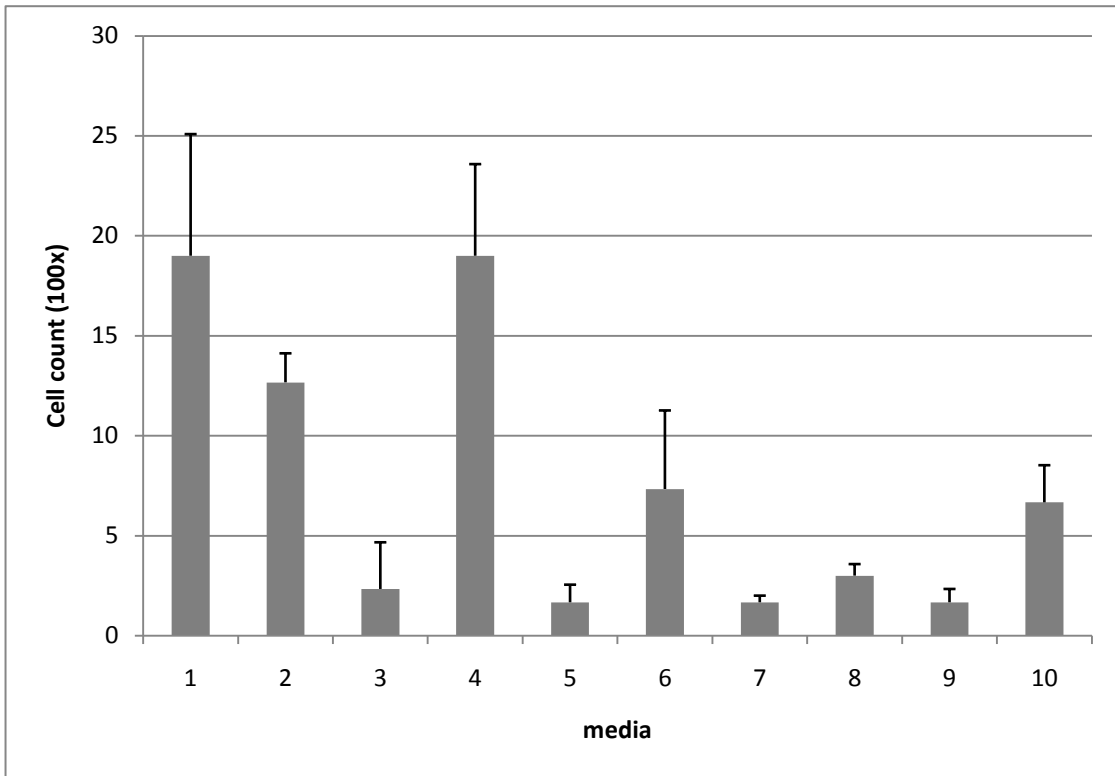


Figure 17. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except a concentration of 5400 MSC's was added to each well. Each media was performed in triplicate. The magnification used in these experiments was 100x, only because there were so few cells in each well. For each well, three fields of vision were counted and averaged together. Each bar represents the averaged values (y-axis). The media used (x-axis) in each condition can be referenced in Table 2. The MSC's used in this experiment were from rabbit #15. Standard error bars are included in the graphs.

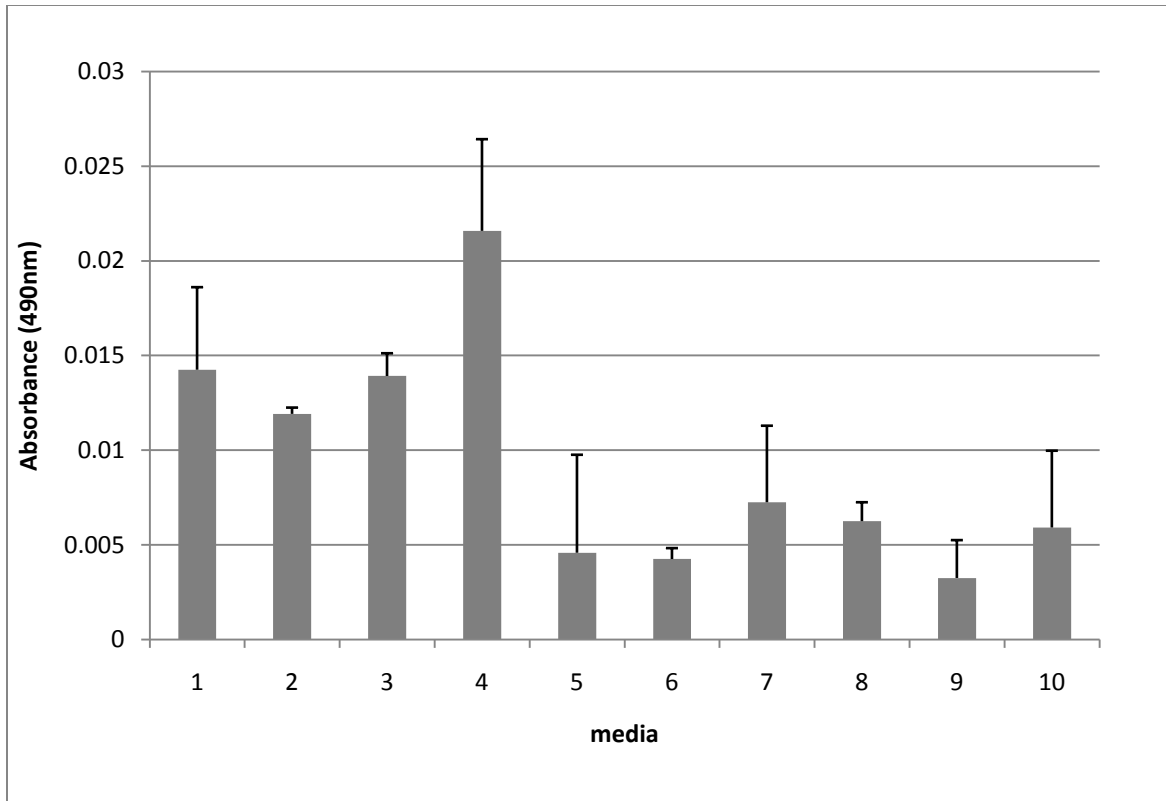


Figure 18. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: The procedure used in this experiment is the same as in Figures 10 and 11. This experiment is a continuation from Figure 15. As a control, four wells contained PBS + 20ul of Cell Titer 96 reagent. Each bar represents the three absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard error bars are included in the graphs.

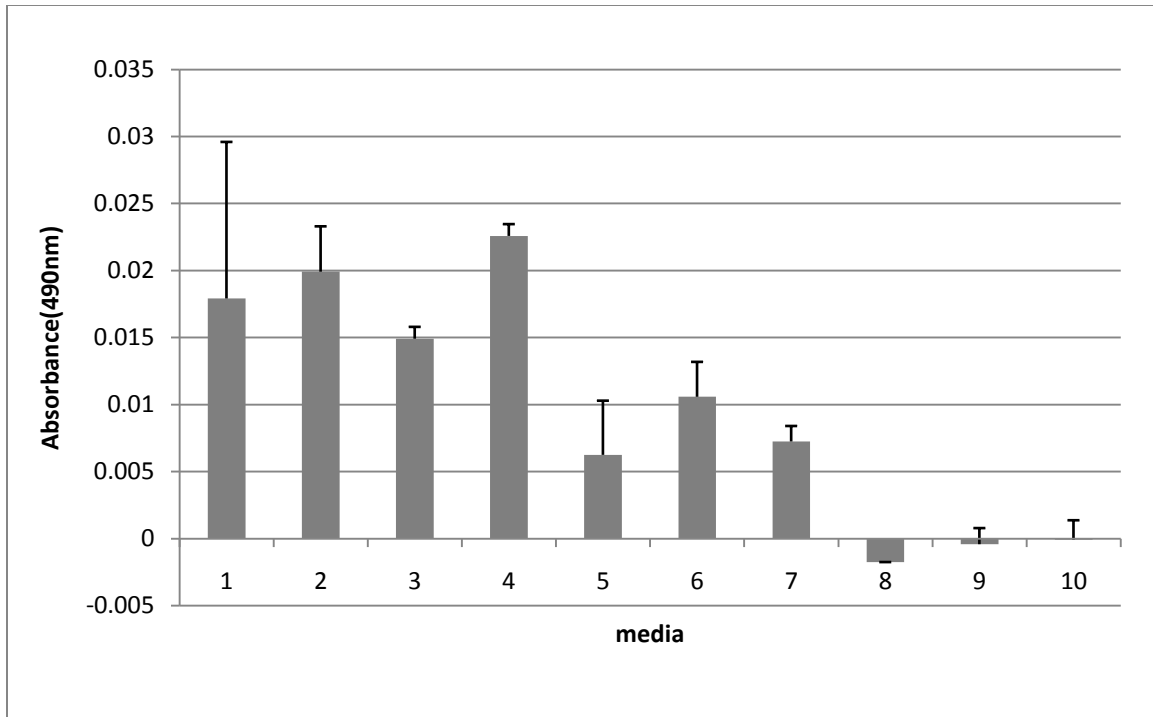


Figure 19. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: The procedure used in this experiment is the same as in Figures 10 and 11. This experiment is a continuation from Figure 16. As a control, four wells contained PBS + 20ul of Cell Titer 96 reagent. Each bar represents the three absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard error bars are included in the graphs.

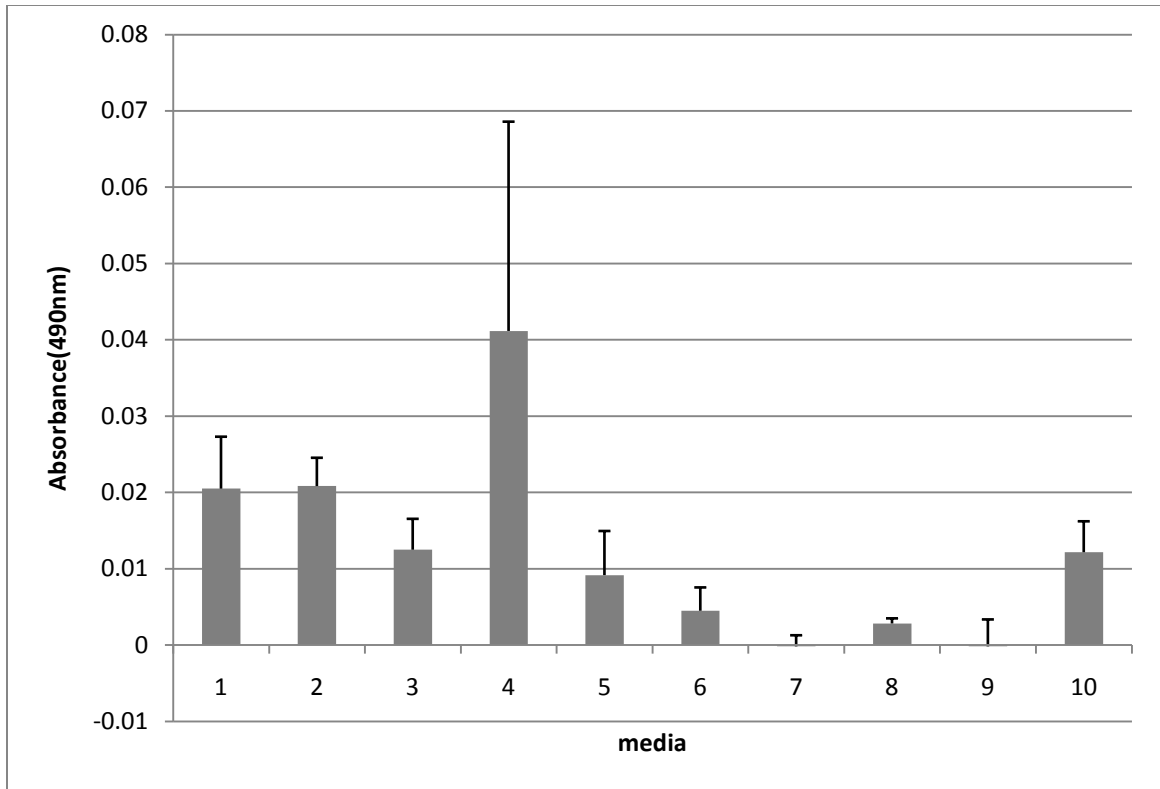


Figure 20. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: The procedure used in this experiment is the same as in Figures 10 and 11. This experiment is a continuation from Figure 17. As a control, four wells contained PBS + 20ul of Cell Titer 96 reagent. Each bar represents the three absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard error bars are included in the graphs.

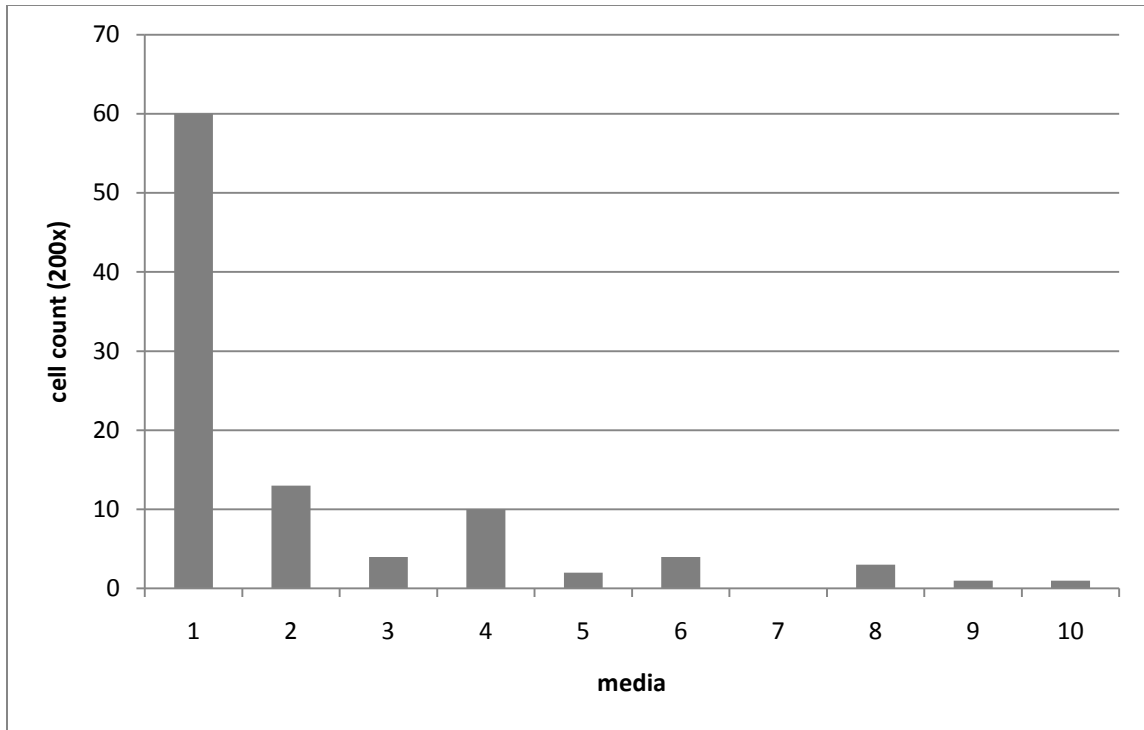


Figure 21. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except to each well, 1000 MSC's were added. Each media was performed in duplicate, but only one well/media was counted at each concentration. The wells used in the cell count were stained with crystal violet to make the cells easier to see. Each bar represents one field of vision at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. Because only one field of vision was counted per media, standard error bars are not included in the graphs. The MSC's used in this experiment were from rabbit #23.

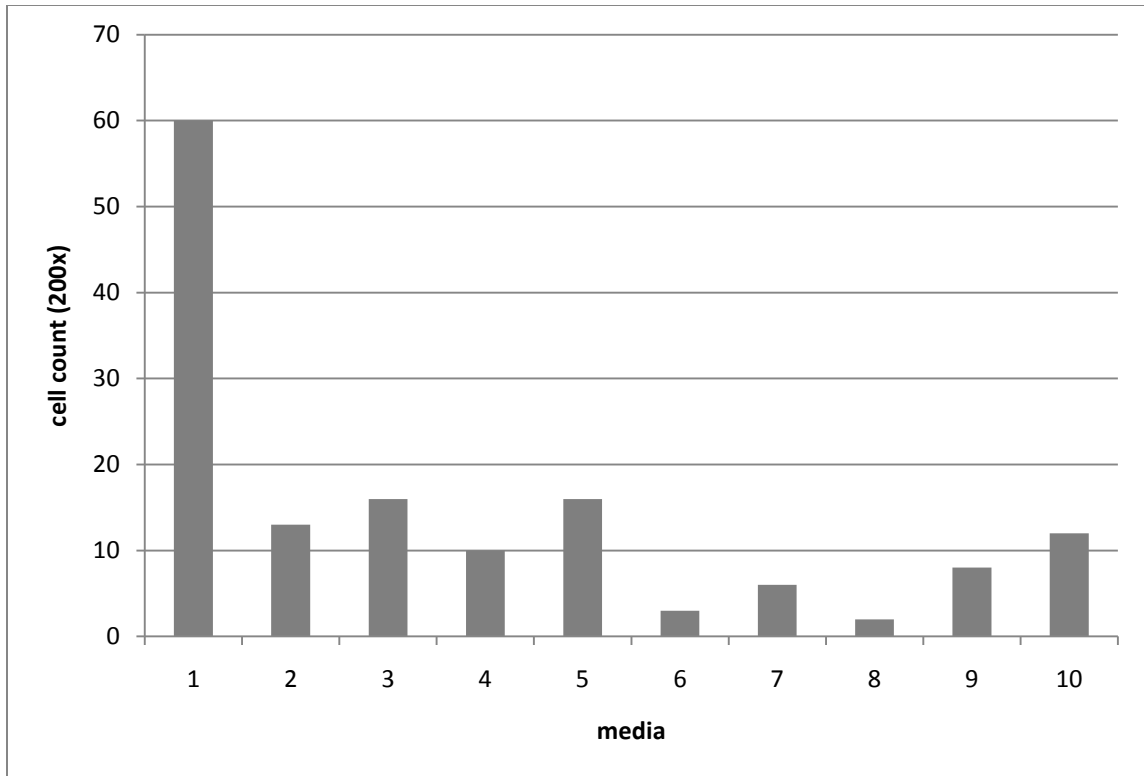


Figure 22. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except to each well, 2700 MSC's were added. Each media was performed in duplicate, but only one well/media was counted at each concentration. The wells used in the cell count were stained with crystal violet to make the cells easier to see. Each bar represents one field of vision at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. Because only one field of vision was counted per media, standard error bars are not included in the graphs. The MSC's used in this experiment were from rabbit #23.

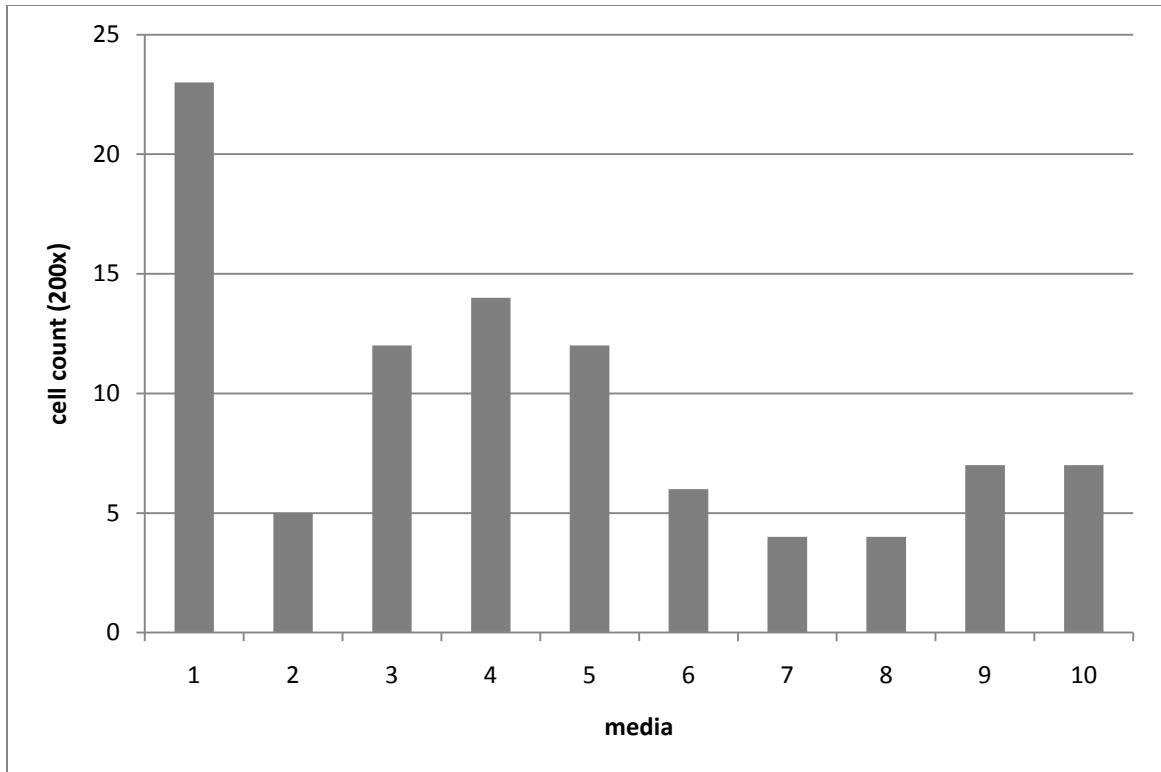


Figure 23. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except to each well, 2700 MSC's were added. Each media was performed in triplicate, but only one well/media was counted at each concentration. The wells used in the cell count were stained with crystal violet to make the cells easier to see. Each bar represents one field of vision at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. Because only one field of vision was counted per media, standard error bars are not included in the graphs. The MSC's used in this experiment were from rabbit #23.

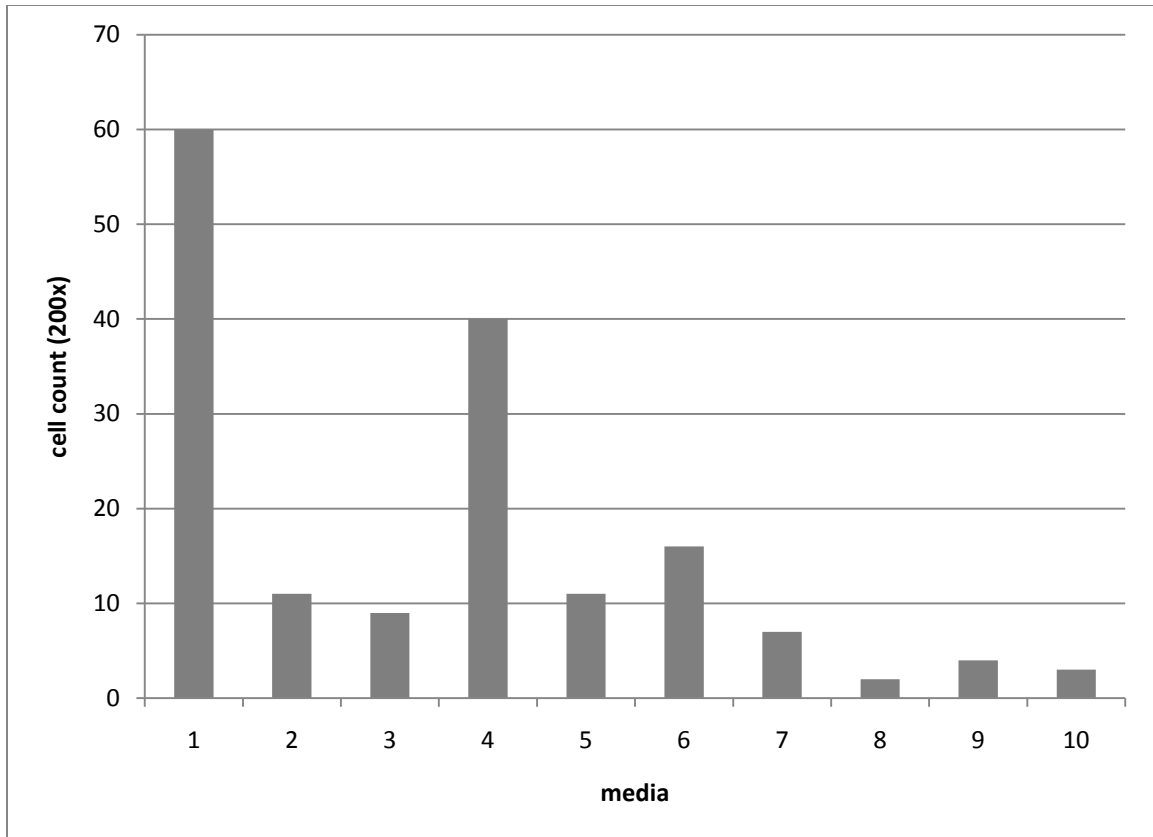


Figure 24. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except to each well, 5400 MSC's were added. Each media was performed in triplicate, but only one well/media was counted at each concentration. The wells used in the cell count were stained with crystal violet to make the cells easier to see. Each bar represents one field of vision at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. Because only one field of vision was counted per media, standard error bars are not included in the graphs. The MSC's used in this experiment were from rabbit #23.

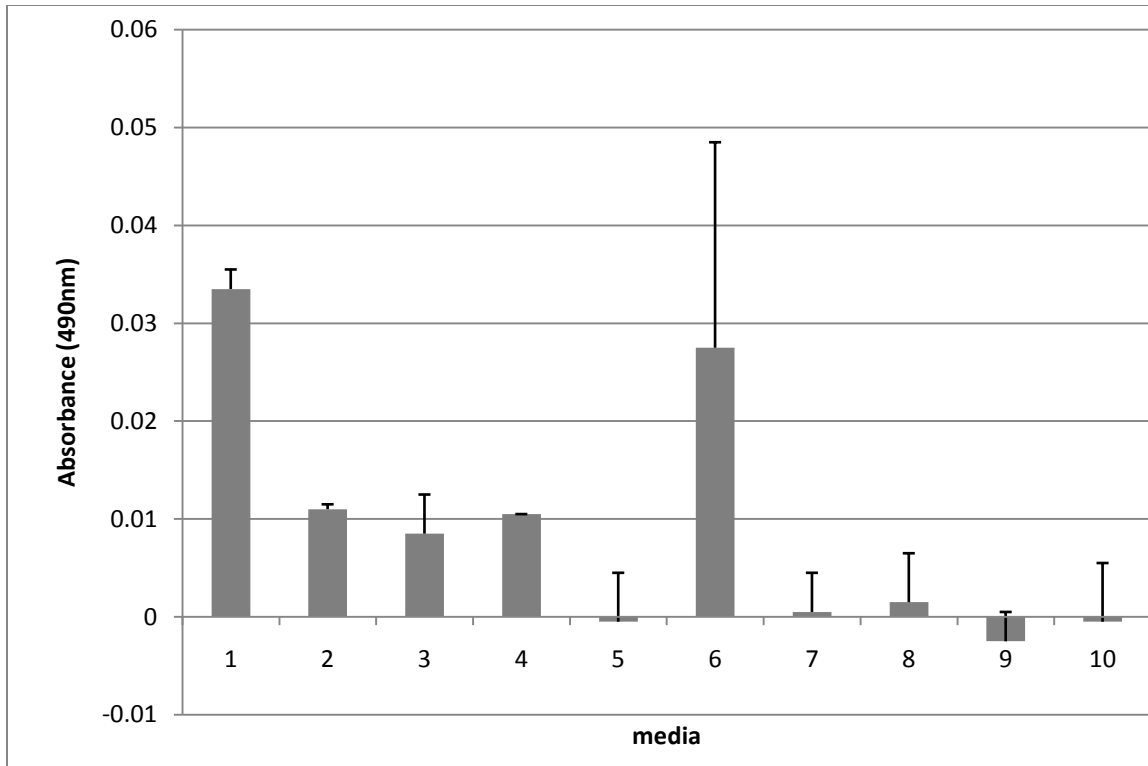


Figure 25. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: The procedure used in this experiment is the same as in Figures 10 and 11. As a control, four wells contained PBS + 20ul of the Cell Titer 96 reagent. This experiment is a continuation from the experiment in Figure 21. Each bar represents the 2 absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard deviation bars are included in the graphs.

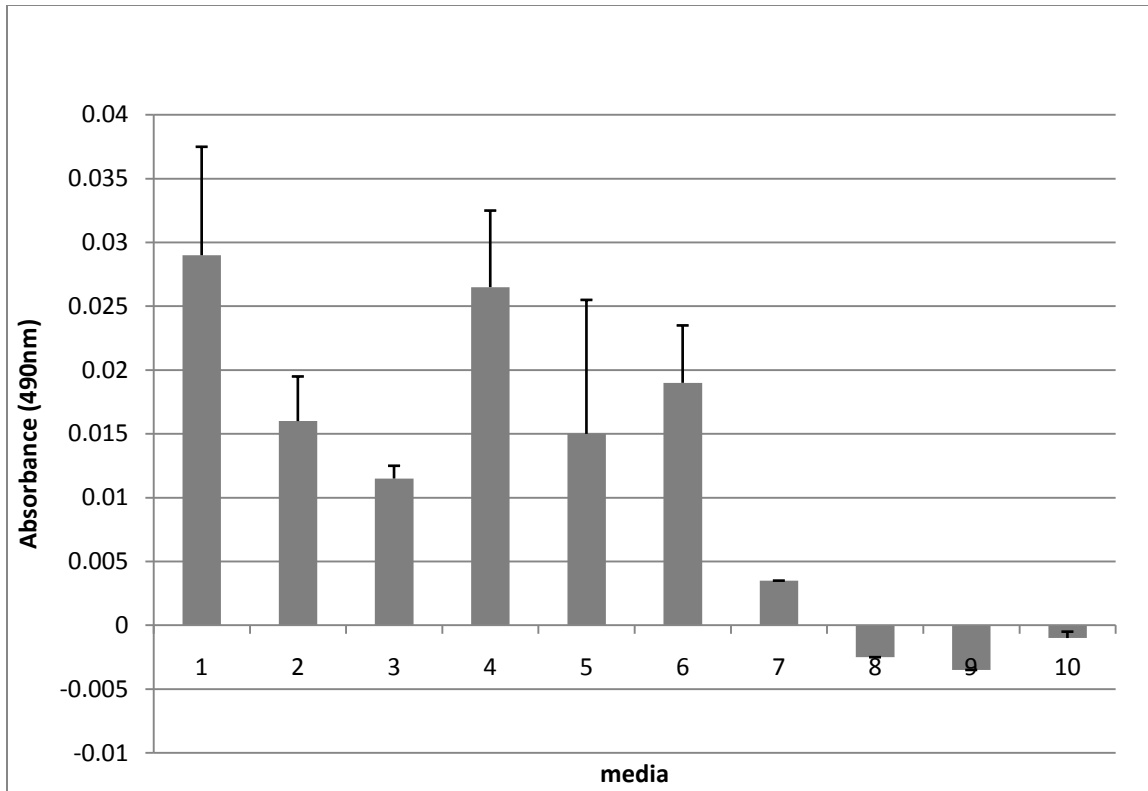


Figure 26. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: The procedure used in this experiment is the same as in Figures 10 and 11. As a control, four wells contained PBS + 20ul of the Cell Titer 96 reagent. This experiment is a continuation from the experiment in Figure 22. Each bar represents the 2 absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard deviation bars are included in the graphs.

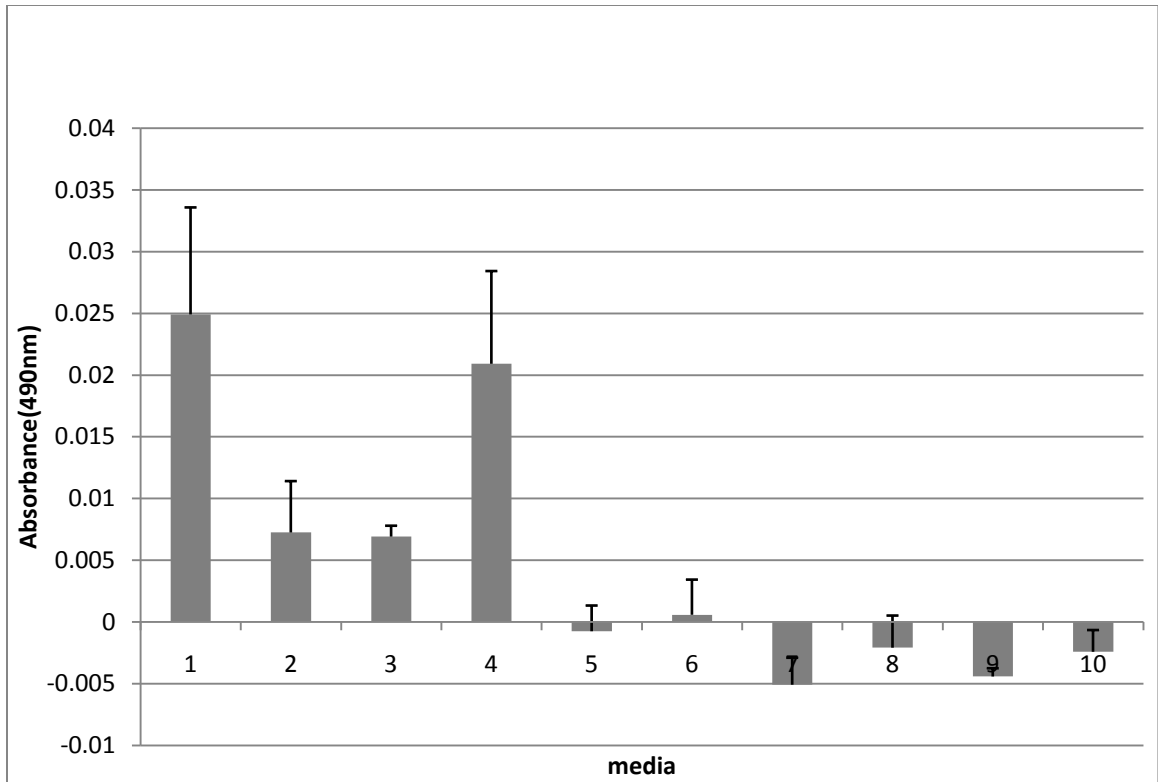


Figure 27. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: The procedure used in this experiment is the same as in Figures 10 and 11. As a control, four wells contained PBS + 20ul of the Cell Titer 96 reagent. This experiment is a continuation from the experiment in Figure 23. Each bar represents the three absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard error bars are included in the graphs.

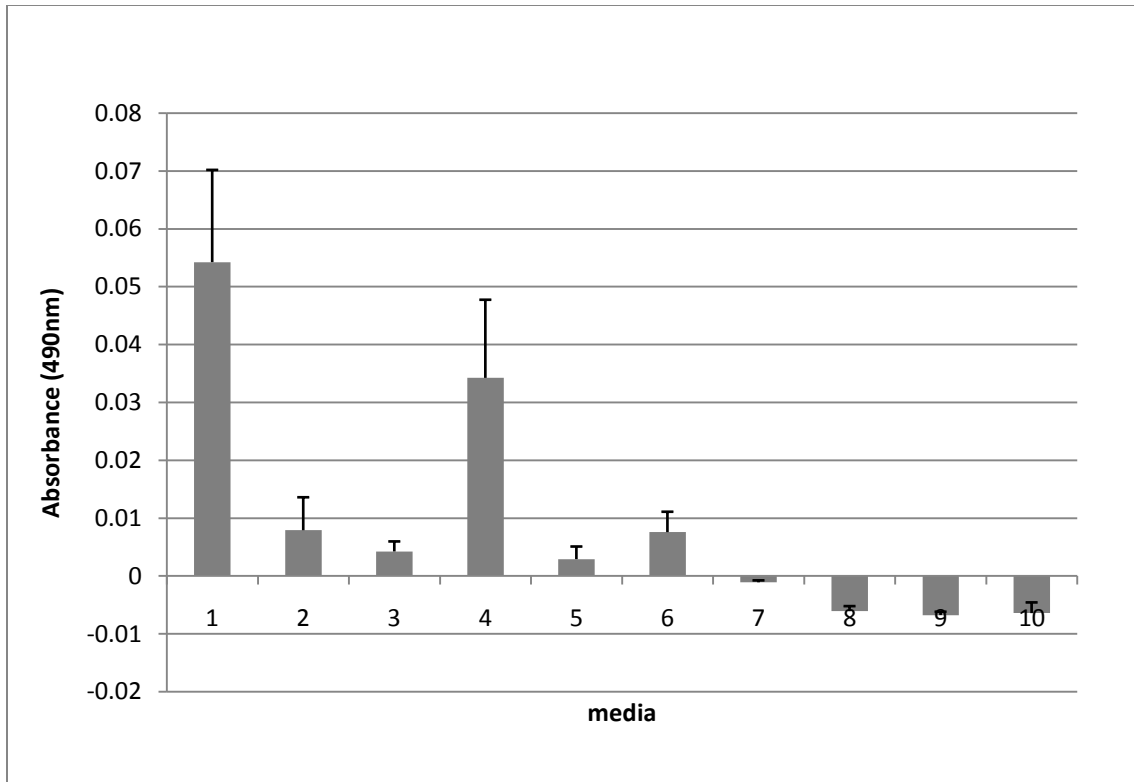


Figure 28. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's: The procedure used in this experiment is the same as in Figures 10 and 11. As a control, four wells contained PBS + 20ul of the Cell Titer 96 reagent. This experiment is a continuation from the experiment in Figure 24. Each bar represents the three absorbance values for each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard error bars are included in the graphs.

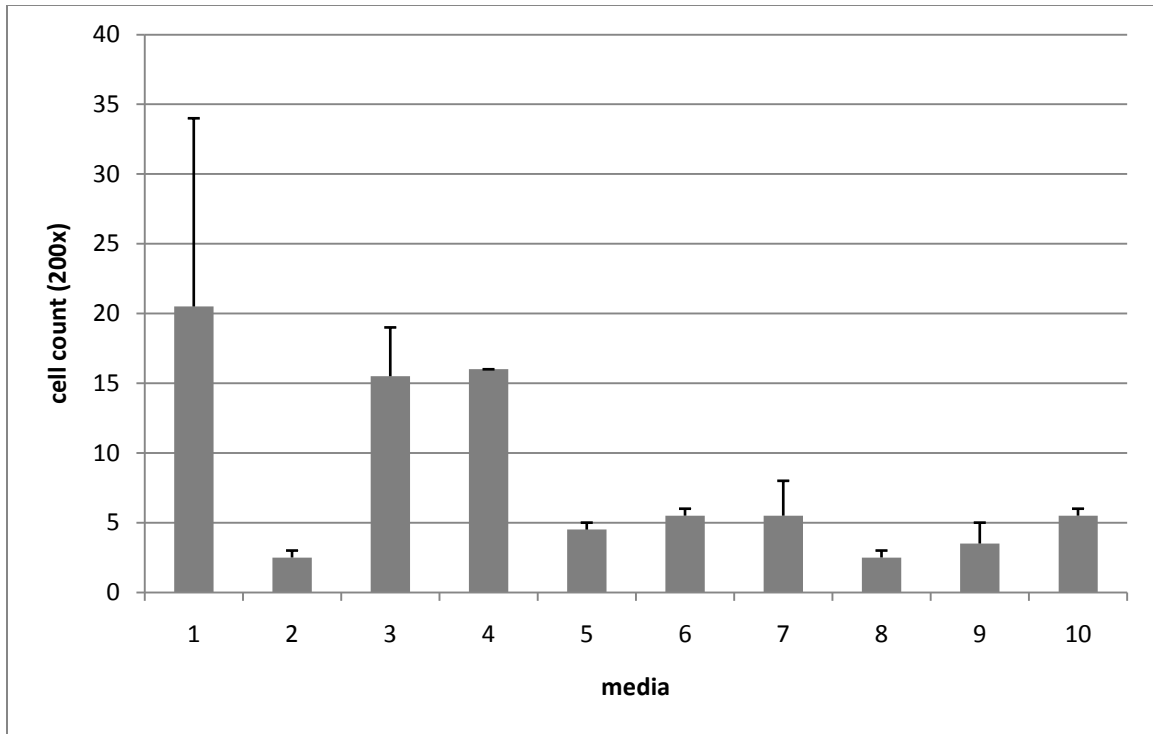


Figure 29. Cell Proliferation assay comparing different types of stem cell media using

rabbit MSC's _Cell Count: The procedure used in this experiment is the same as in Figure 10, except the concentrations of MSC's added to each well was different. To each well, 1000 MSC's was added. Each media was performed in duplicate, and one field of vision was counted from each well. The absorbance was not measured in this experiment. All of the wells were stained with crystal violet to make the MSC's easier to see. Each bar represents two fields of vision (one field of vision/well) averaged together, at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. The MSC's used in this experiment were from rabbit #20. Standard deviation bars are included in the graphs.

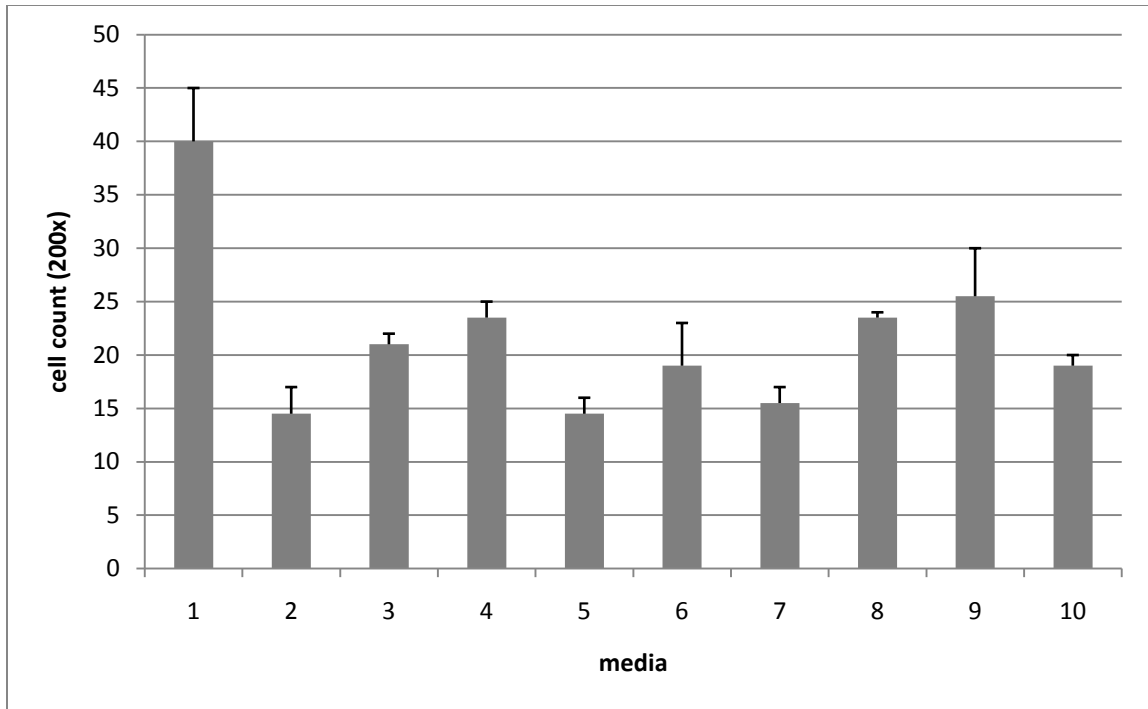


Figure 30. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except to each well, 2700 MSC's was added. Each media was performed in duplicate, and one field of vision was counted from each well. The absorbance was not measured in this experiment. All of the wells were stained with crystal violet to make the MSC's easier to see. Each bar represents 2 fields of vision (one field of vision/well) averaged together, at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. The MSC's used in this experiment were from rabbit #20. Standard deviation bars are included in the graphs.

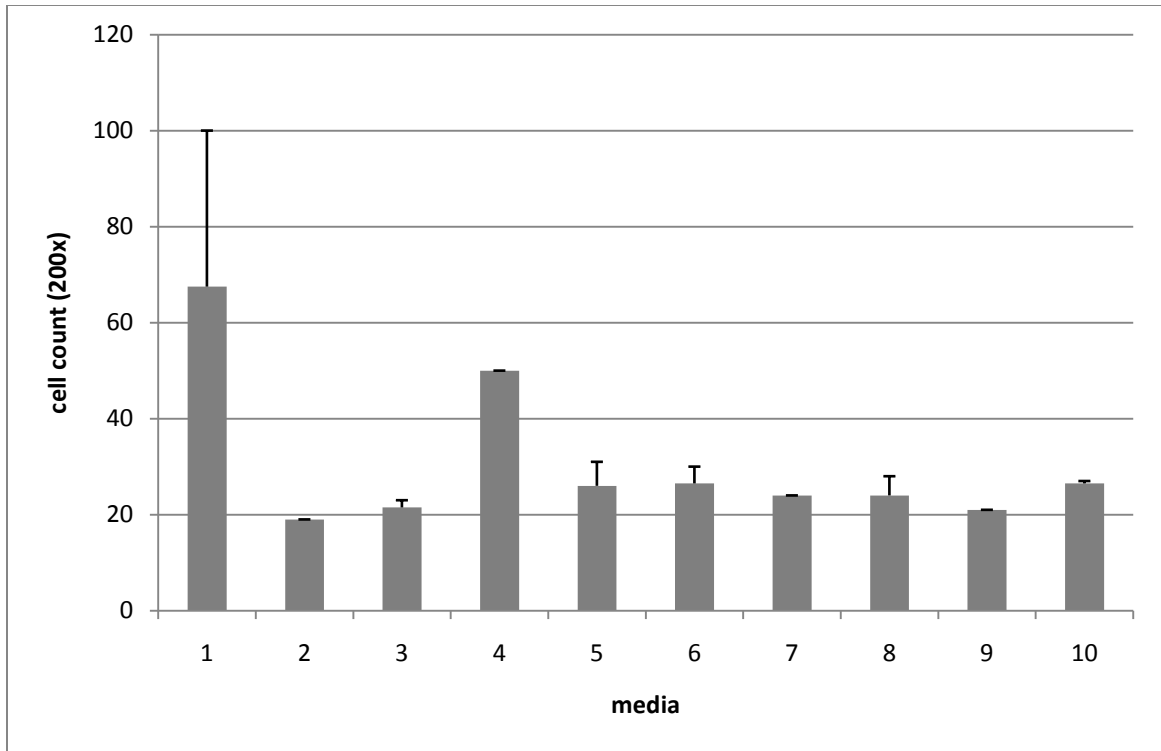


Figure 31. Cell Proliferation assay comparing different types of stem cell media using rabbit MSC's _Cell Count: The procedure used in this experiment is the same as in Figure 10, except to each well, 5400 MSC's was added. Each media was performed in duplicate, and one field of vision was counted from each well. The absorbance was not measured in this experiment. All of the wells were stained with crystal violet to make the MSC's easier to see. Each bar represents two fields of vision (one field of vision/well) averaged together, at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. The MSC's used in this experiment were from rabbit #20. Standard deviation bars are included in the graphs.

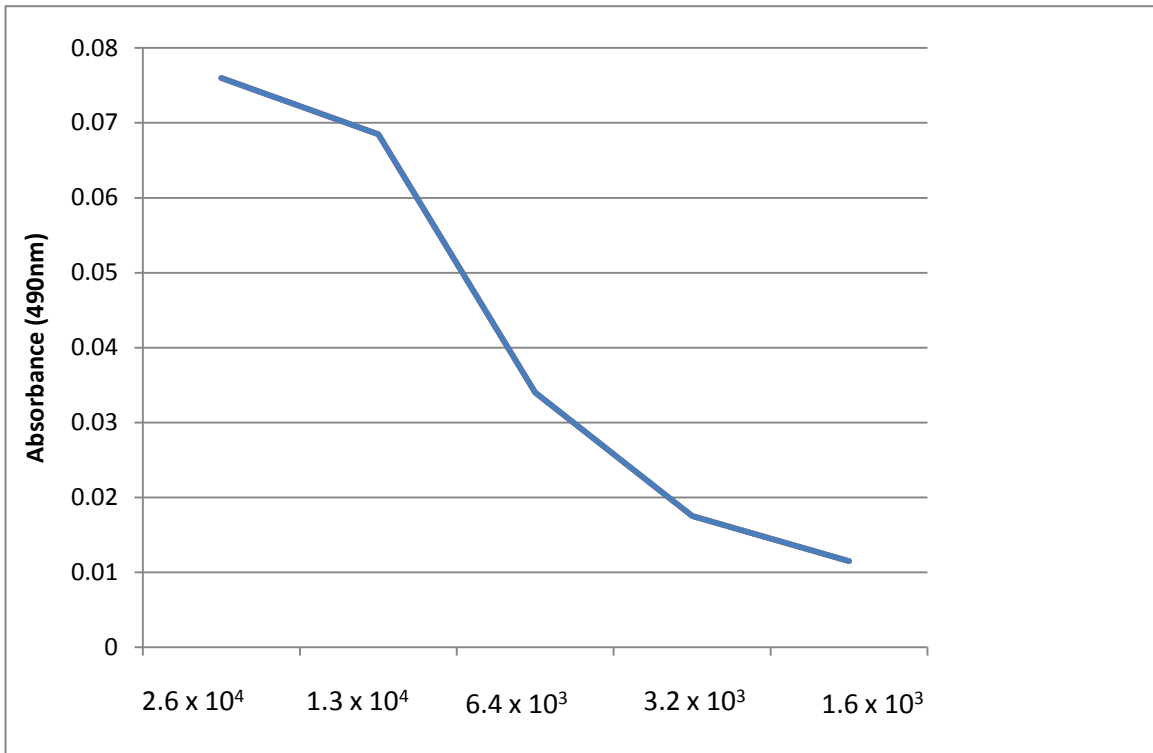


Figure 32. Standard Curve for the Adherence assay using Rat MSC's: This standard curve was performed by using the same procedure as in Figure 3. The MSC media used in this adherence assay with rat MSC's is the same as the media used with the rabbit MSC's (DMEM-High glucose with 10% FCS, 100U/ml penicillin, 100ug/ml streptomycin, 2.5 ug/ml amphotericin B, 1mM of L-glutamine). Untreated tissue culture plates were used in this standard curve. In this standard curve 5.12×10^4 MSC's were added to the first two wells, and serial 2-fold dilutions were performed as previously described. The absorbance reading was taken two hours after the reagent was added. The y-axis indicates the absorbance reading, and the x-axis indicates the number of MSC's in each well.

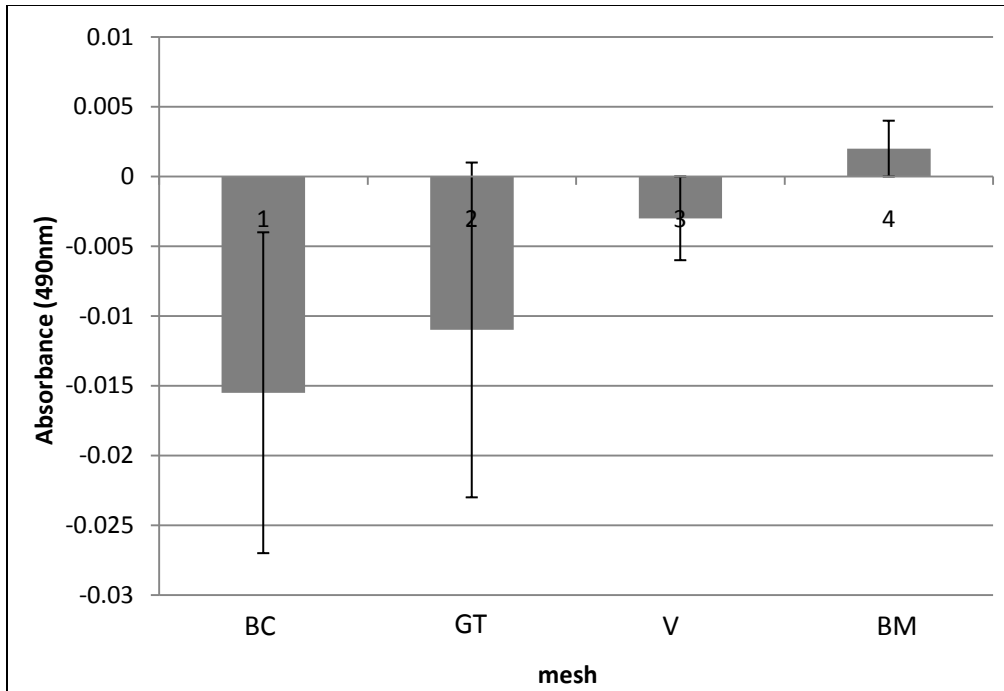


Figure 33. Cell Adhesion Assay: Cell Titer 96 AQ One Solution Cell Proliferation Assay

using Rat MSC's: The procedure used in this experiment is same as in Figure 7, except

the concentration of MSC's added to each mesh was different. In this experiment,

untreated tissue culture plates were used only to make sure the MSC's were sticking to

the mesh instead of the plates. The MSC's used in this experiment were from a rat.

The MSC's were stored in liquid nitrogen until they were used in this experiment. The

wells that contained mesh + MSC's had a total of 2.56×10^4 MSC's (less than the

previous 4×10^4 MSC's), and this was performed in duplicate. The controls used in this

experiment were four wells with PBS + 20ul of the Cell Titer 96 reagent, four wells with

MSC media + 20ul of the Cell Titer 96 reagent, and two wells with only MSC's. The 1st

mesh was the Bard Colla Mend, the 2nd mesh was the Gore-Tex, the 3rd mesh was the

Bard Mesh, and the 4th mesh was the vicryl. Each bar represents the two absorbance

values for each mesh averaged together (y-axis). A standard curve was also performed

in this experiment. Standard deviation bars are included in the graph.

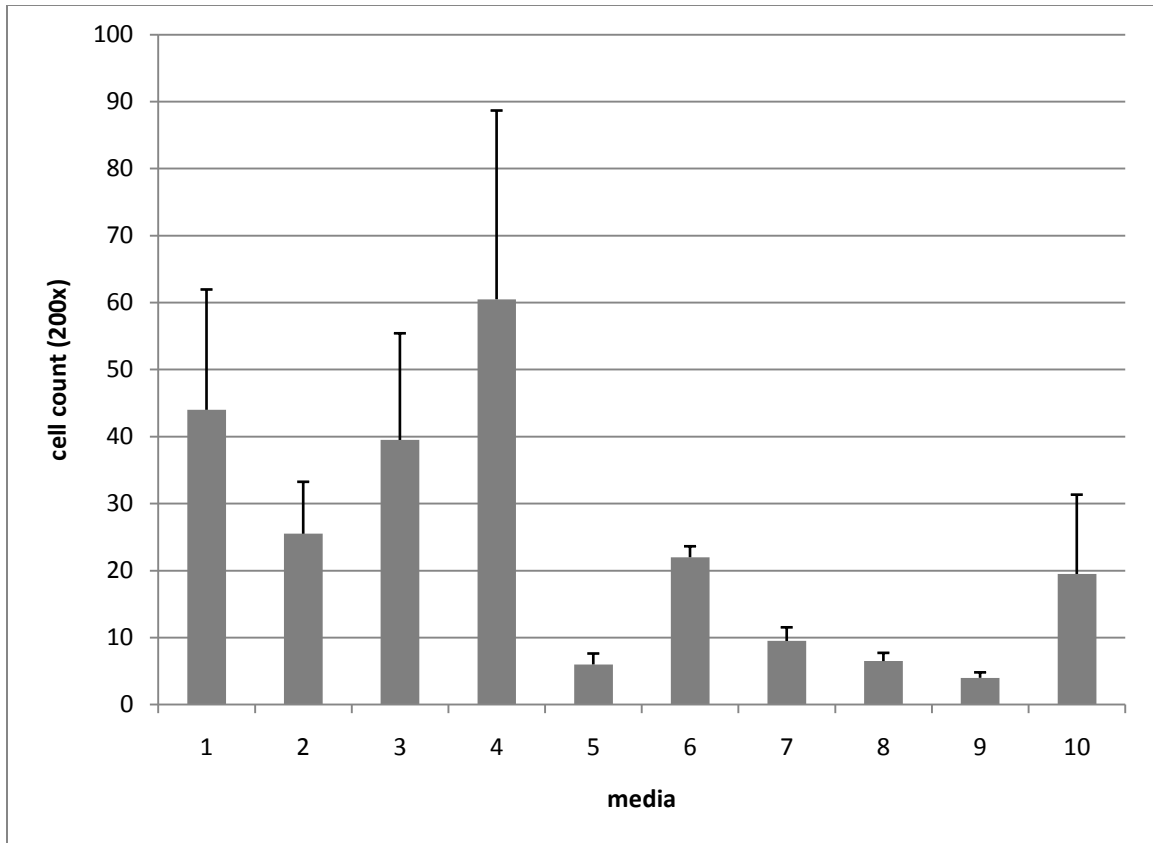


Figure 34. Cell Proliferation assay comparing different types of media using Rat

MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10.

The media used when first plating the MSC's was media "4" (Hams F-12 with 10% FCS, 100U/ml penicillin, 100ug/ml streptomycin, 0.25 ug/ml amphotericin B). To each well, 1000 MSC's were added. Each media was performed in duplicate. The MSC's were stained with crystal violet. Each bar represents two fields of vision (one field of vision/well) averaged together (y-axis), at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. Standard deviation bars are included in the graphs.

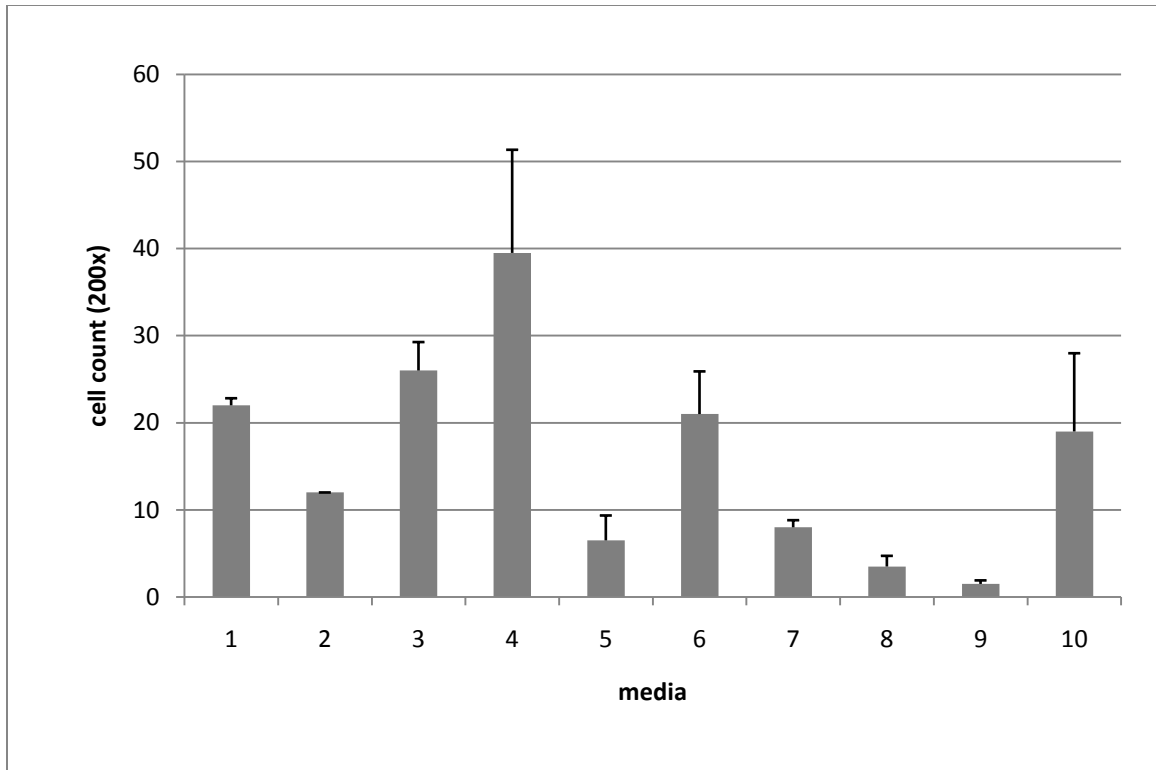


Figure 35. Cell Proliferation assay comparing different types of media using Rat

MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except that 2700 MSC's were added to each well. The media used when first plating the MSC's was media "4" (Hams F-12 with 10% FCS, 100U/ml penicillin, 100ug/ml streptomycin, 0.25 ug/ml amphotericin B). Each media was performed in duplicate. The MSC's were stained with crystal violet. Each bar represents two fields of vision (one field of vision/well) averaged together (y-axis), at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. Standard deviation bars are included in the graphs.

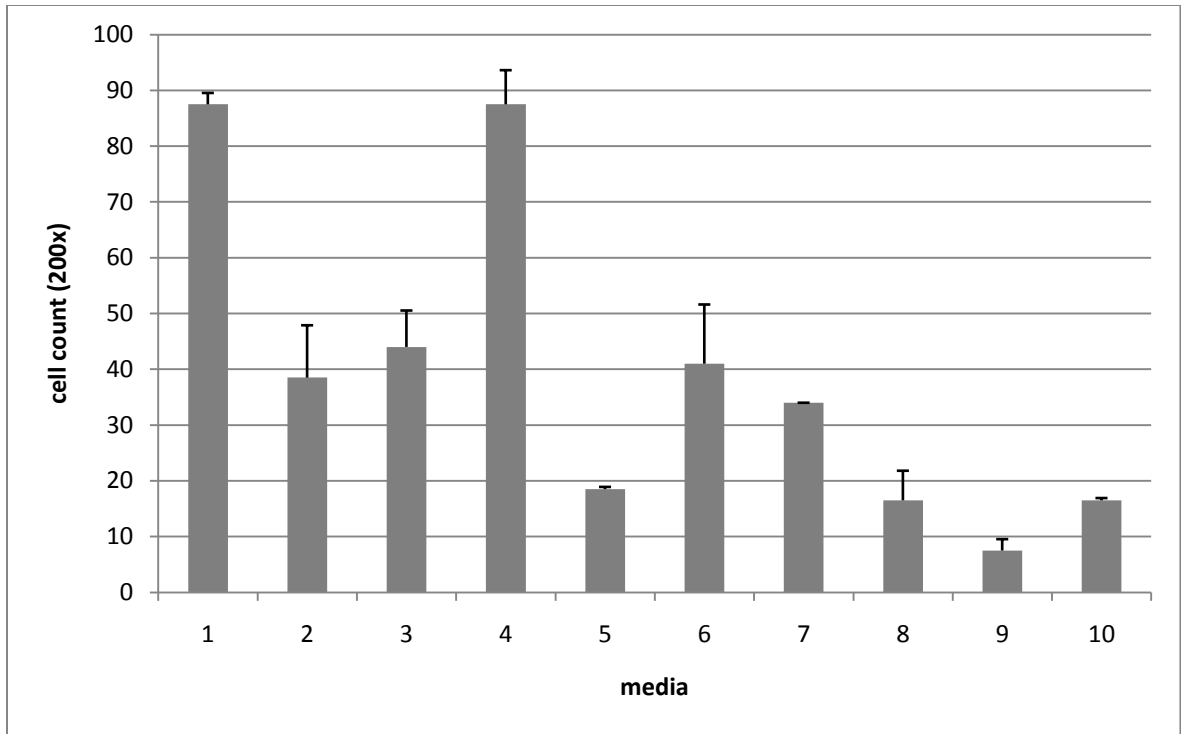


Figure 36. Cell Proliferation assay comparing different types of media using Rat

MSC's_Cell Count: The procedure used in this experiment is the same as in Figure 10, except that 5400 MSC's were added to each well. The media used when first plating the MSC's was media "4" (Hams F-12 with 10% FCS, 100U/ml penicillin, 100ug/ml streptomycin, 0.25 ug/ml amphotericin B). Each media was performed in duplicate. The MSC's were stained with crystal violet. Each bar represents two fields of vision (one field of vision/well) averaged together (y-axis), at the 200x magnification. The media used (x-axis) in each condition can be referenced in Table 2. Standard deviation bars are included in the graphs.

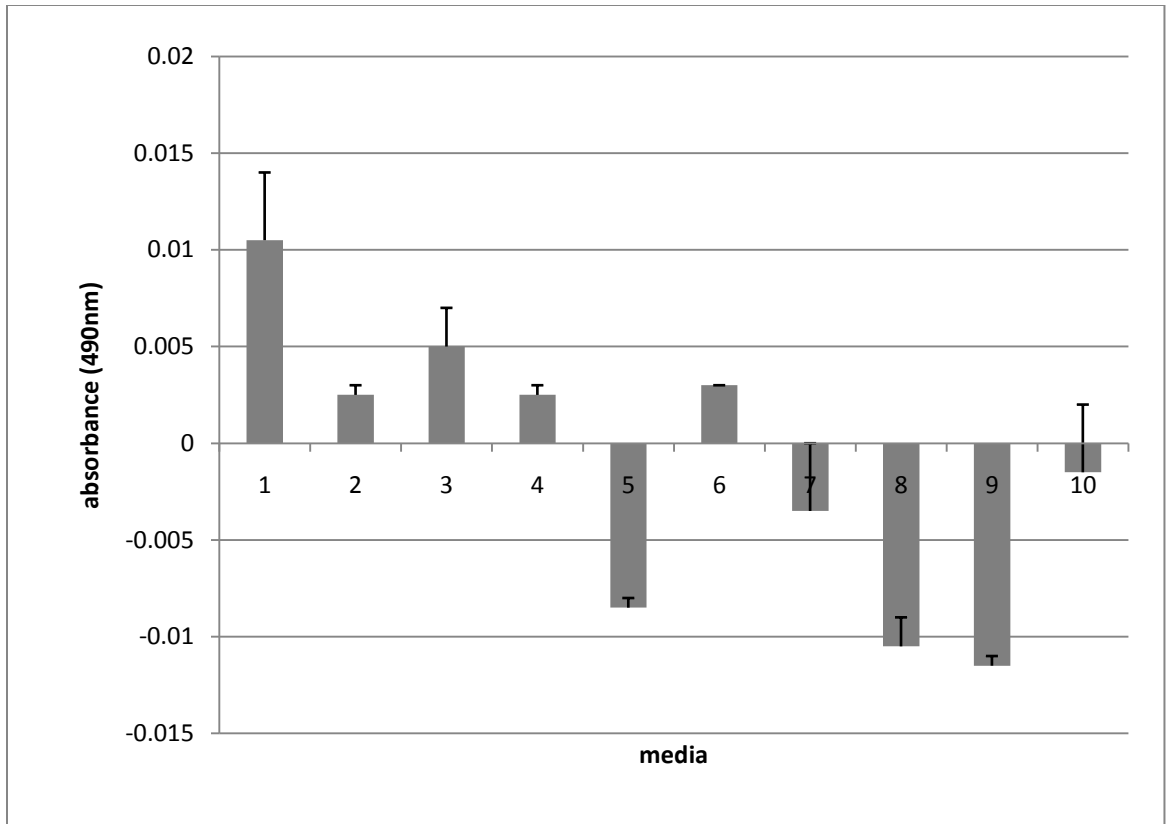


Figure 37. Cell Proliferation assay comparing different types of media using Rat MSC's:

The procedure in this experiment is the same as in Figures 10 and 11. This experiment is a continuation from the experiment in Figures 34. As a control for each plate, four wells contained PBS + 20ul of the Cell Titer 96 reagent. Each bar represents the two absorbance readings from each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard deviation bars are included in the graphs.

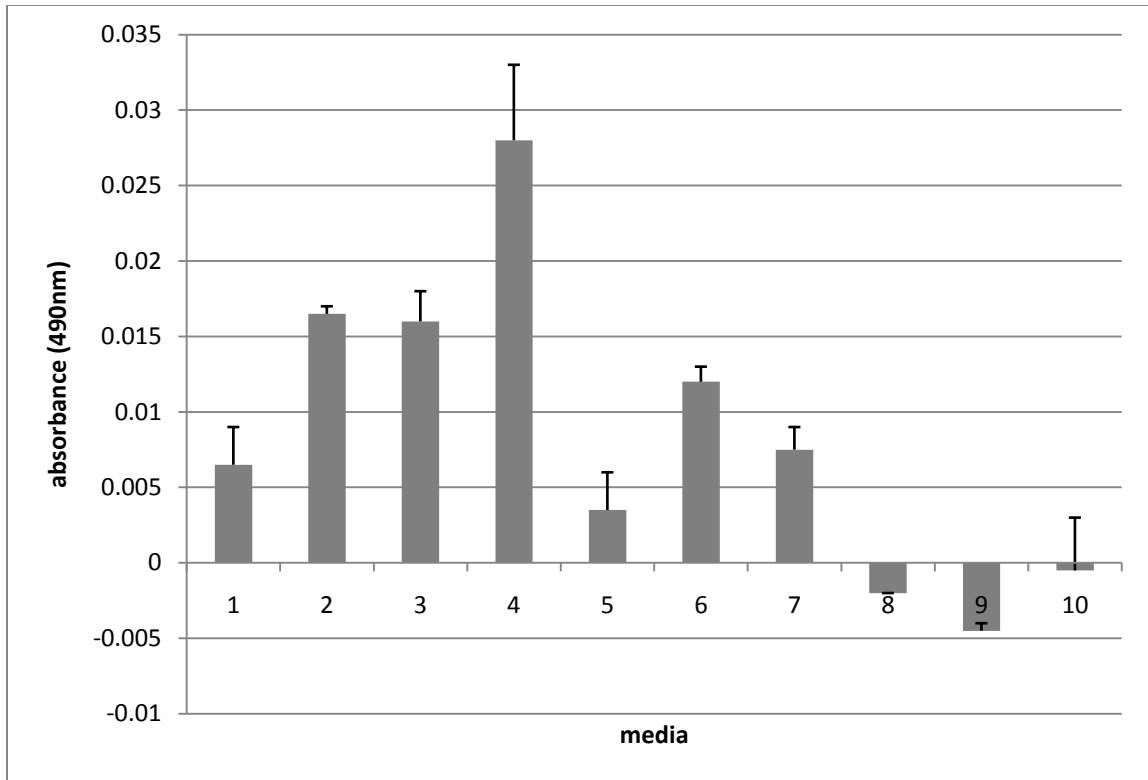


Figure 38. Cell Proliferation assay comparing different types of media using Rat MSC's:

The procedure in this experiment is the same as in Figures 10 and 11. This experiment is a continuation from the experiment in Figure 35. As a control for each plate, four wells contained PBS + 20ul of the Cell Titer 96 reagent. Each bar represents the two absorbance readings from each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard deviation bars are included in the graphs.

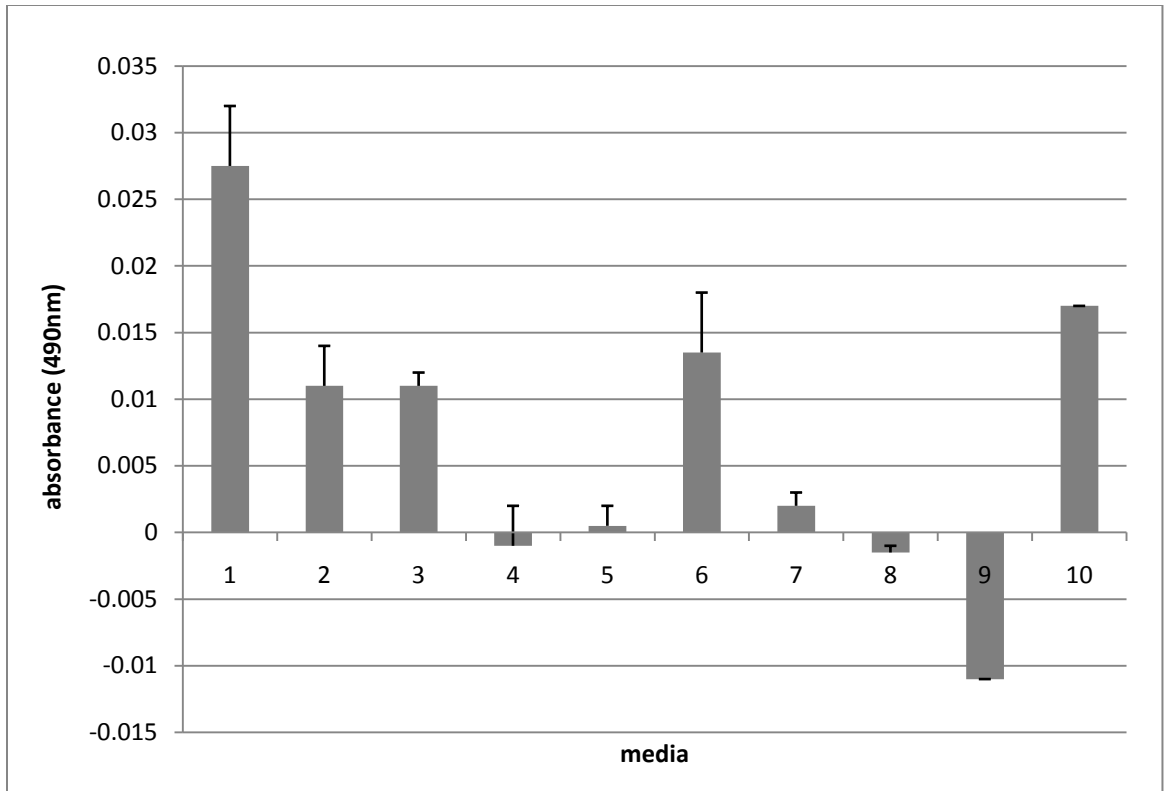


Figure 39. Cell Proliferation assay comparing different types of media using Rat MSC's:

The procedure in this experiment is the same as in Figures 10 and 11. This experiment is a continuation from the experiment in Figure 36. As a control for each plate, four wells contained PBS + 20ul of the Cell Titer 96 reagent. Each bar represents the two absorbance readings from each media averaged together. The y-axis indicates the absorbance values, and the media used (x-axis) in each condition can be referenced in Table 2. Standard error bars are included in the graphs.

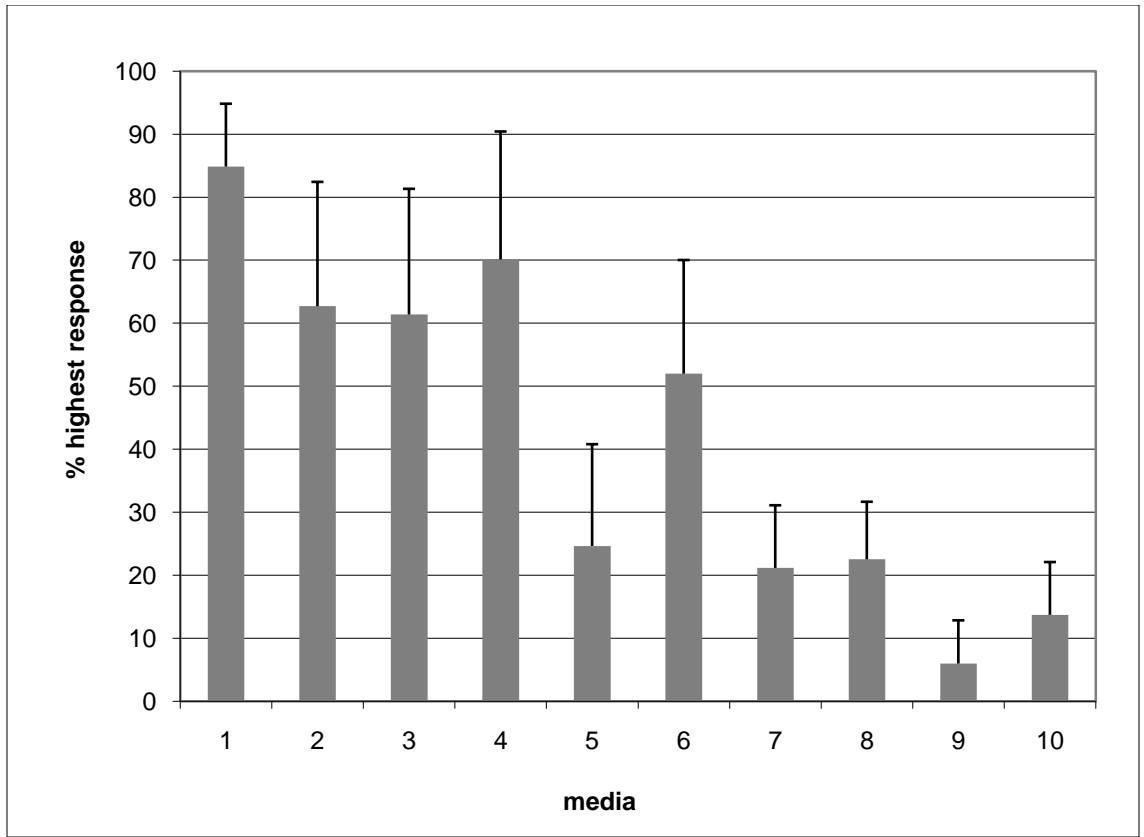


Figure 40. Percent highest for Cell Proliferation assays using Rabbit MSC's at 1000

MSC's: The purpose of calculating the percent highest is to average similar experiments to provide a clear representation of what media produced the highest level of cell proliferation at each concentration. The percent highest was calculated by dividing every value (absorbance value) by the highest value. This was done for each assay. The values calculated at each concentration, for each media were averaged together.

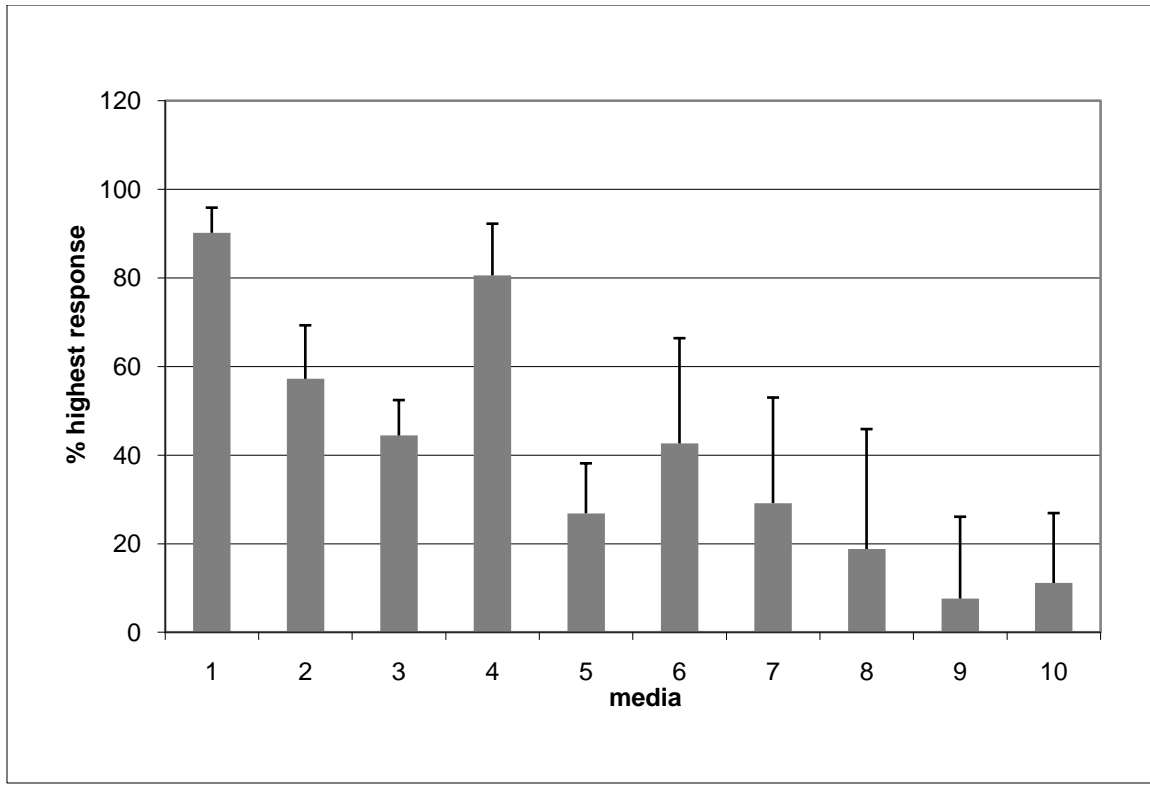


Figure 41. Percent highest for Cell Proliferation assays using Rabbit MSC's at 2700

MSC's: The percent highest was calculated by dividing every value (absorbance value) by the highest value. This was done for each assay. The values calculated at each concentration, for each media were averaged.

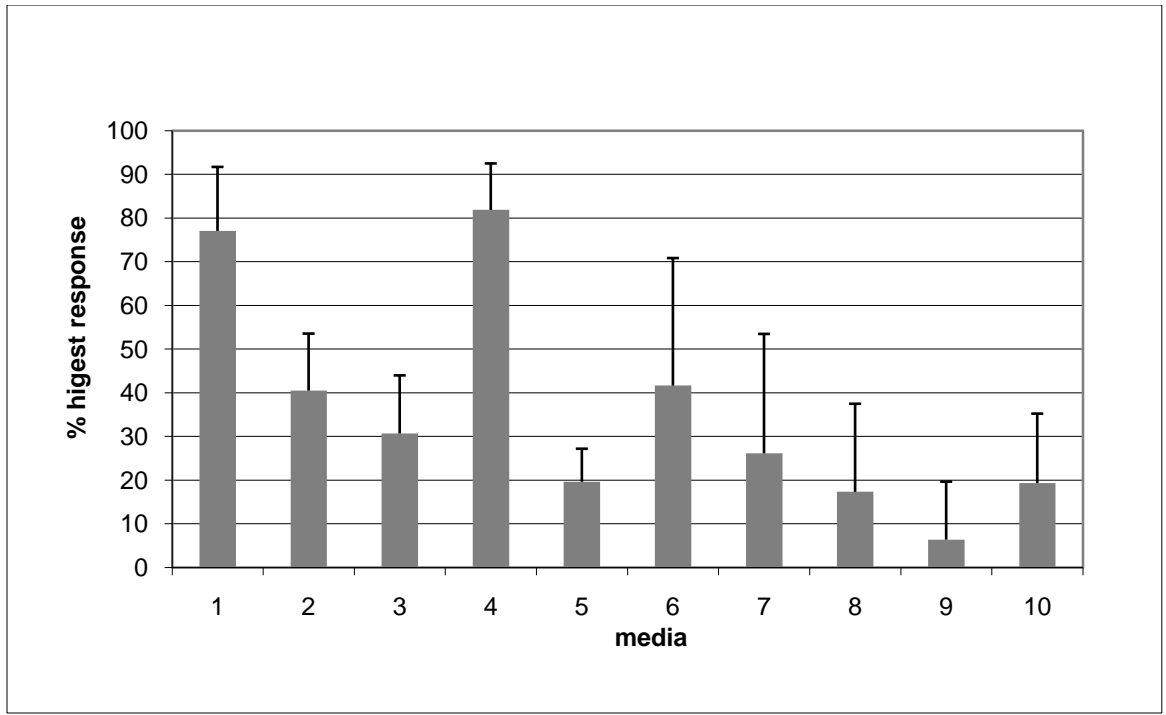


Figure 42. Percent highest for Cell Proliferation assays using Rabbit MSC's at 5400

MSC's: The percent highest was calculated by dividing every value (absorbance value) by the highest value. This was done for each assay. The values calculated at each concentration, for each media were averaged.

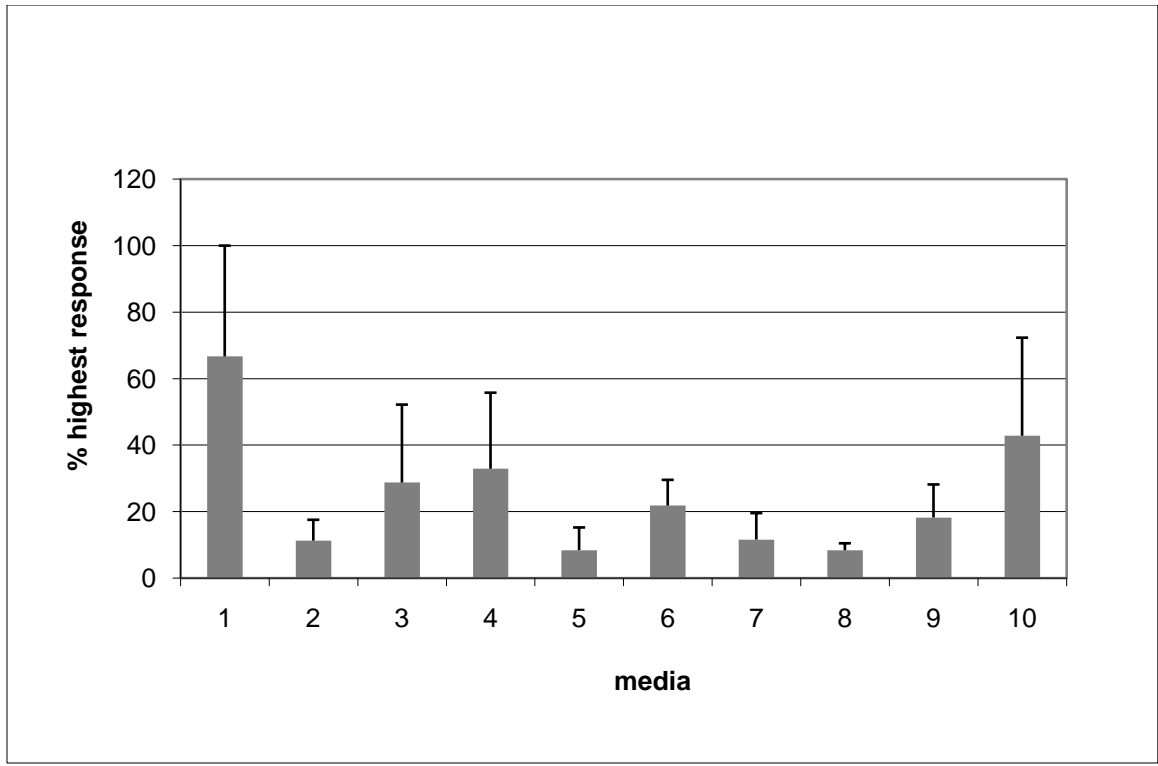


Figure 43. Percent highest Cell Count using Rabbit MSC's at 1000 cells/well: The percent highest was calculated by dividing every value (cell count value) by the highest value. This was done for each assay. The values calculated at each concentration, for each media were averaged.

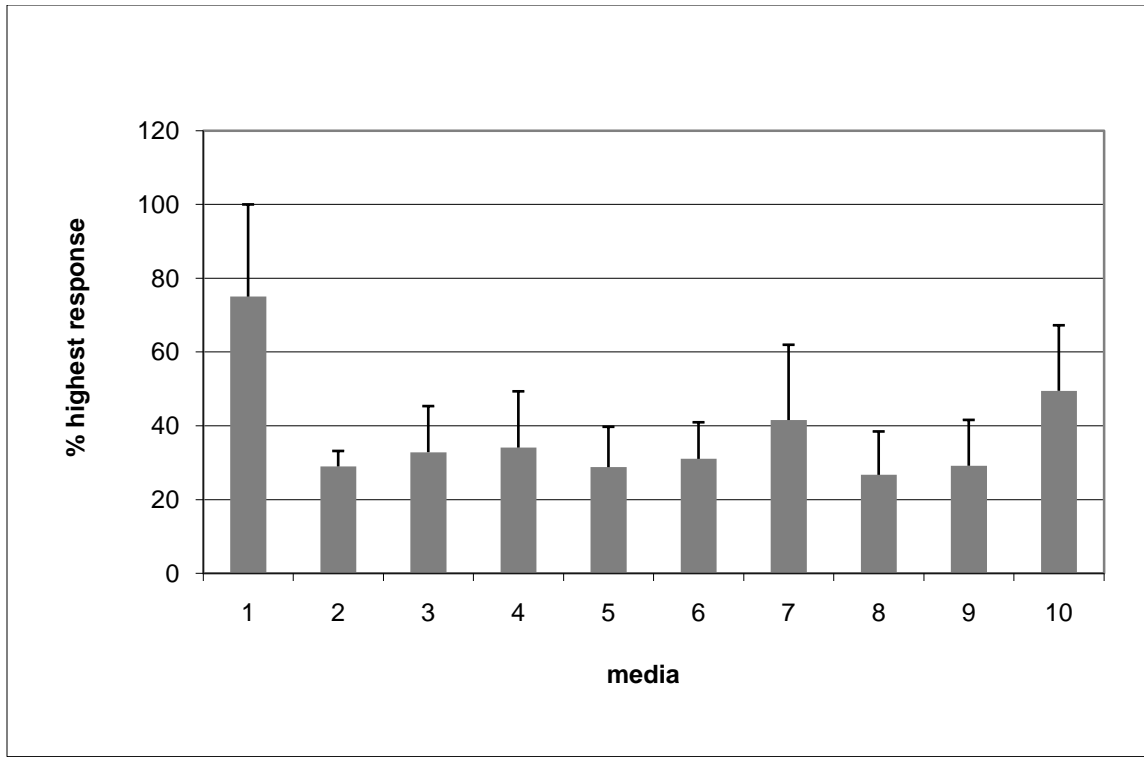


Figure 44. Percent highest Cell Count using Rabbit MSC's at 2700 cells/well: The percent highest was calculated by dividing every value (cell count value) by the highest value. This was done for each assay. The values calculated at each concentration, for each media were averaged.

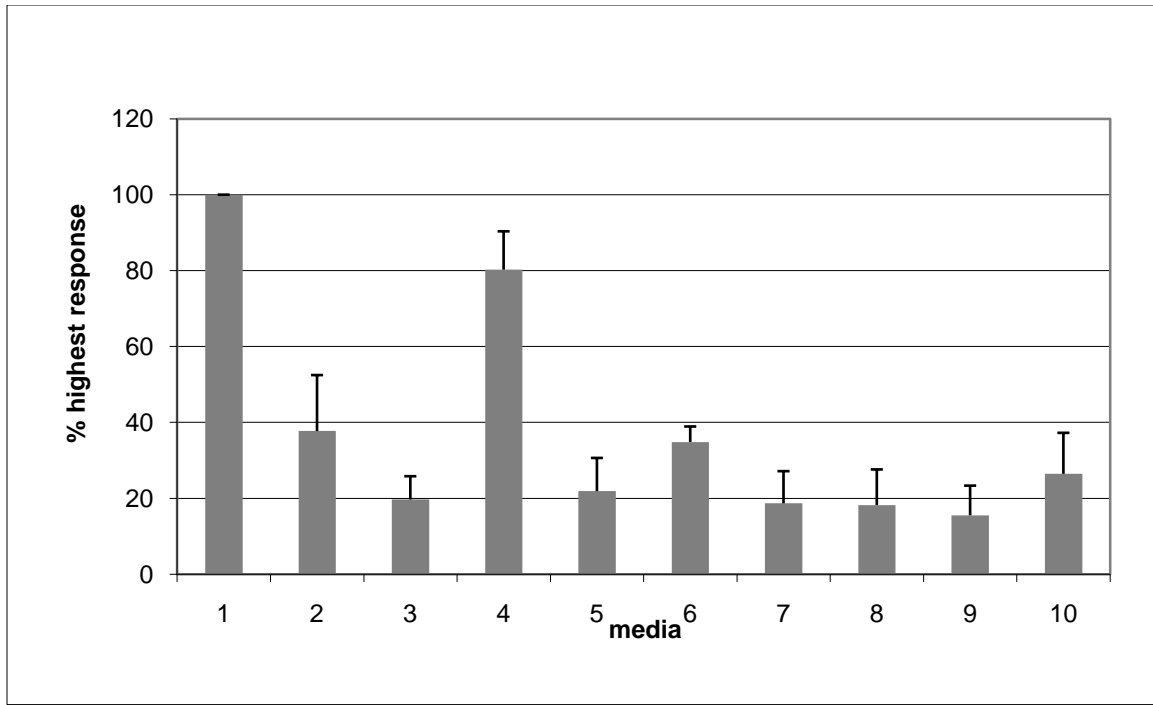


Figure 45. Percent highest Cell Count using Rabbit MSC's at 5400 cells/well: The percent highest was calculated by dividing every value (cell count value) by the highest value. This was done for each assay. The values calculated at each concentration, for each media were averaged.

Preliminary studies using rat MSCs are shown in **Figures 33 through 40**. A standard curve was performed which demonstrated that the colorimetric assay can be used to measure rat MSCs at concentrations as low as 3×10^3 cells/well (**Figure 32**). A single adhesion assay was performed examining rat MSC adherence to mesh materials (**Figure 34**). The data suggests that rat MSCs adhere best to Bard Mesh and bind least well to Bard CollaMend. These are significantly different results from those seen with rabbit MSCs. However, high absorbance in the negative control may have complicated the interpretation of the data and more experiments need to be performed. Rat MSC proliferation assays showed similar results to those seen with rabbit MSCs. Rat cell counts after growth in the various media are shown in **Figures 35** (1000 cells/well), **36** (2700 cells/well), and **37** (5400 cells/well). Rat MSC proliferation measured by the Cell Titer assay are shown in **Figures 38** (1000 cells/well), **39** (2700 cells/well), and **40** (5400 cells/well). While some differences are seen when you compare the rat data to the rabbit data, the same cell culture media (alpha MEM and Ham F12) appear to also support rat MSC growth. Additional experiments will be performed to confirm the media requirements for rat MSCs.

Discussion:

Fascial defects can heal improperly leading to hernia formation. A procedure to alleviate this problem has yet to be discovered. Mesenchymal stromal cells (MSC's) have been shown to enhance the wound healing process in cutaneous and cartilage defects; however, no one has published research using autologous MSC's on fascial defects *in vivo*. MSC's are multipotent cells that are able to differentiate into various cell types. They are thought to be the long-lasting precursors for connective tissue and other specialized tissues, such as bone, muscle, and cartilage. MSC's are not easily rejected and they can divide very rapidly, which is why these cells are becoming extremely popular in the bioengineering field. The purpose of our study was to investigate the success of using MSC's to improve fascial healing.

Other laboratories have shown that MSC's improve healing. Wu and coworkers used BALB/c mice MSC's to test the effects of MSC's in cutaneous repair (Wu et al., 2007). The mice received two bilateral incisions, and each wound received 1 million cells. The test group received MSC's and the control group received dermal fibroblasts. The tissue was sectioned and stained with H & E. The sections were analyzed for the amount of re-epithelialization, tissue regeneration, granulation tissue formation, and angiogenesis. The tissue sections that contained MSC's showed an increase in angiogenesis, and re-epithelialization. Their data also suggests that the injected MSC's play a role in dermal regeneration by contributing to the cells in the skin.

The purpose of the study by Im and coworkers was to test the effects of MSC's

on an osteochondral defect created artificially on the patellar groove, using a rabbit model. Im and coworkers injected MSC's into the defect of the test groups, while the control groups received only media (Im et al., 2001). After 14 weeks of recovery time, the cartilage was removed, sectioned and stained with Hematoxylin and eosin, and Safranin-O/Fast Green. The amount of collagen type II was quantified using immunohistochemistry. The test group showed smooth white tissue which physically resembled articular cartilage. The control groups showed irregular red tissue. When looking at the thickness, appearance, and amount of degeneration at the wound site, the test groups produced significantly thicker cartilage than the control groups. Collagen type II mRNA was found in the regenerated cartilage of the test and control groups. Because some test groups received poor histological scores compared to the control groups, this research group thinks that some MSC's leak out of the sutured defect after implantation. They concluded that MSC's are a good candidate for cartilaginous repair, but there is no guarantee that they will improve healing.

Yan and coworkers compared different types of chondrogenic cell lines and their effects on cartilage repair. Yan and coworkers injected chondrocytes from articular cartilage of a rabbit, MSC's from the bone marrow of a rabbit, fibroblasts from corium tissue of a rabbit, and human stem cells from an umbilical cord, onto cartilage defects (Yan and Yu, 2007). The researchers tried to repair full-thickness cartilage defects by implanting chondrogenic cells, using one type of cell per rabbit. The various cells were impregnated on a PLA matrix, which is a porous- sponge-like material. The PLA/cell combination was implanted at the cartilage defect, and the tissue was histologically

evaluated using the Hematoxylin and Eosin stain 6 or 12 weeks after the surgery. The repaired tissue was significantly better in chondrocyte and MSC groups compared to the human stem cell and fibroblast groups. When comparing the MSC's with the chondrocytes, the MSC's had better differentiated cells, better remodeled subchondral bone, and the contrast between new and original cartilage was less noticeable. This group of researchers concluded that MSC's might be the optimal source of cells for cartilage repair.

The use of MSC's in fascial repair has not been tested before. However, some laboratories are performing similar research, testing the effects of MSC's on wound repair. Han and coworkers looked at bone marrow stromal cells to accelerate wound healing, *in vitro*, and compare these cells to dermal fibroblasts in the areas of cell proliferation, collagen synthesis, and growth factor production (Han et al., 2005). As mentioned previously the bone marrow stroma is the source of MSC's. The cell proliferation was measured using the 3-2-5 diphenyl tetrazolium bromide assay, which measured the absorbance at 570nm. The collagen synthesis was measured using the collagen type 1-terminal propeptide enzyme immunoassay. Growth factor production was measured using an ELISA kit. The results of the study by Han and coworkers indicated that there was not a significant difference in cell proliferation or transforming growth factor- β secretion (Han et al., 2005). However there was increased wound healing activity when using the bone marrow stromal cells in the areas of collagen, basic fibroblast growth factor, and vascular endothelial growth factor production. It is thought that basic fibroblast growth factor and vascular endothelial growth factor- β are

both involved with the angiogenesis during the process of wound healing. This paper provides strong evidence that bone marrow stromal cells have a promising future in wound healing.

As other research groups have shown promising data using MSC's in cutaneous and cartilage repair, the use of MSC's in fascial healing have been tested in this research project. Not only did our research project look at the use of MSC's but we also looked at the effects of platelet rich plasma enhanced fibrin glue, as well as a collagen scaffold. In our study we used platelet rich plasma enhanced fibrin glue to test its effects on MSC's in terms of collagen deposition, collagen remodeling and tensile strength during fascial healing.

It is known that the platelet enriched plasma contains various growth factors such as vascular endothelial growth factor and platelet derived growth factor. Vascular endothelial growth factor stimulates endothelial cell migration and proliferation, which is important during angiogenesis. Platelet-derived growth factor is involved with the formation of granulation tissue, epithelialization, the production of the extracellular matrix, and tissue remodeling, all important processes involved in wound healing. These growth factors enhance MSC growth as well as collagen remodeling. We also used a collagen scaffold because sources have shown that bovine collagen type 1 carries MSC's effectively and helps organize remodeling.

In our study 21 New Zealand white rabbits were anesthetized, 1 midline abdominal incision and 2 bilateral fascial incisions were made on each rabbit. The

defects were closed with nylon sutures, and platelet rich plasma enhanced fibrin glue embedded in collagen with or without MSC's was added to the fascial defects. The control incision received no MSC's, and the test incision received MSC's. The rabbits had 8 weeks to recover, and the incision sites were embedded in paraffin, sectioned, and stained with Masson Trichrome. We measured the collagen deposition in all the slides. Out of the 6 animals, only 3 showed an increase in collagen deposition in the test group when compared to the control group.

The tissue samples were also analyzed biomechanically to measure the tensile strength of the incision site. Each fascial defect was cut into a dumbbell shape, and the tests were performed using an Instron Tensiometer. The tensiometer applied a constant extension rate to the tissue until disruption occurred. Force and tissue disruption were recorded for each tissue specimen, and the data was recorded in a stress-strain plot (not shown). The results showed that the MSC's increased tensile strength at the incision site (Marie, 2009).

The second part of the project was to test the ability of MSC's to adhere to various mesh materials. Other research groups tested various scaffolds, to see which type of vehicle would optimize cell proliferation or cell differentiation. In 2006, Huang and coworkers used adult mesenchymal stem cells and seeded the cells onto collagen type 1-glycoaminoglycan scaffolds (Huang et al., 2006). These researchers wanted to see if the MSC's could undergo osteogenic and chondrogenic differentiation, in a collagen type scaffold, and have the potential to be used in cartilage-bone constructs.

The purpose of this study was to see how a porous 3-D environment, would support the levels of osteogenesis and chondrogenesis of adult rat MSC's. Teixeira and coworkers used a macroporous ceramic scaffold to test for MSC proliferation and adhesion (Teixeira et al., 2007). The MSC's were successfully able to adhere to the scaffold, with an extensive amount of cell proliferation.

Our studies were developed in order to determine the best vehicle for the addition of MSC's to the wound in future studies. The MSC's were incubated with the various mesh materials, and the nonadherent MSC's were washed off. The adherence to mesh was determined microscopically and by using a colorimetric (increased cell number results in increased color in medium) assay. When using the rabbit MSC's we found that the cells were adhered best to Bard CollaMend, and to BardMesh and vicryl the least. Bard CollaMend is a collagen matrix, and it was the most natural and porous mesh material used in this study. When testing the rat MSC's, the only mesh that allowed for any cell adhesion was the Bard Mesh. The assay with rat MSC's was only performed once; therefore this data will be retested in the future.

The third part of this project tested various plating densities to determine which initial concentration of MSC's produces the highest level of cellular proliferation. Similar studies were performed by Bartmann and coworkers who tested various plating densities using human MSC's (Bartmann et al., 2007). A colony forming unit-fibroblast (CFU-F) assay was performed, and the MSC's were seeded at 150 or 375 MSC's/well.

The MSC's used in the assay came from flasks that were originally seeded at 5.6×10^5 , 5.6×10^4 , 5.6×10^3 and 5.6×10^2 densities. The MSC's that originally came from the lower starting densities produced the highest amount of colony forming units. This research group concluded that there is an inverse correlation between plating densities and the amount of cellular proliferation. Our cell count values (Figures 43-45) did not show this trend, however, our absorbance values (40-42) did. The absorbance values using plating densities of either 1000 or 2700 MSC's were higher than the absorbance values using a plating density of 5400 MSC's.

When culturing MSC's, many factors can be altered in order increase the amount of cells proliferation, and to preserve their differentiation capacity (Vaananen, 2005). Factors that have contributed to creating the optimal niche for cells to grow include the quality of serum, the type of basal media, glucose concentration, plating density, and the plastic surface quality (Sotiropoulou et al., 2009). Sotiropoulou and coworkers tested various recipes of MSC media, using human MSC's. Some recipes included a basal media, while other included a more complex media, such as DMEM. Some recipes included a low concentration of glucose, and others included a high concentration. Their results showed that α -MEM with either Glutamax or L-glutamine produced the highest levels of cell proliferation. When culturing the rabbit MSC's at the beginning of the project, we used high amounts of glucose, a good source of carbohydrates which allows a wide range of cell types to grow. We also used 10% fetal calf serum, a source of vitamins, growth factors, and growth inhibitors. The serum also supports the growth of low density cell cultures by increasing the medias

buffering capacity, and protects the cells from mechanical damage. A review of the literature showed that several different types of media are used to culture MSC's and no systematic comparison of media has been performed. We examined several combinations of ingredients to determine which produced the highest level of cell proliferation.

In our study using rabbit MSC, media containing α -MEM, 20% fetal calf serum, 100U/ml of penicillin, 100ug/ml of streptomycin, and 1mM of L-glutamine (media 1) produced the highest levels of cell proliferation, especially at the 1000 and 2700 plating densities. MSC media containing Ham's F-12, 100U/ml of penicillin, 100ug/ml of streptomycin, 0.25ug/ml of amphotericin B (media 4) also produced high levels of cell proliferation, especially at the 5400 plating density. In the past, our lab used MSC media which contained high glucose DMEM, 100U/ml of penicillin, 100ug/ml of streptomycin, 1mM of L-glutamine, 1ml of amphotericin, and 10% fetal calf serum (media E), so this was a significant find for our lab. In our study using rat MSC's media 1 and 4 also produced the highest levels of cell proliferation however this experiment was only performed once, and will require repeated analysis.

In the rabbit model our studies have shown that MSC's increase the tensile strength of the incision site. In some of our experiments MSC addition appeared to increase the collagen deposition at the incision site. Our cell proliferation assay's using rabbit MSC's showed that α -MEM + Hams F-12 are superior growth media, when compared to the media our laboratory was using previously. Our cell adhesion assay

showed that Bard Colla Mend appears to be the best vehicle for the addition of MSC's to an incision in future studies. The next stage in this project will be to extend our studies to a rat model. Preliminary studies of adhesion and cell proliferation assays have been performed using rat MSC's. Studies are ongoing in which rat MSC's have been implanted at the site of surgical incisions. The biomechanical properties, collagen deposition and collagen remodeling, as well as the collagen ratio between collagen type 1 and type 3 will all be analyzed.

References

Amid, P.K., Shulman, A.G., Lichtenstein, I.L. "Selecting Synthetic Mesh for the Repair of Groin Hernia". Postgraduate General Surgery. 4 (1992): 150-155.

Bard Mesh Flat Sheets. [Internet]. [updated 2010]. Davol Inc. Cranston (RI).

Available from:

[http://www.barduk.com/main/product.asp?sectionTypeId=2§ionId=19&productId=](http://www.barduk.com/main/product.asp?sectionTypeId=2§ionId=19&productId=315)

315

Barrientos, Stephan, Stojadinovic, Olivera, Golinko, Michael, S., Brem, Harold, Tomic-Canic, Marjana. "Growth factors and cytokines in wound healing." Wound Repair and Regeneration. 16 (2008): 585-601.

Bartmann, Christina, Ronde, Eva, Schallmoser, Katharina, Purstner, Peter, Lanzer, Gernard, Linkesch, Werner, Strunk, Dirk. "Two steps to functional mesenchymal stromal cells for clinical application". Transfusion. 47 (2007): 1426-1435.

Beets, G.L., van Geldere, D., Baeten, C.G., Go, P.M. "Long-term results of giant

prosthetic reinforcement of the visceral sac for complex recurrent inguinal hernia". Br Journal of Surgery. 83 (1996): 203-206.

Campa, Michael. (2005) "Promega's Cell Titer 96 Aqueous One Solution Cell Proliferation Assay". www.biocompare.com/prorev.

Carson, F.L. "Histotechnology: A Self Instructional Text". (1997). Chicago: American Society of Clinical Pathologists Press.

"Cell Titer 96 Assay One Solution Cell Proliferation Assay: Technical Bulletin". Promega. (2005).

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Krause, D.S., Deans, R.J., Keating, A., Prockop, D.J., Horwitz, E.M., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement". Cytotherapy. 8 (2006): 315-317.

DuBay, Derek, A., Wang, Xue, Adamson, Belinda, Kuzon, William, M., Dennis, Robert, G.,

Franz, Michael, G. "Progressive fascial wound failure impairs subsequent abdominal wall repair: A new animal model of incisional hernia formation." Surgery 137 (2005): 463-71.

Enoch, Stuart, Leaper, David, J. "Basic science of wound healing." Surgery. 26:2 (2007): 31-37.

Eppley, Barry, L., Woodell, Jennifer, E., Higgins, Joel. "Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing". Plastic Reconstructive Surgery. 114 (2004): 1502-8.

Farrell, Eric, O'Brien, Fergal, J., Doyle, Paul, Fischer, Jan, Yannas, Ionnais, Harley, Brendan, A., O'Connell, Brian, Prendergast, Patrick, J., Campbell, Veronica, A. "A Collagen-glycosaminoglycan Scaffold Supports Adult Rat Mesenchymal Stem Cell Differentiation Along Osteogenic and Chondrogenic Routes". Tissue Engineering. 12 (2006): 459-469.

Friedenstein, A.J., Petrakova, K.J., Kurolesova, A.I., Frolova, G.P. Heterotopic of bone marrow: Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation. 2 (1968): 230-247.

Gore Tex Soft Tissue Patch. [Internet]. [updated 2007 July]. Flagstaff (AZ). W. L. Gore & Associates, Inc. Available from: www.goremedical.com/stp/index.

Gregory, Carl, A., Reyes, Emigdio, Whitney, Mandolin, J., Spees, Jeffrey, L. "Enhanced Engraftment of Mesenchymal Stem Cells in a Cutaneous Wound Model by Culture in Allogenic Species-Specific Serum and Administration in Fibrin Constructs". Stem Cells. 24 (2006): 2232-2243.

Greling, D., Clark, R.A.F. "Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix". Journal of Cell Science. 110 (1997): 861-870.

Han, C., Zhang, X., Xu, W., Wang, W., Qian, H., Chen, Y. "Cloning of the nucleostemin gene and its function in transforming human embryonic bone marrow mesenchymal stem cells into F6 tumor cells". International Journal of Molecular Medicine. 16 (2005): 205-213.

Han, Seung-Kyu, Yoon, Tae-Hwan, Lee, Dong-Geun, Lee, Min-Ah, Kim, Woo-Kyung. "Potential of Human Bone Marrow Stromal Cells to Accelerate Wound Healing *in Vitro*". Annals of Plastic Surgery. 55 (2005): 414-419.

Horwitz, Edwin, M. "Mesenchymal Cells Information Summary". (2002).

Huang, Jau-Wen, Chen, Wen-Jer, Liao, Shuen-Kuei, Yang, Chuen-Yung, Lin, Song-Shu, Wu, Chi-Chuan. "Osteoblastic Differentiation of Rabbit Mesenchymal Stem Cells Loaded in a Carrier System of Pluronic F127 and Interpore". Chang Gung Med Journal. 29 (2006): 363-72.

Im, Gun-Il, Kim, Do-Young, Shin, Joo-Ho, Hyun, Cheol-Won, Cho, Won-Ho. "Repair of cartilage defect in the rabbit with cultured mesenchymal stem cells from bone marrow". Journal of Bone and Joint Surgery. 83(2001): 289-94.

Ito, K., Yamada, Y., Naiki, T., Ueda, M. " Simultaneous Implant Placement and Bone Regeneration Around Dental Implants Using Tissue-Engineered Bone with Fibrin Glue, Mesenchymal Stem Cells and Platelet-Rich Plasma". Clinical Oral Implant Research. 17 (2006) 579-586.

Juncosa-Melvin, N., Boivin, Gregory, P., Galloway, Marc, T., Gooch, Cynthia, West, John,

R., Sklenka, Angela, M., Butler, David, L. "Effects of cell-to-collagen ratio in stem cell-seeded constructs for Achilles tendon repair". Tissue Engineering. 12 (2006): 681-689.

Kuo, T., Hung, S., Chuang, C., Chen, C., Shih, Y., Fang, S., Yang, V., Lee, O. "Stem Cell Therapy for Liver Disease: Parameters Governing the Success of Using Bone Marrow Mesenchymal Stem Cells". Gastroenterology. 134(2008): 2111-2121.

LeBlanc, Karl, A. [Internet]. [updated 2008]. Technique Guide. Davol Inc. Cranston (RI). Available from:
<http://www.bardnordic.com/main/product.asp?sectionTypeId=2§ionId=19&productId=273>.

Lennon, Donald, P., Haynesworth, Stephen, E., Bruder, Scott, P., Jaiswal, Neelam, Caplan, Arnold, I. "Human and Animal Mesenchymal Progenitor Cells from Bone Marrow: Identification of Serum for Optimal Selection and Proliferation". In Vitro Cell Developmental Biology. 32 (1996): 602-611.

Lichtenstein, I.L. "The tension-free hernioplasty". The American Journal of Surgery. 157 (1989): 188-193.

Marie, Hazel. "Biomechanical and Elastographic Analysis of Wound Healing Using Mesenchymal Stromal Cell Treated Tissue Following Surgery." (2009).

Marie, Hazel, Fagan, Diana, L., Heffner, Jeremy. "Biomechanical Evaluation of Autologous Mesenchymal Stem Cell Transplantation to Improve Fascial Repair". Proceedings of the ASME 2009 International Mechanical Engineering Congress & Exposition.

Markowicz, M., Heitland, A., Steffens, G.C.M., Neuss, S., Pallua, N. "Human Mesenchymal Stem Cell Implantation and Collagen Modification as a Tool for Tissue Engineering". European Journal of Plastic Surgery. 28 (2005): 284-289.

Masi, L., Franchi, A., Santucci, M., Danielli, D., Arganini, L., Giannone, V., Formigli, L., Benvenuti, S., Janini, A, Beghe, F., Mian, M., Brandi, M.L. "Adhesion, Growth, and Matrix Production by Osteoblasts on Collagen Substrata". Calcified Tissue International. 51 (1992): 202-212.

Morgan, S.J., Darling, D.C. Animal Cell Culture. Bios Scientific Publishers. (1993).

Oreffo, Richard, O.C., Cooper, Cyrus, Mason, Christopher, Clements, Mark.

“Mesenchymal Stem Cells Lineage, Plasticity, and Skeletal Therapeutic Potential”.

Stem Cell Reviews. 1 (2005): 169-178.

Romo, T., Pearson, J., Yalamachili, Zoumalan, R. “Wound Healing”. (2008).

Rosch, R., Junge, K., Schachtrupp, A., Klinge, U., Klosterhalfen, B., Schumpelick, V. “Mesh

Implants in Hernia Repair: Inflammatory Cell Response in a Rat Model”. European

Surgical Research. 35 (2003): 161-166.

Rubio, D., Garcia-Castro, J., Martin, M.C., de la Fuente, R., Cigudosa, J.C., Lloyd, A.C.

“Spontaneous human adult stem cell transformation”. Cancer Research. 65 (2005):

3035-3039.

Schallmoser, Katharina, Bartmann, Christina, Rohde, Eva, *Reinisch, Andreas, Kashofer,*

Karl, Stadelmeyer, Elke, Drexler, Camilla, Lanzer, Gerhard, Linkesch, Werner, Strunk, Dirk.

“Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of

functional mesenchymal stromal cells”. Transfusion. 47 (2007): 1436-1446.

Scott, Jeffrey, R., Fraser, Jenna, M., Al-Beik, Jennifer, Roy, Vincent, Termin, Paul. "Bard CollaMend Implant: Mechanical and Biological Properties Following *in vivo* Implantation". (2007).

Solchaga, Luis, A., Johnstone, Brian, Yoo, Jung, U., Goldberg, Victor, M., Caplan, Arnold, I. "High Variability in Rabbit Bone Marrow-Derived Mesenchymal Cell Preparations". Cell Transplantation. 8 (1999): 511-519.

Sotiropoulou, Panagiota, A., Perez, Sonia, A., Salagianni, Maria, Baxevanis, Constantin, N., Papamichail, Michael. "Characterization of the Optimal Culture Conditions for Clinical Scale Production of Human Mesenchymal Stem Cells." Technology Development. Stem Cells. 24 (2009): 462-471.

Stacey, Alison. *Fundamental Techniques in Cell Culture*. Clevedon (UK). [Updated 2010]. Available at: <http://www.sigmaaldrich.com>.

Teixeira, S., Fernandes, H.M., de Boer, J., Ferraz, M.P., Monteiro, F.J. "Mesenchymal Stem Cell Proliferation and Morphological Analysis on Interconnected Macroporous Structures". Key Engineering Materials. 330-332 (2007): 1129-1132.

Tyrone, John, W., Marcus, Jeffrey, R., Bonomo, Steven, R., Mogford, Jon E., Xia, Yuping, Mustoe, Thomas, A. "Transforming Growth Factor B3 Promotes Fascial Wound Healing in a New Animal Model". Arch Surgery. 135 (2000): 1154-1159.

Vaananen, Kalev, H. "Special Section on Stem Cells: Review Article". Annals of Medicine. 37 (2005): 469-479.

Vicryl (polyglactin 910) woven mesh. [Internet]. [Updated 2009]. Ethicon, Inc. Cornelia (GA). Available at:
[http://www.medcompare.com/details/35889/VICRYL-\(polyglactin-910\)-woven-mesh.html](http://www.medcompare.com/details/35889/VICRYL-(polyglactin-910)-woven-mesh.html).

Wu, Yaojiong, Wang, JianFei, Scott, Paul, G., Tredget, Edwards, E. "Bone Marrow-Derived Stem Cells in Wound Healing: A Review". Wound Repair and Regeneration. 15 (2007): S18-S26.

Wu, Yaojiong, Chen, Liwen, Scott, Paul, G., Treget, Edward, E. "Mesenchymal Stem Cells Enhance Wound Healing Through Differentiation and Angiogenesis". Stem Cells. 25 (2007): 2648-2659.

Yan, Hui, Yu, Changlong. "Repair of Full-Thickness Cartilage Defects With Cells of Different Origin in a Rabbit Model". Arthroscopy: The Journal of Arthroscopic and Related Surgery. 23 (2007): 178-187.

Zarei, Abdolreza, M., Markovic, Boban. Refinement In the Use and Date Analysis of the Promega Cell Titer 96 AQ Non-Radioactive Cell Proliferation Assay. Available at: www.promega.com/enotes/applications/0004/ap0017.htm.

Zhu, Hui, Mitsuhashi, Noboru, Klein, Andrew, Barsky, Lora, W., Weinberg, Kenneth, Barr, Mark, L., Demetriou, Achilles, Wu, Gordon, D. "The Role of the Hyaluronan Receptor CD44 in Mesenchymal Stem Cell Migration in the Extracellular Matrix". Stem Cells. 24 (2006): 928-935.

Zimmerman, Bodo, K., Timpl, Rupert, Kuhn, Klaus. "Intermolecular Cross-Links of Collagen: Participation of the Carboxy-Terminal Nonhelical Region of the α 1-Chain". European Journal of Biochemistry. 35 (1973): 216-221.