The Dissertation Committee for Anesh Prasai certifies that this is the approved version of the following dissertation:

CHARACTERIZATION AND APPLICATION OF ADIPOSE DERIVED STEM CELLS FOLLOWING BURN INJURY

Committee:

Celeste Finnerty (Mentor)

David Herndon (Chair)

Darren Boehning

Marxa Figueiredo

Heinz Redl

Dean, Graduate School

CHARACTERIZATION AND APPLICATION OF ADIPOSE DERIVED STEM CELLS FOLLOWING BURN INJURY

by

ANESH PRASAI (B.S.)

Thesis

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Medical Branch

November, 2016

Dedication

Dedicated to my Buwa (Yadu Bhakta Prasai), Amaa (Jeevan Prasai) and Tuse (Kabita Upreti)

Acknowledgements

My undergraduate studies prepared me for graduate school, while my graduate studies prepared me to be independent. My experience at University of Texas Medical Brach (UTMB) has been a wonderful odyssey and this was only possible because of dedicated mentors, amiable and encouraging colleagues, and my patient family who surrounded me their advice and care during my difficult times.

First, I would like to thank Dr. Celeste C. Finnerty for her constant support, guidance, and encouragement during my time here at the UTMB and the Shriners Hospitals for Children. I can vividly remember the day when I stopped by her office to discuss my intentions of joining her laboratory. Two more students were behind me to visit her, and when I entered her office she was hiding behind the piles of research papers and getting back to work from her maternity leave. One could tell that she had a hectic schedule. Despite her busy schedules, all throughout my studies, Dr. Finnerty was always there to answer my questions or direct me to the right personnel. Dr. Finnerty's personal attributes of hard work, poise, and positive outlook towards life will always motivate me and I wish to strive for the rest of my life.

I'm equally thankful for Dr. David N. Herndon's insightful comments and advice during my meetings and presentations. I feel extremely privileged to work with Dr. Herndon who has inspired me to give the best effort in what I do either in research or when taking care of a patient. If it was not for our past program director and my committee member, Dr. Darren Boehning, I would not be working at Dr. Finnerty's laboratory. After noting my penchant for tissue engineering, he thoughtfully directed me to Dr. Finnerty's lab and since then he has been a source of constant support and to-go person for any kind of help. I am indebted to Dr. Marxa Figueiredo, for her timely advice, understanding nature, invaluable advice during my presentations and meetings, and above all always putting the students first amidst her busy schedule. I am equally

grateful for Dr. Heinz Redl, who helped me polish my project during our committee meetings and during our short reunions at different tissue engineering and burn related conferences.

I would have been lost, if it was not for my colleague and supporter Dr. Amina El Ayadi, who shed a light of encouragement, passion, and knowledge all throughout my graduate studies. Her amiable nature and dedication to science is something I wish to strive for and learn in coming years. I wouldn't have come this far if people working behind the scenes like Michael Wetzel, Ye Yang, John Bergman and Jay Jason had not helped me with different aspects of the project. I will always be thankful for their willingness to help right from the start of this meaningful journey.

Lastly, I feel extremely fortunate and blessed for having loving and indulgent wife Kabita Upreti, who was the ample source of support and hot coffee outside my laboratory. Her understanding nature about the fact that research takes time helped me to be calm and be appreciative of what I have.

CHARACTERIZATION AND APPLICATION OF ADIPOSE DERIVED STEM CELLS FOLLOWING BURN INJURY

Publication No._____

Anesh Prasai, Ph.D.

The University of Texas Medical Branch, 2016

Supervisor: Celeste C. Finnerty

Abstract:

Severe burns induce a prolonged inflammatory response in subcutaneous adipose tissue that modulates signaling in adipose-derived stem cells (ASCs), which hold potential for healing burn wounds or generating skin substitutes. Using a 60% rat scald burn model, we investigated the cellular source of inflammation within adipose tissue and whether inflammation affects ASC fate and function. Endpoints included inflammatory marker expression, expression of ASC-specific cell-surface markers, DNA damage, differentiation potential, and proliferation. Inflammatory marker expression was induced in adipocytes and the SVF at 24 and 48 hours postburn; expression of inflammatory marker mRNA transcripts and protein returned to normal in the SVF isolated 1 week postburn. In enriched ASCs, burns did not alter cell-surface expression of stem cell markers, markers of inflammation, differentiation potential, or proliferative ability. These

results suggest that adipocytes and the SVF, not ASCs, are the main source of inflammation after burns and that ASCs are unaffected by burns or culturing procedures. Following characterization of post-burn ASCs, we evaluated whether topical application of ASCs improves burn wound healing in an ovine burn model. Topical application of ASCs significantly increased the size of the implanted graft, blood flow and vascular endothelial growth factor (VEGF) ($p< 0.05$) and Collagen-1 ($p<0.01$) protein expression in treated animals compared to the controls. Monocyte chemo-attractant protein-1 (MCP-1) mRNA expression was also greater with ASC treatment ($p< 0.05$). ASCs secreted a significant amount of MCP-1 compared to the different cell lines when challenged with lipopolysaccharide (LPS) or 2% burn serum. Human umbilical vein endothelial cells (HUVEC), when treated with 100ng/mL of MCP-1 or 2% of ASC, conditioned media (ASC-CM) increased the expression of MCP-1, VEGF-165 and HIF-1α mRNA transcripts compared to the controls. Fibroblast migration was significantly increased in the presence of ASC-CM or with recombinant MCP-1 peptide ($p<0.001$). ASCs constitutively produce an abundant quantity of MCP-1 and the expression of MCP-1 fluctuates depending upon the microenvironment. This work suggests that post burn ASCs or SVF can be used for cell-based therapy, and that the applied cells stimulate wound healing and angiogenesis pathway potentially via an MCP-1 mediated mechanism.

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Abbreviations:

α-SMA: Alpha-smooth actin muscle

ASCs: Adipose-derived stem cells

bFGF: Basic fibroblast growth factor

bMSC: Bone-marrow derived mesenchymal stem cells

BMPs: Bone morphogenetic proteins

CXCRs: Chemokine C-X-C motif receptors

CEA: Cultured epithelial autografts

ELISA: Enzyme-linked immunosorbent assay

EGF: Epidermal growth factor

FGFs: Fibroblast growth factors

FDA: Food and Drug Administration

GDNF: Glial cell-derived neurotrophic factor

GFP: Green fluorescent protein

GM-CSF: Granulocyte macrophage stimulating growth factor

GMP: Good manufacturing practices

GSBS: Graduate School of Biomedical Science

GVDH: Graft versus host disease

HGF: Hepatocyte growth factor

Hr: Hour

HTS: Hypertrophic scarring

HIFs: Hypoxia-inducible factors

IDO: Indoleamine 2,3 dioxygenase

IACUC: Institutional Animal Care and Use Committee

IFN: Interferon

IL: Interleukin

iNOS: Inducible NO synthase

ISCT: International Society for Stem Cell Therapy

Kda: Kilodalton

LPS: Lipopolysaccharide

MCP-1: Monocyte chemoattractant protein-1

Mg: Milligrams

Min: Minutes

mL: Milliters

^{o}C: Degrees celsius

MHC: Major histocompatibility complex

MMP: Matrix Metalloprotease

NBS: Normal Buffered Solution

MSCs: Mesenchymal stem cells

NK: Natural killer

NO: Nitric oxide

PBS: Phosphate Buffered Solution

PCR: Polymerase chain reaction

PEG2: Prostaglandin E2

PHBV: Poly-3-hydroxybutyrate-co-hydroxyvalerate

PDGF: Platelet-derived growth factor

ROS: Reactive oxygen species

S: Seconds

SDF-1: Stromal cell derived factor-1

SVF: Stromal vascular fraction

TIMP: Tissue inhibitor of metalloprotease

TIE-1: Tyrosine kinase with immunoglobulin-like and EGF-like domains 1

TBSA: Total body surface area

TGF-β : Transforming growth factor beta 1

TNF: Tumor necrosis factor

UTMB: University of Texas Medical Branch

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

WAT: White adipose tissue

Chapter 1: Introduction to adipose-derived stem cells (ASCs)

The eminent German biologist Ernst coined the term “stem cell” in 1868, to define the evolutionary process of unicellular organisms evolving to form multicellular organisms [1]. In 1886, the term stem cell was used to describe the parts of the plant that can grow and regenerate. From these two archaic observations alone, our basic understanding regarding stem cells can be summarized. First, stem cells can differentiate into multiple lineages; second, stem cells can proliferate and maintain their own population, a process known as “self-renewal”. In 1963, the field of stem cell biology took a huge leap when the James Till laboratory showed that different lineages of blood cells originate from special cells in the bone marrow [2]. The potential for stem cells to improve health became apparent in 1968 when a child suffering from a rare immune disease received a bone marrow transplant from his unaffected sister; this child remained healthy throughout adulthood [3]. Until fairly recently, researchers believed that stem cells were found solely in the bone marrow. With the advent of new cell isolation methodologies, and visualization technologies, stem cell-like properties were observed in a variety of tissues including teeth [4], liver [5], blood [6], bone [7], fat [8] and others [9]. Stem cells obtained from tissues such as these are now collectively referred to as adult stem cells. Although each type of the adult stem cell has a set of unique properties, adult stem cells as a whole have tremendous potential to combat various disease states. Here we focus on the adult stem cells isolated from the fat, referred to as adipose-derived stem cells (ASCs).

Zuk et al., at the University of California was the first to identify and characterize ASCs [10]. Initially, ASCs were referred to as processed lipoaspirate (PLA) cells, as the stem cells were isolated from lipoaspirate [10]. Other investigators modified the term PLA to describe the cell populations they were studying, such that confusion ensued with the use of terms such as mesenchymal stem cells derived from adipose tissue (MSADSC) [11], adipose-derived stem cells (ADSCs) [12], adipose-derived adult stromal cells (ADAS) [13], adipose stromal cells (ASC)[14], , pericytes [15], and pre-adipocytes [16]. The International Fat Applied Technology Society (IFATS) established "adipose-derived stem cells" ASCs as a consensus term to describe the cells isolated from subcutaneous fat that adhere to plastic, that are characterized by t the criteria established by the International Society for Cell-based Therapy [17].The term ASC is now used prefentially in place of the myriad names previous derived. In addition to creating unanimity among researchers, the consensus statement also defined the CD markers for ASC characterization, discussed in the characterization section of this chapter.

The discovery of adult mesenchymal stem cells in a plethora of tissues has opened the doors for variety of stem cell-based therapies for clinical use [18]. ASCs are similar to bone marrow-derived mesenchymal stem cells (MSCs). Like MSCs, ASCs can differentiate into adipogenic, chondrogenic, osteogenic, cardiomyogenic, neurogenic, and myogenic, -like cell types [19]. Furthermore, it is relatively easy to isolate up to 5,000 ASCs from one gram of adipose tissue [20-22]. Aside from their capability to differentiate into a different types of cell lineages, ASCs also secrete an abundance of cytokines and growth factors, including interleukin IL-6,7, 8, 11, 12, fibroblast growth factor (FGF), epidermal growth factor(EGF), monocyte chemoattractant protein-1 (MCP-

1) , keratinocyte growth factor(KGF), and macrophage colony-stimulating factor (MC-SF) [23, 24]. The production of these factors may account for the therapeutic effects attributed to ASCs in wound healing [25-27], tissue regeneration [28], angiogenesis [29], and immunomodulation [26].

Identification of ASCs:

As the selection and characterization of the ASC has evolved rapidly, initial studies may not have described the same populations. Great efforts have been made to standarize isolation methods and charaterization criteria. Many protocols have been established for the isolation of ASCs, as different researchers have modified and optimized protocols to isolate ASCs. Five steps are commonly found in the majority of these protocols : a)extensive wash of the tissue with normal buffered solution (NBS), b)enzymatic dissociation of tissue, c)red blood cell lysis, d) centrifugation and wash with NBS, and e)plating. For enzymatic digestion of the tissue, collagenase type 1A is most commonly used, with less wide-spread use of other enzymes such as dispase and hyaluronidase. Rodbell was the first to report the use of collagenase to isolate fat cells from adipose tissue [30] in 1964. Since then, different concentrations of collagenase, ranging from 0.2% to 0.05%, have been used for the isolation of ASCs [31]. Collagenase activity is inhibited, and digestion is ceased by adding an equal volume of ~10% serum containing media. The dissociated cells are then centrifuged (~ 300 G), resulting in three distinct layers: top layer is composed of a lipid layer, while middle layer is the supernatant, and the stromal vascular fraction (SVF) settels at the bottom. The lipid layer is composed of mature adipocytes and lipid droplets, while the SVF is composed of different types of

cells including endothelial cells[32], fibroblasts [33], hematopoietic cells [34], pericytes [35], preadipocytes [36], and adipose-derived stem cells [10].

Culturing the SVF gives rise to an enriched population of ASCs. Because freshly isolated SVF contains many types of cells, the isolation of a particular cell population becomes the next challenge. Selection of special media and manipulation of the culture environment can be used to reduce the cell types being cultured from the SVF [10].

Characterization of ASCs: A panel of cell surface markers is used to define the ASCs and the SVF. For SVF, the most commonly accepted panel of cell surface markers inculde the presence of $CD34^+$ $CD73^+$, $CD90^+$ and $CD105^+$, and the absense of $CD31^-$, $CD45^-$, and $CD235^-$. While for ASCs, the recommended panel includes the presence of $CD36^+$, $CD73^+$, $CD90^+$, $CD105^+$, and $CD106^+$, and absence of $CD106^-$, as described by the International Federation for Adipose Therapeutics and Science (IFATS) [17]. The characterization of ASC, by IFATS encompasses the characterization criteria proposed by greater body of stem cells committee, International Society for Cell-Based Therapy (ISCBT)

ISCBT states that in order to be classified as an stem cell in general, three criteria must be met [17]. Firstly, the cells need to adhere to the plastic cell culture ware. Secondly, cells must be able to differentiate into at least three different lineages namely adipogenic, chondrogenic, and osteogenic. Lastly, the characterized cells must be positive for cell surface marker expression including CD90, CD73, and CD105 and negative for cell surface expression for CD11b, HLA-DR, and CD45. In the absence of a unique set of cell surface markers that are specific for ASCs, these three criteria are applied to ensure that

the same cell population is being isolated and studied. Difficulty in identifying unique cell surface markers for ASCs is compounded by the fact that ASCs share many markers associated with endothelial cells, pericytes, preadipocytes, and even fibroblasts [37]. Furthermore, significant variability of cell surface marker expression by ASCs occurs in relation to the donor age [38, 39] and comorbidities [40], further complicating the comparison of ASCs from multiple individuals. Additional cell surface markers have been proposed for the identification of ASCs (Table 1.1).

Table 1.1: **ASC cell surface markers**

Reference	**Positive markers**	**Negative markers**	**Cell type SVF or ASCs**
Zuk et al.,[8]	$CD44^+$, $CD29^+$, $Stro\text{-}1^+$, 49d (α4 integrin), $CD105^+$	$CD34^-$, $CD106^-$	ASCs
Baer et al.,[41]	No $CD36^+CD34^+$double positive cells but ($CD36^+$ $CD34^-$) and ($CD36^-$ $CD34^+$) populations	$CD36^-$, $CD34^-$	ASCs
Bagloni et al., [42]	$CD34^+$ $CD106^+$	$CD14^-$, $CD31^-$	ASCs
Varma et al., [43]	$CD34^{++}$, $CD117^+$ and HLA-DR^+	$CD34^-$	ASCs
Yoshimura et al., [44]	$CD31^{+/-}$, $CD34^{+/-}$, $CD45^{+/-}$, $CD90^{+/-}$, $CD105^{+/-}$, and $CD146^{+/-}$	$CD45^-$, $CD105^-$, $CD146^-$, $CD31^-$ $CD117^-$	ASCs
Mizuno [45](Review)	$CD9^+$, $CD13^+$, $CD29^+$, $CD44^+$, $CD49d^+$, $CD49e^+$, CD54, $CD55^+$, $CD59^+$, CD73, $CD90^+$, $CD105^+$, $CD106^+$, $CD146^+$, $CD166^+$, HLA I, $Fibronectin^+$, smooth muscle cell-specific alpha actin, $Vimentin^+$, and $Coll\text{-}I^+$	$CD11b^-$, $CD14^-$, $CD19^-$, $CD31^-$, $CD34^-$, $CD45^-$, $CD79a^-$, $CD80^-$, (c-kit)$CD117^-$, $CD133^-$, CD144, HLA-DR, $MyD88^-$, $STRO\text{-}1^-$, and HLA II^-	ASCs
Brzoska et al., [46]	$CD10^+$ $CD13^+$, $CD44^+$, and vimentin+	$CD31^-$, $CD34^-$, $CD45^-$, vWF^- (von Willebrand factor)	ASCs
Lin et al.,[47]	ASCs Located around the niche of vascular region of	$CD34^-$ with culture	SVF and ASCs

	$CD31^+$, $CD34^+$, α-SMA^+, Wnt5a, OCT4, telomerase, $SSEA1^+$, and $STRO\text{-}1^+$		
Traktuev et al., [48]	$CD34^+$, and α-SMA^+	$CD31^-$	ASCs
Araña et al [49]	$MHC\text{-}1^+$, $MHC\text{-}II^{+/-}$, and $CD29^+$	$CD117^-$, $CD31^-$, $CD34^-$	ASCs
Buschman et al., [50]	$CD13^+$, $CD29^+$, $CD44^+$	$CD11b^-$,$D62L^-$, $ICAM\text{-}1^-$ $CD34^-$	ASCs
Katz et al.,[51]	H L A-ABC^+, $CD29^+$, $CD49e^+$, $CD51^+$,$CD49b^{+/-}$,$CD49d^{+/-}$, $CD61^{+/-}$, $CD138^{+/-}$, and $CD140a^{+/-}$	$HLA\text{-}DR^-$, $CD4^-$, $CD8a^-$, $CD11a^-$, $CD11b^-$,$CD11c^-$, $CD18^-$, $CD41a^-$,$CD49f^-$, $CD62L^-$,$CD62P^-$, $CD106^-$, $CD117^-$, $CD133^-$, $CD243^-$, $ABCG2^-$	SVF and ASCs
Baglioni et al., [42]	$CD34^+$, and $CD106^+$	$CD14^-$ and $CD31^-$	ASCs
Sousa et al *(review)[52]*	$CD9^+$, $CD13^+$, $CD29^+$, $CD44^+$, $CD49d^+$, $CD49e^+$, CD54, $CD55^+$, $CD59^+$, CD73, $CD90^+$, $CD105^+$, $CD106^+$, $CD146^+$, $CD166^+$, HLA I, $Fibronectin^+$, smooth muscle cell-specific alpha actin, $Vimentin^+$, and $Coll\text{-}I^+$.	$CD11b^-$, $CD14^-$, $CD19^-$, $CD31^-$, $CD34^-$, $CD45^-$, $CD79a^-$, $CD80^-$, (c-kit)$CD117^-$, $CD133^-$, CD144, HLA-DR, $MyD88^-$, $STRO\text{-}1^-$, and HLA II^-	ASCs
Locke et al *(review)*[53]	$CD9^+$, $CD10^+$, $CD13^+$, $CD29^+$, $CD44^+$, $CD49e^+$, $CD51^+$, $CD55^+$, $CD59^+$, $CD90^+$ and $CD166^+$	$CD11a^-$, $CD11b^-$, $CD11^-$, $CD14^-$, $CD16^-$, $CD18^-$, $CD31^-$, $CD45^-$, $CD50^-$, $CD56^-$, $CD104^-$ and HLA-DR^-	ASCs
Gimble et al [20]	$CD9^+$, $CD10^+$, $CD13^+$, $CD29^+$, $CD44^+$, $CD49^+$, $CD54^+$, $CD105^+$, $CD166^+$, $CD71^+$, $CD73^+$, and $CD90^+$	and $CD146^+$ and $CD11b^-$, $C14^-$, $CD16^-$, $CD18^-$, $CD31^-$, $CD45^-$, $CD50^-$, $D56^-$, $CD62^-$, $CD104^-$, and HLA-DR^-	ASCs
Brzoska et al., [46]	$CD10^+$ $CD13^+$, $CD44^+$, and $vimentin^+$	$CD31^-$, $CD34^-$, $CD45^-$, vWF^- (von Willebrand factor)	ASCs
Lin et al.,[47]	$CD31^+$, $CD34^+$, α-SMA^+, $Wnt5a^+$, $OCT4^+$, telomerase,	$CD34^-$	SVF

	SSEA1^{+}, and STRO-1^{+} and		
Traktuev et al., [48]	CD34^{+} and α-SMA^{+}	CD31^{-}	ASCs
Araña et al [49]	MHC-1$^{+,}$ MHC-II$^{+/-}$, and CD29^{+}	CD117^{-},CD31^{-}, and CD34^{-}	ASCs
Buschman et al., [50]	CD13^{+}, CD29^{+}, and CD44^{+}	CD11b^{-},D62L^{-}, ICAM-1^{-} and CD34^{-}	ASCs
Katz et al.,[51]	H L A-ABC^{+}, CD29^{+}, CD49e^{+}, CD51^{+} ,CD49b$^{+/-}$,CD49d$^{+/-}$, CD61$^{+/-}$, CD138$^{+/-}$, and CD140a$^{+/-}$	HLA-DR^{-}, CD4^{-}, CD8a^{-}, CD11a^{-}, CD11b $^{-}$,CD11c^{-}, CD18^{-}, CD41a^{-} ,CD49f^{-}, CD62L^{-} ,CD62P^{-}, CD106^{-}, CD117^{-}, CD133^{-}, CD243^{-}, and ABCG2^{-}	SVF and ASCs

Autocrine and paracrine activities of ASCs: Stem cell behavior is profoundly influenced by the extracellular niche occupied by the ASC and the growth factors that the stem cells interact with. The dynamic interaction between external stimuli and the stem cells creates signaling cascades that influence self-renewal, cell differentiation, and the production of a plethora of proteins. The external stimuli can include an extracellular matrix, direct cell-cell contact, soluble growth factors, or nutrient levels [54-56] [57].

Although, it was believed that stem cells applied to new areas, such as to the healing wound differentiated into needed cell types [58], there is now a growing consensus that the therapeutic properties of ASCs are derived from the proteins secreted by the stem cells [59-61]. A number of studies have characterized the ASC secretome (Table 2) [62, 63]. Despite the wide variety of stimuli used to elicit protein expression and secretion, and the methods used to profile the secretome of ASCs, the utility of the ASCs secretome for modulating angiogenesis [64], the immune response [65], and wound healing [66] are unambiguously reported in the literature (**figure 1.1** and **table 1.2)**.

Comparative listings of ASCs secretome identified by 2D gel and mass spectrophotometry techniques are reviewed by Kapur et al., [67]. In the study, the authors examined the lists of secretome published by 5 other different researchers and found that 68 proteins expression were common between at least three of the studies, while only 17 proteins were only common between the four of the studies. The difference was attributed to variables like kind of cells being analyzed and the method of secretome preparation and detection. There is an urgent need to unify the culture and characterization technique during the ASCs secretome analysis in order to get the similar results among the researches. In addition to unifying the isolation, culture and characterization, research scientist have also suggested development of effective algorithms to interpret the relationships between numerous data set that exits.

Table 1.2: **Components of the human ASC secretome**

Stimuli	**Growth Factor and Cytokines detected**	**Detection method**	**References**
Hypoxia for 24 hours	VEGF, HGF, GM-CSF, bFGF, and TGF-β	ELISA	Rehman et al., [64]
bFGF and EGF	HGF	ELISA	Kilroy et al., [23]
Lipopolysaccharide	GM-CSF, IL-6, 7,8, 11 and TNF-a	ELISA and RT-PCR	Kilroy et al., [23]
bFGF	VEGF, NGF, SCF and HGF	Bio-plex	Riberio et al., [68]
No stimulation	bFGF, HGF, IFN, IGF, IL-8, SDF-1, TNF-a, VEGF,	ELISA	Li et al., [69]
Co-culture with peripheral blood mononuclear cells	indoleamine 2,3-dioxygenase (IDO) activity and prostaglandin E_2 (PGE_2) and TGF-β PGE_2 and TGF-β	Kynurenine levels and ELISA	Lil et al., [69]
Spheroid formation	MMP9 and MMP13	Western Blot	Cheng et al., [70]
Ex-obese patients	MCP-1	ELISA	Silva et al., [71]

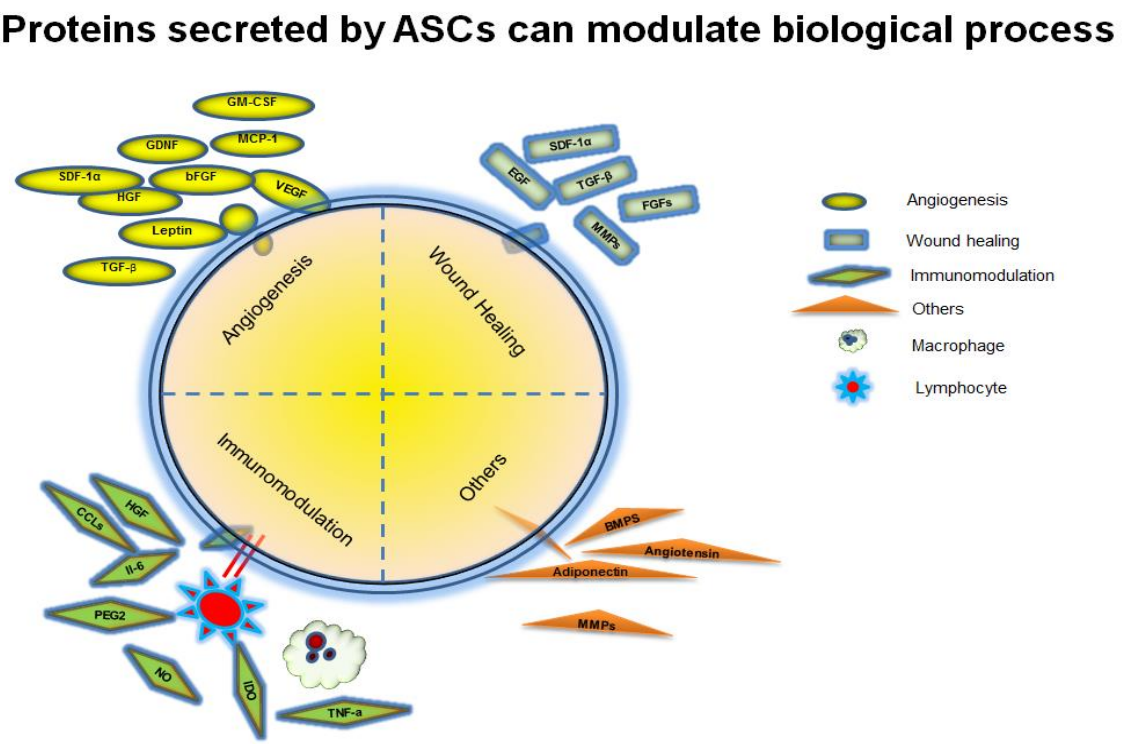


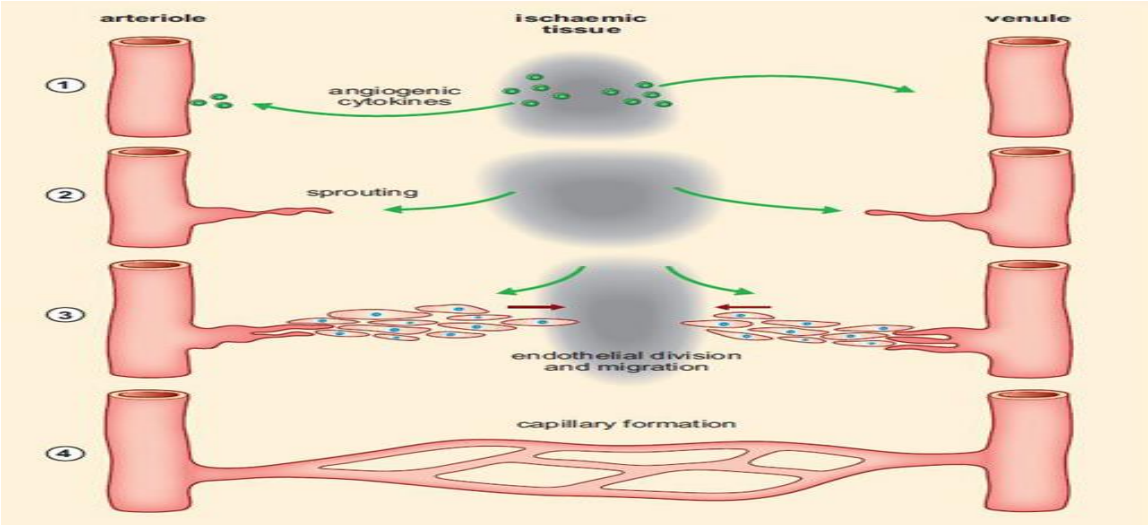
Figure 1.1: **Published secretome of ASCs**

Role of ASCs in wound healing, angiogenesis, and immune system

Angiogenesis:

Angiogenesis is a process of formation of *de novo* capillaries from the pre-existing vessels. Angiogenesis is vital process in all phases of wound healing, which ensures deliver nutrient and variety of cells that regulate the rate of wound healing. Angiogenesis can occur following injury or during the development of tumors or during different disease states. During this process, the cells at affected areas secrete cytokines and enzymes that stimulate the breakdown extracellular matrix, and growth factors such as VEGF and nitric oxide (NO) activates the resident endothelial cells. This leads to activation of sprouting cells including pericytes; endothelial cells proliferate and migrate

to the wound site and form a vessels in the tissues. These cells later organize and form an interconnection link to venules, completing the formation of new capillary network (ILL-1)[72-74].



Illustrations 1: Angiogenesis in ischemic tissue [74]
(Adapted from: http://www.open.edu/openlearn/science-maths-technology/science/biology/introduction-histopathology/content-section-1.7)

In the past, the adipose tissue was long regarded as a quiescent tissue that served as an energy reservoir. There is now an increasing appreciation of the adipose tissue as one of the major endocrine organs influencing processes throughout the body. Before the discovery of stem cells in adipose tissue, researchers were able to identify various angiogenic factors secreted by adipose tissue [75-77]. Thus, it comes to no surprise that ASCs residing in fat depots also have tremendous angiogenic properties and exert their effects through endocrine [78], paracrine [54-56], autocrine [57] and contact dependent pathways [48, 79, 80]. ASCs have been used to modify angiogenic signaling, thereby relieving symptoms attributed arthritis [81, 82], asthma [83], atherosclerosis [84], diabetes [85], osteoporosis [86, 87], cancer [88], obesity [89], brain ischemia [90], heart

ischemia [91, 92], and wound healing [57, 66, 93]. ASCs secrete pro-angiogenic factors including vascular endothelial factor (VEGF) [64], leptin [94], hepatocyte growth factor (HGF) [95], transforming growth factor (TGF-b) [96], fibroblast growth factor (FGF) [97], glial-derived neurotropic factor (GDNF) [98], platelet-derived growth factor (PDGF) [99], bone morphogenetic protein -6 BMP-6 [100], and stromal cell-derived factor-1α (SDF-1α) [97]. VEGF and leptin are the well-studied of these pro-angiogenic factors secreted by ASCs.

ASCs and VEGF: VEGF is one of the most well understood pro-angiogenic growth factors. By binding to tyrosine kinase VEGF receptors (VEGFRs) in the cell membrane, VEGF induces transphosphorylation of the receptor, resulting in vasculogenesis and angiogenesis. The dynamic molecular interactions between ASCs and VEGF have been well-described. Rehman et al. showed that ASCs secrete an abundant amount of VEGF under hypoxic conditions, and that application of conditioned media (CM) obtained from hypoxic ASCs could significantly increase endothelial cell proliferation [64]. In the same study, perfusion of ischemic hindlimbs [64] was improved with application of the ASCs. Similar results were obtained by either ASCs or ASCs CM application in other models of ischemia [101] ,[102] , [103] , [104] , [105] , [106] [97]. Similar to published studies, we also studied the angiogenic effect of ASCs on ovine graft burn wound healing model. Our experiments showed an increase in blood flow in the wounds treated with ASCs. We also demonstrated increased in the expression of VEGF via western blots and ELISA in following the application of ASC (described in **chapter 3**).

ASCs and Leptin: Adipose tissue is regarded as one of the major sources of leptin, thus it comes to no surprise that ASCs secrete abundant quantities of leptin [94, 107]. A recent study suggests that leptin secreted by ASCs acts as a potent angiogenic factor by up-regulating the expression of VEGF receptor R1 (VEGFR-1) and Tyrosine kinase with Immunoglobulin-like and EGF-like domains 1 (Tie-1), two pro-angiogenic receptors, via HIF2-α, but not HIF1-α [94]. In the study, when ASCs were transiently stimulated with hypoxia, leptin mRNA expression was unregulated 30-fold compared to controls, and human umbilical vein endothelial cells (HUVECs) cell lines treated with hypoixa conditioned media had a greater tube formation cells as measured by angiogenesis assay.

In another study, researchers observed that in a combination of hypoxia and leptin, ASCs differentiated into $CD31^{+}$ vascular endothelial cells with expression of nitric oxide synthase mRNA and increased endothelial cell markers including: vascular endothelial VE-Cadherin, vascular endothelial growth factor receptor 2 (VEGFR2), Tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie-2), and von Willebrand factor [107].

During angiogenesis these factors and others guide the breakdown and remodeling of basement membrane and connective tissue. Adipose tissue [108] in general and ASCs in particular [109, 110]have been shown to secrete various proteases and metalloproteinases (MMPs) that are vital for the formation of vascular networks. Song et al., demonstrated that pre-culture of ASCs embedded in collagen type I hydrogel significantly increased the invasion of human umbilical vein endothelial cells (HUVECs) by increasing proteolytic ECM degradation via the action of secreted metalloproteinases (MMPs) rather than VEGF production [111].

Wound healing:

ASC holds great promise in wound healing. Wound healing can be categorized into three dynamic but distinct phases: a) inflammation, b) proliferation, and c)tissue remodeling and maturation. Following an injury, the tissue responds to the injury or insult by constricting the blood vessels and by forming a platelets clot to cease the bleeding. Once the blood flow is ceased, blood vessels dilate and the passage of nutrients, white blood cells, antibodies, enzymes, and other cell types (including stem cells) to an injured area. Inflammation initiates at this stage. Following inflammation, the wound begins to heal through formation of granulation tissue which is composed of fibroblasts and a mixture of extracellular matrix (ECM) like collagen, hyaluronan and elastin. Granulation tissue formation allows new blood vessels to replace damaged ones. The fibroblasts in the wound site aid in the development of new tissue. The last stage is the remodeling stage and can last up to 2 years. During this phase, the dermal tissue is replaced with the new fibroblasts, and other types of cells, angiogenesis ceases and the ECM gets remodeled [112]. ASCs have been shown to aid in all three stages of wound healing [70, 106, 113].

The ASCs extracted from the stromal vascular fraction (SVF) of adipose tissue are rich with angiogenic characteristics and enable cells to differentiate into vascular endothelial cells [114]. ASCs secrete growth factors that modulate the inflammatory response, angiogenesis, and can differentiate into various cell types. The secretory activity of ASCs suggests that there is a beneficial effect when injecting the ASCs in the wound site, which results in the modulation of the wound environment to favor the initiation of wound healing and tissue regeneration [70, 106, 113].

The beneficial effects of ASCs have been investigated in a variety of wound healing models [103] , [104] , [105] , [106]. In the murine excisional wound healing model, human ASCs seeded on a grafted acellular dermal matrix enhanced wound healing and angiogenesis by 7 days post-injury, and ASCs remained viable 2 weeks post grafting as shown by PKH dye staining [115]. This study underscores the important role of ASCs in neovessel formation and the potential use of ASCs in microangiopathies. In a murine soft tissue injury model, ASCs applied on a silk fibroin-chitosan scaffold significantly enhanced wound healing by 8 days and micro-vessel density by 2 weeks post-injury [116]. The engrafted cells expressed fibroblast and epithelial markers suggesting that the ASCs differentiate into fibroblasts, and endothelial and epithelial cells, as needed.

With increase in obesity and type-2 diabetes, non-healing chronic wounds are new clinical problem, with an incidence of 5 to 7 million cases per year in the United States alone [117]. Many of these wounds do not respond to existing therapies. The healing process in chronic wounds is usually perturbed by the perpetuation of one or more of the phases of wound healing; inflammation, proliferation, or remodeling. Chronic inflammation i the injured tissue creates a cascade that disseminates a non-healing state. MSCs regulate the inflammatory phase of wound healing by modulating IL-10 and IL-4 production, suppressing TNF-α, and blocking T-cell proliferation [118]. The proliferation phase is regulated via the secretion of various growth factors such as VEGF, HGF, and PDGF, and by recruitment of keratinocytes, dermal fibroblasts, and host stem cells [119]. The remodeling phase in non-healing wounds is longer than that of regular wounds. MSCs regulate the remodeling phases by controlling the production of TGF-beta and KGF, by regulating matrix metalloprotease MMPs and tissue inhibitors of

metalloproteinases TIMPS, and by regulating collagen deposition. In addition to MSCs, the effects of ASCs in diabetic wound healing has been investigated [57]. Using a Streptozotocin (STZ)-induced diabetes rodent model with full-thickness skin wounds, Kuo et al, reported that ACSs decrease inflammation, accelerate wound healing, and increase blood flow in diabetic wounds through stimulation of angiogenesis and increased tissue regeneration [57]. Both paracrine and autocrine mechanisms are involved in this process.

The use of ASCs derived from diabetic mice was also explored by AL-Fitsi et al, [120]. In their study, they show that the ASCs from animal models of advanced age and from type 1 and 2 diabetes have impaired stromal function as demonstrated by decreased levels of VEGF production, attenuated proliferation, and reduced tubulogenesis compared to normal animals. However, ASCs from aged diabetic mice still retain their angiogenic properties that can stimulate and improve neovascularization of ischemic tissues [120]. Using the rat model of type 2 diabetes and obesity, Kato et al developed a new cell-based therapy for use on a full thickness skin injury. Using fat from normal rats, they isolated ASCs and created a sheet of cells for transplant in to the wounds [121]. ASCs applied in this manner as an artificial skin accelerated wound healing and revascularization. Secreted angiogenic factors from ASCs in the perivascular regions were directly involved in these effects. To sum up, ASCs have been utilized to treat various types of wounds and large human clinical trials will further corroborate the use of ASCs in wound healing.

Immunomodulation:

Stem cells, in general, possess distinctive dual immunomodulatory properties, and can have either immune-suppressive or immune-stimulatory effects [122]. The immunomodulatory phenotypes make stem cells an ideal candidate for use as a cell-based therapy to ameliorate chronic or acute inflammation and autoimmune disorders [123-125] that arise from graft versus host disease (GVHD) [126], allergic abnormalities [83, 127, 128], and others disease conditions [93, 129]. Although there are a number of studies demonstrating the positive effects associated with the application of ASCs, the detailed mechanisms by which these cells modulate immune the system is still under investigation. ASCs are a comparatively new candidate for cell-based therapy compared to bone marrow derived mesenchymal stem cells (bMSCs). Similar to bMSCs, ASCs are immune privileged. Flow cytometry characterization have confirmed that both ASC and bMSC cultured cells do not express the complex HLA-DR (Human leukocyte Antigen-antigen D Related), a ligand for MHC II surface receptor, which plays a significant role in immunogenic response that mediates GVHD condition [43, 52, 130]. Although researchers have reported the expression of HLA-DR [43, 130], along with other hematopoietic related markers including CD11a, CD13, CD14, CD34, CD45, CD86, and CD166 in the SVF cell population [43, 44, 48, 130, 131], expression of these markers were either not present or were reported to be present in insignificant amount in the passaged cells. Passage of SVF enriches the ASC population while eliminating hematopoietic and other cell populations, hence cultured SVF and early passage ASCs are more responsive to T-cells in mixed lymphocyte reactions compared to late passage ASCs [130].

ASCs have been reported to modulate immune cells including macrophages [93, 132], B-cells [133, 134], T-cells [129, 135-137]and others [138-140].

ASCs and Macrophages: Adipose tissue is not only a reservoir for ASCs under normal physiological conditions, but also for a substantial number of resident macrophages referred to as adipose tissue macrophages (ATM) [141]. ATMs occurs with various pathophysiological conditions including obesity [141], diabetes-2 [142], and atherosclerosis [143]. ASCs are most likely to interact with resident ATMs and modulate the tissue homeostasis and disease conditions. Depending upon the tissue niche, macrophage polarization can alter between a pro- or an anti-inflammatory phenotype. M1 macrophages (classically activated) have a pro-inflammatory phenotype while M2 macrophages (alternatively activated) have an anti-inflammatory phenotype. Published studies have corroborated the established perception that as seen with other stem cells [144], ASCs can modulate the phenotype of macrophages, thus favoring the modulation of disease conditions [93, 132, 145, 146]. Most of the published investigations have demonstrated that either the application of ASCs or co-culture of ASCs with M1 macrophage switched the macrophage phenotype from M1 to M2 or suppressed the pro-inflammatory effects of M1 macrophages respectively, leading to a decrease of pro-inflammatory cytokine expression [132, 147]. In summary, ASCs can influence macrophage recruitment, attenuate the macrophage-associated inflammatory response, and modulate macrophage phenotype.

ASCs and Lymphocytes (B and T-cells): ASCs modulate specific and adaptive components of the immune system, including various lymphocyte subsets. Most of the

published studies of interactions between ASCs and B and T-cells describe the relationships that occur in autoimmune disease models or in Graft Versus Host Disease (GVHD) models [148-150]. Applications of ASCs are currently being explored for treating autoimmune diseases including autoimmune hearing loss [136], encephalomyelitis [151, 152], arthritis [134], systemic lupus erythematosus [153], thyroiditis[154], and Crohn's disease [155]. However, few research studies have been directed towards understanding the role of ASCs in modulating inflammation pathways via lymphocytes in other inflammatory disease conditions like asthma, muscle disorders or other diseases besides autoimmune conditions and GVHD [148-150]. Although the exact mechanisms by which ASCs ameliorate autoimmune disease conditions have not been clearly elucidated, both indoleamine 2,3 dioxygenase (IDO) [156]and nitric oxide synthase (iNOS) [157] have been demonstrated to influence proliferation and protein secretion by B and T-cells. Increase in the activity and expression of the enzyme of the enzyme IDO results in the degradation of the essential amino acid tryptophan to kynurenine. Depletion of tryptophan creates an immunosuppressive microenvironment and influences the growth of microbes and T-cells [158]. The presence of IFN-γ and proinflammatory cytokines (e.g. TNF-α) which are primarily found in septic niches are known to increase inducible nitric oxide synthase (iNOS) in stem cells. The increase in nitric oxide (NO) inhibits the proliferation of T-cells. NO produced by stem cells suppresses Stat5 phosphorylation, a crucial pathway vital for T-cell proliferation [157]. In the same study, bMSCs obtained from inducible NOS-/- mice were not able to suppress T-cell proliferation, although bMSCs from wild type mice could [157]. Aside from IDO and NO, other soluble growth factors produced by ASCs, including hepatocyte growth

factor (HGF)[159-161], IL-10[162], IL-6[163], prostaglandin E2 (PEG2)[164, 165], and transforming growth factor β1 (TGF-β1)[166] can also modulate lymphocyte phenotype and function. Although the soluble factors secreted by stem cells can modulate lymphocyte populations, few studies have found no effect of stem cells conditioned media on lymphocyte. Some studies have suggested that CM from stem cells that are not activated has no effect on lymphocytes suggesting that activated stem cell secretes different protein required for the immunomodulatory effects [65, 167] . In summary, ASCs modulate lymphocyte populations via secretion of soluble factors, regulate the proliferation of lymphocytes, and change the cytokine profile from pro-inflammatory to anti-inflammatory, while inducing apoptosis of cytotoxic T-cells or activating regulatory T-cells.

ASCs and natural killer (NK) cells: ASCs are also able to modulate other lymphocytes such as NK cells [138]. A comparative study showed that ASCs modulate NK cells via the IDO pathway, in a manner similar to that found in bMSCs. Expression of the ligand for (DNAX Accessory Molecule-1) DNAM-1 was lower in ASCs, which were less prone to cell-mediated lysis by NK cells compared to bMSCs[168]. ASCs may have NK-like activity as well. In a nude mouse model, ASCs were converted to NK-like cells with anti-tumor properties [169]. In a model of GVHD, ASCs reduced NK cell proliferation and activity [170]. The published findings shed a light on the application of ASCs in modulating NK cells activity to heal tumor or GVHD conditions.

ASCs and Mast cells: Immature mast cells are $CD34^+$ cells produced in bone marrow and then migrate to the tissue where they develop into mature mast cells. Under normal physiological conditions, mast cells are found in most tissues including fat where they

live in harmony with nerve cells and blood vessels. Besides their prominent role in anaphylaxis and allergy , mast cells play a dynamic role in defense against pathogens, wound healing, angiogenesis and immune tolerance[171]. Liu et al., reported the presence of mast cells in white adipose tissue (WAT) where the mast cells participate in diet-induced obesity and glucose intolerance [172]. The WAT acts as a reservoir for mast cell progenitors in a murine model [140]. They are reported to stimulate migration and proliferation while suppressing differentiation of stem cells via PDGF pathway [173]. Mast cells are also found in freshly isolated SVF with CD117 positive expression [174]. ASCs decrease mast cell activity when applied to a pig model of wound healing, resulting in reduced scar formation [175]. Although few studies are conducted to study the interaction between mast cells and ASCs, their co-existing nature in adipose tissue implies that there may be a gap in knowledge in understanding dynamic interactions between two cells.

ASCs and GVHD: GVHD is the major causes of morbidity and mortality following a transplant[176]. The incidence of GVHD is reported to be between 30% and 60% and variables as the age or sex of the patient, source of stem cells, pre-existing disease conditions, and standard of care are recognized for their relationships to increasing the risk of GVHD[177, 178]. While the mortality rate remains as high as 50% [176], immunomodulatory properties of stem cells may help prevent the onset of GVDH [179] [180, 181]. Although the use of ASCs is new in the GVHD field, application of bMSCs was found to ameliorate GVHD in a phase I clinical trial in 1995. Of 23 patients treated with autologous bMSCs, 15 patients reported a beneficial effect [181]. This study was followed by several other small phase I, II, and III clinical trials that tested the use of

bMSCs to prevent GVHD; these studies reported a good response rate to the use of the stem cells despite the high risk-benefit ratio [182-184]. Although a number of *in vitro* and *in vivo* based models corroborate the use of ASCs in treating or preventing GVHD [126, 170, 179, 185], only a few human studies have been conducted in which ASCs were administered to treat GVHD. Of the five patients enrolled in the trial, three patients benefited from the therapy [186]. Although the therapeutic benefits to patients with GVHD are promising, larger studies with assessment of later outcomes are needed to determine whether application of ASCs is safe and efficacious. Large multi-center, randomized controlled trials will further delineate acute and long-term benefits of ASCs in treating GVHD.

Clinical applications:

Currently there are 193 studies registered at the clinicaltrials.gov for the application of cultured ASCs, or SVF in combination with fat tissue, bio-scaffolds or drug interventions [187]. According to this registry, the first clinical trial conducted with "adipose-derived stem cells", began in 2006. Since that time, the number of trials has increased exponentially ([187] **Figure 2**), the studies are subdivided based on their disease conditions. Less than one-fifth of the studies were conducted in the United States, probably due to the strict regulations by which clinical trials are run in the US compared to the Asian countries. The majority of studies using ASCs in human trials are either funded by an industry or university/organization.

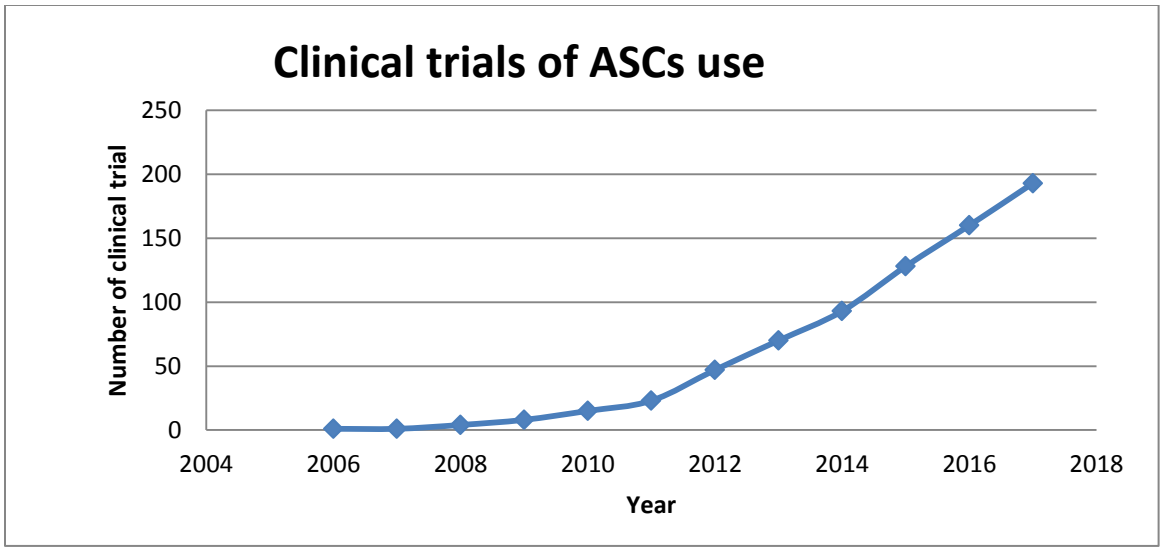


Figure 1.2: **Exponential increase in the clinical trials following the discovery of ASCs** *(Data compiled from clinicaltrials.gov)*

Clinical pplications of ASCs in the post-burn settings:

In 2013 alone, 35 million burn injuries were reported worldwide and resulted in approximately 2.9 million hospitalizations and 238,000 deaths [18, 188]. In patients with large burns, wound coverage options are limited, mainly due to the lack of donor sites. New approaches are needed to cover extensive burn wounds.

We and others believe that ASCs can be used to help heal burn patient. The early first phase, known as shock phase is characterized by hyper-inflammation, a response to tissue damage and necrosis. During this period, patients are challenged by the loss of skin, which helps in temperature regulations and defense from the pathogens. During this period, the burnt tissue is excised and discarded, to quell the inflammation arising from the necrotic tissue. The damaged skin needs to be replaced. The second phase, the hypermetabolic phase of burn injury is characterized by increase metabolic rate, temperature dysregulation, insulin resistance, muscle protein degradation, multi-organ

dysfunction, dampened growth and weak immune protection. This phase can last up to two years depending on the severity of the burn injury [189]. Reprioritization of energy needs can redirect amino acids from muscle tissue to support wound healing. Finally, ASCs can have a positive effect on the remodeling phase of burn injury. Hypertrophic scarring (HTS) is one of the life changing manifestations following the survival of burn injury [190]. Although hypertrophic scarring was characterized as early as 1893, there is a lack of research and little progress has been made in the treatment of this disabling condition [190, 191]. One of the major obstacles in studying HTS is the lack of a definitive animal model. A plethora of animal models including rats, mice, rabbit, sheep and several breeds of pigs have been proposed to study HTS [192-194], nevertheless the female Red Duroc pig is the best available option as the resultant scar is most similar to human HTS [195]. Yun IS showed that local application of ASCs reduces normal scar size and pliability in the Yorkshire pig[196]. At molecular levels, authors observed a decrease in mast cell activity and TGF-β pathway expression [175]. Aside from the changes in lymphocyte populations[196], one of the established characteristics of HTS is the increase in the ratio of type III collagen to type I collagen and a decrease in the activity of matrix metalloproteinases (MMPs) and collagenases [197-199]. Co-culture and conditioned media experiments have suggested that ASCs increase MMP1, MMP2 [113], and mRNA expression of type I procollagen α1 gene in dermal fibroblasts respectively[200]. In another full-thickness burn wound model, authors reported that application of ASCs in the combination with poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) matrix improved wound healing, increased VEGF, bFGF expression, down-regulated expression of TGF-β1 and α-SMA, and increased TGF-β3

mRNA expression – all of which resulted in an anti-scarring phenotype. Similar findings were reported in the full-thickness skin in a rat model, where injection of ASCs under grafted skin resulted in increased levels of VEGF and TGF- β3 and importantly, increased the ratio of collagen I to collagen III [201, 202]. Given these findings, ASCs will most likely have a beneficial role in controlling HTS by accelerating the rate of angiogenesis and wound healing, attenuating inflammation, and most importantly modulating the ECM matrix production following the burn injury. Young et al., [175] injected ASCs in to the wound 50 days post burn and after the scar started forming. ASCs were injected three times with a 10-day interval and the scars examined every 10 days until 100 days after surgery. Scars treated with ASCs had improved color and pliability, decreased scar area, and a reduction in the number of mast cells. ASCs promoted more mature arrangement of collagen in this model probably via the increase of TGFb3 and MMP1 in the early phase and the decrease of a-SMA and TIMP1 in the late phase of scar remodeling [175]. These data suggest that ASCs accelerate wound healing and reduce hypertrophic scarring through autocrine or paracrine regulation of different phases of wound healing or via reduction of fibrosis related pathways including the p38/MAPK signaling pathway as recently suggested [203].

The use of autologous adipose derived stem cells (ASCs) as a cell-based therapy to tackle different phases of burn injuries have has been suggested, however, limited studies have studied the effect of burn injury on the ASCs population. We and others believe that ASCs-based interventions can be highly beneficial for burn patient at different phases during the injury progression (**Figure 1.3)**.

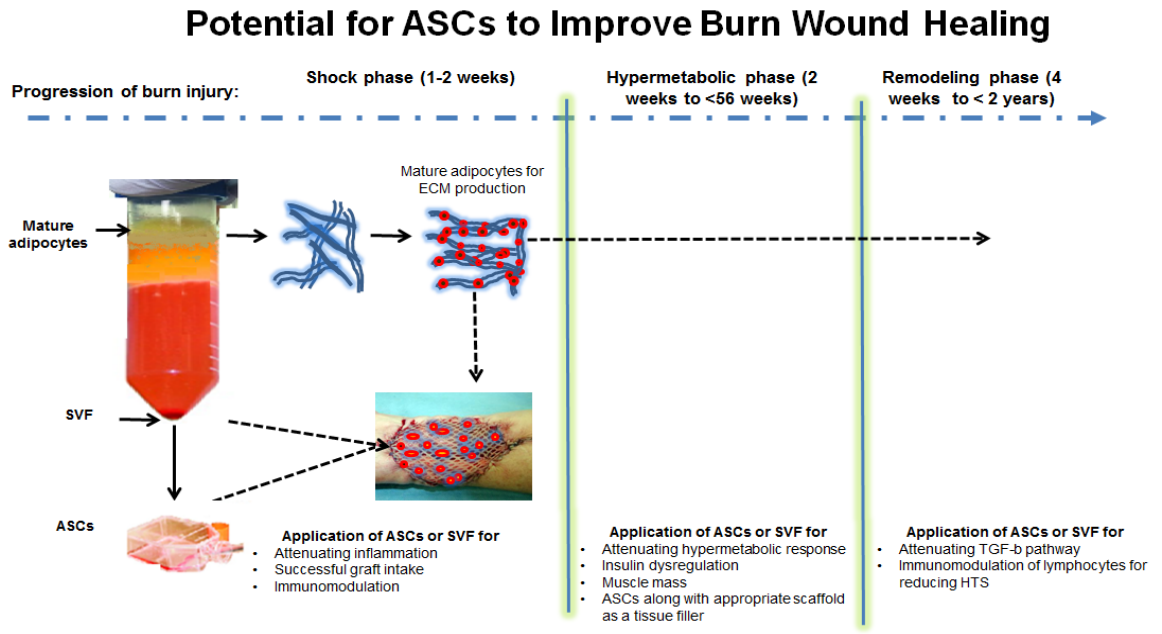


Figure 1.3: **Use of ASCs or SVF to heal burn-wound injury**

However, it is also unknown whether the ASCs isolated from the burn patient retain their stemness including cell surface markers, differentiation potential, proliferation, and cytokine expression. Additionally, although fat tissue is one of the major organs that contributes to inflammation, which component of fat tissue contributes to the inflammatory cascade and how long the inflammation persists in post-burn ASCs or the cells in the surrounding niche have yet to be determined.

These questions will be addressed in Aims 1 and 2 of this study.

<u>Hypothesis and aims</u>

We hypothesized that ASCs retain their stemness in culture following burn injury, and that these cells could be used for the cell-based therapy

We tested this hypothesis in two specific aims:

1. Identify which component of adipose tissue is the major contributor of the post-burn inflammatory response and determine how long the inflammatory cascade persists.

2. Determine whether the cell surface markers, differentiation potential, proliferation, and cytokine expressions are altered in post-burn ASCs.

The data from these two aims will delineate whether ASCs isolated from post-burn adipose tissue can be utilized to heal burn wounds.

Chapter 2: Characterization of adipose derived stem cells (ASCs) following burn injury

Background:

In 2013, 35 million suffered from burn-related injuries worldwide, which resulted in 2.9 million hospitalization and 238,000 deaths. While in the United States, approximately 2 million suffered from burn injuries and 500,000 patients were reported for hospitalization [18, 188]. Depending on the severity of the injury, burn injury is classified into five different categories namely first degree (1^{o}), second degree (2°A), second degree (2°B) third degree (3°) and fourth degree(4°). The 1^{o} and 2°A burn referred to as superficial and superficial partial thickness burn respectively do not require excision and heal usually within 2-3 weeks without scarring. However 2°B, 3° and 4° burn referred to as deep partial thickness burn, full thickness burn, and fourth-degree burn respectively requires a surgical excision and results in contractures, scarring, amputation, functional impairment, and even death.

Severe burn injury, > 40% of total body surface area (TBSA), results in the hypermetabolic, hyperglycemic and hyper-inflammatory state, which significantly increases morbidity and mortality of a burn patient. Following more than 2°B burn, the injured tissue is usually excised and discarded to curb the inflammation arising from the wounded tissue. The discarded tissue usually contains epidermis, dermis, and subcutaneous fat. In the fat depots lie adipose derived stem cells (ASCs). Clinician researchers have proposed that ASCs can be readily obtained from the subcutaneous adipose tissue that is discarded during debridement surgeries following a severe burn injury and that these ASCs can be used for wound healing or tissue regeneration [204-

206]. Additional opportunities for ASC isolation are present during subsequent operations that massively burned patients must undergo and uncover other subcutaneous fat depots. Because coverage options for large burn wounds are limited, autologous ASCs could be applied to severely burned patients to facilitate wound closure; this could be accomplished either directly after harvest of the stromal vascular fraction (SVF) or following manipulation of the enriched ASCs in culture to generate replacement grafts. We have recently shown that a severe burn injury induces a prolonged inflammatory response in subcutaneous adipose tissue isolated from unburned regions [207, 208]. The effects of this inflammatory environment on ASCs are of concern, as inflammatory conditions such as diabetes have been shown to affect the basic properties of MSCs; when transplanted after myocardium infarction, MSCs from diabetic patients have a lower proliferative capacity and a weaker myocardial protective effect than MSCs from non-diabetic patients [209]. Studies on many varieties of stem cells have shown that the fate and function of a stem cell are controlled by factors in the microenvironment of the surrounding niche [210, 211].

Severe burn injury induces a systemic hypermetabolic and inflammatory response that results in elevation of stress hormones and inflammatory cytokines, which can last for several years. Long-term perturbations also occur in non-burned muscle, fat, and skin [212]. In adipose tissue, these responses can last for at least a year [212]. As a result, ASCs that reside at the burn site, those that migrate to the burn site through the circulatory system, and those residing in adipose depots far away from the burn injury are all exposed to altered levels of cytokines and hormones, as well as growth factors

secreted in response to the burn injury. As the niche surrounding the stem cells defines the characteristics of these stem cells and can direct cell function and fate [211], the experiment was designed to determine which cells within the adipose tissue are the source of the inflammation and whether ASCs are affected by the post-burn inflammatory environment.

Using a 60% rat scald burn model, inflammatory responses of adipocytes, the SVF (comprised of multiple cell types including ASCs, inflammatory cells, and pre-adipocytes), and enriched ASCs were analyzed. Endpoints studied in the ASCs included differentiation potential, proliferation, cell surface cluster of differentiation (CD) and inflammatory marker expression, and DNA damage. I also determined whether the time of adipose collection post burn yields ASCs with differing properties, as much of our previous work shows a temporal pattern for the burn-induced elevation of inflammatory mediators both systemically and within adipose tissue.

Materials and methods:

Rat Model of Burn Injury

All animal experiments adhered to the guidelines detailed in the NIH Guide for the Care and Use of Laboratory Animals. The study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX). Male Sprague-Dawley rats weighing 250 g were housed in an animal facility with a 12-hour light and dark cycle for one week to acclimate prior to the initiation of the experiment. All animals received water and food ad libitum and were monitored for the entire study period. Eight animals were included in the control group, while 6 animals were included in each burn group. Prior to the burn injury, 0.05 mg/kg

buprenorphine hydrochloride was administered as analgesia, followed by 40 mg/kg ketamine with 5 mg/kg xylazine (IP) as anesthesia. A 60% total body surface area scald burn was introduced in the manner previously described [213, 214]. Resuscitation was accomplished by intraperitoneal injection of 60 mL/kg Ringer's lactate solution. Buprenorphine hydrochloride was administered every 12 hours to relieve discomfort. Rats were euthanized at 24 hours and 48 hours as well as at 1, 2, and 4 weeks following burn injury. Subcutaneous adipose tissue (0.9–3.0 g) was obtained from burned rats and sham-treated control rats for isolation of adipocytes, SVF, and/or ASCs at these time points.

ASC Isolation

Following its removal, adipose tissue was washed extensively with phosphate-buffered saline (PBS) containing 5% penicillin/streptomycin. The tissue was then minced and incubated with 0.075% collagenase Type IA at 37^{o}C for 60 to 80 minutes with constant shaking. An equal volume of complete media (Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, and 2% antibacterial/antimycotic solution [10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL amphotericin, 8.5 g/L sodium chloride]) was used to inactivate the collagenase. The solution was aspirated and centrifuged at 350 g for 5 minutes; this process separates the cells from the adipose tissue. The layer floating on the top was composed of mature adipocytes; these cells were removed and stored in RNA lysis buffer until analysis by real-time polymerase chain reaction (PCR). The pellet at the bottom of the tube was the SVF, comprised of endothelial cells, fibroblasts, ASCs, immune cells, and other cells. This SVF pellet was reconstituted with PBS and centrifuged at 350 g for 5 minutes. This step was repeated 3 to 4 times until the

supernatant became clear. The pellet was washed with water, and 10X PBS was added to lyse the red blood cells. After these steps, the pellet was resuspended in complete media and filtered through a 70-μm cell strainer. The resulting mixture was divided into two aliquots. The first half was labeled SVF; these cells were preserved in RNA lysis buffer and stored for later analysis. The remaining aliquot was plated on two 100-cm dishes to isolate enriched ASCs. After an 18 hour incubation, the media was aspirated to remove any unattached cells and debris, and complete media was added to the culture dishes. Following 30 additional hours of incubation, one plate of enriched ASCs was treated with RNA lysis buffer, and the lysate was stored for further analysis. The ASCs in the remaining plate were expanded in culture until the fourth passage was reached, and the cells were then used for further experimentation.

Differentiation

ASCs were grown for 4 weeks in complete media with the following components added to induce differentiation into each indicated cell type (**Table 2.1).**

Cell Lineages	Differentiation media
Osteogenic	0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM β-glycerolphosphate, 0.1 μM retinoic acid
Adipogenic	1 μM dexamethasone, 10 μM insulin, 0.5 mM isobutyl-methylxanthine, 200 μM indomethacin
Chondrogenic	6.25 μl/mL insulin, 10 ng/mL TGF- β, 50 nM ascorbate-2-phosphate, 2% fetal bovine serum
Epithelial cells	10 μM all-trans retinoic acid

Table 2.1: Differentiation media composition

Cells were then harvested and fixed for histology or immunocytochemistry. Alternatively, RNA was isolated for real time PCR. Osteogenic cells were stained using alizarin red, adipogenic cells with oil-O-Red, and chondrogenic cells with alcian Blue.

Epithelial cells were subjected to immunohistochemistry for CK-14. Nuclei were visualized with 4',6-diamidino-2-phenylindole.

Real Time PCR

The RNA was isolated using the RNeasy mini kit (Qiagen, Chatsworth, CA) following to the manufacturer's instructions. RNA concentration was quantified using the NanoDrop method (NanoDrop Technologies, Wilmington, DE); reverse transcription reactions were performed with 500 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Laboratories, Hercules, CA). Real-time PCR was performed using SYBR green, while the Step One plus real time PCR system (ThermoFisher Scientific, Waltham, MA) was used for amplification and data collection. PCR conditions were as follows: 95^{o}C for 10 minutes, 40 cycles at 95^{o}C for 15 seconds, and 55^{o}C for 30 seconds. The primer sequences are listed in **Table 2.2**. The delta delta CT method was used to quantify gene expression, which was then normalized to expression of the internal housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cyclophilin A.

Gene	**Forward Primer**	**Reverse Primer**
Adiponectin	5′-AAT CCT GCC CAG TCA TGA AG-3′	5′-GTC CCC TTC CCC ATA CAC TT-3′
ALPB-1	5′-TAAGGGTGACCCAGGAGATG-3′	5′-GGA ACATTGGGGACAGTGAC-3′
Osteonectin	5′-CTGCCACTTCTTTGCGACCA-3′	5′-CTCCAGGCGCTTCTCGTTCTC-3′
Osteopontin	5'-CTG GCA GTG GTT TGC CTT TGC C-3'	5'-CGT CAG ATT CAT CCG AGT TCA C-3'
ChM1	5'-GTGGTCCCACAAGTGAAGGT-3'	5'-TCGACCTCCTTGGTAGCAGT-3'
Collagen II	5'-GAACAACCAGATCGAGAGCA-3'	5'-CTCTCCAAACCAGATGTGCT-3'
CD11b/c	5'-CTGGGAGATGTGAATGGAG-3'	5'-ACTGATGCTGGCTACTGATG-3'
CD73	5'-TCAAATCTGCCTCTGGAAAG-3'	5'-TTCCCCTACCCACTACCTTC-3'
CD90	5'-AGCCAGATGCCTGAAAGAGA-3'	5'- TGATAGAAGGGGGCTGAGAA-3'
CD34	5'-TCTTGGCCAATAGCACAGAACT-3'	5'-TGCAATCAGAGTCTTTCGGGAA-3'
CD105	5'-CTGGAGCAGGGACGTTGT-3'	5'-GCTCCACGCCTTTGACC-3'
Cycophilin A	5'-TATCTGCACTGCCAAGACTGAGTG-3'	5'-CTTCTTGCTGGTCTTGCCATTCC-3'
CK-10	5'-TGGTTCAATGAAAAGAGCAAGGA-3'	5'-GGGATTGTTTCAAGGCCAGTT-3'
CK-14	5'-GGCCTGCTGAGATCAAAGACTAC-3'	5'-CACTGTGGCTGTGAGAATCTTGTT-3'
IL-1β	5'-CACCTTCTTTTCCTTCATCTTTG-3'	5'-GTCGTTGCTTGTCTCCTTGTA-3'
IL-6	5'-CGAGCCCACCAGGAACGAAAGTC-3'	5'-CTGGCTGGAAGTCTCTTGCGGAG-3'

Caspase-1	5’-CACATTGAAGTGCCCAAGCT-3’	5’-TCCAAGTCACAAGACCAGGC-3’
MCP-1	5’-GTTGTTCACACTTGCTGCCT-3’	5’-CTCTGTCATACTGGTCACTTCTAC-3’
NF-kb	5’-GTGCAGAAAGAAGACATTGAGGTG-3’	5’-AGGCTAGGGTCAGCGTATGG-3’
TNF-alpha	5’-TCAGCCTCTTCTCATTCCTGC-3’	5’-TTGGTGGTTTGCTACGACGTG-3’
CD= Cluster of Differentiation, ALBP-1 = adipocyte lipid binding protein-1, ChM1 = Chondromodulin-1		
CK = Cytokeratin		

Table 2.2: Primers for CD markers characterization, differentiation, and inflammation

Flow Cytometry

Fourth passage ASCs were cultured in complete media until sub-confluent prior to flow cytometric analysis (Becton Dickinson FACSCanto cytometer, Franklin Lakes, NJ). Media was removed by washing the ASCs with PBS. The ASCs were then harvested with 0.25% trypsin/EDTA. Cell viability was assessed via propidium iodide staining. Determination of fluorescent cells, dead cells, debris, and background noise was made with FACS DIVA software (Becton Dickinson, Franklin Lakes, NJ). For extracellular staining, the cells were treated with a mixture of ice-cold 1% sodium azide for 30 minutes, washed with PBS, and incubated with 3% bovine serum albumin (BSA) for 30 minutes on ice. Following a PBS wash, aliquots of ASCs were incubated in BSA with the following monoclonal antibodies for flow cytometry: CD73-Alexa 488 (BD Biosciences, San Jose, CA), CD90-Alexa 488 (BioLegend, San Diego, CA), CD11b-Alexa 488 (BioLegend, San Diego, CA), CD34-PE (Santa Cruz Biotechnology, Dallas, TX), CD36-Alexa 488 (BioLegend, San Diego, CA), and CD29-PECy7 (BioLegend, San Diego, CA). For intracellular staining, cells were counted and fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature, washed three times with PBS, and lysed with 90% ice-cold methanol for 30 minutes. The lysed cells were incubated in 3% BSA for 30 minutes and treated with a monoclonal primary antibody to CD105-PE (Bioss Inc., Woburn, MA).

Proliferation

Cells were plated at a density of 5,000 cells per well in xCELLigence® plates (ACEA Biosciences, San Diego, CA). Cellular spreading, attachment, and proliferation were recorded every 15 minutes using the real-time cell electronic sensing (RT-CES)® system (ACEA Biosciences, San Diego, CA), which measures cellular proliferation based on impedance. Cell-sensor impedance was expressed as a random unit called the Cell Index. Data were recorded for 55 hours and then analyzed.

Alkaline Single Cell Micro Gel Electrophoresis (Comet) Assay

Comet assays were performed using the OxiSelect Comet Assay Slides according to the manufacturer's instructions (Cell Biolabs Inc., San Diego, CA). A cellular suspension in PBS (1×10^5 cells per mL) was mixed 1:10 with molten low-melting-point agarose at 37^oC. Seventy-five microliters of this suspension were then placed within each well on the specially prepared microscope slide provided with the kit. Slides were incubated at 4^oC for 15 minutes to allow the agarose to solidify and then were placed in lysis buffer and incubated for 30 minutes at 4^oC in the dark. Denaturation was achieved by transferring the slides to a pre-chilled alkaline electrophoresis buffer (300 mM sodium hydroxide, 1.0 mM EDTA) and incubating for 30 minutes at 4^oC in the dark. The slides were then transferred into a horizontal electrophoresis chamber (BioRad Inc., Hercules, CA) and subjected to electrophoresis at 1 V/cm (30 V, 300 mA) for 15 minutes. Following electrophoresis, slides were washed 3 times with water, once with 70% ethanol, and air-dried. The dried slides were stained with 100 µL of diluted Vista Green

DNA dye (Cell Biolabs Inc., San Diego, CA), and cellular DNA was visualized using an FITC filter-fitted microscope. Comet images were scored visually on a scale of 0 to 4 as described by Collins [215], with 0 representing no damage and 4 representing severe damage.

ELISA

To generate ASC-conditioned media, we cultured fourth passage ASCs in complete media until confluence and the old media was replaced with fresh. After three days, conditioned media (CM) were saved and centrifuged at 300 g for 5 minutes. The resulting supernatants were frozen. The cells were also collected and the total number counted prior to protein extraction and quantification. IL-6, MCP-1, TNF-alpha, and IL-1βeta were measured using ELISAs purchased from R&D Systems, Inc. (Minneapolis, MN).

Western Blot Analysis

Protein was isolated from ASCs at varying times using protein lysis buffer composed of 2 mL 5X lysate buffer (750 mM sodium chloride, 250 mM Tris, 5% Triton X100, 5 mM EDTA), 1 pill of protease inhibitor (Roche, Basel, Switzerland), 100 µL of phosphatase inhibitor cocktail (Sigma, St. Louis, MO), and 66.7 µL of phenylmethylsulfonyl fluoride in 8 mL of water. BCA protein assay was used to measure the total protein concentration, as per manufacturer's instructions (Pierce, Rockford, IL). Following protein measurement, around 40 µg of protein was quenched in buffer composed of 400 mM Tris (pH 6.8), 40% glycerol, 1% bromophenol blue, and 0.8 mL of beta-mercaptoethanol in 10 mL of water. The samples were resolved on a sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, CA) at 150 V for 1 hour. The proteins were then blotted onto a PVDF membrane for 90 minutes at 400 mA. Protein transfer to the membrane was

confirmed by Ponceau staining. The membrane was incubated in 3% BSA for 1 hour at the room temperature and incubated with a primary antibody to NF-κB (Abcam, Cambridge, MA; 1:1000) at 4^{o}C overnight. The blot was washed three times with Tris Buffered Saline T-Tween 20 for 10 minutes and incubated with the HRP-conjugated anti-rabbit secondary antibody (1:5000) for 1 hour at room temperature. The proteins were visualized using Enhanced chemiluminescence Fast Western blotting substrate (Thermo Scientific-Pierce, Rockford, IL) per the manufacturer's instructions. The membranes were then stripped and re-blotted with an anti-GAPDH rat antibody (Cell Signaling Technology, Danvers, MA; 1:1000) for 1 hour at room temperature and HRP-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:5000) for 1 hour at room temperature. The intensity of band was measured using the Image J 1.47 software package (National Institutes of Health, imagej.nih.gov/ij/download/).

Statistical Analysis

Analysis of variance with Tukey's test and paired or unpaired Student's *t* tests were used as appropriate. Data were expressed as the mean ± standard error of the mean, as indicated. Significance was accepted at $p < 0.05$.

Results

The SVF and Adipocytes Are Potential Sources of Post-Burn Inflammation within Adipose Tissue

Messenger RNA expression of inflammatory markers (IL-1β, IL-6, MCP-1, caspase-1, TNF-α, and NF-kB) was measured in freshly isolated adipocytes, the SVF, and enriched

ASCs (Fig. 4). A significant elevation in IL-1β mRNA occurred in adipocytes and the SVF at 24 and 48 hours following burn injury, compared to non-burned controls, ($p=0.037$, Fig. 1B). When compared to expression in non-burned controls, expression of IL-6 mRNA was significantly altered by burn injury ($p=0.009$). In adipocytes, IL-6 mRNA increased while in ASCs it decreased, both at 48 hours after injury ($p=0.009$, Fig. 4B). A significant decrease in MCP-1 mRNA expression was found at 24 and 48 hours post burn in enriched ASCs ($p=0.005$, **Fig. 2.1**). TNF-α mRNA increased significantly in adipocytes at 48 hours following burn injury ($p=0.05$, Fig. 4D). Burn injury did not induce changes in expression of caspase-1 or NF-κB mRNA in any of the cell types, regardless of the time point (Fig. 4E and 4F). In ASCs, protein levels of IL-6, MCP-1, TNF-α, IL-1β, and NF-κB were unaffected by burn injury (data not shown).

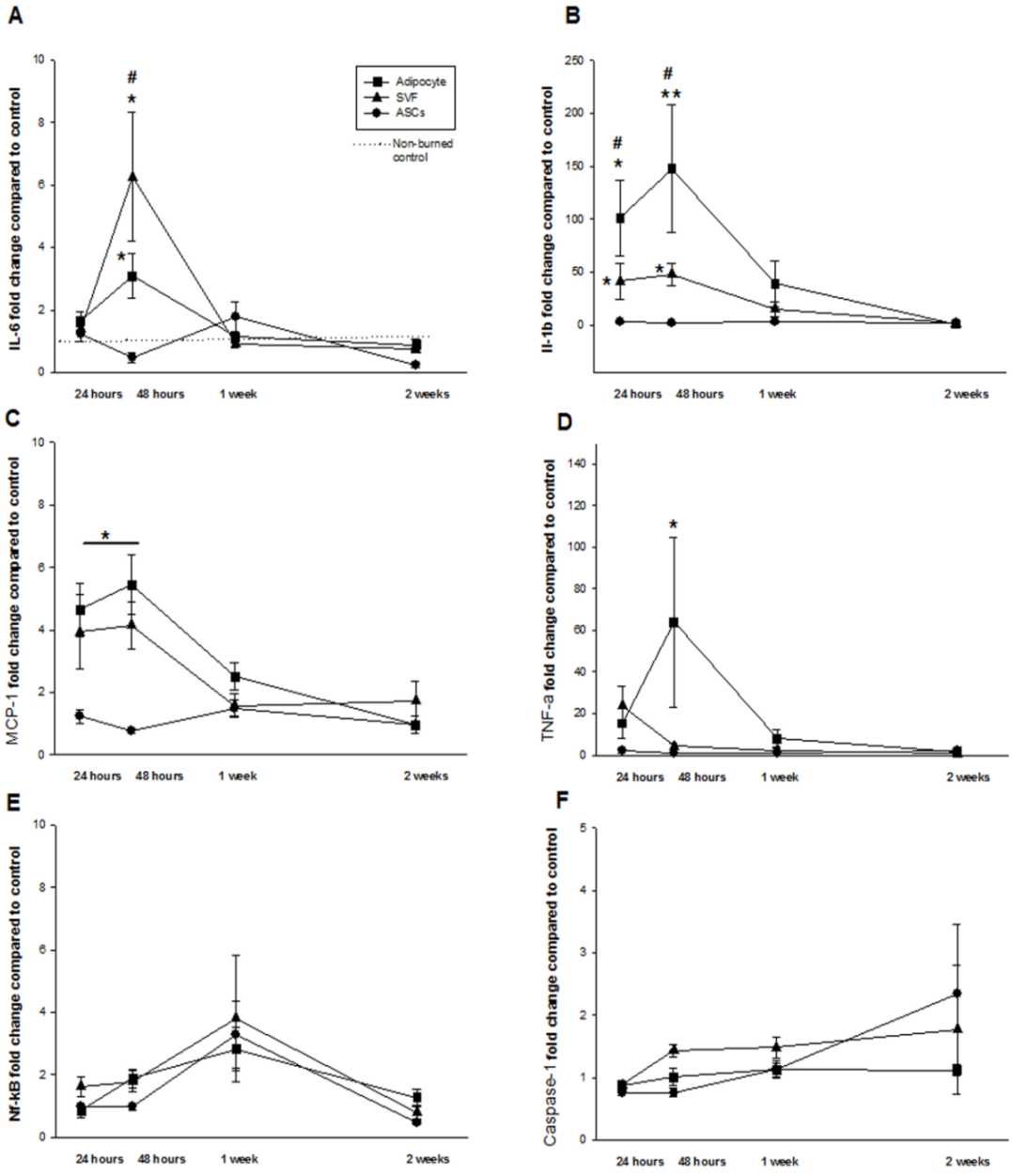


Figure 2.1: Effect of burn injury on cytokine and transcription factor mRNA production by adipocytes, the stromal vascular fraction (SVF), and enriched ASCs. Temporal alterations in expression of **A)** IL-6, **B)** IL-1β, **C)** MCP-1, **D)** TNF-α, **E)** caspase-1, and **F)** NF-κB are shown. Data points represent mean ± SEM of 8 control animals or 6 burned animals (24 hours, 48 hours 1, and 2 weeks post burn*$p < 0.05$ vs. control.

DNA Damage is Detected in the SVF Soon After Burn Injury but Resolves by 72 Hours Post Injury

DNA damage to the cells in the SVF and the enriched ASCs was assessed by comet assay. There was a significant induction of DNA damage in SVF isolated 24 and 48 hours post burn (p=0.05, p=0.005, respectively) (**Fig. 2.2**) compared to non-burned control. This amount of damage correlated to 4 damaged cells per 100 isolated cells. This damage resolved by 72 hours post burn. In cultured ASCs, the level of damage remained the same throughout the 4-week experimental period. Burn injury and subsequent culturing of the ASCs did not induce DNA damage.

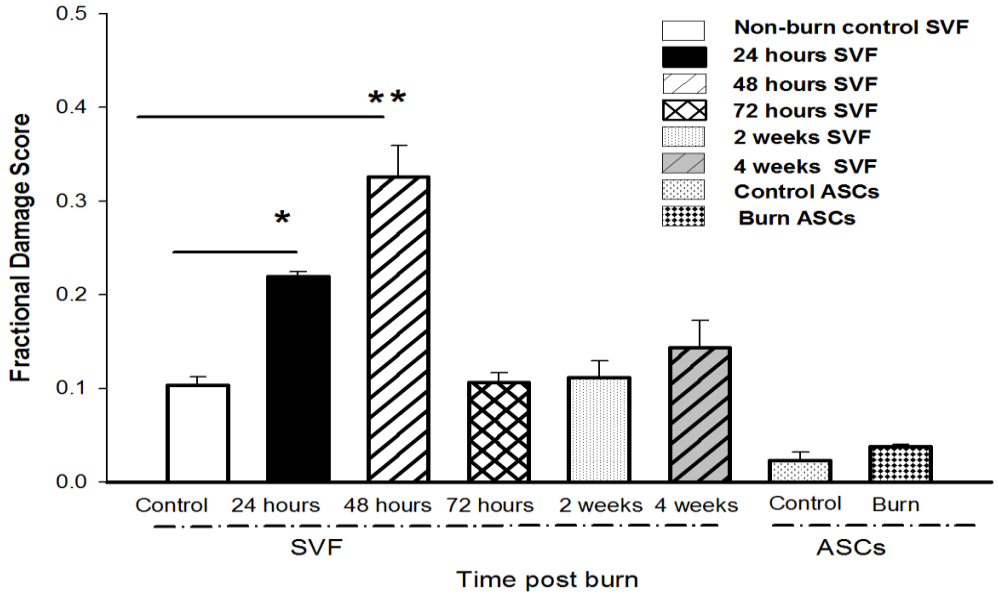


Figure 2.2: Burn injury induces minimal DNA damage in the stromal vascular fraction (SVF) and enriched ASCs. Each bar represents the mean ± SEM of 8 control animals or 6 burned animals (24, 48, or 72 hours, 1, 2, and 4 weeks post burn). *$p < 0.05$ and **$p < 0.005$ vs. control.

Burn Injury Does Not Alter the Differentiation Potential of ASCs

Following isolation and enrichment, ASCs were cultured in media formulated to induce differentiation into adipocytes, osteoblasts, chondrocytes, or epithelial cells. Differentiation into each of these cell types was confirmed by staining with oil O red (adipocytes), alizarin red (osteocytes), or alcian blue (chondrocytes) or by immunofluorescence staining for cytokeratin-14 (epithelial cells) (**Fig. 2.3**). ASCs from burn animals retained their differentiation capacity at all time points examined. The abundance of mRNA specific to each of the differentiated cell types was also measured. As shown in **Fig. 2.4**, we detected no significant differences between differentiated ASCs from non-burned and burned animals in levels of mRNA encoding factors involved in adipogenesis, (adipocyte lipid binding protein-1 and adiponectin [216]), chondrogenesis (chondromodulin 1 and collagen II [217, 218]), osteogenesis (osteopontin and osteonectin [219]), and epithelial differentiation (cytokeratin [CK]-10 and CK-14).

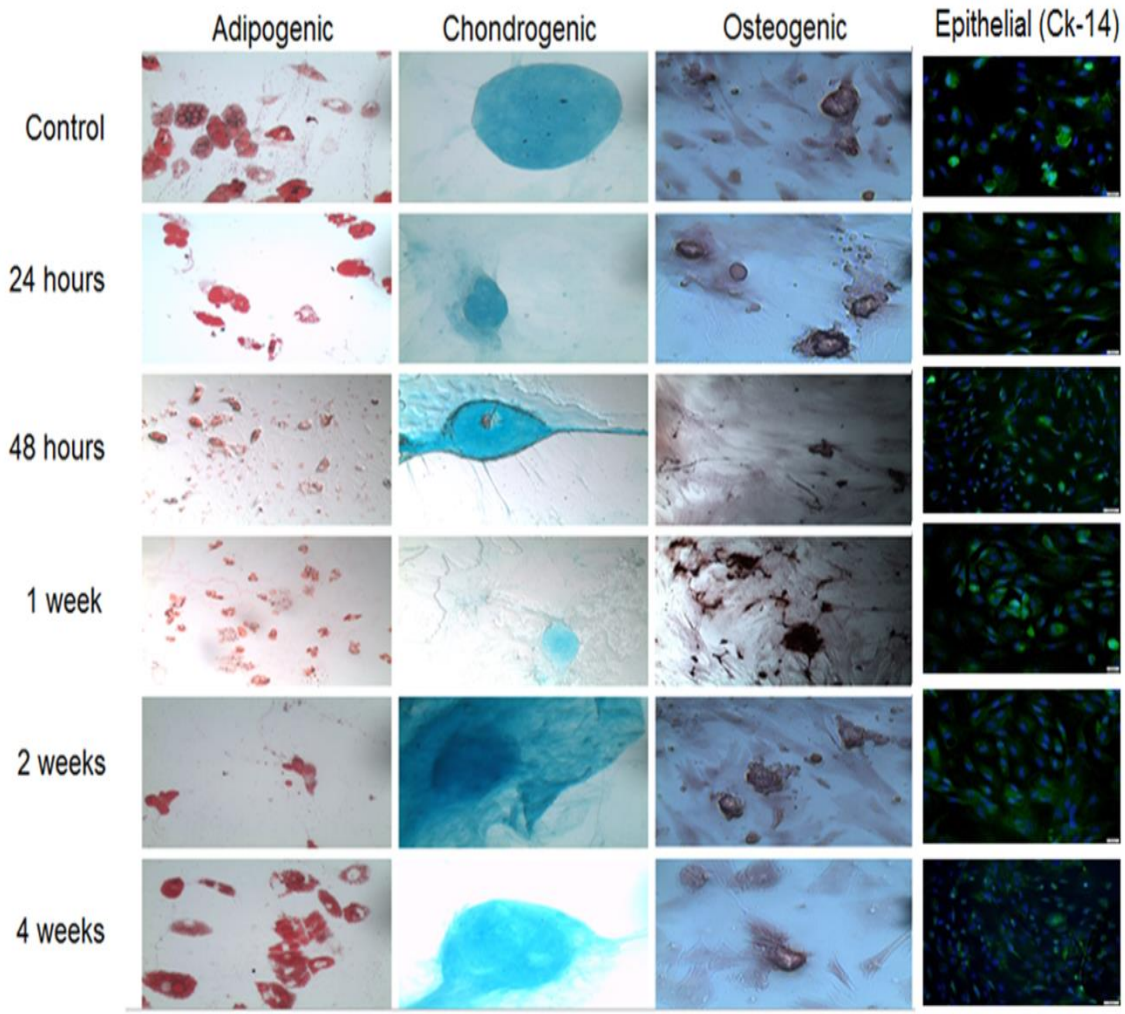


Figure 2.3: Burn injury does not alter the ability of the ASCs to differentiate into adipogenic, chondrogenic, osteogenic, or epithelial lineages. Differentiated ASCs were identified by staining with oil-O-red (adipogenic cells), Alcian blue (chondrogenic cells), or alizarin red (osteogenic cells) or by immunostainning for CK-14 (epithelial cells; green) and counterstaining nuclei with DAPI (blue). Images are shown at 10X magnification.

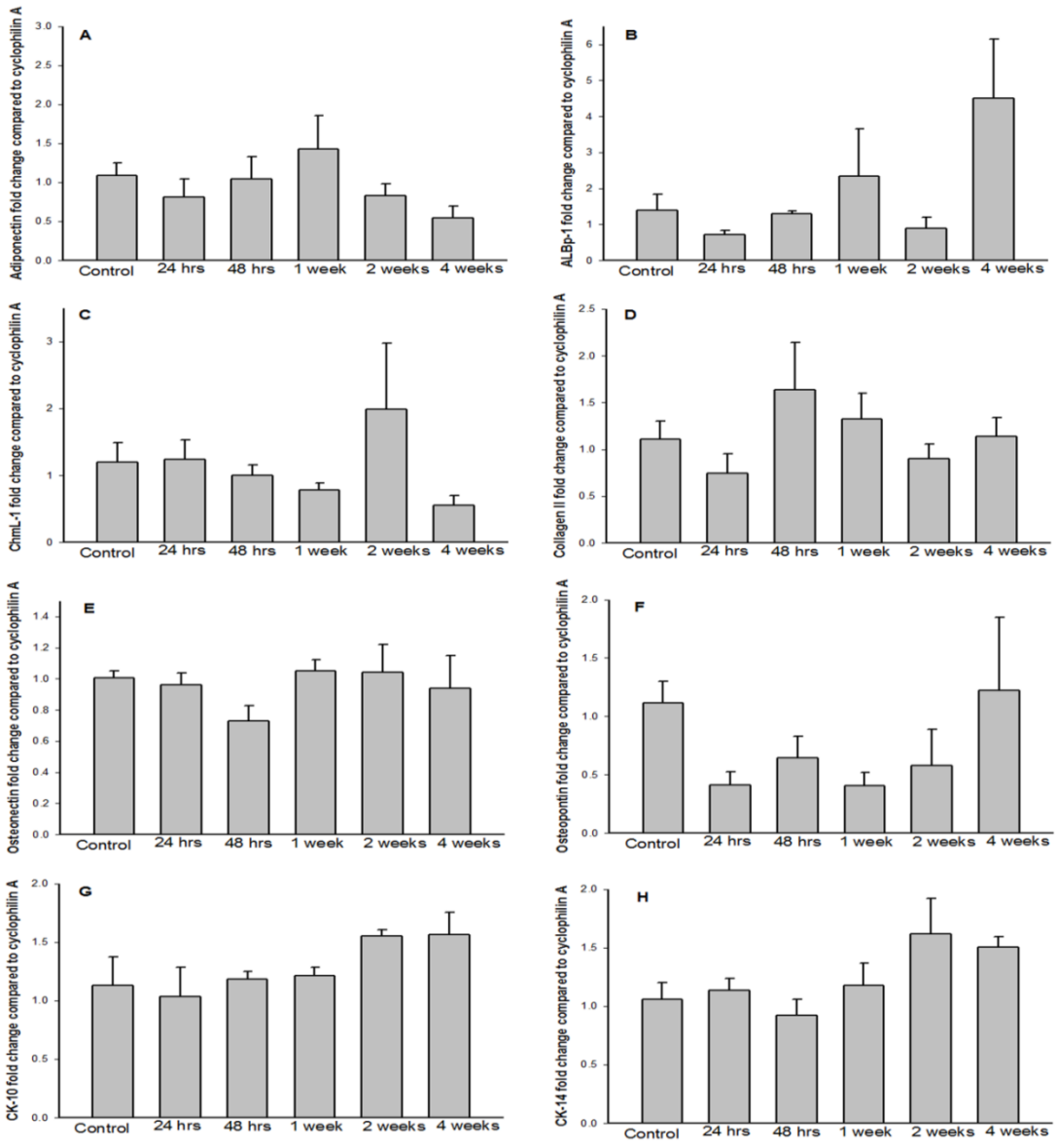


Figure 2.4: Burn injury does not affect the expression of cell type-specific genes in differentiated ASCs. Messenger RNA levels of **A)** adiponectin and **B)** adipocyte lipid binding protein 1 in adipogenic cells, **C)** chondromodulin I and **D)** collagen II in chondrogenic cells, **E)** osteonectin and **F)** osteopontin in osteogenic cells, and **G)** cytokeratin 10 and **H)** cytokeratin 14 in epithelial cells. Each bar represents the mean ± SEM of 8 control animals or 6 burned animals (24 and 48 hours, 1, 2, and 4 weeks post burn).

Post-Burn ASC Populations, as Identified by CD Marker Expression, are Stable

Expression of cell-surface and intracellular CD markers was assessed by flow cytometry. There were no significant differences in the expression of protein levels of CD11b, CD34, CD44, CD105, CD29, CD73, CD90, or CD36 (**Fig. 2.5a and 2.5b**). No differences in the abundance of mRNA encoding these markers was observed. (data not shown).

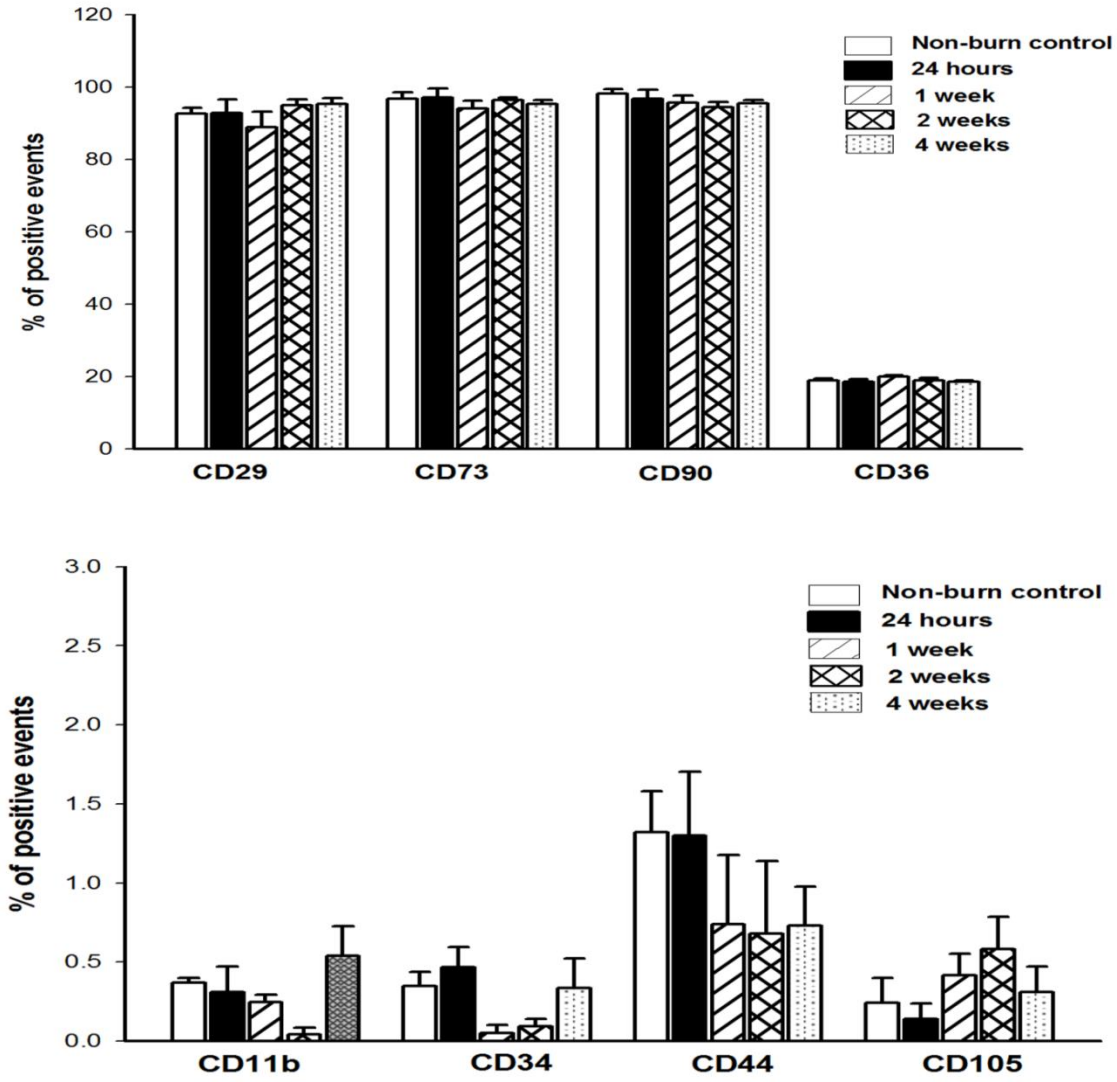


Figure 2.5a and 2.5b: The ASC population is stable following burn injury, as confirmed by CD marker protein levels.

ASC Proliferation is Not Affected by Burn Injury

Measurement of cell proliferation over a 50-hour period via cell impedance showed that cell proliferation was not altered by burn injury (**Fig. 2.6**).

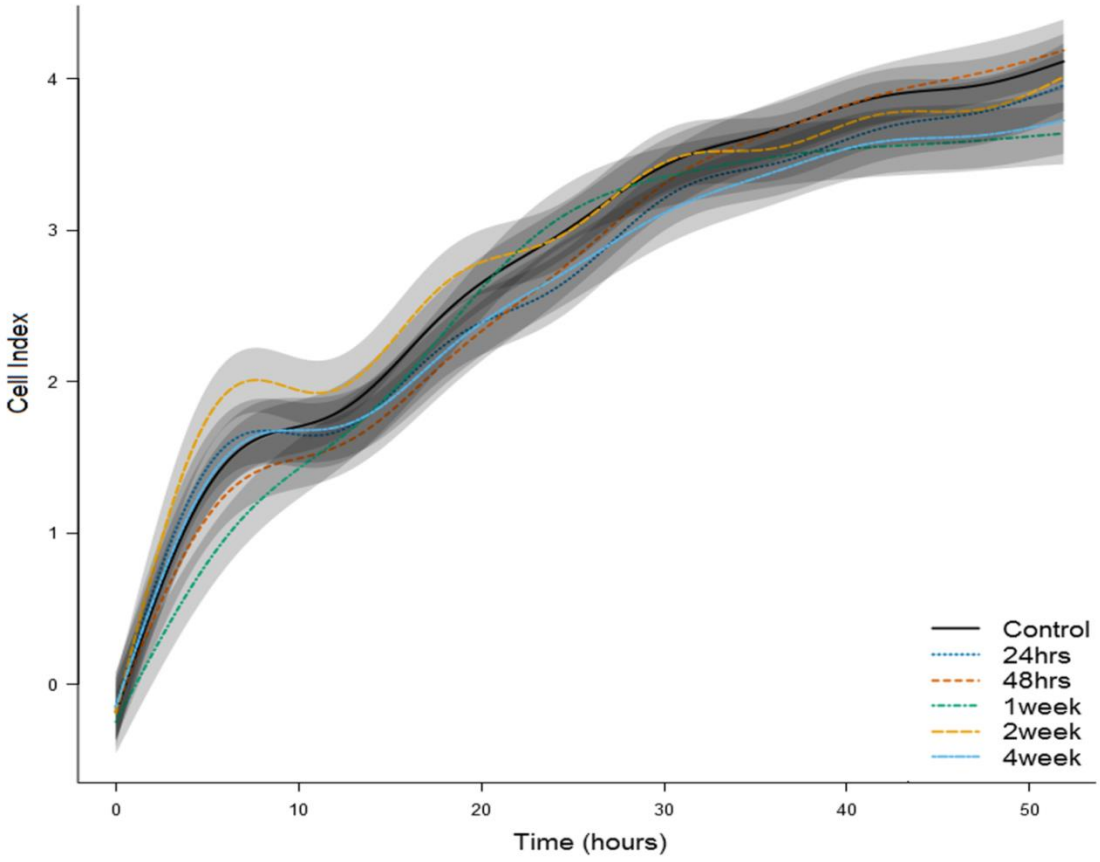


Figure 2.6: Burn injury does not affect proliferation of ASCs. Lines are averages of continuous measurements, and shaded regions around each line indicate SEM. Eight animals were included in the control group and 6 in the burned group (24, 48 hours, 1, 2, and 4 weeks post burn).

Post burns ASCs have similar cytokine expression as control

Cytokine levels of IL-6, MCP-1, TNF-α, IL-1β were measured in the conditioned media using

anti-rat RnD ELISA kit, while the protein levels of NF-κB were measured by western blot. No significance was reached between ASCs conditioned media obtained at various time post burn compared to the non-burn control ASCs. Similarly, no differences were observed in the protein levels of NF-κB between the burn and the non-burn control ASCs (**Fig. 2.7**).

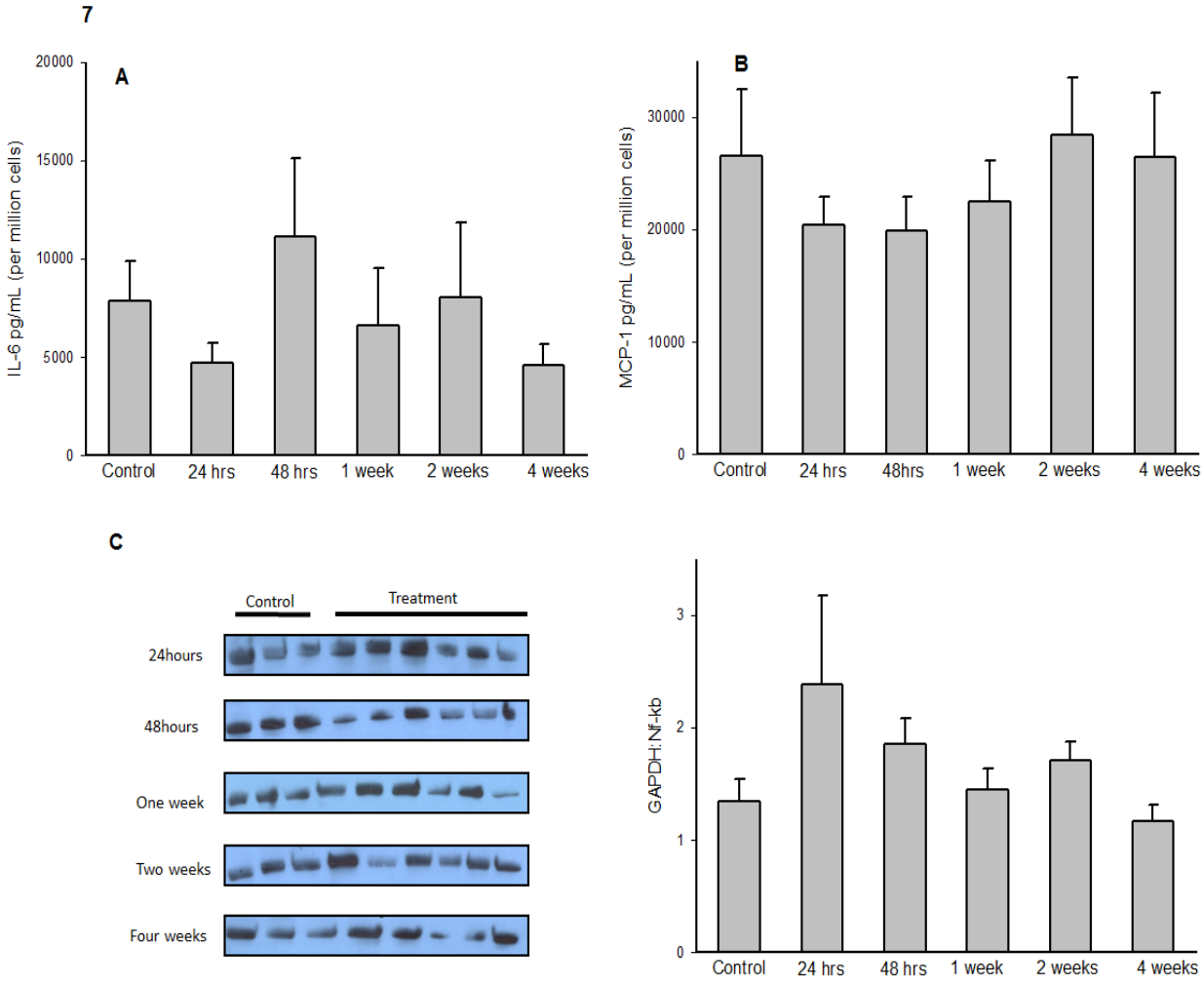


Figure 2.7: Post burns ASCs have similar cytokine expression as a control.ASCs obtained following burn injury expresses parallel levels of cytokines and transcription factors compared to controls, in culture. A and B) Conditioned media secreted cytokines levels in conditioned media A) IL-6, B)MCP-1. IL-1β and TNF-a was below the detection limit C) NF-kB in cultured ASCs

Discussion

Here we have shown that the sources of post-burn inflammation in the adipose tissue include the adipocytes and the stromal vascular fraction. Furthermore, we have shown that the effect of burn injury on the ASCs is relatively small. ASCs can be isolated in great abundance, can differentiate into multiple cell types, and are believed to be safe for autologous and allogeneic transplantation [220]. Because of these qualities, ASC-based therapies have become encouraging approaches in the treatment of myriad diseases [20, 22, 220]. As it is possible to utilize a patient's own ASCs for repair and regeneration following injury or disease, characterization of how these insults affect stem cells that may be utilized for therapeutic purposes is of paramount importance. Inflammation occurs in adipose tissue following injuries such as burns or in association with diseases such as metabolic syndrome or diabetes. By altering the inflammatory milieu where the ASCs reside, it is possible that particular subpopulations of ASCs are selected to proceed toward a different fate or induced to migrate, thereby depleting the tissue of specific subpopulations of ASCs and changing the overall ASC population that could be isolated for clinical use. Therefore, the purpose of our study was two-fold: 1) identify the sources of inflammation in the adipose tissue following a burn injury and 2) determine the effect of burn injury on the fate and function of the ASCs. This was accomplished using the clinically relevant 60% rat scald burn model. Prior work has shown that this model induces a hypermetabolic response similar to that seen in patients with large burn injuries, including increased catabolism and inflammation [213]. Following isolation of the adipose tissue, we were able to obtain adipocytes and SVF for immediate study as well as enriched ASCs using a standard isolation protocol that yielded ASCs for study 48

hours later. These studies show that inflammation comes from adipocytes and the SVF and that ASCs are not inflammatory in culture (nor are they affected by the burn injury and subsequent culturing procedures).

Stem cell fate and function are predominantly determined by dynamic interactions between the stem cells and their environment. Stem cells interact with the ECM, neighboring cells, and secreted proteins, allowing changes in the microenvironment to affect stem cells [210, 211, 221]. In adipose tissue, ASCs and adipocytes are surrounded by factors that facilitate tissue homeostasis under normal conditions. Following bury injury, many pro- and anti-inflammatory cytokines are elevated both systemically and locally within burned and non-burned tissue [212]. In freshly isolated adipocytes and SVF, we showed that IL-1β and IL-6 are elevated in response to burn injury but that levels return to those seen under non-burn conditions between 48 hours and 1 week following the injury. Similarly, TNF-α was elevated in adipocytes but returned to normal levels after burn injury within the same temporal window. We are unable to measure immediate levels of inflammatory mediators in the ASCs, as the isolation protocol necessitates a 48-hour incubation period. Therefore we measured the same markers in enriched, post-isolation ASCs that were cultured in *vitro* for 48 hours. Under these conditions, ASCs from burned animals produced similar levels of inflammatory markers as those from non-burned animals. An additional finding of interest was that the mature adipocytes produced significantly greater amounts of inflammatory markers than the SVF, which contains macrophages and other inflammatory cells. Within adipose tissue, ASCs, mature adipocytes, and other cell types likely function through paracrine signaling [222-224]. These interactions should be studied further to determine the role of this

inflammatory response following burn injury. Additionally, we do not know whether increased DNA damage results in greater cell death within adipose tissue, leading to further increases in the local inflammatory response or whether this damage is repaired. The comet assay was performed to evaluate whether ASCs obtained following burn injury had increased DNA damage. Significantly higher fractional damage scores were recorded in the SVF isolated at 24 and 48 hours post burn than in the non-burn control SVF. Whether these amounts of DNA damage are sufficient to affect tissue function or lead to aberrant cell behavior is unknown.

We were able to isolate ASCs from animals with large burn injuries. ASC identity was confirmed by measuring stemness markers; by differentiating the cells into osteogenic, chondrogenic, adipogenic, and epithelial cells [17]; and by verifying terminal differentiation into these cell types through measurement of cell type-specific gene expression. Burn injury did not change the ability of the ASCs to differentiate into other cell types. It also did not affect ASC proliferation or expression of mRNA for cytokines, growth factors, or CD markers related to burn injury.

Here we used the consensus definition to identify ASCs [17, 225]. Most published studies have shown that CD13, CD90, CD73, CD29, CD36, and CD105 are positive markers for ASCs, while CD34 and CD11b/c are negative markers. Low expression of CD105 expression in rat ASCs could be mainly attributed due to lack of appropriate anti-rat CD105 antibody or an inadequate affinity of the CD105 antibody for the rat homologue as opposed to murine or human CD105. It may also be attributable to downregulation or loss of CD105 in culture [226].

Conclusion

In a model of severe burn injury, ASCs obtained following burns behave similarly to ASCs obtained from non-burned controls. Given the fact that inflammatory markers are elevated and DNA damage is increased 24 to 48 hours post burn, we recommend that ASCs or SVF be used for tissue engineering or wound healing applications in severely burned patients after inflammation resolves. Confirmatory studies utilizing human tissue are needed to determine the timeline for resolution of the inflammation.

Chapter 3: Adipose-derived stem cells (ASCs) improve grafted burn wound

Introduction:

With the fairly recent discovery of stem cells in adipose tissue, the use of adipose derived stem cells (ASCs) to treat various ailments and disease conditions has become more frequent [227]. Before the discovery of ASCs, fat tissue removed during surgical procedures was routinely discarded. The potential utilization of ASCs as a wound healing therapy emerged after the latent stem cell properties of ASCs were disclosed by Zuk and colleagues [8]. With more than 15.6 million cosmetic procedures occurring every year, the procurement and isolation of ASCs have become a relatively easy process, due in part to the abundant availability of these cells [228]. Although many trials of the clinical utility of ASCs are ongoing, [227, 229] [230-232], there is a dearth of work demonstrating how ASCs mediate and accelerate wound healing [24].

ASCs have similar wound healing properties and cytokine expression profiles as those of bone marrow derived mesenchymal stem cells [23, 233]. The mechanisms by which ASCs, applied topically or injected subcutaneously, facilitate and accelerate wound healing are still largely unknown. Histological and flow cytometry studies have suggested that ASCs are located around the vascularized networks in fat tissue, in close proximity to pericytes and endothelial cells[234-236]. These ASCs secrete myriad growth factors involved in wound healing, including interleukin (IL)-6, IL-8, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast grown factor (FGF-2), granulocyte-macrophage colony-stimulating factor (GM-CSF) granulocyte-colony stimulating factor (G-CSF).[23, 24, 64, 237, 238]. Through the action of these secreted

proteins, ASCs can modulate the activities of endothelial cells, fibroblasts, and other resident cells [239-241]. Modulation of endothelial cells by ASCs via paracrine or contact-dependent interactions may result in *de novo* vessel formation. Furthermore, ASCs themselves can undergo endothelial differentiation under certain conditions [107], which may also contribute to the acceleration of wound healing.

In order to elucidate the role of ASCs in wound healing, we used an established model of ovine burn injury. Endpoints included wound closure, epithelialization, blood flow, and expression of trophic factor VEGF. Here we report that the application of ASCs significantly increased wound blood flow by increasing VEGF levels.

MATERIALS AND METHODS:

ASC isolation and culture conditions:

All animal studies were conducted in adherence with the guidelines detailed in the NIH Guide for the Care and Use of Laboratory Animals. The study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch, Galveston, TX, USA.

Adipose tissue was isolated from healthy sheep and washed extensively with PBS containing 5% penicillin/streptomycin. The tissue was then minced and incubated with 0.075% collagenase Type IA at 37^{o}C for 70-90 minutes with constant shaking. Because ovine adipose tissue contains a higher percentage of saturated fat compared to human adipose tissue, the duration of the enzymatic digestion was extended [242, 243]. Following complete digestion, an equal volume of complete media [(Dulbelco's Minimum Essential Medium with 10% FBS and 2% antibacterial/antimycotic solution (10,000IU/mL Penicillin; 10,000ug/mL Streptomycin; 25ug/mL Amphotericin; 8.5g/L

NaCL)] was added in order to inactivate the collagenase. The solution was aspirated and centrifuged at 350 G for 5 minutes in order to separate the cells from the adipose tissue. The cell pellet was reconstituted with PBS and centrifuged at 350 G for 5 minutes. This step was repeated 3 to 4 times until the supernatant became clear. The pellet was then re-suspended in 4.5 mL of water for 30 seconds followed by the addition of 10X PBS in order to lyse the red blood cells. Complete media was then used to re-suspend the pellet and the solution was filtered through a 70 μm cell strainer, suspended in complete media, and washed twice with PBS. The final pellet was seeded into a 25 cm^2 tissue culture flask and incubated in 5% CO_2 at 37ºC. After incubating for 18 hours, the media was replaced, removing the unattached cells and cellular debris. Cells were cultured and passaged until the 2^{nd} passage and frozen down in aliquots. Fourth passage cells were used for the experiments.

Characterization of ASCs: Differentiation and stemness-related marker detection

Ovine ASCs were characterized following the guidelines established by the International Society for Cell Based Therapy [17]. ASCs were differentiated into three different lineages to confirm stemness while flow cytometry and semi-quantitative PCR were used to assess CD marker distribution.

ASC Differentiation:

Following the differentiation into three different lineages, as described below, the stained and differentiated ASCs were photographed using an inverted phase contrast microscope (Leica DFC450) at 10X magnification.

Osteogenic differentiation: In a 6 well plate, 10^4 cells per cm^2 were seeded in complete media. After 24 hours, the media was replaced with osteogenic differentiation media

composed of complete media along with 0.1 µM dexamethasone, 50 uM ascorbate-2-phosphate, 3mM NaH2PO4,[244] 0.1 µM Retinoic acid. Osteogenic differentiation was carried out for 28 days, with media changes performed every three days. Alizarin red staining was used to detect calcium phosphate staining, a marker of osteogenic differentiation.

Chondrogenic differentiation: Cells were plated at a density of 10^5 cells per cm^2 were plated for 24 hours in a complete media. The cells were placed on a rotary shaker at a lower setting for five minutes to concentrate the cells in the middle of the culture plates. After the cultures were confluent, cells were grown in chondrogenic differentiation media (DMEM with 6.25 µl/mL of insulin, 10 ng/mL of TGF-β, 50 nM ascorbate-2-phosphate, and 2% FBS) for 28 days, with media changes performed every 3 days. Alcian blue staining was used to determine the degree of chondrogenic differentiation. [245]

Adipogenic differentiation: In a six well plate, 10^4 cells per cm^2 were plated in a complete media for 24 hours. Differentiation was initiated by replacing complete media with adipogenic differentiation media (complete media with the addition of 1 µM dexamethasone, 10 µM insulin, 0.5 mM isobutyl-methylxanthine, and 200 µM indomethacin). Cells were incubated in this media for 21 days, and the media changed every 3 days. Oil-o-red staining was used to evaluate adipogenic differentiation[8].

Cell surface marker detection via flow cytometry: Fourth passage ASCs were cultured in complete media until the cultures were 70-80% confluent. The cells were harvested with 0.25% trypsin/EDTA. Cell viability was assessed via trypan blue staining. Analysis and determination of fluorescent cells, dead cells, debris, and background noise were made with the BD Accuri™ C6 flow cytometer software. For CD marker staining, the

cells were incubated in ice-cold PBS with 1% sodium azide for 15 minutes, washed three times with PBS, and incubated with 3% BSA for 30 minutes on ice. Following three more PBS washes, aliquots of ASCs were incubated in 1% BSA per the manufacturer's guidelines with fluorescent dyes attached to the primary monoclonal antibodies: CD73, CD90, CD11b, CD34, and CD44.

Cell surface marker detection via semi-quantitative PCR (qPCR):

In addition to flow cytometry, semi-quantitative PCR was used to detect the presence of transcripts for the CD markers associated with the stem cell. Fourth passage ASCs were utilized (commercially available human ASCs and laboratory isolated ovine ASCs). Total RNA was isolated following the manufacturer's protocol (RNA isolation kit, Qiagen, Hilden, Germany). cDNA was made from the total RNA (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA). The expression of stemness marker transcripts was analyzed by semi-quantitative PCR using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma, St. Louis, MO) using a DNA Engine® Peltier Thermal cycler. The primers used for the PCR reaction are listed in table 3.1. The reaction products were electrophoresed on an agarose gel, which was photographed with the Syngene GeneGenius Bio Imaging System for visual comparison.

Gene	Forward	Reverse
Human		
cyclophilin	CTCGAATAAGTTTGACTTGTGTTT	CTAGGCATGGGAGGGAACA
CD34	TGGGCATCGAGGACATCTCT	GATCAAGATGGCCAGCAGGAT
CD11b/c	CTTGCCTTTCACCACCTGAT	TCCCAGGCTCCAGTATTTTG
CD73	CAGACTCATGATGACAGAGG	GAGATGTACAGGATCTTCCC
CD90	CACCAGTCACAGGGACATGA	ACCTACACGTGTGCACTACCA
CD105	CGTGGACAGCATGGACC	GATGCAGGAAGACACTGCTG
CD44	CAGGAAGAAGGATGGATATGG	ATTACTCTGCTGCGTTGTC
Sheep		
cyclophilin	CAT ACA GGT CCT GGC ATC TTG TC	TGC CAT CCA ACC ACT CAG TCT
CD34	TGGGCATCGAGGACATCTCT	GATCAAGATGGCCAGCAGGAT

CD11b/c	CCTTCATCAACACAACCAGAGTGG	CGAGGTGCTCCTAAAACCAAGC
CD73	TGGTCCAGGCCTATGCTTTTG	GGGATGCTGCTGTTGAGAAGAA
CD90	CAGAATACAGCTCCCGAACCAA	CACGTGTAGATCCCCTCATCCTT
CD105	CGGACAGTGACCGTGAAGTTG	TGTTGTGGTTGGCCTCGATTA
CD44	GTGTCGTGTGCCCAGTTATGA	CTCGTCAGAGGTCCCATTTTC

Table 3.1: Primers for human ASCs and ovine ASCs characterization

Ovine model of grafted burn wound healing

Surgical preparation: Seven female sheep, weighing 27-37 kg were housed in an animal facility with a 12 hour light and dark cycle for two weeks to acclimate prior to the initiation of the experiment. All animals received water and food ad libitum and were monitored for the entire study period. After the acclimatization, the animals were chronically instrumented with multiple vascular catheters for the cardiopulmonary hemodynamic monitoring, intermittent blood sampling, core body temperature, and microsphere injection. Briefly, sheep were anesthetized with inhaled isoflurane (Piramal Healthcare Ltd. India) using an inhalation mixture of 2 to 5 vol% in oxygen, via an endotracheal tube. Under aseptic conditions, a 7Fr. Swan-Ganz thermodilution catheter (model 131F7; Edwards Critical Care Division, Irvine, CA) was inserted into the right jugular vein through an 8.5Fr. a percutaneous introducer sheath (Edwards Life- sciences, Irvine, CA) and was placed into the common pulmonary artery. Then, the right femoral artery was cannulated, and a polyvinylchloride catheter (16-gauge, 24-in., Intracath; Becton Dickinson Vascular Access, Sandy, UT) was positioned in the descending aorta. Through a left thoracotomy at the level of the fifth intercostal space, a Silastic catheter (0.062-in. inner diameter, 0.125-in. outer diameter; Dow-Corning, Midland, MI) was positioned in the left atrium. Following the operative procedure, sheep were awakened

and monitored in the ICU in a conscious state for 5-7 days. During this time, they had free access to food and water. Pre and post-surgical analgesia were provided with buprenorphine (0.05 mg/kg, subcutaneous Buprenorphine SR^{TM}, SR Veterinary Technologies, Windsor, CO).

Experimental protocol: After 5-7 days of surgical recovery, sheep were again anesthetized with inhaled isoflurane (as described above for surgical preparation). Anesthesia was then maintained with 2% to 5% isoflurane in 60% O_2. Two burn sites of approximately 25cm^2 (5cm x 5 cm each) were made on the both side of the dorsum of the animal (total 4) with 5 cm between sites and 5 cm from the spine, yielding 2 wounds per animal (~2% of the total body surface area [TBSA]).

Homogenous 3rd-degree flame burns were applied with a Bunsen gas burner on the dorsum until the skin was thoroughly contracted. During and after the burn procedure until the surgery, all sheep were resuscitated with Ringer's lactate solution and had free access to water. Twenty-four hours after injury, the burned skin was excised to the level of the panniculus cavernous according to the clinical early excision procedure established for human patients with third-degree burns under anesthesia with inhaled isoflurane. The average size of the resulting defect was approximately 25 cm^2 for each side. For autograft harvesting, the dorsum of the animal was used. Split-thickness skin grafts (0.4 mm thickness) were harvested by the air dermatome. Wounds were autografted with 2x2 cm skin patches. Each patch was attached by suturing in the center of the wound **(Figure 3.2)**.

After the graft procedure, one of the wound sites was randomly allocated to topical

application of 7 million allogeneic ASCs reconstituted in 1mL of the vehicle (PBS). The contralateral site, (control) was treated with PBS.

The applied ASCs were allowed to equilibrate in the wound site for 10 minutes followed by the application of petroleum jelly. The grafted wounds were then covered with sterile non-adhesive polyurethane sheets with pressure bandages. Animals were hemodynamically monitored daily for one week and the bandage was removed on day 7. Pictures of the wound sites were taken at 7 and 15^{th} days to assess the wound closure. During the study period, wounds were covered with petroleum jelly and polyurethane to keep moist. On the 15^{th} day, sheep were euthanized under deep anesthesia and the wound tissue sampled.

Wound Planimetry: After the initial autografting and during each dressing change, standardized digital photographs of the wound fields were taken. The photographs of each wound site were taken with a calibrated benchmark positioned adjacent to the wound. Photographs were analyzed using planimetrical software (Image J 1.43u, Wayne Rasband, National Institutes of Health, USA) to determine total wound size and the area of grafted skin.

Wound Blood flow measurement using Laser Doppler: The blood flow in the treated and untreated (control) was measured using a LASER Doppler device (PeriFlux System 5000 ModelPF5001, PERIMED), 7 and 15 days after grafting as previously described [246] . Five locations were randomly chosen in each site and the means were analyzed.

Wound blood flow measurement using microsphere injection technique: The wound blood flow was also measured by fluorescent microsphere injection technique. Approximately 5 million (two different colors) stable microspheres (15.0±0.1 μm in

diameter; Interactive Medical Technologies LTD, Los Angeles, CA) were injected into the left atrium of the heart at 0 (before the operation) and 15 days as previously described [247]. Twenty ml of blood was collected from femoral artery within 2 minutes with a Harvard pump (Harvard Apparatus Co. Model 55-1143, South Natick, MA) as a reference. At 15 days, sheep were euthanized and the wound was excised and the number of microspheres was counted in one gram of tissue. Then, the blood flow was calculated as mL per gram tissue per minute.

Ultrasound examination of autografted burn wounds: The epithelization of wounds at the treated and untreated sites were examined with an ultrasound machine (Vevo 2100 High-Resolution imaging system, VisualSonics), 15 days after grafting. The degree of epithelization was scored as follows: score 0 =incomplete epithelialization; score 1=partial epithelialization; score 2= completed epithelialization with irregular and uneven epithelium, and score 3--completed epithelialization with regular and even epithelium. Representative ultra-sound images of the wound healing scores are presented in **Figure 3.3**. Nine locations were randomly chosen for ultrasound measurements and the means were summarized.

Historical epithelial score

The degree of uncovered wound epithelization was also assessed in formalin fixed wound tissue by two independent and masked pathologists using light microscopy at 15 days.

Quantitation of the VEGF protein: The VEGF protein expression in the wound tissue was measured via ELISA. The ELISA was performed according to the manufacturer's instructions (R&D Systems Inc, MN)

Statistical Analysis:

The following statistical tests were used where appropriate: Analysis of variance (ANOVA) followed by Tukey's test; paired or unpaired Student's *t* tests. Data were expressed as the mean ± standard deviation or as the mean ± standard error of the mean. Significance was accepted at $p < 0.05$.

RESULTS:

Characterization of Ovine ASCs:

Cultured ASCs successfully differentiated into adipogenic, chondrogenic, and osteogenic lineages as shown by oil-o-red, Alcian blue, and Alizarin red staining respectively (Fig 11). Due to the lack of ovine antigen recognizing antibodies for CD90, CD73, and CD105, only CD44 was detected on the ASCs via flow cytometry. Stemness-related CD marker gene expression did show positive transcript levels of CD73, CD90, CD105 and CD44 as compared with human ASCs (**Fig 3.1**).

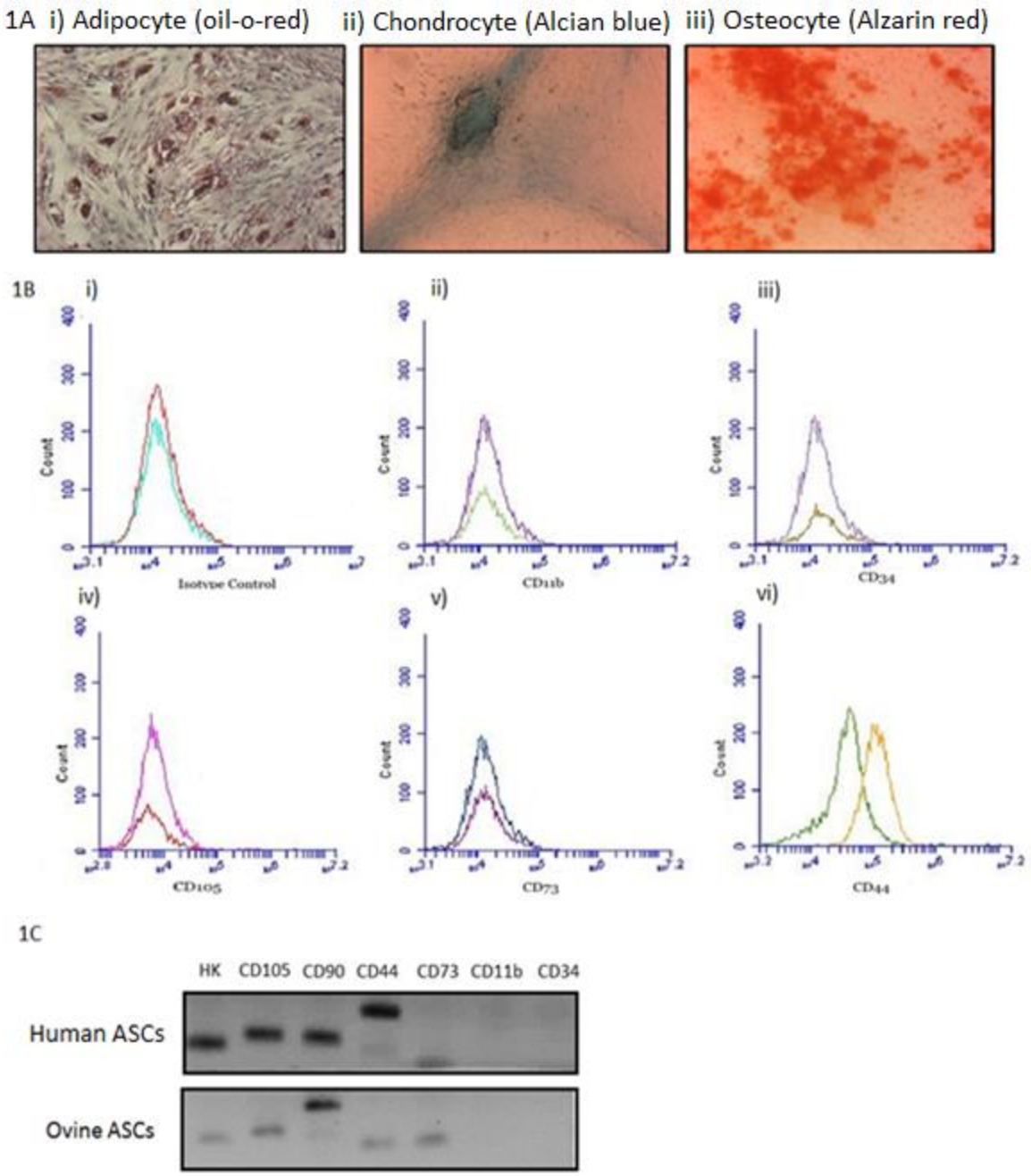


Figure 3.1: Characterization of ovine ASCs A) Adipose derived stem cells were differentiated into three different lineages and stained i) adipocytes with oil-o-red ii) chondrocyte with Alcian blue iii) osteocytes with Alizarin red. B) Characterization of ASCs with flow cytometry i) isotype control ii) CD11b, iii)CD34 iv) CD105 v)CD73 vi)CD44. C) mRNA levels of CD markers of ovine ASCs and commercial human ASCs

ASCs improved wound closure within 15 days:

Planimetry demonstrated that the graft size in ASC-treated animals was significantly greater than in the control animals (3.92 ± 0.89)cm^2 versus 3.22 ± 0.90 cm^2, respectively, p<0.013), with the graft size in the ASC-treated normalized to control ratio equal to 1.23 ± 0.12(p <0.013). The average size of the re-epithelialized area of the wound was 17.73 ± 1.55 cm^2 with ASC treatment compared to 15.76 ± 1.35cm^2 with the controls. (Figure 2C, 2D) The wound epithelialization scores estimated by ultrasound were significantly higher in treated sites (1.71 ± 0.95) vs compared to control sites (0.71 ±0.75), p = 0.05). **(Fig 3.2).**

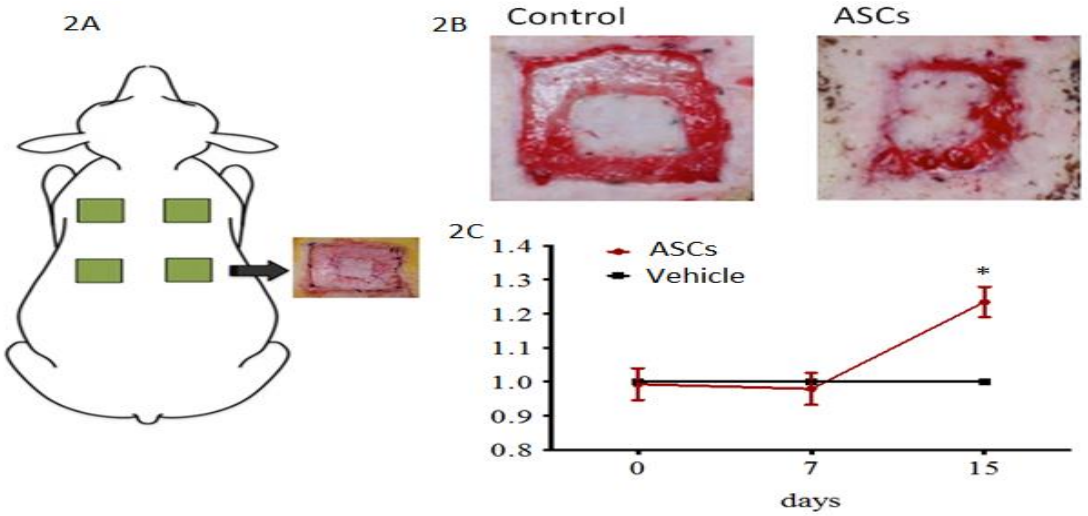


Figure 3.2: Graphical representation and quantification of sheep wound healing. 2A)Full thickness burn was induced in an area of 5x5 cm, with a total of four wounds per animal. 24 hours following burn injury, the burned skin was removed and autografted and stitched with donor skin at the center. Topically 7 million allogeneic ASCs or vehicle(PBS) were added to the wound. 2B) Pictorial representation of wound healing 2C) Wound healing progression at day 7 and 15 following the application of ASCs.

Quantification of wound closure at 7 and 15 days. The graft size is normalized to the controls. Error bar represents standard error of mean (S.E.M.) (n=5, *p< 0.05).

Histological epithelized score:

We show the degree of epithelization of uncovered wound estimated by histological examination in Figure 5. There was no significant difference between ASC-treated wound (44.2 ± 9.2%) and control wound (31.8 ± 7.3%). **(Fig. 3.3)**

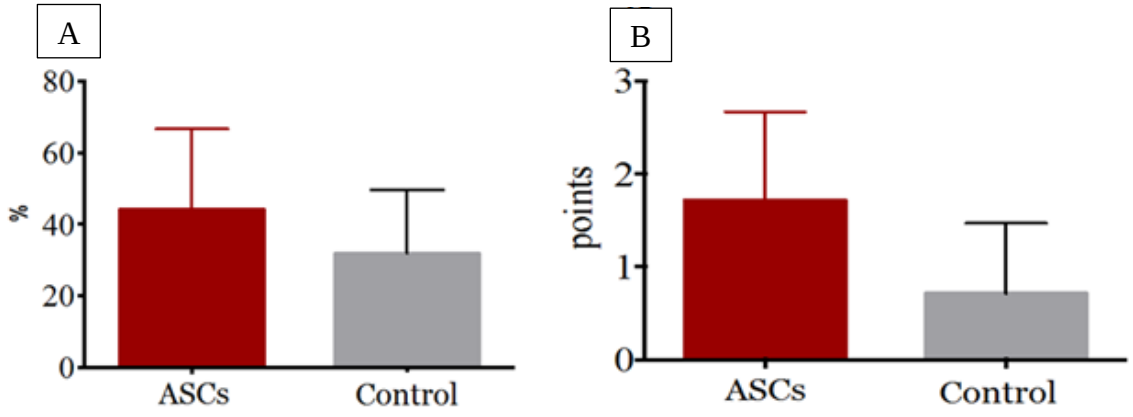


Figure 3.3: Topical application of ASCs increases wound epithelialization. A) Histological analysis of wound epithelization, B) Analysis of wound epithelization using high-resolution ultrasound. Error bar represents standard error of mean (S.E.M.) (n=6, *p< 0.05).

ASCs significantly increased wound blood flow:

Blood flow in ASC-treated wounds, measured by laser Doppler, was normalized to the control. It is significantly greater 7 days post-application in treated vs. control, p<0.0025), and tended to be higher in treated vs. control (p <0.0545) (**Fig 3.4**).

The blood flow measured by microsphere injection technique was (**Fig 3.4**) significantly higher 15 days ($p<0.0169$) vs. control

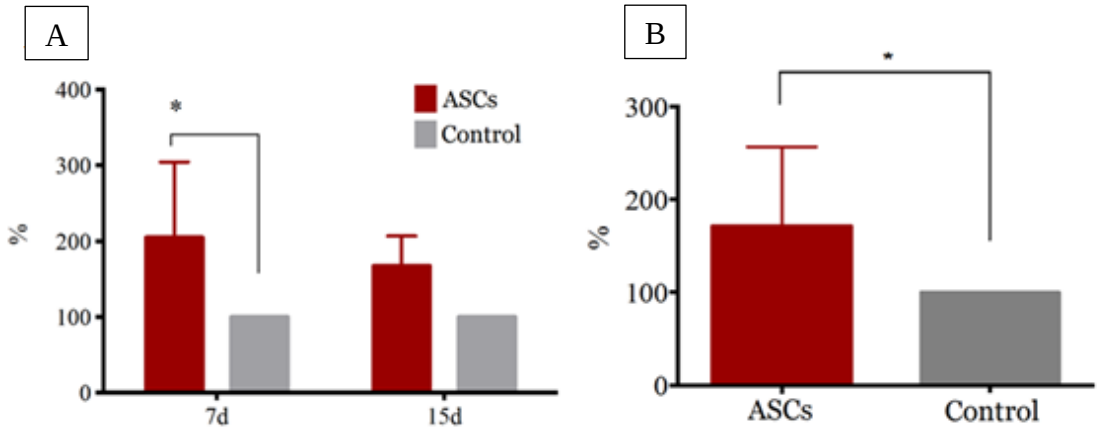


Figure 3.4: ASCs significantly increase blood flow to the wound bed. A) Measurement of blood flow by Laser Doppler technique at day 7 and 15 post-burn. B) Fluorescent microsphere injection technique. Error bar represents standard error of mean (S.E.M.) (n=7, *p< 0.05).

Topical application of ASCs increases wound epithelialization

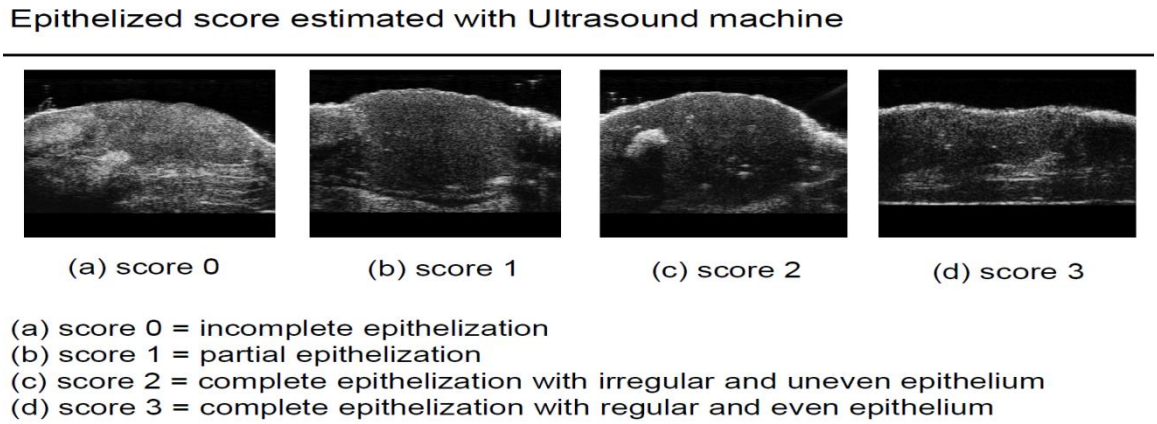


Figure 3.5: Histological epithelialization score used to evaluate wound biopsies

Wound bed VEGF expression is elevated with ASC treatment:

Wound biopsy tissue obtained from the vehicle and ASC-treated wounds 15 days post-application were pooled and VEGF levels were measured. ASC treatment resulted in significantly higher levels of VEGF 21.1 ± 2.5 pg/ml ($p < 0.031$) compared to vehicle treatment 16.1 ± 1.7 pg/ml (**Fig 3.6**). $p<0.031$ using Wilcoxson test

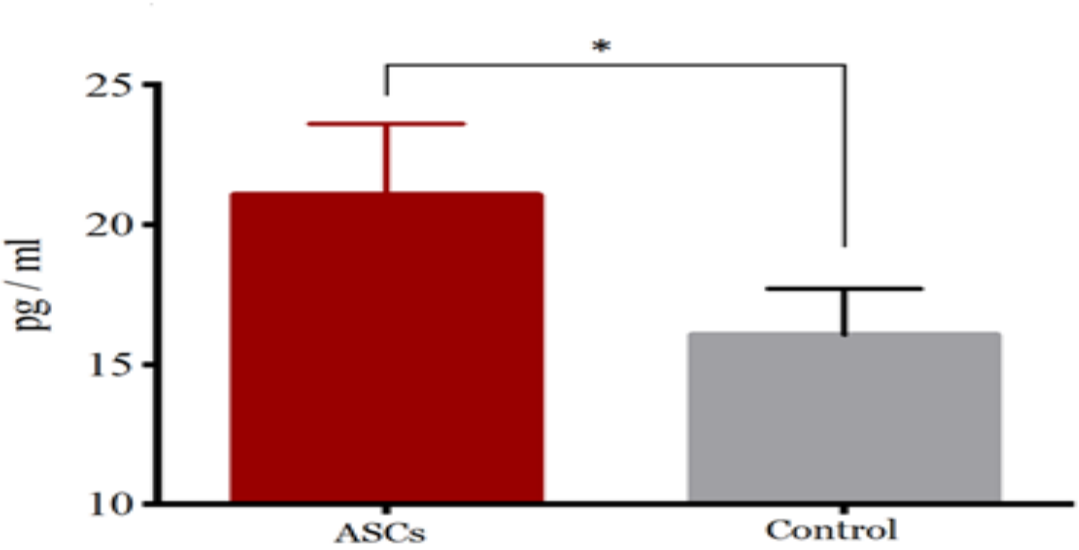


Figure 3.6: Wounds treated with ASCs express significantly higher levels of VEGF. ELISA was performed on skin tissue (epidermis and dermis) obtained from ASCs treated animals and controls. Error bar represents standard error of mean (S.E.M.) (n=7, *$p< 0.05$).

DISCUSSION:

This study was designed to evaluate the potential of allogeneic ASCs in grafted burn wound closure. Here we first demonstrate beneficial effects of topically applied ASCs on grafted burn wound healing in a clinically relevant large animal model. The wound care (escharectomy, wound closure, and daily care) mimicked the clinical care provided to severely burned patients at our own institution. The grafted wound was cared for in a

conscious sheep. Most of the published research elucidating the effects of ASCs on wound healing has utilized either ischemic or diabetic rat models to study vasodilation and neovasculogenesis. However, there is a paucity of research regarding how ASCs or other stem cells modulate the wound environment [248-250]. To our knowledge, this is the first study to investigate the effects of ASCs on grafted burn wound healing.

ASCs are believed to have great potential for improving wound healing. Like mesenchymal stem cells, ASCs can regulate critical processes involved in wound healing, including inflammation [251-253], vascularity [64, 240], and immunity [134, 152]. Severe burn injury induces a hypermetabolic, hypercatabolic state, activating inflammation, vascular abnormalities, and immune suppression. For these reasons, ASCs may be a successful wound healing therapy following a severe burn injury [254, 255].

The major outcome of the study was that ASCs accelerated the growth of grafted skin, increased wound bed blood flow, and promoted expression of the potent growth factor VEGF.

Although, the exact mechanism of how ASCs improved the wound healing is not completely understood, our data suggest that topically applied ASCs may have accelerated the growth of grafted skin by promoting wound bed blood flow via potent growth factor VEGF.

This wound healing model includes the shrinkage of excised burn wounds, the growth of granulation tissue, survival of grafted skin, and epithelization from grafted skin and surround skin.

In ASCs group, although the vascularity in granulation tissue increased and grafted skin were spreading faster than control, epithelization from the surrounding skin did not have a significant difference between two groups. The re-epithelized area was earned almost by grafted skin, not by surrounded skin. Whole wound size also earned by shrinkage, not

by skin epithelization. This might mean ASCs application couldn't salvage the zone of stasis. However, the burned wound was limited to the local site, it should be considered that inflammation was slight and the influence that marginal skin and subcutaneous tissue besides burn were injured by debridement.

Previous studies have elucidated the angiogenic influence of ASCs in different wound healing [250] and ischemic [64, 240] models. The increase in blood flow around the wound sites implies that either there are more blood vessels (via angiogenesis or vasculogenesis) or increased vasodilation; the end result of both of these scenarios is an increase in the blood volume circulating around the wound site. Following severe burn injury, there is extensive damage to the blood vessels. Additionally, there is an increase in demand for oxygen and nutrients to fuel the reparative and remodeling processes, defense mechanisms, and debris clearance, resulting in greater demand for nutrients. The distress signals triggered by a burn injury within the wound niche may have activated the applied ASCs, as stem cells modulate inflammation [256]. Dependent on the surrounding niche, ASCs may change from a pro to anti-inflammatory phenotype [93] [122]. Thus applied ASCs may be induced to release signals which can modulate the behavior of endothelial cells and other neighboring cells. In this case, where there is a significant increase in VEGF, which is involved in angiogenesis and vasodilation.

ASCs secrete growth factors that modulate angiogenesis, including VEGF, HGF, MCP-1, IL-6, and FGF [23, 239]. Dynamic interaction between the ASCs and the niche can stimulate the production of growth factors that drive wound healing and regeneration.

The limitation of the study is that we did not clarify whether the therapeutic effects of ASCs on wound healing are derived from paracrine effects of the ASCs secretome as opposed to the ASCs differentiating into other cell types. Modulating the stem cell secretome may be an avenue to improve the therapeutic outcome in diseases and disorders being treated with stem cells. Future studies are warranted to elucidate the mechanisms underlying how ASCs improved the healing of grafted burn wounds. Nevertheless, our data suggest that topical application of adipose-derived stem cells improved grafted burn wound healing by promoting wound bed blood flow and increased VEGF expression.

Chapter 4: Cytokine monocyte chemoattractant protein-1 (MCP-1) is one of the vital secretomes of adipose-derived stem cell

Introduction:

Delayed or compromised wound healing remains a challenge, especially for patients who are diabetic, severely burned, or those with chronic non-healing wounds. Preclinical and clinical studies of stem cell products for treating debilitating wounds have shown that stem cells and their byproducts can improve would healing [257, 258]. Although several studies suggest that stem cells such as bMSCs migrate and differentiate at the site of wound or insult [58], there is a growing consensus that following the topical application of stem cells, therapeutic effects are derived from the paracrine activities of the stem cells as opposed to the stem cells differentiating into new cell types within their new niche [60, 259, 260]. The application of ASCs or ASC conditioned media (ASC-CM) has resulted in increased angiogenesis and augmentation of collagen synthesis [66, 201, 240, 241]. However, the mechanisms underlying how stem cells, especially adipose derived stem cells (ASCs), modulate the surrounding microenvironment through paracrine signaling to facilitate wound repair remains unclear.

ASCs produce overabundance of growth factors and cytokines including IL-6, FGF, TGF, MCP-1, VEGF, and PDGF - all of which are known to modulate inflammation, the immune response, angiogenesis, and other pathways that are essential for wound healing [23, 60, 64, 99]. As with other stem cells, the proteins secreted by ASCs fluctuate based on the microenvironment, allowing the stem cell to respond to the signals surrounding it [122]. The topical application of ASCs has been shown to increase blood flow to the wound [64, 102, 180, 201]. Additionally, in our ovine model of burn wound healing, we

demonstrated that the topical application of ASCs increased the size of the graft on the wound (chapter 3). A significant increase in VEGF and collagen 1A protein expression was found with ASC application, also a significant increase in MCP-1 mRNA transcript was found in ASCs treated animals compared to the controls.

Among the less studied components of the ASC secretome that aids in wound healing, is monocyte chemoattractant protein-1(MCP-1) known as CCL2 fundamental role in both wound healing and angiogenesis [261-265]. We previously demonstrated that cultured ASCs constitutively produced MCP-1 in abundance (30ng-50ng/mL/million cells),. MCP-1 induces HUVEC cells to form tubes, and increases collagen synthesis by fibroblasts in a dose-dependent manner [266, 267]. Following the topical application of ASCs in the ovine burn wound model, angiogenesis increased; we surmise that this increase is due to ASC-produced MCP-1acting via the cognate receptor of MCP-1 (C-C chemokine receptor type 2 (CCR2)) which is predominantly expressed by endothelial cells. We and others have shown that ASC-CM or co-culture of ASCs with fibroblasts modulates components of the extracellular matrix including collagen levels [231, 268]. MCP-1 has also been reported to modulate fibroblast migration [269]. Significant increases in the implanted graft size, abundance of collagen 1A, VEGF expression, and blood flow in ASC-treated animals may be due to ASC-produced MCP-1.

Given these findings, we hypothesize that the beneficial effects resulting from ASC application to the burn wound is due to increased expression of MCP-1, which may be modulated by the surrounding microenvironment in order to support angiogenesis and tissue remodeling. To elucidate the role of ASC–produced MCP-1 in wound healing and

angiogenesis, we used an established ovine burn wound model and conducted complimentary *in vitro* experiments.

MATERIALS AND METHODS:

Ovine model of burn injury

All animal experiments adhered to the guidelines detailed in the NIH Guide for the Care and Use of Laboratory Animals. The study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch, Galveston, TX, USA. The experiment was conducted as previously described in chapter 3. Following burn injury, wound biopsies were flash frozen and stored at -80^{o}C until subsequent analysis. ASCs were isolated from non-burned animals as previously described in chapters 2 and 3.

Cell culture:

The following cells were purchased: human bone marrow derived stem cells (bMSCs : cat# PCS-500-012™, ATCC, Manassas, VA), human ASCs (IASCs : cat # R7788115, Invitrogen Thermofisher , Tampa, FL), , and primary human neonatal dermal fibroblasts(PCS : cat #PCS-201-010^{TM} , ATCC, Manassas, VA). Primary human ASCs (PASCs) were isolated from a burn patient; primary ovine ASCs were isolated from a non-burned sheep.. Cells were grown and passaged in Dulbecco's Minimum Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and 2% antibacterial/antimycotic solution (10,000IU/mL Penicillin; 10,000ug/mL Streptomycin; 25ug/mL Amphotericin; 8.5g/L NaCl). Fourth passage cells were used for all of the experiments.

Quantitation of MCP-1 in ASC-CM:

Fourth passage cells were plated at a density of 10^4 cells per cm^2 in complete media. After ~80% confluency was reached, the cell culture was serum starved for 12 hours with 2% FBS-containing media. Following serum starvation, cells were treated with 100ng/mL lipopolysaccharide (LPS), 2% burn serum, or 2% FBS media (control) for 24 hours. MCP-1 abundance in the CM was quantitated via ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Preparation of ASC-CM for experimental use:

ASC-CM was used in the scratch assay, migration assay, and angiogenesis assay. Confluent ASCs were grown in complete media containing 10% FBS for 24 hours, and the media harvested. The collected media was centrifuged at 400g for 10 minutes, the supernatant aspirated and then stored at -80^{o}C for future use.

Cell migration via scratch assay:

Plastic culture dishes were coated with 50ug/mL of collagen for 4 hours. Following the removal of the solution, plates were washed with PBS. Fourth passage PCS fibroblasts were seeded in 12 well plates at a density of $1.5x10^5$ cells per well with complete media (DMEM with 10% FBS). One day after the plating, when the cells were confluent, the media was changed to DMEM with 2% FBS for 12 hours. The scratch assay was performed using the Tissue Culture Wounder, US Utility Patent Application Number: US2015/0024425A1, a device with a pipet tip attached at a precise angle, which can move along the tissue culture surface with uniform pressure to make consistent and

reproducible scratches. Following delivery of the scratches, media was replaced with MCP-1 recombinant protein (Abcam cat#73866, Cambridge, MA) at a concentration of 1ug/mL or 100ng/mL, or ASC –CM at a concentration of 2%, 5%, or 10%, or 2% or 10% FBS as controls. Photographs were taken with a Nikon Diaphot 300 microscope equipped with a 10x lens at 0, 2, 4, and 7 hours following the treatment. Total wound closure was measured using the To scratch program (http://www.cselab.ethz). Wound closure was determined by using the following formula:

(area of the wound at experimental time points / area of wound at time 0) x 100%, starting at 100% wound area at time 0.

Real-time PCR:

Total RNA was isolated according to the manufacturer's instructions (RNeasy mini kit, Qiagen, Chatsworth, CA). RNA was quantified (NanoDrop Technologies, Wilmington, DE), and reverse transcription reactions were performed with 500 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was completed using SYBR green while the Step One plus Life Technologies real-time PCR machine was used for amplification and data collection. PCR was carried out at 95 ^{o}C for 10 minutes; with 40 cycles at 95 ^{o}C for 15 seconds, followed by 55 ^{o}C for 30 seconds. The primer sequences are listed in **Table 4.1** and **4.2**. Sheep primers were obtained from published studies of Lloyd et al [270]. The delta delta CT method was used to quantify gene expression. Cyclophilin A and 18s expression was used as internal housekeeping genes.

Table 4.1: Primers for human ASCs and ovine ASCs characterization

Gene	Forward	Reverse
Human		
cyclophilin	CTCGAATAAGTTTGACTTGTGTTT	CTAGGCATGGGAGGGAACA
CD34	TGGGCATCGAGGACATCTCT	GATCAAGATGGCCAGCAGGAT
CD11b/c	CTTGCCTTTCACCACCTGAT	TCCCAGGCTCCAGTATTTTG
CD73	CAGACTCATGATGACAGAGG	GAGATGTACAGGATCTTCCC
CD90	CACCAGTCACAGGGACATGA	ACCTACACGTGTGCACTACCA
CD105	CGTGGACAGCATGGACC	GATGCAGGAAGACACTGCTG
CD44	CAGGAAGAAGGATGGATATGG	ATTACTCTGCTGCGTTGTC
Sheep		
cyclophilin	CAT ACA GGT CCT GGC ATC TTG TC	TGC CAT CCA ACC ACT CAG TCT
CD34	TGGGCATCGAGGACATCTCT	GATCAAGATGGCCAGCAGGAT
CD11b/c	CCTTCATCAACACAACCAGAGTGG	CGAGGTGCTCCTAAAACCAAGC
CD73	TGGTCCAGGCCTATGCTTTTG	GGGATGCTGCTGTTGAGAAGAA
CD90	CAGAATACAGCTCCCGAACCAA	CACGTGTAGATCCCCTCATCCTT
CD105	CGGACAGTGACCGTGAAGTTG	TGTTGTGGTTGGCCTCGATTA
CD44	GTGTCGTGTGCCCAGTTATGA	CTCGTCAGAGGTCCCATTTTC

Table 4.2: Sheep and human primers used for analyzing angiogenesis related markers

Target	Forward	Reverse
Sheep_Cyclophilin	CATACAGGTCCTGGCATCTTGTC	TGCCATCCAACCACTCAGTCT
Sheep_MPC-1	CAAGACCATCCTGGGCAAA	GTCCTGGACCCATTTCAGGTT
Human_MCP-1	CCC CAG TCA CCT GCT GTT AT	AGG TGA CTG GGG CAT TGA TT
Human_VEGF165	CCCTGGCTTTACTGCTGTAC	TCTGAACAAGGCTCACAGTG
Human_HIF-1α	TCTGGATGCTGGTGATTTGG	GTGAATGTGGCCTGTGCAGT
Human_18s	GTAACCCGTTGAACCCCATT	CCATAAAATCGGTAGTAGCG

Migration Assay:

The effect of MCP-1 on cell migration was assessed using the xCELLigence® system (**MANUFACTURER, CITY, STATE**). PCS cell lines were grown in tissue culture plates with 10% FBS, followed by overnight serum starvation in DMEM with 2% FBS. After 12 hours, 4 x 10^4 PCS fibroblasts were seeded in the xCELLigence® cell culture plates, and cells were treated with 10 ng/mL human recombinant protein MCP-1, 2% or 5% ASC-CM, or 2% or 10% FBS in DMEM. To ensure that the effects were due to

MCP-1, the peptide was neutralized by a 1 hour incubation with the MCP-1 neutralizing peptide (ab9669, Cambridge, MA,) following the manufacturer's protocol. Cell migration was monitored every 10 minutes using the real-time cell electronic sensing (RT-CES)® system, which measures the migration based on impedance. Impedance is expressed as an arbitrary unit called the Cell Index. Data was recorded for 15 hours and then analyzed.

Protein Detection via Western Blot:

Whole tissue and cell extracts were fractionated by SDS-PAGE and electrophoresed on 15 wells, with 4-20% gradient Biorad gels. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. After incubation with 5% BSA in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 minutes, the membrane was washed with TBST for 15 minutes three times. The membrane was incubated with antibodies against GAPDH (1:1000), HIF-1α (1:250), VEGF (1:500), collagen 1a (1:500), or MCP-1 (1:20) at 4 °C, overnight. The membranes were washed three times for 15 minutes each, and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti- rabbit or mouse secondary for one hour at room temperature. Blots were then washed with TBST three times and the signal detected with the ECL system (Thermo Pierce, Tampa, FL) according to the manufacturer's protocols.

Statistical Analysis:

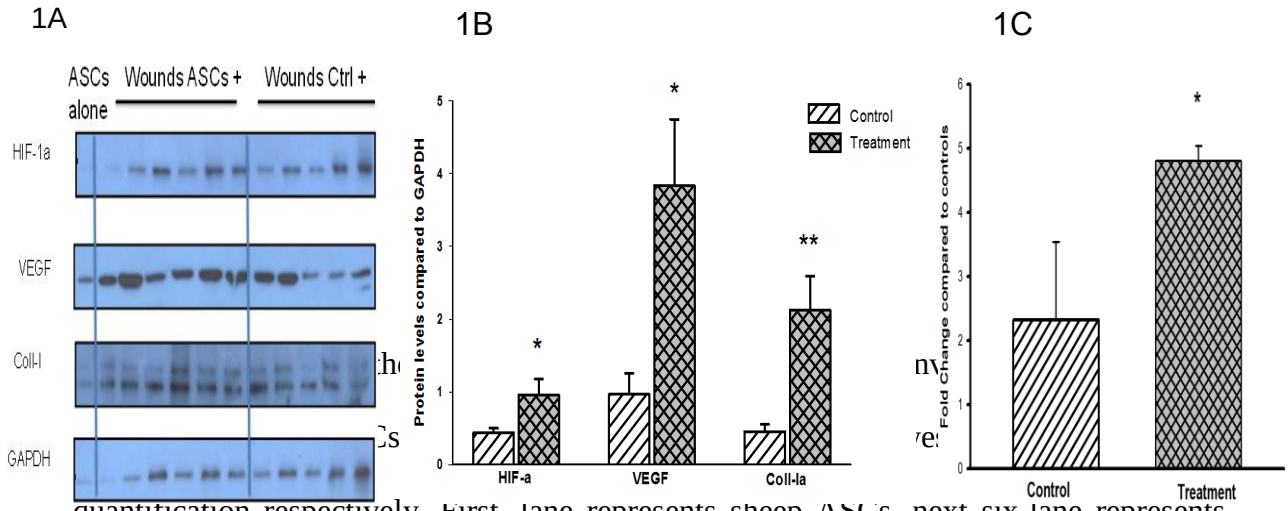
The following statistical tests were used where appropriate: Analysis of variance (ANOVA), followed by Tukey's test; and paired or unpaired Student's *t* tests. Data were expressed as the mean ± standard deviation or as the mean ± standard error of the mean.

Significance was accepted at $p < 0.05$.

RESULTS:

VEGF, HIF-1α, and collagen-1A expression are increased in ASC-treated wounds

Skin tissue biopsies treated with ASCs or controls were measured for HIF-1α, VEGF, and collagen 1A levels. Protein levels were normalized to GAPDH. ASCs treatment significantly increased the expression of HIF-1α ($p<0.05$), VEGF ($p<0.05$), and collagen 1A ($p<0.01$) compared to the controls **(Fig 4.1)** Although a variety of anti-sheep MCP-1 was tested, due to lack of appropriate antibody MCP-1 was not visualized in the western blot. *(Data not shown)* However, mRNA levels of MCP-1 were significantly higher in ASCs treated animals compared to the controls ($p<0.05$) **(Fig 4.1).**



quantification respectively. First, lane represents sheep ASCs, next six-lane represents treatment (n=6) and the remaining 5 lanes represents control (n=5). Error bar represents standard error of mean (*$p< 0.05$, **$p<0.005$). 1C Increase in the mRNA levels of MCP-1 in ASCs treated animals (*$p< 0.05$)

ASC-CM or MCP-1 both increases scratch wound closure

In a previous study (Chapter 3), application of ASCs increased the implanted graft size, which is mainly composed of ECM and dermal fibroblast. To study the effect of ASC-CM and MCP-1 on fibroblast, migration scratch assay was performed using neo-natal human dermal fibroblast cells. Fibroblast wound closure was significantly higher in both the ASC-CM media and MCP-1 treatments at different time points 2 hours ($p<0.05$), 4 hours ($p<0.01$) and 7 hours ($p<0.001$) and concentrations compared to the control (2% FBS only). Control wells scratch area increased initially, but returned to initial area size after 7 hours. MCP-1 increased the wound closure significantly in a dose-dependent manner at 2 and 7 ($p <0.05$)hours following the scratch assay. Although no significance in wound closure was observed the different concentration of ASC-CM treatment, ASC-CM treated cells displayed significantly higher wound closure compared to 10% FBS at all the time points examined ($p<0.05$) (**Fig 4.2).**

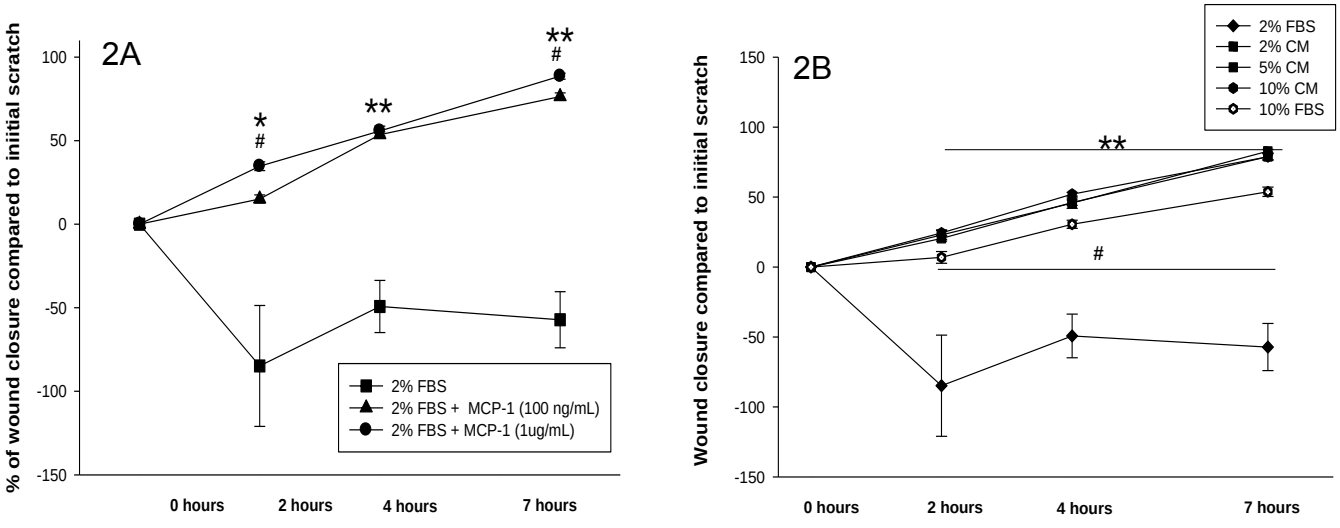


Figure 4.2: MCP-1(A) and ASCs CM (B) decreases the size of the scratch wound area.

Wound closure was significantly higher with all the treatments compared to the control. In fig 2A symbol asterisk (*) represents wound closure of MCP-1 (100 ng/mL) and MCP-1 (1ug/mL) compared to the controls, while hashtag # represents ($p<0.05$) wound closure of MCP-1 (1ug/mL) compared to MCP-1 (100 ng/mL). In fig 2B symbol asterisk (*) represents wound closure of control 2% FBS compared to 2, 5, 10% ASCs CM, while hashtag # represents wound closure of 10% FBS compared to ASCs CM. Error bar represents standard error of mean (n=3, # =$p<0.05$, *=$p<0.01$, **=$p<0.001$).

ASC-CM significantly increased the migration of fibroblast cells

To corroborate the scratch assay study, XCELLigence® system was used to study the effects of ASC-CM or MCP-1 peptide on fibroblast migration. Fibroblast treated with 2% or 5% of ASC-CM had significant increase migration ($p<0.05$) compared to the other treatments including control (2% FBS), positive control (10% FBS), and MCP-1. MCP-1 peptide treated fibroblast had higher migration rate compared to the control as shown by higher cell index. MCP-1 neutralizing antibody decreased the MCP-1 induced migration; however, the significance was not reached **(Fig 4.3).**

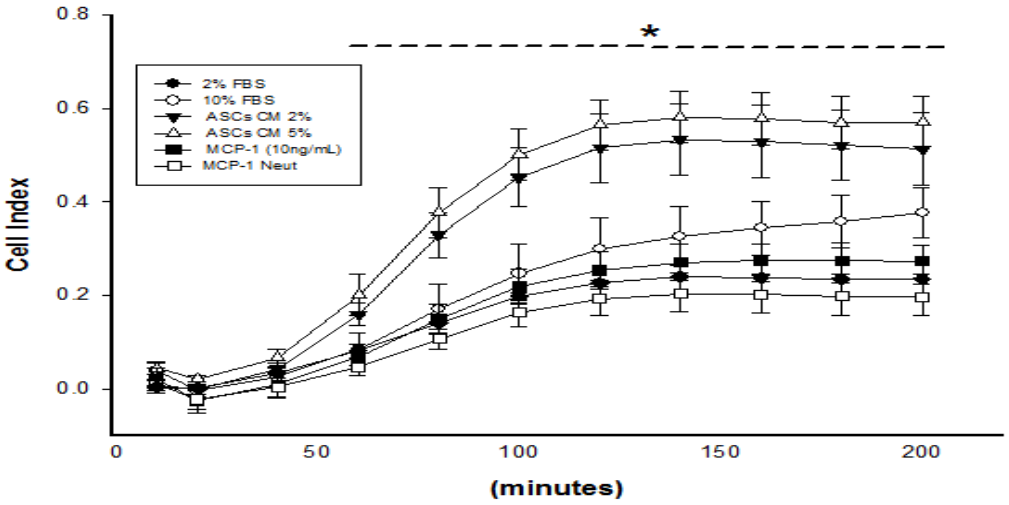


Figure 4.3: ASC-CM or MCP-1 peptide both stimulates fibroblast cells migration. Fibroblast cell lines were treated with ASC-CM (2% and 5%), MCP-1, MCP-1 neutralizing antibody, 10% FBS as a positive control and along with 2% FBS as an internal control (n=3, * =$p<0.05$).

ASC-CM and MCP-1 increased angiogenesis-related genes in HUVEC cell lines.

Application of ASCs or ASC-CM has been associated with an increase in angiogenic pathway. ASC-CM or MCP-1 peptide treatment resulted in significant increase in the mRNA levels of angiogenesis-related genes including VEGF-165 and HIF-1α ($p<0.05$). Interestingly, treatment with MCP-1 peptide or ASC-CM further increased the MCP-1 transcript levels in HUVEC cells ($p<0.05$) **(Fig. 4.4).**

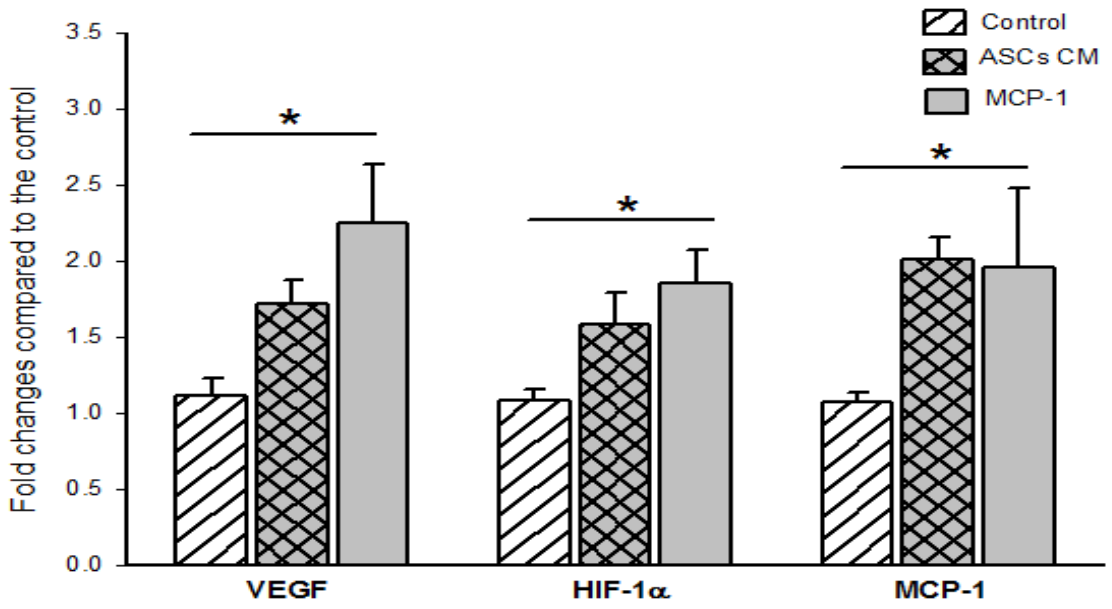


Figure 4.4: MCP-1 and ASC-CM increases angiogenesis-related genes in HUVEC cell line. HUVEC cells were treated for 24 hours with either 100 ng/mL of MCP-1 or 2% of conditioned media (CM) obtained from ASCs. Transcript levels of MCP-1, HIF-1α and VEGF-165 were measured with real-time PCR and compared to 18s as an internal control. Error bar represents standard error of mean (n=3, * =$p<0.05$).

MCP-1 is abundantly expressed by ASCs under normal and challenged conditions

Basal expression of MCP-1 was significantly higher in ASCs obtained from burn patient (PASCs) as well as ASCs purchased from the vendor (IASCs) compared to bMSCs or PCS ($p<0.001$). Following LPS (100ng/mL) challenge, all the cell lines had significantly higher MCP-1 expression compared to the basal levels, except for the fibroblast cell lines. When aforementioned cell lines were challenged with 2% burn serum, both ASCs significantly increased the MCP-1 levels compared to the bMSCs or fibroblast cell lines. Additionally, MCP-1 levels in the ASC-CM grown with 10% FBS, which was used for

scratch assay, migration assay, treatment of HUVEC cells, and angiogenesis assay were also measured. MCP-1 in the 10% FBS ASC-CM was (15115.82 ± 992.5 pg/mL per million cells). **(Fig. 4.5)**

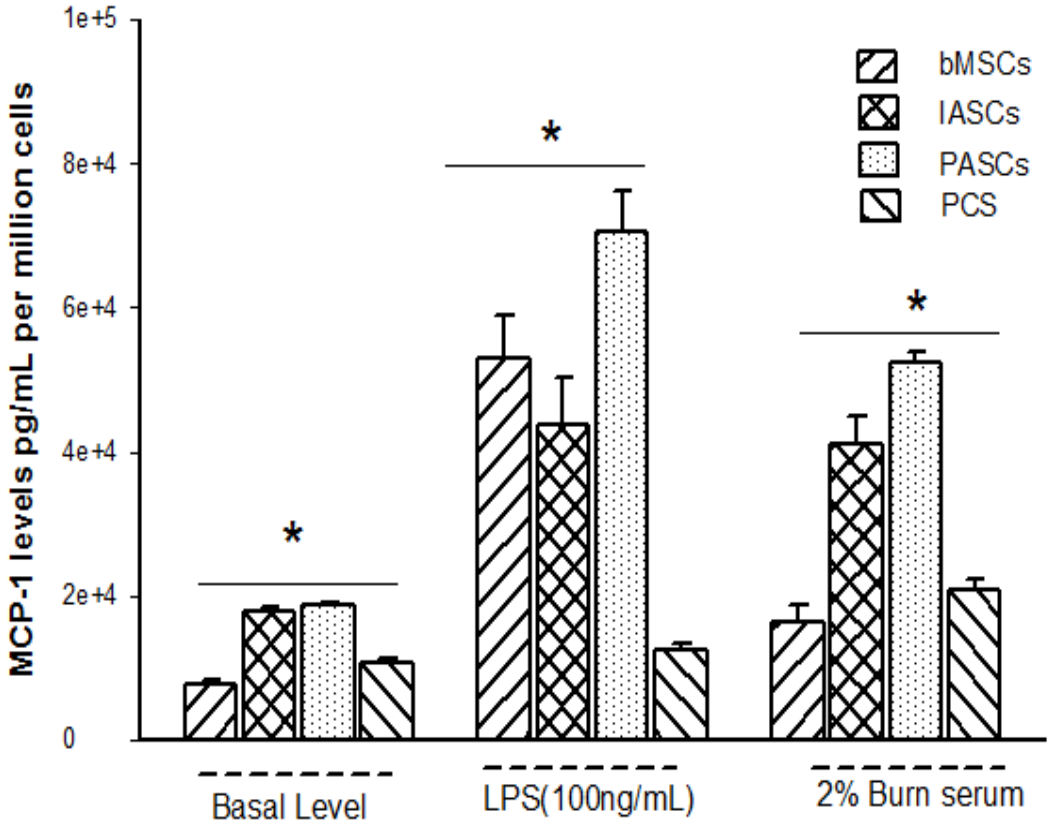


Figure 4.5: MCP-1 is abundantly expressed by ASCs under normal and challenged conditions. bMSCs, PASCs, IASCs, and PCS cell lines were treated with either LPS(100ng/mL) or 2% burn serum. Error bar represents standard error of mean (n=3, * =$p<0.05$).

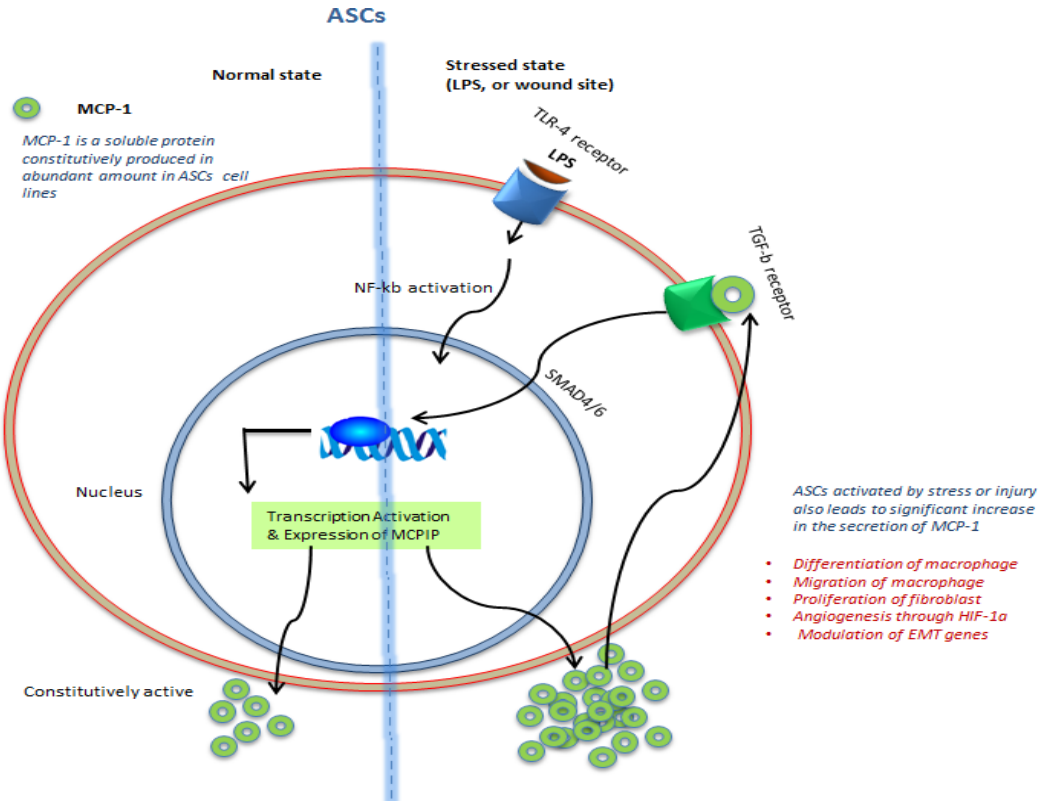


Figure 4.6: Schematic diagram of MCP-1 produced by ASCs

DISCUSSION:

ASCs produce abundant growth factor and cytokines either constitutively or following stimulation such as LPS or HGF [23, 233]. ASC derives its therapeutic properties by modulating a number of growth factors under different niches. In this study, we demonstrated that MCP-1 is one of the vital secretomes of ASCs produced in an abundant quantity that has a vital role in wound healing and angiogenesis. MCP-1 belongs to C-C chemokine family and produced as an inactive soluble factor by a diverse cell types including fibroblast, endothelial, epithelial, astrocytes, smooth muscle including ASCs [261, 271]. Besides the established role in immunity, recently MCP-1 has been documented for its angiogenic capabilities [266, 272, 273]. Niu et al, observed increased

tube formation in HUVEC with increasing concentration of MCP-1 as measured by angiogenesis assay. In the same study, MCP-1 treated HUVEC cells had increased expression of VEGF and HIF-1α via activation of a novel transcription factor, MCP-1 induced protein (MCPIP) [266]. Similarly, in our *in vivo* ovine burn graft model, application of ASCs increased the protein level of VEGF, Coll-1A, and HIF-1α expression. Due to lack of appropriate anti-sheep antibodies, various other growth factors including MCP-1was measured via Real Time PCR in the ASCs treated and control animals. ASCs treated animals had a significant increase in the transcript levels of MCP-1 compared to the controls. Although different anti-sheep antibodies listed in the research studies were tested for MCP-1[274, 275], both of the antibodies either failed to generate a signal or generated non-specific staining *(data not shown)*. To understand the role of MCP-1 and ASC-CM mediated angiogenesis, HUVEC cells were treated with either MCP-1 or ASC-CM, both treatments increased the transcript levels of VEGF-165, HIF-1α as well as MCP-1, a similar observation was reported by Hong et al., in human aortic endothelial cells [273]. Additionally, we also observed a significant increase in the tube formation when HUVEC cells were stimulated with either ASC-CM obtained from hypoxic or normoxic conditions compared to the controls.

MCP-1 or CCL2 is known to bind to two surface receptor proteins CCR2 and CCR4 which are expressed in predominantly in endothelial cells and monocytes and other hematopoietic cells including natural killer (NK) cells [276-279]. Growth factors like VEGF [64], PDGF [280]and TGF-b[281] are constitutively produced by ASCs, and all the aforementioned growth factors are also involved in the production of MCP-1 through positive feedback signaling [273, 282, 283]. MCP-1 has also been shown to modulate its

expression via paracrine and autocrine manner in different cell lines [284, 285]. In our *in vitro* study, treatment of HUVEC cells with ASC-CM or MCP-1 peptide further increased the MCP-1 transcript levels. While in our ovine graft burn wound study, topically applied ASCs resulted in significant increase in transcript levels MCP-1. ASC-produced MCP-1 may have induced more MCP-1 production in the endothelial cells and other cells residing at the wound site, resulting in an accelerated rate of angiogenesis via production of angiogenic factors. Both types of ASCs, (IASCs and PASCs) secreted significantly higher levels of MCP-1 constitutively or when stimulated with 2% burn serum compared to the bMSCs or fibroblast cell lines, indicating that ASCs respond to the burn niche by increasing the production of MCP-1 protein as observed in ovine burn model. Although numerous growth factors and cytokines are produced by ASCs, an increase in angiogenesis signaling following ASC-CM or ASCs application can be partially attributed to the abundance of MCP-1 protein by produced by ASCs either constitutively or when stimulated **(Fig 4.6)**.

Besides angiogenesis, the role of ASC- produced MCP-1 in extracellular matrix production was also studied. Similar to our published studies[268], other researchers have reported that co-culture of ASCs and fibroblasts increase the transcript and the protein levels of a family of collagen genes [66, 200, 268]. Gharaee-Kermani et al, has reported that MCP-1 stimulates fibroblast collagen synthesis by activating TGF-b pathways. In the same study, the increasing concentration of MCP-1 was correlated with an increase in collagen synthesis *in vivo* and *in vitro* model. Quentin et al., observed that MCP-$1^{-/-}$ mice had significantly less (0.0001) amount of hydroxyproline, a precursor molecule for collagen, than those of wild-type mice and the wound closure was significantly delayed

in the MCP knockout mice [265]. Recently, a more relevant study conducted by Bieley's et al on full-thickness burn wound found a significant increase in the adipogenesis, vascularity and transcript levels of collagen type I and III following the application of ASCs. Similar findings were reported by Karim et al.[286, 287]. Although, several studies have reported an increase in the collagen synthesis following ASCs application or co-culture experiments, the molecular mechanisms behind the increase in the collagen levels have not been explored [66, 241]. In our study model, application of ASCs on the ovine burn graft wound model significantly increased the collagen-1A protein levels and MCP-1 transcript levels. While *in vitro* studies, we observed a significant increase in scratch wound closure with the application of ASC-CM or MCP-1 at different concentrations. Migration assay also demonstrated that ASC-CM predominantly increased the fibroblast cell lines migration relatively in a dose-dependent manner compared to the controls or MCP-1 treatment. In the presence of MCP-1 neutralizing antibody, migration was decreased compared to the MCP-1 treatment alone. Significant increase in the migration of fibroblast cells in ASC-CM treated samples could be attributed to the fact that ASC-CM had high levels of MCP-1 expression (15115.82 ± 992.5 pg/mL per million cells) and another possibility is due to the presence of other secretome in ASC-CM. These studies taken together insinuate that ASC-produced MCP-1 has a fundamental role in angiogenesis and wound healing. MCP-1 knockout ASCs cell lines are further warranted to demonstrate the vital role of ASC-produced MCP-1 in angiogenesis and wound healing.

Summary and conclusion

While numerous published studies have demonstrated the versatile use of ASCs to combat different disease conditions, a limited number of these studies have focused on the molecular mechanism behind the therapeutic properties of stem cells. Our novel study suggests that ASCs derived its restorative properties via modulation of secretome, especially MCP-1.

Summary and conclusion:

The overarching goal of this dissertation work was to study the stemness-related changes in the adipose-derived stem cells (ASCs) population following burn injury and to explore the medicinal properties of stem cells in treating burn injury. Although ASCs hold great promise in treating debilitating wounds including burns, it is imperative to check for the changes in post-burn ASCs before their autologous transplantation. Fat tissue, where ASCs resides, is a major source of inflammation in different disease states including burn injury, however, which component of the fat tissue contribute to inflammation and the duration of the inflammatory cascade in post burn ASCs is still unknown. We hypothesized that ASCs retain their stemness-related properties in culture following burn injury and these cells can be applied for autologous cell-based therapies. We tested this hypothesis in two specific aims.

The first research aim was to identify the component of adipose tissue contributes to the inflammatory cascade. This aim was addressed in chapter 2. Using a 60% rat scald burn model, we investigated the cellular source of inflammation within adipose tissue and studied whether inflammation affects ASC fate and function. The stromal vascular fraction (SVF), adipocytes, and ASCs were isolated from adipose tissue at varying times post burn and inflammatory makers were quantified. Burn injury significantly induced expression of inflammatory markers in adipocytes and the SVF at 24 and 48 hours post burn; expression of inflammatory marker mRNA transcripts and protein returned to normal in the SVF isolated one-week post burn. In all the time points, the post-burn inflammatory response was highest in the adipocytes, followed by the SVF and least in the cultured ASCs (Fig 2.1). Comet assay was used to determine the extent of DNA

damage in the SVF and ASCs population post-burn. In our study, we found that SVF isolated at 24 and 48 hours post burn had the highest DNA damage compared to SVF and enriched ASCs at different time points. And the damage was minimal-4 cells damaged in 1000 cells, suggesting that the extent of the damage may be insignificant, thus there may be a suitable window period to obtain SVF or ASCs following burn injury (Fig 2.2).

The second aim was to determine whether the cell surface markers, differentiation potential, proliferation, or cytokine expression were altered in post-burn ASCs. Cell surface markers for post burn ASCs were quantified for the expression of positive markers ($CD29^+$, $CD73^+$, $CD90^+$, and $CD36^+$) and negative markers ($CD11b^-$ and $CD34^-$). As per published literature, we were able to identify ASCs population in post burn adipose tissue and found that burn injury did not induce changes in ASCs population compared to the controls (Fig 2.5). To study the differentiation potential in post burn ASCs, ASCs were differentiated into four lineages including adipogenic, adipogenic, chondrogenic, osteogenic, and epithelial lineages. Differentiation was confirmed by oil-O-red (adipogenic cells), Alcian blue (chondrogenic cells), or alizarin red (osteogenic cells) or by immunostaining for CK-14 (epithelial cells; green) staining. In the same study, RNA was isolated from the differentiated cells and the mRNA expressions of lineage-specific genes were studied. Adipogenic (adiponectin and adipocyte lipid binding protein-1 (ALBP-1)), chondrogenic (Chondromodulin 1 and Collagen II), osteogenic (osteonectin and osteopontin), and epithelial lineages (cytokeratin 10 and 14) related genes expression were studied with real-time PCR. Our study demonstrated that burn injury does not alter the ability of the ASCs to differentiate into different lineages compared to the controls.

Besides differentiation and cell surface markers expression, another equally important aspect of stem cells is the ability to proliferate following the transplantation[58]. We compared the proliferation rate of post-burn ASCs and control using the real-time cell electronic sensing (RT-CES)® system (ACEA Biosciences, San Diego, CA). Post-burn ASCs proliferation over a 50-hour period was comparable to that of controls and no significant difference was observed.

Cytokines and growth factors expression are upregulated following a severe burn injury. To ascertain if we can observe the same phenomenon in cell culture conditions, we compared the expression of cytokines (IL-1β, IL-6, and MCP-1) and growth factor (TNF-α) expression in post-burn ASC conditioned media (ASC-CM) and controls via ELISA. While the expression of transcription factor NF-κB was measured via western blots. Protein levels of IL-1β and TNF-α were undetected in both groups. There was no significant difference in the protein levels of IL-6, MCP-1 and NF-κB between two groups. Interestingly, we observed that in ASC-CM, MCP-1 was secreted in abundant quantity by both control and post-burn ASCs (Fig 2.7).

The usage of autologous stem cells to attenuate adverse physiological conditions including burn and other disease states has garnered a lot of attention from the general public and research experts, however little research has been done to study the changes in the post burn adipose derived stem cells (ASCs) and the stromal vascular fraction (SVF) that may be used as a cell-based therapy to heal burn wounds or to generate skin substitutes. The study in chapter 2 describes dynamic changes in SVF and ASCs and provides a window for the usage of SVF and ASCs isolated from routinely discarded fat tissue. These results suggest that adipocytes and the SVF, not ASCs, are the main source

of inflammation after burns and that ASCs are unaffected by burn injury or culturing procedures. The study also suggests that cells isolated more than 48 hours post injury may be best for cell –based therapy or tissue engineering purposes. This study is significant because it provides much-needed information that can be used as a guide to harnessing the wound healing potential of stem cells following burn injury or other traumatic events. We believe that our findings from Chapter 2 could be translated into the clinic almost immediately and are likely to have a tremendous impact on burn care and other cellular-based therapies.

The foregoing chapters 3 and 4 provide insight into the application and molecular mechanism by which ASCs modulates wound healing. Researchers have explored the use of adipose-derived stem cells (ASCs) as a cell-based therapy to cover wounds in burn patients; however underlying mechanistic aspects are not completely understood. We hypothesized that ASCs would improve post-burn wound healing after eschar excision and grafting by increasing wound blood flow through induction of angiogenesis-related pathways via soluble factors including MCP-1. To test this hypothesis, we used an ovine burn model. A 5 cm^2 full thickness burn wound was induced on each side of the dorsum. After 24 hours, the burned skin was excised and a 2 cm^2 patch of autologous donor skin was grafted with either topical application of 7 million allogeneic characterized ASCs or placebo treatment (PBS). In our study, topical application of ASCs significantly increased wound blood flow as measured by microsphere fluorescence and laser Doppler techniques ($p<0.05$) (Fig 3.4). Potent pro-angiogenic factor VEGF protein expression was also significantly increased in ASCs treated animals compared to the controls (Fig 3.5). Animals treated with ASCs also had an accelerated the patch graft growth and better

granulation (Fig 3.3). In brief, ASCs accelerated grafted skin growth possibly by promoting angiogenesis via soluble factors. In the same study, we also found a significant increase in the protein levels of VEGF, collagen-1A, and HIF-1α levels in ASCs treated animals.

Published literature supports that topical application of stem cells increases blood flow to the wound [64, 102, 180, 201]. However, how ASCs aid in wound healing and angiogenesis is still less understood. Using the ovine model described in chapter 3, we studied different proteins secreted by ASCs that pay a fundamental role in wound healing including MCP-1. From our previous chapter 2 (Fig 2.7), we observed that ASCs constitutively secretes abundant quantity of MCP-1 protein. In our ovine burn model, we found that the transcript level of MCP-1 was significantly higher in ASCs treated animals compared to the controls. Monocyte chemoattractant protein-1(MCP-1) also known as CCL2, has a fundamental role both wound healing and angiogenesis [261-265]. To elucidate the role of ASCs –produced MCP-1 in wound healing and angiogenesis we used an established sheep burn injury model and conducted various *in vitro* experiments.

First, we compared the expression of MCP-1 in ASCs obtained from the burn-injured patient; control ASCs, bone marrow derived stem cells (bMSCs) and fibroblast obtained from the vendor via ELISA. Both ASCs, patient-derived and ASCs obtained from the vendor, had a significantly higher basal levels of MCP-1 compared to bMSCs (bone marrow derived stem cells), and fibroblast cell lines. When different cells lines were challenged with 2% burn serum, both ASCs secreted a significant amount of MCP-1 compared to the controls. To delineate the role of ASCs- produced MCP-1 in angiogenesis, we used human umbilical vein endothelial cells (HUVEC), when treated

with MCP-1 or 2% of ASC-CM both treatments increased the expression of MCP-1, VEGF-165 and HIF-1α at the transcripts levels compared to the controls. Additionally, ASCs CM significantly increased the tube formation of endothelial cells in an angiogenesis assay.

In our *in vivo* ovine sheep study, we observed that application of ASCs increased the expression of MCP-l. Similar results were obtained *in vitro* HUVECs study, following the application of ASCs-CM or MCP-1. Published studies have reported that MCP-1 is regulated via autocrine and paracrine fashion in different cells lines and disease conditions [284, 285]. Following the application of ASCs in the excised burn wound, ASCs may have been stimulated by the wound niche or constitutively produced MCP-1 from ASCs may have upregulated the expression of MCP-1 in different cell lines including the endothelial cells in autocrine and paracrine fashion resulting in increased angiogenesis.

Topical application of ASCs not only improved blood flow but also increased the size of implanted graft. MCP-1 has been reported to increase fibroblast migration, collagen synthesis in different experimental models [261-265] [269]. To understand the role of ASCs-produced MCP-1 in fibroblast migration, scratch assay, and migration assay were conducted. Both assays demonstrated that both MCP-1 and ASCs-CM increased the fibroblast migration. Published studies from our laboratory have also demonstrated that application of ASCs-CM modulates family of collagen genes and other proteases responsible for ECM remodeling [231, 268].

Given these findings, our study implies that ASCs secretes abundant quantity of MCP-1 either constitutively or when stimulated. The ASCs-produced MCP-1 levels fluctuate

depending upon the microenvironment giving ASCs its wound healing and angiogenic properties.

While numerous published studies have demonstrated the versatile use of ASCs to combat different disease conditions, a limited number of these studies have focused on the molecular mechanism behind the therapeutic properties of stem cells. Although MCP-1 knockout ASCs can further corroborate the role of ASCs-produced *in vitro* MCP-1 in wound healing, our initial study suggests that ASCs derive its restorative properties via modulation of secreted proteins, especially MCP-1. α

Conclusion:

Although further studies are warranted to elucidate specific genomic changes in post burn ASCs, these results so far hint that post-burn ASCs may be utilized for cell-based therapy and tissue engineering purposes. Using an established rat scald burn model, we demonstrated that adipocytes and stromal vascular fraction are the sources of persistent inflammation for 48 hours post-injury; while in cultured adipose derived stem cells inflammation is similar to the controls. Additionally, while using ovine burn graft model we also demonstrated that topical application of ASCs increases the rate of wound healing by increasing the genes involved in angiogenic pathways via soluble factor such as MCP-1.

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281. Kim, B.S., K.S. Kang, and S.K. Kang, *Soluble factors from ASCs effectively direct control of chondrogenic fate.* Cell Prolif, 2010. **43**(3): p. 249-61.
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Vita

November 15, 2016

CURRICULUM VITAE

NAME: Anesh Prasai, Ph.D.

PRESENT POSITION AND ADDRESS:

Graduate Assistant
Department of Neuroscience and Cell Biology/ Surgery
The University of Texas Medical Branch

301 University Blvd.
Galveston, Texas 77555-1220

Phone: 409-770-6867
Fax: 409-770-6919

EDUCATION:

May 1994	Warren Wilson College, NC	B.A.
December 2016	University of Texas Medical Branch, Galveston, TX.	Ph.D.

PROFESSIONAL AND TEACHING EXPERIENCE:

2013 – 2015	High School Biomedical Research Program Mentoring, University of Texas Medical Branch, Galveston, TX
2012 – 2013	UTMB Medical Student Summer Research Program, University of Texas Medical Branch, Galveston, TX
2006 – 2010	Teaching assistant for Chemistry, Statistics, Calculus, and Physics Warren Wilson College, Swannanoa, NC

RESEARCH ACTIVITIES:

Area of Research

Isolation, characterization, and application of adipose-derived stem cells for burn wound healing and attenuating post-burn hypertrophic scarring
Beta-adrenergic receptor signaling
Ubiquitination signaling
Development of techniques and protocols
Animal studies and translational research

TEACHING/ TRAINNING:

a. Students/Mentees/Trainees:

i. Post-doctoral fellows:

2016 – present	Janosh Cambiaso-Daniel, M.D.
2016 – present	Gabriel Hundeschagen, M.D.
2014 – 2016	Paul Wurzer, M.D.

2013 – 2014	Padma Nainar, M.D.
2012 – 2015	Jessica Tanksley, M.D.

ii. Ph.D. Degree Students:

2015	Catherine Sampson, 1st year GSBS student, rotation
2015	Ashley Smith, 1st year GSBS student, rotation
2015	Suzan Alharbi, 1st year GSBS student, rotation
2014 -present	Jayson Jay, 1st year GSBS student,
2013-present	Robert Patrick Clayton, 1st year GSBS student
2013	Alice Bittar, 1st year GSBS student, rotation
2012-present	Michael Wetzel, 1st year GSBS student,

iii. Medical students:

2016	Aman Jain, MSSRP
2015	Suhas Kochat
2013	Adam Hensley, MS1

iv. Undergraduate students:

2014	Zachary Seikel, SURP program
2013	Chase Van Gorp, Baylor student / SURP program
2011-2012	Anastasia Offrodile, UTMB PREP program
2011-2012	Aman Jain, Baylor University student

v. High school students:

2016	Raima Siddiqui, Junior, High School Summer Research Program
2016	Zoe George, Junior, High School Summer Research Program
2014-2015	Tania Gonzalez, Junior, Ball High Research Program
2013-2014	Quincy Segal, Senior, Ball High Research Program
2012	Rawena Jacob, sophomore
2010	Aman Jain, senior

MEMBERSHIP IN SCIENTIFIC SOCIETIES/PROFESSIONAL ORGANIZATIONS

2010 – current	Sigma Xi
2010 – current	theiKON, Non-profit Organization, (founding member)
2010 – current	American Chemical Society
2011 – current	Tissue Engineering and Regenerative Medicine International Society
2014 – 2015	Vice-president, Cell Biology program
2011 – 2013	Shock Society

AWARDS / SCHOLARSHIPS / HONORS:

2016	Best Presentation, Cell Biology Program, UTMB, TX
2015	Barbara K. Kolmen scholarship award, UTMB, TX
2014	UTMB Retirees Scholarship Award, UTMB, TX
2014 – 2015	Ball High School Bench Program, Best Mentor, UTMB, TX
2013 – current	Social Service award (2013), Cell Biology Program, UTMB, TX
2014 – 2015	Vice-president, Cell Biology program, UTMB, TX
2009	Beebe International Scholarship, Warren Wilson College, NC
2008	Forbes Scholarship, Warren Wilson College, NC

2007 Robinson International Scholarship, Warren Wilson College, NC
2006 – 2010 Woodbury Annual Scholarship, Warren Wilson College, NC

PUBLISHED:

A. ARTICLES IN PEER-REVIEWED JOURNALS:

1. Bohanon FJ, Wang X, Graham BM, **Prasai A**, Vasudevan SJ, Ding C, Ding Y, Radhakrishnan GL, Rastellini C, Zhou J, Radhakrishnan RS. Enhanced anti-fibrogenic effects of novel oridonin derivative CYD0692 in hepatic stellate cells. //Molecular and Cellular Biochemistry, 2015, Dec;410(1-2):293-300 PMID: 26346163

2. Mascarenhas DD. Elayadi A. Singh BK. **Prasai A.** Hegde SD. Herndon DN. Finnerty CC. Nephrilin peptide modulates a neuroimmune stress response in rodent models of burn trauma and sepsis. International Journal of Burns & Trauma. 3(4):190-200, 2013.

3. Shavkunov, A., Panova, N., **Prasai, A.,** Veselenak, R., Bourne, N., Stoilova-McPhie, S., Laezza, F. Bioluminescence methodology for the detection of protein-protein interactions within the voltage-gated sodium channel macromolecular complex // Assay and Drug Development Technologies, 2012, Apr;10(2), pp. 148-60.

4. Collin F D., **Prasai A** Intrinsic Variability of Beta Lyrae Observed with a Digital SLR Camera// The Society for Astronomical Sciences 28th Annual Symposium on Telescope Science. Held May 19-21, 2009 at Big Bear Lake, CA. Published by the Society for Astronomical Sciences., p.121

5. Mascarenhas DD; El Ayadi A; Wetzel M; **Prasai A**; Mifflin R; Jay J; Herndon DN; Finnerty CC**.** Hepcidin-dependent effects of the Nephrilin peptide on post-burn glycemic control, renal function, fat and lean body mass, and wound healing. Journal of Burn Care and Research. 2016.

B. OTHER:

Thesis/Dissertation

Characterization and application of adipose-derived stem cells following burn injury

C. PUBLISHED ABSTRACTS:

1. Enkhbaatar P, Hiroshi I, **Prasai A**, Malgerud E, Finnerty C**,** Traber L, Herndon D, Traber D. Preclinical evaluation of adipose-derived mesenchymal stem cell efficacy in grafter burn wound healing. Journal of Tissue Engineering and Regenerative Medicine. 6 (Suppl 1) 266, 2012.

2. El Ayadi A, **Prasai A**, Pazdrak K, Bergmann J, Herndon D, Finnerty CC. Burn injury

alters overall protein phosphorylation in adipose tissue. Shock 37 (Suppl 1) 118-119, 2012.

3. **Prasai A**, Pazdrak K, El Ayadi A, Bergmann J, Hegde S, Herndon D, Finnerty CC. Burn injury alters adipose derived stem cell. Shock 37 (Suppl 1)118, 2012.

4. Finnerty CC**, Prasai A**, El Ayadi A, Pazdrak K, Herndon D. Burn induced alterations in adipose derived stem cells. Journal of Tissue Engineering and Regenerative Medicine. 6 (Suppl 1) 269, 2012.

5. El Ayadi A, Offordile AE, Herndon DN, Hegde S, **Prasai A**, Diaz EC, Sousse LE, Jeschke MG, Finnerty CC. IGF-1 reduces the hepatic unfolded protein response in thermally injured animals. Shock. 39(Suppl 2) 50, 2013.

6. Offordile AE, El Ayadi A, Herndon DN, Hegde S, **Prasai A**, Diaz EC, Sousse LE, Jeschke MG, Finnerty CC. Long-term activation of the endoplasmic reticulum stress following burn injury. Shock. 39(Suppl 2)69, 2013.

7. Bohanon FJ, Wang XF, Herndon DN, El Ayadi A, Ding Y, Wetzel M, **Prasai A,** Rastellini C, Finnerty CC, Zhou J, Radhakrishnan R. Oridonin Inhibits Burn Injury-Induced Activation and Inflammatory Signaling of Hepatic Stellate Cells. Journal of the American College of Surgeons. 219 (3) S41-S42, 2014.

8. Mascarenhas D, El Ayadi A, Singh BK, **Prasai A**, Herndon DN, Hegde S, Finnerty CC. Nephrilin peptide modulates a neuroimmune stress response in rodent models of burn trauma and sepsis. Shock41(Suppl 2) 39, 2014.

9. Mifflin R, **Prasai A**, Herndon D, Finnerty C. In vitro analysis of mesenchymal stem cell trafficking. Tissue Engineering Part A. 20 (Suppl 1)S7-S8. 2014.

10. **Prasai A**, Mifflin R, El Ayadi A, Herndon D, Finnerty C. Inflammation decreases under cell culture conditions. Tissue Engineering Part A. 20 (Suppl 1) S47-S48. 2014.

11. Mifflin R, **Prasai A**, Herndon DN, Finnerty CC, Burn Injury Does not Alter the Anti-Inflammatory Phenotype of Cultured Adipose-Tissue Derived Stem Cells. Tissue Engineering Part A. 21(Suppl 1): S86, 2015.

12. Wurzer P, Wetzel MD, **Prasai A**, Branski LK, Parvizi D, Tuca A, Madeo F, Kamolz LP, Herndon DN, Finnerty CC. Spermidine impacts Proliferation and Migration in Human Fibroblasts. Tissue Engineering Part A. 21 (Suppl 1) S139-S140, 2015

13. El Ayadi A, Wang Y, **Prasai A**, Wetzel M, Mifflin R, Herndon DN, Finnerty CC.

Oxandrolone in combination with exercise abrogates burn-induced proteolysis pathways in the diaphragm. Shock 43(6, Suppl 1) 67, 2015.

14. Alharbi S, **Prasai A**, El Ayadi A, Herndon D, Finnerty C. Adipose derived stem cells alter fibroblast extracellular matrix production. Tissue Engineering Part A. 21 (Suppl 1) S94-S95, 2015.

15. El Ayadi A, Wang Y, **Parsai A,** Jay J, Herndon D, Finnerty C. Effects of propranolol on skin angiogenesis following burn injury. Wound Repair and Regeneration. 23 (2) A20. 2015.

16. **Prasai A,** El Ayadi A, Mifflin R, Herndon D, Finnerty C. Burn injury induces inflammation and cell death in adipose derived stem cells (ASCs). Tissue Engineering Part A. 20 (Suppl 1) S87. 2015.

17. Stoilova-McPhie, S., **Prasai, A**., Shavkunov, A.,S., Panova, N., Laezza, F. Differential impact of fgf14 mutations on protein-protein interaction interfaces in macromolecular complexes. *Society for Neuroscience*, 2012 (New Orleans, LA)

18. Shavkunov, A.S., **Prasai, A**., Buzhdygan, T.P., Panova, N., Bourne, N., Veselenak, R., Stoilova-McPhie, S., Laezza, F. Model-based peptide inhibitors of FGF14:Nav channel interface. *Society for Neuroscience*, 2011 (Washington, DC)

19. Stoilova-McPhie, S., Shavkunov, A., Buzhdygan, T., **Prasai, A**., Laezza, F. Modeling of small peptide fragments as inhibitors of the FGF14:Nav channel complex formation. *UT Austin Conference on Learning & Memory*, 2011 (Austin, TX)

20. Shavkunov, A., Buzhdygan, T., **Prasai, A**., Stoilova-McPhie, S., Laezza, F. Modeling of Small Peptide Fragments as Inhibitors of the FGF14:Nav Channel Complex Formation. *Sealy Center 16th Annual Structural Biology Symposium*, 2011 (Galveston, TX)

PUBLICATIONS – SUBMITTED / IN REVIEW:

1. Mascarenhas DD; El Ayadi A; Wetzel M; **Prasai A**; Mifflin R; Jay J; Herndon DN; Finnerty CC**.** Hepcidin-dependent effects of the Nephrilin peptide on post-burn glycemic control, renal function, fat and lean body mass, and wound healing. Journal of Burn Care and Research. 2016.

2. Duan H, El Ayadi A, Herndon DN, **Prasai A**, Wetzel MD, Finnerty CC. JAK / STAT3 signaling contributes to diaphragm atrophy after severe burn injury in rats. Am J Resp and Critical Care Medicine.

3. El Ayadi A; **Prasai A**; Ye Yang; Herndon DN; Finnerty CC Beta 2 Adrenergic Receptor trafficking, degradation, and cell surface expression are altered by traumatic stress. The Journal of Biological Chemistry