

**THE EQUINE MESENCHYMAL STROMAL CELL SECRETOME AS A THERAPY  
FOR CUTANEOUS WOUNDS**

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# THE EQUINE MESENCHYMAL STROMAL CELL SECRETOME AS A THERAPY FOR HORSE CUTANEOUS WOUNDS

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Horses suffer from chronic skin wounds such as exuberant granulation tissue (EGT), also called “proud flesh”, and allergic dermatitis (AD) in part because there are no adequate treatments for these pathologies. Equine mesenchymal stromal cells (MSC) are adult multipotent progenitor cells that can be isolated from a variety of horse tissues and expanded in culture. These cells have been used for decades by clinicians as a biologic treatment for orthopedic injuries of horses, but the mechanisms by which MSC improve healing *in vivo* have not been well-defined. Based on work carried out in other species, we hypothesized that equine MSC can be used as a therapy to promote the healing of horse cutaneous wounds because factors secreted by MSC, collectively called the MSC secretome, act on the wound environment, in ways that stimulate healing. We carried out a series of *in vitro* studies to (i) characterize the secretome of MSC isolated from horse peripheral blood and (ii) determine if the MSC secretome acts on target cell types found in the skin, in ways that suggest it will promote wound healing *in vivo*.

First, I present a description of MSC and a history of equine MSC research and therapy, to provide a context for our experimental work. Then, I review the utility of the horse as a model for cutaneous wound healing, which provides additional justification for our studies. We used various experimental methods to evaluate the composition of the MSC secretome and performed series of *in vitro* assays to determine that specific factors secreted by MSC stimulate equine

dermal fibroblast (DF) migration, a necessary aspect of skin wound healing. We carried out an additional set of experiments showing that MSC secreted factors act on dysregulated equine DF, pushing them toward a more normal phenotype that may be better suited to contribute to wound closure. Finally, we demonstrated that the MSC secretome inhibits the growth of bacterial species found as contaminants in chronic horse skin wounds.

These results show that factors secreted by equine MSC do act on cell types found in horse skin wounds in ways that may lead to improved wound healing *in vivo* and warrant future studies to test the efficacy of the MSC secretome as a treatment for horse skin wound *in vivo*.

## **BIOGRAPHICAL SKETCH**

Rebecca Harman (Becky) arrived at Cornell University in 1988 to begin an undergraduate degree in Natural Resources. When she graduated in 1992, she took on a series of part-time jobs, including milking cows on a dairy, caring for pigs at the Cornell Swine Facility and working at a local small animal veterinary practice, with the goal of finding a job that was both engaging and enjoyable.

Becky soon realized that to do satisfying work, she needed additional formal education, but she was not inspired to follow any particular path. She took a position in the Virology Section at the Animal Health Diagnostic Center (AHDC) at the Cornell College of Veterinary Medicine in 1993, a job that would allow her to take classes at Cornell while learning basic laboratory skills. Very quickly Becky fell in love with the lab environment. For the next 5 years Becky worked at the AHDC and took full advantage of the benefit of taking classes.

In 1998 Becky left the AHDC to take a job as a technician in the research lab of Dr. Susan Quirk in the Animal Science Department at Cornell. Becky flourished in Sue's reproductive physiology lab; she learned how to design experiments, taught herself how to carry out techniques she was unfamiliar with and work with animal models. She continued with her course work and developed a sincere interest in basic science research. Becky had to leave Sue's group in 2007 but was fortunate to be welcomed back to the AHDC, where she worked for 5 more years. During that time, she was encouraged by her current employer and Sue to work on a MS degree in Animal Science through the Employee Degree Program.

Becky moved to Dr. Douglas Antczak's reproductive immunology lab at the Baker Institute for Animal Health at Cornell in 2009. There she worked as a Research Support Specialist, took more classes, and completed her MS degree. In Doug's lab she learned how to

analyze data and present it in a comprehensive way. As Doug invited Becky to attend conferences and meetings, Becky also learned how to write abstracts, prepare posters and present her work to colleagues. The value of collaborations was another important lesson Becky learned during her time in Doug's lab.

When Becky needed to change jobs again in 2013, the timing was perfect for her to start working with Dr. Gerlinde Van de Walle, a newly hired faculty member at the Baker Institute. Gerlinde supported Becky's decision to apply for a PhD program in Biological and Biomedical Sciences, in the field of Immunology and Infectious Disease, which Becky could work on through the Employee Degree Program. Becky was accepted and has spent the past 5 years working as the Lab Manager of Gerlinde's lab, taking classes, and doing the research necessary to fulfill the requirements of a PhD. Gerlinde has proven to be an excellent mentor and has given Becky opportunities to be involved in all aspects of the scientific process, including grant and manuscript preparation, which Becky thoroughly enjoys.

After completing her PhD, Becky will continue to work with Gerlinde. She is looking forward to spending many more years contributing to basic scientific research, taking (even more) classes and developing collaborations with smart, supportive colleagues who make her job engaging and enjoyable every day.

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I would also like to thank the members of my Special Committees. My Master's Degree Committee was comprised of Dr. Susan Quirk, Dr. Douglas Antczak and Dr. David Deitcher. If they had not believed in my ability to do the work necessary to pursue an advanced degree in science, I never would have mustered the confidence to apply to a PhD program. Gerlinde, Doug, Dr. Cynthia Leifer and Dr. Tudorita Tumbor make up my PhD Committee. I am grateful that they have made suggestions to improve my work, and help me seriously think about my future, even though I am a non-traditional student.

Finally, I would like to express my gratitude to Gerlinde and Cornell for the opportunity to participate in the Employee Degree Program. I realize that Gerlinde was under no obligation to let me be in the Program, and that Cornell does not owe this benefit to employees. It has been a privilege for me to work towards a graduate degree while maintaining the benefits of my position as a member of the Cornell staff.

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## LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
Abx	antibiotics
ACTA2	smooth muscle actin
AD	atopic dermatitis
AD-MSC	adipose-derived mesenchymal stromal cells
AM-MSC	amniotic mesenchymal stromal cells
AMP	antimicrobial peptide
ANXA2	annexin A
BM-MSC	bone marrow-derived mesenchymal stromal cells
BrdU	bromodeoxyuridine
CAMP	cathelicidin
CB-MSC	umbilical cord blood-derived mesenchymal stromal cells
CCL	CC chemokine
CDH	cadherin 11
CFPD	cutaneous fibroproliferative disorder
CFU	colony forming units
CM	conditioned medium
CM FT	frozen and thawed conditioned medium
CM HI	boiled conditioned medium
CoCl <sub>2</sub>	cobalt chloride
COL	collagen

CST3	cystatin C
CTS	capthepsin
CxCL	CXC chemokine
DEFB	beta defensin
DF	dermal fibroblasts
DMEM	Dulbecco's modified Eagle medium
ECIS	electric cell-substrate impedance sensing
ECM	extracellular matrix
EGT	exuberant granulation tissue
EGTDF	primary dermal fibroblasts derived from equine exuberant granulation tissue
ESC	embryonic stem cells
EV	extracellular vesicles
FBS	fetal bovine serum
FGF	fibroblast growth factor
FLG	filaggrin
FN	fibronectin
HSC70	heat shock cognate 70
ICC	immunocytochemistry
IFN	interferon
IL	interleukin
ILC-2	type 2 innate lymphoid cells
iPSC	induced pluripotent stem cells
ISCT	International Society of Cellular Therapy

LB	Luria-Bertani
LC-MS/MS	liquid chromatography-mass spectrophotometry
LCN	lipocalin
LDH	lactate dehydrogenase
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MSC	mesenchymal stromal cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBL6	horse dermal fibroblast cells
NLR	neutrophil lymphocyte ratio
NPN	1-N-phenylaphthylamine
NPWT	negative-pressure wound therapy
OPN	osteopontin
PAI	plasminogen activator inhibitor
PB-MSC	peripheral blood-derived mesenchymal stromal cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDT	population doubling time
PGE <sub>2</sub>	prostaglandin E <sub>2</sub>
PI3	elafin
PLAT	tissue plasminogen activator

PLAU	plasminogen activator urokinase
PMN	polymorphonuclear leucocytes
QPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RNAi	double-stranded RNA-mediated interference
RPSA	laminin receptor 1
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDC	syndecan
SDFT	superficial digital flexor tendon
SDH	succinate dehydrogenase complex
SERPINE	plasminogen activator inhibitor
TAZ	tafazzin
TBE	trypan blue exclusion
TBS	tris buffered saline
TGF- $\beta$	transforming growth factor beta
Th2	T helper 2
TIMP	metallopeptidase inhibitor
TLR	toll-like receptor
TNC	tenascin-C
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
YAP	yes-associated protein

**CHAPTER ONE: AN INTRODUCTION TO MESENCHYMAL STROMAL CELLS AND  
REVIEW OF EQUINE MESCHYMAL STROMAL CELL THERAPY**

## **1.1. Introduction**

Our lab studies the potential of the equine of the mesenchymal stromal cell (MSC) secretome, comprised of all factors secreted by MSC, to be used as a biologic therapy for horse cutaneous wounds. The goal of this dissertation is to use *in vitro* assays to determine if MSC secreted factors act on cell types found in the skin in ways that suggest they may be a useful therapy for cutaneous wounds of horses. The long-term goals of this project are to (i) validate equine MSC-secreted factors *in vivo* as a safe and effective therapy for horse skin wounds, (ii) provide equine clinicians with protocols to offer MSC-secreted factors as a therapy to their patients, and (iii) use what is learned while evaluating this therapy for horses, to improve treatments for humans.

This chapter starts with a definition of MSC and a broad review of the isolation, culture and therapeutic use of MSC from other species, primarily humans. It then provides a comprehensive review of the history of equine MSC research and the current therapeutic applications of MSC in equine medicine. The chapter ends with citing specific work supporting the use of MSC-secreted factors as a therapy for skin wounds. This information, along with the review of horse cutaneous wound healing in Chapter Two, sets the stage for both the studies presented in this dissertation and our long-term goals for the project.

## **1.2. The history of MSC culture and MSC as a stem cell therapy**

### *1.2.1. History of MSC culture*

MSC are adult, non-hematopoietic, multipotent progenitors derived from the mesodermal germ layer. These cells can self-renew as well as differentiate into the various cells of the skeletal system including osteocytes, chondrocytes and adipocytes [1]. MSC were first described as fibroblast-like cells isolated from human bone marrow [2], but the term MSC was not coined

until 1991 [3]. Since the original MSC isolation, these cells have been collected from a range of species including mouse, rat, horse, cow, pig, dog and cat. In addition, MSC have been isolated from a variety of adult tissues including bone marrow, adipose tissue, placental tissues, and umbilical cord blood [4]. They are typically isolated from a heterogeneous mixture of cells and purified through culture. In the case of bone marrow-derived MSC (BM-MSC), for example, diluted bone marrow is plated in a tissue culture vessel in appropriate medium. Cultures are maintained over several weeks, with the medium being removed and replaced every few days. Early in the culture period, non-adherent lymphoid cells are washed away, while adherent cells of lymphoid origin eventually die and are removed. The remaining adherent cells, representing the MSC, grow as small, dense colonies with a fibroblast-like morphology. Although individual primary MSC look like fibroblasts, they do not behave as fibroblasts do in primary culture. Primary fibroblasts will spread and cover the surface of a culture vessel, whereas MSC form discrete colonies which need to be dispersed and passaged in order to form a monolayer. Once MSC cultures are established, they proliferate rapidly, allowing for tremendous expansion of MSC lines.

As MSC are derived from heterogeneous populations of cells collected from different tissues and a variety of species, and many laboratory groups isolate MSC using slightly different techniques, there has been debate between researchers about what features define MSC. To set a universal standard for the definition of MSC, the International Society of Cellular Therapy (ISCT) published minimal criteria for characterizing cells as MSC in 2006, based on the following 3 *in vitro* characteristics; (i) MSC must be plastic-adherent when maintained under standard culture conditions, (ii) MSC must express the surface proteins CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14 or CD11b, CD79 $\alpha$  or CD19 and HLA-

DR, and (iii) MSC must have the potential to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* [5] (Table 1.1).

**Table 1.1. Defining features of mesenchymal stromal cells**

<b>Feature</b>	<b>Requirement</b>
morphology	plastic-adherent
surface marker expression	CD105 positive CD73 positive CD 90 positive CD45 negative CD34 negative CD14 negative CD11b negative CD79 $\alpha$ or CD19 negative HLA-DR negative
differentiation potential	osteoblasts adipocytes chondroblasts

Since the majority of what is known about MSC comes from *in vitro* studies, there is an ongoing debate in the field as to whether or not MSC exist as we know them *in vivo* or if they are a by-product of cell culture. It has been proposed that *in vivo*, MSC reside in close association with the vasculature and home to sites of injury in response to cytokine and chemokine signals from wounded tissues. At their homing sites, MSC are thought to differentiate into cells to replace those that were damaged or lost due to injury, and more importantly, to stimulate healing by secreting factors that act on the wound environment. There is evidence that human and equine MSC are indeed perivascular in origin, and may even represent pericytes [6,7]; contractile cells that wrap around blood vessels and serve to regulate both capillary blood flow and the

differentiation of endothelial cells [8], but the proposed natural actions and functions of MSC *in vivo* are largely speculative.

### *1.2.2. MSC as a means for stem cell therapy*

Irrespective of the precise origin of MSC, it is understandable that stem cell biologists have investigated their value as a therapy. Embryonic stem cells (ESC) that exhibit prolonged undifferentiated proliferation potential and are capable of forming cells of all three embryonic germ layers were first derived from mouse embryos nearly 40 years ago [9, 10]. Authors of an early paper describing human ESC lines recognized the therapeutic value of these cells [11], but also realized that although ESC have tremendous potential as regenerative therapies, there are ethical issues associated with collecting cells from embryos. Since then, legal obstructions have limited work that can be done with ESC. In response to the difficulties of obtaining ESC, researchers have developed methods of reprogramming adult human cells to induce them into a pluripotent state [12, 13, 14]. These induced pluripotent stem cells (iPSC) are not plagued with the restrictions placed on ESC, but they have proven to be difficult to generate and depending on the reprogramming methods used may not be appropriate for therapeutic use. The idea that MSC, a naturally-occurring cell type that can be collected non-invasively from adults, may be used as a regenerative therapy makes them an attractive alternative to ESC and iPSC.

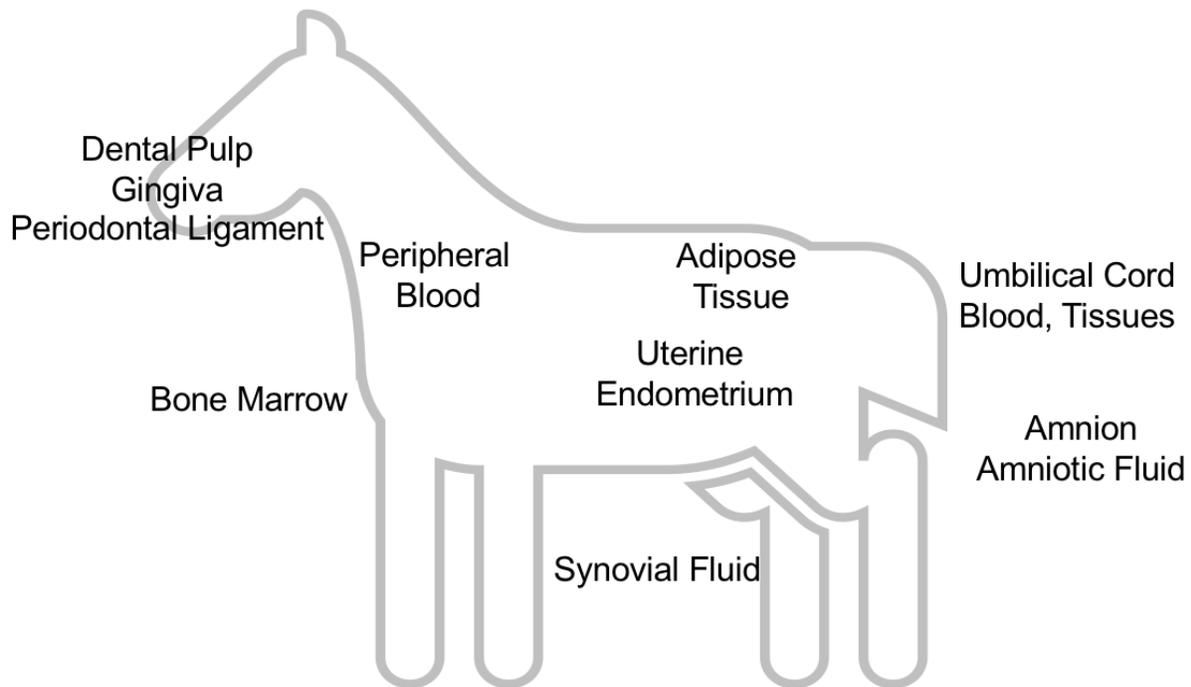
### **1.3. A review of equine MSC research and therapy**

The isolation of equine MSC, and the use of these cells as a therapy for orthopedic injuries, was first described in 2003 [15]. In this study, 500,000 autologous BM-MSCs were injected into a lesion in the superficial digital flexor tendon of one horse. No observable swelling was noted immediately after the procedure, and at 10 days and 6 weeks post-procedure, the animal showed no signs of lameness and there was no increased thickening of tendon as

determined by ultrasonography. The technique was determined “rational and feasible”, but no conclusions could be made based on a study consisting of one animal and one treatment, with no controls. Larger and more complete studies carried out in the past 15 years have established that autologous MSC therapy is not detrimental to horses [16, 17], and the clinical use of autologous MSC to treat orthopedic injuries is now routine. However, fundamental research has not kept pace with practice [18], so there are many gaps in our knowledge of basic equine MSC biology. The following sections highlight past and active areas of equine MSC research, in addition to the current knowledge on equine MSC and what questions remain to be addressed.

### *1.3.1. Isolation, culture and storage of equine MSC*

In the 15 years since equine MSC were first isolated from bone marrow aspirates and expanded in culture [15], numerous publications have described protocols for the isolation of these cells from different tissue sources, methods to culture these cells, and optimal conditions for long-term MSC storage. In addition to bone marrow, equine MSC have been isolated from adipose tissue [19], whole blood [20], synovial fluid [21], uterine endometrium [22], umbilical cord blood and tissues [23, 24] amnion and amniotic fluid [25, 26], dental pulp [27] gingiva and the periodontal ligament [28]. As described above, MSC cultures typically start from a heterogenous population of cells, which over time become essentially “pure” populations of MSC. Steps to enrich for MSC before culture such as applying cells to a density gradient, selectively digesting tissue with enzymes, or taking advantage of cell migration from tissue explants have been reported, depending on the tissue source of origin [29, 30, 31] (Figure 1.1).




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**Figure 1.1. Tissue sources of equine mesenchymal stromal cells (MSC).** Equine MSC from each of the tissue sources indicated above have been reported on.

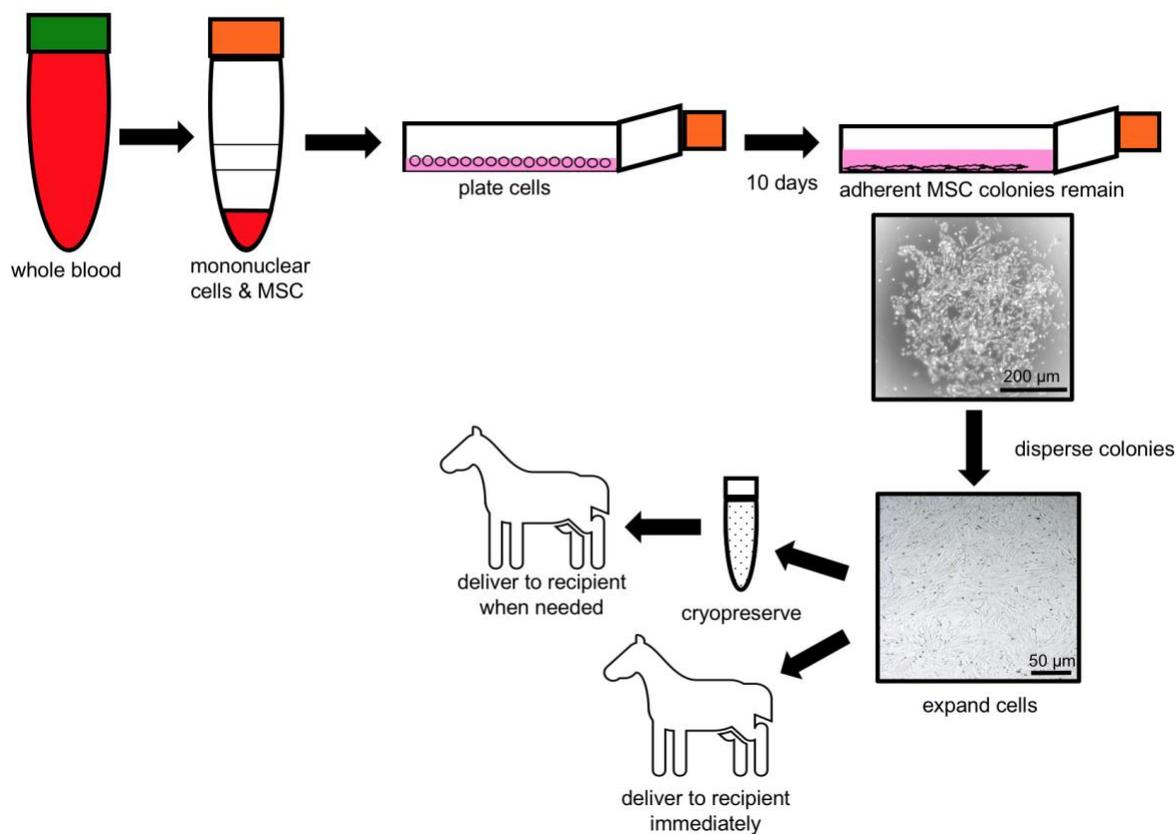
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Standard culture conditions for equine MSC exist, regardless of tissue source of origin. Equine MSC are typically maintained in culture medium containing fetal bovine serum (FBS). This is opposed to human MSC, which are often grown in commercially available serum-free medium, with the goal of standardizing cell products and preventing contamination by pathogens [32]. Studies evaluating the suitability of commercially available serum-free culture medium for equine bone marrow and adipose tissue (AD)-MSC determined that although the equine MSC could be isolated and expanded in serum-free medium, cell morphology, proliferation rate and surface immunophenotype were variable as compared to control equine MSC cultured in medium containing FBS [33, 34]. The authors concluded that serum-free culture conditions are

species-specific, and although currently available serum-free culture products are suitable for human MSC, optimization of serum-free culture conditions are needed if equine MSC are to be grown in the absence of FBS.

Similar to human MSC, researchers recommend passing equine MSC before cell monolayers reach confluency and maintaining the cells in culture for a limited time to avoid unwanted MSC differentiation, which is particularly important for cultures that are being expanded for therapeutic use.

Any cell type that will be used therapeutically is more useful if it can be successfully stored for long periods of time for “off-the-shelf” use. Cryopreservation of MSC is currently the standard method of maintaining cells for future applications. Several groups have compared the viability, proliferation rate, cell morphology, and immunophenotype and differentiation potential, of MSC before and after different methods of cryopreservation [35, 36, 37, 38]. The consensus of these studies is that MSC cryopreserved in medium containing FBS and dimethylsulfoxide maintain their integrity and minimal stem cell qualities after thawing (Figure 1.2)




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**Figure 1.2. Isolation, culture and storage of equine peripheral blood-derived mesenchymal stromal cells (MSC).** Equine MSC can be isolated from peripheral blood (as well as from other tissues), and then purified and expanded in culture. MSC and/or MSC-secreted factors can either be delivered immediately to recipients, or the cells can be cryopreserved for revival, expansion and use when needed.

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### 1.3.2. Characterization of equine MSC

The bulk of equine MSC scientific literature describes studies designed to compare the characteristics of MSC cultures isolated from different tissue sources or animal origins. As described above, equine MSC have been isolated from multiple different tissue sources. In addition, they have been collected from the same tissue source at various body sites, for example; from adipose tissue biopsies collected from the tail head, inguinal region and

mesentery or bone marrow aspirates from the ilium and sternum [39, 40]. In addition, MSC have been isolated from horses suffering from a particular disease [41, 42], as well as from cadavers [43, 44]. The two most common features that have been analyzed for these comparative studies are the surface protein immunoprofile of MSC, and their potential for trilineage differentiation *in vitro* [45, 46, 47, 48, 49]. The majority of equine MSC cultures tested express the surface proteins proposed by the ISCT to be MSC markers and can differentiate into osteoblasts, adipocytes and chondroblasts, but the degree of protein expression and efficiency of differentiation often are variable between cultures obtained from different sources.

Other features of MSC cultures have been examined, depending on the interests of the particular research group, as well as the desired application for MSC. Specifically, susceptibility to senescence [50], proliferation rates [51], migration rates [52], immunomodulatory properties [53, 54], and gene expression patterns [55, 56] have been compared across MSC cultures. Overall, the results from these studies, combined with unpublished observations, are that equine MSC populations are not identical. Differences may be attributed to the tissue source from which the MSC were isolated, age, health and hormonal status of the donor animal and potentially the length of time MSC were in culture prior to characterization. A reasonable conclusion when considering MSC for therapeutic purposes is that different MSC cultures may be more or less appropriate to use as treatments for specific ailments. Before using MSC as therapy, the specific culture should be screened to determine if it expresses and fulfills the desired characteristics.

Despite differences across equine MSC cultures, inherent characteristics are preserved across most, if not all MSC lines. All equine MSC are spindle-shaped cells that grow in monolayers, even if they exhibit slightly variable morphologies. For example, these cells may be very long and slender, or appear more cuboidal with short cytoplasmic extensions [23]. All

equine MSC divide rapidly in culture and are viable for multiple passages in culture without major changes in surface protein expression or differentiation potential. Moreover, they can be cryopreserved and revived, without compromising original surface marker expression or differentiation potential [57].

### *1.3.3. Treatment of tissue injuries with equine MSC*

As noted above, the clinical use of autologous equine MSC to treat orthopedic injuries is now a routine practice. Surprisingly, few scientific reports that document the efficacy of MSC therapy for damaged tissues, have been published since the original study by Smith in 2003. The effects of MSC on ligament and tendon injuries have been the most commonly described; about 20 peer-reviewed manuscripts have been published on this topic [58, 59, 60, 61, 62,17]. Other publications describing the use of MSC *in vivo* as a therapy report on torn menisci [63], damaged peripheral nerves, inflamed laminae [64, 65], and inflamed uterine endometria [66].

Although some of these studies have yielded promising data, for example the significantly increased vascularization at the site of injury upon MSC administration in an induced tendon injury model when compared to control injuries [62], the majority of them led to variable and inconclusive outcomes. Multiple factors contribute to the difficulty researchers face when carrying out experiments designed to test the efficacy of MSC therapy in horses. The evaluation of clinical cases can be useful for obtaining preliminary data, but case reports typically do not compare treatments to controls and often do not describe the treatment of multiple animals that are in comparable situations. Another hurdle is the expense incurred when enrolling large animals in long-term experiments. It is costly to maintain enough animals to include treatment and control conditions and reach statistical significance when analyzing results. An additional challenging aspect of working with veterinary species is that the diversity

between individuals may influence study results in ways that do not impact experiments performed using inbred laboratory species such as mice, rats and rabbits. Careful and creative ways of thinking about experimental set-ups, as well as increased funding are needed to obtain more conclusive results about the efficacy of equine MSC to treat equine tissue injuries.

#### *1.3.4. Safety of allogeneic versus autologous equine MSC therapy*

The wide spread clinical use of autologous equine MSC to treat joint and tendon injuries has led clinicians and clients to trust that MSC therapies are safe. In addition, experimental studies specifically designed to test the safety of MSC administered to horses have determined that the risks of treating horses with autologous MSC are minimal. However, an ideal treatment for any ailment is an off-the-shelf, ready-to-use product and as autologous MSC may not be readily available, researchers have explored the potential of using allogeneic equine MSC therapeutically. In theory, allogeneic MSC can be isolated, expanded in culture, and cryopreserved from donor horses for use in any patient, making them more versatile than autologous MSC.

A recent study designed to test the level of inflammation induced by intramuscular injection of allogeneic equine MSC, showed that the introduction of these cells did not promote significantly more inflammation or tissue damage, as assessed by ultrasound, hematological evaluation and histology compared to a saline injection [67]. A more sophisticated study, evaluating the clinical response of the synovial joint to intra-articular injection of autologous versus allogeneic MSC, found no differences in the examined clinical parameters following the first injection. After the second injection, however, there was a significant adverse clinical response of the joint to allogeneic MSC as compared to autologous MSC, leading the authors to conclude that there is immune recognition of allogeneic MSC upon second exposure [68].

Elegant work by another group showed that major histocompatibility complex (MHC)-incompatible equine MSC induced cytotoxic antibodies to donor MHC antigens in recipient horses after intradermal injection [69]. This led the authors to conclude that this type of response may limit the effectiveness of repeated allogeneic MSC use in a single horse and could lead to undesirable inflammatory responses in recipients. Another study determined that multiple injections of allogeneic MSC do not induce a systemic inflammatory response but do alter lymphocyte subsets in healthy horses [70].

The overall conclusion based on these, and other, studies is that recipient animals do recognize allogeneic MSC, but the type and severity of reaction is variable and may depend on the source of donor MSC, how many times donor MSC are administered, and how donor MSC are delivered.

#### *1.3.5. Mechanisms of action of equine MSC*

Elucidating the mechanisms by which MSC act on target tissues is not only important from a basic scientific perspective but has practical implications as well. If we know how exogenous MSC contribute to healing, we can fine tune MSC therapy to maximize benefits and minimize unwanted side effects [71].

##### *1.3.5.1 Integration of equine MSC at sites of injury*

Some clues to the mechanisms of action of therapeutically-administered MSC have been gathered from studies in which MSC have been tracked *in vivo* after injection. One such study tracked labeled autologous MSC injected at the sites of induced superficial digital flexor tendon (SDFT) lesions for 24 weeks post-injection [72]. The authors reported that the numbers of labeled cells within the tendon lesions decreased over time, and that some of the remaining cells were viable and integrated into the local tissue. In addition, small numbers of labeled cells were

identified in the peripheral blood within 24 hours of injection and could be detected 24 weeks later in contralateral control tendon lesions that had not been injected with cells. The authors, however, did not report on improvement in SDFT lesions during the course of the study. Another group tracked allogeneic MSC injected at the sites of naturally occurring SDFT disease for 8 weeks post-injection. This study detected sporadic labeled cells at the site of injury in 5 out of 7 cases after 8 weeks and found that the MSC injection was tolerated with no side effects in most animals. No untreated control animals were included in the study, so clinical improvement as a result of treatment could not be assessed [60]. Additional studies have also indicated that MSC injected at the site of tendon lesions do sporadically persist at the wound site [61, 73], but none of this work provides evidence that the MSC actually differentiate into the cells needed to rebuild and replace tissues damaged or lost by injury. Evidence from *in vivo* experiments in horses, combined with data collected from other species, suggests that if equine MSC administered therapeutically do indeed contribute to tissue repair, their primary mechanism of action is most likely through secreted factors (as a whole called the secretome, see section 1.4.1), that act on the wound environment to promote healing.

#### *1.3.5.2. Immunomodulatory action of equine MSC*

Based on work carried out in humans and mice, which established an immunomodulatory role for MSC, equine researchers have designed experiments based on hypotheses that equine MSC can affect immune cell function, and as a result alter the course of disease progression or tissue healing. Results from *in vivo* studies in horses, designed to examine the immunomodulatory properties have largely been inconclusive. This is in part because many studies used inadequate numbers of animals, and in part because protocols were not optimized [58, 74]. Select studies however, have convincingly shown that MSC influence the inflammatory

response *in vivo*. When  $1.5 \times 10^7$  MSC were injected into the uterine lumen, the inflammatory response to uterine infusion was diminished as compared to the vehicle control, as assessed by histology [75]. Another study used histologic analysis as well as antibody-binding to determine that inflammation was increased in joints after intra-articular injection of  $1.5 \times 10^7$  MSC as compared to joints receiving the vehicle control [76]. Studies such as these have provided useful information about the dose of MSC needed to generate a response and have proved that MSC can indeed elicit a response *in vivo*, but they were not designed to uncover the mechanistic details of how MSC interact with immune cells.

Work in simpler culture systems has shed light on some of the specific immunomodulatory actions of equine MSC. Umbilical cord blood-derived MSC (CB-MSC) were shown to suppress *in vitro* lymphocyte proliferation [77]. Microvesicles from equine amniotic MSC (AM-MSC) down regulate pro-inflammatory gene expression and decrease the secretion of pro-inflammatory cytokines by endometrial cells and tendon cells [78, 74], and microvesicles as well as conditioned medium from AM-MSC modulate the release of pro-inflammatory cytokines by equine alveolar macrophages [79]. Studies evaluating the immunomodulatory properties of BM-MSC found that these cells can mildly activate lymphocytes *in vitro*, and stimulate them to secrete inflammatory cytokines [80, 81]. BM-MSC can also suppress pre-activated lymphocytes in a prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)-dependent manner [80]. Likewise, a more recent *in vitro* study with BM-MSC that was designed to determine how the inflammatory stimuli toll-like receptor (TLR) ligands and inflammatory macrophages affect the phenotype and function of MSC, found that exposure of MSC to TLR ligands or inflammatory macrophages enhances the ability of MSC to suppress lymphocyte proliferation [82]. The authors speculate that consistent with the inflammatory licensing theory, the idea that

the inflammatory state of the environment directs cells to respond in ways that will temper inflammation, exposure to inflammatory signals activates the immunomodulatory capabilities of MSC.

Collectively, these studies show that equine MSC exert direct immunomodulatory properties on target cells, primarily via paracrine secretion, and that secretion can be manipulated by altering the environment in which MSC reside.

#### *1.3.6. Delivery of equine MSC therapy*

An intriguing aspect of therapeutic development is to determine the most optimal delivery methods for a given therapy. A thorough review of published literature yielded only one manuscript describing the administration of MSC or MSC-secreted factors to horses in any way other than introducing the cells themselves to recipient animals [74]. However, *in vitro* experiments designed to explore MSC delivery methods have recently been performed, with a primary focus on supporting MSC in biomaterials with the goal of maximizing their efficacy in cartilage repair.

##### *1.3.6.1 Scaffold-supported equine MSC for cartilage repair*

For the purpose of cartilage repair, culturing MSC in supportive matrices can provide several benefits. The supportive material provides MSC with three-dimensional contacts that mimic the *in vivo* environment, which may efficiently stimulate the differentiation of MSC into the large numbers of cells with an elastic cartilage phenotype needed for successful transplant. The supportive material may also be rendered bioactive, supplying MSC with chemical signals that promote appropriate differentiation in addition to providing physical support. In either situation, the supportive material may be populated with MSC *in vitro*, and then transplanted into injured joints to serve as an anchor to keep MSC localized to the site of injury *in vivo*. Matrices

that have been tested include gelatin meth acryloyl-based hydrogels [83], decellularized cartilage-derived ECM [83, 84, 85], hyaluronic acid-infused hydrogels [87], collagen-infused hydrogels [88], bacterial cellulose hydrogels [89], as well as combinations of these materials [90]. Bioactive factors of significance have been successfully incorporated in hydrogels along with MSC, with the primary goal of directing MSC to differentiate into functional cartilage. These factors include TGF- $\beta$ 1, bone morphogenic protein-2 (BMP-2) [91], and acid ceramidase [92]. In these studies, various read-outs were used to quantify the differentiation of MSC into functional, elastic, cartilage cells. The authors detected a level of success when compared to controls but proposed future experiments to fine-tune their protocols. This incorporation of bioengineering techniques into the field of equine MSC therapy is a relatively new, but active, area of study that is particularly suited to improve the success of MSC as a treatment for joint injuries.

#### *1.3.6.2 Equine MSC secretome delivery*

To date, there have been few published studies describing the reactions of recipient horses to secreted factors obtained from (i) allogeneic MSC and no reports of (ii) allogeneic MSC encapsulated in biomaterials. The purpose of the latter is to prevent direct cell-cell contact with the recipient in order to avert recognition of foreign material and unwanted inflammatory responses by the recipient, while providing factors secreted by MSC that promote regenerative effects. Our group has carried out experiments to evaluate the efficacy of MSC-secreted factors as well as of microencapsulated MSC *in vitro*, and the results are outlined in the research chapters of this dissertation.

### **1.4. The MSC secretome as a therapy for cutaneous wounds**

#### *1.4.1. The MSC secretome*

The MSC secretome, consisting of all factors secreted by MSC, has been implicated as the most important mechanism through which MSC contribute to tissue repair in various species [93]. MSC-secreted factors include secreted vesicles, such as exosomes, microvesicles and apoptotic bodies [94, 95, 96], as well as free peptides, proteins, and small molecules [97, 98]. Select evidence of the functional impacts of these secreted factors exists *in vivo*. For example, it has been determined that treatment with the human BM-MSC secretome reduces pain and subchondral bone alterations in a mouse model of osteoarthritis [99]. In another mouse study, researchers found that topically administered BM-MSC attenuates clinical symptoms in an allergic conjunctivitis model through multiple cyclooxygenase 2-dependent anti-allergic mechanisms [100]. Finally, a human clinical study showed that exogenously delivered AD-MSC-derived secretome inhibits inflammatory responses of microglia [101]. Collectively, results from these, and many other studies that have been previously compiled in published review articles [102, 93, 103], support the use of MSC-secreted factors as a therapy for various disease conditions.

MSC have been shown to respond to environmental signals by altering their secretion patterns in ways reminiscent of the adaptive and innate immune system responses [104, 105], which further increases the attractiveness of MSC as a biologic therapy since exogenously delivered MSC may react to the recipient environment by secreting factors that promote homeostasis in whatever tissue they home to [106, 1067 108]. Finally, several studies found that MSC can also be “pre” primed *in vitro* to secrete specific products, before their use *in vivo* for therapeutic purposes [109, 110, 111].

Studies to define the functional effects of equine MSC-secreted factors on target cells or tissues have only been initiated over the past 5 years, mainly by our research group. For

example, we and others have shown that equine MSC promote angiogenesis in an *in vitro* model system [112, 113] through the paracrine stimulation of endothelial cells, which causes them to secrete higher levels of vascular endothelial growth factor (VEGF-A) [112]. Another group determined that secreted factors from equine MSC stimulate the migration of corneal fibroblasts in culture, and that one of the factors likely responsible for this effect was transforming growth factor beta-1 (TGF- $\beta$ 1) [114]. We have evaluated the effects of the equine MSC secretome on fibroblasts, as well as bacteria commonly found in horse skin wounds. These results are described in the research chapters presented in this thesis.

#### *1.4.2. MSC as a therapy for cutaneous wounds*

Multiple characteristics of MSC, as outlined above, make them an attractive biologic treatment for skin wounds, and their therapeutic potential to heal skin has been intensively explored in human medicine, including in rodent animal models of wound healing and small-scale human clinical trials. In contrast, the potential of equine MSC to aid in equine wound management has been far less explored and is the basis for this dissertation.

Cutaneous wound healing in humans and horses consists of an overlapping series of highly coordinated cellular responses to injury that serve to (i) stop bleeding, (ii) remove and replace damaged tissues, and (iii) restore epithelial barrier function. Even under the best of circumstances, the repair of adult skin is imperfect, and complications often lead to delayed healing and scarring. As reviewed in Chapter 2, current therapies for cutaneous wound healing are inadequate, so novel alternate and adjunctive treatments are needed.

As mentioned above, the efficacy of MSC and MSC-secreted factors as biologic therapies for human skin wounds has been explored, and evidence suggests that these cells can be useful as a complete, or supplemental treatment for a variety of wound types. The majority of this

research was done with human BM-MSC administered to induced wounds in rodent animal models. For example, a recent study demonstrated that MSC conditioned medium administered to full-thickness skin wounds of rats led to enhanced epithelialization and angiogenesis [115]. Another group used BM-MSC acellular derivatives (which primarily consisted of secreted vesicles) to treat wounds in a diabetic mouse model. Wounds treated with acellular derivatives showed higher degrees of closure at select times post-wounding than wounds treated with a vehicle control. Treated wounds also exhibited a less pronounced inflammatory response, more granulation tissue formation and a higher density of collagen fibers [116]. In addition to small rodent models, pigs have been used to test the efficacy of MSC as a therapy for skin wounds. In one study, porcine BM-MSC were injected into the skin of pigs intradermally just prior to the infliction of full-thickness incisional wounds. After 28 days, the wounds exposed to MSC scored better than the controls and fibroblasts in wounds exposed to MSC were larger than those in control wounds [117].

In addition to studies carried out in these animal models, a number of experiments with human subjects have also provided evidence that MSC can improve skin wound healing. In a small study consisting of 3 patients with chronic wounds, treatment of wounds with bone marrow aspirate, followed by sequential treatments of BM-MSC, led to a decrease in wound size and increase in dermal vascularity and thickness [118]. In another case study, BM-MSC that were applied topically to acute surgical wounds and chronic lower-extremity wounds, were shown to accelerate the healing of acute wounds and decrease the size of chronic wounds at the time of examination, 20 weeks post-treatment [119]. In a relatively large study, 20 chronic, non-healing wounds were treated with BM-MSC seeded in a collagen sponge, ninety percent of the wounds healed completely at the end of the study [120].

The results of these investigations suggest that there is potential for MSC to be administered as a therapy to treat human cutaneous wounds, but larger clinical studies with longer follow-up periods as well as randomized controlled trials are necessary before MSC therapy can be accepted as a clinical practice [121]. Currently, patients are being actively recruited for trials designed to examine the long-term effects of MSC therapy on chronic wounds [ClinicalTrials.gov], which is a step towards bringing this promising treatment to those suffering from non-healing wounds.

### **1.5. Concluding remarks**

The work referenced in this chapter (i) describes the history of MSC culture in species other than horses, (ii) provides a broad history of equine MSC research and therapy, and (iii) briefly describes the use of MSC-secreted factors as a therapy for human skin wounds, providing the rationale for evaluating the potential of MSC-secreted factors to treat horse cutaneous wounds. As outlined in the following research chapters, we designed *in vitro* experiments to assess the effects of the equine MSC secretome on cell types found in the skin. The research described in this dissertation was carried out to support our long-term goals of (i) validating equine MSC-secreted factors *in vivo* as a safe and effective therapy for horse skin wounds, (ii) providing equine clinicians with protocols to offer MSC-secreted factors as a therapy to their patients, and (iii) using what is learned while developing this therapy for horses, to improve treatments for humans.

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## **CHAPER TWO: THE HORSE AS A MODEL FOR CUTANEOUS WOUND HEALING**

This manuscript is under review at *Advances in Wound Care* as Harman RM, Theoret CL, Van de Walle GR. The horse as a model for the study of cutaneous wound healing. 2018

## 2.1. Introduction

The skin is the largest organ of the mammalian body. Skin serves as a protective barrier to foreign pathogens and chemical compounds, regulates body temperature, prevents dehydration, and allows for sensing the environment [1]. As uncompromised skin is necessary for good health and well-being, treatment of skin wounds is of high priority in medicine.

At its 2012 annual meeting, The Wound Healing Society sponsored a symposium on preclinical models of wound healing. This meeting led to the drafting of a consensus statement that “strongly encourages all wound researchers to involve human wound tissue validation studies to make their animal and cell biology studies more translationally and clinically significant” [2]. Indeed, the practice of generating hypotheses based on what is known about wound healing in humans can guide researchers as they design experiments using *relevant* models to appropriately answer the questions at hand. The horse is an underused, yet physiologically relevant model animal that is well-suited to address questions about the mechanistic details of normal and impaired cutaneous wound healing in humans. Horses share anatomical features with humans and naturally suffer from several types of chronic wounds that are reminiscent of human conditions, making them an ideal model for the study of particular skin pathologies. In addition, as caretakers of veterinary species, we are obligated to understand the conditions horses suffer from and attempt to relieve them. With this in mind, horses do not only serve as models for humans, but what is learned from cutaneous wound research in horses will improve veterinary medicine as well. The goals of this review are to (i) describe the common limitations of current wound therapies for humans and horses, that are in part a result of our lack of understanding of wound healing processes, (ii) compare skin physiology and normal wound healing in humans and horses, (iii) propose the horse as a model for two types of chronic wounds

experienced by humans, and (iv) offer evidence that the horse is an appropriate model in which to test novel therapeutics for cutaneous wound management. Based on the information provided in this review, we put the horse forward as a relevant model for wound healing studies, which will benefit both human and veterinary health.

### *2.1.1 Relevance of cutaneous wounds in humans and horses*

More than 11 million people in the United States are negatively affected by acute wounds, inflicted by accidents or surgical procedures yearly [3]. In addition, the prevalence rate of chronic, non-healing wounds in developed countries is estimated to be an approximate 2% of the population [4]. In the United States alone, 6.5 million patients are affected by chronic wounds annually, with an estimated health care cost of \$25 billion [5, 6, 7]. Unfortunately, these numbers are on the rise, corresponding with increasing rates of diabetes and obesity, as well as aging populations, all of which predispose people to suffer from chronic wounds. Indirect costs of cutaneous wounds are associated with skin scarring, with \$12 billion spent annually in the United States to reduce or remove scars [8], as well as with disabilities and lost wages caused by chronic non-healing wounds. In addition to placing an economic burden on individuals and health care systems, skin wounds take an unquantifiable physical and emotional toll on affected patients.

The economic cost of wounds in horses is not as well documented, but skin wounds are among the most common ailments treated by equine practitioners [9]. Moreover, a 2006 USDA report states that ~19-24% of equine euthanasia cases are carried out as a way to humanely deal with non-healing skin wounds or traumatic injury to the skin [10].

### *2.1.2 Current therapies for wound management in humans and horses*

The complexity of the cutaneous wound healing process contributes to difficulties in executing successful treatment protocols when natural wound healing is not adequate. Indeed, it is well-

established that healing of full-thickness skin wounds is far more intricate than implied by the conventional description of the 4 phases of wound healing. It is clear that each of the “phases” (hemostasis, inflammation, proliferation, and remodeling) is made up of interdependent events executed by resident skin cells, migrating immune cells and extracellular matrix (ECM) components, in addition to growth factors, cytokines, and chemokines, released by cells and the surrounding environment [11]. The events required for successful wound healing consist of a cascade that is initiated by wounding and continues until long after wound closure is achieved [12]. Conventional treatments for cutaneous wounds of humans and horses broadly aim to improve the wound bed by keeping it moist, free of pathogens, and adding or removing pressure, depending on the type of wound [13, 14]. These methods have remained largely unchanged over the past decades.

In parallel with our increasing knowledge of wound healing processes in humans, there has been an effort to incorporate increasingly sophisticated treatment strategies based on specific cellular responses to injury. Various recombinant growth factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF), have been used to treat wounds in experimental settings. However, malignancy risks and other problems associated with adding recombinant proteins to cutaneous wounds have been recognized, and these therapies have not yet been fine-tuned for clinical use [15]. Hyperbaric oxygen therapy, which consists of breathing pure oxygen while in a total body chamber where pressure is controlled, is also used as a method to positively influence cells involved in wound healing by altering clotting, cell proliferation, angiogenesis and tissue repair [16]. Although there are few negative side effects to this type of therapy, it is currently not widely used due to the extensive

equipment and technical expertise needed to execute it, and long-term positive results are not well documented [16, 17].

Likewise, equine practitioners have made efforts to refine treatments for cutaneous wounds, based on the growing knowledge of the cellular mechanisms that drive wound repair. The choice of treatment for cutaneous wounds in equine patients depends on the body location of the wound. Wounds on the lower neck and trunk of horses tend to heal by the contraction of muscle below the skin, which draws the edges of the wound together. In contrast, wounds on the upper neck, face and legs close primarily by epithelialization, consisting of the migration of keratinocytes over granulation tissue. Similar to studies for the treatment of human skin wounds, active research is focused on determining the effects of specific proteins, such as VEGF-E and interleukin-10 (IL-10), or oxygen therapy, on the healing of horse wounds [18, 19, 20, 21], but as in human medicine, these treatments are not yet available to equine patients.

Collectively, the expenses associated with cutaneous wounds, as well as the lack of adequate treatment options for both humans and horses, creates the need for (i) basic scientific research designed to understand the mechanisms that drive normal and delayed wound healing, and (ii) the development and testing of targeted adjunctive and novel therapies in both human and veterinary medicine.

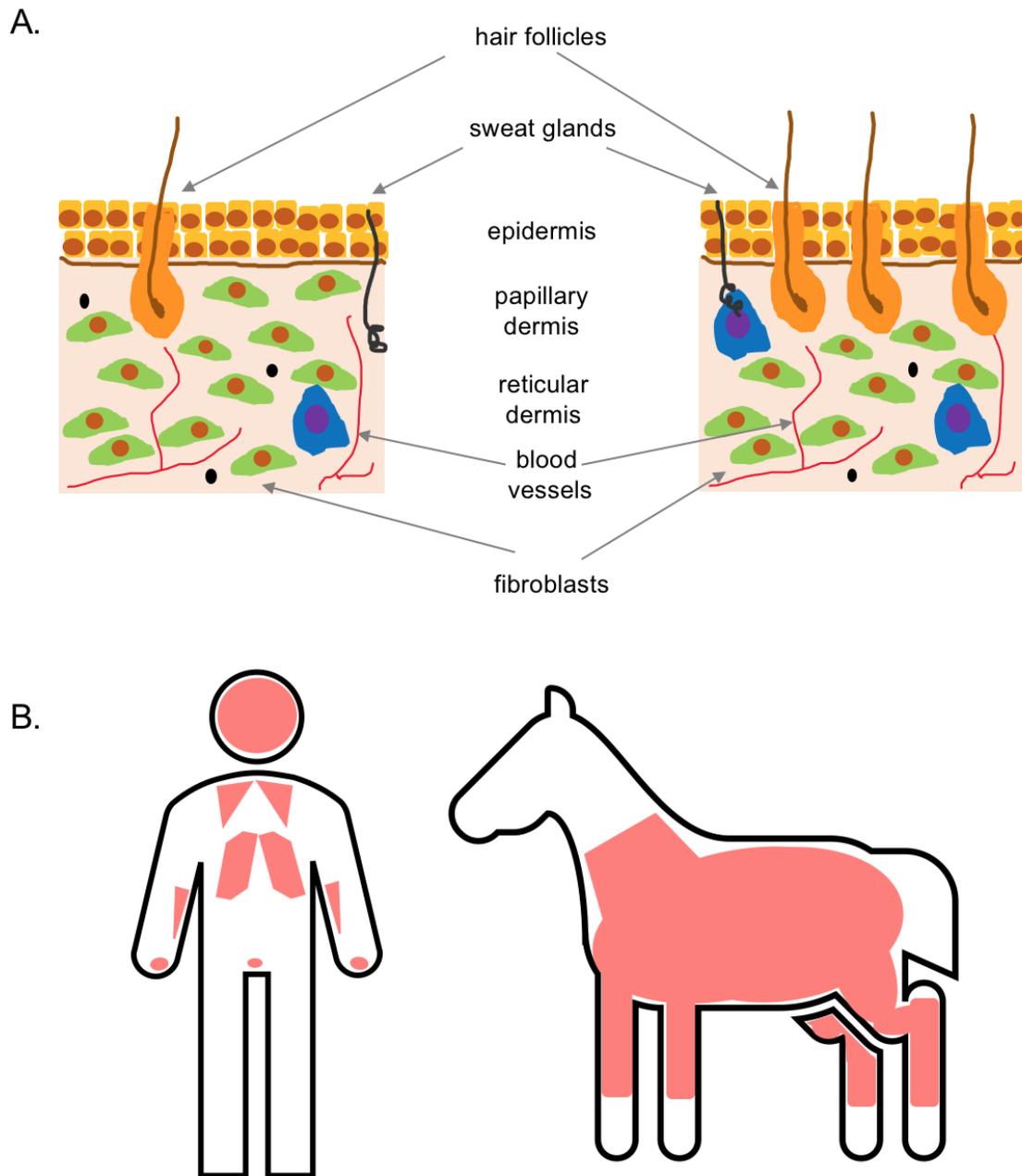
## **2.2. Skin physiology and normal wound healing in humans and horses**

### *2.2.1. Skin physiology*

The skin of humans and horses is comprised of two primary layers which protect internal organs from mechanical damage and invasion by pathogens. The outer epidermis, derived from the embryonic ectoderm, is a thin, cellular layer that provides a waterproof barrier to the

environment. The dermis lies beneath the epidermis and is made up of two regions, the papillary dermis and the reticular dermis. This layer contains fibroblast cells which produce and remodel the dense, collagenous connective tissue that makes up the bulk of the dermal layer. The dermis is also rich with blood vessels, lymphatic vessels, and glands such as sweat and sebaceous glands. Hair follicles originate in this layer as well. Epidermal extensions, referred to as rete ridges or pegs, extend into the dermal layers. Resident and infiltrating immune cells can be found in both layers of the skin. The hypodermis, or superficial fascia, is located below the dermis and primarily consists of fat and connective tissue, but also contains blood vessels. This layer is not considered part of the skin, but rather serves as an insulating layer and helps attach the skin to underlying structures.

The human epidermis is comprised of 4-5 stratified layers of epithelial cells [22], while the horse epidermis is described as being made up of 5 stratified squamous cell layers [23, 24]. Human and horse skin both have a thick dermal layer, which in the human contains sparse hair follicles while the hair follicles in the horse are densely distributed [22, 25] (Figure 2.1A). Human skin thickness is relatively uniform from site-to-site on the body [26], while the thickness of horse skin is variable according to body site [14]. Humans are considered “tight-skinned”, meaning that the skin is firmly attached to the underlying subcutaneous connective tissue [22]. Horses are often also described as “tight-skinned” [27], but since the qualities of horse skin vary across the body, some parts of the horse such as the trunk, may be considered to have “loose-skin”, which is more typical to that of laboratory rodents. Humans and horses each have a disrupted panniculus carnosus; a layer of striated muscle below the hypodermis. In humans, remnants of this muscle are found in the face, hands, chest and scrotum [28], whereas it stretches from the knees to the base of the neck in horses [29] (Figure 2.1B).



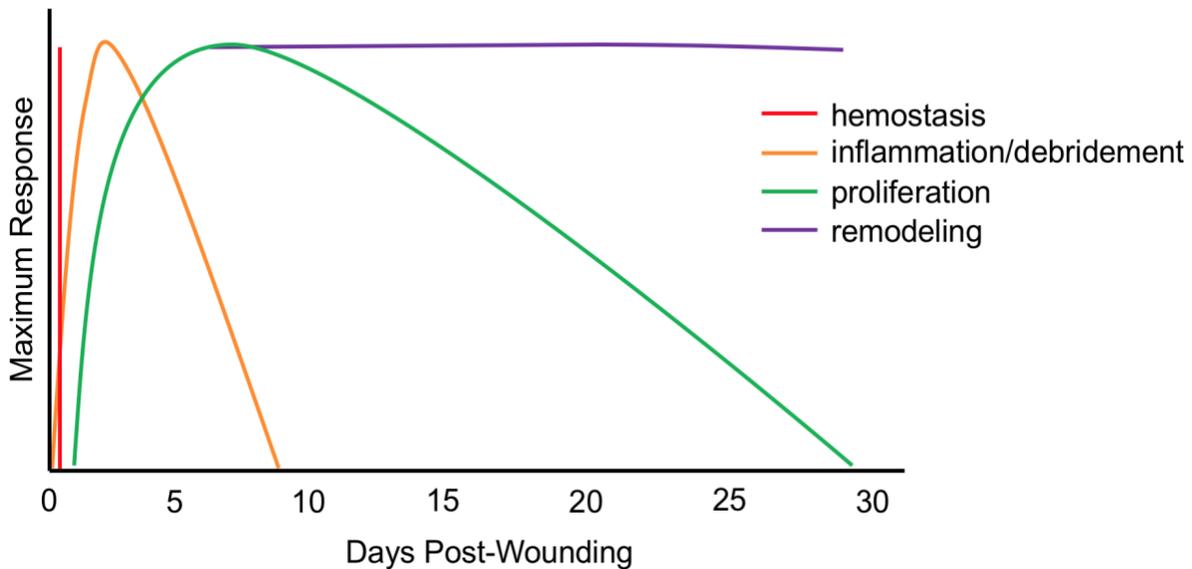

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**Figure 2.1. Human and horse skin physiology.** **A.** Schematic cross sections of human (left) and horse (right) skin. One primary difference between the species is the density of hair follicles. **B.** Distribution of the panniculus carnosus muscle in humans (left) and horses (right).

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### 2.2.2. Normal wound healing

Similar to most mammals, successful skin wound healing in humans and horses involves a network of synchronized biological processes that restores the integrity of the skin after injury. The process of wound healing after full-thickness injury is often divided into four phases (Figure 2.2).



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**Figure 2.2. Stages of normal wound healing in human and horse.** Normal wound healing in humans and horses is comprised of a similar overlapping series of events. Immediately after injury, bleeding is stopped by hemostasis. An intense inflammatory response follows, which serves to clear pathogens and induce proliferation of cells needed to fill the wound space. The remodeling phase continues long after wound closure. In this phase stromal cells coordinate the turnover of extracellular molecules in the wound bed, leading to increasing tissue strength.

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During hemostasis, the immediate response to injury, vasoconstriction and coagulation serve to stop blood loss and to concentrate the growth factors that contribute to wound healing. The subsequent inflammatory or debridement phase, lasting for 3 to 7 days post injury, is characterized by infiltration of immune cells which control contamination, and secrete cytokines

and growth factors that induce the proliferative phase [30]. The proliferative phase continues for at least 2 weeks post injury, during which time epithelial cells migrate from the wound margins to close the wound, and a population of dermal fibroblasts (DF) are activated by tumor necrosis factor alpha (TNF $\alpha$ ), PDGF, transforming growth factor beta 1 (TGF $\beta$ -1) and mechanical stress in the wound bed [31]. Activated DF secrete collagen and other ECM proteins and may differentiate into myofibroblasts, which contribute to wound contraction. Finally, the remodeling phase can proceed for up to a year after initial injury. During this phase, ECM proteins are alternately laid down and degraded, creating an increasingly organized tissue environment [30]. In many cases, however, complete tissue regeneration is not achieved, leading to chronic non-healing wounds or abnormal scar formation. As the details of wound healing have been elucidated, it has become apparent that the four phases of healing are not distinct, rather the events required for successful wound healing are a continuous cascade that is initiated by wounding and continues until long after wound closure is achieved [12].

Due to similarities in skin architecture, aspects of normal cutaneous wound healing in humans and horses are very much alike (Table 2.1).

**Table 2.1. Features of normal wound healing in human and horse**

<b>Feature</b>	<b>Human</b>	<b>Horse</b>
primary surface closure <sup>27,32</sup>	epithelialization	epithelialization (limb), muscular contraction (trunk)
dermal healing <sup>27</sup>	stromal granulation, myofibroblast activity	stromal granulation, myofibroblast activity (limb)
neutrophil:lymphocyte ratio <sup>26</sup>	60:40	60:40
blood oxygen capacity <sup>38</sup>	high	low
ambulatory species <sup>38</sup>	yes	no
platelet composition <sup>39</sup>	high	low

Humans have skin that is firmly attached to the underlying connective tissue and as a result, rely on epithelialization for the primary surface closure of skin wounds, followed by dermal healing via stromal granulation and myofibroblast migration and contraction. This is very similar to how skin healing progresses on horse distal limbs [27]. In contrast, the mechanism of primary wound closure on the horse trunk is more similar to that seen in rodents, which involves contraction of the panniculus carnosus [32]. Another concordance between humans and horses that may contribute to similarities in skin wound healing is the ratio of neutrophils to lymphocytes (NLR) in peripheral blood. Human and horse blood each have a ratio of about 60% neutrophils to 40% lymphocytes, whereas the NLR is much lower in mouse blood [26]. In human medicine, the NLR in blood as well as in tissues is emerging as a measure of subclinical inflammation [33, 34, 35, 36, 37]. Since inflammation is a critical aspect of cutaneous wound healing in mammalian species (Figure 2.2), similar basal NLR may contribute to similarities in this stage of wound healing.

In addition to parallels, there are basic physiological differences between humans and horses that may contribute to variable mechanisms of skin wound healing between these species (Table 2.1). Humans can be classified as stalking predators, whereas horses are flight animals, with hearts capable of going from resting to 300 beats per minute almost instantly. This extreme flight response results in a contraction of the spleen, which releases a reserve of red blood cells into the circulation, increasing the oxygen carrying capacity of blood to levels far higher than are possible in humans [38]. As successful skin wound repair depends on a steady supply of well-oxygenated blood, it stands to reason that the different patterns of blood oxygen availability in humans and horses may result in alternative mechanisms for healing between the species. Another notable difference between humans and horses is that while humans can be immobilized

for months to years with proper nursing care, horses are an ambulatory species that must stand and walk to survive. If horses are recumbent for longer than 72-96 hours, life-threatening complications begin to develop [38]. A subtler difference between the species is the platelet composition of the blood; human blood has a much higher concentration of platelets compared to horse blood [39], which may affect the rate of coagulation and alter the initial inflammatory response to wounding. There are no published reports describing how these basic physiological differences between humans and horses lead to differences in skin wound healing between the species, but an awareness of them may help researchers determine which human skin conditions can be accurately recapitulated in the horse model.

### **2.3. The horse as a model for cutaneous wound healing research**

Researchers have been studying the mechanisms of cutaneous wound healing in humans and horses for decades. For an overview of the models used for human wound healing research, we would like to refer to other reviews [40, 41, 42, 43, 44, 45]. In this review, we will focus on the models, both *in vitro* and *in vivo*, used to study the (patho)physiology of wound healing in horses.

#### *2.3.1. Naturally-occurring chronic skin wounds similar in humans and horses*

Several types of naturally-occurring skin wounds of horses are uniquely similar to conditions experienced by humans, making the horse an ideal animal model in which to study these pathologies. Two such naturally occurring wound types, namely fibroproliferative wounds and wounds resulting from allergic responses, are particularly relevant because the development of these wounds is not well understood and treatment options for both species are inadequate.

##### *2.3.1.1. Fibroproliferative disorders*

The formation of normal granulation tissue is an essential process in successful cutaneous wound healing in both human and horse [46, 47, 27]. Granulation tissue is created by fibroblasts and is primarily composed of type-III collagen. It fills the wound space, forming a barrier between the body and external contaminants, and provides epithelial cells a surface over which to migrate. Granulation tissue also serves as support for the cells that contribute to wound repair. Endothelial cells in the granulation tissue form new capillaries, needed to bring oxygen and immune cells to the wound bed. Fibroblasts secrete ECM molecules, and the enzymes required to break them down during the rounds of ECM remodeling that occur during the course of tissue repair [48]. Fibroproliferative disorders, characterized by the deposition of excessive ECM or granulation tissue, are associated with chronic low-grade inflammation, cellular proliferation and lack of wound closure. Fibroproliferative tissue occurs uniquely in humans, as hypertrophic scars and keloids, and on the distal limbs of horses where it is called proud flesh, or exuberant granulation tissue (EGT). Table 2.2 gives an overview of the key features that are either similar or different between human keloids and equine EGT.

**Table 2.2. Key features of human keloids and equine exuberant granulation tissue (EGT)**

<b>Feature</b>	<b>Keloids</b>	<b>EGT</b>
protrudes beyond wound margins <sup>49,14</sup>	yes	yes
ulceration <sup>54</sup>	yes	yes
epithelial cell coverage <sup>54</sup>	often complete	usually absent
disorganized collagen <sup>54</sup>	yes	yes
tissue macrophages <sup>54</sup>	minimal	minimal
tissue mast cell <sup>54</sup>	minimal	minimal
dermal fibrosis <sup>54</sup>	moderate-severe	moderate-severe
fibroblast proliferation <sup>54</sup>	excessive	excessive
elastic fibers <sup>54</sup>	no	no
granulation tissue <sup>54</sup>	no	yes
dermal vascularity <sup>54</sup>	moderate-severe	minimal
keloidal collagen <sup>54</sup>	yes	no
myofibroblast <sup>54</sup>	yes	no
inflammation <sup>52,55,56,57,58,59</sup>	low-level, sustained	low-level, sustained
microvascular occlusion <sup>54,68</sup>	yes	yes
apoptotic balance <sup>66,67,68</sup>	skewed	skewed
hypoxia <sup>56,69,70</sup>	yes	yes

Keloids are defined as scars that grow continuously and invasively beyond the margins of the original wound, which can range from pea-sized to as large as a football [49]. The quality of life of patients suffering from keloid disease can be reduced due to both physical complications, including severe itching, pain, loss of mobility and infection, as well as psychological impairments, such as embarrassment, lack of self-confidence and depression [50]. There is no single, effective treatment regime, and although many therapies are available, most are associated with extreme high rates of recurrence [51]. Keloid development involves alterations in complex pathways, but the mechanisms of the initiation and regulation of these pathways in the

context of keloid scarring are unknown [52]. Although chronic inflammation has been shown to contribute to keloid formation, details of keloid pathogenesis in the early stages remain poorly understood. In response to trauma, 15-20% of people of Asian, African and Hispanic origins develop keloids [51]. There is evidence of genetic susceptibility to keloid, but again, the underlying pathogenesis is not well-defined.

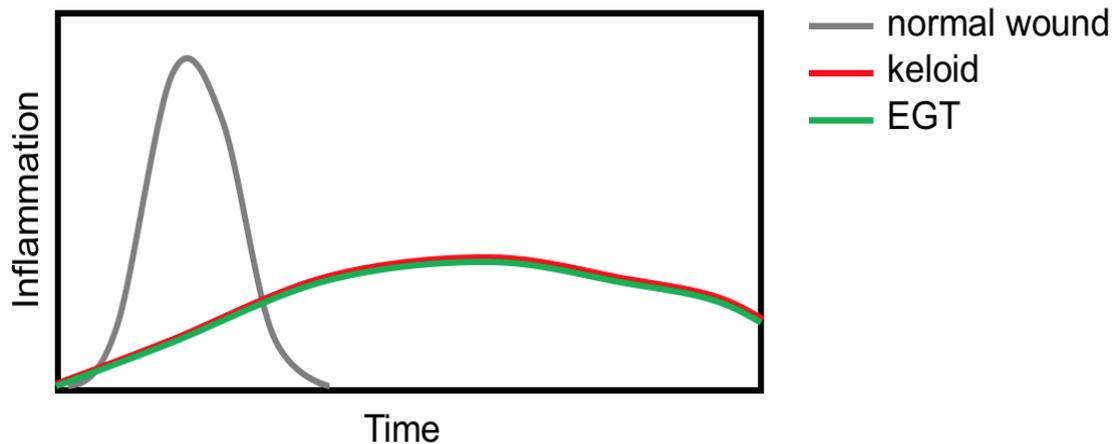
As described above, horse skin wounds heal by distinct mechanisms based on the body site on which they occur. Wounds on the distal limbs that heal by epithelialization usually close slowly and can develop EGT. EGT is visually irregular with a pebbled, moist surface that protrudes beyond the margins of the wound. This phenotype is a result of residual fibrin deposits, disorganized fibroblasts and vasculature, and a lack of epithelial cell coverage [14]. As this tissue is exposed to the environment, it is susceptible to abrasion, secondary infection, and re-injury. Removal of EGT by surgical excision is currently considered the best treatment option, even though the procedure often needs to be performed multiple times before successful wound closure occurs [53]. Interestingly, ponies, which are the same species as horses, but are distinguished from horses based on size (ponies are <1.48 m in height at the withers, while horses are taller) rarely develop EGT in association with limb wounds. The intriguing differences in wound healing between (i) body site in horses and (ii) horses versus ponies, have been the focus of many *in vivo* studies that led to what is known about EGT, and are described in more detail in section 2.3.3.

A comparative histological study of keloids and EGT determined that both tissues are characterized by ulceration, although keloids tend to exhibit substantial epithelial cell coverage, while EGT usually is lacking epithelial cells. Both keloids and EGT also have disorganized, thickened collagen fibers, minimal numbers of tissue macrophages and mast cells, moderate to

severe dermal fibrosis, and excessive numbers of proliferative fibroblasts. Elastic fibers were not observed in either tissue type. Granulation tissue was absent in keloid tissue, but present in EGT. Dermal vascularity was moderate to severe in keloids, and minimal in EGT. Keloidal collagen and myofibroblasts were observed in keloids but not in EGT [54].

Gene and protein expression has been well studied in keloids and EGT, as well as individual cell types isolated from these tissues. Analysis of mRNA and protein from keloids has shown that the expression of TGF- $\beta$ 1, matrix metalloproteinase-1 (MMP1), PDGF, and FGF, are increased in this tissue as compared to normal skin [55, 56]. These cytokines and growth factors directly and indirectly contribute to a prolonged low-level inflammatory phase of wound healing and delay the healing response [52]. Early work measuring growth factor concentrations in homogenates made from horse limb and thoracic wounds demonstrated that TGF- $\beta$ 1 is more highly expressed in limb versus thoracic wounds post-injury, while transforming growth factor  $\beta$ 3 (TGF- $\beta$ 3) and FGF expression patterns are similar irrespective of wound site [57]. Analysis of the inflammatory mediators in the TGF- $\beta$  family in horse and pony wounds showed that while wounds on ponies express higher levels of active TGF- $\beta$  post-wounding regardless of wound location (trunk or limb), wounds on horse limbs that are prone to develop EGT exhibit a sustained low-level expression of this family of growth factors [58]. Building on this work, a study was performed to analyze the function of polymorphonuclear leukocytes (PMN) as well as the local cytokine profiles in horse and pony limb and neck wounds [59]. The results of this study indicated that pony PMN produce higher levels of reactive oxygen species compared to horse PMN, and that TNF $\alpha$ , interleukin-1 (IL-1), and chemoattractants, are produced at higher concentrations in pony wounds than in horse wounds. Collectively, these suggest that an intense, yet temporally limited inflammatory response, supports efficient wound closure, while a

sustained low-level inflammatory response promotes excessive fibrosis in both humans and horses (Figure 2.3).



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**Figure 2.3. Illustration of the inflammatory response in normal wound healing as compared to keloid or exuberant granulation tissue (EGT) development.** Normal wound healing is characterized by an intense and short-lived inflammatory response immediately after wounding. During the development of human keloids or EGT in horses, the inflammatory response is delayed, sub-optimal and does not fully resolve.

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Suppression subtractive hybridization was used to identify genes differentially expressed in the margins of normally healing wounds as compared to intact skin in horses [60]. This work resulted in a list of 226 genes expressed 7 days post wounding. The authors used this data to compare gene expression patterns in biopsies taken from normally healing wounds on horse trunks as opposed to biopsies collected from EGT wounds on horse limbs. Annexin A2 (ANXA2) and MMP1 mRNA was found to be upregulated in trunk but not limb wounds, corresponding to the rapid tissue turnover required for normal wound healing [61]. Another study by the same group examined the expression, both on mRNA and protein levels, of laminin receptor 1 (RPSA) in healing wounds on the bodies and legs of horses [62]. This work showed

upregulation of RPSA in thoracic wounds at 4 and 6 weeks post-wounding, when epithelialization was occurring, while expression in limb wounds was downregulated at post-wounding times. Data on the expression patterns of RPSA, a receptor that binds multiple ligands including a variety of ECM molecules, may help tease out events that favor normal wound healing over the development of excessive fibrotic tissue and chronic wounds. Follow up work showed that OB-cadherin, a protein positively associated with contractile fibroblasts, was higher in thorax than limb wounds [63]. Additionally, osteopontin (OPN), which is essential for proper ECM organization in normal wound healing was also differentially expressed in horse wounds depending on wound site, which the authors suggest may reflect the delayed and low-level inflammatory response characteristic of EGT [64].

Microvascular occlusion, the excessive proliferation of fibroblasts, and a skewed apoptotic balance are all characteristics of keloids and hypertrophic scars in humans and EGT in horses that may contribute to the excessive deposition of ECM [65, 66, 67, 68]. Tissue hypoxia has been implicated as a contributing factor to fibroproliferative diseases in humans, although the underlying mechanisms are not fully understood. Studies in horses indicate that microvascular occlusion leads to a relative state of hypoxia in limb wounds, which may promote the weak, but prolonged inflammatory response characteristic of chronic, fibrotic wounds [69, 70].

Based on the similarities between the two diseases, and the benefits provided by studying cutaneous pathologies in a large animal species (discussed in section 2.3.3), EGT in horses has been proposed as a model for keloids in humans [27].

#### *2.3.1.2. Atopic dermatitis*

Another chronic inflammatory skin disease experienced by both humans and horses is atopic dermatitis (AD). Intense itch and recurrent eczematous lesions, triggered by exposure to

harmless environmental allergens, characterize this increasingly common condition. Clinical signs of AD in humans often develop during childhood and include pruritic lesions, hives, lichenified skin, and staphylococcal colonization [71, 72, 73]. AD is frequently accompanied by asthma and allergic rhinitis in humans [74, 75, 76], and food allergies are another associated condition [77, 78]. As the tendency to have an allergic condition is associated with susceptibility to additional allergic conditions, symptoms often appear in sequence with age, supporting the concept of an ‘atopic march’ [71]. AD in humans is also associated with non-atopic comorbid conditions including other autoimmune-mediated diseases as well as depression and anxiety [79, 80, 81].

Patients with AD often present with ultrastructural abnormalities of the stratum corneum, characterized by dry skin and compromised barrier function. These abnormalities have been linked to mutations in the filaggrin (FLG) gene, that lead to a lack of this protein in the outer layer of the skin [82, 83].

AD in humans has historically been considered IgE-mediated, supported by an exaggerated T helper 2 (Th2) response, [84, 85], but this view is expanding into one in which epidermal barrier disruption, the responses of various T cell subsets, and dysbiosis of the commensal skin microbiota all contribute to the pathology [71]. Although it is proposed that molecules secreted upon damage of the epidermis and products of the immune cells that respond to them are increased in AD lesions, cytokine profiles in skin affected with AD have not been accurately defined. It has been shown, however, that epithelial cells from AD patients express high levels of thymic stromal lymphopoietin (TSLP), a mediator that indirectly stimulates T cells to produce T helper (Th)2 cytokines in other pathologic situations [86]. Increased mRNA levels of the Th2 interleukins IL-4, IL-10, and IL-13, and the Th2-associated cytokine interleukin-22

(IL-22), have all been detected in acute atopic dermatitis lesions when compared to healthy skin [87]. CD4<sup>+</sup> T cells from AD lesions produce IL-4, IL-13, and IL-22, whereas CD8<sup>+</sup> T cells from AD lesions are a source of IL-13, IL-22, IL-17 and interferon-gamma (IFN $\gamma$ ) [88], and type 2 innate lymphoid cells (ILC-2) in AD lesions express IL-4 [89].

AD in horses presents both as a skin and a respiratory disease, but evidence of a progressive atopic march is only anecdotal in horses [90]. Clinical signs of AD in horses include hives and pruritus, and similar to human AD, staphylococcal colonization is commonly found [90]. Food allergies can lead to AD in horses, although other conditions, such as hypersensitivity to proteins in the saliva of biting midges in the genus *Culicoides*, are more commonly associated with equine AD [91]. Ultrastructural abnormalities in lipid lamella and disorganization of the stratum corneum, which resembles skin lesions in human AD, have been reported in horses [92]. The antibody and cytokine profiles in the skin and/or serum of horses that are sensitive to AD have not been described thoroughly, but equine AD has been shown to be an IgE-mediated disease that presents with an increased expression of IL-4 [91, 93, 94]. The horse is currently not actively used as an animal model for human AD, but efforts to increase our knowledge of equine AD and to better define similarities and differences, will provide better insights into the suitability of the horse as a model for AD in humans. Some of the key features, both similar and different, that have been studied in both species are shown in Table 2.3.

**Table 2.3. Key features of human and horse atopic dermatitis (AD)**

Feature	Human	Horse
recurring lesions <sup>71,90</sup>	yes	yes
hives <sup>71,90</sup>	yes	yes
lichenified skin <sup>71,90</sup>	yes	yes
Staphylococcal colonization <sup>72,73,90</sup>	yes	yes
co morbidities <sup>74,75,76,90</sup>	asthma, allergic rhinitis	respiratory disease
allergen triggers <sup>77,78,91</sup>	food, environmental allergens	<i>Culicoides</i> salivary proteins
atopic march <sup>71,90</sup>	yes	anecdotal evidence
abnormalities of stratum corneum <sup>82,83,92</sup>	yes, associated with <i>FLG</i> mutation	yes, cause unknown
IgE-mediated <sup>84,85,91</sup>	yes	yes
Th2 cytokine expression <sup>86,87,93,94</sup>	yes, well defined	yes, not well explored

### 2.3.2. Equine *in vitro* skin wound models

*In vitro* cell culture models have been used extensively to study the characteristics of cells involved in equine cutaneous wound healing, such as resident skin cells and migrating immune cells, as well as their responses to potential treatments.

#### 2.3.2.1. Two-dimensional (2D) cell culture

Because of their simplicity, 2D cell models are heavily used to define basal characteristics of skin cell populations such as cell morphology, population doubling time, gene and protein expression patterns, and cell motility. They are also useful to detect changes in target cells in response to an insult, treatment, or change in culture conditions.

For example, the inherent growth characteristics of DF isolated from the trunk and distal limbs of horses and ponies have been evaluated in a 2D culture system [95]. Results from this study showed that DF isolated from horse limbs grow more slowly than DF isolated from the horse trunk, as well as from pony limb and trunk, and led the authors to conclude that differences

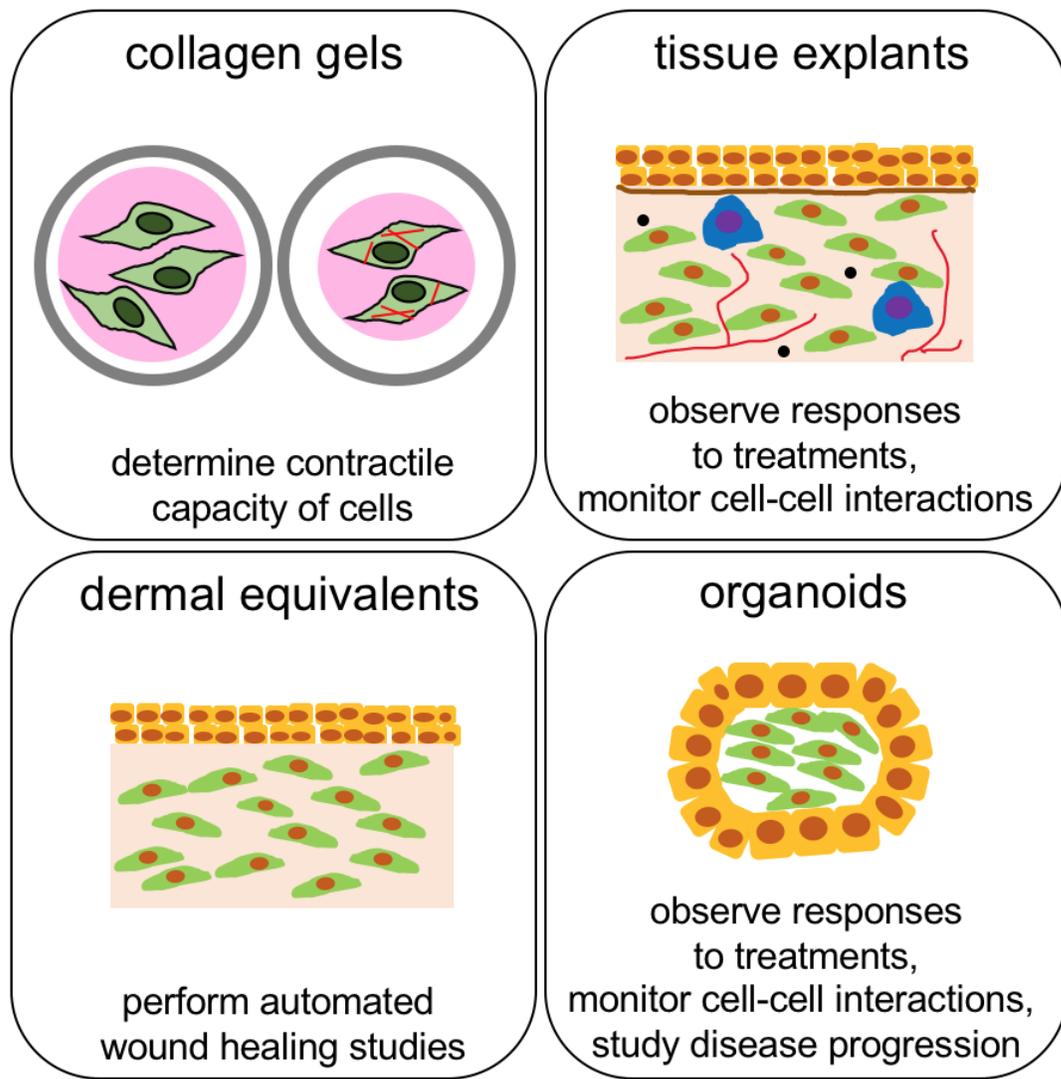
in DF growth may contribute to the development of EGT. Consequently, influencing DF growth could be a potential way to control the development of EGT [95]. Another study from our group compared basic characteristics of DF isolated from healthy horse skin and horse EGT in a 2D monoculture model [96]. This work showed that cultured DF from EGT display a more cuboidal morphology in culture compared to DF cultured from healthy skin, and that DF isolated from EGT have a longer population doubling time, migrate more slowly, and have gene expression profiles that are distinct from those observed in DF isolated from healthy skin. Moreover, EGT-derived DF were found to be more susceptible to busulfan-induced senescence than DF derived from healthy skin [96]. These observations could provide some clues as to why EGT persists *in vivo*. In addition to DF, primary equine keratinocyte cultures [97] and equine digital vein endothelial and smooth muscle cell cultures [98] have been established in 2D culture systems. As these cell types are all involved in skin wound healing *in vivo*, they can be used in future experiments to learn more about the cell-cell and cell-ECM interactions that occur during normal and impaired wound closure.

Migrating immune cells can also be isolated from horse blood, characterized in culture, and then used in various 2D and 3D (see below) co-culture models used to observe cell-cell interactions relevant to wound healing processes. For example, CD4<sup>+</sup> T cells, were purified from horse and pony peripheral blood mononuclear cell preparations and cultured, which allowed for the identification of a subpopulation of FOXP3<sup>+</sup> cells analogous to regulatory T cells in other species [99, 100]. As these cells are known to be dysregulated in human AD and fibroproliferative tissue, they may prove to be a useful tool for *in vitro* wound healing studies with equine cells. Polymorphonuclear cells (PMN) isolated from horses and ponies, and

maintained in culture, showed that (i) reactive oxygen species production of pony PMN is higher than that of horse PMN and (ii) *in vitro* chemotaxis of PMN differs between those isolated from ponies and horses, providing the basis that these cells can contribute to differences observed in horse and pony skin wound healing [101]. Equine monocyte-derived macrophages [102] and mesenchymal stromal cells (MSC) [103, 104] are additional examples of migratory cells that have been characterized using 2D cell culture systems and contribute to skin wound healing.

#### *2.3.2.2. Three-dimensional (3D) cell culture*

A variety of 3D cell culture systems have been developed for the study of equine cutaneous wound healing (Figure 2.4). These models are often considered more anatomically significant than 2D cell culture, as they incorporate both a cellular support system or matrix and/or multiple cell types, which allow for the establishment of cell-matrix and cell-cell contacts that mimic the *in vivo* wound microenvironment [105].



**Figure 2.4. Three-dimensional (3D) equine *in vitro* cell culture models.** Collagen gels are used to determine the contractile capacity of cells, which can be quantified. Tissue explants, which maintain *in vivo* architecture, are useful to observe responses to treatments and monitor cell-cell interactions, but their lifespan *in vitro* is limited. Uniform dermal equivalents are used in automated wound healing studies. Organoids, which have a long lifespan in culture and exhibit a high degree of cellular organization are used to observe responses to treatments, monitor cell-cell interactions and study disease progression.

The contractile capacity of equine DF isolated from the distal limb and buttocks of horses and ponies was determined by taking advantage of relatively simple 3D cell culture models; growing equine DF in both floating and anchored collagen gels [59]. From these studies it was concluded that *in vivo* differences in contraction between wounds on the limbs versus buttocks, and between horses versus ponies, is not caused by the inherent contraction capacity of DF at wound sites, suggesting that the activity of these cells *in vivo* is determined by the wound environment.

Tissue explants are 3D culture models that are perhaps the most directly reminiscent of the *in vivo* environment. For these cultures, biopsies are collected and maintained in culture medium, with or without physical support, for several weeks [106]. These explants are primarily used to compare the responses of tissue to treatments [107, 108], test the toxicity of compounds [109], and monitor cell migration and reepithelization after wounding [110]. While explants (i) contain a greater diversity of cell types than can be incorporated in an engineered 3D model and (ii) maintain the complex architecture of the skin, they have a limited life span in culture. Equine dermal explants have been used as a source of hair follicle-derived stem cells [111], as well as primary keratinocytes [112], and our lab is currently optimizing an equine skin explant model in which to study bacterial biofilm growth in skin wounds (Van de Walle, personal communication).

Dermal equivalent models are another type of 3D cell culture model, often comprised of DF, embedded in a solid ECM, seeded with an “epidermis” of keratinocytes. Additionally, a hypodermis of adipose tissue can be incorporated [113]. Over several weeks in culture, the keratinocytes will form several layers, including the stratum corneum. These models are typically less complex than tissue explants, but since it is possible to generate many uniform

dermal equivalents that may be maintained for a relatively long time in culture, they are considered ideal for automated wound healing studies and toxicity testing [114, 115]. Recently, an equine dermal equivalent model has been developed that consists of “normal” horse DF and keratinocytes, supported by a collagen matrix and resembles equine skin morphologically and structurally [116]. Although not currently available, construction of similar models using cells isolated from EGT or from animals suffering from AD, with or without the addition of immune cells, could prove to be a productive and physiologically relevant way to study pathological wound healing processes *in vitro*.

Finally, organoids are defined as *ex vivo* multicellular fragments containing the major cell types of a particular organ that approximate *in vivo* organization. One strategy for developing organoids is to culture multipotent or pluripotent stem cells in a 3D matrix under conditions that promote self-organization of the cells. As these models achieve a level of cellular organization not possible with dermal equivalents, and they can be maintained in culture longer than tissue explants, they are useful models for the study of pathologies, cell-cell interactions, and drug discovery [117, 118, 119]. Murine and human organoids have been developed from retinal tissue, kidney, liver, intestines, stomach, lung and brain [117], and very recently, from skin [120]. Equine intestinal organoids are currently the only organoid type published [121], although we anticipate that equine organoids from additional tissues, including skin, will be established in coming years.

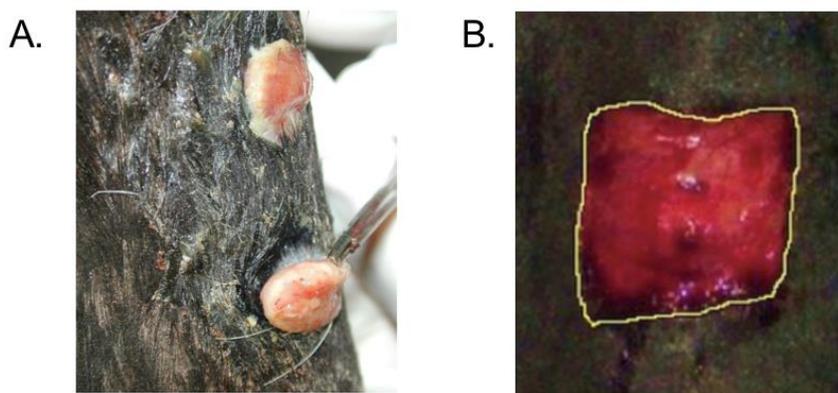
### 2.3.3. Equine *in vivo* skin wound models

*In vivo* studies in horses have provided researchers with a well-controlled environment in which to study conventional therapies such as bandaging and ointments [122, 123, 124], and they have been used to evaluate the efficacy of full-thickness grafts [125], to understand

complications of sores associated with casting [126, 127], and to elucidate the mechanisms responsible for AD [128, 91]. However, the majority of experimentally-induced and controlled horse wound studies have been carried out with the goal of increasing our understanding of the development of cutaneous fibroproliferative disorders.

As described above, horse skin wounds on the distal limbs heal primarily by epithelialization, similar to human wounds. Since horse body wounds tend to heal by contraction, the horse provides a model in which wound healing by different mechanisms can be studied within the same animal, eliminating individual-to-individual variation. Moreover, the size of horses also provides the following experimental benefits: (i) multiple experimentally-induced wounds can be generated at one site on an animal, allowing for controls and treatments to be performed on the same individual and (ii) multiple biopsies of horse wounds can be collected over time, to evaluate healing processes in a way that cannot be performed in humans or small model species.

The horse model for experimentally-induced EGT was developed and validated in 2001 and has since been adopted by many equine researchers [57, 129, 68, 21, 130]. In this model, multiple full thickness 6.25 cm<sup>2</sup> wounds are created on the lower limbs using a scalpel. Wounds can be as close as 1.5 cm apart and are typically arranged in a vertical column. Postoperative bandaging reliably induces the formation of EGT (Figure 2.5A). Full thickness wounds of the same size, inflicted on the thorax or neck, and which do not develop EGT, can serve as controls (Figure 2.5B). This model has allowed for the study of the effects of wound oxygenation and temperature on EGT development [69,70], and the evaluation of gene and protein expression patterns in EGT compared to wounds that heal quickly [61, 62, 63, 64, 131, 132].



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**Figure 2.5. Experimentally inflicted horse skin wounds.** **A.** Exuberant granulation tissue (EGT) induced by bandaging experimentally inflicted wounds on the lower leg of a horse [14]. **B.** Experimentally inflicted wounds on the torso do not develop EGT [129]. *Images from the referenced published work.*

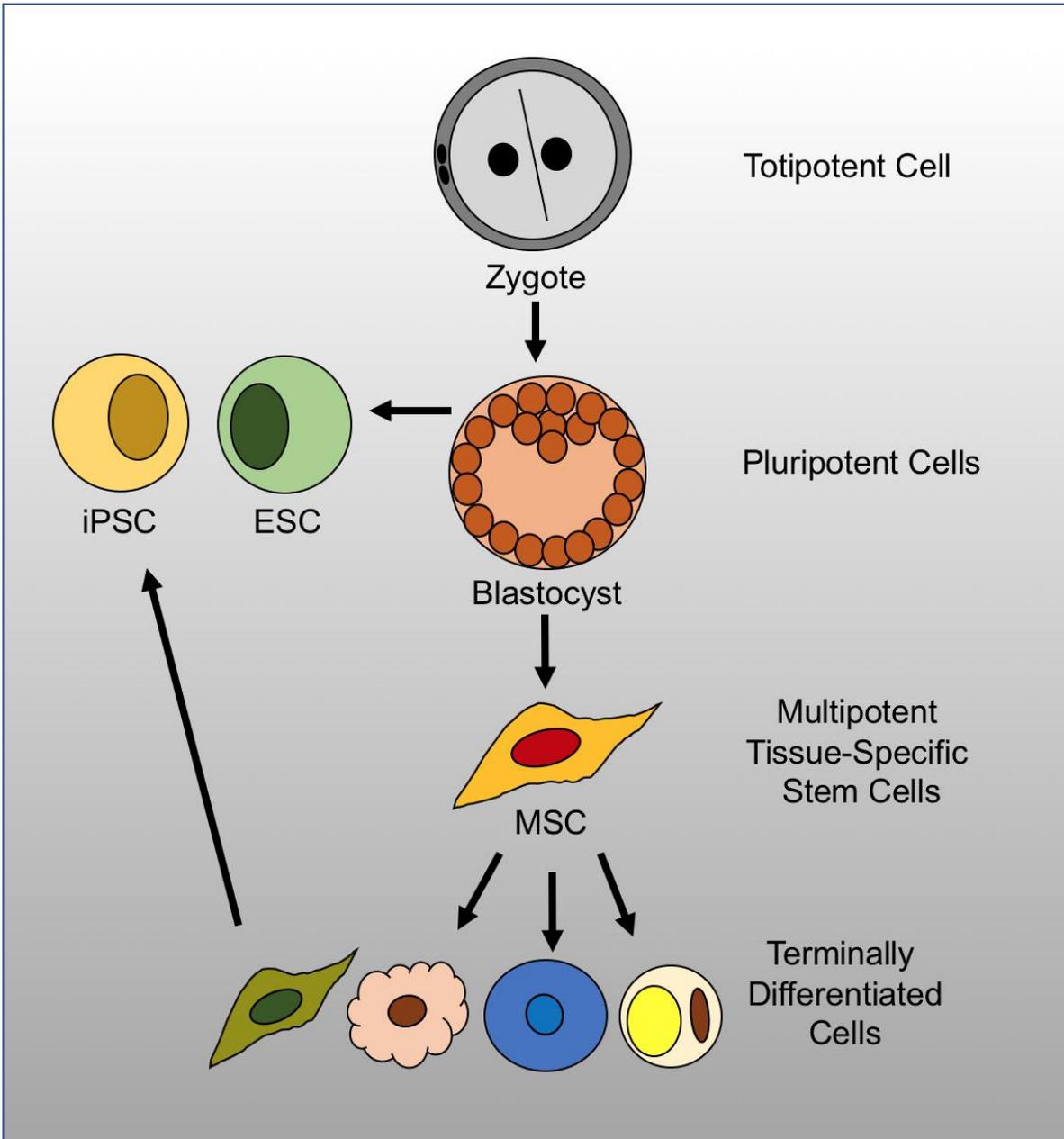
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#### **2.4. The horse as a model to explore novel therapeutics for wound management**

As a large animal, with physiological concordance to humans, the horse may serve not only as a model for the biological processes involved in wound healing, but also as a translational species in which to study novel therapies. Benefits of using an animal the size of a horse to test new wound management therapies include (i) the ability to experimentally inflict well-controlled skin injuries instead of having to rely on human patients enrolled in clinical trials with different stages of chronic wounds and/or additional underlying diseases, and (ii) testing alternate therapies for skin wounds in horses allows for earlier invention than is possible in humans, where unconventional treatments are usually only administered as ‘a last resort’. Finally, progress in the development of novel therapies in an equine model will not only benefit humans, but equine patients as well. The therapies described in this section are currently being developed for both humans and horses.

#### *2.4.1. Stem cell therapies*

Stem cells, or their products, are a type of cell-based therapy that may be used to augment the natural events required for successful wound healing, while avoiding the side effects associated with administering recombinant factors and the limits associated with a technology-heavy procedure [7]. Stem cells are defined as undifferentiated cells that can (i) self-renew, maintaining the stem cell pool, and (ii) differentiate into mature cell types. Stem cells are classified based on their potency, with totipotent stem cells, present during the zygote stage, that can differentiate into all cell types of the body. Pluripotent stem cells, such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), can give rise to almost all of the cells that comprise an organism with exception of placental tissues. Multipotent stem cells are found after birth, and these adult tissue-specific stem cells exhibit the ability to differentiate into any of the cell types that arise from their germ layer of origin (Figure 2.6).



**Figure 2.6. Stem cell hierarchy.** The totipotent cells of the zygote can differentiate into all cell types of the body. The embryonic stem cells (ESC) of the blastocyst are pluripotent stem cells that can give rise to most of the cells making up an organism. Mesenchymal stromal cells (MSC) are considered to be multipotent tissue-specific stem cells, able to differentiate into the cell types making up their specific tissue-of-origin. Terminally differentiated cells can be re-programmed into induced pluripotent stem cells (iPSC), with a potential analogous to that of ESC.

#### 2.4.1.1. Embryonic stem cells (ESC)

Human ESC, with a limitless capacity to self-renew and the ability to differentiate into almost any cell type could be therapeutically valuable, but ethical issues associated with collecting cells from embryos have largely hindered their evaluation for this purpose. Ethical concerns are not as much of a concern when studying ESC isolated from veterinary species, but these cells are currently not actively being used in equine medicine. Putative equine ESC have been described and validated as ECS based on marker expression and differentiation potential, but they did not form teratomas when injected into immunocompromised mice, which is the gold standard for human and murine ESC verification [133]. After this first description, additional studies with equine ESC have been carried out in relation to their potential for treatment of tendon injuries [134, 135, 136, 137], but these cells, to our knowledge, have never been explored as a therapy for cutaneous wounds.

#### 2.4.1.2. Induced pluripotent stem cells (iPSC)

iPSC are adult cells, often skin fibroblasts, that have been re-programmed from a differentiated state into a less-differentiated state, typically by the introduction of transcription factors specific to pluripotent cells [138]. Human iPSC were first derived from adult human DF in 2007 [139]. Since then, they have been differentiated successfully into cardiomyocytes, cells of the skeletal system, hepatocyte-like cells, and cells of the skin, with the goal of generating cells and tissues that can be used for transplantation [140, 141, 142, 143]. In human medicine, the skin has been recognized as an ideal organ for iPSC-based applications. Skin equivalents can be generated from iPSC derived from patients with inherited genetic skin conditions, allowing for the study of these diseases *in vitro* [143]. In addition, disease-causing mutations can be

corrected in iPSC, allowing for the generation of gene-corrected replacement skin for patients suffering from genodermatoses [144].

Equine iPSC have been generated from fetal fibroblasts and keratinocytes. iPSC derived from horse fibroblasts expressed endogenous markers associated with pluripotency, displayed a stable karyotype over long-term culture, and formed teratomas containing all three embryonic germ layers when grafted into mice [145, 146]. Keratinocyte-derived iPSC have been reported to express endogenous pluripotent stem cell markers and to form derivatives of the three germ layers when injected into mice. With the appropriate *in vitro* conditions, they were found to resemble cholinergic motor neurons that were functionally active, based on the ability to generate action potentials. Importantly, these cells could be expanded without the support of feeder cells, a desirable quality if these cells are to be used for biomedical applications [147].

#### *2.4.1.3. Mesenchymal stromal cells (MSC)*

MSC are a type of adult, multipotent progenitor cell that can be readily expanded in culture [148]. These cells actively secrete a rich composite of bioactive factors that may prove to be a safe, easy-to-deliver therapy to improve tissue repair [149]. Human MSC have been actively studied as a therapy for the healing of burn wounds, incisional wounds made by a scalpel during surgery, and excisional wounds generated by the removal of a portion of skin [150, 151, 152,153]. On the NIH Clinical Trials website, there are currently several trials listed to test the efficacy of MSC to promote the healing of diabetic foot wounds as well as burn wounds.

Although human MSC therapy for wound, and other, injuries and diseases has been studied for years, it still is not accepted as a clinical practice. This is in contrast to equine medicine, where MSC are clinically used as a therapy for orthopedic injuries. As a standard practice, equine clinicians isolate MSC from the patient's bone marrow, expand the cells in culture, and then

cryopreserve the cultured cells in the event the patient suffers from a tendon or ligament injury later in life. If and when this happens, these autologous MSC are revived and injected at the site of injury. This general acceptance of MSC therapy by equine practitioners and clients, makes veterinary medicine an ideal setting in which to explore the therapeutic value of MSC for additional types of injuries and disorders.

We have used *in vitro* systems to study the potential of equine MSC derived from peripheral blood, a less invasive source than bone marrow, for wound management. We have shown that these cells secrete factors that stimulate angiogenesis, secrete proteins that promote DF migration, and produce antimicrobial compounds that inhibit the growth of bacteria commonly found in skin wounds [154, 155, 156]. We also found that when exposed to MSC-derived secreted factors, the expression of genes associated with a contractile cell phenotype is altered in EGT-derived DF, but not DF isolated from normal skin, and that these factors do not stimulate the migration of EGT-derived DF as they do for DF derived from normal skin [96]. Finally, we have begun to explore methods of cell encapsulation that may facilitate the delivery of equine MSC secreted factors to skin wounds [103]. Collectively, these *in vitro* studies suggest that secreted factors from equine MSC could promote wound healing *in vivo* and may be used as a treatment option to prevent or reduce proud flesh formation on the lower limbs of horses.

A recent *in vivo* study from the group of Borjesson showed that injection of allogeneic MSC into experimentally-induced distal limb wounds altered gene expression at the wound site and enhanced early healing [130]. The promising results of this preliminary *in vivo* study provides a strong basis for additional *in vivo* studies designed to further evaluate the effects of MSC therapy on skin wounds of the horse.

#### 2.4.2. Platelet concentrates

A fine-tuned balance of growth factors and cytokines is critical to successful wound healing. As these signaling proteins are responsible for stimulating and directing the dynamic cell-cell and cell-ECM interactions that occur during healing, the composition and concentration of growth factors and cytokines varies both across the wound bed and temporally during the healing process. Since growth factors such as PDGF, VEGF, and EGF, and cytokines such as interleukins, IFNs, lymphokines and TNFs are necessary for cutaneous wound healing, their therapeutic potential has been studied. Applying individual growth factors or cytokines to wounds, however, has not proven to be successful. This is not surprising as the wound bed contains a changing array of proteases, enzymes that may immediately destroy specific exogenous proteins, that can inhibit the action of these growth, and other, factors. Conversely, the application of individual growth factors or cytokines to wounds might flood the wound bed with overwhelming signals, which could disable resident cells and prevent them from participating in the healing process [14]. The administration of platelet concentrates, also called platelet-rich plasma or PRP, has been explored as an alternative to treating wounds with individual factors in both human and equine medicine. Platelet concentrates are usually generated by centrifuging the patient's own blood, in order to collect the platelet-rich fraction as well as blood cells that secrete an array of growth factors and cytokines. Human platelet concentrates of various compositions have been classified and characterized based on leukocyte and fibrin content in an attempt to identify features that add therapeutic value [157]. A recent review on PRP adjunct therapy in humans described that PRP (i) improved the healing of acute wounds, (ii) had mild (or no) positive effects on wounds resulting from laser therapy, and (iii) has not been studied in the context of burn wounds extensively enough to draw any conclusions for the treatment of this type of wounds [158]. In horses, several studies suggest that PRP has

beneficial effects on the healing of surgical as well as burn wounds [159, 160], while it has been reported by other studies that topical application of PRP does not accelerate the repair of small granulating wounds on the distal limbs of horses [161]. Clearly, more work is needed to determine what types of platelet concentrates are the most appropriate for the treatment of which types of cutaneous wounds.

#### 2.4.3. Maggot debridement therapy

For centuries, fly larvae have been purposefully used to debride necrotic tissue from human cutaneous wounds, but the efficacy and complete benefits of larval therapy have not been adequately assessed or optimized [162]. The most commonly used flies belong to the family Calliphoridae, with *Lucilia sericata*, the greenbottle blowfly, being the most widely accepted species. Newly emerged, germ-free larvae can be purchased from FDA-approved vendors with a prescription for both human and veterinary use [14]. Historically, it has been accepted that maggots debride wounds, which prepares the wound bed for healing. More recent evidence suggests that larvae may contribute to wound healing in additional ways. *In vitro* studies have shown that maggot secretions can reduce biofilms [163, 164] and digest components of necrotic human tissue [165], implying that they may serve to disinfect wounds. A retrospective analysis designed to describe and assess the results of maggot debridement therapy in horses concluded that, “maggot debridement therapy can be recommended in equids for debridement and enhanced healing and its potent antibacterial action” [166]. Similarly, a survey of veterinarians who used maggot debridement therapy on horse hoof and leg wounds concluded that maggot therapy can serve as a useful treatment [167]. This therapy, especially until fully optimized and proven effective, may be rejected by people as it is considered to be aesthetically unappealing by many. The horse is a reasonable model in which to refine maggot debridement therapy, to elucidate the

mechanisms by which it improves wound healing, and to provide the evidence that it can be a safe and effective therapy for both humans and horses.

#### *2.4.4. Negative pressure therapy*

In human medicine, negative-pressure wound therapy (NPWT) is used as an adjunct to traditional methods for treating a variety of cutaneous wound types. There are several types of NPWT systems, but all are similar in that they involve dressing the wound to create a sealed area that is then subjected to a vacuum. Drainage tubing is also incorporated to allow for the removal of fluids moved to the surface by the vacuum [168]. NPWT promotes second intention healing, by reducing the wound size, removing excess fluid, and improving the wound environment in ways that are not fully understood, but are thought to contribute by modulating cytokine expression in the wound bed towards a profile that favors angiogenesis, ECM remodeling, and deposition of granulation tissue [169, 170]. A recent and comprehensive review of NPWT concluded that it can be used to manage chronic, complex and infected wounds, but that results from randomized control trials are variable [168]. Therefore, additional research focused on understanding the mechanisms of action of NPWT is needed in order to fully take advantage of this promising adjunctive therapy. Several case studies in horses have also concluded that NPWT facilitated second-intention healing and shortened the time to healing [171, 172, 173]. However, and in line with human studies, the mechanisms through which NPWT acts in horses have not been defined. Clinical trials in horses could shed light on how NPWT alters the wound microenvironment, leading to more effective treatments for both humans and horses.

#### *2.4.5. Engineered skin tissue substitutes*

Skin substitutes, grown *ex vivo*, are commercially available for human use. They are used when patients do not have adequate skin available for autografting, and to reduce the risks of

rejection and disease transmission that are associated with allografted skin. Engineered skin substitutes may be used to replace the dermal or epidermal layers and are comprised of various combinations of skin cells and ECM molecules. A recent review of human skin substitutes determined that these products have revolutionized the management of wounds, but that more robust trials are needed to identify optimal skin substitutes for specific types of wounds, as well as to determine what features can be added to improve the intrinsic quality of currently available substitutes [174]. Equine researchers have developed cell culture systems to support the proliferation and differentiation of keratinocytes and to produce skin-equivalents that consist of fibroblasts and an epidermal layer of differentiated keratinocytes [116, 112], but engineered skin substitutes are not currently used in equine medicine.

A major limitation of skin substitutes for both species is that they don't include, glands, blood vessels and nerves. An ideal skin substitute would include a population of stem cells, which could divide and, differentiate under the appropriate environmental conditions into the various cell types found in skin. As mentioned above, equine iPSC have been differentiated successfully into functional neurons [147], suggesting an equine skin substitute could be seeded with these cells in order to form complete structures necessary for full skin regeneration.

## **2.5. Concluding remarks**

Healthy, uncompromised skin is required for both humans and horses to experience a high quality of life. Due to a lack of understanding of the mechanisms that contribute to normal and impaired cutaneous wound healing, current therapies are inadequate for both species. This poses a large burden to the human and veterinary health care systems not only economically, but physically, as a result of the pain and suffering experienced by human and equine patients with

non-healing wounds. The horse is a physiologically relevant large animal species that may serve as a model to answer questions about mechanisms involved in wound healing that effect humans and equids, and to test therapies for both species. We believe that an increase in basic scientific research and clinical studies focusing on skin wound healing in horses will produce data that will benefit both human and veterinary health.

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**CHAPTER THREE: MICROENCAPSULATED EQUINE MESENCHYMAL STROMAL  
CELLS PROMOTE CUTANEOUS WOUND HEALING *IN VITRO***

The results of this study were published in Bussche L\*, Harman RM\*, Syracuse BA, Plante EL, Lu Y, Curtis TM, Ma M, et al. Microencapsulated equine mesenchymal stromal cells promote cutaneous wound healing in vitro. *Stem Cells Res and Therapy* 2015;6:66.

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### 3.1. Summary

The prevalence of impaired cutaneous wound healing is high, and treatment is difficult and often ineffective, leading to negative social and economic impacts for our society. Innovative treatments to improve cutaneous wound healing by promoting complete tissue regeneration are therefore urgently needed. Mesenchymal stromal cells (MSC) have been reported to provide paracrine signals that promote wound healing, but (i) how they exert their effects on target cells is unclear and (ii) a suitable delivery system to supply these MSC-derived secreted factors in a controlled and safe way is unavailable. The present study was designed to provide answers to these questions using the horse as a translational model. Specifically, we aimed to (i) evaluate the *in vitro* effects of equine MSC-derived conditioned medium (CM), containing all factors secreted by MSC, on equine dermal fibroblasts, a cell type critical for successful wound healing, and (ii) explore the potential of microencapsulated equine MSC to deliver CM to wounded cells *in vitro*.

Mesenchymal stem cells (MSC) were isolated from the peripheral blood of healthy horses. Equine dermal fibroblasts from the NBL-6 line were wounded *in vitro*, and cell migration and expression levels of genes involved in wound healing were evaluated after treatment with MSC-CM or NBL6-CM. These assays were then repeated using the CM collected from MSC encapsulated in core-shell hydrogel microcapsules.

Our salient findings were that equine MSC-derived CM stimulated the migration of equine dermal fibroblasts and increased their expression level of genes that positively contribute to wound healing. In addition, we found that equine MSC packaged in core-shell hydrogel microcapsules had similar effects on equine dermal fibroblast migration and gene expression,

indicating that microencapsulation of MSC does not interfere with the release of bioactive factors.

Our results demonstrate that the use of CM from MSC might be a promising new therapy for impaired cutaneous wounds and that encapsulation may be a suitable way to effectively deliver CM to wounded cells *in vivo*.

### **3.2. Introduction**

Cutaneous wound healing is comprised of a network of biological processes, collectively restoring the integrity of the skin after injury. Unfortunately, the ideal outcome of cutaneous wound healing, which encompasses complete tissue regeneration, is often sacrificed in favor of quickly closing a wound with formation of fibrotic scar tissue [1]. Fibrotic scar formation is an undesirable result of cutaneous wound healing, not only for cosmetic reasons, but because scar tissue has compromised mechanical strength and is more sensitive to pain than healthy skin [2]. Treating cutaneous skin wounds and reducing scar tissue causes a financial burden worldwide, with annual expenditures on products designed to minimize scarring exceeding \$5 billion [3].

In order to understand the processes involved in cutaneous wound healing and/or treat fibrotic scar tissue, researchers commonly use laboratory rodents as models. Although relevant information has been obtained from studies using rodents, wound healing in these species does not accurately mimic human tissue regeneration [4-7]. For example, mice primarily heal by contraction due to the presence of the *panniculus carnosus* in their subcutaneous tissues, whereas humans lack this muscle and instead rely on epithelialization to close cutaneous wounds [7]. Due to these differences, there is a critical need for an animal wound model that closely mimics the natural processes of healing and scar formation in humans. Horses may prove to be an ideal species in which to study cutaneous wound healing, since they also depend on epithelial cell

activity to heal skin wounds. In addition, horses and humans both suffer from hypergranulation formation, a pathological process defined as an excess of granulation tissue beyond the amount required to replace the tissue deficit. Hypergranulation tissue is referred to as proud flesh in horses and keloid in humans [8]. These common features make the horse an attractive translational model in which to study the underlying pathogenesis of excessive cutaneous wound healing as well as to evaluate the potential of novel treatments.

Mesenchymal stromal cells (MSCs) are multipotent stromal progenitor cells with regenerative properties that are present in a variety of tissues and organs [9]. MSC actively contribute to regenerative processes, as they are involved in the inflammatory [10], proliferative [11] and remodeling [12] phases of tissue regeneration. Although MSC were originally reported to contribute to tissue repair by trans-differentiating into the specific cell types needed to restore injured tissue, recent data indicate paracrine signaling as the primary mechanism for the regenerative effects of MSC [13,14]. Indeed, we recently identified angiogenic stimulating factors in the conditioned medium (CM) of equine MSC and showed that these factors could induce proliferation and vessel formation of equine endothelial cells *in vitro* [15]. Practically, this implies that the CM obtained from MSC cultures, which contains all factors secreted by MSC, may be used as a “stem cell-free” therapy in regenerative medicine. This type of therapy offers several advantages over cellular MSC cell remedies, including the absence of inherent toxicity, no risk for tumor formation of engrafted cells, and no concerns about allograft-associated immune rejection [16,17]. Previous reports have demonstrated that CM obtained from human MSC cultures can improve cutaneous wound healing, although the underlying mechanisms remain unclear [18,19]. The potential of CM obtained from equine MSC cultures to contribute to wound healing has not been studied to date but is essential in order to take

advantage of the horse as a physiologically relevant translational model in which to study cutaneous wound healing.

An important aspect to take into consideration when proposing the use of MSC-derived CM in regenerative medicine is the development of a suitable delivery system for these secreted products. Indeed, optimal spatial distribution and continuous release of factors at the site of injury are two key components of MSC treatment that may significantly improve clinical outcome. Cell microencapsulation, which involves immobilization of the cells within a polymeric semi-permeable membrane, provides a supportive microenvironment for the cells in which they can proliferate and release bioactive factors while being shielded from the external environment [20,21]. These microcapsules can be injected at the transplantation bed localizing the release of therapeutic factors in a controlled way. A recent study by Xu *et al.* describes the potential use of human MSC encapsulated in biomaterials for the treatment of cutaneous wounds and their initial findings were that cells ‘packaged’ in a gelatin/poly(ethylene glycol) biomatrix mediated the early resolution of inflammatory events and facilitated the proliferative phases in wound healing [22]. To the best of our knowledge, however, the use of microencapsulated equine MSC in equine regenerative medicine has not been explored to date.

Therefore, the aims of the present study were (i) to evaluate the *in vitro* effects of equine MSC-derived CM on equine dermal fibroblasts, a cell type critical for successful wound healing, and (ii) to explore the potential of microencapsulated equine MSC to deliver the critical components of CM to wounded cells *in vitro*. Our salient findings were that equine MSC-derived CM stimulated the migration of equine dermal fibroblasts and increased the expression levels of genes that positively contribute to wound healing in these cells. In addition, we found that equine MSC packaged in core-shell hydrogel microcapsules have similar effects on equine dermal

fibroblast migration and gene expression, suggesting MSC encapsulation may be a suitable way to effectively deliver products secreted by MSC to wounded cells *in vivo*.

### 3.3. Methods

#### *Cells*

Equine mesenchymal stromal cells (MSC) were isolated from the peripheral blood of 3 healthy warmblood mares between 8 and 12 years old, exactly as described previously [15,23]. The blood collection was approved by the Cornell Institutional Animal Care and Use Committee (IACUC # 2014–0038). Briefly, blood was collected from the *v. jugularis* of healthy donor horses, peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation on 1.080 g/mL Percoll (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and subsequently seeded at a density of  $16 \times 10^4$  cells/cm<sup>2</sup> in a T75 flask in culture medium, consisting of low glucose (LG) Dulbecco's modified Eagle medium (DMEM) (Invitrogen), supplemented with 30% fetal bovine serum (FBS) (Atlanta Biological, Flowery Branch, GA),  $10^{-7}$  M low dexamethasone (Sigma Aldrich, St. Louis, MO), 50 µg /mL gentamycin, 1x penicillin-streptomycin, and 2 mM L-glutamine (all from Life Technologies, Grand Island, NY). Cultures were maintained at 37°C with 5% CO<sub>2</sub>. At 70% confluency, cells were removed from flasks using 0.25% trypsin–EDTA and further cultured in expansion medium, which is identical to the culture medium but without dexamethasone. Equine MSC were characterized by immunophenotypical protein profiling using flow cytometry and their potential for trilineage differentiation, exactly as described previously [15]. In essence, equine MSC were confirmed to be positive for CD29, CD44, CD90 and CD105, and negative for CD45, CD79α, MHC II and a monocyte/macrophage marker. The successful trilineage differentiation of MSC toward osteoblasts, chondroblasts and adipocytes was confirmed using a range of histochemical stains.

The equine dermal fibroblast cell line NBL6 (ATCC, Manassas, VA) was cultured in standard medium, consisting of minimal essential medium (MEM, Corning) supplemented with 10% FBS (Atlanta Biochemicals) and 1% penicillin/streptomycin (Invitrogen). Cells were maintained at 37°C with 5% CO<sub>2</sub>.

#### *Generation of conditioned media and pre-treatments*

Conditioned medium (CM) was collected from MSC after 2 days of culture, when cells were 70% confluent. To this end,  $6 \times 10^5$  MSC were seeded in a T75 flask in 8 ml expansion medium. After 48 hours (h), medium was collected, centrifuged twice for 7 minutes at  $300 \times g$  to remove any cellular debris, and used for further experiments. CM from NBL-6 cells was used as a control and was collected after 2 days of culture, exactly as described for MSC-derived CM. For pre-treatment experiments, MSC were seeded in expansion medium supplemented with 10 ng/ml tumor necrosis factor alpha (TNF $\alpha$ ; R&D, Minneapolis, MN), 20 ng/ml interferon gamma (IFN $\gamma$ ; R&D) or 150  $\mu$ M cobalt chloride (CoCl<sub>2</sub>, Sigma). After 24 h of culture, cells were washed twice with phosphate buffered saline (PBS) and fed with 8 ml fresh expansion medium. CM was collected 24 h later, as described above.

#### *Microencapsulated MSC*

For experiments using encapsulated MSC,  $6 \times 10^5$  MSC were seeded per T75 flask in 8 mL expansion medium, and in parallel the same number of cells were encapsulated in double layer microparticles using a multi-fluidic electrostatic cell micropackaging technique [24]. Briefly, type I collagen neutralized by 1 N sodium hydroxide was mixed with MSC in expansion medium for a final concentration of 0.45 mg/mL. Cells supported by collagen were encapsulated in 0.9% (w/v) alginate hydrogel (FMC Biopolymers, Philadelphia, PA) at a concentration of about 37 cells per capsule. Microencapsulated MSC were incubated in a crosslinking bath with

100 mM calcium chloride and 5 mM barium chloride under electrical field strength of 7 kV. Microencapsulated MSC were resuspended in 8 mL expansion medium and maintained at 37°C with 5% CO<sub>2</sub>. Empty core-shell hydrogel microcapsules, containing no cells, were included as negative control. After 48 h, supernatants were collected, centrifuged twice for 7 minutes at 300 × g to remove cellular debris, and used as CM in experiments.

#### *In vitro scratch assays*

NBL-6 cells were seeded in 6-well plates at a density of  $6 \times 10^4$  cells/cm<sup>2</sup>. Upon 90% confluency (~ after 24 h), cells were washed twice with PBS and serum-starved overnight. A linear defect was then inflicted on the monolayer using a 200 µl pipette tip. Culture medium was immediately removed (along with any dislodged cells) and replaced with freshly collected CM, diluted 1:1 in expansion medium. Similar scratch assays were repeated in the presence of 2 µg/mL mitomycin C, which was added at the time of scratch infliction. Reference marks were made across the bottoms of the wells with an ultrafine marker. Images of scratches were captured using a Nikon Diaphot-TMD inverted light microscope with an attached CoHu CCD camera (Nikon, Melville, NY) leaving the reference marks outside the capture image field but within the eye-piece field of view for repeatable orientation of scratches. Photographs of scratches were taken at 0, 24 and 48 h post scratching, and migration distances of cells were calculated in a blinded-manner using ImageJ [25]. Widths of scratches were taken in two places in each of 3 wells scratched per treatment, for a total of 6 measurements per treatment. Scratch width was subtracted from the time 0 scratch widths at the same location to determine cell migration distance. Migration distances within treatments were then averaged to determine overall migration.

#### *Electric cell-substrate impedance sensing*

Cell migration was measured by Electric Cell-substrate Impedance Sensing (ECIS) using the ECIS Model Z instrument with 96 W array station (ECIS, Applied BioPhysics Inc., Troy, NY). Dermal fibroblasts were seeded at a density of  $2 \times 10^5$  cells per well in a 96W1E<sup>+</sup> PET array chip (Applied BioPhysics Inc.) in standard medium. After 24 hours, medium was removed and replaced with serum-free medium for at least 8 hours prior to wounding. A uniform circular defect was created in the cell monolayer by lethal electroporation (1200  $\mu$ A, 40 kHz) for 40 seconds by 350  $\mu$ m diameter circular electrodes located in each well of the array chip. The defect created in the fibroblast monolayer will be referred to as wound throughout the paper, as originally described by Keese *et al.* [26]. Dead cells were detached from the electrode by gently pipetting medium up and down three times before removal. Control CM or MSC-derived CM was then added to appropriate wells, and an alternating current ( $\sim 1$   $\mu$ A, 32 kHz) was applied to the electrodes to measure impedance (Ohms) and monitor wound closure in real-time. As cells migrate to repair the circular wound, they physically re-cover the electrode causing an increase in impedance. The time required for re-covering the electrode was determined as the number of hours it took for the impedance levels in wells with the 350  $\mu$ m circular wound to return to the same values as the impedance levels in control wells in which cells were not wounded. Cell migration was also documented by phase contrast images captured using an Olympus CCX41 inverted microscope equipped with an Olympus LC20 digital camera.

#### *Gene expression analyses*

NBL-6 cells were cultured in  $15 \times 100$  mm culture dishes until confluent. Cells were serum-starved overnight, and scratches were made. After two washes with PBS, 1:1 diluted CM derived from MSC (un-stimulated, stimulated or encapsulated), or control NBL-6 was added to each NBL-6 culture. After 24 h, CM was removed, monolayers were rinsed with PBS and cells

were further cultured for another 24 h. Subsequently, mRNA was extracted from the cells using an RNEasy Plus Kit, according to manufacturer's instructions (QIAGEN, Valencia CA), followed by a DNase digestion using DNase I (Invitrogen). cDNA was synthesized using M-MLV Reverse Transcriptase (USB, Cleveland, OH), according to the manufacturer's protocol. SYBER green-based quantitative polymerase chain reaction (qPCR) assays were carried out on an Applied Biosystems 7500 Fast Real Time PCR instrument (Applied Biosystems, Carlsbad, CA) to determine fold changes of genes of interest in NBL6 cells incubated with CM of MSC compared to NBL6 incubated with control CM. The comparative  $C_t$  method ( $2^{-\Delta\Delta C_t}$ ) was used to quantify gene expression levels where  $\Delta\Delta C_t = \Delta C_t(\text{sample}) - \Delta C_t(\text{reference})$ . The previously validated reference gene SDHA was used to normalize samples [27]. Primers to amplify CCL2, CxCL10, IL8, MMP1, MMP2, TMP1, PLAU, PLAT, PAI1, CTSK, ANXA2, COLIII, COL1, SDC2, SDC4 and fibronectin were designed using Primer3 software, based on horse sequences found in the National Center of Biotechnology Information (NCBI) GenBank, and primer sequences are listed in Table 1. All samples were run in triplicate.

**Table 3.1. Overview of primers used for semi-quantitative RT-PCR**

Gene	Abbreviation	Forward primer (5'-3')	Reverse primer (5'-3')
Succinate dehydrogenase complex, subunit A	SDHA	TCCATCGCATAAGAGCAAAG	GGTGGAACCTGAACGAACTCC
Plasminogen activator urokinase	PLAU	TGTGAGATCACTGGCTTTGG	TGACATTCTGGTGGGAAAC
Annexin A2	ANXA2	GCAGTGTCTGTCACCTCCAG	CCTCCTTCTTGATGCTCTCC
Cathepsin K	CTSK	TCCAGAAGGGAAAACAAGCAC	TCTTATTCCGAGCCATGAGG
CC chemokine 2	CCL2	GGCTCAGCCAGATGCAATTA	GCTTCTTGTCAGCTGCTT
Metallopeptidase 2	MMP2	GAAGGCTGTGTTCTTTGCAG	TCCAGTTAAAGGCAGCATCC
Syndecan 4	SDC4	GCAGCATCTTTGAGAGGACAG	GTTTCTTGCCAGGTCGTAG
Metallopeptidase 1	MMP1	GCCAAATGGACTTCAAGCTG	TAGGAAAGCCGAAGGATCTG
Tissue plasminogen activator	PLAT	AGTTCTTGCTGGCTCCTGTC	ATCGGTATGTTCTGCCCAAG
Interleukin 8	IL8	AGACGCACTCCAAACCTTTC	CAGACCTCAGCTCCGTTGAC
Metallopeptidase inhibitor 1	TIMP1	GCTCCCTGGAACAGTCTGAG	CGTCCACAAGCAATCAGTGTC
Plasminogen activator inhibitor-1	PAI1	CCCTGGAGAGTGAAGTGGAC	CCTGCGATACATGGAGAAGC
Collagen type 1A1	COL1	AAGGACAAGAGGCACGTCTG	GCAGGAAAGTCAGCTGGATG
Collagen type 3	COLIII	GGGTATAGCTGGTCTCTGTC	GCGCTCTTTCTCCTTTAGC
Fibronectin	Fibronectin	AGGTCGTTACTGTGGCAAC	TAATGGGAGACGGTGTAGGG
Syndecan 2	SDC2	GACTCAGAAAGGAACGTGGAC	ATAACTCCGCCAGCAATGAC
CXC chemokine 10	CxCL10	GACTCTGAGTGGAACCTCAAGGAAT	GTGGCAATGATCTCAACACG

### *Cell viability assays*

To ensure mitomycin C was not toxic to NBL-6 fibroblasts, cells were seeded at 10,000 per well in 96 well microplates. At 90% confluency, mitomycin C was added to triplicate wells at 0, 2, 20, 200 and 2000 ng/mL. After 48 h of culture, an MTT *in vitro* toxicology assay (Sigma Aldrich, Saint Louis, MO) was carried out according to manufacturer's instructions and absorbance was measured at 570 nm on a Multiskan EX plate reader (Thermo Fisher Scientific, Waltham MA). Optical densities of wells treated with mitomycin C were compared to those of untreated wells to determine cell viability.

To test the viability of microencapsulated MSC, capsules were dissolved on days 2, 7, 12, 17, and 22 post encapsulation using a solution of 1% EDTA. The released MSC were washed with culture medium, incubated for 3 minutes with trypsin-EDTA to create a single cell suspension, washed with culture medium, and percentage of living cells was determined using the trypan blue exclusion (TBE) assay.

### *Population Doubling Time (PDT) calculations*

For determination of PDT,  $2 \times 10^5$  released MSC were plated in a T25 tissue culture flask and cultured in expansion medium. The PDT was calculated using the following link: [28]. PDTs of microencapsulated MSC were compared to PDTs of non-encapsulated MSC from the same horse at the same passages.

### *Characterization of MSC post encapsulation*

MSC removed from capsules 2 days post encapsulation were plated in T75 tissue culture flasks containing expansion medium until monolayer was 90% confluent. At that time, cells were collected for flow cytometric analyses and seeded for differentiation assays as described previously [15].

### *Proliferation assays*

The effect of mitomycin C on the proliferation of NBL-6 was evaluated using a bromodeoxyuridine (BrdU) proliferation assay kit (Abcam, Cambridge, MA). To this end, NBL-6 were seeded on a 96-well plate at a density of  $5 \times 10^4$  cells/well and incubated with mitomycin C for 48 h. Regular medium was used to establish baseline proliferation. The BrdU proliferation assay kit was used following manufacturer's instructions and the resulting absorbance was read at 450 nm on a Multiskan EX microplate reader using Ascent software (Thermo Scientific, Waltham, MA). Empty wells and wells without BrdU were included as controls.

### *Statistical analyses*

Student's t-test for paired data was used to test for statistically significant differences in cell migration (scratch assays and ECIS), mRNA expression (RT-qPCR), cell viability (TBE and MTT) and BrdU incorporation (ELISA) between untreated and CM-stimulated NBL6. Data given are the mean of 3 replicates and the bars show standard deviations.

To compare data from different individuals, statistical analysis was done by means of the nonparametric Kruskal-Wallis and Dunn's multiple comparison test ( $\alpha = 0.05$ ), and significant differences were considered at  $p < 0.01$ .

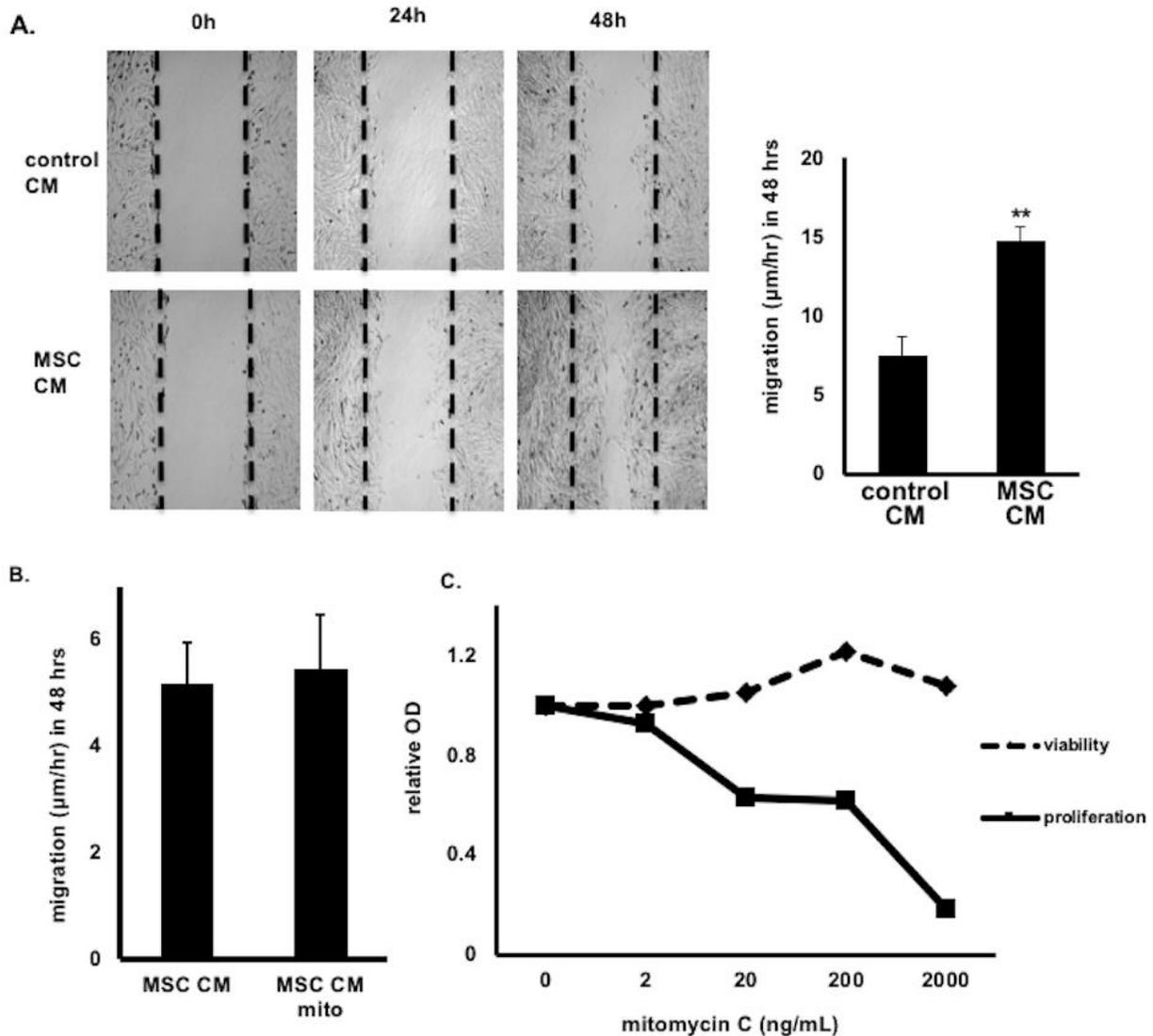
## **3.4. Results**

### *Conditioned medium (CM) from mesenchymal stromal cells (MSC) stimulates migration of dermal fibroblasts in scratch assays*

Since dermal fibroblasts are critically involved in wound healing [29] we decided to study the effects of MSC-derived CM on the migration of these cells using *in vitro* scratch assays [30]. We found that equine dermal fibroblast cells (NBL-6) migrated significantly faster when

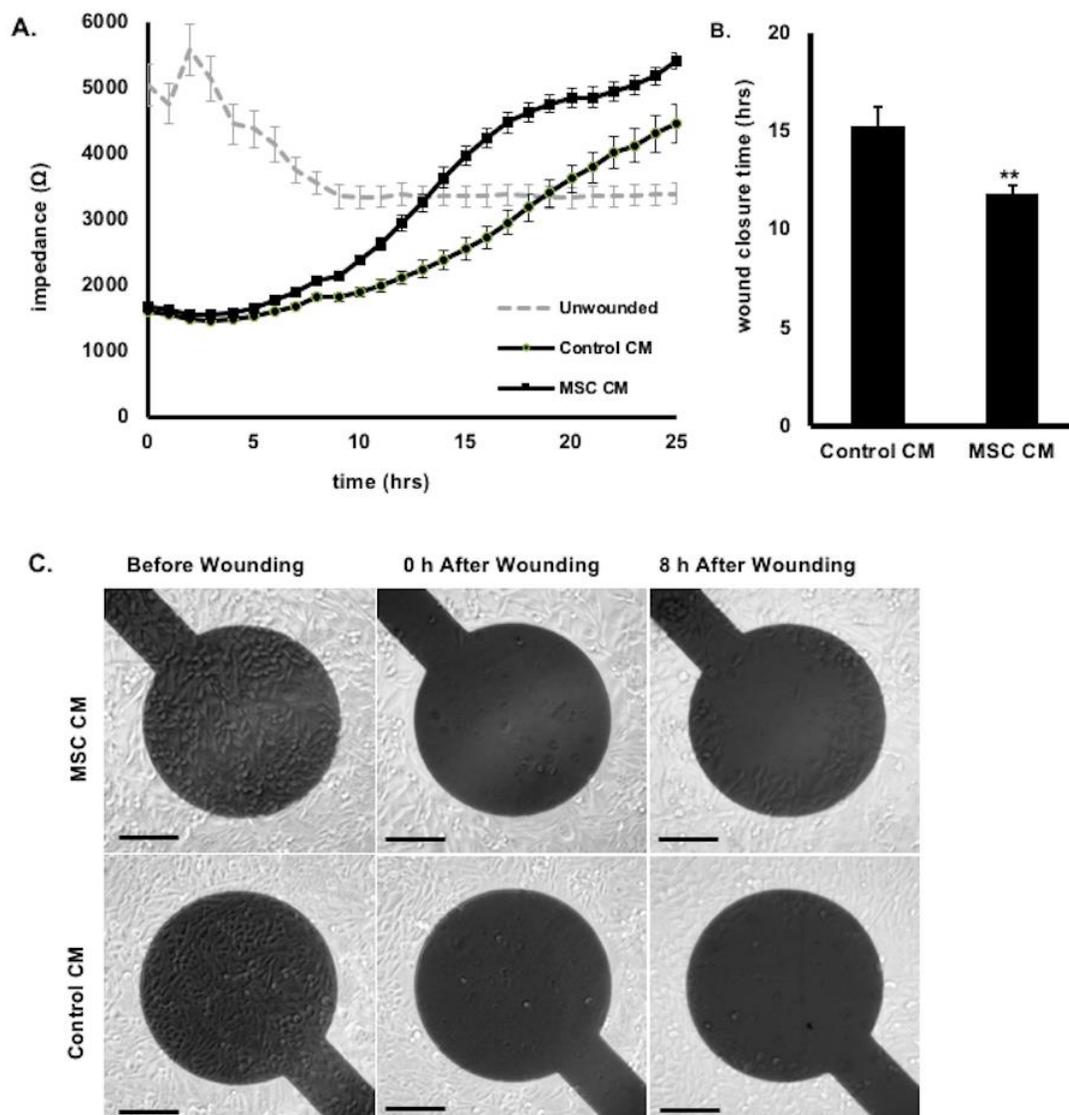
cultured in CM obtained from equine MSC compared to control CM, which was collected from NBL-6 (Figure 3.1A).

Given the high incidence of hypergranulation formation in equine and human wounds [8], it was important to explore whether this increased rate of wound closure was due to enhanced migration and/or increased proliferation of the fibroblasts. Therefore, we repeated the scratch assays in the presence of mitomycin, an inhibitor of proliferation. No differences in migration of NBL-6 cells cultured in MSC-derived CM in the presence or absence of mitomycin were found, indicating that the observed effect in the scratch assays was not due to an increased proliferation of the cells (Figure 3.1B). To control that the mitomycin concentration used for these experiments (ie. 2000 ng/ml) was effective in inhibiting proliferation without affecting cell viability, we performed a BrdU ELISA as well as an MMT assay, respectively (Figure 3.1C).



**Figure 3.1. Mesenchymal stromal cell (MSC) conditioned medium (CM) promotes migration of dermal fibroblasts in scratch assays (n = 3).** **A.** Representative phase contrast images of wounded NBL-6 cells cultured with MSC-derived CM (lower panels) as compared to control CM (upper panels) over a 48-hour period, and migration distances of NBL-6 cells are expressed as  $\mu\text{m/hr}$  in 48 hrs. \*\*:  $P < 0.01$  **B.** Migration of NBL-6 cells cultured with MSC-derived CM as compared to CM from mitomycin C-treated MSC (2000 ng/ml). **C.** Viability (dashed line) and proliferation (solid line) of NBL-6 cells cultured in the presence of various concentrations of mitomycin C.

To corroborate our findings on the increased migration of fibroblasts when exposed to MSC-derived CM, Electric Cell-substrate Impedance Sensing (ECIS) experiments were used to measure *in vitro* wound closure rates [31]. Again, we found that equine dermal fibroblasts migrated significantly faster when cultured in CM obtained from equine MSC compared to control CM with a wound closure of  $11.8 \pm 0.35$  h versus to  $15.3 \pm 0.96$  h, respectively (Figure 3.2A & B). Because the ECIS system creates only a very small wound (350  $\mu\text{m}$ ) that normally heals in 12–18 hours, cell migration rather than proliferation is the major contributor to closure under these conditions (Figure 3.2C). Interestingly, the ending impedance value shown in Figure 3.2A of the MSC CM treated fibroblasts was higher than the control, indicating that addition of the MSC CM may increase either cell-cell or cell-matrix contacts on the healed fibroblast monolayer leading to a higher final impedance.



**Figure 3.2. Mesenchymal stromal cell (MSC) conditioned medium (CM) promotes wound closure using electric cell-substrate impedance sensing (ECIS) (n = 3).** **A.** Wound healing rates of NBL-6 cells cultured with MSC-derived CM or control CM, as determined by electrical impedance in Ohms detected from 0–24 hrs post wounding and **B.** total wound closure time expressed in hrs. \*\*: P < 0.01. **C.** Phase contrast images of wounded NBL-6 cells cultured with MSC-derived CM (upper panels) as compared to control CM (lower panels) over a 24-hour period. Scale bars = 100 μm.

*The CM of MSC induces alterations in the expression of genes involved in wound healing*

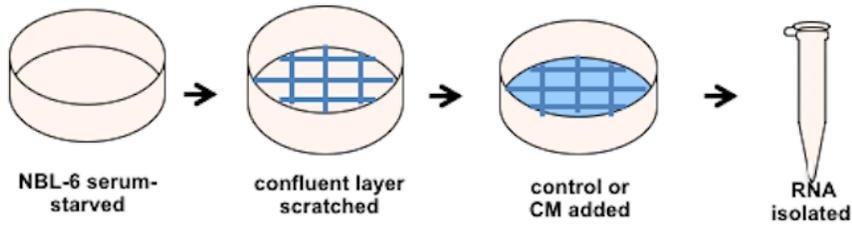
Semi-quantitative RT-PCR analyses were performed to investigate the relative mRNA expression levels of selected inflammatory cytokines, remodeling enzymes, extracellular matrix components and adhesion molecules, all associated with wound repair, in NBL-6 cultured with MSC-derived CM as opposed to control CM. Figure 3.3A shows the experimental set-up for these studies and it was observed that culturing dermal fibroblasts in the CM of MSC differentially regulated mRNA expression of several genes involved in wound healing when compared to gene expression in dermal fibroblasts cultured in control CM (Figure 3.3B). A significant upregulation of the proinflammatory mediator interleukin 8 (IL8) ( $p < 0.05$ ) and a significant downregulation of the chemokine C-X-C motif chemokine 10 (CxCL10) ( $p < 0.01$ ) was observed in dermal fibroblasts cultured in the presence of MSC-derived CM (Figure 3.3B). Gene expression levels of extracellular matrix components and adhesion molecules were not significantly different, with the exception of Collagenase 3 (COLIII) which was significantly lower in dermal fibroblasts cultured in the presence of MSC-derived CM compared to control CM ( $p < 0.05$ ) (Figure 3.3B). Finally, the expression of a variety of remodeling enzymes was upregulated when dermal fibroblasts were cultured in CM, with matrix metalloprotease protein 1 (MMP1) showing a significant higher expression in dermal fibroblasts cultured in the presence of MSC-derived CM compared to control CM ( $p < 0.01$ ) (Figure 3.3B).

*The CM of MSC stimulated with TNF $\alpha$ , IFN $\gamma$ , or COCl $_2$  further alters gene expression in dermal fibroblast cells, but does not result in an increased migration capacity*

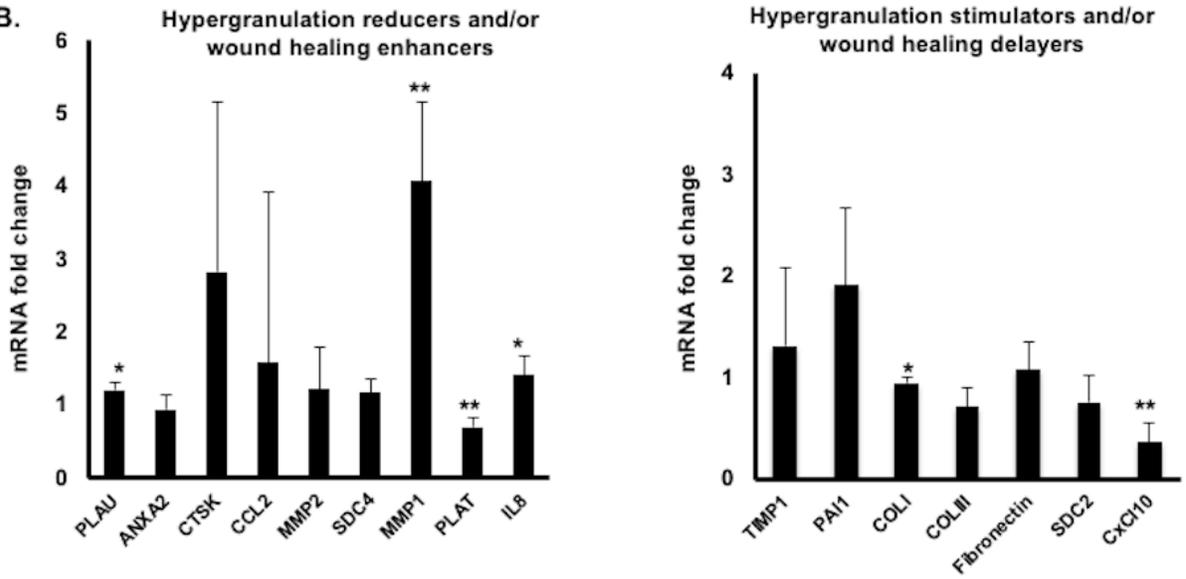
Since it has been reported previously that pre-stimulation of human MSC increased their wound healing properties [32-35], we stimulated our equine MSC with TNF $\alpha$ , IFN $\gamma$  and COCl $_2$  to evaluate the effects on (i) expression of wound healing-related genes in NBL-6 cells and (ii) *in*

*vitro* scratch assays. It was observed that stimulation of MSC with TNF $\alpha$  led to a significant increase in MMP1 ( $p < 0.01$ ) and IL8 ( $p < 0.05$ ) mRNA expression in NBL-6 cells, whereas stimulation with IFN $\gamma$  led to a significant increase in CxCL10 expression ( $p < 0.05$ ), compared to culturing NBL-6 cells with CM of non-stimulated MSC (Figure 3.3C). In contrast, none of the stimulating agents influenced the expression of COLIII and stimulation of MSC with CoCl $_2$  had no effect on the expression of any of the evaluated genes (Figure 3.3C). When evaluating these pre-stimulation treatments in scratch assays, no differences in migration rates were observed between fibroblasts exposed to the stimulated MSC-derived CM versus the non-stimulated MSC-derived CM (Additional file 1: Figure S3.1A&B).

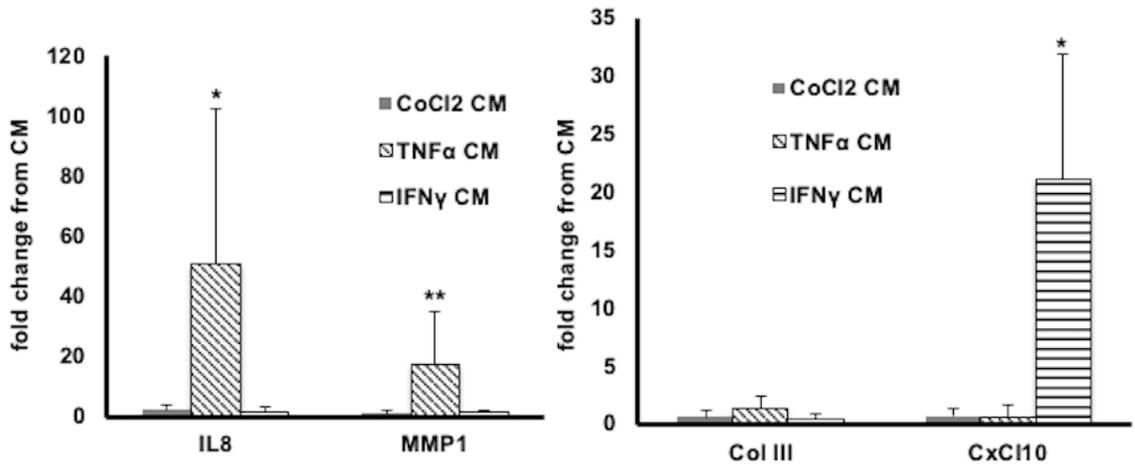
A.



B.



C.



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**Figure 3.3. Mesenchymal stromal cell (MSC) conditioned medium (CM) alters gene expression levels in dermal fibroblasts (n = 3).** **A.** Schematic illustration of NBL-6 treatments for RNA isolation. **B.** Fold-change of mRNA levels, as detected by qRT-PCR, in NBL-6 cells cultured with MSC-derived CM as compared to control CM **C.** or NBL-6 cells cultured with CM from preconditioned MSC as compared to CM from non-preconditioned MSC. Left panel shows genes involved in reduction of hypergranulation and/or enhancement of wound healing and right panel shows genes that stimulate hypergranulation and/or delay wound healing. \*: P < 0.05; \*\*: P < 0.01.

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*The effects of MSC-derived CM on the expression of wound healing-related genes are donor dependent in our study*

When studying the changes in gene expression in more detail, it became clear that potential significant differences in gene expression levels could be masked due to the rather large standard deviations (STDEV) we observed for several tested genes. Since we used the CM of MSC from three different individual horses for our experiments, we reasoned that these large STDEV could possibly be caused by natural inter-horse variations. Therefore, we decided to present the results of the gene expression alterations per individual donor horse instead of using the average (Additional file 2: Figure S3.2A). By presenting the data this way, several observations were made. Firstly, using the non-parametric Kruskal-Wallis test to compare the gene expression data between horses, statistically significant differences between individuals became apparent in the mRNA expression levels of certain genes (Table 3.2). At a p value of < 0.01, there were statistically significant differences for two hypergranulation reducers and/or wound healing enhancers, namely cathepsin K (CTSK) and matrix metalloproteinase-2 (MMP2) (Table 2A). Likewise, statistically significant differences (p < 0.01) between horses were found for four hypergranulation enhancers and/or wound healing reducers, namely plasminogen

activator inhibitor 1 (PAI1), collagenase type III (COLIII), fibronectin and syndecan 2 (SDC2) (Table 2B). Secondly, using the Dunn's post-hoc test it was observed that culturing NBL-6 cells in the MSC-derived CM of Horse B resulted in a significant mean rank difference of  $\geq 6$  for the genes CTSK, MMP2, PAI1 and COLIII, when compared to NBL-6 cells that were cultured in the CM of either Horse A- or Horse C-derived MSC (Additional file 2: Figure S3.2B). Overall, the increased expression levels of CTSK and MMP2, genes advantageous for wound healing, with a simultaneously decreased expression level of PAI1, a gene disadvantageous for wound healing, would make Horse B a better donor for providing MSC with the capacity to promote wound healing than either Horse A or C. Comparing the effects of Horse B MSC-derived MSC on migration of fibroblasts, using scratch assays, with the other two horse MSC samples, revealed no statistically significant differences and all three MSC samples could equally increase NBL-6 migration ( $p < 0.01$ , Additional file 2: Figure S3.2C). Taken together, these data indicate that the expression level pattern in CM-treated dermal fibroblast cells is donor horse MSC-specific in our study.

**Table 3.2. Statistical analyses of gene expression data from individual horses**

<b>A. Hypergranulation reducers and/or wound healing enhancers:</b>			
<b>Gene</b>	<b>Significant difference</b>		
	<b>Horse A versus Horse B</b>	<b>Horse B versus Horse C</b>	<b>Horse A versus Horse C</b>
PLAU	-	-	-
ANXA2	-	Yes*	-
CTSK	-	Yes**	-
CCL2	-	Yes*	-
MMP2	-	Yes**	-
SDC4	-	-	-
MMP1	-	-	-
PLAT	-	-	-
IL8	-	-	Yes*
<b>B. Hypergranulation enhancers and/or wound healing reducers:</b>			
<b>Gene</b>	<b>Significant difference</b>		
	<b>Horse A versus Horse B</b>	<b>Horse B versus Horse C</b>	<b>Horse A versus Horse C</b>
TIMP1	-	Yes*	-
PAI1	-	Yes**	-
COL1	-	-	-
COL3A1	-	Yes**	-
Fibronectin	Yes**	-	-
SDC2	-	-	Yes**

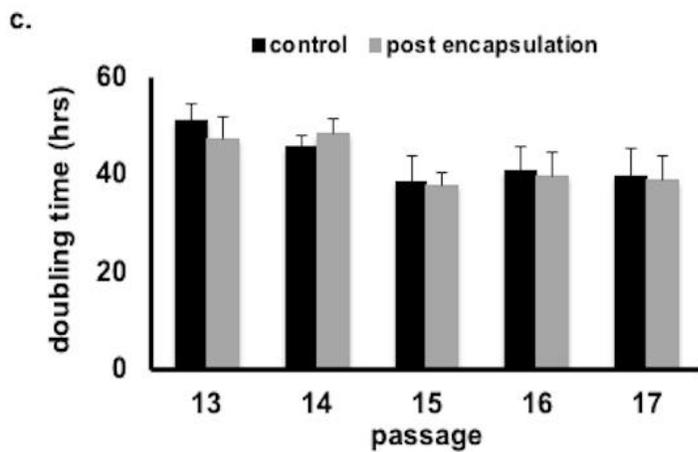
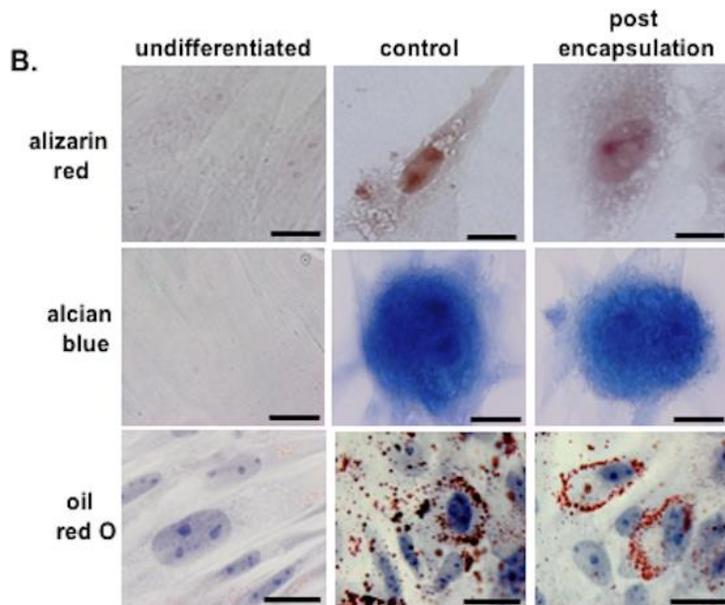
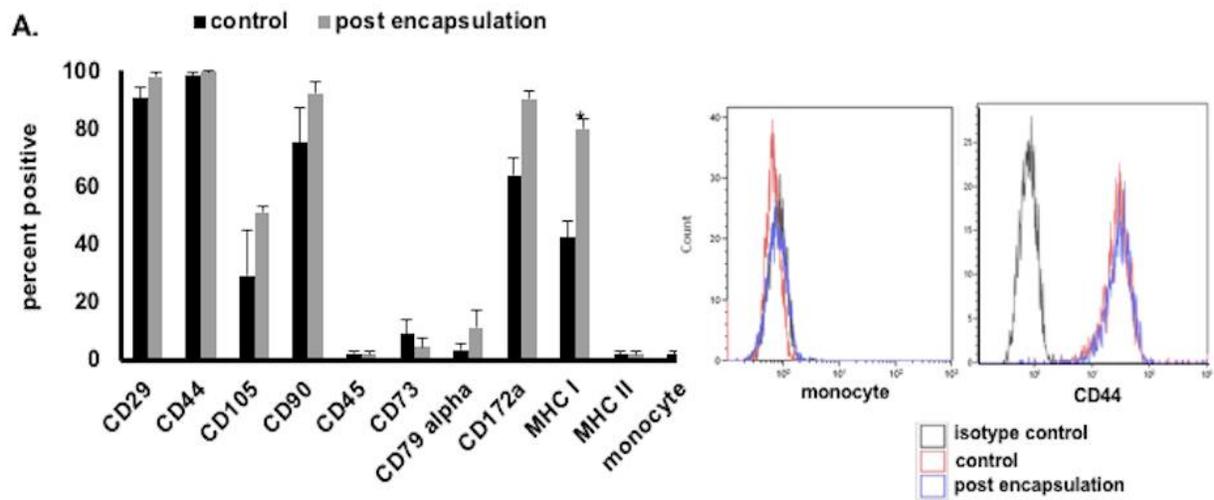
\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ .

*Encapsulated MSC retain stem cell characteristics and remain viable during long-term encapsulation*

After clearly demonstrating that the CM of equine MSC promotes wound healing, we were interested in exploring the potential of microencapsulating MSC as a way to deliver the critical components of CM to wounded cells *in vitro* in a controlled manner.

In a first set of experiments, we encapsulated equine MSC and 2 days later, removed these MSC from their capsules to evaluate whether they retained their stem cell characteristics. Overall, the immunophenotypical profile, as detected by flow cytometric analysis using a commonly used set of cell surface markers [36], did not differ between MSC removed from capsules and MSC that were never encapsulated (Figure 3.4A). There was a significant difference ( $p < 0.05$ ) in the expression of MHC I, but this marker is known to be highly variably

expressed by MSC [37]. In addition, MSC removed from capsules retained the capacity to differentiate into osteocyte, chondrocyte and adipocyte lineages (Figure 3.4B). Moreover, the population-doubling time (PDT) of MSC removed from capsules was virtually identical to MSC from the same source, but never encapsulated, from passage 13 up till passage 17 (end of experiment) (Figure 3.4C).



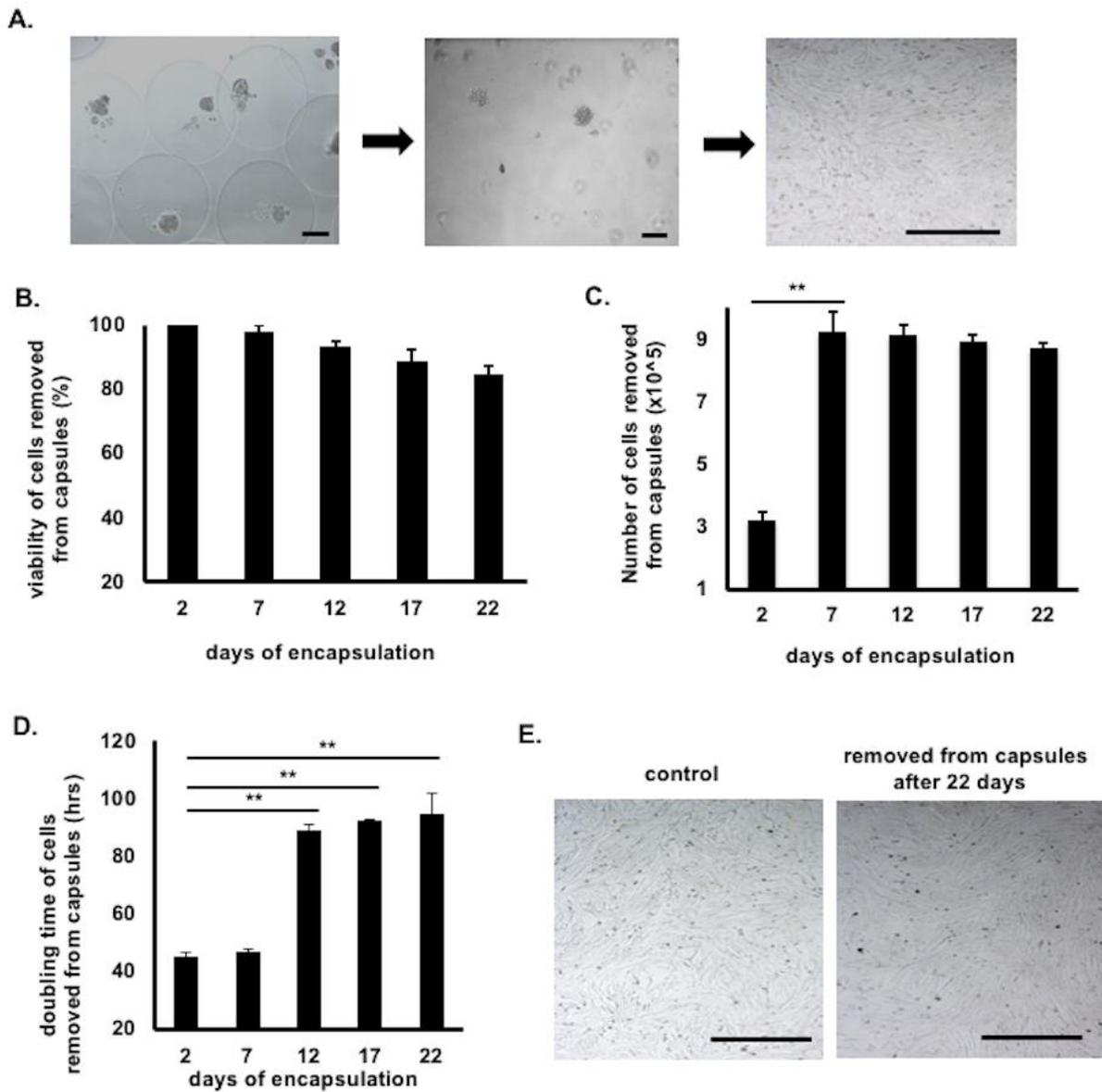
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**Figure 3.4. Mesenchymal stromal cells (MSC) retain stem cell characteristics after microencapsulation (n = 3).** **A.** Expression of surface markers, as detected by flow cytometry, on MSC removed from capsules after 2 days post encapsulation as compared to control MSC that were never encapsulated. Percentage of total cells positive are shown left and representative histograms are shown right. \*:  $P < 0.05$ . **B.** Representative images of MSC stained with alizarin red, alcian blue and oil red O to detect osteocyte, chondrocyte and adipocyte differentiation, respectively. Left column: undifferentiated MSC cultured in expansion medium; center column: control MSC that were never encapsulated cultured in MSC differentiation media; and right column: MSC removed from capsules after 2 days post encapsulation cultured in MSC differentiation media. Scale bars = 10  $\mu\text{m}$ . **C.** Population doubling times (PDTs) of MSC removed from capsules after 2 days post encapsulation as compared to control MSC that were never encapsulated.

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In a second set of experiments, we cultured MSC in capsules for over three weeks in order to test the potential of long-term encapsulation. MSC encapsulated for 2 days were used as the short-term encapsulation control time point (day 2) and compared to MSC encapsulated for successive 5-day intervals (day 7, 12, 17 and 22). At all these different time points, equine MSC were removed from equal numbers of capsules and subsequently plated in culture wells (Figure 3.5A). Before plating, viability was determined using trypan blue exclusion and the number of live cells was determined. Over the course of the experiment, the viability of long-term encapsulated cells decreased slightly compared to short-term encapsulation but did not fall below 80% and this slight decrease was not statistically significant (Figure 3.5B). In addition, the number of viable cells present in the capsules for 7 days was significantly increased compared to 2-day encapsulation, indicating that equine MSC did divide in the capsules, but remained constant thereafter (Figure 3.5C). After plating, and upon confluency of the cells, PDTs were calculated. Cells encapsulated for 7 days showed a similar PDT to that of the short-term, 2-day, encapsulated MSC. In contrast, cells encapsulated for more than 7 days showed a significantly

higher PDT, indicating that it took those cells much longer to form a confluent monolayer (Figure 3.5D). For the duration of this set of experiments, pictures from confluent monolayers were taken daily to confirm that the stem cell morphology was maintained, indicating that the change in PDT was not due to differentiation of the MSC which could result in an altered growth pattern (Figure 3.5E).



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**Figure 3.5. Mesenchymal stromal cells (MSC) remain viable after long-term**

**microencapsulation (n = 3).** **A.** Representative images of MSC encapsulated (left), immediately after removal of capsules (center), and after several days of culture (right) Scale bars = 50  $\mu\text{m}$ . **B.** Viability, as determined by trypan blue exclusion, of MSC removed from capsules at 5-day intervals starting at 2 days post encapsulation. **C.** Numbers of viable cells removed from equal numbers of capsules at 5-day intervals starting at 2 days post encapsulation. \*\*:  $P < 0.01$ . **D.** Population doubling times of MSC removed from capsules at 5-day intervals starting at 2 days post encapsulation. \*\*:  $P < 0.01$ . **E.** Representative phase contrast images of cultured MSC removed from capsules 22 days post encapsulation as compared to control MSC that were never encapsulated. Scale bars = 50  $\mu\text{m}$ .

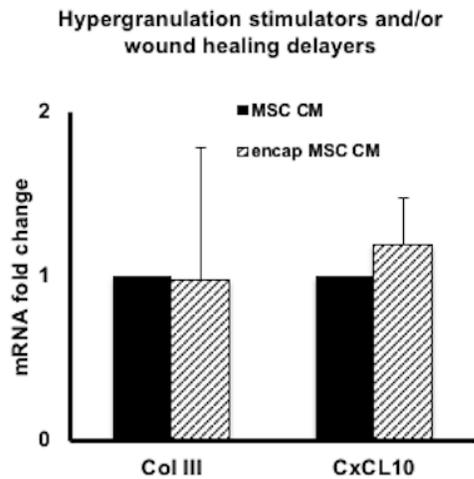
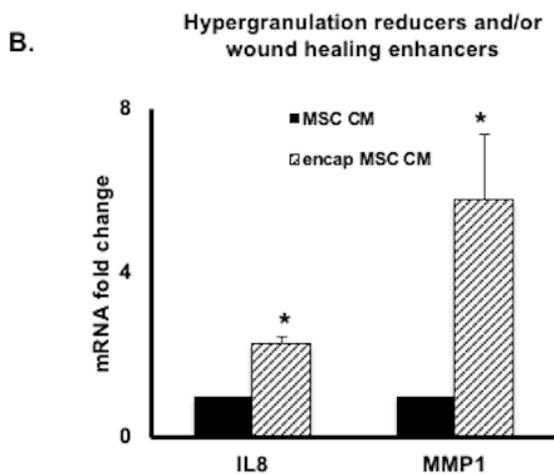
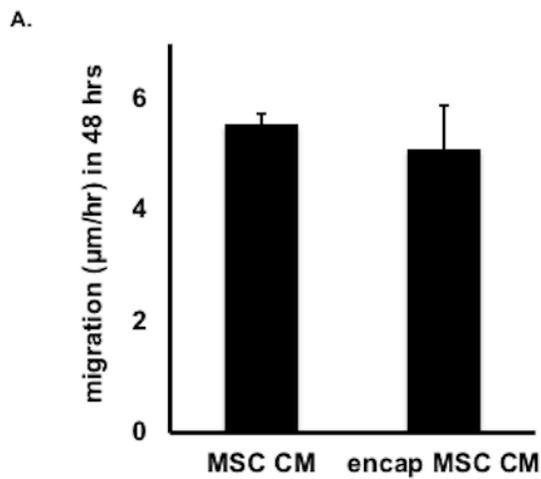
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*Encapsulated MSC-derived CM stimulates migration of dermal fibroblasts and induces alterations in the expression of genes involved in wound healing*

After showing that microencapsulated MSC remain viable and retain general stem cell characteristics, we evaluated the CM from encapsulated MSC in order to determine potential positive effects on dermal fibroblast migration and expression of genes involved in wound healing. To this end, we repeated the scratch assays and gene expression analyses, as previously described, using CM from non-encapsulated MSC (control) and from MSC that were encapsulated and cultured for 2 days.

Using scratch assays, we found that NBL-6 cells had an equally fast migration when cultured in CM obtained from equine MSC compared to CM obtained from encapsulated equine MSC (Figure 3.6A). CM that was collected from 2-day cultured empty capsules was also included as a control and showed a significant slower migration compared to either MSC-derived CM (data not shown). Next, semi-quantitative RT-PCR analyses were performed on NBL-6 cells cultured in CM obtained from non-encapsulated MSC (control, set as 1) and compared to gene expression in NBL-6 exposed to CM obtained from encapsulated MSC. Specifically, the relative

mRNA expression levels of a selected set of genes (ie. IL8, MMP1, ColIII and CxCL10) involved in wound healing were determined. A significant up-regulation of IL8 and MMP1 gene expression was observed in NBL-6 cells cultured in CM obtained from encapsulated equine MSC as compared to NBL-6 cells which were cultured in CM of non-encapsulated MSC (Figure 3.6B). In contrast, the gene expression levels of COLIII and CxCL10 were indistinguishable between NBL-6 cells cultured in either CM from encapsulated MSC or non-encapsulated MSC (Figure 3.6B).



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**Figure 3.6. Conditioned medium (CM) from microencapsulated mesenchymal stromal cells (MSC) promotes NBL-6 migration and alters gene expression (n = 3).** **A.** Migration distances of NBL-6 cells cultured in CM from encapsulated MSC as compared to MSC CM in *in vitro* scratch assays. Data are expressed as  $\mu\text{m/hr}$  in 48 hrs. **B.** Fold-change of mRNA, as detected by qRT-PCR, in NBL-6 cells cultured with encapsulated MSC CM as compared to control MSC CM. Left panel shows genes involved in reduction of hypergranulation and/or enhancement of wound healing and right panel shows genes that stimulate hypergranulation and/or delay wound healing. \*:  $P < 0.05$ .

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### 3.5. Discussion

The present study is the first to evaluate the effects of conditioned medium (CM) from equine mesenchymal stem cells (MSC) on wound-healing properties of equine dermal fibroblasts, and to explore the potential of encapsulated equine MSC to deliver these critical components to equine target cells. Our salient findings were that the CM of equine MSC stimulates the migration of equine dermal fibroblasts *in vitro* and increases the expression levels of genes that positively contribute to wound healing. Importantly, similar results were obtained using CM from MSC that were encapsulated in core-shell hydrogel microcapsules, indicating the potential of microencapsulated MSC to deliver the bioactive factors present in CM.

During our studies on the expression levels of genes involved in wound healing when equine fibroblasts were exposed to MSC-derived CM, two interesting observations were made. First, we found a highly significant, 4-fold, increase in the mRNA expression of the matrix metalloproteinase 1 (MMP1). MMP1, also designated interstitial collagenase or fibroblast collagenase, is a critical remodeling enzyme required for re-epithelialization during wound healing [38,39]. *In vitro* studies have shown that CM from amnion-derived human MSC alters the expression of MMP1 in human dermal fibroblasts [35], which is similar to our present findings with CM from peripheral blood (PB)-derived equine MSC and equine dermal

fibroblasts. Interestingly, the CM collected from MSC that were microencapsulated even further altered the expression of MMP1 as evidenced by a significantly higher MMP1 expression in dermal fibroblasts when exposed to the CM from microencapsulated MSC compared to CM from non-encapsulated MSC (Figure 6B). A similar observation was made for IL8 expression in dermal fibroblasts (Figure 6B), another critical component in cutaneous wound healing known to stimulate migration and proliferation of keratinocytes [40]. Although speculative at this point, we hypothesize that the close contact between the MSC in these capsules and/or the capsule microenvironment changes the secretion pattern of MSC, or alternatively, that the MSC secretome is influenced by adherent (non-encapsulated) versus non-adherent (encapsulated) culture conditions. An increased expression level of MMP1 and IL8 in dermal fibroblasts was also observed when equine MSC were preconditioned using tumor necrosis factor alpha (TNF $\alpha$ ). TNF $\alpha$  has been described to stimulate human MSC to secrete elevated levels of IL-6 and IL8, resulting in accelerated wound healing [32], and our study indicates that IL8 is also upregulated in the target cells exposed to this preconditioned MSC-derived secrete. For the two other agents that we used to precondition equine MSC in our present study, however, we did not observe an upregulation of IL8 nor MMP1 in dermal fibroblasts when exposed to CM from these stimulated MSC. The first agent, CoCl<sub>2</sub>, is a chemical known to mimic hypoxia and our results in our equine model reflect results from a previous study in which no significant alterations were found in the expression levels of MMP1 in human dermal fibroblasts incubated with CM from hypoxic human MSC when compared to CM from normoxic human MSC [35]. In addition, no significant alterations were found in any of the other genes that were tested, ie. IL8, COLIII and CxCL10, when equine fibroblasts were exposed to the CM of CoCl<sub>2</sub>-treated equine MSC. The second agent we used to stimulate equine MSC was interferon gamma (IFN $\gamma$ ), a cytokine known to

stimulate MSC to increase wound healing in aged mice [33] but for which the specific effects on the expression of wound related factors in target cells, like dermal fibroblasts, have not previously been investigated. We found in our present study that equine dermal fibroblasts showed a significant increase in CxCL10 expression when incubated with CM of IFN $\gamma$  pre-conditioned MSC. CxCL10 is a chemokine known to delay wound healing and to disorganize neovascularization [41], and although more in-depth studies are needed, we would like to argue that pretreating MSC with IFN $\gamma$  might not be beneficial as a regenerative treatment to promote cutaneous wound healing.

Secondly, we observed that the expression level pattern in CM-treated dermal fibroblast cells was donor horse MSC-specific. Although our current study is too limited in experimental numbers and is lacking repeated isolations from the same donor horse to draw definite conclusion about donor dependency, our data are the first to indicate a donor-specific effect of MSC-derived CM on skin target cells specifically. This study, together with other studies describing donor variation in the cytokine expression level and *in vitro* bone tissue repair potency of human MSC [42,43], collectively suggest that screening of candidate MSC donors is critical in order to take full advantage of the therapeutic effects of MSC on cutaneous wound healing. Moreover, it is important to consider the specific purpose of the regenerative therapy, as the CM of one donor MSC might prove more beneficial than the CM of another donor MSC, depending on the desired therapeutic effect.

Despite the exciting potential of the MSC-derived CM as an effective cell-free therapeutic treatment in regenerative medicine, a major impediment to its use is the fact that these MSC formulations are not fully optimized in terms of delivery methods. The ability to control secretion of MSC-derived bioactive factors is critical given the limitations of

pharmacokinetics and stability of proteins *in vivo*. To begin exploring the potential of such delivery methods, we microencapsulated our equine MSC using core-shell hydrogel microcapsules. Conventional alginate microcapsules have been used successfully to encapsulate human MSC [44-47]. However, our study demonstrated that this approach is feasible for equine MSC, by using core-shell capsules which were designed to provide a more relevant extracellular environment and to better protect the encapsulated cells [24]. Indeed, we showed that equine MSC survive and divide within these microcapsules and retain their stem-cell characteristics. Importantly, we found that CM collected from these encapsulated equine MSC equally promotes dermal fibroblast migration when compared to the CM from non-encapsulated MSC, suggesting that encapsulating of MSC may be an ideal strategy to control the delivery of secreted products to equine wounds *in vivo*. Based on data we have collected thus far, future experiments are planned in which we will evaluate the healing promoting effects of equine MSC, non-encapsulated as well as microencapsulated, in an *in vivo* horse model, like e.g. the horse model established by the group of Theoret et al. in which skin wounds are experimentally induced to heal both normally as well as with the formation of exuberant granulation tissue [48].

### **3.6. Conclusions**

Mesenchymal stromal cells (MSC) have been reported to provide paracrine signals that promote wound healing, but (i) how they exert their effects on target cells is unclear and (ii) a suitable delivery system to supply these MSC-derived secreted factors in a controlled and safe way is unavailable. The present study was designed to provide answers to these questions using the horse as a translational model. Our results suggest that conditioned medium (CM) of MSC might be a promising new therapy for impaired cutaneous wounds and that microencapsulation may be a suitable way to effectively deliver CM to wounded cells *in vivo*. Moreover, our data

showed that the effects of MSC-derived CM appear to be donor specific, suggesting that a proper screening of candidate MSC donors is critical in order to take full advantage of the therapeutic effects of MSC on cutaneous wound healing.

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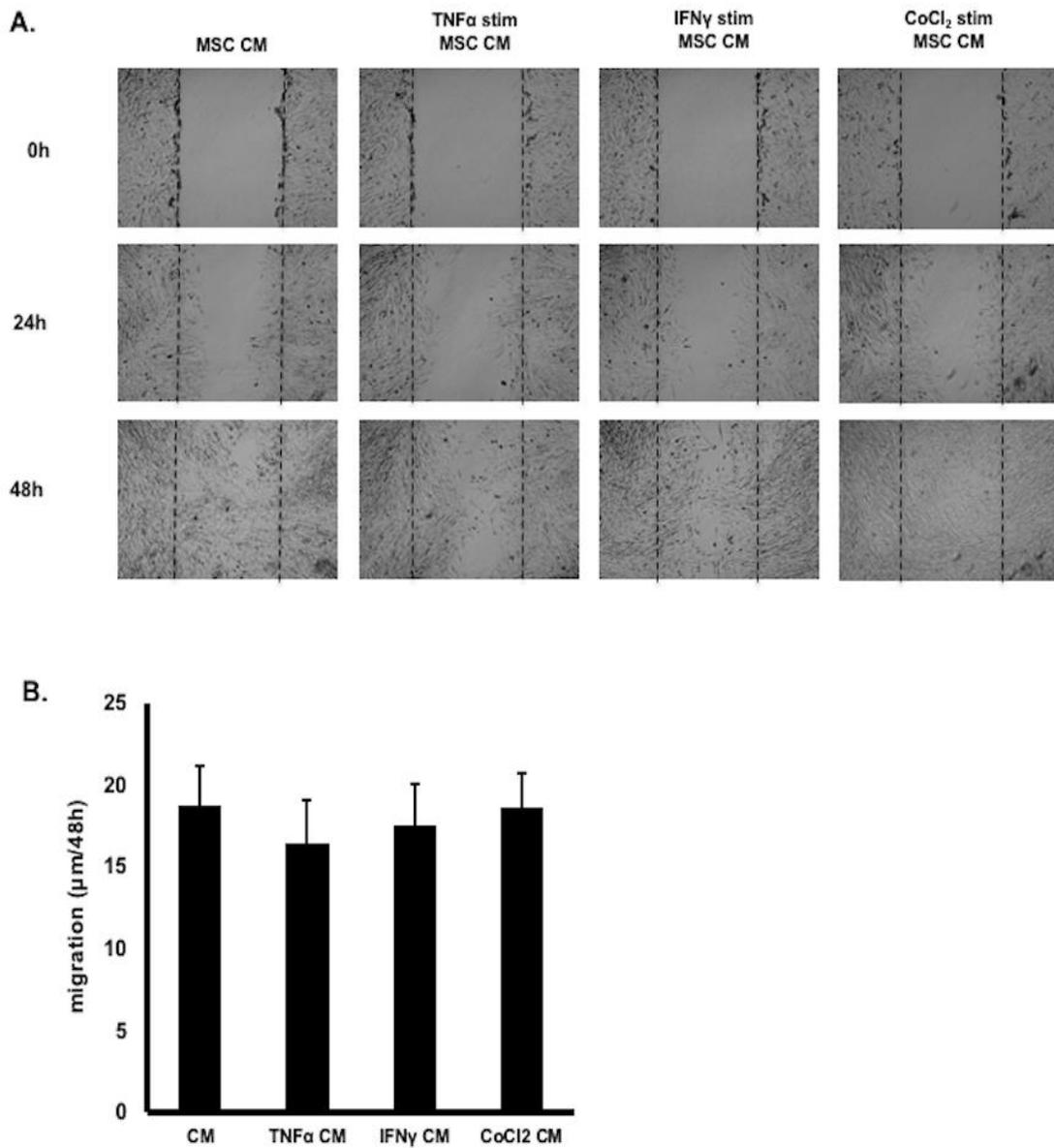
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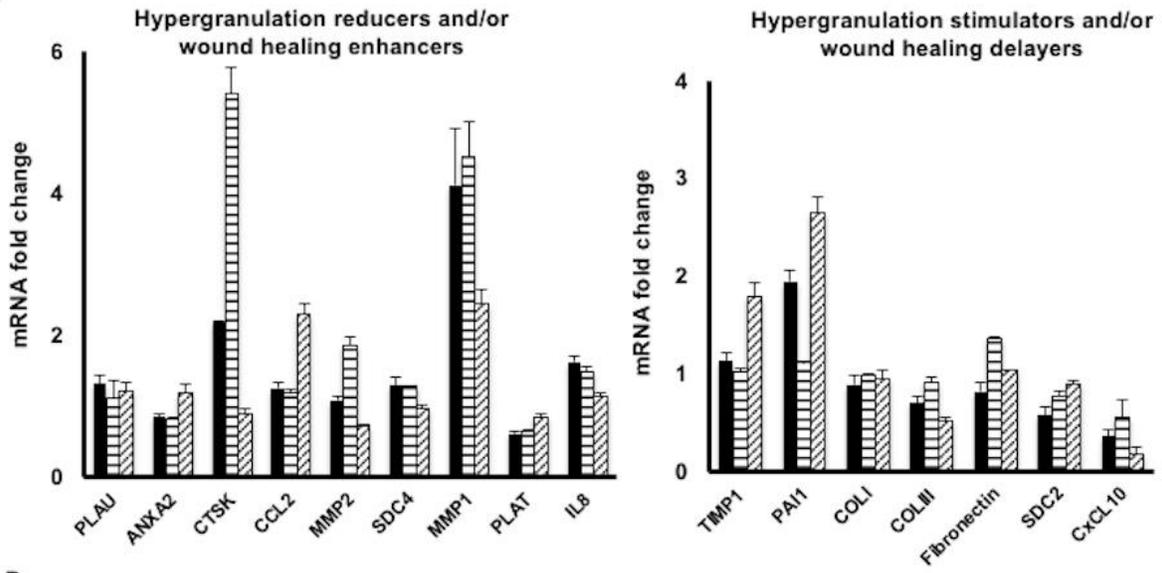
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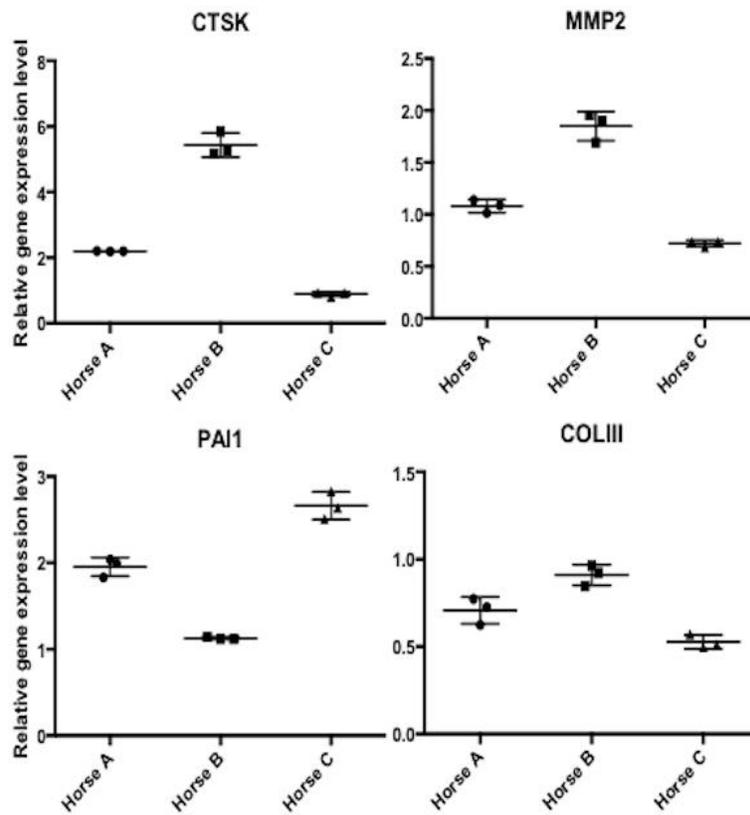


**Figure 3.S1. A. Conditioned medium (CM) from preconditioned mesenchymal stromal cells (MSC) does not affect migration of dermal fibroblasts.** Representative phase contrast images of wounded NBL-6 cells cultured with CM of MSC stimulated with TNF $\alpha$ , IFN $\gamma$  or CoCl $_2$  as compared to control MSC CM at 0, 24 and 48 h. **B.** Migration distances of NBL-6 cells cultured with CM of MSC stimulated with TNF $\alpha$ , IFN $\gamma$  or CoCl $_2$  as compared to control MSC CM. Data are expressed as  $\mu\text{m}/\text{hr}$  in 48 hrs. (n = 3).

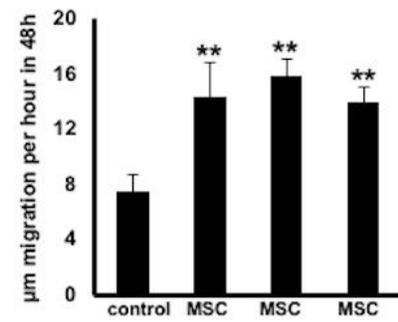
A.



B.



C.



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**Figure 3.S2. Mesenchymal stromal cell (MSC) conditioned medium (CM) alters NBL-6 gene expression in a donor-specific manner. A.** Fold-change of mRNA, as detected by qRT-PCR, in NBL-6 cells cultured with MSC CM from 3 different donor horses as compared to control NBL-6 CM. Left panel shows genes involved in reduction of hypergranulation and/or enhancement of wound healing and right panel shows genes that stimulate hypergranulation and/or delay wound healing. **B.** Expression levels of CTSK and MMP2 (genes involved in reduction of hypergranulation and/or enhancement of wound healing), and PAI1 and ColIII (genes that stimulate hypergranulation and/or delay wound healing) with a significant mean rank difference of  $\geq 6$  upon incubation with Horse B MSC-derived CM. **C.** Migration distances of NBL-6 cells cultured with CM of MSC from 3 different donor horses as compared to control CM. Data are expressed as  $\mu\text{m/hr}$  in 48 hrs. \*\*:  $P < 0.01$ . (n = 3).

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**CHAPTER FOUR: SECRETED FACTORS FROM EQUINE MESENCHYMAL  
STROMAL CELLS DIMINISH THE EFFECTS OF TGF- $\beta$ 1 ON EQUINE DERMAL  
FIBROBLASTS AND ALTER THE PHENOTYPE OF DERMAL FIBROBLASTS  
ISOLATED FROM CUTANEOUS FIBROPROLIFERATIVE WOUNDS**

\*The results of this study were published in Harman RM, Bihun IV, Van de Walle GR. Secreted factors from equine mesenchymal stromal cells diminish the effects of TGF- $\beta$ 1 on equine dermal fibroblasts and alter the phenotype of dermal fibroblasts isolated from cutaneous fibroproliferative wounds: Mesenchymal stromal cell effects on fibroblasts. *Wound Repair Regen.* 2017;25:234–247.

#### **4.1. Summary**

The prevalence of cutaneous fibroproliferative disorders (CFPDs) is high and almost exclusively occurs in humans (keloids and hypertrophic scars) and horses (exuberant granulation tissue), making the horse a valuable translational model for studies on prevention and treatment of human CFPDs. CFPDs arise as a result of dysregulated wound healing characterized by persistently high levels of cytokines, such as transforming growth factor beta 1 (TGF- $\beta$ 1), that contribute to excessive extracellular matrix deposition, and the physical disorganization of dermal fibroblasts. The mesenchymal stromal cell (MSC) secretome, consisting of all factors secreted by MSC, has been shown to promote normal wound healing in both humans and horses, but its potential to treat CFPDs remains largely unexplored. Therefore, the objective of this study was to examine the effects of the equine MSC secretome on equine dermal fibroblasts (DF) influenced by cytokines that contribute to the development of CFPDs. First, primary equine DF were treated with TGF- $\beta$ 1 *in vitro* in the presence or absence of MSC secreted products. We found that MSC secreted products could block TGF- $\beta$ 1-induced changes in DF morphology, proliferation rate, gene expression, and contractile-capacity. We then isolated primary DF from equine exuberant granulation tissue, to evaluate the potential of the MSC secretome to alter the phenotype of cells derived from a complex CFPD environment. These results showed that MSC secreted factors did not change proliferation or migration of these cells but did lead to changes in expression of genes and proteins involved in extracellular matrix remodeling and did affect contractile capacity. These results warrant future studies designed to evaluate the potential of the MSC secretome to minimize the pathologies associated with CFPD *in vivo*.

#### **4.2. Introduction**

Successful cutaneous wound healing involves a network of synchronized biological processes that restore the integrity of the skin after injury. However, this is often not achieved as healing may be sacrificed in favor of quickly filling in a wound with fibrotic tissue [1]. While fibrotic tissue serves to prevent immediate secondary infection by limiting the area exposed to bacteria, it is compromised in terms of mechanical strength and is more sensitive to pain than healthy skin [2]. In addition, if control of fibrotic tissue formation is lost, e.g. due to chronic inflammation, CFPDs may develop. The chronic inflammation characteristic of most CFPDs sustains the production of cytokines, such as TGF- $\beta$ 1, growth factors, angiogenic compounds and proteolytic enzymes that contribute to the continuous deposition of extracellular matrix components. This process progressively alters normal skin architecture and can lead to the accumulation of fibrotic tissue that protrudes beyond the margins of the wound, subjecting it to abrasion, disruption, and secondary infection [3]. The abnormal deposition of cutaneous fibrotic tissue may lead to physical complications, increased health-care costs and psychological problems for affected patients [4].

Laboratory rodents are commonly used to evaluate novel preventative and treatment options for CFPDs. Although these models provide valuable information, wound healing in rodents does not accurately mimic human tissue regeneration [5-8]. Cutaneous wounds in mice heal by contraction of the *panniculus carnosus*, a subcutaneous muscle that humans lack. Humans rely on epithelialization and contraction by myofibroblasts to close cutaneous wounds [9]. There is a need for translational models that closely mimic the natural processes of cutaneous healing and CFPDs in humans. The horse is an ideal model since horses, like humans, depend on epithelial cell activity and contractile fibroblasts to heal cutaneous wounds. In addition, horses and humans are the only mammals known to naturally suffer from pathologic CFPDs during wound closure,

characterized by excessive deposition of extracellular matrix and cessation of healing [10].

Common features of human and equine CFPDs make the horse an attractive translational model in which to study CFPDs as well as to evaluate the potential of novel treatments [11].

MSC are adult multipotent progenitor cells that can differentiate into fat, bone and cartilage. They reside in a variety of tissues and organs, including bone marrow, adipose tissue and blood, and have been shown to actively contribute to healing processes [12-15]. Originally, MSC were believed to contribute to healing by trans-differentiating into cell types needed to restore injured tissue, but now it is generally accepted that paracrine signaling by these cells is the primary mechanism [16]. Cellular products secreted by MSC, collectively referred to as the secretome, instead of MSC themselves are currently being evaluated as a safe and effective “stem cell-free” therapy for healing of wounds [17]. Such an approach offers advantages over traditional cellular MSC therapies in that it avoids (i) recipient immune reactions, (ii) the risk of tumor formation by donor MSC and (iii) ectopic tissue formation from donor MSC [18, 19]. We recently showed that the equine MSC secretome promotes dermal fibroblast (DF) migration and upregulation of genes involved in wound healing *in vitro* [20], but its potential to minimize the pathologies associated with equine CFPDs has not yet been explored.

The aim of the present study was to examine the effects of the secretome, collected as conditioned medium (CM), of equine MSC on primary DF influenced by cytokines that contribute to CFPD. For the first set of experiments we treated DF from normal skin tissue with TGF- $\beta$ 1 in the presence or absence of MSC CM. These experiments allowed us to determine if MSC CM could block the effects of a specific inflammatory cytokine involved in the development of CFPD, by TGF- $\beta$ 1 treatment of a homogeneous population of DF. In the second set of experiments, we isolated DF from equine exuberant granulation tissue to examine the

effects of MSC CM on a heterogeneous population of dysregulated DF derived from a complex CFPD environment.

### **4.3. Methods**

#### *Cells*

Equine MSC were isolated from the peripheral blood of healthy donor horses, as described previously [20-22]. Blood collection was approved by the Cornell Institutional Animal Care and Use Committee (IACUC # 2014-0038). Equine MSC were characterized by immunophenotypical protein profiling using flow cytometry and their potential for trilineage differentiation [21,22]. Equine MSC were confirmed to be positive for CD29, CD44, CD90 and CD105, and negative for CD45, CD79 $\alpha$ , MHC II and a monocyte/macrophage marker. The successful trilineage differentiation of MSC toward osteoblasts, chondroblasts and adipocytes was confirmed using histochemical stains [20, 21]. MSC cultures were maintained at 37°C with 5% CO<sub>2</sub> in culture medium, consisting of low glucose Dulbecco's modified Eagle medium (Corning, Tewksbury, MA), supplemented with 30% fetal bovine serum (Atlanta Biological, Flowery Branch, GA), 1x penicillin-streptomycin and 2 mM L-glutamine (both from Life Technologies, Grand Island, NY). At 75- 80% confluency, cells were removed from flasks using 0.25% trypsin-EDTA and passaged for further culture. MSC used for experiments were between passage 3 to 9.

Normal equine DF cells were derived from the dermis of healthy research horses euthanized for reasons not related to this study. DF were also isolated from equine exuberant granulation tissue (EGTDF) from patients admitted to the College of Veterinary Medicine, Cornell University, with owner consent. For isolating both DF types, a 5 mg piece of tissue was minced and placed in 40 mL standard medium, consisting of Dulbecco's modified Eagle medium, 10%

fetal bovine serum (Atlanta Biochemicals) and 1% penicillin/streptomycin (Invitrogen), supplemented with 0.5 µg/mL Dispase II (Worthington Biochemical, Lakewood, NJ) and filtered through a 22 µm filter. Tissue was incubated for 8 h at 37°C on a stir plate, to obtain in a single cell suspension, which was filtered through a 100 µm cell strainer to remove any undigested tissue. Cells were washed twice in standard medium by centrifugation, counted, and plated at a density of  $1 \times 10^6$  cells per flask in T75 flasks with standard medium. Cultures were maintained at 37°C with 5% CO<sub>2</sub>. At 80% confluency, cells were removed from flasks using 0.25% trypsin-EDTA and passed for further culture. Primary DF and EGTDF used for experiments were passage 5 or lower. For experiments using primary DF and EGTDF, cell lines from 3 independent donors were used for each cell type.

The equine dermal fibroblast cell line NBL-6 (ATTC<sup>®</sup> CCL-57) was cultured in standard medium and maintained at 37°C with 5% CO<sub>2</sub>.

#### *Recombinant proteins and chemicals*

TGF-β1 (R&D Systems, Minneapolis, MN) was used at 10 ng/mL for 48 h to induce changes in DF and EGTDF were exposed to 10 nM staurosporin (EMD Millipore, Billerica MA) or 100 µM busulfan (Sigma Aldrich, St. Louis, MO) for 24 h to induce apoptosis and senescence, respectively.

#### *Generation of CM*

CM was collected from MSC at 80% confluency. For this,  $6 \times 10^5$  MSC were seeded in a T75 flask. After 24 h, medium was removed, the monolayer was rinsed with PBS, and 8 mL standard medium was added. Twenty-four h later, medium was collected, centrifuged twice for 7 min at  $300 \times g$  to remove cellular debris, and diluted 1:1 in standard medium to use as CM for further experiments. For each assay requiring MSC CM, cell lines from 3 donor horses were

used to generate CM. CM from the equine dermal fibroblast cell line NBL-6 cells was collected exactly as described for MSC-derived CM and used as a control for all CM experiments.

#### *Cell viability assays*

To assess cell viability, 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH)-based *in vitro* toxicology assays (Sigma Aldrich, Saint Louis, MO) were carried out according to manufacturer's instructions. Absorbance was measured on a Multiskan EX plate reader using Ascent software (Thermo Fisher Scientific, Waltham MA). Beta-galactosidase activity was measured to determine senescence using a senescence cells histochemical staining kit, according to manufacturer's instructions (Sigma Aldrich, Saint Louis, MO).

#### *Cell proliferation assays*

##### *i) Population doubling time (PDT) calculations*

For the determination of the PDT,  $2 \times 10^5$  primary DF or EGTDF were plated in a T25 tissue culture flask and cultured in standard medium. The PDT was calculated using the following link, [www.doubling-time.com/compute.php](http://www.doubling-time.com/compute.php), on cell isolations from 3 different horses.

##### *ii) Cell counts*

To quantify cell division in response to CM treatments,  $5 \times 10^4$  cells were plated per well in four wells of a 96-well tissue culture plate for each treatment condition and incubated for 24 h. Treatments were then administered for 48 h, culture medium was removed, cell monolayers were rinsed with PBS and 25  $\mu$ l 0.25% trypsin–EDTA was added to each well for 4 minutes. Detached cells were incubated with 25  $\mu$ l Trypan Blue and number of viable cells was counted using a hemocytometer.

##### *iii) ELISA-based detection of cell proliferation*

To evaluate cell proliferation, a bromodeoxyuridine (BrdU) assay kit was used following manufacturer's instructions (Abcam, Cambridge, MA). The resulting absorbance was read on a Multiskan EX microplate reader using Ascent software.

#### *Gene expression analyses*

Fibroblasts were seeded at a density of  $2 \times 10^5$  in T25 tissue culture flasks. After 24 h, culture medium was removed, cell monolayers were rinsed with PBS, and cells were incubated in the appropriate treatment medium for 48 h. Subsequently, mRNA was extracted from the cells using an RNEasy Plus Kit (QIAGEN, Valencia CA) and cDNA was synthesized using M-MLV Reverse Transcriptase (USB, Cleveland, OH), both according to the manufacturer's protocols. SYBER green-based quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays were carried out on an Applied Biosystems 7500 Fast Real Time PCR instrument (Applied Biosystems, Carlsbad, CA) to determine fold changes in gene expression. The comparative  $C_t$  method ( $2^{-\Delta\Delta C_t}$ ) was used to quantify gene expression levels where  $\Delta\Delta C_t = \Delta C_t(\text{sample}) - \Delta C_t(\text{reference})$ . The previously validated reference gene *beta-2-Microglobulin (B2M)* was used to normalize samples [26]. Primers to amplify *fibronectin 1 (FN1)*, *smooth muscle actin (ACTA2)*, *collagen type III alpha 1 (COL3A1)*, *collagen type I alpha 2 (COL1A2)*, *matrix metalloproteinase 1 (MMP1)*, *yes-associated protein 1 (YAP1)*, *tafazzin (TAZ)*, and *plasminogen activator inhibitor 1 (SERPINE1)* were designed using Primer3 software, based on horse sequences found in the National Center of Biotechnology Information (NCBI) GenBank. Primer sequences are listed in Table 1. All samples were run in triplicate.

**Table 4.1. Primers used for semi-quantitative RT-PCR.**

Gene Product	Abbreviation	Forward primer (5'-3')	Reverse primer (5'-3')
Fibronectin 1	<i>FNI</i>	AGGTCGTTACTGTGGGCAAC	TAATGGGAGACGGGTGTAGGG
Smooth Muscle Actin	<i>ACTA2</i>	CCAGCACCATGAAGATCAAG	CTGGAAGGTGGACAGAGAGG
Collagen Type III alpha 1	<i>COL3A1</i>	GACCTGAAATTCTGCCATCC	ACGTGGAACATTCTGAAGGAC
Collagen Type I alpha 2	<i>COL1A2</i>	TCAAGGTTTCCAAGGACCTG	TTCCAGGGTGACCATCTTC
Matrix Metalloproteinase 1	<i>MMP1</i>	GCCAAATGGACTTCAAGCTG	TAGGAAAGCCGAAGGATCTG
Yes-Associated Protein 1	<i>YAP1</i>	TCAGGTCTCTTCTGATGG	GCCAAGAGGTGGTCTTGTTT
Tafazzin	<i>TAZ</i>	CTCCCACTTCTTCAGCTTGG	TGAAGTCCATTCCCTTCTGG
Plasminogen activator inhibitor 1	<i>SERPINE1</i>	CCCTGGAGAGTGAAGTGGAC	CCTGCGATACATGGAGAAGC
Beta-2-Microglobulin	<i>B2M</i>	TCTTTCAGCAAGGACTGGTCTTT	CATCCACACCATTGGGAGTAAA

### *Immunofluorescence analyses*

Cells for immunofluorescence were grown in 24 well culture dishes fitted with 35 mm coverslips, rinsed with PBS and fixed in 4% paraformaldehyde for 10 min. Following 3 rinses with PBS, cells were permeabilized using PBS + 1% Triton-X 100 + 1% bovine serum albumin for 30 min at room temperature (RT). Primary antibodies Alexa 488-conjugated rabbit anti-matrix metalloproteinase 1 (Biorybt, Cambridge, UK), rabbit anti-fibronectin (Sigma Aldrich), mouse anti-smooth muscle actin (Dako, Carpinteria, CA), all at 1:100 dilution in PBS, and Alexa 647-conjugated phalloidin, diluted 1:200 in PBS, were added to the wells for 1 h at 37°C. Wells were rinsed 3 times with PBS, and Alexa 488- or 647-conjugated secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA), diluted 1:100 in PBS, were added to appropriate wells. After 30 min at RT, cells were washed 3 times with PBS and DAPI was added for 5 min. After a PBS wash, coverslips were removed from wells and mounted on slides using mounting medium (Dako, Carpinteria, CA). Cells were examined with a Zeiss LSM confocal microscope (Oberkochen, Germany) and images were captured with a camera controlled by ZEN imaging

software. Anisotropy scores were calculated using the ImageJ plugin FibrilTool (<http://imagej.nih.gov/ij/>).

#### *Western blot analyses*

Cells were lysed in RIPA buffer containing a 1X general protease inhibitor. Protein concentration was determined with a BCA protein assay (Thermo Fisher Scientific, Waltham, MA) prior to gel loading to ensure loading of an equal amount of protein (10 µg). 6X reducing sample buffer was added to yield a final concentration of 1X and lysates were boiled for 10 min at 95°C. Samples were subjected to SDS polyacrylamide gel electrophoresis on a 10% gel and transferred to Immobilon PVDF membranes (Millipore, Billerica, MA) using a transblot turbo system (Biorad, Hercules, CA). Membranes were blocked in 5% bovine serum albumin diluted in Tris buffered saline (TBS) and incubated with mouse anti-smooth muscle actin (Dako), rabbit anti-fibronectin (Sigma Aldrich) or rabbit anti- matrix metalloproteinase 1 (Biorybt) diluted 1:1000 in TBS + 5% bovine serum albumin for 2 h at RT on a rotating platform. Blots were washed for 50 min (10 x 5 min) with TBS-Tween, then incubated with a 1:20,000 dilution of HRP conjugated goat anti-rabbit or HRP conjugated goat anti-mouse (Jackson ImmunoResearch Labs) for 1 h at RT. All blots were washed for 50 min (10 x 5 min) with TBS-Tween and then visualized by chemiluminescence using Clarity Western ECL (BioRad, Hercules, CA). Membranes were probed in parallel with rabbit anti-lamin B1 antibody (Cell Signaling, Danvers, MA), diluted 1:5000, as a control. Gels were imaged on a BioRad ChemiDoc MP system (BioRad) and band intensities were determined using Image Lab software. Intensities of the bands of interest were divided by the intensities of loading control bands to calculate relative band intensity.

#### *Gel contraction assays*

Cells were resuspended at a concentration of  $2 \times 10^6$  cells/mL in EZCol Pure Gel Solution (Sigma Aldrich). Volumes of 500  $\mu$ l cells in collagen solution were pipetted into 6.5 mm transwells fitted in 24-well culture plates and incubated for 30 min at 37°C and 5% CO<sub>2</sub> to allow collagen solution to solidify before 1 mL appropriate medium was added per well. After 5 days, images were taken of the collagen gel and plug diameter was measured in a blinded manner using ImageJ.

#### *In vitro scratch assays*

Scratch assays were carried out, exactly as previously described [20]. Images of scratches were captured using a Nikon Diaphot-TMD inverted light microscope with an attached CoHu CCD camera (Nikon, Melville, NY) leaving the reference marks outside the capture image field but within the eye-piece field of view for repeatable orientation of scratches. Photographs of scratches were taken at 0, 24 and 48 h post scratching, and migration distances of cells were measured in a blinded-manner using ImageJ. Widths of scratches were recorded in two places in each of 3 wells scratched per treatment, for a total of 6 measurements per treatment. Scratch width was subtracted from the time 0 scratch widths at the same location to determine cell migration distance. Migration distances within treatments were then averaged to determine overall migration.

#### *Statistical analyses.*

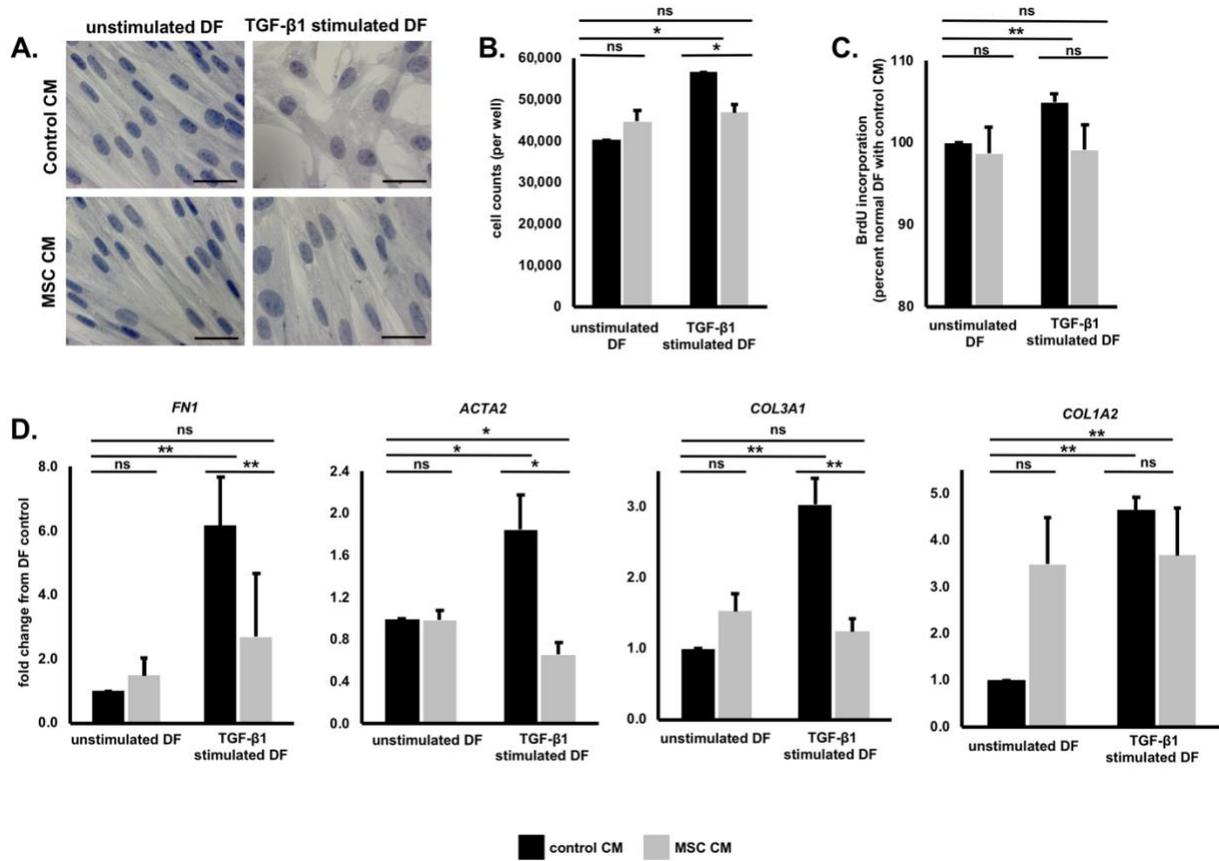
The student's t-test for paired data was used to test for statistically significant differences. When multiple t-tests were performed on a single response variable within an experiment, the Holm-Šídák method was used to counteract the problems associated with multiple comparisons. GraphPad software was used for analysis. Data given are the mean of 3 replicates and the bars show standard deviations.

#### 4.4. Results

*CM from equine MSC inhibits TGF- $\beta$ 1-induced changes in equine DF morphology and proliferation rates but does not alter untreated DF.*

It is documented that treatment of rodent and human DF with TGF- $\beta$ 1 changes cell morphology [23, 24]. Since persistent TGF- $\beta$ 1 expression is a hallmark of CFPD *in vivo* [25], we treated primary cultures of normal equine DF with TGF- $\beta$ 1 in the presence of MSC CM to see if factors secreted by MSC can dampen the effects of TGF- $\beta$ 1, helping DF maintain a phenotype found in healthy skin. First, morphology was assessed by light microscopy of hematoxylin stained cells. It was observed that DF exposed to MSC CM during TGF- $\beta$ 1 stimulation retained their elongated fibroblastic phenotype as seen in unstimulated DF, exposed to either control or MSC CM (Figure 1A). In contrast, TGF- $\beta$ 1 stimulated DF exposed to control CM adapted a more spread-out cell shape, indicative of the transition into a myofibroblast-like phenotype (Figure 4.1A). Since TGF- $\beta$ 1 treatment has been shown previously to increase human DF proliferation in culture [26], we evaluated proliferation to determine if this holds true for equine DF as well. We found that TGF- $\beta$ 1 stimulated DF exhibited a significantly higher proliferation rate when compared to unstimulated DF, as measured by both cell counts (Figure 4.1B,  $P < 0.05$ ) and BrdU ELISA (Figure 4.1C,  $P < 0.01$ ), when exposed to control CM. However, when DF were exposed to MSC CM during TGF- $\beta$ 1 stimulation, the proliferation rate was significantly reduced compared to cells exposed to control CM ( $P < 0.01$  cell counts). MSC CM did not have a positive effect on proliferation rate of unstimulated normal DF when assessed by cell counts and BrdU ELISA (Figure 4.1B & C). To ensure that alterations in cell proliferation were not due to a toxic effect of either TGF- $\beta$ 1 stimulation or MSC CM treatment, LDH release assays were performed. No significant differences in total LDH release between unstimulated and TGF- $\beta$ 1

stimulated DF, exposed to either control CM or MSC CM, were observed (Supplementary Figure 4.1A).

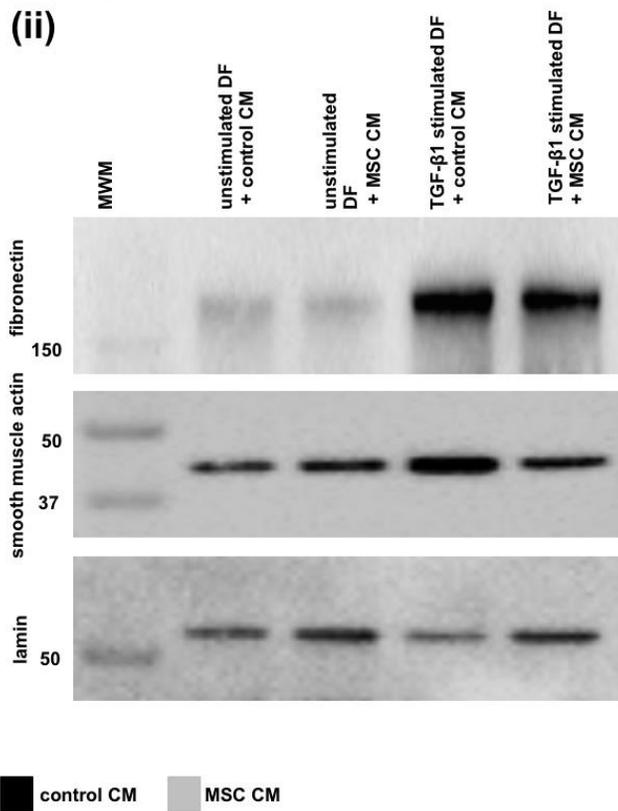
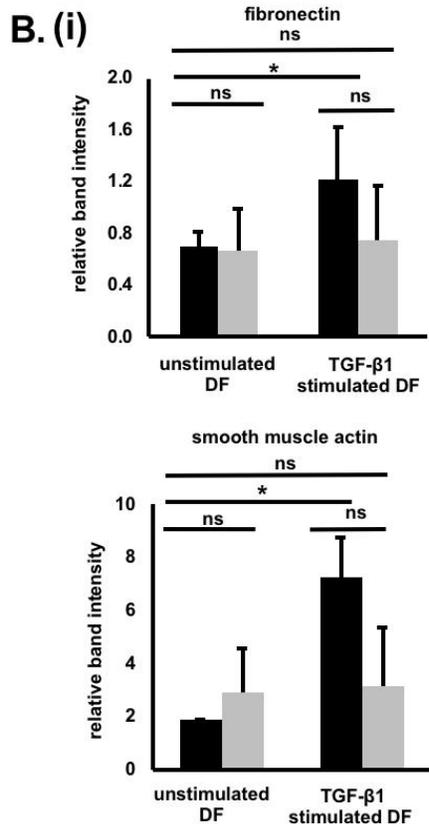
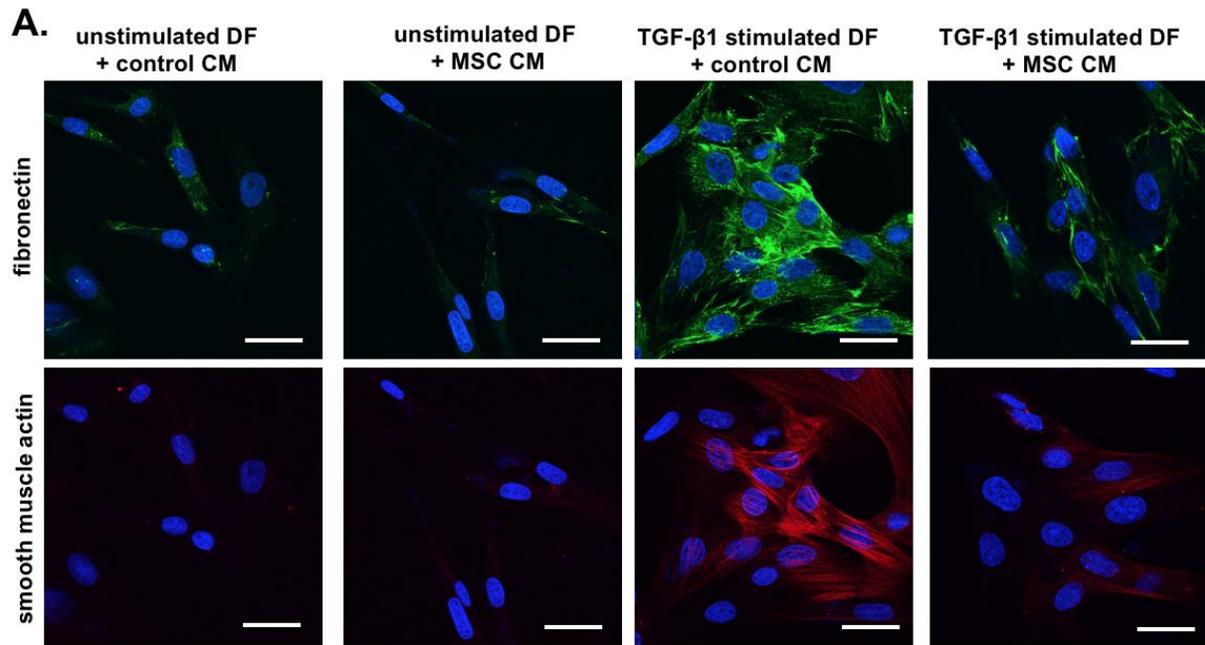


**Figure 4.1. Mesenchymal stromal cell conditioned medium (MSC CM) inhibits TGF-β1 induced changes in dermal fibroblast (DF) morphology, proliferation and gene expression.**

**A.** Morphology of untreated and TGF-β1 treated DF in the presence of control and MSC CM. **B-C.** Proliferation of untreated and TGF-β1 treated DF in the presence of control and MSC CM measured by cell counts (B) and BrdU incorporation (C). **D.** Expression of genes involved in extracellular matrix production and cytoskeleton composition in untreated and TGF-β1 treated DF in the presence of control and MSC CM, as measured by qRT-PCR. n=3, scale bars=20 μm  
\*: P < 0.05, \*\*: P < 0.01.

*CM from MSC inhibits TGF- $\beta$ 1-induced changes in DF gene and protein expression and reduces contractile capacity of these cells.*

Human DF exposed to TGF- $\beta$ 1 exhibit a change in expression of genes and proteins involved in cytoskeletal organization and secreted extracellular matrix components [27]. Using qRT-PCR, we found that expression of the genes *FNI*, *ACTA2*, *COL3A1* and *COL1A2*, were significantly increased in TGF- $\beta$ 1 stimulated DF compared to unstimulated DF when exposed to control CM (Figure 1D,  $P < 0.05$  or  $P < 0.01$ ). In contrast, exposing DF to MSC CM during TGF- $\beta$ 1 stimulation resulted in *FNI* and *COL3A1* gene expression levels that were undistinguishable from unstimulated DF (Figure 1D). *ACTA2* and *COL1A2* gene expression levels did decrease in response to MSC CM, but not to levels that were comparable to unstimulated DF (Figure 1D,  $P < 0.05$  or  $P < 0.01$ ). The MSC CM did not have any effect on gene expression levels in unstimulated normal DF (Figure 1D). Next, expression of fibronectin and smooth muscle actin was followed up on a protein level, using both immunofluorescence and Western blot analyses. Overall, expression patterns of both proteins followed gene expression results. An increase in antibody binding was observed in TGF- $\beta$ 1 stimulated DF as compared to unstimulated DF in immunofluorescence analyses, when exposed to control CM (Figure 4.2A). However, when DF were exposed to MSC CM during TGF- $\beta$ 1 stimulation, the fluorescence signals of both proteins were weak and comparable to unstimulated DF (Figure 4.2A). These results were examined quantitatively by calculating intensities of protein bands detected on Western blot membranes. Stimulated DF exposed to control CM expressed more fibronectin and smooth muscle actin protein than unstimulated DF exposed to control CM. When DF were exposed to MSC CM during TGF- $\beta$ 1 stimulation, levels of both proteins decreased to levels undistinguishable from those of unstimulated DF exposed to control CM (Figure 4.2B).

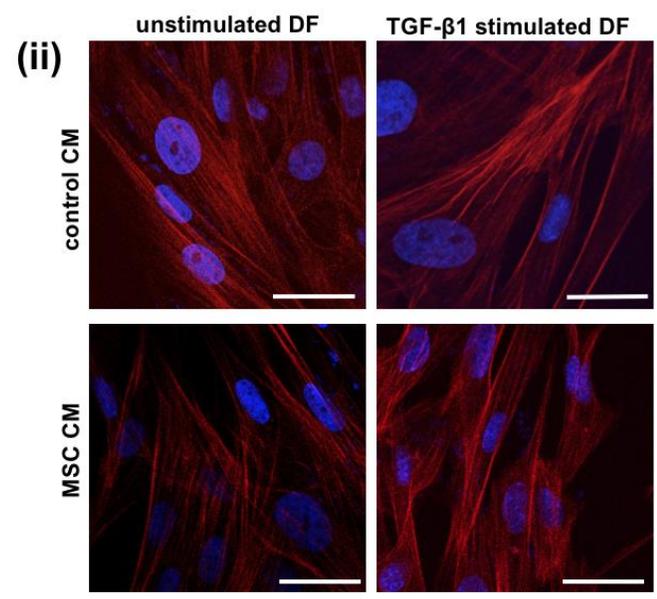
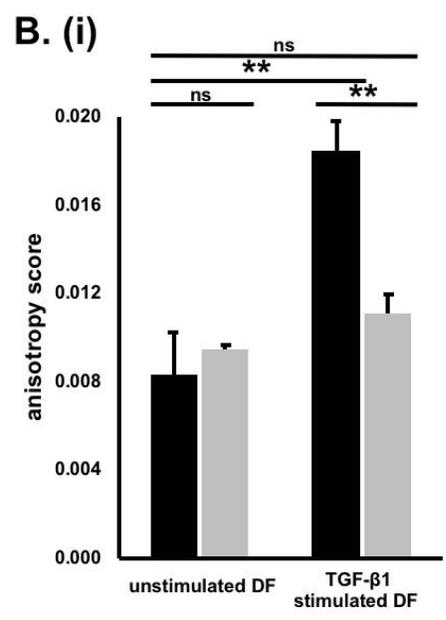
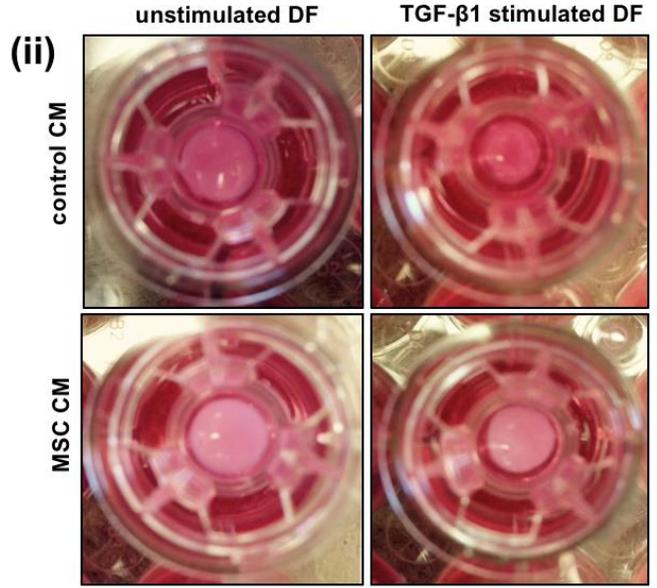
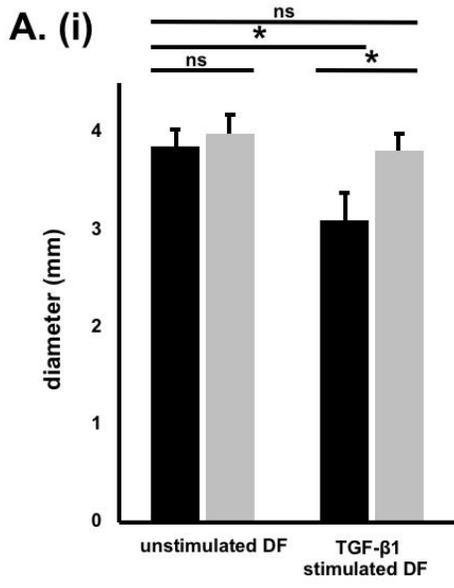


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**Figure 4.2. Mesenchymal stromal cell conditioned medium (MSC CM) inhibits TGF- $\beta$ 1 induced changes in dermal fibroblast (DF) protein expression.** **A.** Fibronectin and smooth muscle actin proteins visualized by immunofluorescent antibody binding. Fibronectin was labeled with Alexa 488 (green), smooth muscle actin labeled with Alexa 647 (red), and nuclei counterstained with DAPI (blue). **B.** Quantification of bands in Western blots (i). Representative images of Western blots for fibronectin (top panel) and smooth muscle actin (middle panel), with loading control (bottom panel) (ii).  $n=3$ , scale bars=20  $\mu\text{m}$ , \*:  $P < 0.05$ .

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Finally, we evaluated the effects of MSC CM on contractility since well-organized actin stress fibers in TGF- $\beta$ 1 stimulated DF contribute to the characteristic contractile phenotype of these cells [28]. DF stimulated with TGF- $\beta$ 1 and cultured in control CM adapted a contractile phenotype as measured by a decreased diameter of collagen plugs (Figure 3A,  $P < 0.05$ ). This change in contractile capacity was not observed in DF stimulated with TGF- $\beta$ 1 in the presence of MSC CM, and MSC CM did not affect the contractile capacity of unstimulated normal DF (Figure 4.3A). To further examine the contractile capacity of unstimulated and TGF- $\beta$ 1-stimulated cells, anisotropy was determined using an F-actin filament staining. Anisotropy is defined as the level of organization of actin filaments and is based on how parallel these filaments are in relation to one another within individual cells [29]. We found that DF treated with TGF- $\beta$ 1 in the presence of control CM had more organized filaments than untreated DF, as reflected by high anisotropy scores (Figure 4.3B,  $P < 0.01$ ). In contrast, DF treated with TGF- $\beta$ 1 in the presence of MSC CM exhibited anisotropy scores indistinguishable from untreated DF (Figure 4.3B), indicating a less organized actin network within each cell. MSC CM did not affect anisotropy of unstimulated normal DF (Figure 4.3B).



■ control CM    ■ MSC CM

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**Figure 4.3. Mesenchymal stromal cell conditioned medium (MSC CM) inhibits TGF- $\beta$ 1 induced contractile phenotype of dermal fibroblasts (DF).** **A.** Quantification of the diameter of collagen plugs containing cells, as measured using ImageJ software (i). Representative images of collagen plugs containing cells (ii). **B.** Quantification of anisotropy scores (directional dependence), as calculated by the ImageJ plugin FibrilTool (i). Representative images of F-actin staining using 647-Phalloidin (ii). n=3, scale bars=20  $\mu$ m, \*: P < 0.05, \*\*: P < 0.01.

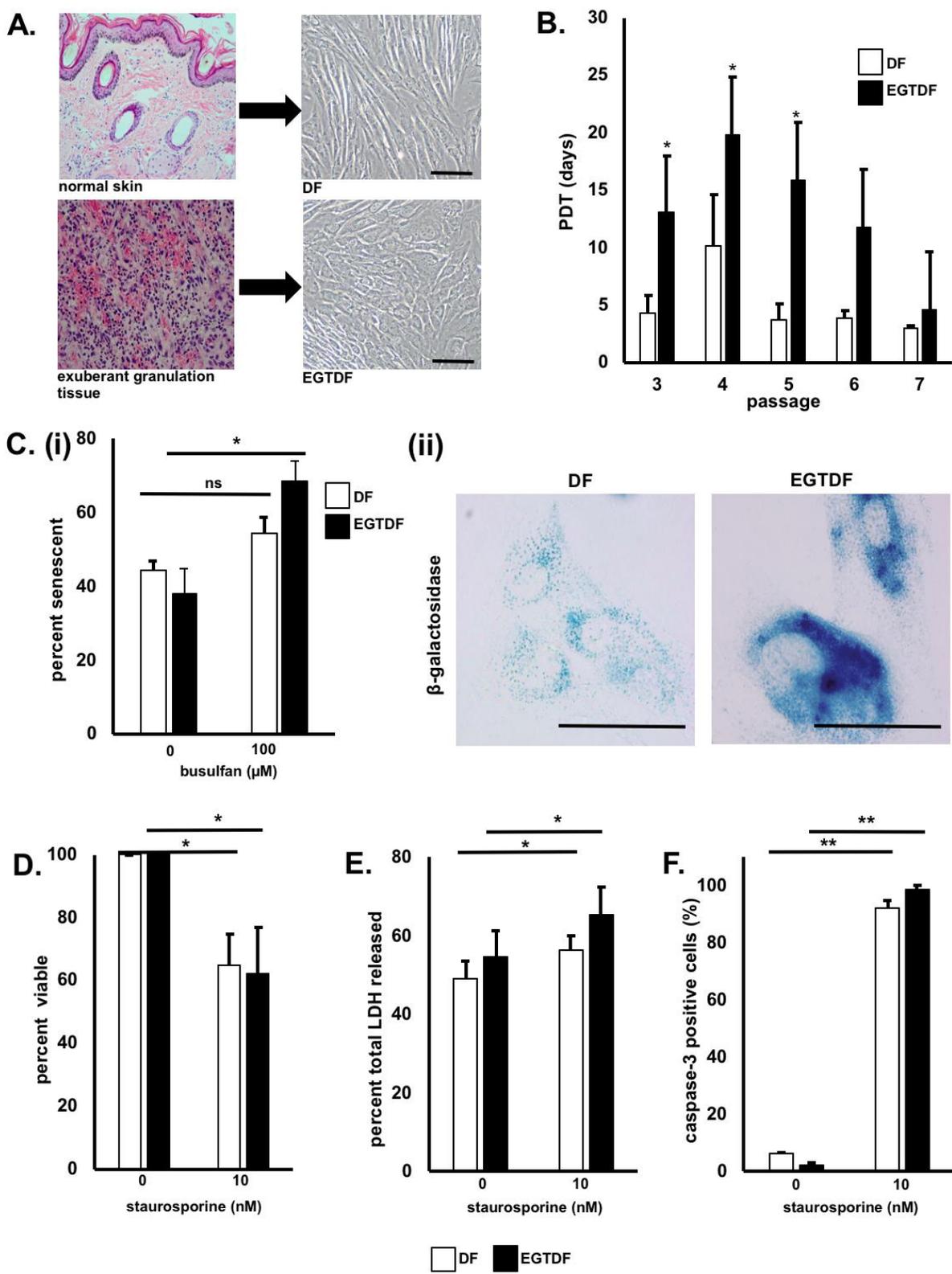
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*DF derived from equine exuberant tissue behave differently in culture than DF derived from healthy equine skin.*

Based on our results showing that MSC CM inhibited the well-documented effects of TGF- $\beta$ 1 on a homogeneous population of DF isolated from healthy skin, we decided to expand our study in order to determine whether MSC CM is also capable of acting on a diverse population of dysregulated primary fibroblasts isolated from exuberant granulation tissue. Before carrying out assays to explore this question however, we needed to determine if EGTFD differed from DF collected from healthy skin, since we observed that MSC CM did not affect the proliferation, normal gene and protein expression or contractile capacity of DF isolated from healthy skin. Our goals for this set of experiments were (i) to determine if EGTFD were dysregulated as compared to DF from healthy skin and (ii) if so, to evaluate the effect of MSC CM on these cells.

Representative hematoxylin-eosin stained histology sections clearly showed that exuberant granulation tissues are disorganized when compared to healthy skin tissue sections (Figure 4.4A, left panels). Specifically, these tissue sections lacked an epithelial layer, did not contain hair follicles or glands, and were highly vascular (Figure 4.4A, left panels). Primary fibroblast cultures established from these tissue samples differed morphologically from DF isolated from healthy skin; EGTFD were more spread out and exhibited a cuboidal shape as compared to normal DF (Figure 4.4A, right panels). We characterized these fibroblast cultures by calculating

PDT and found significantly higher PDT values at passage 3, 4 and 5, indicative of slower growth in EGTDF cultures as compared to normal DF cultures (Figure 4.4B,  $P < 0.05$ ). At later passages, EGTDF started to divide more quickly, as shown by lower PDT values, and a difference in PDT was no longer observed by passage 6 (Figure 4.4B). Since it has been postulated that fibroblasts in exuberant granulation tissue are more resistant to cell death compared to normal DF [30], we decided to expose both types of fibroblasts to busulfan and staurosporin in order to induce senescence and apoptosis, respectively [31]. Exposing fibroblast cultures to busulfan did not significantly increase the level of senescence in normal DF cultures, in contrast to EGTDF cultures, where a significant increase in  $\beta$ -galactosidase-positive cells was observed indicating that EGTDF are more susceptible to senescence than DF (Figure 4.4C,  $P < 0.05$ ). No such difference was observed upon treatment with staurosporin with both types of fibroblast cultures being equally susceptible to apoptosis (Figure 4.4D-F, Supplementary Figure 4.S1B).



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**Figure 4.4. Dermal fibroblasts isolated from normal skin (DF) or exuberant granulation tissue (EGTDF) differ in morphology, population doubling time and susceptibility to senescence, but not susceptibility to apoptosis. A.** Hematoxylin-eosin stained tissue sections from healthy horse skin (upper left) and exuberant granulation tissue (EGT, lower left). Phase contrast images of cultured cells isolated from DF (upper right) and EGTDF (lower right). **B.** Population doubling time of DF and EGTDF over 5 passages in culture (P3 through P7). **C.** Busulfan induced senescent cells in DF and EGTDF, as measured by  $\beta$ -galactosidase activity (i). Representative high-power images of DF and EGTDF treated with busulfan and stained with  $\beta$ -galactosidase (ii). **D-F.** Staurosporine induced apoptosis in DF and EGTDF, as measured by MTT processing (D), LDH release (E) and cells positive for active caspase-3 (F). n=3, scale bars=20  $\mu$ m, \*: P < 0.05, \*\*: P < 0.01.

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After determining that EGTDF behave differently in culture than DF derived from healthy equine skin, we compared this heterogeneous population of cells isolated from clinical biopsies to the relatively homogeneous population of TGF- $\beta$ 1 stimulated DF used in our first set of experiments. Proliferation rates, as well as the expression of *FNI*, *ACTA2*, *COL3A1* and *COL1A2* were directly compared between these two cell types, and normal DF were included for reference. The results indicate that although EGTDF and TGF- $\beta$ 1 stimulated DF differ from normal DF in terms of proliferation rate as measured by BrdU incorporation (Supplementary Figure 4.S2B, P < 0.05 or P < 0.01) and *FNI* and *ACTA2* gene expression (Supplementary Figure 4.S2C, P < 0.05 or P < 0.01), these cell types are not analogous to each other.

*CM from MSC does not alter proliferation or migration of EGTDF but does affect gene and protein expression in EGTDF and reduces their contractile capacity.*

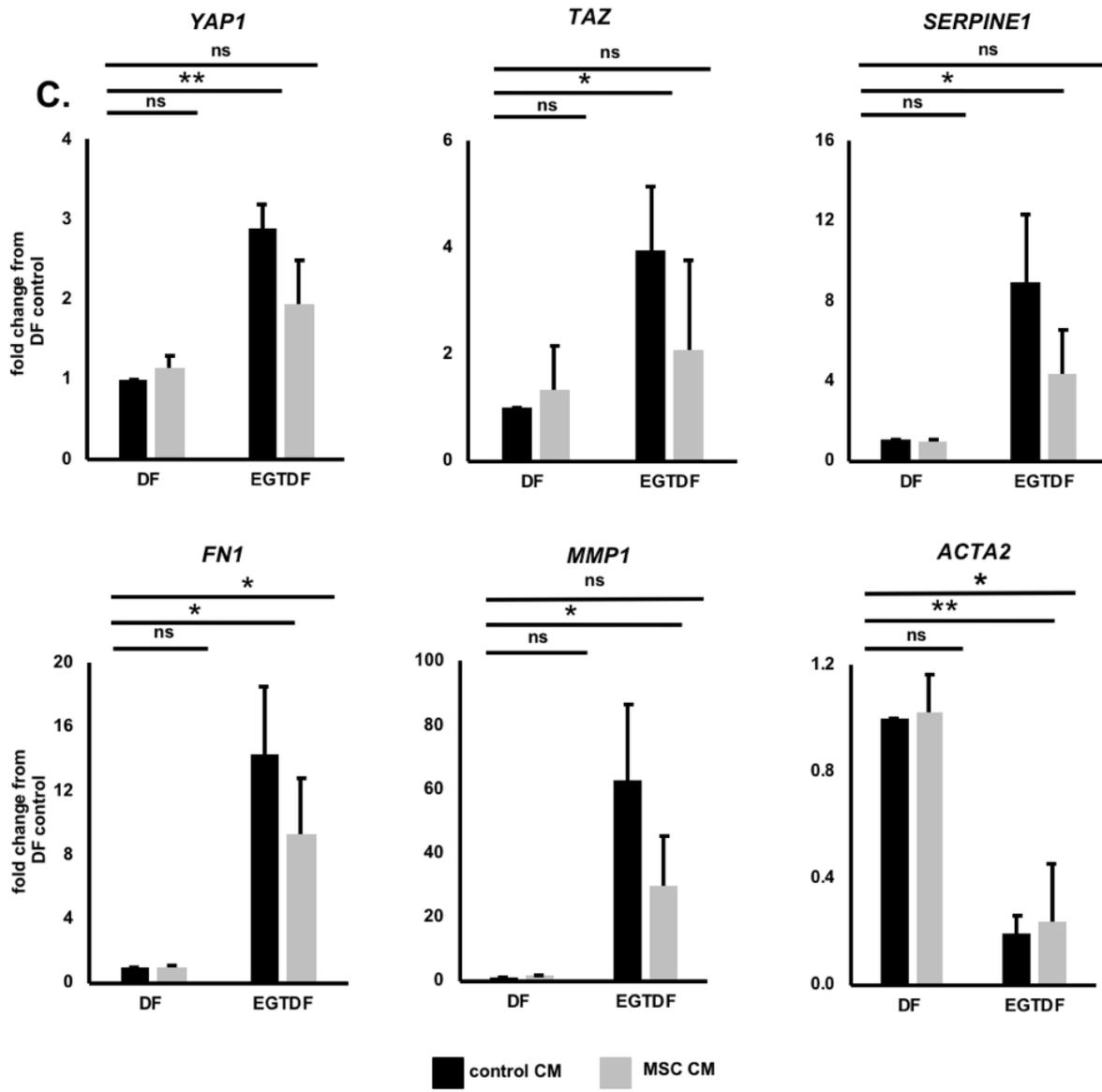
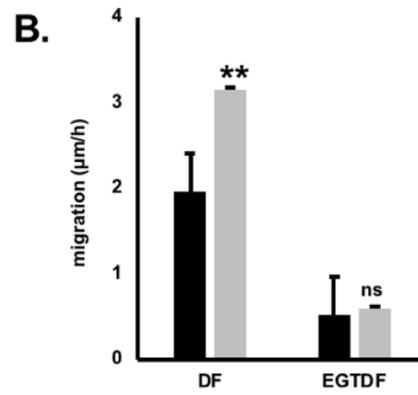
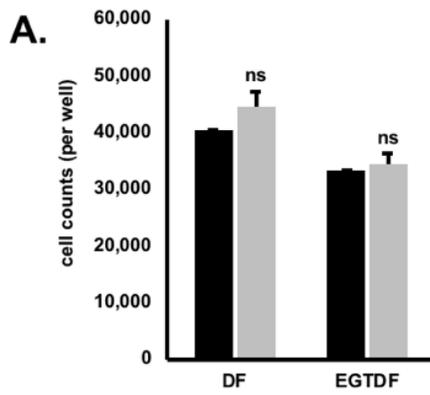
Our lab previously showed that MSC CM does not alter proliferation, but does increase migration of NBL-6 cells, a commercially available equine fibroblast cell line [20]. Repeating those experiments with primary DF cultures confirmed these observations (Figure 4.5A-B, P <

0.01). As with DF, no significant effects on proliferation were observed in EGTDF cultures treated with MSC CM, as measured by cell counts (Figure 4.5A) and BrdU incorporation (Supplementary Figure 4.S1C). However, and in contrast to DF, the migration rate of EGTDF treated with MSC CM did not increase, as measured by scratch assays (Figure 4.5B).

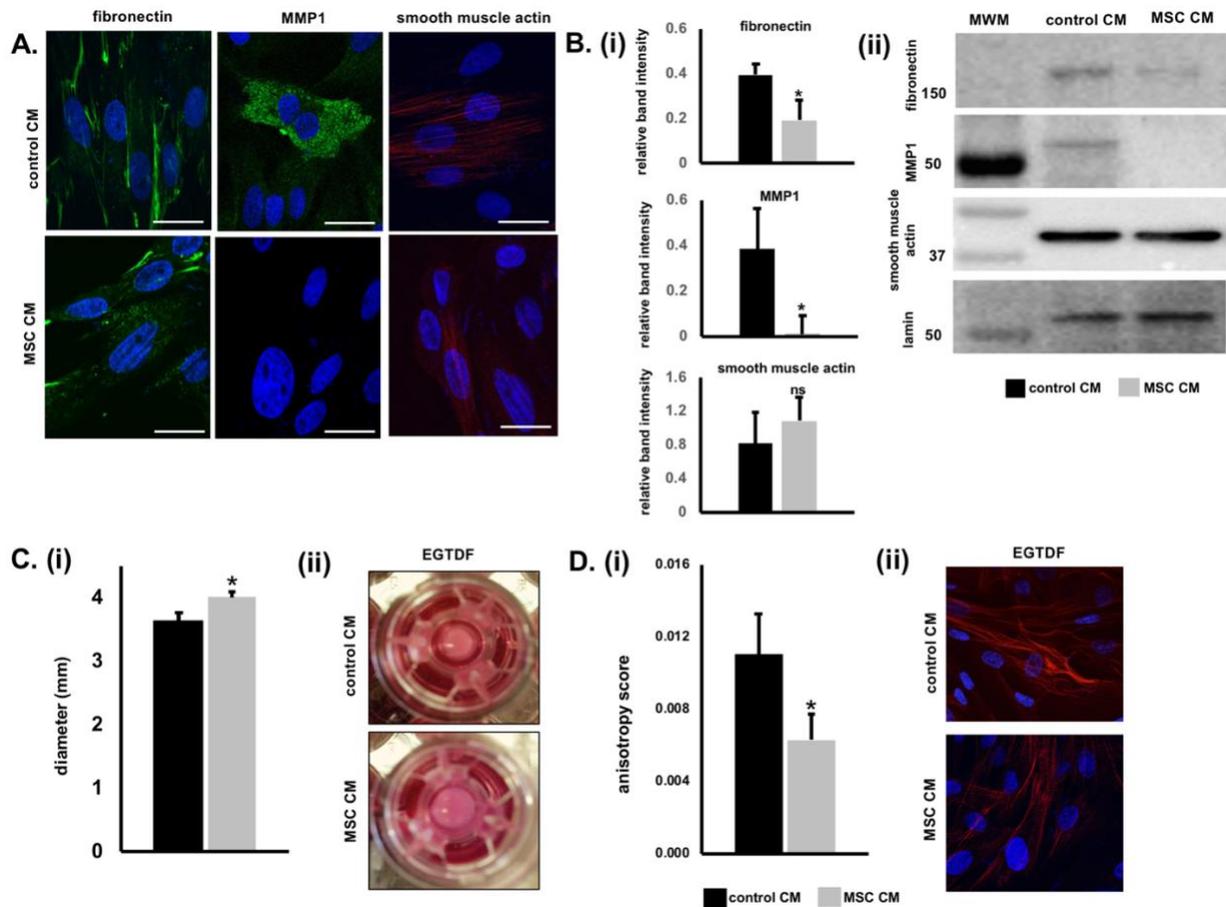
As part of our characterization of EGTDF, we used qRT-PCR to compare the expression of 13 genes we predicted might be differentially expressed between EGTDF and DF based on previous work by other groups [11, 30, 32-38]. Expression of 7 of these genes; *transgelin*, *secreted phosphoprotein 1*, *tumor protein p53*, *decorin*, *transforming growth factor beta 3*, *COL3A1* and *COL1A2* did not differ in EGTDF as compared to DF (data not shown & Supplementary Figure 4.2C). In contrast, we consistently detected significant differences in the expression of 6 genes between DF and EGTDF (Figure 4.5C,  $P < 0.05$  or  $P < 0.01$ ). These genes are involved in cell proliferation and apoptosis (*YAP* and *TAZ*), extracellular matrix remodeling (*SERPINE1*, *FNI* and *MMP1*) and cytoskeletal composition (*ACTA2*). An upregulation of *MMP1* expression has been described previously in both horse and human CFPDs [38,39]. Since the expression of these 6 genes was reliably altered in EGTDF as compared to DF isolated from healthy skin, we decided to use these genes as read-outs to determine if MSC CM could affect a heterogeneous population of dysregulated fibroblasts isolated from an exuberant granulation tissue environment. When cultured in MSC CM, gene expression levels of 4 of the 5 genes up-regulated in EGTDF decreased to levels that were virtually identical to expression levels in DF. The expression of *FNI*, while decreased in the presence of MSC CM, did not reach a level similar to that in DF (Figure 4.5C,  $P < 0.05$ ). The expression level of *ACTA2*, which was lower in EGTDF as compared to DF, did not increase to a level equivalent to that in DF when EGTDF were cultured in MSC CM (Figure 4.5C,  $P < 0.05$ ). In addition, fibronectin and matrix

metallopeptidase 1 protein expression decreased when EGTDF were cultured in MSC CM as compared to control CM, while the protein expression of smooth muscle actin did not change (Figure 4.6A & B,  $P < 0.05$ ).

Finally, we determined whether MSC CM alters contractility in EGTDF cultures. Using gel contraction assays, we found that treatment with MSC CM reduced the contractile nature of EGTDF, as detected by an increased diameter of collagen plugs (Figure 4.6C,  $P < 0.05$ ), and evaluating anisotropy scores showed less organized actin networks in EGTDF treated with MSC CM, as shown by lower anisotropy scores (Figure 4.6D,  $P < 0.05$ ).



**Figure 4.5. Mesenchymal stromal cell conditioned medium (MSC CM) does not affect proliferation of dermal fibroblasts (DF) and exuberant granulation tissue DF (EGTDF), but differentially affects DF and EGTDF migration and induces changes in gene expression in EGTDF. A.** Proliferation of DF and EGTDF treated with MSC CM, as measured by cell counts. For clarity, cell count data has been reformatted from Figure 1. **B.** Migration of DF and EGTDF treated with MSC CM, as measured by *in vitro* scratch assays. **C.** Expression of *YAP1*, *TAZ*, *SERPINE1*, *FNI*, *MMP1* and *ACTA2* in EGTDF compared to normal DF in the presence of MSC CM, as measured by qRT-PCR. n=3, \*: P < 0.05, \*\*: P < 0.01.



**Figure 4.6. Mesenchymal stromal cell conditioned medium (MSC CM) reduces fibronectin and matrix metalloproteinase 1 (MMP1), but not smooth muscle actin, protein expression and reduces contractile capacity in exuberant tissue dermal fibroblasts (EGTDF). A.** Fibronectin, matrix metalloproteinase 1 (MMP1) and smooth muscle actin proteins visualized by

immunofluorescent antibody binding. Proteins of interest were labeled with Alexa 488 (green) or Alexa 647 (red), and nuclei counterstained with DAPI (blue). **B.** Quantification of bands in Western blots (i). Representative images of Western blots for fibronectin (top panel), MMP1 (second panel), smooth muscle actin (third panel) and loading control (bottom panel) (ii). **C.** Quantification of the diameter of collagen plugs containing cells, as measured using ImageJ software (i). Representative images of collagen plugs containing cells (ii). **D.** Quantification of anisotropy scores (directional dependence), as calculated by the ImageJ plugin FibrilTool (i). Representative images of F-actin staining using 647-Phalloidin (ii). n=3, scale bars=20  $\mu$ m, \*: P < 0.05.

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#### **4.5. Discussion**

This study is the first to evaluate the effects of CM from equine MSC on DF treated with TGF- $\beta$ 1 as well as on dysregulated primary DF isolated from equine exuberant granulation tissue. We found that MSC CM significantly dampens the effects of TGF- $\beta$ 1 on DF in terms of cell proliferation, gene expression, and contractile capacity. We also found that MSC CM alters gene and protein expression in EGTDF cultures, as well as reduces the contractile capacity of these cells.

Successful wound healing in humans and horses depends on appropriately oriented contractile cells and cycles of deposition and remodeling of extracellular matrix proteins, each cycle leading to more highly organized, mechanically functional tissue [40]. As DF are the resident skin cells involved in contraction and extracellular matrix remodeling, it is encouraging to find that treatment with MSC CM alters contraction and the expression of extracellular matrix components in DF that are either pushed towards an altered phenotype by TGF- $\beta$ 1 or that are isolated from exuberant granulation tissue. Importantly, we found that the MSC CM had little to no effects on these phenotypical characteristics in normal DF. This pattern of targeting

dysregulated cells while leaving normal cells unaffected, may be one of the most valuable therapeutic features of MSC secreted products.

The two types of dysregulated DF used in this study each had unique characteristics that allowed us to evaluate the potential of MSC CM to reduce CFPD. First, we examined the effects of MSC CM on DF treated with TGF- $\beta$ 1. This approach provided us with a uniform population of cells with a discrete phenotype. Since it is well-documented that TGF- $\beta$ 1 treatment leads to the upregulation of fibronectin, smooth muscle actin and type I and type III collagens, as well as the formation of prominent actin-based contractile fibers [26,27], we chose these features as read-outs for our assays. TGF- $\beta$ 1 is detected at relatively low levels immediately after wounding of the lower limb in horses. During the proliferative phase of lower limb wound healing, however, when granulation tissue is being formed to fill the wound space, TGF- $\beta$ 1 levels in horse limb wounds increase and persist, providing a constant stimulus to DF to proliferate. *In vivo*, this enhanced and persistent DF proliferation ultimately leads to excessive granulation tissue that extends beyond the wound margins and inhibits wound closure [41]. Blocking the activity of TGF- $\beta$ 1, therefore, may reduce the formation of exuberant granulation tissue.

We then proceeded to examine the effects of MSC CM on primary DF isolated from naturally occurring exuberant granulation tissue. Our rationale for this set of experiments was that if MSC CM could affect a heterogeneous population of dysregulated fibroblasts derived from a complex wound environment *in vitro*, it may be effective as a therapy for CFPDs *in vivo*. We characterized these cells before using them in our MSC CM experiments and observed that the morphology of EGTDF from different equine patients was consistently distinct from that of DF isolated from healthy skin. In addition, we found higher PDTs for cultured EGTDF as compared to cultured DF. This was an unexpected result as we predicted that fibroblasts from

exuberant granulation tissue would proliferate more rapidly, resulting in lower doubling time, than those isolated from healthy skin. This suggests that in exuberant granulation tissue, DF may persist in excess due to reasons other than accelerated proliferation. Resistance to senescence or apoptosis might explain why DF can persist *in vivo*, although this was not observed in our *in vitro* studies. Similarly, studies in which samples from experimentally-induced exuberant granulation tissue were examined histologically did not find evidence of increased resistance to cell death when compared to wounded skin that healed normally [30]. Using EGTDF cultures gave us the opportunity to evaluate the effects of the MSC CM on a naturally occurring population of DF derived from a dysregulated wound environment. We found that MSC CM affects the phenotype of EGTDF cultures, causing them to be more like normal DF, suggesting that MSC therapy might be beneficial to reduce CFPD *in vivo*.

A limitation of the current studies is that many of the experiments were carried out in a two-dimensional (2D) culture system. DF and EGTDF grown in this type of system are subject to an environment that is quite different from the tissue the cells were derived from, particularly in terms of cell-cell contacts and substrate rigidity. Because of this, observations made in a 2D culture system do not necessarily reflect what occurs *in vivo*. The contraction experiments described were performed in a three-dimensional (3D) culture system, which more closely resembles the *in vivo* environment. Our results showing that MSC CM affects the contractile capacity of both DF stimulated with TGF- $\beta$ 1 and EGTDF cultured in 3D systems, suggests that MSC CM may indeed influence EGTDF *in vivo*.

Although beyond the scope of this study, we initiated experiments designed to identify the bioactive factors in the MSC CM that might account for the effects observed in our present study. Preliminarily we found, taking a biased approach using commercially available

cytokine, protease and protease-inhibitor antibody arrays (R&D Systems, Minneapolis MN), several proteins of interest that were present in at least two-fold higher concentrations in MSC CM compared to control fibroblast CM (Supplementary Figure 1D). Currently, we are evaluating these proteins in more detail to confirm the role of these factors, which will allow us to optimize future protocols to enhance the secretion of these factors and/or to allow their individual synthesis.

Taken together, this study aimed to evaluate the functional effects of CM from equine peripheral blood-derived MSC on DF influenced by cytokines that contribute to CFPD. Our results indicate that MSC CM does affect these cells, and warrant studies in more complex model systems in order to determine if MSC CM does have the potential to improve CFPDs *in vivo*.

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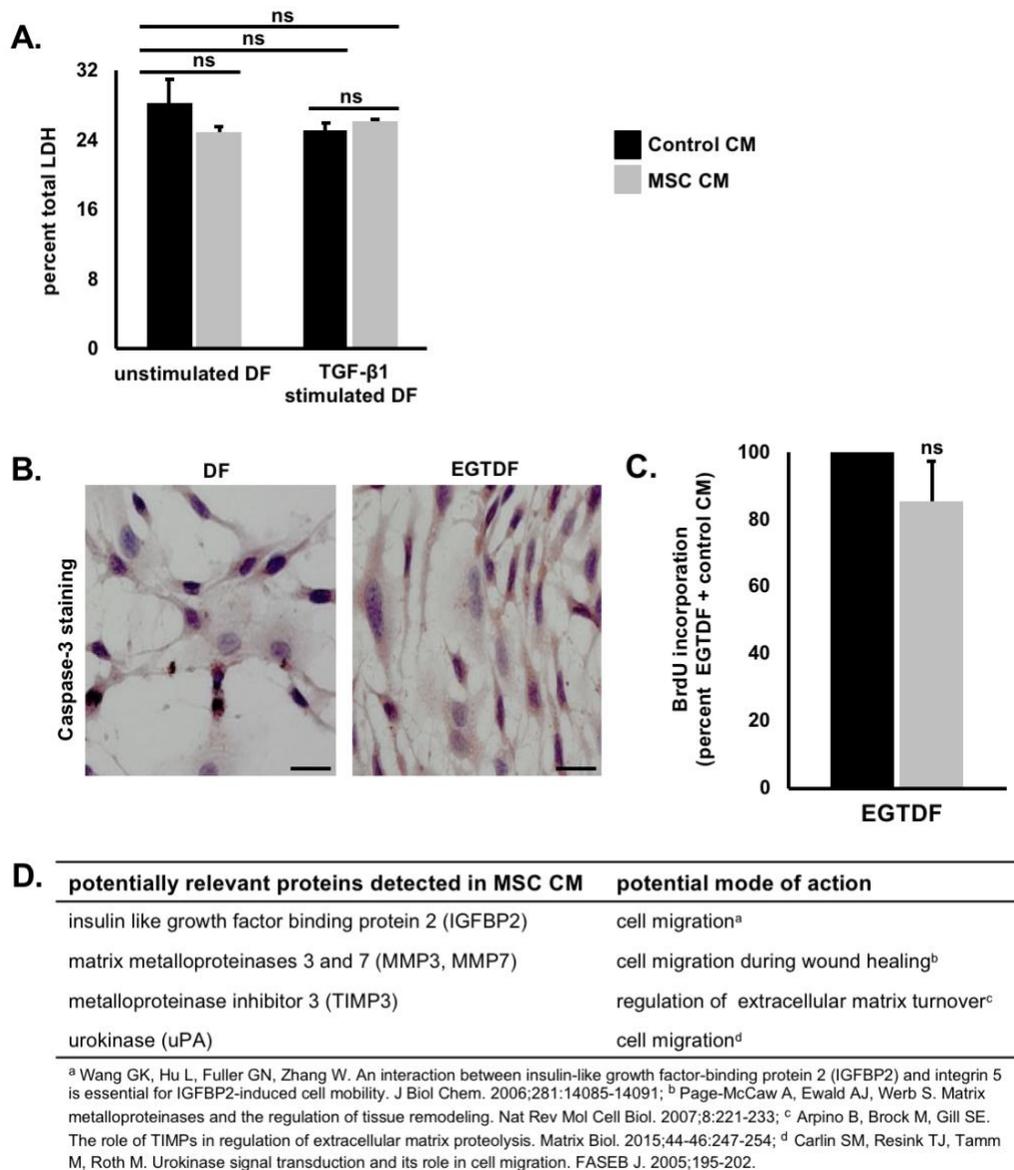
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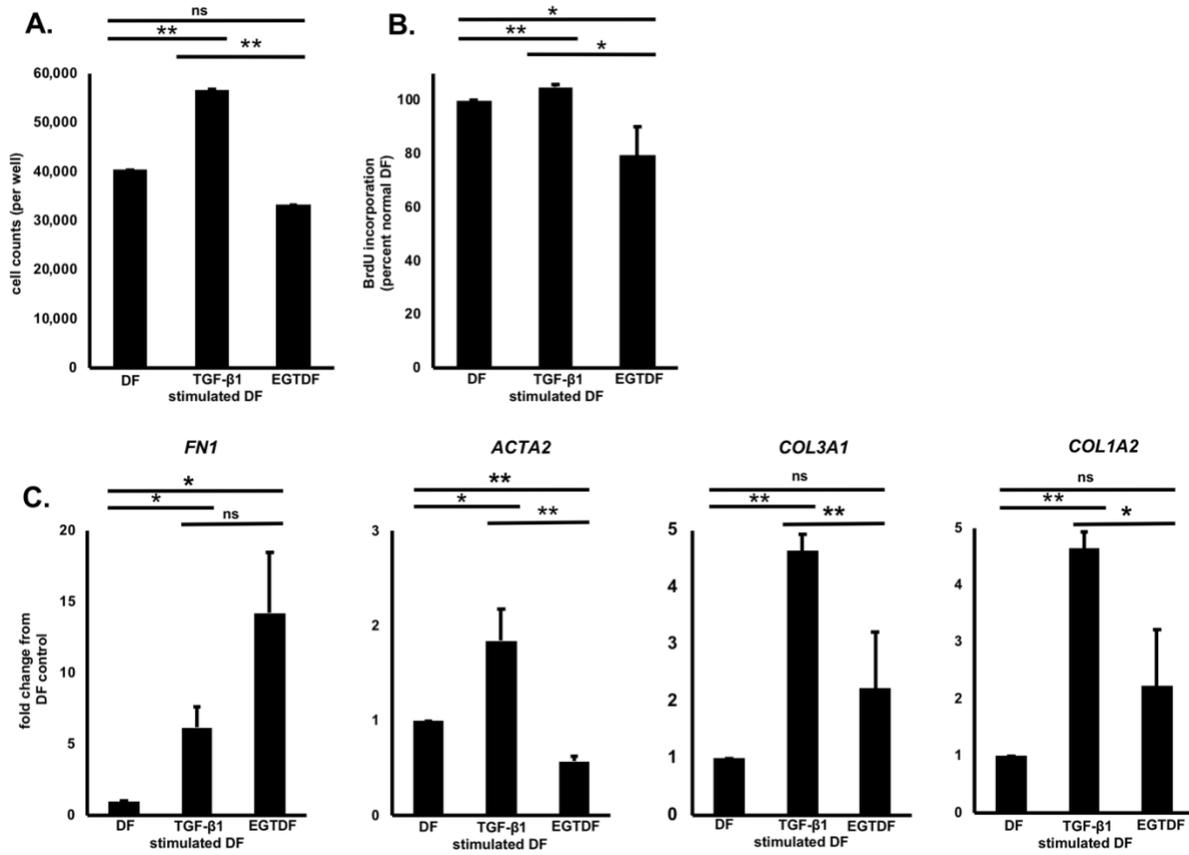
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**Figure 4.S1. Supplementary data.** **A.** TGF- $\beta$ 1 treatment is not toxic to DF as measured by LDH release. **B.** Representative images of normal DF and EGTFD, treated with staurosporine and labeled with an antibody against active caspase-3. **C.** Treatment with MSC CM does not increase EGTFD proliferation as measured by BrdU incorporation. **D.** Potentially relevant proteins detected by antibody arrays in MSC CM that are present in at least two-fold higher concentrations in MSC CM compared to control fibroblast CM. Mode of action and references are also provided. n=3, scale bars=20 $\mu$ m, P < 0.05.



**Figure 4.S2. Comparison of dermal fibroblasts (DF), TGF-β1 treated DF, and exuberant granulation tissue DF (EGTDF).** A-B. Proliferation measured by cell counts (A) and BrdU incorporation (B). C. Expression of genes involved in extracellular matrix production and cytoskeleton composition in DF, TGF-β1 treated DF and EGTDF as measured by qRT-PCR. n=3, \*: P < 0.05, \*\*: P < 0.01.

**CHAPTER FIVE: ANTIMICROBIAL PEPTIDES SECRETED BY EQUINE  
MESENCHYMAL STROMAL CELLS INHIBIT THE GROWTH OF BACTERIA  
COMMONLY FOUND IN SKIN WOUNDS**

The results of this study were published in Harman RM, Yang S, He MK, Van de Walle GR. Antimicrobial peptides secreted by equine mesenchymal stromal cells inhibit the growth of bacteria commonly found in skin wounds. *Stem Cell Res Ther.* 2017;8:157.

## 5.1. Summary

The prevalence of chronic skin wounds in humans is high, and treatment is often complicated by the presence of pathogenic bacteria. Therefore, safe and innovative treatments to reduce the bacterial load in cutaneous wounds are needed. Mesenchymal stromal cells (MSC) are known to provide paracrine signals that act on resident skin cells to promote wound healing, but their potential antibacterial activities are not well described. The present study was designed to examine the antibacterial properties of MSC from horses, as this animal model offers a readily translatable model for MSC therapies in humans. Specifically, we aimed to (i) evaluate the *in vitro* effects of equine MSC on the growth of representative gram negative and gram-positive bacterial species commonly found in skin wounds and (ii) define the mechanisms by which MSC inhibit bacterial growth.

MSC were isolated from the peripheral blood of healthy horses. Gram negative *E. coli* and gram-positive *S. aureus* were cultured in the presence of MSC and MSC conditioned medium (CM), containing all factors secreted by MSC. Bacterial growth was measured by plating bacteria and counting viable colonies or by reading the absorbance of bacterial cultures. Bacterial membrane damage was detected by incorporation of N-phenyl-1-naphthylamine (NPN). Antimicrobial peptide (AMP) gene and protein expression by equine MSC were determined by RT-PCR and Western blot analysis, respectively. Blocking of AMP activity of MSC CM was achieved using AMP-specific antibodies.

We found that equine MSC and MSC CM inhibit the growth of *E. coli* and *S. aureus*, and that MSC CM depolarizes the cell membranes of these bacteria. In addition, we found that equine MSC CM contains AMPs, and blocking these AMPs with antibodies reduces the effects of MSC CM on bacteria.

Our results demonstrate that equine MSC inhibit bacterial growth and secrete factors that compromise the membrane integrity of bacteria commonly found in skin wounds. We also identified four specific AMPs produced by equine MSC. The secretion of AMPs may contribute to the value of MSC as a therapy for cutaneous wounds in both horses and humans.

## **5.2. Introduction**

Chronic cutaneous wounds are a rapidly growing health care burden in human medicine. In the United States, chronic leg and foot ulcers alone affect 2.4-4.5 million people [1,2]. Chronic skin wounds are defined as wounds that do not improve after 4 weeks or do not heal within 8 weeks [3]. Chronic wounds are classified into different categories and are caused by a variety of insults. A common feature of chronic wounds, regardless of initial cause, is colonization by pathogenic bacteria, which leads to an inflammatory host response and delayed wound healing [4,5]. Chronic wounds typically contain a diversity of bacterial species that may interact to form matrices on wound surfaces called biofilms. Biofilms are particularly problematic as they show greater resistance to traditional antibiotics compared to planktonic cells of the same species [6].

Antibiotic-resistant organisms are a challenge for the field of medicine in general, and for health care workers caring for patients with chronic cutaneous wounds in particular [7]. Alternatives to conventional antibiotics are currently being explored, with a focus on finding compounds that can kill bacteria directly rather than by disrupting metabolic activity and proliferation. Bacteria can survive insults to metabolic and proliferative pathways and evolve to avoid them, but they are less likely to become resistant to compounds that kill them directly [8,7]. Naturally-occurring or synthetic antimicrobial peptides (AMPs) could be used for the design of new classes of antibiotics [9-11]. The diverse array of AMPs act through different mechanism, and because many AMPs are bactericidal as opposed to bacteriostatic, it is unlikely

that bacteria will be able to respond to these AMPs by adopting resistance strategies [12-14]. This makes AMPs a promising class of molecules to be explored as novel antimicrobial therapies. A disadvantage of synthetic AMPs as an alternative to conventional antibiotics, however, is that they are costly to generate and purify [15]. Therefore, naturally-occurring AMPs for therapeutic use may be a more practical and cost-effective substitute for traditional antibiotic therapy.

To explore this, a suitable animal model that allows for proper testing of the efficacy of naturally-occurring AMPs to kill bacteria in chronic wounds of humans is warranted. Like humans, horses suffer from naturally-occurring chronic wounds [16,17] and bacterial infection of horse wounds delays the normal healing process by prolonging inflammation, reducing resident skin cell migration, and disrupting extracellular matrix formation [18,5].

Mesenchymal stromal cells (MSCs) are adult multipotent progenitor cells, present in a variety of tissues and organs [19] and contribute to healing processes by participating in the inflammatory, proliferative and remodeling phases of tissue repair [20-22]. Recent data show that paracrine signaling is the primary mechanism by which MSC contribute to tissue repair [23,24]. Our lab has previously demonstrated that equine MSC CM, comprising all factors secreted by MSC, can increase equine dermal fibroblast migration and block the effects of transforming growth factor beta-1 on equine dermal fibroblasts, providing the rationale for their potential in cutaneous wound management [25,26]. The potential of equine MSC to contribute to wound healing by reducing bacterial loads has not been explored to date, although this seems achievable based on previous reports showing that human MSC possess antimicrobial properties such as killing *E. coli* in rodent lung infection models, as well as reducing overall bacterial loads in septic mice [27-29].

Therefore, the aims of the present study were to (i) evaluate the *in vitro* effects of equine MSC on the growth of representative gram negative and gram-positive bacterial species commonly found in skin wounds and (ii) define the mechanisms by which MSC inhibit bacterial growth. Our notable findings were that equine MSC and MSC CM, can inhibit the growth of *E. coli* and *S. aureus* and that MSC CM can depolarize the cell membranes of these bacteria. In addition, we identified four AMPs produced by MSC, and observed that blocking these AMPs in MSC CM with antibodies reduces the effects of MSC CM on bacteria.

### **5.3. Methods**

#### *Cells*

Equine mesenchymal stromal cells (MSC) were isolated from the peripheral blood of 3 healthy warmblood mares between 8 and 12 years old, exactly as described previously [30]. The blood collection was approved by the Cornell Institutional Animal Care and Use Committee (IACUC # 2014-0038). Cells were seeded at a density of  $1.6 \times 10^5$  cells/cm<sup>2</sup> in a T75 flask in culture medium, consisting of low glucose Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 30% fetal bovine serum (FBS) (Atlanta Biological, Flowery Branch, GA),  $10^{-7}$  M low dexamethasone (Sigma Aldrich, St. Louis, MO), 50 µg /mL gentamycin, 1x penicillin-streptomycin (P/S), and 2 mM L-glutamine (all from Life Technologies). Cultures were maintained at 37°C with 5% CO<sub>2</sub>. At 70% confluency, cells were removed from flasks using 0.25% trypsin–EDTA and further cultured in expansion medium, which is identical to the culture medium but without dexamethasone. Equine MSC were characterized by immunophenotypical protein profiling using flow cytometry and their potential for trilineage differentiation, exactly as described previously [31].

The equine dermal fibroblast line NBL6 (ATCC, Manassas, VA) was cultured in standard medium, consisting of DMEM supplemented with 10% FBS and 1x P/S, and maintained at 37°C with 5% CO<sub>2</sub>.

#### *Bacterial cultures*

*Escherichia coli* (*E. coli*) 10536 and *Staphylococcus aureus* (*S. aureus*) 25923 (ATCC) colonies were maintained on Luria-Bertani (LB) agar (Life Technologies) plates at 4°C for up to one month. For each experiment, a colony of the appropriate species was picked and used to inoculate 4 ml LB broth (Life Technologies), which was incubated on a shaker at 200 rpm, overnight at 37°C, in a warm room with ambient air. Overnight cultures were diluted 1:100 in 4 ml LB broth and allowed to incubate, shaking at 200 rpm, at 37°C until cultures reached the exponential growth phase, as determined by the absorbance reading of 1 ml culture at 600 nm using an Ultraspec 2100 pro spectrophotometer (Amersham Pharmacia, Cambridge, UK). Bacteria in the exponential growth phase were used for all experiments, unless stated otherwise.

#### *MSC-bacterial co-cultures*

For experiments in which MSC and bacteria were co-cultured in direct contact with each other, 150,000 MSC or control NBL6 cells were plated per well in 6 well plates in expansion or standard culture medium, respectively. After 24 hours (h), culture medium was removed, cell monolayers were rinsed twice with phosphate buffered saline (PBS) and 1 mL DMEM was added to wells. Bacteria were added at  $1.5 \times 10^6$  per well. Control cultures contained bacteria in plain DMEM or DMEM with 2x P/S without eukaryotic cells. All cultures were incubated for 6 h at 37°C in a warm room with ambient air, while shaking at 100 rpm. The pH of the culture medium was measured at the start and end of the incubation period and remained constant at a pH of 7.5 throughout the experiments. Culture media and cell monolayers, lysed with 1%

saponin (Sigma Aldrich) in distilled water, from each well were transferred to 5 ml tubes, vortexed to evenly distribute bacteria, and subsequently diluted in 10-fold dilutions ranging from 1: 10 to 1: 1,000,000. Three 10 µl drops of each dilution were spotted on LB agar plates and allowed to incubate overnight at 37°C. Bacterial colonies were counted and colony forming units (CFU) per ml were calculated for each treatment.

Transwell experiments were carried out using the same numbers of cells and bacteria as were used for the direct contact co-cultures. For these assays, MSC or NBL6 cells were plated in 0.4 µm transwell inserts (Corning, Oneonta, NY) fitted in 6 well culture plates. Bacteria were added to lower chamber, and after incubation for 6 h at 37°C while shaking at 100 rpm, in a warm room with ambient air, culture medium from the lower chamber was collected for assessment of live bacteria, as described above.

#### *Conditioned medium (CM) collection and treatments*

CM was collected from MSC and NBL6 cells after 2 days of culture, when cells were 70% confluent. To this end,  $6 \times 10^5$  cells were seeded in a T75 flask with expansion medium. After 24 h, medium was removed, cell monolayers were rinsed twice with PBS, and 8 ml DMEM were added. Medium was collected 24 h later, centrifuged twice for 7 min at 300 x g to remove cellular debris, and used for subsequent experiments.

Experiments were also performed with equine MSC CM that was treated as follows: To inactivate large secreted proteins, CM was heat inactivated at 80°C for 30 min or treated with 1 U/ml proteinase K (Qiagen, Valencia, CA) for 6 h at 37 °C before use. To determine if the active factors responsible for the antibacterial effects of MSC CM are biologically stable, CM was frozen and thawed, or lyophilized and reconstituted before being used in assays. To determine the active sub fraction of the CM responsible for inhibiting bacterial growth, CM was filtered

using Amicon Ultra-15 centrifugal filters (EMD Millipore, Darmstadt, Germany), as per manufacturer's instructions, and individual fractions containing secreted factors of specific molecular weights were used for subsequent experiments. To confirm the bioactive roles of identified AMP, CM was incubated with primary rabbit monoclonal antibodies (Ab) against cystatin C (clone EPR4413) or rabbit polyclonal Ab against elafin, lipocalin 2, cathelicidin (Abcam, Cambridge, MA), combined equally for a final concentration of 4  $\mu\text{g/ml}$ , for 1 h prior to the start of the experiments. CM incubated with 4  $\mu\text{g/ml}$  rabbit IgG (Abcam) was used as a control.

#### *CM-bacterial co-cultures*

CM collected as described above, was diluted 1:2 in LB broth for a total volume of 200  $\mu\text{l}$  and plated in triplicate wells of 96 well plates. 500 bacteria were added per well. Plates were incubated shaking at 100 rpm at 37°C, in a warm room with ambient air, for 8 h and 16 h for *E. coli* and *S. aureus*, respectively. Wells containing DMEM diluted 1:2 in LB (negative control) and DMEM diluted 1:2 in LB with 2x P/S (positive control) were also included. For the first experiment, the absorbance of the cultures was read using a 96 well Multiskan EX plate reader (Thermo Fisher Scientific, Waltham, MA) at 600 nm and relative bacterial growth was calculated by comparing the absorbance of the CM wells or antibiotic positive control wells to the absorbance of the DMEM negative control wells. In addition, medium from each well was transferred to 1.5 ml tubes, vortexed to evenly distribute bacteria, and subsequently diluted in 10-fold dilutions, spotted on LB agar plates and allowed to incubate overnight at 37°C as described above. Bacterial colonies were counted and colony forming units (CFU) per ml were calculated for each treatment. By comparing the 2 read-outs (CFU and absorbance), we determined that

absorbance adequately reflected the CFU results, and used it as the read-out for subsequent CM-bacterial co-culture assays.

A subset of CM co-culture experiments was done using bacterial cultures in the post-exponential, stationary, growth phase CM. To this end, stationary bacterial cultures were washed with PBS, and bacterial pellets containing  $1 \times 10^9$  CFU *E. coli* or  $1 \times 10^8$  CFU *S. aureus* were resuspended in either 12.5 mg/ml polymixin B, 50 mg/ml nisin, or MSC CM. After 1 h at 4°C, bacteria were rinsed twice with PBS by centrifugation, diluted in 10-fold dilutions in PBS, spotted on LB agar plates and allowed to incubate overnight at 37°C, as described above. Bacterial colonies were counted and CFU per ml were calculated for each condition.

#### *Biofilm assays*

Biofilm assays were carried out based on a method described by O'Toole [32]. Briefly, 50  $\mu$ l of equine MSC and NBL6 cell CM was pipetted into triplicate wells of u-bottom microtiter plates. DMEM and DMEM with 2x P/S were included as negative and positive controls, respectively. An equal volume of bacteria from cultures in the exponential growth phase was added to each well, and plates were incubated for 72 h to allow for biofilm formation. *E. coli* biofilms were grown at room temperature, *S. aureus* biofilms were grown at 37°C, in a warm room with ambient air. At 24 and 48 h, 50  $\mu$ l CM or control medium was added to appropriate wells. After 72 h, medium was removed from wells and wells were rinsed 2 x with distilled water. *E. coli* biofilms were stained with 2.5% safranin and *S. aureus* biofilms were stained with 0.1% crystal violet for 10 min, after which stains were removed by rinsing biofilms twice with distilled water and allowed to air dry. Dye retained in biofilms was then solubilized using 30% acetic acid and transferred to wells of 96-well flat bottom microtiter plates. Absorbance of solubilized dye was measured at 550 nm on an Infinite 200 pro plate reader.

### *Bacterial membrane depolarization assays*

In brief, CM was added to an equal volume of DMEM in the first column of wells in a 96 well microtiter plate and titered in 1:2 dilutions in DMEM. One-N-phenylaphthylamine (NPN) (Sigma Aldrich) was added to each well for a final concentration of 10  $\mu\text{M}$ , and 50  $\mu\text{l}$  of bacteria from cultures in the exponential growth phase were added. Plates were analyzed immediately in an Infinite 200 pro plate reader (Tecan, Morrisville, NC) using an excitation of 355 nm and emission of 444 nm.

### *Antibody arrays*

A human proteome profiler antibody array, previously shown to cross-react with horse proteins [31, 33] was used, as per manufacturer's instructions (R&D Systems, Minneapolis, MN), to screen CM from equine MSC and equine dermal fibroblasts for the presence of AMPs. Positive signals were visualized using the ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA), normalized to the background, and data were quantified by measuring the sum of the intensities of the pixels within the spot boundary pixel area using image analysis software (Image Laboratory 4.1; Bio- Rad).

### *Reverse transcription-polymerase chain reaction (RT-PCR)*

RNA was extracted from cells using an RNeasy Mini Plus kit (Qiagen) and cDNA was synthesized using M-MLV Reverse Transcriptase (USB, Cleveland, OH), per manufacturers' protocols. Primers were designed using Primer3 software, based on sequences found in the National Center of Biotechnology Information (NCBI) GenBank, and where possible, primer sets spanned an intronic region to prevent amplification of genomic DNA (Table 5.1). RT-PCR using Taq DNA Polymerase (Life Technologies) was performed to amplify the AMP genes cystatin C (*CST3*), elafin (*PI3*), lipocalin 2 (*LCN2*), cathelicidin (*CAMP*) and beta defensin 2

(*DEFB4A*). Beta-2-microglobulin ( $\beta 2M$ ) was included as a reference gene. PCR products were

Gene Product	Abbreviation	Forward primer (5'-3')	Reverse primer (5'-3')
Cystatin C	<i>CST3</i>	TTTCCTGTCACCGTACAGC	GCACAATGTCCGTGGTGAA
Elafin	<i>PI3</i>	GAGAAGGCTGAGTGCCAGAG	ACCAGCGAAATCATCTCCAG
Lipocalin 2	<i>LCN2</i>	TCAAGGATGACCAGTTCCAG	CCTTCCTGAAGAACACGATG
Cathelicidin	<i>CAMP</i>	GGGTAGATGGTCACTGTTGC	AGCCCATTCCTCCTGAAGTC
Beta Defensin 2	<i>DEFB4A</i>	CGTTCCTCGTTGTCTTCCT	CACAGGTGCCAATCTGTTTC
Beta-2-Microglobulin	<i>B2M</i>	GGGCTACTCTCCCTGACTGG	TACCTGCCACACAGGTCAA

run on a 1.5% agarose gel containing GelRed intercalating dye (Thermo Fisher Scientific) at 97 V for 1 h and gels were imaged on a BioRad ChemiDoc MP system (BioRad). Band intensities were measured using BioRad Image Lab software and the intensities of the AMP gene bands were divided by the intensity of reference gene bands, to calculate relative band intensities.

**Table 5.1. Primers used for RT-PCR.**

*Western blot and immunocytochemistry (ICC) analyses*

Western blot analyses were performed, exactly as previously described [26], using the same Ab that were used to block AMP activity in MSC CM, diluted 1:500, followed by HRP-conjugated goat anti rabbit secondary Ab (Jackson ImmunoResearch Labs, West Grove, PA), diluted 1: 20,000.

ICC was performed, exactly as previously described [34], using the same Ab that were used to block AMP activity in MSC CM, diluted 1:100, followed by HRP-conjugated goat anti rabbit secondary Ab, diluted 1: 100.

*Statistical analyses*

The student’s t-test for unpaired data was used to test for statistically significant differences in relative bacterial growth (filtration experiments), and relative band intensities (RT-PCR and Western blot analyses). When multiple t-tests were performed on a single response variable

within an experiment, the Holm-Šídák method was used to counteract the problems associated with multiple comparisons. One-way ANOVA, followed by the Tukey's multiple comparison test was used to determine statistically significant differences in bacterial viability (co-culture experiments, CM experiments and bactericidal vs bacteriostatic effects of MSC CM experiments), relative growth (biofilm, CM experiments excluding filtration experiments, antibody blocking experiments and NPN experiments, excluding filtration experiments). GraphPad software was used for analysis. Data given are the mean of 3 replicates and the bars show standard deviations.

#### **5.4. Results**

##### *Equine mesenchymal stromal cells (MSC) inhibit bacterial growth via paracrine signaling*

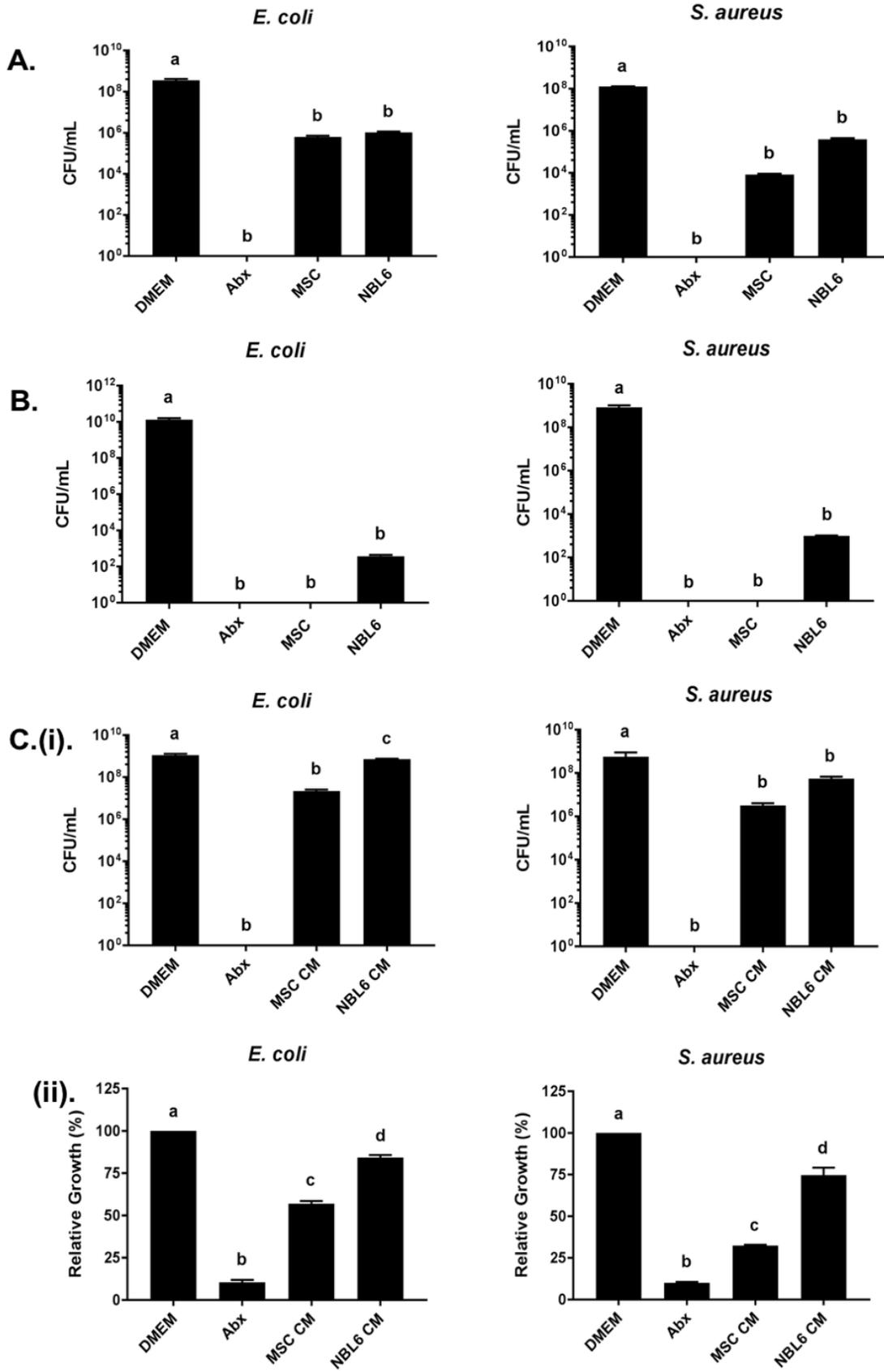
To begin assessing the antimicrobial potential of equine MSC, we designed *in vitro* experiments to determine if MSC can inhibit the growth of representative gram positive and negative bacteria, *E. coli* and *S. aureus*, respectively, that are commonly found in cutaneous wounds. Plain medium (DMEM) and antibiotics were included as negative and positive controls, respectively, and experiments were also performed with the equine dermal fibroblast cell line NBL6, since skin fibroblasts are known to secrete antimicrobial compounds [35-37].

When culturing bacteria in direct contact with equine MSC for 6 hours, like experiments previously carried out by Sung *et al.* with human MSC [38], we found that the growth of both bacterial species was significantly inhibited when compared to DMEM (Figure 5.1A). Based on these encouraging results, we repeated these experiments with equine MSC cultured in transwell inserts to determine if the observed inhibition of bacterial growth was dependent on direct cell-to-cell contact. Equine MSC cultured in transwell inserts were still capable of effectively inhibiting the growth of bacteria within the time frame of the experiment (Figure 5.1B),

indicating that the observed effects are at least in part mediated by factors secreted by equine MSC, similar to what has been reported for human MSC [27,38]. To determine whether equine MSC constitutively secrete factors with antimicrobial properties or if secretion is induced upon bacterial sensing, we cultured bacteria in conditioned medium (CM) collected from MSC and found that the relative bacterial growth, as assessed by CFU after 8 h for *E. coli*, and after 16 h for *S. aureus*, was significantly inhibited in the presence of MSC CM when compared to DMEM (Figure 5.1C(i)). These results were directly compared to an alternative read-out for bacterial growth inhibition, namely the measurement of absorbance of bacterial cultures at 600 nm using a spectrophotometer. Although these two read-outs are not 100% similar (CFU/ml detects only live bacteria within a culture, while absorbance detects all bacteria that grew in the culture regardless of their viability at the time of measurement), the results obtained with absorbance reading reflected the results obtained by calculating CFU/ml (Figure 5.1C(ii)). Since absorbance measurements are less time-consuming and allowed us to perform more technical replicates within each experiment, we decided to use absorbance as a read-out for subsequent CM experiments.

When comparing the results obtained with the equine MSC to the results obtained with the positive antibiotic control (Abx) and the NBL6 cells, we found that the levels of bacterial growth inhibition were not statistically different from the positive antibiotic control or NBL6 cells when we used CFU/ml as a read-out, except that fewer CFU/ml *E. coli* were detected when cultured in MSC CM as compared to NBL6 CM (Figure 5.1A, B & C). When using relative growth as a read out, the results suggest that the levels of bacterial growth inhibition caused by equine MSC CM are situated between the levels of inhibition obtained by Abx and NBL6 cells (Figure 5.1C). This may reflect the mechanism by which MSC secreted factors inhibit bacterial growth.

Taken together, these results show that equine MSC secrete factors that effectively inhibit the growth of both *E. coli* and *S. aureus*, to levels comparable or even greater than those observed with antibiotics or dermal fibroblasts.



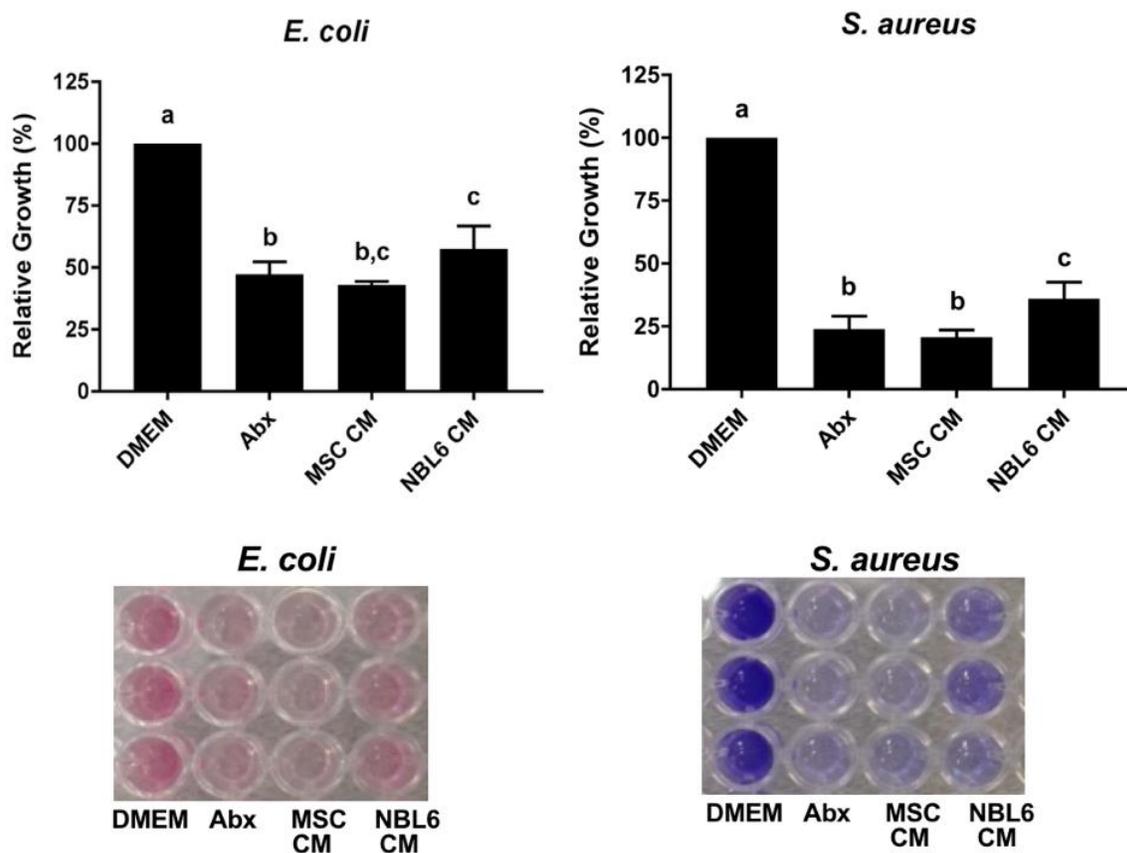
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**Figure 5.1. Equine mesenchymal stromal cells (MSC) inhibit bacterial growth.** **A.** CFU per ml of *E. coli* (left panel) and *S. aureus* (right panel) following culture with DMEM, DMEM with antibiotics (Abx), MSC or NBL6 cells. **B.** CFU per ml of *E. coli* (left panel) and *S. aureus* (right panel) following culture with DMEM, DMEM with antibiotics, MSC or NBL6 cells using transwell inserts. **B.** CFU per ml (i) and relative growth based on absorbance readings at 600 nm (ii) of *E. coli* (left panels) and *S. aureus* (right panels). Cultures were grown in DMEM, DMEM with antibiotics, MSC CM or NBL6 CM each diluted 1:2 in LB broth. Different letters indicate statistically significant ( $P < 0.05$ ) differences.

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*Equine MSC secrete factors that inhibit bacterial biofilm formation*

Because biofilms contribute to the inhibition of cutaneous wound healing [39,7] we assessed the effect of equine MSC CM on biofilm formation using an *in vitro* biofilm assay established by O'Toole [32]. Again, equine MSC CM significantly reduced the growth of both *E. coli* and *S. aureus* biofilms, to an extent that was virtually identical to that observed with the antibiotic control for both types of bacteria, and to levels that differed significantly from NBL6 CM for *S. aureus* (Figure 5.2).

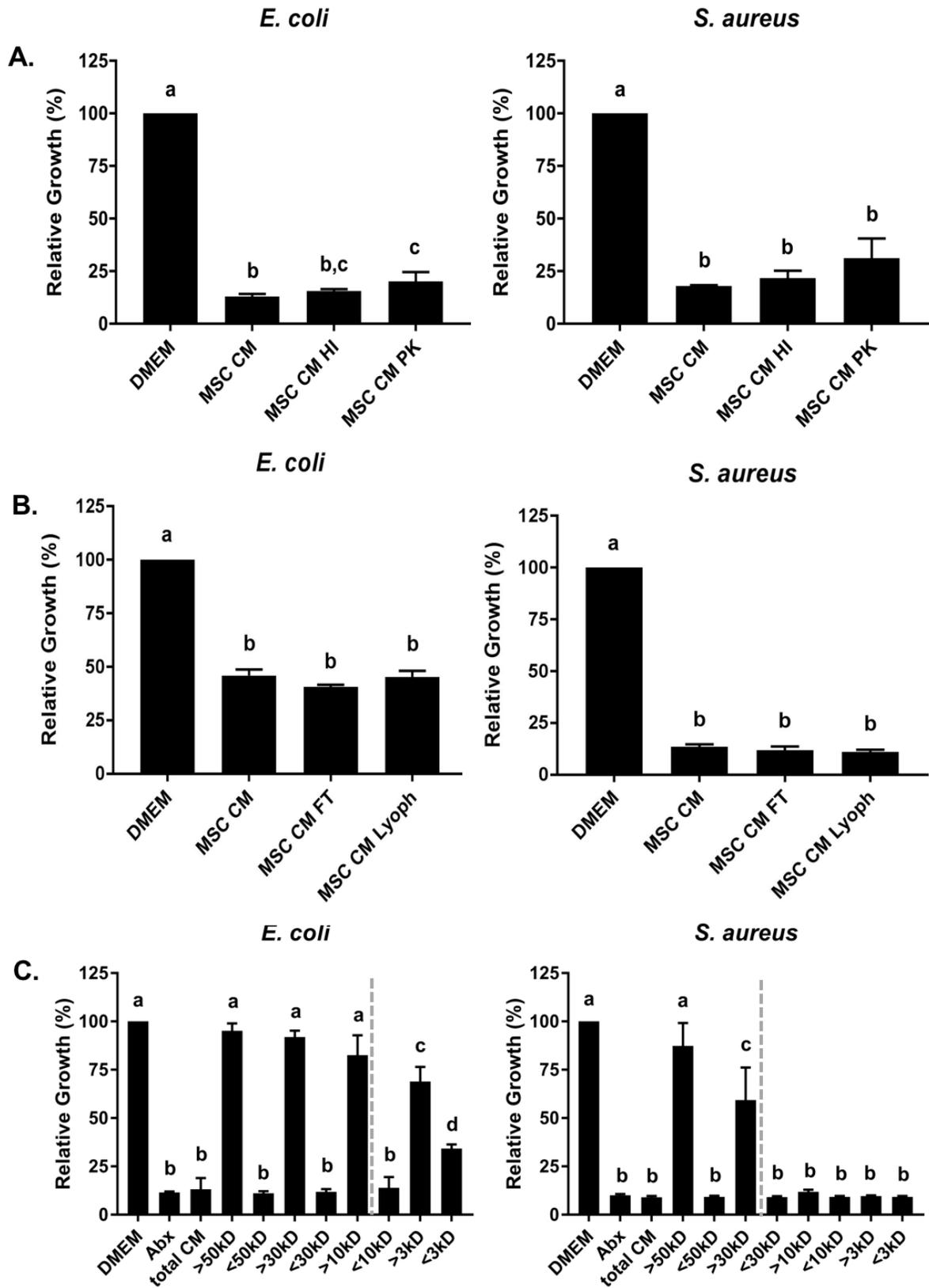


**Figure 5.2. Equine mesenchymal stromal cells (MSC) constitutively secrete factors that inhibit bacterial biofilm formation.** Relative growth of *E. coli* (left panel) and *S. aureus* (right panel) biofilms grown in DMEM, DMEM with Abx, MSC CM or NBL6 CM. Representative images of dye taken up by biofilms are shown below graphs. Different letters indicate statistically significant ( $P < 0.05$ ) differences.  $n = 3$ .

*Equine MSC secrete stable, low molecular weight molecules that inhibit bacterial growth*

To determine the characteristics of the bioactive factors with antimicrobial properties secreted by equine MSC, the following set of experiments was performed. First, we used boiling (heat inactivation = HI) and proteinase K treatment (PK) to inactivate large proteins in the MSC CM, and compared the levels of bacterial growth inhibition to those obtained with untreated

MSC CM. We found that HI CM still significantly inhibited the growth of both types of bacteria when compared to medium control, more specifically to levels indistinguishable from those obtained with the same MSC CM that was left untreated (Figure 5.3A). Similar results were observed for the PK treated CM, although the growth inhibition of *E. coli* was not as robust when compared to the untreated CM (Figure 3A). Next, we froze and thawed, as well as lyophilized and reconstituted, CM before its use in the bacterial assays in order to determine the stability of the active factors. We observed that neither freezing nor lyophilizing significantly altered the growth inhibitory effects of CM on either bacterial species tested, as compared to fresh CM (Figure 5.3B). Finally, we filtered the MSC CM to obtain sub fractions containing secreted factors of specific sizes and found that secreted factors of less than 10 kDa and less than 30 kDa significantly inhibited the growth of *E. coli* and *S. aureus*, respectively (dotted lines, Figure 5.3C).



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**Figure 5.3. Equine mesenchymal stromal cells (MSC) secrete stable, low molecular weight factors that inhibit bacterial growth.**

**A.** Relative growth of *E. coli* (left panel) and *S. aureus* (right panel) based on absorbance readings at 600 nm. Cultures were grown in DMEM, MSC CM, heat inactivated MSC CM (HI) or proteinase K treated CM (PK) each diluted 1:2 in LB broth. **B.** Relative growth of *E. coli* (left panel) and *S. aureus* (right panel) based on absorbance readings at 600 nm. Cultures were grown in DMEM, MSC CM, frozen-thawed MSC CM (FT) or lyophilized-reconstituted CM (Lyoph) each diluted 1:2 in LB broth. **C.** Relative growth of *E. coli* (left panel) and *S. aureus* (right panel) based on absorbance readings at 600 nm. Cultures were grown in DMEM, MSC CM, and MSC CM fractionated by size of secreted factors. Different letters indicate statistically significant ( $P < 0.05$ ) differences.  $n = 3$ .

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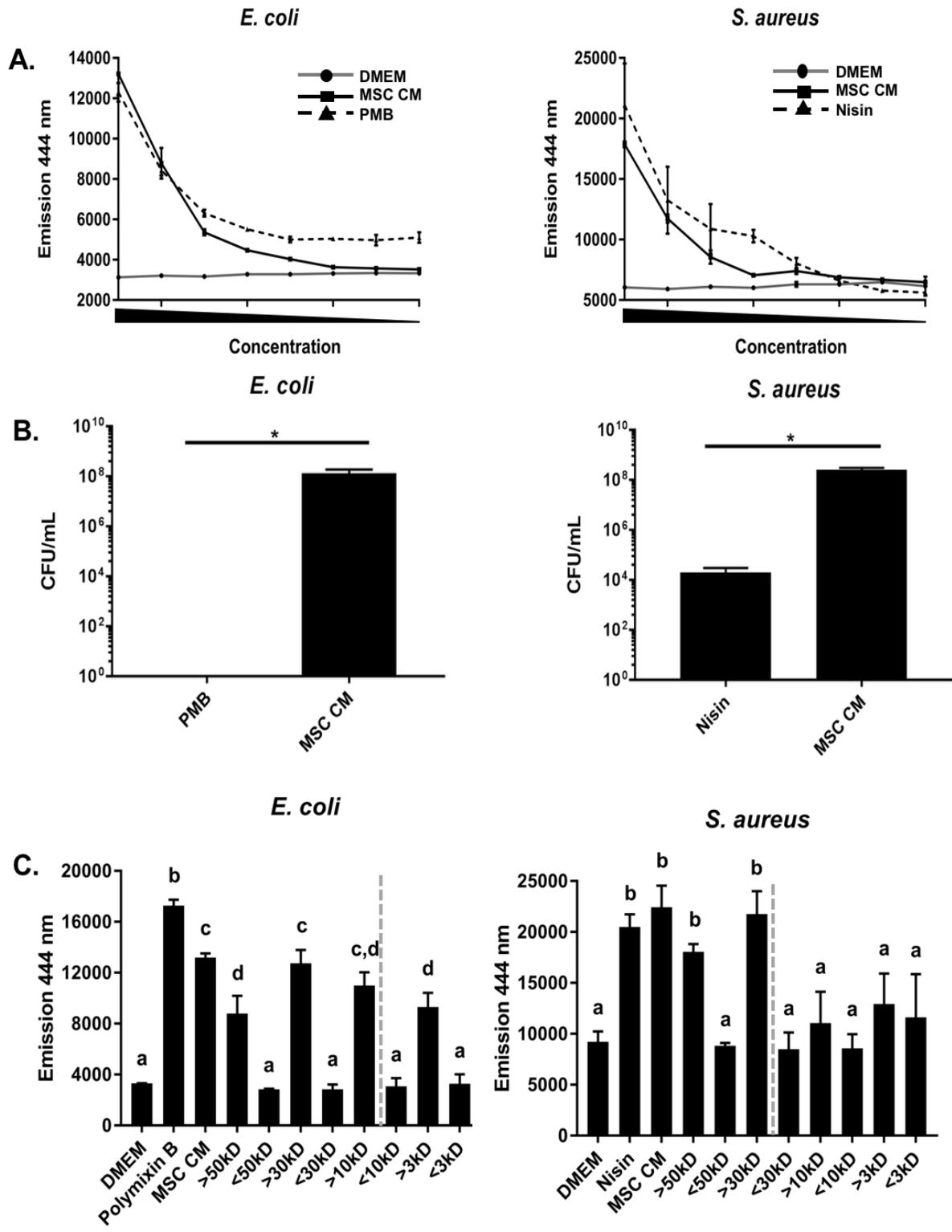
*Equine MSC secrete factors that depolarize bacterial cell membranes, but do not appear to immediately kill bacteria*

In parallel, we decided to evaluate whether the observed MSC CM-mediated bacterial growth inhibition is caused by a membrane depolarizing mechanism. To this end, we exposed bacteria to MSC CM in the presence of 1-N-phenylaphthylamine (NPN), a compound that is excluded by intact outer membranes of bacteria but taken up into the hydrophobic interior of outer membranes that have been depolarized. Consequently, a strong fluorescent NPN signal is correlated with disrupted bacterial membranes [40,41]. The results from these experiments demonstrated that the equine MSC CM causes membrane depolarization of *E. coli* as well as *S. aureus* (i) in a concentration-dependent manner and (ii) to degrees similar to Abx known to depolarize gram negative and gram-positive bacterial membranes; polymixin B and nisin, respectively (Figure 5.4A). Since polymixin B and nisin are bactericidal by killing bacteria shortly after contact [42,43], we decided to evaluate whether the observed equine MSC CM-mediated bacterial membrane damage resulted in similar immediate killing of bacteria. To this end, we directly compared the number of viable bacteria after CM treatment to the numbers

obtained after exposure to these two bactericidal Abx. Based on the difference in number of bacteria between these two types of treatments, with both bactericidal Abx resulting in significantly lower CFU/ml ( $P < 0.05$ , Figure 5.4B), we concluded that treatment of stationary, non-dividing bacteria with equine MSC CM did not immediately kill bacteria to the same extent as the bactericidal control compounds.

We then repeated the NPN experiments with the different CM sub fractions and found that *E. coli* exposed to MSC secreted factors greater than 10 kDa took up more NPN than *E. coli* exposed to MSC secreted factors smaller than 10 kDa, indicating that the bioactive factors that depolarize *E. coli* membranes are most likely 10 kDa or greater in size (dotted line, Figure 5.4C). Likewise, *S. aureus* exposed to MSC secreted factors greater than 30 kDa took up more NPN than *S. aureus* exposed to MSC secreted factors smaller than 30 kDa, indicating that the bioactive factors that depolarize *S. aureus* membranes are most likely 30 kDa or greater in size (dotted line, Figure 5.3C).

Collectively, these data indicate that equine MSC secrete a variety of bioactive factors of different sizes that affect bacteria by various modes-of-action, including membrane depolarization.



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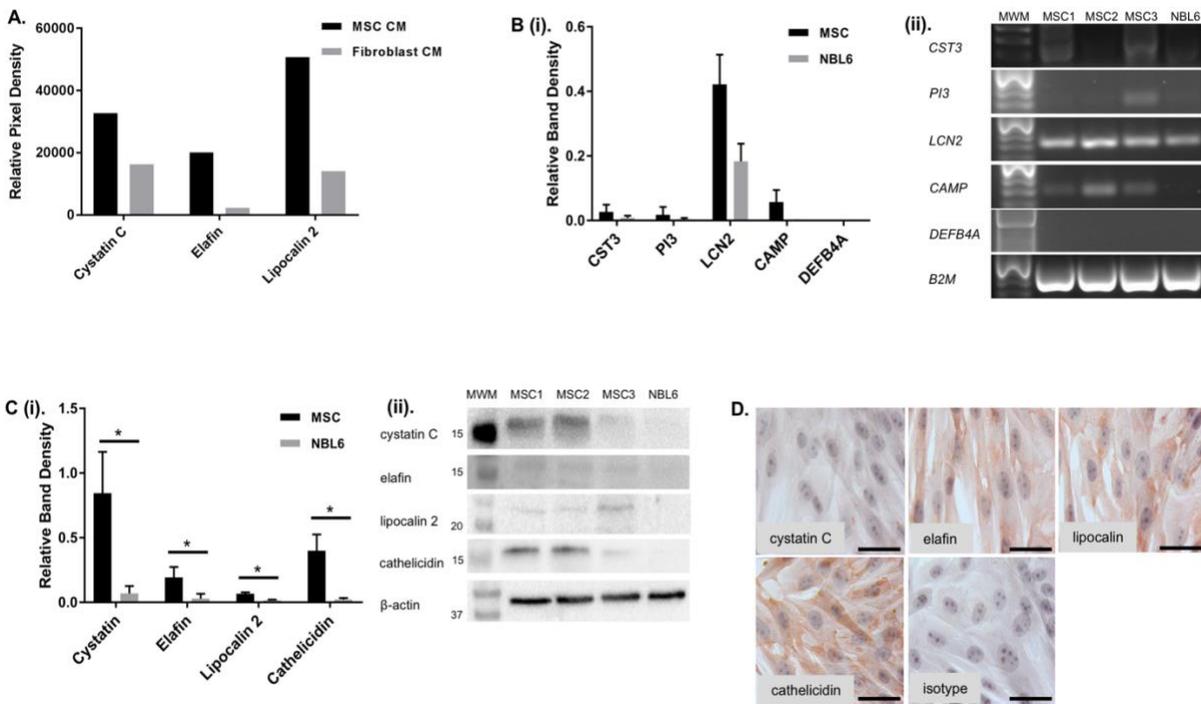
**Figure 5.4. Equine mesenchymal stromal cells (MSC) secrete factors that depolarize bacterial cell membranes, but do not cause immediate cell death.** **A.** Incorporation of NPN into depolarized *E. coli* membranes (left panel) and *S. aureus* membranes (right panel) treated with MSC CM compared to high concentrations of compounds known to depolarize bacterial cell membranes. **B.** CFU per ml of *E. coli* (left panel) and *S. aureus* (right panel) following culture at 4°C with bactericidal compounds polymyxin B (PMB), nisin or MSC CM. \*:  $P < 0.05$  **C.** NPN incorporation by depolarized *E. coli* (left panel) and *S. aureus* (right panel) membranes as measured by fluorescent emission at 444 nm. Bacteria were treated with DMEM, MSC CM, and MSC CM fractionated by size of secreted factors. Different letters indicate statistically significant ( $P < 0.05$ ) differences.  $n = 3$ .

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#### *Equine MSC secrete antimicrobial peptides (AMPs)*

Since AMPs are a class of low molecular weight molecules known to directly kill bacteria by forming pores in bacterial membranes [13,14], we decided to evaluate the secretion of AMPs by equine MSC. We preliminarily screened the equine MSC CM, and a control dermal fibroblast CM for the presence of AMPs using a human proteome profiler array, which can detect the relative levels of over 100 proteins simultaneously ( $n=1$ ). The expression of three AMPs, namely cystatin C, elafin, and lipocalin 2, were readily detected in MSC CM, at higher levels than in the control CM (Figure 5.5A). The expression of these AMPs, as well as the expression of cathelicidin and beta defensin 2 (the most common mammalian AMPs, both of which have previously been described in the horse [12,44]), was then evaluated in the 3 equine MSC cultures that were used for the bacterial assays by RT-PCR using primers that had previously been confirmed by our lab to amplify products of the appropriate lengths in horse cells. Except for the gene *DEFB4A* (encoding beta defensin 2), expression of the AMP transcripts *CST3*, *PII*, *LCN2* and *CAMP* (encoding cystatin C, elafin, lipocalin and cathelicidin, respectively) could be detected (Figure 5.5B). Next, western blot analyses as well as immunocytochemistry (ICC) were

performed to confirm protein expression of those AMPs that were detected on a mRNA level (Figure 5.5C&D). For comparison, we also performed RT-PCR and Western blot analyses on NBL6 cells and found that whereas these cells showed similar expression levels of *CST3*, *PII*, *LCN2* and *CAMP* transcripts (Figure 5.5B), the expression of the corresponding proteins cystatin C, elafin, lipocalin and cathelicidin, was significantly lower in NBL6 compared to MSC cultures (Figure 5.5C). This nicely corresponded with the original preliminary screening of dermal fibroblast CM using the human proteome profiler array (Figure 5.5A).



**Figure 5.5. Equine mesenchymal stromal cells (MSC) express at least four antimicrobial peptides (AMP).** **A.** Detection of three AMPs in MSC CM and control CM as detected by a commercially available antibody array. n = 1. **B.** (i) Relative band densities of five AMP transcripts in equine MSC and NBL6 compared to the reference transcript *B2M* as detected by RT-PCR. (ii) Representative images of PCR products run on agarose gels. Lane 1 = molecular weight marker, Lanes 2, 3 and 4 = equine MSC derived from 3 different horses, Lane 4 = NBL6. **C.** (i) Relative band densities of four AMPs in equine MSC and NBL6 compared to the reference

protein  $\beta$ -actin as detected by Western blot. (ii) Representative images of protein lysates run on polyacrylamide gels, transferred to PVDF membranes and probed with anti-AMP antibodies. Lane 1 = molecular weight marker, Lanes 2, 3 and 4 = equine MSC derived from 3 different horses, Lane 4 = NBL6. **D.** Images of equine MSC labeled with four anti-AMP antibodies (red). Scale bars = 10  $\mu$ m, \*:  $P < 0.05$ ,  $n = 3$ .

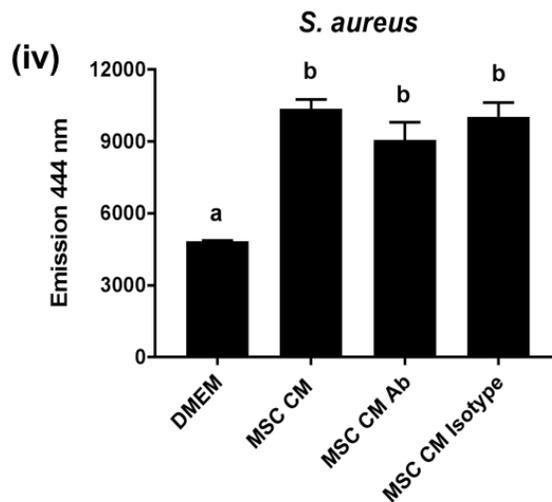
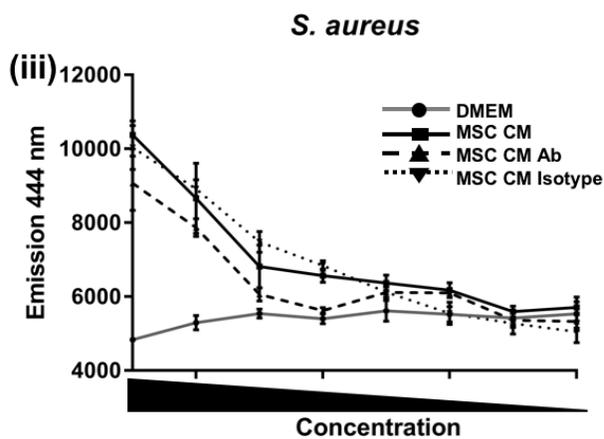
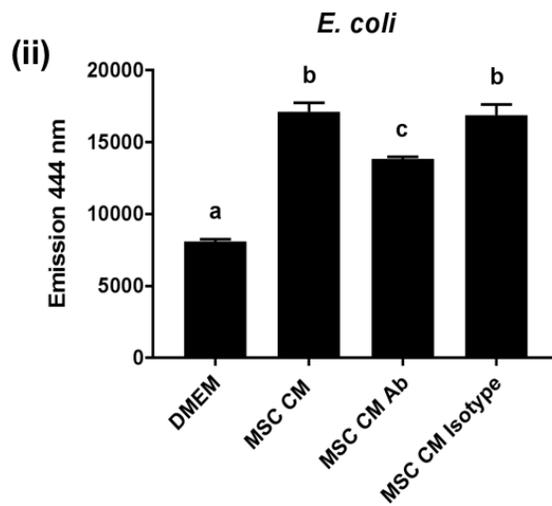
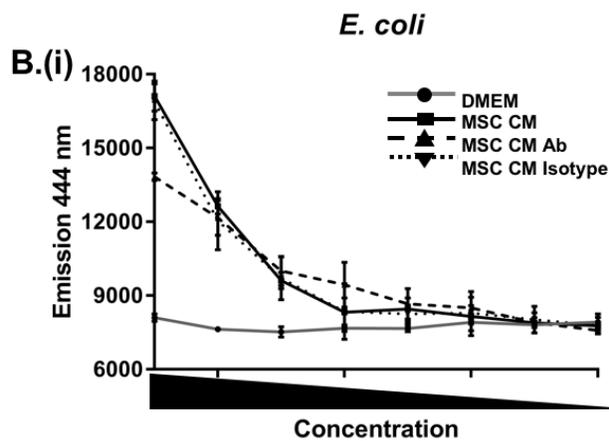
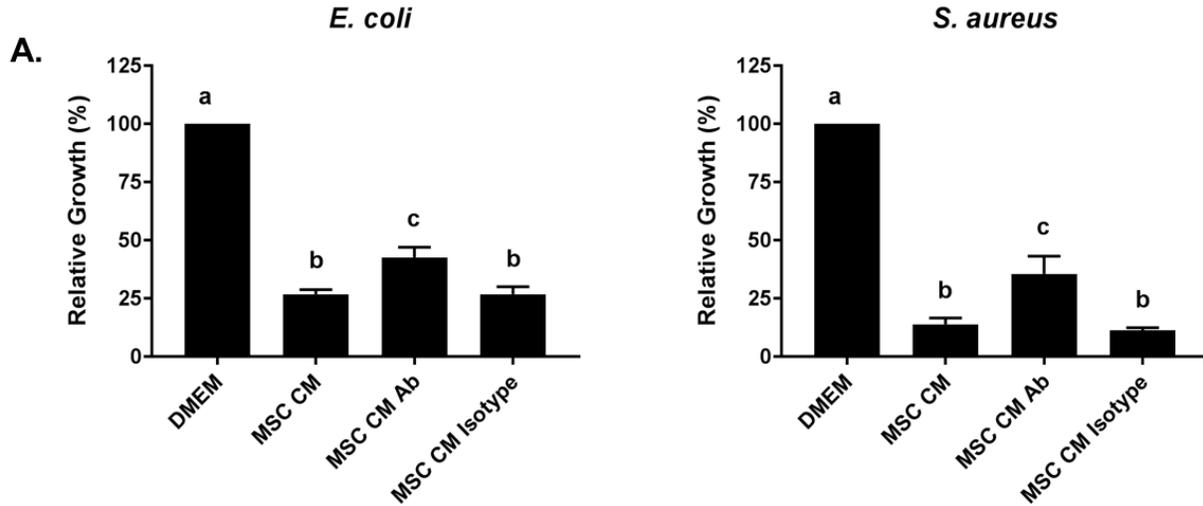
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*The ability of equine MSC to inhibit bacterial growth and induce bacterial membrane damage is greatly reduced by pretreatment of CM with anti-AMP antibodies*

To provide a link between the AMPs detected in equine MSC and the observed antimicrobial effects of the MSC CM, we repeated the bacterial growth inhibition assays using MSC CM that was pretreated with antibodies against these AMPs. Although we found that anti-AMP antibody-pretreated CM still reduced bacterial growth significantly when compared to the DMEM control (Figure 5.6A), the bacterial growth inhibition by pretreated CM was significantly lower compared to untreated MSC CM or CM pretreated with isotype control antibodies (Figure 5.6A). This suggests that cystatin C, elafin, lipocalin and/or cathelicidin are involved in, but not solely responsible for, the MSC CM-mediated growth inhibition of *E. coli* and *S. aureus*. We then corroborated these results by repeating the NPN experiments using antibody-pretreated MSC CM. A significant difference in *E. coli* membrane depolarization was observed compared to the DMEM control that was significantly less pronounced than the effects of untreated MSC CM or CM pretreated with isotype control antibodies (Figure 5.6B (i&ii)). Interestingly, no significant difference in the membrane depolarization of *S. aureus* was observed between the antibody-pretreated MSC CM and untreated MSC CM or CM pretreated with isotype control antibodies, indicating that MSC secreted factors other than cystatin C, elafin, lipocalin or cathelicidin, are responsible for the membrane damage detected in this bacterial species (Figure 5.6B (iii&iv)).

Taken together, these results demonstrate that the equine MSC secreted AMPs cystatin C,

elafin, lipocalin and cathelicidin, are, at least in part, responsible for the observed growth inhibition of *E. coli* and *S. aureus* cultured in MSC CM. In addition, these AMPs also contribute to the membrane damage detected in *E. coli*, but not *S. aureus*.



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**Figure 5.6. Blocking antimicrobial peptide (AMP) activity decreases the effects of mesenchymal stromal cell conditioned medium (MSC CM) on bacteria.** **A.** Relative growth of *E. coli* (left panel) and *S. aureus* (right panel) based on absorbance readings at 600 nm. Cultures were grown in DMEM, MSC CM, MSC CM pre-incubated with anti-AMP antibodies or MSC CM pre-incubated with an isotype control antibody, each diluted 1:2 in LB broth. **B.** Incorporation of NPN into depolarized *E. coli* and *S. aureus* membranes in the presence of decreasing concentrations of DMEM, MSC CM, MSC CM pre-incubated with anti-AMP antibodies or MSC CM pre-incubated with an isotype control antibody as measured by fluorescent emission at 444 nm (i & iii). Emission values from *E. coli* and *S. aureus* with treatments diluted 1:2 (ii & iv). Different letters indicate statistically significant ( $P < 0.05$ ) differences.  $n = 3$ .

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## 5.5. Discussion

This study is the first to demonstrate that equine mesenchymal stromal cells (MSC) possess antibacterial properties by showing that MSC inhibit the growth of *E. coli* and *S. aureus* and depolarize the membranes of these bacteria *in vitro*. Moreover, this study describes the presence of four distinct antimicrobial peptides (AMPs) in the equine MSC secretome, namely cystatin C, elafin, lipocalin 2, and cathelicidin, and demonstrates that these AMPs are at least partially responsible for the antimicrobial effects of MSC.

The MSC-produced AMPs identified in this study represent several classes of antimicrobial compounds, some of which have been documented to be produced by MSC from other sources and species. Cystatin C has a mode-of-action that has not been fully elucidated to date but has been identified as a secreted product of both murine bone marrow-derived and human adipose tissue-derived MSC [45,46]. Lipocalin 2 limits bacterial growth by sequestering iron-containing siderophores and has been identified in the secretome of mouse bone marrow-derived MSC [47]. Cathelicidin is a cationic a that kills bacteria directly by disrupting membrane polarization and

produces a cleavage product (LL-37) that has previously been described as a secreted product of human bone marrow-derived MSC [27,48]. Elafin is known to kill bacteria by disrupting the membrane integrity. To our knowledge, elafin has not been reported as a product of MSC, so this study is the first to report on the production of this AMP by MSC.

Besides these four AMPs, we also evaluated the presence of beta defensin 2 in equine MSC since this AMP (i) has been described to be secreted by human umbilical cord-derived MSC [49,38] and (ii) belongs to a ubiquitous family of AMPs that are found in most mammals, including horses [50,44]. However, and to our surprise, we could not detect beta defensin 2 in equine MSC. This was not due to an improperly functioning of the PCR primers, as we have used the same primers to confirm expression of this AMP transcript in equine keratinocytes (data not shown). To identify the complete spectrum of equine MSC-derived AMPs, we plan to use a more unbiased and global approach (e.g. liquid chromatography–mass spectrometry (LC-MS/MS)) in future experiments.

The bacterial growth inhibition and biofilm assays were run in parallel with an equine dermal fibroblast cell line, based on the knowledge that the skin of most mammals produces AMPs [50,51] and AMP mRNA and proteins have been identified in horse skin [44]. As expected, we found that equine dermal fibroblasts inhibit bacterial growth and produce specific AMPs. To the best of our knowledge, the AMP production by horse skin fibroblasts had not been evaluated previously. Interestingly, the expression of AMPs by the horse dermal fibroblast cell line NBL6 was not equivalent to that of equine MSC, with equine MSC producing higher levels of cystatin C, elafin, lipocalin 2 and cathelicidin. This further emphasizes the therapeutic potential of equine MSC in cutaneous wound management, as the administration of MSC or MSC secreted products

could supplement AMPs expressed at low levels in the skin, therefore expanding the range of AMPs locally present to fight skin infections.

Although the results of this study clearly demonstrate that equine MSC have antimicrobial properties against both *E. coli* and *S. aureus*, there appears to be a difference in the underlying mechanisms targeting each species. Based on the CM fractionation experiments, we demonstrated that secreted factors less than 10 kDa in size are responsible for the observed growth inhibition of *E. coli* whereas secreted factors less than 30 kDa appear to inhibit the growth of *S. aureus*. Moreover, MSC secreted factors greater than 10 kDa contributed to most membrane damage seen in *E. coli*, whereas secreted factors greater than 30 kDa caused membrane damage to *S. aureus*. And although in the current study, we did not fully investigate the exact underlying mechanisms by which equine MSC CM affects different bacteria species, our results do clearly show that MSC secreted factors (i) inhibit bacterial growth and (ii) depolarize the outer membranes of bacteria. It will be interesting, therefore, to study the effects of MSC CM on additional bacterial species commonly found in equine skin wounds, such as the gram negative bacteria *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, and the gram positive *Aerococcus viridians*, *Staphylococcus warneri*, and *Staphylococcus epidermidis* [39], to determine whether there are indeed distinct mechanisms used by equine MSC to target gram positive versus gram negative bacteria. As most of these bacterial species are also found in human skin wounds [52,53], results will be relevant to human as well as veterinary medicine.

Since we found that equine MSC secrete a variety of AMPs that appear effective against both gram positive and negative bacteria, these cells may serve as a broad-spectrum treatment to control bacterial growth and kill bacteria, without leading to resistance. Work by other groups has shown that in addition to controlling bacteria, AMPs and other host defense peptides directly

contribute to wound healing by inducing cell migration and proliferation, promoting angiogenesis, and in general, accelerating the healing process [54]. This dual-function of peptides to promote wound healing, combined with the ease and low cost of isolating MSC and collecting CM, makes MSC CM an ideal biological source for naturally-occurring peptides as well as other factors that promote wound healing. Our data indicating that equine MSC CM can be lyophilized and still retain anti-bacterial activity suggest that using MSC CM therapeutically may be practical as well, providing a stable, off-the-shelf product for clinical use.

Taken together, our group focuses on the potential of equine MSC to be used as a therapy for skin wounds and the data generated in this study suggests that in addition to positively affecting resident skin cells, as we have demonstrated previously [31,25,26], equine MSC may also improve cutaneous wound healing by reducing the bacterial load in wounds. Next, we intend to evaluate the efficacy of equine MSC *in vivo* by assessing how these cells promote skin wound healing and affect bacterial burden, in both acute and chronic wounds.

## **5.6. Conclusions**

Mesenchymal stromal cells (MSC) have been reported to provide paracrine signals that promote cutaneous wound healing, but (i) the effects of equine MSC on the growth of gram negative and gram-positive bacterial species commonly found in skin wounds and (ii) the mechanisms by which equine MSC inhibit bacterial growth had not been explored thus far. The present study is the first to show that equine MSC possess antimicrobial properties by inhibiting the growth of *E. coli* and *S. aureus*, in part by secreting antimicrobial peptides (AMPs) and depolarizing bacterial cell membranes. This antibacterial activity may contribute to the value of MSC as a therapy for chronic cutaneous wounds in both horses and humans, where colonization by pathogenic bacteria commonly inhibits normal healing.

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**CHAPTER SIX: PLASMINOGEN ACTIVATOR INHIBITOR-1 AND TENASCIN-C  
SECRETED BY EQUINE MESENCHYMAL STROMAL CELLS STIMULATE  
DERMAL FIBROBLAST MIGRATION *IN VITRO* AND CONTRIBUTE TO WOUND  
HEALING *IN VIVO***

The results of this study were published in Harman RM, He MK, Zhang S, Van De Walle GR. Plasminogen activator inhibitor-1 and tenascin-C secreted by equine mesenchymal stromal cells stimulate dermal fibroblast migration in vitro and contribute to wound healing in vivo. *Cytherapy*. 2018.

## 6.1. Summary

Impaired cutaneous wound healing is common in humans, and treatments are often ineffective. Based on the significant emotional and economic burden of impaired wound healing, innovative therapies are needed. The potential of mesenchymal stromal cell (MSC) secreted factors to treat cutaneous wounds is an active area of research that is in need of refinement before effective clinical trials can be initiated. The aims of the present study were to (i) study which MSC secreted factors stimulate dermal fibroblast (DF) migration *in vitro* and (ii) evaluate the potential of these factors to promote wound healing *in vivo*.

To this end, MSC were isolated from the peripheral blood of healthy horses, a physiologically relevant large animal model appropriate for translational wound healing studies. Conditioned medium (CM) from cultured equine MSC was analyzed by liquid chromatography-mass spectrophotometry (LC-MS/MS) to identify secreted proteins of interest. Double-stranded *RNA-mediated interference* (RNAi) was used to silence the genes encoding selected proteins, and the effects of CM from these transfected MSC on migration of cultured equine DF cells *in vitro* and full-thickness wounds in mice were evaluated.

We found that MSC-derived plasminogen activator inhibitor-1 (PAI-1) and tenascin-C significantly increased DF migration *in vitro* and improved wound healing *in vivo* by decreasing time to wound closure.

These results suggest that in a complex wound environment, MSC-secreted factors PAI-1 and tenascin-C contribute to the positive effect of therapeutically applied MSC CM on wound healing.

## 6.2. Introduction

Yearly, more than 11 million people in the United States are impacted by acute wounds [1]. When the healing of these wounds is compromised, affected patients may suffer physical and

emotionally, and an economic strain may be put on individuals as well as the health care system. The prevalence rate of chronic non-healing wounds in wealthy countries is 1-2 percent of the population [2]. In the United States alone, 5.7 million patients are affected by chronic wounds annually, at an estimated cost of \$20 billion [3, 4].

The complexity of the cutaneous wound healing process contributes to difficulties in executing successful treatment protocols when natural wound healing is not adequate. It is now well-established that healing of skin wounds is far more involved than the traditional description of the 4 phases of wound healing implies. Indeed, each of the “phases” (hemostasis, inflammation, proliferation, and remodeling) is comprised of interdependent events involving resident skin cells, migrating immune cells, extracellular matrix components, as well as the growth factors, cytokines and chemokines released by cells and the supportive environment [5]. Consequently, the events required for successful wound healing are a continuous cascade that is initiated by wounding and continues until long after wound closure is achieved [6]. Standard treatments for cutaneous wounds aim to improve the wound bed by keeping it moist, free of pathogens, and adding or removing pressure, depending on the type of wound [7]. In recent years, there has been an effort to incorporate more sophisticated treatment strategies based on what has been learned about cellular responses to injury, such as the administration of platelet-derived growth factor, vascular endothelial growth factor and fibroblast growth factor to wounds. However, malignancy risks associated with adding recombinant growth factors to cutaneous wounds have been recognized, and these therapies have not yet been fine-tuned for clinical use [8]. Hyperbaric oxygen therapy, which consists of breathing pure oxygen while in a total body chamber where pressure is controlled, is also being studied as a method to positively influence cells involved in wound healing by altering clotting, cell proliferation, angiogenesis and tissue repair [9]. Although there are few

negative side effects to this therapy, it is currently not widely used as the equipment and technical expertise needed to provide it are not universally available, and long-term positive results are not well documented [9, 10].

Stem cell therapies may be used to augment the natural events required for successful wound healing, while avoiding the side effects associated with administering recombinant growth factors and the limits associated with a technology-heavy procedure like hyperbaric oxygen therapy [11]. Mesenchymal stromal cells (MSC) are adult, multipotent progenitor cells that can be isolated easily from a variety of adult tissues and readily expanded in culture [12]. These cells actively secrete a rich composite of bioactive factors that may prove to be a safe, easy-to-deliver therapy to improve tissue repair [13]. Our laboratory studies MSC isolated from the peripheral blood of horses, a physiologically relevant large animal model appropriate for translational wound healing studies [14, 15]. We have used *in vitro* assays to demonstrate that equine MSC secreted factors act on target cell types present in the skin in ways that suggest they may promote cutaneous wound healing *in vivo*. For example, we demonstrated that equine MSC secrete cytokines and chemokines that promote endothelial cells to form vessel-like structures [16], as well as antimicrobial peptides that inhibit the growth of bacterial commonly found in skin wounds [17]. We also found that conditioned medium (CM) from equine MSC cultures stimulates dermal fibroblast (DF) migration [18], although the bioactive factors responsible for this effect were not identified.

The aims of the present study were to determine (i) which factors secreted by equine MSC promote DF migration *in vitro* and (ii) if these factors also contribute to cutaneous wound healing *in vivo*. We identified MSC-derived plasminogen activator inhibitor-1 (PAI-1) and tenascin-C as stimulators of DF migration *in vitro*. Moreover, using a murine full thickness wound model, we

demonstrated that the presence of these proteins in MSC CM decreased time to wound closure *in vivo*.

### **6.3. Methods**

#### *Cell isolation and culture*

Equine MSC were isolated from the peripheral blood of 3 healthy warmblood mares between 8 and 12 years old and cultured exactly as we described previously [19]. Blood collection was approved by the Cornell Institutional Animal Care and Use Committee (IACUC # 2014-0038). The equine and murine cell lines NBL6 and NIH3T3 (ATCC, Manassas, VA), respectively, were cultured in standard medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1x penicillin-streptomycin and maintained at 37°C with 5% CO<sub>2</sub>.

#### *CM collection and treatments*

CM from untransfected MSC, NBL6 and NIH3T3 cells was collected after 2 days of culture, when cells were 70% confluent, as described previously [18]. CM from cells transfected with small interfering RNA (siRNA) was generated by removing culture medium 24 hours (h) post transfection, rinsing monolayers 2x with phosphate buffered saline (PBS), and adding 2 ml DMEM per well. CM was collected 24 h later. All *in vitro* experiments, except mass spectrometry, were carried out using the CM from MSC isolated from 3 different horses, with technical replicates as described for each assay.

Experiments were also performed with equine MSC CM that was treated as follows: To inactivate large secreted proteins, CM was heat inactivated at 80°C for 30 min or frozen and thawed before use. To determine the active sub fraction of the CM responsible for stimulating fibroblast migration, CM was filtered using Amicon Ultra-15 centrifugal filters (EMD Millipore, Darmstadt,

Germany), as per manufacturer's instructions, and individual fractions containing secreted factors of specific molecular weights were used for subsequent experiments.

#### *Extracellular vesicle (EV) isolation and inhibition*

MSC-derived EV were isolated by centrifugation of CM at 300 xg, 10 min to pellet out cellular debris. Supernatant containing EV was transferred to clean tubes and centrifuged first at 2,000 xg for 30 min, transferred to clean tubes, and then centrifuged 100,000 xg for 60 min to pellet out EV. Supernatant was saved as the EV-free CM fraction, and EV were resuspended in DMEM and centrifuged again at 100,000 xg for 60 min. After discarding the supernatant, EV were resuspended in 16 ml DMEM, equivalent to the starting volume of CM. To inhibit EV formation by MSC, 10  $\mu$ m GW4869 (Sigma Aldrich, St. Louis, MO) was added to MSC cultures during the generation of CM.

#### *Protein identification by Liquid chromatography-mass spectrophotometry (LC-MS/MS)*

In solution digestion was performed following a protocol by Zhang, with slight modification [20]. The protein pellet from 10 mL medium culture was dissolved in 300  $\mu$ L denaturing solution containing 6.0 M guanidine-HCl, and 100 mM PBS pH 7.0, sonicated for 5 min, centrifuged and supernatant was collected. Repeat above extraction three times and a total of 800  $\mu$ L extracts were incubated at 56 °C for 45 min. The denatured sample was reduced with 10 mM DTT and alkylated with 55 mM Iodoacetamide for 1hr in dark at room temperature. Excess Iodoacetamide was quenched with 30 mM DTT for 30mins at room temperature. The samples were diluted to a final volume of 3,775  $\mu$ L with 50mM Tris HCL pH 8.0 to reduce the Guanidine HCL concentration to less than 1M. The samples were digested by adding 25  $\mu$ g trypsin (Promega, Madison, WI) and incubated at 37 °C for 16 hours. Digestions were stopped by the addition of 25  $\mu$ L TFA. The digests were further desalted by solid phase extraction using

Sep-Pack Cartridge (Waters Corporation, Milford, MA) and the eluted peptides were evaporated to dryness by a Speedvac SC110 (Thermo Savant, Milford, MA). The in-solution tryptic digests were reconstituted in 20  $\mu$ L of 0.5% FA for nanoLC-ESI-MS/MS analysis, which was carried out using an Orbitrap Fusion<sup>TM</sup> Tribrid<sup>TM</sup> (Thermo-Fisher Scientific, San Jose, CA) mass spectrometer equipped with a nanospray Flex Ion Source, and coupled with a Dionex UltiMate3000RSLCnano system (Thermo, Sunnyvale, CA) as reported previously [21]. The gel extracted peptide samples (5  $\mu$ L) were injected onto a PepMap C-18 RP nano trap column (5  $\mu$ m, 100  $\mu$ m i.d x 20 mm, Dionex) with nanoViper Fittings at 20  $\mu$ L/min flow rate for rapid sample loading and then separated on a PepMap C-18 RP nano column (3  $\mu$ m, 75  $\mu$ m x 25 cm) at 35  $^{\circ}$ C, and eluted in a 90 min gradient of 5% to 35% acetonitrile (ACN) in 0.1% formic acid at 300 nL/min., followed by a 7 min ramping to 90% ACN-0.1% FA and an 8 min hold at 90% ACN-0.1% FA. The column was re-equilibrated with 0.1% FA for 25 min prior to the next run. The Orbitrap Fusion is operated in positive ion mode with spray voltage set at 1.6 kV and source temperature at 275 $^{\circ}$ C. External calibration for FT, IT and quadrupole mass analyzers was performed. In data-dependent acquisition (DDA) analysis, the instrument was operated using FT mass analyzer in MS scan to select precursor ions followed by 3 second “Top Speed” data-dependent CID ion trap MS/MS scans at 1.6 m/z quadrupole isolation for precursor peptides with multiple charged ions above a threshold ion count of 10,000 and normalized collision energy of 30%. MS survey scans at a resolving power of 120,000 (fwhm at m/z 200), for the mass range of m/z 375-1575. Dynamic exclusion parameters were set at repeat count 1, an exclusion list size of 500, 40 s of exclusion duration with  $\pm$ 10 ppm exclusion mass width. The activation time was 10 ms for CID analysis. All data were acquired under Xcalibur 3.0 operation software (Thermo-Fisher Scientific). The DDA raw files for CID MS/MS only were subjected to database searches

using Proteome Discoverer (PD) 2.2 software (Thermo Fisher Scientific, Bremen, Germany) with the Sequest HT algorithm [22]. The MS/MS spectra were searched against a NCBI database for *Equus caballus*\_RefSeq database that contained 30,927 entries. Oxidation of M, deamidation of N,Q residues and protein N-terminal acetylation were specified as variable modifications; carbamidomethyl C was specified as a static modification. The peptide precursor tolerance was set to 10 ppm and fragment ion tolerance was set to 0.6 Da. Only high confidence peptides defined by Sequest HT with a 1% FDR by Percolator [23] were considered for the peptide identification. The final protein IDs contained protein groups that were filtered with at least 2 peptides per protein. Results were further analyzed using PANTHER [24] and STRING software [25].

#### *Double-stranded RNA-mediated interference (RNAi)*

Custom Silencer Select siRNA targeting equine plasminogen activator inhibitor-1 (*SERPINE1*) and tenascin-C (*TNC*) were designed (Ambion, Foster City, CA). Silencer Select Negative Control #2 siRNA (Ambion) was confirmed to not have complementarity to any equine genes using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and was used as a non-specific (scramble) control. For RNAi,  $3 \times 10^5$  MSC were seeded per well in a 6 well plate, and Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect cells following manufacturer's protocol.

#### *Viability assays*

To assess cell viability, 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH)-based *in vitro* toxicology assays (Sigma Aldrich) were carried out as we previously described [19, 17]. Absorbance was measured on a Multiskan EX plate reader

using Ascent software (Thermo Fisher Scientific). Triplicate wells were assessed for each condition tested.

#### *Reverse transcription-polymerase chain reaction (RT-PCR)*

SYBR green-based quantitative RT-PCR was performed to determine fold change in *SERPINE1* and *TNC* transcripts, and data were analyzed, as previously described [26, 27]. The previously validated reference gene beta-2-Microglobulin (*B2M*) was used to normalize samples [28] and all samples were run in triplicate. Semi-quantitative RT-PCR using Taq DNA Polymerase (Life Technologies) was performed to amplify genes for cell surface receptors of PAI-1 and tenascin-C; integrin alpha-5 (*ITGA5*), integrin beta-1 (*ITGB1*), urokinase receptor (*uPAR*), annexin II (*Annexin II*), epidermal growth factor receptor (*EGFR*), integrin subunit alpha V (*ITGAV*) and integrin beta-3 (*ITGB3*). Primers are shown in Table 6.1.

**Table 6.1. Primers used for RT-PCR.**

Gene Product	Abbreviation	Forward primer (5'-3')	Reverse primer (5'-3')
integrin alpha-5	<i>ITGA5</i>	TGCTCCTAGGTCTGCTCATC	TGGCCCTCAAGTATGTCTC
integrin beta-1	<i>ITGB1</i>	CACTGAAGAGGTGGAGGTTG	GTGTAGTTGGGGTTGCATTC
urokinase receptor	<i>uPAR</i>	GAATAGGCATGCAGGTCATC	TCCCCTCACAGCTGTAACAC
annexin II	<i>Annexin II</i>	AGATATTGCCTTCGCCTACC	TTCCTCTTCACTCCAGCATC
epidermal growth factor receptor	<i>EGFR</i>	GTACCTCAACACCCATC	GCCCTTAGGACAGGAGTAGC
integrin subunit alpha-V	<i>ITGAV</i>	TCGACAGGCTCATATTCTCC	GCAGCTAAAACAGCAAGGTC
integrin beta-3	<i>ITGB3</i>	AGGCACTTAACGACAAGCAG	CTGAGGACAAAGCTGAGGAG
plasminogen activator inhibitor-1	<i>SERPINE1</i>	CCCTGGAGAGTGAAGTGGAC	CCTGCGATACATGGAGAAGC
tenascin	<i>TNC</i>	CGGCCAAGTTTGACCATTAC	TTCCTGTCCAGGTTCAGAC
beta-2-microglobulin	<i>B2M</i>	TCTTTCAGCAAGGACTGGTCTTT	CATCCACACCATTGGGAGTAAA

### *Western blot (WB) analyses*

WB were carried out as we previously described [17,28]. CM was collected from culture wells seeded with identical numbers of cells, to compare protein levels detected in CM to number of cells seeded. Membranes were incubated with rabbit anti-tenascin-C (Millipore) or rabbit anti-PAI-1 (Abcam, Cambridge, MA) antibodies (Abs) diluted 1:1000, and then incubated with HRP conjugated goat anti-rabbit Abs diluted 1:20;000 (Jackson ImmunoResearch Labs). Where appropriate, membranes were probed in parallel with the loading control Abs rabbit anti-fibronectin (Sigma Aldrich) or rabbit anti-beta actin antibody (Abcam), each diluted 1:1000. Blots were visualized by chemiluminescence using Clarity Western ECL (BioRad, Hercules, CA), imaged on a BioRad ChemiDoc MP system (BioRad), and band intensities were determined using Image Lab software. Intensities of the bands of interest were divided by the intensities of loading control bands to calculate relative band intensity.

### *Cell migration assays*

*In vitro* scratch assays with NBL6 or NIH3T3 cells were performed, exactly as we previously described [18]. Photographs of scratches were taken at 0 and 48 h post scratching, and migration distances of cells were calculated in a blinded-manner using ImageJ software (<http://imagej.nih.gov/ij/>). Oris cell migration assays were carried out according to the manufacturer's instructions. Reference wells were used to determine the area with no cells at the time of stopper removal. For analysis, cells were stained with a crystal violet solution (0.5% crystal violet in 20% (v/v) methanol) for 20 min, washed 2x with PBS, and dried overnight. Images of wells were captured using an Olympus inverted light microscope and migrated cells were counted by a blinded-observer. Numbers of cells migrated from duplicate wells were averaged.

### *Cell adhesion assay*

Cell adhesion strength was quantitated using a centrifugation assay that applies a controlled detachment force [29]. Briefly, DF were seeded in replicate 96-well plates and 24 h later, culture medium was removed and replaced with CM for 48 h. Triplicate wells were assessed for each condition assayed. The lid from one plate was replaced by microplate adhesive tape, and centrifuged inverted at 4,000 x g for 5 min to remove loosely adherent cells. Medium was removed from the replicate, non-centrifuged control, plate. All wells were washed once with PBS to remove detached cells and stained with a crystal violet as described above. Crystal violet was solubilized by adding methanol to each well followed by rocking for 30 min, after which absorbance was quantitated spectrophotometrically at 570 nm on a Multiskan EX plate reader. Cell adhesion strength was presented as absorbance of the wells on the centrifuged plate relative to the replicate control wells. Cells were not seeded in the outer two wells of the 96-well plate and treatment group locations were randomized to prevent biasing the adhesion data based on the small changes in centrifugal forces present in different locations of the plate.

#### *Analysis of actin cytoskeleton*

Actin was labeled using Alexa 647-phalloidin, as we previously described [28]. Cells were examined with a Zeiss LSM confocal microscope (Oberkochen, Germany) and images were captured with a camera controlled by ZEN imaging software. Anisotropy scores were calculated using the ImageJ plugin FibrilTool [30].

#### *Mouse full-thickness wound experiments*

These experiments were approved by the Cornell Institutional Animal Care and Use Committee (IACUC # 2017-0116). A 4 mm biopsy punch was used to create two full thickness wounds on the back of 6-8-week-old female C57/B6 mice. Wounds were treated daily with 50  $\mu$ l vehicle control (DMEM) or various CM. Wounds were photographed and measured daily until

complete wound closure. One set of mice, representing each treatment group, was euthanized at day 4 post wounding, and wounded skin was collected, fixed, and stained with hematoxylin and eosin (H&E) for histological analysis.

#### *Statistical analyses*

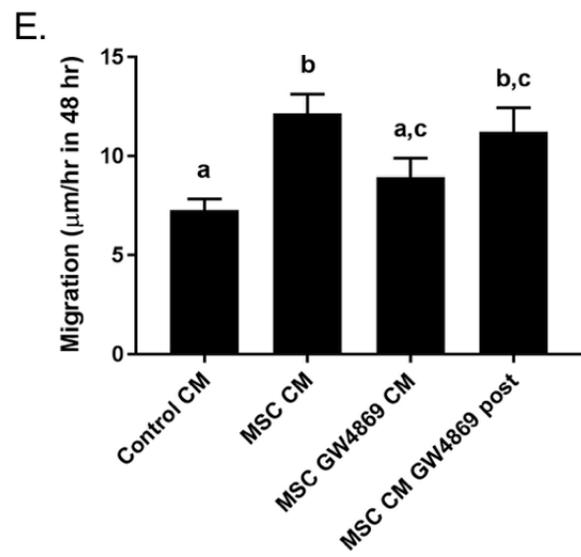
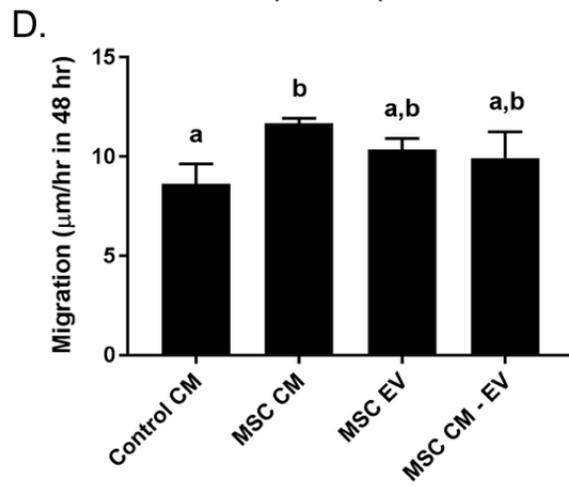
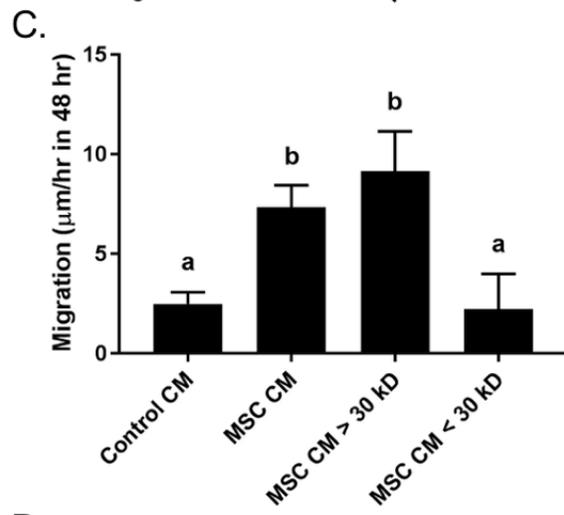
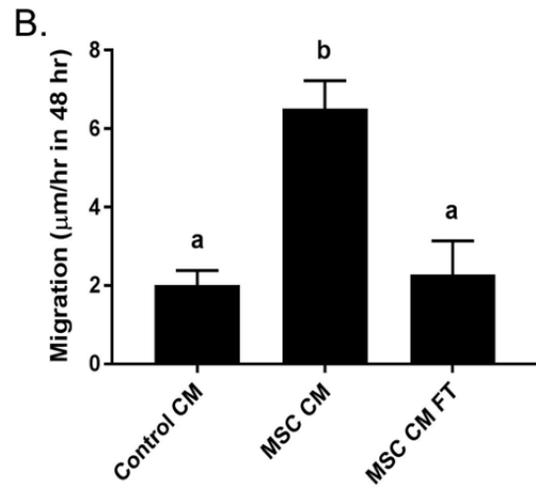
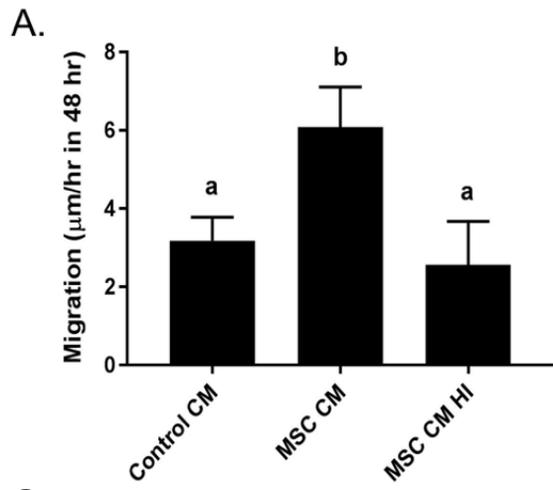
GraphPad software was used to determine statistically significant differences. One-way ANOVA, followed by the Tukey's multiple comparison test was used for all experiments, with exception of experiments where only two conditions were compared. In those cases, the student's t-test for unpaired data was used. Data given are the mean of 3 replicates and the bars show standard deviations. For experiments with multiple comparisons, significance is expressed by letters printed above data points on graphs. The same letter indicates no difference between conditions, while different letters indicate significant differences between conditions. For experiments where only two conditions are compared, significance is expressed by asterisks printed above data points on graphs. P-values are defined in figure legends.

#### **6.4. Results**

*MSC secreted factors that stimulate DF migration in vitro are temperature sensitive and are, at least in part, associated with EV.*

We demonstrated previously that equine MSC CM stimulated DF migration *in vitro* [18]. To explore which MSC secreted factors play a role in this, we first performed experiments to determine the characteristics of the active factors. As we previously reported, DF in the presence of MSC CM migrated more quickly than DF in the presence of control CM (i.e. CM collected from DF) as measured by *in vitro* scratch assays (Figure 1). When MSC CM was boiled (CM HI) or frozen and thawed (CM FT), however, this stimulating effect was abolished (Figure 6.1A&B). This indicates that the active factors in MSC CM are temperature sensitive. Next, we fractionated

MSC CM using 30 kD centrifugal filters, and found that the fraction retained by these filters (i.e. factors > 30 kD) stimulated DF migration to levels comparable to complete MSC CM, whereas the fraction that passed through the filters (i.e. factors < 30 kD) did not (Figure 6.1C). This suggests that the active factors are larger than 30 kD or are engaged in complexes that are larger than 30 kD. Since MSC from other species have been shown to produce EV that are >30 kD and can deliver cargo to target cells [31], we decided to compare the effects of control CM, MSC CM, MSC-derived EV and EV-deficient MSC CM on DF migration. As expected, MSC CM significantly increased DF migration as compared to control CM (Figure 1D). MSC-derived EV as well as EV-deficient MSC CM also increased DF migration as compared to control CM, although this effect didn't reach significance (Figure 6.1D), suggesting that some of the migration-stimulating factors in MSC CM are associated with EV, while others are not. To further explore the potential role of MSC EV in stimulating DF migration, we treated MSC with the EV inhibitor GW4869 and collected CM from these cells. DF migration in the presence of this CM was not statistically different from the migration observed in the presence of control CM and was statistically lower than the migration in the presence of CM collected from untreated MSC (Figure 6.1E), indicating that EV from MSC do, at least in part, deliver factors that promote DF migration. To ensure that the absence of increased migration in the presence of GW4869-treated MSC CM was not due to the presence of residual inhibitor in the CM, we supplemented untreated MSC CM with GW4869 just before it was applied to the DF. This GW4869-supplemented CM did stimulate DF migration to similar levels as non-supplemented CM (Figure 1E), indicating that GW4869 by itself does not inhibit DF migration.



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**Figure 6.1. The mesenchymal stromal cell (MSC) secreted factors that stimulate dermal fibroblast (DF) migration *in vitro* are temperature sensitive and are, at least in part, associated with extracellular vesicles (EV).** As measured by scratch assays, the increase in DF migration induced by culture with MSC conditioned medium (CM): **A. B.** was not observed when MSC CM was heat inactivated or frozen and thawed, **C.** was maintained in the fraction of MSC CM retained by a 30 kD filter, and lost in the fraction that flowed through, and **D. E.** was in part attributable to EV in MSC CM. n = 3; p < 0.05; Different letters above data points indicate significant differences between conditions, the same letter indicates no difference.

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*LC-MS/MS analysis of MSC CM and control CM reveals differences in protein composition.*

To better define the nature of the active factors responsible for DF migration, we decided to perform LC-MS/MS for identification of proteins in medium culture samples. We suspected proteins to be important migration-stimulating active factors based on our findings that the equine MSC secreted factors responsible for promoting DF migration (i) are temperature sensitive and (ii) are either > 30 kD, or in complexes that are > 30 kD. To this end, we submitted the > 30 kD fractions of both MSC CM and control (DF) CM for LC-MS/MS analysis, to identify and compare the protein compositions of each CM. A total of 103 proteins were confidently identified in MSC CM and 92 proteins were confidently identified in control CM. The complete lists can be found in Suppl Table 6S.1. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis by PANTHER revealed that the proteins detected in control CM were associated with 15 biological pathways, whereas the proteins detected in MSC CM were associated with 19 biological pathways (Figure 6.2A). Analysis using STRING indicated that proteins detected in control CM had 49 edges, representing protein-protein associations that are specific and contribute to a common function, while proteins detected in MSC CM had 157 edges (Figure 6.2B). Collectively, these

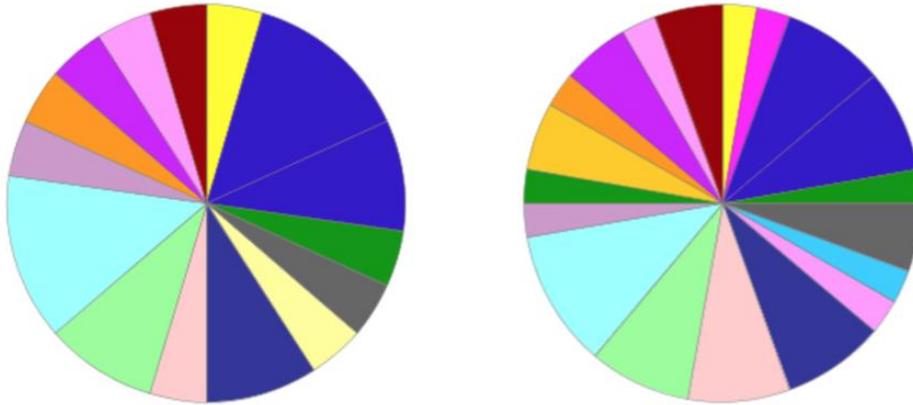
analyses show that MSC CM contains a greater number of proteins and that these proteins are involved in more diverse biological pathways compared to control CM.

Comparative analysis further showed that 43 proteins were uniquely detected in MSC CM (Table 6.2). We performed a literature search on these 43 proteins and prioritized proteins for further analyses based on (i) reported involvement in cell migration and (ii) whether they had previously been described as being secreted by MSC from other species. Two top candidate proteins were PAI-1 and tenascin-C, both of which were reported to contribute to cell motility [32, 33, 34, 35]. PAI-1 has been described as a secretory product of human MSC [36] and tenascin-C mRNA has been detected in MSC isolated from both humans and horses [37]. We first confirmed their presence in the CM from MSC isolated from 3 different horses by Western blot analysis and found higher levels of PAI-1 and tenascin-C in CM from all 3 MSC cultures compared to CM collected from an equal number of DF cultures (Figure 6.2C). In addition, we used RT-PCR to look for transcripts of receptors for these two proteins in DF. We confirmed that DF do express the PAI-1 receptors *ITGA5*, *ITGB1* and *uPAR*. as well as the tenascin-C receptors *Annexin II*, *EGFR*, *ITGAV* and *ITGB3* (Figure 6.3).

A.

Control CM

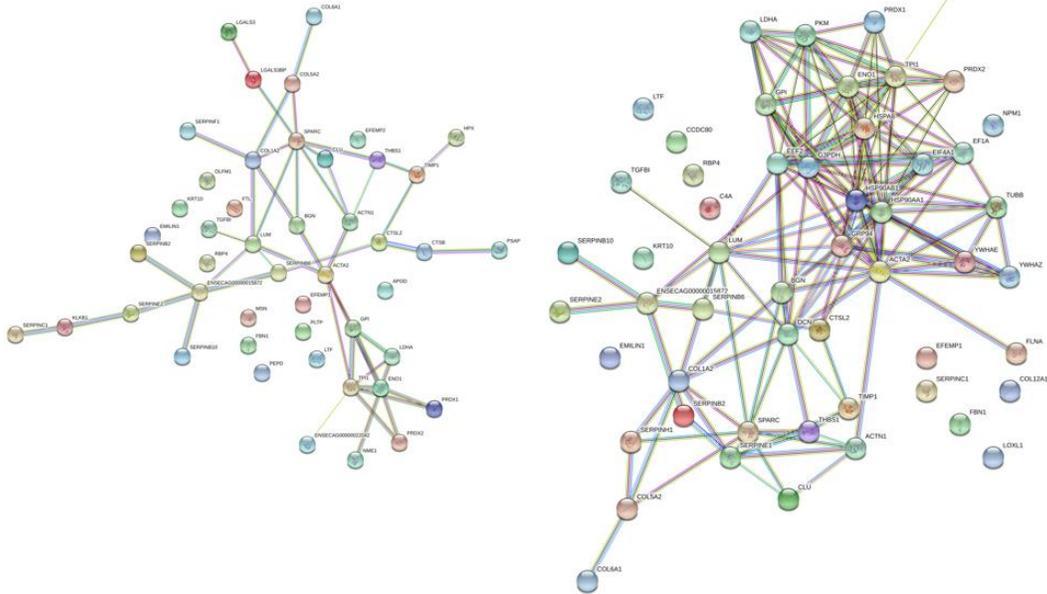
MSC CM



B.

Control CM

MSC CM



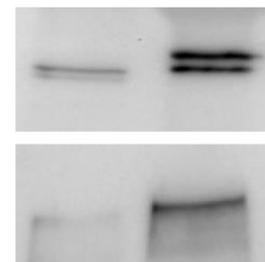
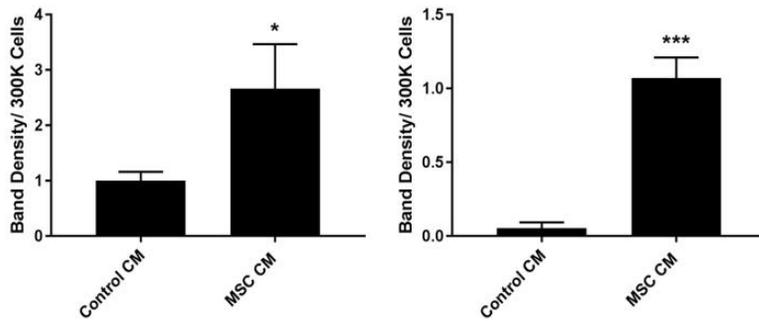
C. (i)

PAI-1

TNC

(ii)

Control CM    MSC CM



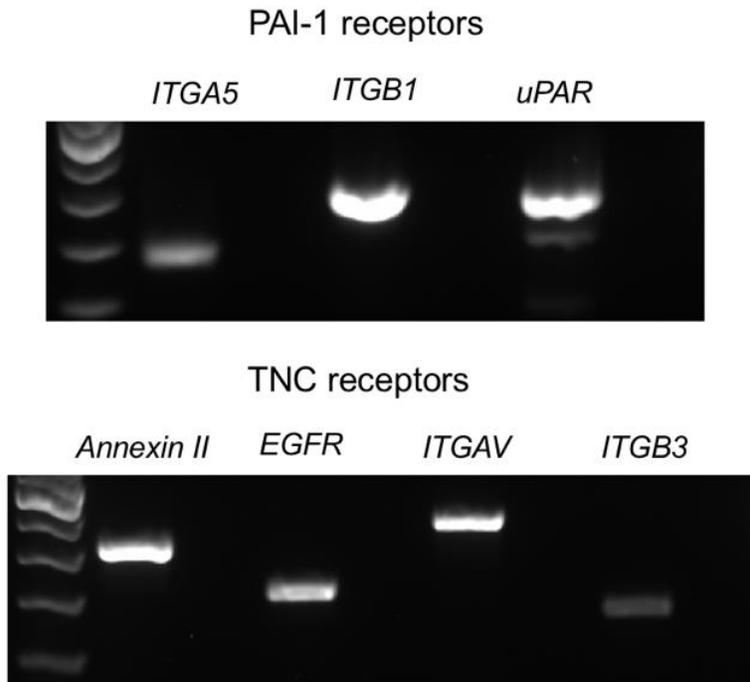
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**Figure 6.2. Mass Spectrophotometric analysis of mesenchymal stromal cell (MSC) conditioned medium (CM) and control CM reveals differences in protein composition. A.** PANTHER pathway analysis indicates that proteins detected in MSC CM are involved in more biological pathways than those detected in control CM. **B.** STRING analysis suggests that proteins detected in MSC CM exhibit more protein-protein associations that are specific and contribute to a common function, than proteins detected in control CM. **C.** Western blots were used to confirm that plasminogen activator inhibitor-1 (PAI-1) and tenascin-C (TNC) are detected in higher levels in MSC CM than in control CM. n = 1 for mass spectrophotometry; n = 3 for Western blots; \* p < 0.05; \*\*\* p < 0.001.

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**Table 6.2. Proteins detected in mesenchymal stromal cell (MSC), but not control, conditioned medium (CM) by mass spectrometry.**

<b>Accession</b>	<b>Description</b>
NP_001075250.1	elongation factor 1-alpha 1 [Equus caballus]
NP_001157345.1	endoplasmic precursor [Equus caballus]
NP_001157328.1	glyceraldehyde-3-phosphate dehydrogenase [Equus caballus]
NP_001075247.1	heat shock cognate 71 kDa protein [Equus caballus]
NP_001157427.1	heat shock protein HSP 90-alpha [Equus caballus]
NP_001075407.1	heat shock protein HSP 90-beta [Equus caballus]
NP_001333133.2	keratin 4 [Equus caballus]
NP_001157446.1	lactotransferrin precursor [Equus caballus]
XP_001504337.1	14-3-3 protein epsilon isoform X1 [Equus caballus]
XP_014583708.1	14-3-3 protein zeta/delta [Equus caballus]
XP_005601929.1	alpha-2-HS-glycoprotein [Equus caballus]
XP_005602671.1	angiotensinogen [Equus caballus]
XP_014586433.1	apolipoprotein B-100 [Equus caballus]
XP_005602045.1	coiled-coil domain-containing protein 80 [Equus caballus]
XP_001914777.1	collagen alpha-1(XII) chain isoform X1 [Equus caballus]
XP_003363907.1	complement C1q tumor necrosis factor-related protein 3 isoform X1 [Equus caballus]
XP_001492943.1	complement C4-A [Equus caballus]
XP_005606524.1	decorin isoform X1 [Equus caballus]
XP_005597812.1	eukaryotic initiation factor 4A-I isoform X1 [Equus caballus]
XP_014592455.1	fibulin-1 [Equus caballus]
XP_014594516.1	filamin-C [Equus caballus]
XP_014589771.1	histone H3.1-like [Equus caballus]
XP_003364388.2	integrin beta-1 isoform X1 [Equus caballus]
XP_014594379.1	laminin subunit beta-1 [Equus caballus]
XP_014595948.1	L-lactate dehydrogenase B chain isoform X1 [Equus caballus]
XP_001915132.1	elongation factor 2 [Equus caballus]
XP_001915328.2	filamin-A [Equus caballus]
XP_014595805.1	glypican-1 [Equus caballus]
XP_014595409.1	histone H2A type 2-C-like [Equus caballus]
XP_001917737.2	lysyl oxidase homolog 1 [Equus caballus]
<b>XP_001916657.1</b>	<b>tenascin [Equus caballus]</b>
XP_014596747.1	neural cell adhesion molecule 1 isoform X1 [Equus caballus]
XP_005615050.2	nucleophosmin isoform X1 [Equus caballus]
XP_001496993.3	peptidyl-prolyl cis-trans isomerase A [Equus caballus]
<b>XP_001492567.3</b>	<b>plasminogen activator inhibitor 1 [Equus caballus]</b>
XP_005603239.1	purine nucleoside phosphorylase-like [Equus caballus]
XP_005614707.2	rab GDP dissociation inhibitor alpha [Equus caballus]
XP_005612091.1	serpin H1 [Equus caballus]
XP_005602120.1	target of Nesh-SH3 isoform X19 [Equus caballus]
XP_005610680.2	tubulin alpha-1D chain [Equus caballus]
XP_001491228.1	tubulin beta chain [Equus caballus]
NP_001137266.1	pyruvate kinase PKM isoform M2 [Equus caballus]
NP_001230074.1	vimentin [Equus caballus]




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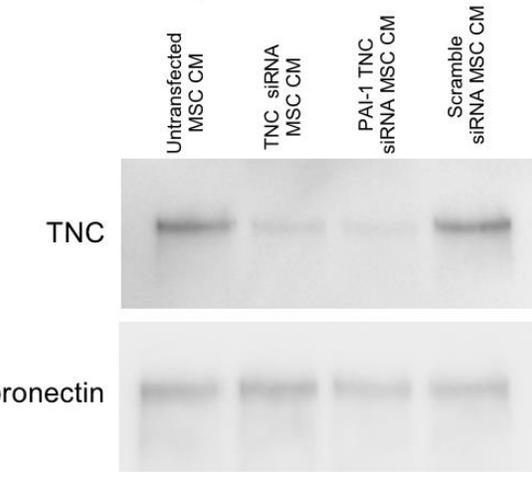
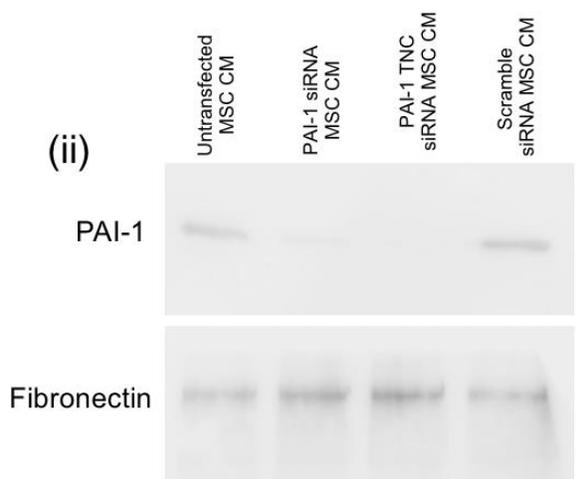
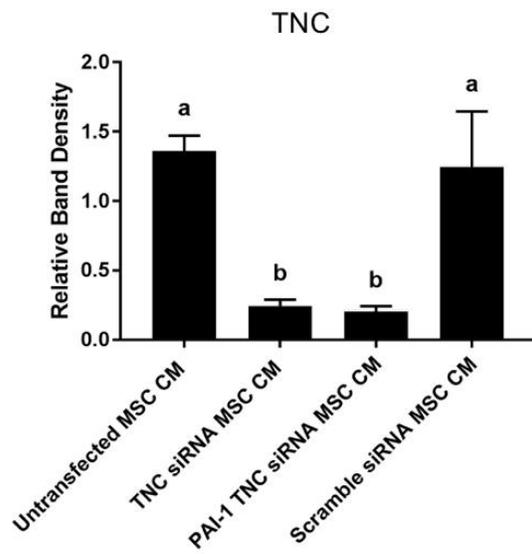
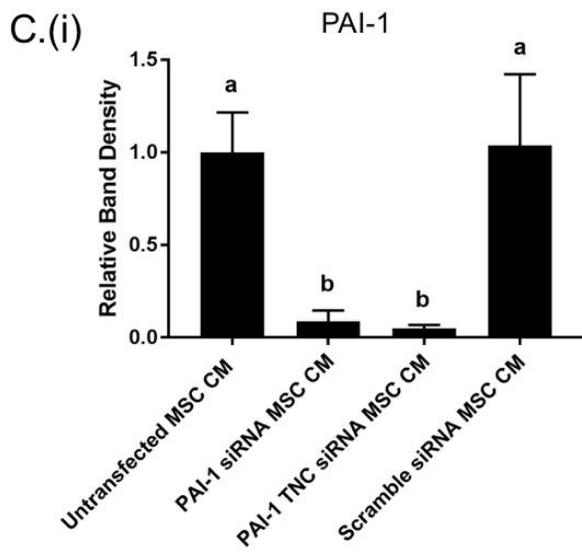
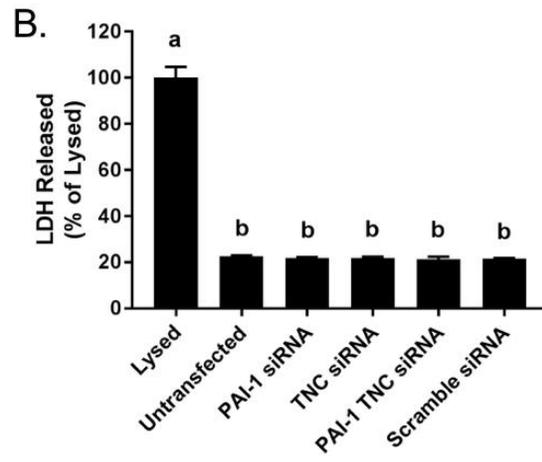
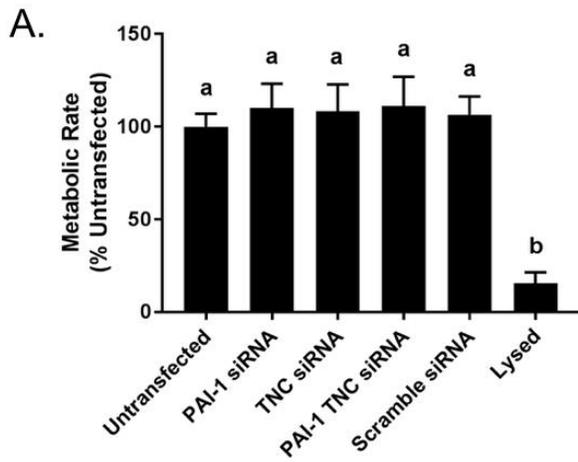
**Figure 6.3. Dermal fibroblasts (DF) express genes corresponding to plasminogen activator inhibitor-1 (PAI-1) and tenascin-C (TNC) receptors.** RT-PCR was used to amplify genes corresponding to PAI-1 and TNC surface receptors in DF. n = 3.

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*RNAi effectively knocks down PAI-1 and tenascin-C in MSC and MSC CM.*

We decided to use gene silencing to study the functional role of PAI-1 and tenascin-C in DF migration. To this end, we transfected equine MSC with siRNAs specific to PAI-1, tenascin-C or both. Significant knock-down of the genes *SERPINE1* and *TNC* was confirmed by qRT-PCR (Suppl Figure 6S.1A) and knock-down of the corresponding proteins (PAI-1 and tenascin-C, respectively) in MSC was confirmed by Western blot (Suppl Figure 6.S1B). Transfected MSC appeared morphologically healthy, and cell metabolism and viability were not negatively affected as determined by MTT metabolism (Figure 6.4A) and LDH release (Figure 6.4B) assays. Importantly, significantly less PAI-1, tenascin-C, or both, were detected in the CM collected from

these transfected MSC (Figure 6.4C). Scrambled control siRNA did not affect the expression of either protein in MSC or MSC CM (Suppl Figure 6S.1B, Figure 6.4C), despite the observation of a slight significant decrease in *SERPINE1* gene expression (Suppl Figure 6S.1A). Taken together, these results indicate that we successfully reduced the expression and secretion of these proteins by MSC.



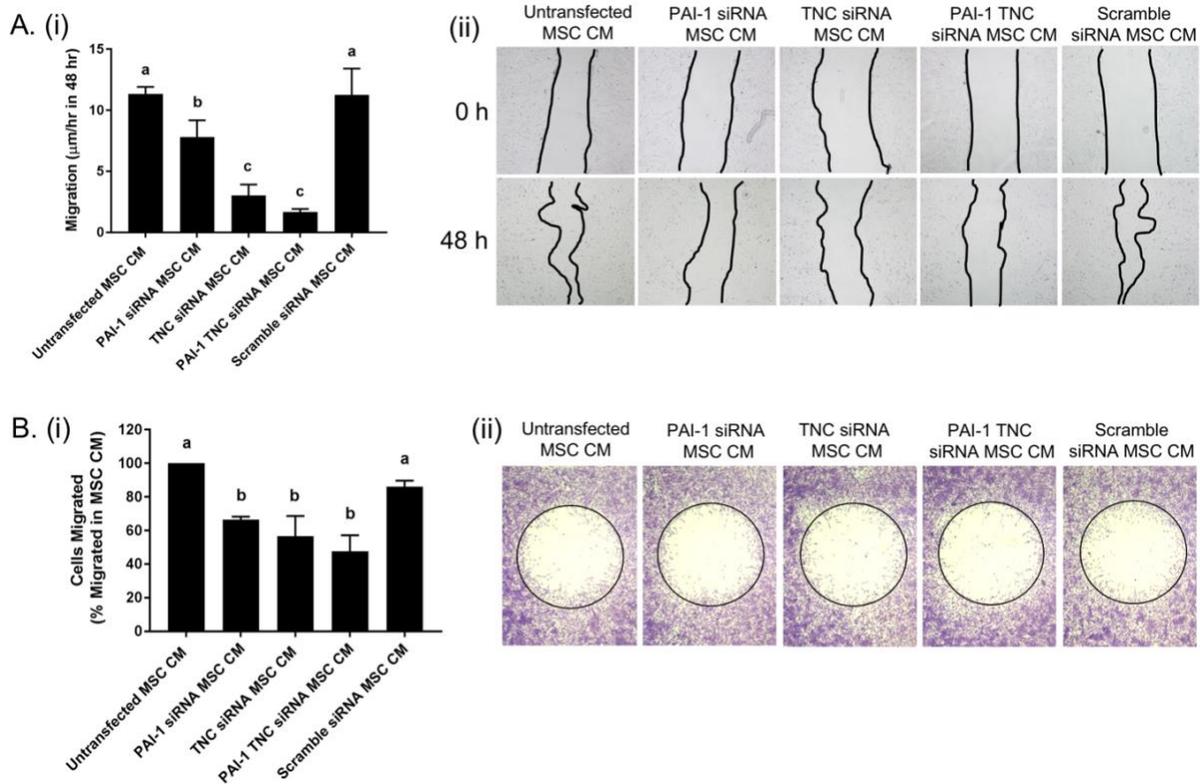
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**Figure 6.4. RNAi does not affect viability of equine mesenchymal stromal cells (MSC) and knocks down plasminogen activator inhibitor-1 (PAI-1) and tenascin-C (TNC) expression in equine MSC conditioned medium (CM).** **A. B.** Following transfection of equine MSC with siRNA cells remained metabolically stable as measured by the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), and did not undergo increased death, as measured by lactate dehydrogenase (LDH) release. **C.** Transfection of MSC with siRNA led to decreased PAI-1 and TNC in MSC CM.  $n = 3$ ;  $p < 0.05$ ; Different letters above data points indicate significant differences between conditions, the same letter indicates no difference.

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*DF migration is decreased using MSC CM with reduced PAI-1 and tenascin-C levels.*

CM from siRNA transfected equine MSC was used in *in vitro* scratch assays. Significantly reduced DF migration was observed using PAI-1-, tenascin-C-, or PAI-1/tenascin-C-deficient CM when compared to CM from untransfected MSC (Figure 6.5A). These results indicate that PAI-1 and tenascin-C are involved in the stimulation of MSC CM-induced fibroblast migration. To corroborate these results, we used the Oris cell migration assay and found that significantly fewer DF migrated into empty spaces in culture wells within 48 h when they were treated with MSC CM lacking PAI-1, tenascin-C, or both, as compared to CM from untransfected MSC (Figure 6.5B). CM from MSC that were transfected with non-specific scramble siRNA did not affect MSC CM-induced DF migration in either assay (Figure 6.5).

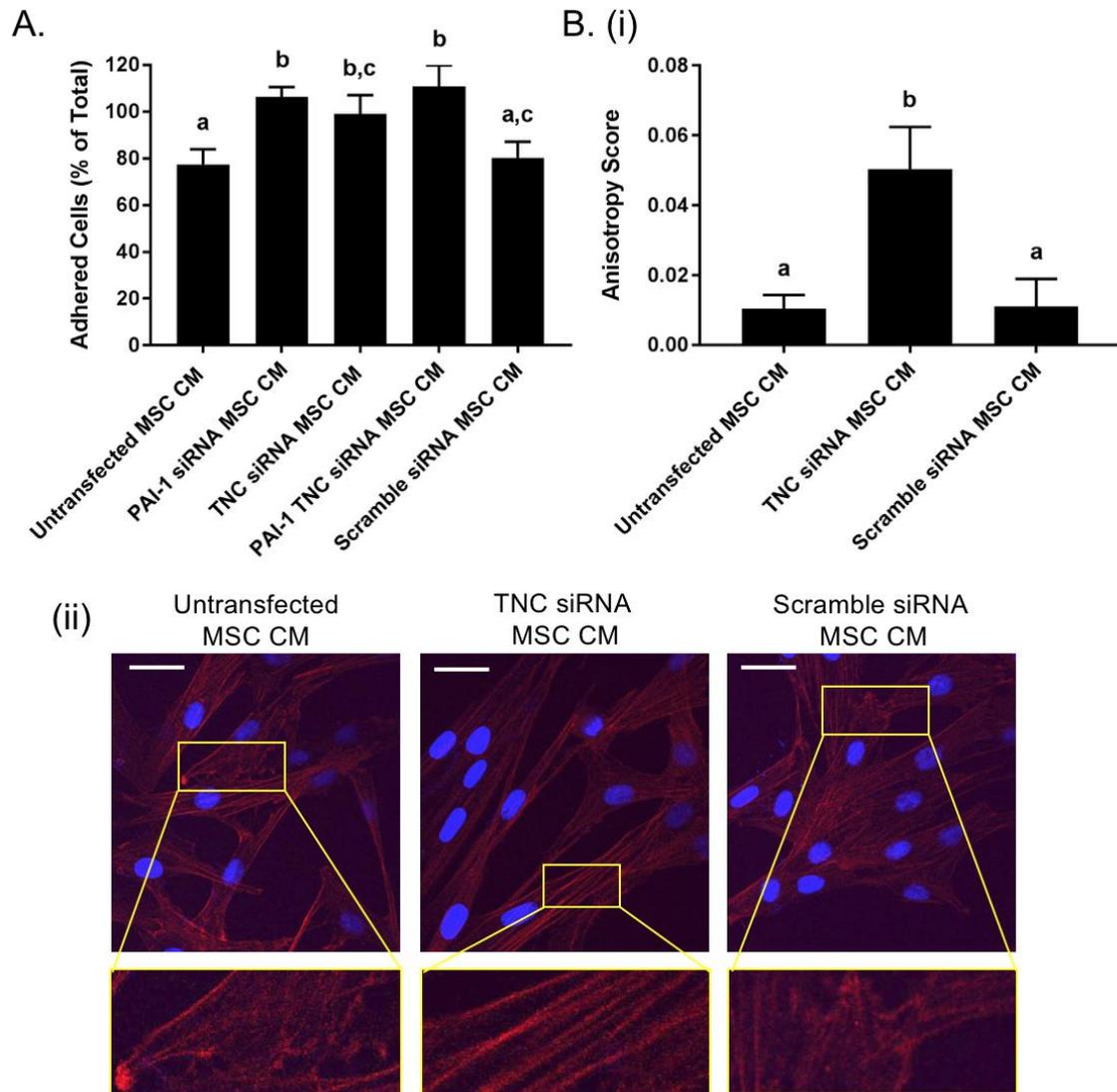


**Figure 6.5. Dermal fibroblast (DF) migration is decreased when plasminogen activator inhibitor-1 (PAI-1), tenascin-C (TNC), or both are reduced in mesenchymal stromal cell (MSC) conditioned medium (CM).** **A.** DF cultured in MSC CM depleted of PAI-1, TNC or both by transfection of siRNA, did not migrate as far in a given period of time as DF cultured in the CM from untransfected MSC as measured by scratch assay and **B.** Oris cell migration assay.  $n = 3$ ;  $p < 0.05$ ; Different letters above data points indicate significant differences between conditions, the same letter indicates no difference.

*DF adhesion is increased using MSC CM with reduced PAI-1 and tenascin-C levels.*

As decreased cell migration is often associated with increased adhesion of cells to substrate, we performed an experiment to determine whether PAI-1 and tenascin-C are involved in DF adhesion to culture wells. A significantly increased DF adhesion was observed using PIA-1-, tenascin-C, or PAI-1/tenascin-C-deficient CM when compared to CM from untransfected or scramble siRNA-transfected MSC (Figure 6.6A). Since a diffuse actin cytoskeleton is a cellular

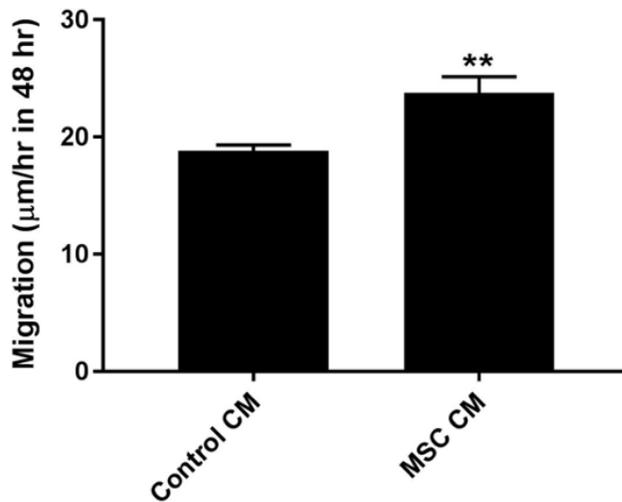
phenotype associated with decreased adhesion and increased migration in the presence of tenascin-C [34], we decided to evaluate whether tenascin-C affects the arrangement of actin filaments in DF by assessing anisotropy. Anisotropy describes the property of actin filaments being directionally dependent, and a low anisotropy score is indicative of a more motile phenotype of cells [30]. Anisotropy scores of DF cultured in tenascin C-deficient CM were significantly higher than those of DF cultured in either complete MSC CM or scramble siRNA-transfected MSC CM, and the actin filaments in these cells adopted a parallel orientation in contrast to the web like actin in DF cultured in either complete MSC CM or scramble siRNA-transfected MSC CM (Figure 6.6B). Together, these results suggest that tenascin-C contributes to the diffuse web like pattern of actin filaments in DF cultured in untransfected MSC CM, which is associated with decreased adhesion and increased migration.



**Figure 6.6. Dermal fibroblast (DF) adhesion to substrate is increased when plasminogen activator inhibitor-1 (PAI-1), tenascin-C (TNC), or both are reduced in mesenchymal stromal cell (MSC) conditioned medium (CM).** **A.** DF cultured in MSC CM depleted of PAI-1, TNC or both by transfection of siRNA, adhered more tightly to substrate than DF cultured in the CM from untransfected MSC as measured by adhesion assay. **B.** Actin filaments of DF cultured in MSC CM depleted of TNC took on a more parallel orientation than actin filaments of DF cultured in the CM from untransfected MSC, as determined by calculating anisotropy scores.  $n = 3$ ;  $p < 0.05$ ; Different letters above data points indicate significant differences between conditions, the same letter indicates no difference.

Wound closure is delayed in a murine full-thickness wound model using MSC CM with reduced PAI-1 and tenascin-C levels.

Based on the role of equine MSC-secreted PAI-1 and tenascin-C in DF migration *in vitro*, we hypothesized these proteins would also promote cutaneous wound healing *in vivo*. To this end, we used the well-established murine full-thickness wound model [38]. First, we confirmed that factors secreted by equine MSC can stimulate the migration of murine target cells by performing a scratch assay on NIH3T3 cells, a cell line derived from the mouse embryonic mesoderm, in the presence of MSC CM (Figure 6.7).



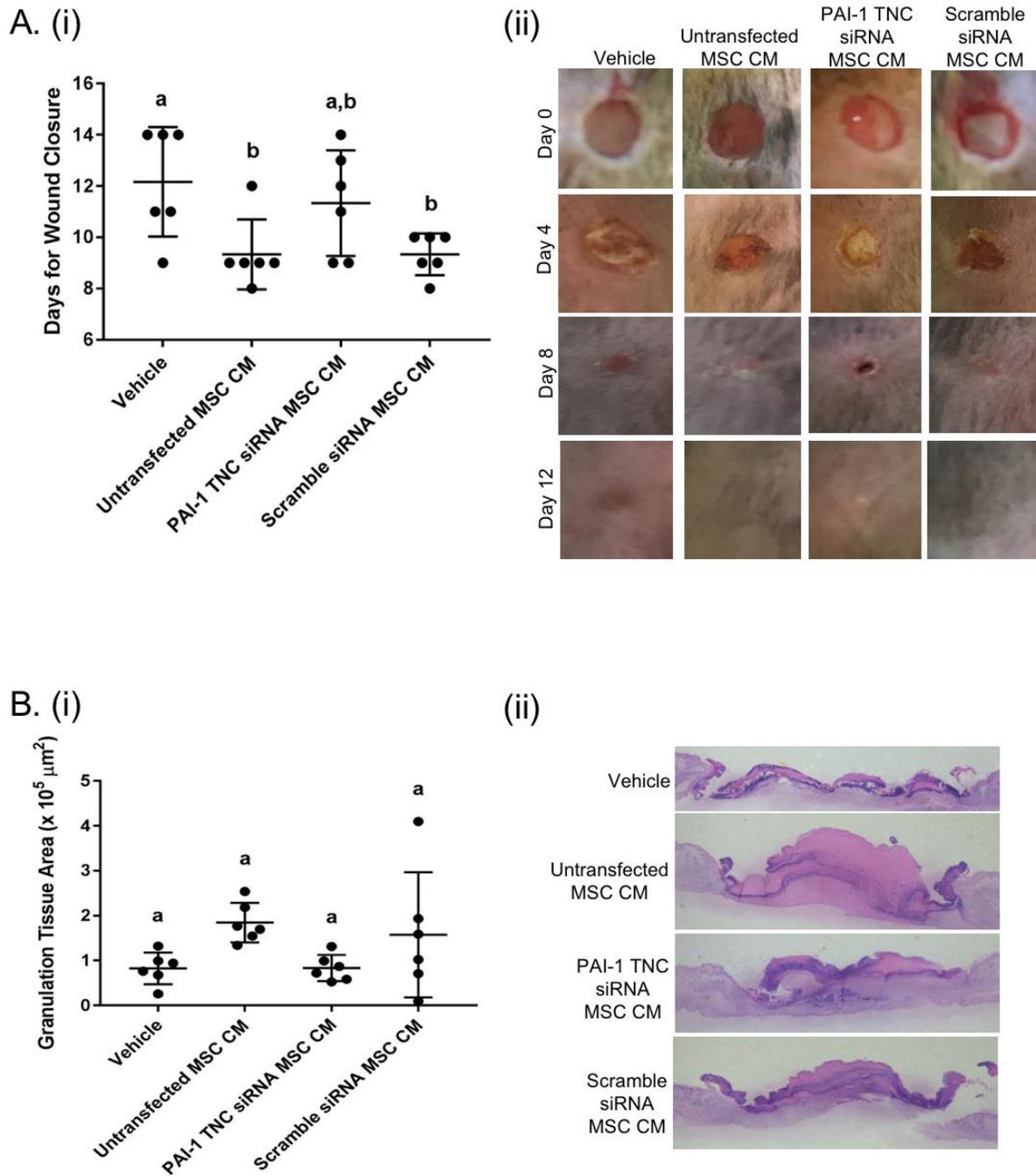
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**Figure 6.7. Factors in mesenchymal stromal cell (MSC) conditioned medium (CM) promote migration of mouse dermal fibroblasts (DF).** Mouse DF cultured in MSC CM migrated farther in a given period of time than mouse DF cultured in control (mouse DF) CM as measured by scratch assay.  $n = 3$ ;  $p < 0.01$ .

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Next, we inflicted two 4 mm full-thickness wounds on the backs of immunocompetent mice using a biopsy punch. Wounds were then treated daily with either a vehicle control (DMEM), complete CM from untransfected MSC, PAI-1/tenascin-C-deficient MSC CM, and CM from

scramble siRNA-transfected MSC. Wounds from one set of mice were photographed and measured daily, until complete wound closure. The other set of mice were euthanized at day 4 post-wounding for histological wound assessment. We found that wounds treated with CM from untransfected MSC closed more quickly compared to wounds treated with the vehicle control, indicating that equine MSC CM promotes wound healing in this model. Interestingly, wounds treated with PAI-1/tenascin-C-deficient MSC CM had a slower wound closure rate compared to wounds treated with CM from untransfected MSC (Figure 6.8A), although this difference did not reach statistical significance. These data suggest that these two proteins are, at least in part, responsible for wound closure *in vivo*. As expected, the closure rate of wounds treated with CM from untransfected MSC was not statistically different from that of treatment with scramble siRNA-transfected MSC CM (Figure 6.8A). Histological analyses of distance between epithelial tongues and number of new capillaries in the wound bed 4 days post-wounding did not reveal any statistically significant differences between treatments (data not shown). A larger granulation tissue area, which is associated with early stages of successful wound closure [1], was measured in the wound beds of wounds treated with untransfected MSC CM as compared to PAI-1/tenascin-C-deficient CM, but this difference did not reach statistical significance (Figure 6.8B).



**Figure 6.8. Wound closure is delayed in a murine full-thickness wound model when plasminogen activator inhibitor-1 (PAI-1) and tenascin-C (TNC) are reduced in mesenchymal stromal cell (MSC) conditioned medium (CM)** **A.** As compared to the vehicle control, wounds treated with MSC CM closed more quickly, while wounds treated with MSC CM depleted of PAI-1 and TNC did not. **B.** Wounds treated with MSC CM appeared to have a

greater amount of granulation tissue than wounds treated with the vehicle control, while wounds treated with MSC CM depleted of PAI-1 and TNC appeared more like those treated with the vehicle control. n = 6 wounds; p < 0.05; Different letters above data points indicate significant differences between conditions, the same letter indicates no difference.

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## 6.5. Discussion

Our research focuses on the potential of equine peripheral blood-derived MSC to be used as a therapy for cutaneous wounds. We study MSC from peripheral blood, as this method of collection is non-invasive as compared to collecting MSC from bone marrow or adipose tissue, two other common sources of equine MSC, and the success rate of MSC isolation from equine peripheral blood is high [47,48].

The present study demonstrates that equine peripheral blood-derived MSC CM (i) stimulates fibroblast migration *in vitro* and (ii) promotes wound healing *in vivo*. Importantly, we identified at least two proteins secreted by these cells, namely PAI-1 and tenascin-C, to be involved in these processes.

We decided to examine the effects of these two proteins in our experimental systems based on their unique expression in MSC CM and the fact that they both have well-established roles in cell migration. PAI-1 is an acute phase glycoprotein, expressed highly in response to elevated cytokines, such as transforming growth factor beta (TGF- $\beta$ ) and epidermal growth factor released at the time of tissue injury. PAI-1 contributes to wound closure by promoting cell migration and granulation tissue formation [39, 32]. Although PAI-1 expression is important during normal wound healing, prolonged or pathologic expression of PAI-1 is associated with fibrotic diseases and excessive collagen deposition. Fibroblasts isolated from keloids as well as those isolated from the skin of scleroderma patients exhibit high levels of PAI-1 as compared to normal skin fibroblasts

[40]. PAI-1 expression must be modulated temporally for successful wound healing, which should be considered when testing the efficacy of MSC-derived PAI-1 as a therapy for cutaneous wounds. The extracellular matrix molecule tenascin-C is highly expressed during normal cutaneous wound healing [35]. It was initially described as a modulator of adhesion, but since has been identified as a molecule involved in cell migration, proliferation, apoptosis, and transdifferentiation. Expression of active tenascin-C is tightly regulated at both the gene and protein levels [41]. This is important as the overexpression of tenascin-C is associated with cancer, often at the invasive front, and has been linked to metastasis [42]. As with PAI-1, the timing of the administration of therapeutic tenascin-C should be optimized to ensure successful wound healing, without undesirable side effects. In addition to PAI-1 and tenascin-C, the secreted molecules lactotransferrin, complement C1q, complement C4, decorin, fibulin, lactate dehydrogenase, lysyl oxidase homolog 1, target of Nesh-SH3, and fibulin 1, were also uniquely detected in the CM from equine MSC. However, with the exception of fibulin 1, which has been shown to reduce migration velocity *in vitro* of breast cancer cells by suppressing cellular interactions with fibronectin [43], these molecules have not been previously described as contributing to cell migration.

In the present study, we demonstrated that EV from MSC do, at least in part, deliver factors that promote DF migration. Although we did not specifically perform LC-MS/MS on MSC-secreted EV, LC-MS/MS of the complete MSC CM revealed proteins that have been identified in EV secreted by various cell types, such as filamin A, the tubulins, and integrin beta-1 [44, 45, 46]. Since these proteins are associated with cell adhesion and migration through interactions with actin, it will be interesting to study the roles of these EV-associated proteins in cell migration and wound healing in more detail. To our knowledge, no data exist to suggest that these molecules can be transported from one cell to integrate and act as cytoskeletal components of a recipient cell.

We have previously shown that factors secreted by equine MSC promote the migration of both an equine DF cell line [18] as well as primary dermal fibroblasts isolated from horses [28]. In the present study, we used equine and mouse cell lines, as well as a mouse model as screening tools to evaluate the efficacy of peripheral blood-derived MSC on wound healing and to study the contribution of PAI-1 and tenascin-C to the wound healing process. The murine full-thickness wound model is a well-established model used to study cell migration, communication between different cell types found in the skin and the roles of specific genes in the healing process [38]. For example, this model has been used in studies to show that (i) ultraviolet radiation inhibits keratinocyte motility [49], (ii) interleukin-33 improves wound closure through increased M2 macrophage polarization [50], and (iii) a hypoxia response element in the vascular endothelial growth factor A promoter is required for optimal granulation tissue formation [51]. Using this mouse model, our present study showed that PAI-1 and tenascin-C secreted by equine MSC decrease wound closure time *in vivo*. Based on these encouraging results, we are now planning to initiate similar experiments in the horse. The horse is an excellent model for human wound healing studies based on the fact that (i) horse skin wounds heal by epithelialization, similar to human skin wounds, and (ii) horses naturally develop chronic wounds that resemble human conditions [15]. Importantly, this large animal model allows for the design of studies with control and treated wounds all in the same animal [52]. In addition, multiple biopsies flanking a wound site can be collected throughout an experiment, allowing for a temporal analysis of wound parameters such as histology, mRNA expression or protein profiles [53]. This type of analysis is not always feasible in smaller animal models such as rodent models. Consequently, the horse model has been used to evaluate various therapies for improving wound healing [52, 54] including a recent study in which

the potential of bone marrow-derived MSC for skin wounds on the lower legs of horses was evaluated [55].

Identifying which factors secreted by MSC act on target cell types found in the skin is important as it may allow to refine the CM generated by MSC in order to optimize therapeutic benefits. First, it allows for the pre-screening of MSC cultures with a high level of bioactive factors of interest. Indeed, we previously found a donor-specific effect of MSC-derived CM on skin target cells [16] and confirmed in the present study that the expression of PAI-1 and tenascin-C mRNA was significantly different in MSC cultures isolated from different horses (Suppl Figure 6S.2A). The observation that MSC from different individuals exhibit different secretion patterns has also been described for human MSC [56]. Second, it allows the evaluation of pre-treatment modalities of MSC that would result in increased expression of bioactive factors of interest [57]. For example, we found that pre-treatment of MSC with TGF $\beta$ -1 significantly increased the level of tenascin-C in CM, as detected by Western blot analyses (Suppl Figure 6S.2B). Although the levels of PAI-1 in MSC CM did not increase using the same treatment conditions (data not shown), it may be possible to induce higher expression of this protein by pre-treating with a different dose of TGF $\beta$ -1, pre-treating for a longer period of time, or pre-treating with a different cytokine or growth factor. Lastly, and although not directly related to MSC therapy *per se*, knowledge on the bioactive factors present in MSC CM could inform the design of recombinant protein therapies, which are being intensively explored for wound healing. [58, 59, 60].

## **6.6. Conclusions**

We previously reported that equine MSC CM stimulates DF migration, but (i) the factors responsible for this effect were not defined and (ii) it was not known whether these factors contribute to wound healing *in vivo*. The present study is the first to show that PAI-1 and tenascin-

C secreted by equine peripheral blood-derived MSC stimulate DF migration, and that these proteins are involved in the promotion of wound healing *in vivo*. Defining the factors secreted by MSC that contribute to cutaneous wound healing may increase the value of MSC as a therapy for skin wounds in both horses and humans.

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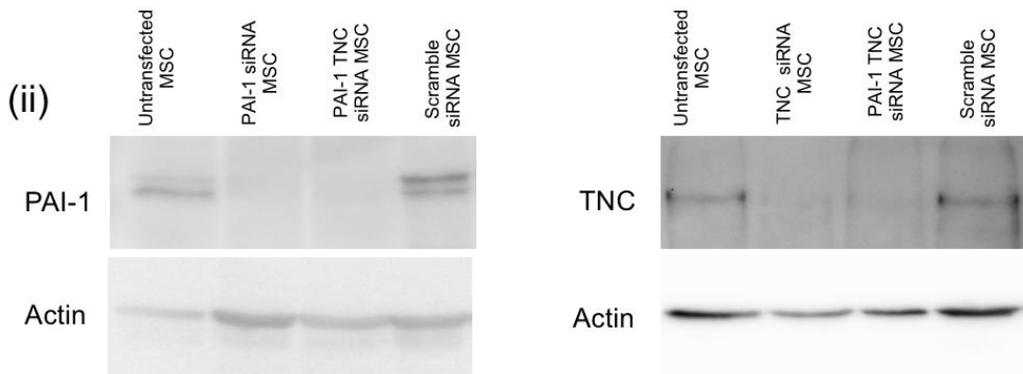
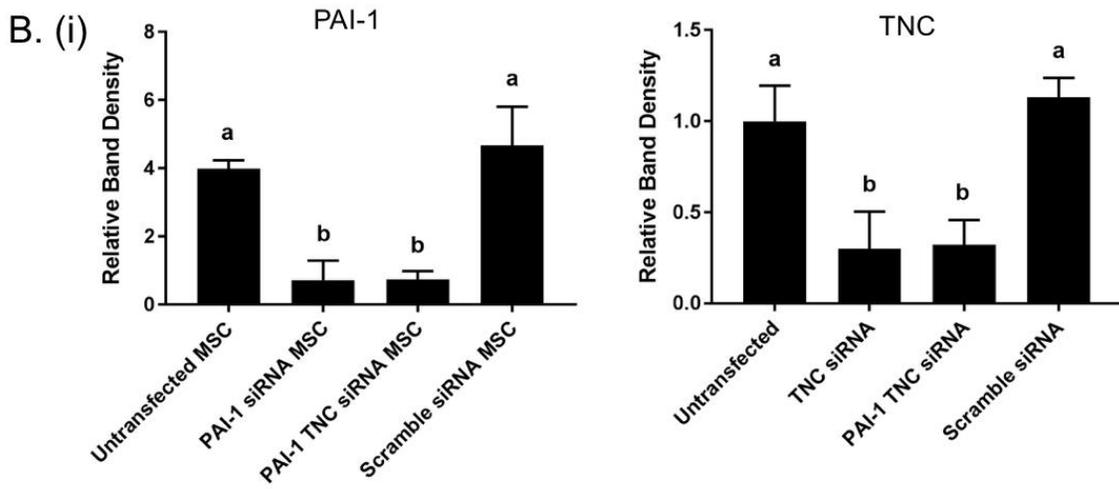
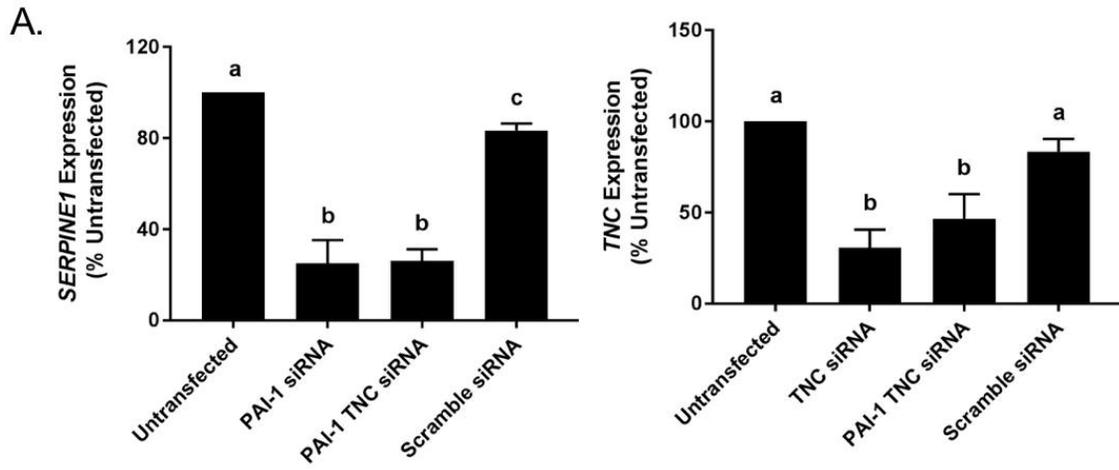
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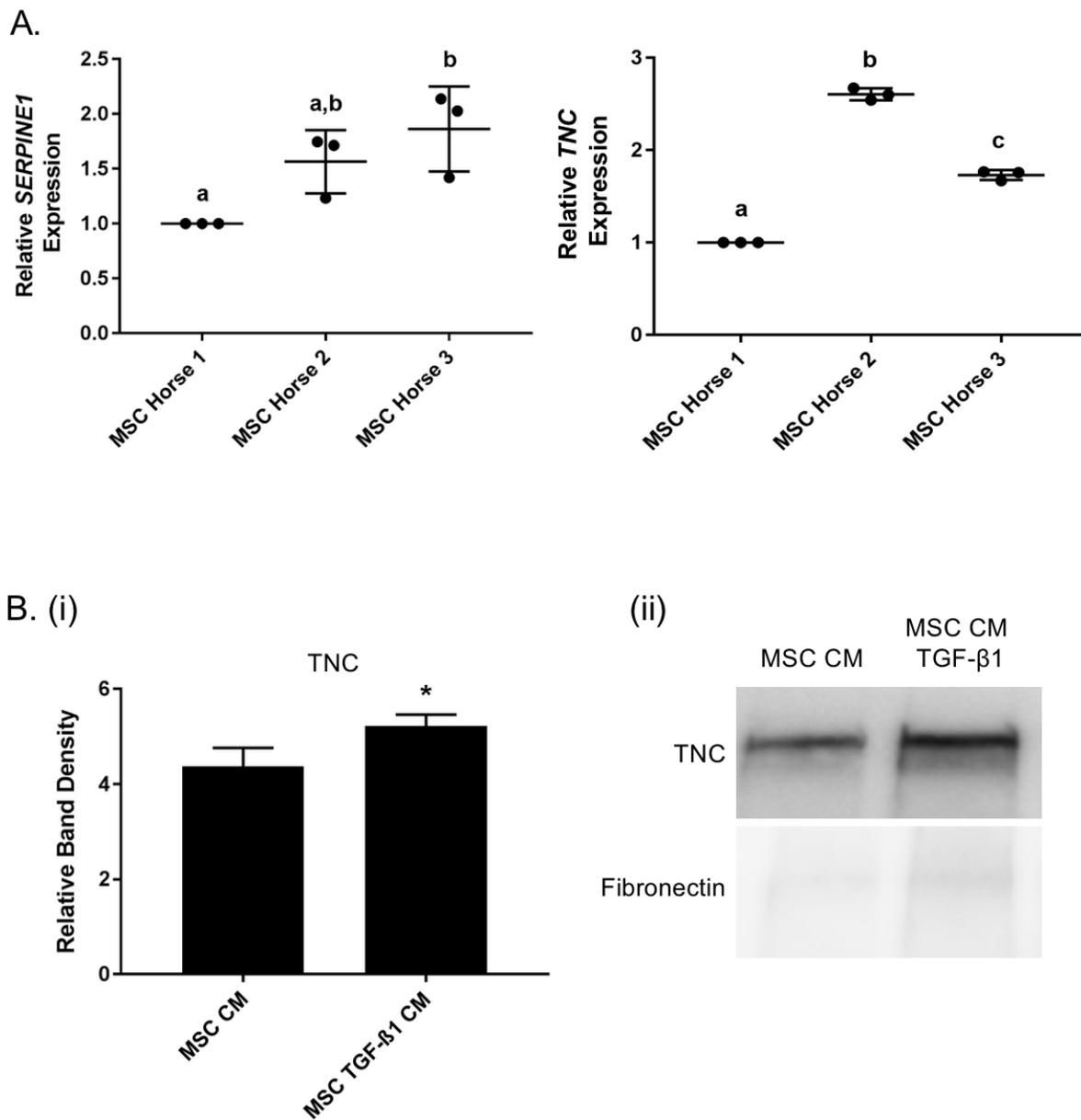
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**Figure 6.S1. RNAi knocks down plasminogen activator inhibitor-1 (PAI-1) and tenascin-C (TNC) expression in equine mesenchymal stromal cells (MSC).** **A.** Following transfection of equine MSC with siRNA; PAI-1 (*SERPINE1*) and TNC (*TNC*) gene expression was reduced as detected by qRT-PCR and **B.** PAI-1 and TNC protein expression was reduced as determined by Western blot.  $n = 3$ ;  $p < 0.05$ ; Different letters above data points indicate significant differences between conditions, the same letter indicates no difference.



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**Figure 6.S2. Variable expression profiles of plasminogen activator inhibitor-1 (*SERPINE1*) and tenascin-C (*TNC*) transcripts, as well as tenascin-C (*TNC*) protein levels in equine mesenchymal stromal cells (MSC).** **A.** MSC cultures from different horses exhibited variable expression of *SERPINE1* and *TNC* as detected by qRT-PCR. **B.** Treatment of MSC with TGF $\beta$ -1 led to increased levels of TNC in MSC CM as detected by Western blot. n = 3; p < 0.05; Different letters above data points indicate significant differences between conditions, the same letter indicates no difference.

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**Table 6S.1. Proteins detected in control (DF) and mesenchymal stromal cell (MSC) conditioned medium (CM).**

**Control (DF) CM**

<b>Accession</b>	<b>Description</b>
NP_001075249.1	lumican precursor [Equus caballus]
NP_001075308.1	biglycan precursor [Equus caballus]
NP_001075413.1	clusterin precursor [Equus caballus]
NP_001075415.2	serotransferrin precursor [Equus caballus]
NP_001075420.1	retinol-binding protein 4 precursor [Equus caballus]
NP_001075422.1	gelsolin [Equus caballus]
NP_001075984.1	metalloproteinase inhibitor 1 precursor [Equus caballus]
NP_001108012.1	ferritin light chain [Equus caballus]
NP_001137424.1	serpin B10 [Equus caballus]
NP_001137425.1	SPARC precursor [Equus caballus]
NP_001137426.1	pigment epithelium-derived factor precursor [Equus caballus]
NP_001138352.1	L-lactate dehydrogenase A chain [Equus caballus]
NP_001156589.2	thrombospondin-2 precursor [Equus caballus]
NP_001157446.1	lactotransferrin precursor [Equus caballus]
NP_001191672.1	nucleoside diphosphate kinase B [Equus caballus]
NP_001254532.1	alpha-enolase [Equus caballus]
NP_001295881.1	thrombospondin-1 precursor [Equus caballus]
NP_001296443.1	selenium-binding protein 1 [Equus caballus]
XP_001488401.2	collagen alpha-1(VI) chain [Equus caballus]
XP_001488418.3	serpin B6 isoform X1 [Equus caballus]
XP_001489154.4	fibronectin [Equus caballus]
XP_001489596.2	anionic trypsin [Equus caballus]
XP_001490291.2	xaa-Pro dipeptidase [Equus caballus]
XP_001490356.3	plasma kallikrein [Equus caballus]
XP_001490657.2	glucose-6-phosphate isomerase [Equus caballus]

XP\_001491000.1 galectin-3-binding protein [Equus caballus]  
 XP\_001492201.5 N-acetylglucosamine-6-sulfatase [Equus caballus]  
 XP\_001492989.1 collagen alpha-2(I) chain [Equus caballus]  
 XP\_001494409.1 cathepsin L2 [Equus caballus]  
 XP\_001494662.3 EGF-containing fibulin-like extracellular matrix protein 2 [Equus caballus]  
 XP\_001497522.2 triosephosphate isomerase [Equus caballus]  
 XP\_001498242.1 cathepsin B [Equus caballus]  
 XP\_001499854.1 keratin, type I cytoskeletal 10-like isoform X1 [Equus caballus]  
 XP\_001500887.1 apolipoprotein D isoform X1 [Equus caballus]  
 XP\_001501867.1 collagen alpha-2(V) chain [Equus caballus]  
 XP\_001502309.1 fibrillin-1 [Equus caballus]  
 XP\_001502604.2 EMILIN-1 [Equus caballus]  
 XP\_001503085.1 actin, aortic smooth muscle [Equus caballus]  
 XP\_001503419.1 phospholipid transfer protein [Equus caballus]  
 XP\_001503814.1 prosaposin isoform X1 [Equus caballus]  
 XP\_001503927.3 thrombospondin-4 [Equus caballus]  
 XP\_001504911.1 moesin [Equus caballus]  
 XP\_001505187.1 purine nucleoside phosphorylase [Equus caballus]  
 XP\_001916726.3 prolow-density lipoprotein receptor-related protein 1 [Equus caballus]  
 XP\_001917632.1 EGF-containing fibulin-like extracellular matrix protein 1 [Equus caballus]  
 XP\_001918096.4 sphingomyelin phosphodiesterase [Equus caballus]  
 XP\_003364083.1 alpha-actinin-1 isoform X1 [Equus caballus]  
 XP\_003364129.1 noelin isoform X1 [Equus caballus]  
 XP\_003365612.1 plasminogen activator inhibitor 2 [Equus caballus]  
 XP\_005598069.1 CD44 antigen isoform X1 [Equus caballus]  
 XP\_005598858.1 fructose-bisphosphate aldolase A [Equus caballus]  
 XP\_005599767.1 transforming growth factor-beta-induced protein ig-h3, partial [Equus caballus]  
 XP\_005600606.1 transketolase [Equus caballus]  
 XP\_005602923.1 pro-cathepsin H [Equus caballus]  
 XP\_005602958.1 chondroitin sulfate proteoglycan 4 [Equus caballus]  
 XP\_005605252.1 galectin-3 [Equus caballus]  
 XP\_005605710.1 collagen alpha-1(XV) chain [Equus caballus]  
 XP\_005607055.1 inter-alpha-trypsin inhibitor heavy chain H5 [Equus caballus]  
 XP\_005607147.1 peroxiredoxin-1 [Equus caballus]  
 XP\_005607578.1 procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 isoform X1 [Equus caballus]  
 XP\_005608557.1 N-acetylgalactosamine-6-sulfatase [Equus caballus]  
 XP\_005609063.1 WD repeat-containing protein 1 [Equus caballus]  
 XP\_005609834.2 laminin subunit gamma-1 [Equus caballus]  
 XP\_005610703.1 glia-derived nexin [Equus caballus]  
 XP\_005611909.1 peroxiredoxin-2 [Equus caballus]

XP_005612174.1	hemopexin [Equus caballus]
XP_014585370.1	procollagen C-endopeptidase enhancer 1 isoform X1 [Equus caballus]
XP_014586066.1	platelet-derived growth factor receptor beta [Equus caballus]
XP_014586837.1	fibulin-2 [Equus caballus]
XP_014587011.1	serotransferrin [Equus caballus]
XP_014587164.1	alpha-actinin-4, partial [Equus caballus]
XP_014588510.1	vinculin isoform X1 [Equus caballus]
XP_014590559.1	CD109 antigen [Equus caballus]
XP_014590916.1	pantetheinase [Equus caballus]
XP_014591200.1	latent-transforming growth factor beta-binding protein 2 [Equus caballus]
XP_014591730.1	collagen alpha-1(V) chain [Equus caballus]
XP_014591905.1	protein disulfide-isomerase [Equus caballus]
XP_014591939.1	neuronal pentraxin-1 [Equus caballus]
XP_014591965.1	collagen alpha-2(VI) chain [Equus caballus]
XP_014592190.1	C-type mannose receptor 2 [Equus caballus]
XP_014592250.1	alpha-N-acetylglucosaminidase [Equus caballus]
XP_014592408.1	collagen alpha-1(I) chain [Equus caballus]
XP_014592730.1	basement membrane-specific heparan sulfate proteoglycan core protein, partial [Equus caballus]
XP_014593897.1	72 kDa type IV collagenase [Equus caballus]
XP_014594947.1	antithrombin-III [Equus caballus]
XP_014595238.1	extracellular matrix protein 1 [Equus caballus]
XP_014595871.1	collagen alpha-3(VI) chain [Equus caballus]
XP_014596179.1	alpha-2-macroglobulin-like [Equus caballus]
XP_014596564.1	complement C3-like [Equus caballus]
XP_014596899.1	lysosomal alpha-mannosidase [Equus caballus]
XP_014597084.1	tripeptidyl-peptidase 1 [Equus caballus]
XP_014597534.1	transcobalamin-2 isoform X1 [Equus caballus]

## MSC CM

Accession	Description
NP_001075247.1	heat shock cognate 71 kDa protein [Equus caballus]
NP_001075249.1	lumican precursor [Equus caballus]
NP_001075250.1	elongation factor 1-alpha 1 [Equus caballus]
NP_001075308.1	biglycan precursor [Equus caballus]
NP_001075407.1	heat shock protein HSP 90-beta [Equus caballus]
NP_001075413.1	clusterin precursor [Equus caballus]
NP_001075420.1	retinol-binding protein 4 precursor [Equus caballus]
NP_001075984.1	metalloproteinase inhibitor 1 precursor [Equus caballus]
NP_001137266.1	pyruvate kinase PKM isoform M2 [Equus caballus]
NP_001137424.1	serpin B10 [Equus caballus]

NP\_001137425.1 SPARC precursor [Equus caballus]  
 NP\_001138352.1 L-lactate dehydrogenase A chain [Equus caballus]  
 NP\_001156589.2 thrombospondin-2 precursor [Equus caballus]  
 NP\_001157328.1 glyceraldehyde-3-phosphate dehydrogenase [Equus caballus]  
 NP\_001157345.1 endoplasmin precursor [Equus caballus]  
 NP\_001157427.1 heat shock protein HSP 90-alpha [Equus caballus]  
 NP\_001157446.1 lactotransferrin precursor [Equus caballus]  
 NP\_001230074.1 vimentin [Equus caballus]  
 NP\_001254532.1 alpha-enolase [Equus caballus]  
 NP\_001295881.1 thrombospondin-1 precursor [Equus caballus]  
 NP\_001333133.2 keratin 4 [Equus caballus]  
 XP\_001488401.2 collagen alpha-1(VI) chain [Equus caballus]  
 XP\_001488418.3 serpin B6 isoform X1 [Equus caballus]  
 XP\_001489154.4 fibronectin [Equus caballus]  
 XP\_001489596.2 anionic trypsin [Equus caballus]  
 XP\_001490657.2 glucose-6-phosphate isomerase [Equus caballus]  
 XP\_001491228.1 tubulin beta chain [Equus caballus]  
 XP\_001492567.3 plasminogen activator inhibitor 1 [Equus caballus]  
 XP\_001492943.1 complement C4-A [Equus caballus]  
 XP\_001492989.1 collagen alpha-2(I) chain [Equus caballus]  
 XP\_001494409.1 cathepsin L2 [Equus caballus]  
 XP\_001496993.3 peptidyl-prolyl cis-trans isomerase A [Equus caballus]  
 XP\_001497522.2 triosephosphate isomerase [Equus caballus]  
 XP\_001499854.1 keratin, type I cytoskeletal 10-like isoform X1 [Equus caballus]  
 XP\_001501867.1 collagen alpha-2(V) chain [Equus caballus]  
 XP\_001502309.1 fibrillin-1 [Equus caballus]  
 XP\_001502604.2 EMILIN-1 [Equus caballus]  
 XP\_001503085.1 actin, aortic smooth muscle [Equus caballus]  
 XP\_001503927.3 thrombospondin-4 [Equus caballus]  
 XP\_001504337.1 14-3-3 protein epsilon isoform X1 [Equus caballus]  
 XP\_001505187.1 purine nucleoside phosphorylase [Equus caballus]  
 XP\_001914777.1 collagen alpha-1(XII) chain isoform X1 [Equus caballus]  
 XP\_001915132.1 elongation factor 2 [Equus caballus]  
 XP\_001915328.2 filamin-A [Equus caballus]  
 XP\_001916657.1 tenascin [Equus caballus]  
 XP\_001916726.3 prolow-density lipoprotein receptor-related protein 1 [Equus caballus]  
 XP\_001917632.1 EGF-containing fibulin-like extracellular matrix protein 1 [Equus caballus]  
 XP\_001917737.2 lysyl oxidase homolog 1 [Equus caballus]  
 XP\_003363907.1 complement C1q tumor necrosis factor-related protein 3 isoform X1 [Equus caballus]  
 XP\_003364083.1 alpha-actinin-1 isoform X1 [Equus caballus]

XP\_003364388.2 integrin beta-1 isoform X1 [Equus caballus]  
 XP\_003365612.1 plasminogen activator inhibitor 2 [Equus caballus]  
 XP\_005597812.1 eukaryotic initiation factor 4A-I isoform X1 [Equus caballus]  
 XP\_005598069.1 CD44 antigen isoform X1 [Equus caballus]  
 XP\_005598858.1 fructose-bisphosphate aldolase A [Equus caballus]  
 XP\_005599767.1 transforming growth factor-beta-induced protein ig-h3, partial [Equus caballus]  
 XP\_005600606.1 transketolase [Equus caballus]  
 XP\_005601929.1 alpha-2-HS-glycoprotein [Equus caballus]  
 XP\_005602045.1 coiled-coil domain-containing protein 80 [Equus caballus]  
 XP\_005602120.1 target of Nesh-SH3 isoform X19 [Equus caballus]  
 XP\_005602671.1 angiotensinogen [Equus caballus]  
 XP\_005603239.1 purine nucleoside phosphorylase-like [Equus caballus]  
 XP\_005606524.1 decorin isoform X1 [Equus caballus]  
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 XP\_005611909.1 peroxiredoxin-2 [Equus caballus]  
 XP\_005612091.1 serpin H1 [Equus caballus]  
 XP\_005614707.2 rab GDP dissociation inhibitor alpha [Equus caballus]  
 XP\_005615050.2 nucleophosmin isoform X1 [Equus caballus]  
 XP\_014583708.1 14-3-3 protein zeta/delta [Equus caballus]  
 XP\_014585370.1 procollagen C-endopeptidase enhancer 1 isoform X1 [Equus caballus]  
 XP\_014586433.1 apolipoprotein B-100 [Equus caballus]  
 XP\_014586837.1 fibulin-2 [Equus caballus]  
 XP\_014587011.1 serotransferrin [Equus caballus]  
 XP\_014587164.1 alpha-actinin-4, partial [Equus caballus]  
 XP\_014588510.1 vinculin isoform X1 [Equus caballus]  
 XP\_014589771.1 histone H3.1-like [Equus caballus]  
 XP\_014590559.1 CD109 antigen [Equus caballus]  
 XP\_014590916.1 pantetheinase [Equus caballus]  
 XP\_014591200.1 latent-transforming growth factor beta-binding protein 2 [Equus caballus]  
 XP\_014591730.1 collagen alpha-1(V) chain [Equus caballus]  
 XP\_014591905.1 protein disulfide-isomerase [Equus caballus]  
 XP\_014591965.1 collagen alpha-2(VI) chain [Equus caballus]  
 XP\_014592190.1 C-type mannose receptor 2 [Equus caballus]  
 XP\_014592408.1 collagen alpha-1(I) chain [Equus caballus]  
 XP\_014592455.1 fibulin-1 [Equus caballus]

XP\_014592730.1 basement membrane-specific heparan sulfate proteoglycan core protein,  
partial [Equus caballus]  
XP\_014593897.1 72 kDa type IV collagenase [Equus caballus]  
XP\_014594379.1 laminin subunit beta-1 [Equus caballus]  
XP\_014594516.1 filamin-C [Equus caballus]  
XP\_014594947.1 antithrombin-III [Equus caballus]  
XP\_014595238.1 extracellular matrix protein 1 [Equus caballus]  
XP\_014595409.1 histone H2A type 2-C-like [Equus caballus]  
XP\_014595805.1 glypican-1 [Equus caballus]  
XP\_014595871.1 collagen alpha-3(VI) chain [Equus caballus]  
XP\_014595948.1 L-lactate dehydrogenase B chain isoform X1 [Equus caballus]  
XP\_014596179.1 alpha-2-macroglobulin-like [Equus caballus]  
XP\_014596564.1 complement C3-like [Equus caballus]  
XP\_014596747.1 neural cell adhesion molecule 1 isoform X1 [Equus caballus]

## **CHAPTER SEVEN: SUMMARY AND FUTURE PROSPECTS**

## 7.1. Summary

The aim of this dissertation was to determine if factors secreted by equine mesenchymal stromal cells (MSC) act on cell types found in the skin, in ways that suggest they may be a useful therapy for cutaneous wounds of horses. The long-term goals of this project are to (i) validate equine MSC-secreted factors *in vivo* as a safe and effective therapy for horse skin wounds, (ii) provide equine clinicians with protocols to offer MSC-secreted factors as treatment to their patients, and (iii) use what is learned while evaluating this therapy for horses, to improve treatments for humans.

The impaired healing of cutaneous wounds is problematic for horses and humans, and some chronic wounds of horses are reminiscent of those in man. Novel therapies developed for horses will not only benefit equine medicine but may fill a need in human medicine as well.

The work described here took advantage of *in vitro* culture methods to assess the effects of equine MSC-secreted factors on cell types found in the skin, and defined which factors were responsible for these effects. In particular, the target cells of interest were dermal fibroblasts (DF), resident skin cells that are tightly regulated during normal wound healing and which may contribute to delayed wound healing when their function is impaired.

Chapter One reviewed the pertinent aspects of MSC biology, as well as the history of equine MSC research and therapy, with the goal of setting the stage for the work described in the following research reports. Several critical points were highlighted regarding stem cells and their regenerative potential that formed the rationale for the research described in this thesis. For example, the therapeutic benefits delivered by MSC from any species are generally a result of factors secreted by MSC that act on the local environment, as opposed to the MSC differentiating into cells that are damaged or lost due to injury. Also, previous studies (mainly carried out in

mice) generated data suggesting that human MSC can improve the healing of human skin wounds. Finally, MSC therapy is an accepted practice in equine medicine, particularly in orthopedics, despite the fact that little is known about how these cells affect wounded tissues. Combined, this information led us to study the potential of equine MSC-secreted factors to be used as a therapy for cutaneous wound healing in horses.

The aim of Chapter Two was to present the horse as a relevant large animal model for human skin wound healing. We first described that nonhealing cutaneous wounds negatively affect both horses and humans, in part because current strategies for wound management are inadequate. We then compared the skin physiology and processes involved in normal wound healing in these two species and described in detail two types of naturally-occurring chronic skin pathologies that are similar in horses and humans; fibroproliferative disorders and atopic dermatitis. We concluded that although there are differences between horse and human skin repair, the many similarities warrant the use of the horse as a model for human normal and impaired wound healing, and so we proposed the horse as a translational model in which to explore novel therapeutics for wound management.

Chapter Three was the first of four published chapters describing our studies with equine MSC. The study was designed to evaluate the effects of MSC-secreted factors delivered as conditioned medium (CM) on equine dermal fibroblasts (DF) and determine if secreted factors from MSC microencapsulated in core-shell hydrogel beads had similar effects. We chose DF as targets because DF migration and specific changes in DF gene expression are critical to successful wound healing. We were motivated to encapsulate MSC, because enveloping MSC in alginate physically separates them from their target cells. We verified that MSC-secreted factors can diffuse through the capsules and affect target cells comparably to the secreted factors from

non-encapsulated MSC. This separation may be relevant when allogeneic donor MSC are delivered to recipients *in vivo*. The lack of cell-cell contact may prevent unwanted immune reactions from the recipient and prevent MSC from integrating into recipient tissue and forming a tumor. Specifically, we found that MSC CM stimulated DF to migrate and skewed the expression patterns of a select panel of DF genes toward those that positively contribute to wound healing. We also found that encapsulating MSC in core-shell hydrogel beads did not alter these effects. From this work, we concluded that MSC CM might serve as a therapy for horse skin wounds, and that encapsulation may be an effective way to deliver MSC CM *in vivo*.

Chapter Four described experiments expanding on our work looking at the effects of MSC CM on equine DF. The long-term goal of this study was to determine if MSC CM might serve as a therapy for a specific class of chronic cutaneous wounds found in horses and humans. In horses, the wounds are called exuberant granulation tissue (EGT) or proud flesh, and in humans they are generally referred to as fibroproliferative tissue. These wounds are characterized by excessive quantities of fibrotic tissue and may be painful and prone to additional complications. Fibroblasts in these wound environments are exposed to prolonged low-levels of inflammatory cytokines and are dysregulated, contributing to wound pathology. The specific aims of our experiments were to assess the effects of MSC CM on fibroblasts dysregulated by the influence of inflammatory mediators, by evaluating the effect of MSC CM on two different “types” of dysregulated DF. First, we treated normal DF with transforming growth factor beta 1 (TGF- $\beta$ 1) in culture, an established method of inducing a fibroproliferative-type phenotype. Second, we isolated fibroblasts from clinical EGT cases. We determined that MSC CM blocked TGF- $\beta$ 1-induced changes in DF morphology, proliferation rate, gene expression, and contractile-capacity. In addition, we found that MSC-secreted factors did not

change proliferation or migration rates of DF isolated from EGT but did lead to changes in expression of genes and proteins involved in extracellular matrix (ECM) remodeling and altered contractile capacity. These results showed that MSC CM changed the phenotype of dysregulated DF in ways that seemingly push them toward a more normal phenotype. From this we concluded that MSC CM might have potential as a novel therapy for fibroproliferative wounds in horses, and potentially in humans as well.

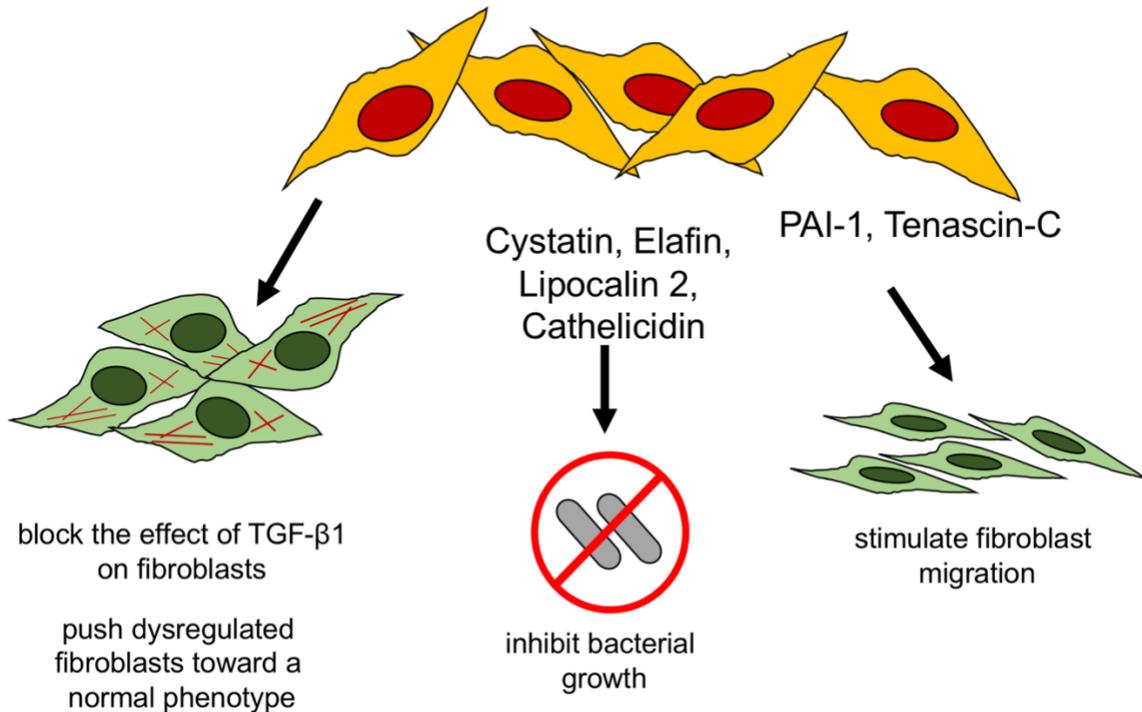
The studies reported in Chapter Five diverted from the effects of MSC CM on DF. For these experiments we evaluated the effects of MSC CM on the growth of bacterial species commonly found as contaminants in equine nonhealing skin wounds; gram-negative *E. coli* and gram-positive *S. aureus*. We found that factors secreted by MSC inhibited the growth of both these bacterial species and that this effect was in part mediated by four specific antimicrobial peptides that we identified in MSC CM; cystatin-C, elafin, lipocalin and cathelicidin. Based on these data, we provided additional evidence for the value of MSC CM as a therapy for cutaneous wounds, as controlling bacterial infection is an important aspect of wound management.

The final experiments outlined in this dissertation were described in Chapter Six. They were designed to determine which MSC-secreted factors stimulate DF migration *in vitro*, as well as to evaluate the potential of those factors to promote wound closure *in vitro*. We were motivated to perform these experiments because (i) we observed previously that the profile of secreted factors by MSC lines isolated from different horses can vary and (ii) we know that the secretory profile of MSC can be manipulated by stimulating MSC with various compounds in culture. Therefore, if we know which secreted factors stimulate DF migration, we can focus our work on MSC lines that are high producers of those factors and/or stimulate MSC to secrete more of the factors of interest. First, we analyzed CM from MSC cultures using liquid

chromatography-mass spectrophotometry (LC-MS/MS) to identify all proteins secreted by MSC. A literature search of directed us to focus on several MSC-secreted proteins that are involved in cell migration. Then, we used double-stranded RNA-mediated interference (RNAi) to silence the genes encoding these proteins in MSC, in order to evaluate the effects of CM from silenced MSC on migration of DF cells *in vitro* and wound closure in mice *in vivo*. We found that MSC-derived plasminogen activator inhibitor-1 (PAI-1) and tenascin-C significantly increased DF migration *in vitro* and decreased time of wound closure *in vivo*. This work is significant, as it is the first demonstration that equine MSC-secreted factors improve skin wound healing *in vivo*, and it is the first to identify specific factors that contribute to this effect.

Based on the results found in these studies, we feel justified in designing experiments to test the efficacy of MSC-secreted factors as a therapy for equine skin wounds *in vivo* (Figure 7.1.)

## Equine Peripheral Blood-Derived Mesenchymal Stromal Cells



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**Figure 7.1. Qualities of equine mesenchymal stromal cell (MSC)-secreted factors defined by work in this dissertation.** Work described in this dissertation was the first to determine that equine MSC secrete specific factors that exert defined effects on target cell types found in horse skin.

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### 7.2. Future prospects

We achieved the goal of this dissertation by determining that factors secreted by equine mesenchymal stromal cells (MSC) do act on cell types found in the skin, in ways that suggest they may be a useful therapy for cutaneous wounds of horses. However, before MSC-secreted factors are used clinically, in veterinary or human medicine, several challenges must be overcome.

The therapeutic potential of MSC has been extensively explored in multiple species by both basic scientists and clinicians since the term MSC was coined, and the idea that these cells could be used as a biology therapy was formally proposed [1]. In close to 40 years of MSC study, thousands of scientific papers have been published describing the effects of MSC on target cells, biological processes, and disease states. Still, MSC therapy is not routinely used in human medicine, and although it is commonly used by equine practitioners to treat orthopedic injuries, there is minimal evidence that MSC therapy is effective. There is no one answer to the question: Why are MSC not yet widely used as a therapy? The paragraphs below outline a brief discussion of challenges faced when developing any cell-based therapy, followed by the suggestion that specific aspects of MSC biology make this cell type particularly difficult to introduce into the clinic.

#### *7.2.1. General challenges of developing a cell-based therapy*

Advancing any therapy from a concept, through laboratory and clinical testing, and finally to patients, is a long, difficult and expensive process [2, 3, 4]. The development of cell-based therapies is no exception, and arguably may be more difficult than other types, due to the inherent qualities of cells and the unpredictability and high cost of cell culture [5].

##### *7.2.1.1. Challenges with production and delivery of cell-based therapies*

Depending on the type of cell-based therapy, delivery route to patients, and frequency of administration, following steps are commonly involved in preparing treatments: cell isolation and enrichment, characterization, expansion, encapsulation or seeding on supportive matrices, freezing, shipping, reviving, and administration to the patient [6]. Cells prepared for therapeutic purposes should be handled under sterile conditions and tested regularly throughout the preparation process to detect unwanted contaminants. In addition, and based on the fact that cells

are dynamic, living entities that can change in response to environmental stimuli, they should be tested regularly to ensure that their desired characteristics are maintained throughout the process and that they remain viable [3]. The balance between producing enough cell-based product to be effective and striving for the shortest production time possible to reduce both the risk of negative environmental impacts on the product and the cost of maintaining cells in culture, is difficult to manage. In addition to meeting the day-to-day demands of maintaining healthy cell cultures, researchers must comply with a slew of regulations, handed down by different governmental agencies to fulfill formal manufacturing requirements, [7].

#### *7.2.1.2. Challenges when validating cell-based therapies in vivo*

A challenge in the development of a cell-based therapy in both human and veterinary medicine, is demonstrating convincing efficacy in large, randomized controlled studies. As many cell-based therapies rely on autologous products, cell-therapy trials often start in patients rather than healthy volunteers. When patients are involved, the traditional divisions between phases of human clinical trials; phase I (safety), phase II (efficacy), and phase III (confirmation of safety and efficacy) can be blurred, and cohort size may be limited [8]. In humans and veterinary species, the lack of clarity as to what is being evaluated during the course of a trial, and small numbers of participants, each weaken the power of study results. In addition, choosing an appropriate patient population for the initial trials is critical, but there is tension between enrolling patients with disease states that are advanced enough to warrant exposure to an experimental and potentially dangerous therapeutic, and those whose conditions are moderate enough that they will respond to therapy [9]. Finally, to critically evaluate both the safety and efficacy of cell-therapy trials, long-term follow up with trial subjects is ideal [5]. Long-term follow up is not only laborious, it can delay reliable conclusions by years to decades. These

hurdles of (i) difficulty in designing trials with clear read-outs, (ii) choosing an appropriate patient population, and (iii) need for long-term follow up, all have contributed to the lack of convincing evidence that many potential cell-based therapies are safe and effective *in vivo*.

### 7.2.2. *The knowledge gap preventing development of effective MSC-based therapies*

As described briefly above, developing any cell-based therapy is a challenging process. MSC however, may be even more difficult to work with than other cell types, as extremely little is known about the origin of these cells, and how they function naturally *in vivo*. Indeed, this lack of knowledge about basic MSC biology contributes to the challenges faced when optimizing these cells for therapy.

MSC were first identified as adherent cells isolated from bone marrow cultures and were originally called colony-forming fibroblast [10]. In 1991 the term MSC was coined [1], and in that same year, MSC cells were identified as STRO-1-positive, glycophorin-A-negative cells in bone marrow, by antibody-based sorting [11]. STRO-1 is the name given to a mouse IgM that was discovered in a hybridoma screen, as an antibody that binds to stromal cells in the bone marrow, but for years its antigen remained undetermined. Glycophorin-A is a marker of erythroid precursors [11]. When STRO-1-positive, glycophorin-A-negative cells were separated from the remainder of the bone marrow components and cultured, it was determined that they were analogous to the colony-forming fibroblast that were isolated by adhesion years earlier. Although it is clear that these cells, named MSC, are present in the stromal compartment of the bone marrow, it is not known if, when, or how, they exit from the bone marrow, where they go if they do exit, how they may change as they develop, and what function they serve *in vivo*. STRO-1 has not proven to be a marker specific for and inclusive of all MSC. Work using the STRO-1 antibody showed that in adult tissues, this marker labels mature endothelial cells, as well as non-

endothelial cells associated with the vasculature, some of which may be pericytes [12, 13, 14]. Additional studies suggest that STRO-1 does not react with cells called MSC that are isolated from tissues other than bone marrow, such as adipose tissue, umbilical cord, umbilical cord blood, peripheral blood [15, 16, 17, 18]. The antigen recognized by STRO-1 was only recently identified as heat shock cognate 70 (HSC70) [19]. HSC70 is a ubiquitously and constitutively expressed protein, but STRO-1 cell surface binding is limited to only a few cell types when measured by flow cytometry [20]. When lysate from a panel of cells with variable STRO-1 surface binding was probed with STRO-1 for Western blot analysis, STRO-1 detected HSC70 in all lysates [19]. It has been proposed that cell surface expression of HSC70 coincides with specific stages of cellular development, but currently there is no convincing data to support this theory [19].

Additional cell surface markers, such as the stage-specific embryonic antigen 4 (SSE-4), the low-affinity nerve growth factor receptor CD271, and the adhesion molecule CD146, have been proposed and examined as markers that might facilitate the isolation of MSC from tissues, or the selection of MSC with high stemness. But cells designated as MSC, isolated from various sources do not uniformly express these proteins either [21]. This raises the possibility that MSC isolated from sources other than bone marrow might not be the same MSC that are detected in bone marrow. However, cells isolated from other tissues are defined as MSC according to the minimal *in vitro* criteria put forth by the International Society of Cellular Therapy (ISCT) in 2006: (i) MSC must be plastic-adherent when maintained under standard culture conditions, (ii) MSC must express the surface proteins CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14 or CD11b, CD79 $\alpha$  or CD19 and HLA-DR, and (iii) MSC must have the potential to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* [22]. Clearly,

these minimal criteria encompass a heterogeneous population of cells termed MSC, that may express different surface markers as a result of being at various stages of differentiation or residing in different tissue environments. Culture conditions have proven to alter MSC surface protein expression as well, which may confound results obtained from *in vitro* studies [23, 24]. In the field of MSC biology, it is currently accepted that there is no single marker that is MSC specific [21], which results in the inability to track these cells and to answer questions about their basic biology. For example: Do all MSC originate in the bone marrow and then exit to populate various peripheral tissues as cells of hematopoietic origin do? The lack of a single marker also makes it challenging to follow MSC *in vivo* to sites of injury. Indeed, it has been suggested that these cells home to injured sites, based on the results of experiments using MSC labeled *ex vivo*, which were then introduced to animals with various pathologies [25, 26, 27, 28]. Seemingly, bone marrow transplant experiments could shed light on MSC origin and fate, as they have for the origin of hematopoietic cells, but studies in mice and data from human transplants overwhelmingly indicate that after bone marrow transplantation, donor MSC engraftment does not occur [29]. Bone marrow and peripheral blood stromal cells detected in human patients post-transplant are recipient, not donor in origin, irrespective of the level of conditioning patients received prior to transplantation [30, 31, 32].

The lack of information about the basic biology of this heterogeneous population of cells named MSC has undoubtedly contributed to the lag in the field of MSC therapeutics. If more was known about how MSC interact with their *in vivo* environment, they could potentially be cultured in more optimal ways and researchers could focus on optimizing their innate potential. The naming of MSC and categorizing the diversity of cells that are MSC by ISCT definition may have also negatively impacted the field. Contradictory findings across laboratory experiments

and between clinical trials are common, and data are often not reproducible between groups. The seemingly inconsistent outcomes of MSC therapy cause researchers and funding agencies to lose interest in pursuing the development of MSC as a useful biologic treatment, which is unfortunate because many of the inconsistencies may be a result of “comparing apples to oranges”. There is great heterogeneity across MSC cultures isolated from different sources, even if they fit the definition of MSC proposed by the ISCT. Until more is known about the origin, development and function of MSC isolated from different tissues *in vivo*, it will be difficult to understand the “results” researchers and clinicians observe *in vitro* and in clinical applications.

### **7.3. Final remarks**

The work presented in this dissertation showed that *in vitro*, equine MSC-secreted factors act on target cell types found in the skin, in ways that suggest they may improve cutaneous wound healing *in vivo*. The next phase of this work, before attempting to move forward with the development of MSC therapies for horse or human skin wounds, might be to take a step back and invest in more tools to study the basic biology of these cells *in vivo*. When we know more about the origin, development and function of MSC, we may finally be successful in developing effective treatments using these cells.

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