IMPROVING THE CLINICAL UTILITY OF MESENCHYMAL STEM CELLS THROUGH ENHANCING CELL SURVIVAL: ENGINEERED AND CELLULAR APPROACHES

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Austin Nuschke, Ph.D.

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For decades, mesenchymal stem cells have been touted as highly promising cells for use in regenerative medicine. Their flexibility in differentiation to other cell types, paracrine abilities in supporting angiogenesis, and modulatory behavior in inflammation have led to researchers applying the cells across a wide variety of treatments, including skin healing, bone regeneration, myocardial infarction repair, and others. However, a fundamental limitation in all MSC therapies has been a lack of survival for implanted cells, preventing them from carrying out their many beneficial functions. We have examined strategies to improve this MSC survival dilemma, hypothesizing that a combination of engineered approaches to activate survival signaling and modulation of autophagy to generate energy for the cell would act to improve MSC survival and function in the face of stressors. Our results show that cell surface restriction of EGFR to constitutively activate downstream survival signaling improves MSC longevity in two systems: physical tethering of soluble EGF on bone regeneration scaffolds, and EGFR tethering through EGF-like repeats of the ECM protein Tenascin C in a polymer for skin wound healing. Additionally, we found that MSCs have a high accumulation of autophagosomes, which are mobilized for degradation during the stress of differentiation and can be tuned to affect MSC function in relation to differentiation. Ultimately, these strategies to alter MSC signaling and function will be of considerable use for attempts to apply the great potential utility of MSCs to wound healing and other contexts in biomedical science.

TABLE OF CONTENTS

PREFACE ..... **XIII**

1.0 INTRODUCTION **1**

1.1 MESENCHYMAL STEM CELLS **2**

1.1.1 Discovery and characteristics **2**

1.1.2 Sources and functions **4**

1.2 WOUND HEALING **7**

1.2.1 Skin wound healing **8**

1.2.1.1 Chronic wounds **10**

1.2.2 Extracellular matrix and wound healing **16**

1.2.3 Matrix in dysrepair **21**

1.3 MESENCHYMAL STEM CELL UTILITY IN WOUND HEALING **24**

1.3.1 Immunomodulation **24**

1.3.2 Paracrine activity and angiogenesis **26**

1.3.3 Reduction in scar formation **28**

1.4 LIMITATIONS IN MSC CLINICAL UTILITY **30**

1.4.1 Inflammation **34**

1.4.2 Nutrient deprivation **35**

1.4.3 Changes in oxygen tension **36**

1.5 STRATEGIES TO IMPROVE MSC SURVIVAL 37

1.5.1 EGFR signaling and cell survival 37

1.5.2 Physical tethering of soluble EGF 38

1.5.3 Tenascin C 41

1.5.3.1 Interaction between Tenascin C and EGFR 43

1.6 AUTOPHAGY AND CELL SURVIVAL 47

1.6.1 The process of autophagy 47

1.6.1.1 Role of autophagy in stress responses and survival 49

1.7 HYPOTHESIS 50

2.0 EGF TETHERED TO B-TRICALCIUM PHOSPHATE BONE SCAFFOLDS VIA A HIGH AFFINITY BINDING PEPTIDE ENHANCES SURVIVAL OF HUMAN MESENCHYMAL STEM CELLS IN MOUSE MODELS 51

2.1 ABSTRACT 51

2.2 INTRODUCTION 52

2.3 MATERIALS AND METHODS 55

2.3.1 Reagents 55

2.3.2 MSC cell culture 55

2.3.3 Cell tracking 56

2.3.4 Binding peptide fabrication 57

2.3.5 Scaffold preparation and seeding 57

2.3.6 Mouse inoculations 58

2.3.7 Plug sectioning 59

2.3.8 Flow cytometry 59

2.3.9 Statistical analyses 60

2.4 RESULTS 60

2.4.1 Tethered-EGF β-TCP scaffolds improve MSC survival in vivo 60

2.4.2 tEGF improves overall cellularity of implanted scaffolds 62

2.5 DISCUSSION 65

3.0 A WOUND HEALING POLYMER UTILIZING THE MATRIKINE TENASCIN C ENHANCES MESENCHYMAL STEM CELL SURVIVAL AND IMPROVES SKIN WOUND HEALING OUTCOMES IN MICE 71

3.1 ABSTRACT 71

3.2 INTRODUCTION 72

3.3 MATERIALS AND METHODS 74

3.3.1 Reagents 74

3.3.2 Cell culture and MSC labeling 75

3.3.3 Polymer fabrication 76

3.3.4 MSC incorporation and polymer application 77

3.3.5 Polymer characterization analyses 77

3.3.6 Mouse procedures 77

3.3.7 Wound tissue extraction and sectioning 78

3.3.8 MSC extraction and flow cytometry analysis 79

3.3.9 Statistical analyses 80

3.4 RESULTS 80

3.4.1 Stable PEG-polymer fabrication and MSC incorporation 80

3.4.2 MSC survival in the TNC-based polymer 82

3.4.3 Wound maturation and collagen content improve with Tenascin C additive 84

3.4.4 Collagen alignment is improved in the wound bed with Tenascin C additive 86

3.4.5 Murine cellular infiltrate is altered with the Tenascin C additive 86

3.5 DISCUSSION 87

4.0 HUMAN MESENCHYMAL STEM CELLS UNDERGO AUTOPHAGY AT THE INITIATION OF DIFFERENTIATION 94

4.1 ABSTRACT 94

4.2 INTRODUCTION 96

4.3 MATERIALS AND METHODS 98

4.3.1 Reagents 98

4.3.2 Cell culture 98

4.3.3 Transmission electron microscopy 100

4.3.4 Osteogenic differentiation 100

4.3.5 Adipogenic differentiation 101

4.3.6 Assessment of autophagosome turnover 102

4.4 RESULTS 103

4.4.1 MSCs contain a large number of mid-stage autophagosomes 103

4.4.2 Autophagy is highly active during the early stages of differentiation 104

4.4.3 Modulation of differentiation outcomes through autophagy regulation 112

4.4.4 Autophagic turnover is halted in MSCs 114

4.4.5 Glucose levels alter autophagy activity in MSCs 117

4.5 DISCUSSION 117

5.0 CONCLUSIONS AND SPECULATIONS 123

5.1 CONSIDERATIONS IN MSC BIOLOGY 124

5.1.1 MSC heterogeneity 124

5.1.2 Autophagy and MSC reprogramming 125

5.2 FUTURE WORK IN MSC SURVIVAL SYSTEMS 126

5.2.1 *In vivo* modeling 126

5.2.2 Functional changes in MSCs 128

5.3 FUNCTIONALIZATION OF OTHER REGENERATIVE SCAFFOLDS 129

5.4 APPROACHES FOR OTHER CELL THERAPIES 131

5.5 COMBINATION APPROACHES FOR CELL SURVIVAL 132

5.6 CONCLUSION 134

APPENDIX A 136

BIBLIOGRAPHY 138

LIST OF TABLES

Table 1. Clinical sources of MSCs 6

Table 2. Chronic wound healing technologies..... 9

Table 3. Clinical trials for MSCs and chronic wounds..... 15

Table 4. Matrix and matricellular proteins during wound repair..... 18

LIST OF FIGURES

Figure 1. Key players during different stages of wound repair 11

Figure 2. Phase transitions in excisional wound healing and disrepair. Wound healing proceeds from the initial homeostatic phase through tissue regeneration and into resolution..... 13

Figure 3. The 'scarring cycle' schematized 23

Figure 4. Various threats multipotential stromal cells face at the delivery site leading to loss of cells 33

Figure 5. Schematic of EGFR survival signaling 40

Figure 6. TNC is protective to MSCs in the face of FasL-induced death..... 45

Figure 7. TNC causes sustained activation of Erk through activation of EGFR 46

Figure 8. Experimental schematic for tEGF plug injections..... 61

Figure 9. Surface tethered EGF improves MSC survival on β-tricalcium phosphate scaffolds ... 64

Figure 10. MSCs show improved survival localized to tEGF-TCP aggregates 65

Figure 11. Improved MSC survival on tEGF scaffolds increases overall cellularity 66

Figure 12. The PEG-collagen polymer with TNC degrades gradually in vitro and integrates into mouse wounds..... 81

Figure 13. The polymer matrix with TNC promotes long term MSC survival. 83

Figure 14. MSC-based polymer yields improved wound healing outcomes 85

Figure 15. Collagen alignment and maturation improves using the MSC-based polymer 88

Figure 16. MSC-based polymer augments murine cell infiltrate in wound beds 89

Figure 17. Mesenchymal stem cells show a relatively high number of autophagosomes 105

Figure 18. High autophagosome concentration is consumed during early immortalized human mesenchymal stem cell differentiation 107

Figure 19. MSC differentiation quickly induces autophagosome clearance. 110

Figure 20. Autophagy is mobilized during differentiation of ihMSCs 112

Figure 21. Early arrested autophagy can be modulated to alter immortalized human mesenchymal stem cell adipogenesis outcomes. 113

Figure 22. Immortalized hMSC show little autophagosome turnover unless stimulated 116

Figure 23. Glucose concentrations alter LC3 expression in MSCs 118

Figure 24. Schematic for arrest and utilization of autophagy in normal MSC function 121

PREFACE

This dissertation is the culmination of many years of work here in Pittsburgh, as well as the result of many more years of preparation and work throughout my entire life. Many people helped make this possible, and I cannot acknowledge that enough. First and foremost, my family has supported me for 26 years and has helped make my life enjoyable and successful. I cannot thank my parents, Dave and Melissa, enough for their emotional, intellectual, and financial support that afforded me the opportunity to get to this point. My siblings, Erik, Greg, and Lora, thank you for being a source of relaxation and fun when I visited home and for being there for me.

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1.0 INTRODUCTION

This chapter includes material excerpted from the following publications:

Nuschke A. Activity of mesenchymal stem cells in therapies for chronic skin wound healing. Organogenesis. 2014;10(1):29-37.

Wells A, Nuschke A, Yates CC. Skin tissue repair: matrix microenvironmental influences. Matrix Biology. 2015;e-pub ahead of print.[1]

Rodrigues M, Yates CC, Nuschke A, Griffith L, Wells A. The Matrikine Tenascin-C Protects Multipotential Stromal Cells/Mesenchymal Stem Cells from Death Cytokines Such as FasL. Tissue Engineering Part A. 2013;19(17-18):1972-1983.

Mesenchymal stem cells (MSCs) have become a widely studied type of cell in the past approximately 15 years, mostly as potentially useful cells for regenerative therapies. These cells have been noted for decades for their multipotent capacity and paracrine activity that proves promising for treatments in bone regeneration, skin wound healing, immunomodulation, and

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many other areas of research. Accordingly, hundreds of clinical trials have been launched investigating patient outcomes in a variety of treatments involving MSCs, from direct injection into wound sites to engineered delivery vehicles for tissue regeneration to slow-release devices for immunoregulation.

While MSCs do indeed have the capacity to be extremely useful across all of these potential functions, a variety of factors combine to make implant sites for the cells very harsh, ultimately leading to limited MSC survival and, consequently, disappointing regenerative outcomes after treatment. As a result, strategies to extend the lifespan of implanted MSCs have become a topic of great interest, particularly for the context of wound healing, where implant environments are particularly harsh, devoid of nutrients, and hyperinflammatory. Our work has examined how the endogenous pathways that promote cell survival might be exploited to that end, improving resistance to cell death stimuli and ultimately improving MSC function and clinical outcomes as a result.

1.1 MESENCHYMAL STEM CELLS

1.1.1 Discovery and characteristics

Mesenchymal stem cells, also referred to as multipotent stromal cells, were first discovered as a progenitor cell population in the bone marrow by Goujon in 1869, who found that autologous bone marrow transplants yielded bone formation upon transplantation into muscle tissue (Goujon 1869). This work was built on by Alexander Maximow in 1924, who found that this same progenitor was able to differentiate into different blood cells, suggesting a

stem-like nature to MSCs (Maximow 1924). In the 1960's, pioneering work from McCulloch and Till showed the clonogenic potential of the mesenchymal progenitor population, work that helped to form an understanding of the nature of stem cell populations in general (Becker, McCulloch et al. 1963; Siminovitch, McCulloch et al. 1963). The inherent ability of these cells to differentiate was definitively established in 1968 then by Tavassoli and Crosby, who showed osteogenic capacity of this progenitor population (Tavassoli and Crosby 1968). From there, Friedenstein showed in a series of studies in the 1970s the characteristics of this population, further demonstrated their osteogenic potential, and their differences from other hematopoietic populations, in particular their adherence to tissue culture plastic that yielded formation of MSC colonies, which still is used as a means of characterizing an isolated primary population (Friedenstein, Petrakova et al. 1968; Friedenstein and Kuralesova 1971; Friedenstein 1976). The plasticity and clonogenicity of this population of mesenchymal progenitors ultimately led to attempts to translate MSC work into clinical therapies, and the first clinical trial attempting to utilize autologous culture-expanded MSCs occurred in 1995, where Lazarus et al intravenously introduced autologous MSCs into patients to examine bone marrow healing outcomes (Lazarus, Haynesworth et al. 1995).

Related to clinical interest in MSCs, perhaps their most notable function is their capacity to differentiate into a variety of cell types. Though the most commonly referenced lineage derived from MSCs includes osteoblast formation (such as Goujon's study in 1869), MSCs are widely acknowledged to form adipogenic and chondrogenic lineages (Pereira, Halford et al. 1995; Jiang, Jahagirdar et al. 2002; Zuk 2002; Corotchi, Popa et al. 2013), MSCs have the capacity to form other cell types such as cardiomyocytes, fibroblastic lineages, and others under

the appropriate culture conditions (Oreffo, Cooper et al. 2005; Mishra, Mishra et al. 2008; Montanari, Dayan et al. 2015; Shen, Wang et al. 2015).

Defining characteristics of MSC populations, despite cell source or culture protocol, is often related to cell surface marker expression. While these markers can vary some depending on tissue source for the MSCs (Table 1), there are some consensus markers that are used to characterize the cells following extraction. In general, it is accepted that expression of CD73, CD90, and CD105 in combination is characteristic of an MSC, as well as a lack of CD34 and CD45 expression. While these markers are almost always used in MSC characterizations, there is some evidence to suggest that surface marker expression is highly sensitive to culture conditions, such as reduced CD105 expression during differentiation or during starvation cultures (Jin, Park et al. 2009; Mark, Kleinsorge et al. 2013). While the other classic defining feature of MSCs is the capacity to differentiate into multiple lineages, there is also evidence to suggest that cell source can affect differentiation potential for any given lineage. For example, umbilical cord-derived MSCs have been cited as having reduced adipogenic capacity (Kern, Eichler et al. 2006), and similarly adipose-derived MSCs have demonstrated reduced chondrogenic differentiation in some studies (Hennig, Lorenz et al. 2007).

1.1.2 Sources and functions

One particular advantage that MSCs have over other cells (both multipotent and otherwise) for use in regenerative medicine is the numerous sources that MSCs can be derived from, albeit with slightly different characteristics. Historically, MSCs have been isolated primarily from bone marrow tissue through density centrifugation (Jiang, Jahagirdar et al. 2002), yielding the classic spindle-shaped/polarized cell that became the standard for MSC work over

the years. Indeed, the telomerase-immortalized cell line used for much of the work in this dissertation (ihMSCs) is a bone marrow-derived line, and for many years much of MSC work was performed on primary or immortalized MSCs from that source. While immortalized and primary cells show some differences in morphology in culture, MSC functions such as differentiation and paracrine activity remain largely comparable.

More recently, it has become evident that MSCs could be derived from more easily-accessed tissues, often in higher number. Perhaps the most widely used alternative source is now adipose-derived MSCs, which are easily harvested from fat tissue to yield MSCs comparable in shape and general behavior to bone marrow-derived cells (Zuk 2002). This source has been more widely studied of late due to the easy of isolation and, more importantly, abundance of available tissue for extraction, as the MSCs are easily isolated from excess tissue from liposuction or resections in plastic surgery (Schreml, Babilas et al. 2009). Other sources include the Wharton's jelly of the umbilical cord, which was a popular source for mothers to freeze cells and maintain an autologous stem cell population for many years due to ease of access in postnatal tissue and potential for therapeutic use later in life. MSCs from this tissue source perform comparably to classical bone marrow-derived MSCs, but have some interesting characteristics such as increased proliferative rate (Baksh, Yao et al. 2007) and improved paracrine response to hypoxia (Nagano, Kimura et al. 2010). The placenta is another maternal source of MSCs that has drawn interest due to the non-invasive means by which MSCs can be harvested, as placenta can be harvested following birth and cells extracted with no additional invasive procedures like bone marrow extraction. These MSCs are interesting in that they have a generally low yield from extraction (compared to abundant yields from adipose tissue, for example), but some evidence has shown

Table 1. Clinical sources of MSCs. An overview of sample tissue sources for mesenchymal stem cells, including the basic means of isolation and culture, common surface marker expression patterns, and general considerations for use in clinical applications as well as characteristics of the cell subtype. For most sources, there is occasionally some debate over consensus for surface markers; the most commonly mentioned ones are cited in this case. Table adapted from (Nuschke 2014).

Cell Source	Isolation source/ method	Sample surface markers		Notes
Bone marrow	Typical bone marrow harvest followed by marrow aspirate density centrifugation/plate adherence with the SVF (Gnecchi and Melo 2009).	+	CD13, CD44, CD73, CD90, CD105, CD29, CD166, CD147	- Most well-characterized and extensively studied population - More painful isolation procedure, inherent risk factors of marrow harvest
		−	CD34, CD45, CD14	- Few cells present in marrow aspirate compared to other methods
Adipose tissue	Processing of residual tissue from liposurgery; tissue digestion/plate adherence to isolate MSC population. Comparatively easy isolation procedure (Arana, Mazo et al. 2013).	+	CD13, CD44, CD73, CD90, CD105, CD9, CD29 , CD106, CD147, CD166 (low)	- Easier isolation method for patient - Abundant and readily available source - Greater number of cells obtained during isolation - Potentially reduced chondrogenic differentiation efficiency (Hennig, Lorenz et al. 2007)
		−	CD34, CD45, CD133, CD144, CD14	
Placenta	Obtainable from amniotic fluid or placental tissue (various), with standard tissue digestion or fluid fraction segregation. Cells obtained via plate adherence (In 't Anker, Scherjon et al. 2004).	+	CD44, CD29, CD105, CD90, CD144, CD166	- Readily available and non-invasive cell source - Potentially improved growth potential and life span (Barlow, Brooke et al. 2008)
		−	CD14, CD34, CD45, CD117	- Substantially low cell yield from isolation, varies with source tissue and individual
Umbilical cord	Can be obtained from cord itself (digested), Wharton's jelly, or cord blood. Density gradient purification or enzymatic digestion, depending on the sour (Zhang, Hirai et al. 2011; Corotchi, Popa et al. 2013).	+	CD73, CD105, CD44, CD29, CD144, CD166	- Generally highly proliferative MSC populations (Baksh, Yao et al. 2007) - Heterogeneous MSCs obtained based on UC source tissue
		−	CD45, CD34, CD14	- Potentially reduced adipogenic capacity (Kern, Eichler et al. 2006) - Debatable expression of classic MSC markers CD90 and CD105 - Respond well to hypoxic conditions (Nagano, Kimura et al. 2010)

Peripheral blood	Mobilization of MSCs into blood (G-CSF injections), collected by density centrifugation and plating of mononuclear cell fraction; fibrin microbeads have also been used for collection (Kassis, Zangi et al. 2006).	+	CD73, CD90, CD105, CD44, CD166	-	Relatively easy to obtain compared to marrow harvest
				-	Historically controversial whether peripheral blood can contain significant population (Kassis, Zangi et al. 2006; Chong, Selvaratnam et al. 2012)
		–	CD34, CD45, CD14	-	Variable number of cells available for isolation in this method

that placental MSCs have improved lifespan and potential for growth (Barlow, Brooke et al. 2008).Other MSC sources ultimately show comparable characteristics, which is always similar marker expression and general behavior, but some differences in differentiation capacity, proliferative behavior, and other cellular characteristics remain.

1.2 WOUND HEALING

Regardless of the cell source, the goal of MSC research is a focus on the utility of the cells in regenerative medicine. Because of their very flexible differentiation capacity and wide array of paracrine abilities, MSCs have been examined in a wide variety of wound healing contexts. Among those numerous clinical contexts, skin wounds are perhaps the most attractive candidates for MSC therapy due to their characteristics that align so well with MSC function. MSCs are theoretically able to help modulate that inflammation that often plagues chronic wounds, can assist with angiogenesis through paracrine secretions such as VEGF, and have the potential to differentiate into fibroblastic lineages if needed in the tissue replacement process. As such, the majority of the following thesis work focused on MSCs in the context of wound healing for bone and, more substantially, skin.

1.2.1 Skin wound healing

Skin wound healing is a complex multi-stage process that orchestrates the reconstitution of the dermal and epidermal layers. In many pathological circumstances such as diabetes or severe burns, the normal wound healing process fails to adequately restore function to the skin, leading to potentially severe complications from ulcers or resulting infections. As the incidence of obesity and resulting diabetes continues to increase in the developed and developing world (CDC 2011), the prevalence of chronic wounds related to these conditions continues to be a major focus of wound care research. In fact, non-healing wounds from these conditions have produced a multi-billion dollar advanced wound care market for technologies aimed at stimulating wound healing in patients that suffer from dysfunctional wound repair, with large projected growth in the near future (Stuart 2007). Most current biological technologies for advanced wound care aim to provide antimicrobial support to the open wound and a matrix scaffold (collagen-based in many cases) for invading cells to reestablish the skin, with some focus on growth factor support of the healing process (Table 2) (Boateng, Matthews et al. 2008; Murphy and Evans 2012). However, patient outcomes in this area remain marginal and these products remain relatively expensive, and thus novel bioengineered approaches to chronic wound repair remain a topic of great interest.

Mesenchymal stem cells (MSCs) are important cells in orchestrating the three main phases of normal wound healing (inflammatory/proliferative/remodeling), directing inflammation and antimicrobial activity and promoting cell migration during epithelial remodeling (Maxson, Lopez et al. 2012). However, recently due to advances in understanding of MSC immunosuppression and secretion of pro-angiogenic factors, MSC-based cell therapy in

combination with matrix scaffold approaches to improve wound healing outcomes has become a potential strategy in treatment of non-healing wounds.

Traditionally, MSCs have long been identified for their ability to migrate to sites of injury in the body and differentiate into several cell lineages such as bone, fat, and cartilage (Pereira, Halford et al. 1995; Pittenger, Mackay et al. 1999; Kassis, Zangi et al. 2006; Kolf, Cho et al. 2007), making them attractive candidates for a variety of cell therapies in recent studies. A variety of easy means of isolating and expanding these cells ex-vivo (bone marrow (Hung, Chen et al. 2002; Corotchi, Popa et al. 2013), adipose tissue (Zuk 2002; Corotchi, Popa et al. 2013), placenta (In 't Anker, Scherjon et al. 2004), peripheral blood (Kassis, Zangi et al. 2006), and others) also makes MSCs useful cells for therapeutic approaches to supplementing tissue regeneration (Table 1). Additionally, these cells have been shown to have notable

Table 2. Chronic wound healing technologies. A sample of current FDA approved bioengineered approaches for advanced wound care, including diabetic wounds and other chronic lesions. Table adapted from (Nuschke 2014).

Technology	Company	Product Summary
Apligraf®	Organogenesis	Bilayered human skin directly applied to a wound. Lower layer contains collagen and fibroblasts, upper layer contains expanded keratinocytes (Falanga and Sabolinski 1999).
Dermagraft®	Shire Regenerative Medicine	Human fibroblasts integrated into a collagen/GAG polymer scaffold (dermal substitute) (Naughton, Mansbridge et al. 1997).
Hydrofiber (Aquacel®)	ConvaTec	Wound hydration system; carboxymethylcellulose fibers that gel upon application to hydrate wound. Silver may be used as an antimicrobial agent (Jones, Bowler et al. 2004).
Bilayer Matrix Wound Dressing®	Integra	Outer antimicrobial silicone layer, with an inner collagen/GAG matrix layer for cell invasion/remodeling (Iorio, Goldstein et al. 2011).
Regranex®	HealthPoint Biotherapeutics	Topical gel (CMC) containing Becaplermin (recombinant PDGF) to stimulate wound healing (Rees, Robson et al. 1999).

immunomodulatory effects on the surrounding environment following transplantation (Krampera, Glennie et al. 2003; Aggarwal and Pittenger 2005; Corcione, Benvenuto et al. 2006; Gebler, Zabel et al. 2012), and can support native cells with the secretion of a variety of pro-survival and pro-migratory cytokines and growth factors (Matsumoto, Omura et al. 2005; Chen, Tredget et al. 2008). As a major problem in chronic wounding is unmitigated inflammation, this characteristic of MSCs has made them good candidates for approaches to cell therapy for chronic wounds in particular.

1.2.1.1 Chronic wounds

The normal wound healing process is characterized by three main phases that lead to efficient reconstitution of a functional dermis/epidermis and revascularized tissue (Figure 1) (Franklin, Adam et al. 1999; Behm, Babilas et al. 2012). Briefly, the inflammatory/hemostasis phase immediately follows wounding, serving to stop bleeding in the wound bed via platelet aggregation and fibrin clot formation. This is followed by invasion of neutrophils and mast cells that follow a chemotactic gradient to clear the wound of dead cells, debris, and residual ECM. The proliferative/tissue replacement phase then proceeds, including fibroblast migration into the wound bed and deposition of new ECM (collagen). VEGF and B-FGF also stimulate de novo angiogenesis in the skin (Knighton, Phillips et al. 1990; Nissen, Polverini et al. 1998; Barrientos, Stojadinovic et al. 2008). Finally, the remodeling process resolves the wound by organizing collagen fibers that formed during fibroblast proliferation in parallel with further removal of fibronectin to increase the strength of the new skin.

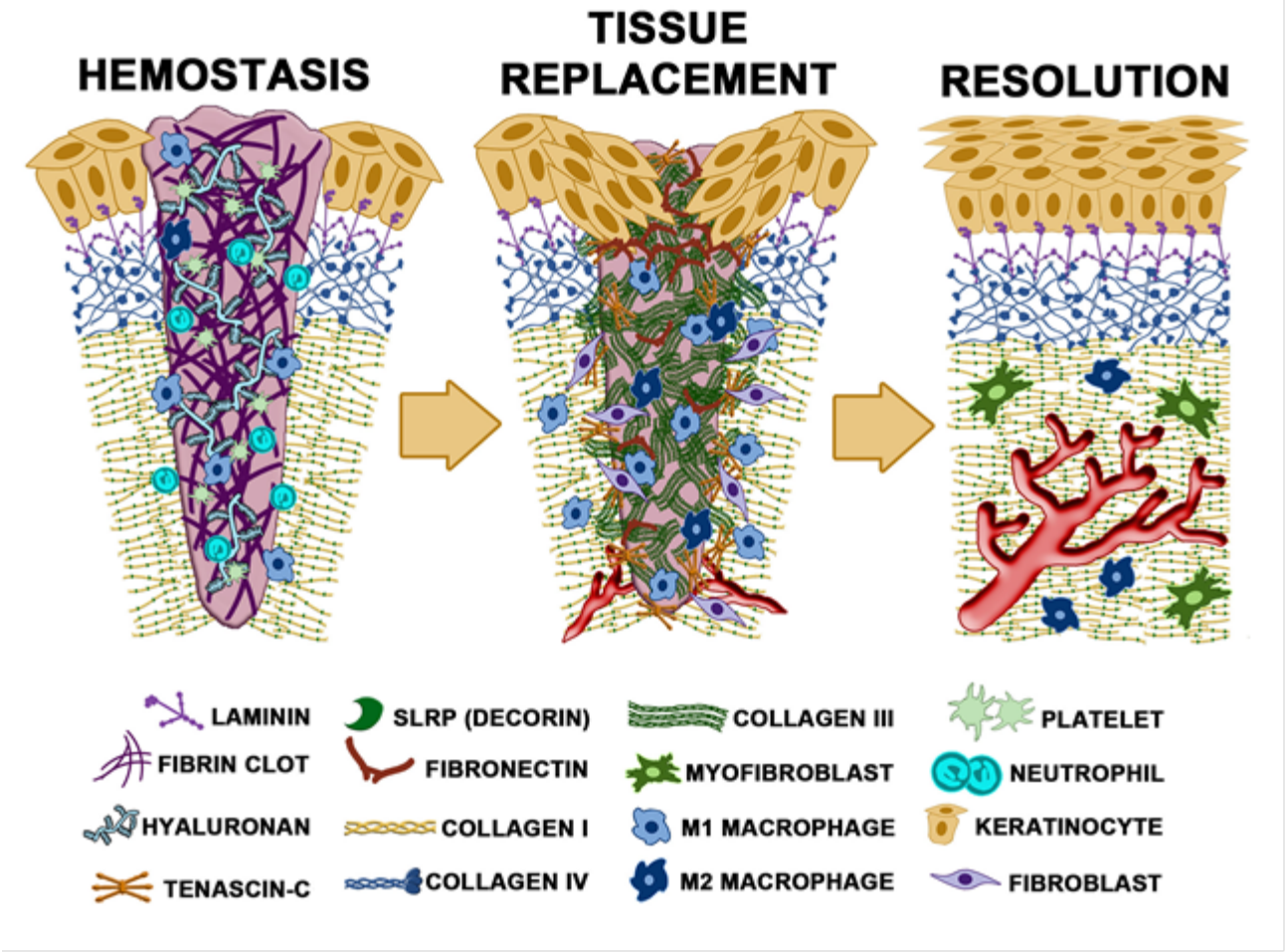

Figure 1. Key players during different stages of wound repair. The hemostasis (day 7), tissue replacement (day 14) and resolving (day 30) phases of a mouse wound are schematized here. Highlighted in each stage (and identified below) are the main cells, soluble signals, and matrix/matricellular components that constitute that stage. The presented are from studies in mice, but similar findings have been reported in human biopsies and specimens. Figure adapted from (Wells, Nuschke et al. 2015).[2]

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A chronic or non-healing wound is essentially a wound that does not progress normally through the wound healing process, resulting in an open laceration of varying degrees of severity (Stadelmann, Digenis et al. 1998; Menke, Ward et al. 2007). These conditions can be cause by a number of various pathophysiological conditions (diabetes (Fahey III, Sadaty et al. 1991), venous stasis ulcer progression (Stanley, Park et al. 1997), and others), though all causes generally lead to a hyper-inflammatory environment, particularly evidenced by the characteristic presence of neutrophils/high MMP activity that leads to high breakdown of new collagen during the wound healing process and inhibition of pro-healing factors (PDGF, TGF-B, and others)

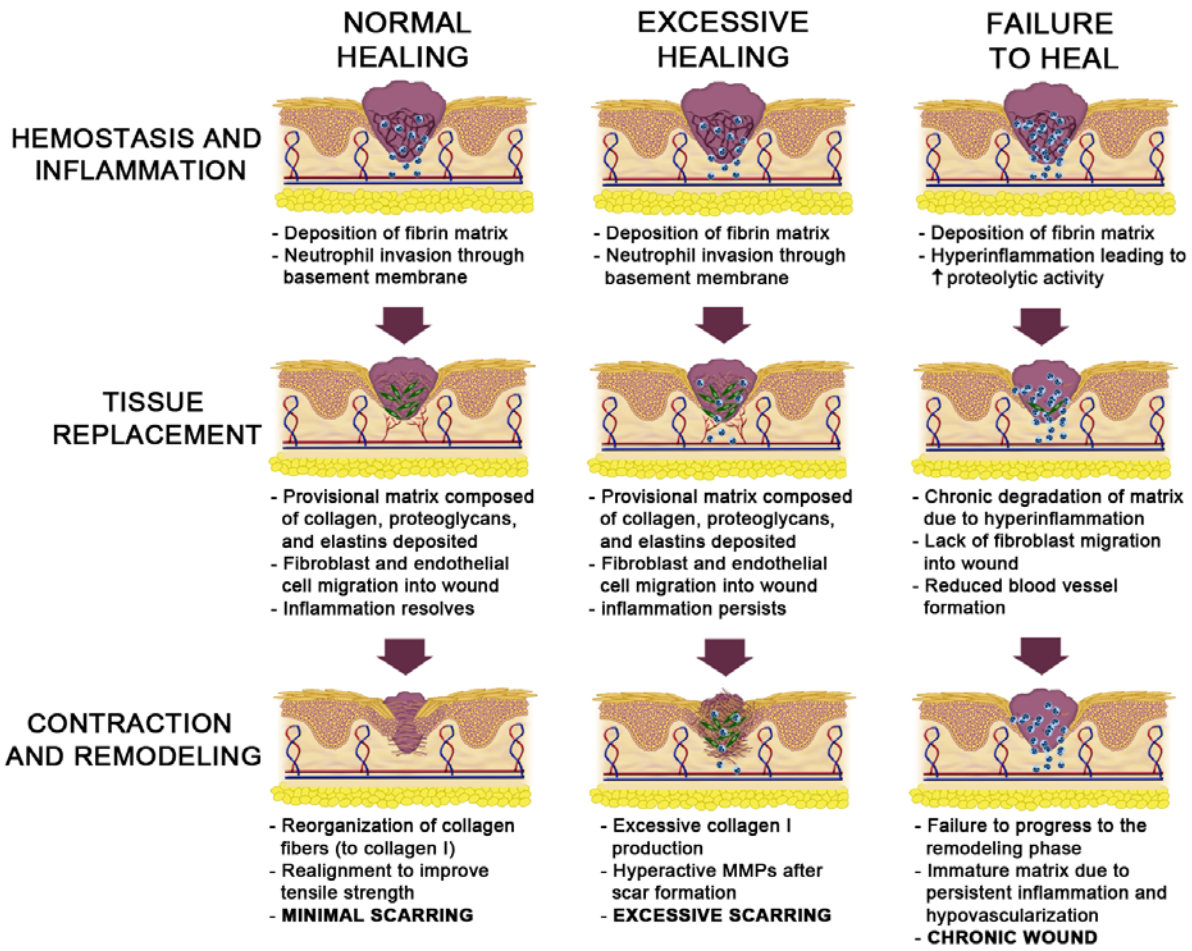

Figure 2. Phase transitions in excisional wound healing and disrepair. Wound healing proceeds from the initial homeostatic phase through tissue regeneration and into resolution. While these phases overlap both temporally and spatially within a wound, the orchestrated progression leads to re-established functioning with minimal scarring (left sequence). If the resolution phase has excessive cellularity and matrix from an over-exuberant tissue replacement phase that lacks stop signals, this is not a stable phenotype. The renewal of an active immature matrix in the resolution phase results in excessive tissue and hypertrophic scars (middle sequence). When the homeostatic phase does not transition towards regeneration, the healing is stalled and the initial, tissue-destructive inflammation persists. This situation leads to a chronic wound or ulcer (right sequence). Figure adapted from (Wells, Nuschke et al. 2015).[3]

(Yager, Zhang et al. 1996; Chen, Ward et al. 1997; Nwomeh, Liang et al. 1999; Pirilä, Korpi et al. 2007) (Figure 2). This excessive inflammation phenotype leads to wounds that cannot resolve under normal circumstances, especially until the inflammation in the wound bed is controlled to a normal level and fibroblasts are able to effectively migrate into the wound space and synthesize new matrix.

Clinically, these wounds present a large problem for wound care specialists globally, with approximately 1-2% prevalence and a greater than 50% recurrence rate for diabetic patients (Boulton, Vileikyte et al. 2005; Ghanassia, Villon et al. 2008). This need has generated a large interest in new treatments for improving patient outcomes in chronic wound therapies. Mesenchymal stem cells, given their immunomodulatory and angiogenic properties, have therefore been studied extensively with regards to cell therapy to supplement wound dressings. With over 500 listed clinical trials for MSC therapies as of September 2015 (clinicaltrials.gov),

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many include studies utilizing MSCs for healing ischemic/diabetic foot ulcers and similar wounds (Table 3).

Ultimately, this interest in MSCs for cell therapies in wound healing revolves around several key aspects, including immunosuppression, angiogenesis stimulation, and scar reduction. As MSCs play a normal role in the wound healing process (as evidenced by ~ 1% of MSCs homing to wound sites upon intravenous injection) (Sasaki, Abe et al. 2008), they are an obvious candidate for study in this context as opposed to embryonic stem cells or other regenerative sources. Recent studies have outlined some successful approaches to promoting wound healing with MSCs, including autologous bone marrow-derived MSCs in fibrin matrix (Falanga, Iwamoto et al. 2007) or, more recently, intramuscular injection of autologous MSCs to improve diabetic wound closure (Wan, Xia et al. 2013). While some trials have been aimed primarily at safety of MSC use for wound healing (Kirana, Stratmann et al. 2012), several clinical trials have shown the potential benefit of MSCs for inclusion in wound healing devices, including improved average rate of wound healing and general limb perfusion after treatment (Lu, Chen et al. 2011) and also improved acute wound healing correlating to the number of injected cells (Falanga, Iwamoto et al. 2007). Despite any effects on healing, there was some doubt as to any reduction in limb amputation rate or relative pain levels among groups, a major consideration for effective therapy in chronic wounds (Lu, Chen et al. 2011). In general, the consensus from completed trials has been an overall improvement in chronic wound closure with application of mesenchymal stem cells, particularly as a part of a matrix delivery system (wound gel, etc.).

Table 3. Clinical trials for MSCs and chronic wounds. A sample of currently listed international clinical trials involving mesenchymal stem cells and applications in advancing chronic wound healing. Data collected from clinicaltrials.gov; reference ID's refer to listings from that database. Table adapted from (Nuschke 2014).

Study	Trial ID	Research Location	Status
Induced Wound Healing by Application of Expanded Bone Marrow Stem Cells in Diabetic Patients With Critical Limb Ischemia	NCT01065337	Ruhr University of Bochum, Bochum, DE	Completed (Kirana, Stratmann et al. 2012)
Human Adipose Derived Mesenchymal Stem Cells for Critical Limb Ischemia in Diabetic Patients	NCT01257776	University Hospital Virgen Macarena, Sevilla, ES	Unknown
Umbilical Cord Mesenchymal Stem Cells Injection for Diabetic Foot	NCT01216865	Qingdao University, Qingdao, CN	Unknown
Autologous Bone Marrow Stem Cell Transplantation for Critical, Limb-threatening Ischemia (BONMOT)	NCT00434616	Franziskus Hospital Berlin Vascular Center, Berlin, DE	Unknown
Autologous Bone Marrow Stem Cell Transfer in Patients With Chronical Critical Limb Ischemia and Diabetic Foot	NCT01232673	University Hospital Ostrava, Ostrava, CZ	Completed (Prochazka, Gumulec et al. 2010)
Study of the Effectiveness of Autologous Bone Marrow-Derived Mesenchymal Stem Cells in Fibrin to Treat Chronic Wounds	NCT01751282	Roger Williams Medical Center, Providence, RI	Completed; Recruiting for next phase (Falanga, Iwamoto et al. 2007)
Comparison of Autologous Mesenchymal Stem Cells and Mononuclear Cells on Diabetic Critical Limb Ischemia and Foot Ulcer	NCT00955669	Third Military Medical University, Chongqing, CN	Completed (Lu, Chen et al. 2011)
The Role of Lipoaspirate Injection in the Treatment of Diabetic Lower Extremity Wounds and Venous Stasis Ulcers	NCT00815217	Washington D.C. Veterans Affairs Medical Center	Unknown
Safety Study of Stem Cells Treatment in Diabetic Foot Ulcers	NCT01686139	Sheba Medical Center, Ramat Gan, IL	Pre-recruitment
Intramuscular Mononuclear Cells and Mesenchymal Stem Cells Transplantation to Treat Chronic Critical Limb Ischemia	NCT01456819	UKM Medical Centre, Kuala Lumpur, MY	Recruiting

1.2.2 Extracellular matrix and wound healing

Another key element to wound healing in all contexts (as well as dynamics of MSC utility in wound healing) is the extracellular matrix. Wound healing is a highly orchestrated process that involves multiple developmental lineages, cell types, and local and systemic effects. Not only do the resident parenchymal cells and their stromal counterparts need to be replaced, but the support structures of the vascular, nervous and immune systems must be re-established. The process has been extensively studied in the skin and mucosal surfaces as these sites are the most often wounded both traumatically and iatrogenically. While most surface wounds heal with near regenerative repair, regaining the vast majority of pre-wound functionality, the ubiquity of such insults, particularly in individuals with comorbidities and advanced age, means that wounds that 'fail to heal' or heal excessively (scarring/keloids) remain major medical issues.

Repair of this tissue system is also the best-described as the skin is readily accessible for both wounding and longitudinal observation with easy, repeated sampling. As most wounds heal with little to no complication, such studies have been undertaken in human volunteers. What has emerged is a process that has been parsed into overlapping stages: initial hemostasis to quickly seal the breach and prevent desiccation and infection (hemostatic phase), tissue regeneration to replace the lost cells (tissue replacement phase), and finally wound resolution to restore the diverse functions of the skin and remodel the new matrix (resolving phase) (Figure 1) (Yates, Hebda et al. 2012).

These phases, which occur at different rates across the wound, have been considered from multiple angles. Many conceptions of wound healing focus on either the cell types, soluble signals, or structures that predominate during each phase. However, the reality is that each of these not only are present but impact each other. For instance, the hemostatic stage includes both

the initial platelet plug to prevent bleeding and form a barrier eschar and the lysate-attracted inflammatory infiltrate of hematopoietically-derived immune cells to prevent infection and limit colonization. The soluble signals present late in this stage herald the invasion and expansion of the formed elements as well as signal to relevant cells in the existing wound space, both of which mark the tissue replacement phase during which the tissue is reformed, but only once the appropriate and supportive matrix is generated. Subsequent to this, various signals released by the maturing structures (functional vessels and redifferentiated epidermis) induce both the quiescence of the cells in the tissue and involution of excess cells (keratinocytes and dermal fibroblasts) and structures (immature vessels), to revert to a relatively avascular and quiescent skin that is reinforced by a matrix suppressive to cell proliferation and migration. This parallels the concept of 'dynamic reciprocity', first described by Bissell et al in 1982 (Bissell, Hall et al. 1982), describing the critical interaction of key components of the healing process whereby each cellular or matrix element signals between one another to that ultimately drives resolution. Specifically, in skin healing the dynamic reciprocity between cells and the surrounding microenvironment has been shown to play a role in many aspects of healing, including differentiation, migration, proliferation, and others (reviewed recently in (Schultz, Davidson et al. 2011)).

An outline of major matrix elements known to be involved in or produced during wound healing can be found in Table 4. The initial fibrinogen-cleaved fibrin clot contains the plasma proteins entrapped during hemostasis. The highly pliant fibrin, accompanied by more adhesive vitronectin, supports the invasion of macrophages and other leukocytes. In addition to serving to limit infection, the proteases produced by the cells breaks down the suppressive boundary matrices that limit subsequent tissue ingrowth. The leukocyte elastases released into the wound

Table 4. Matrix and matricellular proteins during wound repair. For details on many of the molecules noted, please see current reviews (Merline, Schaefer et al. 2009; Midwood, Hussenet et al. 2011; Neill, Schaefer et al. 2012; Stoffels, Zhao et al. 2013; Grahovac and Wells 2014; Halper and Kjaer 2014; Murphy-Ullrich and Sage 2014). Table adapted from (Wells, Nuschke et al. 2015).[4]

Matrix/Matricellular Protein	Phase(s)	Main Producer Cell(s)	Cellular Effects	Notable Cellular Receptors
Collagen I	**Tissue Formation Tissue Remodeling**	Epithelial, Endothelial Fibroblasts, Smooth Muscle	Tissue architecture, Tensile strength Suppresses cell immigration Transitions fibroblasts to myofibroblasts	Integrins (mainly α2β1, α11β1), DDR1 and DDR2, LAIR1, Glycoprotein VI
Collagen III	**Tissue Formation**	Epithelial, Endothelial Fibroblasts	Tissue architecture Promotes fibroblast migration Supports angiogenesis	Integrins (mainly α2β1, α11β1), Glycoprotein VI, LAIR1, DDR1 and DDR2
Fibrinogen/Fibrin	**Homeostasis Tissue Formation**	Platelets Leukocytes, Endothelial (from plasma)	Cell-Cell interaction, Cell-Matrix Platelet –protein, Promotes cell migration	αIIbβ3 integrin, VE-cadherin
Hyaluronan	**Tissue Formation Tissue Remodeling**	Epithelial Fibroblasts Smooth Muscle	Limits matrix stiffness Decreases cell proliferation Induces fibroblast differentiation	CD44, ICAM-1
Fibronectin	**Homeostasis Tissue Formation Tissue Remodeling**	Epithelial, Endothelial Fibroblasts, Leukocytes,	Tissue architecture Promotes cell proliferation and migration Increases opsoninization	Several Integrins (mainly α5β1, avβ3)
Tenascin C	**Tissue Formation**	Epithelial, Fibroblasts	Modulates cell-matrix Anti-adhesive, Promotes migration Promotes proliferation, Angiogenesis	αvβ3 and α9β3 Integrins, annexin II, EGFR
Decorin	**Tissue Formation Tissue Remodeling**	Fibroblasts	Stabilizes collagen matrix Limits cell proliferation Negates growth factor signaling	EGFR, Met, IGF-IR, TLR2/4
Entactin (or nidogen)	**Homeostasis Tissue Formation**	Epithelial, Fibroblasts Neutrophil	Basement membrane component Stabilizes basement membrane	α3β1 Integrin
Laminin	**Tissue Formation Tissue Remodeling**	Epithelial, Endothelial Fibroblasts, Smooth Muscle, Platelets, Monocyte	Basement membrane component Limits cell compartmentalization	67LR, various integrins (α2β1, α3β1, α7β1, α6β4)
Thrombospondin	**Homeostasis Tissue Formation**	Platelets, Fibroblasts, Smooth Muscle cells	Angiogenesis Alters cell proliferation and migration (Both context dependent)	CD36, β1 integrins, IAP-mediated interactions
SPARC	**Tissue Formation**	Fibroblasts	Promotes cell proliferation and migration	α5β1 Integrin, stabilin-1, V-CAM

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bed degrade molecules suppressive to angiogenesis (Li, Brooke et al. 1998; Cowan, Jones et al. 2000) while also affecting fibronectin degradation and protease inhibitor activity in more chronic wounds. In addition, matrix metalloproteinases (MMPs) degrade collagen I that limit stromal migration (Allen, Asnes et al. 2002; Smith, Wells et al. 2006). In this manner, the suppressive small leucine-rich glycoproteins (SLRPs) decorin and lumican are untethered and removed from the adjacent matrix. MMPs also provide other functions to the wound healing process, such as regulating mediators of inflammation via establishment and modification of chemokine gradients (Rodriguez, Morrison et al. 2010) .

The resident cells prime the adjacent ECM allowing for immigration and proliferation of stromal cells, while also producing fibronectin, tenascin, entactin and thrombospondin (in addition to these elements that may pre-exist in the wound bed). The latter matrix elements come together to form a provisional ECM with both adhesive and anti-adhesive properties allowing for optimal locomotion of the stromal cellular components (fibroblasts, endothelial cells and keratinocytes)(Murphy-Ullrich 2001). Tenascin and other proteins are mainly at the edge of the advancing wound front wherein they establish a pro-migratory environment, serving also to enhance inflammatory cell adhesion and migration early in the healing process (Chiquet-Ehrismann, Orend et al. 2014). These matricellular proteins in turn effect growth factor signaling through receptor binding motifs within the proteins themselves (matrikines), which signal via the EGF receptor to induce motility (Swindle, Tran et al. 2001; Iyer, Tran et al. 2008; Grahovac and Wells 2014; Shao, Li et al. 2014) In the same vein, these components also may contain growth factor binding sites that similarly regulate signaling, such as those found in fibronectin (Zhu and Clark 2014). As the fibroblasts immigrate and establish in the wound site,

they produce collagen III and collagen I to provide further structural integrity to the matrix, with fibronectin acting in this important case as a scaffold for collagen deposition (Kadler, Hill et al. 2008). Along with these collagens, SLRPs become present again, limiting the signaling through motility-triggering growth factor receptors (Reed and Iozzo 2002; Neill, Schaefer et al. 2012). At the skin surface, dedifferentiated keratinocytes re-epithelialize the wound by ingrowth on top of this matrix. Behind the leading edge, the contact-inhibited keratinocytes are induced by unknown signals to contribute collagen IV and laminins to the delineating basement membrane, which in turn anchors the keratinocytes by hemidesmosomes, further driving maturation (Gipson, Spurr-Michaud et al. 1988; Clark 1996; Singer and Clark 1999).

The transition to the resolving phase is marked by dramatic involution of the excess cells of all types that appear during the tissue replacement phase. This coincides with the change in the matrix from a supportive, Tenascin C and collagen III-predominant matrix to one in which collagen I and SLRPs constitute the dominant matrix proteins. This stiffer matrix in turn drives the stromal cells to take on a myofibroblast phenotype and further produce fibrillar collagen (Georges, Hui et al. 2007). This combination of collagen fibrils and myofibroblasts results in wound contraction, as the motility signals are channeled to transcellular contractility (Allen, Asnes et al. 2002). This ultimately results in a scar with overly aligned collagen fibrils that regains only about 80% of the strength of the unwounded skin. Still, as the skin regains most functionality and is not overly cellular or collagenous, this is considered as normal wound healing (Figure 2).

1.2.3 Matrix in dysrepair

Dysrepair falls mainly at the two ends in the process – inability to repair the wound (failure to heal) and over-exuberant healing (scarring). Counter-intuitively, both of these medical issues can be traced to excessive matrix turnover. Chronic ulcers are found to have increased activity of MMPs and other matrix-degrading proteases (Raffetto 2009). This has even led to suggestions that protease inhibition be a part of chronic wound treatment (Raffetto 2009). At the other end, we and others have found persistence of MMP activity in hypertrophic scars (Yates, Whaley et al. 2007; Yates, Bodnar et al. 2011; Djafarzadeh, Notohamiprodjo et al. 2013; Rohani and Parks 2015). This does not take into account the failure to initiate healing in persons whose skin is highly compromised due to advanced age and severe metabolic derangements (including persons with malnutrition and widespread metastatic cancer), nor select genetic defects. Furthermore, vascular compromise including advanced arteriosclerotic conditions, such as those found in extremities in long-standing type II diabetes, present barriers to healing that lie outside the matrix issues, and thus require reestablishing adequate blood flow to enable any healing to occur.

Both failure to heal the wound and scarring are marked by matrix turnover disrupting the normal processes. Non-healing ulcers are stalled in matrix generation and maturation. The open wound becomes compromised as it is colonized by the skin microbiome (Grice and Segre 2011; Grice and Segre 2012). Signals from microbiome products maintains a level of hematopoietically-derived immune cell infiltration. Both the leukocytes and microbes produce proteases that degrade the provisional matrix. These protein fragments further attract leukocytes and keep the stromal cells in a synthetic mode, generating matricellular proteins. The initiating event is still unclear, whether it is colonization/infection, excessive inflammatory infiltrate, or

matrix turnover, though the ongoing failure to heal clearly has a matrix component that is critical to the pathological feed-forward loop.

Scarring results from the failure to appropriately terminate the healing process (Figure 2, Figure 3). The presence of excess fibrillar collagen in both hypertrophic scars and keloids belies the active turnover that led to the accumulation. Proteases are found to persist in scar tissues. MMP-2 in particular, strangely in conjunction with its inhibitors TIMP-1 and TIMP-2, is found in human burn and hypertrophic scars (Ulrich, Ulrich et al. 2010), whereas MMP-9 appears to correlate with scar resolution (Reno, Grazianetti et al. 2002). Other MMPs, particularly MMP-1 have been proposed as therapeutics to break down the fibrillar collagen to reduce scars. The reasons for this excessive accumulation of collagen I are uncertain, but once started, the process may be cyclical (Yates, Bodnar et al. 2011). Excessive tissue transglutaminase not only leads to the cross-linking of the collagen fibrils, but also directly or indirectly protect the stromal cells from apoptosis, thereby increasing the synthetic period of scars (Linge, Richardson et al. 2005).

Given the role of inflammation and scarring in extracellular matrix disrepair in wound healing, treatments for chronic or dysregulated wounds must account for these important elements. It is because of these factors that MSCs, which have the potential to modulate this inflammation and attenuate scarring outcomes, have been examined so closely in relation to treatments for skin repair.

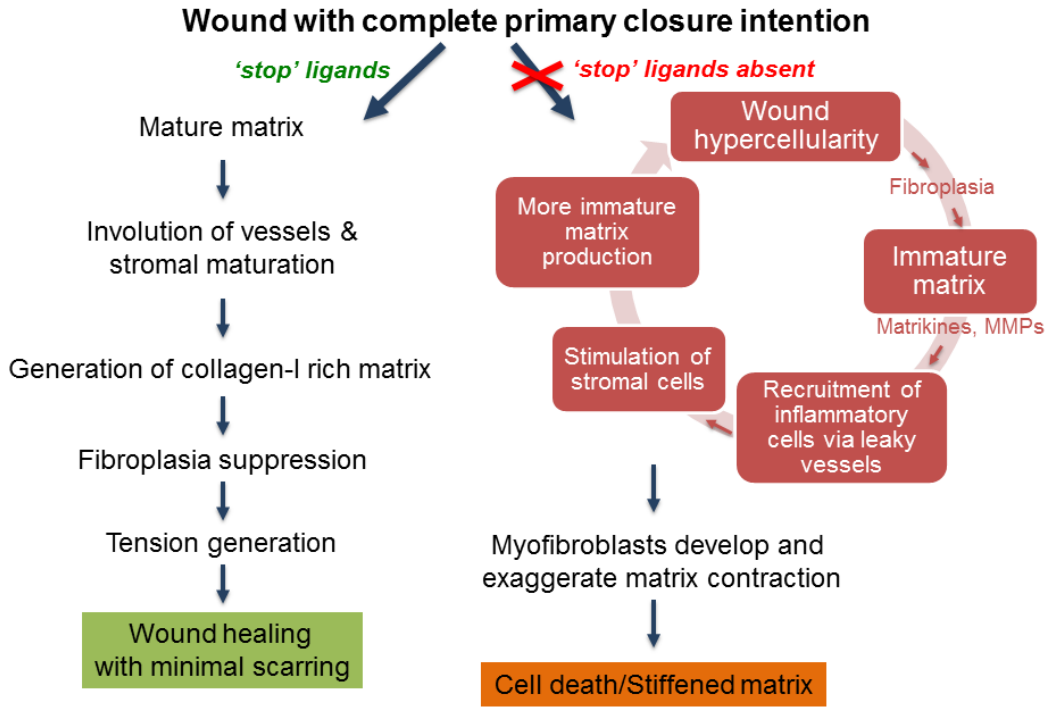

Figure 3. The 'scarring cycle' schematized. Hypertrophic scars are regenerated from a persistence of the tissue replacement phase, in the absence of 'stop signals' such as ligands for CXCR3 or angiopoietins even after full re-epithelialization and seeming 'wound closure' (right arm). This hypercellularity with active matrix turnover re-initiates a chronic inflammatory milieu which in turns drive more immature matrix production, eventually leading to excessive but poorly functional fibrillar collagen. Figure adapted from (Wells, Nuschke et al. 2015).[5]

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1.3 MESENCHYMAL STEM CELL UTILITY IN WOUND HEALING

1.3.1 Immunomodulation

Chronic hyperinflammation in the wound bed is the most substantial barrier to treatment in non-healing wounds and other clinical maladies, as outlined previously. While corticosteroid treatment seems an obvious choice to combat this issue, steroid treatments for wound beds have shown negative impacts on wound repair due to such effects as reduced TGF-β and IGF-1 levels in wound fluid (Wicke, Halliday et al. 2000) and decreased procollagen mRNA synthesis (Perez, Shull et al. 1992). Mesenchymal stem cells have recently been shown to hold a variety of immunomodulatory effects on host immune cells in both wound healing and transplant biology contexts. These characteristics are potentially what make MSCs the most attractive cell type for cell therapy in chronic wounds, as they exert pleiotropic effects on the inflammatory mechanisms to move the wound past static inflammation and fibrosis.

It has been known for some time that donor MSCs are able to suppress host T cell proliferation, a key activity in reducing wound bed inflammation (Beyth, Borovsky et al. 2005). More recently, this was demonstrated to be dependent on MSC induction of IL-10 (Yang, Park et al. 2009) in native T cells and macrophages, as well as TGF-β activity (Nasef, Chapel et al. 2007). Additionally MSCs have been shown to be capable of modulating host TNF-α production to mediate excessive inflammatory effects, and reduce NK cell function in the inflammatory phase, lowering IFN-γ activity in the process (Aggarwal and Pittenger 2005). Conversely, in the later stages of inflammation, active TGF-α is able to stimulate implanted MSCs to produce a variety of pro-healing growth factors and cytokines, including VEGF to stimulate angiogenesis in the wound bed (Wang, Crisostomo et al. 2008). In 2008 Ren et al showed a dependence on

pro-inflammatory factors for these processes to be effective, suggesting a potential time window for application of allogeneic MSCs to be efficient in reducing inflammation (Ren, Zhang et al. 2008).

Importantly, recent research into the immune response to allogeneic MSCs has shown that in most systems, the donor MSCs are 'immunoprivileged' and do not induce a significant response in the host, suggesting that allogeneic cell sources may be possible for chronic wound therapies, where diabetic patients may have already-defective endogenous MSC populations making autologous therapy less than optimal. This characteristic of allogeneic MSCs is crucial in this particular wound environment, where excessive inflammation already drives the chronic phenotype and additional immune response from cell implantation must be as low as possible. However, several studies have shown that this immunoprivileged characteristic is lost as the MSCs differentiate, leading to a gradual host response to the implanted cells (Huang, Sun et al. 2010; Li, Li et al. 2013). Thus, approaches to keeping MSCs undifferentiated may be key in future chronic wound therapies.

MSCs have also recently been identified as having antimicrobial effects, a significant advantage in reducing effects of any contaminants in the wound during injury and treatment (Mei, Haitsma et al. 2010). This was identified in 2008 by Krasnodembskaya and colleagues (Krasnodembskaya, Song et al. 2010) as a mechanism based on secretion of LL-37, a peptide with a wide array of antimicrobial properties including broad spectrum microbial defense via disruption of bacterial cell membranes (Turner, Cho et al. 1998) and directly limiting bacterial macrophage activity via upregulation of chemokine receptors, all while ignoring pro-inflammatory cytokine activation (Scott, Davidson et al. 2002). In terms of cell therapeutics, the concern for reducing infection is great and many products attempt to seal wounds with silicone

barriers to dressings. Silver nanoparticles have also been examined for antimicrobial properties, which can be conveniently included in wound healing gels and allowed to leach into the wound locally (Yates, Whaley et al. 2007; Thawatchai, Seiichi et al. 2008). Combined with MSC antimicrobial activity, this would help to reduce any additional inflammation seen during the healing process.

As a whole, all of the effects produced by MSCs here help to solve the problem of chronic hyperinflammation in the wound bed and advanced wounds such as diabetic ulcers into the next stages of wound healing. MSCs also have potential to improve the common issue of inhibited perfusion that plagues these wounds as well through paracrine activity. Allogeneic application of MSCs in gel-based products for wound healing holds promise for combatting these issues, as has been done in various applications for MSC therapy using fibrin-based gel systems (Bensaid, Triffitt et al. 2003; Catelas, Sese et al. 2006) and related mimetics (Daniel, Nancie et al. 2013).

1.3.2 Paracrine activity and angiogenesis

Revascularization of the wound bed is a crucial stage of the normal wound healing process, where new vessels form as granulation tissue develops to supply blood to the wound area, which is in need of oxygen and nutrients. Endothelial cells therefore need to be able to move through the dermis of the wound and form tubes in the newly developing tissue, a process that is balanced by the growth factor production cascade during wound healing. Mesenchymal stem cells play a normal role in this process as they are recruited to the wound bed following mobilization from endogenous sources (Badiavas, Abedi et al. 2003). The ability of mesenchymal stem cells to promote angiogenesis in vivo is not necessarily unique, as several

other cell types have been shown to been integral in stimulating angiogenesis via cell therapy, such as hematopoietic stem cells (Takakura, Watanabe et al. 2000) or resident cardiac progenitor cells (Chun, O'Brien et al. 2013). However, the unique role of MSCs during normal wound repair and additional effects of MSCs discussed in this review make the application of MSCs to stimulate vessel growth in chronic wounds particularly interesting for future studies in clinical MSC application for wound therapy.

There is evidence that MSCs can differentiate into a variety of skin cell types, contributing to repopulation of the wound bed with normal dermal structure, as well as endothelial cells to yield new vessels (Wu, Chen et al. 2007; Sasaki, Abe et al. 2008). Recently several groups have focused on differentiation of MSCs into endothelial cells, an approach that has potential to be useful in direct transplant into anti-angiogenic environments such as ischemic wound beds. Results have shown endothelial-like cell populations derived from human MSCs in vitro with varying degrees of donor variation (Janeczek Portalska, Leferink et al. 2012; Portalska, Groen et al. 2013), while Bago et al showed similar results for amnion-derived MSCs in glioma tumors (Bago, Alieva et al. 2013). Furthermore, pericytes that stabilize vessel walls and promote vessel maturation during angiogenesis have been shown to be derived from bone marrow populations following injury (Kokovay, Li et al. 2006). Recent evidence suggests that these pericytes in fact represent a sub-population of mesenchymal stem cells that contribute to the healing process (Blocki, Wang et al. 2013). These cells all have the potential to support new vessel growth in a chronic wound bed, a critical aspect of overcoming barriers to current therapies as vessel growth is otherwise inhibited.

Perhaps more important than differentiation, secreted factors also play a substantial role in MSC regulation of angiogenesis in the wound bed. Chronic wounds are often subject to anti-

angiogenic conditions, including reduced growth factor production as a result of increased MMP production in the wound bed, as outlined by Krisp et al recently in a global secretome analysis of wound exudates (Krisp, Jacobsen et al. 2013). MSCs naturally produce a variety of pro-angiogenic factors following recruitment to the wound bed that stimulate endothelial cell proliferation and tube formation in the wound bed, most notably VEGF, a potent stimulator for angiogenesis that is regulated by IL-6 and TGF-α in the wound bed (Herrmann, Weil et al. 2011). Though it has been shown that exogenous VEGF application to wounds can stimulate angiogenesis (Galiano, Tepper et al. 2004), MSCs used in cell therapeutics also have been shown to stimulate EC recruitment and wound healing via VEGF secretion (Wu, Chen et al. 2007; Chen, Tredget et al. 2008) or via pre-differentiation into angiogenic precursors (Roura, Bagó et al. 2012). Ultimately, MSCs are able to stimulate de novo angiogenesis in wound beds upon transplantation, a crucial factor in stimulating healing in chronic wounds that lack this normally due to the hyperinflammatory environment.

1.3.3 Reduction in scar formation

Another consideration in repair of wounds under all circumstances is the formation of scars, caused by deposition of excess ECM by fibroblasts in the wound bed. These structures carry a variety of undesirable consequences, including unsightly appearance on the skin and, more critically, scars lack many of the normal makeup of the skin such as follicles and nerve endings and also do not retain the normal tensile strength of undamaged skin (Singer and Clark 1999). While scar reduction research has been a field in of itself for quite some time, it is a notable consideration for patients with large non-healing ulcers.

As discussed previously, anti-inflammatory mechanisms of MSCs have several effects on fibrotic phenotypes in the wound, and thus play a major role in reducing scar formation following wound healing. Most notably, MSC production of PGE2 drives a variety of changes in the scarring phenotype. PGE2 from MSCs has been shown to increase secretion of IL-10 by T cells and macrophages (Nemeth, Leelahavanichkul et al. 2009), an important anti-inflammatory cytokine in the wound environment. PGE2 secreted by MSCs in response to the inflammatory wound bed plays a crucial role in the healing process, reducing T cell migration and NK cell proliferation during the inflammatory phase (Spaggiari, Capobianco et al. 2008; Bouffi, Bony et al. 2010). The upregulation of IL-10 in the wound by MSCs also has a multitude of effects on general scar formation, including downregulation of IL-6 and IL-8 to reduce collagen production in the wound (Liechty, Kim et al. 2000) and inhibition of neutrophil invasion and macrophage activity to suppress reactive oxygen species (ROS) generation (Sato, Ohshima et al. 1999), all leading to support of regenerative healing in recent experimental scar formation models (Peranteau, Zhang et al. 2008). ROS generation is also affected by nitric oxide secreted by MSCs, acting as a scavenger to prevent the fibrotic activity of the oxygen radicals (Senel, Cetinkale et al. 1997; Schafer and Werner 2008). Though these anti-inflammatory mechanisms are part of normal MSC function following homing to acute wound sites, the hyperinflammatory environment of a chronic wound makes the MSC ability to modulate excessive inflammation and reduce excessive scarring critical. Ultimately, reduced scar formation is not an outcome desired specifically for chronic wounds, but nevertheless is a significant potential benefit of utilizing MSCs to promote closure of such non-healing wounds. Experimentally, recent experimental evidence has shown that MSCs can indeed reduce a fibrotic phenotype in a bleomycin fibrosis

mouse model (Wu, Huang et al. 2013), showing promise for reduced scar formation in future MSC therapeutics.

Mesenchymal stem cells also produce a variety of anti-fibrotic factors throughout the wound healing process. Aside from IL-10, HGF is a major contributor to reduced fibrosis, which has been shown to be effective in advancing clean wound healing in a variety of tissues such as liver (Yu, Lu et al. 2010) and skin (Ono, Yamashita et al. 2004; Satish, Babu et al. 2004). HGF has also been attributed to chronic wounds, with differential regulation of HGF production and presence of c-Met in chronic wound dermis (Conway, Ruge et al. 2007). Specifically in relation to fibrosis, HGF has been demonstrated to reduce TGF-β and collagen production in fibroblasts (Mou, Wang et al. 2009), and also have a multitude of effects on cell recruitment to the wound bed, including endothelial cells and promotion of keratinocyte migration (Bevan, Gherardi et al. 2004). Ultimately, HGF production by transplanted MSCs would yield a more normal state of cell migration and matrix production than what is normally seen in chronic wound beds.

Together in concert with the other immunomodulatory mechanisms of MSC function in wound repair, addition of MSCs to chronic wounds may prove to be an effective means of promoting cleaner healing on a smaller time scale than traditional treatments.

1.4 LIMITATIONS IN MSC CLINICAL UTILITY

In typical cutaneous wound healing, MSCs are mobilized from host sources and home to the site of injury, persisting to support immunomodulation and improved angiogenesis in the wound bed as the skin repairs itself. These host MSCs are able to perform these normal functions

despite somewhat challenging conditions in the wound site, such as hypoxia or lack of nutrients. However, in the case of chronic wounds, the normally-ischemic wound environment becomes even harsher, with excessive inflammation (Khanna, Biswas et al. 2010) and an environment not conducive to angiogenesis compared to normal wounds (Krisp, Jacobsen et al. 2013). Therefore, a significant barrier to successful use of MSCs in any potential cell therapy has been post-implant cell survival in a variety of ischemic injury models. Past studies have shown marginal MSC preservation in various models including cardiac infarct (Wang, Pasha et al. 2013) or cerebral injury (Tang, Cai et al. 2013), but still MSC use in any of these models is limited by MSC death due to the harsh wound environment. As all of the benefits of MSC therapy for any type of wound healing are dependent on cell survival in the wound, strategies to improve this survival following implantation are of interest in future research efforts. Recent studies have examined the effectiveness of preconditioning human MSCs with varying oxygen concentrations or pan-caspase inhibitors to improve the MSC survival response immediately following implantation. Saini et al showed hyperoxic and pan-caspase pre-treatment of the cells substantially decreased MSC apoptosis in a cardiac infarct model, a scenario that produces an ischemic environment for implanted MSCs (Saini, Gumina et al. 2013). Conversely, Chang et al recently demonstrated the advantages of preconditioning MSCs in hypoxia, which was shown to improve the secretory capabilities of the cells (VEGF, HGF, and others), a main benefit of MSC therapy (Chang, Chio et al. 2013). Gene therapy in MSCs has also received some attention, as Wang et al recently showed that adenoviral upregulation of protein kinase G1α improved MSC survival following implantation into a similar cardiac infarct model (Wang, Pasha et al. 2013).

Additional recent studies have examined the possibility of exploiting endogenous signaling pathways in promoting MSC survival in a variety of wound healing contexts. The

possibility of activating pro-survival pathways via matrikine moieties is a relatively novel concept that has been demonstrated to affect MSC signaling during wound healing normally (Tran, Griffith et al. 2004). This research led to the idea that EGFR could be activated by EGF molecules tethered to growth scaffolds, which was shown to improve MSC survival in vitro during cell death assays (Fan, Tamama et al. 2007; Rodrigues, Blair et al. 2013). This system of activating EGFR artificially to promote survival signaling has been applied to several tissue engineered surfaces, and provides the MSCs with a variety of survival advantages that could be used to combat the ischemic wound environment. More recently, Rodrigues et al showed that the matrix protein Tenascin C could produce similar effects in vitro (Rodrigues, Yates et al. 2013). Tenascin is easily incorporated into collagen-based scaffolds, and could potentially be combined with current therapeutic gels to modulate MSC survival. This could be beneficial in scaffold design for MSC delivery to chronic wound beds, as biomaterials used in scaffold design can at times produce a more robust artificial inflammatory response.

Nevertheless, the limited survival seen in many clinical studies utilizing MSCs remains a great challenge. There are several common categories of challenges these cells face upon implantation that may have deleterious effects on cell survival, and thus are factors of great interest in examining MSC utility as it relates to *in vivo* challenges (Figure 4).

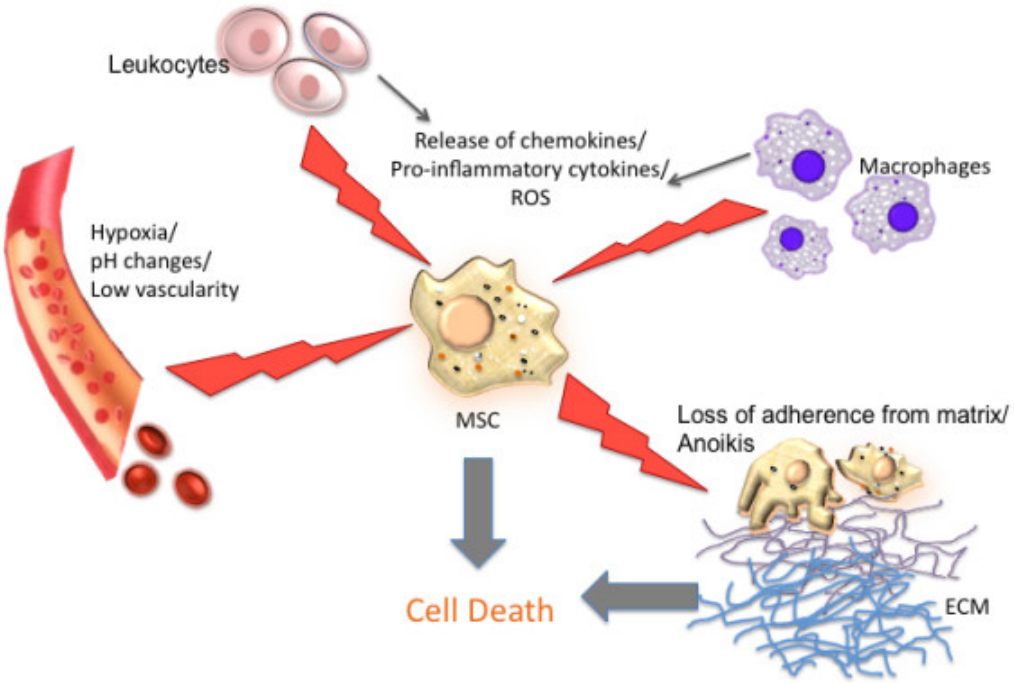

Figure 4. Various threats multipotential stromal cells face at the delivery site leading to loss of cells. Leukocytes and macrophages that are brought into the MSC implantation site as part of the nonspecific inflammatory response can secrete proinflammatory cytokines and chemokines within the first few days of healing, as well as increase cellular stress and generate reactive oxygen species (ROS) that might activate apoptotic cascades in a comparable time frame. Wound and ischemic sites where MSC therapy is usually required are low in vascularity and are hypoxic regions, which might add to MSC cell stress throughout the course of the healing process. Also, lack of attachment of MSCs to the extracellular matrix (ECM) may cause MSCs to detach, undergo anoikis and ultimately lead to cell death. Figure adapted from (Rodrigues, Griffith et al. 2010), Figure 1.[6]

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1.4.1 Inflammation

Despite their immunoprivilege and apparent ability to modulate immune responses (particularly in transplant biology) (Aggarwal and Pittenger 2005), innate and acquired immune responses to MSC implant remain a significant hurdle to survival in the first week of post-implant healing. Given that often times MSCs in chronic wound patients such as diabetics are dysfunctional and not ideal for autologous use (Khan, Akhtar et al. 2011), allogeneic cell sources are frequently leveraged for MSC isolation and expansion prior to treatment. Unsurprisingly, this allogeneic transplant of donor MSCs has been shown to induce a host immune response, including induction of a memory T-cell response (Nauta, Westerhuis et al. 2006)that can lead to rejection of the implanted cells. Some studies have also suggested subsets of MSCs that can be anti- or pro-inflammatory through specific toll-like receptor (TLR)-based downstream polarization of the cells, such as TLR-4 primed MSCs drastically inducing IL-6 production while TLR-3 primed MSCs ablated this effect (Waterman, Tomchuck et al. 2010). Although allogeneic inflammatory responses can be limited by the use of autologous MSC transplants, local inflammation at the implant site is ultimately a significant concern for MSC survival. This is especially true in the case of severe wounds such as chronic skin ulcers, which are typically the areas of interest for MSC implantation. The need for allogeneic MSC treatment is therefore clear in the context of wound healing, as well as strategies to combat cell death from such immune responses.

1.4.2 Nutrient deprivation

Perhaps a more substantially limiting factor in MSC survival is a lack of nutrients and vascular supply in the implant site. This is especially significant due to the relevant uses of MSCs, as sites such as chronic wound beds, non-healing bone defects, and others often have a severely limited angiogenic response. It is a well-established fact that blood supply to wounded tissue is critical to the healing process. Blood flow supplies nutrients (such as oxygen) to the wound site, where cells lining the injured region are metabolically active and require energy. In impaired healing, such as diabetic ulcers of the skin, one of the common pathologies is reduced blood flow to the wound site resulting in a lack of available nutrients, as well as inhibited angiogenesis in general leading to poor regenerative outcomes (Falanga and Sabolinski 1999; Falanga 2005).

Given that MSCs are almost always culture-expanded prior to implant into these sites, the cells have become adapted to abundant nutrient supplies and very little stress. Upon implant into these harsh nutrient-poor conditions, the cells are forced to re-adapt to wound conditions, where blood flow is limited and available nutrient levels, such as oxygen, glutamine, and others, are scarce. In addition, it is often the case that patients receiving these treatments have underlying nutrient deficiencies that propagate to the wounds themselves, such as reduced vitamin levels or carbohydrate depletion (Arnold and Barbul 2006). These factors combine to yield a highly unfavorable environment for the implanted MSCs, and contribute heavily to the limited cell survival cited in many clinical studies using the cells recently.

1.4.3 Changes in oxygen tension

Outside of general nutrient considerations, a major shock that implanted MSCs often face is changes in oxygen tension. Despite the fact that primary MSCs are extracted from tissue at physiologic oxygen tensions (~5% O_2), these cells are almost always plated in culture and expanded for several passages, where they are typically exposed to ambient oxygen conditions (unless a hypoxic chamber is used during culture). As such these cells become adapted to higher oxygen conditions than exist in implant sites; even a physiologic oxygen tension of 7% is vastly lower than the ambient 21% oxygen typically used for cell culture. In general, the effect of oxygen levels on cell culture has been well-documented. Glioblastoma cells, for example, were found to have improved stem-like qualities at physiologic oxygen tension (McCord, Jamal et al. 2009), and primary neurons were shown to have altered mitochondrial function in response to changing oxygen culture levels (Tiede, Cook et al. 2011).

The difference between ambient and physiologic oxygen tensions become even more pronounced in the context of MSC therapies and wound healing, where available oxygen levels are even lower in tissue such as chronic wound beds (5-20 mm Hg) than typical skin (Schreml, Szeimies et al. 2010). Both *in vitro* and *in vivo* studies have shown MSC death from hypoxic conditions, including in a myocardial infarct site, a key area of MSC cell therapy research (Zhu, Chen et al. 2006; Hu, Yu et al. 2008). As such, many researchers have looked to combat this through hypoxic preconditioning of MSCs, which has shown benefit in that it improves MSC proliferation and function (Grayson, Zhao et al. 2007) and also enhances MSC survival (Hu, Yu et al. 2008) and therapeutic potential (Rosova, Dao et al. 2008). Despite the benefit of hypoxic exposure prior to implant, culture-expanded MSCs face a difficult challenge in adapting to

severely low oxygen tensions in wound beds, and strategies to improve survival must account for this factor.

1.5 STRATEGIES TO IMPROVE MSC SURVIVAL

Given the long list of challenges MSCs face upon implantation, and the correlated studies that have shown just how limited MSC survival in fact is *in vivo,* research into both engineered and cellular approaches to improving MSC survival are critical. A number of mechanisms exist already in the cell that are activated in response to stress or death stimuli, helping the cell to redirect metabolic load to key processes for survival, such as the epidermal growth factor receptor (EGFR) signaling cascade. Other mechanisms also exist in the cell for producing energy during times of stress, including the process of autophagy, which recycles debris in the cell and is highly active during starvation or other harsh conditions. These pathways that are already in place and well characterized in the cell biology field are excellent candidates for options to exploit and improve MSC survival, ultimately increasing longevity and MSC utility as a result.

1.5.1 EGFR signaling and cell survival

The epidermal growth factor receptor is a well-studied receptor protein tyrosine kinase that is generally present on all epithelial and stromal cells, as well as some others. The receptor signals a wide array of pathways that affect cellular processes including survival (Zhou, Qiu et al. 2006), migration (Andl, Mizushima et al. 2004), proliferation (Chandra, Lan et al. 2013), and even differentiation in stem cell populations (Platt, Roman et al. 2009). These effects are all

generated from activation of the pathways downstream of EGFR activation, including phospholipase C-γ, the MAP kinase pathway, and phosphatidylinositol 3-kinase as well. Classically, this receptor is bound and then subsequently internalized via clathrin-mediated endocytosis and then degraded or recycled depending on which of its ligands bind (soluble EGF or TGFα, respectively) (Vieira, Lamaze et al. 1996), as is true for most membrane growth factor receptors. This binding event induces activation of downstream signaling events that can promote survival, among other responses, via the Akt and MAPK signaling arms.

Many studies across decades of research into the EGFR cascade have demonstrated its role in promoting cell survival. Perhaps the most commonly described role of EGFR is in cancer cell survival, both as a general characteristic of cancer cells (Weihua, Tsan et al. 2008) and as a valuable target for therapies designed to disrupt cancer cell longevity in chemotherapeutics (Sordella, Bell et al. 2004). Beyond interest in chemotherapies, many studies have also examined the dynamics of EGFR activation in non-cancerous cells, such as cell adhesion-dependent EGFR survival promotion (Moro, Venturino et al. 1998) and, crucially, EGFR-dependent cell survival in the face of stressors such as hypoxic conditions (Wang, McCullough et al. 2000) or in the face of nutrient starvation (Ayuso-Sacido, Moliterno et al. 2010). Ultimately, the role of EGFR in cell survival signaling is very well understood and the cascade is an excellent option for designing methods to promote survival in the face of death stimuli.

1.5.2 Physical tethering of soluble EGF

Given the important role of endogenous EGFR signaling in promoting resistance to cell death, our group has pursued exploitation of this pathway as a means to improve MSC survival

and, consequently, potential MSC utility in wound healing. One such approach involves physical tethering of EGFR ligands (e.g. soluble EGF) directly to growth surfaces such as culture dishes or scaffolds. With the EGFR ligand physically bound to the seeding site, EGFR can be activated with a normal soluble ligand binding event, but the activated receptor complex remains physically bound to the cell surface. This prevents the internalization and subsequent degradation of EGFR, allowing for constitutive and long term activation of downstream EGFR signaling that is not stoichiometrically limited by available ligands or transient in nature (Figure 5), generally without feedback desensitization due to the lack of internalization and receptor level feedback over time. This approach of growth factor tethering has seen some investigation into improving cell survival,

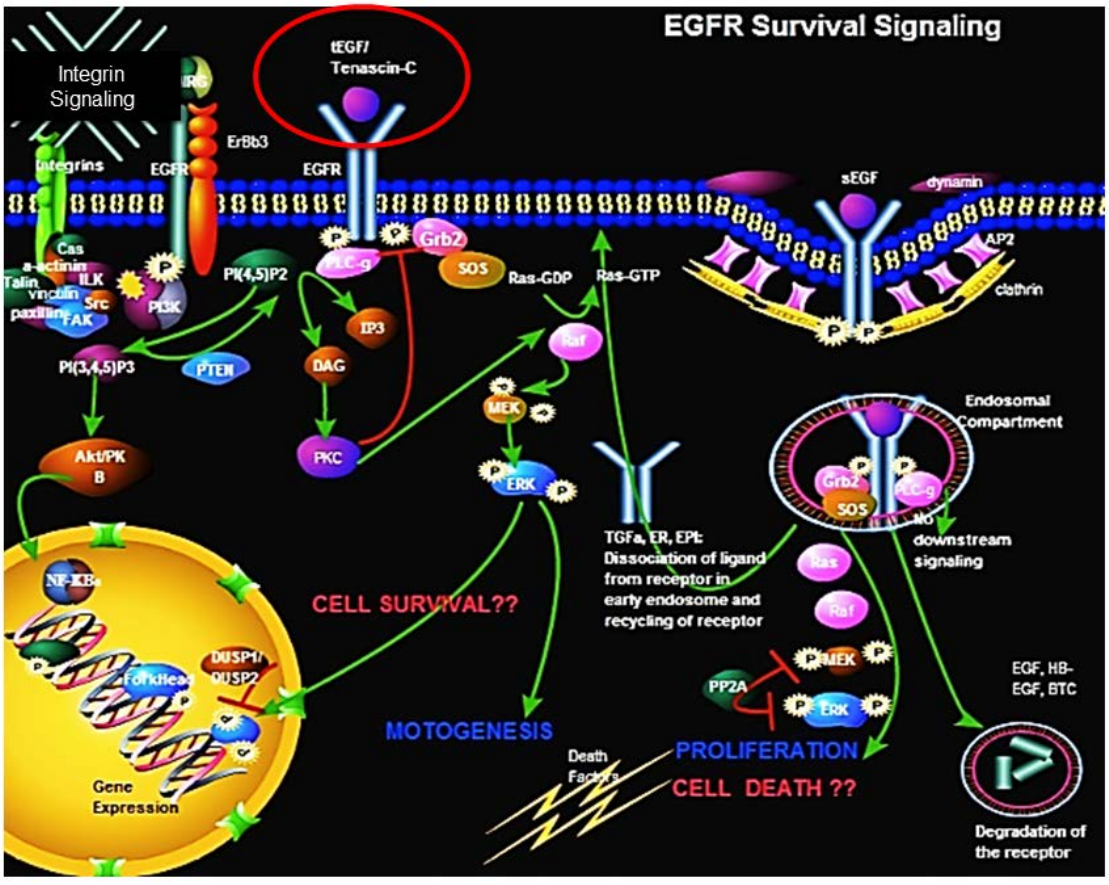

Figure 5. Schematic of EGFR survival signaling. Classically, soluble EGFR ligands bind and activate the EGF receptor at the cell surface, which induces clathrin-mediated internalization of the activated receptor complex (right). This is then trafficked for degradation by the lysosome, or recycled to the cell surface, depending on the soluble ligand. In systems for EGFR tethering, a binding peptide bound to a soluble ligand or signaling domain embedded in the extracellular matrix holds the activated EGFR complex at the cell surface, preventing this internalization and degradation and promoting long term downstream EGFR signaling (left).

such as smooth muscle cells (Lequoy, Liberelle et al. 2014), as well as research into targeted delivery of therapeutics through cargo tethering through similar methods (Wang, Bruggeman et al. 2014; Assal, Mizuguchi et al. 2015).

As our group is interested in strategies to improve MSC survival, we have taken this approach of soluble EGF tethering and applied it previously to culture systems *in vitro* to demonstrate the effect of constitutive EGFR activation on MSC death resistance. In 2007, Fan et al showed that covalently modifying a culture surface with tethered soluble EGF provided MSCs with a survival advantage in the face of Fas ligand treatment, showing also that the effect was not reproducible with normal soluble EGF treatment and demonstrating surface restriction of the EGFR via immunofluorescence (Fan, Tamama et al. 2007). This work was built on by Platt et al in 2008, who showed this same EGF tethering system improved the osteogenic capacity of MSCs *in vitro* (Platt, Roman et al. 2009). Rodrigues et al expanded this work in 2013 by showing that the protective effects of tethered EGF extended also to MSCs that had undergone differentiation, suggesting the survival advantage of this approach can extend beyond undifferentiated MSCs in culture (Rodrigues, Blair et al. 2013). Most recently, efforts have been made to functionalize this system to clinically relevant scaffolds or systems for regeneration. To that end, Alvarez and Rivera developed a binding peptide that is able to fuse to β-Tricalcium

phosphate (TCP), a relevant scaffold for bone regeneration (Alvarez, Rivera et al. 2015). They were able to demonstrate stable binding of the tethering peptide to the scaffold, as well as associated activation of downstream EGFR signaling for MSCs on the scaffold and an improved proliferative capacity as well in comparison to unbound scaffold controls.

Ultimately, this approach of functionalizing surfaces for cell seeding with a tethered EGFR ligand holds great promise for extending the lifespan of implanted cells. With the binding peptide specifically applied to TCP scaffolds, this approach stands ready for *in vivo* testing and studies to examine MSC response to appropriate stressors in the context of constitutive EGFR activation. Furthermore, this model is able to be adapted to a wide variety of scaffold surfaces with adjustments to the binding peptide, and great potential for improving implanted cell survival exists as a result.

1.5.3 Tenascin C

Cell signaling through growth factor receptors has long been a known contributor to many critical cell functions such as cell proliferation, migration, differentiation, and survival. Classically, these receptors are activated via soluble ligands, such as spliceoforms of vascular endothelial growth factor (VEGF) acting on one of its three receptors or EGFR being activated by its major ligands, EGF or TGFα (Cross, Dixelius et al. 2003; Jorissen, Walker et al. 2003). Recently, however, studies of matrikine tissue specificity showed that matrikines activate growth factors. Specifically, our group pioneered the discovery of the ability of the matrikine Tenascin C to bind and activate EGFR, activating a variety of relevant cell signaling pathways (Swindle, Tran et al. 2001). This has expanded into biomaterials research dedicated to surface restriction of

receptor signaling via growth factor tethering to constitutively activate survival pathways via EGFR (Rodrigues, Blair et al. 2013). By designing scaffolds that incorporate matrikines that can bind growth factor receptors to stimulate cell survival, the diverse benefits of MSC therapy are more easily accessed as the cell is able to utilize its secretome or differentiate to support the transplant site.

Tenascin C is a multimeric matri-cellular protein composed of two identical trimers that combine via disulfide bonds at Tenascin assembly domains at the N-terminus to form a hexamer structure (Kammerer, Schulthess et al. 1998). Of note is the motif distal to the N-terminus at the assembly domain that contains domain repeats (14.5 per arm (Jones and Jones 2000)), some of which can interact with the EGF receptor, commonly referred to as EGF-like repeats (EGFL). These repeats were classically established as anti-adhesive moieties for interactions with fibroblasts and neuronal glia (Ghert, Qi et al. 2001), but more recently have been studied for their ability to repeatedly activate the EGF receptor in a variety of cells (Swindle, Tran et al. 2001). Further distal to the EGFL is a group of fibronectin-like repeats that also play a role in integrin binding (Leahy, Hendrickson et al. 1992; Schnapp, Hatch et al. 1995), though we have showed this interaction is less relevant to survival signaling in MSCs (Rodrigues, Yates et al. 2013). TNC is incorporated into the normal extracellular matrix via interaction with an existing fibronectin template and interactions with $\alpha 5/\beta 1$ integrins in the extracellular space.

Physiologically, Tenascin C is a glycoprotein that forms part of the normal extracellular matrix, playing a tightly regulated role in several processes including wound healing, inflammation, and tumorigenesis. TNC is globally expressed at sites of inflammation, including skin damage (Betz, Nerlich et al. 1993), lung epithelium (Gueders, Hirst et al. 2010), and muscle tendon (Riley, Harrall et al. 1996). It also is expressed in tumor matrix and has been shown to

play a role in tumor proliferation/invasion, due at least in part to the EGF-like repeats conserved in the TNC structure (Juuti, Nordling et al. 2004; Grahovac, Becker et al. 2012). As a universally expressed matrikine, TNC is a potentially relevant protein for use in bioengineered scaffold devices for cell delivery to sites of wounding or inflammation.

1.5.3.1 Interaction between Tenascin C and EGFR

A key characteristic of Tenascin C is its comparative affinity for the EGF receptor, which has been found to be distinctly low (K_D = 74 uM (Iyer, Tran et al. 2007)) in comparison with typical EGF-EGFR interaction (K_D = ~20 nM (Zhou, Felder et al. 1993; Domagala, Konstantopoulos et al. 2000)). This low affinity leads to restriction of the interaction to the cell surface, as EGFL binding does not provide enough stability to initiate receptor internalization (Iyer, Tran et al. 2007). Despite this low affinity, the high number of EGFL present in endogenous Tenascin C allows for repeated low-affinity binding of the EGFR, creating a 'high-avidity/low-affinity' interaction with the receptor. This subsequently leads to persistent activation of EGFR signaling, and therefore persistent survival signaling, while the receptor is within range of the EGFL moiety. This contrasts with typical soluble EGF activation of EGFR (such as in the physical tethering described previously), which is a stable interaction that leads to receptor internalization and degradation (as is common in growth factor receptor signaling). The ability of persistent EGFR activation to promote MSC survival has been explored previously, where we have shown that tethering EGF molecules to a growth surface constitutively activates EFGR and allows MSCs to resist death signals *in vitro* (Rodrigues, Blair et al. 2013) (Figure 6).

This activation correlated with upregulation of downstream signaling events in the EGFR cascade, including upregulated Erk phosphorylation (Figure 7).

Because of Tenascin C's ubiquitous presence in physiological wound healing and unique ability to transiently and repeatedly bind EGFR to activate cell survival, it is an excellent candidate to be included in scaffold designs for seeding cells that are typically subject to frequent cell death, in the same vein as the physical EGF tethering described previously. As MSC therapies continue to struggle with rampant cell death at the implant site, this protein presents a potential method to extend MSC survival and yield better patient outcomes through constitutive EGFR activation, similar to the EGF tethering strategy proposed for solid scaffolds. As with the physical EGF tethering approach, this system must be functionalized to *in vivo* wound healing contexts to demonstrate true efficacy in extending implanted cell life span. To that end, matrix-based polymers or soft scaffolds incorporating TNC have been a topic of great interest to our group in recent years, in an effort to apply MSCs to chronic skin wound healing contexts.

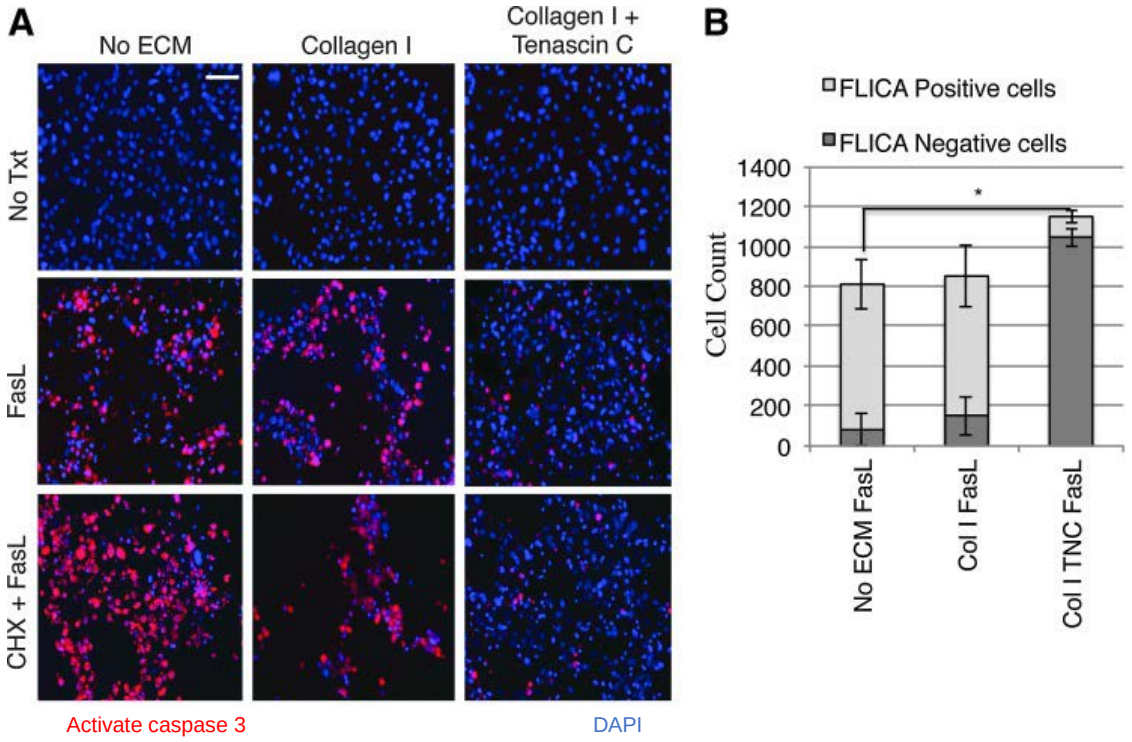

Figure 6. TNC is protective to MSCs in the face of FasL-induced death. Fluorescent inhibitor of caspase activity (FLICA)-stained primary human bone marrow-derived multipotential stromal cells (prhMSC) 12 h post-treatment with FasL/cycloheximide (CHX) and FasL on indicated surfaces (scale bar 50 µm) (A). Number of FLICA-positive and negative prhMSC grown on indicated surfaces after a 12-h treatment with FasL (*p<0.01) (B). TUNEL-stained MSCs 12 h post-treatment with FasL/CHX and FasL on indicated surfaces (scale bar 10 µm). Figure adapted from (Rodrigues, Yates et al. 2013).[7]

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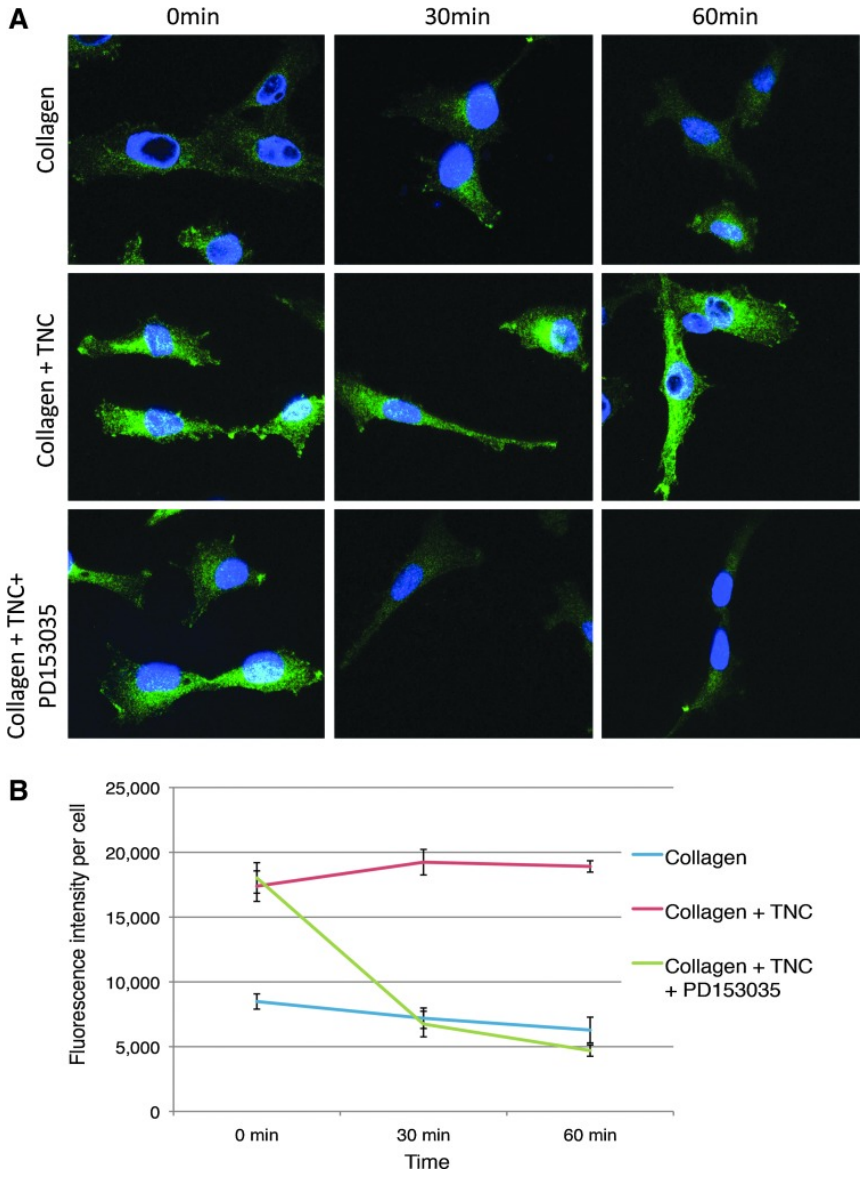

Figure 7. TNC causes sustained activation of Erk through activation of EGFR. Immunofluorescence for phosphorylated Erk (green fluorescence) in MSCs grown on either Col I or Col I and TNC, at times 0, 30, and 60 min, in the presence or absence of the EGFR inhibitor PD153035, shows sustained Erk activity by TNC dependent on EGFR activation (scale bar: 10 µm) (A). Fluorescence intensity of a single MSC, across 10 cells quantified by ImageJ (B). Figure adapted from (Rodrigues, Yates et al. 2013).[8]

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1.6 AUTOPHAGY AND CELL SURVIVAL

While the role of signaling pathways related to cell survival (such as EGFR, as discussed) are critical for approaches to promote longevity of implanted cells, another side to cell survival is recycling machinery in the cell that can respond to stressors to generate energy for the cell and improve resistance to death stimuli. One key process in that area is autophagy, a major process by which virtually all cells respond to stress via recycling of cellular debris and organelles. While this process is widely understood on a molecular level, and has been studied extensively as a stress response pathway in many contexts, studies of autophagy in progenitor cells remain relatively sparse. Given autophagy's key role in factors such as nutrient deprivation or differentiation, it is a pathway of great interest in relation to improving MSC survival long term *in vivo*.

1.6.1 The process of autophagy

Macroautophagy (referred to hereafter as 'autophagy') is the process by which a cell encapsulates and degrades cellular debris, allowing for recycling of amino acids and other key components for cellular health. This process is highly conserved among mammalian cells and also among organisms such as yeast. Canonically, autophagy proceeds via formation of autophagosomes in the cytosol, or membrane-bound structures sequestering the material targeted for degradation (damaged organelles, etc.) in the cytosol (Levine and Klionsky 2004; Meijer and Codogno 2004; Mizushima and Levine 2010). These autophagosomes are then trafficked to the lysosome, where they fuse to form an autolysosome and allow the acidic environment of the lysosome to degrade the debris. Given the wide array of potential targets for autophagic

degradation, the resulting degradation products such as amino acids and other biochemical components become available for re-use in biosynthesis, including de novo protein production. Autophagy in general is therefore often induced during periods of cellular stress, when metabolic demand becomes high and cells shift energy resources toward survival.

The main tool for tracking autophagy in biomedical science is the microtubule-associated protein 1A/1B-light chain 3 (LC3), which has been used extensively to track the formation and degradation of autophagosomes in the cell. Cytosolic LC3 exists as the isoform LC3I, which is present in most cell types at comparable concentrations throughout the cell. As autophagosomes form in the cytosol to traffic debris for degradation, cytosolic LC3I converts to the isoform LC3II, which is found specifically in autophagosome membranes (Kabeya, Mizushima et al. 2000. As such, LC3II punctae forming in the cell has classically been used as a means to assess autophagy activity in a cell, with increased activity correlating with a higher degree of LC3 puncta formation. Comparably, the relative levels of LC3I and II expression (typically assessed via western blot) can be used to determine changes in autophagic cycling over time, assuming a relatively constant stoichiometric relationship between the two isoforms (Klionsky, Abeliovich et al. 2008). Perhaps the most useful tool for monitoring autophagic turnover is tracking of LC3 with a tandem GFP-RFP reporter, which can be used in live cell experiments to directly assess autophagy. As LC3II is expressed in the autophagosome membranes, both reporters of the tandem marker fluoresce, yielding a yellow signal upon merge. However, as the autophagosome is trafficked to the lysosome, the GFP signal is dissipated by the acidic environment while the RFP signal persists (Kimura, Noda et al. 2007; Zhou, Zhong et al. 2012). This makes the tandem reporter a powerful tool for monitoring turnover in real time, as opposed to retroactive analysis on relative LC3 levels or puncta formation.

1.6.1.1 Role of autophagy in stress responses and survival

In the context of cell survival, the process of autophagy is key in providing building blocks for new protein synthesis. Several studies have outlined the role of autophagy in the cell survival response, and even have correlated this with MSCs in particular. It has been suggested that, more specifically, a starvation stimulus is one of the main drivers of degradative macroautophagy in mammalian cells (Levine and Klionsky 2004). In particular, several groups have identified specific stimuli or associated starvation pathways directly related to autophagy. In 2002, Talloczy et al found that the starvation-induced kinase Gcn2 (among other downstream targets) were essential for a starvation-induced autophagic response. (Talloczy, Jiang et al. 2002). More specifically, given the role of amino acid availability in autophagic products, it was found that available amino acid levels themselves regulated autophagic flux (van Sluijters, Dubbelhuis et al. 2000). Autophagy has also been widely associated with response to oxidative stress in many cell types (Kiffin, Christian et al. 2004; Lemasters 2005), as well as nutrient starvation (Kabeya, Mizushima et al. 2000; Komatsu, Waguri et al. 2005). Autophagy has also more recently been examined in the context of MSCs, with groups reporting a time-dependent and highly-coordinated role for autophagy in MSC differentiation (Oliver, Hue et al. 2012; Pantovic, Krstic et al. 2013), as well as a specific role of autophagy during serum deprivation and hypoxia in MSCs (Zhang, Yang et al. 2012).

There is ultimately a clear correlation between factors that affect autophagy and factors that affect MSCs in wound environments, including available nutrients, oxidative stress, and others. The MSC autophagy phenotype is therefore a key consideration for the overarching goal of improving MSC survival in the face of stressors, and as such analysis of autophagosome

morphology and turnover under normal and metabolically demanding conditions is of great interest. Additionally, MSC autophagy might ultimately be a candidate for targeted modulation to improve the cellular response to a particular stressor, and could also work in concert with other approaches to extending MSC lifespan, such as EGFR tethering.

1.7 HYPOTHESIS

In brief, the challenges mesenchymal stem cells face upon implantation into a wound site create a harsh environment that severely limits their clinical utility in most contexts. Novel strategies to extend their lifespan are crucial if the true therapeutic potential of MSCs is to be harnessed and made to work in the many contexts of wound healing. To that end, the following work as a whole was designed to build on previous research from our group aiming to alleviate MSC death *in vivo*. We have examined MSC survival from three distinct angles: physical EGFR ligand tethering, EGFR tethering through matrikine signaling, and exploiting the natural use of MSC autophagy in response to stressors. Our approaches to EGFR tethering have shown improvements in MSC survival *in vitro, and we thus hypothesized that re-engineering EGFR tethering to a solid (TCP) scaffold and a matrix-derived (Tenascin C) polymer both would improve long term MSC survival following implant in vivo, improving correlated healing outcomes in the wound space.* Additionally, given the important role of autophagy in cellular stress responses, *we hypothesized that modulating autophagy via drug pre-treatment would alter MSC response to stressors and alter long term MSC function under duress*. Ultimately the combination of these two distinct cellular processes may prove highly beneficial in approaches to improving the clinical utility of MSCs in cell therapies.

2.0 EGF TETHERED TO B-TRICALCIUM PHOSPHATE BONE SCAFFOLDS VIA A HIGH AFFINITY BINDING PEPTIDE ENHANCES SURVIVAL OF HUMAN MESENCHYMAL STEM CELLS IN MOUSE MODELS

2.1 ABSTRACT

Mesenchymal stem cells/multipotent stromal cells (MSCs) are attractive candidates for use in cell therapies for wound healing due to their ability to differentiate into a variety of cell lineages (bone, fat, cartilage) and support the wound environment with cytokines and immunomodulatory activity. However, usage of these cells is often limited by poor cell survival upon implantation into a wound environment, where the cells are faced with challenges such as ROS generation, host inflammation, and a poor vasculature and nutrient supply. Thus, strategies to improve MSC survival are needed to enable these cells to be part of regeneration. We have previously established a system in vitro for improving MSC survival by tethering EGF (tEGF) molecules to surfaces to restrict EGFR signaling to the cell surface, which is preferential for survival over proliferation.

Here, we applied this system to tricalcium phosphate (TCP) particle scaffolds, clinically used in bone healing, tethering EGF to the scaffold surface and seeding MSCs to simulate a potential surface for bone repair. Human primary MSCs seeded on TCP were injected into the perifascial space of immunocompetent mice with or without tEGF attached to the surface for up

to 21 days, subjecting the cells to nutrient deprivation and inflammation comparable to a hostile wound environment.

We found that tethering EGF to the TCP scaffolds yielded approximately a 4-fold increase in MSC survival compared to non-EGF scaffolds at 21 days, as well as significant improvements in survival in the short term at 2 and 7 days implantation. The tEGF scaffolds also yielded improved cellularity over control TCP scaffolds, in part due to improved MSC survival. Overall, our approach to sustaining EGFR signaling reduced MSC death in vivo and as a result may be useful for future cell therapies where MSCs typically die upon implantation.

2.2 INTRODUCTION

The use of mesenchymal stem cells (MSCs) in regenerative medicine has become a topic of great interest in recent years, both in the fields of tissue regeneration and transplant biology. MSCs are naturally able to home to sites of injury and contribute to the tissue repair process via differentiation or paracrine activity, as has been seen in processes such as skin wound healing (Sasaki, Abe et al. 2008) and bone regeneration (Kitaori, Ito et al. 2009), among others. Building on their natural role in such healing responses, MSCs have been studied in the clinical context of tissue regeneration and cell therapy for many years now due to their ability to differentiate into multiple cell types (Mackay, Beck et al. 1998; Pittenger, Mackay et al. 1999; Toma, Pittenger et al. 2002). In addition, MSCs secrete a variety of supportive cytokines that promote regenerative processes, particularly factors that induce neovascularization (VEGF-α, IGF-1, etc.), a key factor in successful regeneration in any tissue (Chen, Tredget et al. 2008; Wang, Crisostomo et al. 2008). These cells also have a documented immunoprivilege in an undifferentiated state, as well

as the ability to modulate the local host inflammatory response itself, allowing them to resist rejection in an allotransplant or xenotransplant and quell the host reaction to the implanted scaffolds, tissue, or other relevant agent (Aggarwal and Pittenger 2005; Ren, Zhang et al. 2008; Gebler, Zabel et al. 2012).

All contexts in which MSCs are proposed for regeneration therapeutically involve implantation of MSCs into wound sites or tissues, with the goal of inducing MSC differentiation or exploiting the MSC secretome to improve the regenerative outcome for the patient. However, in all of these contexts, implanted MSCs are immediately subjected to harsh conditions in the wound site or degenerative tissue; that is, a hypoxic and ischemic environment that yields poor cell survival. This has been cited in several studies across different models of MSC-based regeneration, including bone repair or myocardial infarct regeneration (Hu, Yu et al. 2008; Giannoni, Scaglione et al. 2010; Zimmermann, Gierloff et al. 2011). These studies have reported less than 50% survival of implanted cells after just the first 48 hours, with virtually no detectable MSCs remaining after 1-2 weeks. As both MSC paracrine activity and differentiation require time to prove effective, this limited cell survival is a major hurdle to effective use of MSCs across cell therapies in general. This has resulted in efforts to improve the survival of implanted MSCs to improve their therapeutic efficacy.

The epidermal growth factor receptor (EGFR), upon activation of its tyrosine kinase, activates a multitude of downstream signaling pathways including survival-associated signaling through the Akt and MAPK pathways (Wang, McCullough et al. 2000; Junttila, Li et al. 2008). How the cell decides between proliferation, migration, and survival dominating depends in large part on the cellular temporo-spatial aspects of the active kinase; signaling from the endosome favors proliferation (Haugh, Huang et al. 1999; Pennock and Wang 2003) whereas cell surface

signaling is preferential for motility (Haugh, Schooler et al. 1999; Iyer, Tran et al. 2008) and survival (Fan, Tamama et al. 2007). We have established a system of tethering soluble EGF (tEGF) to MSC growth surfaces in vitro that prevents this receptor internalization and subsequent cessation of EGFR signaling, leading to constitutive activation of EGFR signaling. This approach of covalently tethering EGF has led to in vitro improvement of MSC survival in the face of death stimuli such as Fas ligand (Fan, Tamama et al. 2007; Rodrigues, Blair et al. 2013) while maintaining the functional capacity of the MSCs to differentiate (Platt, Roman et al. 2009). This type of survival signaling should be applicable to healing situations, but the tEGF needs to be functionalized to materials compatible with *in vivo* wound contexts.

In this study, we applied this system of tethered EGF to a clinically relevant scaffold for bone healing, β-Tricalcium phosphate (β-TCP), by using a high affinity multimeric binding peptide to link soluble EGF to the scaffold. β-TCP is already used as a filler material for bone defects requiring alternatives to standard bone grafts (Meadows 2002; Ogose, Kondo et al. 2006), as well as in cell therapies as a common scaffold for cell seeding in cell-based osteogenic therapies (Takahashi, Yamamoto et al. 2005; Yuan, Cui et al. 2007). To examine the ability of tEGF to improve both short term and long term stem cell survival on this scaffold, we seeded primary human bone marrow-derived MSCs onto β-TCP with EGF tethered to its surface and injected the scaffolds into the perifascial space of immunocompetent mice in a collagen-I/Matrigel plug. We found that utilizing surface tethered EGF improved overall MSC survival in both the short and long term (with respect to the MSC lifespan), as well as increasing overall plug cellularity and angiogenic character. Cellularity of implanted plugs was also improved in the EGF-tethered scaffolds. Overall, we show here that surface tethering of EGF to clinically

relevant scaffolds for stem cell therapy is possible, and improves the ability of implanted cells to persist and ultimately provide their therapeutic effect to the wound environment.

2.3 MATERIALS AND METHODS

2.3.1 Reagents

For all cell culture, α-MEM [15-012-CV] was obtained from Mediatech (Manassas, VA), FBS [S11550H] was obtained from Atlanta Biologicals (Flowery Branch, GA), and L-glutamine [25030-081] was obtained from Thermo Fisher/Gibco (Grand Island, NE). CM-DiI cell tracker [C7000] used in culture was obtained from Life Technologies (Grand Island). For injectable plug formation, growth factor-reduced/phenol red-free Matrigel [356231] was obtained from BD (Franklin Lakes, NJ), and rat tail collagen I [354236] was also obtained from BD at 3.84 mg/mL concentration. For plug digestion and cell processing for flow, Liberase TL [0540102001] was obtained from Roche (Indianapolis, IN) and 40 μM filters [352340] for cell filtration were obtained from BD.

2.3.2 MSC cell culture

Primary human bone marrow-derived mesenchymal stem cells were obtained from the NIH-funded core facility run by Dr. Darwin Prockop at Texas A&M. As characterized by the repository, this MSC population was from a male 24 year old donor and showed 64-66% percent

CFUs upon analysis of recovered P1 plated cells. The MSCs showed a doubling period of approximately 24 hours through three passages of low density expansion testing, with positive results for both osteogenic and adipogenic differentiation protocols as well. Surface marker analyses demonstrated typical MSC characteristics, with 98.7% positive for CD105, 99.0% positive for CD90, and 98.3% positive for CD73, in addition to 0.19% positive for CD34 and 0.16% positive for CD45, as expected. For this study, proliferation media consisted of α-MEM without deoxyribonucleotides or ribonucleotides, supplemented with 2 mM L-glutamine and 16.5% FBS. Cells obtained from the Prockop repository were allowed to recover overnight in an excess of proliferation media at 37°C, and then passaged up to passage 3 for all studies. Cells were passaged at approximately 70% confluence.

2.3.3 Cell tracking

Primary MSCs were tracked using a red CM-DiI cell tracker 24 hours prior to detachment. To track the cells, MSCs were grown to ~70% confluence at passage 3. Cell tracker at 1 mg/mL in DMSO was diluted to 1 µM in PBS at 37°C, and 10 mL of working solution was added to each 75 cm^2 culture dish and incubated at 37°C for 5 minutes. Dishes were then moved to a 4°C incubation for a further 15 minutes. Cells were then washed in warm PBS extensively and standard MSC growth medium was then re-added to each dish. After 12 hours red fluorescence at the MSC cell membranes was confirmed in each dish prior to use *in vivo*.

2.3.4 Binding peptide fabrication

The high affinity linker peptide used to bind soluble EGF to the TCP surfaces was fabricated by Jaime Rivera in the Griffith laboratory at the Massachusetts Institute of Technology. Details of peptide fabrication, stability, and interaction with MSCs in culture can be found in more detail in (Alvarez, Rivera et al. 2015). Briefly, a human EGF sequence was inserted into a pMAL expression cassette via PCR mutagenesis to yield a library of multimer insertions fused to EGF. This protein was expressed in *E. coli* and induced with IPTG to yield protein for harvest via maltose binding protein affinity chromatography and purified using ultrafiltration and sterile syringe filtration. Reported purity of the full-length binding peptide ranged from 75% to 90%. Protein concentration was measured via Nanodrop ND-2000 and provided to our group at the University of Pittsburgh for use *in vivo*.

2.3.5 Scaffold preparation and seeding

β-tricalcium phosphate powder consisting of 63-106 μM β-TCP particles (average 82 μM) was used for all studies as a scaffold for MSC seeding. For each individual plug, 10 mg of β-TCP powder was weighed and placed into separate tubes. For the experimental tEGF group, 10 mg β-TCP was incubated with 200 μL of 2 μM BTCPbp-C1-EGF solution for 24 hours at 4°C. Following incubation, the residual protein solution was aspirated and, for those scaffolds receiving MSCs, CM-DiI tracked MSCs were trypsinized and added individually to both the tEGF and untreated β-TCP sample tubes at a concentration of $1.5x10^6$ MSCs in 1 mL proliferation media per sample. Individual samples were rotated at 37°C for 90 minutes to allow

attachment of the MSCs to the β-TCP scaffolds as previously described (Kuznetsov, Cherman et al. 2011; Kuznetsov, Mankani et al. 2013). Following cell attachment, samples were gently spun to collect all β-TCP particles, and proliferation media was aspirated. Both MSC and non-MSC TCP and tEGF-TCP samples were then gently mixed with a 1:1 volume mixture (0.5 mL each; 1 mL total plug volume) of growth factor-reduced Matrigel and collagen I (3.84 mg/mL) to form a viscous injectable plug, as previously described. Fully mixed scaffold preparations were placed on ice to prevent gelation prior to injection.

2.3.6 Mouse inoculations

8-10 week old immunocompetent FVB mice were obtained from Jackson Laboratories, ME, USA. For injection, the collagen I/Matrigel mixtures containing TCP (described in the previous section) were transferred into the perifascial space above the quadriceps on each leg, with 1 mL of gel injected transcutaneously via 21 gauge needle on the anesthetized mice. Plugs solidified immediately in vivo and were extracted at each time point following mouse euthanization via CO_2 asphyxiation. All mice were housed on a 12 hour light/dark cycle, and given food and water as needed. Mouse wounding and sacrificing was performed in accordance with Animal Welfare Act regulations, and full garb with mask, gloves, and gown was utilized during all animal exposure. All animal care was performed in accordance with the VA Healthcare System and IACUC guidelines.

2.3.7 Plug sectioning

Collagen-I/Matrigel plugs were extracted after 2, 7, and 21 days of incubation in the perifascial space of each mouse. Half of each plug was fixed in 4% paraformaldehyde for 30 minutes, rinsed in PBS, and transferred to 30% sucrose for overnight incubation at 4°C. Plugs were then frozen in liquid nitrogen and cryosectioned at the Center for Biologic Imaging at the University of Pittsburgh to observe presence of the CM-DiI-tracked MSCs throughout the plugs.

2.3.8 Flow cytometry

Collagen-I/Matrigel plugs were extracted after 2, 7, and 21 days of incubation in the perifascial space of each mouse. Half of each plug was placed in 1 mL Liberase-TL, for 90 minutes at 37°C to break down the collagen matrix holding the cells. After 90 minutes, the scaffold/plug mix was homogenized via pipetting and washed through a 40 μM mesh filter into collecting tubes. The single cell suspension was subjected to a cell count, and $2x10^5$ cells from each sample (or the maximum available) was divided into plastic flow cytometry tubes, with two separate tubes used for both an MSC panel and a murine inflammation panel. Cells were washed in a 0.1% FBS in PBS flow buffer and blocked for non-Fc mediated interactions for 20 minutes on ice using anti-CD16/CD32 (BD Biosciences #553142). Cells were washed again and stained with an MSC antibody panel, including MSC markers PE-Cy7 CD73 (BD Biosciences #550257) and APC CD90 (BD Biosciences #559869), and a separate inflammatory panel including CD11b+ (BD Biosciences #557672), Nk1.1+ (BD Biosciences #550627), and CD3e+ (BD Biosciences #555276) cells. Cell populations were analyzed using a BD LSRII flow cytometer,

assaying for the presence of MSCs via double positivity for CD73 and CD90 at all time points, as well as presence of each individual inflammatory marker.

2.3.9 Statistical analyses

For all statistical analyses, a student's t-test was used to compare tEGF conditions with the non-tethered controls, including number of surviving MSCs and cellularity of plugs. P values less than 0.05 were considered significant.

2.4 RESULTS

2.4.1 Tethered-EGF β-TCP scaffolds improve MSC survival in vivo

To assess the ability of tEGF to improve immediate and long-term MSC survival in the face of *in vivo* barriers to cell longevity, we applied tEGF to clinically relevant β-TCP particles and seeded MSCs on these scaffolds mixed into a gel plug for injection into the perifascial space of immunocompetent mice above the quadriceps muscle (Figure 8). At each time point, mice were euthanized and half of each gel plug was fixed and cryosectioned to determine the distribution of the TCP particles and MSCs throughout each gel scaffold, as well as monitor the survival of the CM-DiI tracked MSCs over time (Figure 9). Sections showed a generally even distribution of MSCs throughout all implants, with tracked MSCs localizing in the vicinity of the tricalcium phosphate particle aggregates, as expected

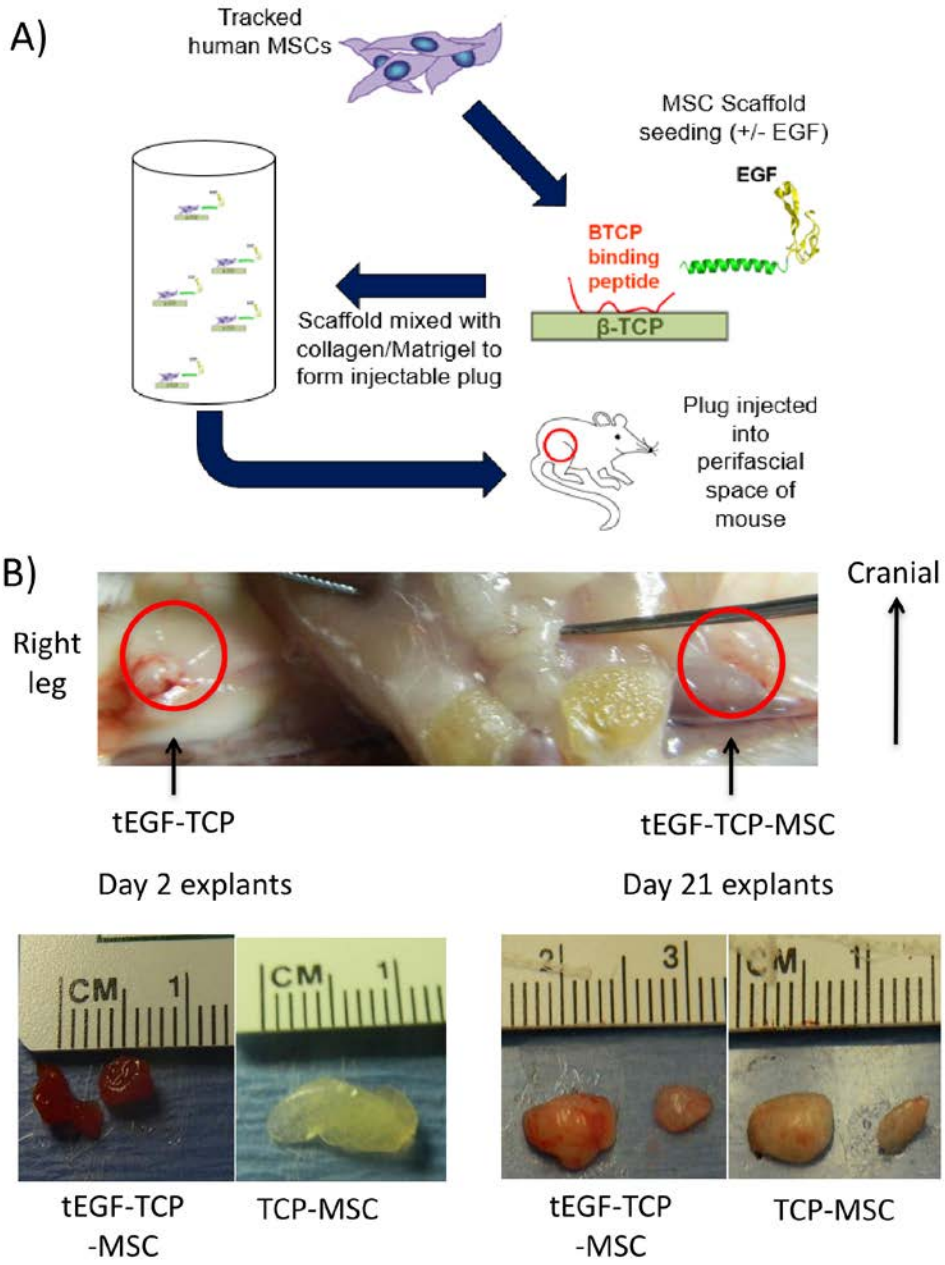

Figure 8. Experimental schematic for tEGF plug injections. Tracked (CM-DiI) primary bone marrow-derived human MSCs were seeded onto tricalcium phosphate particles with or without soluble EGF tethered to the scaffold surface via rotation in proliferation media (A; also see methods). The seeded TCP scaffolds were mixed into a 1:1 collagen I/Matrigel gel mixture and injected into the perifacial space of FVB mice above the quadricep (B). Plugs were extracted on days 2, 7, and 21 for cryosection and flow cytometry analysis.

(Figure 10). Importantly, fluorescent signal from the tracked MSCs was more prevalent in the tEGF-modified TCP conditions compared to the standard TCP scaffold conditions, suggesting an improvement in MSC survival in these scaffolds. tEGF scaffolds also improved the apparent angiogenic character of the plugs, as can be seen in the extracted plugs in Figure 8b. Correlated with improved survival, this might suggest that MSCs retain paracrine capacity to induce angiogenesis while seeded on tEGF scaffolds.

MSC survival was quantified using collagenase digestion of the other half of each extracted plug with subsequent flow cytometry analysis of MSC presence using common human MSC markers CD73 and CD90 (Figure 9a). Percent positive MSC populations were derived from total cell harvests from all plugs at the aforementioned time points, and MSC percentages from flow cytometry were then applied to the total cell count from each plug to estimate the number of MSCs present in each half-plug. Over the course of two experiments, results show that tEGF-TCP scaffolds provide a distinct survival advantages to implanted MSCs at all assayed time points. Days 3 and 7 showed an approximately 4-fold improvement in remaining MSCs over the TCP scaffold alone, with $4.0x10^5$ +/- $2.9x10^4$ and $3.90x10^5$ +/- $6.5x10^4$ MSCs surviving at each time point, compared to $9.53x10^4$ +/- $2.2x10^4$ and $1.08x10^5$ +/- $5.4x10^4$, respectively (40% and 39% survival of the initial 1 million implanted cells). This survival effect is perhaps most notable in the long term, where a significant portion of the MSCs persist out to 21 days; $2.92x10^5$, or 29% of the initial implanted population.

2.4.2 tEGF improves overall cellularity of implanted scaffolds

Cellularity is often a point of interest in regenerative scaffolds, owing to the importance of invading CD31+ cells and proliferation of any native cells that might be participating in the

regenerative response. We assessed total cell counts from extracted plugs during the processing of each plug for flow analysis via automated cell counting. We found that tEGF included on the TCP scaffolds significantly improved the total number of isolated cells within each plug, particularly at days 7 and 21 where the tEGF conditions show a drastic increase in cellularity, well above the cellularity that would come from the implanted MSCs alone (Figure 11). This increased cellularity represented an almost two to three-fold increase over the unmodified TCP scaffolds; we showed $1.89x10^5$ +/- $3.0x10^4$ cells in total on tEGF particles compared to $1.04x10^5$ +/- $1.7x10^4$ on TCP alone at day 3, and $3.89x10^6$ +/- $1.33x10^5$ on tEGF particles at day 7 compared to $1.74x10^6$ +/- $2.82x10^5$ on TCP alone. Notably, this result was concurrent with an increase in the apparent vascularity of each plug, as described in Figure 1. TCP scaffolds without tEGF showed a slight upward trend in cellularity over 21 days, roughly doubling the initial 1 million identified cells at the day 2 time point. Additionally, the majority of the increase in cellularity came after the day 2 time point at days 7 and 21, well beyond the associated peak inflammatory response for the injected plugs.

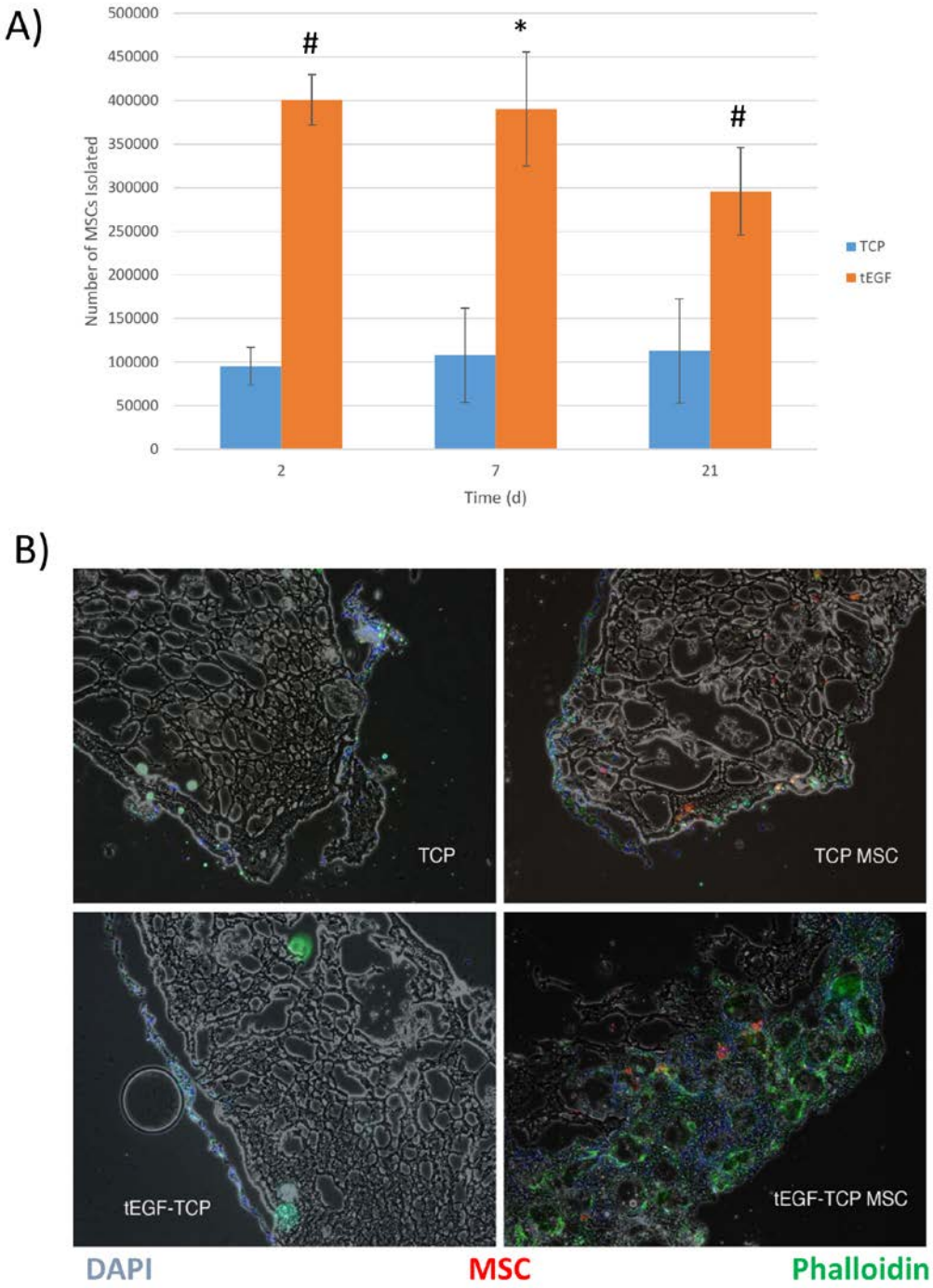

Figure 9. Surface tethered EGF improves MSC survival on β-tricalcium phosphate scaffolds. Extracted plugs were digested and cells were isolated for flow cytometry analysis, using MSC markers CD73 and CD90 to identify the human MSCs (A). Half of each plug was also cryosectioned and analyzed for presence of general cells (DAPI, actin stains) and visual presence of MSCs (B). A student's t-test was performed to compare significance of MSC survival, *p<0.01, #p<0.05.

2.5 DISCUSSION

Our group has previously examined the benefit of EGF tethering largely *in vitro*, showing enhanced survival in the face of Fas ligand challenge (Platt, Roman et al. 2009; Rodrigues, Blair et al. 2013; Rodrigues, Yates et al. 2013). To translate this to *in vivo* benefit, recent strides have been made to develop clinically relevant vehicles for EGF tethering, specifically a peptide that is able to provide a stable binding between soluble EGF and a tricalcium phosphate surface for

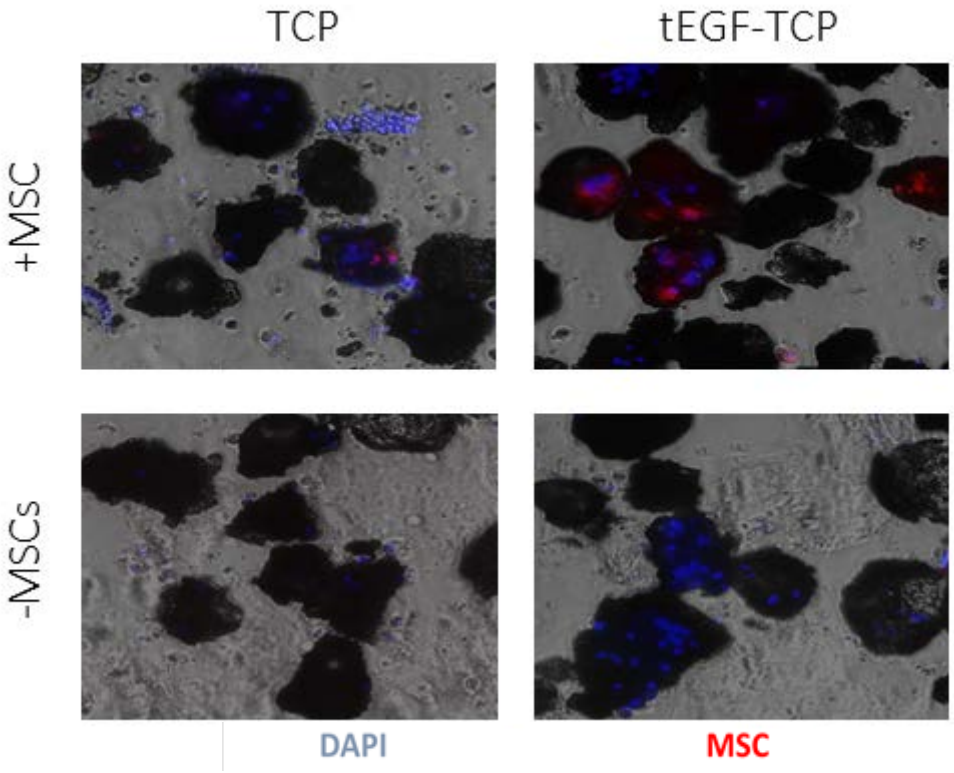

Figure 10. MSCs show improved survival localized to tEGF-TCP aggregates. TCP particles extracted from plugs at day 7 were resuspended in PBS and imaged in suspension for presence of CM-DiI signal localized to TCP particles. –MSC controls were included to isolate CM-DiI signal specific to MSC populations. DAPI in blue, MSCs labeled with CM-DiI in red.

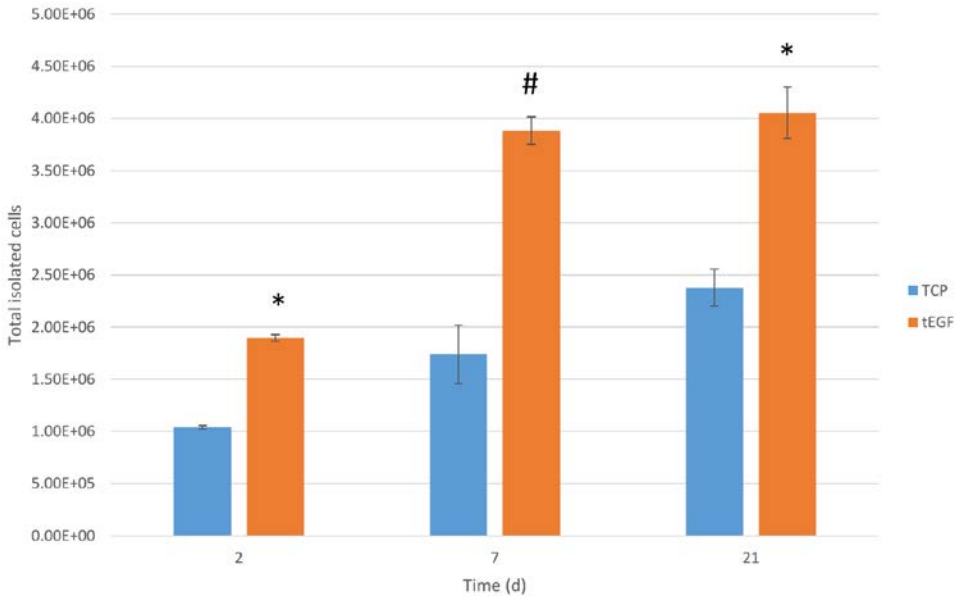

Figure 11. Improved MSC survival on tEGF scaffolds increases overall cellularity. Digested plugs from each time point were counted using a Sceptre cell counter, and total cell counts were recorded and extrapolated to yield an estimated cell count for the entire extracted plug at each time point. Cell counts are reported here as those estimates, prior to MSC identification and thus including the whole cell extract from the mouse, as well as any MSCs left in the plug. A student's t-test was performed to compare significance of changes in cellularity across the three weeks of incubation, *p<0.01, #p<0.05.

bone regeneration. Here, we have built on work by Alvarez and Rivera in designing this peptide and examining effects *in vivo* (Alvarez, Rivera et al. 2015), attempting to challenge MSCs in an implant site and hypothesizing that EGF tethering would provide a protective effect in this context. Results from this study demonstrate the utility of surface tethering soluble EGF to scaffolds for MSC implantation in an environment that is sufficiently challenging to MSCs. Our chosen implant site, the perifascial space above the quadricep, provided an environment largely devoid of blood flow or a nutrient supply, and highly hypoxic compared to the culture environment, thus being similar to a wound bed in which vessels are absent until repair. We also

subjected the cells to any relevant innate and acquired immune response, as we used immunocompetent mice for our xenotransplant.

Our data shows that a modest concentration of surface-tethered EGF is able to keep up to 30% of implanted MSCs alive even at day 21 following injection into a region generally devoid of vessel growth and nutrients for the cells (Figure 9); significantly higher than some clinical studies have shown for typical MSC survival *in vivo* (Hu, Yu et al. 2008; Giannoni, Scaglione et al. 2010; Zimmermann, Gierloff et al. 2011). Though a majority of the cells have still indeed died at the end of this study, a three week lifespan is sufficient for these cells to have started and proceeded well into the process of differentiation, which is one of the critical processes for the specific bone healing context. Additionally, this approach has been shown to protect differentiating MSCs long enough to form a new cell type, while the differentiated cells become ultimately more resistant to cell death (Rodrigues, Blair et al. 2013). Though we have shown that tEGF substrates have a positive effect on the osteogenic potential of MSCs *in* vitro (Platt, Roman et al. 2009), follow up studies are necessary to look further at the efficiency of MSC osteodifferentiation *in vivo* and translate improved survival to a possible clinical benefit.

Analyses of total cell counts from extracted plugs in this study showed improved cellularity in those scaffolds with the tEGF binding peptide, including all extracted murine and human cells. While this is obviously partially due to the improved survival of MSCs on these scaffolds as reported in Figure 10, this data also highlights the likelihood that MSCs maintain paracrine function with the constitutive EGFR activation induced on our tEGF scaffolds. For example, MSCs have long been known to be important in inducing angiogenesis locally via VEGF activity, improving vessel formation in tube formation assays (Duffy, Ahsan et al. 2009) and also showing increased angiogenesis in implant sites *in vivo* in a wound healing context

(Wu, Chen et al. 2007). Our group has previously shown *in vitro* that sustained EGFR activation through tethering approaches may improve paracrine function of MSCs through upregulation of growth factor and/or cytokine production (Tamama, Kawasaki et al. 2010). Future studies with EGF tethering and MSCs must discern the effects of EGFR tethering on the MSC secretome and associated outcomes in the implant site, both beneficial and otherwise.

Given its role in a wide array of cellular processes, it stands to reason that our persistent activation of EGFR would have effects beyond enhanced cell survival. While survival signaling via EGFR functions primarily through the PI3K/Akt signaling arm (Wang, McCullough et al. 2000; Downward 2004), there are other aspects to EGFR signaling that might benefit the MSCs in a clinical context. A variety of studies in EGFR tethering have shown, for example, enhanced MSC proliferation in concert with the improved survival we have seen in this study (Platt, Wilder et al. 2009; Alvarez, Rivera et al. 2015). While enhanced proliferation may help to simply increase the number of available MSCs post-implantation and improve overall survival outcomes, increased proliferation of MSCs long term may assist with MSC-derived osteoblast migration into a bone defect site or other comparable scenarios where proliferation is of importance. Additionally, cell motility is intimately tied into the EGFR signaling cascade (Chen, Xie et al. 1994; Xie, Pallero et al. 1998), and a multitude of studies have shown the effects of EGFR activation on enhanced migration. Although surface restricted EGFR signaling has shown to yield outcomes for improved survival preferentially over migration (Platt, Wilder et al. 2009), the benefits of any enhanced MSC motility from this construct may assist with cell engraftment and ultimately regenerative outcomes in the implant site.

A limitation of this study is undoubtedly conclusions that can be drawn from the perifascial implant site chosen here. Though the true clinical relevance of tricalcium phosphate

biomaterials is bone remodeling, and thus an implant site in a long bone defect is perhaps the most relevant implant site of interest, we chose here to instead model an avascular and ischemic site by injecting the scaffolds via Matrigel into the perifascial site described in Figure 8. We ultimately did this to avoid the limited bone healing conclusions that could be drawn from a relatively different load-bearing architecture in the murine femur or other appendage site, and similarly avoided a cranial or maxillary site due to the differing architecture in comparison to typical non-union bone defect sites such as the leg. As a result, most of our conclusions here are limited to the survival of MSCs in general and less focused on the osteogenic potential of the TCP scaffolds. In addition, the limited size of the injected plugs allowed for little analysis of the actual identity of the infiltrating cells that contributed to the cellularity increases described in this study. Given the potential role of MSCs in inducing this invasion via paracrine secretions such as VEGF or immunomodulatory cytokines, future studies will need to elucidate precisely what effects our demonstrated extended survival have on both the immune infiltrate as well as the formation of vascular networks in the implant site.

Due to its relatively straightforward design and the ubiquitous expression of EGFR on many cell types, the EGF tethering system has a wide array of potential applications in regenerative medicine. Growth factor tethering (and specifically EGF tethering) has been used in a variety of contexts, including improving survival of other cell types such as vascular smooth muscle cells (Lequoy, Liberelle et al. 2014) or targeted therapeutic growth factor delivery (Wang, Bruggeman et al. 2014; Assal, Mizuguchi et al. 2015). In the context of MSC clinical utility, although this study analyzed the effects of tEGF on a construct designed primarily for bone regeneration, the applications of EGF tethering may also extend outside of tricalcium phosphate scaffolds and bone repair in general. As discussed previously, a variety of clinical

trials have shown marginal MSC survival after a few days post-implantation, including cardiac, skin, and bone regeneration. In any of these contexts, EGF tethered to the applicable scaffold might improve the survival of seeded cells to benefit the implant site, including MSCs and other non-MSC therapeutics.

3.0 A WOUND HEALING POLYMER UTILIZING THE MATRIKINE TENASCIN C ENHANCES MESENCHYMAL STEM CELL SURVIVAL AND IMPROVES SKIN WOUND HEALING OUTCOMES IN MICE

3.1 ABSTRACT

Mesenchymal stem cells remain of great interest in regenerative medicine due to their ability to home to sites of injury, differentiate into a variety of relevant lineages, and modulate inflammation and angiogenesis through paracrine activity. As such, many clinical studies have endeavored to use MSCs in wound healing or other relevant clinical contexts that align with available MSC functions. Many of these studies have found that despite the great promise of MSC therapy, cell survival upon implant is highly limited and greatly reduces the therapeutic potential of MSCs. Thus, strategies to improve MSC survival are of great interest in an effort to improve upon current clinical outcomes from MSC therapy.

The matrikine Tenascin C, a protein expressed often at the edges of a healing wound, contains unique EGF-like repeats that are able to bind EGFR at low affinity and induce downstream pro-survival signaling without inducing receptor internalization. Previously, we have shown that this protein is able to protect MSCs from induced cell death *in vitro*. In this study, we applied Tenascin C to a matrix-based polymer for skin wound healing, incorporating human MSCs into the polymer prior to application to mouse wound beds. We found that the

TNC-based polymer was able to promote MSC survival long term *in vivo*, leading to associated improvements in wound healing such as dermal maturation and collagen content. Ultimately, this matrikine-based approach to improving MSC survival may be of great use across a variety of cell therapies utilizing matrices as delivery vehicles for cells.

3.2 INTRODUCTION

Mesenchymal stem cells/multipotent stromal cells (MSCs) have long been identified as excellent candidates for regenerative skin therapies due to their ability to support angiogenesis and modulate inflammation through paracrine activity, as well as differentiate into fibroblast lineages and support tissue replacement (Pittenger, Mackay et al. 1999; Wu, Chen et al. 2007; Sorrell and Caplan 2010; Jackson, Nesti et al. 2012). As such, MSCs have been studied extensively as candidates for novel treatments in chronic wound healing, where current therapies still leave much room for improvement. However, MSCs implanted into harsh wound environments, such as the hyperinflammatory and devascularized chronic wound bed (Menke, Ward et al. 2007), have shown extremely limited survival, often times surviving for 2-3 days prior to death. The clinical utility of these MSCs in wound healing is therefore limited severely by inadequate cell survival, when paracrine activity or differentiation both require days to weeks to yield effective changes in the healing process.

Strategies to improve upon MSC survival in engineered devices for cell delivery to the wound site have therefore become a topic of great interest. Many cells take advantage of endogenous signaling pathways or cellular machinery to promote cell survival and/or resist cell death in harsh conditions. One such key pathway is the epidermal growth factor receptor (EGFR)

pathway, which can promote cell survival via the Akt and MAPK signaling arms (Chen, Xie et al. 1994; Jorissen, Walker et al. 2003). Canonically, ligand binding of EGFR by soluble EGF (or other ligands) induces dimerization of the EGFR heterodimer, followed by activation of downstream signaling and subsequent internalization of the activated receptor complex. This complex is then either targeted for degradation by the lysosome or recycled back to the cell surface for further activation as available (Yamazaki, Zaal et al. 2002; Jiang, Huang et al. 2003). Consequently, survival signaling through the EGFR is transient and stoichiometrically limited by available soluble ligands in the extracellular space, which leads to short-term downstream activation of survival signaling.

To exploit EGFR signaling for long term cell survival, methods to prevent receptor internalization are necessary. The matrikine Tenascin C is an extracellular matrix protein that is normally expressed during development and at sites of injury on the periphery of wounds to promote fibroblast migration and inflammatory cell infiltration into the damaged tissue (Latijnhouwers, Bergers et al. 1996; Jones and Jones 2000). A notable element of TNC is EGF-like repeats contained in each arm of the TNC hexamer, where a subset of the 14.5 repeats are able to bind the EGFR at a distinctly low affinity (Leahy, Hendrickson et al. 1992; Swindle, Tran et al. 2001; Iyer, Tran et al. 2007). Because of this specialized binding interaction, matrices containing TNC are able to bind EGFR with high avidity, activating downstream signaling without inducing receptor internalization due to the low affinity. Our group has previously shown that sustained EGFR activation through TNC-coated culture surfaces can promote MSC resistance to Fas ligand-induced cell death *in vitro,* with constitutive activation of downstream signaling pathways (Iyer, Tran et al. 2007; Iyer, Tran et al. 2008; Rodrigues, Yates et al. 2013).

Functionally, this system should be able to also promote MSC resistance to cell death *in vivo*, but must be functionalized to appropriate matrix-based approaches for wound healing.

In this study, we applied our system of TNC-based EGFR tethering to a polyethylene glycol-based polymer for skin wound healing. We tuned the polymer to incorporate a TNC/collagen I coating for MSC seeding, followed by integration of primary MSC cultures into the polymer dressing. This treatment was applied to two mouse models, wild type and CXCR3 -/-, which are an impaired wound healing model through excessive scarring and delayed wound healing (Huen and Wells 2012). We found that the polymer with TNC incorporated was able to keep over 40% of implanted MSCs alive after 3 weeks in the wound bed, a significant improvement over many clinical studies involving MSCs. In addition, we found a correlated improvement in wound healing outcomes in both mouse models as a result of the polymer treatment, including more rapid wound maturation and collagen production/alignment, and altered infiltration of murine cells in the wound healing response. This TNC-based approach shows promise for improving MSC utility in matrix-based scaffolds for cell therapy across multiple wound healing contexts.

3.3 MATERIALS AND METHODS

3.3.1 Reagents

For all cell culture, α-MEM [15-012-CV] was obtained from Mediatech (Manassas, VA), FBS [S11550H] was obtained from Atlanta Biologicals (Flowery Branch, GA), and L-glutamine

[25030-081] was obtained from Thermo Fisher/Gibco (Grand Island, NE). CM-DiI cell tracker [C7000] used in culture was obtained from Life Technologies (Grand Island), and infared cell tracker was also obtained from Life Technologies [D12731]. For plate coating, rat tail collagen I [354236] was obtained from BD at 4.41 mg/mL concentration and Tenascin C was obtained from EMD Millipore [CC065] at 100 µg/mL. $Na_2B_4O_7$ for prepolymer solvent was obtained from Sigma [S9640-25G], and $AgNO_3$ and $(NH_4)_2S_2O_8$ for prepolymer catalyst solutions were obtained directly from the polymer manufacturer, CM-Tec (Newark, DE). For plug digestion and cell processing for flow, Liberase TL [0540102001] was obtained from Roche (Indianapolis, IN) and 40 µM filters [352340] for cell filtration were obtained from BD.

3.3.2 Cell culture and MSC labeling

Primary bone marrow-derived MSCs were obtained from the NIH-funded MSC repository at Texas A&M under the direction of Dr. Darwin Prockop. These cells are certified and analyzed for appropriate surface marker expression/differentiation potential by the repository prior to shipment. As characterized by the repository, this MSC population was from a male 24 year old donor and showed 64-66% percent CFUs upon analysis of recovered P1 plated cells. The MSCs showed a doubling period of approximately 24 hours through three passages of low density expansion testing, with positive results for both osteogenic and adipogenic differentiation protocols as well. Surface marker analyses demonstrated typical MSC characteristics, with 98.7% positive for CD105, 99.0% positive for CD90, and 98.3% positive for CD73, in addition to 0.19% positive for CD34 and 0.16% positive for CD45, as expected. All primary MSCs were cultured in α-MEM supplemented with 17.5% triple dialyzed FBS and L-glutamine. P1 MSCs

were expanded at 37°C to approximately 70% confluence, when they were passaged until P3 to allow for sufficient cell numbers for implant.

For cell tracking, CM-DiI (Thermo Fisher) [C7000] was resuspended at 1 mg/mL in DMSO as a stock solution, and then diluted to 1 µM in warm PBS, where it was left on cells at 37°C for 5 minutes followed by 15 minutes at 4°C. The flask was then washed and incubated in proliferation media overnight. Fluorescent signal from MSCs in culture was confirmed prior to use in experiments. For infared tracking of implanted cells, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide (Life Technologies) [D12731] was added directly to culture media at 1 µM from a stock solution of 1 mg/mL. Labeling solution was incubated on the adherent MSCs for 30 minutes, followed by replacement with normal proliferation media.

3.3.3 Polymer fabrication

The polyethylene glycol/LDI-DOPA polymer used for all wound healing assays was assembled in concordance with previous procedures from our group (Babu, Zhang et al. 2006; Yates, Whaley et al. 2007). Prepolymer (PEG400-(LDI-DOPA)$_2$) was obtained in bulk from CM-Tec (Newark, DE). Briefly, a stock solution for redox catalysis was prepared as a combination of 14.4 mM aqueous $AgNO_3$ and 7.1 mM aqueous $(NH_4)_2S_2O_8$, and solvent for the prepolymer was prepared as 0.1 M aqueous $B_4Na_2O_7$. Prepolymer was dissolved in 500 µL $B_4Na_2O_7$ solvent in a Teflon dish for 5 minutes, and 7 µL of $AgNO_3$/$(NH_4)_2S_2O_8$ catalyst solution per 0.4 g prepolymer was then directly added to the dissolved prepolymer for mixing. The redox catalysis was allowed to proceed for 20 minutes, after which the formed polymer was deposited into coated plates for cell incorporation or directly applied to mouse wounds.

3.3.4 MSC incorporation and polymer application

Passage 3 primary bone marrow-derived MSCs were expanded and subsequently harvested via trypsinization. The cells were combined into one tube and counted using a Scepter cell counter (EMD Millipore), and 1 million cells were re-seeded into each well of a 6-well plate, with each well left pre-coated with 1 μg/cm^2 of rat tail collagen I and human Tenascin C (one or both, depending on the condition). After 12 hours of incubation in the coated plates, 0.4 g of redox-catalyzed polymer was directly added to each well, and the contents of the well was gently scraped and mixed together using a cell scraper. The cellularized polymer was then transferred directly to the mouse wounds and allowed to incorporate over 60 minutes. For non-MSC treatments, catalyzed polymer was directly applied to the wound bed following preparation.

3.3.5 Polymer characterization analyses

For *in vitro* assessment of the polymer, degradation assays and cell incorporation assays were performed. Degradation was allowed to proceed with 0.4 g formed polymer in 2 mL PBS at 37°C over 10 days, with a daily scoring of remaining polymer area as a means of analyzing degradation. Cells tracked with CM-DiI label were visualized via fluorescent microscopy within the polymer immediately after incorporation.

3.3.6 Mouse procedures

Wild type FVB mice and CXCR3 -/- mice were obtained from Jackson Laboratories, ME, USA. 8 mm punch biopsy wounds were formed on the back of each mouse, one wound per

subject. Mice were either left untreated as controls or treated with varying degrees of polymer formation/MSC incorporation, 0.4 g formed polymer per 8 mm wound. All mice were housed on a 12 hour light/dark cycle, and given food and water as needed. Mouse wounding and sacrificing was performed in accordance with Animal Welfare Act regulations, and full garb with mask, gloves, and gown was utilized during all animal exposure. All animal care was performed in accordance with the VA Healthcare System and IACUC guidelines.

3.3.7 Wound tissue extraction and sectioning

Mice were treated with polymer (+/- TNC, +/- MSCs) for both short term experiments for MSC survival and long term assays for wound healing outcomes. For short term time points (3, 7, 21 days), mice were euthanized via CO_2 asphyxiation and tissue in the 8 mm punch biopsy site was extracted, including unwounded tissue surrounding the wound bed. Wounds were cut in half, and tissue was fixed in formalin and paraffin-embedded, afterwards being transported to a core pathology laboratory for sectioning, including standard procedures for H&E and Trichrome stains.

For Picrosirius Red birefringence under polarized light, distribution of fibrils in terms of thickness (cross-sectional area) and arrangement in terms of length of the collagen scars were quantitatively analyzed by using Metamorph analysis. Polarization microscopy reveals tightly packed thick and long fibrils of type 1 collagen as either bright red-orange intense birefringence in tissue and thin short loose fibrils as yellow-green. Percent staining of mature fibers was determined by comparing the total staining intensity of the birefringence (area of staining summed for intensity of pixel) of wound biopsies compared with the biopsies of the contralateral unwounded skin.

Wound healing was analyzed in a double-blinded manner by veterinary pathologist Dr. Joe Newsome, Dept of Pathology, UPMC using a scoring scale to quantify healing outcomes. Epidermal healing was quantified on a 4 point whole-number scale, where 0= no migration, 1= partial migration, 2= complete migration with partial keratinization, 3= complete keratinization, and 4= hypertrophic epidermis. Dermal healing was scored on a similar 4 point scale, where 0= no healing, 1= inflammatory infiltrate, 2= granulation tissue present- fibroplasias and angiogenesis, 3=collagen deposition replacing granulation tissue >50%, and 4= hypertrophic fibrotic response.

3.3.8 MSC extraction and flow cytometry analysis

For short term MSC survival analyses and inflammatory panels, wounded tissue (with a small portion of surrounding unwounded skin) was extracted per above procedures, and allowed to digest for 2 hours in Liberase at 37°C to allow for cell release from the skin. Whole cell extracts were counted and separated into flow cytometry tubes at $2x10^5$ cells per tube. Samples were blocked for non-specific Fc mediated interactions via CD16/32 [BD 553142] for 20 minutes on ice, and then probed with antibodies for human markers or murine inflammatory markers. For MSC survival, HLA-ABC [BD 560169] was used as a single probe for human cells in the xenotransplant model. For murine inflammation, the same cell extracts were probed for CD3e [BD 553063] and CD31 [BD 550274] positivity to assess T cell populations and potential angiogenesis.

3.3.9 Statistical analyses

For wound healing outcomes (wound maturation, dermal maturation, collagen content) a two-way student's t-test was performed for direct comparisons of treatments, with p values <0.05 considered statistically significant. For inflammatory infiltrate, one-way ANOVA of each mouse model group was performed for all three conditions, with $p < 0.05$ considered significant.

3.4 RESULTS

3.4.1 Stable PEG-polymer fabrication and MSC incorporation

The PEG-DOPA/collagen polymer was first subjected to *in vitro* assessment to determine its suitability for MSC incorporation and wound application. The standard polymer formulation was prepared in 0.5 g batches and allowed to degrade in warm PBS at 37°C over 10 days, and polymer degradation was assessed as deterioration of the whole bulk polymer via area measurement (Figure 12a/b). The fully formed polymer with TNC showed stable and more controlled degradation than either the polymer prior to redox catalysis (prepolymer) or the polymer without TNC incorporated. Whole polymer images from day 8 of degradation reflected this trend (Figure 12b). We also assessed both the ability of MSCs to incorporate into the functional polymer and also assessed integration into the wound site post-addition of MSCs. MSC incorporation analysis showed a relatively even and concentrated presence of MSCs (tracked red; Figure 12c) following mixing. As shown in Figure 12d, the fully cellularized

polymer also integrated well into the wound beds, absorbing into the tissue after approximately 60 minutes.

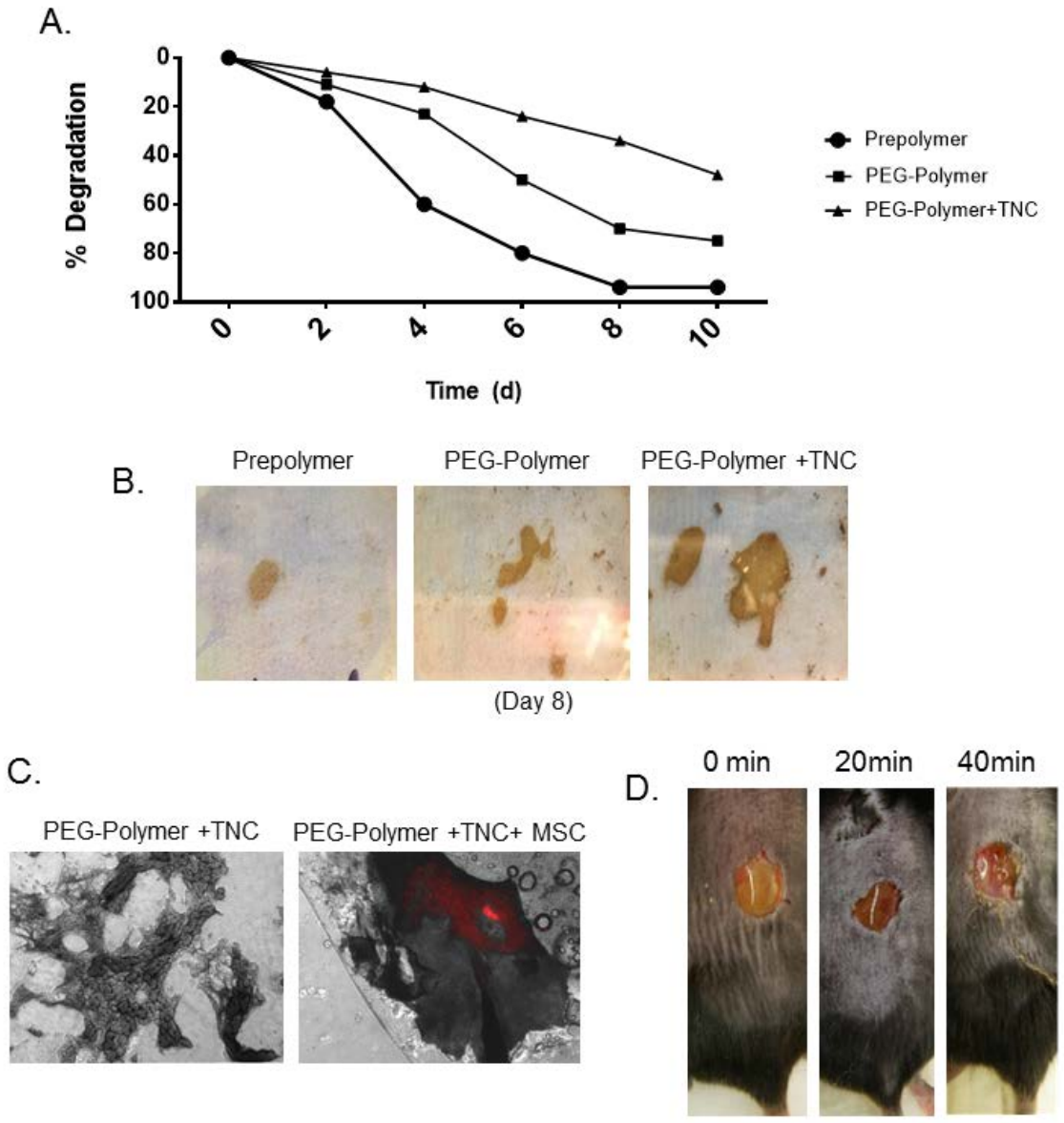

Figure 12. The PEG-collagen polymer with TNC degrades gradually in vitro and integrates into mouse wounds. The PEG-collagen polymer was assessed in vitro for degradation in PBS over 10 days as a function of preparation state (prepolymer, polymer alone, or polymer + TNC) (A), with percent degradation assessed as a size of the original polymer aggregate (B; Day 8 shown). For MSC incorporation testing, cell presence was monitored via CM-DiI cell tracker on MSCs prior to addition to the polymer (C; Day 8 shown).

10X images 200 μM. For polymer application, the functional polymer showed adequate integration into wound beds after 40-60 minutes (D).

3.4.2 MSC survival in the TNC-based polymer

MSC utility in the wound bed is highly dependent on adequate implanted cell survival over time. To assess this, we excised untreated and MSC/polymer-treated wounds over 21 days of wound healing and digested the tissue to release whole cell extracts into solution. From there, cells were probed for human leukocyte antigen (HLA) positivity to assess the presence of MSCs in each wound. Results show that in both the wild type and CXCR3 knockout mouse models, MSCs were able to survive at a detectable level out to 21 days, well above the reported clinical standard for survival *in vivo* of no more than a few days (Figure 13) (Hu, Yu et al. 2008; Giannoni, Scaglione et al. 2010; Zimmermann, Gierloff et al. 2011). Specifically, at day 21 we found roughly 40% of the original 1 million MSCs remained detectable. Notably, the estimated number of MSCs (derived from percent HLA-positive cells multiplied by cell counts) were higher in the first week than the implanted level of 1 million cells, which may account for the high percentage of surviving cells at the end of the study. Results also showed a decreased apparent MSC survival at day 7 in the knockout mice, despite consistent survival levels at the other time points.

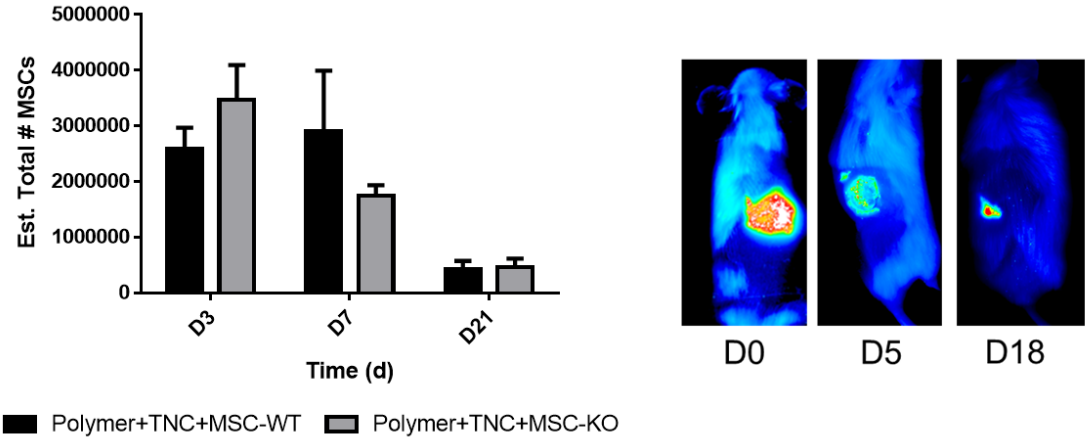

Figure 13. The polymer matrix with TNC promotes long term MSC survival. Cells were extracted from mouse wounds via collagenase digestion and probed with a human-specific HLA antibody to detect human MSCs via flow cytometry. MSC survival in the wound bed was assessed for both wild type and CXCR3 -/- mice. An estimated total number of MSCs extracted was determined by applying %HLA+ back to the whole cell count. As a confirmation, MSCs were tracked prior to polymer application with an infrared membrane label, and wound areas were imaged directly on wild type mice using a LI-COR Odyssey imaging system to detect the IR label. Shown here are three representative images over 18 days. N=3-5 for flow cytometry analyses; n=1 for IR tracking confirmation.

We additionally tracked the implanted MSCs via infrared labeling and direct detection via Licor Odyssey analysis (Figure 13), with a small n=1 as a confirmatory method. We found that signal from the cells was visibly detectable out to 18 days, comparable to data obtained from the primary experiment. This confirmation suggests that the TNC-based polymer for wound healing was indeed able to promote an adequate level of MSC survival *in vivo*.

3.4.3 Wound maturation and collagen content improve with Tenascin C additive

In addition to survival of implanted cells, wound healing outcomes in the mice were assessed as a function of polymer treatment. Tissue sections were treated with both H&E and Trichrome stains to assess the maturation of the wound healing process and collagen content changes in the wound bed (Figure 14). A double-blinded scoring analysis of H&E sections showed a faster overall wound maturation and specific dermal maturation in both the wild type and knockout models, with improved scores at all time points in polymer treated mice over untreated controls (Figure 14a/b). Notably, in the knockout model wound healing scores never reached healing levels in the untreated wild type mice, even at 60 days; treatment with the MSC polymer improved the healing response to the untreated wild type control level in both assessments, even outscoring the wild type model in some cases.

Collagen content was also assessed via Metamorph analysis of trichrome staining normalized to control unwounded skin (Figure 14c/d). The normalized data showed that polymer-treated wounds in both wild type and knockout models had an overall improvement in collagen content at all time points, visibly evident in the representative sections shown here. The knockout mice lagged behind early in collagen content, but improved to the level of the treated wild type model after approximately 21 days. Trichrome sections also seemed to show an improvement in early collagen alignment outcomes, visible in the day 7 sections presented here. Though a potential concern with increased collagen production may be a tipping of the balance towards keloid formation or undesirable scarring outcomes, we did not observe any deleterious effects of the improved collagen content in the long-term wounds prior to excision.

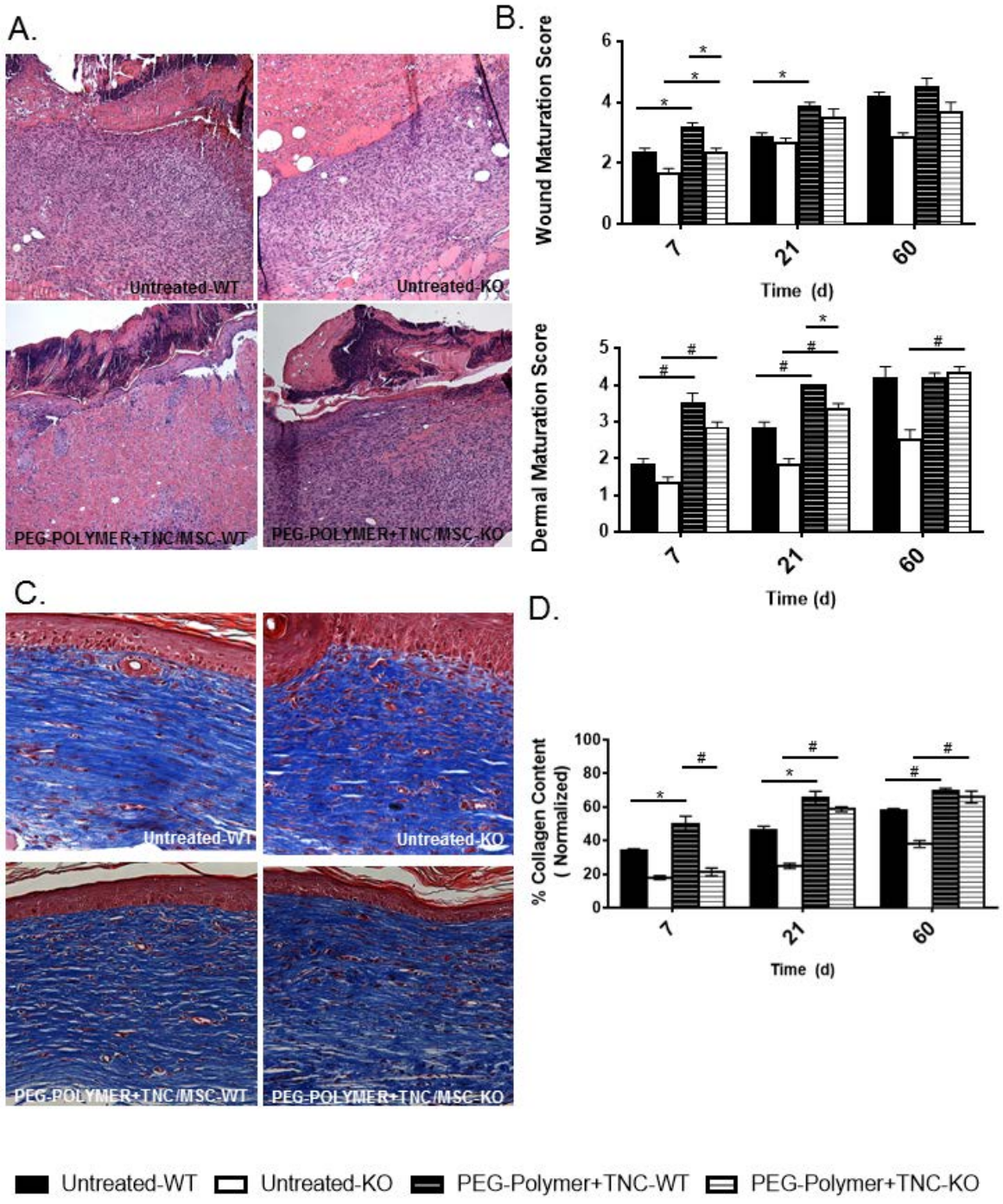

Figure 14. MSC-based polymer yields improved wound healing outcomes. Full thickness sections of mouse wounds across 60 days were assessed via H&E stain (A; Day 7 shown) and quantified for whole wound maturation and dermal maturation (B; Day 7 shown) by a veterinary pathologist in a double-blinded analysis. Both wild type and CXCR3 -/- mice were assessed (for scoring method, see experimental

procedures). Wound tissue was also sectioned and treated with a trichrome stain to assess matrix content and organization at the same time points (C; Day 60 shown). Total percent collagen content was analyzed through a Metamorph analysis normalized to unwounded tissue (D). N=3. *p<0.05, #p<0.01.

3.4.4 Collagen alignment is improved in the wound bed with Tenascin C additive

To further investigate collagen alignment and maturity in the wound bed following polymer treatment, sections were also probed with picrosirius red stain (Figure 15). Metamorph quantification showed an improved maturity/alignment in both wild type and knockout models over 90 days, with more pronounced improvements in alignment in the knockout mice. Additionally, appearance of collagen I (red), indicative of a more mature wound, was more prevalent in the MSC treated mice (D60 shown here; Figure 15a). Differences in maturity were again more noticeable in the knockout mice, where wound healing is impaired and more collagen III (green), indicative of a wound in a more immature state, is present in the wound bed in general. Particularly in the CXCR3 -/- mice, the polymer + TNC condition without MSCs did seem to demonstrate some improvement in collagen alignment despite the lack of cellular treatment.

3.4.5 Murine cellular infiltrate is altered with the Tenascin C additive

To assess any role of potential paracrine activity from MSCs surviving in the wound bed, we assessed CD31+ cell infiltration as a metric of angiogenesis and CD3e+ infiltration as an assessment of murine inflammatory response in relation to T cell invasion (Figure 16). Flow

cytometry analysis showed that inflammatory infiltrate early in the wound healing response was statistically unaffected in general by treatment, although trends did show that polymer application tended to blunt CD3+ cell invasion and both day 3 and day 7 during the peak inflammatory response (Figure 16a). In general, results did now show much change as a result of including MSCs in the polymer for CD3+ cell results, although CXCR3 -/- mice did demonstrate a trend towards reduced infiltration early at day 3, albeit not statistically significant.

CD31+ cell infiltration, as a method for assessing angiogenic potential in the wound bed, did show marked changes as a result of polymer treatment (Figure 16b). Both wild type and knockout mice demonstrated an approximately two-fold increased presence of CD31+ cells after 3 days, with relatively comparable levels to the untreated mice at day 21. Day 7 results also shown a trend towards increased CD31+ cell invasion in both models, although the knockout mice alone demonstrated statistical significance as the +MSC condition for the wild type model reverted to a lower level of invasion at day 7. Again, there did not appear to be much of a change as a result of MSC presence in the wound bed, suggesting the polymer itself (and perhaps Tenascin C) acted as a paracrine attractant for the invading murine cells.

3.5 DISCUSSION

Mesenchymal stem cells are widely studied in the context of wound healing as potential sources of paracrine benefit to the wound healing process as well as sources of progenitor populations for differentiation. However, limited MSC survival upon implantation into the harsh wound environment has yielded limited success with MSC therapies for wound healing to this

point. This fundamental issue has led to research into strategies for extending the lifespan of MSCs *in vivo*, and consequently improving any associated outcomes in wound healing as a result

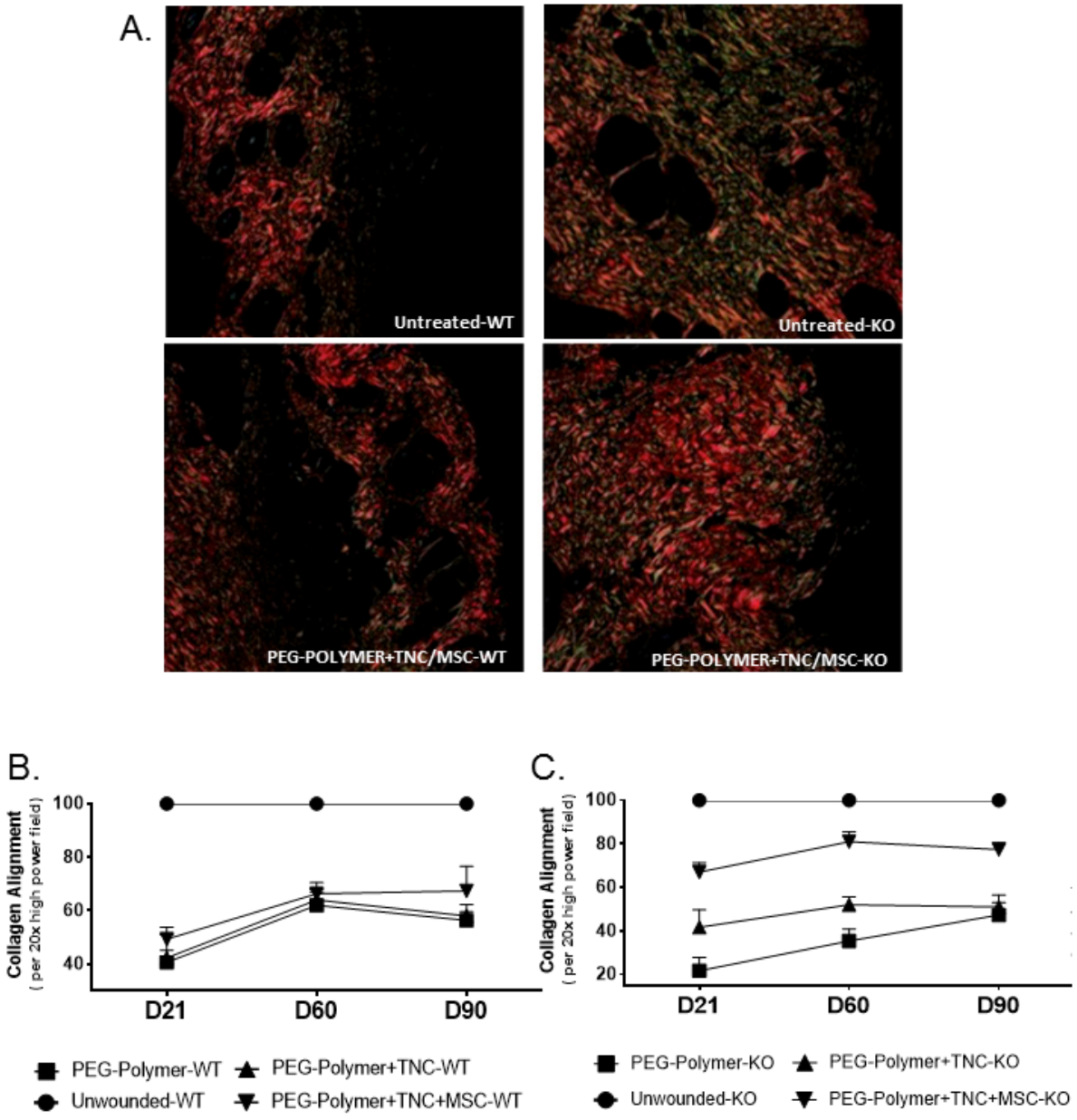

Figure 15. Collagen alignment and maturation improves using the MSC-based polymer. Full thickness mouse skin sections from wound beds were excised over 60 days and probed with picrosirius red stain to assess collagen outcomes in the wounds (A; D60 shown) for both wild type and CXCR3 -/- mice. PS Red was scored for collagen alignment in a double-blinded fashion by a veterinary pathologist. N=3.

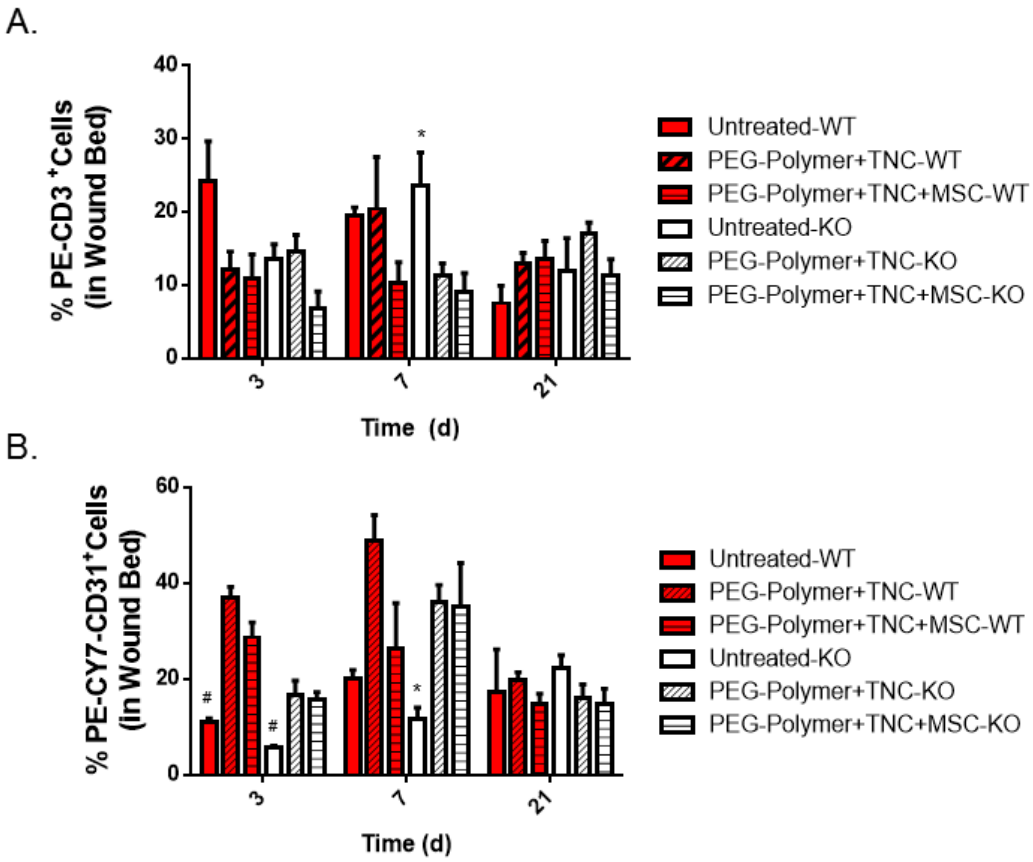

Figure 16. MSC-based polymer augments murine cell infiltrate in wound beds. Cell extracts from mouse wounds in both wild type and CXCR3 -/- mice were probed for the presence of CD3e+ (A) and CD31+ (B) cells to assess inflammatory and angiogenic infiltrate over the first 21 days of wound repair. Treatment conditions analyzed for murine infiltrate were untreated mice, mice treated with a TNC-based polymer only, and mice treated with the TNC-based polymer combined with primary human MSCs. N=3-5. *p<0.05, #p<0.01.

of a higher number of available MSCs. To that end, our group has worked to exploit the EGFR signaling cascade in MSCs to promote survival signaling and improve resistance to death stimuli. We have previously utilized the matrikine Tenascin C to this effect, taking advantage of EGF-

like repeats in the TNC hexamer that are able to bind EGFR at a low affinity but high avidity, allowing for surface-restricted and constitutive EGFR activation. Here, we applied this protein to a polymer for skin wound healing, allowing MSCs to be exposed to the survival signaling *in vivo* and promote healing in mice. We found that the TNC-based polymer allowed for extensive survival of the implanted MSCs, with over 40% of the original population surviving at 21 days. This led to improved wound healing outcomes in wild type and CXCR3 knockout mice, with enhanced dermal maturation, collagen content, and collagen alignment in the wound beds in long-term assessments.

Matrix-based approaches to wound healing are not a particularly novel innovation, and many commercially available treatments for chronic wound dressings utilize fabricated or existing matrices to that effect. This has previously included collagen-based dressings, including decellularized collagen matrices in concert with glycosaminoglycans (Iorio, Goldstein et al. 2011), bilayered matrices incorporating fibroblasts and keratinocytes to jump-start the healing process (Falanga and Sabolinski 1999), and others. While these approaches to provide the host tissue with space for native cells to migrate and engraft into the new wound matrix, they ultimately fail to address many of the key pathologies for chronic wound healing, such as hyperinflammation or reduced angiogenesis (Stadelmann, Digenis et al. 1998; Falanga 2005; Kirsner, Marston et al. 2012; Krisp, Jacobsen et al. 2013). Thus, novel treatments to address these factors have become necessary in the next generation of wound healing strategies. We have investigated mesenchymal stem cells for this purpose due to their widely acknowledged role in normal wound healing, as well as capacity to modulate the chronic inflammation and avascularity that plague such wounds (Wu, Chen et al. 2007; Chen, Tredget et al. 2008; Stoff, Rivera et al. 2009; Sorrell and Caplan 2010; Jackson, Nesti et al. 2012; Wan, Xia et al. 2013).

While MSCs undoubtedly hold great promise for improving on existing matrix-based approaches to wound healing, a fundamental limitation in all MSC therapies has been limited cell survival upon implant due to the harsh nature of the wound environments. Due to the matrix-based character of the wound healing polymer (and other cited treatments for wound healing), a logical additive to our treatment were proteins that might assist with improving survival of implanted cells. We utilized the EGF-like repeats of Tenascin C here for this purpose, which are able to bind EGFR at low affinity but high avidity to induce pro-survival signaling constitutively. While we showed here that this approach can in fact promote long term survival of implanted cells, this treatment strategy also affords any migrating resident cells the opportunity to survive much longer than expected in the wound bed. Indeed, a major factor contributing to the chronic nature of diabetic ulcers and other comparable conditions is the lack of healthy fibroblasts and/or keratinocytes lining the wound bed, which are subjected to the same conditions that implanted MSCs face *in vivo* (Bucalo, Eaglstein et al. 1993; Hehenberger, Kratz et al. 1998; Phillips, al-Amoudi et al. 1998). By incorporating an abundance of Tenascin C, it is probable that these cells (fibroblasts, keratinocytes, CD31+ cells, and others) will have the ability to invade and engraft in the same matrix, ideally surviving on the same time scale and thus allowing a more complete healing response. By the same token, invading inflammatory cells might also be able to exploit the TNC infrastructure to resist anti-inflammatory strategies, reinforcing the importance of maintaining the immunomodulatory MSC population at high quality in the long term.

A limitation of this study is the well-acknowledged limitations of murine skin wound healing. As can be seen even in our data in this study, mouse wounds rarely fail to heal and, in fact, mostly heal at a rapid pace, entering the remodeling phase within a few weeks at most. Long term outcomes that are analyzed such as our day 60 or day 90 time points here provide

little insight beyond potential scarring implications, as seen in the CXCR3-/- model used by our group. As such, while we do show an improved rate of healing and collagen maturation in mouse wounds treated with our polymer, we have not here delineated effects in a 'chronic wound' model or one with a delayed healing response in general. As chronic wound models are quite limited in scope in murine systems, future studies scaling this MSC-based polymer for chronic wound therapies will require an examination of more appropriate wound healing models, such as porcine flaps or contaminated wound sections to attempt to truly inhibit the healing process. We have also here only examined bone marrow-derived MSCs, whereas many groups have transitioned to looking at adipose-derived MSCs for skin wound therapy due to easing the burden of translation to humans and potential differences in paracrine profiles of the two subpopulations. Future studies examining the optimal conditions for MSC success in this wound healing polymer must include an exploration of the MSC subtype most suited for the TNC approach to extending survival while also leading to the most desirable wound healing outcomes.

In addition to the functional importance of MSCs in our treatment strategy, the delivery polymer must also encapsulate the other necessary aspects of complete wound healing. Previously, we have shown that this PEG polymer is able to promote wound healing in comparable mouse models (Yates, Whaley et al. 2007), also leveraging an $AgNO_3/(NH_4)_2S_2O_8$ catalyst system for polymerization for this purpose. Silver has a well-documented antimicrobial role (Kumar and Munstedt 2005; Kim, Kuk et al. 2007), and as such we originally applied this as the rationale for a silver nitrate-based redox catalysis method. Silver ions in solution as part of the catalysis process are ultimately able to act in a dual fashion as both a catalyst and an antimicrobial agent, which we showed was functional in previous studies with this polymer (Babu, Zhang et al. 2006; Yates, Whaley et al. 2007). In concert with the wound healing matrix

and MSC-based therapeutic for reducing hyperinflammation and inducing a more robust wound healing response, this polymer as whole appears to encapsulate the entire wound healing process and holds great promise for scaling to chronic wound therapies in the future.

4.0 HUMAN MESENCHYMAL STEM CELLS UNDERGO AUTOPHAGY AT THE INITIATION OF DIFFERENTIATION

This chapter contains material largely excerpted from:

Nuschke A, Rodrigues M, Stolz DB, Chu CT, Griffith L, Wells A. Human mesenchymal stem cells/multipotent stromal cells consume accumulated autophagosomes early in differentiation. Stem Cell Research & Therapy. 2014;5(6):140.[9]

4.1 ABSTRACT

Bone marrow mesenchymal stem cells/multipotent stromal cells (MSC) are recruited to sites of injury and subsequently support regeneration through differentiation or paracrine activity. During periods of stress such as wound site implant or differentiation, MSCs are subjected to a variety of stressors that might activate pathways to improve cell survival and generate energy. In this study, we monitored MSC autophagy in response to the process of differentiation.

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MSC autophagosome structures were observed using transmission electron microscopy and a tandem GFP-RFP autophagic flux reporter to monitor LC3 turnover in real time. MSCs were differentiated using standard osteogenic and adipogenic media, and autophagy was examined during short term and long term differentiation via immunoblots for LC3-I and II. Autophagy was modulated during differentiation using rapamycin and bafilomycin treatments to disrupt the autophagosome balance during the early stages of the differentiation process, and differentiation was monitored in the long term using Von Kossa and Oil Red O staining, as well as qPCR analysis of typical differentiation markers.

We found that undifferentiated MSCs showed an accumulation of a large number of undegraded autophagic vacuoles, with little autophagic turnover. Stimulation of autophagy with rapamycin led to rapid degradation of these autophagosomes and greatly increased rough endoplasmic reticulum size. Upon induction of osteogenic differentiation, MSC expression of LC3 II, a common autophagosome marker, was lost within 12 hours, consistent with increased turnover. However, during adipogenic differentiation, drug treatment to alter the autophagosome balance during early differentiation led to changes in differentiation efficiency, with inhibited adipocyte formation following rapamycin treatment and accelerated fat accumulation following autophagosome blockade by bafilomycin.

Our findings suggest that MSCs exist in a state of arrested autophagy with high autophagosome accumulation, and are poised to rapidly undergo autophagic degradation. This phenotype is highly sensitive and a balance of autophagy appears to be key in efficient MSC differentiation and function, as evidenced by our results implicating autophagic flux in early osteogenesis and adipogenesis.

4.2 INTRODUCTION

Mesenchymal stem cells/multipotent stromal cells (MSCs) have the ability to migrate into sites of injury, self-renew and differentiate, as well as release trophic and growth factors (Pereira, Halford et al. 1995; Pittenger, Mackay et al. 1999; Bianco, Riminucci et al. 2001; Kim, Yoo et al. 2005). These activities combine to bring about post-injury tissue regeneration, making them prime candidates for use in regenerative medicine, including repair of tissues such as bone and cartilage. For purposes of therapy, MSCs are often implanted into wound beds devoid of nutrients and oxygen, and high in reactive oxygen species and pro-inflammatory/pro-death cytokines, which lead to a rapid loss of these cells (Zhu, Chen et al. 2006; Kim, Meliton et al. 2010; Rodrigues, Griffith et al. 2010; Rodrigues, Turner et al. 2012). However, endogenous MSCs contribute to wound healing, despite being subject to the harsh wound microenvironment, suggesting that MSCs have an innate mechanism of adapting to an environment low in nutrients. In other situations, MSCs also face highly demanding conditions during the process of expansion and differentiation, where the cells are used to generate new tissue; this has been studied in the context of myocardial repair, epidermal skin healing, and many others (Falanga, Iwamoto et al. 2007; Lu, Chen et al. 2011; Wang, Pasha et al. 2013; Williams, Suncion et al. 2013). In either case, cellular mechanisms that can help the cells prime themselves to efficiently overcome these high metabolic demands would be advantageous to the cell on an innate level and also as potential mechanisms to exploit to improve clinical outcomes.

Macroautophagy, a conserved form of autophagy (and called simply autophagy hereafter), is a catabolic process of ‘self-eating’ or cannibalism wherein starving cells fuel themselves by forming double membranous vacuoles called ‘autophagosomes’ that sequester and degrade cytoplasmic material upon fusion with lysosomes. Traditionally, autophagy has been

considered a means of recycling cellular components during times of nutrient starvation, and indeed autophagosome formation is prevalent in cells under nutrient deprivation and hypoxia (Papandreou, Lim et al. 2008; Rabinowitz and White 2010). Additionally, autophagy plays a role in cellular differentiation such as mitochondrial clearance during erythrocyte differentiation or fat droplet deposition during adipocyte differentiation (Mizushima and Levine 2010). Previous studies have found autophagosomes to be present in MSCs (Oliver, Hue et al. 2012; Zhang, Yang et al. 2012; Pantovic, Krstic et al. 2013), at a level higher than many differentiated cells. This suggested that the autophagosomes are altered during differentiation.

In this study we queried whether autophagosomes play a role during MSC differentiation and function, and thus could be potentially modulated to affect the differentiation process. We used transmission electron microscopy and the autophagosome marker LC3 II to determine that autophagosomes were more prevalent in the MSCs than the differentiated cells, with the cells being filled with autophagosomes. Using a tandem fluorescent reporter to examine autophagic flux, we found that in MSCs under normal conditions *in vitro* these autophagosomes had not fused with lysosomes and therefore weren't being degraded or recycled. Additionally, a forced release from this hold on autophagy led to rapid loss of autophagosomes accompanied by expansion of the rough endoplasmic reticulum indicative of cellular reprogramming. We further studied differentiating MSCs, showing activation of autophagy during early differentiation and altered differentiation outcomes when autophagy was modulated during the same time period. Our results suggest a mechanism by which MSCs are arrested in mid-autophagy while being maintained as multipotent cells, allowing for rapid generation of autophagosome degradation products when needed. This function could allow the cells to adapt to challenging conditions in wound healing, and ultimately may be a hallmark of a stem cell.

4.3 MATERIALS AND METHODS

4.3.1 Reagents

DMEM [10-014-CV] was obtained from CellGro (Manassas, VA). RNase Minikit [74104] and Quantitect Reverse Transcription Kit [205311] were obtained from Qiagen (Valencia, CA), and Brilliant SYBR Green qPCR Master Mix [600548] was from Agilent Technologies (Santa Clara, CA). Bafilomycin A1 from S. griseus [B1793] was obtained from Sigma-Aldrich (St. Louis, MO) and tamoxifen citrate [54965-24-1] from ICN Biomed (Irvine, CA).

For immunoblotting, rabbit polyclonal LC3 antibody [NB100-2331] was obtained from Novus Biologicals (Littleton, CO) and goat anti-rabbit IgG secondary antibody [A9169] was from Sigma-Aldrich (St. Louis, MO). Housekeeping gene anti-actin produced in rabbit [A2668] was from Sigma-Aldrich. Protein ladder for all immunoblots was a Full Range Rainbow marker [RPN800E] from GE Life Sciences (Pittsburgh, PA).

4.3.2 Cell culture

Primary human bone marrow-derived mesenchymal stem cells (prhMSC) were obtained from Dr. Darwin Prockop at the NIH-funded Stem Cell Repository at Texas A&M University. As characterized by the repository, this MSC population was from a male 24 year old donor and showed 64-66% percent CFUs upon analysis of recovered P1 plated cells. The MSCs showed a doubling period of approximately 24 hours through three passages of low density expansion testing, with positive results for both osteogenic and adipogenic differentiation protocols as well.

Surface marker analyses demonstrated typical MSC characteristics, with 98.7% positive for CD105, 99.0% positive for CD90, and 98.3% positive for CD73, in addition to 0.19% positive for CD34 and 0.16% positive for CD45, as expected. For experiments in this study, cells were maintained in α-MEM without, supplemented with 16.5% FBS (Atlanta Biologicals, Lawrenceville, GA), 100 units/ml Penicillin/Streptomycin and 2mM L-glutamine. Cells between passages 1 and 3 were used for experiments. A second human bone marrow-derived cell line, immortalized using hTERT (imhMSC), a kind gift from Dr. Junya Toguchida's lab, Kyoto University, Japan, was also used for studies. Proliferation media comprising DMEM with 10% FBS (Gemini Bio-Products, Sacramento, CA), containing 1 mM sodium pyruvate, 1mM L-glutamine, 1 μM non-essential amino acids, and 100 units/mL penicillin-streptomycin was used.

To account for changes in oxygen concentration, cells were either grown in incubators at ambient air conditions (21% oxygen) or Biospherix incubators at 4% oxygen. At no point in the culture of cells at 4% oxygen were the cells exposed to ambient oxygen conditions, as the microscopes were also contained in the Biospherix chamber.

For osteogenic differentiation media, proliferation media was supplanted with 100nM dexamethasone, 100 μM β -glycerophosphate, and 50 μM L-ascorbic acid. Cells were maintained in osteogenic differentiation media at either 21% or 4% oxygen for up to 30 days. For adipogenic differentiation, the hMSC adipogenic bulletkit [PT-3004] from Lonza (Hopkinton, MA) was used throughout differentiation, with induction and maintenance media switched every three days.

4.3.3 Transmission electron microscopy

MSCs were grown to confluence in a 6 well plate either in 5.5 mM glucose and left untreated or treated with 5 μM rapamycin or 5 nM bafilomycin for 1 hour or 4 hours. After treatment, cells were fixed in 2% glutaraldehyde in PBS, washed in PBS and processed as previously described (Stolz, Ross et al. 1999). 70 nm sections were imaged on a Jeol JEM-1101 transmission electron microscope, and autophagosomes were captured at different magnifications as described in the results.

4.3.4 Osteogenic differentiation

Immortalized hMSC (up to passage 15) were incubated in osteogenic DMEM media for 72 hours (short term) or 30 days (long term) at 21% or 4% oxygen. In short term studies, protein lysates were collected after 1, 3, 6, 12, 24, 48, and 72 hours differentiation. Conditions were included to modulate autophagy using 5 μM rapamycin or 5 nM bafilomycin for the first three hours of differentiation to control short term autophagy outcomes. For long term studies, samples were collected after 0, 10, 20, and 30 days, with hydroxyapatite deposition probed by von Kossa stain. Briefly, samples were washed with PBS without calcium or magnesium, fixed in 4% paraformaldehyde, treated with 1% silver nitrate solution and incubated under UV light for 10 minutes. Wells were washed with sodium thiosulfate overnight and imaged under transmitted light.

RNA was isolated from MSCs grown in osteogenic media on days 0, 10, 20 and 30 using RNeasy kit (Qiagen, Valencia, CA). After treatment with genomic DNA wipeout buffer, 1ug template RNA was reverse transcribed into cDNA using Quantitect cDNA kit (Qiagen, Valencia,

CA). Quantitative PCR was performed using 1 μl cDNA for all samples with GAPDH as control, 12.5 μL of Brilliant SYBYR green master mix (Agilent Technologies, Santa Clara, CA) and 3.75 μL forward and reverse primers. Primers for Runx2 and Osteocalcin, markers for early and late osteogenesis, were used to probe for osteogenesis. Forward and reverse primer sequences were:

GAPDH Forward Primer: 5'-GAGTCAACGGATTTGGTCGT-3'

GAPDH Reverse Primer: 5'-TTCATTTTGGAGGGATCTCG-3'

Runx2 Forward Primer 5'-CCTCGGAGAGGTACCAGATG-3'

Runx2 Reverse Primer 5'- TTCCCGAGGTCCATCTACTG-3'

Osteocalcin Forward Primer 5'-GTTTATTTGGGAGCAGCTGGGATG-3'

Osteocalcin Reverse Primer 5'-GTTTATTTGGGAGCAGCTGGGATG-3'

4.3.5 Adipogenic differentiation

Immortalized hMSC (up to passage 10) were grown to confluence in standard DMEM (see above) at 21% oxygen. Samples were then treated with adipogenic differentiation media (Lonza, see above) and assayed for fat droplet formation after 10, 20, and 30 days. To monitor the effects of modulating early autophagy on the long term differentiation process, separate groups were treated with 5 μM rapamycin or 5 nM bafilomycin for the first three hours of differentiation, after which normal adipogenic media was used for the rest of the 30 day study.

At each time point, fat droplet accumulation was assayed using Oil Red O staining. For the Oil Red O reagent, stock solutions were prepared using 3 mg/mL Oil Red O [O-0625] (Sigma) in isopropanol. Working solutions consisted of 3:2 Oil Red O stock in deionized water. For staining, cells were fixed in 4% paraformaldehyde, washed, and treated with 60% ethanol for

5 minutes. Oil Red O stain was then added for 5 minutes, and samples were imaged for fat droplet formation, with droplets appearing red under transmitted light.

4.3.6 Assessment of autophagosome turnover

Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used to transfect MSCs with an mRFP-GFP tandem fluorescently tagged LC3 plasmid (tfLC3), a kind gift from Dr. Tamotsu Yoshimori's lab, Osaka University, Japan (Kimura, Noda et al. 2007). The plasmid is designed in a manner that both RFP and GFP are expressed in autophagosomes on LC3 until fusion with the lysosome. Once in the acidic compartments of the lysosome, GFP- fails to fluoresce, while RFP-LC3 continues to generate a fluorescent signal. For transfection, 1µg of plasmid was diluted in Optimem and mixed in a 1:1 ratio with Lipofectamine 2000 diluted 1:25 in Optimem. This mixture was incubated for 20 minutes at room temperature, and then added to MSCs grown to 70% confluence in 5.5 mM or 25 mM glucose media without FBS. MSCs were allowed to sit in this mix for 6 hours, after which cells were placed in complete media. Media without FBS was used instead of Optimem due to MSC death in the presence of Optimem alone. Empty vector control was used in assessment of the GFP-RFP LC3 fluorescent signal prior to treating the cells.

Cells were left untreated, to probe for basal autophagosome recycling, or treated with 5 µM rapamycin (LC Laboratories, Woburn, MA), or 5 nM bafilomycin [B1793-2UG] (Sigma) for 15 minutes or 1 hr and imaged for RFP and GFP fluorescence. While yellow (merge of GFP and RFP) puncta indicate autophagosomes prior to fusion with lysosomes, red puncta indicate autophagosomes post fusion with lysosomes (Kimura, Noda et al. 2007).

4.4 RESULTS

4.4.1 MSCs contain a large number of mid-stage autophagosomes

We have earlier reported that MSC generate reactive oxygen species (ROS) even though being largely glycolytic (Funes, Quintero et al. 2007; Pattappa, Heywood et al. 2011; Rodrigues, Turner et al. 2012). As such, we examined the mitochondrial content of immortalized human MSCs (ihMSC) using transmission electron microscopy (TEM). Interestingly, we found a large number of mitochondria, but unexpectedly these were at least partially encapsulated in autophagosomes (Figure 17). The autophagosomes do not appear to have fused with lysosomes and the ratio of early autophagosomes to late/'empty' ones is abnormally high (Figure 17), thus seeming to have accumulated in a partial arrest prior to lysosomal degradation. In contrast, treatment with rapamycin for 1 hour, a drug that inhibits mTOR signaling and stimulates autophagy (Shigemitsu, Tsujishita et al. 1999), led to rapid clearance of previous autophagosome clusters that also coincided with an increase in rough endoplasmic reticulum size (Figure 17). After 4 hours of rapamycin treatment, the remaining autophagosomes seem to have cleared completely, and the rough endoplasmic reticulum size returned to a more normal state (data not shown). There appears to be early stages of new autophagosome formation after 4 hours of rapamycin stimulation, consistent with a new cycle of autophagosome generation. This pattern of autophagosome utilization was not present in untreated MSCs.

Analysis of primary human MSCs (prhMSC) shows a similar phenotype, though not as striking as what is seen with the telomerase-immortalized cells (Figure 17). Notably, the smaller cells in prhMSC cultures were the ones that showed the higher autophagosome concentrations, in contrast with a more constant high concentration in the immortalized cells. Treatment with

rapamycin for 1 hour led to autophagosome degradation as seen in the ihMSCs; rough endoplasmic reticulum (RER) enlargement was also noted though again to a lesser extent than in the immortalized MSC (Figure 17).

4.4.2 Autophagy is highly active during the early stages of differentiation

Recent evidence has suggested that autophagy occurs in MSCs, and more importantly plays a role in a critical early period (approximately 3 hours) during the differentiation process (Pantovic, Krstic et al. 2013). We first looked at how MSCs responded to the first few days of differentiation with respect to changes in LC3, a common marker for autophagosome degradation. Immortalized MSCs were grown to confluence and exposed to standard osteogenic differentiation media for 72 hours, with lysates collected for immunoblot analysis of LC3 at various time points (Figure 18a). To examine changes in LC3 compared to standard drug treatments to modulate autophagy, we also included treatments of rapamycin and bafilomycin (a drug that inhibits autophagy) in separate groups to control MSC autophagy during the aforementioned critical window, followed by replacement of the media with the normal differentiation media (Figure 18a).

About 15 autophagy-related proteins act in a hierarchical manner to bring about initiation, nucleation, elongation, and recycling of the autophagosomes (Codogno, Mehrpour et al. 2012). The mammalian microtubule associated protein-1 light chain 3 (LC3) is part of the elongation-closure machinery of autophagosomes. It is either present in a form unconjugated to lipids called LC3 I that is widely distributed in the cytoplasm, or in a form conjugated to the lipid phosphotidylethanolamine (PE) and bound to both membranes of the mature autophagosome called LC3 II (Kabeya, Mizushima et al. 2000). LC3 II is a commonly used marker for

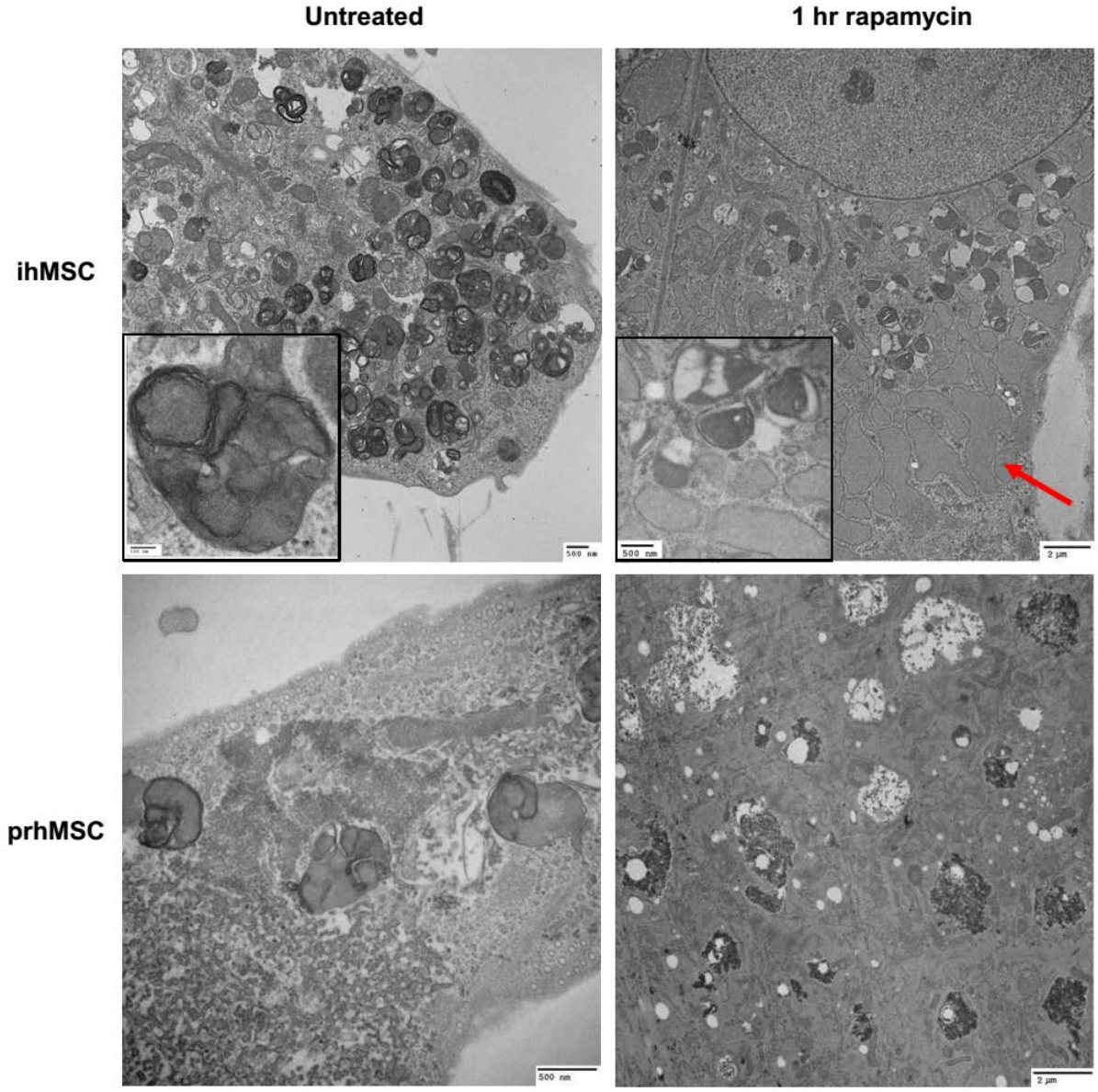

Figure 17. Mesenchymal stem cells show a relatively high number of autophagosomes. Immortalized human MSCs grown at standard culture conditions (1 g/L glucose, 10% fetal bovine serum, or FBS) or following treatment with 5 µM rapamycin for 1 hour were subjected to transmission electron microscopy analysis at 15,000× magnification (top). A 150,000× image of a late-stage autolysosome is included with the control treatment (top left inset), as is a 40,000× magnification of residual autophagosome structures in the rapamycin treatment (top right inset). Primary human MSCs were analyzed for autophagosome formation at

standard conditions (1 g/L glucose, 16.5% FBS) at 40,000× (bottom left) and following 5 μM rapamycin treatment for 1 hour at 10,000× (bottom right). Figure adapted from (Nuschke, Rodrigues et al. 2014).

autophagosomes since it migrates to a lower position during electrophoresis (Klionsky, Abeliovich et al. 2008). LC3 immunoblotting is generally assessed by the relative changes in LC3II (lower band) over time, signifying the degradation of autophagosomes by the lysosome, which decreases LC3II that has been localized to the autophagosome membrane. Osteogenic differentiation results in Figure 16 a and b show a marked and rapid reduction LC3II, decreasing from a high initial level across the course of treatment, with a notable reduction in LC3II just after 3 hours of differentiation stimulus (with relatively constant cytosolic LC3I, as seen in the blots in Figure 18a and quantitated by densitometry across repeat experiments in Figure 18b). There was also an apparent recovery following the initial activation of autophagic flux, as cytosolic LC3I levels increased at 24 hours and a subsequent partial recovery of LC3II levels was observed (Figure 18B).

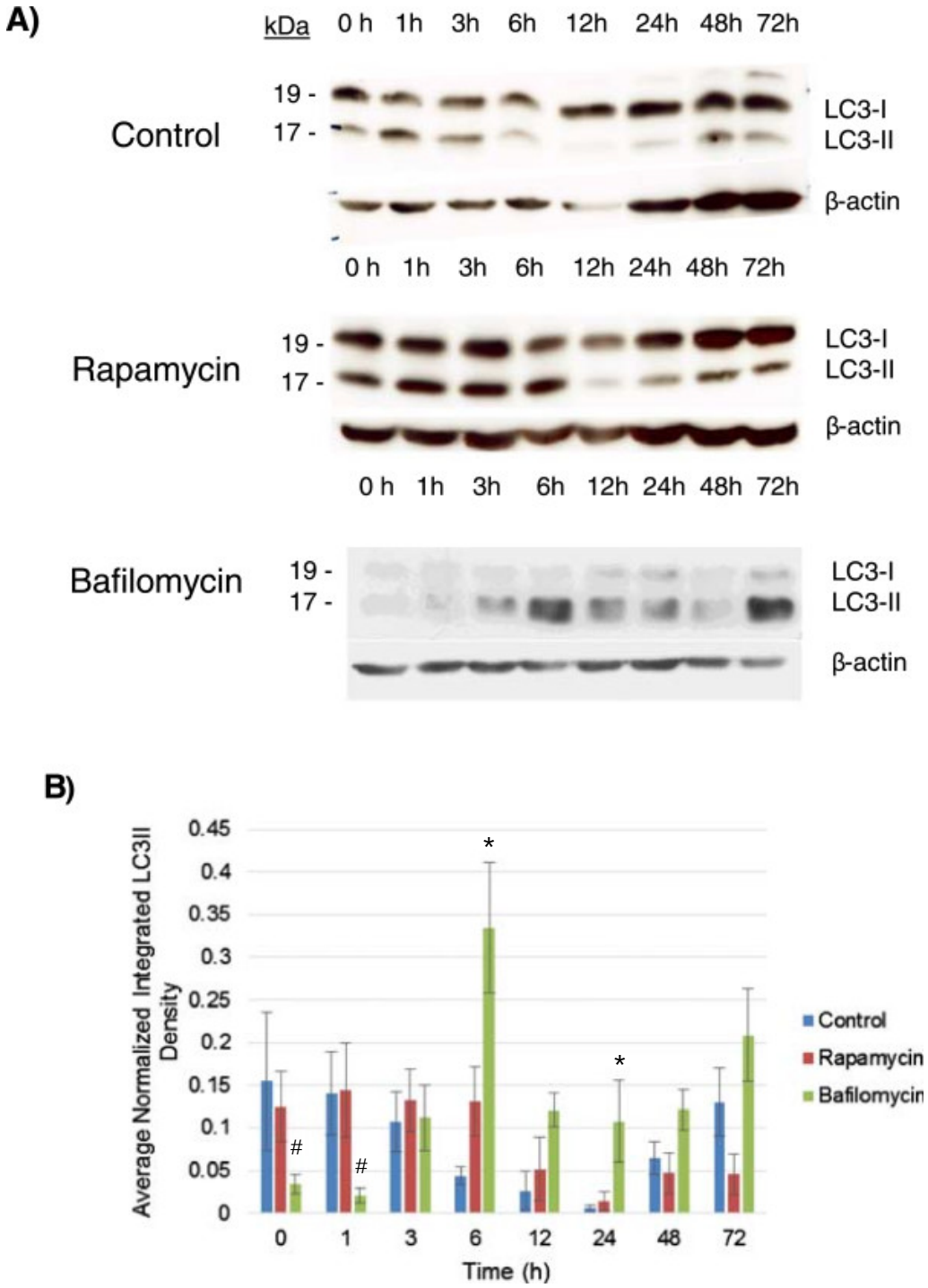

Figure 18. High autophagosome concentration is consumed during early immortalized human mesenchymal stem cell differentiation. (A) Immortalized human mesenchymal stem cells were differentiated under osteogenic conditions (see Materials and methods) and assayed for changes in LC3I and LC3II during a 72-hour window. Cells were differentiated under standard conditions (top) or with addition of 5 µM rapamycin (middle) or 5 nM bafilomycin (bottom) for the first 3 hours of differentiation to modulate

autophagy. Immunoblots were performed for LC3 at the indicated time points to assess autophagosome degradation via relative changes in LC3II (lower band; 17 kDa). Studies were repeated three times with similar trends seen consistently. (B) Average standardized densities normalized by the sum of replicates were quantified via densitometry to measure autophagosome accumulation (LC3II bands) across three separate differentiations. Average values as standardized to β-actin are reported here. *p<0.05, #p<0.01. Figure adapted from (Nuschke, Rodrigues et al. 2014).

This control differentiation showed a trend similar to the rapamycin-treated cells, which showed a similar rapid clearance of LC3II and subsequent re-accumulation of LC3 after 24 hours of differentiation. This effect was inhibited by bafilomycin treatment, which limited LC3II turnover and generally inhibited LC3II degradation, most notably at the 6-hour mark when the average LC3II density was greatly increased in the bafilomycin treatment compared with the control and rapamycin treatments. Bafilomycin treatment also led to a distinctly higher average LC3II level later in the time course, suggesting a potentially higher available number of autophagosomes in the long term in the MSCs following a brief bafilomycin treatment at the onset of differentiation.

TEM analysis of MSCs subjected to osteogenic differentiation also confirmed these results (Figure 19). MSCs subjected to osteogeneic conditions showed a clearance of accumulated autophagosomes within three hours, comparable to what was seen with rapamycin stimulation in Figure 17. This was, again, correlated with a larger rough endoplasmic reticulum, as expected from an increase in autophagosome degradation and associate protein synthesis changes.

Given the long-term nature of MSC differentiation, and also the need for energy generation during potential stressors such as hypoxic conditions, we also assayed how LC3

levels were affected during differentiation of MSCs into osteoblasts over 30 days in standard culture (21%), or more rapidly under the more physiologically relevant (4%) oxygen concentrations. Differentiation proceeded as expected, with hydroxyapatite deposition seen using Von Kossa stain after 10 days at 4% oxygen and after 20 days at 21% oxygen (Figure 20). qPCR analysis of early and late osteogenesis markers (Runx2 and Osteocalcin) also confirmed this, with more rapid differentiation seen at 4% oxygen concentrations. In terms of the autophagy phenotype, autophagosome marker LC3II was drastically affected by differentiation, with LC3II levels falling by 10 days at 4% O_2 and 20 days in 21% O_2. LC3 expression on the whole also diminished during differentiation, as seen particularly in the more robust differentiation at 4% oxygen that resulted in reduced band intensity for both LC3I and LC3II, suggesting turnover of both LC3 pools with resultant decreases in autophagic potential as the cells form osteoblasts.

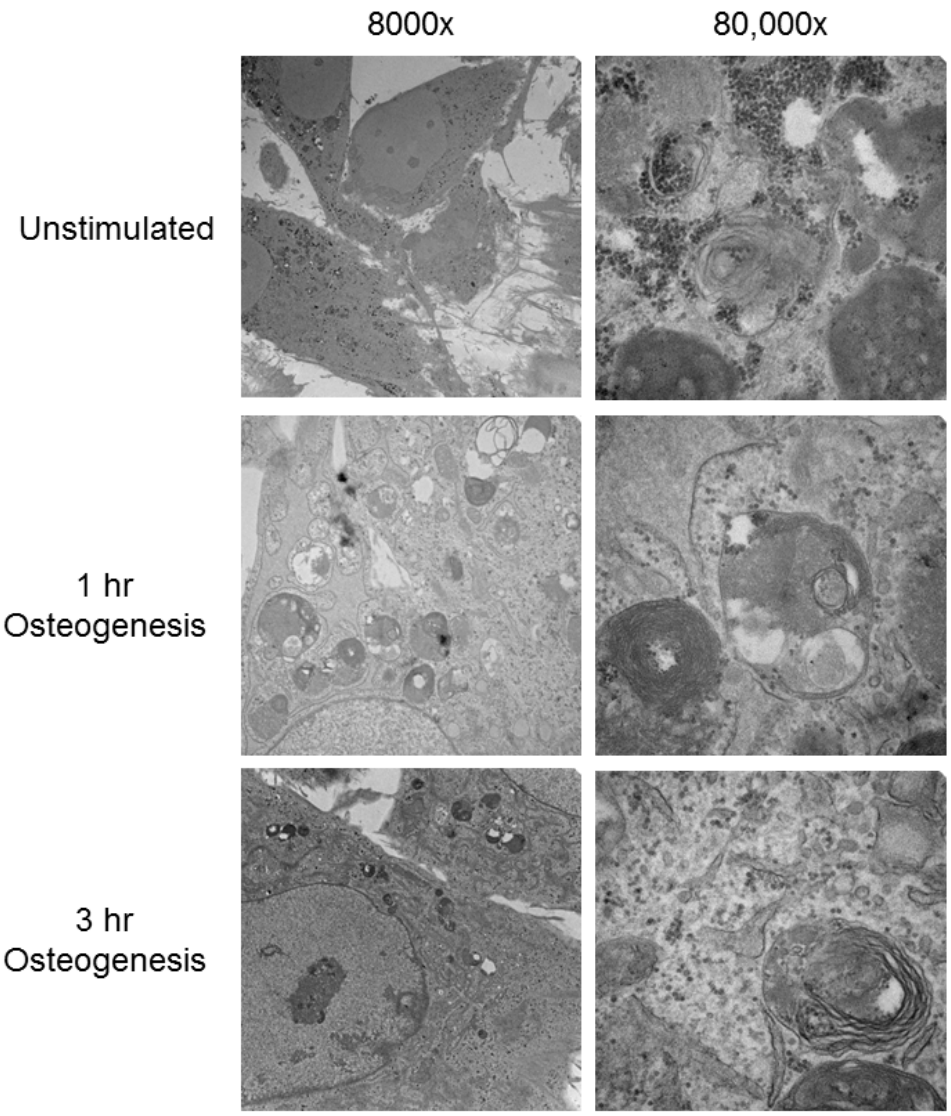

Figure 19. MSC differentiation quickly induces autophagosome clearance. Immortalized human MSCs grown at standard culture conditions (1 g/L glucose, 10% FBS) or following osteogenic differentiation for 1 and 3 hours were examined via TEM at 8000 and 80,000x Shown are representative images of cell morphology for all three conditions over three experiments.

Differences in LC3 reduction seem to be directly related to oxygen concentration, which clearly has an effect on the rate of bone deposition, both early and late-stage. Differentiation was much more rapid at 4% oxygen, which coincided with the more rapid LC3II reduction. This result suggests that MSCs may release from their unfused autophagosome state during times of

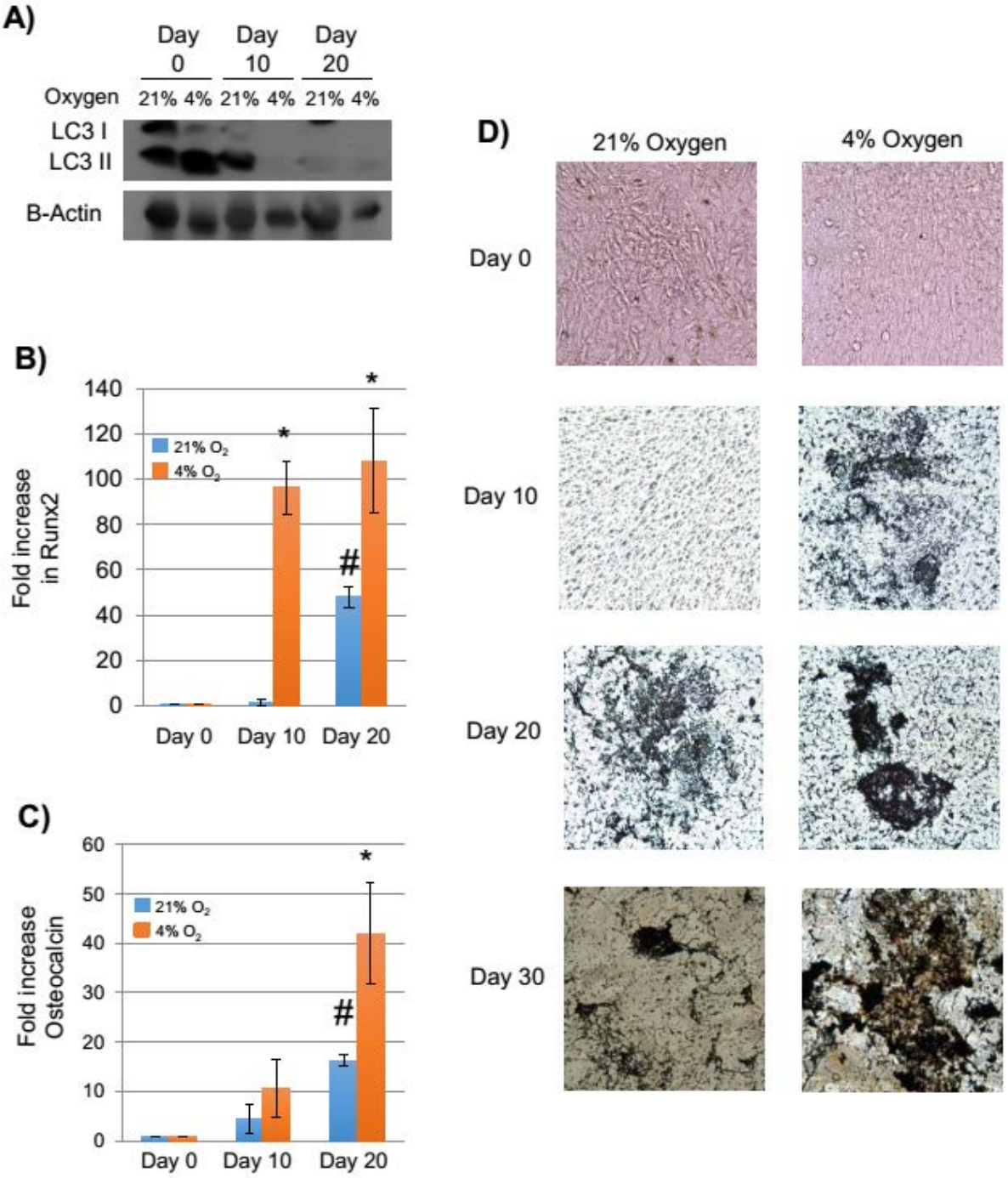

Figure 20. Autophagy is mobilized during differentiation of ihMSCs. Cultured ihMSCs were stimulated to undergo osteogenesis to form osteoblasts for 30 days at 21% and 4% oxygen. LC3I and II levels were assessed via western blot every 10 days (A), and levels of Runx2 (B) and Osteocalcin (C), markers of early and late osteogenesis, respectively, were assayed via qPCR (21% oxygen – blue; 4% oxygen – red). A Von Kossa stain was employed every 10 days to visualize osteodeposition at both oxygen concentrations via light microscopy at 200x magnification (D). Shown are representative of experiments repeated at least three times, with statistical significance noted for difference from day 0 and between the two different ambient oxygen concentrations. Figure adapted from (Nuschke, Rodrigues et al. 2014).

high energy demand, such as during differentiation. Ultimately, the 'pre-autophagy' phenotype is seen only in functional and undifferentiated MSCs, and may be a hallmark of such a cell that is utilized in a stressful *in vitro* environment.

4.4.3 Modulation of differentiation outcomes through autophagy regulation

Data in Figures 18-20 suggest a key role for autophagy in MSC differentiation, particularly during the early critical period as the cells initiate the differentiation process. Earlier work demonstrated limited adipose tissue in mice lacking the critical effector *Atg7* in adipocytes (Singh, Xiang et al. 2009; Zhang, Goldman et al. 2009). We hypothesized that the nature of autophagy's apparent utility in differentiation would lead to altered differentiation outcomes if autophagy was modulated in the key early window of the process in general and thus examined a second differentiation pathway of adipogenesis. To test this, we differentiated immortalized MSCs into adipocytes using commercial adipogenic media over the course of 30 days, in the face of 5 μM rapamycin or 5 nM bafilomycin treatments during the first 3 hours of differentiation (as in Figure 18) to alter the autophagic balance at the onset of differentiation.

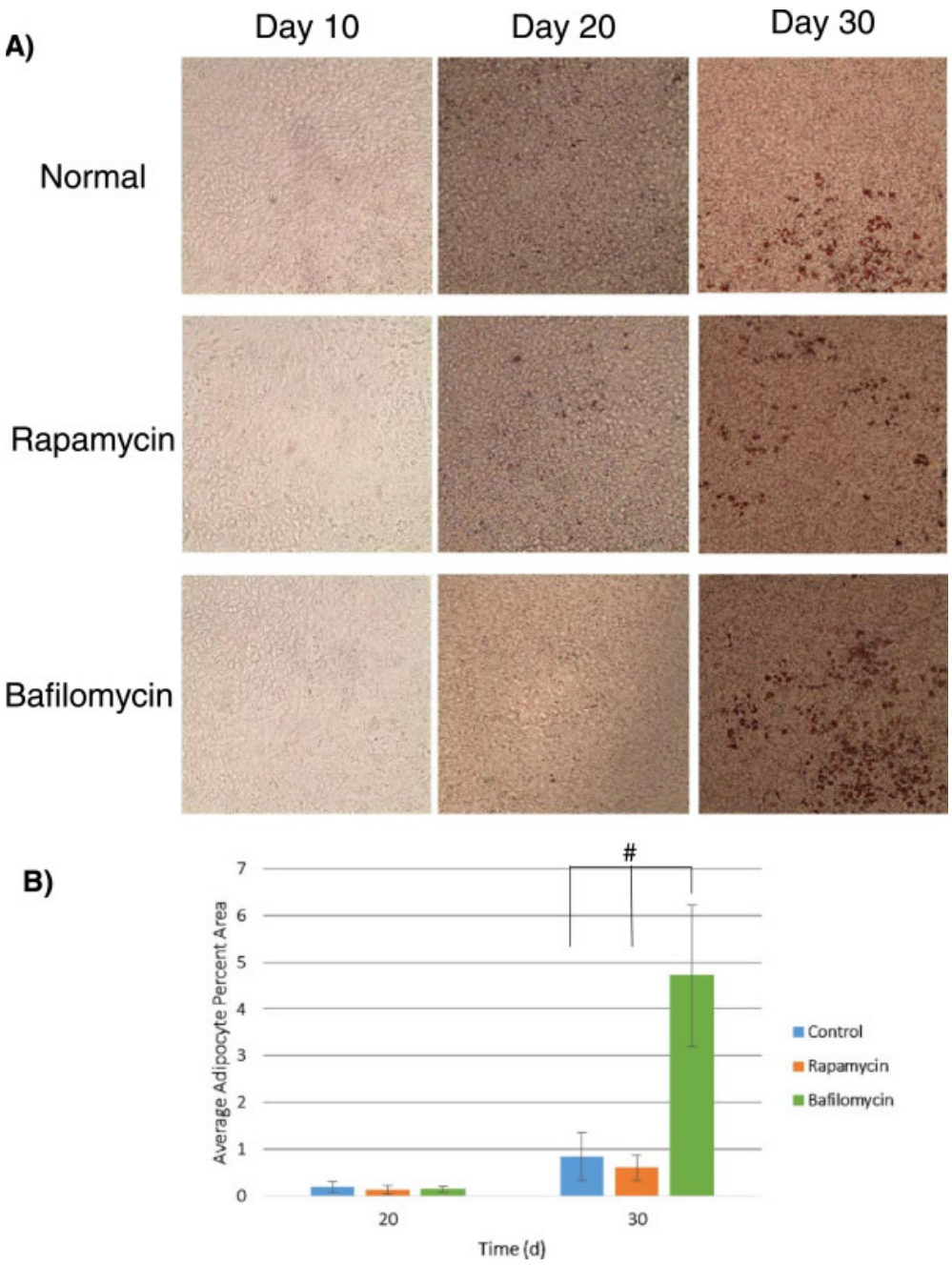

Figure 21. Early arrested autophagy can be modulated to alter immortalized human mesenchymal stem cell adipogenesis outcomes. (A) Immortalized human mesenchymal stem cells differentiated under adipogenic conditions were left untreated or treated with 5 µM rapamycin or 5 nM bafilomycin for the first 3 hours of differentiation. Differentiation outcomes were monitored via changes in fat droplet accumulation by using an Oil Red O stain. (B) Fat droplet percentage area in individual images was quantified by using

thresholded images to monitor adipocyte formation. Analysis was performed by using representative images from eight-image fields across two studies (# P <0.05). Figure adapted from (Nuschke, Rodrigues et al. 2014).

Surprisingly, fat droplet accumulation via Oil Red O staining showed that bafilomycin treatment to block the autophagosome utilization early in differentiation led to a more robust differentiation/fat droplet accumulation over 30 days in the MSCs (Figure 21a). Quantification of fat droplets on day 20 showed little difference among treatments, and this was presumably due to the low amount of mature adipogenesis in general to this time point (Figure 21b). However, at day 30 when substantial fat accumulation was seen in culture, adipocyte percentage area quantification showed a significant increase in adipocyte accumulation in the bafilomycin-treated condition. This stands in contrast to the rapamycin treatment, which showed a slight decrement (that was not statistically significant) compared with the control differentiation. Thus, our results show that briefly delaying the induction of autophagy in adipogenic differentiation improves long-term differentiation outcomes and suggests a potential key role for autophagy and the balance of autophagosome accumulation/cycling in MSCs early in commitment to lineage. Additionally, we show here that autophagy plays a role across multiple differentiation pathways, suggesting a key role in general MSC physiology and potential clinical utility in many contexts.

4.4.4 Autophagic turnover is halted in MSCs

MSCs analyzed by TEM showed a high concentration of autophagosome structures (Figure 17). However, single time points seen in TEM images did not reveal whether these led to degradation of the contents. The absence of late stage autophagosomes hinted at an arrest in

cycling, but nothing definitive could be garnered by these snapshots. Given the apparent dynamic role of autophagy in differentiation and the rapid change in phenotype seen in these cells, we examined the baseline status of any autophagic turnover in normal MSCs. To examine maturation and flux of autophagosomes over time, we used a tandem GFP-RFP LC3 plasmid to monitor the local pH environment of LC3II puncta. This plasmid tags LC3 in the cell with both GFP and RFP, and thus when LC3II is expressed present in non-acidified autophagic structures, the green and red co-localize to show a yellow tag. However, the acidic environment necessary for lysosomal degradation of the autophagosome quenches the GFP signal, and thus, any autophagosomes that have fused with active lysosomes will show only the RFP signal. This therefore represents a useful tool for monitoring autophagosome turnover in real time (Kimura, Noda et al. 2007).

Immortalized MSCs showed very little loss of GFP signal over time, indicating a lack of turnover of LC3II and suggesting arrested autophagic degradation in the cells (Figure 22). The yellow signal from GFP/RFP co-localization was quickly lost following 15 minutes of treatment with rapamycin to induce autophagy, and almost all GFP signal was quenched after 1 hour in rapamycin. Furthermore, treatment with bafilomycin to halt autophagy showed no appreciable effect on fluorescent signal in the ihMSCs. Given that bafilomycin halts autophagy by preventing lysosomal fusion and degradation, this phenotype would be expected in cells that already are arrested in maturation prior to forming acidified autolysosomes. Ultimately, little turnover of GFP signal (and thus autophagosome recycling) is seen unless stimulated to with rapamycin. This suggests that, while MSCs do have a significant level of basal autophagosome formation, a great deal of these autophagosomes appear to be arrested in the middle of the autophagic process prior to lysosomal degradation. The cells are poised to be rapidly released from this state when

cues are received signaling a need for autophagic degradation products (as occurs with mTOR inhibition).

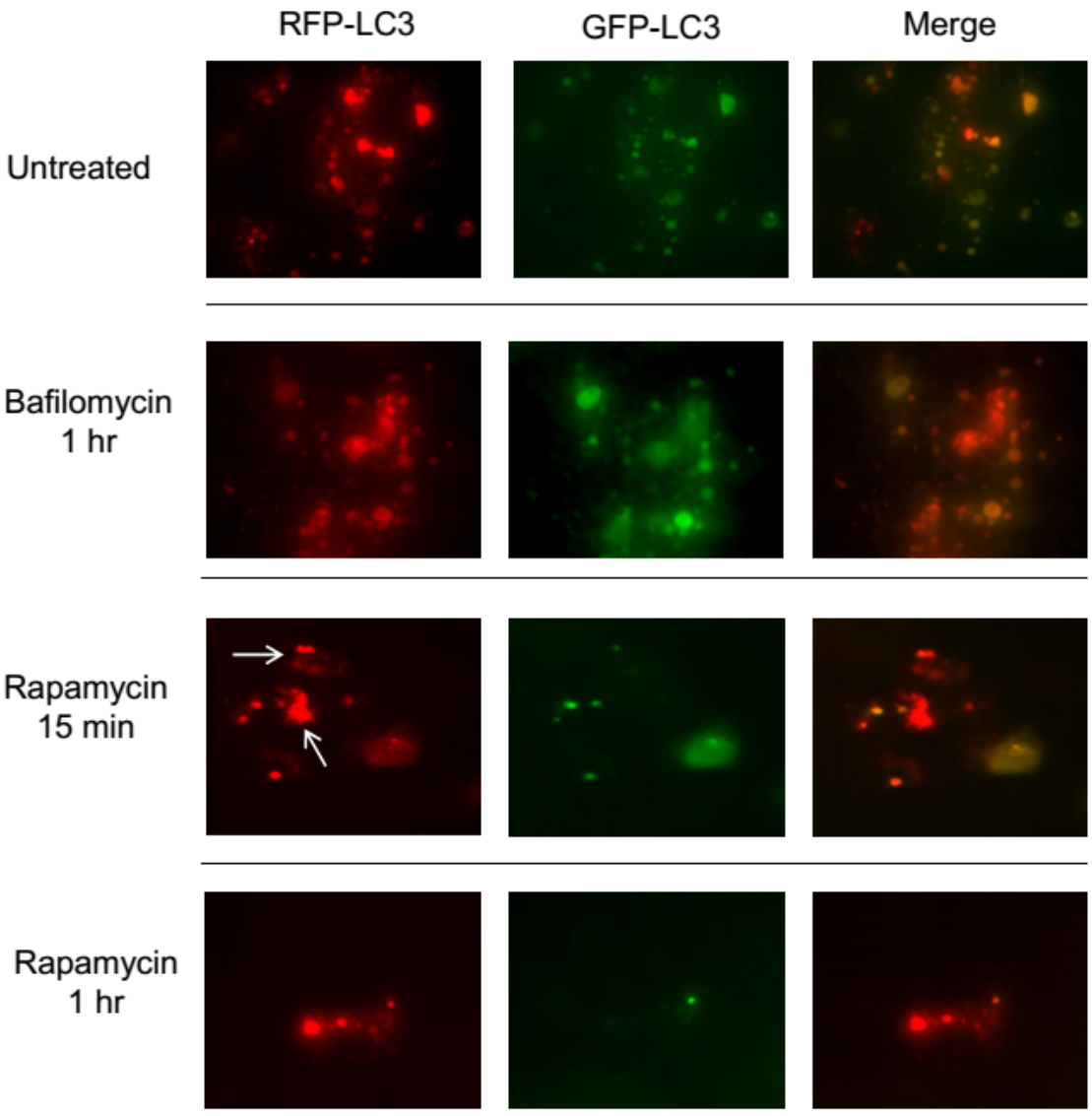

Figure 22. Immortalized hMSC show little autophagosome turnover unless stimulated. Immortalized hMSCs grown under normal culture conditions were tagged with a tandem GFP-RFP LC3 via viral transfection (see methods). ihMSC were then stimulated with bafilomycin to halt autophagy for 1 hour, or with rapamycin to induce autophagy for 15 minutes and 1 hour. Shown are representative of experiments repeated at least three times. Figure adapted from (Nuschke, Rodrigues et al. 2014).

4.4.5 Glucose levels alter autophagy activity in MSCs

In addition to a differentiation stimulus, we also examined the effects that varying nutrient conditions had on autophagy in immortalized MSCs. With comparable culture conditions to previous differentiation experiments, we assessed LC3 levels in the MSCs via western blot following a shift from high (4.5 g/L) glucose to low (1.0 g/L) glucose DMEM expansion media, and vice versa. We found that shifting the cells to a hyperglycemic stress reduced LC3 levels in general, essentially clearing the cell of autophagosomes after 48 hours as evidenced by the lack of LC3II expression in the MSCs (Figure 23). Conversely, a shift back to lower glucose conditions induced the opposite effect in the same time frame, leading to a re-accumulation of autophagosomes as shown by the reappearance of LC3II over time. In contrast, we found that irrespective of glucose concentration, oxygen tension alone did not appear to alter LC3 expression in the cells in the same time frame (Figure 23), suggesting nutrient effects on MSC autophagy may be limited to specific nutrients or conditions.

4.5 DISCUSSION

Macroautophagy exists to quickly generate energy substrates during cellular stress due to nutrient deprivation, to remove unnecessary organelles during differentiation state changes, and/or to generate precursors for macromolecular syntheses. Understanding of its role, and even its existence, in stem cell biology remains nascent, particularly in MSC. Previous studies of autophagy in stem cells in general have shown a potentially interesting role of the autophagic

processes in normal cell function and differentiation. Embryonic stem cells, for example, have been claimed to have constitutive levels of autophagy based on an upregulation of autophagy proteins early during differentiation (Tra, Gong et al. 2011). Additionally, reprogramming of somatic cells to induced pluripotent stem cells has been shown to involve the

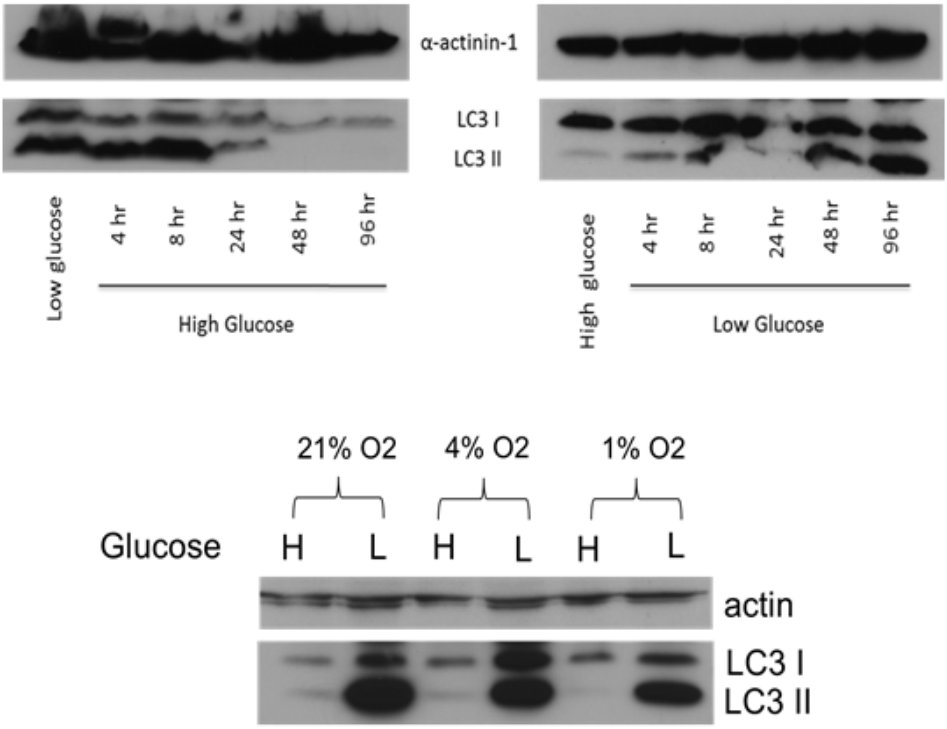

Figure 23. Glucose concentrations alter LC3 expression in MSCs. Immortalized hMSCs were cultured in low (1 g/L) and high (4.5 g/L) glucose DMEM for 72 hours and subsequently changed to high or low glucose media, respectively. Changes in LC3 in response to glucose concentration were assayed over 96 hours (top) via western blot. ihMSCs were also cultured in high and low glucose media for 96 hours at ambient oxygen and at 4% and 1% oxygen tensions and again assessed for LC3 expression via western blot. Studies were repeated three times with similar trends seen consistently.

process of autophagy and its signaling pathways (Chen, Shen et al. 2011; Menendez, Vellon et al. 2011). Limited study of MSC autophagy has shown a significant constitutive level of autophagy-associated proteins upregulated in these cell, with potential early activation and, later, loss of the phenotype during differentiation (Oliver, Hue et al. 2012; Pantovic, Krstic et al. 2013). Additionally, production of the autophagic machinery has been shown to have a protective role during MSC starvation (Zhang, Yang et al. 2012). These reports all delineate a possible role of the process in generating energy or precursors for stem cells during times of stressed metabolic demand, such as differentiation, reprogramming, or starvation. Herein, we show that in an undifferentiated state, mesenchymal stem cells present an arrest in autophagy prior to autophagosome degradation by the lysosome. This effect is seen in both immortalized and primary cell lines, though to a less dramatic extent in the primary cells. Most importantly, the autophagic phenotype is lost during cellular differentiation.

Given the high concentration of autophagosomes in MSCs, the key role in differentiation, and the general lack of autophagic turnover at a basal state, we propose a model wherein mesenchymal stem cells exist in a state of “arrested” autophagy, where an unusually high concentration of autophagosomes sit in the cells prior to lysosomal degradation (Figure 24). This arrested phenotype could be used to rapidly generate amino acid building blocks when necessary via autophagy, as indicated by RER enlargement following rapamycin stimulation in Figure 1. Though this RER enlargement is less pronounced in the primary cells, the mechanism is still present and could be less dramatic due to fewer autophagosomes present at baseline in primary cells. For example, cells undergoing differentiation may take advantage of this phenotype to rapidly precursors during the demanding differentiation process in a wound arena with limited

perfusion, or might also take advantage of these arrested energy stores for situations where the cells are challenged to survive, such as in clinical cell therapy settings including chronic skin wounds or myocardial defects.

A halted autophagic process may in fact be a hallmark of undifferentiated MSCs. Moreover, this novel autophagic characteristic of MSCs may play a key role in their physiology during wound healing. Cells in a wound environment are subject to a wide variety of stressors and challenges to their survival, making mechanisms like autophagy key for allowing stem cells to exist long enough to proliferate and differentiate effectively into the target cell type. In arresting autophagy before lysosomal degradation, MSCs have a method to rapidly release substrates for lysosomal degradation, generating energy and anabolic resources for the cell, as seen in the accompanying rough endoplasmic reticulum expansion. Additionally, though glycolysis is significant in MSCs (Funes, Quintero et al. 2007; Mylotte, Duffy et al. 2008), it is possible that by resynthesizing mitochondria via this mechanism, the MSC present the machinery for rapid macromolecule synthesis in nutrient-rich environments, while providing materials for autophagic degradation and recycling of macromolecular building blocks. This mechanism may play a key role in how MSCs function in a wound bed, and could also be manipulated to promote cell survival further in cell transplant scenarios.

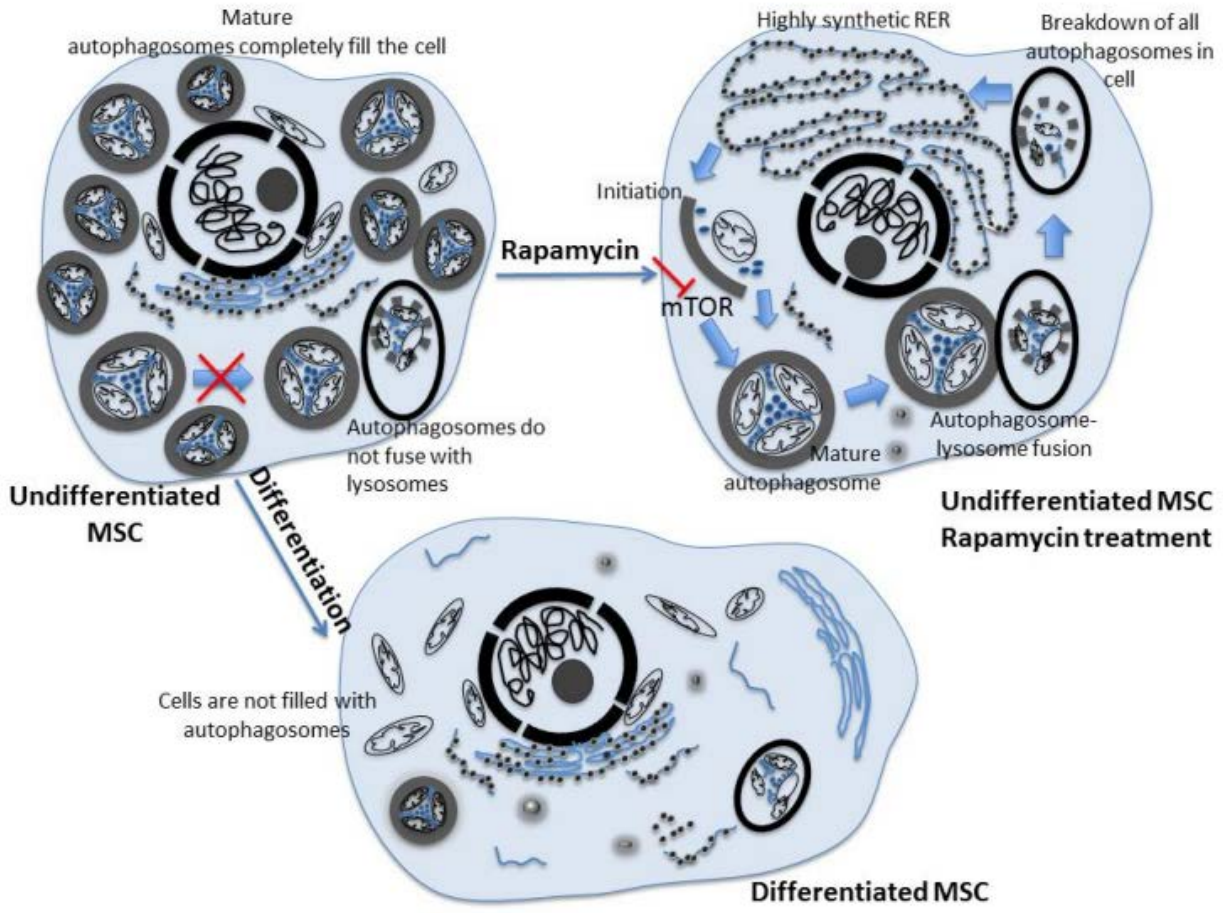

Figure 24. Schematic for arrest and utilization of autophagy in normal MSC function. As evidenced by TEM images and GFP-RFP tracking of LC3 in this study, undifferentiated mesenchymal stem cells appear to exist at a state of high basal autophagy, with many autophagosomes in a state of arrest in the cytosol (left). Artificial activation of autophagosome degradation with rapamycin (right) leads to a rapid clearance of existing autophagosomes in the cell, with subsequent rough ER enlargement, suggesting a potential advantage for MSCs to mobilize autophagy during times of high energy demand. Thus, during such a time such as differentiation or nutrient starvation (bottom), a similar phenotype is observed in the cells whereby arrested autophagosomes in the cell are mobilized and degraded, allowing the MSCs to proceed in the differentiation process or potentially resist negative effects of stressors. Figure adapted from (Nuschke, Rodrigues et al. 2014).

Given the role of autophagy activation early in MSC differentiation that we have discussed here, our results showing the altered differentiation outcomes present an interesting possibility for affecting MSC differentiation across a variety of scenarios. Here, we showed inhibiting the autophagic flux activated by MSCs at the initiation of differentiation with a modest bafilomycin treatment helped the cells produce a more robust adipogenic differentiation over the course of 30 days. This suggests that holding the cells from cycling autophagosomes too early in the process may be beneficial to particular differentiation pathways, and furthermore placing cells in a pro-autophagy environment such as nutrient stress or hypoxia might hinder adipogenic differentiation if the MSCs are unable to maintain the autophagosome balance and activate cycling at the appropriate time. Additionally, this suggests that drugs to modulate autophagy might be tangentially used to affect desired differentiation outcomes in culture settings, or also *in vivo* if a differentiation is desired in a clinical setting. Further study of this phenotype across various differentiation pathways is warranted, particularly establishing the specific pathway of activation that links differentiation and autophagy activation and also examining the mechanism of autophagosome accumulation that is the underlying key to this unique activity of MSCs.

5.0 CONCLUSIONS AND SPECULATIONS

Despite the great clinical promise of mesenchymal stem cells, whether it be for differentiation, angiogenic signaling, or immunomodulation, highly compromised survival upon implantion into harsh wound sites continues to limit their utility in regenerative medicine. Surprisingly, although the number of studies and clinical trials leveraging MSCs for therapy continues to rise, very few studies accurately monitor the fate of the cells upon implant. Those that do often use *in vivo* fluorescence to track the presence of implanted cells, which can be quantified to a degree but doesn't provide any information on the health of the cells or actual numbers surviving (likely due to the expensive nature of animal studies in the trials). Accordingly, despite the MSC survival issue discussed at length in this work, very little research has addressed this fundamental problem with MSC therapy (and cell therapies in general). While some studies have evaluated the role of nutrients, oxygen, and delivery vehicles as factors in cell survival, these studies remain descriptive in nature and have not ultimately provided a functional solution to the problem at hand (Mangi, Noiseux et al. 2003; Tang, Tang et al. 2005; Potier, Ferreira et al. 2007; Hu, Yu et al. 2008).

Two important cellular processes in resisting death stimuli are downstream EGFR signaling, which promotes cell survival through Erk and Akt signaling arms, and autophagy, which helps to resist cell death through recycling of cellular debris to generate biochemical building blocks for the cell. We have shown with this work the ability to exploit both of these

processes separately, through constitutive EGFR activation via ligand tethering to a scaffold or in the matrix, and through autophagy via modulation of autophagic flux during times of stress (such as differentiation). Though this work has demonstrated efficacy from exploiting these pathways in these contexts, there remains much work to be done building on these studies, as well as great promise for adapting this work to other areas of regenerative medicine in the future.

5.1 CONSIDERATIONS IN MSC BIOLOGY

5.1.1 MSC heterogeneity

A concern in most studies of MSC biology and function is always the heterogeneous nature of MSC populations upon extraction from the bone marrow or other tissue of interest. This concern is well known among those studying MSCs, and is easily seen even by examining surface marker expression patterns among different isolates, which often have varying expression profiles to a degree, particularly among different MSC subtypes. This is also a potential concern for our studies here, where a variety of potential functional aspects of both our survival approaches and modulations for autophagy may be affected by MSC heterogeneity. We did examine both immortalized and primary MSCs for most experiments in our studies, but focused mostly on bone marrow-derived cells and also on primary cells from a single repository. As such, an important consideration for future studies will be examining both as many donors as possible, as well as a variety of other MSC sources to delineate any issues of note related to these studies. For example, we found a high concentration of autophagosomes in our studied MSC

populations here (both immortalized and primary), which undoubtedly was part of the reason our approaches towards modulating autophagy were so effective in altering long term differentiation outcomes. Though autophagy almost certainly plays a role in all MSC stress responses, we will need to see if this autophagosomes accumulation is comparable in adipose-derived cells, and if so, how comparable the effects of our bafilomycin pre-treatments are on adipocyte formation over time.

5.1.2 Autophagy and MSC reprogramming

In the work described in this dissertation, we examined the role of autophagy in MSC response to stressors and, in particular, the ability of autophagy modulation to alter long term differentiation outcomes in the cells. While these results hold interesting implications for MSC survival and potential modulations for improving MSC function under duress, there are other potentially interesting functions for autophagy that may be interrelated with these findings. For example, it has been acknowledged that the specific changes in autophagy among various stem cell populations can differ greatly, such as upregulation or downregulation of autophagy during differentiation (reviewed extensively in (Pan, Cai et al. 2013)). Autophagy has also been noted to play a role in stem cell (induced pluripotent cell) reprogramming, wherein deletion of key autophagy genes such as Atg5 or Atg7 inhibited the capacity of the cells to be reprogrammed to a pluripotent state. In concurrence with this, the early stages of the reprogramming protocol lead to a brief induction of the autophagic machinery, in the time frame of 1-3 days (Wang, Xia et al. 2013). Though this has been less thoroughly studied in MSCs, there is a clear role for autophagy in the reprogramming and differentiation process, particularly as these processes are induced. While we did not specifically examine the effects of autophagy on upregulation of such genes as

Oct4 or Nanog to correlate our findings with a reprogramming phenotype, it is possible that our approaches to modulating autophagy for differentiation may be relevant in other MSC general functions. Future studies examining these effects are of interest as we integrate our approaches to continue onward to establish optimal conditions for MSC function upon implant *in vivo*.

5.2 FUTURE WORK IN MSC SURVIVAL SYSTEMS

5.2.1 *In vivo* modeling

The research discussed in this dissertation largely focused on mouse models, including implant into a perifascial ischemic site for an MSC survival challenge on bone scaffolds, and application to a murine skin wound via polymer encapsulation. In both cases, the models used for the MSC survival challenge have somewhat limited translational significance, and thus our systems must ultimately be scaled to more relevant models moving forward.

In the context of bone regeneration, MSC applications are most widely associated with regeneration of non-healing long bone defects, such as in the leg or other load bearing contexts. We ultimately avoided direct implantation into murine bone due to the different architecture and, more critically, different load-bearing nature of mouse appendages compared to human (mostly due to the bipedal form of humans). As such, correlated regenerative outcomes in bone healing such as osteoblast formation, *de novo* bone tissue engraftment, and load-bearing capacity of any new tissue are better suited for higher order animals, such as dogs or pigs. Scaling of TCP particles to the size of a non-healing bone defect in either of these models is an important next step, including appropriate changes in methods for tethering peptide fabrication and MSC

incorporation. Additionally, the fate of differentiating MSCs must be tracked in further studies, as here we largely focused on the simple presence of surviving CD73/CD90+ cells. We have demonstrated that EGF tethering can enhance the osteogenic capacity of bone marrow derived-MSCs (Platt, Roman et al. 2009), but have not examined the *in vivo* fate of osteoblasts in the TCP-tEGF system, an important aspect of MSC function in the context of bone. For instance it is quite possible that signals that promote survival of the MSCs might limit survival of the differentiated cells.

In addition, the model used for skin wound healing here is also limited in scope. Punch biopsy wounds in mice, while slightly impaired in CXCR3 -/- animals, remains quite different from a non-healing human wound in both speed of closure as well as underlying pathologies. The pig is a preferred model animal due to the similarity in the architecture and the presence of auxiliary structures of the skin. While many insights have been garnered, the challenges of genetic manipulations in the porcine model have promoted the use of suboptimal rodent models, wherein genetically altered animals and tissues are more easily generated. As the skin in rodents is only loosely connected to the underlying fascias, the largest part of healing of full thickness wounds occurs by primary contraction; partial thickness wounds are a challenge due to the thinness of the skin and are thus not representative given the small amounts of tissue to regenerate. To overcome this major role of contraction, wounds may be splinted in rodents, but again such a situation artificially imposes constraints absent in human wounds with the tension leading to excessive collagen deposition and thus a hypertrophic scar (Guo, Carter et al. 2011; Suarez, Syed et al. 2014).

In the case of chronic wounds, tissue insults in diabetic mice and other animals (either genetic variants or by killing of beta cells) do heal well even though with a slight delay

compared to normal littermates. As such, an oft-used chronic wound model remains the porcine skin flap model, which maintains the similar architecture to the human skin while generating avascular/ischemic regions to mimic a chronic wound (Boateng, Matthews et al. 2008). However, ultimately surgical generation of avascular flaps does not represent these wounds so much as compromised pedicles and muscle flaps in humans. Rather, in human diabetic and chronic wounds the vascular compromise occurs at the small arteriole level, and not usually from limited arterial supply. Ultimately, skin organ cultures using decellularized human skin or bioreactor-based approaches for modeling chronic wound environments may be necessary for establishing the utility of our approach to maintaining MSC survival in a true hyperinflammatory and avascular wound bed.

5.2.2 Functional changes in MSCs

The tEGF and TNC studies performed for this thesis work largely evaluated the actual physical survival of MSCs in the implant sites in mice, with some focus on related outcomes such as extract cellularity or wound healing improvements as a result of treatment. Given the ubiquitous role of EGFR in a wide array of signaling pathways, a key aspect of this work that must be built on is changes in MSC function during exposure to our systems for constitutive EGFR activation. Previous work from our group and from Dr. Tamama has outlined both the importance of paracrine secretions from MSCs, as well as the potential effects of EGFR activation on altering the MSC secretome (Tamama, Kawasaki et al. 2010; Tamama and Kerpedjieva 2012). However, despite our work in improving survival and ensuring ability of the MSCs to differentiate, a major area of focus going forward must be correlating improved MSC survival with any changes in paracrine gene expression or, conversely, identifying any

deleterious side effects of constitutive EGFR activation. To that end, global analyses of relevant growth factors and cytokines in wound healing (VEGF, IL-6 and IL-8, TGF-β, and others) are an important next step in examining both Tenascin C and tEGF culture surfaces, and will strengthen the correlation between improved MSC survival and improved wound healing that we demonstrated in our polymer approach to wound healing in this work.

5.3 FUNCTIONALIZATION OF OTHER REGENERATIVE SCAFFOLDS

Throughout this work we have demonstrated the potential clinical utility of EGFR tethering in two specific contexts: physical EGF tethering to tricalcium phosphate particles, and EGFR surface retention through EGF-like repeats in Tenascin C. Perhaps the most promising prospects for this work in the future relate to the flexibility of both of these approaches in improving cell survival in general. Our work here generally focused on wound healing scaffolds; tricalcium phosphate is a widely used biomaterial for filling bone defects and as a scaffold for cell delivery (Kitsugi, Yamamuro et al. 1993; Kasten, Luginbühl et al. 2003; Wang, Fan et al. 2010), and polyethylene glycol-based matrices for skin, which have seen wide use across many bioengineering approaches (Halstenberg, Panitch et al. 2002; Holland, Tessmar et al. 2004).

A major promising direction for this work, therefore, is applying EGF tethering to different systems for regenerative medicine, both with different cell types and in different tissues of interest. The list of scaffolds that have been explored for application in cell therapy is quite long; since the late 1990's, studies have been undertaken evaluating such materials as polyglycolic acid for cartilage repair (Vunjak-Novakovic, Obradovic et al. 1998), poly(lactic-coglycolic acid) as a different material for bone regeneration(Holy, Shoichet et al. 2000), and

more advance materials such as carbon nanotube-based networks as seeding surfaces for cells (Correa-Duarte, Wagner et al. 2004). The more rigid nature of these materials affords them the possibility of being covalently modified with a comparable binding peptide for EGF or other growth factors in a similar fashion to TCP in our study here, allowing for a great diversity in applications for improving cell survival (or other growth factor-based outcomes).

The same principal can be applied to more matrix-based or "soft" regenerative scaffolds in relation to the matrikine signaling from Tenascin C. As mentioned previously, collagen scaffolds for wound repair are extremely common, and have accordingly seen translation into commercial products such as bilayered matrices as flexible dressings for skin wounds (Table 2). As Tenascin C is easily obtained in recombinant form and integrated into existing matrices, such as in our work on the wound healing polymer described in this thesis, TNC promises to be a widely-applicable method for improving cell survival. This is perhaps highly relevant in skin wound healing, where implanted MSC survival is limited, but also survival of invading resident fibroblasts and keratinocytes that are required for adequate wound closure. Redesign of existing treatments (such as bilayered matrices or hydrofiber dressings) or future matrix-based therapeutics to incorporate Tenascin C may alleviate some of that cell death and change the nature of wound repair in the near future. Additionally, further work in identifying the key EGF-like repeats involved in binding the EGFR may assist with concentrating the bioactive portions of the protein and further enhance the therapeutic potential of the approach.

5.4 APPROACHES FOR OTHER CELL THERAPIES

Though the utility of EGFR tethering and modulation of autophagy has been demonstrated here in mesenchymal stem cells, the issue of limited cell survival in cell therapies is far from limited to MSCs alone. While MSCs are perhaps the most widely used progenitor cell in regenerative medicine in the United States, various fields have also utilized a wide array of primary cells in therapies with limited cell survival and poor patient outcomes despite great therapeutic promise. This has been particularly true in neuroregeneration, where clinical research has shown limited survival of Schwaan cells in spinal cord transplant (Patel, Joseph et al. 2010) or, more notably, highly limited survival of implanted neuronal cells or stem cell-derived glia in efforts to treat Parkinson's (Emerich, Winn et al. 1992; Lindvall and Kokaia 2006). Myocardial infarct also remains an area of great interest in cellular therapeutics, and while MSCs are most often used for treatment (survival of which is low, as previously discussed), efforts have also been made using autologous myoblasts (Pagani, DerSimonian et al. 2003), cardiomyocytes (stem cell-derived or otherwise) (Yamamoto, Sakakibara et al. 2003; Laflamme, Chen et al. 2007), or mononuclear bone marrow-derived cells (Tateishi-Yuyama, Matsubara et al. 2002).

The unifying factor among all of these different cell types is the common expression of EGFR. As noted previously, the EGFR is a widely expressed receptor among many cell types, including MSCs and, more importantly, these cell types showing limited survival upon implant (Ling, Wu et al. 2005; Schreier, Rabe et al. 2013; Lee and Sun 2015). Importantly the effectiveness of tethering EGFR signaling appears not to be dependent on receptor levels, with MSCs showing protection at just some 5000 EGFR/cell while fibroblasts are also protected at 100,000/cell or so; however at higher levels of receptors the number of available tethers may need to be titrated to avoid excess activation. Growth factor tethering has seen relatively limited

yet diverse applications in the past 15 years, primarily as a means for delivering a particular growth factor to a targeted site as opposed to a means to affect a local cell seeded on the same delivery vehicle. Given the relatively straightforward means for functionalizing different scaffolds and the flexibility in using physical tethering or matrikine signaling for solid or matrix-based scaffolds, respectively, the potential for applying EGF tethering to other cell types and/or scaffolds is robust. Injectable matrices for engraftment in myocardial defect sites, perhaps the most widely studied area of MSC regeneration, are easily redesigned for incorporation of TNC and, consequently, improved cardiomyocte survival in the ischemic infarct site. Neuronal death in spinal cord therapies, as cited previously, could also be improved through growth factor tethering in the scaffold for neuron delivery. The potential for improving regenerative outcomes is ultimately limited only by the biomaterials engineering needed to re-functionalize the growth factor tethering system to new biomaterials, and thus holds great promise for future cell therapies.

5.5 COMBINATION APPROACHES FOR CELL SURVIVAL

Despite the individual role of both EGFR signaling and autophagy that we have demonstrated throughout this work, interplay between these pathways may be of great use in improving on this work in future studies. The connection between autophagy and EGFR is fairly well-documented, with EGFR inhibition being linked to an induction of autophagy (primarily in cancer cells in relation to chemotherapeutics) (Fung, Chen et al. 2012; Wei, Zou et al. 2013). Here, we have shown that EGFR stimulation through our tethering systems improves MSC survival, while also showing that a transient modulation of autophagy improves MSC response to

stressors such as a differentiation stimulus, and that autophagy is induced rapidly under stressful conditions such as nutrient deprivation. These approaches both show promise individually as strategies for improving long term MSC function *in vivo*, but perhaps more interesting is the possibility for exploiting the cross-talk between these processes to even further enhance therapeutic benefit. For example, slow-release devices for delivering modulators of autophagy might be embedded into tEGF-TCP scaffolds to both induce long term survival signaling as well as delay the induction of autophagy to enhance the differentiation outcomes, as described in Chapter 4 of this work. This effect may happen naturally given the cited role of EGFR inhibition in autophagy stimulation, and must be examined further as we integrate these approaches in the near future.

Beyond EGFR signaling and autophagy, other mechanisms exist that might be exploited to promote cell survival. Ultimately, many groups have undertaken work dedicated to improving cell survival, and more specifically, to improving MSC survival and associated clinical outcomes. Perhaps the most exciting and timely possibility for building on this work is design of combination approaches that leverage both our work on EGFR activation here and other strategies to improve MSC survival. One easily-applied combination approach includes preconditioning methods, which have shown some benefit for MSCs in terms of hypoxic preconditioning (Rosova, Dao et al. 2008; Saini, Gumina et al. 2013) as well as other preconditioning regimens, such as TGF-α treatment to improve cardio-regenerative outcomes (Herrmann, Wang et al. 2010) or pharmacological preconditioning with trimetazidine to improve resistance to oxidative stress (Wisel, Khan et al. 2009). In either case, appropriate preconditioning followed by seeding of MSCs in an EGFR-tethering construct prior to implant may drastically extend MSC lifespan and improve patient outcomes, particularly in hypoxic

wound beds. Gene therapy has also shown some promise, with heme oxygenase-1 (an anti-apoptotic enzyme) transfection in a hypoxia-inducible system proving to be beneficial for MSC survival in infarct sites (Tang, Tang et al. 2005). Though gene therapy is less ideal due to the regulatory concerns associated with approval for such approaches, this may also be a promising system for a combination approach with preconditioning and EGFR-tethering systems. Ultimately, the flexibility of our tethering systems, ubiquitous expression of EGFR, and common autophagy behaviors among different cell types present a wide variety of possibilities for improving MSC survival and function in wound healing, as well as functionalizing new systems and approaches for cell therapy in general in the future.

5.6 CONCLUSION

In conclusion, we have demonstrated with this work the *in vivo* efficacy of two methods for constitutive cell survival signaling, physical EGF tethering to scaffolds and matrikine tethering through Tenascin C. These approaches were both able to maintain MSC survival in ischemic conditions for up to 21 days, far greater than the typical level in clinical MSC use. In addition, we have demonstrated the key role for autophagy in MSC function and response to stressors such as differentiation or nutrient changes. This autophagic machinery is also highly tunable, as evidenced by the ability to modulate differentiation outcomes through treatments to alter autophagy.

Ultimately the utility of these findings has great significance in the field of regenerative medicine. As MSCs continue to be a large focus in cell therapy research, the fundamental limitation of implanted cell survival will remain a hurdle to success. The flexibility and efficacy

of our approaches here present opportunities for adaptation of existing cell therapeutics or considerations for future treatments involving EGFR activation. Furthermore, the interplay between EGFR and autophagy may yet present opportunities for further extending the lifespan of implanted MSCs, in combination with other methods for improved function such as preconditioning approaches. Translational application of these findings may ultimately improve MSC utility in cell therapy and greatly improve associated patient outcomes across a wide variety of clinical contexts.

APPENDIX A

ABBREVIATIONS

α-MEM – Alpha modification of eagle medium

DMEM – Dulbecco's modified eagle medium

EGFL – EGF-like repeats

EGFR – Epidermal growth factor receptor

Erk – Extracellular-signal-regulated kinases

ihMSC – Immortalized human MSCs

LC3 – Microtubule associated protein-1 light chain 3

MAPK – Mitogen-activated protein kinase 1

MSC – Mesenchymal stem cell/multipotent stromal cell

mTOR – Mammalian target of rapamycin

prhMSC – Primary human MSCs

PS Red – Picrosirius red

TCP – Tricalcium phosphate

tEGF – Surface-tethered EGF

TEM – Transmission electron microscopy

TNC – Tenascin C

RER – Rough endoplasmic reticulum

SLRP - small leucine-rich glycoproteins

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