The Imprinting Effects of Mechanical Environment on the Fibrogenesis of Mesenchymal Stem Cells

by

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Institute of Biomaterials and Biomedical Engineering
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Abstract

When routine repair mechanisms fail to regenerate severe burn wounds, mesenchymal stem cell therapy is considered. However, engrafted mesenchymal stem cells are prone to become myofibroblasts when exposed to high mechanical tension and pro-fibrotic cytokines in the wound microenvironment. Myofibroblast activity increases wound stiffness and activates healthy precursor cells into destructive phenotype, resulting in pathological remodelling and hypertrophic scarring. Using soft silicone substrates with near-physiological stiffness, I tested the hypothesis that myofibroblast characteristics acquired by mesenchymal stem cells in cell culture are preserved by microRNA modifications typical for fibrosis and demonstrated that priming mesenchymal stem cells on soft substrates protect them from subsequent activation and that the mechanically propagated myofibroblast memory is mediated by miR-21. This study aims to demonstrate that suppressing myofibroblast activation will maximize and prolong the beneficial regenerative effects of mesenchymal stem cells while terminating harmful and excessive tissue remodelling characteristic for fibrosis upon engraftment.
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List of Abbreviations

α-SMA  α-smooth muscle actin
BM     bone marrow
BM-MSC bone marrow-derived mesenchymal stem cells
CFU-F  colony forming unit-fibroblast
ECM    extracellular matrix
ED-A FN ED-A splice variant of fibronectin
FN     fibronectin
FA     focal adhesion
FAK    focal adhesion kinase
KLF-6  krüppel-like factor-6
MF     myofibroblast
miRNA  microRNA
MMP    matrix metalloproteinase
MRTF-A myocardin-related transcription factor-a
MSC    mesenchymal stem cell
PDMS   polydimethylsiloxane
PDGF   platelet derived growth factor
RISC   RNA interference-induced silencing complex
ROCK   rho-associated protein kinase
TGF-β1 transforming growth factor-β1
TGFβRII transforming growth factor-β receptor type II
TCP    tissue culture plastic
Chapter I: Statement of the Problem

I.1 Rationale

One of the major challenges in mesenchymal stem cell (MSC) therapy for tissue regeneration is to control MSC activation into fibrogenic myofibroblasts (MFs). The highly contractile and synthetic activities of MFs are considered beneficial for initial wound closure and connective tissue repair if their action is terminated properly by apoptotic clearance (Hinz, 2010; Hinz et al., 2012). However, persistence of MFs frequently contributes to continued tissue contracture and fibrosis (Hinz, 2007). Likewise, the occurrence of the MSC-MF phenotype is of particular harm and its persistence can potentially jeopardize the success of regenerative approaches. The conditions leading to the transition from acute to persistent MF activation are currently unclear. However overwhelming evidence points towards a “MF memory” as fibrotic signatures of pathological MFs at the protein, gene, and transcriptional level can be retained over several passages in culture (Larsson et al., 2008). In addition, previous works from our lab have demonstrated that primary lung fibroblasts explanted directly onto fibrotic stiff substrates and sub-cultured continuously on these substrates acquire a MF memory that is retained regardless of subsequent stress reduction by transferring to soft substrates. Similarly, priming lung fibroblasts on soft substrates protect them from MF activation by increased mechanical stress on stiffer substrates (Balestrini et al., 2012). It is unknown whether this mechanically induced MF memory also exists in MSCs and what regulatory mechanisms propagates the mechanically primed MF character.

MicroRNAs (MiRNAs) have gained recognition as critical regulators of many cellular and molecular processes through their action on targeted mRNA degradation or translational repression (Chau and Brenner, 2011; Collino et al., 2011; Greco and Rameshwar, 2007; Guo et al., 2011; Jiang et al., 2010; Lim et al., 2010; Xia and Hui, 2012). Their aberrant expression and dysregulation have been associated with a broad range of developmental abnormalities and diseases (Chau and Brenner, 2011; Jiang et al., 2010). Although profiling studies have established their role in fibrosis, the response of miRNAs to mechanical environment is largely unknown (Chau and Brenner, 2011; Jiang et al., 2010). By assessing the expression levels of select candidate miRNAs over the duration of mechanical priming and validating their functional roles by knockdown and overexpression, this study aims to fill the gap in knowledge
regarding the mechanically regulated miRNA expression underlying MSC-to-MF activation and persistence.

I.2 Long-term Aim

Suppressing MF activation through priming will maximize and prolong the beneficial regenerative effects of MSCs while terminating harmful and excessive tissue remodelling characteristic for fibrosis upon engraftment.

I.3 Hypothesis

MF characteristics acquired by MSCs in cell culture are preserved by miRNA modifications that are typical for fibrosis.

I.4 Objectives and Specific Aims

Objective 1: To test if the mechanical conditions of cell culture can be modulated to prime MSCs towards or protect MSCs against the MF fate.

Specific Aim 1.1: Isolation and purification of rat BM-MSCs.

To model soft normal skin and stiff fibrotic tissue, silicone rubber substrates with an elastic modulus of 5 kPa and 100 kPa, respectively, will be produced. Because MSCs are traditionally expanded on tissue-culture plastic (TCP) and direct isolation on soft polymer substrates is novel for these cells, multiple control experiments are necessary to ensure that the inherent surface or mechanical properties of silicone elastomers do not permanently alter MSCs. Bone marrow-derived cell mixtures will be subjected to magnetic cell sorting to enrich for mesenchymal stromal cells using specific surface markers. Lineage induction experiments will be performed to ensure that the obtained MSCs are multipotent. For functionalization of substrates, different ECM surface coatings will be tested to rule out that ECM-specific changes are masking true mechanical responses.

Specific Aim 1.2: Mechanical priming of MSCs and evaluation of MF character.
The enriched MSCs will be primed for an additional three passages before switching to the respective other substrate stiffness for two more passages. Before and after the divergence, characteristics of the MF phenotype will be assessed to determine if MSCs indeed acquire a mechanical memory.

**Objective 2:** To assess if transcriptional control through miRNAs propagates the mechanically primed MF character of MSCs.

Specific Aim 2.1: Expression of selected fibrosis-associated miRNA candidates.

The predicted downstream targets of a list of fibrosis-associated miRNAs will be examined and potential candidates that mediate MF memory will be selected. Their expression profiles over the duration of priming will be characterized to determine if miRNAs are mechanically responsive and if the mechanically induced memory is also preserved on a transcriptional level.

Specific Aim 2.2: miRNA knockdown and overexpression.

If the expression levels of one or more candidate miRNAs correlate with the MSC-MF phenotype, they will be interfered with mimics or inhibitors to further investigate their direct involvement in regulating the mechanically propagated MF character and their role in mediating memory.
Chapter II: Introduction

II.1 Tissue Engineering and Regenerative Medicine for Cutaneous Wound Repair

II.1.1 The impact of burn injuries

Tissue engineering is a rapidly advancing field that combines the principles and expertise of bioengineering and life sciences to understand tissue structure-function relationship under physiological conditions, with the ultimate goal to rescue or improve tissue integrity and functionality under pathological conditions (Burd et al., 2007). One of the major applications of tissue engineering is the treatment of cutaneous wounds and associated complications – an extremely costly health care burden that exceeds $20 billion annually in the US (Chen et al., 2009). Skin is the largest organ of the body and serves as a protective barrier against various injurious agents (Clark et al., 2007). Loss of skin integrity, most commonly caused by thermal injury, can lead to substantial physiological imbalance that can result in disability and morbidity (Clark et al., 2007). Over 2 million people suffer from thermal injury and its consequent infections each year. Despite efforts to improve wound care and burn resuscitation, the mortality rate remains as high as 10% (Branski et al., 2009; Butler et al., 2010). In surviving patients, the significant functional and aesthetic impairment as a consequence of dramatic wound contractures can have enormous impact on their quality of life.

Major treatment goals for severe burns include rapid wound closure, restoration of barrier function, and benign scar formation (Branski et al., 2009). However, thermal injury elicits a chain of systemic responses that complicates and hinders the development of specific treatment. The pathophysiology under such circumstances is complex and consists of three rapidly evolving stages: an early shock phase involving the release of cytokines and resultant major immune dysfunction; a hypermetabolic phase where inflammation persists and progresses to multiorgan failure; and a late pathological remodelling phase ultimately leading to hypertrophic scarring (Branski et al., 2009; Butler et al., 2010; Leclerc et al., 2011).
II.1.2 Skin Anatomy

The challenges in cutaneous wound treatment stem from the complexity of skin, which is a multilayered organ composed of multiple cell types embedded in a three-dimensional extracellular matrix (ECM) of both fibrillar and non-fibrillar components (Branski et al., 2009; Burd et al., 2007; Chen et al., 2009). The outer epidermal layer is a stratified, matrix-free, and avascular epithelium consisted mainly of keratinocytes organized in layers according to their respective stages of maturation (McLafferty et al., 2012). The epidermis extends from the basement membrane and protects the body from external environment through an outermost layer of keratinized dead cells. Epithelial stem cells present on the basement membrane are the major regulators of epidermal turnover as they undergo continuous self-renewal and unidirectional differentiation(Chen et al., 2009). Dermis lies in between epidermis and hypodermis and confers both mechanical strength and resilience to the skin, due to its rich ECM content (Chen et al., 2009). In addition, dermis contains skin appendages, extensive nerve networks, and vasculature that supplies nutrients to the epidermis (Chen et al., 2009; McLafferty et al., 2012). Lastly the hypodermis or subcutaneous tissue underneath provides insulation via adipose tissue (Chen et al., 2009; McLafferty et al., 2012). Due to its high level of complexity, repair of compromised skin requires ordered integration of cellular and molecular events including cell proliferation, differentiation, migration, ECM synthesis and deposition, angiogenesis, as well as regeneration of nerves and appendages (Branski et al., 2009; Chen et al., 2009). Stem cells are intended to be used to regenerate different parts of the skin after injury. MSCs in particular play an important role in reconstituting the damaged connective tissue as they have been shown to replenish the dermal fibroblast population and secrete ECM components and growth factors that facilitate regeneration (Fathke et al., 2004).

II.1.3 Cutaneous Wound Repair

Physiological wound healing occurs is a sequence of three interconnected phases: inflammation, proliferation, and maturation. Immediately following injury, a cascade of events is triggered to rapidly rescue the disrupted mechanical integrity of skin, often at the expense of losing tissue homeostasis and functionality, resulting in scar formation (Hinz, 2007). The repair cascade begins when dead cells or cellular debris caused by the injury triggers the release of inflammatory mediators that initiate blood coagulation, leading to formation of fibrin clot that
acts as a temporary physical barrier and development of a provisional ECM (Chen et al., 2009; Duffield et al., 2012). At the same time, increased vasodilation and enhanced matrix metalloproteinase (MMP) activity lead to improved vessel permeability and basement membrane disruption, respectively, which all together result in cell recruitment/migration (Baum and Arpey, 2005; Duffield et al., 2012). The recruited inflammatory cells secrete a cocktail of growth factors and cytokines, most notably vascular endothelial growth factor, platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1, and interleukins which work in synergy to promote the proliferation of vascular endothelial cells and fibroblasts – a defining feature of the second, proliferation phase (Duffield et al., 2012; Wynn, 2007). Recruited and locally proliferating fibroblasts secrete ECM components that partially fill in the wound and gradually replace the provisional fibrin matrix with collagen. As ECM mechanical tension increases and under the influence of profibrotic cytokine TGF-β1, myofibroblast (MF) differentiation also occurs, giving rise to the most prominent cell phenotype present in the maturation phase of the healing wound (Hinz, 2007).

MFs are derived from multiple origins including but not limited to resident fibroblasts, pericytes, circulating fibrocytes, local and bone marrow (BM) derived mesenchymal stem cells (BM-MSCs), as well as epithelial/endothelial cells through the process of epithelial/endothelial-to-mesenchymal transition (Hinz, 2010; Hinz et al., 2007; Micallef et al., 2012). MFs contribute to the repair of damaged connective tissue by synthesizing and remodelling the collagenous ECM. In addition to collagen type I, MFs also de novo synthesize the ED-A splice variant of fibronectin (ED-A FN) during wound healing (Hinz, 2007). Other than its role as a structural protein, ED-A FN has been shown to precede α-smooth muscle actin (α-SMA) expression and is essential for the induction of MF features (Serini et al., 1998). Another hallmark of MFs is de novo expression and incorporation of α-SMA into neo-formed in vivo stress fibers (Hinz, 2007; Hinz, 2010; Hinz et al., 2001). Using this highly contractile apparatus, MFs generate characteristic irreversible and long-lived isometric contractions that physically pull the edges of the wound inward to facilitate faster closure (Duffield et al., 2012; Hinz, 2010). Taken together, the contractile and secretory activities of MFs rapidly restore mechanical coherence of injured skin with partial compromise in functionality, as evident by tissue deformation and loss of elastic properties. Importantly, when the reparative process occurs in a finely orchestrated manner under physiological conditions, MFs disappear by apoptosis (Hinz, 2010; Hinz and Gabbiani, 2010).
At the same time, increased endothelial cell proliferation and progenitor cell recruitment lead to angiogenesis, providing nutrients to keratinocytes as they proliferate and migrate to the surface of newly formed granulation tissue (Chen et al., 2009). And lastly, the final maturation stage goes on for a prolonged period of time as further remodeling events are necessary to regain the elasticity and tensile strength of repaired skin (Chen et al., 2009). The intricate networks of events and the vast number of players involved in cutaneous wound healing imply that dysregulation at any level can have tremendous impact on the repair outcome (Branski et al., 2009). A classic example of such is pathological remodelling and the resulting tissue dysfunction.

II.1.4 The Role of MFs in Pathological Remodelling and Fibrosis

When tissue encounters repetitive injury or prolonged inflammation as in the case of severe burns, MF activities become sustained, dysregulated, and excessive, leading to pathological remodelling (Duffield et al., 2012; Wynn, 2007). The end result is hypertrophic scarring in the case of cutaneous wound repair or fibrosis in other internal organs. This process can be explained by a self-perpetuating cycle of MF activation and persistence, initiated by the synergistic effect of TGF-β1 and other proinflammatory cytokines present in the wound microenvironment such as tumor necrosis factor-α and interleukin-1β (Duffield et al., 2012; Micallef et al., 2012). During pathological remodelling, MFs continuously synthesize structural components such as collagen and remodel the surrounding ECM by secreting matrix cross-linking enzymes and modulating the balance of MMPs and their tissue inhibitors (Duffield et al., 2012; Micallef et al., 2012). MF contraction and remodelling of the ECM gradually increase tissue stiffness, resulting in permanent alteration of both ECM composition and mechanical properties (Duffield et al., 2012).

Moreover the contractile forces produced in stress fibers are transmitted to the ECM at sites of cell-ECM adhesions that are termed focal adhesions (FAs)- highly dynamic mechanosensitive structures that respond to ECM stiffening through changes in their size, shape, composition, and number as cells spread (Hinz, 2006; Hinz, 2010) (Figure 1). Mechanical stress fosters the development of specialized ‘supermature’ FAs (suFAs) consisting of cytoplasmic proteins vinculin, paxillin and tensin and transmembrane receptors integrins αvβ3 and α5β1 (Hinz, 2006). These molecular mediators actively transmit extracellular forces to
internal cytoskeletal tension through a variety of mechanotransduction pathways in addition to recruiting other signaling molecules to relay the signal further downstream (Holle and Engler, 2011). In other words, suFAs are platforms that allow force transmission to and perception from the rigid ECM, leading to the generation of matched, greater intracellular tension via formation of more pronounced α-SMA-positive stress fibers (Hinz, 2010; Holle and Engler, 2011; Wells, 2008). In a rat model of skin wound healing, increasing tissue tension by wound splinting accelerates the expression of α-SMA whereas tension release has the opposite effects (Hinz et al., 2001). A similar result is obtained by stretching human burn scar tissue in situ (Junker et al., 2008). In in vitro culture systems, α-SMA expression in MFs is augmented on stiff polymer substrates while suppressed on soft polymer substrates (Goffin et al., 2006; Solon et al., 2007).

Figure 1  **The feedback loop of MF activation and persistence in wound healing.**

Inflammatory signals released after injury activate fibroblasts and other precursor cells to spread and remodel the provisional wound ECM. The resulting increase in stiffness drives the formation of proto-MFs characterized by small FAs and cytoplasmic-actin stress fibers. TGF-β1 present in the local niche stimulates the expression and neo-incorporation of α-SMA into stress fibers to generate greater contractile forces, leading to FA supermaturation. Differentiated MFs produce an even stiffer ECM that drives further MF activation in a positive feedback loop. Source: Hinz. J Invest Dermatol 2007. 127:526-37 (Hinz, 2007)
In addition to changing MFs’ contractile properties and internal tension by cytoskeleton rearrangements, augmented extracellular tension contributes to the increased TGF-β1 activity. TGFβ1 activation from latent deposits in the ECM can occur through integrin-mediated traction force (Wipff et al., 2007). Because efficient TGFβ1 activation by MFs depends on a mechanically resistant ECM, tissue stiffening further drives the vicious fibrotic cycle by pumping more ‘fuels’, in this case profibrotic cytokines, into the system.

In summary, the intricate interplay of biochemical and mechanical cues in the wound microenvironment promotes and sustains the MF phenotype, which in turn leads to the clinical presentations of pathological remodelling. Understanding the mechanisms and conditions leading to the transition from acute to persistent MF activation is of critical importance in connective tissue repair and it forms the overarching theme of my thesis study.

II.1.5 Tissue Engineering for Severe Burns

In normal healing, when wound size and severity are within body’s physiological repair capacity, stem cell therapy is not necessary and body-inherent stem cellular resources can be mobilized in addition to differentiated cells. For example, epidermal stem cells present in the basal layer and resident epidermal stem cells found surrounding the hair follicle region can contribute to the repair and regeneration of damaged epidermis by producing keratinocytes that rebuild skin’s barrier properties (Shi et al., 2006). Furthermore, MSCs in the BM are known to multiply immediately following burn injury, circulate, and migrate to the site of injury to reconstitute the compromised skin. However, in the case of severe burns where more than 60% of the total body surface is destroyed, many complications arise. Not only the aforementioned local stem cell reservoir is exhausted, MSCs in the BM are also suppressed due to severe sepsis and toxicity to silver sulfadiazine – a topical antibacterial treatment for burn wound (Chen et al., 2009). Eventually, the damage is too severe to be repaired by routine mechanisms alone and other regenerative approaches are often considered (Butler et al., 2010).

Traditional strategies such as autografting are well-established but not applicable to healing severe burns as intact host donor skin is very limited (Butler et al., 2010). Allografts can serve as temporary shield but eventually will slough off due to immune rejection after a week (Butler et al., 2010). To circumvent this limitation, tissue engineering approaches based on cell therapy or using decellularized ECM have been explored extensively. Cell therapy by definition is the
administration of living cells of autologous or allogeneic origins through local or systemic delivery mode to replace, reconstruct, and regenerate the compromised tissue (Burd et al., 2007; Leclerc et al., 2011; Shi et al., 2006). The healing success is significantly improved when the burn wound is rapidly covered with cells (Wu et al., 2012). Expanding keratinocytes harvested from patients into cultured epidermis is one option, but not without limitations. Devoid of dermis and its physical support, the thin epidermal layer is very fragile and difficult to handle. Alternatively, a bilayered construct consisted of fibroblasts/ECM network and keratinocytes to simulate dermis and epidermis is equally problematic as immune rejection eventually kicks in (Chen et al., 2009). In the case of implanted matrices, although they serve as templates to facilitate vascularization and other critical cellular processes in place of the deficient native ECM, they must be covered with autografts eventually. Overall, the high cost and lack of effectiveness prevent these engineered skin substitutes from becoming widely applicable (Chen et al., 2009). Due to these limitations, my thesis study explores the possibility of harvesting MSCs and preserving their regenerative potential for the purpose of cell therapy as an alternative to treat severe burns.

II.2 MSCs for Cell Therapy

II.2.1 Clinical applications of MSCs

The realization of the tremendous therapeutic potentials of stem cells has fostered rapid development in stem cell research and the consequent surge in related clinical trials. A 2011 review by Trounson et al. documented 123 ongoing clinical trials involving the use of MSCs, most of which are Phase I studies (Trounson et al., 2011). This number has nearly tripled in the past two years to an impressive 368, according to a recent access to the public clinical trials database http://clinicaltrials.gov. MSC therapy has revolutionized and continues to impact the treatment of a wide range of diseases including cancer, cardiovascular diseases, diabetes, bone/cartilage diseases, gastrointestinal diseases, immune rejection/autoimmune diseases, and neurodegenerative diseases (Trounson et al., 2011). Because fibroblast donor sites left after severe burns are not sufficient to satisfy tissue's demand for cells, MSCs are being explored as a potential therapeutic source under such circumstances (Auger et al., 2009; Larouche et al., 2009).
II.2.2 MSC Biology

MSCs in culture are defined as adherent, clonogenic, non-phagocytic cells that can give rise to colony-forming unit fibroblasts (CFU-F) (Pittenger et al., 1999). They exhibit self-renewal capacity and multilineage differentiation potentials (Baksh et al., 2004; Kolf et al., 2007). MSC research is complicated by the absence of one definitive marker. There is consensus, however, that BM-MSCs do not express the erythrocyte marker Ter-119, endothelial marker CD31, and a number of hematopoietic markers such as CD5, CD45R, CD11b, and Gr1 (Baksh et al., 2004; Kolf et al., 2007). Rat BM-MSCs are the major cell type utilized in my study and have been shown to co-express CD29 (β1-integrin), CD54 (ICAM-1), and CD90 (Thy-1) (Dezawa et al., 2004). Other commonly identified MSC surface markers include CD44 (HA receptor), CD73 (SH3/4), CD105 (endoglin), CD106 (VCAM), and stro-1 (Baksh et al., 2004; Kolf et al., 2007).

II.2.3 MSCs as a Potential Source for Cell Therapy

MSCs have been isolated from a variety of organs including BM, adipose tissue, the tunica adventitia of arteries and veins (Caplan, 2009; Corselli et al., 2012), umbilical cord blood (Lu et al., 2006) and Wharton’s jelly (Wang et al., 2004b). MSCs have generated great excitement and promise as potential candidates for cell-based therapies, owing to many of their intrinsic properties. With the appropriate combination of inducing factors, MSCs are capable of chondrogenesis, osteogenesis, adipogenesis, myogenesis, and tenogenesis in vitro (Caplan, 2007; Wu et al., 2007). In vivo MSCs have been shown to differentiate into various tissue-specific cells in response to tissue-specific cues (Sasaki et al., 2008). MSCs produce a broad array of paracrine factors that modulate numerous cellular and molecular processes (Caplan, 2007; Wu et al., 2007). They are capable of inhibiting T cell proliferation and recognition by inhibiting the production of tumor necrosis factor-α and interferon-γ, thereby effectively attenuating local inflammatory responses (Caplan, 2007; Mezey, 2011). Moreover, the complications associated with immune rejection of allogenic tissue can be avoided since autologous MSCs can be directly obtained from patients with relative ease (Baksh et al., 2004). Overall, the combinatory action of their multipotency, trophic activity, and immunosuppressive benefits render MSCs an ideal cell type for therapeutic purpose. In vivo MSCs have been demonstrated to engraft into site of injury and differentiate into a variety of connective tissue progenitor cells to regenerate damaged myocardium, reduce bleomycin-induced lung fibrosis, and rescue neurological abnormalities, etc. (Wu et al., 2007).
With regard to cutaneous wound repair, the paracrine properties of MSCs have been shown to support the growth and differentiation of keratinocytes and promote migration of many other cell types critical for wound repair (Leclerc et al., 2011). Furthermore, MSC-conditioned media is known to enhance fibroblast survival and synthetic activity while suppressing oxidative stress and matrix degradation, thereby facilitating dermal wound regeneration (Jeon et al., 2010). In addition to paracrine support, Sasaki et al. demonstrated that MSCs not only can be induced to transdifferentiate into keratin 14-positive keratinocytes \textit{in vitro}, but also are capable of migrating into site of injury, producing other functional cell progenies when intravenously injected in a mice model of skin wound (Sasaki et al., 2008). In particular, the GFP-tagged MSCs were found to differentiate into GFP-positive endothelial cells, pericytes, and to lesser extent macrophages/monocytes (Sasaki et al., 2008). More evidence of MSC therapeutic benefits came from animal studies showing that locally injected MSCs facilitate benign scar formation as well as recovery of dermal architecture and tensile strength (Leclerc et al., 2011). Taking together their modulatory functions and differentiation potential (Figure 2), MSCs are perfect candidates to support cutaneous wound repair and thus ideal to be studied in my project.

**Figure 2**  \textit{MSCs can influence cutaneous regeneration by distinct mechanisms.}
MSCs can enhance cutaneous wound repair and regeneration by suppressing inflammation, modulating differentiation of major cell types in the skin, promoting benign scar formation, and promoting angiogenesis. Source: Jackson et al. Stem Cell Res Ther 2012. 3:20(Jackson et al., 2012).
II.2.4 MSC-to-MF Activation is a Possible Risk for the Success of MSC Therapy

Although MSC therapy has generated great excitement and promising results in many preliminary studies, the long-standing controversy remains, mainly because MSC plasticity can be a double-edged sword (Bianco et al., 2013). On the one side, MSCs can home to and regenerate the damaged organ by attaining the characteristics of tissue-specific cells. On the flip side, the hostile wound microenvironment harbours harmful stimuli that could potentially drive MSC differentiation into destructive cell phenotype (Mishra et al., 2009). One potential outcome of failed regenerative intervention is teratoma formation. Human BM-MSCs have been shown to assume the characteristics of carcinoma-associated fibroblasts when exposed to tumor-conditioned media in culture and promote tumor cell growth both in vitro and in vivo (Mishra et al., 2009). Another potential fate of MSCs that is often neglected is their activation into MFs.

Like any other mesenchymal cell, MSCs are prone to MF differentiation in pro-fibrotic conditions (Cai et al., 2001; Park et al., 2011; Wang et al., 2004a). For the treatment of burn wounds, which are often complicated by dramatic tissue contractures and stiffening due to the actions of dermis-derived MFs, this MSC-MF phenotype is of particular damage and may not deliver the intended therapeutic benefits. Using an in vitro co-culture model, keloid-derived fibroblasts or associated humoral factors were found to induce MSC chemoattraction and differentiation into MFs characterized by abundant stress fibers and production of collagen bundles (Akino et al., 2008). The possibility of MSC-to-MF activation has also generated considerable discrepancy in the reported outcome of MSC therapy. For example, in a rabbit model of corneal wound healing, systemically administered MSCs were capable of homing to the injured site and contributed to improved healing by differentiating to MFs (Ye et al., 2006). Conversely, in a mouse skin wound model, the highly contractile activities of MSC-MFs impaired the functionality of the engineered graft by causing powerful contractions in the collagen scaffolds (Kinner et al., 2002; Schneider et al., 2008).

To explain this discrepancy, one study analyzed the success of BM-MSC transplantation for the treatment of irradiation-induced lung injury at two different time points. MSCs injected immediately after injury successfully differentiated into functional lung epithelial and endothelial cells, resulting in enhanced repair (Yan et al., 2007). On the contrary, MSCs delivered two months after injury when fibrosis started to develop became activated to MFs.
that further contributed to fibrosis progression (Yan et al., 2007). Collectively, a number of similar studies suggest the critical importance to control the timing of MSC transplantation. Immediately after organ damage before the onset of pathological remodelling, the microenvironment is vastly different from later fibrotic stage. However by the time the first signs of fibrosis are brought to the attention of clinicians, the situation has more than often progressed past the optimal time frame for cell therapy under most circumstances (Hinz et al., 2012). The balance between beneficial repair and detrimental tissue contracture by MFs is delicate and it is the persistence of MFs in fibrosis that makes the difference (Hinz et al., 2012; Micallef et al., 2012). My thesis study therefore sets out to investigate the biomechanical mechanisms underlying MSC-to-MF transition, with the ultimate goal of attenuating permanent MF activation and delivering the intended therapeutic benefits of MSCs.

**II.3 Cells Sense and Respond to their Physical Environment – Stiffness Matters**

*II.3.1 The Stiffness of Tissues*

Because MFs are known to be mechanically activated, it is important to understand the physical environment they interact with. The physical landscape of an organism is vastly diverse and dynamically evolving during development and disease progression. This complex environment harbours biomechanical cues that regulate cellular responses and feedbacks that in turn actively reshape this landscape, as often observed when injuries occur (Moore et al., 2010). Tissues vary in their inherent mechanical properties, which are in part characterized by Young’s modulus or elastic modulus E with the dimension Pascal (Pa). E is defined as the ratio of stress, or applied force per unit area, over the resulting strain (Moore et al., 2010). The stiffness of tissues in multicellular organisms spans from 0.1 Pa in brain to 30,000 kPa in cartilage and bone (Moore et al., 2010; Solon et al., 2007). Moreover, in response to injury, the physiological stiffness of organs changes dramatically as well as during the progression of diseases such as fibrosis, atherosclerosis, and calcific aortic stenosis. For example, the Young’s modulus of provisional ECM during early stage of wound repair ranges from 0.01 to 1.0 kPa (Rhee and Grinnell, 2007). As the synthetic and contractile activities of MF increase over time, this value is elevated to approximately 50~100 kPa (Olsen et al., 2011).
II.3.2 Mechanical Cues Govern MSC Fate Decisions

The change in tissue stiffness during repair and remodelling has tremendous impact on MSC fate. For example, when injected into the infarcted/scarred myocardium which is high in mechanical tension, undesirable differentiation of MSCs leads to the formation of encapsulated structures containing calcification and ossification (Breitbach et al., 2007). This study highlights the potential deleterious aspects of MSC plasticity and seriously questions the clinical safety of MSCs, especially when our understanding of their interaction with physical environment is limited.

The effects of soluble factors and genetic regulators on cell fate commitment have been characterized extensively. However, it is now becoming clear that physical properties of the microenvironment play a far more powerful role than previously appreciated. During embryonic development, stiffness is temporally and spatially modulated as ECM undergoes dynamic changes in composition. This biomechanical information is extracted and processed by early progenitor cells to make the appropriate fate decisions (Reilly and Engler, 2010). In fact, stem cells have been shown to be more sensitive to mechanical perturbation than their differentiated counterparts. Under small, continuous oscillatory stress, the spreading response of stem cells is much greater than that of a differentiated cell, potentially due to their intrinsically soft material property (Chowdhury et al., 2010).

Many studies have examined the responses of stem cells in a controlled physical environment. The seminal work by Engler and colleagues demonstrated that compliant polymer substrates that recapitulate the stiffness of native tissues can direct MSC differentiation into neurogenic, myogenic, or osteogenic cell types (Engler et al., 2006). Another study investigating MSC self-renewal was able to show that soft polyacrylamide substrates with stiffness value similar to that of the BM attenuate cell cycle progression whereas stiff substrates stimulate MSCs to re-enter the cell cycle. These quiescent MSCs remain capable of proliferation and terminal differentiation. The authors went on to postulate that the native marrow niche harbours critical mechanical cues that sequester MSCs in their quiescence state and allows for the maintenance of a MSC reservoir for a long period (Guilak et al., 2009; Winer et al., 2009). Although the precise molecular mechanisms by which extracellular tension is translated into functional changes in gene expression to ultimately affect lineage specification are still under intense investigation, mounting evidence suggests
that common mechanotransduction pathways elaborated further in the ensuing sections are implicated. More specifically, RhoA and non-muscle myosin II isoforms have been shown to be associated with MSC lineage specification by regulating actin-myosin generated intracellular tension (Engler et al., 2006; McBeath et al., 2004).

The important conclusion from the aforementioned examples and many other similar studies is that mechanical stimuli can act equally potent as soluble, differentiation inducing growth factors at driving stem cell fate decisions and may even override the biochemical cues under certain circumstances. Therefore it is critical to assess how changes in matrix stiffness in my study can affect the MSC-MF phenotype – an often ignored fate of MSCs.

II.3.3 Principle Mechanisms of Cell Mechanotransduction

Cells embedded in the ECM of various tissues are highly mechanosensitive and can process the biomechanical cues into corresponding adaptive changes in cellular structure or function. The process of translating biophysical information from the surrounding environment into biochemical or bioelectrical signals is termed mechanotransduction and is often classified into ‘outside-in’ and ‘inside-out’ sensing mechanisms (Holle and Engler, 2011; Jaalouk and Lammerding, 2009). Adaptive changes can be short-term responses that manifest as organization of cytoskeleton, modulation of intracellular tension, adhesion, spreading, or migration. Alternatively, the forces can be transmitted from the ECM via the cytoskeleton to the nuclear interior to affect gene expression, ultimately leading to long-term changes in protein synthesis, proliferation, or differentiation (Chan et al., 2010; Jaalouk and Lammerding, 2009). Impaired mechanotransduction or disruption in the relay sequel of force transmission can result in mechanobiology diseases and contribute to other processes such as tumorigenesis and metastasis (Jaalouk and Lammerding, 2009; Yu et al., 2011).

The relationship between a cell and its substrate can be understood as a system of two mechanical springs connected in series. As a cell spreads and pulls on a stiff substrate, its internal stiffness will increase. When the threshold is reached where a cell’s internal tension matches the stiffness of the substrate, any further incremental stress applied to the system will lead to substrate deformation, which can be visualized and quantified in vitro. If the substrate is stiff enough, spreading will stop completely and the cell organizes its cytoskeleton and adhesions (Solon et al., 2007). Historically, cell biology research has focused mainly on
studying the effects and dissecting the pathways of soluble factors, however increasing evidence suggests that mechanosensing pathways can act in concert with or even override the signals from soluble stimuli to direct cellular processes (Janmey et al., 2009; Solon et al., 2007). This notion has profound implications for therapeutic applications. Our ability to better understand mechanosensing pathways, better engineer artificial ECMs, and better manipulate cell properties to our advantage may improve the outcomes of clinical treatments (Carver and Goldsmith, 2013; Guilak et al., 2009). Therefore using skin-soft silicone elastomers, I investigated the possibility of generating MSCs that are shielded from MF activation and therefore could alleviate detrimental tissue contractions in burn wound healing.

II.3.4 Key Players and Possible Mechanisms of Mechatrontransduction

While the ECM conveys passive physical tension, cells actively probe their underlying substrate by applying traction force and changing membrane tension in response to changes in stiffness (Engler et al., 2006; Kobayashi and Sokabe, 2010). Biomechanical information about the cell exterior is transmitted to the inner contractile machinery at sites of specialized adhesions on the surface of plasma membrane, commonly known as FAs \textit{in vitro}. In accordance, cells tune their internal stiffness to match that of their substrate, ultimately reaching a critical threshold tension that promotes FA supermaturation, a process used to describe the elongation of FAs to suFAs and the associated compositional changes (Hinz, 2006). The \textit{in vivo} counterpart of suFAs is termed ‘fibronexus’, historically used to characterize the co-alignment of intracellular microfilament bundles with extracellular FN fibrils (Hinz, 2006). Integrins are the key mediators that facilitate attachment to a wide range of ECM molecules by recognizing a specific RGD (Arg-Gly-Asp) motif. They are transmembrane receptors composed of an α and a β subunit, which can assemble into 24 different heterodimers currently identified in vertebrates (Moore et al., 2010). Although integrins lack intrinsic enzymatic activity, they are implicated in mechanosensing as a physical linkage between the actin cytoskeleton and ECM. More importantly, the cytoplasmic domain of integrins serves as binding site for a plethora of signaling molecules via direct or indirect mechanisms (Carver and Goldsmith, 2013).
Many possible mechanisms are responsible for the conversion of biomechanical signals into biochemical signals downstream of integrins. Stress induced integrin clustering can lead to the activation of focal adhesion kinase (FAK), which in turn activates other signalling molecules via direct phosphorylation or acts as a binding scaffold itself to recruit more proteins (Carver and Goldsmith, 2013). Furthermore, when cells encounter resilience from the ECM, the molecular forces are transmitted to mechanosensitive FA proteins, causing their unfolding and exposure of binding domains to relay the signals onwards (Jaalouk and Lammerding, 2009; Moore et al., 2010; Vogel, 2006). A classic example of such is talin, an integrin binding protein in FA complexes. Talins are responsive to low stretching forces and can expose up to 11 domains to attract other binding partners such as vinculin. This interaction is extremely sensitive as in vitro force experiments demonstrated that unfolding of a single talin rod can activate vinculin binding (Chowdhury et al., 2010; del Rio et al., 2009). Vinculin itself is force-dependent and has been postulated to allow the binding of mitogen-activated protein kinase 1, or MAPK1, which activates a cascade of signaling events that affect numerous cellular processes ranging from proliferation to differentiation (Holle and Engler, 2011).

Rho/Rho-associated protein kinase (ROCK) signaling pathway is another extensively characterized mechanism through which mechanotransduction occurs. Guanidine exchange factors bind to integrins and catalyze various Rho GTPases, most commonly RhoA and Rac. Depending on the amount of force, RhoA further phosphorylates and to varying degrees activates ROCK, a kinase that modulates actin nucleation, stress fibers organization, and cell contractility (Holle and Engler, 2011). Again, this mechanism illustrates the importance of the ECM-FA-cytoskeleton axis in the transmission of molecular forces.

Mechanosensitive channels, otherwise known as stretch activated channels, participate in mechanotransduction by increasing the permeability to soluble ions, especially intracellular Ca$^{2+}$ (Kobayashi and Sokabe, 2010). Externally applied mechanical stress has been shown to increase the cytosolic Ca$^{2+}$ concentration, which in turn induces contractility via phosphorylation of myosin light chain kinase (Holle and Engler, 2011). Moreover, increasing substrate rigidity leads to higher amplitude of spontaneous Ca$^{2+}$ oscillations, which influences actin cytoskeleton organization by modulating the activities of actin-associating proteins. This pathway is thought to be implicated in the migration of cells towards substrate of higher stiffness, a process also known as ‘durotaxis’ (Kobayashi and Sokabe, 2010; Lo et al., 2000).
In addition to the three major mechanosensing pathways summarized in Figure 3, other potential mechanisms are under investigation. Changes in chromatin conformation due to intracellular deformations potentially lead to altered access to transcription machinery (Jaalouk and Lammerding, 2009). More recently, YAP and TAZ transcriptional regulators have attracted significant attention. ECM stiffness has been shown to increase the level of YAP/TAZ and promote their translocation from the cytoplasm to nucleus. A number of crucial transcription factors involved in lineage specification and profibrotic signalling are modulated by this nuclear translocation event as a consequence. Connective tissue growth factor and TGF-β1 are among the transcriptional targets of YAP/TAZ down the signaling cascade (Calvo et al., 2013; Dupont et al., 2011; Maller et al., 2013; Raghunathan et al., 2013) – another example illustrating the complexity of the vicious fibrogenic cycle. Physical stress, which in itself is a potent stimulus, activates additional transcriptional regulators through mechano-transducing pathways to modulate the expression of MF-related genes. And the cycle goes on. For example, in a ROCK dependent manner, mechanical tension causes the nuclear

Figure 3  Schematic depicting the three major possible mechanosensing pathways.

translocation of myocardin-related transcription factor-a (MRTF-A) from the cytoplasm, which directly upregulates the transcription of Col1a2 and Col3a1 genes (Small et al., 2010). Moreover, the intronic CArG box element upstream of α-SMA promoter is regulated by the binding activity of serum response factor and the mechanosensitive co-factors MRTF-A and –B, resulting in the enhanced contractile function (Crider et al., 2011). Taken together, the transcription of many marker genes associated with fibrosis is mechanically regulated to produce and sustain the MF phenotype.

Some or all of these mechanotransducing mechanisms and players are potentially implicated in the stiffness-induced MSC-to-MF differentiation process in my thesis study.

### II.4 Potential Transcriptional Regulations Leading to the MF Phenotype

#### II.4.1 Can Cells Memorize Mechanosensed Information?

Our lab has developed systems to isolate and culture cells on silicone elastomers with stiffness values ranging from one to several hundred kPa (Wipff et al., 2007). Using this culture system, we have previously published data indicating that cells can memorize the mechanosensed information. Lung fibroblasts explanted directly onto fibrotic stiff substrates and sub-cultured on these substrates acquire a MF memory that is retained regardless of subsequent stress reduction by transferring to soft substrates. Similarly, priming lung fibroblasts on soft substrates prevent them from being activated by increased mechanical stress on stiffer substrates (Balestrini et al., 2012). Moreover, genome-wide analysis revealed that the pathological MF phenotype in idiopathic pulmonary fibrosis-derived primary lung fibroblasts persists up to nine sub-cultivations in vitro, as reflected in the organized changes in gene regulation and translation (Larsson et al., 2008).

Collectively, fibrotic signatures at the protein, gene, and transcriptional level can be retained over several passages in culture, pointing towards a “MF memory” that may be mediated by epigenetic changes including DNA methylation, histone modifications, and regulation of select genes by miRNAs (Hinz et al., 2012). These changes alter the chromatin architecture as well as the accessibility to and processing of certain genes without changing the DNA sequence itself, leading to preservation of gene expression changes after cell division (Arnsdorf et al., 2010).
II.4.2 Modifications at the DNA level

DNA methylation is mediated by DNA methyltransferases (DNMT) and occurs on the cytosine residue of CpG islands, which usually reside in sites of transcription initiation (Hu et al., 2010; Lorenzen et al., 2012; Mann et al., 2007). By either directly blocking transcription factor binding or indirectly inducing a more condensed chromatin structure through recruitment of histone-modifying enzymes, DNA methylation contributes to transcriptional repression of target genes (Lorenzen et al., 2012). Several lines of evidence suggest that DNA methylation not only directly regulates the MF phenotype by affecting the cell cytoskeleton, but also modulates soluble factors that control MF differentiation. For example in quiescent fibroblasts, the CpG islands in the promoter region of α-SMA are 80% methylated. Global inhibition of DNMT activity results in dramatically elevated α-SMA expression level whereas overexpression of DNMT causes suppression of α-SMA (Hu et al., 2010). The level of methyl-CpG-binding proteins such as MeCP2 has also been shown to increase in α-SMA positive MFs (Mann et al., 2007). Through a more indirect route, the promoter region of PDGF receptor β, a known regulator of differentiation and ECM synthesis, is demethylated during embryonic stem cell differentiation (Hewitt et al., 2012).

Histone modifications enable the switch between an active euchromatin where DNA is accessible to the transcriptional machinery and an inactive heterochromatin. Histone deacetylases (HDACs) directly contribute to the formation of transcription-resistant heterochromatin and are implicated in fibrogenesis in various ways. For example, HDAC4 is involved in the upregulation of TGF-β1 mediated α-SMA expression (Glenisson et al., 2007) and silencing of MMPs (Qin and Han, 2010), thereby contributing to the progression of fibrosis.

II.4.3 miRNAs in Fibrosis

Epigenetic regulation is the heritable alteration in chromatin structure and consequent changes in accessibility to gene transcription without affecting the DNA template. Although transcriptional regulation through miRNAs is not epigenetic in its classic sense, it is often categorized with this group because miRNAs simultaneously modulate many downstream gene targets – much similar to the effects of heterochromatin formation at a certain gene region.
Approximately 90% of the genomic DNA consists of non-coding RNAs, revealed by high-throughput analysis of the transcriptome. The 1~2% protein encoding portion of the genome is extensively and dynamically regulated by small non-coding RNAs including miRNAs. They play an important role during development and in maintaining normal tissue homeostasis (Lorenzen et al., 2012). MiRNAs control a wide range of cellular and molecular processes. Aberrant miRNA expression and perturbation in miRNA processing pathway can lead to the development of many diseases (Davis et al., 2008; Jiang et al., 2010; Stefani and Slack, 2008).

![miRNA processing pathway](image)

**Figure 4** *miRNA processing pathway.*

MiRNAs are encoded in the intronic regions and transcribed by RNA polymerase II to produce primary miRNAs (pri-miRs). Initial cleavage of pri-miRs by Drosha yields 65-nucleotide-long, stem-loop structures called precursor miRNAs (pre-miRs), which are further processed into mature 22-nucleotide-long RNA duplexes of guide strand and passage strand. MiRs are loaded onto RNA interference-induced silencing complex (RISC) to mediate gene repression. Source: Chau and Brenner. Hepatology 2011. 53:4-6 (Chau and Brenner, 2011).

40~50% of mammalian mRNA is regulated by miRNAs (Collino et al., 2011), which are highly conserved non-coding RNAs of approximately 22-nucleotide long. MiRNAs are encoded in the intronic regions of genome and are transcribed into primary miRs (pri-miR) by RNA polymerase II (Chau and Brenner, 2011). The cleavage of pri-miRs by ribonuclease III enzyme Drosha in the nucleus releases the stem-loop-structured precursor miRs (pre-miRs) of approximately 65-nucleotide-long (*Figure 4*) (Chau and Brenner, 2011; Lorenzen et al., 2012).
Following exportin- and Ran-GTP-mediated translocation into the cytoplasm, pre-miRs are further processed to double-stranded mature miRNAs consisted of a guide and a passenger strand. While the passenger strand is later degraded, the guide strand is incorporated into an argonaute-containing RNA interference-induced silencing complex (RISC) (Chau and Brenner, 2011; Lorenzen et al., 2012). The 5’ seed-regions of miRNAs recognize and bind to the 3’ untranslated regions of target transcripts to facilitate target degradation or translational inhibition, depending on whether there is perfect complementarity (Chau and Brenner, 2011). MiRNAs and miRISCs are enriched in cytoplasmic storage foci known as processing bodies, or P-bodies. P-bodies are where degradation of mRNAs occurs (Collino et al., 2011).

Approximately 20 miRNAs have been shown to play a role in fibrogenesis and MF activation in various organs and cell systems (Table 1). Most commonly reported are pro-fibrotic miR-21 and anti-fibrotic miR-19, -29, and -200. The expression of miR-21 is upregulated in cardiac, pulmonary, liver, and renal fibrosis through mechanisms involving the TGF-β1 pathway (Bonci, 2010; Chau and Brenner, 2011; Jazbutyte and Thum, 2010; Liu et al., 2010). The promoter activity of miR-21 is enhanced in response to pSmad3, a downstream effector of TGF-β1 signaling (Zhong et al., 2011). Elevated miR-21 in turn represses a negative regulator of the pro-fibrotic pathway, Smad7, via direct target binding and degradation, resulting in a feed-forward loop that potentiates the effects of TGF-β1 (Liu et al., 2010). Treatment with miR-21 mimic potentiates the effects of TGF-β1, as the transcript levels of collagen I, FN, and α-SMA increase significantly (Zhong et al., 2011).

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<th>Fibrosis Related miRNAs</th>
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**Table 1.** List of miRNAs known to be associated with fibrosis in various organs and systems.

The highlighted miRNAs are most extensively studied in fibroproliferative diseases and are the major candidates to be examined in my thesis study.
In contrast, miR-19b directly regulates the expression of TGF-β receptor II, Smad3, and α-SMA and is present at a reduced level in liver fibrosis (Lakner et al., 2012). Similarly, loss of miR-29 family is correlated with renal fibrosis, liver fibrosis, cardiac infarction, and multiple sclerosis since miR-29a/b/c not only target a multitude of ECM associated genes, but also regulate the expression of TGF-β1 and PDGF (Chau and Brenner, 2011; Cushing et al., 2011; Wang et al., 2012; Xiao et al., 2012). Lastly, miR-200 attenuates the TGF-β1 induced upregulation of FN and α-SMA. The level of miR-200a/b/c is significantly reduced in both a mouse model of experimentally induced pulmonary fibrosis and patients with idiopathic pulmonary fibrosis (Yang et al., 2012). Whether any of these fibrosis-associated miRNAs are regulated in MSCs upon culture and whether substrate stiffness affects miRNA expression in MFs are currently unknown. In my study, I will examine the response of select fibrosis-associated miRNAs to changes in mechanical stimulus and investigate their role in mediating mechanically propagated MF memory.

Chapter III: Materials and Methods

III.1 Fabrication and Functionalization of Soft Substrates

Deformable silicone elastomers of varying stiffness were prepared from polydimethylsiloxane (PDMS) by altering the base-to-curing agent ratios. PDMS silicone substrates were chosen as opposed to other polymers because they are non-toxic, tunable and optically clear (Wipff et al., 2007). Moreover, substrate deformations, or wrinkles can be visualized and quantified to putatively assess cell contractility. The polymer was cast into plastic tissue culture dishes (TPP, Techno Plastic Products, Switzerland) and spun to form a uniform thin layer of approximately 50 μm with a spin processor (SPIN150, SPS-Europe B.V, Putten, the Netherlands). Fully polymerized substrates were treated with plasma oxygen (PE-100, Plasma Etch, NV, USA) to create a hydrophilic surface. To allow cell attachment, the etched substrates were sterilized with 10 min UV light exposure and coated with FN, pronectin, or gelatin solution (all at 2 μg/cm²) overnight at 37°C. The coating solution was removed prior to cell seeding.
### III.2 MSC Isolation and Culture

Primary MSCs were obtained from the BM of 5-week old male C57BL/6 or FVB mice and Wistar rats (Charles River Breeding Laboratories, Inc., Wilmington, MA, USA) using standard isolation (Polisetti et al., 2010). Animals were euthanized with CO\(_2\) followed by cervical dislocation. Both femurs were taken and cleaned of attached muscles. The BM was flushed out using a syringe, suspended, and centrifuged. The cell pellet was then re-suspended and divided into equal parts and seeded onto substrates of varying stiffness and/or TCP. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA).

After an initial expansion passage, mouse MSCs were purified by negative sorting using anti-CD5, CD45, CD11b, Gr-1, Ter-119 and CD45R conjugated magnetic beads (Miltenyi Biotec) using an established protocol (Itoh and Aubin, 2009). To circumvent the limitation of rat cell surface markers, rat MSCs were enriched by positive selection (Figure 5) using anti-CD54 and CD-90 conjugated magnetic beads (Zhang and Chan, 2010). Cell proliferation was monitored and cells were counted. To offset the difference in proliferation rate on soft versus stiff substrate, the seeding density of rat MSCs on soft substrates was 1000 cells/cm\(^2\) and 1500 cells/cm\(^2\) on stiff substrates. Cells were grown near confluent, trypsinized, resuspended in medium and plated again onto respective substrates for sub-culturing and subsequent analyses.

![Enrichment of MSCs by magnetic sorting based on positive selection](image)

**Figure 5** Enrichment of MSCs by magnetic sorting based on positive selection.

Total cell suspensions were incubated with biotinylated antibodies against CD54 and CD90. A secondary incubation with anti-biotin microbeads retained the positive cells inside the column within the magnetic field whereas un-labelled lineage negative cells were eluted. The positive fraction containing MSCs was obtained by removing the column away from the magnet. Scheme modified from Miltenyi Biotec.
III.3 MSC Differentiation

Adipogenic Differentiation: Soft- or stiff-primed MSCs were seeded in plastic 6-well plates at a density of 10,000 cells/cm². Following complete cell attachment overnight, MSCs were induced with adipogenic medium – standard culture medium supplemented with 0.5 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 μg/mL insulin (Sigma-Aldrich, St. Louis, MO, USA) for 14 days with frequent media change. At the end of the induction, MSCs were fixed with 3% paraformaldehyde (PFA) for one hour and incubated with 0.3% Oil Red O solution for 2 hours. The cultures were then washed carefully with PBS to get rid of background staining.

Osteogenic Differentiation: Soft- or stiff-primed MSCs were seeded in plastic 6-well plates at a density of 2,500 cells/cm². Following complete cell attachment overnight, MSCs were induced with osteogenic medium – standard culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid (Sigma-Aldrich) for 14 days. At the end of the induction, MSCs were fixed with 3% PFA and stained with 0.5% Alizarin Red solution. Careful washing was carried out to avoid dislodging of calcium deposits.

III.4 Immunofluorescence and Microscopy

MSCs from different assays were washed with PBS, fixed with 3% PFA, and permeabilized with 0.2 % Triton X-100 for further processing. Cells were incubated with primary antibodies directed against α-SMA (mouse IgG2a, a kind gift from Dr. Giulio Gabbiani, University of Geneva) and ED-A FN (mouse IgG1, Santa Cruz Biotechnology, Inc. CA, USA). Corresponding anti-mouse TRITC and FITC secondary antibodies (Southern Biotechnology Associates, Inc., AL, USA; Sigma Aldrich) were used. Nuclei were stained with DAPI (Sigma Aldrich) and F-actin with Phalloidin-Alexa Fluor ®647 (Invitrogen, Carlsbad, CA, USA).

Images were acquired with an inverted microscope (Axiovert 135M, Carl Zeiss, Germany) equipped with a CCD camera (Orca R2, Hamamatsu, Guelph, ON, Canada) or an upright Axio Imager microscope platform (Carl Zeiss). All figures were assembled using Adobe Photoshop.
III.5 Western Blotting

SDS-PAGE separation gels of 6%, 10% were prepared depending on protein sizes. 6% gels were used to analyze the expression of higher molecular weight proteins including Collagen I, ED-A FN, TGF-βRII, total FN, and vinculin, along with vimentin for loading control. 10% gels were used to analyze the expression of α-SMA and vimentin.

MSCs from different assays were scraped into a standard lysis buffer with protease inhibitor cocktail (Complete Mini, Roche, Bath, UK) and further lysed with a syringe. Total protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes using wet transfer at 100 V, 500 mAmperes for 1.5 hours. The nitrocellulose membranes were then blocked with 5% milk in TBS-T buffer (1X TBS, 0.1% Triton-X 100), followed by overnight incubation with primary antibodies used for immunofluorescence staining in addition to collagen I (rabbit polyclonal IgG, Acris, San Diago, CA, USA), TGFβ receptor II (TGFβRII) (rabbit polyclonal IgG, Santa Cruz Biotechnology, Inc.), total FN (rabbit polyclonal IgG, Sigma Aldrich), vinculin (mouse IgG1, Sigma Aldrich), and vimentin (mouse IgG1, Sigma Aldrich). After a subsequent 1 hour incubation with IRDye® goat anti-mouse or anti-rabbit secondary antibodies, the blots were visualized and images were acquired with Li-Cor Odyssey Clx system (Li-Cor Biosciences, Lincoln, NE, USA). Quantification of the blot was performed using the Li-Cor Image Studio 2.0 (Li-Cor Biosciences) and the band densities were normalized to housekeeping vimentin expression (total and cytoskeletal protein).

III.6 Quantitative Real-Time PCR (qRT-PCR) for miRNA Expression

Total RNA including miRNAs and other small non-coding RNAs longer than 18 nucleotides was extracted using Qiagen miRNeasy Mini Kit (Invitrogen, Carlsbad, CA, USA). Consistently for all assays, 500 ng of RNA was reversed transcribed using miScript II RT Kit (Invitrogen). The resulting cDNA was diluted per instruction and an input cDNA of 5 ng per reaction was used across all qRT-PCR experiments. MiR-19, 21, 29, and 200 specific miScript Primer Assays (Invitrogen) along with the Universal upstream primers contained in the miScript SYBR Green PCR Kit (Invitrogen) were used for real-time reactions on a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA). All reactions were performed in technical triplicates. Relative quantification was performed using the ΔΔCt method where the miR expression data were normalized to that of an endogenous control RNU6 (Invitrogen).
III.7 Transfection of miR-21 Mimics and Inhibitors

Soft- and stiff-primed MSCs at P3 were seeded on respective substrates and allowed to proliferate for 3 days to reach an optimal density for transfection. Soft-primed MSCs on soft substrates were transfected with 20 nM miR-21 mimics via lipofection using HiPerfect Transfection Reagent (Invitrogen). Stiff-primed MSCs on stiff substrates were transfected with 200 nM miR-21 inhibitors (Invitrogen). In both conditions, a mock transfection was performed using the transfection reagent alone. There was also a non-transfected control for comparison on either substrate.

48 hours after transfection, total RNA was collected from all conditions and reverse transcribed as previously described. The relative expression levels of miR-21 in all experimental groups were quantified. Another set of MSCs transfected in the same manner were also immunofluorescence stained to assess effects of overexpression or knockdown on the level of α-SMA positive stress fibers.

III.8 Statistical Analysis

Data were presented as mean±standard deviation. Where applicable, two-tailed paired t-tests were performed and differences were considered to be statistically significant if p<0.05.
Chapter IV: Results

IV.1 Isolation and Purification of Mouse BM-MSCs

My first objective is to test whether the mechanical conditions of cell culture can be modulated to prime BM-derived MSCs towards or protect MSCs against the MF fate. BM-MSCs in comparison to MSCs from other organ sources are relatively easy to isolate, but they only account for 0.001~0.01% of the total nucleated cell population in the marrow stroma (Wu et al., 2012). Conventionally, the colony forming, TCP-adherent BM-MSCs are selected and subsequently expanded in culture for an extended period of time to achieve a sufficient cell number for therapeutic applications. However, the supra-physiological stiffness of TCP likely induces permanent changes in MSCs that undermine their therapeutic potential. Our lab has developed systems to isolate and culture cells over multiple passages on silicone substrates with physiologically relevant stiffness values ranging from 1 kPa to several hundreds and are able to fine-tune the fibrogenic response over a gradient of stiffness (Wipff et al., 2007). Using these novel substrates, we have shown that continued exposure to fibrosis-stiff culture surfaces primed lung fibroblasts towards the MF phenotype, and the preservation of this stiffness-dependent fibrotic response is independent of a sustained stiffness stimulus (Balestrini et al., 2012). In order to assess whether a similar mechanical memory is present in MSCs, it was important to first of all establish soft-primed and stiff-primed MSCs that are initiated directly on skin-soft (5 kPa) and fibrotic-stiff (100 kPa) substrates, as opposed to cell lines that are preconditioned to TCP. The nature of the project as well as its constant demand for fresh batches of BM denied the option of directly isolating and priming MSCs of human origin, due to limited access to clinical samples.

As an initial attempt to obtain primary MSCs, the BM from C57BL/6 mice was aspirated and the total cell suspension was plated directly onto soft and stiff silicone substrates or TCP as a standard control. Mouse BM-MSCs were allowed to arise and proliferate through colony formation over time. However, in all three conditions, hematopoietic cells, being the main stromal population of the BM remained strongly adherent to both the co-cultured MSCs and their underlying substrates. A number of different strategies to remove these contaminants were not successful, including frequent PBS wash, physical agitation, and trypsinization for multiple passages. F-actin staining marked the outline of small, round immune cells that
attached directly to the α-SMA expressing MSCs, which were clearly distinct in size and morphology (Figure 6). Although MSCs showed enhanced incorporation of α-SMA into stress fibers as stiffness increased (Figure 6), the immune cells in such close proximity may exert trophic effects that overpower the mechanical stimuli.

![Figure 6](image)

**Figure 6** *Heterogeneity in mouse BM stromal cell culture.*

Mouse BM aspirate was plated onto silicone substrates of 5 kPa and 100 kPa, and TCP. The stromal population was allowed to expand for 1 passage to enrich for MSCs. After an additional 2 passages, cells in each condition were fixed and immunostained for α-SMA, F-actin (Phalloidin), and nuclei (DAPI). F-actin marked the outline of the small round immune cells, which were adherent and in direct contact with the larger MSCs. The nuclei size and morphology between the two populations were clearly distinct as well. The chromatin content of MSCs was more loosely packed and diffused in nuclei of larger size. MSCs expressed α-SMA and differed in the extent of stress fiber incorporation on substrates with increasing stiffness.

To eliminate this undesirable confounding factor, magnetic cell sorting based on negative selection was introduced. A repertoire of antibodies that target T cells (CD5), B cells (CD5/CD45R), dendritic cells (CD45R/Gr1), monocytes/macrophages (CD11b), granulocytes (Gr1), neutrophils (Gr1), and erythrocytes (Ter-119) were selected to magnetically deplete nearly all contaminating immune cells. The net yield of purified MSCs derived from the input
stromal cells of 20 animals was on a mere scale of $10^4$ cells and the percentage yields were consistently lower than 5% across all conditions (2.1% on average) despite efforts to optimize culture conditions and antibody concentration. The MSC-containing cell fractions were eluted and subsequently re-plated onto corresponding culture substrates. Immediately after cell attachment and spreading, it was observed that the immuno-depleted culture was much more homogeneous as compared to unsorted total stromal population following trypsinization alone. However, the enriched MSCs showed extremely slow growth kinetics and in certain cases failed to survive past the second day.

It has been reported that using similar method of isolation by negative selection, the yield of MSCs from the FVB/N mouse strain is 23±8% (Baddoo et al., 2003), which is considerably higher than my MSC recovery rate using C57BL/6 mice. However, switching to this particular strain of mice did not boost the net yield beyond $10^5$ cells/20 animals. Another reported alternative to address the low proliferation rate of immuno-depleted MSCs is to supplement the culture with fibroblast growth factor (FGF)-2 (Baddoo et al., 2003). However, the confounding effect of FGF-2 on MF phenotype and its known interplay with TGF-β1 may dilute the effect of substrate stiffness and influence the outcome of priming (Cushing et al., 2008). Overall, the low yield and slow growth rate of purified mouse BM-MSCs failed to meet the project’s demands for large cell quantity, requiring a different and more appropriate animal model.

### IV.2 Isolation, Enrichment, and Characterization of Rat BM-MSCs

To circumvent the technical challenges discussed previously, a rat model was used to establish primary MSCs instead. Using a similar isolation technique as described for mouse BM-MSCs, I was able to successfully enrich and expand BM-MSCs from Wistar strain rats. Due to limitation of available antibodies against rat cell-surface markers, I employed magnetic cell sorting based on positive selection for CD54 and CD90, which was previously established by others to replace immuno-depletion (Zhang and Chan, 2010). Initially positively labelled MSCs were directly purified from the raw BM aspirate at a net yield of 4.5%. However, the resulting cell number was not sufficiently high to initiate the priming study and purified MSCs at this stage had extremely low proliferation rate. As a result, the freshly isolated stromal cells were expanded in culture on respective substrates for one passage (P0) as a first step toward enrichment of MSCs.
In contrast to the TCP-adherent cultures established from murine BM, which was a mixture of mesenchymal and hematopoietic cell types, isolating BM-MSCs of rat origin based on plastic adherence and colony formation is a standard practice as rat hematopoietic cells can be gradually depleted in culture with each successive media change (Polisetti et al., 2010).

Using the standard TCP culture as control, I recorded and compared stromal cell growth dynamics on silicone substrates versus on TCP and observed three distinct features: First, hematopoietic cells preferentially adhered to silicone surfaces irrespective of stiffness whereas...
very few hematopoietic cells attached to TCP. PBS washings or media changes eliminated most hematopoietic cells with relative ease on TCP while those on silicone substrates remained strongly adherent. Secondly, continuous monitoring of the culture revealed more rapid colony formation and expansion on polymer substrates as small satellite colonies rapidly merged into bigger and denser colonies. In contrast, TCP cultures contained sparse colonies of significantly smaller size, as visualized by vimentin positive MSCs (Figure 7A). I performed a dilution series to better resolve the difference and found that the CFU numbers were lower on TCP at all chosen dilution ratios (1:2, 1:4, and 1:8) (Figure 7B). Finally, the higher proliferation rate of colony forming MSCs on silicone substrates relative to that on TCP led to significantly higher cell yield per unit area at the end of expansion (Figure 7C).

Hematopoietic cells were completely absent from TCP cultures within one additional passage (Figure S1). However, it took considerably longer for the cultures on silicone to become enriched and homogeneous for MSCs (Figure S1). In order to eliminate the potentially confounding trophic effects from contaminating cells already at an early time point, positive selection for CD54 and CD90 was introduced immediately after the initial expansion. This procedure effectively eliminated nearly all hematopoietic cells, as evident by the disappearance of cells with small, round morphology (Figure 8A). The purified MSCs acquired a well-spread polarized morphology and formed FAs that transmitted considerable contractile force to deform the underlying soft substrate (5 kPa), visible as substrate wrinkles that spanned the entire substrate surface under MSCs (Figure 8A). Because direct isolation of MSCs on tunable silicone substrates without expansion on plastic is a new method, I also validated the functionality of the enriched MSCs by demonstrating their capacity to undergo adipogenesis and osteogenesis, as shown by Oil Red O and Alizarin Red staining, respectively (Figure 8B). The extents of adipogenic or osteogenic differentiation were comparable between soft- and stiff-initiated cultures, indicating that stiffness variation did not selectively favor one particular population of MSCs versus another. However, this observation based on histochemical staining needs to be confirmed using more quantitative methods.
IV.3 Evaluation of MSC Growth and MF Character on Various ECM Surface Coatings

In contrast to commercially available TCP vessels, which allow rapid attachment and spreading without functionalization with ECM coating, cells adhere poorly to plasma-treated silicone surface and require ECM coating to provide adhesive ligands. Initially, MSCs were isolated and expanded on substrates functionalized with FN – the most extensively characterized surface coating in the lab. However, we needed to ensure that responses to stiffness change do not occur exclusively on or due to the underlying FN. Hence, following the enrichment and characterization of BM-MSCs, I assessed the growth kinetics of these cells when seeded on different types of ECM proteins and tested how well they respond to stiffness change on each respective ECM substratum.
Four ECM proteins commonly used to functionalize biomaterial surface were tested and compared. FN and type I collagen are major components of the ECM that allow proper cellular functions including adhesion, proliferation, migration, and differentiation. Gelatin is the water-soluble derivative of collagen. Pronectin is a genetically engineered protein polymer that incorporates multiple copies of cell attachment epitope RGD from FN, which enables proper cell attachment and signalling.

All four ECM coatings promoted MSCs attachment and expansion, albeit clear differences in cell morphology, proliferation rate, and contractile property. Collagen was immediately eliminated because of the clear phenotypic difference between MSCs on collagen coated soft and stiff substrates, the former being elongated while the latter rhomboid (Figure S2). In addition and only observed with collagen coating, soft MSCs clustered into multiple foci of cell aggregates. MSCs grown on FN coated substrates appeared much larger in size compared to those on pronectin or gelatin (Figure 9A) and doubled at 3-times higher rates. The initial MSC plating densities were thus adjusted for all cultures to achieve confluence within the same time frame. In addition, in FN coated culture every cell deformed the soft 5 kPa substrate and there was overall higher wrinkle content (~3 fold) – an indicator of higher contractility. These observations were confirmed by differences at the level of immunofluorescence and Western Blotting. MSCs cultured on FN contained more α-SMA positive stress fibers (Figure 9A) and the overall SMA protein expression level was higher (Figure 9B) compared to gelatine and pronectin cultures. However, there was a clear difference in the extents of α-SMA incorporation into stress fibers and its expression levels between soft 5 kPa and stiff 100 kPa substrates, independently of the type of coating (Figure 9). Based on these preliminary observations, I chose FN as the ECM substratum for the ensuing priming study due to two main concerns. Taking into consideration the therapeutic relevance of this study, rapid expansion of MSCs is critical for implantation as a large number of cells are required to cover and regenerate the wounded area. The slow growth rate on projecting or gelatin was not ideal for this purpose and may become an impediment to testing the culture system in a surgical animal model for regeneration later on. From a technical perspective, slow growth rates will influence the timeline of priming and would need to be compensated by higher seeding density. Moreover, the baseline α-SMA expression level on pronectin and gelatin coated stiff substrates was very low. Any further manipulation to downregulate α-SMA may not be clearly detected.
To ensure that deposition of FN proteins on substrates with varying stiffness was similar in all following experiments, I immunofluorescence labelled the FN surface coating in control experiments. All cell-free substrates exhibited a uniform layer of FN after the coating procedure regardless of stiffness (data not shown). Hence, all observed effects were due to mechanical differences and not due to differential coating – a process which may elicit distinct fibrotic response pertaining to a particular stiffness and mask the true mechanical response.

**Figure 9**  *MSC-to-MF transition on substrates with different ECM coatings.*

MSCs initially expanded on FN-coated substrates for 2 passages were seeded onto substrates with corresponding stiffness coated with FN, pronectin, or gelatin. The plating density on pronectin and gelatin was 3 times higher than that on FN (3000 cells/cm² vs. 1000 cells/cm²) to offset the difference in proliferation rate. (A) After 4 days under respective culture conditions, cells were fixed and immunostained for α-SMA and nuclei (DAPI). MSCs appeared larger and contained overall more α-SMA positive stress fibers on FN-coated substrates of either stiffness. On all three ECM coatings, there was clearly increased incorporation of α-SMA into stress fibers on the 100 kPa substrates. (B) Total cell lysates from the 4-day cultures were collected and analyzed with Western Blotting. When loaded with the same 5 μg of total protein lysate, α-SMA expression was higher on FN-coated substrates. Different expression levels on soft versus stiff substrate were evident regardless of the coating type.
IV.4 Expression of α-SMA over Multiple Passages on Different Culture Substrates

Having demonstrated that stiffness influenced α-SMA localization and expression, I then monitored how such changes propagate over passages. Using BM-MSC culture on FN coated TCP as standard control; I found that a clear difference in α-SMA positive stress fiber density was established after four passages under respective culture conditions. MSCs continuously cultured on 100 kPa and TCP contained much denser and more prominent stress fibers and had much larger spreading area, i.e. a more pronounced MF phenotype. The α-SMA expression levels were progressively elevated on stiff substrates but were lower on soft substrates across all four passages. BM-MSCs initiated on TCP expressed initially higher levels of α-SMA and maintained elevated expression throughout (Figure 10).

![Image](image.png)

**Figure 10**  
Assessment of α-SMA expression over the duration of mechanical priming.  
Continuous culturing on 5 kPa and 100 kPa substrates or TCP for 4 passages established the MF character to varying degree. Total cell lysates were collected at each passage from all culture conditions for up to 4 passages and analyzed using Western Blotting. The expression levels of all groups are normalized relative to that of P1 5 kPa. MSCs continuously cultured on 5 kPa substrates had suppressed α-SMA expression whereas the level progressively increased on 100 kPa. TCP as standard control did not show altered α-SMA expression level and remained high throughout.
IV.5 MSCs Acquired Mechanical Memory upon Priming

Following the assessment of MF characteristics, mechanical priming of BM-MSCs was carried out according to the scheme shown in Figure 11.Briefly, the enriched MSC fractions were primed for an additional three passages before switching to the respective other substrate for two additional passages. Before and after the divergence, the localization of α-SMA in stress fibers and the distribution of ED-A FN were assessed using immunofluorescence and the expression of MF associated markers across all experimental conditions were compared using Western blotting.

![Figure 11](image)

**Figure 11  Experimental setup of mechanical priming.**
Rat BM-MSC cultures were initiated and sub-cultured on 5 kPa and 100 kPa silicone substrates for four passages before switching to the respective other substrate stiffness for an additional two passages.

To examine the outcome of mechanical priming, I compared four experimental conditions:

- **Soft control (5T5):** BM-MSCs cultured exclusively on soft substrates to model healthy condition;

- **Stiff control (100T100):** BM-MSCs cultured exclusively on stiff substrates to model fibrotic condition;

- **Soft-primed (5T100):** BM-MSCs explanted and sub-cultured on soft substrates for four passages and then transferred to stiff substrates to model *de novo* exposure of transplanted MSCs to fibrotic-stiff zones;

- **Stiff-primed (100T5):** BM-MSCs explanted and sub-cultured on stiff substrates for four passages and then transferred to soft substrates to model reduced stress.
Immunostained MSCs in stiff control and stiff-primed groups exhibited more pronounced MF characteristics and had higher incorporation of α-SMA into stress fibers compared to soft control and soft-primed MSCs that appeared much smaller in size and were less α-SMA positive (Figure 12). Stiff-primed MSCs generated prominent wrinkles on the soft substrates post-switching. In many instances, the cell contractile forces were so high that the surface plasma layer was torn, exposing the hydrophobic polymer underneath. These pronounced wrinkles or substrate deformations were even visible in immunofluorescence as black lines that intersected and segmented the stress fibers perpendicularly (Figure 12, white arrows).

**Figure 12** Effect of mechanical priming in MSCs – Immunofluorescence.

Following the priming setup, the MSC-MF character was assessed prior to and after substrate switching. Cells were fixed and immunostained for α-SMA, ED-A FN, and nuclei (DAPI). MSCs derived from the soft substrate cultures (5T5 and 5T100) appear smaller in size, contained less dense stress fibers, and produced more ED-A FN. MSCs derived from the stiff substrate cultures (100T100 and 100T5) are larger, contain much denser and prominent stress fibers, and produced low levels of ED-A FN.
In addition to actin organization, primed MSCs also maintain their respective level of ED-A FN production after substrate switching as shown in immunofluorescence (Figure 12) and Western blotting (Figure 13). Despite the increase in mechanical stress conferred by the stiffer substrates, α-SMA expression levels in the soft-primed group remained low after switching to stiff substrates and were comparable to soft control (Figure 13).

**Figure 13  Effect of mechanical priming in MSCs – Western Blotting.**

Total cell lysates were collected before and after priming and analyzed using Western Blotting for the expression of multiple MF-related markers. One passage after substrate switching, the expression levels of α-SMA, ED-A FN, and collagen in soft-primed (ST100) and stiff-primed (100T5) MSCs remained similar to their controls after substrate switching, as shown by densitometric quantification (Student’s t-test, p<0.05).
These results demonstrated the protective effect of soft substrate culture against subsequent MF activation. Similarly, preservation of MF phenotype after stiff-priming was verified as stress reduction did not significantly lower α-SMA expression level in the stiff-primed group (Figure 13). Other MF markers including ECM proteins were also examined as they were not carried over with pass aging and may be more indicative of mechanical memory. Similar priming effects were observed at the level of FN (both ED-A and total), collagen I, and to a lesser extent TGFβRII (Figure 13). Intriguingly, these markers were expressed at a lower level on stiff substrates despite the well-developed MF phenotype.

In addition to protein expression pattern and profile, difference in proliferation rates was also preserved through priming. Prior to substrate switching, MSCs cultured on soft substrates doubled at a moderately faster rate and often began to form cell-cell contacts while the other condition was still sub-confluent. In order to circumvent this issue and for both conditions to reach the same degree of confluence at the end of each passage, the seeding density was adjusted accordingly to compensate for the slower growth on stiff substrates. Interestingly after switching to the new substrates, primed MSCs maintained their original growth pace. More specifically, soft-primed MSCs continued to proliferate at a faster rate despite being on stiffer substrates whereas stiff-primed exhibited continued slower growth.

Collectively, these data indicate a mechanical memory acquired by MSCs over passages on respective substrates, which is retained regardless of the subsequent mechanical stimulus.

Although continuous priming on soft substrates suppressed MF phenotype, the process did not selectively expand a population of MSCs resistant to MSC-to-MF differentiation. Importantly, soft-primed MSCs were not only protected from stress elevation but also protected from activation by profibrotic cytokine TGF-β1 (Figure 14). However, when switched to TCP and followed with TGF-β1 treatment, the combination of both high mechanical tension and cytokine stimulation synergistically drove the MSCs towards the MF fate (Figure 14). Hence, soft primed MSCs retained their potential to acquire MF characteristics, when provided with pro-fibrotic stimuli on stiff culture substrates.
IV.6 MiR-21 potentially mediates the mechanically induced memory

Having demonstrated the persistence or attenuation of MF character by stiff or soft priming, respectively, my second objective was to investigate whether the regulation via miRNAs acts as possible mechanism to preserve this mechanically induced memory. To date, a number of miRNAs have been associated with fibrosis through profiling studies (Hinz et al., 2012). Dysregulation of miRNA expression in fibroproliferative diseases in comparison to normal healthy conditions make them an attractive and novel pool of therapeutic targets and biomarkers. However, their response to mechanical stimuli and their implications in MSC-to-MF activation have not yet been studied. Hence, it is important to investigate how changes in substrate stiffness can lead to modifications in miRNA expression that potentiate MF persistence and fibrogenesis.
Based on computational predictions using TargetScan (www.targetscan.org), I examined the potential downstream mRNA targets of a number of commonly studied fibrosis-related miRNAs and narrowed the candidate list down to miR-19a, -21, -29b, and -200b, all of which have multiple targets known to be involved in processes such as ECM synthesis and remodelling or pro-fibrotic cytokine signalling. Examples include various collagen isoforms, fibrillins, MMPs, FA molecules, TGF-β receptors, and small GTPases responsible for mechanotransduction. Preliminary tests were conducted to validate if these candidate miRNAs are indeed present in rat BM-MSCs. MiR-200b was immediately eliminated from the list due to its low expression level, which may compromise knockdown studies. Of the remaining candidates, miR-21 is known to act pro-fibrotic whereas miR-19a and miR-29b are anti-fibrotic.

**Figure 15** Expression of miRNAs on soft and stiff substrates over time.

Total RNA from each condition was collected over the priming study, reverse-transcribed and the expressions of miR-21, -19, and -29 were monitored. MiR expression levels were first normalized to endogenous control RNU6 in all experimental conditions and then expressed as relative levels to P1 5 kPa. MiR-21 level on stiff substrates gradually increased over passages but was suppressed on soft substrates. Soft-primed (5T100) and stiff-primed (100T5) groups maintained similar miR-21 expression levels as their respective controls (5T5 and 100T100). (B) In contrast, stiffness did not influence the expression profile of miR-19 or miR-29 (p<0.05).
Following the same cell culture scheme elaborated above, the expression levels of the remaining miRNA candidates were examined using qRT-PCR over the duration of the priming experiment. The expressions of miR-19a and miR-29b were not affected by changes in stiffness and were not pursued in my further experiments. However, the levels of miR-21 increased progressively over passages on stiff substrates and remained elevated two passages after substrate switching. In contrast, the overall miR-21 expression was suppressed upon soft priming and transferring to stiff substrates did not lead to an increase in miR-21 level (Figure 15). These data collectively suggest that miR-21 is indeed mechanically responsive and that the effects of mechanical priming also exist at the transcriptional level.

Interestingly, the trend of miR-21 expression very closely followed that of the α-SMA expression profile over time. This correlation prompted us to establish a direct link between miR-21 expression and MF character. As shown in Figure 16, transfection with miR-21 mimics increased the overall miR-21 expression level by approximately 37-fold, resulting in increased α-SMA incorporation into stress fibers. A maximum of 3-fold knockdown was achieved by transfecting stiff-primed MSCs with miR-21 inhibitors/antisense oligonucleotides before any further down-regulation began to affect cell survival. A decrease in α-SMA positive stress fiber density was observed as a result. However, further more quantitative methods are necessary to confirm the effects of up- and down-regulation.

With the preliminary finding that differential miR-21 expression underlies the MSC-MF character, the next logical step is to investigate whether miR-21 is responsible for preserving the mechanically propagated MF memory. To this purpose, miR-21 expression levels established on respective substrates were disrupted using mimics or inhibitors to presumably interfere with memory. The outcome of this manipulation was assessed two weeks after substrate switching, with the expectation that the stiffness of new substrates would redirect MSC fate and re-establish miR-21 expression profiles accordingly. Two separate sets of preliminary experiments were carried out, with non-targeting siRNA (NT) or non-transected controls (C) as normalization standards, respectively. More specifically, three days prior to substrate switching, soft-primed MSCs were transfected with miR-21 mimics to achieve an up-regulation of 27-fold (Figure 17A) and 41-fold (Figure 17B) when compared to their relative controls - 5 NT and 5 C. Following the same scheme, the transfected MSCs were plated onto soft (5 MI→5) or stiff (5 MI→100) substrates and maintained for additional two passages.
Meanwhile the negative control MSCs (5 NT and 5 C) were continuously passaged onto soft substrates and served to normalize miR-21 expression. Two weeks after substrate switching when the effects of mimics fully subsided (theoretically last for 72 hrs) and the expression levels were re-established according to respective substrates, the miR-21 level in soft (5 MI→ 5) MSCs was reduced to about the same level as the negative controls, as predicted. Unexpectedly,
MSCs exposed to higher mechanical tension (5 MI→100) also showed reduced miR-21 level comparable to that of the soft (5 MI→5) rather than elevated expression commonly observed on stiff substrates. In the case of stiff-primed MSCs, transfection with miR-21 inhibitors resulted in a down-regulation of approximately 5-fold in both sets of experiments prior to substrate switching, thereby restoring miR-21 expression back to baseline levels (Figure 17).

Figure 17  Effect of miR-21 overexpression and knockdown on mechanical memory.

Manipulation of miR-21 expression level prior to substrate switching partially disrupted the memory. The effects of miR-21 mimics and inhibitors were assessed near the end of P3 to confirm the success of overexpression (A: 27-fold; B: 41-fold) and knockdown (A and B: 5-fold). The transfected MSCs were subsequently re-plated onto soft or stiff substrates for two additional passages to investigate whether disruption of miR-21 expression affected mechanical memory on a transcriptional level. Although overexpression of miR-21 in soft-primed MSCs did not appear to disrupt memory, down-regulation in stiff-primed MSCs restored miR-21 expression to baseline level and allowed new expression levels to re-establish on different substrates. KD-MSCs switched to the soft substrates had lower miR-21 level than KD-MSCs on stiff substrates. Two biological replicates were performed, using MSCs transfected with non-targeting control (NT) or non-transfected MSCs (C) as control conditions to normalize miR-21 expression, respectively.
During the subsequent two passages when the expression levels were re-established and stabilized on corresponding new substrates, the down-regulated MSCs, having their memory erased, built up their miR-21 level again. As predicted, MSCs re-exposed to stiff substrates (100 KD→ 100) showed greater elevation in expression that nearly approached the negative controls (100 NT and 100 C) while MSCs switched to the soft substrates (100 KD→ 5) had only modest increase. These preliminary data further strengthen the argument that miR-21 is mechanically regulated. However, to fully understand the role of miR-21 in mediating MF memory, detailed examination of MF character upon manipulation with mimics/inhibitors is necessary. Notably, although similar data trends were obtained in separate experiments using NT saran transfected MSCs (Figure 17A) or non-transfected MSCs (Figure 17B) to normalize, a control study revealed that NT siRNA itself confounded the results of priming and disrupted MF memory (data not shown). Therefore, other transfection techniques and alternatives need be explored to circumvent this limitation.

Chapter V: Discussion

The clinical use of MSCs has been explored extensively in the context of repair and regeneration due to their therapeutic benefits including immunoregulatory functions, differentiation potentials, and trophic effects. However, many critical questions remain unanswered. The conditions leading to their malignant transformation, the short and long term interactions of implanted MSCs with the local microenvironment, the optimal culture systems that can preserve their therapeutic potentials are just a few among many, not to mention the current lack of standardization in cell harvesting and preparation. These challenges, if not addressed, will have tremendous impact on the clinical outcome of MSC-based therapy. This thesis study examined how the mechanical conditions of cell culture influence the fibrogenic behaviour of MSCs and my main findings are:

1) Primed MSCs maintain mechanically propagated MF character even after stress reduction. Soft-primed MSCs are protected from MF activation by subsequent stress elevation.

2) MiR-21 expression is mechanically regulated and potentially mediates the MF memory.

3) Soft polymer substrate offers many advantages as compared to TCP, including rapid colony formation/expansion, enhanced adhesion, and suppression of MF character.
V.1 MSCs Acquire a Mechanical Memory upon Priming

The major finding of my thesis study is that upon switching to their respective substrates, primed MSCs maintained their growth, phenotype, contractility, and synthetic activity (Figure 12, Figure 13). Soft-primed MSCs had overall higher proliferation rate, smaller size, lower level of α-SMA expression/α-SMA positive stress fibers, weaker contractility, and higher ED-A/collagen production and maintained these features regardless of increased stress.

Irrespective of the type of ECM surface coating, MSCs exhibited features of MFs on stiff substrates (Figure 9), suggesting that it is the mechanical property of the substrate that drives MF differentiation rather than features pertaining to particular ECM ligands. The growth and phenotypic changes of MSCs continuously cultured on their respective substrates or TCP were traced for a period of four passages. Interestingly, the difference in α-SMA expression only began to be established at P3 (Figure 10) whereas the difference in α-SMA positive stress fiber level can be detected much earlier (data not shown). This discrepancy can be explained by the fact that organization of actin is a rapid process in immediate response to mechanical stimulation. In brief, ECM strain activates ROCK, which directly regulates actin nucleation, elongation, and stress fiber stabilization in addition to activating LIM kinase and cofilin to affect actin organization (Holle and Engler, 2011). Alternatively, mechanosensitive ion channels allow the influx and oscillations of Ca^{2+} in response to stress and modulate the activities of actin binding proteins (Kobayashi and Sokabe, 2010). These short term responses affect the cellular localization of α-SMA rather than its synthesis, which takes considerable time and many more signaling events. In other words, the cytosolic pools of non-polymerized actin can be shifted rapidly to allow production of matching intracellular tension according to the substrate stiffness(Solon et al., 2007). This response is not necessarily reflected at the protein expression level.

In line with the idea of internal stiffness matching to exterior, α-SMA expression levels on soft substrates increased moderately at the beginning of priming but remained low onwards. This phenomenon exemplifies a dynamic and reciprocal process whereby MSCs sense their soft surrounding and attempt to adjust its cytoskeletal tension accordingly until that balance is soon reached. In contrast on stiff substrates, MSCs encounter much higher ECM strain and continue to tune their internal stiffness until reaching their physical limit to reinforce the cytoskeleton, evident by the gradual increase in α-SMA expression level. This limit is reflected
in the eventually equalized α-SMA expression levels between stiff substrate and TCP. Importantly, the preservation of α-SMA is not simply due to the delay in its synthesis as the status of α-SMA positive stress fiber organization supports the protein expression data. Although actin polymerization and reorganization are highly dynamic and rapid, soft-primed MSCs maintained their relatively smaller size and lower stress fiber density upon substrate switching. Stiff-primed MSCs were larger and contained more stress fibers regardless of stress reduction. This finding suggests the existence of mechanisms that stabilize the acting structure or actin binding protein activities in addition to affecting α-SMA on the transcriptional level.

In addition to α-SMA, the expression of other MF-related proteins was also preserved. The ED-A domain of FN has been implicated in fibrosis and MF differentiation (Serini et al., 1998). Insertion of the ED-A domain into the FN protein can cause conformational changes that expose binding sites such as the RGD motif (Manabe et al., 1997) to increase integrin binding and downstream signaling events. Increased production of ED-A FN is characteristic for MFs. My findings however contradict this notion as MSCs on stiff substrates produced less ED-A FN despite their well-developed MF phenotype. In addition, the production of collagen I on stiff substrate was also lower, which is counterintuitive as well. To explain this discrepancy with the literature, one possible theory is that MSCs on stiff substrates were much more spread and proliferated at a lower rate. This could potentially affect the deposition of ECM proteins, which may require a certain threshold density to kick start the cross-linking process. Even though the growth difference was accounted for by adjusting the seeding density, at any given time and assuming the same degree of confluence, the number of stiff MSCs per unit area was lower than the number of soft MSCs on respective substrates simply due to the difference in spreading area.

One may argue that the priming effect is due to permanent alterations to MSCs or a selective process whereby certain cell populations are favored on certain substrates. However, this does not appear to be the case because soft- and stiff-primed MSCs are equally capable of adipogenesis and osteogenesis after three passages on respective culture substrates (Figure 8). Hence, MSCs cultured in non-inductive normal media were not determined to any specific lineage on substrates of certain stiffness. Also supporting this finding, Chen and coworkers have produced micro-molded PDMS micropost arrays of different height to generate varying substrate rigidity (Fu et al., 2010). MSCs assume a well-spread morphology with prominent
stress fibers on short microposts (high effective stiffness) whereas MSCs on long microposts (low effective stiffness) display a round morphology. Despite the differences in cell shape, FA size, and cytoskeletal tension, MSCs did not express differentiation markers at any micropost rigidity when given basal growth medium alone, suggesting that stiffness is not sufficient to induce lineage specification. However, when cultured in medium containing both adipogenic and oestrogenic differentiation cues, round or spread MSCs are capable of differentiating into both lineages on respective substrates, albeit a strong shift in the balance - with soft microposts favoring adipogenesis and rigid microposts favoring osteogenesis. The authors explained this observation with very early cytoskeletal rearrangement pre-determining MSC fate because initial disruption of cytoskeletal tension decreases the osteogenic response when examined at a later time point (Fu et al., 2010).

In my study soft- and stiff-primed MSCs were incubated in exclusively adipogenic or osteogenic media following standard induction protocols on TCP (Pittenger et al., 1999; Polisetti et al., 2010) rather than on soft or stiff substrates. Under this controlled condition, no difference in the extent of differentiation was observed, although more quantitative molecular characterization is lacking. It is possible that on their respective substrates, soft- and stiff-primed MSCs can be induced to differentiate to varying degree. However, the endpoint of my study is not to direct MSCs towards certain lineage, but to generate MSCs that are protected from MF activation while unaltered in their multilineage differentiation potential.

The fact that my primed MSCs remained fate-undecided on different stiff substrates is in somewhat contradiction to a study by Engler and coworkers. In this seminal work, MSCs were shown to differentiate along neurogenic, myogenic, or osteogenic lineages when cultured on brain-soft, muscle-stiff, or bone-stiff polyacrylamide gels, respectively (Engler et al., 2006). However, the resulting progenies are not terminally differentiated; neurogenic MSCs on soft substrates are still capable of expressing myogenic and osteogenic markers when given corresponding soluble factors, although they do become less plastic after continuous culturing on soft substrates for three weeks (Engler et al., 2006). Although the study established a direct link between ECM elasticity and stem cell fate, an alternative interpretation of the findings could be that cell shape regulates lineage commitment. Elongated, branched MSCs engage a specific set of ligands to activate neurogenic programs further downstream. Spindle-shaped MSCs become myogenic and spread-out MSCs are osteogenic. In contrast, the soft- and stiff-
primed MSCs in my study did not show distinctive difference in cell shape. Although soft-grown MSCs were smaller in size, cells in both priming groups displayed rhomboid and well-spread morphology similar to MSCs on TCP. Shape-directed lineage commitment has been shown by using micropatterning technique suggesting that cell shape itself is a critical switch that controls lineage commitment (McBeath et al., 2004). As often observed, sparsely seeded MSCs appear better-spread and exhibit more prominent stress fibers compared to high density culture. MSCs of such morphology are prone to osteogenesis. By controlling the degree of cell spreading, round MSCs are found to preferentially undergo adipogenesis when cultured in bipotential differentiation medium. This process appears to be dependent on cytoskeletal tension as constitutively expressing active RhoA in round adipogenesis-prone MSCs induces osteogenic shift. Conversely, disrupting actin assembly with Cytochalasin D or treatment with ROCK inhibitor and myosin II inhibitor abrogates such shift, indicating that actin myosin generated intracellular tension is implicated in this cell shape-mediated lineage commitment (McBeath et al., 2004).

Although stiff-primed MSCs are likely higher in intracellular tension than soft-primed MSCs, tension did not lead to activation of differentiation programs in my study, at least not permanently. Another possible explanation for the contradictory findings to Engler et al. is that specific ECM ligand-receptor interactions influence cell fate decisions. It has been postulated that full-length ECM proteins can engage different types of integrins and drive competing intracellular signaling pathways that may ultimately lead to an undirected promiscuous outcome or a specific defined outcome (Martino et al., 2009). For example, ECM fragments with increased specificity for α5β3integrins significantly enhance MSC osteogenesis (Martino et al., 2009). Consistently, it is likely that the collagen used to coat polyacrylamide gels (Engler et al., 2006) initiates vastly different integrin binding events from the FN coating in my study, resulting in different net outcome. I excluded collagen from my study after initial tests with four different ECM coatings to avoid confounding phenotypic difference on soft versus stiff substrate (Figure S2). MSCs grown on soft, collagen coated silicone elastomers appeared elongated and had long extensions – not dissimilar to ‘Engler’s MSCs’.

Alternatively, the discrepancy can be attributed to the inherent physical properties of different types of polymer substrates employed in the two studies. It has been reported that human MSCs are equally capable of undergoing osteogenesis and to lesser degree adipogenesis
across a range of stiffness values on PDMS substrates. In contrast, MSCs seeded on soft polyacrylamide hydrogels are poorly spread and preferentially undergoes adipogenesis whereas stiff hydrogels favors osteogenesis (Trappmann et al., 2012). A detailed examination of hydrogel surface topography using scanning electron microscopy reveals significant variation in porosity as stiffness changes. More specifically, pore size varies inversely with stiffness, due to the differences in network crosslinks. And porosity in turn affects the distance between collagen anchoring points, leading to variation in adhesion strength. The mechanical feedback that MSCs sense then translates into biochemical signals that govern their fate decisions. In other words, the loosely bound ECM on soft hydrogels fails to provide enough mechanical strength to induce proper integrin clustering events and impairs the activation of crucial signaling pathways – MAPK in particular (Trappmann et al., 2012). As a result, the differentiation response on hydrogels is likely not due to substrate stiffness, but rather confounded by porosity difference. However, this effect is not observed with PDMS substrate used in my study.

Another indication that priming is not a selective process is the fact that soft-primed MSCs retained their ability to differentiate into MFs when exposed to a combination of pro-fibrotic stimuli (Figure 14). Prior work from our lab has demonstrated that latent TGF-β1 can be mechanically activated from ECM stores independent of proteolytic cleavage. This process is mediated by MF contraction and requires a mechanically strained ECM. More specifically and relevant to my current study, the release of active TGF-β1 is enhanced on fibrotic-stiff substrates (Wipff et al., 2007). Hence, it is possible that soft-primed MSCs, deficient in α-SMA positive stress fibers, lack the proper contractile machinery to generate sufficient force for the activation of latent TGF-β1. Moreover, a less resistant ECM on soft substrate suppresses effective TGF-β1 release. As a consequence, MF activation cannot be sustained by autocrine signaling on soft substrates and the fibrogenic cycle is interrupted. Even when soft-primed MSCs were transferred to stiff substrates, they still lack the contractility to mechanically activate TGF-β1 and therefore remain suppressed. However, soft-primed MSCs are not generally deficient in their ability to become MFs. When active TGF-β1 was supplemented together with elevated stress (TCP substrates), the priming brake was released to resume the MF cycle. It is unknown yet and of interest to investigate whether combined treatment with
TGF-β1 receptor blocker and stress reduction on soft substrates can lead to de-differentiation of the stiff-primed MSCs.

V.2 MiR-21 is Mechanically Responsive and May Regulate α-SMA Expression

Although the fundamental role of miRNAs in the development and progression of fibroproliferative diseases has been well established, research is lacking with regard to whether and how they respond to biomechanical cues. Except for one study that implicates miR-365 in chondrocyte proliferation and differentiation in response to cyclic loading, there is currently no indication that mechanical environment can alter the expression levels of miRNAs leading to fibrosis. The novel, second main finding of my study is that substrate stiffness alone influences the expression levels of miR-21, which may regulate the MSC-MF phenotype via direct or indirect mechanisms potentially involving the regulation of actin-organization, stability, and turnover, as well as actin synthesis.

Prior work investigating the role of miRNAs in pulmonary hypertension identified miR-21 as a key player through network-based prediction approach. MiR-21 is found to be upregulated in pulmonary tissue from rodent and human models of pulmonary hypertension, potentially due to severe hypoxia. Importantly on a molecular level, this study established that miR-21 directly repressed the expression of RhoB (Parikh et al., 2012). RhoB belongs to the same small GTPase family as the previously discussed RhoA. In fact, the three isoforms RhoA/B/C share 85% amino acid sequence homology despite their different roles in regulating cellular behaviour. RhoA is known to mediate actomyosin contractility whereas RhoB is implicated in cytokine trafficking (Wheeler and Ridley, 2004). Intriguingly, simultaneous knockdown of RhoA and RhoC does not completely suppress actin stress fiber formation. Rather, silencing of RhoA leads to the post-transcriptional stabilization and upregulation of RhoB without affecting RhoB mRNA levels. This phenomenon can be explained by the fact that the RhoGTPase binding partner RhoGDIα is rate-limiting and can only be made available to RhoB when RhoA is suppressed (Ho et al., 2008). The same regulatory mechanism may equally apply to the situation where RhoB is transcriptionally silenced – by an endogenous regulator miR-21 rather than an exogenously introduced siRNA. To put into context, miR-21 mediated degradation of RhoB may cause an upregulation in total and active RhoA. In a RhoA/ROCK dependent manner, stress fiber formation is enhanced and α-SMA transcription is activated upon nuclear
translocation of MRTF-A, resulting in a more pronounced MSC-MF phenotype. This possible explanation for the observed correlation between miR-21 expression and α-SMA expression/α-SMA positive stress fiber formation is testable in future experiments.

Although direct association of α-SMA expression with miR-21 levels has been reported, the effect was studied in the context of TGF-β1 signaling and attributed to the effects and regulations of signaling molecules Smad3 and Smad7 (Liu et al., 2010; Zhong et al., 2011). How miR-21 activates α-SMA transcription in a TGF-β1 independent manner is currently unknown. Another potential alternative theory to explain the link between miR-21 and α-SMA levels involves Krüppel-like factor-6 (KLF-6), which is among the predicted targets of miR-21. A splice variant of KLF-6 has been implicated in liver fibrosis by directly regulating the levels of collagen production (Ghiassi-Nejad et al., 2012). In addition, the progressive activation of hepatic stellate cells in culture signified by increased level of α-SMA has been correlated with decreased KLF-6 (Ghiassi-Nejad et al., 2012). Hence, KLF-6 is an attractive candidate for the missing link between miR-21 and α-SMA expression during MF activation. It is likely that targeted degradation/repression of KLF-6 by miR-21 causes the transcriptional upregulation of pro-fibrotic mRNAs such as α-SMA, resulting in MF activation.

The fact that mechanical priming affects multiple cellular processes ranging from proliferation to actin organization suggests the presence of some upstream regulator that oversees these activities. Because the target recognition sequence resided in the seed region of miRNAs is only about 7 or 8 nucleotide-long, a single miRNA can act on a vast array of downstream gene targets implicated in multiple signaling pathways (Chau and Brenner, 2011). Therefore, miRNA is an attractive candidate for mediating the mechanically propagated MF memory. Importantly, memory is penetrating to the transcriptional level as miR-21 expression is also preserved upon substrate switching (Figure 15). This observation may seem contradictory to the finding that miR-21 is mechanosensitive. One possible explanation is that sustained activation of a fibrotic program upon stiffness priming may trigger a feedback response to maintain miR-21 expression and override the mechanical stimulus. To support this hypothesis, I found that when miR-21 expression was restored to baseline level by interfering with inhibitors, the memory on a transcriptional level was erased and the feedback mechanism broke down. As a consequence, according to the new substrates, MSCs built up their respective expression level – lower on soft substrates and higher on stiff substrates (Figure 17).
V.3 Advantages of Harvesting MSCs on Soft Culture Substrate - Practical Implications

Irrespective of their source, primary MSCs have to be sub-cultured and expanded to obtain a sufficient cell number for therapeutic applications. Traditionally this procedure is performed on TCP (Pittenger et al., 1999). However, the supra-physiological stiffness of TCP could potentially induce permanent changes that program MSCs towards the MF phenotype. Several lines of evidence support the development of MSC-MF character. MSCs in vitro on TCP develop a well-spread morphology and contractile stress fibers that are characteristic of MFs (Hinz and Gabbiani, 2010). When cultured continuously on TCP or treated with tumor-conditioned media containing fibrogenic cues, MSCs are known to upregulate α-SMA expression and increase their contractility (Cai et al., 2001; Kinner et al., 2002). Similarly, MSC-to-MF transition occurs when MSCs are maintained on rigid culture substrates and/or treated with profibrotic cytokine TGF-β1 (Park et al., 2011). Furthermore, proteomic profiling studies investigating the global effects of TGF-β1 on MSCs revealed the activation of a MF differentiation program involving altered expression of cytoskeletal proteins, ECM-related proteins, membrane proteins, and metabolic enzymes (Wang et al., 2004a). Although the contractile and synthetic activities of MFs are considered beneficial in the early stage of wound repair, their persistence can lead to detrimental contractions, tissue structure disruptions, and functional impairment. Especially in the context of MSC therapy for severe burns, activation of MSC-MF phenotype may exacerbate the wound contractures and hypertrophic scarring instead of delivering the intended therapeutic benefits.

As opposed to TCP, substrates with physiologically relevant stiffness values offer several advantages. First of all, culture expansion on soft substrates generated MSCs that are protected from permanent MF activation while unaltered in their differentiation potential. In striking contrast, enriched MSCs that are sub-cultured on TCP for six passages have been shown to lead to calcification when injected into infarcted myocardium (Wen et al., 2012). Secondly, silicone elastomers, irrespective of stiffness, fostered better and faster initial attachment of stromal cells and more rapid colony formation/expansion as compared to TCP. The overall difference in colony size, number, and formation kinetics (Figure 7) could be explained by the substrates’ ability to preserve cohorts of hematopoietic cells, which are interspersed within the colony, feeding their mesenchymal neighbors with trophic factors and promoting faster colony formation. Other evidence supporting this role of hematopoietic cells came from observation
that following enrichment of MSCs and depletion of hematopoietic cells, the proliferation rate
difference gradually saturated.

An alternative explanation is that the inherent surface and mechanical properties of silicone
substrates enable better initial attachment for mesenchymal colony starter cells, thus allowing
signals or feedback mechanisms between neighboring mesenchymal cells to reinforce each
other and drive the rapid proliferation. In contrast, the few fibroblastic cells that attached to
TCP remained largely in isolation, resulting in slow growth and impaired clonogenic expansion.
This finding has important implications for MSC harvesting in preparation for implantation,
the goal of which is always to obtain cells in large quantities in a short amount of time. MSCs
in their native environment are likely supported and nourished by surrounding hematopoietic
cells. Maintaining this relationship in culture immediately following isolation may benefit the
MSCs at least temporarily, as was achieved by using polymer substrates. However, long-term
exposure to trophic factors likely induces phenotypic changes in MSCs that are not desired.
Overall, the suppressed MF character, high proliferation rate and rapid colony formation
collectively imply that soft silicone substrates are an ideal alternative to “standard” TCP
culture establishment.
V.4 Conclusion and Significance

MSC therapy has generated great excitement and promising results in wound repair and regeneration. Although a lot has been learned with respect to their intricate interplay with surrounding niche, many more questions emerged. In order for MSCs to maintain and deliver their therapeutic benefits, it is of critical importance to understand not only the effects of in vitro culture on cell fate decisions, but also the long term interactions with host microenvironment upon implantation.

Adding to the expanding body of literature that recognizes the role of physical environment on MSC behaviour, this study provides a molecular mechanism of how the mechanical environment acts as powerful stimulus to influence cellular and molecular processes associated with fibrosis. I was able to demonstrate that MSCs acquire a MF memory, which is potentially propagated through miR-21 – a mechanosensitive transcriptional regulator. Importantly using soft silicone elastomers, I was able to harvest and establish soft-primed MSCs that exhibit many advantages compared to traditional TCP-initiated cultures. The rapid clonogenic expansion, high proliferative potential, and protection from subsequent MF activation make these primed cells an idea source for therapy to properly repair and regenerate severe burn wounds instead of exacerbating harmful remodelling. In addition, MSCs have been manipulated using miRNA technology to control the expression of transcription factors that regulate self-renewal and differentiation. As an example using such technology, the expression of the tumor suppressor protein menin can be controlled to direct stem cells to either osteogenesis or myogenesis in animal models(Aziz et al., 2009). The finding that inhibiting miR-21 expression reduced α-SMA incorporation into stress fibers has significant implications for therapeutic applications. By either direct injecting into fibrotic scar or interfering in culture, miR-21 antisense oligonucleotides may have promising clinical future at attenuating detrimental MF persistence. These approaches will greatly enhance the success of wound healing and improve the quality of life of burn victims.

This study demonstrates the principle in tissue engineering and regeneration that by manipulating how regenerative cells perceive the physical environment, it is possible to direct their fate to our benefits and to attain desirable characteristics for therapeutic applications. In addition, the ability to produce durable alterations in gene expression would allow MSCs to maintain their desired qualities over the long time.
V.5 Future Perspectives

The functional role of miR-21 in mediating the MF memory needs to be validated by assessing if overexpression or downregulation can abrogate the mechanically propagated MF character. Moreover, it will be interesting to look into the effect of priming on KLF-6 expression and to test the hypothesis that miR-21 upregulates α-SMA expression by causing targeted degradation of KLF-6. With this information, we would come full circle to demonstrate that mechanically induced miRNA expression transcriptionally regulates MF activation and persistence. Furthermore, the pathways through which the mechanical microenvironment influences epigenetic modulations are currently unknown and evidence in this area is scarce. Arnsdorf et al. were the first to demonstrate that mechanical stimulation by oscillatory fluid flow alters DNA methylation status, ultimately resulting in osteogenic differentiation of MSCs (Arnsdorf et al., 2010). An alternative mechanism underlying MF memory could be mechanically regulated DNMT expression, which has been directly linked to the level of α-SMA expression (Hu et al., 2010; Mann et al., 2007). This possibility was examined during the course of my study and preliminary results (data not shown) indicate that DNMT expression was elevated on soft substrates. However, future studies are necessary to establish the link between mechanical environment, DNMT, and MSC-MF memory. Lastly, in the long run, the beneficial effects of soft-primed MSCs can be tested functionally in an in vivo rat model of skin wound healing to see if scarring can be reduced.
Chapter VI: Supplementary Figures

**Figure S 2. Unsorted MSCs on silicone substrates and TCP.**

MSCs obtained from P0 expansion were trypsinized and plated again onto respective substrates and TCP without magnetic sorting. Trypsinization successfully eliminated hematopoietic cells on TCP as the culture became much more homogeneous. In contrast, contaminating cells remained on soft and stiff substrates.

**Figure S 1. MSCs exhibited distinct morphology on collagen coated soft or stiff substrates.**

MSCs cultured on collagen-coated soft substrates were elongated and lacked contractile machinery. In addition, they had a tendency to cluster together to form dense islands. In contrast, MSCs on collagen-coated stiff substrates were well-spread and characterized by dense α-SMA positive stress fiber bundles. This phenotypic change is specific to collagen coated substrates and not observed on any other type of ECM coating.
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