The Regulation of Human Dermal Fibroblasts by Mast Cells *In Vitro*

by

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Abstract

Background: Dermal fibroproliferative disorders are forms of aberrant cutaneous wound healing, which can lead to the formation of hypertrophic scars (HTS). HTS develop after prolonged healing of deep dermal burns and are associated with excessive inflammation. HTS are characterized by exaggerated cell migration, increased fibroblast proliferation, and up-regulated secretion of cytokines and extracellular matrix proteins. Fibroblasts, especially from the deep layers, are involved during the remodeling process and the excessive biosynthesis of extracellular matrix proteins. Mast cells (MC) have been implicated in fibrotic diseases and HTS development as they are present in significantly greater numbers than in normal skin and appear to degranulate and release pro-inflammatory as well as pro-fibrotic mediators in response to injury. Thus, we hypothesized that activated MC regulate dermal fibroblasts and play a significant role in HTS development.

Methods: Fibroblasts were isolated from human HTS tissue and site-matched normal skin, and the superficial and deep layers of normal human skin. The fibroblasts were co-cultured with MC (LAD2) with or without stimulation with substance P. Fibroblast proliferation was quantified by cell counting. Collagen production was measured using the 4-hydroxyproline assay. Myofibroblast differentiation was assessed by flow cytometry. Matrix metalloproteinase-1 and 2, and transforming growth factor-beta 1 were quantified by Simple WES. Simple WES and ELISA measured decorin in the fibroblast lysate and cell culture medium. Fibroblast-populated collagen contraction was examined using a collagen lattice assay.

Results: Activated MC significantly increased cell proliferation and reduced collagen production in normal skin fibroblasts (NS Fb), as well as superficial and deep dermal fibroblasts (DF). Myofibroblast differentiation was reduced significantly in NS Fb co-cultured with MC.

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Activated MC significantly lowered decorin expression in the DF. Also, the levels of decorin in the medium of the NS Fb, and in the layered fibroblasts were significantly down-regulated after co-culturing with activated MC. Likewise, activated MC significantly decreased MMP-1 expression in the DF, and lowered MMP-2 in NS Fb. Activated MC significantly decreased DF-populated collagen gel contraction compared to the controls.

Conclusions: In this study, activated MC regulated dermal fibroblasts, and exerted an important modulation of DF *in vitro*. Our results suggest the relation of activated MC and DF may be one of the key components for HTS development.

Preface

In this research, collecting human skin tissues from donors were necessary to extract primary fibroblast cell lines. The human skin tissues were obtained from HTS and site-matched normal skin from burn patients as well as from healthy patients who had an abdominoplasty. The University approved the collection of human tissues of Alberta Research Ethics Board on September 17th, 2011 (protocol number Pro00023826).

This thesis is an original work of Josue Rodrigues Silva. I was responsible for designing and completing the experiments, collecting and analyzing the results, and writing the thesis. Dr. Jie Ding and Dr. Edward E. Tredget assisted with the design of the experiments and analysis of results. The committee members assisted with the thesis editing and modifications.

Dedication

I want to dedicate this work to my love, my partner and my best friend. Fernanda, I would not be able to do it without you.

Also, I would like to thank my children, Thomas and Clara, for making my life so colorful and bright.

I want to thank my parents, Fernanda and Manoel, for your unconditional love, support, and kind words of encouragement.

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Josue Rodrigues Silva

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List of Abbreviations

α-SMA	Alpha smooth muscle actin
CTGF	Connective tissue growth factor
DALY	Disability-adjusted life years
DMEM	Dulbecco's modified eagle's medium
DF	Deep fibroblasts
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Fb	Fibroblasts
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FcεRI	High-affinity IgE receptor
GAG	Glycosaminoglycan
HMC-1	Human mast cell 1
HTS	Hypertrophic scar(s)
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL	Interleukin
LAD2	Laboratory of allergic diseases 2
LAP	Latency Associated Peptide

Mast cells	MC
MC_{TC}/MC_T	Mast cell expressing tryptase-chymase / mast cell expressing tryptase
MMP	Matrix metalloproteinase
NK cells	Natural killer cells
NS	Normal skin
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
SCF	Stem cell factor
SF	Superficial fibroblasts
SP	Substance P
TBSA	Total body surface area
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
YLD	Years lived with disabilities

Chapter 1   Introduction

Burn incidence around the world is high. It carries a deep burden in the patient's life and the health system. According to the report from the World Health Organization in 2004, there are approximately 11 million of new cases of burn injuries every year worldwide, and a recent update in 2016 from the same organization estimates that 265,000 of those cases end in death [1, 2]. The most recent repository from the American Burn Association, analyzing the last 10 years, presented a small decline in the mortality rate, from 3.9 % to 3.0 % in males and 4.1 % to 2.9 % in females [3]. It was only with the implementation of burn centers and the improvement in the management and care of the burn patients that the decline of the death ratio was possible [4, 5]. Nevertheless, the analysis from the World Health Organization in 2004 showed burns as one of the leading causes of disability [1, 6].

Burn injury is one of the leading causes of hypertrophic scar (HTS) formation. Prevalence studies show that approximately 70% of the burn patients will develop HTS [7, 8]. As mortality from burn injury has improved, research deepens into understanding the mechanisms behind HTS development. This chapter will review the literature about the concepts, consequences, and treatments of burn injury. It will also highlight the main cellular and molecular components of HTS formation before discussing the project based on the hypothesis that mast cells (MC) can regulate dermal fibroblasts.

1.1 The skin structure

The skin tissue is part of the integumentary system, which is structurally formed by different types of cells, hair, glands, and others [9-11]. Immune cells complement the barrier defense. Additionally, the skin also takes part in temperature regulation, excretory, and sensory functions. The skin accounts for up to 15 % of the body mass. Anatomically it is divided into

three layers: epidermis, the most external part; the dermis, the portion located in the middle; and hypodermis, or subcutaneous tissue [9, 10, 12]. The epidermis is avascular, and the nutrients flow from the dermal capillaries. It is mainly composed by keratinocytes. These cells form the five layers of the epidermis, also called Stratum. In the deepest layer, new cells are formed, and as they move up, tonofilaments provide strength. Finally, the external layer is constituted by dead cells and keratin. The other cells in the epidermis are melanocytes, Langerhans cells, and Merkel cells [10, 12]. Melanocytes are responsible for producing melanin and giving the skin color. Interestingly, melanocytes are present in similar quantity in different races. The difference in skin color is due to the amount of melanin produced by these cells. Although the epidermis is constituted with more keratinocytes than melanocytes, melanocytes share the melanin produced with keratinocytes, forming a considerably homogeneous skin color [10]. Langerhans cells are the first sentinels of immune defense in the skin. Lastly, Merkel cells are part of the sensorial system involved in the touch sensation.

The dermis is divided into two regions: papillary and reticular [10]. The papillary region has capillaries. The reticular region is rich in collagen bundles and elastic fibers, forming a strong support for the skin. The main cells in the dermis are fibroblasts. They are responsible for organizing the extracellular matrix (ECM) and giving structure to the dermis. As part of the defense mechanisms, macrophages and MC can be found in this area.

Vessels, nerves, and adipocytes from the subcutaneous tissue. Finally, the appendages in the skin are the pilosebaceous units formed by the hair, sebaceous glands, arrector pili muscles, and the apocrine and eccrine sweat glands.

1.2 Wound healing and hypertrophic scar formation

The wound healing process is divided into four phases: hemostasis, inflammation, proliferation, and remodeling (**Figure 1.1**) [13-15]. In the normal wound healing process, the hemostasis phase, which lasts approximately 15 minutes, starts with vascular constriction induced by prostaglandins, circulating epinephrine, and thromboxane to stop the bleeding [16]. Concomitantly, clotting is induced by platelets and controlled by MC-derived tryptase-heparin complexes [16, 17]. Vascular permeability will be induced by the histamine and other cytokines released by MC marking the transition to the inflammation phase [13, 17]. At the beginning of the inflammation phase, growth factors, and cytokines act as chemoattractants for immune cells. Marked by redness, edema, and pain, the inflammation phase lasts around 5 days. It evolves with the presence of recruited immune cells such as MC, monocytes, T lymphocytes, and neutrophils in the wound site. These cells secrete a vast array of growth factors and cytokines. Consequently, the growth factors stimulate cell proliferation and fibroblast migration preparing for the next phase [13, 15]. The proliferation phase is noted for fibroblast proliferation and reepithelization with keratinocytes. Fibroblasts produce collagen to reform the ECM. Also, they differentiate into myofibroblasts, which act by helping wound closure. The proliferation phase lasts up to 6 weeks. Finally, enzymes such as matrix metalloproteinases (MMPs) and mechanisms such as the cell death control remodel the ECM by degrading collagen and promoting apoptosis, respectively.

During the abnormal scarring process, the HTS will be formed due to a dysregulated response in each phase [14, 18, 19]. The hemostasis phase starts right after the injury. High levels of fibronectin remain for an extended period [20, 21]. Fibronectin has been shown to spread the platelets in the wound, helping cell migration, and adhesion. The inflammation phase is prolonged, and it is characterized by increased expression of cytokines such as transforming

growth factor (TGF), platelet-derived growth factor (PDGF) and interleukins (IL, e.g., IL-4 and IL-10). Immune cells such as macrophages and MC, already present in skin, secrete several pro-inflammatory and pro-fibrotic cytokines [14, 22, 23]. Additionally, neutrophils and lymphocytes migrate to the wound site, increasing the inflammatory response. Burn injuries that affect deep layers of the skin have this inflammatory response accentuated, and consequently, they are more prone to develop HTS [18].

In HTS formation, the proliferation phase is marked by persistent overproduction of collagen, fibronectin, and glycosaminoglycan (GAG) [13, 24]. Skin fibroblasts and keratinocytes become highly active, and the proliferation is increased [18]. The changes in the interactions between Langerhans cells with keratinocytes during scar formation are linked to HTS formation [25]. IL-1α, produced by both types of cells, is found to be down-regulated in HTS. This imbalance is associated with abnormal ECM formation. Proteoglycan dysregulation is found in HTS. Decorin is decreased in the HTS, while versican is increased [26, 27]. Also, collagenase activity and production are affected. In the HTS, MMP-1 and MMP-9 are down-regulated compared to normal skin [13, 28]. However, MMP-2 expression in the HTS and blood of burn patients were increased compared to controls [29, 30].

The injury depth of wounds can determine the outcomes of wound healing [31]. Superficial wounds usually heal in a short period of two weeks, and no surgical intervention is required [31, 32]. Deep wounds are more prone to cause HTS and are associated with longer healing time. The HTS characteristically occurs when the burn injury is deeper than 0.5 mm inside the reticular dermis zone (**Figure 1.2**) [31, 33]. One possible reason for the development of HTS is the difference between superficial fibroblasts (SF) in the papillary dermis and deep fibroblasts (DF) in the reticular dermis [34]. Studies have shown that DF express more pro-

fibrotic characteristics such as lower levels of decorin and higher levels of TGF-β, connective tissue growth factor (CTGF), and collagen compared to SF [23, 31]. If the burn injury is deep enough to damage the papillary dermis, the DF are the ones in the wound site. Consequently, it is possible that they are the ones responsible for rearranging the wound.

1.3 Burn disabilities

Thousands of patients develop disabilities every year due to burn injury. According to the Institute for Health Metrics and Evaluation, the onus of burn disabilities has not changed much in the last 20 years; this includes environmental, behavioral and occupational risks (not specified in details) [35]. The years lived with disability (YLDs) is the measurement of the prevalence multiplied by the time lost with that condition [36, 37]. The burden of the disabilities is calculated by the sum of YLDs and the years of life lost due to premature death by that disorder; the unit is called disability-adjusted life years (DALYs). From 1995 to 2015, the YLDs associated with burns went from 13.67 to 8.92 YLDs per 100,000 patients [35]. In the same period, the burn DALYs has reduced from 29.55 to 16.52 per 100,000 patients. Although those numbers are an important representation of the burn injuries, the real numbers may actually be higher. A considerable proportion of the cases happen in low- and middle-income countries such as Angola and India, two of the countries with the highest numbers of DALYs and YLDs associated with burn injuries, where the health system is not well developed, and the health information system is not adequately established [35, 38-42].

1.3.1 Physical and psychological disabilities

The physical and psychological trauma caused by burn injuries have a lasting impact on the patient's quality of life [43]. By evaluating the psychological and somatic symptoms behind the evolution of the scar, Bock et al. in 2006 and Mazharinia et al. in 2007 concluded there are

severe consequences in the life quality of these patients [44, 45]. As part of these studies, different points were assessed to show how detrimental the consequences of burns can be. They found important repercussions in many aspects: pain or pruritus; emotional fragility, reduced daily activities, difficulty with interpersonal relationships, lack of focus at work or school, poor self-esteem, and other psychological impairments.

In many cases, patients suffer from psychological trauma [46]. Patients, especially children, have difficulties in returning to their normal life. Scars and deformities in the skin can affect their self-esteem and confidence. Moreover, during the healing process, the long treatments, the pain, and their appearance influence them emotionally. According to Zeitlin, in a follow up in children that suffered burn injuries, about 20 % of these patients remembered the incident, and up to 25 % had memories from the hospitalization [47]. These results were mainly attributed to the age of the child, especially those over 3 years old. In the same study, Zetlin showed that more than 20 % of the patients that suffered burn during childhood developed a fear of fire or hot water. Also, more than 40 % of these patients became cautious and hypervigilant, which persisted as they grew up. During adolescence, around 20 % of the patients had difficulty in dealing with the opposite sex. Also, the remaining scars impact their self-confidence. In adults, a review by Sheffield et al. found that only around 10 % of the patients received psychiatric support [43]. The need for psychotherapy was highly associated with the long-term treatment of the wound.

Consequences can also be seen in the physical trauma from burn injuries. Burn scars range from small cosmetic problems to important contractures [27]. Superficial lesions in the skin can cause discoloration, pain, tenderness, pruritus, and changes in skin texture [18]. In deeper lesions, starting from the superficial partial-thickness (second-degree), the scars can

drastically compromise skin structure. As a consequence of the damage, the patient may suffer from significant pain in the early recovery period. Moreover, an erythematous, raised, and pruritic scar can develop (**Figure 1.3 A**) [26]. Additionally, it disfigures the appearance of the patient, is associated with burn alopecia in the damaged area and promotes changes in the skin like stiffness and rough texture.

In some instances, wound contraction is increased during the healing process. It can cause other negative outcomes such as joint contractures [48-50]. The scar is bulky and has an inelastic texture. If the scar occurs in joints (especially extremities) and face, it will limit movements and cause anatomical deformities (**Figure 1.3 B**) [51]. Structures like eyelids can be constricted becoming narrowed and immobilized. When considering the relevance of the area affected by burns, hands and arms are the body parts that are most exposed to the risk of this injury, accounting for 45 % of the cases [31]. From new adventurers, such as children exploring the home environment, to adults with their occupational and behavioral injuries, hands and arms hold almost half of the functional impairments. This kind of injury carries such importance that the American Burn Association considers mandatory referral of these cases to a burn center.

1.3.2 Burn assessment

Over the years and with the evolution of knowledge about the scarring process, scar scales were created to help with earlier diagnosis [52]. Since the treatment applied depends on the classification of the wound, it was imperative to create reliable and fast tools to be used. The depth classification, Wallace's rule of 9, the Lund and Browder's chart, and the total body surface area (TBSA) are only used for quick assessments [53]. The depth classification divides the burn injury into three degrees: 1) first-degree burn or epithelial burn is the most superficial lesion; 2) second-degree burn involves up to the dermis. It can be subdivided in superficial

second-degree burn affecting the papillary dermis, and deep second-degree burn involving up to the reticular dermis; 3) third-degree burn is considered a full thickness burn. The Wallace's rule of 9 applies the area of burn damage in percentage in the body, and it is used mainly in adults. The Lund and Browder's chart also expresses the area of burn injury in percentage and can be used to define the injury in children. Both methods provide the TBSA. They classify and determine the severity of the burn, and help define the initial treatment. Less than 10 % and 15 % of TBSA are considered minor burns in children and adults, respectively. Major burns are up to 30 % and 35 %. Finally, critical burns are anything more than 30 % in children and 35 % in adults. From Garcia-Velasco in 1978 to Singer in 2007, scar scales evolved and went through adaptations [54]. Currently, the Patient and Observer Scar Assessment and the modified Vancouver Scar Scale are the ones mostly used [54, 55]. Assessment for scar diagnosis can be divided mainly into four groups: color, including vascularization and pigmentation; metric values such as volume, height, and extent; biomechanical properties (i.e., elasticity and stiffness); and hydration. Color evaluation can be very subjective and therefore, requires the support of different techniques such as laser, colorimetry, and computer analysis of photographs. Measures of scar volume require the application of ultrasound. These measurements are hard to acquire because accessibility to this equipment is difficult since it is expensive and time-consuming to use. The clinical assessment of depth of the burn and the risk of developing severe scars are based on the surgeon's experience and has an accuracy of around 70 % [32, 56]. Thus, different methods and tools should be used in combination to improve the accuracy of the diagnosis of burn depth and scarring potential.

1.4 Burn rehabilitation, scar prevention, and treatments

Even with advances in the care and rehabilitation of burn patients, the functional and aesthetic consequences remain a significant problem [19]. Treatments are often time-consuming and uncomfortable for patients [57]. Severe injuries might require many sessions of surgery. Surgeries in the early stages of the recovery aim to remove damaged and necrotic tissue. Once the healing process is over, surgery will be used to repair some areas by removing the formed scars. Also, it will be used to restore motor functions due to contractures. However, surgical interventions do not always evolve with good results. Furthermore, they are also associated with recurrence [18, 57]. Other procedures have also been used to improve the outcomes of HTS such as corticosteroid injection, compression therapy, splitting, serial casting and laser therapy [31, 57, 58]. Each of these procedures will act in specific aspects of the HTS. Corticosteroids inhibit fibroblast proliferation and can reduce inflammation. Compression therapy accelerates scar maturation. Splinting and serial casting have their importance in preventing contractures. Lasers reduce scar size and erythema. However, these treatment modalities are time-consuming, often uncomfortable.

Ideally, the patients are treated carefully to avoid complications. The treatments include prophylactic, current, and emerging therapies [13]. Pressure therapy is indicated as prophylaxis of HTS in a burn injury. It should be 23-24 hours per day for over 6 months [13, 19, 31]. Another treatment used to prevent HTS formation is silicone gel sheeting. It is applied 2 weeks after the wound healing started, and the treatment will last for 2 months. Flavonoid gels and creams have been used for HTS, but their results are not well established [13]. In the current therapies, surgical revision is the traditional treatment and can include methods such as linear excision or grafting. Corticosteroids are considered a second-line treatment for HTS in early stages when

other therapies have not been successful. The treatment regime is an intralesional injection in the papillary dermis per month over 3 months, and it can be extended to several months as needed. However, extended use of corticosteroid therapy can cause skin atrophy and telangiectasia [13, 59]. Limited for small scars, cryotherapy is done with liquid nitrogen spray, and it may cause blistering and local pain [13, 19]. Laser therapy has shown to be efficient in young HTS [13, 51]. They are classified in nonablative selective (e.g., pulse-dye laser), and ablative nonselective (e.g., CO_2 laser). They can be applied 2 to 6 sessions every 2 to 6 weeks. Finally, the emerging therapies there are the interferon as a promising therapy, and 5-Fluorouracil injections with good results for inflamed HTS.

1.5 Cellular basis of HTS formation

<u>**1.5.1**</u> <u>Fibroblasts and myofibroblasts</u>

Fibroblasts are present in different organs with specific attributes. Dermal fibroblasts are very important for skin physiology [60]. They are the most abundant cells in the dermis. In the skin, they have two subpopulations with considerably different characteristics. One population, SF, is located in the papillary region of the dermis, and the second one, DF, resides in the reticular region [34, 60]. Fibroblasts, the most common dermal cells that synthesize collagen, are involved in the biosynthesis of ECM. During reepithelization, fibroblasts produce fibroblast growth factor 7 and 10 (FGF-7 and 10), IL-6 and insulin-like growth factor 1 (IGF-1) [18]. The ECM deposition is controlled by CTGF, TGF-β and IGF-1 [18, 31]. While neovascularization can be influenced by vascular endothelial growth factor (VEGF) and CTGF, ECM remodeling is guided by TGF-β and PDGF. Alteration in the wound physiology will lead to an imbalance in proteoglycan production by fibroblasts [18]. In HTS, decorin was found reduced to 75 %, contrasting with up-regulation of versican [61]. Also, DF produce less decorin and more

versican, which indicates the involvement of fibroblasts from reticular dermis in the HTS formation [62].

HTS is characterized by having more myofibroblasts than normal skin [63]. The fibroblasts undergo differentiation into proto-myofibroblasts, and later, into myofibroblasts [64, 65]. These specialized cells express alpha-smooth muscle actin (α-SMA) and contribute to wound contraction. TGF-β1 induces α-SMA production in association with fibronectin, which helps explain the increased number of myofibroblasts in the HTS since both are up-regulated there [64, 66, 67]. More recently, versican, another up-regulated molecule in the HTS, has been suggested to help in fibroblast differentiation [68]. The mechanism appeared to involve TGF stimulation because the SMAD pathway was activated. At the end of the wound repair, if the tissue does not require the contractile, then myofibroblasts are degraded by apoptosis [63]. In HTS, where the degradation of myofibroblasts does not occur properly, some treatments have been proposed. In 2001, patients received treatment with interferon-alpha-2b (IFN-α2b) [57]. Results suggested the treatment reduced the number of myofibroblasts by inducing apoptosis. The contractile force of myofibroblasts is well known, and it is an important mechanism for wound healing progress. However, in HTS, this function is exaggerated. Thus, the cytoskeletal elements of the cells will continue to be targeted for research and development of possible treatments [65].

1.5.2 Keratinocytes

Keratinocytes are the cells that compose the first barrier of the skin and create the epidermis. Keratinocytes work together with fibroblasts to form the basement membrane of the epidermis. [60]. Both secrete components, while keratinocytes arrange them. Keratinocytes are responsible for producing collagen type IV and VII, laminins, perlecan, TGF, ILs, and more.

Cooperation with fibroblasts is vital for skin physiology. Co-culture of keratinocytes and fibroblasts has shown to increase collagen IV production by both, and TGF-β2 expression by fibroblasts [69, 70]. Communication of these two cells correlates with the amount of keratinocyte growth factor 1 (KGF-1) [71]. Consequently, the up-regulation of KGF-1 can cause hyperproliferative epidermis. The absence of keratinocytes after the burn injury will cause an imbalance in the skin homeostasis due to late epithelialization and lack of communication with fibroblasts [25, 34, 72].

1.5.3 Mast Cells

MC are intricate immune cells which have a complex and vast quantity of mediators and many possible pathways of activity [18, 73]. It is conceivable that MC orchestrate fibrosis by acting indirectly with their chemotaxins and recruiting other cells to act (**Figure 1.4**) [17]. Previous research has found a higher density of MC in the human HTS samples compared to human normal skin samples [74, 75]. These results suggested a relationship between MC and HTS development.

MC have been implicated in fibrotic diseases in different tissues. For example, IL-13 secreted by MC was associated with lung contraction and fibrosis [76]. Also, tryptase, a protease produced by MC, was isolated from lungs and applied on dermal fibroblasts *in vitro*, and stimulated fibroblast proliferation and collagen type I production [77].

MC are rich in granules and vesicles filled with a diversity of substances. The trafficking of these mediators is controlled by exocytosis and can be triggered by many different ways [73]. The exocytosis of different molecules depends on the type of stimuli. Also, mediator secretion can happen with or without degranulation. For example, neuropeptides such as substance P (SP) can stimulate the secretion of cytokines and chemokines through degranulation by the activation

of the natural killer receptors [78]. By contrast, lipopolysaccharides (LPS) can stimulate secretion of cytokines and chemokines without degranulation by activating the toll-like receptor 4 (TLR4) [79]. The granules and vesicles contain amines, proteoglycans, proteases, enzymes, and cytokines. Histamine, TGF-β, tumor necrosis factor-alpha (TNF-α), IL-4, IL-13, and tryptase have been associated with HTS formation [18].

The gap junction is an essential connection for communication between MC and fibroblasts. Gap junctions of mouse fibroblasts and MC increased collagen lattice contraction [80]. After determining the gap junction with coupling index through staining with Lucifer yellow and rhodamine (Rh)-dextran, a control group was treated with uncoupler trifluoromethyl ketone to inhibit the gap junction. Results showed that inhibition of gap junctions between MC and fibroblast reduced collagen contraction compared to the experimental group that had gap junctions maintained. In another experiment, rat MC and human fibroblasts increased the pro-fibrotic activity *in vitro* reaffirming the gap junction relevance [81]. MC were pre-treated with calcein and co-cultured with fibroblasts. The communication between both cells was confirmed by the passage of calcein from MC to fibroblasts. An experimental group was treated with an inhibitor of the fatty acid amide to inhibit the gap junctions. In this study, fibroblasts co-cultured with MC increased collagen production and myofibroblast differentiation compared to co-cultured fibroblasts with MC that had the gap junctions blocked.

Human MC can be classified into MC_T and MC_{TC} according to the proteases they express (tryptase and chymase). Previous studies have shown that MC may differentiate into MC_T or MC_{TC} depending on the stimuli [73, 82]. The stimuli can originate from infection, inflammation, or cell migration through the tissue.

1.5.4 Neutrophils

The neutrophils are granulocytes derived from myeloid stem cells. They infiltrate the wound at the beginning of the inflammatory phase [28]. Right after the hemostasis, neutrophils are attracted to the wound by the products of fibrinolysis [13, 18, 61]. These cells, once in the wound, secrete proinflammatory cytokines (IL-1α, IL-1β, IL-6, TNF-α), the collagenase MMP-8, and the gelatinase MMP-9 [18, 28, 83]. Therefore, neutrophils also influence the remodeling phase of wound healing. The neutrophils clean the wound of bacteria and detritus by phagocytosis [84]. Another previous finding was that after severe burns, neutrophils expressed elevated heat shock proteins which increased oxidative stress and down-regulated apoptosis, therefore leading to the prolonged presence of these cells in the wound [85, 86].

1.5.5 Monocytes

Secreted by macrophages and fibroblasts, the monocyte chemotactic protein-1 is an important chemokine that recruits monocytes to the wound site [50]. Other chemoattractants can also contribute to bringing monocytes from the bone marrow to the wound site. Monocytes respond to TGF-β1 and activated stromal cell-derived factor 1/ C-X-C chemokine receptor type 4 (SDF-1/CXCR4) pathway [31]. Once in the skin, monocytes differentiate into macrophages [87]. Recently, the SDF-1/CXCR4 pathway has been the focus of research. SDF-1 was up-regulated in burn patients' serum and HTS, which would continuously recruit CD14+ CXCR4+ cells and prolong the inflammation [50, 51]. A CXCR4 antagonist was tested to reduce monocytes trafficking into the wound in the human HTS-like nude mouse model, and as a consequence, the HTS characteristics were attenuated [88].

1.5.6 Macrophages

Macrophages play many roles in wound repair with their wide range of cytokines and phagocytic capacity [87]. Macrophages act from the inflammation phase. They stimulate revascularization, influence ECM organization, and skin reepithelialization [15, 18]. Macrophage fosters the transition from the inflammation phase to the proliferation phase by stimulating fibroblasts and keratinocytes [31]. During this transition between phases, they produce cytokines that control cell adhesion and migration. They release a considerable amount of cytokines: IL-1α, IL-1β, IL-6, TNF-α, TGF-β, FGF2, IGF-1, IL-1, FGF10, PDGF, and VEGF. Macrophages have been directly associated with HTS formation by overstimulating fibroblasts [89]. Macrophages increased secretion of TGF-β, FGF2, and IGF-1, which induced excessive collagen production by fibroblasts [18, 25]. The immune cell communication causes an interesting complementary loop of stimuli and activation. Macrophages and dendritic cells stimulate T lymphocytes to differentiate into T-helper 1, 2, 3 17, and T regulatory cells [50]. Then, the differentiated T lymphocytes activate macrophages into two subpopulations [61, 87, 89]. The first subpopulation called classically activated macrophages (M1) can be activated by T-helper 1 cell products, and they seem to act as an anti-fibrotic agent in the early phases of the wound healing. The second population is called alternatively activated (M2). They are activated by T-helper 2 cell products and act as a pro-fibrotic promoter [87]. Considerably more research is underway regarding macrophages, especially the fluctuation of subtypes during the wound healing process.

1.5.7 Lymphocytes

Lymphocytes are highly concentrated in HTS compared to the level found in the normal skin [18, 90]. T-lymphocytes are attracted to the wound by IL-15, and chemokines CXCL8 and CCL2. Also, they respond to SDF-1 chemoattractant stimuli [51, 91]. Once in the wound, lymphocytes are activated and release interleukins (e.g., IL-4, IL-5, IL-6, IL-10, IL-13, and IL-

31) that impact the inflammation and ECM deposition. During an inflammatory response, T helper-1 cells can reduce fibrogenesis, while T helper-2 cells are associated with increased pro-fibrotic cytokines capable of stimulating fibroblasts to produce collagen and fibronectin [13, 61]. Additionally, T helper-1 cells produce IFN-γ that inhibit T helper-2 cells in producing IL-4 (which is a potent pro-fibrotic cytokine) and stimulate macrophages to produce IL-12, an anti-fibrotic cytokine [13, 18, 31]. Similarly to other cells involved in the wound healing, T-cells are also a source of TGF-β1 [50, 62, 91].

1.6 Molecular basis of HTS formation

1.6.1 Growth factors

TGF-α is a protein from the epidermal growth factor (EGF) family [18, 92]. Macrophages and keratinocytes produce it in the wound area. Once TGF-α binds to the EGF receptor, it initiates cell proliferation in the wound healing process [93-95].

TGF-β is a cytokine from the transforming growth factor superfamily [96]. Three isoforms have been well documented: TGF-β1, TGF-β2, and TGF-β3 [97]. It is produced by different cells such as macrophages, MC, platelets, fibrocytes, and fibroblasts. With a vast range of action, TGF-β controls cell proliferation, differentiation, homeostasis, cell cycle, and more [13, 84]. On the other hand, a malfunction could also cause abnormal wound healing process such as HTS [96]. Previous experiments showed higher expression of TGF-β1 in HTS [50, 87]. In a sequence of stressors over the burn injury, followed by the inflammatory process and wound healing, the constant production and activation of TGF-β1 is suggested to differentiate fibroblasts into myofibroblasts [98]. It contributes to HTS formation by producing extra collagen and increasing contraction [85, 98]. M2 macrophages also produce large amounts of TGF-β1 that is associated with HTS development [89]. Additionally, researchers have found a higher

expression of TGF-β1 produced by DF compared to SF [62, 85]. Because of the broad capabilities of TGF-β, it has been targeted for treatment in different fields, including in HTS formation [61, 84]. In the skin, TGF-β3 is the only cytokine from the family that has been suggested to have anti-scarring function [18, 50, 51]. Treatments with intradermal injection of TGF-β3 improved the macro characteristics of the scars [84]. Although TGF-β3 (commercially called Avotermin or Juvista) did well through the clinical trial phases I and II, it failed in advanced clinical trials and did not reach the clinical market [99-101].

PDGF is a disulfide-bonded dimeric glycoprotein that belongs to the group of growth factors, and it has five isoforms (AA, AB, BB, CC, and DD) [51, 102, 103]. It has been correlated with the pathophysiology of HTS [25]. It was first discovered in platelets, but later also found produced by macrophages, MC, keratinocytes, fibrocytes, and fibroblasts [18, 104]. PDGF affects the inflammation phase, neovascularization, and ECM deposition. Along with TGF-β1, PDGF has been shown to help myofibroblast differentiation *in vitro* [91]. The first three isoforms have been implicated in that process, together with stimulating collagen formation and cell adhesion in fibrotic diseases such as lung fibrosis [19, 105, 106].

Secreted by platelets, macrophages, MC, and fibroblasts, EGF is a small molecule with the function best represented by reepithelialization [107]. It helps to recruit cells, especially keratinocytes and fibroblasts from surrounding the wound, to accelerate wound closure [13, 107]. Another role of EGF is its capacity to control the production of MMPs by fibroblasts [108-110]. Previously, tests involving SP and EGF increased proliferation of fibroblasts *in vitro* [111].

VEGF including six different growth factors (A, B, C, D, E and placenta growth factor), is produced by MC, platelets, neutrophils, macrophages, keratinocytes, and fibroblasts, and stimulates neovascularization [18, 112]. Similarly to other growth factors, VEGF also works as a

chemoattractant for pericytes and smooth muscle cells that help neovascularization [107]. MC can secrete VEGF by selective release without degranulation, and it acts mainly as a vasodilator [113]. VEGF is one of many cytokines and growth factors capable of transcriptionally activating MMPs [83]. Interestingly, VEGF was found in higher concentration in SF compared to DF in culture [34].

IGF-1 works together with growth hormone to modulate insulin [114]. It is secreted by platelets, T-cells, fibroblasts, and macrophages, and it can stimulate cell recruitment into the wound [13, 15, 107]. IGF-1 has been shown to increase keratinocyte mobility *in vitro*. Also, it stimulates fibroblasts and myofibroblasts to produce collagen [14, 22, 115, 116]. IGF-1 working synergistically with EGF increases keratinocyte proliferation [22]. Along with TGF-β1, the expression of IGF-1 was found higher in the HTS compared with site-matched normal skin [31, 117]. It was proposed that since sebaceous and sweat glands store IGF-1, once the burn injury damages these structures, IGF-1 will be released in the wound and stimulate pro-fibrotic characteristics on fibroblasts.

CTGF is produced by fibroblasts and endothelial cells. Together with TGF-β1, CTGF increases collagen production, myofibroblast differentiation, and cell proliferation [18, 19, 31, 118]. This molecule is present in fibrotic diseases such as scleroderma, and it has been associated with HTS development [22, 119]. This idea of CTGF as a factor for HTS formation was supported when researchers found that CTGF was increased in the HTS fibroblasts compared with normal fibroblasts [120]. Later, they showed that normal fibroblasts stimulated with TGF-β1 increased CTGF expression, which is similar to what is found in HTS formation. Another inference is about the location of the fibroblasts in the skin and their differences. DF expressed more CTGF than SF from normal human skin [51, 62]. Also, CTGF has been implicated as a

regulator of MMPs and tissue inhibitors of metalloproteinases (TIMPs), and it indirectly works on angiogenesis by stimulating VEGF production [121].

FGF is a big family of 22 members produced by many cells such as fibroblasts, neutrophils, and macrophages [14, 22]. All FGF are associated with cell proliferation from different tissues except FGF-7, which seems to be exclusive to epithelial cells [122]. FGF-1 and FGF-2 are associated with angiogenesis, and FGF-2 is highly present in the wound healing process [22]. In the proliferation of fibroblasts, FGF-1 facilitates the cell cycle to enter the G_1 phase and enhance mitosis [14]. Like many other growth factors, FGFs have been targeted for treatment or tested as treatment. Basic FGF (bFGF) was used to increase neovascularization in a model for skin, although it showed an enhanced trend, no significant improvement was identified in this study [123].

1.6.2 Proteoglycans

Proteoglycans such as decorin and versican have been widely studied and analyzed in wound healing. Decorin, the main proteoglycan in the skin, regulates TGF-β1 and interacts with collagen, playing an essential role in fibrillogenesis and cell proliferation. It is produced and secreted by fibroblasts and epithelial cells and has been associated with HTS when it is down-regulated [49, 97, 124, 125]. Initially, it was demonstrated that HTS tissue expressed less decorin than site-matched normal skin from burn patients [26, 27]. It was found to be suppressed in HTS for approximately 1 year [49]. Decorin organizes the ECM by attaching to the collagen type 1 C-terminus (the name was given due to how this protein "decorates" collagen) [126-128]. It also binds to other types of collagen, but most importantly, it binds to TGF-β1 and helps control its activity [13]. In a study to quantify decorin in the skin from two different age groups, young patients (20-30 years old) expressed more decorin than aged patients (>80 years old) and the

GAG size of the decorin in the second group was smaller [129]. Perhaps this could be one of the causes of poor healing in older patients. To improve the expression of decorin in the tissue and avoid HTS formation, researchers treated mice with calcium channel blocker (verapamil), interferon and corticosteroid (kenacort) in a nude mouse model with human skin graft [130]. Decorin gene expression was significantly increased compared to the untreated group.

Versican, in contrast with decorin, is highly present in HTS [27]. Versican is a strong hydrophilic molecule. It was implicated in disarranging the fibrils of ECM contributing to HTS formation. [61, 68, 131]. Versican also stimulates fibroblast differentiation into myofibroblasts. This molecule was initially described as a facilitator of cell adhesion, but it was versatile in its capabilities, and hence, the name versican was suggested [132]. Versican can be found in four different isoforms (V1, V2, V3, and V4), three with GAG and V3 without it. Versican interacts with cell-surface proteins such as CD44, EGF receptor, and integrins, and it also connects to ECM proteins such as fibronectin, fibrillin, and hyaluronan [133]. Overexpression of V1 stimulates fibroblast differentiation in culture [68]. In a comparison between normal skin and HTS tissue, versican IHC staining was strongly present in the HTS while it was found in low expression in the normal tissue [14, 27]. Versican was up-regulated in DF compared to SF, suggesting DF is likely responsible for HTS formation [34, 60, 62].

1.6.3 Histamine

Mainly produced by MC and basophils, histamine is famous for its roles in allergic responses and vasodilatation [84, 134, 135]. In HTS, it is possibly the most important cause of the redness and the itching symptom [19]. In heart remodeling diseases, histamine was highly present, especially associated with edema. Histamine is present in the plasma, and perhaps in the wound site, is increased right after the burn injury and peaks around 1 hour after the injury [136].

This is likely correlated with the necessity of vasodilation to help cell migration into the wound site. Another function of histamine is to stimulate the production of growth factors by fibroblasts such as FGF-2 [137]. Histamine stimulates wound closure (scratch model) by keratinocyte migration and reduces apoptosis *in vitro* [138]. In a wound healing study, histamine has shown to influence fibroblast proliferation and cell migration *in vitro* [139]. Dermal fibroblasts stimulated with histamine *in vitro* changed toll-like receptors (TLRs) expression [140]. While TLR4, 5 and 10 were up-regulated, histamine decreased the expression of TLR 2, 3, 6, 7, 8 and 9. This information crosslinks with other research where TLRs are associated with HTS formation, especially the inflammatory response induced by TLR4 [112].

1.6.4 Proteases

Tryptase and chymase are proteases produced by MC. Much work has been done to understand these two enzymes and their relation with fibroblasts in fibrosis development. Both have been shown to activate TGF-β1 [141]. Tryptase was said to be responsible for helping wound repair, acting as a chemoattractant for immune cells (especially macrophages), being a proinflammatory molecule, and stimulating fibroblast mitogenesis and collagen production [142]. Dermal fibroblast proliferation was increased after dose-dependent addition of tryptase in culture [143]. Moreover, tryptase from activated human MC increased α-SMA expression in fibroblasts and increased collagen lattice contraction [144]. Also, human mast cell-1 (HMC-1) sonicates or isolated tryptase increased fibroblast proliferation and up-regulated type 1 collagen synthesis of dermal fibroblasts and procollagen type 1 c-peptide in the supernatant [77, 145]. Compared to normal skin tissue, tryptase was found at a constant level, while chymase was found significantly reduced in new and old scars [146].

Chymase also has been related to inflammatory stimuli, fibroblast proliferation, vascular permeability (by breaking epithelial cells junctional complexes) and fibrosis [113, 147, 148]. In a guinea pig model that received human chymase, increased eosinophils and neutrophils in the skin was observed [149]. Another experiment using a chemotaxis chamber technique, chymase was responsible for promoting chemotactic activity in monocytes and neutrophils [150]. Human intestinal smooth muscle cells were cultured with purified dermal chymase, and procollagen was cleaved to a fibril-form collagen product [151]. In HTS formation, chymase continues to be investigated.

1.6.5 Matrix metalloproteinases (MMPs)

MMPs are a big family of 25 enzymes present in the ECM [13]. Growth factors and cytokines are responsible for stimulating MMP production by fibroblasts and keratinocytes [18]. Directly associated with TIMPs, an imbalance between these two types of proteins can lead to scars [19, 50]. Collagenases and gelatinases are MMP subtypes. They are responsible for the organization and degradation of ECM [28, 83, 152]. MMP-1 is a collagenase enzyme that is down-regulated in HTS [153]. Its main activity is to cleave collagen type III, and secondarily it can digest gelatin and other collagen types (VII, VIII, and X) [28]. To complement the ECM structure regulation, MMP-2 and MMP-9 cleave collagen types IV, V, VII, and X. The gelatinase MMP-2, in a study done with quantitative zymography, was found up-regulated in HTS compared to normal skin [29]. Also, MMP-2 can cleave the latency-associated protein (LAP) portion of TGF-β1 and activate it [154]. MMP-9 is another gelatinase that is down-regulated in HTS [29]. Interestingly, mice are known to not develop scars. Scientists have shown that MMP-9 is up-regulated during the wound healing period in mice [155], and it was postulated that high levels of MMP-9 could explain how these animals have wound healing without forming

scars. Recently our lab has shown that M2 macrophages reduced MMP-1 activity in human dermal fibroblasts [89]. Since M1 macrophages are involved in normal wound healing by stimulating MMP-1 production by fibroblasts, targeting M2 specifically for treatment might normalize MMP-1 expression and activity.

1.6.6 Substance P

The neuropeptide SP has been used in the study of fibrosis, and it was found to stimulate fibroblast differentiation [156-159]. Previously, SP showed the capacity to act as a chemoattractant for monocytes, neutrophils, and fibroblasts [160]. SP is regularly used for MC degranulation experiments. In a study where shock and psychological stress were applied to mice, SP expression associated with MC degranulation was increased, suggesting that stressed neurons can secrete enough SP to activate MC [161].

Interestingly, not only neurons but also monocytes and macrophages can produce SP [162, 163]. Researchers have measured the level of SP in normal skin, burned skin wound, and HTS tissues from patients [164]. They found a higher concentration of SP in the HTS, suggesting its possible importance in the pathophysiology of HTS development. MC degranulation stimulated by SP through the neurokinin receptor activation is capable of stimulating the secretion of cytokines, chemokines, and lysosome enzymes [73].

1.7. Research summary and thesis formulation

With an array of growth factors and cytokines, MC can stimulate fibroblast proliferation. In a dose-dependent manner, tryptase increased the fibroblast proliferation rate [143]. Proteases activated TGF-β1, which is a mitogenic factor for fibroblasts [141]. Histamine increases FGF-2 production, as previously described, and augments fibroblast proliferation [22].

Type I and type III collagen are present in the skin. Their ratio during the scarring process varies, and an imbalance between both leads to HTS [18, 165, 166]. As outlined above, the direct application of tryptase stimulated fibroblasts to produce collagen type I [77].

Myofibroblasts are responsible for wound contraction. They are present in normal wound healing, and their numbers are increased in HTS [63-65]. The influence of histamine or protease-activated TGF-β1 on fibroblasts induces myofibroblast differentiation [18, 141, 144].

During the development of wound healing, an imbalance of the physiological protein profile leads to the HTS formation. TGF-β1 is up-regulated, while decorin is down-regulated in the HTS [61]. In a human scar model in nude mice, treatment with INF-α2b up-regulated decorin expression [130]. Since MC are known to produce IFN-α and IFN-β [113], it is possible that decorin expression in fibroblasts would increase after co-culture with activated MC.

Moreover, it is possible that the impact of MC mediators on fibroblasts could interfere in the ECM organization by reducing MMP production. The growth factor EGF can mediate MMP production by fibroblasts [109, 110]. Therefore, MC can contribute to change the MMP production, since MC is a source of EGF [167].

Exaggerated skin contraction is a characteristic of HTS [19, 52]. The communication between MC and fibroblasts through gap junctions increases collagen lattice contraction [80, 81]. Examining fibroblast-mediated collagen contraction in co-cultures with MC would provide some insight into scar development.

Previously, studies have shown that DF and HTS fibroblasts express more pro-fibrotic characteristics than SF and normal fibroblasts, respectively [33, 62]. SF and DF are phenotypically different, and the DF are suspected of contributing to HTS formation. Also, as

mentioned before, the HTS skin showed higher MC density compared to normal skin. Therefore, it is possible that MC might influence the pro-fibrotic characteristics of DF or cause some changes in normal skin fibroblasts similar to HTS fibroblasts.

Given this background, we hypothesized that activated MC regulate deep dermal fibroblasts similarly to HTS characteristics.

In this research, we investigated the effects of activated MC on human dermal fibroblasts from normal skin and compared to HTS skin fibroblasts as a control. Also, we tested the effect of activated mast cells in layered fibroblasts and compared the response of superficial and deep fibroblasts. We tested fibroblast proliferation, collagen production, myofibroblast differentiation, collagen gel contraction, and production of TGF-β1, decorin, and MMPs. Normal skin fibroblasts were cultured with activated MC and compared to HTS fibroblasts.

1.8. Figures
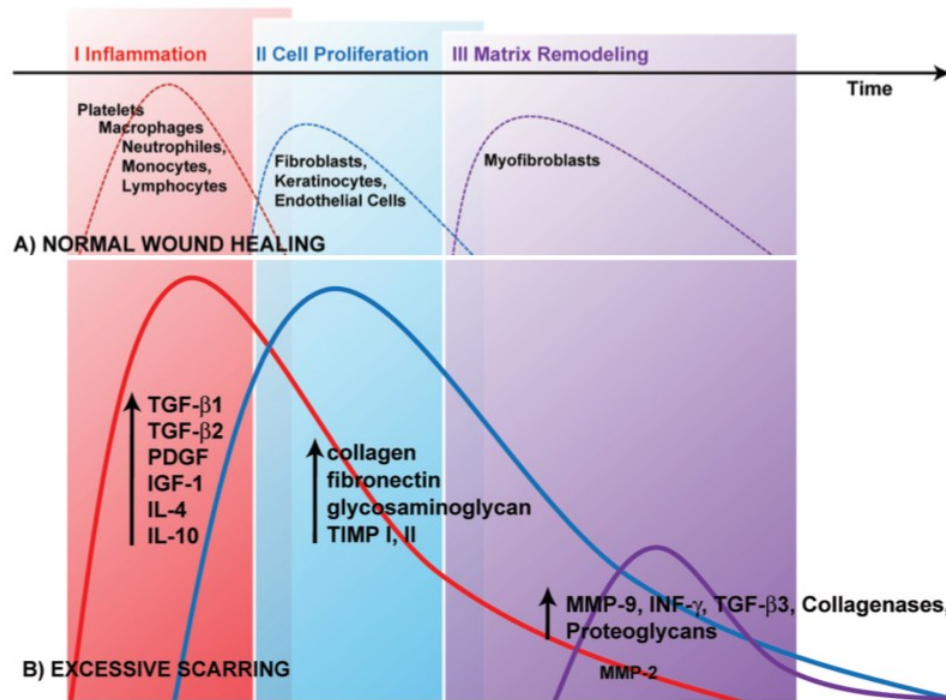
Figure 1.1. The wound healing process.

(A) The normal wound healing is characterized by a short inflammation phase and with the recruitment of immune cells [13]. The second phase is associated with fibroblast, keratinocyte, and endothelial cell proliferation. Lastly, the matrix remodeling phase is an equilibrium between biosynthesis and degradation. (B) Excessive scar formation with exacerbated inflammatory activation overlapping with the cell proliferation phase and late remodeling phase.

Figure 1.2. Wound depth and its relation with HTS formation.

Representative image from human skin [31]. The skin is presented with two subpopulations of fibroblasts. These cells are divided by a virtual critical depth around 0.56 mm. If the skin wound is deeper than the critical depth, it is likely to heal with HTS.

Figure 1.3. Hypertrophic scars.

(A) Erythematous, raised, and thickened scars [50]. (B) Scars after burn injuries on limbs and face restraining movements [51].
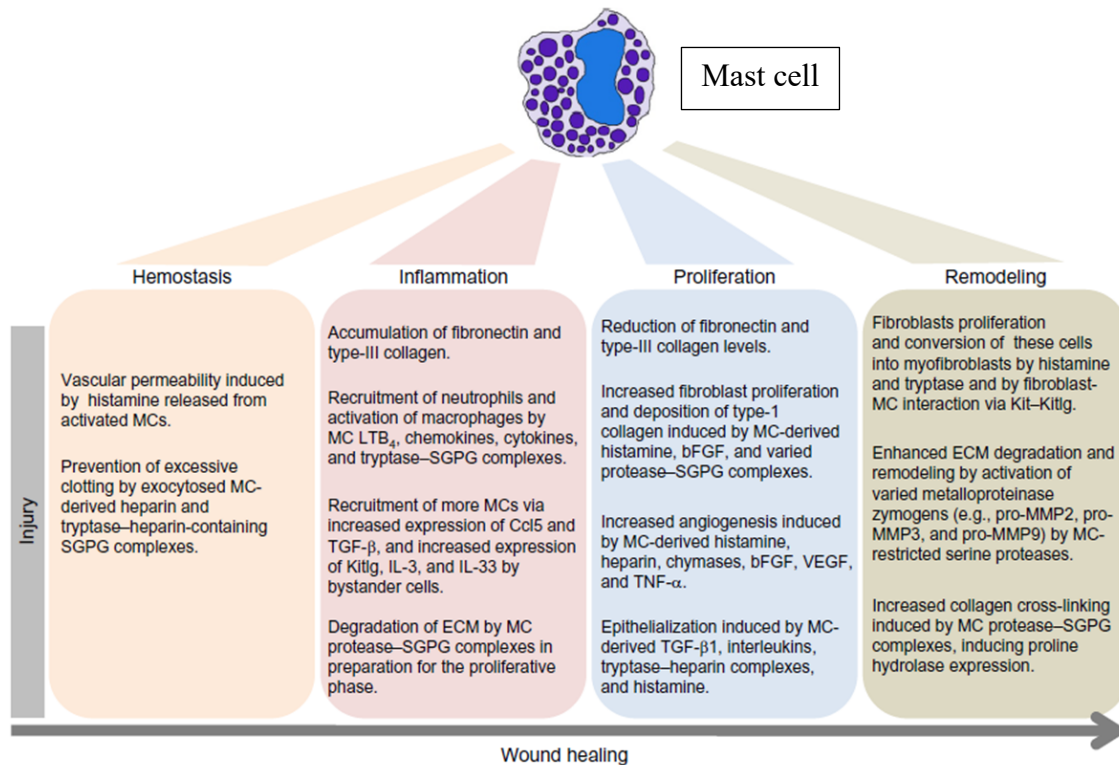
Figure 1.4. Mast cells and wound healing.

The roles of MC in the phases of wound healing [17]. During the hemostasis phase, histamine secreted will cause vascular permeability. Also, MC secrete heparin and tryptase. These two molecules will connect to serglycin proteoglycans (SGPG) and form complexes that control clotting. In the inflammation phase, MC secrete cytokines and growth factors that recruit more immune cells to the wound site. Next, MC stimulate fibroblast proliferation by secreting histamine and proteases. Lastly, during the remodeling phase, MC proteases activate MMPs to help the ECM degradation.

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Chapter 2   The Regulation of Human Dermal Fibroblasts by Mast Cells *In Vitro*

2.1 Introduction

Millions of patients develop scars every year due to trauma, surgeries, burns, lacerations, abrasions, piercings, and vaccinations [1]. Aberrant wound healing can lead to the formation of hypertrophic scars (HTS) [2]. HTS can be painful, pruritic, and erythematous. They are raised, thickened, and elevated above the level of normal skin, but typically are confined to the borders of the original injury. They may cause contractures of the tissues, and usually undergo partial resolution over time. HTS characteristically occur during the prolonged healing process in deep dermal burns. They will progress with excessive inflammation, associated with exaggerated immune cell migration, increased fibroblast proliferation, overproduction of collagen, and abnormal secretion of growth factors and cytokines [3, 4].

In the HTS, fibroblasts are the major cells involved during the remodeling process and the excessive biosynthesis of ECM. Mast cells (MC), which are well known to play a role in allergy, are filled with many granules rich in histamine and proteases and are also linked to wound healing disorders [1, 5]. MC have been implicated in fibrotic diseases in different tissues and the development of HTS [6-11]. By degranulating in response to injury, MC release pro-inflammatory and pro-fibrotic mediators. The presence of increased numbers of MC in the skin, lungs, heart, kidney, during the wound healing remodeling process, has been well documented [12, 13]. However, the challenge is in determining the role MC play in the development of HTS and the mechanism by which they promote the changes in the tissue, or more specifically, in the fibroblasts. MC are intricate defense cells which contain a vast quantity of mediators and many possible activation pathways that could be implicated in HTS formation [7, 14]. Previous work

conducted in our laboratory has demonstrated that fibroblasts from deep dermal layers closely resemble fibroblasts derived from HTS as compared to superficial layers of the dermis [15].

It is our hypothesis that activated MC have an essential role in transforming dermal fibroblasts similar to HTS fibroblasts (HTS Fb) by selectively increasing their pro-fibrotic features of deep dermal fibroblasts as compared to superficial dermal fibroblasts.

2.2 Materials and Methods

2.2.1 Fibroblast isolation and culture

Human skin was collected from patients following approval of the University of Alberta Ethics Committee, (protocol number Pro00023826) with informed consent from the donors. For this study, skin biopsies were used to extract paired dermal fibroblasts from HTS and site-matched normal skin from seven burn patients. Layered fibroblasts from the superficial and deep dermis of normal skin were extracted from seven patients that underwent elective abdominoplasty for cosmetic purposes.

Fibroblasts were isolated and cultured following established protocols in our laboratory [15, 16]. For the paired fibroblasts, patient biopsies were washed with PBS, and immersed in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10 % fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific Inc.). The hypodermis was removed with sterile scissors. The tissue was submerged in dispase II solution 2.5 U/ml (Sigma-Aldrich Canada Co., Oakville, ON) and kept at 4 °C for 20 hours to detach epidermis from the dermis. The solution was aspirated, and tissue washed with PBS. The dermis was minced into small pieces inside a 50 ml conical tube, and 10 ml of collagenase type I solution 40 U/ml (Sigma-Aldrich Canada Co.) was

added and kept for 1 hour in a shaker incubator (Marshall Innova 4,000, New Brunswick Scientific Co., Enfield, CT) at 37°C. The cells were pelleted by centrifugation (Beckman centrifuge, Palo Alto, CA) at 1,000 rpm for 10 minutes at room temperature. The supernatant was aspirated, and cells were washed with 10 ml of DMEM twice to remove ECM fibrils and debris. Cells suspended in 10 ml of DMEM were seeded in the T75 flasks. Cells were incubated (Thermo Fisher Scientific Inc.) at 37 °C, 5 % CO_2, and 95 % air. The medium was changed every 48 hours. Cells were cultured until 80 % cell confluency was achieved, they were then detached from the flask with 3 ml of 1xTrypsin/EDTA (0.25 %, wt./vol.), and reseeded into a new flask.

For fibroblasts from different skin layers, abdominoplasty skin tissue was washed with PBS. The process for harvesting the fibroblasts from different layers followed the previously described protocol [15]. Briefly, the dermatome (Padgett Instruments, Plainsboro, NJ) was set to 0.5 mm, and the tissue was cut into five layers. The first layer of the dermis was used to collect the superficial fibroblasts, while the fifth and last layer was used for collecting deep fibroblasts. Middle layers were discarded. Any fat tissue was removed with sterile scissors. The superficial layer was submerged in dispase solution and kept at 4°C for 20 hours to detach epidermis from the first layer of the dermis. The superficial and deep layers of the dermis went through the same protocol described previously for the paired fibroblast isolation.

2.2.2 LAD2 mast cells

Laboratory of Allergic Diseases 2 (LAD2) is a mast cell line generously provided by Dr. Dean Befus, University of Alberta, Edmonton, AB. MC were cultured following the protocol developed by Kirshenbaum [17]. Cryovials with 10^7 cells per 1.5 ml of pZerve cryopreservative solution (Sigma-Aldrich Canada Co.) were thawed, and cell viability was checked. Procedures continued if the viability of the cells was higher than 80 %. MC were kept in pZerve with the

addition of 100 µl prepared StemPro-34 (Thermo Fisher Scientific Inc.) containing 200 ng/ml recombinant human stem cell factor (rhSCF) (PeproTech, Rocky Hill, NJ). Cells were divided in a 6 well plate and placed in the shaker platform (Boekell shaker) to oscillate cells at 60 rpm for 6 hours at room temperature. Every 30 minutes, the cells were checked for clumps or debris and gently dispersed as needed. After 6 hours, an additional 500 µl StemPro-34 was added containing 200 ng/ml rhSCF and transferred to the incubator (Thermo Fisher Scientific Inc.) at 37 °C, 5 % CO_2 and 95 % air. The medium was changed within the first 24 hours by centrifugation at 1,000 rpm for 5 minutes at room temperature (Beckman centrifuge, Palo Alto, CA).

Fifty % of the medium was replaced every 7 days. Cells were kept to a ratio of 10 ml of medium per 1 million cells. The media contained 100 ng/ml of rhSCF to support cell viability and growth. Cells were kept growing for no more than 11 months following the manufacturer's recommendation to not use the same stock for more than 12 months. During the study, LAD2 were tested for mycoplasma twice and were found free of infection.

2.2.3 Co-culture of fibroblasts and MC

Preliminary conditions were tested to determine the MC cell line and substance P (SP) concentration (**Figure A-1**). SP is a neuropeptide secreted by neurons and macrophages in the skin, and it is well established as an MC degranulation stimulator [18-20]. Also, initial tests were performed to define the media ratio and the Fb/MC ratio for co-culture (**Figure A-2**). Fibroblasts at passage 3 were seeded at a density of 100,000 cells in the T25 flask with 3 ml of DMEM and incubated at 37 °C, 5 % CO_2, and 95 % air for 24 hours to allow the fibroblasts to acclimatize and attach to the flask (**Figure 2.1**). The media was changed to 3 ml of StemPro/DMEM in 2:3 ratio and 100,000 MC were loaded to the co-culture for another 24 hours. SP was added to the

medium at a final concentration of 10 µM to activate the MC and then incubated for 48 hours before fibroblasts were collected for analysis.

The experimental groups consisted of untreated normal skin fibroblasts (NS Fb); NS Fb and SP (NS Fb+SP); NS Fb and unstimulated MC (NS Fb+MC); NS Fb and activated MC with SP as the experimental group (NS Fb+SP+MC); and HTS Fb. In addition, superficial fibroblasts (SF) and deep fibroblasts (DF) were treated in the same fashion as NS Fb groups.

2.2.4 Cell proliferation assay

After the co-culture with fibroblasts, MC were removed by gently washing out with PBS. Fibroblasts were detached with trypsin-EDTA (0.25 %, wt./vol.) after 2 minutes incubation at 37°C, 5% CO_2, 95% air. Cells were placed in 50 ml conical tubes, and 10 ml of DMEM was added to neutralize the trypsin. Fibroblasts were centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded, and cells were resuspended with 1 ml of PBS. Finally, cells were stained with trypan blue, and the living cell number was counted using a TC10^{TM} Automated Cell Counter (Bio-Rad, Hercules, CA).

2.2.5 Hydroxyproline colorimetric assay

Collagen production was tested using hydroxyproline colorimetric assay kit (Sigma-Aldrich Canada Co.) following the company's protocol. Supernatants of the co-culture medium were collected and stored at -20°C and the fibroblast numbers were counted. All samples were thawed, and the experiment was performed in duplicates. A standard curve was created with hydroxyproline standard with 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1.0 µg/well. For the sample preparation, 100 µl of samples were added into pressure-tight polypropylene vials with polytetrafluoroethylene-lined cap. The samples were hydrolyzed with 100 µl of concentrated

hydrochloric acid ~12 M (Sigma-Aldrich Canada Co.) at 120°C for 3 hours. After cooling at room temperature, 4 mg of activated charcoal was added to absorb colored impurities, and samples were centrifuged at 10,000 × g for 3 minutes. Fifty μl of supernatant was transferred to 96 well plates and dried under vacuum at 60°C in a convection oven (APT Line VD, Binder GmbH, Bohemia, NY) for 2 hours. One hundred μl of the chloramine T buffer mixture was added, and the oxidized samples were allowed to cool at room temperature for 5 minutes. One hundred μl of 4-(dimethylamino) benzaldehyde reagent was added to each well and incubated for 90 minutes at 60 °C to form the chromophore. Absorbance was measured at 560 nm (A560) using a Varioskan Lux plate reader (Thermo Fisher Scientific Inc.). The 4-hydroxyproline concentration was calculated using the formula $C = Sa/Sv$ where Sa is the amount of hydroxyproline in the sample (μg) from standard curve; Sv is the sample volume (μL) added into the wells; C is the concentration of hydroxyproline in the sample. The result was corrected for cell number.

2.2.6 Flow Cytometry for myofibroblast differentiation

Fibroblasts were blocked with 10% FBS for 15 minutes to reduce non-specific staining, followed by cell fixation with 2 % formaldehyde for 10 minutes, and permeabilized with 0.05 % saponin (Sigma-Aldrich Canada Co.) for 10 minutes. Samples were washed with 2 % BSA/PBS. Cells were incubated in a solution of PE-conjugated mouse anti-human α-SMA antibody (R&D Systems Inc., Minneapolis, MN) 1:10 concentration and rabbit anti-human tryptase (Abcam, Cambridge, UK) 1:100 dilution in 2 % BSA/PBS for 30 minutes on ice and protected from light. Isotype negative controls were a 1:10 dilution in 2 % BSA/PBS of mouse anti-human PE-conjugated IgG2a isotype control (MACS® Miltenyi Biotec Inc., Auburn, CA) and 1:100 IgG from rabbit serum (Sigma-Aldrich Canada Co.). APC-conjugated goat anti-rabbit secondary

antibody (Santa Cruz Biotechnology, Dallas, TX) was added at 1:100 dilution in 2% BSA/PBS for 30 minutes on ice and protected from light. Cells were washed with PBS and analyzed by Attune NxT Flow Cytometer (Thermo Fisher Scientific Inc.). Analyzed groups included unstained cells as blank control, isotype control, and the samples stained for both antibodies. Tryptase was included in the staining process to certify that collected cells did not include MC in the samples. Results were analyzed by FlowJo v10 software.

2.2.7 Simple WES assay for TGF-β1, decorin, MMP-1, and MMP-2 protein expression

Fibroblasts were collected after MC were washed away from the co-culture with PBS. The cells were lysed with 100 μl of 1xRIPA buffer (Millipore Ltd., Canada, ON) and 1 μl proteinase inhibitors cocktail containing 104 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.085 mM of aprotinin, 4 mM bestatin, 1.4 mM N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide, 2 mM leupeptin, and 1.5 mM pepstatin A (Sigma-Aldrich Canada Co.). With a 28 gauge needle, the solution was aspirated back and forward ten times. The samples were kept on ice, vortexed for 30 sec every 5 minutes for a total three times over 15 minutes. The tubes were centrifuged at 12,000 rpm for 20 minutes at 4 °C, and the supernatant was collected. The total protein was measured by the Pierce[TM] BCA protein assay (Thermo Fisher Scientific Inc.) following the standard protocol provided by the company. The primary antibodies used were goat anti-human TGF-β1 (R&D Systems Inc.), mouse anti-human decorin (R&D Systems Inc.), mouse anti-human MMP-1 (R&D Systems Inc.) and goat anti human MMP-2 (R&D Systems Inc.). For secondary antibodies, goat anti-mouse horseradish peroxidase-conjugated antibodies (Protein simple, San Jose, CA) and rabbit anti-goat horseradish peroxidase-conjugated (R&D Systems Inc.) were used. Following the company's protocol for 12-230 kDa separation system, fluorescent master mix diluted four-fold was added to the lysate

(1,000 µg/ml of protein solution). Along with the biotinylated ladder, the cell lysates were denatured in a water bath at 95 °C for 5 minutes. The lysate samples, ladder, primary and secondary antibodies, antibody diluent, and luminol-peroxide mix, were loaded in the plate. Using the Simple WES machine parameters (Protein Simple), the incubation time was set at 60 minutes for the primary antibody, and 30 minutes for the secondary antibody. The results were detected by chemiluminescence peaks and quantified using the software Compass for Simple WES (version 3.1.8). Final results were normalized by the cell numbers from each sample used to prepare the lysates. The calculation was done with the following formula: (total protein from the lysate / cell number) x (WES signal / protein loaded) = chemiluminescence (chem)/cell.

2.2.8 Enzyme-linked immunosorbent assay **(**ELISA) for decorin

Level of decorin in the cell culture medium was determined by ELISA following the company's protocol (R&D Systems Inc.). The 96-well ELISA plate was pre-coated with 100 µl of decorin capture antibody per well, and it was left at room temperature overnight. The plate was washed with 0.05 % tween 20 in PBS before it was blocked with 300 µl of 1 % BSA in PBS in each well and incubated at room temperature for 1 hour followed by washing with 0.05 % tween 20 in PBS. 100 µl of the samples were added and incubated for 2 hours at room temperature. After incubation, the plate was rewashed. One hundred µl of the decorin detection antibody was added to each well, covered with a new adhesive strip and incubated for 2 hours at room temperature. Aspiration and washing of the wells were repeated three times. One hundred µl of the working dilution of streptavidin-horseradish peroxidase was added, then incubated for 20 minutes at room temperature and protected from light. After aspiration and washing, 100 µl of substrate solution was added to each well and incubated for 20 minutes at room temperature. Fifty µl of stop solution was added to each well. Varioskan Lux plate reader (Thermo Fisher

Scientific Inc.) was used to read the optical density of each well at 450 nm and 540 nm. Readings at 540 nm were subtracted from the readings at 450 nm for wavelength correction. Decorin concentration was calculated based on the standard curve. Final results were normalized for the cell number of each sample previously counted.

2.2.9 Collagen lattice contraction assay

The collagen lattice assay was performed in a combination of our laboratory protocol with the protocol from the company. Fibroblasts were collected after co-culture with MC. The collagen gel lattice was prepared with $5x10^4$ fibroblasts in 485 μl of DMEM, 15 μl of 7.5 % $NaHCO_3$ solution, 100 μl of 10x PBS and 400 μl of Cultrex 3D Culture Matrix Rat Collagen I (R&D Systems Inc.). One ml of fibroblast-populated collagen gel lattice was added in a 12-well plate. Gel polymerization was complete after 2 hours in the incubator at 37 °C, 5 % CO_2, and 95 % air. The gel was detached with 22 gauge needle, and 2 ml of DMEM was added gently to detach the gel and avoid bubbles. Each sample was conducted in duplicate. On a lightbox, pictures were taken every 24 hours for 4 days. The lattice diameter was measured by Fiji ImageJ, and the gel contraction is expressed in percentage of the original size.

2.2.10 Statistical analysis

The analysis was performed with GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Comparison within the normal skin fibroblasts (NS Fb) was made by Repeated Measures One-Way ANOVA followed by Tukey's post-hoc test. Comparison between NS Fb and HTS Fb was made by Repeated Measures One-Way ANOVA and Sidak's post-hoc test. Comparison within-groups and between-groups of the superficial and deep layers of skin groups were made by Repeated Measures Two-Way ANOVA and Sidak's post-hoc test. Results are

expressed in the graphs as mean ± standard deviation (SD) and p value <0.05 was considered significant.

2.3 Results

2.3.1 Fibroblast proliferation was increased by MC

To determine the influence of MC on fibroblast proliferation, NS Fb were co-cultured with MC that were activated with SP. Activated MC significantly increased NS Fb numbers compared to the untreated NS Fb (4.66×10^5 vs. 2.73×10^5) (**Figure 2.2 A**). Also, activated MC increased NS Fb numbers to levels similar to HTS Fb (4.66×10^5 vs. 4.32×10^5). Not activated mast cells significantly increased NS Fb proliferation compared to untreated (3.64×10^5 vs. 2.73×10^5).

Activated MC significantly increased superficial fibroblast (SF) numbers compared to the untreated SF (6.57×10^5 vs. 3.54×10^5) (**Figure 2.2 B**). Non-activated mast cells significantly increased SF proliferation compared to untreated (4.55×10^5 vs. 3.54×10^5). Activated MC significantly increased deep fibroblast (DF) numbers compared to untreated DF (4.18×10^5 vs. 2.33×10^5). SF had a significantly greater cell number than DF, and comparison between SF and DF in the other condition had similar results.

2.3.2 Collagen production was reduced by activated MC

Collagen production was determined using the hydroxyproline colorimetric assay. Hydroxyproline is an important collagen component. It is mainly found in the collagen structure. Therefore, it can be used as a collagen production indicator. Activated MC significantly decreased collagen production by NS Fb compared to untreated NS Fb (0.55 vs. 0.99 pg/ml/cell) (**Figure 2.3 A**).

Activated MC significantly decreased the collagen production by SF compared to untreated SF (0.32 vs. 0.62 pg/ml/cell) (**Figure 2.3 B**). In parallel, activated MC significantly decreased the collagen production by DF compared to untreated DF (0.48 vs. 0.96 pg/ml/cell). SF had significantly lower collagen production than DF (0.62 vs. 0.96 pg/ml/cell), but this distinction was not present in MC co-cultures.

2.3.3 Myofibroblast differentiation in the NS Fb was reduced by MC

To assess fibroblast differentiation into myofibroblasts, cells were stained for α-SMA and quantified by flow cytometry. Activated or non-activated MC, significantly decreased the number of α-SMA positive cells in NS Fb compared to untreated NS Fb (1.00 % and 1.04 % vs. 1.68 %, respectively) (**Figure 2.4 A**).

Although there was a trend towards a reduction of the α-SMA positive cells in the co-culture of activated MC with SF and DF compared to untreated SF and DF, no significant differences were observed (**Figure 2.4 B**).

2.3.4 TGF-β1 expression in the lysate did not change with MC

Analyzed by Simple WES, activated MC did not change TGF-β1 expression in NS Fb compared to untreated NS Fb (**Figure 2.5 A**). Although it was not statistically significant, HTS Fb produced more TGF-β1 than untreated NS Fb and NS Fb co-cultured with activated MC. No significant difference was found among all conditioned groups of the SF and DF (**Figure 2.5 B**).

2.3.5 MMP-1 expression in DF lysate was reduced by MC

Activated MC did not change the expression of MMP-1 in the NS Fb (**Figure 2.6 A**). No difference was found in the MMP-1 expression in any condition of NS Fb compared to HTS. Activated or non-activated MC significantly decreased MMP-1 expression in DF compared to

untreated DF (1.00 and 1.43 vs. 2.62 chem/cell, respectively). A significant difference in MMP-1 expression was found between SF and DF (1.30 vs. 2.62 chem/cell) (**Figure 2.6 B**).

2.3.6 MMP-2 expression in NS Fb lysate was reduced by activated MC

Activated MC significantly decreased NS Fb MMP-2 expression compared to the untreated NS Fb (58.38 vs. 32.70 chem/cell). (**Figure 2.7 A**). HTS produced significantly less MMP-2 than untreated NS Fb (25.70 vs. 58.38 chem/cell). There were no significant changes found in the SF and DF groups (**Figure 2.7 B**).

2.3.7 Decorin expression in DF lysate was reduced by MC

Decorin expression in the lysate from NS Fb co-cultured with activated MC decreased compared to untreated NS Fb, although not significantly (**Figure 2.8 A**). HTS Fb expressed significantly less decorin than untreated Fb (80.75 vs. 221.80). Activated MC significantly decreased decorin expression in DF compared to untreated DF (169.30 vs. 379.40 chem/cell). (**Figure 2.8 B**).

2.3.8 Decorin secretion was reduced in NS Fb and DF media by MC, and it was reduced in SF media by activated MC

The decorin secreted by fibroblasts in the medium was measured by ELISA. Activated or not MC significantly reduced the decorin secretion in NS Fb compared to untreated NS Fb (1.97 and 2.00 vs. 3.37 pg/ml/cell, respectively) (**Figure 2.9 A).** Also, activated or not MC significantly decreased decorin secretion compared to HTS Fb (1.97 and 2.00 vs. 4.56 pg/ml/cell, respectively).

Activated MC significantly decreased decorin secretion in SF compared to untreated SF (2.03 vs. 4.48 pg/ml/cell) (**Figure 2.9 B**). Additionally, activated or not activated MC

significantly decreased the decorin secretion in DF compared to untreated DF (2.89 and 4.10 vs. 6.60 pg/ml/cell, respectively).

2.3.9 The collagen lattice contraction was reduced in DF previously cultured with activated MC

Fibroblast-mediated gel contraction was measured by collagen lattice contraction assay. There was a decreasing trend in the gel contraction in NS Fb cultured with activated or not MC, but not statistically significant (**Figure 2.10 A).** HTS showed a similar pattern of gel contraction over time as the other samples.

Not activated MC significantly reduced collagen gel contraction of SF compared to untreated SF at 96 hours (34.6 vs. 29.2 % of the original size) (**Figure 2.10 B**). For the DF, activated MC significantly decreased collagen gel contraction compared to untreated DF at 24 hours (69.2 vs. 57.0 %), at 72 hours (41.9 vs. 33.3 %) and at 96 hours (30.9 vs. 24.9 %). Still in the DF group, not activated MC significantly reduced collagen contraction compared to untreated DF at 72 hours (40.4 vs. 36.2 %) and at 96 hours (30.5 vs. 24.9 %). Comparison between SF and DF showed that SF induced significantly less gel contraction than DF at 24 hours (66.3 vs. 57.0 %), at 72 hours (39.2 vs. 33.3 %), and at 96 hours (29.2 vs. 24.9 %).

2.4 Discussion

HTS is a complication of burn injury, formed once the injury damages a critical depth of the skin, associated with an increased inflammatory response [2, 21-23]. Researchers over the years have been investigating the cells and molecules that are associated with HTS formation [21, 24-31]. Currently, the field has expanded to uncover the mechanisms of how HTS evolve and to test new treatments in animal models [32-36].

The presence of MC as a pivotal player in scar formation has been described in the skin and other organs in the development of fibrosis. MC are multifunctional cells in the skin. They contain many growth factors, enzymes, cytokines, and chemokines, which broaden their function [14, 37]. Their interactions with DF have been suggested as a reason for prolonged inflammation and the development of HTS [15, 26, 38-40].

In this study, activated MC were tested to determine if they can regulate normal dermal fibroblasts and if superficial and deep dermal fibroblasts would respond differently to activated MC *in vitro*. The results would provide a better understanding of how MC regulate fibroblasts in wound healing of damaged skin and potentially offer new treatment strategies for deep burns and other injuries to the skin to prevent and treat fibroproliferative disorders.

From previous findings in the literature, fibroblast proliferation *in vitro* was stimulated by different substances produced by MC, including tryptase, chymase, and histamine [29, 30, 37, 41-44]. In our co-culture system, the presence of non-activated MC significantly increased fibroblast proliferation compared to control. This was likely associated with the normal secretory flux from constitutive exocytosis [45]. MC cytokine secretion can occur without the need for activation [14]. Known as constitutive exocytosis, MC secrete their molecules in the absence of defined stimuli. However, in our experiments, with activated MC, a significant increase was noted in fibroblast proliferation in NS Fb to reach similar rates as HTS Fb. The same was observed in both SF and DF.

MC secrete their molecules through the regulated exocytosis known as degranulation. In this case, MC degranulate after a specific stimulus such as activation by SP. Hence, this augmentation in the proliferation with the activation of the MC was probably related to the degranulation of large vesicles containing growth factors, proteinases, and histamine. Also, our

results confirmed previous results where untreated SF showed a faster proliferation rate compared to untreated DF [15].

HTS are known to contain larger amounts of collagen I and III than normal skin [1, 2, 46]. Tryptase from human lung tissue has shown to increase type I collagen production in human dermal fibroblasts *in vitro* [41]. In this previous study, direct exposure to tryptase increased total collagen production without controlling for proliferative effects. However, in contrast with the direct effects of tryptase on collagen production by fibroblasts, our study showed a significant reduction in collagen production on a per-cell basis. Activated MC had similar effects on both SF and DF, down-regulating collagen production, which differs from what is expected in the development of HTS. Finally, untreated DF produced more collagen than untreated SF, was expected based on previously published data by our group [15]. Differences found in our results are likely associated with the total collagen production being computed by cell number. Also, collagen was measured by colorimetric assessment of hydroxyproline in the supernatant. In this case, we did not differentiate the collagen type that were being produced. Therefore, we cannot determine if, even with the reduction of total collagen production, the specific type of collagen was up- or down-regulated.

HTS tissues are known to contain more myofibroblasts than normal skin tissues [47]. Histamine and TGF-β1 were found to stimulate myofibroblast differentiation [7, 48, 49]. However, our results showed significantly fewer α-SMA positive cells after NS Fb were co-cultured with activated MC. In this study, fibroblast proliferation was significantly increased after stimulation by activated MC in 48 hours. Therefore, it is possible that the fibroblasts were in an accelerated cell cycle that did not allow cell differentiation in this time frame of the co-culture. In SF and DF, there was also a reduction of myofibroblast differentiation with activated

MC. However, it was not statistically significant, which may be due to the variability between cell lines or the down-regulation of myofibroblast differentiation during the increased mitogenesis induced by activated MC.

In previous literature, it is well documented that TGF-β1 is overexpressed in the HTS tissues [1, 2, 50]. No literature was found associating MC with stimulating TGF-β1 production in NS Fb. However, M2 macrophages, which are also associated with HTS formation, have been shown to stimulate TGF-β1 production in NS Fb [32]. Therefore, we considered the possibility that activated MC would exert similar effects since macrophages and MC share similarities in molecular profile [7, 14, 51, 52]. Although it was not statically significant, a trend can be noted showing that HTS Fb produce more TGF-β1 than NS Fb. Activated MC did not influence TGF-β1 expression in NS Fb, SF, and DF. The unexpected results could be associated with the method applied. Although measuring secreted TGF-β1 in the supernatant could provide a better view of the effect of MC on its production and secretion, the difficulty in measuring TGF-β1 secreted in the medium would be to identify if it was produced by MC or fibroblasts. Another possibility is that after 48 hours of post-MC activation, the stimuli of fibroblasts to produce TGF-β1 has passed, and production was normalized. Therefore, measuring the TGF-β1 protein expression by Simple WES, or the mRNA levels by a reverse transcription-polymerase chain reaction in different time points after the MC activation could provide a better understanding of its effect on fibroblasts.

In this study, one of the objectives was to investigate the regulation of MMPs production in fibroblasts by MC. MMPs are proteins found in the ECM with an essential role in wound remodeling and regeneration [53]. MMP-1 was reported to be down-regulated in the HTS. [54]. Interestingly, MMP-2 was found up-regulated in burn patients' sera, and MMP-2 activity was

found to be increased in HTS compared to normal tissue [55, 56]. These results were suggested to be a physiological mechanism to improve the remodeling process, even though it could not counter the higher production of collagen in the HTS development. In our experiments protein expression was measured in the cell lysates by Simple WES. Activated MC did not significantly change MMP-1 in the NS Fb, although a reducing trend was observed. Similarly, MMP-1 expression was lower in the HTS Fb compared to untreated NS Fb, but again, not statistically significant. These results could be related to the time point when they were tested. In the layered fibroblasts, we were able to detect important changes. DF expressed significantly more MMP-1 than SF. Activated MC regulation was more prominent on the DF than on SF. DF co-cultured with activated MC had MMP-1 expression significantly reduced compared to untreated DF.

Contrasting with the literature, our results showed a significantly lower expression of MMP-2 in the HTS Fb compared to NS Fb. This could be associated with the method applied. While we tested in the cell lysate at 48 hours, previous researchers checked MMP-2 expression in the sera and MMP-2 activity in the tissue. Activated MC significantly decreased MMP-2 expression on NS Fb compared to untreated NS Fb. However, activated MC did not regulate evident changes in the SF or DF groups.

It is possible that at any point after stimulation by activated MC, MMPs protein expression inside the fibroblasts could have varied. Therefore, a better understanding of the effects of activated MC on MMPs production in fibroblasts could be developed by assessing MMPs mRNA expression over time by reverse transcription-polymerase chain reaction. Complementary information about the protein level overtime in the lysate could be acquired by Simple WES.

In HTS, decorin has been found to be down-regulated compared to normal skin [21, 57-59]. In previous literature, in the human HTS animal model treated with interferon (IFN-α), decorin was up-regulated [33]. Given the fact that MC are known to produce IFN-α and IFN-β [29, 60], it is possible that activated MC might regulate decorin production by dermal fibroblasts. First, we measured decorin from the cell lysate by Simple WES. With a small but statistically insignificant trend, activated MC reduced decorin expression in the untreated NS Fb. Our results confirmed that untreated NS Fb express significantly more decorin than HTS Fb. Once again, activated MC exerted more influence on DF than on SF. Activated MC significantly down-regulated decorin expression on DF compared to untreated DF. To confirm these results, ELISA was used to quantify decorin in the supernatant. After NS Fb were co-cultured with MC, the levels of decorin were significantly reduced compared to untreated controls independent of the MC activation state. This result suggests that MC can interfere in decorin expression by secreting factors in a manner independent of classical degranulation. In SF there was a significant reduction of decorin secretion with activated MC, while in DF there was a significant reduction of decorin with MC independent of activation. Co-culture with activated MC exerted pro-fibrotic characteristics in decorin expression.

HTS is known to be rigid and contracted [22, 61]. Also, in previous studies, MC have been shown to increase collagen gel contraction [62-65]. Our results showed no significant difference between any conditions of NS Fb in gel contraction studies, as well as NS Fb compared to HTS Fb. A trend over time can be observed of activated MC reducing SF collagen contraction compared to untreated SF, although it was not significant. However, DF pre-cultured with activated MC significantly decreased collagen gel contraction. DF also contracted the gel lattice at a significantly faster rate than SF, as we have reported previously [15]. The apparent

differences in our results compared to the literature may be in part attributed to the fact that other investigations treated the fibroblasts embedded in the lattice with the MC directly or the conditioned media of the MC to stimulate the myofibroblast contraction. In our research, fibroblasts were stimulated with MC during the co-culture and collected after 48 hours, separately from MC, for studies of only the fibroblasts on the contraction of collagen lattices.

Overall, in this study, activated MC regulated pro-fibrotic characteristic on NS Fb by significantly increasing fibroblast proliferation. Additionally, MC decreased decorin secretion similarly to what is found in HTS tissues. Moreover, activated MC promoted significant reductions in myofibroblast differentiation and collagen production compared to control fibroblasts. Down-regulation of myofibroblast differentiation and collagen production can lead to an abnormal healing process with longer time to wound repair and closure. Therefore, this can extend the proliferation phase in wound healing, which can also translate in later over-production of collagen associated with the increased fibroblasts population. Activated MC did not have a direct influence in the NS Fb on TGF-β1 expression after 48 hours. However, this result represents only a single time point in the production of this protein, and perhaps do not show the effect of activated MC on fibroblasts concerning TGF-β1 production. Activated MC had a major impact on MMP-2 expression by reducing its expression in NS Fb. The down-regulation of MMP-2 in a wound healing scenario would weaken the capability for the tissue to rearrange the ECM. In cases where deep wounds occur, these results suggest that the effects of activated MC in DF could be one of the causes for HTS development. In the DF, activated MC increased proliferation, which is compatible with what is found in HTS. Also, collagen production was reduced, which in combination with decreased collagen gel contraction, suggest that activated

MC depress the abilities of DF to close the wound. Finally, by decreasing MMP1 and down-regulating decorin in DF, activated MC would compromise the scar remodeling process.

2.5 Conclusion

In this study, activated MC played a regulatory role in dermal fibroblasts. In some experiments, not activated MC was sufficient to promote changes. However, once MC were activated, a more prominent fibroblast regulation was observed. Activated MC stimulated NS Fb proliferation, down-regulated collagen production, and lowered MMP-2 expression. Also, activated or not MC reduced the decorin secretion, and decreased myofibroblast differentiation in the NS Fb.

MC exerted certain stimulation in SF, but MC modulation was considerably more important in DF. Activated MC regulated SF and DF by increasing proliferation, and decreasing collagen production. However, in DF, activated MC down-regulated MMP-1 expression, reduced decorin expression and secretion, and reduced collagen gel contraction. Taken together, our results suggest that the relation of activated MC and DF is one of the key components for HTS development.

2.6 Figures
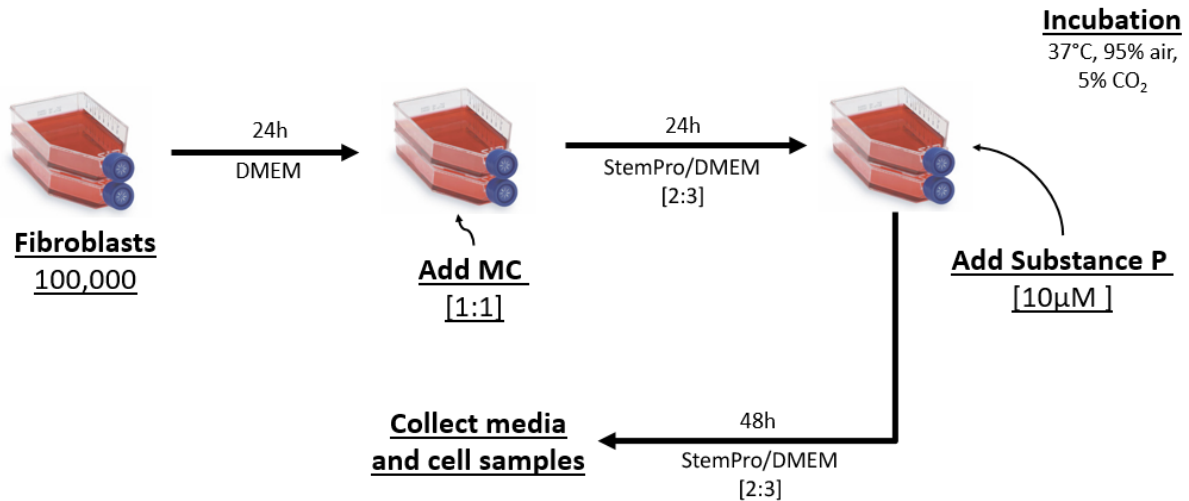

The co-culture model

Figure 2.1. Fibroblast and MC co-culture model.

One hundred thousand fibroblasts cultured from hypertrophic scars, site-matched normal skin, or the superficial and deep layers of normal skin dermis were cultured in the T25 flask with 3 ml of DMEM in the incubator (37 °C, 5 % CO_2, and 95 % air) for 24 hours. The media was changed on the second day with StemPro and DMEM in a ratio of 2:3. On the same day, the same number of MC was loaded for co-culture. Twenty-four hours after co-culture, SP was added at a concentration of 10 µM to stimulate MC degranulation. After another 48hours, MC were washed off, and fibroblasts were collected for further analysis.
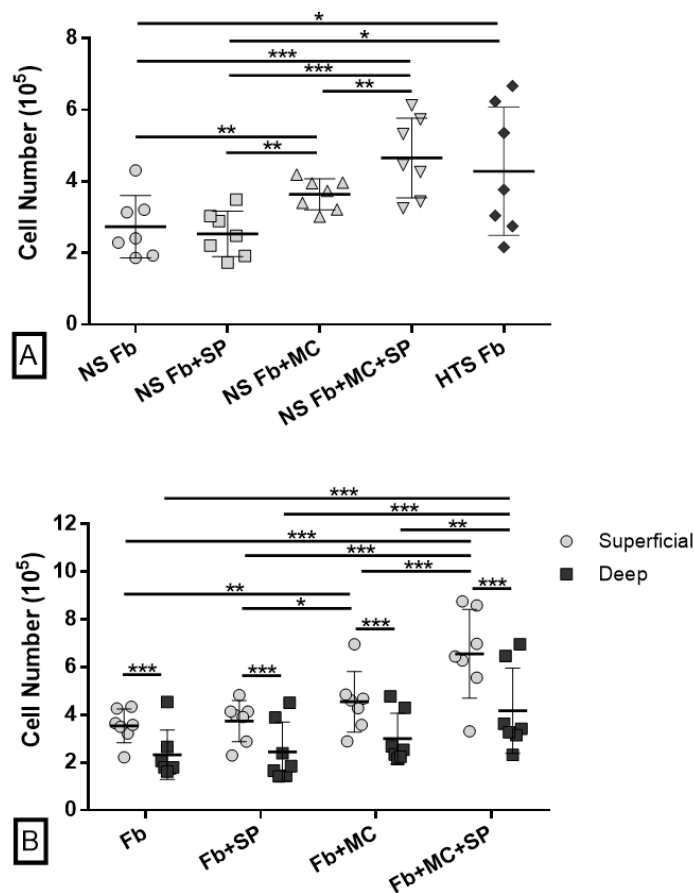
Figure 2.2. Dermal fibroblast proliferation.

Dermal fibroblasts were seeded at a concentration of 100,000 per flask and were co-cultured for 48 hours with the indicated conditions. Collected fibroblasts were counted by the cell counter machine, and only live cells were included. The cell number is displayed as mean $\pm$ SD. (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=7. (B) Similar experiments were performed using SF and DF. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=7.
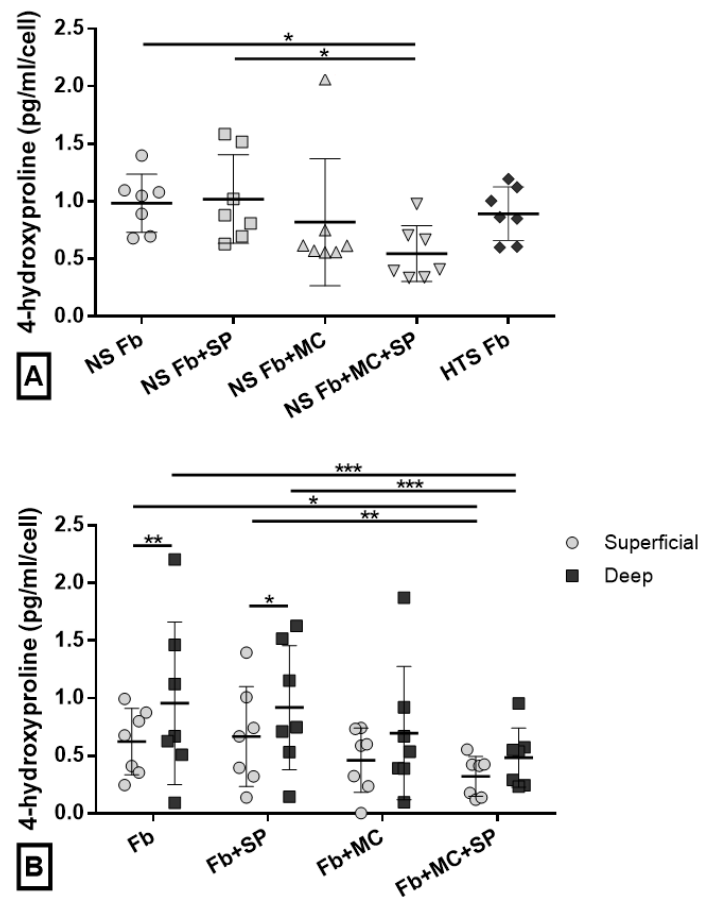
Figure 2.3. Dermal fibroblasts collagen production.

Dermal fibroblasts were cultured for 48 hours after introduction of each condition. The medium was collected, and collagen in the supernatant of fibroblasts was assessed by the 4-hydroxyproline assay kit. The results are displayed as the mean ± SD of pg/ml/cell. (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb. * $p < 0.05$, n=7. (B) Similar experiments were performed using SF and DF. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n=7.
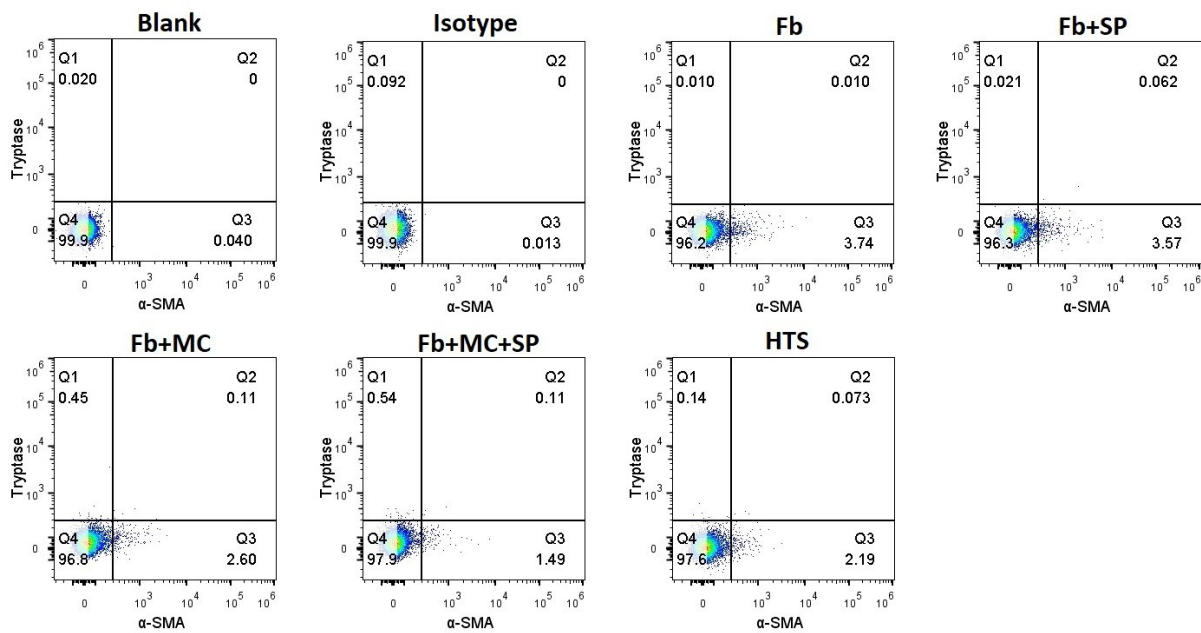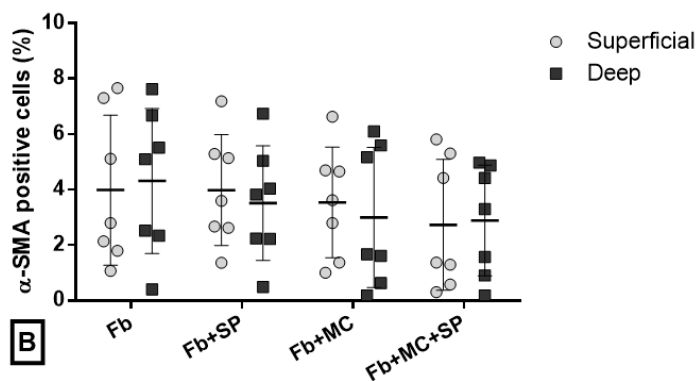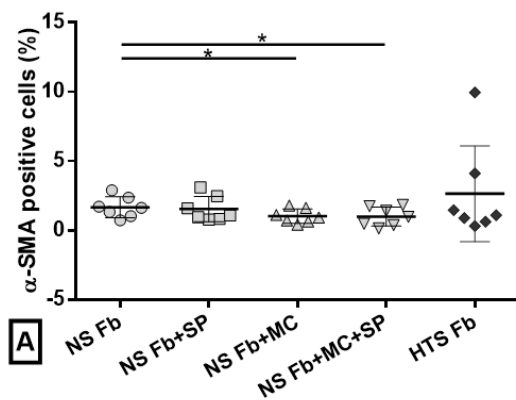
Figure 2.4. Dermal fibroblast differentiation into myofibroblast.

Dermal fibroblasts were co-cultured for 48 hours. Collected fibroblasts were stained for α-SMA and tryptase. The α-SMA-expressing cells were quantified by flow cytometry. The results are displayed as mean ± SD (% of total fibroblasts). (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb. * $p<0.05$, n=7. (B) Similar experiments were performed using SF and DF, n=7. (C) Representative images of the flow cytometry samples of the NS Fb group and HTS Fb.
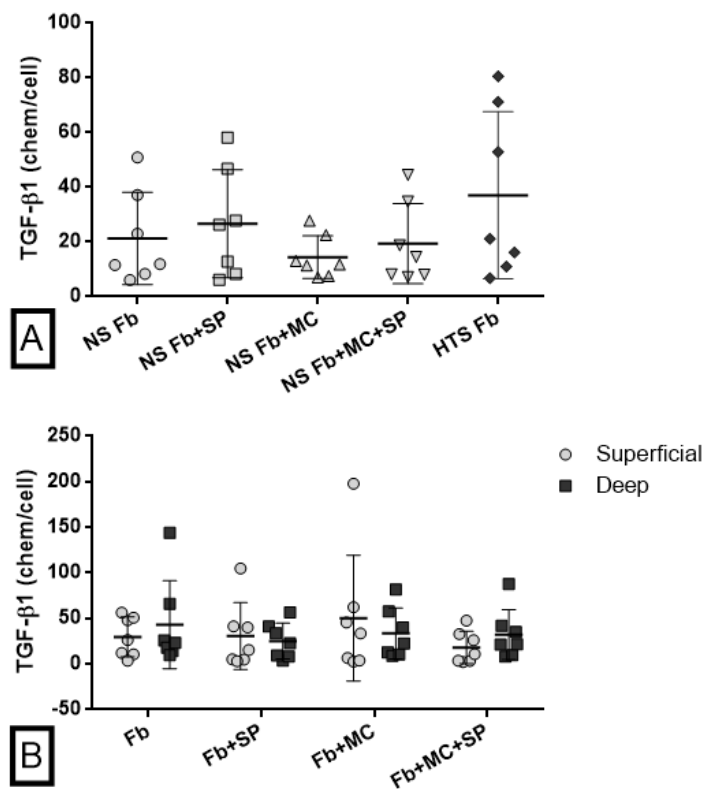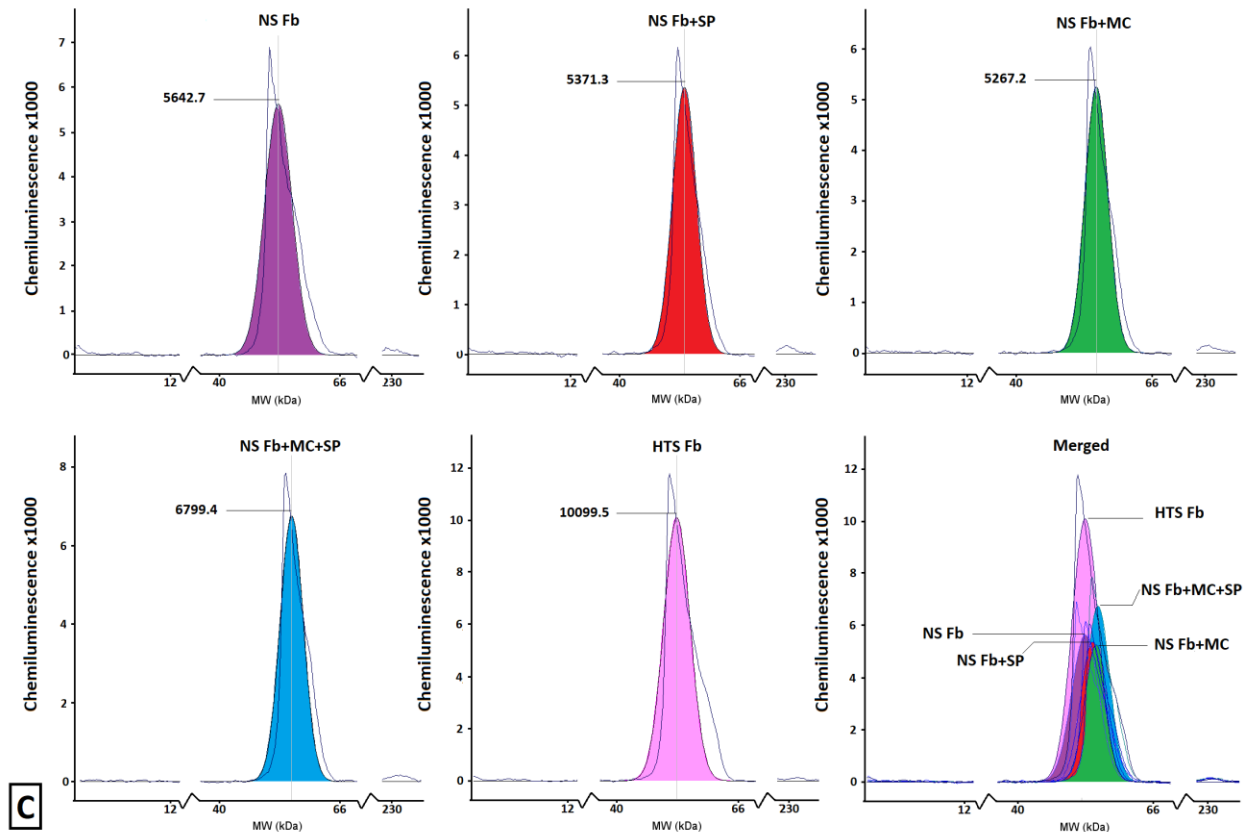
Figure 2.5. TGF-β1 expression in dermal fibroblasts.

Collected fibroblasts were lysed, and total protein concentration was measured by the Pierce[TM] BCA protein assay. TGF-β1 was assessed by Simple WES. The TGF-β1 protein expression is displayed as mean ± SD in chem/cell. (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb, n=7. (B) The SF or DF conditions are the untreated Fb, Fb with SP, and Fb co-cultured with MC (activated by SP or not), n=7. (C) Representative image of the TGF-β1 expression in the NS Fb after co-cultured with MC (activated by SP or not) and SP, and NS or HTS Fb without any treatment.
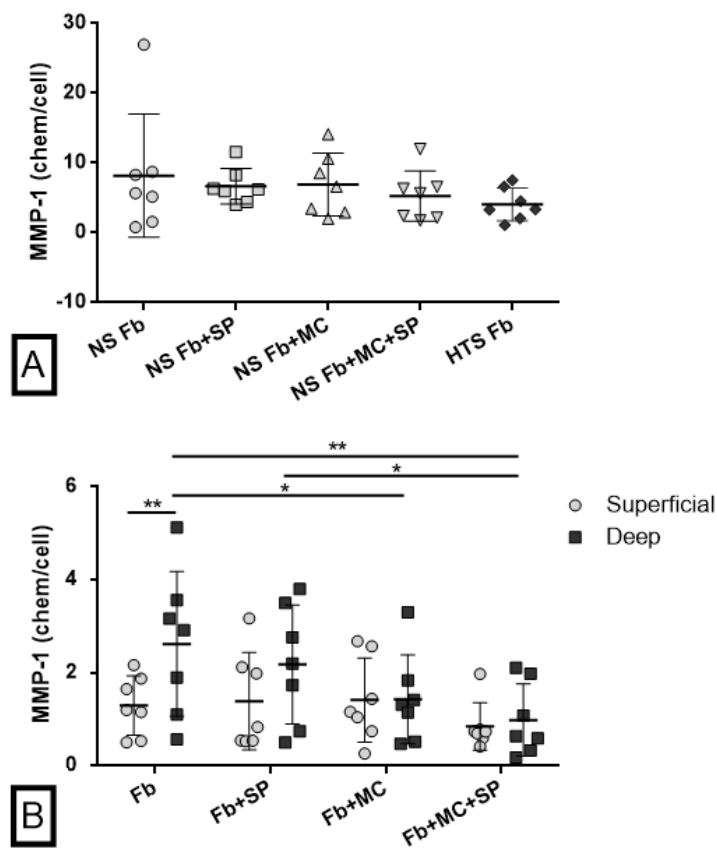
Figure 2.6. MMP-1 expression in dermal fibroblasts.

Dermal fibroblasts were co-cultured for 48 hours and collected for analysis. Fibroblasts were lysed and total protein measured by the Pierce[TM] BCA protein assay, and MMP-1 was assessed by Simple WES. Results are displayed as mean ± SD in chem/cell. (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb, n=7. (B) The SF or DF conditions are the untreated Fb, Fb with SP, and Fb co-cultured with MC (activated by SP or not). n=7. * $p<0.05$, ** $p<0.01$, n=7.
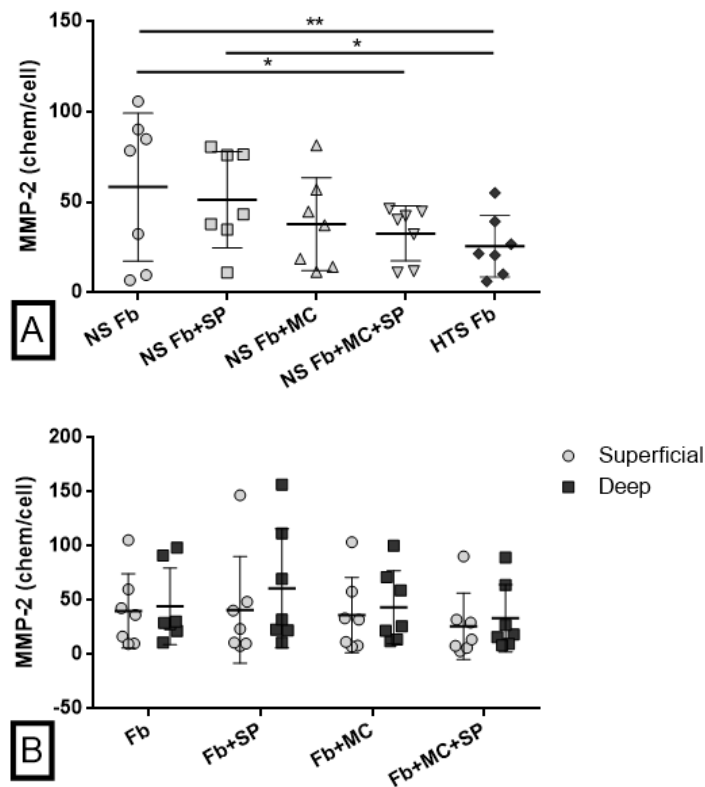
Figure 2.7. MMP-2 expression in dermal fibroblasts.

Dermal fibroblasts were co-cultured for 48 hours and collected for analysis. Fibroblasts were lysed and total protein measured by the Pierce^{TM} BCA protein assay, then MMP-2 was assessed by Simple WES. Results are displayed as mean ± SD in chem/cell. (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb. * $p < 0.05$, ** $p < 0.01$, n=7. (B) The SF or DF conditions are the untreated Fb, Fb with SP, and Fb co-cultured with MC (activated by SP or not), n=7.
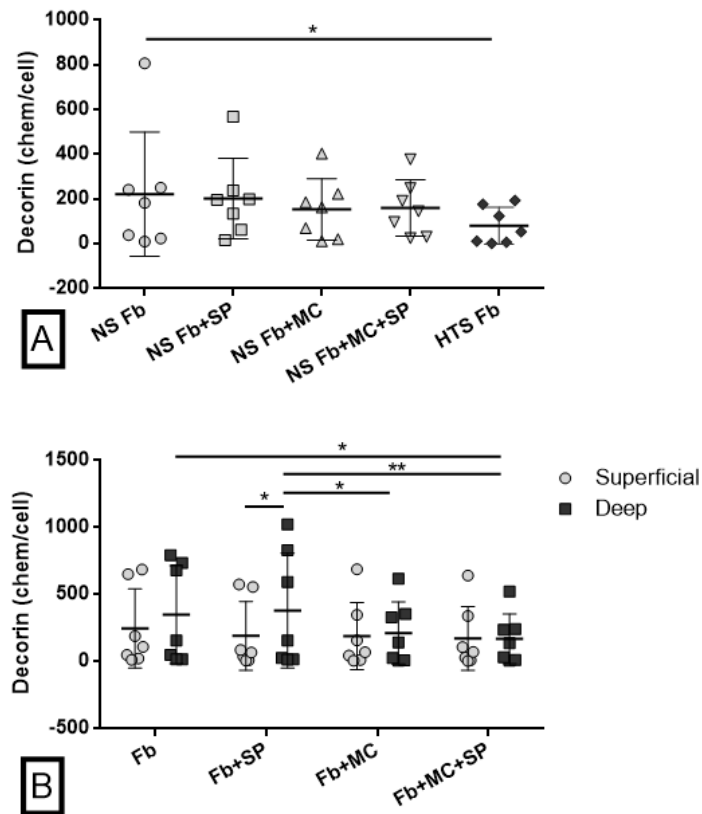
Figure 2.8. Decorin expression in dermal fibroblasts.

Dermal fibroblasts were co-cultured for 48 hours and collected for analysis. Fibroblasts were lysed and total protein measured by the Pierce[TM] BCA protein assay, then decorin was assessed by Simple WES. Results are displayed as mean ± SD in chem/cell. (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb. * $p<0.05$, n=7. (B) The SF or DF conditions are the untreated Fb, Fb with SP, and Fb co-cultured with MC (activated by SP or not). * $p<0.05$, ** $p<0.01$, n=7.
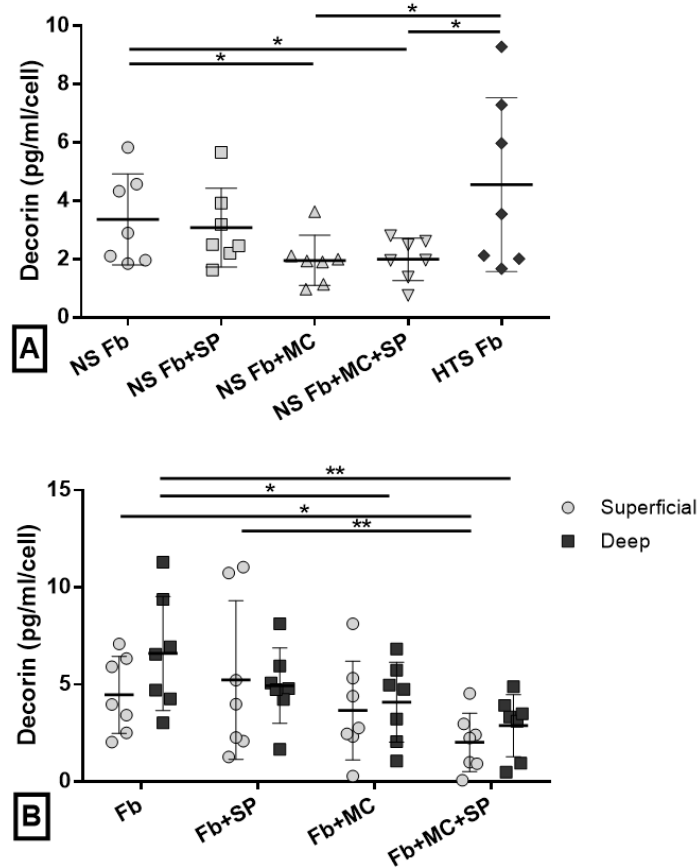
Figure 2.9. Decorin secretion by dermal fibroblasts.

Decorin secreted into the supernatant by fibroblasts after co-cultured with MC were measured by ELISA, and results are displayed as mean ± SD in pg/ml/cell. (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb. * $p<0.05$, n=7. (B) The SF or DF conditions are the untreated Fb, Fb with SP, and Fb co-cultured with MC (activated by SP or not). * $p<0.05$, ** $p<0.01$, n=7.
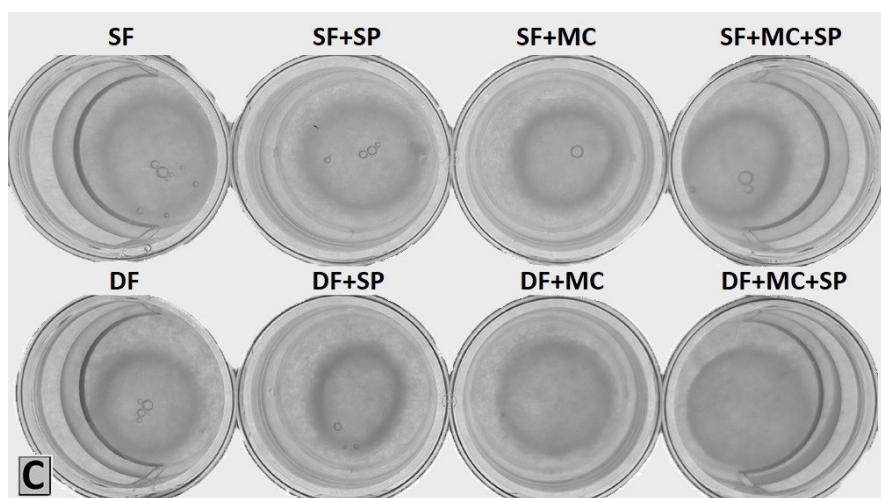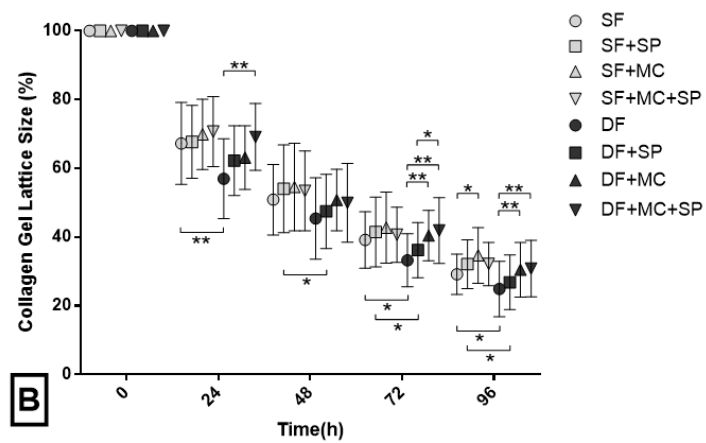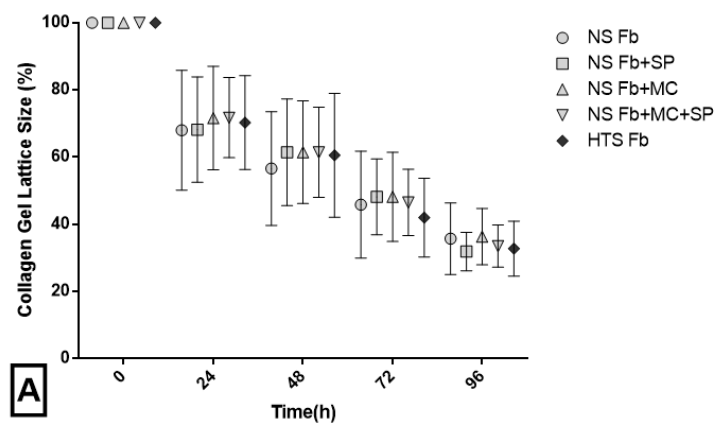
Figure 2.10. Fibroblast-populated collagen gel contraction assay.

Dermal fibroblasts were co-cultured with MC for 48 hours. Collected fibroblasts (MC-free) were seeded into the collagen gel lattice. The gel contraction was monitored over time, and pictures were taken every 24 hours. Results are displayed as mean $\pm$ SD by size in % of the original size. (A) The conditions are represented by the untreated NS Fb, NS Fb with SP, NS Fb co-cultured with MC (activated by SP or not), and the HTS Fb, n=7. (B) The SF or DF conditions are the untreated Fb, Fb with SP, and Fb co-cultured with MC (activated by SP or not). * $p<0.05$, ** $p<0.01$, n=7. (C) Representative images of SF or DF-populated collagen gel lattice at 96 hours.

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Chapter 3.   Conclusions and future directions

In this study, the effects of activated MC with SP on human dermal fibroblasts were examined in our co-culture model. Normal dermal fibroblasts were used to receive the treatments, and the results were compared to site matched HTS Fb. Additionally, the different responses from fibroblasts from two matched populations, papillary (superficial) and reticular (deep) dermis, were tested for the effects of activated MC.

In our hypothesis, activated MC would promote pro-fibrotic response on DF in culture (**Figure 3.1**). Activated MC regulated dermal fibroblasts in almost all testing. The fact that we could not find significant changes in the TGF-β1 expression in the lysate is very likely related to the time point that was tested. Testing TGF-β1 before and right after the stimulation, additionally to the results that we have, would provide a better understanding regarding how TGF-β1 expression fluctuate in a time course. Also, we verified that, although activated MC regulate SF, activated MC exerted a more expressive modulation on DF. Finally, not activated MC reduced MMP-1 expression and decorin secretion on DF. However, activated MC had a stronger effect on DF decreasing the expression of these two molecules compared to not activated MC.

Part of the results corroborated with previous publications (**Figure 3.2**). In contrast, we also found results inconsistent with our expectations. In the HTS what we see is higher collagen production, more myofibroblasts, and more contraction. It is possible that the differences in the results from what we expected are due to the time point when these experiments were performed, or to the fact that we corrected the results to the cell numbers. However, it is important to consider that with reduction of collagen production, reduction of myofibroblasts differentiation and decreased collagen contraction, it will prolong the wound closure. Consequently, if a wound

takes longer to heal, it is more prone to develop HTS [1]. Therefore more experiments are needed to confirm and extend the results.

Additional experiments could provide a further explanation for our results. For example, testing TIMPs in the Simple WES would give the opportunity to check the ratio of MMP/TIMPs to correlate it with MMPs activity, wound healing, and ECM organization [2-5]. Also measuring the expression of decorin, and MMPs in different time points would provide a broader understanding of protein expression overtime after the activated MC stimuli. For future experiments, further exploration of the regulation of fibroblast proliferation by activated MC could be tested. For example, TGF-β1, which is found in high concentration in the HTS, up-regulates cyclin-dependent kinase inhibitors (CKI) such as p21, p27, and p53 [6-8]. These CKI are responsible for regulating cell proliferation and cell death (apoptosis) [9, 10] and could be studied in co-cultures.

In the field, there is very few research that used LAD2 as MC source for their experiments compared to HMC-1. HMC-1 are a very well tested and experimented MC cell line. However, they are considered immature MC [11]. LAD2 have a better potential to mimic the MC found in the skin. This could also have been another factor for having different results from what we found in the literature.

This research was valuable because we confirmed that activated MC could regulate dermal fibroblasts and that DF are key cells for the HTS formation. Additionally, we tested a co-culture model of human dermal fibroblasts and MC. With this model, we can stimulate MC to degranulate and explore different regulatory mechanisms on dermal fibroblasts. In the future, it could be a useful model to test different pathways or to test new drugs that may improve burn injury healing.

3.1 Figures

➢ **Activated mast cells**

	NS Fb	SF	DF
Proliferation	↑	↑	(↑↑)
Collagen production	↓	↓	↓
MMP-1			(↓↓)
MMP-2	↓		
Decorin (lysate)			↓
Decorin (media)	↓	↓	(↓↓)
Myofibroblast differentiation	↓		
Collagen contraction			(↓)

Figure 3.1. Summary of activated MC effect on dermal fibroblasts.

Summary of the results for every experiment comparing the changes that activated MC regulated on dermal fibroblasts. Blues circles represent results that not activated MC also regulated DF. The two arrows in the DF group for proliferation, MMP-1, and decorin in the media shows that, although not activated MC also regulated DF, activated MC exerted a more expressive regulation on these cells.

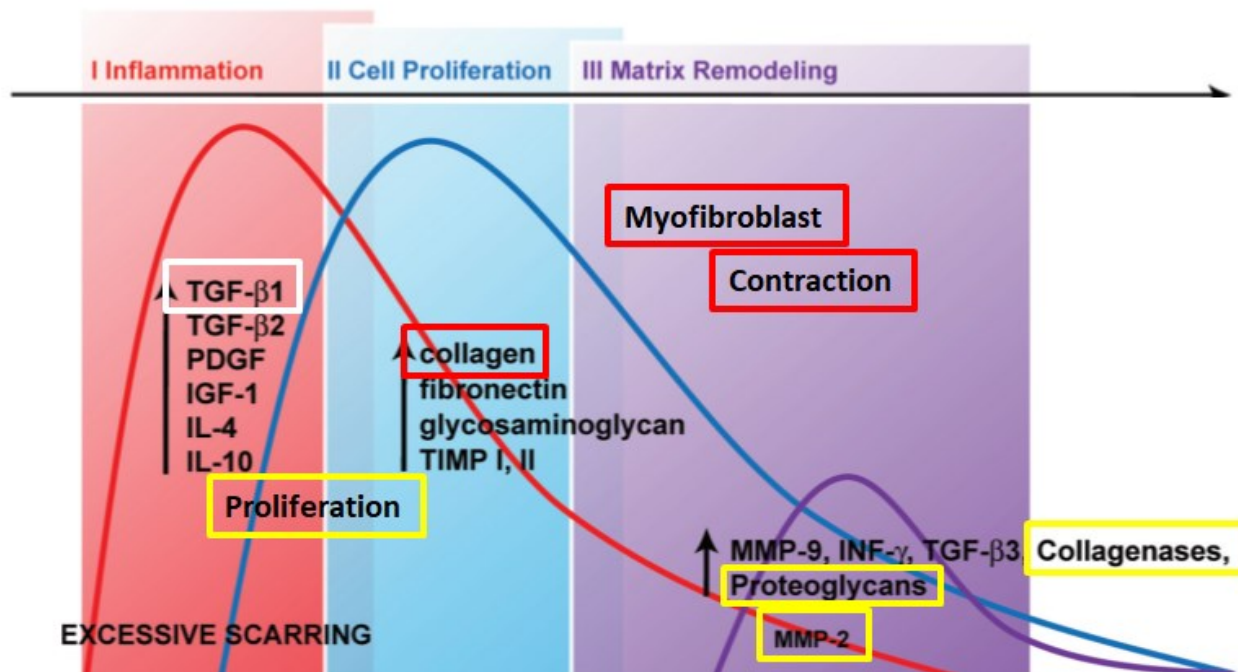**Figure 3.2. Results in perspective.**

Image adapted with our results highlighted [12]. Yellow boxes represent the results that were regulated by activated MC and represent what is found in the HTS. The white box shows the only result that was not significantly different after co-culture with MC. Red boxes are the results that were regulated by activated MC, but they are contrary to what is found in the HTS.

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Appendices

     Appendix 1. To test function and to define the concentration of substance P to use, β-hexosaminidase secretion using the chromogenic test was performed [1-4]. Concentrations of SP lower than 50 nM have been shown to generate MC membrane response, but no degranulation occurred [5]. Therefore, for this thesis, the terms degranulation and activation was used interchangeably referring to the SP stimuli received by MC followed by exocytosis of granules. HMC-1 and LAD2 were tested by the β-hexosaminidase assay. The initial selection of SP concentrations for MC activation was based on published literature [5-8]. The initial set of SP concentrations was 0.1 μM, 1.0 μM, and 10 μM plus no treatment with SP and assays were performed in triplicate. Results are expressed by the percentage of β-hexosaminidase released per condition. The minimal range of concentrations of SP to achieve β-hexosaminidase release of at least 20 % was determined. While HMC-1 performed below the minimal range, LAD2 achieved maximum degranulation with SP concentration at 10 μM (Figure A-1.A). The second set of LAD2 was tested to verify β-hexosaminidase maximum secretion and to determine the best SP concentration to activate LAD2. Using SP concentrations at 2 μM, 5 μM, 10 μM, 20 μM, and 40 μM, LAD2 activation started at 5 μM, and maximum degranulation response was achieved at 10 μM (Figure A-1.B). With these results, LAD2 was selected along with the SP concentration of 10 μM to activate MC degranulation.
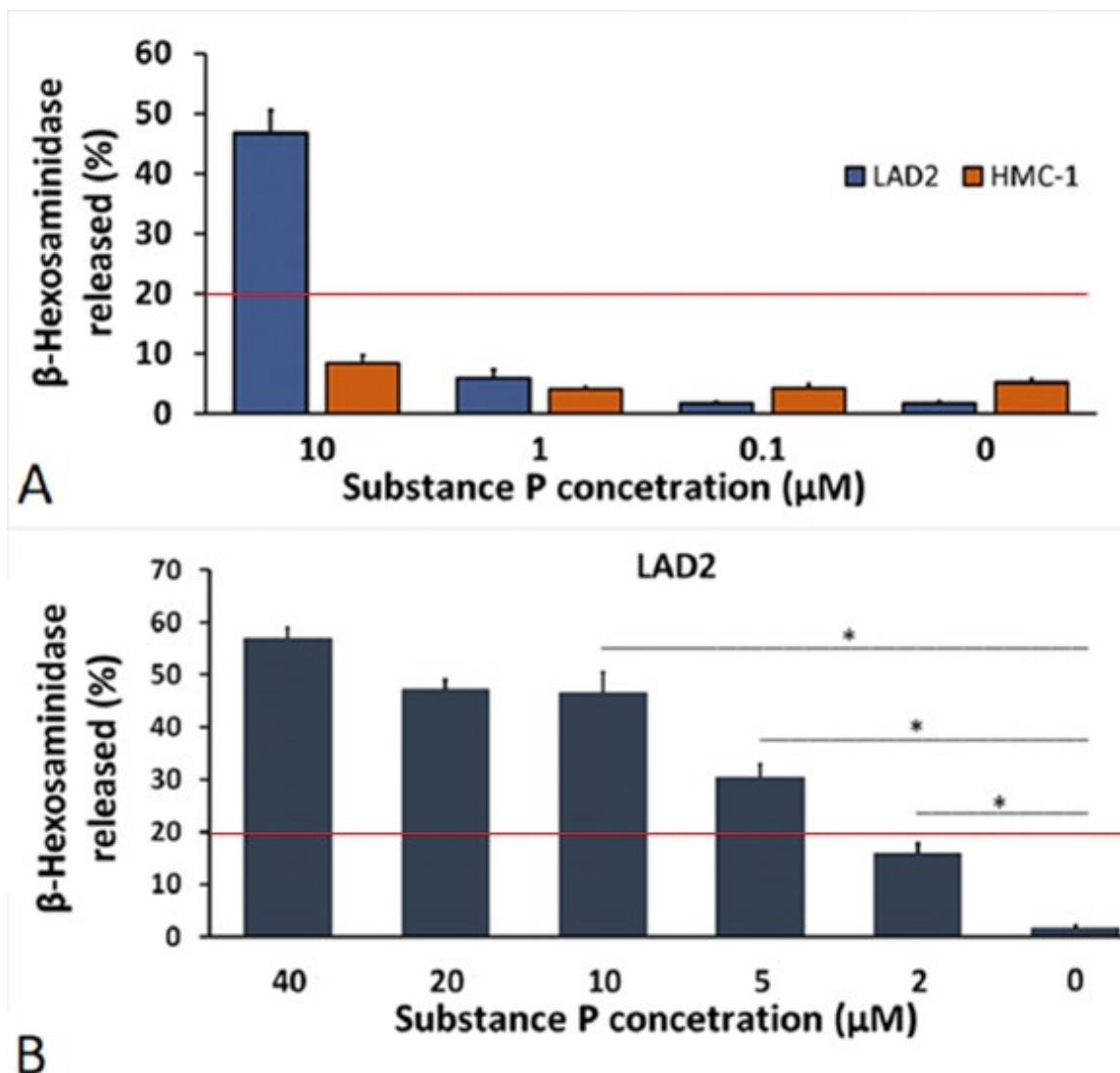
Figure A-1. MC degranulation assay. The β-Hexosaminidase assay was performed on HMC-1 and LAD2 to assess cell activation and optimum SP concentration. A) Initial tests with [SP] at 0.1 μM, 1.0 μM and 10 μM (n=3). B) LAD2 were tested for a new set of [SP] 2 μM, 5 μM, 10 μM, 20 μM and 40 μM (* $p<0.05$ n=3).

Appendix 2. The next step was to optimize the culture conditions for both cells cultured together. A set of six different medium combinations were tested with the following ratios of StemPro/DMEM: 1:0, 1:1, 3:2, 4:1, 1:4 and 2:3, and experiments were performed in triplicates. The cells were observed for five days. Ratios containing more StemPro than DMEM caused

clumping, cell death, and poor growth (Figure A-2.A). Ratios with more DMEM improved the fibroblast proliferation to a standard doubling rate after 48 hours. For the co-culture model, the initial cell ratio was based on our preliminary results, where Fb/MC ratio of 4:1 in the normal skin was observed, and it was also based on published literature [8-11]. Further experiments included Fb/MC ratios 1:1 and 2:1 (Figure A-2.B). After five days of co-culture, 1:1 ratio showed favorable fibroblast growth. Hence, the cell ratio chosen for this study was 1:1.
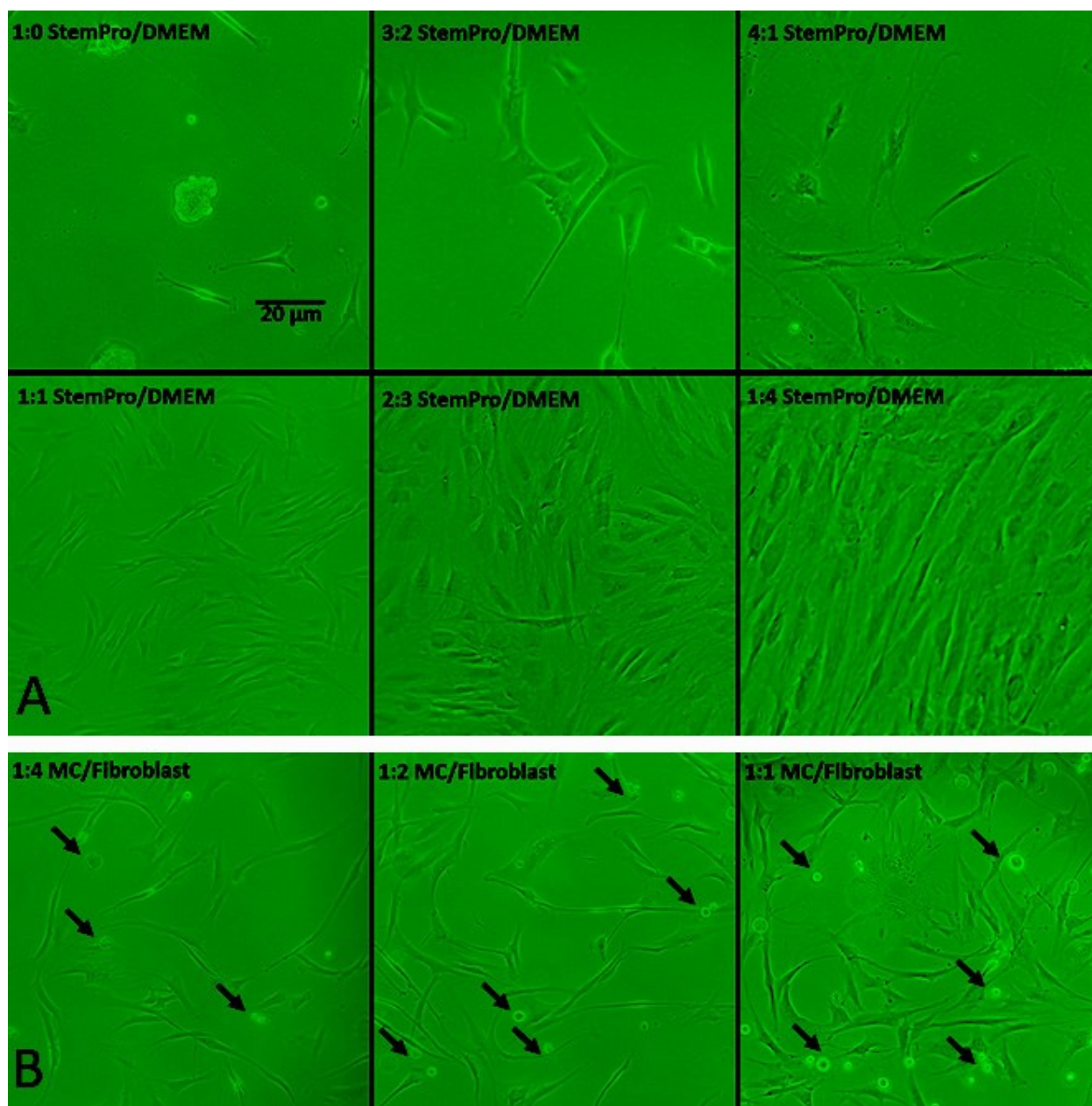
Figure A-2. Media and cell ratio tests. A) Different media ratios between StemPro and DMEM were used to culture fibroblasts (1:0, 1:1, 3:2, 4:1, 1:4, and 2:3). Cell development and growth were monitored over five days (n=3). (B) Fb/MC were seeded at 4:1, 2:1 and 1:1 cell ratio and observed during five days (Arrows pointing to MC in the co-culture; n=3).

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