A Novel Nude Mouse Model of Hypertrophic Scarring Using Scratched Full-Thickness Human Skin Grafts

by

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Abstract

Introduction: Hypertrophic scar (HTS) is a dermal form of fibroproliferative disorder that develops following deep skin injury. This dermal fibrosis can cause deformities, functional disabilities, and aesthetic disfigurements. The pathophysiology of HTS is not understood due in part, to the lack of an ideal animal model. We hypothesize that human skin with dermal wounds of a depth known to reproducibly cause HTS once grafted onto athymic nude mice will develop a raised elevated scar similar to HTS seen in humans.

Objective: Our aim is to develop a representative animal model of human HTS and to explore the cellular origin of the fibrosis either locally derived from the deep dermal human fibroblasts or from the bone marrow derived monocytes of the mouse.

Methods: Thirty-six nude mice were grafted with full thickness human skin with deep dermal scratch wound before or 2 weeks after grafting. The scratch on the human skin grafts was made using a specially designed jig creates a wound > 0.6 mm in depth which has been demonstrated to be the depth of injury in human skin volunteers beyond which HTS consistently develops. Deep dermal wounds in the human skin grafts transplanted on to the dorsal surface of athymic mice were morphologically analyzed by digital photography. Mice were euthanized at one, two, and three months postoperatively before histological analysis of scar thickness, collagen synthesis and fiber orientation, mast cells, macrophages, human leukocyte antigen-ABC, and myofibroblasts was performed. Morphologic persistence of

human fibroblasts in the raised elevated scar was documented up to one-year post engraftment using human anti-ABC antibodies.

Results: The mice developed red, raised, and firm scars in the scratched xenografts with more contraction, increased recruitment of macrophages and myofibroblasts as compared to the xenografts without deep dermal scratch wound. Scar thickness and collagen bundle orientation and morphology were resembled that seen in human HTS in the deep dermal wounds within the engrafted human skin on the dorsum of the mice. The fibrotic scars in the wounded human skin were morphologically and histologically similar to human HTS and human skin epithelial cells and fibroblasts persisted in the remodelling tissues for at least one year post-engraftment.

Conclusion: Thus, deep dermal injury in human skin retains its profibrotic nature after transplantation, affording a novel model for the assessment of therapies for the treatment of human fibroproliferative disorders of the skin.

Preface

This thesis is an original work by Saad M. Alrobaiea. The research project, on which this thesis is based, received research ethical approval from the Animal Care and Use Committee for Health Science in University of Alberta, Project name "A Novel Nude Mouse Model of Hypertrophic Scarring Using Scratched Full-Thickness Human Skin Grafts", No. AUP00000344, September 21, 2014.

Dedication

To my beloved parents Mohammad Alrobaiea & Mashael Albarrak who taught me

diligence in work and ambition for success,

To my lovely sisters Norah, Sarah, Reem and my dear brother Saud

With love

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List of abbreviations

ASCs	Adipose-derived stem cells
ANOVA	Analysis of variance
BALB	Bagg albino laboratory bred
BSA	Bovine serum albumin
CD	Cluster of differentiation
C-GAG	Collagen-glycosaminoglycan
CSS	Cultured skin substitute
CTGF	Connective tissue growth factor
DAB	Diaminobenzidine
DAPI	4'6-diamidino-2-phenylindole
DF	Deep fibroblasts
DWS	Deep wound scar
HTS	Hypertrophic scar
ECM	Extracellular matrix
FPD	Fibroproliferative disease
FISH	Fluorescence in situ hybridization
FTSG	Full thickness skin graft
FITC	Fluorescein isothiocyanate
FOX	Forkhead box

GFP	Green fluorescent protein
Gp	Glycoprotein
H&E	Hematoxylin and Eosin
HLA	Human leukocyte antigen
IFN	Interferon
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
LED	light-emitting diode
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinases
NO	Nitric oxide
NOS	Nitric oxide synthase
PDL	Pulsed dye laser
PDGF	Platelet derived growth factor
SF	Superficial fibroblasts
SLRP	Small leucine-rich proteoglycan
STSG	Split thickness skin graft
SWS	Superficial wound scar
TBS	Tris buffered saline
TGF- β	Transforming growth factor-β
TIEG	Transforming growth factor-β inducible early gene

VEGF	Vascular endothelial growth factor
5-FC	5-flurouracil
α-SMA	α-smooth muscle actin

Chapter 1

1.1 Skin anatomy and physiology

Skin is the largest organ in human's body. It is not only serving as an outer covering it is far beyond that. As an organ it has important functions to regulate and maintain the hemostasis of the body [1]. The skin is divided into three layers, namely the epidermis, dermis and hypodermis (Figure 1-1). The epidermis has five layers, which are as follows in the order of the superficial to deep epidermis: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale; however, the epidermis itself exhibits variable levels of thickness in different parts of the body, where it is thinnest in the eyelid and thickest on the palms and soles[2,3]. Keratinocytes form up to 95% of the epidermis and the deepest layer of the epidermis, the stratum basale, is where keratinocytes are regenerated. The epidermis also contains melanocytes, Langerhans cells and Merkel cells, in addition to skin appendages like hair follicles, the pores of sebaceous (oil) glands, eccrine (sweat) glands and apocrine glands [4].

The dermis is the second layer of the skin. It is divided into papillary or superficial dermis and reticular or deep dermis in which injuries that reach to the deep dermis have been shown to develop abnormal scars[5]. The dermis contains most of the skin appendages and receives the major blood supply to the skin. Most of the dermis is made up by the ECM proteins including collagen and elastin, which give the mechanical support and strength for the skin and its appendages in contrast to epidermis which has a small amounts of ECM [4].

The hypodermis is the deepest and thickest layer of the skin that consists mostly of adipose tissue. It is shown to be missing in some parts of the body where is the skin is very thin like in eyelids thus, it is not considered to be a true skin component. Large vessels and nerves reside in it and it acts as a shock absorber in addition to its critical role in energy storage and thermal insulation for the body [3,4].

The skin serves as a physical barrier that protects the body from exogenous hazards and trauma, and is considered part of the natural innate immune system [6]. In addition, it maintains and regulates body temperature through hydration and lubrication, in part through the secretions of its appendages. The skin is also considered a sensory organ, perceiving pain, touch and temperature stimuli from the external environment. Furthermore, the skin has a metabolic function: It is crucial for activation of vitamin D, which maintains the hemostatic level of calcium in the body[7]. Neuroimmunoendocrine functions of the skin have been reported in which neuropeptides have a role in preservation of the tissue integrity and regulate inflammatory response in the skin [8].

1.2 Wound healing

Skin wound healing is a dynamic and complex process that requires the integration of many inflammatory cells with growth factors and cytokines. Normal or mature scar is the endpoint of the skin wound repair that leads to restoration of new tissue but this type of scar lacks the same structural and functional integrity of the original undamaged skin and aesthetic appearance of the normal skin [9].

Skin wound healing has four overlapping phases: the hemostasis phase, inflammation phase, proliferation phase and remodeling (maturation) phase. Hemostasis is the initial phase that occurs immediately after the insult. Platelets are crucial cells in this phase and the whole healing process. In addition to their role in controlling bleeding and hemostasis, they release multiple cytokines and chemokines that attract and activate inflammatory cells. Platelets become activated and form the initial clot by interacting with glycoprotein IIb/IIIa (GpIIb/IIIa) proteins, which are integrin complexes found on the surface of platelets that bind to the damaged subendothelium. This clot serves as a temporary extracellular matrix for cell migration to the wound site. In addition, activated platelets will trigger the intrinsic and extrinsic coagulation cascades [10].

The inflammatory phase begins by influx of the inflammatory cells to the wound site. Neutrophils are the earliest leukocytes that arrive in the first 24 hours of the injury followed by the macrophages, which are attracted in part after neutrophil apoptosis before finally lymphocytes. Macrophages reach the wound two days after the injury and stay until the end of the inflammatory period [9]. Macrophages have been reported to be key cells in wound healing and their role is not only to remove bacteria and debris from the wound, but they also have the ability to induce reepithelialization and granulation tissue formation, cytokine production, wound contraction as well as playing a role in the formation of new blood vessel at the injured site [11]. Disruption or prolongation of this phase can cause impaired healing and/or chronic wounds that often results in more immature scarring [12,13].

The proliferation phase of wound healing starts two to three days after the injury and it is marked by the presence of fibroblasts which enter the wound site before the end of the inflammatory phase [9,10]. This phase of normal wound repair is characterized by neovascularization, collagen deposition, granulation tissue formation, reepithelialization and concludes with wound contraction. The new blood vessel formation occurs together with fibroblasts and epithelial cell proliferation and serves to provide these cells with nutrients and oxygen but gives rise to the erythematous appearance of the tissue during repair. Granulation tissue serves as primitive tissue that consists of new blood vessels, inflammatory cells, fibroblasts and endothelial cells that covers the wound bed [14]. Fibroblasts form temporary ECM to support cell ingrowth. Fibroblasts also produce collagen, which is necessary to increase the wound strength as the wound in this point of time is only held together with fibrin-fibronectin clot which does not have that much strength to resist traumatic injury [9]. At first the wound contracts without the presence of myofibroblasts but fibroblasts stimulated by growth factors will differentiate into myofibroblasts which facilitate wound contraction in the later stages of healing [9].

Keratinocytes from the wound edges and skin appendages like hair follicles and sebaceous glands migrates to the wound to reepithelialize it in addition to dissolving the clot, debris and provisional matrix by secreting the plasminogen activator and collagenase. Keratinocytes will continue to migrate from the edges of the wound until they meet and stop their migration and start to form and attach to a new basement membrane before they

undergo morphological changes that resemble the mature non-migrating resident keratinocytes [9,10].

The remodeling phase starts when type III collagen that is abundance in early wound healing is replaced with type I collagen and the disorganized collagen fibers are rearranged and aligned along lines of tensile strength. In addition many remaining cells from the previous phases undergo apoptosis that results in an increase of the tensile strength in the tissue. This phase is the longest phase in wound healing and its length depends on the size and depth of the injury [9,10]. It leads to the final wound appearance where successful maturation of the healing wound requires a delicate balance between collagen synthesis and degradation. Thus, fibrosis can result from an imbalance between collagen synthesis and degradation in this phase that will lead to hypertrophic scars (HTS) or keloid formation [13].

1.3 Overview of hypertrophic scar (HTS) in the skin

Hypertrophic scar (HTS) is a dermal form of fibroproliferative disease in which the biomechanical features of normal tissue is disturbed due to changes in the composition of the extracellular matrix (ECM) and its organization. These diseases involve many human tissues including pulmonary fibrosis, myelofibrosis, vascular atherosclerosis, liver cirrhosis and rheumatoid arthritis[15].

HTS is an abnormal scar that develops clinical features of elevated, hyperemic, and pruritic scar that is confined to the boundaries of the original injury [12]. HTS usually develops after deep dermal injury or severe thermal injuries that lead to extensive skin loss. It may be

linear in shape which mostly happens after deep surgical incision or widespread as in cases following burn injuries [16].

In addition to disfigurement and the loss of the aesthetic appearance of the skin, HTSs can lead to considerable morbidity and disability, especially if they occur over the joints (Figure 1-2). This is considered an important reason for the unpleasant and prolonged rehabilitation period, particularly in patients who have survived life-threatening injuries; this is especially a problem in children and dark-skinned people, in whom pathological scarring occurs more commonly[9,10,17]. Disruption of daily activities, anxiety and depression may be long-term sequelae of HTS, in addition to post-traumatic stress reactions and loss of self-esteem, which can diminish quality of life in these patients [14,18].

Clinical observation shows that HTSs are uncommon in superficial, shallow wounds or in areas of the human body such as the eyelids, scalp, palm of the hand and sole of the foot; in contrast, HTSs usually occur on the face, neck, trunk, back and extremities, which suggests that scarring susceptibility may be related to anatomic structures in the deep dermis[1,9]. In addition, it has been observed that HTSs rarely develop in denervated areas of the skin), and they commonly develop in children and patents with dark skin types [9,19].

The formation of an HTS is due to interaction of many cell types, producing numerous cytokines and growth factors, making it a very complex process. Knowing the exact role of these factors and controlling their activity may be helpful in preventing and treating HTS and other fibroproliferative disorders [9,10,20]. Despite the extensive studies on the cellular and molecular biology of HTS, their pathophysiology is still not well understood. One of the

main reasons for the limited understanding of this form of fibrosis is the lack of a representative and functional animal model of HTS that can assist in the investigation of the exact mechanisms that cause abnormal scarring in vivo and testing of the effectiveness of novel forms of treatment [9,10,21].

Despite the improvements in survival among individuals with extensive thermal injuries over recent years with the advancements in antibiotics, fluid resuscitation and specialized burn centers, the incidence of post-burn scarring has not improved concomitantly[13,22]. Deitch et al. investigated the development of HTS in burn patients and found that approximately 30% of burned sites in dark-skinned patients form HTSs, whereas the prevalence of HTSs in white patients was approximately 15%. Other studies have compared the prevalence of HTS formation in skin-grafted sites in both pediatric and adult patients, and shown that more than 75% of these sites become hypertrophied in children [23,24].

1.4 Possible mechanisms of hypertrophic scarring

1.4.1 Role of deep dermal fibroblasts

Fibroblasts are abundant cells of connective tissue and they have an extremely important role in wound healing. They continuously produce components of the ECM that maintain the physical integrity of the connective tissue. There are two characteristics feature of fibroblast activation which are the phenotypic modification and substantial proliferation [25]. Many cells and cytokines are contributing to HTS formation however the dermal fibroblasts are key cells in this process. HTS is a dermal form of fibroproliferative disorders and

persistence of myofibroblasts in the tissue is responsible in part for the pathological fibrosis by increasing matrix synthesis and contraction of the tissue [23]. Furthermore there are increased numbers of fibroblasts and myofbroblasts in HTS tissues in comparison with the normal skin and mature scars which supports their role in fibrosis [26].

There are three subpopulation of fibroblasts in vitro that show various features when cultured separately [2]. Two of these fibroblasts sub-populations are located in the papillary and reticular dermis that are known as superficial (SF) and deep fibroblasts (DF) however; the third sub-population is associated with hair follicles. It is reported that there are differences in physical and biochemical features between superficial and deep fibroblasts including size, packing, proliferation rate and the production of type I and III procollagen [27]. Within the normal skin there are significant differences between the SF and the DF in terms of molecular and physical characteristics. The DF proliferate at slower rates and produce more TGF-β1 and are also found to produce more collagen but less collagenase in comparison to the SF [28,29]. In addition deep dermal fibroblasts within normal skin contain more α-SMA and these α-SMA-positive cells contract collagen to a greater extent, which suggests more evidence of the fibrotic nature of deep dermal fibroblasts [29]. Moreover injuries to the superficial dermis heal with little or no scar and the deep wounds usually lead to development of HTS [30].

Myofibroblasts are crucial cells for dermal wound healing by inducing contraction and granulation tissue formation. Myofibroblasts are observed transiently in the wound during normal healing process and their presence indicates active contraction phase of the healing

wound [31]. After the contraction phase has stopped few myofibroblasts are detected in normal scarring. This is mediated by apoptosis of the cells between the period of the granulation tissue to mature scar formation [26].

Cell migration assays performed on SF and DF to test the difference in fibroblasts migration from each layer of the skin showed the number of migrating cells from SF is higher than the DF. In addition the SF appears to be smaller, bipolar in shape and stretched in one direction, where cell processes extend from one cell to another indicating migration compared with the DF that appears to be large and flat with multiple cell processes extending from many cell borders indicating a stationary behavior.[32,33].

1.4.2   Role of Transforming Growth Factor beta (TGF- β)

There are three isoforms of TGF□β, which are TGF-β1, TGF-β2 and TGF-β3 which are encoded by different genes and all of them are present in wound healing process [34]. TGF-β1 expression is decreased by the influence of the non-fibrotic cytokine TGF-β3 and the balance between them may have an important role in scar formation [35]. High expression of TGF-β3 was seen in fetal fibroblasts which heals without scaring [36]. In addition the ratio of TGF-β3 to TGF-β1 was higher in the non-scarring gingival wound model [37].

The development of HTS is often linked to the over-expression of TGF-β1[38]. It has been reported that the level of TGF-β1 is found to be elevated in the serum of recovering burn patients and HTS tissue stains more intensely for this cytokine than do normal skin samples or mature scars [39]. TGF-β1 is produced by all the major cell types participating in

wound repair including T-lymphocytes, macrophages, smooth muscle cells, endothelial cells, fibroblasts, epithelial cells and fibrocytes [38,40]. Fibroblasts are the predominant cell type involved in healing wounds and may provide much of the TGF-β1 in active HTS especially as its synthesis can be auto-induced in these cells [41]. It was reported that fibroblasts from HTS produce higher amounts of TGF-β1 than fibroblasts site-matched pairs from normal skin tissue. In addition fibroblasts from deeper dermal layers progressively produce increasing amounts of TGF-β1 as compared to superficial dermal layers [29]. This data suggests that fibroblasts from the deeper dermal layers resemble fibroblasts in HTS in terms of TGF-β1 production and as such may contribute substantially to the cell population in HTS.

1.4.3 Role of Small Leucine-Rich Proteoglycans (SLRPs)

Decorin and fibromodulin are members of the small leucine-rich proteoglycans (SLRPs) family that present in the ECM in which they interact with collagen, modifying their arrangement, orientation and deposition in the ECM [42].

Apoptosis is a critical step in wound repair that leads to a decrease in the number of fibroblasts in granulation tissue, which in turn reduces scar formation by lowering the deposition of ECM. The lack of apoptotic stimuli to some extent may play a role in HTS formation [43]. Decorin is a proteoglycan that has been shown to induce cell growth suppression and apoptosis in many cell types by interacting with cell surface receptors [44]. It has been found that HTS tissue has lower decorin level than normal skin [45]. Decreased amounts of decorin may contribute to HTS formation in many ways. Lack of decorin may

result in thick unorganized collagen fibers [46]. Also the activity of fibrotic TGF-β1 increases if the decorin level is reduced[47]. Furthermore, the lack of decorin in HTS leads to reduction of myofibroblasts apoptosis in granulation tissue that normally occurs during wound healing [45]. Taken together these findings support an important role of decorin in HTS formation.

Immunofluorescence staining of SLRPs from different layers of normal skin showed decrease expression of decorin and fibromodulin in deep layer fibroblast compared with the superficial fibroblasts, suggesting that down-regulation of decorin and fibromodulin expression occurs in the fibroblasts of the deep dermal wounds [45].

1.4.4 Role of Nitric Oxide and Nitric Oxide Synthase

Nitric oxide (NO) is a crucial cellular signaling molecule in the human body that is involved in many physiological and pathological processes, such as controlling neovascularization and vascular tone, airway contraction, gastrointestinal peristalsis, insulin secretion and antimicrobial activity [48-51]. Furthermore, it is also involved in the development of the nervous system and acts as a neurotransmitter [48]. NO also contributes to immune system regulation and it had an anti-proliferative functions [52].

The production of NO is controlled by nitric oxide synthase (NOS) that has three isoforms, two of them are mainly expressed which are endothelial cNOS and neuronal cNOS and their activities depend on the elevation of intracellular Ca^{2+} and extrinsic calmodulin. The third isoform is not expressed in normal conditions and its activity is independent from the level of intracellular Ca^{+2}. iNOS is induced in many cells by inflammatory cytokines and

bacterial lipopolysaccharide (LPS) [52]. NO has a significant role in wound healing by its vasodilator activity and anti-proliferative function [53]. A Sufficient amount of NO produced by endothelial cells, fibroblasts and leukocytes maintains the vascular tone and proliferation rate to restore the integrity of the injured skin. It has been reported that the TGF-β suppresses NO production and HTS express more TGF-β than normal skin [54,55].

Site-matched biopsy samples from HTS and normal skin taken from the same patient show both normal skin and HTS fibroblasts produce NO without any stimulation. However, HTS fibroblasts produce lower amounts of NO compared to normal skin [52]. Fibroblasts from normal and HTS skin were stimulated with interferon-γ (IFN-γ) and lipopolysaccharides (LPS) alone or in combination for 96 hours revealed that there was no significant stimulation for NO production when IFN-γ and LPS used alone but, there was significant increase in NO production from both normal and HTS fibroblasts when stimulated by combination of IFN-γ and LPS together [52]. There was a concentration-dependent response and the proportion of this increase was similar for both HTS and normal skin fibroblasts. The mRNA for cNOS and iNOS were both expressed in normal and HTS fibroblasts and also present on HTS and normal skin fibroblast however HTS fibroblast showed lower staining intensity than the normal fibroblasts. In addition, the endothelial cNOS expression was decreased in HTS. The above data suggest decreasing cNOS expression and NO production play a role in increasing the cellularity of the wound leading to HTS formation.

1.4.5   Role of collagenase

Collagenase is a member of the matrix metalloproteinases (MMP) family that starts the process of collagen degradation [56]. During wound healing collagenase has an important role in collagen deposition and connective tissue remodeling [57]. Abergel et al. [58] reported that collagenase activity in keloid fibroblasts in three out of six patients were below the average of their normal controls. Insulin-like growth factor 1 (IGF-1) and TGF-β1 have been reported to be highly expressed in HTS in which they influence the collagen deposition, and reduce collagenase activity [57].

1.4.6   Role of Insulin-like Growth Factor 1 (IGF-1)

The development of HTS may be due to interaction of many cells with cytokines and growth factors like IGF-1, and it is suggested that high level of IGF-1 may lead to increased amounts of type I and type III procollagen in post-burn HTS [20]. Hypertrophic scar samples taken from patient who suffered from burn injury was analyzed using Northern blot where the mRNA expression for IGF-1 is markedly higher in HTS than the normal control skin. In addition, treating fibroblasts with IGF-1 caused an increase in expression of type I and type III procollagen mRNA. Thus the persistence of IGF-1 in post-burn HTS contributes to ECM accumulation, which is evident in all fibroproliferative diseases including HTS [57].

1.5   Current managements of HTS

Dermal fibroprolifirative disorders are challenging diseases to treat. The current treatment approaches for HTS and other fibrotic conditions are very controversial and no standardized method for treating HTS [12,59]. In addition the current treatments of HTS require

prolonged periods of time to show their effect and are expensive, however most are not highly effective.

There are different treatment modalities that have been used in wound care and fibroproliferative disorders of the skin. The decision to use a specific modality often relies on the site and size of the injury and patients age, compliance and comorbidities of the patient, in addition to the time from the original injury. The outcomes can often be predicted by the depth of injury into the dermis based on the time required to heal the wound [5,16]. On this basis preventive treatments can be used preemptively to reduce contractures and HTS formation. Theses approaches can be classified to conservative wound management, medical treatments and surgical interventions [60,61].

Pressure garments, silicone gel sheets, transparent facemasks and serial casting have been used in treating HTS and are considered conservative treatment approaches. The theory of pressure garments mode of action is considered to be via creating a low oxygen environment that may lead to decrease fibroblast proliferation and increasing myofibroblast apoptosis and reducing collagen synthesis [12,62]. Pressure garments can be customized to fit the patient's measurements and need to be worn for 24 hours daily. The ideal pressure to observe an effect of the garments is unknown, but it has been reported that 5-15 mmHg shows good clinical results [63]. However controlled studies of burn patients showed that there is no statistically significant benefit of the pressure garments between patient treated with or without them despite the cost of the pressure garments, inconvenience and discomfort [64].

Another form of conservative way to treat skin contracture and dermal fibroproliferative is silicone gel sheets [12,65]. These sheets can be worn for at least 12 hours daily and exert their effect by reducing evaporation from the stratum corneum thereby maintaining its optimal hydration [66]. Furthermore silicone gel acts by increasing the temperature of the HTS, which enhances the activity of collagenase that leads to remodeling of collagen in the ECM. Skin reactions including contact dermatitis may develop with silicone gel sheets as a complication of treatment [12].

Transparent facemasks are used in patients who suffer from facial burn injuries. They are relatively easy to use and more socially accepted as they don't cover the face in public [61,67]. Serial casting is used as well in treating HTS specially scars that is over joints to prevent or minimize contracture deformities [68].

Intralesional corticosteroid injections have been used widely in the treatment of dermal fibroproliferative disorders, which can be used alone or combined with other treatments [12,69]. These injections exert their effect by decreasing fibroblast proliferation and inducing fibroblast apoptosis. In addition they reduce mast cells in HTS which helps decrease pruritus [60,70]. Subcutaneous tissue atrophy, hypopigmentation and telangiectasia are undesirable side effects of corticosteroid injections [71,72].

Interferon with all its isoforms (α,β,γ) have been shown to reduce fibrosis by decreasing collagen and ECM production through reduced TGF-β level [73]. Interestingly it has been found that collagenase activity increases after treating HTS fibroblasts with interferon α2b suggesting a superior role of it more than interferon γ as HTS treatment [74]. However the use

of interferon has side effects including fever, chills, myalgia, headache, fatigue and night sweats which limits their use [12,75].

The effect of the pyrimidine analog 5-flurouracil (5-FC) on skin fibroproliferative disorders has been investigated and it shows some promising effect when it is used as intralesional injections [76]. It acts by increasing fibroblasts apoptosis and decreasing collagen production. It can be combined with corticosteroids in which this combination has been reported to have better effect than using either of them alone in addition to reducing their side effects [69].

Pulsed dye laser (PDL) has been also tested for treating HTS and shows varying effect in which some investigators reported that there was 57% improvement of the scar after the first session of treatment and it increase to 83% after the second session whereas other investigators reported that there was no significant effect of it [77-79].

Radiation therapy has been considered as treatment modality for HTS by inducing fibroblast apoptosis. It has been reported to have high efficacy if it is used after surgical excision compared with using it alone [80]. Radiation-induced malignancy is the main restriction of use although few cases have been reported to exert this side effect[81]. Furthermore radiotherapy is contraindicated in children as well in tissues with a high carcinogenic potential like thyroid and breast [61,82].

Surgical excision of the HTS usually is considered if the nonsurgical therapy fails to treat or minimize HTS and in severe cases that led to impaired function due to skin

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contracture [75]. It has high recurrence rates especially if it is not accompanied with other treatments such as intralesional corticosteroid or radiotherapy [1,12,80,83-85].

With the advanced technology and tissue engineering cultured skin substitute (CSS) has been developed which make a huge advantage in the field of reconstruction and wound care. In some severe cases of extensive skin loss or third degree burns a suitable donor site for autografting is not available, therefore CSS is the most favorable option of treatment in these cases. CSS containing autologous keratinocytes and fibroblasts seeded on an artificial ECM made of collagen-glycosaminoglycan (C-GAG) shows a great potential in skin repair since more promising clinical results have been reported [86,87].

1.6   Animal models of HTS

The lack of an animal model of HTS is a main obstacle that limits the full understanding of the pathophysiology of the skin abnormal scarring [21]. Dermal fibroproliferation is unique for humans and animals do not normally form HTS, which make the development of animal scar model difficult and more challenging. The development of an ideal animal model is important to study the histological, molecular and cellular basis of hypertrophic scarring, to understand the pathophysiology that leads to the development of pathological scars. Moreover, ideal animal model of HTS will enable investigations of the effectiveness newer preventive and therapeutic modalities to reduce or treat HTS and to test their safety before using it in humans [88]. An ideal animal model should resemble human HTS in its morphological appearance, clinical symptoms, histological and cellular biology. Several

models have been developed aiming to look like human HTS which includes rabbit ear model[89], Red Duroc porcine model[90] and athymic nude mouse model [91-93] in which each of these models has its advantages and limitations.

1.6.1 Rabbit ear model

It was observed that surgical scar in rabbit ears would last for months which resembled human HTS histologically and grossly. These observation lead to using of the adult female New Zealand white rabbit was shown to form reproducible HTS in their ears which could last for 280 days [89,94]. This model is used currently by many researchers to examine the effectiveness of many wound modulating agents [95-98]. In addition rabbits have decreasing severity of hypertrophic scarring with increasing age, which is a similar feature of the HTS in humans in which its incidence is reduced among older as compared with young patients. These features suggest that rabbit ear model demonstrates some similarities with human HTS. However, the creation of this wound relies on the injury to the underlying cartilage which also becomes thickened and shows irregular disorganized chondrocyte proliferation that is markedly different from the thermal injuries that cause human HTS making this model suboptimal to study the cellular mechanisms of human HTS formation [21].

1.6.2 Porcine model

The female Red Duroc pig has been reported to be the animal that has the most similar skin to the human skin in regards of epidermal thickness, dermal-epidermal thickness ratio,

hair follicle pattern and collagen content [99]. In addition the similar but not identical wound healing process of the Duroc pig compared to human when creating a deep dermal wound to specific sites of the pig has shown to give rise of thick scar that last approximately to five months [90]. Furthermore the Duroc pig HTS model is similar to the human HTS in terms of the expression of the small leucine-rich proteoglycan decorin and versican in addition it has the same insulin-like growth factor 1 (IGF-1) and myofibroblast levels. Also it shows a similar pattern of angiogenesis which gives more validity to this model [100,101].

However the difficulty in housing, handling, caring of the animal and the length of the study adds significant cost to use this model in addition, the Duroc pigs are expensive since they are not common in the food industry [1,90,102,103] thus, limiting the use of the pig as HTS models. Currently this model is used in the wound dressing researches [21].

1.6.3   Athymic nude mouse model

These mice are genetically designed to have absent thymus gland which makes them lack the T-cells in and their immune system unable to do its function such as delayed hypersensitivity responses, killing viruses and most importantly xenograft rejection. Thus, these nude mice become widely used in the immunological, oncological and transplant researches but they have to be used under pathogen free conditions as they have a high susceptibility to infection by viruses and other microorganisms [104]. They have the nickname of nude mice as result of the absence of the outer furry cover due to twisting and coiling of the hair fibers within the follicle infundibulum giving them their appearance of hairlessness

due to the Forkhead box protein N1 (FOXN1) mutation that is required for thymic epithelium development and pleotropic effect on hair follicles [105].

Nude mice have been used extensively in wound healing researches. The initial experiments involve the transplanting of human HTS tissue or keloid tissue to the nude mice which after transplantation of the HTS and keloid start to regress which represent the final stage of them and disable studying the initiating molecular and cellular factors that lead to this scarring due to incomplete vascularization of the transplanted tissue and absence of epidermal-dermal interaction with the transplanted tissue [106]. Then Yang et al.[91] transplanted human full thickness skin graft to the back of the nude mice that showed without even thermal injury the mice start to developed red, raised and thick scar that resemble clinically human HTS and it was better than transplanting the fibroproliferative scar itself to the mice in which the epidermal–dermal interaction was present in addition to the good vascularity to the graft. The HTS-like graft was been able to be followed and persist for 135 days and cellular and histological examination of the scar was similar to human hypertrophic scarring in terms of collagen deposition. .

Recently the wound healing research lab in the University of Alberta were able to characterize this model more to develop a promising technique using the athymic nude mice by transplanting split thickness skin graft taken from female patients who underwent elective abdominoplasty surgery and transplanting it into the back of nude mice. This led to the development of red, raised and hard scar that looks like human HTS and has clinical, cellular, histological and immunehistochemical characteristics of human HTS 30 days post

transplantation and employed mice with autografts as controls (Figure 1-3) [93]. The transplanted skin survival was confirmed histologically by the presence of positive staining of HLA-ABC in all the grafts in all time points of the study which gives the ability to test prophylactic and therapeutic treatment on human skin without using human subjects in addition to being the first model to show the changes of SLRPs expression similar to the expression level observed in human HTS. However it is not clear if this model is fully analogues to the human hypertrophic scarring process.

1.6.4 Dermal scratch wound in human volunteers

Dunkin et al.[5] designed a novel jigsaw to produce standardized dermal scratch wound that is deep in one end and superficial in the other end to know if there is a relation between depth of the injury and scar formation. The length of the wound was 51.3 ± 0.6 mm and maximum depth of the wound is 1.6mm. 113 healthy volunteers participated in the study and wound was created on the lateral aspect of the hip along with the relaxed skin tension, midway between anterior superior iliac spine and grater trochanter under local anesthesia in sterile condition. The subjects were reviewed weekly for one month then at 6,10,18,24 and 36 weeks and the scar progression were assessed with digital photography and high frequency ultrasound scanning. That showed with wound healing progress a fibrous scar developed at the deep end whereas no scar visible at the superficial end of the wound. This study defined a threshold of depth in which fibrotic scar developed and it was calculated to be 0.56 ± 0.03 mm or 33.1% of the lateral hip skin thickness and these numbers were calculated by simple

trigonometry and high frequency ultrasound. However the cellular and molecular aspect of the wound healing from this wound was not examined.

Another study done in the wound healing research lab in the University of Alberta aimed to show the relation of the depth of injury and HTS formation and analyzing the deep dermal fibroblasts and their role in this fibrotic process [45]. This study aimed to identify the expression and localization of factors which regulate wound healing including decorin, fibromodulin, TGF-β1 and TGF-β3 in an experimental human scratch wound and also from fibroblasts from superficial and deep layer of normal skin. A specially designed jig using a no 11 scalpel blade was created after the original description of human dermal scratch model by Dunkin et al.[5] and after obtaining an informed written consent from a burn patient an incisional wound was made using the jig located midway between anterior superior iliac spine and greater trochanter under general anesthesia in sterile condition in the operating room at the time of split thickness skin graft harvesting. The incision was 6 cm long, and the maximum depth of the wound was 3 mm. Around 70 days post wounding the superficial wound healed with minimal scaring (SWS) while the deep wound at the other end healed with red, raised and hard scar (DWS) that also confined to the boundaries of the wound, which grossly resemble HTS (Figure 1-4). In addition the DWS at microscopic level shows thicker epithelium, hypercellularity, hypervascularity and bulky collagen bundle fibers compared with SWS, which indicate that the DWS developed microscopic features of HTS. These data indicate that deep dermal injuries usually lead to development of fibrotic scar that have clinical and cellular characteristic similar to HTS and this warrants further studies.

What limits the use of this kind of the study is the ethical issues around the participating human volunteers in the study and creating a scar that may have the potential to be fibrotic in addition to the compliances of these volunteers to be followed up during the study period which may have extended time. In addition the volunteer health condition must be taken into consideration in which the one with chronic diseases or family history of abnormal scaring cannot be fit for the study.

1.7   Conclusion and formulation of research question

HTS is a pathological scar that follows deep injuries and is characterized to be hyperemic, elevated, itchy scar that remains intact to its original borders and has deposition of excess ECM. The pathophysiology of HTS remains unclear, which makes it difficult to treat. Many cells and growth factors have been linked to HTS. From the previous studies we showed that fibroblasts from different layers of the skin have distinctive features in which the DF proliferate at a slower rate and produce more TGF-β1 and CTGF compared with SF. This review showed that DF produce more versican but less decorin and fibromdulin. In addition to producing more collagen but less collagenase compared with SF these data suggest that DF may resemble HTS fibroblasts. Understanding the integration between the inflammatory cells and cytokines and targeting the molecules involved in wound healing my give rise to a novel preventive and therapeutic modality for HTS.

Several treatment modalities have been proposed and used in treating HTS and their effect varies from one patient to another, which is also due to the lack of a functional animal model in which these treatment effects could be tested and to determine their effectiveness.

A major undesirable outcome of post-burn wound healing is HTS formation, which is due to excessive accumulation of ECM that has a different organization and composition compared with normal skin. Two-thirds of patients who have burn injuries develop HTS that results in a long unpleasant rehabilitation period and considerable disfiguring and functional disabilities. The relationship of the depth of the wound and the formation of HTS is clinically observed and showed that the superficial wounds usually heals with no or minimal scarring in contrast to deep wounds which are more likely to develop elevated, red and hard scars that are confined to the original injury borders resembling HTS. In addition it has been reported that there is a threshold of dermal depth in which injury beyond that level will cause healing with fibrotic scarring

This review of the literature revealed that HTS is a clinical problem whos pathophysiology remains not well understood and the critical challenge to study it is the absence of an ideal animal model that has the same clinical, histological, cellular and molecular features of human HTS that can aid in the study and investigation of the pathophysiological process that lead to abnormal scarring. In addition to help testing the effectiveness a new therapeutic modality to reduce HTS. Despite the development of some animal models, an ideal one has not yet been developed.

This project aims to develop an animal model that explores the relation between the depth of the injury and HTS formation using athymic nude mice. Knowing that scar occurs at a critical depth in humans and grafting human skin over athymic nude mice forms a graft that is similar to HTS we hypothesize that scratch full thickness human skin grafted it onto the back of a nude mouse will form a scar over the graft and represent human HTS morphologically and histologically. We will observe the differences between grafts with scratched human skin before and after grafting and grafts with no scratch.

Thirty-six athymic nude mice were used in this study; they are euthanized to harvest the grafts after 1, 2 and 3 months post-grafting. At each time point, the mice divided into three groups according to the graft type, namely a non-scratch group, a scratch before grafting group and a scratch after grafting group (Figure 1-5).

After harvesting the grafts, hematoxylin and Eosin (H&E) staining was done to assess the grafts cellularity and vascularity. Masson's trichrome and picrosirius red stainings are performed to observe the collagen organization and production in addition to Immunohistochemistry staining for myofibroblasts, macrophages, and mast cells. In addition, HLA-ABC staining was done to assess the grafts survival.

Success in development of this model will become a useful tool to study the mechanism of hypertrophic scarring. To be a reproducible model with more acceptable morphological manifestations similar HTS that is based on a mechanism known to develop fibrosis in human skin are great advantages of this model. In addition to facilitate the study of the molecular and cellular biology that lead to hypertrophic scarring and the exact role of the

fibrotic cells, cytokines and growth factors that lead to HTS. Importantly it will be of great value to test the effectiveness of novel anti fibrotic treatments for HTS and fibroproliferative diseases.
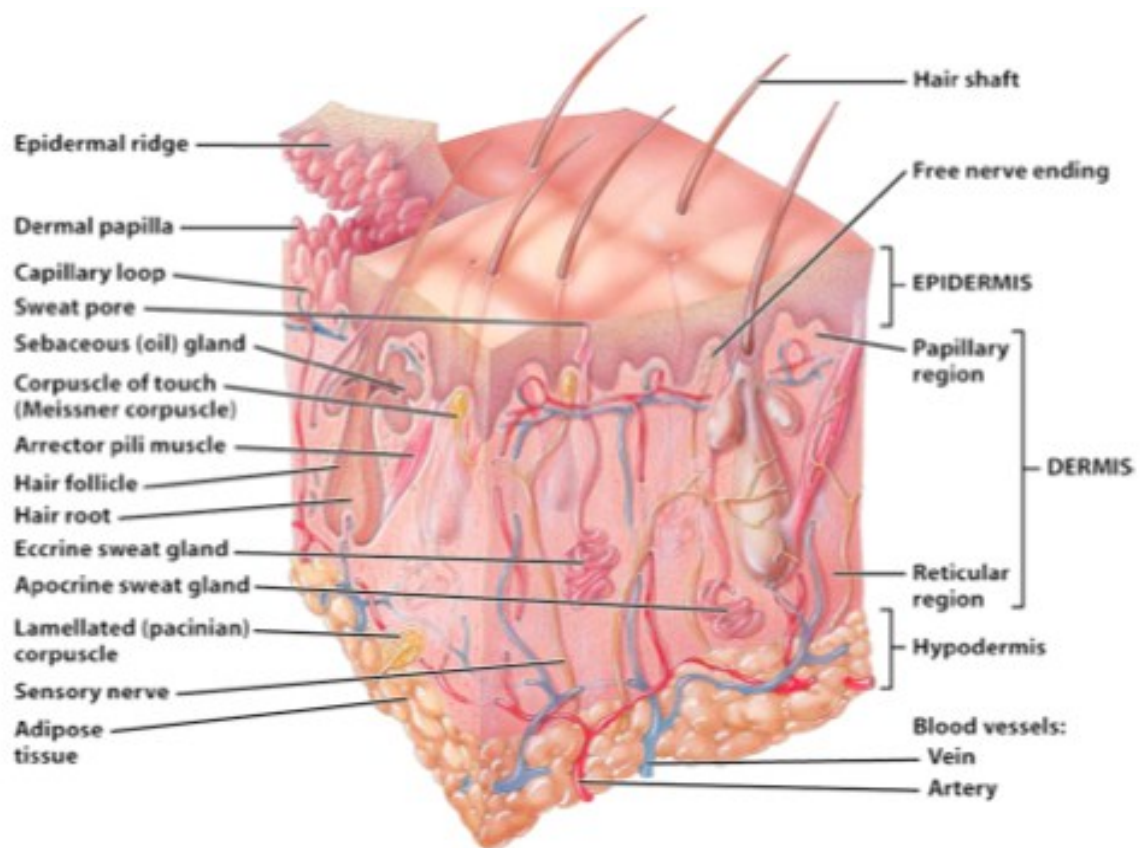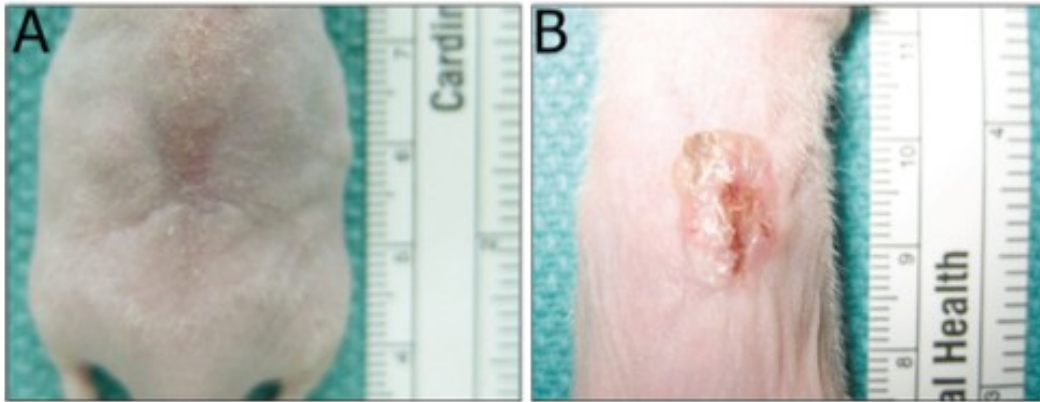
Sectional view of skin and subcutaneous layer

Figure 1-1 Skin anatomy

Structure of the section showing the different layers of normal human skin[1]

Figure 1-2 Hypertrophic scar and contracture

Hypertrophic scarring in 34-year-old man, 8 months after 60% total-body-surface-area burn of face, upper extremities, and hands[107]

FTSG   **STSG**

Figure 1-3 Morphological manifestations the HTS-like nude mouse

(A) Nude mouse with transplanted autograft skin (B) Nude mouse with transplanted split thickness human xenograft[93].
Morphological observation of athymic nude mice with transplanted full thickness human skin (FTSG) and split thickness human skin grafts (STSG)[92]
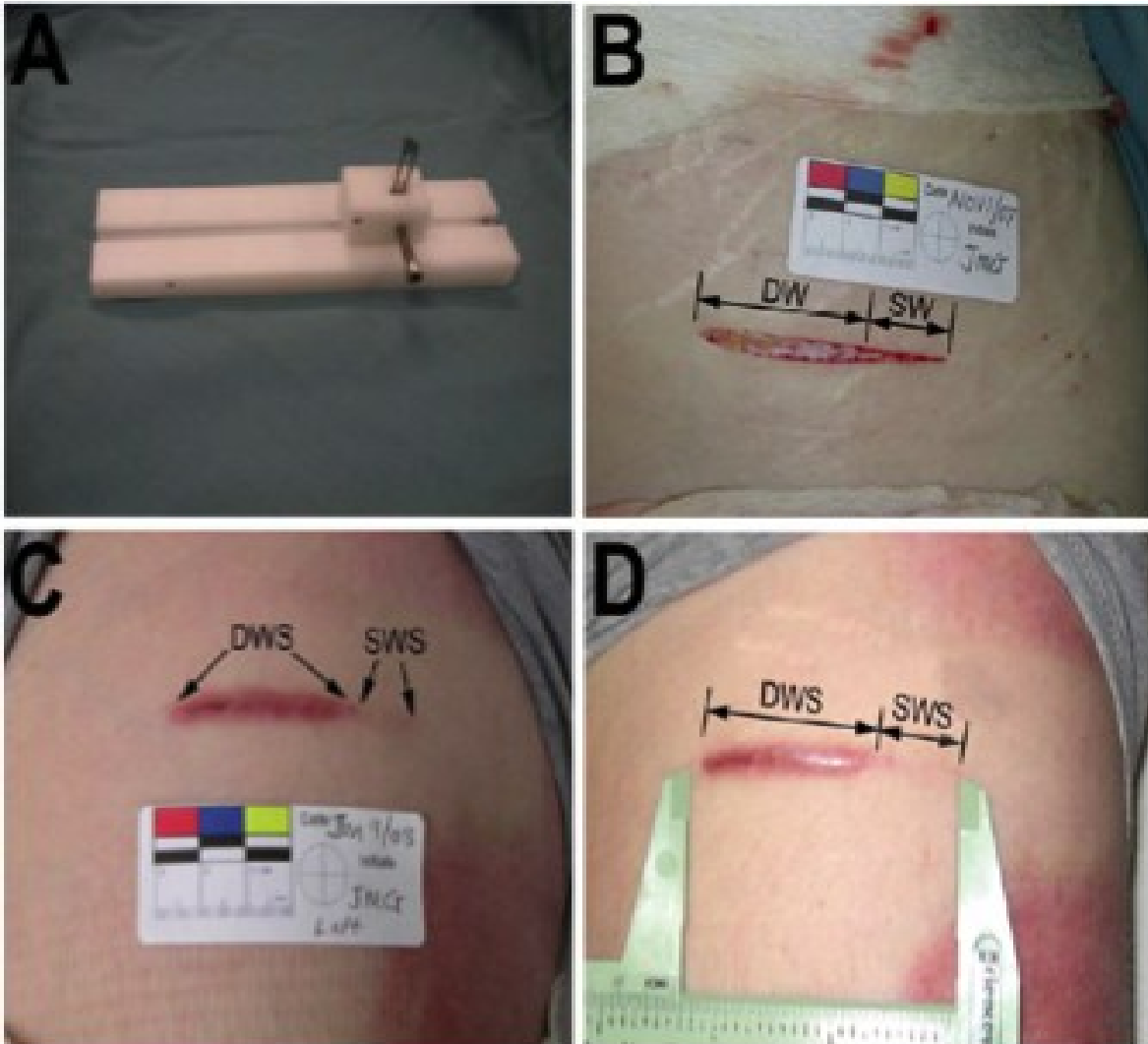
Figure 1-4 Scratch wound healing according to wound depth

(A) Jig used for the creation of scratch wound model. (B), Wound created on the anterior thigh at day 0. (C), Scratch wound 70 days after the wound. (D), Deep and superficial wound scar showing the DWS developed HTS and SWS shows minimal scar. DW, deep wound; SW, superficial wound; DWS, deep wound scar; SWS, superficial wound scar[45].
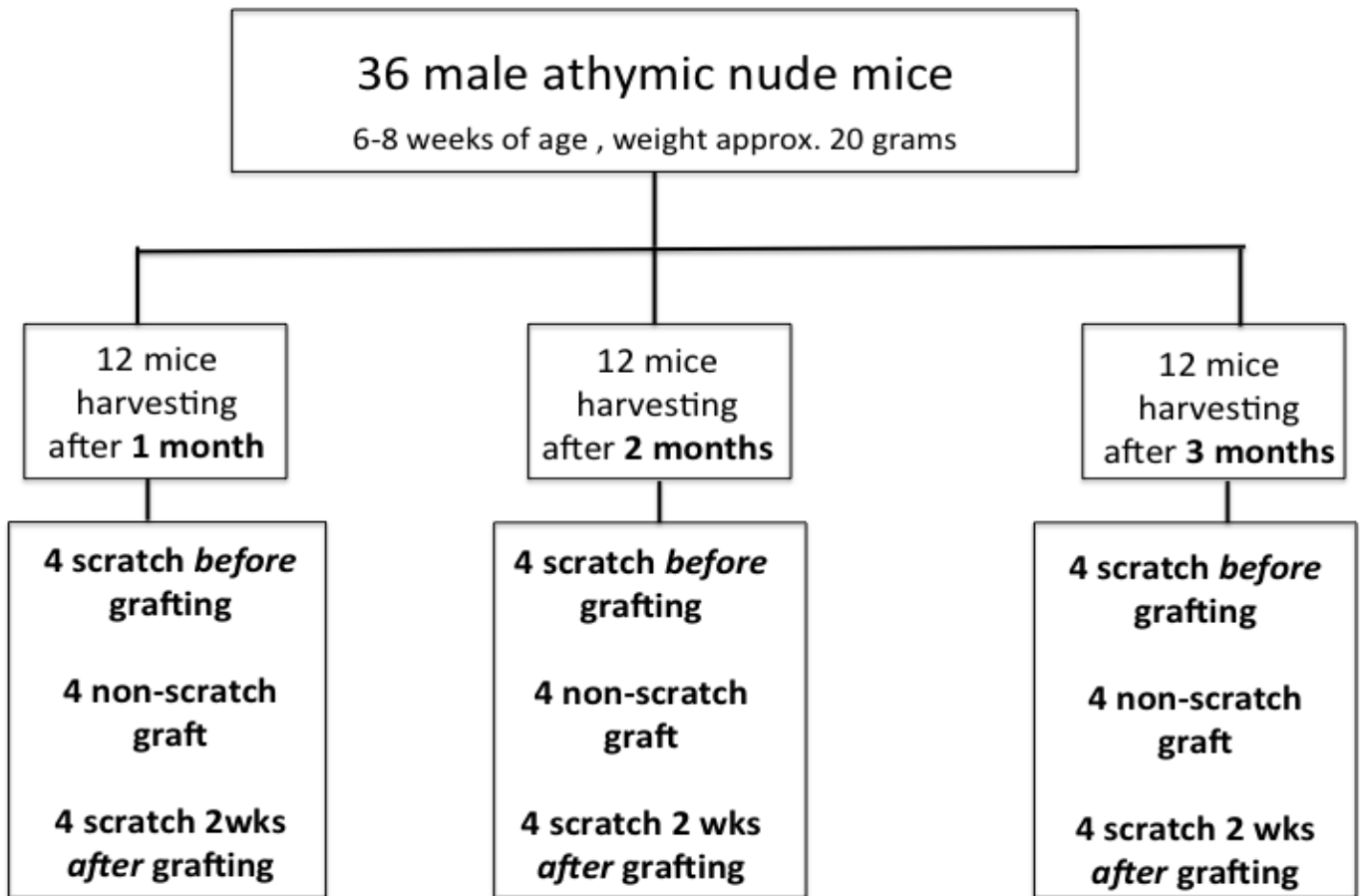
Figure 1-5 Study design

2 Chapter 2: A Novel Nude Mouse Model of Hypertrophic Scarring Using Scratched Full-Thickness Human Skin Grafts

2.1 Introduction

Skin wound healing is an extremely complex process that involves the reactions and interactions of inflammatory cells, growth factors, and cytokines [108]. This orchestrated process can be divided into four overlapped phases starting with hemostasis, followed by inflammation and proliferation, and ends by maturation including collagen remodelling to restore the damaged skin integrity through formation of a mature scar [9,108,109]. Hypertrophic scar (HTS) is a dermal form of fibroproliferative disease (FPD) that develops after burns and deep skin injuries or even planned surgical wounds[24]. It presents as hyperemic, elevated, firm scar not exceeding the boundaries of the original site of injury [72]. These scars often cause contracture deformities leading to permanent disabilities and aesthetic disfigurement and prolonged period of hospitalization and rehabilitation[110]. Increased cell proliferation, excess blood vessel formation, collagen deposition, and thin collagen fibers disorganized in the dermis in addition to the presence of □-smooth muscle actin (□-SMA)-expressing cells are the main histological features of HTS. Imbalance in the synthesis and degradation of extracellular matrix (ECM) components by fibroblast leads to the development of dermal fibrosis typical of HTS[15].

Despite many proposed treatments to date, there are still few if any reliable, efficient, forms of therapy for HTS. In addition, the current treatments of HTS requires prolonged

periods to be effective and tends to be expensive with considerable side effects [12,59]. One major investigative hurdle in dermal FPD is the lack of an ideal animal model, which responds to injury similar to humans[21,111].

Duncan et al. have described a progressively deep dermal injury in the lateral thigh skin of 113 human volunteers, which heals without scar in the superficial regions of the wound. However, in the deeper regions of the progressively deep injury a red raised HTS develops when the wounds exceeded 0.56 mm in depth[5]. Honardoust confirmed these findings and described increased TGF-β, large molecular weight proteoglycans and less decorin and type II TGF-β receptors in the deeper tissues typical of immature human HTS [45]. Fibroblasts from these deeper layers of the skin have been found to possess many or most features of HTS fibroblasts as compared to superficial dermal fibroblasts and site-matched normal skin fibroblasts. In contrast, considerable evidence supports the importance of bone marrow derived monocytes and fibrocytes in the development of HTS [30], However it is unclear the relative importance of resident fibroblasts in the deep dermis of the skin versus infiltrating immune cells in the development of HTS and their persistence in the remodelling scar over time.

Unfortunately few representative animal models of HTS exist currently and many investigators have questioned the value of therapies developed and tested in rabbits and pigs which do not appear to translate into effective therapies for dermal fibrosis in humans [89,90]. Thus, by creating wounds in human skin which are known to reliably develop HTS and transplanting the skin to athymic mice we seek to answer questions about the quantitative

importance of deep dermal fibroblasts resident in human skin and to clearly identify bone marrow derived cells in the fibrotic tissues and their role in the developing scar. Thus, it is our goal to develop this model to improve our understanding dermal fibrosis and allow for the development of novel and safe anti-fibrotic treatments that will reliably translate into effective therapies for fibrotic disorders in human skin [112].

2.2   Materials and Methods

2.2.1   Preparation of Skin Grafts

Full-thickness human skin samples were obtained from female patients who underwent elective abdominoplasty procedure following informed, written consent. Full thickness skin grafts were obtained from the lower abdominal skin, avoiding the damaged areas that contained stretch marks. Excessive subcutaneous fat was removed manually with curved iris scissors, before cutting it into 3.0×1.5 cm grafts with a scalpel. Thereafter a scratch wound was made on each graft using a no.11 scalpel blade in a specially designed modification of a jig originally described by Dunkin et al[5] as illustrated in (Figure 2-1). The modified jig creates a 2 cm long wound that is > 0.6 mm deep in each of the grafts. Thereafter the grafts were stored in sterile normal saline at 4°C until the time of grafting.

2.2.2   Transplantation of Skin Grafts

All animal experiments were performed using protocols approved by the University of Alberta Animal Care and Use Committee meeting the standards of the Canadian Council

on Animal Care. The mice were housed in a virus antibody-free biocontainment facility for the entire experiment and conditioned for two weeks before experimentation.

Thirty-six male athymic nude mice NU(NCr)-Foxn1[nu] at four weeks of age weighing ~25 grams were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). The animals were divided into three groups: mice grafted with human skin without scratch wound, mice grafted with human skin with scratch wound before grafting, and mice that were anesthetized before performing the scratch procedure in the engrafted human skin at two weeks after transplantation. There were 4 mice in each of the experimental groups.

In the prone position under isoflurane anesthetic (Halocarbon Laboratories, River Edge, NJ), the dorsal skin surface was disinfected with iodine. A 3.0 x 1.5 cm skin area was marked using a prefabricated plastic template as previously described [93]. The full thickness skin was excised using surgical scissors, leaving the panniculus carnosus intact. Then, full thickness human skin grafts with or without scratch wound were transplanted and sutured with 4-0 silk suture (Ethicon©, Somerville, NJ) using a tie-over bolus dressing technique with a non-adherent petrolatum (Xeroform™, Covidien, Mansfield, MA) and dry gauze to ensure adherence of the graft to the wound bed as previously described [92,93,113]. All animals received narcotic analgesia (Hydromorphone HP 10 diluted to 0.05 mg/mL, Sandoz, Boucherville, QC) subcutaneously for pain management following grafting. The dressing and the stitches were removed two weeks after the surgery. At this time, a scratch wound was made in the xenografted human skin tissue in 12 mice using the previously described jig for each of 3 time points. The graft morphology was monitored weekly by digital photography

using standardized conditions, wherein distance from the animal, lighting, and exposure were constant. Animals were euthanized at one, two, and three months post-grafting to harvest the human skin xenografts for histology and immohistochemistry analysis.

In separate experiments, two athymic nude mice were grafted with skin samples from an African-American female patient who underwent an elective abdominoplasty. Scratch wounds using the above-mentioned technique were made before grafting onto the back of nude mice to clinically observe the persistence of the dark melanin containing keratinocytes and dermal elements of the scratched grafts for one year after transplantation.

2.2.3 Histologic Analysis of Human Skin Xenografts

Biopsies were harvested from xenografts unscratched skin, or skin xenografts with scratch wound before or after transplantation at all three time points. Each was divided into three parts. The first part was fixed in 10% formalin (Zinc Formal Fixx™, Thermo Scientific, Pittsburgh, PA) for 24 hours, processed, and embedded in paraffin. These blocks were cut to 5 μm sections, mounted on glass slides and subjected to hematoxylin and eosin (H&E), Masson's trichrome, α-SMA, toluidine blue and picrosirius red staining. The second part was embedded and frozen in cryomatrix (Shandon Cryomatrix™, Thermo Scientific, Pittsburgh, PA). The frozen blocks were cut to 10 μm sections and mounted on glass slides that were used for macrophage and human leukocyte antigen (HLA)-ABC staining. The third part was snap frozen at −80°C for PCR and collagen production analysis.

2.2.4 Measurements of the Wound Area

As described previously [113] using the weekly photographs taken with a ruler in standardized conditions to document wound healing and scar formation, ImageJ software (National Institutes of Health, Bethesda, MD) was used to measure the graft area in the photos to assess the wound area over time.

2.2.5 Histological Analysis of Graft Thickness

Graft thickness was evaluated in hematoxylin and eosin (H&E) staining viewed under 100× magnification using bright field microscopy. The distance from the stratum corneum to the dermal-fat junction was measured using ImageJ software (National Institutes of Health, Bethesda, MD) in five random high powered fields in sections from each time point.

2.2.6 Immunohistochemical Analysis of Macrophages

As previously reported [92], frozen sections were warmed at room temperature for 30 minutes, fixed in ice-cold acetone for 5 minutes, then air-dried for 10 minutes. After blocking with 10% bovine serum albumin (BSA) (Sigma-Aldrich Inc., St. Louis, MO) for 1 hour, endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Sections were incubated with primary antibodies of rat anti-mouse F4/80 (eBioscience, San Diego, CA) at 1:100 dilution in 1% BSA overnight at 4°C. Thereafter, the secondary antibody, biotinylated rabbit anti-rat immunoglobulin G (IgG) (Dako, Glostrup, Denmark), was applied at 1:500 dilution in 1% BSA for 30 minutes at room temperature. Secondary detection was done after

incubation with VECTASTAIN® Elite avidin-biotin complex (ABC reagent) for 30 minutes, washing the slides with peroxidase substrate, 3, 3′-diaminobenzidine (DAB) (Vector Laboratories Inc). Finally, counterstaining was performed using hematoxylin and the sections were dehydrated through five changes of increasing concentrations of ethanol before mounting the slides with permount (Fisher Scientific Company, Fair Lawn, NJ) and covering with glass coverslips. For negative controls, staining with the primary antibody was replaced with 1% BSA. F4/80 positive macrophages were counted in five random HPFs under ×200 magnification in all sections.

2.2.7 Immunohistochemical Staining for α-SMA Expressing Myofibroblasts

Paraffin embedded human xenografts sections were used to stain for α-SMA as previously reported [93,113]. Sections were deparaffinized and rehydrated in five decreasing gradients of ethanol, then treated for antigen retrieval with 0.05% trypsin before blocking with 10% BSA for one hour. Thereafter, sections were incubated with primary antibody of rabbit anti-αSMA (Millipore, Billerica, MA), 1:1000 dilution in 1% BSA overnight at 4°C. The secondary antibody of goat anti-rabbit immunoglobulin G (IgG) (Dako, Glostrup, Denmark) was applied at 1:500 dilution in 1% BSA for 30 minutes at room temperature. Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) was applied for 30 minutes and washed with peroxidase substrate 3, 3′-diaminobenzidine (DAB) substrates (Vector Laboratories Burlingame, CA) before counterstaining with hematoxylin. Dehydration through five changes of increasing gradient ethanol was made before mounting the slides

with permount (Fisher Scientific Company, Fair Lawn, NJ) and glass coverslips. Staining with primary antibody was omitted for staining control resulting in negative staining. Brown positively stained cells were counted in five random HPFs under ×200 magnification in all sections.

2.2.8 Toluidine Blue Staining for Mast Cells

Toluidine blue staining was used to detect mast cells as previously described[92,93]. Paraffin sections were deparaffinized and rehydrated through five changes of decreasing gradient ethanol. Sections were then incubated with toluidine blue (Toluidine Blue O, Fisher Scientific Company, Fair Lawn, NJ) solution for two to three minutes, followed by washing in distilled water. Finally, sections underwent dehydration by five changes of increasing gradient ethanol before clearing them with two changes of xylene and mounting with permount (Fisher Scientific Company, Fair Lawn, NJ) and glass coverslips. Positively stained red purple mast cells were quantified in five random HPFs in all sections.

2.2.9 Masson's Trichrome Staining for Collagen Bundle

Masson's trichrome staining was used to detect collagen bundles in the dermis. Paraffin-embedded sections of human HTS, human normal skin, and skin graft biopsies from mice were deparaffinized and rehydrated before staining in Weigert's iron hematoxylin, Biebrich scarlet acid, and phosphomolybdic-phosphotungstic acid for 10 minutes each. The sections were then transferred directly to aniline blue and stained for 10 minutes further.

After rinsing in distilled water and differentiating in 1% acetic acid for five minutes, the sections were dehydrated and mounted. Collagen fibers were visualized in green, nuclei in black, and keratin in red under bright field microscope.

2.2.10 Picrosirius Red Staining for Collagen Orientation

Using paraffin embedded sections as previously described [93,114], picrosirius red staining was used to assess the birefringence of the collagen fibers of human HTS, human normal skin, and human xenograft biopsies from mice. Briefly, sections were deparaffinized and rehydrated through five changes of decreasing gradient ethanol before incubating them in Sirius red (Sigma-Aldrich Inc., St. Louis, MO) and picric acid (Sigma-Aldrich Inc., St. Louis, MO) solution for 1 hour at room temperature. Thereafter, sections were washed in two changes of acidified water, dehydrated in 100% ethanol, and cleared in xylene. The slides were then mounted with permount (Fisher Scientific Company, Fair Lawn, NJ) and covered with glass coverslips, then examined using a polarizing microscope (AxioIm- ager.A1, Carl Ziess MicroImaging Inc., Thornwood, NY).

2.2.11 Human HLA-ABC Staining for Evaluation of Human Skin Survival in the Mouse Body

To assess the human skin survival as previously reported [93], frozen sections were warmed at room temperature for 30 minutes, fixed in ice-cold acetone for 5 minutes, then air-dried for 10 minutes. After blocking with 10% bovine serum albumin (BSA), sections were

incubated overnight at 4°C with FITC-labeled anti-human HLA-ABC antibody (Accurate Chemical & Scientific Corp., Westbury, NY) diluted to 1:50 in 1% BSA. Samples were washed with Tris-buffered saline (TBS) before mounting. The mounted slides were stained with ProLong Gold antifade reagent with DAPI (Life Technolo- gies, Carlsbad, CA) and sealed with coverslips and examined with fluorescence microscopy Zeiss AxioImager M2 instrument (Jena, Germany). Primary antibody was omitted resulting in negative staining.

2.2.12 Statistical Analysis

Four mice for each time point and each group were used. Statistical analysis was performed using analysis of variance (ANOVA) with Tukey multiple comparison in Prism 6 for Mac (GraphPad Software, Inc.). Data were expressed as mean ± standard error (SE), with significance set at P-value ≤ 0.05

2.3 Results

2.3.1 Morphological observations

The transplanted human skin grafts were clinically viable throughout the experiment. After removing the dressing from the mice, the xenografts were soft and pink, and the scratch area healed. At two weeks, the scratch area was completely closed and the whole xenograft had started to become elevated and hardened compared to the surrounding mouse skin. Furthermore, at two months after the procedure, the xenografts started turning red, raised and indurated more than the normal mouse skin. These features persisted to the end of the study

showing features of HTS and the scratched area over the grafts healed similar to adjacent grafted human skin. Nevertheless, the scratch wound grafts, whether before or after grafting, contracted more than the non-scratch wound grafts (Figure 2-2). The two scratched xenografts from an African-American skin sample healed in the same fashion and the scratched wounds healed with same color of the adjacent human skin and were viable for one year after grafting (Figure 2-3). This showed a morphological evidence of human cell viability and their contribution in the development of the fibrotic xenografts.

The total wound area of engrafted tissues as quantitated and displayed in (Figure 2-4) suggested that the wound area of the grafts continued to contract in all the groups over time. There was no significant difference between the three groups at the first month post-transplantation. However, the scratch wounds before and after grafting resulted in more graft contraction compared to the non-scratched skin grafts at 2 months post-transplantation: (1.83 ± 0.073 cm^2 and 1.88 ± 0.054 cm^2 vs. 2.51 ± 0.011 cm^2, respectively $P < 0.05$) and at 3 months post-transplantation (1.68 ± 0.076 cm^2 and 1.63 ± 0.15 cm^2 vs. 2.12 ± 0.07 cm^2, respectively $P < 0.05$).

2.3.2 Scratched grafts developed similar histologic characteristics of HTS

Quantification of xenografted human skin tissue showed a gradual increase in xenograft thickness of all the groups over time. The thickness of xenografts from mice grafted with the scratched human skin performed either before or after grafting peaked at two months, then decreased, while the non-scratched xenografts continued to show a steady

increase in thickness (Figure 2-5). The skin grafts in the deep dermal scratch performed before and after grafting the mice were thicker at two months as compared to the intact non-scratched grafts (2.80 ± 0.19 and 2.9 ± 0.13 vs. 1.68 ± 0.15 mm, respectively $P < 0.05$) post engraftment. Nevertheless, the presence of a deep dermal injury in the skin grafts, whether performed either before or after grafting, resulted in no significant difference in dermal thickness and fibrosis compared to intact non-scratched grafts at one and three months post engraftment.

Dermal collagen organization and accumulation were assessed using Masson's Trichrome stain in xenografts from all time points (Figure 2-6), which demonstrated that the normal basket-weave pattern of the collagen fibers seen in normal human skin was replaced with thin collagen fibers that were oriented parallel to the skin surface in all the grafts, which is similar to human HTS. Using picrosirius red staining and polarized light microscopy collagen fibers in the human skin grafts appeared thin with yellow-orange birefringence similar to human HTS in contrast to the collagen fibers in the normal human skin, which are thicker with a basket-weave morphology (Figure 2-7).

2.3.3 Scratching the grafts increase α-SMA expressing myofibroblasts formation

Immunohistochemistry staining for □-SMA detected and quantified myofibroblast formation in xenografts in all time points (Figure 2-8A). A high density of spindle-shaped cells was present in the xenografts at each time point, similar to human HTS but different from normal human skin, where the α-SMA expressing cells appeared to be vascular smooth

muscle cells associated with endothelium [12]. Quantification of the numbers of the α-SMA-expressing cells in the scratch wounds before or after grafting respectively demonstrated significantly increased α-SMA-expressing cells at two months as compared to non-scratched wound xenografts (35.0 ± 1.5 and 33.8 ± 2.3 versus 22.8 ± 2.3 cells per HPF, respectively $P < 0.05$) which may be related to the higher contractility of the skin grafts seen in the scratch wound grafts which was significantly different at the peak of contraction at two months post transplantation. Furthermore, the scratch after grafting xenografts showed more α-SMA-expressing cells than non-scratched xenografts at 3 months post transplantation (20.4 ± 1.8 versus 13.0 ± 1.1 cells per HPF, p= 0.0142) (Figure 2-8B).

2.3.4   Retention of human skin cells post-transplantation

Staining for HLA-ABC was used for the detection of the surviving of human cells in the skin grafts on the back of the mice where HLA-ABC antigens was used to detect nucleated human cells. Using direct immunofluorescent staining for HLA-ABC in skin grafted tissues at all time points, retention of green staining patterns in the epidermal and dermal cells of the grafts was similar to the staining seen in fresh human skin compared to the negative control mouse skin where little or no staining was present. This confirms the retention of viable human cellular elements in the tissues engrafted for several months post transplantation even in the grafts which sustained the deep dermal scratch performed prior to or at two weeks post transplantation (Figure 2-9) rather than a gradual replacement of human cells in the skin grafts with mouse cells infiltrating from the wound bed.

2.3.5 Scratch wounds increase macrophage infiltration in human skin xenografts

Immunohistochemistry staining for F4/80 was used to detect mouse macrophages in all tissue sections from all time points (Figure 2-10A) where increased density of F4/80-expressing cells was apparent in all grafts from all time points compared to the normal mouse skin. Quantification of the macrophages number in xenografts revealed that within the first month, the xenografts with the deep dermal scratch wounds performed before or after grafting had a higher number of macrophages than the non-scratch wound grafts (11.4 ± 1.47 and 12.2 ± 1.42 vs 7.0 ± 0.83 in 5 HPFs, respectively P < 0.05). At two months after grafting, macrophages showed the highest number in all groups; however, there was no significant difference between them. At three months after grafting, macrophages in the scratch wound xenografts, whither before or after transplantation were higher than the non-scratch wound xenografts (10.2 ± 0.66 and 10.2 ± 1.4 vs 6.0 ± 0.54, respectively P < 0.05) (Figure 2-10B). This suggests a persistent inflammatory response caused by the deep dermal scratch wound, which is a feature fibroproliferative disorders, such as human HTS [9,12].

2.3.6 Mast cells in the human skin xenografts

Increased mast cell density have been reported in human HTS [115], as well as in animal models of HTS[92,93], which has been suggested to account for the pruritus and pain in fibrotic tissues associated with mast cells degranulation[116]. Using toluidine blue staining, mast cells were significantly increased in xenografts at all time points (Figure 2-11A) as compared to

the normal human skin. Mast cell numbers peaked at two months, then significantly decreased at three months for all the groups. The mast cell counts for the non-scratch group were $(10.0 \pm 1.54, 15.60 \pm 0.68,$ and $6.20 \pm 1.4)$ mast cells per HPF respectively after one, two, and three months as compared to the scratch before grafting group with the deep dermal scratch performed prior to transplantation $(10.6 \pm 2.12$ and 14.6 ± 0.92 and $6.40 \pm 0.51)$ and the deep dermal scratch after transplantation $(14.0 \pm 0.71$ and 19.0 ± 0.45 and $8.20 \pm 2.1)$. However, there was no significant difference in the mast cell count between the scratch wound grafts, whether before or after grafting, and the non-scratched grafts in all time points (Figure 2-11B).

2.4 Discussion

Despite considerable research in skin wound healing, the pathogenesis of dermal fibrosis is still poorly understood[9]. The lack of an ideal animal model representative of human dermal fibrosis limits our understanding on the underlying mechanisms of fibrosis and hinders the accuracy and applicability in testing the effectiveness and safety of novel anti-fibrotic therapies. Unfortunately HTS develops only in human and not animal skin which makes the reliability of therapies effective in animal tissues using commonly used models of scarring to date such as the rabbit ear [89] or the dorsum of the red Duroc pig, of questionable translational value for human fibroproliferative disorders [101].

Various reports attempt to describe the animal models of HTS, including the rabbit ear model[89], by making a deep incision overlying the ear cartilage of the rabbit that

developed inflamed and hypertrophied scar. The ability of making many scars in each ear and using multiple treatments are the main advantages of this model [117,118]. The wound environment in this model is different from that of the dermal fibrosis, wherein the ear cartilage, as well as the dermis, undergoes hypertrophy which contributes to scar thickness but it not truly representative of dermal scarring in humans where perichondral hypertrophy is not a interactive factor in the scar humans develop.

Because pig skin has been found to more closely resemble human tissues in terms of the lack of contraction due to an absence of panniculous carnosis and the dependence on external sources of Vitamin C to support collagen metabolism, the female red Duroc pig have been used to develop an HTS-like model by making deep wounds onto the back of the animal which results in a thick scar[101]. However, this scar is not erythematous and raised typical features of human HTS but instead depressed, making it morphologically dissimilar to human HTS in addition to being an expensive and more difficult to handle model, which limits its use.

Recently the use of subcutaneous osmotic pumps in nude mice delivering bleomycin have resulted in the development of a fibrotic scar over the back of the animals[119] which has some features similar to human HTS. However, the need for continuous delivery of bleomycin for the development of fibrosis which resolves quickly after discontinuing the drug makes the model worthy of consideration but the tissues affected are murine, the morphologic features differ significantly from human HTS and the testing of antifibrotic therapies will be complicated and difficult in this model.

The athymic nude mouse model was established by Yang et al.[91] by transplanting full thickness human skin onto the back of the mice. This procedure developed a graft that had features similar to that of human HTS. This model has been modified more by transplanting split thickness skin graft that also developed similar characteristics of human HTS [92,93]. Because HTS occurs after injury to a critical depth in the dermis of human skin [5], we attempted to test the deep dermal scratch model validated in humans to develop a reproducible typical HTS to the skin when the injury exceeds 0.56 mm in depth in the dermis, to similarly injured human skin either before or after transplantation onto the athymic nude mouse. Deep dermal injury to the human skin either before or after transplantation successfully engrafts and subsequently developed thickened raised scars, which persisted beyond one year. Re-epithelialization occurred by migration of adjacent human epithelial cells in the engrafted human skin similar to human skin injuries [120]. The raised, thickened dermal scar that develops after deep dermal injury in the transplanted human skin contains substantial numbers of fibroblasts, mast cells, myofibroblasts, and macrophages very typical of human HTS [121], and while some of these cells stained for human ABC antigen in the skin, others may arise from the bone marrow of the mouse which is yet to be conclusively determined.

In the past bone morrow derived cells such as fibrocytes, macrophages, and mast cells have been confirmed present in the healing human skin engrafted on the nude mice, which likely play an important regulatory role in scar formation[122]. While many of these immunologic cells are likely derived from the mouse, murine immunology is well

characterized as compared to other animal species and resembles human immune responses, which further adds to the unique features of the model which are similar to human responses to injury [121].

Prolonged inflammation is highly associated with dermal scarring [121]. Inflammatory cells infiltration is essential for wound repair; however, increased amounts and activity of the macrophage, especially in the maturation phase, could cause an increase in ECM deposition and lead to fibrotic scar[123]. In this study, increased macrophages infiltration was observed in all the grafts as compared to normal skin. However, scratching the grafts before and after transplanting induced more macrophage infiltration to the grafts that were significantly observed at three months, indicating prolonged inflammation where the increased numbers of macrophages likely lead to increased ECM deposition though paracrine cytokine production similar to human HTS [41]. The macrophage is an important immune cell in wound healing. It has multiple functions during skin repair, including phagocytosis, antigen presenting, and cytokines and chemokines production, which promote angiogenesis, fibroblasts proliferation, and collagen synthesis [124]. It is a key cell that coordinates the wound healing process throughout the multiple phases [123,125]. It has been found that mice genetically engineered to produce non-functional macrophages at different stages of healing have inadequate wound repair due to a delay in re-epithelialization and neovascularization in addition to improper granulation tissue formation after depletion of macrophages during the inflammatory phase of wound healing [125,126]. Depletion of macrophages during the maturation phase did not show any significant delay in wound repair [127]. However, alternative techniques exist for timely

depletion of macrophages such that the role of macrophage function during different phases of scar formation after deep dermal injury to the human skin transplanted onto athymic mice as described herein will be helpful to understand more their effects in HTS formation as well as to test novel drugs targeting macrophages.

Other bone marrow derived cells, which are important in fibrotic disorders, include mast cells, which were increased significantly in all three types of xenografts by two months post transplantation as compared to normal skin. However, there was no significant difference among the scratched and non-scratched grafts at all time points. Mast cells have several roles in wound healing. They release many mediators from their granules after being stimulated by skin injury[116]. They promote inflammation, reepithelialisation, and increased vascular permeability and angiogenesis [128]. Moreover, histamine increases fibroblast proliferation and its differentiation into contractile myofibroblast[129]. Several studies have shown mast cells involvement in scar formation by affecting collagen maturation and remodelling [130,131]. Although increased mast cell number and activity occurs in HTS [132] lower mast cell numbers have been found in scarless regenerative wound healings such as fetal wounds and oral mucosal wounds[133,134]. It has been reported that wounds in mast cell deficient mice heal with minimal scar tissue compared to the wild type suggesting that a significant role of mast cells exists during fibrotic scar formation in this model [133,135].

An important unique feature of the deep dermal scratch in this model is the significantly increased number of α–SMA expressing myofibroblasts observed at two months post-transplantation as compared to the intact human skin without the deep dermal injury.

This finding correlates with the increase in wound contraction and reduced graft area observed as compared to the non-scratched model. The increase in scar thickness also suggests an increase in the synthesis and deposition of ECM [136]. Myofibroblasts have an important role in wound healing by inducing wound closure, collagen secretion, and reorganizing ECM [137]. They may originate from several cell types, such as pericytes, chondrocytes, osteoblasts, and circulating fibrocytes; however, most appear to arise from local connective tissue fibroblasts [138]. The differentiation of myofibroblasts from the local fibroblast is likely induced by the transforming growth factor-β1(TGF-β1) which has been reported to increase the expression of myofibroblasts; whereas, reduction in transforming growth factor-β inducible early gene (TIEG) knock out mice decreases both wound contraction and myofibroblast infiltration in tissues supporting the importance of TGF-β1 for myofibroblasts differentiation, wound contraction and fibrotic matrix accumulation [139]. Previously, we have described increased bone marrow derived fibrocytes in the transplanted human skin in the nude mouse model which can be an additional source of TGF-β for indirect stimulation of myofibroblast differentiation [92], or also directly forming the myofibroblastic phenotype from fibrocytes recruited to the site of tissue injury [140]. In normal wound healing, the myofibroblasts undergo apoptosis and disappear from the granulation tissue after wound closure; however, in HTS, these cells fail to undergo apoptosis and continue to be present in the fibrotic tissue similar to our findings. This persistent expression of α-SMA myofibroblasts in all the time points in the mouse model is a consistent feature of human HTS [141].

Thus the presence of increased numbers of bone marrow derived macrophages, mast cells and fibrocytes associated with the increase in dermal fibrosis in the deep dermal scratch model suggests that an important role exists for systemically derived cells in the fibrosis in human skin. This is in addition to the local activation of unique HTS-like fibroblasts present in the deep dermis, which we have previously shown to very closely resemble the fibroblasts found in HTS as compared to fibroblasts found in the upper layers of the dermis or in site-matched normal skin [30,45]. Bone marrow derived cells appear to be recruited to the injured tissue in this model and they may promote angiogenesis, cytokines and chemokines production, and ECM deposition as described in other models of wound healing[142,143] In the future, this model may permit ongoing investigation into the relative importance of locally derived fibroblasts versus bone marrow derived cells in human fibrosis so that more effective antifibrotic strategies can be developed [143]. The deep dermal scratch will facilitate topical application of antifibrotic agents to determine the effectiveness of local versus systemic antifibrotic therapies. The creation of the deep dermal scratch in the human skin performed before transplantation results in fibrosis very similar to that which occurs following deep dermal scratch after the human skin has been successfully grafted. This will increase the usefulness of the model where the deep dermal scratch created *ex vivo* prior to transplantation is much easier to perform and safer than injuring the mice *in vivo* and avoids the need to burn or create other injuries to the animals *in vivo* thereby minimizing the suffering of animals used to study human fibrotic disorders.

2.5   Conclusion

In conclusion, we observed that human skin grafts transplanted to athymic mice with a deep dermal injury created either ex vivo or in vivo developed a thicked contracted fibrotic scar with similar morphologic and histologic features of human HTS. In addition, human cellular elements are retained in the fibrotic tissues after transplantation. As compared to the non-scratched model of human skin on the transplanted in the athymic model previously described, the deep dermal injury had increased wound contraction, scar thickness and fibrosis and prolonged inflammatory cell infiltration including macrophages, mast cells and α-SMA expressing myofibroblasts. Because creating the deep dermal injury in the transplanted human skin either before or after transplantation lead to very similar degrees of fibrosis, creating deep dermal injury to human skin ex vivo prior to transplantation will afford a representative, affordable, ethical and practical model for studying the local and systemic factors involved in scarring in human skin tissues in the future. The fibrosis which develops and persists in human skin in this animal model will also facilitate the investigation of both local topical and systemic novel antifibrotic therapies with likely improved translational effectiveness in human dermal fibrotic conditions including HTS.
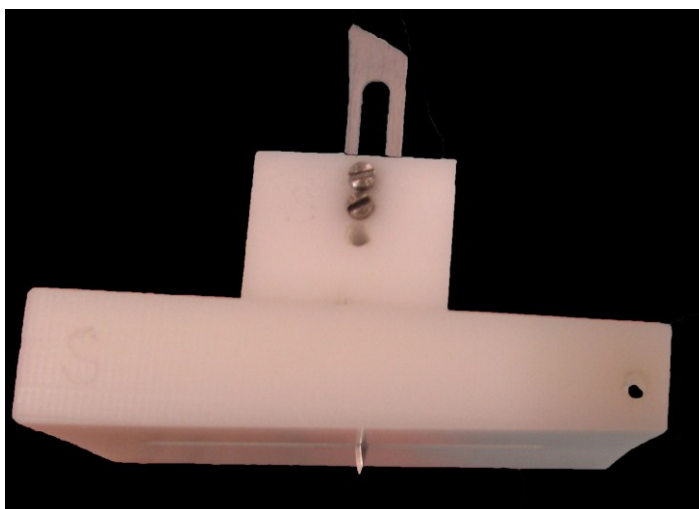
Figure 2-1 The jigsaw used to make the scratch wound
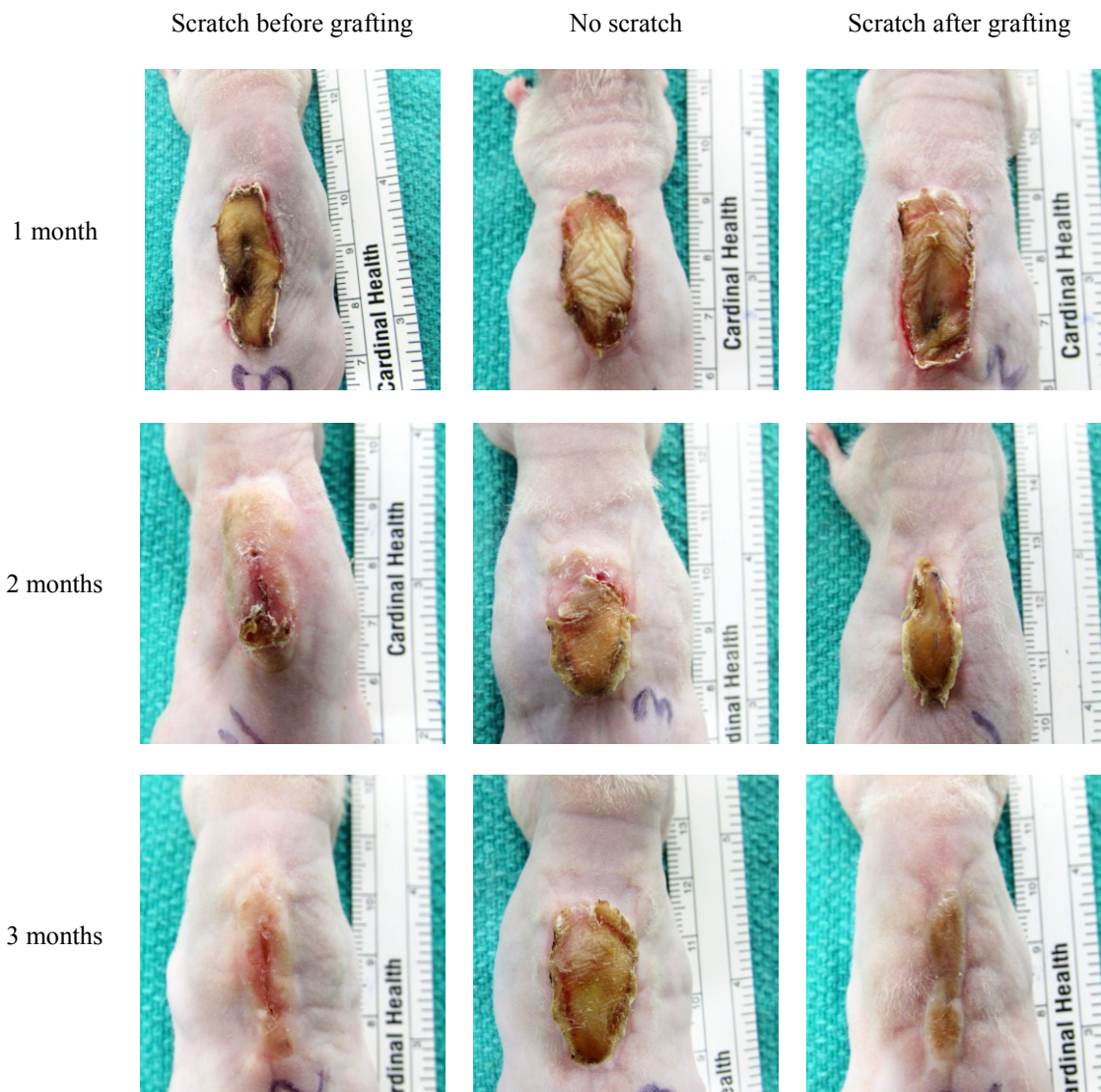
Figure 2-2 Scratched grafts shows similar morphological features of HTS

Morphological observation of the grafts development over time showing the red, elevated grafts compared to the mouse skin. In addition, the scratched grafts (before or after grafting) contract more than the ones without scratch.

2 weeks post grafting   1 year post grafting

Figure 2-3 Morphological observations of the dark skin grafts after 1 year

Two athymic nude mice were grafted with human dark skin xenograft with scratch wound before grafting. The graft survived and the scratch in it healed preserving the color of the human skin for one year after grafting.
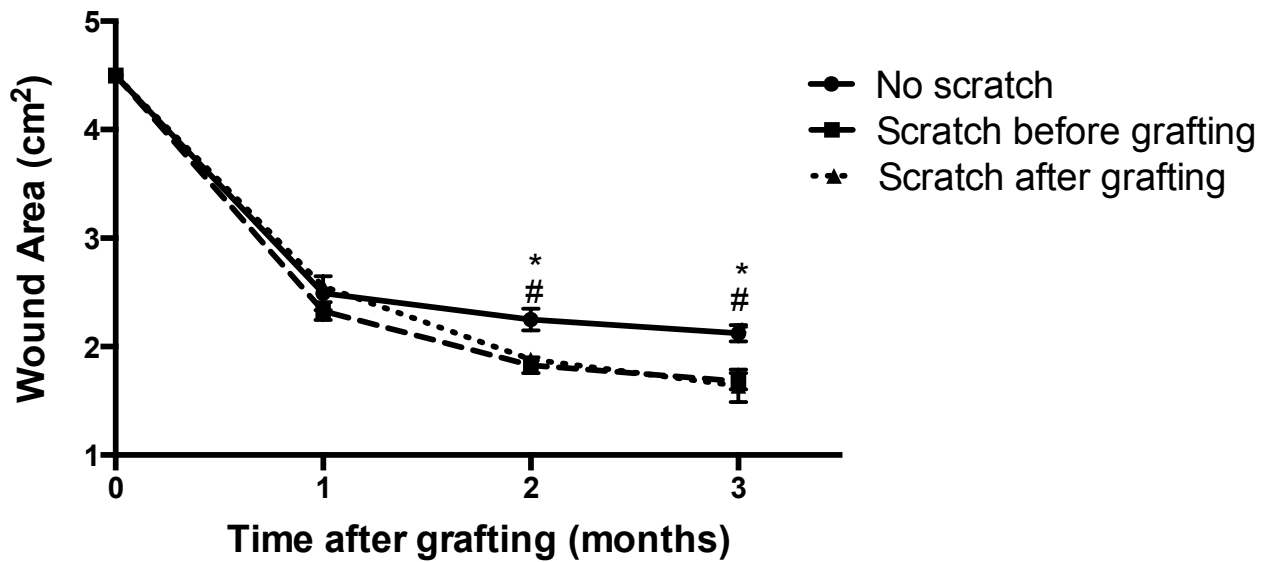
Figure 2-4 Scratch wound cause more graft contraction

Representative image of circled wound area and the quantification of the wound areas over time after grafting measured using ImageJ software. No scratch grafts (circle), scratch before grafting (square) and scratch after grafting (triangle). # $P< 0.05$ compared between the scratch before grafting and no scratch group in the same time point. * $P< 0.05$ compared between the scratch after grafting and no scratch group in the same time. Results are expressed as the (mean $\pm$ SE, n=4).
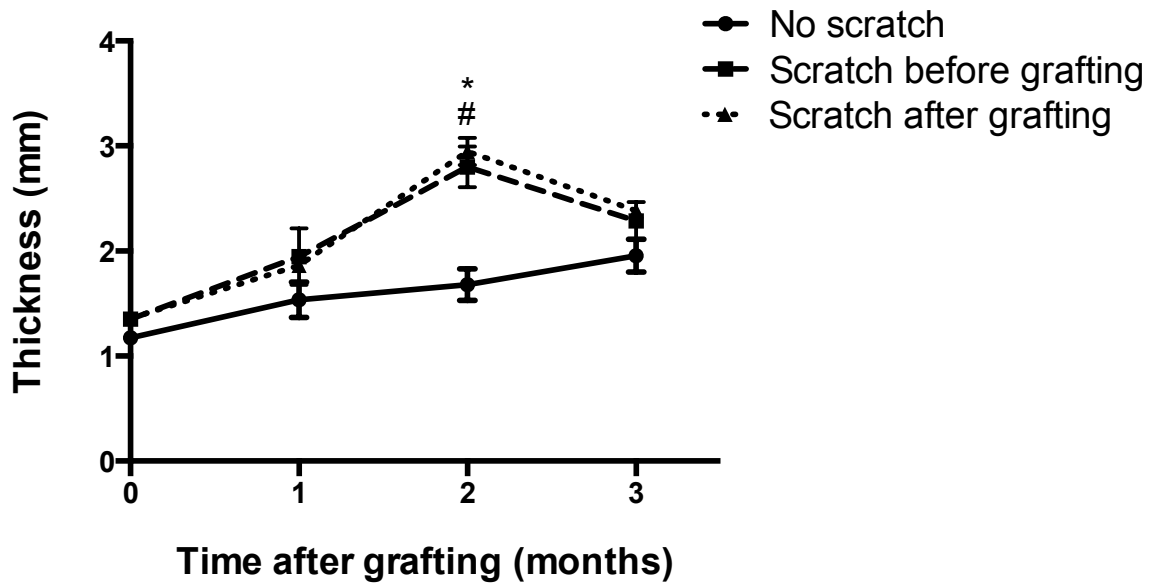
Figure 2-5 Scratch wounds (before and after grafting) increase graft thickness in 2 months then decreased

Dermal thickness of the grafted skin, the distance was measured from the stratum corneum to the dermal-fat junction in five randomly selected site/field. No scratch grafts (circle), scratch before grafting (square) and scratch after grafting (triangle). # $P< 0.05$ compared between the scratch before grafting and no scratch group at the same time point. * $P< 0.05$ compared between the scratch after grafting and no scratch group at the same time. Results are expressed as the (mean ± SE, n=4).
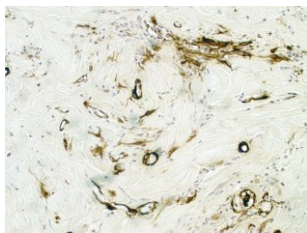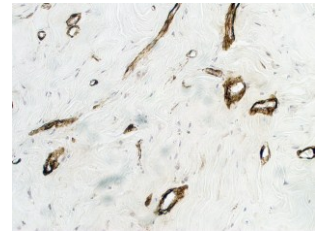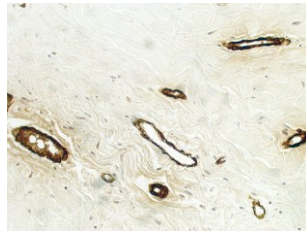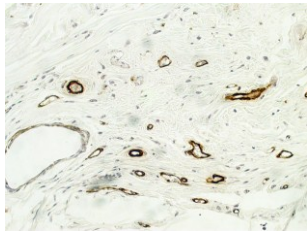
Normal skin   HTS

Scratch before grafting   No scratch   Scratch after grafting

1 month

2 months

3 months
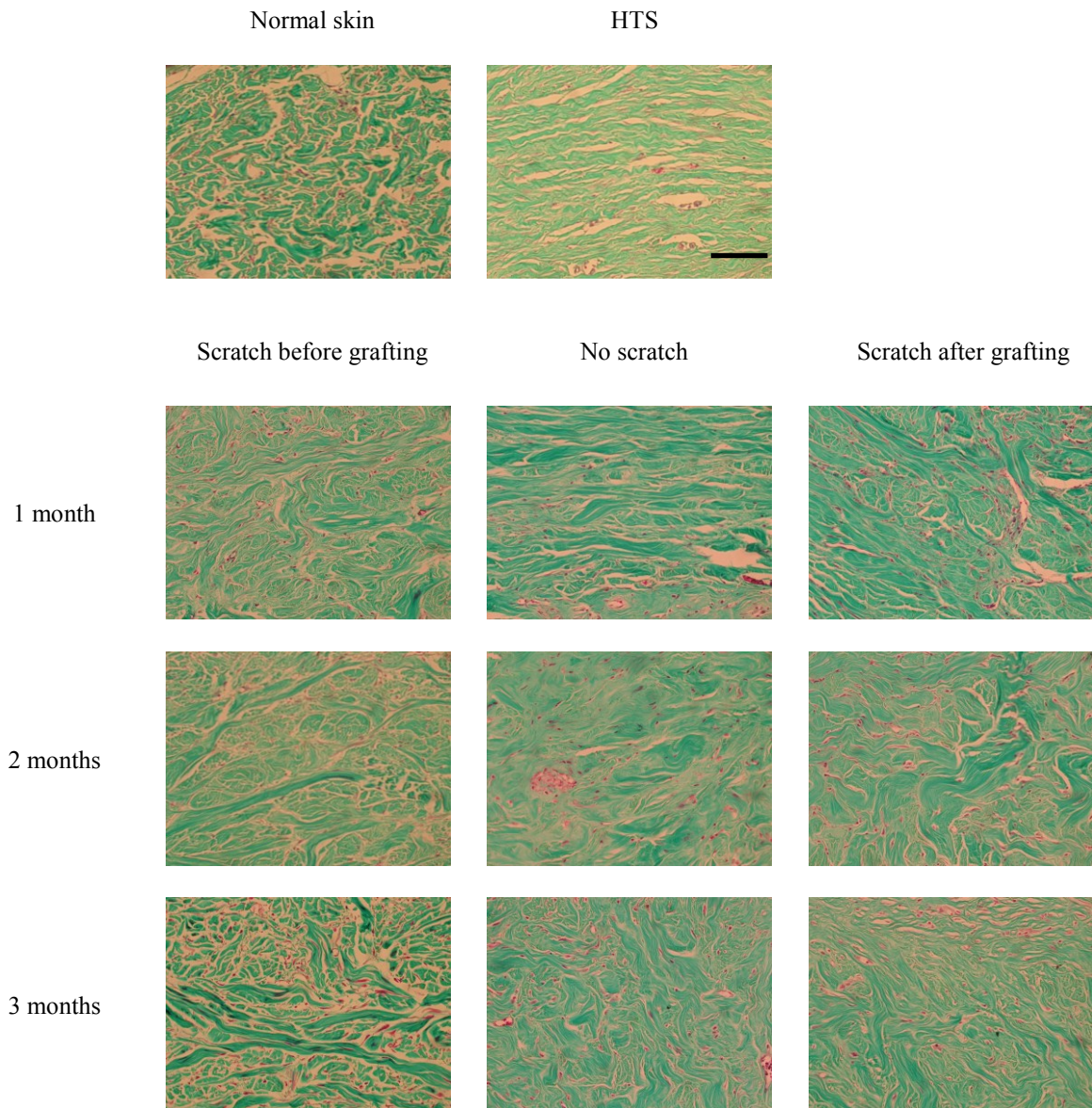
Figure 2-6 Masson's Trichrome staining of the xenografts displays similar collagen orientation to HTS

Representative images of Masson's trichrome staining tissue sections of human normal skin, human hypertrophic scar (HTS) and xenografs from all time points. Showing basket-weave collagen bundle in normal skin in contrast to the whorled collagen bundle of xenografts that are consistent with HTS. Scale bar=100μm.
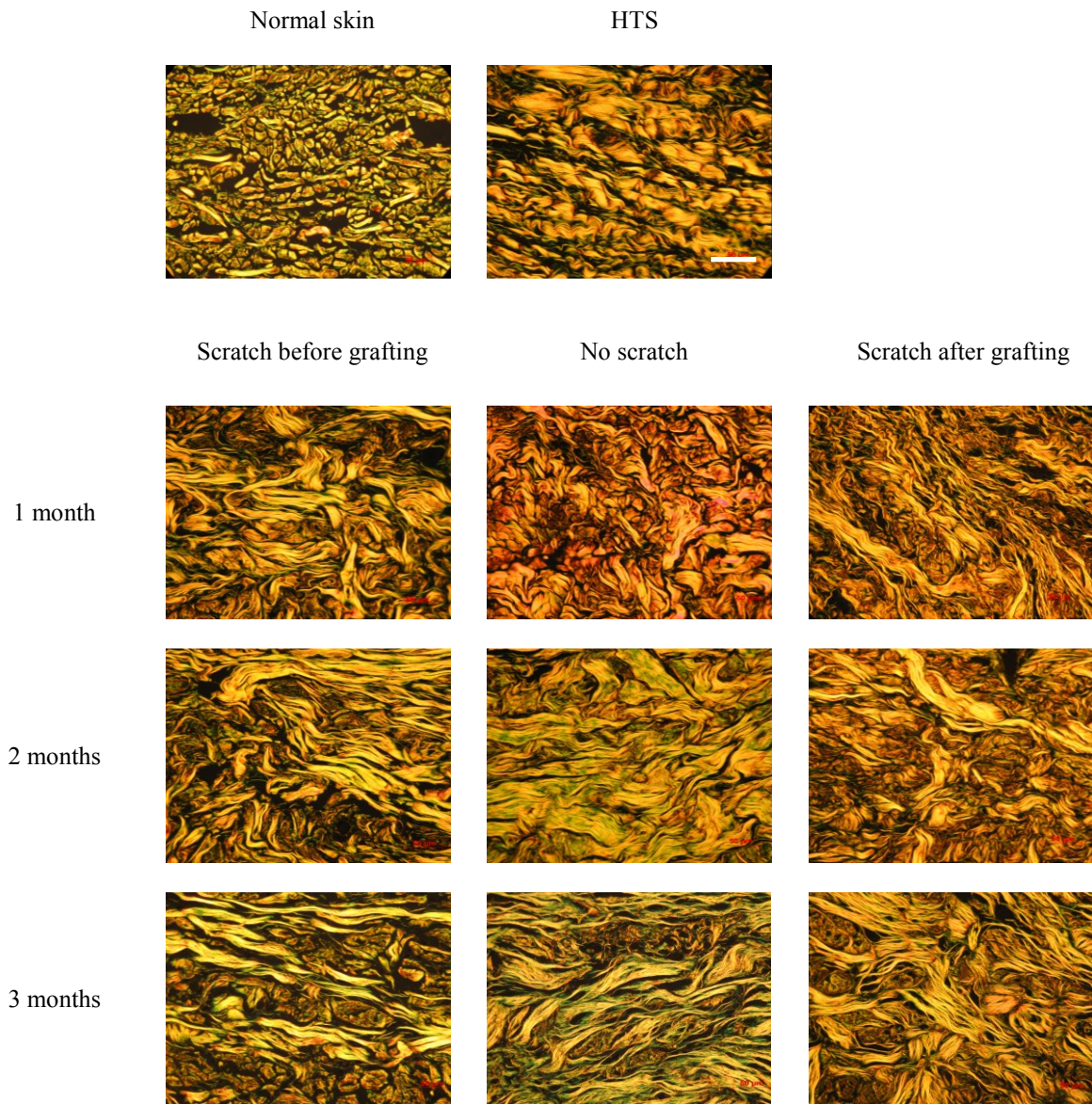
Figure 2-7 Picrosirus red staining of the xenografts displays similar collagen morphology to HTS

Representative images of a picrosirius red stained sections of human normal skin, human hypertrophic scar (HTS) and xenografs from all time points. Showing randomly oriented, thick with basket weave pattern collagen fiber in normal human skin. Compared to the thin fibers that are oriented parallel to the skin surface of the xenografts similar to HTS. Scale bar=50μm
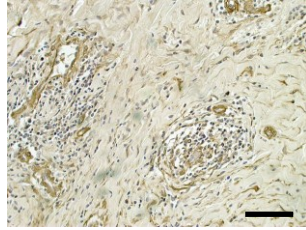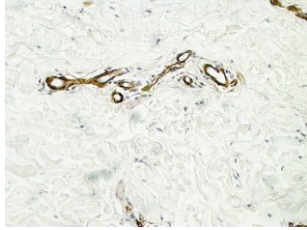
A

Normal skin　　　　HTS

Scratch before grafting　　　　No scratch　　　　Scratch after grafting
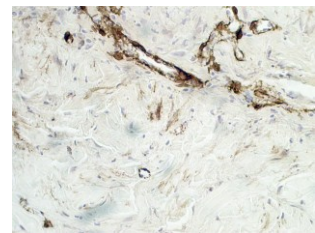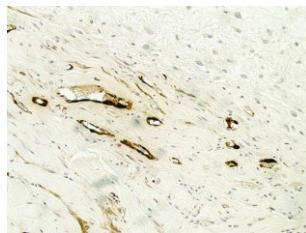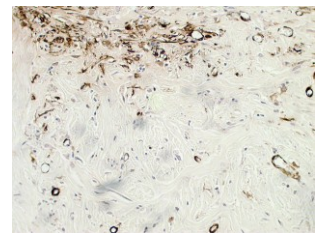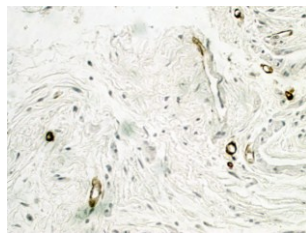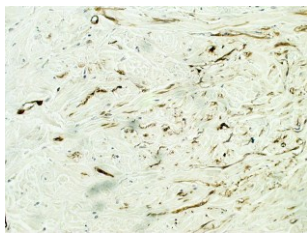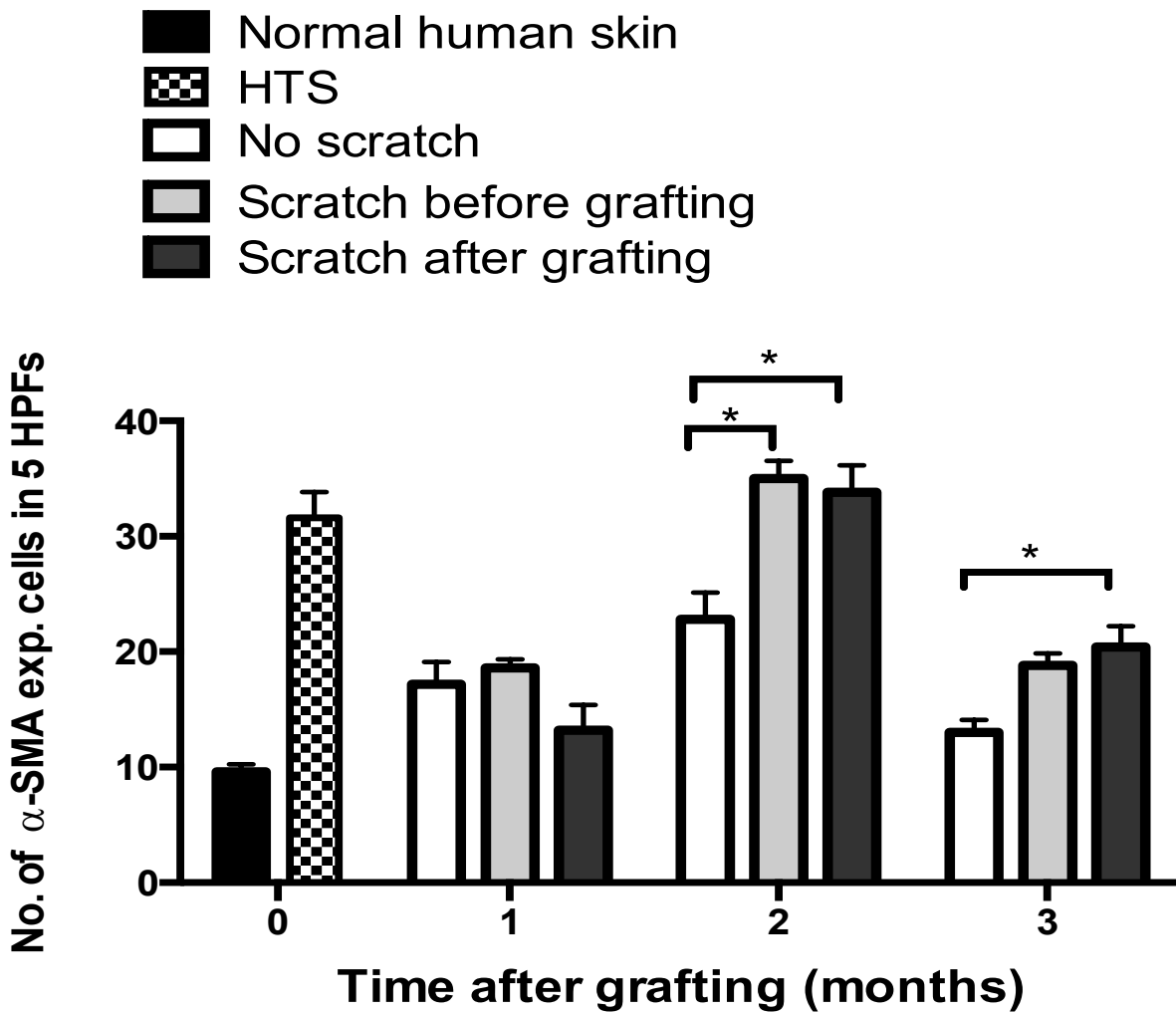
1 month

2 months

3 months

B

Figure 2-8 Scratching the grafts (before and after) increase myofibroblast formation

    (A) Representative image of an α-smooth muscle actin (α-SMA) stained section of human normal skin, HTS and transplanted xenografts at all time points showing a positive brown staining indicating the presence of myofibroblasts throughout the dermis consistent with HTS. Normal human dermis stains positive only around blood vessels. Scale bar=50μm

    (B) Quantification of α-SMA expressing cells in the grafts counted in ×200 magnification of five random high-power fields. Data expressed as mean ± SE (N= 4, * $p < 0.05$)

Human skin

Mouse skin

Scratch before grafting   No scratch   Scratch after grafting
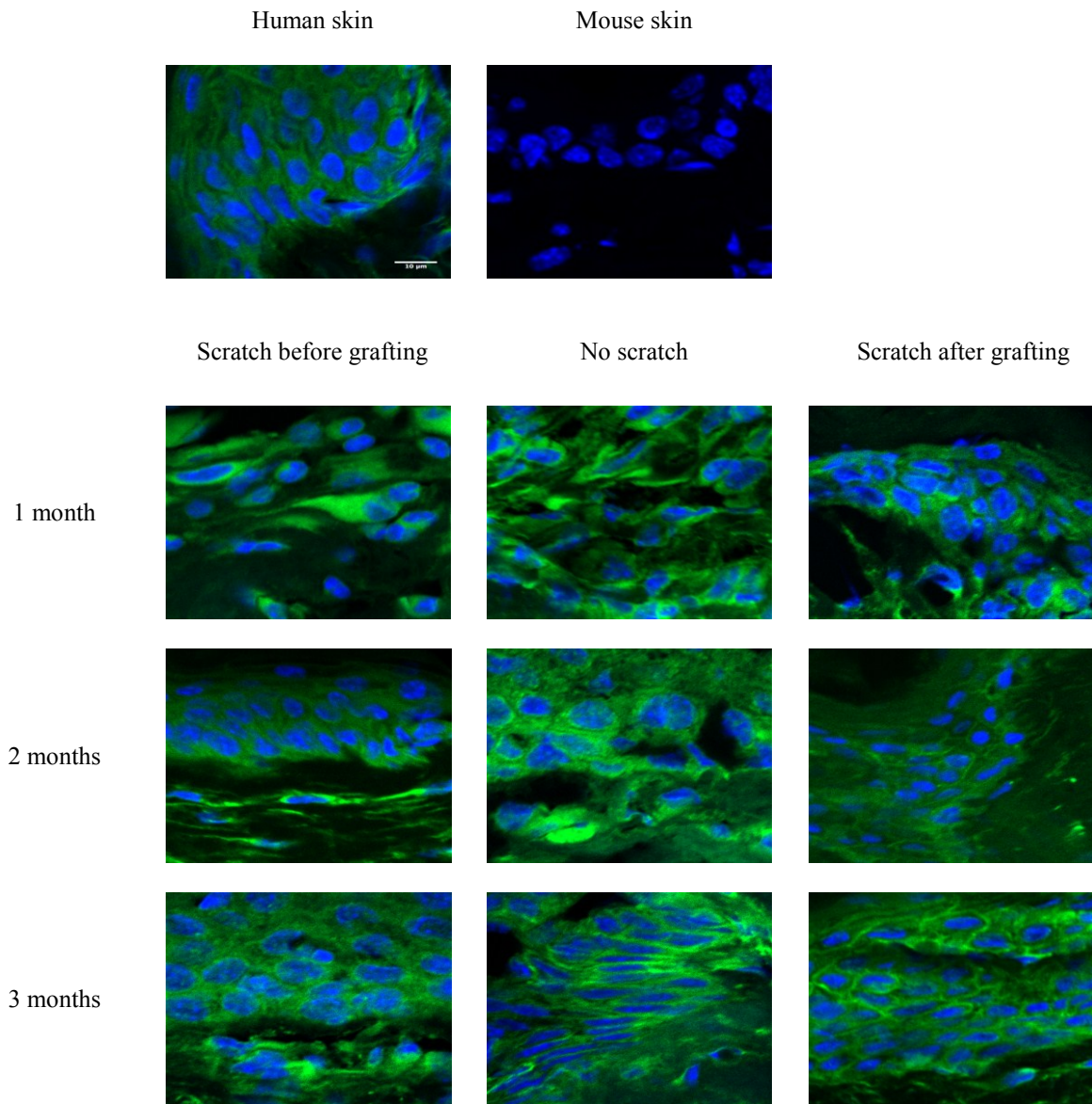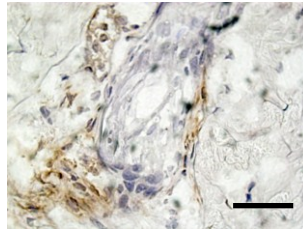
1 month

2 months

3 months

Figure 2-9 Anti human HLA-ABC staining demonstrating retention of human cell post-transplantation

Representative images of anti-human FITC human leukocyte antigen-ABC antibody stained sections confirm survival of transplanted human xenografts at all time points. Showing positive staining with green, net-like, immunofluorescent staining pattern that is consistent with normal human skin compared to normal mouse skin, which has no visible green staining (negative control). Scale bar=10μm
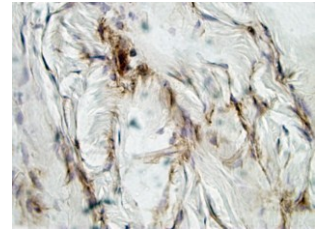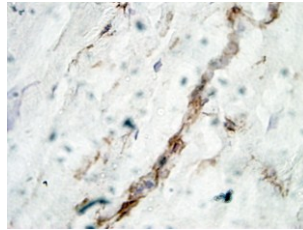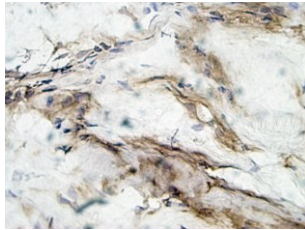
A

Normal mouse skin
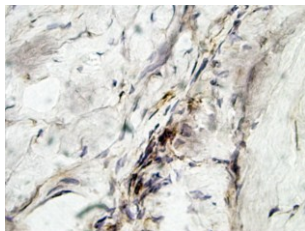
	Scratch before grafting	No scratch	Scratch after grafting
1 month			
2 months			
3 months			
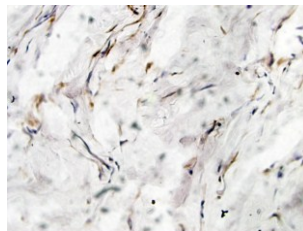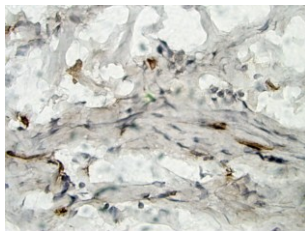
B
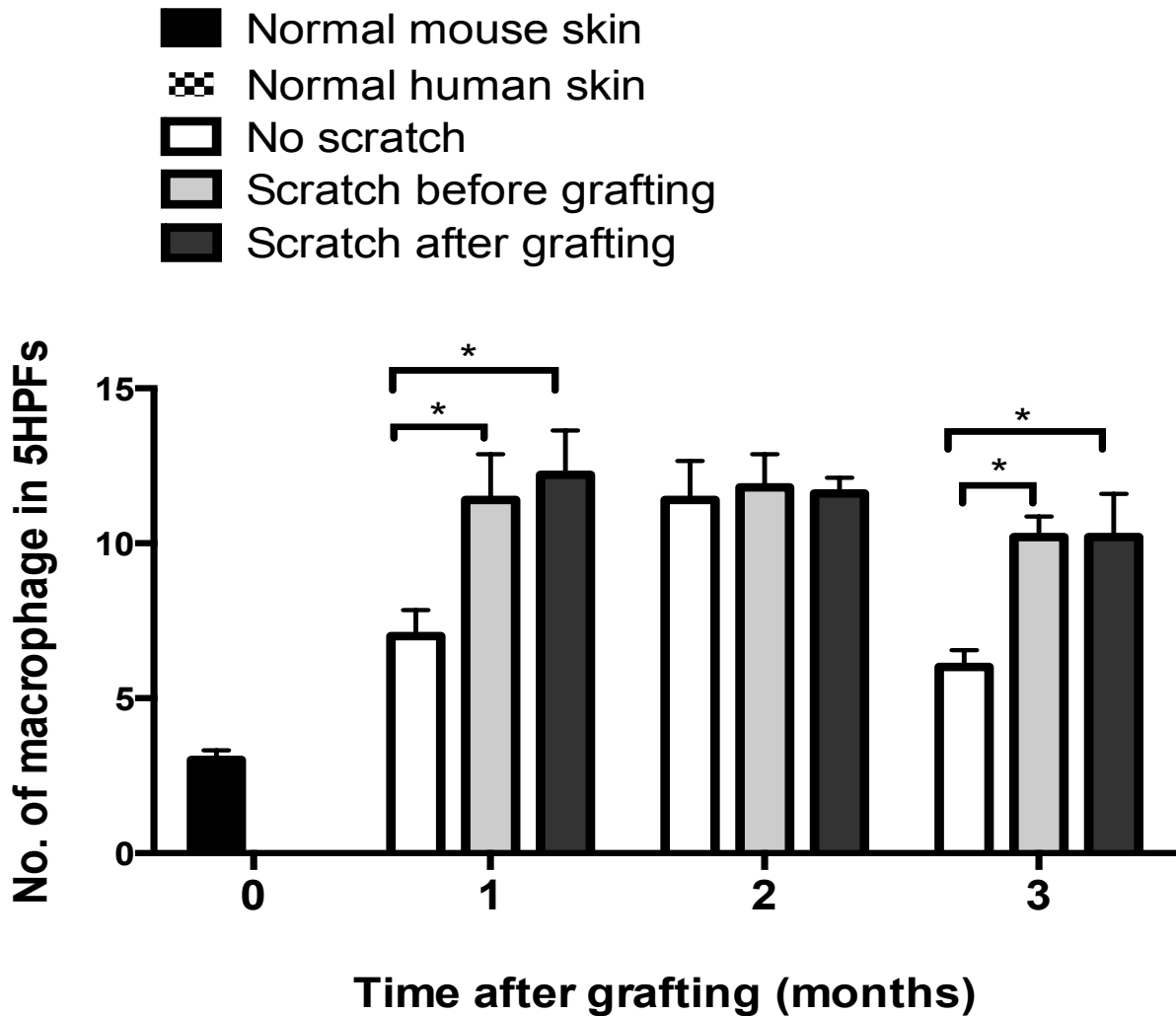
Figure 2-10 Scratching (before and after grafting) increase macrophage infiltration in human skin xenografts

(A) Immunohistochemistry staining using an anti-F4/80 antibody showing macrophages infiltration in normal mouse skin and transplanted xenografts in all time points. Scale bar=50μm

(B) Quantification of macrophages in the grafts counted in ×200 magnification of five random high-power fields. Data expressed as mean ± SE (N= 4, * $p < 0.05$).
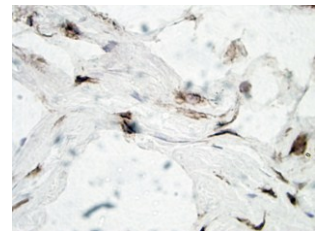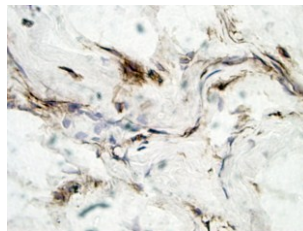
A

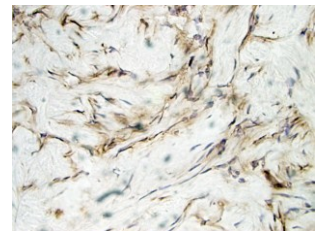|	Normal skin	HTS
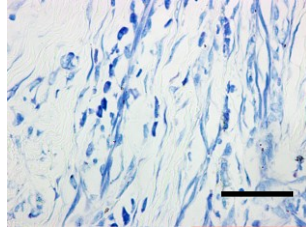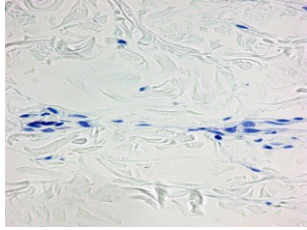
	Scratch before grafting	No scratch	Scratch after grafting
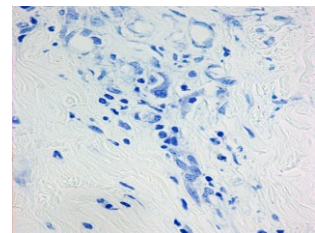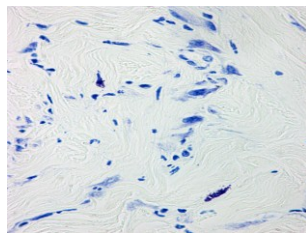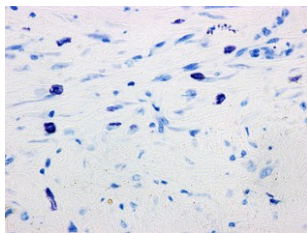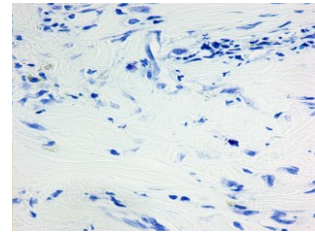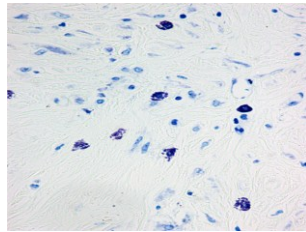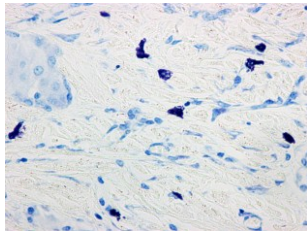
1 month
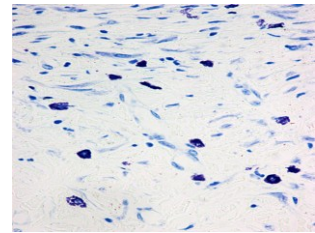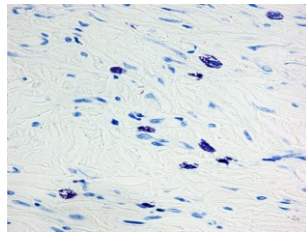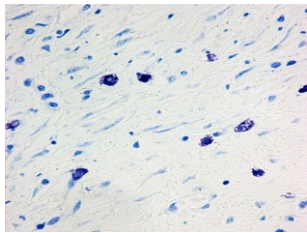
2 months

3 months

B
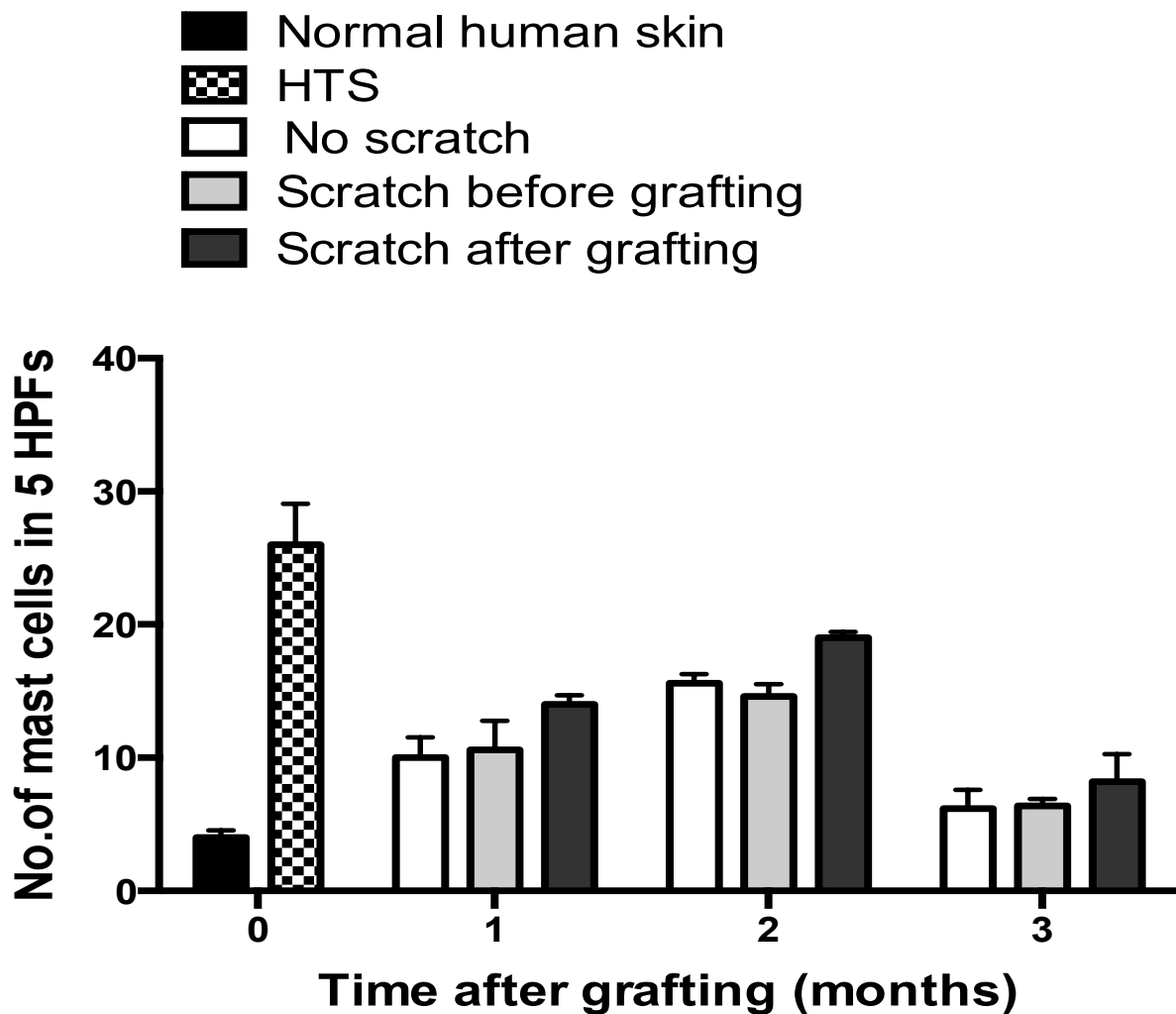
Figure 2-11 Toluidine blue staining showed increase mast cells at two months followed by gradual decrease in all xenografts

(A) Representative images of a toluidine blue stained section of human normal skin, HTS and transplanted xenografts at all time points showing an increased density red purple staining mast cells similar to human HTS compared to fewer mast cells seen in normal human skin. Scale bar=50μm

(B) Quantification of mast cells in the grafts counted in ×200 magnification of five random high-power fields. Data expressed as mean ± SE (N= 4)

3 Chapter 3

3.1 Conclusion and Future Directions

Wound healing is a complex physiological process that aids in restoring the shape and, more importantly, the function of an organ [10]. This orchestrated process involves the interaction of many inflammatory and cellular mediators; it also requires the optimal synthesis and degradation of ECM proteins [9]. The subsequent result of wound repair is mature scar formation, [144] and any anomalies in this process lead to pathological scar formation [72].

HTS is a unique human dermal FPD that develops following trauma, deep skin injury, burns, and even surgical scars [145]. This chronic dermal fibrosis leads to loss of normal skin function, disabilities, limitations in joint mobility by skin contracture in addition to undesired aesthetic disfigurement, which negatively impacts the quality of life of the affected person [146,147]. HTS is difficult to treat because of its poorly understood pathophysiology and the lack of an ideal animal model to study it, as the disease is unique to humans [148].

Here, we attempt to develop an animal model for HTS to expand our knowledge of dermal fibrosis and to develop novel treatment approaches. We showed that scratching human full thickness skin graft (FTSG) to a critical depth and grafting it onto the back of nude mice resulted in grafts that simulated HTS morphologically and histologically.

There is some variation in this model, which may be attributed to the use of outbreed athymic nude mice instead of the inbreed (BALB)/c-nu/nu nude mice used earlier and to the delicate and challenging new technique that we used to scratch the human skin using a

jigsaw. Nevertheless, we have shown that human FTSG with preexisting injury (before grafting) and injury (after grafting) can survive and heal on the back of nude mice. This novel animal model will help researchers investigate HTS and dermal wound healing.

We found that human scratched grafts survived throughout the experiment with HLA-ABC, which suggests the presence of human cells in all the scratched grafts. Identifying and quantifying these human cells will substantially contribute to our knowledge and future research avenues. It will help us investigate and understand the roles of human dermal cells and mouse cells in the formation fibrotic xenografts. Fluorescence in situ hybridization (FISH) is a potentially beneficial technique for use in this model [149,150]. It will help quantify the cells in the xenografts and allow the identification of the X chromosome containing cells from human female donor's skin and Y chromosome containing cells from the recipient male mice. This will aid to identify the exact human cell location within the scratched graft and to assess their proliferation after transplantation.

The use of nude mouse with the ubiquitous transgenic green fluorescent protein (GFP) will be of great value in the study the fibrosis using this model [151]. This bright green color of the mouse organs including the skin that can be detected using a simple blue–light-emitting diode (LED) flashlight or highly specific equipment for whole-body imaging such as the Olympus OV100 (Olympus Corp.) [152,153]. It will help to elucidate the interaction between the grafted human skin and the mouse skin at macroscopic and microscopic levels. Fluorescein labeled anti-fibrotic treatments on the GFP nude mice will enable real-time

tracking of the drug site of action and quantification of the drug interaction with the human graft.

Adipose-derived stem cells (ASCs) would be an interesting treatment modality to test in this model [154]. ASCs are abundant sources of pluripotent stem cells and can differentiate into different lineage, which can be easily isolated from fat tissues and cryopreserved up to six months [155]. Many studies discuss the potential role of ASCs in wound healing and show the accelerated healing of excisional wounds in nude mice [156-158]. ASCs contribute to wound healing by direct differentiation to dermal fibroblasts or by secreting factors such as transforming growth factor-β1 (TGF-β1), insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF) [159,160]. Therefore, isolating ASCs from the skin tissue of patients who have undergone elective abdominoplasty and infuse them on nude mice either locally or systematically would be an interesting method to peruse and study the effect of ASCs in the grafts and their potential role as novel treatment approach to HTS.

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