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Towards defining fibroblast phenotypes in cutaneous scarring: CD90 (Thy-1), CD34 and SMA expression in dermal scar fibroblasts

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TOWARDS DEFINING FIBROBLAST PHENOTYPES IN CUTANEOUS SCARRING: CD90 (THY-1), CD34 AND SMA EXPRESSION IN DERMAL SCAR FIBROBLASTS

by

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The fear of the LORD is the beginning of wisdom

Proverbs 9:10 [KJV]

DEDICATION

I dedicate this work to my beloved wife, Djavila Amari. You have given so much of yourself to allow me to pursue this training and I am unendingly in your debt.

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TOWARDS DEFINING FIBROBLAST

PHENOTYPES IN CUTANEOUS SCARRING:

CD90 (THY-1), CD34 AND SMA EXPRESSION

IN DERMAL SCAR FIBROBLASTS

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ABSTRACT

Background: Cutaneous scarring is a reparative response to wounding in an attempt to restore homeostasis. Pathologic scars include hypertrophic and keloidal scars. No defined dermal scar fibroblast phenotype has been described. This study examines for such a phenotype, looking at expression patterns and spatial relationships of CD90, CD34 and smooth muscle actin (SMA) expressing fibroblasts in cutaneous scars. Additionally, this work investigates for evidence of scar fibroblast transition from the background $CD34^+$ stromal cell network. It also delineates a timeline for the appearance/disappearance of this phenotype in physiologic scarring. Finally, it assesses the relative contributions of $CD90^+$ and SMA^+ fibroblasts to scar collagenization.

Methods: 117 scars were classified as reparative (n=47), hypertrophic (n= 40) or keloidal (n=30). Where possible, scar age was calculated. Immunohistochemistry with CD90, CD34 and SMA was performed on all scars. Double-staining immunohistochemistry for CD90/CD34 was applied to all scars assessing for the presence of dual $CD90^+/CD34^+$ transitioning cells. Double-color immunofluorescence was also performed to further

identify transition. A subset of scars was double stained with CD90/SMA to evaluate spatial relationships. Additional scars were double-stained with CD90/procollagen-1 or SMA/procollagen-1 to assess for active collagen synthesis. Expression was graded as diffuse, focal/rare (i.e. minority) and negative.

Results: A $CD90^{diffuse}/CD34^{negative/minority}$ pattern was the most commonly observed phenotype among all scars. SMA expression was variable. Transitioning $CD90^{+}/CD34^{+}$ fibroblasts were observed in 90.6% of scars. In reparative scars, a $CD90^{diffuse}/CD34^{negative/minority}$ phenotype was time-limited, developing within 48 hours and reverting to a $CD34^{diffuse}$ state at 160–180 days. Many pathologic scars exhibited prolonged $CD90^{diffuse}$ expression. Both $CD90^{+}$ fibroblasts and myofibroblasts express procollagen-1. $CD90^{+}$ fibroblasts contributed more cells to scar mass than myofibroblasts. When spatial relationships were examined, myofibroblasts exclusively localized to $CD90^{+}$ areas and exhibited CD90 double-positivity. CD90 expression was not limited to SMA^{+} zones.

Conclusions: Scar fibroblasts predominantly exhibit a $CD90^{diffuse}/CD34^{negative/minority}$ phenotype. These $CD90^{+}$ fibroblasts likely transition from the background $CD34^{+}$ network. This phenotype is reversible in reparative scars, but is prolonged in some pathologic scars. Both $CD90^{+}$ fibroblasts and myofibroblasts collagenize scars. The co-localization of myofibroblasts to CD90-rich areas and CD90 dual-positivity may suggest a common origin.

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LIST OF ABBREVIATIONS

ECM……...Extracellular matrix

ED-A FN……... Alternatively spliced ED-A containing fibronectin

EnMT……... Endothelial-mesenchymal transition

EpMT…….. Epithelial-mesenchymal transition

GPI…… Glyophosphatidylinositol

IF…….. Immunofluorescence

IHC……...Immunohistochemistry/immunohistochemical

LAP…….Latency associated peptide

LLC……..Large latent complex

LTBP……..Latent transforming growth factor-beta binding protein

MMP……..Matrix metalloproteinase

PC-1……..Procollagen I

PDGF……..Platelet derived growth factor

SLC…….. Small latent complex

SMA……. Smooth muscle actin

TGF-β…… Transforming growth factor-beta

VEG-F……..... Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

Cutaneous scarring is a reparative fibrotic response of the skin to wounding, with the aim to restore barrier integrity and homeostatic functionality. While simple reparative scarring differs from true regeneration in its imperfect reproduction of texture, color, elasticity and depending on the depth and extent of injury, cutaneous adnexae, it is a physiologic rather than pathologic process which most frequently results in adequate restoration of function, and often acceptable aesthetic results. As with all normal reparative processes however, an excessive or inadequate response leads to aberrant outcomes. In skin repair an excessive scarring response results in the development of a pathologic scar. The two main types of pathologic scars are hypertrophic and keloidal scars (keloids) which differ from simple reparative scars in their bulky clinical appearance, prolonged course and histopathological features.

Despite extensive and important work done in the field of wound healing, a comprehensive understanding of the complex nature of skin repair is lacking and while well-established phases of wound healing have been elucidated, aspects of the cellular and molecular biology/pathology of cutaneous restoration and aberrant scarring remain unknown. One such information-gap is the absence of a well-defined dermal "wound-healing" fibroblast phenotype beyond the traditional smooth muscle actin (SMA) expressing myofibroblast. As will be discussed in the literature review, recent work in various organ systems, including the skin, point to the existence of a population of

fibroblasts expressing the glycosylphosphatidylinositol (GPI) anchored protein CD90 (Thy-1). While the exact role of these cells in fibrotic disease seems to vary among organ systems, there is mounting evidence that in the skin, they are associated with a pro-fibrotic state, though specific work regarding their contribution of cutaneous scarring has yet to be undertaken. Additionally, while scar fibroblast origin has been studied in other organ systems, data regarding their origin in cutaneous fibrosis is incomplete and inconclusive. In this regard, only rare investigators have given attention to the role of the quiescent background $CD34^+$ fibroblastic stromal cell network consistently present in normal skin, as a resident source for wound healing fibroblasts.

In an attempt to delineate a scar fibroblast phenotype, this study examines the changes in fibroblast protein expression phenotypes among all three categories of cutaneous scars and compares these patterns with uninjured skin and between each scar type. Specifically, it investigates for the presence of a $CD90^+$ fibroblast population, looks for evidence suggestive of local transition from the background $CD34^+$ reticular network, assesses for variations in protein expression patterns and attempts to elucidate the natural history of CD90 and CD34 expression in scars, by demonstrating a timeline for their commencement and disappearance in reparative scar fibroblasts. Also addressed is the relationship, if any, between these fibroblasts and classical SMA^+ myofibroblasts, the currently lauded main-player in fibrotic disease.

Chapter two highlights the background clinical and pathologic basis for defining scars as

simple reparative versus pathologic and briefly reviews the relevant literature related to fibroblast expression of the evaluated proteins with respect to wound healing and fibrosis. Chapter three outlines the hypotheses to be tested and proposes a hypothetical model of reparative scarring predicted from existing data and investigator driven postulation while chapter four will review the relevant materials and the study methodology. Results and the discussion of their interpretation and relevance will be presented in chapters five and six respectively with chapter seven discussing limitations and the final chapter will summarize the overarching themes explicated herein.

CHAPTER TWO: BACKGROUND AND REVIEW OF THE LITERATURE

2.1 Wound healing and physiologic scarring

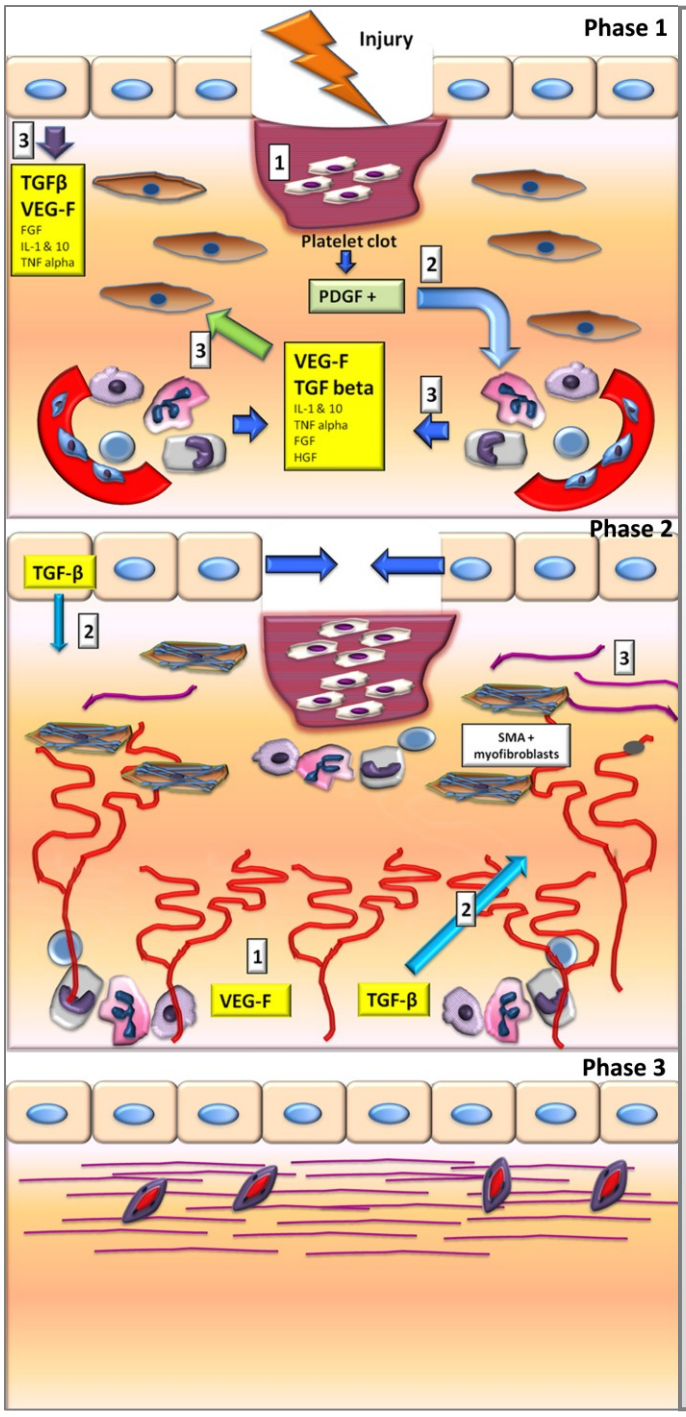
Cutaneous scarring is an extra-uterine physiologic fibrotic response to wounding. Unlike early gestational age fetuses, children and adults with dermal injury heal with some degree of fibrosis (Rowlatt, 1979). Fibrosis is an attempt to return the damaged dermis to its pre-wound state and while significant variation in cicatrix quality exists, the vast majority of injuries heal without significant functional or devastating aesthetic complications. These simple reparative scars are often asymptomatic and are characterized by a flat appearance and time dependent diminishing of erythema and other signs of inflammation. The adjacent uninjured skin typically does not exhibit abnormal texture, color or changes in elasticity as the reparative process is largely limited to the specific area of injury. These scars are the product of successful and well regulated (though partially overlapping) stages of wound healing, classically divided into inflammatory, proliferative and remodeling/maturation phases, which are briefly reviewed (**Figure 1**).

The inflammatory stage occurs immediately after wounding and begins with the formation of the platelet plug. In addition to its hemostatic properties, the platelet plug is a key source of cytokines, in particular, platelet derived growth factor (PDGF) and to a lesser degree transforming growth factor beta (TGF-β). Release of these cytokines results in the influx of inflammatory cells (initially neutrophils and later

monocytes/macrophages) as well as the activation of quiescent resident fibroblasts (Childs & Murthy, 2017; Goldman, 2004). Along with an antimicrobial function, the inflammatory cells themselves function as a source of numerous cytokines and growth factors including vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β), important for angiogenesis, fibroblastic differentiation and transition to the proliferative phase (Gurtner, Werner, Barrandon, & Longaker, 2008).

The proliferative phase begins at around 48 hours and is characterized by keratinocyte migration, vascular proliferation and later by fibroblast production of new extracellular matrix (ECM) composed primarily of collagen. Important for wound contracture is the conversion of fibroblasts to contractile myofibroblasts, primarily under the influence of TGF-β and in response to increasing ECM tension (Gurtner et al., 2008; Werner, Krieg, & Smola, 2007). This phase correlates histologically to granulation tissue (early proliferation) and the characteristic cellular scar (established proliferation).

The remodeling phase begins at approximately 2 weeks, lasting up to 12 months. It is characterized by a delicate balance between fibroblast/myofibroblast production of the ECM and its breakdown by proteolytic enzymes, particularly matrix metalloproteinases (Nwomeh, Liang, Diegelmann, Cohen, & Yager, 1998). As the ECM returns to its pre-wound tension, activated fibroblasts/myofibroblasts disappear from the wound (classically thought to be achieved via apoptosis) and this phase histologically correlates with the relatively acellular “aged” scar.



Inflammatory Phase

1. **Wounding leads to development of the platelet plug**
2. **Release of cytokines including PDGF attracts inflammatory cells to wound**
3. **Inflammatory cells and activated keratinocytes release additional cytokines including VEGF and TGF-β, with TGF-β activating quiescent fibroblasts**

Proliferative Phase

1. **Under the influence of VEG-F, angiogenesis occurs contributing the vascular component of granulation tissue**
2. **TGF-β and increasing ECM tension initiate myofibroblastic differentiation with expression of SMA**
3. **Fibroblasts put down ECM primarily as collagen. Re-epithelialization is also occurring at this time (dark blue arrowheads)**

Remodeling/Maturation Phase

- **Low-cellularity scar with parallel collagen bundles and vertically oriented blood vessels.**

Figure 1. Phases of wound healing

2.2 Pathologic scarring: hypertrophic scars and keloids

Hypertrophic scars and keloids are similar but separate clinical entities characterized by an elevated and bulky clinical appearance. The primary clinical difference between these lesions is their relationship to the scar-line of the initial wound. Hypertrophic scars remain confined within the margins of the initial injury, while the expansile keloid extends beyond this margin. Additional clinical differences are highlighted in **Table 1**.

Traditionally, histolopathologic differences between hypertrophic scars and keloids also exist (**Table 1**). Hypertrophic scars are composed of well circumscribed nodules and fascicles of plump fibroblasts with excessive but thin fibrillary collagen and rare to absent formation of thick glassy haphazard collagen bundles (keloidal collagen). Alternatively, keloids are defined by the presence of well-developed keloidal collagen, often with poorly defined borders, and in mature lesions, broad pauci-cellular areas with scattered, large fibroblasts. Histopathologic overlap however is commonly encountered in routine practice, and in conjunction with equivocal clinical data ("hypertrophic scar versus keloid"), may render precise scar classification difficult. In keeping with this, there exists a body of literature suggesting that these scars are not distinct entities but rather, are different manifestations of the same disease based on the overlapping clinical, histopathologic and proposed cellular/molecular pathophysiology (Atiyeh, Costagliola, & Hayek, 2005; Huang, Akashai, Hyakusoku, & Ogawa, 2014; Kose & Waseem, 2008; Lee, Yang, Chao, & Wong, 2004).

While the pathophysiology of hypertrophic scars and keloids is incompletely understood (**Figure 2**), a failure of the normal regulation of wound healing leading to an exaggerated response is likely, though the specific predisposing factors and molecular milieu which determines which pathologic scar variant will manifest are unclear. Specific abnormalities in the remodeling phase leading to an imbalance between ECM production and removal has long been proposed as a mechanism for bulky scar formation. Decreased expression and activity of the collagenase MMP-1 in keloids and hypertrophic scars has observed, though counter-intuitively, increases in MMP-2 in pathologic scars has also been demonstrated (Imaizumi et al., 2009; Lee, Trowbridge, Ayoub, & Agrawal, 2015). Additional reported differences in the extracellular matrix of hypertrophic scars and keloids include a preponderance of immature type III collagen in the former and a haphazard mixture of types I and III collagen in the latter (Bailey et al., 1975; Mari et al., 2016).

While wound healing phase-specific pathology may occur, evidence exists that pathologic scar fibroblasts may be intrinsically different from normal dermal fibroblasts and are therefore pathological in all phases of wound repair (Ashcroft, Syed, & Bayat, 2013; Kischer et al., 1989; Suarez, Syed, Alonso-Rasgado, & Bayat, 2015). In this regard, an emerging hypothesis is that a failure of fibroblast apoptosis leads to the prolonged presence of ECM producing cells, accounting for the characteristic excessive collagen production. Suggested pathomechanisms for both hypertrophic scars and keloids include down regulation of pro-apoptosis-related genes, p53 mutations, resistance to fas-

mediated apoptosis and elevations in the expression of the anti-apoptotic protein bcl-2 (Linge et al., 2005; Lu, Gao, Ogawa, Hyakusoku, & Ou, 2007; Moulin et al., 2004; Saed et al., 1998).

Differences in the cellular components of hypertrophic scars and keloids have largely focused on defining the presence or absence of myofibroblasts, with inconsistent results. While multiple studies highlight the increased number myofibroblasts in hypertrophic scars compared with keloids, others report significant positivity in the latter (Ehrlich et al., 1994; Lee & Vijayasingam, 1995; Lee et al., 2004). From a clinical perspective however, it is conceivable that keloids should have lower numbers of contractile myofibroblasts, since ineffective contracture intuitively seems pathomechanistically plausible in these expansile scars.

An important advancement in the current pathophysiologic models of fibrosis is the focus on pro-fibrotic cytokine/growth factor signaling pathways. It is likely that an excessive pro-inflammatory/pro-fibrotic cytokine milieu plays an important role in the development of a pathologic scar (Armour, Scott, & Tredget, 2007). While numerous cytokines/growth factors are proposed to contribute to the development of hypertrophic scars/keloids, transforming growth factor beta (TGF-β) is the best studied and is reviewed below (**Figure 3**).

TGF-β has 3 isoforms and of these, β_1 and β_2 appear to be profibrotic while the β_3 isoform

signaling may result in diminished scarring (Finnson, Arany, & Philip, 2013). TGF-β is produced by a number of cell types including keratinocytes, macrophages and fibroblasts themselves. Intracellular TGF-β is synthesized as an inactive form and is subsequently bound to the TGF-β latency associated peptide (LAP) becoming the small latent complex (SLC). The SLC is cleaved and becomes covalently bound to latent TGF-β binding protein (LTBP) forming the large latent complex (LLC). Once secreted into the extracellular space, the LLC interacts with components of the extracellular matrix and microfibrils. Proteolysis leads to the LLC becoming solubilized. Soluble LLC can attach to target cell surfaces via interaction of LAP with integrins. Protease cleavage or conformational change based on $\alpha_v\beta_6$ integrin binding of LAP releases free/active TGF-β allowing for receptor binding and activation of downstream intracellular Smad proteins which enter the nucleus and induce transcription of profibrotic sequences (Biernacka, Dobaczewski, & Frangogiannis, 2011; Finnson, McLean, Di Guglielmo, & Philip, 2013; Ten Dijke & Arthur, 2007). Secretion and activation TGF-β is essential for normal wound healing, though excessive or prolonged expression leads to fibrosis. TGF-β secretion increases production of ECM components including fibronectin and collagens while inhibiting MMPs and increasing expression of MMP inhibitor proteins (Barrientos, Stojadinovic, Golinko, Brem, & Tomic-Canic, 2008). Additionally, TGF-β is a potent inducer in fibroblasts of a myofibroblastic phenotype important for normal scar contraction but also postulated to be a major source of the excessive ECM production and abnormal remodeling typical of multiple fibrosing diseases including pulmonary, hepatic and renal fibrotic disease (Hinz et al., 2012). Numerous reports highlighting an increased

expression of TGF-β and/or its receptors in fibroblasts from hypertrophic scars and keloids have be published, providing further evidence for its role in cutaneous fibrosing diseases (Bettinger, Yager, Diegelmann, & Cohen, 1996; Finnson, McLean, et al., 2013; Fujiwara, Muragaki, & Ooshima, 2005; Lee et al., 1999; Schmid, Itin, Cherry, Bi, & Cox, 1998; Wang et al., 2000).

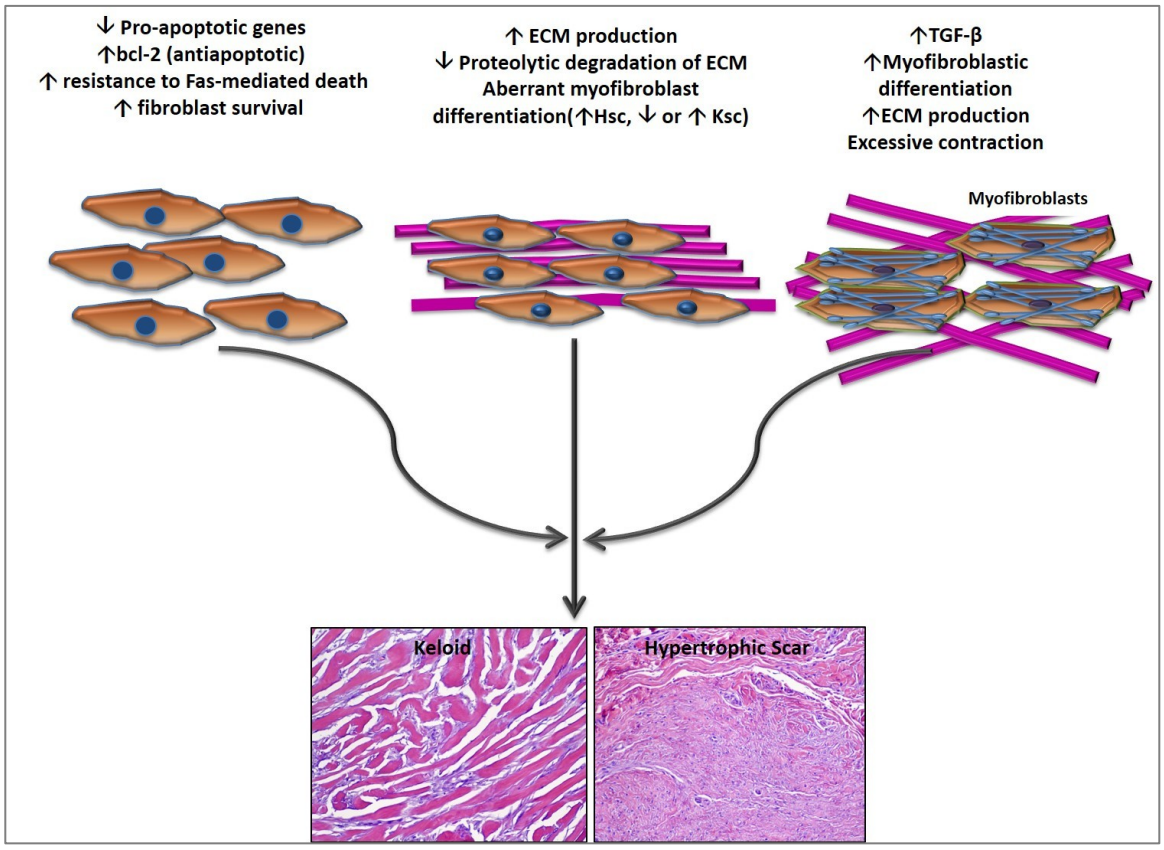


Figure 2. A summary of the proposed pathomechanistic pathways for pathologic scar formation

Clinical & histopathologic features	Hypertrophic scar	Keloidal scar
Racial predisposition	None	Patients of African ancestry(Kelly, 1988)
Area of predilection	High skin tension	High skin tension and low skin tension (earlobes)
Pre-existing injury	Almost always	Sometimes though spontaneous lesions occur(Jfri, Rajeh, & Karkashan, 2015)
Onset	Immediately after injury	Often insidious
Pain/erythema	Often	Often
Relationship to index wound margin	Confined to index wound margin	If injury associated, extends beyond wound margin
Spontaneous resolution	Occasionally	Unusual
Conventional histology	○ Well defined fascicles or nodules of plump fibroblasts ○ Fibrillary collagen with little keloidal collagen ○ SMA rich nodules	○ Poorly defined margins ○ Abundant glassy keloidal collagen ○ Lack SMA rich nodules (Ehrlich et al., 1994)

Table 1. Comparison of the clinical and histologic features of hypertrophic scars and keloids

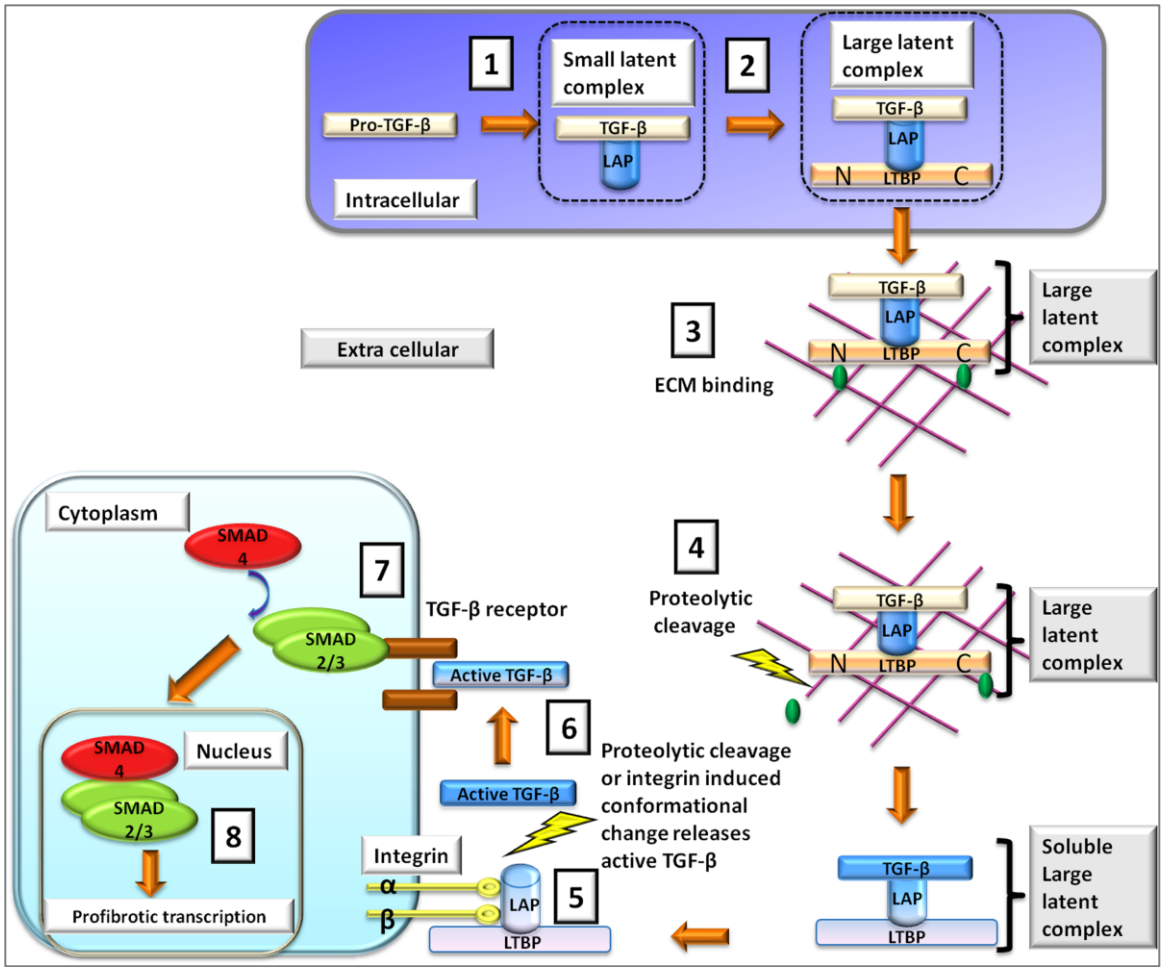


Figure 3. Transforming growth factor beta signaling [1] Inactive intracellular TGF-β binds to LAP (SLC), and later **[2]** is covalently attached to LTBP (LLC), with subsequent secretion into the extracellular space. **[3]** Extra cellular LLC binds to the ECM at its N-terminal and with microfibrils at the C-terminal. **[4]** Proteolytic cleavage of LTBP allows for solubilization of the LLC. **[5]** Soluble LLC attaches to its target cell via integrin binding and **[6]** further proteolytic cleavage of LAP or integrin associated conformational changes, results in release of active TGF-β. **[7]** Active TGF-β binds to its receptor and via SMAD signaling, induces **[8]** transcription of profibrotic sequences. SLC, small latent complex; LLC large latent complex

2.3 Regarding the origin of scar fibroblasts

Multiple theories have been put forward to explain the origin of the scar fibroblast. Some of the major theories include activation of quiescent resident progenitor cells (reviewed below), transformation of circulating $CD34^+$ hematopoietic "fibrocytes" to tissue fibroblasts and epithelial/endothelial mesenchymal transition (EpMT/EnMT) (Bucala, Spiegel, Chesney, Hogan, & Cerami, 1994; Kuwahara et al., 2016; Nazari et al., 2016).

Activation of quiescent resident mesenchymal/stromal cells represents the classical view of scar fibroblast origin. Evidence exists that resident stromal/mesenchymal cells act as a source of active fibroblasts/myofibroblasts in various organ systems including the liver, kidney, lung and the heart (Furtado, Costa, & Rosenthal, 2016; Iwaisako, Brenner, & Kisseleva, 2012; Picard, Baum, Vogetseder, Kaissling, & Le Hir, 2008; Xia et al., 2014). Interestingly, despite the long acknowledged existence of a background $CD34^+$ stromal cell network in skin, little attention has been paid to its role as a potential source of scar fibroblasts in cutaneous fibrosis.

In the skin, a population of $CD34^+$ fibroblastic cells are present in the dermal stroma as well as in a periappendegeal and perivascular location (Ceafalan, Gherghiceanu, Popescu, & Simionescu, 2012; Díaz-Flores et al., 2015). Various names have been attributed to these cells including telocytes, stromal fibroblastic cells, fibrocytic cells and dendrocytes among others (Díaz-Flores et al., 2014). To avoid confusing terminology, we will use the

term $CD34^+$ fibroblastic stromal cells/network. Ultrastructurally, these cells are characterized by a small triangular cell body and numerous long cytoplasmic processes, termed telopodes, which are involved in frequent cell-cell interactions with neighboring telopodes as well as other cell types, accounting for the extensive and intricate network noted with immunohistochemical staining for CD34 as viewed in a two-dimensional plane on standard histologic sections (Manole & Simionescu, 2016). While their biologic function is not completely understood, in the physiologic state, these low-proliferative capacity cells are proposed to contribute to architectural homeostasis, to have a role in vascular biology, to act as nurse cells for mesenchymal stem cells and importantly serve as a mesenchymal progenitor cell reservoir (Ceafalan et al., 2012; Díaz-Flores et al., 2015). Supporting this latter concept is the observed presumed transition of activated gut $CD34^+$ fibroblastic stromal cells to myofibroblasts in granulation intestinal tissue, with concurrent loss of CD34 expression (Díaz-Flores et al., 2015). While the majority of this work has been performed in extracutaneous tissue, a recent study presented evidence that in the fibrosing cutaneous disease scleroderma, pathologic $CD90^+$ fibroblasts (see below) are derived from the background $CD34^+$ fibroblastic stromal cell network. In addition to loss of CD34 and neoexpression of CD90, dual positive fibroblasts expressing both CD34 and CD90 were observed with a double-stain immunohistochemistry protocol and via immunofluorescence studies. These cells were interpreted as disease associated fibroblasts caught in transition and the authors suggested a reciprocal pattern of expression for these markers, with cells at the transitional poles being either $CD34^+/CD90^-$ or $CD90^+/CD34^-$ (Nazari et al., 2016). This finding is of paramount

importance to the genesis of this study's hypotheses. Assuming potential analogy of fibrosis pathways in scleroderma and cutaneous scarring, we propose that evidence of this stromal → mesenchymal transition should also be found in dermal scars and if present, is compatible with a mesenchymal progenitor function of the $CD34^+$ fibroblastic stromal cell network and specifically, supports the hypothesis that this network is a significant (and possibly the foremost) source of wound healing/scar fibroblasts in the skin.

2.5 The CD90 (Thy-1) expressing fibroblast: what do we know?

While determination of fibroblast origin is an important step adding to a more comprehensive understanding of cutaneous scar physiology/pathophysiology, of additional importance is the delineation of a reproducible cutaneous scar fibroblast phenotype. The CD90 expressing fibroblast has recently been described as playing various roles in fibrotic conditions involving different organ systems. These fibroblasts are typically not found in the quiescent dermis with the exception being in the perivascular and periadnexal adventitia (Nazari et al., 2016). While their role in skin disease is largely unexplored, the aforementioned research indicates that CD90 expressing fibroblasts are present in cutaneous sclerosing diseases such as scleroderma. Additionally, they observed the presence of these cells in benign fibrohistiocytic tumors and in a subset of scars (Nazari et al., 2016). CD90 is a glycosylphosphatidylinositol (GPI) anchored cell surface protein of the immunoglobulin superfamily (Williams &

Gagnon, 1982). Although no one clear biologic function has been elucidated, it is postulated to activate a number of signaling pathways, though given its cell surface location without an intracytoplasmic tail, how it does so is currently unexplained. With respect to scar formation, CD90 has been shown to activate TGF-β signaling an important pathway in fibrogenesis (**Figure 3**) (Rege & Hagood, 2006). Curiously, CD90 expressing fibroblasts may have different roles relating to fibrosis depending on the tissue involved. To this point, a $CD90^{+}$ phenotype appears to be pro-fibrotic in the orbit (Grave's ophthalmopathy), kidney (renal tubulointerstitial fibrosis) and in cardiac fibrosis, but protective against fibrosis in the lung (interstitial pulmonary fibrosis) (Brandau et al., 2015; Hagood et al., 2005; Hudon-David, Bouzeghrane, Couture, & Thibault, 2007; Yuasa et al., 2013). It is also noteworthy, that baseline fibroblast CD90 expression varies among organ systems with unperturbed dermal stromal fibroblasts being $CD90^{-}$ while a $CD90^{+}$ phenotype is observed in the majority resident resting lung fibroblasts and a mixture of $CD90^{+/-}$ cells in present the myometrium and kidney (Hagood et al., 2005; Koumas, King, Critchley, Kelly, & Phipps, 2001; Rege & Hagood, 2006). These data support the growing notion of tissue specific cellular functioning, where cells (in this case fibroblasts) exhibiting overlapping protein expression phenotypes have varying biologic roles dependent on the tissue of origin (Slany et al., 2014). It also underscores the importance of studying fibroblast biology in the tissue of interest, as extrapolation of results from experiments performed in other organ systems may yield incorrect conclusions.

When the function of CD90 was specifically examined in dermal fibroblasts with regards to its role in fibroblast proliferation and regulation of apoptosis, CD90 expression was associated with less fibroblast proliferation and more apoptosis compared to fibroblasts from $CD90^{-/-}$ knockout mice suggesting an important role in regulation of fibroblast growth and homeostasis (Schmidt et al., 2015). The authors additionally examined for the effect of CD90 expression on fibroblast differentiation and function, demonstrating that CD90 expression was associated with significantly increased levels of bioactive TGF-β and mRNA expression of α-SMA, the extra domain A splice variant of fibronectin (ED-A FN), collagen I (α1) and collagen III (α1). Interestingly, when fibroblasts from $CD90^{-/-}$ knockout mice were seeded on immobilized, recombinant Thy-1 coupled to an IgG Fc for 72 hours, a significant decrease in proliferation, and increased mRNA expression of α-SMA, ED-A splice variant of fibronectin, collagen I (α1) and levels of bioactive TGF-β were observed (Schmidt et al., 2015). These results convincingly suggest that in skin fibroblasts, CD90 is actively involved in ECM production (specifically the laying down of collagen and ED-A FN) fibroblast-myofibroblast differentiation, regulation of fibroblast proliferation and control of fibroblast apoptosis.

2.6 The SMA positive myofibroblast

The myofibroblast is a fibroblast which has acquired contractile stress fibers composed of cytoplasmic actins. These stress fibers allow for cell migration and increased contractility. Persistent activation leads to the development of a specific actin isoform, α-SMA, which allows for more effective contractility (Hinz, 2007; Hinz et al., 2007). SMA positivity is the main immunohistochemical method of identifying myofibroblasts. As myofibroblasts are typically absent in non-fibrotic tissue, the origin of myofibroblasts has been a topic of considerable interest. Like with other dermal fibroblasts, local transformation of quiescent resident fibroblasts, trans-differentiation of epithelial or endothelial cells and a circulating fibrocyte precursor represent the main theories of myofibroblast origin (Hinz et al., 2007). More recent work has suggested pericyte, macrophage or adipocyte origins for fibrosis associated myofibroblasts (Humphreys et al., 2010; Martins et al., 2015; Meng et al., 2016). While all are interesting hypotheses, for the purpose of this study, only the first will be reviewed in detail.

Myofibroblastic differentiation of quiescent fibroblasts is thought to require a combination of increased mechanical stress within the ECM, cytokine stimulation and interaction with specific ECM proteins (Hinz, 2010; Hinz et al., 2007). After wounding, various cytokines and growth factors activate fibroblasts (**Figure 1**) which begin production of ECM components leading to a disruption of its pre-injury cross-linked structure, and an increase in ECM stiffness (Wipff & Hinz, 2009). The resultant

mechanical challenge induces the formation of cytoplasmic actin stress fibers as discussed above. In conjunction with this mechanoinduction differentiation pathway, TGF-β secreted from macrophages, keratinocytes and resident fibroblasts themselves, acts a potent cytokine inducer of a myofibroblastic phenotype (Gabbiani, 2003). It is interesting that the previously detailed study and others suggests that CD90 expression may be a prerequisite for optimal TGF-β secretion, acting as an upstream inducer of myofibroblast differentiation. This upstream role is supported by evidence suggesting CD90 expression occurs prior to SMA^+ myofibroblast differentiation (Koumas, Smith, Feldon, Blumberg, & Phipps, 2003; Schmidt et al., 2015; Yuasa et al., 2013). Finally, the interaction of fibroblasts with TGF-β dependent ECM protein ED-A splice variant of fibronectin (ED-A FN) seems to precede and be necessary for induction of a myofibroblastic phenotype. Again, the upstream role of CD90 in expression of ED-A FN has been described, intimately linking this protein to the major fibroblast → myofibroblast differentiation pathways. After ECM stability and re-epithelialization has been achieved, myofibroblasts disappear from the wound bed traditionally explained by massive fibroblast apoptosis (also linked to CD90 expression) (Gabbiani, 2003). Previous research indicates that myofibroblasts appear by day 6 and are completely absent after day 30, coinciding with the presence of increased apoptotic figures (Darby, Skalli, & Gabbiani, 1990).

It should be mentioned that pathologic fibrosis has, in many conditions (including scars), been attributed primarily to the abnormal persistence of the myofibroblast (Badid,

Mounier, Costa, & Desmoulière, 2000; Darby & Hewitson, 2007; Kis, Liu, & Hagood, 2011; Santucci, Borgognoni, Reali, & Gabbiani, 2001; Sarrazy, Billet, Micallef, Coulomb, & Desmoulière, 2011; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002; Wynn, 2008). While their presence and remodeling/contractile role in these conditions are widely accepted, the evidence supporting their role as the main contributors to production of the ECM and scar collagenization characteristic of all fibrotic pathology is less clear.

CHAPTER THREE: AIMS AND HYPOTHESES

3.1 Study Aims

The basic aims of this study are to define scar fibroblast phenotypes in cutaneous scars, and to determine similarities or differences between these protein expression patterns in reparative, hypertrophic and keloidal scars. In specific, it examines for the expression patterns of CD90 (Thy-1), CD34 and SMA within these scar groups, and attempts to delineate the spatial relationships between these various fibroblast populations and the comparative contributions of each fibroblast-type to scar mass. This study also examines for evidence of fibroblast transitioning from the background $CD34^+$ stromal cell network, using the presence/absence of fibroblasts exhibiting double positivity for the various protein markers. Evaluation will be performed with double-stained immunohistochemistry and immunofluorescence studies. Additionally, this work aims to delineate a timeline for the loss/neo-expression of the various markers after wounding and explores for evidence of reversion to the quiescent pre-wounding state or persistence of an activated scar fibroblast phenotype. Finally, contribution to ECM production by the various fibroblast-types, as it relates to scar collagenization, will be evaluated for via expression of procollagen-1. Based on the literature reviewed in the previous chapter and investigator driven postulation, a hypothetical model of scarring was developed and is represented in **Figure 4**.

3.2 Proposed hypothetical model of fibroblast transitions in cutaneous scarring

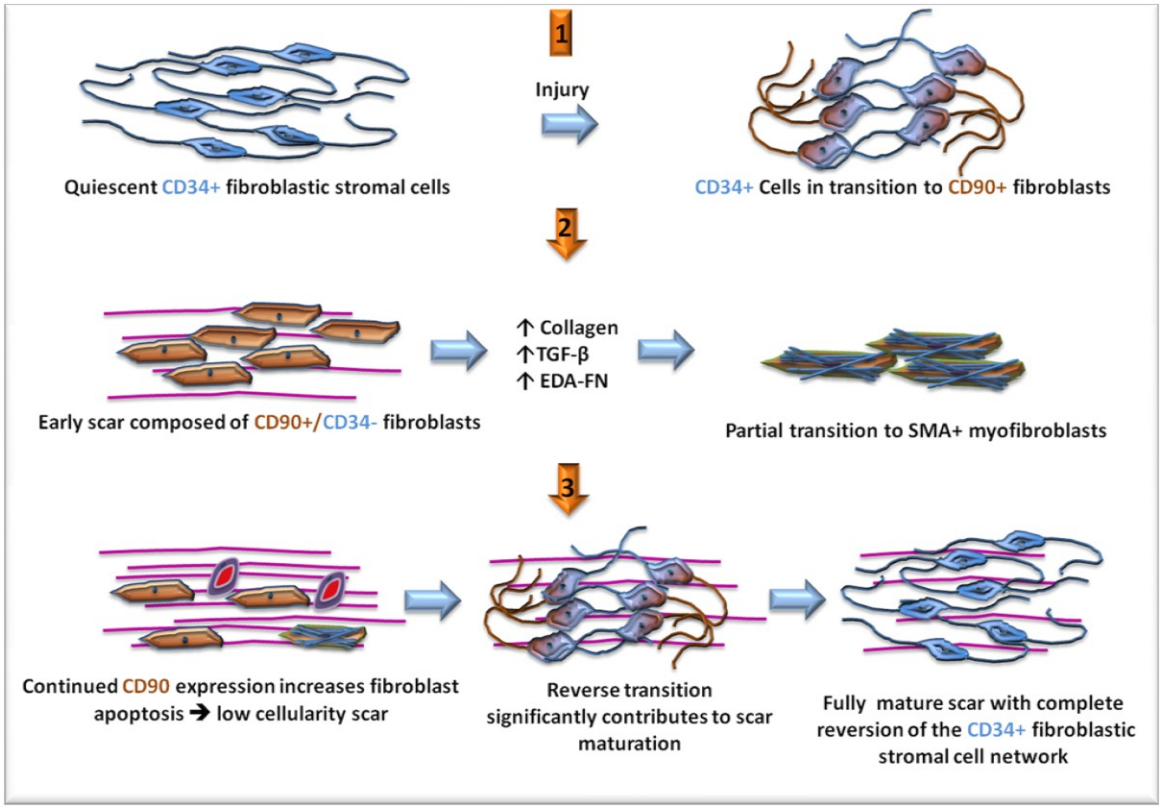


Figure 4. Hypothetical model of fibroblast transitions in simple reparative scars
[1] After injury, quiescent background $CD34^+$ fibroblastic stromal cells undergo a change in protein expression profile and begin to express CD90. **[2]** $CD90^+/CD34^-$ fibroblasts lay down collagen and increase TFG-β and EDA-FN leading to development of the well-established scar. Resultant cytokine stimulation and changes to ECM mechanics induces partial myofibroblastic transition. **[3]** CD90 expression regulates fibroblast apoptosis/proliferation leading to a low-cellularity scar. When ECM homeostasis is achieved, re-transition to $CD34^+/CD90^-$ fibroblastic stromal cell network begins with complete reversion in the mature scar

CHAPTER 4: MATERIALS AND METHODOLOGY

4.1 Specimen selection, scar classification and scar aging

Boston Medical Center's institutional review board approved all procedures and data analysis. Specimens diagnostically coded as "scar", "hypertrophic scar" or "keloid" were identified and then retrieved via the database of Skin Pathology Laboratory at Boston University School of Medicine. Standard hematoxylin and eosin (H&E) sections were reviewed for histologic inclusion criteria (see below) and specimens were classified as simple reparative scars (n=47), hypertrophic scars (n=40) or keloidal scars (=30). Additionally, specimens exhibiting normal skin from uninvolved adjacent tissue sections in excision specimens containing simple reparative scars were identified for control purposes (n=10).

As all simple reparative scars selected were from excision specimens of previously biopsied lesions, an accurate scar age could be calculated for these specimens by subtracting the date of initial biopsy performed from the date of the excision. Where the information was available, exact scar age was also calculated for hypertrophic scars (n=23) and keloids (n=13), though in many instances no clinical data was provided to assist with aging the scars. Where clinical data regarding the age of the scar was available but not explicit, the minimum age was recorded. As such, a hypertrophic scar/keloid recorded as "present for many years" or "present for greater than six months" was

recorded at its minimum age of 365 days or 180 days respectively. Scar age was recorded in days, and subsequently categorized as <30 days (<1 month), 30–89 days (1–3 months), 90–179 days (3–6 months) and >180 days (> 6 months).

4.2 Histologic criteria for scar classification

Simple reparative scars (n=47)

Classification as a simple reparative (**Figure 5**) scar relied on fulfillment of the following criteria:

i. Excision specimens/recurrence biopsy specimens

ii. Increased fibroblast proliferation and collagen production (early/proliferating scars)

OR

Parallel dermal fibrosis and low cellularity with vertically oriented blood vessels ("aged" histologic appearance)

iii. No large nodular or broad fascicular component

iv. No keloidal collagen formation

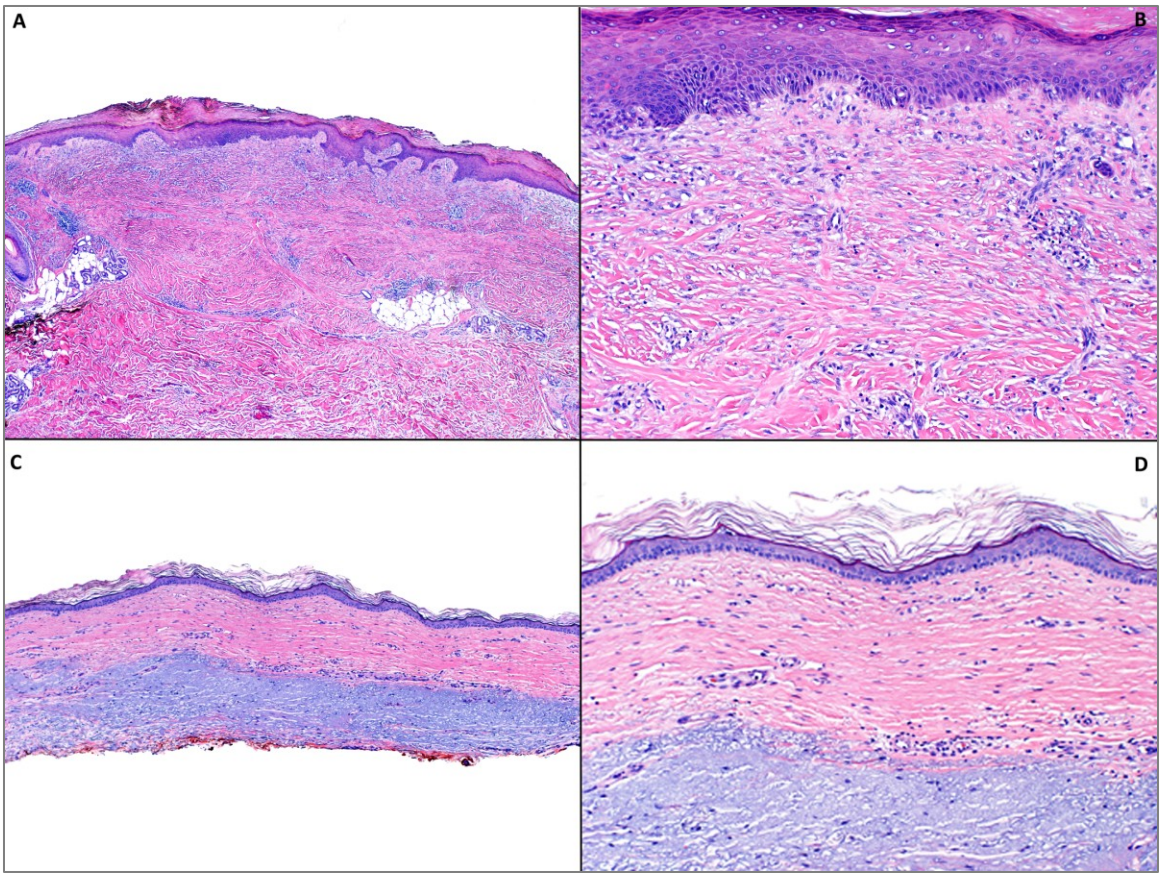


Figure 5. Histologic appearance of simple reparative scars Early cellular scars (A–B) demonstrate increased numbers of plump fibroblasts in addition to parallelly arranged collagen bundles and vertically oriented blood vessels. Note similar architectural abnormalities but low-cellularity in histologically "aged" scars (C–D). *Original magnification: A, C X20; B, D X100*

Hypertrophic scars (n=40)

Classification as a hypertrophic scar (**Figure 6**) relied on fulfillment of the following criteria:

i. Large well defined fibroblast rich nodules or large fascicles with fibrillar collagen (active/cellular histologic appearance)

OR

Well defined nodular/fascicular arrangement of fibrotic collagen and scar-type vasculature (aged histologic appearance)

ii. Absent keloidal collagen

OR

If keloidal collagen present, should represent a minor component of the total scar (<10%) and be limited to center of the scar nodule/fascicle

Note: While classically hypertrophic scars are taught to be completely devoid of keloidal collagen, in daily practice, focal keloidal collagen is frequently noted in otherwise typical hypertrophic scars. Because of this, lesions with an extremely well defined nodular/fascicular architecture characteristic of a hypertrophic scars but with a small amount of keloidal collagen were classified in this study as the former, though note was made of the presence/absence of focal keloidal collagen

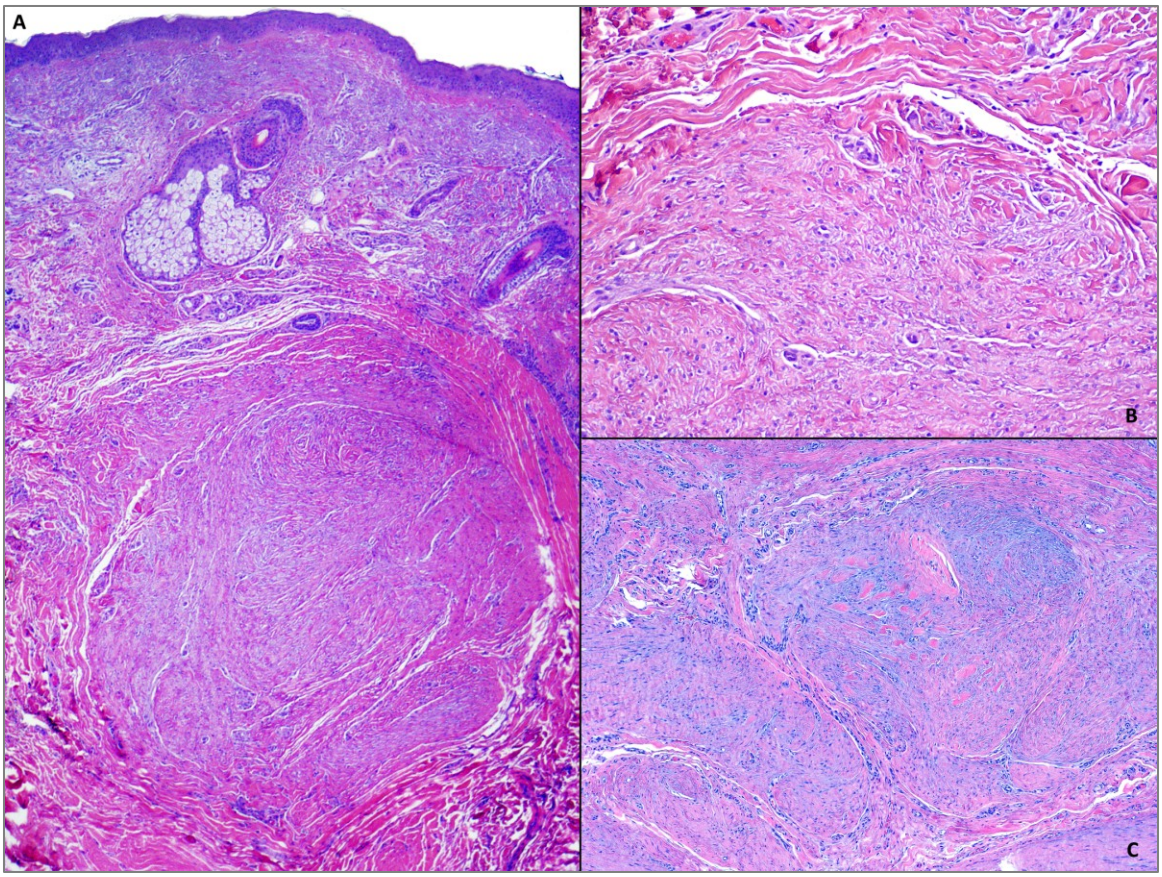


Figure 6. Histologic features of hypertrophic scars A well-defined fibroblastic nodule in the deep dermis characteristic of a hypertrophic scar (A–C). Note focal keloidal collagen in an otherwise typical hypertrophic scar nodule (C). *Original magnification: A x20; B X100; C X40*

Keloidal scars (n=30)

Classification as a keloid (**Figure 7**) relied on fulfillment of the following criteria:

i. Expansive nodule with >10% composed of typical keloidal collagen
ii. Poorly defined borders with proliferative "tongues" at the specimen margins

OR

Extensive keloidal collagen but with a somewhat well-defined nodular/fascicular architecture reminiscent of hypertrophic scar-like architecture

Note: Similar to the above caveat for hypertrophic scars, occasional keloids were encountered with an architecture more reminiscent of a hypertrophic scar but with such extensive keloidal collagen, that histopathologic designation of hypertrophic scar would be difficult based on current diagnostic convention. As such these lesions were classified as keloids but the presence of typical versus hypertrophic scar-like architecture was recorded as a sub-categorization.

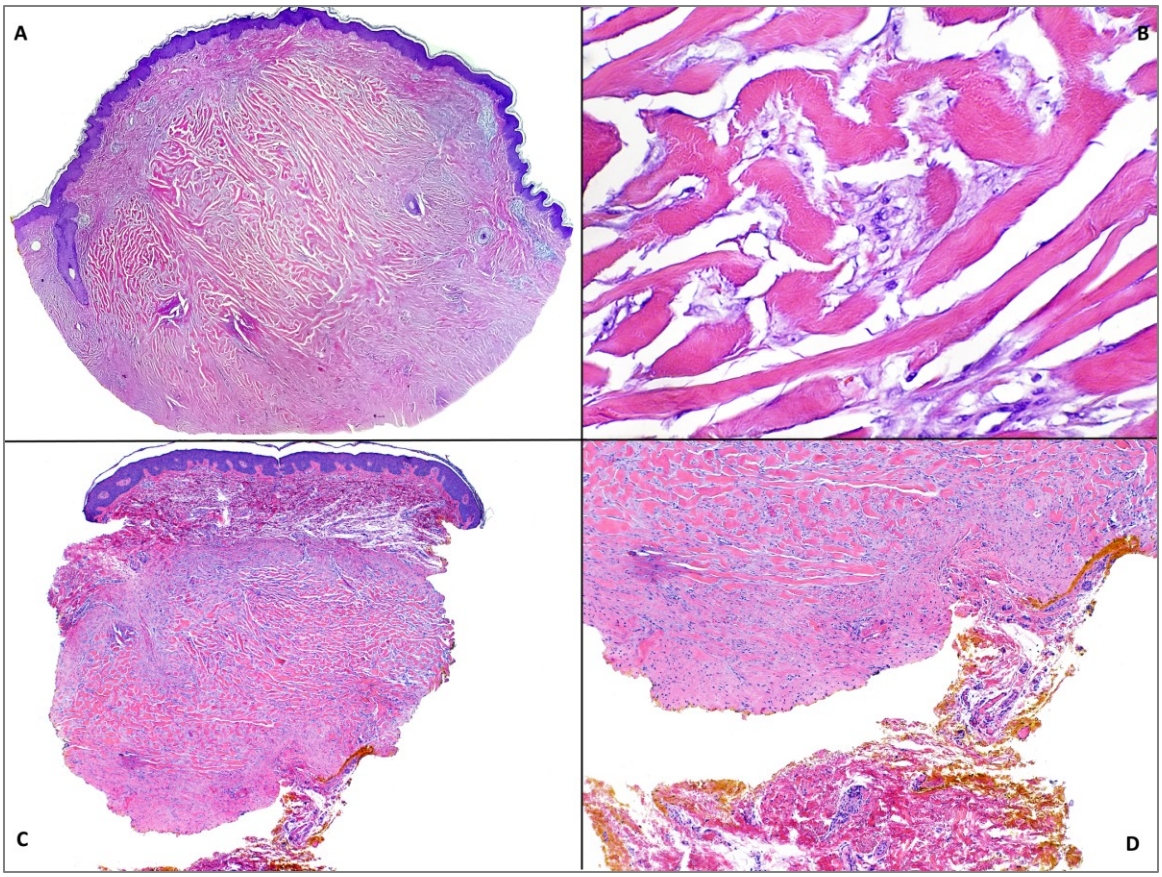


Figure 7. Histologic appearance of keloidal scars Note the expansile nature of a keloid with typical architecture (A–B), with a characteristic elevated appearance compared with surrounding normal skin and an infiltrating base (A). Large amounts of disorganized, eosinophilic collagen (keloidal collagen) are present (B) along with scattered large fibroblasts (B). Occasional extensively collagenized keloids do not elevate the skin and have a relatively circumscribed architecture somewhat reminiscent of a hypertrophic scar (C–D). Note the keloidal collagen and a rim of normal dermis subjacent to the scar, highlighting its relative circumscription (D). *Original magnification: A, C X20; BX200; C X40*

4.3 Immunohistochemistry and immunofluorescence protocols

All immunohistochemistry/immunofluorescence (IHC/IF) was performed on formalin fixed paraffin embedded tissue. Double and single staining protocols (modified from those described by Nazari et al., 2016) were applied to all specimens. After deparaffinization, hear antigen retrieval was performed with Tris-Ethylenediaminetetraacetic acid (EDTA) at pH 9.0. Subsequent protein blocking was performed with 3% H_2O_2 followed by endogenous peroxidase and alkaline phosphatase blockade with either BloxAll (Vector Labs®, Burlingame, CA) or dual endogenous enzyme block (Dako Inc.® Denmark). Further blocking with 2.5% normal horse serum was performed prior to primary antibody incubation. Primary antibodies utilized in this study (see **Table 2** for dilutions) included monoclonal rabbit anti-human CD90 (clone: EPR3132, Abcam®), monoclonal mouse anti-human CD34 (clone: QBEnd 10, Dako®), monoclonal mouse anti-human SMA (clone: 1A4, Dako®) and monoclonal rat anti-human procollagen type 1, *N*-terminal (clone: M-58, Chemicon®). Horse anti-rabbit/mouse/rat (mouse adsorbed as necessary) immunoglobulin (horseradish peroxidase [HRP]/alkaline phosphatase) polymer detection kits were appropriately utilized (ImmPress™, Vector Labs® Burlingame, CA) and chromogen development was performed with either AMEC red (HRP, ImmPact Vector labs®, Burlingame, CA) or HighDef™ blue (alkaline phosphatase Enzo Life Sciences Inc. ®). In the case of the double-staining protocol, quenching with H_2O_2 was performed after chromogen development of the first primary antibody and prior to incubation with the second. Dual

CD90/CD34 staining was performed on all specimens to assess for single cell dual positivity as evidence of mesenchymal transition. Additionally, all samples were evaluated for SMA expression. SMA positivity was primarily assessed via a single stain protocol but a subset of specimens was double-stained with CD90/SMA (n=26) to examine for single cell dual positivity and more accurately appraise the spatial relationship between fibroblasts expressing these two proteins. Similarly, a subset of specimens was double stained with procollagen-1 (PC-1)/CD90 (n=18) or PC-1/SMA (n=8) in order to demonstrate the presence of collagen synthesis and to determine the relative contribution of the various fibroblast phenotypes to ECM production.

Double-stained IF with CD90/CD34 was performed on 3 reparative scars and a similar protocol was adhered to with the exception of polymer detection which was performed with Alexa Fluor 488 and Alexa Fluor 594 tyramide reagents (Thermo Fisher Scientific®, Waltham, MA). IF imaging was executed using confocal laser microscopy (FlouView FV10i, Olympus® Waltham, MA).

Antibody	Dilution	Incubation
CD90 (Thy-1) **[EPR3132, Abcam®]**	1:1200 (IHC*) 1:3000 (IF*)	Overnight (12 hours) Overnight (12 hours)
CD34 **[QBEnd 10, Dako®]**	1:400 (IHC) 1:2500 (IF)	60 minutes 90 minutes
SMA **1A4, Dako®**	1:500 (IHC)	60 minutes
Procollagen type 1 **M-58, Chemicon®**	1:300 (IHC)	30 minutes

Table 2 Dilutions and incubation times for primary antibodies

***** IHC, immunohistochemistry; IF immunofluorescence

4.4 Histologic grading of protein expression

In addition to recording dichotomous positive or negative expression of a given marker, for a more complete analysis, protein expression was also graded using the following system:

i. *Diffuse expression*: >50% of fibroblasts within main body of the scar express protein
ii. *Focal expression*: <50% of fibroblasts within main body of the scar express protein but more than rare single cell positivity
iii. *Rare expression*: scattered single cell positivity within the main body of the scar
iv. *Negative*: No positivity within main body of the scar

With regards to fibroblast dual marker expression (CD90/CD34, CD90/SMA, CD90/PC-1 and SMA/PC-1), positivity was recorded only when clear double-positivity within a single cell was observed. For ease of statistical analysis, the presence/absence of a specific marker, the presence/absence of dual positive cells and categories of expression were assigned a numerical value ranging such that dichotomous 'present/absent' expression was recorded as '0' or '1' respectively and expression patterns were graded as 0–3 (negative → diffuse expression). It should be specifically mentioned that with regards to dual CD34/CD90 expression, double positive cells identified only at the periphery of scars (but not within the main scar) were interpreted as cells in-transition and would be recorded as present dual positivity but in the absence of expression in the main body of fibrosis, general scar expression of CD34 would be recorded as negative.

4.5 Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS version 23). As described above categorical/dichotomous variables were generated based on scar type (reparative/hypertrophic/keloidal scar) the presence or absence of a particular IHC marker (CD90/CD34/SMA/PC-1), the expression pattern of that marker, presence or absence of dual positive CD90/CD34 fibroblasts. Additional variables were created for the subset of specimens on which additional double-staining for CD90/SMA, CD90/PC-1 and SMA/PC-1 was performed, including variables designed to reflect spatial relationships and the relative contribution of each fibroblast type within a given

scar (e.g. CD90+ fibroblasts greater than/less than/equal to SMA+ fibroblasts, PC-1 expression limited to/extending beyond SMA rich areas etc.). Categorical variables were also constructed for combination of expression patterns (e.g. CD90 $^{\textbf{diffuse}}$/CD34 $^{\textbf{focal/rare/absent}}$, CD34$^{\textbf{diffuse}}$/CD90$^{\textbf{focal/rare/absent}}$ etc.). Where scar age was available, specimens were categorized as described above (see **Section 4.1**). Specimens with missing data (limited to ages of hypertrophic scars and keloids) were not included in the relevant analyses.

For scars categorized as hypertrophic or keloidal, additional dichotomous variables were created reflecting the presence or absence of keloidal collagen and the architectural pattern (typical-keloid vs. hypertrophic scar-like) respectively (see **Section 4.2**).

Descriptive statistics were generated for all variables and displayed in tabular or graphical form. All variables in this study were assigned numerical values and analyzed as categorical data. Association between variables was evaluated for using Chi-squared test of independence and where appropriate Fisher's exact test. Results were considered significant at $p \leq 0.05$.

CHAPTER 5 RESULTS

Overall, 117 specimens met the criteria for inclusion in this study, with 47 being classified as simple reparative scars, 40 as hypertrophic scars and 30 as keloidal scars. The results of the tested hypotheses are detailed below (**Table 3)**.

5.1 In normal skin, the reticular dermal stroma exhibits a $CD34^{diffuse}CD90^{negative}$ phenotype with $CD90^{+}$ cells localized to the perivascular and periadnexal adventitial compartments

When normal appearing skin was evaluated for a population of $CD34^{+}$ or $CD90^{+}$ fibroblasts (n=10), the reticular dermal stromal compartment uniformly exhibited a $CD34^{diffuse}/CD90^{negative}$ phenotype (**Figure 8**). The $CD34^{+}$ cells formed a dense and intricate network of interconnecting dendritic processes, consistent with the previously described $CD34^{+}$ fibroblastic stromal cell network with its numerous long telopodes and multifocal cell-cell connections.(Manole & Simionescu, 2016) While these fibroblastic cells were ubiquitous, they lacked the distinct parallel architectural arrangement characteristic of scar fibroblasts, in keeping with their quiescent state. In the perivascular, perifollicular and peri-eccrine adventitia, an admixture of $CD34^{+}$ and $CD90^{+}$ fibroblasts (sharply delimited to these compartments) was observed. In the perivascular and perifollicular locations, occasional double-positive CD34/CD90 fibroblasts were noted.

These findings are consistent with those previously reported for expression patterns of CD90 and CD34 in the skin.(Nazari et al., 2016)

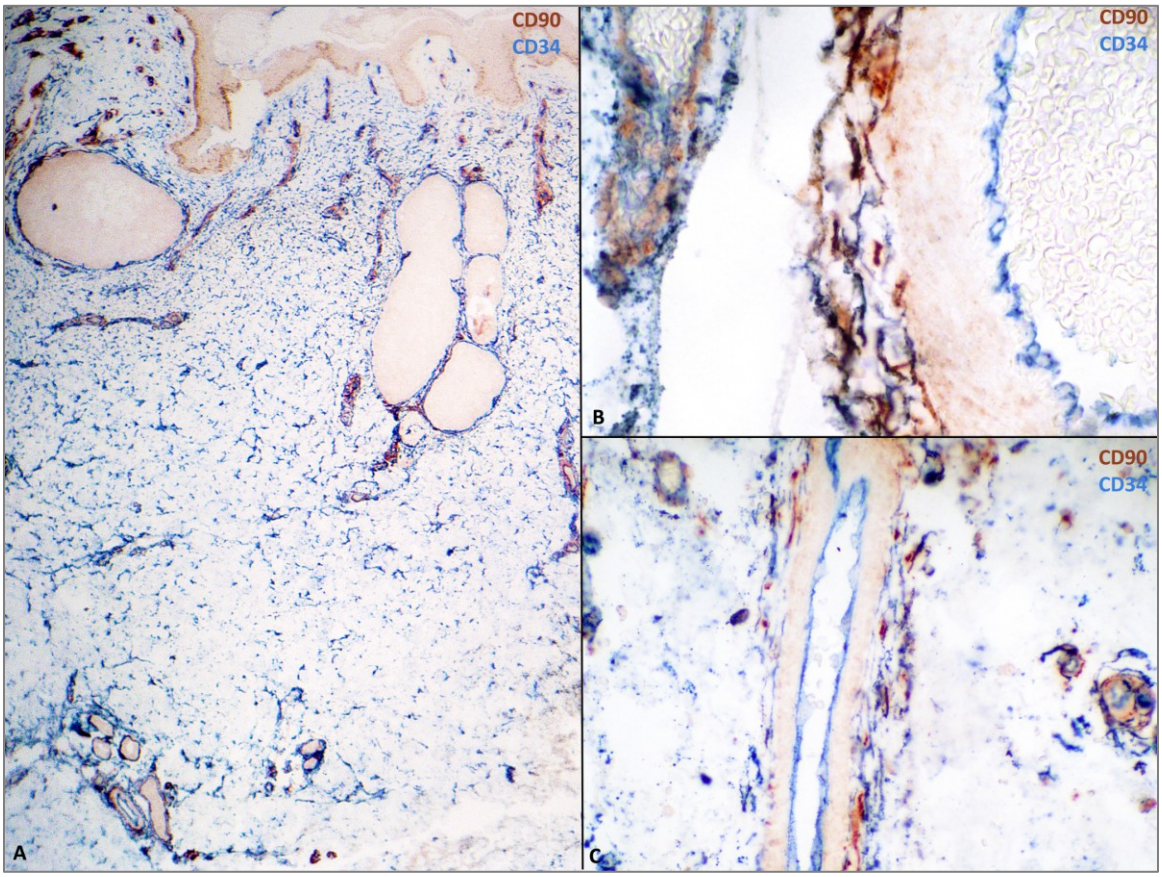


Figure 8. Regional expression of CD34 and CD90 in normal human skin The normal dermis is characterized by diffuse CD34 positivity corresponding to the resident stromal fibroblastic cell network. These quiescent fibroblasts form an intricate interstitial network, with increased concentration around adnexae (A). CD90 expressing fibroblasts are absent in the unperturbed interstitial reticular dermis but positive cells are noted within the periadnexal (B) and perivascular (C) adventia. Note dual CD90/CD34 expressing fibroblasts in a perifollicular and perivascular location (B, C). *Original magnification A X20; B X200; C X100*

5.2 Simple reparative scars, hypertrophic scars and keloids are most commonly characterized by a CD90^{diffuse}/CD34$^{negative/minority}$ fibroblast phenotype

When scar specimens (n=117) were evaluated for the presence of CD90 expressing fibroblasts within the main body of the scar, such a population was identified in 96.6% (n=113) of all scars examined. When individual scar types (reparative, hypertrophic and keloidal) were examined for fibroblast CD90 expression, reparative, hypertrophic and keloidal scars exhibited a population of CD90^{+} fibroblasts within the scar in 93.6% (n=44), 97.5% (n=39) and 100% (n=30) of specimens respectively (**Table 3**). There was no significant association between presence of a CD90^{+} fibroblast population and scar type (*p=0.3*). When CD90 expression pattern (diffuse/focal/rare/negative) was evaluated, a CD90^{diffuse} pattern was observed in 88.9% (n=104) of all scars examined. Scar-type group analysis revealed 80.9% (n=38) of reparative scars, 90% (n=36) of hypertrophic scars and 100% (n=30) of keloids demonstrated a CD90^{diffuse} expression pattern (**Figure 9)**. In scars with a CD90^{diffuse} expression pattern, the histologic outline of the fibrosis was clearly delineated by the interface of scar CD90^{+} fibroblasts and the junction of the background CD34^{+} fibroblastic stromal cell network, imparting an inverse imprint appearance (**Figure 10**). While there was no statistically significant association between CD90 expression pattern and scar type (*p=0.14*), it was interesting to note that keloids never demonstrated a CD90$^{negative/minority}$ phenotype [a "minority" phenotype refers to scars where true positivity is noted, but only in a minority of fibroblasts. It is a composite designation representing a combination of specimens exhibiting a rare/focal expression

pattern]. This pattern was however observed in both reparative and hypertrophic scars, though it was more common in reparative scars (19.1%, n=9) than in hypertrophic scars (10%, n=4) (**Figure 9**). Based on the proposed model of fibroblast transitions in scarring (see **Section 3.2, Figure 4**), the presence of a $CD90^{negative/minority}$ population in reparative scars is consistent with the concept that as fibroblasts become inactive (trending towards the pre-wound state) they are characterized by a loss of CD90. The observation that a subset of hypertrophic scars also exhibit such a population, correlates with the known natural history of spontaneous (albeit delayed) involution witnessed in occasional scars of this subtype. The lack of a $CD90^{negative/minority}$ in keloids is also congruent with their clinical course, typified by a tendency to indefinite growth.

When the presence of $CD34^{+}$ fibroblasts within the main scar body (defined bands/nodules/fascicles of fibrosis) was examined for, overall 83.8% (n=97) of all scars had a negative or only focally/rarely identified (minority phenotype) CD34 population. When CD34 expression patterns were examined among individual histologic scar type groups, a negative/minority CD34 expression pattern was also the most commonly observed pattern, accounting for 82.9% of all scars (n=97), 80.9% (n=38) of simple reparative scars, 72.5% (n=29) of hypertrophic scars and 100% (n=30) of keloids (**Figure 11)**. Taken together, among all scars and within each histologic scar group, a $CD90^{diffuse}/CD34^{negative/minority}$ expression pattern is the most commonly observed scar fibroblast phenotype.

	Reparative Scars n=47 (%)	Hypertrophic Scars n=40 (%)	Keloidal Scars n=30 (%)	Total n=117 (%)
CD90 + fibroblasts within scar body (any positivity)	93.6 (n=44)	97.5 (n=39)	100 (n=30)	96.6 (n=113)
CD34+ fibroblasts within scar body (any positivity)	25.5 (n=12)	60.0 (n=24)	53.3 (n=16)	44.4 (n=52)
SMA+ myofibroblasts within scar body (any positivity)	66.0 (n=31)	75.0 (n=30)	90.0 (n=27)	75.2 (n=88)
C90 Expression pattern				
○ **CD90 ^{Diffuse}**	***80.9 (n=38)***	***90.0 (n=36)***	***100.0 (n=30)***	***88.9 (n=104)***
○ **CD90 $^{Negative/minority♦}$**	***19.1 (n=9)***	***10.0 (n=4)***	***0***	***11.1 (n=13)***
– **CD90 ^{Negative}**	6.4 (n=3)	2.5 (n=1)	0	3.4 (n=4)
– **CD90 ^{Rare}**	10.6 (n=5)	2.5 (n=1)	0	5.1 (n=6)
– **CD90 ^{Focal}**	2.1 (n=1)	5.0 (n=2)	0	2.6 (n=3)
CD34 Expression pattern				
○ **CD34 ^{Diffuse}**	***17 (n=8)***	***27.5 (n=11)***	***0***	***16.2 (n=19)***
○ **CD34 $^{Negative/minority♦}$**	***83.0 (n=39)***	***72.5 (n=29)***	***100.0 (n=30)***	***83.8 (n=98)***
– **CD34 ^{Negative}**	76.6 (n=36)	40.0 (n=16)	46.7 (n=14)	56.4 (n=66)
– **CD34 ^{Rare}**	0	2.5 (n=1)	16.7 (n=5)	5.1 (n=6)
– **CD34 ^{Focal}**	6.4 (n=3)	30.0 (n=12)	36.7 (n=11)	22.2 (n=26)
SMA Expression pattern				
○ **SMA ^{Diffuse}**	***36.2 (n=17)***	***52.5 (n=21)***	***40.0 (n=12)***	***42.7 (n=50)***
○ **SMA $^{Negative/minority♦}$**	***63.8 (n=30)***	***47.5 (n=19)***	***60.0 (n=18)***	***57.3 (n=67)***
– **SMA ^{Negative}**	34.0 (n=16)	25.0 (n=10)	10.0 (n=3)	24.8 (n=29)
– **SMA ^{Rare}**	12.8 (n=6)	5.0 (n=2)	13.3 (n=4)	10.3 (n=12)
– **SMA ^{Focal}**	17.0 (n=8)	17.5 (n=7)	36.7 (n=11)	22.2 (n=26)
Dual CD90+/CD34+ fibroblasts	85.1 (n=40)	92.5 (n=37)	96.7 (n=29)	90.6 (n=106)
Keloidal collagen present	N/A	35.0 (n=14)	100.0 (n=30)	N/A
Hypertrophic Scar-like architecture	N/A	N/A	36.7 (n=11)	N/A

Table 3. Summarized descriptive statistics of reparative, hypertrophic and keloidal scars

♦ A "minority" phenotype includes a combination of rare/focal expression patterns

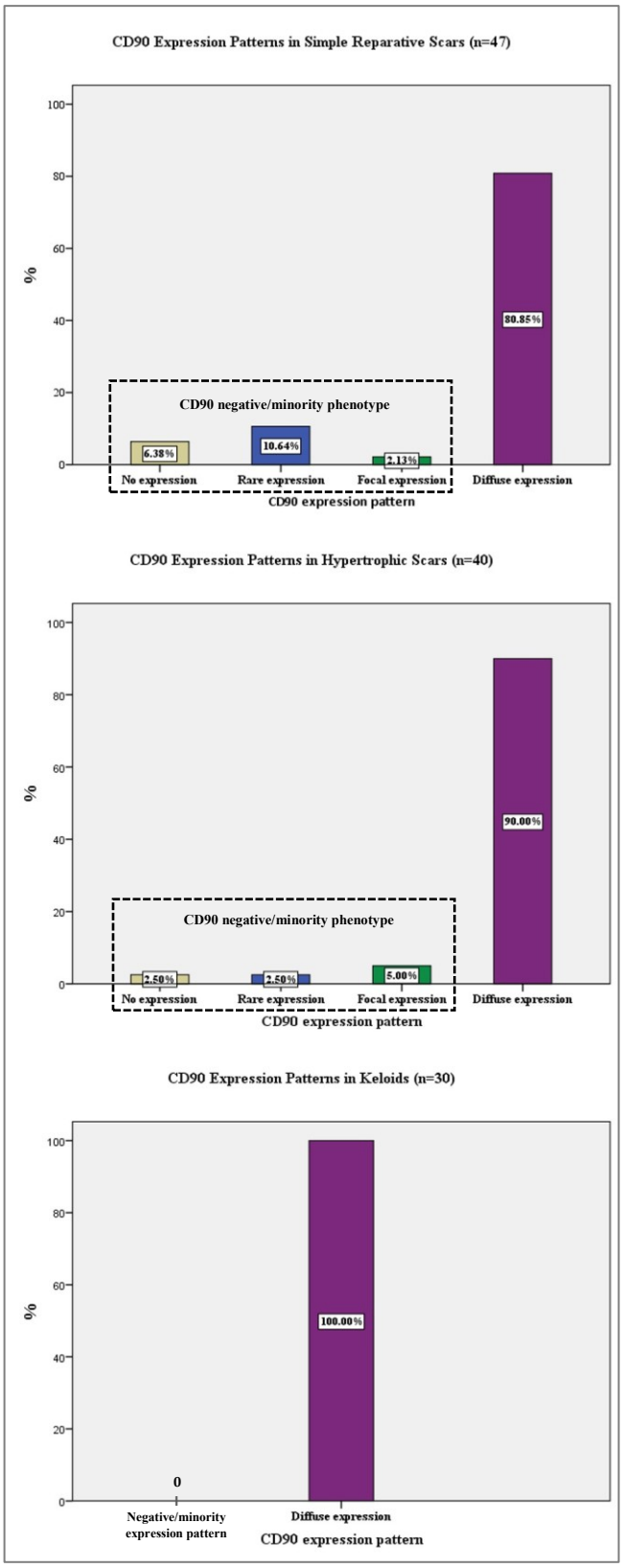


Figure 9. CD90 expression patterns based on scar type
While diffuse CD90 expression was the most pattern in all scar groups, a CD90$^{\text{negative/minority}\blacklozenge}$ pattern (boxed) was never observed in keloids and more likely in reparative scars (19.1%, n=9) than hypertrophic scars (10%, n=4). There was no statistically significant association between CD90 expression pattern and scar type ($p=0.14$)
(♦ Minority phenotype defined as any scar with a rare/focal expression pattern)

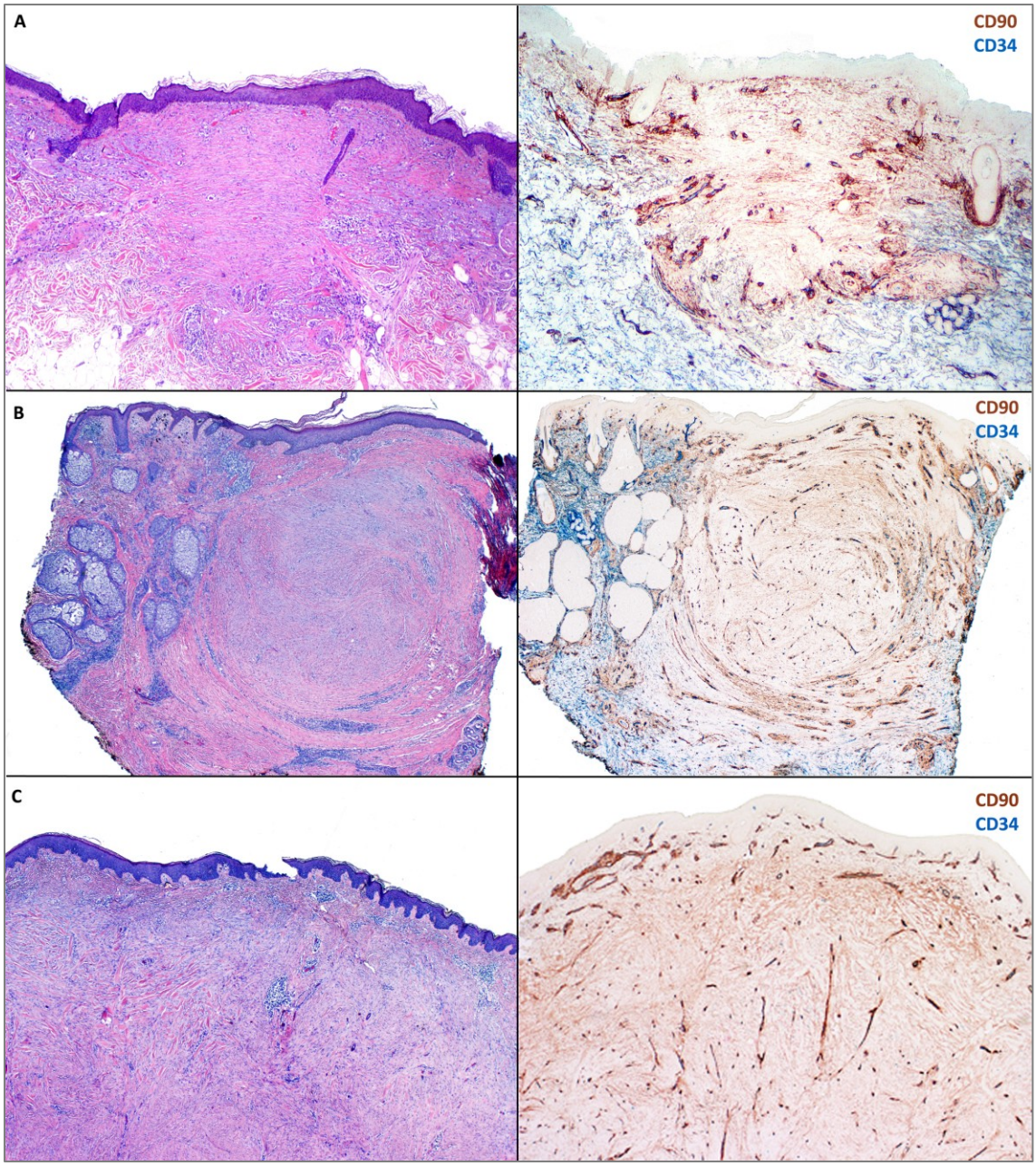


Figure 10. A CD90^{diffuse}/CD34$^{-/minority}$ phenotype in cutaneous scars This was the most commonly observed pattern in reparative scars (A), hypertrophic scars (B) and keloids (C). Note frequently identified "inverse imprint" relationship, between CD90 and CD34 clearly highlighting the main scar body (A, B)**.** *Original magnification A–C X20*

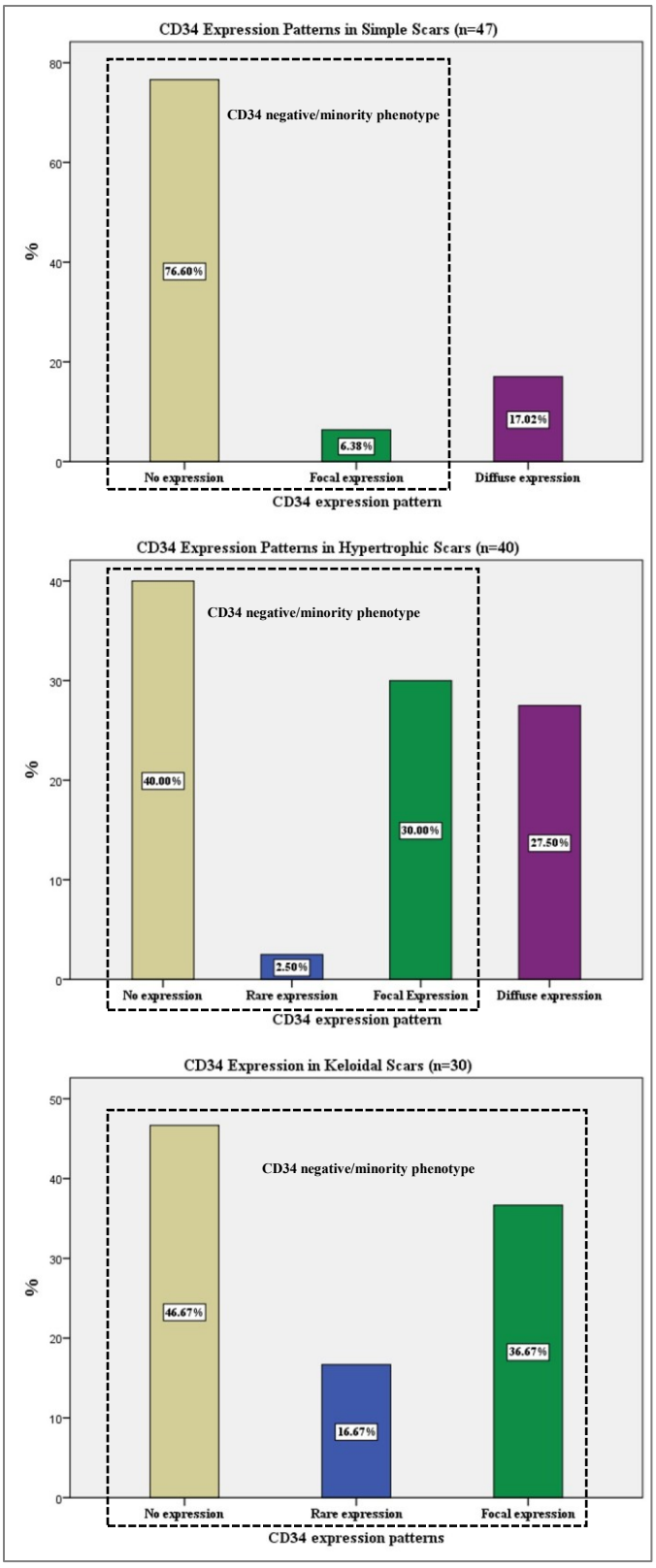


Figure 11. CD34 expression patterns based on scar type
Among all scar groups, a CD34$^{\text{negative/minority}\blacklozenge}$ (boxed) was the most common expression pattern (83.8%, n=98), although diffuse CD34 expression (a CD34 revertant state) was observed in a subset of simple reparative (17%, n=8) and hypertrophic scars (27.5%, n=11), but never in keloids. There was a statistically significant association between CD34 expression pattern and scar type ($p<0.0001$). Absent expression pattern categories in the adjacent graphs indicate that no scar in that group expressed the missing pattern (n=0)
(⬥ Minority phenotype is defined as any scar with a rare/focal expression pattern)

Diffuse expression
Focal expression
Rare expression
No expression

5.2 All scar types demonstrate CD90/CD34 dual positive fibroblasts suggestive of local transition from the background CD34+ fibroblastic stromal cell network

As previously detailed, this study hypothesizes that the background $CD34^+$ fibroblastic stromal cell network serves as a mesenchymal progenitor pool which after injury loses its CD34 expression in exchange for CD90 expression. One method of evaluating for this transition would be to assess for the presence of fibroblasts expressing both proteins within a single cell via immunohistochemistry (IHC) and/or immunofluorescence (IF).

With IHC, dual CD90/CD34 positive fibroblasts were identified in 90.6% (n=106) of all scars examined and were found in 85.1% (n=40) of reparative scars, 92.5% (n=37) of hypertrophic scars and 96.7% (n=29) of keloids (**Figures 12, 13)**. In the majority of specimens, CD34 expression was identified at the scar periphery corresponding to the interface between the unaffected background $CD34^+$ fibroblastic cell network and scar fibrosis, congruent with the notion that $CD90^+$ fibroblasts are derived from the surrounding quiescent network. These double positive fibroblasts were easily identified in the vast majority of cases. When double color immunofluorescence was performed on three reparative scars to assess for dual positive fibroblasts, numerous cells clearly displaying both proteins within a single cellular process were frequently observed (**Figure 14**). The morphology of these cells was consistent with either fully mature scar fibroblasts or exhibited delicate dendritic processes characteristic of stromal fibroblastic cell telopodes. Like with IHC evaluation, dual-positive cells were predominantly

identified at the boundary of the main scar ($CD90^{\mathbf{diffuse}}$ with no CD34 co-expression) and the uninvolved dermal stromal network ($CD34^{\mathbf{diffuse}}$ with CD90 co-expression). While vascular endothelium expresses CD34 and the surrounding perivascular adventitia and pericytes may express CD90, misinterpretation of vessels as fibroblasts is improbable as vessel morphology and distribution are clearly unalike that of a fibroblast, an easily appreciable distinction. Overall these findings are interpreted as evidence supporting the proposed CD34→CD90 transition hypothesis.

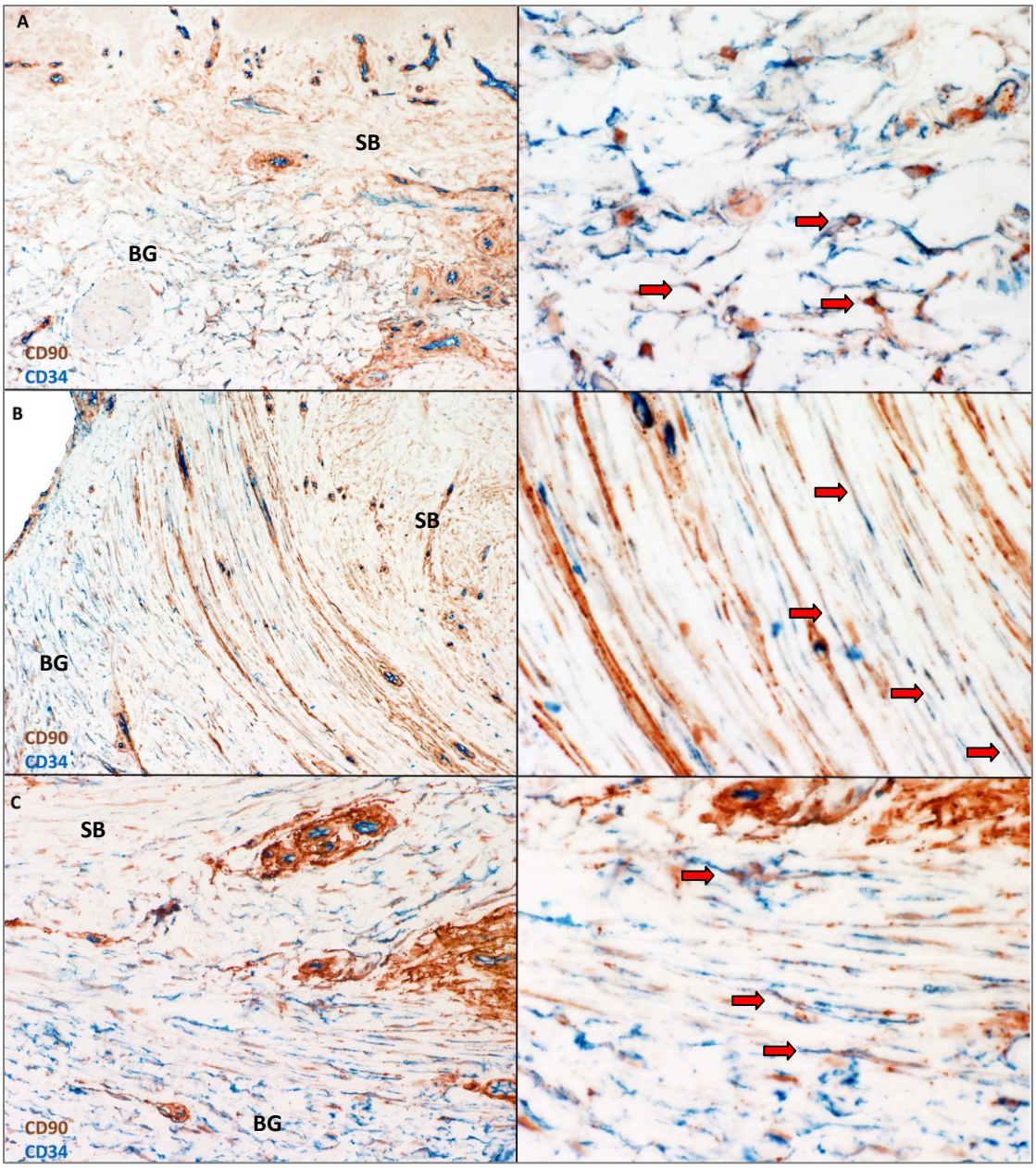


Figure 12. Examples of dual expressing CD90^{+}/CD34^{+} fibroblasts in cutaneous scars Fibroblasts expressing both CD90 and CD34 within a single cell (arrows). Dual expressing cells were most commonly seen at the interface between the main scar body (SB) and the background CD34+ stromal cell network (BG). These co-expressing cells were present in reparative (A), hypertrophic (B) and keloidal scars (C). *Original magnification A, B X40 (L), X200 (R); C X100 (L), X200 (R)*

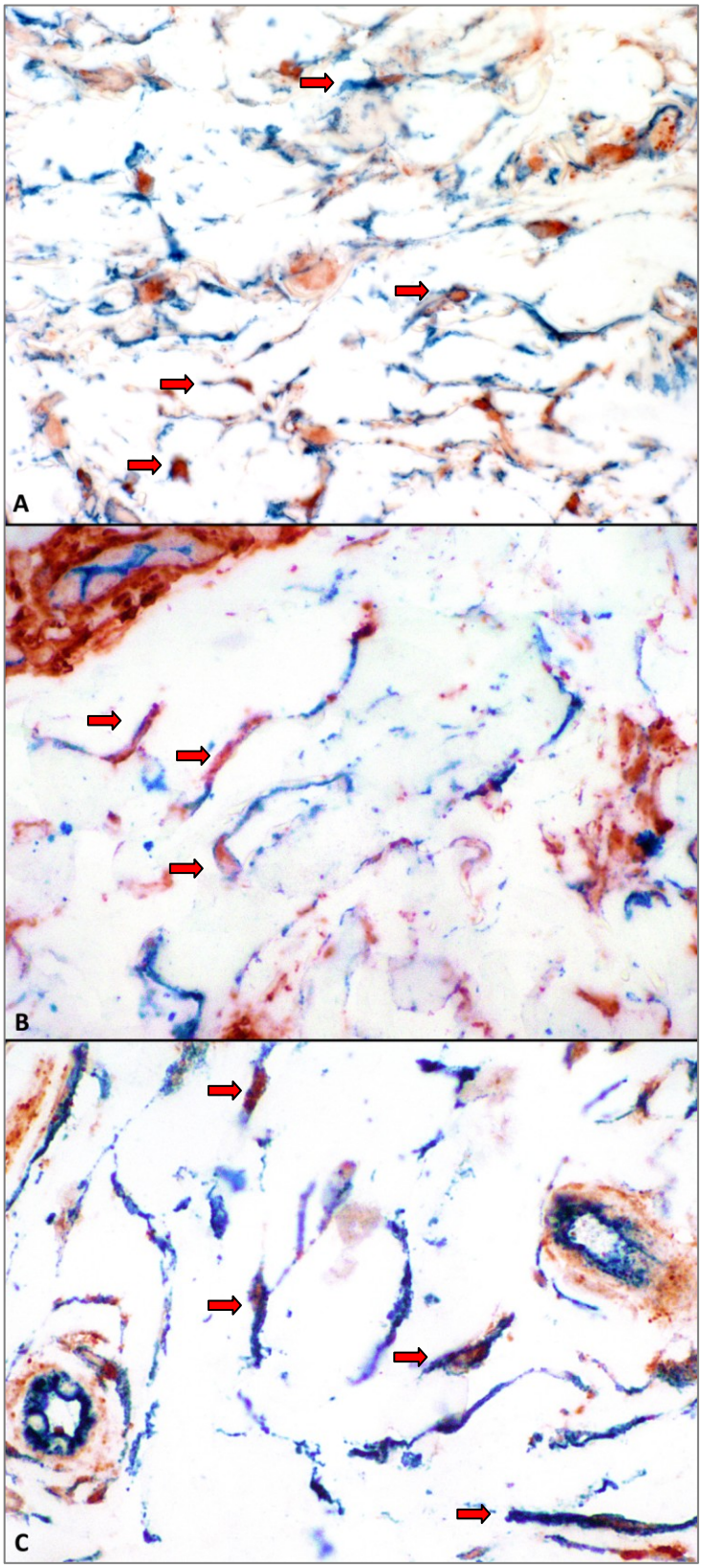


Figure 13. High magnification examples of dual expressing $CD90^{+}/CD34^{+}$ fibroblasts
Note high power examples of fibroblasts expressing both CD90 and CD34 within a single cell (arrows) in a reparative scar (A), a hypertrophic scar (B) and a keloid (C). *Original magnification A-C x400*

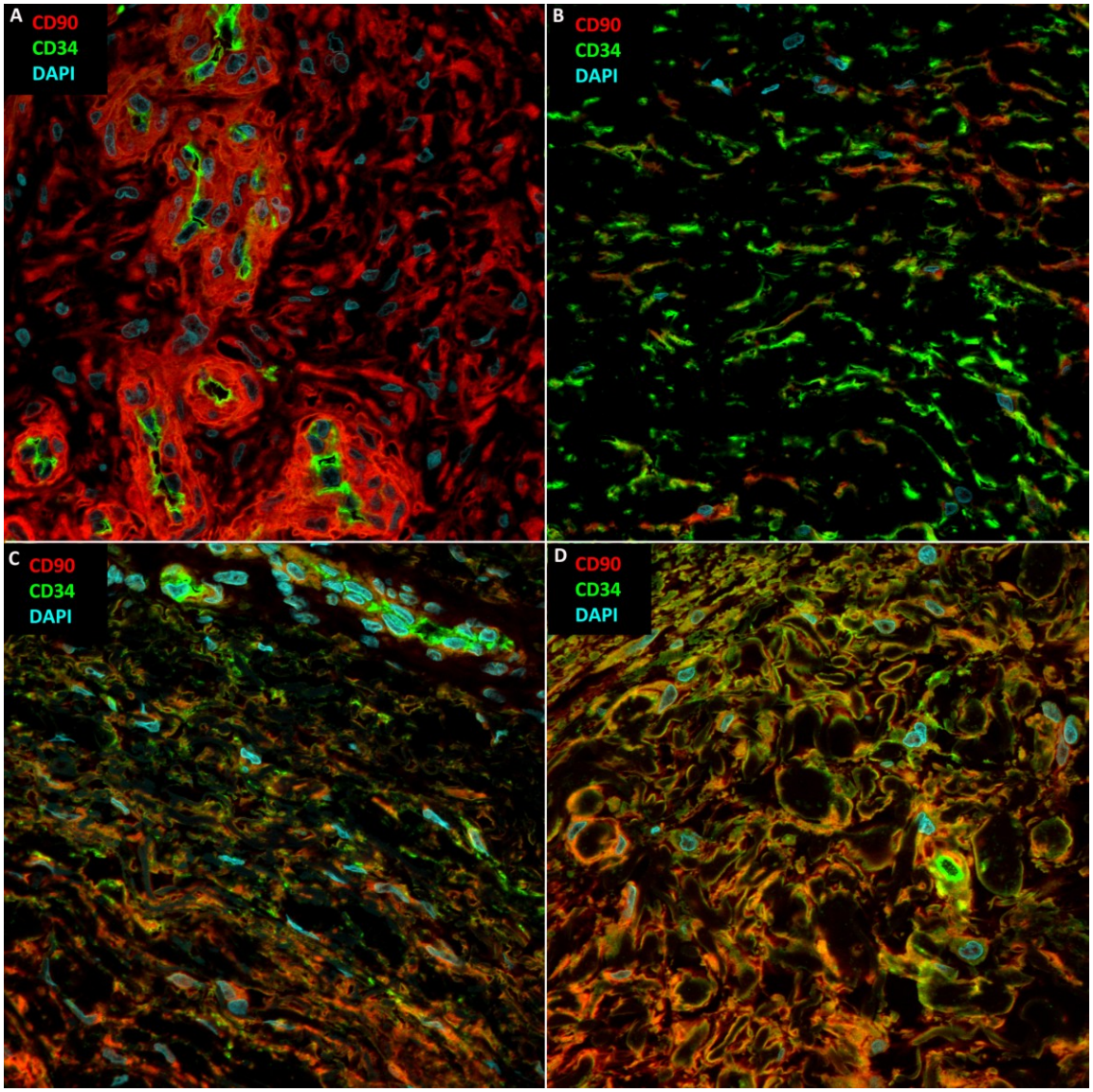


Figure 14. Dual expressing CD90^{+}/CD34^{+} fibroblasts in cutaneous scars confirmed with double color immunofluorescence
An example of the CD90$^{\text{diffuse}}$/CD34$^{\text{negative}}$ phenotype observed in the main scar body (A). Note dual expressing CD90^{+}/CD34^{+} fibroblasts at the scar-background interface (B–C) with some examples demonstrating extensive dual expression (C, D). *Original magnification A–D X600*

5.3 In simple reparative scarring, a CD90^{diffuse}/CD34$^{negative/minority}$ phenotype is a time-limited and reversible phenomenon eventuating in return of the homoeostatic CD34^{diffuse}/CD90^{negative} state: a "CD34 revertant phenotype"

The goal of cutaneous repair is achievement of as close an approximation of the pre-wounded tissue state as possible. This study hypothesizes that in physiologic scarring, when maximal repair has occurred, the scar fibroblast protein expression returns to the pre-injury phenotype. We also propose that in the non-pathologic state, scar reversion would follow a general and reproducible time-line. In order to test this hypothesis, reparative scars were dated according to their age in days and further grouped into age categories as follows: <30 days (< 1 month), 30–89 days (1–3 months), 90–179 days (3–6 months) and > 180 days (>6 months) (see **Section 4.1**). When CD90 expression was assessed for based on scar age (**Figure 15)**, all scars younger than 89 days (~ 3 months) were characterized by a CD90^{diffuse} expression pattern. Scars ranging between 90–179 days (3–6 months) could still express CD90 diffusely, but 2 scars in this category demonstrated a negative/minority profile. At greater than 160 days, no scar had a CD90^{diffuse} pattern with all exhibiting a CD90$^{negative/minority}$ phenotype (only negative staining or rare single cell positivity was seen in these cases, **Figure 16**). Interestingly, when a 2-day old wound was examined for CD90 expression, diffuse transition to CD90 had already occurred. Fascinatingly, widespread dermal CD90 expression was noted far beyond the lateral and deep margins of the visible wound, extending deeply into the septae and lobules of the underlying subcutis and even involving the adjacent tissue

section with an intact epidermis and no evidence of dermal injury (**Figure 17)**. A similar diffuse pattern was also noted in scars aged 8, 14 and up to ~30 days. This pattern contrasts sharply with the inverse area imprint appearance seen in well-developed scars (**Figure 10**) and raises the possibility that early transition is less discriminatory, affecting the large regions of involved and clinicopathologically uninvolved skin and subjacent adipose tissue. Together, these data suggest that CD90 expression in fibroblasts appears rapidly after wounding (as early as 48 hours) in a diffuse, non-scar-outlined pattern which may persist up until 1 month, followed by diffuse CD90 expression within a well-outlined scar (inverse imprint pattern) up until ~3+ months of age. Between 3–6 months of age, some scars begin to lose their CD90 expression with subsequent significant loss of CD90 expression occurring in scars >160–180 days of age.

When CD34 expression was examined for based on scar age, the inverse expression pattern (as anticipated) was observed. All scars younger than 90 days had a $CD34^{\textbf{negative/minority}}$ pattern, 2 scars between 90–179 days displayed a revertant $CD34^{\textbf{diffuse}}$ pattern, the youngest of which was 98 days. All 7 scars older than 160 days (6 scars > 180 days) demonstrated a $CD34^{\textbf{diffuse}}$. In keeping the reciprocal staining pattern of CD34 and CD90 and the age-dependent pattern of expression observed for CD90, scars lose CD34 rapidly after wounding and continue to express a $CD34^{\textbf{negative/minority}}$ phenotype until between 90–180 days when there seems to be the beginning of the return of the pre-wound $CD34^{\textbf{diffuse}}$ state. Complete return of a $CD34^{\textbf{diffuse}}$ appears to occur after 160–180 days in reparative scars. Furthermore, the disappearance CD34 mirrors the initial

appearance of CD90 and likewise the reappearance of CD34 corresponds to the disappearance of CD90, providing further evidence of the reciprocal nature of these proteins (**Figure 21)**. In simple reparative scars, CD90 expression pattern was significantly associated with CD34 expression pattern ($p<0.0001$). This reappearance of CD34, felt to represent the maximal return to pre-injury status, is hereafter termed a "CD34 revertant state". The histologic appearance of the revertant state is one of a low-cellularity scar with retention of its cicatrized collagen architecture (**Figure 16- C**). As expected, in simple reparative scars there exists a statistically significant association between both CD90 and CD34 expression patterns and scar age ($p<0.0001$).

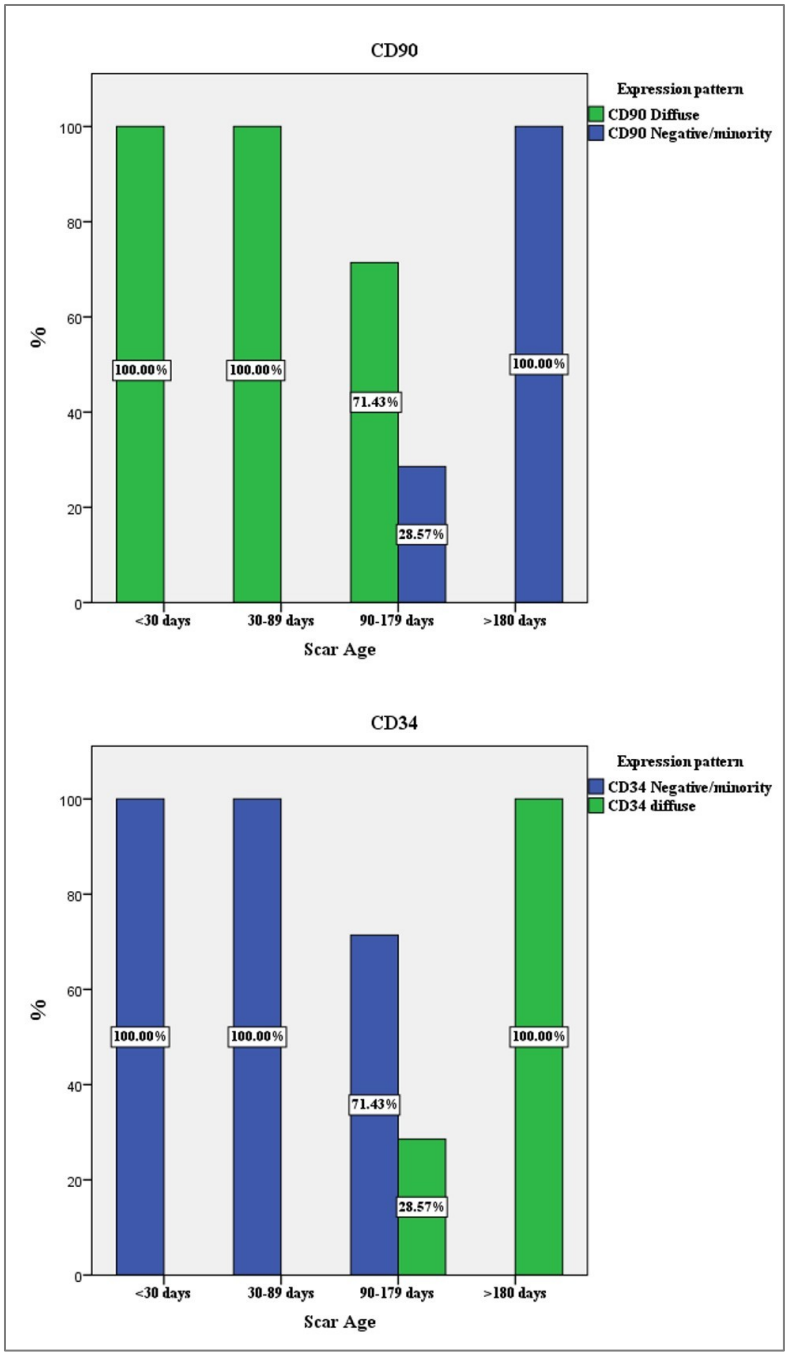


Figure 15. Age based expression of CD90 and CD34 in reparative scars
All scars younger than 90 days exhibited a $CD90^{diffuse}CD34^{-/minority}$ phenotype. Loss of a diffuse CD90 pattern is accompanied by a return of diffuse CD34 positivity (a CD34 revertant state), and was observed in all scars> 160 days (time point not indicated). Scar age was significantly associated with expression patterns of both CD90 and CD34 ($p<0.0001$)
(Minority phenotype is defined as any scar with a rare/focal expression pattern)

Diffuse

Negative/ minority

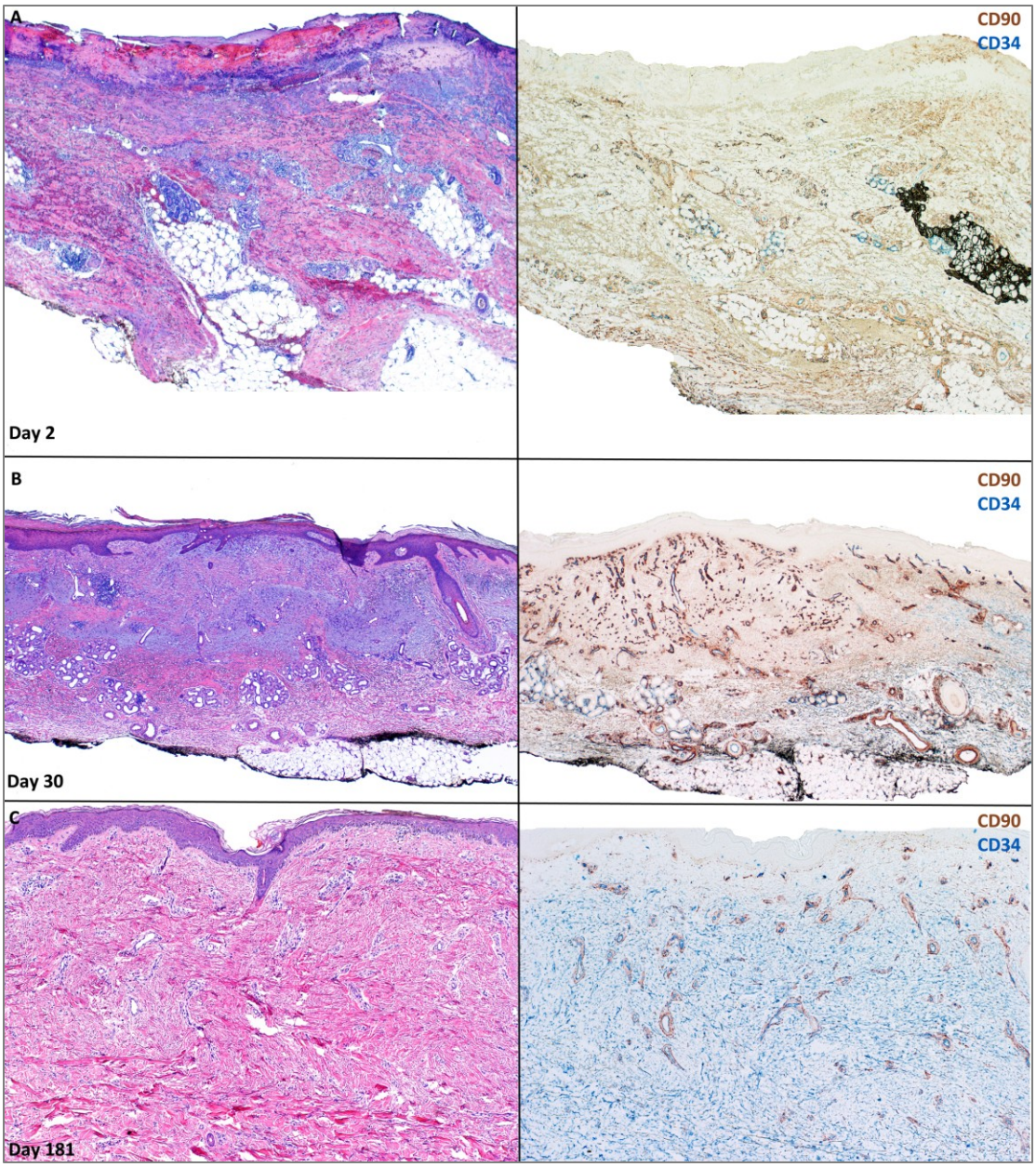


Figure 16. Stages of scar fibroblast activation and reversion As early as 48 hours after wounding there is loss of CD34 and extensive expression of CD90 (A, see **Figure 17**). This phase may last until approximately 30 days and is followed by diffuse CD90 expression delimited to a well-defined scar, lasting until 160 days (B). Loss of CD90 positivity and diffuse re-expression of CD34 occurs as early 98 days and in all scars between 160–180 days (C). *Original magnification A, B X20; C x40*

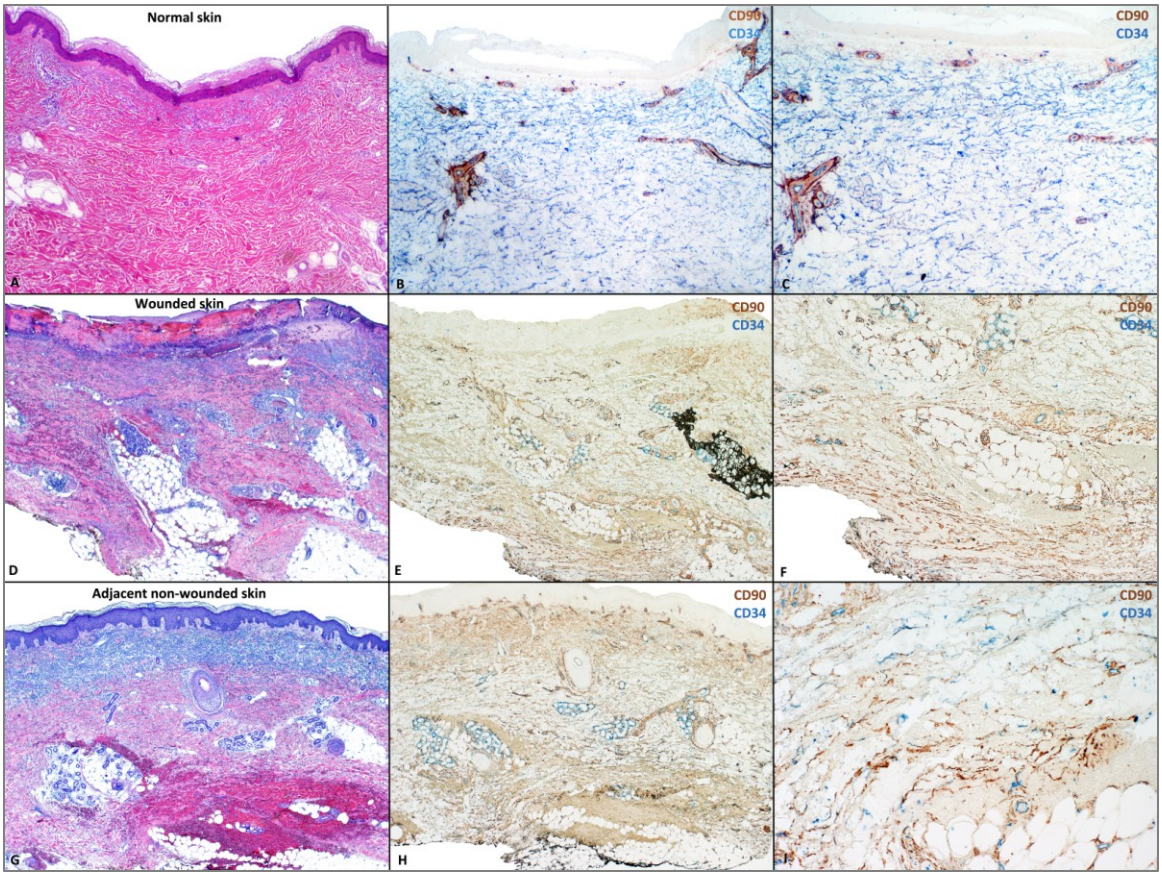


Figure 17. Loss of CD34 and extensive indiscriminate expression of CD90 48 hours after injury Note pan-dermal loss of CD34 and concurrent expression of CD90 in injured skin (D–F) compared with normal skin (A–C) with transition extending far beyond the visible wound (E, F) and even involving the fat septae (F). Fascinatingly, adjacent tissue sections (~ 3mm away) with undamaged intact epidermis (G) also exhibited a loss of CD34 and neo-expression of CD90 (H, I) extending into the subcutis (I). *Original magnification A, B, D, E, G, H X20; C, F, I X40*

5.4 A CD34 revertant ($CD34^{diffuse}$) phenotype is observed in reparative scars and hypertrophic scars, but not in keloids

A revertant phenotype was examined for in pathologic scars. Interestingly, a revertant phenotype was also observed in 27.5% (n=11) of hypertrophic scars and this finding is consistent with the clinical resolution reported to occur in a subset of hypertrophic scars (**Figure 18)**. A revertant phenotype was not observed in any keloid. As expected based on these observations and those detailed above, there was a statistically significant association (*p=<0.0001*) between CD34 expression pattern and histologic scar type and specifically, between histologic scar type and a revertant phenotype ($CD34^{diffuse}$ pattern) (*p=0.*014). These data support the suggestion of CD34 reversion as a biologic phenomenon occurring in reparative scars and a subset of histologically aged hypertrophic scars. The finding that a revertant state was not observed in keloids is also compatible with their known natural history of persistence. Additionally, when CD90 expression pattern was evaluated in scars which exhibited a CD34 revertant phenotype, a peculiar difference in the CD90 expression patterns was detected. 8 of 9 revertant reparative scars demonstrated a $CD90^{negative/minority}$ /$CD34^{diffuse}$ reversion pattern whereas 7 of 11 revertant hypertrophic scars displayed the opposite $CD90^{diffuse}$/$CD34^{diffuse}$ pattern. These findings raise the possibility of a difference in reversion pathways between reparative and pathologic scars.

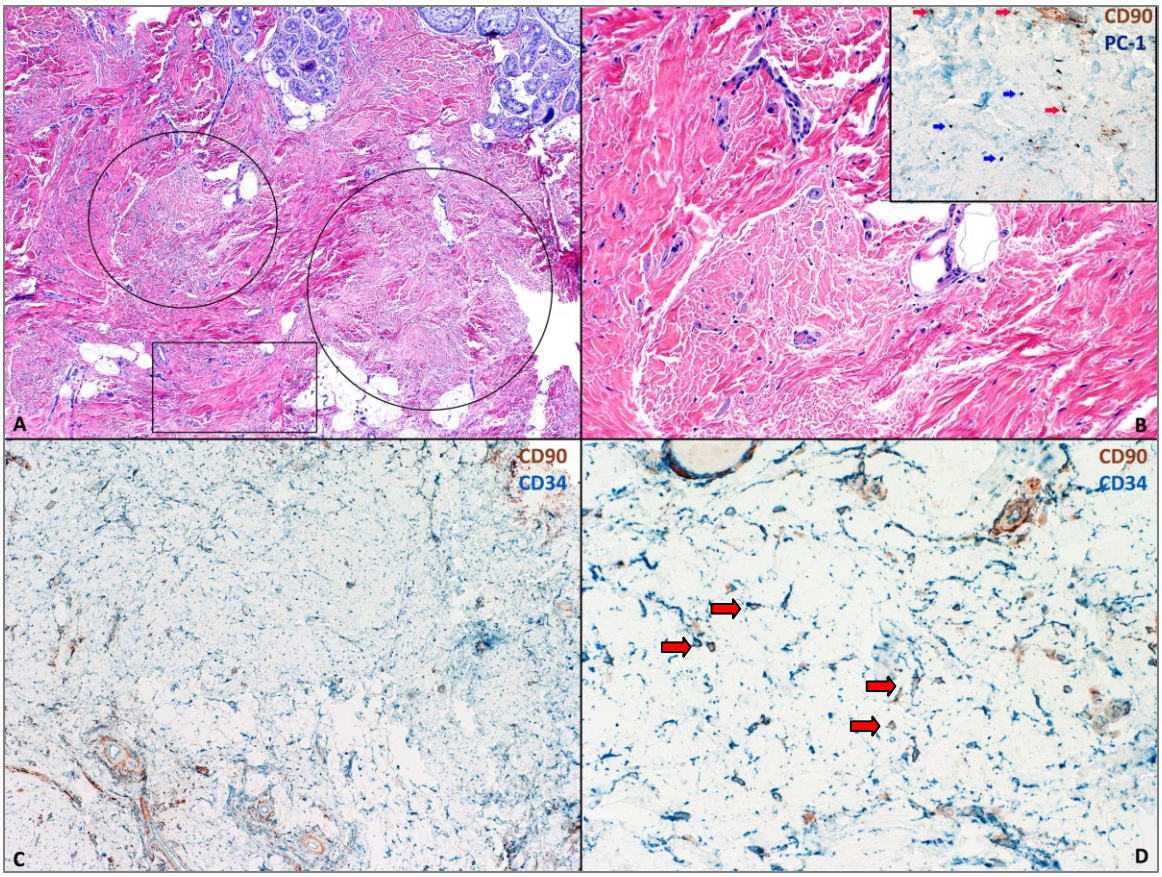


Figure 18. A revertant hypertrophic scar Note the low-cellularity nodules (A, circled) and fibrous fascicles (A, boxed) characteristic of aged hypertrophic scars. Higher magnification of a nodule reveals rare fibroblasts, some of which are $CD90^{+}$ (B inset, red arrows) and many which are procollagen I $(PC\text{-}1)^{+}$ indicative of active collagen synthesis (B inset, blue arrows). There is however generalized return of CD34 positivity (CD34 revertance, C), though many of the $CD34^{+}$ fibroblasts continue to express CD90 (D). *Original magnification A, C X40; B, D X200*

5.5 Keloids and a subset of hypertrophic scars are characterized by a prolonged CD90^{diffuse} fibroblast phenotype

As discussed above, in simple reparative scars complete reversion is associated with loss of CD90 and re-expression of CD34. While clinical data allowing for scar aging was lacking in a number of hypertrophic scars and keloids, we were able to date significant subset of these entities. In total 23 hypertrophic scars were dated, with ages ranging from 32 days to 1098 days. Twelve hypertrophic scars aged 180–1098 days were identified, and of these, nine (75%) displayed a CD90^{diffuse} pattern. Thirteen keloids were dated and twelve were older had ages ranging between 300–3128 days. All keloids exhibited a CD90^{diffuse} pattern. These data suggest that a subset hypertrophic scars and keloids are characterized by a prolongation of their CD90^{diffuse} phenotype, potentially serving as further evidence that these entities may be diseases on a spectrum, but with keloids having more profound delay in losing their active fibroblast phenotype.

5.6 Reparative and pathologic scars contain SMA+ myofibroblasts in varying proportions

Next the presence of SMA expression in fibroblasts (classical myofibroblasts) and the associated expression patterns were examined for. Overall, among all scar groups, 75.2% (n=88) expressed SMA to some degree. In simple reparative scars, a near equal proportion of scars displayed a SMA^{diffuse} pattern (36.2%, n=17) or absent SMA

expression (34.78%, n=16). The remaining scars exhibited focal or rare SMA positivity therefore a SMA $^{\textbf{negative/minority}}$ phenotype was the most commonly observed pattern in simple reparative scars (63.8%). In hypertrophic scars 52.5% (n=21) exhibited a SMA$^{\textbf{diffuse}}$ pattern with 17.5% (n=7) exhibiting focal positivity, 5.0% (n=2) demonstrating only rare single cells and 25% (n=10) were entirely negative for SMA. Among keloidal scars, 40% (n=12) diffusely expressed SMA, while 36.7% (n=11) exhibited focal SMA expression, 13.3% (n= 4) rare SMA expression and 10% (n=3) having no detectable SMA expression. A SMA$^{\textbf{negative/minority}}$ phenotype (60%) was the most commonly observed pattern in keloids (**Figure 19**). Overall however, there was no significant association between scar type and SMA expression pattern (*p=0.085*)

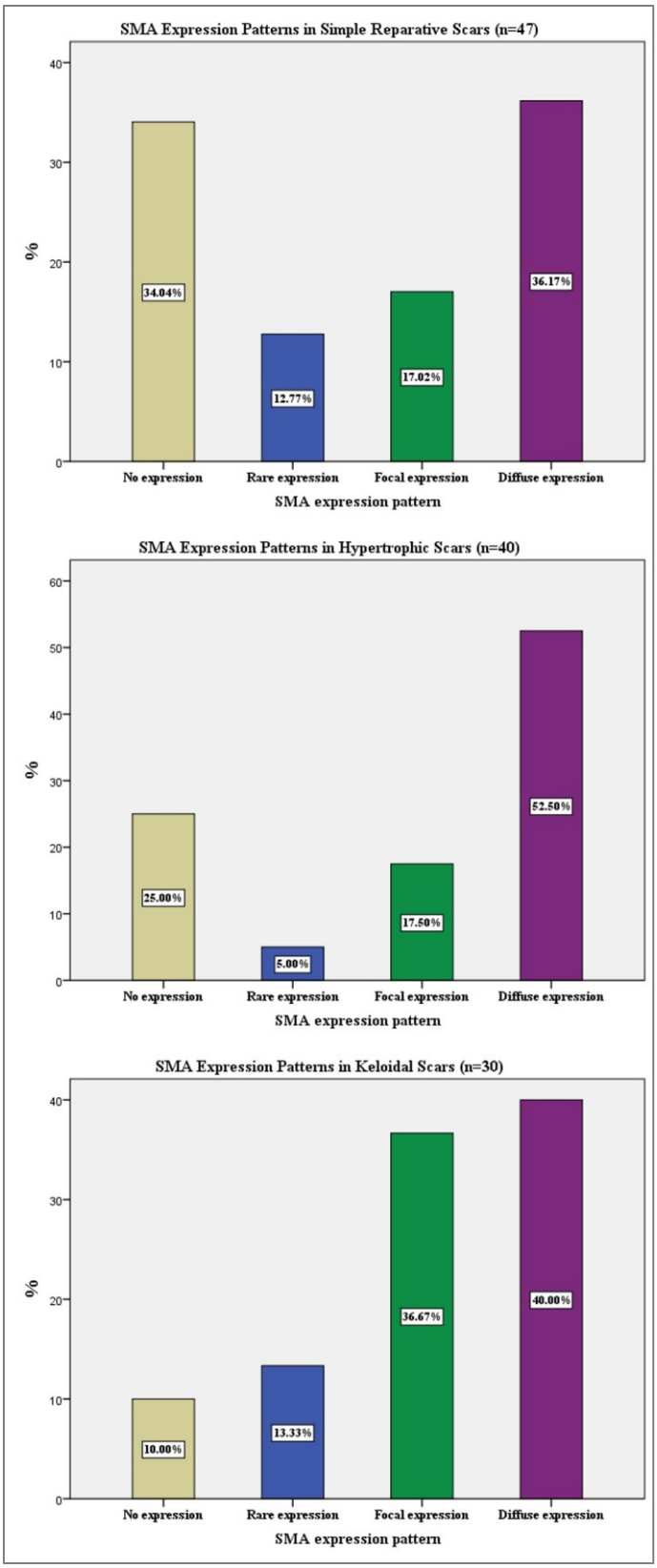


Figure 19. SMA expression patterns based on scar type
All scar types were more likely than not to demonstrate SMA+ myofibroblasts to some degree. Unlike CD90 however, diffuse fibroblastic expression of SMA was not observed in the overwhelming majority of cases. A SMA$^{-/\text{minority positive}\blacklozenge}$ phenotype was the most commonly observed pattern in simple reparative scars (63.8%, n=30) and keloids (60%, n=18). Hypertrophic scars displayed diffuse SMA positivity (52.5% n=21) in almost equal proportions to a negative/minority pattern. There was no significant association between SMA expression pattern and scar type (*p=0.085)*
(⬥ Minority phenotype is defined as any scar with a rare/focal expression pattern)

Diffuse expression
Focal expression
Rare expression
No expression

5.7 In simple reparative scarring, the presence of SMA^+ myofibroblasts is a time-limited and reversible phenomenon

Like CD90 expressing fibroblasts, in physiologic scarring, classical SMA^+ myofibroblasts also appear and disappear from scars in a time dependent manner. 87.1% (n=27) of scars between 0–89 days (0–3 months) had identifiable SMA+ myofibroblasts with 48.4% of scars expressing SMA diffusely (n=15). Interestingly, none of the scars between 90–180 days expressed SMA diffusely with 71.43% (n=5) having no identifiable SMA^+ fibroblasts (**Figure 20**). No reparative scar older than 160 days exhibited SMA^+ myofibroblasts (n=7). The association between SMA expression pattern and scar age in reparative scars was statistically significant at $p<0.0001$. The pattern of SMA expression and loss thereof closely approximated the pattern noted for CD90 expression, though $CD90^{diffuse}$ pattern continued to be seen in the majority of scars between 90–180 days, suggesting that CD90 expression may be retained longer than SMA expression in physiologic scarring. Loss of SMA expression and a $CD90^{diffuse}$ pattern coincided with reversion to a CD34 positive state (**Figure 21)**.

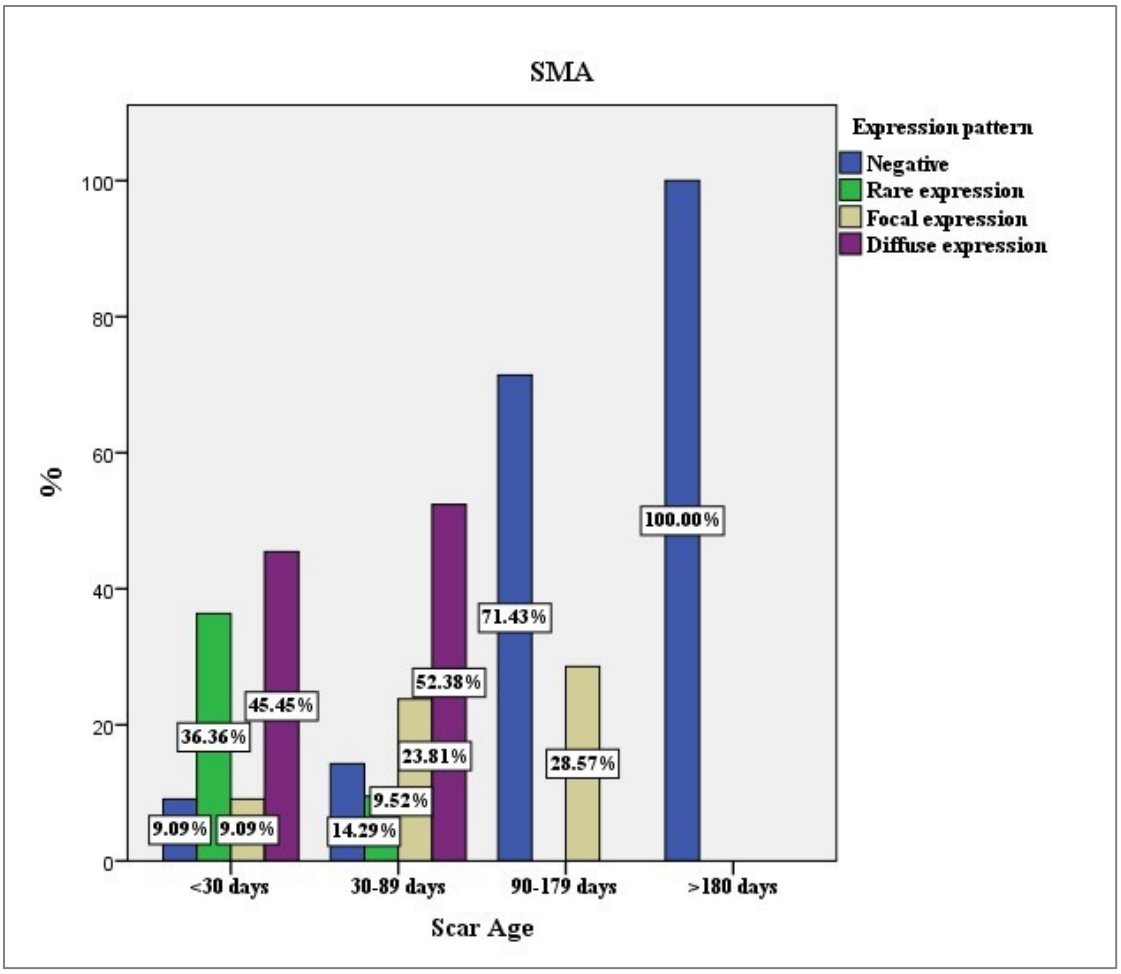


Figure 20. Age based expression of SMA in reparative scars Like CD90 and CD34, the presence of SMA expressing myofibroblasts in reparative scars is time dependent. SMA expression is focal to absent in scars older than 90 days. The SMA expression timeline resembles that of CD90. There was a statistically significant association between scar age and SMA expression pattern ($p < 0.0001$)

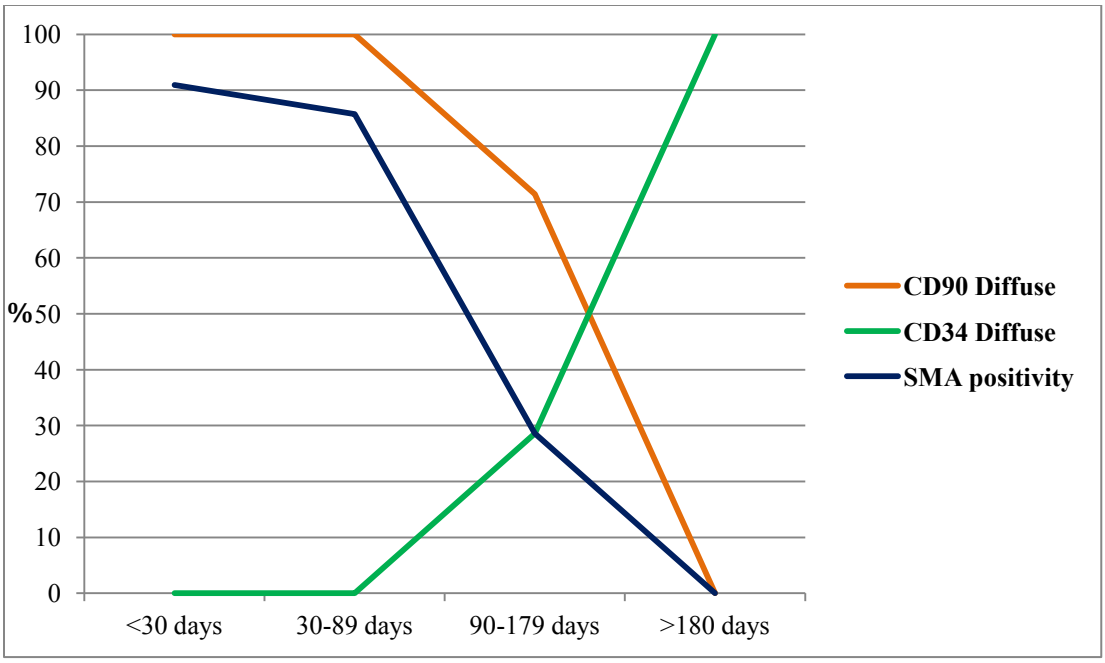


Figure 21. Relative time course for appearance/disappearance of CD90, CD34 and SMA in reparative scars All scars are diffusely CD90 positive until 90 days, after which CD90 expression decreases and is absent in scars older than 160–180 days. SMA positivity closely resembles CD90 expression, although decrease in SMA expression begins earlier that CD90. The loss of a diffuse CD90 expression pattern and SMA positivity corresponds to the re-expression of CD34 (reversion)

5.8 Many hypertrophic scars are characterized by persistence of SMA+ myofibroblasts

When the relationship between SMA expression and (available) scar age was examined for in hypertrophic scars, 9 of the 12 scars (75%) older than 180 days still contained SMA+ myofibroblasts and 6 displayed diffuse positivity. Compared with reparative scars, hypertrophic scars appear to be characterized by a persistence of SMA+ myofibroblasts. Though the gaps in scar-age data for hypertrophic scars preclude comprehensive delineation of an SMA expression pattern time-line, it is interesting to note, that the 3 dated hypertrophic scars which had lost their SMA expression were all older than 500 days. These data suggest that SMA expression, while prolonged is not necessarily permanent, though a marked delay in its disappearance may characterize this scar type.

5.8 In keloids, diffuse SMA expression is associated with a hypertrophic scar-like architecture

When the presence and fibroblast expression pattern of SMA was examined in keloids, a clear dichotomy was present with 60% (n=18) exhibiting a $SMA^{negative/minority}$ pattern and 40% (n=12) diffusely expressing SMA. Taken into account the conflicting data in the literature regarding myofibroblast presence in keloids, a predictor for variations in SMA expression patterns was sought. Of the 12 keloidal scars with a $SMA^{diffuse}$ phenotype, 10 (83.33%) exhibited a hypertrophic scar-like architecture (**Figure 22**). Additionally, 90.9%

(10/11) keloids with a hypertrophic scar-like architecture exhibited diffuse SMA expression. Unsurprisingly, there was a statistically significant association between SMA expression pattern and keloidal architecture (*p<0.0001*).

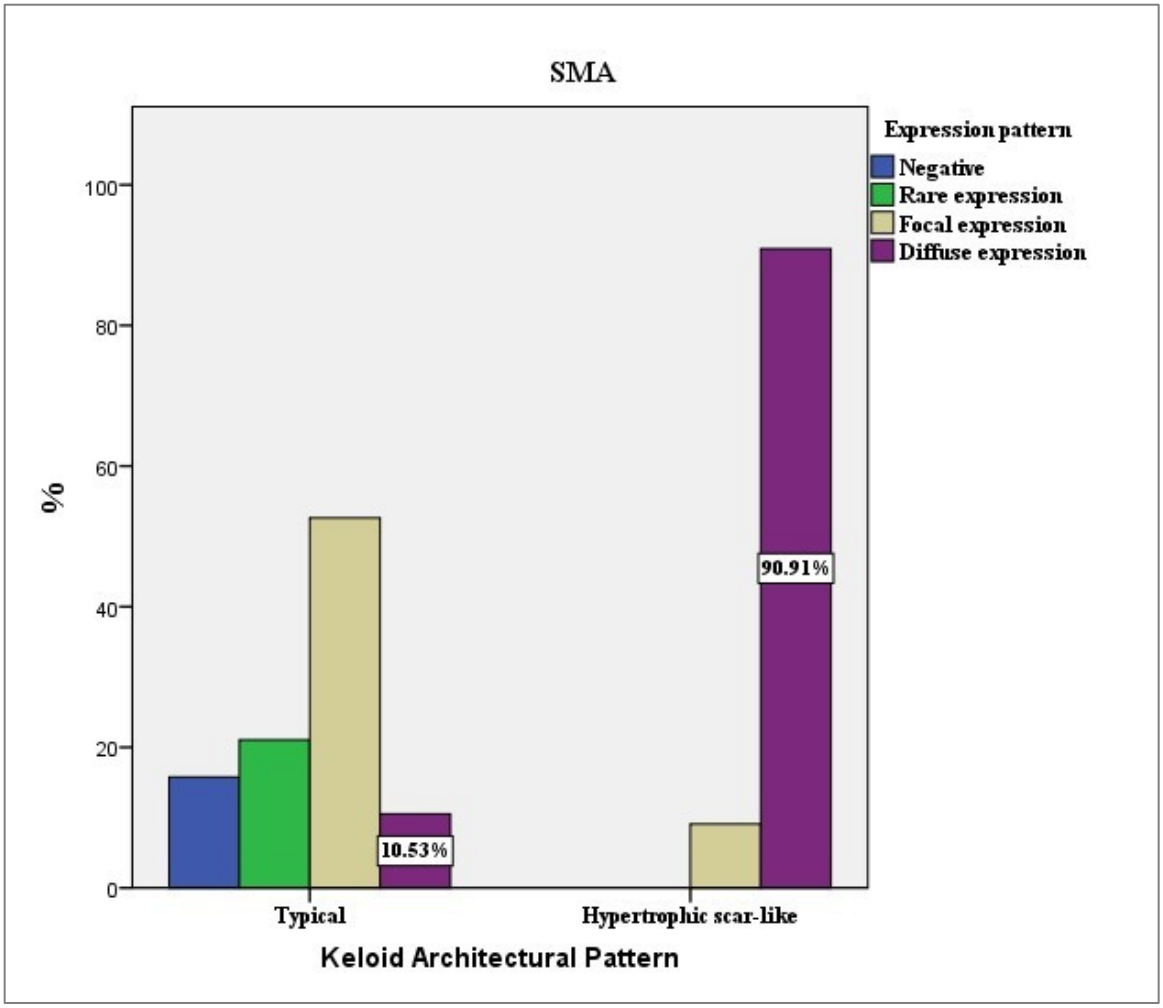


Figure 22. SMA expression pattern based on keloidal architecture
A SMA^{diffuse} expression pattern occurred much more commonly in keloids with a hypertrophic scar-like architecture (91 %, n=10), but was only rarely seen in cases with the typical expansile architecture (11%, n=2). Keloid architecture was significantly associated with SMA expression pattern (*p<0.0001)*

5.9 In reparative, hypertrophic and keloidal scars, CD90$^+$ fibroblasts contribute more cells to the main scar body compared with classical SMA$^+$ myofibroblasts

In order to assess the relative contribution to the scar of CD90+ fibroblasts and SMA$^+$ classical myofibroblasts, all scars expressing both markers were evaluated (n=88). Of these, 94.3% (n=83) demonstrated scars with CD90$^+$ fibroblasts predominating over SMA$^+$ myofibroblasts. Three cases (5.7%) demonstrated an approximately equal proportion. No scar demonstrated SMA$^+$ myofibroblast predominance. These findings suggest that in many scars, the bulk of scar fibroblasts are CD90$^+$ with a less prominent component of classical SMA$^+$ fibroblasts (**Figures 23, 24 part-1 & 25**).

5.10 SMA$^+$ myofibroblasts exclusively co-localize to CD90$^+$ fibroblast rich areas, while CD90$^+$ fibroblasts are not limited to SMA$^+$ myofibroblasts rich areas

In addition to relative contributions by area positive, the spatial relationship between fibroblasts expressing both markers was evaluated. Specifically, we wished to address whether SMA$^+$ myofibroblasts exist in a spatially independent manner within scars or alternatively are exclusively zonally co-localized with CD90 expressing fibroblast-rich areas. Eighty-seven (98.9%) of specimens containing any myofibroblasts, demonstrated classical SMA$^+$ myofibroblasts exclusively within to CD90$^+$ fibroblast-rich areas (**Figures 23 & 24 part-1)**. A single hypertrophic scar demonstrated a tiny isolated focus of myofibroblastic differentiation immediately adjacent to a CD90$^+$ fibroblast-rich area.

CD90 expression typically involved the entire scar and occurred independently of SMA expression. These findings appear to suggest that while CD90 expression occurs in the absence of SMA expression, expression of SMA may be dependent on CD90 expression.

5.11 SMA^+ myofibroblasts extensively co-express CD90

Twenty-six specimens (8 reparative, 7 hypertrophic and 11 keloidal scars) were double-stained with CD90/SMA to accurately assess for dual expressing cells. All samples exhibited extensive dual CD90/SMA positivity. Grossly, the vast majority of SMA^+ myofibroblasts co-expressed CD90 (**Figures 23-D & 24 part-1-D**). The pattern of overlap was often distinctive, with SMA exhibiting a membranous pattern, forming a rim around $CD90^+$ cells, which exhibit both a membranous and cytoplasmic pattern. Taken together with the delimited overlap of SMA rich zones to CD90 rich zones, and the overwhelmingly greater prevalence of $CD90^+$ fibroblasts in the scar, these findings are compatible with the hypothesis that CD90 expression is associated with myofibroblastic differentiation. The copious dual positivity also raises the possibility that myofibroblasts are derived from a subset population of $CD90^+$ fibroblasts and if this is so, perhaps ultimately are derived from the resident $CD34^+$ fibroblastic cell network.

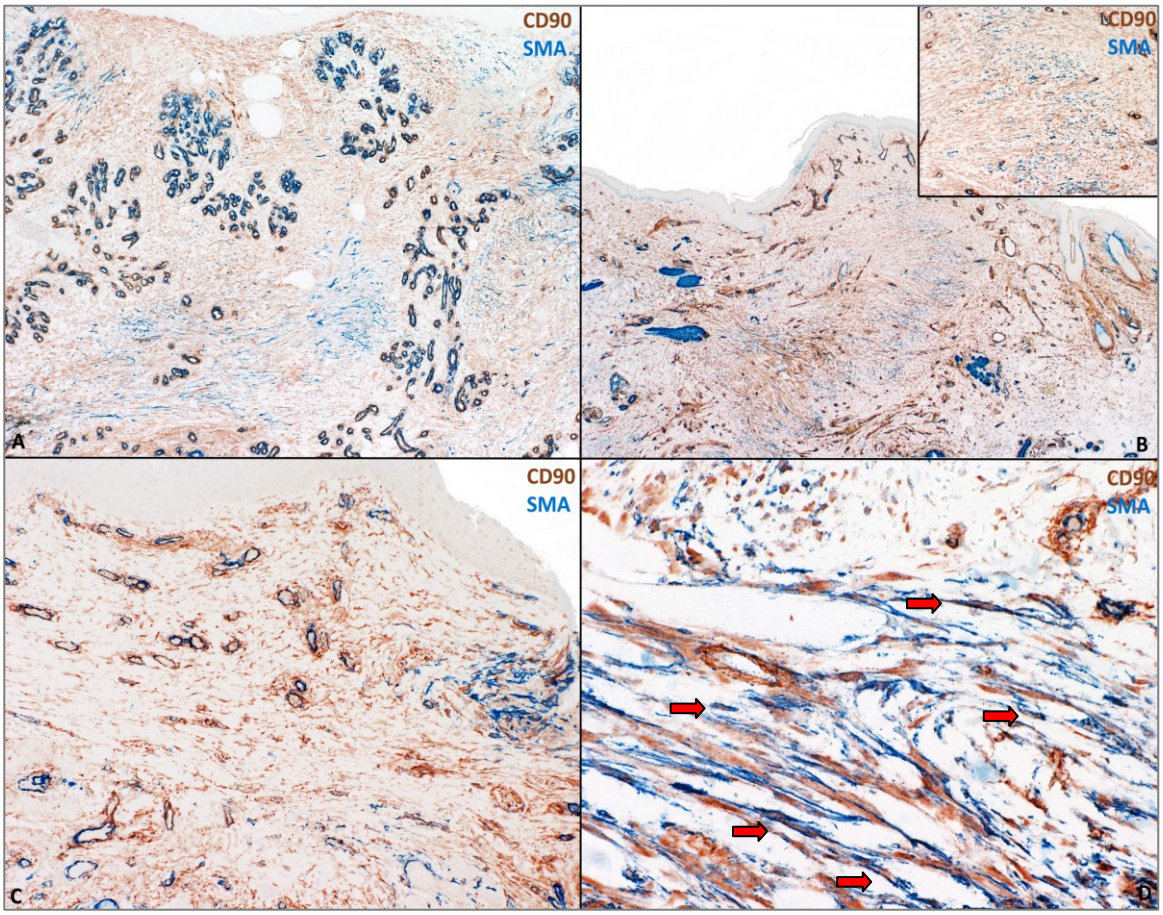


Figure 23. Relative contributions to reparative scar mass and spatial relationship of CD90 expressing fibroblasts and SMA^{+} myofibroblasts In 94.3% of all myofibroblast containing scars, CD90^{+} fibroblasts predominated over classical SMA^{+} myofibroblasts (A–C) including those with a SMA$^{\mathbf{diffuse}}$ pattern (A, B). Additionally, SMA rich areas are contained within CD90 rich areas, but CD90 expression is independent of SMA (A–C). Note extensive CD90 expression of SMA^{+} myofibroblasts, with SMA exhibiting a membranous pattern, rimming CD90^{+} cells (D, arrows). *Original magnification A, B X20; B inset, C X40; D X200*

5.12 In cutaneous scars CD90$^+$ fibroblasts contribute to scar collagenization more extensively and independently of SMA+ myofibroblasts

In order to ascertain whether CD90$^+$ fibroblasts are biologically active with regards to ECM/collagen production, 26 scars were stained for procollagen-1(PC-1) expression. Eighteen of these were dual stained with CD90 and 8 with SMA. For relative spatial comparison, PC-1/CD90 specimens were compared with their previously performed SMA stained counterpart and likewise, PC-1/SMA specimens were compared with tissue previously stained with CD90. All specimens demonstrated PC-1 expression spatially matched to CD90 fibroblast rich areas. In CD90/PC-1 double-stained tissue, extensive co-expression was visualized confirming that CD90 cells are actively involved in collagen production. While PC-1 expression was seen in SMA rich areas, it was never limited to these areas, extending beyond these zones and when compared with the corresponding CD90 stain, in all cases, PC-1 expression co-localized with CD90 rich zones (**Figures 24 part-2 & 25**). In all SMA/PC-1 double-stained tissue, dual marker expression was easily identified consistent with the previously widely accepted significant collagen synthesizing function of myofibroblasts. Nevertheless, in all samples examined, the CD90$^+$ fibroblast rather than the SMA$^+$ myofibroblast was the major contributor (by area) to scar collagenization, doing so independently of SMA expression.

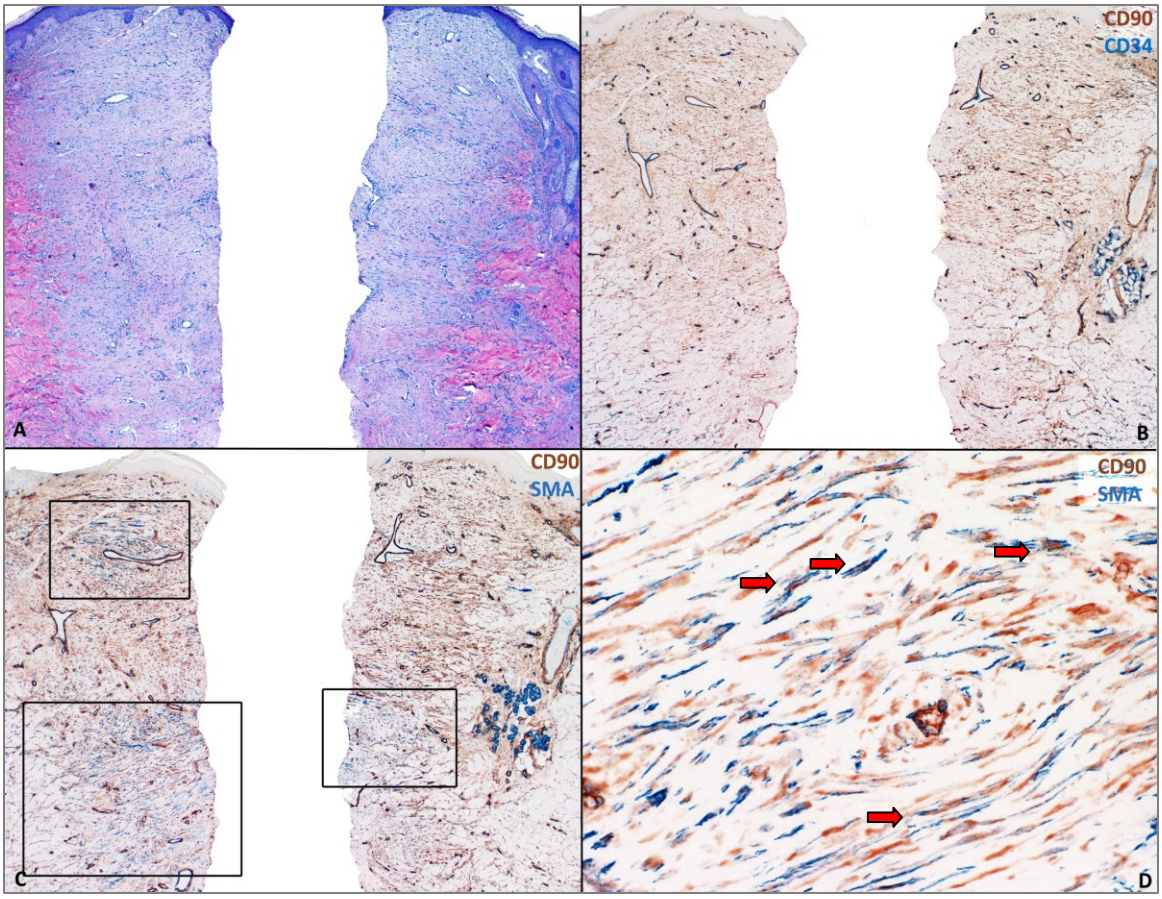


Figure 24 Part-1. Comparison of CD90 rich and SMA rich areas in a reparative scar
In this vertically oriented scar, CD90 is expressed diffusely throughout the scar (B) predominating over SMA rich areas (C, boxed). SMA^{+} areas are contained within CD90 rich zones (C, D) and typical extensive co-expression of CD90 is noted (D, arrows).
Original magnification A–C X20; D X200

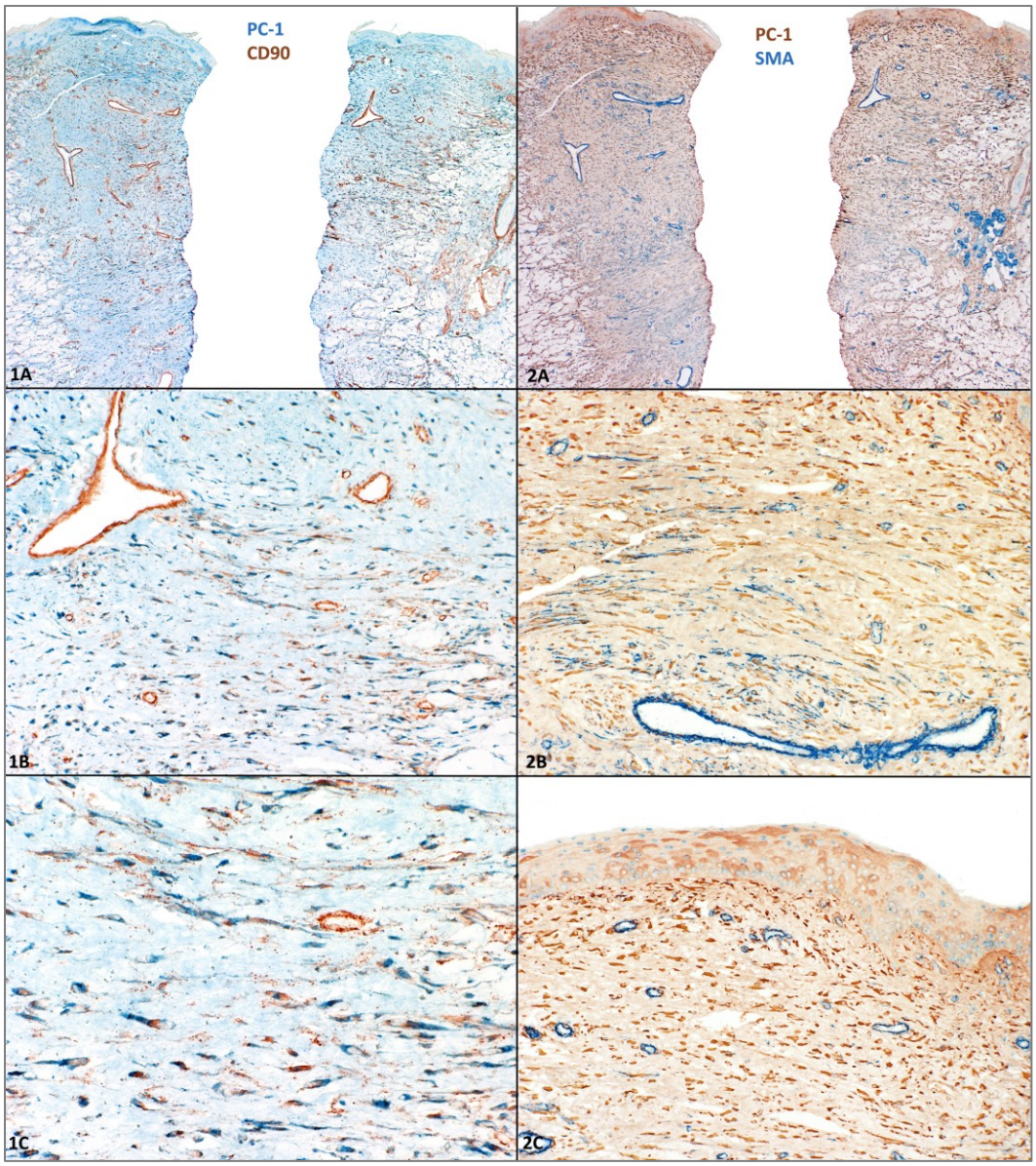


Figure 24 Part-2. Relative contribution of CD90^{+} vs. SMA^{+} fibroblasts to scar collagenization Staining with procollagen I (PC-1, **blue**) and CD90 (**brown**) (1A–C) shows diffuse PC-1 positivity in the scar (1A, B), spatially corresponding with CD90 rich zones (Figure 24 part 1, panel B) and marked dual positivity with CD90 (1C). PC-1 (**brown**) and SMA (**blue**) (2A–C) staining demonstrates collagen synthesis in SMA rich areas (2B) but also extensively in zones without identifiable myofibroblasts (2C), but which co-localize with CD90 rich areas (**Figure 24 Part-1, panel B**). *Original magnification 1A, 2A X20; 1B, 2B X100; 1C x200; 2C x 40*

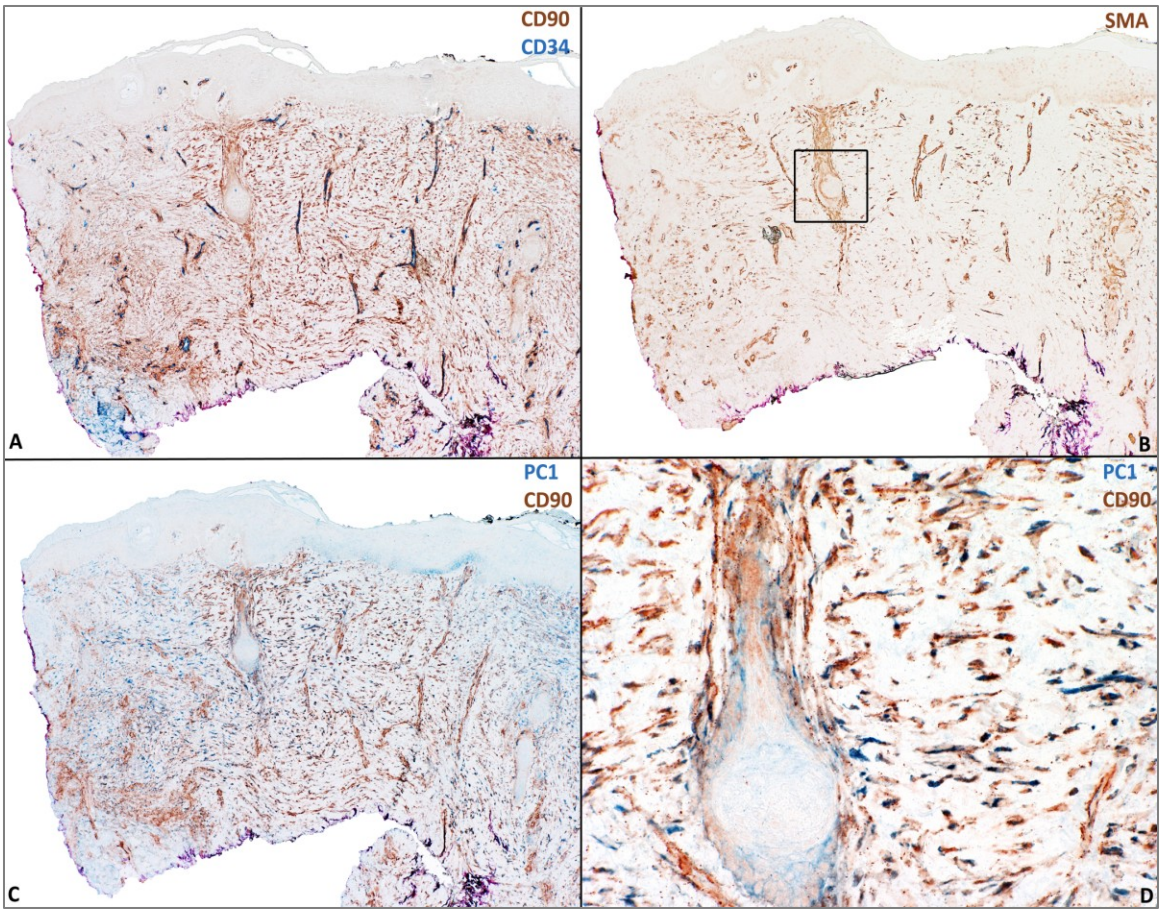


Figure 25. CD90^{+}/SMA^{-} fibroblasts contribute more to reparative scar collagenization than do classical SMA+ myofibroblasts
Note diffuse CD90 positivity (A) and only focal SMA positivity (B). There is diffuse procollagen I (PC-1) positivity corresponding to a CD90 rich distribution (C). Note CD90^{+} cells from a SMA poor zone (B, boxed) synthesizing collagen as indicated by co-expression of PC-1. *Original magnification A–C X40; D X200*

CHAPTER 6 DISCUSSION

Fibroblast biology is a rapidly expanding field in medicine. It is estimated that 45% of deaths in the United States are associated with fibrotic disorders (Wynn, 2004). Furthermore, no consistently effective anti-fibrotic therapy currently exists and therefore further understanding of the mechanisms of fibrosis is of tremendous importance to improving survival and quality of human life. The skin provides an easily accessible tissue for evaluating fibrosis with well-defined fibrotic states and although findings in one organ system are not necessarily generalizable to other systems, any information expanding our current grasp of these complex states increases the chance of developing effective therapeutic and reversal modalities. This research aimed to help define scar fibroblast phenotypes and protein expression patterns in reparative, hypertrophic and keloidal scars.

Based on existing literature demonstrating a profibrotic role for $CD90^+$ fibroblasts in different tissues and evidence suggesting they may originate from the background $CD34^+$ fibroblastic stromal cell network, a hypothetical model for cutaneous scarring was developed. This model proposes that after wounding, resident fibroblastic stromal cells lose their CD34 positivity and begin to express CD90, transitioning from quiescence to active wound healing fibroblasts. Some of these fibroblasts go on to influence the development of classical SMA^+ myofibroblasts or themselves undergo myofibroblastic transition. As wounds heal and ECM homeostasis ensues, active fibroblasts re-transition to dormancy, losing their CD90 positivity and reverting to CD34 positive state. A model for

pathologic scarring suggests that for various reasons, scar fibroblast remain in an active state and are unable to regain or have delayed reversion to their pre-wound CD34+ state. The findings of this study support the plausibility of these models.

As shown in the results, active scars are characterized by a $CD90^{\textbf{diffuse}}$/ $CD34^{\textbf{negative/minority}}$ phenotype with variable SMA positivity. This was observed in reparative scars as well as in both categories of pathologic scars. In conjunction with their role as cellular components of the scar mass, $CD90^{+}$ fibroblasts are also integrally involved in scar collagenization across scar categories, as evidenced by extensive co-expression of PC-1 (procollagen-I). The utilized anti-PC-1 antibody recognizes the *N*-terminal of procollagen (type I collagen precursor peptide) which is present in cells actively producing collagen but is cleaved prior to assembly of mature cross-linked collagen (Bateman, Boot-Handford, & Lamandé, 2009; Krustrup, Rossen, & Thomsen, 2006). In the skin positivity is generally limited to fibroblasts and is therefore used as supplemental marker of fibroblastic lineage (Krustrup et al., 2006).

The repeatedly observed $CD90^{\textbf{diffuse}}$/$CD34^{\textbf{negative/minority}}$ phenotype underscores the apparent expression reciprocity between CD90 and CD34, with polar differentiated fibroblasts within scars expressing one in exchange for the other. Dual expression is however observed primarily at the active scar interface with surrounding uninvolved dermis or in old scars reverting to their pre-injury $CD34^{\textbf{diffuse}}$/$CD90^{\textbf{negative}}$ phenotype. These double-positive cells are interpreted as being in a transitional state of differentiation

between latency and activation and were observed in the vast majority of reparative and pathologic scars. The idea of scar fibroblasts originating from a local mesenchymal progenitor pool has already been discussed in the literature review, and these findings further support the existence of such a process in skin biology/pathology. Given the existence of these transitional cells, while other sources of fibroblasts including circulating fibrocytes and fibroblastic transformation of either epithelial or endothelial cells likely play some role in wound healing, we suggest that the ubiquitous dermal fibroblastic $CD34^+$ stromal cell network containing a large supply of potential wound-healing fibroblasts is, by local proximity and quantity alone, a more likely candidate for the primary source of scar fibroblasts. Moreover, the predominantly scar-periphery location of these co-expressing cells favors a local transition theory, as no easily explainable reason exists why fibrocytes (also exhibiting CD34) arriving from the circulation would almost exclusively congregate at the scar-normal interface. An additional observation lending support to a local transition theory, is the speed of phenotype transition with extensive pan-dermal and subcutaneous replacement of the $CD34^+$ network by CD90 expression as early as 48 hours. Such rapid repopulation in the distribution of the preexisting resident stromal network by either circulating fibroblasts of endo/epithelial trans-differentiation seems unlikely. Importantly, with the possible exception of newly wounded skin, the presence of dual expressing cells can provide evidence supporting the existence of transition, but cannot determine the direction of the transition. Therefore, scars expressing dual positivity at their margin may be evolving towards either pole, one conversion extending fibrosis and the other reverting. Taken together, this study advances the view

that local transition from the background $CD34^{+}$ fibroblastic stromal cell network (possibly with contributions from other precursors) represents a likely source of active scar fibroblasts.

As predicted in our hypothetical model of physiologic/reparative scarring, the $CD90^{\mathbf{diffuse}}/CD34^{\mathbf{negative/minority}}$ phenotype is time-limited, and complete scar maturation culminates with return of the $CD34^{\mathbf{diffuse}}/CD90^{\mathbf{negative}}$ network, a process we term a "CD34-revertant" or simply "revertant" phenotype. In this study, scars were classified as (successfully) revertant only when they expressed CD34 in >50%. It is important to note that many scars including keloids demonstrated rare or focal CD34 positivity within the scar suggesting possible attempted, but unsuccessful (at the time of evaluation) reversion. While reversion was seen as early as 98 days, in general, it was observed in all scars older than 160 days. Fascinatingly, scars may exhibit two revertant phases. As noted in the results, scars between 2 days and as old as 28 days exhibited wide-spread loss of CD34 and diffuse neo-expression of CD90 in an indiscriminate distribution, involving the deep dermis, subcutaneous tissue and even adjacent uninjured skin. This pattern contrasts sharply with the well-outlined $CD90^{+}$ fibrosis characterizing older scars. Potentially, the excessive cytokine/growth factor storm associated with acute injury induces this unselective transition but as scars enter the 1 month period with tighter regulation of cytokine release, the redundant CD90 expressing cells which will not contribute to the defined scar re-transition to their pre-injury $CD34^{+}$ state. This "early" CD34 reversion is followed by scar proliferation, maturation and eventually, late/complete reversion between

160–180 days. This data provides convincing evidence of the time-limited and reversible nature of the CD90$^{\textbf{diffuse}}$/CD34$^{\textbf{negative/minority}}$ phenotype.

In pathologic scars, we found that a subset of hypertrophic scars greater than 180 days in age (predominantly older than one year), still lacked a revertant phenotype and are therefore associated with a prolongation of a CD90$^{\textbf{diffuse}}$CD34$^{\textbf{negative/minority}}$ phenotype. In fact, based on the aging methodology (see **Section 4.1**) where scars with non-explicit clinical data were assigned their minimum age, underestimation of pathologic scar-age has likely occurred, such that the prolongation of diffuse CD90 expression may be even more dramatic than is currently apparent. Reversion can however occur in hypertrophic scars, and such scars were identified in thus study, accounting for nearly one-third of the samples evaluated. While the day age was not known for most of the revertant scars, the youngest revertant scar was age 280 days. Interestingly many of these CD34-revertant scars continued to express CD90, unlike their reparative scar counterparts, and could additionally, retain their collagen synthesizing function (**Figure 18, inset**). This disconnect in simultaneous and bilateral CD90/CD34 reversion, highlights the aberrant prolongation of CD90 expression in this scar type. The observation of four CD90$^{\textbf{negative/minority}}$ revertant hypertrophic scars does however suggest that bilateral reversion can occur, though the timeline is currently undetermined. It is unclear what factors account for this reversion heterogeneity in hypertrophic scars, allowing for some scars to re-enter a physiologic track and eventually regress while others display a non-revertant, active phenotype extremely late into their lifespan (observed up until 1098 days

in this series). Eleven of 12 keloids for which an age was calculable were 300 days or older and all exhibited a non-revertant CD90$^{\textbf{diffuse}}$CD34$^{\textbf{negative/minority}}$ phenotype. Keloids appear to be characterized by a lack of tendency for reversion and prolongation of an active phenotype. The exact mechanism by which a prolongation of a CD90$^{\textbf{diffuse}}$CD34$^{\textbf{negative/minority}}$ contributes to the clincopathologic features of hypertrophic scars and keloids is unknown. Possibly the observed prolongation of CD90 expression represents a failure of its physiologic functioning as it relates to self-regulation. As discussed before, CD90 signaling in dermal fibroblasts regulates fibroblast growth and increases fibroblast apoptosis preventing excessive fibroblast proliferation.(Schmidt et al., 2015) It is plausible that failure in this self-regulating apoptotic pathway leads to unregulated fibroblast activity characteristic of pathologic scarring. Compatible with this theory is the various mechanisms of resistance to apoptosis described in both hypertrophic scars and keloids (Linge et al., 2005; Lu et al., 2007; Saed et al., 1998). As detailed previously, CD90 expression is also associated with increased active TGF-β levels and collagen production (Koumas et al., 2003; Schmidt et al., 2015). Failure therefore of CD90-related apoptosis in the presence of an intact TGF-β release pathway, could result in its protracted and excessive release, accounting for the TGF-β hyper-elevation reported in keloids and hypertrophic scars (Bettinger et al., 1996; Wang et al., 2000.). Furthermore, as demonstrated, these fibroblasts are actively involved in collagen synthesis so that the existence of unremitting CD90 expression would likely over-collagenize scars. It is thus possible, that the exuberant collagen production seen in pathologic scarring may be an indirect consequence of failed apoptosis and aberrant cell-longevity rather than a primary

intrinsic propensity of pathologic scar fibroblasts to over-produce the ECM. Conceivably, via an unknown mechanism, hypertrophic scars are able to overcome this hypothetical failure and regain their physiologic trajectory albeit in a delayed fashion. Conversely, failure to re-enter a physiologic track may lead to an ancient but active hypertrophic scar or an infiltrative keloid. An alternative hypothesis is that aberrations in one or multiple profibrotic cellular/molecular pathways may exist and extended CD90 positivity is protective in an effort to induce apoptosis and fibroblast differentiation, with similar resultant hyper-collagenization as a side-effect of persistent expression. As proof these concepts is beyond the scope of this work, additional studies are needed to test these hypotheses.

The final goal of this work was to examine the relationship between classical SMA^+ myofibroblasts and $CD90^+$ fibroblasts. As detailed previously, the presence of myofibroblasts in fibrosing conditions is well accepted and these cells have been purported as the main effector cell in fibrosis. While somewhat iconoclastic, this study finds that though myofibroblasts are significant producers of collagen and are present to varying degrees in all scar types, they were never more plentiful than $CD90^+$ fibroblasts, contributing less cellular mass and new collagen to the scar than their $CD90^+$ counterparts. Indeed, collagen production, as implied by PC-1 positivity was never limited to SMA+ areas but always extended to co-localize with CD90 rich areas. Additionally, SMA positivity was, except in one case, limited to areas of CD90 positivity and the majority of these myofibroblasts also dually expressed CD90. Again, these findings are consistent

with an upstream role of CD90 in the myofibroblastic differentiation pathway. The exuberant CD90/SMA positivity further raises the possibility that myofibroblasts are directly derived from a subset of $CD90^+$ fibroblasts. Ultimately, if SMA expressing fibroblasts are derived from the $CD90^+$ fibroblasts, then myofibroblasts are also potentially derived from the background $CD34^+$ stromal cell network. Whether or not the observed $CD90^+$ fibroblasts represent what have been previously described as protomyfibroblasts, remains to be seen, but if in fact they are these cells, myofibroblast differentiation (as suggested by their name) seems not to be their exclusive or predominant role as the majority do not progress to a fully evolved phenotype (Falke, Gholizadeh, Goldschmeding, Kok, & Nguyen, 2015; McAnulty, 2007; Otranto et al., 2012; Tomasek et al., 2002). While SMA^+ myofibroblasts likely play a significant role in profibrotic disease, this role may be shared with other fibroblasts and may in actuality, be dependent on them.

One area however in which classical myofibroblasts are unparalleled in their contribution is scar contractility. In the current study, though myofibroblasts were present in all scar types, diffuse SMA expression was clustered with well contracted scar types i.e. reparative scars, hypertrophic scars (with their well-defined hyper-contracted nodules and fascicles) and in keloids with a hypertrophic scar-like (non-infiltrative) architecture. Perhaps those cases classified as keloid based on the extensive keloidal collagen component but with a relatively well defined nodular architecture (suggestive of functional myofibroblastic contractile ability) may exhibit clinical features more similar to hypertrophic scars (with regards to their tendency to continuously expand far beyond the index scar-line), though

studies with stronger clincopathologic correlation are needed to confirm this suggestion. The converse seems to be histologically accurate however, with the vast majority of keloids displaying a $SMA^{negative/minority}$ phenotype exhibiting large sizes and infiltrative borders presumably related to a lack of myofibroblastic contractile ability. As a whole, myofibroblasts along with CD90 fibroblasts are significant contributors to scar physiology and pathology.

Finally, the evolving evidence for the role of CD90 in fibroblast proliferation, control of apoptosis, TGF-β regulation, myofibroblast differentiation and scar collagenization, suggests this molecule as a potential therapeutic target for the management of cutaneous fibrosis. Therapies decreasing its expression or inducing its reversal to a quiescent state may provide an efficacious weapon against these potentially devastating and commonly treatment-resistant diseases.

CHAPTER 7 STUDY LIMITATIONS

7.1 Study limitations

Retrospective immunohistochemistry based experiments are always limited by the inability to see a single specimen as it progresses through its physiology/pathology, with a positive/negative finding representing only a snapshot in time of one particular scar, giving little indication of what the result might have been a short time before or after tissue harvesting. As such, definitive proof that a specific scar type always or never exhibits a particular phenotype cannot be achieved via this method. Nevertheless, the findings presented provide valuable evidence on the way to definitive proof.

While overall, the number of specimens evaluated was adequate, it was difficult to collect large numbers of scars at a specific age interval to strengthen the generalizability of the timelines for reversion. Additionally, information regarding the exact age of many pathologic scars was unavailable, preventing further analysis of this data.

CHAPTER 8 CONCLUSION

In summary, this study has examined fibroblast phenotypes with regards to the relationship between expression of CD90, SMA and CD34 in simple reparative scars, hypertrophic scars and keloids. The results define a common $CD90^{\textbf{diffuse}}/CD34^{\textbf{negative/minority}}$ phenotype as the most common in active scars of all types. The findings also show that this expression pattern is transient in physiologic scars and that pathologic scars are defined to various degrees by its persistence. This study also provides additional evidence to build on the pre-exiting theory that CD90 expressing scar fibroblasts are derived from the resident $CD34^{+}$ fibroblastic stromal cell network. It shows that $CD90^{+}$ fibroblasts are active participants in ECM production as it relates to laying down of collagen. Additionally investigated was the relationship and relative contributions of $CD90^{+}$ fibroblasts and myofibroblasts with the results suggesting that $CD90^{+}$ fibroblasts may contribute more to collagen production and scar mass than do classical myofibroblasts. Myofibroblasts however appear to retain their preeminent role in scar contracture.

Overall, the findings presented in this study help to define a fibroblast phenotype in cutaneous scarring and adds to the existing scientific data regarding local fibroblast transitions and the relative roles of various fibroblasts in the genesis of a physiologic or pathologic scar.

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