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Distinct fibroblasts in scars and regeneration

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Author Comments:	

Distinct fibroblasts in scars and regeneration

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Abstract

The skin is home to a collection of fibroblastic cell types from varying embryonic origins. These varying fibroblastic lineages display unique genetic programs and *in vivo* functions. Studying the diversity of fibroblastic cells is emerging as an important area for cutaneous biology, wound repair and regenerative medicine. In this mini-review we discuss the distinct embryonic origins, microenvironments, and transcriptomic profiles of fibroblastic lineages, and how these varying lineages shape the skin's wound response across injury depths, anatomic locations, and developmental time to promote either scarring or regeneration. We outline how the development of single cell sequencing has led to our improved understanding of fibroblastic lineages at the molecular level and discuss existing challenges and future outlook on developing regenerative therapies that are based on this emerging field of eclectic fibroblasts.

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Introduction

Lower taxonomic groups regenerate a perfect tissue replica after injury. Mammals, and humans, on the other hand, have evolved additional repair mechanisms replacing regeneration with tissue scarring. Scars form a quick seal that closes open wounds, reducing the risk of infection and increasing chances of survival. However, this comes at a cost of replacement with dense plugs of matrix that severely restrict tissue biomechanics, function and physiological performance.

The pathways underlying the tissue response to regenerate or patching with scars, hold tremendous potential for novel, targeted therapeutic interventions. However, the pivotal signals for choosing either path remain a biological puzzle. Several recent papers have explored the scar-regeneration paradigm of skin, indicating that differing communities of fibroblasts underlie the skin's diverse repair responses. This mini-review discusses the current state of knowledge on heterogeneous fibroblastic cells, viewed primarily through skin, and how studying the diversity of fibroblast communities can advance the development of novel treatment options to promote regenerative repair and reduce scar formation.

Distinct microenvironments for scarring and regenerative fibroblasts

The skin of mammals and humans is composed of an outer epidermal layer that encloses a multi-layered connective tissue structure. Dermal connective tissue is sub-divided into upper (papillary) dermal connective tissue, lower (reticular) dermal connective tissue, and a deeper hypodermal connective tissue rich in adipocytes, blood vessels and peripheral nerves. In mice, this deep hypodermal connective tissue is further separated by a muscle layer termed panniculus carnosus (PC), which further separates hypodermal connective tissue from the underlying subcutaneous fascia. In humans where PC muscle is minimally present and is not a ubiquitous feature of the skin, the fascia connective tissue layers are continuous with the upper hypodermal layers. Murine and human skin are structurally different, but both maintain distinct dermal connective tissue compartments across the dorsal-ventral axis.

The presence of distinct fibroblast communities across the skin's different connective tissue layers has been known for several decades [1]. However, the ability to isolate, image and study diverse behaviors and functions of fibroblast subsets in their native skin microenvironments has evolved with more modern techniques that shed light on these functionally diverse populations. Driskell et al., published one of the first papers demonstrating the existence of distinct embryonic sources and cellular lineages for skin fibroblasts that are spatially and functionally unique [2]. Through embryonic genetic lineage tracing, Driskell and colleagues determined three different embryonic lineages of fibroblasts and demonstrated that each lineage has a distinct spatial location and gene marker expression; namely dipeptidyl peptidase 4 (*DPP4*, also termed CD26; a cell surface receptor that marks fibroblasts of the papillary dermis), delta-like non-canonical notch ligand 1 (*Dlk1*; characterizing fibroblasts within the reticular layers), and stem cell antigen 1 (*Sca1*; a cell surface receptor marking fibroblasts within the hypodermis) [2].

A similar spatial restriction of distinct fibroblast subsets has been documented in human skin. Korosec and colleagues reported that fibroblast activation protein (FAP) and Thy-1 cell surface antigen (CD90) double positivity ($FAP^{+}CD90^{+}$) defines human upper papillary fibroblasts, whereas a $FAP^{-}CD90^{+}$ phenotype defines the lower reticular and hypodermal-restricted populations of fibroblasts [3]. A separate study by Philippeos et al. looks into

human breast skin suggested that CD39 defines papillary dermal fibroblasts, while CD36 defines a reticular and hypodermal fibroblast population. The authors identified two major populations of human dermal fibroblasts: The first, a population defined by $CD90^{+}CD39^{+}CD26^{-}$ cell surface expression that enriches for upper dermal connective tissue compartments. This fibroblastic population expresses high levels of type VI collagen alpha 5 chain (*COL6A5*), and functions in supporting epithelial differentiation and rete ridge formation. A separate $CD90^{+}CD36^{+}$ fibroblast population was found to be enriched in lower dermal connective tissue, and apears to play a more active role in scar formation [4].

Our research group has more recently identified an additional fibroblast population that resides within the lowest connective tissue layers, termed as superficial fascia. Fascia fibroblasts were highlighted as the major wound fibroblast subset that contribute to scar formation, mobilizing the connective tissue of the fascia into open skin wounds. Genetic ablation of fascia fibroblasts or placement of an impermeable film to create a physical barrier preventing their migration into wound sites, led to chronic open wounds devoid of scars [5]. Further studies on human skin have demonstrated that fibroblasts from the human skin dermo-hypodermal junction, where the fascia connective tissue resides, are distinct from dermal papillary and reticular fibroblasts, and exhibit a specific molecular profile related to extracellular matrix (ECM) organization and remodeling, and closely related to the cellular profile associated with skin fibrosis [6].

Whereas recent studies show that the physical numbers of fibroblasts remain stable under homeostatic conditions and during aging [7], wounding elicits mass movement of fascia fibroblasts, which in turn changes wound fibroblast composition. Perhaps indicating that physical numbers remain stable but the phenotypic profile or relative ratio of certain fibroblast populations changes over time. Such changes in the composition of fibroblast communities with age, could have its relevance to frailty, and the propensity to chronic wound responses seen under disease and with increasing age.

Developmental origins of scar and regenerative fibroblasts

Fibroblast subtypes can be classified based on their unique embryonic origins, through genetic lineage tracing approaches. For example, the connective tissue covering the head and neck regions develop from fibroblasts that originate from the embryonic neural crest, whereas skin connective tissue covering our torso develops from fibroblasts originating from embryonic paraxial mesoderm (also known as dermomyotome). Moreover, connective tissues of internal organs are derived, to a large degree, from fibroblasts originating from lateral plate mesoderm. Indeed, we and others have shown that fibroblasts coming from different embryonic origins display different cellular and physiological properties during homeostatic conditions and during wound repair [8].

For example, lineage tracing techniques have demonstrated that scar formation in skin wounds can be attributed to a single fibroblastic cell lineage, a phenotype not modulated by its microenvironment, producing scars even upon transplantation into a remote location. In the backskin of mice, genetic lineage tracing of somatic progenitors that express Engrailed-1 (*En1*) transcription factor give rise to a fibroblastic cell lineage (termed EPFs). EPFs have been found to provide the bulk scarring outcomes after skin injury in both splinted wound models and in the stroma that develops in a melanoma tumor model in the back-skin [9]. Similarly, in the ventral skin, paired related homeobox 1 (*Prrx1*) defines progenitors that give rise to a separate lineage of fibroblasts responsible for the fibrotic

outcomes in chest and belly skin [10,11]. Intriguingly, both fibroblast lineages appear to be located within the fascia connective tissue compartment [5,11], potentially linking functional contributions of fibroblast lineages with distinct anatomic locations in skin.

Two additional embryonic populations of fibroblasts have been discovered. The first is a population defined by the simple absence of En1 expression in their progenitors (termed ENFs) and that also reside in the backskin, as intermingled cells with EPFs. Interestingly, the mammalian backskin undergoes a phenotypic shift in its response to injury, from scarless regeneration at fetal stages, to scarring at peri- and postnatal stages of development, or between second to third trimester in humans. During this period of skin development, there is a significant compositional change in fibroblastic populations. The two functionally distinct fibroblast lineages of EPFs and ENFs undergo a lineage succession during development from an ENF-predominant dermis during embryonic and fetal development, to an EPF-predominant dermis at peri- and postnatal stages. This compositional change underlies the phenotypic shift in the skin's response to injury, from regeneration-to-scarring [12]. Whereas EPFs contribute to scar formation in adult wounds, ENFs are responsible for developing the healthy connective tissue organization of the dermis, and are involved in the scarless wound healing seen in wounds that are inflicted at fetal stages of development.

Scarless wound healing within adult mammals is rare, but is exemplified within the buccal mucosa of the oral cavity. Healing within this soft tissue is characterized by rapid epithelization, and a reduced period of inflammation after the initial injury. The differences in healing between dermal and oral tissue has been demonstrated to be a complex interplay resulting from the environmental milieu created within the oral cavity by the presence of saliva [13], a muted angiogenic response upon wounding [14] and a distinct fibroblast molecular profile and phenotype, including the presence of long telomeres and a resistance to fibrotic triggers such as transforming growth factor beta 1 [15,16]. Here a defined lineage of fibroblasts is based on Wnt1 expression in its progenitors (termed as WPFs for Wnt1-lineage positive fibroblasts). WPFs are central to the regeneration of a healthy, porous connective tissue organization [9].

Scarring and regenerative fibroblasts are transcriptionally distinct

The development of molecular profiling approaches such as single cell RNA-sequencing (scRNA-seq) techniques and the ability to computationally analyze thousands of cells has led to a significant advance in understanding of fibroblast heterogeneity [17]. ScRNA-seq has provided an unprecedented insight into the molecular characteristics of each fibroblast population, distinguishing molecular pathways that are represented within regenerating and scarring type fibroblasts. Computational tools that are based on single cell analysis, such as pseudotime and RNA velocity analyses assisted researchers in uncovering divergent fibroblast development trajectories. This is accomplished computationally by either placing cells along a continuous gene expression path representative of the evolution of transcriptional change [18], or by describing the rate of gene expression change based on the ratio between unspliced pre-mRNAs and spliced mature mRNAs [19,20], respectively.

Tabib and colleagues performed the first comprehensive scRNA-seq study to investigate fibroblast heterogeneity in healthy human skin from forearm [21]. The authors determined two major subsets of human skin fibroblasts. The most abundant subset is characterized by co-expression of secreted frizzled-related protein 2 (SFRP2) and CD26 a cell surface

receptor that serves as a co-stimulatory molecule for T cells with dipeptidyl peptidase activity in its extracellular domain. SFRP2^{+}CD26^{+} fibroblasts are elongated bipolar cells that can be found distributed between collagen bundles, and express higher levels of common matrix genes, including type I collagen, fibrillin, and fibronectin. The other subset expresses flavin containing mono-oxygenase 1 (FMO1) and lymphocyte-specific protein 1 (LSP1). FMO1^{+} fibroblasts have a larger cell volume compared to SFRP2^{+}CD26^{+} cells, enlarged nuclei, express lower levels of *COL1A1* and *COL1A2*, and show negative regulation of cell migration and motility by gene ontology analysis [21]. The authors also identified five minor fibroblast subsets, each uniquely expressing different genetic signatures, for example *CRABP1*, *COL11A1*, *FMO2*, *PRG4*, or *C2ORF40*. One important distinction that stems from this single cell transcriptomic data is that there is no recognizable stratification between reticular, papillary, hypodermal, or fascia fibroblasts. All of the above, mentioned subsets are found throughout the dermis and do not appear to be regionally restricted [21]. This concept is supported by a recent study on human skin fibroblasts using scRNA-seq, which reported that CD26^{+} transcripts are in fact present in fibroblasts across all dermal layers and are not regionally restricted [22].

ScRNA-seq analysis was recently conducted on mouse large excisional wounds. Large wounds to the backskin induce hair follicle regeneration as opposed to scarring, and serves an additional model to study the scar-regeneration paradigm of skin. By performing scRNA-seq of large wounds Guerrero-Juarez and colleagues identified a fibroblast population in large wounds that is enriched for retinoic acid binding protein *CRABP1* [23], a marker of upper wound cells [24]. Intriguingly, *CRABP1*$^{+}$ wound fibroblasts share gene expression signatures with papillary fibroblasts from unwounded mouse skin, indicating that large wounds regenerate, rather than scar, by recruiting *CRABP1*$^{+}$ papillary fibroblasts. Furthermore, by immunofluorescence staining, CRABP1^{+}/PDGFRa^{+} cells were enriched beneath the epidermis, while CRABP1^{-}/PDGFRa^{+} cells primarily localized to the lower dermis [23]. This study indicates that CRABP1^{+} and CRABP1^{-} defines two subsets of wound fibroblasts that directly map to the papillary and lower reticular dermal compartments, respectively.

One additional intriguing finding that is emerging from scRNA-seq data is that clear genetic distinctions evident between skin fibroblast communities appears to undergo progressive loss of distinction with skin aging. scRNA-seq in combined with long-term lineage tracing studies have shown that the markers in neonatal mouse skin that allow identification of different clusters, *CD26* for papillary fibroblasts, *Dlk1* for reticular fibroblasts, and *Sca1* for hypodermal fibroblasts, are lost with age. Also, the distinguishing transcriptome profiles of the signaling pathways of these populations appear to be lost [25]. Similar conclusions were drawn in human dermal fibroblasts, indicating the partial loss of cellular identity may occur during the aging process [26]. Still, molecular profiling without protein validation should be cautiously interpreted. Does loss of distinctive genetic signature with ageing correlate with loss of phenotypic clarity at the protein and functional level? It would be interesting to know the translational relevance of changes at the mRNA level to fibroblast functions.

Molecular profiling has also been used to directly link transcriptomic features of disease-associated fibroblasts to their pathological phenotype and function, and how this differs from “normal” fibroblasts. For example, Dupuytren's contractures are characterized by fibrosis of the fascia connective tissue underlying the skin of the palm and fingers [27]. The etiology of Dupuytren’s contractures has been unclear until a recent scRNA-seq study

uncovered a unique Intercellular Adhesion Molecule 1 (*ICAM1*$^{+}$) fibroblast subset isolated from Dupuytren's nodules, as a key driver of inflammation whose feedback sustains fibroblastic activation and fibrosis. The *ICAM1*$^{+}$ fibroblasts secrete high levels of interleukin (IL) 6 and IL8 and exhibit a direct chemotactic activity to promote immune cell recruitment. This population is expanded in developing and immune-cell rich stages of fibrosis in Dupuytren's nodules [28]. Another example where molecular profiling of has been used to understand disease pathophysiology, is in the context of atopic dermatitis, a commonly recognized inflammatory disease. Recently, He and colleagues proposed that a fibroblast subpopulation characterized by *COL6A5*, *COL18A1*, chemokine (C-C motif) ligand 2 (*CCL2*) and *CCL19* expression is specific to these skin lesions, and that this fibroblast subpopulation is critical in the development of atopic dermatitis [29].

The above data indicates that 26 distinct fibroblastic cell types (summarized in Table 1) inhabit diverse skin locations. These different populations of fibroblasts emerge from various embryonic sources, express unique genetic markers, inhabit distinct microenvironments and most important, exhibit distinct functions *in vivo* (Table 1). The heterogeneity of fibroblastic cell types underlies the skin's diverse scar and regeneration responses seen across developmental time, between different anatomic skin locations and across injury depth.

Table 1. Unique features of heterogeneous skin fibroblasts

Fibroblast subsets	Species	Developmental stage	Anatomic location	Niche	Surface marker	Lineage	Transcriptomic feature	Phenotype	Reference
PDGFRa+CD26+	mouse	neonatal		papillary dermis	CD26			regenerative	[2]
PDGFRa+Dlk1+	mouse	neonatal		reticular dermis	Dlk1			scarring	[2]
PDGFRa+Sca1+	mouse	neonatal		hypodermis/fascia	Sca1			scarring	[2]
FAP+CD90-	human	adult	abdominal & breast	papillary dermis	FAP+CD90-		PDPN, NTN1	regenerative	[3]
FAP-CD90+	human	adult	abdominal & breast	reticular dermis	FAP-CD90+		ACTA2, MGP, PPARg, CD36	scarring	[3]
CD90+CD39+CD26-	human	adult		papillary dermis	CD39+CD26-		COL6A5	regenerative	[4]
CD90+CD36+	human	adult		reticular dermis	CD36+			scarring	[4]
fascia EPFs	mouse	embryonic to adult	dorsal	fascia		En1+		scarring	[5]
fascia fibroblasts	human	adult	abdominal & breast	fascia				scarring	[6]
adventitia Sca1+	mouse	adult	blood vessel	adventitia	Sca1			scarring	[30]
Sca1+CD34+CD29+	mouse	adult	dorsal	wound	Sca1+CD34+CD29+	En1+		scarring	[31]
ADAM12+PDGFRa+	mouse	adult	ear			ADAM12+		scarring	[32]
EPFs	mouse	embryonic to adult	dorsal			En1+		scarring	[9,12]
ENFs	mouse	embryonic to adult	dorsal			En1-		regenerative	[9,12]
WPFs	mouse	embryonic to adult	oral			Wnt1+		regenerative	[9]
PPFs	mouse	embryonic to adult	ventral			Prrx1+		scarring	[10,11]
PNFs	mouse	embryonic to adult	ventral			Prrx1-		regenerative	[10,11]
SFRP2+CD26+	human	adult	forearm				SFRP2, CD26	scarring	[21]
FMO1+LSP1+	human	adult	forearm				FMO1, LSP1	regenerative	[21]
CRABP1-PDGFRa+	mouse	adult	dorsal	wound			CRABP1+	scarring	[23]
CRABP1+PDGFRa+	mouse	adult	dorsal	wound			CRABP1-	regenerative	[23]
Wnt5	mouse	adult	dorsal	wound			Wnt5	wound repair	[33]
Churc1	mouse	adult	ventral	wound			Churc1	wound repair	[33]
Msx1	mouse	adult	facial	wound			Msx1	wound repair	[33]
ICAM1+	human	adult	hand	Dupuytren's nodules			IL-6, IL-8	scarring	[28]
COL6A5+COL18A1+	human	adult		atopic dermatitis lesion			CCL2, CCL19	inflammatory	[29]

Translational approaches for scarless regeneration

Recent findings of different lineages of fibroblasts with varying roles during the healing process, directly impact both basic and clinical paradigms for wound repair, regeneration, and various cell transplantation strategies in the context of regenerative medicine. This includes, for example, the design of skin substitutes for severe skin defects originating from burns, accidents, congenital diseases, tumors, or chronic ulcers, and which require corrective surgery. Today's standard of skin care constructs includes collagen scaffolds that leave patients with disfiguring and debilitating scars. Clear delineation of fibroblast subsets, and their roles during tissue repair is crucial in the development of novel skin grafts with patient's own supportive fibroblasts, enabling superior 'scarless' regenerative outcomes. In addition, the delineation of the molecular makeup of individual fibroblast subsets that drive scar and regeneration, will serve as basis in developing pro-regenerative and anti-scarring therapies that precisely target fibrotic mechanisms or promote regenerative outcomes following injury. Based on the delineation of fibroblastic subsets, we propose two overarching interventive strategies to reduce scarring and enhance regenerative repair after skin injury (Figure 1).

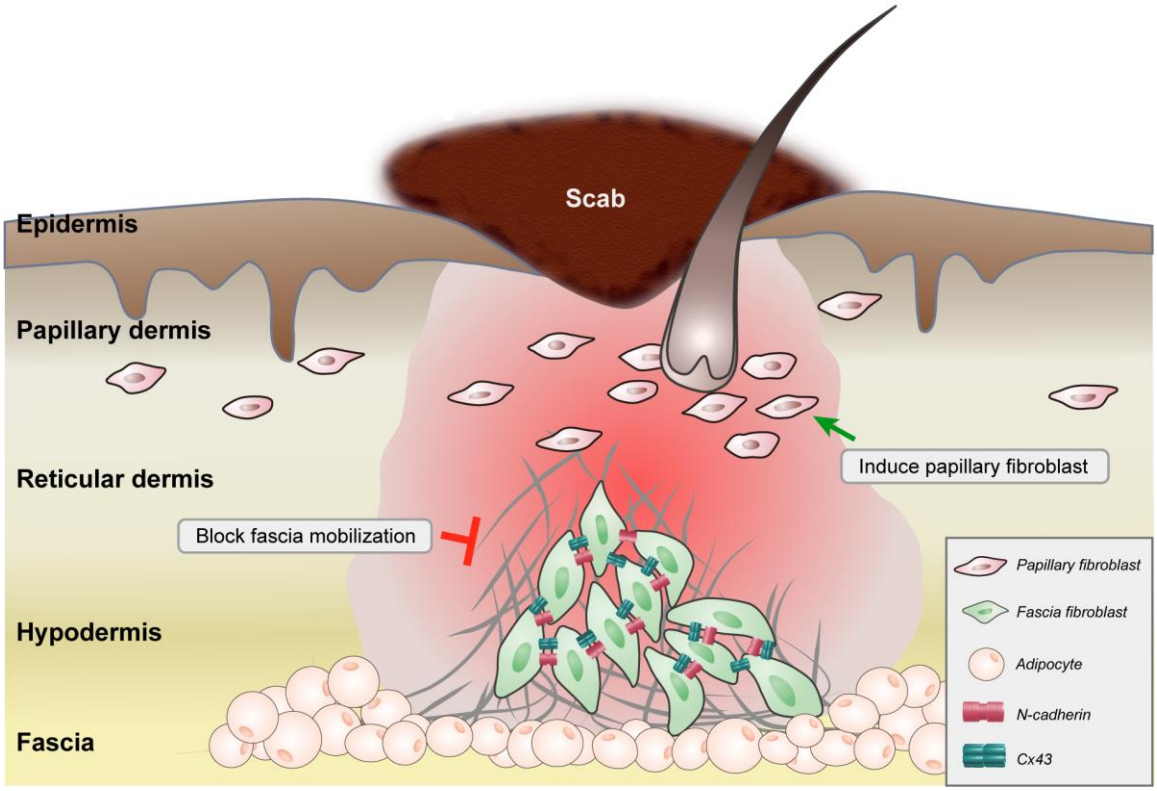

Figure 1. Translational approaches to reduce scar and promote regeneration by blockage of fascia mobilization and induction of papillary fibroblasts.

1). Preventing scars by blocking fascia mobilization

Fibrosis and scarring are recognized as the excessive deposition of ECM. Previous translational paradigms aimed at inhibiting *de novo* ECM deposition from persistently activated myofibroblasts in the wound bed. Over the past couple of years, accumulating evidence has shown that wound ECM also comes from the mobilization of fascia connective tissue. Both *de novo* ECM deposition and movements of pre-existing ECM,

together, play significant roles in scar development including its maturation. Of note - anatomic skin locations that have minimal or non-existent fascia tissue, such as the genitalia respond to injury with minimal scar formation [34]. Whereas the chest and backskin have thick and multi-layered fascia tissues, and it is these anatomic sites that severely scar over. Physically blocking fascia movements by placing synthetic films at precise skin locations could, in theory, minimize human scar formation, although at the risk of adversely delaying wound healing. It could therefore be envisaged that a potential first therapeutic approach would be targeting upstream events that drive fascia ECM movements into wounds. From the biophysical point of view, fascia is essential for transmission of the biomechanical forces such as tension and stretching. Fascia fibroblasts act as a rheostat to buffer the mechanical loads imposed on skin by actively regulating their cytoskeletal organization to adaptively change their morphology and cellular alignment [35]. Such tissue movements imposed by cytoskeletal responses appear to be specific to the fascia, and does not occur in more densely packed dermal connective tissue layers [36]. Fascia ECM is pulled into wounds from the coordinated, collective migrations of resident fascia fibroblasts [5,37]. These collective movements are largely dependent on intercellular communication and adhesion between adjacent fibroblasts, mediated by the N-cadherin adherens junctions and Connexin43 gap junction. Inhibiting N-cadherin or Connexin43 through genetic ablation or chemical inhibition are extremely effective in breaking the cell-cell interactions needed by fascia fibroblasts to mobilize ECM into wounds, thereby reducing scars. Both anti-N-Cadherin and anti-Connexin43 approaches have shown promising anti-scarring effects in small rodent models [37,38].

2). Triggering regeneration with papillary fibroblasts

A completely different route to regeneration would be focusing on the regenerative fibroblasts themselves. Using scRNA-seq, Phan and colleagues have identified a subset of neonatal papillary fibroblasts that express the canonical Wnt transcription factor *Lef1*. These papillary fibroblasts have a distinct transcriptomic profile compared to fibroblasts in reticular, hypodermal or fascia connective tissue layers. *Lef1* expression was found only in developing skin (up to postnatal day 2) but was turned off in juvenile homeostatic skin (postnatal day 21), when the skin scars over. However, forced constitutive activation of *Lef1* in wound fibroblasts of adult transgenic mice led to regeneration of large hair follicles with arrector pili, and to an improved functional recovery of skin compared to small non-functional hair follicles, which normally form in the center of large skin wounds [39]. Comparative analysis of single cell transcriptomics of follicle regenerating wounds and hairless wounds has revealed that scarring wounds have a high proportion of reticular fibroblasts, while regenerating wounds contain a higher proportion of papillary fibroblasts [40]. These findings indicate that the re-acquirement of regenerative traits seen in neonatal wounds could potentially be realized by adoptive transfer or enhancement of their function or physical numbers in wounds.

Challenges and future perspectives

Research into fibroblast heterogeneity has tremendous translational potential and it would need to overcome several challenges before it could be realized towards a wide range of regenerative therapies. For example, with the fast-developing single cell multi-omics, identifying and purifying distinct fibroblast subsets with new markers becomes feasible

and constantly highlights new fibroblastic populations, but it also highlights the subjective interpretation of single cell multi-omic datasets. Up until now, very little overlap has been seen within fibroblast subsets, across different datasets [41]. Furthermore, the fast-expanding lists of differentiated fibroblastic cell types and lineages adds to the obscurity of how these lineages are interconnected. We don't know whether transcriptionally heterogeneous fibroblast subpopulations represent truly distinct cell types or just diverse cellular states along an activation continuum.

Since molecular profiling does not fully explain how transcriptionally distinct populations act in a functional way that is different to promote scarring or regeneration, one stepping stone would be to link transcriptional heterogeneity with physiological function. Such a functional linkage would enable a universal nomenclature for fibroblastic stromal cells.

As we know more about the cellular origins, distinct cellular states of fibroblasts, the precise master regulators of each cell state and the unique functions of each fibroblast type, we grow closer towards realizing a complete genealogy tree, reminiscent of hematopoiesis, which would help refine missing open questions on how heterogeneous fibroblasts relate to one another, and open up targeted strategies for wound repair and regenerative medicine alike. Harnessing the power of diverse fibroblasts therefore holds tremendous opportunities to human health and disease, potentially instilling regeneration back to human injured tissues.

Conflict of interest statement

Nothing declared.

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