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Wound healing, fibroblast heterogeneity, and fibrosis

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Abstract

Fibroblasts are highly dynamic cells that play a central role in tissue repair and fibrosis. However, the mechanisms by which they contribute to both physiologic and pathologic states of extracellular matrix deposition and remodeling are just starting to be understood. In this review article, we discuss the current state of knowledge in fibroblast biology and heterogeneity, with a primary focus on the role of fibroblasts in skin wound repair. We also consider emerging techniques in the field, which enable an increasingly nuanced and contextualized understanding of these complex systems, and evaluate limitations of existing methodologies and knowledge. Collectively, this review spotlights a diverse body of research examining an often-overlooked cell type - the fibroblast - and its critical functions in wound repair and beyond.

Abstract

Talbott et al discuss the current state of knowledge in fibroblast biology and heterogeneity, primarily focusing on fibroblasts in skin wound repair. They also consider emerging techniques in the field and evaluate limitations of existing methodologies and knowledge.

Keywords

Wound healing; fibrosis; regeneration; fibroblasts; fibroblast heterogeneity

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Introduction

Broadly, fibroblasts are the cells of the body's connective tissue responsible for producing and remodeling extracellular matrix (Lynch and Watt, 2018). Fibroblasts are not merely passive "bystander" cells, but rather are a highly active cell type with diverse and dynamic functions throughout the body and its development. Fibroblasts play critical roles in development/growth, homeostasis, and the injury response; they are vital in forming and maintaining the structure of virtually every organ, and are key contributors to the repair process and formation of new tissue following tissue damage (Plikus et al., 2021). In recent years, it has become increasingly well understood that fibroblasts are an extremely heterogeneous cell type, exhibiting significant phenotypic and functional variability between and even within tissues (Griffin et al., 2020; Lynch and Watt, 2018). These cells are also highly plastic (LeBleu and Neilson, 2020), making their study both challenging and fascinating, as they are capable of contributing to a wide array of cellular and tissue-level processes through highly varied mechanisms.

Fibroblasts have been most deeply studied in the skin, as they play an especially critical role in this organ's physiologic and pathologic functioning (desJardins-Park et al., 2018; Gurtner et al., 2008). As the body's largest organ and its first line of defense against the external environment, the skin must meet an unusually high demand for structural resiliency. The skin's cells must be able to repair not only the day-to-day wear and tear caused by constant exposure to environmental stressors (e.g., sun damage) but also more significant wounds resulting from burns, lacerations, or other traumatic injuries. Wound repair is a complex, multi-stage, highly orchestrated process in which fibroblasts play multiple critical roles, via both signaling to other key wound cell types and direct closure and filling-in of the defect/injury site (Gurtner *et al.*, 2008).

Adult wound healing results in formation of a scar, which consists of nonfunctional fibrotic tissue. Scarring is just one (acute) example of fibrosis, a process that can occur anywhere in the body and which results in replacement of native tissue with dense connective tissue, ultimately leading to loss of normal tissue function. Collectively, fibroses are estimated to be responsible for up to 45% of all deaths in the industrialized world (Henderson et al., 2020). As the primary downstream mediators of scarring and fibrosis, fibroblasts are an active target of research seeking to improve wound healing and organ fibrosis outcomes. While this review primarily focuses on fibroblasts' roles in the skin and its wound healing, insights into fibroblast biology and heterogeneity gained from the skin may ultimately translate to other organs and fibrotic processes.

Overview of wound repair

As the body's main external barrier, the skin is a frequent site of tissue damage. Following injury, the skin undergoes a complex wound repair reaction involving many cell types, cytokines, and molecular mediators (Eming et al., 2014). Skin wound repair involves interactions of multiple cell types, with fibroblasts playing key roles later in healing. Following hemostasis, during which the intrinsic and extrinsic coagulation pathways are initiated to prevent blood loss, immune cells are recruited into the injury site, where

they help to sterilize the wound and remove debris (Eming *et al.*, 2014). The release of chemokines by platelets and early inflammatory cells in the wound attracts macrophages (derived from circulating monocytes), one of the key immune players during skin repair. They, too, engulf necrotic cellular debris and pathogenic material from the wound site. A vital feature of macrophages is their dramatic ability to tailor their responses to specific environmental stimuli, displaying diversity and plasticity in the response to tissue damage (Gordon and Taylor, 2005). Wound macrophages were once classically divided into two main subsets – M1 (“classically activated”) macrophages, implicated in pro-inflammatory events; and M2 (“alternative activated”) macrophages, thought to be anti-inflammatory and pro-regenerative (Krzyszczuk *et al.*, 2018) – and the deletion of specific macrophage populations was shown to dramatically alter the outcome of fibrotic reactions (Lucas *et al.*, 2010). However, our modern understanding of macrophage and monocyte heterogeneity highlights that these cells, like fibroblasts, exhibit considerable plasticity and heterogeneity in function and origins, with some subtypes specific to particular disease states (Auffray *et al.*, 2007; Hanna *et al.*, 2011; Kohyama *et al.*, 2009; Okabe and Medzhitov, 2014; Satoh *et al.*, 2013; Satoh *et al.*, 2017).

The early recruitment and activity of inflammatory cells and platelets activates and directs the migration of fibroblasts to the wound site around days 5–7 (Bussone, 2017). Fibroblasts are the primary cell type responsible for synthesizing and depositing new ECM to repair the skin’s structural framework (Bussone, 2017). Subsets of fibroblasts recruited to the wound differentiate into myofibroblasts under the influence of mechanical tension and cytokines such as TGF- β . Myofibroblasts are responsible for wound contracture following injury. While in rare instances (such as fetal wound healing (desJardins-Park *et al.*, 2019)) native-like ECM is regenerated, in the context of typical postnatal wound repair, profound activation of fibroblasts by the wound cytokine/inflammatory milieu leads to deposition of excess, poorly-ordered matrix, resulting in fibrosis (scarring) (Seki *et al.*, 2007; Szabo *et al.*, 2007). Although communication between macrophages and fibroblasts has been implicated in driving both regenerative and fibrotic wound outcomes, the specific signaling pathways and spatiotemporal coordination involved in this cell crosstalk and its divergent influences remain underexplored.

Circulating fibroblasts (also known as fibrocytes), a group of bone marrow-derived mesenchymal progenitor cells (Abe *et al.*, 2001), have been reported to migrate to the injury bed in response to cytokine stimuli and differentiate into contractile fibroblasts under the influence of IL-4, IL-13, and interferon (IFN)- γ (Shao *et al.*, 2008), but these likely represent a small minority of all fibroblasts in skin wound healing (Grieb *et al.*, 2011). A similar phenomenon has been described in several other tissues including liver, lung, heart, blood vessels, and cornea (Haudek *et al.*, 2006; Lassance *et al.*, 2018; Mori *et al.*, 2005; Phillips *et al.*, 2004; Reich *et al.*, 2013; Yan *et al.*, 2016). Although fibrocytes appear to arise from a hematopoietic lineage, their exact progenitor is unknown and the stimuli for fibrocyte recruitment and differentiation are not well defined (de Oliveira and Wilson, 2020). Given the limited knowledge into fibrocyte origins and characteristics, the extent of their functional contribution to acute fibrosis is unclear.

Finally, during the last stage of wound repair, the remodeling phase, fibroblasts continue to crosslink and turn over the initially deposited provisional ECM. They replace some collagen type III with collagen type I and structurally modify the initially deposited granulation tissue, causing strengthening and stiffening of the ECM over time to ultimately form the mature scar (Hinz, 2007). Remodeling is the longest and least well understood phase of wound repair and can last from weeks to months or even years.

Overview of modern wound healing research

Given the complexities of the wound repair process, it is unsurprising that a wide range of experimental models exist to study the biology of wound healing. These range from simple *in vitro* models involving isolated wound cell types, to more complex three-dimensional *in vitro* models incorporating multiple cell types, to *in vivo* animal models of wound repair with varying degrees of faithfulness to human wound biology (Table 1).

In vitro models

In vitro platforms of varying complexity have been used over many years to study the behavior of defined wound cell populations. Fibroblast *in vitro* studies are limited by the fact that fibroblasts undergo significant, pro-fibrotic changes, with broad shifts in marker expression, and adopt a mechanically-activated, myofibroblast phenotype over time in culture independent of other stimuli (Baranyi et al., 2019; Bayreuther et al., 1988; Masur et al., 1996; Walmsley et al., 2015). Three-dimensional (3D) models are of growing interest and may more accurately recapitulate *in vivo* biology (Duval et al., 2017), often incorporating multiple cell types (though none replicate all relevant wound healing cell types). Layered spheroids (Ebner-Peking et al., 2021) (most commonly applied to the study of skin cancers (Klicks et al., 2019; Randall et al., 2018)) are low cost and relatively reproducible, but lack the air-liquid interface (ALI) inherent to the native skin environment (Klicks et al., 2017). More commonly used are layered models consisting of cells grown on a preformed scaffold. While these models can incorporate ALI culture and the scaffolds can be tuned to the mechanical and chemical properties of skin, they are limited by fibroblasts' tendency to contract and/or degrade the scaffold over time (Randall *et al.*, 2018), which may suggest that fibroblasts are still supra-physiologically "activated" in these models. The most truly biomimetic models are arguably organotypic culture with *ex vivo* culture of intact skin biopsies, which theoretically contain all relevant cell types in their native organization; however, these suffer from practical issues such as donor-to-donor variability and availability of samples, and lack an *in vivo*-like mechanical environment (Randall *et al.*, 2018). Overall, *in vitro* fibroblast studies are severely hampered by their inability to simultaneously recapitulate multiple key factors that contribute to skin healing *in vivo*, including physical tension, inflammation, epithelial-mesenchymal signaling, and hemostasis (Liang et al., 2007).

In vivo models

Animal models are ultimately critical to capture the spatial and temporal complexity of wound repair and other fibrotic processes. Mice are the most often-used animal model for

the study of wound healing and fibroses (with other rodents, including rats, less frequently used), with various models aiming to recapitulate excisional healing, incisional healing, ear and scalp wounds, chronic pressure ulcers, and hypertrophic scarring. The primary criticism/limitation of mice as a model for human wound repair is that – because of their loose skin and subcutaneous panniculus carnosus muscle, which largely lacks an analog in humans (Wong et al., 2011b) – unencumbered mouse wounds on the dorsal or ventral surfaces will contract rapidly and heal in a fraction of the time as that of tight-skinned humans (whose skin is under greater tension and adhered to underlying tissue) (Galiano et al., 2004). Contraction also results in a substantially smaller scar, with up to 90% of the wound area closing by contraction (Zomer and Trentin, 2018), rather than the entire wound being filled by formation of new (granulation) tissue (Galiano *et al.*, 2004). One approach to circumvent this issue is to physically prevent wound contraction using a silicone splint affixed to the skin around the wound, resulting in healing through granulation and re-epithelialization of the wound bed in a process and on a timeline that better mimics human healing (Chen et al., 2013; Galiano *et al.*, 2004; Wong *et al.*, 2011b).

Alternatively, full-thickness wounds may be produced on the dorsal tail skin; these do not exhibit contraction and instead heal by secondary intention, with epithelium advancing over granulation tissue. Because of their relatively delayed healing sequence, dorsal tail wounds have been proposed as an experimental model for delayed wound closure (Falanga et al., 2004). Other rodent models, such as the rabbit ear punch model, rely on underlying cartilage as a “natural splint” to prevent wound contraction (Masson-Meyers et al., 2020). This wound model has also been used extensively to study healing mechanisms in MRL/MpJ “superhealer” mice, which display relatively rapid re-epithelialization of ear punch wounds and regenerative healing with little fibrosis compared to wild type mice (Heydemann, 2012). (In contrast to their ear punch regeneration, splinted dorsal wounds in MRL/MpJ heal via scarring, similar to wild type mice (Colwell et al., 2006).) It should be noted, however, that site-specific differences in skin architecture between these distinct wound models may limit translational relevance to the dorsal and ventral skin surfaces.

A popular *unsplinted* wound model is the wound-induced hair follicle neogenesis (WIHN) model, in which very large wounds (2.25 cm^2 in adult mice) heal via substantial contraction with re-epithelialization in the wound center; these wounds regrow sparse hair follicles in the center of the wound (Ito et al., 2007; Wang et al., 2015), though on a background of fibrotic/scar-like ECM (Mascharak et al., 2022), indicating that WIHN healing likely does not represent complete wound regeneration.

While the above-mentioned models all employ excisional wounds, incisional wound models are also used (Ansell et al., 2014); simple incisions in mice tend to heal with extremely fine/minimal scars, but mechanically-loaded wound models have been developed which, by applying tension across mouse incisional wounds, approximate the mechanical stress experienced by healing human wounds and yield hypertrophic scarring resembling that commonly observed in humans (Aarabi et al., 2007). Parabiosis models represent another useful tool in the study of mouse wound healing, in which the blood supply of two mice is surgically joined to establish cross-circulation (Wong *et al.*, 2011b); such models have been

used to establish roles for circulating versus local factors and cells in wound repair (Hamou et al., 2009; Song et al., 2010; Wong *et al.*, 2011b).

While mouse studies have played an important role in informing our current understanding of fibrotic disease mechanisms, including the role of key molecular pathways (e.g., PDGF, TGF- β , and Wnt signaling), cell types (e.g., alpha-smooth muscle actin [α -SMA]⁺ myofibroblasts), and cellular interactions (e.g., fibroblast-inflammatory cell and fibroblast-epithelial cell crosstalk), to date, few effective antifibrotic therapies have translated from mouse studies. Much of this disparity relates to fundamental structural and functional differences in mouse and human skin (Figure 1). In addition to the biomechanical differences between mice and humans (tight versus loose skin) outlined above, mouse skin is substantially thinner than human skin (<25 μ m versus >100 μ m), contains relatively less reticular (deep) dermal tissue, and lacks the rete ridges and (outside of the paws) sweat glands that are found in human skin (Zomer and Trentin, 2018). Further, mouse skin contains a much higher density of hair follicles than most of the human body (implying a higher density of stem cells and progenitors, which may have important repercussions for injury repair outcomes). Mouse wounds are also immunologically distinct from human wounds, containing relatively greater proportions of lymphocytes (including $\gamma\delta$ T-cells, which are absent from human skin (Li et al., 2018)) and unique cytokines (including chemokine [C-C motif] ligand 6 [Ccl6], a critical mediator of fibroblast mechanotransduction signaling in scars and tumors (Coelho et al., 2007; Demircioglu et al., 2020)). Several of these structural and functional differences relate to cell and molecular processes implicated in WIHN, a process without an analog in humans (Gay et al., 2013).

Due to these fundamental limitations of mouse wound healing research, large animal models also play an important role in discovery and translation. Pigs have the most anatomically and physiologically similar skin to humans, with similar epidermal and dermal thickness, relative hairlessness, and, importantly, tight/fixated skin with similar mechanical properties to humans (Masson-Meyers *et al.*, 2020). Red Duroc pigs in particular are thought to be the ideal animal model for human wound healing, as they exhibit human-like wound healing and have a similar propensity for scarring (Wong *et al.*, 2011b). Porcine tissues have also been shown to have similar responses to substances such as growth factors (Swindle et al., 2012), an important translational consideration. Key limitations of pigs, as with any large animal model, are that they are substantially more expensive to maintain, experimental procedures are inherently more complicated (typically requiring trained anesthesia/surgery personnel), reagents and methods are much less well developed than for mice (Zomer and Trentin, 2018), and although limited transgenic porcine models have been generated, these remain far from widespread application (Crociara et al., 2019; Yang and Wu, 2018). While these barriers are collectively prohibitive for the use of pigs in early-stage discovery, their biological similarities to humans make swine a critical model for filling the “translational gap” between mouse research and human clinical trials.

Xenotransplantation models can also be valuable tools for wound healing research. In these models, which have been used since at least the 1970s, human skin is transplanted onto mice, then wounded and its healing studied (Demarchez et al., 1986; Reed and Manning, 1973). These models are highly useful as they allow for the study of human cells’ response

to wounding (Borrelli et al., 2021). However, it can be challenging to separate contributions of human cells within the graft from those of mouse cells in the surrounding tissue; some studies have suggested that mouse cells deposit the early granulation tissue that initially fills the xenograft wound (which is then re-invaded and replaced by human fibroblasts from the graft), though this may depend on the precise nature (e.g., thickness) of the grafted skin (Rossio-Pasquier et al., 1999). Another fundamental limitation is that xenograft recipients are necessarily immune-compromised (typically nude/athymic/T cell-deficient mice) to prevent rejection (Demarchez *et al.*, 1986), which could alter the inflammatory response to wounding.

Finally, numerous models exist for the study of abnormal or impaired wound healing issues common in humans, such as chronic wounds due to diabetes, pressure, ischemia, or reperfusion, which do not naturally occur in animals (Grada et al., 2018). The study of impaired diabetic wound repair (which leads to chronic/non-healing wounds in humans) commonly employs different diabetic mouse models. While these exhibit delayed wound healing, they largely fail to recapitulate all aspects of diabetic wound repair, likely because human diabetes is typically multifactorial (compared to mouse diabetes models relying on single-gene manipulation) and, in fact, genes altered in diabetes models may independently affect wound repair (e.g., leptin may alter wound angiogenesis) (Fang et al., 2010; Grada *et al.*, 2018).

Review of current literature

What is a fibroblast?

Overview of fibroblasts—Fibroblasts were initially reported in the 1800s, first by Virchow who described them as “spindle-shaped cells of the connective tissue,” and later by Ziegler who was the first to use the term “fibroblast” to describe the cells that deposit new connective tissue in wounds (Molenaar, 2003; Muhl et al., 2020b; Plikus *et al.*, 2021). Fibroblasts are the most common cells in connective tissue throughout the body and are responsible for producing, depositing, and integrating the proteins of the ECM (Lynch and Watt, 2018), which consists of both fibrous/structural proteins and “ground substance,” a hydrated gel of molecules such as glycosaminoglycans (GAGs) and proteoglycans (Kendall and Feghali-Bostwick, 2014; Ravikanth et al., 2011). While largely nonproliferative in the absence of stimulation (e.g., injury) (LeBleu and Neilson, 2020), fibroblasts are far from functionally quiescent. In both homeostasis and tissue repair/fibrosis, fibroblasts not only secrete but also continuously turn over and remodel the ECM, via production of enzymes that cross-link (e.g., lysyl oxidase [Lox]) or promote or inhibit the degradation of (e.g., matrix metalloproteinases [MMPs] or tissue inhibitors of metalloproteinases [TIMPs], respectively) ECM proteins (Kendall and Feghali-Bostwick, 2014; Lu et al., 2011; Shaw and Rognoni, 2020). Fibroblasts are also a non-terminally differentiated and relatively plastic cell type and, beyond their direct functions in producing and maintaining ECM, are critical to a host of other processes such as inflammation, cancer progression, and angiogenesis (Kendall and Feghali-Bostwick, 2014; Shaw and Rognoni, 2020).

Experimental definitions of fibroblasts—Experimentally, fibroblasts can be defined in several ways, each with advantages and shortcomings. Classically, fibroblasts were considered to be cells that adhered in culture (Griffin *et al.*, 2020; Lynch and Watt, 2018), and were isolated based on their relatively easy propagation on passaging (Plikus *et al.*, 2021); however, other cell types can also adhere to tissue culture plastic, including macrophages and endothelial cells (Fleit *et al.*, 1984; Kelley *et al.*, 1987; Relou *et al.*, 1998). Morphologically, fibroblasts are typically elongated, spindle-shaped cells, but upon activation (e.g., adherence to plastic substrate, wound stimuli) can spread out and become stellate (Ravikanth *et al.*, 2011).

Unfortunately, no universal fibroblast markers have been identified; while some markers are considered “typical” of fibroblasts (for instance, ECM components such as vimentin or procollagen Ia2 chain), these are neither specific nor necessarily expressed by all fibroblasts, though some context-specific fibroblast markers have been defined (e.g., cardiac fibroblasts are thought to express discoidin domain receptor 2 [DDR2]) (Lynch and Watt, 2018). Many studies do use common “fibroblast markers” such as platelet-derived growth factor receptor alpha (PDGFRA) to prospectively define or isolate fibroblasts (Buechler *et al.*, 2021; Driskell *et al.*, 2013; Muhl *et al.*, 2020b; Philippeos *et al.*, 2018). However, this marker is not specific to fibroblasts (e.g., also expressed by cells of the central nervous system (Zhu *et al.*, 2014)) and, critically, we and others have found that not all fibroblasts – whether in the dermis or other tissues – express PDGFRA (Biasin *et al.*, 2020; Guerrero-Juarez *et al.*, 2019; Mascharak *et al.*, 2021a; Xie *et al.*, 2018). A contrasting approach to defining fibroblasts is lineage exclusion, in which, rather than relying on positive selection based on defined markers, fibroblasts are isolated based on their *lack* of expression of hematopoietic and other non-fibroblast cell lineage markers (Walmsley *et al.*, 2015); of course, a possible drawback of this approach is that it could fail to effectively exclude every non-fibroblast cell and thus be overly inclusive.

Transcriptomic analysis (e.g., scRNA-seq) allows fibroblasts to be classified based on broader patterns of gene expression, rather than positive or negative expression of a single marker. Fibroblasts can generally be distinguished by transcriptomic patterns corresponding to their function (i.e., a transcriptomic profile dominated by ECM genes), and may also express “typical” fibroblast markers such as *CD34* and *Pdgfra* (Muhl *et al.*, 2020b). However, large-scale transcriptomic studies have found that there is no single fibroblast transcriptomic identity or marker; considerable transcriptional diversity exists among fibroblasts (particularly between different organs), with significant variability in expression between different fibroblast clusters in terms of specific types and quantities of ECM genes/modifiers, as well as other genes, expressed (Kendall and Feghali-Bostwick, 2014; Muhl *et al.*, 2020b). These studies suggest that panels of multiple markers, rather than a single or small number of genes, may be needed to transcriptomically define the fibroblast identity.

Defining fibroblasts across organs—Fibroblast transcriptional identities are particularly variable between different organs (Muhl *et al.*, 2020b). A recent large-scale transcriptomic profiling of fibroblasts from 16 mouse tissues by Buechler *et al.* revealed various functional fibroblast identities (e.g., adventitial, parenchymal, alveolar), each with distinct expression patterns of known fibroblast-associated genes; thus, fibroblast

specialization across organs was reflected in differential expression of a limited set of core, defining signaling pathways (e.g., nuclear factor kappa B [NF- κ B], tumor necrosis factor [TNF], Wnt) (Buechler *et al.*, 2021). However, while single-cell transcriptomic analysis allows for more nuanced classification and identification of fibroblasts, even this method may miss some heterogeneity among fibroblasts; for instance, a single transcriptionally-defined population could contain multiple epigenetically distinct subpopulations that might respond differently to a given stimulus (Lynch and Watt, 2018). Further, experimentally, relying on transcriptomic profiles to define fibroblast identity precludes prospective isolation of fibroblasts or fibroblast subtypes on this basis, as transcriptomic identity does not necessarily correlate directly to surface marker profiles or other phenotypic features that can be leveraged experimentally. Overall, the existing body of research demonstrates that many challenges remain in the classification of fibroblasts – including the lack of a unified definition of a fibroblast – and highlights the importance of incorporating multiple analytical lenses and balancing broad versus restrictive definitions of this diverse cell type.

Fibroblast heterogeneity in fibrosis

Over 160 years after Virchow first described apoptosis-resistant, matrix-producing cells of the connective tissue (Molenaar, 2003), the scientific community has come to appreciate the remarkable functional heterogeneity and organ-specific specialization of fibroblasts. Fibroblast heterogeneity can be broadly described according to developmental origin and spatial location.

Fibroblast heterogeneity according to developmental origin—The majority of fibroblasts in the body are embryonically derived from paraxial and lateral plate mesoderm precursors (Thulabandu *et al.*, 2018), while fibroblasts in craniofacial tissues derive from neural crest mesenchyme (Wong *et al.*, 2006) – thus, a first level of fibroblast heterogeneity is introduced during development. These differing embryonic origins may in turn influence fibrotic behavior in postnatal life: neural crest-derived, *Wnt1* lineage-positive fibroblasts (WPFs) mediate scarless healing of the oral mucosa, while scarring healing in the skin of the trunk is accomplished by paraxial mesoderm-derived, *Engrailed-1* (*En1*) lineage-positive fibroblasts (EPFs) in the dorsal skin (Rinkevich *et al.*, 2015), and lateral plate mesoderm-derived, *Paired related homeobox 1* (*Prrx1*) lineage-positive fibroblasts (PPFs) in the ventral skin (Leavitt *et al.*, 2020) (Figure 1). Whereas EPFs and PPFs are responsible for the vast majority of fibrosis following injury, radiation, and tumor desmoplasia, their counterpart fibroblast populations – *En1* and *Prrx1* lineage-negative fibroblasts (ENFs and PNFs, respectively) – are non-fibrogenic and permit regenerative healing (Jiang *et al.*, 2018; Mascharak *et al.*, 2021a) (Figure 2). This behavior is cell-intrinsic: EPFs or PPFs transplanted into the normally non-scarring oral mucosa retain their fibrogenic behavior in response to wounding, while non-scarring WPFs, ENFs, or PNFs transplanted into dorsal or ventral skin retain their non-fibrogenic phenotype (Mascharak *et al.*, 2021a; Rinkevich *et al.*, 2015). It was recently shown that certain postnatal dermal fibroblasts in mice re-activate *En1* expression in response to wound mechanical forces, and that blocking this reactivation via mechanotransduction inhibition yields ENF-mediated wound regeneration with recovery of hair follicles, glands, and normal matrix ultrastructure (Mascharak *et al.*,

2021a). Thus, embryonically-defined fibroblast lineage hierarchies may be continuously reinforced through postnatal reactivation of developmental gene programs.

In addition to the aforementioned fibrogenic and non-fibrogenic (EPF/ENF and PPF/PNF) lineages, skin fibroblasts have also been differentiated into papillary, reticular, and hypodermal subpopulations on the basis of lineage, surface marker expression, and location within the mouse dermis (Driskell *et al.*, 2013; Driskell and Watt, 2015; Phan *et al.*, 2020) (Figure 1). Several mesenchymal cell subpopulations have also been identified in close association with hair follicles, including dermal papillae fibroblasts (which are Sox2/Lef1/Crabp1⁺ and induce hair follicle formation and cycling), dermal sheath fibroblasts (which are α -SMA/Itga5/Itga8⁺ and harbor self-renewing hair follicle dermal stem cells), and arrector pili fibroblasts (which are CD26⁻Itga8⁺ and involved in piloerection); these hair follicle-associated fibroblasts develop from papillary dermal condensates via Wnt and Shh pathway activation (Lim *et al.*, 2018; Noramly *et al.*, 1999; Phan *et al.*, 2020; Zhang *et al.*, 2009). Consistent with their superficial location within the dermis, papillary fibroblasts (CD26⁺Lrig⁺Sca1⁻) descended from *Blimp1* lineage-positive, Dlk1⁻Lrig⁺ progenitors are closely associated with the overlying epithelium (Driskell *et al.*, 2013). Supporting fibroblasts' anatomically-specific roles within the dermis, transplantation of mixed dermal cell suspensions lacking papillary fibroblasts fails to reconstitute either papillary dermal ECM or hair follicles, highlighting the importance of these specific fibroblasts for hair follicle formation (Driskell *et al.*, 2013). In contrast, reticular (Dlk1⁺Sca1⁻) and hypodermal (CD24⁺Dlk1⁻Sca1⁺) fibroblasts, which are descended from *Blimp1* lineage-negative, Dlk1⁺ progenitors, reside deeper within the dermis and contribute to the vast majority of early fibrosis and adipocyte regeneration during wound healing (Driskell *et al.*, 2013; Plikus *et al.*, 2017) (Figure 2). By dominating the wound repair process and effectively excluding papillary dermal fibroblasts (which typically do not participate until later in healing, where they play a more minor role), these deeper skin fibroblast populations thereby preclude hair follicle regeneration and instead promote formation of the “bare area” characteristic of a scar.

Single-cell sequencing supports that distinct – yet non-exclusive and highly dynamic – transcriptomic and epigenomic profiles exist for papillary versus reticular lineage fibroblasts (Foster *et al.*, 2021; Thompson *et al.*, 2021). For example, CD26 has been reported as a marker for pro-regenerative papillary fibroblasts in neonatal healing and WIHN, while Dlk1 is expressed by reticular fibroblasts; however, scATAC-seq of neonatal mouse wounds revealed that chromatin regions encoding both CD26 (*Dpp4*) and *Dlk1* are accessible across multiple fibroblast clusters, rather than exclusive to one (Thompson *et al.*, 2021). Further, CD26 is also expressed by 94% of EPFs (which are found across all dermal layers) and by *En1*-reactivating reticular fibroblasts in the dorsal skin in response to wound mechanical forces (Jiang *et al.*, 2018; Mascharak *et al.*, 2021a; Rinkevich *et al.*, 2015). Such discrepancies highlight the difficulty of reconciling fibroblast heterogeneity simultaneously through micro-anatomical and lineage-based lenses, and these comparisons may be further confounded by differences in the experimental model systems used (e.g., unstented large wounds in WIHN versus smaller stented wounds). It will be important for future studies to establish how much of fibroblasts' developmentally-encoded behavior (e.g., EPFs' propensity for scarring, papillary fibroblasts' association with follicular development

and WIHN) depends on experimental context (size of the wound relative to total body surface area, age and breed of mouse, presence or lack of stenting mechanical force, etc.).

Fibroblast heterogeneity according to spatial location—Fibroblasts from different body sites have shown to display distinct gene expression profiles, highlighting fibroblast topographical heterogeneity. Such position-dependent differences in gene expression profiles (e.g., pattern formation, cell-cell signaling, matrix remodeling) along the anterior/posterior, proximal/distal, and dermal/non-dermal axes have found to reflect Hox gene expression (Rinn et al., 2008). The differentiation and specialization of mesenchymal progenitors over the course of development, and their interactions with developing endoderm- and ectoderm-derived structures, in turn yields fibroblast “organotypicity,” or organ-specific fibroblast heterogeneity.

A recent transcriptomic profiling of mouse heart, skeletal muscle, colon, and bladder, found that fewer than 20% of fibroblast-enriched genes were shared between these four organs (Muhl *et al.*, 2020b). Differences in expression were particularly evident for genes comprising the matrisome, supporting the concept that fibroblast ECM production is tailored to the unique properties of each organ. Nonetheless, overarching commonalities may exist between fibroblasts of distinct origins: recently, a fibroblast transcriptional “atlas,” built from 230,000 fibroblasts across 17 mouse and human tissues in homeostatic and perturbed states (such as infection, injury, and fibrosis), revealed ten conserved gene transcriptional clusters reflecting differential enrichment of NF- κ B, TNF, Ccl19, and Wnt signaling pathway genes (Buechler *et al.*, 2021). Interestingly, two of these clusters – characterized by *Col15a1* and *Pi16* expression, respectively – were each represented in nearly all assayed tissues. These clusters were proposed by the authors to represent two “universal” fibroblast phenotypes: one basement membrane-secreting (expressing *Col15a1*, *Col4a1*, and *Hspg2*), and one with a “resource” stem-like phenotype (expressing *Pi16*, *Dpp4*, and *Ly6c1*). Thus, it is possible that transcriptional “tailoring” of organ-specific fibroblast subtypes occurs within the context of limited fundamental, conserved fibroblast gene expression programs that persist across diverse tissues and conditions.

Myofibroblast heterogeneity—In response to Wnt, TGF- β , interleukin, TNF, and PDGF signaling within the wound environment, fibroblasts from various niches (including the dermis, perivascular region, and adipose tissue) transition into myofibroblasts, dramatically increasing their expression of ECM and contractile proteins (such as α -SMA – encoded by the *Acta2* gene – and myosins) (Klinkhammer et al., 2018; Murdaca et al., 2014; Piersma et al., 2015) (Figure 2). Like their non-activated fibroblast counterparts in homeostasis, myofibroblasts are heterogeneous and can be further segmented by scRNA-seq into transcriptionally distinct subpopulations. For example, scRNA-seq of mouse wound *Acta2*⁺ myofibroblasts revealed 12 populations, which could be further classified into *Pdgfra*^{high}/*Pdgfrb*^{low} (nine subclusters, including *Crabp1*⁺ subpopulations located under epithelium and *Crabp1*⁻ subpopulations in the deep dermis) and *Pdgfra*^{low}/*Pdgfrb*^{high} (three subclusters, evenly distributed throughout the dermis) subtypes (Plikus *et al.*, 2017). Another study that combined scRNA-seq with genetic lineage tracing differentiated mouse α -SMA/collagen type I (col-I)⁺ myofibroblasts into two subtypes – so-called “adipocyte precursors”

(CD26^{high}CD29⁺) and CD29^{high} cells (CD26^{low}CD29^{high}) – with unique transcriptomes and varying propensities for regenerating adipose tissue in concert with CD301b⁺ macrophages (Shook et al., 2018). Thus, dermal myofibroblasts likely also represent a heterogeneous cell population with diverse origins, phenotypes, and microenvironmental influences.

Human skin fibroblast heterogeneity—Efforts to fully characterize dermal fibroblast heterogeneity in humans are complicated by limited tissue availability, donor site-specific differences in mechanics and skin structure (as well as individual donor-to-donor variability), and generally poor correlation with known mouse fibroblast markers (Harn et al., 2021; Philippeos *et al.*, 2018). Nonetheless, cell surface markers have been proposed to distinguish papillary (FAP⁺Thy1⁻) and reticular (FAP⁺Thy1⁺) fibroblasts in human skin (Korosec et al., 2019) (Figure 1). Like their counterparts in the mouse dermis, FAP⁺Thy1⁻ human papillary fibroblasts were found to be incapable of regenerating adipocytes, while FAP⁺Thy1⁺ human reticular fibroblasts expressed high levels of *ACTA2*, *PPARG*, and *CD36* and readily differentiated into adipocytes, consistent with the pro-fibrotic reticular fibroblast phenotype previously reported in mice. Single-cell transcriptomic profiling has also revealed five transcriptionally distinct fibroblast subpopulations present in human skin, including one localized to the upper dermis (*COL6A5*⁺), one in the deep dermis (*CD36*⁺ preadipocytes), and three in the reticular dermis (including two previously uncharacterized populations) (Philippeos *et al.*, 2018). A separate study distinguished two major fibroblast clusters (one *SFRP2/DPP4*⁺ and one *FMO2/LSPI*⁺), with five minor clusters (including one expressing *CRABP1*) (Tabib et al., 2018). It should be noted, however, that these studies utilized cells derived from unwounded skin, indicating that these fibroblast subdivisions may or may not be relevant in the context of wounding; further, the fibroblast populations' distinct gene expression signatures were largely lost following cell culture (Tabib *et al.*, 2018; Walmsley *et al.*, 2015), again highlighting a fundamental limitation of the *in vitro* study of fibroblasts. Transcriptomic profiling of scar, keloid, and scleroderma skin tissues has reportedly reproduced papillary, reticular, mesenchymal, and pro-inflammatory fibroblast subpopulations, with *POSTN*⁺ “mesenchymal” fibroblasts implicated in keloid formation (Deng et al., 2021); however, these subtype designations were based in gene expression signatures derived from *unwounded* skin (Solé-Boldo et al., 2020), further underscoring a need for additional studies specifically characterizing the heterogeneity of fibrotic skin fibroblasts.

Visceral organ fibroblast heterogeneity—Substantial intra-organ heterogeneity also exists in fibroblasts of the visceral organs (e.g., liver, lung, and colon). The liver is capable of a remarkable degree of regeneration, but chronic injury – for instance, prolonged chemical, autoimmune, or malignant insult – can result in cirrhosis, a fibrotic process mediated by myofibroblasts. The cellular origins of scarring liver myofibroblasts are controversial; these pro-fibrotic cells have alternately been proposed to be derived from resident hepatic stellate cells (expressing vitamin A metabolism-related proteins) (Bataller and Brenner, 2005; Geerts, 2001) or portal fibroblasts (*Col15a1/elasticin/Entpd2*⁺) (Beaussier et al., 2007; Wells et al., 2004), bone marrow-derived mesenchymal cells (Forbes et al., 2004; Russo et al., 2006), or epithelial-to-mesenchymal transition of liver hepatocytes

(though recent fate mapping results contest this hypothesis) (Chu et al., 2011; Zeisberg and Duffield, 2010).

The differing contributing populations identified by these studies may be the product of differences in experimental models: myofibroblasts responding to carbon tetrachloride hepatotoxic injury were, reportedly, primarily of stellate cell origin (Zhang et al., 2016), while myofibroblasts generated following cholestatic injury from bile duct ligation were found to originate from portal fibroblasts (Dobie et al., 2019). In an acute non-alcoholic steatohepatitis (NASH) mouse model, Dobie et al. revealed central vein-associated hepatic stellate cells to be the main culprit of liver fibrosis, with Lysophosphatidic Acid Receptor 1 (LPAR1) as a potential anti therapeutic target (Dobie *et al.*, 2019). Stellate cells were further revealed to serve as the source of liver fibrosis via IL11/IL11R α and TGF- β signaling in an additional scRNA-seq mouse atlas of NASH pathogenesis (Xiong et al., 2019). To obtain a better understanding of liver fibrosis pathogenesis, Ramachandran et al. characterized the transcriptome of 100,000 single human cells from healthy and cirrhotic human livers. Interactome analysis identified PDGFR α ⁺, collagen-producing mesenchymal cells as a key contributor to fibrosis through several intra-scar pro-fibrogenic pathways including TNF Receptor Superfamily Member 12A (TNFRSF12A), PDGFR, and Notch signaling (Ramachandran et al., 2019). Single-cell RNA-seq has also revealed distinct transcriptomic profiles for stellate cells and portal fibroblasts (Dobie *et al.*, 2019; Krenkel et al., 2019) as well as heterogeneity *within* the stellate cell population, with transcriptional profiles depending on distance from the central veins as well as injury state (Dobie *et al.*, 2019). Such heterogeneity may have direct translational implications, as targeted inhibition of central vein-associated *Lpar1*⁺ stellate cells (the dominant injury-responsive cell in a study utilizing chemical liver injury) was shown to mitigate fibrosis (Dobie *et al.*, 2019). Mechanical forces – specifically, Hippo signaling via Yes-associated protein (YAP) – have also been implicated in stellate cell-mediated fibrogenesis, and targeted pharmacological inhibition of YAP/TEAD was found to impede cirrhosis in a chemical liver injury model (Dechêne et al., 2010; Manmadhan and Ehmer, 2019; Mannaerts et al., 2015; Martin et al., 2016).

Like the liver, the lung parenchyma is capable of regeneration, due to its robust population of type II pneumocyte stem cells. However, loss of these stem cells or prolonged injury by agents such as bleomycin can instead induce pulmonary fibrosis, a pathologic process with progressive, devastating clinical consequences. Single-cell transcriptional profiling of normal and fibrotic (bleomycin-treated) mouse lungs has revealed multiple distinct cell subpopulations, including *Acta2*⁺ myofibroblasts, *Coll3a1* and *Coll4a1*-expressing fibroblasts, lipofibroblasts (which are necessary for proper function of type II pneumocytes and clinically implicated in idiopathic pulmonary fibrosis [IPF]), mesothelial cells (which line the pleura), and mesenchymal progenitors (which give rise to the aforementioned cell types during lung morphogenesis), as well as a *Pdgfrb*^{high} subpopulation that specifically emerges in fibrogenesis (El Agha et al., 2017a; Valenzi et al., 2019; Xie *et al.*, 2018). Lineage-tracing experiments and *in silico* differentiation trajectory analysis also suggest that *Tbx4* lineage-positive mesenchymal progenitors contribute to nearly all α -SMA⁺ myofibroblasts in adult lung injury (Arora et al., 2012; Xie et al., 2016), suggesting that, as in the skin, injury in the lung may induce reactivation of developmental pathways and

contributions from multiple mesodermal lineages. As with dermal fibroblasts and stellate cells of the liver, Hippo mechanotransduction signaling has been shown to drive fibroblast activation, ECM deposition, and tissue stiffening in the lung in the context of bleomycin injury or IPF; accordingly, indirect inhibition of YAP/TAZ signaling (via fibroblast-specific dopamine receptor D1 antagonism) was found to ameliorate pulmonary fibrosis (Haak et al., 2019). Across multiple organs, activation of YAP/TAZ signaling promotes fibrosis, and NUA family kinase 1 has been recently shown to act as a profibrotic kinase driving a positive feedback loop in concert with TGF- β and YAP/TAZ activation (Zhang et al., 2022).

In contrast to the liver and lungs, cardiac tissue does not have the ability to regenerate following acute or chronic injury after roughly the first week of life (Lam and Sadek, 2018) and instead responds with devastating fibrosis. Fibroblasts derived from epicardium (see section on epithelial-to-mesenchymal transition, below) appear to be the main effector cells of cardiac fibrosis and are activated following injury (Davis and Molkentin, 2014). In a similar approach to studies performed in the skin, lineage-tracing and single-cell -omics have been applied to healthy and fibrotic cardiac tissue to identify novel fibroblast subpopulations and lineages, though the functional importance of cardiac fibroblast heterogeneity is not yet clear (Ali et al., 2014; Muhl et al., 2020a; Wang et al., 2022). Such approaches have, however, successfully identified possible key molecular regulators of cardiac fibrosis. Single-cell profiling of infarcted cardiac tissue in mice revealed a fibrotic signature in Collagen Triple Helix Repeat Containing 1 (*Cthrc1*)-expressing fibroblasts within scars (Ruiz-Villalba et al., 2020). Furthermore, fibroblasts with a similar signature were identified in heart tissues from patients with myocardial infarcts and dilated cardiomyopathy (Ruiz-Villalba et al., 2020). The transcription factor *Aebp1* has also been identified as a crucial cardiac fibrosis regulator in α -SMA⁺ myofibroblasts in human dilated cardiomyopathy and ischemic cardiomyopathy (Rao et al., 2021). Post-transcriptional control of cardiac wound healing by MBNL1 has recently been implicated in regulating fibroblast state plasticity during cardiac wound healing (Bugg et al., 2022). Suppression of the Hippo pathway has been found to play an antifibrotic role in the heart: Xiao et al. found that *Lats1* and *Lats2* mutant resting cardiac fibroblasts transitioned to a myofibroblast-like cell state (Xiao et al., 2019). In contrast, *Lats1/2* deletion led to a relentless pro-fibrotic and pro-inflammatory cascade that ultimately resulted in organ failure. Reducing levels of YAP/TAZ effectors in *Lats1/2* mutants attenuated the fibrotic phenotype following infarction. Thus, Hippo signaling appears to regulate cardiac fibroblast fate transition upon injury. Finally, recent evidence suggests that the oxygen sensing in the post-ischemic heart plays a critical role in regulating proliferation of cardiac fibroblasts, with Hif-1 α opposing fibroblast proliferation through regulation of mitochondrial reactive oxygen species (ROS) (Janbandhu et al., 2022).

Fibroblast plasticity in fibrosis

While fibroblasts clearly exhibit heterogeneity by lineage, phenotypic markers, and transcriptomic profiles, the notion of distinct fibroblast identities is complicated by these cells' plasticity and propensity for interconversion with other cell types. A canonical example of fibroblast plasticity potentially implicated in fibrosis is the phenomenon of epithelial-to-mesenchymal transition (EMT), which involves epithelial cells losing their

typical cell-cell adhesions and apical-basal polarity to generate motile “fibroblastoid” cells (Kalluri and Neilson, 2003). Type I EMT refers to EMT that occurs during the process of development: for example, PDGF and TGF- β signaling in the developing heart induces expression of *Sox9*, *Snail*, and *Slug*, causing epicardial epithelial cells to convert into cardiac fibroblasts (Gittenberger-de Groot et al., 1998; von Gise and Pu, 2012). Type II EMT, in contrast, occurs during wound healing: in the heart, mirroring the developmental EMT process, EMT of epicardial cells following injury contributes fibroblasts and coronary smooth muscle cells to damaged cardiac tissue (Lepilina et al., 2006; Stone et al., 2016). Similarly, mechanical tension, NF- κ B, TGF- β , and Wnt signaling may lead to generation of α -SMA⁺ myofibroblasts from injury-responsive epithelial progenitors in renal, liver, and pulmonary fibrosis (Wynn and Ramalingam, 2012). Targeting EMT may represent an anti-fibrotic strategy: EMT was reversed by using viral delivery of transcription factors to reprogram myofibroblasts into hepatocyte-like cells (expressing *Foxa3*, *Gata4*, *Hnf1a*, and *Hnf4a*) (Rezvani et al., 2016; Song et al., 2016) and cardiomyocytes (expressing *Gata4*, *Mef2c*, *Tbx5*), which could hold promise for ameliorating liver cirrhosis and cardiac fibrosis (Miyamoto et al., 2018). Although the transdifferentiation of epithelial cells to fibroblasts may represent the origin of a specific subset of myofibroblasts, many questions remain unanswered regarding EMT (Lovisa, 2021). The molecular pathways that contribute to the activation of EMT and this process’s orchestration at a transcriptional level remain to be elucidated, including during skin fibrosis.

Plasticity is also evident in the contributions of cells from various mesenchymal compartments to scarring. In the dermis, the lower (reticular) lineage of fibroblasts was initially implicated as the source of α -SMA⁺ myofibroblasts for fibrosis (Driskell *et al.*, 2013); however, more recently, it was shown that all neonatal fibroblast populations defined by scATAC-seq have accessible chromatin for canonical myofibroblast markers (*Acta2*, *Tagln*, and *Tgfbr2*) (Thompson et al., 2021), suggesting the potential for greater plasticity than previously appreciated. Conversion of adipocytes, pericytes, fascial fibroblasts, and circulating hematopoietic cells into myofibroblasts has also been implicated in mouse skin and lung fibroses (Abe *et al.*, 2001; Correa-Gallegos et al., 2019; Di Carlo and Peduto, 2018; Jiang et al., 2020; Shook et al., 2020). Furthermore, reversal of these cell-type transformations may be involved in regeneration of skin and lung parenchyma. In the context of WIHN, myofibroblasts were found to terminate their contractile behavior and convert into adipocytes in response to bone morphogenetic protein (BMP) ligands secreted by regenerated hair follicles (Plikus *et al.*, 2017; Shook *et al.*, 2018). Similarly, loss of myofibroblast-to-lipofibroblast conversion is a hallmark of IPF (El Agha et al., 2017b; Habel and Hogaboam, 2017). It should be noted that reports of these cell-type interconversions have largely been derived from studies in highly-engineered mouse model systems; thus, while promising, further work is needed to establish their relevance to human tissue repair and fibrotic disease.

Another important functional consideration complicating fibroblast plasticity during skin repair is that the skin is naturally the first line of defense against environmental assaults. The skin must constantly respond to insults – whether significant injuries, akin to experimentally-induced or surgical wounds, or more minor tissue damage such as sun exposure over a human’s lifetime – to maintain its homeostatic functioning. Experimentally,

this can be challenging as skin states may be significantly affected by model organisms' behavioral tendencies, such as fighting or self-injury in mice. Furthermore, the biology of wound repair and skin can be impacted by tissue's history of prior injury. Naik et al. illustrated that epidermal stem cells (EpSCs) have a prolonged memory to acute inflammation: these cells maintained increased chromatin accessibility of specific stress-response genes following injury, with some sites persisting up to 180 days after initial injury (Naik et al., 2017). Upon secondary insult, these “memory” genes were transcribed rapidly. Thus, the capacity of the EpSCs to “learn” from their prior experience of injury seems to prime skin to respond to subsequent injury more efficiently. Similarly, Gonzales et al. found that EpSCs developed epigenetic adaptations in wound repair-related genes following injury, allowing them to later transcribe stored wound memory genes involved in inflammation, cytoskeletal reorganization, and cell migration upon secondary injury for an accelerated wound healing response (Gonzales et al., 2021). It will be of interest for future studies to determine whether fibroblasts can similarly develop and maintain cellular “memory” to augment repeated responses to injury stimuli.

In attempting to reconcile fibroblast heterogeneity with plasticity, several themes emerge. First, developmental origin (e.g., lineage) and microenvironmental context (e.g., ECM stiffness, local inflammatory cytokines, cell-cell interactions) likely converge to determine fibroblast behavior. Second, unlike the unidirectional differentiation hierarchies of well-defined systems such as the hematopoietic cell lineage, fibroblasts and other mesenchymal populations seem to relate in a more complex, dynamic network that is highly context-dependent and variable by experimental model. The particular mode of tissue damage and specific model organism (e.g., neonatal versus aged mouse) may dictate which fibroblast subpopulations dominate the injury response and, accordingly, the final outcome of injury. One example of such context dependence in mice is the partially-regenerative healing by papillary fibroblasts observed in large contracting wounds (WIHN) compared to scarring healing by *En1*-activating reticular fibroblasts found in smaller stented wounds (Mascharak et al., 2021a; Rinkevich et al., 2015; Thompson et al., 2021). Third, importantly, fibroblast heterogeneity at rest does not correspond to fibroblast heterogeneity in injury; tissue damage may initiate multiple, distinct differentiation trajectories as various mesenchymal compartments and/or fibroblast subtypes are mobilized. Finally, molecular markers or cell subpopulations determined to have functional significance in lower organisms often do not have corollaries in human tissues, highlighting the critical role of model systems and organisms that more accurately approximate human fibrotic disease mechanisms.

Contributions from other cell types

The pivotal role of immune cells in fibrosis and wound repair has led to interest in interrogating and understanding the cross-talk between immune cells and fibroblasts, with a particular focus on macrophage-fibroblast interactions. Multiple studies have attempted to identify macrophage subpopulations capable of mitigating pro-fibrotic fibroblast activity. One macrophage-fibroblast co-culture study revealed that CD163 overexpression in human primary macrophages promoted a less-fibrotic fibroblast phenotype and supported a “pro-resolution” wound molecular milieu (Ferreira et al., 2020). Transplantation of a CD301b-expressing macrophage subpopulation into partially-healed (POD 3) mouse wounds was also

found to increase fibroblast proliferation in and repopulation of wounds, consistent with these cells representing a reparative, anti-inflammatory macrophage phenotype (Shook et al., 2016). Collectively, the identification of signaling pathways governing fibroblast-immune cross-talk may ultimately allow for manipulation of the wound microenvironment toward skin regeneration. Canonical Wnt signaling has been implicated in fibrogenic myofibroblast activity in multiple tissues (Burgy and Königshoff, 2018; Piersma *et al.*, 2015) and may also play a role in immune cell-fibroblast interactions in fibrosis. Gay et al. demonstrated that Wnt-related immune activity is associated with dermal scarring in mouse WIHN, with macrophages in late wounds found to phagocytize the dermal Wnt inhibitor SFRP4 to establish persistent Wnt activity and drive skin fibrosis (Gay et al., 2020). Hippo signaling may also be relevant to such bidirectional fibroblast-macrophage crosstalk, as recent studies suggest that YAP mechanotransduction signaling in fibroblasts elevates expression of colony stimulating factor 1 (Csf1), a lineage-specific growth factor for macrophages (Zhou et al., 2022; Zhou et al., 2018).

Another important factor to consider when studying fibroblast heterogeneity and wound healing is the role of hair follicle populations. The skin contains hair follicles, which are not static but rather actively cycle throughout the mammalian life cycle (Houschyar et al., 2020). This fact represents a potential confounding factor in experiments relating to the skin, as, for instance, wound-relevant biology can differ based on hair follicle stage (Matsuzaki and Yoshizato, 1998; Morgan, 2014). Further, hair follicles represent important stem and progenitor cell niches with a unique cellular composition compared to the rest of the skin; these cells are known to contribute to wound repair (Ito et al., 2005; Morgun and Vorotelyak, 2020). As humans are relatively hairless compared to mice, effects of hair follicle cells and molecular signaling on wound repair may be more pronounced in common (rodent) experimental models but relatively less relevant in human wound repair, posing a translational barrier. While studies have examined hair follicle cell populations at the single-cell level in both mice (Ge et al., 2020; Joost et al., 2016) and humans (Takahashi et al., 2020), these studies largely focused on development and/or homeostasis and few studies have looked explicitly or in depth at these cells, and their potential interactions with fibroblasts and other cell populations, in wound repair and fibrosis.

Although these studies are encouraging, such approaches are limited to the evaluation of the few already-known pathways and cell types that drive skin fibrosis. However, novel computational analytic methods may allow for broader interrogation of cell-cell interactions. A scRNA-seq tool called CellChat was recently developed to capture interactions between fibroblasts and other cell types in the skin during embryonic development and adult skin wound healing (Jin et al., 2021). This tool uses known ligand-receptor interactions to computationally predict interactions between cell populations based on single-cell transcriptomic datasets. In this study, 60 significant ligand-receptor pairs were identified in 25 cell groups in mouse wounded skin (POD 12). CellChat identified a complex, widespread, and highly influential network of TGF- β signaling involving wound fibroblasts, with multiple ligands identified as driving fibroblast-fibroblast, fibroblast-myeloid, and fibroblast-endothelial interactions. In contrast, outgoing non-canonical Wnt signaling was only identified in one population of fibroblasts and primarily drove fibroblast-to-fibroblast communication. In this study, CellChat analysis also highlighted that communication

patterns between embryonic skin and adult wounds differed dramatically. While no signaling pathway was entirely unique to either embryonic skin or adult wound repair, the relative strength of each signaling network differed by context, with fibroblast growth factor (FGF), nerve growth factor (NGF), and Wnt signaling dominating embryonic skin development and TGF- β , galectin, and epidermal growth factor (EGF) predominating during adult wound repair. Overall, newly-developed scRNA-seq tools to assess global communication patterns among diverse wound cell types may be useful in identifying pathways for cell crosstalk that can be targeted to modulate fibrosis.

Future research directions

Molecular tools in the study of wound repair

Cutting-edge molecular tools continue to shed new light on the complex mechanisms of skin biology, wound repair, and fibrosis. Novel methods are being applied to study wound healing at multiple molecular “levels,” from epigenomic to transcriptomic to proteomic to tissue level (Figure 3). These include methods for characterizing the wound milieu at single-cell resolution and increasingly high-resolution methods for spatial interrogation of wound processes. However, these methods have key limitations to consider when interpreting and contextualizing data derived from them, which should be addressed in future studies to define fibroblast heterogeneity more universally.

Species-specific differences—Regardless of the specific methods used for analysis, species-specific differences must always be considered and reconciled. Fibroblast subpopulations in homeostatic mouse skin have now been characterized by numerous studies using lineage-tracing and immunohistochemical techniques (Driskell and Watt, 2015). Several markers have been identified as discriminating papillary and reticular mouse skin fibroblasts, including *Dpp4* (Korosec *et al.*, 2019), *netrin-1* (*Ntn1*), *podoplanin* (*Pdpn*), and *matrix Gla protein* (*Mgp*) (Janson *et al.*, 2012; Nauroy *et al.*, 2017; Reynolds *et al.*, 2021; Sorrell and Caplan, 2009). However, attempts to apply these gene markers to human scRNA-seq datasets have displayed minimal cluster specificity (Vorstandlechner *et al.*, 2020), highlighting the fact that fibroblast cluster-defining markers may be species-specific and cross-species conclusions must be tempered. Additionally, animal studies often rely on subjective analysis of histologic images, which can be prone to bias. Quantitative and increasingly automated image processing approaches may help to streamline and remove subjective bias from wound and fibrosis analyses; for instance, machine learning analysis of ECM and cellular interaction patterns, clinical datasets, and -omics data is increasingly being used to establish diagnostic and prognostic signatures of fibrotic disease (Mascharak *et al.*, 2021a; Mäkelä *et al.*, 2021; Showalter *et al.*, 2021).

Differences in computational methods—A specific challenge of scRNA-seq, one of the most commonly used methods for interrogation of fibroblast heterogeneity, is low capture efficiency and high dropout rate, with relatively few mRNA molecules ultimately analyzed per captured cell (Liu and Trapnell, 2016). This leads to noisier and more variable data than prior methods such as bulk RNA-seq, which can provide substantial challenges for computational scRNA-seq analysis (Poirion *et al.*, 2016). Initial data

analysis requires clustering transcriptionally similar cells using computer-based methods (e.g., nearest-neighbor analysis); following the identification and annotation of cluster groups, distinct cluster gene signatures can be identified. However, resolution of individual clusters is highly subjective, depending on both the user and specific methods employed (Kharchenko, 2021). To date, there have been diverse efforts to cluster human and mouse fibroblast populations at the single-cell level, which have identified anywhere from three to twelve distinct fibroblast subpopulations (He et al., 2020; Solé-Boldo *et al.*, 2020; Tabib *et al.*, 2018); it is not yet clear how much of this variability results from true biological differences (e.g., wounded versus unwounded skin) and how much is a product of differing computational/analysis methods. Future methods to enable increased uniformity of cluster configuration between studies may aid in the increasingly robust identification of fibroblast subpopulations and integration of findings and conclusions across multiple studies.

Inference of cellular trajectories—A fundamental limitation of current sequencing methods is that protocols are designed to capture a static snapshot of cell states from a specific biological state or point in time (for instance, unwounded skin, or a given postoperative day in wound healing). This is clearly an issue for the study of a process like skin repair, which is highly dynamic, involves multiple biologically discrete but temporally overlapping stages, and may be misunderstood by simply analyzing a single timepoint. Multiple scRNA-seq analysis methods have been developed with the goal of deducing dynamic processes from these static/single-state datasets, including Monocle, a tool for establishing “trajectories” of changing gene expression among cells within a scRNA-seq dataset. However, these tools require caution in use and interpretation: for instance, they typically attempt to incorporate all analyzed cells within the same trajectories, possibly including cells that do not actually participate in the process of interest (Kharchenko, 2021). Further, even the most advanced computational methods cannot compensate for biological sampling limitations, such as a limited range of conditions or postoperative timepoints examined. Trajectory analysis methods may be most powerful when combined with experimental sampling across multiple distinct biological states, such as a recent study which employed multiple high-throughput methods (including scRNA-seq) combined with differentiation/trajectory analyses to establish dynamic trajectories of wound repair in both fibrosis and regeneration (Mascharak *et al.*, 2022). Future longitudinal studies, incorporating samples collected at multiple timepoints and during distinct phases of dynamic processes such as wound repair, will enable more robust and powerful conclusions to be drawn from trajectory analyses and may provide a more nuanced understanding of molecular regulators that govern cell fate decisions in and outside the skin. Such approaches may also be bolstered by advancements in methods for real-time analysis of fibroblast and wound biology, such as improved *in vitro* models (e.g., functional organoid models with both epidermal and dermal components (Lee and Koehler, 2021; Lee et al., 2020)) and intravital microscopy (Jiang *et al.*, 2020). Inference of cellular trajectories will also benefit from improved methods for lineage tracing, which conventionally rely on expression of reporter molecules under control of site-specific recombinases (e.g., Cre) but lack single-cell resolution. Genetic barcoding is a promising approach to bypass this limitation, whereby cell lineage may be linked to single-cell -omics data by expression of unique heritable genetic sequences (e.g., polylox and CRISPR barcodes) (Kalhor et al., 2018; Pei et al., 2017).

Loss of spatial information—The spatial organization of skin is critical to its function, with skin containing distinct anatomical layers (containing heterogeneous fibroblast populations) and defined structures such as hair follicles and glands (Driskell and Watt, 2015). Although existing single-cell analyses are useful in providing high-resolution molecular information from a cellular perspective, a significant drawback is that all spatial information is lost, as the tissue must be homogenized for individual cells to be processed through the sequencing pipeline. Thus, conclusions about the functional contributions of specific, isolated fibroblast subpopulations are inherently correlative. Transcriptomic and proteomic platforms such as Visium and CODEX, respectively, have introduced a spatial component to traditional RNA-sequencing or surface marker-based studies of cellular heterogeneity (Black et al., 2021; Foster *et al.*, 2021; Goltsev et al., 2018). Such spatially-informed approaches may provide a deeper understanding of the mechanisms that govern fibroblast behavior during wound repair. To date, one study has examined the spatial transcriptomic profile of wounds, using Visium spatial transcriptomic sequencing (10x Genomics) to analyze mouse wounds over time (POD 2, 7, and 14) (Foster *et al.*, 2021). The epidermal, dermal, and hypodermal layers were confirmed to cluster distinctly based on anatomically-specific transcriptional programs. Characteristic patterns of gene expression were confirmed for key known wound cell types within the spatial context of wounds; for example, *Krt6b* expression marked keratinocytes found superficially in the skin; *Pdgfra* was expressed by fibroblasts within the dermis; and *Msr1* distinguished macrophages within the skin. At POD 14, there were clear spatial distinctions between transcriptomic profiles of the superficial versus deep regions of the dermis (e.g., *Timp1* was expressed in the basal dermis and *Thbs2* in the apical scar region). High-throughput spatial transcriptomic sequencing and other spatially-informed analyses (e.g., RNAscope multiplexed *in situ* hybridization) have yet to be widely applied in the fields of wound healing and fibroblast research, but they may hold promise for future studies examining spatially-restricted processes relevant to wound repair (e.g., epithelial-mesenchymal interactions at the epidermis-dermis junction, or hair follicle regeneration in wounds).

Multi-omic approaches—As is clear from a review of the literature, individual methods have advanced significantly and applications of novel techniques have meaningfully furthered the study of wound healing and fibroblast biology in recent years. However, the above challenges, and the lack of unified conclusions drawn from existing research (as evidenced by the relative dearth of translational advancements in the clinical wound healing space), underscore the hazards of overreliance on any single research tool or methodology in the complex study of fibroblast heterogeneity, wound repair, and fibrosis. Particularly promising moving forward are approaches that combine multiple analytic modalities, such as single-cell sequencing and spatial mapping or transcriptomic and epigenomic sequencing, as these can provide increased depth of insight compared to any single data type (Abbasi et al., 2020; Phan et al., 2021). Multi-omic approaches may be facilitated by tagging harvested cells with barcode oligonucleotide-labeled hashing antibodies, which allows for data to be linked, at the biological replicate level, across multiple assays (for example, paired scRNA-seq and scATAC-seq) (Foster *et al.*, 2021; Mascharak *et al.*, 2022; Thompson et al., 2021). This approach enables imputation of datasets onto one another and direct correlation of epigenomic, transcriptomic, proteomic, or other features, allowing for increasingly precise

definition of fibroblast states (Foster *et al.*, 2021; Granja *et al.*, 2021; Mascharak *et al.*, 2022).

Toward translation

This exploration of the current literature clearly shows that wound healing and fibroblast biology are an active and exciting area of research. New discoveries are regularly being made that shed light on the fundamental mechanisms of physiology and pathology in the skin. Overall, owing in large part to fundamental differences between model organism (e.g., mouse) and human biology, as well as limitations of individual methodologies and studies, translational progress has been modest. To date, no targeted molecular therapies to promote wound repair, prevent scarring, or drive wound regeneration exist (and therapeutic options for fibroses in other organs are similarly limited). However, taking a step back, patterns can be drawn from the robust body of wound healing and fibroblast literature that hold significant translational promise. While individual studies have critical limitations, a more robust approach may be to look for emergent patterns across multiple studies, which may represent more fundamental truths about the drivers of fibrosis.

A promising “case study” that speaks to this strategy is the example of tissue mechanics and mechanotransduction in scarring, which has emerged over numerous studies (Figure 4) (Barnes *et al.*, 2018; Harn *et al.*, 2019; Mascharak *et al.*, 2021b; Ogawa, 2011). *In vitro* evidence has long supported the ability of stiff substrates, such as tissue culture plastic, to activate contractile and pro-fibrotic molecular fibroblast programming. *In vivo*, the importance of skin tension in scarring is supported by “experiments of nature” – for instance, early-gestation fetal skin has extremely low resting tension and heals without a scar (Hu *et al.*, 2018; Larson *et al.*, 2010), as do extremely loose-skinned *Acomys* (African spiny) mice (Seifert *et al.*, 2012). It is also demonstrated by studies showing both that increasing tension across a wound increases scarring (Aarabi *et al.*, 2007; Gurtner *et al.*, 2011; He *et al.*, 2021; Son and Hinz, 2021) and, conversely, that either decreasing tension or inhibiting cellular mechanical signaling (mechanotransduction) reduces scarring in wounds (Chen *et al.*, 2021; Gurtner *et al.*, 2011; Ma *et al.*, 2018; Mascharak *et al.*, 2021a; Wong *et al.*, 2013; Wong *et al.*, 2011a) as well as split-thickness skin grafts (Chen *et al.*, 2022). Providing the ultimate translational validation, two randomized controlled clinical trials showed that tension offloading of human surgical wounds (following either abdominoplasty or scar revision operations) significantly reduced scarring (Lim *et al.*, 2014; Longaker *et al.*, 2014). The trajectory of knowledge in this “case study” – moving from *in vitro* evidence, to small and then large animal studies, ultimately to the clinic – highlights the possibilities for basic knowledge of fibroblast biology to ultimately pave the way for novel therapies. Further supporting a conserved role for mechanical signaling in fibrosis, mechanotransduction has been shown to be involved and/or dysregulated in other organs – including liver and lung – and in aging as well as injury states (Angelini *et al.*, 2020; Dechêne *et al.*, 2010; Haak *et al.*, 2019; Manmadhan and Ehmer, 2019; Mannaerts *et al.*, 2015; Martin *et al.*, 2016), which further implies that mechanics-targeted approaches may be similarly effective in preventing fibrosis in diverse organs other than the skin.

While no other pathway has been as thoroughly translated in the context of wound healing and scarring, similar patterns certainly emerge when the literature is taken as a whole – for instance, the importance of inflammation in fibrosis. This scenario may be more complex, as both “positive” (i.e., pro-regenerative/anti-fibrotic) and “negative” (pro-fibrotic) examples and forms of inflammation exist, precluding blanket inhibition of inflammation or ablation of immune cells as a viable target for blocking fibrosis. However, as studies continue to establish the precise mechanisms by which inflammatory cells communicate with other fibrosis-relevant cell types (including distinct subtypes of fibroblasts), and their dynamics throughout fibrosis development and resolution, similarly promising translational approaches may be identified which could be effective in targeting fibrosis in higher organisms and, ultimately, humans. It is critical for researchers to bear in mind not only how we can advance our fundamental understanding of molecular and cellular biology, but also how we can continually integrate and contextualize knowledge to draw increasingly powerful conclusions from the vast existing body of work.

Conclusions

Fibroblasts are a central cellular contributor to the skin and other organs, with dynamic roles in homeostasis, wound repair, and fibrosis. Long dismissed as relatively passive “bystander” or “filler” cells, fibroblasts are increasingly appreciated as critical mediators and governors of both physiologic and pathologic ECM deposition and remodeling. These cells have also been shown in recent years to possess impressive plasticity and heterogeneity, with phenotypically and functionally distinct subtypes of fibroblasts contributing to different anatomical sites and biological processes. Many key questions – including the full extent of fibroblast heterogeneity both in and outside of the skin, how fibroblast states change when perturbed (e.g., by injury), and how these cells are influenced by interactions with other skin and wound cell types – remain to be addressed, but emerging research techniques promise to shed further light on these fascinating cells in coming years.

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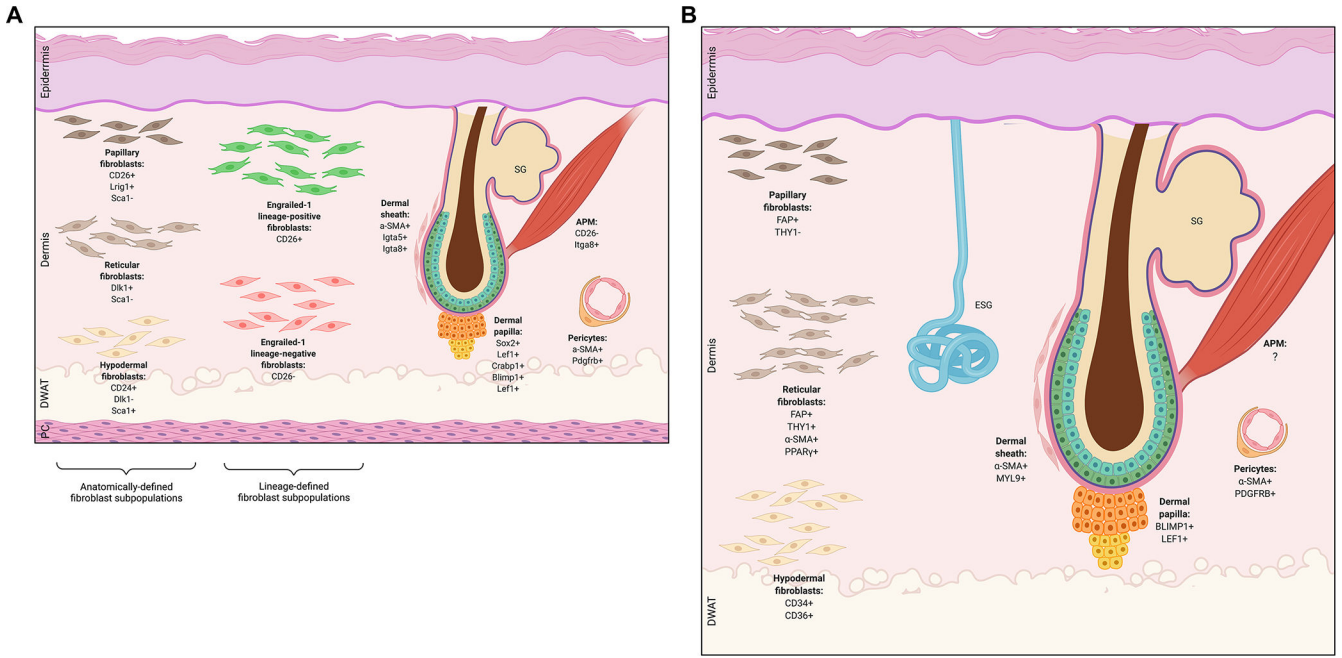


Figure 1: Differences in the architecture of mouse versus human skin.

A) Depiction of mouse skin structure, which includes a panniculus carnosus (PC) muscle layer under the hypodermis. **B)** Depiction of human skin structure. Human skin differs from mouse skin in that it is thicker overall, has sparser hair follicles (HF), and contains eccrine sweat glands (ESG) which are not found in mice outside the skin of the paws. For both mouse and human skin schematics, various mesenchymal subpopulations and characteristic markers are shown, including microanatomically defined fibroblast lineages (papillary, reticular, hypodermal fibroblasts), functionally defined fibroblast lineages (*Engrailed-1* lineage-positive and lineage-negative fibroblasts), HF-associated fibroblasts (dermal sheath, papilla, arrector pili), and pericytes. SG, sebaceous gland; DWAT, dermal white adipose tissue; APM, arrector pili muscle.

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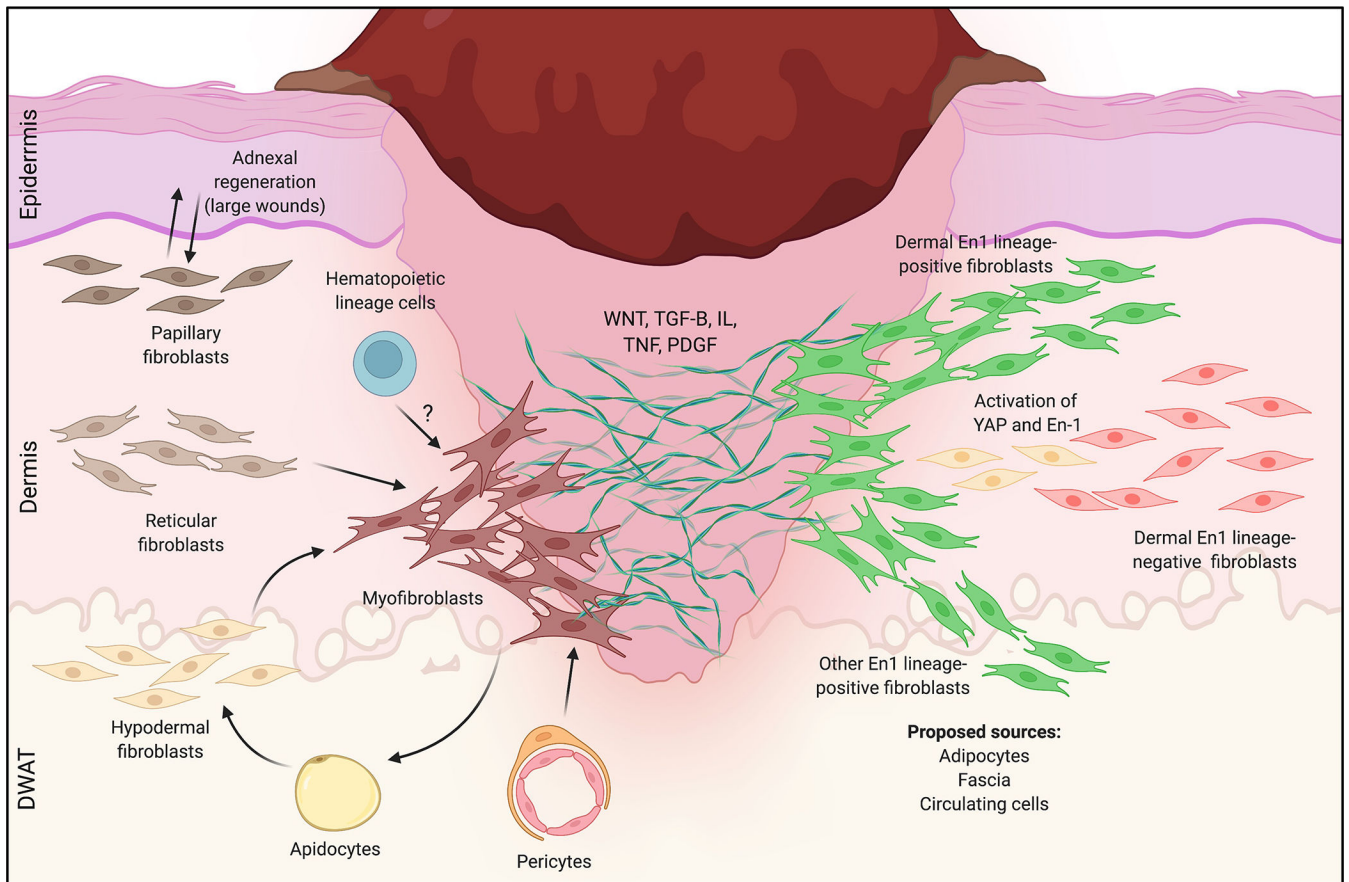


Figure 2: Fibroblast heterogeneity in mouse skin wound healing.

Left: Heterogeneous responses to wounding for the three microanatomically-defined lineages of fibroblasts (papillary, reticular, hypodermal). In small wounds, reticular (middle) and hypodermal (bottom) lineages dominate, giving rise to scarring myofibroblasts. In larger wounds (e.g., WIHN), papillary fibroblasts (top) preferentially interact with epidermal elements to drive sparse adnexal regeneration, and a subset of reticular layer-derived myofibroblasts regenerates adipocytes. Pericytes (bottom middle) and possibly circulating hematopoietic cells (“fibrocytes”, top middle) may also generate scarring myofibroblasts.

Right: Heterogeneity in wound healing responses by *Engrailed-1* (*En-1*) lineage-positive (green, EPF) and -negative (red, ENF) fibroblasts. EPFs are responsible for the vast majority of dorsal skin scarring in response to wounding, while ENFs do not contribute to fibrosis; a subset of ENFs reactivates *En-1* to contribute postnatally-derived scarring EPFs. Proposed sources of scarring EPFs include the dermis (all layers), adipose tissue, circulating cells, and fascia. How these different lineages of fibroblasts (papillary, reticular, adipose, EPFs/ENFs, etc.) overlap and/or give rise to one another remains unclear.

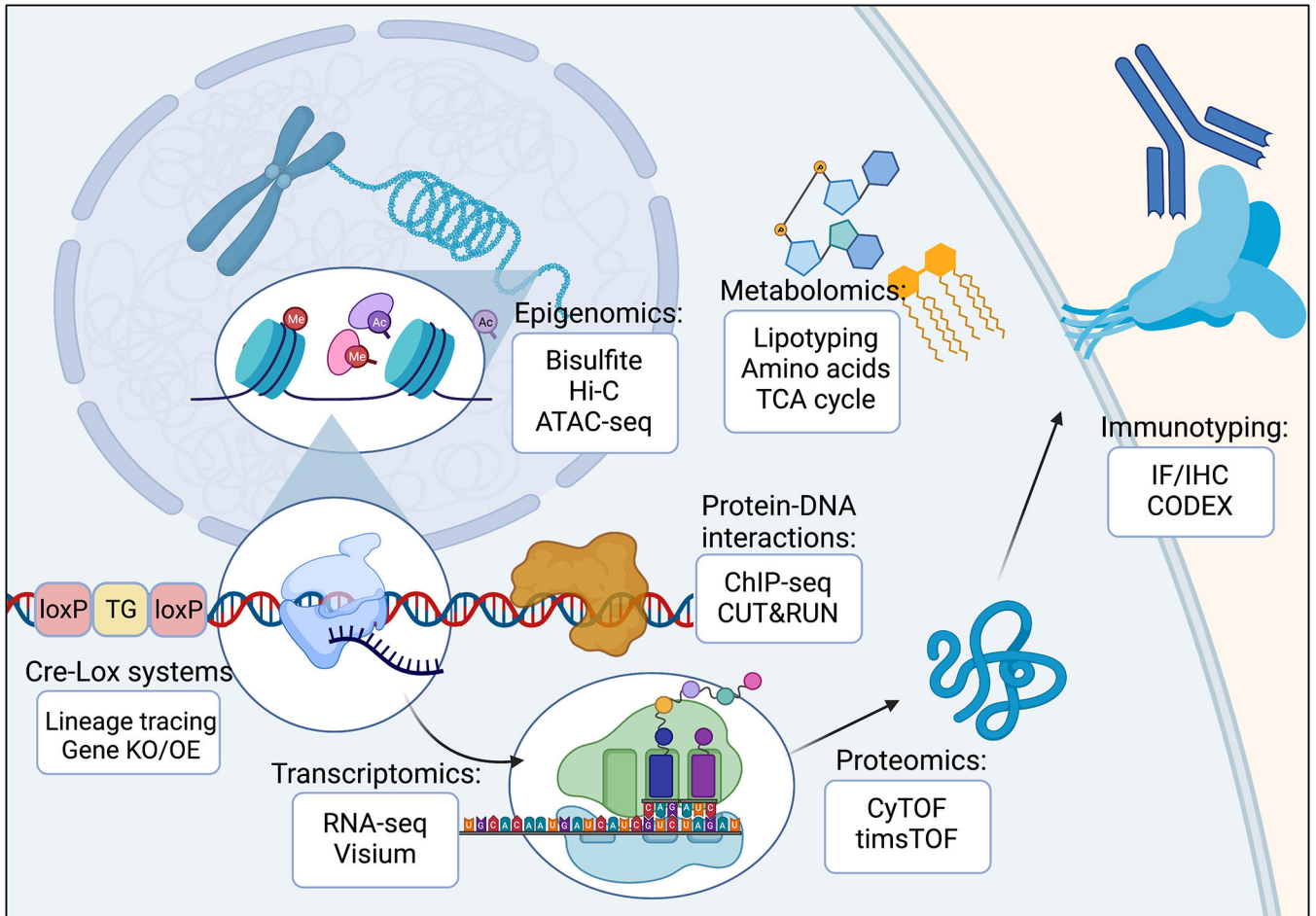


Figure 3: Levels of molecular regulation of fibroblast behavior.

Fibroblast behavior depends on the complex interplay of many factors, including these cells' epigenomic, transcriptomic, and proteomic profiles. Modern technologies are able to interrogate fibroblasts at each of these molecular levels to better understand fibroblast heterogeneity and functioning.

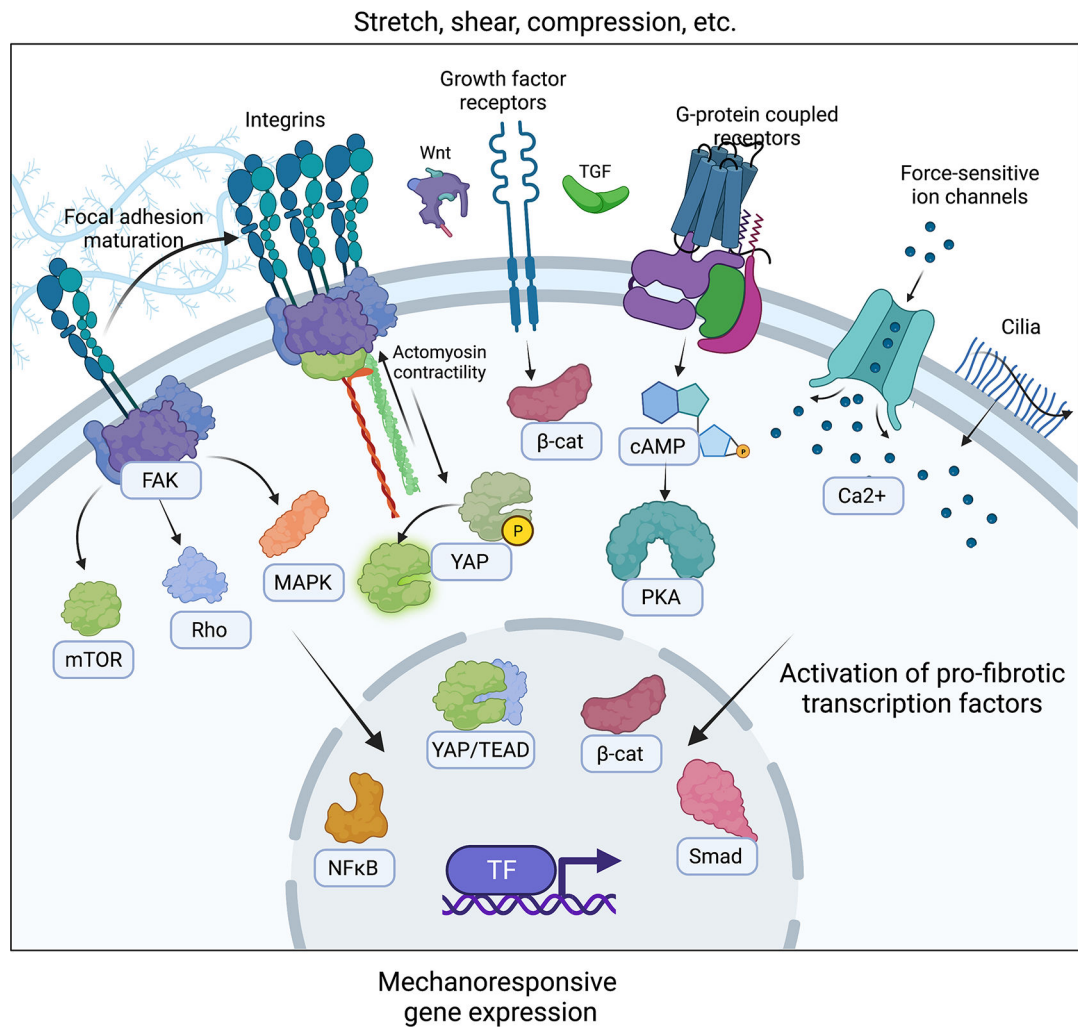


Figure 4: Mechanisms of mechanotransduction signaling.

Schematic depicting the diverse mechanisms and signaling pathways/cascades by which fibroblasts and other mechanosensitive cell types may communicate changes in their mechanical environment to molecular phenotypic changes. Multiple levels of evidence exist to show that fibroblasts activate pro-fibrotic machinery in response to mechanical stress, and that fibroblast mechanical signaling can be targeted to mitigate fibrosis.

Table 1:

Comparison of models for studying wound repair.

Model	Strengths	Limitations
<i>In vitro</i> monolayer models	<ul style="list-style-type: none"> • Ease of use • Amenable to genetic manipulation 	<ul style="list-style-type: none"> • Does not reconstitute the complex 3D environment and cell-cell and cell-matrix interactions of the skin. • Fibroblasts undergo pro-fibrotic shift <i>in vitro</i>
Three-dimensional culture systems (spheroid, organotypic, <i>ex vivo</i>)	<ul style="list-style-type: none"> • Ease of use • Amenable to genetic manipulation and tuning of native-like substrate properties (e.g., stiffness) • In <i>ex vivo</i> culture, contain all relevant cell types in native organization 	<ul style="list-style-type: none"> • Most models do not fully reconstitute all cell-cell and cell-matrix interactions, or functionality, of the skin. • Fibroblasts undergo pro-fibrotic shift <i>in vitro</i>. • May require use of exogenous growth factors or chemical inhibitors in culture media.
Mouse excisional/incisional <i>in vivo</i> wound models	<ul style="list-style-type: none"> • Ease of use, replicability • Genetically dissectible using transgenic animals • Allows for lineage tracing of fibroblast populations. • Can test potential anti-fibrotic agents. 	<ul style="list-style-type: none"> • Loose skinned animal model with a panniculus carnosus muscle, which is not analogous with human wound healing.
Wound-induced hair neogenesis (WIHN)	<ul style="list-style-type: none"> • In vivo model of skin regeneration, with recovery of sparse follicles in the scar center. 	<ul style="list-style-type: none"> • No known correlates outside of rodents. • Limited degree of regeneration, with follicles emerging against a scar background.
Mouse xenograft <i>in vivo</i> models	<ul style="list-style-type: none"> • Ease of use • Provides initial understanding into human wound healing. • Can test potential anti-fibrotic agents. 	<ul style="list-style-type: none"> • Limited insight into immune-fibroblast interactions. • Surgical expertise required to overcome rejection of human skin grafts. • Donor tissue availability for xenografting.
Porcine <i>in vivo</i> models	<ul style="list-style-type: none"> • Anatomically and physiologically similar to human skin (especially red Duroc pigs) 	<ul style="list-style-type: none"> • Expensive • Transgenic animals are limited • Experimental procedures require surgical and anesthetic expertise.