

Fibroblast heterogeneity: implications for human disease

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Fibroblasts synthesize the extracellular matrix of connective tissue and play an essential role in maintaining the structural integrity of most tissues. Researchers have long suspected that fibroblasts exhibit functional specialization according to their organ of origin, body site, and spatial location. In recent years, a number of approaches have revealed the existence of fibroblast subtypes in mice. Here, we discuss fibroblast heterogeneity with a focus on the mammalian dermis, which has proven an accessible and tractable system for the dissection of these relationships. We begin by considering differences in fibroblast identity according to anatomical site of origin. Subsequently, we discuss new results relating to the existence of multiple fibroblast subtypes within the mouse dermis. We consider the developmental origin of fibroblasts and how this influences heterogeneity and lineage restriction. We discuss the mechanisms by which fibroblast heterogeneity arises, including intrinsic specification by transcriptional regulatory networks and epigenetic factors in combination with extrinsic effects of the spatial context within tissue. Finally, we discuss how fibroblast heterogeneity may provide insights into pathological states including wound healing, fibrotic diseases, and aging. Our evolving understanding suggests that ex vivo expansion or in vivo inhibition of specific fibroblast subtypes may have important therapeutic applications.

Introduction

A paradigm of the metazoan body plan is the combination of epithelial and mesenchymal elements into structured three-dimensional organs. Fibroblasts are cells that synthesize and integrate structural proteins such as collagen and elastin into the extracellular matrix (ECM) of most mesenchymal tissues (1–4). It has long been believed that fibroblasts exhibit differing functional identities (5), and in recent years, evidence has emerged to support this concept. Fibroblasts are characterized by the expression of markers including vimentin and procollagen I α 2 chain (1). However, these markers are not considered specific for fibroblasts (6), and it has not been demonstrated that they are expressed on all fibroblast types.

Fibroblast heterogeneity can be considered according to stage of development, tissue of origin, or tissue microenvironment. The mammalian skin has proven a particularly tractable tissue in which to address these questions. The epidermis, a stratified squamous epithelium, overlies the dermis, a mesenchymal tissue (Figure 1). In addition to fibroblasts and ECM components, the dermis, in common with other mesenchymal tissues, incorporates a wide range of other cell types, including blood vessel components (endothelial cells, smooth muscle cells, and blood vessel-associated fibroblasts) (7), eccrine and apocrine sweat glands, lymphatic vessel components, neurons (8), sensory receptors, and tissue-res-

ident immune cells (9, 10). These other cell types are sufficiently numerous that fibroblasts represent a minority cell population in the adult dermis.

A simple definition of cellular identity is that it represents the set of macromolecules present within a cell at a specific point in time. However, this definition omits the possibility that cells with identical gene expression patterns at a particular time point can respond differently to an external stimulus or differentiate spontaneously because of epigenetic or other factors. A further complication is that spatial location with respect to ECM components and other cells may play a role in the maintenance of a specific cellular identity, and these signals can be lost when cells are extracted from the tissue. As a consequence of these factors, the dissection of cellular identity and lineage relationships is inherently more challenging for cells that constitute solid tissues than for cells of the hemopoietic system.

Here, we discuss emerging concepts relating to cellular identity and lineage relationships of fibroblasts. We begin by discussing fibroblast heterogeneity according to tissue and anatomical site of origin. Subsequently, we examine evidence in support of the concept that individual tissues, such as the dermis, comprise multiple fibroblast subtypes. We consider to what extent differences are specified intrinsically by transcriptional regulatory networks and epigenetic mechanisms versus by extrinsic factors that depend on the spatial context within the tissue, such as secreted factors, cell-cell communication, and interactions with the ECM.

While full details of the mechanisms governing fibroblast cellular identity remain to be determined, it is likely that specific fibroblast subtypes will play key roles in wound healing, in diseases characterized by excessive fibrosis, in cancer stroma, and

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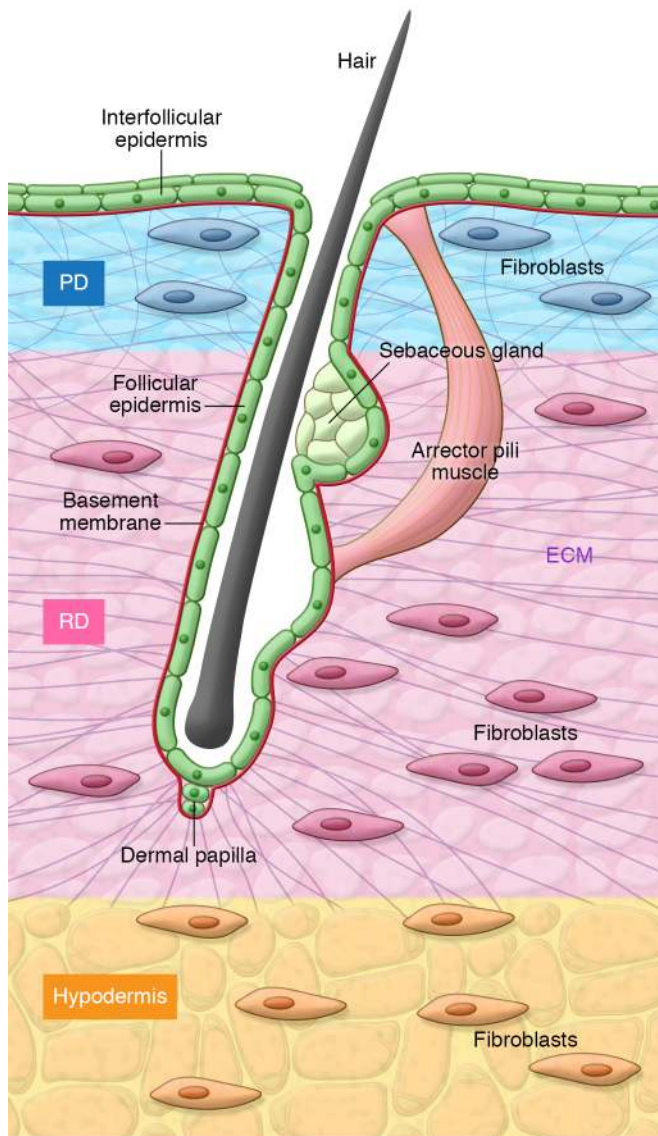


Figure 1. Structure of murine dorsal skin. The skin has two layers, the epidermis and the dermis. The epidermis is a stratified squamous epithelium with associated adnexal structures such as hair follicles and sebaceous glands. It is separated from the underlying connective tissue, the dermis, by a basement membrane (red). The three dermal layers are the papillary dermis (PD), reticular dermis (RD), and hypodermis/white adipose tissue. The dermal papilla and arrector pili muscle constitute two specialized populations of dermal mesenchymal cells. Adapted with permission from *Science* (143).

(15) are correlated with differing patterns of Hox gene expression that reflect the developmental origin of the cells (16, 17). Therefore, differences in the behavior of fibroblasts from different body sites likely reflect a combination of intrinsic differences and the role of factors such as mechanical stress that differs between body regions (22).

Fibroblasts derived from the bone marrow, which are the cells that adhere when plated in culture, are worthy of special consideration. This population of fibroblasts have the capacity to differentiate into multiple lineages (23) and have been termed mesenchymal stem or stromal cells (MSCs). Initial reports suggested that MSCs can contribute to the dermis (24–26), but subsequent studies do not appear to support this finding (27–29). Interestingly, clinical trials have suggested that the infusion of MSCs into the blood may ameliorate several diseases, such as graft-versus-host disease (30, 31). However, there has been no demonstration that these MSCs stably engraft, and it has been proposed that any beneficial effects are achieved by release of paracrine factors (32).

Recent results support the concept that pericytes — blood vessel-associated fibroblasts — are the *in vivo* counterpart of MSCs (33). Pericytes are associated with and encircle vascular channels in most tissues (34, 35). A number of markers have been proposed for the identification of pericytes, including NG2 (35) and RGS5 (36). It remains unclear whether different subtypes of pericytes are associated with arteries, veins, and lymphatics. Within muscle, it appears that pericytes have the capability to differentiate to muscle fibers (37), but at the present time there is no evidence that pericytes have the capability to differentiate into other dermal lineages. A specific subset of ADAM12-expressing perivascular fibroblasts present in skin and muscle are activated by wounding and contribute to collagen overproduction in the scar (38). Genetic ablation of these cells reduces collagen production and scarring (38).

Cardiac fibroblasts constitute the largest cellular population within the heart (11) and are located throughout the myocardium, interspersed between myocytes (11). They exhibit specific expression of discoidin domain receptor 2 (DDR2) (39) and play a critical role in the regulation of normal myocardial function in addition to the pathological remodeling that accompanies hypertension and heart failure. It has long been recognized that the effects of angiotensin II signaling on cardiac fibroblasts and myocytes lead to hypertrophy and fibrosis (11). Proinflammatory signaling by IL-1 β and TNF- α also leads to pathological alterations in collagen synthesis by cardiac fibroblasts that accompany myocardial failure (40). Fibroblasts are also integral to the structure of the lung, and, as for the heart, the effects of proinflammatory cytokines such as IL-1 α on pulmonary fibroblasts play an important role in the progression of fibrotic lung disease (41).

potentially in tissue aging. This suggests the possibility that *in vivo* inhibition of specific fibroblast subtypes or cellular therapy consisting of *ex vivo* expansion and subsequent reintroduction may have therapeutic benefit in the treatment of such conditions.

Fibroblast heterogeneity according to anatomical origin

Mesenchymal-derived connective tissues including heart (11), lung (12), gastrointestinal tract (13), and muscle (14) contain fibroblasts that fulfill specialized functions (15). Differences in gene expression have been demonstrated between dermal and nondermal fibroblasts (16, 17), and fibroblasts derived from different anatomical sites have differing developmental origins, including the neural crest, lateral plate mesoderm, and dermatomyotome (18–20). Moreover, there are significant differences in the architecture of the dermis at different body sites, which are associated with a differing potential for disease processes such as the formation of keloid scars (21). Differences in morphology and behavior in cultured fibroblasts derived from different body sites

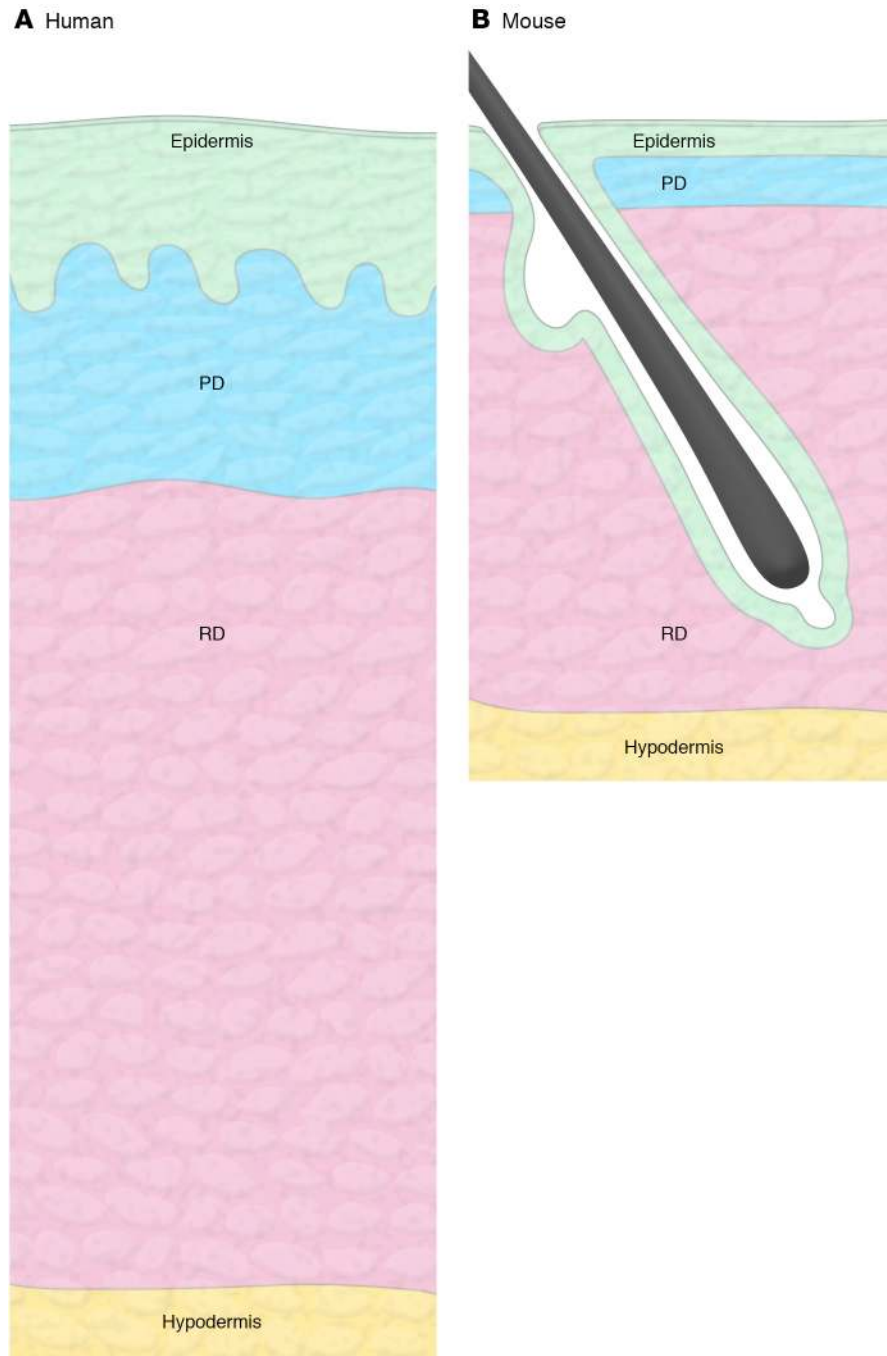


Figure 2. Comparison of human and murine skin. The interfollicular epidermis and dermis are thicker in human than in mouse skin. In most body sites mice possess a higher density of hair follicles. In human skin the boundary between the epidermis and dermis undulates, whereas it is flat in mouse skin. Adapted with permission from *Cold Spring Harbor Perspectives in Biology* (4).

Thus far we have considered differences in fibroblasts arising from distinct anatomical sites; however, as we shall discuss, multiple, functionally distinct fibroblast subtypes can arise from a single tissue. This is best understood in the dermis. Although fibroblast heterogeneity in other organs has yet to be investigated in detail, it seems likely that it exists. For example, fibroblasts derived from kidney cortical and medullary regions exhibit differences in cell culture and in their responses to mitogenic stimulation (42, 43).

Fibroblast identity differs between papillary and reticular dermis

The mammalian dermis represents an archetypal mesenchymal tissue largely composed of ECM elements, primarily type I and type III collagens, but also other collagen subtypes, proteoglycans, and elastin (4). It serves as a structural scaffold, and a basement membrane enriched in type IV collagen (44) separates it from the epidermis, a stratified squamous epithelium that forms the outermost layer of the skin. The dermis is penetrated by numerous appendageal structures, including hair follicles and sweat glands, which manifest as specialized invaginations of the epithelium, for which the basement membrane is in continuity with the interfollicular epidermis (Figure 1). The detailed microstructure of the dermis differs between human and mouse skin. In humans, there is a relatively low density of hair follicles on body sites outside of the scalp, with intervening interfollicular epidermis exhibiting invaginations known as rete ridges. In mice, the density of hair is higher and rete ridges are not prominent (Figure 2).

The dermis has distinct layers that are readily identified histologically: the papillary dermis is the superficial layer of the dermis that lies immediately below the epidermis (Figure 1). This is composed of dense but poorly oriented collagen bundles that provide structural support to the overlying basement membrane (4). Beneath this layer is the reticular dermis, which comprises directionally oriented, thicker collagen bundles with intervening spaces (45, 46). It has long been recognized that there are differences in the composition of the ECM components at the ultrastructural level that constitute the papillary and reticular dermis. This has led to the hypothesis that ECM composition reflects differences in fibroblast identity in the tissue compartments.

Microarray analysis of cultured fibroblasts derived from mechanically separated dermis has revealed differences in gene expression (47); however, subsequent studies have yet to reproduce proposed markers

of papillary and reticular layers. Dermatome experiments of this nature are inherently limited, since the level of sectioning cannot be precisely controlled, and therefore the possibility that reticular fibroblasts are included in the papillary sample cannot be excluded. Furthermore, since they are studied in the form of explant cultures, it is not clear to what extent in vitro selection for specific cell subpopulations plays a role and to what degree blood vessel-associated fibroblasts, known as pericytes, contribute.

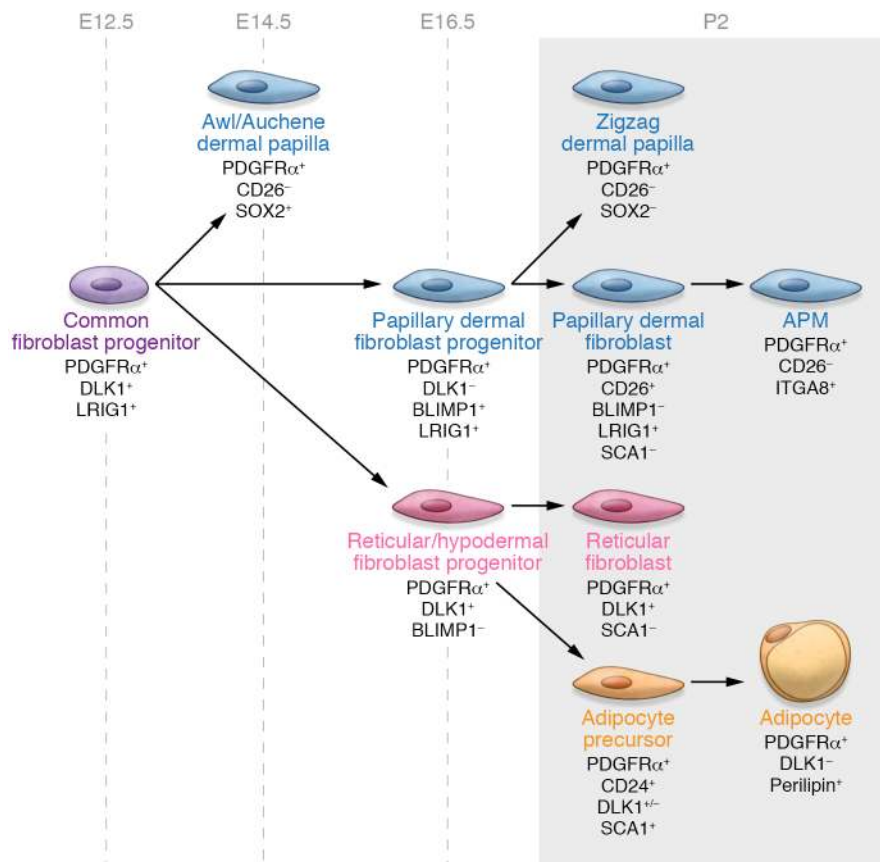


Figure 3. Mouse dermal fibroblast lineages. Dermal fibroblasts derive from common fibroblast progenitor cells and differentiate into specific lineages by postnatal day 2 (P2). These subtypes display distinct functions. For example, papillary fibroblasts are essential in the coordination of the hair cycle and the formation of hair follicles after injury, and reticular fibroblasts mediate early wound repair responses. Adapted with permission from *Trends in Cell Biology* (6). APM, arrector pili muscle.

papillary fibroblasts, reticular fibroblasts, and dermal papillae are derived from a multipotent mesenchymal progenitor that is present at embryonic day 12.5 (E12.5), with lineage commitment occurring by E16.5 (56). At postnatal day 2, papillary, reticular, and hypodermal markers can be differentiated according to the expression of cell surface markers including CD26, SCA1, and DLK1, with CD26 marking the upper dermal lineage (ref. 56 and Figure 3). Papillary fibroblasts are required for new hair follicle formation following wounding, whereas reticular fibroblasts mediate the early events in wound repair and express so-called fibroblast activation markers such as α -smooth muscle actin (α SMA) (56).

A subsequent study identified fibroblast subtypes in the mouse dermis according to the expression of Engrailed-1 (En1) and CD26. The authors found that the CD26⁺En1⁺ population is responsible for dermal development and fibrosis and also contributes to the cancer stroma (57). The differences observed between these two studies with respect to CD26 expression highlight that the expression of lineage markers can be dynamic, varying according to stage of development even where lineage identity is maintained. Further studies are needed to establish whether similar lineages are present in the human dermis.

Immunohistochemical studies have demonstrated differences in the expression of collagen subtypes and proteoglycans between papillary and reticular dermis (45, 48–50), although interpretation of these studies is complicated by differences in the density of fibroblasts and ECM elements in different regions of the dermis. Papillary and reticular fibroblasts can be isolated by explant culture of mechanically sectioned (dermatomed) human dermis. Explant fibroblast cultures derived from these layers exhibit differences in a number of parameters, including rate of cell division, contraction, and the expression of various collagens and proteoglycans (5, 51, 52). Cultured fibroblasts derived from the papillary dermis support the formation of a normal stratified epidermis in three-dimensional organotypic culture more effectively than fibroblasts from the reticular dermis (47, 53, 54). This may reflect differences in both secreted factors and ECM elements (55); however, as noted above, these observations are potentially confounded by selection inherent in explant cultures and by contamination with other fibroblast subpopulations, including pericytes.

More recently, we and others demonstrated the presence of functional dermal fibroblast subtypes in the mouse dermis. Using PDGFR α as a pan-fibroblast marker, lineage tracing experiments revealed that dermal fibroblast populations including

Additional fibroblast subtypes present within the dermis

Two separate populations of fibroblasts are associated with hair follicles (Figure 1). Hair follicle dermal papilla (FDP) fibroblasts play a central role in the development of hair and the coordination of the hair cycle (58, 59). Both primary and cultured FDP fibroblasts have the capability to induce the development of epithelial hair follicle elements at ectopic sites in the skin (60–63). In the mouse, dermal papillae are derived from a common fibroblast progenitor that is present at E12.5 (56). Lineage tracing of *Sox2-Cre* FDP fibroblasts reveals that these cells have only a minority contribution to wound healing (64). However, there is some evidence that in certain circumstances dermal papilla (FDP) fibroblasts can differentiate to other cell types, including nerve and cartilage (65). A second population of hair follicle-associated fibroblasts, dermal sheath (DS) fibroblasts, encapsulate the hair shaft external to epithelial components of the hair follicle, from which they are separated by a basement membrane termed the glassy membrane.

A number of markers have been described for human and mouse FDP and DS fibroblasts, including CD133 (in human and mouse) and nonspecific alkaline phosphatase (in human) (66–68). Marker expression changes according to stage of the hair cycle (69–72) and, in the mouse, depends on the hair follicle type, with

Sox2 expressed in all dermal papillae from E16.5 but confined to guard, awl, and auchene hairs from E18.5 (70). Variation has also been observed in the expression of markers for hair follicles according to anatomical site of origin; for example, the expression of androgen receptors is higher in regions of the scalp that are susceptible to male pattern hair loss (73).

Additional populations of fibroblasts within the dermis include pericytes, preadipocytes, and myofibroblasts. As discussed in detail above, pericytes are associated with and encircle vascular channels (34, 35). Wound healing in the adult organism is characterized by the formation of an ECM-rich scar that lacks normal cutaneous appendages such as hair and sweat glands (74). Wounding leads initially to the formation of a fibrin-rich plug, followed by the accumulation of leukocytes and the migration of α SMA⁺ myofibroblasts from adjacent dermis and underlying adipose tissue into the wound (75–78). Subsequently, the infiltrating α SMA⁺ fibroblasts develop a contractile phenotype (79). Lineage tracing in the mouse demonstrates that the initial wave of fibroblast migration is derived from lower-lineage (i.e., reticular) fibroblasts (56).

Preadipocytes are fibroblasts with the capacity to differentiate to adipocytes. These are enriched in the lower dermis (80). In humans, they express the CD36 cell surface marker (81). Lineage tracing of preadipocytes with Cre-labeled adiponectin reveals that preadipocytes proliferate and mature adipocytes migrate to contribute to wound repair (82). Preadipocytes are differentiated from other fibroblasts by the expression of ZFP423, a zinc finger transcription factor (83). Peroxisome proliferator-activated receptor gamma 2 (PPAR γ 2) is a master transcriptional regulator of the subsequent adipogenic differentiation program (84).

Interestingly, a recent study has demonstrated that myofibroblasts have the capacity to transdifferentiate to adipocytes in response to bone morphogenetic protein (BMP) signals arising from hair follicles in the healing wound (85), suggesting that preadipocytes are not the only cell type that can give rise to mature fat cells in the dermis.

Intrinsic mechanisms specifying fibroblast cellular identity

Cellular identity is determined both by intrinsic mechanisms, including transcriptional regulatory networks (86) and epigenetic processes, and by extrinsic factors, including cell-cell signaling, soluble signaling mediators, or ECM elements. A key role for intrinsic mechanisms in the specification of fibroblast identity is demonstrated by classic heterotopic transplantation experiments, which show an instructive role of mesenchyme in specifying the identity of overlying epithelial tissues. The culture of dental epithelium from chick embryos overlying dermal mesenchyme led to the development of epidermis including cutaneous appendages (87). Similar results were seen with the ability of wing mesenchyme to induce the development of feathers in heterotopic tissues (88).

Consistent with these findings, fibroblasts derived from palms and soles are able to induce the expression of keratin 9 in nonpalmoplantar keratinocytes, but this is not the case for fibroblasts derived from nonpalmoplantar sites (89). Furthermore, studies of hair transplantation demonstrate that follicles taken from the posterior scalp retain their developmental identity when transplanted

to anterior scalp (90), and FDP fibroblasts maintain hair induction capability for both mouse and human, at least for early passages in culture (71, 91, 92).

In most cases, the details of transcriptional regulatory networks governing specification of fibroblast subtypes are unknown. However, the identity of certain key transcriptional regulators has been established. Sox2 appears to play a key role in the specification of certain hair follicle types within the mouse dermis. Cre-mediated ablation of Sox2 in FDP cells leads to a reduction in awl/auchene hairs and an increase in zigzag hairs in mice (93). Conversely, expression of Sox18 is required for the formation of zigzag hairs (94). Interestingly, Sox2⁺ FDP fibroblasts maintain their identity in culture (71), consistent with intrinsic differences in cellular identity.

Extrinsic factors governing fibroblast cellular identity

Extrinsic factors governing cell fate include cell-cell contact, secreted signaling factors, and interactions with ECM elements. The exposure of a particular cell to these influences will depend on spatial context within the tissue. Coculture experiments reveal that keratinocytes instruct fibroblasts to produce a range of cytokines, including IL-6 and FGF7 (95, 96). IL-1 has been identified as an inducing factor in these coculture experiments (96, 97). Conversely, factors secreted by fibroblasts influence the growth and differentiation of keratinocytes, indicating reciprocal cell-cell interaction (98). Additionally, ECM context has an important impact on fibroblast cellular identity. Fibroblasts cultured as a monolayer exhibit differences in collagen expression and morphology in comparison with fibroblasts in three-dimensional culture. There are also differences observed between collagen and fibrin gels (99–101). An instructive role of ECM elements in cellular identity is supported by tissue engineering strategies in which the ECM of decellularized organs is repopulated with cells (102, 103). While the full details of these interactions remain to be discovered, it is possible that a dynamic interplay between ECM signaling and production is important in the maintenance of fibroblast cellular identity in tissues.

With regard to specific signaling pathways that are implicated in the regulation of fibroblast cell identity, the emerging picture is of a complex interplay of multiple signaling pathways that synergistically regulate fibroblast identity and function within the dermis. Preeminent among these is the Wnt pathway. Epidermal Wnt signaling is required for hair follicle morphogenesis (92, 104). Epidermal overexpression of the Wnt downstream effector β -catenin in all basal epidermal keratinocytes via the keratin 14 (*K14*) promoter leads to de novo hair follicle morphogenesis in adult skin; this is accompanied by extensive remodeling of the dermis ECM (105). These effects are mediated by both TGF- β and Hedgehog signaling (106) and influence the lower and upper dermal fibroblast lineages, respectively. Pharmacological deletion or genetic ablation of these pathways prevents the response to epidermal Wnt/ β -catenin overexpression (106).

Other signaling pathways, including secreted BMPs and insulin-like growth factors, play a role in mediating the effects of keratinocyte-specific β -catenin expression, leading to an increase in adipogenesis. Conversely, inhibition of Wnt/ β -catenin signal-

ing in the epidermis impairs adipogenesis (107). Interestingly, it appears that the consequences of Wnt/ β -catenin are cell type-specific. β -Catenin stabilization in dermal fibroblasts during development leads to fibrosis and impairs adipogenesis (108). Wnt/ β -catenin expression in the adult dermis inhibits de novo hair follicle formation in healing wounds, and postnatal ablation of β -catenin in dermal fibroblasts enhances new hair formation (109).

Additional signaling pathways that have been implicated in the regulation of dermal fibroblasts include the Notch pathway, which has a role in the regulation of inflammation and hair follicle morphogenesis (110, 111). TGF- β signaling also plays a key role in wound healing and scar formation (3). BMP signaling has been implicated in the regulation of hair follicle morphogenesis (112–114). Furthermore, BMP signaling is required for the transdifferentiation of myofibroblasts to adipocytes in healing wounds (85).

Dysregulation of Wnt signaling is observed in a number of conditions that are characterized by aberrant or excessive fibrosis. The profibrotic effects of TGF- β are mediated through canonical Wnt signaling via a decreased expression of the Wnt antagonist Dickkopf-1 (115). A role for Wnt signaling has also been seen in renal fibrosis (116), idiopathic pulmonary fibrosis (117), and the increased fibrosis that is observed to accompany muscle regeneration with aging (118).

Fibroblasts play a critical role in maintaining the structural integrity of the dermis. To fulfill this function, there must exist a mechanism for fibroblasts to adapt and respond to mechanical stress. The details of these processes are emerging. YAP signaling via the Hippo pathway has been implicated in the regulation of ECM stiffness (119). Furthermore, a role for focal adhesion kinase (FAK) signaling has been demonstrated in the response of wound repair to mechanical stress (22). FAK signaling is triggered by cutaneous injury and signals through ERK to induce the expression of monocyte chemoattractant protein-1 (MCP-1), a profibrotic chemokine.

Therapeutic implications

The emerging concept that mammalian tissues, such as the dermis, contain fibroblasts with differing functional identities has profound implications for the understanding of a wide range of pathological states, including diseases characterized by excessive fibrosis such as scarring, scleroderma, cancer, and potentially including chronological aging. It is likely that fibroblast subtypes identified in mice will be conserved in the human, and this is the subject of ongoing investigation.

In contrast to adult mammals, wounds in the fetus heal without scarring (120, 121). An understanding of the role of fibroblast subtypes in this process may suggest strategies to reduce scarring in adult organisms. Scar-free healing is observed up until 24 weeks of human gestation and is conserved across many mammalian species (121). Studies examining human fetal skin grafts to immunodeficient mice indicate that this is an intrinsic property of the fetal skin (122), possibly mediated by differences in the fibroblast subtypes present. Interestingly, epidermal activation of canonical Wnt signaling in the basal layer of mouse epidermis leads to remodeling of the adult dermis to a neonatal-like state with formation of new hair follicles (105). Wnt signaling is also required for regeneration of nails and digits in neonatal mice (29, 123) and may contribute to differences in wound healing between the fetus and adult.

The involvement of specific fibroblast lineages may have particular relevance to diseases characterized by excessive fibrosis and scarring such as scleroderma, keloids, graft-versus-host disease, and pulmonary fibrosis. Keloidal scarring is characterized by excessive fibroblast proliferation and ECM deposition (124, 125), and keloid-derived fibroblasts exhibit differences in gene expression and behavior in culture compared with fibroblasts from healthy dermis (126, 127). It has been proposed that these disease-associated alterations in fibroblast behavior reflect epigenetic alterations in gene expression patterns, and in keeping with this, fibroblasts in rheumatoid arthritis exhibit differences in DNA methylation and histone acetylation (128). Fibroblasts derived from patients with scleroderma exhibited differences in methylation of the *FLII* promoter (129). Epigenetic differences were also observed between keloidal and control fibroblasts (130). Histone deacetylase inhibitors were able to normalize to some extent the differences in gene expression seen with fibroblasts derived from the airways of patients with severe pulmonary hypertension (131).

Epithelial tumors consist of a mixture of epithelial elements, fibroblasts, and ECM components. Cancer-associated fibroblasts (CAFs) play an important role in evolution of solid tumors. There is an extensive crosstalk between CAFs and the epithelial elements of tumors (132), wherein fibroblasts associated with later stages of malignancy exhibit different phenotypes. Interestingly, CAFs exhibit an increased expression of α SMA (1), which may reflect alterations in the composition of fibroblast subtypes. YAP signaling as a consequence of mechanical stress also plays an important role in remodeling of the ECM that accompanies carcinogenesis. There is evidence for heterogeneity of CAFs (133), but the extent to which these differences reflect fibroblast subtypes present in normal tissue remains to be determined, and further characterization of fibroblast subtypes may increase our understanding of these questions.

Aging is associated with characteristic changes in the structure of the dermis, including dermal thinning and flattening of the dermal-epidermal junction, with loss of the normal rete ridges (134, 135). Interestingly, this appears to preferentially affect the papillary dermis: alterations in the behavior of papillary dermal fibroblasts derived from older donors have been observed in three-dimensional cultures (136). Changes are also prominent in other components of connective tissue such as the large blood vessels. In the skin, proliferation of fibroblasts occurs during mouse embryonic development; however, subsequent growth of the animal is not associated with further fibroblast proliferation and is instead achieved simply by an increase in the ECM volume between adjacent fibroblasts (109). This is consistent with the observation that the rate of fibroblast division in the adult dermis is very low in the steady state (137). It may be that during aging this relative paucity of fibroblasts in the adult is insufficient to maintain the structural integrity of the ECM. Alternatively, it may be that there are age-associated alterations in the composition of fibroblast subtypes within the dermis.

A goal of regenerative medicine is the restoration of normal form and function either via cellular therapies in vivo or by the reconstitution of functional organs ex vivo. Efforts to accomplish the latter have led to attempts to reconstitute intact organs via the

reintroduction of cellular populations to decellularized ECM templates (102, 103). This has proven challenging, and future efforts may be informed by a better understanding of the fibroblast subtypes present in these tissues. There have also been attempts to prepare organotypic skin substitutes comprising both fibroblasts and keratinocytes for grafting onto chronic wounds or other sites (138). It is recognized that, *in vitro*, fibroblasts derived from the upper dermis better support the formation of a structurally and functionally normal epidermis (47, 53, 54), and it is likely that an understanding of fibroblast subtypes present in human dermis will facilitate the prospective isolation of cells for the repopulation of dermal templates of this nature.

A detailed characterization of fibroblast subtypes in the human in combination with an understanding of the differential contribution of these subtypes to human disease may offer novel strategies for the treatment of diseases. In this context, it could be envisaged that the action of deleterious fibroblast subtypes could be inhibited, perhaps via inhibition of signaling pathways specific to these subtypes or via monoclonal antibody-mediated deletion. In support of this concept, experimentally reducing the number of fibroblasts during wound healing in the mouse can reduce the degree of fibrosis (38). Alternatively, beneficial subtypes may be expanded *ex vivo*, perhaps via manipulation of cell signaling pathways. Trials of fibroblast cell therapy are already under way for the treatment of poorly healing ulcers (139) and epidermolysis bullosa (140). Notably, papillary fibroblasts appear to be more effective when used in the construction of tissue-engineered skin substitutes (141).

Conclusions and outstanding questions

The emerging picture from the research outlined in this Review is that fibroblasts represent a family of related cell types with differing functions in the formation and maintenance of ECM in connective tissues and play a key role in coordinating the function of other cell types within the tissue. The understanding of fibroblast heterogeneity is most advanced for the mammalian dermis, because this is a particularly tractable tissue for experimental studies. However, it is probable that these principles are common to other vertebrate tissues including the intestine, lung, heart, and muscle.

Many details of the mechanisms that establish and maintain vertebrate cellular identity remain unclear, and the outstanding questions in the field of fibroblast cell biology reflect this. It is presently unclear to what extent differences in fibroblast cellular identity are specified intrinsically versus reflecting a consequence of spatial context within the tissue such as cell-cell contacts and signaling. Intrinsically specified differences in fibroblast functional identity likely reflect a combination of transcriptional regulatory networks with epigenetic mechanisms. At present, in most cases we do not know the full details of the transcriptional regulatory networks that dictate the identity of fibroblast subtypes.

The importance of extrinsic signals in the maintenance of cellular identity is of particular relevance to the *ex vivo* expansion of fibroblasts in culture. There is evidence that fibroblasts exhibit a significant shift in the expression of cell surface markers in the transition to cell culture (142). This may reflect an influence of extrinsic signals that are required for the maintenance of fibroblast cell identity, which will be lost on transition to culture. Furthermore, since minimal fibroblast cell division occurs *in vivo*, it may be that the powerful mitogenic stimuli encountered in cell culture lead to dramatic alterations in cellular identity. These issues are of considerable relevance to efforts to develop fibroblast therapies, particularly as *ex vivo* strategies will have a higher complexity for regulatory approval and likely a higher cost. Furthermore, they are important for the study of human disease, since many studies have sought to infer disease mechanisms through the study of cultured patient-derived fibroblasts.

Another unresolved question is the degree of plasticity that exists in fibroblast cell identity — can one type of fibroblast differentiate to another given the appropriate stimuli, or is there a hierarchy of fibroblasts exhibiting progressively restricted differentiation potential? To what extent do mesenchymal stem/stromal cells correspond to fibroblast subtypes present elsewhere in the body, and do they have the capacity to differentiate into other fibroblast subtypes and incorporate into tissues?

It is not clear to what extent fibroblast subtypes identified in the mouse dermis are conserved in the human. To understand the role that fibroblast subtypes play in human disease, markers that permit subtype identification in human histological sections and permit the prospective isolation of these subtypes by flow cytometry will be required. Previous studies of human dermis have attempted to define markers that distinguish fibroblasts cultured separately from the papillary and reticular dermis (47). However, this approach has not yielded cell surface markers that permit the prospective isolation of fibroblast subpopulations directly from the skin. These issues are areas of active investigation, and it is likely that recent advancements in the area of single-cell-based transcriptional and epigenetic analysis will catalyze a greater understanding.

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