

# Fibrocytes, Wound Healing, and Corneal Fibrosis

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**PURPOSE.** This review highlights the roles of fibrocytes—their origin, markers, regulation and functions—including contributions to corneal wound healing and fibrosis.

**METHODS.** Literature review.

**RESULTS.** Peripheral blood fibroblast-like cells, called fibrocytes, are primarily generated as mature collagen-producing cells in the bone marrow. They are likely derived from the myeloid lineage, although the exact precursor remains unknown. Fibrocytes are identified by a combination of expressed markers, such as simultaneous expression of CD34 or CD45 or CD11b and collagen type I or collagen type III. Fibrocytes migrate into the wound from the blood where they participate in pathogen clearance, tissue regeneration, wound closure and angiogenesis. Transforming growth factor beta 1 (TGF- $\beta_1$ ) and adiponectin induce expression of  $\alpha$ -smooth muscle actin and extracellular matrix proteins through activation of Smad3 and adenosine monophosphate-activated protein kinase pathways, respectively. Fibrocytes are important contributors to the cornea wound healing response and there are several mechanisms through which fibrocytes contribute to fibrosis in the cornea and other organs, such as their differentiation into myofibroblasts, production of matrix metalloproteinase, secretion of tissue inhibitor of metalloproteinase, and release of TGF- $\beta_1$ . In some tissues, fibrocytes may also contribute to the basement membrane regeneration and to the resolution of fibrosis.

**CONCLUSIONS.** New methods that block fibrocyte generation, fibrocyte migration, and their differentiation into myofibroblasts, as well as their production of matrix metalloproteinases, tissue inhibitor of metalloproteinase, and TGF- $\beta_1$ , have therapeutic potential to reduce the accumulation of collagens, maintain tissue integrity and retard or prevent the development of fibrosis.

**Keywords:** fibrocytes, fibrosis, corneal stroma, transforming growth factor beta, myofibroblasts

Current medical management of corneal scarring after injury, infection or surgery relies on the application of topical corticosteroids and, in some situations topical mitomycin C, to minimize the corneal scarring response.<sup>1</sup> However, these drugs often have limited efficacy and have been associated with long-term complications, such as glaucoma, cataract formation, and corneoscleral melting.<sup>1</sup> When these medications fail and vision compromising corneal scarring occurs, the treatment options are surgical procedures such as phototherapeutic keratectomy, lamellar keratoplasty, or penetrating keratoplasty. Corneal transplants require tissue donors and long-term medical care and may end in graft rejection. Therefore more effective approaches to prevent or ameliorate corneal fibrosis are needed.

The wound healing response to injury (traumatic, infectious, or surgical) involves a complex cascade of events that aims to rapidly repair the damaged tissue.<sup>2,3</sup> Myofibroblasts are a heterogeneous population of cell types that have distinct functions throughout the repair process, including

the deposition of Extracellular matrix (ECM) components, which when excessive may result in organ fibrosis and dysfunction.<sup>4,5</sup>

The concept that myofibroblasts may originate from different sources goes back many decades. However, in 1994 Bucala and his colleagues<sup>6</sup> describe a population of bone marrow-derived, fibroblast-like cells circulating in the blood that have the capacity to enter into injured tissues and differentiate into myofibroblasts. Those authors coined the term “fibrocytes” in recognition of these cells’ collagen production (fibro-) and their discovery that they circulate in the blood (-cyte).<sup>6,7</sup>

More recently, fibrocytes have been defined as spindle-shaped cells with oval nuclei, that express markers of hematopoietic progenitor cells, leukocytes and mesenchymal cells,<sup>8</sup> and that contribute to several functions involved in the healing response and angiogenesis<sup>9,10</sup> that can progress to pathological fibrosis in organs such as the lung,<sup>11</sup> liver,<sup>12</sup> kidney,<sup>13,14</sup> heart,<sup>15</sup> blood vessel,<sup>16</sup> skin,<sup>17</sup> and eyes.<sup>18</sup>

These bone marrow–derived cells have recently become a focus of corneal wound healing in the cornea and likely contribute to fibrotic processes in the retina, vitreous, and other structures in the eye.

This review will summarize what is currently known about the origin of fibrocytes in several different organs, their cellular characteristics, the molecular mechanisms underlying their recruitment to wounds, their differentiation into myofibroblasts, and the potential role of fibrocytes in corneal healing and fibrosis. It is important to keep in mind that some characteristics of fibrocytes discussed could be tissue or species specific.

## FIBROCYTE ORIGIN

It is widely accepted that fibrocytes arise from bone marrow cells. Lassance et al.,<sup>18</sup> demonstrate, using green fluorescent protein (GFP) chimeric mice, that cells derived from bone marrow give rise to fibrocytes that enter the cornea after injury. Similarly, in an experiment using sex-mismatched bone marrow chimeric mice (female), Mori et al.<sup>17</sup> found the Y chromosome (from transplanted male bone marrow) in the nuclei of the majority of fibrocytes. Although these and many other studies provide direct evidence that fibrocytes arise from bone marrow-derived cells, the exact progenitor cell to fibrocytes remains unknown.

Bone marrow consists of stroma and stem cells, which are single cells with the ability to self-renew, as well as to develop into multipotent progenitor cells that subsequently generate different mature cell types. There are 2 types of stem cells in the bone marrow: hematopoietic stem cells (HSCs), and mesenchymal stem cells (MSCs). The former contribute to the formation of hematopoietic,<sup>19</sup> as well non-hematopoietic,<sup>12</sup> mature cells. The latter are involved in producing mesenchymal tissue lineages and have the potential to differentiate into many cells associated with bone, cartilage, tendon, muscle, fat, and bone marrow stroma.<sup>20,21</sup>

Russo et al.<sup>12</sup> show that both MSCs and HSCs serve as bone marrow sources of hepatic myofibroblasts, and interestingly, MSCs appear to be the predominant source of myofibroblasts in the injured liver (53% of myofibroblasts arose from MSCs vs. only 8.2% from HSCs). Although MSCs give rise to myofibroblasts, they do not appear to be precursor cells to fibrocytes because circulating or tissue-resident mesenchymal stem cells and mesenchymal progenitor cells do not express CD34 (hematopoietic progenitor cell marker) or CD45 (leukocyte common marker), which are widely accepted as the most important markers for identifying fibrocytes.<sup>22</sup> Moreover, although the human MSCs residing in the bone marrow express CD34, CD45, and CXCR4 to varying degrees, they remain negative for the monocyte markers of fibrocytes.<sup>20,23</sup>

Conversely, there is strong evidence supporting fibrocyte origin from hematopoietic progenitor cells. Fibrocytes, as well as pluripotent and multipotent human hematopoietic cells, express the CD34 marker.<sup>6</sup> The CD34 marker has been also reported to be expressed by capillary endothelium,<sup>6</sup> but the absence of von Willebrand antigen in fibrocytes argue against their endothelial origin.<sup>8</sup> Fibrocytes also express the pan-myeloid antigen marker CD13, reinforcing the theory that hematopoietic progenitor cells (likely myeloid progenitor cells) are the source of fibrocytes rather than MSCs.<sup>24,25</sup> Varcoe et al.<sup>16</sup> show that circulating labeled leukocytes infiltrate tissue injured *in vivo* and progressively acquire the fibrocyte phenotype (CD34<sup>+</sup>CD45<sup>+</sup>vimentin<sup>+</sup>),

which further supports the hypothesis that fibrocytes arise from hematopoietic cells.

Regarding the myeloid hypothesis of fibrocyte origin; fibrocytes appear to originate from either a common progenitor cell shared between monocytes and fibrocytes, or they arise directly from monocytes. *In vitro* experiments demonstrate that either peripheral blood mononuclear cells (PBMC) or purified CD14<sup>+</sup> cells (likely monocytes) can give rise to fibrocytes.<sup>8,9,26–28</sup> Some evidence supports the hypothesis that there is more than one progenitor cell for fibrocytes. Curnow et al.<sup>9</sup> show that PBMCs and purified CD14<sup>+</sup> cells each have a unique differentiation response to the presence or absence of serum, resulting in 2 different types of fibrocytes. Phillips et al.<sup>11</sup> also show that there are distinct fibrocyte populations, and therefore they could arise from different precursor cells.

Some evidence, however, argues against the monocyte origin of fibrocytes. Bucala et al.<sup>6</sup> show that fibrocytes do not express nonspecific esterases that are expressed in monocytes. In the process of monocytes' maturation from monoblasts to promonocytes, CD34 expression is lost.<sup>29</sup> Monocytes are therefore CD34<sup>–</sup> cells, distinguishing them from fibrocytes. In addition, fibrocytes are considered by most investigators to be CD14<sup>–</sup> cells, whereas monocytes express the CD14 marker.<sup>27</sup>

Although these studies in general discredit the theory of fibrocyte origin from monocytes, it cannot be totally excluded. Monocytes could differentiate into fibrocytes and regain expression of CD34 while losing CD14 expression. This process parallels the one found in the differentiation from classical monocytes (proinflammatory phenotype) into nonclassical monocytes (prohealing phenotype), where nonclassical monocytes re-express CD16 while decreasing CD14 expression.<sup>30</sup> Fibrocytes also express other monocyte markers such as CD11a, CD11b, CD32, and CD64, reinforcing their possible origin from monocytes.<sup>25</sup> It is well known that macrophages originate from monocytes, and Hashimoto et al.<sup>31</sup> show that both macrophages and fibrocytes express Hck and Lyn—Src family kinases—the expression of which is often cell lineage specific.

Identification of the location where fibrocyte differentiation occurs is as important as identification of the progenitor cell(s) responsible for the creation of fibrocytes. Fibrocyte differentiation has been reported to predominantly occur within the bone marrow, although there is support for other sites. Thus Phillips et al.<sup>11</sup> demonstrate a dramatic increase in the number of fibrocytes in the bone marrow after induction of murine lung fibrosis, suggesting that the bone marrow is the primary site of differentiation of fibrocytes. Assuming that monocytes are the precursor cells of fibrocytes, Reich et al.<sup>14</sup> studied the localization of monocyte-to-fibrocytes differentiation. These authors show that inhibiting monocytes migration from the bone marrow after unilateral ureteral obstruction in a mouse model does not reduce the number of circulating fibrocytes. They conclude that fibrocytes migrate from bone marrow as predifferentiated cells—reinforcing that bone marrow is the primary location of fibrocytes differentiation.<sup>14</sup> Monocyte differentiation into fibrocytes has been found to take approximately 10 days *in vitro*, whereas fibrocytes can be found by 1 day after epithelial-stromal injury to the cornea,<sup>18</sup> suggesting that either local differentiation of fibrocytes from monocytes takes a shorter amount of time or they are present in the blood ready to respond to injuries by moving into tissues and

differentiating into myofibroblasts when appropriate signals are delivered.

In addition to fibrocyte differentiation in the bone marrow, there is some evidence also to support extramedullary differentiation of fibrocytes. Hematopoietic progenitor cells recirculate between peripheral blood and bone marrow,<sup>19</sup> and *in vitro* cultivation of blood cells has been shown to give rise to fibrocytes.<sup>27,28</sup> Thus the possibility of fibrocytes precursor cells being present in the bloodstream and locally differentiating into fibrocytes is feasible. Moreover, Varcoe et al.<sup>16</sup> use labeled hematopoietic cells to show that circulating leukocytes can infiltrate the wound site *in vivo* and differentiate into fibrocytes.

Serum amyloid P (SAP) is an inhibitor of fibrocyte differentiation present at wound sites and in the bloodstream that impairs the extramedullary differentiation of fibrocytes.<sup>32</sup> In disorders associated with high circulating levels of TGF- $\beta_1$  (a strong promoter of fibrocyte differentiation)<sup>33</sup> or with low levels of SAP,<sup>32</sup> fibrocyte differentiation within the blood circulation is facilitated. In addition, wounded tissues undergoing repair have been found to have a progressive reduction in levels of SAP over time, enabling local infiltrating leukocytes to differentiate toward the fibrocyte phenotype.<sup>32</sup> These findings suggest that although fibrocyte differentiation likely occurs predominantly in the bone marrow, fibrocytes may also be generated within the circulating blood or within the injured tissue. A potential model showing fibrocyte precursors present in the bone marrow or blood, and subsequent differentiation into fibrocytes prior to migration into the wounded tissue or, alternatively, fibrocyte precursors may directly migrate to the wounded tissue and undergo local differentiation is illustrated in [Figure 1](#).

In summary, the predominance of evidence supports the hypothesis that fibrocytes are primarily generated as mature collagen-producing cells in the bone marrow and are likely derived from the myeloid lineage. However, the identity of the specific bone marrow-derived cell that give rise to fibrocytes remains uncertain. Under certain conditions, such as low serum levels of SAP and high circulating levels of TGF- $\beta_1$ , fibrocytes may also originate in the bloodstream. Finally, during the wound healing response in some tissues, fibrocytes may also locally differentiate from monocytes.

## FIBROCYTES MARKERS

During the hematopoietic maturation process for fibrocytes from their progenitor cells, there are changes in the pattern of gene expression for marker proteins expressed on the cell surface, which facilitates identification of intermediate cell populations.<sup>29</sup> Unfortunately, there is no single specific standardized marker for mature fibrocytes. Most studies have discriminated fibrocytes from other cells based on their expression of some combination of cell surface markers related to hematopoietic stem/progenitor cells (CD34), leukocytes (CD45RO and leukocyte-specific protein-1), monocytes (CD11a, CD11b, CD32 and CD64), and fibroblasts (collagen type I, collagen type III, fibronectin, and vimentin).<sup>6–8,27,34</sup>

Although fibrocytes do express some monocyte markers, they are negative for nonspecific esterases, as well as the monocyte/macrophage-specific markers CD14 and CD16. Also, unlike monocytes, fibrocytes produce collagen type I and collagen type III and express CD34.<sup>24,27,35</sup> Fibrocytes can also be distinguished from dendritic cells because they do not express typical surface proteins of dendritic cells

or their precursors (such as CD1a, CD10 and CD83).<sup>27</sup> In addition, fibrocytes do not express the B-cell marker CD19 or T-cell markers CD3, CD4, CD8, CD25 or CD56.<sup>25</sup> Fibroblasts express stromal markers similar to fibrocytes; however, fibroblasts do not express the hematopoietic markers found on fibrocytes.<sup>27</sup>

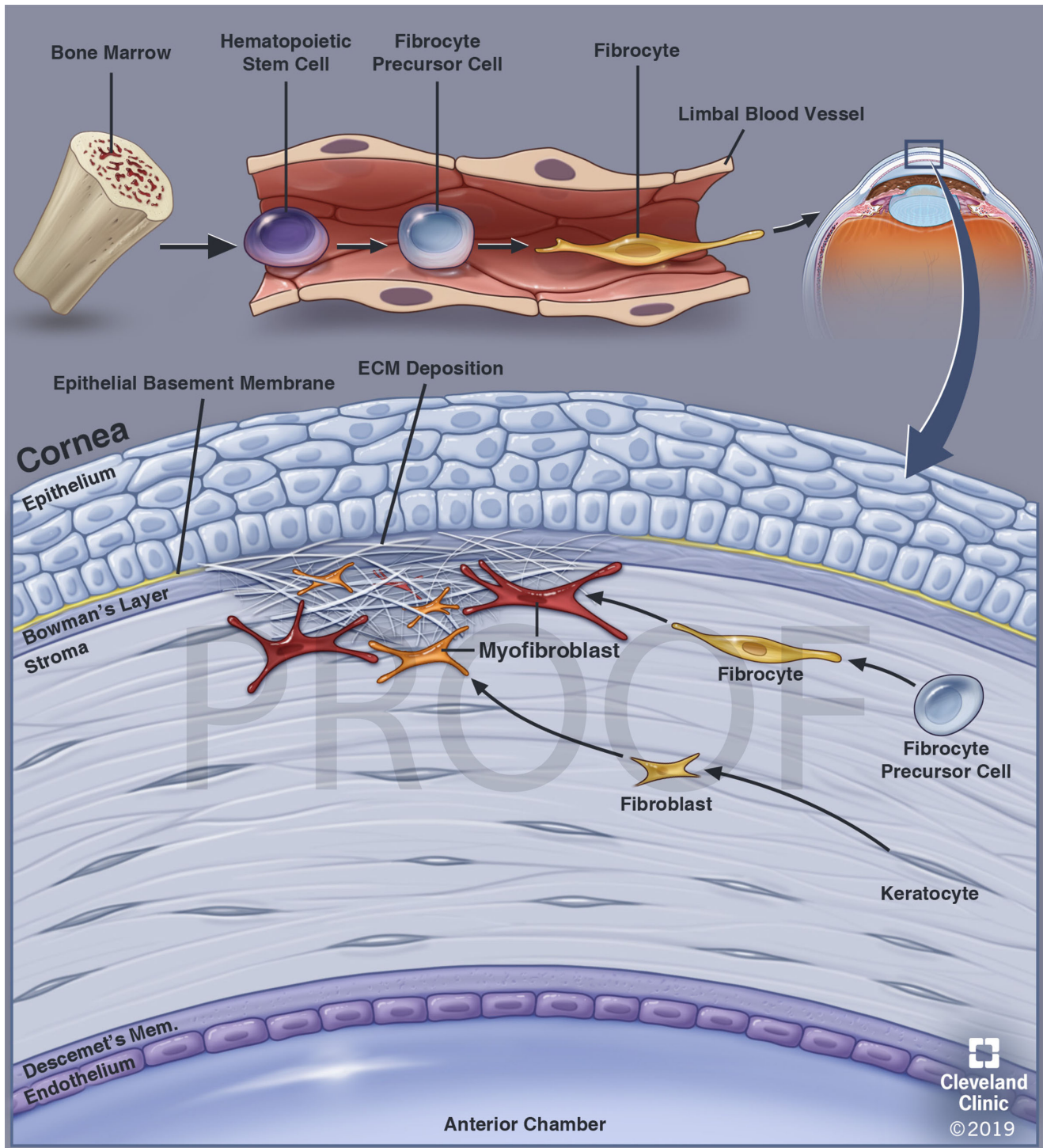
Fibrocytes also express the chemokine receptors CCR2, CCR3, CCR5, CCR7, CXCR4, CXCR6, and CX3CR1, host defense Fc receptors for immunoglobulin G (CD32 and CD64), antigen presentation and costimulatory molecules of lymphocyte activation (major histocompatibility complex [MHC] class I and II/CD80 and CD86), and cell surface enzymes (CD10 and CD13).<sup>24,25,27,36–38</sup> The complete list of fibrocytes markers is presented in [Table 1](#).<sup>8,24,25,39</sup>

In the majority of studies reported, fibrocytes were identified by a combination of expressed markers, for example, the simultaneous expression of CD34 or CD45 or CD11b and collagen type I or collagen type III.<sup>7,26,38,40</sup> In other studies, the combination of CD34 or CXCR4 and CD45 or LSP-1 plus intracellular collagen (type I or type III) has also been considered an accurate criterion to identify fibrocytes.<sup>8</sup>

Pilling et al.<sup>8</sup> warn that, even using these combination of markers, fibrocytes may not be fully distinguished from other cell types because macrophages may also express CD34 and produce or ingest collagen.<sup>8</sup> Vimentin, another marker used to identify fibrocytes, is also expressed by macrophages.<sup>9</sup> Distinction of fibrocytes from other hematopoietic cells is likewise problematic, especially in fibrotic tissues where there are high levels of extracellular collagen, because fibrocytes produce rather low levels of collagen.<sup>41</sup> In addition, the use of CD34, CD45, or CXCR4 for identifying fibrocytes can lead to an underestimation of fibrocyte numbers because these cells are known to show progressive loss of CD34 (earlier) and CD45 (later), as well as CXCR4, as they differentiate into mature myofibroblasts.<sup>7,9,11,17,42</sup> Furthermore, the distinction of myofibroblasts that arose from fibrocytes from myofibroblasts that arose from resident fibroblast may also be difficult using CD34, CD45 or CXCR4 markers because fibrocytes may lose expression of 1 or more of these markers during their differentiation into myofibroblast<sup>7,9,11,17,42</sup>, although retention of CD45 has been noted in at least some myofibroblasts in corneal fibrosis.<sup>43</sup> Pilling et al.<sup>8</sup> suggest that by using DAPI, PM-2K, CD32 and collagen antibodies, fibrocytes may be correctly discriminated from macrophages (PM-2K and CD32 positive) and from fibroblasts (collagen positive but PM-2K and CD32 negative) because of fibrocyte expression of CD32 and collagen and absence of PM-2K. They also have shown that CD49c can be used to differentiate early fibrocytes (CD49c negative) from late-differentiating fibrocytes (CD49c positive).<sup>8</sup> These difficulties in discriminating fibrocytes can generate conflicting reports regarding fibrocyte involvement in wound healing and fibrosis in different organs, but given current knowledge, this, at least to some extent, is unavoidable. A better consensus about a precise combination of markers for distinguish fibrocytes remains an elusive goal.

## FACTORS THAT PROMOTE AND INHIBIT FIBROCYTE DIFFERENTIATION

Several factors have been reported to promote or inhibit fibrocyte differentiation, including multiple cytokines, growth factors, immunoglobulin and other substances.

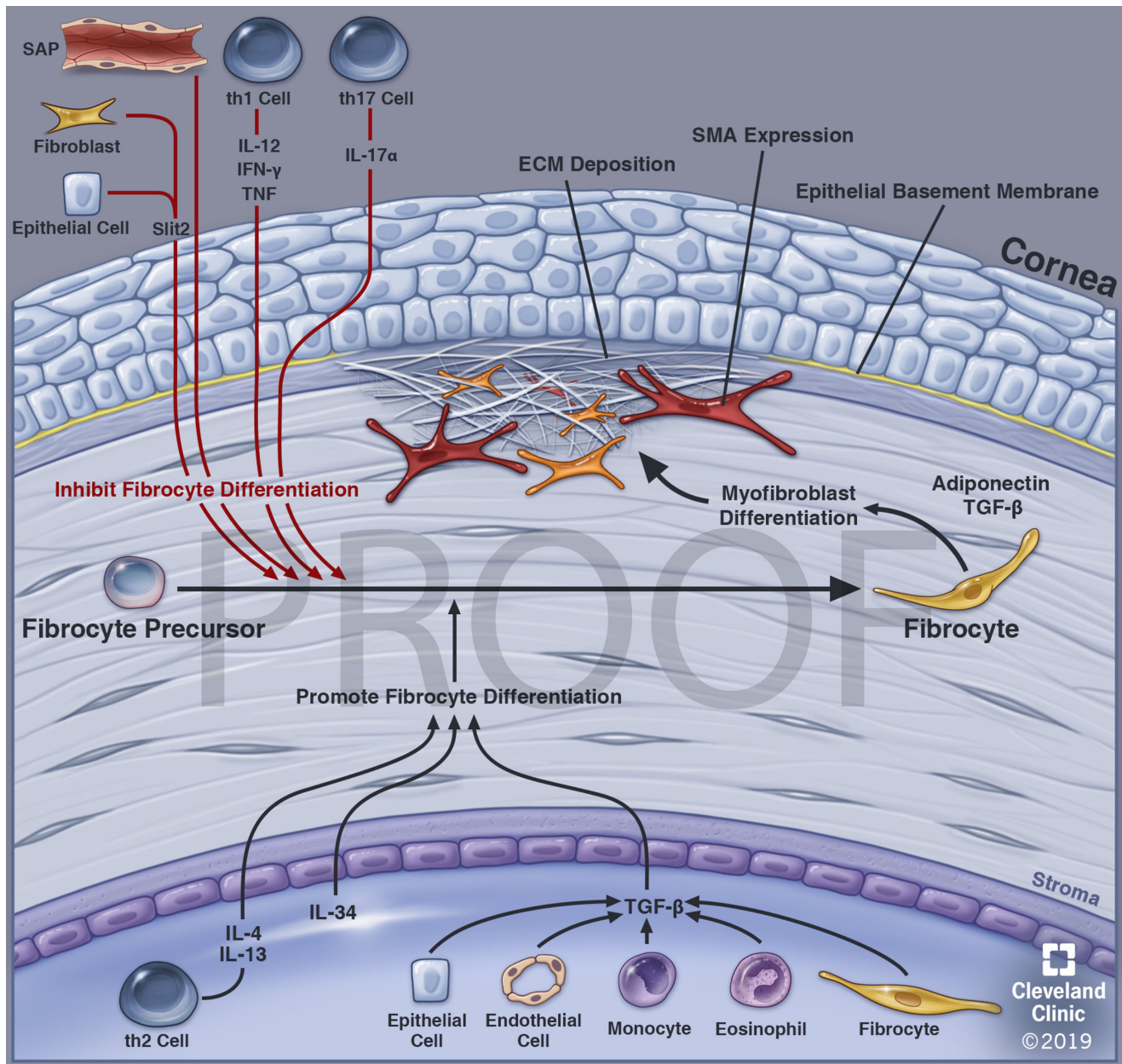


**FIGURE 1.** Schematic diagram showing the 2 best characterized precursors to myofibroblasts in the cornea. Bone marrow–derived hematopoietic stem cells give rise to fibrocyte precursors that develop into fibrocytes primarily within the bone marrow, but fibrocyte precursors in the blood and in the cornea can locally differentiate into fibrocytes and undergo TGF- $\beta_1$ -modulated development into myofibroblasts. Keratocytes develop into corneal fibroblasts and similarly undergo TGF- $\beta_1$ - and PDGF-modulated development into myofibroblasts. Illustration by Brandon Stelter. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2019. All Rights Reserved.

These factors act to promote or inhibit fibrocyte differentiation (Fig. 2).

T-cells are well-characterized regulators of bone marrow-derived fibrocyte activation.<sup>26</sup> Naïve CD4<sup>+</sup> T-cells can differentiate into T helper (Th) 1, Th2 or Th17 cells—

which are characterized and distinguished based on their functions and signature cytokine profiles.<sup>44</sup> Th1 cells produce Th1 cytokines such as interleukin-12, interferon gamma, and tumor necrosis factor (TNF)—all of which promote classical activation of macrophages, contributing to



**FIGURE 2.** Schematic diagram showing key modulators that promote and inhibit the development of fibrocyte precursors into fibrocytes. TGF- $\beta_1$  produced by corneal epithelial cells and endothelial cells, as well as immune cells, and present in aqueous humor, drives the development of SMA+ myofibroblasts from fibrocytes when TGF- $\beta_1$  levels are sufficiently high in the stroma after injuries. Illustration by Brandon Stelter. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2019. All Rights Reserved.

inflammation and elimination of intracellular pathogens,<sup>13,45,46</sup> but inhibit fibrocyte differentiation. Th17 cytokines, such as IL-17A, also have been shown to inhibit fibrocyte differentiation.<sup>24</sup> In contrast, Th2 cells produce cytokines such as IL-4 and IL-13, both of which induce alternative activation of macrophages and promote the monocyte-to-fibroblast transition.<sup>13,45,46</sup> Niedermeier et al.<sup>26</sup> suggest that the interaction between Th2 cytokines and fibrocytes precursor cells occurs via a mTOR-PI3 kinase dependent pathway. Zhong et al.<sup>36</sup> demonstrate that IL-4 facilitates the *in vitro* differentiation of fibrocytes by increasing the activity and expression of store-operated Ca<sup>2+</sup> entry (SOCE) channels. In their studies, blockage of

SOCE channels with SKF-96365 significantly inhibits IL-4-induced differentiation of fibrocytes.<sup>36</sup> Yan et al.<sup>47</sup> show that Th2 cytokines induce the JAK3/STAT6 signaling pathway and stimulate bone marrow-derived fibroblast activation, which has been shown to play a role in the development of interstitial renal fibrosis. These authors further confirm the role of STAT6 signaling in myeloid fibroblast activation by using CP690550 (a JAK3-specific inhibitor) and STAT6 deficiency in bone marrow chimeric experiments.<sup>47</sup>

IL-34 is a newly identified cytokine that activates the colony-stimulating factor-1 receptor (CSF-1R).<sup>48</sup> Galligan and Fish<sup>49</sup> show that *in vitro* fibrocyte precursors treated with IL-34 induce the proliferation of fibrocytes—mediated by the

TABLE 1. Markers Expressed by Fibrocytes

Marker	Expression
Stem cell/progenitor markers	
CD34	++
CD105	++
Leukocyte markers	
CD45RO	++
LSP-1	++
CD90	–
Monocyte markers	
CD11a	++
CD11b	++
CD11c	++
CD13	++
CD14	+/-
CD16	+/-
CD32	++
CD64	++
Macrophage markers	
25F9	+
S100A8/A9	+
PM-2K	–
CD163	+/-
CD206	+/-
Dendritic cell markers	
CD1a	–
CD10	–
CD83	–
B-cell markers	
CD19	–
T-cell markers	
CD3	–
CD4	–
CD8	–
CD25	–
CD56	–
MHC molecules	
Class I	++
Class II	++
Co-stimulatory molecules	
CD40	+
CD80	+
CD86	++
Integrins	
CD18	++
CD29	++
CD49a	+
CD49b	++
CD49c	–
CD49d	+/-
CD49e	++
CD49f	–
CD61	++
CD103	–
$\alpha 4\beta 7$	–
Chemokine receptors	
CCR1, CCR3, CCR4, CCR5, CCR7, CCR9, CXCR1, CXCR3, CXCR4, CX3CR1	+ / ++
CCR2, CXCR6	+ / -
Cell enzymes	
CD10	++
CD13	++
CD172a	++
FAP	++
Prolyl-4-hydroxylase	++
MMP-1	–

TABLE 1. Continued

Marker	Expression
MMP-2	++
MMP-7	+
MMP-8	++
MMP-9	++
ECM proteins	
Collagen I/III/IV	+
Collagen V	++
Vimentin	+
Fibronectin	+/-
Glycosaminoglycans	
Perlecan	++
Versican	++
Hyaluronan	++
Decorin	+

The symbols represent no expression (–), high or increasing level of expression (++), (+), and conflicting reports of expression (+/-). Based on published data.<sup>8,24,25,39</sup> LSP-1, lymphocyte-specific protein 1; S100A8/A9, calprotectin (heterocomplex of the two S100 calcium binding proteins, S100A8 and S100A9); CCR, CC-chemokine receptor; CXCR, CXC-chemokine receptor; FAP, fibroblast activation protein.

activation of cognate CSF-R1s on the fibrocytes. Moreover, they demonstrate that rheumatoid arthritis patients have elevated levels of IL-34, which are independent predictors of radiographic progression of the disease and simultaneously increase the numbers of fibrocytes in the bloodstream. These findings suggest that IL-34 contributes to fibrocyte proliferation in the development of rheumatoid arthritis and may also play a role in the pathogenesis of other fibrotic diseases.<sup>49</sup>

TGF- $\beta_1$  is likely the most important fibrogenic and growth-regulating cytokine involved in wound healing in many organs. It is well established that TGF- $\beta_1$  promotes the differentiation of fibrocytes toward the myofibroblast phenotype, including expression of alpha-smooth muscle actin ( $\alpha$ -SMA), along with stimulation by endothelin-1 (ET-1).<sup>27,50</sup> No expression of  $\alpha$ -SMA is found in freshly purified fibrocytes<sup>6,11</sup>, but Mori et al.<sup>17</sup> note that in skin 61.4% of fibrocytes become  $\alpha$ -SMA positive 7 days after wounding. This new maturing cell population down-regulates the expression of CD34, CD45, and CXCR4,<sup>7,9,11,17,42</sup> while producing much more collagen and fibronectin than the fibrocyte precursors.<sup>50</sup> Mori et al.<sup>17</sup> also find that only 21% of myofibroblasts that originated from fibrocytes remain positive for CD34 at day 7 after injury in skin. Several cell types produce TGF- $\beta_1$ —such as monocytes, macrophages, eosinophil, epithelial cells, endothelial cells—as well as fibrocytes themselves.<sup>25</sup> TGF- $\beta_1$  is secreted in its latent form bound to a latency-associated peptide.<sup>42</sup> Once secreted, this complex interacts with other proteins termed *latent TGF- $\beta$  binding proteins*, and the complex is anchored in the ECM.<sup>42</sup> Proteolytic cleavage of latency-associated peptide, latent TGF- $\beta$  binding proteins, or ECM proteins by a number of proteases, including MMP-9, appears to be a key to the release of TGF- $\beta_1$ .<sup>42</sup> Chiang et al.<sup>42</sup> show that R1R2, a peptide that reduces the proteolytic activity of MMP-9, prevents fibrocyte differentiation to myofibroblasts by blocking TGF- $\beta_1$  activation mediated by MMP-9. Yang et al.<sup>47</sup> use in vitro and in vivo techniques to examine how TGF- $\beta_1$  induces the expression of  $\alpha$ -SMA and ECM proteins by fibrocytes. Their results indicate that bone marrow-derived

fibroblast activation occurs by the Smad3 stimulation pathway.<sup>47</sup> However, Smad3 deficiency does not completely abolish fibrocytes activation and ECM protein expression *in vivo*. This suggests that other factors may be involved in the activation of fibrocytes by TGF- $\beta$ <sub>1</sub>.<sup>47</sup>

Yang et al.<sup>51</sup> suggest that adiponectin, an adipocyte-specific protein, is a critical regulator of monocyte-to-fibroblast transition in renal fibrosis. In their studies, genetic deletion or deficiency of adiponectin reduce the expression of profibrotic chemokines and cytokines, bone marrow-derived fibroblast accumulation, myofibroblast activation, and the production of ECM protein in the kidney after obstructive injury. Adiponectin is found to stimulate  $\alpha$ -SMA and ECM protein expression in bone marrow-derived monocytes via activation of adenosine monophosphate-activated protein kinase (AMPK).<sup>51</sup> They also note that AMPK inhibition with a pharmacologic inhibitor (compound C) or dominant negative AMPK- $\alpha$ 1 attenuates adiponectin-induced expression of  $\alpha$ -SMA and ECM proteins.<sup>51</sup>

The stimulation of receptors expressed by fibrocyte precursors by aggregated IgG or SAP has also been shown to inhibit the development of fibrocytes.<sup>15,32,52</sup> In the presence of serum, fibrocytes require up to 2 weeks to differentiate, whereas in absence of serum this process is accelerated to 3 days.<sup>32</sup> Pilling et al.<sup>32</sup> were able to isolate the factor found in serum and plasma that is responsible for preventing the rapid appearance of fibrocytes, and identify it as SAP. This molecule belongs to the pentraxin family of autacoids and binds to receptors for the Fc gamma portion of IgG (Fc $\gamma$ RI or CD64 and Fc $\gamma$ RII or CD32).<sup>15,32</sup> High levels of SAP are found at wounded tissue sites during the early phase of the inflammatory response.<sup>32</sup> In contrast, low levels of SAP are found in wounded tissues at later stages of the healing process due to a rapid clearing or inactivation process, thereby allowing monocytes to differentiate into fibrocytes to participate in tissue regeneration.<sup>32</sup> The serum of patients with systemic fibrotic diseases is reported to have lower levels of SAP, and this serum has lower capacity to inhibit fibrocyte differentiation than serum from healthy individuals *in vitro*.<sup>32</sup> Clinical trials show that SAP injections reduce the decline in organ function in pulmonary fibrosis and myelofibrosis patients.<sup>53</sup> In spite of SAP injections having markedly reduced the number of proliferative fibrocytes, and preventing fibrosis, SAP has not been shown to suppress inflammation or chemokine expression.<sup>15</sup> Zhong et al.<sup>36</sup> clarify the mechanism of action of SAP and demonstrate that SAP inhibits IL-4-induced differentiation of fibrocytes due to its SAP inhibiting effect on SOCE channels.

Pilling et al.<sup>54</sup> show that fibroblasts, as well as epithelium cells, secrete a signal (Slit2) that prevents entering monocytes from becoming fibrocytes in healthy tissue. However, in the injured tissue, there are low levels of Slit2, which facilitates fibrocyte differentiation and development of fibrotic lesions.<sup>54</sup> Moreover, injections of Slit2 prevented fibrosis in a mouse model of lung fibrosis.<sup>54</sup>

## FIBROCYTE MIGRATION

Fibrocyte recruitment into wounded tissues includes multiple steps, beginning with the exit of precursor cells, as predifferentiated collagen-producing cells, from the bone marrow into the blood, passage from peripheral blood vessels into the injured tissue—where they complete their differentiation and contribute to the healing process. Fibrocytes or fibrocyte precursors comprise only 0.1%–0.5% of

nonerythrocytic cells circulating in the blood<sup>27</sup> but are approximately 10% of the cells that infiltrate into a wound.<sup>6</sup> Ling et al.<sup>55</sup> demonstrate that the peak of fibrocyte recruitment to the blood and injured site occurs 3 and 5 days after the injury, respectively. According to a previous study,<sup>39</sup> transmigration of fibrocytes through the alveolar epithelial basement membrane is associated with the proteolytic activity of MMP-2 and MMP-9, in contrast to their migration in the tissue (collagen type I-coated Boyden chambers) which is thought to related to MMP-8 activity. Cell motility is guided by physical and chemical cues given by the surrounding environment.<sup>56</sup> Thus fibrocytes have been shown to express several relevant chemokine receptors, including CCR2, CCR3, CCR5, CCR7, CXCR4, CXCR6, and CX3CR1.<sup>24,25,36–38</sup> Fibrocytes also express numerous molecules related to cell adhesion and cell–cell interactions, such as CD9, CD11a, CD11b, CD11c, CD18, CD43, CD164, LSP1, CD34, CD29, CD44, CD81, the CD49 complex, and CD81.<sup>25</sup> Although all of these receptors have been identified on fibrocyte surfaces, they are not expressed simultaneously. For example, Phillips et al.<sup>11</sup> show that there are at least 2 phenotypically distinct fibrocytes populations in a lung model (CD45+ Col I+ CXCR4+ and CD45+ Col I+ CCR7+). Sakai et al.<sup>10</sup> also describe the presence of CCR7- CXCR4- and CCR2+ collagen-producing cells in a mouse renal fibrosis model.

CCR7 is a receptor for CCL19 and CCL21 (also known as *secondary lymphoid tissue chemokine* [SLC], 6CKine, Exodus-2, and TCA-4)<sup>27</sup> that has been shown to be involved in the attraction of T-cells and mature dendritic cells<sup>57</sup> and is expressed at sites of inflammation mediated by TNF-1.<sup>58</sup> Abe et al.<sup>27</sup> demonstrate that CCL21 contributes to *in vitro* migration of fibrocytes, whereas CXCL12 (the only chemokine known to bind to CXCR4) does not. They also observe CCL21 expression by the vascular endothelium within the wound sites, suggesting that fibrocytes migrate into early wound sites in part due to an interaction between vascular endothelium-derived CCL21 and CCR7+ fibrocytes.<sup>27</sup> Moreover, Sakai et al.<sup>10</sup> show that CCR7+ fibrocytes infiltrate the mouse kidney via CCL21+ vessels, contributing to the pathogenesis of renal fibrosis after induced ureteral obstruction. They found more than a 50% reduction in the number of fibrocytes in kidneys when mice are treated with anti-CCL21 antibodies or have CCR7 deficiency. They conclude that CCL21/CCR7 signaling is the major pathway attracting fibrocytes into the kidney in their model.<sup>10</sup> Studies done with patients with chronic obstructive asthma have shown enhanced expression of CCR7 by fibrocytes and increased levels of CCL19 by the bronchial epithelium, suggesting the participation of CCR7/CCL19 in fibrocyte attraction not only in animal and *in vitro* models but also in human models.<sup>59</sup>

Phillips et al.<sup>11</sup> found conflicting results regarding the ability of CXCR4+ fibrocytes to migrate in response to CXCL12. They suggest that the trafficking of CXCR4+ fibrocytes appears predominant over CCR7+ fibrocytes in lungs of bleomycin-treated mice.<sup>11</sup> They show that there is a gradient of CXCL12 between the lung and plasma of bleomycin-treated mice, which they hypothesize promotes the recruitment of CXCR4+ fibrocytes to the fibrotic lung.<sup>11</sup> They also found a similar, but weaker, gradient for CCL21 (ligand of CCR7). Using an *in vitro* chemotaxis assays, they confirm that CD45+ Col I+ CXCR4+ fibrocytes migrate in a way that is guided by the CXCL12 gradient *in vitro*.<sup>11</sup> In later studies,<sup>11</sup> specific anti-CXCL12 antibody block the recruitment of CXCR4+ fibrocytes into the lung and attenuate

**TABLE 2.** Chemokines and Respective Receptors that Act for the Recruitment of Fibrocyte and their Relevance on Organ Fibrosis.

Chemokine	Receptor	Model of Study	Fibrocytes Reduction Caused by an Antagonist or Absence	Effect of Antagonist or Absence in Reducing Fibrosis	References
CCL19 and CCL21	CCR7	Renal fibrosis induced by ureteral obstruction	Anti-CCL21 Ab: ~54% CCR7-null mice: ~61%	Anti-CCL21 Ab: ~53% reduction CCR7-null mice: ~58% reduction	Sakai et al <sup>10</sup>
CXCL12	CXCR4	Lung fibrosis induced by bleomycin-treatment	Anti-CXCL12 Ab: ~45%	Anti-CXCL12 Ab: significantly reduced fibrosis (% of reduction no shown)	Phillips et al <sup>11</sup>
CCL2	CCR2	Renal fibrosis induced by ureteral obstruction	Anti-CCR2 Ab: 0%CCR2-null mice: ~45%	Anti-CCR2 Ab: No effect on collagen-I expression CCR2-null mice: 20-30% reduction	Reich et al <sup>14</sup>
CCL2	CCR2	Renal fibrosis induced by ureteral obstruction	CCR2-null mice: ~45%	CCR2-null mice: ~50% reduction	Xia et al <sup>63</sup>
CCL5 CCL11 CCL24	CCR3 and CCR5 CCR3 and CCR5 CCR3	Chemotaxis assay of isolated fibrocytes from patients with asthma	Anti-CCL5 + Anti-CCL11 + Anti-CCL24 Abs: 53.1%	No shown	Isgro et al <sup>62</sup>
CXCL16	CXCR6	Renal fibrosis induced by ureteral obstruction	CXCR6-null mice: ~50%	CXCR6-null mice: ~50% reduction	Xia et al <sup>37</sup>
CXCL16	CXCR6	Renal fibrosis induced by ureteral obstruction	CXCR6-null mice:50-60%	CXCR6-null mice: ~50% reduction	Chen et al <sup>64</sup>

CCL CC-chemokine ligand; CXCL CXC-chemokine ligand; CCR CC-chemokine receptor; CXCR CXC-chemokine receptor.

fibrosis. Moreover, patients with an exacerbation of their asthma showed strong CXCL12 expression on the epithelium concomitantly with enhanced fibrocytes expression of CXCR4.<sup>59</sup> Fibrocytes were recently implicated in the inflammation associated with necrotizing enterocolitis in neonates and are most likely recruited to the intestine through the CXCR4/CXCL12 axis.<sup>60</sup>

CCR2 is a receptor for the chemokine (C-C motif) ligand 2 (CCL2), which is also called *monocyte chemoattractant protein-1*.<sup>25</sup> Reich et al.<sup>14</sup> investigate the role of CCR2 in the migration of fibrocytes in a murine model of obstructed kidney using CCR2-null mice. They noted that wild and CCR2  $-/-$  type mice had equal numbers of fibrocytes in the peripheral blood, but only CCR2  $-/-$  mice display a marked reduction of fibrocytes in the obstructed kidney.<sup>14</sup> These authors conclude that fibrocytes do not require CCR2 to leave the bone marrow, whereas their migration into the kidney is dependent on CCR2 receptor.<sup>14</sup> Conversely, Singh et al.<sup>61</sup> report increased concentrations of CCL2 in the plasma of patients with asthma, and suggested that the gradient modification led to fibrocyte recruitment into the blood from bone marrow. They also found that CCL2 mediated fibrocyte migration and potentially contributed to the development of airway smooth muscle hyperplasia in asthma.<sup>61</sup>

Chemotactic factors CCL5, CCL11, and CCL24 are found to be released by epithelial cells and to contribute to the pathogenesis of asthma.<sup>62</sup> CCL5 and CCL11 bind to CCR3 and CCR5, whereas CCL24 selectively binds to CCR3.<sup>62</sup> Patients with severe asthma show increased concentrations of CCL5, CCL11, and CCL24 in the sputum and fibrocytes isolated

from their blood exhibit increased expression of CCR3 and CCR5.<sup>62</sup> The fibrocyte migration was reduced 53% when anti-CCL5, anti-CCL11, and anti-CCL24 antibodies are used together with the sputum of severe asthma patients in a chemotaxis assay study.<sup>62</sup>

Xia et al.<sup>37</sup> show that the chemokine receptor CXCR6, a receptor for CXCL16, also contributes to recruitment of bone marrow-derived fibroblast precursors in renal fibrosis. Kidney tubular epithelial cells are found to produce CXCL16 mediate by TNF- $\alpha$  stimulation.<sup>63</sup> Genetic disruption of CXCR6 is demonstrated to reduce the recruitment of bone marrow-derived fibroblast precursors into the kidney and to decrease the development of renal fibrosis.<sup>37</sup>

Therefore, in response to injury, inflammatory cells, endothelial cells, epithelial cells, fibroblasts, and myofibroblasts have the capacity to produce CCL21, CCL19, CXCL12, CCL2, CCL5, CCL11, CCL24, and CXCL16, depending on the organ and specific pathological process. These chemokines act concertedly to recruit fibrocytes via interaction with their respective receptors (summarized in the Table 2).<sup>10,11,14,62-64</sup>

## FIBROCYTE FUNCTIONS IN WOUND HEALING

Fibrocytes play a pivotal role in wound healing and tissue repair processes in many organs.<sup>40</sup> They are unique cells that exhibit the proinflammatory properties of macrophages and the tissue remodeling properties of fibroblasts. Fibrocytes contribute to wound healing by numerous mechanisms: (I) by acting as antigen-presenting cells capable of stimulating



**TABLE 3.** Cytokines, Chemokines and Growth Factors Produced by Fibrocytes

Factor	Comments
Cytokines	
TNF- $\alpha$	Constitutive; increases under IL-1 $\beta$ stimulation
IL-6	Under IL-1 $\beta$ or TNF- $\alpha$ stimulation
IL-10	Constitutive; increases under IL-1 $\beta$ or TNF- $\alpha$ stimulation
IL-1 $\beta$	Constitutive
Chemokines	
CCL2	Constitutive; increases under TGF- $\beta$ 1 or IL-1 $\beta$ stimulation
CCL3	Constitutive; increases under TGF- $\beta$ 1 or IL-1 $\beta$ stimulation
CCL4	Constitutive; increases under TGF- $\beta$ 1 or IL-1 $\beta$ stimulation
CXCL1	Constitutive; increases under TGF- $\beta$ 1 or IL-1 $\beta$ stimulation
CXCL2	Constitutive; increases under TGF- $\beta$ 1 or IL-1 $\beta$ stimulation
CXCL8	Constitutive; increases under TGF- $\beta$ 1 or IL-1 $\beta$ stimulation
Growth factors	
TGF- $\beta$ 1	Constitutive
CTGF	Constitutive
M-CSF	Constitutive
IGF-1	Constitutive
Angiogenin	Constitutive
PDGF-AA	Constitutive; increases under IL-1 $\beta$ stimulation
PDGF-BB	Constitutive
FGF2	Constitutive; increases under hypoxia
VEGF-A	Constitutive
Others	
Periostin	Constitutive; increases under TGF- $\beta$ 1 stimulation

Based on published data.<sup>35,40,60,74–76</sup> CCL2, CC-chemokine ligand 2, also known as monocyte chemoattractant protein-1; CCL3, CC-chemokine ligand 3, also known as macrophage inflammatory protein 1- $\alpha$ ; CCL4, CC-chemokine ligand 4, also known as macrophage inflammatory protein 1- $\beta$ ; CXCL1, CXC-chemokine ligand 1, also known as GRO alpha; CXCL2, CXC-chemokine ligand 2, also known as macrophage inflammatory protein 2; CXCL8, CXC-chemokine ligand 8, also known as IL-8; CTGF, connective tissue growth factor; M-CSF, macrophage colony-stimulating factor; IGF-1, insulin-like growth factor-1; FGF2, fibroblast growth factor 2; VEGF, vascular endothelial growth factor.

T-cell-mediated immunity<sup>40</sup>; (II) by directly depleting pathogens through the release of extracellular traps, lysosomal peptides,<sup>65</sup> as well as by recently demonstrated phagocytic activity<sup>66</sup>; (III) by producing cytokines, chemokines, and growth factors important for wound repair (see Table 3)<sup>35,67,68</sup>; (IV) by secreting extracellular matrix proteins and glycosaminoglycans (see Table 1); (V) by promoting wound closure via  $\alpha$ -SMA-mediated contraction<sup>27</sup>; (VI) by promoting angiogenesis,<sup>67,68</sup> via secretion of growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor-2 and vascular endothelial growth factor; and (VII) by transforming into other mesenchymal cell types, in addition to fibroblasts and myofibroblasts, such as adipocytes, that contribute to new-tissue formation.<sup>69</sup>

Wound healing is classically divided into hemostasis, inflammation, proliferation, and remodeling. Fibrocytes appear to contribute to the innate immune response of the inflammatory phase, as well as in angiogenesis, collagen deposition, and wound contraction of the proliferation phase. In addition, fibrocytes may play a role in tissue remodeling during the maturation phase of wound healing. In the course of the inflammatory phase, damaged and dead cells are cleared from the tissues, along with bacteria and other pathogens. In this phase, there is a heightened Th1 immune response over the Th2 response, resulting in stimulation of fibrocyte innate immune functions. Nemzek et al.<sup>70</sup> show that the transfer of fibrocytes to mice with septic bacterial peritonitis enhanced the mouse survival and reduced the bacterial burden, reinforcing fibrocyte contributions to pathogen clearance. After the acute phase of inflammation is completed and the proliferation phase begins, there is an accelerated Th2 response over Th1 and upregulation of TGF- $\beta$ 1, promoting the monocyte-to-fibrocyte transition and differentiation of fibrocytes into myofibroblasts. These myofibroblasts deposit high levels of ECM components such as collagen type I and collagen type III that contribute to reinforcing the injured tissue and even the growth of new tissue. Chesney et al.<sup>35</sup> demonstrate that IL-1 $\beta$  acts on fibrocytes, stimulating a transition between a remodeling phenotype and a proinflammatory phenotype. During the maturation phase of wound healing, the cells that are no longer needed are removed by apoptosis, and the collagens are degraded or realigned along tension lines, a process that may also involve fibrocytes.<sup>39,71</sup> Many details regarding fibrocyte functions and their interactions with other cells during the wound healing remain to be characterized, including the role of fibrocytes in pathogen depletion and desirable mesenchymal properties that may someday be used to regenerate injured tissues and organs.<sup>72</sup>

## THE ROLE OF FIBROCYTES IN SCARRING AND FIBROSIS

Fibrocytes appear to be capable of a primary role similar to local tissue fibroblasts in response to injury—such as production of collagen and other ECM proteins. However, conflicting reports have been published about their capacity to produce ECM components. Schmidt et al.<sup>50</sup> report that fibrocytes are immature mesenchymal cells that do not produce large amounts of ECM components. Higashiyama et al.,<sup>41</sup> using a specific and sensitive experimental system that detects exclusively BM-derived collagen-producing cells, conclude that BM-derived cells have a limited role in collagen production. Conversely, Bellini et al.<sup>73</sup> demonstrate that under Th2 cytokine stimulation, fibrocytes produce high levels of collagenous and non-collagenous matrix components. Some of these discrepancies could relate to tissue-specific differences or the point in differentiation fibrocytes are studied.

There are other ways in which fibrocytes promote fibrosis. Fibrocytes secrete a unique profile of cytokines, chemokines, and growth factors (Table 3) that regulate the tracking and activation of surrounding cells and stimulate the deposition of connective tissue components in the injured tissue.<sup>35,40,60,74–76</sup> Thus fibrocytes promote fibroblast and macrophage migration by paracrine signaling via the chemokines CXCL8<sup>70</sup> and CCL2,<sup>10</sup> respectively. Fibrocytes can also act as antigen-presenting cells and induce

significant T-cell proliferation.<sup>40</sup> Fibrocytes secrete MMP-2 and MMP-9, which degrade the subendothelial basement membrane of the capillary vessels and thereby facilitate the influx of fibrocytes into the wounded tissue.<sup>39,42</sup> Moreover, MMP-9 secreted by fibrocytes appears to catalyze the release of active TGF- $\beta_1$  from a latent complex, which promotes myofibroblast proliferation and differentiation.<sup>42</sup> Driven by TGF- $\beta_1$ , the fibrocytes may differentiate into myofibroblasts, which are recognized as the effector cells of fibrogenesis.<sup>11,12,15,77-79</sup> Finally, Kuroda et al.<sup>80</sup> suggest that fibrocytes promote colon fibrosis by inhibiting collagen degradation through the production of TIMP-1.

In some tissues fibrocytes have been found to contribute to the resolution of fibrosis. Fibrocytes produce noninterstitial collagens, perlecan, versican, and hyaluronan, and consequently contribute to the timely regeneration of basement membranes that has been associated with the resolution of fibrosis in some tissues.<sup>71,81</sup> The MMPs secreted by fibrocytes may participate in the degradation of collagens in fibrotic tissues.<sup>39</sup> Bianchetti et al.<sup>71</sup> also show that fibrocytes degrade collagenous proteins through an Endo180-mediated pathway that contributes to collagen turnover and ECM remodeling.

## FIBROCYTES IN CORNEAL WOUND HEALING AND FIBROSIS

The cornea is the transparent anterior wall of the eye that, along with the lens, focuses light on the retina.<sup>82</sup> Fibrocytes have been shown to participate in the cornea wound healing response to injury that can lead to stromal fibrosis. The immunohistochemical study of fibrocytes in the cornea is challenging since an important marker for detection of fibrocytes is collagen type I,<sup>6</sup> and the normal corneal stroma itself contains large amounts of collagen type I.<sup>18</sup> Fibrocytes themselves typically produce low amounts of collagen.<sup>71</sup> Thus Lassance et al.<sup>18</sup> find intense stromal staining for collagen type I in corneas—which interferes with the tracking of collagen type I expressed by fibrocytes. De Roo et al.<sup>83</sup> report they can identify fibrocytes in the cornea by immunohistochemistry for the collagen type I marker. Flow cytometry<sup>24</sup> or use of fluorescent-labeled collagen<sup>41</sup> can be used to identify fibrocytes in the corneal stroma, although flow cytometry is not able to show tissue anatomy and the localization of fibrocytes in the wound healing process.

After many types of injuries of the cornea, including trauma, infection and surgery, depending on the severity, induced surface irregularity, and likely genetic factors, the wound healing process results in development of mature myofibroblasts and fibrosis in the normally transparent connective tissue stroma.<sup>82</sup> Damage of basement membrane (epithelial basement membrane or Descemet's endothelial basement membrane) and delayed or aborted regeneration underlies the development of corneal fibrosis.<sup>81</sup> Defective BM permits TGF- $\beta_1$ , PDGF, and likely other modulators, derived from tears and epithelium or aqueous humor and endothelium, to penetrate the stroma at sustained levels necessary to drive the development of precursor cells into myofibroblasts that generate disordered extracellular matrix and fibrosis.<sup>81,84</sup>

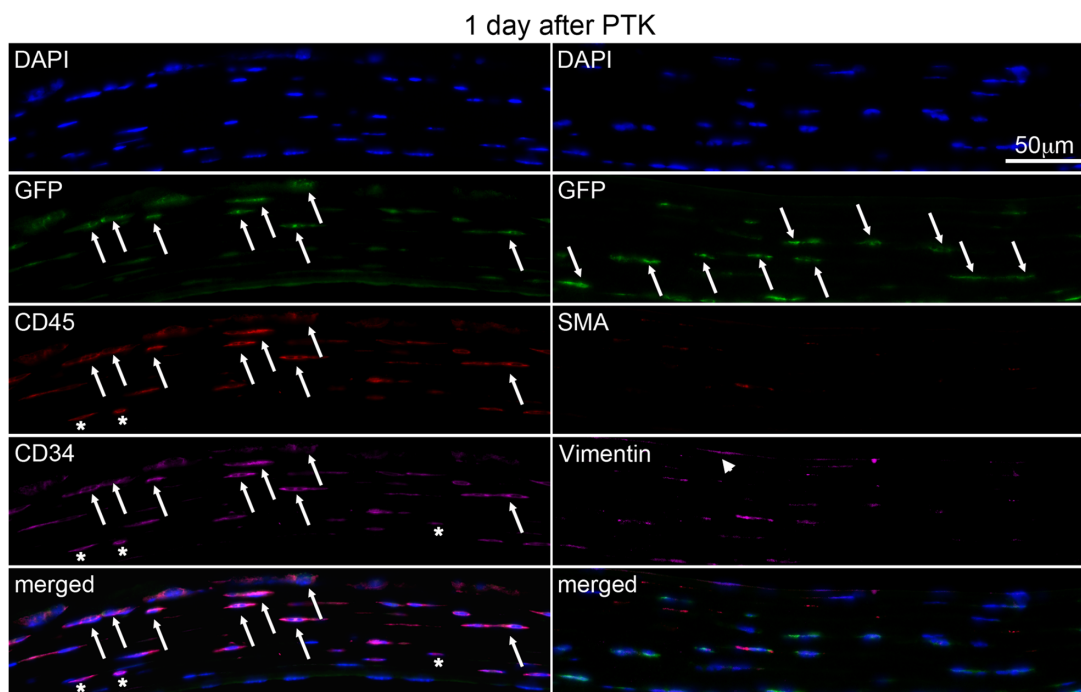
Myofibroblasts can develop in the cornea directly from corneal precursor cells (namely the keratocytes) via corneal fibroblasts, as well as from bone marrow-derived cells (fibrocytes).<sup>18,78,84,85</sup> Schwann cells<sup>86</sup> and epithelial cells (through

the epithelium-mesenchymal transition)<sup>87-89</sup> have also been identified as possible progenitors of corneal myofibroblasts, but very little is known about the potential role of the latter progenitors in corneal wound healing. Studies that used GFP chimeric mice have demonstrated conclusively that corneal myofibroblasts arise from both bone marrow-derived cells and keratocytes, with the ratio of each in a wounded cornea varying from 30% to 70% depending on the injury and individual animal.<sup>18,78</sup> Another animal study also supports bone marrow-derived cells being precursors to corneal myofibroblasts.<sup>90</sup> That study shows that inhibition of differentiation of local monocytes into fibrocytes—by administering subconjunctival SAP—significantly decreases myofibroblast generation in rabbit corneas at 1 month after photorefractive keratectomy (PRK) injury.

Lassance et al.<sup>18</sup> show (Fig. 3) that hematopoietic cells immediately migrate into cornea from limbal blood vessels after injury, resulting in a large influx of these cells into the mouse corneal stroma using chimeric mice with bone marrow transplants from green fluorescent protein (GFP)+ mice. Fibrocytes are found to correspond to approximately 24% of the hematopoietic cells that infiltrated the wounded cornea at 4 days after injury.<sup>18</sup> At this time point, 50%–70% of  $\alpha$ SMA+ cells in each corneal section are GFP+, indicating that these myofibroblast precursors and myofibroblasts likely differentiate from fibrocytes. Prior studies that also use GFP chimeric mice to demonstrate many myofibroblasts in fibrotic corneas originate from bone marrow-derived cells (likely fibrocytes).<sup>78,84</sup>

Normal versus defective BM regeneration likely relates to the type and severity of the stromal injury and results from inadequate production or incorrect localization of BM components—including laminins, nidogens, perlecan, and collagen type IV.<sup>82,91</sup> Corneal epithelial injury triggers release of IL-1 and apoptosis of the underlying keratocytes mediated by Fas-Fas ligand system in a manner that is proportional to the size of the original injury.<sup>92</sup> Extensive loss of keratocytes delays BM regeneration because of a deficiency or inadequate localization of some BM components contributed by keratocytes to the EBM.<sup>82,93</sup> Bianchetti et al.<sup>71</sup> suggest that fibrocytes may also contribute to a thickening of the EBM by also producing BM components; however, more work is needed to validate this contribution to corneal BM regeneration. In addition, injuries that produce corneal stromal surface irregularity promote fibrosis by mechanically impeding BM regeneration—and thereby augmenting TGF- $\beta_1$  and PDGF penetration into the stroma to drive myofibroblast development and their persistence.<sup>82,94</sup>

After corneal infection, trauma or surgery, keratocytes in proximity to the injury are “activated” by released growth factors, such as TGF- $\beta_1$  to transform into “corneal fibroblasts” that neither express the keratocyte marker keratocan nor the myofibroblast marker  $\alpha$ SMA.<sup>82</sup> Moreover, bone marrow progenitor cells are also stimulated by growth factors to differentiate into fibrocytes.<sup>18</sup> Corneal fibroblasts and fibrocytes secrete relatively small amounts of disordered ECM into the stroma and produce mild stromal opacity.<sup>82</sup> Fibrocytes have been shown to produce MMPs with possible roles in homing and cell migration, contributing to organ fibrosis, and contradictorily, might also help to reorganize the disordered ECM.<sup>39,71</sup> Corneal fibroblasts and fibrocytes may possibly revert to their respective progenitor cells as the wound healing response subsides in the stroma, but once they are deprived of an ongoing adequate supply of TGF- $\beta_1$ , these fibrocytes, fibrocyte progenitors, and corneal



**FIGURE 3.** Multiplex immunohistochemistry (Left column DAPI, GFP, CD45 and CD34 and right column DAPI, GFP, SMA and vimentin) of the cornea in chimeric mouse with green fluorescent protein-expression in bone marrow-derived cells at 1 day after irregular phototherapeutic keratectomy used to generate corneal fibrosis at 1 month after surgery. Large numbers of bone marrow-derived GFP+ cells (arrows) were present in corneal stroma. Virtually no GFP+ cells are noted in unwounded corneas (not shown). Many of these GFP+ cells co-express CD45 (arrows) or CD34 (arrows). There were no cells positive for the myofibroblast marker  $\alpha$ -SMA at this time point. In the merged panel on the bottom left, many GFP+ cells are also CD45+ and CD34+, and these likely are fibrocytes (arrows). Some stromal cells are vimentin+ (arrowheads), and these cells also likely include fibrocytes. Asterisks indicate a few cells that were CD34+ or CD45+ that were GFP-, which could result from maximum 95%–98% chimerization of the mice using these methods. Blue is DAPI staining of cell nuclei. Merged is the overlay of all images in that column. Negative control IHC was performed for all antigens without primary antibody and no staining was detected (not shown). Magnification  $\times 400$ . Republished with permission from Lassance L, Marino GK, Medeiros CS, Thangavadiel S, Wilson SE. Fibrocyte migration, differentiation and apoptosis during the corneal wound healing response to injury. *Exp Eye Res.* 2018;170:177-187. Copyright © 2019 Elsevier Ltd.

fibroblasts may undergo apoptosis.<sup>18</sup> Further studies are needed to assess whether these cells can revert back to their progenitor cells in vivo. In contrast, sustained exposure to TGF- $\beta_1$  drives corneal fibroblasts and fibrocytes to differentiate into mature myofibroblasts—which markedly reduce normal corneal stromal transparency because (1) they themselves are opaque due to diminished crystallin protein production<sup>85,95</sup> and (2) they produce large amounts of disordered extracellular matrix<sup>82,85</sup> that alters the normal regular structure of the corneal stromal fibrils that is critical to corneal stromal transparency.

During this differentiation toward the mature myofibroblast phenotype, keratocytes lose the expression of keratocan,<sup>82</sup> whereas fibrocytes lose the expression of the CD34 marker,<sup>17</sup> and possibly the CD45 marker (although some mature corneal myofibroblasts retain CD-45 expression),<sup>43</sup> confounding the identification of which myofibroblasts arose from corneal fibroblasts versus fibrocytes. Even though the bone marrow-derived myofibroblasts and corneal fibroblast-derived myofibroblasts are thought to correspond to the same spectrum of cells, preliminary proteomic studies have found that they have distinct profiles of protein production (Paramananda S, et al. IOVS 2019; ARVO E-Abstract 5254). There is limited in vitro evidence that corneal-derived myofibroblasts can transdifferentiate back to corneal fibroblasts after resolution of the corneal wound healing response,<sup>82,96</sup> but nothing is known about

whether it is possible for bone marrow-derived myofibroblasts to transdifferentiate back to fibrocytes as fibrosis resolves.

Even though many keratocytes transition to corneal fibroblasts and large numbers of fibrocytes enter the cornea within the first few hours after high correction PRK or microbial keratitis, few  $\alpha$ SMA+ myofibroblasts appear in these corneas until two to three weeks after the initial injury.<sup>18,81</sup> This delay is likely related to the TGF- $\beta_1$ -dependent developmental program these precursors must go through before they become mature  $\alpha$ SMA+ myofibroblasts that produce large amounts of disordered extracellular matrix materials that contribute to fibrosis. We hypothesize that this time required for mature myofibroblast development is a protective mechanism to ensure that large numbers of fibrosis-producing myofibroblasts do not develop when they are not truly needed. In humans, the developmental delay is often even longer. For example, when scarring fibrosis develops after PRK, it is frequently not evident until at least 3 months after the surgery.

The survival of myofibroblasts is dependent on an ongoing adequate source of TGF- $\beta_1$ , which suppresses myofibroblast apoptosis induced by paracrine or autocrine secretion of IL-1.<sup>82,97</sup> IL-1 is secreted by myofibroblasts themselves or by adjacent cells—including corneal fibroblasts, keratocytes and inflammatory cells (such as monocytes).<sup>82,97</sup> IL-1 release occurs during the resolution of stromal fibrosis

and triggers myfibroblast apoptosis when TGF- $\beta_1$  levels are low.<sup>82,97,98</sup> Barbosa et al.,<sup>99</sup> using IL-1 receptor I knockout mice, show that SMA+ myfibroblast density is higher and cell apoptosis in the anterior stroma is lower in the IL-1RI knockout mice compared with control mice at 1 month and 3 and 6 months after fibrosis-producing irregular phototherapeutic keratectomy—demonstrating the importance of IL-1 in the resolution of fibrosis.

Thus corneal fibrosis may resolve over a period of months to years if the inciting injury is eliminated and the EBM regenerates, leading to apoptosis of myfibroblasts and reorganization of disordered extracellular matrix by the repopulating keratocytes. Most scarred corneas undergoing fibrosis resolution exhibit areas of clearing called “lacunae” where the EBM has regenerated and underlying myfibroblasts undergo apoptosis.<sup>82</sup> Over time, these lacunae tend to enlarge and coalesce as more of the surrounding BM regenerates, fibrosis disappears, and full transparency of the cornea can possibly be restored.<sup>81</sup>

Fibrocytes have an important role in corneal wound healing when they rapidly migrate to the site of injury and differentiate into mature  $\alpha$ SMA-expressing myfibroblasts to maintain the integrity of the tissue, contract the wound, and prevent vision-threatening corneal perforation.<sup>18</sup> Thus myfibroblasts have a vital function in the response to injury, but when that response becomes excessive, it leads to pathological corneal fibrosis that itself impairs vision.<sup>18</sup>

## CONCLUSION

Fibrocytes are a leukocyte subpopulation that resides primarily in the bone marrow, but enter the circulation and migrate to sites of tissue injury in response to chemokine signals. In the wounded tissue, fibrocytes exhibit marked plasticity to ensure a more proinflammatory and pro-repair phenotype. An imbalance between the Th1 and Th2 immune responses, resulting in an exacerbated Th2 response and upregulation of TGF- $\beta_1$  leads to monocyte-to-fibrocyte transition and further precursor differentiation into mature myfibroblasts—resulting in high ECM component deposition and organ fibrosis. By contrast, the Th1 immune response over the Th2 response seems to stimulate innate immune function of fibrocytes, contributing to the removal of pathogens.

Besides the important role of fibrocytes in differentiation into myfibroblasts, there are several other roles fibrocytes may play in corneal fibrosis and fibrosis in other organs. Fibrocytes perpetuate an inflammatory cycle by facilitating the recruitment and activation of inflammatory cells. Moreover, fibrocytes produce MMPs that appears to promote the release of TGF- $\beta_1$  from normal ECM, further contributing to myfibroblast differentiation. Additionally, fibrocytes themselves produce TGF- $\beta_1$  and other modulators that enhance myfibroblast proliferation and ECM production. New drugs that interfere with the fibrocyte generation, fibrocyte migration and their eventual differentiation into myfibroblasts, as well as their production of MMPs and TGF- $\beta_1$ , have therapeutic potential to reducing the accumulation of collagens and maintenance of tissue integrity.

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