**Molecular and Translational Medicine**
*Series Editors:* William B. Coleman · Gregory J. Tsongalis

Monte S. Willis · Cecelia C. Yates
Jonathan C. Schisler *Editors*

# Fibrosis in Disease

## An Organ-Based Guide to Disease Pathophysiology and Therapeutic Considerations

Humana Press

# Molecular and Translational Medicine

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*We dedicate this book to the vast number of patients struggling with the numerous diseases with primary fibrosis. While the therapeutic options are extraordinarily limited, in editing this book, it is clear that the future is bright, with new and exciting approaches already on the horizon. We thank our mentors for their tireless inspiration and guiding light in the face of uncertainties and risk – Charles Jennette, MD; Cynthia Lander, PhD; Lynda F. Bonewald, PhD; Marion E. Couch, MD, PhD, MBA; Chris Newgard, PhD; Cam Patterson, MD, MBA; Timothy Turner, PhD; Mark E. Sobel, MD, PhD; and Clayton C. Yates, PhD. It would be an honor to have passed any fraction of their enthusiasm, guidance, and unwavering encouragement they were so generous to share with us.*

*To Tina, Connor, and Declan,*

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*I dedicate this book to all of my science and English teachers throughout my education, most notably the late Father Ebenhoeh:*

*How many moles?*

Jonathan

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# Introduction

Systemic and organ-specific human fibrotic disorders, the loss of physiological organ structure and function, are a worldwide leading cause of morbidity and mortality and can affect all tissues and organ systems. Fibrotic disorders or fibroproliferative diseases result from chronic tissue damage or out-of-control wound healing response, thereby resulting in a vast number of conditions in which it is the primary factor as well as the large number consequence of the disease. The spectrum of these disorders includes, but is not limited to, pulmonary fibrosis, systemic sclerosis, liver cirrhosis, cardiovascular disease, and progressive kidney disease. This range of diseases also encompass pathophysiologies not readily recognizably related, such as macular degeneration and cancer metastasis. Thereby approximately, 100 million patients have fibrosis-related tissue damage; in some cases, this causes progressive and life-threatening disease outcomes. Remarkable progress in elucidating the pathogenesis of these common diseases with fibrotic components as the scope of fibrotic disorders and their etiological manifestations is diverse, yet they share underlying factors. Most notably, these factors include the complex and intrinsically linked relationship between inflammation and extracellular matrix remodeling. As the importance of inflammation and fibrosis is apparent in the long-term recovery and treatment of diseases, effective anti-fibrotic therapies targeting the underlying ongoing disease processes are lacking.

Despite the advances in the discovery of organ-specific mechanisms of disease and therapeutic targets efforts, success remains indefinable for a multitude of reasons. Among these reasons is the lack of understanding or defining (i) early detection, (ii) reversibility mechanisms, and (iii) extracellular matrix and inflammation crosstalk. The complexity of discovering and applying therapies to fibroproliferative disease may be due to the diversity of the systems the pathogenesis of disease itself involves. By nature, fibroproliferative diseases are interdisciplinary, involving multiple cell types (organ-specific epithelial cells), immune cells, endothelial cells, and fibroblasts. Bone marrow, cytokines and chemokines, and organ-specific pathologies further speckle both the clinical and scientific disciplines in such a way that communication is often limited to the clinical or scientific tribes we live in, despite the most significant access to information known to man available today.

Therefore, the primary focus of this text is to bring together authors from diverse clinical, scientific, and therapeutic backgrounds for readers to more fully appreciate the current state of the science in organ fibrosis. We also hope the reader recognizes the impact and potential opportunities gained from identifying well-defined mechanisms in other organ systems. This book comprehensibly makes available the advances in the discovery of preclinical therapeutic targets (at least 20+ to date) involving TGF-beta (and other cytokines), transcription factors, and downstream kinases; it's important to recognize both the broader impact and potential opportunities that exist even today.

## Fibrotic Signaling in the Skin

There is a broad spectrum of human fibrotic skin diseases: nephrogenic fibrosing dermopathy, mixed connective tissue diseases, scleromyxedema, eosinophilic fasciitis, and most notably systemic sclerosis (SSc). The initiating factors for this process in the early stages are believed to be driven by vascular injury, immune dysfunction, and an autoimmune response. The immune dysfunction, resulting in a dysregulated inflammatory response, and vascular injury result from pathologic wound healing. *Chapter* 1 introduces the chemokines and chemokine receptor signaling that influences cellular activities after tissue injury and alternation in the chemokine response that leads to fibrosis. *Chapter* 2 dissects the underlining mechanisms that drive the pathogenesis of systemic sclerosis while comprehensively analyzing human and animals to study this disease. *Chapter* 3 outlines cellular aspects of skin fibrosis, specifically the changing macrophage phenotypes in skin fibrosis and the influence that macrophage ontogeny, epigenetic factors, heterogeneity, and the microenvironment exert on these phenotypes.

## Fibrotic Signaling in the Lung

Lung fibrosis is characterized as a progressive scarring of the lung parenchyma, which ultimately leads to impaired gas exchange, respiratory failure, and death. *Chapter* 4 describes the signaling mechanisms that occur in lung fibrosis while highlighting the three principal cellular actors in the fibrotic lung: the epithelial cell, the macrophage, and the fibroblast. *Chapter* 5 explores the extracellular matrix element of fibrosis and discusses the major matrikines identified in the mouse and human lung as well as proposes how targeting these molecules could be used in treating these diseases. *Chapter* 6 underlines the less well-studied cell type, mast cells, and their characteristic function that influences the progression of pulmonary fibrosis has not been pursued. *Chapter* 7 describes the advances in our understanding of the heterogenous pericyte phenotype, including its distribution and origin that contributes to their involvement in both ECM remodeling and inflammation features

of fibrosis. *Chapter* 8 circumscribes the emerging therapeutic targets and therapies in idiopathic pulmonary fibrosis. *Chapter* 9 describes the changes in the matrix microenvironment in chronic lung diseases and how these changes amplify and sustain pathological lung fibrosis, which has limited the effectiveness of current therapies.

## Fibrotic Signaling in Heart

Most etiologies of heart disease involve pathological myocardial remodeling that produces excessive deposition of extracellular matrix (ECM) proteins by cardiac fibroblasts (CFs), which reduces tissue compliance and accelerates the progression to heart failure. *Chapter* 10 introduces fibrosis in the heart, specifically the signaling pathways that are associated with fibrosis and cardiomyopathies. Despite the numerous etiologies of cardiomyopathies, it is essentially a fibrotic disease that ultimately disrupts cardiac output. Several of these pathways are further explored in depth, including the WNT signaling pathway, in *Chapter* 11, and matrix metalloproteinases that modify the extracellular fibrotic matrix in the heart, in *Chapter* 12. The remodeling of the extracellular matrix in the heart produces peptides that may be used diagnostically or even therapeutically, concepts explored in *Chapter* 13.

## Fibrotic Signaling in the Vasculature, Liver, Kidney, and Eye

The role of fibrosis in other organ systems reveals several commonalities to the pathophysiology of fibrosis observed in the skin, lung, and heart. In disease conditions, the plasticity of vascular cells results in the transformation of different cell type into fibroblast-like cells, contributing to vascular stiffness, as discussed in *Chapter* 14. Recent insights into how the ECM in the liver changes in the development and progression of cirrhosis revealed insight into the reversibility of liver fibrosis, profoundly impacting the development of new therapies for cirrhosis, as well as tumorigenesis, explored in *Chapter* 15. The balance between fibrosis and angiogenesis highlights the complex interplay between adaptive and maladaptive processes that occur in the pathology of chronic kidney disease, as detailed in *Chapter* 16. Macular degeneration is hallmarked by the accumulation of nonfunctional, fibrotic tissue, exemplifying the pathological effects of excessive and aberrant would repair. *Chapter* 17 highlights how the recent advances in our understanding the balance between angiogenesis and inflammation revealed new approaches in pharmacotherapies targeting fibrosis in macular degeneration.

# Part I
# Skin, Cell Injury, and Wound Healing

# Chapter 1
# The Role of Chemokines in Fibrotic Dermal Remodeling and Wound Healing


**Zariel I. Johnson, Christopher Mahoney, Jun Heo, Erin Frankel, Dana R. Julian, and Cecelia C. Yates**

## Introduction

Skin is the largest organ in the adult body and serves many crucial roles in health and disease. It is part of the integumentary organ system, which also includes hair, nails, glands, and nerves. The skin is responsible for protecting from pathogens by acting as a physical barrier. The outer layer of skin prevents excessive water loss to enable homeostasis of electrolyte balance. Specialized neurons provide feedback to sense stimulation by temperature, pressure, and touch. The lower fatty layer of skin acts as an energy reserve and provides padding. Thus, the integrity of the skin and its composite layers is critical for normal function of the organism.

Injury to the skin comes in many forms and is repaired through a sequence of overlapping, ordered phases that restore barrier function and, in cases of complete healing, biomechanical properties. Healing involves sealing the wound through hemostasis, preventing infection and attracting cells through inflammation, regenerating lost tissue and permanently closing the wound, and remodeling the initial

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replacement tissue. Alterations in this healing cascade can lead to the development of fibrotic or scar tissue that lacks biological functions and/or mechanical properties of healthy skin.

Chemokines are a family of small cytokines initially identified as chemoattractant molecules. Over time, further study has shown these molecules have essential roles in many areas of physiology ranging from promoting differentiation to influencing metastasis. Ligands may signal through cognate receptors or exert their actions through interaction with the extracellular matrix. Promiscuous binding of ligands paired with the ability of receptors to form oligomers results in a vast diversity of signaling outputs.

At the beginning of wound healing, the pro-inflammatory and pro-angiogenic chemokines play an essential role by attracting immune and endothelial cells. This process allows for the growth of the vascular supply needed to support extracellular matrix synthesis by fibroblasts and migration of keratinocytes to re-epithelialize the wound area. Following the formation of this granulation tissue, there is a switch to angiostatic chemokines, which halt angiogenesis and cause the regression of the overabundant blood supply. These same chemokines have proved essential to stop the migration of keratinocytes and promote maturation of the dermis and epidermis.

## Normal Dermal Architecture and Function

Skin is the largest organ in the body and comprises about 15% of the adult body weight [1]. The skin is the body's primary physical barrier with the outside world and plays essential roles in immunity and homeostasis. It must continuously sense and respond to environmental changes across the entire body.

The skin is composed of three layers: the epidermis, dermis, and hypodermis (Fig. 1.1). Within each layer, there exists a multitude of individual structures, including: hair follicles, sweat glands, nerves, connective tissue, adipose tissue, and blood vessels [2]. The relative proportion of layer depth varies with the body area to match form to function. For example, the epidermis is much thicker on surfaces that experience friction, such as the soles of the feet, than other areas. The epidermis is the outermost layer of skin and is a stratified, squamous epithelium layer made up primarily of keratinocytes, which produce keratin, and dendritic cells. Other cell types present in this layer include melanocytes, Langerhans cells, and Merkel cells, each with specialized functions. Specific structures found in the epidermis include sweat glands, pilosebaceous follicles, and, on the distal phalanges, nails. The epidermis can be further separated into two anatomical layers: the regenerative layer and the outer stratum corneum. The dermis is the next deepest layer of skin and is made up of relatively few cells that reside in an abundant extracellular matrix. Present in this layer are cells such as fibroblasts, monocytes, Langerhans cells, lymphocytes, and vascular-related cells and structures including sweat glands, nerve endings, hair follicles, and blood vessels. These cells account for only about 10% of the tissue con-

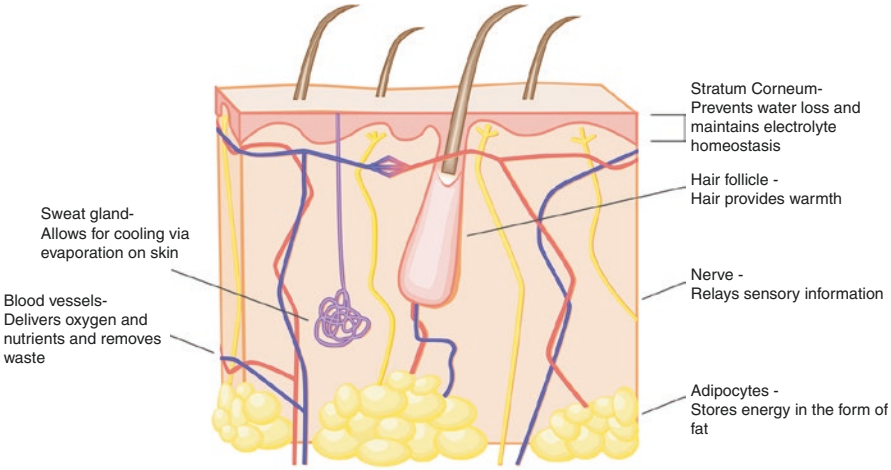


**Fig. 1.1** Normal skin architecture is critical to meet functional needs of the human body. Each layer of skin has biomechanical properties and structures that allow it to perform specific functions, including immune defense, homeostasis, and sensory functions. Chemokines, through interaction with their receptors and the extracellular matrix, play a vital role in skin repair following injury and regeneration of the complex structures depicted here

tent, while the remaining 90% is mostly collagen type I and elastin. The rich vascular supply of the dermis is essential to deliver nutrients and other circulatory products to the epidermis, which does not have its own blood supply. Finally, the deepest layer of skin is the subcutaneous layer, also known as the fatty layer. The most abundant cell type in this layer is the adipocyte, which stores energy in the form of fat, followed by the fibroblasts and macrophages.

The skin serves many vital functions for organism survival. Perhaps the most critical function is acting as a barrier to infection. The outermost layer of the epidermis, the stratum corneum, consists of several layers of squamous epithelial cells. This cell layer is continuously undergoing cell desquamation, which sloughs bacteria with it before being replaced by fresh cells. The oil glands of the skin also secrete fatty acids and produce an acidic pH, making it difficult for many bacteria to colonize the skin. However, a person can become prone to infection if an injury is present, leaving gaps for microbes to penetrate deep into tissue. Skin is also critical for maintenance of water and electrolyte balance. It retains fluids along with electrolytes, but also can secrete them through sweat; this balance between retention and secretion of water and electrolytes allows for proper osmotic balance of tissue. The skin plays a crucial role in maintaining the temperature of the body. When the body gets overheated, the sweat glands release perspiration, which then absorbs heat and evaporates from the surface. The blood vessels will also dilate to increase surface area and dissipate heat outward. When the body becomes cold, shivering occurs to generate heat from the muscles. In addition, blood vessels constrict, and more blood flow is directed towards vital organs and away from face and apendages. The skin is crucial not only for regulating but also for providing insulation, sensing tempera-

ture, and other stimuli, such as pain and pressure. The skin is loaded with sensory receptors including the Meissner's corpuscle, the Pacinian corpuscle, the Ruffini corpuscle, the hair root plexus, and free nerve endings. These sensory receptors allow for the sensation of touch, heat, and pain. Along with acting as an energy reserve, the subcutaneous layer of skin is essential for insulating the body to maintain temperature homeostasis and provides mechanical padding. Finally, the skin is critical for the synthesis of Vitamin D. Vitamin D is produced via the stimulation of provitamin D3 from ultraviolet radiation from the sun. Provitamin D3 photolyzes into provitamin D3, which then isomerizes to Vitamin D3 (Holick Pincus 1987).

## Dermal Wounds

A wound is a disturbance in the normal anatomic structure and function of the tissue. Wounds come in many forms and may include non-penetrating wounds that do not break the skin (abrasions and contusions), penetrating wounds, or other wounds (burns, frostbite, electrical wounds). For the remainder of this chapter, we will focus on features of dermal wounds such as penetrating wounds and burn injury. These wounds may result from pathologic processes beginning internally or externally, and they may also be acute or chronic [3]. Acute wounds usually proceed through an orderly and relatively quick reparative process that results in, at least to some extent, restoration of the native anatomy and functional integrity. On the other hand, chronic wounds often fail to heal through such an ordered healing process and often do not allow the tissue to regain its original features and integrity [4, 5]. Many environmental and genetic factors influence the rate and quality of dermal healing. These include, but are not limited to, age, nutrient availability at the wound site, lifestyle factors, use of medications, comorbidities, and infection [6–8].

Dermal wounds can heal through primary, secondary, or tertiary intention. The primary intention, also known as primary closure, generally takes place within the first few hours following wounding and involves bringing the two edges of the wound together also known as approximation. This process typically requires mechanical means such as adhesive strips, sutures, staples, or clips. Wounds that heal through primary intention usually close rapidly and have minimal scarring. In cases where the wound edges cannot be brought together, wounds are left open and allowed to close by secondary intention. In this process, wound contraction and re-epithelialization lead to closure, albeit at a slower rate than in primary intention. Often, wounds that require healing by secondary intention are large, traumatic wounds. Finally, tertiary intention occurs in wounds that cannot approximate the edges within 3–6 days, often in cases of inadequate blood supply or infection.

The process of wound healing requires precise coordination of signaling, both temporally and spatially. On one end of the spectrum, too little healing results in non-healing, open wounds that are susceptible to infection. At the other end, over-activation of the healing response results in a buildup of scar tissue and have detrimental effects on adjacent heathly functional tissue.

## Dermal Wound Healing Is a Complex, Multistep Process that Involves Many Cell Types and Secreted Factors

Dermal wound healing encompasses three critical, overlapping phases that include activities of various cell types and secreted factors. The stages are essential to seal the wound and prevent water loss and infection (hemostasis/inflammation), regenerate lost tissue (tissue replacement/proliferation), and remodel the provisional extracellular matrix to restore native tissue function (resolution). The specific roles of chemokine signaling in these stages of healing will be discussed in more detail later in this chapter.

The primary objective of the hemostasis and inflammation phase is to halt blood loss and begin the process of cell migration to the wounded area. The inflammatory/hemostasis phase takes place in the initial 24 hours and can last up to 2 days. Signs of inflammation such as local edema and redness characterize this phase. In the inflammatory/hemostasis phase, blood that leaked during the initial injury coagulates through the aggregation of thrombocytes and platelets in a fibrin network, which is the product of thrombin cleaving fibrinogen. This network serves three primary roles: to act as a temporary barrier to infection, to form a provisional matrix for the migration of incoming cells, and to stimulate fibroblast proliferation. Soon after injury, neutrophils and other inflammatory cells traffic to the site. These cells release lysosomal enzymes that facilitate the breakdown and engulfment of damaged tissue. As this phase continues, activation of the complement system and platelet degranulation attract neutrophils, which produce reactive oxygen species and clean up cell debris. With time, monocytes are also attracted to the wound site where they become polarized to various phenotypes. They can then contribute to phagocytosis and release multiple secreted factors to promote proliferation and angiogenesis [9]. These activated macrophages play an essential role in initiating the second step of healing through the secretion of cytokines, pro-angiogenic factors, and fibrogenic factors.

In the tissue replacement/proliferation phase, the wound must begin to close, usually through a combination of contraction and proliferation of fibroblasts. This phase spans from roughly 48 h to 2 weeks post-injury [10]. An essential component of this process includes angiogenesis, which allows for the transport of oxygen, nutrients, and immune cells (via blood) to the wound bed [11]. The proliferation of local and recruited fibroblasts and the production of extracellular matrix components result in granulation tissue. These fibroblasts are highly synthetic producers of collagen and other extracellular matrix components. At the same time, keratinocytes migrate to re-epithelialize the wound. Contraction will bring the edges closer together, thus decreasing the area that will require re-epithelization.

In the final resolution phase, the provisional extracellular matrix (ECM) that has been produced by fibroblasts is remodeled to match the characteristics of the native tissue. This phase can begin weeks after the injury and last for more than a year afterward. The constituents of the provisional ECM, mainly composed of type 3 collagen, are broken down, reorganized, and resynthesized, and the content of type

1 collagen is increased. Compared to the granulation tissue, the resulting ECM is less abundant in cellular material and blood supply but better matches the biological and mechanical attributes of unwounded skin. Inflammation that took place during the early phases of injury is resolved during this phase as well. The migration of keratinocytes that were previously essential to re-epithelialize the wound is halted.

While dermal wounds sustained at the fetal stage can heal perfectly, adult dermal wounds often result in scarred or fibrotic tissue [12, 13]. In cases of large initial wounds, the resulting scars can be painful and disfiguring [14]. Scarring results when the wound does not correctly move from the proliferative phase to the resolution phase [15]. In skin, scarring may be classified by clinical phenotypes that include atrophic, hypertrophic, and keloid scars. Atrophic scars are flat areas that are depressed compared to the area of skin surrounding the scar [16]. On the other hand, raised scars include hypertrophic and keloid scars. While hypertrophic scars remain within the area bounded by the original wound, keloid scars are characterized by the fact that they extend beyond the border of the wound margin. Similar to other scars, keloids demonstrate increased collagen content and proliferation of fibroblasts compared to normal tissue [17, 18]. Scar tissue is in many ways inferior to healthy skin. While it can regain the barrier functions imperative for preventing infection and water loss, this scar tissue is mechanically less suitable than its healthy counterpart. Traditionally, biomechanics of skin tissue are evaluated based on the tensile failure, which is a measurement of the skin's ability to oppose rupturing. Recently, investigators have begun to assess more physiologically relevant parameters of skin biomechanics. Scar tissue is much less failure resistant and less compliant and has slower relaxation time [19].

## The Discovery of Chemokines as Soluble Signaling Molecules

Historically, chemokines have been referred to by many names. These include small inducible secreted (SIS) family of cytokines, small inducible gene (SIG) family of cytokines, small cytokine (SCY) family of cytokines, platelet factor 4 (PF4) superfamily, and intracrine cytokine family [20–24]. Advancements in protein purification and molecular cloning in the early 1980s set the stage for the birth of chemokine research. In late 1986, Teizo Yoshimura and Kouji Matsushima isolated and purified a chemoattractant protein from the supernatant of media used to culture peripheral blood mononuclear cells (PMBCs) that had been activated by lipopolysaccharide (LPS) [25]. The protein was named monocyte-derived neutrophil chemotactic factor (MDNCF) for its activity and source. Shortly after, Ettore Appella's laboratory identified the N-terminal amino acid sequence of MDNCF. This discovery led to the observation that the N-terminus of MDNCF was highly similar to inflammation-related proteins that would later be classified as chemokines, including β-hemoglobin, platelet factor 4 (PF4), and interferon-gamma-inducible protein (IP) 10 [26].

In 1987, Yoshimura and Matsushima published their findings simultaneously along with the laboratories of Marco Baggiolini and Jo Van Damme, who had

purified the same protein [27, 28]. Because MDNCF acted as a chemoattractant for a subset of T lymphocytes, it was at this point renamed as interleukin-8 (IL-8) [29]. The Leonard and Oppenheim laboratories purified additional chemokines from the supernatant of LPS-activated PBMCs [30, 31] as other groups began to discover several chemokine family members. Oppenheim began to call these proteins chemokines, an abbreviated form of "chemoattractant cytokines."

Early on, chemokines were most recognized for their action on the innate and adaptive immune systems through modulating leukocyte communication and migration. Over time, various studies uncovered additional functions including hematopoiesis, angiogenesis, and oncogenesis. Some chemokines can co-stimulate T-cell differentiation, enhance survival of immune cells, or even act as antimicrobial peptides in peripheral epithelial tissues [32, 33].

Chemokines may be constitutively produced or induced rapidly under certain conditions. Some chemokines with homeostatic roles are produced and secreted at all times. These may be involved in immune surveillance or localization of immune cells. Those that promote inflammation are typically induced in response to some stimulus. These chemokines usually induce trafficking of leukocytes to the site of inflammation or infection and activate cells that will be critical for wound healing.

## The Chemokine Family Tree

Chemokines are a large family of cytokines characterized by their ability to bind heparin, their small size, and the presence of four highly conserved cysteine amino acids. Although considerable homology between chemokines exists at the amino acid and overall structural level, they are required to transmit various signaling cues needed for a myriad of downstream functions. The 50 chemokines identified to date fall into four categories (C, CC, CXC, and CX3C), and with variability in splicing and enzymatic cleavage of the N- and C-termini results in hundreds of different molecules. In general, these chemokines signal through activation of G-protein-coupled receptors (GPCRs), atypical/alternative chemokine receptors (ACRs), or interaction with GAGs. In most cases, a single chemokine is recognized by multiple GPCRs, contributing to the essential diversity of the chemokine signaling system. Chemokine GPCRs can function as dimers or even higher-order oligomers, further adding to this complexity [34].

The more than 50 chemokines identified are divided into four categories (C, CC, CXC, and CX3C), based on the characteristic pattern of cysteine residues in proximity to the amino terminus of the mature proteins [35]. Chemokines in the CC family have the first two cysteine amino acids adjacent to one another. Those in the CXC family have a single, variable amino acid dividing these two cysteines. The CX3C group (CX3CL1) has three amino acids between these cysteines. Finally, the C family (XCL1 and XCL2) only has two of the usual four cysteines.

The tertiary structure of chemokines is well conserved. A flexible unfolded N-terminal acts as the signaling domain. Next, the core domain is made up of an

N-loop, a three-stranded β-sheet, and a C-terminal helix. For many of the CXC chemokines, the β-sheets act as an interface for dimerization. This results in a functional dimer consisting of six β-sheets and two α-helices. For CC family chemokines, dimerization uses residues near the N-terminus and overlapping with the CC cysteines [35]. CX3CL1 most commonly exists as a monomer and XCL1 forms a head-to-tail dimer [36, 37]. Interestingly, monomer forms of chemokines can bind their cognate receptors and activate downstream signaling [38, 39]. However, the body of evidence supports that chemokine oligomerization required interaction with GAGs.

Several amino acid motifs are critical for chemokine function. CXC chemokines can be categorized based on containing a three-amino-acid motif known as the ELR motif (Glu-Leu-Arg) at the N-terminus [40]. Classically, those lacking the ELR motif (CXCL4, CXCL9, and CXCL10) have been considered angiostatic, and those containing the motif (CXCL5, CXCL6, CXCL8, and others) have been considered angiogenic [41]. Chemokines containing the ELR motif bind to CXCR2, while those lacking the motif bind to CXCR3. Specific aspartic acid residues of chemokines can also be necessary for receptor activation. For example, Asp2.63, Asp4.50, and Asp6.58 are key for ligand binding with the CXCR4 receptor [42].

## Chemokine Receptors and Signaling

Chemokine receptors, which are mainly seven transmembrane G-protein-coupled receptors (GPCRs) that belong to the class A rhodopsin-like family, primarily mediate chemokine signaling [43]. GPCRs make up approximately 3% of genes in the human genome and are vital for signaling ranging from the sensation of light and taste to the regulation of blood pressure [44]. GPCRs contain three extracellular loops (ECLs) and three intracellular loops (ICLs) that link α-helices. A disulfide bond between ECLs 1 and 2 restricts movement of the α-helices. When activated by agonist binding, GPCRs undergo structural changes on the cytoplasmic face, which catalyze the exchange of GDP on the alpha subunit of the heterotrimeric G protein [45]. In turn, the alpha and β-gamma subunits are free to dissociate and bind to downstream effector molecules, commonly cAMP, IP3, and others. Chemokine-stimulated GPCRs can trigger several downstream effects that eventually lead to actin polarization, changes in cell shape, and directed cell locomotion. Stimulation of the Gαi subunit causes the activation of calcium channels and inhibition of adenyl cyclases and cyclic adenosine monophosphate (cAMP) production. The G beta-gamma subunits are required for chemotaxis. The activation of these subunits triggers signaling effectors including GPCR kinases (GRKs), ion channels, and phospholipase C-β (PLC-B). PLC-B catalyzes PIP3 to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 initiates a release of calcium from endoplasmic reticulum (ER) stores, while DAG can activate protein kinase C (PKC), a kinase involved in receptor regulation. Furthermore, both Gα and Gβγ subunits can

independently activate phosphoinositide 3-kinase (PI3K), resulting in the activation of the kinases Akt and the mitogen-activated proteins kinases (MAPKs).

In addition to GPCRs, alternative/atypical chemokine receptors (ACRs) can bind chemokines, leading to various outcomes. These receptors, while structurally similar to GPCRs, utilize different downstream signaling cascades [46]. These receptors do not contain the canonical DRYLAIV motif in the second intracellular loop, preventing them from binding G proteins [47]. However, ACRs internalize their chemokine ligands, a process termed "interceptors" [48]. Cognate chemokine-ACR binding can lead to various outcomes. While some leads to chemokine degradation [49], others lead to chemokine transport [50]. Finally, these ACRs can act in concert with GPCRs through dimerization. There are at least five members of this group including the Duffy antigen receptor for chemokines (DARC), D6, CXCR7, and CC chemokine receptor-like 1 and 2 (CCRL1 and CCRL2) [46].

In addition to transmitting signals through membrane-spanning receptors, there is growing evidence that chemokines influence cellular activities via interaction with extracellular matrix components, particularly glycosaminoglycans (GAGs) [51]. GAGs are long, unbranched polysaccharide structures composed of repeated disaccharide units. They are found attached to protein cores of proteoglycans or can be shed into the extracellular matrix (ECM). GAGs can influence chemokine signaling through the immobilization of chemokines, which results in the formation of "haptotactic" chemokine gradients. These concentration gradients then direct the migration of receptor-bearing cells [35]. GAGs immobilize chemokines on the endothelial surface using chemokine ligand sugar moieties. This ensures the directionality of chemotactic signals for leukocytes [52]. Chemokines that cannot attach to endothelial GAGs are either dispersed from the production site or diluted to concentrations below the receptor-activating threshold, consequently reducing leukocyte extravasation into tissues such as skin which has very high GAG content.

Chemokines play essential roles in both health and disease. They are known to regulate leukocyte migration, inflammation, angiogenesis, organ development, autoimmunity, and tumorigenesis. Many of these activities have particular relevance to normal dermal healing and are crucial to dysfunctional repair that results in the persistence of chronic wounds or the development of fibrotic outcomes. Specifically, chemokine signaling orchestrates the recruitment of specialized leukocytes to the wound, epithelization, angiogenesis, and ultimate tissue remodeling (Fig. 1.2).

## Chemokines in the Hemostasis and Inflammation Stages of Wound Healing

Chemokines are crucial during this stage for inducing the recruitment of various cell types to the wound site. These chemokines induce migration and proliferation along with promoting the cellular debris clearance.

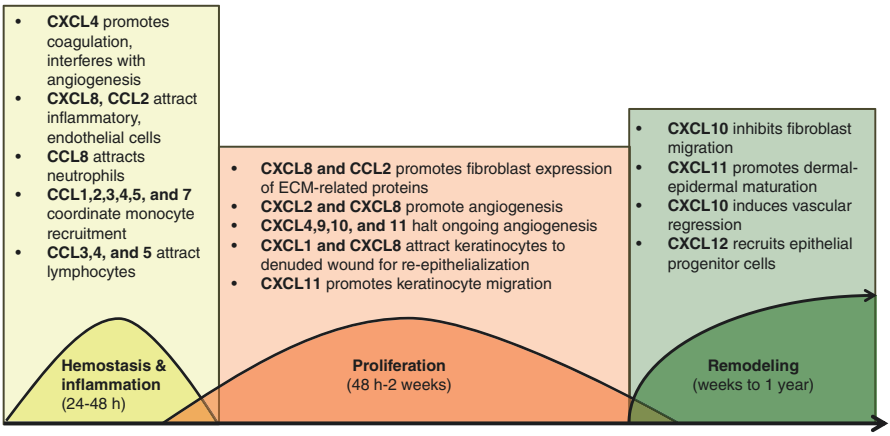


**Fig. 1.2** Predominant chemokine and chemokine receptor activities vary with wound healing phase. In the immediate period following wounding, CXCL4, CXCL8, and CCL2 regulate the actions of endothelial cells while CCL1, 2, 3, 4, 5, 7, and 8 attract various immune cells to the site of tissue injury, promoting the inflammatory response. In the proliferative phase, CXCL8 and CCL2 promote the synthesis of extracellular matrix (ECM) components by fibroblasts, while CXCL1, CXCL8, and CXCL11 direct the migration of keratinocytes to close the wound. ELR motif-containing chemokines CXCL2 and CXCL8 promote angiogenesis early in the proliferative phase, and a slightly later increase in the angiostatic chemokines CXCL4, CXCL9, CXCL10, and CXCL11 prevents ongoing angiogenesis. Finally, in the remodeling phase, the new tissue must be reorganized to meet the biomechanical needs of the tissue. The CXCR3 ligands CXCL10 and CXCL11 act on multiple cell types to signal the end of the remodeling phase, while CXCL12 induces the migration of epithelial progenitor cells to the newly formed tissue. CXCL1 (GROa) C-X-C motif-containing ligand 1, CXCL2 (GRO2) C-X-C motif-containing ligand 2, CXCL4 (PF4) C-X-C motif-containing ligand 4, CXCL8 (IL8) C-X-C motif-containing ligand 8, CXCL9 (MIG) C-X-C motif-containing ligand 9, CXCL10 (IP-10) C-X-C motif-containing ligand 10, CXCL11 (I-TAC) C-X-C motif-containing ligand 11, CXCL12 (SDF-1) C-X-C motif-containing ligand 12, CCL1 CC motif chemokine ligand 1, CCL2 (MCP-1) CC motif chemokine ligand 2, CCL3 (MIP-1α) CC motif chemokine ligand 3, CCL4 (LAG1) CC motif chemokine ligand 4, CCL5 (RANTES) CC motif chemokine ligand 5, CCL7 (MCP-3) CC motif chemokine ligand 7, CCL8 (MCP-2) CC motif chemokine ligand 8

The chemokine CXCL4 plays an important role in the initial process of coagulation and inhibits the formation of blood vessels from occurring too early in the healing process. CXCL4 weakly attracts immune cells, but strongly impacts hemostasis through multiple mechanisms. In the normal coagulation cascade, heparin binds to and activates antithrombin III through a conformational change. Activated antithrombin III then inactivates thrombin and other proteases. Thrombin promotes platelet activation and coagulation through the conversion of fibrinogen to fibrin. CXCL4 can prevent the formation of this heparin/antithrombin III complex, thus slowing the process of thrombin inactivation and ultimately promoting coagulation [53]. At the same time, CXCL4 acts to suppress coagulation through inhibition of Hageman factor [54, 55] and stimulation of activated protein C, an inhibitor of clotting factors [56]. Angiogenesis is the formation of new blood vessels from existing

microvessels. This process is critical for wound healing. CXCL4 acts against angiogenesis through inhibiting both proliferation and migration of endothelial cells [57]. CXCL4 interferes with the key pro-angiogenic factors VEGF and bFGF [58, 59]. In another mode of action, CXCL4 binds and activates the CXCR3B receptor, leading to endothelial cell apoptosis [60]. Finally, CXCL4 can alter the course of angiogenesis through interaction with integrin proteins, which act as adhesion receptors on endothelial cells. CXCL4 has been shown to inhibit endothelial cell adhesion and migration on fibronectin and vitronectin through interaction with avb3, avb5, and a5b1 integrins [61]. CXCL4 also acts by attracting and activating granulocytes by promoting the release of multiple lysosomal enzymes by neutrophils [62]. CXCL4 suppresses hematopoiesis through inhibition of both the proliferation and maturation of megakaryocyte progenitor cells [63, 64]. Also, CXCL4 promotes the adhesion and quiescence of progenitor cells through crosstalk with chondroitin sulfate molecules and other pro-hematopoiesis chemokines [65]. Other chemokines play a role in controlling the migration of incoming cells. Platelet degranulation during this phase results in the release of cytokines including TGF-β, PDGF, and VEGF that act on macrophages, keratinocytes, and fibroblasts. In response, these cells produce chemokines CXCL8 and CCL2 to induce migration of inflammatory and endothelial cells to the wound area.

Various growth factors, cytokines, and chemokines coordinate inflammation that takes place in this phase, which together attract multiple cell types to begin the process of healing. It is mostly the job of CXC chemokines that are stored in blood platelets and released upon injury to initiate this response. Early on, activated platelets release pro-migratory factors that attract leukocytes, mainly neutrophils, to the wound to eliminate pathogens [66]. Neutrophil expression of CXCR2 primarily coordinates this process, which attracts them in response to CXCL8 [67]. To potentiate these effects, arriving neutrophils secrete CXCL8, resulting in the recruitment of more neutrophils. Along with pro-migratory functions, CXCL8 inhibits apoptosis of neutrophils that have been attracted [68] and increases endothelial permeability to allow leukocyte migration [69].

Following the initial neutrophil response, the primary leukocyte cell type switches to monocyte lineages, partially due to the effects of IL-6-mediated signaling [66, 70]. Several CC family chemokines, including CCL1, CCL2, CCL3, CCL4, CCL5, and CCL7, coordinate the recruitment of monocytes [71]. Monocytes express CCR2, the receptor for CCL2, a chemokine, which is produced by both neutrophils and macrophages themselves [72]. Triggered by surrounding chemokines and cytokines, monocytes polarize into different macrophage phenotypes, which act to remove cell debris, present antigens, and produce various secreted factors. Similar to these monocytes, chemokines regulate the migration of mast cells, which express multiple receptors [73]. Mast cells produce proinflammatory factors upon degranulation and can release chemokines to stimulate fibroblast proliferation.

Finally, lymphocytes are recruited to the wound to modulate the immune response. CCL3, CCL4, and CCL5 attract lymphocytes to the wound [67]. B lymphocytes produce antibodies, and T lymphocytes produce vital cytokines and interact with antigen-presenting cells to provide host defense.

## Chemokines in the Proliferative Stage of Wound Healing

Toward the end of the inflammatory phase, the wounded area must begin to regenerate new tissue. This is achieved largely through the proliferation of resident fibroblasts, which produce the constituent elements of the dermal extracellular matrix. As growth factors activate fibroblasts, they take on a contractile phenotype that aids in wound closure. It is also crucial that angiogenesis takes place to support the growth and synthetic capacity of these fibroblasts. Thus, endothelial cells must be attracted to the wound for this phase to progress. Together, these endothelial cells, fibroblasts, and extracellular matrix form the granulation tissue. In turn, this new tissue allows for the migration of basal keratinocytes from the wound edge into the center of the wounded area. In this phase, chemokines influence migration of cells and tissue remodeling processes.

Fibroblasts can both produce chemokines as well as respond to them. TGF-β and PDGF from macrophages and activated platelets stimulate fibroblasts which then produce and secrete extracellular matrix proteins. CCL2, for instance, can promote fibroblast expression of TGF-β and collagen or enzymes related to matrix turnover such as MMP-1 and TIMP-1 [74, 75]. Similarly, the chicken ortholog of human CXCL8 induces fibroblasts to produce extracellular matrix components tenascin, fibronectin, and collagen and pushes them toward a contractile phenotype known as the myofibroblasts [76]. These myofibroblasts secrete alpha-smooth muscle actin along with other ECM components, allowing for cell contraction to aid in wound closure.

Angiogenesis is crucial to support the synthetic capabilities of fibroblasts and to deliver nutrients to the healed tissue over time. Cells in the wound area, including fibroblasts, macrophages, and keratinocytes, express the key growth factor VEGF. Proteolytic enzymes, such as MMPs, produced by fibroblasts in the wounded area also promote endothelial cell migration by degrading the ECM that they must pass through. As with all steps of the wound healing process, it is vital that angiogenesis stops at the appropriate stage when fibroblast granulation tissue has replenished the wound. An essential regulator of this process is a switch in the expression of pro-angiogenic to angiostatic chemokines is an important regulator of this process. ELR motif-containing chemokines, such as CXCL8 and CXCL2, are induced after wounding but begin to shut off after that [66]. In turn, chemokines lacking the ELR motif, including CXCL9, CXCL4, CXCL10, and CXCL11 start to increase at this time.

Re-epithelialization of the wound requires the correctly timed proliferation and migration of keratinocytes from the edges of the wound. Factors released at the wound edge trigger the proliferation of keratinocytes, including EGF, TGF-β, and FGF [77]. Chemokines that play a part in this process include CXCL8 and CXCL1. Keratinocytes, which express the receptor CXCR2, are attracted toward the denuded area of the tissue through the high expression of CXCL8 and CXCL1 [71]. In fact, studies in mice that do not express CXCR2 show functional deficits in re-epithelialization [78]. Basal keratinocytes also express CXCL11, a CXCR3 ligand. Secretion of CXCL11 promotes the migration of undifferentiated keratinocytes into the wounded area through activation of calpain [79, 80]. Mice deficient in CXCR3 signaling showed signs of impaired wound healing, including delayed re-epithelialization and impaired basement membrane formation [81].

## Chemokines in the Remodeling Stage of Wound Healing

The proliferative phase leaves behind a wound that is now sealed from environmental insult but that lacks the mechanical and biological features of native tissue. This results from reorganization of the provisional extracellular matrix and loss of cellularity, required for the earlier proliferative stage. The fibroblast-produced matrix must be broken down and reorganized, and collagen fibers must be appropriately cross-linked to regain tissue integrity. Fibroblasts are responsible for the synthesis of new extracellular matrix components and the enzymes to break down the provisional matrix. Fibroblasts synthesize ECM components that characterize mature tissue, such as fibronectin and collagens I and III [82].

The resolution phase included loss of cellularity, due to apoptosis and decreased migration by both fibroblasts and endothelial cells. During this phase, the myofibroblasts that became differentiated during the proliferative phase die through apoptosis [83]. The CXCR3 ligand chemokines CXCL9, CXCL10, and CXCL11 are especially crucial in the dermal and epidermal maturation phases. The cytokines IFN-γ and TNF-α induce these three angiostatic chemokines present in the wound during the resolution phase. CXCL10 inhibits EGF-dependent migration of fibroblasts by inhibiting the activity of calpain, which is required for detachment of the trailing edge of these cells [84]. CXCL11 is required for dermis-epidermis maturation and the development of mature basement membrane [81].

Regression of the neovasculature mainly stems from apoptosis of endothelial cells and action of the angiostatic chemokines [85, 86]. These chemokines exert their function specifically through the CXCR3-B receptor, a variant of the CXCR3 receptor expressed on endothelial cells [60]. Transduction through this signaling axis results in the inhibition of endothelial cell proliferation through a caspase-3-mediated mechanism. At the same time, CXCL10 binding to CXCR3 results in the dissociation of microvessels that have already assembled through activation of PKA and inhibition of m-calpain [85]. Thus, disruption of this intricate signaling axis leads to defects in wound repair in animal models of CXCR3-signaling deficiency (Fig. 1.3). CXCR3 null mice display wounds with hypercellular, fragile, collagen-deficient dermis [87]. Wounds in CXCR3 null mice have excessively high levels of disorganized collagen and MMP-9, which is associated with immature ECM [88]. It is largely fibroblast CXCR3 expression that dictates this healing response. When wild-type fibroblasts are transplanted into CXCR3 null mice following wounding, significant improvements in healing are observed, illustrating the importance of fibroblast CXCR3 [89].

Stem cell populations are critical for regaining native tissue structures, such as the hair follicle, during tissue repair. Chemokines can guide both mesenchymal stem cells (MSCs) and endothelial cells (EPCs) to sites of injury. Migration of MSCs is influenced by a wide array of chemokines and receptors, while CXCL12 acts as a major chemoattractant for EPCs [90, 91].

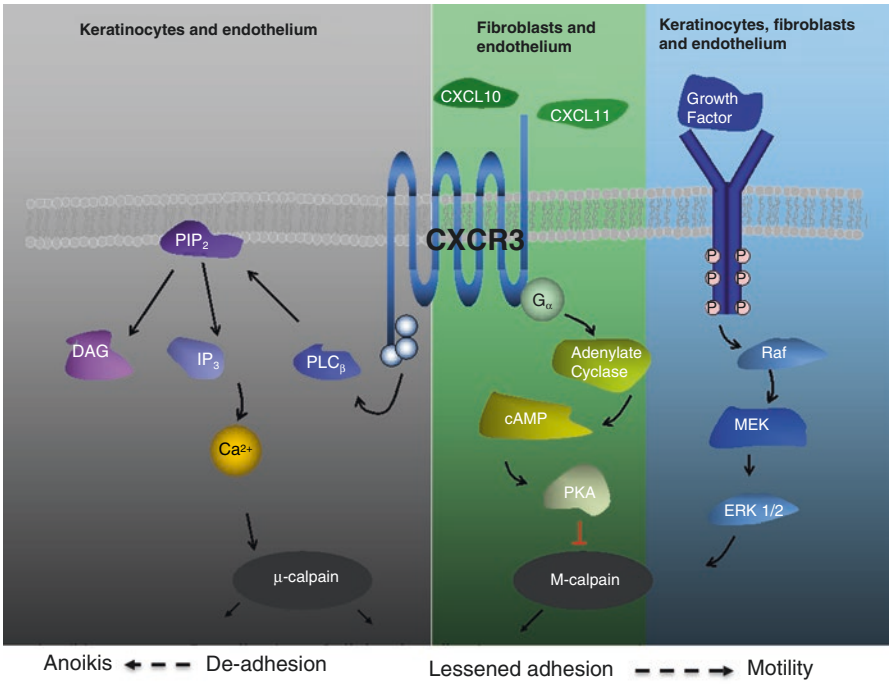


**Fig. 1.3** CXCR3 signaling axis during wound repair. The CXCR3 receptor is a seven transmembrane domain G-protein-coupled receptor. This signaling system is extant in human and rodent with the receptor being ubiquitous, and the ligands regulated temporally and spatially. Keratinocytes, fibroblasts, and endothelial cells express the CXCR3 receptor. Endothelial cells of the neovasculature produce CXCL10/IP-10 and appear in the dermis, while redifferentiating keratinocytes behind the leading edge of the wound express CXCL11/IP-9. These secreted peptide factors, both CXC chemokines that lack the canonical N-terminal sequence ELR (glutamic acid-leucine-arginine), bind in common to the ubiquitous CXCR3 chemokine receptor. CXCR3 signaling blocks growth factor-induced fibroblast and endothelial cell motility by suppressing m-calpain activation. In contrast, these chemokines do not block the motility of dedifferentiated keratinocytes but rather increase their motility via lessened adhesiveness that shifts the cell into the most permissive adhesion/contractility state and thus promotes motility and in turn more rapid re-epithelialization. For endothelial cells, in which the β3 integrin predominates, CXCR3 activation of calpain1/μ-calpain leads to detachment and anoikis

## Chemokines in the Fibrotic Phase of Healing

Chemokines are thought to be key regulators of pathophysiological hallmarks of tissue fibrosis. Several "major-cluster" chemokines are shown to be critical for tissue fibrosis and inflammation. There are subsets of chemokines whose genes are located in large clusters at particular chromosomal locations. This clustering is thought to indicate that the functionality and differential expression of chemokines in different tissues can be a part of a customized inflammatory response as previously mentioned. The two major clusters of CC (cysteine-cysteine) chemokine

genes and CXC genes have been identified as critical for tissue fibrosis (Table 1.1) [92]**.** Specifically, in Systemic Sclerosis (SSc), there is evidence of genetic association between the CXCL8 gene polymorphism and increased risk [35]. Increased presence of chemokines (i.e., CCL17, CCL22, CCL27) in the serum of patients with SSc has also been noted to contribute to skin sclerosis. CXC chemokines are also increased in the serum of patients with SSc, such as CXCL9 and CXCL10, which have an angiostatic role and are highly expressed in the skin of SSc patients compared to controls [85, 86]. Although chemokines have been identified in fibrosis, the gene regulation that translates the biological is still being explored.

Among this proposed regulation is the extracellular matrix-chemokine interactions fundamental to all phases of wound healing and are not limited to dermal maturation. Cells are regulated by chemokines to synthesize ECM proteins to meet the demands of the active remodeling wound environment [81, 87, 88]. Fibrosis results from the failure to transition from the regenerative phase to the resolving phase. The wound maintains a pro-stimulatory matrix with fibronectin and tenascin-C featured prominently and is relatively devoid of the suppressive SLRPs [88]. Along with the immature matrix, levels of proteases are elevated. These enzymes degrade the newly deposited collagen and generate chemotactic fragments that recruit inflammatory and stromal cells. This is discussed extensively in Chaps. 5 and 10. Robust data supports the notion that immunomodulated chemokine systems are mediators of both inflammatory and resident stromal cells. Resident cells,

**Table 1.1** Chemokine ligands, receptors, and their involvement in tissue fibrosis

| | Receptor | | |
|---|---|---|---|
| Chemokine | Agonist | Antagonist | Disease involved in |
| CXC family | | | |
| CXCL4 | CXCR3-B | | Liver fibrosis |
| | | | Scleroderma (SSc), renal interstitial fibrosis |
| CXCL4L | CXCR3-B | | Liver fibrosis, renal interstitial fibrosis |
| CXCL9 | CXCR3 | CCR3 | Liver fibrosis |
| | | | Scleroderma |
| CXCL10 | CXCR3 | CCR3 | Liver fibrosis |
| | | | Scleroderma |
| CXCL11 | CXCR3, CXCR7 | CCR3, CCR5 | Liver fibrosis |
| | | | Scleroderma |
| CXCL12 | CXCR4, CXCR7 | | Pulmonary fibrosis |
| | | | Scleroderma |
| CC family | | | |
| CCL2 | CCR2 | | Scleroderma |
| | | | Cardiac fibrosis |
| CCL5 | CCR1, CCR3, CCR5 | | Liver fibrosis |
| CCL17 | CCR4 | | Skin sclerosis |
| CCL22 | CCR4 | | Skin sclerosis |
| CCL27 | CCR10 | | Skin sclerosis |

specifically fibroblasts and endothelial cells, are central for replication and matrix synthesis, degradation, and vascular remodeling, all of which are characteristic of processes of fibrosis, and it is likely chemokines plays a significant role in the regulation of the cell matrix.

## Conclusion

The skin is a highly complex organ required for protection, homeostasis, and sensation. It is crucial that skin injuries heal in a quick and ordered fashion for the survival of the organism. Prompt and coordinated skin repair occurring in distinct healing phases must be completed to restore tissue structure and function.

The multiplicity of signaling that can be achieved by chemokines lends itself well to regulating these processes. Chemokines are a diverse family of secreted proteins, which can shape cell activities through surface receptors and functional interactions with extracellular matrix components. In the early steps of wound healing, pro-inflammatory and pro-angiogenic chemokines are required to attract immune cells and prime the area for the growth of granulation tissue and re-epithelialization. Later on, it is mainly chemokines that lack the ELR motif, which turn off these activities and allow for remodeling to proceed. The requirement for tight control of these systems is born out in the phenotypes of animals that lack the necessary signaling molecules and receptors.

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# Chapter 2
# Fibrosis and Immune Dysregulation in Systemic Sclerosis


**Yahya Argobi and Gideon P. Smith**

## Abbreviations

| | |
|---|---|
| ACEi | Angiotensin-converting enzyme inhibitor |
| AP-1 | Transcription factor activator protein-1 |
| ARA | RNA polymerase III antibody |
| AT1R | Angiotensin II type 1 receptor |
| ATX | Autotaxin |
| BAFF | B-cell-activating factor |
| BLM | Bleomycin |
| cGVHD | Chronic graft-versus-host disease |
| Col3a1 | Type III collagen |
| CTGF | Connective tissue growth factor |
| CTLA-4 | T lymphocyte-associated antigen 4 |
| DAMPs | Damage-associated molecular patterns |
| ECM | Extracellular matrix |
| ET-1 | Endothelin-1 |
| ETAR | Endothelin-1 type A receptor |
| Fli1 | Friend leukemia integration 1 |
| FN-1 | Fibronectin |
| Fra-2 | Fos-related antigen-2 |
| IFN | Interferon |
| ILD | Interstitial lung disease |
| IPF | Idiopathic pulmonary fibrosis |
| JAKs | Janus kinases |
| Klf5 | Kruppel-like factor 5 |

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| | |
|---|---|
| LPA | Lysophosphatidic acid |
| MMP | Metalloproteinases |
| mRSS | Modified Rodnan skin score |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PDGF | Platelet-derived growth factor |
| PDGFR | Platelet-derived growth factor receptor |
| PPAR- | Peroxisome proliferator-activated receptor gamma |
| ROS | Reactive oxygen species |
| RP | Raynaud's phenomenon |
| RXR | Retinoid X receptor |
| Scl-70 | Topoisomerase 1 |
| SMA | Smooth muscle actin |
| SSc | Systemic sclerosis |
| STATs | Transducers and activators of transcription |
| TGF-B | Transforming growth factor-B |
| Th | T helper cell |
| TLRs | Toll-like receptors |
| TNF | Tumor necrosis factor |
| Treg | Regulatory T cell |
| TSK-1 | Tight skin 1 |
| TSK-2 | Tight skin 2 |
| uPAR | Urokinase-type plasminogen activator receptor |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VEGF | Vascular endothelial growth factor |

## Introduction

### *Epidemiology and Clinical Background*

Systemic sclerosis (SSc) is a chronic connective tissue disease of unknown origin, characterized by vascular abnormalities, autoimmunity, and excessive sclerosis of the skin and other internal organs. The prevalence of SSc is 150–300 cases per million. SSc is more common in women than in men [1]. SSc is subcategorized into two major clinical subtypes: limited and diffuse. Limited SSc is characterized by skin hardening that is limited to the fingers, hands, and face. In contrast, in late-stage diffuse SSc, generalized skin hardening occurs. However, phenotypically diffuse SSc may be difficult to distinguish from the limited form in early disease as it also starts in the fingers and hands before it progressively spreads, with time involving the forearms, arms, trunk, face, and lower extremities. Although the most common symptom at presentation and diagnosis is when sclerosis develops in the fingers and hands, many patients experience a prodromal phase of non-pitting edema and erythema before skin hardening development. All SSc patients experience Raynaud's phenomenon (RP), an episodic vascular spasm, most commonly of the hands, and usually cold-induced. This is manifested by color changes of the affected part, classically

described as a three-phase sequence of white, blue, and red, although many SSc patients have only two out of this sequence. Some patients will also experience RP in the toes, ears, nose, or nipples and can have other triggers such as emotional stress. SSc patients are more likely to experience digital ulceration and ischemia with Raynaud's attacks than patients with primary RP. In limited SSc, patients often present with a long history of RP, often decades. In contrast, in patients with diffuse SSc, the onset of RP typically occurs within a year of the onset of skin sclerosis [2]. SSc has a heterogeneous clinical presentation which reflects the complex pathogenesis. It is associated with variable involvement of major internal organs including the lungs, kidneys, gastrointestinal tract, and heart. In the past renal crisis was a significant cause of mortality. However, this has diminished with greater knowledge of its screening and use of angiotensin-converting enzyme inhibitors (ACEi). At present, pulmonary fibrosis and pulmonary arterial hypertension are the leading causes of death in SSc. Recent advances have been made in SSc pathogenesis and treatment. However, current therapies are disappointing, and the mortality rate is still high. The recent studies in animal models and the progress in revealing possible pathophysiologic abnormalities in SSc have led to a new era of promising targeted therapy, likely moving the field away from general immunosuppressive therapy and more toward specific anti-cytokine therapy with better efficacy and lower rates of side effects [3].

## *Pathogenesis: Genetic and Environmental Factors*

SSc pathogenesis is characterized by three main pathways: endothelial injury and vasculopathy, inflammation and immune imbalance, and fibroblast activation and sclerosis (Fig. 2.1). The temporal relation between these events is still not clear, but recent investigations suggested that endothelial injury is the initiating process leading to vasculopathy with subsequent sclerosis accompanied by autoimmunity [4]. This would fit with the clinical observation that Raynaud's often occurs very early in disease before significant organ morbidity.

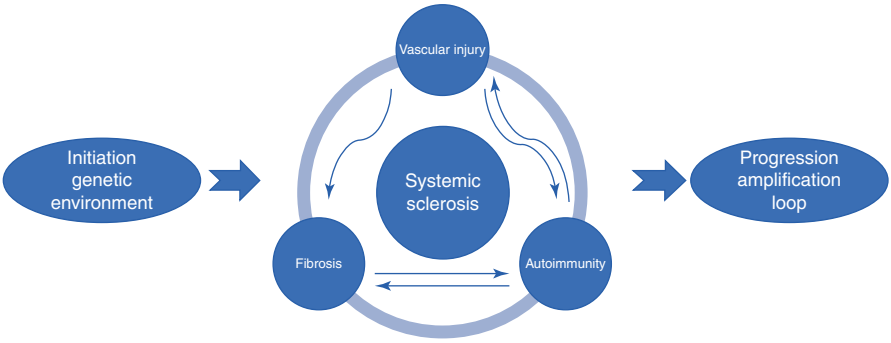


**Fig. 2.1** Etiopathogenesis of systemic sclerosis. The pathogenesis of SSc is believed to be initiated by a vascular injury, followed by an autoimmune response leading to skin and internal organ sclerosis. Tissue sclerosis will result in more tissue injury and generate amplification loops

SSc appears to occur in genetically susceptible individuals, and there is some evidence to suggest that its expression is influenced by environmental factors. Evidence for a genetic predisposition comes from studies showing specific human leukocyte antigens and single nucleotide polymorphisms. However, concordance rates were found to be extremely low in monozygotic twins [5]. The genetic factors are therefore insufficient to completely explain the development of SSc, suggesting environmental factors also play a role in SSc via, e.g., DNA methylation, histone modification, and miRNA regulation. Epigenetics could thus explain the link between inherited genetic factors and environmental factors.

Indeed, occupational exposure to crystalline silica and solvents has been strongly associated with SSc. Exposure to vinyl chloride is similar to SSc, with associated Raynaud's phenomenon, skin thickening, and acro-osteolysis. However, in most cases the trigger for SSc is still unknown but research remains ongoing [6–8].

## Animal Models

While each of the SSc animal models helps delineate one or more potential pathways, due to the complexity of the clinical disease, there is no single model that accurately recapitulates all of the aspects of SSc pathogenesis. Most commonly, models lack the pathological cascade of SSc from initial vascular injury to resultant tissue sclerosis associated with immune imbalance. Older models, including the bleomycin-induced fibrosis mouse and the tight skin mouse, focused mainly on skin fibrosis and sclerosis in SSc. Although UCD-200 and UCD-206 chicken models recapitulate most aspects of human SSc pathogenesis (vasculopathy, inflammation, autoantibodies, and subsequent fibrosis), due to the cost and difficulty of working with larger models and the limited knowledge of its molecular basis, it is less widely adopted than the mouse models.

Newer models like urokinase-type plasminogen activator receptor-deficient mice and double heterozygous mice for friend leukemia integration 1 (Fli1) and Kruppel-like factor 5 (Klf5) genes are useful in studying the underlying vasculopathy. In the following section, some of the models that contributed to our understanding of SSc pathogenesis are discussed [9, 10].

### *Bleomycin-Induced Skin Fibrosis Mouse Model*

The bleomycin-induced skin fibrosis model was established by Yamamoto et al. [11]. It is among the widest used models and used extensively for skin and lung fibrosis in SSc. Bleomycin (BLM) is a chemotherapeutic agent used in the treatment of Hodgkin's lymphoma, squamous cell carcinomas, testicular cancer, and malignant pleural effusion. BLM is inactivated by a cytosolic cysteine proteinase enzyme, bleomycin hydrolase, which is widely distributed in normal tissues with the exception of the skin and lungs [12]. BLM was noted to induce lung fibrosis after

intratracheal administration during cancer treatment. Repeated high doses of subcutaneous injections of BLM have been used to induce lung fibrosis, in addition to dermal fibrosis, in mice. Subcutaneous injections of BLM induced dermal sclerosis, which was histologically characterized by thickened homogenous collagen bundles, thickened vascular walls, and inflammatory infiltrates. The skin sclerosis was apparent only histologically but not so obvious clinically. Transforming growth factor-β (TGF-β) was expressed in the sclerotic dermis and mononuclear cells mainly consisted of CD4 positive T cells and macrophages [11]. BLM induces similar histologic changes to SSc including activation of fibroblasts, dermal sclerosis, myofibroblasts, collagen accumulation, infiltration of mononuclear cells into the lesional skin, and development of antinuclear antibodies [9, 10]. BLM may also induce reactive oxygen species (ROS) leading to endothelial vascular injury followed by inflammation and subsequent secretion of profibrotic cytokines. Zhou CF. et al. showed that BLM induces the epithelial-mesenchymal transition in the sclerotic skin. They also found that blocking of ROS production by N-acetyl-L-cysteine, a well-known direct scavenger of oxygen free radicals, significantly attenuated BLM-induced oxidative injury, epithelial-mesenchymal transition, and collagen production. However, N-acetyl-L-cysteine didn't significantly inhibit TGF-β expression in BLM-induced skin sclerosis, suggesting that antioxidants may be a potential therapeutic agent for BLM-induced skin sclerosis [12]. BLM also activates fibroblast and leads to collagen accumulation [13]. Autoreactive CD4 T cells have been implicated in autoimmunity induction in the BLM mouse model [14]. A recent study found that BLM enhanced TGF-β1 expression, suppressed adipogenesis through TGF-β1, and promoted adipocyte fibrosis. This study suggested that adipocytes may have an inhibitory effect on the progression of fibrosis [15].

## *Tight Skin-1 Mouse Model*

The tight skin (Tsk-1) mouse is another widely adopted model of SSc, characterized by a partial duplication within the fibrillin gene. Although the homozygous Tsk-1 mice are embryonic lethal, the heterozygous Tsk-1 is associated with dermal sclerosis, activation of fibroblast, and collagen synthesis [16]. Skin sclerosis involves the deposition of collagen fibrils and microfibrils. Extracellular microfibrils were increased in the dermis in localized and systemic sclerosis [17]. The altered fibrillin leads to TGF-β cascade activation and subsequent increased collagen production, but the detailed molecular mechanisms underlying the activation of fibroblasts remain unknown. Altered fibrillin is associated with altered extracellular matrix (ECM) composition, including collagen fibrils and microfibrils deposition, and with increased numbers of dermal fibroblasts containing high levels of procollagen mRNA. Lemaire et al. found that increased deposition of type I collagen into the ECM independent of both altered Colα1(I) mRNA expression and type I collagen secretion. They also found that the deposition of microfibril-associated glycoprotein 2, a protein which is associated with fibrillin 1, was also altered in the skin of patients with SSc [18]. Tsk-1 is associated with autoantibodies such as anti-DNA

topoisomerase-1 but lacks an early inflammatory infiltrate. Thus, the Tsk-1 model features are slightly different from those in SSc. Of note, thickening occurs in the Tsk-1 hypodermis, rather than in dermis itself. The histologic changes in the lungs are of more similar to emphysema rather than pulmonary fibrosis. Also, there are no vascular changes detectable in Tsk-1 mice models in comparison with SSc [16].

## *Tight Skin-2 Mouse Model*

This model was generated in 1995 by administration of ethylnitrosourea, characterized by a gain-of-function missense mutation in Type III collagen (*Col3a1*), located on chromosome 1 [19, 20]. Tight skin-1 (Tsk-2) mice showed increased collagen content and expression of type I collagen genes. Histologically, there was marked thickening of the dermis and excessive deposition of thick collagen fibers, which extended deeply into the subdermal adipose tissue [19]. In contrast with Tsk-1, a mononuclear cell infiltrate was present in the lower dermis and in the adipose tissue septa. Studying autoimmunity in Tsk-2 showed that 93% of the Tsk-2/+ models were positive for anti-Scl70, 82% for anti-centromere, 5% for anti-RNP/Sm, and none for anti-RNA-polymerase II Abs [21]. As for Tsk-1, it lacks vascular changes. Tsk-1 and Tsk-2 models were used in studies of both skin and lung fibrosis in SSc.

## *Fibrosis in Transgenic Mice with Wnt/B-Catenin Gain of Function*

The Wnt/β-catenin pathway is implicated in fibrotic diseases including liver fibrosis, renal interstitial fibrosis, idiopathic pulmonary fibrosis, and SSc [22–25]. Fibroblasts from different tissues respond to Wnt/β-catenin signaling. Studies suggested that Wnt/β-catenin activates fibroblast and myofibroblast and leads to collagen synthesis. Mice with forced activation of Wnt/β-catenin demonstrated increased myofibroblasts and induction of fibrosis. Inhibition of Wnt/β-catenin on mice ameliorated fibrosis. This model is used to study mechanisms and treatments of myofibroblast-driven fibrosis which may or may not be as relevant in SSc [25, 26] but may be more appropriate in diseases such as nephrogenic systemic fibrosis.

## *Urokinase-Type Plasminogen Activator Receptor-Deficient Mice*

Manetti et al. reported this mouse model in 2014. Urokinase-type plasminogen activator receptor (uPAR) plays a vital role in the fibrinolytic system, ECM remodeling, and angiogenesis. It is also known as (CD87 antigen) and expressed by monocytes, endothelial cells, and fibroblasts. It is important in fibroblast-to-myofibroblast

transition and accumulation of ECM components in the skin. Patients with SSc have decreased expression of uPAR in the skin. uPAR has been implicated in endothelial cleavage and in SSc vasculopathy. $uPAR^{-/-}$ mice showed increased dermal thickness and accumulation of thickened closely packed collagen bundles, in addition to abundant perivascular collagen accumulation in the dermis. This model demonstrated a marked increase in myofibroblast counts and strong expression of profibrotic cytokines including TGF-β, connective tissue growth factor (CTGF), and endothelin-1 (ET-1) in the dermis. Subcutaneous fat tissue was partly replaced by connective tissue with perivascular fibrosis and perivascular inflammatory cell infiltrates. $uPAR^{-/-}$ mice also had a reduced number of dermal microvessels and increased apoptosis of endothelial cells. This model is associated with the development of dermal sclerosis, pulmonary fibrosis, and peripheral vasculopathy but lacks the immunological aspect of this disease. This model closely reproduces pathological features of SSc and is therefore a promising model to study the interplay between vascular changes and tissue fibrosis [27].

### *Fos-Related Antigen-2 Transgenic Mice*

Fos-related antigen-2 (Fra-2) is a member of the Fos family, a component to the transcription factor activator protein-1 (AP-1). The AP-1 is a dimeric transcription factor composed of the Fos (c-Fos, Fra-1, Fra-2, FosB) and Jun (c-Jun, JunB, JunD) subfamilies. AP-1 regulates various biological activities including cell proliferation, apoptosis, inflammation, wound healing, and cancer by binding to the AP-1 sites located in the promoters of target genes [28]. Fos-related antigen-2 expression is markedly increased in the skin tissue of SSc animal models, including BLM-induced mice and Tsk-1 mice. The expression of Fra-2 was increased in skin biopsies of SSc patients compared with healthy controls, especially in endothelial and vascular smooth muscle cells. Fra-2 transgenic mice initially developed apoptosis of capillary endothelial cells at around age 9 weeks followed by a severe loss of small blood vessels in the skin that is paralleled by progressive skin fibrosis at 12 weeks of age [29]. Pulmonary arterial occlusion occurs at 12 weeks of age, followed by pulmonary fibrosis in another few weeks [28, 29]. Therefore, pulmonary involvement of Fra2 transgenic mice is similar to the pulmonary interstitial and vascular changes of SSc. However, there was no pulmonary occlusive venopathy detected in this model. In SSc lesional skin, myofibroblasts express Fra-2. Silencing Fra-2 normalizes type I collagen production in these cells suggesting a Fra-2 role in activating fibroblasts in SSc. Stimulation with TGF-β and platelet-derived growth factor (PDGF) increased the expression of Fra-2 in SSc fibroblasts and induced DNA binding of Fra-2. Deletion of Fra-2 decreased the stimulatory effects of TGF-β and PDGF and subsequently decreased collagen production from SSc fibroblasts [30]. In addition to the skin and lung, the cardiac tissue in Fra-2 transgenic mice displayed histological features similar to SSc myocardial tissue including cardiomyocyte damage with contraction band necrosis, endothelial cell apoptosis, decreased capillaries,

myofibroblastic differentiation, and collagen accumulation in the myocardium. Furthermore, overexpression of Fra-2 in the myocardium was observed in autopsy subjects with SSc [31]. The Fra-2 model is similar to SSc in both vasculopathy and fibrosis, and like the uPAR model, it is a promising model to study the interplay between vascular changes and tissue fibrosis in SSc; although it is important to point out that Fra-2 also lacks the immunological aspect of SSc.

### *Double Heterozygous Mice for Friend Leukemia Integration 1 and Kruppel-Like Factor 5 Genes*

This model was developed by Asano (2015). Fli1 is an inhibitor of TGF-β and subsequently collagen 1 expression. Friend leukemia integration 1 (Fli1) expression is repressed in SSc fibroblasts and endothelial cells. Kruppel-like factor 5 (KLF5) is a potent repressor of the CTGF gene, and its deficiency leads to the induction of CTGF expression. Fli1 deficiency alone does not affect the transcription of the CTGF gene, but simultaneous downregulation of KLF5 and Fli1 synergistically stimulates the CTGF gene transcription [32]. Mice with a homozygous deletion of both Klf5 and Fli1 die in utero. However, mice with heterozygous deficiency of both Klf5 and Fli1 spontaneously develop features of SSc. $Fli1^{-/-}$ murine embryonic fibroblasts exhibited a robust expression of fibrillar collagens. $Klf5^{+/-}$ and $Fli1^{+/-}$ mice showed a marked increase in dermal thickness and collagen content. The dermal collagen fibrils were irregular and thick, and mRNA levels of the Col1a1, Col1a2, and CTGF genes were significantly increased in $Klf5^{+/-}$and $Fli1^{+/-}$ mice compared with wild-type mice. A decrease in the number of subcutaneous vascular density was observed in 2-month-old mice. Also, reduced arteriolar blood flow velocity and interstitial hypoxia was evident in the skin of $Klf5^{+/-}$ and $Fli1^{+/-}$ mice. Additionally, pulmonary arterioles develop markedly thickened vascular walls with the expansion of alpha-smooth muscle actin (α-SMA)-positive cells at 8 months of age [33]. Interestingly, these mice are associated with B-cell activation and autoantibody production. $Klf5^{+/-}$ and $Fli1^{+/-}$ mice sequentially develop immune activation, vasculopathy, and tissue fibrosis, as seen in patients with SSc. This mouse model recapitulates the main features of SSc and could be useful to study SSc pathogenesis and to develop new drugs.

## Vascular Injury

Many authors believe that vascular injury is the primary player in the pathogenesis of SSc [34, 35] and several studies implicated ROS as a primary mediator of disease. ROS are important in different metabolic pathways, including endothelial vascular injury, immune dysregulation, and stimulation of TGF-β with resultant collagen production and fibrosis. Vascular injury leads to vascular tone

dysfunction, ischemia, and tissue hypoxia. Studies suggested that overproduction of ET-1 may underlie endothelial injury in SSc [34]. ET-1 is a potent vasoconstrictor peptide produced by vascular endothelial cells and is a mediator of vascular proliferation, inflammation, and fibrosis. ET-1 plasma levels were found to be elevated in SSc patients and levels correlated with the number of digital ulcers [35–38]. This led to the consideration of bosentan as a therapy for scleroderma. Bosentan is an ET-receptor antagonist. Two randomized clinical trials showed that bosentan reduced the number of new digital ulcers in patients with SSc [39, 40].

Stimulating autoantibodies against both angiotensin II type 1 receptor (AT1R) and endothelin-1 type A receptor (ETAR) were reported in SSc [41]. These receptors are expressed on endothelial cells and fibroblasts and can induce TGF-β production, IL-8, and vascular cell adhesion molecule-1 (VCAM-1). The latter leads to T-cell chemotaxis and collagen production, and this pathway represents a potential link between vascular injury, immune dysregulation, and fibrosis. These effects are reduced by addition of AT1R and ETAR blockers [42].

Another potential link is vascular endothelial growth factor (VEGF), a potent proangiogenic molecule. Vascular endothelial growth factor is elevated in SSc patient sera and skin biopsies [43]. Mouse models show that high levels of VEGF induced fibrosis in inflammatory and noninflammatory stages of SSc, with insufficient angiogenesis [44]. Derrett Smith et al. reproduced the SSc-pulmonary arterial hypertension (PAH) by inhibiting VEGF, in a mouse model characterized by a ligand-dependent upregulation of transforming growth factor (TGF), suggesting the link between TGF and VEGF signaling [45]. Treatment with bevacizumab, an antibody against VEGF, prevented the bleomycin-induced dermal fibrosis [46].

## Sclerosis

Sclerosis is the accumulation of ECM components in the dermis of the skin and tissues of other organs (Fig. 2.2). ECM is composed mainly of collagen, elastin, hyaluronic acid, proteoglycans, heparin, and chondroitin sulfates, among other molecules. ECM provides structural support and is important in cell signaling, communication, and adhesion. Sclerosis is determined by two main processes, the synthesis and the degradation of the ECM, with collagen being the most abundant component in sclerosis. ECM synthesis is mainly conducted by activated fibroblasts, whereas ECM degradation is performed by metalloproteinases (MMP) that lead to collagen cleavage [47]. Anti-MMP-1 antibodies and anti-MMP-3 antibodies were found in SSc, inhibiting MMP-1 and MMP-3 collagenase activity, respectively, and subsequently reducing ECM degradation and lead to accumulation of ECM [48, 49]. Profibrotic cytokines implicated in SSc include TGF-β, CTGF, plasminogen activator inhibitor-1 (PAI-1), fibronectin 1 (FN-1), and IL-6 [50–53].

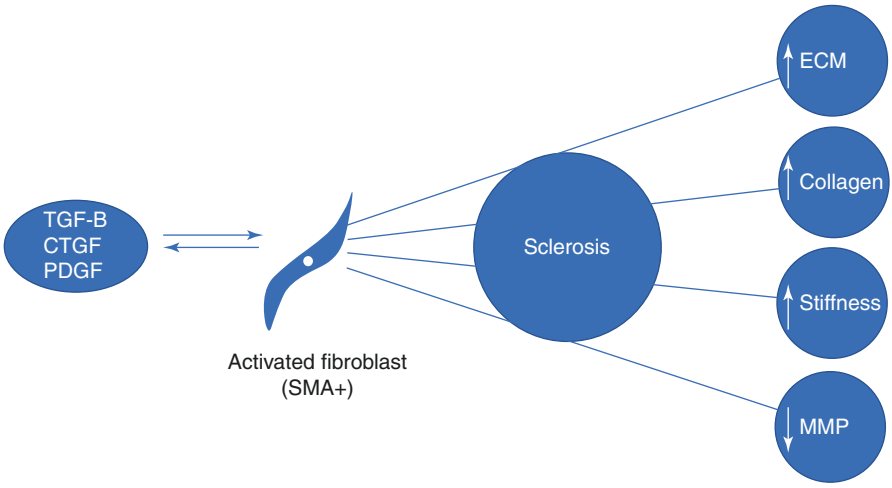


**Fig. 2.2** Accumulation of ECM components in tissues leads to sclerosis. Fibroblasts are activated (express alpha-smooth muscle actin) by TGF-β, CTGF, and PDGF. Activated fibroblasts stimulate collagen production, inhibit MMP, and result in tissue and organ sclerosis

## Cell Types Involved in Systemic Sclerosis

### *Fibroblasts*

Fibroblasts are the most common cells found in the connective tissues of most organs. They are spindle-shaped cells that express vimentin but not desmin or α-SMA. Fibroblasts are activated by cytokines and growth factors, developmental pathways, ET-1, and thrombin. They can secrete cytokines and chemokines and express cell surface integrin adhesion molecules and receptors for TGF-β, PDGF, and CTGF [54]. Sclerosis in SSc results primarily from activated fibroblasts and myofibroblasts rather than from overproliferation of fibroblasts. The overproduction of type I collagen in SSc results mainly from the abnormally activated transcription of *COL1A1* [55]. Increased deposition of collagen I, III, V, and VII are found in the dermis of SSc, along with elevated lysyl hydroxylase and lysyl oxidase levels (enzymes that catalyze posttranslational collagen) [56–59]. The effect of TGF-β on SSc patient lesional skin fibroblasts acts to transdifferentiate them into myofibroblasts. Myofibroblasts are specialized fibroblasts that acquire characteristics of smooth muscle cells, including the expression of α-SMA. Myofibroblasts can also transdifferentiate from other cells including pericytes, endothelial cells, fibrocytes, and adipocytes (Fig. 2.3). They are considered the principal effector cells in fibrosis. They produce collagen and lead to increased ECM deposition and contractile tension within fibrotic tissues [60]. They are essential for connective tissue homeostasis and wound repair. Under normal circumstances they resolve from the site of injury. However, in fibrotic conditions, including SSc, they persist. Histological

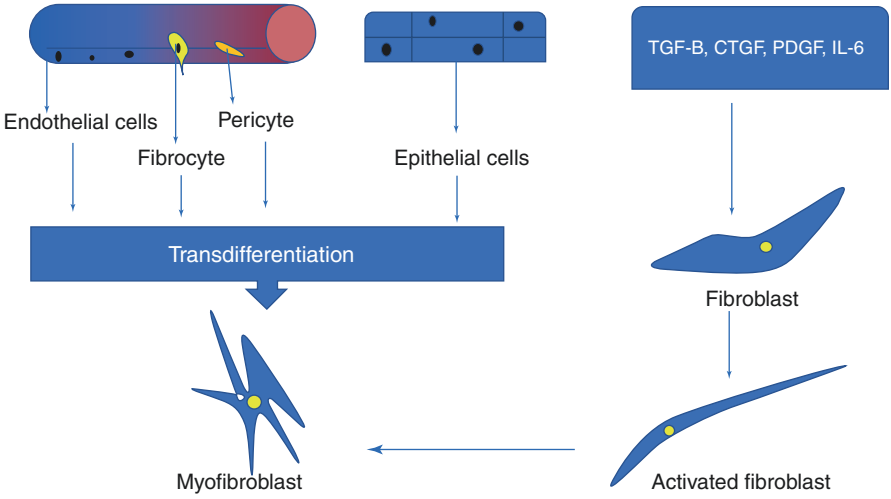


**Fig. 2.3** Myofibroblasts are the primary effector cells in systemic sclerosis. Myofibroblasts originate from activated fibroblasts and can also transdifferentiates from other cells including pericytes, fibrocytes, endothelial cells, and epithelial cells

analysis of lesional skin and fibrotic tissues from SSc patients showed an abundance of myofibroblasts [61]. A primary vascular injury along with immune response can cause persistent fibroblast activation and profibrotic events with subsequent sclerosis. Once initiated, sclerosis can escalate leading to progressive injury through amplification loops of profibrotic processes. This vicious circle generates more tissue damage, accumulation of damage-associated molecular patterns (DAMPs), increased dermal stiffness, hypoxia, and ROS, all of which lead to further fibroblast activation and myofibroblast transdifferentiation [62].

## *Macrophage*

Research over the past decade revealed strong evidence that macrophages/monocytes are involved in the pathogenesis of SSc; however, their exact role in the disease is still not fully clear. The presence of macrophage signature observed in the skin, blood, and lungs of SSc patients is quite obvious [139]. Macrophages are important effector cells that can influence the immune system. They are classified into M1 (classic)- and M2 (alternative)-activated macrophages. The classic M1 macrophage has enhanced microbicidal and tumoricidal activity, associated with interferon (IFN)-γ and tumor necrosis factor (TNF) signals, and secretes inflammatory cytokines. The M2 alternatively activated macrophage has anti-parasitic response and associated with Th2 environment and Il-4 secretion [140]. M1 macrophages can result in robust inflammation and tissue injury, whereas M2 macrophages are normally involved in wound repair and can induce fibrosis [139]. M1 macrophage has

many markers including CD64, CD86, TLR2, TLR4, IL-12, and IL-6. M2 macrophage also has many markers including macrophage scavenger receptor 1, CCR2, CD204, CD163, IL-10, CCL22, CCL18, CCL13, and CCL17 [139]. Macrophages were detected between collagen bundles and around skin adnexa and blood vessels in SSc patients [141]. In the lesional skin from SSc patients, the number of CD163+ cells and CD204+ cells between the collagen fibers was significantly higher than that in healthy controls. Flow cytometry showed that the population of CD14+ cells was larger in the peripheral blood mononuclear cells derived from SSc patients [142]. CD14 is mainly expressed by macrophages, although it can also be expressed by other cells including neutrophils and dendritic cells. Mathes et al. studied global chemokine expression in systemic sclerosis and found that chemokines, CCL18, CCL19, and CXCL13, were upregulated in SSc skin. Interestingly, CCL19 expression correlated with vascular inflammation in SSc skin. CCL19 might play a role in the recruitment of macrophages and immune cells in the SSc skin [143]. CCL2 has been implicated in SSc pathogenesis; it was reported to be higher in sera of SSc patients and upregulated in SSc patient lesional skin. Additionally, it was strongly correlated with the modified Rodnan skin score (mRSS) [144, 145]. The above evidence of macrophage involvement in SSc pathogenesis, particularly with M2-activated macrophages, should encourage further research in this area.

## *B Cells*

B cells are responsible for antibody and cytokine production, antigen presentation, and immunoregulation. B cells are believed to play a central role in the pathogenesis of SSc. B-cell functional abnormalities have been implicated in the onset and progression of many systemic autoimmune disorders including SSc. Lesional skin and affected lung tissues from patients with SSc showed B-cell infiltration. Also, B-cell-related genes were upregulated in SSc skin lesions [146]. B cells serve as antigen-presenting cells, which can also induce dendritic cell maturation through cell contact and promote profibrotic Th2 differentiation [147].

Autoantibody production is one of the earliest signs of SSc. In addition to autoantibody production, hypergammaglobulinemia and polyclonal B-cell hyperactivity are observed in SSc patients. Around 95% of patients with SSc have specific autoantibodies against intranuclear antigens including topoisomerase 1 (Scl-70), centromere, and the RNA polymerase III (ARA). Those autoantibodies are diagnostic, but they are unlikely to be pathogenic. They mainly reflect T-cell activity against their antigenic targets. Recent studies demonstrated that B cells not only produce these autoantibodies but are also chronically activated and secrete cytokines that may contribute to vascular injury and collagen production in SSc patients [148].

CD19 expression is regulated during the B-cell activation and correlates positively and with the production of autoantibodies. CD20 levels are normal in SSc. CD19 expression levels are higher on B cells from SSc patients. However, the increase in CD19 expression is small (20%) [4]. Autoantibody levels are

spontaneously increased in CD19-transgenic mice, and they are generally reduced in CD19-deficient mice [149]. BLM-treated wild-type mice developed dermal and lung fibrosis, hypergammaglobulinemia, anti-topo I production, and the profibrotic cytokines interleukin-6 and TGF-β1, all of which were inhibited by CD19 deficiency [150]. A phase I multicenter, randomized, double-blind, placebo-controlled study using MEDI-551, an investigational humanized monoclonal antibody that targets CD19 and mediates antibody-dependent and cell-mediated cytotoxicity of B cells, included 28 subjects with SSc. The drug was well-tolerated and safe; however, one patient died from renal crises believed to be unrelated to the drug. B-cell depletion and plasma cell depletion were achieved and were dose-dependent. A mild clinical effect was observed, mild improvement of Rodnan skin score (mRSS) in affected skin [151]. A prior study using the same drug in SSc identified reduced B-cell and plasma cell gene signatures which correlated with reduced collagen gene expression in skin lesions [152]. Further well-designed, randomized, double-blind, placebo-controlled clinical studies are required to clarify the potential of this drug as a therapeutic option in SSc.

B-cell-activating factor (BAFF) is a member of the TNF superfamily and exerts important homeostatic functions on B cells including maturation, activation, and survival [153]. Serum BAFF levels were elevated in TSK+ mice, and a BAFF antagonist inhibited the development of skin sclerosis and the autoantibody production. The BAFF antagonist suppressed the upregulated expression of profibrotic cytokines including IL-6 and IL-10 [154]. François et al. reported elevated BAFF in IPF patient bronchoalveolar lavage. In addition, BAFF levels were elevated in BLM-induced lung fibrosis, and BAFF inhibition attenuated pulmonary fibrosis and IL-1β levels. BLM-induced BAFF expression and lung fibrosis both were IL-1β and IL-17A dependent in that mouse model, suggesting that BAFF act as an amplifier of IL-17A production by T cells [155].

B cells also negatively regulate the immune reaction through specific regulatory B cells. Regulatory B cells inhibit inflammation and differentiation of TH1 and TH17 cells by inducing Tregs and production of IL-10. Regulatory B cells were reduced in SSc patients and inversely related to SSc activity [156, 157].

B-cell-depleting therapy with an anti-CD20 monoclonal antibody (rituximab) in SSc has shown limited improvement in skin and lung fibrosis in human studies. B-cell depletion with anti-CD20 monoclonal antibody in newborn TSK mice reduced skin fibrosis, autoantibody production, and hypergammaglobulinemia but not in adult mice with established disease, suggesting that B-cell depletion was beneficial after early disease onset in Tsk mice during the inflammatory phase but not so much in the late fibrotic phase [158]. A large, multicenter, observational trial using the EUSTAR database showed beneficial effects of rituximab (RTX) on skin and lung fibrosis in patients with SSc compared with matched-control SSc [159]. This study included 63 patients treated with RTX. The primary analysis was the Rodnan skin score (mRSS) change from baseline to follow-up. The secondary analysis was the change in FVC and safety measures. There were 46/63 SSc patients, 35 had diffuse SSc and 11 limited SSc, and the disease duration was 5 (3–10) years.

After a follow-up of 7 (5–9) months, the mRSS decreased from 18.1 ± 1.6 to 14.4 ± 1.5 ($p = 0.0002$). In patients with severe diffuse SSc ($n = 25$), the mean mRSS decreased from 26.6 ± 1.4 to 20.3 ± 1.8 ($p = 0.0001$) after 6 (5–9) months follow-up. This study showed a promising therapeutic effect of RTX on skin and lung sclerosis in SSc patients; a prospective randomized, double-blinded trial is needed to confirm the efficacy of RTX in SSc.

## *T Cells*

Accumulating evidence suggests that SSc is a T-cell disease. T helper (Th) cells were observed predominantly around blood vessels in the dermis of SSc patients. However, Th cells represent nonhomogeneous subpopulations of Th1, Th2, Th9, Th17, and regulatory T (Treg) cells [171]. Th cells are important mediators of the immune response in fibrotic diseases (Fig. 2.4). Th1 cells secrete cytokines including IFN-γ, IL-2, and TNF-α. They promote pro-inflammatory cell-mediated immune responses. On the other hand, Th2 cells secrete cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13. They are responsible for humoral-mediated immunity. Some Th2 cytokines, including IL-4, IL-13, and TGF-β, participate in ECM accumulation by activating collagen deposition, whereas Th1 secretes IFN-γ which has an opposite

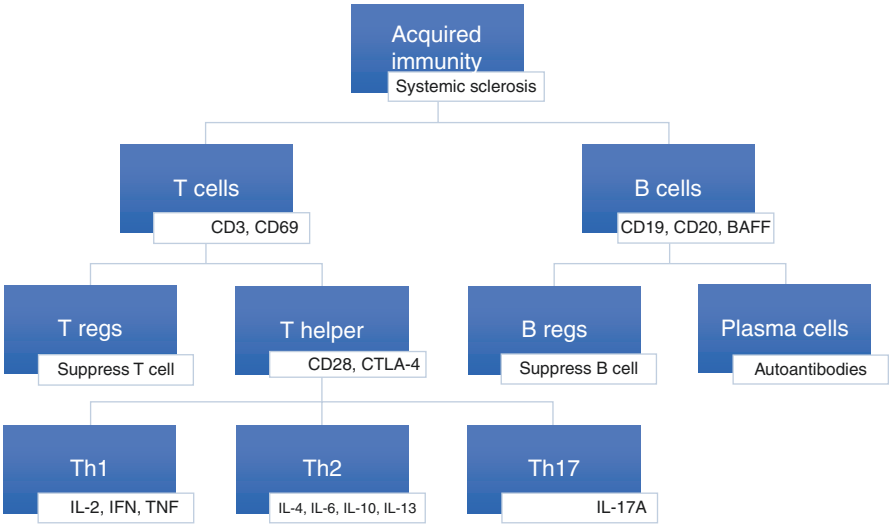


**Fig. 2.4** Systemic sclerosis is considered a T-cell disease. Systemic sclerosis (SSc) is regarded as a T-cell disease. However, B cells (lymphocytes) play an essential role in the initiation and progression of the disease. B-cell depletion from rituximab has shown some therapeutic effect in SSc patients. Th2 cytokines stimulate ECM production, while Th1 through IFN secretion has an antifibrotic effect. Recent studies identified dysfunctional Tregs and Th17 in the pathogenesis of SSc (see text for details)

response [172]. T-cell abnormalities from patients with SSc are contradictory. Some studies have shown decreased levels of T cells, while other studies showed increased levels of both T-cell subsets. However, most studies suggest involvement of T cells in SSc pathogenesis. Activation of T cells occurs early in the course of SSc, even before microscopic evidence of fibrosis. Skin biopsy specimens from patients with SSc had increased expression of CD3, a T-cell marker, and CD69, an early T-cell activation marker. The expression of those markers was more enhanced in the early inflammatory phase of the disease suggesting that T cells play a key role in SSc initiation [173]. A recent cohort study on 47 SSc patients showed a reduction in the number of total lymphocytes, total T cells, and CD8+ and activated/memory CD8+ T cells in the peripheral blood from patients with SSc compared to healthy controls. This study suggests that T cells and CD8+ T cells may have a relevant role in the pathogenesis of SSc [174]. In the early stage of SSc and before sclerosis occurs, the affected tissue exhibits an inflammatory infiltrate composed of macrophages, mast cells, and T cells, suggesting that T cells play a role in the initiation of the disease. Some studies demonstrate that CD4+ T cells were the predominant cell type in the inflammatory infiltrate of the lesional skin in SSc patients [175, 176]. Another recent study found that CD8+ T cells are numerous in the sclerotic skin of SSc patients in the early stage of the disease, whereas CD4+ T cells predominated in the late stage of the disease and strongly suggested that IL-13 and CD8+ T cells play an important role in dermal sclerosis [177]. Skin biopsies and sera from SSc patients after 7–8 years of disease onset did not show significant expansion of total T cell and CD4+ and CD8+ T cell [178]. Increased numbers of memory T cells were observed in lung biopsies from SSc patients with alveolitis and interstitial lung fibrosis [179]. In SSc, T cells respond to an unknown antigen and differentiate to a Th2 type. This Th2 shift leads to the secretion of many cytokines including IL-4, IL-13, and IL-6, which induce fibrosis, either directly or indirectly, stimulate macrophages and the secretion of TGF-β, and increase ECM deposition in the affected tissues [180, 181].

Recent studies explored the important role of Treg cells in SSc. Tregs are a subset of T cells that counterbalance the activity of T helper cells. Some studies report decreased Treg numbers in SSc, while other studies demonstrated an increased number of Tregs. However, most studies suggested that Tregs are dysfunctional in SSc irrespective of their number. MacDonald et al. reported differentiation of human Treg cells into Th2 cytokine-producing cells in sera and skin biopsies of SSc patients [182].

Current immunosuppressive strategies to treat patients with systemic sclerosis are largely disappointing. However, targeted therapies based on better understanding of the pathogenesis may lead to improved outcomes. Targeting T-cell activation may suppress autoimmunity associated with SSc and subsequent tissue sclerosis. Abatacept is a soluble fusion protein that consists of the extracellular domain of human cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) linked to the modified Fc portion of human immunoglobulin G1. Thus, abatacept inhibits T-cell activation by binding to CD80 and CD86 and blocking interaction with CD28. Levels of soluble CTLA-4 (sCTLA-4) were elevated in the sera from patients with the

diffuse SSc, and sCTLA-4 correlated with the extent of skin sclerosis. Furthermore, patients with elevated sCTLA-4 levels had a shorter disease duration and more frequent presence of digital pitting scars [183]. Abatacept inhibited dermal fibrosis in experimental chronic graft-versus-host disease (cGVHD) models. Abatacept treatment started on the first day of BLM challenge prevented the induction of BLM-induced dermal sclerosis and reduced dermal thickness of established BLM-induced sclerosis. There was a marked reduction in T-cell, B-cell, and monocyte infiltration and IL-6 and IL-10 levels in lesional skin in mice treated with abatacept. And to prove its effects on T cells, abatacept did not exert antifibrotic properties in CB17-SCID mice challenged with BLM, but it has been reported that dermal fibrosis was successfully induced in these mice lacking T cells. Additionally, abatacept exerts no antifibrotic effect in Tsk-1 mice, a noninflammatory model of SSc [184]. These findings suggest that abatacept exerts antifibrotic action by inhibiting T cells. Recently, a placebo-controlled randomized trial of abatacept in patients with diffuse cutaneous systemic sclerosis was published. In this study, ten subjects were randomized to abatacept ($n = 7$) or placebo ($n = 3$). In the abatacept group, disease duration from first non-Raynaud's symptom was $2.4 \pm 1.6$ years. Five out of seven patients (71%) randomized to abatacept and one out of three patients (33%) randomized to placebo experienced ≥30% improvement in Rodnan skin score (mRSS) [185]. Larger studies on patients with early SSc disease are warranted to further determine the safety and effectiveness of this drug.

## Signaling Mediators

### *TGF-β*

TGF-β is a pleiotropic cytokine that is considered the principal regulator of both wound healing and pathological fibrosis and is a potent profibrotic molecule considered the primary mediator of skin sclerosis in SSc. Most cells are capable of both secreting and responding to TGF-β including macrophages and fibroblasts. TGF-β is secreted as latent precursors bound to the latency-associated peptide. Binding of the latent TGF-β to integrins activates TGF-β and subsequent fibroblast activation [63]. Integrins especially αVβ3 and αVβ5 are overexpressed in fibroblasts in SSc. TGF-β mediates its effects through binding of the TGF-β receptors, and downstream signaling occurs through the canonical Smad pathway leading to gene transcription [64]. Activation of fibroblasts by TGF-β results in stimulation of collagen synthesis, production of CTGF, upregulation of matrix gene expression, and myofibroblast transformation.

Bortezomib, a drug that inhibits TGF-β signaling, prevented BLM-induced lung and skin fibrosis in mice [65]. Interestingly, a recent study on mice showed that systemic blockade of TGF-β with high doses of a blocking monoclonal antibody had no protective effect against pulmonary fibrosis [66]. However, there was partial protection against pulmonary vasculopathy [66]. In 2014, the FDA approved

pirfenidone and nintedanib, for the treatment of patients with idiopathic pulmonary fibrosis. Both drugs act by downregulating the expression or intracellular signaling by TGF-β [67–69]. An open-label phase II study of pirfenidone in patients with SSc-associated interstitial lung disease (ILD) showed improvement of ILD with changes in skin thickening [70]. Pirfenidone gel used in 12 patients with localized scleroderma had positive responses in both the inflammatory and fibrotic phases [71]. Nintedanib inhibited the endogenous activation of SSc fibroblasts and prevented BLM-induced skin fibrosis [72]. These two drugs are useful in idiopathic pulmonary fibrosis (IPF); further studies are needed to investigate their therapeutic effects in SSc-ILD and SSc-dermal sclerosis.

### *Connective Tissue Growth Factor*

Connective tissue growth factor (CTGF), also known as CCN2, is a cysteine-rich secreted protein of the CCN family of immediate-early genes. CTGF was found to be overexpressed in many conditions including liver cirrhosis, keloids and hypertrophic scars, pulmonary hypertension and fibrosis, and SSc [73]. TGF-β is thought to induce fibrosis in the early stage, while CTGF maintains tissue fibrosis in the chronic stage. CTGF is one of the downstream mediators of TGF-β, mediating TGF-β-profibrotic effect. It promotes fibroblast proliferation and collagen and ECM production [74]. Also, it plays a role in cellular adhesion, cell migration, and angiogenesis [75, 76]. CTGF expression was found elevated in serum and skin lesions of patients with SSc, and its serum level correlates with the extent of skin sclerosis and severity of lung fibrosis [77]. In a transgenic mouse model, CTGF maintained TGF-β-induced skin fibrosis by sustaining COL1A2 promoter activation and increasing the number of activated fibroblasts [78]. Xiao et al. showed that silencing CTGF expression via RNA interference led to inhibition of CTGF and subsequent inhibition of collagen I and collagen III [79]. TGF-β has broad activities, and its blockage might lead to many side effects. Therefore, targeting downstream mediators including CTGF could be a better therapeutic approach for SSc.

### *Platelet-Derived Growth Factor*

Platelet-derived growth factor (PDGF) is a primary mitogen and chemoattractant that regulates the proliferation, migration, survival, and differentiation of mesenchymal progenitor cells that give rise to diverse types of stromal cells. It is secreted by a variety of cells including platelets, monocytes, and vascular endothelial cells. Mesenchyme-derived cells, fibroblasts, smooth muscle cells, and pericytes, are all responsive to PDGF. PDGF binding induces PDGF receptor (PDGFR) tyrosine kinase activity, activating downstream signaling pathways including Ras/MAPK, PI3K/AKT, PLCγ/PKC, Src family kinases, and STATs [80]. PDGF signaling is

associated with human diseases including cancer, atherosclerosis, and fibrosis [81]. PDGF plays a critical role in stimulating the replication, survival, and migration of myofibroblasts during the pathogenesis of fibrotic diseases and is implicated in the pathogenesis of scleroderma. Remarkably, PDGF is almost undetectable in healthy skin or lung [82]. Levels of PDGF were elevated in bronchoalveolar lavage fluid from SSc patients [83] and downregulation of PDGFR mRNA resulted in blockade of the PDGF signaling and inhibited fibroblast transdifferentiation to myofibroblasts in skin lesions of SSc patients [84]. Baroni et al. reported that stimulatory autoantibodies to the PDGFR were found in all 47 patients with SSc in the study. These antibodies selectively induced Ha-Ras-ERK1/2 and ROS accumulation. Furthermore, PDGFR autoantibodies stimulated type I collagen gene expression and myofibroblast phenotype conversion in normal human primary fibroblasts [85]. Makino et al. evaluated the potential efficacy of crenolanib as a potential therapeutic agent for SSc using patient-derived dermal fibroblasts and a murine model of angiotensin II-induced skin and heart fibrosis. Crenolanib besylate is a well-tolerated, selective inhibitor of type III tyrosine kinases (PDGFRα, PDGFRβ, and FMS-like tyrosine kinase 3); it has been clinically evaluated for treatment of glioma and gastrointestinal stromal tumors. Crenolanib resulted in reduced skin and heart fibrosis in vivo by inhibiting accumulation of PDGFR-positive collagen-producing fibroblasts. Additionally, crenolanib effectively inhibited proliferation and migration of SSc and healthy control fibroblasts and attenuated TGF-β-induced expression of CTGF [86]. Since crenolanib is already used in clinical trials with good safety records, it has potential as a therapeutic agent for patients with SSc. PDGF signaling pathways were activated in pulmonary vessels of Fra-2 transgenic mice. Treatment with nilotinib prevented the development of proliferative vasculopathy and lung fibrosis in Fra-2 transgenic mice [87]. Nilotinib is a second-generation tyrosine kinase inhibitor that was developed for patients with treatment-refractory chronic myeloid leukemia and with intolerance to imatinib. A recent open-label, pilot clinical trial was performed on patients with early diffuse SSc treated with nilotinib. Patients had early and active diffuse SSc with median disease duration of 0.7 years. The Rodnan skin score (mRSS) improved by a mean of 4.2 points (16%) at 6 months and by 6.3 points (23%) at 12 months in seven patients who completed the study. Two patients withdrew from the study due to mild QT prolongation; other than those complications, the drug was well-tolerated. Large, multicenter, double-blind, randomized controlled trials are needed to assess the efficacy and safety of this drug in this complex disease [88].

## *Adenosine*

Adenosine, an endogenous purine nucleoside, plays a vital role in inflammation, tissue remodeling, and fibrosis. It has a pathogenic role in human SSc and the BLM-induced dermal fibrosis model, stimulating dermal fibroblasts to increase collagen production and dermal fibrosis via adenosine receptor ($A_{2A}R$) activation [89]. A small study showed that expression of adenosine $A_{2A}R$ is increased threefold in dermal fibroblasts isolated from SSc patients ($n = 5$) to healthy controls ($n = 4$) [90]. A

pharmacological $A_{2A}R$ receptor antagonist prevented the development of dermal fibrosis by reducing dermal collagen in a mouse model with elevated tissue adenosine [91]. Adenosine is generated from adenine nucleotide intracellularly or from released ATP by nucleoside triphosphate diphosphohydrolase (CD39) and ecto-5′-nucleotidase (CD73) extracellularly. CD39- and/or CD73-deficient mice treated with BLM had decreased dermal myofibroblasts and collagen content [92]. Therefore, targeting adenosine production and ($A_{2A}R$) activation may be a useful therapy in SSc.

## *Lysophosphatidic Acid*

Lysophosphatidic acid (LPA) is a bioactive lipid molecule essential for a variety of cellular and developmental processes including motility, chemotaxis, cell cycle progression, viability, and wound healing [93]. The dysregulation of LPA signaling is implicated in cancer, atherosclerosis, ischemia, and fibrosis. LPA is highly selective and specific to a class of G protein-coupled receptors called LPA receptors ($LPA_{1–6}$) [94]. $LPA_1$ plays a key role in lung and kidney fibrosis [95, 96]. A study using a BLM-induced mouse model showed that LPA1 KO mice were markedly resistant to dermal fibrosis. This study also showed that pharmacological antagonism of $LPA_1$ attenuated BLM-induced dermal fibrosis [97]. Autotaxin (ATX) is an enzyme that hydrolyzes the abundant extracellular lysophosphatidylcholine to synthesize LPA and free choline. A recent report identified elevated dermal ATX in a bleomycin-induced model. These studies identified that the use of an ATX inhibitor led to markedly attenuated BLM-induced dermal fibrosis when treatment was initiated before or after the development of fibrosis. Interestingly, the same study found that LPA induces IL-6 expression, and IL-6, in turn, induces ATX expression, leading to additional LPA production in SSc dermal fibroblasts compared with control dermal fibroblasts [98]. $LPA_1$ antagonists are promising drugs in SSc treatment, and human trials are woefully needed.

## *Peroxisome Proliferator-Activated Receptor-γ*

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a nuclear hormone receptor that plays a key role in regulating a wide variety of physiological functions including lipid metabolism, glucose homeostasis, inflammation and innate immunity, and, more recently, connective tissue homeostasis. PPAR-γ is as ligand-activated transcription factor that is critical in regulating mesenchymal cell biology and tissue remodeling, expressed in many cells including adipocytes, macrophages, T cells, B cells, platelets, epithelial cells, endothelial cells, smooth muscle cells, and fibroblasts. PPAR-γ functions as a heterodimer with the retinoic acid-inducible retinoid X receptor (RXR), translocating to the nucleus where it binds to PPAR response elements (PPRE) to induce gene transcription [99]. Research over the past decade implicated PPAR-γ in fibrosis and SSc. PPAR-γ is an important component of the antifibrotic defense mechanism in human tissues, as such abnormalities in PPAR-γ

are associated with fibrosis in various organs including the skin, lung, liver, heart, kidney, and pancreas. PPAR-γ expression is regulated by multiple cytokines [100], for example, TGF-β is a potent inhibitor of PPAR-γ expression in fibroblasts [101]. In addition, TNF-α, IFN-γ, IL-1, IL-13, Wnt ligands, leptin, and CTGF, all suppress PPAR-γ expression [102–106]. On the other hand, fatty acids, eicosapentaenoic acids, docosahexaenoic acids, nitrolinoleic acid, and LPA activate PPAR-γ expression [107]. TGF-β negatively regulated the expression and function of PPAR-γ, whereas PPAR-γ ligands directly disrupted TGF-β signal transduction and suppressed TGF-β production [108]. PPAR-γ has potent antifibrotic effects, and its expression and activity are impaired in patients with SSc. Recent studies investigated the effect of PPAR-γ agonism in SSc-cultured fibroblasts and mouse models and confirmed its antifibrotic action as a potential new therapy for SSc [109, 110]. Adiponectin, which is a sensitive and specific index of PPAR-γ activity, inversely correlated with skin fibrosis in sera from SSc patients. Also, skin biopsies had reduced adiponectin mRNA expression, which was inversely correlated with the Rodnan skin score. A PPAR-γ agonist resulted in marked induction of adiponectin expression in explanted mesenchymal cells in vitro [111]. PPAR-γ-deficient mice showed enhanced responsiveness to BLM-induced sclerosis, with increased dermal thickness, increased collagen production, and a greater appearance of myofibroblasts, and enhanced Smad3 phosphorylation. Additionally, fibroblasts in PPAR-γ-deficient mice had enhanced sensitivity to the TGF-β-mediated increase in expression of α-SMA and type I collagen [112]. PPAR-γ agonist reduced TGF-β and attenuated renal interstitial fibrosis and inflammation in tubulointerstitial fibrosis mouse model [113]. Wei et al. reported decreased PPAR-γ levels in SSc skin and lung biopsies. Decreased PPAR-γ was identified in unstimulated fibroblasts explanted from the lesional skin. Expression of PPAR-γ target genes in lesional skin was reduced. In addition, TGF-β suppressed PPAR-γ expression in normal fibroblasts and completely prevented adipogenic differentiation in both subcutaneous preadipocytes and dermal fibroblasts [114]. Rosiglitazone is an insulin-sensitizing agent widely in type 2 diabetes mellitus treatment and exerts its biological effects partially via PPAR-γ. In BLM-induced sclerosis mice, rosiglitazone attenuated the severity of dermal sclerosis, collagen accumulation, subcutaneous lipoatrophy, and inflammation. Furthermore, rosiglitazone decreased tissue myofibroblasts and downregulated TGF-β in lesional skin [115]. Taken together, these studies indicate that PPAR-γ agonists, already in clinical use for the treatment of type 2 diabetes mellitus, attenuated fibrosis, suggesting their potential as promising antifibrotic drugs in SSc.

## *Retinoids*

Retinoids are vitamin A derivatives and have been used therapeutically in dermatological conditions since the 1970s, most commonly in the treatment of acne and hyperkeratotic diseases. Retinoid receptors are important in the regulation of cell metabolism, cell growth, and immunity and are targets in cancer therapy [116]. They also play a role in collagen metabolism and remodeling by inhibiting collagen

transcription and production [117]. Several case reports and case series showed improvement of skin thickening in SSc patients treated with retinoids. However, those studies were limited by their small size, and we still lack randomized controlled trials [116]. Toyama et al. investigated the effects of tamibarotene on dermal fibrosis, an agonist for retinoic acid receptor α/β used for the treatment of acute promyelocytic leukemia relapsed from or refractory to all-trans-retinoic acid. The study showed attenuated dermal fibrosis in BLM-treated mice and attenuated hypodermal fibrosis in tight skin-1 mice. Tamibarotene also suppressed several profibrotic molecules in lesional skin of BLM-treated mice including TGF-β, CTGF, IL-4, IL-10, IL-13, and IL-17A. In vitro, tamibarotene reversed the profibrotic phenotype of treated dermal fibroblasts and suppressed ICAM-1 expression in endothelial cells [118]. This suggests that tamibarotene may be a useful therapy in SSc.

## *Innate Immunity Signaling*

The innate immune system is activated in the onset phase and the progression of SSc. Innate immune signaling is implicated not only in the immune dysregulation associated with SSc but also in tissue fibrosis. Monocytes and macrophages among other inflammatory cells are recruited to sites of injury. Toll-like receptors (TLRs) are important factors in eliciting this immune response when signaled by microbial or viral antigens, but they also recognize endogenous molecules and have been implicated in autoimmune diseases. TLRs are activated in SSc and trigger an inflammatory reaction and collagen production [119, 120]. Different types of TLR have been implicated in SSc pathogenesis including TLR2, TLR4, TLR5, TLR8, TLR9, and TLR10. TLR4 was elevated in sera and skin biopsies of patients with SSc [121]. Bhattacharyya et al. investigated TLR4 in SSc, in vivo and in vitro, and found that TLR4 increased the sensitivity of fibroblasts to transforming growth factor-β1 and enhanced collagen synthesis [122]. In a subsequent study, dermal and lung fibrosis was attenuated in BLM-treated TLR4 knockout mice [123]. TLR9 was found in over 60% of myofibroblasts in SSc lesional skin biopsies. TLR9 activates TGF-β and induces collagen production [124]. TLR5 may have suppressive effects on collagen expression. TLR5 and TLR10 expression was increased in SSc fibroblasts in vitro and in vivo, probably via the TGF-β/Smad3 activation [125]. Targeting TLRs by neutralizing antibodies might be possible, but only for those on the cell surface, including TLR2, TLR4, and TLR5. Small molecule antagonists are another option to target TLRs. However, these inhibitors might block multiple TLRs and therefore give rise to unwanted immunosuppression [126]. TLRs induce secretion of inflammatory cytokines including TNF and IL-6, which have proven to be excellent targets for rheumatoid arthritis and other inflammatory diseases. Ibudilast (AV4II), a TLR4 antagonist, suppressed pro-inflammatory cytokines including TNF-α and IL-6 in neuroinflammation [127]. Antibodies that target TLR4, NI-0101, and IA6 (Novimmune, Geneva, Switzerland) are being investigated for the treatment of acute and chronic inflammation [128]. Further research is needed to understand the role of TLRs in fibrosis and possibly to develop a targeted therapy.

## *Interferon*

Increased expression and activation of interferon (IFN)-regulated genes were detected in the peripheral blood and skin biopsy samples of patients with SSc. Type I IFNs including IFN-α, IFN-β, and IFN-ω alleviate the effects of viral and bacterial infections in the innate immune system. IFNs are important in immune cell differentiation and proliferation and inflammatory cytokine production [129]. Activated type I IFN system was implicated in autoimmune diseases including systemic lupus erythematosus (SLE) and Sjögren syndrome. Recently, activated type I IFN system was detected in the sera of SSc patients. Digital ulcers including digital loss were associated with increased serum levels of IFN alpha. The plasmacytoid dendritic cells were the main source of IFN alpha production [130]. Also, serum IFN-γ levels were higher in SSc patients when compared to controls. Higher serum IFN-γ levels are associated with pulmonary hypertension in SSc patients [131]. IFN-λ1, also known as IL-29, is a newly described cytokine and member of type III interferon family and is also associated with SSc. IFN-λ1 is known for its immune-regulatory, antiviral, antiproliferative, and antitumor functions. IFN-λ1 and IFN-γ levels in SSc patients were higher than those in healthy individuals [132]. Progressive pulmonary fibrosis in SSc-related ILD is associated with upregulated expression of TGF-β and IFN-regulated genes [133]. The development of SSc was reported in patients after IFN-α therapy [134]. A randomized, placebo-controlled, double-blind trial, where 35 patients with early scleroderma received subcutaneous injections of either IFN-α or placebo, showed that IFN-α treatment in SSc is harmful. There was a greater improvement in the skin score in the placebo group, and there was also a greater deterioration in lung function in the IFN-α group [135]. Taken together, targeting IFNs, IFN-α in particular, may be useful in treating SSc patients. Sifalimumab, a fully human immunoglobulin $G_1$ κ monoclonal antibody that binds to and neutralizes most IFN-α subtypes, was recently evaluated in a phase IIb study in patients with moderate to severe SLE and showed broad efficacy and could be a promising therapy for refractory SLE [136]. Anifrolumab (also known as MEDI-546) is an investigational human IgG1κ mAb directed against subunit 1 of the type I IFN receptor and may also be a useful therapy [137]. A recent phase I open-label clinical trial was completed in 34 adult SSc patients who received anifrolumab. The expression of upregulated type I IFN-related genes in skin and blood cells was normalized. The drug was safe and well-tolerated in this study, but given the importance of type IFNs against viral infections, further clinical studies are needed to ensure its safety and efficacy. A subsequent study of anifrolumab administration in 47 SSc patients is associated with downregulation of T-cell-associated proteins and upregulation of type III collagen degradation marker. This study showed suppressive effects of anifrolumab on T-cell activation and collagen accumulation and could lead to progress in treating SSc and other autoimmune diseases [138]. A larger, double-blind, placebo-controlled trial might approve its usefulness in SSc.

### *Interleukin-6*

Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates cellular proliferation, activation, and differentiation of numerous cell types. It has been involved in many biological effects including immunoregulation, T-cell differentiation, angiogenesis, and osteoclast formation and is synthesized by a wide variety of cells. Elevated IL-6 expression is found in a variety of diseases, including RA, Castleman's disease, and osteoporosis [160, 161]. IL-6 signaling is implicated in many fibrotic diseases including renal fibrosis, GVHD, idiopathic lung fibrosis, liver fibrosis, keloids, and SSc [162, 163]. IL-6 signaling consists of two distinct modes: classical IL-6 signaling and IL-6 trans-signaling. IL-6 receptor (IL-6R), also known as CD126, contains the ligand-binding site, which is transmembrane IL-6R or soluble IL-6R. IL-6 trans-signaling was implicated in rheumatoid arthritis, in Crohn's disease, and recently in SSc [160]**.** The classical IL-6 signaling occurs in lymphocytes and hepatocytes which express IL-6R and gp130. However, neither IL-6R nor IL-6 binds to gp130 alone. IL-6R is restricted to hepatocytes and leucocytes. However, gp130 receptor is expressed on multiple cell types including the liver, lung, placenta, spleen, kidney, and heart [161]. The IL-6 trans-signaling occurs when the soluble IL-6R, released by proteolytic cleavage of membrane-bound IL-6R or by translation from an alternatively spliced mRNA, binds with IL-6 through gp130 and forms an agonistic complex. This complex initiates a signal transduction cascade through Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) [164]. Classical and trans-signaling of IL-6, both lead to activation of STAT3. STAT3 plays a role in the regulation of various cellular processes, including development and proliferation. Once they are activated, they form a homodimer and translocate into the nucleus where they bind the DNA and activate transcription of target genes [160, 164]. Although STAT3 is implicated in fibrosis and collagen production, the mechanism is still not clear. A recent study showed that STAT3 enhanced TGF-β signaling [165]. Khan et al. identified elevated IL-6 in SSc patients, which suggests that elevated serum IL-6 levels may identify a subgroup of diffuse SSc cases with poor outcome in terms of skin involvement at 3 years from disease onset. This study also showed the role of IL-6 trans-signaling in inducing dermal sclerosis via direct activation of dermal fibroblasts [166].

Elevated IL-6 levels are found in SSc serum and lesional skin, especially in the early stages of the disease. Also, IL-6 levels correlated tightly with skin thickness scores. Serum IL-6 level was predictive of early disease progression and mortality in SSc-ILD [167, 168]. Targeting IL-6 by both passive and active immunization prevented the development of bleomycin-induced dermal fibrosis in Tsk1 mice [169]. Tocilizumab (anti-IL-6 receptor monoclonal antibody) has proved to be an effective therapy for various autoimmune inflammatory diseases such as rheumatoid arthritis, polyarticular and systemic juvenile idiopathic arthritis, and multicentric Castleman's disease [167]. Recently, a phase II, double-blind, placebo-controlled randomized trial of tocilizumab (162 mg administered subcutaneously per week) in

87 patients with early SSc (<5 years from first non-Raynaud's symptom) has been published. The study showed that skin sclerosis did not differ between groups but did decrease more in the tocilizumab group than in the placebo group, and lung function showed a clinically relevant improvement [170].

## *Interleukin-4*

Interleukin-4 (IL-4) is mainly produced by Th2 cells but also can be secreted by other cells including natural killer T cells, mast cells, eosinophils, and basophils [186]. Recently, IL-4 was implicated in the development of SSc. IL-4 binding results in JAK1 and JAK3 activation, which result in phosphorylation of the cytoplasmic tyrosines, followed by STAT6 phosphorylation and activation, leading to gene transcription [187]. Activation of JAK-STAT pathway induces Th2 cell differentiation and proliferation, synthesis of immunoglobulin E, macrophage activation, and fibroblast collagen production [172]. IL-4 is a profibrotic cytokine involved in the fibrotic process, activates the fibroblasts, induces myofibroblasts, and stimulates ECM production and deposition [172]. Serum IL-4 level was higher in patients with SSc than in the controls [188]. IL-4 promotes collagen synthesis by increased transcription of the collagen mRNA in cultured human fibroblasts [189]. Kodera et al. reported that disrupting of one or both IL-4 alleles rescues homozygous TSK/TSK mice that generally die by day 9 of gestation. IL-4 mediates fibrosis at least in part by increasing the expression of the TGF-β mRNA in fibroblasts [190]. Neutralizing anti-lL-4 antibodies to Tsk/+ mice prevented the development of dermal sclerosis, leading to normalization of dermal collagen content [191]. A recent study in cardiac fibrosis found that the number of fibroblasts and macrophages was reduced after neutralizing IL-4, suggesting that IL-4 may exert its profibrotic effect via mechanisms that involve actions on both fibroblasts and macrophages [192].

## *Interleukin-13*

Interleukin-13 (IL-13) is a Th2 cytokine that promotes IgE class switching and inhibits pro-inflammatory cytokines. IL-13 is also an essential mediator in fibrosis and has been involved in many fibrotic diseases including interstitial lung disease, liver cirrhosis, and SSc [172]. Binding of IL-13 and its receptor activates JAK-STAT and TYK-STAT pathways, followed by STAT6 phosphorylation and activation, which lead to changes in gene transcription that induces epithelial cell mucus metaplasia, airway smooth muscle contraction, IgE synthesis, and macrophage activation [193]. IL-13 serum levels were higher in the sera of patients with SSc [194]. IL-13 stimulates collagen I production in primary cultured airway

fibroblasts by activating JAK/STAT6 signal pathway in a BLM-induced model [195]. IL-13 was found to be a potent stimulator and activator of TGF-β1 in vivo and also demonstrated that its fibrogenic effects are mediated, in great extent, by TGF-β activation [196]. Skin lesion levels of both IL-13 and its receptor were increased and correlated with SSc progression, in the murine model of BLM-induced sclerosis [197]. Imatinib mesylate, a tyrosine kinase inhibitor with a demonstrated activity against c-Abl, c-kit, and PDGFR, has been disappointing in SSc treatment [198]. A human anti-IL-13 antibody (CAT-354) was developed and being investigated in adults with asthma. Research of anti-Il13 antibody on mouse model and human SSc is needed [199].

### *Interleukin-17*

T helper 17 (Th17) cells are a subset of T cells implicated in the pathogenesis of fibrogenic, inflammatory, and autoimmune diseases, which produce IL-17A and IL-17F, in addition to IL-21, IL-22, IL-23, and IL-26 [200]. IL-17 is a pro-inflammatory cytokine with effects on epithelial, endothelial, and fibroblast cells [201]. Patients with SSc have increased frequencies of circulating Th17 cells. However, the circulating IL-17 was not detectable, which is similar to rheumatoid arthritis and psoriasis, two autoimmune diseases in which IL-17 play a key role in their pathogenesis. Levels of Th17 inducing cytokines including IL-6, IL-1α, and IL-23 were higher in the sera of SSc patients [202]. Yang et al. found that IL-17 is involved in fibroblast growth and collagen production. These studies also suggested that increased circulating Th17 cells and increased infiltration of IL-17 in skin lesions may be associated with SSc progression [203]. Upregulation of IL-17A mRNA was found in SSc patients compared with healthy individuals. IL-17A is derived from SSc patient serum-mediated endothelial cell inflammation by upregulating chemokines and adhesion molecules, suggesting that it might play a key role in the endothelial injury in SSc [171]. Another study showed IL-17A derived from patients with SSc was observed to induce proliferation, collagen synthesis, and migration of dermal vascular smooth muscle cells via ERK1/2 signaling pathway, suggesting that IL-17A and ERK1/2 might be promising therapeutic targets for the treatment of SSc [204]. A study in a BLM-induced murine model of systemic sclerosis showed high infiltrating Th17 cells in the skin and lung and higher levels of serum IL-17A. There was positive correlation between the levels of IL-17A expression, skin and lung inflammatory scores, and skin fibrosis. It also showed that IL-17A enhanced lung fibroblast proliferation and type I collagen, TGF-β, and IL-6 expression in vitro, which were attenuated by treatment with anti-IL-17A [205]. Therefore, it is tempting to try secukinumab, a fully human monoclonal antibody against interleukin-17A, in SSc. Secukinumab is FDA approved for plaque psoriasis, psoriatic arthritis, and ankylosing spondylitis with excellent therapeutic results.

## Conclusion

Immunosuppressive medications currently remain the mainstay in the management of systemic sclerosis. However, they have only a modest effect on patient outcomes in comparison with other connective tissue diseases like systemic lupus erythematosus and are a blunt instrument for the suppression of immune activation. For this reason, the search for new therapeutics remains of high importance in systemic sclerosis. Thankfully, animal models identified new targets in all three pathways that are believed to be important in the pathogenesis of clinical systemic sclerosis: vasculopathy, immune dysregulation, and sclerosis (Table 2.1). In addition, although some targets, like the TLR, remain mostly unexplored, therapies that target many of these pathways are already developed for use in other diseases. While it is hopeful that one of these proves useful, just as the disease itself has various manifestations, focused studies on different parts of these pathways in different patients may be necessary as our understanding of the underlying factors in their phenotypic differences emerges. Continued study on all fronts, therefore, remains important, but new therapy seems more likely now than in any other point in the history of this research.

**Table 2.1** Summary of recent therapeutic targets in systemic sclerosis

| Target | Drug |
|---|---|
| ROS | Antioxidants [12] |
| PDGF | Crenolanib, nilotinib, other tyrosine kinase inhibitors [86–88] |
| TGF-β and its signaling | Bortezomib, pirfenidone, nintedanib [65–72] |
| VEGF | Bevacizumab [46] |
| Adenosine | $A_{2A}R$ antagonist [91] |
| $LPA_1$ | $LPA_1$ antagonist, ATX antagonist [98] |
| PPAR-γ | PPAR agonists, glitazones [115] |
| Retinoid receptor α/β | Tamibarotene [118] |
| INF | Sifalimumab, anifrolumab [137, 138] |
| B cell | Rituximab, anti-CD19, BAFF antagonist [151, 154, 158, 159, 164] |
| IL-4 | Anti-IL-4 antibody [191, 192] |
| IL-6 | Tocilizumab [169, 170] |
| IL-13 | Anti-IL-13 antibody (CAT-354) [199] |
| IL-17A | Cosentyx [205] |
| T cell | Abatacept, CTLA-4 blocker [184, 185] |

Recent research in SSc has led to better understanding of signaling pathways in SSc. Targeting specific cytokines in SSc may result in effective novel therapy. Summarized below are the most recent targeted cytokines and their clinical trials

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# Chapter 3
# Macrophage Plasticity in Skin Fibrosis


**Melanie Rodrigues and Clark A. Bonham**

## Introduction

Macrophages are a heterogeneous group of immune cells of myeloid lineage that participate in myriad roles throughout the body, including host defense, clearance of debris, tissue repair, and matrix remodeling [1]. In most adult tissues, they can be tissue resident [2, 3], persisting from embryonic erythro-myeloid progenitors [4, 5], or they can differentiate from circulating monocytes derived from the bone marrow following injury [6–8]. Tissue-resident macrophages can also self-renew to maintain their numbers [9] and exhibit a long half-life [10]. Notably, in response to varying signals in the tissue microenvironment, macrophages can acquire unique characteristics that are important for homeostasis and repair. Aberrations in macrophage function have a causal association with several diseases, including fibrosis [11].

Since the 1990s, macrophages have been classified as polarized cells developing into two major pathways [12]. The classical pathway, associated with interferon-γ (IFN- γ) and lipopolysaccharide (LPS) stimuli, leads to the formation of M1 macrophages [13]. These macrophages activate intracellular STAT1, release pro-inflammatory cytokines such as TNF-α and interleukins IL-6 and IL-12, and are responsible for defense against pathogens [12]. Alternatively, macrophages can be activated in response to IL-4 stimulation and STAT6 activation to yield M2 macrophages with roles in tissue repair, angiogenesis, and cell proliferation [14].

The M1/M2 model may not be the most accurate paradigm regarding macrophage function. This is because the dichotomous subsets have largely been identified based on in vitro studies [12, 13]. It is now known that macrophages in vivo modify their role based on the need of the tissue and display a propensity for plasticity, often simultaneously expressing markers indicative of both M1 and M2 subtypes [15]. Thus, macrophage differentiation is not terminal.

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The polarization model also classifies any macrophage that is divergent from a pro-inflammatory cell as an M2 macrophage, leading to a variety of macrophage subsets being designated to this alternate group. Under this alternate category are macrophage subsets that can deposit or remodel the extracellular matrix (ECM), perform angiogenesis, release growth factors, or perform combinations of these activities, exhibiting heterogeneity in both their transcriptional machinery and function [16]. Thus, in a tissue milieu, where several signaling pathways are activated in concert, there can be several macrophage subsets that exist simultaneously but are oversimplistically grouped into one category of alternatively activated macrophages and studied as one population. Whether all macrophages are inherently alike with subsets resulting from adaptation in response to spatiotemporal signals or whether some macrophages have a greater predisposition to a particular function remains unknown.

Skin repair is a complex process involving a multitude of cell types and diverse signals from the microenvironment in overlapping stages of inflammation, neovascularization, growth, reepithelialization, and remodeling [17, 18]. In response to these altering spatiotemporal signals, macrophages demonstrate unique functions [16]. In the early inflammatory stage, macrophages are primed into a pro-inflammatory and microbicidal state in response to damage- and pathogen-associated molecular patterns (DAMPs and PAMPs) [19, 20]. The pro-inflammatory macrophages can exacerbate the innate immune response by increasing the recruitment of classical monocytes [21, 22]. These macrophages engulf and rapidly kill bacteria within phagosomes, a nutrient-limiting environment high in reactive oxygen species and reactive nitrogen species [23] (Fig. 3.1). Pro-inflammatory macrophages also eliminate expended neutrophils and matrix debris and switch the wound environment from an inflammatory state to one promoting growth [24, 25]. The resolution of the inflammatory stage by macrophages is critical to the healing response, and if neutrophils are not eliminated in a timely manner, a chronic wound will result [26, 27].

During neovascularization, macrophages release vascular endothelial growth factor (VEGF) which is critical for endothelial cell proliferation, migration, and tubulization [28]. They also participate in vessel anastomosis by fusing nascent endothelial vessels and connecting them to the systemic vasculature [29, 30]. Interestingly, due to their involvement in new vessel formation, these monocytes and macrophages are commonly mistaken for endothelial progenitor cells [31–34]. Concurrent with neovascularization, fibroblast to myofibroblast transition occurs in the wound, and macrophages can stimulate ECM deposition by myofibroblasts [35, 36]. Macrophages can also acquire properties of fibroblasts and deposit ECM themselves [37]. Excessive ECM deposition by macrophages is evident in pathological fibrosis [38]. Finally, in the remodeling stage, macrophages take on the distinct role of phagocytizing excessive numbers of cells and remodeling the ECM to revert the skin into a state of homeostasis. An improperly remodeled wound can lead to a persistent proliferative state and fibrosis.

Scarless skin repair is observed only in the early embryo [17]. Adult skin, in contrast, almost always heals with a scar [39]. In certain cases, there can be excessive scarring, as seen in hypertrophic scars (HTS), resulting in fibrosis. Skin fibrosis is common following surgical procedures, traumatic injuries, radiation, and burn injuries [1, 2] and involves thickening and hardening of the skin, eventually leading to dermal contractures that affect joint movement, function, and disfigurement.

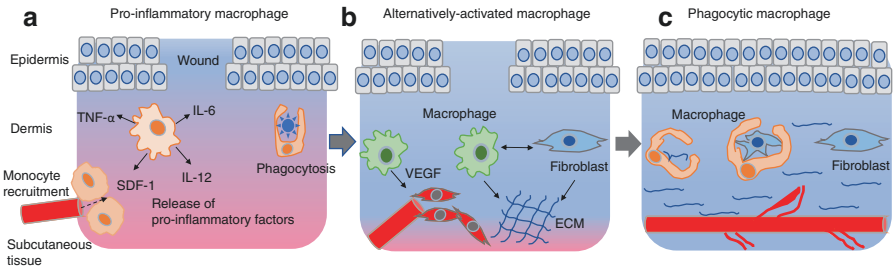


**Fig. 3.1** Macrophages in the wound healing response. Macrophages acquire various functions during the progression of wound healing. (**a**) In the inflammatory phase, macrophages release pro-inflammatory cytokines such as IL-6, IL-12, TNF-α, and SDF-1 that recruit more monocytes into the wound to heighten the antimicrobial response. These macrophages are also actively performing phagocytosis and eliminating bacteria, cell debris, and cells undergoing apoptosis such as neutrophils. (**b**) When the wound transitions into the growth and proliferation phase, macrophages release VEGF that activates endothelial cells to perform angiogenesis. Macrophages also interact with fibroblasts, activating ECM deposition by fibroblasts. They can also deposit ECM themselves. (**c**) In the remodeling stage of healing, macrophages phagocytize excessive ECM and cells which are no longer required for the wound healing response such as myofibroblasts, resolving the healing state and transitioning the wounded skin into homeostasis. Aberrations in these cellular responses, especially during ECM deposition and remodeling, can lead to the onset of fibrosis. ECM extracellular matrix, IL-6 interleukin-6, IL-12 interleukin-12, SDF-1 stromal cell-derived factor 1, TNF-α tumor necrosis factor alpha, VEGF vascular endothelial growth factor

The pathological changes that lead to skin fibrosis have been attributed to alterations in mechanical forces, signaling pathways, cellular responses, and extracellular matrix remodeling during wound healing [40, 41]. Skin fibrosis can also manifest as keloids, which are raised scars that grow beyond the wound boundaries [42]. These are notable in darker skinned individuals such as those of Hispanic, Asian, and Black heritage. There is also progressive fibrosis of the skin in scleroderma, a connective tissue disease correlated with macrophage aberrations. The underlying causes that lead to fibrosis in scleroderma are still being investigated [43, 44].

In this chapter, we describe the various unique functions that macrophages exhibit during wound healing and alterations in these characteristics that can result in skin fibrosis. We describe macrophage heterogeneity and hypothesize how various macrophage subsets might be derived in the skin. Finally, we compare macrophage responses in skin fibrosis to fibrosis in other pathological states such as the foreign body reaction and fibrosis of the liver and lung.

## Mechanotransduction and Macrophage Activation in Hypertrophic Scar Formation

Injury to the skin from trauma, burns, radiation, and surgery often results in HTS formation [1, 2]. The sequelae of HTS include airway edema, speech and swallowing dysfunction, sensory defects, disfigurement, and psychological distress to the patient [1, 2]. Children are especially susceptible to contact burn wounds that are

deep partial thickness or full thickness, which almost always result in excessive HTS formation. While full thickness burns can be surgically treated using skin grafts, there currently are no standardized treatment options for patients with deep partial thickness injuries [5]. Thus, there is an urgent need to understand the mechanisms underlying HTS so that effective therapies can be developed.

Scar tissue is in a constitutively active proliferative phase [45]. While there can be several factors that shift wound healing into scar formation, it has become apparent that alterations in mechanical forces can bring about changes in cellular responses leading to chronic inflammation, excessive ECM deposition, and decreased apoptosis of cells [40]. These mechanisms deemed "mechanotransduction" pathways underlie abnormalities in wound healing. At the systemic and organ level, mechanical forces are active in several inflammatory diseases such as atherosclerosis [46], ventilator-induced lung injury [47], vascular stenosis [48], biomedical implant-tissue interactions [49, 50], and arthritis [51]. At the cellular level, mechanical strain is known to initiate a cycle of biochemical signaling that contributes to progressive inflammation in vascular disease [46], strain-induced lung injury, and musculoskeletal disease [39, 52–54].

During human skin repair, fibrosis has been found to be a direct biological response for restoring mechanical equilibrium across the wound. Elevated levels of distractive forces during the healing process are counteracted by the deposition of stiffer and stronger scar ECM so that skin integrity and mechanical homeostasis can be reestablished [55, 56]. Clinical trials have demonstrated that off-loading of profibrotic tensile forces during the remodeling phase of repair results in markedly less hypertrophic scarring [55, 56]. The off-loaded wounds do not display wound dehiscence or delayed healing, and no negative clinical consequences on dermal strength have been observed. It is important to understand the cellular and molecular factors that underlie these clinical observations.

The most well-studied mechanotransduction pathway in the skin is the integrin-focal adhesion kinase (FAK) pathway, which is involved in several other fibroproliferative states such as hepatic fibrosis [57], cardiac hypertrophy [58], atherosclerosis [59], and pulmonary fibrosis [60]. In the skin, FAK is expressed in keratinocytes and fibroblasts [40, 61–65]. Following injury to the skin and in the presence of mechanical forces, excessive activation of FAK leads to HTS [39, 40, 55, 66, 67]. Notably, FAK knockout mice or the application of FAK inhibitor to healing wounds reduces HTS by modulating numerous downstream components involved in fibrogenic responses, such as PI3K/Akt and mitogen-activated protein kinases (MAPK) [64, 65, 68–76]. It is important to reflect, however, that the skin contains cell types other than fibroblasts and keratinocytes. Immune cells are the major constituents of the wound during the initial stages of healing, with influences throughout wound healing, and aberrations in these cells might have a significant role to play in scar formation [77].

To understand the role of immune cells in HTS, FAK knockout mice were subjected to mechanical strain. FAK-KO mice produced lesser monocyte chemoattractant protein 1 (MCP1) resulting in fewer macrophages in the wound and reduced scarring of the skin [40]. Microarray analysis of a well-established murine HTS

model recently compared gene expression of mechanically strained wounds compared to unstrained control wounds [67]. A cellular subset was identified in the mechanically strained wounds, which was enriched in inflammatory cell genes, indicating that the cells were distinct from keratinocytes or fibroblasts [67].

To identify inflammatory cells contributing to the scar response, T-cell deficient mice were wounded and then subjected to mechanical strain. They identified a ninefold reduction in HTS, due to the inability of these mice to recruit monocytes and activate macrophages [67]. Mechanically strained wounds in the T-cell deficient mice also showed lesser fibroblast and epidermal proliferation [67]. These murine findings suggest that mechanical forces in the healing wound alter macrophage and T-cell-regulated pathways, which in turn affect fibroblast and keratinocyte activity and induce fibrotic responses in the skin [78, 79].

## Macrophage Alterations in the Early and Proliferating Wound with Implications in Hypertrophic Scar Formation

Since macrophages are central to almost every stage of the wound healing response, there has been an effort to understand alterations in their function that may result in HTS. Most studies have focused on characterizing alterations in macrophages in the initial stage of wound repair based on the hypothesis that a heightened early inflammatory response is causative for fibrosis. However, macrophage depletion studies have indicated that this is not the case. Pro-inflammatory macrophages are necessary for the early stages for normal repair, while excessive or reduced pro-inflammatory macrophages are associated with HTS [78–80].

This is predominantly because, in the inflammatory stage, macrophages are required for phagocytosis, pro-inflammatory cytokine release, and ROS production to eliminate microbial activity [23–25, 81]. More importantly, macrophages are essential for the engulfment and elimination of expended neutrophils, which ends the inflammatory response in the wound and alters the wound milieu into a pro-regenerative environment [82]. When macrophages are depleted during the inflammatory stage, there is an increased influx of neutrophils in the wound as a compensatory response. These neutrophils persist in the wound, and the inflammatory state continues into the subsequent stages of healing, leading to delayed wound closure [83, 84].

It will be noteworthy to determine if all pro-inflammatory macrophages persist and acquire a neovascular profile in preparation for the next phase of healing. It is possible that separate subsets of macrophages are responsible for the regenerative phase, and these subsets may result from differences in their monocyte precursors. Monocytes, like their descendants, are highly plastic cells, and different populations of these immune cells have been identified. In humans, "classical" monocytes express the surface marker CD14 and can further be divided into subsets based on the expression of CD16 [9]. In mice, classical monocytes are $LY6C^{hi}CX3CR1^{lo}CD43^{lo}$ tissue [9]. These monocytes are mainly involved in pro-inflammatory cytokine

release and give rise to pro-inflammatory macrophages or dendritic cells, which have antigen-presenting capacity (Fig. 3.2).

In humans, "nonclassical" monocytes are found to be CD14$^{lo}$CD16+, and in mice, they are LY6C$^{lo}$CX3CR1$^{hi}$CD43$^{hi}$ [9]. Since nonclassical monocytes are temporally observed in injured or infected tissue after the classical monocyte response, they are hypothesized to differentiate from classical monocytes [85, 86]. These cells are found to continually survey the endothelium on the luminal side, searching for any breaks or injuries [87–89]. Upon detecting an injury, nonclassical monocytes first recruit neutrophils to remove debris [89] and then settle in the perivascular niche where they serve as progenitors for alternatively activated macrophages, supporting vascular growth and remodeling [89]. Pro-angiogenic macrophages have not been found to differentiate into endothelial cells in vivo [31], although they can acquire endothelial markers such as CD31 and VEGFR2 in vitro in the presence of endothelial cell differentiation media [90, 91]. Instead, these alternatively activated macrophages release pro-angiogenic growth factors such as VEGF that activate endothelial cell proliferation and tubulization [32, 92].

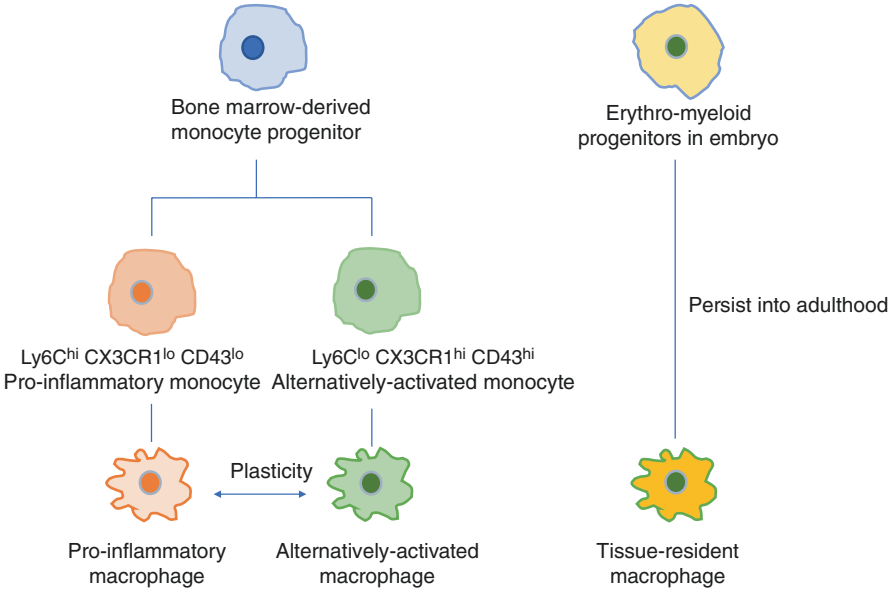


**Fig. 3.2** Macrophage ontogeny and cellular responses. In response to skin injury, circulating bone marrow monocytes are recruited to the wound and result in a significant number of macrophages. Classically activated monocytes with surface markers Ly6Chi CX3CR1lo CD43lo are hypothesized to give rise to pro-inflammatory macrophages. The nonclassical monocytes Ly6C$^{lo}$ CX3CR1$^{hi}$ CD43$^{hi}$ give rise to the alternatively activated macrophages. Both subsets of macrophages display a propensity for plasticity. Tissues have a separate resident macrophage population, but how this cellular population differs in surface marker expression and function from monocyte-derived macrophages remains unknown. CX3CR1 CXC3 chemokine receptor 1, CD43 leukosialin/sialophorin

The existence of at least two phenotypically and functionally distinct monocyte subpopulations in mice and humans suggests evolutionary conservation of monocyte and macrophage heterogeneity. There are likely other distinct monocytes subpopulations that remain to be identified. More recently, an atypical monocyte called the segregated nucleus containing atypical monocyte (SatM) has been found to be critical in developing skin fibrosis [93]. These monocytes are Ceacam1+Msr1+Ly6C-F4/80-Mac1+ and share certain characteristics of granulocytes yet are distinct. SatM does not produce transforming growth factor β (TGF-β), which is an important mediator in converting fibroblasts to collagen-depositing, contractile myofibroblasts [93]. However, SatM induces fibroblasts to deposit collagen by producing tumor necrosis factorα (TNFα) [93].

During the growth stage of healing, some macrophages have been found to acquire mesenchymal properties and can deposit ECM, particularly collagen I. These macrophages, called fibrocytes, can lose their hematopoietic markers as they acquire a mesenchymal phenotype. Growth factors such as TGF-β1, which are known to be essential for the fibroblast to myofibroblast transition, can upregulate the expression of collagen I, α smooth muscle actin (α SMA), tissue inhibitor of metalloproteinase 1 (TIMP-1), and VEGF in these macrophages, indicative of mesenchymal differentiation [32, 94]. What causes some macrophages to become fibrocytes, and the extent of their role in skin fibrosis, remains unknown. Since macrophages can produce TGF-β1 themselves, it is possible that they initiate autocrine responses for their differentiation [37, 95, 96].

Depletion of macrophages during neovascularization and in the growth stage has been found to result in severe hemorrhage and chronic wounds, implying that alternate macrophages are required for the normal healing response [97]. Since macrophage depletion may not be an effective therapeutic solution, it is important to understand the microenvironmental factors that can exert influences on macrophages, so that these alterations may be targeted. Chemokines such as stromal cell-derived factor 1 (SDF-1/CXCL12) are important for monocyte recruitment [37], while colony stimulating factor (CSF-1) is essential for resident macrophage activation [98]. Depletion of these factors in the wound reduces monocyte recruitment and macrophage numbers and consequently decreases new vessel formation [31, 99].

The microenvironment also influences the epigenetics of macrophages, such as in shaping regulatory regions and gene activity [100]. These epigenetic studies suggest that the developmental origins of macrophages are less important in establishing their identity compared to their tissue microenvironment [100, 101]. The microenvironment may also influence the repertoire of transcription factors in macrophages and adjoining cells. PU.1, a transcription factor from the Etx family, for example, is necessary for maintaining the cell-type-specific H3K4me1 region within several enhancer regions in macrophages. Retroviral expression of PU.1 in fibroblasts induces fibroblasts to acquire macrophage markers and macrophage characteristics, indicating that transcriptional machinery of cells can be re-wired by the acquisition of novel regulatory regions [102]. Modulating epigenetic alterations in macrophages might hold the key to treating several diseases, including fibrosis [103].

## Macrophage Activation During Wound Remodeling and Its Association with Hypertrophic Scar Formation

The remodeling stage of wound healing determines if the wound will result in a scar or will recur. In this phase, there is regression of the nascent vasculature, removal of expendable cells, and subsequent reorganization of the ECM. From a cellular perspective, the role of myofibroblasts shifts from ECM production into ECM remodeling. To achieve this, myofibroblasts synthesize matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) [104, 105], which act in concert to replace collagen III within the granulation tissue to collagen I, seen in the intact uninjured skin. Subsequently, these myofibroblasts need to revert into fibroblasts of the homeostatic skin, and excessive myofibroblasts need to undergo apoptosis and removal. If the myofibroblasts and other expendable cells within the remodeling skin do not undergo apoptosis, HTS tend to form [106].

Macrophages are the key phagocytizing cells during wound remodeling and responsible for eliminating myofibroblasts. This implies that the alternate macrophages prevalent during the growth phase of healing need to be replaced again by their phagocytizing counterparts. If this transition into the phagocytizing subtype does not occur, activated macrophages that persist in the remodeling wound will continue to produce growth factors and stimulate fibroblasts to produce ECM, advancing the growth phase of healing and leading to fibrosis [107]. These profibrotic factors include TGF-β, CCL17, CCL22, connective tissue growth factor (CTGF), and insulin growth factor 1 (IGF1) [107, 108].

Alteration in the fibroblast phenotype during remodeling is also critical in shifting the balance of healing toward fibrosis. Fibroblasts in skin fibrosis overexpress the CD47 “don’t eat me” signal [109], which prevents them from being recognized and phagocytized by macrophages, a process similar to macrophage evasion in several solid tumors [110]. Increased expression of CD47 in fibroblasts occurs via the c-Jun pathway [109]. Treating fibrotic samples with the CD47 antibody has been shown to effectively treat tumors and diseases such as atherosclerosis in animal models by blocking “don’t eat me” signals on diseases cells [111–114]. A similar approach may promote macrophage clearance of fibroblasts in skin fibrosis.

Macrophages may also acquire a “fibrolytic” function during remodeling. In this new role, macrophages synthesize various MMPs and TIMPs that allow for ECM degradation and prevention of excessive degradation, respectively. In the lung, depletion of macrophages in the late stage of healing has been found to delay the clearance of scar due to the reduced presence of MMPs [115]. A similar mechanism might be activated in the skin where an imbalance between TIMP and MMP expression would lead to abnormal ECM modification and fibrosis [116, 117].

In addition to the phagocytic and fibrolytic activity, macrophages are also actively involved in signaling within the remodeling wound. Importantly, macrophages are a source of the anti-fibrotic ligands CXCL10/IP-10 and CXCL11/IP-9 [118, 119], which bind and signal the CXCR3 pathway to initiate the “stop healing”

response [120]. CXCR3 is upregulated on several cells in the remodeling wound, including endothelial cells and fibroblasts. CXCR3 activation of endothelial cells allows for the neovessels generated during the healing response to be pruned into stable and well-perfused blood vessels that can resume homeostasis [121]. This CXCR3-mediated blood vessel regression is found to occur via PKA-mediated inhibition of m-calpain [122].

Similarly, fibroblasts activate the CXCR3 pathway during the remodeling stage to reduce motility and ECM deposition [123]. Wounds in CXCR3 knockout mice lead to HTS [120], but this scar response can be reverted by introducing fibroblasts or mesenchymal stem cells with CXCR3-signaling activity [124, 125]. CXCL10 and CXCL11 also prime macrophages into a pro-inflammatory state [126], which has been implicated in several inflammatory diseases. This pathway, therefore, might be critical to the conversion of alternate macrophages to the phagocytic and fibrolytic macrophage phenotype. Uncovering other signaling pathways that are preferentially expressed during wound remodeling and the involvement of macrophages in this signaling response might generate a better understanding of why some wounds undergo dystrophic healing such as in keloids and HTS.

## Macrophages in Scleroderma

Scleroderma is an overarching term which includes the fibrotic conditions of localized scleroderma and systemic sclerosis (SSc). While localized scleroderma consists of fibrotic lesions limited to the skin and subcutaneous tissue, SSc is a connective tissue disorder with fibrosis and vascular changes in the skin and multiple visceral organs. Only in rare cases of SSc (called sine scleroderma) is there visceral organ damage without skin disease. There are three clinical stages of the disease. The first phase called the edematous phase manifests as stiff and puffy fingers and hands [127]. The second indurative phase is characterized by sclerodactyly, which is the tightening and thickening of the skin [127]. In the third phase, characterized by atrophy, the skin may soften, resulting in skin lesions and ulcers which are recurrent [128]. The organ-based manifestations of SSc can include lung fibrosis, pulmonary arterial hypertension, renal failure, and gastrointestinal complications [129].

The etiology of these conditions remains unclear. It has long been suggested that scleroderma results from the upregulation of certain chemokines, which recruit and activate leukocytes, particularly macrophages and CD4+ T-cells [130–132]. This is followed by an excessive synthesis of ECM. However, it remains to be determined how the activation of immune cells causes the overproduction of ECM components, particularly type 1 collagen. Recent studies demonstrate that an alternatively activated macrophage subset is abundant in the disease [133, 134], which may exert influences on fibroblast deposition of ECM. It is also possible that the alternatively activated macrophages deposit ECM themselves.

Analysis of tissue specimens from patients with SSc has demonstrated that an upregulated production of the cytokines monocyte chemoattractant protein 1 (MCP1) and macrophage inflammatory protein 1 (MIP1) is responsible for the recruitment of monocytes/macrophages to sites of inflammation [132]. The monocytes are predominantly CD-14 monocytes, and the macrophages which are subsequently formed are the most abundant mononuclear cells in the skin lesions, even surpassing CD4+ T-cell counts [43]. This mechanism of chemokine-induced macrophage recruitment is evidenced in several other pathological conditions including atherosclerosis, rheumatoid arthritis, interstitial lung disease, and glomerulonephritis [135, 136]. The chemokines are expressed in the keratinocytes, vascular endothelial cells, and macrophages of the sclerotic patient skin [132].

As early as 1987, the "advanced differentiation" and "activation changes" of SSc monocytes were found to differ from normal monocytes [137]. With the understanding of macrophage heterogeneity, it is now known that alternatively activated macrophages with CD163 and CD204 expression, derived from an increased recruitment of CD14+ monocytes, are present in SSc patient skin [134]. The CD14+ and CD163+ monocytes deposit collagen, and their numbers increase as the patients age [138]. Moreover, the skin CD163+ macrophages display high levels of CCL19, which is most likely responsible for recruitment of more monocytes into the skin [139]. Several groups have now independently demonstrated higher CD163 in the serum of patients with SSc, which directly correlates with severity in the skin disease in these patients [140–144].

Genome-wide studies have linked inflammatory changes, particularly increased chemokine production and alterations in vascular remodeling to the severity of fibrosis in scleroderma [145]. A total of 1800 genes from monocytes and 863 genes from CD4+ T-cells are differentially expressed in scleroderma patients [146]. One of the most differentially expressed genes in the purified monocytes was IFN-induced protein 44 (IFI44), a gene which is induced in response to type I IFNs. High levels of this gene were not found in any normal patient samples. Interestingly, the source of the interferons was found to be the vascular and perivascular cells in the skin tissue of patients with scleroderma [146]. Other IFN-regulated genes have also been found to be differentially expressed in CD14+ monocytes from scleroderma patients [147, 148].

Patients with scleroderma display increased circulating cytokine levels of inflammatory mediators such as TNF-alpha, IL1-beta, ICAM-1, and IL-6, as well as markers of vascular injury such as VCAM-1, VEGF, and von Willebrand factor [148]. The immune-induced fibrotic gene expression signature and pro-fibrotic macrophages are evidenced in multiple tissues of patients affected by SSc [149]. However, the pro-fibrotic macrophage signatures are distinct in each organ [149], suggesting either the involvement of tissue-resident macrophages or the complex alterations brought about by the microenvironment on the same type of recruited monocyte. A more thorough understanding of the pathways that govern activation of monocytes and macrophages in scleroderma will be useful in unraveling biomarkers as diagnostics and develop therapies for this fibrotic disease.

## Macrophages in the Foreign Body Response and Fibrosis

Although biomaterials may be inert and non-toxic, once implanted in the body, they can elicit adverse reactions at the material surface, leading to fibrous encapsulation of the implanted material. Placement of biomaterials in the subcutaneous space leads to injury of the skin and concomitant wound healing. However, there are molecular and cellular events exclusively activated in response to the foreign body. Simplistically, implantation of the biomaterial leads to adsorption of blood proteins on the material surface, which activates an innate immune response, particularly the recruitment of monocytes and activation of macrophages [150, 151]. These macrophages fuse with each other to form a unique morphological variant called the multinucleated giant cells or the foreign body giant cells (FBGC) [152, 153]. The FBGCs persist around the non-phagocytosable material throughout its lifetime [154], leading to a chronic inflammatory state or biofouling and resulting in fibrosis.

The formation of the fibrotic capsule is a bimodal process, where the first phase involves activation of cellular responses to limit or neutralize the foreign body and the second phase activates the reparative process to restore injured tissue by the formation of scar [155]. Immediately following implantation, components of the clotting pathway adsorb onto the surface of the foreign body by the Vroman effect, where the highest motility proteins arrive first and are replaced by proteins which bind to the surface with a higher affinity [151]. The proteins include albumin, fibrinogen, fibronectin, vitronectin, thrombospondin, SPARC, and complements [156]. Along with the thrombus, these proteins form a provisional matrix surrounding the implant. Chemokines, cytokines, and bioactive agents get entrapped in the matrix and recruit the first responders, the neutrophils, following which monocytes are recruited [155].

The monocytes are found to be nonclassical, giving rise to alternatively activated macrophages [157]. However, the adherent monocyte-derived macrophages are differentially activated for cytokine production and phenotypic expression depending on the surface chemistry of the biomaterial. In general, flat and smooth surfaced biomaterials exhibit a thin layer of macrophages with lesser fibrosis compared to rough, high surface-to-volume fabrics, particulates, and porous implants, which feature greater numbers of macrophages and foreign body giant cells [155]. Macrophages bind the provisional matrix through β1 and β2 integrins, and these integrins are found to be necessary and sufficient for macrophage fusion and formation of the FBGCs [158] (Fig. 3.3).

The biomaterial-adherent FBGCs can be quite large, with dozens to even hundreds of nuclei and measure approximately 1 mm in diameter. They have been found occupying up to 25% of implant surface area on retrieved materials [155], which appear to be pitted and cracked in regions associated with FBGCs, most likely due to the release of reactive oxygen species and other cytokines from the FBGCs [159]. Thus, the presence of FBGCs is viewed as an undesirable response to

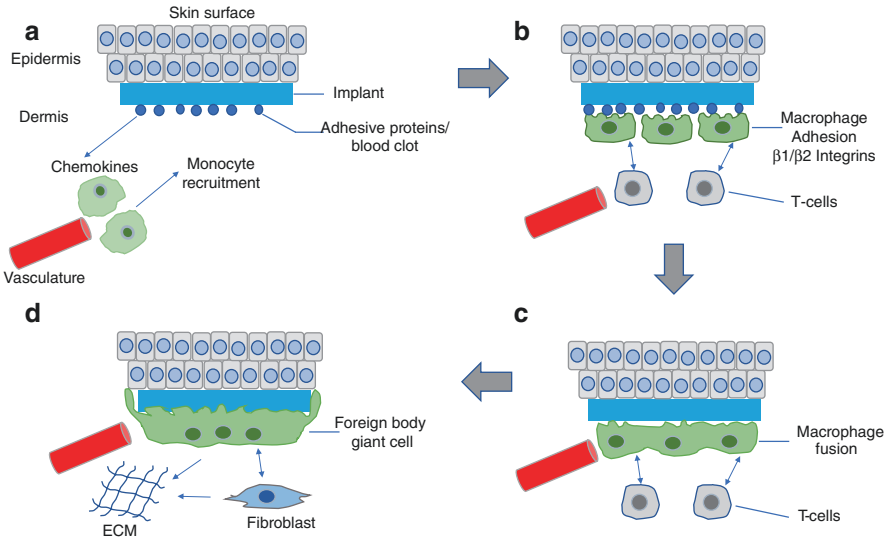


**Fig. 3.3** Macrophages and fibrosis during the foreign body response. (**a**) Biomaterial implantation in the body adsorbs various proteins and clotting factors on its surface. Hemostatic plugs (blood clots) then form, which release chemokines and recruit monocytes from the bone marrow. (**b**) Macrophages adhere to the foreign body through integrins β1 and β2. (**c**) Interactions with T-cells promote macrophage fusion and formation of the multinucleated foreign body giant cell. (**d**) These large FBGCs attempt to phagocytize the foreign body, release pro-inflammatory cytokines to degrade the foreign body, and interact with fibroblasts to deposit excessive ECM surrounding the biomaterial. Together, these interactions lead to the formation of a fibrotic capsule. FBGC foreign body giant cell, ECM extracellular matrix

the biomaterial, and ways to specifically target and eliminate these cells are being actively studied. However, there currently are no known surface markers that can distinguish FBGCs, and their large size limits their isolation by traditional methods such as fluorescent-activated cell sorting. This makes it difficult to isolate and characterize these cells from in vivo samples.

The cytokines Il-4 and IL-13, which are known mediators of the alternative-macrophage response, have been found to be essential in macrophage fusion and formation of the FBGC, but the source of these cytokines in the implant site remains unclear [160, 161]. It is hypothesized that T-cells produce these mediators which drive macrophage fusion [155] since they appear together with monocyte-derived macrophages in the chronic inflammatory phase [162]. Interestingly, the recruitment of T-cells into the implant site is not found to elicit an adaptive immune response [162]; instead, their recruitment is non-specific and might occur in response to the various cytokines released by macrophages.

Studies of lymphocyte and macrophage co-culture systems in the presence of implant materials have helped delineate the role of T-cells at the implant site. Over 90% of T-lymphocytes were found to associate with adherent macrophages and not with the biomaterial surface, resulting in enhanced lymphocyte proliferation [155, 163]. Hydrophilic and neutral surfaces were selective for CD4+ T-cell interactions,

and hydrophobic surfaces were selective for CD8+ T-cell interactions [155]. Interestingly, IFN-γ was produced only in the T-cell macrophage co-cultures and not in cultures with either of the cell types grown by themselves [155]. Furthermore, T-cells specifically induced pro-inflammatory cytokine production in macrophages, including IL-1β, TNF-α, IL-6, IL-8, and MIP-1β, but did not influence on the production of anti-inflammatory factors [164]. This in vitro data is supported by clinical findings where activated T-lymphocytes adherent to macrophages have been found on left ventricular assist device (LVAD) surfaces retrieved from patients [165].

How important then is the presence of T-cells in macrophage activation and the foreign body response? Lymphocyte-deficient mice implanted with biomaterials used to evaluate this. These mice displayed a foreign body response and FBGCs comparable to normal mice, both in terms of morphology and extent of fibrosis [166]. IL-4 was not detected in the implants of T-cell deficient mice [62]. However, IL-13 levels were comparable between the T-cell-deficient and normal mice indicating that other cells such as natural killer cells, mast cells, basophils, and eosinophils can produce these cytokines and may lead to a compensatory macrophage activation response in the absence of T-cells [167].

In addition to mediating the immune and phagocytic response, pro-fibrotic cytokines such as TGF-β and IL-1 released from macrophages and FBGCs regulate the proliferation and ECM deposition by fibroblasts [151]. Furthermore, macrophages and FBGCs in the implant may deposit collagen themselves, but the activation of these pathways needs further elucidation. Overall, these findings validate a central role for macrophages in the foreign body fibrosis response.

## Tissue-Resident Macrophage Responses in Other Forms of Fibrosis: Lessons from the Lung and Liver

The differences between embryonic-derived and adult, monocyte-derived macrophages in skin healing and fibrosis remain unclear. This is predominantly because there are no known surface markers that have been discovered that can distinguish between these two subtypes in the skin. However, fate-mapping studies have revealed tissue-resident macrophages function in other tissues and their responses within these tissues during fibrosis. Here, we will briefly describe tissue-resident macrophages in lung and liver fibrosis to generate ways of unraveling the function of these macrophages during skin repair.

### *Lung Fibrosis*

Macrophages represent the most numerous set of immune cells within the lungs under homeostasis. The two most prominent populations are the alveolar macrophages (AMs) and the interstitial macrophages (IMs). The two populations are distinguished by their differing expression of integrins [168]. AMs are found in the

airway lumen and are characterized by high CD11c expression and no CD11b expression [168]. Studies have found that AMs are a long-lived, self-sustaining cell population that colonizes their respective space within a few days of birth. Additionally, circulating monocytes are minimally recruited to maintain the AM population during homeostasis [2]. Conversely, IMs are found in the parenchyma and maintain high CD11b expression and low CD11c expression [168]. The heterogeneity of varying macrophage populations thus has potential implications for the development of disease in the lungs, such as fibrosis.

Idiopathic pulmonary fibrosis (IPF) is a common form of interstitial lung disease (ILD), characterized by excessive deposition of collagen and extracellular matrix components into the interstitial space of the lung [169]. This results in the fibrotic remodeling of the lung's structure. IPF occurs typically in adults over 55 years of age, causing impaired gas exchange, shortness of breath, and even death in affected individuals. Genetics, exposure to certain harmful chemicals, and prior fibrotic or lung illness all increase the risk of development of IPF. Bleomycin, amiodarone, and methotrexate cause lesions within the lung. While much of the pathogenesis of IPF remains unknown, it is known that abnormal regeneration within the lung plays a critical role. Initially, chronic inflammation was believed to be the underlying cause of fibrosis within the lung, though more recent studies have dismissed this hypothesis, as anti-inflammatories tend to have a negligible effect on those suffering from IPF [170].

Macrophages work in close concordance with myofibroblasts during wound regeneration, and as such, regulate fibrosis through their secretion of chemical mediators [171, 172]. AMs within the lung secrete MMPs essential to the breakdown of the ECM [173]. Distinguishing between tissue-resident AMs and monocyte-derived AMs is important to elucidate the exact role of AMs in the fibrotic process, as they are phenotypically unique. Murine studies using a bleomycin-induced lung injury model have displayed that tissue-resident AM deletion does not affect the severity of the resulting fibrosis [174]. In contrast, deletion of monocyte-derived AMs led to less severe cases of fibrosis. Transcriptomic analysis displayed significant differences in gene expression between the two populations [174]. These findings suggest that there may be other factors beyond macrophage plasticity that underlie the ability to perform varying roles.

## *Liver Fibrosis*

Hepatic fibrosis is characterized by excessive deposition of ECM components following tissue repair. Common causes include alcohol abuse, hepatitis C infection, and nonalcoholic fatty liver disease. Inflammation activates myofibroblasts, which produce and deposit ECM components essential to wound repair. Dysregulated regeneration of the liver following injury results in the fibrous lesions that characterize hepatic fibrosis and can lead to further complications. More severe cases can result in cirrhosis, liver failure, and portal hypertension, which typically require liver transplantation to be attenuated.

Hepatic macrophages are essential to the different phases of regeneration in the liver. Similar to other macrophage populations, hepatic macrophages display high levels of heterogeneity and plasticity. As such, they partake in various roles during tissue repair, including the destruction of foreign bodies, induction of cytokines and growth factors, and regulation of both inflammatory and anti-inflammatory responses. These differing roles result from their expansive heterogeneity, which is in part attributed to their divergent heritages. Tissue-resident hepatic macrophages, known as Kupffer cells (KCs), are of a self-renewing line of embryo-derived cells and are the most numerous population of tissue-resident macrophages throughout the entire body [2, 175]. These seem to have different functions than their monocyte-derived counterparts. Like other groups of macrophages involved in fibrosis, however, hepatic macrophages often exhibit markers for both M1 and M2 phenotypes, making their exact roles in the fibrotic response difficult to discern. Thus, scholars propose to classify hepatic macrophages based upon their heritage rather than their surface markers.

The liver is in constant contact with antigens and foreign bodies due to its physiological role and proximity to the intestines and portal vein [176]. KCs serve to prevent unnecessary immunological action and to maintain homeostasis. Located within the lumen of liver sinusoids, KCs closely monitor the bloodstream for any harmful substances. KCs maintain a wide variety of surface receptors, including SR-A1, SR-AII, mannose, and Fc-γ receptors, which allow for the detection of molecules for phagocytosis [176]. This prevents any potential harm from befalling the liver and, subsequently, an inflammatory response from occurring. An array of heterogeneity is displayed even within the Kupffer cell population. Subtypes take on different phenotypes and roles dependent on their microenvironment, with different localizations displaying dissimilar surface markers and sizes [177].

KCs function primarily as defenders during homeostasis. Following injury, however, they assume a pro-inflammatory role. Capitalizing on their proximity to other cell types, KCs can instigate inflammation through secretion of signaling molecules. For instance, KCs can secrete the chemokine CXCL16 to recruit natural killer T-cells (NKTs), which in turn secrete mediators to recruit and activate other macrophages [178]. Furthermore, studies suggest that KCs produce reactive oxygen species (ROS) and cytokines, such as IL-6 and TNF-α, which serve to activate hepatic stellate cells (HSCs) [179, 180]. HSCs comprise a population of perisinusoidal cells local to the liver that maintain a wide variety of roles, including ECM upkeep [181].

Following injury, HSCs are activated by KC secreted mediators and adopt an α-smooth muscle actin positive myofibroblast profile [182, 183]. These HSC-derived myofibroblasts are perhaps the primary source of collagen-1 deposition following injury, though KCs have been shown to assist as well [180]. Additionally, matrix metalloproteinase 13 (MMP13) was found to be almost exclusively expressed in regions KC inhabited regions of fibrotic tissue, suggesting that KCs play a major role in MMP production, and thus, ECM degradation [184]. Following the subsequent removal of KCs, significantly reduced MMP13 expression is seen, along with an attenuation of fibrosis [184, 185].

KCs are also believed to induce apoptosis to resolve the inflammatory response. Animal studies have shown that individuals with mild liver injury display both high levels of M2 KCs and KC apoptosis [186]. Programmed cell death was found exclusively in M1 designated KCs, sparing both M2 KCs and other hepatocytes [186]. A positive correlation between M1 KC apoptosis and attenuation of liver fibrosis was observed. Further studies using conditioned medium examined the effects of pro-M2 stimuli on M1 KC populations. M2 polarized KCs by IL-4, adiponectin, or resveratrol exhibited pro-apoptotic behavior toward M1 KCs. IL-10 was found to be the primary mediator of M2 regulated M1 apoptosis [186].

In the absence of inflammation, hepatic macrophages are mostly comprised of resident Kupffer cells. Following injury and inflammatory response, however, monocytes are recruited extensively for differentiation into macrophages. The exact roles of monocyte-derived macrophages within hepatic fibrosis are mostly unknown, though murine studies have observed that targeted deletion of these cells (marked by CD11b+ F4/80+) yields an ameliorated fibrotic response [187]. This was characterized by reductions in scarring and myofibroblast activation.

## Conclusion

As the outermost layer of the human body, the skin is subject to numerous environmental insults including ultraviolet radiation, rapid changes in temperature and moisture, and mechanical force. From an evolutionary perspective, delays in wound healing make the organism vulnerable to further damage and infection. Since scarless embryonic healing may be a more energy intensive and slower process [188], adult skin heals by scar formation to prevent the onset of a chronic wound. While regenerative healing of adult skin, without scar formation, might represent the ideal form of healing, this outcome is rarely observed in the adult skin.

Following burn injury, radiation and traumatic injuries, keloid formation, or in scleroderma, the wounded tissue continues to remain in a proliferative and activated state, with active deposition of extracellular matrix even when the wound is expected to heal. This may result in excessive scar formation, which can sometimes exceed the boundary of the original injury, causing disfigurement and psychological distress to the patient. While excessive scarring or skin fibrosis has traditionally been attributed to fibroblast dysfunction, specifically myofibroblast activation and excessive deposition of ECM by distinct fibroblast subsets [41, 189], there has been an attempt more recently to understand the contribution of immune cells to this undesirable wound outcome.

Several immune cells play an active role in wound healing. Immediately after injury, mast cells, which are otherwise engaged in the allergic response, can release cytokines, vasodilatory and vascular permeability factors, and proteases that enhance the recruitment of immune cells, mainly neutrophils into the wound [190]. Neutrophils eliminate pathogenic threats by releasing toxic granules, producing reactive oxygen species, initiating phagocytosis, and generating neutrophil

extracellular traps (NETs) [18]. Macrophages are subsequently activated and persist until the wound heals performing various functions as needed by the altering wound microenvironment and exerting influences on other cell types.

In the early wound, macrophages produce pro-inflammatory cytokines to eliminate bacteria and perform phagocytosis to eliminate expended neutrophils. They can also present antigens to T-cells in the skin, although these functions are usually designated to Langerhans cells in the epidermis and dendritic cells in the dermis [18]. Once the inflammatory state resolves, macrophages acquire a more regenerative function where they influence endothelial cells to perform angiogenesis and fibroblasts to deposit ECM [16]. They can also acquire mesenchymal properties and deposit ECM themselves. Such macrophages are called fibrocytes and may contribute to the fibrotic state [37]. In the final remodeling state, macrophages reacquire a phagocytic state to engulf cells such as myofibroblasts which are expendable to the wound as healing resolves. Macrophages also acquire a fibrolytic profile where excessive ECM is remodeled to bring the skin back into the homeostatic state.

Skin fibrosis is associated with an excessive influx of monocytes and macrophages [93]. However, depletion of the entire macrophage pool during the inflammatory and growth phases does not enhance the healing response as macrophage-induced responses are necessary for healing [97]. There have been attempts to modulate mechanical forces in the healing wound [67], chemokines such as SDF-1 and MCP-1 in the microenvironment [31, 37, 99], and epigenetic alterations of macrophages [100, 101] to subsequently modulate their behavior.

Interestingly, macrophage dysfunction is present in a wide range of fibrotic states, including those induced with scleroderma and implanted biomaterials. In scleroderma skin lesions, activated monocytes and macrophages are the most abundant mononuclear cells [43], recruited in response to cytokines released by vascular and perivascular cells [146]. Macrophages from skin specimens of scleroderma patients are found to be alternatively activated, expressing the surface marker CD163 [134]. They can influence fibroblast collagen deposition and deposit ECM themselves [138], but their pro-fibrotic profiles are distinct in each organ in patients affected by scleroderma, indicating the role of the microenvironment in modulating this cell type [149].

The foreign body reaction is a unique model to study macrophages in fibrosis since it involves macrophage fusion and the formation of multinucleated FBGCs that persist around the implant for its lifetime, attempting to phagocytize the implant [152, 153]. Retrieved implants display monocytes, macrophages, and FBGCs adhered to the implant with local destruction most likely due to the release of ROS and inflammatory cytokines [159]. The monocytes and macrophages closely interact with other immune cells, especially T-cells at the implant site which aid in their activation, fusion, and release of cytokines [155, 163, 164]. FBGCs have remained enigmatic as their enormous size makes them difficult to isolate, and it remains to be seen how their formation and persistence relate to accumulation of excessive ECM and skin fibrosis.

Thus, macrophage activation and their acquisition of unique characteristics are evidenced in various forms of skin fibrosis; yet several questions regarding the

mechanisms underlying their alterations remain unknown. One of the most ambiguous is how the ontogeny of macrophages relates to their function. Tissue-resident macrophages may modulate their behavior differently from circulating monocyte-derived macrophages recruited in response to injury. While distinctions in these subsets have not yet been clarified in the skin, ongoing studies analyzing various macrophage subsets in lung and liver fibrosis are underway. These studies may provide cues to understanding distinct macrophage responses in skin fibrosis. Ultimately a comprehensive understanding of macrophage plasticity and heterogeneity will be needed to develop diagnostics and therapies for skin fibrosis.

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# Part II
# Lung

# Chapter 4
# Fibrotic Signaling in the Lung

**Justin A. Dutta, Harinath Bahudhanapati, Jiangning Tan, Alon Goldblum, and Daniel J. Kass**

## Introduction

### *Idiopathic Pulmonary Fibrosis: Definition and Clinical Concepts*

Idiopathic pulmonary fibrosis (IPF) is defined as a chronic fibrosing interstitial pneumonia—a disease characterized by lung injury, the infiltration of fibroblasts, and the deposition of extracellular matrix. This cascade of events leads to the destruction of normal alveolar architecture, compromised gas exchange, and ultimately respiratory failure and death. Epidemiologic data suggest that the incidence of IPF ranges from 3–20 per 100,000 and a prevalence of 10–60 per 100,000 (reviewed in [1]). After several clinical trial failures, two drugs, pirfenidone and nintedanib, were approved by the US Food and Drug Administration for the treatment of IPF [2]. These drugs are effective in slowing down the rate of lung function deterioration, but they are not considered cures [2]. Ongoing research is necessary to understand fibrosis at the cellular and molecular levels to identify new targets for therapy. For in-depth reviews of the clinical aspects of IPF, readers are encouraged to read these outstanding reviews [1, 3, 4].

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## *Lung Injury and Repair*

There are several important pathologic concepts to consider when thinking about signaling in the fibrotic lung. First is the injury. There are no consensus data to suggest what that initial injury actually is. Many speculate that the disease exists for years before symptoms are apparent. A growing literature even suggests that *interstitial lung abnormalities*, a potential radiologic manifestation of early disease, may represent the first lesions of IPF [5, 6]. But while the injurious stimulus is unknown, the conventional thinking about IPF is that the injurious stimulus represents a disease of *epithelial* injury. Environmental associations with IPF include cigarette smoke and exposure to stone, metal, or organic dusts [7]. The nature of these stimuli supports the concept of epithelial injury since the respiratory epithelium is continuous with the external environment. Second is the host. Genetic causes seem to be most closely associated with epithelial cell phenotypes. Several genes associated with surfactant synthesis and therefore limited to expression in type II alveolar epithelial cells appear to be responsible for several cases of familial interstitial pneumonia, or what some people call *familial* IPF [8]. Mutations in the telomerase complex, which account for up to 20% of so-called familial IPF [9–11], were modeled in vivo and suggest that lung epithelial cells with telomere defects are deficient in their capacity to repair.

To conceptualize lung repair, it is important to consider the roles of different lung cell types. Central to this paradigm are the epithelial cells, which are needed to repopulate the epithelium and to maintain a proper gas exchange surface. The type II alveolar epithelial cell, the so-called defender of the alveolus [12], acts like the stem cell of the alveolar space and gives rise to type I alveolar epithelial cells [13–15], the cells responsible for normal gas exchange. Normal repair leads to reestablishment of a normal epithelium. In pulmonary fibrosis, however, epithelial repair is either insufficient or fails. Some researchers suggested that the type II alveolar epithelial cell achieves some degree of intermediate phenotypes that fails to repopulate the epithelium and leads to an interaction with the critical effector cell of IPF, the fibroblast. This is the crux of pulmonary fibrosis: the epithelial injury in IPF results in a pathologic interaction between epithelial cells and fibroblasts and macrophages that leads to the fibrotic phenotype hallmarked in IPF with fibroblast accumulation increased matrix deposition. So why does one form of injury, as in IPF or bleomycin lung injury, lead to fibrosis, whereas another injury such as influenza typically does not? Traits specific to the epithelial cells do not completely explain this dichotomy. For example, telomere dysfunction is not only associated with IPF but also emphysema [16, 17]. Both processes injure epithelial cells, but while influenza resolves, IPF does not. This difference is poorly understood, but it is the understanding of this difference that underlies the study of pulmonary fibrosis.

## *Lessons from Therapy*

Though many chemical agents are effective in decreasing experimental pulmonary fibrosis, modeled most consistently by bleomycin, only two agents have been found to be effective in IPF patients. In November 2014, the US Food and Drug Administration (US FDA) approved pirfenidone and nintedanib as treatments for IPF [2, 18]. These medications, independently, have been shown to slow the rate of deterioration of pulmonary function as measured by forced vital capacity (FVC) [3, 19]. Where do these drugs act? Nintedanib was shown to inhibit receptor tyrosine kinase signaling by PDGF, FGF, and VEGF [20]. The effect on these three receptor pathways appears to be important since the PDGF signaling inhibitor imatinib alone had previously failed in clinical trial [2, 21]. Pirfenidone was shown to inhibit TGFβ or p38 signaling [22], but other effects [23, 24] were noted and suggest that the therapeutic benefit of these agents may not clearly relate to the conventional mechanisms. Furthermore, in the case of nintedanib, inhibition of FGF signaling may actually be deleterious, as the FGF pathway is critical to lung epithelial cell development [2]. These data highlight one of the most critical considerations when discussing fibrotic signaling, that the lung is a "cocktail" of cells [2]. The cells in the lung have different tasks, bidirectional signaling, and different spatial relationships, and, therefore, the signaling events in each cell type differ and have unique effects on the fibrotic phenotype (Fig. 4.1). So while nintedanib, which actually does *something* in IPF, may inhibit FGF signaling in the lung epithelial cell compartment, this

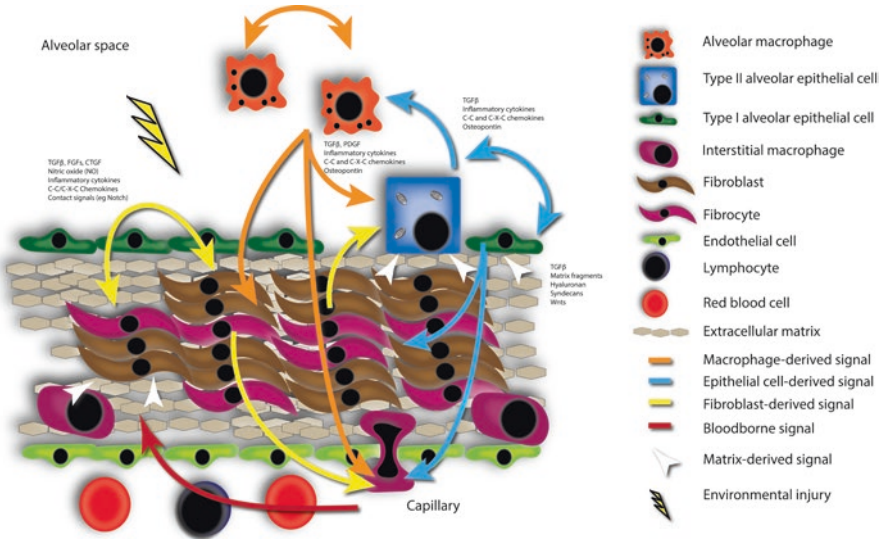


**Fig. 4.1** Schematic representation of intercellular signaling in pulmonary fibrosis. Interactions between cells and between the microenvironment are critical to effecting fibrosis. In addition, signaling in the lung is affected by environmental stimuli and distant blood-borne signals

effect may not be relevant for the overall clinical effectiveness of this medication. As noted above, many chemical agents were found to be effective in animal models of pulmonary fibrosis and in in vitro assays that model fibrotic phenotypes. These agents helped elucidate many of the signaling pathways in the relevant cell types. However, caveat lector, it remains to be determined if the signaling pathways described below are critical to the pathogenesis of IPF.

The three main and most studied cell types in the lung are epithelial cells, fibroblasts, and macrophages. In the following sections, the fibrotic phenotype of each cell type is described. For example, in fibroblasts, the pathologic phenotypes include myofibroblast differentiation, deposition of matrix, proliferation, and resistance to apoptosis. In epithelial cells and macrophages, the same categorizations are more complicated. Far greater attention has been paid to the fibroblast likely due to the ease of isolation and culturing of lung fibroblasts, in contrast to lung epithelial cells and lung macrophages.

Although the focus here is on mechanisms within the lung, observations in other organs including the heart, kidney, liver, and skin may be generalizable to the lung. Both human and animal studies are included throughout the text and are distinguished where appropriate.

## Epithelial Cell Signaling in Pulmonary Fibrosis

### *Epithelial Cell Integrity*

Epithelial cell integrity is necessary for maintaining an adequate gas exchange surface and for preventing injury, especially fibrotic injury. Approaches that deplete the mouse lung of epithelial cells can cause spontaneous fibrosis [25]. This has led to the idea that an exposed basement membrane, a consequence of epithelial loss, is a potent fibrotic stimulus. Telomere dysfunction in epithelial cells has also been associated with fibrosis, possibly through a mechanism of “stem cell failure” leading to an inability to repopulate a normal gas exchange surface [26]. Epithelial cell apoptosis, associated with silencing of suppressor of cytokine signaling 1 (SOCS1, which inhibits STAT signaling through the Janus kinases), can cause alveolar epithelial cell apoptosis [27]. Signaling induced by lysophosphatidic acid (LPA) via its receptor LPA1 is necessary for bleomycin-induced pulmonary fibrosis [28], which may be in part due to induction of apoptosis in epithelial cells [29]. LPA1 induces downstream signaling via G proteins (reviewed in [30]). The mechanism of apoptosis induced by LPA1 is not clear, but signaling via ROCK1 and ROCK2 may be involved, as ROCK1-/ROCK2-haploinsufficient mice are protected from epithelial cell apoptosis induced by bleomycin [31]. Deficiency of LPA2 also attenuated epithelial cell apoptosis and bleomycin-induced pulmonary fibrosis [32].

Epithelial cell integrity is critical for physiologic function. Misfolded surfactant proteins are associated with the induction of the unfolded protein response and endoplasmic reticulum (ER) stress (reviewed in [33]) in epithelial cells. Induction of ER

stress in alveolar epithelial cells can be inhibited by Smad and Src inhibition. This mechanism was modeled by alveolar epithelial cell-specific deletion of the tyrosine phosphatase Shp2 [34], which is associated with decreased surfactant protein expression, disorganized lamellar bodies, epithelial cell apoptosis, and inflammation-free pulmonary fibrosis. This pathophysiological response may be mediated in part by the failure of FGF signaling, known to be critical for epithelial cell health and to prevent fibrosis [35–37]. More recent data suggest that the loss of PTEN-induced putative kinase 1 (PINK1) and subsequent mitochondrial dysfunction in type II alveolar epithelial cells is a critical determinant of IPF [38]. Silencing of PINK1 in A549 cells, or the presence of free mitochondrial DNA, promoted TGFβ and FGF2 gene expression, suggesting that epithelial cells may be the source of fibrotic signals. Very recently, delivery of thyroid hormone restored normal mitochondrial energetics in lung epithelial cells and protected against several models of pulmonary fibrosis [39].

## *Epithelial-Mesenchymal Transition*

TGFβ signaling plays a key role in regulating fibrotic phenotypes in epithelial cells. In the absence of TGFβ signaling in epithelial cells, animals are protected from experimental pulmonary fibrosis [40, 41]. TGFβ is central to epithelial-mesenchymal transition (EMT), a recognized biologic process in cancer and development by which epithelial cells lose intercellular adhesion protein expression and assume a more mesenchymal phenotype, which was thought to represent a significant source of fibroblasts in IPF (reference [42], cited 856 times). Since the first publication of EMT using human data, there was a meteoric rise of EMT citations which peaked in 2012 and in many ways dominated the pulmonary fibrosis landscape. The number of citations fell off dramatically in 2012 following the publication of a study, using elegant lineage tracing experiments that suggested that bleomycin injury showed no evidence of mesenchymal cells from epithelial origins [13]. However as of 2012, publication of EMT studies in pulmonary fibrosis is on the rise again. This pattern reflects an evolution of the understanding of EMT from a strict definition, positing epithelial cells as a direct source of fibroblasts, to a more nuanced interpretation of EMT that includes epithelial-mesenchymal *interactions* and *responses*. The signaling events associated with the effect of EMT on this phenotype, which is consistently observed in vitro to be TGFβ-responsive, may also be associated with other critical epithelial cell effects in pulmonary fibrosis.

EMT is thought to be a TGFβ-driven process [42–48], and it is most closely associated with Smad-dependent pathways. Matrix, independent of culturing with recombinant TGFβ, induced nuclear localization of Smad3, an effect that was inhibited by the TGFβR1 inhibitor, SB431542 [45]. Inhibition of EMT may occur by suppression of Smad-related pathways. For example, hepatocyte growth factor (HGF) induced expression of the TGFβ signaling inhibitor, Smad7, and induced nuclear export of Smad7, via a mitogen-activated protein kinase (MAPK)-dependent mechanism [43]. All-trans-retinoic acid decreased EMT by decreasing activation of Smad3 [47].

Several different pathways, intersecting or running in parallel to TGFβ, are necessary for EMT. A549 cells, lung cancer cells that are surrogates for type II alveolar epithelial cells, can be stimulated in vitro with inflammatory cytokines to undergo EMT, a process blocked by Rho kinase (ROCK) inhibition [44]. Hyaluronan expression is increased in culture epithelial cells in response to stretch and can induce EMT via a MyD88-dependent mechanism [49]. This process leads to induction of Wnt target gene Wnt-inducible signaling protein 1 (Wisp1). Blockade of Wisp1 prevented stretch-induced EMT, suggesting a critical role for Wnt signaling in this process. Alveolar epithelial cell expression of the α3 integrin is necessary for EMT, in vitro and in response to bleomycin injury, through phosphorylation of β-catenin at tyrosine 654 (Y654) and complexing with p-Smad2 [50]. β-Catenin phosphorylation induced by TGFβ is Src kinase-dependent [51]. Src kinase inhibition (which inhibits activation of β-catenin) or raising Axin levels in the cell via inhibition of the enzyme tankyrase (which leads to Axin degradation, inhibiting the nuclear translocation of β-catenin) can inhibit EMT. Inhibition of Wnt signaling with ICG-001 inhibited expression of α-SMA and β-catenin occupancy of the α-SMA promoter [46]. Wnt-induced EMT appears to be JNK1-dependent [52]. Together, these data suggest critical interactions between TGFβ signaling, the Wnt pathway, and EMT. Loss of the endogenous Akt inhibitor PTEN or expression of a dominant-negative isoform increased EMT in vitro and in vivo following bleomycin injury [53]. While not a study of EMT per se, expression of miR-375 inhibited Wnt/β-catenin AEC differentiation [54]. This suggests that Wnt/β-catenin signaling is a part of much broader lung epithelial cell differentiation program. Activation of Notch signaling increased EMT and epithelial cell migratory capacity [55]. This could be inhibited by Notch blockade. Notch signaling increased phosphorylation of Smad3 after 48 h of epithelial overexpression of the Notch intracellular domain (NICD) and increased epithelial expression of TGFβ1. The durable effect of Smad3 suggests that EMT induced by Notch could be the result of autocrine/paracrine stimulation by activated TGFβ. However, Notch-dependent sites were necessary for transcription of the myofibroblast marker α-smooth muscle actin (α-SMA).

## *Epithelial Cells as Sources of Intercellular Signals*

If EMT is *not* associated with the accumulation of myofibroblasts, are these cells secretorily active? Epithelial cells are sources of connective tissue growth factor (CTGF), a factor dependent on Rho signaling [56]. Wnt/β-catenin signaling in epithelial cells may be an important source of IL-1β, IL-6 [57], and the IL-10 family member, IL-22 [58]. Epithelial cells may also be the source of the monocyte/macrophage chemoattractant CCL2/MCP-1 [59]. TERT or TERC-/- mice are characterized by enhanced expression of IL-1, IL-6, CXCL15 (human IL-8 homolog), IL-10, and CCL2 [58]. Type II alveolar epithelial cells are sources of the secreted IL-6 family receptor CRLF1, which signals in an autocrine fashion by ciliary

neurotrophic factor receptor (CNTFR) to recruit T-cells to the bleomycin-injured lung [60]. Epithelial cells that are deficient in G proteins $G\alpha_q$ and $G\alpha_{11}$ have decreased TGFβ activation but increased IL-33 expression, which promote M2 type alveolar macrophage accumulation [61], discussed in greater detail below. Alveolar epithelial cells may be a source of osteopontin [62], which may be deleterious in IPF [63]. In alveolar epithelial cells, the transgenic expression of TGFα, a member of the epidermal growth factor (EGF) family, can cause pulmonary fibrosis [64].

## *Epithelial Cell Migration*

Focal adhesion kinase (FAK), which is necessary for cell migration, is associated with the ability of epithelial cells to migrate to areas of injury, one of the critical functions of epithelial cells. Epithelial-specific deletion of FAK leads to epithelial cell apoptosis and enhanced bleomycin injury [65]. In an influenza injury model (non-fibrosis), Tp63-expressing stem cells in the bronchiolar epithelium proliferate and migrate to areas of denuded alveolar epithelium [66]. These cells express Krt5 and ΔNp63, a non-transactivating isoform of Tp63 [67]. Enhanced Notch signaling in these cells led to proliferation but suppressed surfactant protein C expression and was regarded as "inferior" in terms of restoring normal lung function [68]. The authors found that hypoxia-induced HIF1α activity was partly responsible and that rapid type II alveolar epithelial cell differentiation could be induced by either silencing HIF1α or by enhancing Wnt/β-catenin signaling, suggesting that Wnt/β-catenin signaling is essential for normal alveolar reconstitution in lung injury.

## *Newer Technologies and Recently Described Signaling Pathways in Epithelial Cells*

The broad application of single cell RNA-Seq has permitted an unparalleled level of granularity for the analysis of human lung epithelial cells in both health and disease. A recent study in IPF epithelial cells demonstrated aberrant activation of several signaling pathways including HIPPO/YAP, P53, WNT, and AKT/PI3K [69]. Profiling of the mesenchymal cells in the alveolar niche suggests a critical role for FGF and STAT3 signaling in the target epithelial cell [70], although this does not exclude other sources of STAT3-activating ligands [60]. STAT3 is critical for several protective epithelial cell phenotypes including protection from hyperoxia [71], pneumonia [72], and epithelial cell migration [73] and for proper handling of surfactant [74], but remarkably, not fibrosis. Future challenges in the field include developing better methods for ex vivo culture methods and better definition of the important fibrotic readouts from epithelial cells in culture. Observations in lung epithelial cells have been limited to animal models of fibrosis, and the focus has certainly been on

alveolar epithelial cells. The discovery of *MUC5B*, the so-called IPF gene, ignited interest in the role played by airway epithelial cells in fibrosis [75–77].

## Macrophages

The signaling events in macrophages that contribute to pulmonary fibrosis are unclear; however, targeting macrophages can shape fibrosis in the animal models, suggesting that macrophages may represent another therapeutic target. For example, depleting macrophages with clodronate protect mice from TGFβ-induced pulmonary fibrosis [78]. Macrophage depletion via diphtheria toxin decreased fibrosis in the *Schistosoma mansoni* model of fibrosis [79]. So while macrophages are important, there are several challenges in studying macrophage biology in the lung, for example, the anatomic source of macrophages. Alveolar macrophages may be "tissue-resident," derived from fetal progenitors, and are not dependent on monocyte homing to the lung [80]. The second source of macrophages are the monocyte-derived population, which migrate into the lung and subsequently differentiate [80]. Another major challenge is the role of macrophage polarization and in particular on the so-called "M2" macrophage. The M2 macrophage has been defined as "alternatively" activated [81], as opposed to the "classically" activated M1 macrophage, and constitutes several subphenotypes of macrophages that are associated with IL-4, IL-13, TGFβ, and IL-10 stimulation (reviewed in [82]). Macrophages are traditionally defined by their upstream signals, by activation of the relevant transcription factors, and by what they produce. The M1 macrophage is critical to the response to infection and is stimulated by IFNγ and lipopolysaccharide, requires IRF5, and makes iNOS, IL-12, and IL-23 [83]. The transition from the M1 to the M2 macrophage is sometimes referred to as "resolving" or "healing" [81]. Of particular interest is the observation that the M2 macrophage is a local source of TGFβ and may help to propagate fibrosis [83]. The M2 macrophage is defined, in large part, by expression of the enzyme arginase 1 (Arg1) because of its tight association with IL-4/IL-13 stimulation [84]. In IPF, macrophage polarization to the M2 phenotype appears to drive the dreaded IPF exacerbation [85]. Therefore, the vast majority of studies of macrophage signaling have focused on the events that transition toward the M2 phenotype. Macrophage researchers have proposed uniform nomenclature requirements for ongoing studies [81], but for the purpose of this review, we will employ the M1 and M2 terminology, commonly employed in the literature.

In general, the guiding principle is that stress-dependent polarization to the M1 phenotype, e.g., with TNFα, may protect from experimental fibrosis [86]. In contrast, deficiencies in M1 signaling that shift toward the M2 phenotype are associated with fibrosis. The M2 phenotype is well-modeled by *Schistosoma mansoni* infection, which leaves parasite eggs in the lung and induces a macrophage-rich focus, stimulated by Th2 cytokines and characterized by M2 activation, expression of Arg1, and expression of chitinase-like molecules [87]. Deficiency of *Map3k8* which is necessary for the expression of inflammatory cytokines downstream of toll-like

receptor, IL-1β, or TNFα is required for Th1 responses and leads to unchecked M2 activation and worsens *Schistosoma mansoni*-induced organ fibrosis [87]. Differential signaling through Akt isoforms appears to be a critical "fork in the road" for macrophage polarization and fibrosis phenotypes. Akt1-/- mice exhibited increased macrophage apoptosis and were protected from bleomycin-induced pulmonary fibrosis [88]. Akt2-/- mice were protected from bleomycin injury, and normal fibrosis could be restored by adoptive transfer of wild-type macrophages or by administration of IL-13, suggesting that the role of the M2 macrophage is important in this model [89]. IL-33, which signals through MyD88, stimulated Akt2-/- macrophages, which exhibited impaired production of IL-13 and TGFβ [89]. IL-33 polarized macrophages to alternative activation, and accordingly, IL-33-/- mice are protected from fibrosis [90]. Prolonged phosphorylation of STAT6 and increased fibrotic gene expression in response to IL-4/IL-13 in macrophages are observed with increased expression of miR-142-5p and targeting of SOCS1 [91]. Rac1, a small GTPase member of the Ras family associated with cytoskeletal organization, regulates MMP9 expression in macrophages by inhibition of AP-1 and SPI-1 [92]. Conditional deletion of Shp2 in macrophages and MMP28 expression [93] predisposed to alternative activation and enhanced bleomycin-induced injury [94]. IRAK-M-/- mice exhibited enhanced bleomycin-induced injury, presumably related to enhanced polarization to the alternatively activated phenotype [95].

Blockade of CCL2 signaling prevents fibrosis modeled by macrophage-specific deletion in Hermansky-Pudlak mice [59], which models the rare genetic disease of oculocutaneous albinism, blindness, and interstitial lung disease. CCL2 signaling may also be critical to the phenotype observed in C-C motif chemokine receptor 4 (CCR4) null mice, where M1 activation in response to bleomycin was impaired and protected against fibrosis [96].

Novel signals may be associated with shifts toward the M2 phenotype in fibrosis. Human macrophages exposed to asbestos have greater expression of MARCO (macrophage scavenger receptor with collagenous structure) and polarization [97]. In addition, MARCO-/- animals exhibited preferentially to the M1 phenotype and exhibited significantly to lower TGFb levels in the BAL [97].

ErbB4 is a member of the EGF receptor family of tyrosine kinases. ErbB4-dependent signaling in macrophages suppressed cytokine production in macrophages, downstream of the PI3K and STAT3 pathways [98]. Loss of ErbB4 exacerbated pathologies in several models of fibrosis, suggesting that fibrosis modeled simply on the M1 and M2 dichotomy may be more complicated, given that M1-mediated inflammation in this model appears to be pro-fibrotic. Loss of β-catenin in myeloid cells did not protect from acute phase fibrosis but did facilitate resolution [99]. This intriguing observation suggests that β-catenin signaling in macrophages could lead to the differentiation of an alveolar macrophage that prevents resolution of fibrosis [99].

Stimulated macrophages are the sources of many important and bioactive signals. IL-4 and IL-13 stimulation of macrophages can increase expression of IGF-1 [100]. Interaction with CD44 on alveolar macrophages with hyaluronan of specific lengths activated inflammatory gene [101] expression. It has been known that

human macrophages are sources of pro-fibrotic soluble signaling factors including PDGF [102, 103], MCP1/CCL2 [104], and fibronectin fragments [105]. Macrophage-specific overexpression of syndecan-2, a heparan sulfate proteoglycan, protects against pulmonary fibrosis, possibly by promoting TGFβR internalization in fibroblasts [92]. And as noted above, macrophage-derived products are associated with IPF exacerbations [85].

## Fibroblasts

In contrast to epithelial cells and macrophages, fibroblasts are much easier to propagate in culture and can "remember" their origin; that is to say, fibroblasts maintain their phenotypes (normal versus diseased) even after several passages in culture. In terms of fibrotic signaling events, there are limited readouts, but they are well-characterized. These signaling qualities of fibroblasts provide an outstanding in vitro model system for the fibrotic phenotype. However, single cell RNA-Seq experiments, as well as flow sorting experiments with lineage tracing and staining, demonstrated that fibroblasts in vivo are not a monolithic population and likely can be categorized into several subphenotypes like macrophages and T-cells [13, 106]. Traditional cell culture-based methods likely miss the heterogeneity of these cells and their possibly distinct signaling mechanisms. While this question is not a focus of this review, there is intense debate within the fibrosis community as to the cell of origin of the myofibroblast.

Principally, research has focused on myofibroblast differentiation including synthesis and deposition of matrix, proliferation, resistance to apoptosis, and more recently, to their role as shaping the inflammatory infiltrate. Myofibroblasts can be grown not only on tissue culture plastic but also on diverse substrata including collagen of different degrees of stiffness [107] and decellularized lungs [108], providing a model system to determine how these cells react to their environments. Intercellular communication with fibroblasts is clearly bidirectional. They signal to and receive signals from epithelial cells, inflammatory cells, and each other. The signals may be local, derived from nearby cells or from the matrix, but it appears that they may be distant as well, since fibroblasts can respond to endocrine inputs and can call cells from the periphery.

### *The Centrality of TGFβ and Smad-Dependent Mechanisms to Myofibroblast Differentiation*

TGFβ is the central signaling molecule in pulmonary fibrosis. Latent TGFβ, bound to the latency-associated peptide (LAP), can be released and activated by cellular mechanical forces [109]. TGFβ can also be activated by matrix metalloproteinases (MMP), such as MMP9 [110]. TGFβ, and all of its at least 30 family members in

humans, bind two type I receptors, which propagate the signal, and two type II receptors, which activate the process [111]. So-called canonical signaling involves receptor-phosphorylated Smads (such as Smads2 and 3) to form active transcription factor complexes including Smad4 [111]. Noncanonical signaling includes signals that propagate via MAP kinases, PI3K, IKK, and Rho kinase signaling among others [111]. Lactic acid increases in IPF patients, presumably by hypoxia, can lead to local lactic acid production, which can activate latent TGFb [112]. Loss of TGFβ signaling by conditional knockout of TGFβR2 in resident fibroblasts protected from bleomycin-induced pulmonary fibrosis [113]. Myofibroblast differentiation is characterized by the expression of α-smooth muscle actin (*ACTA2*). Smads, particularly Smad3, are rate limiting for myofibroblast differentiation in vitro, and Smad-binding elements are necessary for the transcription of α-SMA [114–116]. Cell-penetrating peptides that block Smad3 inhibited myofibroblast differentiation in human fibroblasts [117]. MicroRNA-mediated effects on Smads have important effects on myofibroblast differentiation. MicroRNA (miR)-21 increased α-SMA expression, possibly by direct binding to and degradation of inhibitory Smad7 [118]. More recently, miR-27a-3p inhibited myofibroblast differentiation via blockade of α-SMA and Smads2 and 4 [119]. MiR-101 could decrease experimental pulmonary fibrosis and myofibroblast differentiation by decreasing Smad2/Smad3 signaling by depletion of TGFβR1 [120]. The potent antifibrotic hormone relaxin, via stimulation of G-protein-coupled receptors, promoted expression of NOS2 (iNOS) in fibroblasts. Elaboration of paracrine nitric oxide (NO) leads to a cGMP/protein kinase G-dependent inactivation of Rho/ROCK, ultimately leading to cellular relaxation [121]. NO inhibited pro-fibrotic TGFβ signaling by blocking phosphorylation of Smad2 [122]. In vitro, IPF fibroblasts, compared to donor controls, exhibited lower expression of RXFP1, the receptor for circulating relaxin (encoded by *Rln2*), via TGFβ and Smad signaling, and impaired in vitro sensitivity to a relaxin-like agonist [123]. Sensitivity to relaxin could be restored by enhancing expression of RXFP1.

As in epithelial cells, one critical mediator of Smad signing is focal adhesion kinase (FAK), which merits special attention. FAK is a non-receptor cytoplasmic tyrosine kinase that resides at focal adhesions, where integrins cluster [124]. FAK activation by receptor tyrosine kinases or G-protein-coupled receptors results in docking of Src and ultimately signaling via MAP kinases, PI3 kinase, and Rho [124]. Pharmacologic inhibition of FAK can block the effect of TGFβ on myofibroblast differentiation [125]. Interestingly, prostaglandin E2 (PGE2) can block myofibroblast differentiation induced by TGFβ, not by blockade of Smad activation, but rather by blocking phosphorylation of FAK [126]. Pro-fibrotic gene expression required cell adhesion and was dependent on JNK via FAK signaling [127]. Pharmacologic [128] or PPARγ ligand [129] blockade of PI3K can block fibroblast proliferation and expression of α-SMA. In the case of PPARγ ligands, TGFβ-induced phosphorylation of Akt was blocked independent of p38 or PTEN but was dependent on FAK [129]. FAK-related nonkinase, a structurally related protein, has been found to inhibit TGFb-induced myofibroblast differentiation via negative regulation of TGFb-mediated activation of downstream effectors such as FAK, Rho, and Smad3 [130].

## *Smad-Independent Mechanisms in Myofibroblast Differentiation*

Myofibroblast differentiation is associated with Smad-*independent* mechanisms, including the MAPK pathways. TNFα, also by Smad-independent mechanisms, blocked α-SMA expression induced by TGFβ via ERK- and Egr1-dependent mechanisms [131]. Cav1 expression in fibroblasts suppressed TGFβ-induced matrix deposition via JNK [132].

TGFβ-induced expression of NADPH oxidase 4 leads to cellular $H_2O_2$ generation, which is necessary for myofibroblast differentiation [133]. Silencing of NOX4 or blockade of $H_2O^2$ blocks the effect of TGFβ. Myofibroblast differentiation via colchicine was blocked by Rho kinase inhibition or by an SRF inhibitor independent of Smads [134]. PGE2 blocked myofibroblast differentiation by reducing nuclear translocation of serum response factor and megakaryoblastic leukemia (translocation) 1 (SRF-MKL1) complexes, mediated by reduced expression of SRF via a p38-dependent mechanism [135]. Expression of an activated serum response factor (SRF) could suppress myofibroblast differentiation [136], another example of Smad-independent blockade of α-SMA expression and myofibroblast differentiation induced by TGFβ.

## *Other TGFβ-Dependent Pathways Involved in Myofibroblast Differentiation*

Fibroblasts can shed off soluble ephrinB2 by TGFβ-stimulated ADAM10 and can promote fibroblast chemotaxis and activation; furthermore, mice lacking ephrinB2 are protected from experimental pulmonary and skin fibrosis [137]. This protection occurs through a MKL1-/MRTFA-dependent mechanism [138].

Endothelin-1 (ET-1) was found to induce myofibroblast differentiation by a PI3K-/Akt-dependent mechanism [139] and mediates resistance to apoptosis [140, 141]. TGFβ increased signaling via the MAPKs, but in this study, only JNK inhibition prevented myofibroblast differentiation [142]. TGFβ signaling in lung fibroblasts may work synergistically with ERK signaling induced by basic FGF [143].

## *Mechanosensing and Responsiveness to Matrix*

The role of the matrix and of mechanical forces in activated fibrotic signaling in the lung is well-established, perhaps best exemplified by the mechanism of TGFβ activation by mechanical forces and interactions with the αvβ6 integrin [109]. As noted above, decellularized lung can be employed as a model culture system to test the effects of the matrix and matrix stiffness [144]. Matrix fragments are potentially

biomarkers of IPF disease severity [145]. The nature of the matrix appears to be the primary driver of the fibrotic phenotype [107].

Responsiveness to matrix stiffness is a hallmark of myofibroblast differentiation. Transient receptor potential vanilloid 4 (TRPV4) is activated in response to changes of membrane stiffness and was found to regulate α-SMA expression by a Smad-independent mechanism; however, increased nuclear translocation of the α-SMA coactivator MRTF-A was observed [146]. TRPV4 pharmacologic inhibition and silencing could block α-SMA expression, and TRPV4-/- animals were protected from bleomycin injury [146]. Smad2/Smad3 dependence of myofibroblast differentiation can be shifted to Smad1 by glucocorticoid treatment [147], known to be dangerous in IPF [148].

There is an increased recognition of the impact that mechanical forces from the cell's microenvironment have on cell signaling pathways. This increased focus on "mechanosensing," particularly in the context of pulmonary fibrosis, has led to the creation of a National Heart, Lung, and Blood Institute Workshop that helps guide the research community on mechanobiology in pulmonary fibrosis [149]. Interactions with matrix in pulmonary fibrosis are well-known. Hyaluronan, a major extracellular matrix component, is a non-sulfated glycosaminoglycan [150] and interacts with toll-like receptors 2 and 4. Hyaluronan protected lung epithelial cells from apoptosis associated with lung injury, in a MyD88-dependent process [151]. Hippo signaling via the transcriptional coactivators YAP and TAZ has emerged as a key signaling pathway. When grown on stiff, but not on compliant, matrices, YAP and TAZ accumulate in the nucleus and affect key fibroblast functions including proliferation and matrix synthesis [152]. TAZ heterozygotes are protected from pulmonary fibrosis [153]. TAZ reinforced TGFb-driven signals and increased expression of CTGF via a TAZ response element [153]. Mechanosensing by the alpha6 integrin can confer an invasive phenotype to the fibrotic fibroblast, via ROCK-mediated activation of Fos and Jun [138].

## *Rho/ROCK*

Arrangement of actin filaments occurs as a result of myofibroblast differentiation and is regulated by the Rho-associated coiled-coil forming protein kinase (ROCK) family of serine/threonine kinases [154]. ROCK isoforms 1 and 2 are activated by Rho GTPases downstream of several G-protein-coupled receptors and ligand-receptor tyrosine kinase interactions [154]. Inhibition of Rho/ROCK by fasudil, via the transcription factor MKL1, protects animals from pulmonary fibrosis and prevents TGFb-induced myofibroblast differentiation [155]. ROCK1- or ROCK2-haploinsufficient mice were protected from bleomycin injury, which included decreased myofibroblast differentiation and decreased epithelial cell apoptosis [31]. ROCK inhibitors are currently in clinical trials for fibrotic diseases, including IPF (https://clinicaltrials.gov/ct2/show/NCT02688647, disclosure: Dr. Kass is a sub-investigator in this pharmaceutical-sponsored trial).

## *Fibroblast Proliferation and Resistance to Apoptosis*

In contrast to TGFβ, PDGF is a powerful mitogen for lung fibroblasts [156–161]. PDGFRα or PDGFRβ are expressed on lung fibroblasts or pericytes, respectively, with pericytes possibly representing the cell of origin for the lung myofibroblast (reviewed in [162]). IGF1 stimulates myofibroblast differentiation and matrix synthesis [163]. Basic FGF (or FGF-2) induces myofibroblast differentiation in vitro, but FGF knockout animals are characterized by impaired epithelial recovery [164]. TGFβ-induced proliferation may also be JNK-dependent [165]. Expression of KGF (FGF7) in normal lung fibroblasts is JNK-dependent, and KGF expression is impaired in IPF fibroblasts [166].

The final common pathway in IPF is the accumulation of activated fibroblasts in the lung parenchyma. The source of fibroblasts in IPF is unclear but likely includes a combination of recruitment of circulating fibroblast precursors [167, 168] and proliferation of resident lung mesenchymal cells [169]. In contrast to epithelial cells in IPF, fibroblasts in IPF lungs "paradoxically" demonstrate little apoptosis [170] despite an environment in the lung that is hostile to cell survival [171, 172]. Ex vivo, fibroblasts from IPF lungs are resistant to pro-apoptotic stimuli compared to normal human fibroblasts [173]. Persistence of fibroblasts in fibrotic lungs may be due to soluble survival factors that protect cells against various forms of apoptosis [174]. Pro-survival Akt signaling, by means of pathologically reduced expression of phosphatase and tensin homolog (PTEN) [175], is activated in IPF myofibroblasts and leads to a phenotype of highly proliferative cells [176]. Further, pathologic activation of PI3K/Akt in IPF fibroblasts occurs downstream of interaction with extracellular polymerized collagen [176]. Indeed, PTEN+/− animals exhibit increased fibrosis in response to bleomycin [176]. In addition, pharmacologic inhibition of PI3K prevents fibrosis induced by transgenic expression of TGF-α [177]. Collectively, these data show that in IPF, fibroblasts proliferate and evade apoptosis by mechanisms that include stimulation by growth factors, interactions with matrix, and activation of pro-survival pathways.

Oncostatin M, a member of the IL-6 family, via STAT3 induced fibroblast chemotaxis and myofibroblast differentiation [178]. However, IL-6 was cytostatic for normal lung fibroblast via STAT3, but a shift in expression to ERK signaling in IPF led to proliferation [179]. Differential sensitivity to Fas-induced apoptosis was observed because of a similar mechanism [180, 181].

## *Fibroblasts as Sources and Targets of Inflammatory Chemokines*

IPF is a disease classically defined as *independent* of inflammation [182]. However, in studies of both experimental and idiopathic pulmonary fibrosis [183–188], there is now increased recognition of a role for inflammation [3]. Furthermore, recent data support the concept that mesenchymal cells in the lung are immunologically active and can act as potent regulators of the character and extent of the

inflammatory infiltrate in the lung [189–191]. IPF fibroblasts were shown to be a potent source of CXCL12, a chemoattractant for T-cells and possibly for bone marrow-derived matrix-producing cells [192]. CXCL12 is chemoattractant for lymphocytes [193] via the G-protein-coupled receptor CXCR4 and, to a lesser extent, CXCR7 [194]. CXCL12, also known as stromal-derived factor 1 (SDF-1), is expressed in fibroblasts [195]. In fibrosis, CXCL12 appears to drive the accumulation of so-called fibrocytes, bone marrow-derived collagen-producing cells [167, 168]. Neutralization of CXCL12 signaling attenuates bleomycin-induced pulmonary fibrosis [196]. Expression of CXCL12 is regulated by noncanonical NF-κB2 signaling [197]. TGFβ increased the expression and promoted nuclear translocation of p65, which was blocked by silencing of Smad3 [198]. CXCL12 induced CTGF expression via ERK, JNK, and AP-1 [199]. CCL18 stimulated matrix synthesis, which could be blocked by ERK but not p38 signaling. It should be noted that blood levels of CCL18 is a powerful predictor of outcome in IPF and in SSc [135, 185].

Several studies implicate relatively less studied fibroblast-derived chemokines in the pathogenesis of IPF. CXCL6 is a fibroblast-derived chemokine [200, 201]. CXCL6, also known as granulocyte chemotactic protein (GCP)-2, signals through CXCR1 and CXCR2 and is a known chemoattractant for neutrophils. The role of CXCL6 has yet to be elucidated in fibrosis. CXCL6 is expressed in dermal fibroblasts in SSc, and serum levels of CXCL6 correlate with the degree of dermal and pulmonary fibrosis [202]. CXCL6 expression was induced by pro-fibrotic stimuli including CTGF [203] and low molecular weight hyaluronan [204]. While the chemotactic activity of CXCL6 is well-characterized, CXCL6 may act independently via CXCR2 in fibroblasts to induce a senescent and pro-survival phenotype [205]. CCL7 is increased in the blood of SSc patients and is associated with the extent of skin and lung fibrosis [206]. CCL7 expression is increased in UIP [207, 208]. CCL7 is fibroblast-derived [209, 210]. While CCL7 is classically studied in the context of monocyte chemotaxis via CCR2 [211], CCL7 may work directly on fibroblasts and work synergistically with TGFβ to promote matrix synthesis [212].

## *Wnt/β-Catenin Signaling*

There is ample evidence of "aberrant" Wnt/β-catenin signaling in IPF [213], but the critical question is, is Wnt/β-catenin signaling always pathogenic [214]? Focusing solely on the effect of Wnt on fibroblasts, noncanonical Wnt signaling via Wnt5a upregulated in IPF and stimulated fibroblast proliferation and resistance to apoptosis [215], and nuclear localization of β-catenin activated proliferation but not myofibroblast differentiation [216]. Wnt3a induced expression of α-SMA and collagen, but these effects were reversed by Dickkopf-1 (DKK1) [217]. Foxd1+ cells in the mouse lung expanded after bleomycin-induced injury and were enriched in Wnt signaling-associated transcripts [218]. Low α2β1 integrin is associated with more β-catenin activity and promotes proliferation [219]. Pharmacologic inhibition of or silencing of GSK-3 (glycogen synthase kinase 3), a constitutively active serine/threonine kinase, which inhibits Wnt signaling as one of its functions, could block

TGFβ-induced expression of α-SMA, independent of ERK, but mediated by phosphorylation of CREB [220].

## Endothelial Cells

It is important to consider other cells in the lung that in general have received less attention than the aforementioned cell types. While the signaling events were not clearly elucidated in this study, CXCR7 expression, which can transduce signals from CXCL12 as opposed to CXCR4, is associated with lung repair [221]. Endothelial-mesenchymal transition (EndoMT) has received considerable attention [222–224]. TBX4+ cells differentiated into myofibroblasts in animal models, and before injury, several TBX4+ cells were endothelial cells. It is possible that TBX4 expression is not dependent on TGFbeta1 but rather on TGFbeta2. This has not been fully explicated [225]. Lymphatic endothelial cells may be an important source of PDGF in pulmonary fibrosis [226].

## Lymphocytes

The role of lymphocytes in IPF is controversial. As noted above, and in the literature [60, 184, 192], lymphocytes and inflammatory mediators can certainly shape the fibrotic phenotype, both in experimental animals and in patients. Perhaps the best indicator of a role for lymphocytes in fibrosis is the number of non-IPF interstitial lung diseases that are associated with autoimmunity (e.g., systemic sclerosis or the antisynthetase syndromes) and with dysregulation of T-cell pathways (hypersensitivity pneumonitis) [227]. The failure of patients to respond to corticosteroid therapy and the lack of lymphocytes seen in the biopsies of patients with IPF were critical to the notion that led many to consider IPF to be a disease free of inflammation [182]. However, this idea has shifted [3]. To date, there is little data on lymphocyte signaling pathways in IPF. Interferon-γ went to a clinical trial for IPF and failed [228]. It is not clear what was being targeted, but new clinical trials that address inflammation in IPF hold promise that there will be a greater study of lymphocytes in the future.

## Conclusions

The central challenge of studying signaling in the fibrotic lung is to know which cell and which pathways are most important. A great deal is known about fibrotic signaling in fibroblasts, but there is comparatively less in macrophages and epithelial cells and even far less in lymphocytes and endothelial cells. Growing pharmaceutical interest in IPF—in addition to approved drugs, increasing sophistication of cell

culture methods and omics technologies, and ex vivo modeling of fibrotic lung diseases—suggests that the future is bright for IPF research.

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# Chapter 5
# Pulmonary Matrikines: Origin, Function, and Contribution to Fibrotic and Non-fibrotic Lung Disease

**Gautam George, Janice Walker, and Ross Summer**

## Introduction

The extracellular matrix (ECM) of the lung is a latticelike structure comprised of interlocking proteins that provide structural support, facilitate intercellular communication, and give direction to immune cells trafficking in and out of the lung [1]. In many organs, including the lung, the ECM is a highly dynamic compartment that undergoes frequent remodeling through both increased synthesis and degradation of its core components [1, 2]. These ECM changes lead to complex, bidirectional responses often referred as "dynamic reciprocity," which describes the way in which changes in the composition of the ECM can, at first, directly alter the behavior of cells by activating cell surface receptors but ultimately influence cell behavior by altering the production of ECM proteins. While the balance between synthesis and degradation is usually tightly regulated, dramatic changes in lung ECM composition occur during organ development, aging, and a wide range of pathological respiratory conditions [3–5].

A notable consequence of ECM remodeling is that proteolytic processing leads to the liberation of potent bioactive peptide fragments into the surrounding environment [6]. These small peptide fragments were initially termed "matrikines" by Maquart et al. and have been identified in virtually all organs under pathological conditions [7]. Matrikines exert their effects on a wide range of cell types and appear to have particularly important roles in regulating immune cell behavior and

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coordinating tissue repair responses [8, 9]. In this chapter, we will discuss the emerging evidence linking matrikines to lung biology, including reviewing the mechanisms by which these molecules are formed and elicit their downstream responses. We will also discuss the role of matrikines in the pathogenesis of several respiratory disorders and postulate how these molecules could be targeted to treat human disease.

**What Makes a Matrikine a Matrikine?** There is no uniform consensus definition of what defines a matrikine; however, the general rule is that three principle criteria must be fulfilled for an ECM-derived peptide to be called a matrikine. First and foremost, all matrikines are derived from a parent ECM molecule, such as collagen, hyaluronan, elastin, or laminin. Second, all matrikines must be produced by a biologically relevant form of enzymatic or chemical digestion, as opposed to some contrived experimental method seen only in laboratory settings. Finally, all matrikines must exhibit biological actions distinct from their parent ECM molecule, and these actions must be displayed in cells that typically reside within proximity of the cell from which they are derived [7–9]. With this criterion in mind, this chapter will focus exclusively on pulmonary matrikines, whereas other small bioactive molecules found in the ECM, such as cytokines, chemokines, and growth factors, will not be discussed further in this chapter.

**What Is the Primary Source of Lung Matrikines?** As the above discussion connotes, all matrikines are derived from structural elements of the "matrisome." The matrisome is a term used to describe every component of the ECM including fibrillar and non-fibrillar proteins (glycoproteins and proteoglycans) as well as the enzymes (e.g., metalloproteases) that participate in their remodeling [10, 11]. Although qualitative differences are found in the matrisomes of different tissues, most matrisome components are shared across a broad range of organs. Additionally, while over 80 different types of proteins are found in most matrisomes, at present, it appears that only a few ECM proteins liberate matrikines. In this section, we will discuss the four major ECM proteins recognized to release matrikines in the lung [11–13]. There are multiple sites in the lung where matrikine production is frequent (Fig. 5.1).

*Collagen* Collagen is by far the most abundant ECM molecule in the lung and is probably the most significant contributor to the production of matrikines. Collagen is found in virtually all regions of the lung, including the interstitial spaces, and the areas surrounding airways and blood vessels [14]. Moreover, collagen is a molecule that undergoes frequent turnover, suggesting that matrikines emanating from this molecule could impart biological effects under both homeostatic and pathological conditions [15]. Although collagen I and III predominate, many other collagen molecules exist in the lung, and all collagen molecules possess a similar primary structure comprised of a series of Gly-Pro-X repeats (with X usually being a proline). This core structure enables collagen molecules to adopt a unique right-handed helical conformation, which supports the formation of other more structurally sophisticated molecules such as collagen fibrils and fibrillar collagen. Collagen fibrils are

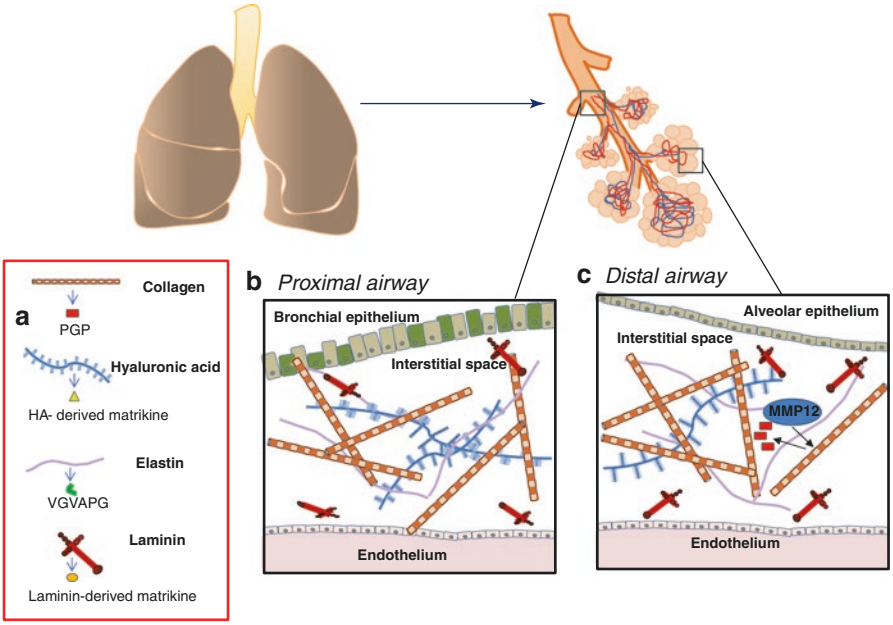


**Fig. 5.1** Origin of major lung matrikines. Square inserts depict proximal and distal regions of the lung matrisome. (**a**) Graphical key to the four major extracellular matrix molecules and matrikines liberated in the lung. (**b**) Proximal and (**c**) distal regions of the lung, respectively

formed when two α1 chains and one α2 chain coalesce to form a tightly wound trimeric protein complex, whereas fibrillar collagen is made when these fibrils combine to form thick rope-like structures. Importantly, collagen fibrils and fibrillar collagen are both extremely resistant to enzymatic degradation, which is presumably necessary to limit unwanted collagen matrikine production under homeostatic conditions [14–18].

*Elastin* Elastin is another ECM protein known to liberate matrikines in the lung [12]. Elastin is found in virtually all vertebrate tissues and is most abundant in arteries, ligaments, and the lung. When fibroblasts, smooth muscle cells, or endothelial cells secrete tropoelastin, the tropoelastin coalesces to form large polymer-like structures (elastin). Elastin fibers are extremely resilient molecules and are capable of stretching to extraordinary lengths (140% of their resting length), thereby enabling the lung and other elastin-rich tissues to withstand years of mechanical strain [13]. Importantly, elastin production ceases after birth in both the rodent and human lung, which means that its turnover is limited almost exclusively to pathological states that cause elastin destruction (e.g., emphysema) [18, 19].

*Hyaluronan* Hyaluronan (HA) is the third ECM molecule known to liberate bioactive molecules in the lung. HA is an anionic glycosaminoglycan found throughout the ECM of the lung, including in basement membranes and the

pulmonary interstitial spaces [20]. HA is synthesized as a large polysaccharide composed of repeating units of N-acetyl-d-glucosamine and d-glucuronic acid [21]. Like elastin, HA is capable of deforming in many directions but also serves other important functions such as acting as a lubricant for tissues, an absorber of extracellular water, and a scavenger of reactive oxygen species. In humans, HA degradation is most commonly mediated by one of six naturally occurring hyaluronidase enzymes. However, exogenous hyaluronidases derived from bacterial species can also contribute to HA degradation in some cases [22].

*Laminins* More recent evidence indicates that laminin ECM proteins can also liberate matrikines in the lung. Laminin proteins are large-molecular-weight heterotrimeric glycoproteins found exclusively in basement membranes and hemidesmosomes (cell/matrix adhesion structures located at the basal aspect of some epithelial cells) [23]. Laminins are composed of three distinct chains called α, β, and γ chains and are assigned a three-number code based on their constituents (e.g., laminin-332). The C-terminus of laminin proteins has been shown to interact with the plasma membrane of some cells, while the N-terminal domain binds directly to other ECM components, thereby enabling laminin to serve as a bridging molecule between structure cells and other ECM proteins. Given that most laminin proteins are located in basement membranes (BMs), it is not surprising that matrikines emanating from these molecules have been most closely linked to influencing the behavior of airway epithelial cells [24].

## Formation and Function of Pulmonary Matrikines

A large number of matrikines have been identified in mouse and human tissues, and, collectively, these molecules exhibit a diverse array of biological functions. A list of only those matrikines abundantly expressed in the lung with well-defined biological functions is summarized in Table 5.1. Additional matrikines are discussed in *Chapter* 10.

*Collagen-Derived Matrikines* Existing evidence suggests that collagen-derived matrikines arise mostly from fibrillar collagen (e.g., collagen I) in the lung. Moreover, the predominant bioactive matrikine emanating from lung collagen is a tripeptide fragment containing 3–5 proline, glycine, and proline (PGP) repeats [35]. While Pfister and colleagues were the first to show that PGP fragments possess potent neutrophil chemotaxis activity in an ocular injury model, it is now appreciated that PGP exhibits similar biological functions in the lung and various other tissues [36]. Interestingly, this chemotactic activity is mostly dependent on acetylation of PGP, which likely provides additional control over the regulation of this matrikine's activity. Acetylation of PGP imparts stronger agonist activity on the CXCR1 and CXCR2 chemokine receptors, which also induce IL-8-mediated neutrophil chemotaxis in human tissues [37]. Currently, PGP is thought to be formed

**Table 5.1** Matrikines in the lung

| ECM | Enzyme(s) | Matrikine | Biological function |
|---|---|---|---|
| Collagen | MMP-8, MMP-9, Prolyl endopeptidase (PE) | Proline-glycine-proline (PGP) | Pro-inflammatory chemokines that can activate cell signaling via CXC-chemokine receptor 2 (CXCR2) [25]; act as neutrophil chemoattractants especially N-ac-PGP [26] |
| | | N-terminal acetylated PGP (N-ac-PGP) | |
| Elastin | MMP-1, MMP-3, MMP12 (macrophage elastase), neutrophil elastase | VGVAPG | Chemotactic for fibroblasts and monocytes [27]; modulates T-cell response in emphysema in mouse model [28] |
| Hyaluronan | Hyaluronidase | Low- and high-molecular-weight-derived HA matrikines | Low-molecular-weight (LMW) HA is pro-inflammatory [29]; LMW-HA can induce MMP-9 and MMP-12 to promote cytokine production [30] |
| Laminin | MMP-2 (for laminin-111) | 60 kDa fragment (derived from laminin-111) | Laminin-111-derived fragment can modulate epithelial-mesenchymal transition in embryonic stem cells [31] |
| | MMP-3, MMP-12, MMP-14, MMP-19 MMP-20, neutrophil elastase (for laminin-332) | $\gamma_2$ ectodomain | $\gamma_2$ ectodomain is chemotactic for neutrophils [32]; can modulate epithelial cell migration [33], morphology [34], and epithelial regeneration during alveologenesis [13] |

Table created from PubMed search (keywords: matrikine, laminin fragment, elastin, collagen) and adapted from Gaggar and Weathington [12]

by a two-step process that involves the activity of matrix metalloproteinases (MMPs) (mostly MMP8 and MMP9) as well as a serine prolyl endopeptidase. Notably, this two-step process may serve as another layer of protection that helps to reduce unwanted PGP production [35, 36, 38].

*Elastin-Derived Matrikines* Senior and colleagues were the first to demonstrate that matrikines are liberated from elastin molecules in the lung and that these bioactive substances possess potent monocyte chemotaxis activity in vitro and in vivo [27]. Moreover, it was determined that the biological activity of elastin matrikines was mediated, in large part, by a small peptide fragment (less than 50 kDa) containing multiple VGVAPG subunits [39] and that MMP12 and other metalloproteinases mediated elastin fragmentation. In these and other studies, VGVAPG not only promoted monocyte recruitment, but it also induced T lymphocyte and fibroblast chemotaxis. Moreover, VGVAPG can increase the activity of macrophage elastase (aka MMP12), suggesting that VGVAPG production is capable of triggering an autofeedback loop that might drive further matrikine production. To date, the only receptor known to engage VGVAPG is the S-gal receptor, suggesting that modulating this interaction could prove useful for augmenting or inhibiting VGVAPG activity in vivo [18, 19, 40].

*Hyaluronan-Derived Matrikines* In contrast to other ECM molecules, HA degradation has been shown to liberate both low- and high-molecular-weight matrikines, which have distinct functional activities [20]. For example, low-molecular-weight HA-derived matrikines (<100 kDa) exhibit immune-related functions, as judged by their ability to induce MMP9 and MMP12 activity and augment pro-inflammatory cytokine production [30]. In contrast, high-molecular-weight HA-derived matrikines possess limited immune regulatory properties but instead appear to enhance epithelial integrity by increasing lung epithelial cell growth and proliferation. Thus, the influence of HA-derived matrikines on tissues is likely dictated by the extent of proteolysis by hyaluronidases (Hyals), with more extensive proteolysis liberating smaller peptide fragments with pro-inflammatory properties [22, 29]. That said, this hypothesis has yet to be proven, and the mechanisms responsible for regulating the production of low- versus high-molecular-weight HA matrikines remain unclear.

*Laminin-Derived Matrikines* Although the role of laminin-derived matrikines in the lung is still poorly understood, existing evidence indicates that these molecules possess mitogenic activity on lung epithelial cells. Furthermore, the ability of laminin-derived matrikines to act as mimetics of the hormone called epidermal growth factor appears to drive these effects. For example, the cleavage of the $\gamma_2$ chain of laminin-332 by MMPs liberates a small bioactive fragment that possesses EGF-like properties and can augment epithelial regeneration during alveologenesis [13]. Additionally, other bioactive matrikines liberated from laminin also exhibit immune-related properties in some contexts. One example comes from studies in which laminin was exposed to neutrophil elastase and led to small bioactive fragment production that augmented neutrophil chemotaxis in vitro [32, 34]. Currently, neither the structure of laminin-derived matrikines nor the receptors in which they engage have yet to be uncovered.

## Matrikines in Lung Health and Disease

Given the ability of pulmonary matrikines to influence immune cell infiltration and regulate the growth of epithelial cells, it is not surprising that matrikine activity correlates with the development and progression of a wide range of respiratory diseases. In the following section, we will discuss the emerging evidence that links matrikines to the pathobiology of several respiratory diseases [41, 42].

## Pulmonary Fibrosis

Pulmonary fibrosis refers to a group of conditions that cause respiratory dysfunction by scarring the distal airspaces. These conditions can arise from direct pulmonary insults (drugs, radiation, or environmental exposures) or develop in association with other disorders like autoimmune conditions, chronic pulmonary infections, or

granulomatous diseases. Occasionally, pulmonary fibrosis can develop without any identifiable explanation, and conditions arising in this way are classified as idiopathic pulmonary disorders [43].

Although the mechanisms leading to pulmonary fibrosis likely differ depending on underlying etiology, it is generally believed that scar tissue formation occurs only when lung progenitors fail to regenerate the distal pulmonary epithelium after injury. In turn, this triggers the initiation of wound healing responses, presumably as an attempt to restore barrier function lost in response to epithelial injury [44]. Importantly, this wound healing response is associated with both the production of new ECM proteins and also the degradation and remodeling of existing ECM molecules, the latter of which is mediated by numerous collagenases, elastases, and matrix metalloproteinases [45, 46].

The major ECM component produced during scar tissue formation is collagen. In fact, collagen is estimated to represent over 90% of scar tissue mass in all tissues. As previously described, collagen is a significant source of matrikine production through the liberation of PGP [35]. Because PGP is known to have potent neutrophil chemotactic activity, its production is hypothesized to exacerbate fibrotic lung disease [47]. However, in recent work, it was shown that administering PGP or a peptide analog to mice led to a reduction in pulmonary fibrosis induced by bleomycin [48]. These findings suggest that PGP may possess additional functions or that neutrophil infiltration induced by this matrikine has protective effects on the lung.

In addition to collagen, proteoglycans and glycosaminoglycans, including HA, are also synthesized and degraded in pulmonary fibrosis [49]. Although the role of HA matrikines in pulmonary fibrosis is not well understood, HA synthase overexpression is sufficient to induce fibrotic remodeling in some tissues. These findings suggest, at least in part, that HA production and the matrikines liberated from this molecule contribute directly to the development of fibrotic remodeling in the lung [50, 51]. However, because HA also liberates matrikines with pro-growth properties, therapeutic strategies designed to inhibit hyaluronidase activity entirely may not yield predictable results if used in the treatment of fibrotic conditions.

## Chronic Obstructive Pulmonary Disease (COPD)

COPD is one of the most common respiratory diseases and is the third leading cause of death in adults in the USA. COPD is a condition that results most commonly from chronic tobacco smoke exposure but is linked to other environmental exposures in a minority of cases. Classically, COPD is a term used to describe two distinct pathological states, namely, chronic bronchitis and emphysema. Although neither is considered to be a fibrotic pulmonary disorder, tissue remodeling is a prominent feature of moderate-sized airways in patients with chronic bronchitis. Moreover, emphysema is a disease characterized by massive loss of ECM components, particularly elastin, which contributes directly to the loss of elastic recoil and dilation of distal pulmonary air sacs [52].

In COPD, chronic environmental exposures are thought to contribute to disease by inflaming the airways and inducing immune-mediated tissue destruction. An essential contributor to tissue injury in this disease is neutrophils, which are known to infiltrate the walls and airways of COPD patients. While neutrophils are a crucial first line of defense against microbial attack, these cells can also damage tissues by releasing of a wide range of toxic substances including many of which that are involved in degrading ECM proteins.

The most studied matrikine in COPD is the collagen-derived PGP fragment [53]. For example, O'Reilly et al. showed PGP levels are markedly increased in the sputum from patients with COPD when compared to healthy controls or individuals with asthma [54]. Elevated serum PGP levels are present in COPD patients. Moreover, a recent multicenter clinical trial showed that sputum PGP levels were highest during exacerbations and levels tended to increase right before the onset of symptoms, suggesting that PGP production may contribute to the pathobiology of acute exacerbations. Consistent with this notion, treatment of COPD exacerbations with antibiotics (azithromycin) not only improved acute COPD symptoms but also reduced neutrophil infiltration and decreased sputum PGP levels [35, 38].

Because loss of elastic recoil characterizes emphysema, it makes sense that matrikines liberated from elastin might contribute to the development of disease [18]. Consistent with this line of reasoning, elastin degradation products are known to be markedly increased in the lungs of mice exposed to cigarette smoke and in the urine of patients with COPD. Moreover, antagonizing elastin fragment formation can abrogate both macrophage accumulation and airspace enlargement in a mouse model of cigarette-induced emphysema [55].

## Cystic Fibrosis

Cystic fibrosis (CF) is one of the most common genetic diseases in people of Northern European descent. Functional defects in a transmembrane conductance regulatory (CFTR) protein causes CF, by altering the chloride transport across the surface of epithelial cells. CFTR mutations cause a decrease in chloride secretion, leading to various downstream consequences including an increase in the viscosity of airway secretions [56]. In the lung, changes to mucus viscosity have been shown to compromise ciliary function in the airways, leading to further problems such as inspissated secretions and enhanced susceptibility to bacterial respiratory infections.

As the name implies, CF often leads to tissue remodeling from the destructive, inflammatory responses to recurrent infections. There are few studies investigating the role of matrikines in the pathobiology of CF. One recent example is by Gaggar et al. who examined levels of PGP in the sputum of patients with CF patients and moderate-severe lung disease [57]. Though the sample size was small, PGP levels were found to be significantly increased in eight out of ten patients with CF when compared to healthy controls. Because elevated proteolytic PGP enzyme activity is present in CF patient lungs, as evidenced by elevated sputum MMP-9 and neutrophil elastase, it is likely that increased proteolytic activity contributes to PGP

production. This hypothesis is further supported by recent in vitro studies demonstrating that PGP production can be induced by incubating collagen I or collagen II with sputum samples from CF patients [57]. Currently, it is unclear whether PGP is merely a biomarker in CF or contributes to the progression of disease [57].

## Acute Respiratory Distress Syndrome and Mechanical Ventilation

Acute respiratory distress syndrome (ARDS) is a severe form of lung injury that develops in association with acute medical conditions such as bacterial pneumonia, sepsis, pancreatitis, or major trauma [58]. ARDS results in widespread pulmonary inflammation that causes innate immune-mediated damage to the alveolar epithelium and endothelium of the lung [59]. There is the significant and sustained recruitment of neutrophils and macrophages in this disease. Tissue remodeling occurs in severe cases of ARDS, which correlates with elevated levels of various MMPs in the BAL fluid [60–62].

To date, the role of matrikines in the pathobiology of ARDS has not been extensively studied. However, it has been demonstrated that levels of PGP are elevated in the BAL fluid in this disease. Mechanistically, PGP is thought to augment neutrophil chemotaxis. However, emerging evidence also suggests that PGP may contribute to disease by exacerbating pulmonary vascular leakage. For example, it has been shown that binding of PGP to the chemokine receptor CXCR2 on endothelial cells activates inflammatory pathways that augment endothelial permeability. Conversely, reducing PGP levels has been shown to attenuate agonist-induced pulmonary edema in mice.

Finally, patients with ARDS often require mechanical ventilation to support their breathing while the lung is in the process of healing [59]. However, mechanical ventilation can also propagate lung injury if not utilized appropriately. Current strategies aim to minimize damage by limiting air sac overdistension while also reducing the number of times alveolar units collapse between each breath. Failure to apply these principles has been shown to increase the production of ECM proteins and the enzymes that lead to matrikine production, suggesting that matrikines liberated in response to the treatment of ARDS might also contribute to ongoing disease progression [63–66].

## Potential Therapeutic Strategies for Blocking Matrikine Activity In Vivo

Therapeutic targeting of matrikines has been employed in various fields outside the lung, including dermatology [67]. For example, in dermatology, peptide molecules such as glycine-histidine-lysine, glycine-glutamate-lysine-glycine, and lysine-threonine-threonine-lysine-serine, which are matrikines relevant to that field, have been

used extensively as a treatment to augment skin rejuvenation [68]. As such, it is reasonable to assume that therapeutic interventions targeting matrikines may hold promise in the treatment of respiratory diseases even though no such therapy currently exists.

Currently, the most likely target of therapeutic intervention would be to block PGP activity given its connection to various lung disorders including COPD, cystic fibrosis, and pulmonary fibrosis. However, ample evidence exists to suggest that targeting other matrikines might also be clinically useful in some respiratory conditions. One logical approach to blocking matrikine activity would be to deliver small peptide inhibitors of specific matrikines directly into the lung. Consistent with this approach, a tetramerized arginine-threonine-arginine (RTR) peptide antagonist has been used effectively to reduce neutrophil influx and ameliorate tissue injury in an alkali-induced ocular injury model in rodents [36, 69, 70]. Alternatively, inhibiting matrikine activity could be achieved by blocking the enzymes involved in their production, although this approach would have numerous off-target effects given the role of MMPs in regulating many biological processes. Finally, it is also reasonable to assume that inhibiting matrikine activity by interfering with the binding of matrikines to their receptors may be a potential pharmacological approach, as blocking receptor-ligand interactions is a strategy that has been employed to treat many other diseases and conditions [25, 71].

## Conclusion

In summary, matrikines are small bioactive peptides derived from chemical or enzymatic breakdown of larger extracellular matrix proteins. These peptides serve a wide range of functions but predominately act to regulate immune and reparative functions. Emerging evidence indicates matrikines play an essential role in the pathobiology of many pulmonary disorders, suggesting that targeting these molecules may be an effective strategy for reducing the onset and progression of respiratory diseases. Ongoing research in this area is needed to advance these concepts to the bedside.

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# Chapter 6
# The Role of Mast Cells in the Pathophysiology of Pulmonary Fibrosis


**Chiko Shimbori, Chandak Upagupta, Paul Forsythe, and Martin Kolb**

## Introduction

Idiopathic pulmonary fibrosis (IPF), the most common form of the idiopathic interstitial pneumonias, is a chronic and fatal disease with a poorly understood pathogenesis. Recently, two drugs, nintedanib and pirfenidone, were approved by FDA for IPF treatment [1–3]. While serving as an important milestone in the treatment of IPF, nintedanib and pirfenidone treatments slow disease progression, limited to slowing, but not stopping disease progression. Accumulating evidence suggests that the clinical course and proposed molecular mechanism of IPF are heterogeneous, implying a more complicated pathophysiology [4]. Therefore, more dynamic therapies are required, focusing on the cellular and molecular pathogenesis of IPF. IPF is characterized by progressive fibroblast and myofibroblast activation, causing extensive and disordered deposition of extracellular matrix (ECM). This leads to destruction of the alveolar architecture, resulting in a relentless decline of

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pulmonary function [1–3]. Fibrosis, or the process of fibrogenesis, is the indispensable response of the host to protect itself and provide wound healing. Myofibroblasts are responsible for the aberrant ECM deposition and contractile force and are therefore believed to play a major role in the disease progression. Myofibroblasts are rarely found in a healthy tissue. They are greatly upregulated in responding to injury and play a critical role in the wound healing response [5]. During the normal wound healing process, once the active phase of repair is finished, myofibroblasts will slowly disappear via apoptosis, and most of the scar matrix will eventually resolve. In IPF, however, the fibrotic response is persistent as the myofibroblasts appear to be resistant to apoptosis [6]. These dysregulated myofibroblasts persist and nourish the fibrotic process, contributing to the excessive deposition of ECM. This aberrant ECM deposition destroys the structural integrity of the lung and causes abnormal biomechanical and biochemical characteristics [7–12]. There is increasing evidence that the microenvironments created within the ECM affect the behavior of structural pulmonary cells leading to a vicious cycle of fibrosis [6, 13–16].

Mast cells originate from CD34-expressing hematopoietic stem cells in the bone marrow [17]. Although mast cells are best known as major effector cells in allergic and acute inflammatory diseases, mast cells have also been associated with pulmonary fibrosis. Mast cells have long been known to accumulate within the lungs of human and rodent pulmonary fibrosis [18–20] (Fig. 6.1). Furthermore, along with inflammatory mediators, mast cells release profibrotic mediators which stimulate fibroblasts and myofibroblasts [18–20]. Interestingly, mast cells have not been investigated as extensively as other cells, and their role in the progression of pulmonary fibrosis has not been fully understood. In this chapter we address the role of mast cells in the pathophysiology of pulmonary fibrosis and propose their potential as a therapeutic target for pulmonary fibrosis.

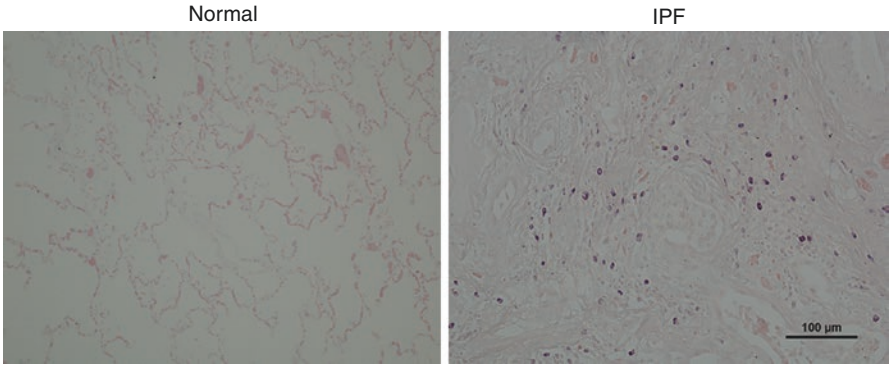


**Fig. 6.1** Increased mast cell infiltrations in fibrotic parenchymal IPF lung. Toluidine blue staining shows that an IPF lung expresses more mast cells than the healthy human lung

## Mast Cell Biology

### *Origin and Tissue Distribution of Mast Cells*

Mast cells are multifunctional tissue-resident cells participating in innate and adaptive immune responses and mediate inflammation as well as contribute to the tissue-remodeling process. The mast cell was first described in 1878 by Paul Ehrlich based on the metachromatic staining such as toluidine blue. Mast cells are long-lived granulated cells derived from hematopoietic precursors; such mast cell progenitors ordinarily are found only in small numbers in the blood and complete their differentiation and maturation in the local tissues [21–23]. Mast cells are widely distributed throughout different tissues in vertebrates. They are especially common near surfaces exposed to the external environment such as the skin, airways, intestine, and genitourinary tracts, where they function as sentinel cells in host defense [24–27]. Although the precise mechanism of the recruitment of mast cell progenitors is unclear, chemotactic factors such as chemokines, leukotrienes, and integrins are believed to play an important role [28, 29]. The integrins α4β7 and α4β1 are expressed on mast cell progenitors and are required for mast cell trafficking. However, they are downregulated as these cells mature [28, 30]. Like cells in the monocyte lineage, mature mast cells located in the tissues can proliferate in response to appropriate stimulation [24, 27]. Stem cell factor (SCF), the ligand for c-kit, is produced by structural cells in the tissues (and also by mast cells) and plays a crucial role in mast cell localization, development, survival, migration, and function within tissues [21–23]. Other growth factors that have been shown to influence mast cell growth and survival include interleukin (IL)-3, IL-4, IL-9, IL-10, IL-33, and transforming growth factor (TGF)-β1, most of which are produced by an autocrine mechanism [22]. Mast cells are distributed throughout nearly all tissues and often close to potential targets of their mediators such as epithelium and glands, smooth muscle, fibroblasts, blood and lymphatic vessels, and nerves [31].

### *Mast Cell Phenotypic Heterogeneity*

Mast cells can be subcategorized into populations defined by anatomical location and/or mediator content [24, 32]. Mast cell-specific proteases, including tryptase and chymase, are major constituents of mature mast cell granules, and mast cells are subdivided into two major subsets depending on their tryptase and chymase content. The major tryptases in humans are the βI-, βII-, and βIII-tryptases and the enzymatically inactive α-tryptase. Alleles of α-tryptase and βI-tryptase are encoded at one genetic locus, whereas the βII and βIII allelic variants are encoded at a neighboring locus. In mice, the dominating tryptases of mast cell granules are mouse mast cell protease 6 (mMCP6) and mMCP7, of which mMCP6 is likely the most homologous

to human β-tryptases. In addition to these tryptases, both humans and rodents express a monomeric transmembrane tryptase (γ-tryptase) [31]. With respect to chymase, humans express only one mast cell chymase (which is encoded by CMA1). By contrast, the chymase locus of rodents has expanded considerably and encodes the four major chymases that are expressed by mature mouse mast cells: mMCP1, mMCP2, mMCP4, and mMCP5. On the basis of tissue location and substrate specificity and affinity for proteoglycans, mMCP4 is probably the functional homologue of human chymase, although mMCP5 has a closer phylogenetic relationship to human chymase. However, unlike human chymase and mMCP4, which both have chymotrypsin-like substrate specificity, mMCP5 has elastase-like cleavage properties [31]. The profile of protease, chymase, and tryptase expression has been used to define subsets of mast cells. In humans, mast cells can be subdivided into the $MC_T$ subset, which expresses tryptases only, and the $MC_{TC}$ subset, which expresses tryptases and chymase. In rodents, mast cells are classified as either connective tissue mast cell (CTMC) or mucosal mast cell (MMC) types on the basis of the expression profile of mast cell-specific proteases. $MC_{TC}$ or CTMC, as the name implies, is mainly located in the connective tissues of the intestinal submucosa, the peritoneal cavity, and surrounding blood vessels and in the skin. In contrast, $MC_T$ or MMC is usually found in the mucosal tissues of the lung and intestine and mainly expresses tryptase [22]. Thus, mast cells were subcategorized into populations defined by anatomical location and/or mediator content. However, although this system of mast cell classification has been useful, it remains relatively simplistic. Kitamura et al. reported that MMC and CTMC phenotypes are reversible in certain microenvironmental conditions, and transdifferentiation between these two phenotypes has been seen in rodent mast cells [33]. Interestingly, given that mast cell maturation occurs in peripheral tissues, mast cell heterogeneity in response to both genetic and environmental factors in different tissues is presumably caused by microenvironmental conditions. The cells can then be positioned phenotypically to express a broad spectrum of types, kinetics, and/or magnitude of secretory functions [24, 32]. Therefore, a central hypothesis is that the heterogeneity of mast cells in tissues is much more diverse than merely two polarized phenotypes ($MC_T$ and $MC_{TC}$ or MMC and CTMC) and is somewhat dynamically changing in accordance with environmental conditions.

## *Mast Cell-Derived Mediators*

Mast cells contain various bioactive mediators, which include inflammatory and also profibrotic mediators. Mast cells can store and release these mediators upon degranulation and/or secrete de novo a broad spectrum of biologically active mediators. Following the stimulation, mast cells rapidly release mediators that are pre-stored in the cytoplasmic granules, into the extracellular space (Fig. 6.2). Some examples of these mediators include vasoactive amines (histamine and serotonin), neutral proteases (tryptases, chymases), proteoglycans (e.g., heparin), and some cytokines and growth factors (Fig. 6.2 and Table 6.1). The second class of secreted products is generated by the de novo synthesis of proinflammatory lipid mediators, such as

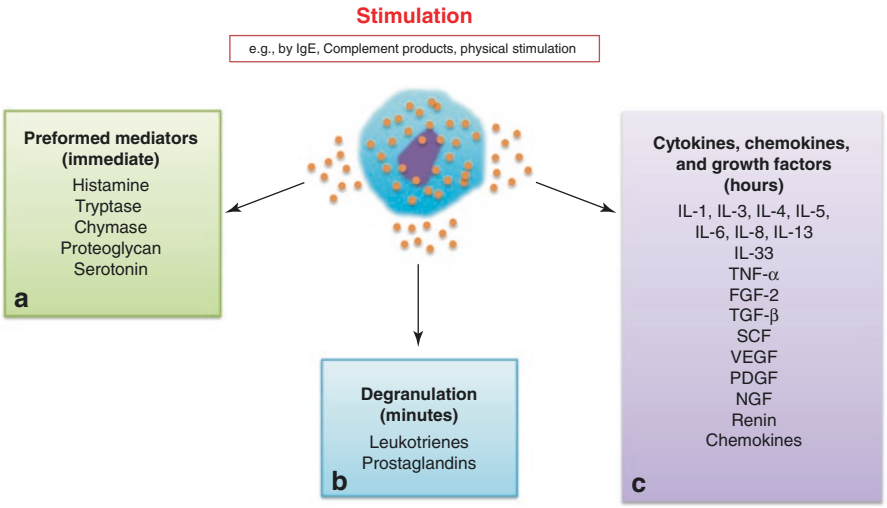


**Fig. 6.2** Mast cell mediators released upon stimulation. When mast cells are activated by IgE, complement products, physical stimulation, etc., they release various (**a**) bioactive mediators derived from immediate degranulation, (**b**) eicosanoids synthesized de novo within minutes of activation, and (**c**) numerous cytokines, chemokines, and growth factors synthesized de novo within hours of activation

**Table 6.1** Mast cell mediators

| Mediators class | Mediators |
|---|---|
| Biogenic amines | Histamine, serotonin (5-HT), dopamine, polyamine |
| Lysosomal enzymes | β-Hexosaminidase, β-glucuronidase |
| | β-D-galactosidase, arylsulphatase A |
| | Cathepsins C, B, L, D, and E |
| Mast cell-specific proteases | Tryptase, chymase, carboxypeptidase A3 (CPA3) |
| Non-mast-cell-specific proteases | Cathepsin G, MMP9, active caspase 3, ADAMTS5, granzyme B, renin |
| Lipid mediators | Prostaglandin D2 and E2, leukotrienes B4 and C4, and platelet activating factor |
| Proteoglycans | Serglycin (heparin and chondroitin sulfate) |
| Cytokines | TNF-α, TSLP, IL-4, IL-5, IL-6, IL-13, IL-33, IFN |
| Chemokines | RANTES (CCL5), eotaxin (CCL11), IL-8 |
| | (CXCL8), MCP-1 (CCL2), MCP-3 (CCL7) |
| | MCP-4 |
| Growth factors | TGF-β, FGF-2, VEGF, PDGF, NGF, GMCSF, SCF |

prostaglandins and leukotrienes (Fig. 6.2 and Table 6.1). Furthermore, mast cells are also able to synthesize and secrete a large number of growth factors, cytokines, and chemokines, e.g., IL-1, IL-6, IL-10, IL-13, IL-33, TNF-α, VEGF, PDGF, FGF-2, TGF-β, and many others (Fig. 6.2 and Table 6.1). The specific secretory factors and amounts of these mediators are dependent on the phenotype of the mast cells and the nature of the stimulus inducing mast cell activation [23, 24, 32, 34, 35]. Following

activation, mast cells are unique in that they replenish their granules, usually within a few weeks of activation [36]. The local homeostatic cytokine milieu of a tissue modulates the precise granule components, allowing mast cells to adapt to their local environment to mount a tissue appropriate inflammatory response as discussed above [32]. This ability to regranulate allows mast cells to tailor the composition of their granules and thus be more prepared for the specific challenges [26]. These mast cell-derived mediators have also been shown to have various effects on the function of diverse immune or structural cells, findings which indicate that mast cells have the potential to influence diverse biological responses including tissue remodeling and fibrosis [20]. We will discuss each profibrotic factors in the next chapter.

## Mast Cell and Pulmonary Fibrosis

### *Fibroblast and Mast Cells*

During fibrogenesis, at the end of the proliferation phase and the start of the maturation/remodeling phase, fibroblasts differentiate into myofibroblasts, which are distinguished by α-smooth muscle actin (αSMA) expression [37]. Resident tissue fibroblast is one of the most prevalent sources for myofibroblasts wherein myofibroblast differentiation of fibroblasts can be induced by treatment with TGF-β and other cytokines [38]. Mast cells have been implicated in fibroblast activation and myofibroblast transdifferentiation (Fig. 6.3). For example, the proliferation and

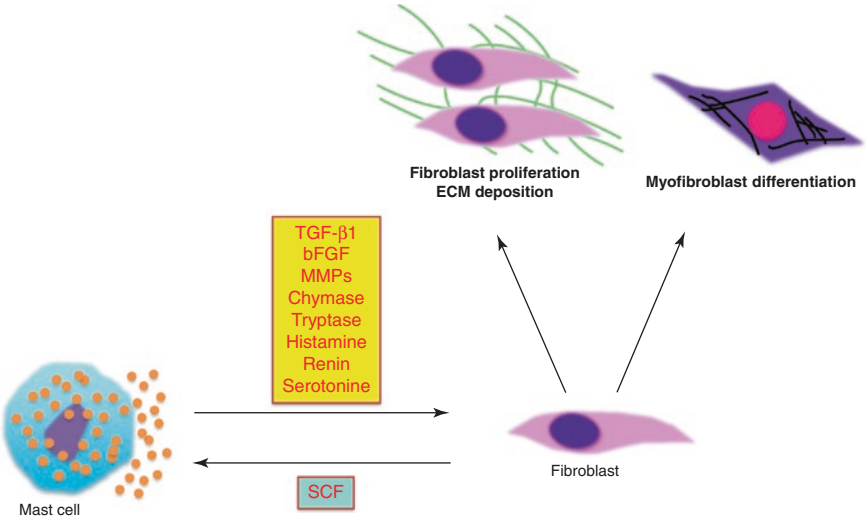


**Fig. 6.3** Mast cell and fibroblast interaction. Mast cell degranulation results in the release of fibrogenic mediators (e.g., TGF-β1), leading to activation, proliferation, and differentiation of pulmonary fibroblasts, which contribute to further ECM production in the lung

collagen production of 3T3 mouse fibroblasts were increased by co-culturing with rat peritoneal mast cells [39]. Furthermore, the exposure of these fibroblasts to the HMC-1 mast cell line resulted in increased expression of αSMA- and fibroblast-driven contraction of a collagen gel [40]. Several mast cell-derived mediators contribute to this fibroblast-mast cell interaction; details of each mediator will be described below [40–44]. In pulmonary fibrosis, human lung mast cells develop the $MC_{TC}$ phenotype, and their number correlates with the accumulation of myofibroblasts expressing αSMA [45]. On the other hand, fibroblasts also exert effects on mast cell function and phenotype. Co-culturing of mast cells and fibroblasts induced a change in mast cell phenotype, from MMT to CTMC [46, 47]. Fibroblasts induce mast cell proliferation through a release of SCF [43]. Previous studies reported that fibrotic disease-derived fibroblasts produce more SCF than normal fibroblasts [48, 49]. These findings suggest that mast cells and fibroblasts interact with each other, and this contact may contribute to the development of a profibrotic environment and disease progression. In the next section, we will discuss mast cell-derived profibrotic mediators.

## *Mast Cell Products as Profibrotic Mediators*

The functions of mast cells are dependent on their ability to secrete a diverse array of biologically active compounds [24]. Some of these mast cell-derived mediators have been seen to be profibrotic (Table 6.1).

### Histamine

Histamine is one of the most well-known mast cell-derived biogenic amines and is present in all subtypes of mast cells across all species. Histamine is a pleiotropic mediator, which exerts its biological effects through the activation of four different G-protein-coupled receptor subtypes (H1-4R) [50]. Histamine stimulates proliferation of mouse, rat, or normal human lung fibroblasts [41, 42, 51, 52]. Histamine also induces proliferation and collagen synthesis in human skin fibroblasts [53]. Furthermore, the histamine receptor H1R is expressed in human and rat lung fibroblasts, and the histamine-induced fibroblast activation leads to increased proliferation and synthesis of TGF-β and collagen via H1R [44]. Moreover, Kohyama and coworkers [54] demonstrated in vitro that the H4R mediates the profibrotic effects of histamine on human fetal lung fibroblasts. In fact, the histamine effect on potentiating fibronectin-induced lung fibroblast migration was blocked by a selective H4R antagonist. Furthermore, Lucarini et al. recently revealed that H4R antagonist also inhibited bleomycin-induced lung inflammation and fibrosis [55]. These results suggest that the histamine H4R could be a novel therapeutic target for the treatment of lung inflammatory and fibrotic disease.

### Tryptase

Tryptase is a mast cell-specific protease and is highly expressed by mast cells. Tryptase stimulates collagen I synthesis and proliferation of normal human lung fibroblasts [56, 57]. Tryptase-induced human fetal and lung fibroblast proliferation occurs through the activation of the protease-activated receptor (PAR-2) [58]. A more recent study showed that PAR-2 siRNA inhibited tryptase induction of collagen and fibronectin synthesis by human lung fibroblasts [43]. Tryptase levels are increased in human bronchoalveolar lavage fluid (BALF), serum, and lung homogenate of IPF patients [43, 59, 60]. Furthermore, PAR-2 levels are increased in the fibroblasts derived from IPF lungs, therefore sensitizing fibroblasts to mast cell-derived tryptase [61]. In vivo studies, however, have not been consistent. One study reported that PAR-2-deficient mice inhibited bleomycin-induced pulmonary fibrosis [62]. However, another study found no protective effect in a similar model [63]. A pharmacological approach such as PAR2 inhibitor will be helpful to evaluate the effect of tryptase in vivo.

### Chymase

Chymase is another mast cell-specific protease that is highly expressed by mast cells. Mast cell chymase activates procollagen peptidase by processing procollagen to fibril-forming collagen, therefore potentially regulating collagen biosynthesis [64]. In vivo experiments have confirmed that chymase can cause lung fibrosis in paraquat- or bleomycin-induced pulmonary fibrosis models in hamsters [65, 66]. Mast cell-derived chymase promotes proliferation and collagen synthesis via TGF-β1/Smad activation in rat cardiac fibroblasts [67]. A chymase inhibitor is protected against silica-induced pulmonary fibrosis in mice [68] and bleomycin-induced fibrosis in both mice and hamster models [69, 70]. In humans, dogs, sheep, monkeys, and hamsters, a subtype of chymase, α-chymase, is capable of converting angiotensin (Ang) I to Ang II. On the other hand, β-chymase, which is expressed in rats and mice, is unable to convert Ang I–II [71]. Inhibition of chymase causes a 90% reduction in Ang II-generating activities in the homogenates of human arteries. These findings suggest that Ang II production may be predominantly chymase-dependent in the pathophysiology associated with mast cell activation and angiotensinogen upregulation in humans. Previous studies have revealed that Ang II plays an important role in pulmonary fibrosis [72]. Furthermore, Lang et al. have shown that chymase generates Ang II and enhances collagen expression in human lung fibroblast cultures [73]. The role of angiotensin in pulmonary fibrosis will be discussed later in this section. The experimental evidence convincingly suggests that chymase may contribute directly to the pathogenesis of pulmonary fibrosis or indirectly via the Ang II axis [74].

### Transforming Growth Factor-β

Transforming growth factor (TGF)-β is a growth factor known to regulate various cellular processes such as cell growth, proliferation, differentiation, and apoptosis [75]. Three major isoforms of this growth factor have been identified: TGF-β1, TGF-β2, and TGF-β3, with TGF-β1 being the most closely implicated in the development of IPF [75]. TGF-β1 overexpression induces persistent pulmonary fibrosis in rodents, while Smad3 KO mice are protected from TGF-β1- and bleomycin-induced pulmonary fibrosis [76, 77]. TGF-β1 activates many fibrogenic pathways inducing fibroblast proliferation, collagen production, and differentiation into myofibroblasts [18, 75]. Myofibroblasts can originate from a variety of precursor cells, such as fibroblasts, endothelial cells, pericytes, fibrocytes, and epithelial cell via mesenchymal transition (EMT), in all of which TGF-β1 plays a major role [10, 78]. In addition to myofibroblast differentiation, TGF-β1 also regulates the characteristic of other cell types. For example, TGF-β1 promotes M2 macrophage polarization, a known profibrotic phenotype that ultimately produces more TGF-β1 [79]. TGF-β1, therefore, plays a major role in the development and also the exponential progression of pulmonary fibrosis. Both human IPF and experimental models of pulmonary fibrosis are characterized by an upregulation of TGF-β1 [80]; moreover, inhibition of TGF-β1 signaling interferes with the development of experimental lung fibrosis [81]. Most of the new pharmacological agents for pulmonary fibrosis target TGF-β1 signaling pathways.

#### Chymase and Transforming Growth Factor-*β* Activation

TGF-β is secreted by mast cells in an inactive form. The inactive latent TGF-β requires activation before having any biological effect. The precursor form of TGF-β consists of an active TGF-β protein attached to a latency-associated peptide (LAP) to create the small latent complex (SLC). The SLC then binds to the latent TGF-β binding protein (LTBP) found at the ECM, to form the large latent complex (LLC). The LLC is unable to bind to the TGF-β receptors and is therefore inactive. TGF-β is activated when the SLC is cleaved to release the bioactive TGF-β homodimer. This activation of TGF-β can proceed through multiple context-dependent mechanisms, which include activation via acidic pH, matrix metalloproteinases (MMPs), plasmin, reactive oxygen species, and mechanical stress [12, 82–84]. In addition to these mechanisms, mast cell-derived chymase, which is a chymotrypsin serine protease, can also immediately activate latent TGF-β1 complexes by disrupting the noncovalent interaction between the LAP and active TGF-β1 [67, 85, 86]. It has been shown that the TGF-β1 concentration increases in human fibroblast culture supernatants following exposure to chymase, while the addition of a chymase inhibitor abrogates this effect [69, 70, 86]. Moreover, chymase supplementation results in increased proliferation of human fibroblasts, whereas it is inhibited by

TGF-β-neutralizing antibodies, suggesting the role of chymase in TGF-β-mediated fibroblast proliferation [86]. These studies show that chymase induces fibroblast proliferation and collagen synthesis through TGF-β1 activation [67, 86]. With the crucial role of the TGF-β1 signaling pathway in pulmonary fibrosis, it is tempting to speculate that mast cell chymase-dependent TGF-β1 activities are of particular importance in the pathological symphony of pulmonary fibrosis.

### Basic Fibroblast Growth Factor/Platelet-Derived Growth Factor/Vascular Endothelial Growth Factor

Basic fibroblast growth factor (bFGF or FGF-2), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) are potent profibrotic factors, which all signal through tyrosine kinase receptors [87]. Mast cells are one of the main sources of FGF-2, which is a potent mitogenic factor for alveolar type II cell, smooth muscle cells, fibroblasts, and myofibroblasts [88, 89]. FGF-2 is believed to be pro-fibrotic because of its mitogenic effects on fibroblasts and myofibroblasts [89, 90]. In primary human lung fibroblasts, administration of TGF-β1 induces FGF receptors and the expression and secretion of FGF-2 into culture media [89, 91, 92]. Furthermore, TGF-β1-induced fibroblast proliferation, myofibroblast differentiation, and FGF-2 secretion are all inhibited by FGF2-neutralizing antibodies, suggesting a cooperative mechanism between FGF2 and TGF-β1 [93, 94]. In vivo, abrogation of FGF signaling reduced pulmonary fibrosis and improves survival in bleomycin-treated mice [95]. Furthermore, FGF-2 antisense inhibited bleomycin-induced pulmonary fibrosis in rats [96]. The most recent study using FGF2 knockout mice in the bleomycin model of pulmonary fibrosis highlighted the role of FGF-2 in contributing to the initiation, but not the development of fibrosis [97]. FGF-2 has been detected in the lung tissue and BALF of IPF patients [98]. Immunohistochemistry shows FGF-2 localization within mast cells in IPF lungs [88]. PDGF induces fibroblast chemotaxis, fibroblast proliferation, and fibroblast-mediated tissue matrix contraction [99]. Due to these effects, PDGF is also believed to play a role in the pathogenesis of fibrotic diseases [100]. Furthermore, PDGF is important in inducing the secretion of growth factors and ECM components in fibroblasts, causing fibroblast proliferation and the production of fibronectin by both normal and fibrotic lung fibroblasts [101]. PDGF is also an important factor in the migration of myofibroblasts [102]. Expression of PDGF-B from an adenoviral vector, or administration of recombinant human PDGF-BB delivered intratracheally into rat lungs, produces histopathologic features of fibrosis [103]. Additionally, PDGF and PDGF receptors are expressed in the early stage but not the late stage of IPF [104]. VEGF is a key regulator of angiogenesis, which has been implicated in the pathogenesis of fibrotic lung diseases, including IPF. Fibroblasts play a critical role in angiogenesis, and fibroblast-derived matrix proteins and cytokines are essential for endothelial cell-mediated lumen formation [105]. Interestingly, dermal fibroblasts from systemic scleroderma patients overexpress VEGF in response to

autocrine TGF-β signaling [106]. This VEGF could play a role in the vascular damage that in turn promotes fibroblast activation, thereby supporting fibrosis [106]. In IPF, the serum VEGF level may reflect the severity of the disease and offer clinical benefits to predict the disease progression [107]. Nintedanib, one of the FDA-approved anti-fibrotic drugs, is a small molecule inhibitor targeting the tyrosine kinase receptors of PDGF, FGF-2, and VEGF [87, 108].

### C-C Motif Chemokine Ligand 2

C-C motif chemokine ligand 2 (CCL2 aka MCP-1) is a chemokine that signals through the CCR2 receptor, displaying chemotactic activity for immune cells such as monocytes. CCL2 can stimulate fibroblast collagen production via upregulation of TGF-β expression [109]. Furthermore, the role of CCL2 in fibrosis is in its ability to attract fibrocytes, bone marrow-derived mesenchymal progenitors, and M2 macrophage subtype as seen within IL-10-induced pulmonary fibrosis in mice [110].

### Interleukin-13

The Th2 cytokine interleukin-13 (IL-13) is a profibrotic mediator that is thought to promote fibrosis via both TGF-β-dependent and TGF-β-independent mechanisms. IL-13 can induce TGF-β production and activation in murine bleomycin-induced pulmonary fibrosis and human airway fibroblasts, and this is thought to involve IL-13Rα2-induced remodeling [111, 112]. IL-13 can also directly promote fibrosis by stimulating proliferation or collagen production by lung fibroblasts, as well as differentiation into myofibroblasts [113, 114]. Recent in vivo studies showed that targeting IL-13 could inhibit pulmonary fibrosis in a radiation-induced lung fibrosis model or a humanized SCID idiopathic pulmonary fibrosis model [115, 116].

### Interleukin-33

Interleukin-33 (IL-33) is a member of the interleukin-1 (IL-1) cytokine family. IL-33 needs the specific receptor ST2 (membrane-bound receptor) and IL-1 receptor accessory protein heterodimer for its binding. IL-33 is a crucial regulator of mast cell activation [117, 118]. It has recently been reported that IL-33 potentiates bleomycin-induced lung injury in an undefined but ST2-independent manner [119]. The expression of IL-33 mRNA is also increased in IPF lung tissue [119]. IL-33 also polarizes M2 macrophage subtype to produce IL-13 and TGF-β1 [120]. Although the role of IL-33 as a cytokine in the fibrotic process is unknown, IL-33 may be a novel profibrogenic cytokine that signals through ST2 to promote the initiation and progression of pulmonary fibrosis.

### Cysteinyl Leukotrienes

After the immediate mast cell degranulation response, the arachidonic acid-dependent inflammatory mediators, such as cysteinyl leukotriene ($LTC_4$, $LTD_4$, and $LTE_4$), are rapidly produced and released from mast cells due to enzymatic, rather than transcriptional changes within mast cells [25]. These lipid mediators contribute to diverse biological responses, such as local vascular permeability, tissue edema, the recruitment of inflammatory cells, and fibrosis development [121]. Several in vitro studies have shown that leukotrienes act directly on lung fibroblasts or myofibroblasts to promote their migration, proliferation, and collagen synthesis [122–124]. Furthermore, $LTD_4$ induces proliferation of both murine and human fibrocytes [125]. Fibrocytes are capable of producing CysLTs and also express cysteinyl leukotriene receptor (CysLTR) 1 and CysLTR2, which are regulated through autocrine or paracrine secretion of these lipid mediators [125]. Animal studies also indicate the contribution of CysLTs in pulmonary fibrosis. Mice that are genetically deficient in 5-lipoxygenase (5-LO), an enzyme necessary for leukotrienes generation, are protected from bleomycin-induced pulmonary fibrosis [126]. In addition, treatment with CysLTR1 antagonists limited bleomycin- and silica-induced pulmonary fibrosis [127–130].

### Renin/Angiotensin

Mast cells are one of the sources of renin. Protein levels of renin are increased in the lungs of bleomycin-induced fibrosis [44]. Mast cell-derived renin induces proliferation, the release of TGF-β, and collagen synthesis in human lung fibroblasts via Ang II AT1 receptor [44]. Following mast cell degranulation, renin that is released into the interstitial space triggers local production of Ang II, by cleaving available angiotensinogen. Several in vivo and ex vivo studies have revealed that Ang II plays an important role in pulmonary fibrosis [72]. The purpose of Ang II in bleomycin-induced pulmonary fibrosis has been shown through angiotensin-converting enzyme (ACE) inhibitors and ANG II receptor blockers (ARBs), which reduced fibrogenesis [131–134]. These findings are consistent with the conclusion that collagen deposition is absent in the lungs of bleomycin-treated Ang II AT1 receptor (AT1R) knockout mice [131]. TGF-β also stimulates the expression of Ang II AT1R in lung fibroblasts [135]. Königshoff et al. demonstrated that Ang II exerts a mitogenic activity on human lung fibroblasts through AT1R [136]. The profibrotic effects of Ang II also occur through its induction of procollagen production in human lung fibroblasts via AT1R activation and, at least in part, via the autocrine action of TGF-β [137]. In the lung tissue of IPF patients, there is an increase of Ang peptides and AT1R and AT2 expressions [136].

### Proteoglycan

In mast cells, proteoglycans of the serglycin type, heparin and chondroitin sulfate, are the dominant species and are found in large quantities within mast cell granules [31]. In connective tissue mast cells, the heparin type is dominant, whereas in mucosal mast cells, chondroitin sulfate is the main proteoglycan species [31]. The aberrant deposition of proteoglycans is an early and prominent feature of fibrosis [138]. This abnormal accumulation of proteoglycans has been shown to occur in both human and animal pulmonary fibrosis [139–142]. A recent study has shown that abnormal regulation of proteoglycans plays a vital role in the pathophysiology of fibrosis. A proteoglycan synthesis inhibitor attenuated TGF-β1-induced fibroblast proliferation and myofibroblast differentiation [143]. These studies explain that abnormal proteoglycan deposition is a part of the exacerbated accumulation of ECM constituents during the fibrotic process. Furthermore, heparin is known to have an important role in stabilizing FGF-2 and potentiating its signaling pathway [144].

### Serotonin

The molecule serotonin (5-hydroxytryptamine or 5-HT) is involved in numerous biological processes including fibrosis. In the lung, platelets, neuroendocrine cells, and mast cells are significant sources of 5HT [145]. 5-HT stimulates the proliferation of rat pulmonary arterial fibroblasts [146]. In IPF patient lungs, 5-HT receptors, 5-HT1A/B, and 5-HT2A/B are increased and localized to fibroblasts, whereas 5-HT2B is primarily found within the lung epithelium [147]. Furthermore, 5-HT2A/B antagonist, terguride, is a potent repressor of fibroblast TGFβ-1 expression, and when administered in vivo, the drug improved lung function and decreased fibrosis in established bleomycin-induced pulmonary fibrosis [147]. A recent study also showed that 5-HT2B receptor antagonists prevented myofibroblast differentiation, in vitro and in vivo, with a subsequent effect on collagen deposition in bleomycin-induced pulmonary fibrosis [148], and 5-HT7 receptor antagonist also attenuated bleomycin-induced fibrosis in mice [149].

### Matrix Metalloproteinase-9

Mast cells produce MMP-9, also known as gelatinase B, which regulates ECM turnover [150]. However, MMPs including MMP-9 show controversial effects within fibrosis development [151, 152]. Traditionally, MMPs have been considered antifibrogenic factors due to their proteolytic degradation of extracellular matrix [151]. MMP-9 is associated with the breakdown of lung parenchyma in COPD patients [153]. However, in vitro studies showed that MMP-9 neutralized antibody reduced

TGF-β1-induced EMT [154]. Recent studies have shown that MMP-9 is also implicated in the initiation and progression of fibrosis through tubular cell EMT as well as activation of resident fibroblasts, endothelial-mesenchymal transition (EndoMT), and pericyte-myofibroblast transdifferentiation [155]. Furthermore, MMP-9 is also known to activate TGF-β [156]. MMP-9 is also increased in IPF lungs where it is expressed by alveolar epithelial cells, macrophages, neutrophils, and fibroblasts in fibroblastic foci [157]. Moreover, it was shown that both tryptase and chymase are capable of activating MMPs, thereby contributing to ECM turnover [158, 159].

## *Mast Cell Phenotype Plasticity in Pulmonary Fibrosis*

As we discussed earlier, mast cells exhibit a heterogenic phenotype and are sensitive to their surrounding microenvironment. The exact mechanism of how mast cells change their characteristics in response to the environment is not entirely known. Recent accumulated evidence implies that fibrotic milieu of ECM may contribute to the "tune" mast cell phenotype [32, 160, 161]. Although mucosal $MC_T$ is the most frequent phenotype in the airway of healthy lungs [60], $MC_{TC}$ is increased in IPF lungs, and their number correlates with the degree of fibrosis or decline in lung function [60]. Furthermore, $MC_{TC}$ express TGF-β1, and TGF-β1-positive mast cells are increased in IPF lung and skin fibrosis in secondary lymphedema patients [60, 160]. In vitro studies also indicate that fibroblasts contribute to the phenotype switching in mouse mast cells between MMC and CTMC [46, 47]. These findings suggest that the profibrotic ECM induces a profibrotic phenotype in the mast cells, increasing TGF-β1 content and release. Although the mechanism of the mast cell phenotype changing is not fully elucidated, mast cell phenotype plasticity may be a possible target for a mast cell-related treatment in pulmonary fibrosis.

## *Mechanism of Mast Cell Activation in Pulmonary Fibrosis*

Activated mast cell responses are diverse and not unitary. These responses to different stimuli can be influenced by intrinsic and microenvironmental factors that affect the expression or functionality of surface receptors and/or signaling molecules [32]. The most studied method of mast cell activation is the allergic reaction, an adaptive immune response mediated by the high-affinity IgE receptor on the mast cell surface [24], although stimulation of other receptors, such as toll-like receptors (TLRs) and the IL-33 receptor ST2, can also result in mast cell activation [18]. Neuropeptides, other cytokines, growth factors, toxins, basic compounds, complement, immune complexes, certain drugs, as well as physical stimuli activate mast cells [24]. Several cytokines and growth factors induce the fibrotic microenvironment, and these biochemical changes promote mast cell activation. Furthermore, the physical stiffness

in pulmonary fibrosis contributes to the mast cell activation. The biomechanical changes, such as lung stiffness, are increased in the lungs of IPF and animal pulmonary fibrosis models [7–9, 12]. A recent in vitro study showed that mast cells are activated in the setting of a rigid ECM [162]. It is possible that mechanical stress-induced mast cell activation may contribute to TGF-β1 activation, which forms a vicious profibrotic cycle in the fibrotic lung. Therefore, these biochemical and biomechanical alterations in the fibrotic lung may induce further mast cell activation.

## *Mast Cells in Idiopathic Pulmonary Fibrosis*

Mast cells and their association with IPF is both an old and new topic of research. Increased numbers of mast cells have long been known to be present in the fibrotic area in IPF lungs, especially within the parenchyma [60, 74, 163–167] (Fig. 6.1 and Table 6.2). The increase of mast cell-derived mediators, histamine and tryptase, is also increased in the BALF from IPF patients [59, 168, 169] (Table 6.2). Interestingly, recent studies highlight that mast cells also undergo phenotypic changes, which may indicate disease progression or severity. A study showed that the specific $MC_{TC}$, phenotype, is associated with lung function and degree of fibrosis in IPF patients [60]. Furthermore, a study found a negative correlation between tryptase in BALF and lung function and prognosis [59]. Although Cha et al. reported that mast cells and chymase might indeed be elevated in IPF and the increased mast cell density in IPF could predict slower disease progression, chymase-positive cells were detected as mast cells in this study [167]. These findings indicate that mast cell populations may be heterogenic depending on the disease severity or progression, notably that chymase-positive mast cells may be increased in moderate IPF and tryptase-positive mast cells may be increased in advanced IPF. Therefore, diversity in mast cell phenotype depending on the disease severity may explain the discrepancy in mast cell numbers or related mediators and disease severity. Further studies are required.

## *Mast Cell and Pulmonary Fibrosis Animal Model*

Mast cell accumulation was examined in the lung parenchyma in different rodent experimental pulmonary fibrosis models using bleomycin, silica, and active-TGFβ1 overexpression. Although these models have increased mast cell (as does IPF), the degree of mast cell contribution is not fully elucidated. One of the possible difficulties to examine the role of mast cells in vivo is because mast cell-specific drugs or mast cell-deficient models have not been developed. In this section, we will review previous in vivo studies, highlight some of the advantages and disadvantages of each study, and discuss the direction of further investigations.

**Table 6.2** Mast cell in IPF patients

| Material | Observation | Method | References |
|---|---|---|---|
| Lung tissue from fibrotic lung diseases | Increase mast cells in fibrotic lung | Lung histology with metachromatic staining | Kawanami et al. [163] |
| BALF from IPF | Increase of histamine in IPF BALF, but not asthma, sarcoidosis | Histamine assay in BALF | Rankin et al. [168] |
| Lung tissue from IPF | Increase of histamine in IPF BALF | Histamine assay in BALF | Casale et al. [169] |
| Lung tissue from IPF | Increase of mast cell number in the IPF lung but also in nonfibrotic disease | Lung histology | Fortoul and Barrios [164] |
| Lung tissue from IPF | Increase of mast cell number in the IPF lung compared to control lungs. Most of mast cells were degranulated | Lung histology with tryptase immunohistochemistry | Hunt et al. [165] |
| Lung tissue from IPF | Increase of c-kit, tryptase, chymase-positive cells in the IPF lung | Lung histology with c-kit and tryptase immunofluorescence and PCR for c-kit, tryptase, chymase in human lung-derived mast cell | Edwards et al. [166] |
| Lung tissue from IPF | Increase of tryptase in IPF BALF, and it has a poorer outcome | Tryptase antibody-fluoroimmunocap method in BALF | Kawatani et al. [59] |
| Lung tissue from IPF | Increase of MCTC-type mast cells in IPF lung showed positive correlation with the degree of fibrosis and negative correlation with lung function<br>TGF-β-positive mast cells are increased in IPF fibrotic area | Lung histology with tryptase, chymase, and TGF-β1 immunohistochemistry and immunofluorescence | Andersson et al. [60] |
| Lung tissue from IPF | Increase of chymase-positive mast cells in IPF lung. This correlated with a slower rate of decline in forced vital capacity | Lung histology with chymase immunohistochemistry | Cha et al. [167] |
| Lung tissue and BALF from IPF | Increase of tryptase and transmembrane SCF in IPF lung.<br>Transmembrane SCF but not soluble SCF mRNA was increased in fibroblast isolated from IPF lung | Lung histology with toluidine blue staining, immunohistochemistry of tryptase and SCF. Western blot analysis of tryptase and SCF protein levels in lung homogenates.<br>RT-qPCR for SCF in BALF and fibroblasts | Wygrecka et al. [43] |

### Pharmacological Approach

Pharmacological approach is important because of the clinical application. A pharmacological target for the mast cells is usually mast cell stabilizers or drugs targeting mast cell-derived mediators (Table 6.3). In vivo pharmacological studies targeting mast cell or mast cell-derived mediators also show the importance of these mast cell-related mediators in pulmonary fibrosis. Mast cell stabilizers, such as cromoglycate, tranilast, and ketotifen, are considered to inhibit mast cell activation by stabilizing membranes; however, their molecular targets are not fully understood [35]. Tranilast attenuated bleomycin-induced pulmonary fibrosis in mice [170]. Another mast cell stabilizer, cromoglycate, prevented pulmonary allotransplantation-induced lung fibrosis [171]. Mast cell-specific proteases targeting drugs show promising effects on pulmonary fibrosis (Table 6.3). An inhibitor of chymase was protective against induction of fibrosis induced by bleomycin in the mouse model [69] and in hamsters [70] as well as in silica-induced pulmonary fibrosis in mice [68]. Other non-mast cell-specific targets are histamine, IL-13, serotonin, CysLTs, FGF2, PDGF, and VEGF (Table 6.3). Recently, Lucarini et al. revealed that H4R antagonist inhibited bleomycin-induced inflammation and fibrosis [55]. Recent in vivo studies showed that targeting IL-13 could attenuate established pulmonary fibrosis in both radiation-induced lung fibrosis model and humanized SCID idiopathic pulmonary fibrosis model [115, 116]. 5-lipoxygenase inhibitor or cysteinyl leukotriene type 1 receptor blocker also prevented bleomycin- or silica-induced pulmonary fibrosis in mice [127–130]. AT1R antagonists are protected from pulmonary fibrosis by instilling bleomycin [131–135]. Another recent study showed that 5-HT2B receptor antagonists prevented myofibroblast differentiation, in vitro and in vivo, with subsequent effect on collagen deposition in bleomycin-induced pulmonary fibrosis [148]. 5-HT2A/B and 5-HT7 receptor antagonists showed therapeutic impact in bleomycin-induced pulmonary fibrosis [147, 149]. Inhibiting FGF-2 protected bleomycin-induced pulmonary fibrosis in rats [96, 97], and tyrosine kinase inhibitor of PDGFR, FGFR, and VEGFR, nintedanib, showed significant therapeutic effect in bleomycin- or silica-induced pulmonary fibrosis [87, 108]. These studies indicate that mast cells and mast cell-derived mediators have a potent profibrotic effect and contribute to fibrosis development and can be a possible target for fibrosis treatment.

### Mast Cell-Deficient Model

The use of mast cell-deficient animals has elucidated the role of mast cells in pulmonary fibrosis. To date, mice whose sole abnormality is a specific lack of all populations of mast cells have not been reported. Although there are many different types of mast cell-deficient models, c-kit-mutant mice, such as WBB6F1-KitW/W-v mice and C57BL/6-KitW-sh/W-sh mice, are most common [32, 172, 173]. For the study of pulmonary fibrosis, constitutive mast cell-deficient mice were used; c-kit-mutant mice such as WBB6F1-KitW/W-v, C57BL/6-KitW-sh/W-sh, and Ws/Ws rats or

**Table 6.3** Pharmacological animal studies

| Drug | Animal | Model and examined day | Treatment | Observation | References |
|---|---|---|---|---|---|
| *Mast cell stabilizers* | | | | | |
| Tranilast p.o. 10 and 20 mg/kg/day | Female ICR mice | Bleomycin, i.t. day 35 | Preventive | Attenuates fibrosis | Mori et al. [170] |
| Cromoglycate: s.c, 150 mg/kg | Male Wistar Kyoto and Fischer 344 rats | Allotransplantation and aspiration of gastric fluid-induced lung fibrosis, examined at day 56 | Preventive | Attenuates fibrosis | Chang et al. [171] |
| *Mast cell-specific protease inhibitor* | | | | | |
| Chymase inhibitor, SUN C8077: i.p. five times a week | Male ICR mice | Bleomycin, i.t. day 14 | Preventive | Attenuates fibrosis | Tomimori et al. [69] |
| Chymase inhibitor NK3201: p.o. 30 mg/kg/day | Male Syrian hamsters | Bleomycin, i.t. day 28 | Preventive | Attenuates fibrosis | Sakaguchi et al. [70] |
| Chymase inhibitor TY-51469: osmotic pump 0.1 or 1.0 mg/kg/day | Male ICR mice | Silica i.t. day 21 | Preventive | Attenuates fibrosis | Takato et al. [68] |
| *Mast cell-related inhibitor* | | | | | |
| H4R antagonist 40 mg/kg osmotic micropumps | Male C57BL/6 mice | Bleomycin i.t. day 21 | Preventive | Attenuates fibrosis | Lucarini et al. [55] |
| Anti-IL-13 IgG antibody: i.p. 0.5 mg per mouse weekly, 3–8 weeks | Female c57BL/6NcR mice | Radiation-induced lung fibrosis, five daily fractions of 6Gy, examined at day 56 | Therapeutic | Attenuates fibrosis | Chung et al. [116] |
| IL-13 inhibitor, tralokinumab: p.o. 3 mg/kg/day | Female C.B-17-scid-beige (C.B-17SCID/bg) mice | Humanized SCID idiopathic pulmonary fibrosis model, examined at day 63 | Therapeutic | Attenuates fibrosis | Murray et al. [115] |
| FGF-2 antisense | Rats | Bleomycin, i.t. examined at day 28 | Preventive | Attenuates fibrosis | Chen et al. [96] |
| 5-LO inhibitor, zileuton, p.o., 50 mg/kg; CysLT1 receptor MK-571: osmotic pump | Male CD mice | Bleomycin, i.t. day 7 | Preventive | Attenuates fibrosis | Failla et al. [127] |
| CysLT1 receptor blocker montelukast, s.c., 1.0 mg/kg | Male C57BL/6 mice | Bleomycin, i.t. day 14 | Preventive | Attenuates fibrosis | Izumo et al. [128] |

**Table 6.3** (continued)

| Drug | Animal | Model and examined day | Treatment | Observation | References |
|---|---|---|---|---|---|
| CysLT1 receptor blocker, montelukast: s.c. 10 mg/kg | Female C57BL/6 | Bleomycin, i.t. day 14 | Preventive | Attenuates fibrosis | Shimbori et al. [129] |
| CysLT1 receptor blocker, pranlukast: p.o. 30 mg/kg/day | Female C57BL/6 | Silica, i.t. day 70 | Preventive | Attenuates fibrosis | Shimbori et al. [130] |
| AT1 antagonist, candesartan cilexetil: drinking water | Male Sprague-Dawley rats | Bleomycin, i.t. day 21 | Preventive | Attenuates fibrosis | Otsuka et al. [132] |
| AT1 receptor antagonist, losartan: i.p. 10 mg/kg/day | C57BL/6 mice | Bleomycin, i.t. day 14 | Preventive | Attenuates fibrosis | Li et al. [131] |
| AT1 receptor antagonist, losartan: p.o. 50 mg/kg/day | Male Sprague-Dawley rats | Bleomycin, i.t. day 15 | Preventive | Attenuates fibrosis | Molina-Molina et al. [133] |
| AT1-specific antagonist olmesartan medoxomil p.o. 0.1 or 1 mg/kg/day AT2-specific antagonist | Male ICR mice | Bleomycin, i.t. day 14 | Preventive | Attenuates fibrosis | Waseda et al. [134] |
| PD123319: osmotic pumps 0.5 or 5 mg/kg/day | | | | | |
| 5-HT2B receptor antagonist EXT5, p.o., 30 mg/kg or EXT9, p.o., 30 mg/kg | Female C57/Bl6 mice | Bleomycin, s.c. three times/week for 2 weeks, day 14 | Preventive | Attenuates fibrosis | Lofdahl et al. [148] |
| 5-HTR2A/B antagonist, terguride: i.p. 0.4 or 1.2 mg/kg i.p. twice/day | Female C57BL/6 mice | Bleomycin, i.t. day 28 | Therapeutic | Attenuates fibrosis | Konigshoff et al. [147] |
| 5-HTR2A/B antagonist terguride i.p. 1.2 mg/kg/day; 5-HT7 receptor antagonist SB-269970 i.p. 1 mg/kg day | Male albino rats | Bleomycin, 5 mg/kg, i.t. day 21 | Therapeutic | Attenuates fibrosis | Tawfik and Makary [149] |

non-c-kit-mutant mast cell-deficient mice such as mi/mi mouse (the MITF encoded by the mutant mi locus deletes one of four consecutive arginines in the basic domain) were employed. These constitutive mast cell-deficient mice were used, and some studies applied mast cell reconstitution to confirm mast cell-derived effects (Table 6.4). O'Brien-Ladner et al. showed that bleomycin-induced pulmonary fibrosis was attenuated in mutant mi/mi mice [174]. Brown et al. showed impaired lung inflammation and collagen deposition in KitW-sh/W-sh mice in a model of pulmonary fibrosis induced by instilling silica [175]. A more recent study reported that mast cell-deficient mice (WBB6F1-W/Wv) were protected against bleomycin-induced pulmonary fibrosis, which lung mast cell reconstitution restored the bleomycin-induced pulmonary fibrosis [44]. In addition, SCF-deficient mice are protected from bleomycin-induced pulmonary fibrosis, and therapeutic anti-SCF treatment also inhibits bleomycin-induced lung fibrosis in mice [176]. Although the

**Table 6.4** Mast cell-deficient animal studies

| Animal | Mutant | Model and examined day | Observation | Reference |
|---|---|---|---|---|
| *Mast cell deficient* | | | | |
| Mouse | mi/mi | Bleomycin, i.p. day 42 | Attenuated pulmonary fibrosis | O'Brien-Ladner et al. [174] |
| Rat | Ws/Ws | Bleomycin, i.t. day 42 | The fibrosis was more severe in Ws/Ws rats than in control normal (+/+) rats | Okazaki et al. [178] |
| Mouse | WBB6F1-W/Wv | Bleomycin i.v. | No effect on pulmonary fibrosis | Mori et al. [177] |
| Mouse | KitW-sh/W-sh | Silica, i.t. 3 months | Attenuated pulmonary fibrosis | Brown et al. [175] |
| Mouse | WBB6F1-W/Wv | Bleomycin, i.t. day 14 | Attenuated pulmonary fibrosis. Mast cell reconstitution (isolated from the lung) restored the fibrosis | Veerappan et al. [44] |
| Mouse | W sh/W sh | Bleomycin, i.n. day 7,14, 28 | Pulmonary fibrosis was reduced at day 7 but not at days 14 and 28. Mast cell reconstitution (isolated from the bone marrow) restored pulmonary fibrosis at day 7 but not days 14 and 28 | Reber et al. [179] |
| *Chymase deficient* | | | | |
| Mouse | MCPT4-deficient | Bleomycin, i.n. day 7,14, 28 | Pulmonary fibrosis was reduced at day 7 but not at days 14 and 28 | Reber et al. [179] |
| *SCF deficient* | | | | |
| Mouse | WCB6F1/J KitlSl KitlSld | Bleomycin, i.n. day 21 | Attenuated pulmonary fibrosis | Ding et al. [176] |
| *PAR2 deficient* | | | | |
| Mouse | PAR2 deficient | Bleomycin, i.t. day 14 | No effect on pulmonary fibrosis | Su and Matthay [63] |
| Mouse | PAR2 deficient | Bleomycin, i.n. day 14 | Attenuated pulmonary fibrosis | Lin et al. [62] |

participation of mast cells seems limited in their study and mast cells are not the only cells responsive to SCF, SCF-c-Kit axis may be an interesting target for pulmonary fibrosis. In contrast, other reports failed to show any implication of mast cells in bleomycin-induced fibrosis using mast cell-deficient WBB6F1-KitW/W-v mice and Ws/Ws rats [177, 178]. Reber et al. used mast cell-deficient KitW-sh/W-sh mice and engraftment of these mice with bone marrowderived mast cells to demonstrate that mast cells can amplify the acute lung injury induced by administration of bleomycin but did not modulate fibrosis progression [179]. They also found that mast cells and the mast cell-derived chymase MCPT4 may contribute to the fibrosis initiation, but not the progression of fibrosis using Mcpt4−/− mice, which is lacking mMCP4 [179]. In PAR-2-deficient mice studies, one study reported that PAR-2-deficient mice are not affected by bleomycin-induced pulmonary fibrosis, while the other study showed preventive effects in bleomycin-induced pulmonary fibrosis [62, 63]. These findings suggest that previous in vivo studies using genetic depletion models are not consistent in elucidating the role of mast cells in pulmonary fibrosis.

### Future Direction of Animal Study

Animal models are indispensable in investigating the contribution of mast cells to pulmonary fibrosis and in determining their potential as therapeutic targets. Although the precise reason for this discrepancy in animal studies, especially in mast cell-deficient studies, is unknown, there are several potential reasons to consider. First, the animal species and experimental models are varied in each study. Most studies employ murine models of pulmonary fibrosis for mast cell investigations. However, the mast cell population and distribution in the healthy mouse is different from other vertebrates, including humans [180]. Mast cells are rarely found within the pulmonary parenchyma and airways in the mice and are located predominantly around the main-stem bronchi. On the contrary, larger vertebrates, including rats, have mast cell in the peripheral bronchioles and lung parenchyma [180]. For experimental pulmonary fibrosis model, the bleomycin-induced model is most commonly used. Although the bleomycin model is well characterized, it is an acute injury model and associated with an intense inflammatory response in the early stages of the model. Furthermore, bleomycin-induced fibrosis by single intratracheal injection can resolve after 3 or 4 weeks, and it is not a persistent model of fibrosis [181, 182]. Since IPF is a heterogenic disease with persistent fibrosis, using a non-bleomycin model, such as TGF-β1 and the silica, age-induced, humanized model of fibrosis, may be useful to evaluate the precise role of mast cells. Therefore, we must be careful when using mouse models for mast cell analysis in the lung.

Second, most of the pharmacological and genetically modified mice studies evaluated preventative effects, but not the therapeutic effects (Table 6.3). To better apply these findings to a more clinically relevant setting, it is necessary to also assess the efficacy of the drug therapeutically, after the establishment of the disease [183]. Preventive effects are essential to evaluate the role of mast cells in the initiation of disease. However, this does not provide the evidence related to the role of mast cells in disease progression.

One of the difficulties of studying mast cells in vivo is that there is no mast cell-specific drug or mast cell-deficient model. Mast cell stabilizers also affect the functions of other cell types [184]. Mast cells are also known to contain hundreds of mediators, and mast cells have heterogeneity depending on microenvironment as we discussed above. These findings suggest that targeting single mast cell-related mediators will not be enough to block the mast cell-induced profibrotic influence. In mast cell-deficient mice, one potential problem is that the constitutive deficiency of the target during development may induce different biological responses. Mast cell-deficient rodents also have non-specific abnormalities other than mast cell depletion [32]. For example, c-kit-mutant mice feature complex alterations of the immune system and other organ systems, including a virtually complete lack of germ cells, deficiency of interstitial cells of Cajal, lack of cutaneous melanocytes, a moderate anemia, cardiomegaly, and insulin resistance [32, 185–187]. Non-c-kit-mutant mast cell-deficient mice, mi/mi mouse, also show unspecific alterations like microphthalmia, depletion of pigment in both hair and eyes, osteopetrosis, and a decrease in the number of mast cells [188]. It has already been noted that conclusions made using genetically modified mast cell-deficient mice depend highly on the mouse strain background, the nature of the mutation resulting in a mast cell deficiency, and the severity of the model used [173]. It is possible that other cell types can also mediate inflammation and fibrosis and may overlap with the mast cell function in pulmonary fibrosis development. Although mast cell reconstitution to mast cell-deficient animals is one of the ways to identify the role of mast cell, cultured or isolated mast cells may have an altered phenotype compared to within the lung tissue, especially because mast cells are highly sensitive to the surrounding microenvironment. One promising approach is conditional mast cell knockout mice model. There are two models using diphtheria toxin (DT)-based conditional deletion system with kit-independent manner: Mcpt5-Cre mice and Mas-TRECK [173, 189, 190]. Mcpt5-Cre expresses Cre under the control of the Mcpt5 promoter, and Mas-TRECK uses Il4 enhancer elements previously shown to be specific for IL-4 production in mast cells [173, 189, 190]. In these mice, repeating DT treatment induced mast cell deficiency in peritoneal, skin, stomach, and intestine [173, 189–191]. Although there is no report about lung mast cell expression, this inducible mast cell-deficient model will be a powerful tool to evaluate the role of mast cells.

## *Mast Cell and Pulmonary Hypertension*

The development of pulmonary hypertension (PH) as a secondary complication of IPF is well documented, occurring in as many as 32–85% of patients [192]. The pathobiology of PH is heterogeneous and is characterized by multiple phenotypes such as vasoconstriction, dysregulated pulmonary vascular remodeling, increased angiogenesis, and inflammation [193]. In advanced IPF, the incidence of PH rises markedly, suggesting that PH and IPF are strongly linked, where PH may accelerate the fibrosis [194]. Total serum tryptase has been found to be higher in idiopathic pulmonary artery hypertension (IPAH) than in controls [195]. Mast cells

accumulated around the remodeled vessels, specifically perivascular mast cell accumulations. Mast cell numbers were increased, and a majority of them were degranulated within the lungs of PH patients and PH-induced rats [195–197]. Mast cells release biogenic amines, including serotonin, histamine, and chymase, which play a crucial role in pulmonary arterial or vein vasoconstriction and smooth muscle cell proliferation [198–200]. Mast cells also release various angiogenic mediators, such as VEGF, FGF-2, PDGF, histamine, heparin, tryptase, and chymase, among others [201]. Mechanistically, multiple mast cell activities may contribute to the dysfunctional angiogenesis observed in PH. IPF lungs show a heterogeneous distribution of vessels with a decrease in vascular density in fibrotic areas and an increase in the tissue adjacent to fibrosis, and it has been proposed that this heterogeneity may at one site support fibroproliferation, but other sites block normal repair mechanisms [202]. These mast cell mediators can act at various stages of angiogenesis [203]. The proximity of mast cells to blood vessels in tissues associated with angiogenesis has long suggested a relationship between mast cells and angiogenesis. Therefore, mast cells may play a part in the PH pathophysiology. Interestingly, PH and vascular remodeling were significantly attenuated in PH models rats treated with stabilizers and chymase inhibitor or in mast cell-deficient Ws/Ws rats [196, 197, 204]. Tyrosine kinase inhibitors, such as imatinib, improved pulmonary vascular remodeling by reducing perivascular accumulation of c-kit-positive cells, mainly bone marrow-derived progenitor cells but also mast cells in pulmonary arteries of mice exposed to chronic hypoxia [205]. In a small ancillary study, it was found that the magnitude of the serum tryptase decreases after imatinib therapy correlated with the reduction in pulmonary vascular resistance [206]. Together these studies indicate that the molecular and cellular mechanisms of PH and IPF are linked proximately through mast cells.

## *Mast Cell and Cough*

Coughing is one of the primary symptoms in IPF patients. A chronic cough is a distressing and disabling symptom with a significant impact on quality of life [207]. A recent study using a validated cough counter showed that IPF patients cough as frequent as patients with cough hypersensitivity syndrome and significantly more than asthmatics [208]. Interestingly, cough in IPF is more prevalent in patients who have never smoked and in patients with an advanced stage of IPF. Furthermore, there is evidence that coughing is an independent predictor of disease progression and may predict prognosis or need for transplantation [209]. Although the precise mechanism of the cough in IPF is unclear, sensory afferent nerves, Aδ fibers, and c fibers are believed to play an important role. It is known that patients with IPF have an enhanced cough reflex sensitivity to inhaled capsaicin and that induced sputum from patients with IPF contains higher levels of the neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) than controls [210]. Sensory nerves express NGF receptor, TrkA, and BDNF receptor, TrkB. Therefore, these neurotrophins are believed to contribute to the cough response [211]. NGF, BDGF, TrkA, and TrkB are significantly increased in IPF

sputum, BALF, and lung tissue [211]. Mast cells can release NGF [212]. Mast cells are often found near nerves and nerves within the lung, therefore suggesting they may function as an intermediate cell between the nervous and immune system [213]. In the respiratory tract, activation of nociceptors leads to sneezing, coughing, dyspnea, and reflex bronchospasm and secretions. TRPV1 (transient receptor potential) receptor which are expressed on c-fiber are also involved in the cough reflex. Interestingly, PAR2 the only member of the PAR family that can be activated by mast cell tryptase and is predominantly co-expressed with TRPV1, and PAR2 activation sensitized TRPV1 activity [214]. Intriguingly, peripheral nerve terminals can activate mast cell by releasing substance P and corticotropin-releasing factor, thereby creating a positive feedback loop for ongoing mast cell activation and nociceptor sensitization [214]. Furthermore, the cough reflex is also induced by mechanical force through a mechanosensor [215]. In IPF lungs, the aberrant deposition of ECM makes the lung stiffer and induces further mechanical force [12], and mast cells also play a role in this process as we described earlier. Overall, mast cells may contribute to the IPF cough reflux directly and indirectly. A Phase 2 clinical trial of a mast cell stabilizer for treating chronic cough in patients with IPF is currently ongoing.

## Mast Cell as a Pulmonary Fibrosis Drug Target

The literature review presented here shows the strong correlation between mast cell accumulation and pulmonary fibrosis development. To further investigate the possibility of mast cells as drug targets for IPF and pulmonary fibrosis, we will discuss the possible mast cell-related drug target in this section (Fig. 6.4). Mast cell stabilizers are the most basic pharmacological approach. The advantages of this drug are the ability to target mast cell themselves and their wide clinical use in allergic diseases. However, stabilizers just suppress mast cells but do not delete them. In the fibrosis milieu, ECM and surrounding cells provide profibrotic growth factors and induce stiffness. Therefore, mast cells may be continuously exposed to these biochemical and mechanical stimuli counteracting the action of stabilizers which, additionally, may have reduced access to mast cells in the fibrotic lung. Alternative avenues are thus required to inhibit mast cell function in pulmonary fibrosis, developing drugs targeting mast cell-derived mediators. There are many promising targets in mast cell-derived mediators; therefore, it may be advantageous to combine inhibitors with multiple mediators of interest. Furthermore, it would be essential to address the profibrotic phenotype of mast cell granule contents by proteomics or genomics studies, to detect target molecules. Another potential target is the recruitment of mast cell progenitors to the lung tissue. The mechanism of mast cell infiltration is not fully understood, but it is suggested that mast cell infiltration is regulated by α4β1 and α4β7 integrins [28, 30]. Several chemotactic factors (chemokines, leukotrienes, etc.) and adhesion molecule-1 (CADM1) through activation of the CXCR3/CXCL10 axis also contribute to mast cell progenitor recruitment into the lung [29]. The role of chemotactic factors in activating mast cells is an emerging


**Fig. 6.4** The targets to inhibit mast cell functions. This graph illustrates multiple strategies to inhibit mast cell function targeting different mechanisms. (*1*) The mast cell-stabilizing drugs (e.g., cromoglycate, ketotifen, tranilast) block the release of mast cell granules following the activation. (*2*) Mast cell inhibitors that block specific mast cell mediators, such as histamine, chymase, tryptase, serotonin, renin, IL-33, and leukotrienes. (*3*) Inhibitors that target mass cell recruitment into the lungs, targeting the mast cell interactions with integrins and chemokines. (*4*) Targeting inhibitory signaling in mast cells, such as immunoreceptor tyrosine-based inhibitions (ITIMs). (*5*) Modulating extracellular matrix characteristic by reducing mechanical stress is also a possible target to regulate mast cells. (*6*) Combination therapy of mast cell-targeted drugs and existing antifibrotic drugs

area of research critical for further elucidating the role of mast cells in disease. Inhibitory receptors on mast cells may be another exciting therapeutic target to consider [19]. Mast cells express receptors with immune receptor tyrosine-based inhibition motifs (ITIMs). Upon activation, ITIM receptors recruit phosphatases that dephosphorylate critical signaling molecules preventing mast cell activation [216]. ITIM activation is enhanced when co-ligated with Fcε RI, KIT, or both, which then inhibits both of these signaling pathways. A possible therapeutic strategy for preventing mast cell activation on the basis of these receptors is to target the ITIM

receptor and Fcε RI or KIT with bispecific antibodies [217]. Another target is the interaction of the profibrotic ECM and mast cells. The current study showed that the ECM microenvironment has significant impact on mast cell function and phenotype. Therefore, adjusting this profibrotic ECM microenvironment would provide an important avenue for fibrosis therapy. Mast cells are sensitive to mechanical stimuli; therefore, attenuating lung stiffness by inhibiting cross-linking enzymes [218, 219] or mechanotransduction [220, 221] may also be an attractive therapeutic target, in addition to directly targeting mast cells. Finally, because of the complex heterogeneity of IPF pathophysiology, combination therapy of mast cell-targeted drugs and existing anti-fibrotic drugs (pirfenidone and nintedanib) or other novel drugs may be a productive therapeutic approach.

## Conclusion

In summary, accumulating evidence illustrates that mast cells function as profibrotic factors. Mast cell may contribute to the fibrotic chain reaction following the cross-talk with ECM and other types of cells such as mesenchymal cells (Fig. 6.5). IPF is a heterogenic disease in which mast cells play different roles depending on the stage

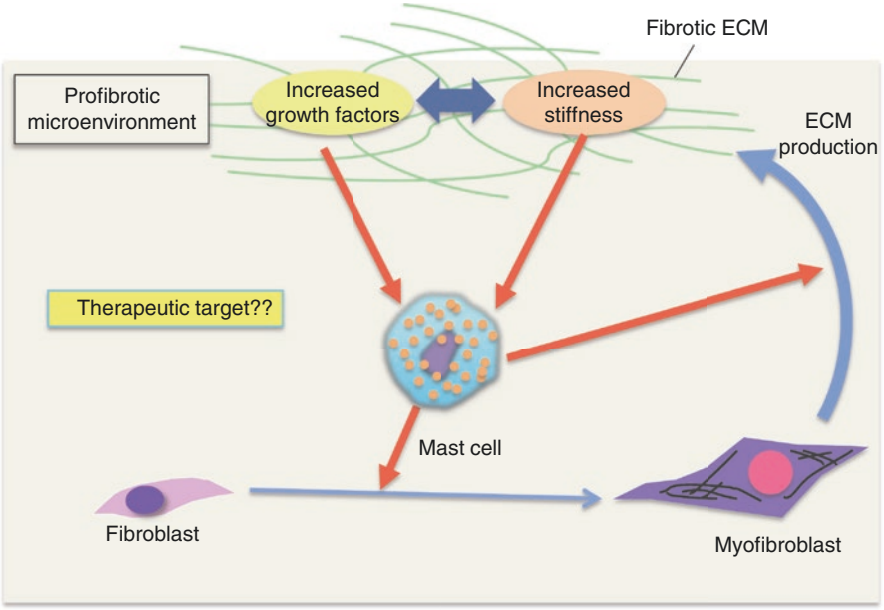


**Fig. 6.5** The possible role of mast cells in pulmonary fibrosis development. Mast cells are affected by the biochemical and biomechanical features of profibrotic ECM. These fibrotic microenvironments induce fibrotic phenotype of mast cells. Mast cells can activate fibroblasts and myofibroblasts and enhance further ECM deposition. Thus, mast cells are possible to contribute to the vicious cycle of pulmonary fibrosis progression

of the disease or local and systemic milieu. Although our understanding of mast cell development has increased, there are significant gaps in knowledge of the ontogeny of mast cells, especially how mast cells achieve the impressive heterogeneity that they exhibit in peripheral tissues. Indeed, recent studies postulate that this heterogeneity of mast cell is much more extensive than currently appreciated, especially at the level of tissue-specific receptor expression and molecular mechanisms underlying microenvironmental conditioning, including epigenetic controls on gene expression and function. Studying the relationships between mast cells and ECM microenvironments and the interaction with other cells will help our understanding of the mechanisms of disease progression in pulmonary fibrosis, and lead to the development of novel therapeutic strategies for fibrotic diseases, particularly IPF.

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# Chapter 7
# Pericytes and T Cells in Lung Injury and Fibroproliferation


**Alexander Birbrair, Pedro Henrique Dias Moura Prazeres, Daniel Clark Files, and Osvaldo Delbono**

## Introduction

The respiratory system is essentially an external organ, constantly exposed to the external environment. As such, it is in contact with any number of antigens and chemical agents that can injure the upper (nasal cavity, pharynx, and larynx) or lower (trachea, bronchi, and lungs) respiratory tract [1]. Injuries to the lung parenchyma are particularly harmful, as the parenchyma is the site of gas (oxygen and carbon dioxide) exchange. Acute or chronic injuries to the lung result in acute or chronic hypoxemic or hypercapnic respiratory failure, respectively. While there are several structural and pathologic mechanisms that contribute to respiratory failure, some lung injuries result in a progressive fibroproliferative response that leads to respiratory failure and death.

While many lung diseases often result in some degree of fibroproliferation, two common lung disorders where fibroproliferation is the primary pathophysiological driver of disease are acute respiratory distress syndrome (ARDS) [2, 3] and idiopathic interstitial pneumonia, particularly idiopathic pulmonary fibrosis (IPF) [4]. These diseases differ completely in clinical presentation, with ARDS resulting from acute lung injury (hours to days) and respiratory failure, whereas idiopathic

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pulmonary fibrosis occurs over months to years as a result from either chronic injury or an abnormal and progressive aberrant host response to an acute injury. While the clinical presentation and treatment of these diseases differ, lung fibroproliferation is the common pathophysiology that mediates respiratory failure in both of these conditions.

## *Acute Respiratory Distress Syndrome*

ARDS affects at least 200,000 persons per year in the United States alone and carries an acute mortality risk of 30–40% [5]. ARDS can occur via direct or indirect injuries to the lung. Examples of direct injuries include aspiration of gastric fluids, pneumonia, toxic inhalations, drowning, or burns [6, 7], whereas indirect lung injuries can occur secondary to severe trauma, sepsis, blood transfusions, and pancreatitis [8]. Direct or indirect injuries result in damage to the lung epithelium or endothelium resulting in a cascade of events leading to acute respiratory failure [2].

An acute and a resolution phase characterizes ARDS pathophysiology following the inciting injury. In the acute phase, a protein-rich exudate floods the alveoli mediating damage to both the alveolar epithelial and the vascular endothelium, compromising the integrity of the functional lung unit [2]. The major purpose of the resolution phase is to re-establish lung homeostasis by removing proteins, fluids, and dead cell debris from the alveolar airspace, activating type II pneumocytes to regenerate type I pneumocytes, and restoring the architecture of the lung unit [2]. Patients that survive ARDS have ongoing problems, including skeletal muscle weakness and neurocognitive and psychiatric impairment leading to reduced quality of life, hospital readmissions, and increased mortality risk [9].

In patients that fail to resolve lung injury, a dangerous fibroproliferative phase ensues [10, 11]. Fibrosis is initiated if the immune response fails to remove the trigger or if lung damage progresses faster than the body can repair it [12]. In ARDS, extracellular matrix proteins accumulate, predominantly collagen, and lead to reduced pulmonary compliance and ongoing hypoxemia [13]. These patients often fail to liberate from mechanical ventilation and die from chronic respiratory failure and multi-organ failure. Mechanical ventilation with higher tidal volumes can independently contribute to ongoing lung injury, known as ventilator-induced lung injury (VILI), where fibroproliferation in the lung was also observed [14].

## *Idiopathic Pulmonary Fibrosis*

IPF is the most common and severe form of the idiopathic interstitial pneumonia, with an incidence of 5–16 cases per 100,000 persons or up to 50,000 patients per year in the United States [15]. Similar to ARDS, IPF incidence increases with age [5, 16]. However, in contrast to ARDS, the clinical presentation of IPF is insidious over a period of months to years, as patients present with chronic hypoxemic

respiratory failure. Most patients diagnosed with IPF die within 2–5 years of diagnosis, although there are variable rates of disease progression. Recently approved new treatments hold promise for improving outcomes of these patients [17–19].

The pathophysiology of IPF is thought to result from varying combinations of environmental, aging, and/or genetic factors that lead to alveolar injury susceptibility. Endogenous or exogenous irritants may injure the lung chronically, or an abnormal host repair ensues, leading to lung fibroproliferation, ultimately leading to chronic hypoxemic respiratory failure and death.

A better understanding of the cells and mechanisms that underlie lung fibroproliferation in ARDS and IPF will open new avenues for treatment for these debilitating disorders. Here, we consider the role of pericytes in the lungs as possible cellular targets for more effective treatments for these debilitating disorders. The location of pericytes in the lung and their known role in vascular structural integrity and fibroproliferation make pericytes interesting and relatively unexplored cellular targets for fibrotic lung diseases.

## Pericytes

In 1923, Karl Zimmermann noted the contractile properties of a population of cells he named *pericytes* because they were located around blood vessels [20]. Morphological identification of pericytes has been the mainstay in the past, which is still a useful approach. Electron and early light microscopy identified elongated cell bodies with a prominent nucleus and cytoplasmic processes that embrace endothelial cells [21, 22]. Typically, pericytes are found surrounding nonmuscular microvessels, capillaries, and postcapillary venules, embedded in the basement membrane, where they partially envelop and attach to endothelial cells by focal contacts named *peg-to-socket junctions* [23]. Pericytes are widely distributed, since microvessels are present in most organs, and are responsible for regulating blood flow, supporting angiogenesis, and serving as cell progenitors, depending on the organ [24].

Although morphology is important in identifying pericytes, several other cell types have similar morphological characteristics. To avoid confusion, scientists started to consider both anatomical location and biochemical markers, such as cell membrane and intracellular proteins. These molecular markers are also useful in tracing genetic lineage, using methodologies that identify the cells that give rise to pericytes and those into which pericytes differentiate [25]. However, identifying pericytes by their markers is quite tricky. Depending on their embryonic origin and the host organ, pericytes may display a vast number of markers [26], possibly related to their specific functions. For example, pericytes that help to regulate the blood flow in, and contractility of, microvessels express contractile proteins like alpha-smooth muscle actin (α-SMA) [27] and, as mural cells (pericytes and vascular smooth muscle cells), provide the physical integrity microvessels require [28–30], while pericytes that serve as nervous cell progenitors express neural cell markers like NG2 [31]. Other markers used to identify pericytes include platelet-derived

growth factor receptor-β (PDGFR-β) [32, 33], aminopeptidase N (CD13) [34], and nerve/glial antigen-2 (NG2) proteoglycan (CSPG4) [35, 36].

The most well-known functions of pericytes are related to maintaining organ homeostasis. During angiogenesis, pericytes dissociate from endothelial cells, momentarily destabilizing the blood vessel and allowing endothelial sprouting into the tissue. Growth factor signaling (PDGF, VEGF, and TGF) then recruits the pericytes to stabilize the newly formed vessel [32, 37, 38]. Pericytes have antifibrinolytic properties and play a role in coagulation [39, 40]. Pericytes also affect immune responses in the central nervous system [41–43] and promote T-cell proliferation and activation [44–46]. Pathologically, pericytes may respond to angiogenic signals from tumors, thus supporting tumor progression and sometimes facilitating metastasis [47]. Pericytes also participate in adipogenesis, converting to adipocytes in response to injury, and fibrotic scar tissue through several mechanisms that lead to collagen deposition and fibroblast proliferation [29].

Pericytes are heterogeneous with regard to phenotype, distribution, and origin [30, 48, 49]. Zimmerman distinguished three types based on their location in the blood vessels: precapillary, true-capillary, and postcapillary [29, 50]. Precapillary pericytes have circular branches that tend to wrap around the vessel and express varying amounts of α-smooth muscle actin [51]. True-capillary pericytes are spindle-shaped with many short secondary processes and extend mainly along the vessels' long axis. They do not express α-smooth muscle actin [51]. Postcapillary pericytes are shorter and stellate and cover the abluminal surface of postcapillaries [29].

The number and density of pericytes vary by organ, possibly linked to the organ's blood pressure and the number of large vessels that serve it. These variations contribute to different pericyte-endothelial cell ratios. In the lungs, it is 1:10; in the central nervous system, 1:1; and in skeletal muscle, 1:100. This heterogeneous distribution has raised questions about whether lung pericytes have functions distinct from those in other organs, and several groups are studying the specific roles they play in normal and injured lungs [28, 52]. Some suggest that the more pericytes in a tissue, the higher its blood pressure and the more controlled are its vessels [28], which may explain why there are more pericytes identified on vessels of larger diameter [30].

Pericytes also differ in their origins [53]. Lineage-tracing studies indicate that forebrain pericytes have a neuroectodermal origin [54]. In sharp contrast, in most other organs explored, pericytes are derived from the mesoderm, more specifically from the sclerotomal compartment [55–62]. The exact origins of pericytes in the lung remain unknown.

Pericyte heterogeneity is exemplified by their marker expression profiles [29]. For example, pericytes localized on venules express desmin and αSMA, while those on capillaries express desmin but not usually αSMA [36, 63]. ATP-sensitive potassium channel Kir6.1 is undetectable in pericytes in the skin and heart but highly expressed in brain pericytes [64]. In the spinal cord, pericytes that express the glutamate-aspartate transporter (GLAST) differ from those that express desmin and αSMA [65]. Bone marrow has both sinusoid-associated leptin receptor (LEPR)+ and LEPR- pericytes [66]. In the skin, NG2- and NG2+ pericytes have been

described. Additionally, two populations of pericytes were identified in several organs, including the lungs, based on nestin-GFP expression [67, 68]. Using a nestin-GFP/NG2-DsRed bi-genic mouse, nestin-GFP-/NG2-DsRed+ (type I) and nestin-GFP+/NG2-DsRed+ (type II) pericytes were found in close proximity to endothelial cells [68]. Discovering the functions of specific pericyte subtypes in the lungs will help target the most appropriate cells for treating various pulmonary diseases.

## Physiology of Pericytes in the Lungs

In the gas exchange process, carbon dioxide and oxygen diffuse into the microvasculature from the alveolar airspace at the terminations of ducts, or sacs, whose walls contain elastic fibers coated with types I and II pneumocytes [69, 70]. Type I pneumocytes have a squamous (flat) morphology and cover 90–95% of the alveolar surface, so they are responsible for maintaining the equilibrium of fluids in the airspace; their occlusive junctions prevent tissue fluids from leaking into it [71]. However, pneumocytes cannot replicate and are very susceptible to the toxic agents that compromise alveolar functions in cases of injury, and type II pneumocytes must form type I pneumocytes to re-establish homeostasis [72, 73]. Although crucial for the recovery of type I pneumocytes, type II pneumocytes cover less than 5% of the alveolar surface due to their cuboidal morphology [74]. Type II pneumocytes play an important physiological role in the secretion of pulmonary surfactant, a substance that decreases alveolar surface tension. Surfactant prevents the alveolar sacs from collapsing at the moment of expiration and maintains the optimal fluidity of the surface that facilitates gas diffusion to and from the alveolar airspace [75–77] (Fig. 7.1).

Efficient gas exchange requires alveolar fluid balance but also control of the pulmonary blood flow [78] mediated by cardiac output and several other factors, including body position (i.e., upright or supine) [79]. Within the microvasculature of the alveolar sacs, more refined regulation is required. For example, the resistance and permeability of pulmonary arterioles vary with the integrity of the endothelial and subjacent cell layers [80]. Pulmonary arterioles and small-diameter vessels, including venules and capillaries, integrate the vascular bed of the alveolar sacs, where controlling blood flow is crucial because it is the region where the major function of the organ is carried out. The vascular bed that surrounds the alveolar sacs serves two main purposes: structural support and gas exchange [81–83].

Previous studies demonstrated that pericytes are critical for vascular development and support. Boström and colleagues used animal models to show that the formation of functional alveoli depends on platelet-derived growth factor alpha (PDGFα) signaling. Without the action of this growth factor during embryonic development, vascular bed formation compromise ensues, and newborn knockout mice had smaller lungs with malformed alveoli [84]. Pericytes express PDGF receptors – in fact, they are biochemical markers used to identify them – and PDGF singling contributes to angiogenesis both in organ development and homeostasis.

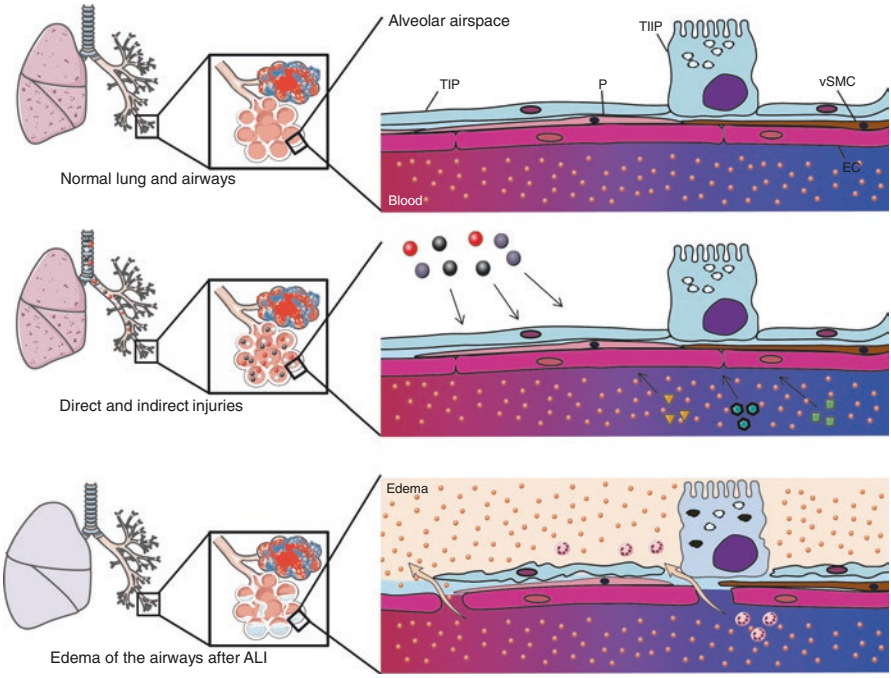


**Fig. 7.1** Direct and indirect injuries to the lung. Types I and II pneumocytes (TIP and TIIP) compose the lung epithelium and maintain alveolar structure. On the other side of the blood-air interface, the microvasculature is composed of endothelial cells (EC) and perivascular cells, such as pericytes (P) and vascular smooth muscle cells (vSMC). Lung injury begins with direct damage to the lung epithelium that causes inflammatory components to destabilize vascular endothelial cells and indirectly damage the lungs. As a result of either direct or indirect injuries, vascular and perivascular cells become loosely attached, allowing proteins, fluids, and immune cells to leak into the alveolar airspace and damage type I and II pneumocytes

Therefore, pericyte recruitment via PDGF signaling influences the formation of a stable vascular bed that, in the lungs, leads to the development of functional alveolar sacs [24, 85]. Although staining shows that both pericyte subtypes co-localize with two classical pericyte markers, PDGFRβ and CD146, at least in the skeletal muscle, only type I expresses the adipogenic progenitor marker PDGFRα [68, 85, 86]. Whether the same is true in the lungs remains unknown, but if so, type I pericytes may exclusively control the functions related to this receptor.

The gas exchange also depends on a stable vascular bed through which pulmonary capillaries transport venous blood from the right heart to the alveolar sacs [87] and then oxygenated blood to the left atrium and systemic circulation [88]. Pericytes help to control blood flow in two ways: (1) regulating vessel permeability to optimize gas exchange and (2) providing contractile force to the vascular bed, assuring that flow decreases and subsequently increases before and after gas exchange, respectively [89, 90]. Type I pneumocytes control permeability within the airspace; the pulmonary surfactant controls fluidity. However, vascular and perivascular cells also participate by preventing leaks from the bloodstream into the airspace and

ensuring proper gas diffusion both ways [91, 92]. Endothelial cells lining the vascular-facing surface of vessel walls primarily control blood vessel permeability [93]. Small-diameter vessels have fewer endothelial cells, resulting in the formation of small spaces (termed *fenestrations*) [94], possibly allowing fluid to leak out. Endothelial cell fenestrations are involved with cell migration into the tissue during inflammatory responses [95]. Pericytes provide a remedy. Because of their characteristic morphology and increased localization around small vessels, pericytes provide an extra layer of cellular support, which if injured could theoretically lead to endovascular leak [96]. Also, perivascular cells control the contractility of pulmonary capillaries, which have no or only a thin muscular layer [97]. Another perivascular-like cell type with contractile properties are vascular smooth muscle cells that surround small-diameter vessels [98], but Edelman and colleagues established that pericytes could express contractile proteins, such as desmin, α-smooth muscle actin, and myosin, and thereby potentially regulate blood flow in pulmonary capillaries by vasoactive signaling [99]. Future studies are needed to determine whether regulation of blood flow and vascular permeability and contraction is unique to pericyte subtypes.

Although pericytes can function as mural cells in pulmonary capillaries, some researchers point to other cell populations that reside in the lungs and may stabilize alveolar sac structure [104], such as fibroblasts [100], mesenchymal stem cells, smooth muscle cells, adventitial cells [101], and macrophages [102, 103]. Like pericytes, resident fibroblasts and myofibroblasts surround vessels, and these cell types are difficult to distinguish based on morphology alone [105, 106]. Biochemical markers are also problematic since other cell types express pericyte markers, and even in pericytes, their expression varies with developmental stage [49]. For instance, PDGFRβ is a known marker of such cell types as fibroblasts [100, 107], while NG2 proteoglycan can be expressed in macrophages [108]. Pericytes that do not express NG2 were also recently described [109]. Note that sometimes, perivascular cells can be distinguished by the circumstances under which they appear, and classical electron microscopy studies reveal that in contrast to other perivascular cells, pericytes localize under the vascular basal lamina [110]. Whether all functions attributed to perivascular cells in the lungs correspond to pericytes remains unclear. Combining pericyte molecular markers with immunolabeling of the basal lamina in genetic lineage-tracing models in the lungs will clarify several open questions in pulmonary biology.

In a recent article, Lefrançais and colleagues demonstrated that the lung is a reservoir for hematopoietic stem cells (HSCs). These progenitors can migrate out of the lungs, repopulate the bone marrow, and contribute to many hematopoietic cells [111]. In adult bone marrow, pericytes form a special niche for quiescent HSCs, promoting the dormancy essential for their maintenance [66, 112]. Whether pericytes contribute to HSC maintenance in the lungs remains unknown.

Rock and colleagues pointed out that pericytes and resident fibroblasts comprise 10–20% of all lung cells [113]. They both contribute to the structure of the lung parenchyma, but their participation differs [113]. Fibroblasts are the most common cell in all connective tissues, mainly producing extracellular matrix and collagen [114]. They are critical to wound healing and the formation of an organ's stroma

[115] but are strongly correlated with pathological processes. Fibroblasts initiate inflammatory responses, play roles in tumor growth and resistance, and may compromise function in various organs, including the lungs, by supporting the substitution of scar-like connective tissue for functional tissue [114, 116]. Pericytes, on the other hand, are quite plastic. Because their distribution around the body is so heterogeneous, their origin, even within the same tissue, may also be heterogeneous, which may explain their variable markers and functions [53]. Marriott and colleagues determined that pericytes in the lungs are part of a large population of mesenchymal stem cells and expressed, among a vast number of markers, NG2 and αSMA markers, which are related to neural and contractile cells, respectively. These cells played different roles in the lungs than other cells derived from the same embryonic origin [49, 117, 118].

## Pericyte Involvement in Fibrosis

Fibrosis is characterized by the excessive deposition of fibrous connective tissue (*fibroproliferation* or *fibrodeposition*) as a reparative or healing process. Without removal of the injurious agent, fibrosis may become pathological. If the tissue damage is too extensive, the injury may result in the remodeling of the organ's architecture [119–121]. Sometimes this remodeling is harmless; for example, after a cut or bruise, scar tissue, the result of fibrodeposition, can replace the epithelium without compromising homeostasis in any body functions. At other times, the scar tissue formation can compromise the organ's entire function, leading to systemic impairment or death, depending on the extent of the scar and the organ, for instance, the lungs or kidneys.

Fibrodeposition is not a simple process, requiring well-organized cell recruitment, differentiation, signaling, and protein deposition. The resulting mass of scar tissue is composed of various cell types and proteins that replace the organ's functional units [122]. Key among them are myofibroblasts, well-known producers of extracellular matrix proteins, such as collagen, glycoproteins, and proteoglycans [116, 123, 124], that primarily produce collagen when activated by autocrine and/or paracrine signals, such as transforming growth factor beta (TGF-β) [125]. In the aberrant wound healing observed in IPF, myofibroblast proliferation increases, and they express high levels of actin, especially α-SMA, and myosin, which enhances the connection of myofibroblast with the extracellular matrix, contributing to the contractile properties that characterize scar tissue [114, 126, 127]. Identifying the cells that originate myofibroblasts might allow the arrest of fibrosis, or even reverse fibrosis, in certain disease conditions [128]; however, recent findings demonstrate that the origins of myofibroblasts may be heterogeneous, even within a single tissue [129].

Studies of antifibrotic drugs have tested their effects on endothelial cells [130], epithelial cells [131–133], circulating progenitor cells [134–139], resident fibroblasts [140], and pericytes [29] from multiple tissues. Our knowledge of cellular complexity in the lungs has improved, but the biological processes of fibrous tissue deposition here are not fully understood. The inflammatory response in ARDS and

IPF may stimulate pericytes to differentiate into myofibroblasts and fibroblasts; pericyte differentiation is known to increase the number of cells producing extracellular matrix and collagen deposition [141]. Moreover, when pericytes differentiate, their morphology changes, and they detach from the endothelial cell layer [142]. Therefore, during fibrodeposition in the lungs, pericytes not only cause remodeling in the parenchyma but also destabilize the pulmonary capillaries, compromising gas exchange and the stability of the alveolar sacs [143, 144].

Data in a murine model of ARDS suggests that some degree of fibroproliferation is part of normal lung injury repair [145]. Several researchers define pericyte participation in fibrodeposition as an "organ-dependent" process, and its role in some organs, like the lungs, is still under debate. Using different animal models of cholestatic, toxic, and fatty liver diseases, researchers demonstrated that liver-resident pericytes (called *hepatic stellate cells*) are the main source of collagen and play an important role in fibrodeposition [146–148]. Dulauroy and colleagues showed that after acute injury, skeletal muscles generated scar tissue with the active participation of collagen-producing pericytes [149]. In kidney fibrosis, the contribution of pericytes is not well stablished. Using a model of angiotensin-II-induced renal fibrosis, Faulkner and colleagues showed that fibrogenic cells derive from perivascular cells later identified as pericytes by Humphreys and colleagues [150, 151]. In contrast, LeBleu and colleagues found that ablating pericytes did not alter the level of kidney fibrosis [152].

Pericyte contribution to fibrous tissue in the lungs is also controversial. Rock and colleagues found that pericytes proliferated after lung lesion but did not produce fibrogenic cells after injuries induced by bleomycin [111]. In contrast, a recent study using lineage-tracing mapping showed that FoxD1+ pericytes do contribute to pulmonary fibrogenesis [105]. The use of different transgenic mouse models might explain this discrepancy. Rock et al. [113] used the inducible NG2-CreER transgenic mouse, which has a very low recombination efficiency; thus, the labeling does not include the whole pericyte population. As lung pericytes are heterogeneous, and at least two subtypes have been described, perhaps only a fraction of these cells contribute to pulmonary fibrosis. Indeed, using bi-genic nestin-GFP/NG2-DsRed mice, type I (nestin-GFP$^{-}$/NG2-DsRed$^{+}$/PDGFR$\beta^{+}$), but not type II (nestin-GFP$^{+}$/NG2-DsRed$^{+}$/PDGFR$\beta^{+}$), pericytes were found to contribute to collagen production in the lungs after bleomycin-induced injury [68]. Fibrous tissue formation depends on several molecular processes, including TGFβ signaling, supporting the idea that growth factors play an essential role [153]. Strikingly, a recent study demonstrated that perivascular Gli1+ cells in a pericyte niche adjacent to endothelial cells in the lungs expand and significantly contribute to αSMA+ myofibroblasts after pulmonary injury [117]. The overlap between Gli1+ cells and previously described pericyte populations in the lungs remains unclear.

Although several studies indicate that pericytes do participate in lung, kidney, liver, and spinal cord fibrodeposition after injury [65, 100, 113], the exact cellular and molecular mechanisms remain unknown. Whether pericytes influence fibroproliferation only directly by producing extracellular matrix proteins or also stimulate other cells to differentiate into myofibroblasts that act as immune regulators, modulating cells that favor the proliferative process, is an open question.

## Interaction Between Pericytes and the Immune System

The vascular events of inflammation mark the lung parenchyma's first response to injury. Due to increased permeability of the microvessels in the blood-air barrier, a protein-rich fluid floods the alveolar airspace that damages both types I and II pneumocytes after injury [154, 155]. Damage to type I pneumocytes disrupts the integrity of the blood-air barrier and allows interstitial fluids, proteins, immune cells especially neutrophils, and fibroblasts to leak into the airspace [156]. Damage to type II pneumocytes decreases pulmonary surfactant production, possibly inactivated by the amount of fluids in the airspace, and compromises regeneration of type I [155, 157]. These are the first events of vascular and blood-air barrier destabilization leading to clinical ARDS onset. Inflammation is not triggered to increase organ damage [158]; rather, inflammation is an attempt to restore normal function in the lung and to heal wounds. The immune system controls the main events of inflammation – vascular alterations, extravasation of plasma proteins, and cell migration – that lead to organ remodeling [159]. The remodeling may be irreversible if the initial insult is not removed or if inflammation cannot resolve; the tissue then becomes compromised and is replaced by fibrotic scar tissue [160].

Pericytes in the pulmonary capillaries usually regulate the endothelial cell layer via paracrine signaling, controlling the diffusion of proteins and cells through the vessel walls. During the inflammatory response, endothelial cells retract, and pericytes cover the resulting gaps, preventing proteins and cells in the bloodstream from escaping [95]. Recent studies demonstrated that the presence of pro-inflammatory cytokines, such as IL-2, mediates a conformation change to pericytes around the leaky vessel [161]. Pericytes re-establish their junctions with endothelial cells, allowing plasma components with a high protein concentration [162] to spill into the airspace and interact with collagen and other extracellular matrix components, leading to the release of more cytokines, growth factors, and chemoattractant factors [163, 164].

In ARDS, the cells that respond first to injuries are neutrophils, rapidly invading the lung parenchyma, and once inside the alveolar airspace, neutrophils produce cytokines and pro-inflammatory mediators that affect the integrity of the alveolar sacs, compromising type I and II alveolar epithelial cells [165]. In addition to vascular permeability, pericytes contribute directly to neutrophil migration from the vessels into the tissue. Once neutrophils have penetrated the endothelial cell layer, direct contact with the pericytes' basement membrane relaxes the perivascular cytoskeleton via inhibition of intracellular signaling RhoA/ROCK, changing the conformation of pericytes that direct neutrophils to regions that express low quantities of extracellular matrix proteins [165, 166]. The expression of intercellular adhesion molecule-1 (ICAM-1), macrophage antigen-1 (Mac-1), and leukocyte function-associated antigen-1 (LFA-1) on pericytes facilitates the transmigration of immune cells. Pericytes expressing ICAM-1 and the chemoattractant MIF were shown to attract and activate neutrophils and macrophages as well as facilitate their trafficking. Pericytes also participate in the immune response by enhancing the functions of neutrophils and macrophages in the interstitial space [167] (Fig. 7.2).

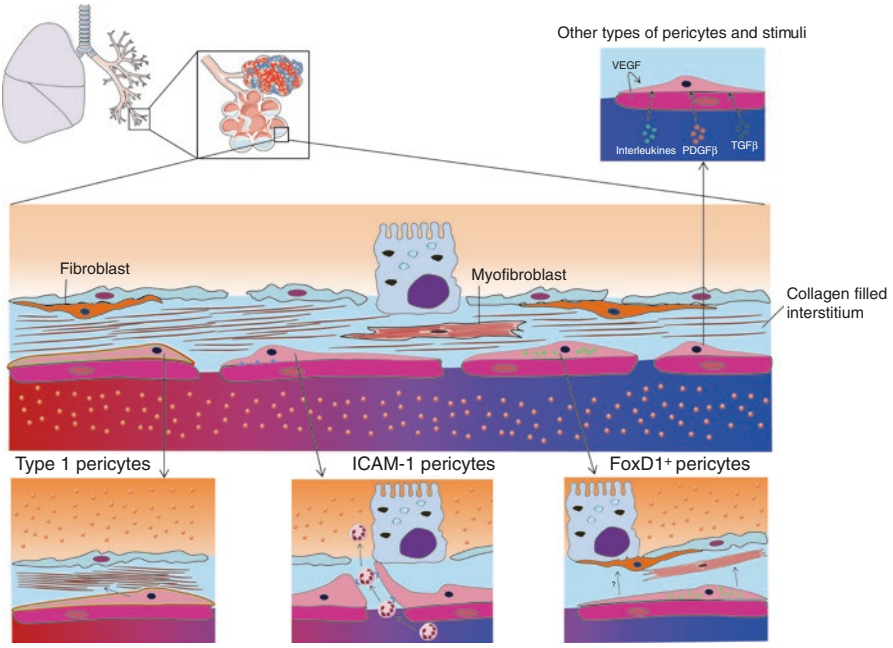


**Fig. 7.2** Heterogeneity of pericytes in the blood-air interface and their role in lung fibrodeposition. At least three types of pericytes influence fibrodeposition in the lungs. Type I is the source of type I collagen, which, when deposited in the interstitium, remodels the tissue's architecture. Pericytes expressing the adhesion molecule ICAM-1 direct and support neutrophil migration from the bloodstream to the alveolar airspace. FoxD1+ pericytes can differentiate into myofibroblasts that express αSMA. The overlap between the two distinct pericyte populations described so far remains unknown. The hypotheses that there might be other pericyte subtypes and that pericytes might be stimulated by TGFβ, PDGF, interleukins, and VEGF to support fibrodeposition have not been confirmed

## Pericytes and T Cells in Lung Fibrosis

In ARDS, multiple variables, including the nature, duration, and intensity of the aggression and the individual patient's response, influence the development of the inflammation, driving the resolution process along different pathways [168]. The ideal outcome after injury is complete resolution, the restoration of the organ's normal architecture with no or little compromise of lung function [169]. When an injury ends rapidly, or if the tissue sustains little damage, macrophages mediated the removal of cell debris, and the lymphatic system reabsorbs edema fluid, resulting in complete restoration [169, 170].

Persistent injury leads to extensive tissue damage [171] in organs like the lungs where the regeneration rate is low, and in conditions such as fibroproliferative ARDS and IPF associated with substantial exudate and fibrin deposition, the inflammatory and late immune system responses cannot remove or resolve the injury, perpetuating fibrosis [172]. In both ARDS and IPF, we do not fully know what

causes tissue fibrosis [173]. Certain cell types seem pivotal. Researchers identified many cell types, including, but not limited to, fibroblasts and myofibroblasts, which participate in the direct deposition of matrix proteins or differentiate into matrix-secreting cells by either inhibiting or stimulating signaling [174]. Pericytes are recruited to wounded tissue by PDGF signaling and can produce collagen. Several groups suggested, and fate-mapping studies confirmed, that in cases of acute tissue injury, subsets of pericytes detach from the perivascular space and differentiate into myofibroblasts, making a key contribution to the formation of scar tissue [149]. Note that all groups agree that pericytes alone are not responsible for the resultant fibrosis in these conditions [153].

Pericytes may contribute to fibrosis in other ways [141]. Lung injury resolution requires a microenvironment that enables cells, such as fibroblasts and pericytes, to develop and expand [175]. Pericytes physically help immune cell migration into the injured tissue through the endothelial cell layer, but whether immune cells communicate with type I pericytes to maintain the microenvironmental conditions that support fibrosis remains unknown.

The first cells to invade the lung parenchyma in ARDS are neutrophils. These cells release cytokines that attract, and possibly activate, pericytes. Once the inflammatory response is established, T cells (both Th1 and Th2) are recruited to the damaged site [176]. Th1 cells secrete IFN-γ to directly suppress fibroblasts' synthesis of collagen and control the rates of collagen degradation by regulating metalloproteinases (MMP) in the extracellular matrix [177]. While T cells clearly play an important role in controlling collagen deposition in the injured lung, they do not contribute to the resolution process [176, 178]. No one has examined whether they inhibit pericyte-dependent fibrogenic responses.

In the later stages of ARDS, lymphocytes mobilize to the damaged tissue. Inflammatory mediators secreted by CD4+ T cells strongly influence extracellular matrix deposition and tissue remodeling [179, 180]. The cytokine secretion profile of CD4+ T cells determines their classification [181], with T helper 1 (Th1) cells secreting relatively large amounts of interferon-γ (IFN-γ) and other pro-inflammatory cytokines, such as IL-2 and tumor necrosis factor-α (TNF-α), as defense mechanisms associated with infectious diseases and phagocytosis [182]. In contrast, T helper 2 (Th2) cells secrete IL-4, IL-5, and IL-13 and are associated with immunoglobulin E (IgE) production and immune reactions mediated by mononuclear cells, as observed in the initial stages of ARDS [183]. These two subpopulations of CD4+ cells are mutually antagonistic: IFN-γ inhibits Th2 cells, while IL-10 inhibits Th1 cells [176, 184, 185]. Th2 cells also play an important role in collagen deposition [186]. They control collagen synthesis by regulating the expression of tissue inhibitors of matrix metalloproteinase (TIMP). The main cytokines Th2 cells secrete (IL-4, IL-5, IL-13) enhance collagen deposition [187]. IL-10, a cytokine related to the Th2 response, is crucial to the fibrosis process. Secreted by T regulatory cells, macrophages, and dendritic cells, it inhibits Th1 cells and cells to secrete IL-13, which activates fibrogenic cells, leading to collagen deposition and fibrosis [176, 188]. T regulatory cells have been shown in animal models of ARDS to mediate active lung injury resolution and regulate collagen removal in the late phase [145, 189]. Future studies should explore pericyte and immune cell interactions in lung injury and resolution.

## Therapeutic Options

Mortality from ARDS has decreased significantly since the original description of this syndrome in the 1970s [2, 190]. Much of this decrease can be attributed to overall improvements in the care of critically ill patients and the use of low tidal volume ventilation strategies [191]. Other emerging treatments include the use of early neuromuscular blockade and prone positioning [192, 193]. Despite these improvements, there are no specific lung-targeted pharmacologic therapies to facilitate lung injury resolution in ARDS. Additional cellular and molecular pathways that might be involved in the development of lung fibrosis must be identified as possible therapeutic targets.

Based on the results of promising randomized controlled trials showing reduced lung function decline, the FDA approved two pharmaceuticals nintedanib and pirfenidone for the treatment of IPF in 2014, opening new potential avenues of treatment for this debilitating disease [194, 195]. While the mechanism of action of these drugs is incompletely understood, nintedanib is a tyrosine kinase inhibitor and targets PDGFRα, which is expressed by pericytes as mentioned above [196]. It is unknown if one of the mechanisms of benefit of nintedanib in IPF occurs via pericyte involvement. Another recent study showed that the small-molecule Gli inhibitor GANT61 reversed the fibrosis phenotype in bone marrow by impairing the expansion of Gli1+ myofibroblasts [197]. Since Gli1 labels a significant portion of lung pericytes, a GANT61 drug might be able to inhibit pulmonary pericyte-derived fibrosis.

## Conclusion

Although a subpopulation of pericytes may play a central role in lung disease, their contribution under physiologic conditions remains unknown. From a drug development perspective, pericytes provide a cellular target with a consistent molecular repertoire and response to signals. The challenge will lie in limiting the deleterious functions of pericytes while preserving the healthy ones.

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# Chapter 8
# Emerging Therapeutic Targets and Therapies in Idiopathic Pulmonary Fibrosis

**Vineela Parvathaneni, Snehal K. Shukla, and Vivek Gupta**

## Introduction

The human respiratory system is primarily comprised of airways and lungs which work efficiently in sync to promote efficient gaseous exchange. The airway anatomy consists of extrathoracic and intrathoracic segments which involve the conduction of air to the alveolar surface. The lungs and airways remove carbon dioxide (CO2) produced after metabolism while supplying oxygen to the tissues through exhalation and inhalation processes [1]. Incoming air into the respiratory system flows through the upper airways (nose, mouth, larynx), conducting airway(trachea, bronchi, bronchioles) and alveolar airways [2]. The lung is the most perseverant organ in the body contracting about 20 times a minute and involves the exchange of gases during breathing [3]. The mechanical process of breathing is vital for the gaseous exchange of oxygen and carbon dioxide as the blood circulates through the lung [4]. All the organs of the respiratory system function in a complex processes, and their individual functional integrity is very important for proper gaseous exchange and in turn efficient functioning of the human body [5] (see Fig. 8.1).

While the tissues or the organs of the respiratory system, especially lungs, provide a vast surface area which is instrumental in efficient gaseous exchange and drug absorption, this attribute also inadvertently exposes the respiratory system to the outside environment and hence imposes higher potential for injury and infections. Any injury or infections to any of the components of the respiratory system will affect the basic function of the respiratory system, leading to respiratory disorders. Airways dysfunction can cause obstructive lung disorders like asthma and bronchitis, whereas parenchymal lung injury will lead to restrictive lung diseases [6].

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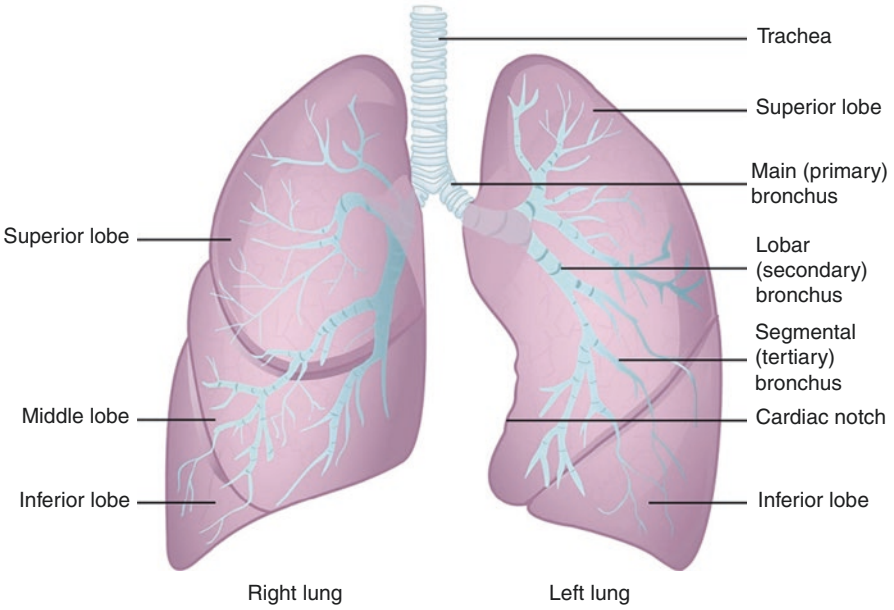


**Fig. 8.1** Human lung anatomy. (**a**) Each lobe consists of bronchopulmonary segments. (**b**) Each segment receives air from tertiary bronchus and blood by its artery. (**c**) Cardiac notch allows space for the heart. (Adapted from: Gross Anatomy of the Lungs (2018, January 1) [188])

One of the major classes of restrictive lung diseases is known as "interstitial lung diseases (ILD)." ILD is a large group of more than 200 lung diseases, most of them rare, characterized by involvement of lung interstitium. One of the primary disorders classified as ILD is idiopathic pulmonary fibrosis (IPF), a fatal disorder characterized by the continued decline in lung function. The ATS/ERS classification defines IPF as "a specific form of chronic fibrosing interstitial pneumonia of unknown etiology, limited to the lungs and associated with the histological entity of usual interstitial pneumonia" [7]. Potential causes and mechanisms underlying the disease condition are majorly unknown, and hence the term "idiopathic" has been used. IPF is also sometimes known as "usual interstitial pneumonia (UIP)", due to progressive scarring of both lungs. IPF is a progressive disorder characterized by alveolar epithelial cell injury, the formation of myofibroblast foci, and buildup of extracellular matrix in the lung parenchyma leading to scarring (thickening) of the lungs [8].

IPF is generally characterized by altered wound healing. This lung disease is found to have a genetic background making it incurable like cancer [9]. The cellular and molecular aberrances are common in this disease. IPF is generally a disease of the elderly with meager survival rates, mostly attributed to late diagnosis and general symptoms. While few specific medications to treat IPF exist today, the emphasis is mostly on abolishing disease symptoms and not on treatment, which is due to idiopathic nature of the disease. Numerous clinical trials are still in progress to develop a medication to treat this disease. Multiple studies have identified the possible pathways underlying in the pathophysiology of idiopathic pulmonary

fibrosis. The pathogenesis of the disease is not clear yet today, and most perceptions are based on the tissue pathology, resulting in lung fibrosis in animal models. Clinical observations suggest striking differences on how disease develops in different patient populations, with few patients experiencing a relatively gradual diminishing of the lung function and others with acute collapses and rapid exacerbation of the disease. These differences suggest inherent differences in pathogenic mechanisms and pathways responsible for IPF development. This discrepancy has convoluted the clinical trial design and the identification of potential IPF therapies. The cause for pulmonary fibrosis today is unclear in most cases, leading them to be identified as idiopathic pulmonary fibrosis. Idiopathic pulmonary fibrosis (IPF) usually affects middle-aged and older adults. The course of IPF varies among different individuals, with some developing the disease very quickly while others have a slow disease progression. While IPF may be cured quickly in some patients, it may last longer or lead to death in other patients. As the lung tissue thickens in IPF, the ability to move oxygen into the bloodstream diminishes. As a result, the brain and other organs don't get the oxygen they need. The common causes of death due to IPF include respiratory failure, pulmonary hypertension, heart failure, pulmonary embolism, pneumonia, and lung cancer. Idiopathic pulmonary fibrosis (IPF) is a devastating disease with an average life expectancy from diagnosis of 2.5 years with 5-year survival of between 20% and 40% [10]. Physiologically, IPF can compromise oxygen diffusion, lung function, and is typically fatal [11]. The accumulation of collagen (fibrosis) in the lung observed in IPF leads to shortness of breath and fatigue with exertion secondary to the reduced oxygen transfer [12]. Typical early symptoms of IPF include shortness of breath during periods of activity and a dry cough. Additionally, patients with IPF have symptoms of weight loss and fatigue. In later-stage IPF, enlargement or clubbing of the fingertips may occur. As the disease progresses, shortness of breath may even occur at rest [13]. The pathobiology of IPF is heterogeneous and is thought to include alveolar epithelial cells and fibroblast apoptosis and an aberrant wound healing process after injury to these cells [14].

The major risk factors for IPF development include environmental factors such as exposure of inhaled toxins, genetic mutations, and the presence of another co-occurring disorder like gastroesophageal reflux disease [11]. Even though many studies have investigated the pathogenesis of IPF, there is limited understanding of the biology leading to the increased need of thorough clinical studies in this area [15]. Moreover, since IPF is a disease of elderly people, it is more difficult to treat due to the presence of other co-occurring diseases such as pulmonary hypertension and lung cancer. The pathophysiology of IPF fibrosis involves signaling events in fibroblasts that mediated proliferation and differentiation into myofibroblast, in addition to infiltrating inflammatory [16]. For example, fibroblasts have toll-like receptors (TLRs) which act as pattern recognition receptors and act as a link between the innate and adaptive immune responses and control wound healing and the fibrotic response directly. TLRs promote tissue repair or fibrosis in many disease settings, albeit with profound differences depending on the cellular microenvironment [17]. The biological processes underlying IPF are thought to reflect an aberrant reparative response to repetitive alveolar epithelial injury in a genetically susceptible aging individual [18]. The cardiovascular manifestations of IPF include

pulmonary hypertension, heart failure, and coronary artery disease [19]. Comparisons between IPF and normal lungs are summarized in Fig. 8.2.

Recent studies of scleroderma have identified links between pulmonary fibrosis found in scleroderma and the pathogenesis of the skin manifestations of scleroderma that suggest inflammatory immune cells play a significant role. These findings from the research are demonstrating that targeting immune cells can reduce adverse effects and the technology applied to correlate the data directly with patient samples is offering a promising approach to personalized medicine. This group of

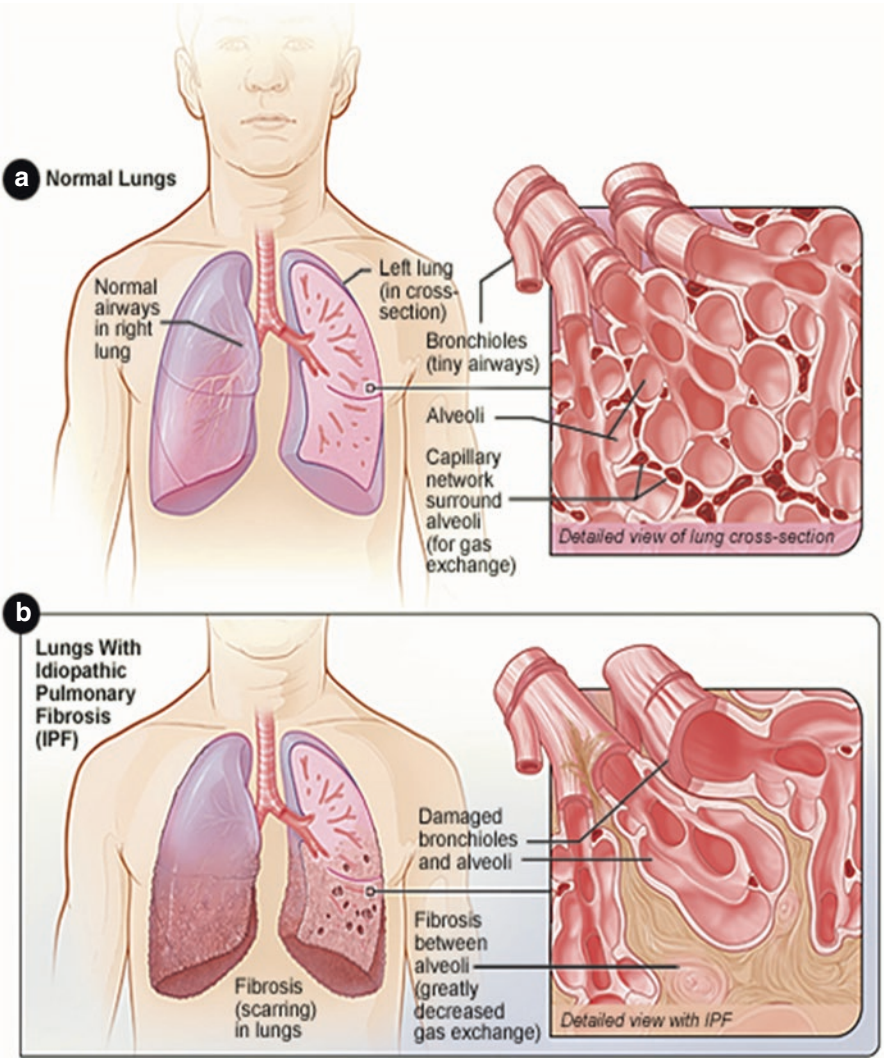


**Fig. 8.2** Comparison of normal lung and IPF affected lung. The comparison has been shown between the lung and airways in (**a**) normal and (**b**) diseased condition. The inset image describes in detail about the lung airways and air sacs being affected by IPF. (Adapted from: Adapted from File:Ipf NIH.jpg. (2016, November 29) [189])

scientists has utilized next-generation sequencing technologies and novel animal models to conduct their studies [20]. In addition, they have correlated the animal model data with the patient samples wherein they have traced the immune cells throughout the progression of IPF to find out the role of immune cells in the disease progression. It was also discovered that monocyte-derived macrophages were the key driver for the disease development in IPF. Both scleroderma and IPF are fatal diseases found to have common symptoms such as scarring and hardening of the tissues, hence proving scleroderma to be closely related to IPF, thus leading to discovery of a new target for IPF treatment.

The initial treatment for IPF includes primarily non-specific anti-inflammatory agents such as corticosteroids since IPF progression is perceived as mainly involving inflammatory pathways. Recently fibrinolytic agents such as pirfenidone (Esbriet®), an anti-inflammatory agent, and nintedanib (Ofev®), a tyrosine kinase inhibitor, are reported to alter fibrosis and the aberrant alveolar wound repair processes [21]. These antifibrotic agents have gained much importance due to their multi-target mechanism of action involving inhibition of different growth factor receptors, suppression of TGF-β, and blocking mitogenic effects of profibrotic cytokines [22], as the IPF involves multiple disease-causing pathways in its pathogenesis and progression [23]. While these abovementioned agents have proven beneficial in preventing further deterioration, they are limited in their utility in reversing the pathophysiological processes of IPF, emphasizing the unmet need of new agents for IPF therapy. In addition to developing drugs targeting symptoms and disease progression, a comprehensive study of genetic factors involved in IPF pathogenesis and strategies to target those genetic changes or facts could also have an enormous potential to further develop effective therapies. Recent advances in understanding the multiple interrelated pathogenic pathways underlying IPF have identified various molecular phenotypes resulting from complex interactions among genetic, epigenetic, transcriptional, posttranscriptional, metabolic, and environmental factors [24]. They have been known to confer variable susceptibility to the condition differing risks of rapid progression and altered responses to therapy. Unfortunately, there are a few properly designed studies that have examined the efficacy of treatment options for IPF [25]. Current lines of IPF research are mainly focused on molecular genetics of pathologic events occurring at the epithelial-mesenchymal interface of the alveolus [26].

## Epidemiology of IPF

The reported prevalence and incidence of idiopathic pulmonary fibrosis (IPF) varies and depends upon how the data was ascertained and reported and the population age and geographic location. As a general rule, men are slightly more affected by IPF than women [27]. In a study of 147 patients, the incidence of acute IPF exacerbations has been estimated to be 8.5% per year [28]. Idiopathic pulmonary fibrosis primarily affects smokers over the age of 50, which may be helpful in delineating the relationship between tobacco use and the pathogenesis of interstitial lung disease (ILD) and IPF [29].

Idiopathic pulmonary fibrosis is the most commonly encountered ILD in pulmonology practices. Its overall incidence and prevalence, however, are unclear. Geographically, most studies come from the United States and Europe (United Kingdom, Czech Republic, Norway, Finland, Greece, and Turkey), with only two studies reported from Asia (Japan and Taiwan) [30]. Since most of these studies included patients diagnosed prior to the redefinition of IPF in 2000, their accuracy in line with today's definitions is unclear. Strategies to ascertain cases within the source population varied considerably, leading to additional variability. These strategies varied from the exclusive reliance on diagnostic codes to surveys of clinicians at subspecialty clinics (low diagnostic sensitivity) to a combination of searching administrative data and focused review of medical records (potentially improving diagnostic sensitivity and specificity). With these issues in mind, the published IPF prevalence ranges from 0.7 per 100,000 in Taiwan to 63.0 per 100,000 in the United States, and the published incidence has ranged from 0.6 per 100,000 person years to 17.4 per 100,000 person years [31, 32]. While significant, it is unclear how much of this variation among studies is due to geographic or demographic differences in the risk of IPF [11].

In a study conducted in the United Kingdom, the underlying cause of death due to lung fibrosis has increased from 1979 to year 2000 [30]. Using insurance claim data, this study identified the incidence of death secondary to lung fibrosis as 16.3 per 100,000 person years (6.8 per 100,000 person years for narrow definition)in 2000, as compared to 2.14 per 100,000 person years in 1979 and 2.72 per 100,000 person years in 1991 [30, 33]. When local hospital records in Norway were studied, the overall IPF incidence was found to be 4.3 per 100,000 person years [30]. Another study of countries outside the United States and Europe estimated IPF incidence to be 1.2 per 100,000 person years, with a prevalence of 1.7 per 100,000 person years [30]. The higher IPF incidence rate compared to prevalence (how widespread the disease is overall) may explain the increased involvement of risk factors resulting in a higher incidence over a period. By considering all the data above, the risk factors involved in the pathogenesis of IPF can be studied and explained. The variation between countries across different studies may reflect a transition in IPF incidence across the world from high to low incidence. Understanding why these trends are occurring may provide useful insights into the cause or causes of the disease [34]. Idiopathic pulmonary fibrosis is invariably a progressive disease, with clinical courses varying considerably between individuals, with episodes of severe acute respiratory deterioration (acute exacerbations) being unpredictable [35].

## Pathogenesis of IPF

The overall prognosis of patients with IPF is poor, and each patient demonstrates variable rates of disease of progression and symptoms, and a multitude of factors have to be considered to understand the disease pathogenesis [36]. A pictorial representation of all the pathways involved in IPF pathogenesis is described in Fig. 8.3 [37]. Here we briefly outline these pathways. For a more in-depth discussion on the pathogenesis of IPF, readers are directed to a highly cited recent review [33].

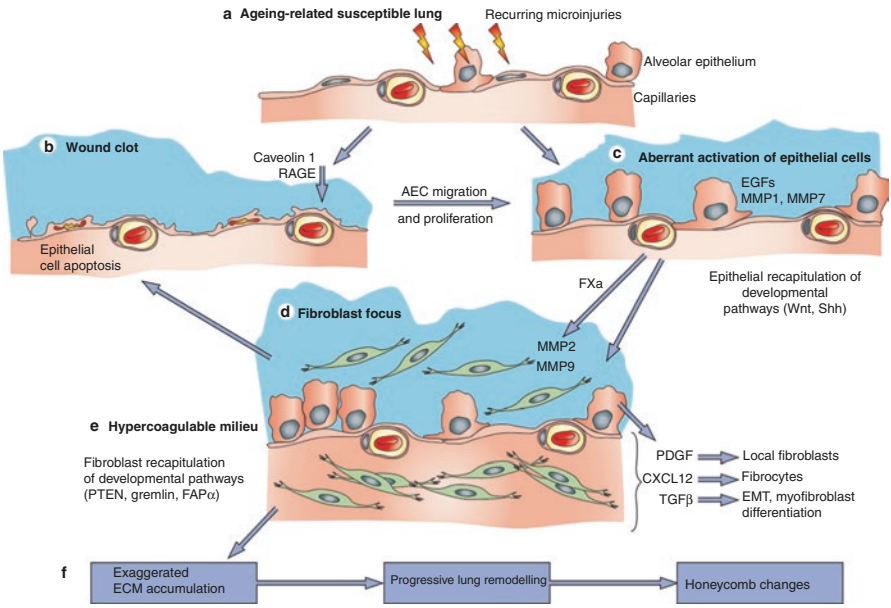


**Fig. 8.3** Overall molecular mechanisms involved in the development of idiopathic pulmonary fibrosis. (**a**) Age-related lung susceptibility is related to the repetitive microinjuries which induce cell death in the lung. (**b**) Wound clot: microinjuries cause fibrinogen release and formation of wound clot. (**c**) Aberrant activation of epithelial cells: The wound clot is followed by the AEC migration and proliferation causing activation of epithelial cells and release of MMP-1 and MMP-7 causing further contribution to cell migration. (**d**) Fibroblast focus: The MMP-2 and MMP-9 activate TGF-β and disrupt the basement membrane. (**e**) Hypercoagulable milieu: Assembling of TF-FVIIa-FX complex enhancing fibrogenic milieu. (**f**) Exaggerated ECM accumulation: Myofibroblasts secrete fibrillar collagens mainly and provoke epithelial apoptosis which lead to lung remodeling, scars along with MMP-1 secretion provoking the honeycomb cyst formation. TF tissue factor, TGF-β transforming growth factor, FVIIa factor VIIa, FX factor X, MMP matrix metalloproteinase. (Adapted from: King et al. [37])

## *Mechanism of Wound Repair*

Any injury in the alveolar structures stimulates the repair process, wherein dead and damaged cells are replaced by the new ones, a phenomenon essential for growth and survival. Before understanding IPF, it is essential to understand the mechanism involving wound repair. This mechanism can be divided into four [4] parts: (1) *clotting/coagulation phase*; (2) *leukocyte entry to site of injury*; (3) *fibroblast proliferation, activation,* and *migration*; and (4) *wound contraction*, illustrated in Fig. 8.4 and detailed below.

*Part 1. Clotting/coagulation phase* The alveolar structures in the lung stimulate the release of inflammatory mediators from the epithelial/endothelial cells in response to injury, causing the activation of antifibrinolytic-coagulation cascade and development of extracellular matrix, and this phase is known as *clotting/coagulation phase*

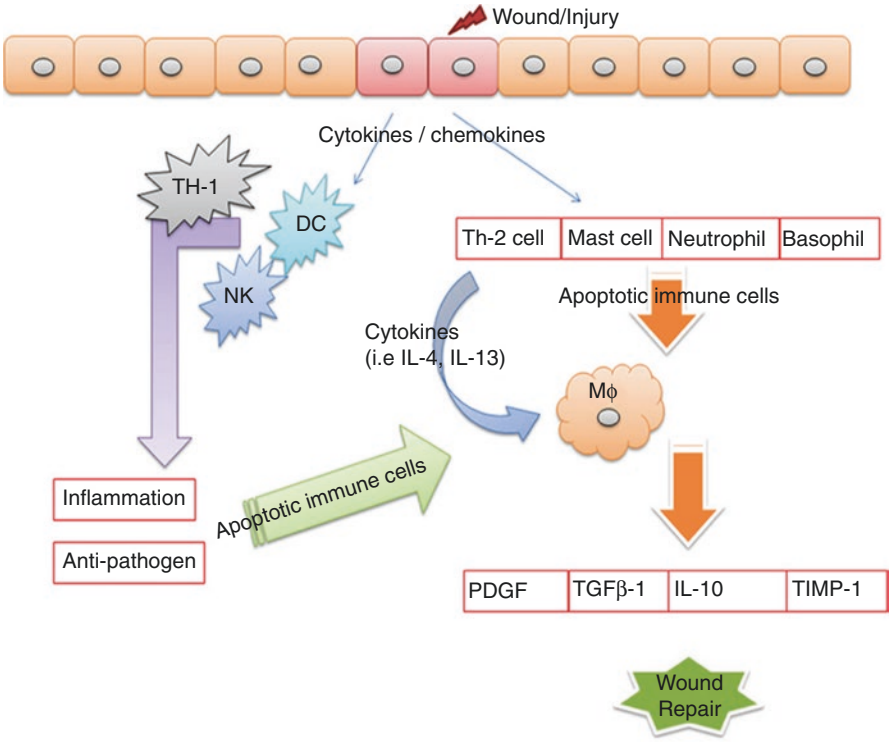


**Fig. 8.4** Mechanism of wound repair. (**a**) Clotting/coagulation phase: The endothelial/epithelial cells cause the release of adhesion molecules MCP-1 (monocyte chemotactic proteins), PDGF (platelet-derived growth factor), and ET-1 (endothelin) in response to injury. (**b**) Inflammatory cell migration: release of interleukins (IL), integrins, and interferon-γ (INF-γ) chemokines and cytokines from lymphocytes, while macrophages release other inflammatory mediators such as neutrophils, natural killer (NK) cells, and T cells. (**c**) Fibroblast proliferation, activation, and migration: The activation of fibroblast leads to recruitment of endothelial cells via EMT pathway for promoting wound repair. (**d**) Wound contraction: The wound contracts toward the center; however, unregulated repair mechanism can lead to the formation of scarring/fibrosis. (Adapted from: Wu et al. [190])

[32]. The cascade initiates the activation of platelets and blood clot formation process leading to dilation of blood vessels and increased permeability.

*Part 2. Leukocyte entry to site of injury* This results into entry of leukocytes (neutrophils, macrophages, lymphocytes, and eosinophils) to the site of injury which releases chemokines and cytokines such as IL-1β, TNF, IL-13, and TGF-β which helps to eliminate the debris and dead cells from the site and trigger the proliferation of fibroblasts contributing to the inflammatory cell migration phase [38].

*Part 3. Fibroblast proliferation, activation, migration* The third phase, i.e., fibroblast proliferation, activation, and migration, witnesses the proliferation and differentiation of fibroblasts in conjunction with the migrated fibrocytes from the bone marrow into myofibroblasts which secrete extracellular matrix components.

The fibroblast and myofibroblasts can also be recruited from the process known as *epithelial mesenchymal transition* (*EMT*) [39]. The endothelin system specifically endothelin 1 is known to be associated with the cellular transformation and replication, thereby promoting the development of the wound repair [40].

*Part 4. Found contraction* In the final stage of tissue remodeling and resolution phase, the edges of the wound come toward the center facilitating the epithelial/endothelial cells to proliferate and repair the damage tissue using the extracellular matrix component – this process is referred to as wound contraction [39].

## *What Promotes Fibrosis?*

Fibrosis results from the continuous (or multiple repeating cycles) irritation resulting in epithelial cell injury and activation. Repetition of fibroblast proliferation, migration, and activation results in the dysregulated generation of myofibroblasts and fibroblasts producing exaggerated extracellular matrix, leading to an impaired wound repair process [39, 41].

## *Inflammatory Pathways*

Idiopathic pulmonary fibrosis is historically considered a disease involving the progressive activation of inflammatory pathways and the resulting immune mediators. Hence, therapies have focused on modulating the immune system to effectively treat disease. However, the pathology of IPF affected lungs does not substantiate the concept that the immune system underlies the disease as one might expect. Specifically, the pathology of IPF-afflicted lungs does not demonstrate substantial inflammatory infiltrates, and IPF does not generally respond well to immunosuppressants [42]. Moreover, recent studies investigating IPF pathogenesis have actually proposed that the *disease is a consequence of fibroblast dysfunction rather than of uncontrolled inflammation* [42]. In studies of the biological samples collected from IPF patients during exacerbations of disease, little evidence supporting the presence/significance of inflammation were found. The presence of non-specific intestinal pneumonia can be seen in surgical lung biopsy samples of IPF patients, which suggests the significance of inflammation in IPF [42]. In contrast, some of the studies of lung biopsies from patients identified pro-inflammatory mediators and cytokines, while an influx of inflammatory cells was observed in bronchoalveolar fluids (BAL) during exacerbations [36]. The role of inflammation in human pulmonary fibrosis is controversial, and despite of this ambiguity, the role of inflammation has been addressed as a significant factor [42]. The presence of an extensive human data supports the inflammation but fails to respond to the immunosuppressant therapy making it critical to identify the role of inflammation in fibrosis. From the pathology of biological samples of IPF patients, it was evident that inflammation is indeed a critical factor in IPF.

Bringardner et al. have asserted that inflammation is a critical factor in IPF and proposed five probable mechanisms by which inflammation contributes to IPF pathogenesis: (1) direct inflammatory hypothesis, (2) matrix hypothesis, (3) growth factor receptor hypothesis, (4) plasticity hypothesis, and (5) the vascular hypothesis as described in detail below [42].

1. *Direct inflammation hypothesis.* The direct inflammation hypothesis proposes that inflammatory cells directly damage the lung in IPF through the release of elastases. In addition, cytokines and growth factors exaggerate the process. Evidence for this hypothesis comes from the presence of pro-inflammatory cytokines. Along with elevated cytokines involved in the activation of monocytes and neutrophils (e.g., macrophage inflammatory protein 1α and interleukin-8) in IPF patient lung tissue [42] as compared to healthy volunteers [43, 44], elevated levels of macrophage colony-stimulating factor (M-CSF) have been identified in IPF patient BAL fluid [45, 46], with M-CSF directed mononuclear phagocyte recruitment reported in a bleomycin model of murine lung fibrosis [42]. As cell recruitment is one of the way through which inflammation takes place, these observations suggest involvement of inflammatory pathway directly in the disease progression. Inflammatory cells are involved to produce chemokines and cytokines to direct repair and act as significant producers and activators of profibrotic proteins like tissue growth factor β (TGF-β) and connective tissue growth factor, the combination of which amplifies and bolsters lung fibrosis, through inducing expression and release of collagen from tissue fibroblasts. Earlier, the lymphocytes, eosinophils, and neutrophils were found to be decisive contributors to the pathology observed in lungs of IPF patients [9].
2. *Matrix hypothesis.* According to matrix hypothesis, inflammatory mediators are released as a result of a remote injury and are trapped in the pulmonary extracellular matrix. This results in a dysregulated wound repair leading to fibroblast activation and subsequent production of collagens in the pathogenesis of IPF. Evidence for this hypothesis comes from the matrix deposition and fibroblast foci in IPF patients resulting in cell recruitment and activation [9].
3. *Growth factor hypothesis***.** In the growth factor – receptor hypothesis of IPF – some cell types with growth factor receptors multiply unhampered, sequentially leading to activation and amplification of the inflammatory cascade [42]. These growth factor receptors are upregulated in the presence of glucocorticoids. Growth factors such as M-CSF and insulin-like growth factor are elevated in the presence of dexamethasone, hence leading to cell proliferation which is unaffected by the use of immunosuppressants, which in turn explains the failure of immunosuppressant therapy in IPF treatment. Since steroids upregulate M-CSF receptors on cells similar to macrophages, this in turn results in an increased cell population sensitization and elevated cytokine secretion, subsequently activating pro-inflammatory cascades [9].
4. *Plasticity hypothesis.* The plasticity hypothesis of IPF asserts that numerous cell types have the capability to differentiate into other cell types. Phenotypic transformation of monocytes into macrophage and epithelial cells into mesenchymal

cells are two examples for the differentiation underlying the plasticity hypothesis of IPF [9].

5. *Vascular hypothesis.* The vascular hypothesis of IPF patients supposes that increased d-dimers and possible microvascular injury underlie the pathophysiology of IPF. Maintaining vascular integrity and patency is essential and is achieved through hemostasis. Pathological vessel occlusion or bleeding occurs due to an imbalance between thrombus formation and fibrinolysis. Protease-activated receptors play critical roles in mediating thrombin-induced fibroblast proliferation and extracellular matrix deposition. The profibrotic potential of coagulation factors in experimental models of pulmonary fibrosis is well documented as is antibody deposition in the lung vascular endothelium with endothelial cell necrosis [47, 48]. The deposited immunoglobulin G (IgG) antibody drives the continuity of human monocytes through M-CSF production. Taken together, these findings suggest the initiation of inflammatory cascade mediated via antibodies once the lung vascular endothelium is injured. Subsequently, autoantibodies activate fibroblasts and drive cellular growth and differentiation through M-CSF-dependent pathways. There is plausible epidemiological evidence of an interrelation between thrombotic vascular events and IPF that is supported by biological demonstration of a local and systemic prothrombotic state, corresponding with disease severity and clinical outcomes [49]. The manipulation of coagulation system plays a role in IPF pathogenesis. This explains the significance of another strategy of novel anticoagulant therapy which has potential antifibrotic properties in addition to their anticoagulant effects [9]. The involvement of coagulation factors in the pathogenesis of fibrosis is summarized in Fig. 8.5 [37].

Rho kinase (ROCK) signaling pathways are involved in profibrotic responses of epithelial and endothelial cells to tissue injury. The profibrotic responses of multiple cells types suggest that the Rho kinases are focal points in pulmonary fibrosis, through which many upstream signals induce profibrotic downstream responses [50]. This has led to the idea that ROCK inhibition may be a particularly potent therapeutic target for pulmonary fibrosis [50]. The combined effect of fibroblast hyperplasia and reduced expression of apoptotic mechanisms in IPF can further escalate the fibrotic response. Together, these signaling pathways illustrate a cascade of failed regulatory mechanisms leading to the excess secretion of cytokines, chemokines, and growth factors, which terminates in a wound healing response which is continues beyond what is needed (fibrosis) [11]. Impaired efferocytosis, the process by which dead and dying cells (apoptotic and/or necrotic) are removed by phagocytic cells, contributes to the pathogenesis of chronic lung diseases, including emphysema and cystic fibrosis. It has been hypothesized that efferocytosis reduced in alveolar macrophages isolated from subjects with IPF [51]. Willis et al. have suggested that alveolar epithelial cells (AECs) undergo epithelial to mesenchymal transition (EMT) when chronically exposed to TGF-β1, raising the possibility that epithelial cells may serve as a novel source of myofibroblasts in IPF [52].

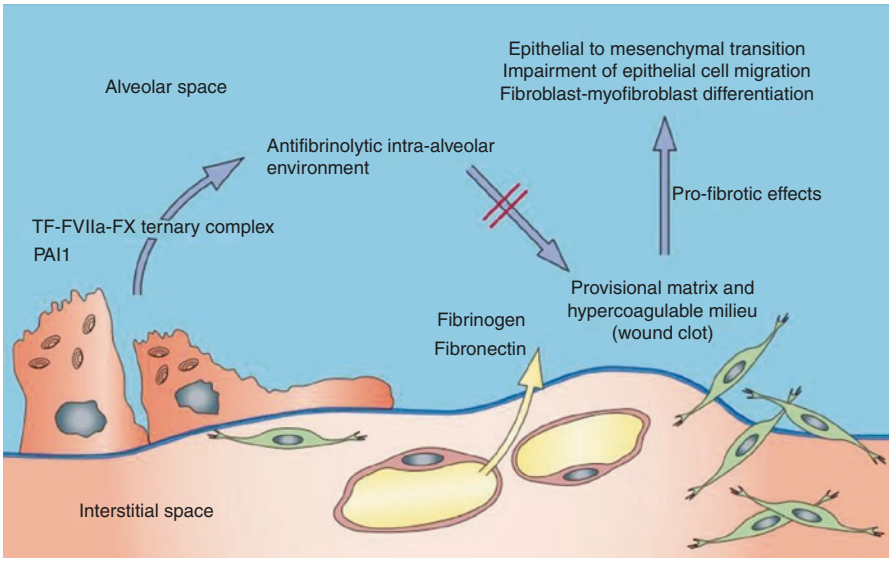


**Fig. 8.5** Vascular hypothesis of fibrotic development and role of coagulation factors in IPF pathogenesis. (**a**) Fibrinogen and fibronectin are released after lung injury with wound clot formation. (**b**) Formation of TF-FVIIa-FX ternary complex formation and PAI 1 secretion by abnormally activated epithelial cells causing cleavage of fibrinogen to fibrin in turn and creation of an antifibrinolytic microenvironment. (**c**) Wound clot persistence and active proteases trigger EMT. TF tissue factor, FVIIa factor VIIa, FX factor X, EMT epithelial mesenchymal transition. (Adapted from: King et al. [37])

## Etiological Factors

### *Environmental Factors*

Both basic science and animal studies provide evidence for the pathogenesis of IPF due to inhalation of lung disease-causing agents. Long-term exposure to toxins and pollutants (silica dust, asbestos fibers, hard metal dusts, vegetable and animal dust, coal dust, grain dust, bird and animal droppings) can significantly contribute to causing lung disease [53]. This may not be too surprising, given that asbestosis and silicosis are known to cause fibrotic lung disease, and may explain the higher possibility of IPF progression with direct exposure to those agents [54]. Exposure to occupational toxins and pollutants has been associated with IPF. Occupational factors associated with IPF have been found in farming, livestock hairdressing, exposure to metal dust, raising birds, stone cutting/polishing, and exposure to vegetable dust/animal dust [54]. Additionally, an interaction between smoking and exposure to livestock and farming with IPF has been identified as has the increase in risk in presence of dusty environment [54].

Among these risk factors associated with IPF, a history of cigarette smoking seems to be the most strongly associated risk factor in IPF. Cigarette smoking is one of the most discerned risk factors for IPF progression and has deleterious response

on survival of patients with IPF [55]. In a recent meta-analysis of observational studies examining environmental and occupational risk factors for IPF, significantly increased risk for IPF was associated with cigarette smoking and exposure to cigarette smoking [56]. Several environmental exposures are also associated with an increased risk of developing IPF. Occupational factors, primarily metal and wood dust exposure, adjusted for age and smoking, have been found to be significantly associated with IPF pathogenesis and progression. Particulate matter present in the cigarette smoke is deliberated to exacerbate lung pathology through altering both the oxidant/antioxidant balance and protease/antiprotease balance in the lung [55]. Particulate matter also promotes cellular apoptosis and necrosis and generating DNA and lipid intermediates, thus exacerbating inflammation [55]. As the IPF is recognized as a disease of aging, it was hypothesized that smoking might be a factor to the development of lung diseases, in an age-dependent manner. Despite the identification of those mechanisms involved, exact pathways causing IPF due to smoking are unclear till today. Hence, numerous insights and research efforts are needed to identify the possible and potential mechanisms to develop novel therapeutic approaches to treat smoking-induced IPF [57]. The majority of IPF patients have a history of cigarette smoking. From case-control studies, smoking increases the risk of IPF, with odds ratio (OR) from 1.11 to 3.23, suggesting a definitive role for smoking in initiation and progression of lung fibrosis [5].

## *Co-occurring Disorders*

Comorbidities such as gastroesophageal reflux (GERD), cardiovascular diseases, and others will complicate IPF and significantly impact clinical course and prognosis of the disease [58].

### Gastroesophageal Reflux Disease

Gastroesophageal reflux (GER) causes chronic microaspiration of gastric contents, causing repetitive lung injury and resulting pulmonary fibrosis in susceptible individuals. The idea that GERD and IPF may be linked was reported by Gnanapandithan et al. who described a patient with achalasia and GER who went on to develop IPF. After developing GER, her clinical course rapidly worsened, punctuated by acute exacerbations of IPF despite best efforts to manage the GERD by inhibiting acid production [59].

Since patients with IPF have a high prevalence of gastroesophageal reflux, it has been hypothesized that GERD may be associated with the pathogenesis and progression of IPF. Some have attributed GERD as a cofactor in the development and progression of the IPF [42]. While GERD is very common in the general population, it is unclear why only a very small proportion develops pulmonary fibrosis. One argument against the causal relationship between GERD and IPF is that the increased gastric content reflux may be secondary to the mechanical effects of IPF

including poor lung compliance, distortion of mediastinal anatomy, and weakening of the lower esophageal sphincter [60]. In IPF patients, the sphincter in the lower esophagus will not work properly and let the contents of stomach enter upper esophagus and also to enter lungs. This microaspiration is the main driving force aggravating IPF through lung fibrosis and ultimately declining the lung function. While the causal relationship between GERD and IPF has not been established, there are clear mechanisms by which GERD may be a contributing factor and should be considered in treating IPF patients with GERD.

#### Pulmonary Hypertension

Pulmonary hypertension (PH) is a well-recognized complication of IPF, with underlying pathogenesis characterized as overexuberant fibro-proliferative process [61]. While a causal link between PH and IPF has not been established, there are overlapping mediators between the pathogenesis of the two diseases. These mediators include endothelin 1 (ET-1), transforming growth factor (TGF)-β, prostaglandin (PG) E2, bone morphogenetic protein receptor type 2 (BMPR2), adenosine signaling, hyaluronan, and IL-6, which have established roles in progression of fibrosis and the development of PH and IPF [62].

#### Cardiovascular Disease

Cardiovascular diseases such as ischemic heart disease and cerebrovascular disease constitute an important group of comorbid conditions affecting those with IPF [63]. Studies by Hubbard et al. have identified that IPF patients have a marked relative increase in the risk of vascular disease, which should be considered during the routine care of these patients [64]. The causal links between the two, however, are not known.

#### Osteoporosis

Caffarelli et al. have assessed the prevalence of osteoporosis and fragility fracture in a population of adults with IPF. They identified that IPF patients had a high prevalence of fragility with vertebral fractures [65]. The causal links between the tow, however, are not known.

### *Genetic Factors*

Multiple in vitro experiments by several research groups have reported mechanisms involved in the induction of ER stress and the impact of ER stress in developing lung fibrosis. One study of both exon 4 and L188Q SFTPC mutations identified that ER stress is accompanied by increased alveolar epithelial cell (AEC) death [66].

Mutant pro-SP-C (surfactant protein C) was identified as one of the causes of ER stress development through surfactant protein C (SFTPC) mutation [66]. Hence, aberrant protein processing has become another possible target in the pathogenesis of lung fibrosis that is being further investigated. The striving of AECs to heal the injury may underlie the activation of the unfolded protein response (UPR) and ER stress in the development of lung fibrosis [14].

Linkage analysis and candidate gene approaches have identified four genes that cause inherited forms of IPF. Mutations in SFTPC, SFTPA2, TERT, and TERC genes that encode the surfactant protein C, surfactant protein A2, the telomerase reverse transcriptase, and the RNA component of telomerase have been shown to be linked to disease, respectively. Mutations in SFTPC and SFTPA2 cause increased endoplasmic reticulum stress in type II alveolar epithelial cells, which mutations in TERT and TERC cause IPF by shortening telomere lengths resulting in an accelerated aging and death of lung stem cells. The penetrance of pulmonary fibrosis in TERT mutation carriers is 40% in subjects (mean age, 51), with penetrance increasing with advanced age and in males, with positive associations with fibrogenic environmental exposures [67]. These findings highlight the presence of two unrelated biologic processes related to ER stress and telomerase function in the pathogenesis of IPF related to the increases in age-related prevalence and highlight the importance of epithelial cell injury and dysfunction in the development of lung fibrosis [68].

The endoplasmic reticulum (ER) is mainly responsible for folding of secretory proteins. The secretory proteins are delivered to the ER and undergo proper folding. But in the presence of ER stress due to various factors like metabolic stress, calcium depletion, mutations in the proteins, or diminished energy stores, the unfolded protein response (UPR) comes into action. It improves protein folding and prevents cell death due to accumulation of misfolded proteins. Several studies have been conducted to ascertain the involvement of ER stress and UPR pathways in the progression of IPF. In most of the cases, the IPF is characterized by the prominent alveolar epithelial cell apoptosis (as summarized in Fig. 8.6) [68]. In addition to endoplasmic reticulum stress and the unfolded protein response, DNA damage and repair pathways and cellular senescence were identified by many genetic approaches which might provide new therapeutic targets in fibrotic lung diseases. Kropski et al. have showed induction of ER stress by expression of mutant human L188Q SFTPC in type II AECs or by tunicamycin administration, which further led to fibrotic remodeling in response to low-dose bleomycin that was more severe than in mice without ER stress. L188Q SFTPC mice showed an increased number of TUNEL-positive type II AECs and increased expression of caspase-3 following bleomycin exposure, suggesting that ER-stress-mediated induction of AEC apoptosis associated to the development of fibrosis [55].

Idiopathic pulmonary fibrosis is a recognized feature of a multisystem inherited disease called dyskeratosis congenita (DC), which caused by mutations in components of the telomere maintenance pathway. It is estimated that 10–15% of IPF cases are caused by mutations in the telomerase pathway. The presence of short telomeres in sporadic IPF patients in the absence of telomere mutations suggests telomere dysfunction as a main feature of the disease process. Some studies have concluded that genes associated with the DC disease are also involved in development of lung

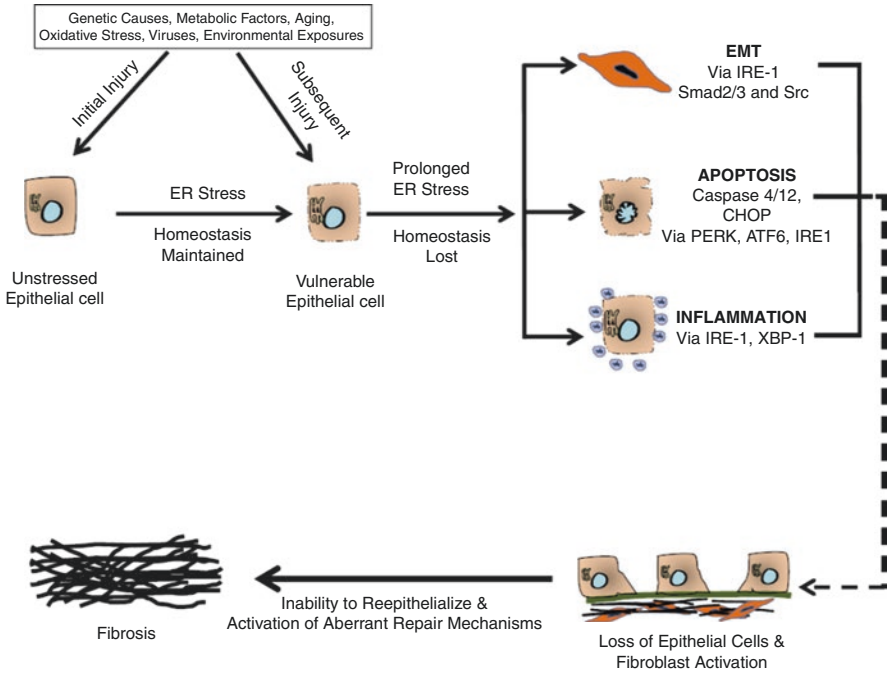


**Fig. 8.6** Mechanisms by which ER stress is contributing to IPF development. (**a**) Genetic causes and exposure to injurious agents and other factors induce the ER stress. (**b**) Activation of EMT, apoptosis, or inflammatory pathways. (**c**) Activation of fibroblasts. (**d**) Activation of aberrant repair mechanisms in turn causing fibrosis. (Adapted from: Tanjore et al. [191])

fibrosis, including TERT and TERC mutations [55]. Furthermore, these telomerase mutations suggest aberrant AEC changes, which is the important manifestation in lung fibrosis pathogenesis. When telomeres shorten to reach a critical threshold, they can be sensed as double-stranded DNA breaks, triggering a DNA-damage response. Activation of DNA-damage pathways leads to apoptosis or cell senescence. Transforming growth factor-β (TGF-β) signaling and activation of the Wnt-β-catenin pathway promote EMT as well as myofibroblast differentiation in vitro [69]. These are several other mechanisms revealed to be involved in the IPF through different pathways. In 2011, a large genome-wide linkage study of IPF patients identified a common polymorphism in the promoter of the gene encoding a mucin (MUC5B) that was associated with many fold increase of the risk in IPF patients [69]. Further advances in genomic science are essential to investigate the additional genes responsible for IPF to highlight new pathways [68].

Active transforming growth factor-β (TGF-β) levels are increased in the lungs of patients with IPF. All three isoforms (TGF-β1, TGF-β2, and TGF-β3) of inactive TGF-β are synthesized and secreted bound to the latency-associated peptide (LAP). Normally, TGF-β is inactive and bound to LAP. During the progression of lung fibrosis, AECs express higher levels of the integrin αvβ6, which can bind to the arginine-glycine-aspartate (RGD) sequence of LAP [70]. Binding of αvβ6 is

restricted to the TGF-β1 and TGF-β3 isoforms [70]. Activation of epithelial cells expressing αvβ6 through binding of mediators such as thrombin to their receptors causes actin/myosin-mediated contraction of the epithelial cell [70]. This contraction pulls on the latent TGF-β tethered to the epithelial cell via binding of LAP to the integrin αvβ6. This retraction induces TGF-β activation and signaling in localized, spatially restricted lung regions [70].

## *Infectious Agents*

Like the previously discussed environmental risk factors for triggering lung fibrosis (e.g., dust, pollution, asbestos, and gastric aspirate), the susceptibility of the patient to infection and the failure of host defense also contributes to IPF disease progression. Both bacterial and viral infections may influence IPF outcomes either as exacerbating agents or initiators of disease [71]. Many IPF patients are hospitalized for pulmonary infections [72]. The key to understanding the pathogenesis of chronic lung disease may reside in deciphering the complex interactions between the host, pathogen, and resident microbiota during stable disease and exacerbations.

Viruses have been identified (both protein and DNA) from IPF patient lung tissue, including the Epstein-Barr virus (EBV) and human herpesvirus (HHV) which locally infect the AECs. Identification of the viral infection is often difficult as the immunosuppressive therapy given to treat patients often obscures the diagnosis. This situation leads to mixed results supporting viral infections as an etiological factor for IPF [41]. Parainfluenza 1 (Sendai) and influenza A virus pneumonitis have been reported to cause alveolitis and parenchymal changes causing deposition of collagen and hence damaging the lungs [73].

The co-localization of the virus with markers of ER stress and apoptosis suggest mechanisms by which viruses may be linked to the development of IPF. Activation of ER stress and apoptosis has been identified in the development of both IPF and the pulmonary fibrosis-associated rare genetic disorder Hermansky-Pudlak syndrome [74]. In a murine bleomycin model, chronic murine gammaherpesvirus 68 (MHV-68) infection resulted in the deposition of collagen, increased tumor growth factor (TGF)-β expression, and the altered synthesis of surfactant proteins. Similarly, MHV-68-induced pulmonary fibrosis in aged mice is associated with upregulation of the potent profibrotic growth factor, TGF-β. These findings open a new path to develop an antiviral therapy that may be effective in IPF. Stabilization of IPF by administering antiviral therapy has been described in some case reports [59]. Another study investigating IPF lung biopsies has identified Herpesvirus saimiri DNA [74]. In 2003, Folcik et al. demonstrated that the idiopathic pulmonary fibrosis is associated with strong co-expression of four herpes virus saimiri-associated proteins [74]. Together, these several lines of evidence suggest that viral infections may contribute to the development and/or progression of IPF by multiple mechanisms, including the activation of ER stress and increased TGF-β expression.

Lung infections with the bacteria *Staphylococcus aureus* and *Klebsiella pneumoniae* are known to cause IPF after sequential viral infection [75]. The presence of bacterial pathogens such as *Haemophilus*, *Streptococcus*, *Moraxella*, and *Pseudomonas* species has been identified in bronchoalveolar lavage (BAL) cultures for 8 of 22 stable IPF patients [76]. Another recent study of the lung microbiome to establish the pathogenesis of IPF identified that the presence of *Staphylococcus* and *Streptococcus* genera was significantly associated with IPF severity. Further investigation is necessary to clarify the role of bacteria in IPF. Infections have long been suspected to play a role in IPF and could potentially be targets for future therapies [5]. These studies are a timely reminder of the complex relationship between microorganisms and the respiratory tract, which remains to be fully elucidated [77].

## *Other Drugs or Medications*

Idiopathic pulmonary fibrosis was hypothesized to be complicated by the presence of other drugs. As such, multiple studies have been carried out to assess an increased risk of IPF secondary to the presence of medications. Antidepressant drugs, specifically tricyclic antidepressants, were found to exhibit some association with IPF [78]. Other evidence supporting a link between medications and IPF was provided through radiolabeling studies, whereby clomipramine accumulation in the lung was demonstrated to be significant [78]. Other studies have reported that nonsteroidal anti-inflammatory agents exhibit marginal significance to increase the risk of IPF while asserting the laxatives, antihistamines, barbiturates, and antianginal drugs as nonfactors to increase the risk of IPF [79]. To develop therapies for IPF, a more precise understanding is needed of the relationships between the specific molecular pathways that are activated during each stage of IPF. This understanding will inform which stage of the disease therapies target as well as whether targeting one stage of the disease is sufficient to slow progression or whether simultaneous targeting of molecular pathways in each stage of the disease is required for effective therapy [70].

## *Age*

Aging is a driving force of IPF, which we eluded to in our previous section discussing the link between telomerase activity and IPF progression. IPF generally occurs more often with age in genetically susceptible individuals who may be exposed to environmental risks factors. Idiopathic pulmonary fibrosis occurs mainly in middle-aged people and increases remarkably with aging. While the mechanisms involved in disease progression through aging are uncertain, aging is identified as a strong

risk factor [80]. One well-established underlying factor with aging may be the attenuation of the immune system, which allows damage through infections. In addition, the increase in genetic changes that occur with age may contribute to the development of IPF. These changes may lead to the failure of apoptotic mechanisms and result in the accumulation of dysfunctional organelles and molecules in the cells and generation of excess fibrous tissue [81].

## Therapeutic Targets

### *Alveolar Epithelial Cell Injury*

Idiopathic pulmonary fibrosis may be generalized as an abnormal wound healing inflammatory disease since it involves injury to the alveolar epithelial cells (AECs), which then triggers a cascade of dysregulated epithelial-fibroblast generation [82]. This then may lead to multiple cycles of epithelial cell injury, which then triggers the release of growth factors (e.g., TGF-β1), cytokines, and matrix metalloproteinase (MMPs) leading to mesenchymal cell activation/proliferation, formation of fibroblastic foci, transformation of fibroblasts to myofibroblasts, and extracellular matrix deposition, terminating in parenchymal destruction [83]. After the injury for the repair of epithelial endothelium, type 2 AECs differentiate into type 1 AECs. It has been hypothesized that this process is altered in IPF, due to loss of epithelial architecture, extracellular matrix changes, and the mesenchymal transition process [84]. Given the importance of AECs in this process, attention has been given to identifying specific AEC genes that may prove to be therapeutic targets.

#### Genetic Basis of Alveolar Epithelial Cell Injury

Multiple genes have been identified in AECs, which may be related to the fibrotic process and represent therapeutic targets in future studies.

MUC5B

The mucin 5B (MUC5B) is a gel-forming mucin produced in bronchial epithelial cells providing mucosal host defense. Overexpressing MUC5B deregulates mucosal host repair process and causes a decrease in lung clearance and hence excessive injury in prolonged run, thus resulting into fibrosis. It is thought that the MUC5B gene interferes with the type II alveolar cells, diminishing the reepithelization process and enhancing the alveolar collapse along with fibrosis of adjacent bronchoalveolar units [85].

ELMOD2

In a genome-wide study performed on six Finnish families suffering from familial idiopathic pulmonary fibrosis (IPF), five loci of interest were identified [86]. These included the engulfment and motility (ELMO) domain containing 2 (ELMOD2) and LOC152586 genes, identified as functionally uncharacterized genes [86]. The lungs from IPF patients had significantly decreased ELMOD2 mRNA expression compared to healthy control lungs, leading to the idea that it was a novel gene candidate for IPF therapy [86]. Another study demonstrated that EMLOD2 regulates the interferon-related antiviral responses, wherein a decrease in the response of interferon was observed with respect to viral infection [87].

HLA

Polymorphisms in human leukocyte antigen (HLA) have been associated with IPF, in addition to degenerative, autoimmune, and communicable diseases [88]. Both class I and class II HLA proteins are segmented into HLA-A, HLA-B, and HLA-C antigens (class I) and HLA class II subtypes HLA-DR, DQ, and DP. While HLA class I is expressed on the surface of most mammalian cells, HLA class II is associated with regulation of T-cell -dependent immune responses and found to be expressed by specific cell types such as dendritic cells, macrophages, and fibroblasts [89]. Studies investigating the role of HLA class I and class II genes in patients with IPF identified an increased HLA class II alleleDRB1*1501 in the IPF patients, suggesting evidence that immunogenic processes are involved in IPF progression [90]. Despite several studies, it has not been possible to establish a relationship between the HLA class II allele and IPF [91].

## *WNT/β-Catenin Pathway*

The wingless-related integration site (WNT)/β-catenin pathway has a key role in tissue development, regulation, homeostasis, regeneration, and wound repair [92]. In the canonical Wnt signaling pathway found in normal lung tissues, the β-catenin protein is phosphorylated due to its interface with axin, adenomatous polyposis coli (APC), and glycogen synthase kinase (Gsk)-3β, ultimately leading to degradation of β-catenin (Fig. 8.7). The WNT/β-catenin pathway in the IPF tissues represents the activation of membrane receptors such as the frizzled (Fzd) or the low-density lipoprotein receptor-related proteins (Lrp) 5 and 6. This involves the phosphorylation of Lrp6 by Gsk-3β and casein kinase γ in the cytoplasm causing the inactivation of Gsk-3β [93]. Thus, it prevents the phosphorylation of β-catenin and accumulation of hypo-phosphorylated β-catenin in the cytoplasm. The β-catenin gets subsequently translocated in the nucleus triggering the target gene expression by interacting with members of the T-cell-specific transcription factor/lymphoid enhancer-binding factor (Tcf/Lef) family [94]. The involvement of Wnt pathway was demonstrated

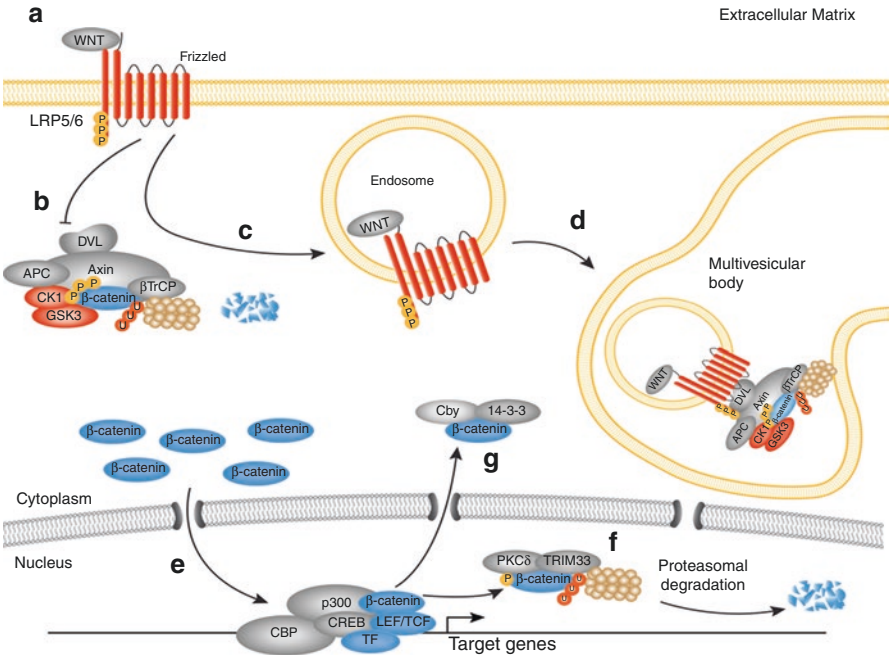


**Fig. 8.7** Wnt signaling pathway in IPF. (**a**) Wnt ligand binds with frizzled receptor and in association with lipoprotein receptor-related protein (LRP) forms a complex. (**b**) The destruction complex consisting of disheveled (DVL) Axi, adenomatous polyposis coli (APC), glycogen synthase kinase $_3$ (GSK3), and casein kinase 1(CK1) is inhibited to perform ubiquitination resulting in the accumulation of cytoplasmic β-catenin. (**c**) The clusters of complexes are taken up by endosomes leading to sequestering of destruction complex followed by inhibition of GSK3. (**d**) The GSK3 is transported to multivesicular bodies where it protects β-catenin from proteasomal degradation. (**e**) The newly produced β-catenin enters the nucleus from the cytoplasm and interacts with transcription factors such as LEf/TCB and co-factors like p300 and CBP. (**f**) The phosphorylation of β-catenin via protein kinase C (PKC) and tripartite motif (TRIM)33 may lead to termination of the pathway. (**g**) The sequestering of 14-3-3 and Chiibby (Cby) in the cytoplasm may also lead to inhibition of β-catenin activation. (Adapted from: Piersma et al. [93])

wherein the abnormal expression of β-catenin along with other target genes such as cyclin-D1 and metalloproteinase matrilysin (MMP-7) has been recorded to provide support to the development [95]. Also, Zuo et al. established the Wnt target genes such as matrilysin (MMP-7) and fibronectin contributing to the development of pulmonary fibrosis (Fig. 8.7) [96].

## Genetic Basis

Matrix metalloproteinases (MMPs) are endopeptidases which can degrade extracellular matrix proteins and broadly speaking are antagonized by tissue inhibitors of MMPs (TIMPs). The balance between MMPs and TIMPs are thought to regulate the balance of ECM degradation and synthesis, a process essential to avoid

fibrogenesis. Matrix metalloproteinase-1 and MMP-7 are significantly upregulated in IPF, which leads to an excessive breakdown of the extracellular matrix protein, and their deposition in the lungs results into thickening of the alveolar tissues leading to difficulty in the oxygen exchange at those sites [97].

### *Epithelial-Mesenchymal Transition Pathway*

The epithelial-mesenchymal transition (EMT) pathway describes the transformation of epithelial cells into cells with mesenchymal characteristics, including their change in shape, increased motility, and expression of mesenchymal markers such as N-cadherin (CDH2), vimentin (VIM), and α-smooth muscle actin (ACTA2) [98]. This transition results in the development of the fibroblast and myofibroblast. The proliferative changes in the fibroblast play important roles in the pathogenesis of IPF. In healthy fibroblasts, the β1 integrin binds to polymerized type 1 collagen, cascading the activation of tumor suppressor phosphatase and tensin homolog (PTEN), thus leading to suppression of PI3K-Akt-S6K1 pathway and preventing the fibroblast proliferation. In contrast, IPF fibroblasts display a pathological pattern of β1 integrin expression resulting into low PTEN activity and uncontrolled activation of PI3K-Akt-S6K1 pathway, resulting in excessive fibroblast proliferation and progressive accumulation in the lungs [39]. This observation was supported by studies of mice expressing β-galactosidase in the lung epithelial cells, which provided evidence of alveolar epithelial cells as progenitors of fibroblasts and the extracellular matrix playing a vital role in the epithelial trans-differentiation during fibrogenesis (Fig. 8.8) [99].

Transforming growth factor-β (TGF-β) is released immediately after cell injury as a pro-inflammatory response which serves as a chemotaxis signal for neutrophils and fibroblasts. The role of TGF-β switches during the injury repair and the resolution phase, where it regulates matrix protein synthesis, inhibits type II alveolar epithelial cell (AEC) proliferation, and promotes the proliferation of fibroblasts to myofibroblasts by also supporting the EMT pathway [97]. The increased TGF-β expression in IPF lungs suggests it may be an important therapeutic target for IPF [82]. For example, transforming growth factor (TGF)-β3, lymphoid enhancer factor-1 (LEF-1), and Slug, a TGF-β target gene, are upregulated in IPF, and hence they could be studied as potential targets for the treatment of IPF [41].

## Therapies for Idiopathic Pulmonary Fibrosis

Some patients will have IPF in a stable condition for longer time periods, while others may experience a rapid progression, and another group may experience diminished symptoms and disease with fluctuations between periods of stability and worsening symptoms. Hence, the treatment strategies for pulmonary fibrosis are

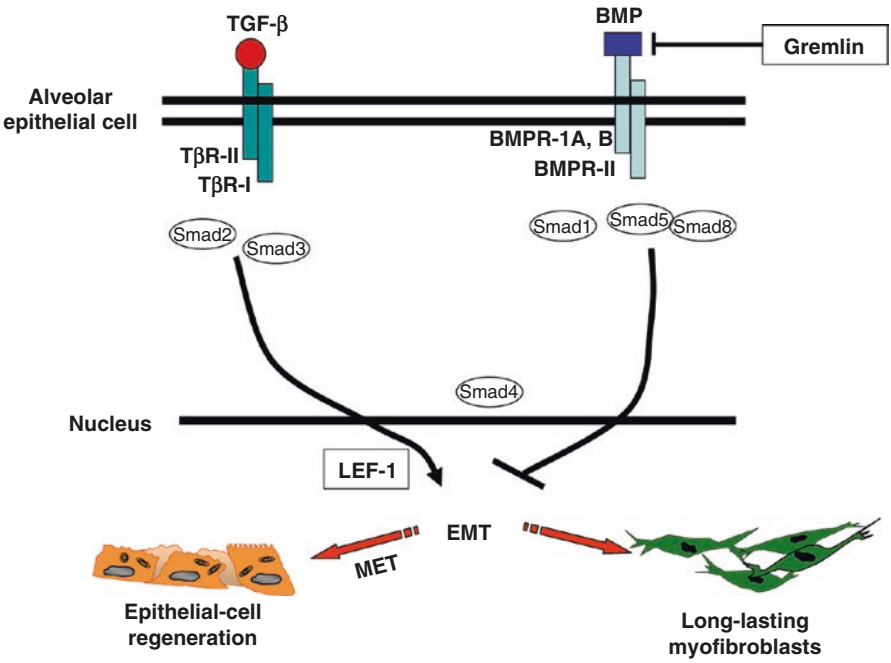


**Fig. 8.8** The EMT pathway in IPF. The IPF leads to upregulation of TGF-β3, gremlin, and LEF-1 while downregulation of BMP-2. This TGF-β upregulation is mediated by Smad2 and Smad3, and the BMP is mediated via Smad1, Smad 5, and Smad8. The increase in BMPs within the nucleus may oppose the TGF-β-induced EMT along with initiation of mesenchymal to epithelial transition (MET) process. However, dysregulated TGF-β/BMP pathway may cause fibrotic phenotype. (Adapted from: Selman et al. [41])

highly individualized, based upon medical history and other important conditions [100]. Interpretation of recent international and national European guideline updates and treatment recommendations, available clinical data from published and ongoing trials investigating potential pharmacological agents, and individual patient's preferences must be considered in the clinical management of IPF [101].

## *Traditional Therapy*

Idiopathic pulmonary fibrosis is associated with the fibro-proliferation predominantly more than inflammation, which may explain the poor responses to therapies targeting inflammation [102]. These poor responses illustrate our limited understanding of IPF pathophysiology. The strategies toward treatment of IPF have been widely studied and are significantly been modified over decades, as illustrated in Table 8.1 [103].

**Table 8.1** Recommended guidelines for IPF treatment

| Type | Mechanism | Agent |
|---|---|---|
| Strong recommendation against use | Anticoagulant | Warfarin[a] |
| | Combination | Prednisone + azathioprine + N-acetylcysteine[b] |
| | Selective endothelin receptor antagonist | Ambrisentan[b] |
| | Tyrosine kinase inhibitor with one target | Imatinib[b] |
| Conditional recommendation for use | Tyrosine kinase inhibitor with multiple targets | Nintedanib[a] |
| | Antifibrotic | Pirfenidone[a] |
| | Antacid therapy | Proton pump inhibitors and H2-receptor blockers[c] |
| Conditional recommendation against use | Dual endothelin receptor antagonists | Macitentan, bosentan[b] |
| | Mucolytic (antioxidant) | *N*-acetylcysteine monotherapy[b] |
| | Anti-pulmonary hypertension therapy for idiopathic pulmonary fibrosis-associated pulmonary hypertension | Reassessment of the previous recommendation was deferred |
| | Phosphodiesterase-5 inhibitor | Sildenafil[a] |
| Formulation of a recommendation was deferred | Lung transplantation | Single vs. bilateral lung transplantation |

From Raghu et al. [103]
[a]Moderate confidence in effect estimates
[b]Low confidence in effect estimates
[c]Very low confidence in effect estimates

## Corticosteroids and Immunomodulators

Treatment for IPF initially focused on symptomatic relief using corticosteroids with or without immunosuppressive drugs [104]. This strategy was consistent with the hypothesis that managing inflammation may reduce the progression of the disease [105]. The use of corticosteroids like prednisone alone was used in high dose for producing an effect. However, the effect observed was not significantly different from non-treated control subjects. Moreover, the severe and nonreversible side effects associated with the corticosteroids have made their use in humans contentious [106, 107]. Despite this, it had been thought that "no patient with IPF should be denied a trial of corticosteroids" since there were limited treatment options apart from the lung transplantation [108]. As advances in IPF treatments have progressed, the usage of the corticosteroids along with immunosuppressants like azathioprine and cyclophosphamide followed. Comparative studies of prednisone alone and in combination with either cyclophosphamide or azathioprine have not demonstrated any significant increases in patient survival compared to prednisone alone [109]. The suppression of bone marrow function associated with such immunosuppressants may increase the risk of associated infections [110].

### Anticoagulants

The mechanism of wound repair in IPF involves activation of the coagulation cascade along with anti-fibrinolytic activity which activates the clotting/coagulation pathway. Thrombin plays a role in lung fibrosis as proliferating fibroblasts deposit collagen due to decreased protein C concentration [111, 112]. This is consistent with what is seen in bleomycin-induced lung fibrosis in mice, where increased deaths were found secondary to low protein C levels [113]. Anticoagulants such as heparin and urokinase type plasminogen activator decrease the bleomycin-induced fibrosis in rabbits [114]. This leads to clinical trials to understand the effect of anticoagulants in 56 IPF patients using warfarin [115]. While this study reported an observed reduction in mortality [115], studies that followed did not consistently replicate these findings. For example, when warfarin was administered as a treatment therapy to 97 IPF patients, their condition worsened clinically compared to control subjects [116]. Thus, the use of anticoagulants in the treatment of IPF is not clearly a viable treatment option.

### Antifibrotic Agents

The advancement in our understanding of IPF pathophysiology has led to the development of new treatment options which have relatively fewer observed side effects compared to previous options. Antifibrotic therapies that inhibit fibroblast proliferation and induce fibroblast apoptosis have shown great potential in treating IPF. Colchicine was the earliest compound tested for its antifibrotic effect and impact upon IPF [110, 117]. Colchicine significantly inhibits fibrosis by blocking fibroblast collagen synthesis [111]. To establish the effects of colchicine compared to prednisone in humans, a study was performed in 26 patients which demonstrated that colchicine treatment resulted in a better disease outcome compared to prednisone, with minimal treatment associated side effects [118, 119]. This helped to establish the efficacy of antifibrotic agents in the treatment of IPF.

Another antifibrotic treatment that has been tested is D-penicillamine. D-penicillamine is a collagen antagonist which inhibits both collagen synthesis while suppressing fibroblast proliferation. When D-penicillamine was tested for its efficacy in treating IPF in bleomycin-induced fibrosis rats, it was found to significantly reduce collagen accumulation and suppress the fibrosis [120]. These findings were supported by another study of D-penicillamine where radiation-induced hydroxyproline accumulation in the lungs of rats was condensed [121]. Subsequently, clinical trials comparing the effect of combination of D-penicillamine with prednisone (vs. prednisone alone) in 56 IPF patients found no significant differences between the groups [122].

## *Advancement in IPF Therapies: Novel Treatment Approaches*

### Antifibrotic Agents

Pirfenidone [5-methyl-1-phenyl-2-(1H)-pyridone] is an FDA-approved antifibrotic agent for the treatment of IPF. Pirfenidone inhibits IPF progression by suppressing TGF-β, decreasing collagen synthesis and extracellular matrix formation, while blocking the mitogenic effects of profibrotic cytokines [22]. Pirfenidone is hypothesized to reduce oxidative stress and lung fibroblast proliferation [123]. A preclinical study of bleomycin-induced pulmonary fibrosis in hamsters found that pirfenidone suppressed TGF-β gene transcription. Pirfenidone also inhibits the platelet-derived growth factor (PGDFR)-induced cellular hyper-proliferation [124]. When a combination therapy of pirfenidone with prednisolone was compared to pirfenidone alone in paraquat-induced pulmonary fibrosis in rats, the combination therapy was found to have more potent therapeutic effects than pirfenidone alone [125]. In an international randomized, double-blind phase III clinical study (CAPACITY), 779 patients were followed for 72 weeks and confirmed the phase II trial of pirfenidone, showing a favorable improvement in outcomes [126]. In a randomized, controlled phase III trial of pirfenidone in IPF patients, pirfenidone was well tolerated by patients, but photosensitivity, a well-known side effect of pirfenidone, was observed [127].

Interferon-γ (IFN-γ) is an endogenous cytokine involved in regulation of TGF-β, which functionally restricts fibroblast proliferation and collagen synthesis. In a study of bleomycin-induced lung fibrosis in rat, IFN-γ1b was found to downregulate TGF-β1 transcription [128]. Ziesche et al. performed a long-term study on small group of patients with less advanced pulmonary disease, not clearly defined as IPF [129]. When these patients were treated with a combination of interferon-γ-1b with prednisolone (at doses without clinical effects when given alone), they responded significantly to this combination treatment, while glucocorticoid alone cannot be effective [129]. In bleomycin-induced pulmonary fibrosis in rats, INF-γ administered intratracheally inhibited collagen accumulation by suppressing collagen synthesis and hence inhibiting pulmonary fibrosis [130].

Etanercept is a tumor necrosis factor-alpha (TNF-α) receptor antagonist widely used for the treatment of rheumatoid arthritis. TNF-α supports fibrogenesis and is also known to be found in higher amounts in IPF patient lungs [131]. The role of TNF-α in fibrosis has been studied in bleomycin-induced fibrosis in mice, where increased TNF production is associated with excessive collagen accumulation, alveolar damage, and fibroblast proliferation [132]. In a randomized, double-blind, placebo-controlled clinical study, etanercept was found to be well tolerated by the patients; however, no significant improvement was observed in the disease condition after 48 weeks of treatment [133].

Imatinib is an effective tyrosine kinase inhibitor of platelet-derived growth factor (PDGF) receptors and Kit receptor that is FDA approved for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors [134]. Imatinib is well-known for inhibitory effects against the TGF-β-induced extracellular matrix proliferation,

thus impeding the lung fibrosis. This is supported by studies in bleomycin-induced pulmonary fibrosis in mice where imatinib suppressed the TGF-β signaling so as to diminish the fibrosis [135]. Other studies support imatinib as a potential candidate for treatment of pulmonary fibrosis, whereby imatinib significantly suppresses bleomycin-induced pulmonary fibrosis by inhibiting mesenchymal cell proliferation [136, 137]. In a study comparing the inhibitory effect of TGF-β-induced differentiation among imatinib mesylate, nintedanib, and SB-431542 (transforming growth factor (TGF)-β receptor I kinase inhibitor) in primary human bronchial fibroblasts, imatinib mesylate was found to be non-efficacious when compared to other two molecules [138]. In a randomized, double-blind clinical trial carried out in 119 patients for 96 weeks of testing imatinib in pulmonary fibrosis, treatment did not have an impact on the patient lung function or median survival [139].

Nintedanib (BIBF 1120) is an FDA-approved drug for the treatment of IPF known to inhibit three different tyrosine kinase receptors including platelet-derived growth factor receptor alpha (PDGFRα), vascular endothelial growth factor receptors (VEGFR-1, VEGFR-2, and VEGFR-3), and fibroblast growth factor receptor 1 (FGFR-1). Preclinical trials initially used a molecule named BIBF-1000, which belongs to the same class of drug as nintedanib (aka BIBF-1120) [138]. When BIBF-1000 was tested in bleomycin-induced fibrosis in rats, it significantly reduced collagen deposition and inhibited profibrotic gene expression resulting in significantly less fibrosis [140]. To further investigate the mechanism of action of nintedanib, studies were carried out in bleomycin- or silica-induced fibrosis and human lung fibroblasts [141]. In these studies, nintedanib inhibited human lung fibroblast proliferation and in animal models demonstrated both anti-inflammatory and antifibrotic activity [141]. In a phase II clinical trial conducted on 432 patients comparing nintedanib with placebo controls, nintedanib-treated patients demonstrated a reduction in disease progression along and an improved quality of life [142]. In phase II and phase III clinical studies testing the safety and efficacy of nintedanib for long-term treatment, nintedanib was associated with diarrhea as a major adverse effect but was still considered a safe and effective IPF treatment [143].

### Antioxidants

N-acetylcysteine (NAC) is an FDA-approved mucolytic drug used in IPF owing to its antioxidant effect. Since IPF patient lungs are deficient in the naturally occurring antioxidant glutathione, there is an imbalance in the levels of oxidants and antioxidants. Providing antioxidant activity via N-acetylcysteine is one mechanism that helps in treating IPF [144]. In a small study performed to evaluate the efficacy of N-acetylcysteine in IPF patients by increasing glutathione levels, it was observed to be safe with minimal side effects [144]. A preclinical study supporting the use of NAC to treat silica-induced lung fibrosis in rats found that it reduced reactive oxygen species (ROS) [145]. Further, a double-blind, randomized, placebo-controlled multicenter study performed on 182 patients demonstrated the effectiveness of a high oral dose of NAC in combination with the standard therapy (i.e., prednisolone

and azathioprine) compared to NAC alone which established that NAC reduces pulmonary fibrosis progression [146]. Another clinical study assessed the combination of prednisolone, azathioprine, and NAC (triple therapy) and found that this combination has substantial impact upon pulmonary fibrosis apart from the safety of the drug [147]. Delivery of the NAC to achieve better administration has been tested in conjunction with stem cells. Using a bleomycin-induced fibrosis of mouse and human fibroblasts, human embryonic mesenchymal cells pretreated with NAC were tested for their impact on lung fibrosis [148]. When human embryonic mesenchymal cells were pre-treated with NAC, an increase in the efficacy of human embryonic mesenchymal cells were seen along with a significant reduction in fibrosis and lung inflammation [148].

### Angiotensin System Inhibitor

The angiotensin converting enzyme (ACE) inhibitor captopril is widely used to treat hypertension and is known to indirectly impact renin. Renin has recently been reported as an angiotensin-independent profibrotic mediator of lung fibrosis, known to cause increased collagen and TGF-β production [149]. One of the earliest studies identifying the action of captopril in the prevention of fibrosis was performed in radiation-induced pulmonary dysfunction in rats. These studies identified that captopril reduced collagen accumulation along with mast cell accumulation in the lungs, resulting in amelioration of the pulmonary fibrosis [150]. In other studies performed on rats with radiation-induced endothelial cell dysfunction and pulmonary fibrosis, captopril was found to impact disease progress, thus being a potential candidate for consideration as modifiers of pneumotoxicity [151]. When ACE inhibitors were given to treat bleomycin-induced pulmonary fibrosis, their potential treatment for lung fibrosis was seen [152]. To understand the antifibrotic mechanism of captopril, human lung fibroblasts were assessed in the presence of mitogenic stimuli, where a dose-dependent diminution in fibroblast proliferation was seen [153]. The underlying mechanism involved in pulmonary fibrosis attenuation was found to be related to Fas-induced apoptosis in a human lung epithelial cell line [154]. A concentration-dependent inhibition of the pulmonary fibrosis was observed wherein the lung epithelial cell apoptosis was promoted by captopril [154]. Thus, captopril can be considered as an important candidate in the treatment of the IPF.

Losartan is a well-known angiotensin II type1 receptor antagonist. Angiotensin II is involved in fibroblast production and procollagen synthesis indirectly through angiotensin I activation [155]. Thus, angiotensin II inhibition was initially hypothesized to be a potential treatment alternative. Support for this has been published in a bleomycin-induced fibrosis in rats treated with losartan [156]. In a pilot study, 12 IPF patients were treated with losartan, which was found to improve disease progression over the 12 months of treatment [157]. A phase II clinical trial of the drug is currently in progress to evaluate the safety and efficacy during the treatment [158].

### Targeting Endothelin Receptors

The dual endothelin receptor antagonist bosentan is approved for the treatment of the pulmonary hypertension. Biopsy analysis of IPF lungs identified an increased endothelin-1 expression, suggesting its role in the pathophysiology of IPF [159]. These findings lead to the idea of using endothelin receptor antagonists as a potential treatment of pulmonary fibrosis. An early preclinical study in bleomycin-induced pulmonary fibrosis in rats tested the efficacy of bosentan in IPF [160]. They identified that endothelin-1 receptor antagonism with bosentan reduced fibrosis [160]. Two multinational, randomized controlled clinical trials, namely, BUILD-1 and BUILD-3, are conducted to confirm the efficacy of bosentan in 158 and 616IPF patients, with patients receiving either bosentan or placebo. In both trials, the bosentan was well tolerated among, but efficacy was not justified, thus ending its use in IPF [161, 162]. Newer endothelin receptor antagonists such as ambrisentan and macitentan have been subjected to phase II double-blind, randomized placebo-controlled clinical trial evaluation, but the results were not promising; hence the studies were terminated [163].

### Incapacitating Dysregulated MMP Activity

Matrix metalloproteinases (MMPs) are a group of enzymes that can stimulate fibroblast proliferation when dysregulated. MMP dysregulation can also result in extracellular matrix (ECM) accumulation and plays an important role in the development of pulmonary fibrosis [164]. Hence, inhibition of MMPs may turn out to be a prospective treatment for IPF.

Doxycycline is a potent MMP inhibitor widely used as an antibiotic. It has been demonstrated that doxycycline attenuates the progression of pulmonary fibrosis in bleomycin-induced fibrosis in mouse model [165]. Doxycycline has also shown similar effects in human fibroblast cells via inhibiting MMP, TGF-β, and collagen synthesis [166]. Clinical trials using doxycycline as a treatment alternative for IPF patients have found it to be safe and effective [167, 168]. Additional trials are needed with larger groups of patients for further efficacy conclusions to be made.

## *Prospective Future Therapies*

### Antacid Therapy

Abnormal gastroesophageal reflux (GER) is prevalent in about 87% in IPF patients. As discussed previously, GER may be attributed of being an important etiological factor for IPF. When proton pump inhibitors are administered to IPF patients, they do attenuate GER [169]. GER is also a risk factor for microaspiration, hence leading to a relationship with IPF [170]. The relationship between GER and IPF can be

supported by the study reported to possess greater GER rate in IPF patients compared to non-IPF patient [171]. A study was performed in 204 patients to understand the relationship between GER variable and survival time in IPF patients. They identified that GER and microaspiration play pathogenic role in IPF and GER therapies such as proton pump inhibitors, and histamine 2 blockers attenuated the radiologic fibrosis, increasing the survival time of patients [172].

### Stem Cell Treatment

The use of stem cells has been frequently explored in the past decade as a treatment of a variety of disorders. In the context of IPF therapies, the use of stem cells as a treatment option arises from the fact that the alveolar epithelial cells need refurbishing after the lung injury in IPF, which is supported by the mesenchymal stem cells (MSCs) owing to their properties of differentiation into endothelial and epithelial cells, reducing inflammation and helping in epithelial tissue repair process [173]. The use of MSCs has been shown to reduce disease in bleomycin-induced fibrosis in mice by blocking TNF-α and interleukin-1 [174]. Similar results were obtained when umbilical cord-derived MSCs and bone marrow-derived MSCs were used to treat bleomycin-induced lung injury in mice [175, 176]. Not enough clinical trial data are available for stem cell therapy to make any conclusions on their efficacy in IPF. However, a case report of a 56-year-old man with IPF on long-term oxygen therapy has been published after umbilical cord-derived MSCs were administered [177]. These studies demonstrated that the stem cell therapy was associated with a positive effect including an improved quality of life and lung function after 12 months [177]. Thus, the use of MSCs in IPF may prove to be a potential IPF treatment once sufficient human trials data become available.

## *Novel IPF Therapies*

The effect of the small molecule ICG-001, a selective inhibitor of Wnt signaling (Wnt/β-catenin/CBP-driven transcription), has been tested as a therapy in bleomycin treated rats and found that it had the ability to reverse developed fibrosis [178]. Other studies also support the role of Wnt signaling in pulmonary fibrosis. When bleomycin-treated rats were given WNT1 inducible signaling pathway protein 1 (WISP1)-neutralizing antibodies, the development of fibrosis was attenuated [179]. In another study of bleomycin-induced pulmonary fibrosis in rodents, the effect of blocking Wnt/β catenin pathway was assessed by knocking down β catenin (via β catenin siRNA siRNA) [180]. They identified that blocking the Wnt pathway attenuated pulmonary fibrosis [180]. Together these studies illustrate the utility of targeting Wnt signaling in the development of experimental IPF.

Bone morphogenetic proteins (BMPs) are known to antagonize the TGF-β-induced EMT by attenuating the TGF-β-induced Smad-dependent cell signaling. A

decrease in the expression of BMPs has been observed in the IPF lungs which supports for the increase in the EMT and thus deteriorating the lung architecture [41]. The use of BMPs may therefore be another potential therapy in IPF, which is currently untested to our knowledge.

MicroRNA replacement therapy has been tested in IPF. Using a single-stranded RNA, termed "miR-29b Psh-match," bleomycin-induced pulmonary fibrosis rats were treated compared to its double-stranded counterpart [181]. Treatment with the single-stranded RNA showed higher suppression of the disease and less side effects as otherwise associated with the use of miRNAs, thus leading it to be effective for drug treatment [181].

## *Non-pharmacologic Therapies*

### Lung Transplantation

Lung transplantation is a well-accepted treatment for IPF with a median survival posttransplantation estimated to be about 4.5 years [182]. The number of IPF patients receiving bilateral lung transplant has increased as compared to single lung transplant due to the increased survival rate [183]. The safety of lung transplantation after antifibrotic treatment can be a matter of concern. One study analyzed the impact of previously administered antifibrotic therapy on post lung transplantation survival rate in 62 IPF patients [184]. They observed that the use of antifibrotic agents (pirfenidone or nintedanib) did not raise the surgical complication or mortality rate [184].Nevertheless, lung transplantation is associated with complications of posttransplantation resulting in decreased survival rate. This can be accredited to the factors such as infections due to the use of immunomodulators, rejection of the organ by the body, and airway stenosis [131].

### Pulmonary Rehabilitation

Pulmonary rehabilitation has not been widely explored as a therapy for IPF, yet it can play a vital role in improving symptoms and disease progression. Pulmonary rehabilitation involves a training program of aerobic conditioning, strength and flexibility training, educational seminars, nutritional interventions, and psychosocial consultation [185]. Pulmonary rehabilitation has been shown to improve the quality of life in patients posttreatment. When the effect of pulmonary rehabilitation was assessed in a group of 13 patients and compared to 15 patient controls, they found that rehabilitation improved exercise capacity and health-related quality of life parameters compared to the control group [186]. Without medications pulmonary rehabilitation helps to accomplish less dyspnea, less depression, less anxiety in patients with chronic obstructive pulmonary disease and IPF, and leads to better quality of life measures [187].

## Concluding Remarks

The mechanisms involved in IPF pathogenesis and progression are complex, making it difficult to develop a specific treatment. In addition, the disease progression and symptoms vary among patient populations due to diverse disease pathology, which demands a more personalized design of therapies. For an effective IPF treatment, more investigations are needed to understand the pathogenesis of IPF and develop a novel therapy to prevent the progression of the disease. The knowledge of disease and preclinical/clinical study landscape is very crucial in facilitating new therapies. Therapies should be based on the disease pathogenesis present. For example, when IPF is aggravated and worsens in the presence of other disorders like GERD or cardiovascular diseases, a specific medication should specifically target these mechanisms to retard further disease progression. The use of antacids and proton pump inhibitors should become the first choice where treatment of GERD is essential. Similarly, as respiratory infections are also one of the factors to cause IPF, identifying and treating those infections are also gaining importance. Two FDA-approved drugs named pirfenidone and nintedanib have been successful in providing symptomatic relief against IPF, but still the pursuit for an effective delivery system remains to be explored. As the discovery of a new chemical entity or evening repurposing an established drug for IPF requires a huge investment of time, money, and manpower, opting for another delivery system may be a boon in disguise. Moving from the traditional formulation toward the novel drug delivery systems such as lipid-based drug delivery systems, specific targeted drug delivery system or advanced pulmonary delivery may prove to be a valuable step in various pathological conditions including pulmonary fibrosis. This would help in reducing the dose, dosing frequency, as well as the side effects resulting into better patient compliance.

The path toward development of a newer delivery system is arduous owing to implementation of large-scale manufacturing which is obstructed due to its own challenges. Also, a significant role in treating IPF that involves patient rehabilitation and patient counseling is vital because it helps guide patients toward treatment while helping them regain their hope and faith toward life.

Lastly, identifying newer targets that can lead to a better treatment is warranted but will be challenging, as the IPF pathogenesis is highly complex and predominantly unresolved, particularly in humans. Even though numerous studies have been negotiated to investigate and to decipher the exact pathogenesis of IPF, still a thorough and continuous study is critical in the future.

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# Chapter 9
# Dynamic Reciprocity: The Role of the Extracellular Matrix Microenvironment in Amplifying and Sustaining Pathological Lung Fibrosis

**Janette K. Burgess, Kirsten Muizer, Corry-Anke Brandsma, and Irene H. Heijink**

## Abbreviations

| | |
|---|---|
| ADSCs | Adipose-derived MSCs |
| ASM | Airway smooth muscle |
| ATI | Alveolar type I cells |
| ATII | Alveolar type II cells |
| BAL | Bronchoalveolar lavage |
| BM | Basement membrane |
| BM-MSCs | Bone marrow-derived MSCs |
| BOS | Bronchiolitis obliterans syndrome |
| COPD | Chronic obstructive pulmonary disease |
| DAMPs | Damage-associated molecular patterns |
| ECM | Extracellular matrix |
| EMT | Epithelial-to-mesenchymal transition |
| ER | Endoplasmic reticulum |
| FGF | Fibroblast growth factor |
| HGF | Hepatocyte growth factor |
| HSPG | Heparin sulfate proteoglycan |
| IGF | Insulin-like growth factor |
| IPF | Idiopathic pulmonary disease |
| MMPs | Matrix metalloproteinases |
| MSCs | Mesenchymal stromal/stem cells |

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| | |
|---|---|
| PDGF | Platelet-derived growth factor |
| PG | Proteoglycan |
| $PGE_2$ | Prostaglandin $E_2$ |
| SFPTC | Pro-surfactant protein C |
| TGF | Transforming growth factor |
| UC-MSCs | Umbilical cord-derived MSCs |
| VEGF | Vascular endothelial growth factor |
| WNT | Wingless/integrase-1 |
| YAP | Yes-associated protein |
| α-SMA | Alpha-smooth muscle actin |

## Introduction

The architecture of the lung consists of airways, parenchyma, and a vascular system, providing blood supply for the lung tissues as well as constituting the main component for gas exchange. The extracellular matrix (ECM) contains the building blocks of all these components, providing physical support and stability. Until recently, ECM has been regarded as an inert structure. However, it is a diverse and dynamic mesh of proteins, glycoproteins, and lipids that provides both structural integrity to every organ in the body and regulates intercellular communication and controls tissue and organ development. Spatial and temporal expression of matricellular proteins generates an environment that facilitates these functions. Significantly, the regulatory role of ECM can become unbalanced, causing detrimental changes to the architecture and functionality of organs. These changes are evident in the tissue remodeling characteristics in all compartments in the lungs, in airways in asthma and chronic obstructive pulmonary disease (COPD) (fibrosis) and in parenchymal tissue, with tissue destruction in the emphysema phenotype of COPD and thickening in idiopathic pulmonary fibrosis (IPF) (fibrosis), leading to symptomatic disease. The focus of this chapter is to describe the changes in the matrix microenvironment in the lung in chronic disease and the resultant role these changes have in amplifying and sustaining pathological lung fibrosis.

## The Extracellular Matrix of the Lung

The extracellular matrix (ECM) is a tissue-specific structure consisting of connective tissue and residing cells that is essential for normal (healthy) organ function. The ECM is a highly dynamic structure [1], in which more than 150 different components have been identified in humans (reviewed in [2, 3]) that are assembled, following a complex endogenous program, to form a network with distinct spatial organization [4, 5]. It provides structural support as well as biochemical and biomechanical stimuli to the residing and infiltrating cells. As such it regulates many processes including cell adherence, motility and growth/proliferation, cellular differentiation,

and wound healing [5, 6]. In light of this, tight interchange between cells and the ECM in which they reside the potential for pathological outcomes when the ECM environment is disrupted in disease is significant. The dynamic reciprocity of the cell:ECM interchange has recently emerged as a pivotal factor in the initiation and progression of chronic lung diseases [3, 7].

In the lungs, the ECM is restricted to two compartments, the basement membrane and the interstitial spaces. In the basement membrane (BM), the ECM presents as a thin layer beneath all epithelial and endothelial cell layers, whereas in the interstitial spaces, the ECM is present between the alveoli and the blood vessels, forming the parenchyma [8]. The lung ECM needs to protect cells against the endless stretch and strain of breathing but also needs to be useful in the exchange of oxygen and carbon dioxide [9]. The main components of the lung ECM are collagen and elastin, which are both fibrous proteins; fibronectin and laminin, which are both adhesive proteins; and proteoglycans and glycosaminoglycans, which form a hydrated polysaccharide gel in which all other components are embedded. Collagen IV, a non-fibrillar collagen, and laminin are the main BM ECM proteins, whereas the fibrillar collagens I, II, III, V, and XI, elastin, fibronectin, and the proteoglycans are the main components of the interstitial ECM in the lung [10–14]. The fibrillar proteins are responsible for the tensile strength of the lung, while the elastic fibers provide elastic recoil. Changes in the ratio, composition, or cross-linking of these proteins can have a substantial impact on the behavior and stiffness of the ECM. Cross-linking of these proteins is mediated by nonenzymatic and enzymatic pathways, including enzymes of the lysyl oxidase (LOX) and tissue transglutaminase (tTG) families [15, 16].

Resident interstitial fibroblasts and activated myofibroblasts are the primary sources producing lung ECM proteins, and they are also the primary effector cells during injury and repair. Additional important sources of ECM are the airway smooth muscle and airway epithelial cells [7, 8].

## Main Components of the Lung ECM

### *Collagen*

Collagen fibers are the main components of the ECM. They are produced by fibroblasts and are responsible for the tensile strength of the ECM. There are 28 types of collagens, but the main subtypes of collagen in the lung ECM are types I, II, III, IV, V, and VI. Each type has a distinct characterization; while collagen type I is, for instance, a thick and rigid fiber, type III is more flexible [9]. Although collagens are mainly addressed as collagen fibers, not all collagen types are fibrous. Collagen types I–III are fibrous collagens, and IV–VI are nonfibrous collagens. All types are expressed heterogeneous, and together they form a mesh of collagen. Which collagen types are expressed differs per ECM type. Collagen types I and III are mainly seen in the interstitial spaces, while type IV is a vital component of the BM. However,

expression of different collagen types does not only differ in ECM layer but also in the tissue region in the lung. For example, the large bronchioles consist mainly of types I and III, while the alveoli consist of III, IV, and V [5, 9, 17–20].

### *Elastin*

Smooth muscle cells and fibroblasts residing in the lung ECM synthesize the protein tropoelastin, which is cross-linked to make elastin. Together with microfibrils, the elastin proteins in turn cross-link into elastin fibers. Elastin fibers are highly flexible and resilient and are responsible for the intrinsic recoil property of the lung tissue [5, 9, 17, 18].

### *Fibronectin and Laminin*

Fibroblasts produce the fibrous proteins fibronectin and laminin in the lung. Their main function in the lung is to regulate cell adhesion, although they are often incorrectly thought to contribute to ECM strength. Fibronectin functions as an extracellular mechano-regulator; it has binding sites for the cells of the epithelium (integrin binding sites), ECM components (like collagen, fibrin, and proteoglycans), and secreted molecules (like growth factors). By attaching to cell and matrix and being able to stretch out multiple times its size, it can signal cells regarding environmental changes and cause the cells to, for example, change their morphology and motility [5, 17, 21]. Laminin has a similar function to fibronectin: it attaches cells to their BM [9, 18].

### *Glycosaminoglycans and Proteoglycans*

Repeating disaccharides make up the long polysaccharide glycosaminoglycans (GAGs). There are two types of GAGs: non-sulfated and sulfated. The most abundant non-sulfated GAG is hyaluronic acid. It is synthesized by mesenchymal cells, and it is an important stabilizer of the loose ECM [9]. Important sulfated GAGs are heparan sulfate (and its modified form heparin), chondroitin sulfate, keratin sulfate, and dermatan sulfate. Sulfated GAGs can form proteoglycans (PGs) by attaching to a protein core [22].

While proteoglycans are mainly responsible for maintaining the tissue hydration, there are different types of PGs, which all have specific functions. In lung tissue, there are three main PGs distinguished based on the GAGs that are formed around the core protein [9, 18, 22]:

### Versican

Versican is a PG which contains chondroitin sulfate, primarily located in the interstitium in regions where there are no collagen and elastin fibers. Its exact function is unclear, but it is thought to be important in tissue hydration and cell-matrix interactions.

### Perlecan

Perlecan is a PG containing heparan sulfate located primarily in the vascular BM. Perlecan functions as a filtration barrier by interacting with collagen IV. This barrier limits the flow of macromolecules and cells between tissues. It also regulates ECM repair and remodeling processes by being able to bind to growth factors, cytokines, and proteinases.

### Decorin

Decorin is a PG containing dermatan sulfate located in both the interstitium and the epithelial BM. Decorin is a multifunctional regulatory molecule known for its influence in collagen fibrillogenesis by binding to and, by doing so, "decorating" collagen fibers, hence its name. More importantly, decorin has an anti-fibrotic role by its ability to inhibit the growth factor TGF-β through binding to it, ultimately suppressing proliferation and activation of fibroblasts [23, 24].

### Tenascin-C

Tenascin-C is a large hexametrical glycoprotein, comprised of multiple binding domains. These domains can bind to cells, ECM components, and pathogens. In the lungs, tenascin-C is expressed upon injury of the epithelium and is responsible for the migration of fibroblasts toward the site of injury. By binding to fibroblasts as well as different ECM components like fibronectin, it can change cellular adhesion, contractility, and motility and organize ECM remodeling. This drives fibroblast migration and activation at the injury site and promotes tissue repair. After the epithelium is repaired, tenascin-C is degraded, and secretion is stopped [25–27].

## *Matrix Metalloproteinases*

To maintain tissue homeostasis, ECM molecules do not only have to be produced, but they also have to be degraded. Degradation of ECM molecules is the job of endogenous proteases. The main type of proteases in the ECM is the matrix

metalloproteinases (MMPs), which are produced by fibroblasts and inflammatory cells. Their activity is highly regulated, considering that too much MMPs causes a lot of tissue damage by excessive ECM degradation. MMPs are secreted in a latent form and are only activated by proteolytic cleavage. Their activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs). MMPs and TIMPs bind in a one-on-one stoichiometry, and the balance between these components is essential for maintenance of ECM homeostasis [9, 18, 28].

## Matrix Alterations in Lung Disease

### *Asthma*

Characteristically, asthma is associated with ECM changes in all lung compartments [7], with the most distinctive being thickening of the basement membrane (BM) [29] (Fig. 9.1a). The accumulation of collagens I, III, and IV and fibronectin in the subepithelial lamina reticularis associates with this BM thickening [30]. BM thickening develops early in the disease and persists during asthma remission [31, 32]. It is remarkable that increased BM thickening is not associated with duration of the disease or disease severity [31].

The ECM changes in asthma vary depending on the lung compartment, severity of the disease, and control of the disease [7, 33]. It is important to note that access to the airway tissue in asthma is difficult and restricted to bronchial and transbronchial biopsies, except for the studies investigating fatal asthma for which autopsy material is available. In fatal asthma, alterations in fibronectin and MMP-9 and MMP-12 content were found in the ASM of the large airways as well as in the small airways. In addition, increased elastin content was detected in the ASM of large airways, whereas changes in collagen I and III content were identified in the small and not in large airways [34, 35]. Together with changes in PG deposition in fatal asthma that are most pronounced in the small airways [36], these studies show clear ECM changes in the small airways in asthma implying their involvement in asthma pathophysiology. Biopsy studies assessing the central airways have demonstrated important changes in the ECM when comparing severe to mild-moderate asthma, such as increased collagen deposition and an increase in ASM mass [37, 38]. Furthermore, significant PG changes have been found with respect to asthma control in central airways and lung parenchyma with increases in collagen, versican, and decorin in patients with uncontrolled versus those with controlled asthma [39].

### *COPD*

Abnormal lung tissue remodeling is an important hallmark of COPD [40]. The constant damage by noxious gases and oxidative stress in COPD leads to chronic activation of the repair mechanisms and inflammatory processes, resulting in

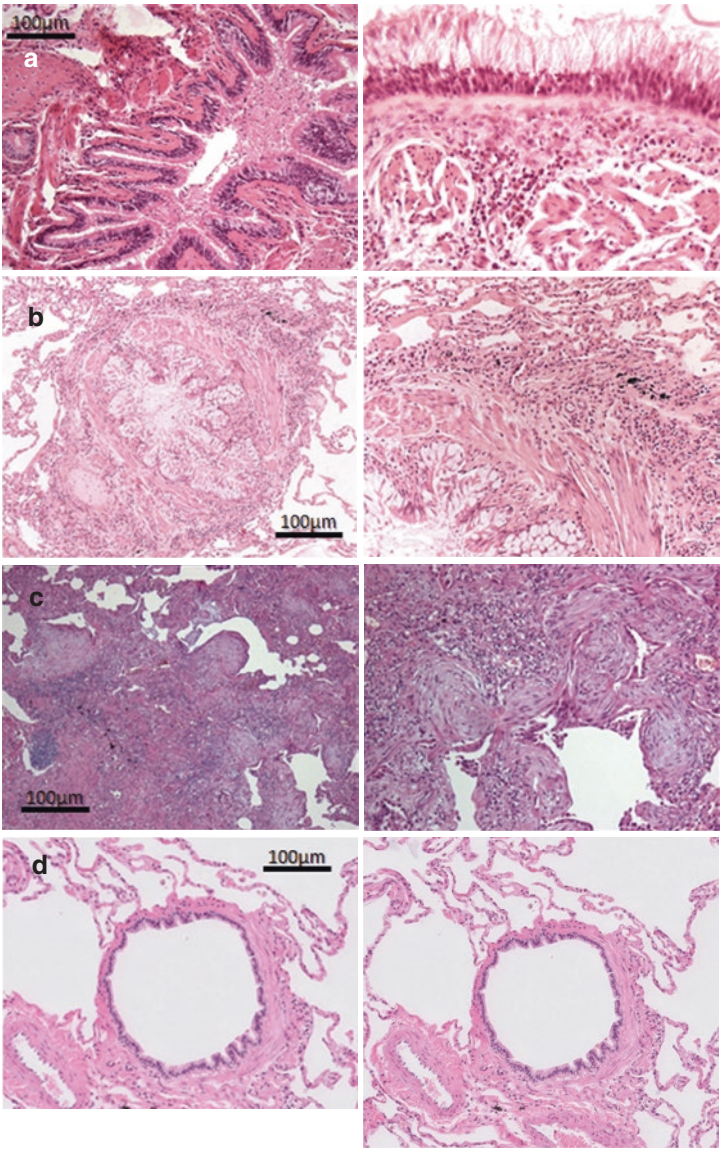


**Fig. 9.1** Pathological changes in fibrotic lung diseases. Photomicrographs of human lung tissue sections from (**a**) individuals with asthma, (**b**) individuals with chronic obstructive pulmonary disease (COPD), and (**c**) individuals with idiopathic pulmonary fibrosis (IPF) (**d**) illustrates normal lung structure from a healthy donor. Right-hand panels illustrate higher magnification images from the same pathology. Tissues were obtained from the tissue archive within the Department of Pathology and Medical Biology at the University Medical Center Groningen

aberrant tissue repair and remodeling. This abnormal response is not limited to a specific lung compartment in COPD but present throughout the lungs and varies between different regions in the lung. In the large and small airways, excessive ECM deposition underlies the observed airway wall fibrosis (Fig. 9.1b), whereas in the lung parenchyma, ECM degradation by proteolysis occurs which is not sufficiently repaired, resulting in emphysema. The underlying causes of these different remodeling processes in lung regions are poorly understood.

In healthy tissue, there is a balance between proteases, such as MMPs and neutrophil elastase (a protease specifically cleaving elastin), and antiproteases, such as TIMPs and α-1 antitrypsin (an antiprotease inhibiting neutrophil elastase) [9, 41, 42]. However, in COPD there is an imbalance, with an increase in MMP-2, MMP-9, and MMP-12 by macrophages in COPD [43, 44], thus exceeding the amount of antiproteases present. This leads to degradation of (healthy) ECM components, especially elastin, which ultimately leads to emphysema [41, 45, 46].

Several studies have shown a reduction in the elastic fibers in COPD patients [47, 48] as well as abnormalities in the ultrastructure with disrupted fibers and signs of disturbed elastogenesis [49]. However, gene expression studies have shown the opposite effect in COPD, with increased elastin gene expression in severe COPD [44] and upregulation of several elastogenesis-related genes, including elastin and fibulin-5, in COPD lungs [50]. This increase at the gene expression level and decreased presence of elastin and elastin fiber abnormalities in COPD suggests a defect in the formation and repair of elastic fibers in COPD.

Of interest, the reduction in elastic fibers is also observed in the small and large airways [48]. This indicates that loss of elastic fibers contributes to airway obstruction in COPD not only via disruption of alveolar attachments to the airways but also via loss of structural support of the airways.

Total collagen deposition has been shown to be increased in GOLD II and IV COPD in both the small airways and the parenchyma [51]. However, when assessing specific types of collagen, the results are quite variable. The chronic inflammation and fibrosis in the small airways are assumed to underlie the excessive deposition of collagens I and III as alterations (increases and decreases) in collagen expression are present in COPD (Table 9.1). Some of these observed differences may result from differences in quantification methods and also the tissue source variability and time of sampling.

**Table 9.1** Changes in expression levels of collagen types I, III, and IV in COPD patients

| | Small airways | | Lung parenchyma | Airway[a] |
|---|---|---|---|---|
| Collagen type | Mild/moderate | Severe | Mild/moderate | Mild to severe |
| I | ↓ [48] | ↓ [52] | ↑ [48] | ↑ [53] |
| | ↑ [52, 54] | | | |
| III | ↓ [52] | ↓ [52] | ↔ [48] | ↑ [54] |
| | ↔ [48] | | | |
| IV | ↔ [48] | | ↔ [48] | ↑ [54] |

[a]In these articles it was not defined which part of the lung was used for this analysis.

Of interest, recent imaging techniques using second harmonic generation microscopy have shown structural changes in collagen fibrils, with more disorganized collagen fibrils in COPD [55, 56]. These functional changes are consistent with a reduction in the expression of the PGs decorin and biglycan, important collagen-cross-linking molecules, in severe COPD [57, 58]. Next to changes in decorin and biglycan, alterations in versican have also been shown. An apparent increase in protein level was found in patients suffering from moderate to severe COPD, compared to control. However, data was inconsistent when comparing expression levels of versican in mild to moderate COPD with controls [43, 48, 53, 59]. It was suggested that high versican levels cause a decrease in elastin fibers, as versican inhibits elastin-binding protein, a key protein in elastic fiber assembly [43].

Fibronectin and laminin deposition was demonstrated to be increased in the small airways in COPD, possibly contributing to airway obstruction, but not in the parenchyma [48, 54]. Other studies showed no change in laminin protein expression in the small airways in moderate COPD compared to controls [57, 60].

## *IPF*

Extensive ECM deposition characterizes IPF in the alveolar regions of the lung leading to a complete distortion of the lung architecture (Fig. 9.1c). A distinctive characteristic feature of IPF compared to other interstitial lung diseases is the presence of fibroblastic foci. These foci consist of accumulated myofibroblasts that are located close to regions with alveolar epithelial cell disruption and are considered the primary source of the ECM proteins deposited during the pathogenic process. Early studies have demonstrated the deposition of collagens, mainly types I and III, and fibronectin in the alveolar regions in chronic fibrotic lung disease. The presence of fibroblastic foci was shown within the airspaces, outside the destroyed lamina propria [61]. These findings are in line with the current theory that repetitive alveolar epithelial injury is underlying the pathogenesis of IPF [62]. Versican is a critical ECM protein with an enhanced presence within these fibroblastic foci. Whereas the versican-rich areas are low in mature collagen, they stain positive for type 1 procollagen, indicating early collagen synthesis and active ECM remodeling, a process that may be driven by versican [63]. Next to versican, tenascin-C is also increased in fibroblast foci [64].

Increased numbers of elastic fibers in the fibrotic regions of IPF patient lungs correlate with the amount of collagen deposited as well as with disease prognosis [65]. This indicates that, although elastic fibers are needed for elastic recoil of the lungs, excessive amounts have opposite effects and increase stiffness of the lungs.

How specific these ECM changes are for IPF versus other interstitial lung diseases remains to be established. On the gene expression level, no differences were observed in tenascin, fibronectin, and versican expression when comparing IPF and chronic hypersensitivity pneumonitis [64]. Another intriguing question is whether the increased ECM deposition in IPF actively contributes to disease

pathology or is "only" a result of the active fibrotic response in IPF. Recent findings from Parker et al. suggest that the remodeled ECM is more than a bystander and activates a pro-fibrotic feedback loop between fibroblasts and the lung matrix, with a potential role for microRNA-29. Intervention in this loop may provide new avenues for pharmacologic intervention in IPF [66].

## Matrix-Driven Cellular Responses

The evidence is accumulating that the ECM is biologically active; it influences cell functionality directly through direct bidirectional communication between the ECM components and cells and also indirectly as a reservoir for growth factors and chemoattractants such as cytokines and chemokines [67, 68] (Fig. 9.2).

Endogenous degradation of the ECM releases fragments, referred to as matrikines that have also been recognized to have biological entities in their own rights (see *Chapter* 6) (reviewed in [12, 69]). Upon release matrikines can undergo changes in their secondary or tertiary structure, resulting in exposure of previously inaccessible sites within the amino acid sequence that can expose active sites to drive new biological functions (reviewed in [70–73]). These matrikines can bind to different receptors on resident cells or cells migrating through the lung tissue to induce a

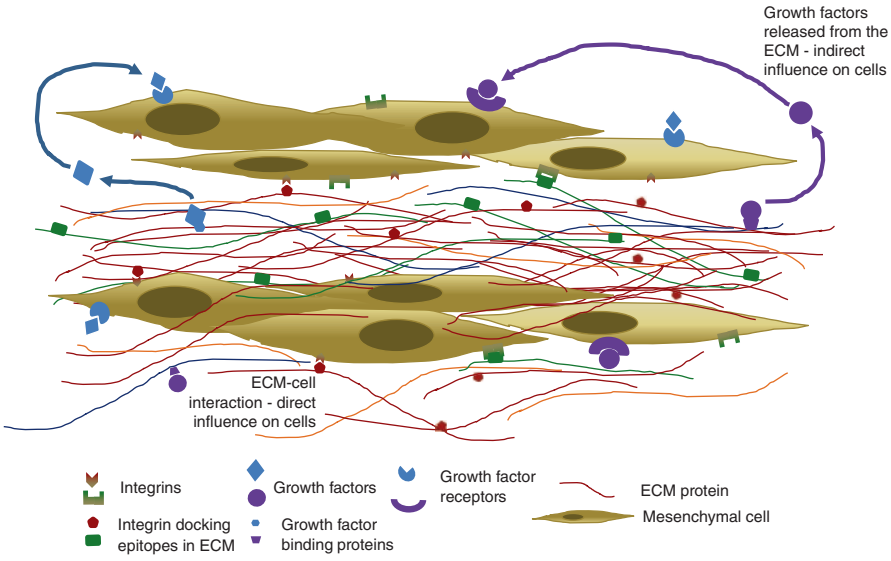


**Fig. 9.2** The influence of the ECM on cells within the microenvironment. The ECM can have a direct effect on cellular response through direct interaction with cell surface receptors, particularly integrins. Alternatively cells can be influenced indirectly by the ECM through the release of growth factors that are anchored in the ECM via growth factor-binding proteins. Released growth factors interact with appropriate receptors in the cell surface to initiate intracellular signaling cascades

range of cellular responses; the responses are driven by the cell type, the cellular activation state, and other factors within the microenvironment of the cell. These responses can contribute to both the inflammatory and fibrotic phases of lung disease. Of particular interest recently has been the pro-inflammatory/immunogenic role of matrikines [74] and their potential to activate specific pattern recognition receptors. In this role, they are recognized as damage-associated molecular patterns (DAMPs) which have been highlighted as having a potential role in COPD [75].

ECM proteins incorporated in the matrix also directly interact with cells via cell surface receptors, particularly integrins [76]. Specific combinations of the heterodimeric integrin alpha and beta chains create the diversity within the integrin family, providing the specificity for binding to the various ECM proteins. Specific integrins recognize defined epitopes on individual ECM proteins, allowing for specificity within the ECM-cell interactions in different locations with tissues [77]. Conformational changes in the ECM proteins occur when ECM proteins change from a soluble to a matrix-incorporated form, which in some cases results in rearrangement of the integrin recognition epitope such that a different integrin will now recognize the protein.

In in vitro studies, individual ECM proteins have been reported to influence mesenchymal cellular attachment, proliferation, migration, rate of wound closure, and pro-inflammatory responses (cytokine output) [78–83].

An important place of cross talk between the ECM and the epithelial cells and fibroblasts in the lung is the epithelial-mesenchymal trophic unit (EMTU). Evans and colleagues first described the EMTU as a thin layer of fibroblasts in the ECM in close contact with the epithelial cells [84]. They suggested that the close bidirectional communication between cells and matrix in the EMTU is responsible for the maintenance of airway homeostasis. This close contact allows the cells to efficiently and quickly control inflammatory reactions and repair and remodeling processes when an injury occurs [84–86]. In the EMTU there is contact between the epithelium and fibroblasts through small cytoplasmic extensions, enabling them to communicate [85].

## *Migration*

The movement (migration) of cells is dependent on the cues the cells receive through their interactions with the complex tissue environments in which they are located. Several mechanisms can drive cell migration, which all involves actomyosin-driven changes in cell body shape [87]. A cell extends protrusions from its leading edge and adheres them to epitopes in the surrounding tissue to enact migration. Following this action, it retracts the rear end of the cell body, resulting in its translocation [88]. As such, the confirmation of the tissue in which a cell resides profoundly influences migration. In this way, the density of adhesion ligands, the topography (shape), and the stiffness of the ECM have an essential role in the regulation of cell migration [89, 90].

Individual ECM components have been shown to influence cell migration differentially. Parameswaran et al. reported that fibronectin, collagen V, and collagen III induce strong migration of human airway myocytes, while collagen I, laminin, and elastin induce strong adherence of the cells but limit their movement [80]. Similarly, the small leucine-rich repeat proteins decorin and biglycan, which are important proteins for maintaining the structure of lung connective tissue, induced fibroblast cytoskeletal changes which resulted in increased cellular migration [91].

## *Proliferation*

The ECM also has a major influence on the cellular proliferative response. Disruption of the interaction of cells with their surrounding ECM, through blockade of integrin binding, starves their growth potential and promotes the induction of apoptosis [92, 93]. Major structural proteins in the ECM regulate cellular proliferation, with collagen I and fibronectin promoting proliferation, while laminin reduced the proliferative response [94]. In addition, several lung disorders have increased amounts of versican (reviewed in [95]), which regulates fibroblast proliferation [96]. Biglycan and decorin also have several important roles in the regulation of cell proliferation. The central core proteoglycan backbone common to both biglycan and decorin acts as a binding repository for cytokines such as transforming growth factor (TGF)-α [97], while the finer architecture of the PGs side chains also can interact with cytokines and growth factors. The binding of growth factors by glycosaminoglycan side chains that are rich in L-iduronate has been suggested to be a negative regulatory mechanism through which these ECM proteins inhibit proliferation of fibroblasts [98, 99]. However, while heparin sulfate proteoglycan (HSPG) is recognized for its antiproliferative effects, treating fibroblasts with a combination of HSPG and platelet-derived growth factor (PDGF)-BB enhanced the mitogenic effect. Thus, the ECM itself regulates cell proliferation through complex effects including modifying the availability of epitopes for direct interactions with cellular receptors and through the regulation of the availability of cytokines and growth factors, both mechanisms which may initiate stimulatory and/or inhibitory signals for cells [100].

## *Cellular Responses to ECM Stiffness*

Changes in the ECM fiber structure and rigidity (stiffness) are pivotal for the maintenance of the tissue elasticity and structure but also central in controlling cellular responses. Stiffening of the ECM as a result of tissue remodeling, particularly during the development of fibrosis, influences cell adhesion, migration, and proliferation [101]. Fibroblasts increase the area of contact per cell, enhance actin stress fiber

formation, and develop larger focal adhesion complexes when in contact with substrates of increased stiffness. Fibroblast proliferation rates increase as their substrate becomes stiffer. The relocation of the nuclear transcriptional regulator YAP (yes-associated protein) from the cytoplasm to the nucleus is the critical signaling event in response to increasing substrate stiffness [102]. In vitro, fibroblasts grown on matrices representing the pathological stiffness of diseased fibrotic tissues have an accumulation of YAP in the nucleus, but this is not seen on matrices of physiological stiffness. These findings suggest the translocation of YAP to be involved in the events leading to the amplification and sustaining of pathological fibrosis [103].

## Dynamic Reciprocity in the Lung

### *Alterations in the ECM and Consequences for Disease*

As we begin to understand the alterations in the profiles of ECM proteins in diseased lung tissues, there is now a need to investigate the functional significance of these changes. It is still debated whether chronic inflammation in lung disorders triggers an aberrant tissue repair or indeed if the altered ECM can drive the aberrant inflammatory response. At least, fibrotic events are not merely seen as a consequence of inflammation, and both events are now thought to act in parallel [104–106].

How the composition of the ECM contributes to disease pathology in the airways is an emerging focus in the field. While remodeling of the airways (structural changes including alterations in the epithelial cells, smooth muscle cells, blood vessels, and the ECM) has long been recognized as a feature of the asthmatic airway [107], it is now also being characterized in other airway pathologies (reviewed in [108]) (Fig. 9.3).

Matrices deposited by airway smooth muscle (ASM) cells derived from asthmatic and non-asthmatic individuals, which have been profiled to contain different ECM proteins [109], alter the cellular responses of ASM cells seeded onto these matrices, with regard to proliferative, migratory, and wound healing capacity [109–111]. Similarly, the matrices deposited by ASM cells derived from asthmatic and non-asthmatic donors regulate inflammatory cell responses, with regard to directing cellular migratory patterns [112]. Matrices deposited by human lung fibroblasts in response to exposure to cigarette smoke are also pro-proliferative [82]. These data demonstrate that the ECM in the airway tissues of patients with fibrotic lung diseases can dictate cellular behaviors and add to or modulate the disease pathology.

As our understanding of the remodeling events, particularly the changes in the ECM profile in the region of disease focal points in the lung expand, we will be able to focus more accurately on the development of novel therapeutics designed to target the airway remodeling.

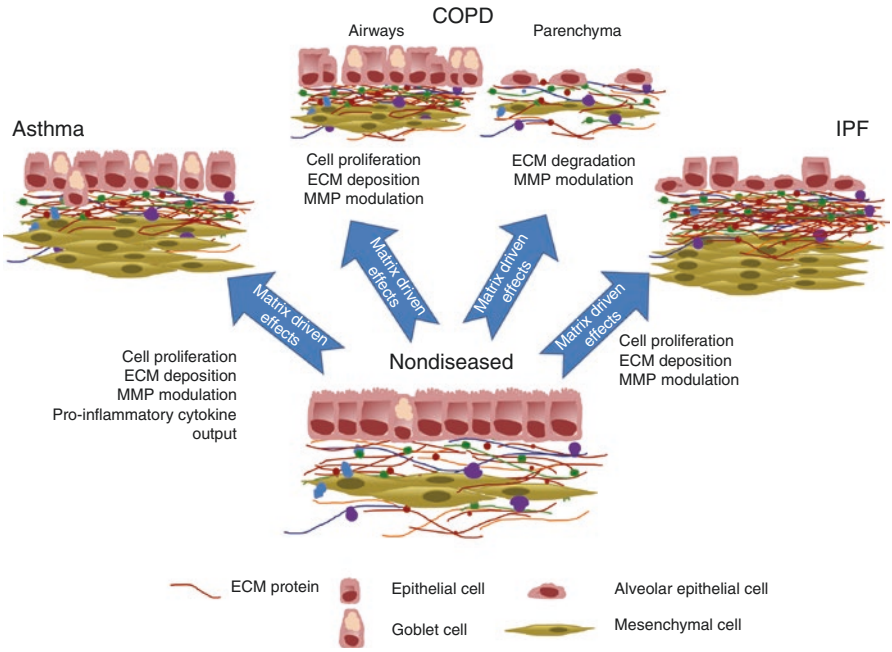


**Fig. 9.3** The dynamic reciprocity of the ECM in lung disease. Alterations in the ECM in asthma, COPD, and IPF drive processes that contribute to the structural changes that are recognized in these disease pathologies. The ECM is an active contributor to the disease underlying mechanisms

## *The ECM in Asthma*

There is accumulating evidence that the interplay between ASM cells and ECM proteins not only influences the ECM in the microenvironment, but also the proliferative, migratory, and synthetic responses of the ASM cells, all of which are functions that link integrally to the structural remodeling process in the airway wall in asthma [81, 109, 112–115].

ASM cells produce and secrete ECM proteins and matrix metalloproteinases (MMPs), which potentially influence the ECM microenvironment surrounding the cells. In vitro studies have shown ASM cells have the capacity to secrete and deposit many ECM proteins including collagens (I, II, IV, V), fibronectin, elastin, laminins (β1, γ1, β2, α1), decorin, chondroitin sulfate, perlecan, versican, and thrombospondin [109, 116–118].

Transforming growth factor (TGF)-β, a pro-fibrogenic growth factor that has repeatedly been implicated in airway remodeling in asthma and other fibrotic lung diseases, is released by cells in a pro-form bound to a latent binding protein [119]. This complex is anchored in the ECM, providing a reservoir of this growth factor that can be released on demand. Active TGF-β is released from the ECM depot through a mechanism involving mechano-activation and integrin αvβ5 on ASM

cells [120]. Among its many functions, TGF-β induces the production of ECM proteins by ASM cells [121–123]. Therefore, through the regulation of TGF-β activation, the ECM contributes to the maintenance of homeostasis within tissues by orchestrating the balance of the matrix production and degradation. The disruption of the ECM may be a key driver for the perpetuation of the fibrotic process.

Fibroblasts are also a target cell for ECM-modulated effects in lung disease. A distinct subset of elongated fibroblasts was described in the bronchoalveolar lavage (BAL) of mild asthmatics but not in healthy controls. The asthmatic-derived elongated fibroblasts produced higher amounts of biglycan, decorin, and versican than those obtained from bronchial biopsies from the same patients. While the study did not show a direct causal relationship, they did report that the asthmatic-derived elongated fibroblasts migrated twice as far as the fibroblasts originating from bronchial biopsies from the same patients, suggesting that the altered ECM profile contributed to the migratory phenotype of the elongated fibroblasts [124]. It is interesting to note that similar data have been reported for fibroblasts from donors with systemic sclerosis [125].

## *The ECM in COPD*

A picture is beginning to emerge of the changes in the ECM in COPD patients; however this is not as advanced as that for asthma as yet. Regional-specific ECM changes within the lungs are being identified: through which our understanding of the impact of the ECM on pathophysiological consequences will be advanced. Whether the changes in the ECM have a positive or a negative impact on the functional output of the lung is controversial (reviewed in [126]). Peribronchial fibrosis in milder COPD has been suggested to be protective against airway narrowing [127]. However, it has also been postulated that the thickened airway wall, resulting from the deposition of ECM proteins and changes in the cellular composition around the airway, might also be protective against excessive airway narrowing [128].

The deposition of ECM proteins in COPD patients' lung tissues is regarded as being predominantly driven by fibroblasts. Hallgren and colleagues reported significantly higher versican production from distal lung fibroblasts derived from COPD patients than those from controls [59], which reflects the observations in tissue made by Merrilees et al. [43]. Merrilees and colleagues postulated that the presence of versican may inhibit the formation of elastin fibers.

Other variances in ECM production by fibroblasts from COPD donors include a lack of difference in biglycan production between fibroblasts isolated from the central and distal airways of COPD patients, where fibroblast differences are observed compared to control donors. In contrast, central fibroblasts from COPD patients produce less perlecan compared to those from control donors [59, 129], which reflects the data seen in lung tissue from pulmonary emphysema patients [57]. Of interest, expression of decorin is not different between the fibroblasts from healthy

controls or COPD patients from either lung compartment [59], which also reflects findings in the tissue of patients [48]. ASM cells derived from COPD patients have also been reported to deposit differential perlecan compared to non-diseased donor ASM cells [130].

In the early stages of COPD, the reduced fibroblasts and epithelium contact may liberate fibroblasts from the inhibitory control exerted by the epithelium, eventually contributing to airway wall fibrosis [85]. Using an in vitro model of the EMTU comprising the co-culture of airway epithelial cells and lung fibroblasts, we recently demonstrated that airway epithelial cells induce a pro-inflammatory phenotype in lung fibroblasts with decreased ECM production via the release of IL-1α [131]. This IL1α-dependent effect on fibroblasts was enhanced by cigarette smoke-exposed COPD-derived epithelial cells, suggesting aberrant cross talk in COPD.

## *The ECM in Interstitial Lung Fibrosis*

In fibroblasts derived from patients with pulmonary fibrosis, those fibroblast populations that were producers of high levels of hyaluronan and decorin had lower proliferative rates than those with low levels of these ECM proteins [132]. In addition, after lung transplantation, fibroblast proliferation rates were negatively correlated with the production of perlecan and decorin, which has been suggested to be an early indication of ongoing remodeling, and these levels may act as potential markers for bronchiolitis obliterans syndrome (BOS) [133]. Indeed, the structure of perlecan in IPF has been shown to be highly sulfated, with increased levels of GAGs on the HSPG of perlecan in the areas of fibrosis. It has been suggested that the highly sulfated composition of perlecan enhances the growth factor binding capacity which contributes to the lung tissue remodeling in fibrosis [134].

In IPF the fibrotic deposits in the lung tissue are considered to be both a cause of and also a consequence of fibroblast activation. Not only is the ECM altered in the fibrotic lung [135], but the tissue is more rigid than non-diseased lung tissue [136]. It is also interesting to note that not only the ECM is stiffer in pulmonary fibrotic diseases, but also the fibroblasts are stiffer [137]. In a murine model of bleomycin-induced lung fibrosis, alterations in the fibroblast phenotype from a quiescent state to a progressively active cell with increased proliferation rates and enhanced matrix production accompanied the induced increase in local tissue stiffness. The mechanism underlying these changes was suggested to be driven by the suppression of the fibroblast production of cyclooxygenase-2 and prostaglandin $E_2$ ($PGE_2$), which resulted in a disruption of the autocrine inhibitory mechanism opposing fibrogenesis [138].

Changing the stiffness of the substrate on which cells are grown has profound effects on cellular behaviors, which are relevant for lung fibrosis. Alterations in the proliferative and contractile responses are seen between fibroblasts from healthy controls and patients with IPF when these cells are cultured on stiff matrices. Strikingly, the cellular responses to $PGE_2$ are also different between the healthy control and IPF fibroblasts in the rigid matrices. These differences disappeared

when the fibroblasts were moved to a softer matrix environment, although the IPF fibroblasts retained some of their resistance to the anti-fibrotic effects of $PGE_2$ [139].

The decellularized lung matrices from IPF patients, which are stiffer than the matrices derived from healthy controls [136], can direct the behavior of cells seeded on these matrices. These fibrotic matrices establish a feedback loop that induces the mesenchymal cells within this microenvironment to transcribe ECM proteins, thereby driving the cells to further pathologically remodel the ECM [66].

## Potential to Break the Vicious Cycle of Dynamic Reciprocity

### *Role of Stem/Progenitor Cells in the Matrix-Driven Pathology of Pulmonary Fibrosis*

Epithelial cell injury is thought to be a crucial driver in the pathogenesis of fibrosis in the lung. In this respect, emerging evidence suggests that the regeneration of alveolar epithelium in IPF is impaired by cellular senescence and stem cell exhaustion, which may subsequently contribute to the observed matrix abnormalities in IPF [140]. Impaired reepithelization may promote fibroblast and myofibroblast responses, leading to tissue remodeling and deposition of ECM molecules such as collagen [140]. Telomere shortening, one of the hallmarks of cellular senescence, in alveolar epithelium may impair autophagy, increase endoplasmic reticulum (ER) stress, and lead to incorrect processing of pro-surfactant protein C (SFPTC), triggering EMT and pro-fibrotic responses [140, 141]. Indeed, mutations in telomerase have been reported to underlie the inheritance of IPF in a subset of patients with documented family history of the disease [142], while short telomeres, which limit tissue renewal capacity in the lung, are a risk factor for pulmonary fibrosis [143]. Furthermore, alveolar epithelial cells isolated from the lungs of patients with IPF display abnormalities, with higher mRNA expression of mesenchymal markers such as type I collagen and α-SMA [144].

Recent reports from animal models support the vital role for stem/progenitor cell aging in lung fibrosis, specifically senescence of the alveolar type II (ATII) cells. ATII cells are cuboidal-shaped and characterized by the expression of surfactant proteins, which maintain the structural integrity and reduce surface tension in the alveoli by producing surfactants. In addition, ATII cells serve as progenitor cells of ATI cells, the cells responsible for respiratory gas exchange, contributing to the regeneration of alveolar tissue upon injury by reepithelialization. Telomerases maintain the progenitor function of ATII cells, while telomerase abnormalities impair their reparative function [141]. In a mouse model, telomere dysfunction in ATII cells was shown to induce epithelial senescence which was accompanied by increased numbers of α-SMA-positive mesenchymal cells, with increased levels of TGF-β, leading to increased collagen deposition and fibrosis [145]. Thus, exhaustion of ATII cells may be crucial in the development of pulmonary fibrosis.

Resident niches of epithelial progenitor cells expressing SFPTC/Scgb1a, p63/KT5/Scgb1a1, and α6β4/E-cadherin have renewal capacity like that of ATII cells in murine and human lungs [146–148], being capable of differentiating into both ATII and ATI cells [147]. During these processes, stromal support is essential, both for maintenance and renewal of the alveolar epithelium. Here, mesenchymal stromal/stem cells (MSCs) are of particular interest. MSCs are multipotent stem cells that are present in various tissues in the adult body, including bone marrow, skeletal muscle, umbilical cord, and adipose tissue. While their niche and microenvironment within the human lung have not been characterized extensively, resident MSCs are also present in the human lung [149, 150]. MSC can differentiate into cells from different lineages, including adipocytes, osteoblasts, chondrocytes, and myofibroblasts. Although several studies suggest that MSCs can express epithelial markers upon differentiation in vitro, it is still under debate whether MSCs can differentiate into functional ATII or ATI cells in vivo. Nonetheless, MSCs can support alveolar epithelial maintenance and repair via anti-apoptotic, tissue-protective, and regenerative activities, producing a wide range of growth factors. These include vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF)s, insulin-like growth factor (IGF), wingless/integrase-1 (WNT) ligands, Notch and Ang-1, and matrix molecules such as elastin and laminin to promote site-specific epithelial cell responses. In vitro, MSCs have been shown to facilitate elastic fiber regeneration, thus supporting neo-alveolarization [151]. Mouse models have demonstrated that elastin is critical for the formation of alveoli, and decreased elastin deposition results in defective alveolarization [152]. Laminin contributes to the maintenance of an alveolar epithelial phenotype and allows alveolar progenitor cells to proliferate [147]. In addition, MSCs have anti-fibrotic and immunomodulatory activities and secrete MMPs, tissue inhibitors of metalloproteinase (TIMPs), micro-RNAs, and anti-inflammatory factors into the damaged microenvironment, affecting the injured tissue [140]. Based on this broad array of characteristics, MSCs are predicted to have a regenerative potential for lung tissue injury and have captured particular attention for the anticipation of cell-based therapies in IPF (Fig. 9.4).

## *Preclinical Studies with Stem Cells in IPF*

Various exogenous stem cells have been used in animal models to investigate their potential in lung fibrosis, including MSCs, lung stem/progenitor cells, and ATII cell-differentiated embryonic stem cells. MSCs constitute the most widely described stem cells for regenerative medicine purposes, based on their paracrine reparative function. In addition, their release of anti-inflammatory factors suppresses allograft rejection and protects against inflammation-induced injury. MSCs also home to sites of tissue injury, and when administered intravenously, MSCs are predominantly found in the lung, followed by the liver and then the other organs [153]. Therefore,

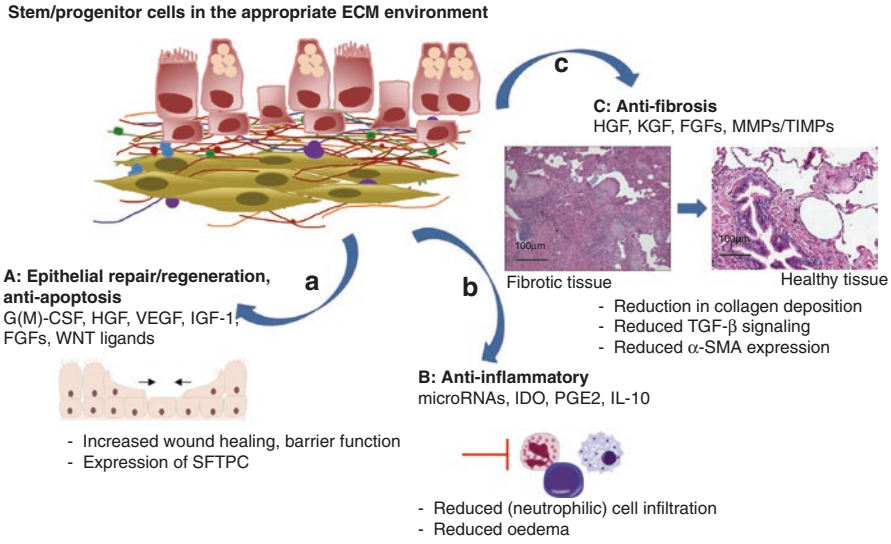


**Fig. 9.4** Potential of stem cell-based therapy in lung fibrosis. The potential of mesenchymal stem/progenitor cells for reversing the aberrant fibrotic response in lung diseases, with (**a**) epithelial repair/regeneration and anti-apoptosis capacity, (**b**) anti-inflammatory capacity, (**c**) anti-fibrosis capacity. *FGF* fibroblast growth factor, *G(M)-CSF* granulocyte (macrophage) colony-stimulating factor, *HGF* hepatocyte growth factor, *IDO* indoleamine 2,3-dioxygenase, *IGF-1* insulin-like growth factor 1, *KGF* keratinocyte growth factor, *MMPs/TIMPs* metalloproteinases/tissue inhibitor of metalloproteinases, *PGE2* prostaglandin E2, *SFTPC* surfactant protein C, *α-SMA* α-smooth muscle actin, *TGF-β* transforming growth factor-β, *VEGF* vascular endothelial growth factor, *WNT* wingless/integrase-1. Tissues were obtained from the tissue archive within the Department of Pathology and Medical Biology at the University Medical Center Groningen

cell-based therapies with MSCs have been suggested for the treatment of fibrotic lung disease. Some, but not all, preclinical studies in animal models of pulmonary fibrosis suggest that MSCs might indeed be effective in the treatment of IPF.

Several models have used bone marrow-derived MSCs (BM-MSCs), adipose-derived MSCs (ADSCs), or umbilical cord-derived MSCs (UC-MSCs) in bleomycin-induced pulmonary fibrosis, which upon intratracheal installation induces severe inflammation followed by fibrotic scarring in a temporal pattern over a 28–35-day period [154, 155]. Using this model of IPF, several groups have shown that the administration of MSCs ameliorates bleomycin-induced lung injury in mice [156–163], as reviewed by Alvarez and co-workers and Toonkel and co-workers [140, 153]. Findings from Ortiz and co-workers and Lee and co-workers showed that intravenous administration of BM-MSCs reduces bleomycin-induced lung edema, (neutrophilic) inflammation, collagen deposition, and overall mortality [156, 161]. Similar effects were observed upon intravenous administration of UC-MSCs in this model, reducing inflammation, TGF-β expression, and collagen deposition, while increasing MMP-2 expression [158]. Additionally, ADSCs were shown to reduce fibrosis/collagen content, epithelial apoptosis, and TGF-β expression upon

bleomycin-induced injury [160, 163, 164], to protect against body weight loss and increase survival rates [164]. In the latter study, a decrease in pro-fibrotic genes such as MMPs and FGFs was observed upon intravenous administration of ADSCs on days 3, 6, and 9 following bleomycin installation [164], which may be the consequence of the ameliorating effects on inflammation and/or epithelial damage.

Indeed, the protective effects of MSCs have been attributed to the suppression of inflammation and triggering production of reparative growth factors, including G-CSF and GM-CSF [157]. The intratracheal administration of bleomycin acts in three stages [165]. During the 1st week, bleomycin-induced cytotoxicity results in alveolar epithelial apoptosis and necrosis, which is followed by lung recruitment of neutrophils and macrophages. During the 2nd and 3rd week after bleomycin treatment, aberrant repair and tissue remodeling lead to collagen deposition and fibrosis [165]. Ortiz and co-workers were the first to demonstrate the protective effect of BM-MSCs on bleomycin-induced lung injury but also showed that the timing of administration is crucial [156]. When male BM-MSCs were injected in female mice upon bleomycin installation, decreased MMP and collagen mRNA expression in lungs was observed. However, no protective effects were observed when BM-MSCs were administered 7 days after the bleomycin challenge. Especially the immunomodulatory effects of BM-MSC are thought to contribute to the protective effects against lung damage, while the use of BM-MSC during the fibrotic phases of the repair response can be detrimental due to the production of growth factors, ECM molecules, and MMPs [165]. Along the same line, in a mouse model of radiation-induced lung fibrosis, the administration of BM-MSCs exerted protective effects at early time points, i.e., within hours after injury, while more engraftment in the lung interstitium and expression of myofibroblasts markers were observed after 60–120 days [165]. Nonetheless, MSCs also express various pro-fibrotic factors, and the administration during early stages of the disease may be essential. Because IPF is frequently only detected during later stages of the disease, caution is warranted for the use of MSCs as a cell-based therapy for IPF.

Other models beyond bleomycin-induced lung fibrosis have been used to determine the efficacy of MSCs. For instance, in a model of radiotherapy, thoracic irradiation (15 Gy of x-rays) resulted in complete disruption of the alveolar structure. Administration of ADSCs via the tail vein within 2 h after irradiation attenuated alveolar collagen deposition on day 28, as well as pro-inflammatory cytokine production (day 1–28), expression of pro-fibrotic factors including TGF-β (day 7–28), and apoptosis in lung tissue (day 3–28) [166]. In a rat model of silica-induced fibrosis, intravenous injection of BM-MSCs on days 1 and 4 after intratracheal silica installation restored alveolar epithelial damage and reduced collagen deposition at 15 and 30 days [167]. In ovalbumin (OVA)-sensitized mice, human BM-MSCs were intravenously injected twice after the OVA challenge protocol and found to reverse subepithelial collagen deposition in the airways as well as total lung collagen deposition [168]. In rats exposed to cigarette smoke 1 h daily for 56 days, intravenous administration of human BM-MSCs on days 29 and 42 reduced airway fibrosis. Similar but more pronounced effects were observed when induced pluripotent stem cells (iPSCs) instead of BM-MSCs were used [169].

Indeed, in addition to MSCs, iPSCs, lung stem/progenitor cells, and ATII cell-differentiated embryonic stem cells are being considered for regenerative strategies aimed at restoring fibrotic pathologies in the lung matrix. Embryonic cell-derived ATII cells have been transplanted into lung tissue in an acute bleomycin-induced model of lung injury in mice, showing beneficial effects on body weight recovery, arterial blood oxygen saturation, collagen deposition, and increased survival when administered one to two days after bleomycin-induced injury [170]. ATII cells differentiated from mouse iPSCs were intratracheally administered 24 h after installation of bleomycin, resulting in decreased inflammation and collagen deposition. Additionally, Zhou and co-workers demonstrated that mouse iPSCs, when delivered intratracheally 24 after installation of bleomycin, significantly reduce edema, collagen deposition, inflammatory mediators, and activation of TGF-β signaling and upregulate epithelial marker E-cadherin in lung tissue 3 weeks after installation of bleomycin [171, 172]. In a rat model, ATII cells were transplanted into bleomycin-damaged lungs at days 3, 7, and 15, which also reduced collagen deposition and severity of fibrosis at day 21 after bleomycin installation [173]. Because ATII cells produce less pro-fibrotic factors than MSCs, less detrimental, pro-fibrotic effects are to be expected from the administration of ATII-differentiated cells compared to MSCs, although more studies are warranted, e.g., on their impact during the fibrotic phase of lung injury.

A complicating factor for the use of all types of autologous stem cells is that IPF development is related to aging. Aging has been shown to impair MSC function in mice, with lower expression of inflammatory mediators [174], reduced proliferation [140], and impaired regenerative functions [175]. Similarly, aging and cellular senescence may result in exhaustion of other stem/progenitor cell types. As IPF is predominantly present in the elderly, the use of allogeneic stem cells, especially from younger individuals, may be of interest, although murine studies have shown that ADSCs may be less susceptible to aging-induced deficiencies [176]. Because of their immune-suppressive properties and being immune privileged, especially MSC-based therapies may qualify best for allogeneic stem cell therapy. Another option to overcome immunological limitations is the use of secreted factors in conditioned medium instead of stem cell-based therapy. For instance, the effect of the secretome of iPSCs has been investigated in a rat bleomycin model. Here, 24-h serum-free conditioned medium from iPSCs was instilled intratracheally 7 days after treatment with bleomycin. Another 7 days later, a marked improvement in fibrosis as assessed by histology and soluble collagen content was observed [177].

## *Clinical Trials with Stem Cells in IPF*

To date, accumulating evidence from clinical trials shows that intravenous or endobronchial transplantation of MSCs in humans is safe. Moreover, MSCs have been reported to exert beneficial effects in phase 1 and 2 clinical trials in immune-mediated diseases, such as graft-versus-host disease and Crohn's disease [178]. In a phase 1b, nonrandomized clinical trial in 14 IPF patients, there were no serious

adverse events within 12 months after 3 endobronchial infusions of autologous ADSCs [179]. Similar results were observed in a phase 1 safety clinical trial of nine IPF patients treated with a single intravenous injection of BM-MSCs during a follow-up of 15 months [180]. However, evidence for a beneficial role of transplanted MSCs in clinical trials with patients with lung fibrosis is currently lacking [181]. The challenge is considerable, because of the extensive abnormalities in the ECM of IPF patients that need to be restored. Recently, a case report showed that intravenous administration of human UC-MSCs in a 56-year-old man with IPF who was receiving long-term oxygen therapy, with a 12-month follow-up, found that the patient had a reduction in oxygen therapy, an increase in FEV1/FVC ratio, and improvement in their quality of life (St George's Respiratory Questionnaire; SGQR) and physical performance, as assessed by the 6-min walking test [182]. A single-center, randomized phase 1b study with allogeneic placenta-derived MSCs in patients with moderate to severe IPF and follow-up of 6 months showed that MSCs were well tolerated and had minor side effects. Upon intravenous injection of MSCs, a transient 1% (0–2%) fall in oxygen saturation was observed after 15 min, but there were no changes in hemodynamics. At 6 months, FVC, diffusion lung capacity for carbon monoxide ($DL_{CO}$), 6-min walking distance, and CT fibrosis score were unchanged compared with baseline [183]. Autologous ADSCs were also used in a prospective, nonrandomized placebo-controlled phase 1b clinical trial with 3 intrabronchial infusions in 14 IPF patients with mild to moderate disease. Again, no serious adverse events were observed, and ADSC-treated patients did not deteriorate in both functional parameters and indicators of quality of life. On the other hand, there were no significant improvements in functional parameters (FVC, $DL_{CO}$) or 6-min walking distance. Thus, although administration of MSCs in IPF patients during advanced stages of the disease does not result in adverse effects with respect to the fibrosis score within 6–12 months, no beneficial effects have been observed so far. Additional preclinical and ex vivo studies will be required to assess the optimal route of administration, the source of MSCs, dosage, and frequency of installation and possibly also new insight into the improvement of MSC engraftment, survival, and functioning of donated MSCs.

Furthermore, the reciprocal influence of these MSC-induced changes in the ECM environment on the responses and reparative capacity of endogenous MSCs and other stem cell types has not been examined to date. The lack of knowledge of this effect will need to be addressed as we try to move forward toward making cell-based therapies a reality in lung fibrosis.

## Conclusion

Until now, curative options for fibrotic lung diseases have not existed, with the possible exception of lung transplantation for a limited group of patients. The recent approval of the first anti-fibrotic drugs for lung diseases [184, 185], as well as the increasing development of immunomodulatory, biological, and anti-fibrotic

therapies, including cell-based therapies, has added to the complexity of therapeutic decision-making in this field. Currently, not only are clinical indicators restricted, but especially disease informative biomarkers are absent, or at the very least limited, necessitating an urgent need to understand each patient's pathophysiological processes better. A greater understanding of the dynamic interchange of the ECM in lung fibrosis will enhance not only our knowledge of the mechanisms underlying these detrimental pathologies but will provide opportunities for novel approaches to improve the quality of life from millions of individuals around the world.

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# Part III
# Heart

# Chapter 10
# Fibrotic Signaling in Cardiomyopathies

**Saranya Ravi, Monte S. Willis, and Jonathan C. Schisler**

## Classification, Etiology, and Pathophysiology of Cardiomyopathies

### *Dilated Cardiomyopathy*

Dilated cardiomyopathy (DCM) is a disorder of the heart muscle, most often within the left ventricle. The ventricle is enlarged and weak, resulting in poor blood pumping capacity (Fig. 10.1). A conclusive diagnosis of DCM requires the presence of dilated left ventricle or both ventricles along with deficiency in contraction of the ventricle [1]. Many factors can cause DCM including genetics, ischemic heart disease, hypertension, infections, alcohol, and toxins [2, 3]. Clinical studies have revealed a higher occurrence of ischemia-related DCM compared to nonischemic causes [4]. DCM can result in progressive heart failure due to compromised systolic function, arrhythmias, cardiomegaly, and thromboembolic events and cause sudden death [5, 6].

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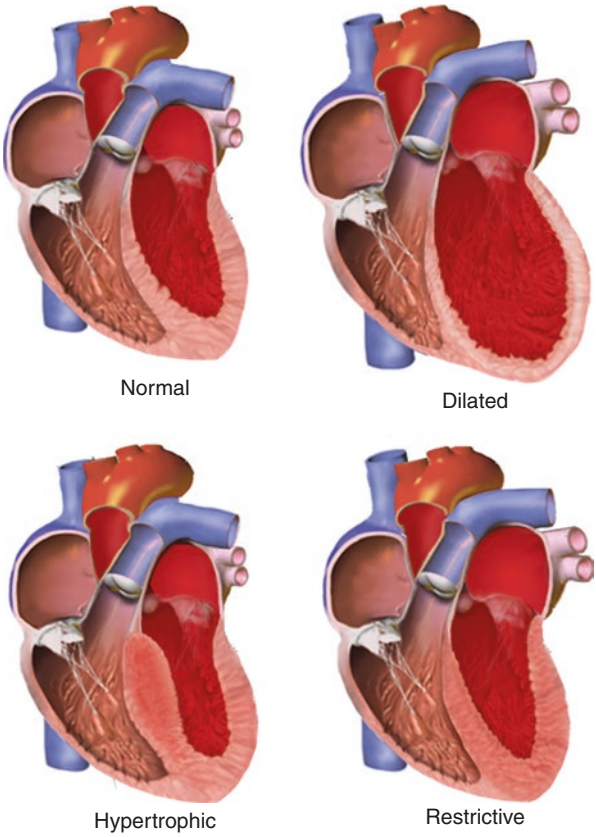


**Fig. 10.1** Structural categories of cardiomyopathies. Compared to a normal heart, dilated cardiomyopathies are characterized by an enlarged left ventricle. Hypertrophic cardiomyopathies result from increased wall thickness of the left ventricle. Restrictive forms of cardiomyopathies are caused by wall stiffening and abnormal ventricular filling. (Image source unmodified from Nicholas Patchett, CC BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0/))

Patients with ischemic heart disease also have some level of coronary artery disease that contributes to the ischemia. Ischemia promotes left ventricular remodeling and subsequent dilation of the ventricle and weakening of the muscle [7]. This hypoperfusion of the heart can trigger cardiomyocyte death, an integral part of ventricular remodeling that leads to a reduction in wall thickness and increases in diameter of the ventricle [8–10]. Subsequently, the remaining cardiomyocytes undergo compensatory hypertrophy [11, 12]. Additionally, as ventricular remodeling progresses, matrix metalloproteinases (MMPs) break down the extracellular matrix that supports the cardiomyocytes, leading to additional ventricular dilation and weakening [13, 14]. In response to the loss of cardiomyocyte mass, fibrosis is activated as a protective mechanism which leads to stiffening of the heart [15–18].

## *Hypertrophic Cardiomyopathy*

Hypertrophic cardiomyopathy (HCM) is defined as hypertrophy of the myocardium, notably left ventricular hypertrophy in the absence of hypertension or aortic stenosis (Fig. 10.1). The primary cause of HCM is genetic, and evidence suggests that HCM affects 1 in 500 adults, making it the most commonly occurring genetic cardiovascular

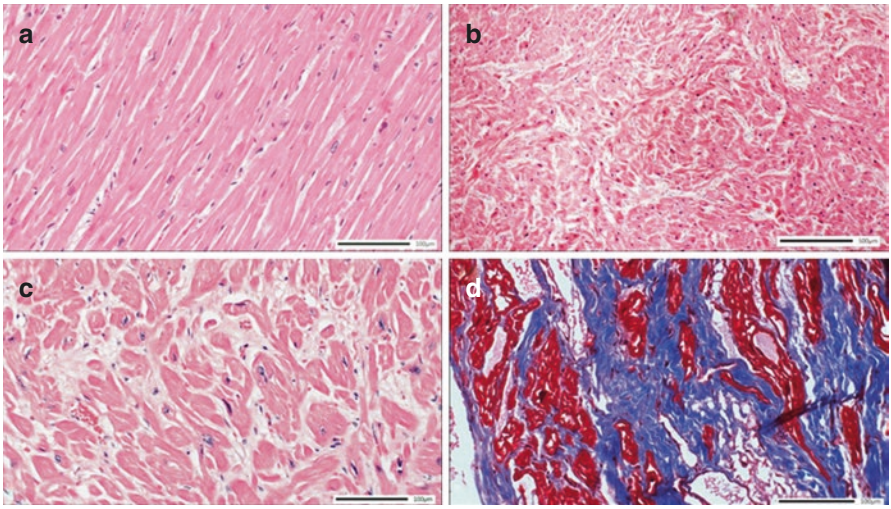


**Fig. 10.2** Histology of hypertrophic cardiomyopathy. (**a**) H&E-stained normal myocardial section. (**b**) H&E-stained myocardial section from patient with HCM, 4× magnification. (**c**) H&E-stained myocardial section from patient with HCM, 20× magnification. HCM sections display disorganized myocardial arrangement. (**d**) Masson's trichrome-stained myocardial section from patient with HCM, 20× magnification. Blue staining indicates fibrosis. (Marian and Braunwald [30])

disease [19, 20]. While hypertension and aortic stenosis can lead to left ventricular hypertrophy, the diagnosis of HCM is excluded if these pathologies are present [21]. HCM can lead to left ventricle outflow obstruction, mitral regurgitation, myocardial ischemia, diastolic dysfunction, and in some cases sudden cardiac death [22, 23].

A number of genes with several hundreds of mutations have been identified in HCM patients [24]. The most common genes are those that encode myocardial contractile proteins or the cardiac sarcomere [24, 25] including β-myosin heavy chain (MYH7), myosin-binding protein C (MYBPC3), cardiac troponin T (TNNT2), cardiac troponin I (TNNI3), α-tropomyosin (TPM1), cardiac α-actin (ACTC1), myosin regulatory light chain (MYL2), essential myosin light chain (MYL3), and cysteine- and glycine-rich protein 3 (CSRP3) [26, 27]. Causal mutations in these genes result in hypertrophied and disorganized cardiomyocytes as well as an increase in the proliferation of fibroblasts leading to interstitial fibrosis [28, 29] (Fig. 10.2). These pathological changes contribute to the cardiac dysfunction exhibited in patients with HCM. In addition to structural proteins, other genes that encode mitochondrial proteins, calcium handling proteins, as well as genes implicated in Fabry disease and glycogen storage diseases confer susceptibility to HCM [24] (Fig. 10.2, [30]).

## *Arrhythmogenic Right Ventricular Cardiomyopathy*

Arrhythmogenic right ventricular cardiomyopathy (ARVC), also called arrhythmogenic right ventricular dysplasia, is predominantly a right ventricular heart muscle disease. Studies estimated that ARVC occurs in 1 in 5000 to 1 in 2000 people in the

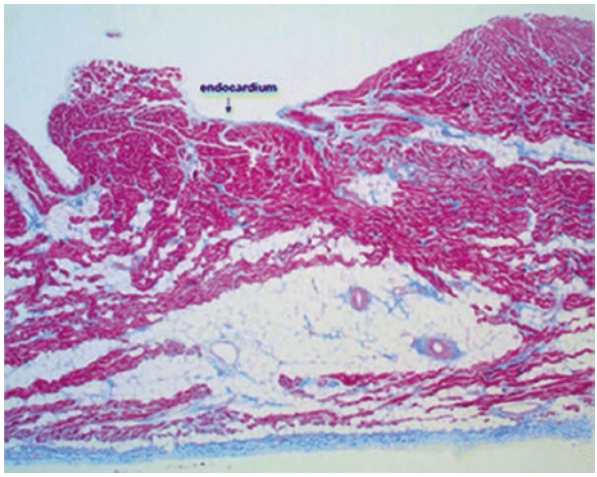


**Fig. 10.3** AZAN staining of human right ventricle depicting cardiomyocytes (red), fibrosis (blue), and adipocytes (white). (Cox and Hauer [46])

general population and is often attributed to a genetic defect [31, 32]. Mutations in cardiac desmosomes are causative in ARVC [33–37]. Hallmarks of ARVC include right ventricle dilation and myocardial thinning [38, 39] as well as the presence of T-lymphocyte infiltration, myocyte death, and replacement of right ventricle myocardium with fibrofatty tissue [38, 40]. ARVC presents clinically as palpitations and ventricular arrhythmias and can also result in sudden death [39, 41, 42].

The desmosome is an intercellular adhesion junction that anchors the cell membrane to intermediate filaments of the cytoskeleton, providing structural integrity [43]. In the heart, the desmosomes connect and hold in place the cardiomyocytes. Causal genes for ARVC are desmoplakin (DSP), plakophilin 2 (PKP2), desmoglein 2 (DSG2), and desmocollin 2 (DSC2) [33–37]. These mutations in desmosome-related proteins disrupt cellular junctions and lead to cardiomyocyte death (Fig. 10.3). Desmosome mutations also activate adipogenic and fibrotic pathways, resulting in replacement of myocardium with fibrofatty tissue (Fig. 10.3) [44, 45] (Fig. 10.3, [46]).

## *Restrictive Cardiomyopathy*

Restrictive cardiomyopathy (RCM) is a disease characterized by stiffening of ventricular walls, resulting in an abnormal ventricular filling (Fig. 10.1). This disease can affect either left or right ventricles. However, the systolic function is usually normal. There are several causes of RCM, which can be either genetic or acquired [47–49]. Among the causes of ventricular wall stiffening is the extensive interstitial fibrosis detected in patients with RCM [47]. Amyloidosis, sarcoidosis, endomyocardial fibrosis, storage diseases such as Fabry disease, Gaucher disease, and hemochromatosis, as well as mutations in sarcomeric proteins, and diabetic

cardiomyopathy are other known causes of RCM [47, 48]. Given the full range of underlying conditions that lead to RCM, there are geographic differences in the occurrence and cause of the disease. RCM leads to peripheral edema, fatigue, exercise intolerance, elevated central venous pressure, and heart failure [47, 48].

## *Desminopathies*

Mutations in desmin, alphaB-crystallin, and other proteins that associate with desmin can lead to desmin-related cardiomyopathy [50, 51]. Desmin is an intermediate filament protein that is required for the structural integrity of the cardiac muscle. In the heart, desmin is vital to connect both the sarcomere with the extracellular matrix, as well as for connecting adjacent sarcomeres [52]. Mutations in desmin and desmin-associated genes disrupt the structure and alignment of myofibrils and lead to the deposition of inclusion bodies within the cardiomyocytes (Fig. 10.4). These cytoplasmic inclusion bodies consist of misfolded desmin and alphaB-crystallin [51, 53, 54]. Desminopathies are associated with a diagnosis of DCM, RCM, and HCM [55–61]. Other histological findings in desmin-related cardiomyopathy include cardiomyocyte hypertrophy, mitochondrial disorganization and swelling, and extensive interstitial fibrosis [52, 62] (Fig. 10.4 modified from [63]).

## *Diabetic Cardiomyopathy*

Diabetic cardiomyopathy (DiCM) is defined as ventricular dysfunction in the absence of any recognized causes such as coronary artery disease or hypertension [64]. Diabetes increases the risk of heart failure in both men and women independent of other comorbidities [65]. Poor glycemic control leads to left ventricular hypertrophy, toxicity associated with accumulation of lipids in fibroblasts and cardiomyocytes, interstitial fibrosis, and increased death of cardiomyocytes, fibroblasts, and endothelial cells. Diabetic cardiomyopathy results in both systolic and diastolic dysfunction, impaired heart contractility, and heart failure [66].

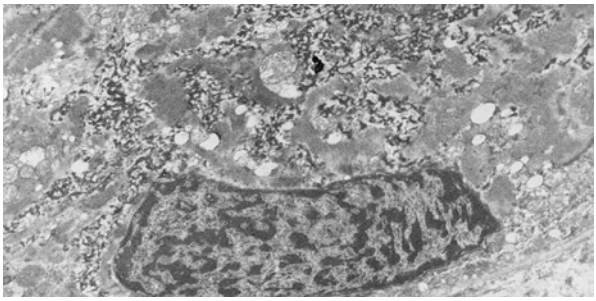

**Fig. 10.4** Transmission electron micrograph images of human cardiac muscle. Biopsy from a 27-year-old male with restrictive cardiomyopathy. Desmin deposits are found in the intermyofibrillar region. (Arbustini et al. [63])

Remarkably, the pathophysiology of these cardiomyopathies with diverse etiologies reveals that fibrosis is a prominent component in cardiomyopathies and likely contributes to the pathologies observed. Next, we discuss the role of cardiomyocytes and fibroblasts in the fibrotic response.

## *Mouse Models of Cardiomyopathies*

Rodent models, in particular Mus musculus, are often used to study the etiology of cardiomyopathies and are used as preclinical models to test therapies. These models often utilze various genetic manipulations including knockout (gene silencing), knockin (gene editing), or transgene expression. The targeted gene or experimental treatemnt as well as the resulting cardiomyopathy classification are summarized in Table 10.1, encompassing models of DCM, HCM, ARVC, and DiCM.

**Table 10.1** Mouse models of cardiomyopathies. The genetic manipulation or experimental treatment (Model) to induce the indicated classification of cardiomyopathy (Disease): DCM (dilated cardiomyopathy), HCM (hypertrophic cardiomyopathy), DiCM (diabetic cardiomyopathy), RCM (restrictive cardiomyopathy), and ARVC (arrhythmogenic right ventricular cardiomyopathy). When known, the pathways implicated in the disease are indicated.

| Model | Disease | Pathway |
|---|---|---|
| S532P mutation in α-myosin heavy chain (MHC) – mouse | DCM [270] | |
| F764L mutation in α-myosin heavy chain (MHC) – mouse | DCM [270] | |
| Dystrophin/utrophin knockout – mouse | DCM [271] | |
| Muscle LIM protein knockout – mouse | DCM [272] | |
| Overexpression Gαq – mouse | DCM [273] | |
| Feeding of furazolidone for 14 days – turkey poults | DCM [274] | |
| Abcg5 mutation in exon 10 from G to A – mouse | DCM [275] | |
| Expression of dominant-negative form of CREB transcription factor (CREBA133) – mouse | DCM [276] | |
| Overexpression of TNFα in the heart – mouse | DCM [277] | TNFα |
| Expression of human retinoic acid receptor-α under the control of β-myosin heavy chain promoter – mouse | DCM [278] | |
| Tropomodulin overexpression – mouse | DCM [279] | |
| Mutation in immunoglobulin mu binding protein 2 – mouse | DCM [280] | |
| Cardiac-specific overexpression of myocyte enhancer factor 2 (MEF2) transcription factors MEF2A and MEF2C – mouse | DCM [281] | Calcineurin, MAPK |
| Expression of mutant KDEL receptor – mouse | DCM [282] | |
| Cardiac-specific knockout of Cypher – mouse | DCM [283] | |
| Cardiac-specific expression of ZASP S196L mutation – mouse | DCM [284] | |
| Overexpression of N-cadherin – mouse | DCM [285] | |
| Overexpression of E-cadherin – mouse | DCM [285] | |
| Constitutively active Rac1 in the myocardium – mouse | DCM [286] | Rho-like GTPases |
| Calreticulin overexpression in the heart – mouse | DCM [287] | |

| Model | Disease | Pathway |
|---|---|---|
| Cardiac-specific overexpression of calsequestrin – mouse | DCM [288] | |
| Autoantibodies targeted against β1-adrenoreceptor – mouse | DCM [289] | |
| Cardiomyocyte-specific overexpression of $\alpha$-$_{1B}$ adrenergic receptor – mouse | DCM [290] | |
| Ventricle-specific deletion of Erb-B2 receptor tyrosine kinase 2 – mouse | DCM [291] | |
| Deletion of Mn superoxide dismutase – mouse | DCM [292] | |
| Knockout of PD-1 receptor – mouse | DCM [293] | |
| Infection with encephalomyocarditis virus – mouse | DCM [294] | |
| Dominant-negative mutation of vasodilator-stimulated phosphoprotein (VASP) and mammalian enabled (Mena) in the heart – mouse | DCM [295] | |
| Expression of dominant-negative neuron-restrictive silencer factor in the heart – mouse | DCM [296] | |
| Cardiomyocyte deletion of vinculin – mouse | DCM [297] | |
| Non-obese diabetic mice expressing human MHC class II molecule DQ8 and deletion of the mouse equivalent gene IAβ – mouse | DiCM, DCM [298] | |
| Cardiac-specific overexpression of PPARγ – mouse | DCM [299] | |
| E361G mutation in alpha-cardiac actin gene (ACTC) – mouse | HCM [300] | |
| R403Q mutation in α-myosin heavy chain (MHC) – mouse | HCM [301] | |
| R400Q mutation in β-myosin heavy chain – rabbit | HCM [302] | |
| Truncated troponin T missing exon 16 – mouse | HCM [303] | |
| R92Q troponin T mutation – mouse | HCM [304] | |
| Cardiac troponin T mutations: R92L, R92W, R94L, A104V, R130C, E163R, S179F, E244D – mouse | HCM [305] | |
| Mutations in myosin-binding protein C – mouse | HCM [306, 307] | |
| D175N mutation in α-tropomyosin – mouse | HCM [308] | |
| Naturally occurring deletion of exon 1 of the delta-sarcoglycan gene – hamster | HCM, DCM [309] | |
| Spontaneously occurring HCM in cats | Familial HCM [310] | |
| α-Myosin heavy chains lacking the light chain binding domain – mouse | HCM [311] | |
| Inducible troponin T Q92 switch on switch off – mouse | HCM [312] | |
| R45G troponin I mutation – mouse | HCM [313] | |
| D166V mutation in ventricular myosin regulatory light chain – mouse | HCM [314] | |
| R193H troponin I mutation – mouse | RCM [315] | |
| R145W troponin I mutation – mouse | RCM [316] | |
| Mutation desmocollin-2 – zebra fish | ARVC [317] | |
| Spontaneously occurring mutations in ryanodine receptor (RyR2) – canine | ARVC [318] | |
| Knockin of human RyR2 mutation R176Q – mouse | ARVC [319] | |

(continued)

**Table 10.1** (continued)

| Model | Disease | Pathway |
|---|---|---|
| Mutation in laminin receptor 1 – mouse | ARVC [320] | |
| V30M, Q90R, W233X, and R2834H mutations in desmoplakin – mouse | ARVC [321] | |
| Knockout of desmoplakin – mouse | ARVC [322] | Wnt/β-catenin |
| R735X mutation in plakophilin 2 – mouse | ARVC [323] | |
| Plakoglobin knockout – mouse | ARVC [324] | |
| Plakoglobin knockdown – zebra fish | ARVC [325] | Wnt/β-catenin |
| Streptozotocin-induced type I diabetes – mouse, rat | DiCM [326] | |
| Alloxan-induced diabetes – mouse, rat, rabbit | DiCM [327] | |
| Overexpression of calmodulin (OVE26 mouse) – mouse | DiCM [328] | |
| Heterozygous mutation in insulin 2 (Akita mouse) – mouse | DiCM [329] | |
| Mutation in leptin gene (ob/ob mouse) – mouse | DiCM [330] | |
| Leptin receptor mutation (db/db mouse) – mouse | DiCM [331] | |
| Missense mutation in rat leptin receptor (ZDF rat) – rat | DiCM [332] | |
| Cardiac-specific overexpression of PPARα – mouse | DiCM [333] | |
| Cardiac-specific overexpression of long-chain acyl-CoA synthetase – mouse | DiCM [334] | |
| Heart muscle-specific expression of human lipoprotein lipase – mouse | DiCM [335] | |
| Cardiac-specific overexpression of fatty acid transport protein 1 – mouse | DiCM [336] | |
| Overexpression of protein kinase C β2 in the myocardium – mouse | DiCM [337] | |
| Ablation of brown adipose tissue (UCP-DAT mouse) – mouse | DiCM [338] | |
| Cardiomyocyte-selective insulin receptor knockout – mouse | DiCM [339] | |
| Cardiac-specific deletion of GLUT4 – mouse | DiCM [340] | |
| Deficiency of adipose triglyceride lipase – mouse | DiCM [341] | |
| Lack of PDK1 in cardiac muscle – mouse | DiCM [342] | |
| Expression of dominant-negative mutant phosphoinositide 3-kinase (PI3K) in the heart leading to downregulation of PI3K – mouse | DiCM [343] | |
| Knockout of glucokinase in the liver – mouse | DiCM [344] | |

## Cell Types Involved in Cardiac Fibrosis

Fibrosis is the process of thickening and scarring as a result of deposition of extracellular matrix proteins by fibroblasts. Fibrosis in the heart leads to stiffening of the cardiac muscle which can uncouple the mechano-electric system, increasing the risk of arrhythmias and causing heart failure [67, 68]. Fibroblasts are cells responsible for production of extracellular matrix components (ECM) such as collagen types I, III, IV, V, and VI, laminin, and elastin [69]. Fibroblasts also play an

essential role in signaling by secreting cytokines, proteases, and growth factors [70] as well as producing MMPs that break down ECM structures [71]. There is more than one source of fibroblasts in the heart. The first is resident fibroblasts present in the myocardium [68, 72]. In addition, endothelial cells from the vasculature can undergo an endothelial-mesenchymal transition (EMT) upon pro-fibrotic signals and take on a fibroblast-like phenotype [68, 72]. Perivascular cells from the cardiac vasculature have also shown to acquire a fibroblast-like phenotype via the production of collagen. Finally, bone marrow-derived progenitor cells and monocytes are both believed to be cell types that contribute to the fibroblast population within the heart [68, 72].

Fibrosis occurs in patterns of reactive interstitial fibrosis and replacement fibrosis. In reactive fibrosis deposits, accumulation of ECM in the interstitial areas occurs as an adaptive mechanism to maintain the pressure-producing capacity of the heart. Cardiac pressure overload, diabetes, and aging result in an interstitial fibrosis pattern [73, 74]. This type of reactive fibrosis occurs in early stages of the disease, and as the pathology progresses and cardiomyocyte hypertrophy and death occur, replacement fibrosis can be observed [75].

During ischemia, mechanical stress, or other injuries to the heart, the pro-fibrotic signals cause the differentiation of fibroblasts to myofibroblasts [76]. These myofibroblasts increase the production and secretion of ECM matrix components and have increased expression of alpha-smooth muscle actin protein (αSMA). This "differentiated" state of fibroblasts is associated with cardiac fibrosis and pathogenesis. The expression of αSMA is required for contractility of the myofibroblasts, which aids in wound healing; however, this can contribute to arrhythmias in the heart [77]. Myofibroblast MMP production results in the breakdown of ECM proteins which in turn triggers a fibrotic response [78]. Impaired cardiac function due to fibrosis limits oxygen supply to the myocardium, which can also lead to impairment of cardiac muscle function [79].

Crosstalk between fibroblasts and cardiomyocytes is important in cardiac muscle homeostasis [80]. Exosomes play a critical role in this communication between fibroblasts and cardiomyocytes. Exosomes are formed in the cellular endosomal network. Inward blebbing of the endosomal plasma membrane leads to the formation of early endosomes. A second event occurs wherein many of these early endosomes are captured in another section of the endosomal membrane leading to the formation of late endosomes or multivesicular bodies (MVBs). MVBs can then fuse the cell membrane and are expelled into the extracellular space [81]. Studies have shown that stimulation of fibroblasts with angiotensin II (AngII) increases exosome production by fibroblasts. In turn, these exosomes lead to an increase in the expression of AngII in cardiomyocytes, which is a potent hypertrophic signal for these cells [82]. Myofibroblasts secrete multiple proteins that can lead to cardiomyocyte hypertrophy, for example, the transcription factor Krüppel-like factor 5 (KLF5), which induces a transcriptional hypertrophic program in cardiomyocytes [83]. Another paracrine effector secreted by myofibroblasts that trigger cardiomyocyte hypertrophy is the microRNA miR-21 [84]. A study showed that fibroblast-derived exosomes

are enriched in miR-21, which are subsequently taken up by cardiomyocytes, and trigger the decreased expression of sorbin and SH3 domain-containing protein 2 (SORBS2) and PDZ and LIM domain 5 (PDLIM5), resulting in a hypertrophic response in the cardiomyocytes.

Crosstalk can occur in the opposite direction as well. Hypertrophic stimuli toward cardiomyocytes can activate apoptosis signal-regulating kinase 1 (ASK1), leading to necrotic and apoptotic cardiomyocyte death [85, 86]. As previously discussed, cardiomyocyte death triggers a robust proliferative response in the fibroblasts. Additionally, cardiomyocytes themselves can act as a trigger of fibrotic response. Cardiomyocyte-specific deletion of Krüppel-like factor 6 (KLF6) in a mouse model increased fibrosis after infusion of AngII [87].

## Fibrotic Signaling Pathways in Ischemic Heart Disease-Related Cardiomyopathy

### *TGFβ*

Transforming growth factor β (TGFβ) is a cytokine that enhances the production of extracellular matrix proteins many different organ systems [88, 89]. There are three different isoforms of TGFβ – TGFβ1, TGFβ2, and TGFβ3 – which have distinct expression patterns in different organs. All three isoforms are expressed in the heart. In particular, expression of TGFβ is seen in fibroblasts, endothelial cells, vascular smooth muscle cells, and macrophages [90].

The fibrosis and collagen deposition observed in HCM and DCM is thought to be associated with TGFβ activity. TGFβ expression level is 2.5 times greater in the myocardium of HCM hearts [91], and plasma concentrations of TGFβ are two times higher in patients who were diagnosed with idiopathic dilated cardiomyopathy compared to healthy controls [92].

Ischemic injuries and myocardial infarction trigger the upregulation of TGFβ activity. After an infarct occurs, the myocardium undergoes a healing process which consists of three distinct phases – an inflammatory phase, a proliferative phase, and the maturation phase [93]. During the initial phase of wound healing, platelets are hypothesized to be the source of TGFβ. Even low concentrations of TGFβ have a very powerful chemotactic effect, recruiting monocytes, lymphocytes, and neutrophils to the wound area [94, 95]. The neutrophils and macrophages facilitate clearance of damaged or necrotic cardiomyocytes. In the next phase of wound healing, the inflammatory cascade is inhibited, and fibroblasts and endothelial cells are recruited to the wound area [93, 96]. Remarkably, TGFβ also plays a role in the suppression of inflammation. The balance between the pro- and anti-inflammatory actions of TGFβ is controlled by the environment of other cytokines and chemokines expressed in the tissue [95, 97]. During the proliferative phase, macrophages and fibroblasts are the primary sources of TGFβ [93]. TGFβ is responsible for the differentiation of fibroblasts into myofibroblasts, which then secrete ECM proteins

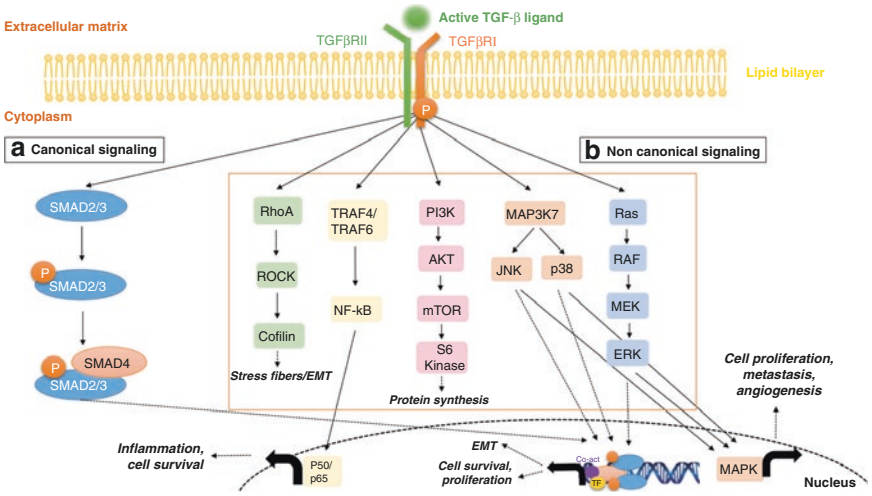


**Fig. 10.5** Canonical and noncanonical TGFβ signaling pathways. (**a**) Canonical signaling pathway: TGFβ ligands bind to TGFβRII, which in turn activates TGFβRI. TGFβRI-regulated SMAD2/3 proteins are phosphorylated at their C-terminal serine residues and form complexes with SMAD4 (co-SMAD), initiating a number of biological processes through transcriptional regulation of target genes. (**b**) Noncanonical signaling pathways: the TGFβ receptor complex transmits its signal through other factors, such as the mitogen-activated protein kinases (MAPKs), phosphatidylinositide 3-kinase (PI3K), TNF receptor-associated factor 4/6 (TRAF4/6), and Rho family of small GTPases. Activated MAPKs can exert transcriptional regulation either through direct interaction with the nuclear SMAD protein complex or via other downstream proteins. Moreover, activated JNK/p38/ERK act in concert with SMADs to regulate cellular function. RhoA/ROCK can be activated by TGFβ to induce actin stress fiber formation during EMT via a non-transcriptional mechanism. TGFβ can activate PI3K and AKT by inducing a physical interaction between the PI3K p85 subunit and the receptor complex leading to translational responses via mTOR/S6kinase activation. TGFβ activation of the TRAF proteins can initiate nuclear factor-κB (NF-κB) signaling activity, leading to the inflammatory response among other processes. The arrows indicate activation/signaling direction of the respective pathway. (Image source unmodified from doi.org/10.3390/jcm6010007, CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/))

responsible for filling in the void left by the necrotic cardiomyocytes. In conjunction with ECM synthesis, TGFβ also suppresses the expression of MMPs. Finally, during the maturation phase, the differentiated myofibroblasts undergo fibrosis leaving behind a scar made up of collagen [98].

TGFβ signaling is initiated when the ligand (TGFβ) binds to the TGFβ type II receptor dimer extracellularly (Fig. 10.5). This binding recruits the TGFβ type I receptor dimer [99, 100]. Both the type I and type II receptors are serine/threonine kinases consisting of extracellular, transmembrane, and intracellular domains. The type II receptor kinase then phosphorylates the cytoplasmic side of the type I receptor, forming the activated TGF complex [99, 100].

TGFβ receptor activation can signal through Smad and non-Smad downstream pathways (Fig. 10.5). The Smad proteins consist of three families of

proteins – receptor-activated Smads (R-Smads), co-mediator Smads, and inhibitory Smads [101]. The activated TGFβ receptor complex can directly phosphorylate the R-Smad, Smad2. Phosphorylated Smad2 binds to Smad3 and Smad4, and this complex localizes to the nucleus where it can bind to the regulatory regions of target extracellular matrix proteins, initiating their transcription and thereby upregulating the expression of these genes [100, 101] (Fig. 10.5, [102]).

### *ERK MAPK Pathway*

The ERK MAPK pathway is an example of a non-Smad pathway regulated by TGFβ. Binding of TGFβ to its receptors can lead to phosphorylation of tyrosine residues on both the type I and type II receptors [103]. Tyrosine phosphorylated type I receptor can phosphorylate ShcA. Phosphorylated ShcA can associate with Grb2/Sos proteins [104]. Recruitment of Sos to the plasma membrane catalyzes the exchange of GDP for GTP in the Ras protein, thereby activating it [105, 106]. Ras activation leads to activation of Raf, which phosphorylates and actives MEK, which in turn directly phosphorylates and activates ERK (Fig. 10.5). ERK translocates to the nucleus where it phosphorylates a number of transcription factors, including AP-1, leading to an increased production of extracellular matrix proteins such as collagen [107].

### *JNK/p38 Pathway*

Activation of the JNK and p38 pathway through TGFβ begins with the association of TRAF6 with the TGFβ receptor complex [108, 109]. This leads to activation of RING finger E3 ligase and polyubiquitination of K63 on TRAF6. This leads to recruitment of TAK1 (TGFβ-activated kinase 1) and polyubiquitination [110, 111]. TAK1 is a MAP3K, which can phosphorylate and activate MKKs – MKK4 and MKK3/6. MKK4 phosphorylates and activates JNK, while MKK3/6 phosphorylates p38 [111]. Both p38 and JNK phosphorylate c-Jun which, as seen with ERK signaling mentioned above (Fig. 10.5), activates the transcription factor AP-1, stimulating the production of extracellular matrix proteins [112, 113]. Another transcription factor that is activated by p38 activity is ATF-2, which has a significant role in the transcription of pro-inflammatory mediators. ATF-2 can also form a transcription complex with Smad 2,3,4, further driving the pro-fibrotic response [114]. Another role of the JNK/p38 pathway signaling through the ATF-2 transcription factor is to stimulate EMT, increasing the expression of fibrotic mediators [115].

### *PI3K/AKT Pathway*

Binding of TGFβ to its receptor and assembly of the TGFβ receptor complex activate PI3K and phosphorylation of its downstream effector AKT (Fig. 10.5) [116]. AKT activates mTOR cis phosphorylation at Ser2448. This triggers a phosphorylation

cascade, leading to the phosphorylation of S6K (ribosomal protein S6 kinase) and 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1), both of which are required for protein synthesis involved in EMT [106, 117, 118]. The increase in PI3K/AKT singling via mTOR increased the expression of collagen I in dermal fibroblasts [119], highlighting how this pathway plays a dual role in EMT and by directing the production of collagen, both contributing to increased fibrosis (Fig. 10.5, [120]).

### *Rho-Like GTPases*

The family of Rho-like GTPases, RhoA, Rac, and Cdc42 are essential signaling proteins in the regulation of EMT [121]. RhoA is a small GTPase that controls cytoskeletal organization and dynamics regulated by TGFβ. The activation of the Smad pathway by TGFβ leads to the transcription and translation of NET1, an activator of RhoA [122]. RhoA activation activates the kinase ROCK1 which phosphorylates LIM kinase, leading to phosphorylation of cofilin. Phosphorylation of cofilin inhibits actin filament disassembly, resulting in the formation of actin stress fibers, an event that promotes EMT [122]. While TGFβ activates RhoA, surprisingly it also plays a role in degrading RhoA. RhoA interacts with the TGFβ receptor complex at tight junctions, wherein the TGFβ type II receptor phosphorylates Par6 [111, 123]. Phosphorylated Par6 recruits Smurf1, and this complex regulates the ubiquitination and degradation of RhoA which causes the disintegration of tight junctions, thereby leading to EMT [111, 123]. Both the activation and degradation of RhoA through TGFβ are required for the pro-fibrotic response stimulated by EMT.

TGFβ is also responsible for the activation of Cdc42 and Rac1. Cdc42 is recruited to the TGFβ receptor complex, which recruits a number of different proteins including Rac1 exchange factors, PAK-1, and occludin. The interaction of occludin with TGFβ receptor type I encourages the localization of the type I receptor to the tight junction, which leads to loss of the tight junction integrity and ultimately EMT [111, 124].

### *Wnt/β-Catenin*

Wnts are secreted lipoglycoproteins that bind to the transmembrane receptor Frizzled (Fz). In the absence of Wnt ligands, a cytoplasmic destruction complex actively degrades the signaling protein β-catenin (Fig. 10.6). This destruction complex consists of Axin, adenomatous polyposis coli (APC), GSK-3 and casein kinase 1 (CK1), and the E3-ubiquitin ligase βTrCP. In the active state, this complex leads to the phosphorylation and subsequent ubiquitination and degradation of β-catenin by the proteasome [125]. This process is antagonized when Wnt ligands bind to the cell surface Fz receptor. Wnt-Fz binding recruits the co-receptors lipoprotein-receptor-related proteins (LRP)5 and LRP6 [126, 127]. In the cytosol, the LRP co-receptors are phosphorylated by GSK3 and CK1, leading to the activation of the Fz-LRP complex [126, 128, 129]. This activated Fz-LRP complex sequesters the GSK3/Axin destruction complex, thus preventing the

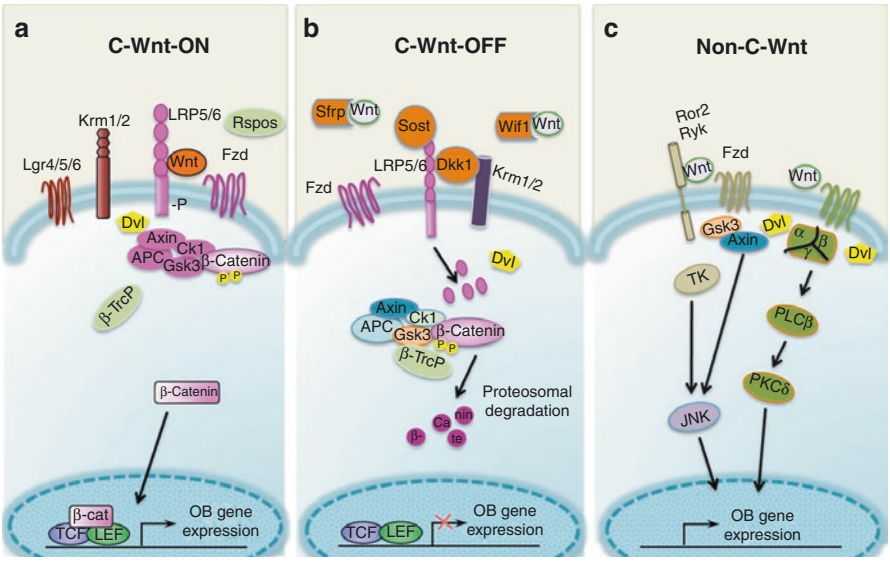


**Fig. 10.6** Wnt/β-catenin pathway. (**a**) In the Wnt On state, Wnt binds to Frizzled receptor activating LRP5/6 causing the recruitment of Dvl and the destruction complex to the plasma membrane and excluding βTrCP, which prevents the proteasomal degradation of β-catenin. β-Catenin then translocates to the nucleus and associates with transcription factor TCF/LEF to stimulate gene transcription. (**b**) The β-catenin destruction complex: in the Wnt Off state, the destruction complex consisting of Axin, APC, GSK-3 and CK1, and βTrCP targets β-catenin for destruction by the proteasome. (**c**) Noncanonical WNT signaling triggers its effects through alternative pathways including WNT/Rho-Rac- and WNT/G-protein-coupled receptors

ubiquitination and degradation of β-catenin [130]. Subsequently, β-catenin translocates to the nucleus and associates with transcription factors T-cell factor (TCF) and lymphoid enhancer-binding factor-1 (Lef-1) leading to changes in the expression of fibrotic genes (Fig. 10.6) [131, 132]. Activation of Wnt/β-catenin increases the synthesis of collagen, α-smooth muscle actin, and differentiation of fibroblasts to myofibroblasts [133–136].

It should be noted that there is crosstalk between the Wnt/β-catenin and TGFβ pathways. Wnt signaling is repressed in LRP5-deficient mice, leading to decreased TGFβ expression and fibrosis [137]. TGFβ activity reportedly increases singling through Wnt by downregulating DKK1, a Wnt pathway inhibitor [138] (Fig. 10.6, [139]).

## *YAP/TAZ Pathway*

Mechanical signals such as extracellular matrix stiffness and cell contact regulate the YAP/TAZ pathway. The YAP/TAZ pathway is the mammalian analog of the Hippo pathway in *Drosophila* (Fig. 10.7). The first step in this signaling cascade is the association of the cytoplasmic kinases MST1 (mammalian Ste20-like kinase1)

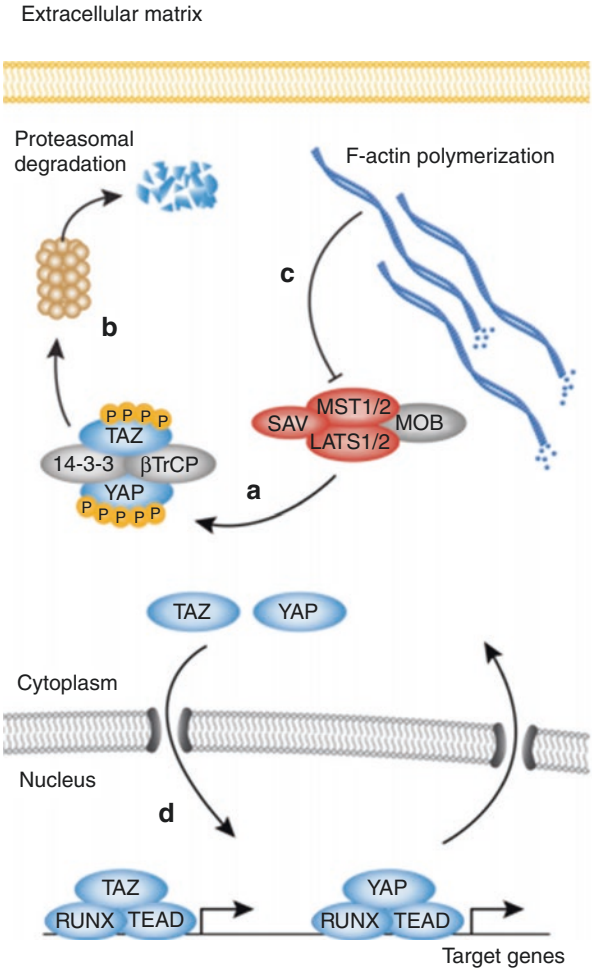


**Fig. 10.7** YAP/TAZ signaling pathway. (**a**) YAP and TAZ are phosphorylated by the Hippo kinase complex consisting of MST1/2, Salvador (Sav), and LATS1/2. (**b**) Phosphorylated YAP/TAZ is sequestered in the cytoplasm by 14-3-3 proteins and βTrCP and is targeted for degradation. (**c**) F-actin polymerization inhibits the formation of the Hippo kinase complex. (**d**) YAP and TAZ translocate to nucleus and associate with transcription factors. (Image source unmodified from doi.org/10.3389/fmed.2015.00059, CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/))

and MST2 with Salvador [140]. This enzyme complex can phosphorylate the MOB1A/B subunits of LATS1 (large tumor suppressor kinase 1) and LATS2. The activated LATS1/2 complex phosphorylates YAP and TAZ, which then associate with 14-3-3 proteins and βTrCP. βTrCP polyubiquitinates and targets YAP and TAZ for proteasomal degradation [127, 141]. When cells are grown on a compliant surface or grown under confluent conditions promoting cell-cell contacts, F-actin polymerization is increased, stimulating the dephosphorylation of YAP and TAZ, which in turn leads to nuclear translocation of YAP and TAZ [140, 142, 143]. In the nucleus, YAP and TAZ bind to transcription factors such as TEA DNA-binding domain (TEAD), inducing the expression of collagen and α-smooth muscle actin (Fig. 10.7) [144, 145].

Not surprisingly, there is crosstalk between the YAP/TAZ, TGFβ, and Wnt/β-Zcatenin pathways. YAP- and TAZ-deficient fibroblasts produce less ECM proteins and express lower amounts of myofibroblast markers in response to TGFβ

stimulation [144]. YAP can bind to Smad7, which has a high affinity for the TGFβ type I receptor and can, therefore, inhibit TGFβ signaling [146]. Furthermore, TAZ interacts with Smad2/3/4 complexes and can dictate the localization of a TAZ/Smad complex. At low levels of TAZ, the Smad complex and TAZ localize to the nucleus, initiating pro-fibrotic transcription gene expression, whereas, at higher levels of TAZ, the Smad complex and TAZ remain in the cytoplasm [147].

TAZ communicates with the Wnt/β-catenin pathway by binding to phosphorylated β-catenin. This results in both proteins being degraded by the proteasome. However, when Wnt signaling is activated, dephosphorylated TAZ is unable to bind β-catenin; thereby, both these proteins can translocate to the nucleus and activate transcription programming. Similarly, phosphorylated YAP can bind to β-catenin and prevent translocation to the nucleus [148] (Fig. 10.7, [127]).

## *AMPK*

5′ adenosine monophosphate-activated protein kinase or AMPK is an energy sensor that plays a crucial role in maintaining energy homeostasis. AMPK plays a protective role in the pathology of cardiac fibrosis. AMPK is activated when the ratio of AMP to ATP increases and stimulates the production of ATP [149]. AMPK is a heterotrimeric protein complex consisting of β and γ subunits and the catalytic α subunit [150]. These subunits have multiple isoforms, and in the heart, the isoforms that are expressed are α1/2, β1/2, and γ1/2 [151]. Binding of AMP to the γ subunit allows for the α subunit to be phosphorylated at Thr172 [152]. The kinases that facilitate phosphorylation and activation of AMPK are liver kinase B1 (LKB1), calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) and TAK1 [153]. Activated AMPK phosphorylates and inactivates acetyl-CoA-carboxylase (ACC), which increases fatty acid oxidation. AMPK activation also enhances ATP production through glycolysis [154]. AMPK activates 6-phosphofructo-2-kinase (PFK2), increasing levels of fructose 2,6-bisphosphate, thereby stimulating 6-phosphofructo-1-kinase, an enzyme that regulates glycolytic flux [155]. While activated AMPK promotes ATP production, it also inhibits mTOR protein synthesis to spare cellular energy expenditure [156].

AMPK plays a vital role in the heart during ischemia-reperfusion injury. During ischemia, the lack of oxygen and diminished ATP production activates AMPK, stimulating glycolysis allowing the production of ATP in reduced oxygen conditions. This shift toward glucose utilization (oxidation) during ischemia is important to preserve cardiac function and minimize cell death [157]. During the reperfusion phase, as oxygen availability increases, the activated AMPK facilitates fatty acid oxidation and quick metabolic recovery of the tissues. Fatty acid oxidation suppresses glucose oxidation. However, glycolysis remains active. This uncouples glucose oxidation from glycolysis, which can lead to acidosis and hypercalcemia and hypernatremia which can reduce the contractile ability of the heart [154]. Thereby, AMPK has both a

protective and, perhaps, maladaptive role in cardiac function in ischemia-reperfusion injury. However, in ischemia-reperfusion studies in mice lacking AMPKα2, levels of ATP and glycolysis are downregulated, and greater infarct size is observed, suggesting that AMPK functions predominantly as a protective factor in the heart [158].

Likewise, in a model of pressure overload in mice lacking AMPKα2, there is a robust increase in cardiomyocyte size and fibrosis. In these AMPK α2 knockout animals, activities of downstream mTOR targets, p70S6K, and 4EBP1 were increased [159], with mTOR activation leading to increased fibrosis, as previously described. Interestingly, the AMPK activator pioglitazone decreased fibrosis in a model of pressure overload [160, 161], and AMPK activation with AICAR reduced interstitial fibrosis and hypertrophy after ischemia-reperfusion injury in mouse hearts [162], suggesting that AMPK is a potential therapeutic target for diseases involving cardiac fibrosis.

Additionally, there is crosstalk between the AMPK and TGFβ signaling pathways that may play a role in the protective effects afforded by AMPK activation. Activation of AMPK reduced Smad3-dependent transcriptional activity and inhibited the differentiation of fibroblasts to myofibroblasts [163]. Further, AMPK activity reduced the interaction of Smad3 with the transcriptional coactivator p300 and caused proteasomal degradation of p300 [164]. Activation of AMPK also reduced TGFβ1 levels by decreasing the expression of hepatocyte nuclear factor 4 alpha (HNF-4α), a potent transcriptional activator of TGFβ1 [165].

## *Renin Angiotensin System (RAS)*

The renin angiotensin system plays an important part in hypertension-induced cardiac fibrosis. Expression of angiotensin II (AngII) is induced when perfusion pressure in the kidney precipitously falls and AngII mediated increased water retention and increased extracellular volume. Studies have demonstrated a strong interaction between AngII and the TGFβ pathway. Administration of AngII in rats induced the expression of TGFβ mRNA in myocardial tissue [166–169]. Additionally, TGFβ activity is required for AngII-dependent cardiomyocyte hypertrophy. Blocking TGFβ activity using an antibody inhibited AngII-mediated cardiomyocyte hypertrophy in cultured cardiomyocytes [167]. AngII binds and signals through its receptors, AngII type 1 ($AT_1$) and AngII type 2 ($AT_2$). AngII infusion in mice lacking the $AT_2$ gene showed marked decrease in cardiac fibrosis [170]; likewise, treating rats with an $AT_1$ receptor antagonist decreased TGFβ expression and cardiac fibrosis [169, 171, 172].

AngII can activate several of the pathways previously discussed. For example, the binding of AngII to the $AT_1$ receptor activates Smad and RhoA proteins. AngII directly phosphorylates Smad2 and increases the nuclear translocation of Smad2 and Smad4 [173]. AngII signaling through $AT_1$ receptor also activates RhoA [174]. Smad and RhoA activation both lead to the expression of connective tissue growth factor (CTGF), which mediates the production of ECM proteins in fibroblasts

[173, 175, 176]. Further, AngII also activates the p38 MAPK pathway and activation of the AP-1 transcription factor. AP-1 is responsible for the production of ECM proteins as well TGFβ itself, which further propagates fibrosis [169]. NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a transcription factor that was shown to be activated by AngII, and activation of NF-κB increased the expression of collagen I and CTGF, thereby enhancing cardiac fibrosis [177].

## *Na/K-ATPase*

The Na/K-ATPase is a transmembrane protein that maintains cellular ion homeostasis by transporting $Na^+$ and $K^+$ across the membrane via ATP hydrolysis. Apart from playing a role in ion transport, the Na/K-ATPase also plays a role as a signal transducer, activating numerous pathways, some of which include fibrosis-related signaling [178–180]. A group of digitalis drugs such as ouabain and digoxin inhibit the activity of Na/K-ATPase and are used to treating heart failure and arrhythmias [181]. Recently, endogenously occurring digitalis-like compounds ouabain and marinobufagenin (MBG) were detected in the circulation of mammals, including humans [182–185]. Infusion of MBG in rats increased cardiac fibrosis, and when an antibody was used to immunize rats against MBG, the fibrotic response was attenuated [180]. Several pathways may contribute to the effect of MBG. For example, MBG activated the ERK pathway, which as previously discussed, is a pro-fibrotic signaling pathway [180]. Another study revealed that MBG administration in rats activates Src kinase, which plays a role in activating Smad3 and AKT pathways [179]. Finally, MBG was shown to activate PKCdelta, which phosphorylates and degraded Friend leukemia integration-1 (Fli-1), a transcription factor that negatively regulates the expression of collagen-1; MBG also increases collagen-1 levels [178].

## *Calcineurin NF-AT*

Calcineurin is a serine/threonine phosphatase that controls the activity of the NF-AT transcription factors. The NF-AT (nuclear factors of activated T cells) transcription factors regulate a number of genes involved in inflammation, neuronal development and cardiac hypertrophy [186, 187]. Calcineurin consists of the catalytic subunit calcineurin A, and the calcium-binding proteins calcineurin B and calmodulin [188, 189]. When intracellular calcium levels rise, calcineurin is activated and is able to dephosphorylate and activate NF-AT, which can then translocate to the nucleus [190]. Calcineurin activates NF-AT3, which interacts with GATA4, a transcription factor that drives cardiac hypertrophy and extensive interstitial fibrosis [191]. Additionally, NF-AT3 null mice have a decreased hypertrophic responsive following AngII infusion and pressure overload [192]. The NF-AT transcription factor associates with AP-1 and controls the expression of target genes [190], including the transcription of a number of ECM proteins as previously discussed.

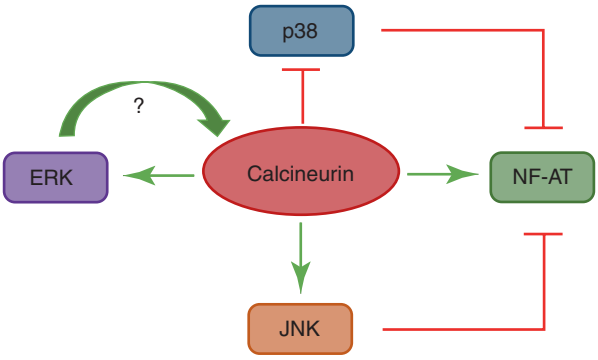


**Fig. 10.8** Crosstalk between calcineurin/NF-At pathway and the MAPK pathway. The catalytic subunit calcineurin A can dephosphorylate NF-AT transcription factors. Calcineurin also phosphorylates and activates JNK and ERK kinases while inhibiting p38 activity. ERK has also shown to directly activate calcineurin through an unknown mechanism

The MAPK signaling pathways p38, JNK, and ERK interact with the calcineurin pathway and can modulate the cardiac fibrotic response (Fig. 10.8). Activation of calcineurin causes activation of ERK and JNK through phosphorylation [193]. On the other hand, calcineurin inhibits p38 activity via upregulation of a phosphatase MKP-1. ERK activation reciprocally enhances calcineurin activation and increased NF-AT4 translocation to the nucleus in cardiomyocytes [194]. Additionally, ERK can directly phosphorylate and inhibit the activity of NF-AT1 in COS cells, suggesting that the effects of ERK on the calcineurin NF-AT pathway are cell type- and environment-specific [195]. JNK can also directly phosphorylate NF-AT1, NF-AT2, and NF-AT3 [195–197]. Further, p38 can phosphorylate NF-AT1, NF-AT2, and NF-AT4 [195, 196, 198]. Thereby, both JNK and p38 negatively regulate NF-AT calcineurin activity.

## *FGF23*

Fibroblast growth factor 23 (FGF23) is a hormone secreted by osteocytes that plays a role in the regulation of phosphate homeostasis and vitamin D metabolism. Elevated levels of FGF23 patients with chronic kidney disease were associated with left ventricular hypertrophy [199]. In isolated rat cardiomyocytes, exposure to FGF23 led to hypertrophy by activation of the calcineurin NF-AT pathway. Consistently, in mice, intramyocardial overexpression of FGF23 by injection of an adeno-associated virus increased levels of fibrosis following ischemia-reperfusion or myocardial infarction [199]. Immunohistochemical staining also revealed that FGF23 overexpressing mice had higher expression of TGFβ and β-catenin following ischemia-reperfusion and myocardial infarction compared to control animals [200]. These data suggest that FGF23 plays a role in myocardial fibrosis through activation of calcineurin/NF-AT and β-catenin pathways.

## *Calpain*

Calpains are serine proteases that can be activated by calcium influx in the cytosol or by phosphorylation through ERK [201]. Increased calpain activity can promote cardiac hypertrophy and fibrosis [202]. Calpains degrade IκBα, an inhibitor of NF-κB, allowing the translocation of NF-κB to the nucleus where it initiates expression of genes involved in fibrosis and hypertrophy [203]. Calpains also activate calcineurin NF-AT by degrading an autoinhibitory domain on calcineurin or by cleaving a calcineurin inhibitor cain/cabin1 [204], promoting NF-AT-mediated fibrotic signaling. Further, calpains have also been shown to directly activate latent TGFβ to its active form by cleaving the latency-associated peptide [205].

## *Ubiquitin Ligases*

The ubiquitin-proteasome system maintains protein homeostasis by targeting and degrading specific proteins. Ubiquitin ligases appear to have a protective role in cardiac fibrosis. Carboxyl terminus of heat shock protein 70-interacting protein (CHIP) is an E3 ubiquitin ligase and protein chaperone and has shown to have a protective role in AngII-induced cardiac fibrosis. Overexpression of CHIP in mouse heart decreased phosphorylation of p38, JNK, and p65 (a subunit of the NF-κB transcription complex) resulting in attenuation of cardiac fibrosis [206]. Furthermore, CHIP is necessary for protection against pressure overload-induced fibrosis in part through its chaperone activity toward AMPK, increasing ATP production and inhibiting protein synthesis [207].

Atrogin-1 is a protein that is part of a multi-subunit E3 ubiquitin ligase complex, which inhibits calcineurin and increases the activity of the Foxo1 and Foxo3a transcription factors [208, 209]. Transgenic mice which express atrogin-1 in the heart have less interstitial fibrosis and hypertrophy following pressure overload or voluntary exercise than control mice [208, 209]. Atrogin-1 expression leads to the ubiquitination and degradation of calcineurin A, which in turn downregulates translocation of NF-AT4 to the nucleus [208]. Foxo1 and Foxo3a are transcription factors that decrease the cardiac hypertrophic response [210]. When Akt phosphorylates Foxo1 and Foxo3a, they are sequestered in the cytoplasm and are unable to perform their transcriptional activity [211]. Atrogin-1 blocks the Akt-dependent phosphorylation of these transcription factors. Further, atrogin-1 acts as a coactivator of Foxo1 and Foxo3a transcription factors through polyubiquitination [209].

Mutations in muscle-specific ubiquitin ligase (MuRF1) are a cause of hypertrophic cardiomyopathy [212, 213]. As a ubiquitin ligase, MuRF1 plays an important role in protein quality control of cardiac myosin-binding protein C (cMyBP-C) [214]. Interestingly, mutations in cMyBP-C have been identified as a common cause of hypertrophic cardiomyopathy [215, 216]. These studies provide further evidence of the link between MuRF1 activity and cMyBP-C in cardiomyopathy.

### *Tumor Necrosis Factor-α (TNFα)*

TNFα is a cytokine produced by endothelial cells, monocytes, macrophages, fibroblasts, and cardiomyocytes. TNFα exerts both protective and damaging effects toward cardiac function [217–219]. TNFα binds to and signals through its receptors TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Signaling through the TNF receptor 1 (TNFR1) can induce cardiac fibrosis [217]. Binding of TNFα to TNFR1 induces receptor trimerization and activation of the receptors' cytoplasmic domains [220]. Downstream, this trimerization event leads to the recruitment of TAK1 and activation of JNK and p38 signaling. JNK and p38 activation, in turn, lead to the activation of the AP-1 transcription factor [220, 221] and fibrotic signaling, as previously discussed.

## Treatment of Fibrosis in Cardiomyopathy

### *Antihypertensives*

The use of antihypertensive drugs, such as ACE inhibitors, angiotensin II receptor blockers, beta-blockers, and calcium channel blockers, is recommended in treating dilated, hypertrophic, restrictive, and arrhythmogenic right ventricular cardiomyopathies [222–225]. Since the renin angiotensin system (RAS) plays an important role in the activation of fibrosis, various classes of RAS inhibitors have been tested in humans. Treatment with the angiotensin-converting enzyme (ACE) inhibitor, lisinopril; AngII receptor blockers (ARBs), such as losartan; and aldosterone antagonists, including spironolactone and eplerenone, lead to decreased fibrosis and fibrotic markers in patients with cardiac dysfunction [226–229]. The utility of an AngII vaccine was tested in rat model of myocardial infarction (MI). Immunization of rats with a peptide vaccine against AngII led to a decrease in collagen content in the heart post MI [230].

### *Ivabradine*

Ivabradine is a heart rate-lowering drug used in the treatment of heart failure. This drug was shown to be protective in dilated cardiomyopathy and is associated with the inhibition of myocardial fibrosis [231–233].

### *Anti-inflammatory Agents*

A number of pro-inflammatory cytokines such as TGFβ, TNFα, IL-11, IL-6, and IL17 associate with cardiac fibrosis. Granulocyte colony-stimulating factor (G-CSF) infusion in a mouse model of pressure overload increased IL-1β levels and decreased

cardiac fibrosis [234]. Mice deficient in the TNF receptor 1 and receptor 2 failed to develop cardiac fibrosis in response to AngII infusion [217]. Further studies demonstrated that knocking out TNF receptor 1 expression protects the mouse heart against remodeling after coronary ligation [235]. Together, these studies suggest that targeting the TNF receptor 1 is a potential therapeutic strategy to target cardiac fibrosis in cardiomyopathy.

There has been mixed data regarding the therapeutic efficacy of IL-11 in fibrosis. In a mouse study, fibroblast-specific expression of IL-11, or administration of IL-11, promoted cardiac fibrosis [236]. Conversely, IL-11 administration after coronary ligation attenuated cardiac fibrosis response [237]. Continued work on IL-11 is needed to elucidate its utility as a therapeutic.

Antagonizing TGFβ is an obvious approach in the treatment of fibrosis in cardiomyopathies. Pirfenidone is a TGFβ inhibitor approved for the treatment of idiopathic pulmonary fibrosis [238]. In an animal model of pressure overload, administration of pirfenidone reduced cardiac fibrosis, decreased collagen-1 expression, and attenuated p38 phosphorylation [239]. Pirfenidone is currently being tested for safety and efficacy for use in human patients with cardiac conditions. Tranilast is another TGFβ inhibitor presently in use in Japan to treat asthma, allergic rhinitis, atopic dermatitis, and other skin disorders [240]. In mouse models of diabetic cardiomyopathy, studies have shown that treatment with tranilast decreased fibrosis, along with inhibition of Smad2 phosphorylation and decreased activation of the ERK MAPK pathway [241, 242].

### *Fenofibrate*

Fenofibrate, a PPAR-α agonist, is a cholesterol-lowering agent used to treat hypercholesterolemia. Animal studies demonstrated that fenofibrate decreases cardiac fibrosis in animals with diabetes [243, 244]. More recently, fenofibrate has been shown to be valuable in treating chagasic cardiomyopathy. Chagas disease is caused by a parasitic infection that can lead to chagasic dilated cardiomyopathy. In mice infected with the parasite, a combination of fenofibrate and benznidazole (a trypanocidal drug) led to decreased cardiac fibrosis and improved cardiac function [245].

### *Calcineurin Inhibitors*

Cyclosporin A and tacrolimus are immunosuppressant drugs used in treating various inflammatory conditions as well in preventing organ rejection posttransplantation. Cyclosporin A and tacrolimus inhibit the calcineurin/NF-AT pathway, and studies have evaluated their potential in treating hypertrophic cardiomyopathy. Calcineurin inhibition can decrease cardiac hypertrophy in animal models [246–249]; however, other studies demonstrated that calcineurin inhibition worsens cardiac hypertrophy and fibrosis [250, 251]. Calcineurin inhibitors associated with

hypertrophic and dilated cardiomyopathy in transplant patients, thereby warranting further studies into this approach for treatment [252, 253].

### *Polyphenols*

Flavonoids and non-flavonoids belonging to the polyphenol family were shown to have anti-fibrotic properties in both animal and cell studies (reviewed in [254]). Flavonols such as quercetin, taxifolin, and isorhamnetin decreased cardiac fibrosis in mouse and rat models [255–257], likely through inhibiting the TGFβ signaling pathway [255, 257]. Resveratrol administration suppresses cardiac fibrosis in animal models of diabetic cardiomyopathy and cardiomyopathy caused by Duchenne muscular dystrophy [258, 259].

### *Sildenafil*

Sildenafil is a vasodilator used in treating pulmonary artery hypertension and erectile dysfunction. Studies in mice showed that sildenafil improved cardiac function and decreased fibrosis in a model of Duchenne muscular dystrophy [260]. However, in patients with Duchenne muscular dystrophy cardiomyopathies, no beneficial effects were observed; rather, a worsening of cardiac function was observed [261]. In another mouse study, tadalafil, which has a similar mechanism to sildenafil, but a longer half-life, delayed the onset of cardiomyopathy in Duchenne muscular dystrophy. The authors also reported significant decrease in interstitial and perivascular fibrosis [262].

### *MAPK Inhibitors*

Mutations in the lamin A/C gene (LMNA) lead to the development of dilated cardiomyopathy. LMNA cardiomyopathy is characterized by increased ERK1/2 and JNK signaling [263, 264]. In mice with LMNA mutations, the administration of either the ERK1/2 inhibitor PD98059 or the JNK inhibitor SP600125 improved cardiac function and decreased myocardial fibrosis [265].

Mice overexpressing $\beta_1$-adrenergic receptor and $\beta_2$-adrenergic receptor develop cardiomyopathy accompanied by decreased ejection fraction and fibrosis [266]. To inhibit p38 activation, these mice were crossed with a transgenic mouse that expressed dominant-negative p38α. Only the mice overexpressing the β2-adrenergic receptor with p38 inhibition improved ejection fraction and decreased fibrosis [266]. These studies provide a framework in testing the utility of MAPK inhibition in treating the fibrosis and cardiac dysfunction related to cardiomyopathies.

### *Stem Cell Therapy*

The goal of stem cell therapy in treating cardiomyopathies is to stimulate the regeneration and replacement of dead or damaged myocardium. In a rat model of dilated cardiomyopathy, intravenous injection of cultured human mesenchymal stem cells decreased cardiac fibrosis [267]. Studies to test the utility of autologous bone marrow-derived stem cells to treat adult and pediatric patients with cardiomyopathy are ongoing in clinical trials. The safety and feasibility of this method were validated through these studies, but no information on fibrosis was reported [268, 269].

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# Chapter 11
# WNT Signaling and Cardiac Fibrosis


**Gentian Lluri and Arjun Deb**

## Introduction

Through decades of research, our understanding of cardiac injury, repair, fibrosis, and the specific pathways involved has greatly expanded. There are several types of cells activated and recruited to the heart following an injury. Regardless of the type of injury, fibrosis represents a common end result [1–4]. These cells interact with each other directly (cellular contact) or indirectly (cytokines and other signaling molecules), and it is through such communications that the response to injury is affected. Our understanding of such interactions is critical in designing and developing novel therapeutic agents to treat a variety of cardiac conditions.

Undoubtedly, there are many signaling pathways that regulate such complex interactions. Among these, Wnt signaling pathways play an extremely important role in orchestrating an elaborate response to cardiac injury and promoting cardiac fibrosis. This chapter focuses not only on the pathways that promote fibrosis but also on exciting insights on the modulation and regression of pathological fibrosis. Although the most up-to-date information is presented, it is possible that by the time this book is published, new knowledge not available presently will continue to unfold.

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## WNT Signaling Pathway

Since the discovery of the first Wnt gene (wingless-related integration site) which at the time in 1982 was called int-1, at least 19 members have been characterized in the family of Wnt proteins. The term "Wnt" is derived from a combination of wingless (a *Drosophila* segment polarity gene) and int-1 (a mouse proto-oncogene), both of which encode similar secretory proteins [5]. These proteins are secreted into the extracellular space and subsequently undergo posttranslational modifications by the resident endoplasmic reticulum acetyltransferase porcupine [6]. There are two main mechanisms by which Wnts exert their effects: canonical (beta-catenin-dependent) pathway and noncanonical (beta-catenin-independent) pathway [7]. Beta-catenin is the central molecule in the canonical pathway. It is present throughout the animal kingdom with two diverse functions. First, it mediates the Wnt signaling pathway, and second it is an integral part of the cadherin-catenin cell adhesion complex [8]. The noncanonical pathway can be further divided into calcium and the planar cell polarity (PCP) pathways.

### *Canonical Pathway*

In the canonical pathway, Wnt binds to the Frizzled receptors. These receptors are seven transmembrane proteins, where the large N-terminal cysteine-rich extracellular domain participates to Wnt ligands. In addition, these proteins have three intracellular loops and a C-terminal domain that participates in downstream signaling in the cytoplasm [9]. There are at least ten different Frizzled receptors that have been identified in mammals to date [10]. In addition, lipoprotein receptor-related protein (LRP) 5/6 is part of the receptor complex [11]. In the absence of Wnt activation, a beta-catenin destruction complex is created in the cytoplasm. This complex is composed of several proteins including adenomatous polyposis coli (Apc) protein, casein kinase 1alpha (CK1alpha), axin, glycogen synthase kinase 3 (GSK3), and beta-catenin. The formation of this complex leads to beta-catenin sequential phosphorylation (Fig. 11.1a). Phosphorylated beta-catenin is released from the complex and bound by the E3 ubiquitin ligase receptor, β-transducin repeat-containing protein (β-TrCP), and subsequent degradation. The result of this process is the lowering of the concentration of cytoplasmic beta-catenin content.

Binding of Wnt to Frz and LRP5/6 initiates a signaling cascade where glycogen synthase kinase 4beta (GSK3beta)-mediated phosphorylation of beta-catenin is inhibited. Initially, the binding of Wnt to Frz and LRP5/6 leads to the recruitment of dishevelled (Dvl) protein. Subsequently, the destruction complex of proteins moves toward the plasma membrane and binds to the receptor complex. The canonical signaling pathway is unique in that the activation of Wnt/Fz signaling leads to the inhibition of beta-catenin degradation rather than its production (Fig. 11.1b). This results in higher levels of beta-catenin inside the cell, where of beta-catenin can migrate to the nucleus and activate the process of gene transcription. Alternatively, beta-catenin can move to the cell adhesion complex, and it is not currently known what mechanisms lead to

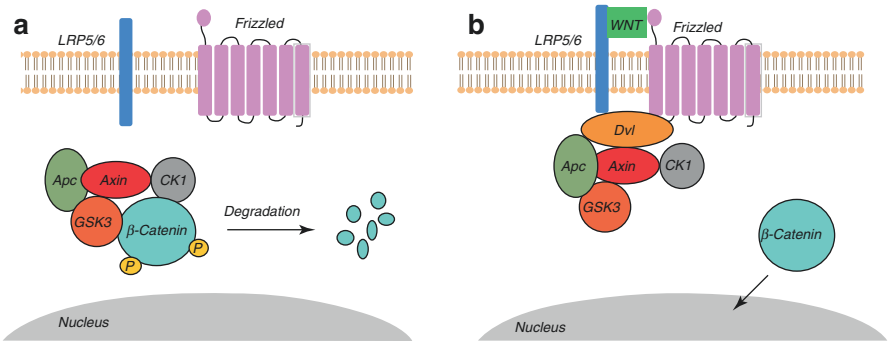


**Fig. 11.1** Canonical Wnt signaling. (**a**) In the absence of Wnt, beta-catenin is prepared for degradation by the destruction complex, which is composed of axin, adenomatous polyposis coli (Apc), and casein kinase 1 (CK1), and it is phosphorylated by GSK-3. (**b**) In the presence of Wnt ligand with Frizzled and LRP5/6 receptor, dishevelled protein is recruited, which results in the inactivation of the destruction complex and subsequent stabilization of beta-catenin, which enters the nucleus to modulate several transcription factors

migration of beta-catenin to the cell adhesion complex vs nuclear translocation [12]. However, the half-life of nuclear beta-catenin is much shorter (minutes) compared to the beta-catenin in the cell adhesion complex, which is very stable.

There are other mechanisms by which Wnt activation leads to cytoplasmic beta-catenin accumulation. Currently, it is not fully understood what the biological significance of these mechanisms are, but they include Wnt-induced degradation of axin and inhibition of GSK3 through the phosphorylated cytoplasmic tail of LRP5/6. This inhibition leads to the internalization of the signalosome into multivesicular bodies, protecting beta-catenin from degradation [13]. Next, beta-catenin migrates into the nucleus and transcriptionally activates the expression of targeted genes. With regard to nuclear translocation, most proteins require interaction with chaperones to enter the nucleus, given the nucleus is surrounded by a relatively protein-impermeable membrane. However, the transport of beta-catenin across the nuclear membrane occurs via direct interaction with the nuclear core complex [14]. Once, inside the nucleus, beta-catenin activates the transcription factor 4 (TCF)/lymphoid enhancer-binding factor (LEF) group of transcription factors to initiate Wnt-mediated gene transcription [15]. In addition, the receptor tyrosine kinase families Ror and Ryk have been proposed as receptors for several Wnts [16]. The Ror1 and Ror2 members of the Ror family are single-pass transmembrane receptors composed of a cysteine-rich extracellular domain and tyrosine kinase and proline-rich intracellular domains [17].

## *Noncanonical Pathway*

In the noncanonical pathways, the downstream effects of Wnt signaling are independent of beta-catenin (Fig. 11.2). Such pathways are utilized mostly by the Wnt5a class members and consist of two primary signaling mechanisms: (1) protein

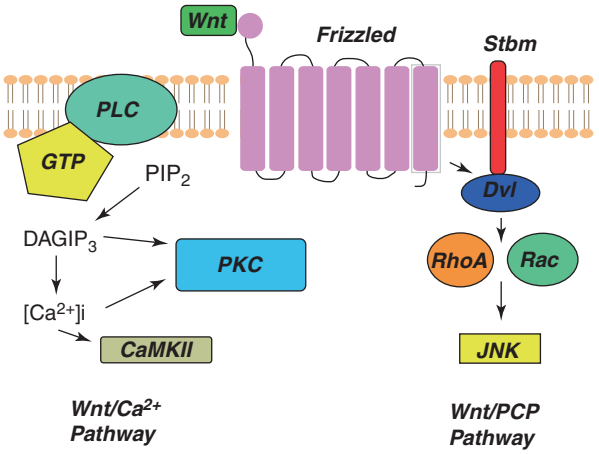


**Fig. 11.2** Noncanonical Wnt signaling. The Wnt/$Ca^{2+}$ is initiated following Wnt activation of Frizzled receptors causing G-protein-dependent calcium release, thereby activating CaMKII and PKC. The Wnt/PCP signaling is initiated via interaction of Frizzled and strabismus receptors which, via dishevelled protein, leads to activation of JNK via RhoA/Rac activation

kinase C (PKC)-mediated Wnt/$Ca^{2+}$ signaling pathway and (2) the planar cell polarity (PCP) pathway. In the Wnt/$Ca^{2+}$ pathway, Wnt enhances PKC activity via a heterotrimeric G-protein-linked, calcium-dependent mechanism which also involves calmodulin-dependent kinase II (CamKII), and it leads to the dephosphorylation of the transcription factor of activated T-cells (NFAT) [18]. Subsequently, NFAT translocates into nucleus where it activated NFAT-responsive genes [19]. Second, the Wnt/plan cell polarity (PCP) signaling pathway although not fully understood at present time is thought to act via the activation of cytoplasmic Dvl via Wnt/Fz interactions with transmembrane receptor strabismus (Stbm). This leads to the recruitment of several small GTPases of the Rho family (RhoA and Rac) and activation of c-Jun N-terminal kinase (JNK). This mechanism is especially critical during development where processes from cell polarization to anterior-posterior body axis elongation allow the tissue and the organs to take their final shape.

## Cardiac Fibrosis

Cardiac injury, whether acute or chronic, includes a wide variety of pathologies such as acute tissue ischemia, chronic hypertrophy, cardiac arrhythmias, as well as valvular dysfunction. Regardless of the insult, the heart responds in a characteristic way, where endothelial dysfunction, inflammation, as well as fibrosis, all play critical roles. Cardiac insults are characterized by loss of cardiac muscle, which is replaced by fibroblasts and increased fibrous extracellular matrix proteins. During the repair process, cardiac fibroblasts are recruited and proliferate. Fibroblast proliferation is a hallmark feature of cardiac fibrosis [20, 21]. Normally, cardiac fibroblasts, of mesenchymal origin, reside in the cardiac interstitium. The compensatory role of fibroblasts in the normal heart is to maintain the hemostasis of the extracellular matrix, serving as a scaffold for supporting all the other cardiac cell types as

well as facilitating electromechanical transduction. Once activated by the injury, cardiac fibroblasts express a variety of growth factors and cytokines. These secreted factors affect fibrosis by stimulating the differentiation of fibroblasts into myofibroblasts [22, 23]. Although the role of Wnt signaling is well established to play an important role in this process [1], there are several coexisting signaling pathways, via mediators such as transforming growth factor beta (TGF-β), interleukin 18 (IL-18), or matrix metalloproteinases (MMPs), that are involved in the pathogenesis of cardiac fibrosis [24], and these additional pathways are a topic in *Chapter* 12.

## WNT Signaling in Myocardial Infarction

Myocardial infarction (MI) affects more than seven million of people each year. A partially or completely occluded coronary artery results in impaired blood flow and deprivation of the myocardium of oxygen and nutrients [25]. The most common cause of an MI is the progression and rupture of an atherosclerotic plaque into a coronary artery; however, other etiologies such as coronary vasospasm and embolus into a coronary artery can lead to cardiac injury and MI. Uninjured cardiac tissue is composed of several types of cells including cardiomyocytes, fibroblasts, smooth muscle cells, as well as endothelial cell and epicardial cells. Although cardiomyocytes make up the vast majority of cardiac mass, the fibroblasts constitute 2/3 of the total number of the cardiac cells. However, following a myocardial infarction, the delicate balance between cardiac cells is altered. The heart has limited regenerative properties, hence there is substantial cardiac muscle loss following an MI, and cardiac healing response has several similarities to the wound healing process found in skin and scar formation. Following the acute injury, polymorphonuclear cells are seen to infiltrate the injured region within the first 24 h, followed by monocyte/macrophages during the subsequent 48 h. The main function of these cells is to clear the necrotic tissue from the infarcted area, a prerequisite step in the repair process. Within 1 week, there is a rapid proliferation of cardiac fibroblasts with collagen deposition in the infarcted area, leading to the formation of granulation tissue. The granulation tissue is rich in newly formed blood vessels and myofibroblasts, derived from resident fibroblasts, fibrocytes, or epicardial cells [26]. Myofibroblasts have dual properties: contraction and synthesis of extracellular matrix. These properties make myofibroblasts uniquely positioned in the formation of a strong and resistant tissue following the loss of cardiac cells [27]. Overtime, there is an increase on the extracellular matrix cross-linking, ultimately leading to the replacement of granulation tissue by scar tissue [28].

Although Wnt activity in the heart is low during normal physiological conditions in an adult heart, this is not the case during remodeling of the heart under pathological conditions [28]. Wnt genes are differentially expressed in a MI mouse model. For example, following surgical ligation of a coronary artery, upregulation of *Wnt2*, *Wnt4*, *Wnt10b*, and *Wnt11* and downregulation of *Wnt7b* occur [29–31]. In a neonatal heart, when MI was caused by cryoinjury, several Wnts including *Wnt3a*, *Wnt4*,

*Wnt5b*, *6*, *Wnt8a*, *Wnt9b*, and *Wnt10b* were upregulated in the myocardium of the injured hearts. In a separate study, *Wnt5* and *Wnt9a* were upregulated in the epicardial layer of the injured heart [32]. A combination of the age of the heart (neonatal vs adult) and type of injury (ligation vs cryoinjury) appears to modulate the pattern of Wnt activation. Such findings suggest that specific pattern of Wnt expression following cardiac injury effects the recovery process. *Wnt11* overexpression in a MI mouse model led to a reduction in the infarct size [30]. Also, overexpression of *Wnt10b* in cardiomyocytes following injury leads to improved ventricular function [29]. Alternatively, direct application of Wnt3a protein in the border zone of the infarct area has been shown to have negative effects [33].

## WNT Signaling in Cardiac Hypertrophy

Hypertrophy of the cardiomyocytes is an adaptive response to several external mechanical and neurohormonal stimuli, among which include pressure overload, MI, and beta-adrenergic stimulation. Hypertrophy is associated with cardiac remodeling, impaired cardiac function, and ultimately congestive heart failure [34, 35]. Both canonical and noncanonical Wnt signaling are activated in the hypertrophic heart, as shown by increased *Wnt3a* and *Wnt5a* expression [36]. Primary cardiomyocytes exposed to Wnt5a resulted in cardiomyocyte growth, and the growth effect was mediated through the Wnt component Dapper-1 [37]. Similarly, Dapper-1 is the mediator of the hypertrophic effects of Wnt3a as well [38]. Conversely, Wnt5a has beneficial effects that suppress cardiomyocyte growth in the right ventricle in a hypoxia-induced pulmonary hypertension mouse model [39]. In addition, Frizzled2 is involved in cardiac hypertrophy. More than 20 years ago, it was shown that aortic banding in rats lead to the immediate and sustained increase levels of Frizzled2 [40]. This was confirmed in other cardiac hypertrophy animal models where a positive correlation between Frizzled2 and LV mass exists [41]. In the right ventricle, hypertrophy in a pulmonary hypertension animal model is associated with Wnt/beta-catenin activation, and blockage of Wnt-LRP5/6 complex attenuated RV hypertrophy [42].

## WNT Signaling in Heart Failure

Heart failure is a major health problem of global significance. In the United States, heart failure has reached epidemic proportions, and it is estimated to have a prevalence of more than 40% by year 2030 with one in two patients with heart failure expected to die within 5 years [43]. The current therapies for heart failure are aimed at relieving symptoms and attenuating the further decline of patients affected by this condition. These therapies are neither curative nor do they completely prevent heart failure from developing. Wnt signaling was shown to affect adverse cardiac remodeling and subsequent progression of heart failure, suggesting that inhibiting this

pathway could be beneficial to the treatment of heart failure. As such, these interventions could potentially target either the extracellular, cytoplasmic, or nuclear portions of the signaling cascade.

As described above, overexpression of *Wnt10b* and *Wnt11* lead to the attenuation of MI-dependent cardiac damage by reducing infarct size and fibrosis [29, 30]. In addition, inactivation of Wnt signaling with GNF-6231, a porcupine inhibitor, improved cardiac function and reduced fibrosis by suppressing Axin2 and beta-catenin levels [44]. Also, the inhibition of Frizzled receptors following MI attenuated the infarct size and inhibited heart failure progression [45, 46]. Hence, a general approach of Wnt signaling inhibition can either retard or prevent development of heart failure following a myocardial infarction event. Secreted Frizzled-related protein 1 (SFRP1) also has cardioprotective effects following an MI. Accordingly, the absence of SFRP1 causes cardiac fibrosis, hypertrophy, and reduced cardiac function, whereas elevated levels of SFRP1 have the opposite effect [47]. Patients with heart failure have low levels of SPRF1 at baseline, and these levels are attenuated following the placement of left ventricular assist device [48]. In addition, there are several other isoforms of SFRP; proapoptotic SFRPs (SFRP3 and SFRP4) are upregulated in heart failure, but not SFRP1 or SFRP2 [49]. Indeed, there is a correlation of SFRP3 levels and adverse outcomes in patients with heart failure [50]. This correlation is not linear, meaning that extreme low, as well as extreme high, levels of SFRP3 are associated with adverse events and poor outcomes [51].

In addition to the extracellular components of Wnt signaling pathway in heart failure described above, the intracellular (cytoplasmic) components also play a distinct role. Dishevelled-1 overexpression is associated with increased mortality in a mouse model [34, 52]. Also, mice deficient of GSK3alpha and GSK3beta have severely abnormal myocardium, secondary to DNA damage, and apoptosis, with subsequent fibrosis and hypertrophy [53]. The downstream effect of GSK3beta signaling in heart failure appears not to be related to Wnt signaling, but through the PI3K/Akt pathway [54, 55].

Third, beta-catenin nuclear signaling is another site of Wnt action involved in heart failure. Cardiac cells from patients with heart failure (ischemic and nonischemic) as well as cardiomyocytes of failing hearts from a mouse cardiomyopathy model had elevated nuclear levels of beta-catenin [56]. Also, in a spontaneously hypertensive heart failure rat model, the nuclear levels of beta-catenin are significantly upregulated, whereas beta-catenin levels in the intercalated disk regions were reduced. This balance between the nuclear and intercalated disk levels of beta-catenin correlated with a shift from compensated hypertrophy to decompensated heart failure [57].

## WNT Signaling in Cardiac Arrhythmias

Few unique features of cardiomyocytes are their attachment to each other and their ability to act synchronously. This is achieved partially through intercalated disks which are highly specialized structures providing mechanical and electrical

coordination. Each of these disk structures are composed of three parts. First are the desmosomes, whose function is to anchor the adjacent cells. Second are the adherens junctions, which connect the actin filaments to the cell adhesion complex, and third are the gap junctions, which are critically important in the electrical and mechanical coordination of cardiomyocytes [58]. Both desmosomes and adherens junctions include catenin family members, independent of their role in Wnt signaling. In addition, gap junctions are composed of connexin (Cx) proteins, usually comprised of six Cx43 proteins in a ventricular gap junction, allowing for the rapid transmission of the action potentials [59]. In addition, Cx40 and Cx45 are found in the gap junctions of cells in the His bundle, Purkinje system, and other fast-conducting tissue [60]. Wnt/beta-catenin signaling affects Cx43 expression, for example, when an increase of Cx43 expression was seen with Wnt/beta-catenin activation in rat cardiomyocytes [61]. In addition, reduced levels of beta-catenin and Cx43 along with remodeling of gap junctions were observed in a cardiomyopathy mouse model prone to arrhythmias [62]. The link between Wnt/beta-signaling and Cx43 expression was also observed in mouse model with conduction abnormalities [63], further establishing the link between Wnt and Cx43.

Ample evidence also exists on the role of Wnt signaling in arrhythmogenic right ventricular cardiomyopathy (ARVC). This condition is an autosomal dominant inherited cardiac disease, associated with ventricular arrhythmias and heart failure, which are the main cause of death in young patients carrying the condition. Histologically, ARVC is characterized by the deposition of fibrous and adipose tissue in the myocardium [64]. It is considered a desmosomal disease, as the mutations that affect desmosomal proteins have been detected in a number of affected patients [65–67]. In vitro experiments demonstrated that decreasing desmoplakin levels result in the translocation of plakoglobin into the nucleus, which competes with beta-catenin. This leads to a reduction to Wnt/beta-catenin signaling, inducing an upregulation of fibrogenic and adipogenic genes. Data from in vivo mouse models had consistent results with a role for Wnt signaling in ARVC, where alterations of the desmoplakin gene and cardiac-specific silencing of beta-catenin lead to an ARVC phenotype [68, 69]. Further support on the role of Wnt/beta-catenin signaling in ARVC was provided by genetic fate-mapping experiments, demonstrating that adipocytes in ARVC hearts are derived from second heart field progenitor cells, in which Wnt/beta-catenin signaling attenuation drives these cells to an adipogenic fate [70]. More recently, GSK3 was found to be redistributed in the intercalated disks in a mouse model with abnormal desmosomal protein. Treatment with a GSK3 inhibitor lead to reduced fibrosis and inflammation and improved ventricular function in this model, paving the way for a possible role of GSK3 as a novel therapeutic target in ARVC [71].

## WNT Signaling in Valvular Disease

Normal vertebrate hearts have four valves: aortic and pulmonary semilunar valves at the arterial pole in addition to the mitral and tricuspid valves, which separate the atria and ventricles. Adequate and coordinated opening/closing of these valves are

required for efficient unidirectional blood flow [72]. The cusps of the semilunar valves and the leaflets of the atrioventricular (mitral and tricuspid) valves consist of complex connective tissue. These valves are covered by endocardial endothelial cells with interspersed valve interstitial cells (VICs). There are three stratified extracellular matrix (ECM) layers, namely, the fibrosa (rich in collagen), spongiosa (rich in proteoglycan), and ventricularis (rich in elastin) [73].

Valvular dysfunction is a common cause of cardiac dysfunction secondary to hypertrophy, fibrosis, and ultimately heart failure. Valvular defects can be categorized in stenotic or regurgitant defects. One of the valves commonly affected by stenosis is the aortic valve, seen in ~20% of patients older than 65 years old [74]. Congenital bicuspid aortic valve defects are the most common congenial heart condition in adults, affecting 1–2% of the general population, and have a much higher prevalence of aortic valve stenosis [75]. The main cause of valvular stenosis is calcification, which leads to reduced mobility of the leaflets. The process of calcification in the valves bears many similarities to bone formation [76, 77]. The role of Wnt/beta-catenin signaling in bone formation is well studied and established. Several Wnt genes are activated in precursors of osteoblasts, and beta-catenin is critical for the differentiation of mature osteoblasts [78]. Also, the bone density is affected by LRP5 mutations, highlighting the role of Wnt/betacatenin pathway in bone density [79, 80].

In addition to calcific valvular stenosis, the Wnt/beta-catenin pathway is also linked to myxomatous mitral valve disease and subsequent regurgitation. Clinically, valvular regurgitation is commonly seen in the mitral valve where regurgitation is found in ~10% in patients older than 75 years old. The most common cause of mitral valve regurgitation is myxomatous mitral valve [81]. Common findings include thickened redundant mitral valve leaflets, with degradation of collagen and elastin and deposition of proteoglycans, and reactivation of Wnt/beta-signaling pathway [82]. Valvular interstitial cells activate the transcription factor Sox9 in the absence of beta-catenin signaling, leading to upregulation of chondrogenic genes [83]. Axin2 negatively affects Wnt/beta-catenin signaling, and an indirect alteration of Wnt/beta-catenin signaling by inactivation of Axin2 leads to myxomatous mitral valve disease, highlighting the role of Wnt/beta-catenin pathway in normal valve function [84].

## WNT Signaling in Pulmonary Arterial Hypertension

Elevated pulmonary pressures with subsequent right-sided heart failure, and ultimately death, are the hallmark features of pulmonary arterial hypertension (PAH) [85]. The most characteristic pathological finding of PAH is the loss of small vessels and wall thickening, secondary to smooth muscle proliferation, resulting in luminal space loss and an increase in pulmonary vascular resistance [86]. Given the known role of Wnt signaling in angiogenesis and cell growth, it stands to reason that such signaling could play a role in pathology of PAH. Indeed, the) first studies of Wnt signaling in PAH suggested that PAH upregulates the noncanonical pathway genes, such as *Wnt11*, *Rho kinase*, *dishevelled*, and *Daam* [87]. Simultaneously, it

was discovered that the protective role of bone morphogenic protein (BMP) on the pulmonary endothelium is dependent on the Wnt signaling pathway [88]. Mutations in bone morphogenetic protein (BMP) receptor II (*BMPRII*) result in idiopathic PAH. Apoptosis of pulmonary artery endothelial cell was seen in patients with *BMPRII* mutations. Also, proliferation of vascular smooth muscle cells is associated with enhanced levels of active beta-catenin in patients with PAH [89]. Furthermore, the severity as) well as the progression of PAH is associated with the dysregulation of miRNAs which affect WNT/beta-catenin signaling pathways [90]. Finally, several Wnt family genes such as *FZD4*, *FZD5*, and *CTNNB1* as well as downstream targets such as *CCDN1* and *VEGFA* are upregulated in end-stage PAH [90].

## Conclusion and Future Perspectives

In recent years, there has been a robust increase in the number of studies focused on elucidating the characteristics of Wnt signaling in general cellular signaling as well as within the cardiovascular system in particular. Wnt proteins actively participate in a variety of biological processes, including proliferation, differentiation, adhesion, and others. As described in this chapter, there is ample evidence that Wnt signaling pathways play critical roles in several cardiovascular diseases. Although there are still areas of uncertainty in the signaling cascade, it stands to reason that a comprehensive understanding of these pathways will allow us to better understand the disease process and ultimately target these pathways to modulate the progression of the disease.

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# Chapter 12
# Matrix Metalloproteinase-9-Dependent Mechanisms of Reduced Contractility and Increased Stiffness in the Aging Heart

**Merry L. Lindsey and Lisandra E. de Castro Brás**

## Introduction

With aging, the myocardium undergoes a series of adaptations at the molecular, cellular, and tissue scales. Over a wide range of animal species and humans, a constant distinguishing feature of cardiac aging is reduced myocardial reserve capacity – the inability to handle stress above and beyond the normal scope [10, 49]. In the absence of a second injury, cardiac performance is usually sufficient to maintain normal day-to-day operations. When superimposed on an increased workload, however, diminished cardiac reserve is readily apparent.

The well-documented physiological adaptation to age in humans and rodents includes decreased sympathetic signaling and decreased heart rate variability [51, 65, 68, 70]. The mass of the left ventricle (LV) increases with age, due to increased wall thickness, and there is prolonged systolic contraction and diastolic relaxation that occur before myocardial performance is affected [52]. Cardiac aging by itself yields a slight, but relevant, decrease in LV physiology. For example, ejection fraction declines from 70% for young 2–8-month-old mice to 60% for senescent 24–38-month-old mice [51].

Physiological changes in cardiovascular function associated with aging, in both humans and rats, include higher afterload and impaired vasodilation, which, when combined, increase LV wall stress and induction of hypertrophic signaling pathways

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in cardiomyocytes [70]. While hypertension is prevalent in humans and rats, hypertension does not naturally occur with aging in mice. Despite the lack of increased blood pressure, mice develop cardiomyocyte hypertrophy, indicating that intrinsic myocardial changes have direct roles in shifting myocyte phenotype [84]. When cardiomyocyte hypertrophy develops, there is increased oxygen and energy demand due to the increased cell size. The formation of a low-oxygen environment, in turn, stimulates free radical production, resulting in oxidative stress that can damage cellular constituents [76, 82].

In response to age-related changes, cardiomyocytes release proinflammatory cytokines and chemokines that stimulate an increase in the number of macrophages in the LV [20, 60]. Macrophages are a rich source of additional cytokines, chemokines, and growth factors, as well as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). In older humans who otherwise have no evidence of cardiovascular disease, the myocardium has an increased MMP/TIMP ratio [11]. Aging myocardial tissue similarly demonstrates increased MMP/TIMP ratios in a variety of animal models [43, 52]. In aging mice, increased MMP activity is associated with inflammation, extracellular matrix (ECM) deposition, and a reduction in angiogenesis capacity [84].

At the cellular level, aging induces DNA damage and alters protein structure and organelle function; of these changes, the development of mitochondrial dysfunction with aging is the best studied [71]. Molecular changes translate to impairing normal cell physiology, including the upregulation of apoptosis or necrosis signaling that leads to cardiomyocyte death [44]. In the ECM, collagen accumulates with aging, demonstrated in reports of age-associated myocardial fibrosis in mice, rats, dogs, sheep, and humans [1, 13, 15, 21, 31, 34, 38, 54]. In this chapter, we summarize the current knowledge on aging effects on cardiac MMPs and TIMPs (MMP-9 in particular) and the cell types that regulate or are regulated by MMP-9.

## Aging Effects on Myocardial and Circulating MMP-9 Expression

MMPs are a family of enzymes that proteolyze components of the ECM. With a few exceptions, MMPs are secreted as pro-forms, with an inhibitory pro-peptide domain bonded by a cysteine residue to the $Zn^{2+}$ ion present in the catalytic domain. The classical cysteine switch mechanism of MMP activation involves cleavage of the pro-domain to expose the catalytic domain. There are caveats to the activation process, as MMPs can have activity without pro-domain cleavage, occurring in the presence of substrate [45]. Therefore, previous attempts to measure MMP activity were simplistic as analytical methods only targeted the active form. Likewise, MMPs are endogenously inhibited by TIMPs, and effects to focus on MMP to TIMP ratios were simplistic in assuming a one MMP to one TIMP scenario. Of the MMPs measured in cardiac aging, MMP-9 is the most studied and well characterized.

Aging effects on MMP-9 and TIMP-1 are summarized in Table 12.1. In mouse plasma, MMP-9 positively associates with age and with monocyte chemotactic protein-1 (MCP-1) [85]. MCP-1 stimulates MMP-9 secretion directly through the extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK)-mediated pathways [89]. The list of substrates processed by MMP-9 includes a wide range of ECM proteins, as well as a variety of cytokines, growth factors, and other MMPs. A partial list of MMP-9 substrates is shown in Table 12.2. ECM protein substrates include collagens; fibronectin; laminins; MMP-2, MMP-9, and MMP-13; osteopontin; and periostin, while non-ECM substrates include endothelin-1; interleukin (IL)-1β, interleukin-6, and interleukins-8; tumor necrosis factor-α (TNF-α); and vascular endothelial growth factor (VEGF) [19, 29, 53, 69]. A number of intracellular substrates have also been identified, including citrate synthase [18, 24].

Evidence to support the concept that MMP-9 is a major mediator of age-related increased LV stiffness is strong [64]. MMP-9 is robustly expressed by leukocytes (including neutrophils, macrophages, and lymphocytes), with low expression in cardiomyocytes [40]. Macrophage-derived MMP-9 was also implicated in cardiac aging [74]. MMP-9 levels doubled in the LV of older mice, and plasma MMP-9

**Table 12.1** Changes in MMP-9 and TIMP-1 in the plasma or left ventricle with aging [60]

| | Age effect | RNA/protein | Source | Species | References |
|---|---|---|---|---|---|
| MMP-9 | ↓ | Protein | Plasma | Human | [11] |
| | ↑ | Protein | Plasma | C57BL/6 J mice | [20] |
| | ↑ | Protein | Plasma | C57BL/6 J mice | [85] |
| | ↑ | Soluble protein | LV | C57BL/6 J mice | [20] |
| | ↑ | mRNA | LV | C57BL/6 mice | [75] |
| | ↑ | mRNA | LV | C57BL/6 J mice | [21] |
| | ↑ | Soluble protein | LV | C57BL/6 J mice | [21] |
| | ↑ | mRNA | LV | C57BL/6 J mice | [84] |
| TIMP-1 | ↑ | Soluble protein | Plasma | Human | [11] |
| | ↑ | Soluble protein | Plasma | C57BL/6 J mice | [20] |

↑ increased, ↓ decreased

**Table 12.2** A list of MMP-9 substrates

| Extracellular matrix | Cytokines, growth factors, and intracellular proteins |
|---|---|
| Collagens | Citrate synthase |
| Fibronectin | Endothelin-1 |
| Laminins | Interleukin (IL)-1β, interleukin-6, and interleukin-8 |
| MMP-2, MMP-9, and MMP-13 | Tumor necrosis factor-α (TNF-α) |
| Osteopontin | Vascular endothelial growth factor (VEGF) |
| Periostin | |

concentrations positively correlated with LV end-diastolic dimensions [20]. The expression of cadherin 1, integrin $\alpha_V$, and TIMP-3 is reduced in aging MMP-9 null mice, accompanied by increased angiogenesis and decreased cardiomyocyte hypertrophy [84]. In contrast, MMP-28 deletion amplifies inflammation, and of note, MMP-9 is elevated in the absence of MMP-28, suggesting cross talk among MMPs [55, 56, 64]. Likewise, transgenic overexpression of macrophage-derived MMP-9 induces greater age-dependent cardiac hypertrophy and vessel rarefaction phenotype, with enhanced cardiac inflammation and fibrosis [74].

## Aging Effects of MMP-9 on Cardiomyocyte Cell Physiology

LV physiology is dictated by changes in cardiomyocyte physiology. With age, the cardiomyocyte undergoes a series of quantitative and qualitative changes, with an increase in cell size characterizing the early initial response. Whether measured as cell volume, cross-sectional area, or cell length, all indices of myocyte hypertrophy increase with age and yield a concomitant reduction in inter-cardiomyocyte space [5, 84]. Along with cell size, aging increases the number of cardiomyocytes that are multinucleated [4, 63]. The increase in myocyte hypertrophy can yield an insufficient oxygen supply [48]. While reports that hypertrophic myocardium is less efficient and uses more oxygen, other reports have identified that hypertrophic myocardium is more efficient and uses less oxygen [35]. The observed differences in efficiency and oxygen utilization could be accounted for by the type of hypertrophic stimuli utilized (physiological vs. pathological), making additional studies necessary to define how hypertrophy affects energy demands during aging. Hypoxia induces expression of hypoxia-inducible factor 1 and is a potent stimulus for the upregulation of angiogenic signaling. Cardiomyocytes also contribute to ECM turnover by expressing collagen type IV [30]; MMP-2, MMP-9, and MMP-14; and all four TIMPs [9, 67, 79].

In addition to increases in size with age, cardiomyocytes undergo shifts at the organelle level. For example, as mitochondrial DNA mutations accumulate, protein oxidation is enhanced, resulting in decreased bioenergetic efficiency. Increased reactive oxygen species (ROS) enhances both myocyte apoptosis and inflammation [58]. The genetic inhibition of the mammalian target of rapamycin (mTOR), a serine-threonine kinase intracellular energy sensor, enhances both mitochondrial function and life span in mice [32, 83]. In addition to mitochondria, calcium signaling is impaired with age. Reductions in SERCA2 activity, the sarcoplasmic reticulum $Ca^{2+}$ pump regulating myocyte contraction and relaxation by regulating intracellular $Ca^{2+}$ stores, occur in the aging myocardium [46]. In total, all combined changes occurring in and around myocytes with age reduce cell numbers in animal models and humans [3, 62]. In humans, the aging myocardium loses approximately 38 million cardiomyocyte nuclei per year, by one estimate [62].

Circulating endocrine hormones influence cardiomyocyte function, making the endocrine contribution to cardiac aging a significant factor with age. In particular,

the renin-angiotensin system (RAS) is a strong contributor to myocyte cell physiology. Angiotensin II (Ang II) and angiotensin-converting enzyme (ACE) increase with age in the myocardium [23, 50]. Ang II directly stimulates myocyte hypertrophy, fibrosis, apoptosis, LV stiffness, and LV diastolic dysfunction [26]. The ACE inhibitor enalapril or the Ang II type 1 receptor antagonist losartan can both ameliorate age-related effects on the heart [7]. ACE inhibitors also inhibit MMP-9 activity by directly interacting with the catalytic domain, which partially explains beneficial effects on cardiac aging [87, 88].

## Aging Effects of MMP-9 on Myocardial Endothelial Cell Physiology

Multiple endothelial dysfunction markers increase with age. Markers of aging endothelium include impaired nitric oxide (NO) bioavailability that diminishes vasodilation, decreased endothelial NO synthase (eNOS) expression, and increased ROS generation including superoxide radical ($O_2^-$) [14, 37]. ROS rapidly scavenges NO, further decreasing bioavailability and generating other free radicals, including reactive nitrogen species. ROS can uncouple eNOS by depleting tetrahydrobiopterin (BH4) stores, and uncoupled eNOS converts L-arginine to $O_2^-$ instead of NO to create a positive feedback loop [91]. ROS and reactive nitrogen species both promote protein structural abnormalities by reacting with specific amino acid residues, resulting in an increased protein oxidation or nitrosylation to either enhance or reduce activity. Of note, free radicals can disrupt the cysteine switch to activate MMPs, resulting in an active full-length enzyme [22]. Non-sequestered ROS resulting from excessive ROS production activates nuclear factor-kappa B (NFκB) to shift endothelial cell gene expression to a proinflammatory state [27]. In addition, ROS can activate the TNF-α signaling pathway [37]. Oxidative stress can accelerate endothelial cell senescence by blocking the proliferative response to mitotic stimuli [77]. Overall, aging has multiple diverse effects on endothelial cell structure and function.

One index of endothelial cell physiology status is the vascular permeability index; if the endothelial cell is performing, permeability should be very low in the myocardium. Vascular permeability increases in the myocardium of 15–1-8month-old compared to 6–8-month-old mice [84]. This increase is concomitant with increased expression of cadherin-1, a transmembrane protein that forms adherens junctions among endothelial cells. The increase in cadherin-1 may indicate an attempt to preserve vascular permeability. Expression of the αV integrin decreases with age, along with decreases in vessel numbers. Deleting MMP-9 blunts the vascular permeability phenotype in 15–18-month-old mice, illustrating that MMP-9 has a direct role in maintaining vessel integrity [84].

Vascular endothelial growth factor (VEGF) is an essential component of angiogenesis signaling; VEGF stimulates endothelial cell proliferation, migration, and tube formation [84]. Of note, VEGF mRNA increases in the myocardium of both 15–18-month-old wild-type and MMP-9 null mice, yet vessel numbers assessed by

*Griffonia simplicifolia* lectin I staining increase with age only in the MMP-9 null group [84]. This finding indicates there is an age-related disconnect between angiogenic mediators released by myocardial cells and their ability to influence vessel numbers [84]. Coronary blood flow is lower in the LV of 30-month-old rats compared to 6-month-old rats, consistent with lower vessel numbers [47]. Reduced blood perfusion, neovessel formation, and vasodilation generate an aging environment that can maintain basal function but has reduced reserve potential.

## Aging Effects of MMP-9 on Cardiac Macrophage Cell Physiology

Cardiac aging can be characterized as a chronic low inflammatory state [33]. Mitochondrial DNA released from dying cells is one initiator that both stimulates inflammatory gene expression in endogenous cells and serves as a direct chemoattractant for macrophages. Mitochondrial DNA is not methylated and therefore can trigger macrophage activation by binding to Toll-like receptors [71]. Beyond macrophage numbers, LV macrophage polarization is also affected by aging [55]. MMP-9 deletion suppresses the shift in polarization by elevating CD206 and Fizz1 on macrophages, which prevents the $F4/80^{+}CD206^{+}$ M2 cell shift to $F4/80^{+}CD206^{-}$ M1 cells [55]. Deletion of the secreted protein acidic and rich in cysteine (SPARC) also reduces the age-associated increase in macrophage numbers [75]. In peritoneal macrophages stimulated with SPARC recombinant protein, M1 pro-inflammatory polarization markers (Ccl3, Ccl5, TNF-α, and IL-12) increase, while M2 anti-inflammatory polarization markers (Arg1 and Mrc1) reduce [75]. MMP-9, therefore, is a key mediator of cardiac inflammation.

## Aging Effects of MMP-9 on Collagen Turnover

With age, there is a subtle but significant accumulation of myocardial ECM proteins, including fibrillar components such as collagen, matricellular proteins such as SPARC, and integrins [61]. Total collagen content is the sum of all collagen types (e.g., I, III, IV, V, VI) and includes the full complement of protein forms that reflect ECM quality (e.g., full length, proteolytically fragmented, or posttranslationally modified). Collagen subtypes can vary in tridimensional structure, physicochemical properties, and roles. Of the collagens in the myocardium, the most abundant are collagens I and III, with collagen I representing 85 ± 5% and collagen III representing 11 ± 4% of the total collagen composition in young adult nonhuman primates [81].

Collagen content in the mouse increases from 1–2% of total LV area in young to 2–4% of total LV area in old mice [21, 51]. As a comparison, myocardial infarction increases collagen content in the scar to 65% of total LV area by 1 month after

induction [80]. In rats, while LV amount in 1-month-old rats is higher (6%), the amount increases to a similar degree, doubling to 12% by 22–26 month of age; this increased collagen content both in young and old rats is reflective of a much higher fibroblast count in rats compared to mice [31]. Additionally, collagen fibril numbers increase, as do collagen fibril diameter [34].

While total collagen accumulation after injury is species specific, the increase in collagen with age is consistent across species. In humans with no history of cardiovascular disease, autopsies show myocardial collagen contents of 4% in 20–25-year-old young individuals, and this increases to 6% in 67–87-year-old individuals [34]. Compared to younger subjects, hearts from 80-year-old individuals had higher collagen I to collagen III ratios, due to changes in both numerator and denominator [59]. The shift in the collagen I to collagen III ratio provides an intrinsic cardiac mechanism of increased LV stiffness, independent of vascular changes [34, 59]. While collagen I is characterized by high tensile strength, collagen III is more distensible [61]. Opposite to protein levels, collagen I, collagen III, fibronectin, and β1 integrin mRNAs decrease in the LV with age [21, 38, 57]. This tells us that collagen accumulation with age is due to posttranscriptional modifications at the protein level rather than increased transcription [61].

In addition to collagen type, the amount of cross-linking can also increase LV stiffness without affecting collagen amount [39]. Hydroxylysyl pyridinoline is elevated in the aging rat LV, reflecting increases in collagen cross-linking [73]. Fibroblasts synthesize and secrete collagen into the extracellular space as a procollagen, where further processing results in mature collagen fibril [66]. SPARC regulates collagen cross-linking and is expressed by cardiac fibroblasts, cardiomyocytes, endothelial cells, and macrophages [12, 75]. In the LV, SPARC increases with age and correlates with LV diastolic stiffness measurements and insoluble collagen content. SPARC deletion blunts the aging effects on LV collagen [13]. Aging SPARC null mice (18–29 month old) show reduced collagen III and IV expression and macrophage numbers compared to aging wild-type control mice [25, 75]. Lysyl oxidase (LOX) is an enzyme whose activity produces covalent cross-linking of collagen fibrils to increase tensile strength and prevent degradation by proteases [8]. LOX-induced collagen cross-linking is increased in the LV of old rats [72].

Nonenzymatic reactions between proteins and sugar residues generate advanced glycation end products (AGEs), and AGEs can covalently bind to each other to form protein-protein cross-links among a variety of proteins, particularly long-lived protein such as ECM components, including collagen, laminin, and elastin [36]. AGE plasma concentrations in elderly humans link to the extent of diastolic dysfunction [16]. In dogs, treatment with an AGE cross-link breaker reduces age-related diastolic dysfunction [6]. In addition to collagen, modifications in fibronectin folding occur with age [2]. Increased stretching can partially unfold the secondary structure of fibronectin, which may shift cell binding recognition sites [2]. In summary, multiple mechanisms regulate ECM composition, which changes with age and contributes to the age-associated changes in collagen.

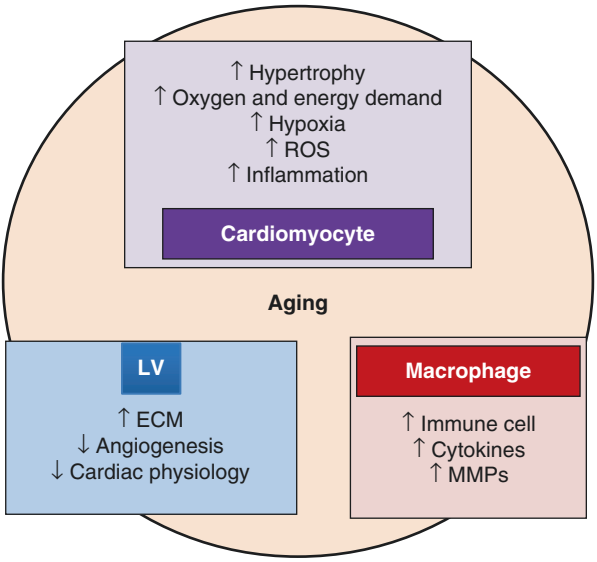


**Fig. 12.1** The spiral of cardiac aging. Aging induces cardiomyocyte hypertrophy, which increases oxygen demand which results in hypoxia. Hypoxia stimulates the generation of reactive oxygen species (ROS) and subsequent release of proinflammatory factors. The increase in inflammatory proteins stimulates macrophage infiltration, and macrophages, in turn, secrete cytokines and matrix metalloproteinases (MMPs), which leads to increased extracellular matrix (ECM) turnover. In the LV, there is a concomitant decrease in angiogenesis, which further stimulates hypoxia and the development of cardiac dysfunction

## Future Directions and Conclusion

The myocardium undergoes multiple cellular and extracellular responses during aging, leading to increased LV stress and diastolic dysfunction (Fig. 12.1). Of the mechanisms regulating the makeup of the extracellular matrix and collagen deposition, MMP-9 signaling is a critical juncture point in aging. Future studies targeting how MMP-9 activity could be modified to prevent or slow the development of the cardiac aging phenotype are warranted. Aging resets baseline values to a new homeostatic point, and experiments examining how MMP-9 delays or prevents this shift may improve cardiac aging.

Proteomics has been successfully used to catalog MMP-9 substrates, and such unbiased explorations help elucidate the molecular and cell signaling pathways that converge at MMP-9 and identify useful bioindicators of cardiac aging [17, 28, 56, 64]. Likewise, genomics identifies gene pathways important in aging [42, 86]. Next-generation sequencing provides global evaluation of the LV and aging-relevant cell types (e.g., cardiomyocytes, endothelial cells, macrophages, and fibroblasts). With the accumulation of big data, developing computational models will be useful to understand how intra- and intercellular communication signaling pathways are altered during the life span [90]. Understanding the

interconnections among myocyte hypertrophy, endothelial cell vascularization, macrophage inflammation, and fibroblast ECM accumulation is an important future avenue of exploration [41, 78].

While this review focused on age-related changes in MMP-9 signaling, the development of similar strategies will identify how other MMP family members that increase with aging are mechanistically linked to the changes observed. In conclusion, understanding the dynamic MMP-9 signaling alterations that occur over the time continuum of cardiac aging provides us with novel insight into the mechanisms of the aging process, with implications for both aging physiology and aging superimposed on pathophysiology.

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# Chapter 13
# Using Peptidomics to Identify Extracellular Matrix-Derived Peptides as Novel Therapeutics for Cardiac Disease


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## Introduction

Peptidomics is the systematic, comprehensive, and qualitative/quantitative proteomic evaluation of endogenous peptides in biological samples, collected at a chosen time point and spatial location. Peptidomics can be used to identify and validate all endogenous peptides in a biological sample and to compare expression levels of the peptides of interest. Mass spectrometry (MS)-based approaches, compared to a ligand-binding assay, improve and expand significantly the analysis of peptides by increasing specificity and sensitivity and allowing for the unbiased identification of peptides. As a result, peptidomics has been applied to the comprehensive mapping of food protein digestion [1–3], drug efficacy and toxicity [4–6], biomarker discovery [7–9], and the study of soluble polypeptides, including signaling molecules like cytokines, growth factors, and neuropeptides [10, 11]. Additionally, peptidomics is a discovery tool that can be used to determine the function of unknown peptides [12, 13], to study physiological (homeostasis) conditions such as protein turnover [14, 15], and to investigate disease-specific proteolytic cleavage to inform on disease states [16, 17]. Thus, peptidomics is an important branch of proteomics that bridges the gap between proteomics and metabolomics. Since it being coined in the late

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1990s [18], the term peptidomics (or peptidome) has been used in over 1200 publications listed on PubMed. This attests to the rapid evolution and continuous growth of this technique.

Three major categories of naturally occurring peptides exist: (1) ribosomal, synthetized by mRNA translation [19]; (2) non-ribosomal, synthesized using a modular protein complex (mostly found in plants, fungi, and unicellular organisms) [20]; and (3) proteolytic products, formed by proteolysis of proteins either during protein turnover or actively degraded on a pathological setting [21]. This chapter will focus on the analysis of the third type of naturally occurring peptides in cardiac disease. A common cardiac disease studied is myocardial infarction (MI), where the injury induces cardiac tissue to progress through several healing phases, ranging from the early clearance of damaged cells and matrix debris, the subsequent proliferation and migration of cells (fibroblasts) to synthesize and deposit new matrix, vessel formation to support the new cell population, and finally extracellular matrix (ECM) remodeling [22]. The importance of the remodeling is clear when the cardiac ECM's roles are appreciated. Cardiac ECM provides structural and mechanical support to the myocardium, directs signal transduction within the cell, and modulates cell-cell and cell-substrate interactions [23]. During MI, there is extensive extracellular protein turnover as ECM is degraded to clear damaged/necrotic cells and *de novo* ECM is synthesized to form an infarct scar [24]. During these phases, numerous biologically active endogenous peptides are generated that are physiologically beneficial [25]. For example, a naturally generated cardiac collagen type I peptide following MI can reduce fibrosis and stimulate angiogenesis in a mouse MI model, with a net reduction in left ventricular (LV) dilation, demonstrating its potential as a treatment tool [26]. Others have used peptides derived from fibronectin and collagen type IV to promote higher levels of angiogenesis and arteriogenesis in an ischemia/reperfusion rat model [27]. The beneficial effect of ECM peptides is, however, not limited to the cardiac field. For example, peptides derived from collagen type IV, laminin, fibronectin, and thrombospondin have proven to be effective metastasis inhibitors [28–30].

The ultimate goal of research in the MI filed is to discover treatments that can prevent, limit, or reverse the adverse myocardial remodeling that leads to LV dilation and impairment of LV physiology. The myocardial remodeling occurring after MI and the quality of the resulting scar depend on the balance between ECM degradation and deposition. While excess degradation can progress into LV aneurysms or rupture, excessive ECM deposition can result in myocardial stiffness that can lead to the development of heart failure (HF) or arrhythmias [31–33]. Evaluating ECM turnover post-MI, and therefore the ECM peptidome, is important for a complete understanding of LV remodeling and to find new mechanisms to limit adverse remodeling.

## Basics of Peptidomics Research

Peptides are commonly distinguished from proteins by an arbitrary molecular weight cutoff of 50 amino acids [34, 35]. However, small proteins such as insulin (51 amino acids) are very close to the peptide upper limit [36], and some peptides

like the Alzheimer's beta-peptide (39–43 amino acids) could be considered a protein, based on the size criteria [37]. Therefore, a more specific and current definition is that a peptide is a poly-amino acid molecule without a tertiary structure [34].

Peptidomic studies have the potential to provide insight into the tissue/organ response to stimuli such as cardiac injury. Such direct functional studies are critical at a time when protein function is often unknown and inferred by its similarity to a known gene sequence. The issue with using inferences is that it does not take into consideration the unique properties of each protein, particularly with respect to fragmentation products that can result in new biological functions. However, to date, there have been only a few peptidomic studies on the cardiac field, likely as a result of technical challenges and the need for a specific set of expertise. Moreover, current proteomic methods are often not suitable for studying the generation of endogenous peptides [38]. A major difference between peptidomics and proteomics is that peptidomics does not require enzymatic digestion of the sample before examination in the mass spectrometer (Fig. 13.1). As a result, peptidomics may be considered technically easier than proteomics, but this is, however, a misconception as several factors make peptidomics more challenging than proteomics, including sample preparation and data analysis.

Identification and quantitation of peptides from complex biological matrices using MS-based approaches typically require selective enrichment and systematic peptide extraction to achieve successful analysis. Rather than analyzing a sample to identify which intact proteins are present, peptidomics studies endogenous protein fragment generation. As in all MS studies, sample preparation is critical for successful peptidomic studies [39]. In peptidomics, the first and most critical step in sample

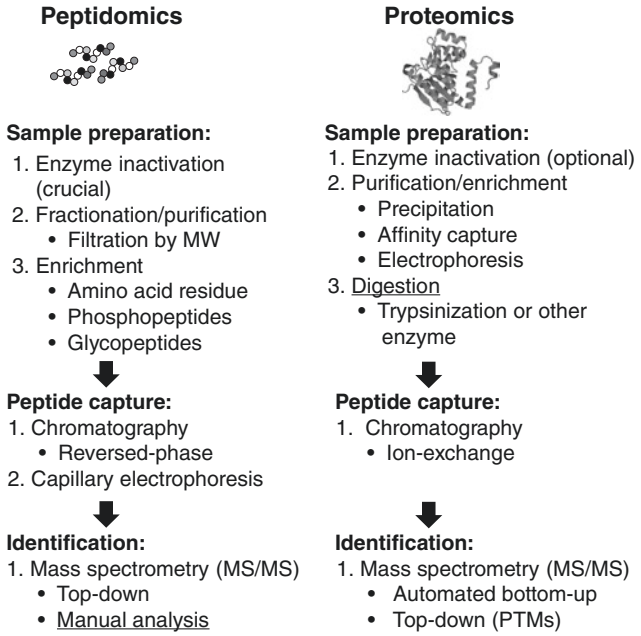


**Fig. 13.1** Summary of technical differences between peptidomic and proteomic methodologies. Underlined steps represent methods unique to that approach

preparation is enzyme inactivation. Peptides are then purified, in contrast to proteins that are commonly digested into tryptic fragments (bottom-up approaches), and then analyzed [40]. The second step of sample preparation usually involves removal of the most abundant proteins, as well as removal of proteins >10–20 kDa [41, 42]. Conventional techniques used to achieve this utilize molecular weight cutoffs membrane filtration and targeted precipitation [25]. However, these methods come with known limitations, including partial loss of peptides, partial contamination from untargeted fractions, or possible peptide aggregation [43].

The diverse physicochemical properties of peptides, including a wide range of sizes, hydrophobicity, net charge, and posttranslational modifications (PTMs), make the study of peptides challenging [25]. Consequently, isolating the peptidome of a complex mixture with a single purification method can be difficult, and multiple peptide purification methods are often used sequentially on the same sample [44]. A variety of enrichment methods, which can be used individually or to complement filtration and/or precipitation, were developed to extract specific peptide fractions. Peptides can be purified using methods targeting specific amino acid residues, such as cysteine [45, 46], methionine [47], or tryptophan [48]. Phosphopeptides can also be fractionated using approaches based on their differential interaction with metals, like adenosine triphosphate (metal carrier) [49], titanium dioxide [50, 51], and more recently nanocomposites [52]. A variety of methods are used to obtain glycopeptide-enriched samples, including chromatography-based extractions using different solid phases. These include microcrystalline cellulose [53, 54], zwitterionic materials [55, 56], and click maltose [57, 58]; boronic-based chemicals [45, 59]; hydrazine affinity [60–64]; and lectin-mediated affinity [65, 66].

Peptidomics employs a wide variety of MS techniques and mass spectrometers. We do not cover all of these techniques here, as they are shared between peptidomics and proteomics and reviewed elsewhere [11, 67, 68]. Detected peptides can be identified and validated by tandem mass spectrometry, by *de novo* sequencing, or by online database searches using curated protein sequence databases. While protein identification in proteomics relies on automated analyses of MS data using several tryptic fragments, in peptidomics, MS/MS data of a single peptide is used for identification [40]. Furthermore, in peptidomic analyses, we need to include all potential modifications that shift masses and allow for a range of cleavage sites and PTMs, which significantly affect automatic identifications. Thus, a top-down approach is performed, whereby the MS/MS spectra are manually evaluated to verify that the assignments of peptides are accurate [69–71]. Ongoing technological improvements in the areas of MS and bioinformatics are expected to facilitate peptidomics throughput.

## ECM Peptides for MI Treatment

Repairing cardiac tissue remains one of the most challenging goals in the cardiac field. Our labs and others have sought to better understand the development of adverse LV remodeling that can lead to HF using ECM-derived peptides. The Lee

Lab used polymers derived from ECM proteins, for example, fibrin or collagen type I, and polymers modified with ECM-derived functional moieties, to induce angiogenesis enhancing the formation of new capillaries and functional arterioles in the MI region [72–74]. These studies had positive results but came with the limitation of using direct injection or implantation into the infarcted area, which can result in off-target complications. Moreover, these polymers are too large to pass through capillaries and therefore cannot be delivered intravenously (i.v.) [75].

To overcome this limitation, Mihardja and colleagues chemically conjugated peptides to an anti-myosin heavy-chain antibody to allow for i.v. administration and specifically target the infarcted area, which resulted in a dramatic angiogenic response post-MI [27]. Another way to overcome limitations inherent to the use of polymers is to use relevant functional groups derived from the ECM proteins, i.e., ECM-derived peptides. We identified a collagen type I C-terminus fragment (C-peptide 1158/59) that is produced during normal ECM turnover but is quickly degraded by matrix metalloproteinase (MMP)-9 in a mouse MI model. A mechanistic examination revealed that this endogenous C-1158/59 peptide had beneficial effects *in vitro* and *in vivo*. In humans, we analyzed plasma samples from patients taken 24–48 h post-MI. Increased plasma levels of C-1158/59 negatively correlated with LV filling [26], suggesting that therapeutic elevation of C-1158/59 could have a beneficial effect on disease progression. To assess its therapeutic potential, we synthesized a short peptide mimetic of the C-1158/59 cleavage site (p1158/59) and delivered p1158/59 through an osmotic pump continuously for 7 days post-MI. p1158/59 treatment resulted in less LV dilation, and collagen I deposition (i.e., less fibrosis), with concomitant, increased collagen III expression and enhanced angiogenesis [26]. The shift in collagen I/collagen III ratio may be the mechanism behind the preservation of LV geometry observed in the p1158/59 treated group. The next section will focus on peptides that specifically target cardiac fibrosis.

## Anti-fibrotic Peptides

Cardiac fibrosis occurs following MI and is a benchmark of congestive heart failure. Several peptides have shown anti-fibrotic activity and potential beneficial effects in the treatment or attenuation of cardiac pathologies (Fig. 13.2) [26, 76]. Atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) display anti-fibrotic properties, with CNP being the most potent as well as showing additional anti-hypertrophic effects in cultured cardiac fibroblasts and cardiomyocytes [77, 78]. In an MI rat model, CNP markedly attenuated LV dilation, cardiomyocyte hypertrophy, and collagen volume fraction in the non-infarcted region [76]. Ghrelin, a growth hormone-releasing peptide, was used as a treatment to improve cardiac performance in rats with chronic heart failure and resulted in increased cardiac output and LV fractional shortening as well as reduced collagen deposition [79]. Additionally, an intravenous bolus infusion of human ghrelin decreased mean arterial pressure and increased cardiac and stroke volume indexes in patients with chronic heart failure [80].

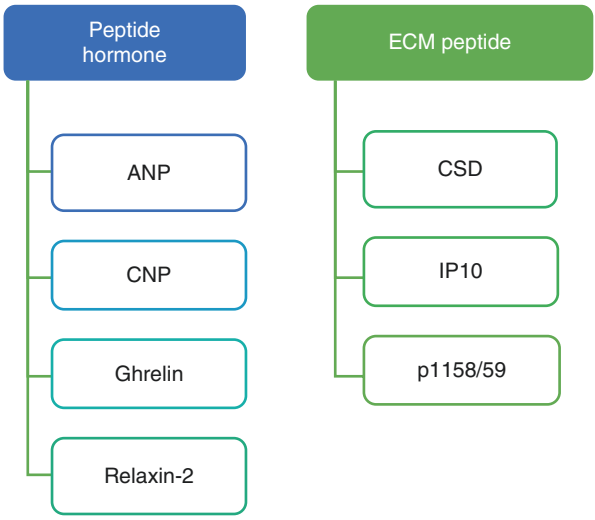


**Fig. 13.2** Schematic representation of extracellular peptides with anti-fibrotic activity. ANP atrial natriuretic peptide, CNP C-type natriuretic peptide, CSD caveolin-1 surrogate peptide, IP10 CXCL10 agonist

Relaxin-2 is a peptide hormone that exerts beneficial anti-fibrotic and anti-inflammatory effects in diverse models of cardiovascular disease, including MI [81]. Relaxin-2 treatment inhibited the MI-induced progression of cardiac fibrosis in a mouse model, with a parallel reduction in transforming growth factor (TGF)-β1 expression, myofibroblast differentiation, and cardiomyocyte apoptosis [82]. IP10 peptide, a C-X-C motif chemokine 10 agonist, reduced expression of α-smooth muscle actin, collagen type I, fibronectin, and tenascin C in a co-culture of cardiomyocytes and cardiac fibroblast post-MI [83].

More recently, caveolin-1 surrogate peptide (CSD) was tested in mice with congestive heart failure. Animals that received CSD daily (4–6 weeks) displayed improved ventricular function (increased ejection fraction, stroke volume, and cardiac output and reduced wall thickness) and decreased levels of collagen type I, collagen chaperone heat shock protein 47, and fibronectin [84]. A therapy that can inhibit cardiac fibrosis from progressing would not only preserve cardiac function but also prevent heart failure. Despite successful preclinical trials using anti-fibrotic peptides, currently, there are no efficient clinical therapies available.

## ECM Peptides as HF Biomarkers

According to the NIH, a biomarker is a characteristic that can be measured and evaluated as an indicator of normal biologic processes, pathologic processes, or pharmacologic responses to therapeutic intervention [85]. Easy-to-monitor biomarkers that can predict progression to HF would be an important advancement to risk-stratify affected patients and to serve as a biological tool to guide HF therapy. Because peptides can easily migrate between cellular and tissue compartments,

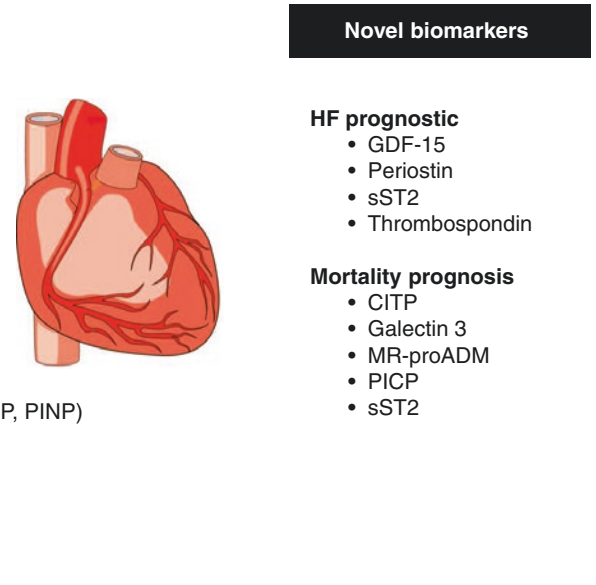


**Fig. 13.3** Biomarkers currently used clinically to diagnose heart failure (left panel) and recently identified biomarkers of heart failure prognosis (right). BNP B-type natriuretic peptide, NT-proBNP N-terminal proBNP, MT-proANP mid-regional pro-atrial natriuretic peptide, sST2 soluble ST2, CITP collagen type I carboxy-terminal telopeptide, PICP collagen type I carboxy-terminal propeptide, PINP procollagen type I N-terminal propeptide, PIIINP procollagen type III amino-terminal propeptide, GDF-15 growth differentiation factor 15, MR-proADM mid-regional pro adrenomedullin

several pathogenic processes associate with peptide changes (composition and abundance) in different body fluids (plasma, serum, urine, saliva) [86]. These reasons make peptides an attractive target for biomarker discovery.

HF occurs when the heart cannot maintain adequate output to the peripheral tissues or can do so only at increased filling pressures [87]. Abnormalities in LV function and neurohormonal regulation are major features of this condition [88]. HF can occur secondary to either systolic or diastolic dysfunction. Systolic dysfunction is characterized by reduced contractility and decreased pump function, whereas diastolic dysfunction entails impaired diastolic filling and increased chamber stiffness [87, 88]. Biomarkers of HF typically refer to proteins, peptides, or other molecules measured in the circulation (Fig. 13.3). Biomarkers of HF are normal components of pathways related to regulation of the neurohormonal system, myocardial injury, LV dysfunction, and cardiac remodeling [89]. Currently, B-type natriuretic peptide (BNP) and N-terminal proBNP (NT-proBNP) are the gold standard biomarkers in HF diagnosis and prognosis [90]. More recent biomarkers, such as mid-regional pro-atrial natriuretic peptide, mid-regional pro-adrenomedullin, cardiac troponins (troponin I and troponin T), endothelin-1, and peptide arginine vasopressin, have shown potential for clinical application [89, 91–96].

Plasma ECM peptides for HF prognosis include galectin-3, ST2, and growth differentiation factor 15 (GDF-15). Galectin-3 has been approved by the Food and Drug Administration (FDA) for the prediction of poor outcome in HF patients in combination with clinical assessment [97]. Upon myocardial injury, macrophages secrete galectin-3 under the influence of mediators such as osteopontin [98], a matricellular protein which expression increases markedly under a variety of pathophysiological conditions of the heart [99]. A study in 240 HF patients by Lok et al., described galectin-3 as an independent marker for LV remodeling and mortality in patients with chronic HF [100]. In this study, patients that showed a reduction in LV end-diastolic volume (EDV) over time (~ 9 years follow-up) had lower levels of galectin-3 at entry compared to patients in whom the LVEDV was stable or increased. Importantly, this was not observed in the levels of NT-proBNP. Additionally, galectin-3 levels positively correlated to changes in LVEDV and were a predictor of mortality after long-term follow-up [100].

Suppression of tumorigenicity 2 (ST2) is a member of the interleukin-1 receptor family that has two main isoforms: transmembrane or cellular (ST2L) and soluble or circulating (sST2) [101]. sST2 is released from cardiomyocytes and fibroblasts after mechanical strain and was identified as a novel biomarker of cardiac stress, fibrosis, and remodeling [102]. The ligand of ST2 is interleukin (IL)-33, known to participate in signaling that leads to a reduction of tissue fibrosis and myocyte hypertrophy in mechanically strained hearts [103]. Circulating sST2 acts as a decoy receptor, thereby blocking the beneficial effects that occur when IL-33 attempts to bind to ST2L and leading to cardiac hypertrophy, fibrosis, and ventricular dysfunction [104]. The study Pro-Brain Natriuretic Peptide Investigation of Dyspnea in the Emergency Department (PRIDE) was the first to assess sST2 in 593 HF patients [105]. PRIDE reported that even though sST2 levels were higher among those patients with acute HF, NT-proBNP was superior for diagnosis of acute HF. However, sST2 $\geq$0.20 ng/ml strongly predicted death at 1 year [105]. Other studies on acute HF patients demonstrated that sST2 measured on hospital admission strongly associates with the measures of HF severity and poor outcome [106–109]. Overall, sST2 seems to provide independent and additive prognostic information to clinical variables and well-established cardiovascular biomarkers.

A prospective cohort study analyzed 92 biomarkers in 847 consecutive patients from the Västmanland Myocardial Infarction Study (median follow-up 6.9 years). GDF-15 was one of the biomarkers most strongly linked to all-cause mortality [110]. GDF-15 is a potent inflammatory cytokine that is released during physiological stress from the peripheral vasculature and is a mediator of myocardial fibrosis [111]. Another prospective study measured plasma GDF-15 and NT-proBNP in 916 patients with HF with reduced ejection fraction (HFrEF, EF < 50%; $n$ = 730) and preserved EF (HFpEF, EF $\geq$ 50%; $n$ = 186) [112]. While NT-proBNP was lower in HFpEF than HFrEF, GDF-15 was elevated in both groups. During a median follow-up of 23 months, GD-F15 provided incremental prognostic value when added to other clinical predictors (NT-proBNP and troponins) [112].

Collagen peptides have been used as biomarkers of collagen synthesis and degradation for decades. Even though collagen peptides are not commonly used to

assess progression to HF in the clinic, they are the gold standard to assess myocardial fibrosis, which is a pathophysiological mechanism common to a wide range of cardiovascular diseases. The Optimizing Congestive Heart Failure Outpatient Clinic (OPTIMAL) project assessed the efficacy of collagen type I carboxy-terminal telopeptide (CITP) and propeptide (PICP) to predict long-term mortality in elderly patients with HFrEF [113]. The data show CITP and PICP to be independent predictors for all-cause and cardiovascular mortality, giving evidence that disturbances to collagen type I metabolism (excessive degradation) have independent prognostic implications for long-term mortality in patients with HFrEF.

Hoffman and colleagues measured collagen peptide biomarkers in 56 patients with systemic right ventricular dysfunction. The group measured serum levels of procollagen type III amino-terminal propeptide (PIIINP), CITP, procollagen type I N-terminal propeptide (PINP), and NT-proBNP in patients with D-transposition of the great arteries and healthy controls [114]. PIIINP, CITP, PINP, and NT-proBNP were all elevated in the diseased patients. They concluded that PIIINP might be a good indicator of systemic right ventricular remodeling and for reduced longitudinal systolic function. Similarly, Liu et al. assessed ECM biomarkers in a patient with diabetic cardiomyopathy and reported PIIINP levels to directly correlate with diastolic dysfunction [115].

Other ECM-derived peptides that currently are in the preclinical phases and have a strong correlation to heart failure are ECM degradation products such as collagens, periostin, osteopontin, and thrombospondin. As mentioned above, C-1158/59 – a collagen type I-derived peptide – levels are reduced in MI patients, compared to healthy controls, and negatively correlated with E/e' ratios, suggesting that lower levels of C-1158/59 correlate with reduced cardiac function due to elevated filling pressures [26].

After MI, the soluble ECM periostin is robustly expressed [116]. One recent study identified elevated periostin levels circulating in 123 patients (45 MI, 45 stable coronary artery disease, and 31 controls) [117]. The authors identified an association between decreased periostin blood levels and predicted cardiac function 3 months after acute MI. A possible mechanism for periostin cardiac effects is via cardiomyocyte stimulation. Periostin has been shown to induce reentry of differentiated cardiomyocytes into the cell cycle by integrin activation and improve heart function after MI in a rat model [118].

Circulating osteopontin robustly increases after myocardial infarction, suggesting its role in post-MI remodeling of the left ventricle [119]. We demonstrated in recent studies that osteopontin-derived peptides generated by MMP-9 increase cardiac fibroblast migration after wounding [120]. MMP-9 strongly correlates with worse patient MI outcomes [121], and this study demonstrated that different MMP-9 cleavage sites generate osteopontin peptides with distinct biological functions. *In vivo* studies focused on the osteopontin peptidome after MI and/or HF are necessary to fully understand the cardiac roles of this matricellular protein.

Thrombospondins are a matricellular family of multifunctional proteins involved in ECM synthesis and deposition, cell-ECM interactions, and tissue remodeling (under homeostasis and pathological conditions) [122]. Thrombospondin expression

increases in response to cardiac injury and is an active player in progression to HF [123, 124]. Peptides derived from thrombospondin 1 (TSP1) mimic the antiangiogenic properties of full-length TSP1 [125] and inhibit platelet aggregation [126], which may prove useful in the treatment of thrombosis. TSP1 is also involved in the activation of TGF-β, which leads to excessive fibrosis in several cardiovascular diseases [127, 128].

## Conclusions

Through the analysis of blood or urine samples from patients, the field of clinical peptidomics has evolved rapidly due to its high sensitivity and utility in biomarker discovery. Endogenous peptides provide a wide range of functions and have great potential as drugs, drug targets, or biomarkers (Table 13.1). The ECM plays a critical role in the maintenance as well as in the regulation of the myocardium, particularly as a response to stress or injury. ECM-derived peptides that are formed during cardiac remodeling can interact with cells and play an active role in intercellular signaling, to control cell behavior that is critical to the repair process. Additionally, the

**Table 13.1** Examples of peptides and small proteins in the human blood and their physiological relevance in cardiovascular disease

| Peptide | Molecular source | Molecular mass (~kDa) | Physiological relevance | Reference |
|---|---|---|---|---|
| Atrial natriuretic peptide | Peptide hormone | 16.7 | Anti-fibrotic, vasodilator | [77] |
| B-type natriuretic peptide | Peptide hormone | 34.6 | HF diagnostic | [90] |
| CSD | Caveolin-1 | 2.7 | Anti-fibrotic | [84] |
| C-type natriuretic peptide | N/A | 13.2 | Anti-fibrotic, anti-hypertrophic | [76–78] |
| Galectin-3 | N/A | 26.2 | HF prognostic: LV remodeling and mortality | [97, 100] |
| Ghrelin | Peptide hormone | 3.3 | Anti-fibrotic | [79, 80] |
| GDF-15 | N/A | 34.1 | HF mortality predictor | [11, 112] |
| Hep I | Collagen type IV | 1.6 | Pro-angiogenic | [27] |
| Hep III | Collagen type IV | 1.8 | Pro-angiogenic | [27] |
| IP10 | CXCL10 | 8.6 | Anti-fibrotic | [83] |
| N-terminal proBNP | Peptide hormone | 8.4 | HF diagnostic | [90] |
| p1158/59 | Collagen type I | 1.4 | Anti-fibrotic, pro-angiogenic | [26] |
| Relaxin-2 | Peptide hormone | 6.0 | Anti-fibrotic, anti-inflammatory | [81, 82] |
| RGD | Fibronectin | 1.3 | Pro-angiogenic | [27] |
| sST2 | ST2 | 38.5 | HF mortality predictor | [102, 105–109] |

presence and concentrations of ECM peptides in the blood act as surrogate indicators of ECM degradation and remodeling, both signature features of myocardial repair after MI and LV remodeling during HF. Interestingly, the development of synthetic ECM peptides (mimics of endogenous ECM peptides) recently emerged as a novel approach to elucidate the interaction of native ECM molecules with cells and ECM-ECM interactions. This will help us to further understand how the ECM regulates the myocardial environment. In conclusion, ECM peptides have not only the potential to be informative biomarkers of HF but also to develop into innovative strategies to treat heart failure due to their varied biological activities and cell-interacting capabilities.

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# Part IV
# Vasculature, Kidney, Liver, and Organ Fibrosis/Metastasis

# Chapter 14
# Vascular Fibrosis and Disease


**Mengxue Zhang, Bowen Wang, K. Craig Kent, and Lian-Wang Guo**

## Introduction

Cardiovascular disease is the number one cause of death worldwide [1]. The majority of cardiovascular disease incidences stem from insufficient blood supply to organs and tissues. This is acutely problematic in the heart and brain where cardiovascular disease gives rise to heart attacks and strokes, respectively. Atherosclerosis is the primary cause of obstructed circulation due to the buildup of cholesterol-rich plaques in the artery [2]. Artery stiffness, most often aging-associated, can alter the circulation as well. Standard interventions to recanalize the vascular system mainly include balloon angioplasty (and stenting) and bypass using autologous vein or

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prosthetic grafts [3]. A great number of these treatments fail because of recurrent diseases, namely, restenosis (re-narrowing) of arteries after angioplasty and stenosis of grafts. In all major vascular diseases (Fig. 14.1), fibrosis of the vasculature (herein termed vascular fibrosis) plays an important etiological role [4].

Fibrosis is often defined as a dysregulated wound-healing response. The hallmark of fibrosis is excessive deposition of extracellular matrix (ECM) [5]. Fibrosis occurs in different organs (or tissues) with distinct features [6]. For example, arterial stiffness (further discussed below) represents a type of vascular fibrosis promoted mainly by the change of the vasculature-specific smooth muscle cells to a phenotype that is highly ECM-synthetic. On the other hand, there is strong and compelling evidence for the concept that some core fibrogenic mechanisms and signaling pathways are shared by various organs [7]. Sustained myofibroblastic activation has been observed across many fibrotic pathologies, and the transforming growth factor (TGFβ)/Smad axis is widely recognized as a commanding pathway driving the differentiation of myofibroblasts from a variety of cell types [8].

In this chapter, we provide an overview of the underlying cellular and molecular mechanisms of fibrosis in major vascular diseases, as well as emerging epigenetic mechanisms involved in vascular fibrosis. As many excellent reviews on fibrosis exist, we set our focus specifically on vascular fibrosis. The relevance of lung and heart fibrosis is mentioned, but additional details on fibrosis in these organs can be found in Chaps. 5 and 11, respectively.

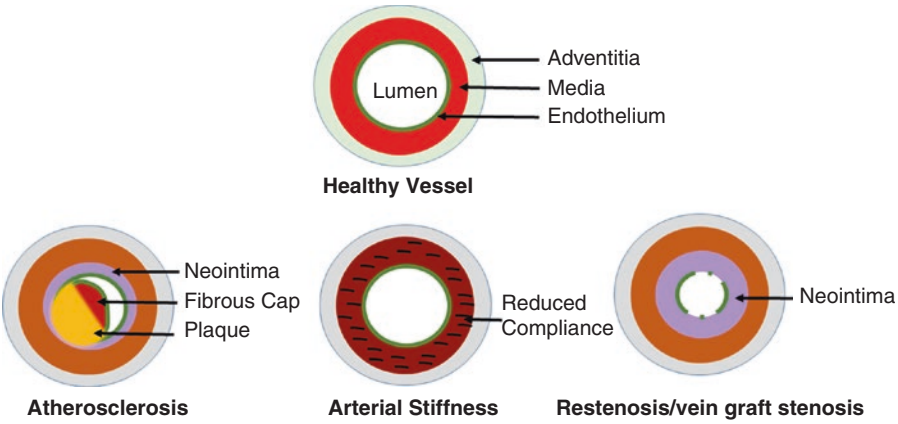


**Fig. 14.1** Schematic of major vascular pathologies with fibrosis involved. The normal vascular wall is composed of three layers (color coded), intima/endothelium, media, and adventitia, where three major types of vascular cells, endothelial cells, smooth muscle cells, and fibroblasts, respectively, reside. These different types of cells all can transform to myofibroblasts or myofibroblast-like cells contributing to various forms of vessel wall remodeling, e.g., the formation of atherosclerotic plaque and its fibrous cap, increased smooth muscle cell and medial stiffness in aged arteries, or neointimal hyperplasia and constrictive remodeling that result in post-angioplasty arterial restenosis or vein graft stenosis. The dashed green circle represents damaged or dysfunctional endothelium

## Fibrosis in Vascular Diseases

As commonly observed in other fibrotic disorders, fibrosis in the vascular system is a fundamental biological process in response to tissue damage or other signaling cues such as inflammation [9]. Although initially beneficial, if unchecked, the fibrotic process can turn pathophysiological, resulting in excessive ECM remodeling and scar formation that either causes or exacerbates vascular diseases. The role of fibrosis in vascular pathobiology is both temporally and spatially variable, as described below.

### *Atherosclerosis*

Atherosclerotic arteries, caused either by narrowed lumen or precipitating thrombi, reduce blood flow to the heart, brain, or lower extremities. Aside from plaques and neointima in the arterial wall, constrictive geometric vessel remodeling, i.e., reduced overall size, is important and, in some cases, a critical contributor to luminal narrowing (Fig. 14.1). It is well recognized that fibrosis is the primary cause of constrictive remodeling [10]. Analyses of numerous postmortem samples of severe atherosclerosis have shown substantial constriction of the vessel size accompanied by accumulation of various ECM proteins [11]. However, at early stages of atherosclerosis, the artery may undergo enlargement to compensate for the lumen size reduced by plaque formation. This process is known as adaptive remodeling or the Glagov phenomenon [12]. Thus, fibrotic constrictive remodeling of atherosclerotic vessels is not typical until advanced stages of disease progression when plaques occupy greater than 40% of the lumen area [12]. Fibrosis in the plaque can be beneficial, as ECM deposition could stabilize the fibrous cap and shoulder area of the plaque, thereby preventing plaque rupture and thrombosis [13].

### *Restenosis*

Following angioplasty, a procedure to reopen atherosclerotic arteries, restenosis can occur (Fig. 14.1). Restenosis can occur even with stent implantation, i.e., in-stent restenosis, as the neointimal lesion can grow through stent struts. All tunica layers of the restenotic arterial wall can harbor fibrosis. In the neointima, ECM accounts for an increasing portion of the lesion as the disease progresses [14]. It is unclear if accumulation of ECM is the cause or consequence of neointimal proliferation or if ECM accumulation is part of a feed-forward pathologic cycle. However, it has been well documented that fibrosis and consequential constrictive remodeling critically contribute to restenosis [15].

### *Graft Failure*

Surgery using autologous vein or prosthetic grafts to bypass occluded vessels is a common management strategy for many patients. These procedures include coronary artery bypass grafting (CABG) [16], femoral-popliteal bypass surgery (fempop) [17], and arteriovenous fistula [18]. It is estimated that ~25% of vein grafts fail due to stenosis within the first 12–18 months after grafting [19]. Fibrosis plays a biphasic role in vein grafts. Adapting to arterial blood flow, newly grafted veins undergo extensive fibrotic remodeling (or arterialization) that results in thickened media and adventitia and hence are physically strengthened to withstand arterial blood pressure. Inadequate fibrosis at this stage could lead to failure of graft maturation [20]. However, under constant stimulation of the arterial type of hemodynamics, excessive fibrosis contributes to constrictive remodeling and neointimal hyperplasia in the inner vessel wall [21]. These two processes can both prompt graft occlusion.

### *Arterial Stiffness*

Arterial stiffness is a key alteration during aging that leads to cardiovascular morbidity and mortality [22]. Typically observed in hypertension and measured by increase of pulse wave velocity, arterial stiffness often manifests as reduced elasticity, compliance, and distensibility. While inflammation, calcification, and endothelial dysfunction can all contribute to arterial stiffness, fibrosis plays a central role. Biomechanical properties of large arteries are mostly determined by the quantity and quality of collagen and elastin [23]. While crosslinked collagen fibers (especially type I) are stiff and strong, elastin fibers confer elasticity. Arteries become stiffer as the physical rigidity of fibrillar collagen predominates over the remaining elastin following age-associated degradation of elastin fibers. Evidence indicates that arterial stiffness is also associated with increased collagen expression and crosslinking, a relative excess of type-I over type-III collagen fibers, and altered collagen fiber orientation, all characteristic of fibrosis [24].

## Cell Types Involved in Vascular Fibrosis

Similar to lung and heart fibrosis [25], a key etiology in vascular fibrosis is increased population and activity of myofibroblasts, which display exaggerated ECM production [26]. Three distinct major cell types reside in the three layers of the vessel wall: fibroblasts in the tunica adventitia (outer layer), smooth muscle cells (SMCs) in the tunica media (middle layer), and endothelial cells (ECs) forming the endothelium (inner layer) [27]. In response to pathogenic cues, all of the vascular resident cells,

as well as circulating progenitor cells and inflammatory cells (e.g., macrophages), are capable of transforming into myofibroblasts or myofibroblast-like cells contributing to vascular fibrosis.

### *Fibroblasts*

Under pro-fibrotic conditions, fibroblasts in the vascular adventitia undergo myofibroblast transformation. As myofibroblasts represent the apex of the fibrotic cell phenotype, resident fibroblasts are the central mediators of vascular fibrosis [28]. Fibroblasts, especially with a myofibroblastic phenotype, are the workhorse driving ECM deposition not only in the adventitia but also in the media as well as in the neointima [29]. Experiments using animal models indicate that myofibroblasts differentiated from fibroblasts can migrate from the adventitia to the neointima [30], consistent with their presence in the neointimal lesion of human restenotic arteries [31]. In the case of atherosclerosis, however, strong evidence supports that myofibroblast-like cells in the plaque fibrous cap are not derived from adventitial fibroblasts, but rather from other cell types such as SMCs and ECs [32].

### *Smooth Muscle Cells*

Similar to pulmonary fibrosis, SMCs play an important role in vascular fibrosis [33]. It is well acknowledged that SMCs are not terminally differentiated. In response to injury or other stimuli, mature SMCs can dedifferentiate and acquire new phenotypes that resemble multiple other lineages such as myofibroblasts [34]. These transformed SMCs become highly synthetic, i.e., active in producing ECM proteins [35]. Recent studies have shown that, instead of differentiating into myofibroblasts, SMCs only transform to myofibroblast-like cells via a mechanism distinct from that underlying the adventitial fibroblast-to-myofibroblast transformation [36, 37]. SMCs contribute to vascular fibrosis most prominently in atherosclerosis and restenosis. Recent lineage-tracing studies [33, 38] provided compelling evidence indicating that the majority of the active ECM-producing cells in the plaque fibrous cap and neointimal lesion are originated from resident SMCs. This conclusion is also supported by evidence from human atherosclerotic plaques [39].

### *Endothelial Cells*

Epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndoMT) have long been shown to contribute to cardiac, pulmonary, liver, and renal fibrosis [40]. The role of EndoMT in vascular fibrosis and associated

diseases is less well documented. Recent studies using EC-specific lineage tracing and knockout animal models demonstrate that EndoMT is crucial to the pathogenesis of atherosclerosis [41] and graft failure [42, 43]. Specifically, vascular resident ECs contributed to fibrosis via EndoMT and/or transformation into myofibroblast-like and SMC-like phenotypes.

## *Fibroblast Progenitor Cells*

Various types of circulating and resident progenitor cells are capable of differentiating into fibroblasts or myofibroblasts following vascular injury. It is well established that mesenchymal stem cells and fibrocytes, found in the vessel wall and the circulation, respectively, can differentiate into fibroblasts [44, 45]. Sca-1(+) vascular progenitor cells, which are adventitial residents [46], were shown to contribute to the pathophysiology of atherosclerosis, arterial restenosis, and vein graft stenosis by differentiating into fibroblast-like cells [47]. A recent report suggested that circulating progenitor cells are the major source of fibrogenic cells participating in aortic fibrosis induced by hypertension. Another recent report found that resident multipotent vascular stem cells could differentiate into all the cell types in the neointimal lesion in an animal model of restenosis [48]. However, this discovery was later refuted by a lineage-tracing study [49], which demonstrated that the majority of the cells in the mouse atherosclerotic plaque (including neointima) originated from the arterial wall-resident SMCs. Moreover, recent lineage-tracing studies in different organs overall supported the assertion that the main sources of myofibroblasts, the master cellular driver of fibrosis, are resident mesenchymal cells such as fibroblasts and pericytes [50]. Consensus on the delineation of the relative contributions of various stem or progenitor cells to vascular fibrosis awaits further clarification and is likely dependent on specific disease contexts.

## *Inflammatory Cells*

Leukocyte activation and inflammation are common in vascular diseases. Leukocytes factor into vascular fibrosis largely in an ancillary fashion, e.g., secretion of profibrotic factors and also production of proteases that degrade ECM proteins [51]. However, as suggested in recent studies, monocytes could also contribute to fibrosis as the origin of fibrocytes, which are capable of differentiating into fibroblasts [52]. Different subtypes of monocytes variably impact the fibrotic process [53]. Specifically, in atherosclerosis associated with unstable plaques, M1 macrophages prominently accumulate in the fibrous cap region, whereas M2 macrophages are more abundant in the adventitial layer [54]. It is important to note that inhibition of pro-inflammatory reactions may yield undesirable outcomes given the complexity of disease mechanisms. For example, blocking IL-17A could effectively prevent the development of atherosclerotic plaque [55], but this treatment impairs fibrosis, thus inevitably leading to increased plaque instability and risk of rupture [56, 57].

## ECM Remodeling

The ECM is the network of diverse proteins and sugars that surround cells. ECM homeostasis is essential for development, normal tissue functions, and wound healing [58]. Imbalanced ECM remodeling in the vascular wall, e.g., excessive ECM protein deposition and crosslinking, can lead to vascular fibrosis. In healthy vessels, collagen (mainly type I and type III) and elastin account for ~60% and ~30% of total ECM proteins, respectively. The decomposition of elastin fibers and an increase in the stiffer collagen fibers often occur in vascular fibrosis. Fragmented elastin fibers are often present in human atherosclerotic tissues [59], angioplasty-injured arteries [60], and also stenotic vein grafts [61, 62]. Growing evidence indicates that it is not only the increase of total collagen but also changes in subtype ratios, crosslinking levels, and organization of collagen fibers that collectively contribute to vascular fibrosis. While age-related arterial stiffness commonly has an increase of type-I versus type-III collagen (less stiff than the former), recent studies suggest that the opposite may benefit adaptive remodeling in a restenosis model [63]. In addition, excessive collagen crosslinking is present in vascular fibrosis and constrictive remodeling [64]. Expression of other ECM proteins, such as tenascin [65], osteopontin [66], periostin [67], and hyaluronan [68], is increased in diseased vessels, contributing to vascular fibrosis in animal models. Taken together, all these changes collectively give rise to aberrant ECM remodeling and vascular fibrosis.

## Key Molecular Pathways Participating in Vascular Fibrosis

### *MMPs and TIMPs*

In healthy tissues, ECM production and degradation determine ECM homeostasis. If dysregulated, excessive synthesis, assembly, and crosslinking of ECM proteins can lead to altered biochemical and biomechanical matrix properties and, ultimately, fibrosis [69]. Various matrix metalloproteinases (MMPs) regulate the turnover of collagen and other ECM proteins, as do tissue inhibitors of metalloproteinases (TIMPs). The imbalance between these two antagonistic groups could lead to vascular fibrosis or other pathologies [70]. Retrospective analysis of clinical specimens revealed a close association of increased TIMP1 expression with arterial stiffness and calcification [71]. On the other hand, atherosclerotic plaques in diabetic subjects associated with a decrease in TIMP3 expression [72], coinciding with increased activities of MMPs 1, 8, 9, and 13 and plaque instability [73]. Other ECM proteases and their inhibitors were identified to participate in vascular fibrosis. These include a disintegrin and metalloproteinases (ADAMs) [74], as well as cathepsins [75, 76], whose dysregulation contributes to the pro-fibrotic signaling in atherogenesis [77] and restenosis [78]. Aside from the proteases and their inhibitors, lysyl oxidases, which catalyze collagen crosslinking, are linked to fibrotic processes. The ECM-sculpting proteins are regulated by a variety of pathways (e.g., TGFβ), at multiple levels, including expression and enzymatic activity.

## *TGFβ*

TGFβ is a master regulator of fibrosis [79]. Various vascular diseases are characterized by abnormal activation of canonical (Smad-dependent) and noncanonical (Smad-independent) TGFβ signaling pathways [80]. Similar to the mechanisms in cardiac and pulmonary fibrosis, the TGFβ/Smad3 signaling axis plays a pivotal role in vascular fibrosis [81] via acting on all three major vascular cell types (Fig. 14.2). It is well established that the TGFβ/Smad3 signaling is the primary driver of the transformation of adventitial fibroblasts to myofibroblasts [82]. In SMCs, elevation

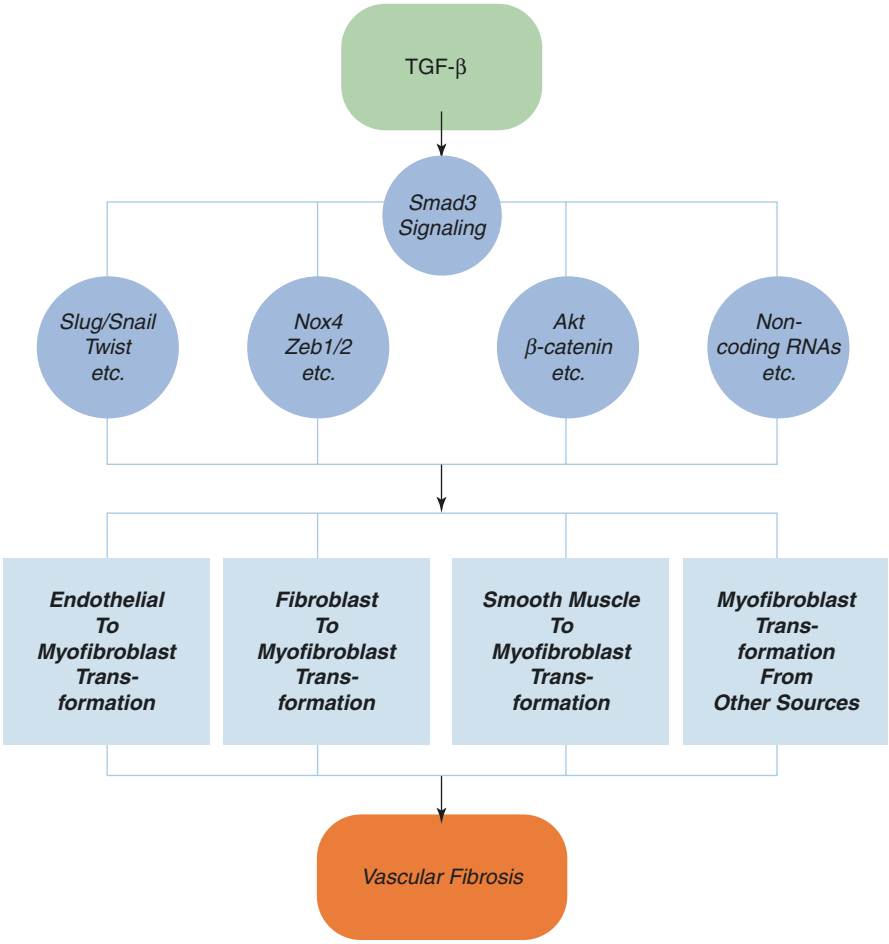


**Fig. 14.2** TGFβ/Smad3 signaling in vascular fibrosis. This is an overview of TGFβ/Smad3-activated signaling pathways and the associated transformation of vascular and nonvascular cells into myofibroblast or myofibroblast-like cells

of both TGFβ and Smad3 leads to cell dedifferentiation and a synthetic phenotype and consequently increased ECM production [83, 84]. In addition, TGFβ signaling was reported to suppress MMP2 expression in SMCs, further potentiating the pro-fibrotic effect of TGFβ [85]. Paradoxically, in a rat model of restenosis, TGFβ/Smad3 upregulation leads to adaptive (or expansive) rather than constrictive arterial remodeling. The underlying mechanism may involve increased secretion of connective tissue growth factor (CTGF) from SMCs which, in a paracrine fashion, alters production of type-I and type-III collagen in adventitial fibroblasts in favor of adaptive remodeling [63, 86]. TGFβ/Smad3 also plays a role in EndoMT. A recent study identified that activation of the Slug/Snail pathway via TGFβ/Smad3 drives EndoMT and ultimately myofibroblast-like phenotypes that contribute to vascular fibrosis [42]. Although TGFβ is a well-established fibrogenic factor, in view of its pleiotropic effects and the complex reciprocal influences between vascular diseases and fibrosis, challenges remain to elucidate the specific TGFβ-mediated mechanisms in various disease and intervention contexts.

### *Angiotensin II*

Aside from its classic role in promoting renal fibrosis and hypertension, angiotensin II and associated pathways are also important players in vascular fibrosis. Numerous studies in animal models demonstrated that injury in the vessel wall leads to increased activity of angiotensin-converting enzyme (ACE), concomitant with increases in angiotensin II [87, 88]. Angiotensin II is a potent inducer of fibroblast-to-myofibroblast transformation or myofibroblast-like phenotypes of SMCs [89]. Multiple signaling pathways are involved, including the reactive oxygen species (ROS)/NADPH oxidase (NOX) signaling axis [90] and protein kinase C pathways [90, 91]. Activation of angiotensin II receptors (AT1 and AT2) can cross-activate other pro-fibrotic pathways such as TGFβ, CTGF, and TNFα, hence further exacerbating vascular fibrosis [92, 93].

### *CTGF*

CTGF is another potent pro-fibrotic factor often found to be upregulated in fibrotic tissues [94]. CTGF expression was positively correlated with plaque stability in atherosclerotic patients, consistent with its role in strengthening the fibrous cap [95]. However, there is an inverse correlation between CTGF level and vein graft patency, likely due to constrictive remodeling caused by excessive pro-fibrotic CTGF signaling [96]. On the other hand, in an animal model of angioplasty-induced restenosis, CTGF gene transfer in the carotid artery produced an unexpected beneficial outcome of adaptive remodeling, despite significant accumulation of collagen

in the adventitia. This adaptive remodeling was a likely result from the TGFβ/Smad3-activated paracrine CTGF signaling produced by SMCs and transduced to adventitial fibroblasts [86].

### *TNFα*

TNFα is a potent pro-inflammatory cytokine that is elevated in patients with vascular disorders. Surgical injury alone in animal models stimulates the secretion of TNFα, contributing to vascular fibrosis and constrictive remodeling [97, 98]. In cultured cells, TNFα promotes ECM synthesis in all the major vascular cell types. Specifically, TNFα stimulates several pro-fibrotic transformations such as the transformation of fibroblasts to myofibroblasts [99] and the transformation of SMCs and ECs (via EndoMT) to myofibroblast-like cells [100, 101].

## Therapeutic Strategies for Vascular Fibrosis

Despite extensive mechanistic and preclinical studies, effective treatments specifically designed to treat fibrotic conditions are still lacking in the clinic [102]. Existing therapeutics for cardiovascular disease, in principle, could ameliorate vascular fibrosis. These include beta blockers, inhibitors for ACE and angiotensin receptor and endothelin-A receptor, statins, glitazones, and rapamycin [103]. However, to date, these drugs have had mixed results in vascular disease. For example, infliximab (antibody for TNFα) mitigated arterial fibrosis and stiffness in patients with rheumatoid arthritis [104] but at the same time worsened atherosclerosis [105].

The molecular pathways participating in vascular fibrosis represent potential targets for intervention [106]. Inhibitors or neutralizing antibodies for the TGFβ signaling pathways have shown promising outcomes in multiple preclinical models of vascular fibrosis [107, 108]. Anti-CTGF antibodies were shown to be effective in ameliorating vascular fibrosis and stiffness in diabetic models [109]. Manipulation of ECM protease activities represents another promising strategy for treatment of vascular fibrosis. Other therapeutics, such as nitric oxide [110], carbon monoxide [111], and antioxidants [112], are known to be antifibrotic as well. In addition, some emerging antifibrotic agents currently in clinical trials for cancer, such as bone morphogenetic protein-7 (BMP7) and polypeptide hormone relaxin, have beneficial effects in multiple models of fibrotic disease [113, 114]. While promising, these potential therapeutics require thorough safety and efficacy analysis via optimized preclinical and clinical tests [96]. These studies are warranted, considering the interwoven relationships between vascular fibrosis and various vascular diseases and comorbidities that complicate therapeutic outcomes.

## Emerging Importance of Epigenetics in Vascular Fibrosis

Epigenetics refers to potentially heritable changes in gene expression without involving a DNA sequence change. Epigenetic mechanisms have been implicated in almost all pathophysiological processes [115]. It is increasingly acknowledged that epigenetic dysregulation plays a profound role in vascular fibrosis and diseases. Thus, targeting epigenetic mechanisms represents a new avenue to identify effective treatments for vascular fibrosis. Here we describe three major epigenetic mechanisms.

### *Noncoding RNAs*

Unlike mRNA that is translated into proteins, noncoding RNAs are regulators of gene expression [116]. The best known noncoding RNAs include microRNAs (miRNAs), long noncoding RNAs, and circular RNAs. miRNAs are endogenous epigenetic modulators and have been extensively studied in vascular diseases. Delivery of synthetic miRNAs or anti-miRNAs demonstrated great potential in mitigating disease progression. miR-15 [117], miR-21 [118], miR-24 [119], and miR-29 [120] are the most prominent regulators that strongly influence ECM deposition and fibrosis in various organs, including arteries. Dysregulation of miR-15, miR-29, and miR-210 occurs in the fibrous cap of plaques in atherosclerotic patients [121]. Reducing miR-210 expression improved fibrous cap stability in animals [121]. The underlying mechanism may involve inhibition of the SMC transformation to a synthetic, myofibroblast-like phenotype. Moreover, in injury-induced restenosis models, miR-21 [122], miR-24 [123], miR-29 [124], and miR-143/145 [125] strongly influence SMC transformation and also geometric vascular remodeling. miR-155 was shown to negatively regulate angiotensin II-induced adventitial fibroblast activation [126], which could potentially benefit the prevention or treatment of aortic fibrosis and stiffness in hypertension patients.

### *DNA Modification*

DNA methylation, particularly at the CpG islands located upstream of gene transcription start sites, typically leads to repressed gene transcription via recruitment of silencing protein complexes [127]. Multiple vascular diseases are associated with aberrant DNA methylation, e.g., in dedifferentiated SMCs in the fibrous cap of atherosclerotic plaques [128]. In a hyperhomocysteinemia model, inhibition of DNA methylation by pharmacologically blocking DNA methyltransferases effectively ameliorated ECM deposition and aortic stiffness [129]. Ten-eleven translocation-2 (TET2), an enzyme that facilitates DNA demethylation, blocked SMC transformation to a synthetic, myofibroblast-like phenotype [130].

### *Histone Modification*

Amino acids in the unstructured tail regions of histones can undergo an array of chemical modifications, including acetylation, methylation, phosphorylation, sumoylation, citrullination, and ubiquitination. These epigenetic marks, especially acetylation and methylation, can substantially alter the transcriptional accessibility of genes and also serve as docking sites for transcriptional regulators [131]. The SMCs and adventitial fibroblasts undergoing transformation to the synthetic/myofibroblastic phenotype showed aberrant histone methylation and acetylation [99, 132]. Inhibition of histone deacetylase 3 (HDAC3) stabilized the fibrous cap of atherosclerotic plaque in animal models [133]. Recently, the bromo- and extra-terminal domain (BET) family of epigenetic "readers" (binding to acetylated histone) was implicated in liver [134], pulmonary [135], and pancreatic fibrosis [136]. Systemic delivery of a BET inhibitor ameliorated cardiac fibrosis [137]. Our published and unpublished data also suggests that targeting BET family members such as BRD4 could effectively inhibit the myofibroblast-like phenotype of SMCs and ECs and ameliorate lumen narrowing in angioplastied arteries and also vein grafts in rats [138].

Overall, epigenetic mechanisms underlying vascular fibrotic processes are poorly understood. More research in this area would refresh the momentum in identifying novel targets and interventional methods for efficacious and safe treatment of vascular fibrosis.

## Concluding Remarks

Fibrosis is a key player in the development and progression of vascular diseases. While the etiology of fibrosis varies widely in different disease contexts, aberrant ECM remodeling is the common feature produced principally by a myofibroblastic phenotype. Thus, vascular fibrosis is the collective result of contributions from vascular and nonvascular cell types that transform into myofibroblasts or myofibroblast-like cells. Although great strides were made over the past decade in understanding fibrosis in various diseases, there remains a lack of effective clinical treatments targeting fibrosis. More in-depth research is therefore warranted to uncover novel fibrogenic mechanisms and to dissect the complex relationships between fibrosis and concomitant vascular disorders. As fibrosis leads to numerous diseases, progress in optimizing antifibrotic treatments in the vascular system will have broad impacts on public health.

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# Chapter 15
# Liver Fibrosis: Current Approaches and Future Directions for Diagnosis and Treatment


**Jennifer Y. Chen, Dhruv Thakar, and Tammy T. Chang**

## Epidemiology and Burden of Disease

Cirrhosis is the 5th leading cause of death in the United States and the 13th leading cause of death worldwide [1, 2], resulting in one million deaths per year worldwide and 33,000 deaths per year in the United States [3]. In addition, an estimated 19,500 deaths per year are attributed to hepatocellular carcinoma, which occurs more frequently among patients with cirrhosis [4]. In the United States, cirrhosis ranks eighth in economic cost burden [2] with annual direct costs estimated at greater than $2 billion and indirect costs exceeding $10 billion [5]. The incidence and prevalence of cirrhosis are difficult to estimate because the majority of patients are asymptomatic during the early stages of disease.

Patients with cirrhosis are classified into two main prognostic stages: compensated or decompensated disease. Patients who develop conditions such as variceal hemorrhage, ascites, or hepatic encephalopathy are classified to have decompensated cirrhosis; those without clinical complications are classified as having

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compensated cirrhosis. The Child-Turcotte-Pugh classification is used clinically to stratify patients with cirrhosis. Those that belong to Child's class A are compensated, whereas those in Child's classes B and C are decompensated. The average life expectancy from the time of diagnosis varies from 13 years, for patients with compensated disease, to only 2 years in patients with decompensated cirrhosis [6]. The risk of death in patients with compensated versus decompensated cirrhosis is 4.7 versus 9.7 times higher than the general population, respectively [7].

## Etiologies of Liver Fibrosis

Development of liver fibrosis and, ultimately, cirrhosis is the final common pathway of any chronic liver disease. The most common etiologies of chronic liver disease in the United States are alcoholic liver disease, chronic hepatitis C infection, and nonalcoholic fatty liver disease. Together, these three diseases account for approximately 80% of the disease etiologies in individuals with end-stage liver failure awaiting liver transplantation between 2004 and 2013 [8].

Nonalcoholic fatty liver disease (NAFLD), defined as the presence of steatosis in ≥5% of hepatocytes in individuals who do not consume excessive alcohol, affects 80–100 million people in the United States and is the most common cause of chronic liver dysfunction [9]. The rising prevalence of NAFLD, currently 20–30% worldwide, is linked to the obesity epidemic that has engulfed the United States and the world. More than a third of the US population is now obese, and trends indicate that prevalence of obesity will continue to increase [10]. NAFLD is a manifestation of the metabolic syndrome and coexists with obesity, type 2 diabetes, insulin resistance, dyslipidemia, and cardiovascular disease. About 10% of people with NAFLD will develop a progressive form of the disease termed nonalcoholic steatohepatitis (NASH), characterized by ballooning hepatocyte degeneration and inflammation. NASH can progress to fibrosis, cirrhosis, liver failure, and hepatocellular carcinoma [11]. Although a minority of people with fatty liver disease develop NASH, it is the only indication for liver transplantation that is rapidly increasing in frequency and thus predicted to become the most common cause of end-stage liver disease requiring transplantation in the United States [12, 13].

Other etiologies of liver fibrosis include chronic viral hepatitis B infection, hemochromatosis, autoimmune hepatitis, primary and secondary biliary cirrhosis, primary sclerosing cholangitis, Wilson disease, alpha-1 antitrypsin deficiency, celiac disease, polycystic liver disease, idiopathic portal fibrosis, idiopathic adulthood ductopenia, granulomatous liver disease, veno-occlusive disease, hereditary hemorrhagic telangiectasia, and right-sided heart failure. In addition, medications (e.g., methotrexate) and infection (e.g., echinococcosis) can result in cirrhosis. In approximately 85–90% of patients, a specific etiology for liver fibrosis is identified [14]. Common laboratory tests ordered to identify potential causes of chronic liver disease are displayed in Table 15.1.

**Table 15.1** Common laboratory tests obtained to determine the etiology of liver disease

| Disease etiology | Laboratory tests |
|---|---|
| Autoimmune hepatitis | Antinuclear antibody, anti-smooth muscle antibody, immunoglobulin G |
| Primary biliary cirrhosis | Anti-mitochondrial antibody |
| Wilson disease | Ceruloplasmin, 24 h urinary copper excretion |
| Alpha-1 antitrypsin deficiency | Alpha-1 antitrypsin |
| Hemochromatosis | Iron, ferritin, total iron binding capacity; if suggestive, screening for mutations associated with hemochromatosis |
| Chronic hepatitis B infection | Hepatitis B surface antigen, hepatitis B surface antibody |
| Chronic hepatitis C infection | Hepatitis C antibody, hepatitis C viral load if antibody positive |
| Celiac disease | Anti-tissue transglutaminase antibody |

## Histopathological Changes in the Fibrotic Liver

The liver receives a dual blood supply from the hepatic artery and the portal vein. The hepatic artery delivers highly oxygenated blood from the celiac trunk of the aorta. The portal venous system carries blood from the esophagus, stomach, small and large intestine, pancreas, gallbladder, and spleen to the liver and constitutes 75% of total hepatic blood flow. Hepatic artery and portal vein derived blood mix in the hepatic sinusoids that are permeable vascular channels lined by fenestrated endothelial cells, allowing transport of macromolecules to hepatocytes. Within the sinusoid, there exists the space of Disse, which is located extraluminal to the endothelial cell and adjacent to the hepatocyte. Hepatic stellate cells and Kupffer cells lie within the space of Disse and play important roles in fibrogenesis (Fig. 15.1).

The extracellular matrix of the normal liver is unique in structure and composition in several ways. The space of Disse separates epithelial hepatocytes from the fenestrated sinusoidal endothelium and contains a basement membrane-like matrix that lacks the typical electron-dense structure of basement membranes in other tissues [16–18]. The low density of the liver basement membrane-like structure is critical for allowing easy bidirectional macromolecular diffusion between blood and liver cells and for maintaining the differentiated function of hepatocytes [18, 19]. Collagen IV, a non-fibrillar collagen that is a major component of most tissue basement membranes, is present in the normal space of Disse in the form of α1α1α2(IV) heterotrimers [20]. It is controversial whether laminin, another extracellular matrix protein that is typically found in basement membranes, is present in the normal liver. Some reports suggest that the space of Disse is devoid of laminin [21], while others report that both collagen IV and laminin are present [19]. The absence of an identifiable basement membrane structure, despite the presence of both collagen IV and laminin, may be explained by the absence of nidogen, a glycoprotein that typically

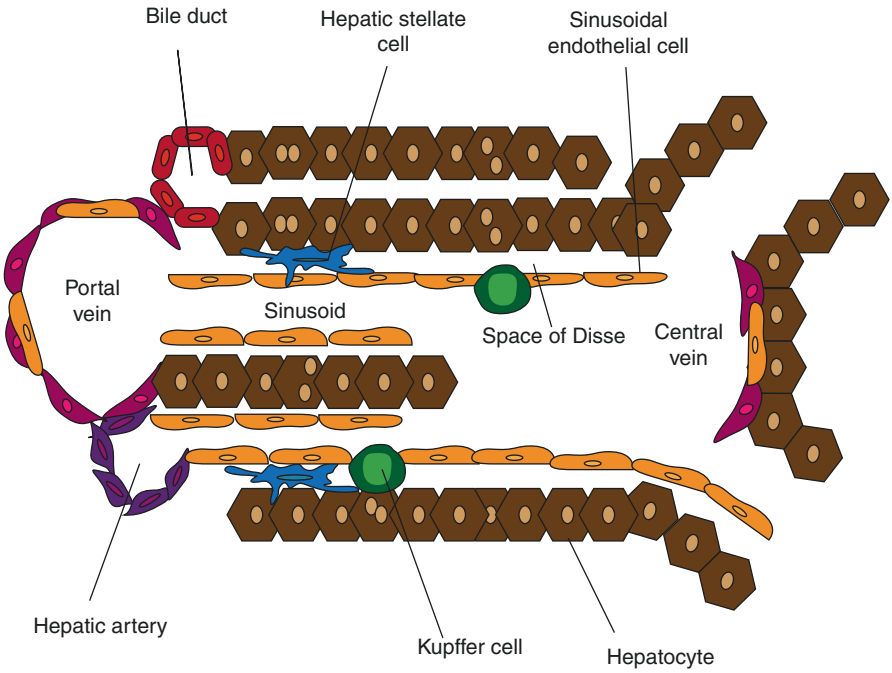


**Fig. 15.1** Microarchitecture and cellular components of hepatic sinusoids and the location of the space of Disse. (Reprinted with permission from [15])

functions to bridge collagen IV and laminin networks in basement membranes [19]. On the other hand, the space of Disse contains collagen type I and fibronectin, which are not typical constituents of basement membranes [18, 22–24]. Another isoform of collagen, collagen XVIII, has also been localized to the perisinusoidal zones in the liver [25–27]. Due to the high amounts of collagen XVIII, the liver is the major source of the collagen XVIII in the body. Additionally, in the normal liver, the heparan sulfate proteoglycans perlecan and agrin are found in small amounts.

Although the normal liver space of Disse consists of a low-density extracellular matrix, during fibrosis the sparse matrix is progressively replaced by a continuous interstitial-like matrix with the accumulation of fibrillar collagens, particularly collagens I and III [20, 28–30]. This process, called capillarization of the sinusoids, is accompanied by a loss of fenestration of the sinusoidal endothelium and physical changes in the hepatocytes that lose their microvilli [28, 31]. Fibrosis is also associated with markedly elevated amounts of perlecan and agrin [32–34]. These changes are hypothesized to adversely affect hepatocyte viability during progression toward liver cirrhosis [35].

When fibrosis advances to cirrhosis, the normal architecture of the liver is significantly disrupted by the formation of increasingly dense fibrous septa of fibronectin and interstitial collagens I, III, V, and VI. Histologically, cirrhosis is characterized by the formation of fibrotic septa, distortion of hepatic vascular

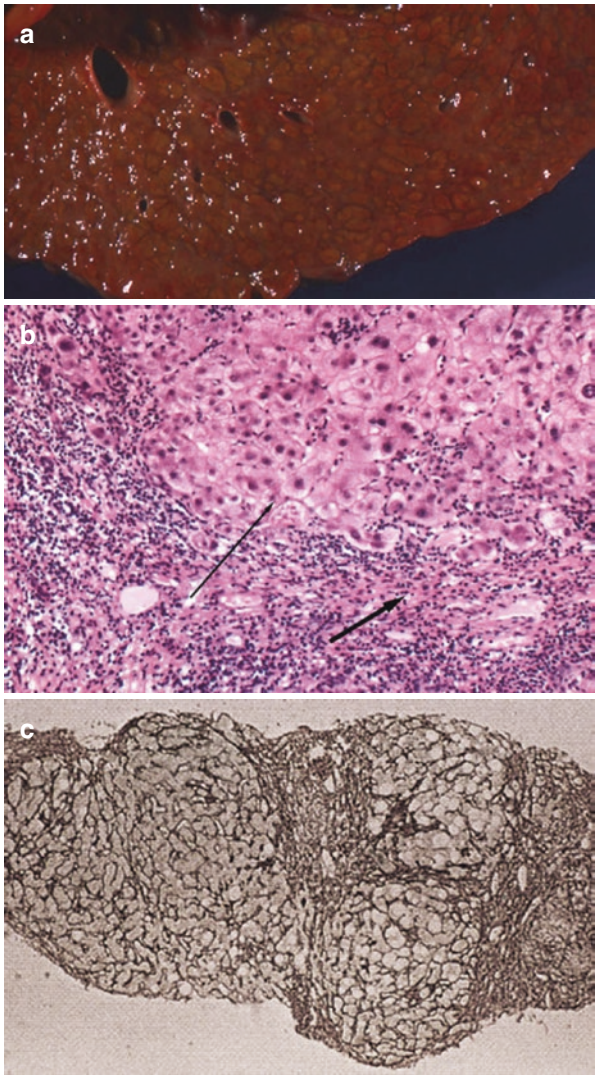


**Fig. 15.2** Gross appearance and histological features of the cirrhotic liver. (**a**) Gross specimen showing the cut surface of a cirrhotic liver with nodular appearance. (**b**) Hematoxylin and eosin stain of a liver biopsy from a cirrhotic patient showing a regenerative nodule (*thin arrow*), fibrotic septa (*thick arrow*), and inflammation. (**c**) Reticulin stain of a cirrhotic liver showing regenerative nodules outlined by fibrotic septa. (Reprinted with permission from [36])

architecture, and development of regenerative nodules (Fig. 15.2). The formation of a continuous basement in the space of Disse is characteristic of cirrhosis [18]. In the cirrhotic liver, sinusoidal cells and hepatocytes synthesize and secrete laminin [37, 38], which leads to a marked increase in laminin deposition in the space of Disse, eventually forming a continuous basement membrane with fibrillar collagens and perlecan [20, 21]. The increased extracellular matrix deposition during cirrhosis impairs the normal exchange of soluble proteins and fluids between sinusoidal blood and adjacent liver cells, which is a major contributor to hepatic failure [39].

### *Clinical Pathology Classification Systems*

There are several clinical histologic scoring systems used to characterize chronic liver disease progression that differ according to etiology. The Ishak and METAVIR scores are used to assess chronic hepatitis, and they include descriptions of degree of fibrosis and necro-inflammatory activity. The Ishak score includes several stages for describing fibrosis, which allows for documentation of small changes in fibrosis progression. The nonalcoholic fatty liver activity score (NAS) is utilized to evaluate patients with NAFLD/NASH, and the components of the score include steatosis, lobular inflammation, and ballooning. The NAS ranges from 0 to 8, with scores of 5–8 considered diagnostic of NASH [40].

## Clinical Diagnosis of Liver Fibrosis

Clinicians still consider liver biopsy, performed by percutaneous, laparoscopic, or transjugular approaches, to be the gold standard for the diagnosis of liver fibrosis. However, liver biopsy is invasive and subject to sampling error [41]. As such, development of accurate and noninvasive diagnostic tools for liver fibrosis is an active area of laboratory and clinical investigation. A liver biopsy may be unnecessary to confirm diagnosis, if clinical, laboratory, and radiographic data support the diagnosis of cirrhosis.

### *Serum Biomarkers*

Numerous serum biomarker panels were developed for the diagnosis of liver fibrosis, but there remains no consensus as to which indices are most clinically useful [42–44]. Direct biomarkers are measures of serum proteins indicative of the accumulation and turnover of the liver extracellular matrix and are used to estimate the extent of fibrogenesis. An example of a direct biomarker panel is the enhanced liver fibrosis (ELF) test, which measures hyaluronic acid, tissue inhibitor of metalloproteinase 1 (TIMP-1), and amino-terminal pro-peptide of procollagen type III (PIIINP) [45]. Indirect biomarkers of liver fibrosis are serum markers that change as a result of functional alterations of the liver, hepatocellular damage, and inflammation. An example of an indirect measure of liver fibrosis is the aspartate aminotransferase (AST)/platelet ratio index (APRI), which can be calculated from two commonly obtained laboratory tests [46]. The FibroTest, also known as BioPredictive in the EU and FibroSURE in the United States, is an extensively studied proprietary indirect biomarker panel that is composed of alpha-2-macroglobulin, haptoglobin, apolipoprotein A1, bilirubin, and gamma-glutamyl transferase [47, 48]. In general, serum biomarkers are better at predicting advanced stages of fibrosis and less accurate in distinguishing earlier stages of fibrosis from no fibrosis (Table 15.2) [42–44].

**Table 15.2** Sensitivity and specificity of serum biomarker tests to exclude fibrosis and diagnose cirrhosis

| Test name | Biomarker type | Biomarkers | Exclude fibrosis | | Diagnose cirrhosis | |
|---|---|---|---|---|---|---|
| | | | Sensitivity (%) | Specificity (%) | Sensitivity (%) | Specificity (%) |
| Enhanced liver fibrosis (ELF) | Direct | HA, TIMP-1, PIIINP | 90 | 55 | 83 | 97 |
| AST/platelet ratio index (APRI) | Indirect | AST, platelets | 95 | 27 | 51 | 94 |
| FibroTest | Indirect | α2M, haptoglobin, ApoA1, bilirubin, GGT | 89 | 53 | 50 | 93 |

*HA* hyaluronic acid, *TIMP-1* tissue inhibitor of metalloproteinase 1, *PIIINP* amino-terminal propeptide of procollagen type III, *AST* aspartate aminotransferase, *α2M* alpha-2-macroglobulin, *ApoA1* apolipoprotein A1, *GGT* gamma-glutamyl transferase

## *Conventional Imaging Modalities*

Conventional imaging techniques are only able to detect advanced stages of liver fibrosis. Ultrasonography is well-tolerated, and diagnostic features of cirrhosis include a small nodular liver, right lobe atrophy, and hypertrophy of the caudate or left lobes. Ultrasonography has been reported to have a sensitivity of 91% and a specificity of 94% for the diagnosis of cirrhosis [49]. Ultrasonography can also provide information regarding portal hypertension, such as flow and diameter within the portal vein. Computed tomography (CT) can also be used to identify findings suggestive of cirrhosis, including liver nodularity and caudate lobe hypertrophy. Magnetic resonance imaging (MRI) can provide information regarding fat content for diagnosis of steatosis [50] and hepatic iron concentration for determining iron overload [51], in addition to information regarding liver size. Other diagnostic features obtained by these imaging modalities are indicative of advanced fibrosis and represent sequelae of decompensated cirrhosis, including ascites, varices, splenomegaly, hepatic or portal vein thrombosis, the presence of porto-collateral circulation, or the finding of reversal of flow within the portal system. Splenomegaly is a sensitive but nonspecific sign of portal hypertension [52].

## *Novel Technologies: Elastography and Liver Stiffness*

Increased liver tissue stiffness is a hallmark of cirrhosis. Accordingly, noninvasive imaging modalities have been developed to quantify liver stiffness as a surrogate marker of fibrosis. Transient elastography (TE), also known as FibroScan, utilizes

pulse-echo ultrasound to measure liver stiffness, and a threshold of greater than 20 kilopascals (kPa) is associated with a diagnostic accuracy of over 90% [53]. Magnetic resonance elastography (MRE) provides data of larger areas of the liver compared to TE and has been shown to be more accurate than TE [54].

Transient elastography and MRE are increasingly being used clinically to determine a patient's liver stiffness as a proxy for fibrosis and to aid in clinical decision-making. The relative liver stiffness determined by these techniques correlates well with the histological severity grade of fibrosis. TE, expressing stiffness in elastic modulus (E), estimates normal human liver stiffness to be around 5 kPa and grade 4 cirrhotic livers to be 15–20 kPa [55]. MRE, expressing stiffness in shear storage modulus (G'), estimates normal human liver stiffness to be 2 kPa and places the matrix stiffness cutoff for grade 4 cirrhotic livers at >5 kPa [56, 57]. The advantage of these noninvasive techniques is that they evaluate the rigidity of large regions of the liver, thereby avoiding sampling error that is inherent in assessing fibrosis by liver biopsy. However, stiffness values obtained are extrapolated from the response of the liver to shear waves and, therefore, represent relative values and do not accurately reflect the absolute mechanical tissue stiffness. Direct mechanical testing of liver tissue by rheometry suggests that normal liver stiffness is around 400 Pa and fibrotic liver stiffness ranges around 2 kPa [58, 59]. Atomic force microscopy (AFM), which measures microscale matrix rigidity, shows that increased matrix stiffness in fibrotic livers is a local phenomenon near fibrotic tracts and regions distant from the deposition of aligned collagen approaches the stiffness of normal liver. AFM determined the absolute rigidity of normal mouse liver matrix to be approximately 150 Pa and the stiffness of areas near fibrotic tracts in fibrotic livers to range between 1 and 6 kPa [60].

Determining the absolute matrix stiffness of a fibrotic liver may be clinically important as there is evidence that increased matrix rigidity directly inhibits hepatocyte functions. Therefore, increased matrix stiffness may be a key mechanism by which fibrosis causes liver dysfunction [60]. The relative tissue stiffness reported by elastography does not reflect the absolute matrix stiffness hepatocytes experience at a cellular level. Therefore, current elastography techniques may not be sensitive enough to determine the changes in liver stiffness that will impact hepatic function and clinical outcomes. In addition, elastography does not distinguish between tissue stiffness due to perfusion pressure produced by portal hypertension versus matrix rigidity associated with increased collagen deposition. It is currently unknown whether different physiological inducers of tissue rigidity have differing effects on hepatocyte function. Likewise, the relative contribution of each source of stiffness to the overall rigidity of fibrotic livers is also unknown. Moreover, liver fibrosis is not a homogenous process at the tissue level; even in advanced fibrosis, where there are bridging fibrotic bands, areas of relatively less collagen deposition remain, suggesting that a hepatocyte's response to matrix rigidity is likely specific to its immediate local microenvironment. If hepatocytes exhibit suppressed functions at a threshold level of matrix stiffness, the overall averaged stiffness of the liver may be less clinically important to predicting

hepatic functional outcomes than the proportion of liver volume that has reached a certain threshold stiffness. The experience with incorporating TE and MRE into clinical practice is evolving. There remain confounding factors whether changes in elastography correlate with changes in fibrosis and uncertainty whether liver stiffness measurements are predictive of clinical outcome [61]. Additional research is required to investigate the relationship between fibrotic liver stiffness and hepatocyte function and determine whether elastography may be used to prognosticate liver function.

## Clinical Presentation

Early-stage fibrosis is largely asymptomatic. Patients with advanced liver fibrosis present with liver metabolic dysfunction and clinical sequelae of portal hypertension.

### *Pathophysiology of Portal Hypertension*

In patients with cirrhosis, portal hypertension results from alterations in portal vascular pressure as described by Ohm's law ($\Delta P = F \times R$), where the pressure gradient in the portal circulation ($\Delta P$) is a function of portal flow ($F$) and resistance ($R$). In cirrhosis, increased intrahepatic resistance and hyperdynamic portal circulation result in portal hypertension and its clinical sequelae. The increased resistance occurs in the setting of structural mechanisms, including collagen deposition, vascular distortion, and microthrombi. In addition, increased intrahepatic vascular tone, which occurs as a result of reduced nitric oxide availability and endothelial cell dysfunction, contributes to the increased resistance [62]. Hyperdynamic portal circulation, or increase in portal venous inflow, is induced by multiple factors, including peripheral and splanchnic vasodilation, increased cardiac output, and reduced mean arterial pressure. Splanchnic vasodilation leads to activation of neurohumoral and vasoconstrictive systems mediated by norepinephrine, angiotensin II, and antidiuretic hormone, resulting in sodium and water retention and increased blood volume. Therapies to reduce portal hypertension include medications that cause splanchnic vasoconstriction, such as nonselective beta-blockers, vasopressin, and somatostatin.

### *Measurement of Portal Pressure*

Portal hypertension is measured by obtaining a hepatic venous pressure gradient (HVPG) through catheterization of the hepatic vein. The HVPG is defined as the difference between the wedged hepatic vein pressure and the free hepatic vein pressure. Normal portal pressure is 5 mmHg or less. In patients with cirrhosis, HVPG

>10 mmHg predicts the development of varices, HVPG >12 mmHg predicts variceal bleeding, and HVPG >16 mm Hg indicates a higher risk of death [63]. Patients who attain a reduction in HVPG to less than 12 mmHg or an overall reduction of 20% with medical therapy are less likely to develop complications of portal hypertension, including variceal bleeding, ascites, and encephalopathy [52].

## *Variceal Bleeding*

Variceal bleeding contributes to the morbidity and mortality in patients with cirrhosis. Variceal formation results from the development of portosystemic collateral pathways that shunt the blood away from the liver to reduce portal venous pressure. The esophagus, stomach, and rectum are common areas in the gastrointestinal tract in which portal hypertension manifests as varices (Fig. 15.3). Gastroesophageal varices are present in approximately 30–40% of patients with compensated disease and up to 85% of those with decompensated disease [64]. Esophageal varices develop at a rate of 5–8% per year [65], and progression from small to large varices occurs at a rate of 10–12% per year [66]. The 6-week mortality associated with an acute variceal hemorrhage ranges between 15% and 25% [67].

At the time of diagnosis of cirrhosis, patients usually undergo endoscopy to determine the presence and size of varices. Recent studies show that patients with a liver stiffness <20 kPa, as determined by TE, and a platelet count >150,000/mm$^3$ have a low risk (<5%) of having high-risk varices, suggesting that endoscopy may be avoided in this subset of patients [68]. In patients undergoing endoscopy who have medium or large esophageal varices, the treatment options include endoscopic variceal ligation or medical therapy with nonselective beta-blockers. Endoscopic variceal ligation consists of placement of rubber bands on variceal columns that leads to localized mucosal and submucosal necrosis. Esophageal ulcerations may occur at the site of band ligation, and these ulcers have the potential to bleed. Ligation is repeated until all varices are obliterated. Studies show that the rates of gastrointestinal bleeding and mortality do not differ between endoscopic variceal ligation and medical therapy [69, 70].

Patients who present with variceal bleeding are treated with intravenous vasoactive drugs to reduce portal pressure, such as octreotide or terlipressin, as well as endoscopic therapy. The type of endoscopic therapy indicated depends on the location of the varix. Esophageal varices are treated with endoscopic variceal ligation. Gastric varices have been reported in up to 20% of patients with cirrhosis [52] and are classified according to their location. Gastroesophageal varices type 1 (GOV1) are esophageal varices extending into the lesser curvature, and gastroesophageal varices type 2 (GOV2) are those extending into the fundus. Isolated gastric varices type 1 (IGV1) are located in the fundus, and isolated gastric varices type 2 (IGV2) are located elsewhere in the stomach. In patients with bleeding GOV1, treatment options include endoscopic variceal ligation or injection with cyanoacrylate glue.

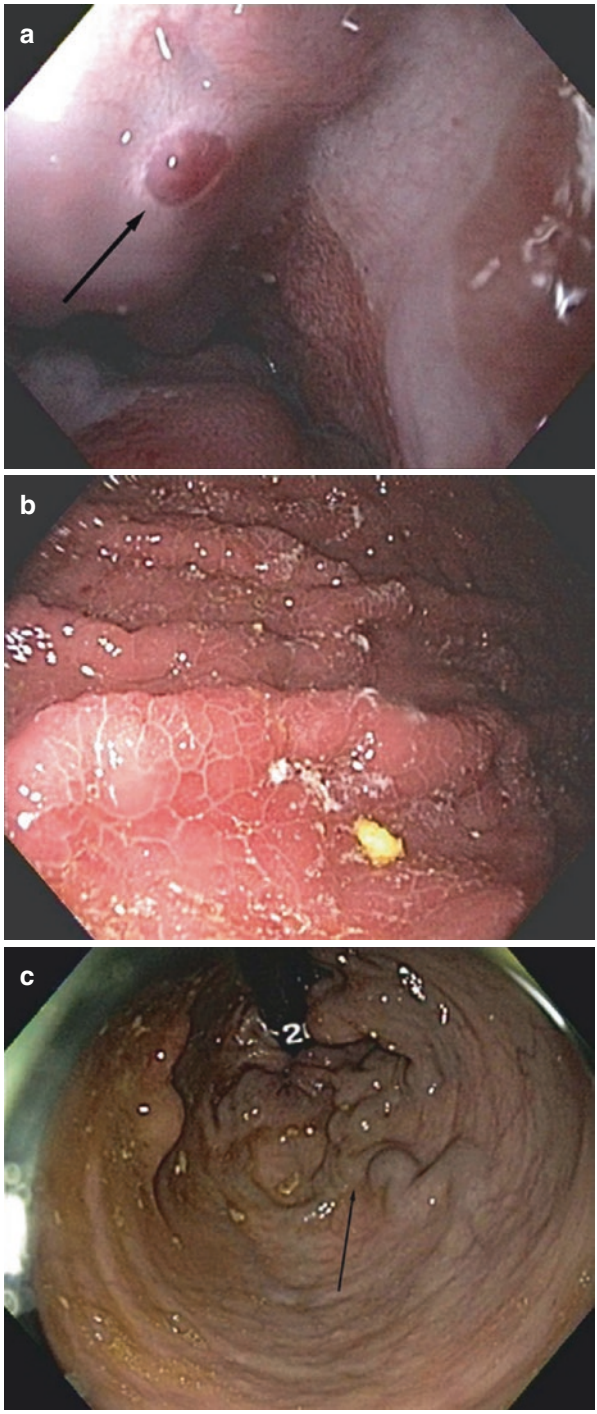


**Fig. 15.3** Endoscopic manifestations of portal hypertension in the gastrointestinal tract. (**a**) Large varices in the distal esophagus with a "nipple sign" (*arrow*) showing evidence of recent bleeding. (**b**) Hyperemic edematous mucosa in the stomach with a "fish scale" appearance characteristic of portal gastropathy. (**c**) Dilated internal hemorrhoidal veins in the rectum that are varices (*arrow*). (Reprinted with permission from [36])

Fundal varices (GOV2 and IGV1) have a higher rebleeding rate, and the recommended treatment is transjugular intrahepatic portosystemic shunt (TIPS) in which a stent is placed between the hepatic and portal veins. TIPS decreases portal pressure and is indicated for patients who experience recurrent variceal hemorrhage despite endoscopic variceal ligation. In addition, early TIPS within 72 h of hospital admission may be beneficial for patients with advanced cirrhosis who present with acute variceal bleeding [71].

Patients with cirrhosis presenting with variceal bleeding are at high risk of developing bacterial infections. The use of antibiotic prophylaxis is associated with a decrease in the development of infections, recurrent hemorrhage, and death [72]. Patients with acute bleeding should be transfused with packed red blood cells when the hemoglobin falls below 7 g/dL, with the goal of maintaining hemoglobin between 7 and 9 g/dL [52]. A randomized clinical study showed that this transfusion strategy is associated with increased survival in patients with Child's class A or B cirrhosis, compared with a more liberal transfusion strategy that aimed to maintain hemoglobin above 9 g/dL [73]. Moreover, patients who recover from acute variceal hemorrhage are at a high risk for rebleeding, which is associated with a mortality rate of up to 33% [52]. Endoscopic variceal ligation and medical therapy (propranolol or nadolol) combination therapy was shown to be more effective than ligation alone in preventing recurrent bleeding [74].

## *Ascites*

Ascites, the accumulation of fluid within the peritoneal cavity, is the most common complication of cirrhosis. The onset of ascites is associated with a 1-year mortality rate of 20% [6]. Treatment involves restriction of salt from the diet and use of diuretic medications, furosemide and spironolactone, which increase salt and water excretion into the urine. In refractory cases of ascites, treatment options include the addition of midodrine, which was shown to reduce ascites and improve systemic hemodynamics [75]. Additional approaches for the treatment of refractory ascites include TIPS placement. Patients with refractory ascites should also be considered for liver transplantation.

Spontaneous bacterial peritonitis is an infection of ascitic fluid without evidence for a secondary source. Development of spontaneous bacterial peritonitis is associated with a poor prognosis, with 30% of patients dying within a month and an additional 30% within 1 year. Clinical symptoms include fever, abdominal pain, and altered mental status. Spontaneous bacterial peritonitis is diagnosed by an absolute polymorphonuclear leukocyte count of 250 cells or greater or a positive bacterial culture from the ascitic fluid. Intravenous antibiotic and albumin therapy has been shown to reduce the risk of renal failure and death in patients with spontaneous bacterial peritonitis [76]. All patients with a prior episode of spontaneous bacterial peritonitis should be maintained on prophylactic oral antibiotics as secondary prevention.

### *Hepatic Encephalopathy*

Hepatic encephalopathy (HE) is a neuropsychiatric disorder in patients with cirrhosis. HE is characterized by changes in personality, motor function, level of consciousness, and cognition. The development of HE is associated with a 1-year mortality rate as high as 64% [77]. Patients who develop encephalopathy in the setting of preserved liver function should undergo imaging to evaluate for the presence of portosystemic shunts, because embolization of large shunts has been shown to be effective in a subset of patients [78]. The pathogenesis of HE is not completely understood, but several neurotoxins have been implicated. The best characterized neurotoxin is ammonia. Produced by the colon, ammonia enters the portal circulation and is converted into glutamine by the liver, preventing ammonia from entering the systemic circulation. In the setting of advanced liver disease, decreased hepatocyte function and portosystemic shunting lead to increased systemic circulating ammonia, which interferes with brain function in several ways. Studies suggest that hyperammonemia may induce astrocyte swelling, impair blood to brain transport of amino acids, and alter neuronal electrical activity. Arterial hyperammonemia is observed in 90% of patients with HE, but the serum levels are not sensitive or specific for the diagnosis of HE. Additional toxins implicated in the pathogenesis of HE include short-chain fatty acids, mercaptans, aromatic amino acids, and manganese. The recommended treatment for the prevention of recurrent encephalopathy is lactulose, a synthetic disaccharide metabolized by the colon to inhibit ammonia production and trap fecal ammonia. Lactulose was associated with a reduced risk of recurrent encephalopathy compared to placebo (20% vs 47%) [79]. When added to lactulose therapy, rifaximin, a poorly absorbed antibiotic, has been shown to reduce the risk of recurrence from 46% to 21% [80].

## Treatment of Liver Fibrosis

Current treatment for liver fibrosis consists of supportive care and management of the sequelae of decompensated cirrhosis as discussed above. Until recently, liver fibrosis was thought to be a chronically progressive disease that was irreversible [81]. Effective treatments of chronic hepatitis B and C infection demonstrated that removal of agents causing injury to the liver can lead to the reversal of fibrosis [82–84]. Several novel drugs directly targeting various aspects of the fibrogenesis pathway are now in clinical trials (Table 15.3).

For patients who progress to end-stage liver failure as a consequence of cirrhosis, liver transplantation is the only treatment available. Donor organs are allocated according to the Model for End-stage Liver Disease (MELD) score. For patients with hepatocellular carcinoma, there are several systems for liver transplant listing, including the Milan criteria [85] and the UCSF criteria [86]. Although the clinical

**Table 15.3** Targeted anti-fibrotic agents in clinical trials

| Drug | Target | Mechanism | ClinicalTrials.gov identifier |
|---|---|---|---|
| Simtuzumab (GS-6624) | Lysyl oxidase 2 neutralizing monoclonal antibody | Inhibit cross-linkage of collagen fibers | NCT01452308 |
| Losartan | Angiotensin II type 1 receptor antagonist | Inhibit HSC activation | NCT00298714 |
| Emricasan (IDN-6556) | Caspase inhibitor | Inhibit hepatocyte apoptosis | NCT02138253, NCT03205345 |
| ND-L02-s0201 (BMS-986263) | Vitamin A-coupled lipid nanoparticle containing siRNA against heat shock protein 47 | Inhibit formation of collagen | NCT02227459, NCT03420768 |
| GR-MD-02 | Galectin-3 antagonist | Inhibit TGFβ-mediated HSC activation | NCT02421094 |
| Cenicriviroc | C-C chemokine receptor (CCR)2-CCR5 antagonist | Inhibit migration and fibrogenesis | NCT02217475, NCT03028740 |
| GS-9674 | Farnesoid X receptor agonist | Inhibit HSC activation | NCT03449446 |
| Obeticholic acid | Farnesoid X receptor agonist | Inhibit HSC activation | NCT02548351 |

outcomes for patient who undergoes liver transplant are good, the effectiveness of transplantation as a treatment for cirrhosis is limited by the critical shortage of donor organs [87]. Development of therapeutic adjuncts or alternatives to liver transplantation through regenerative medicine and tissue engineering approaches are active areas of laboratory investigation.

## Hepatic Stellate Cells: Central Regulators of Liver Fibrogenesis

Originally identified by von Kupffer in 1876, hepatic stellate cells (HSCs) are located in the space of Disse and represent approximately 15% of the total number of resident cells in normal liver [88]. In their quiescent state, HSCs store retinyl esters in cytoplasmic lipid droplets [89]. Following liver injury, HSCs become activated and transdifferentiate into fibrogenic myofibroblasts. This change is characterized by the loss of lipid droplets; accumulation of contractile filaments, such as α-smooth muscle actin; and proliferation. Although additional cell types have been identified that contribute to hepatic fibrogenesis, including portal fibroblasts and sinusoidal endothelial cells, fate-tracing studies have shown that activated HSCs are the major source of extracellular matrix in parenchymal and cholestatic liver injury [90, 91].

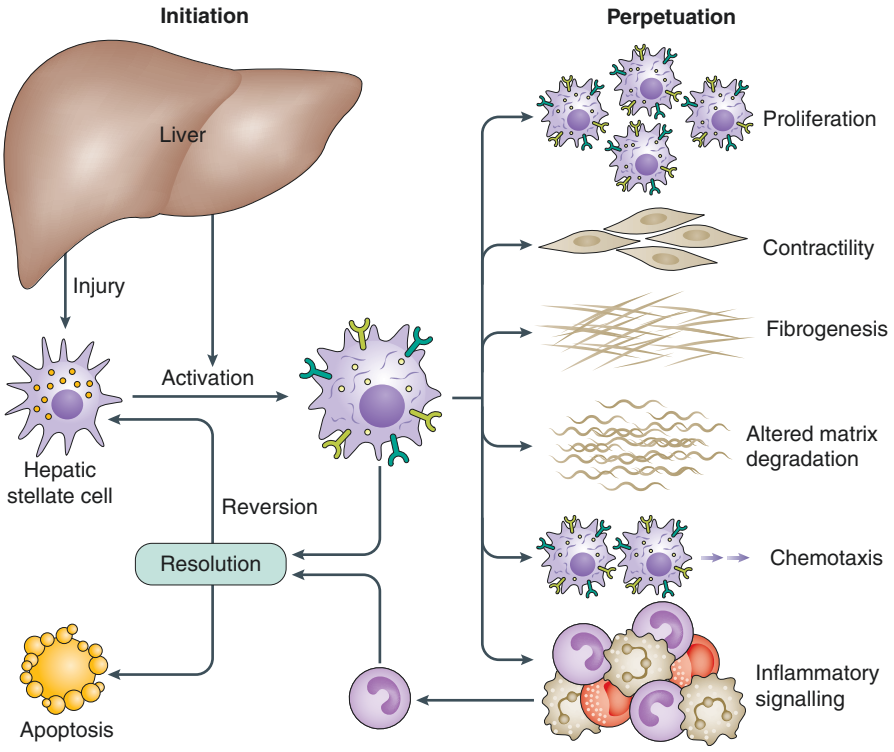


**Fig. 15.4** Initiation, perpetuation, and resolution of hepatic stellate cell activation. (Reprinted with permission from [92])

HSC activation consists of two phases, initiation and perpetuation (Fig. 15.4). During the initiation phase, paracrine stimulation from neighboring cell types, including platelets, endothelial cells, Kupffer cells, and hepatocytes, results in early changes in gene expression and phenotype that render HSCs more responsive to other stimuli. Platelets produce several fibrogenic cytokines and growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ), and epidermal growth factor (EGF). In addition, injury-induced hepatocyte apoptosis promotes HSC activation [93, 94]. During the perpetuation phase, autocrine and paracrine stimulations maintain the activated HSC phenotype. HSCs primed by stimuli become responsive to growth factors and cytokines, leading to HSC retinoid loss, proliferation, chemotaxis, contractility, altered matrix degradation, inflammatory signaling, and fibrogenesis. The cumulative effect is deposition of fibrotic extracellular matrix.

HSCs can be distinguished from other liver cell types by several markers, including platelet-derived growth factor receptor-β (PDGFRβ), desmin, glial fibrillary acidic protein (GFAP), and lecithin retinol acyltransferase (LRAT). Conditional deletion of these genes was utilized to target HSCs in mouse models of liver disease [95].

### *Cytokines and Chemokines*

TGFβ, an important pro-fibrotic cytokine, is produced in its latent form by several cell types in the liver, including HSCs, platelets, and Kupffer cells. When bound to its receptor, SMAD proteins are phosphorylated and activated, leading to transcription of collagen types I and III [92]. TGFβ activates several pathways that promote HSC activation, including the mitogen-activated protein kinase (MAPK) and c-Jun *N*-terminal kinase (JNK) pathways [96, 97]. Several factors were identified that regulate TGFβ-mediated myofibroblast activation, including galectin-3. Inhibitors of galectin promoted fibrosis regression in a rat model [98] and are currently being investigated in a clinical trial (Table 15.3).

Other pro-fibrotic cytokines include PDGF, which drives HSC proliferation and migration, and connective tissue growth factor (CTGF), which stimulates HSC proliferation, migration, adhesion, survival, and extracellular matrix production [92].

### *Receptor-Mediated Activation*

HSCs express a myriad of receptors, several of which can be targeted to reduce HSC activation and hepatic fibrosis. HSCs express integrins that mediate communication between the cytoskeleton and extracellular matrix. Integrins regulate the activation of TGFβ and HSC-specific deletion of integrin αv resulted in reduced fibrosis in a mouse model [99].

HSCs also express G-protein-coupled receptors, including C-C chemokine receptors (CCRs), cannabinoid receptors 1 and 2, and angiotensin II type 1 receptor (AT1R). Chemokine receptors CCR2 and CCR5 were implicated in fibrogenesis through promotion of macrophage recruitment and HSC activation [100–103]. A dual CCR2-CCR5 antagonist, cenicriviroc, is currently being investigated in clinical trials for patients with NASH (Table 15.3). Cannabinoid receptor 1 promotes fibrosis, whereas cannabinoid receptor 2 has anti-fibrogenic effects [104, 105]. AT1R and its ligand, angiotensin II, promote HSC activation and fibrosis through phosphorylation of Janus kinase 2 [106].

Toll-like receptors (TLRs) are a class of proteins that play an important role in the innate immune system. TLRs recognize structurally conserved damage-associated molecular patterns (DAMPs) released following hepatocyte injury. Activated HSCs express TLR2, TLR3, TLR4, TLR7, and TLR9. TLR4 activation induced chemotaxis of Kupffer cells and promotes TGFβ-induced HSC activation [107]. TLR2 promoted the activation of the inflammasome, resulting in NASH progression in a mouse model [108].

Finally, HSCs express nuclear receptors such as the farnesoid X receptor (FXR), liver X receptor (LXR), peroxisome proliferator-activated receptor gamma (PPARγ), vitamin D receptor (VDR), and nuclear receptor subfamily 4 group A member 1

(NR4A1). FXR inhibits HSC activation [109], and FXR agonists, such as obeticholic acid, improve NAS score and fibrosis stage in patients with NASH [110]. Activation of PPARγ induces HSC inactivation [111], and a dual PPARα-PPARδ agonist improved NASH in a large clinical trial [112]. VDR ligands reduce HSC activation mediated by TGFβ and reduce hepatic fibrosis [113].

### *Additional Pathways*

Several signaling pathways regulate HSC activation, including the Hedgehog pathway and the Hippo pathway. Inhibition of the Hedgehog pathway leads to decreased HSC activation and reduced hepatic fibrosis [114]. The Hippo pathway is a kinase cascade that results in phosphorylation and inactivation of the transcriptional coactivator yes-associated protein (YAP). YAP inhibition inactivates HSCs and reduces fibrosis [115–118]. The mechanism by which YAP regulates HSC activity is not completely understood.

Regulation of HSC activation may be mediated by microRNAs, including miR-21 [119] and miR-221 [120], as well as histone modifications regulated by myocardin-related transcription factor A (MRTF-A) [121]. In addition, methyl-CpG-binding protein 2 (MECP2) regulates epigenetic signaling by suppressing PPARγ transcription, resulting in increased HSC activation and fibrosis [122].

### *HSC Clearance Following Injury*

In light of clinical data highlighting the regression of fibrosis in patients with liver disease, recent studies elucidated the fate of HSCs following cessation of injury. HSC clearance occurs through apoptosis, senescence, and reversion (Fig. 15.4). Apoptosis, a form of programmed cell death, occurs during resolution of liver injury and results in reduced numbers of activated HSCs. This process is mediated by death receptors expressed by activated HSCs, including first apoptosis signal (FAS) receptor, tumor necrosis factor receptor 1 (TNFR1), neurotrophin receptor p75 (p75NTR), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors [123]. HSCs undergo senescence, which refers to cell-cycle arrest after reaching a finite proliferative limit. HSC senescence occurs in a p53-dependent manner and results in decreased numbers of activated HSCs and reduced expression of extracellular matrix proteins [124]. Fate-tracing studies in mice demonstrated that HSCs can also undergo reversion to an inactivated phenotype [125, 126]. Approximately 50% of activated HSCs undergo reversion after cessation of liver injury. Interestingly, these HSCs do not return to the quiescent state but exist as inactivated HSCs that are primed for reactivation in response to another injury [125, 126].

## Liver Fibrosis and Cancer

Fibroblasts, depending on their activation state, are involved in wound healing, mediating pathological tissue fibrosis, and inducing a desmoplastic reaction in the tumor microenvironment (Fig. 15.5) [127]. HSCs, being the fibroblasts of the liver, are involved in all of these processes within the liver. Indeed, stellate cells are critical in regulating liver regeneration after injury [88, 128], and they are chief producers of fibrotic extracellular matrix during liver fibrogenesis [90, 91]. HSCs also play an important role in modulating the tumor microenvironment in the liver during cancer development and progression [129, 130]. The liver is an interesting model organ to investigate the role of the tumor microenvironment because fibrosis-related effects likely contribute to the development of both primary liver cancers and metastatic liver lesions from other primary sites.

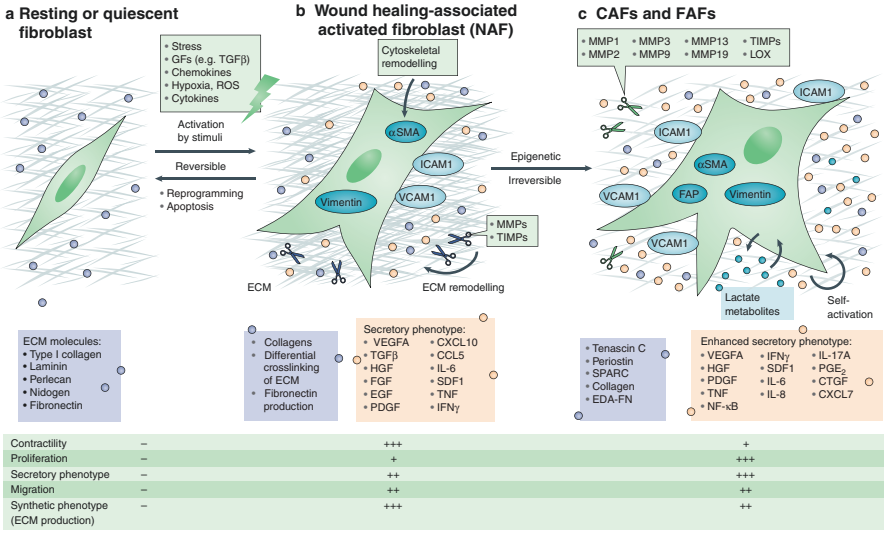


**Fig. 15.5** Functional and phenotypic differences between normal activated fibroblasts (*NAFs*), fibrosis-associated fibroblasts (*FAFs*), and cancer-associated fibroblasts (*CAFs*). (Reprinted with permission from [127]) CCL5, C-C motif chemokine ligand 5 (also known as RANTES); CTGF, connective tissue growth factor; CXCL, C-X-C motif chemokine ligand; EDA-FN, extradomain A-fibronectin; EGF, epidermal growth factor; FAP, fibroblast activation protein; FGF, fibroblast growth factor; GFs, growth factors; HGF, hepatocyte growth factor; ICAM1, intercellular adhesion molecule 1; IFNγ, interferon-γ; IL, interleukin; LOX, lysyl oxidase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; $PGE_2$, prostaglandin $E_2$; ROS, reactive oxygen species; SDF1, stromal cell-derived factor 1; TGFβ, transforming growth factor-β; TIMPs, tissue inhibitors of metalloproteinases; TNF, tumor necrosis factor; VEGFA, vascular endothelial growth factor A; VCAM1, vascular adhesion molecule 1

### *Hepatocellular Carcinoma*

Hepatocellular carcinoma (HCC) is an aggressive primary liver tumor with a poor prognosis. Worldwide, HCC is the fifth most common cancer and the third most common cause of cancer deaths [131]. HCC is one of the few cancers for which incidence is increasing [132]. HCC most commonly occurs in the setting of chronic liver disease, in which cycles of necrosis, inflammation, and tissue repair lead to progression of nodular lesions. Importantly, HCC is strongly associated with liver fibrosis, and 90% of HCCs arise within cirrhotic livers [133]. The oncogenic process begins with a benign liver regenerative nodule, progressing to a low-grade dysplastic nodule, then to a high-grade dysplastic nodule, and ultimately HCC [134]. The earliest mutations in this progression are telomerase promoter mutations that induce telomerase reactivation [135]. Subsequent oncogenic mutations that promote HCC progression are numerous, and the molecular signatures of the tumors fall into two broad subtypes. The aggressive subtype is characterized by activation of cell proliferation signals, including the p53, Ras/ERK, and Akt/mTOR pathways. The less aggressive subtype is characterized by the activation of Wnt/β-catenin signaling [136]. HCC is distinguished from high-grade dysplastic nodules by the invasion of neoplastic cells into the stroma of portal tracts [134]. Early HCC may be treated by surgical resection or by liver transplantation. Current chemotherapeutic and targeted molecular therapeutic options are limited and ineffective. Most HCC patients (85%) present with advanced disease and have median survival times of less than 1 year [137].

Although cirrhosis is one of the most important risk factors for developing HCC [138], the nature of the association between liver fibrosis and HCC is not established. Whether fibrosis actively promotes the development of HCC or is simply a by-product of persistent inflammation related to ongoing hepatocellular injury is controversial. Increased tissue stiffness in fibrotic livers may create an environment permissive for HCC development through several mechanisms. Greater matrix stiffness in fibrotic liver can activate HSCs and portal fibroblasts to produce and deposit fibrillar collagens that promote fibrosis progression [139, 140]. Stiffened extracellular matrix stimulates epithelial cell integrin signaling and cytoskeletal contractility, leading to enhanced proliferation and invasiveness of premalignant and malignant cells [141]. In HCC, increased integrin signaling has been shown to promote tumorigenesis by stimulating cell motility [142] and inhibiting apoptotic pathways [143]. Increased matrix stiffness stimulates the proliferation and chemotherapeutic resistance of HCC cell lines [144]. Focal adhesion kinase (FAK) signaling is required for the progression of a c-Met/β-catenin-driven in vivo mouse model of HCC [145], suggesting that the stiffened matrix of a fibrotic liver may activate FAK signaling to promote HCC progression.

Importantly, increased matrix rigidity has a direct inhibitory effect on expression of hepatocyte nuclear factor 4 alpha (HNF4α) [60], a master transcriptional regulator

of hepatic function and a tumor suppressor. Inhibition of HNF4α stimulates hepatocyte proliferation [146] and induces expression of mesenchymal genes [147]. HNF4α expression is inhibited in fibrotic livers, and forced re-expression slows the progression of fibrosis [148, 149]. Similarly, HNF4α expression is reduced in HCC, and forced re-expression inhibits tumor progression [150–154]. These findings suggest that increased matrix rigidity in fibrotic livers may create an environment permissive for HCC development by decreasing HNF4α expression in hepatocytes. Moreover, HNF4α expression in primary hepatocytes cultured on stiff matrix is increased by inhibition of Rho/Rho-associated protein kinase signaling, indicating a critical role of the mechano-signal transduction networks in modulating HNF4α activity in response to tissue stiffness [60].

## *Liver Metastases*

The liver is the most common distant organ site for tumor metastases. While any primary tumor may metastasize to the liver, primary cancers that most frequently present with liver metastases are gastrointestinal, breast, lung, neuroendocrine, and melanoma [155]. Two theories have been proposed to explain why certain tumors have propensity to metastasize to specific organs. The "seed and soil" hypothesis proposed by Paget in 1889 postulates that certain tumor cells (the "seeds") have affinity for the microenvironment of certain organs (the "soil") and that metastases occur when seed and soil are compatible. Alternatively, Ewing proposed in 1929 that metastatic spread was determined by mechanical factors related to the vascular system [156]. The two theories are not mutually exclusive, and both mechanisms are likely important in promoting the establishment of metastatic lesions within the liver. The sinusoidal capillary system of the liver can act as a great sieve to entrap circulating tumor cells and facilitate cancer cell infiltration into the liver parenchyma. In addition, stromal cells such as HSCs may provide a prometastatic microenvironment by promoting tumor cell proliferation, recruiting neovascularization, and suppressing antitumor immunity [129].

Cancer-associated fibroblasts (CAFs) are myofibroblasts that associate with tumors and play a role in remodeling the tumor stroma. CAFs appear to have a distinct phenotype from resting and normal activated fibroblasts involved in wound healing [127] (Fig. 15.5). CAFs may enhance tumorigenesis by inducing cancer cell invasion and angiogenesis. YAP, part of the Hippo pathway and a mechano-signal responsive transcriptional coactivator, is activated in CAFs and promotes cancer cell invasion, extracellular matrix stiffening, and angiogenesis [157]. These findings give functional significance to the desmoplastic reaction surrounding many tumor types and suggest that the stroma may be a key regulator of tumor progression. In addition, the desmoplastic tumor stroma may act as a physical barrier to the efficient delivery of cytotoxic chemotherapeutic agents in the treatment of cancers [158]. While there is evidence that the stiffened tumor microenvironment may be protumorigenic, there is also evidence that the role of the stiffened tumor stroma may

be more complex. Ablating the peri-tumoral stroma by targeting Hedgehog signaling enhanced cancer cell killing by chemotherapy in preclinical studies [159]. However, targeting CAFs in human pancreatic cancer led to accelerated disease progression and halted clinical trials [160]. Additional studies showed that depletion of CAFs and inhibition of Sonic Hedgehog led to accelerated pancreatic cancer progression and more aggressive disease [161, 162]. These studies suggest that the tumor stroma may have both pro- and antitumor properties that are context dependent. Therefore, rather than ablating the tumor stroma in devising novel treatments for cancer, perhaps it is more important to "reeducate" the stroma to be more antitumorigenic [163]. The role of CAFs in the regulation of cancer progression remains an important ongoing area of laboratory investigation.

It is not yet established whether pre-existing liver fibrosis promotes or inhibits the development of liver metastases from other primary sites. There is evidence that activated HSCs become CAFs and promote tumorigenesis by secreting growth factors, remodeling the stroma, promoting angiogenesis, and suppressing the antitumor response [129]. It is possible that activated HSC and cancer cell cross talk results in a feed-forward loop that enhances metastatic tumor growth. On the other hand, there are decades of clinical observational studies suggesting that fibrotic livers are less prone to developing metastases and that the fibrotic liver microenvironment may be poor "soil" for the metastatic implants [164]. An alternative explanation as to why metastases in fibrotic livers are less observed may be that cancer patients with liver fibrosis have shorter life-spans and die before the development of clinical metastases [165]. In addition, it is also possible that because the fibrotic liver is such good "soil" for primary liver cancer development, the chances of finding primary liver tumors in a fibrotic liver are much greater than finding metastatic cancer, thereby giving the impression that fibrotic liver is less prone to metastases. Because the liver is such an important organ for the development of primary cancers as well as secondary spread of metastases, improved understanding of the role of the liver microenvironment in modulating tumor progression is critical for advancing cancer treatment research.

## Conclusion

Liver fibrosis is a significant source of human disease, morbidity, and mortality. Shifting patterns in etiological factors indicate that NAFLD/NASH will become a dominant cause of liver fibrosis and cirrhosis worldwide. Advances in understanding the role of extracellular matrix stiffening and the molecular basis of HSC activation in liver fibrosis have led to novel diagnostic modalities and development of targeted anti-fibrotic therapies. Diagnostic tools and treatment options for liver fibrosis are continuing to evolve and are being informed by basic discoveries from the laboratory and outcomes of clinical trials. Determining how the fibrotic liver microenvironment may regulate hepatocyte function and tumorigenesis remains important areas of investigation.

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# Chapter 16
# Tipping the Balance from Angiogenesis to Fibrosis in Chronic Kidney Disease

**Yosuke Hirakawa, Tetsuhiro Tanaka, and Masaomi Nangaku**

## Peritubular Capillary Rarefaction and Hypoxia in CKD

### *Characteristics of the Renal Vasculature*

Despite receiving as much as 20% of the blood pumped out from the heart, the kidneys are naturally one of the most hypoxic organs [1, 2]. One important reason for this hypoxia is that the kidney has a unique vasculature system. The renal artery, the feeding artery of a kidney, branches into the arcuate arteries, which run vertically and parallel with the veins, and finally into the afferent arteriole. An afferent arteriole transitions into a glomerular loop and an efferent arteriole. In the glomerulus, epithelial cells known as podocytes form a barrier known as the slit diaphragm that filters water, electrolytes, and metabolites into primitive urine from the blood. The peritubular capillary (PTC) originates from the cortical efferent arterioles and ends in the renal interlobular veins, which finally converge into the renal vein (Fig. 16.1). In this vasculature system, the glomeruli and PTC participate in substance transport. The glomeruli are unique structures that consist of endothelial cells, podocytes, and mesangial cells, all of which are closely related to filtration. The PTC delivers oxygen and nutrients to tubular cells and receives the reabsorbed water, electrolytes, amino acids, and metabolites.

In this complicated vasculature, an oxygen shunt exists between the arteries and veins that run closely parallel and causes the physiological low oxygen tension in the kidney. The existence of the oxygen shunt was first proposed in 1990 and was recently argued by calculations using a three-dimensional computational model since the computational simulation results depend on the assumption of vessel

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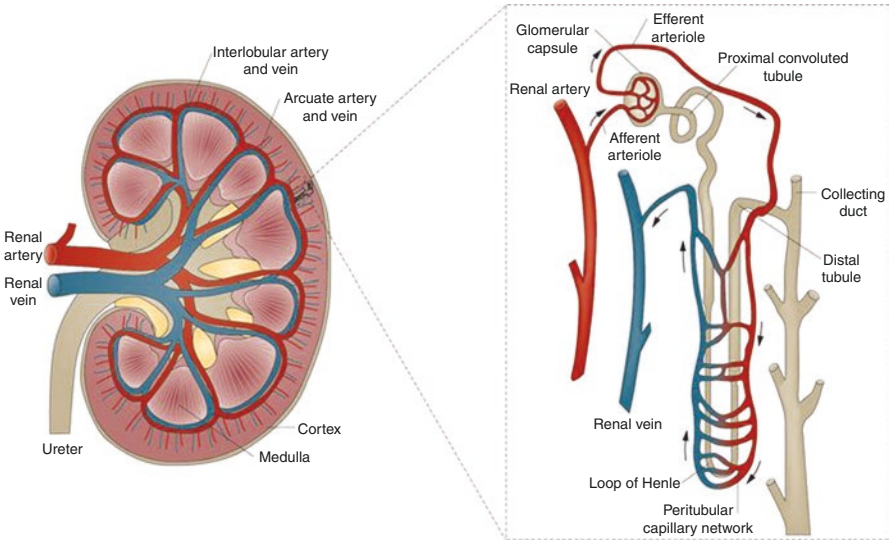


**Fig. 16.1** Renal vasculature system. The renal artery branches into the arcuate arteries, which finally branch into the afferent arteriole. An afferent arteriole transitions into a glomerular loop and an efferent arteriole, which divide into the PTC plexus. The PTC surrounds the tubules and ends in the renal interlobular veins, which ultimately converge into the renal vein. PTC, peritubular capillary. (Reprinted with permission from the Nature Publishing Group © Mimura and Nangaku [3])

distance [4–6]. Partly because of the oxygen shunt, an oxygen gradation exists in the kidney, that is, higher in the cortex and lower in the medulla. The oxygen gradation is proven using pimonidazole. Pimonidazole binds to the thiol group in intracellular proteins under hypoxic conditions (<15 mmHg of oxygen tension), and immunohistochemistry of the pimonidazole–protein adduct in the kidney revealed that pimonidazole binds to an intracellular protein, mainly in the corticomedullary junction, indicating lower oxygen tension in the corticomedullary junction. The existence of an oxygen gradient in the kidney was also proven using an oxygen microelectrode, although the absolute value of oxygen tension varies by species. In mice, oxygen tension of the renal cortex and medulla are reportedly 50 mmHg and 30 mmHg, respectively, while the oxygen tension in the renal cortex of sheep is <40 mmHg [7, 8]. Renal hypoxia aggravates CKD, with multiple mechanisms leading to renal tubulointerstitial hypoxia in CKD. The mechanisms can be divided into two categories: an increase in oxygen demand and a decrease in oxygen supply. Increases in oxygen demand in tubular cells are shown in animal models including the remnant kidney, hypertension, and diabetic kidney disease (DKD) [9–12]. Increases in reactive oxygen species, which induces mitochondrial dysfunction and sodium reabsorption using adenosine triphosphate as the energy source, are thought to increase the oxygen demand. The mechanisms of the decreased oxygen supply for the tubular cells in CKD include PTC rarefaction (e.g., decreased PTC density), decreased PTC flow, glomerular capillary bed impairments, and anemia, mainly via insufficient erythropoietin production [13]. Of these, PTC rarefaction is closely

related to renal hypoxia. In general, a decrease in capillary density increases the distance from the capillary to the cells. Since oxygen transport is dependent on passive diffusion, a cell distant from the capillary should suffer from hypoxia because of limited oxygen supply. The existence of PTC rarefaction in human CKD is proven. Immunohistochemistry of platelet-endothelial cell adhesion molecule-1/CD31 as an endothelial marker revealed that PTC decreases in human DKD and Finnish congenital nephrotic syndrome, a hereditary nephrotic syndrome caused by mutations in the *NPHS1* gene encoding for nephrin [14, 15]. In these human samples, levels of vascular endothelial growth factor (VEGF), a well-known angiogenic factor, were also decreased with PTC rarefaction. This finding implies that renal hypoxia in CKD is at least partly due to insufficient VEGF activation since increased VEGF expression mediates hypoxia via the stabilizing the hypoxia-inducible factor (HIF). Multiple animal models demonstrate PTC rarefaction including acute kidney injury, obstructive nephropathy, remnant kidney, and obesity-induced nephropathy [16–19]. Therefore, PTC rarefaction is now widely recognized as a pathological hallmark of CKD. The importance of PTC rarefaction is also emphasized in the acute kidney injury (AKI) to CKD continuum. Most patients who survive AKI have a complete restoration of renal function. However, a recent epidemiological study and animal experiments indicated that AKI episodes frequently result in subsequent CKD in the long term [20]. After severe AKI, a decreased total PTC perfusion (due to a decrease in PTC number, decreased individual capillary area, and decreased total cortical perfusion area) correlates with renal function after AKI recovery [21]. PTC rarefaction results in hypoxia, which plays a pivotal role in the AKI to CKD transition. As mentioned above, the existence of PTC rarefaction is supposed to determine renal prognosis. The mechanisms of PTC rarefaction are complicated since there are many angiogenic factors involved in PTC maintenance.

## *Importance of Angiogenic Factors in CKD*

### VEGF/Soluble fms-like Tyrosine Kinase

VEGF, the most well-known angiogenic signaling molecule, acts mainly on vascular endothelial cells and promotes angiogenesis. The VEGF family is composed of four proteins: VEGF-A, VEGF-B, VEGF-C, and VEGF-D. Of them, VEGF-A is the dominant member, with several splicing isoforms in humans (VEGF-A, VEGF121, VEGF165, VEGF165b, VEGF189, and VEGF206) and mice (VEGF120, VEGF164, and VEGF188) [22]. Differences in isoelectric point and affinity for heparin differentiate VEGF isoforms, which are important characteristics determining VEGF isoform bioavailability [23]. VEGF binds to VEGF receptor-2 (VEGFR-2; also known as fetal liver kinase-1), resulting in the enhancement of downstream signaling. There is another receptor for VEGF, VEGFR-1 (also known as *fms*-like tyrosine kinase [FLT-1]); however, its exact role is unknown. VEGFR-1 acts as a decoy receptor for VEGF; thus, it is thought to play an anti-angiogenic role. However, in

the kidney, soluble Flt-1 (sFlt-1) plays several roles other than angiogenesis as described later. In the healthy kidney, VEGF is expressed in podocytes and tubular cells in the renal medulla [24, 25], while endothelial cells of the preglomerular vessels and PTC25 express VEGFR-1 and VEGFR-2 [26]. The importance of VEGF in CKD progression is supported by a study that demonstrated an association between the VEGF-460 polymorphism and progression to CKD stage 5, although the mechanism of how this polymorphism affects the clinical course of CKD is unknown [27]. Since promoting angiogenesis is an important role of VEGF, the correlation between VEGF and PTC density in CKD was of great interest. Although PTC rarefaction is a hallmark of CKD and, thus, impaired angiogenesis must occur, few studies show VEGF downregulation in CKD; the fact that the VEGF concentration is unchanged in a CKD model might be considered an impaired response to hypoxia [19]. Indeed, using a clinical biopsy specimen, renal VEGF expression was decreased in patients with advanced CKD compared with patients in whom the renal function was maintained [28]. On the other hand, serum VEGF concentrations are increased in CKD patients, indicating a nonrenal origin [29]. Conversely, increased VEGF expression is also considered pathogenic. VEGF expression increases in the podocytes of patients in the early stage of DKD, which is supposed to correlate with glomerular endothelial cell proliferation [30]. VEGFR expression is suppressed in endothelial cells of patients with DKD, indicating that an increased VEGF level does not result in activation of the downstream pathway of VEGFR in the endothelium. Autocrine VEGF has been shown to affect podocytes, neighboring cells of non-endothelial origin [31]. Considering that the inhibition of VEGF signaling slows DKD progression, the upregulation of VEGF in podocytes induces glomerulopathy, likely via VEGF signaling activation in the podocytes [32–34].

The importance of maintaining VEGF expression was revealed using gene manipulation. Systemic homozygous and heterozygous deletions of the *VEGF* gene resulted in prenatal death. Thus, an inducible or cell-specific knockout models are necessary to study [35, 36]. Inducible systemic *VEGF* knockout with doxycycline (dox) resulted in thrombotic macroangiopathy [37], which is assumed to be a result of endothelial dysfunction. This result is consistent with the fact that the clinical use of VEGF inhibitors against malignant tumor triggers various glomerulonephritis, and the most frequent pathological aspect is thrombotic macroangiopathy, followed by minimal change disease, which is thought to result from endothelial dysfunction and podocytopathy, respectively [37–40]. Cell type-specific VEGF knockout models are necessary to exclude the possibility that VEGF originating outside kidney is involved. Investigators created podocyte-specific VEGF knockout animals since VEGF expression is abundant in podocytes within the kidney. However, podocyte-specific homozygous knockout of the *VEGF* gene resulted in perinatal lethality probably, likely due to congenital nephrotic syndrome [41]. The glomeruli in these mice contained podocytes but lacked capillary loops, indicating that VEGF secreted from podocytes was vital for normal vascular structure within the glomeruli. Podocyte-specific heterozygous *VEGF* knockout mice can live longer but displayed proteinuria and endothelial fenestration loss, which finally produced foot process effacement. Podocyte-specific dox-inducible *VEGF* knockdown resulted in acute kidney injury

in adult mice with the disruption of $\alpha_v\beta_3$ integrin signaling, finally resulting in mesangiolysis, decreased endothelial fenestration, and foot process effacement in podocytes [42]. Together, these studies illustrate the need for VEGF expression in the development and maintenance of glomerular structure and filtration barrier.

On the other hand, extremely high VEGF levels can also cause renal damage. Transgene overexpression of VEGF165 in rabbits represents microaneurysms within the glomeruli in the early phase and tuft collapse accompanied by cyst formation in the late phase [43]. Selective stimulation of VEGFR2 induced mesangial expansion and tubular damage when combined with uninephrectomy and endothelial nitric oxide synthase (NOS) knockout [44]. Mice overexpressing VEGF164 in podocytes displayed collapsing nephropathy at 5 days of age [41]. Podocyte-specific tet-inducible VEGF164 overexpression resulted in glomerular basement membrane thickening, mesangial expansion, and podocyte effacement, all of which resemble pathological finding of early DKD [45]. In this experiment, the correlation of VEGF and proteinuria demonstrates its pathogenicity in vivo [45]. The same transgene induced nodular glomerulosclerosis in endothelial NOS knockout mice, and this mouse model resembled human DKD in the point that glomerular nodules extensively overexpressed collagen IV and laminin like Kimmelstiel–Wilson nodular glomerulosclerosis without diabetic milieu [46]. These results indicate that the extreme activation of VEGF signaling in podocytes impairs endothelial and epithelial function in the glomeruli (Fig. 16.2). Conversely, moderate human VEGF165

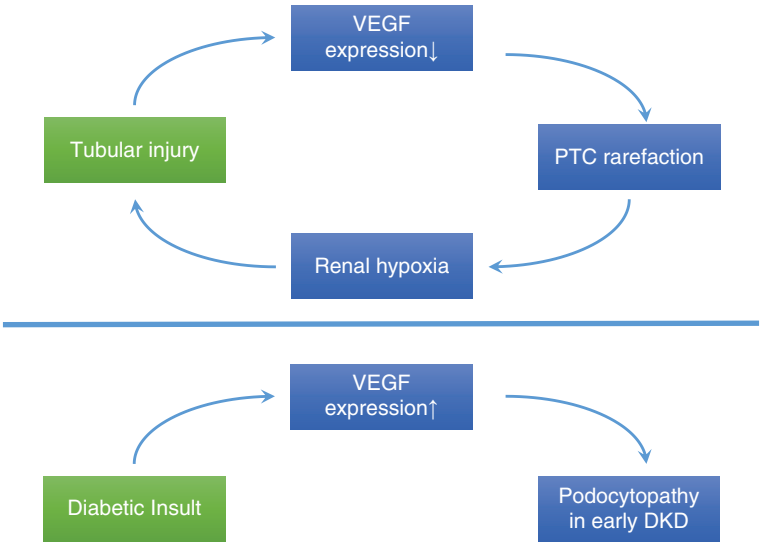


**Fig. 16.2** Effect of VEGF on tubular damage and diabetic insult. Tubular injury leads to VEGF downregulation, which contributes to PTC rarefaction, which aggravates renal hypoxia and exacerbates the tubular injury. In the diabetic milieu, increased glomerular VEGF expression is seen with DKD and mediated podocyte injury. DKD, diabetic kidney disease; PTC, peritubular capillary; VEGF, vascular endothelial growth factor. (Original figure as per authors)

expression in mice protects podocytes from puromycin aminonucleoside nephropathy, indicating that appropriate VEGF induction might be advantageous in podocytes in an autocrine manner [47]. Additionally, tet-inducible tubule-specific VEGF164 overexpression in mice also leads to focal interstitial fibrosis, tubular cyst formation, and glomerular enlargement, demonstrating extremely high VEGF damage levels in tubular cells [48]. Since the pathological characteristics of VEGF-induced glomerulopathy resemble those of early DKD, inhibiting VEGF may be a promising strategy in early DKD. Soluble VEGFR-1 (sFlt-1) is a splice variant of VEGFR-1 that lacks the transmembrane domain and the intracellular tyrosine kinase domain and binds to VEGF with the same affinity and specificity as VEGFR-1; thus, it has potent inhibitory effects against VEGFR. The increased serum sFlt-1 concentration in remnant kidney model rats might explain the endothelial dysfunction in CKD patients via VEGF signaling blockade [49]. Hence, interventions that restore serum VEGFR-1 levels can retard DKD progression. Ku and colleagues revealed that inducible sFlt-1 overexpression in podocytes ameliorated diabetic glomerular injuries such as mesangial expansion, glomerular basement membrane thickening, and foot process effacement in mice with streptozotocin-induced diabetes [32]. Another report revealed that an increased serum sFlt-1 level by the intramuscular administration of adeno-associated virus-1 encoding human sFlt-1 reduced albuminuria but exacerbated interstitial damage in *db/db* mice [33]. These results indicate that sFlt-1 have a protective effect on podocytes in the diabetic milieu and do not harm glomeruli as observed in VEGF knockout diabetic models, while blockade of the VEGF signaling pathway is harmful in the tubulointerstitial compartment. Regarding glomerulonephritis, Hara et al. revealed that blockade of VEGF by human sFlt-1 overexpression in muscle aggravated nephrotoxic serum-induced glomerulonephritis [50]. Jin and colleagues reported that podocyte-specific sFlt-1 deletion resulted in podocytopathy and that sFlt-1 binds to lipid rafts on the cell surface and regulates pericyte function, which might lead to podocyte protection [51]. Despite a detailed elucidation of podocyte-protective effect in the diabetic condition, the effect of sFlt-1 on CKD progression other than podocytopathy has not been well-determined. The increases in serum sFlt-1 concentrations seen in patients with preeclampsia or CKD illustrate its potential importance in the pathogenesis of these diseases [49, 52, 53]. Both CKD-induced atherosclerosis in CKD patients and experimental animal models have elevated sFlt-1 [49, 54, 55]. While these findings suggest that sFlt-1 is related to atherosclerosis in CKD, it remains to be proven directly.

### Angiopoietin-Tie Signaling

Angiopoietin-Tie signaling plays an important role in the control of vascular quiescence [56]. Initially identified as orphan receptors, Tie receptors were later found to be agonists and antagonists pf angiopoietins (Ang). The Ang proteins are composed of four isoforms, Ang1–Ang4. Although the role of Ang3 and Ang4 is not known, Ang1 is reported to be a Tie2 agonist, while Ang2 a competitive antagonist.

Ang1 and Ang2 were secreted by cells via a paracrine and an autocrine manner, respectively. The fact that the expression level of Ang2, but not Ang1, was driven by VEGF administration and hypoxia implicates that Ang2 is vital for angiogenesis [57]. The clustering of Ang1 and Tie2 leads to the stimulation of survival signals, endothelial cell adhesion to the matrix, increased vascular integrity, and the inhibition of vascular permeability, all of which ultimately contribute to maintaining vascular quiescence [58]. Ang2 activates endothelial cells from the quiescent state. In response to the VEGF gradient, sprouting vascular endothelial cells release Ang2 and antagonize Ang1–Tie2 signaling in proximate mural cells, followed by mural cell detachment and further sprouting. The VEGF gradient also induces tip cell upregulation of Delta-like protein 4 and activates Notch in stalk cells. Notch downregulates VEGFR-2, thereby blunting the response to VEGF and allowing tip cells to lead during sprouting (Fig. 16.3). In CKD, the Ang1–Ang2 balance is changed. David et al. reported that the plasma concentration of Ang1 and Ang2 was decreased and increased, respectively, in hemodialysis patients in a cross-sectional study [59]. They also revealed that a higher plasma Ang2 concentration was correlated with severe coronary artery scores assessed with percutaneous transluminal coronary angiography and severe peripheral artery disease scores assessed with ultrasonography. The same group also clarified that plasma Ang2 concentration increases as GFR decreased using inulin clearance technique [60], while a high plasma Ang2 level was an independent predictor of all-cause mortality in

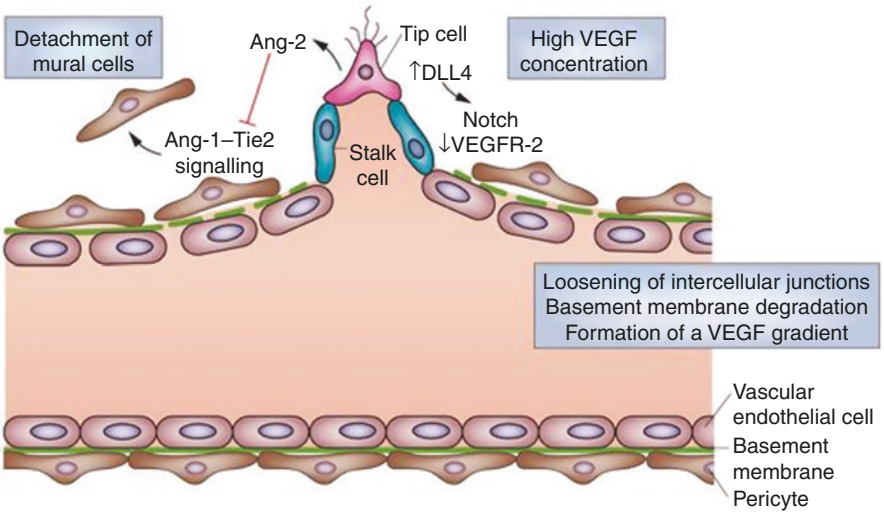


**Fig. 16.3** Ang-Tie signaling in angiogenesis. A high VEGF concentration around tip cells leads to Ang2 release of Ang2. Released Ang2 antagonizes Ang1–Tie2 signaling resulting in detachment of pericytes. Also, in response to VEGF gradient, the tip cells upregulate DLL4, which activates Notch in stalk cells, finally resulting in reduction in the susceptibility to VEGF signaling. Ang, angiopoietin; DLL4, Delta-like protein 4; Tie, angiopoietin 1 receptor; VEGF, vascular endothelial growth factor. (Reprinted with permission from the Nature Publishing Group © Tanaka and Nangaku [23])

advanced CKD patients [61]. Another group showed that plasma Ang2 level was independently associated with albuminuria in CKD stage G3–G5 patients [62]. Tsai et al. reported the following: a study including 621 CKD stage G3–G5 patients revealed that patients with high Ang2 levels had a rapid decline in renal function defined as the slope of estimated glomerular filtration (eGFR) <−5 mL/min/1.73 $m^2$/year [63]. Another study with 270 patients with stage G3–G5 CKD identified high plasma Ang2 levels and left ventricular hypertrophy correlated, while low plasma Ang1 level and left atrium dilatation correlated [64]. These results indicate that plasma Ang2 is a marker of vascular disease.

Ang1 has a role in the pathogenesis of atherosclerosis in an experimental atherosclerotic model [65]. Ang1 overexpression promotes atherosclerosis by increasing serum concentrations of VEGF and monocyte chemoattractant protein-1, increasing circulating inflammatory monocytes, and promoting monocyte/macrophage retention in atherosclerotic plaques in $ApoE^{-/-}$ mice. From this result, the suppression of plasma concentrations of Ang1 in CKD patients might represent a defensive mechanism against atherosclerosis. The next question is whether Ang-Tie signaling affects renal fibrosis. In mice, PTC rarefaction is associated with a decrease in Ang1 and an increase in Ang2 shown in a nephrotoxic serum model [66]. In a murine unilateral ureteral obstruction (UUO) model, the PTC rarefaction occurred in accordance with renal interstitial fibrosis and was restored by Ang1 overexpression by adenovirus transfection, probably via restoring insufficient Tie2 signaling [67]. Like VEGF, Ang1 is also expressed in podocytes and thus affects podocytes. In an early model of DKD induced by streptozotocin administration, a decrease in glomerular Ang1 expression occurred while Ang2 was unchanged. Podocyte-specific dox-inducible overexpression of Ang1 protected the glomeruli and alleviated albuminuria but suppressed diabetes-induced epithelial proliferation [68]. Another group reported that dox-inducible tubule-specific overexpression of Ang1 ameliorated renal fibrosis in a murine UUO model and restored PTC [69]. Long et al. revealed that Ang1 overexpression by adenovirus transfection indeed stabilized PTC but worsened fibrosis in a folic acid nephropathy model [70]. These results implicate the possibility that the restoration of Ang1 expression in podocytes and tubules ameliorates podocytopathy and PTC rarefaction, respectively.

Experimental atherosclerosis models demonstrate the importance of Ang2. Ang2 can stimulate nitric oxide release from endothelial cells in a Tie2-dependent manner, while Ang2 overexpression in ApoE knockout ($ApoE^{-/-}$) mice reduced atherosclerotic plaque size [71]. Conversely, Ang2 neutralization by blocking antibody led to decreased fatty streak formation and plasma triglyceride in hypercholesterolemic (low-density lipoprotein $receptor^{-/-}$ apolipoprotein $B^{100/100}$) mice [72]. Meanwhile, the administration of a specific Ang2 inhibitor ameliorated increased expression of proinflammatory and profibrotic cytokines in the aorta in a murine model of remnant kidney [73]. The exact reasons for the dissociation of the loss-of-function and gain-of-function study are not known. The effect of Ang2 is not limited to atherosclerosis; Ang2 appears to also control liver regeneration [74]. In the early phase after partial hepatectomy, Ang2 is downregulated, resulting in reduced transforming growth factor (TGF)-β1 production in liver sinusoidal endothelial cells, which enables hepatocyte proliferation. In the late

angiogenic phase, the recovery of endothelial Ang2 expression enabled angiogenesis. It is noteworthy that Ang2, a potent angiogenic factor, influences liver regeneration via TGF-β, a major fibrogenic factor; in liver fibrogenesis, the spatiotemporal control of Ang2 results in successful regeneration, not fibrogenesis. To employ Ang2 as a therapeutic target in CKD, a better understanding of the Ang2 regulatory mechanisms in normal and diseased kidneys is needed.

### Endoglin

Endoglin, also known as CD105, is a co-receptor for TGF-β1 and TGF-β3 and believed to be involved in vascular homeostasis given its association with endothelial NOS. Endoglin is highly expressed in proliferating vascular endothelial cells and related with vascular diseases. In 2006, Venkatesha and colleagues investigated the role of endoglin in preeclampsia [75]. They demonstrated increased serum endoglin concentration and endoglin expression in the placenta of preeclampsia patients and that the co-administration of endoglin and sFlt-1 resulted in HELLP syndrome in pregnant rats. Endoglin impaired the binding of TGF-β1 to its receptors, which was assumed to explain the molecular mechanism of its in vivo effect. Serum concentrations of endoglin and sFlt-1 are a predictor of preeclampsia [76]. Therefore, endoglin is an important molecule in the pathogenesis of preeclampsia, whose hallmark is endothelial dysfunction.

Genetic manipulation of the endoglin gene illustrates its role in vascular diseases. Heterozygous knockout of endoglin resulted in a larger infarct or atrophic area in a murine model of permanent distal middle cerebral artery occlusion. The association between polymorphism of the endoglin gene and worse outcomes in arteriovenous malformation illustrates the importance of endoglin in human cerebral infarction [77]. On the other hand, heterozygous knockout of endoglin also resulted in improved survival in a right ventricular pressure overload model [78]. Since endoglin expression changes in monocyte–macrophage differentiation, macrophage-specific endoglin knockout mice displayed delayed immune responses and increased bacterial infection but prolonged survival after lipopolysaccharide-induced peritonitis [79]. These results indicate that whether the effect of suppression of endoglin signaling protects organs depends on the disease. Thus, what about kidney disease? The pathological role of endoglin in CKD progression is currently not known. Serum endoglin concentrations are not influenced by eGFR [80], which indicates that endoglin is not a marker of CKD. Meanwhile, reported increased endoglin concentrations in tubular injury after ischemia/reperfusion and the attenuation of ischemia-/reperfusion-induced renal dysfunction with endoglin haploinsufficiency suggest a link [81]. Endoglin also affected the progression of fibrosis in a murine UUO model. Transgenic mice, which ubiquitously overexpress human L-endoglin, the longer and most abundant isoform of endoglin, displayed higher collagen I accumulation in the UUO kidney [82]. In these transgenic mice, the TGF/Smad pathway was enhanced, which was considered the cause of the fibrosis aggravation. On the other hand, ubiquitous overexpression of human S-endoglin, the shorter isoform, alleviated fibrosis after UUO

in mice [83]. These results implicate that the measurement of total endoglin might be misleading, but discrimination among these isoforms is needed to elucidate the importance and precise pathogenic role of endoglin in human CKD.

### Angiogenin

Angiogenin is a HIF downstream protein and an angiogenic factor whose suppression is related to impaired angiogenesis in diabetes mellitus [84, 85]. The mechanistic role of angiogenin was shown using angiogenin knockout mice, which shows aggravation of endoplasmic reticulum (ER) stress-induced nephropathy compared to wild-type controls [86]. Another piece of evidence is that a high urinary angiogenin concentration was reported to be a risk factor for graft failure in kidney transplant recipients [87]. This increase in urinary angiogenin in the poor outcomes group was considered a result of high ER stress in tubular cells. Since angiogenin also has transfer RNase activity, these results might not be completely due to the angiogenic properties of angiogenin [88].

### Thrombospondin-1

Thrombospondin-1 (TSP-1) is an endogenous multifunctional matricellular glycoprotein and exerts as a major activator of TGF-β and an angiogenic inhibitor [18, 89]. TSP-1 is expressed very weakly in the healthy renal cortex, with increased expression identified in renal diseases. TSP-1 knockout alleviated renal damage in a UUO model [90], obesity-related nephropathy [91], an Alport's syndrome murine model [92], and adriamycin-induced nephropathy [93]. Since TSP-1 is an activator of TGF-β, which plays a pivotal role in renal fibrosis, the beneficial effects of TSP-1 knockout may be linked to mechanisms involving TGF-β signaling blockade. However, TSP-1 could be protective by other mechanisms in renal diseases. TSP-1 increases reactive oxygen species in vascular smooth muscle cells [94] and limits vasodilation in a reactive oxygen species-dependent manner [95]. Thus, the in vivo effect of TSP-1 might, at least in part, derive from endothelial dysfunction. Indeed, a report employing TSP-1 short heparin RNA indicated that the renoprotective effect of knockdown of TSP-1 resulted in PTC restoration [96].

## Importance of Hypoxia in CKD Progression

### *Evidence and Importance of Renal Hypoxia in Human CKD*

Change in these angiogenic factors is involved in PTC rarefaction, which aggravates renal hypoxia. It is now widely accepted that renal hypoxia aggravates CKD progression, and several findings of clinical studies support this notion. First, a high

hemoglobin concentration is a better prognostic factor in CKD patients. A decrease in hemoglobin, the carrier of oxygen molecules in the blood, results in lower tissue oxygenation, which must aggravate renal hypoxia. In fact, a decrease in hemoglobin worsens renal hypoxia in animal models [2, 97]. Tsubakihara et al. showed that the maintenance of a higher hemoglobin level (11–13 g/dL) with darbepoetin-α, an erythropoiesis-stimulating agent (ESA), resulted in better renal prognosis than a lower hemoglobin level (9–11 g/dL) [98]. On the other hand, Palmer et al. performed a meta-analysis and reported that a higher hemoglobin target was not correlated with a better renal prognosis but associated with higher risks of stroke, hypertension, and vascular access thrombosis [99]. A clue that elucidates the source of this discrepancy might be the time of ESA initiation [100]. Gouva et al. reported that the early initiation of erythropoietin-α resulted in better renal prognosis [101], although a limitation of this study is its small patient number. Akizawa et al. reported that only the early initiation of ESA treatment reduced the risk of renal events without increased adverse effects [102]. Taken together, the maintenance of a high hemoglobin level may retard CKD progression with early intervention; these results may also stem from the pleiotropic effect of ESA [103]. Second, the incidence of CKD is higher in diseases that trigger hypoxemia. Acute exposure to high altitude induces diuresis and natriuresis with accompanying potassium and bicarbonate excretion [104]. People living at altitudes higher than 3500 m in Tibet are at high risk of albuminuria and hypertension [105].

These studies indicate that hypobaric hypoxia has an effect on renal reabsorption function, although how high altitude affects CKD progression is not known. Nocturnal hypoxemia, mainly induced by sleep apnea syndrome, is frequent in patients with CKD [106]. On the other hand, Iseki et al. evaluated 4056 patients and reported that patients with a sleep-related breathing disorder are at high prevalence of CKD [107]. Van Gestel et al. researched 3358 vascular surgery patients and showed a relationship between CKD and moderate chronic obstructive pulmonary disease (COPD), which frequently causes hypoxemia. Chen et al. examined a cohort of 23,217 patients with COPD and a matched control group and revealed that COPD patients had a 1.6 times higher risk of CKD than those without COPD [108]. These results indicate that long-term systemic hypoxemia, intermittent or continuous, provokes or aggravates CKD. The third line of evidence involves the study blood oxygen level–dependent magnetic resonance imaging (BOLD-MRI). BOLD-MRI is an MRI-based technique in which deoxyhemoglobin is utilized as an oxygen sensor [109]. Several clinical studies using BOLD-MRI demonstrate that the concentration of deoxyhemoglobin correlates with the slope of the T2* signal. One landmark study by Inoue and colleagues in 2011 [110] examined 142 patients with DKD or CKD without diabetes and revealed that the kidneys of the latter displayed renal hypoxia. Conversely, patients with DKD did not have a correlation between eGFR and renal oxygenation. This result was challenged by the report by Michaely et al. which examined 280 CKD patients and showed no relationship between eGFR and renal oxygenation by BOLD-MRI [111]. This lack of a correlation was also demonstrated in another report [112]. This discrepancy might be explained by the difference of patients' background, especially the prevalence of diabetes mellitus; the

report by Michaely et al. did not show the prevalence of diabetes mellitus. Pruijm et al. studied a total of 195 patients in control, CKD, and arterial hypertension groups and showed no difference in renal oxygenation at rest; however, after furosemide administration, the arterial hypertension group and CKD group displayed renal hypoxia, indicating the existence of a difference in reserved capacity in renal oxygenation [113]. Recently, a new method for assessing BOLD-MRI, the concentric objects technique, was proposed and resulted in the successful detection of renal hypoxia in CKD patients [114, 115]. Taken together, the BOLD-MRI result implies that renal hypoxia really occurs in CKD; however, the next problem is that whether the difference in BOLD-MRI values can explain later CKD progression; therefore, a longitudinal study is desired [116]. The evidence mentioned above supports that renal hypoxia exists in human CKD patients and aggravates CKD progression, whose pathological hallmark is interstitial fibrosis and PTC rarefaction.

## *Cellular Response to Hypoxia*

HIF, a master regulator of adaptive cellular response against hypoxia [3, 13], is considered a factor that mainly connects renal hypoxia and renal fibrosis. HIF consists of two subunits, HIF-α and HIF-β, and involves three isoforms of HIF-α, namely, HIF-1α, HIF-2α, and HIF-3α. Both HIF-α and HIF-β are members of the basic helix-loop-helix PER/ARNT/SIM family of transcription factors. HIF-1α is the first isoform originally identified as a high-affinity DNA binding protein in the *EPO* gene [117]. In the kidney, HIF-1α is dominantly expressed in tubular cells, especially in the medulla, while interstitial and endothelial cells express HIF-2α [118, 119]. The expression pattern of HIF-3α remains elusive.

In the normoxic condition, translated HIF-α undergoes hydroxylation at the proline residues by prolyl hydroxylase domain (PHD), which is followed by ubiquitination by von Hippel–Lindau disease tumor suppressor (VHL), finally resulting in proteasomal degradation. The rate-limiting step of this HIF-α degradation is PHD hydroxylation; thus, the overall rate of HIF-α degradation is determined by the PHD hydroxylation activity, that is, proportional to oxygen concentration unless enzymatic PHD activity changes. In hypoxic conditions, HIF-α escapes oxygen-dependent hydroxylation, resulting in cytosolic accumulation. When HIF-α becomes abundant in the cytosol, HIF-α forms a heterodimer with HIF-β, whose expression is independent of oxygen concentration. This heterodimer translocates into the nucleus, binds to hypoxia-responsive elements containing the –(A/G)CGTG– motif, and transactivates 100–200 of the target genes involved in several pathways such as angiogenesis, erythropoiesis, and energy metabolism. HIF also induces epigenetic changes to enhance the expression of downstream genes [120] (Fig. 16.4). In vascular endothelial cells, HIF-1α binds to only transcriptional starting sites (TSS) and enhancer 1 (−35 kbp from TSS) in the enhancer region of the *SLC2A3* gene, which codes glucose transporter 3, under normoxic conditions. In the hypoxic condition, additional HIF-1α binding occurs in enhancer 2 (−24 kbp from TSS), which recruits

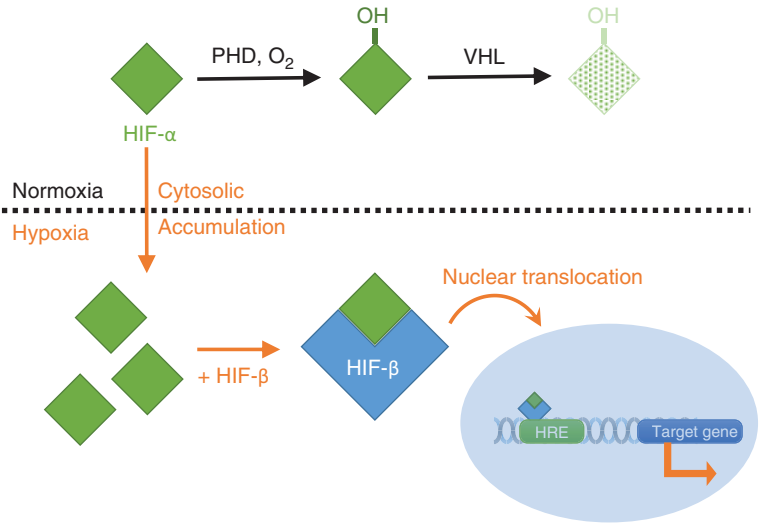


**Fig. 16.4** Oxygen-dependent regulation of HIF. In normoxic conditions, HIF-α is hydroxylated in the proline residue by PHD, resulting in proteasomal degradation by VHL via ubiquitination. In the hypoxic condition, HIF-α escapes degradation and accumulates in the cytosol. Next, HIF-β binds to HIF-α to create a heterodimer and then enters the nucleus and works as a transcription factor. HIF, hypoxia-inducible factor; PHD, proline hydroxylase domain; VHL, von Hippel–Lindau tumor suppressor protein. (Original figure as per authors)

lysine (K)-specific demethylase 3A and leads to robust *SLC2A3* upregulation. These changes help cells survive under hypoxic conditions; angiogenesis and erythropoiesis increase the tissue oxygen supply via increases in vascular density and increases in hemoglobin-bound oxygen, respectively, and a switch in energy metabolism from oxidative phosphorylation to anaerobic glycolysis. Therefore, HIF activation is protective against CKD progression [121, 122].

## *Origin of Renal Myofibroblasts and the Effect of Hypoxia on Their Characteristics*

In organ fibrosis, an increase in the extracellular matrix results from myofibroblast increase or activation. Therefore, the origin of renal fibroblasts has been a matter of concern and remains controversial. The proposed mechanisms of an increase in myofibroblasts in the fibrotic kidney include an increase in resident fibroblasts, dedifferentiation from pericytes, engraftment of bone marrow-derived cells, epithelial–mesenchymal transition (EMT), and endothelial–mesenchymal transition (Fig. 16.5) [123]. Of these, resident fibroblast involvement in myofibroblasts in renal fibrosis may be the predominant mechanism. In the normal kidney, fibroblasts reside ubiquitously in the renal interstitium. A recent study revealed that at least a

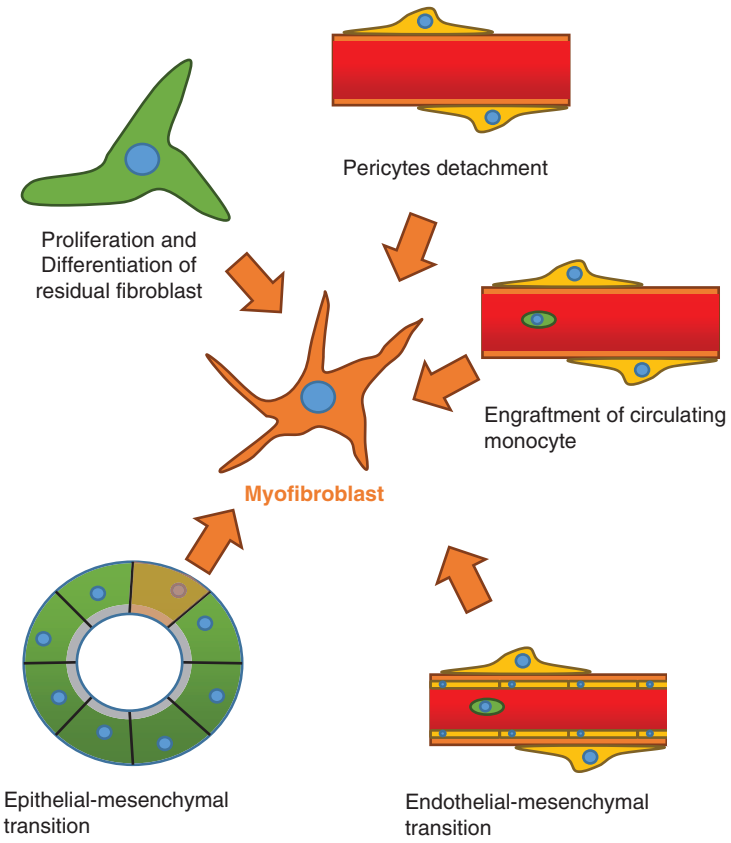


**Fig. 16.5** Origin of myofibroblasts in the kidney. There are many proposed origins of the myofibroblasts in the kidney. Residual fibroblasts and pericytes are dominant candidates for renal myofibroblast creation, while tubular cells may be involved in fibrosis via the partial epithelial-mesenchymal transition. Circulating monocyte engraftment and the endothelial–mesenchymal transition are other sources of renal myofibroblasts. (Original figure as per authors)

certain subpopulation of myofibroblast has potential to produce erythropoietin (EPO) in a hypoxic condition [124]. The same group indicated that renal EPO-producing (REP) cells, which do not currently produce EPO, can transform to myofibroblasts with pathogenic signals such as inflammation [125]. Interestingly, the behavior of such myofibroblasts is HIF-dependent: PHD knockout in REP cell-derived myofibroblasts restored EPO production and reduced fibrosis- and inflammation-related genes in a UUO model [126].

Pericytes are another important source of myofibroblasts in a fibrotic kidney model. Pericytes are the cells that wrap endothelial tubes and capillaries and have the potential to dedifferentiate into myofibroblasts with cytokines such as VEGF, Ang2, and platelet-derived growth factor [127]. Since VEGF and Ang2 are HIF downstream proteins, renal hypoxia is likely related to pericyte detachment. Duffield

and colleagues revealed that myofibroblasts in a renal injury model originated from pericytes [128]. The importance of pericyte-derived myofibroblasts is seen when fibroblast growth factor-inducible 14 (Fn14) and its ligand, TNF-related weak inducer of apoptosis (TWEAK), are altered [129]. Fn14 signaling had the potential for vasoconstriction and detachment of pericytes from the capillaries, and systemic knockout of Fn14 and neutralization of TWEAK attenuated renal fibrosis in a UUO model and Alport's syndrome model of mice, respectively. VEGFR2 blockade prevented differentiation to myofibroblasts, and the differentiation led to a switch in VEGF isomer secretion, that is, downregulation of VEGF164 and upregulation of VEGF120 and VEGF188 [130]. Considering that VEGF164 is an angiogenic isomer and VEGF120 and VEGF188 are dysangiogenic isomers, the differentiation from pericytes to myofibroblasts likely disrupts PTC homeostasis. In addition to pericyte detachment, the disruption of NLRP3 inflammasome activation in pericytes leads to amelioration in fibrosis [131], indicating that pericytes or myofibroblasts of pericyte origin played a role in inflammation and fibrogenesis [132]. Pericytes likely have functions in microvascular stability, which emphasizes their role in PTC homeostasis. The importance of $Gli1^+$ pericytes in PTC homeostasis was recently reported [133]. Following ischemia/reperfusion injury, the distance between Gli1-positive pericytes and endothelial cells increased, and the genetic ablation of pericytes alone led to PTC rarefaction.

One more proposed source of renal myofibroblasts in the fibrotic kidney is from EMT, a phenomenon in which epithelial cells dedifferentiate into mesenchymal cells; in the kidney, it means transformation from tubular cells, especially proximal tubular cells, into myofibroblasts. EMT is readily observed in cultured cells, and a number of studies point to the role of hypoxia as a driver [134], and two representative EMT inducers, snail family zinc finger 1 (Snail1), which is encoded by *SNAI1* gene, and Twist family bHLH transcription factor 1 (Twist), which is encoded by the *Twist1* gene, are downstream of HIF [135–138]. Meanwhile, a fate-mapping method revealed that only a limited number of myofibroblasts in the interstitium stem from renal proximal tubular cells [139].

## Angiogenesis-Oriented Therapies in CKD

### *Drug Therapies*

#### VEGF-Based Therapies

Since maintenance of vascular density, especially PTC density, has been expected as a treatment target of CKD, an increasing emphasis on maintaining sufficient angiogenesis by the administration of angiogenic factors has arisen. The effect of VEGF therapies has been most investigated. The milestone study by Johnson and colleagues [140] showed the efficacy of VEGF-based therapy. They administered VEGF121, the only isoform of VEGF that can result in an effective plasma level

when peritoneally administrated [141, 142], to a rat remnant kidney model and demonstrated that VEGF121 administration reduced blood urea nitrogen, preserved PTC, and alleviated collagen III deposition. Subsequently, the efficacy of VEGF121 administration on PTC preservation was demonstrated in a rat post-acute kidney injury CKD model induced by renal ischemia/reperfusion and in a porcine renal artery stenosis model [143–145]. Chade et al. insisted that one limitation of VEGF121 is the insufficient effect on renal damage, partially according to its short lifetime in vivo [146]. Therefore, they prolonged the plasma lifetime of VEGF by fusion with a biopolymer-stabilized elastin-like polypeptide and showed that the administration of this fusion protein maintained vascular density and ameliorated glomerular filtration rate in a swine renovascular disease model. The effect of exogenous VEGF165, the most abundant isoform of VEGF in humans, has also been studied. Suppression of VEGF165 by DNA aptamer, which can bind to rat VEGF164, aggravated Thy-1-induced glomerulonephritis but not puromycin aminonucleoside nephropathy or passive Heymann nephritis [147]. Conversely, the intraperitoneal administration of VEGF165 ameliorated renal dysfunction and proteinuria in a Thy-1/habu snake venom GN model in rats [148] and nephrotoxic serum-induced necrotizing and crescentic glomerulonephritis [149]. These renoprotective effects of VEGF121 and VEGF165 are likely due to PTC stabilization. Meanwhile, as previously described, VEGF has an injurious effect on podocytes, especially in early DKD. To this end, an inhibitory splicing variant, VEGF165b, is a promising therapeutic choice. VEGF165b is a splicing variant in which replacement of exon 8a by exon 8b occurs and competitively inhibits VEGF165 binding to its receptor [150]. The study of Denys–Drash syndrome, a genetic glomerulopathy caused by a mutation in Wilms' tumor protein-coding gene, illustrates the importance of VEGF165b [151]. In patients with Denys-Drash syndrome, podocytes expressed high VEGF165 levels but lacked VEGF165b expression, and this increased VEGF165/VEGF165b ratio may be the mechanism of glomerulopathy. Both exogenous VEGF165b administration and podocyte-specific VEGF165b overexpression alleviated diabetic changes in glomeruli such as albuminuria and glomerular basement membrane widening [34]. On the other hand, one report showed that podocyte-specific VEGF165b overexpression in mice resulted in no macroscopic changes in the glomeruli but a marked reduction in endothelial fenestration number and size [152]. These results indicated that VEGF165b should have a role in podocyte protection, which is consistent with the fact that an increase in VEGF results in podocytopathy, although its effect on endothelial cells needs to be better understood. Considerable attention should be paid to this issue since VEGF plays two roles in CKD progression: protective for PTC stabilization but injurious against podocytes. Moreover, the fact that the clinical use of VEGF inhibitor triggers minimal change disease, which means podocytopathy, indicates that extreme VEGF suppression degrees must be avoided to obtain the podocyte-protective effect. Thus, a future challenge should be the elucidation of the proper range of VEGF activation/suppression to each CKD origin.

### HIF Stabilizer

HIF stabilization is supposed to be a promising therapeutic strategy against CKD [153] since HIF–PHD inhibition, and HIF activation has yielded encouraging results in animal models. The administration of cobalt chloride, a traditional HIF–PHD inhibitor, ameliorated tubular injury and PTC rarefaction with VEGF upregulation in a remnant kidney model in rats [154]. The renoprotective effect was also seen in a Thy-1.1-induced progressive glomerulonephritis model, although in this model amelioration of PTC was not observed by cobalt chloride administration [122]. Cobalt chloride also ameliorates a DKD model. In spontaneous hypertensive type 2 diabetes rats, cobalt chloride administration alleviated tubulointerstitial fibrosis and PTC rarefaction with HIF upregulation [10]. Nordquist and colleagues reported that the administration of cobalt chloride resulted in partial reversal of renal hypoxia of STZ-induced diabetic rats by cobalt chloride administration, followed by a reduction of albuminuria and alleviation of interstitial fibrosis [121]. It is true that some of these protective effects of HIF stabilization are derived from protection against oxidative stress, but the renoprotective mechanism of the pharmacological inhibition of HIF–PHD by cobalt chloride is multifactorial, and PTC maintenance via the upregulation of angiogenic factors such as VEGF likely plays a role. Erythropoietin is one of the most famous downstream genes of HIF; thus, HIF stabilization is a candidate pharmacological target against renal anemia. Severe side effects prevent cobalt chloride from being used in clinical patients, which has led to the development of novel HIF–PHD inhibitors recently synthesized and undergoing clinical trials for renal anemia [155]. In the near future, HIF–PHD inhibitors may be utilized to treat CKD progression.

However, several studies have raised concerns about the fibrogenic role of HIF. In addition to promoting EMT, HIF cooperates with TGF-β, which plays a crucial role in tissue remodeling and fibrosis [156, 157]. Many studies have investigated the mechanism by which HIF-1 and TGF-β interact. Smad3, a transcriptional factor that transmits downstream signals from the TGF-β receptor, had a synergistic effect with HIF-1α in genes that contain sites co-occupied by HIF-α and Smad3, such as collagen type I alpha 1 [156]. This synergistic effect partly occurs via the mammalian target of rapamycin complex 1 (mTORC1) in mesangial cells, indicating the importance of the Smad3–mTORC1–HIF-1 axis in glomerular matrix expansion [158]. However, HIF's fibrotic role may partly depend on the species. One example is connective tissue growth factor (CTGF), a fibrogenic gene. In mice, the hypoxia-induced *Ctgf* gene expression mediated by HIF-1 [159]. Conversely, the *CTGF* gene in human cultured cells is downregulated under hypoxic conditions [160]. One of the explanations is a species difference in the promoter region of the *CTGF* gene, that is, HRE is detected in the mouse promoter region of *Ctgf* but not in the human *CTGF* gene. Therefore, the hypoxic induction of CTGF may not occur in all species and caution is required in extrapolating the results of animal experiments to human CKD.

Gene manipulation of the HIF pathway also yields conflicting results. Kimura et al. showed that tubular-specific knockout of the *VHL* gene resulted in stable HIF

expression in tubular cells, followed by an exacerbation of renal fibrosis in a remnant kidney model, an aging model, and unilateral UUO [161]. This result is consistent with the result that interstitial fibrosis was exacerbated in UUO kidneys in tubule-specific *Hif1-a*-KO mice from the same group [162]. On the other hand, inducible tubular-specific PHD2 knockout resulted in PTC restoration and tubular injury alleviation in high-fat-diet-induced nephropathy [19]. These conflicting results suggest that, in addition to differences in CKD models, the identification of the proper timing and extent of HIF accumulation by disease is essential for appropriate pharmaceutical HIF activation in CKD. Indeed, one report showed that timing administration of L-mimosine, another PHD inhibitor, had a therapeutic effect on a remnant kidney model; in this paper, the effect of L-mimosine on anti-fibrosis and PTC maintenance was greater if L-mimosine was started 4 weeks after surgery, not 2 or 8 weeks [163].

## *Cell Therapies*

### Endothelial Progenitor Cells

The administration of a range of progenitor cells was shown to be a promising strategy to restore PTC. Endothelial progenitor cells (EPCs) are a self-renewing cell population that can differentiate into functional endothelial cells and contribute to the maintenance of the vascular system since the number of circulating EPCs correlates with cardiovascular risks and EPCs from high-risk patients displayed a senescence phenotype [164]. CD34+ EPC number and function were reduced in CKD patients [165, 166]. The importance of circulating CD34+ EPCs in CKD has recently been described in an animal model of critical limb ischemia. When remnant kidney model mice underwent ligation of femoral and saphenous arteries to induce unilateral hind limb ischemia, their recovery was attenuated [167]. The administration of AST-120, an adsorbent of indoxyl sulfate (IS), partially reversed the worsening effect of the remnant kidney model itself and of the oral administration of indole, a precursor of IS. An in vitro assay also revealed that IS inhibited migration and tube formation in human EPCs. Given that IS is a uremic toxin, an increased serum IS concentration likely plays a role in EPC dysfunction in CKD patients and is accountable for ischemic hind limb aggravation. Therefore, the administration of EPCs, especially healthy ones, is thought to be protective in CKD, at least against vascular complications.

Since PTC rarefaction is a major hallmark and an aggravating factor of CKD, the effect of EPC administration in kidney disease has also been studied. In a porcine renal artery stenosis model, the administration of autologous EPCs attenuated PTC rarefaction and fibrosis [168]. In a rat remnant kidney model, renal blood flow restoration resulted from the administration of autologous EPCs (collected before CKD induction) [169]. Therefore, EPC administration can be a promising strategy of CKD progression or preventing cardiovascular disease in CKD. However, a matter of concern is whether patient-derived EPCs can work in a similar way to those of

healthy donors: in the animal model experiment, the EPC collection prior to the accumulation of uremic toxins. In addition to a decreased number of EPCs, poor mobilization, low viability, engraftment dysfunction, and impaired differentiation are involved in EPC incompetence in CKD patients [170].

### Mesenchymal Stem Cells

Adult stem cells originating from the mesoderm are called mesenchymal stem cells (MSCs). MSCs reside in many tissues including bone marrow and adipose tissues. Since many cells of the nephron are of mesenchymal origin [171], the administration of MSCs derived from bone marrow or adipose tissues are considered a promising strategy for mediating preservation of microvascular density like EPCs [172]. In 2007, Kunter et al. examined the effect of MSC administration from rat bone marrow in a rat Thy-1.1 mesangioproliferative glomerulonephritis model [173]. They showed an early beneficial effect of MSC administration; however, long-term observations were not pursued, in part, due to maldifferentiation of MSCs to adipocytes. Therefore, they insisted that the key to applying MSCs to therapeutics is the suppression of maldifferentiation. Afterward, MSCs residing in the perivascular niche is shown to play an important role in organ fibrosis [174], and it is now widely known that MSCs do not always play a beneficial role in inhibiting fibrosis. Two recent studies showed the effectiveness of MSC administration in a UUO model. Gregorini et al. demonstrated that the administration of MSCs from rat bone marrow alleviated fibrosis in 21 days after UUO surgery [175]. Huuskes et al. reported that the administration of human bone marrow-derived MSCs had a beneficial effect on UUO when combined with serelaxin, a recombinant human relaxin-2 that inhibits TGF-β signaling and promotes angiogenesis [176]. They demonstrated that MSCs accumulated in the injured kidney, which might accordingly suppress the maldifferentiation. In an experimental renal artery stenosis model, the administration of MSCs improved VEGF expression accompanied by PTC density restoration [177]. The protective mechanism of MSCs is proven to be different from that of EPCs, while suppression of the inflammatory response and apoptosis is greater than those of EPCs [178].

### Renal Progenitor Cells/Induced Pluripotent Cells

Since progenitor cell-induced therapies are supposed to be effective for maintaining PTC and inhibiting fibrosis, renal progenitor cells should be an option. The use of induced pluripotent stem cells (iPSC) is a promising strategy in regenerative medicine recently applied to progenitor cell-based therapy. Two reports indicated that iPSC-derived renal progenitor cell administration ameliorated acute kidney injury by cisplatin nephrotoxicity and ischemia/reperfusion injury, with direct engraftment identified in the repair process [179, 180]. Acquiring iPSC from patients is minimally invasive; thus, the future application of iPSC-based therapy to renal diseases is likely.

## Adjustment of the Balance Between Angiogenesis and Fibrosis

As already mentioned, renal hypoxia enhances CKD progression, while HIF stabilization can, at least in proper situations, suppress CKD progression. There seems to be a dissociation at a glance, and the difference is likely derived from insufficient HIF activation compared to the extent of renal hypoxia. One example is proven in EPO production. Chiang et al. reported that IS suppresses the nuclear accumulation of HIF and subsequent EPO production in cultured cells and rat kidney without changes on renal histology [181]. Later, the mechanism of IS-induced insufficient nuclear HIF accumulation was mediated by the aryl hydrocarbon receptor (AhR) [182]. IS can activate AhR, which forms a dimer with an HIF-β subunit to induce downstream gene expression; therefore, increased AhR signaling with IS results in HIF-β occupation and inhibits HIF-α dimerization with HIF-β (Fig. 16.6). There is another HIF-suppressing mechanism of IS. IS has been shown to reduce other expression of HIF downstream genes including VEGF in the hypoxic condition, and this incommensurate expression of HIF downstream genes is mediated by Cbp/p300-interacting transactivator with Glu-/Asp-rich carboxy-terminal domain 2 [183]. This IS-induced suppression of HIF downstream signals was also shown in remnant kidney, isoproterenol-induced heart failure, and ischemic hind limb models [167,

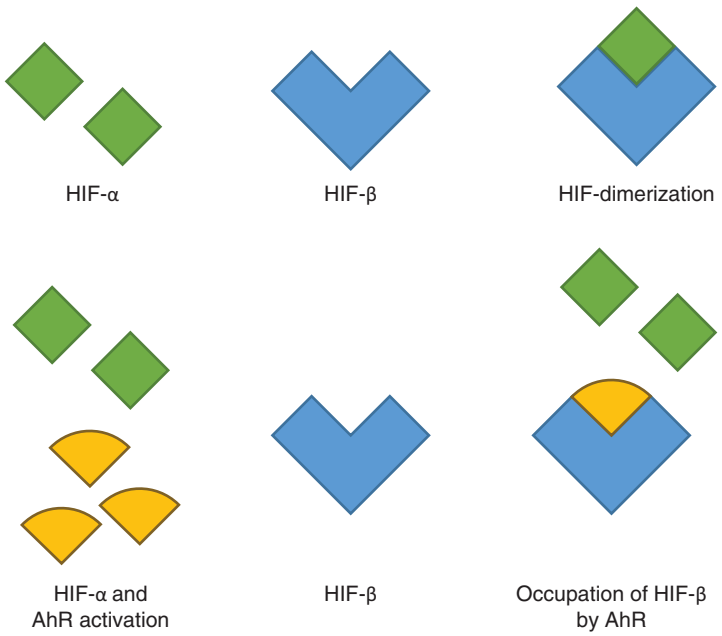


**Fig. 16.6** Insufficient HIF activation via AhR upregulation. In the normal state, HIF-α and HIF-β form a heterodimer. When AhR is activated, it accumulates in the cytosol and forms a heterodimer with HIF-β, resulting in the inhibition of HIF-α and HIF-β heterodimerization. AhR, aryl hydrocarbon receptor; HIF, hypoxia-inducible factor. (Original figure as per authors)

183]. In addition, AST-120, an oral absorbent of indole, an IS precursor, alleviates renal hypoxia itself in a remnant kidney model [184]. Therefore, AST-120 has the potential to retard CKD progression in an experimental animal model. Since the use of AST-120 is approved in advanced CKD patients in Asian countries, its effect in CKD patients is a matter of concern; however, it remains controversial [185–189]. Increased ER stress is another possible mechanism of insufficient HIF activation in CKD. Chiang et al. revealed that renal EPO production was inhibited by the ER stress inducer tunicamycin and that the mechanism of this suppression is the existence of activating transcription factor 4, a transcriptional factor for the unfolded protein response pathway binding site in the enhancer region of the *EPO* gene [190]. Since ER stress and CKD development and progression are closely related, this ER stress-induced suppression of HIF downstream genes may occur in clinical CKD. Therefore, treatment against uremic toxin accumulation and enhanced ER stress might be a clue for the adjusted balance between angiogenesis and fibrosis.

## Future Perspectives

PTC loss and interstitial fibrosis are hallmarks of pathology of CKD, and the subsequent emergence of renal hypoxia accelerates CKD progression. This vicious cycle was widely accepted; however, serious questions in this cycle remain such as the mechanism of insufficient angiogenesis, time of emergence of renal hypoxia, and exact role of angiogenic factors in the kidney. Moreover, HIF has two sides – a protective role against capillary rarefaction via angiogenic factors and a fibrogenic role via the direct enhancement of TGF-β signaling and increase in myofibroblasts, and a detailed understanding of the timing and the context in which HIF works mainly as an angiogenic factor versus a fibrogenic factor has not been achieved. A better understanding of these problems will help us determine what governs the balance between angiogenesis and fibrosis. Another problem is that partly because of the labor-intensive nature of obtaining renal tissue from patients, insufficient knowledge has been obtained about human CKD to determine whether it is reasonable to extrapolate the findings of animal experiments to clinical CKD. Resolving these questions should yield knowledge that guides our future exploration of angiogenesis-based therapies for CKD.

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# Chapter 17
# Fibrotic Remodeling in Exudative (Wet) Macular Degeneration


**Joseph N. Martel, Vincent Q. Nguyen, and Andrew W. Eller**

## Introduction

The visual pathway is intricate and involves the concerted action of many complex intraocular structures and the central nervous system. Any pathological process along the visual pathway can lead to distorted or diminished vision. The light processing structures in the eye are analogous to capturing an image using a camera with film or a digital chip. The anterior ocular structures focus the light onto the retina which is responsible for processing the light energy and transmitting a signal to the brain.

Light from the environment is refracted principally by the cornea and the intraocular lens, and the refracted light is focused on the retina as it transverses through the vitreous in the posterior segment of the eye. The vitreous consists of a relatively transparent gelatinous structure composed mainly of serous fluid, collagen, and hyaluronic acid. The vasculature of the central retinal artery and the capillary vasculature of the underlying choroid nourish the light processing retina, which lines the inner wall of the eye. The retina absorbs photons of light via photoreceptors, generating electric impulses which are then transmitted along the optic nerve to the visual cortex in the occipital lobe.

The retina is the light processing center inside the eye. The retina is an energy-intense, highly metabolic, and oxygen-consuming intraocular tissue. It consists of

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a synchronized network of cellular components and axons that collectively capture light and transduce an electrical impulse to the optic nerve. A specialized region of the retina, known as the macula, has densely packed cone photoreceptors that enable high spatial visual acuity and color vision. In a highly metabolic, oxygen-intense activity, vitamin A-derived light-sensitive molecules bound to opsin within the photoreceptors process the light energy. The underlying retinal pigment epithelium (RPE) assists with recycling of photoreceptor degradative products, contributes to the blood-ocular barrier, and maintains the subneurosensory retinal space. The basement membrane of the RPE, known as Bruch's membrane, is composed of collagen and elastic fibers. The RPE and Bruch's membrane, therefore, serve to protect and maintain the neurosensory retina. The cellular structures of the retina cannot regenerate. Thus, once there is retinal cellular degeneration or irreparable cellular injury, retinal functional deficits often persist long-term.

In the eye, aberrant wound healing and fibrosis can affect the clarity of the visual axis depending on the ocular tissue involved. For example, fibrosis in the cornea can cause corneal opacification and thereby prohibit light from entering the eye. Retinal fibrovascular scarring and gliosis from ischemia and neovascularization on its surface can cause tractional retinal detachments and poorly functioning retinal tissue. In exudative or neovascular age-related macular degeneration (AMD), subretinal fibrosis results in damage and impaired function of the macular region of the retina resulting in severe visual impairment with difficulty discerning fine details in vision and color vision. Naturally, understanding and targeting pathways that influence the development of subretinal fibrosis and precursor lesions such as choroidal neovascularization are of interest to scientists and clinicians. In this chapter, we will review the pathobiology of exudative AMD as it relates to the pathogenesis of subretinal fibrosis.

## Age-Related Macular Degeneration

Age-related macular degeneration is a bilateral progressive retinal degenerative disorder in the geriatric population and the leading cause of irreversible visual impairment in the developed world. The burden of the disease continues to grow, as the already relatively high prevalence of AMD is expected to nearly double by the year 2050. A vision-threatening subtype of AMD characterized by choroidal neovascularization (CNV) generally responds to treatment with vascular endothelial growth factor (VEGF) inhibition, but a significant proportion of patients develop severe macular dysfunction either from inadequate treatment, persistent disease activity refractory to VEGF inhibition, or progressive atrophy and/or subretinal fibrosis despite control of CNV. Macular subretinal fibrosis results in damage to the outer retinal structures essential for light-induced signal transduction to the inner retinal cells and represents the end-stage sequelae of choroidal neovascularization in exudative macular degeneration.

Visual acuity is generally preserved in the earlier nonexudative stage of AMD. With time, accumulation of focal deposits of lipo-glyco-proteinaceous, extracellular material or "drusen," are typically seen in Bruch's membrane. The distribution and size of drusen varies and is found to be concordant to an individual's risk of progression to the late stages of the disease. Along with drusen, the other hallmark features of AMD include retinal pigment epithelium (RPE) abnormalities, which signify the degeneration of the RPE and photoreceptors. In a healthy eye, the RPE is a monolayer of pigmented cells located between the neurosensory retina and the choroid that plays a critical role in the maintenance of visual function. The RPE dysfunction and the accumulation of drusen can lead to thickening of Bruch's membrane and result in decreased diffusion of oxygen from the choriocapillaris to the photoreceptors. These changes to the outer retina stimulate CNV formation. Although the precise mechanism for the development and progression of AMD is yet to be elucidated, the development of unchecked exudative maculopathy usually results in a precipitous decline in vision. Approximately 15% of AMD patients will experience the exudative form of the disease, which is characterized by the development of CNV, retinal pigment epithelial detachments (PED), retinal pigment epithelial tears, retinal hemorrhages, and fibrovascular-induced hypertrophic scarring. Patients with exudative AMD may experience decreased vision, distortion of the vision, or central scotomata.

Fibrosis, typically in the subretinal space, is seen in the end-stage cicatricial form of exudative AMD and shares molecular mechanisms of fibrosis similar to other organs of the body. This "disciform scar," characteristic at the end-stage of disease, refers to the round phenotypic appearance seen clinically (Fig. 17.1). Clinically, the disciform scar usually results in a central scotoma and loss of all fine details in vision with patient reliance thereafter on the rudimentary peripheral vision. Before chronic intraocular VEGF inhibition became the therapeutic mainstay, early treat-

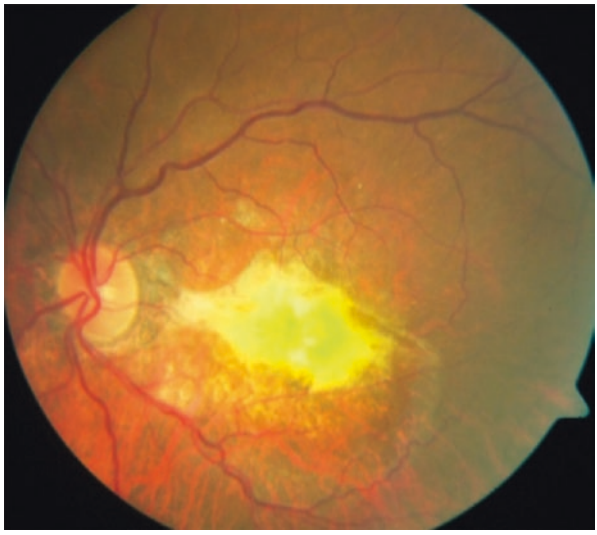

**Fig. 17.1** Fundus photograph of end-stage exudative AMD with macular subretinal fibrosis (disciform scar) and atrophy. (Source: All images are from clinical patients of Dr. Martel)

ments targeting choroidal neovascularization with surgical removal of choroidal neovascular membranes, laser photocoagulation therapy, and photodynamic therapy (PDT) often caused collateral damage to less affected retinal tissue and offered inadequate long-term CNV suppression.

Since the mid-2000s, clinical treatment of exudative AMD has transformed with the advent of intravitreal pharmacotherapy with chronic VEGF inhibition aimed at CNV suppression. These agents reduce the risk of severe vision loss; however, unsuccessful treatment outcomes have often been attributed to the progression of subretinal fibrosis or progressive retinal atrophy. Ideally, AMD therapy directed at the retinal "aging" process itself would prevent retinal degeneration. However, such a treatment has been elusive. VEGF inhibition alone remains an imperfect treatment for AMD, with clinicians and investigators interested in improving visual outcomes further. Thus, investigations into other therapeutic agents aimed at influencing other mediators of vision loss in AMD are of interest.

## Antecedent Events to Subretinal Fibrosis

One of the cardinal features of wound healing is angiogenesis. New vessels are designed to assist in the repair of injured tissue, to increase local oxygen supply, and to recruit inflammatory cells to the damaged tissue. In exudative AMD, CNV develops in the subretinal and/or sub-RPE space, leading to hemorrhage and exudative changes, which in turn stimulates the development of subretinal fibrosis. This process is characterized by proliferation and cellular infiltration of various types. Retinal pigment epithelium, glial cells, fibroblasts, myofibroblast-like cells, and macrophages, interacting with inflammatory cytokines and growth factors, result in remodeling of the chorioretinal tissue extracellular matrix. Because of the complexity of the cellular interactions and the numerous mediators, no useful therapeutic options for subretinal fibrosis in retinal disease currently exist.

Although the precise mechanism of CNV development is unclear, degenerative changes in Bruch's membrane with an accumulation of drusen and thickening of Bruch's membrane contribute to a proangiogenic environment. Additionally, increased vascular permeability, and vasodilation accompanied by migration and proliferation of endothelial cells result in the formation of a neovascular vascular network. The interplay of proangiogenic mediators including VEGF, fibroblast growth factor (FGF), transforming growth factor (TGF), and angiopoietin with downregulation of antiangiogenic mediators result in the development of a mature choroidal neovascular membrane. The neovascularization originates in the choroid and extends through the damaged Bruch's membrane toward the retina.

In nature's attempt to heal Bruch's membrane, the process of wound repair becomes aberrant. Fibroblasts accompany the abnormal choroidal neovascular vessels that spontaneously bleed and leak fluid into the retina and within the subneurosensory retinal space. Clinically, fluorescein angiography is helpful in determining the location and activity of choroidal neovascularization. On fluorescein

angiography, CNV is identified by leakage of fluorescein dye into the retinal tissue represented by focal hyperfluorescence corresponding to the site of CNV (Fig. 17.3).

The spontaneous bleeding and fluid leakage from neovascular blood vessels damage the outer retinal cellular structures including the RPE and photoreceptors. Large subretinal hemorrhages are known to have a poor visual prognosis (Fig. 17.2). Animal models of subretinal hemorrhage have demonstrated the damage in the retina. For example, Glatt and Machemer's work involving autologous blood injection into the subretinal space of rabbits showed early photoreceptor edema within 24 h, severe damage to the outer nuclear layer at 7 days, and photoreceptor degeneration. Additionally, Toth et al. demonstrated the importance of photoreceptor toxicity from fibrin degradation products in a cat model.

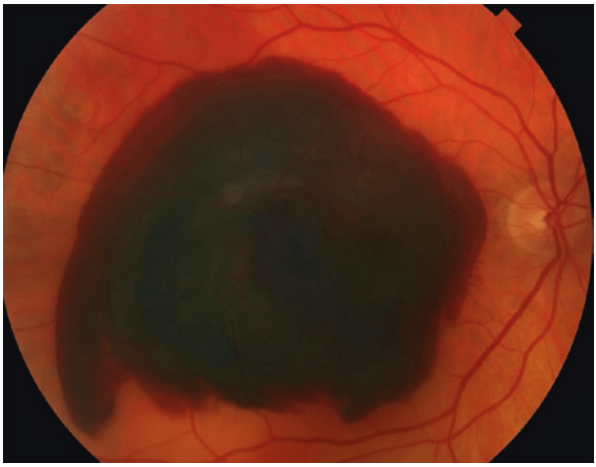

**Fig. 17.2** Fundus photograph of a patient with exudative AMD who developed a massive submacular hemorrhage from choroidal neovascularization. (Source: All images are from clinical patients of Dr. Martel)

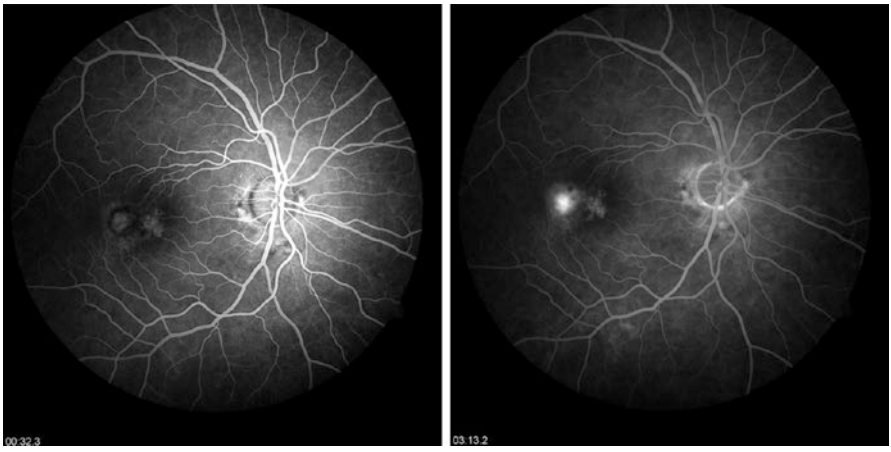

**Fig. 17.3** Fluorescein angiography of a patient with exudative AMD showing fluorescein leakage at the site of choroidal neovascularization in the central macular region of the right eye. (Source: All images are from clinical patients of Dr. Martel)

## Inflammation in Age-Related Macular Degeneration

The immune-privileged properties of the eye allow for transplantation of the cornea, for example, without the need for systemic immunosuppression as in other organs. Specialized immune defenses along with the blood-ocular barrier bestow the immune-privileged status of the retina. Despite the immune-privileged properties of the eye, ocular inflammation correlates with chorioretinal diseases, including AMD.

Although clinical trials of drugs targeting immune effectors have failed to alter the course of AMD, inflammation plays a critical role in the disease pathogenesis. As the macula degenerates, aberrant inflammatory mediators activate the immune system locally in the macular region. Genetic variants of immune modulators such as complement factors, cytokines, chemokines, as well as cellular mediators of innate and adaptive immunity are associated with the development and progression of AMD. Choroidal endothelial cells and invading immune cells have been shown to be responsive to complement activation products and can modulate interactions between monocytes and lymphocytes. Cytokines promoted by complement activation pathways have been shown to promote neovascularization and cell death.

### *Complement Pathway*

Complement pathway activation is known to be important in pulmonary and renal fibrosis and likely also plays a role in intraocular fibrotic changes. Genetic evidence from genome-wide association studies and genetic variant analyses suggests that the complement system, in particular the alternative complement system, is dysregulated in AMD. Components of the complement system have been found in drusen and have been shown to contribute to vascular endothelial growth factor (VEGF) expression, one of the main drivers of choroidal neovascularization. Indeed, several variants of proteins in the complement system such as complement factor H (CFH), complement factor B (CFB), and complement components 2, 3, and 5 (C2, C3, C5) are associated with the progression of or protection against AMD. The strongest association with AMD involves a polymorphism of CFH, which may be involved in up to 50% of AMD patients. Although there remains considerable enthusiasm in complement-targeted therapeutics, the historical clinical trial failures of these drug targets have simply reinforced the lack of understanding of how the complement interactions influence atrophic progression and subretinal fibrosis in AMD.

### *Innate Immune System*

There is clear evidence from animal models demonstrating a complex role of macrophages and microglia in both preventing and encouraging exudative AMD. Microglia and macrophages are innate immune cells that function to initiate inflammatory

responses, clear debris, and remodel tissue. Microglia are specialized cells that typically reside in the inner retina and the central nervous system possessing the ability to migrate into the subretinal space in response to inflammatory stimuli. Several studies have identified the presence of macrophages in AMD, in or around drusen, and at areas of retinal atrophy and choroidal neovascular membranes.

Some investigators have shown that macrophages from CNV tissue express VEGF. Macrophages can also induce proliferation and migration of vascular endothelial cells by cytokines accelerating angiogenesis and CNV formation. Macrophages can display different phenotypes as well: the M1 phenotype which is proinflammatory and more common in retinal macular atrophy or the M2 phenotype, which is anti-inflammatory, angiogenic, and is more common in the exudative AMD subtype.

## *Cytokines and Growth Factors*

Choroidal neovascularization develops in growth factor and cytokine-rich environments, in tandem with proangiogenic molecules, most notably VEGF. While VEGF is the main proangiogenic target in clinical practice, other inflammatory mediators are thought to be contributory to CNV formation. Some of the key mediators include transforming growth factor (TGF)-β, fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and tumor necrosis factor-α (TNF-α). The source of these cytokines may be RPE, infiltrating macrophages, or fibroblasts.

Chemokine receptors on various cells function to direct sites of inflammation, and their associated downstream effectors suggest that many inflammatory-related pathways contribute to the pathogenesis of AMD. In animal models expressing chemokine receptor deficiency such as CCL2, CCR2, and CX3C deficiency, phenotypic characteristics similar to AMD are present. Moreover, a chemokine-dependent immune function may have a role in photoreceptor apoptosis. In terms of CNV formation, chemokine receptors have been shown to be important. An eosinophil/mast cell chemokine receptor, CCR3, has been expressed on human CNV membranes. Both CCR3 and the chemokine CXCL8, also known as interleukin-8 (IL-8), have both been implicated in angiogenesis and are associated with AMD.

In clinical practice, intravitreal pharmacotherapy therapy targeting CNV is the mainstay of treating exudative AMD. Currently, the primary target of the intravitreal pharmacotherapy is VEGF, but other angiogenic modulator drugs targeting PDGF, FGF, and TGF are under investigation. Combined inhibition of VEGF and other growth factors or mediators of angiogenesis may be more effective than anti-VEGF alone. A phase 3 clinical trial in 2016 investigating combination therapy with anti-VEGF intravitreal pharmacotherapy combined with intravitreal pegpleranib (Fovista) anti-PDGF compared to anti-VEGF monotherapy failed to meet the primary endpoint of mean change in visual acuity at 12 months. Despite the dampened enthusiasm for targeting PDGF and other growth factors, PDGF and related growth factors are still being investigated as a potential target for reducing the incidence of subretinal fibrosis in exudative AMD.

## Subretinal Fibrosis

Subretinal fibrosis in exudative AMD shares common molecular and cellular components of scar formation with other organs in the body. Inflammatory and profibrotic cells migrate and proliferate at the Bruch's membrane–RPE complex producing extracellular matrix and clearing cellular damage and debris. Angiogenesis and the development of choroidal neovascularization, sometimes termed choroidal neovascular membranes (CNVM), are associated with these events in the retina. Repeated inflammation and tissue reorganization result in irreversible scar formation.

The generation of subretinal fibrous tissue is complicated, and multiple cell types, proteins, and products are involved. As mentioned previously, the various cytokines and growth factors produced by RPE, infiltrating macrophages, or fibroblasts are thought to be contributory to the pathogenesis of subretinal fibrosis. When the fibrous tissue becomes apparent clinically, the CNV/fibrous subretinal fibrotic tissue complex may be called a disciform scar. Disciform scars may continue to grow, with new areas of neovascularization proliferating along the edges of previously unaffected regions of the retina. An essential clinical imaging tool often used in the diagnosis and surveillance of AMD patients, optical coherence tomography (OCT) imaging, allows in vivo visualization of the subretinal fibrosis by visualizing subretinal hyper-reflective material (Fig. 17.4).

Histologically, fibrous tissue not typically apparent on ophthalmoscopy clinical examination accompanies a choroidal neovascular membrane (CNVM). The CNVM consists of connective tissue components admixed with cellular components. These cellular components include damaged RPE and photoreceptors, vascular endothelial cells, macrophages, myofibroblasts, and fibroblast-like cells. This CNVM fibrovascular tissue complex may be beneath the RPE, termed type I CNV,

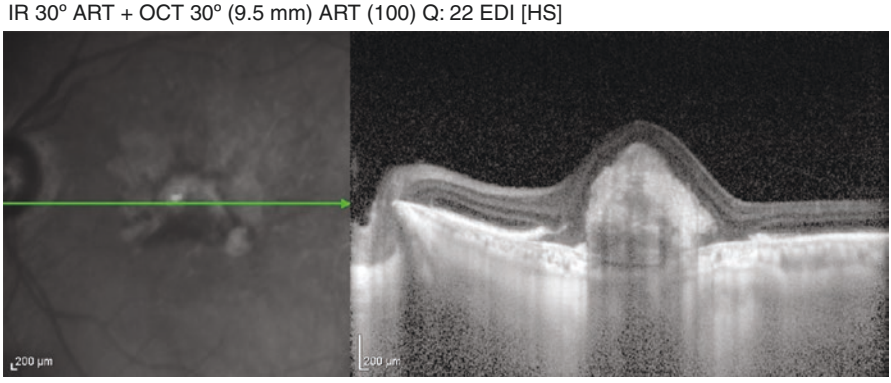


**Fig. 17.4** Optical coherence tomography imaging of the left eye of a patient with exudative AMD demonstrating subretinal hyper-reflective material consistent with subretinal fibrosis. Standard cross sectional analysis view, OCT on Heidelberg Spectralis. (Source: All images are from clinical patients of Dr. Martel)

or between the RPE and the photoreceptors, termed type II CNV. The principal location of the choroidal neovascularization either beneath the RPE or within the subneurosensory retinal space may have implications for disease progression and outcome. CNVM histology has also demonstrated that development of fibrous scar is accompanied by an increase in apoptosis and a decrease in cellularity, suggesting that subretinal fibrosis may evolve along with regression of CNV in exudative AMD. The variable responses to anti-VEGF therapy among patients may be attributable to differences in the CNV morphology, phenotype, and location of CNV growth within the chorioretinal tissue.

The subretinal fibrosis itself is composed of a matrix of collagen (mainly type I and IV with lesser amounts of types III, V, and VI collagen) and fibronectin, with alpha-smooth muscle actin (α-SMA) and cytokeratin. Macrophages can directly produce extracellular matrix including type I collagen, which is responsible for maintaining the integrity of subretinal fibrous tissue and fibronectin. Perhaps one of the critical initial steps of subretinal fibrosis is an epithelial-mesenchymal transition of RPE cells. Alpha-B crystallin is an essential regulator of epithelial-mesenchymal transition, acting as a molecular chaperone for SMAD4 and as its potential therapeutic target for preventing subretinal fibrosis development in exudative AMD. Alpha-B crystallin has also been shown to influence angiogenesis.

Various cytokines and growth factors have been shown capable of triggering the epithelial-mesenchymal transition of the RPE, resulting in the conversion to myofibroblasts, which can further promote fibrotic activities such as cell proliferation, migration, and extracellular matrix remodeling. Matricellular proteins such as thrombospondin 1 (TSP1), tenascin-C, and osteonectin are present in CNV and are thought to regulate fibrosis, binding to growth factor receptors or integrins on the cell surface. Connective tissue growth factor (CTGF) is transcriptionally activated by several factors including transforming growth factor-β (TGF-β). CTGF stimulates fibroblast proliferation, migration, adhesion, and extracellular matrix formation, and its overproduction is thought to play a role in pathways that lead to fibrosis. In the eye, CTGF is correlated with pathologic fibrosis in vitreoretinal disorders; its role in subretinal fibrosis development in exudative AMD is unclear. There remain multiple components and pathways involved in subretinal fibrosis that has yet been fully elucidated. As we better understand the mechanisms of fibrosis in AMD, new therapies and treatments can be explored.

## Clinical Implications in AMD

AMD is the most common cause of severe visual impairment in developed countries. Since the hallmark feature of exudative AMD is choroidal neovascularization, and subfoveal CNV subretinal fibrosis is common, the CNV lesion has been the target of interest to many investigators. Although the previous clinical course of inevitable and often abrupt decline in vision is improving with the advent of anti-VEGF therapy, most patients nevertheless lose vision from exudative AMD albeit at

a much slower pace. Even with treatment, only about 30% of exudative AMD patients retain their original level of vision after 5 years.

Since anti-VEGF therapy only allows short-term suppression of choroidal neovascular activity and the exudative maculopathy, there is the need for chronic administration of intravitreal anti-VEGF pharmacotherapy to slow down the disease progression. Prompt initiation of anti-VEGF therapy may be beneficial since the development of subretinal fibrosis is associated with a longer interval between diagnosis of exudative AMD and treatment with anti-VEGF drugs. However, even with prompt initiation of anti-VEGF therapy, subretinal fibrosis may nonetheless develop or progress. Once subretinal fibrosis and scarring develop, no retinal regenerative treatments for reversing the end-stage maculopathy exist.

Therapeutic strategies aimed at inhibition of subretinal fibrosis are an active area of investigation. Although chronic suppression of the neovascularization seems to reduce the risk of subretinal fibrosis and end-stage maculopathy markedly, there remains a considerable portion of the AMD population that nonetheless experience severe loss of sight. This may be due to anti-VEGF agents serving a more important role in decreasing permeability or leakage of neovascular vessels rather than inducing neovascular regression. The molecular mechanism and effect of VEGF inhibition on pro-fibrotic factors associated with subretinal fibrosis remain unclear. Moreover, there are likely other mediators of CNV formation and exudation.

With emerging therapies on the horizon, early detection of subtle maculopathy in the future will have increasing importance. Advances in ocular imaging with adaptive optics technology and optical coherence tomography imaging with optical coherence tomography angiography may allow better quantification and objective evaluation of subretinal fibrosis and neovascularization. Advancements in in-vivo ocular coherence tomography imaging such as polarization-sensitive optical coherence tomography imaging may offer a unique method of quantifying and following subretinal fibrosis in vivo over time. These retinal imaging technologies may, therefore, allow optimized disease management and evaluation of emerging therapeutic strategies.

In the eye, subretinal fibrosis is a manifestation of aberrant wound repair and is the characteristic end-stage subretinal lesion in exudative AMD. While there have been great strides in the development of anti-VEGF pharmacotherapies, further investigation is necessary to alter the course of this disease. The interplay of various proangiogenic and inflammatory mediators is influential in exudative AMD and the associated subretinal fibrosis.

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