Cellular Mechanisms of Tissue Fibrosis. 6. Purinergic signaling and response in fibroblasts and tissue fibrosis

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Lu D, Insel PA. Cellular Mechanisms of Tissue Fibrosis. 6. Purinergic signaling and response in fibroblasts and tissue fibrosis. Am J Physiol Cell Physiol 306: C779–C788, 2014. First published December 18, 2013; doi:10.1152/ajpcell.00381.2013.—Tissue fibrosis occurs as a result of the dysregulation of extracellular matrix (ECM) synthesis. Tissue fibroblasts, resident cells responsible for the synthesis and turnover of ECM, are regulated via numerous hormonal and mechanical signals. The release of intracellular nucleotides and their resultant autocrine/paracrine signaling have been shown to play key roles in the homeostatic maintenance of tissue remodeling and in fibrotic response post-injury. Extracellular nucleotides signal through P2 nucleotide and P1 adenosine receptors to activate signaling networks that regulate the proliferation and activity of fibroblasts, which, in turn, influence tissue structure and pathologic remodeling. An important component in the signaling and functional responses of fibroblasts to extracellular ATP and adenosine is the expression and activity of ectonucleotidases that attenuate nucleotide-mediated signaling, and thereby integrate P2 receptor- and subsequent adenosine receptor-initiated responses. Results of studies of the mechanisms of cellular nucleotide release and the effects of this autocrine/paracrine signaling axis on fibroblast-to-myofibroblast conversion and the fibrotic phenotype have advanced understanding of tissue remodeling and fibrosis. This review summarizes recent findings related to purinergic signaling in the regulation of fibroblasts and the development of tissue fibrosis in the heart, lungs, liver, and kidney.

fibrosis; purinergic; P2X; P2Y; ATP; adenosine

THE SYNTHESIS AND TURNOVER of the extracellular matrix (ECM) is essential for normal tissue organization and function, both during development and adaptive homeostasis, as well as in the tissue remodeling that occurs after injury. As detailed in other articles in this Theme series and Call for Papers (6, 6a, 53, 88, 95, 142), tissue fibroblasts are the predominant cell type responsible for the generation, maintenance, and degradation of the ECM. Excessive or abnormal signaling by growth factors, such as transforming growth factor (TGF)-β and angiotensin II (ANG II), which stimulate fibroblast proliferation, migration, and profibrogenic activity, typically underlies the development of tissue fibrosis (21, 86, 110). TGF-β and ANG II stimulate the synthesis of collagens and other matrix proteins, increase expression of α-smooth muscle actin (α-SMA), a contractile protein and hallmark of profibrogenic fibroblast activation, and increase the expression of numerous profibrotic markers (Table 1), which include plasminogen activator inhibitor (PAI)-1, connective tissue growth factor (CTGF, also known as CCN2, a member of the Cyr61, CTGF, Nov family of cysteine-rich matricellular secreted proteins), interleukin-6 and monocyte chemotactic protein (MCP)-1 (2, 52, 110). In addition to the well-known profibrotic roles of TGF-β and ANG II, recent work has implicated extracellular nucleotides as important autocrine/paracrine signals that regulate fibroblast homeostasis.

Extracellular Adenosine Triphosphate and Other Nucleotides As Signaling Molecules

Adenosine triphosphate (ATP), the ubiquitous source of energy in cells, achieves typical intracellular concentrations in the low millimolar range (58). In addition, ATP can be released from cells and then impact on cellular signaling and function. Burnstock was the first to show the release of ATP in his studies of nerves in the autonomic nervous system (125), yet 40 years later the precise roles for ATP and other extracellular nucleotides in signal transduction and cell regulation are still not fully understood. It is clear, however, that signaling by extracellular nucleotides plays important roles in virtually all tissues and organ systems (18, 139).

What is generally termed the “purinergic signaling” cascade is initiated by the release of cellular nucleotides from intracellular vesicles or the cytoplasm (139). Studies examining the pericellular space of airway epithelial cells have detected ATP in nanomolar concentrations around resting cells, but physical stimulation can increase pericellular ATP concentrations up to ~1,000-fold (9, 104), concentrations sufficient to activate nucleotide receptors on the cell surface (1, 41, 85).

The release of nucleotides and the downstream signaling that results from the activation of plasma membrane nucleotide receptors on the cells from which nucleotides are released or on nearby cells can produce autocrine or paracrine effects, respectively, that alter cell and tissue homeostasis. Such effects include helping to establish the basal “set points” of signal transduction and functional activities (33, 93, 106). An important response influenced by nucleotide signaling that has garnered substantial recent interest is the regulation of tissue remodeling and, in pathological states, of tissue fibrosis. This review will discuss nucleotide release and the receptors that mediate its signaling, and in particular, the effects of purinergic signaling on fibroblasts and tissue fibrosis.

Mechanisms of ATP Release: Implications For the Regulation of Fibrosis

Mechanisms for cellular nucleotide release have been the subject of a number of comprehensive reviews (19, 33, 84, 85, 89, 128). Here, we briefly summarize the pathways relevant to tissue remodeling and fibrosis.

Tissue injury can trigger damage to the plasma membrane, apoptosis, or necrosis, all of which can lead to the release of
intracellular ATP and other nucleotides (e.g., UTP, ADP, UDP), which can initiate the recruitment of inflammatory cells, such as polymorphonuclear neutrophils and macrophages, as well as platelet aggregation (26, 41, 66). Such findings have provided evidence that ATP released in response to cell injury is a chemotactic signal involved in wound repair and resolution as well as scar formation.

Aside from cell damage-promoted release of nucleotides, gap junction channels have been identified as a major route of cellular ATP release. Connexins (Cxs) are a family of plasma membrane-spanning proteins that form hexameric oligomers (connexons) constituting one-half of a gap junction channel (56, 89, 117). Relatively nonselective transport of signaling molecules of <1 kDa in size can occur through these hemichannels (89), which form a ~1.4–1.8 nM diameter pore through which intracellular molecules can pass into the pericellular and extracellular spaces (80, 120).

Similar to Cxs, the pannexin (Panx) family are membrane-spanning proteins consisting of three isoforms, Panx-1, -2, and -3, which are thought to share functional homology with Cx proteins (107). Panx-1, which is expressed in most tissues, is the best understood of the three isoforms and has been implicated in the formation of hemichannels that can function as major conduits of ATP release (107). While understanding of the gating mechanisms of Cx and Panx hemichannels is incomplete, they include changes in membrane potential, low extracellular Ca\textsuperscript{2+} concentrations as well as mechanical stimulation that can promote hemichannel opening (7, 54, 117, 126).

Together, these various mechanisms of ATP release initiate an autocrine/paracrine signaling cascade that can regulate the fibrotic tone and cellular response of tissue fibroblasts. This profibrotic signaling axis occurs in several organs, including the heart (13, 94, 101), lung (12, 113), kidney (21), and liver (95, 128). These ATP-initiated signaling pathways involve the activation of a diverse family of nucleotide receptors (to be described in the next section). Furthermore, hydrolysis of ATP to adenosine, a reaction catalyzed by cell surface enzymes (33, 139, 148), activates adenosine receptors, adding further complexity to the mechanisms controlling cellular homeostasis (Fig. 1).

### Nucleotide Receptors: Role in Tissue Fibroblasts

There are two types of nucleotide receptors: P2X and P2Y receptors. Expressed in nearly all mammalian tissues, the P2X receptors shown in parentheses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression (↑ or ↓) in Fibrotic Tissues</th>
</tr>
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<tbody>
<tr>
<td>α-Smooth muscle actin (α-SMA)</td>
<td>↑ (63)</td>
</tr>
<tr>
<td>Collagen I</td>
<td>↑ (8, 20)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>↑ (31)</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor (PAI)-1</td>
<td>↑ (52, 111)</td>
</tr>
<tr>
<td>Connective tissue growth factor (CTGF/CCN2)</td>
<td>↑ (70)</td>
</tr>
<tr>
<td>Transforming growth factor (TGF)-β</td>
<td>↑ (17, 86, 115)</td>
</tr>
<tr>
<td>Cysteine-rich angiogenic inducer 61 (CYR61/CCN1)</td>
<td>↑ (70)</td>
</tr>
<tr>
<td>Secreted protein acidic and rich in cysteine (SPARC)</td>
<td>↑ (36, 96)</td>
</tr>
<tr>
<td>Lysyl oxidase (LOX)</td>
<td>↑ (91)</td>
</tr>
<tr>
<td>Periostin</td>
<td>↑ (87)</td>
</tr>
<tr>
<td>Monocyte chemotactic protein (MCP)-1</td>
<td>↑ (35)</td>
</tr>
<tr>
<td>Interleukin (IL)-6</td>
<td>↑ (46)</td>
</tr>
<tr>
<td>IL-33</td>
<td>↑ (73, 147)</td>
</tr>
<tr>
<td>sST-2</td>
<td>↑ (73, 147)</td>
</tr>
<tr>
<td>Thrombospondin-1</td>
<td>↑ (137)</td>
</tr>
<tr>
<td>Relaxin</td>
<td>↑ (118, 143)</td>
</tr>
<tr>
<td>Epac1</td>
<td>↓ (140)</td>
</tr>
<tr>
<td>Snail</td>
<td>↑ (133)</td>
</tr>
<tr>
<td>Slug</td>
<td>↑ (133)</td>
</tr>
</tbody>
</table>

References shown in parentheses.

![Fig. 1. Nucleotide and adenosine receptor-mediated signaling pathways activated in response to cellular nucleotide (e.g., ATP) release.](Fig1.png)
receptors are ATP-gated ion channels of which there are seven subtypes, P2X<sub>1-7</sub> (16, 82, 102). P2X receptor subunits are composed of two transmembrane domains, a single extracellular loop and intracellular amino- and carboxy-termini. Association of the subunits to form a trimer generates a functional P2X receptor (77). The affinity of P2X receptors for ATP and ATP-derived analogs is in the low micromolar range; other nucleotides or adenosine bind weakly or not at all (71, 78). ATP binding to P2X receptors opens their nonselective channel, allowing the passage of mono- and divalent cations. As a consequence, membrane potential changes and intracellular Ca<sup>2+</sup> concentrations increase, leading to the stimulation of Ca<sup>2+</sup>-dependent intracellular signaling pathways (71, 78).

The P2Y receptor subfamily consists of eight 7-transmembrane/G protein-coupled receptors (GPCRs) that respond to a variety of extracellular nucleotides and have distinct patterns of coupling to different heterotrimeric G proteins. Unlike P2X receptors that show exquisite selectivity for ATP as their physiologic agonist, various P2Y receptors respond to both ATP and other nucleotides (e.g., P2Y<sub>2</sub> to UTP) or preferentially to nucleotides other than ATP (Table 2). The G<sub>i</sub>-coupled P2Y receptors, P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub>, signal via the phospholipase C pathway and its products diacylglycerol and inositol triphosphate, which activate protein kinase C and increase intracellular Ca<sup>2+</sup>, respectively (129). P2Y<sub>11</sub> is the only P2Y receptor that couples to Gi, and activates adenylyl cyclase (AC), whereas the remaining P2Y receptors couple to Gi and inhibit AC activity (129). The role of β-arrestins in the signaling and responses of P2Y receptors is not well defined (64, 98, 119).

The P2 receptor subtype(s) that mediate fibrotic responses vary among tissues and cell types. Owing to tissue-specific receptor expression, subtype-specific ligand binding and, possibly, restricted membrane localization (72, 108, 127), the net effect of P2 receptor activation is likely the integration of numerous, parallel signaling pathways.

In the following sections, we describe the contribution of purinergic signaling in the regulation of tissue fibroblasts and the development of fibrosis in several major organ systems (heart, lungs, liver, and kidney).

Heart

In the myocardium, the ECM and collagen lattice, composed primarily of collagen types I and III, are essential for maintaining normal cardiac electrical conductance, myocyte contraction, and myocardial integrity (8, 43). Cardiac fibrosis leads to decreased myocardial compliance, arrhythmias, diastolic dysfunction, and accompanying heart failure (34).

ATP, ADP, UTP, and UDP can be released from endothelial cells, cardiomyocytes, and fibroblasts in the heart following myocardial infarction (83, 132), vascular shear forces (83), hypoxia (30, 37, 50), and pressure overload (101). The released nucleotides act extracellularly to regulate cardiomyocyte hypertrophic responses and to promote fibroblast activity.

Cx and Panx hemichannels play a major role in nucleotide release in the heart. The release of ATP and UDP by mouse cardiomyocytes subjected to mechanical stretch occurs via Panx-1 hemichannels (101). Such release can stimulate the transformation of fibroblasts to activated (profibrogenic) myofibroblasts via paracrine ATP signaling (37, 101). We have shown that ATP is released from adult rat cardiac fibroblasts by hypotonic challenge (which likely produces mechanical stretching of the plasma membrane), a response that depends on Cx-43 and Cx-45 hemichannels (93, 94).

The fate of released ATP includes its binding and autocrine/paracrine activation of P2Y receptors on cardiac fibroblasts (65, 99, 100). P2Y<sub>2</sub> receptors are highly expressed in adult rat ventricular cardiac fibroblasts (121), and activation of these receptors by ATP and UTP is strongly proinflammatory, in terms of increasing synthesis of ECM proteins and of many of the genes and proteins associated with a profibrogenic phenotype (Table 1) (14, 37, 93, 94). P2Y<sub>2</sub> receptors have an important role in the homeostasis of cardiac fibroblasts: constitutive P2Y<sub>2</sub> signaling contributes to the establishment of the “set point” of fibrotic activity in cardiac fibroblasts (33, 93). In addition to P2Y<sub>2</sub> receptors, ATP activates P2X<sub>4</sub> and P2X<sub>7</sub> receptors on cardiac fibroblasts to stimulate cell proliferation in an Akt and ERK1/2-dependent manner (25).

Profibrotic effects from UDP-mediated activation of P2Y<sub>6</sub> receptors have been described by Nishida et al. (97). Activation of P2Y<sub>6</sub> receptors by the autocrine/paracrine release of UDP initiates a fibrotic response that involves the upregulation of CTGF, TGF-β, periostin, and other profibrogenic factors. Treatment with apyrase, which hydrolyzes extracellular nucleotides, strongly reduces basal fibrogenic activity of cardiac fibroblasts (94). Moreover, the enhanced release of cellular nucleotides in pathologic conditions, such as pressure or volume overload, ischemia, hypoxia, or myocardial infarction, may help trigger fibrotic response in the myocardium. Thus, acute increases in interstitial nucleotide concentration following cardiac injury may contribute to remodeling and wound healing responses following myocardial infarction, as well as being a possible mechanism, which when dysregulated, leads to cardiac fibrosis (i.e., excessive scarring and stiffness) (Fig. 2).

Lung

Several different types of fibrosis occur in the lungs. These include fibrotic changes in the interstitium that is located between alveolae, airways, and vascular components, as well as fibrotic changes that can occur in the airways and in pulmonary vessels. Inflammatory events can contribute to fibrotic lung disease, but the role of inflammation is controversial (136). The greatest extent of fibrosis in humans occurs in idiopathic pulmonary fibrosis (IPF), a disease of unknown etiology and for which there is a lack of effective therapies (6a,

Table 2. P2Y receptors, their preferred ligands, and G protein coupling

<table>
<thead>
<tr>
<th>P2Y Receptor</th>
<th>Preferred Ligand (EC&lt;sub&gt;50&lt;/sub&gt;, μM)</th>
<th>Coupling</th>
</tr>
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<tbody>
<tr>
<td>P2Y&lt;sub&gt;1&lt;/sub&gt;</td>
<td>ADP (1) &gt; ATP (4)</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>UTP (0.14) &gt; ATP (0.23)</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;4&lt;/sub&gt;</td>
<td>UTP (2.5) [directive: UTP (2.6) &gt; ATP (1.8)]</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;6&lt;/sub&gt;</td>
<td>UDP (0.300) &gt; UTP (6) &gt; ADP (30)</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;11&lt;/sub&gt;</td>
<td>ATP (17.4)</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;/G&lt;sub&gt;q&lt;/sub&gt;</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;12&lt;/sub&gt;</td>
<td>ADP (60.7)</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;13&lt;/sub&gt;</td>
<td>ADP (60) &gt; ATP (261)</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;14&lt;/sub&gt;</td>
<td>UDP-glucose (80) &gt; UDP-galactose (124)</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Numbers in parentheses are EC<sub>50</sub> values (in μM). Data from Refs. 1 and 3.
Bronchoalveolar lavage (BAL) fluid of patients with IPF contains a higher concentration of ATP than does BAL fluid from control subjects; similarly, BAL fluid from mice whose airways are treated with bleomycin (an agent used to induce a model of pulmonary fibrosis) has a higher ATP concentration than does BAL fluid from control animals (113). Treatment with ATP enhances and with apyrase reduces inflammatory cell recruitment of bleomycin-treated mice. Lung inflammation, expression of fibrotic markers, and collagen content are lower in P2X7 receptor-deficient than in wild-type (WT) mice treated with bleomycin, thus implicating ATP release from bleomycin-injured lung cells as a “danger signal” that acts via P2X7 receptors to promote lung fibrosis (113).

Other data suggest that PKC may contribute to the action of calcium and P2X7 receptors in airway epithelial cells and promote fibrosis in the bleomycin model (12). Inflammation in the lung can also induce release of ATP from airway epithelial cells; this release appears to occur by calcium-dependent vesicular mechanisms but not via Panx-1 (105).

Information regarding purinergic receptors in the lungs has been recently reviewed (19). Direct evidence for the presence of purinergic receptors in lung fibroblasts has been most clearly shown in studies of fibroblasts grown in explant cultures of excised lung parenchyma from WT mice and mice that have a knockout of P2Y2 receptors (65): In fibroblasts of WT mice, nucleotides (with a rank-order of potency: UTP = ATP > ADP > UDP) increase intracellular Ca\(^{2+}\) concentration and inositol phosphate generation from phosphoinositides, responses that are absent in fibroblasts of P2Y2-knockout mice. P2Y2 thus appears to be the only functionally expressed nucleotide receptor in mouse lung fibroblasts. In human pulmonary fibroblasts, activation of P2Y receptors (by ATP, ADP, or UTP) increases calcium waves through ryanodine-insensitive channels and can enhance the expression of P2Y4 receptors, TGF-β, collagen A1, and fibronectin (68).

Studies with a different type of lung fibroblast, i.e., adventitial fibroblasts from the bovine lung pulmonary artery, have shown that acute hypoxia (3% O\(_2\)) induces a ~2-fold increase in the release of ATP within 10 min, that persistent increases in ATP release last at least 24 h, and that chronic hypoxia (for 14 to 30 days) attenuates extracellular ATP hydrolysis by ectonucleotidase(s) (50, 123). In addition, those investigators found that ATP, UTP, ADP-βS, and MeSATP increase [\(^{3}\)H]thymidine incorporation in fibroblasts, a response that is additively increased by hypoxia. Hypoxia promotes the conversion of adventitial fibroblasts to myofibroblasts. In addition, ATP...
and/or hypoxia synergistically increases mitogen-induced DNA synthesis but P2 receptor antagonists [suramin, cibacron blue 3G-A, and pyridoxal phosphate-6-azophenyl-2'-4'-disulfonic acid (PPADS)] and apyrase (the nucleotide hydrolytic enzyme) attenuate ATP and hypoxia-induced DNA synthesis. Such findings suggest that P2 receptor activation by hypoxia may result from the release of endogenous ATP. ATP and hypoxia induce the expression and activation of the Egr-1 transcription factor and stimulate proliferation of adventitial fibroblasts via a mechanism that appears to involve multiple signaling components, including Goxi, PI3K, Akt, mTOR, p70 S6kinase, and ERK1/2 (51). Taken together [and as recently reviewed (124)], such data imply that hypoxia in the lung enhances the growth of adventitial fibroblasts and their transformation to myofibroblasts through the release of ATP, autocrine/paracrine activation of P2 receptors, and altered ATP hydrolysis. Such effects may contribute to the pathophysiology of pulmonary arterial hypertension.

A key question with respect to ATP and lung fibrosis relates to the precise identity of the nucleotide or metabolic product(s) that promote fibrosis. Substantial work has 1) implicated the ATP hydrolytic product adenosine as being profibrotic for lung fibroblasts and in promoting lung fibrosis in experimental animal models and in patients with IPF or with chronic obstructive pulmonary disease (COPD), and 2) suggested that increased expression of enzymes involved in the generation of adenosine generation from ATP and increased expression of A2B receptors, which may contribute to profibrotic effects of adenosine in the lung, occur in such patients (28, 74, 144–146).

Kidney

Renal fibrosis can lead to deteriorating organ function and eventually to renal failure. While TGF-β and ANG II are major regulators of ECM deposition and profibrotic remodeling in the kidney, there is much evidence that links nucleotide and adenosine signaling to the progression of renal fibrosis.

In the kidney, ATP release can be induced by hypotonic stimulation of tubular epithelial cells (134), sympathetic and adrenergic stimulation of the renal cortex (130), and activation of endothelial and smooth muscle cells (122). Cells in the nephron and renal vasculature express P2Y and P2X receptors that mediate numerous physiological actions, including glomerular pressure and renal vascular tone (11, 109, 112, 122).

Mesangial cells and renal fibroblasts, which are key contributors to the formation of ECM in the kidney, produce collagen, fibronectin, and other matrix proteins in response to TGF-β and ANG II signaling (116, 122). Additionally, activation of P2Y2 and P2Y4 receptors on mesangial cells stimulates their proliferation (60), and P2X7 activation increases mesangial cell collagen synthesis (122) and interstitial fibrosis in vivo (55). Solini et al. (122) observed different effects of P2Y and P2X7 receptor activation and reported that UTP-mediated stimulation of P2Y2 receptors inhibits collagen synthesis. This pathway counterbalances P2X7 activation and highlights the contrasting effects that occur with different nucleotides and the responses they promote through activation of various purinergic receptors.

In cardiac fibroblasts, a pronounced upregulation of PAI-1 occurs in response to stimulation of P2Y5 receptors by either ATP or UTP (14, 94). This is notable because PAI-1 upregulation accompanies renal fibrosis and inhibits plasmin activation, thereby blocking the subsequent activation of proteases that degrade ECM (111). PAI-1-knockout mice are resistant to the fibrosis that results from ureteral obstruction, as manifested by less synthesis of collagen, TGF-β, and α-SMA than occurs in control mice (103).

In addition to the action of resident renal fibroblasts and mesangial cells in contributing to renal fibrosis, renal tubular epithelial cells can undergo epithelial-to-mesenchymal transition (EMT) in response to injury, inflammation, and TGF-β signaling (21, 141). The contribution of EMT to renal fibrosis is controversial but has been readily demonstrated in cell culture (81). Cells that have undergone EMT have increased expression of matrix proteins, disrupted epithelial layers, and can be a precursor to pathologic scar formation (21, 67, 141). Limited data have suggested that extracellular nucleotides may promote EMT in renal epithelial cells (135). Adenosine A2A receptor activation has been found to inhibit renal inflammation and fibrosis by attenuating collagen deposition (48) and may also inhibit profibrotic EMT of renal tubular epithelial cells (138). Using the model of unilateral ureteral obstruction to induce renal fibrosis, Xiao et al. (138) have reported that A2A activation suppresses an upregulated expression of α-SMA while restoring expression of the epithelial marker E-cadherin.

Liver

Numerous cell types in the liver express purinergic receptors, which can regulate functions that include glycogen metabolism, bile secretion, vascular tone, and hepatic remodeling (128). ATP can be released by hepatocytes via vesicular exocytosis in response to mechanical forces and osmotic stress, resulting in more than a 30-fold increase in extracellular ATP concentrations (44, 49). Furthermore, the release of ATP and other nucleotides by vascular endothelial cells and biliary epithelial cells can act as a paracrine signal that can activate profibrogenic cells (10).

Hepaticstellate cells (HSCs) in the perisinusoidal space and portal fibroblasts are the major cell types responsible for ECM synthesis and turnover in the liver (15, 40, 95). The contribution of the P2Y family of nucleotide receptors to liver fibrosis is better understood than that of P2X receptors, about which little is known. P2Y2, P2Y4, and P2Y6 receptors are expressed by rat HSCs, which respond to nucleotides, including UDP, by transforming into myofibroblasts that have greater expression of collagen and α-SMA than do resting HSCs (40). Blockade of P2 receptors with PPADS inhibits HSC proliferation and blunts ECM synthesis in a rodent model of CCl4-induced liver fibrosis (38). Furthermore, P2Y2 activation stimulates the recruitment of neutrophils into the liver and causes hepatocyte death (5). In keeping with these data, P2Y2-knockout mice are protected from liver damage and necrosis in response to experimentally (concanavalin A)-induced hepatitis or acetaminophen-promoted liver injury (5).

The importance of nucleotide signaling in liver fibrosis has been demonstrated by experiments that have examined the effects of increasing extracellular nucleotide concentrations. Inhibiting the NTPases responsible for the hydrolysis and termination of nucleotide-mediated signaling exacerbates pathological remodeling and fibrosis (69), illustrating the profi-
brotic consequences of sustained nucleotide signaling (to be described further in the next section).

The hydrolysis of ATP by membrane-bound enzymes is a major source of extracellular adenosine generation and has functional significance in the liver (114). Adenosine A2A receptor activation activates HSCs and stimulates collagen production, promoting hepatic fibrosis (23), while antagonism of those receptors is protective against CCl4 and ethanol-induced liver fibrosis (27). In both primary HSCs and LX-2 cells (a cell line), A2A receptor activation upregulates the synthesis of collagens I and III in a PKA/ERK- and p38 MAPK-dependent manner, respectively (24).

Current evidence suggests that profibrotic nucleotide signaling in the liver involves the activation of both P2 purinergic receptors and P1 adenosine receptors (10, 39). The generation of extracellular adenosine and the subsequent activation of P1 receptors is dependent on the activity of extracellular NTPases, which integrate ATP/ADP and adenosine signaling and their functional consequences (114).

### Hydrolysis of Extracellular Nucleotides: Role of NTPases

The lifetime of extracellular nucleotides, hydrolysis of which occurs by endogenously expressed nucleotidases, is another essential factor in the nucleotide signaling cascade. Such nucleotidases not only hydrolyze nucleotides and terminate ATP/UTP-mediated signaling but also can initiate ADP/UDP and adenosine-mediated signaling pathways (139). Ectonucleoside triphosphate diphosphohydrolases (ENTPDs) are endogenous Ca2+/Mg2+-dependent nucleotidases that hydrolyze tri- and diphosphate nucleotides into their monophosphate forms. Of the eight ENTPD isozymes (ENTPD1–8), four (ENTPD1–3, 8) are cell surface-localized (148). ENTPD-1 and -2 (CD39 and CD39L1, respectively) are the most studied subtypes; ENTPDs have roles in regulating inflammation, platelet activity, and vascular tone (4, 75, 76). Although ENTPDs do not hydrolyze nucleotide monophosphates, the sequential action of membrane-localized 5’-nucleotidases, such as CD73, on AMP, a product of ENTPD action, generates adenosine, which is an agonist for several GPCRs (A1–4 receptors) (22, 29, 32, 148). Increasing evidence indicates that ENTPD activity is an important aspect of P2 signaling in tissue fibrosis that is able to attenuate nucleotide-dependent responses and help initiate adenosine-mediated remodeling pathways (42, 90, 114).

ENTPD activity has been implicated in contributing to cardiac protection postischemia, likely by facilitating the generation of adenosine, which is cardioprotective (79). Köhler et al. (79) demonstrated that ENTPD1-deficient mice or mice treated with an ENTPD inhibitor were more susceptible to ischemic injury, largely as a consequence of impaired generation of adenosine. Conversely, hydrolysis of extracellular ATP with the nucleotidase apyrase increased adenosine generation and reduced infarct size after ischemic insult (79). We recently described the profibrotic effects of inhibiting ENTPD activity in cardiac fibroblasts (93). ENTPD inhibition increased cardiac fibroblast collagen synthesis and α-SMA expression by enhancing nucleotide (P2Y2 receptor) signaling while simultaneously diminishing counterbalancing anti-fibrotic adenosine-mediated (in particular, A2B receptor) signaling pathways (Fig. 2).

Similar mechanisms may regulate ischemic protection in the kidney. ENTPD1 inhibition decreases adenosine generation by ATP released during renal ischemia, thereby exacerbating ischemic injury (57). Thus, in both the heart and kidney, profibrotic nucleotide signaling and anti-fibrotic adenosine signaling (which, as noted above, may also blunt EMT of renal epithelial cells) represent dual points of control in the regulation of organ remodeling. These recent findings suggest that ENTPDs play a key role in controlling the extent of fibrosis via their ability to degrade nucleotides and ultimately, lead to the generation of adenosine.

Contrary to what is observed in the heart, adenosine signaling is profibrotic in the lung: adenosine initiates lung inflammation and promotes fibroblast-to-myofibroblast conversion (19). Adenosine and adenosine receptor levels are elevated in patients with asthma and COPD and contribute to pulmonary fibrosis (28). Mice that lack adenosine deaminase, the enzyme that mediates adenosine clearance, have pulmonary inflammation, fibrosis, myofibroblast proliferation, and alveolar damage as a result of chronically elevated adenosine signaling (28). Unlike in the heart and kidney (but more akin to what occurs in the liver), in the lung, activation of both ATP and adenosine receptors appears to contribute to myofibroblast conversion and the development of fibrosis (28, 113).

Although less is known about the roles of ENTPDs in the liver, ENTPD2 activity in liver portal fibroblasts attenuates bile duct epithelial cell proliferation (69). Loss of portal fibroblast ENTPD2 expression increases epithelial cell proliferation, implying the existence of a paracrine mechanism that involves nucleotide signaling and hydrolysis in the regulation of epithelial cell homeostasis (69). ENTPDs may also be involved in HSC and portal fibroblast proliferation and activation, potentially leading to the development of liver fibrosis by perpetuating purinergic signaling pathways (114).

Thus, results from studies in several tissues implicate ENTPD activity as likely an essential regulatory element in the progression and regulation of fibroblast homeostasis, myofibroblast conversion, and tissue remodeling.

### Perspectives and Future Directions

Substantial recent progress illustrates the ubiquitous role of extracellular ATP and other nucleotides in regulating a diverse range of responses, which include inflammation (113), neutrophil migration (26), cellular homeostasis (33, 93), and organ remodeling. In most cases the precise receptors and pathways mediating such responses are not fully defined, but it is clear that cellular nucleotide release and subsequent autocrine/paracrine signaling are essential mediators of tissue homeostasis and organ function.

In addition to the systems described here, other data indicate the involvement of signaling by nucleotides and adenosine in the development of dermal fibrosis (45), pancreatic fibrosis (61, 131), and in the proliferation of cancer-associated fibroblasts (59, 62, 97). Thus, the mechanisms underlying organ remodeling may also prove applicable to tumor fibroblast physiology, leading to the production and maintenance of the stroma that surrounds malignant cells. Targeting these fibroblasts may thus provide a novel therapeutic strategy in cancer treatment.
Together, these discoveries highlight the importance of understanding the signaling pathways invoked by extracellular nucleotides and adenosine in the regulation of fibroblasts and tissue fibrosis. It is evident that extracellular nucleotides and adenosine are not simply “innocent bystander” signaling molecules, but instead are integral autocrine/paracrine messengers that have essential roles in tissue homeostasis, normal and pathological organ remodeling, and response to injury. Much is still unknown about this signaling axis, which for each tissue encompasses multiple stimuli and mechanisms of cellular nucleotide release, different subsets of receptors, NTPases, and downstream signaling cascades that govern cellular responses. We believe that efforts to elucidate these mechanisms will greatly expand understanding of organ homeostasis, tissue remodeling, and fibrosis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
D.L. and P.A.I. prepared figures; D.L. and P.A.I. drafted manuscript; D.L. and P.A.I. approved final version of manuscript.

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