COMPREHENSIVE INVITED REVIEWS

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Hyaluronan, Transforming Growth Factor β, and Extra Domain A-Fibronectin: A Fibrotic Triad

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Significance: Inflammation is a critical aspect of injury repair. Nonresolving inflammation, however, is perpetuated by the local generation of extracellular matrix-derived damage-associated molecular pattern molecules (DAMPs), such as the extra domain A (EDA) isoform of fibronectin and hyaluronic acid (HA) that promote the eventual acquisition of a fibrotic response. DAMPs contribute to the inflammatory environment by engaging Toll-like, integrin, and CD44 receptors while stimulating transforming growth factor (TGF)-β signaling to activate a fibroinflammatory genomic program leading to the development of chronic disease.
Recent Advances: Signaling through TLR4, CD44, and the TGF-β pathways impact the amplitude and duration of the innate immune response to endogenous DAMPs synthesized in the context of tissue injury. New evidence indicates that crosstalk among these three networks regulates phase transitions as well as the repertoire of expressed genes in the wound healing program determining, thereby, repair outcomes. Clarifying the molecular mechanisms underlying pathway integration is necessary for the development of novel therapeutics to address the spectrum of fibroproliferative diseases that result from maladaptive tissue repair.
Critical Issues: There is an increasing appreciation for the role of DAMPs as causative factors in human fibroinflammatory disease regardless of organ site. Defining the involved intermediates essential for the development of targeted therapies is a daunting effort, however, since various classes of DAMPs activate different direct and indirect signaling pathways. Cooperation between two matrix-derived DAMPs, HA, and the EDA isoform of fibronectin, is discussed in this review as is their synergy with the TGF-β network. This information may identify nodes of signal intersection amenable to therapeutic intervention.
Future Directions: Clarifying mechanisms underlying the DAMP/growth factor signaling nexus may provide opportunities to engineer the fibroinflammatory response to injury and, thereby, wound healing outcomes. The identification of shared and unique DAMP/growth factor-activated pathways is critical to the design of optimized tissue repair therapies while preserving the host response to bacterial pathogens.

Keywords: hyaluronic acid, TGF-β, ED-fibronectin, TLR4, inflammation, fibrosis

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SCOPE AND SIGNIFICANCE

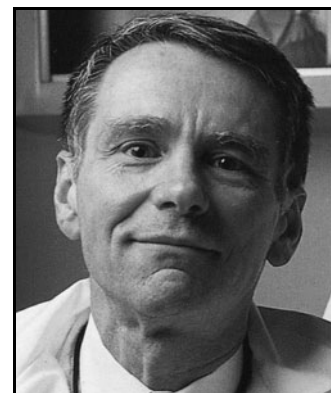Wound healing occurs in a continuum of overlapping phases (*i.e.*, coagulation, inflammation, proliferation, and resolution) in which multiple cell types recognize and orchestrate the restoration of damaged tissue.[1,2] The synthesis, deposition, and long-term reorganization of the trauma site stromal matrix, largely by cohorts of

injury-activated epithelial cells, resident fibroblasts, and recruited vascular pericytes, provides the structural support for cell proliferation and migration within the wound field and determines repair outcomes.[3,4] The remodeled extracellular matrix (ECM) also sequesters growth factors and cytokines that direct the genomic repair program while generating mechanical cues that control cell function, wound contraction, and eventual tissue restructuring.[5,6]

Failure to properly coordinate the repertoire of responses between cells and their ECM has pathological consequences ranging from chronic inflammation and deficient healing to exuberant repair, excessive scarring, and fibrosis.[7–9] The recent adaptation of systems or network approaches provides a window into the complexity of the inflammatory and scar-forming stages following tissue injury while highlighting the underlying pathways, molecular mechanisms, and potential therapeutic targets.[10] Such studies organize wound-impacted genes/proteins into functional categories or nodes providing opportunities to dissect the dynamics of nodal composition and interconnectivity to the wound repair program.

Factors released from dying or damaged cells at the site of injury (*e.g.*, DNA, histones, high mobility group protein B1, heat shock proteins, ATP, interleukin-1 alpha) promote an innate immune response by functioning as Category IA damage-associated molecular pattern (DAMPs) molecules or Alarmins.[11,12] An additional subclass (IIA) of host-derived DAMPs include fragments of ECM molecules as well as the transforming growth factor (TGF)-β1-induced matrix molecules biglycan, decorin, versican, tenascin C, hyaluronic acid (HA), and the extra domain A (EDA) isoform of fibronectin (FnEDA).[13–17] Many DAMPs are endogenous toll-like receptor (TLR) agonists, or signal through other receptors that stimulate the rapid synthesis and release of proinflammatory cytokines and chemokines which, in turn, promote trafficking of immune cells (*e.g.*, neutrophils, macrophages) to the injured tissue to initiate and sustain the process of sterile inflammation.[13,18–21]

While recruitment of immunocompetent cells to the wound site (in response to platelet/mast cell degranulation and locally generated DAMPs) is required for proper healing, nonresolving or chronic inflammation results in the eventual development of fibrotic disease.[22–26] TLR4 is, perhaps, among the most promiscuous of the ECM DAMP-binding TLRs with regard to the diversity of ECM ligands recognized that contribute to the establishment and persistence of the inflammatory reaction to tissue injury[11] and a critical contributor to the repair process. DAMP-type TLR4 ligands control the inflammatory and subsequent fibrotic responses in sterile cutaneous or ischemic wounds.[27] TLR4 expression is elevated in the wound edge epithelial cohort in a mouse model of skin injury[28] and is likely key to the repair process since excisional wound closure is delayed in mutant TLR4 mice.[29] Recent data support the requirement for the TLR4-p38/JNK pathway in the regulation of inflammation and wound resolution as the presence of a nonfunctional receptor, or interference with TLR4 signaling, blunted both processes.[28]

HA and FnEDA, are prominent among the ECM DAMPs that signal through TLR4 and impact healing in their dual capacity as upregulated targets and modulators of the TGF-β response.[30,31] This review focuses on the complex interactions among the DAMPs HA/FnEDA, their receptor systems, the innate immune response, and the TGF-β-signaling pathway in normal and pathological wound healing. Data suggest a model whereby the TGF-β pathway cooperates with the DAMP/TLR4 network to promote a maladaptive profibrotic response to tissue trauma.

TRANSLATIONAL RELEVANCE

DAMP- and growth factor-activated signaling networks intersect during tissue injury to impact the expression of a proinflammatory/profibrotic genomic program and, thereby, healing outcomes. Identification of the interacting elements, the pathways involved, and the nodes of intersection provide a roadmap of potential therapeutic targets for the treatment of fibroinflammatory disorders.

CLINICAL RELEVANCE

Persistent or nonresolving inflammation due to tissue injury triggers eventual development of tissue fibrosis and organ dysfunction. There are limited treatment options for patients with fibrotic disease, which is often progressive due to the establishment of feed-forward loops. A major clinical challenge, therefore, is the design of specific therapies to attenuate the pathophysiological consequences of DAMP/growth factor collaboration while retaining the protective host response to microbial pathogens.

DISCUSSION
HA and FnEDA: transitioning from the ECM to a DAMP

HA and HA receptors–linkages to TLR4 signaling. The role of the ECM in injury resolution is

complex with distinct roles in tissue fibrosis and regeneration. Cutaneous burns in the adult, for example, heal as a scar, which is generally devoid of hair follicles and sweat glands. Fetal wounds, in contrast, undergo a repair process in which the skin architecture is regenerated with no scarring. Fetal wounds are rich in high-molecular-weight (HMW) HA, which appears to promote regenerative healing and decrease fibrosis by diminishing the inflammatory response,[32] likely as a consequence of decreased levels of the proinflammatory cytokines, IL-8 and IL-6, and increases in the anti-inflammatory IL-10.[33,34]

In adults, cutaneous injuries heal along a continuum spanning normal healing to progression along a pathological, fibrotic pathway resulting in the development of hypertrophic scars and keloids. Hypertrophic scars are raised and stiff due to increased numbers of myofibroblast cells and changes in the deposition and organization of collagen. If cellular proliferation and inflammation persist, keloids are formed which extend beyond the original wound margins resulting in disfigurement and, in extreme cases, can lead to loss of function.[33] The mechanisms regulating the pathway of tissue repair along a regenerative or scarring pathway are not well understood, but the ECM and, in particular, HA plays an important role in both tissue regeneration and pathological scarring.

HA is a nonsulfated, straight-chain glycosaminoglycan (GAG), consisting of a repeating disaccharide of glucuronic acid and N-acetyl glucosamine, and the only GAG not attached to protein.[35,36] Following tissue injury and in response to wound-induced factors (*e.g.*, TGF-β), HA is synthesized by fibroblasts, where it provides a scaffold to support cell proliferation and migration as well as promote innate immune responses. This high-negatively charged hydrophilic GAG maintains dermal hydration by regulating water balance and osmotic pressure while acting as a sieve to exclude macromolecules and prevent scarring.[37,38]

HA is synthesized on the inner leaflet of the plasma membrane by HA synthases (HAS) and transported into the extracellular compartment (Fig. 1). In the cutaneous matrix, HA has a relatively short half-life due to its rapid catabolism by the cell surface hyaluronidases HYAL1 and 2.[39,40] HYAL2 is a lipid raft glycosylphosphatidylinositol-linked enzyme that degrades HA into small oligosaccharides for endocytosis by the raft-associated HA receptor CD44 and subsequent lysosomal degradation by HYAL1.[41,42] HA processing and the regulation of HYAL activity are critical to HA function which, in turn, is dependent on HA size.[43]

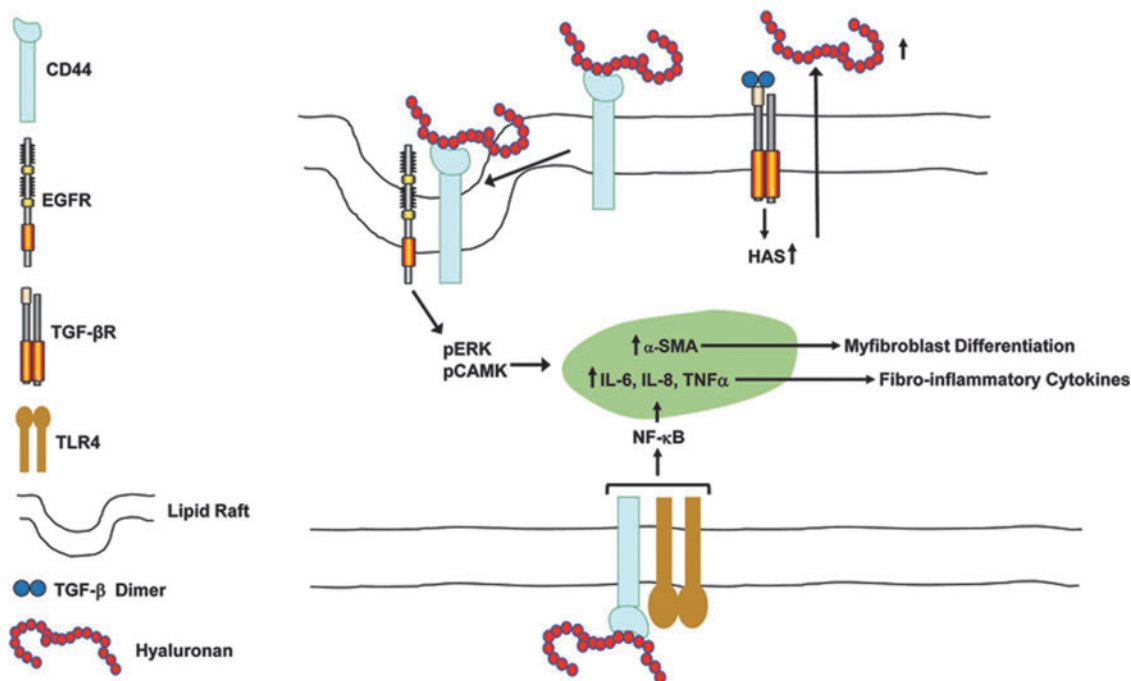The biological roles of HA (*e.g.*, regulation of ECM biophysical properties, mechanical signaling, tissue

Figure 1. Coordinate regulation of fibroinflammation by TGF-β and hyaluronan. TGF-β induces the expression of HAS resulting in the increased synthesis and release of hyaluronan. Binding of hyaluronan to CD44 results in the formation of a lipid raft-localized CD44/EGFR complex leading to the activation of ERK and CAM kinases, which regulate expression of α-SMA and myofibroblast differentiation. Hyaluronan occupancy of CD44 also promotes the TLR4-dependent induction of NF-κB-dependent proinflammatory cytokines, including IL-6, IL-8, and TNFα. α-SMA, α-smooth muscle cell actin; EGFR, epidermal growth factor receptor; HAS, hyaluronic acid synthase; TGF-β, transforming growth factor beta.

inflammation) are dictated by molecular weight and differential interaction with several cell surface-binding proteins, the hyaladherins, including CD44 and the receptor for hyaluronan-mediated motility (RHAMM).[15,36,44,45] CD44 is the best characterized of the HA receptors and essential for cutaneous wound repair where it regulates keratinocyte adhesion, motility, proliferation, differentiation, and survival likely through an association with the actin cytoskeleton and downstream adaptor molecules.[46,47]

Binding of HA to CD44 activates several major signaling effectors, including MAP and CAM kinases, AKT, NF-κB, Rho GTPases, and Src[47–49] (Fig. 1), as well as inducing expression of several pathophysiologically important microRNAs, including miR-21.[40,50] The specific pathway engaged is linked to CD44 function as a coreceptor for TLR2, TLR4, EGFR, or c-met.[15,20,44,45,51] Outcomes are dictated, however, by the nature of the formed ligand/receptor complex. HMW HA (>1,000 kDa) occupancy of CD44 downregulates inflammation and angiogenesis while promoting homeostasis, consistent with findings implicating an antiscarring role for HMW HA likely through an IL-10/HA synthase I axis.[32,52]

While the mechanism is unclear, HMW HA/CD44 interactions increase trafficking of TGF-β receptors to lipid rafts increasing, thereby, receptor turnover and antagonizing TGF-β1-dependent profibrotic SMAD signaling.[53] The interaction of low-molecular-weight (LMW) HA (<5 kDa) with CD44, in contrast, did not exhibit similar antagonism and usually activates a TLR4-induced proinflammatory and proangiogenic program although CD44 also mediates the endocytic clearance of LMW HA, thereby dampening the immune response.[54,55] Failure to remove LMW HA from the wound microenvironment (generated by hyaluronidase-mediated fragmentation of HMW HA) leads to persistent TLR-dependent NF-κB activation and the continued release of inflammatory mediators such as IL-6, IL-1β, and TNFα,[56,57] while CD44 signaling dictated by HA size differentially regulates keratinocyte biological activities.[47] Collectively, these findings suggest that HA-directed therapeutic modalities may have clinical applicability for the treatment of epidermal dysfunction and anomalies of cutaneous wound repair.

While HA/CD44/TLR4 inflammatory responses are regulated by NF-κB, the molecular events underlying NF-κB activation by HA are incompletely understood but may depend on both HA size and recruitment of the TLR4 coreceptor MD2 and the TLR4 adaptor myeloid differentiation factor (MyD88) and their downstream signaling intermediates to CD44/TLR complexes.[51,58] The effects, however, appear cell type as well as context dependent. The downstream consequences of HA/CD44 binding is a function of HA mass, or extent of degradation, which impacts the type of receptor engaged and/or clustered and, thereby, the associated signaling pathways.[59] In general, HMW HA preferentially binds CD44, smaller fragments occupy both CD44 and RHAMM, and the smallest activate TLR2 and TLR4, as well as modulate the ability of larger HA species to complex with CD44 and/or RHAMM by acting as competitive inhibitors.[45,60] In the skin, however, large HA and somewhat smaller molecular mass HA fragments can bind CD44 with the pathway effectors and transcriptional read-outs dependent on the differentiation status of the involved cell types.[61]

The genomic program engaged appears to be dependent on the nature of the specific HA ligand/CD44 complex formed. As is the case with CD44, binding of HA to RHAMM stimulates cell migration and modulates adhesion by facilitating the formation of linkages between CD44 and/or receptor tyrosine kinases (RTKs) with the cytoskeleton, promoting Src/ERK-FAK activation of RhoA/PKCε/NF-κB/Stat3 or Rac1/MAPK/AP-1/p53-p63 signaling to regulate focal adhesion turnover.[62,63] RHAMM-dependent motility, for example, requires association with CD44 and RTKs (e.g., EGFR1).[64,65] RHAMM is upregulated by TGF-β at the site of injury, where it stimulates healing through mobilization of several pathways to coordinate inflammation and fibrogenesis.[65–67] Indeed, loss of RHAMM negatively impacts both CD44 signaling and cutaneous wound repair.[65,68] CD44 is also post-translationally modified and alternatively spliced, moreover, giving rise to several isoforms with distinct functions, which increases the spectrum of potential ligands and coreceptor partners.[69] These diverse activities underscore the complexities involved in clarifying the role(s) of HA in wound repair,[67] a challenge further complicated by the differing functions of the various isoforms of CD44 and RHAMM.[40,70]

Fn: the EDA isoform and TLR4 signaling. Fn is a ubiquitous, multifunctional glycoprotein found as a soluble dimer in the plasma and in a polymerized form in the ECM.[71] Fn is organized into independently folded protein domains (Types I, II, and III), each with specific functional activities. ECM Fn provides both physical support and a scaffold to transduce biochemical as well as mechanical cues that dictate cell behavior.[5,72,73] Remodeling of the

Fn matrix in response to tissue injury or as a consequence of disease pathology involves, in large part, the synthesis of the EDA (also known as EIIIA) isoform of Fn (FnEDA). FnEDA derives from alternative transcript splicing to include an additional Type III domain, known as **E**xtra **D**omain-**A**.[74] Differential cell type-specific pathways downstream of TGF-β also regulate FnEDA splicing and, thereby, FnEDA levels.

Activation of PI3K/AKT/mTOR signaling in mouse embryonic fibroblasts, perhaps by TGF-β-induced downregulation of the phosphatase and tensin homolog on chromosome 10 (PTEN) or suppression of PTEN activity by Ser[380]/Thr[382,383] phosphorylation,[75,76] facilitates mobilization of the splicing factor SF2/ASF (also known as SRSF1), thereby increasing expression of FnEDA.[77] The molecular mechanism controlling alternative mRNA splicing of the EDA exon of Fn depends on spliceosome assembly and RNA secondary structure, as well as the serine/arginine (SR)-rich family of proteins.[78] Signaling from both TLR4 and the $\alpha_4\beta_1$ integrin, moreover, appears required for the FnEDA-dependent expression of fibroinflammatory cytokines suggesting that regulating FnEDA splicing, and thereby EDA-initiated TLR4 activation, may represent a novel strategy for the treatment of fibrosis and disorders that derive from excessive tissue remodeling.[79,80]

Under normal conditions, expression of FnEDA is restricted to early development and adult wound healing.[81] Wound fluid, in fact, is enriched in FnEDA as well as Fn fragments.[82] The development of FnEDA-deficient mice confirmed the involvement of the EDA isoform in injury resolution, inflammation, and tissue fibrosis.[83–85] While the role of FnEDA in the healing process may be multifunctional, wound site factors (*e.g.,* TGF-β) stimulate fibroblasts to synthesize FnEDA, which is required for the conversion of fibroblasts to the contractile myofibroblastic phenotype.[86,87] Dysregulated TGF-β signaling during the repair process, however, promotes high levels of FnEDA synthesis, enhanced myofibroblast persistence, and pathologic ECM accumulation substantially altering tissue mechanics leading to excessive tissue scarring and organ dysfunction.[reviewed in 85,88]

The EDA domain of Fn functions as a DAMP to activate TLR4 signaling in immune cells and human dermal fibroblasts resulting in the increased expression of several fibroinflammatory cytokines, including IL-8 and TNFα.[13,89,90] In dermal fibroblasts, this response is dependent on the $\alpha_4\beta_1$ integrin serving as a coreceptor.[79] The molecular basis of $\alpha_4\beta_1$/TLR4 activation and induction of fibroinflammatory genes by FnEDA is not well un-

derstood. There is no evidence as yet for the formation of physical complexes between $\alpha_4\beta_1$ and TLR4. It is also not known whether the EDA domain binds directly to the TLR4 or if TLR4 is transactivated through integrin-initiated signals.

The EDA domain additionally mobilizes TLR4-dependent proliferative responses in keratinocytes[21] and FnEDA is upregulated in the fibrotic skin of scleroderma patients, in mice with bleomycin-induced cutaneous fibrosis, as well as in keloid scars.[90,91] EDA activates TLR4 signaling either as the individual type III domain or in the context of the intact molecule[90,92] stimulating synthesis of collagen and α-smooth muscle cell actin (α-SMA) in skin fibroblasts.

Collectively, these findings implicate the TLR4-EDA axis in the control of a complex proinflammatory/profibrotic genomic program[90] and suggests that, as the primary source of FnEDA, fibroblasts coordinate both TLR4- and $\alpha_4\beta_1$-dependent autocrine loops that fuel tissue inflammation and subsequent fibrosis (Fig. 2). In the vascular system, for example, FnEDA facilitates the switch of smooth muscle cells to the synthetic phenotype characterized by increased cell proliferation and migration. This FnEDA-mediated differentiation process, which leads to vascular hyperplasia, is dependent on TLR4 and integrin receptors.[93]

Integrin/EDA cooperativity

Regulation of gene expression. Cell surface integrins are single-pass transmembrane heterodimeric receptors that couple structural and matricellular elements of the ECM to the intracellular compartment. Integrin signaling is bidirectional allowing cells to respond to changes in the stroma while regulating this process through control of integrin activation. Among the considerable repertoire of dimeric integrin receptors, only $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_9\beta_1$ recognize the EDA domain; little is known, however, regarding the signaling pathways they impact or their role in the development of inflammation and subsequent fibrotic disease.[94,95]

The EDGIHEL motif in the EDA C-C′ loop is involved in the binding of $\alpha_9\beta_1$ and $\alpha_4\beta_1$[31,95] with Asp[41]/Gly[42] specifically required for site occupancy. The $\alpha_4\beta_7$-binding sequence is not yet confirmed although complex formation between integrin $\alpha_4\beta_7$ and FnEDA promotes myofibroblast differentiation through the activation of FAK and ERK, the generation of increased cellular contractility and expression of α-SMA as well as collagen.[94] It should be acknowledged, however, that α-SMA is an inconsistent biomarker of the contractile, collagen-expressing, fibroblastic phenotype and,
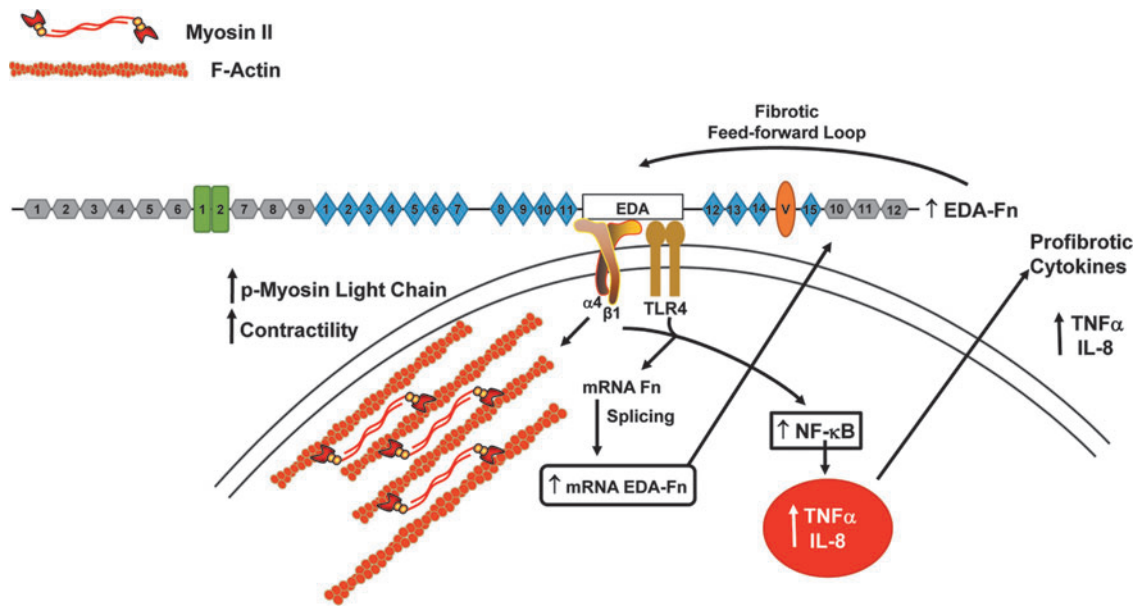
Figure 2. The TLR4 and $\alpha_4\beta_1$ integrin receptors regulate an EDA fibronectin-dependent feed-forward fibrotic loop. Binding of the EDA domain of fibronectin to the $\alpha_4\beta_1$ integrin receptor on dermal fibroblasts stimulates cellular contractility by promoting the formation of actin microfilaments and the phosphorylation of myosin light chain.[97] Interaction of the EDA with a functional complex of TLR4/$\alpha_4\beta_1$ receptors activates NF-κB-dependent transcription of profibrotic cytokines while regulating fibronectin mRNA splicing to increase the fraction of newly synthesized fibronectin containing the EDA domain, thus creating an EDA fibronectin feed-forward loop.[79] EDA, extra domain A.

therefore, may be a consequence rather than a causative factor in myofibroblast differentiation.

FnEDA occupancy of the $\alpha_9\beta_1$ integrin receptor on certain cell types (*e.g.*, colorectal, renal, pulmonary, hepatic, and epithelium) stimulates an epithelial-to-mesenchymal transition, or perhaps a more appropriately designated "plastic" response, that contributes to the myofibroblastic pool within the tumor desmoplastic tissue.[96] In dermal fibroblasts, $\alpha_4\beta_1$ recognition of its binding site on EDA increases actin stress fiber assembly, myosin light-chain phosphorylation, Fn synthesis, and construction of a higher-order 3-dimensional Fn matrix.[97] It appears, however, that $\alpha_4\beta_1$ is necessary but not sufficient in and of itself to function as a network hub in the genomic proinflammatory program. Indeed, coordinate signaling from both TLR4 and the $\alpha_4\beta_1$ integrin is required for the EDA-dependent expression of fibroinflammatory cytokines as well as the increase in the proportion of newly synthesized Fn containing the EDA splice variant (Fig. 2).[79] These data suggest that interactions between the EDA domain of Fn and EDA-recognizing integrins contributes to the acquisition of a fibrogenic phenotype by inducing the expression of genes that impact myofibroblast differentiation.

EDA domain in TGF-β activation. TGF-β regulates myofibroblastic conversion and wound-site ECM remodeling[98] highlighting a key role for TGF-β in tissue repair and the need to define mechanisms of TGF-β activation as one interventional approach to modulate healing outcomes. The three mammalian TGF-β isoforms, however, differ in their ability to direct fibrotic vs. scarless cutaneous healing,[99] suggesting that they have fundamentally distinct mechanisms of action. The TGF-β1, 2 and 3 proteins, consisting of the dimeric growth factor and latency-associated peptide (LAP) domains, interact within the endoplasmic reticulum with the latent TGF-β-binding protein (LTBP) through disulfide bond formation between LAP and LTBP.[100] Furin-directed cleavage of LAP occurs in the Golgi before extracellular secretion of this ternary large latent complex (TGF-β/LAP/LTBP), where the four LTBP isoforms possess variable affinities for elements of the ECM structural network.[101]

The use of genetically deficient mice and mapping of the interacting regions suggests that ECM docking of LTBP-3 and LTBP-4 occurs on fibrillin-1 microfibrils, whereas LTBP-1 interacts with the Fn network.[101,102] LTBP-1 has a greater affinity for FnEDA compared with FnEDB or Fn without the EDA/B splice variants and the EDA domain facilitates the docking of LTBP-1 to the fibroblast ECM.[31,101] Indeed, interference with EDA domain function attenuates both LTBP-1 binding to FnEDA and TGF-β1 activation.[103] Complicating the actual identification of LTBP docking sites in the ECM, however, is the changing dynamics of bind-

ing partners (*e.g.,* from Fn to fibrillin-1 or even fibulin) and the suggestion that heparin and heparan sulfate proteoglycans might facilitate Fn/LTBP-1 interactions, while promoting LTBP-1 multimerization and mechanical force-dependent TGF-β activation.[104,105] Nevertheless, the construction of such multicomponent complexes forms the basis for TGF-β activation in the wound field. This has considerable implications since myofibroblast differentiation, a critical cell type in the wound repair program, requires a microenvironment rich in biologically active TGF-β, a progressively noncompliant stromal matrix and the expression and accumulation of FnEDA.[31,103]

While certain proteinases (*e.g.,* MMP-2, MMP-9, plasmin) liberate TGF-β upon cleavage of the sensitive hinge region in LAP, other models suggest nonproteolytic mechanisms whereby multiple integrins that share the α_V subunit (*e.g.,* $\alpha_V\beta_1$, β_3, β_5, β_6, β_8), and bind the arginine–glycine–aspartic acid (RGD) motif in the N-terminal region of LAP, generate contractile forces with ECM-anchored LTBPs.[86,103,104,106–108] The resulting tension induces a conformational change to the latent TGF-β1 complex releasing, and thereby activating, the TGF-β1 or TGF-β3 dimer[104,109,110] without the need for participating proteases (Fig. 3). A different mode of

liberation is likely involved for TGF-β2 since the TGF-β2 LAP does not possess an RGD site. Alternatively, integrins $\alpha_V\beta_6$ and $\alpha_V\beta_3$ may bind to both the latent TGF-β1 complex and proteinases, simultaneously distorting the LAP cage and providing protease access to the hinge cleavage site.

While $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_8\beta_1$ also recognize the RGD site, it appears that α_V integrins are specifically poised to liberate TGF-β1 or TGF-β3.[111] The role of $\alpha_V\beta_8$ in TGF-β activation, however, may be fundamentally different from other α_V integrins. The $\alpha_V\beta_8$ cytoplasmic tail does not engage the actin microfilament network and, therefore, cannot generate Rho/RhoA-dependent contractile force to free the LAP-caged TGF-β dimer relying instead on proteolytic activity or alternative mechanisms of LAP-associated TGF-β activation.[112,113] EDA/integrin binding may also enhance interactions between the LAP RGD motif and α_V integrins suggesting that FnEDA may actually provide a platform for the generation of tractional force to promote TGF-β release.[31]

ECM remodeling and/or maturation during tissue repair or increased tensional stress as a consequence of accumulating FnEDA at the injury site may also contribute to a resetting of the threshold of TGB-β1 activation.[31,114] These findings support
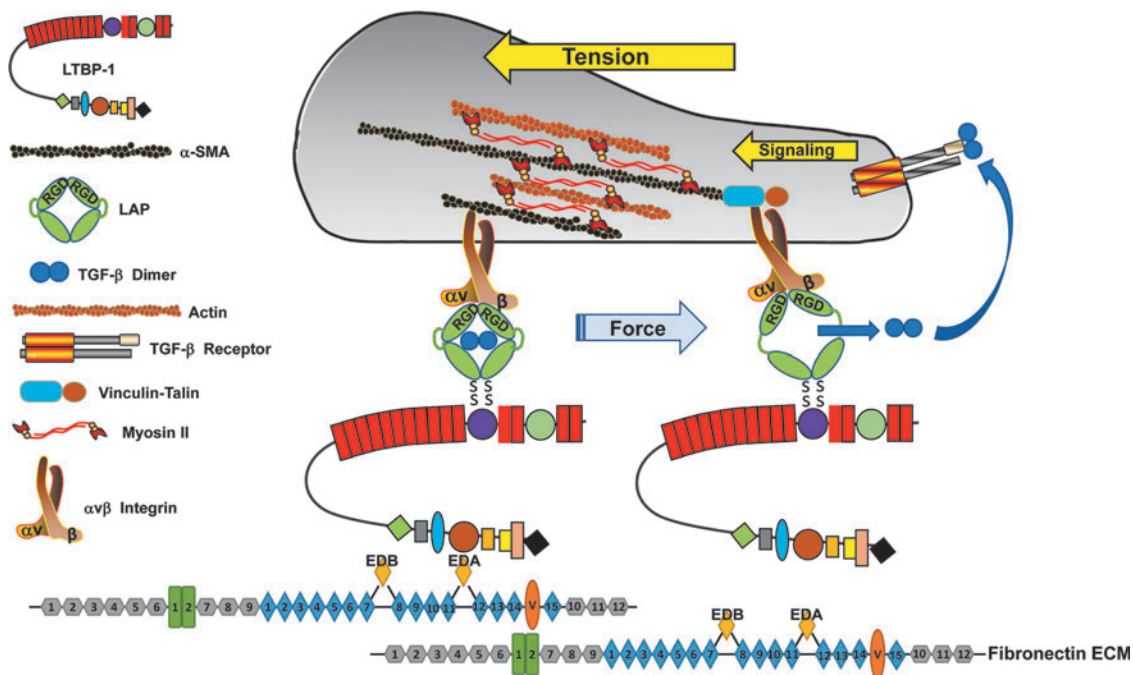

Figure 3. A model of tension-dependent activation of TGF-β upon release from the LAP cage. The ternary large latent (LTBP/TGF-β/LAP) complex forms a bridge between an α_V integrin bound to the RGD site on the latency-associated peptide and LTBP-1 tethered to the fibronectin-rich ECM. Actinomyosin-based contractility generates mechanical tension within this ternary complex inducing a conformational change in the LAP that releases the now-active TGF-β dimer.[derived from 104] Very recent findings using cryoelectron microscopy to probe LAP:TGF-β complex interactions with the $\alpha_V\beta_8$ integrin suggest, however, the existence of an alternative mechanism of TGF-β activation that does not necessitate release of dimeric TGF-β from the LAP.[113] ECM, extracellular matrix; LAP, latency-associated peptide; LTBP, latent GFF-β-binding protein.

a more complex mechanism for continued TGF-β1 signaling and initiation of fibrotic disease and places the TGF-β induction of FnEDA expression as a critical element in a TGF-β/FnEDA/α_V integrin-positive feed-forward loop.[31] Loss of elasticity and progressive ECM stiffness further stimulate expression of FnEDA while increasing the colocalization of LTBP-1 with FnEDA, integrin/ LAP engagement, and cellular force generation collectively augmenting the ongoing conversion of latent to bioactive TGF-β1.[22,115] This increasingly noncompliant TGF-β1-rich microenvironment promotes myofibroblastic differentiation and persistence while mobilizing the HIPPO pathway mechanosensitive effectors YAP and TAZ that, in turn, reinforce expression of genes encoding profibrotic factors.[116–118] A point may be reached when progressive fibrosis becomes self-sustaining, involving both cell autonomous and ECM-driven mechanisms, resulting in the creation of a feed-forward mechanosensitive circuit and a permanent change in the mechanical properties of the supporting stroma that exacerbates disease progression.[119]

Integration of EDA/HA/TLR4 and TGF-β signaling

TGF-β/HA synergy. The TGF-β-directed transition of dermal fibroblasts to the myofibroblastic phenotype is required for wound contraction, collagen deposition, and scar formation,[120] a process regulated by and dependent on both FnEDA and HA.[20,30] TGF-β increases HA levels in the wound bed by inducing the synthesis of HAS, in large part, through the involvement of MAP kinases[121] and HA appears involved in the subsequent fibrotic response.[122] The endogenous synthesis and pericellular organization of polymerized HA is required for myofibroblast conversion as addition of exogenous HA does not promote differentiation.[123]

Induction and maintenance of the myofibroblast phenotype also requires the HA receptor, CD44. While the mechanism is unclear, TGF-β promotes complex formation between EGFR1 and CD44.[124] In response to TGF-β1 stimulation, CD44 translocates into lipid rafts where it colocalizes with the epidermal growth factor receptor (EGFR) to activate a signaling cascade involving ERK1/2 and Ca^{2+}/calmodulin kinase II (Fig. 1); both kinases are essential for myofibroblast differentiation.[124] Since TGF-β1 activates *Src* kinase-dependent EGFR^{Y845} phosphorylation and downstream signaling,[125,126] and HA binding to CD44 similarly promotes Src activation,[49] it appears that TGF-β1/HA cooperation culminates in EGFR→MAP kinase pathway activation that, in the setting of chronically ele-

vated TGF-β1 levels, may promote maladaptive wound repair.

While HA facilitates TGF-β1-induced myofibroblast differentiation through the formation of HA/ CD44 complexes promoting CD44 cellular relocation, HA/CD44 interactions may also downregulate TGF-β1signaling by trafficking the TGF-β receptor (TGF-βR) to caveolae-rich membrane rafts[53] facilitating, thereby, receptor degradation.[127] Additionally, the HA-dependent formation of CD44/ TLR4 complexes contributes to the development of a fibrotic environment by increasing the expression of several cytokines, including TGF-β.[51,128] Clearly, there are complex controls on the intensity and duration of TGF-β1 signaling at several levels in the wound repair program.

Although TGF-β1 transactivates the EGFR and, thereby, its downstream targets AKT and ERK1/2, the myofibroblast phenotype persists after TGF-β removal due to establishment of an autocrine TGF-β/HA-dependent feed-forward loop that promotes tissue fibrosis.[129] Continued maintenance of the myofibroblast phenotype leads to excessive matrix deposition, ECM crosslinking, tissue stiffening, and fibrotic disease. CD44 similarly upregulates synthesis of α-SMA through an actin/MRTF pathway, which is independent of both TGF-β and HA, however, suggesting that the role of CD44 and HA in myofibroblast differentiation is quite complicated. These differential activities of CD44 are not well understood and likely depend on the specific cell types and involved tissue.

PTEN is a target of EDA/HA/TLR4 and TGF-β signaling

TLR4 activation by the FnEDA domain, either as the isolated type III module or in the context of the intact or fragmented FnEDA molecule, induces the expression of several proinflammatory and profibrotic genes that can impair or promote wound healing.[13,14,79,89,130,131] This response involves the MyD88 adapter-like (Mal) protein/MyD88 pathway, downstream of TLR4, to activate NF-κB target genes and likely reflects the nature of the receptor complex (*e.g.*, TLR4 vs. TLR4+coreceptors), the signaling intermediates engaged and the specific repertoire of inflammatory/profibrotic effectors expressed. TLR4 may function as a molecular "switch," binding endogenous DAMPs (*e.g.*, EDA) to activate a repair program while downregulating the TGF-β1 inhibitory pseudoreceptor Bambi (through the same MyD88/NF-κB pathway).[132] Bambi reduction, in turn, sensitizes cells to TGF-β1 in the immediate DAMP-rich microenvironment as well as to EDA-mediated TGF-β1 upregulation promoting persistent

expression of a subset of proinflammatory/profibrotic target genes, including FnEDA, creating a sustaining feed-forward TLR4→TGF-β1→ FnEDA→TLR4 loop that culminates in fibrosis and compromised tissue function (Figs. 4 and 5).

The basis for crosstalk between the TGF-β1 and TLR4 networks in cutaneous pathophysiology is not well defined although recent data suggest significant interaction between the TGF-β1/TLR4 and PTEN pathways in the control of the fibrotic phenotype. PTEN is the principle negative regulator of the PI3K/Akt pathway and a critical factor in several fibrotic disorders. PTEN expression is attenuated in various models of injury-induced tissue scarring, while PTEN deficiency drives cutaneous and renal fibrosis.[76,133–135] Importantly, TLR4 signaling downregulates PTEN levels resulting in fibroblast commitment to a profibrotic phenotype[136] and TGF-β1-initiated expression of fibrotic genes is enhanced by PTEN depletion.[135] PTEN silencing, moreover, cooperates with TGF-β1 to further stimulate induction of the SMAD3-/p53-dependent TGF-β1 profibrotic signature genes, CCN2, SERPINE1, and FnEDA.[134,135]

The mechanism of PTEN reduction in the setting of fibrosis appears due to increased HA/TLR signaling and/or elevated TGF-β1 levels in the injury field.[134,136] TLR4 activation (by lipopolysaccharide, LPS) stimulates miR-718 expression (in macrophages), which impacts PI3K/Akt signaling by targeting PTEN and promoting Akt phosphorylation.[137] pAkt, in turn, downmodulates the expression of TLR4 and several of its signaling effectors through let-7e exerting, thereby, multilevel negative regulation to the TLR4 pathway. Whether TLR4-mobilizing DAMPs utilize the same or different intermediates is not known, but the involvement of DAMP-induced microRNAs in PTEN control is firmly established. In this regard, HA (and likely smaller MW fragments as well) bind to CD44 promoting RhoA/ROCK and NF-κB/Stat signaling while inducing expression of several microRNAs, including the PTEN suppressor miR-21, initiating acquisition of a proinflammatory program.[40,61,138] TGF-β1 also stimulates miR-21 transcription which, in turn, reduces PTEN levels by direct binding to its 3' untranslated region,[139] although miR-21 has targets other than PTEN. Collectively, these data indicate that microRNAs induced upon activation of TLR4, HA/CD44, and TGF-β1 signaling attenuate PTEN levels in both dermal and nondermal cells likely impacting acti-
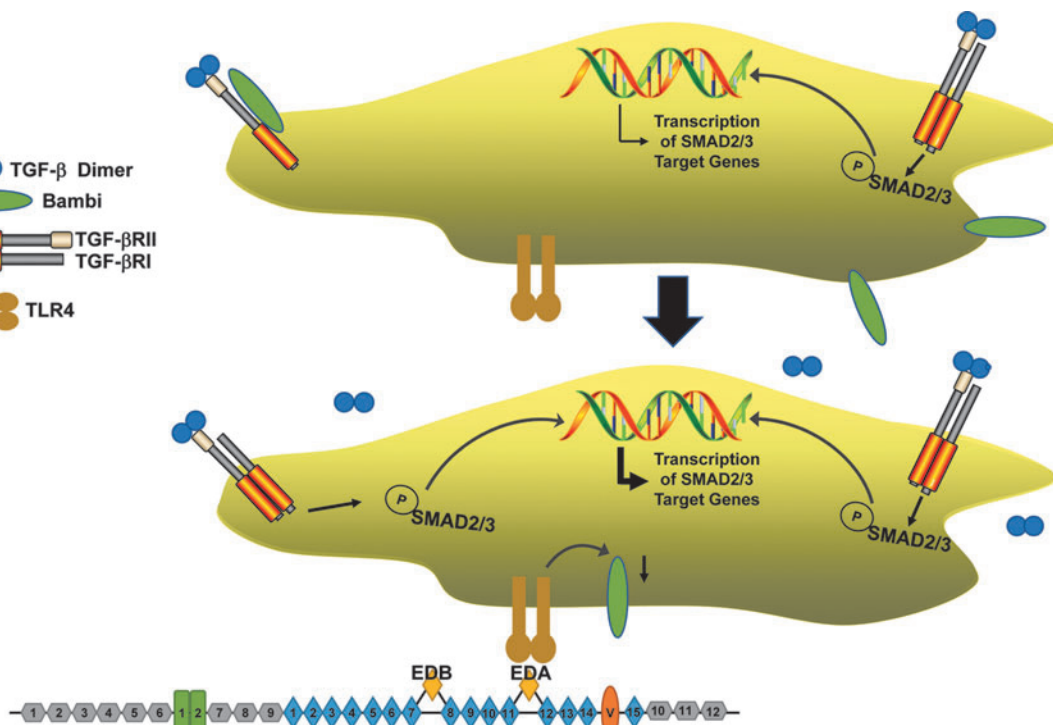

Figure 4. TLR4 activation enhances TGF-β signaling and expression of TGF-β target genes. In unstimulated quiescent cells (*top*), basal production of active TGF-β engages cell surface TGF-β receptors resulting in SMAD phosphorylation and low-level transcription of SMAD-responsive genes. A fraction of TGF-βRII complexes with the TGF-β pseudoreceptor Bambi instead of TGF-βRI rendering the type II receptor signaling incompetent.[(e.g., 156,157)] Upon engagement of TLR4 with EDA (*bottom*), increased generation of active TGF-β1 coupled with Bambi downregulation sensitizes cells to TGF-β1 in the immediate microenvironment increasing TGF-βR signaling and the expression of SMAD target genes. A significant faction of profibrotic factors is induced specifically by SMAD2/3.
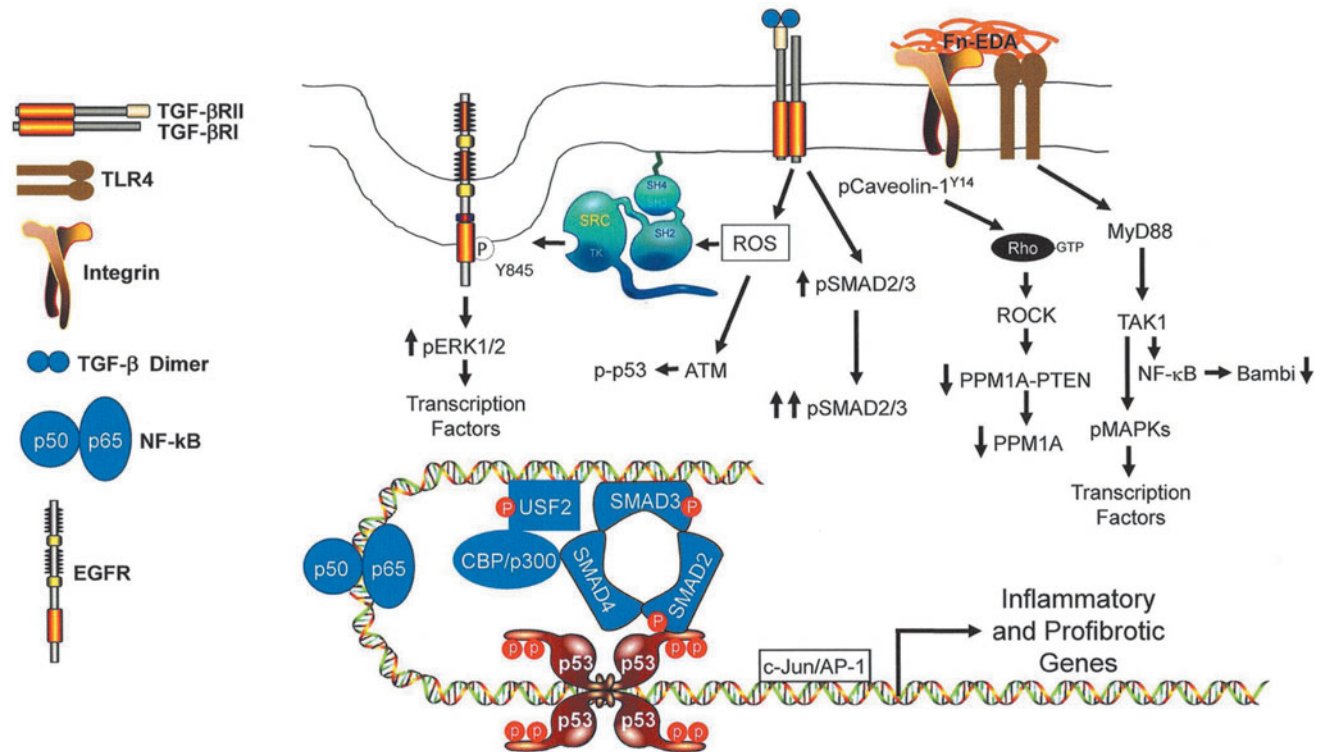
Figure 5. Model illustrating TLR4-dependent signaling events that impact the expression of inflammatory and profibrotic genes. EDA stimulation of TLR4 signaling, perhaps in cooperation with the $\alpha_4\beta_1$ integrin, engages the MyD88 adaptor protein pathway mobilizing TGF-β-activated kinase 1 (TAK1), also known as MAP3K7. TAK1, in turn, activates NF-κB resulting in the NF-κB-mediated downregulation of Bambi,[156,157] enhancing thereby TGF-β signaling while increasing NF-κB(p50/p65)-dependent transcription of inflammatory genes. Bambi suppression, particularly in the context of increased TGF-β synthesis and/or release enhances TGF-βR-dependent SMAD2/3 phosphorylation while increasing the cellular levels of ROS. ROS signaling stimulates ATM-induced p53 phosphorylation, *src* kinase transactivation of the EGFR at Y845 and src-dependent phosphorylation of caveolin-1 at the Y14 site.[142] EGFR-activated ERK1/2 and TAK1-stimulated MAPKs target transcription factors (*e.g.*, NF-κB, USF, AP-1) and chromatin remodeling proteins (*e.g.*, CB/p300) that cooperate with SMADs and p53 to influence expression of a genomic proinflammatory/profibrotic program. TGF-β1 also activates the RhoA/ROCK pathway, likely by promoting src kinase-induced caveolin-1 Y14 phosphorylation and Rho-GTP loading[142] that downregulates both PTEN and PPM1A levels contributing to the persistence of SMAD2/3 phosphorylation and transcription of profibrotic genes. ROS, reactive oxygen species.

vation of a proinflammatory/fibrogenic program with significant implications as to tissue repair outcomes.

PTEN/PPM1A interactions: regulation of SMAD activity.   Recent findings provide considerable insight into the fibroinflammatory consequences of PTEN downregulation. PTEN depletion reduces the levels of PPM1A, a C-terminal SMAD2/3 phosphatase,[140] while promoting SMAD3 phosphorylation and nuclear localization, transactivation of profibrotic genes and secretion of SMAD3-dependent fibrotic factors.[135,141] PPM1A overexpression attenuates fibrogenesis in murine fibroblasts treated with the TLR4-activator LPS, while persistent TGF-β1 stimulation decreases PPM1A levels through Rho/ROCK pathway activation maintaining, thereby, SMAD3-dependent transcription of profibrotic signature genes.[141–143] PTEN may complex, moreover, with PPM1A in human fibroblasts,[144] suggesting that

PTEN is required for PPM1A stabilization and/or function. PPM1A destabilization or loss of function due to PTEN downregulation has implications with regard to TLR4 proinflammatory signaling since, in addition to targeting pSMAD2/3, PPM1A also functions as a RelA $S^{536,276}$ phosphatase, thereby inhibiting NF-κB activation.[145]

PPM1A suppression, similar to PTEN deficiency, increases SMAD3 phosphorylation and stimulates expression of fibrotic genes while PPM1A overexpression inhibits both events.[146] These findings suggest that PTEN is an upstream regulator of PPM1A in dysfunctional tissue repair and implicate PPM1A as a novel repressor of the SMAD3 fibrotic response. TGF-β1 appears to attenuate PPM1A and PTEN expression through protein ubiquitination and subsequent degradation since the proteasome inhibitor MG132 rescues PPM1A and PTEN expression, even in the presence of TGF-β1.[134] While the mechanism is unclear, signaling through TLR4 or the

TGF-βR as well as through HA/CD44 complexes stimulates transcription of the PTEN-targeting microRNA miR-21, and PTEN deficiency has the same outcome as PPM1A knockdown (*i.e.*, maintenance of SMAD3 phosphorylation and induction of profibrotic genes).[134,135]

TGF-β1-initiated Src kinase-dependent caveolin-1 Y14 phosphorylation is a critical event in RhoA/ROCK-mediated suppression of nuclear PPM1A levels maintaining, thereby, SMAD2/3-dependent transcription of profibrotic genes.[142] PTEN activity and cellular location, moreover, are also regulated by Rho kinases and ROCK can phosphorylate PTEN.[147,148] One possibility is that PTEN phosphorylation dissociates PTEN-PPM1A complexes resulting in PPM1A degradation, thereby, retaining SMAD transcriptional activity.[142] Thus, depending on the actual magnitude and duration of the stimulus (*e.g.*, DAMPs and/or TGF-β1), PTEN may function as a rheostat to influence the amplitude and kinetics of the inflammatory response to tissue injury. Clarifying molecular pathways downstream of PTEN in tissue injury may lead to the identification of novel mechanistically relevant and translationally accessible targets underlying TLR4/TGF-β1 signaling to transcriptional controls on disease-causative genes.

CONCLUSIONS

The available data provide for a hypothetical model whereby cooperation among the HA/CD44, EDA/TLR4, and TGF-β1 signaling pathways converge to regulate the wound-initiated DAMP-dependent sterile fibroinflammatory response and, thereby, repair outcomes (Figs. 1 and 5). While, the DAMPs FnEDA and HA are generated at the site of injury, TGF-β1 is released by degranulated platelets, as well as produced by infiltrating immune cells and local epithelial and fibroblastic elements. FnEDA downregulates Bambi, perhaps through formation of NF-κB p50/HDAC1 complexes to repress Bambi transcription[145] making cells more responsive to TGF-β1 in the wound field, resulting in the increased expression of TGF-β1 target profibrotic genes. The net outcome of this reprogramming promotes a gradual increase in tissue rigidity facilitating the tension-induced unfolding of the FnEDA molecule and exposing the EDA domain for TLR4 activation.

Although it is not clear if other endogenous DAMP-like TLR4 ligands similarly augment TGF-β1 signaling and transcription of fibroinflammatory genes, bacterial LPS also enhances cellular sensitivity to TGF-β1 through Bambi downregulation, while pro-

moting tissue fibrosis.[149] Microbial contamination, biofilm formation and prolonged inflammation are significant contributors to the pathophysiology of burn-associated hypertrophic scarring, in which dermal fibroblasts likely regulate the amplitude and duration of the LPS → TLR4-initiated inflammatory response.[150] While inflammation drives wound repair and regeneration, chronic inflammation leads to the development of pathologic fibrosis,[23] perhaps through induction of osteopontin expression by injury-site fibroblasts.[26] In this context, osteopontin appears to inhibit the rate of cutaneous injury repair and triggers hypergranulation and subsequent fibrosis.[26] Similarly, topical application of PDGF-βB to chronic ulcers accelerates healing but may also foster the development of excessive granulation tissue and scarring as part of increased osteopontin expression. Indeed, downregulation of osteopontin may be one mechanism whereby Gleevec reduces pulmonary and dermal fibrosis.[26]

LPS-TLR4 interactions, moreover, require a src kinase-activated EGFR to induce NF-κB-directed expression of inflammatory genes[151–153] suggesting extensive crosstalk between the TLR4 and EGFR pathways. NF-κB signaling in response to EGF, moreover, requires both EGFR and TLR4 activity and TLR4-induced NF-κB mobilization following LPS stimulation is EGFR dependent.[151] Src family kinases are required for NF-κB activation by EGF and LPS while the induced proinflammatory cytokine response to LPS is attenuated by the EGFR inhibitor Erlotinib.[151] Similar EGFR/TLR4 crosstalk exists in response to Fn-derived DAMPs in human dermal fibroblasts resulting in TLR4 signaling. Collectively, these findings suggest that pharmacologic approaches that target the EGFR and/or src kinases may have therapeutic efficacy in regulating DAMP-initiated TGF-β1 hypersensitivity and fibroproliferative disease.[131]

TGF-β1 also promotes the synthesis of the EDA splice variant of Fn by increasing expression of the splicing regulatory protein SRp40 thus initiating a profibrotic feed-forward loop.[154] TGF-β1-induced FnEDA production, moreover, is dependent on PI3 kinase-AKT signaling.[155] Since the HA/CD44, EDA/TLR4, and TGF-β1 pathways each induce miR-21 transcription and PTEN downregulation, the subsequent increase in AKT activity would additionally reinforce FnEDA expression and progressive scar formation. Whether inhibiting the generation of FnEDA alternative splicing has therapeutic utility in the context of maladaptive wound repair is an innovative approach to healing anomalies.

AUTHOR DISCLOSURE AND GHOST WRITING

The authors do not have any commercial conflicts of interest. The article was written exclusively by Drs. McKeown-Longo and Higgins; no ghostwriters were involved.

TAKE-HOME MESSAGES

- Fibrosis is a frequent pathophysiological consequence of chronic inflammation due to tissue injury.
- DAMPs generated at the site of injury are TLR and CD44 agonists that stimulate the production of proinflammatory cytokines and sustain the inflammatory response.
- TLR4 functions as a molecular switch, binding the EDA domain of Fn to activate transcription of NF-κB-regulated target genes while intersecting with growth factor (EGF, TGF-β)-signaling pathways.
- The DAMP-rich microenvironment sensitizes cells to TGF-β1 in the immediate injury field due to TLR4-mediated downregulation of the TGF-β pseudoreceptor Bambi.
- TLR4 induction of inflammatory cytokines and stimulated expression of profibrotic factors as a result of an increase in TGF-β1 signaling may create a sustained TLR4 $\rightarrow$ TGF-β1 $\rightarrow$ FnEDA $\rightarrow$ TLR4 feed-forward loop that culminates in excessive scarring and tissue dysfunction.

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Abbreviations and Acronyms

CK2	= Casein kinase 2
DAMP	= damage-associated molecular pattern
ECM	= extracellular matrix
EDA	= extra domain A
EGFR	= epidermal growth factor receptor
FnEDA	= EDA isoform of fibronectin
GAG	= glycosaminoglycan
HA	= hyaluronic acid
HAS	= HA synthase
HMW	= high molecular weight
HYAL	= hyaluronidase
IL	= interleukin
LAP	= latency-associated peptide
LMW	= low-molecular-weight
LPS	= lipopolysaccharide
LTBP	= latent GFF-β-binding protein
MAP	= mitogen-activated protein kinase
MMP	= matrix metalloproteinase
MYD88	= myeloid differentiation factor 88
PTEN	= phosphatase and tensin homolog
RGD	= arginine–glycine–aspartic acid
RHAMM	= receptor for hyaluronan-mediated motility
ROS	= reactive oxygen species
TGF-β	= transforming growth factor beta
TLR	= toll-like receptor
TNF	= tumor necrosis factor
TRK	= receptor tyrosine kinase
α-SMA	= α-smooth muscle cell actin