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Linking Cell Structure to Gene Regulation: Signaling Events and Expression Controls on the Model Genes PAI-1 and CTGF

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Introduction

External physical forces as well as internal constraints imposed by the microtubule, microfilament and intermediate filament cytoskeletal networks, junctional complexes and integrin-extracellular matrix (ECM) interactions are major determinants of cell structure and function [e.g., 1–3]. Indeed, several basic processes including cell cycle transit, DNA synthesis and apoptosis are profoundly influenced by changes in cellular structural organization [4–9]. The vasculature, for example, is constantly subjected to a continuum of hemodynamic stimuli (e.g., shear strain, flow disturbances, mechanical or pulsile stretch) that alter cytoskeletal dynamics, organization and associated signaling pathways. These same mechanical forces impact expression of genes that, in turn, modulate cell proliferation, migration and ECM synthesis/deposition resulting in the development of tissue-specific pathologies (e.g., focal atherosclerosis) [reviewed in 10–14]. Prominent among the repertoire of fibrosis-promoting proteins implicated in vascular fibroproliferative disease are the matricellular proteins plasminogen activator inhibitor inhibitor-1 (PAI-1, SERPINE1) and connective tissue growth factor (CTGF) [reviewed in 15,16]. Importantly, the transcriptional control networks for both genes are exquisitely sensitive to cytoskeletal perturbations [16]. The continued definition of pathways and mechanisms involved in vascular cell shape-deformation responses may well define new, translationally-relevant, targets for the treatment of vascular disorders.

Mechanosensitive Signaling: The Vascular Model

The available evidence suggests that, upon appropriate mechanical stimuli, integrins are mobilized to orchestrate cellular responses in coordination with (a) growth factor receptors (e.g., those that bind epidermal growth factor [EGFR], transforming growth factor-β [TGF-βR], vascular endothelial growth factor [VEGFR] family ligands), (b) cadherin junctional complexes and (c) clues from the ECM [10,17–21]. Integrins, in fact, are focal points for recruitment of signaling molecules (e.g., focal adhesion kinase [FAK]) to ECM contact sites in shear stress-induced endothelial cell migration [22]. The functional and spatial associations between non-receptor tyrosine kinases (e.g., $pp125^{FAK}$, $pp60^{c\text{-}src}$), moreover, increase with an applied mechanical load reminiscent of those induced by integrin-mediated cell adhesion [8, 12,23,24]. Since these same signaling intermediates ($pp125^{FAK}$, $pp60^{c\text{-}src}$) also lie in the main

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path for mechanical force transfer (i.e., regions enriched in the cytostructural proteins paxcillin, actin and tensin), it is apparent that focal adhesion complexes can potentially translate load deformation stresses into specific biological responses as a consequence of this cytoskeletal "wiring" [8,25]. Indeed, mobilization of FAK, pp60$^{c\text{-}src}$, and Grb2, to focal adhesions under conditions of varying cellular tensional forces engages downstream cascades (i.e., involving mitogen-activated protein [MAP] kinases and the small GTPases Ras, Rac, and Rho) similar to those stimulated by integrin-mediated matrix attachment [26]. Such mechanical forces may effectively cluster, or initiate conformational changes to, integrin receptors with recruitment of structural proteins at the focal adhesion complex. As part of this response, pp125^{FAK} partitions to the cytoskeletal framework and is tyrosine phosphorylated at Tyr-397 leading to recruitment of pp60$^{c\text{-}src}$, a key kinase in load-dependent signal transduction [27]. The pp60$^{c\text{-}src}$ kinase is also activated by mechanical deformation, albeit with different kinetics than that induced by growth factors such as EGF [28]. The association of pp60$^{c\text{-}src}$ with pp125^{FAK} at focal adhesions further stimulates pp125^{FAK} phosphorylation at Tyr-925, creating a binding site for Grb2. The adaptor protein Shc is tyrosine phosphorylated in endothelial cells in response to shear stress, binds to Grb2 by an SH2-dependent mechanism [29] facilitating, thereby, the assembly of a tripartite Shc/Grb2/Sos complex resulting in subsequent Ras GTPase activation. MAP kinase pathways in vascular smooth muscle cells (VSMC) similarly function via pp125^{FAK}/pp60$^{c\text{-}src}$/Grb2 interactions with Ras as a downstream target [27,30–32]. This has important adaptive consequences as both the extracellular signal-regulated kinase (ERK) and c-*Jun*-associated kinase (JNK) pathways are activated in a FAK-dependent manner, at least in the endothelium, in response to mechanical stimulation [27,29]. Cyclic stretch also rapidly activates p38 MAP kinase in VSMC which requires both the small GTPases Ras and Rac since expression of dominant-negative Ras or Rac constructs attenuates p38 phosphorylation as well as stretch-mediated VSMC migration/proliferation [33]. Stress-related ERK activation may further modulate cellular mechanical properties by regulating caldesmon, suggesting a direct effect on the contractile properties of the vascular wall [34].

Mechanical perturbation of cell structure may also activate small GTPases such as Rho, Rac or Cdc42 [35]. Indeed, the Rho kinases (Rho-associated coiled-coil forming kinases; ROCK1/2 which are the major immediate downstream targets of RhoA) and mDia are particularly important elements and impact critical functions including cytoskeletal organization, contractility, motility and gene expression [reviewed in 36] and may well be accessable targets for the clinical management of cardiovascular disease [e.g., 37]. Rho GTPases cycle between active GTP-bound and inactive GDP-bound states which are regulated by guanine nucleotide exchanges factors (GEF) and GTPases-activating proteins (GAPs) [38–41]. It appears that tight control of the temporal/spatial activation of Rho GTPases likely provides for the physiological adjustment to different cycles or amplitude of mechanical forces commonly encountered in the vascular system [e.g., 42]. The complex molecular details of mechanotransduction leading to Rho GTPase signaling, however, are only partially understood. While p190RhoGAP regulates capillary network formation by integrating mechanical and chemical signaling pathways, through the likely downstream intermediates Rho kinases, mDia, LIM-kinase, PAK1 and other small GTPases [36,43], it also functions as an integrator of Rac and RhoA cross-talk, which have opposing effects in the control of endothelial cell morphology [44]. The available evidence, in fact, supports a model wherein Rho→mDia signaling results in Rac activation via a Src-dependent mechanism with suppression of this pathway by ROCK activity [36].

Cell Shape-Dependent Metabolic Controls: Genomic Responses to Cytoskeletal Deformation

Experimental approaches that specifically perturb cell structure (e.g., multidirectional force application, cadherin- or integrin-interfering antibodies, cytoskeletal-active drugs, expression of mutant or cell type "unrelated" cytoskeletal elements, substrate-modulation of cell

morphology by plating on poly-HEMA-coated surfaces or on complex micro-patterned adhesive substrates) provide accessible models to probe deformation-sensitive signaling pathways and their target genes [e.g., 2,3,24,25,45–55]. Such studies highlight the effects of cell shape on transcriptional outputs as well as the resultant phenotypic response. The use of rather novel "phosphotyrosine reporters", that measure fluorescence of YFP fused to multiple SH2 domains derived from the c-Src tyrosine kinase, and specific microtubule disrupting agents, moreover, confirmed the dynamic nature of cytoskeletal framework-anchored signaling events (e.g., involving $pp125^{FAK}$, paxillin, vinculin, $pp60^{c\text{-}src}$) [56].

Distortion of cell morphology with cytoskeleton-targeting drugs has emerged as an important approach to the identification of cell shape-responsive genes as well as, in some cases, the involved signaling pathways [57–61]. Protein-protein interaction mapping revealed that the cellular signaling apparatus is networked with the cytoskeletal framework and, therefore, is highly sensitive to shape perturbation [e.g., 3]. It has become apparent in recent years, moreover, that the expression of certain genes is particularly responsive to changes in cellular configuration regardless of the basis for the cell deformation event. Thus, actin and microtubule cytoskeletal networks, key regulators of cell morphology, integrate and transduce intracellular signals provided by cues from the extracellular matrix, cell-cell interactions and growth factors [1,24,62,63]. Disruption of either the microtubule network (e.g., with colchicine or nocodazole) or cellular microfilaments (e.g., with cytochalasin D or latrunculin B), however, also constitutes effective inductive stimuli leading to changes in gene transcription largely as a consequence of altered signaling events [16,61,64–67]. Transcriptional outputs induced by cell deformation, quite unexpectedly, actually proved to be rather limited when compared to the global reprogramming of gene expression that typically accompanies exposure to serum or individual growth factors [64,65,68–77]. Microarray analyses, in fact, identified only several dozen shape deformation-sensitive genes in endothelial cells and VSMC exposed to steady laminar or turbulent shear stress, among the most prominent of which were PAI-1 and CTGF [e.g., 78,79]. When endothelial cells are exposed to non-uniform shear stress, CTGF is upregulated in a RhoA-dependent manner [80]. However, comparison of the partial inhibition by Rho kinase inhibitors compared to the much stronger effect of statins, which interfere with isoprenylation of multiple signaling molecules among them different small GTPases, indicates involvement of additional signaling molecules in shear stress-mediated induction of CTGF. Remodeling of static cultures of HUVEC to adopt to laminar flow as observed in straight areas of vessels promotes down-regulation of CTGF [80,81]. Downregulation was attributed to the transcription factor KLF2 (Kruppel-like factor 2) [81], which is also involved in the rearrangement of actin fibers in the presence of laminar shear stress [82] The latter effects were linked to an activation of RhoA. The apparent discrepancy of activation of RhoA and downregulation of CTGF needs further investigation and may be resolved by a closer analysis of time-dependent effects of KLF2.

Induced PAI-1 gene expression in each instance, furthermore, correlated with specific stress-associated restructuring within the cellular microfilament system [45,53,54,79,83]. While uncertainties regarding the threshold of expression change, cell type universality of response, kinetics of induced changes and the actual number of sequences assessed, the transcription of genes that encode proteins involved in tissue remodeling processes (e.g., CTGF, PAI-1, several metalloproteinases, urokinase plasminogen activator [uPA], vascular endothelial growth factor, cyclooxygenase, fibronectin, collagen-1) is closely associated with dynamic changes in cellular morphology and shape-altering physiologic processes (Figure 1) [53,60,65,72–77, 78,84–86]. Indeed, targeted reorganization of cell morphology with cytoskeletal-disrupting drugs does, in fact, transcriptionally impact several genes in this subset implicating the cytoskeleton in the signaling apparatus [60,64]. Analysis of dose response, a critical aspect in such assessments, has implicated a "threshold" CD concentration required for dramatic increases in PAI-1 expression that appears different for individual cells types [e.g., 61,83].

Since CD also effectively increased PAI-1 synthesis in suspended cells and colchicine significantly induced PAI-1 expression in adherent cells without the same effect on cellular aborization [45,61,83], it would appear that PAI-1 gene control is more closely associated with changes in cytoskeletal structure than with overall cell shape perturbation. This interpretation is consistent with the realization that cytoskeletal modifications may relieve growth state-dependent constraints on particular signaling events (i.e., Rho-GEFs, SMADs) that impact downstream transcriptional-level controls as these particular effectors are normally microtubule-anchored, at least in unstimulated cells [3,39,63,87]. Disruption of the microtubule network with colchicine or nocodazole, in fact, stimulates several pathways and activates Rho-GEFs leading to Rho-GTP loading with subsequent effects on PAI-1 and CTGF expression [39,65,88,89] as well as SMAD phosphorylation [16,90] (Figure 2). Interestingly, the Rho-ROCK pathway utilizes SMAD3 as a transcriptional activator for PAI-1 (but not for CTGF) induction in colchicine-treated cells while SMAD3 is required for both PAI-1 and CTGF expression in TGF-β1-stimulated VSMC [16]. These findings highlight what appears to be the stimulus-specific engagement of signaling pathways that regulate the expression of these two, clinically-important, profibrotic genes. In microvascular endothelial cells, RhoA-ROCK signaling is essential for upregulation of CTGF by various microtubule disrupting agents, among them combretastatin A4, a tumor vessel-targeting drug [91]. Activation of FoxO transcription factors by inhibition of phosphatidyl inositol 3-kinase further increased the stimulating effect of microtubule disruption. The genomic effects of cytoskeletal deformation are, thus, modulated by additional factors. Indeed, microtubule disruption by combretastatin A4 induced CTGF expression more strongly when endothelial cells were cultured under hypoxic conditions compared to normoxic conditions [92].

Transactivation of Growth Factor Receptors and Downstream Signaling in Response to Cytoskeletal Deformation

EGFR activation and engagement of downstream MAP kinases (e.g., ERK1/2) is a common response to microtubule destabilizing drugs resulting in specific changes in gene expression [16,60,61,64,77,93,94] (Figure 2). EGFR phosphorylation upon microtubule disruption requires generation of reactive oxygen species (ROS) [e.g., 16], in sharp contrast to EGFR activation by native ligands [reviewed in 95]. Vascular cell shape perturbation by cytoskeletal deformation, moreover, involves engagement of at least a subset of receptor and non-receptor tyrosine kinase cascades (e.g., EGFR, Src) leading to gene reprogramming (e.g., in the case of PAI-1 and CTGF) [16,64]. Src kinase involvement, furthermore, is necessary for ERK1/2 activation as well as for PAI-1 and uPA transcription suggesting that cellular deformation-initiated Src signaling is a critical element in the expression of cell shape-sensitive genes [16,60,61,64,74,96–98]. ERK1/2 activation downstream of EGFR/Src induction, however, does not play a major role in CTGF expression (unlike PAI-1 and uPA induction) by microtubule disruption in VSMC indicating that further bifurcation of the signaling pathway downstream of the EGFR [16]. Despite reports that fibroblasts produce TGF-β transcripts upon cytoskeletal disruption [99], colchicine-induced PAI-1 and CTGF expression in VSMC is independent of autocrine release of TGF-β ligands or TGF-βR activity [16,61]. Indeed, microtubule disruption initiates SMAD2/3 phosphorylation, albeit in a delayed manner (2 hours) and in sharp contrast to the rapid (within 15 minutes) and robust SMAD2/3 activation following TGF-β1 stimulation This is consistent with findings that SMAD2/3 can be activated by TGF-βR-independent mechanisms (e.g., via MSP1; a component of the mitotic check point kinase) [16,90,100,101]. SMAD3, moreover, is essential for PAI-1 expression in both TGF-β1- and colchicine-treated VSMC [16] and, in most cells, for CTGF induction upon TGF-β1 addition [reviewed in 15]. Nevertheless, while there is evidence for ligand-independent activation of growth factor receptors as a consequence of cell deformation, the release of

soluble ligands in response to mechanical forces can also activate signaling events in the vasculature [102,103] further complicating delineation of the underlying mechanisms.

Molecular Mechanisms of Gene Control in Response to Cell Shape Perturbation

While there is ample evidence that members of the Rho family impact gene expression, the underlying molecular mechanisms, particularly those involving interplay with cellular structural elements, are only partially understood. The linkage between cytoskeletal remodeling and gene regulation, moreover, largely focus on RhoA signaling and its downstream effectors ROCK and mDia leading to increases in cellular F-actin structures and a corresponding decrease in monomeric actin. Monomeric or G-actin levels profoundly affect the activity of certain serum response factor (SRF)-responsive genes. In this regard, target genes activated by RhoA→actin→MAL→SRF signaling (e.g. CTGF or vinculin) differ from those which are activated via the MEK→ERK→TCF→SRF pathway [104,105]. The PAI-1 and CTGF genes display an interesting, albeit complex, dichotomy in expression control mechanisms by agents that alter actin/microfilament dynamics. Changes in the ratio of G- to F-actin affect CTGF expression as illustrated by the induction of CTGF by jasplakinolide, a drug that recruits monomeric G-actin into higher order F-actin structures, and the reduction in CTGF levels by latrunculin-mediated disruption of F-actin microfilaments [65]. Monomeric actin binds to and sequesters (in the cytoplasm) MAL/MRTF-A/MKL-1, a required cofactor of SRF, and in doing so interferes with the transcription of a subset of genes among them CTGF [67,106–108]. CD and swinholide A actually disrupt actin-MAL interactions [57], thus, stimulating SRF transcriptional activity and, thereby, leading to the induction of SRF-responsive genes (e.g., CTGF) (Figure 3). SRF targets genes with single or multiple copies of the SRF-binding element (the CArG box) and a CArG-like motif at position −3791 is present in the CTGF promoter [67]. CArG box-containing SRF response genes frequently have an adjacent ETS motif that is recognized by the ELK family of SRF co-factors. Two distinct subsets of co-activators, thus, modulate SRF activity. The MAL-like proteins, that are regulated by Rho GTPases, and monomeric actin and the TCF (ternary complex factor) family of Ets domain proteins (e.g., ELK-1, SAP-1, NET), that are activated by MAP kinases. Actin-regulated SRF-dependent gene expression is also subject to negative controls by MAP kinase (ERK) signaling [109,110] and high nuclear levels of monomeric actin [reviewed in 57]. While nuclear G-actin sequesters MAL, making this co-factor unavailable to SRF, ERK-mediated MAL phosphorylation, in contrast, promotes its nuclear export [109]. The PAI-1 gene, however, does not possess a CArG motif and is, thus, unlikely to be a direct SRF target. While TCFs regulate immediate early genes, including c-*fos*, *egr-1*, or *junB*, in association with SRF, PAI-1 expression is apparently SRF independent [111]. Indeed, SRF does not bind to the PAI-1 promoter region that recruits the TCF member Net and there are no obvious consensus SRF motifs in a 300 bp scan region either upstream or downstream of the Net site. Whether SRF binds to a more distal site with subsequent interaction with Net through extended spatial flexibility, however, cannot be excluded although the evidence for SRF independence of PAI-1 expression includes siRNA and ChIP [111].

Summary and Significance

Mechanosensory signaling pathways play a crucial role in vascular cell migration, proliferation and differentiation as well as disease progression [10,13,14,21]. The “tensegrity model”, for example, suggests that the plasma membrane is hardwired to the nucleus via cytoskeletal networks facilitating signal propagation in response to mechanical stimuli originating from either ECM modifications or changes in tensional forces due to alterations of blood flow [reviewed in 8,14]. Recent in vivo studies suggest that not all stress responses result in equivalent outcomes. Vascular endothelial cells can distinguish between laminar shear and

disturbed flow; these stimuli initiate two different biological responses. Laminar shear-induced mechanisms are associated with less inflammation and oxidative stress leading to an atheroprotective phenotype; disturbed flow, in contrast, results in significantly greater inflammation and increased oxidative stress, thereby, acerbating the atherosclerotic phenotype [21,112,113]. PAI-1 expression is augmented in response to mechanical forces such as shear stress. In terms of gene expression, CTGF belongs to a select group of genes which are suppressed by laminar flow but increased in areas of non-uniform shear stress [80], whereas others (e.g., endothelial NO synthetase) are regulated in the opposite direction. Since PAI-1 and CTGF contribute to the pathogenesis of cardiovascular disease and fibrosis, clarifying mechanisms associated with the disturbed flow leading to genomic reprogramming are crucial for identification of novel therapeutic targets. Moreover, the potential importance of morphology-linked controls on the transcription of profibrotic genes such as PAI-1 and CTGF are underscored by their marked induction in mechanical- and hypoxia-stressed, as well as growth factor-stimulated, cells [e.g.,15,69,78,85] and the obvious changes in cytoskeletal dynamics associated with these shape-altering physiological processes. It is increasingly apparent that microtubule and microfilament networks integrate signaling originating from integrin and growth factor receptors (largely due to direct interactions) and activate kinase cascades leading to alterations in gene expression. Several of the involved intermediates (e.g., SMADs, Rho-GEFs), moreover, are sequestered on the cytoskeleton in an “inactive state” and cell shape modifications due to fluctuating mechanical forces could dissociate some of these from their cytoskeletal anchors thus alleviating the “repressive state” leading to the downstream signaling. Not surprisingly, drug-based actin and tubulin cytoskeletal modifications lead cell deformation-sensitive genetic changes including PAI-1 and CTGF induction. Interestingly, comparative analysis of PAI-1 and CTGF expression in response to microtubule disruption in vascular smooth muscle cells highlights a complex network of both unique (e.g., SRF, ERK, SMAD) and common (e.g., Rho, EGFR) signaling elements in gene transcription. Given the emerging importance of these profibrogenic factors, further clarification of the involved pathways could yield novel therapeutic tools and targets to modulate the pahtophysiology of cardiovascular disease.

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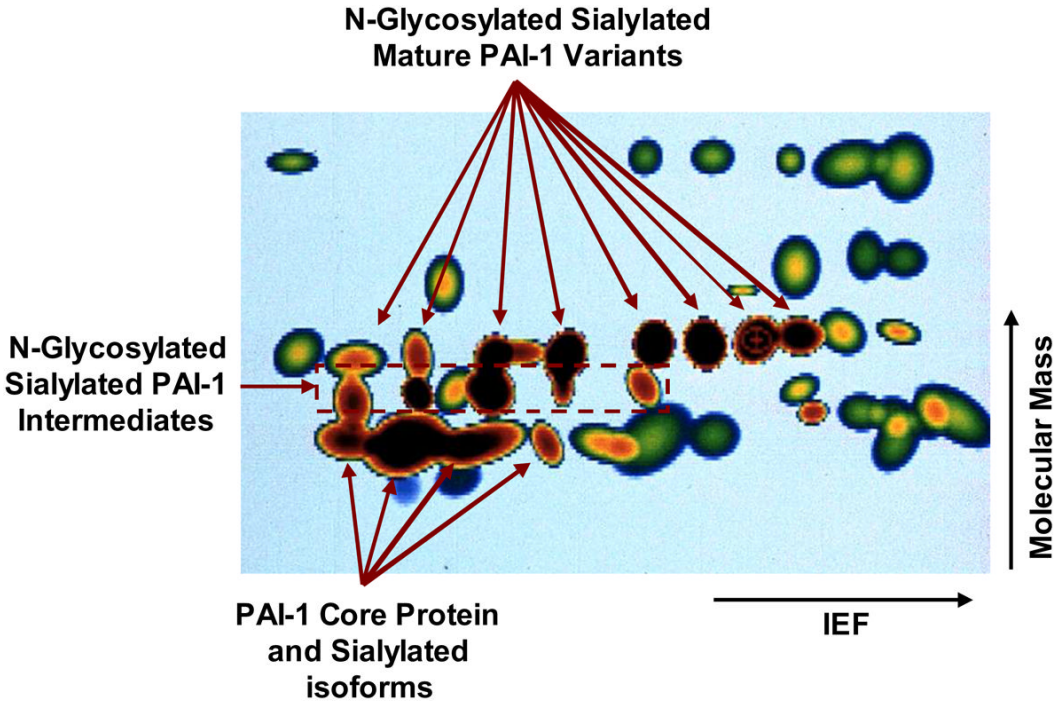

Figure 1. Proteomic mapping of cytochalasin D-induced PAI-1 expression in rat kidney epithelial cells

The ^{35}S-methionine-labeled, saponin-resistant fraction, of control and CD-stimulated rat kidney epithelial cells was separated by 2-D gel electrophoresis and the constitutent proteins visualized by fluorography. Computer-generated spot profiling was used to superimposition common control/CD-treated cellular proteins (green or green with red core spots) with proteins unique to CD-stimulated cells (dark brown spots). Combined 2-D electrophoresis/ immunoblotting identified the CD-induced (dark brown microheterogeneous protein group) as the various glycosylated isoforms of PAI-1 [51]. Directions of isoelectric focusing (IEF) and molecular mass separations are indicated.

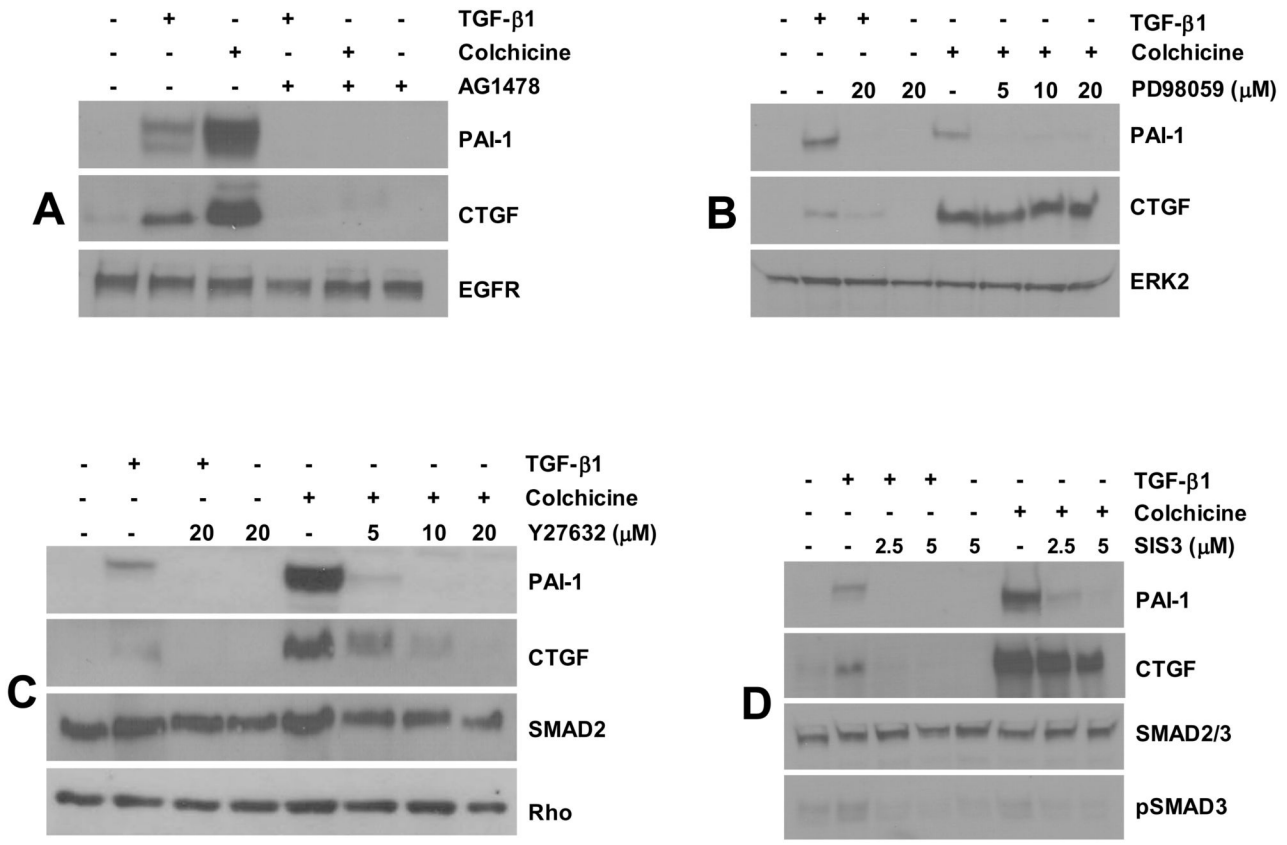

Figure 2. Signaling pathways involved in PAI-1 and CTGF expression in vascular smooth muscle cells

PAI-1 and CTGF expression in response to colchicine as well as TGF-β1 (serving as a classic "inducer") is virtually eliminated by AG1478 pretreatment (at a concentration of 2.5 μM) implicating EGFR in cell deformation induced signaling pathway **(A)**. While PAI-1 induction by microtubule deformation or TGF-β1 is sensitive to the MEK inhibitor PD98059 at the lowest concentration tested (5 μM), CTGF expression, in contrast, is unaffected by even the highest concentration of PD98059 (20 μM) **(B).** To investigate the signaling role of ROCK, a major downstream effector of RhoA, vascular smooth muscle cells were pretreated with Y-27632 prior to colchicine or growth factor stimulation. PAI-1 and CTGF induction in response to both stimuli is dose-dependently blocked by Y-27632 with virtual ablation of expression at concentrations of 10–20 μM (**C**). Pretreatment with SIS3, a specific SMAD3 inhibitor, eliminated PAI-1 expression (but not CTGF) upon microtubule disruption and, as expected, addition of TGF-β1 **(D)**. In all instances, treatment with colchicine or TGF-β1 utilized concentrations of 10 μM and 1 ng/ml, respectively.

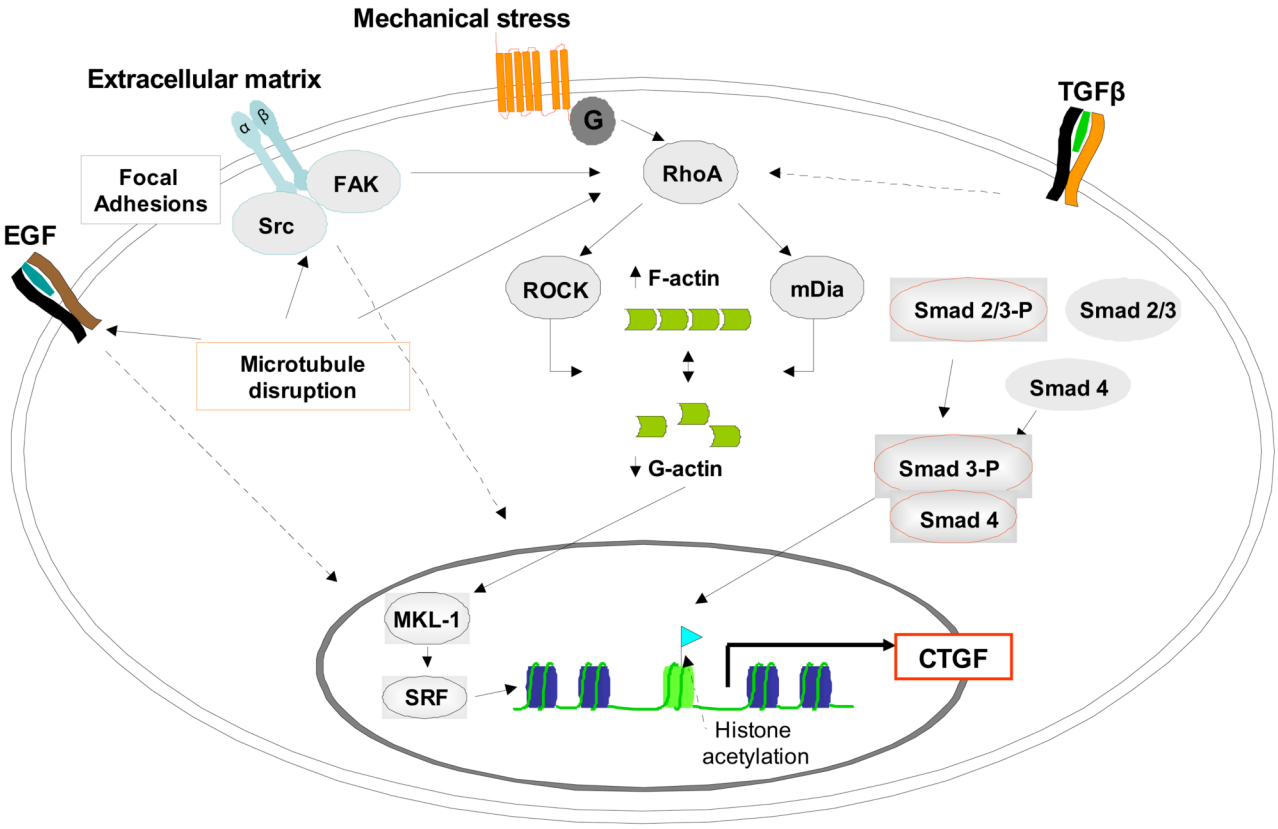

Figure 3. Simplified model of signaling pathways involved in the regulation of CTGF expression by cell shape deformation

Pathways are based on data obtained in different cell types (refer to text) and may not always occur simultanously. For the sake of clarity, several kinase pathways, e.g. p38 MAPK, PI-3K/AKT or PKC, have been omitted, although they contribute to the network of interacting pathways translating morphological alterations into gene expression.