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On Valve Interstitial Cell Signaling: The Link Between Multiscale Mechanics and Mechanobiology

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Abstract

Heart valves function in one of the most mechanically demanding environments in the body to ensure unidirectional blood flow. The resident valve interstitial cells respond to this mechanical environment and maintain the structure and integrity of the heart valve tissues to preserve homeostasis. While the mechanics of organ-tissue-cell heart valve function has progressed, the intracellular signaling network downstream of mechanical stimuli has not been fully elucidated. This has hindered efforts to both understand heart valve mechanobiology and rationally identify drug targets for treating valve disease. In the present work, we review the current literature on VIC mechanobiology and then propose mechanistic mathematical modeling of the mechanically-stimulated VIC signaling response to comprehend the coupling between VIC mechanobiology and valve mechanics.

Keywords

Valve interstitial cell; Mechanobiology; Cell signaling; Systems biology

INTRODUCTION

Opening and closing to ensure the unidirectional blood flow throughout the heart, heart valves are remarkable structures with a mechanically-driven physiological function. Alterations in valve deformation can stimulate growth and remodeling processes that attempt to maintain homeostasis and unidirectional blood flow, as exemplified during pregnancy.[89] However, the valve is not always able to fully accommodate atypical mechanical loading,

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CONFLICTS OF INTEREST

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as in ischemic mitral regurgitation (IMR) that prevents the mitral valve (MV) from fully closing[88] or the congenital development of a bicuspid (BAV) vs. tricuspid aortic valve (TAV).[56] Thus, the ability of heart valves to appropriately respond to their dynamic mechanical environment is crucial for preserving homeostasis, adapting during development, and preventing disease.

Loss of normal physiological function results in surgical valve repair or replacement. However, these surgical therapies do not allow for valves to grow with pediatric patients, are not durable enough to last throughout the remaining lifespan of middle-aged adults, and are ill-suited for most elderly patients since many are not ideal surgical candidates. Moreover, tissue engineering approaches that seek to produce living valve structures have not yet produced scaffolds suitable for use in humans.[114] Pharmaceutical treatments for combating valve disease are a promising alternative or adjuvant to current surgical therapies. However, there is a critical need for understanding how potential pharmaceutical therapies that work on the protein or gene level would propagate up to the biomechanics of valve function.

The resident heart valve interstitial cells (VICs) are crucial for preserving the functional mechanical properties of heart valves in response to both biochemical and mechanical stimuli. VICs are typically fibroblast-like under homeostatic conditions, but can be activated to a myofibroblast phenotype with increased contractility and alpha-smooth muscle actin (αSMA) expression as well as elevated extracellular matrix (ECM) production and remodeling capabilities under a wide variety of stimuli. Although other cell types such as valve endothelial cells[98] and infiltrating immune cells[85] also contribute to valve physiology and pathophysiology, myofibroblasts are the anticipated therapeutic target in many diseases characterized by excessive ECM production and remodeling,[103] including various heart valve diseases. However, targeting the signal transduction network responsible for myofibroblast activation is non-trivial since this activation involves virtually all major signaling pathways.[113] Aberrant mechanotransduction alone can trigger VIC myofibroblast activation[106] and the altered ECM production and remodeling capabilities of myofibroblasts subsequently creates an altered local mechanical environment. Thus, altered mechanotransduction either in response to macroscopic changes in valve deformation or microscopic changes due to local ECM production and remodeling due to myofibroblast activation is a critical component of the signaling network underlying valve diseases.

Computational modeling of valve interstitial cell signaling is an attractive way for comprehension of this vast signaling network and prediction of disease-relevant perturbations to that network. Purely experimental approaches that link external mechanical perturbations to changes in VIC gene/protein expression (e.g., References[58,73]) have not led to the identification of suitable drug targets for valve disease due, in part, to the lack of incorporated prior knowledge of the underlying signaling network responsible for the nonlinear input-output responses. Moreover, computational modeling frameworks can effectively integrate information from multiple experimental studies, connect *in vitro* and *in vivo* observations, and traverse disparate spatial and temporal scales. The importance of mechanotransduction in VIC phenotype and macroscopic valve mechanics to valve physiological function suggest a crucial need for understanding the impact of mechanical perturbations on all relevant spatial scales. This paper reviews the current literature of VIC

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mechanotransduction and provides a path forward for integrating this knowledge of VIC biology to valve physiological function through computational modeling.

ORGAN TO VIC MECHANICAL STIMULATION: MOTIVATIONS FROM THE MITRAL VALVE

Heart valves experience complex and dynamic tension, shear, and flexure over the course of a single cardiac cycle that impact valve behavior in a multiscale fashion from the organ to cellular scales. Moreover, the valve ECM is a dense network of collagen, elastin, and glycosaminoglycans, with material properties more similar to the dense planar connective tissues of the musculoskeletal system than other cardiovascular structures (e.g. blood vessels, myocardium).[91] Using the MV as a case study, details of the VIC microenvironment have been elucidated *via* focused ion beam scanning electron microscopy.[5] These three-dimensional (3D) reconstructions revealed an intimate integration of VICs within the surrounding collagen fibers and elastin inter-lamellar networks through their cytoplasmic extensions. Findings suggest that each VIC mechanically only interacts with the nearest 3–4 collagen and elastin fibers. This key observation suggests that in developing multiscale MV models, each VIC can be considered a mechanically integral part of the local fiber ensemble and is only influenced by more distant structures by the connecting ECM.

A well-established ovine model of IMR with myocardial infarction (MI) results in significant plastic radial deformations of the MV leaflet accompanied by altered ECM composition.[49] However, no changes in effective collagen fiber modulus were observed. A transcriptomic time course indicated that genes induced by yes-associated protein (YAP) were elevated at 4 weeks post-MI (indicating elevated mechanotransduction) and genes related to ECM organization were downregulated at 4 weeks post-MI when IMR occurred. All transcriptomic changes returning to baseline by 8 weeks post-MI. This multiscale study suggested that IMR induces plastic (i.e. permanent) deformation of the MV with neither functional damage to the collagen fibers nor changes in total tissue mass. Thus, it appears that VIC deformation alone likely plays an important role in altering VIC phenotype and remodeling. However, there remains a substantial disconnect between heart valve organ-level biomechanics and the accompanying cell-level phenomena.

As a step in addressing this issue, a multiscale computational model of the MV that links VIC to organ-level MV biomechanical behaviors was used to simulate changes in VIC deformation following surgical MV repair by undersized ring annuloplasty (URA).[3] A planar biaxial bioreactor system was used to cyclically-stretch explanted MV leaflet tissue, emulating the *in vivo* changes in loading following URA surgical repair. This simulation-directed experimental investigation revealed that post-URA surgical repair deformations resulted in decreased VIC activation and collagen mass fraction. These results are consistent with the hypothesis that reduced VIC-deformation-mediated maintenance of the MV leaflet tissue can reduce the physical stimuli required for leaflet tissue homeostasis. While there have been great strides in scaling from the organ- to cell-level to understand the fundamental processes of valve remodeling, there remains a critical need to simulate and predict how

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physiological and pathophysiological mechanical stimuli at the VIC cell membrane are converted into gene expression changes and altered VIC phenotype.

INTERACTIONS WITH THE MECHANICAL MICRO: ENVIRONMENT AND MATRIX MOLECULES

VICs sense the mechanical stimuli discussed in the previous section through cell-ECM and cell-cell[52,62] interactions as well as through release of growth factors sequestered in the ECM. In turn, these mechanical stimuli and growth factors can induce production of ECM components, proteases, and remodeling enzymes. This feedback loop where changes in valve mechanics lead to production of proteins that change valve mechanics has been a widely studied area for understanding the effects of different matrix epitopes and mechanical stimuli on VIC phenotype.

The native 3D VIC microenvironment is very complex,[5] and VIC deformation within this native environment under dynamic conditions remains largely unknown. Moreover, perturbation of native tissue systems and visualization remain quite restricted, warranting the use of *in vitro* platforms for elucidating the effects of different ECM components on VIC phenotype. Coating tissue culture poly-styrene (TCPS) with matrix molecules or peptides provides the simplest *in vitro* platforms for investigating the influence of matrix cues on VIC phenotype. Collagen-coated TCPS has been one way to overcome stiffness-induced VIC activation in culture,[82,90] although this is more relevant to the commonly used porcine VICs than to human VICs.[81] Coating TCPS with heparin, and to a lesser extent fibronectin, increases VIC myofibroblast activation over collagen coatings.[25] Heparin can stimulate VICs to express TGFβ and sequester this de novo TGFβ in close proximity to the VICs in culture.[25] Fibrin coatings induce calcific nodule formation and increase αSMA expression over uncoated TCPS, whereas fibronectin coatings inhibited nodule formation and αSMA expression compared to untreated TCPS.[10,42]

A variety of two-dimensional (2D) hydrogel systems functionalized with matrix-derived proteins and peptides have been used to separate the effects of matrix chemistry and stiffness. Hydrogels used for studying VICs vary in complexity from simple coatings on polyacrylamide gels[8,67,99] to highly extensible poly(ethylene glycol) (PEG) hydrogels that can be modified to present different epitopes for attachment,[39,111] incorporate protease-degradable crosslinkers,[39] present growth factors,[95] and dynamically modulate matrix stiffness.[106] The threshold for stiffness-induced activation is approximately 15 kPa[57,99] and a wide range of stiffnesses have been explored *in vitro* for investigating physiological and pathophysiological stiffness regimes in combination with various biochemical stimuli (Fig. 1).[8,24,39,57,67,97,99,106,111]

Encapsulating VICs in 3D hydrogel systems better mimics the native mechanical environment of the valve in vivo. Porcine VICs cultured in soft PEG gels with compressive modulus of 0.39 kPa exhibit gene expression profiles that more closely resemble that of freshly isolated VICs than VICs cultured on these hydrogels.[69] VICs express less αSMA when cultured in vs. on collagen hydrogels, but absolute αSMA levels and hydrogel compaction resembles that of aortic smooth muscle cells.[15] In addition to the tunable

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features for culturing VICs on hydrogels, VICs can be encapsulated in layered hydrogels[101] or hydrogel-fiber composites[83,102] to better emulate the structure of native heart valves. However, myofibroblast activation increases with decreasing stiffness when VICs are encapsulated within hydrogels,[48,68] possibly due to the decreased void volume immediately around each cell. Although more work is needed to uncover this counterintuitive relationship between activation and stiffness, such hydrogel systems can be used to modify integrin binding epitopes[42] or study the effects of biochemical stimuli on VIC activation[9,29,38] in a geometry that more faithfully represents the *in vivo* environment.

Although controlling the local tissue stiffness provides a useful starting point for investigating VIC response to mechanical stimuli, VICs experience a dynamic, cyclic mechanical microenvironment *in vivo*. Although high-throughput screening platforms have been used to investigate layer-specific VIC differences in the context of different cyclical loadings and TGFβ administration,[74] cyclic deformation is typically applied through tissue culture bioreactors of native valves or cell culture on deformable silicone membranes coated with ECM peptides, proteins, or hydrogels.[100] These systems can stimulate VICs with common sinusoidal cycling or more complex waveforms that mimic *in vivo* deformations.[7] Cyclic stretch increases collagen production in native tissue,[4,6,7] and the maximum amplitude of deformation can be used to preserve homeostasis and promote quiescence or simulate pathological scenarios by increasing or decreasing the maximum strain.[4] Notably, experimental studies seeking to mimic *in vivo* VIC cyclic deformation should also incorporate the effect of pre-strain (seen in the minimum strain amplitude).[3,65]

MECHANOBIOLOGY MECHANISMS IN VICS

Specific details of VIC myofibroblast activation may exhibit disease- and species-specific differences, but the current literature on VIC activation is too primitive to characterize such differences and evaluate their importance on heart valve function. Thus, our review centers on mechanobiological signaling mechanisms likely applicable to VIC myofibroblast activation in a variety of human valve diseases.

Integrins, Cadherins, and Mechanosensitive Ion Channels

Detailed mechanical properties of the cytoskeleton components have been described elsewhere.[76] However, much of the dynamic mechanical stimuli that alter VIC phenotype are processed by VICs and converted into biochemical reactions at the cell membrane via integrin and cadherin complexes as well as mechanosensitive ion channels.

Integrins facilitate cellular recognition of the surrounding ECM and connect extracellular biomechanics with intracellular actin/myosin-dependent biomechanics. Integrins are comprised of α and β subunits that form heterodimers (Fig. 2), where the α subunit plays a greater role in recognition of specific ECM motifs and the β subunit interacts with intracellular proteins to initiate signaling events.[105] Upon integrin activation, the cytoplasmic β tail binds to adaptor proteins such as talins that connect integrins to the actin cytoskeleton. Notably, the ECM-integrin-talin-actin complex exhibits multiple points of force regulation: (1) the ECM-integrin bond exhibits catch-bond behavior, (2) dissociation of the talin-actin bond exhibits slip-bond behavior, (3) talin contains crytpic binding sites for

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vinculin that are unveiled by force, and (4) vinculin binding to talin and actin stabilizes the talin-actin bond. The combination of these force-sensitive behaviors in these ECM-integrin-talin-actin complexes creates a “mechanical clutch” (Fig. 3).[18]

VICs express a wide variety of integrin complexes that facilitate their role in remodeling the ECM in different areas of the leaflet. VICs express $\alpha_2\beta_1$ to bind DGEA motifs on collagen.[63,96,111] VICs cultured on collagen are resistant to calcification; however, blocking $\alpha_2\beta_1$ or culture on DGEA alone does not alter calcification potential, pointing to another integrin dimer for this collagen-mediated inhibition of nodule formation.[42] VICs also express α_V[9,111] and $\alpha_5\beta_1$[42] integrin for binding to RGD motifs in fibronectin, collagen, fibrin, laminin, and other ECM proteins. Finally, VICs express non-integrin membrane proteins that bind the ECM, including the 67-kDa LN receptor that binds laminin and collagen[42,111] as well as CD44 that binds hyaluronic acid and matricellular proteins.[8,80] The many receptor-ECM complexes that can form *in vivo* creates a complex coupling between VICs and their environment that is only beginning to be unveiled.

Cadherins facilitate mechanosensing between cells within the heart valve. Traditional cadherins form homotypic Ca^{2+}-dependent connections between neighboring cells in a Ca^{2+} manner (Fig. 2). Moderate levels of force cause α-catenin to unravel in an analogous manner to talin, allowing vinculin to stabilize the linkage between α-catenin and actin.[72]. Cadherin connections also exhibit force-senstive dissociation and the cadherin-11 contacts between myofibroblastic VICs can withstand higher forces than the cadherin-2 contacts between quiescent VICs.[52,79] Moreover, cadherin-11 connections have a higher affinity for Ca^{2+} than cadherin-2 connections. This combination of differences in Ca^{2+} and force sensitivity likely change cell-cell connections from transient, infrequent events[79] to stable aggregation in end-stage calcific aortic valve disease.

Mechanosensitive ion channels regulate ion transport, including Ca^{2+}, in response to altered mechanics (Fig. 2). Static biaxial strain induces rapid intracellular accumulation Ca^{2+}.[54] Moreover, calcium-channel inhibitor $GdCl_3$ significantly slows Ca^{2+} accumulation and this accumulation is not seen when Ca^{2+}-free media is used.[54] VICs cultured on soft quiescence-promoting hydrogels and stiff, activation-promoting hydrogels exhibit similar levels of pFAK/FAK despite differences in αSMA expression. However, αSMA levels and stress fiber formation can be reduced with the mechano-sensitive ion channel inhibitor GsMTX when VICs are cultured on supra-physiologically stiff TCPS, indicating VICs sense material stiffness primarily through mecahno-sensitive ion channels.

Rho-Family GTPases Control the Actin Cytoskeleton

Rho-family GTPases are activated downstream of integrins, cadherins, and ion channels. These proteins switch between inactive, GDP-bound and active, GTP-bound forms and are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs catalyze the exchange of GDP for GTP to promote signaling and GAPs hydrolyze GTP to reduce signaling. RhoA signaling through mammalian Diaphanous (mDIA) and Rho-associated protein kinase (ROCK) catalyzes actin stress fiber polymerization and myosin-generated tension, respectively.[45,59] This mechanically-stimulated RhoA pathway is illustrated in Fig. 4.

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RhoA-family GTPases regulate both myofibroblast activation, ECM remodeling, and mineralization in VICs. In porcine VICs, nodule-promoting environments caused by TCPS and fibrin coatings lead to elevated RhoA and ROCK activation compared with collagen or fibronectin coatings.[43] Forcing RhoA activation with oleoyl-L-α-lysophosphatidic acid (LPA) induces ROCK activation and αSMA localization to stress fibers.[43] Decreasing RhoA activation with polyunsaturated fatty acids or C3 transferase leads to an increase G-/F-actin, promoting VIC quiescence.[110] Decreased RhoA activation following polyunsaturated fatty acid treatment also leads to reductions in proteins and post-translational modifications that regulate the actin cytoskeleton including cofilin phosphorylated on S3, total cofilin, profilin, and phosphorylated myosin phosphatase.[110] Total and phosphorylated cofilin are elevated in activated VICs, facilitating stress fiber formation and stability.[77] RhoA/ROCK activity is necessary for the expression of ENPP1 and induction of mineralization *in vitro* in response to 15% cyclic strain (emulating pathophysiological stretching) and mineralization media.[13] Moreover, stenotic BAVs exhibit elevated ENPP1 expression over stenotic TAVs, and ENPP1 is preferentially located in the high strain region of the BAV raphe.[13] Expression of dominant negative RhoA decreases expression of αSMA in control media and decreases expression of COL1A1, COL3A1, MMP9, ALP, TGFB1, and RUNX2 in both control and osteogenic media during *in vitro* cyclic stretch.[34]

Although RhoA activity in VICs is closely tied to stress fiber formation and αSMA expression, RhoA is not the only GTPase that affects VIC phenotype. Acute cyclic stretch promotes RhoA/ROCK activation[13,40] whereas chronic cyclic stretch promotes Rac1 activation via a FilGAP-dependent mechanism.[40] siRNA for Cdc42 stops VIC proliferation and caused them to detach from the support.[11] Although RhoA and Rac1 are linked to basal CTGF expression, the increased expression of CTGF in response to cyclic stretch is linked to RhoC activity.[11]] Thus, Rho-family GTPases beyond RhoA contribute to VIC phenotypic changes.

Myocardin-Related Transcription Factor A (MRTFA) Rregulates αSMA Expression

Many smooth muscle cell—proteins, including αSMA, are regulated by myocardin-related transcription factor A (MRTFA). Increased stress fiber formation frees MRTFA from cytoplasm sequestration by monomeric actin. MRTFA subsequently enters the nucleus, binds its co-transcription factor serum response factor (SRF), and promotes the expression of cytoskeleton-related genes including ACTA2/αSMA (Fig. 5).[104] Therefore, MRTFA provides a crucical feedback mechanism regulating myofibroblast activation where a decrease in the monomeric, G-actin pool through increased polymerization induces MRTFAmediated αSMA expression. MRTFA is the key transcription factor governing VIC expression of αSMA. Calcified valves express MRTFA, smooth muscle mysoin heavy chain, and αSMA at elevated levels, but not myocardin.[61] Stimulation with cyclic stretch[11] or TGFβ[61] leads to MRTFA accumulation in the nucleus. Inhibiting RhoA activation and actin stress fiber formation with polyunsaturated fatty acids decrease nuclear translocation of MRTFA.[110] Thus, MRTFA regualtes αSMA expression in VICs.

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Mechanosensing Via YAP

Activation of canonical Hippo signaling through MST and LATS phosphorylates YAP on multiple residues, with S127P sequestering YAP in the cytoplasm and S381P facilitating ubiquitination and degradation.[115] Exclusion of YAP from the nucleus limits organ size and cell proliferation. Upon nuclear accumulation, YAP binds to co-transcriptional regulators TEAD1/4 to stimulate the expression of cytoskeletal, proliferative, and antiapoptotic genes.[35] YAP nuclear localization in response to mechanical stimuli requires RhoA and myosin-generated cytoskeletal tension and can be regulated by both LATS-dependent and LATS-independent mechanisms (Fig. 5)[21,30] Notably, force can directly influence YAP nuclear translocation by regulating transport through nuclear pores [32]. In cancer-associated and normal fibroblasts, cell contractility induced by MRTF/SRF and TGFβ signaling induced by YAP/TEAD are mutually dependent on the ability of both transcription complexes to control cytoskeletal dynamics.[35]

YAP is a key transcription factor involved in mechanotransduction in the heart valve. CTGF expression in static human mitral VIC cultures decreases with siRNA targeting RhoA or Rac1,[11] indicating these GTPases affect YAP nuclear translocation. A recent study of gene expression changes in an ovine model of ischemic mitral regurgitation found that YAP-induced genes were some of the most upregulated as the valve is stretched in this pathology.[49] In valves explanted from patients with AI or AS, VICs with nuclear YAP staining were preferentially located near stiff calcific occlusions. Moreover, when culturing these human VICs on collagen-coated PA gels, YAP is preferentially localized in the nucleus at 15 and 23 kPa for AI- and AS-derived VICs, respectively.[93] YAP phosphorylation on Ser127 could be increased by ROCK inhibition, decreasing expression of the YAP target genes ANKRD1, CTGF, and CYR61. When porcine VICs are cultured on two-dimensional PEG hydrogels, YAP is preferentially located in the nucleus at stiffnesses above 5 kPa and focal adhesion size is correlated with YAP nuclear localization.[70] The slight difference in the stiffness threshold for YAP localization is likely due to a combination of differences in techniques to measure stiffness, signaling through RGD- vs. collagen-bound integrins, and organism of origin. αSMA also decreased in response to ROCK inhibition although not as strongly as canonical YAP-target genes; thus, the effects of YAP on αSMA are likely indirect[93] and connected by cytoskeletal dynamics.[35]

Akt and β-Catenin Mediate VIC Activation

Activation of Akt via phosphorylation downstream of phosp-hatidylinositol-4,5-bisphosphate 3-kinase (PI3K) is a major mechanism regulating VIC phenotype in response to various stimuli. PI3K and subsequent Akt activation occurs downstream of integrininduced FAK activation[19] and Ca^{2+}-induced protein kinase C (PKC) activation.[37] *In vitro* culture on TCPS increases pAkt/Akt compared to freshly isolated porcine VICs and PI3K inhibition reduces the number of myofibroblasts and prevents nodule formation on stiff PEG hydrogels or TCPS.[107] Cyclic stretch also induces Akt phosphorylation.[60] *In vitro*, H_2O_2-mediated RUNX2 accumulation over 24h is mediated by pAkt.[14] However Akt and pAkt were reduced in human valves explanted for CAVD[31] and elevated pAkt in response to osteopontin-CD44 inhibits BMP4-induced calcium deposition.[80] FGF-2 also increases pAkt,[44,60] but counteracts TGFβ-induced VIC myofibroblast activation.[26,38] Akt1

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is a negative regulator of the nuclear factor-κB (NF-κB) pathway and phosphate-induced expression of IL6 in aortic VICs.[31] Thus, altered Akt activation is associated with both homeostatic-preserving and pathological processes in VICs.

β-catenin nuclear localization is another mechanism promoting VIC myofibroblast activation. Although β-catenin is typically considered part of the Wnt pathway, other Wnt-independent pathways can lead to nuclear accumulation of β-catenin and subsequent transcription events. In particular, phosphorylated Akt can inhibit β-catenin degradation and cytoplasmic sequestration via phosphorylation of glycogen synthase kinase β on Ser9.[71] Inhibition of GSK3α/β phosphorylation reduced ALP activity and calcium deposition induced by lipoprotein (a) (Lp(a)).[112] Loss of β-catenin signaling in a Periostin/Cre mouse model leads to the expression of a chondrogenic gene program and excessive proteoglycan accumulation downstream of Sox9.[33] In porcine VICs, β-catenin nuclear localization can be induced either with TGFβ or changes in matrix stiffness and siRNA targeting β-catenin reduces TGFβ-induced expression of αSMA.[20] Cyclic stretch also increases expression of β-catenin.[17] β-catenin interacts with the MRTFA promoter in human breast cancer cells,[46] providing a possible link between mechanical stimuli—induced β-catenin nuclear localization and subsequent αSMA expression in VICs.

Mitogen-Activated Protein Kinase (MAPK) Cascades

VIC myofibroblast activation also proceeds through various mitogen-activated protein kinase (MAPK) cascades. Canonical MAPK activation involves a triple kinase activation scheme where the MAP kinase kinase kinase (MAP3K) phosphorylates the MAP kinase kinase (MAP2K) which subsequently phosphorylates the MAPK. Active MAPKs then lead to phosphorylation of transcription factors that localize to the nucleus and promote their genetic targets. Three major MAPK cascades have been described: extracellular signal-related kinase 1/2 (ERK1/2), p38, and c-Jun NH_2-terminal kinase (JNK). These MAPK cascades and their role in VIC myofibroblast activation are discussed below.

ERK1/2 are key MAPKs affecting VIC phenotypic activation. TGFβ-induced dystrophic calcification proceeds through an ERK-dependent mechanism,[41,52] and blockade of ERK phosphorylation inhibits cadherin-11 expression but increases αSMA expression.[52] This increase in αSMA expression is likely due to the competition for SRF between the Ets-family of transcription factors (activated by ERK1/2) and MRTFA.[78,108]] Moreover, ERK1/2 activation is a key mediator of FGF2-mediated inhibition of TGFβ-induced myofibroblast activation in VICs.[26] However, inhibition of MKK1/2 phosphorylation, and subsequent ERK1/2 activation, induced by lipoprotein a (Lp(a)) increases calcium deposition.[112] The contradictory role for ERK1/2 activation observed for TGFβ-induced vs. Lp(a)-induced calcium deposition warrants further investigation.

Multiple external signals converge on p38 activation and VIC myofibroblast activation. p38 is activated when VICs are stimulated with angiotensin II,[84] Lp(a),[112] TGFβ,[53] BMP-6,[1] and CXCL9.[1] Inhibition of p38 phosphorylation decreases calcium deposition[53,112] and myofibroblast activation.[1,53] p38 is also activated by human serum extracted immediately prior to transcathetar aortic valve replacement.[1] Clearly, p38 is an important modulator of

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VIC activation, but the precise mechanistic details downstream of p38 activation have not yet been documented in VICs.

MAKING SENSE OF MECHANOBIOLOGY *VIA* MATHEMATICAL MODELING

The mechanically-regulated signaling network regulating VIC phenotype is large and complex. A schematic of the key mechanically-directed signaling modules discussed above is presented in Fig. 5, but many of the details of each module are currently unknown. The details of this signaling network are obfuscated by the complex ECM in heart valves[5] that transmits stresses between the tissue- and cell-levels in the valve and its evolution over time due to VIC-directed remodeling. Moreover, myofibroblast activation can also be stimulated by cytokines, matricellular proteins, oxidative stress, *etc*. further increasing the complexity of the signaling network. In fact, myofibroblast activation involves most known signaling pathways[113] and significant cross-talk between these pathways creates a dense signaling network rather than isolated parallel pathways; for instance, in other cell types, YAP can bind β-catenin,[55] ROCK can affect Akt activation *via* PTEN,[66] and TGFβ-induced myofibroblast activation requires engagement of b_3 integrins[36] and the mechanosensitive ion channel TRPV4.[86] This VIC signaling network is also extensively regulated by miRNAs,[22] increasing the complexity of the signaling network beyond protein-protein interactions. Moreover, the effects of individual proteins such as Akt and ERK1/2 exhibit a context dependence in VICs where the activation level of an individual protein is linked to conflicting phenotypic outcomes in response to different stimuli. The combination of many active signaling pathways, their interactions, and the possible context-dependence of individual proteins makes it difficult to determine key mechanisms governing VIC phenotypic changes and modulate them pharmaceutically.

Mechanistic modeling of the signaling network governing VIC activation is a promising way to comprehend the complex signaling network. Models of isolated signaling pathways relevant to VIC activation have revealed emergent dynamic behaviors, including the bistability of Rho/Rac activation,[16] oscillations in NF-κB,[75] and ultrasensitivity in MAPK activation.[50] If these isolated pathways can exhibit such rich dynamical features, what dynamics will emerge in the entire myofibroblast activation network? These multi-input, multi-output, nonlinear processes are impossible to fully comprehend through logic or “pictorial models” alone and the failure to rationally design pharmaceutical treatments for valve disease is likely due to the lack of understanding the system as a whole.[94]

The integration of mathematics with experimental biology and physiology has provided foundational results in cardiovascular biology, with notable examples by William Harvey in 1628 and AV Hill in 1909. William Harvey combined experimental observations on the one-way valves in the veins of the human arm with a mathematical analysis showing that the human heart pumps more blood per hour than is present in the body.[23] This integration of experiments with mathematics allowed Harvey to conclude that blood could not originate from the consumption of food and that blood does not ebb and flow in the arteries and veins; rather, blood flows unidirectionally and there must be small vessels that connect the arteries and veins. The first documented visualization of capillaries under a microscope would not be documented for more than fifty years.[23] A student of JN Langley,

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the mathematically-inclined AV Hill stimulated contraction of frog rectus *abdominis* with nicotine. Using reversible mass-action kinetics, Hill demonstrated his equilibrium results fit the now well-known sigmoidal concentration-effect curve, concluding that his results showed “very strong evidence in favour of a combination between nicotine and some constituent in the muscle”.[47,87] Further mathematical studies contributed to the idea of these mathematical/theoretical receptors, even though the molecular counterparts would not be purified, sequenced, and cloned until the mid 1970s.[87]

While the use of mathematics and computation in biology is not new, the explosion of “-omics” data has transformed the study of intracellular signaling pathways from a data poor to data rich environment, facilitating mechanistic modeling of signaling processes and a systems-level understanding. It is important to distinguish mechanistic models from statistical data analysis techniques as the latter is not sufficient for determining how components interact and change dynamically and, more importantly, predict the results of new experiments. Thus, the collection of data alone through “-omics” technologies does not provide a systems-level understanding. In fact, “the distinguishing feature of systems biology is its combination of computational models and quantitative experimental data as a means to generate formal representations of biological process and discover network-level (‘emergent’) properties not evident from the study of individual components”,[94] placing the mechanistic model informed by experimental data at the cornerstone of systems biology. A mechanistic model provides a conceptual framework that can be used to understand the data and design new experiments that add to this knowledge.[28,94] This is more important for mechanobiological investigations since physical forces typically reversibly modulate the 3D confirmation of proteins (e.g., the unveiling of cryptic binding sites in talin[27] or the regulation of YAP transport through nuclear pore complexes[32]) and these protein structural changes are not detected through typical proteomics investigations[12]

Heirarchical and multiscale modeling has the potential to explain valve-level (patho-)physiological mechanics from the cell-level mechanobiological interactions between multiple signaling pathways, multiple cells, and cells with their immediate microenvironment. Multiscale models are needed because valve biomechanics occurs at multiple length scales[92] and the evaluation of putative drugs and drug targets on valve biomechanics is inherently a multiscale problem.[94] Existing multiscale frameworks have provided a crucial first step in this direction, connecting VIC and leaflet deformations[51,64,109] and connecting altered hemodynamics to VIC activation and calcification in the context of calcific aortic valve disease.[2] However, none of these models connects down to the level of the VIC signaling network. Since pharmaceuticals act on the proteins in the signaling network and efficacious drugs and drug targets have not yet been identified for heart valve disease, integrating cell signaling within a multiscale computational framework of valve mechanics provides a path forward to understand how perturbations to this signaling network impact valve-level biomechanics.

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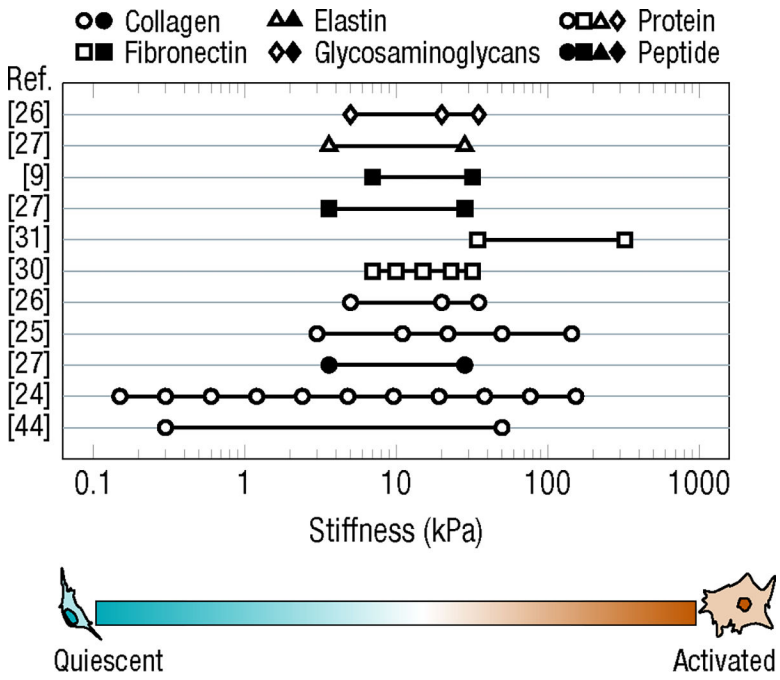

FIGURE 1. Reports on the effects of substrate stiffness in the context of various ECM motifs to explore the mechanically-driven VIC phenotype transition in 2D.

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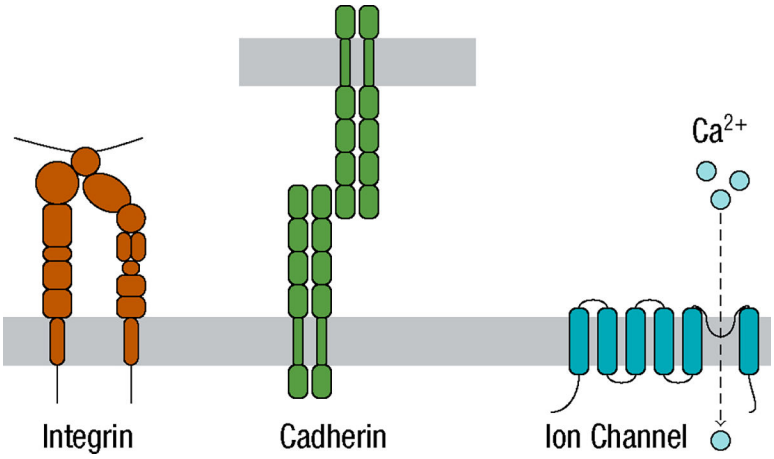

FIGURE 2. Integrins, cadherins, and mechanosensitive ion channels allow VICs to respond to their mechanical microenvironment.

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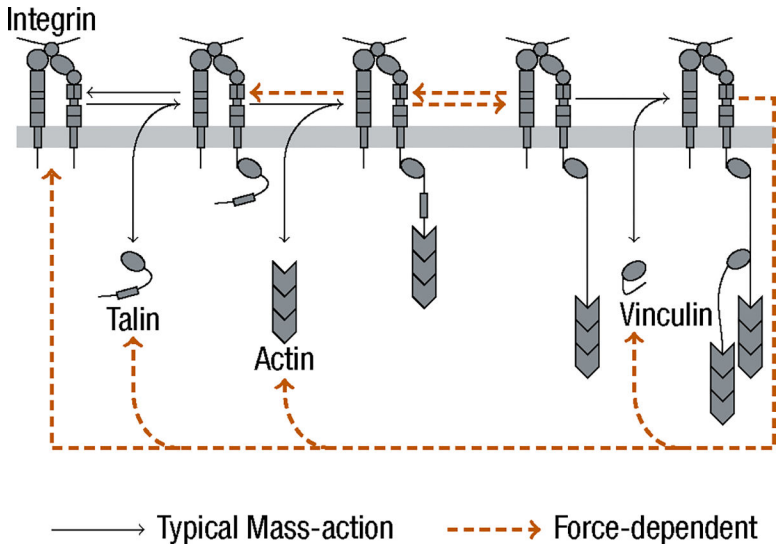

FIGURE 3. Illustration of the "molecular clutch" model of integrin engagement first proposed in Reference 18. The weakest force-sensitive bond moves from the bond between talin and actin to the bond between ECM and integrin upon vinculin stabilization. Cadherin activation can me similarly illustrated.

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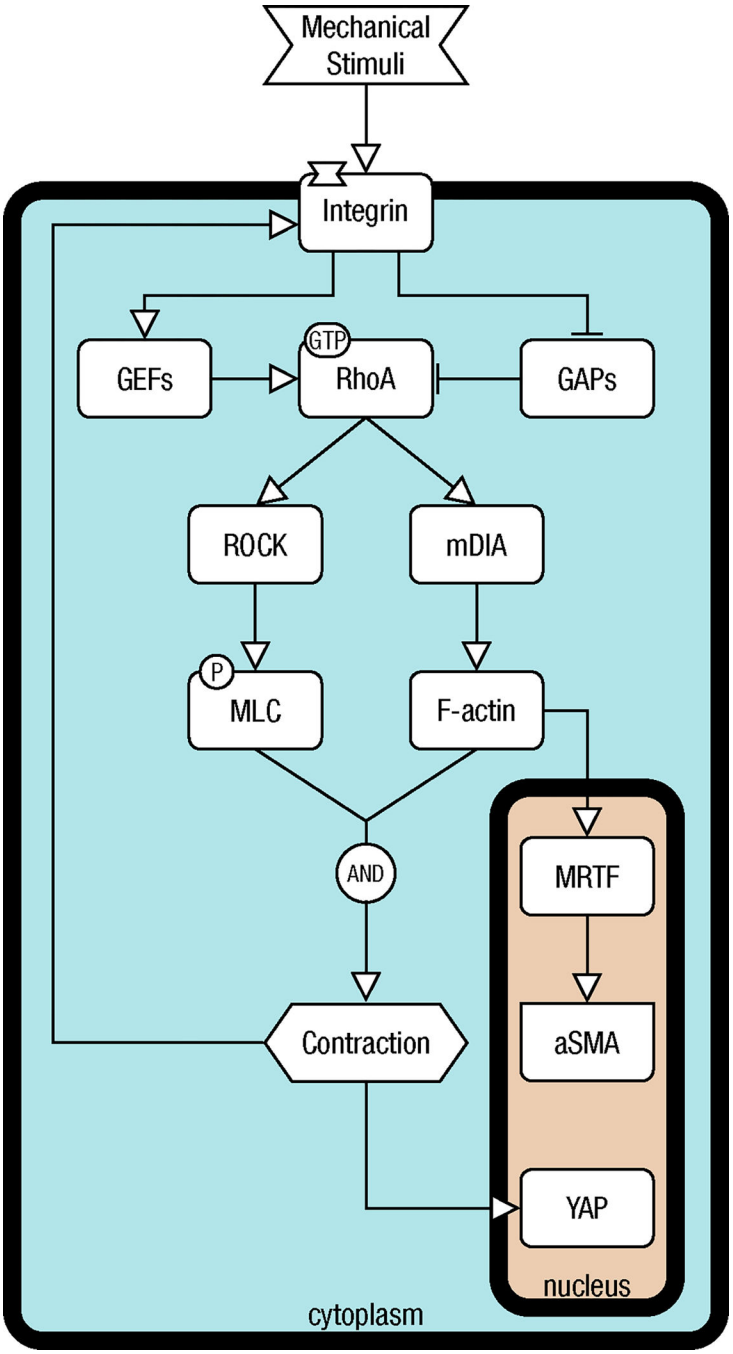

FIGURE 4. Illustration of the RhoA-dependent pathways downstream of integrin activation that lead to MRTF and YAP nuclear localization as well as αSMA gene expression in VICs. Most of the intricacies are currently unknown, so many details have been omitted.

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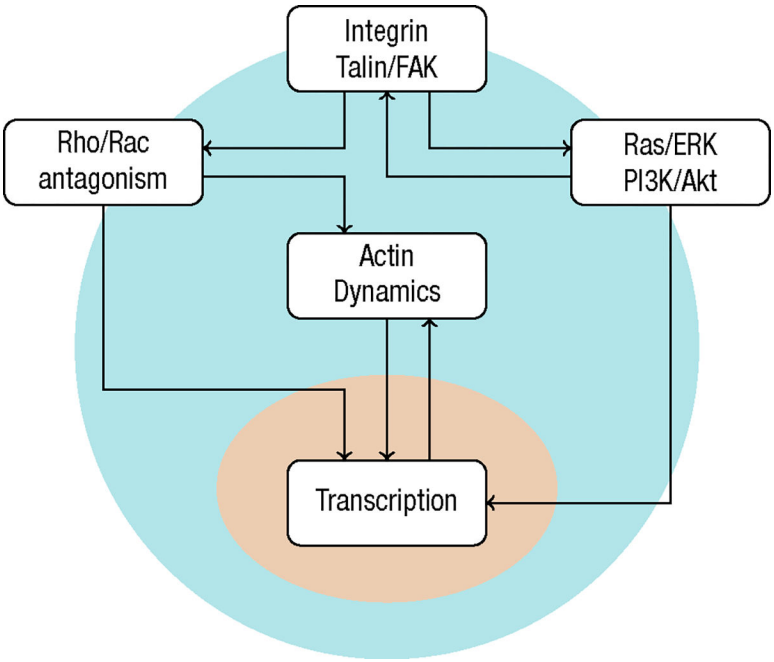

FIGURE 5. Illustration of the key signaling modules involved in VIC mechanobiology.