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Mechanical Forces Reshape Differentiation Cues That Guide Cardiomyogenesis

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Abstract

Soluble morphogen gradients have long been studied in the context of heart specification and patterning. However, recent data have begun to challenge the notion that long-standing in vivo observations are driven solely by these gradients alone. Evidence from multiple biological models, from stem cells to ex vivo biophysical assays, now supports a role for mechanical forces in not only modulating cell behavior but also inducing it de novo in a process termed mechanotransduction. Structural proteins that connect the cell to its niche, for example, integrins and cadherins, and that couple to other growth factor receptors, either directly or indirectly, seem to mediate these changes, although specific mechanistic details are still being elucidated. In this review, we summarize how the wingless (Wnt), transforming growth factor-β, and bone morphogenetic protein signaling pathways affect cardiomyogenesis and then highlight the interplay between each pathway and mechanical forces. In addition, we will outline the role of integrins and cadherins during cardiac development. For each, we will describe how the interplay could change multiple processes during cardiomyogenesis, including the specification of undifferentiated cells, the establishment of heart patterns to accomplish tube and chamber formation, or the maturation of myocytes in the fully formed heart.

Keywords

cadherins; heart; integrins; stem cells; transforming growth factor

Development from single fertilized cells into the diversity we enjoy in the animal kingdom is a highly conserved process driven by robust signaling gradients. Remarkably, similar gradients create simple patterns like the body axis[1] as well as complex ones such as fruit fly wing development[2] by controlling an equally diverse set of cell behaviors, for example, migration, proliferation, and specialization.[2] These behaviors, in turn, are affected by spatiotemporal changes in ligand composition, gradient strength and magnitude, and combinations of agonists and antagonists (which has been more completely reviewed elsewhere[3]). For myocardium specifically, many highly specialized cardiac lineages arise from a set of common progenitors but specify based on differences in the factors to which

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they were exposed.[4–6] These descriptions primarily rely on soluble cues, but a growing body of literature suggests that specification and subsequent tissue patterns can also arise from physical changes. Whether examined in vitro or in vivo, these cues come in many forms; generally they can be categorized as passive stimuli that occur within the microenvironment surrounding each lineage[7–9] or as active forces that are directly applied to cells by other cells or fluids.[10–13] For the former case, cells must directly probe the environment through myosin-mediated contractions to sense changes in their surroundings and use the mechanical feedback it provides to elicit changes in cell behavior.[14,15] Conversely the latter case requires the cell to remodel internally or migrate to transmit the load,[16] a process that could activate similar molecular mechanisms.[15] Regardless of their type and mechanism, these cues can be studied in model organisms[10] or built into protocols to direct stem cell specification or to test their influence more generally as a cue on cell behavior.[17] Although significant evidence from developmental biology exists on soluble gradients, there is growing evidence using unique physical assays ex vivo and engineered microenvironments in vitro that mechanical cues and their interplay with chemical signals influence specification, which we will focus on here.

Although we will summarize what is currently known about the influence that forces and their transduction have on cardiomyogenesis, it would be naive to presume that these pathways are distinct, nonintersecting, or orthogonal to each other, whether or not you limit yourself to physical stimuli only. So whether used separately or in combination with the plethora of soluble morphogen gradients, data described here will suggest a more complex integration of signals such that stimuli are not additive but rather can be compensatory or antagonistic.[18,19] We will provide further examples where physical cues, as with their molecular biology counterparts, can differentially regulate a process depending on the specific lineage or stage of the cell. As an example at the outset of this discussion, changes in wall stiffness can influence tube looping when presented during tube formation[11] but in adulthood could lead to fibrosis.[20] Just as with soluble factors, physical stimuli can vary with space and time[9] and must be transmitted between myocytes and transduced to the nucleus to be interpreted.[15,21] So in the following sections, we will address how mechanical forces drive lineage specification of early progenitors and their subsequent differentiation, specifically focusing on the interaction of forces and established signaling pathways.

Mechanical Cues in Early Myocardial Specification: Gastrulation and Mesoderm Formation

Cardiomyogenesis in vivo is governed by spatiotemporally defined signaling pathways that regulate transcription factor (TCF) expression to drive myocardial specification, differentiation, and maturation.[5,6] In concert with these chemical signals, mechanical cues act on the developing embryo throughout the process of cardiac development. In this first section, we will focus on the earliest specification decisions in model organisms and how mechanics regulates these decisions. Murine models indicate cardiac specification as early as embryonic day 6.5 (E6.5) where a population of cardiac precursor cells residing in the lateral posterior epiblast develops before primitive streak (PS) formation[22,23] and gastrulation. As epiblast cells migrate through the PS, they undergo epithelial–mesenchymal

transition (EMT) to form early mesoderm, which will undergo a series of morphological changes, beginning with formation of the cardiac crescent, to develop into the heart. After EMT and formation of nascent mesoderm, irreversible myocardial commitment occurs,[22,24] indicating that cues during this transition, either chemical or physical, drive cardiac determination. To better orient this discussion, Figure 1 highlights developmental stages of the heart and when in time signaling pathways and mechanics are prevalent. Mechanical signals play a vital role throughout the entirety of the process, beginning in early cardiomyogenesis by initiating gastrulation and contributing to the establishment of chemical gradients that drive early axis development and subsequent cell specification, commitment, and determination. Two such examples of the mechanical influence in early myocardial specification are cell tension–induced tissue invagination and mechanically generated fluid flow–driven chemical gradients. Both are described in the following section and summarized in Figure 2.

In *Drosophila melanogaster* morphogenesis, gastrulation begins after the apical constriction of a population of cells at the ventral midline.[25] The apical surface of the cells shrinks in size because the basal surface increases, creating tension at the cell surface that triggers tissue invagination. Expression of TCFs Twist and Snail induce apical constriction during which myosin II localizes to the apical surface of the cells to generate contractile pulses necessary for cell shape remodeling.[26,27] Snail triggers contraction, whereas Twist prevents relaxation, creating a ratchet-like process to achieve constriction, and embryos mutant in either *snail* or *twist* do not gastrulate properly. It has been proposed that Twist expression during gastrulation is maintained not by direct biochemical signals, such as *snail* expression, but instead by the mechanical cues that occur during PS formation.[28] Ectopic expression of Twist can be induced by mechanically deforming *Drosophila* embryo, demonstrating its mechanosensitivity.[29] In addition, in *Drosophila* mutants lacking Snail expression that consequently exhibit abnormal gastrulation, mechanical indentation rescues mesoderm invagination via the Twist signaling pathway and gastrulation proceeds.[30] This mechanically induced rescue indicates that it is the contractile pulses generated by Snail and not a Snail-mediated intracellular signaling mechanism that enables gastrulation to proceed. Twist induces the expression and secretion of folded gastrulation, a critical protein leading to myosin II apical localization and constriction.[31,32] In the absence of mechanical force, folded gastrulation is endocytosed, its signaling pathway is not activated and myosin II localization does not occur.[30] However, in the presence of mechanical force applied by either mechanical indentation or Snail-induced pulsatile contraction, folded gastrulation endocytosis is inhibited and it remains in the extracellular space.

After EMT markers, the earliest myocardial regulators, for example, *Mesp1/2* and *Fgf8*, are expressed by the gastrulating mesoderm and mediate cardiac precursor cell migration through the PS[33,34]; interruption in expression of either of these genes results in abnormal heart development.[35,36] In the developing chick embryo, *Fgf8* expression is symmetrical across the PS but becomes asymmetrical by Hamburger Hamilton stage 5, residing primarily in the right side of the PS node as the mesodermal layer forms.[37] The origin of embryonic asymmetries are debated with some evidence indicating that early lineage bias drive later asymmetries, whereas other evidence argues for a stochastic, environmentally driven

model.[38,39] In addition to the chemical cues known to regulate PS node asymmetry, mechanical forces can also influence the patterning of developmental gradients. For example, during mouse development, Fgf8 signaling induces secretion of sonic hedgehog and retinoic acid containing vesicles; both are factors that initiate embryonic tissue patterning required for proper mesodermal development.[40] Leftward sonic hedgehog and retinoic acid gradients across the node are then created by fluid flow, termed nodal flow, that is generated by cilia forces on nodal cells (Figure 2).[41] Disruption of nodal flow in knockout mice was embryonic lethal and created a variety of developmental abnormalities, including pericardial sac ballooning and reversed heart tube looping.[42] In chick PS where nodal cells do not express cilia and there is no extraembryonic space through which nodal flow could occur, asymmetry is established by cell migration across the node rather than by transport of secreted factors.[43] Cells exhibit a brief leftward myosin II–dependent migration at Hamburger Hamilton stage 4 that is required for the lateralization of Fgf8- and sonic hedgehog–expressing cells at Hamburger Hamilton stage 5.[44] Thus, regardless of the system and mechanism, it would seem that mechanical signals play a role in the establishment of early Fgf8 and sonic hedgehog signaling gradients that drive pattern formation in the embryonic heart, and in some cases, for example, Fgf8, morphogens can feed back to initiate mechanically controlled pattern formation.

Because these niche are dominated by cell–cell contact, a discussion of early events must include cadherins, a homo-philic class of cell adhesion receptors that bind to neighboring cells and create stable mechanical linkages. Cadherins have been implicated in regulating early expression of *Fgf1* and *Mesp1/2* in the gastrulating embryo to control epiblast cell migration when they ingress into the PS and begin to form the primitive heart tube.[33,34] In addition, cadherins act as mechanosensors by initiating intracellular signaling cascades in response to changes in tension to transduce mechanical information about the surrounding environment.[45] Cadherin-mediated cell adhesion and its associated signaling pathways play a variety of vital roles in tissue morphogenesis and development, cardiomyogenesis, and cardiac disease.[46–48] These functions will be described further in the following sections, but taken together, these data form the basis for how mechanical forces could reshape cardiomyogenesis.

Mechanical Regulation of Signaling Pathways During Cardiomyogenesis

Multiple defined signaling pathways interact during development in exceedingly complex temporal and spatial patterns to drive cardiomyogenesis. These pathways include those that are initiated by soluble cues such as cytokines and growth factors, and those initiated by cell adhesive contacts with the extracellular matrix (ECM) and other cells. Foremost among the soluble signaling pathways are the Wingless (Wnt) and transforming growth factor β (TGFβ) superfamily pathways, whereas the major adhesive pathways include integrin- and cadherin-mediated signaling. Each of these act on cardiomyogenesis at multiple time points and in multiple capacities to orchestrate the process of mesoderm formation, cardiac specification, and differentiation. In the following sections, we will describe the influence of these pathways on cardiomyogenesis and provide examples of an additional layer of complexity in the process in which mechanical forces play a role in modulating the signaling pathways.

Wnt Signaling During Cardiac Development

There are 12 different Wnt growth factor family members, each acting on a variety of receptors at precise points in developmental time and space.[49] Wnts modulate numerous steps during cardiomyogenesis and their actions can be biphasic, for example, activation before gastrulation promotes mesoderm formation, early expression postgastrulation decreases cardiac progenitor cell production, and later activation improves expansion.[50–52] During early cardiac mesoderm specification, Wnt inhibition in the endodermal layer induces diffusible factor production that promotes expression of markers of cardiac lineage, such as *Nkx2.5* and *Tbx5*, in the nascent mesoderm.[53] After mesoderm specification, activation of the Wnt/β–catenin pathway in cardiovascular progenitors located in the secondary heart field leads to their accumulation and inhibits cardiomyocyte differentiation, indicating an important role for the pathway in cardiac progenitor cell maintenance.[54] Wnt3a inhibits terminal differentiation of cardiomyocytes and in order for cardiac progenitor cells to proceed down the cardiomyocyte lineage, the Wnt/β–catenin pathway must be inhibited by extracellular signaling such as regulatory Notch1 or insulin-like growth factors–binding protein-4.[50,55–57]

Wnt acts through several signaling pathways summarized in Figure 3A. Signaling is initiated by binding to Frizzled family receptors. In canonical Wnt signaling, the expression of Wnt-associated genes is mediated by cytosolic β-catenin accumulation, which initiates its translocation into the nucleus where it binds the TCF/lymphoid enhancer–binding factor family of TCFs to alter gene expression (Figure 3A; nucleus). Without Wnt activation of Frizzled, β-catenin binds its destruction complex consisting of axin, adenomatous polyposis coli, and glycogen synthase kinase 3 β. Once associated with its destruction complex, β-catenin is phosphorylated and targeted for degradation, and cytosolic accumulation is inhibited. However, Wnt binding to Frizzled induces the disassembly of the axin/adenomatous polyposis coli/glycogen synthase kinase 3 β destruction complex and cytosolic β-catenin is stabilized, allowing for translocation into the nucleus. Once localized, it alters transcription of genes such as *Brachyury* and *Mesp1* during mesoderm induction and *Isl1*, *Flk1*, and *Nkx2.5* during cardiac progenitor specification and differentiation.[49] In noncanonical Wnt signaling, Frizzled binding activates GTPases RhoA and Rac that mediate contractility and cytoskeletal reorganization, and alter gene expression via c-Jun N-terminal kinase activation of transcriptional regulator, activator protein 1.[58] In myocardial development, noncanonical Wnt and cytoskeletal reorganization have been implicated in migration and polarization of cardiac progenitor cells during gastrulation and cardiac morphogenesis.[59,60] In addition, noncanonical Wnt ligands, Wnt2 and Wnt11, act through c-Jun N-terminal kinase/activator protein 1 to positively influence cardiac differentiation.[61,62]

This complex Wnt signaling that orchestrates cardiomyogenesis is influenced by cell adhesions and cadherin expression, which play a role in mesoderm specification and cardiac differentiation.[49,63] The canonical Wnt signaling pathway interacts bidirectionally with cadherin-mediated signaling to regulate cell migration and cell adhesion during development and cardiac morphogenesis.[49,64] Mechanical forces may modulate expression of adhesions and the Wnt/β-catenin signaling cascade (Figure 3A; orange), which in turn can influence cell mechanics by altering cytoskeletal organization and contractility.[65–68]

Mechanical Forces Modulate the Wnt Pathway During Gastrulation

Adherens junctions, composed of homophilic cadherin binding between 2 adjacent cells, are sites of mechanical linkage between the cells and modulate tissue surface tension, which linearly increases with cadherin expression.[69,70] Cadherins are sensitive to mechanical signals and in response to increased mechanical tension, will remodel cortical F-actin to increase junctional stiffness.[71] Cadherins are linked to F-actin through interactions with β-catenin, which associates with α-catenin and vinculin, 2 mechanosensitive proteins that bind F-actin. Dynamic reorganization of the actin cytoskeleton and modulation of tissue surface tension are essential during development and morphogenesis, implying a crucial role for cadherins and adherens junctions.[66,69,72] For example, as a requirement of cardiac precursor cell migration out of the PS during gastrulation, cells must downregulate expression of E-cadherin to break or remodel cell–cell contacts and allow motility through the PS to form nascent mesoderm (Figure 3B). Fgf8 and Mesp1 signaling generates a reduction in E-cadherin expression by blocking its transcription via repressors, such as Snail.[73,74] Snail knockout embryos exhibit abnormal mesoderm morphology and fail to undergo complete EMT, likely because E-cadherin downregulation does not occur.[75]

Studies examining the differentiation of embryonic stem cells (ESCs) have verified the important role of E-cadherin–mediated cell–cell contacts in the formation of mesoderm and cardiac differentiation, and indicate that Wnt signaling, via β-catenin, plays a key role in the process.[76–79] To initiate ESC differentiation, proliferating cells can be forced to aggregate into embryoid bodies using various methods, such as hanging drop, microwell aggregation, or rotary suspension techniques. In the hanging drop method, small volumes of media containing cells are placed on a petri dish lid, the lid is inverted, and the cells aggregrate within the droplet because of forces created by the liquid surface tension. Alternately, in microwell aggregation, cell suspensions are placed into microwells of defined size and forced to aggregate because of constrictions of the size of the well. The rotary suspension culture technique drives embryoid body formation of ESCs in suspension by submitting them to orbital motion. Each of these forced aggregation techniques generates more homogenous embryoid bodies more efficiently than passive embryoid body formation using liquid suspension culture.[78,80–82] Interestingly, forced aggregation techniques improve differentiation into the cardiac lineage as evidenced by a higher number of spontaneously contracting embryoid bodies within days of formation, increased early expression of mesoderm marker, *Brachyury T*, and a later increase in gene expression of cardiac markers *Nkx2.5*, α-*MHC*, *MLC-2v*, *TNNT2*, *MYL7*, and *Mef2c*.[79,82] In both rotary suspension and microwell cultures, there is an accompanying increase in expression of E-cadherin, indicative of the improved cell–cell contacts created between the aggregated cells.[76,77] Forced aggregation increased early nuclear translocation of β-catenin and increased TCF/lymphoid enhancer–binding factor activity, suggesting that the cellular adhesion dynamics altered the Wnt/β-catenin pathway and downstream transcription of cardiac genes, and plays a role in coordinating EMT and cardiac differentiation.

The intersecting cadherin/β-catenin and Wnt/β-catenin pathways demonstrate the interplay of mechanotransduction and traditional autocrine/paracrine signaling that occurs during development. Before gastrulation, epiblast tissue exhibits a compact, tightly packed cell

morphology, formed as a result of proliferation and migration occurring during morphogenesis that is thought to be heavily influenced by mechanical forces, inducing cells to create a large number of E-cadherin–mediated mechanical cell linkages.[65,66] Wnt signaling from the underlying endoderm tissue initiates E-cadherin downregulation during EMT by turning on transcription of *Mesp1*, which activates the repressor Snail to reduce E-cadherin transcription.[74,83] Consequently, as membrane-localized E-cadherin decreases, its bound β-catenin is released and added to the existing pool of intracellular β-catenin, increasing translocation into the nucleus to alter gene expression (Figure 3). This signal feeds into the Wnt/β-catenin cascade, increasing transcription of Wnt-induced genes, including *Mesp1* among others, which can also activate expression of *Dkk1*, a Wnt pathway inhibitor that promotes cardiac differentiation.[84,85] Thus, mechanical interactions during epiblast development prime the tissue to respond to endoderm-originating Wnt signals during EMT by enriching the membrane-bound pool of β-catenin. Furthermore, loss of cadherin-based mechanical linkages induced by Wnt signaling creates a cascade that feeds forward into the canonical Wnt pathway to enhance cardiac differentiation by Wnt inhibition. This pathway, illustrated in Figure 3B, highlights the exceptional complexity of the coordinated signals that occur during cardiac specification and differentiation that rely on both opposing types of signals (physical versus chemical) and modes of application (spatial versus temporal).

Mechanical Forces Modulate the Wnt Pathway During Tube Formation

After gastrulation, cardiac precursor cells migrate and fuse to form a linear heart tube, which later loops into a C- then S-shape that produces the 4 chambers of the heart (Figure 1). During cardiac morphogenesis, N-cadherin cell adhesions are required for heart tube fusion,[86] and noncanonical Wnt signaling can modulate the expression of cadherins to drive this process.[87] Both noncanonical Wnt5a and Wnt11 signaling pathways have been shown to play a role in cardiac morpho-genesis, and it is likely because of the alteration of passive mechanical linkages via N-cadherin that these pathways act. Wnt11-null mice and primary cardiomyoblasts exhibit abnormal N-cadherin and β-catenin expression characterized by a delocalization from the cell membrane and loss of colocalization between the 2 proteins.[88] These cell adhesion abnormalities result in defects in the cardiac outflow tract and altered cell polarity in mouse Wnt11-null mutants,[89] and linear heart tube defects in *Xenopus* Wnt11 loss of function experiments.[62] Similarly, Wnt5a-null mice exhibit abnormalities in the cardiac outflow tract and do not survive postnatally because of gross organ defects.[90]

These examples describing the importance and role of Wnt signaling, especially as it relates to modulating cadherins, suggest that traditional views of development in which the process is primarily driven by chemical signaling, should be amended to include a role for mechanical influences and interactions. Conversely, passive mechanical forces transduced to cells through their adhesion proteins can both modulate and be modulated by Wnt signaling cascades to shape the fate of cardiomyogenesis at the earliest stages of gastrulation through cardiac fate specification, differentiation, and maturation.

TGFβ Superfamily Signaling During Cardiac Development

During development, the TGFβ growth factor superfamily (consisting of >30 family members) generates an assortment of spatial gradients necessary to drive axis formation and patterning of the embryo.[91] In this section, we will briefly outline this pathway and in a subsequent section describe its mechanical sensitivities as described in Figure 4.

In mouse models, TGFβ family signaling begins in the epi-blast to establish the first embryonic axis and continues to play a role throughout organogenesis and onwards, with continuing roles in adult tissue homeostasis. This diversity of function relies on the variety of family members, which include TGFβs, bone morphogenetic proteins (BMPs), and Nodal, and each of these 3 influence cardiomyogenesis. Mesoderm induction in mouse begins with the initiation of Nodal expression in the epiblast at E5 that activates BMP4 expression in the extra-embryonic ectoderm, which in turn induces Wnt3 expression in the epiblast.[92,93] Wnt3 then activates canonical Wnt signaling to drive EMT and later cardiomyogenesis.[49,92,93] Knockout mouse models demonstrate the importance of TGFβ super-family signaling in early cardiomyogenesis, with Nodal and BMP4 homozygous deletions leading to lethality between E7.5 and E9.5 with a failure to undergo normal gastrulation, PS formation, and mesoderm induction.[94,95] Although Nodal seems to primarily mediate early mesoderm formation, BMP signaling continues play a critical role in cardiac specification and differentiation after gastrulation. Mice with homozygous BMP2 deletions survive past gastrulation, but die by E10.5 and before that time point exhibit delayed cardiac morphogenesis and ectopic localization of the heart tube, which never develops past the linear tube stage.[96] In addition, forced expression of BMP2 in chick embryos resulted in ectopic expression of cardiac markers *Nkx2.5* and *GATA-4*, whereas inhibition of BMP signaling with antagonist noggin blocked cardiac mesoderm differentiation.[97,98] Although BMP signaling acts as a positive cue for cardiac differentiation, an early transient inhibition with noggin is necessary to induce cardiomyocyte differentiation in ESCs, indicating a biphasic regulatory role in cardiogenesis.[99] After cardiac progenitors migrate and fuse to form the linear heart tube, BMP signaling continues to influence cardiac morphogenesis, with BMP6, BMP7, and BMP10 orchestrating heart tube looping and chamber formation, evidenced by the absence or hypoplasticity of ventricles in transgenic mice models lacking expression of these BMPs.[100,101]

Similarly to the BMP branch of the TGFβ superfamily, the TGFβ branch also mediates cardiomyogenesis, but in some instances it inhibits cardiomyocyte differentiation while pushing cardiac progenitors toward smooth muscle and endothelial lineages, and in other instances it promotes differentiation to the cardiomyocyte lineage. Mouse models of TGFβ1 and TGFβ2 gene knockout both exhibit early lethality and cardiac defects.[102] Like BMP, TGFβ2 creates a biphasic influence on cardiomyogenesis, with early expression acting as a positive cue for cardiac mesoderm formation and later expression acting as an inhibitor of cardiac differentiation. Early during cardiac specification, Nodal activates TGFβ2 and the 2 signals act in concert to promote formation of cardiac mesoderm.[103] Although Nodal undergoes autoinhibition after its early expression, TGFβ2 expression persists and continues on to mediate cardiomyogenesis. Inhibition of TGFβ2 with RNA interference enhances

expression of cardiac marker *Myh2*, demonstrating its role in blocking cardiomyocyte differentiation, whereas treatment with recombinant TGFβ2 upregulates expression of *Pecam1* and *Myh11*, markers of endothelial and smooth muscle cells. However, in cell models of cardiomyocyte differentiation, TGFβ1 promotes cardiac differentiation by increasing expression of cardiac markers and contractile proteins, further illustrating the biphasic control of TGFβ over cardiomyocyte differentiation.[104,105]

The importance of spatial and temporal modulation of TGFβ superfamily signaling during cardiomyogenesis and morphogenesis indicates that a complex regulatory system must be established to orchestrate the diverse effects of the pathways. Modulation of BMP occurs on multiple levels, including post-translational processing, extracellular antagonism, and intracellular inhibition. After translation, enzymes cleave secreted, inactive BMP precursors to produce the active form that can bind its receptor and initiate downstream effects.[106,107] Many extracellular proteins, such as noggin, can bind BMP to impede or promote receptor binding, and modulation of receptor availability via internalization or degradation can alter transduction.[108–111] Along with soluble regulators of the TGFβ superfamily, components of the ECM modulate signaling by sequestering TGFβ family members. ECM proteins can interact with both BMPs and TGFβs via their heparin-binding sites or prodomains, and these interactions regulate their availability and consequent downstream signaling.[112,113] For example, in *Drosophila* development, the gradient of BMP analog decapentaplegic is maintained through interactions with type-IV collagen.[114] Decapentaplegic secreted from cells in the dorsal ectoderm binds type-IV collagen and limits its active range, where it maintains germline stem cell populations and regulates dorsoventral axis patterning. In addition, the BMP4 signaling range increases when its ECM-binding prodomain is cleaved, demonstrating the importance of ECM sequestration in establishing BMP gradients.[115] Release of these ligands from the ECM can be initiated by both chemical or mechanical stimulation, which will be discussed in detail in the following section.[116]

Mechanical Regulation of TGFβ and BMP Signaling During Cardiac Development

In addition to the chemical regulation of TGFβ superfamily signaling, mechanical forces also modulate the pathways by extracellular and intracellular mechanisms, summarized in Figure 4, and may play a role in driving cardiomyogenesis. BMP signaling is mediated by mechanical forces at multiple stages during the signaling pathway, including BMP receptor availability and transcriptional regulation of BMP ligands and antagonists.[117] Receptor internalization via endocytosis or degradation inhibits BMP signaling,[118] and cardiomyocyte differentiation requires transient periods of BMP inhibition, indicating that receptor internalization may mediate this process.[99] In addition, stem cell models of cardiomyogenesis demonstrate that small molecule degradation of TGFβ receptors promote cardiomyocyte differentiation.[108] Endocytosis can be mediated by a variety of mechanical stimuli including cell membrane tension and mechanical deformation and thus, it may be proposed that mechanical forces occurring during morphogenesis may mediate TGFβ and BMP receptor endocytosis to mediate progenitor differentiation (Figure 4B).[119] In fact, passive mechanical forces generated by ECM elasticity modulate BMP signaling through

BMP receptor endocytosis and alter lineage specification of stem cells; however, it should be noted that mechanically induced endocytosis is not specific to the BMP signaling pathway and modulates cell membrane trafficking generally.[120] At the transcriptional level, mechanical forces can alter several members of the BMP pathway (Figure 4A). Changes in BMP ligand expression and their mothers against decapentaplegic homolog (SMAD) family effectors on compliant ECM are myosin-dependent, indicating that they are mechanosensitive.[121] Dynamic mechanical stretch applied to cultured neonatal rat cardiomyocytes upregulates BMP2 expression while transiently downregulating BMP4 expression.[122] These changes in BMP expression were accompanied by an increase in cardiac differentiation marker GATA-4, suggesting that the BMP pathway may transduce forces to mediate cardiomyocyte differentiation.

Studies of ECM sequestration and release of TGFβ1 have revealed complex mechanisms that include both chemical and mechanical control of liberation.[116,123] TGFβ1 is synthesized in its inactive form, in which it is associated with a latency-associated protein. During post-translational processing, the complex is covalently liked via disulfide bonding to a latent TGFβ-binding protein before secretion into the extracellular space. Once there, the latent TGFβ-binding protein interacts with deposited ECM proteins fibronectin, fibrillin and vitronectin to maintain an inactive pool of TGFβ1 that can later be liberated in its active form to induce various actions on the surrounding cells.[124,125] Common methods of activation rely on release of TGFβ1 from its latency-associated protein and latent TGFβ-binding protein through proteolytic/enzymatic cleavage via matrix metalloprotinases, proprotein convertases, or glycosidases, and work in the past decade has also revealed a role for integrin-dependent activation of latent TGFβ1.[116] Integrins are transmembrane proteins that associate extracellularly with components of the ECM and intracellularly with components of the cytoskeleton. They are considered to be mediators of force transduction between the inside and outside of the cell, and they are vital players in a variety of processes during development, including migration, differentiation, and morphogenesis.[126,127] Integrins can mediate both proteolytic and mechanically induced release of TGFβ1. Both matrix metalloprotinases and latent TGFβ-binding proteins bind integrins, indicating that integrins may catalyze proteolytic cleavage of TGFβ1 by bringing it into close association with its cleavage enzyme.[128–130] However, integrin-mediated liberation of TGFβ1 can also occur nonenzymatically, with mechanical forces initiating release. In this model, latency-associated protein is a mechanosensitive protein that, in response to contractile forces transmitted through integrins, unfolds into a conformation that does not support binding with TGFβ1, thus releasing it into the extracellular space and enabling receptor binding (Figure 4C).[131–133] Force-induced release of TGFβ1 has been well characterized in the context of myofibroblast differentiation in wound healing and cardiac fibrosis.[131,134,135] In the later example, contraction of cardiac fibroblasts activates latent TGFβ1 from the ECM and induces differentiation of myofibroblasts that promotes fibrosis in damaged cardiac tissue. However, similarly to the biphasic effects of TGFβ1 discussed above, mechanically induced TGFβ signaling also enacts differing effects depending on the target cell population and the temporal window of application. For example, cyclic strain applied to ESCs induces SMAD2/3 activation and plays a role in maintaining pluripotency.[136,137] To date, the influence of mechanical forces on TGFβ1 signaling has not been studied in cardiac

progenitor cell differentiation; however, because TGFβ1 plays a role in cardiomyocyte, vascular smooth muscle, and endothelial cell differentiation during development, it is possible that the contractile forces occurring during cardiac morphogenesis may also regulate TGFβ1 release and action during cardiomyogenesis in a similar fashion.

Integrin Regulation of Cardiac Development

Integrins are transmembrane receptors that link ECM and cytoskeleton to mediate tissue structure and function. Composed of heterodimers of α and β subunits that act as receptors for ECM proteins, integrins transduce mechanical information from the cellular environment into the cell (outside–in) and from inside the cell back out (inside–out).[138] Transduction begins with ECM ligand binding and subsequent initiation of the accumulation of several proteins on the cytoplasmic region of integrin dimers to form focal adhesion (FA) complexes. These proteins include adaptor proteins that structurally link integrins to the cytoskeleton by binding F-actin, scaffolding proteins that bind additional FA proteins, and signaling molecules that propagate intracellular signaling cascades. In the myocardium, many of the integrin-associated FA proteins and downstream signaling result from chemical changes that have been associated with cardiac development and disease.[139] However, most are also influenced by mechanics; the impact of both are highlighted in Figure 5 and described here for their chemical changes and in the next section for their mechanical changes.

Integrins and their ECM adhesions are vital for organism formation and function, and they coordinate many events during development, such as proliferation, differentiation, and migration. In mammals, >18 α subunits and 8 β subunits have been identified, with the myocardium expressing a small subset of these; however, integrin expression patterns are dynamic, adding to their diversity of function, and turnover can be mediated by mechanical forces.[139–142] Numerous knockout studies illustrate the importance of integrins during cardiomyogenesis, particularly the α4, α5, and β1 subunits. Animals null for α4 integrin die by E15.5 and exhibit cardiac defects including absence of epicardium and coronary vessels, cardiac hemorrhage, and abnormal epicardial progenitor migration.[143] Homozygous knockout of α5 expression is embryonic lethal by E11, interferes with mesodermal axis patterning, and embryos develop abnormal heart morphologies, including dys-functional cardiac looping and outflow tract formation.[144,145] In the heart, β1 integrin is the main β subunit that dimerizes with all of the expressed α subunits. Global homozygous knockout of β1 integrin is embryonic lethal by E5.5, whereas cardiac-specific homozygous knockout leads to a variety of cardiac defects.[146–148] In addition, ESCs lacking β1 integrin exhibit a delayed expression of cardiac markers *MHC*, *MLC-2V*, and *ANF* after the onset of cardiac differentiation, aberrant sarcomeric architecture, and abnormal specification to atrial, ventricular, and sinusnodal type cells.[149]

A large body of work demonstrates that integrin-mediated signaling mediates cardiac specification and differentiation. Studies using cell models of cardiomyogenesis indicate that ECM composition and the activation of integrins play a major role in the process. For example, ESCs cultured in 3-dimensional (3D) ECM constructs based on laminin or vitronectin differentiate more efficiently into cardiomyocytes than ESCs cultured on 2D

ECM substrates.[150,151] In 3D culture, there are more points of contact between the cell and ECM, more FAs and possibly enhanced integrin signaling, supporting the important role that integrins play in development. Furthermore, ESCs differentiate more efficiently into cardiomyocytes when cultured on native heart ECM than on commercial ECM mixtures or isolated ECM components.[152,153] Even in the absence of supplemental growth factors commonly used to drive cardiomyogenesis, native ECM is able to induce cardiac differentiation, indicating that cell signaling pathways initiated by integrin activation via ECM binding are stronger differentiation cues than soluble signals.[153]

In ESCs deficient in β1 integrin expression, early cardiomyocyte differentiation was delayed, but ultimately proceeded to result in beating cardiomyocytes, albeit with impaired functional properties.[149,154,155] These mutant cells exhibited an accompanying upregulation of β3 integrin, suggesting a compensatory role that enabled early cardiac specification and differentiation.[156] However, these cell lines exhibit abnormalities in terminal differentiation, indicating the importance of β1 integrin-mediated signaling in the differentiation process. Integrin-linked kinase, an intracellular signaling molecule bound to β integrin subunits, is directly activated by integrins and initiates downstream responses that regulate cell survival, proliferation, and differentiation.[157] Fetal myocar-dial cells overexpressing integrin-linked kinase produce more cardioblast aggregates than wild-type cells, and knockdown of integrin-linked kinase expression with RNA interference reduces cardioblast generation, verifying the importance of integrin signaling in cardiomyogenesis.[158] In addition to β integrins, multiple α integrin subunits are expressed in ESC-derived cardiomyocytes, for example, α3, α5, α6, α7, α11, and αψ, depending on the matrix on which they are cultured, with expression levels of some subunits such as α6 correlating to cardiac marker expression, indicating a role in cardiac differentiation.[159,160]

Integrin-Mediated Mechanotransduction in Cardiac Development

During myocardial development, several mechanical forces, both active and passive, are generated. ECM secreted and assembled by progenitor cells gives the developing tissues structural integrity and results in changes in tissue stiffness. Because myocardial tissue begins to fold during morphogenesis and with the onset of chamber pumping, cardiac progenitors, and later cardiomyocytes are submitted to stretching forces. Both of these active and passive forces are mediated by integrin-based cell adhesions. Integrins act as mechanotransductors to pass these signals to the inside of the cell and alter cell function. In addition to the soluble signaling pathways initiated by integrin–ECM interactions and FA complex assembly, direct mechanical linkages exist between the ECM and the nucleus that are thought to alter gene expression by propagating mechanical waves to the nuclear membrane to directly remodel chromatin or open nuclear pores and modify nuclear transport (Figure 5).[161] In addition, the soluble signaling pathways themselves can be activated based on force applied through integrins and the FA complexes. Several mechanosensitive adaptor proteins exist that contain cryptic-binding sites for kinases that are uncovered in the presence of force to allow phosphorylation and downstream signaling to proceed.[138] In this fashion, cyclic stretch activates multiple pathways in cardiomyocytes to mediate processes, such as cell survival, cell–cell communication, structural reorganization, and electrophysiological functioning.[162]

During development, cardiac progenitors respond to both passive and active mechanical cues, demonstrating the importance of these forces in cardiomyogenesis. Because the myocardium develops, it transitions from a soft mesoderm tissue (≈500 kPa) to an intermediate stiffness (≈11 kPa). Embryonic cardiomyocytes cultured on soft ECM substrates that mimic the mechanical properties of mature heart tissue develop more organized sarcomeres and exhibit more mature functional behavior than cells on softer or stiffer matrices.[7,8] However, other work using human ESCs indicated that cells are sensitive to passive mechanical cues only during early cardiac specification, with substrate stiffness affecting mesoderm induction, but not cardiomyocyte differentiation after that time point.[163] Culture on dynamically stiffening hydrogels that mimic in vivo cardiac tissue stiffening further improves cardiomyocyte differentiation and structural organization over culture on static myogenic hydrogels.[9] This slow stiffening occurs during the course of several hours, similar to the time course observed in vivo; however, additional in vitro studies have demonstrated that more rapid mechanical perturbations also affect cardiomyocyte differentiation and maturation. In some cases, cyclic mechanical strain improved cell survival and cardiomyogenesis,[13,164] but impaired cardiomyocyte differentiation in others.[165] These conflicting results may arise artificially based on experimental design (time course, stimulation frequency, etc.) but may also reflect differences in cell type (mouse versus human) or maturation stage (pluripotent versus specified).

Mechanistic studies have implicated cell adhesive contacts as mediators of force transduction. Several intracellular signaling pathways associated with integrins such as the phosphoinositide 3-kinase/protein kinase B and p38 mitogen-activated protein kinase pathways were found to be upregulated in response to dynamic stiffening, suggesting that integrins may be mediators of the external mechanical cues leading to improved cardiomyocyte differentiation and maturation.[14] Integrin internalization via endocytosis is enhanced on softer substrates; thus, as cardiac tissue stiffens during development, integrin signaling may be enhanced because of a greater number of receptors present at the cell surface.[120] In addition, integrin expression is upregulated in response to mechanical stimulation, indicating that forces improve cell adhesion and FA assembly, potentially increasing downstream FA-mediated signaling to modify cardiomyogenesis.[140,164] However, future work to clarify the impact of both passive and dynamic forces remains to be done to gain a full understanding of the role of mechanics in cardiomyogenesis.

Perspectives: Implications for Disease and Tissue Regeneration

Continued work in clarifying the role of mechanical signaling in myocardial development has the potential to largely broaden our understanding of the origin of congenital and adult heart disease and dysfunction, as well as to affect future therapies aimed at treating these disorders. Abnormal mechanical cues during development and reduced expression of mechanosensitive receptors or proteins can lead to heart deformations or the emergence of cardiomyopathies. For example, as described above, knockout of various integrin receptors and consequently, cell–ECM interactions that maintain cell shape and tension cues, can create several cardiac defects. In addition, mutations in force-sensitive cytoskeletal proteins such as vinculin, titan, integrin-linked kinase, dystrophin, or sarcoglycan are associated with the development of dilated or hypertrophic cardiomyopathies.[166] Cytoskeletal maintenance

of cell shape drives the healthy function of cardiomyocytes, but when cytoskeletal remodeling occurs in response to changing mechanical forces (ie, pressure or volume overload), pathological states develop.[21] To develop efficacious treatments for these diseases, we must consider not only the biophysical or biochemical contributions to the disease state in isolation but also examine how both of these pathways feed into each other to create the complex network of signaling that occurs physiologically.

Because the adult heart holds a limited capacity for regeneration, efforts to generate myocardial cells and tissue from human pluripotent stem cells have garnered significant attention in recent years. These engineered cells and tissues have potential uses as regenerative therapies or as improved in vitro platforms for drug discovery and testing. Ongoing work has established highly efficient cardiomyocyte differentiation protocols based on modulation of FGF, Wnt, TGFβ, or BMP pathways (reviewed in Burridge et al[167]), but these methods produce cardiomyocyte populations that contain large heterogeneity in structural and functional maturation. For this reason, novel techniques to engineer cardiomyocytes and cardiac tissue based on a variety of physical signals are being investigated in an attempt to generate mature cells and tissues that more faithfully recapitulate the in vivo myocardium.[168,169] As described in this review and elsewhere,[65,170] mechanical forces play a significant role in in vivo development and cardiomyo-genesis, which suggests that similar physical cues may aid in the ex vivo production of cardiomyocytes and promote their organization into artificial cardiac tissues and organoids. In fact, development of tissue culture platforms that investigate the effects of substrate elasticity,[163,171] stretching, and shearing[13,172,173] has indicated that these systems have potential to improve the efficiency of human pluripotent stem cell differentiation into mature cardiomyocytes. In addition, mechanical stimulation of engineered cardiac tissues can improve their function and these grafts have the capacity to repair damaged heart tissue in vivo.[174] Thus, by leveraging the important roles that mechanical cues play at multiple stages during cardiomyogenesis, we can engineer grafts from the cellular to the tissue level and create more effective regenerative strategies for cardiac disease and dysfunction.

Conclusions and Future Directions

Multicellular organisms use a wide array of signaling cues to govern their elaborate patterning and organization from the most basic level of a single cell up to the complex interworking of organ systems. Beginning with the appearance of the first cardiac precursors in the pregastrulation epiblast and continuing through the morphological organization of the beating 4-chambered heart, both soluble cues and biophysical signals regulate cardiomyogenesis. In this review, we have summarized several major chemical pathways contributing to cardiac development and proposed multiple points at which mechanical cues intersect these well-studied pathways to mediate the process. Although the study of mechanotransduction is a relatively new one, great strides have been made in uncovering the function of biophysical influences on cardiac development, function, and disease. However, many of the biochemical–biophysical interactions discussed in this review have yet to be thoroughly characterized and require future investigation to fully unravel their role in cardiomyogenesis. An example of this discussed above is that although cyclic stretch has been shown to release latent TGFβ ligands from the ECM to influence myofibroblast

differentiation, its influence on cardiac progenitor differentiation has not been investigated. Multiple TGFβ pathways influence cardiomyocyte differentiation, so although it has not been shown yet, it can be assumed that cyclic stretch might act in the same way to regulate differentiation. Although cyclic stretch improves cardiomyocyte differentiation, its actions are not limited to TGFβ signaling. Other instances of TGFβ's mechanical influence include integrin or cadherin signaling; further studies should also help to clarify the complex role of biophysical interactions with these signaling pathways. Beyond TGFβ, the other signaling pathways described here are equally likely to have complex biochemical–biophysical interactions, both during development and in an adult context. Yet by exploiting knowledge gained about the mechanotransduction pathways activated during development specifically, we have the opportunity to generate novel regenerative strategies for disease intervention. For example, by using applied cyclic stretch to populations of cultured cardiomyocytes and modulating mechotransduction pathways in the cell, more mature cardiomyocytes may be produced that can then be used for cell transplantation therapies, in vitro modeling or drug discovery. Ultimately, by uncovering the ways in which mechanical forces influence development and adding this knowledge to the prevailing chemical gradient-based views, we will create a more complete picture of mammalian cardiomyogenesis. Advances in this direction will aid future work toward clarifying the healthy development and function of myocardial tissue and assist in creating an understanding of pathological and disease states.

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Nonstandard Abbreviations and Acronyms

BMP	bone morphogenetic protein
ECM	extracellular matrix
EMT	epithelial–mesenchymal transition
ESCs	embryonic stem cells
FA	focal adhesion
PS	primitive streak
TCF	transcription factor
TGFβ	transforming growth factor β
Wnt	wingless

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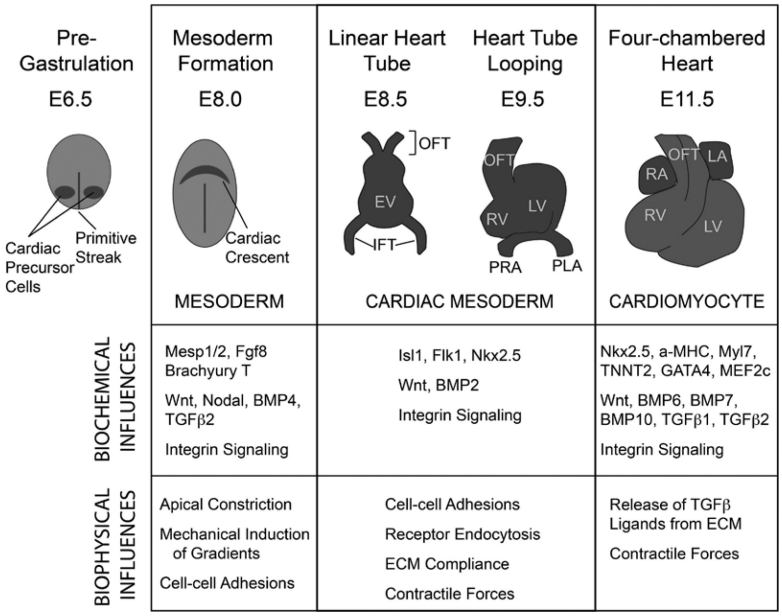

Figure 1. Cardiomyogenesis during stages of development

Overview of the stages of cardiomyogenesis from the first appearance of cardiac precursor cells in the pregastrulation epiblast through the emergence of the 4-chambered heart. The embryonic (E) days noted at each stage reflect mouse development. Both biochemical and biophysical influences on cardiomyogenesis are summarized at each developmental stage. BMP, bone morphogenetic protein; ECM, extracellular matrix; EV, early ventricle; IFT, inflow tract; LA, left aorta; LV, left ventricle; OFT, outflow tract; PLA, primitive left aorta; PRA, primitive right aorta; RA, right aorta; RV, right ventricle; and TGFβ, transforming growth factor-β.

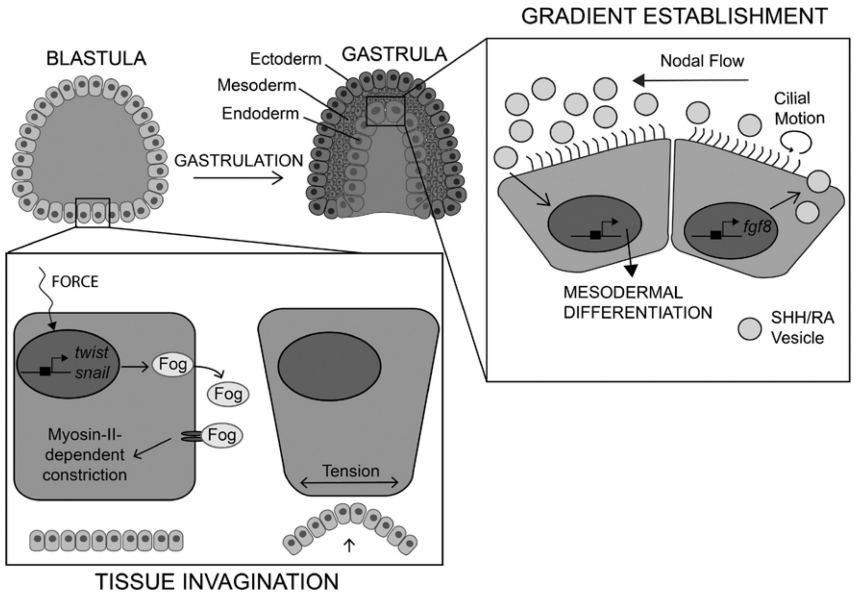

Figure 2. Influence of mechanical forces during gastrulation and early mesoderm formation

Schematic illustration depicting the role of mechanical forces in initiation of tissue invagination during gastrulation (**left**) and establishment of chemical gradients during mesoderm development (**right**). Mechanical force induces expression of *twist* and *snail* genes that mediate folded gastrulation (Fog) secretion and receptor binding to activate myosin II–dependent cell constriction. This generates tension at the cell surface that drives invagination at the onset of gastrulation. Asymmetrical gradients of sonic hedgehog (SHH) and retinoic acid (RA) are created as nodal cilia rotate to generate nodal flow to drive SHH/RA-containing vesicles to the left region of the primitive streak node.

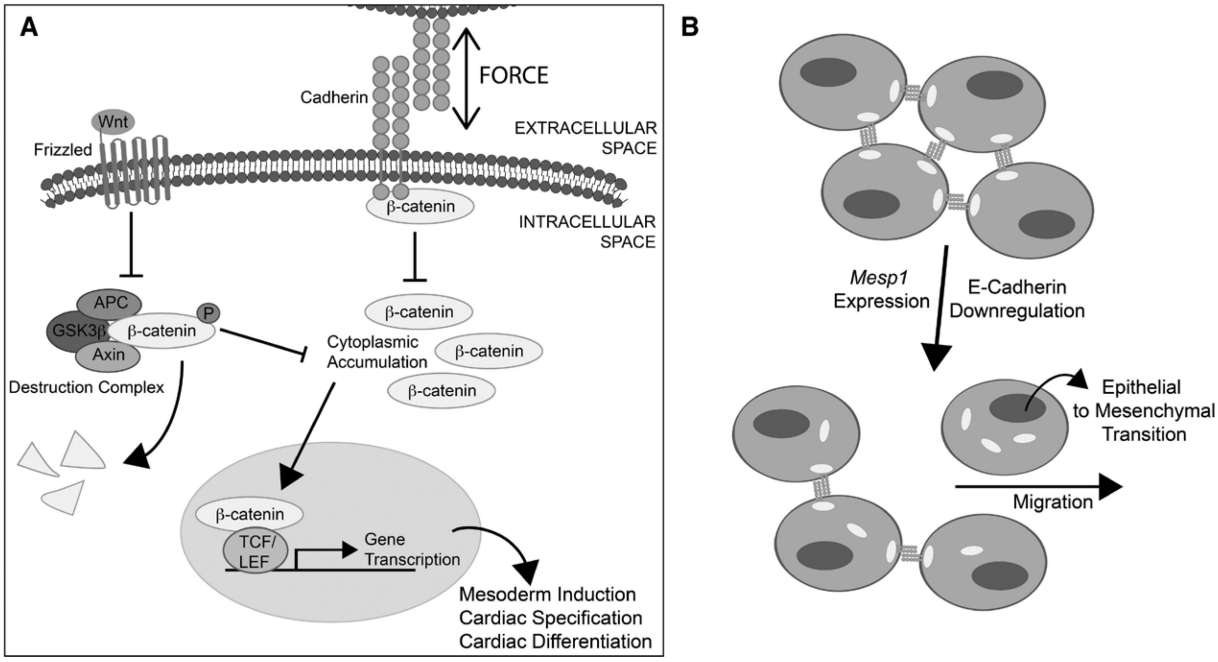

Figure 3. Force-induced modulation of Wnt signaling during cardiomyogenesis

A, Schematic representation of Wnt signaling cascade and cadherin-based mechanical interactions between adjacent cells. Wnt ligand signaling through its Frizzled receptor inhibits the proteolytic degradation of β-catenin by its destruction complex, enabling cytoplasmic accumulation. Increased cytoplasmic β-catenin drives nuclear translocation and initiates expression of Wnt-related genes to modulate cardiomyogenesis. Cadherins interact with the Wnt pathway by binding β-catenin to block its cytoplasmic accumulation and subsequent nuclear translocation. B, Illustration of epithelial–mesenchymal transition (EMT) driving myocyte specification and migration. The loss of E-cadherin–based mechanical connections between cells enables cell migration and increases intracellular β-catenin pools to drive expression of EMT-related genes. APC indicates adenomatous polyposis coli; GSK3β, glycogen synthase kinase 3 β; LEF, lymphoid enhancer–binding factor; and TCF, transcription factor.

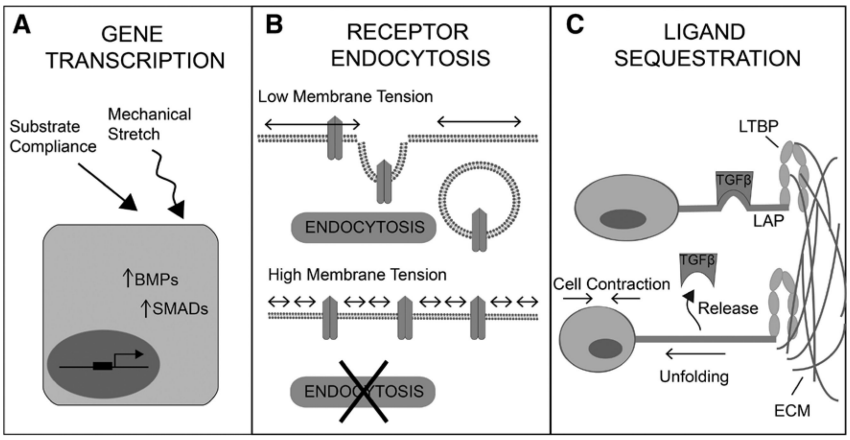

Figure 4. Role of mechanical forces in regulation of transforming growth factor-β(TGFβ) superfamily signaling

Biophysical forces regulate TGFβ superfamily signaling at multiple points in the pathway. **A**, Both passive and active mechanical cues alter gene expression of bone morphogenetic protein (BMP) ligands and mothers against decapentaplegic homolog (SMAD) effector proteins. **B**, Membrane tension generated by mechanical interactions with the extracellular matrix (ECM) drives TGFβ receptor endocytosis. **C**, TGFβ ligands are sequestered by the ECM through interactions with latency-associated protein (LAP) and latent TGFβ-binding protein (LTBP). Cell contraction initiates ligand release by unfolding mechanosensitive LAP to interfere with LAP–TGFβ binding.

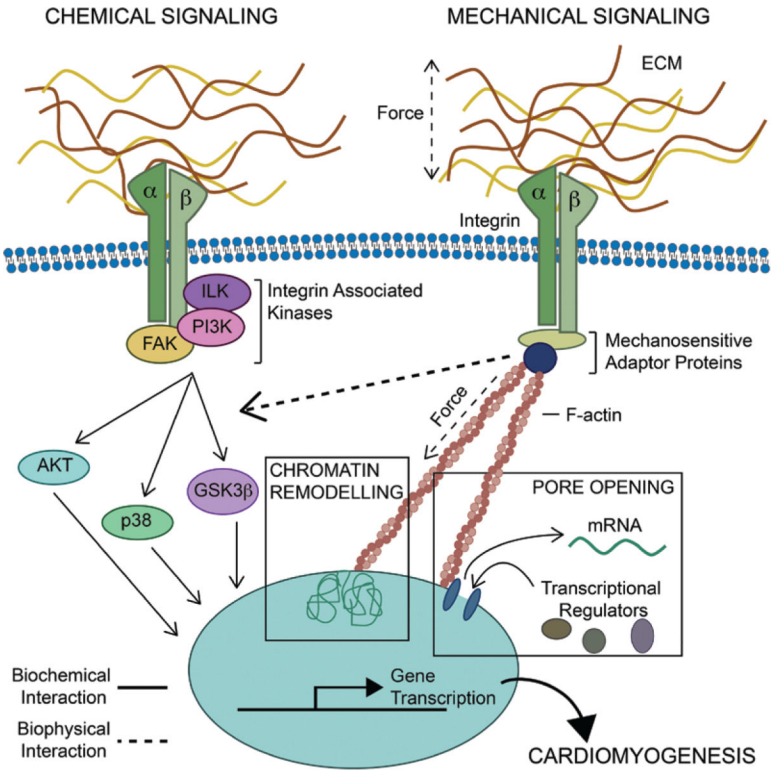

Figure 5. Interplay of chemical and mechanical signals that underlie cardiac specification and development

Schematic representation of integrin-mediated signaling specifically detailing how chemical signals (**left**), for example, integrin ligation, drive focal adhesion formation, and downstream events leading to transcriptional changes. Mechanical signals (**right**) are mediated by actomyosin contractions. Both influence transcriptional changes via common mechanisms diagrammed at the bottom of the illustration, including chromatin remodeling and changes in nuclear pore complex diffusion among others. Dash and solid lines indicate the direction of biophysical and biochemical interactions, respectively. AKT indicates protein kinase B; ECM, extracellular matrix; FAK, focal adhesion kinase; GSK, glycogen synthase kinase; ILK, integrin-linked kinase; and PI3K, phosphoinositide 3-kinase.