Mechanical Forces Reshape Differentiation Cues That Guide Cardiomyogenesis

Cassandra L. Happe, Adam J. Engler

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. (Circ Res. 2016;118:296-310. DOI: 10.1161/CIRCRESAHA.115.305139.)

Key Words: 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 as well as complex ones such as fruit fly wing development by controlling an equally diverse set of cell behaviors, for example, migration, proliferation, and specialization. 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). 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 they were exposed. 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 or as active forces that are directly applied to cells by other cells or fluids. 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
Nonstandard Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>epithelial–mesenchymal transition</td>
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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. 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) when a population of cardiac precursor cells residing in the lateral posterior epiblast develops before primitive streak (PS) formation22,23 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 occurs20,24 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 constriction, 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.33 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; interruption in expression of either of these genes results in abnormal heart development. 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. 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. 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. 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). 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. 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. 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.

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 homophilic 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.30,34 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 1.53–56 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.51,62

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.60,64 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.69,71 Cadherins are sensitive to mechanical signals and in response to increased mechanical tension, will remodel cortical F-actin to increase junctional stiffness.72 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
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. Snail knockout embryos exhibit abnormal mesoderm morphology and fail to undergo complete EMT, likely because E-cadherin downregulation does not occur.

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. 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 aggregate 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. 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 α-MHC, MLC-2v, TNNT2, MYL7, and Mef2c. 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. 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. 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.
decreases, its bound β-catenin is released and added to the exis-
ting pool of intracellular β-catenin, increasing translocation
into the nucleus to alter gene expression (Figure 3). This sig-
nal feeds into the Wnt/β-catenin cascade, increasing transcrip-
tion of Wnt-induced genes, including Mesp1 among others,
which can also activate expression of Dkk1, a Wnt pathway
inhibitor that promotes cardiac differentiation.85,86 Thus, me-
chanical 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 in-
duced by Wnt signaling creates a cascade that feeds forward
into the canonical Wnt pathway to enhance cardiac differentia-
tion by Wnt inhibition. This pathway, illustrated in Figure 3B,
highlights the exceptional complexity of the coordinated sig-
als 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 sig-
naling 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 abnor-
nal N-cadherin and β-catenin expression characterized by a
delocalization from the cell membrane and loss of colocaliza-
tion between the 2 proteins.88 These cell adhesion abnormali-
ties 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 car-
diac 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 cadher-
ins, 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 in-
teractions. 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 assort-
ment of spatial gradients necessary to drive axis formation and
patterning of the embryo.90 In this section, we will briefly outline
this pathway and in a subsequent section describe its mechan-
sical 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 re-
lies 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
epiplast 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 to 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 devel-
ops 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 me-
soder differentiation.97,98 Although BMP signaling acts as a
positive cue for cardiac differentiation, an early transient inhi-
bition with noggin is necessary to induce cardiomyocyte dif-
ferentiation 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 influ-
ence cardiac morphogenesis, with BMP6, BMP7, and BMP10
orchestrating heart tube looping and chamber formation, evi-
denced by the absence or hypoplasticity of ventricles in trans-
genetic mice models lacking expression of these BMPs.100,101

Similarly to the BMP branch of the TGFβ superfam-
ily, 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 dif-
ferentiation 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 car-
diac mesoderm.103 Although Nodal undergoes autoinhibition
after its early expression, TGFβ2 expression persists and con-
tinues on to mediate cardiomyogenesis. Inhibition of TGFβ2
with RNA interference enhances expression of cardiac marker
Myh2, demonstrating its role in blocking cardiomyocyte differ-
entiation, whereas treatment with recombinant TGFβ2 upregu-
lates expression of Pecam1 and Myh11, markers of en-
dothelial and smooth muscle cells. However, in cell models of
Growth factor-β (TGFβ) superfamily signaling is regulated by mechanical forces during cardiomyogenesis.不斷的机械应力可以抑制或激活TGFβ和BMP信号通路，从而影响心肌细胞分化。这些机械刺激可以调控TGFβ和BMP信号通路，促进心肌细胞分化，也可能调节心肌细胞的形态和功能。
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. Integrins can mediate both proteolytic and mechanically induced release of TGFβ1. Both matrix metalloproteinases 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. 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 TGFβ1 binding to the ECM and latent TGFβ-binding protein through proteolytic/ enzymatic cleavage via matrix metalloproteinases, proprotein convertases, or glycosidases, and work in the past decade has also revealed a role for integrin-dependent activation of latent TGFβ1. 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. Integrins can mediate both proteolytic and mechanically induced release of TGFβ1. Both matrix metalloproteinases 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. 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). Force-induced release of TGFβ1 has been well characterized in the context of myofibroblast differentiation in wound healing and cardiac fibrosis. 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. 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). 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. 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..

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. Homozygous knockout of α5 expression is embryonic lethal by E11, interferes with mesodermal axis patterning, and embryonic development abnormal heart morphologies, including dysmorphogenesis.143–145 In ESCs, the 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 cardiogenic-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 cardiac architecture, and abnormal specification to atrial, ventricular, and sinus nodal 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. 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.155

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. These mutant cells exhibited an accompanying upregulation of β3 integrin, suggesting a compensatory role that enabled early cardiac specification and differentiation. 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 myocardial 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 α10, 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 secretion and assembly 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 mechanotransducers 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 adapter 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. 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. 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. 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. 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, but impaired cardiomyocyte differentiation in others. 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. 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. 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. 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, titin, integrin-linked kinase, dystrophin, or sarcoglycan are associated with the development of dilated or hypertrophic cardiomyopathies. 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. 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), 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. As described in this review and elsewhere, mechanical forces play a significant role in vivo development and cardiomyogenesis, 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, stretching, and shearing 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. 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 and 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 mechanotransduction 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.

Sources of Funding
We thank the National Institutes of Health and American Heart Association for funding support (R01AG045428 to A.J. Engler and 15POST25720070 to C.L. Happe).

Disclosures
None.

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Circ Res. 2016;118:296-310
doi: 10.1161/CIRCRESAHA.115.305139
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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