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Signaling Pathways and Gene Regulatory Networks in Cardiomyocyte Differentiation

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Strategies for harnessing stem cells as a source to treat cell loss in heart disease are the subject of intense research. Human pluripotent stem cells (hPSCs) can be expanded extensively *in vitro* and therefore can potentially provide sufficient quantities of patient-specific differentiated cardiomyocytes. Although multiple stimuli direct heart development, the differentiation process is driven in large part by signaling activity. The engineering of hPSCs to heart cell progeny has extensively relied on establishing proper combinations of soluble signals, which target genetic programs thereby inducing cardiomyocyte specification. Pertinent differentiation strategies have relied as a template on the development of embryonic heart in multiple model organisms. Here, information on the regulation of cardiomyocyte development from *in vivo* genetic and embryological studies is critically reviewed. A fresh interpretation is provided of *in vivo* and *in vitro* data on signaling pathways and gene regulatory networks (GRNs) underlying cardiopoiesis. The state-of-the-art understanding of signaling pathways and GRNs presented here can inform the design and optimization of methods for the engineering of tissues for heart therapies.

Introduction

STEM CELLS HAVE the potential to play pivotal roles in the development of heart therapies. Heart diseases are consistently ranked as a top cause of morbidity and mortality (causing approximately one out of every nine deaths) in the United States.[1] Besides preventive measures, successful therapies against the loss of cardiomyocytes due to infarction have been difficult to develop especially considering the limited regenerative capacity of these cells. Although heart transplantation effectively reconstitutes the heart function, widespread applicability of this therapeutic modality is severely hindered by the lack of sufficient donor organs. Furthermore, several complications are associated with immunosuppressive regimens to reduce transplant rejection.

Toward this end, stem cells can be directed along heart muscle cell lineages evidencing their potential for use as a source of cellular material for the repair of damaged myocardium. Recent developments in cell reprogramming technology also demonstrate the conversion of one mature somatic cell type into pluripotent stem cells (PSCs)[2,3] or other differentiated cells of interest (transdifferentiation) including cardiomyocytes[4] offering an excellent venue for noninvasively obtaining autologous cells. Heart cells produced via transdifferentiation will also have to be amenable to controlled proliferation so that the cells can be expanded (e.g., *ex vivo*) to clinically relevant quantities before their therapeutic use and become senescent like native cardiomyocytes once residing in the host. Adult stem cells such as skeletal myoblasts, bone marrow-derived progenitors, and heart-residing progenitors also hold the potential for repairing the damaged myocardium and some have recently reached clinical trials for treating heart maladies,[5] although the mechanisms of healing are unknown.[6] Adult stem cells can be autologous; however, they are difficult to isolate, many times requiring invasive approaches, and have lower proliferative capacity compared with PSCs. Furthermore, whether the immunogenicity profile of these cells is preserved after culture and differentiation is debatable. Application of different adult stem cell types for reconstituting heart function are reviewed elsewhere.[7–9] On the other hand, human PSCs (hPSCs) including human embryonic stem cells (hESCs) and human induced PSCs (hiPSCs) can be propagated extensively indicating their suitability for en masse propagation and differentiation. hPSCs differentiate into various cell types of all germ layers including cardiomyocytes[10] in large, clinically relevant quantities. Moreover, hiPSCs can be derived from adult cells (e.g., skin

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fibroblasts and peripheral blood mononuclear cells) with minimally invasive procedures and in a patient-specific fashion without the ethical issues pertinent to the derivation of hESCs.

Yet, the generation of cardiac precursors and different cardiac muscle cell-types—especially types with direct clinical relevance such as ventricular cells—from hPSCs would require a deeper understanding of the processes involved in the embryonic cardiomyocyte development, which serves as a template for protocols of *in vitro* differentiation of hPSCs. Central to the commitment of stem and progenitor cells are gene transcriptional networks driving the adoption of a specific phenotype and conferring appropriate functional characteristics. Equally important are signal transduction pathways orchestrating the intercellular communication via soluble and/or cell–cell contact signals. Last, the epigenetic profile of cells influences how transcriptional networks respond to signaling cascades with directing cell differentiation.

In this review, we have researched and compiled current information on the physiological regulation of heart and cardiomyocyte formation from *in vivo* genetic and embryological studies of various model organisms. The detailed molecular description of signaling pathways and their respective differentiation mechanisms can be valuable guides for the design and optimization of cardiac differentiation methods possessing various industrially- and clinically desirable features such as reproducible production of progenitor or specific cardiac cell type to certain levels of maturity, improved efficiency and yield of cardiomyocytes obtained, and generalizable methods across diverse hPSC lines.

Wnt Signaling in Cardiomyocyte Differentiation

Wnt ligands are widely expressed at various stages of embryonic development including the epiblast cells and extraembryonic tissues during anterior-posterior axis formation,[11] primitive streak formation, epithelial-to-mesenchymal transition, mesoderm and ectoderm patterning,[12,13] and later during lineage specification. Wnt gradients are persistent across the epiblast and local modulation of Wnt also occurs by factors secreted from adjoining cell layers. Both the canonical and noncanonical Wnt signaling pathways are involved during various stages of cardiac differentiation, with overlapping and distinct roles. In general, the canonical Wnt pathway refers to β-catenin-driven pathways that are activated by Wnt-1, Wnt-2, Wnt-3A, Wnt-8A, Wnt-8B, Wnt-8C, Wnt-10A, and Wnt-10B or small molecules (GSK3β inhibitors BIO, LiCl, and CHIR99021). As a result, the cytoplasmic pool of β-catenin increases allow its nuclear translocation and subsequent interaction with TCF/LEF1 (transcription factor [TF] genes/lymphoid enhancer-binding factor 1) for inducing gene expression or even inhibition of gene repression by certain TCFs.[14] The noncanonical Wnt pathways refer to the planar cell polarity and Wnt/Ca^{2+} pathways, which do not involve β-catenin, and are rather activated by noncanonical Wnt ligands such as Wnt4, Wnt5A, Wnt5B, Wnt6, Wnt7A, Wnt7B, and Wnt11.[15,16]

Drosophila studies initially showed that the Wingless (WNT1 ortholog) and the β-catenin ortholog armadillo are required during a brief period after gastrulation for cardiac specification[17,18] and this requirement is separate from and succeeds Wingless-induced neural segmentation occurring via Sloppy paired[13,19] (close homolog is Brain Factor 1/Blimp-1).

Wnt3 gradients in mice are established early (Fig. 1) and play an essential role for processes such as the formation of anterior-posterior axis, primitive streak, mesoderm, and node required for subsequent cardiac differentiation.[11,20] Detailed examination with mouse ESCs reveals that $Flk1^+$ mesodermal precursors do not emerge when Wnt/β-catenin activity is suppressed by soluble Dkk1.[21] The endogenous activity, which occurs by upregulation of Wnt ligands during differentiating mouse embryonic stem cells (mESCs) is sufficient for the induction of mesoderm and endoderm.

Canonical Wnt signaling is indispensable during early stages of *in vitro* cardiogenic differentiation of P19CL6 mouse embryonic carcinoma cells.[22] This may suggest that

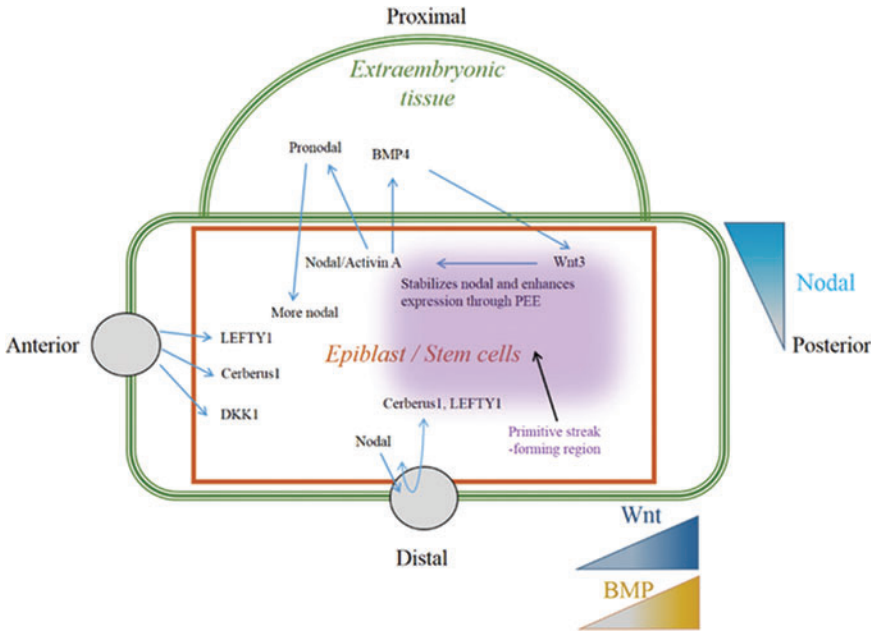

FIG. 1. A model of signaling activity and gradients in mammals during the early stage of embryonic development provides clues for designing the initial stages of hPSC-to-cardiomyocyte differentiation. hPSC, human pluripotent stem cell; PEE, proximal epiblast enhancer. Color images available online at www.liebertpub.com/teb

mesoderm formation via Wnt/β-catenin is an essential initial step in cardiomyocyte differentiation. The PI3K/Akt pathway activates the Wnt3A ligand and promotes cardiopoiesis, whereas PI3K inhibition results in GSK3β-induced degradation of β-catenin[23] alluding to a connection between PI3K and endogenous Wnt/β-catenin signaling activation.

An important TF for mesoderm development and patterning is Brachyury (T), which is expressed in the mesoderm, node, notochordal plate, and notochord during gastrulation. Brachyury is a target of Wnt/β-catenin featuring Wnt responsive elements on its promoter in *Xenopus*,[24] zebrafish,[25] and mouse.[26–28] A study to examine whether *T* is directly transcribed by canonical Wnt signaling in mice found that LEF1 is not essential for the initiation of *T* expression, but it is required for stable maintenance of its expression.[29] In turn, T upregulates *WNT3A*, *FGF8*, and *AXIN2* in mESCs and hESCs[30] indicating an evolutionarily conserved, positive autoregulatory loop for T expression. In mice, T induces MesP1, which marks cardiac progenitors.[31]

After the formation of T-expressing primitive streak and mesoderm, inhibition of Wnt/β-catenin signaling promotes specification toward cardiac anlage. Inhibition of Wnt3A and Wnt8C in the anterior lateral plate mesoderm (LPM) is required for heart formation in chick embryos, and these can also induce cardiac differentiation of posterior lateral mesoderm that would otherwise form blood.[32] Wnt/β-catenin is utilized in mouse iPSCs along with bone morphogenetic protein 4 (BMP4) and Activin A to bring about a PDGFRα$^+$/FLK1$^-$ cell population representing paraxial mesoderm lineages from which tissues such as the bone, cartilage, and skeletal muscle are formed.[33] Similarly, in *Xenopus*, inhibition of Wnt3A and Wnt8 (canonical Wnts) but not Wnt5A nor Wnt11 (noncanonical Wnts) leads to cardiac formation in dorsoanterior mesoderm by the adjacent endoderm and Spemann organizer.[34] Specifically, blocking of β-catenin-mediated Wnt signaling by using DKK1 or Crescent, induces noncardiopoietic ventral mesoderm to heart cells in *Xenopus* embryos. Wnt3A and Wnt1 secreted from the neural tube are also shown to inhibit cardiac formation in adjacent anterior paraxial mesoderm in chick embryos.[35] This suggests that canonical Wnt signaling may act as a switch that either promotes or inhibits cardiac differentiation of mesodermal cells. Unlike the initial commitment to mesoderm cells, further differentiation along cardiac cell lineages requires suppression of canonical Wnt activity.

In *Xenopus*, DKK1 acts on endoderm cells inducing Hex, which in turn triggers paracrine factors to direct adjoining cells toward cardiac fate.[36] Although viewed as an endodermal marker, Sox17 is critical in mediating the expression of Hex in mESCs and coaxes Eomes- and T-expressing mesoderm to cells expressing MesP1, MesP2, and Nkx2.5 thereby pushing the commitment to cardiomyocytes.[37] Other studies in mice have shown that deletion of β-catenin in definitive endoderm can result in ectopic heart formation along the anterior-posterior axis and also convert endoderm to heart.[38] Therefore, inhibition of canonical Wnt pathway induces cardiac differentiation of different mesoderm regions and the endoderm but the precise mechanism still remains under investigation.

Interestingly, Wnt11, a noncanonical Wnt, can induce cardiac cell differentiation in various mesendoderm regions including (noncardiogenic) posterior mesoderm.[39] Wnt11 is found to be essential for cardiac formation in *Xenopus* mediated by protein kinase C and Jun amino-terminal kinase (JNK) and independent of β-catenin.[40] It has been suggested that Wnt11 may act transiently after formation of the heart field to inhibit canonical Wnt signaling. Further cardiac formation proceeds after subsequent antagonism of Wnt11.[41] Other noncanonical Wnts (Wnt5A, Wnt5B, and Wnt4) have also been shown to promote heart formation.[40] Both Wnt5A and Wnt11 contribute to secondary heart field (SHF) progenitor development in mice by suppressing canonical Wnt signaling.[42] When Wnt activation at late stage of differentiation inhibits cardiogenesis, it correlates with Wnt5A and BMP4 downregulation[43] supporting the inverse relationship between canonical and noncanonical Wnt pathways during cardiomyocyte differentiation.

These observations from the embryonic development of model organisms have informed protocols of mESC and hESC differentiation *in vitro*. Primitive streak formation from mESCs requires TGFβ/activin and Wnt/β-catenin signaling[44] with anterior streak formed by high levels of activin, whereas low-level activin A (1 ng/mL) or Wnt3A alone (100 ng/mL) induces posterior streak genes (at the expense of anterior streak) but the sufficiency for mesoderm formation is not conclusive. Wnt/β-catenin is required initially for cardiac differentiation of mESCs at a gastrulation-like stage[37] cultured as embryoid bodies (EBs).[45,46] The same signal should be inhibited subsequently for adoption of heart cell phenotypes. Moreover, Wnt11 treatment also increases cardiac differentiation in these cells. In monolayer differentiation of mESCs, Wnt3A inhibits cardiac differentiation of FLK1- and CXCR4-expressing mesoderm cells, whereas Wnt inhibitors enhance the process.[47] Biphasic application of Wnt/β-catenin is also demonstrated in the induction of hESCs and hiPSCs to cardiomyocytes.[48]

Although the C-terminus of DKK1 induces ectopic heart formation in noncardiogenic mesoderm by inhibition of canonical Wnt pathway, the N-terminus functions independently contributing to chordal mesoderm formation.[49] Rather, Wnt inhibition in hPSCs by small molecules is an efficient mechanism to specify cardiac fate from mesoderm[50–52] and some of these molecules may preferentially specify ventricular cardiomyocytes. Small molecules reported to date are generally stable and inexpensive. Thus, protocols utilizing these may provide tangible advantages over methods based on physiological ligands for the generation of therapeutic cardiomyocytes.[53] In conclusion, Wnt/β-catenin pathway activity is essential but not sufficient for initial commitment of hPSCs to cardiomyocytes. After mesoderm formation, Wnt/β-catenin inhibition can exclusively and efficiently specify cells to cardiomyocyte fates. A summary of the action of Wnt (and of other pathways) is provided in Table 1. Considering that Wnt/β-catenin induces posterior streak mesoderm in mice and is required for both mesoderm and endoderm formation, the identification of mechanisms to select mesoderm over endoderm can potentially augment the efficiency and purity of cardiomyocyte generation.

The BMP Pathway and Cardiomyogenesis

BMP signal transduction involves a subset of TGF-β superfamily ligands (such as BMP2, BMP4, and BMP5-8),

Table 1. Summary of Signaling Pathways Participating in Heart Development

Pathway	*Model systems tested*	*General effects on cardiogenesis*
Wnt	*Drosophila,* chick, mouse, *Xenopus,* zebrafish, mESCs, hPSCs	Early activation of Wnt/β-catenin essential for cardiac mesoderm when combined with other appropriate mechanisms. Inhibiton of Wnt/β-catenin or transient activation of noncanonical Wnts for heart differentiation from mesoderm.
BMP	*Drosophila*, chick, *Xenopus*, zebrafish, mice, mESCs, hPSCs	In early development, inhibits neuroectoderm and promotes mesoderm over endoderm. Cardiac specification of mesoderm and heart organogenesis Transient inhibition in a relatively early stage for cardiogenesis
Nodal	Drosophila, chick, *Xenopus*, zebrafish, mice, mESCs, hPSCs	Specification of endoderm that secretes cardiogenic factors Cardiac mesoderm formation Possible role in mesoderm-to-cardiac differentiation
FGF	Chick, mice, *Xenopus*	Synergizes with nodal/activin A signaling for mesendoderm patterning and mesoderm formation Synergizes with BMPs for mesoderm-to-cardiac differentiation
Notch	Mice, mESCs	Activated by canonical Wnt preventing cardiac mesoderm and inducing somitic differentiation Promotes cardiovascular mesoderm to cardiomyocytes
Sonic hedgehog	Zebrafish, mice, hESCs	Might promote mesoderm to cardiac lineages over somitic lineage Transient role in secondary heart field and myocardium expansion. Hence, may be utilized to fine-tune cardiac cell types or increase yield.
Retinoic acid	Zebrafish, chick, mice, mESCs, human cardiac mesenchymal stromal cells	Control of cardiac progenitor size, regulate side population of progenitors, and stimulate FGF signaling to induce fetal cardiomyocyte formation Secondary heart field development and ventricular cell proliferation Pleiotropic roles in subpopulations of all three germ layers and also various cell types

BMP, bone morphogenetic protein; FGF, fibroblast growth factor; hESCs, human embryonic stem cells; hPSCs, human pluripotent stem cells; mESCs, mouse embryonic stem cells.

which bind to specific cell surface receptors leading to the activation of Smad-1, -5, and -8, which often form complexes with Smad-4 for nuclear translocation and transcriptional activity. BMP-elicited responses vary by cell type or state and cross-talk with other pathways including Nodal/activin signaling (Fig. 1) since both cascades compete for Smad-4. BMPs represses Nodal by sequestration of the shared Smad-4[54] or by promoting the formation of Smad-5/-2 complexes disrupting the Nodal autoregulatory pathway with Smad-2, -4, and Foxh1.[55] The fibroblast growth factor (FGF), Erk, p38, and JNK/mitogen-activated protein kinase (MAPK) pathways phosphorylate Smad-1 contributing to its degradation. There are also interactions with the Wnt/β-catenin pathway since Wnt3A can stabilize and prolong Smad-1 signal by preventing GSK-3- and MAPK-mediated Smad-1 degradation.[56] In fact, a wingless-like effect is observed in *Drosophila* upon mutation of the GSK-3 phosphorylation site in a Smad-1/-5/-8 homolog.[57] In contrast, Smad-1 binds to the canonical Wnt protein dishevelled, inhibiting Wnt/β-catenin signaling in mesenchymal stem cells.[58] Signaling through BMP-Smads also inhibits the transcription-repressive activity of several Smad-interacting proteins such as ZEB1/SIP1 and ZEB2 and occasionally synergize with them to specify and control spatial boundaries within the developing embryo.

BMPs disrupt ESC self-renewal resulting in differentiation.[59] Endogenous BMP2 and its receptors are activated in hESCs under spontaneous differentiation conditions and may act as a positive feedback loop for extra-embryonic differentiation.[60] Inhibition of BMP signaling by Noggin results in neuroectoderm but not in mesoderm or extraembryonic lineages. Lower concentration and short-term BMP4 treatment contributes to the emergence of mesodermal lineages.[61] Therefore, BMP signaling affects early decisions between neuroectoderm, mesoderm, and extraembryonic fates but does not promote endoderm specification.

In the stage 6 chick embryo, combinatorial coculture experiments of different embryonic regions revealed that only the anterior lateral plate endoderm (LPE) induces cardiac differentiation of the anterior LPM,[62] whereas earlier (stages 4–5) anterior LPE and central endoderm cells induce noncardiogenic posterior primitive streak cells toward cardiomyocytes.[63] Cardiogenic paracrine factors expressed in the LPE are identified to be mainly BMPs but also activin A, FGFs, and vitamin A transport proteins (see below; Fig. 2).[64] BMP signaling is also essential for cardiac differentiation as shown in *Bmp2*$^{-/-}$ mice.[65] In fact, BMP2/4 from the adjoining anterior LPE are responsible for cardiac differentiation of the anterior LPM and nonprecardiac anterior medial mesoderm cells during stages 5–7 of chick development.[66] Combined treatment with FGF-4 and BMP-2 (Fig. 2) directs nonprecardiac mesoderm cells (in stage 6 chick embryos) to heart[67] and promotes cell survival/proliferation.[68]

However, depending on end cell type of interest, optimization of FGF and BMP signaling for developing cardiac differentiation protocols will require concomitant study of

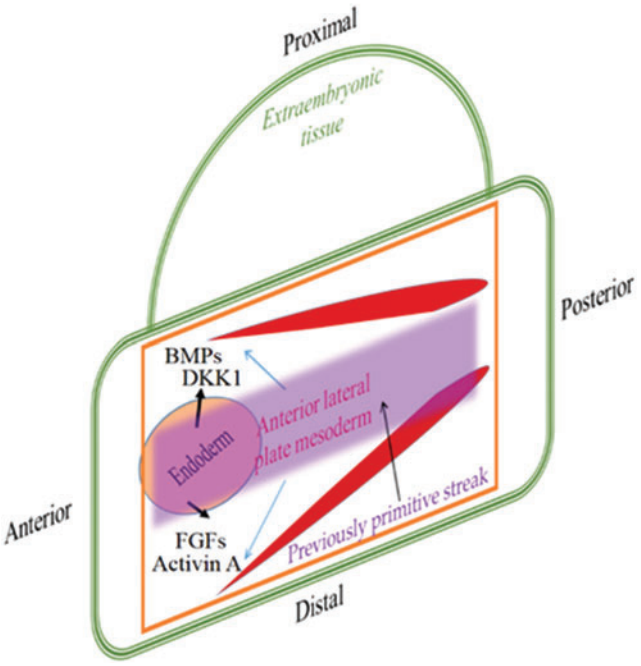

FIG. 2. Local paracrine signaling in the cardiac-forming region of mesoderm in mammals in stages subsequent to those shown in the previous figure. Anterior lateral plate endoderm secretes paracrine factors that induce the emergence of cardiac progenitors in the anterior lateral plate mesoderm. BMP, bone morphogenetic protein; FGF, fibroblast growth factor. Color images available online at www.liebertpub.com/teb

the inhibition of endogenous signaling pathways. More specifically, the LPM consists of both primary and SHF progenitors with defined spatial boundaries. Primary heart field progenitors fuse to form the primary heart tube, which eventually gives rise to the left and right atria, left ventricle, atriovenal inflow tract and atrioventricular canal. Concurrently, SHF progenitors continue to proliferate and extend the heart tube at the arterial pole for forming the right ventricle, ventricular outflow tract, and smooth muscle cells connecting to the aorta and pulmonary trunk.[69] Inhibition of BMP signaling[70] (and activation of other pathways[71]) promotes SHF progenitor expansion suggesting ways of increasing ventricular cell yields during *in vitro* hPSC differentiation.

After fusion of the heart tube, BMP signaling is active in the myocardium and endocardium as evidenced by phospho-Smad1 activity prevalent in these regions.[72] In fact, the switch for SHF progenitors from proliferation to their differentiation requires BMP activation[73] (and inhibition of FGF) to possibly form ventricular myocardium while preventing smooth muscle differentiation that would occur in the presence of FGF8.[74] Moreover, asymmetric BMP activity is observed (activated Smad1 only on right side of developing myocardium) and believed to occur by mechanisms other than ligand expression because BMP7 is still expressed throughout the heart during this stage.

BMP-2 is expressed in the primitive myocardium and in adjacent mesoderm cells with subsequent expression in peripheral myocardial cells of the atrioventricular canal (E7.5–E9.5).[65,75] BMP induces TFs such as *Gata4*, *Mef2c*, and *Nkx2.5*.[76,77] Induction of these cardiac TFs by BMP is occurring via the TGF-β-activated kinase-1 (TAK-1) based on evidence from P19CL6 mouse embryonic carcinoma cells.[78] *Tbx2/3* is activated by BMP for heart (and limb) development in cells expressing *Isl1*.[79] Interestingly, BMP-Smad1 promotes the expression of *Tbx20* but not *Tbx5* and *MHC* (myosin heavy chain) in *Xenopus*, zebrafish, and mice by activating noncanonical Smad-binding elements.[80] Synergy between Smad-1/4 and TAK1 effectors of the BMP pathway in P19CL6 cells leads to the transcription of the activating transcription factor-2 (ATF-2), which stimulates β-MHC expression resulting in terminal cardiac differentiation.[81]

Zebrafish differentiation also requires BMP signals for development of myocardial cells from the SHF.[73] *In vivo* studies show that Wnt inhibition is specifically necessary in regions of high BMP activity,[32] indicating a combined Wnt(−) and BMP(+) requirement during later stages of heart development. As discussed above, Wnt/β-catenin activation contributes to expansion of SHF progenitors, and therefore this pathway would need to be inhibited during BMP-induced differentiation of cardiac progenitors. During vascular development and vein formation, BMPs provide angiogenesis cues.[82] In fact, activin A treatment relies on BMP4 to direct T^+ cells to hematopoietic lineages.[83]

Since heart and vascular lineages originate from mesoderm, it is important to note that the timing and context of BMP signaling is critical for efficient cardiac differentiation. BMP signaling also directs extraembryonic differentiation and formation of other mesodermal organs. For example, for hESC differentiation, 0.5 ng/mL BMP4 is used during the first 24 h, followed by 10 ng/mL BMP4 in combination with 3 ng/mL activin A and FGF2 for 3 days before treatment with DKK1 and VEGF.[84] Others, however, reported the use of 10 ng/mL BMP4 from day 0–3 along with 25 ng/mL activin A from day 1–3 after which inhibitors of canonical Wnt are added.[53] Thus, more detailed investigation is warranted of the timing and concentration of BMP ligands in cardiomyocyte differentiation under a comparable platform of chemically defined media.

Nodal/Activin Signaling During Heart Formation

Nodal gradients are present in epiblast cells during embryo development and are controlled by extraembryonic tissues. The regulation is mediated by high Nodal production in epiblast cells, timed secretion of Nodal antagonists by regions of the visceral endoderm, and autoregulatory feedback loops involving BMP4 and Wnt3 in proximal extraembryonic tissue (Fig. 1).[85–87] The gradients are critical for mesendoderm patterning and lineage specification. The requirement of Nodal signaling for proper left-right asymmetric development of heart, vasculature, lungs, and stomach has been demonstrated via loss-of-function studies in mouse embryos.[88] Suppressing Nodal activity through deletion of Smad-2 or both Smad-2 and -3 affects the proper specification of axial mesendoderm.[89,90] Interestingly, Smad-$4^{-/-}$ mouse epiblasts fail to pattern anterior streak but heart formation is not affected.[91] Smad-4-dependent TGF-β (Smad-2/3)-induced cell actions are categorized as antiproliferative and migratory.[92] Nodal/activin induces multiple mesoderm regions in a concentration-dependent fashion and is sensitive to synergy with FGF in *Xenopus*.[93] Brachyury expression is understood to reflect and transduce these morphogenetic

effects of Nodal/activin on mesoderm formation in *Xenopus*, and the promoter of *Xbra2* has concentration-dependent responsive elements to activin that are also affected by FGF.[94] Low concentrations of activin in combination with FGF induce *Xbra*, whereas high activin concentrations inhibit *Xbra* expression via Goosecoid (*Gsc*), Mix.1, and Xotx2.[95]

Therefore, not only does Nodal signaling regulate the formation of anterior mesoderm and endoderm, which secrete cardiogenic factors, but also plays a direct role in cardiac mesoderm formation. Moreover, the LPE in chick that secretes cardiogenic factors such as BMPs, Wnt inhibitors, and FGFs also secretes activin A,[64] indicating another possible engagement of Nodal in mesoderm-to-cardiomyocyte differentiation (Fig. 2).

Activin A has been used in the first stage of cardiac differentiation of hESCs at high concentration (100 ng/mL) in combination with Wnt3A (100 ng/mL)[96] but investigation of the effects of lower activin A concentrations on mesoderm formation has not been reported to date. During intermediate stages of mesoderm formation and differentiation to cardiomyocytes, fine modulation of combinations of activin A and BMP4 concentrations has been suggested to influence cardiogenesis.[97] However, whether nodal/activin improves the differentiation efficiency and formation of particular cardiac cell types is unclear necessitating studies with various concentrations of activin A alone or in combination with other ligands (e.g., Wnts and BMPs) in early and late stages of hPSC differentiation.

FGF Signaling in Cardiomyocyte Differentiation

FGF signaling is mediated via MAPK and PI3K pathways in hESCs, wherein the PI3K contributes to maintenance of pluripotency possibly by enabling nuclear localization of β-catenin.[98] FGFs are involved in mesendoderm, mesoderm, and endoderm fate decisions in human and mouse PSCs.[99,100] For example, during early mesoderm formation in *Xenopus* FGF signaling combined with Nodal induces T, which features FGF response elements in its promoter.[93,94] Moreover, BMP signaling synergizes with FGF signaling by upregulating FGFR3 to induce T expression leading to mesodermal fates.[101]

In the chick, endoderm cells adjacent to anterior LPM cells secrete BMP-2, FGF-1, and FGF-4,[66,67,102] which are required in combination for cardiac differentiation, and furthermore can coax posterior mesoderm cells to cardiac cells. Indeed, endoderm-originating FGF-1, -4 (stage 11–15), and FGF-2 (stage 9 onward) emerge in the cardiac-forming region promoting proliferation and cardiac differentiation.[102] Other FGFs such as FGF-2/-4 in combination with BMP-2/-4 induce cardiogenesis in noncardiac mesoderm.[68] Cardiac neural crest regulates FGF-8 in endoderm adjacent to cardiogenic mesoderm.[103] The secreted FGF-8 induces NKX2.5 and MEF2C in cells already exposed to BMP2.[104] BMP-2 and FGF-8 induce cardiac formation from the SHF in a similar process as that in primary heart field.[70] Similarly, FGF signaling is required for the addition of outflow tract myocardium from the SHF (reviewed in Dyer and Kirby[105]). FGFs and FGFR2 are also reported to induce Sox2, which inhibits Wnt signaling in adjacent cells for cardiac formation.[106]

FGF signaling may be active during *in vitro* differentiation of hPSCs to cardiomyocytes especially in the presence of other ligands. However, systematic studies with various concentrations and durations of hPSC exposure to FGF ligands are lacking. Such studies can bring to reality promising benefits from the inclusion of FGFs for efficient cardiogenic differentiation.

The Role of Notch Pathway in Cardiac Differentiation

Notch signaling is well known for its role in somitic mesoderm differentiation occurring proximally to the heart-forming region. The Notch ligand Delta-like1 is upregulated by canonical Wnt signaling during somite formation.[107] In mESCs, Notch signaling transmits BMP and Wnt-related signals (including secreted frizzled-related proteins or SFRPs) for cardiac specification and can respecify FLK1-positive hemangioblasts to cardiac fate.[108] Direct induction of mesoderm-to-heart differentiation in hPSC culture occurs with the inhibition of the canonical Wnt pathway that may also contribute to inhibit somite differentiation by suppressing Notch signaling. Therefore, active Notch may be undesirable during intermediate stages of cardiopoiesis (e.g., mesoderm formation) but promotes subsequent improved conversion of cardiovascular mesoderm to cardiomyocytes.

Sonic Hedgehog Signaling and Heart Formation

Dorsal midline-originating Sonic Hedgehog (Shh) induces Smad-1 in zebrafish somites[109] rendering them sensitive to BMP-2/-4 signaling.[73] To that end, inhibition of Shh might improve specification of cardiomyocytes over somitic tissues from mesoderm. However, Shh also contributes to the SHF progenitors[110] transiently for generating sufficient myocardium and smooth muscle. The requirement of Shh for arterial pole development by the right side of SHF is reviewed in Dyer and Kirby.[105] In fact, purmorphamine (a Shh agonist) has been used while inhibiting Wnt/β-catenin and nodal pathways for mesoderm-to-cardiac cell differentiation[51] highlighting that inclusion of modulators of Shh signaling may benefit *in vitro* cardiac differentiation of hPSCs.

Retinoic Acid in Heart Muscle Differentiation

Retinoic acid (RA), a metabolite of vitamin A/retinol, is believed to act primarily in a paracrine manner by binding to two families of nuclear RA receptors (the RA receptors or RARs:RARα, RARβ, and RARγ and the retinoid X receptors or RXRs: RXRα, RXRβ, and RXRγ), which directly regulate transcription. All-*trans*-RA is an abundant form of RA that binds both to RARs and RXRs. The *cis* isomer of RA (9-*cis*-RA), which is typically undetectable during development (detected in the adult mouse pancreas[111]), binds only to RXRs.[112] Almost all embryonic cells express at least one of these nuclear receptors making them responsive to RA. Given this fact, regulation of RA signaling is principally controlled through enzymes producing or inactivating retinoids. RA has several roles in vertebrate embryogenesis including anterior-posterior patterning via Hox genes and development of the posterior neuroectoderm, foregut endoderm, and trunk mesoderm while at later stages RA influences the development of the eye, olfactory regions, and other organs.[113]

In heart development, RA effects are mainly mediated through the RARα and RXRα.[114] At early stages, RA signaling is thought to control progenitor size as increasing RA

concentrations produce decreasing heart populations in chicken and zebrafish embryos[115,116] and micromolar concentrations result in no heart formation. Later on, RA participates in the establishment of the second heart field (typically Isl-1$^+$) cell populations, which will subsequently give rise to atrial cells.[117,118] In ventricular cells, RA contributes to their proliferation as shown in cultured ventricular sections.[119] Even endogenous RA was shown to regulate a side population (effluxing the Hoechst 33342 dye) of heart progenitor cells and stimulate fetal cardiomyocyte differentiation in mice by stimulating FGF signaling.[120]

It should be noted that RA has been used for the differentiation of mESCs[121,122] but its effects on hPSC differentiation are not well understood.[123] The utility of RA to control cardiomyocyte yield is confounded due to its pleiotropic effects in the specification of several other lineages (e.g., adipocytes,[124] neuronal cells[125]). Yet, RA has been used as part of a serum-containing cocktail for reprogramming human cardiac mesenchymal stromal cells into cardiovascular precursors[126] and a recent report[127] showed RA-dependent specification of hPSCs to atrial- or ventricular-like cells (see *In Vitro* Methods for Cardiac Differentiation of hPSCs section).

Heart Development and Gene Regulatory Networks

Heart development relies on coordinated genetic programs regulated by signaling cascades whose activity is stimulated by extracellular ligands in the tissue microenvironment. Central to these programs are TFs and their target genes many of which encode proteins for cardiomyocyte functions. The cardiogenic differentiation of stem cells *in vitro* can be segmented into stages[7] involving the sequential transition of pluripotent cells (expressing Nanog, Oct4, and Sox2) through mesoderm (T, Eomes and MesP1) and cardiac precursors (Gata4, Nkx2.5, TBX5, and MEF2) to heart cells (MYH6, MYH7, and α-cardiac actin). Based on developed analytical methods (e.g., microarrays) and currently evolving next generation sequencing technologies (e.g., RNA seq, microRNA (miRNA) seq, and chromatin immunoprecipitation [ChIP] seq), massive amounts of data have become available allowing the interrogation of the transcriptional programs operating during the PSC specification to diverse lineages including cardiomyocytes. An example is shown in Figure 3A, where using publicly available datasets from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo), a principal component analysis was performed of transcriptomic profiles for cells at different stages of mouse cardiac differentiation. Each stage is characterized by a distinct phenotype and gene expression pattern where PC1 and PC2 represent the top two significant dimensions of genes showing differential expression during cardiac differentiation.

During mesoderm formation, the pluripotency TFs Nanog, Oct4, and Sox2 are downregulated while T expression is activated. The expression of T is directly linked to canonical Wnt signaling activity, at least in mice where the T promoter features TCF/LEF binding sites[26,27] although similar direct evidence is lacking today for the human gene promoter. Interestingly, T and Oct4 are co-expressed in mouse epiblast stem cells derived from mouse postimplantation embryos and T$^+$ cells could revert to a T$^-$ state suggestive of their plasticity during mesoderm specification.[128]

Brachyury forms a negative feedback loop with MesP1[31,129] in the mouse embryo. While the expression of MesP1 is upregulated by T early on, T subsequently suppresses MesP1[130] controlling mesoderm fate commitment. MesP1 also suppresses Sox17 and Foxa2 expression thereby inhibiting the specification toward endoderm, which can originate from T$^+$ cell populations (mesendoderm).[130] The expression of Gata4, Gata6, Tbx20, Hand2, and Nkx2.5 is promoted upon activation of MesP1 in an autoregulatory loop involving negative feedback with Ripply2.[130] BMP signaling also induces Gata4, Mef2c, and Nkx2.5[76,77] while Tbx2/3 is activated in Isl1$^+$ cells.[79] Although not cardiac-specific, Isl1 activates the expression of Foxh1[131] and Mef2C[132] directing development to right ventricle and outflow tract (SHF).[133,134]

In particular Nkx2.5, the mammalian homolog of *Drosophila* tinman, is one of the earliest TFs expressed in cardiac progenitor cells as a target of Gata4 and Smad1/4.[135,136] Downstream genes of Nkx2.5 include the atrial natriuretic peptide (ANP; also known as atrial natriuretic factor [ANF]),[137] cardiac α-actin,[138] GATA6,[139] HAND1,[140] and Mef2C.[131] Gata1 is suppressed by Nkx2.5 coaxing the commitment of progenitors along cardiac instead of hematopoietic cell lineages.[141] The expression of MEF2 genes correlates with Hand genes as essential regulators for cardiac myogenesis and right ventricular development.[142] Cooperation between Smad-1/4 and TAK-1 transducers of BMP signaling activates ATF-2 that transcribes β-MHC.[81]

NKX2.5 also acts in combination with other TFs such as GATA4 and TBX5 to generate functional cardiomyocytes from hESCs.[143,144] For instance, the human ANF promoter is synergistically activated by NKX2.5, TBX5, and GATA4.[145] Through direct interaction between them, Nkx2.5 and Gata4 directly target several cardiac genes including α-cardiac actin[146] and cardiac-restricted ankyrin repeat protein (CARP).[147] Last, Nkx2.5 with Tbx5 directly binds to the promoter of the mouse natriuretic peptide precursor type A (Nppa).[148]

Thus, the complexity of gene regulatory networks governing cardiogenesis is attributed not only to the large number of genes involved but also to the intricate combinatorial effects and feedback/feedforward loops of TFs and co-TFs. Microarray and high-throughput RNA sequencing have become major means of extracting transcriptomic information, which is used for the construction and better understanding of GRNs.[149] It should be noted that the methods for inferring and analyzing GRNs undergo continuous refinements especially with respect to the detection of false positive or negative gene interaction. Despite the shortcomings however, bioinformatics approaches provide valuable directions for studying cell fate decision processes at single gene level during heart differentiation of stem cells and predicting the adoption of cell phenotypes at systems level particularly after targeting genes (e.g., via siRNA/shRNA, ectopic expression, etc.). One such example is shown in Figure 3B where a putative GRN is generated by the ARACNE algorithm[150] from temporally serial microarray datasets taken from mESCs to cells at the E8.5 stage.

In Vitro Methods for Cardiac Differentiation of hPSCs

Initial efforts on the cardiac differentiation of hPSCs *in vitro* involved the formation and culture of EBs in the

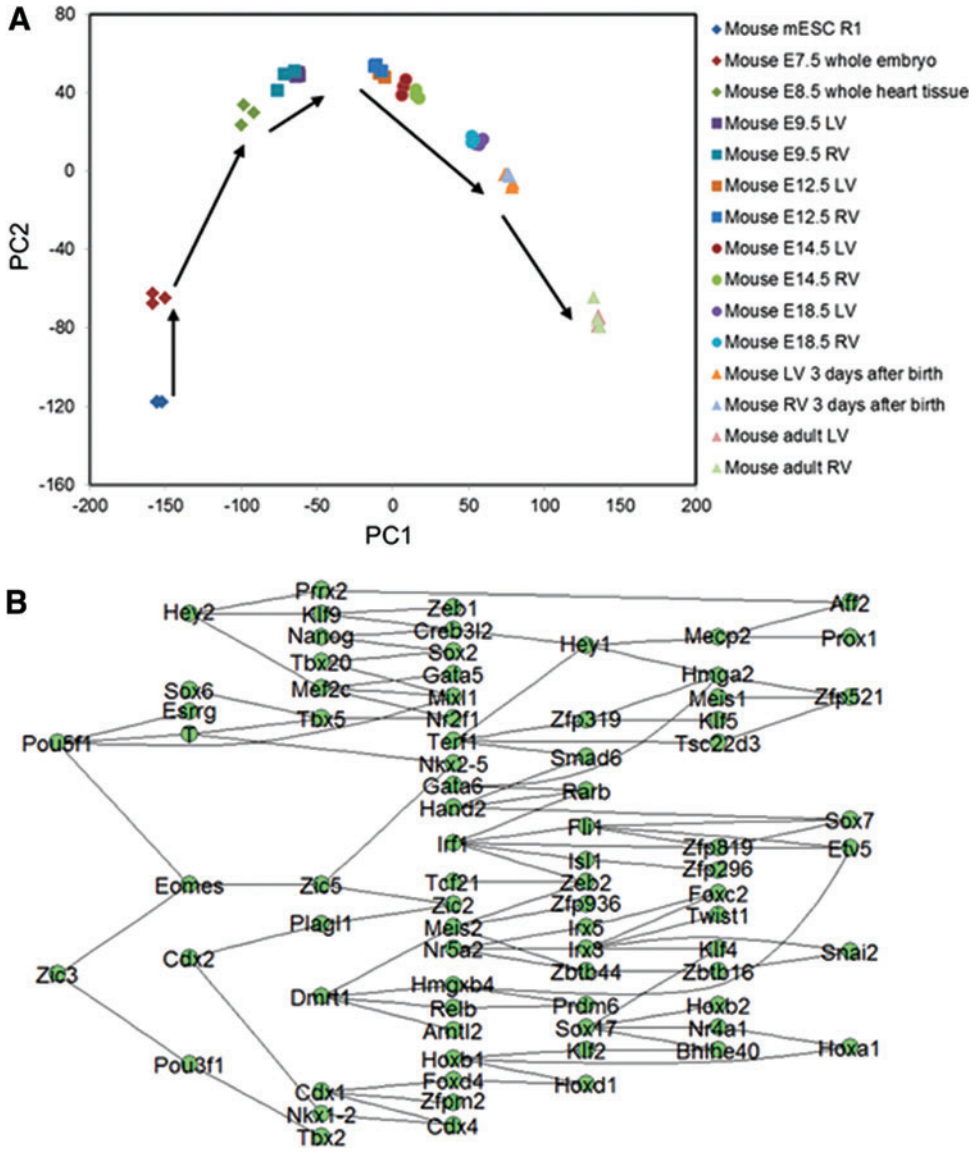

FIG. 3. **(A)** Principal component analysis (PCA) of gene expression microarray data during mouse cardiogenesis. LV, left ventricular; RV, right ventricular. **(B)** An example of a putative gene regulatory network generated using the ARACNE algorithm[150] was shown. Eighty most differentially expressed transcription factors from mouse embryonic stem cell to cells at the E8.5 stage were selected for network inference. The original dataset is available at the NCBI GEO database (GSE51483[175]). Bioconductor packages (Biobase and GEOquery) and the prcomp function in R were implemented for data processing and PCA. The R packages Minet[176] and Rgraphviz were used for gene network inference (ARACNE algorithm) and visualization. Color images available online at www.liebertpub.com/teb

presence of serum.[123,151] Given the undefined composition of serum, these protocols were characterized by low reproducibility and the obtained cell populations were highly heterogeneous with minute fractions of cardiomyocyte-like cells.

With clinical applications of stem cell-derived cardiomyocytes in mind, these issues motivated further investigation into efficient methods utilizing chemically defined media devoid of animal-sourced components. Such directed differentiation protocols for the cardiogenesis of hPSCs often comprise two or more steps of specification utilizing physiologically relevant growth factors and other media ingredients. Of importance in these protocols are also the culture configuration (e.g., monolayer, multilayer, and 3D/EBs), shape, and dimensions of scaffolding, physical parameters (strain and shear), and other variables (e.g., culture surface and stress) of the *in vitro* microenvironment. These steps typically recapitulate aspects of the native hierarchical process of lineage development from pluripotent and germ layer progenitors to specialized cell types.

In an EB protocol by Yang *et al.*,[84] aggregate formation is initiated with 0.5 ng/mL BMP4 in StemPro34-based media for 24 h. The subsequent steps involve treatment with BMP4, FGF2, and Activin A at 10 ng/mL or lower for 4 days, VEGF and DKK1 for the next 4 days, followed by VEGF, DKK1, and FGF2 for the final 6 days. On the 6th day of differentiation, EBs are dissociated into dispersed cells, which are sorted by flow-activated cell sorting after co-staining with KDR and cKIT antibodies. A cardiogenic subpopulation (KDR^{low}/$C\text{-}KIT^{neg}$) is replated for further differentiation. Approximately, 55–60% of the differentiated cells express cardiac-Troponin T (cTnT). In this protocol, the concentrations of Activin A and BMP4 used for specification of different hPSCs are particular to each line and are obtained by fine-tuning in response to KDR/PDGFRa subpopulations. Such adjustments in factor concentrations are required for certain cell lines exhibiting lower efficiencies.[97]

Fewer factors are used in a monolayer protocol with Wnt3A (100 ng/mL) and Activin A (100 ng/mL) during the

first 24 h followed by BMP4 (10 ng/mL) for 4 days in media containing B27 supplement.[96] However, the differentiation efficiency is lower since roughly 25–30% of the cells stained positive for sarcomeric MHC. In comparison, a more recent method with increased differentiation efficiency was described also using the B27 supplement.[48] Between 80% and 98% differentiated cells co-expressed the cardiac markers MLC2a and cTnT. In this protocol, the canonical Wnt pathway was activated by inhibiting GSK3β using shRNA or small molecules for 24 h, and the same pathway is inhibited on day 3 with small molecules such as IWP2/4. Biphasic modulation of canonical Wnt pathway significantly increases the differentiation efficiency and reduces the length of cardiac differentiation with spontaneously contracting cells emerging as early as day 7/8 in several studies.[48,52,152] Optimal outcomes are typically attained when the time for such modulation of Wnt/β-catenin is adjusted by 1–3 days (Fig. 4) and may require to be suited to other factors used, composition of media, and cell line in question.[52,152,153]

Cardiac differentiation protocols have continued to improve in recent years moving toward the use of small molecules, higher efficiencies and reproducibility, and simplicity. Yet, methods to obtain different cardiac cell types remain a challenge, and most protocols yield mixtures of cells displaying atrial, ventricular, and nodal attributes, which are more precisely determined by electrophysiology and the expression of MLC2a or MLC2v after allowing for ''maturation'' over several weeks beyond growth factor treatment.[52] Appropriate incorporation of activin A, BMP4 ligands, and various Wnt/β-catenin inhibitors (e.g., IWPs, IWRs, DKK1, KY02111, N11474, XAV939, Wnt-C59, etc.) might be alluding toward selection between atrial and ventricular cells[53,152] although more work is needed to substantiate these possibilities. Another hurdle in the current differentiation protocols is that the generated cardiomyocytes display characteristics and electrophysiological attributes of the fetal heart and methods for maturation of these cells into adult-like cardiomyocytes need to be researched. As already mentioned, the different cardiac differentiation protocols are based on the activation of the canonical Wnt pathway initially and its inhibition subsequently. Wnt/β-catenin modulation alone appears to be sufficient, yet activin A and BMP4 have also been used in the early stages. It is argued[48,96] that activin A and BMP4 may modulate endogenous Wnt activity, yet separate requirements and fine-tuning of these pathways are demonstrated by others.[84,97] For the Wnt pathway, appropriate selection of timing and duration has proven to be critical, whereas for the activin A and BMP4 cascades, the concentrations, combination, and duration of treatment appear to affect the differentiation. Whether the different pathways function through separate or similar mechanisms requires more complex analysis that takes into account context-dependent outcomes. Moreover, it would be important to address whether the different pathways participate in different aspects of cardiogenesis with the possibility of utilizing them to obtain specific cardiac cell types.

In vitro differentiation of hPSCs to cardiac muscle cells yields mixtures of noncardiac cells and ventricular-, atrial-, and nodal-like cells hampering their application in myocardial repair. Increasing effort is directed toward understanding better the signals that induce cardiac subtype specification but standardized conditions are still lacking. Retinoid signaling, for instance, may regulate the atrial versus ventricular differentiation of hPSCs although more studies are warranted. Human H7 ESCs initially differentiated with BMP4, bFGF, and activin A were further (from day 4 on) treated with Noggin and either RA or the RA antagonist BMS-189453 (RAi).[127] The addition of RAi led to an increase on $cTNT^+$ cells from 50% with Noggin only or Noggin and RA to 73% by day 14. More importantly, cells exposed to RAi expressed higher levels of ventricular cell-specific genes *IRX4* and *MLC2V* and 83% of the cells ($n=23$) exhibited ventricular-like action potentials (APs). In contrast, differentiation with Noggin and RA resulted in cells with atrial markers (ANF and MLC-2A) and atrial-like APs (93% of 19 cells assayed). Others have reported that directed differentiation of hPSCs with small molecule inhibitors of Wnt signaling (e.g., IWR-1) preferentially yields ventricular cardiomyocytes (100% out of 26 cells) compared to only half of the cells (out of 31) displaying ventricular

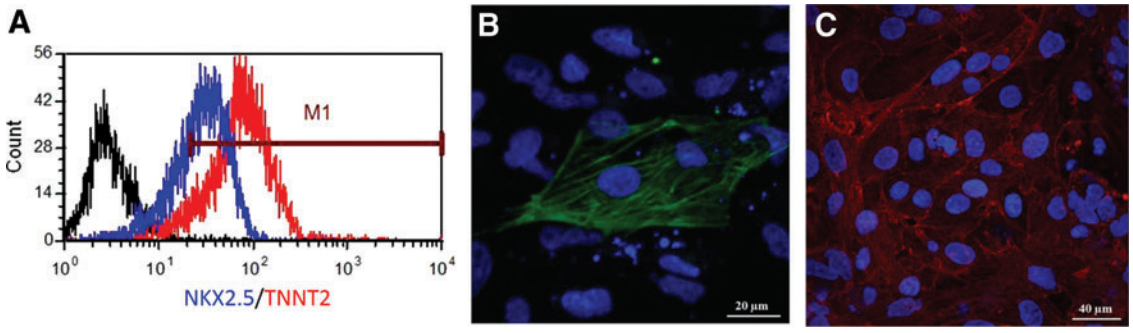

FIG. 4. **(A)** Flow cytometry of differentiated hiPS (IMR90)-4-DL-1 hiPSCs to cardiomyocytes in defined medium using transient inhibition of BMP signaling at an intermediate stage. Almost 68% of the cells expressed NKX2.5 (*blue curve*) and 91% expressed TNNT2 (cardiac Troponin T; *red curve*). *Black curve* corresponds to isotype control. Cardiac cell markers are also probed by immunofluorescence in HUES9 hESCs coaxed toward cardiomyocytes in defined medium supplemented with a Wnt/β-catenin signaling inhibitor after initial induction to mesoderm. Staining is shown against **(B)** TNNT2 (*green*) and **(C)** ACTN1 (α-actinin; *red*). Nuclear DNA staining with DAPI (*blue*) is also shown. Bars: **(B)** 20 µm, **(C)** 40 µm. hESCs, human embryonic stem cells; hiPSCs, human induced PSCs. Color images available online at www.liebertpub.com/teb

cell attributes when treated with DKK-1.[53] However, how the IWR-1 promotes cardiac cell subtype commitment is not understood and others using similar small molecules have reported heterogeneous cardiac cell populations.[48] Subtype selection during commitment may be facilitated by prior enrichment of the differentiating cell population. Glucose-depleted, lactate-supplemented culture medium selects for cardiomyocytes, which metabolize lactate for energy supply while other noncardiac cells die out.[154] A 4-day treatment of differentiating hiPSCs with lactate-containing/glucose-devoid culture medium resulted not only in improved differentiation efficiency (95% $cTNT^+$ cells vs. 63% for control cultures) but also in the reduction of line-to-line variability (ranging from 33% to 92% of $cTNT^+$ cells for five hiPSC lines).[155] Similar enrichment of hPSC-derived cardiomyocytes has been demonstrated in spinner flask cultures.[156]

Another vexing issue is the phenotypic immaturity of hPSC-derived cardiomyocytes including the lack of fully formed sacromeric elements and multinucleation, and their suboptimal Ca^{+2} handling. For their *in vivo* maturation, cardiomyocytes experience mechanical loads and electrical activity influencing contractile function, protein synthesis, cell size, and overall tissue remodeling. *In vitro* recapitulation of hPSC-cardiomyocyte maturation has been attempted mainly by culturing differentiating cells for protracted periods in 3D configurations (e.g., EBs), under mechanical strain (e.g., cyclic loads) or electrical stimulation, and with non-cardiac cells. hPSC-cardiac cells maintained for 80–120 days have greater myofibril density and alignment and a 10-fold higher fraction of multinucleated cells when compared with cells maintained for up to 40 days.[157] Late-stage cells also exhibit upregulated MYH6 and MYH7 (α- and β-MHC) and improved contraction. Given that after 4 weeks of differentiation, the cells reach a rather stable transcriptomic state in 2D and 3D cultures, it is hypothesized that additional time is necessary for functional and ultrastructural maturation.[158] Differentiation can be further enhanced in 3D cultures by culturing cells in bovine (type I) collagen/Matrigel hydrogels yielding cells (2–3 weeks) expressing cardiac genes at comparable levels to the adult left ventricle.[159] Fibrin-based hydrogels, biodegradable scaffolds, and scaffold-free constructs have also been used for promoting maturation of hPSC-heart muscle cells.[160–162] Interestingly, noncardiomyocytes, which are typically found in differentiating cell cultures and are considered undesirable, may contribute to the electrophysiological maturation of hPSC-cardiomyocytes.[163] The presence of noncardiomyocytes (defined as all cells in culture not expressing cardiac cell markers) in EBs results in higher AP amplitude, more hyperpolarized maximum diastolic potentials, and faster maximal upstroke velocities for stem cell-derived cardiomyocytes during 20–60 days of differentiation. In fact, these attributes of electrophysiological maturation were lost when the hPSC-cardiomyocytes were isolated at 20 days of commitment and cultivated without noncardiac cells but were regained after adding back non-cardiomyocytes to the differentiation cultures. Elucidating the identity of these noncardiomyocytes will be necessary for adoption of this method for hPSC-derived heart cell maturation.

Beyond soluble signals, other intracellular factors and processes are also important and have been shown to modulate the cardiogenic differentiation outcome. The potential role(s) of miRNAs during cardiac development and their current utility in *in vitro* differentiation of hPSCs to heart cells are reviewed elsewhere.[164–168] As our knowledge broadens on miRNAs in mechanisms of heart formation, function, disease, and reprogramming or transdifferentiation, new possibilities will emerge for bioprocessing of cardiomyocytes for autologous cell therapy and novel preventive[169–171] and regenerative[166,172] therapeutic methods. Exosomes also emerge as vehicles for information transfer (DNA, RNA, small RNA, and proteins) among cells in the adult heart and *in vitro* suggesting potential roles during hPSC differentiation.[173] Approaches combining multiple stimuli such as miRNAs, signaling activation, and transcriptional regulation are expected to overcome the current challenges for clinical application such as cardiomyocyte maturation, cell type (e.g., ventricular cells) enrichment, and selection, with direct benefits to the therapeutic potential of the transplanted cells.

Conclusions

From our review of the signaling pathways and associated genetic programs emerges a complex picture of processes directing heart cell differentiation. The mode of action of some cascades, such as the biphasic role of canonical Wnt, is well established. However, many of the signaling partners in cardiogenesis operate in nonlinear manner forming networks making challenging to decipher the complete regulatory structure governing heart precursor cell specification. From a hPSC differentiation viewpoint, understanding the complex signaling and GRN activities in their entirety may not be obligatory with recent studies showing that simpler protocols may be effective in generating cardiomyocyte-like cells with attributes sufficient for particular purposes such as drug screening. Several methods have been successfully employed for producing cardiomyocytes involving Wnt/β-catenin modulation and treatments with BMPs and activin A.[48,52,53,84,96,97,152]

Deeper understanding, however, will be essential for generating cardiomyocytes from hPSCs for cell therapies given that the resulting cells should resemble native cardiomyocytes closely for functional integration with the host tissues. Comprehensive investigations are still required that consider sequential combinations of signaling pathways and take into account physiological mechanisms of these pathways as described here. Utilizing chemically defined media will significantly facilitate the assessment of different signaling pathways and targeted GRNs in cardiomyocyte differentiation. Clinical applications also dictate the scalable, xeno-free generation of cardiomyocytes from hPSCs. In the same context, the roles of epigenetic changes and miRNAs have only recently started to be addressed.[174] The success in generating cardiac mesoderm progeny calls for systematic experimental designs focusing on the controlled specification to distinct heart cell types such as ventricular cardiomyocytes, and the impartation with high fidelity of attributes of corresponding native cell populations. Given the rapid progress thus far in the development of efficient strategies for the production of heart cells, we anticipate the realization of more hPSC-derived cardiac cell applications envisioned in regenerative medicine.

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Disclosure Statement

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