Continuous Antagonism by Dkk1 Counter Activates Canonical Wnt Signaling and Promotes Cardiomyocyte Differentiation of Embryonic Stem Cells

Meena Rai,^{1,2} Joel M. Walthall,^{1,2} Jianyong Hu,^{1,2} and Antonis K. Hatzopoulos^{1,2}

Embryonic stem (ES) cells give rise to mesodermal progenitors that differentiate to hematopoietic and cardiovascular cells. The wnt signaling pathway plays multiple roles in cardiovascular development through a network of intracellular effectors. To monitor global changes in wnt signaling during ES cell differentiation, we generated independent ES cell lines carrying the *luciferase* gene under promoters that uniquely respond to specific wnt pathway branches. Our results show that successive, mutually exclusive waves of noncanonical and canonical wnt signaling precede mesoderm differentiation. Blocking the initial noncanonical JNK/AP-1 signaling with SP60125 aborts cardiovascular differentiation and promotes hematopoiesis, whereas interference with the subsequent peak of canonical wnt signaling using Dkk1 has the opposite effect. Dkk1 blockade triggers counter mechanisms that lead to delayed and extended activation of canonical wnt signaling and mesoderm differentiation that appear to favor the cardiomyocytic lineage at the expense of hematopoietic cells. The cardiomyocytic yield can be further enhanced by overexpression of Wnt11 leading to approximately 95-fold enrichment in contracting cells. Our results suggest that the initial noncanonical wnt signaling is necessary for subsequent activation of canonical signaling and that the latter operates under a regulatory loop which responds to suppression with hyperactivation of compensatory mechanisms. This model provides new insights on wnt signaling during ES cell differentiation and points to a method to induce cardiomyocytic differentiation without precise timing of wnt signaling manipulation. Taking into account the heterogeneity of pluripotent cells, these findings might present an advantage to enhance the cardiogenic potential of stem cells.

Introduction

EMBRYONIC STEM (ES) CELLS differentiate to a variety of cell types providing a practical system to obtain cells for functional studies and regenerative therapies [1,2]. The ES cells model is particularly pertinent for generating cells of the cardiovascular and hematopoietic systems, because these cells emerge at the initial stages of embryonic development and, accordingly, appear early during the ES cell differentiation process [3–8]. However, the heterogeneity of the normal differentiation capacity of pluripotent ES cells necessitates timely manipulation of individual signaling pathways in order to enrich the yield of specific cell populations with desired characteristics [9].

Wnt (wingless-type MMTV integration site) signaling in mammals is mediated by the interaction of 19 distinct ligands with 10 Frizzled (Fzd) 7-transmembrane receptors [10]. Receptor activation regulates a battery of cellular processes including transcriptional activation of select target genes, proliferation, differentiation, migration, and polarity

[11]. Wnt signaling has been traditionally divided into 3 branches, the canonical wnt branch, whose major downstream mediator is β -catenin, and 2 noncanonical branches, the first modulating the activity of Rho and JNK kinases, the second leading to increased intracellular levels of Ca^{2+} and subsequent activation of calcineurin and nuclear factor of activated T-cells (NF-AT) transcription factors [10].

An important component of wnt signaling is the existence of coreceptors such as members of the low-density lipoprotein-related proteins (LRP) that are required for canonical signaling, and secreted antagonists that interfere with the binding of wnt ligands to their receptors. Wnt antagonists are mainly of 2 types, those that allow binding of ligands to Frz receptors, but hinder the interaction between Frz and LRP partners such as members of the Dickkopf (Dkk) family, and, secreted frizzled-related proteins (sFRPs) that sequester wnt ligands in the extracellular space preventing binding to receptors [12]. Consequently, Dkk proteins block canonical signaling, which depends on LRPs coreceptors, and channel receptor activation to

¹Division of Cardiovascular Medicine, Department of Medicine and ²Department of Cell and Developmental Biology, Vanderbilt University, Nashville, Tennessee.

noncanonical wnt pathways. In contrast, sFRPs are expected to block all signaling branches.

Wnt proteins have pleiotropic effects on cardiac morphogenesis and cardiomyocyte differentiation [13–15]. During development, canonical wnt signaling activation before mesoderm formation enhances cardiomyocytic differentiation. In contrast, canonical wnt signaling hinders cardiomyogenesis after gastrulation, thus suggesting a biphasic role in cardiac differentiation. Accordingly, Dkk1 has the opposite effects, that is, promoting cardiogenesis at late stages, but blocking it early on [16]. Manipulation of wnt signaling during ES cell differentiation can either hinder or enhance cardiac differentiation, often leading to apparently conflicting effects, thus suggesting that responses may vary depending on the stem cell line used, culture conditions, as well as the timing and duration of wnt signaling stimulation or inhibition [14,16–23].

Our previous genomic analyses identified clusters of coordinately expressed genes during ES cell differentiation that include, in order of timing of expression, (1) genes which take part in the formation of the 3 embryonic germ layers during gastrulation, that is, *Cerberus like 1 homolog*, *T-brachyury*, and *Wnt3*; (2) molecular regulators of mesoderm development, including *Bmp2*, *Bmp5*, *Vegfr2*, and *Foxf1*; (3) genes linked to hematopoiesis such as *Gata1* and *hemoglobins*; (4) transcripts involved in cardiac development, for example, *Mef2c*, *Myh4*, and *cardiac Troponin T2*; and (5) genes associated with neuronal development, such as *Neurog1*, *Neurog2*, and *EphA5* [24]. To place changes in wnt signaling within the framework of this orchestrated differentiation process, we generated 3 independent ES cell lines, each carrying the *luciferase* gene under a promoter that responds to activation of a separate pathway branch activation, namely canonical/ β -catenin, Ca^{2+} /NF-AT, and JNK/AP-1.

Our results show that noncanonical wnt signaling is activated at the onset of ES cell differentiation before the formation of cells representing the 3 embryonic germ layers. Noncanonical activity is followed by a strong transient induction of canonical wnt signaling that coincides with expression of gastrulation markers and extends to the early differentiation stages of mesodermal progenitor cells. We found that blockade of the initial noncanonical JNK/AP-1 activity hinders differentiation of endothelial cells and cardiomyocytes and favors hematopoiesis. In contrast, antagonism of canonical wnt signaling by Dkk1 has the opposite effect enhancing expression of cardiomyocytic and endothelial cell markers at the expense of hematopoiesis specific genes. Although Dkk1 initially blocks canonical wnt activity, it eventually leads to a delayed and prolonged counter activation of canonical wnt signaling that shifts the time window marked by the appearance of mesodermal progenitor cells. Thus, in certain pluripotent ES cell lines, it is possible to stimulate cardiovascular cells through constant antagonism of canonical wnt signaling by Dkk1.

Materials and Methods

ES cell culture and differentiation

CGR8 ES cells were grown in GMEM medium (Sigma-Aldrich; www.sigmaaldrich.com) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μ M β -mercaptoethanol,

and 100 U/mL LIF (ESGRO-Chemicon; www.millipore.com). Cells were maintained at 20%–70% confluence on 10 cm tissue culture plates to preserve the undifferentiated phenotype. ES cell differentiation was performed by using the hanging-drop method [25]. Briefly, ES cells were dissociated with 0.05% trypsin (Mediatech, Manassas, VA; http://cellgro.com) for 3 min and diluted in embryoid body (EB) media [20% FBS, 2 mM-glutamine, 0.1 mM MEM essential amino acids, and 100 μ M β -mercaptoethanol in IMDM (Gibco; www.invitrogen.com)] at a concentration of 2.5×10^4 cells/mL. Twenty microliters drops of 500 ES cells each were spotted on the inside lid surface of a 10 cm bacterial Petri dish. At day 2, EBs were transferred to suspension cultures until day 4, and then 30 EBs per well were allowed to attach onto 0.2% gelatin-coated 12-well tissue culture plates. Recombinant mouse DKK1 (R&D Systems; www.rndsystems.com) was reconstituted with $1 \times$ phosphate-buffered saline (PBS) and used at a concentration of 500 ng/mL. JNK inhibitor II SP60125 (Calbiochem; www.emdchemicals.com) was used at 10 μ M.

RNA preparation and quantitative real-time-polymerase chain reaction analysis

Total RNA was extracted from ES cells by using the RNeasy kit (Qiagen; www.qiagen.com) and reverse-transcribed into cDNA as described [26]. For quantitative PCR, 20 ng of cDNA was amplified with the iQ SYBR Green Supermix kit on an icycler (BioRad; www.bio-rad.com) by using β -actin and/or GAPDH as controls. Relative gene expression levels were quantified by using the formula $2^{(-\Delta\Delta C_t)}$ as described [27]. Primer sequences are included in Supplementary Table S1 (available online at www.liebertonline.com/scd).

Fluorescence-activated cell sorting

EBs created using the T-Brachyury-GFP and Nkx2.5-GFP cell lines [5,6] were washed in $1 \times$ PBS and dissociated with 0.25% Trypsin for 5 min at 37°C and dispersed to single cells by gentle pipetting. Cells were centrifuged for 3.5 min at 3,500 rpm, resuspended in 500 μ L $1 \times$ PBS containing 1:1,000 dilution of propidium iodide for viability staining, and analyzed by flow cytometry. For fluorescence-activated cell sorting (FACS) analysis of day 14 EBs, we used 0.2 mg/mL Collagenase type 2 (Worthington; www.worthington-biochem.com) for 10–15 min at 37°C and stained for 30–60 min at room temperature with the α -actinin mouse monoclonal antibody (Sigma; 1:2,000 dilution). Cells were washed twice with FACS buffer (5% FBS in $1 \times$ PBS containing 0.5% saponin) and resuspended in FACS buffer containing goat anti-mouse IgG Alexa Fluor 488 secondary antibody (diluted 1:200; Invitrogen; www.invitrogen.com). Cells were incubated in the dark at room temperature for 30 min and washed thrice with FACS buffer before being analyzed.

Luciferase assays

ES cells undergoing differentiation in hanging drops were washed with $1 \times$ PBS, spun and dissolved in 100 μ L $1 \times$ reporter lysis buffer (Promega; www.promega.com). Samples were pipetted to homogenize lysates and incubated for 10 min at room temperature. Thirty microliters of each sample was added to a Luciferase assay plate in duplicate and processed as recommended by the manufacturer. Plates

were read by using a Modulus Microplate luminometer (Turner Biosystems; www.promega.com). Values were standardized to protein concentrations determined with the BCA assay (Pierce; www.piercenet.com).

Immunofluorescence

CGR8 cells were cultured and differentiated as just described. On day 4, 5 EBs/well were transferred to chambered slides. On day 6 for β -catenin, and day 14 for α -actinin, cells were washed with 1 $\times$ PBS and fixed with 4% paraformaldehyde for 15 min at room temperature, washed thrice with 1 $\times$ PBS for 5 min each, permeabilized with 0.2% Triton X-100 in 1 $\times$ PBS for 30 min at room temperature, and washed again thrice with 1 $\times$ PBS. Samples were blocked with 1 mg/mL bovine serum albumin (BSA) in 1 $\times$ PBS for 30 min at room temperature followed by primary antibody incubation overnight at 4°C. Next day, cells were washed thrice with 1 $\times$ PBS, incubated with secondary antibodies for 1 h at room temperature, and washed with 1 $\times$ PBS. Slides were then sealed with mounting medium (Vector Laboratories; www.vectorlabs.com), and images were captured with a LSM 510 inverted confocal microscope (Zeiss; www.zeiss.com). Primary antibodies used for immunofluorescence (IF) were as follows: monoclonal Mouse anti- α -actinin (Sigma; 1:1,600 dilution) and monoclonal Mouse anti- β -catenin (Sigma; 1:800 dilution). Secondary antibodies were: Goat anti-Mouse Cy3-conjugated at 1:400 dilution (Jackson ImmunoResearch Laboratories; www.jacksonimmuno.com) and Donkey anti-Mouse Alexa488 at 1:400 dilution (Invitrogen). To visualize and quantify cells, we stained with the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) (1:5,000 dilution; Invitrogen) to mark cellular nuclei.

Generation of stable ES cell lines

We used the Lipofectamine 2000 reagent (Invitrogen) to transfect CGR8 cells and generate stable clones with (1) the *Myh6*-Wnt11 plasmid that was created by digesting the *Myh6* (*a*-MHC) gene promoter-polyA hGH cloning vector (kindly provided by Dr. J. Robbins [28]) with *Sall* and *HindIII* restriction enzymes. The Wnt11 full-length open reading frame fragment was obtained by PCR using CGR8 differentiation day 12 cDNA as template and primers with attached *Sall* and *HindIII* sites as follows: 5'-GTGTCGACGGATGGCCTCC TCCGAG-3' and 5'-TGAAGCTTTATCTAGATCCG GTGGA TCCTACC-3' (restriction sites are underlined). Successful cloning was confirmed by restriction enzyme digests and sequencing; (2) The *Myh6*-DsRed2-Nuc, which was obtained by PCR using plasmid pDsRed2-Nuc (Clontech) as template and primers with attached *Sall* and *HindIII* sites: 5'-GTGTCGAC GGATGGCCTCCCTCCGAG-3' and 5'-TGAAGCTTTATC TAGA TCCGGTGGATCCTACC-3'; (3) the SuperTOPFlash vector (Addgene; www.addgene.org) that has the firefly *luciferase* gene reporter under the control of 7 tandem T-Cell factor/lymphoid enhancer factor (TCF/LEF) binding sites; (4) the JNK/AP-1 reporter (SuperArray Bioscience Corp.; www.sabiosciences.com) that carries the *luciferase* gene under the control of tandem repeats of the Tissue Plasminogen A-response element; and (5) the NF-AT reporter (SuperArray Bioscience Corp) that has the *luciferase* reporter gene under the control of tandem repeats of the NF-AT consensus binding site. ES cells were transfected with 8 μ g of linearized plasmid

when cultures reached 50% confluence, and positive clones were selected with 200 μ g/mL G418, or 150 μ g/mL Hygromycin for 10 days. Single colonies were picked, expanded, and genotyped to confirm vector incorporation.

Statistical analysis

Error bars represent average $\pm$ SE. Statistical analyses were performed by using Student's *t*-test.

Results

Wnt signaling pathway activity during differentiation of mouse ES cells

To monitor the activity of the various branches of the wnt signaling pathway, we generated stable ES cell clones of the germline-competent CGR8 line [29] carrying the reporter *luciferase* gene under the control of either tandem repeats of the TCF/LEF binding sites that respond to canonical/ β -catenin signaling, tandem repeats of the TPA response elements that are activated by JNK/AP-1, or tandem repeats of the NF-AT consensus binding site that is induced by mobilized NF-AT factors. Engineered CGR8 cells with 1 of the 3 indicator constructs were then dissociated and allowed to form EBs in hanging-drop suspensions until day 4. At this stage, EBs were attached to gelatin-coated plates for further differentiation. Treatment regimes were refreshed every second culture day.

The results show that undifferentiated ES cells have low, but detectable levels of canonical/ β -catenin and moderate levels of JNK/AP-1 activity (Fig. 1A). At the onset of differentiation, there is an immediate drop in canonical wnt activity that mirrors a sharp increase in noncanonical/JNK/AP-1 signaling (Fig. 1A). After these initial changes in wnt signaling, at day 3 of differentiation, there is a steep and transient peak in canonical wnt signaling accompanied by a parallel decrease in the JNK/AP-1 branch (Fig. 1A). After day 4, measurable levels of Luciferase activity in both indicator lines decline to close-to-background levels, possibly reflecting the plurality of codifferentiating cell lineages and, thus, the small percentage of cells with active wnt signaling at late differentiation time points.

NF-AT-driven Luciferase activity (not shown) followed, in general, the pattern of JNK/AP-1, but was considerably lower by 3 orders of magnitude compared with canonical wnt signaling maxima and 2 orders of magnitude compared with JNK/AP-1, thus suggesting that JNK/AP-1 activation is the predominant noncanonical branch during these stages. Although such differences might reflect the relative strengths of the artificial reporter constructs and not necessarily endogenous levels of activation, because of the low readings of NF-AT-driven Luciferase activity, we focused further analyses on the canonical/ β -catenin and noncanonical/JNK/AP-1 pathways.

To better visualize the activity of JNK/AP-1 and canonical/ β -catenin signaling in relation to the ES cell differentiation processes, we plotted the expression of their respective targets together with the expression profiles of gene markers for various lineages using the FunGenES database (<http://biit.cs.ut.ee/fungenes>) [24]. As shown in Fig. 1B, the noncanonical phase is marked by induction in the expression levels of several genes involved in the JNK/AP-1 pathway such as *Myb*, *Mapk10*

FIG. 1. Activity of wnt pathways during mouse ES cell differentiation. CCR8 cells carrying the *luciferase* gene under the control of various wnt signaling branches were allowed to differentiate in hanging drops. **(A)** Luciferase assays using protein samples from successive differentiation days reveal alternating signaling peaks of canonical/ β -catenin and non-canonical/JNK/AP-1 activity. Values are mean $\pm$ SE of 3 independent experiments. **(B)** Expression profiles of genes representing targets of JNK/AP-1 (*Mapk10*, *Myb*, and *Pten*) and β -catenin (*Axin2*) and representative genes of key ES differentiation steps such as gastrulation (*T*, *Wnt3*, *Mesp1*), mesoderm formation (*Bmp2*, *Bmp4*, and *Hand1*), endothelial differentiation (*Smad6*, *Vegfr2*), cardiac progenitors (*Isl1*), hemopoiesis (*Gata1*, *Hbb-bh1*), and cardiac cells (*Mef2c*, *Myocd*, *Myh6*, and *Myh7*) using the Search Tool of the FunGenES database. Canonical wnt signaling overlaps with the gastrulation phase and induction of mesodermal progenitor cells. *Bmp2*, bone morphogenetic protein 2; *Bmp4*, bone morphogenetic protein 4; *Gata1*, GATA binding protein 1; *Hbb-bh1*, hemoglobin Z, beta-like embryonic chain; *Isl1*, Islet 1; *Mapk10*, mitogen-activated protein kinase 10 (or *Jnk3*); *Mef2c*, myocyte enhancer factor 2c; *Mesp1*, mesoderm posterior 1; *Myb*, myeloblastosis oncogene; *Myh6*, α myosin heavy chain; *Myh7*, β myosin heavy chain; *Myocd*, myocardin; *Pten*, phosphatase and tensin homolog; *T*, T-brachyury; *Vegfr2*, vascular endothelial growth factor receptor 2 (or *Flk1*). ES, embryonic stem.

(*Jnk3*), and *Pten*, whereas the canonical phase is marked by the canonical wnt signaling target *Axin2*. The temporary peak of canonical wnt activity parallels the strong transient induction of genes involved in the gastrulation process during development (eg, *T-brachyury* and *Wnt3*), a stage that precedes the appearance of early mesodermal markers. Canonical wnt activity extends to and partially overlaps with the early stages of mesodermal differentiation (marked by *Bmp2*, *Mesp1*) when cardiovascular progenitors form and begin to differentiate at days 4 and 5. Canonical wnt activity levels temper off by the time hematopoiesis (*Gata-1*, *Hbb-gh1*) and cardiogenesis (*Mef2c*, *Myh7*) commence (Fig. 1B).

Briefly, our results show sequential, mutually exclusive waves of JNK/AP-1 and canonical/ β -catenin wnt signaling up to day 4 of ES cell differentiation with canonical/ β -catenin after JNK/AP-1 activity. High noncanonical/JNK/AP-1 activity in the first 2 days of ES cell differentiation precedes the appearance of cells representative of ectoderm, mesoderm, and endoderm, whereas canonical wnt activity overlaps with the emergence of progenitor cells for the 3 germ layers and early mesoderm differentiation.

Interference with distinct wnt signaling branches at their corresponding peak windows alters the differentiation of mesodermal lineages in opposite ways

To test how interference with the various wnt pathway branches affects ES cell differentiation, we applied the JNK inhibitor SP60125 at days 0–2 and the canonical wnt antagonist Dkk1 at days 2–4 that correspond to peak stages of JNK/AP-1 and canonical wnt signaling, respectively. SP60125 abolished JNK phosphorylation in differentiating ES cells as expected, thus indicating a blockade of the JNK/AP-1 pathway (not shown).

SP60125 application at 0–2 results in strong suppression (20-fold) of cardiogenic and endothelial differentiation assessed at day 10, whereas hematopoiesis- and endoderm-specific genes are induced by 3.5 and 2-fold, respectively (Fig. 2A). In contrast, treatment with Dkk1 during the peak of canonical wnt activity between days 2 and 4 induces cardiac (17-fold) and endothelial (2-fold) genes and suppresses hematopoietic (2-fold) and endodermal markers (5-fold, Fig. 2A). There is also a modest 1.5-fold induction in neuronal genes such as *tubulin* β 3 in Dkk1-treated EBs and no major changes in RNA samples from EBs treated with SP60125. Thus, it appears that interference with the initial JNK/AP-1 or the subsequent canonical wnt activity has opposite effects on the differentiation of mesodermal and endodermal lineages.

Taken together, our results suggest that the original JNK activity is necessary for properly carrying out subsequent differentiation steps, as inhibition with SP60125 practically abolishes myocardial and endothelial gene expression favoring instead hematopoietic and endodermal differentiation. Conversely, Dkk1 interference suppresses hematopoietic and endodermal lineages and induces endothelial and cardiomyocyte-specific gene markers.

Continuous suppression of wnt signaling by Dkk1 promotes cardiomyocyte differentiation

To test how interference with the JNK/AP-1 and canonical/ β -catenin wnt signaling affects cardiomyocyte

FIG. 2. Interference with wnt signaling branches at specific time windows affects the differentiation of multiple lineages. Differentiating CGR8 ES cells were treated with the JNK inhibitor SP60125 or the canonical wnt antagonist Dkk1 at various time windows as indicated. **(A)** qRT-PCR analysis of day 10 EB RNA samples shows that blockade of the JNK/AP-1 pathway with SP60125 during its peak at days 0–2 of differentiation results in strong suppression of cardiomyocyte (*Myh7*) and endothelial (*CD31*, *Cdh5*) differentiation in favor of hematopoiesis (*Hbb-y*) and endoderm (*Afp*)-specific genes. Suppression of canonical wnt activity during peak signaling at days 2–4 of differentiation conversely induces cardiac and endothelial genes at the expense of hematopoietic and endodermal markers. Dkk1 treatment also leads to a moderate induction of the neuronal marker *Tubb3*. qRT-PCR values are the mean $\pm$ SE of 3 separate experiments. **(B)** Timing of inhibition of the JNK/AP-1 or canonical wnt signaling pathways at different time windows has varying effects on cardiogenesis. qRT-PCR values are the mean $\pm$ SE of 3 separate experiments. **(C)** Confocal images of day 14 EBs, cultured with or without continuous Dkk1 treatment and stained with antibodies recognizing the sarcomeric protein α -actinin. Dkk1-treated cells demonstrate an increase in the number and size of cardiomyocyte colonies compared with control. Cell nuclei were labeled with DAPI. **(D)** FACS analysis of dissociated day 14 EBs stained with α -actinin antibody reveals a 60-fold increase in cardiomyocytes in samples continuously treated with Dkk1. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$ compared with controls. *Afp*, α fetoprotein; *CD31*, platelet endothelial cell adhesion molecule 1 or Pecam1; *Cdh5*, vascular endothelial cadherin; *Hbb-y*, hemoglobin Y, beta-like embryonic chain; *Tubb3*, Tubulin β 3; qRT-PCR, quantitative real-time-polymerase chain reaction; FACS, fluorescence-activated cell sorting; EB, embryoid body.

differentiation throughout the differentiation process, we applied SP60125 or Dkk1 between 0–2, 2–4, 4–10, and 0–10 days of differentiation.

The results show that although JNK blockade at its peak suppresses cardiomyocyte differentiation, application of SP60125 during the surge of canonical wnt activity at days 2–4 induces expression levels of cardiac-specific genes by 5-fold (Fig. 2B). The positive effect of JNK inhibition at days 2–4 appears to override the negative effect on days 0–2, as addition at days 0–4 and 0–10 has less of an impact on cardiogenesis (Fig. 2B).

In contrast to JNK inhibition that shows negative and positive effects on cardiomyocytic differentiation consistent with the corresponding endogenous high and low JNK/AP-1 activities, respectively, the results of Dkk1 application follow a more complex pattern. For example, addition at days 0–2 and after day 4 does not influence cardiac differentiation. Conversely, addition of Dkk1 during the peak of canonical activity between days 2 and 4 strongly induces cardiogenesis, and the same outcome is observed in the remaining 2 treatments that straddle this time point, namely Dkk1 addition between days 0–4 and 0–10 (Fig. 2B). Actually, continuous Dkk1 presence during ES cell differentiation produces the highest induction of *Myh6* on day 10 of differentiation at approximately 30-fold, as measured by quantitative real-time–polymerase chain reaction (qRT-PCR) analysis.

To correlate the induction of the cardiac-specific *Myh6* gene at the RNA level to the yield of cardiomyocytes, we stained control untreated and Dkk1-treated EBs with an antibody against the sarcomeric protein α -actinin. IF analysis shows a strong increase in the number of α -actinin-positive cells with sarcomeric structures that coincide with contracting areas, thus suggesting that they represent cardiomyocytes (Fig. 2C). To quantify the increase of α -actinin-positive cardiomyocytes further, control and Dkk1-treated EBs were dissociated, stained with the α -actinin antibodies, and analyzed by FACS. The results demonstrate that the percentage of α -actinin-positive cells increases from approximately 0.2% in control cultures to 12.5% with Dkk1 treatment, an increase of approximately 60-fold, in line with the molecular data that show approximately 35-fold induction in *Myh6* expression and 25-fold in *Myh7* (Fig. 2A, B, D).

Continuous exposure of differentiating ES cells to Dkk1 expands the original time window of noncanonical activity, but triggers delayed canonical wnt activation

Since canonical wnt signaling is required for both mesoderm specification and expansion of cardiac progenitor cells, the enrichment of cardiomyocytes in differentiating ES cells cultured under Dkk1 was apparently contradictory to the expected outcome. To further investigate the molecular basis of this phenomenon, we tested wnt pathway activities by using the CGR8 Luciferase indicator ES cell lines in the continuous presence of Dkk1 during the differentiation process. The results show that addition of Dkk1 extends the window of JNK/AP-1 for approximately 2 days, from days 0–2 to 0–4 of differentiation, but noncanonical signaling decreases thereafter (Fig. 3A). Moreover, the inclusion of Dkk1 initially reduces canonical wnt activity, but canonical wnt activity springs back at later stages even in the presence of Dkk1. Briefly, continu-

ous Dkk1 application does not eliminate the pattern of JNK/AP-1 followed by canonical/ β -catenin sequence seen in control differentiating ES cells. Instead, it creates a second window of JNK/AP-1 activity which is followed by a delayed peak of canonical/ β -catenin signaling.

To test whether canonical wnt activity changes in the indicator lines extend to typical gene targets of the 2 wnt branches, we isolated RNA samples from the corresponding differentiation stages. qRT-PCR analysis showed that transcripts of known canonical wnt gene targets such as *Axin2* and *Lef1* are downregulated at day 3 in Dkk1-treated cultures, but are induced at day 5 of differentiation compared to untreated cells (Fig. 3B). Correspondingly, the expression of JNK/AP-1 pathway associated genes such as *Myb* and *Pten* show higher expression levels at day 3 Dkk1-treated cells than controls. The delayed activation of canonical wnt signaling also leads to increased levels of cytoplasmic and membrane-associated β -catenin as seen in IF staining of day 6 EBs (Fig. 3C). Thus, it appears that persistent challenge of canonical wnt signaling with Dkk1 leads to activation of regulatory loops that overcome the original suppression. The effect of this reaction counteracts Dkk1 antagonism, thus leading to net activation of canonical wnt signaling at later differentiation stages.

Exposure of differentiating ES cells to Dkk1 delays and expands mesoderm differentiation

As described in Fig. 1, canonical wnt activation during ES cell differentiation coincides with induction of transcripts that have been implicated in the gastrulation phase of development which is represented in day 3 EBs. To investigate how time displacement of canonical wnt signaling affects this stage, we isolated and tested RNA samples for genes, the expression of which follows the peak of canonical wnt signaling. As seen in Fig. 4A, expression of *T-brachyury*, *Cerberus homolog-like 1*, *Wnt3*, *Wnt5a*, and *Wnt 8a* is suppressed in the presence of Dkk1 at day 3. However, in all cases, expression strongly recovers after a delay of 1–2 days.

To test the effect of Dkk1 treatment on T-brachyury⁺ cells, we used the *T-brachyury-GFP* ES cell line [6]. Control, untreated T-brachyury GFP cells follow a similar, but slower differentiation program than CGR8 cells, with T-brachyury⁺ cells appearing at day 5 of differentiation instead of day 3 in CGR8 cells. Nevertheless, continuous exposure of *T-brachyury-GFP* ES cells to Dkk1 also relocated the peak of T-brachyury⁺ cells by 2 days, from day 5 to 7 (Fig. 4B). Thus, canonical wnt antagonism with Dkk1 temporarily suspends, but does not abolish the emergence of T-brachyury⁺ cells in 2 independently derived ES cell lines with distinct paces of differentiation and peaks of mesodermal gene induction.

Continuous exposure of differentiating ES cells to Dkk1 induces cardiogenic progenitor cells

Previous studies have shown 2 waves of mesodermal progenitor cells, an early wave that predominantly gives rise to hematopoietic and endothelial cells, and a second wave that yields cardiomyocytes, as well as endothelial and smooth muscle cells [30,31]. Moreover, it was also shown that cardiogenic progenitor cell expansion depends on canonical wnt signaling [18]. The fact that Dkk1 antagonism leads to delayed induction of canonical wnt signaling during cardiogenic progenitor cell specification and growth raised

FIG. 3. Continuous exposure of differentiating ES cells to Dkk1 expands the time window of noncanonical activity, but triggers delayed canonical wnt activation. CGR8 ES cells were cultured with or without continuous exposure to Dkk1 and analyzed for wnt pathway activity. (A) Luciferase assays using protein samples from successive differentiation days reveal that addition of Dkk1 extends the duration of JNK/AP-1 signaling generating a second peak of activity around day 3 (left graph). Dkk1 initially suppresses canonical wnt activity, but eventually results in delayed activation of canonical wnt signaling (right graph). Values are mean $\pm$ SE of 3 independent experiments. (B) qRT-PCR analysis reveals a downregulation of canonical wnt gene targets and an induction in JNK/AP-1 pathway genes at day 3 of differentiation in Dkk1-treated EBs as compared with controls. In contrast, day 5 Dkk1-treated EBs show increased expression of canonical wnt gene targets and a decrease in JNK/AP-1 pathway-related genes. Values are mean $\pm$ SE of 3 separate experiments. (C) IF analysis of day 6 EBs stained with an antibody recognizing β -catenin reveals increased levels of cytoplasmic and membrane-associated β -catenin in samples treated with Dkk1 at days 0–6 of differentiation as compared with untreated controls. * P < 0.05; ** P < 0.005; *** P < 0.0005 compared with controls. IF, immunofluorescence.

the possibility that the observed cardiogenic stimulation is due to the expansion of cardiac progenitor cells.

In agreement with this idea, we found that Dkk1 treatment causes a delayed and prolonged induction in the expression levels of cardiac progenitor cell (CPC) markers such as *Nkx2.5*, *Isl1*, *Myocardin*, and *Mesp1* (Fig. 5A). Further, we found a 5–6-fold enrichment of *Nkx2.5*⁺ cells at day 6 of differentiation using the *Nkx2.5*-EGFP ES cell line [5] (Fig. 5B). A subset of early cardiac markers such as *Isl1* and *Mesp1* show a transient induction that follows the pattern of canonical wnt signaling activity, whereas *Myocardin* and *Nkx2.5* show long-term persistent high expression throughout the tested time points (Fig. 5A). Although the significance of this split is unknown, it might reflect the persistence of select early cardiac-specific genes in cardiomyocytes. In addition, although the *Mesp1* and *Isl1* levels are lower in Dkk1-treated cells, their expression windows are prolonged compared with control, untreated differentiating cells. Taken together, our results suggest that

the delayed canonical wnt activation, because of the presence of Dkk1, increases differentiation of CPCs.

Successive noncanonical/canonical wnt pathway activation is necessary for subsequent canonical wnt signaling and cardiac differentiation

As just described, JNK/AP-1 activity comes before canonical wnt signaling (Fig. 2A). Dkk1 application creates a second peak of JNK/AP-1 activity that subsides when the delayed canonical wnt activation takes place (Fig. 3A). To test whether this second peak of JNK/AP-1 pathway activation that precedes canonical activity in Dkk1-treated EBs is also important for the delayed canonical wnt activation and cardiomyocyte differentiation, we treated Dkk1-exposed EBs with SP60125 at days 2–4. As shown in Fig. 6, SP60125 treatment during the time window of the second, Dkk1-induced JNK/AP-1 peak attenuates the induction of mesodermal markers (*T-brachyury*,

FIG. 4. Continuous exposure of differentiating ES cells to Dkk1 delays and expands mesoderm differentiation. Differentiating CGR8 and *T-brachyury*-GFP ES cells were continuously treated with Dkk1 and analyzed by qRT-PCR and FACS analysis, respectively. **(A)** qRT-PCR analysis shows that the expression of genes which are associated with gastrulation and mesoderm formation follow the canonical wnt signaling pattern, that is, their expression is delayed, but not aborted in the presence of Dkk1. qRT-PCR values are the mean $\pm$ SE of 3–4 separate experiments. **(B)** FACS analysis of dissociated EBs formed using the *T-brachyury*-GFP cells at days 5 and 7 reveals a delayed emergence of *T-brachyury*⁺ cells in EBs treated with Dkk1 (left). The right graph is a quantification of *T-brachyury*-GFP cells at successive differentiation stages between days 3 and 10 with and without Dkk1 treatment. Values are mean $\pm$ SE of 3 separate experiments. Abbreviations: *Cer1*, Cerberus-like 1.

Wnt3, and *Wnt8a*), markers for cardiogenic differentiation (*Mesp1*, *Myocardin*, and *Nkx2.5*), and the subsequent appearance of cardiomyocyte-specific genes *Myh6* and *Myh7*. Conversely, application of SP60125 at days 0–2 has minor effects on the cardiogenic differentiation of Dkk1-treated cells. These results suggest that the JNK/AP-1 activity immediately before canonical wnt signaling plays a role in the subsequent mesodermal and cardiogenic differentiation.

Dkk1 induces *Wnt11* that further promotes cardiogenic differentiation

The results just described show that persistent antagonism by Dkk1 leads to a number of permanent gene expression

changes such as sustained *Nkx2.5* and *Myocardin* expression beyond the typical time windows in control ES cells. However, the fact that *Nkx2.5*⁺ CPCs are expanded by 5-fold, but cardiomyocytes by nearly 60-fold, suggested that additional changes, taking place concurrently or subsequent to CPC expansion, further enhance cardiomyocytic differentiation. Among the wnt factors that are induced by Dkk1 is *Wnt11*, which is known to stimulate the growth and differentiation of cardiomyocytes during development and ES cell differentiation [15] (Fig. 7A).

To test the effects of higher *Wnt11* expression in conjunction with Dkk1 application on cardiomyocytic differentiation, we cloned the *Wnt11* cDNA under the cardiac-specific *Myh6* promoter [28] and generated stable CGR8 ES

FIG. 5. Continuous exposure of differentiating ES cells to Dkk1 induces cardiogenic progenitor cells. Differentiating CGR8 ES cells were treated with Dkk1 and analyzed by qRT-PCR and FACS analysis. **(A)** qRT-PCR analysis of RNA samples from Dkk1-treated EBs reveals a transient, delayed induction of certain early cardiac markers (*Mesp1*, *Isl1*), and a long-term induction of others (*Myocd*, *Nkx2.5*), thus indicating that delayed canonical wnt activation favors the differentiation of cardiac progenitor cells. Values are mean $\pm$ SE of 3–4 separate experiments. The *Mesp1* expression data are shown in logarithmic scale. **(B)** FACS analysis of the *Nkx2.5*-EGFP ES cell line at day 6 of differentiation shows a 5–6-fold increase in cardiac progenitor cells in EBs continuously treated with Dkk1 as compared with controls.

cell lines. *Myh6*-*Wnt11* ES cells were also engineered with the nuclear DsRed fluorescent protein gene (*DsRed-Nuc*) under the same *Myh6* promoter. The results show that *Wnt11* increases *Myh6* gene expression by 23-fold compared with 33-fold by Dkk1 treatment alone. Combinatorial treatment of *Myh6*-*Wnt11* ES cells with Dkk1 throughout differentiation augments *Myh6* expression levels by 95-fold (Fig. 7B). The increase in *Myh6* gene expression corresponds to higher yields of red-fluorescence-labeled cardiac cells (Fig. 7C). These results suggest that combinatorial hyperactivation of canonical wnt signaling and enhancement of cardiogenic mesoderm can be further stimulated by *Wnt11* to enrich cultures of contracting cardiomyocytes during ES cell differentiation.

Discussion

Pluripotent ES cells offer a practically unlimited source of cardiomyocytes to overcome some of the experimental obstacles associated with adult cells [4–7,25]. The possibility to generate ES-like induced pluripotent stem (iPS) cells from adult somatic tissues opens further opportunities to isolate, analyze, and genetically modify cardiomyocytes from human patients [32–34]. However, these approaches necessitate the ability to obtain sufficient numbers of cardiac cells for both experimental analyses and clinical applications [9,35,36]. Methods to achieve this goal have been developed

and depend on timely manipulation of signaling pathways, favorable extracellular matrix conditions, hypoxia, or manipulation of EB size [37–39].

However, it becomes increasingly evident that individual ES cell lines have variable differentiation capacities in culture or show a wide range of differentiation timetables [34,40]. Therefore, lineage-specific differentiation regimes that depend on precise manipulation of particular steps would need to be customized for individual cell lines based on landmark differentiation points. A case in point is the contribution of the wnt signaling pathway in cardiomyocytic differentiation. Inactivation of various wnt morphogens or pathway modulators shows that they regulate multiple stages of cardiogenesis [41–44]. Studies in zebrafish suggested a biphasic requirement that was also seen in differentiating ES cells [16]. However, it appears that the role of various wnt signaling branches is more complex and dynamic. For example, it was shown that canonical Wnt signaling, mediated by *Wnt3a*, is required for formation of endodermal and mesodermal cells [45,46]. After this step, canonical Wnt/ β -catenin needs to be tempered by specific wnt ligand antagonists such as sFRP-1 and sFRP-5 in order for cardiac specification to proceed [47]. Noncanonical signaling initiated by *Wnt5a* and *Wnt11* promotes this stage. Once CPCs become specified, then canonical wnt/ β -catenin signaling driven by *Wnt2b* is again required to stimulate proliferation and expansion of CPCs [18,19]. Finally,

FIG. 6. Successive noncanonical/canonical wnt pathway activation is necessary for subsequent canonical wnt signaling and cardiac differentiation. RNA analysis of differentiating CGR8 ES cells treated with Dkk1 and SP60125. Addition of SP60125 on days 2–4 just before the delayed canonical wnt activity in Dkk1-treated cells reduces cardiac marker gene expression at day 10 of differentiation. qRT-PCR values are the mean $\pm$ SE of 3 separate experiments. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; # $P = 0.06$ compared with controls.

a blockade of canonical and concomitant activation of non-canonical wnt signaling mainly by Wnt11 pushes differentiation of CPCs to cardiomyocytes [15,16]. During embryonic development and ES cell differentiation, these events take place within a narrow time window, thus making it difficult to time with precision proper manipulation of wnt signaling at corresponding peaks and valleys of activity.

Our data show that changes in wnt signaling take place immediately, at the onset of ES cell differentiation, starting with a downregulation of canonical signaling and a surge in JNK/AP-1 activity which lasts around 48 h in CGR8 cells. After this stage, there is a decrease in JNK/AP-1 activity followed by a sharp increase in canonical activity that is also maintained for approximately 48 h. The wnt signaling waves appear to mirror each other with canonical wnt activity maxima corresponding to minima of noncanonical activity and vice versa. The preceding noncanonical activity is necessary for the subsequent induction of canonical activity, as blocking JNK/AP-1 attenuates the ensuing canonical signaling.

The sequential arrangement of noncanonical/canonical signaling is required for proper differentiation of ES cells. Although interference with either branch at its maximum activity time window does not abolish ES cell differentiation, it results in opposite effects on lineage development. Specifically, JNK/AP-1 blockade reduces cardiogenic and endothelial differentiation with a concurrent increase in hematopoiesis and endoderm differentiation. In contrast, interference with canonical signaling with Dkk1 suppresses hematopoietic and endodermal lineages and induces endothelial and cardiomyocyte-specific gene markers. The last outcome, although consistent with our results, is in contrast to results obtained in another ES cell line showing that continuous application of Dkk1 blocks cardiogenesis [45]. It is likely that individual ES cell lines respond differently to various manipulations of specific signaling cascades. Consistent with this possibility, besides reacting in distinct ways to Dkk1 treatment, the 2 ES cell lines also respond differently to the BMP antagonist Noggin [45,48,49].

At the molecular level, JNK/AP-1 blockade moderates, but does not abolish canonical wnt signaling during mesoderm differentiation. Conversely, Dkk1 abolishes canonical wnt activity during the original mesoderm differentiation stages, but leads to increased canonical signaling during subsequent steps. These changes in canonical wnt activity lead to corresponding variations in *T-brachyury* expression as well as other markers that are involved in the generation of cells representative of the 3 embryonic germ layers. It has been postulated that 2 waves of *T-brachyury* expressing cells arise during ES cell differentiation, the first giving rise to blood and endothelial cells and the second to cardiomyocytes, smooth muscle, and endothelial cells. Therefore, it is likely that the delayed activation of canonical wnt signaling favors the expansion of the second wave of cardiogenic *T-brachyury* cells at the expense of the first.

Our results also show that both branches of wnt signaling are wired to counteract inhibition, as both JNK/AP-1 and canonical/ β -catenin inhibition lead to subsequent activation of compensatory mechanisms that restore JNK/AP-1 and canonical/ β -catenin signaling. Therefore, depending on their timing and duration, inhibitory signals might lead to apparently conflicting data regarding the effects of wnt signaling on ES cell differentiation to cardiovascular lineages. In this light, it appears that a critical determining factor is the time window of canonical wnt activity that coincides with *T-brachyury* induction and other gastrulation stage genes. Since different ES cell lines display slower or faster paces of differentiation with variable *T-brachyury* expression, it is likely that similar timings of treatment regimes could lead to

FIG. 7. Dkk1 induces Wnt11 that further expands cardiogenic differentiation. CGR8 cells, transfected with *Myh6-DsRed-Nuc* and with or without *Myh6-Wnt11*, were treated with Dkk1 and analyzed for expression of cardiac genes and by fluorescence microscopy. **(A)** qRT-PCR shows that *Wnt11* is induced at day 10 of ES cell differentiation in EBs treated with Dkk1 between days 0 and 10. Values are mean $\pm$ SE of 3 separate experiments. **(B)** Heightened Wnt11 expression in combination with addition of Dkk1 yields a significant increase in *Myh6* gene expression in day 10 EBs. Values are mean $\pm$ SE of 3 separate experiments. Fold induction rates are indicated. **(C)** Fluorescence microscopy of day 10 EBs shows that Wnt11 overexpression and Dkk1 addition augment the yield of cardiomyocytes marked by nuclear DsRed protein (top 4 panels). α -Actinin IF staining corroborates the increased numbers of cardiomyocytes in Wnt11 expressing EBs treated with Dkk1 at day 14 of differentiation. * $P < 0.05$; ** $P < 0.005$ compared with controls.

opposite outcomes in individual cell lines. As a result, it is possible that both inhibition and activation of canonical wnt signaling have eventually the same outcome.

These results might be relevant for the adult situation as well where both inhibition and stimulation of canonical wnt signaling have been shown to have beneficial effects [36,50–55]. It is possible that blocking wnt signaling in adult cardiac cells also triggers regulatory loops that lead to the opposite of predicted effects [56].

In conclusion, our results suggest an interconnection between canonical and noncanonical pathway branches, the

existence of strong regulatory loops that counteract interference leading to unexpected outcomes in signaling directions, and that continuous antagonism of canonical wnt signaling creates a counter reaction increasing the number of cardiogenic progenitor cells and the expression of wnt ligands such as Wnt11, which favor cardiogenesis. Combinatorial Dkk1/Wnt11 application further enhances cardiogenic output. These findings could enhance future exploitation of ES cells and iPS cells to study the molecular and cellular bases of cardiovascular diseases and to develop strategies for myocardial regeneration.

Acknowledgments

The authors would like to thank the Vanderbilt FACS Core for expert assistance in cell analysis. This work was supported by NIH grants HL083958 and HL100398 to A.K.H., and Institutional Support from Vanderbilt Medical Center.

Author Disclosure Statement

No competing financial interests exist

References

- Doetschman TC, H Eistetter, M Katz, W Schmidt and R Kemler. (1985). The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol* 87:27–45.
- Keller G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev* 19:1129–1155.
- Sachinidis A, BK Fleischmann, E Kolossov, M Wartenberg, H Sauer and J Hescheler. (2003). Cardiac specific differentiation of mouse embryonic stem cells. *Cardiovasc Res* 58:278–291.
- Moretti A, L Caron, A Nakano, JT Lam, A Bernshausen, Y Chen, Y Qyang, L Bu, M Sasaki, et al. (2006). Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* 127:1151–1165.
- Wu SM, Y Fujiwara, SM Cibulsky, DE Clapham, CL Lien, TM Schultheiss and SH Orkin. (2006). Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* 127:1137–1150.
- Kattman SJ, TL Huber and GM Keller. (2006). Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell* 11:723–732.
- Yang L, MH Soonpaa, ED Adler, TK Roepke, SJ Kattman, M Kennedy, E Henckaerts, K Bonham, GW Abbott, et al. (2008). Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 453:524–528.
- Dambrot C, R Passier, D Atsma and CL Mummery. (2011). Cardiomyocyte differentiation of pluripotent stem cells and their use as cardiac disease models. *Biochem J* 434:25–35.
- Chien KR, IJ Domian and KK Parker. (2008). Cardiogenesis and the complex biology of regenerative cardiovascular medicine. *Science* 322:1494–1497.
- Rao TP and M Kuhl. (2010). An updated overview on Wnt signaling pathways: a prelude for more. *Circ Res* 106:1798–1806.
- Logan CY and R Nusse. (2004). The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 20:781–810.
- MacDonald BT, K Tamai and X He. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 17:9–26.
- Vincent SD and ME Buckingham. (2010). How to make a heart: the origin and regulation of cardiac progenitor cells. *Curr Top Dev Biol* 90:1–41.
- Gessert S and M Kuhl. (2010). The multiple phases and faces of wnt signaling during cardiac differentiation and development. *Circ Res* 107:186–199.
- Pandur P, M Lasche, LM Eisenberg and M Kuhl. (2002). Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* 418:636–641.
- Ueno S, G Weidinger, T Osugi, AD Kohn, JL Golob, L Pabon, H Reinecke, RT Moon and CE Murry. (2007). Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc Natl Acad Sci U S A* 104:9685–9690.
- Nakamura T, M Sano, Z Songyang and MD Schneider. (2003). A Wnt- and beta - catenin-dependent pathway for mammalian cardiac myogenesis. *Proc Natl Acad Sci U S A* 100:5834–5839.
- Qyang Y, S Martin-Puig, M Chiravuri, S Chen, H Xu, L Bu, X Jiang, L Lin, A Granger, et al. (2007). The renewal and differentiation of Isl1+ cardiovascular progenitors are controlled by a Wnt/beta-catenin pathway. *Cell Stem Cell* 1:165–179.
- Wang H, JB Gilner, VL Bautch, DZ Wang, BJ Wainwright, SL Kirby and C Patterson. (2007). Wnt2 coordinates the commitment of mesoderm to hematopoietic, endothelial, and cardiac lineages in embryoid bodies. *J Biol Chem* 282:782–791.
- Wang H, J Hao and CC Hong. (2011). Cardiac induction of embryonic stem cells by a small molecule inhibitor of Wnt/beta-catenin signaling. *ACS Chem Biol* 6:192–197.
- Paige SL, T Osugi, OK Afanasiev, L Pabon, H Reinecke and CE Murry. (2010). Endogenous Wnt/beta-catenin signaling is required for cardiac differentiation in human embryonic stem cells. *PLoS One* 5:e11134.
- Weisel KC, HG Kopp, MA Moore, L Studer and T Barberi. (2010). Wnt1 overexpression leads to enforced cardiomyogenesis and inhibition of hematopoiesis in murine embryonic stem cells. *Stem Cells Dev* 19:745–751.
- David R, C Brenner, J Stieber, F Schwarz, S Brunner, M Vollmer, E Mentele, J Muller-Hocker, S Kitajima, et al. (2008). MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. *Nat Cell Biol* 10:338–345.
- Schulz H, R Kolde, P Adler, I Aksoy, K Anastassiadis, M Bader, N Billon, H Boeuf, PY Bourillot, et al. (2009). The FunGenES database: a genomics resource for mouse embryonic stem cell differentiation. *PLoS One* 4:e6804.
- Hescheler J, BK Fleischmann, S Lentini, VA Maltsev, J Rohwedel, AM Wobus and K Addicks. (1997). Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. *Cardiovasc Res* 36:149–162.
- Beck H, M Semisch, C Culmsee, N Plesnila and AK Hatzopoulos. (2008). Egr-1 regulates expression of the glial scar component phosphacan in astrocytes after experimental stroke. *Am J Pathol* 173:77–92.
- Livak KJ and TD Schmittgen. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402–408.
- Subramaniam A, WK Jones, J Gulick, S Wert, J Neumann and J Robbins. (1991). Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. *J Biol Chem* 266:24613–24620.
- Mountford P, B Zevnik, A Duwel, J Nichols, M Li, C Dani, M Robertson, I Chambers and A Smith. (1994). Dicotronic targeting constructs: reporters and modifiers of mammalian gene expression. *Proc Natl Acad Sci U S A* 91:4303–4307.
- Misfeldt AM, SC Boyle, KL Tompkins, VL Bautch, PA Labosky and HS Baldwin. (2009). Endocardial cells are a distinct endothelial lineage derived from Flk1+ multipotent cardiovascular progenitors. *Dev Biol* 333:78–89.

31. Kouskoff V, G Lacaud, S Schwantz, HJ Fehling and G Keller. (2005). Sequential development of hematopoietic and cardiac mesoderm during embryonic stem cell differentiation. *Proc Natl Acad Sci U S A* 102:13170–13175.
32. Okita K, T Ichisaka and S Yamanaka. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature* 448:313–317.
33. Wernig M, A Meissner, R Foreman, T Brambrink, M Ku, K Hochedlinger, BE Bernstein and R Jaenisch. (2007). *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448:318–324.
34. Zhang J, GF Wilson, AG Soerens, CH Koonce, J Yu, SP Palecek, JA Thomson and TJ Kamp. (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ Res* 104:e30–e41.
35. Nelson TJ, A Martinez-Fernandez, S Yamada, C Perez-Terzic, Y Ikeda and A Terzic. (2009). Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. *Circulation* 120:408–416.
36. Boudoulas KD and AK Hatzopoulos. (2009). Cardiac repair and regeneration: the Rubik's cube of cell therapy for heart disease. *Dis Model Mech* 2:344–358.
37. Bauwens CL, R Peerani, S Niebruegge, KA Woodhouse, E Kumacheva, M Husain and PW Zandstra. (2008). Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells* 26:2300–2310.
38. Niebruegge S, CL Bauwens, R Peerani, N Thavandiran, S Masse, E Sevaptisidis, K Nanthakumar, K Woodhouse, M Husain, E Kumacheva and PW Zandstra. (2009). Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. *Biotechnol Bioeng* 102:493–507.
39. Baharvand H, M Azarnia, K Parivar and SK Ashtiani. (2005). The effect of extracellular matrix on embryonic stem cell-derived cardiomyocytes. *J Mol Cell Cardiol* 38:495–503.
40. Yoshida Y and S Yamanaka. (2011). iPS cells: a source of cardiac regeneration. *J Mol Cell Cardiol* 50:327–332.
41. Kwon C, KR Cordes and D Srivastava. (2008). Wnt/beta-catenin signaling acts at multiple developmental stages to promote mammalian cardiogenesis. *Cell Cycle* 7:3815–3818.
42. Eisenberg LM and CA Eisenberg. (2006). Wnt signal transduction and the formation of the myocardium. *Dev Biol* 293:305–315.
43. Cohen ED, Y Tian and EE Morrisey. (2008). Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. *Development* 135:789–798.
44. Huelsken J, R Vogel, V Brinkmann, B Erdmann, C Birchmeier and W Birchmeier. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. *J Cell Biol* 148:567–578.
45. Lindsley RC, JG Gill, M Kyba, TL Murphy and KM Murphy. (2006). Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* 133:3787–3796.
46. Yamaguchi TP, S Takada, Y Yoshikawa, N Wu and AP McMahon. (1999). T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. *Genes Dev* 13:3185–3190.
47. Chen VC, R Stull, D Joo, X Cheng and G Keller. (2008). Notch signaling respecifies the hemangioblast to a cardiac fate. *Nat Biotechnol* 26:1169–1178.
48. Yuasa S, Y Itabashi, U Koshimizu, T Tanaka, K Sugimura, M Kinoshita, F Hattori, S Fukami, T Shimazaki, et al. (2005). Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. *Nat Biotechnol* 23:607–611.
49. Hao J, MA Daleo, CK Murphy, PB Yu, JN Ho, J Hu, RT Peterson, AK Hatzopoulos and CC Hong. (2008). Dorsomorphin, a selective small molecule inhibitor of BMP signaling, promotes cardiomyogenesis in embryonic stem cells. *PLoS One* 3:e2904.
50. Blankensteijn JD. (2000). Mortality and morbidity rates after conventional abdominal aortic aneurysm repair. *Semin Interv Cardiol* 5:7–13.
51. Barandon L, T Couffignal, J Ezan, P Dufourcq, P Costet, P Alzieu, L Leroux, C Moreau, D Dare and C Duplaa. (2003). Reduction of infarct size and prevention of cardiac rupture in transgenic mice overexpressing FrzA. *Circulation* 108:2282–2289.
52. Mirotsoy M, Z Zhang, A Deb, L Zhang, M Gnecci, N Noiseux, H Mu, A Pachori and V Dzau. (2007). Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci U S A* 104:1643–1648.
53. Alfaro MP, M Pagni, A Vincent, J Atkinson, MF Hill, J Cates, JM Davidson, J Rottman, E Lee and PP Young. (2008). The Wnt modulator sFRP2 enhances mesenchymal stem cell engraftment, granulation tissue formation and myocardial repair. *Proc Natl Acad Sci U S A* 105:18366–18371.
54. Kobayashi K, M Luo, Y Zhang, DC Wilkes, G Ge, T Grieskamp, C Yamada, TC Liu, G Huang, et al. (2009). Secreted Frizzled-related protein 2 is a procollagen C proteinase enhancer with a role in fibrosis associated with myocardial infarction. *Nat Cell Biol* 11:46–55.
55. Hahn JY, HJ Cho, JW Bae, HS Yuk, KI Kim, KW Park, BK Koo, IH Chae, CS Shin, et al. (2006). Beta-catenin overexpression reduces myocardial infarct size through differential effects on cardiomyocytes and cardiac fibroblasts. *J Biol Chem* 281:30979–30989.
56. Aisagbonhi O, M Rai, S Ryzhov, N Atria, I Feoktistov and AK Hatzopoulos. (2011). Experimental myocardial infarction triggers canonical Wnt signaling and endothelial-to-mesenchymal transition. *Dis Model Mech* 4:469–483.

Address correspondence to:

Dr. Antonis K. Hatzopoulos
Division of Cardiovascular Medicine
Department of Medicine
Vanderbilt University
Nashville, TN 37232-6300

E-mail: antonis.hatzopoulos@vanderbilt.edu

Received for publication June 24, 2011

Accepted after revision August 18, 2011

Prepublished on Liebert Instant Online August 23, 2011