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Role of Smad- and Wnt-Dependent Pathways in Embryonic Cardiac Development

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ABSTRACT

The development of the heart is essential for embryogenesis and precedes development of other organs. However, the mechanisms involved in embryonic cardiac development are ill-defined. Recent evidence suggests that Smad and Wnt signaling pathways are important in stem cell fate determination and their commitment to cardiovascular differentiation. We have previously reported that bone morphogenetic proteins (BMP)-2, -5, and -7 and fibroblast growth factors (FGF)-2 and -4 secreted from the adjoining endodermal cells favor cardiac differentiation in murine embryonic stem (ES) cells. Here, we demonstrate that BMP-2, -5, and -7 stimulate receptor-activated Smad1, 5, and 8, which in turn causes oligomerization of Smad4 in the nucleus. We further delineate the role of Wnt signaling pathway as evidenced by induction of Wnt3 and Wnt8b, stimulation of FRP-1, inhibition of GSK-B, accumulation of cytosolic β-catenin, and transcription of target genes, including c-*myc* and cyclin-D1. We also ascertained the specificity of BMP- and Wnt-evoked activation of signaling cascades. Our data are consistent with the hypothesis that BMP-dependent activation of transcription factors including GATA-4, Nkx2.5, and MEF-2C augments cardiac differentiation mediated by cooperative control of Smad and Wnt signaling pathways. Our results provide a solid foundation for further study of the biochemistry of cardiac differentiation from stem cells.

INTRODUCTION

EMBRYONIC STEM (ES) CELLS are a population of multipotent, self-renewing cells that are derived from the epiblast of mammalian blastocyst embryos and retain this developmental identity even after prolonged culture in vitro (1). The recent isolation and culture of human embryonic stem (hES) cells (2) attracts two important considerations. The first is their potential application in regenerative medicine, and the second is their experimental usefulness in delineating important developmental signaling pathways, and the potential they offer for regenerative medicine, as well as the delineation of the events involved in early human development and the signaling pathways activated during development. Pluripotent ES cells can be induced to differentiate into a myriad of tissues belonging to the three germ layers, including ectoderm, mesoderm and endoderm in vitro after aggregation into three-dimensional structures termed embryoid bodies (EBs) (3). EBs give rise to a variety of specialized cell types, including cardiomyocytes, that manifest by the appearance of spontaneously contracting foci (4). Thus, cardiac differentiation of EBs is a suitable in vitro model to study the signaling pathways in heart development from mouse ES cells.

The heart is one of the first organs to develop in a developing embryo and ensures the distribution of vital nutrients within the growing organism. Truly, the well-orchestrated morphological and molecular events that result in the formation of this complex organ are intriguing. Although the cardiac-promoting role of many growth factors has been thoroughly investigated, our current knowledge about regulatory events leading to heart formation is mostly based on previously characterized transcription factors that belong to very different gene families. One

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of the biggest challenges now is to identify molecules that regulate these cardiac transcription factors and to understand their intertwined regulatory relationships.

Dissecting the genetic pathways involved in cardiogenesis has revealed crucial roles for members of various growth factor families in this process. For example, the combination of bone morphogenetic proteins 2/4 (BMP-2/4) belonging to the transforming growth factor-β (TGF-β) family, and fibroblast growth factor-4 (FGF-4) is capable of converting avian posterior mesoderm, which normally does not contribute to the heart, into cardiac tissue (5,6). Similarly, FGF/FGFR signaling also plays important functions in heart formation and development (7).

Likewise, various growth factors appear to exert effects in early hepatogenesis through similar tissue interactions. Studies have shown that the cardiogenic mesoderm, which is transiently opposed to the prospective hepatic endoderm, provides a signal that induces liver progenitors in the endoderm (8). Although this inductive property of the precardiac mesoderm was necessary for determining the hepatic lineage commitment, it was not sufficient for hepatocyte differentiation. Furthermore, during induction of the endoderm with the intervention of precardiac mesoderm via reciprocal signaling, GATA-4 and HNF-3B are key transcription factors that lead to induction of downstream effectors of hepatic differentiation (9).

Members of the TGF-β family control growth and differentiation and have important functions during embryonic development (10,11). BMPs are a subclass of the TGF-β superfamily active in the developing heart (12). BMPs bind to and activate different serine/threonine kinase receptors (BMPR-I and BMPR-II). Upon activation, BMP receptors recruit and phosphorylate several receptor-regulated Smad transcription factors (Smad1, Smad5, or Smad-8), which then interact with Smad4, followed by translocation of the heteromeric Smad complex to the nucleus where it associates with other transcription factors to activate specific BMP-responsive genes (13–15). Three classes of Smads have been defined: the receptor-regulated Smads (R-Smads); the co-Smads (Smad-4), and the inhibitory Smads (I-Smads). Smad proteins are recruited to specific target genes via their interactions with distinct transcriptional cofactors. Investigating the mechanism that mediates the selective response of the embryonic mesoderm to BMP signaling is likely to provide invaluable insight into the molecular basis of cardiac specification, which is poorly understood. Furthermore, a separate pathway involving the TGF-β-activated kinase 1 (TAK1) and p38 mitogen-activated protein kinase (MAPK) is reportedly activated by BMPs in some cells (16,17).

In contrast to the well-investigated roles of the signaling molecules described above, the function of Wnt family members in vertebrate cardiogenesis is currently under investigation. Wnt/Wg genes, related to *wingless* in *Drosophila*, encode a number of secreted proteins that play critical roles in the development of many organisms, especially in cell fate and patterning (18–20). Once Wnt molecules have bound to their receptors, the cytosolic phosphoprotein Dsh or Frz becomes activated, which, in turn, leads to inactivation of GSK-3β. Inhibition of GSK-3 leads to elevated levels of cytosolic β-catenin. GSK-3 inhibits the Wnt pathway, by phosphorylating amino-terminal β-catenin residues, directing β-catenin toward the degradation pathway. In regulating the stability of cytosolic β-catenin, GSK-3 is accompanied by at least three different molecules: adenomatous polyposis coli (APC), Axin (also known as conductin), and GSK-3 binding protein (GBP). APC contains a β-catenin, as well as a GSK-3 binding domain (21). But the role of Wnt proteins in heart development is complex, which arises from the existence of different Wnt signal transduction cascades. To date, only Wnt-3, Wnt-8, and Wnt-11 have been ascribed a promoting role in cardiac differentiation from the mouse embryonic carcinoma stem cell line P19 (22,23). However, this does not exclude positive regulatory roles for other Wnt proteins expressed in cardiac tissue. Targeted gene disruptions of different Wnt genes in mice suggest that the functions of these proteins are interchangeable.

Almost all of the reports on the reciprocal signaling of the endodermal derivatives in cardiogenesis are based on tissue explant systems in chick, quail, or *Xenopus*. In our previous study, we have shown a simple and effective strategy for co-differentiation of mouse ES cells into cardiac myocytes and hepatocyte-like cells without using exemplary cardiotrophic factors and also without employing an in vitro co-culture system. We have also demonstrated that BMPs and FGFs secreted from the surrounding hepatocyte-like cells play the role of intrinsic signals in differentiation of cardiomyocytes (24).

In light of the above considerations, we undertook the present study to define further the obligatory role of Smad molecules as effectors of downstream events leading to cardiac differentiation from mouse ES cells. The activation of Wnt signaling pathway in cardiogenic development is implicated by our results.

MATERIALS AND METHODS

Mouse ES cell culture, co-differentiation of cardiomyocytes, and hepatocyte-like cells

J1 mouse ES cells (ATCC, Manassas, VA) were grown on a mitotically inactivated (mitomycin-c) mouse embryonic fibroblast feeder layer. The medium consisted of Dulbecco's modified Eagle medium (DMEM) (no-pyruvate,

high-glucose formulation; GIBCO-BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; Hyclone, Logan, UT); 1× nonessential amino acids, 2.0 mM glutamine, 1,000 U/ml mouse LIF/ESGRO®, 100 μM 2-mercaptoethanol, 100 U penicillin, and 100 μg/ml streptomycin (all from GIBCO-BRL). This medium inhibits differentiation of ES cells and is used until differentiation is initiated. To initiate differentiation, cells were first cultured on 0.1% gelatin- (Sigma, St. Louis, MO) coated plates without feeder layers. For differentiation of ES cells, EBs were generated by the hanging drop method in suspension culture for 4 days in the absence of leukemia inhibitory factor (LIF). After their generation, 10–15 EBs were seeded onto 35-mm tissue culture plates (Nunc, Roskilde, Denmark) precoated with 0.5% poly-L-lysine (Sigma) in DMEM medium supplemented with 15% FBS, 50 nM basic FGF (R&D Systems, Minneapolis, MN). Rhythmic beating of EB outgrowths, surrounded by oval-shaped hepatocyte-like cells, was observed on days 8–9 of differentiation, and the cells continued to beat for more than 30 days in culture.

RNA extraction and RT-PCR analysis

Total RNA was isolated by the TRIzol method (Invitrogen) according to the manufacturer's protocol. A total of 1 μg of RNA treated with RNase-OUT ribonuclease inhibitor (Invitrogen, Carlsbad, CA) was used for cDNA synthesis. Reverse-transcription using Superscript reverse transcriptase-II (Invitrogen) and oligo(dT) (Invitrogen) was carried out to prime the reaction. PCR primers were selected to distinguish between cDNA and genomic DNA by using individual primers specific for different exons. cDNA (2 μl) was amplified by PCR using Abgene 2× PCR master mix (Abgene: Advanced Biotechnologies, Ltd., Epsom, Surrey, UK) and appropriate primers (see Table 1). The expression of genes such as GAPDH, TTR, AFP, albumin, cTnT, β-MHC, MLC-2v, Smad-1 and -5, Smad-4, GATA-4, Nkx2.5, MEF-2C, Wnt8b, Wnt3, β-catenin, c-*myc*, cyclin-D1, BMP-2, BMP-5, and FGFR-4 were checked. For all of the genes, PCR was performed for 35 cycles, consisting of an initial denaturation at 94°C for 1 min, then 94°C for 30 sec, an annealing temperature of the respective gene primer for 45 sec (for T_m values of individual primers, see Table 1), 72°C for 1 min, and was terminated by final extension at 72°C for 5 min.

Immunochemistry

The differentiated cells were tested for cytoplasmic and nuclear markers by immunofluorescence analysis as described in our earlier report (24). Briefly, EB outgrowths were mechanically dissected, enzymatically dispersed using trypsin-EDTA (0.5% trypsin, 0.53 mM EDTA; Life Technologies, Inc., Grand Island, NY) for 5 min at 37°C; plated on poly-L-lysine-coated two-well-chambered glass slides, and incubated for 48 h. After fixing the cells with 4% paraformaldehyde, the cells were permeabilized with 0.2% Triton X-100 for 5 min at room temperature. The cells were then blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 1 h at room temperature and were incubated with primary antibody solutions (Smad-4, Santa Cruz, Santa Cruz, CA; β-catenin, Santa Cruz; β-MHC, Chemicon, Temecula, CA) diluted with 1% BSA in PBS overnight at 4°C followed by incubation with secondary antibody solution, coupled with a fluorescent label fluorescein isothiocyanate (FITC) at room temperature for 1 h on a rocker. Additionally, cells were counterstained with DAPI (1 μg/ml; Sigma). The slides

TABLE 1. DETAILS OF PRIMER USED

Name of gene	*Annealing temp (C°)*	*Primer sequence (5′-3′)*	*Product size (bp)*
Smad-1/5	63	ATGAATGTGACCAGCTTGTTT	349
		CTGCTTGGAACCAAATGGGAA	
Smad-4	55	AAGGTGGGGAAAGTGAAAC	250
		ATGCTTTAGTTCATTCTTGTG	
Wnt-3	60	ACACTTGAGCAGAACGGATACA	207
		TGGATACAGCAGGTTGGTAGG	
Wnt-8b	55	AATGTCTGACTTGAAATGAAA	190
		AATGGTTAGAAGAGGTTGGC	
β-Catenin	62	GCCTGCAGAACTCCAGAAAG	135
		GTGGCAAAAACATCAACGTG	
Cyclin-D1	62	TCTCCTGCTACCGCACAAC	749
		TTCCTCCACTTCCCCCTC	
c-*myc*	60	CGCGCCCAGTGAGGATATC	281
		CCACATACAGTCCTGGATGAT	

For details of the other primer pairs used, please refer to our previous report (24).

were then mounted with DPX mountant and examined under an inverted fluorescence microscope (Nikon Eclipse E600, Kanagawa, Japan).

Cell fractionation and western blotting of EB outgrowths

Spontaneously beating EB outgrowths, each comprising of at least 10^3 cells, were harvested, washed twice with PBS, and then lysed using M-PER mammalian cell extraction buffer (Pierce, Rockford, IL). Whole-cell lysates were centrifuged at 120,000 $\times$ *g* for 30 min at 4°C, and the resulting supernatant leaving the debris was collected. Protein concentrations were determined using Bradford method (BioRad, Hercules, CA), and 25-μg samples of soluble proteins were boiled in 2% sodium dodecyl sulfate (SDS) sample buffer (with 1 mM β-mercaptoethanol) for 5 min, then separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels and electrically transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corp., Mumbai, India). Blots were stained with Red Ponceau-S stain to verify the loading and transfer of the proteins, blocked with 10% skim milk in tris-buffered saline-Tween (TBST), and sequentially probed with a 1:500 dilution of indicated primary antibodies, including Smad-1, -5, and -8, Smad-4, Wnt-3, Wnt-8b, FRP-1, GSK-3β, β-catenin (Santa Cruz) overnight at 4°C. Bound antibody was visualized using appropriate secondary horseradish peroxidase (HRP) or alkaline phosphatase (ALP) conjugates. The signals were developed using a 3,3-diaminobenzidine tetrahydrochloride (DAB) kit (Vector Laboratories, Burlingame, CA) and or BCIP/NBT alkaline phosphatase substrate tablets (Sigma).

RESULTS

Differentiation of cardiomyocytes interspersed with hepatocyte-like cells

In our earlier report, we showed the generation of spontaneously beating cardiomyocytes surrounded by hepatocyte-like cells. Using the same methodology, we used phase-contrast microscopy, which revealed the appearance of spontaneously beating EB outgrowths at days 8–10 of differentiation (Fig. 1A). The rate of the contraction of the cells was recorded to be at its maximum on the 15th day of differentiation, and the cardiomyocytes were able to retain the contractility up to 30 days or longer in culture. Polyhedral to oval-shaped hepatocyte-like cells were confirmed to be present surrounding the cardiac bodies (Fig. 1B). The identity of both of the cell types was ascertained by reverse transcriptase (RT)-PCR (Fig. 1C) using tissue-specific markers like transthyretin (TTR), alpha feto-protein (AFP), and albumin for liver-specific gene expression and cTnT, β-MHC, and MLC-2V for heart-specific gene expression respectively.

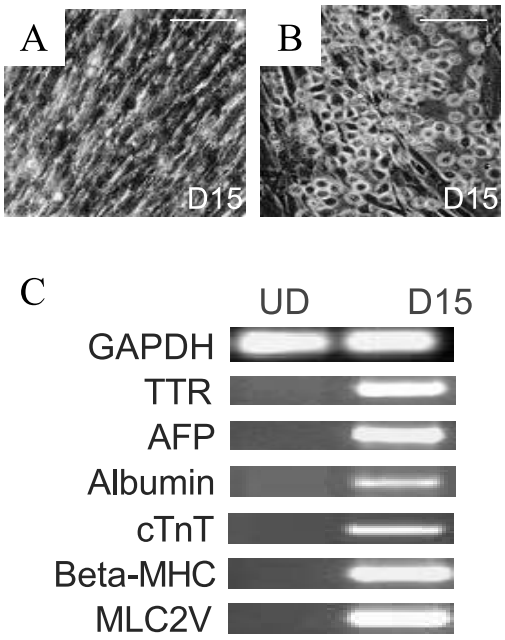

FIG. 1. Co-differentiation of cardiomyocytes and hepatocyte-like cells from mouse ES cells. Phase-contrast micrographs showing day-15 contracting cardiomyocytes (**A**) and oval-shaped hepatocyte-like cells (**B**). Scale bars, 50 μm. (**C**) Gene expression analysis of differentiated cells with liver and cardiac tissue-specific primers including TTR, AFP, albumin, cTnT, β-MHC, and MLC-2V, respectively. GAPDH is used as a housekeeping gene control.

Signaling mechanisms induced by BMP-2, -5, and -7 are mediated through Smad-1, -5, and -8

Smad proteins are important mediators of signaling induced by TGF-β family members. It is also known that Smad-1, -5, and -8 are involved in the TGF-β pathway evoked by BMP and it subsequently binds to the serine/threonine kinase receptors BMPR types I and II. Therefore, we looked for the activation status of these proteins in in vitro cardiogenesis in response to the intrinsic signals like BMPs and FGFs. In our earlier experiments, we have reported the up-regulation in mRNA levels of BMP-2, -5, and -7 along with FGFR-2 and -4 stimulated by the adjacent hepatocyte-like cells. We further investigated the molecular mechanisms behind the initial activation of these signaling molecules. In our study, we have used noggin, a potent inhibitor of the BMP/Smad pathway. Noggin at high concentration is capable of binding BMP ligand with high affinity and can abolish BMP activity by blocking its binding to a cognate cell-surface receptor (25). Therefore, we treated the cells with 500 ng/ml of noggin/Fc chimeric protein (Sigma) on the 4th day of differentiation to antagonize the effect of

intrinsic BMP signaling and to examine the changes in the downstream events. We had observed that lower concentrations of noggin (100 or 200 ng/ml) were not able to block BMP activity (data not shown). The EB outgrowths were harvested and used for immunofluorescence and gene expression analysis, as described in the earlier report. For western blotting, the cells were lysed in M-PER mammalian cell extraction buffer. Equal amounts of proteins were separated by electrophoresis and were transferred to a PVDF membrane. The membranes were then probed with suitable antibodies. The actin levels were used as loading controls.

We observed enhanced expression of the Smad-1, -5, and -8 and Smad-4 on the 15th day of differentiation in comparison to the undifferentiated mouse ES cells (Fig. 2A,B). After the treatment of the cells with noggin chimeric protein, the up-regulation of Smad proteins was reverted back to basal levels, as determined by both gene expression and protein analysis (Fig. 2A,B). Furthermore, a positive expression of cytoplasmic Smad-4 protein in day 15 differentiated cells was demonstrated by immunostaining with less or no expression in the cells exposed to noggin (Fig. 2C,D,E). Mouse embryonic fibroblast (MEF) cells were used as a negative control (Fig. 2F).

The results clearly indicate that in response to BMP-2, -5, and -7 secreted by the hepatocyte-like cells, BMP receptors recruit Smad-1, -5, and -8, which in turn translocate to the nucleus and activate the common Smad, Smad-4. Blocking BMP signaling by application of exogenous noggin reverses the regulation of the Smad proteins and its downstream effectors.

Activation of cardiogenic transcription factors by Smad-4 in the nucleus

Upon activation, receptor-activated Smad proteins (Smad-1, -5, -8) are translocated to the nucleus and interact with the common Smad (Smad-4). In the next step, Smad-4 oligomer binds to the DNA of the target genes like GATA-4, Nkx2.5, and MEF-2C, which thereafter play a pivotal role in specification of the cardiac fate and morphogenesis of the heart.

Hence, we examined the levels of GAT-4, Nkx2.5, and

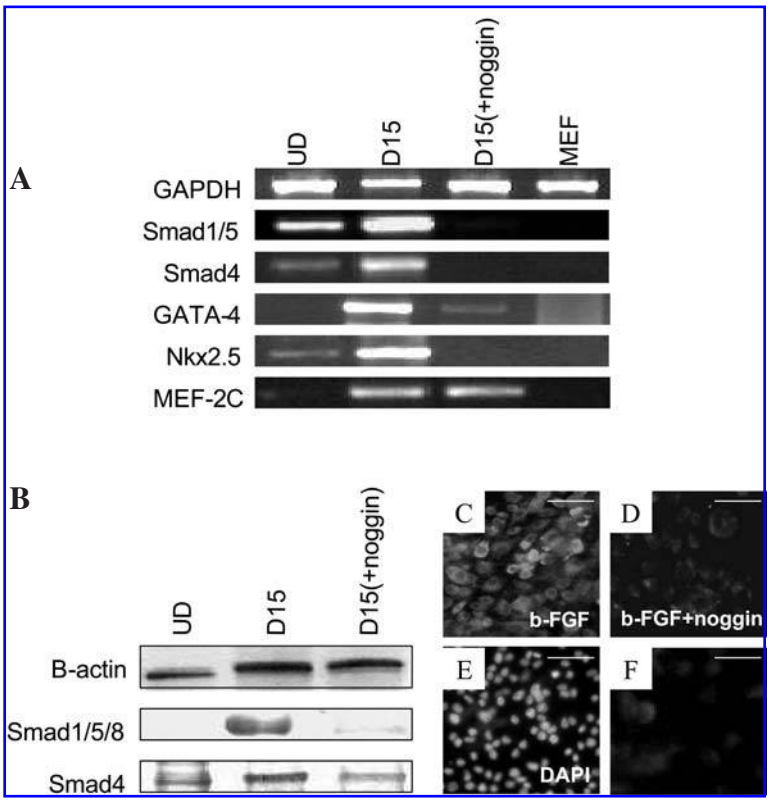

FIG. 2. Differential activation of Smad proteins in cardiac differentiation. (**A**) RT-PCR analysis shows the modulation of Smad-1 and -5, Smad-4, and the subsequent up-regulation of their downstream effectors like GATA-4, Nkx2.5, and MEF-2C. (**B**) The response of Smad-1, -5, and -8, and Smad-4 proteins to the intrinsic BMP signaling could be reversed by the application of noggin, a potent inhibitor of BMP ligand as detected by western blotting. The intensities of the protein expression were normalized against signals obtained with β-actin. (**C**) Immunochemistry shows cytoplasmic localization of Smad-4 in day-15 differentiated cells whereas cells treated with noggin (**D**) were devoid of positively stained cells. (**E**) Presence of cells was confirmed by counterstaining with the nuclear stain DAPI (1 μg/μl). (**F**) MEF cells were stained with goat anti-Smad-4 immunoglobulinG (IgG) to ascertain the specificity of the antibody. Scale bars, 50 μm.

MEF-2C mRNA in the cells before and after exposure to noggin. We observed that there was a transient increase in the expression of these three transcription factors coincident with the onset of cardiac differentiation and was reversible with the treatment of the BMP antagonist. These data indicate that BMPs favor the commitment of mouse ES cells into cardiac phenotype via Smad proteins.

Wnt/β-catenin pathway is activated at the early stage of differentiation

Upon Wnt signaling, the constitutive kinase activity of GSK-3β is inhibited and allows the accumulation of β-catenin in the nucleus. The frizzled form of the transmembrane proteins functions as a receptor for the Wnt family members and aids in the regulation of intracellular levels of β-catenin. β-Catenin is essential for the transcriptional activity of TCF/lymphoid enhancing factor (LEF). TCF-1 and LEF-1 have been shown to be expressed in largely overlapping, complex patterns during embryogenesis. Among the number of TCF/LEF target genes identified (26), we concentrated on c-*myc* and cyclin-D1, both of which have important implications in understanding the role of Wnt signaling.

We first used RT-PCR analysis to detect the temporal changes of Wnt-3 and Wnt-8b in the differentiated cells. Wnt-3 and Wnt-8b exhibited early expression in the undifferentiated cells. However, both were induced to elevated levels within 15 days of differentiation (Fig. 3A). Likewise, data from protein analysis by western blotting support the gene expression results (Fig. 3B). Furthermore, FRP-1, a secreted frizzled-related protein, which

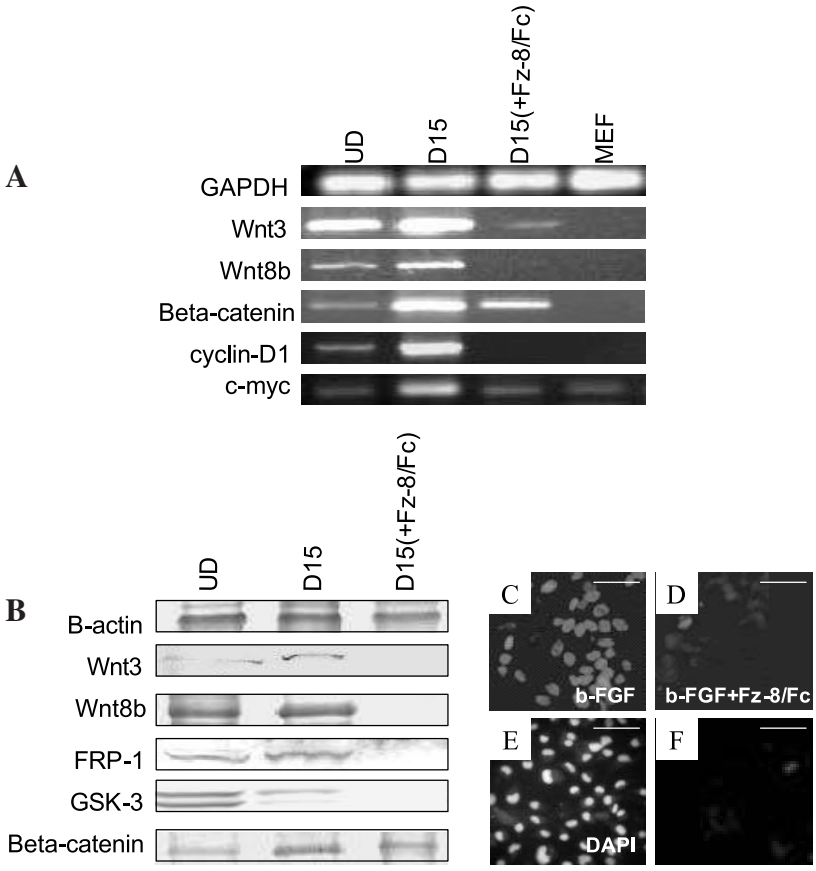

FIG. 3. Wnt signaling mediates in vitro cardiogenesis from mouse ES cells. (**A**) RT-PCR analysis confirms the activation of Wnt-3, Wnt-8b, and β-catenin followed by subsequent upregulation of the TCF/LEF target genes like c-*myc* and cyclin-D1. (**B**) Likewise, protein analysis by western blotting supported the transcriptional profiling. FRP-1, being a receptor to Wnt family members, is up-regulated followed by the down-regulation of GSK-3 in two isoforms, GSK-3α, and GSK-3β (52 and 40 kD). Upon addition, Fz-8/Fc chimeric protein, being an inhibitor of Wnt, was able to block the Wnt signaling and the downstream events thereafter, as determined by the identifying the reduced levels of the related proteins. (**C**) β-Catenin, the key player in canonical Wnt signaling, is shown to accumulate in the cells on the 15th day of differentiation and was reduced to lower levels post Fz-8/Fc treatment as detected by immunochemistry (**D**). (**E**) Cells were similarly counterstained with DAPI (1 μg/μl). (**F**) MEF was used to evaluate the specificity of goat anti-β-catenin IgG. Scale bars, 50 μm.

acts as receptor to Wnt family of proteins, was shown to be up-regulated on day 15 of differentiation (Fig. 3B). We extended our examination to check the regulation of GSK-3, a serine/threonine-directed kinase in this system. Intriguingly, we observed the presence of two related forms of GSK-3, GSK-3α and GSK-3β by western blotting in the undifferentiated cells through the differentiation of cardiomyocytes surrounded by hepatocyte-like cells (Fig. 3B).

To assess whether functional signaling is activated at the time of Wnt induction, we examined cytosolic β-catenin, the crux of the canonical Wnt signal transduction pathway (22,27). Wnt stimulated the accumulation of soluble β-catenin (Fig. 3A,B). Simultaneous treatment with 500 ng/ml Fz-8/Fc chimeric protein (R&D Systems, USA), an antagonist for Wnt-8a and potentially for other Wnt proteins (22), decreased β-catenin to a basal level (Fig. 3A,B), as evidenced by RT-PCR and western blotting of whole-cell lysates. This indicates that the accumulation of β-catenin is regulated by an autocrine or paracrine circuit in the system, involving endogenous Wnt proteins. Conversely, as expected, β-catenin was decreased upon inhibition of Wnt signaling, as determined by immunochemistry. As a third criterion, to confirm the activation of the canonical Wnt pathway, TCF/LEF-dependent transcription of the target genes like c-*myc* and cyclin-D1 was evaluated. Recruitment of Wnt proteins induced a considerable increase in the mRNA levels of both the genes on day 15 of differentiation (Fig. 3A).

Wnt/β-catenin signaling is required to enhance cardiac differentiation

To understand the possible role of Wnt-mediated signaling in early cardiogenesis, we further monitored differentiation induced by the adjoining endodermal cells, with or without Fz-8/Fc. Treatment with soluble Wnt inhibitor prevented GATA-4, Nkx2.5, MEF-2C, and β-MHC induction at least through day 15 of differentiation (Fig. 4A). Likewise, Fz-8/Fc inhibited the expression of BMP-2, BMP-5, and FGFR-4 (Fig. 4A), which indicates that the Wnt pathway lies upstream to the induction of these cardiac differentiation factors. We extended our examination to ascertain the direct implication of Wnt induction in cardiac differentiation. We counted the number of beating regions and found that there was a significant reduction in the total number of contracting cardiac bodies on days 9 through 15 in differentiation after application of Fz-8/Fc (Fig. 4B). Although, there was also a small decrease in the beating rate in terms of the beats/minute, no major alteration in the phenotype of the beating cardiomyocytes was observed. Furthermore, immunofluorescence analysis shows that β-MHC, a marker for mature cardiac muscle, is comprehensively expressed in day-15 beating cardiomyocytes but disappears with the exposure of Fz-8/Fc inhibitor (Fig. 4C,D). Thus, Wnt signaling plays a critical role not only in early cardiogenesis but also in the formation of mature spontaneously beating cardiomyocytes from ES cells.

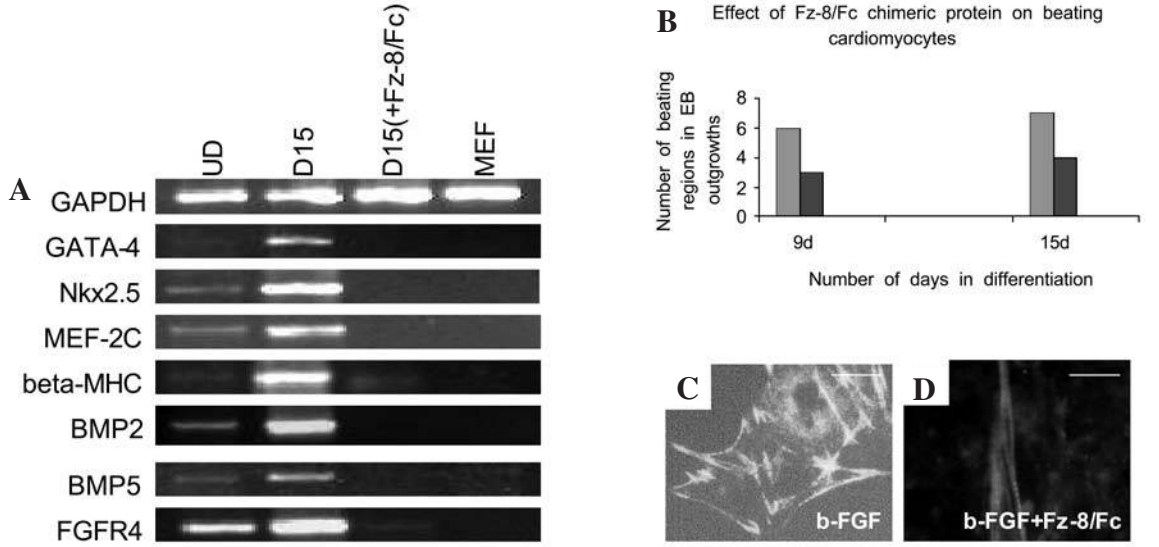

FIG. 4. Activation of Wnt signaling modulates the regulation of its target genes. (**A**) By RT-PCR analysis, a transient increase in the mRNA levels of the cardiogenic transcription factors like GATA-4, Nkx2.5, MEF-2C, and β-MHC was demonstrated. Interestingly, Fz-8/Fc was also seen to have an inducing effect on BMP-2, BMP-5, and FGFR-4 expression. (**B**) Effect of Fz-8/Fc inhibitor on the number of beating regions. (**C**) Immunofluorescence analysis shows presence of β-MHC protein in day-15 cardiomyocytes whereas (**D**) little or no positive expression of the same protein could be detected post Fz-8 treatment. Scale bars, 50 μm.

DISCUSSION

The heart is perhaps the first organ to be formed in the vertebrate embryo. Development and acquisition of a cardiac fate by embryonal mesodermal cells is a fundamental step in heart formation, which is crucial for further development. Several discrete steps initiate heart development in general. Commitment to cardiac fate results from inductive interactions during gastrulation. In amphibian and avian embryos, the endoderm adjacent to the mesodermal cardiac precursors is the source of instructive signaling capable of specifying a cardiac fate (28–30). Likewise, most of the recent work on the prospective role of endoderm-derived growth factors belonging to the TGF-β superfamily in cardiac development has been performed in avian species or primitive species, such as chick, quail, and *Xenopus* (12,31). Therefore, it is important to elucidate when, where, and how mesodermal cells are instructed to assume the cardiac fate for understanding the entire body of mechanisms that operate later in heart development. To date, this problem remains elusive and undefined.

In our earlier report, we demonstrated that during differentiation of ES cells, the hepatocyte-like cells appearing adjacent to the spontaneously beating "cardiac bodies" play an important role in regulating the fate of the ES cells toward the cardiac phenotype by providing necessary intrinsic signals in form of BMPs and FGFs (24). Prior to this, exogenous application of BMP-2 and -4 was demonstrated to induce commitment of ES cells to cardiac differentiation within EBs (32). Conversely, we demonstrated the differentiation of spontaneously beating cardiomyocytes authenticated by the expression of cardiac tissue-specific genes, including cTnT, β-MHC, and MLC-2V (Fig. 1A,C) without using exogenous BMPs or any other cardiotropic factors.

The Smad signaling pathway is critical for transmitting TGF-β superfamily signals from the cell surface to the nucleus (33,34). TGF-β family members initiate their cellular responses by binding to distinct receptors with intrinsic serine/threonine kinase activity and activation of specific downstream intracellular effects termed Smad proteins. Smad proteins relay the signal from the cell membranes to the nucleus, where they effect the transcription of target genes by recruiting co-activators and co-repressors to a wide array of DNA-binding partners (Fig. 5). The different members of the TGF-β family make use of specific Smad proteins to achieve specificity (15). BMP usually is shown to induce Smad-1, -5, and -8 as its downstream effector, whereas TGF-β_1 is commonly known to stimulate Smad-2 and -3. Thus, we looked at the activity of Smad-1, -5, and -8 in our in vitro system. As expected, we noticed increased levels of Smad-7, -5, and -8 and the common Smad Smad-4 (Fig. 2A,B), which is in concurrence with the earlier reports. Furthermore, we checked the mRNA and protein levels of the cardiogenic transcription factors like GATA-4, Nkx2.5, and MEF-2C as a downstream event. These transcription factors are known to activate the promoters of several cardiac genes, such as myosin light chain, troponin T, tropinin I, β-MHC, and ANP. Interestingly, we observed a significant increase in these target molecules, which may be consequential in inducing formation of beating cardiomyocytes.

As a third criterion, we verified the involvement of BMPs in this signaling pathway. To do so, we used a noggin chimeric protein to block the BMP activity on day 4 of differentiation and checked the endogenous BMP levels (data not shown) and the downstream regulators of BMP at progressive days of differentiation. We observed that noggin was able to recover the elevated levels of Smad-1, -5, and -8, Smad-4, and the target genes such as GATA-4, Nkx2.5, and MEF-2C to basal levels (Fig. 2A–C) as studied by RT-PCR, western blotting, and immunofluorescence analyses. Our results indicate that during differentiation in response to BMPs secreted by the adjacent hepatocyte-like cells, Smad proteins are recruited, thus activating the key cardiogenic transcription factors favoring formation of spontaneously beating cardiomyocytes. However, experiments are in progress to show the specific binding of Smad oligomers to the DNA-binding proteins.

BMP has been reported to activate the transcription of Wnt genes in certain cells (35,36). Recent studies in chick embryos revealed that Wnt signaling could dictate biological fate in which BMP signaling is required but not sufficient, such as neural inhibition of ectoderm, neural crest induction, and apoptosis (37–39). In addition, Hussein and co-workers have delineated the molecular mechanism underlying the induction of Msx2 promoter by cooperative activation of Wnt and BMP signaling pathways through the interaction of Smad-4 with the LEF1 transcription factor (40). On the basis of these reports, we have speculated that convergence of the BMP and Wnt signaling pathways is also required to enhance the expression of cardiogenic transcription factors such as GATA-4, Nkx2.5, and MEF-2C, which in turn may induce cardiac differentiation.

Activation of the Wnt/β-catenin signaling cascade was an early event in cardiac differentiation of pluripotent J1 mouse ES cells, as measured by Wnt-3 and Wnt-8b induction, FRP-1 stimulation, GSK-3β down-inhibition, accumulation of β-catenin, and transcription of TCF/LEF target genes including c-*myc* and cyclin-D1 (Fig. 3A–C). Blocking Wnt receptor interactions with soluble Fz proteins largely or completely blocked the cardiogenic pathway, including induction of the earliest markers of cardiac differentiation like GATA-4, Nkx2.5, MEF-2C, and even β-MHC, a late cardiac muscle-specific marker (Fig. 4A). The effect of the activation of these early-stage cardiac transcription factors on cardiac differentiation was

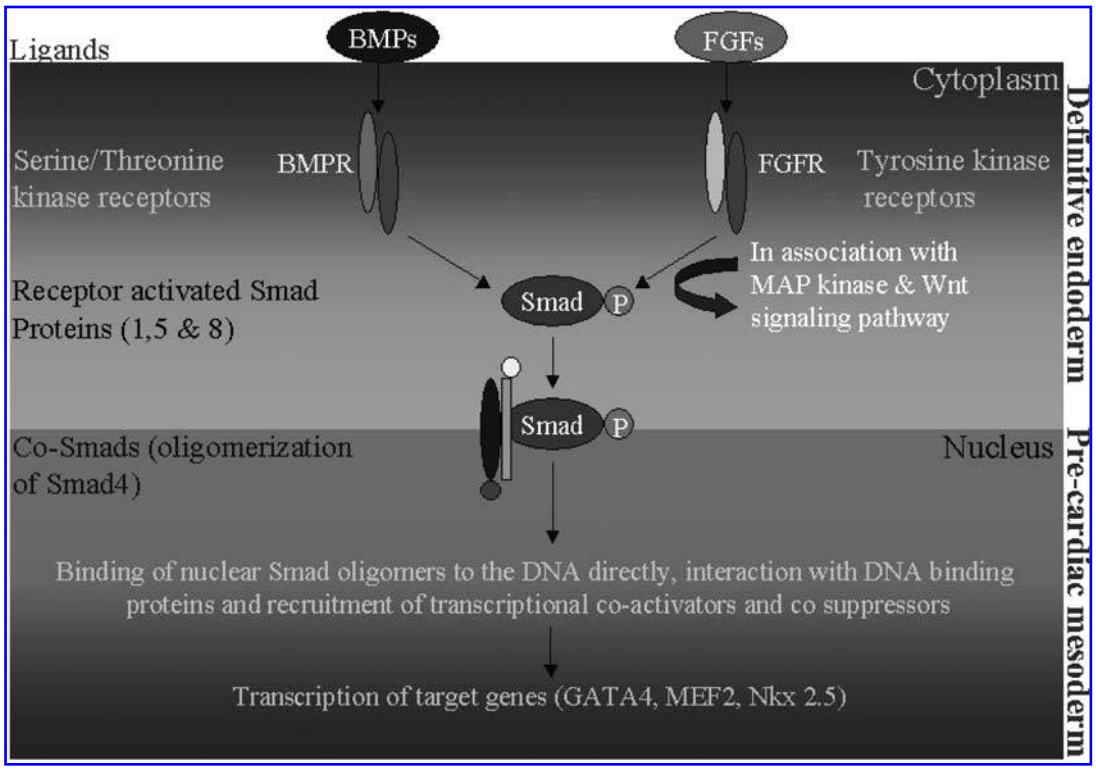

FIG. 5. Schematic illustration of representative signaling pathways in cardiac myogenesis. Sequential activation of various genes/proteins at different stages of the Smad signaling cascade is elucidated, which ultimately leads to the regulation of the target genes in early cardiogenesis like GATA-4, Nkx2.5, and MEF-2C.

ascertained by the decrease in the number of beating regions (Fig. 4B) and subsequent depletion of β-MHC protein expression (Fig. 4C,D) with Fz-8/Fc chimeric protein. Thus, endogenous Wnt proteins mediate cardiogenesis in mouse ES cells and do so via the canonical β-catenin pathway. Fz-8 protein itself had an inducing effect on BMP-2, BMP-5, and FGFR-4, indicating an indirect role of endogenous BMPs in this system. However, our conclusions differ from inhibitory roles found for Wnt-3a and Wnt-8 in *Xenopus* and chicks (41–43). We emphasize that apart from potential phylogenetic dissimilarities, the studies also differ inherently (cultured cells vs. explants and embryos) in the stage of maturation, perturbations, and diversity of the cell types present. But, our results are in agreement with the report by Pandur and co-workers, showing the stimulation of cardiogenesis by Wnt-11 via the noncanonical pathway in *Xenopus* and P19 EC cells (23). Therefore, BMPs via Smad signaling and Wnt proteins via accumulation of cytosolic β-catenin exert a tight control over mammalian cardiogenesis at least in mouse ES cells.

Hence, this dual regulation of Wnt and BMP appears to be frequent in mammalian development. Moreover, Wnt proteins and BMPs are expressed in many overlapping tissues and, being morphogens, this is consistent with the idea that a formation of a gradient of ligand would result in graded expression of common target genes. But, given the extraordinary diversity and overlapping expression of the Wnt ligands and receptors, a complete genetic analysis of the Wnt family, associated downstream events and interactions, even confined to the potential involvement of Wnt proteins in cardiogenesis is far from being straightforward and comprehensive.

CONCLUSION

The present study was undertaken to understand the molecular mechanisms behind the inductive role of endodermal derivatives in early cardiogenesis of mouse ES cells. In summary, our results demonstrate the BMP-2, -5, and -7 and FGF-2 and -4 secreted by the hepatocyte-like cells mediate the simultaneous activation of Smad and Wnt signal transduction pathways and their downstream events, leading to enhancement of cardiac myogenesis. However, the function of endogenous Smad and Wnt proteins emerge more clearly from inhibitor studies. To our knowledge, this is the first report where we have elucidated the obligatory role of interactive signaling pathways in in vitro cardiogenesis, thereby posing im-

portant questions for ES cell biology and mammalian cardiac development. Further experiments to identify other players and their relationships in these pathways are in progress.

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