Wnt/frizzled-2 Signaling Induces Aggregation and Adhesion among Cardiac Myocytes by Increased Cadherin–β-Catenin Complex

Toshihiko Toyofuku,* Zhang Hong,‡ Tsunehiko Kuzuya,‡ Michihiko Tada,‡ and Masatsugu Hori*

*Department of Internal Medicine and Therapeutics and ‡Department of Pathology and Pathophysiology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan

Abstract. *Wingless* is known to be required for induction of cardiac mesoderm in *Drosophila*, but the function of Wnt family proteins, vertebrate homologues of *wingless*, in cardiac myocytes remains unknown. When medium conditioned by HEK293 cells overexpressing Wnt-3a or -5a was applied to cultured neonatal cardiac myocytes, Wnt proteins induced myocyte aggregation in the presence of fibroblasts, concomitant with increases in β-catenin and N-cadherin in the myocytes and with E- and M-cadherins in the fibroblasts. The aggregation was inhibited by anti–N-cadherin antibody and induced by constitutively active β-catenin, but was unaffected by dominant negative and dominant positive T cell factor (TCF) mutants. Thus, increased stabilization of complexed cadherin–β-catenin in both cell types appears crucial for the morphological effect of Wnt on cardiac myocytes. Furthermore, myocytes overexpressing a dominant negative frizzled-2, but not a dominant negative frizzled-4, failed to aggregate in response to Wnt, indicating frizzled-2 to be the predominant receptor mediating aggregation. By contrast, analysis of bromodeoxyuridine incorporation and transcription of various cardiogenetic markers showed Wnt to have little or no impact on cell proliferation or differentiation. These findings suggest that a Wnt–frizzled-2 signaling pathway is centrally involved in the morphological arrangement of cardiac myocytes in neonatal heart through stabilization of complexed cadherin–β-catenin.

Key words: Wnt • frizzled • cardiac myocytes • cadherin • β-catenin

Introduction

Wnt genes encode glycoproteins that, when secreted, act as autocrine or paracrine factors (Burrus and McMahon, 1995) affecting morphogenic events during embryonic and postembryonic development (for review see Moon et al., 1997). In addition, their activation by mouse mammary tumor virus (MMTV) proviral DNA has implicated Wnt genes in the ontogenesis of MMTV-induced mammary tumors (for review see Nusse and Varmus, 1992). More broadly, Wnt genes have been grouped into functional classes based on assays performed in both *Xenopus* embryos (Du et al., 1995) and mammalian cell lines (Wong et al., 1994). For example, ectopic expression of *Xenopus* Wnt (*X*wnt)-1, -3a, -8, and -8b induces formation of a secondary axis, whereas ectopic expression of *X*wnt-5a, -4, and -11 induces morphological movement during gastrulation without altering cell fate (Moon et al., 1993; Cui et al., 1995; Du et al., 1995). Mouse Wnt genes are grouped in a similar manner: Wnt-1, -3a, -7A, and -8 transform mammary C57MG cells, whereas Wnt-4 and -5a lack transforming activity (Wong et al., 1994; Shimizu et al., 1997).

The first insights into the mechanism of Wnt signal transduction came from the discovery of several fly genes exhibiting mutant phenotypes consistent with defects in *wingless* (Wg) signaling, and from studies of vertebrate counterparts that shed light on the biochemical relationship between gene products (for review see Cadigan and Nusse, 1997; Brown and Moon, 1998). For instance, association of Wnt-1, the vertebrate homologue of Wg, and members of the frizzled protein family leads to activation of Disheveled (Dsh) protein. Activated Dsh inhibits glycogen synthase kinase-3β (GSK-3β),[1] increasing cytosolic β-catenin levels as a consequence of decreased GSK-3β–mediated degradation. β-Catenin can then interact with

Address correspondence to Toshihiko Toyofuku, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: 06-6879-3273. Fax: 06-6879-3279. E-mail: toyofuku@mr-path.med.osaka-u.ac.jp

[1]*Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CRD, cysteine-rich domain; ECM, extracellular matrix; EGFP, enhanced GFP; GFP, green fluorescent protein; GPI, glycophosphatidylinositol; GSK, glycogen synthase kinase; GST, glutathione-S-transferase; LEF, lymphoid enhancer factor; MHC, myosin heavy chain α/β; RT, reverse transcriptase; TCF, T cell factor.

© The Rockefeller University Press, 0021-9525/2000/07/225/17 $5.00

members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of architectural transcription factors in the nucleus, inducing transcription of Wnt-responsive genes (for reviews see Nusse 1997; Miller et al., 1999). This type of Wnt-1 signal transduction is required for Wnt-induced cell transformation, although alternative Wnt and frizzled signaling pathways also have been observed recently (Rocheleau et al., 1997; Slusarski et al., 1997; Strutt et al., 1997; Sheldahl et al., 1999).

Cardiac development has been studied extensively and found to be regulated in a highly integrated manner. In *Drosophila* hearts, Wg is absolutely necessary for induction of cardiac but not visceral mesoderm; however, no vertebrate Wnt genes have been shown to be directly involved in cardiac development to date (Wu et al., 1995; for review see Bodmer and Venkatesh, 1998). Nonetheless, despite numerous morphological differences between vertebrate and invertebrate species, many molecular mechanisms orchestrating individual developmental processes are remarkably conserved. Transcriptional analysis revealed expression of mRNA encoding Wnt-5a in rat neonatal cardiac myocytes, and encoding frizzled-2 and frizzled-4 in both cardiac myocytes and fibroblasts (this study), making it likely that Wnt genes are involved in vertebrate cardiac development.

In this report, we describe the use of cultured cells isolated from rat neonatal hearts as a model system to assess the role of Wnt genes in the growth and behavior of cardiac myocytes. When Wnt-3a and -5a, which differ in their capacity to transform cells, were applied, both induced aggregation of cardiac myocytes that was dependent on the presence of fibroblasts, suggesting two possible effects of Wnt proteins on fetal hearts: morphological movement and proliferation of cardiac myocytes. By interfering with the activity of these genes through the use of specific antibodies and dominant negative constructs, we obtained evidence that in both myocytes and fibroblasts Wnt-5a signaling mediated by frizzled increases levels of intracellular β-catenin, which in turn stabilizes cadherin at intercellular junctions necessary for cell–cell adhesion. On the other hand, proliferation and cardiomyogenesis of so-called precursor cells in neonatal hearts is unaffected. Thus, the morphological effects of Wnt on cardiac myocytes are apparently related to the enhanced homophilic adhesion mediated by cadherin.

Materials and Methods

Cultured Cells and Cell Lines

Rat neonatal cardiac myocytes were prepared as previously described (Iwaki et al., 1990). In brief, the hearts were isolated from 1-d-old HLA-Wistar rats, the ventricles were minced, and the cells were dispersed by digestion with 0.1% collagenase at 37°C. The dispersed cells were resuspended in high glucose DME supplemented with 10% FCS and preplated onto culture dishes for 30 min to remove fibroblasts. Nonadhesive cells were collected, concentrated in PBS ($\sim 2 \times 10^7$/ml), and placed on the top of a discontinuous Percoll gradient (1.060/1.086 g/ml) made up in buffer containing 116 mM NaCl, 20 mM Hepes, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, and 0.8 mM $MgSO_4$, (pH 7.35). Centrifugation at 400 *g* for 30 min yielded myocardial cultures containing >95% myocytes, as assessed by immunofluorescent assay using an anticardiac myosin heavy chain antibody β. Fibroblasts on culture dishes were passaged, diluting them fourfold, every four days. After three passages, resultant cultures were ~100% fibroblasts, as assessed by immunofluorescent assay using an anti-vimentin antibody. For 24 h before each experiment, isolated cardiac myocytes ($\sim 10^5$ cells/ml) were cultured with or without fibroblasts ($\sim 10^5$ cells/ml) on glass coverslips coated with poly-L-lysine, collagen, fibronectin, or l-laminine (Sigma-Aldrich) in high glucose DME supplemented with 10% FCS and maintained at 37°C under an atmosphere of 5% CO_2/95% air. The cultures were then washed and incubated in either conditioned medium containing Wnt protein or FCS free medium.

HEK293 and C2C12 cells were grown in high glucose DME supplemented with 10% FCS and penicillin at 37°C under an atmosphere containing 5% CO_2/95% air.

Construction of cDNAs

Wnt-3a and Wnt-5a cDNAs. Full-length mouse Wnt-3a (amino acids 352; Roelink and Nusse, 1991) and Wnt-5a cDNAs (amino acids 379; Gavin et al., 1990) were amplified by reverse transcriptase (RT)-PCR using mRNA isolated from mouse lungs and hearts respectively. To create c-Myc–tagged constructs, an antisense PCR primer for the COOH-terminal domain was designed that deleted the endogenous stop codon and replaced it with the c-Myc sequence. The resultant sequence at the COOH termini of the constructs was GSEQKLISEEDL, an epitope for mouse monoclonal anti–c-Myc IgG antibody (Calbiochem-Novabiochem). To create a V5-tagged construct, an antisense PCR primer for the COOH-terminal domain was designed that deleted the endogenous stop codon and replaced it with the XbaI restriction site at the 5′ end. When ligated into pcDNA3.1/V5-HisA expression vector (Invitrogen), the resultant sequence at the COOH terminus of Wnt-5a contained GKPIPNPL-LGLDST, which was later detected using anti-V5 mouse monoclonal antibody (Invitrogen). After verifying the nucleotide sequences, the cDNAs encoding c-Myc–tagged Wnt-3a and -5a were ligated into the pcDNA3 mammalian expression vector (Invitrogen).

Frizzled-2 and Frizzled-4 cDNAs and Frizzled-glycophosphatidylinositol Chimera cDNAs. The frizzled gene family, homologues of *Drosophila* gene *frizzled*, have been identified as the receptors for Wg and Wnt (Bhanot et al., 1996). Full-length rat frizzled-2 cDNA (amino acids 570; Chan et al., 1992) and mouse frizzled-4 cDNA (amino acids 537; Wang et al., 1996) were amplified by RT-PCR using mRNA isolated from mouse hearts. To create FLAG-tagged constructs, an antisense PCR primer for the COOH-terminal domain was designed that deleted the endogenous stop codon and replaced it with a FLAG sequence. The resultant sequence at the COOH termini of the constructs was GSDYKDDDDKN, an epitope for mouse monoclonal anti-FLAG IgG antibody (Eastman Kodak Co.).

The frizzled gene sequence predicts a protein with an extracellular NH_2-terminal cysteine-rich domain (CRD) and seven transmembrane segments. It has been proposed that the CRD constitutes all or part of the ligand-binding domain (Wang et al., 1996), and cell-surface expression of isolated CRD was found to confer Wnt protein binding activity (Bhanot et al., 1996). Using the protocol described by Bhanot et al. (1996), we used PCR to create frizzled-glycophosphatidylinositol (GPI) chimeras, composed of the CDR (the first 372 amino acids of frizzled-2 or the first 365 amino acids of frizzled-4), a c-Myc epitope, the COOH-terminal 40 amino acids of decay-activating factor, and a GPI-anchored protein (Caras and Weddell, 1989). After verifying the nucleotide sequences, the cDNAs encoding FLAG-tagged frizzleds and frizzled-GPI chimeras were ligated into pcDNA3 for subsequent transfection.

N-Cadherin and Deleted Cadherin cDNAs. Cadherins are Ca^{2+}-dependent adhesion molecules, which in association with α- and β-catenin constitute the major components of adherent junctions in vertebrates. Full-length N-cadherin cDNA (amino acids 907; Tamura et al., 1998) was amplified by RT-PCR using mRNA isolated from mouse hearts. Cadherin is composed of an extracellular domain that contains the Ca^{2+}-sensitive, homophilic binding sites, a transmembrane domain, and a cytoplasmic domain that interacts with α- and β-catenin (for review see Nagafuchi et al., 1993). In mammalian cells responding to Wnt-1, the increased steady-state levels of catenins is primarily due to a selective increase in the amount of uncomplexed, monomeric β-catenin (Papkoff et al., 1996). To detect uncomplexed β-catenin, we used PCR to create a construct consisting of the last 158 amino acids of N-cadherin, including the entire cytoplasmic domain, fused with GST epitope (GST-ΔNcad), which could be used to precipitate the uncomplexed β-catenin. After verifying the nucleotide sequences, the PCR fragment was ligated, in-frame, downstream of the GST coding region in pGEX-3X (Amersham Pharmacia Biotech).

The role of cell–cell adhesion in cardiac development has been in-

vestigated previously using antibody raised against cadherin (Linask and Lash, 1988; Soler and Knudsen, 1994; Linask et al., 1997). To test whether anti-cadherin antibody directly affects cell–cell adhesion by blocking N-cadherin at the cell surface, we performed antibody-inhibition experiments using GST-fused cadherin. In accordance with information from the manufacturer (Santa Cruz Biotechnology, Inc.) indicating that anti–N-cadherin antibody was produced using amino acids 163–181 of the N-cadherin, we used PCR to create a construct covering amino acids 163–181 of N-cadherin fused with GST epitope (GST-ΔNcad), which could then be used to block formation of the antibody–antigen complex. After verifying the nucleotide sequences, the PCR fragment was ligated, in-frame, downstream of the GST coding region in pGEX-3X.

β-Catenin and Deleted β-Catenin cDNAs. β-Catenin is the most downstream component of the Wnt-1 signal transduction pathway (Orsulic and Peifer, 1996; Miller and Moon, 1997). Full-length mouse β-catenin cDNA (781 amino acids; Butz et al., 1992) was amplified by RT-PCR using mRNA isolated from mouse hearts. To construct an EGFP (enhanced green fluorescent protein)–fused construct, an antisense PCR primer of the COOH-terminal domain was designed that deleted the endogenous stop codon and replaced it with an SacII site at the 5′ end. When ligated into pEGFP-N3 expression vector (CLONTECH Laboratories, Inc.), the resultant sequence at the COOH terminus of β-catenin contained the EGFP sequence, which was subsequently detected using rabbit antiserum raised against Aequorea Victoria GFP (Molecular Probes).

The β-catenin NH_2-terminal domain contains the GSK-3β phosphorylation sites and 13 imperfect repeats for interaction with adenomatous polyposis coli, LEF/TCF, and cadherin (Orsulic and Peifer, 1996; Miller and Moon, 1997). Deletion of the first 90 amino acids of β-catenin, lacking GSK-3β phosphorylation sites, results in its accumulation and in activation of signal transduction (Yost et al., 1996; Zhu and Watt, 1999). Thus, to construct a constitutively active β-catenin (Δβ-catenin), an NH_2-terminal domain lacking the first 90 amino acids was created by PCR. After verifying the nucleotide sequences, the full-length cDNAs encoding β-catenin or Δβ-catenin were ligated into the pEGFP-N3 mammalian expression vector.

TCF and Mutant TCF cDNAs. TCF (LEF) transcription factors mediate signaling from *Wingless*/Wnt proteins by recruiting β-catenin to serve as a transcriptional coactivator (for review see Nusse, 1997). After amplifying TCF-4 cDNA (447 amino acids; Lee et al., 1999) using RT-PCR with mRNA isolated from mouse hearts, we created a V5-fused TCF construct using the same procedure as described above for Wnt-5a.

TCF contains an NH_2-terminal β-catenin binding domain as well as a centrally located high mobility group domain, which serves as a DNA binding site (Behrens et al., 1996; Roose et al., 1998). Deletion of the β-catenin binding domain of TCF-1 blocks Wnt-dependent axis formation in *Xenopus* embryo (Behrens et al., 1996; Molenaar et al., 1996); therefore, to construct a dominant negative TCF-4 mutant (ΔN-TCF), an NH_2-terminal domain lacking the first 55 amino acids was created. A dominant positive TCF-4 mutant (TA-TCF), in which the NH_2 terminus of TCF-4 (amino acids 1–55) was replaced with the COOH-terminal transactivation domain of β-catenin (amino acids 694–781), was constructed according to previous studies (van de Wetering et al., 1997; Roose et al., 1998; Vleminckx et al., 1999). The chimeric protein, composed of truncated LEF-1 and the transactivation domain of β-catenin, induced secondary axis formation in *Xenopus* embryo in the absence of Wnt (Vleminckx et al., 1999). After verifying the nucleotide sequences, the full-length cDNAs encoding V5-fused or mutant TCF were ligated into pcDNA3.1/V5-HisA expression vector.

Immunoblot Analysis

Cardiac myocytes and fibroblasts were lysed in lysis buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 100 mM sodium orthovanadate, and Protease Inhibitor Cocktail (Boehringer). The lysates were then solubilized in SDS loading buffer, subjected to SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose blots were incubated with primary antibody against the corresponding target proteins. Mouse monoclonal anti–c-Myc antibody was used to detect c-Myc–tagged Wnt proteins; mouse monoclonal anti–E-cadherin (Progen Biotechnik GMBH), goat polyclonal anti–N-cadherin, goat polyclonal anti–M-cadherin, goat polyclonal anti–P-cadherin, rabbit polyclonal anti–α-catenin, goat polyclonal anti–γ-catenin (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti–β-catenin antibodies (Transduction Laboratories), and anti-V5 antibody (Invitrogen) were used to detect the corresponding proteins. The blots were then washed three times with TBS containing 0.1% Tween 20, incubated with peroxidase-labeled, affinity-purified antibody against the primary antibodies (Kirkegaard & Perry Laboratories Inc.), washed again, and developed using an enhanced chemiluminescence (ECL) Western blotting system (Amersham Pharmacia Biotech).

Immunofluorescent Analysis

Cells grown on glass coverslides were fixed for 10 min in 3% paraformaldehyde and then permeabilized for an additional 10 min with 0.1% Triton X-100. After blocking with 5% bovine serum albumin in PBS for 30 min, the cells were incubated for 2 h with primary antibody against the respective target proteins. Mouse monoclonal anti-cardiac myosin heavy chain α/β (MHC) and goat polyclonal anti–human vimentin antibodies (Chemicon International Inc.) were used to identify cardiac myocytes and fibroblasts, respectively. Anti-cardiac MHC antibody–antigen complexes were visualized by incubation for 1 h with FITC-conjugated, affinity-purified anti–mouse IgG (Zymed Laboratories), while anti-vimentin antibody–antigen complexes were visualized using rhodamine-conjugated, affinity-purified anti–goat IgG (Chemicon International Inc.). Mouse monoclonal anti-FLAG antibody was used to localize FLAG-tagged frizzleds; mouse monoclonal anti–c-Myc antibody was used to localize frizzled-GPI chimeras; rabbit anti-GFP antiserum was used to localize β-catenins; anti-V5 antibody was used to localize TCFs; and mouse monoclonal anti-bromodeoxyuridine (BrdU) antibody (Boehringer) to assess BrdU (Sigma-Aldrich) incorporation into DNA. Each of the primary antibody–antigen complexes was visualized using an FITC-conjugated secondary antibody against the corresponding primary antibody. After washing with PBS, cells on coverslides were mounted in Permafluor aqueous mounting medium (Immunotech) and photographed on a (Olympus) Provis AX80 microscope equipped with the appropriate filters.

Luciferase Assays

An oligonucleotide containing three copies of the TCF consensus sequence (CCTTTGATC) or a mutant thereof (CCTTTGGCC), cloned into a minimal HSV-tk promoter-luciferase vector yielded modified tk-TOP and modified tk-FOP according to the previous studies (van de Wetering et al., 1997; Roose et al., 1998; Vleminckx et al., 1999). For transient transfection, cardiac myocytes were cotransfected by electroporation with the various combinations of plasmids: 1.0 μg of TCF4 construct or β-catenin construct in pcDNA3; 0.3 μg of tk-TOP or tk-FOP; and 0.03 μg of pRL-TK vector as a *renilla* luciferase control vector. Luciferase activity was determined using a dual luciferase reporter assay system (Promega). Cells were harvested 24 h after the transfection, and lysed in lysis buffer. The *firefly* luciferase activities of each of tk-TOP and tk-FOP and the *renilla* luciferase activities of pRL-CMV were measured in the same sample by a luminometer. Transfection efficiency of each sample was normalized by the activity of *renilla* luciferase activity.

Pulse–Chase Labeling

Preconfluent cells were starved by incubation in labeling medium that lacked methionine and cysteine for 1 h at 37°C. Cells were then incubated with fresh labeling medium containing 150 μCi/ml ^{35}S-methionine and -cysteine (specific activity >1,180 Ci/mmol; ICN Biomedicals) for 30 min at 37°C. The labeling medium was removed and the cells were washed three times with ice-cold PBS. For the 0 h time point, cells were lysed immediately. For the chase period, normal grow medium supplemented with 5 mM cysteine and methionine (Sigma-Aldrich) was added to the cells. Equal amounts of protein lysates were precipitated with anti–β-catenin antibodies, and separated by 10% SDS-PAGE. The gels were then incubated for 30 min with Amplify (Amersham Pharmacia Biotech), dried, and exposed to Kodak X-OMAT x-ray film (Eastman Kodak Co.) at −80°C.

Generation of Cell Lines Overexpressing Wnt-3a and Wnt-5a

HEK293 cells and C2C12 cells were transfected with the pcDNA3 vector containing either the Wnt-3a or Wnt-5a cDNA using the calcium phosphate precipitation technique; stable cell lines overexpressing either Wnt-3a or Wnt-5a were established by subsequent selection with 800 mg/ml G418 (Sigma-Aldrich). The transfectants were grown for selection in DME containing 400 μg/ml G418. Each selected clone was analyzed by Northern blot and immunoblot analyses.

Generation of Cardiac Myocytes Overexpressing the Frizzled–GPI Chimera

Cardiac myocytes were cotransfected with vector containing CD4 (Clark et al., 1987) and a vector containing one of the frizzled, frizzled–GPI chimera, β-catenin mutant, or TCF mutant cDNAs using electroporation methods with a Gene Pulser Transfection Apparatus (Bio-Rad Laboratories). The molar ratio of CD4 to the cotransfected constructs was optimized to be 1:5. Cells expressing CD4 were isolated by immunomagnetic separation using mouse CD4 Dynabeads (Dynal), and then detached from the beads by using mouse CD4 DETACHaBEAD (Dynal). In brief, 12 h after transfection, transfectants ($\sim 10^7$ cells/ml) were suspended in PBS and incubated for 20 min at 4°C with the Dynabeads at a final concentration of 4×10^7 beads/ml. Cells bound to the beads were collected by magnet and isolated with washing several times with PBS. One unit of DETACHaBEAD, a polyclonal antibody that reacts with the Fab fragments of monoclonal anti-CD4 antibodies, was then incubated with the beads for 45 min at 20°C, which detached the cells from the beads, yielding a population of cells expressing CD4 ($\sim 10^5$ cells/ml). When the localization of the transfected constructs within the isolated cardiac myocytes was assessed by immunofluorescence microscopy, it was found that almost all of isolated $CD4^+$ cardiac myocytes expressed the cotransfected constructs at a ratio of 1:5. Immediately after immunomagnetic separation, the isolated cardiac myocytes were cocultured with fibroblasts with or without Wnt proteins.

Cell Proliferation Assay

Cultured cells were labeled with 20 μM BrdU (Sigma-Aldrich) for 1 or 12 h, and then fixed in paraformaldehyde. After denaturing the DNA in 2 M HCl, cells were neutralized with 0.1 M borate buffer (pH 8.5), and BrdU was detected by using a monoclonal anti-BrdU antibody, followed by an FITC-conjugated anti–mouse IgG.

Expression and Purification of GST-fused Proteins

Control GST and GST-cadherin proteins were expressed in bacteria induced with 0.1 mM IPTG at 37°C. The cells were then pelleted, resuspended in cold PBS with 1% Triton X-100 and lysed by mild sonication. The lysates were centrifuged at 10,000 *g* for 5 min, after which the supernatants were collected and incubated with glutathione Sepharose 4B beads (Amersham Pharmacia Biotech). After extensive washing with PBS, the GST-fused proteins were eluted with 5 mM of glutathione. These GST-fused proteins were used for the antibody-inhibition experiment using cultured cells and affinity binding assay.

Affinity Binding Assay

The GST-cadherin proteins that bound to the glutathione Sepharose 4B beads were extensively washed, first with PBS and then with binding buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 5% BSA, and protease inhibitor cocktail). Approximately 100 μl of GST-cadherin proteins, bound to glutathione beads, were then incubated in a rotating vessel with 100 μg of total cell lysates from cardiac myocytes or fibroblasts for 2 h at 4°C. The beads were then extensively washed with binding buffer. Finally, associated proteins were eluted with SDS sample buffer and subjected to SDS-PAGE. After transfer to nitrocellulose membranes, immunoblot analysis was performed using mouse monoclonal anti–β-catenin IgG. Primary antibody–antigen complexes were visualized using enhanced chemiluminescence assay kit (Amersham Pharmacia Biotech) and peroxidase-conjugated secondary antibodies against mouse IgG antibodies.

Northern Blot Analysis

After total RNA was extracted from cardiac myocytes, the mRNA was isolated using an Oligotex-dT30 mRNA purification kit (Takara), electrophoretically separated in a 1.2% agarose–formaldehyde gel, and then capillary blotted onto nitrocellulose. The blots were then hybridized with ^{32}P-labeled probes and examined by autoradiography.

Analysis of RNA by RT-PCR

RNA was extracted from cardiac myocytes and fibroblasts. To control for DNA contamination, we omitted reverse transcriptase from the synthesis reaction. Transcripts were detected by semiquantitative RT-PCR. The PCR protocol consisted of 25 cycles at 96°C for 30 s, 54–62°C for 15 s, depending on the melting temperature of the primers, and 72°C for 1 min. The specificity of the amplified product was confirmed by Southern blot and hybridization to a corresponding cDNA or internal oligonucleotide probe. Moreover, cDNA used in each PCR reaction was shown to be in the linear range of the signal by generating a dose–response curve carried for each set of primers. The following pairs of primers were designed: 5′-GGC GAT GGC TCC TCT CGG-3′ and 5′-CTT AAG CCT CCT TAC CAG-3′ for Wnt-3a (sequence data available from EMBL/GenBank/DDBJ under accession number X56842); 5′-CTC GCC ATG AAG AAG CCC-3′ and 5′-CAA CCA GTC CCG AGG CAG-3′ for Wnt-5a (sequence data available from EMBL/GenBank/DDBJ under accession number M89798); 5′-ATG CAC CTC GAG GCC CGC-3′ and 5′-GAG GAC GCG GGC CTC GAG-3′ for frizzled-2 (sequence data available from EMBL/GenBank/DDBJ under accession number L02530); 5′-GCT CTG GCC ATG GCC TGG-3′ and ATT GCT TCC CAC GGA GTG-3′ for frizzled-4 (sequence data available from EMBL/GenBank/DDBJ under accession number U43317); 5′-TGT ACA AAG AGA CTG TCT-3′ and 5′-GTT TCT TGA TGA CTG GTA-3′ for TCF-1 (sequence data available from EMBL/GenBank/DDBJ under accession number X61385); 5′-ATG GTG TCC AAG CTC ACG-3′ and 5′-CTT TTG TCT GTC ATG TTT-3′ for TCF-2 (sequence data available from EMBL/GenBank/DDBJ under accession number NM_009330); 5′-CAG CTC GGT GGT GGC CGC-3′ and 5′-GTC AGC GGG TGC ATG TGA-3′ for TCF-3 (sequence data available from EMBL/GenBank/DDBJ under accession number AJ223069); 5′-TGA ACG GCG GTG GAG GAG-3′ and 5′-CGG GGT GAA GTG TTC ATT-3′ for TCF-4 (sequence data available from EMBL/GenBank/DDBJ under accession number AJ223070); 5′-TGT TGG GAA ATA CGG AAC GAA-3′ and 5′-GCT TAA GCT AAT CGC CGA GTG-3′ for Nkx2.5 (sequence data available from EMBL/GenBank/DDBJ under accession number U85046); 5′-GAT GGG ACG GGA CAC TAC CTG-3′ and 5′-GCT GAT TAC GCG GTG ATT ATG-3′ for GATA-4 (sequence data available from EMBL/GenBank/DDBJ under accession number X75415); 5′-CAC GCA TAA TGG ATG AGA GGA ACC GAC and 5′-ACA TCC CAC TTG CAC TGC CGG TAC TTG for MEF-2a (sequence data available from EMBL/GenBank/DDBJ under accession number U30823); 5′-ATG TCT GGA CGT GGC AAG GGT GG-3′ and 5′-CGA ATC CGT AGA GAG TGC GGC CC-3′ for Histone-H4 (sequence data available from EMBL/GenBank/DDBJ under accession number U62672); 5′-TGC AGG AGA TGA TCG ACG AAG and 5′-TGC AGG AGA TGA TCG ACG AAG for Troponin C (accession number M29793); 5′-ATC CAG CTC AGC CAT GCC AAC CGT ATG and 5′-TGG CCT TCT CCT CTG CGT TCC TAC ACT for β-MHC (sequence data available from EMBL/GenBank/DDBJ under accession number M74752); and 5′-AGG CGA GAC AAG GGA GAA CAC GGC ATC and 5′-GCT GTC TCT GGG CCA TTT CCT CCG ACT for BNP (sequence data available from EMBL/GenBank/DDBJ under accession number D16497).

Estimation of the Morphological Effect of Wnt on Cardiac Myocytes

We found Wnt on cardiac myocytes and found that cardiac myocytes cocultured with fibroblasts aggregate in the presence of Wnt proteins. To compare this effect among various experiments, we defined criteria with which to assess aggregation. Cardiac myocytes were considered to be "aggregation-plus" if they formed wide-based, polyp-like structures that were sharply demarcated from other cell masses. On the other hand, myocytes were considered to be "aggregation-minus" if they appeared in a flat monolayer lacking demarcation, even if the cells were sometimes clustered. Whether cells were aggregation-plus or -minus could be assessed readily under the microscope without immunostaining. To quantify our findings, we dissected out the demarcated aggregates, dispersed each aggregate in one well of a 96-well plate, and then stained with anti-cardiac MHC antibody followed by FITC-labeled anti–mouse IgG. Numbers of FITC-positive cells in each well were then counted.

Results

Expression of Wnt and Frizzled Gene Families in Cardiac Myocytes and Fibroblasts

Substantial expression of Wnt-5a and frizzled-2 and -4 is known to take place in mouse hearts (for review see

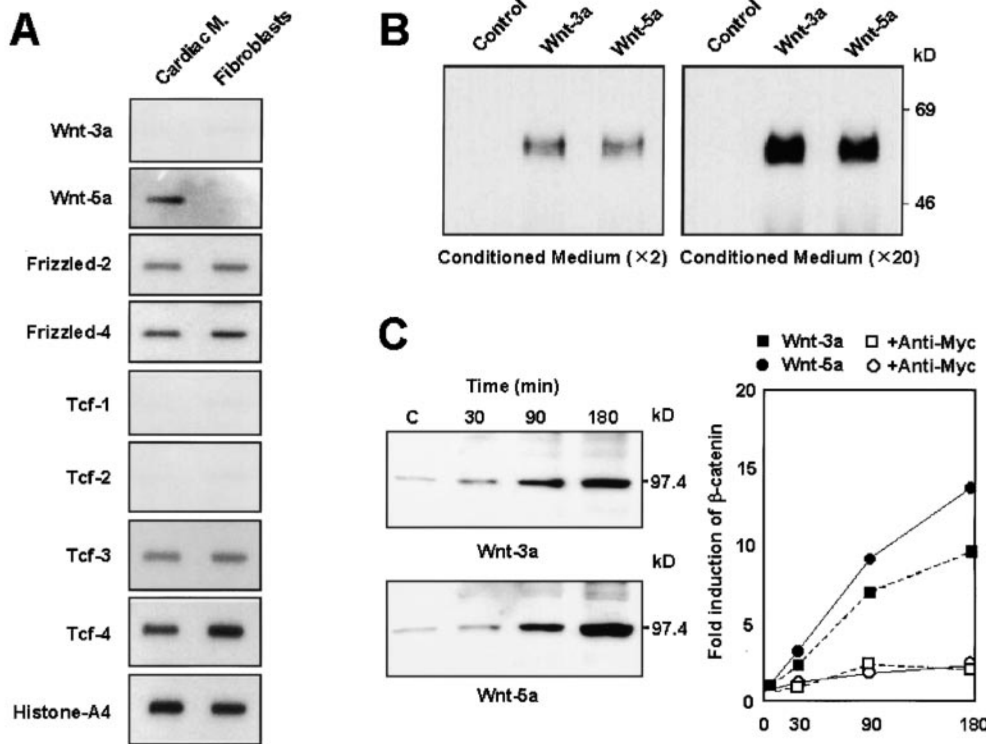


Figure 1. Expression of Wnt, frizzled, and Tcf genes and secretion of biologically active Wnt proteins by HEK293 cells. (A) Transcripts for Wnt-3a and -5a, frizzled-2 and -4, and Tcf-1, -2, -3, and -4 were analyzed by RT-PCR. Samples of total RNA (1 μg) were extracted from cardiac myocytes and fibroblasts, and RT-PCR was performed as described in Materials and Methods. The specificity of the amplified band was confirmed by Southern blot hybridization to corresponding ^{32}P-labeled oligonucleotide probes. Three independent batches of RNA from different experiments gave the same results as shown here. (B) Levels of Wnt-3a and -5a were determined by immunoblotting with anti–c-Myc antibody. Conditioned medium from HEK293 cells transfected with Wnt-3a or -5a were concentrated at 2-fold (left) and 20-fold (right) and were subjected to the SDS-PAGE. (C) Levels of β-catenin were determined with anti–β-catenin antibody. HEK293 cells were incubated with Wnt protein containing medium for time intervals ranging from 30 to 180 min. Collected cells were lysed and total proteins were subjected to SDS-PAGE (left). Time course of β-catenin accumulation was quantified by densitometry (left).

Brown and Moon, 1998), whereas *X*Lef-1 and –3 were identified in *Xenopus* hearts (Molenaar et al., 1998). To determine which cell type expresses these genes, transcription of the Wnt, frizzled, and TCF genes was analyzed by RT-PCR using mRNA from neonatal cardiac myocytes and fibroblasts. Wnt-5a was detected in cardiac myocytes, but not in fibroblasts, whereas frizzled-2 and -4, and Tcf-3 and -4 were detected in both cell types (Fig. 1 A).

Morphological Effects of Wnt-3a and -5a on Cardiac Myocytes

To obtain biologically active Wnt proteins, we established stable cell lines expressing Wnt-3a and -5a in HEK293 and C2C12 cells. After incubating the cells (~2 × 10^7 cells/ml) in the absence of FCS, they were removed by centrifugation at 2,000 *g*. The conditioned medium was collected, cleared of insoluble material by ultracentrifugation (100,000 *g* for 1.5 h), and concentrated 10-fold using a Centriprep 10 column (Amicon). Although there have been reports that Wnt proteins secreted from cultured cells are present in the extracellular matrix (ECM) rather than in the culture medium (Bradley and Brown, 1990; Papkoff and Schryver, 1990), our immunoblot analysis showed c-Myc–tagged Wnt proteins to be in the supernatant (Fig. 1 B). To test whether biologically active Wnt proteins could be detected in the conditioned medium, we incubated HEF293 cells with either Wnt-3a or -5a containing medium (Fig. 1 C). Levels of β-catenin were immediately increased after 30 min incubation of both media, and then reached the maximum levels after 180 min, whereas the presence of anti–c-Myc antibodies in the conditioned medium blocked the accumulation of β-catenin. Therefore, we decided to use conditioned medium in this study, as other investigators have successfully done in the past (van Leeuwen et al., 1994; Bradley and Brown, 1995).

Conditioned medium containing Wnt-3a or -5a was then added to cardiac myocytes plated on poly-L-lysine–coated coverslips in the presence or absence of fibroblasts (Fig. 2). Although application of conditioned medium evoked no morphological changes in either cardiac myocytes or fibroblasts plated alone (data not shown), when myocytes were cocultured with fibroblasts (~10^5 cells/ml each), they began aggregating within 1 d after application, forming ellipsoid bodies (up to 2–3 mm) on top of the fibroblast sheet within 3–4 d. When the effect was quantified by dissecting the demarcated aggregates, dispersing them in respective wells of a 96-well plate, and staining with anti-cardiac MHC antibody followed by FITC-labeled anti–mouse IgG, 50–70 FITC-labeled cells were found in each aggregate (Fig. 3 B). Addition of anti–c-Myc antibody (200 μg/ml) to the conditioned medium blocked the aggregation of cardiac myocytes, confirming that the observed effects were mediated by the tagged Wnt proteins secreted into the conditioned medium. The aggregated myocyte mass beat synchronously at 60–80/min, in contrast to the asyn-

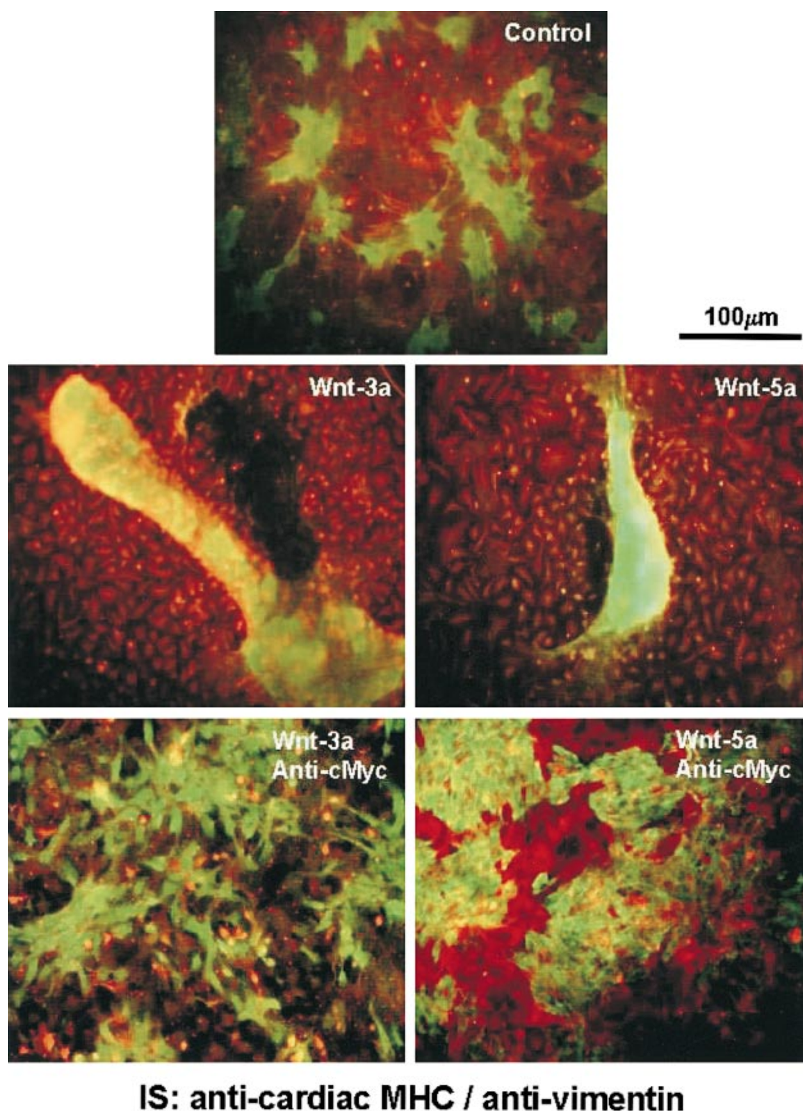


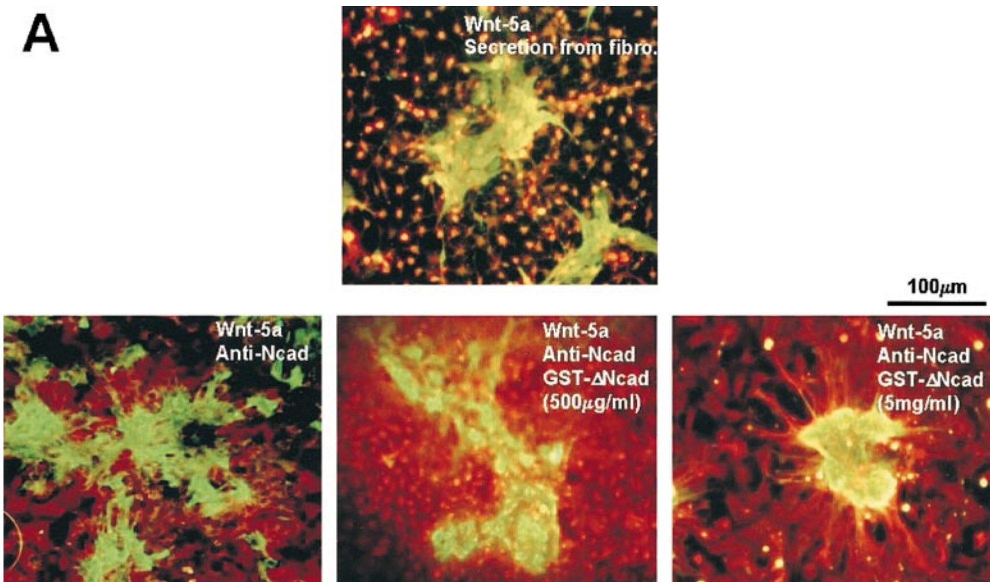
Figure 2. Wnt proteins induced the aggregation of cardiac myocytes in the presence of fibroblasts. Cardiac myocytes and fibroblasts were incubated for 3 d in control medium, conditioned medium containing Wnt-3a or -5a, or conditioned medium containing Wnt protein and anti–c-Myc antibody (200 μg/ml). Cardiac myocytes and fibroblasts were respectively immunostained with mouse anticardiac myosin heavy chain IgG and goat antivimentin IgG, followed by incubation with FITC-conjugated anti–mouse IgG and rhodamine-conjugated anti–goat IgG. Note that myocytes aggregate in the Wnt-3a– and -5a–containing media, although not in the presence of anti–c-Myc antibodies (200 μg/ml).

chronous beating of control cells, which suggests formation of gap junctions between aggregated myocytes.

Role of Fibroblasts in the Morphological Changes Induced by Wnt Proteins in Cardiac Myocytes

Having shown that the presence of fibroblasts promotes Wnt-induced aggregation of cardiac myocytes, we next assessed which factors might be important for this morphological effect. We found that aggregation was unaffected by the material coating the coverslips on which the myocytes were grown. As with poly-L-lysine, cardiac myocytes plated on collagen, laminin, or fibronectin only aggregated when cocultured with fibroblasts (data not shown). Moreover, incubating cardiac myocytes in Wnt-containing medium further conditioned for 12 h by fibroblasts did not induce formation of aggregated masses (Fig. 3 A). Therefore, it appears that direct contact with fibroblasts is key for promoting aggregation of cardiac myocytes under the influence of Wnt proteins.

It has been proposed that cell–cell adhesion mediated by cadherins as well as cell-ECM interactions mediated by integrins are important for morphogenesis during development (for review see Gumbiner, 1996; Radice et al., 1997), and indeed anti-cadherin antibody has been used previously to study cardiac development (Linask and Lash, 1988; Soler and Knudsen, 1994; Linask et al., 1997). Similarly, we added antibodies raised against N-, E-, M-, and P-cadherins and integrin-β to respective samples of Wnt-containing medium and then applied them to 2-d-old cardiac myocyte/fibroblast cocultures. We found that only anti–N-cadherin antibody caused aggregated myocyte masses to revert to clusters of flattened cells; the others had little or no effect on the myocyte clusters (Fig. 3, A and B). The effect of anti–N-cadherin antibody was dose-dependently blocked by the GST-fused extracellular region of N-cadherin (GST-ΔNcad), which presumably competed with endogenous N-cadherin for binding antibody (Fig. 3 A), indicating that formation of cardiac myocyte aggregates is specifically dependent on N-cadherin–mediated cell–cell adhesion, and interactions with ECM were not involved.



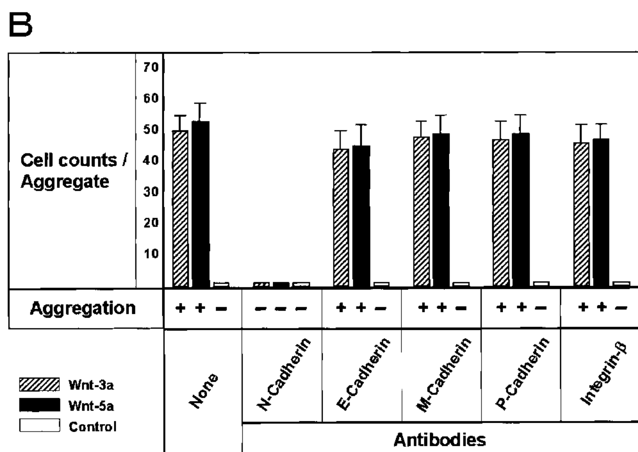


Figure 3. Anti–N-cadherin antibodies inhibited the Wnt-induced aggregation of cardiac myocytes. (A) Representative immunocytochemical staining of cardiac myocytes and fibroblasts incubated for 2 d in Wnt-containing medium, followed by addition of anti–N-cadherin antibodies (100 μg/ml) and incubation for an additional 2 d. Myocytes and fibroblasts were then respectively immunostained as described in Fig. 2. The aggregation of cardiac myocytes was prevented by anti–N-cadherin antibodies, but this effect was blocked by coincubated GST-ΔNcad in a dose-dependent manner. (B) Histogram summarizing the data collected by counting cardiac myocytes in each aggregate. Cardiac myocytes and fibroblasts were incubated in control (white bar), Wnt-3a–containing (hatched bar), or Wnt-5a–containing medium (black bar) for 2 d, followed by addition of the indicated antibodies (100 μg/ml) for 2 d. Judgement of aggregation-plus or -minus in each experiment was performed under microscopy without immunostaining. To count cells in aggregate, each aggregate of cardiac myocytes was excised with a fine glass pipette, dispersed in one well of 96-well plate, and immunostained, and then the FITC-positive cells were counted. 20 aggregates were quantified in three independent experiments. Note that only anti–N-cadherin antibody had an effect on aggregated cardiac myocytes.

Subsequent immunoblot analysis showed that N-cadherin is the predominant cadherin in cardiac myocytes, while E-cadherin predominates in fibroblasts (Fig. 4). Furthermore, incubation of cardiac myocytes and fibroblasts in Wnt-containing medium for 2 d induced distinct patterns of cadherin expression in the two cell types: protein level of N-cadherin was increased in cardiac myocytes, whereas those of E- and M-cadherin were increased in fibroblasts (Fig. 4 A). Densitometric estimation of three independent experiments indicated a 2.0 ± 0.4-fold increase in N-cadherin levels of cardiac myocytes, and 2.2 ± 0.4-fold and 5.9 ± 1.0-fold increase in E- and M-cadherin levels of fibroblasts, respectively. Nevertheless, Northern blots showed that levels of mRNA encoding these cadherins were not affected by incubation with Wnt (Fig. 4 B), suggesting that Wnt increases the stability of cadherins in both cell types without inducing gene transcription, which is consistent with the effect of Wnt-1 on mammalian cell lines (Hinck et al., 1994). Since cell–cell adhesion is established by homophilic binding between cadherins, the increased levels of distinct sets of cadherin in cardiac myocytes and fibroblasts leads to the strengthening of interaction of cardiac myocytes with one another.

Intracellular Signal Transduction of Wnt Proteins in Cardiac Myocytes

Frizzled-2 and -4 are the predominant frizzled genes expressed in both cardiac myocytes and fibroblasts. To test whether a particular frizzled protein functions as an endogenous Wnt-5a receptor, we created dominant-negative constructs of frizzled-2 and -4. Because we were unable to generate stable cultures of cardiac myocytes overexpressing a frizzled–GPI chimera, a transient expression strategy was used. Cardiac myocytes were cotransfected with vectors containing CD4 or frizzled–GPI chimera at a molar ratio of 1:5. At this ratio, 20–30% of live cardiac cells expressed CD4. After immunomagnetic isolation of the $CD4^+$ cells, it was determined that virtually all $CD4^+$ cells also expressed the frizzled or frizzled–GPI chimera at the cell surface (Fig. 5 A).

As exogenously introduced frizzleds and frizzled–GPI

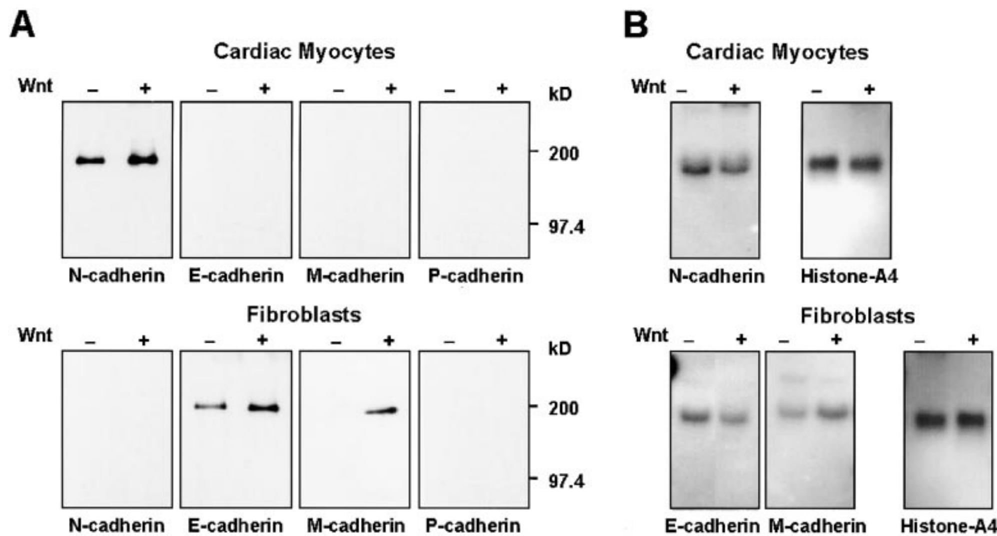


Figure 4. Wnt proteins increased protein levels of cadherins without changes in mRNA levels. Total cell protein and mRNA were extracted from cardiac myocytes and fibroblasts incubated in control or in Wnt-containing medium. (A) Levels of N-, E-, M-, and P-cadherins were determined by immunoblotting equivalent amounts of total cell protein with the specific antibody against each cadherin. Molecular mass standards are shown on the right. (B) Levels of N-, E-, M-, and P-cadherin mRNAs were determined by Northern blot using equivalent amounts of mRNA with corresponding ^{32}P-labeled oligonucleotide probes.

chimeras compete with the extracellular CRD of endogenous frizzleds for the binding of Wnt, increased frizzled expression might be expected to facilitate association of Wnt with cell surfaces. Wnt-5a–V5 cDNA was transiently transfected into cardiac myocytes overexpressing either frizzleds or frizzled-GPI chimeras, and the localization of expressed Wnt proteins then was detected by immunostaining. Wnt proteins were detected at the surface and in the nucleus of cells overexpressing frizzled-2 or frizzled-2–GPI chimera, whereas Wnt proteins were detected mainly in the nucleus of cells overexpressing frizzled-4 or frizzled-4–GPI chimera (Fig. 5 B, top). This result suggested that the signals at the cell surface of cells expressing frizzled-2 and frizzled-2–GPI chimera represent the specific binding of Wnt-5a–V5 proteins to the extracellular CRD of frizzled-2. To clarify this notion, we used antibody against secreted Wnt-5a-V5 to inhibit its binding to the cell surface receptors. As shown in Fig. 5 B, bottom, incubation of anti-V5 antibody eliminated Wnt proteins localized at the surface of cells overexpressing frizzled-2 or frizzled-2–GPI chimera, although incubation with anti-V5 antibody did not affect the Wnt proteins in the nucleus in all types of cells. This result indicated that overexpression of frizzled-2 or frizzled-2–GPI chimera, but not frizzled-4 or frizzled-4–GPI, stabilized Wnt-5a at, or recruited it to, the cell surface. Since endogenous frizzled-2 should also bind Wnt-5a, differences in Wnt-5a signaling between cells expressing frizzled-2–GPI and frizzled-4–GPI should correspond to the differences in bound Wnt-5a between cells expressing only endogenous frizzled-2 and those expressing endogenous frizzled-2 plus frizzled-2–GPI. Thus, the frizzled-2–GPI chimera lacking the cytoplasmic domain, which is essential for the Wnt signal transduction, may function as a dominant-negative construct.

When the isolated transfectants were cocultured with fibroblasts in Wnt-5a–containing medium, cardiac myocytes expressing the frizzled-2–GPI chimera failed to aggregate, in contrast to myocytes expressing the frizzled-4–GPI chimera (Fig. 5 C). The cell counts were 20–30 per aggregate among cultures of transfectants subjected to immunomagnetic isolation in the Wnt-containing medium (Fig. 6), which was only about half of that among cultures of transfectants not subjected to immunomagnetic isolation (Fig. 3 B). Although the reason for this difference is unclear, we speculate that immunomagnetic isolation may disturb the mobility of the transfected cells. Therefore, we compared cell counts per aggregate among groups subjected to the same experimental protocol and found that in Wnt-5a– or Wnt-3a–containing medium, overexpression of frizzled-2–GPI inhibited aggregation, as compared to cultures only expressing endogenous frizzleds (Fig. 6). Thus, interaction between Wnt-5a/Wnt-3a and frizzled-2 appears crucial for the morphological effect of Wnt-5a on cardiac myocytes.

Given that cell–cell adhesion mediated by N-cadherin plays a key role in the morphological effect of Wnt, interaction of cadherin and β-catenin or plakoglobin (γ-catenin) should be a component of this effect. Immunoblot analysis showed that β-catenin is the predominant catenin in cardiac myocytes and fibroblasts. Furthermore, incubating cardiac myocytes and fibroblasts in Wnt-containing medium for 2 d increased protein levels of β-catenin in both cell types, and levels of γ-catenin were slightly increased (Fig. 7 A). Since β-catenin and γ-catenin mRNA levels were unaffected by Wnt proteins (Fig. 7 B), the observed increases in β- and γ-catenin should have been the product of increased protein stability, rather than increased protein synthesis. Incubation of cardiac myocytes with Wnt proteins increased levels of β-catenin complexed with cadherin, which was detected by immunoprecipitation using anti–N-cadherin antibody, and increased levels of free β-catenin, which was detected by affinity binding assay using the cytoplasmic β-catenin binding region of N-cadherin fused to GST (Fig. 7 C). Incubating fibroblasts with Wnt proteins yielded similar results (Fig. 7, A, B, and

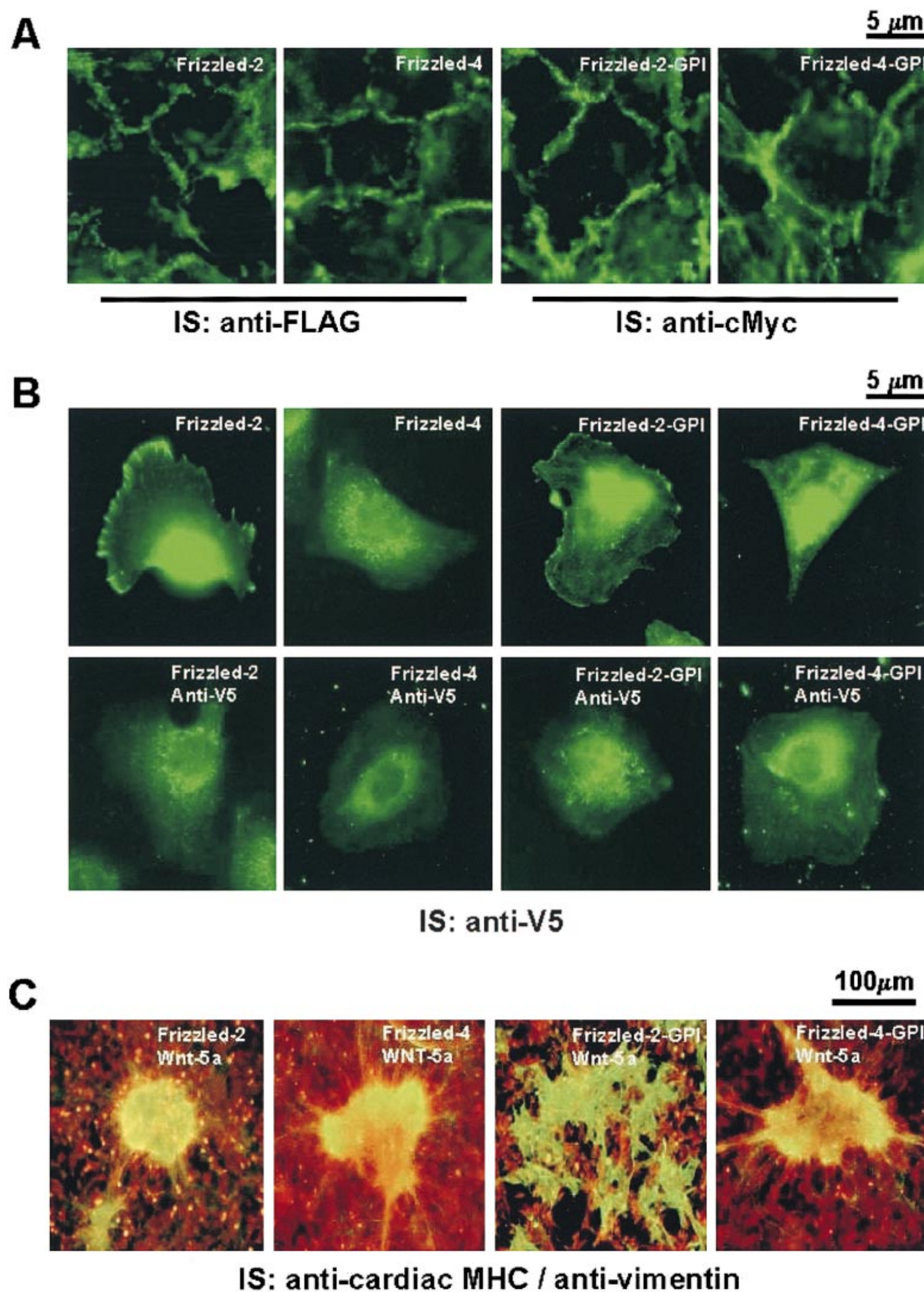


Figure 5. Frizzled-2 functioned as a Wnt receptor in the process of cardiac myocytes aggregation. Cardiac myocytes were cotransfected with vector containing CD4 and vector containing frizzled-2 or -4, or frizzled-2– or -4–GPI chimeras as described in Materials and Methods. (A) Cells expressing CD4 were isolated, after which the transfectants were subjected to immunostaining. Frizzled-2 and -4 and frizzled-2– and -4–GPI chimeras were detected with anti-FLAG and anti–c-Myc antibodies, respectively, followed by labeling with FITC-conjugated anti–mouse IgG. Frizzled-2 and -4 and frizzled-2– and -4–GPI chimera proteins were localized at the cell membranes of transfected cells. (B) Cells expressing frizzled-2 and -4 or frizzled-2– and -4–GPI chimera were transiently transfected with Wnt-5a–V5 construct. Wnt-5a was then detected using an anti-V5 antibody, followed by labeling with FITC-conjugated anti–mouse IgG. (Top) Wnt-5a was detected at the cell surfaces and the nuclei of cells expressing frizzled-2 and frizzled-2–GPI chimera, whereas Wnt-5a was only detected in the nuclei of cells expressing frizzled-4 and frizzled-4–GPI chimera. (Bottom) Incubation of anti-V5 antibody eliminated the signals at the cell surfaces of cells expressing frizzled-2 and frizzled-2–GPI, but did not affect the signals in the nucleus of all types of cells. (C) Immunocytochemical staining of cardiac myocytes cultured in Wnt-5a–containing medium for 3 d in the presence of fibroblasts. In contrast to other cell types, cells expressing frizzled-2–GPI chimera failed to aggregate.

C). The finding that levels of both free N-cadherin and N-cadherin–complexed β-catenin were increased by Wnt proteins confirmed the idea that Wnt proteins strengthen cell–cell adhesion by increasing formation of cadherin–catenin complexes at those sites (Bradley et al., 1993; Hinck et al., 1994).

We also examined the effect of introducing a constitutively active form of β-catenin (Δβ-catenin) into cardiac myocytes. The NH_2 terminus of β-catenin contains several GSK-3β phosphorylation sites, which facilitate the rapid degradation of β-catenin (Rubinfeld et al., 1996; Yost et al., 1996). Wild-type β-catenin has a very short half-life and

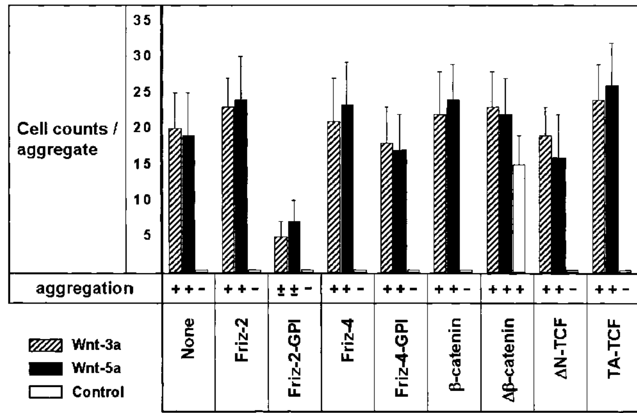


Figure 6. Histogram summarizing the data collected by counting cardiac myocytes in each aggregate. Isolated cardiac myocytes and fibroblasts were incubated in control medium (white bar), Wnt-3a–containing medium (hatched bar), or Wnt-5a–containing medium (black bar) for 3 d. Judgement of aggregation-plus or -minus in each experiment was performed under microscopy without immunostaining. To count cells in aggregate, each aggregate of cardiac myocytes was excised with a fine glass pipette, dispersed in one well of a 96-well plate, and immunostained. Numbers of FITC-positive cells were counted in each well. 20 aggregates were quantified in three independent experiments.

deletion or mutations of the GSK-3β phosphorylation sites at the NH_2 terminus results in accumulation of stable β-catenin in the cytoplasm (Gat et al., 1998; Zhu and Watt, 1999). To determine the half-life of β-catenin and Δβ-catenin in the transfected cells, transfected cells were starved for 1 h, pulse-labeled with [^{35}S]cysteine and [^{35}S]methionine for 30 min, and chased in radioactive-free medium for up to 4 h (Fig. 8 C). As predicted, endogenous and transfected β-catenin had a half-life of less than 1 h, whereas Δβ-catenin was stable throughout the time period examined. Cardiac myocytes were cotransfected with CD4 and β-catenin constructs and then immunomagnetically isolated. β-Catenin and Δβ-catenin were mainly localized at cell–cell interfaces and in a few nuclei (Fig. 8 A), indicating that both proteins are involved in the formation of cadherin–catenin complexes. Overexpression of Δβ-catenin in cardiac myocytes induced their aggregation, even in the absence of Wnt proteins (Fig. 8 B); the cell counts were 15 ± 3 per aggregate, which is significantly higher than in the absence of Wnt (Fig. 6).

Δβ-Catenin lacks the first 90 amino acids but should nonetheless bind to both N-cadherin and α-catenin (Orsulic and Peifer, 1996). To confirm that Δβ-catenin participates in the formation of cadherin–catenin complexes, lysates of cardiac myocytes expressing β-catenin or Δβ-

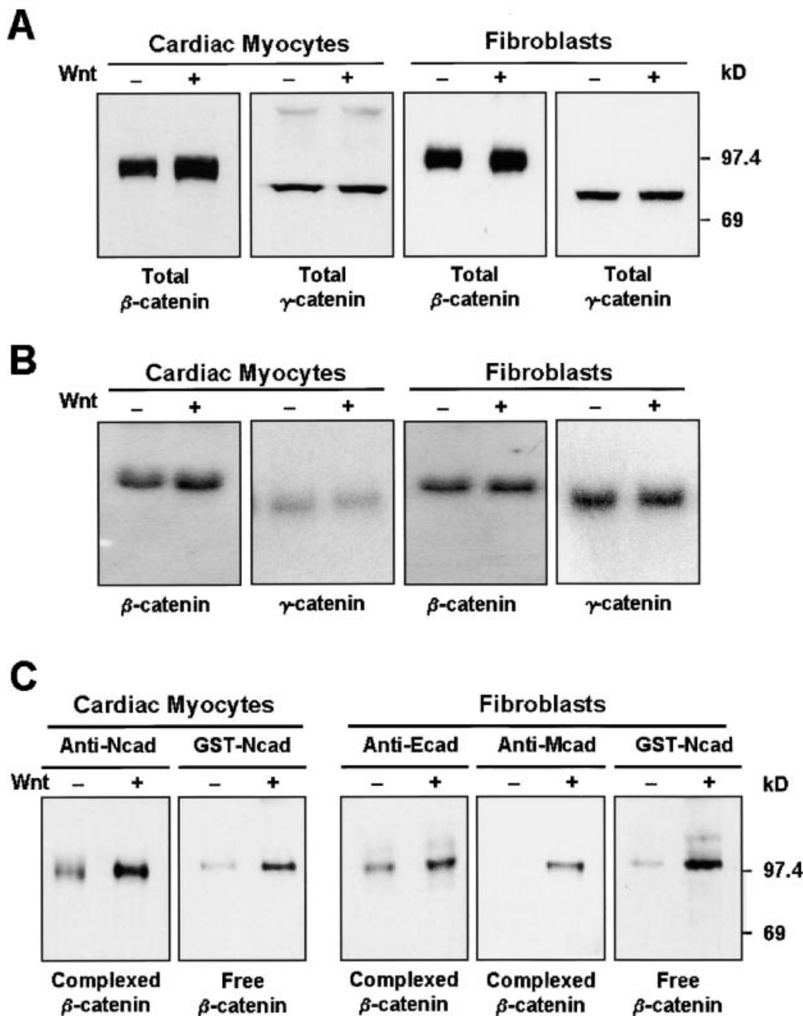


Figure 7. Wnt proteins increased β-catenin levels in cardiac myocytes and fibroblasts. (A) Total cell protein was extracted from cardiac myocytes and fibroblasts incubated in control or Wnt-containing medium. Levels of β- and γ-catenin were determined by immunoblotting equivalent amounts of total cell protein with anti–β-catenin and –γ-catenin antibodies, respectively. (B) Levels of β- and γ-catenin mRNA were determined by Northern blot using equivalent amounts of mRNA with corresponding ^{32}P-labeled oligonucleotide probes. (C) For determination of β-catenin–cadherin complex, equivalent amounts of total cell protein were immunoprecipitated with antibody against N- or E-cadherin; the precipitate was subjected to SDS-PAGE, followed by immunoblotting with anti–β-catenin antibody. For determination of free β-catenin, equivalent amounts of total cell protein were affinity precipitated using a GST-fusion protein containing the cytoplasmic domain of N-cadherin; the precipitate was subjected to SDS-PAGE, followed by immunoblotting with anti–β-catenin antibody. Molecular mass standards are shown on the right.

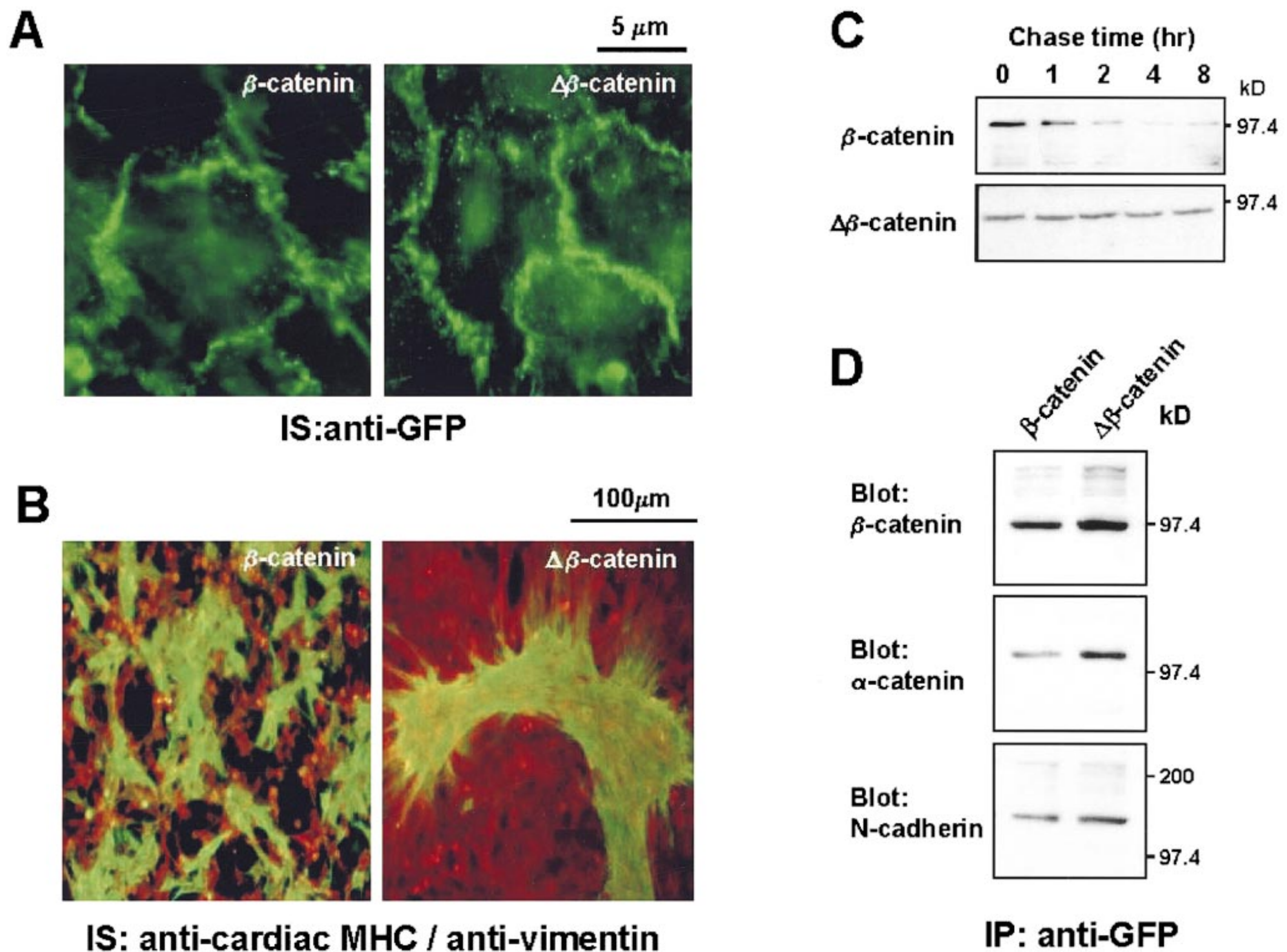


Figure 8. Δβ-Catenin–induced cardiac myocyte aggregation is independent of Wnt signaling. Cardiac myocytes were cotransfected with vector containing CD4 and vector containing either the β-catenin or Δβ-catenin as described in Materials and Methods. (A) Cells expressing CD4 were isolated, after which the transfectants were subjected to immunostaining. The β-catenin and Δβ-catenin were detected with anti-GFP antibody, followed by labeling with FITC-conjugated anti–mouse IgG. β-Catenin and Δβ-catenin were localized mainly to the cell membrane, although a few were seen in the nucleus. (B) Immunocytochemical staining of cardiac myocytes cultured in Wnt-5a–containing medium for 3 d in the presence of fibroblasts. Cells expressing Δβ-catenin aggregated even in the absence of Wnt proteins. (C) Transfected cells were starved, pulse-labeled with [^{35}S]cysteine and [^{35}S]methionine for 30 min, chased in nonradioactive medium for the number of hours shown, and immunoprecipitated with anti–β-catenin antibodies. (D) Lysates of cells expressing β-catenin or Δβ-catenin were immunoprecipitated with anti-GFP antibody, and the associated proteins were subjected to immunoblot using antibodies against β-catenin, α-catenin, or N-cadherin.

catenin were immunoprecipitated with anti-GFP antibodies, and the associated proteins were immunoblotted with antibodies against β-catenin, N-cadherin, or α-catenin. We found that in cells expressing Δβ-catenin, association of N-cadherin and α-catenin was greater than in cells expressing β-catenin (Fig. 8 D). Densitometric estimation of three independent experiments indicated a 2.2 ± 0.3-fold increase in N-cadherin and 1.8 ± 0.4-fold increase in α-catenin. These results suggested that stabilization of β-catenin promotes myocyte aggregation by increasing formation of N-cadherin–catenin complexes.

Although increased free β-catenin can contribute to increased stability of the cadherin/catenin complex (Hinck et al., 1994), when associated with TCF free β-catenin can also serve a signal transduction function in response to Wnt (for review see Nusse, 1997). To test the possibility that the morphological effects of Wnt require TCF target gene expression, dominant negative and dominant positive TCF mutants were constructed. The transactivation activity of TCF constructs were analyzed by measuring luciferase activity of the tk-TOP or tk-FOP reporter plasmids cotransfected into cardiac myocytes (Fig. 9). Luciferase expression of tk-TOP is driven by three copies of a consensus TCF motif and a minimal HSV-tk promoter, while tk-FOP contains mutated, non-functional TCF motif. Upon overexpression of TCF-4, tk-TOP activity increased by ~2.8-fold, probably because TCF-4 could recruit endogenous β-catenin. In contrast, no stimulation was seen in ΔN-TCF, confirming the previous result that the NH_2-terminal domain is essential for the transactivation of TCF (Behrens et al., 1996; Roose et al., 1998). Overexpression of TA-TCF resulted in a 17.5-fold stim-

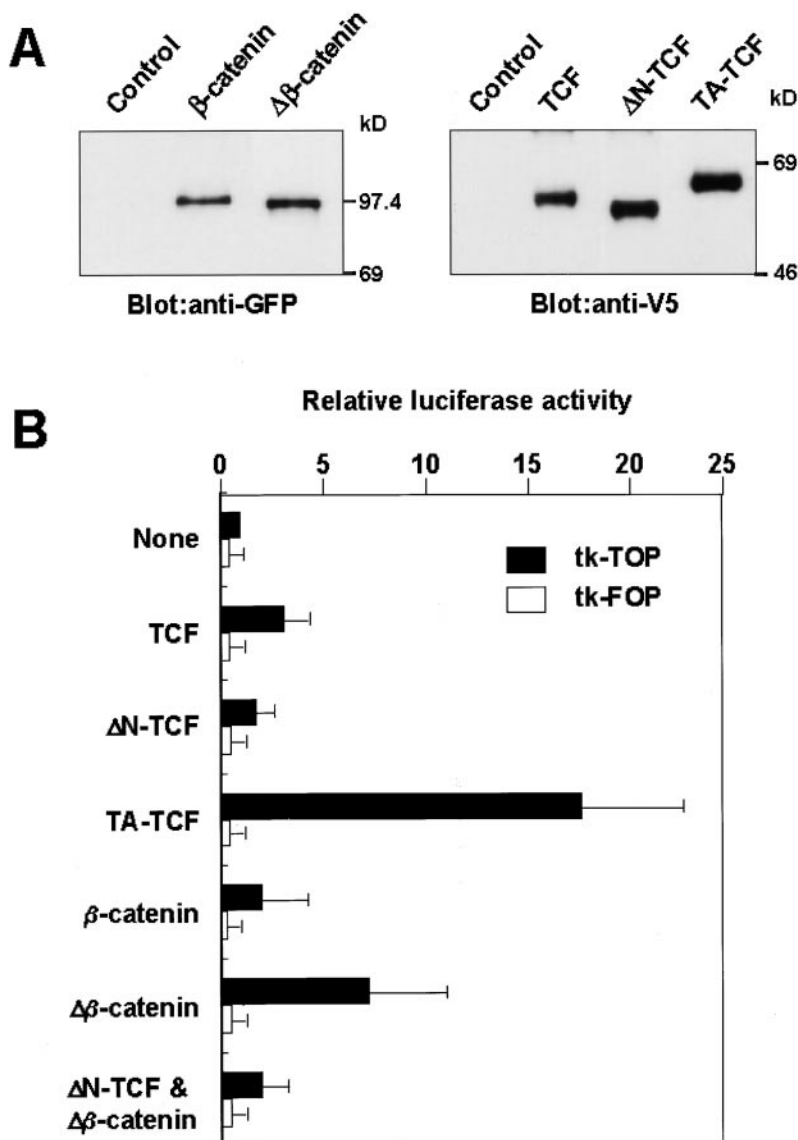


Figure 9. TCF and β-catenin function as sequence-specific transcriptional activators in cardiac myocytes. (A) Levels of β-catenin and TCF constructs expressed in cardiac myocytes were detected by immunoblotting with anti-GFP and anti-V5 antibodies, respectively. (B) Reporter gene activation in cardiac myocytes transfected with either TCF or β-catenin constructs and either tk-TOP or tk-FOP plasmids were gained as relative luciferase activities.

ulation of luciferase activity, indicating TA-TCF as a dominant positive construct. None of the various TCF constructs activated the tk-FOP activity. In comparison, overexpression of β-catenin increased tk-TOP activity by 2.3-fold, whereas overexpression of Δβ-catenin increased by 7-fold, indicating that overexpressed β-catenin and Δβ-catenin recruit endogenous TCF to activate the tk-TOP activity; the differences of activation levels may correspond to the differences of stability between β-catenin and Δβ-catenin as shown in Fig. 8 B. When the cardiac myocytes were co-transfected with ΔN-TCF and Δβ-catenin at the molar ratio of 5:1, ΔN-TCF suppressed the Δβ-catenin–induced activation of the tk-TOP activity from 7-fold to 2.5-fold. Since ΔN-TCF retains the DNA-binding high mobility group domain, this result indicated that ΔN-TCF functions as a dominant negative construct to compete with endogenous TCF for the TCF-binding motif of tk-TOP gene.

The transfected TCF proteins in the cardiac myocytes were detected in the nuclei of the transfectants (Fig. 10 A). Introduction of ΔN-TCF did not inhibit aggregation of cardiac myocytes in Wnt-containing medium, and expression of TA-TCF did not induce their aggregation in the absence of Wnt (Fig. 10 B). Although it is not clear whether TA-TCF functions as the dominant-positive construct on all target genes in cardiac myocytes, when considered together with the findings that incubation with Wnt had no effect on transcription of cadherin or β-catenin mRNA in cardiac myocytes or fibroblasts, it is plausible that TCF target gene expression may be not involved in the morphological effect of Wnt on cardiac myocytes.

Effects of Wnt Proteins on the Proliferation and Differentiation of Cardiac Myocytes

By analyzing BrdU incorporation, we also examined whether Wnt affects the proliferation or differentiation of cardiac myocytes or fibroblasts by regulating gene expression. Whether cardiac myocytes were incubated with BrdU for 1 or 12 h, no nuclear staining was observed, with or without Wnt proteins (Fig. 11 A). In contrast, numbers of BrdU-labeled nuclei increased time-dependently in fi-

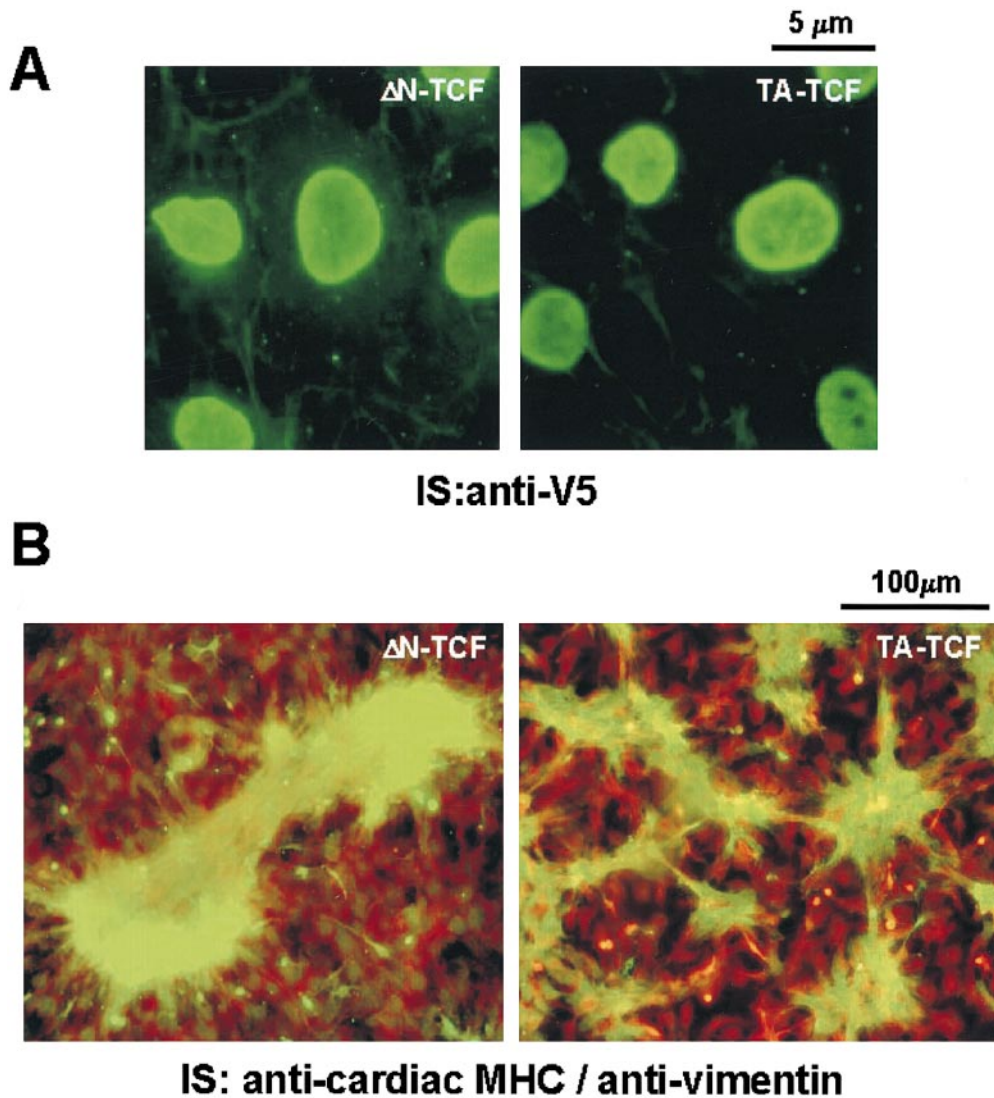


Figure 10. TCF is not involved in cardiac myocyte aggregation. Cardiac myocytes were cotransfected with vector containing CD4 and vector containing either ΔN-TCF or TA-TCF as described in Materials and Methods. (A) Cells expressing CD4 were isolated, after which the transfectants were subjected to immunostaining. ΔN-TCF and TA-TCF were detected using anti-V5 antibody, followed by labeling with FITC-conjugated anti–mouse IgG, and were localized primarily in the nuclei. (B) Immunocytochemical staining of cardiac myocytes cultured in Wnt-5a–containing medium for 3 d in the presence of fibroblasts. Cells expressing dominant-negative ΔN-TCF aggregated in Wnt-containing medium, whereas cells expressing dominant-positive TA-TCF failed to aggregate in the absence of Wnt proteins.

broblasts, although Wnt had no effect on the number of BrdU-labeled nuclei (Fig. 11 B), indicating that Wnt did not induce cell proliferation.

To test whether Wnt induces differentiation of so-called precardiac cells in the neonatal heart, semiquantitative RT-PCR of selected cardio-specific genes was performed. In contrast to other experiments in which a discontinuous Percoll gradient was used to purify cardiac myocytes from other cells types, we separated cells according to their ability to adhere to culture plates, hoping to retain precardiac cells, if any were present, in one or the other cell pool. Eventually, the nonadherent pool contained mainly cardiac myocytes, and the adherent pool contained mainly fibroblasts. Semiquantitative RT-PCR of the gene transcripts expressed early in cardiogenesis, including mRNAs encoding GATA-4 (Heikinheimo et al., 1994), Nkx2.5 (Lints et al., 1993) and MEF-2C (Lints et al., 1993; Edmondson et al., 1994) showed that, although all three markers were constitutively expressed in the cardiac myocyte-rich pool, none were affected by Wnt proteins (Fig. 12). A trace amount of GATA-4 mRNA was detected in the fibroblast-rich pool, but it, too, was unaffected by Wnt proteins. Similarly, terminal differentiation markers, including transcripts for contractile proteins cTpC and β-MHC and for the peptide hormone, BNP, were exclusively expressed in the cardiac myocytes–rich pool and were unaffected by Wnt proteins (Fig 12). Although we cannot rule out the possibility that small differences between gene transcripts could be masked by RT-PCR, Wnts probably have little if any effect on the transcriptional regulation of cardio-specific genes in cells derived from fetal hearts.

Discussion

Heart development in vertebrates and *Drosophila* is initiated by bilaterally symmetrical primordia that may be of equivalent embryological origin: the anterior lateral plate mesoderm in vertebrates and the dorsal mesoderm in arthropods. These mesodermal progenitors then merge, forming a heart tube at the ventral midline in the case of the former or in the dorsal midline in the case of the latter. In *Drosophila*, Wg is known primarily for its ectoderm patterning function (for review see Nusse and Varmus, 1992; Perrimon, 1994) and for specifying neuroblast iden-

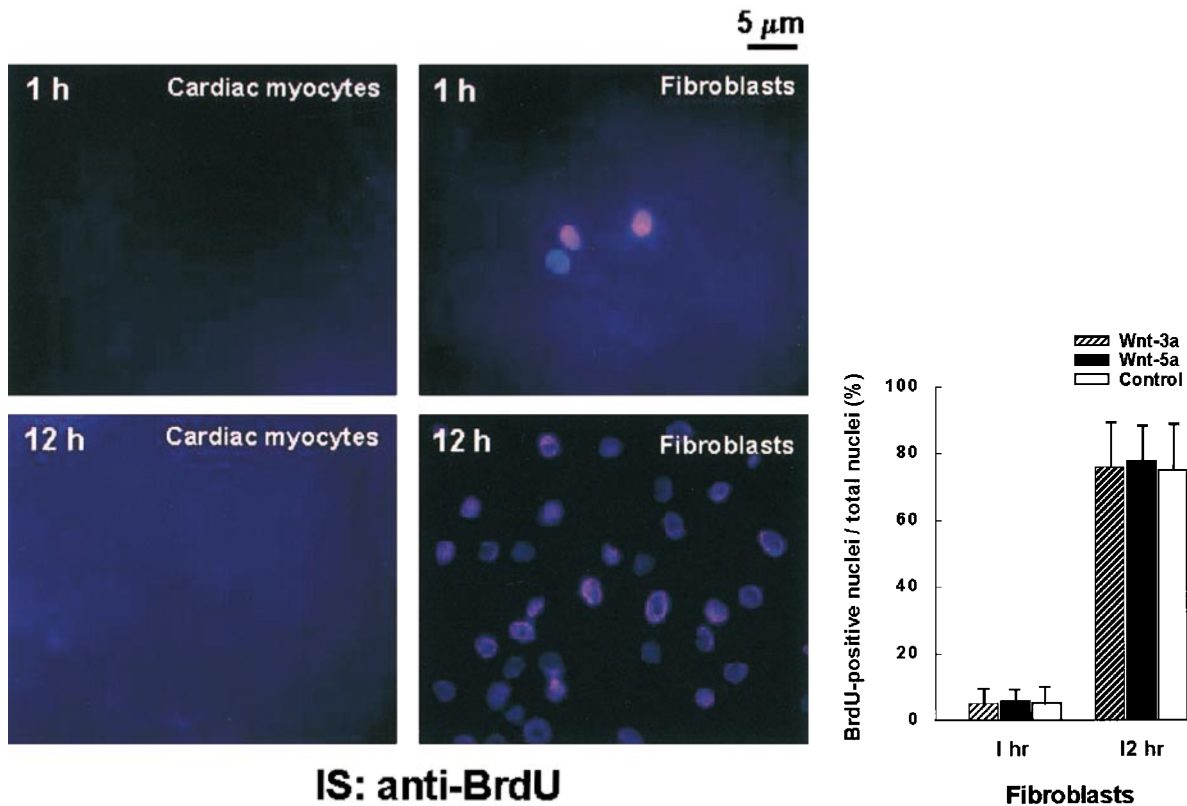


Figure 11. BrdU incorporation into cardiac myocytes and fibroblasts was unaffected by Wnt proteins. (A) Representative immunocytochemical staining of cardiac myocytes and fibroblasts incubated in Wnt-5a–containing medium for 3 d and then with 20 μM BrdU in the same medium for either 1 or 12 h. BrdU-labeled cells were immunostained with anti-BrdU IgG, followed by FITC-conjugated anti–mouse IgG. (B) Histogram showing the frequency of BrdU-labeled nuclei in fibroblasts incubated in control (white bar), Wnt-3a–containing (hatched bar), or Wnt-5a–containing (black bar) medium for either 1 or 12 h.

tity (Chu-LaGraff and Doe, 1993). In addition, at the stage that dorsal mesoderm is subdivided into somatic, visceral, and cardiac mesoderm, Wg is required for induction of cardiac, but not visceral, mesoderm (Wu et al., 1995; Park et al., 1996, 1998). The product of *decapentaplegic* (dpp), a member of the TGF-β superfamily secreted from ectoderm, was also found to induce visceral and cardiac mesoderm (Staehling-Hampton et al., 1994; Frasch, 1995). The concerted actions of Wg and dpp thus appear to be required for determination of cardiac cell fate in *Drosophila.* On the other hand, no vertebrate Wnt genes involved in cardiogenesis have yet been found, even though some have been detected in the developing mammalian heart (Monkley et al., 1996). Therefore, we hypothesize that in mammalian hearts the central role of Wnt may be to exert a morphological rather than a cardiogenetic effect.

We observed that rat cardiac myocytes secrete Wnt-5a, which promotes their aggregation in the presence of fibroblasts. Using dominant negative constructs of frizzled-2 and -4, we further showed that the receptor mediating the morphological effects of Wnt-3a and -5a was frizzled-2. There is ample evidence that the biological effects of Wnt can be mediated by coexpressed frizzled functioning as the Wnt receptor. For instance, in *Xenopus* before midblastula transition, overexpression of Wnt-1, -3a, -8, and -8b induces axis duplication by stabilizing β-catenin (Moon et al., 1993; Cui et al., 1995; Du et al., 1995), whereas overexpression of Wnt-5a does not, and may even antagonize induction of axis duplication by Wnt-1 (Torres et al., 1996). But when human frizzled-5 is coexpressed, it serves as a receptor for *X*wnt-5, mediating axis duplication (He et al., 1997). After midblastula transition, the same overexpression of Wnt-5a stabilizes β-catenin (Larabell et al., 1997), which might be explained by the presence of newly synthesized Wnt receptors allowing Wnt-5a to activate an intracellular pathway that stabilizes β-catenin. Moreover, in *zebrafish* embryos, frizzled-2 causes an increase in the release of intracellular Ca^{2+} which is enhanced by *X*wnt-5a (Slusarski et al., 1997). Thus, Wnt-5a and other Wnts may couple to multiple frizzleds that may in turn signal along multiple pathways (for review see Miller et al., 1999).

The biological effects of *Wg* are also regionally regulated by the distribution and level of frizzled expression in *Drosophila* (Cadigan et al., 1998), which limits the effective range of diffusion of secreted *Wg* proteins to, at most, 20 cell diameters from their secretion site (Zecca et al., 1996). Regarding the functional role of the Wnt/frizzled signaling pathway in mammalian heart, it is noteworthy that frizzled-2 is induced in infarcted and hypertrophied hearts in rodents (Blankesteijn et al., 1996, 1997). In that

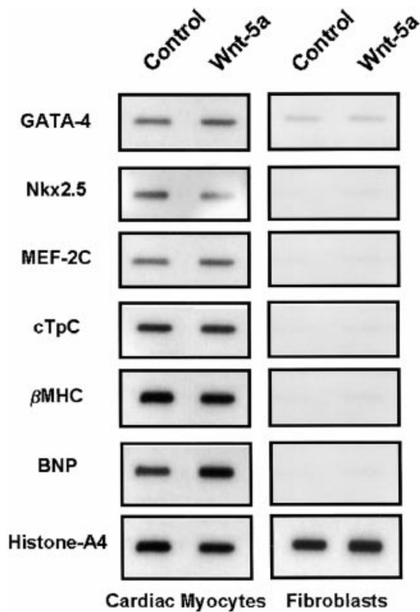


Figure 12. Wnt proteins did not change transcripts for markers of early cardiogenesis and terminal differentiation by using semiquantitative RT-PCR. Samples of total RNA (1 μg) were extracted from cardiac myocytes incubated with control or W5a-containing medium, and RT-PCR was performed as described in Materials and Methods. In all cases, the specificity of the amplified band was confirmed by Southern blot hybridization to corresponding ^{32}P-labeled oligonucleotide probes. Three independent batches of RNA from different experiments gave the same results as shown here.

context, our findings suggest that Wnt-5a may play a role in remodeling injured hearts through binding to increased frizzled-2.

The interaction between catenins and cadherin is known to be crucial for the formation and strength of Ca^{2+}-dependent, cell–cell adhesions (for review see Kemler, 1992; Nagafuchi et al., 1993). β-Catenin is a component of adhesion junctions and has been shown to physically associate with the cadherin cytoplasmic domain. We observed that modulation of β-catenin by Wnt-3a and -5a was in concert with similar changes in the abundance of cadherin in both cardiac myocytes and fibroblasts. Increases in complexed β-catenin, immunoprecipitated from these cells using antibodies against N- and E-cadherin, confirmed that β-catenin and cadherin colocalize at cell–cell interfaces; that is, the adhesion between cells was cadherin-based. Consistent with that conclusion, anti–N-cadherin antibodies prevented the morphological effects of Wnt proteins. In earlier studies using suspensions of PC-12, C57MG, and AtT20 mammalian cell lines, overexpression of Wnt-1 induced Ca^{2+}-dependent reaggregation in parallel with increased expression of cadherins and β-catenin (Bradley et al., 1993; Hinck et al., 1994). Neither study, nor ours, showed an increase in mRNA of cadherin nor β-catenin, indicating the posttranscriptional stability of two proteins by Wnts. Thus, cell–cell adhesion between cardiac myocytes is apparently strengthened by Wnt-evoked stabilization of cadherin–β-catenin complexes, thereby inducing aggregation of cardiac myocytes. This notion is supported by the finding that overexpression of constitutively active β-catenin promoted aggregation of cardiac myocytes, even without Wnt.

Catenins also function in cadherin-independent signaling pathways that regulate differentiation and cell proliferation (for review see Cadigan and Nusse, 1997). Free β-catenin is known to interact with TCF-1/LEF1 transcription factors and to activate target genes (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). For example, Wnt-1 increases levels of homeobox genes such as mouse *engrailed-1*, which is important for vertebrate neural development (Danielian and McMahon, 1996). Furthermore, injection of TCF mutants into *Xenopus* embryo blocks both endogenous axis formation and the ability of ectopic β-catenin to induce a secondary axis formation (Molenaar et al., 1996). By contrast, in this study, overexpression of neither a dominant-negative nor a dominant-positive TCF mutant affected aggregation, indicating that the β-catenin/TCF gene activation pathway has little or no impact on Wnt-induced stabilization of the cadherin–β-catenin complex.

We also examined whether Wnt proteins might induce proliferation and/or differentiation via a β-catenin/TCF gene activation pathway in neonatal heart cells. However, BrdU-labeling and semiquantitative RT-PCR analysis of markers of early cardiogenesis (GATA-4, Nkx2.5, and MEF-2C) and terminal differentiation (Troponin-C, β-MHC, and BNP) both indicated that Wnt has little if any effect on the transcriptional regulation of cardio-specific genes in cells derived from fetal hearts and suggested that there are few if any precardiac cells capable of differentiating in response to Wnt.

The morphological changes evoked by Wnt in cardiac myocytes required the presence of fibroblasts. Plating cardiac myocytes on various matrices, including l-laminine, fibronectin, and collagen, or culturing the cells in fibroblast-conditioned, Wnt-containing medium had no effect on aggregation, suggesting that direct contact between myocytes and fibroblasts is necessary for the induction of aggregation. Although the molecular mechanism remains unknown, one possible explanation may be derived from the finding that different cadherin isoforms are induced in cardiac myocytes and fibroblasts. Since Wnt proteins induced N-cadherin in cardiac myocytes, and E- and M-cadherin in fibroblasts, homotypic cell–cell adhesion between myocytes and fibroblasts should be strengthened by increasing homophilic binding of N- and E-cadherin, respectively. It may be that strengthening homotypic cell–cell adhesion distinguishes cardiac myocytes from fibroblasts, enabling their subsequent aggregation on the surface of fibroblasts.

Although the specific role of Wnt-mediated cell adhesion in the formation of various organ compartments is not known, an effect of Wnt on the regulation of intercellular gap junctions has been demonstrated in *Xenopus* embryos, in vivo (Olson et al., 1991). Cadherin-mediated cell adhesion appears to be a prerequisite for gap junction formation in several cell types (Jongen et al., 1991; Gumbiner et al., 1988), and the abundance of gap junctions can be regulated by both cadherin (Mege et al., 1988) and Wnt (Meyer et al., 1997; van der Heyden et al., 1998). In our study, electrophysiological experiments carried out in our

laboratory have thus far shown gap junctional conductance to be unaffected by the morphological changes induced in cardiac myocytes by Wnt (data not shown).

Tissue formation requires coordinated cell proliferation and morphogenetic movement of groups of cells (for review see Gerhart and Keller, 1986). During these events, the integrity of cell groups is maintained by cell–cell adhesion, but at the same time, the cells must proliferate and must slide past or dissociate from other groups of cells (for review see Gumbiner, 1996). The induction of differentiation during the formation of skeletal muscle is necessarily preceded by aggregation of precursor cells (Edwards et al., 1983; Skerjanc et al., 1994). This requirement for close contact between similar cells during skeletal muscle myogenesis is known as the community effect, which is important for differentiation of somites, cell lines, and embryonic stem cells into skeletal muscle (for review see Gurdon et al., 1993; Kato and Gurdon, 1994; Skerjanc et al., 1994; Cossu et al., 1995). Cadherin-mediated adhesion has been implicated in the community effect, as well as in skeletal muscle differentiation (for review see Gurdon et al., 1993; George-Weinstein et al., 1997). Similarly, at an early stage of vertebrate heart development, the N-cadherin/β-catenin complex is involved in demarcating the boundary separating ventral and dorsal mesoderm, and it is within the resultant clusters of N-cadherin–containing dorsal mesoderm that the commitment and phenotypic differentiation of cardiac myocytes proceeds (Linask, 1992; Linask et al., 1997). The developmental stage of these events is analogous to the stage at which Wg induces cardiac mesoderm in *Drosophila* (Wu et al., 1995; Park et al., 1996). Therefore, we hypothesize that by modulating the interaction of cadherin and catenin, Wnt proteins play a central role in the morphogenetic translocation of cardiac precursor cells during development of the heart-forming region.

We thank Dr. A. Neil Barclay at the University of Oxford for providing us with a CD4 cDNA clone.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

Submitted: 16 November 1999
Revised: 25 May 2000
Accepted: 30 May 2000

References

Behrens, J., J.P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, and W. Birchmeier. 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature.* 382:638–642.

Bhanot, P., M. Brink, C.H. Samos, J.C. Hsieh, Y. Wang, J.P. Macke, D. Andrew, J. Nathans, and R. Nusse. 1996. A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature.* 382:225–230.

Blankesteijn, W.M., Y.P. Essers Janssen, M.M. Ulrich, and J.F. Smits. 1996. Increased expression of a homologue of *Drosophila* tissue polarity gene "frizzled" in left ventricular hypertrophy in the rat, as identified by subtractive hybridization. *J. Mol. Cell. Cardiol.* 28:1187–1191.

Blankesteijn, W.M., Y.P. Essers Janssen, M.J. Verluyten, M.J. Daemen, and J.F. Smits. 1997. A homologue of *Drosophila* tissue polarity gene frizzled is expressed in migrating myofibroblasts in the infarcted rat heart. *Nat. Med.* 3:541–544.

Bodmer, R., and T.V. Venkatesh. 1998. Heart development in *Drosophila* and vertebrates: conservation of molecular mechanisms. *Dev. Genet.* 22:181–186.

Bradley, R.S., and A.M. Brown. 1990. The proto-oncogene int-1 encodes a secreted protein associated with the extracellular matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1569–1575.

Bradley, R.S., and A.M. Brown. 1995. A soluble form of Wnt-1 protein with mitogenic activity on mammary epithelial cells. *Mol. Cell. Biol.* 15:4616–4622.

Bradley, R.S., P. Cowin, and A.M. Brown. 1993. Expression of Wnt-1 in PC12 cells results in modulation of plakoglobin and E-cadherin and increased cellular adhesion. *J. Cell Biol.* 123:1857–1865.

Brown, J.D., and R.T. Moon. 1998. Wnt signaling: why is everything so negative? *Curr. Opin. Cell Biol.* 10:182–187.

Burrus, L.W., and A.P. McMahon. 1995. Biochemical analysis of murine Wnt proteins reveals both shared and distinct properties. *Exp. Cell Res.* 220:363–373.

Butz, S., J. Stappert, H. Weissig, and R. Kemler. 1992. Plakoglobin and beta-catenin: distinct but closely related. *Science.* 257:1142–1144.

Cadigan, K.M., M.P. Fish, E.J. Rulifson, and R. Nusse. 1998. Wingless repression of *Drosophila* frizzled 2 expression shapes the Wingless morphogen gradient in the wing. *Cell.* 93:767–777.

Cadigan, K.M., and R. Nusse. 1997. Wnt signaling: a common theme in animal development. *Genes Dev.* 11:3286–3305.

Caras, I.W., and G.N. Weddell. 1989. Signal peptide for protein secretion directing glycophospholipid membrane anchor attachment. *Science.* 243:1196–1198.

Chan, S.D., D.B. Karpf, M.E. Fowlkes, M. Hooks, M.S. Bradley, V. Vuong, T. Bambino, M.Y. Liu, C.D. Arnaud, G.J. Strewler, et al. 1992. Two homologs of the *Drosophila* polarity gene frizzled (fz) are widely expressed in mammalian tissues. *J. Biol Chem.* 267:25202–25207.

Chu LaGraff, Q., and C.Q. Doe. 1993. Neuroblast specification and formation regulated by wingless in the *Drosophila* CNS. *Science.* 261:1594–1597.

Clark, S.J., W.A. Jefferies, A.N. Barclay, J. Gagnon, and A.F. Williams. 1987. Peptide and nucleotide sequences of rat CD4 (W3/25) antigen: evidence for derivation from a structure with four immunoglobulin-related domains. *Proc. Natl. Acad. Sci. USA.* 84:1649–1653.

Cossu, G., R. Kelly, S. Di Donna, E. Vivarelli, and M. Buckingham. 1995. Myoblast differentiation during mammalian somitogenesis is dependent upon a community effect. *Proc. Natl. Acad. Sci. USA.* 92:2254–2258.

Cui, Y., J.D. Brown, R.T. Moon, and J.L. Christian. 1995. Xwnt-8b: a maternally expressed *Xenopus* Wnt gene with a potential role in establishing the dorsoventral axis. *Development.* 121:2177–2186.

Danielian, P.S., and A.P. McMahon. 1996. Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. *Nature.* 383:332–334.

Du, S.J., S.M. Purcell, J.L. Christian, L.L. McGrew, and R.T. Moon. 1995. Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in *Xenopus* embryos. *Mol. Cell. Biol.* 15:2625–2634.

Edmondson, D.G., G.E. Lyons, J.F. Martin, and E.N. Olson. 1994. Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development.* 120:1251–1263.

Edwards, M.K., J.F. Harris, and M.W. McBurney. 1983. Induced muscle differentiation in an embryonal carcinoma cell line. *Mol. Cell. Biol.* 3:2280–2286.

Frasch, M. 1995. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature.* 374:464–467.

Gat, U., R. DasGupta, L. Degenstein, and E. Fuchs. 1998. De novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell.* 95:605–614.

Gavin, B.J., J.A. McMahon, and A.P. McMahon. 1990. Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. *Genes Dev.* 4:2319–2332.

George-Weinstein, M., J. Gerhart, J. Blitz, E. Simak, and K.A. Knudsen. 1997. N-cadherin promotes the commitment and differentiation of skeletal muscle precursor cells. *Dev. Biol.* 185:14–24.

Gerhart, J., and R. Keller. 1986. Region-specific cell activities in amphibian gastrulation. *Annu. Rev. Cell Biol.* 2:201–229.

Gumbiner, B., B. Stevenson, and A. Grimaldi. 1988. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* 107:1575–1587.

Gumbiner, B.M. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell.* 84:345–357.

Gurdon, J.B., P. Lemaire, and K. Kato. 1993. Community effects and related phenomena in development. *Cell.* 75:831–834.

He, X., J.P. Saint Jeannet, Y. Wang, J. Nathans, I. Dawid, and H. Varmus. 1997. A member of the Frizzled protein family mediating axis induction by Wnt-5A. *Science.* 275:1652–1654.

Heikinheimo, M., J.M. Scandrett, and D.B. Wilson. 1994. Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev. Biol.* 164:361–373.

Hinck, L., W.J. Nelson, and J. Papkoff. 1994. Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing beta-catenin binding to the cell adhesion protein cadherin. *J. Cell Biol.* 124:729–741.

Iwaki, K., V.P. Sukhatme, H.E. Shubeita, and K.R. Chien. 1990. Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. fos/jun expression is associated with sarcomere assembly; Egr-1 induction is primarily an alpha 1-mediated response. *J. Biol Chem.* 265:13809–13817.

Jongen, W.M., D.J. Fitzgerald, M. Asamoto, C. Piccoli, T.J. Slaga, D. Gros, M. Takeichi, and H. Yamasaki. 1991. Regulation of connexin 43-mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. *J. Cell Biol.* 114:545–555.

Kato, K., and J.B. Gurdon. 1994. An inhibitory effect of *Xenopus* gastrula ectoderm on muscle cell differentiation and its role for dorsoventral patterning of mesoderm. *Dev. Biol.* 163:222–229.

Kemler, R. 1992. Classical cadherins. *Semin. Cell Biol.* 3:149–155.

Larabell, C.A., M. Torres, B.A. Rowning, C. Yost, J.R. Miller, M. Wu, D.

Kimelman, and R.T. Moon. 1997. Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* 136:1123–1136.

Lee, Y.J., B. Swencki, S. Shoichet, and R.A. Shivdasani. 1999. A possible role for the high mobility group box transcription factor Tcf-4 in vertebrate gut epithelial cell differentiation. *J. Biol Chem.* 274:1566–1572.

Linask, K.K. 1992. N-cadherin localization in early heart development and polar expression of Na+,K(+)-ATPase, and integrin during pericardial coelom formation and epithelialization of the differentiating myocardium. *Dev. Biol.* 151:213–224.

Linask, K.K., K.A. Knudsen, and Y.H. Gui. 1997. N-cadherin-catenin interaction: necessary component of cardiac cell compartmentalization during early vertebrate heart development. *Dev. Biol.* 185:148–164.

Linask, K.K., and J.W. Lash. 1988. A role for fibronectin in the migration of avian precardiac cells. I. Dose-dependent effects of fibronectin antibody. *Dev. Biol.* 129:315–323.

Lints, T.J., L.M. Parsons, L. Hartley, I. Lyons, and R.P. Harvey. 1993. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants [published erratum appears in *Development.* 1993 Nov;119(3):969]. *Development.* 119:419–431.

Mege, R.M., F. Matsuzaki, W.J. Gallin, J.I. Goldberg, B.A. Cunningham, and G.M. Edelman. 1988. Construction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 85:7274–7278.

Meyer, R.A., M.F. Cohen, S. Recalde, J. Zakany, S.M. Bell, W.J. Scott, Jr., and C.W. Lo. 1997. Developmental regulation and asymmetric expression of the gene encoding Cx43 gap junctions in the mouse limb bud. *Dev. Genet.* 21: 290–300.

Miller, J.R., A.M. Hocking, J.D. Brown, and R.T. Moon. 1999. Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca^{2+} pathways. *Oncogene.* 18:7860–7872.

Miller, J.R., and R.T. Moon. 1997. Analysis of the signaling activities of localization mutants of beta-catenin during axis specification in *Xenopus*. *J. Cell Biol.* 139:229–243.

Molenaar, M., J. Roose, J. Peterson, S. Venanzi, H. Clevers, and O. Destree. 1998. Differential expression of the HMG box transcription factors XTcf-3 and XLef-1 during early xenopus development. *Mech. Dev.* 75:151–154.

Molenaar, M., M. van de Wetering, M. Oosterwegel, J. Peterson Maduro, S. Godsave, V. Korinek, J. Roose, O. Destree, and H. Clevers. 1996. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell.* 86:391–399.

Monkley, S.J., S.J. Delaney, D.J. Pennisi, J.H. Christiansen, and B.J. Wainwright. 1996. Targeted disruption of the Wnt2 gene results in placentation defects. *Development.* 122:3343–3353.

Moon, R.T., J.D. Brown, and M. Torres. 1997. WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.* 13:157–162.

Moon, R.T., J.L. Christian, R.M. Campbell, L.L. McGrew, A.A. DeMarais, M. Torres, C.J. Lai, D.J. Olson, and G.M. Kelly. 1993. Dissecting Wnt signalling pathways and Wnt-sensitive developmental processes through transient misexpression analyses in embryos of *Xenopus laevis*. *Dev. Suppl.* 85–94.

Nagafuchi, A., S. Tsukita, and M. Takeichi. 1993. Transmembrane control of cadherin-mediated cell-cell adhesion. *Semin. Cell Biol.* 4:175–181.

Nusse, R. 1997. A versatile transcriptional effector of Wingless signaling. *Cell.* 89:321–323.

Nusse, R., and H.E. Varmus. 1992. Wnt genes. *Cell.* 69:1073–1087.

Olson, D.J., J.L. Christian, and R.T. Moon. 1991. Effect of wnt-1 and related proteins on gap junctional communication in *Xenopus* embryos. *Science.* 252:1173–1176.

Orsulic, S., and M. Peifer. 1996. An in vivo structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. *J. Cell Biol.* 134:1283–1300.

Papkoff, J., B. Rubinfeld, B. Schryver, and P. Polakis. 1996. Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. *Mol. Cell. Biol.* 16:2128–2134.

Papkoff, J., and B. Schryver. 1990. Secreted int-1 protein is associated with the cell surface. *Mol. Cell. Biol.* 10:2723–2730.

Park, M., T.V. Venkatesh, and R. Bodmer. 1998. Dual role for the zeste-white3/shaggy-encoded kinase in mesoderm and heart development of *Drosophila*. *Dev. Genet.* 22:201–211.

Park, M., X. Wu, K. Golden, J.D. Axelrod, and R. Bodmer. 1996. The wingless signaling pathway is directly involved in *Drosophila* heart development. *Dev. Biol.* 177:104–116.

Perrimon, N. 1994. The genetic basis of patterned baldness in *Drosophila*. *Cell.* 76:781–784.

Radice, G.L., H. Rayburn, H. Matsunami, K.A. Knudsen, M. Takeichi, and R.O. Hynes. 1997. Developmental defects in mouse embryos lacking N-cadherin. *Dev. Biol.* 181:64–78.

Rocheleau, C.E., W.D. Downs, R. Lin, C. Wittmann, Y. Bei, Y.H. Cha, M. Ali, J.R. Priess, and C.C. Mello. 1997. Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell.* 90:707–716.

Roelink, H., and R. Nusse. 1991. Expression of two members of the Wnt family during mouse development—restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* 5:381–388.

Roose, J., M. Molenaar, J. Peterson, J. Hurenkamp, H. Brantjes, P. Moerer, M. van de Wetering, O. Destree, and H. Clevers. 1998. The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature.* 395:608–612.

Rubinfeld, B., I. Albert, E. Porfiri, C. Fiol, S. Munemitsu, and P. Polakis. 1996. Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex. *Science.* 272:1023–1026.

Sheldahl, L.C., M. Park, C.C. Malbon, and R.T. Moon. 1999. Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. *Curr. Biol.* 9:695–698.

Shimizu, H., M.A. Julius, M. Giarre, Z. Zheng, A.M. Brown, and J. Kitajewski. 1997. Transformation by Wnt family proteins correlates with regulation of beta-catenin. *Cell Growth Differ.* 8:1349–1358.

Skerjanc, I.S., R.S. Slack, and M.W. McBurney. 1994. Cellular aggregation enhances MyoD-directed skeletal myogenesis in embryonal carcinoma cells. *Mol. Cell. Biol.* 14:8451–8459.

Slusarski, D.C., V.G. Corces, and R.T. Moon. 1997. Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature.* 390:410–413.

Soler, A.P., and K.A. Knudsen. 1994. N-cadherin involvement in cardiac myocyte interaction and myofibrillogenesis. *Dev. Biol.* 162:9–17.

Staehling Hampton, K., F.M. Hoffmann, M.K. Baylies, E. Rushton, and M. Bate. 1994. dpp induces mesodermal gene expression in *Drosophila*. *Nature.* 372:783–786.

Strutt, D.I., U. Weber, and M. Mlodzik. 1997. The role of RhoA in tissue polarity and Frizzled signalling. *Nature.* 387:292–295.

Tamura, K., W.S. Shan, W.A. Hendrickson, D.R. Colman, and L. Shapiro. 1998. Structure-function analysis of cell adhesion by neural (N-) cadherin. *Neuron.* 20:1153–1163.

Torres, M.A., J.A. Yang Snyder, S.M. Purcell, A.A. DeMarais, L.L. McGrew, and R.T. Moon. 1996. Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early *Xenopus* development. *J. Cell Biol.* 133:1123–1137.

van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Loureiro, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin, and H. Clevers. 1997. Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell.* 88:789–799.

van der Heyden, M.A., M.B. Rook, M.M. Hermans, G. Rijksen, J. Boonstra, L.H. Defize, and O.H. Destree. 1998. Identification of connexin43 as a functional target for Wnt signalling. *J. Cell Sci.* 111:1741–1749.

van Leeuwen, F., C.H. Samos, and R. Nusse. 1994. Biological activity of soluble wingless protein in cultured *Drosophila* imaginal disc cells. *Nature.* 368:342–344.

Vleminckx, K., R. Kemler, and A. Hecht. 1999. The C-terminal transactivation domain of beta-catenin is necessary and sufficient for signaling by the LEF-1/beta-catenin complex in *Xenopus laevis*. *Mech. Dev.* 81:65–74.

Wang, Y., J.P. Macke, B.S. Abella, K. Andreasson, P. Worley, D.J. Gilbert, N.G. Copeland, N.A. Jenkins, and J. Nathans. 1996. A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene frizzled. *J. Biol Chem.* 271:4468–4476.

Wong, G.T., B.J. Gavin, and A.P. McMahon. 1994. Differential transformation of mammary epithelial cells by Wnt genes. *Mol. Cell. Biol.* 14:6278–6286.

Wu, X., K. Golden, and R. Bodmer. 1995. Heart development in *Drosophila* requires the segment polarity gene wingless. *Dev. Biol.* 169:619–628.

Yost, C., M. Torres, J.R. Miller, E. Huang, D. Kimelman, and R.T. Moon. 1996. The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. *Genes Dev.* 10:1443–1454.

Zecca, M., K. Basler, and G. Struhl. 1996. Direct and long-range action of a wingless morphogen gradient. *Cell.* 87:833–844.

Zhu, A.J., and F.M. Watt. 1999. Beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. *Development.* 126:2285–2298.