Noncanonical Wnt11 Signaling Is Sufficient to Induce Cardiomyogenic Differentiation in Unfractionated Bone Marrow Mononuclear Cells

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Background—Despite the frequent clinical use of adult unfractionated bone marrow mononuclear cells (BMMNCs) for cardiac repair, whether these cells are capable of undergoing cardiomyogenic differentiation in vitro remains uncertain. In addition, the role of Wnt signaling in cardiomyogenic differentiation of adult cells is unclear.

Methods and Results—Unfractionated BMMNCs were isolated from adult mice via Ficoll-Paque density-gradient centrifugation and cultured in the presence of Wnt3a or Wnt11. In control BMMNCs, Wnt11 was not expressed, whereas the expression of markers of pluripotency (Oct-4 and Nanog), as well as that of Wnt3a and β-catenin, decreased progressively during culture. Exposure to Wnt3a rescued β-catenin expression and markedly increased the expression of Oct-4 and Nanog, concomitant with increased cell proliferation and CD45 expression. In contrast, exposure to ectopically expressed noncanonical Wnt11 markedly decreased the expression of Oct-4 and Nanog and induced mRNA expression (quantitative real-time reverse-transcription polymerase chain reaction) of cardiac-specific genes (Nkx2.5, GATA-4, atrial natriuretic peptide, α- and β-myosin heavy chain, and cardiac troponin T) by day 3 with subsequent progression to a pattern characteristic of the cardiac fetal gene program. After 21 days, 27.6±0.6% and 29.6±1.4% of BMMNCs expressed the cardiac-specific antigens cardiac myosin heavy chain and cardiac troponin T, respectively (immunocytochemistry), indicating cardiomyogenic lineage commitment. Wnt11-induced cardiomyogenic-specific expression was completely abolished by the protein kinase C inhibitor bisindolylmaleimide I, partially abolished by the c-Jun-N-terminal kinase inhibitor SP600125, and attenuated by the Wnt inhibitor Dickkopf-1.

Conclusions—In adult density-gradient separated BMMNCs, canonical Wnt3a promotes stemness, proliferation, and hematopoietic commitment, whereas noncanonical signaling via Wnt11 induces robust cardiomyogenic differentiation in a protein kinase C– and c-Jun-N-terminal kinase–dependent manner. (Circulation. 2008;117:2241-2252.)

Key Words: bone marrow cells • c-Jun N-terminal kinase • cell differentiation • protein kinase C • stem cells • Wnt-3a protein • Wnt11 protein

The bone marrow (BM) is an easily accessible source of autologous adult stem and progenitor cells, and mounting evidence supports the potential utility of BM-derived cells (BMCs) for somatic tissue repair.1–3 The mononuclear fraction of BMCs (unfractionated BMMNCs) represents a phenotypically heterogeneous mixed population of committed and uncommitted cells. Preclinical studies using an enriched subpopulation of BMMNCs have revealed the ability of BMCs to restore structure and function in dead myocardium.4 Because of this and because of the ease of preparation, BMMNCs are currently the most widely used and readily available cells for cardiac repair in humans. However, because tracking cardiomyocytic differentiation is not clinically feasible, the mechanism of the salubrious effects of BMMNCs is controversial, and it is not known whether unfractionated BMMNCs acquire a cardiac phenotype once implanted in humans. Although the cardiomyogenic potential of various subsets of BMCs and other adult primitive cells has been investigated, the ability of unfractionated BMMNCs to undergo cardiac commitment remains unclear.

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Assessing the cardiogenic potential of unfractionated BMMNCs would necessitate identification of stimuli capable
of inducing cardiac differentiation of these cells. Much of our understanding of in vitro cardiomyogenic differentiation derives from studies performed in embryonic stem cells. From these data, one can infer that this process, to some extent, is dependent on the same molecular cues that direct in vivo cardiac development. In particular, members of the Wnt (Drosophila, wingless-related proteins) family of secreted molecules, namely Wnt3a and Wnt11, function to regulate vertebrate cardiac morphogenesis, and both are able to activate cardiogenesis in cultured murine P19 embryonal carcinoma cells, albeit via dissimilar signaling pathways. Whereas canonical Wnt3a signaling leads to Wnt/ß-catenin–dependent activation of Wnt-responsive genes, Wnt11-mediated noncanonical signaling uses protein kinase C (PKC) and c-Jun-N-terminal kinase (JNK) for the activation of Wnt-responsive genes. Although Wnt-mediated myogenic induction has been reported, it remains unclear whether Wnt3a or Wnt11 can independently induce cardiac differentiation in adult cells.

To elucidate this issue, we investigated the effects of Wnt3a and Wnt11 on cardiac lineage commitment of unfractionated BMMNCs. We also investigated the impact of these proteins on BMMNC proliferation, expression of markers of pluripotency, and reciprocal regulation of signaling. Our results indicate that in unfractionated BMMNCs, Wnt3a promotes hematopoietic commitment and stemness, whereas Wnt11 induces cardiac lineage commitment in a PKC-dependent manner.

Methods

The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, publication No. NIH 86-23). A detailed description of all materials and methods is provided in the online-only Data Supplement.

Animals

Adult C57BL/6 mice (age, 6 to 10 weeks; body weight, 20 to 25 g) were used for this study. Donor mice for each experimental group were obtained from the same litter.

Generation of Stably Transfected Wnt11/293 Cell Sublines

The Wnt11 expression vector (pWnt11/cDNA3) was a gracious gift from Dr Leonard M. Eisenberg (Medical University of South Carolina, Charleston). To generate Wnt11-conditioned medium (Wnt11-CM) and empty vector control medium (control), HEK-293 cells underwent transfection with pWnt11/cDNA3 (Wnt11-CM) or the empty pcDNA3 vector (control) via LipofectAMINE. Stable transfectants (designated Wnt11/293-1 and Wnt11/293-2) were selected on the basis of their resistance to Genetacin (G418), and expression of Wnt11 in stable transfectants and its presence in the CM were verified by Western blotting.

Isolation and Culture of BMMNCs

The overall scheme for all cell culture experiments is depicted in Figure I of the online-only Data Supplement. Unfractionated (density-gradient separated) BMMNCs were isolated from adult C57BL/6 mice by Ficoll-Paque density-gradient centrifugation as previously described. For all experiments, unfractionated BMMNCs were seeded on fibronectin-coated plates (or coverslips) (n=5×10⁵ cells per well) in the lower chamber of a dual-chamber 6-well tissue culture plate (Transwell coculture system). To ensure maximal attachment of BMMNCs, cultures were maintained in complete medium for 10 days at 37°C. A single medium change was performed at day 6. Attachment of all BMMNC subfractions was complete by day 10, whereupon a paucity of cells remained unattached.

Induction of Differentiation

Starting on day 10, inserts bearing cells with the empty vector pcDNA3/293 (control) or Wnt11/293-2 cells (Wnt11-CM) were placed atop BMMNCs cultured in the lower chamber of the aforementioned dual-chamber system (online-only Data Supplement Figure IA). Thus, during coculture for 21 days, BMMNCs were separated from Wnt11-expressing Wnt11/293-2 cells in the upper chamber by a semipermeable polyester membrane (pore size, 0.4 μm). This eliminated the possibility of cell fusion or cell–cell contact as a requirement for Wnt11-mediated effects. BMMNCs also were cultured in the presence of Wnt3a (recombinant Wnt3a protein added to the medium, 150 ng/mL).

Cytomorphological Evaluation of Cell Types

Photomicrographs of cells acquired at different stages of culture were evaluated for cellular morphology by a cytopathologist in a blinded manner.

Assessment of Cellular Proliferation

In separate experiments, unfractionated BMMNCs (n=1×10⁶) were cultured as above and monitored by phase-contrast microscopy for up to 21 days. Cell number and viability were determined on days 8, 16, and 21.

Immunocytochemistry

Cultured BMMNCs and control Wnt11/293-2 cells were fixed and permeabilized on glass coverslips. Hematopoietic cells were defined by the expression of the panhematopoietic marker CD45. The expression of Oct-4, a marker of pluripotent cells, also was evaluated by immunostaining. Cardiomyocytic differentiation was recognized by the presence of cardiac-specific proteins (cardiac troponin T [cTnT], cardiac myosin heavy chain [cMyHC], and connexin-43). Immunocytochemistry was performed according to published methods using the primary and secondary antibodies specified in the online-only Data Supplement. All immunocytochemical photomicrographs were acquired with an LSM 510 confocal microscope (Zeiss).

Assessment of Cardiac Differentiation

For quantitative assessment of cardiomyocytic differentiation, an average of 2400 cells per plate were counted. Multiple fields from all 4 quarters of the plate were systematically evaluated. The number of cells expressing cardiac-specific intracellular contractile proteins in each field was calculated; the total number of cells was calculated from nuclear staining with DAPI. In addition, cells were concomitantly stained with markers specific for various noncardiac lineages (skeletal muscle [myogenin], neural [nestin, GFAP], myeloid [CD11b, Gr-1], and others). Cells were counted as positive when they were positive for cardiac-specific contractile proteins and negative for all of these markers. Thus, we used a rigorous method to assess cardiac differentiation in vitro. The percentage of cells differentiating into cardiomyocytes was calculated as the number of cells positive for proteins divided by the total number of nuclei in the field. These experiments were performed in triplicate. As a confirmatory measure, the expression of cardiac troponin I (cTnI) was determined by ELISA (as described below) after culture in Wnt11-CM with and without the Wnt inhibitor Dickkopf-1 (Dkk-1) (online-only Data Supplement Figure IC).

Assessment of mRNA Expression by Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

cDNA was generated from freshly isolated (baseline) BMMNCs and at 0, 3, 7, and 21 days of culture. Cell viability was assessed by trypan blue exclusion before RNA extraction. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis for all the genes of interest (α-myosin heavy chain [α-MyHC], β-MyHC, cTnT, Nkx2.5, GATA-4, atrial natriuretic peptide [ANP],
β-catenin, Oct-4, Nanog) and 18S rRNA was performed in triplicate using QuantiTect SYBR Green Real-Time PCR kits using validated primer pairs (Qiagen) and published methods.20

Assessment of Wnt Signaling
To investigate noncanonical signaling pathways, freshly isolated unfractonated BMMNCs were cultured in Wnt11-CM or in empty vector (control) medium in the presence or absence of the PKC inhibitor bisindolylmaleimide I (BIM; 1 μmol/L) and JNK (SP600125; 10 μmol/L) inhibitors (online-only Data Supplement Figure IB). Expression of Wnt3a and β-catenin was measured by qRT-PCR to investigate the effect of control, Wnt3a medium, and Wnt11-CM on canonical signaling.

Western Immunoblotting and Quantitative ELISA
Western analysis of Wnt11 expression was performed according to published methods.19 Western immunoblotting for phospho-SAPK/JNK (Thr183/Tyr185) was performed as previously described.21 The expression of cTnI in proteins isolated from homogenized cultured cells was quantified with a commercially available ELISA kit (Life Diagnostics, West Chester, Pa) according to published methods.22

Statistical Analysis
Data are expressed as mean±SEM. Differences were analyzed with the unpaired Student t test or ANOVA (1 way or repeated measures) as appropriate. After ANOVA, pairwise comparisons were performed with the Student t test using the Bonferroni correction. Statistical significance was defined as P<0.05. When the Bonferroni correction was used, the significance level was adjusted by dividing 0.05 by the number of comparisons performed.

All authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Generation of Cell Lines With Ectopic Expression of Wnt11
Two Wnt11/293 transfected cell lines (Wnt11/293-1 and Wnt11/293-2) were generated to achieve ectopic overexpression of Wnt11. Both lines demonstrated robust constitutive expression levels of Wnt11 protein in cellular extracts as well as in media, confirming release of Wnt11 from Wnt11/293 cells (Figure 1A). The protein levels of the active (soluble) form of Wnt11, distinct from the inactive matrix-associated form,18 in the medium were ~30% of the cellular levels (Figure 1B), similar to previous studies that used Wnt11 expression systems.14 Although Wnt11 expression was higher in Wnt11/293-2 at the mRNA level (Figure 1C), no significant difference was observed in Wnt11 expression between Wnt11/293-1 and Wnt11/293-2 cells at the protein level (Figure 1B). Wnt11/293-2 cells were selected for the Wnt11-CM experiments in this study.

Morphology of Unfractionated BMMNCs
Before Culture
Unfractionated BMMNCs exhibited considerable morphological variation 24 hours after plating (online-only Data Supplement Figure II). The majority of cells were round and large with eccentric nuclei (promyeloictytic precursors) or with a high nuclear-to-cytoplasmic ratio, characteristic of myelo-blastic hematopoietic precursors, whereas a few cells displayed early granulocytic (myelocytic and metamyelocytic) features.23

Effect of Wnt3a and Wnt11 on the Expression of Markers of Pluripotency in Unfractionated BMMNCs in Culture
Because Wnt3a and Wnt11 are known to influence both differentiation and renewal of various stem cell populations,15,23,24 we investigated the effect of these proteins on the expression patterns of well-known markers of pluripotency that
are associated with an undifferentiated state (Oct-4 and Nanog). Culturing BMMNCs for 21 days in control medium exerted a significant negative effect on the intrinsic pluripotency of these cells, as evidenced by a 264±0.2-fold decrease in the expression of Oct-4 and Nanog, respectively (P<0.001 for both; Figure 2A and 2B). Culturing BMMNCs in Wnt11-CM further enhanced this downregulation of Oct-4 and Nanog expression (352±0.2-fold and 471±0.2-fold decrease, respectively, compared with baseline; P<0.001 for both; Figure 2A and 2B). In contrast, Wnt3a markedly enhanced the expression of Oct-4 and Nanog (177±0.4-fold and 158±0.6-fold increase, respectively, compared with baseline; P<0.001 for both; Figure 2A and 2B). Moreover, immunocytochemical assessment revealed a marked increase in the number of Oct-4–positive BMMNCs after culture in the presence of Wnt3a compared with untreated control BMMNCs, indicating that Wnt3a increases the number of cells expressing markers of pluripotency (Figure 2C and 2D and online-only Data Supplement Figure III).

**Effect of Wnt3a and Wnt11 on Proliferation of Unfractionated BMMNCs During Culture**

In unfractionated BMMNCs cultured in control medium, only minimal proliferation was noted until the later stages of culture (Figure 2E). In BMMNCs cultured in Wnt11-CM, the proliferation rate was similar to that in control medium at all time points. Conversely, starting on day 8, the increase in cell number was significantly greater in BMMNCs cultured in Wnt3a (110±0.1-fold versus 12±0.1-fold in control and 14±0.1-fold in Wnt11-CM on day 16, P<0.001 for both; 297±0.1-fold versus 93±0.9-fold in control and 101±0.1-fold in Wnt11-CM on day 21, P<0.001 for both; Figure 2E), indicating that Wnt3a promotes proliferation of unfractionated BMMNCs.

**Wnt3a Preserves the Hematopoietic Potential of Cultured Unfractionated BMMNCs**

Consistent with the decrease in levels of Oct-4 and Nanog, control BMMNCs displayed considerable morphological hetero-

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**Figure 2.** Expression of markers of pluripotency and proliferation rates in cultured unfractionated BMMNCs. qRT-PCR analysis of Oct-4 (A) and Nanog (B) mRNA expression in freshly isolated unfractionated BMMNCs (baseline) and in BMMNCs cultured for 21 days with empty vector (control), Wnt3a, or Wnt11-CM. mRNA levels in unfractionated BMMNCs after 21 days of culture are normalized to 18S rRNA (internal control) and expressed as fold difference relative to baseline (calibrator). Assays were performed in triplicate. Data are mean±SEM from 3 independent experiments. C and D. Representative merged confocal images of BMMNCs after 21 days of culture in Wnt3a show nuclear localization of Oct-4. E. Days of proliferation are indicated on the x axis; fold increase in number of cells is indicated on the y axis. Cultures were initiated with a total of 10⁴ cells (0 on the graph) in each group. Scale bar=25 μm.
Wnt11 Activates Cardiomyogenesis in Cultured Unfractionated BMMNCs

After 21 days of culture in Wnt11-CM, cellular morphology changed from the small and large round cells (online-only Data Supplement Figure II) to predominantly elongated, rod-shaped, and elliptical cells (Figure 4A and 4D). Immunocytochemical analysis revealed a cardiac phenotype in BMMNCs cultured in Wnt11-CM for 21 days, as evidenced by the positivity for cardiac-specific structural proteins (cMyHC and cTnT; Figure 4C and 4F, respectively) and connexin-43 (Figure 4G through 4M) and negativity for markers for noncardiac lineages, including the skeletal muscle marker myogenin (Figure 4N through 4Q) and others.19 Interestingly, although BMMNCs expressed cardiac-specific antigens and acquired a cardiomyocytic morphology, a sarcomeric structure was not apparent in our cultures; moreover, the vast majority of these cells remained mononucleated and, for the most part, did not fuse or form networks (Figure 4), ostensibly indicating a less mature phenotype. Importantly, after 21 days, only BMMNCs cultured in Wnt11-CM demonstrated cardiomyogenic differentiation (Figure 5A through 5D), with 27.6±0.6% and 29.6±1.4% of all cells staining positive for cMyHC and cTnT, respectively (Figure 5Q). Wnt11-expressing Wnt11/293-2 cells showed no evidence of cardiomyogenic differentiation (Figure 5M through 5P), indicating that the Wnt11-induced cardiac differentiation was specific for BMMNCs. Of note, we observed the expression of myeloid lineage markers CD11b and Gr-1 in very small (and similar) numbers of cultured BMMNCs in both Wnt11-CM and control medium (data not shown).

Next, we assessed whether Wnt proteins were capable of activating the cardiac gene program in cultured BMMNCs. qRT-PCR analysis of BMMNCs during 21 days of culture revealed that Wnt11-CM activated the cardiac gene program by day 3 of exposure, as evidenced by expression of early markers of cardiomyogenesis (Nkx2.5, GATA-4, and ANP) and cardiac contractile proteins (cTnT, α-MyHC, and β-MyHC) (Figure 6A through 6F). Notably, no cardiac-specific transcripts were detected in BMMNCs before culture in Wnt11-CM (day 0), after 21 days of culture in the presence of the empty vector.
(pcDNA3–293 cells), or in the presence of Wnt3a (data not shown). Compared with the levels at day 3, the respective mRNA levels of cardiac-specific markers at days 7 and 21 increased as follows: Nkx2.5, 9.7±0.3-fold and 31.9±0.4-fold (P<0.001 for both; Figure 6A); GATA-4, 11.1±0.3-fold and 72.3±0.3-fold (P<0.001 for both; Figure 6B); ANP, 293.1±0.3-fold and 1139.9±0.3-fold (P<0.001 for both; Figure 6C); cTnT, 17.8±0.4-fold and 192.6±0.4-fold (P<0.001 for both; Figure 6D); and β-MHC, 51.7±0.3-fold and 422.6±0.3-fold (P<0.001 for both; Figure 6F). Interestingly, although

Figure 4. Morphological and phenotypic features of unfractionated BMMNCs cultured for 21 days in Wnt11-CM. A and D, Representative transmission images show cells at various stages of differentiation with morphologies characteristic of differentiating cardiomyocytes: enlarged elliptical shape (white arrows), some with short cytoplasmic processes (white arrowhead); rod-shaped (asterisk); or elongated (black arrowhead). B, C, E, and F, Confocal microscopic images of these same BMMNCs show expression of the cardiac-specific contractile proteins cMyHC and cTnT (C and F, respectively; green fluorescence), indicating cardiomyogenic differentiation. G through I, Confocal images of BMMNCs after 21 days of culture in Wnt11-CM demonstrate expression of connexin-43 (H and I; green). J through M, Colocalization (M) of connexin-43 (K and M, green) and cTnT (L and M, red). BMMNCs cultured under the same conditions underwent coimmunostaining and revealed cTnT expression (P and Q; green fluorescence) and the absence of the skeletal muscle–specific protein myogenin (O). All nuclei are stained with DAPI (blue). Scale bar=25 μm.
levels of all transcripts, including \( \beta \)-MyHC, showed a significant progressive increase at each time point, \( \alpha \)-MyHC expression increased significantly at day 7 (5.9 ± 0.3-fold; \( P \leq 0.001 \); Figure 6E) but remained essentially unchanged thereafter. This pattern of gene expression is consistent with the temporal sequence of cardiomyogenic gene expression described in embryoid bodies and during fetal development.27–29 A 367% increase in cTnI expression by ELISA in BMMNCs cultured in Wnt11-CM...
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Wnt3a and Wnt11-CM exerted opposite effects on the expression of both Wnt3a and Wnt11. Interestingly, ectopic overexpression of Wnt11 also modified the effect of Wnt proteins on Wnt and β-catenin–mediated canonical pathway, recruited by Wnt3a without Dkk-1. After 21 days of culture, although Dkk-1 had little effect by itself, it reduced Wnt11-induced expression of cTnI in BMMNCs (Figure 7C). Together, these data suggest that although Wnt11 attenuates Wnt/β-catenin signaling at the cell surface, at the subcellular level, upstream of β-catenin, and at the level of β-catenin itself, inhibition of canonical signaling diminishes the full cardiomyogenic potential of Wnt11/noncanonical signaling.

Figure 6. Wnt11-induced activation of the cardiac gene program in unfractionated BMMNCs. Cells were cultured for 21 days either in the presence of 150 ng/mL recombinant Wnt3a protein or in coculture with Wnt11-secreting cells (Wnt11/293-2) or pcDNA3/293 empty vector (control) cells. Quantitative assessment of expression of cardiac-specific transcription factors (GATA-4 and Nkx2.5), ANP, and cardiac contractile proteins (cTnT, α- and β-MyHC isoforms) was performed by qRT-PCR at days 0, 3, 7, and 21. A through F, No expression of cardiac-specific markers was detected before induction of BMMNCs (day 0). No expression of cardiac-specific markers was observed in control and Wnt3a-treated BMMNCs during 21 days of culture (data not shown). mRNA levels in unfractionated BMMNCs cultured in Wnt11-CM are normalized to 18S rRNA (internal control) and expressed as fold differences relative to first detectable mRNA expression after induction (day 3). All samples were run in triplicate. Data are mean±SEM of 3 independent experiments. *P<0.001 vs respective levels on day 3; #P<0.001 vs respective levels on day 7.

Effect of Wnt Proteins and Wnt Inhibitors on Wnt3a, β-Catenin, and cTnI Expression

The β-catenin–mediated canonical pathway, recruited by Wnt3a and other members of the Wnt1 family of molecules, is distinct from the noncanonical signaling pathways used by the Wnt5 family, which includes Wnt11. However, because recent reports have described crosstalk between Wnt11/noncanonical and canonical signaling pathways, we first examined the effect of Wnt proteins on Wnt and β-catenin transcriptional activity in freshly isolated and cultured unfractionated BMMNCs. No Wnt11 expression was detected in freshly isolated and cultured unfractionated BMMNCs. No Wnt11 expression was detected in freshly isolated and cultured unfractionated BMMNCs. No expression of cardiac-specific markers was detected before induction of BMMNCs (day 0). No expression of cardiac-specific markers was observed in control and Wnt3a-treated BMMNCs during 21 days of culture (data not shown). mRNA levels in unfractionated BMMNCs cultured in Wnt11-CM are normalized to 18S rRNA (internal control) and expressed as fold differences relative to first detectable mRNA expression after induction (day 3). All samples were run in triplicate. Data are mean±SEM of 3 independent experiments. *P<0.001 vs respective levels on day 3; #P<0.001 vs respective levels on day 7.

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Next, we investigated whether a well-established antagonist of Wnt signaling, Dkk-1 (a soluble inhibitor of canonical signaling that blocks Wnt signaling at the cell surface), would influence the cardiomyogenic commitment of cultured BMMNCs. BMMNCs were cultured in Wnt11-CM with or without Dkk-1. After 21 days of culture, although Dkk-1 had little effect by itself, it reduced Wnt11-induced expression of cTnI in BMMNCs (Figure 7C). Together, these data suggest that although Wnt11 attenuates Wnt/β-catenin signaling at the cell surface, at the subcellular level, upstream of β-catenin, and at the level of β-catenin itself, inhibition of canonical signaling diminishes the full cardiomyogenic potential of Wnt11/noncanonical signaling.

Activation of the Cardiac Gene Program Depends on Wnt11/PKC Signaling

Although the precise mechanisms involved in noncanonical Wnt pathways remain to be delineated, PKC and JNK are generally considered to be the primary mediators of noncanonical Wnt signaling during cardiogenesis. Because PKC is upstream of JNK in this signaling pathway, we tested the effect of Wnt11-CM on PKC-mediated JNK phosphorylation. Cell viability was estimated to be >98% before mRNA expression analysis. No cardiac-specific gene expression was observed in BMMNCs cultured in control (empty vector) medium (data not shown). Pharmacological inhibition of PKC with BIM completely blocked Wnt11-induced JNK phosphorylation in BMMNCs (Figure 8A) and abolished cardiac gene expression (Figure 8B). Interestingly, although JNK inhibition via SP600125 completely abolished Wnt11-induced expression of cardiac contractile proteins in BMMNCs, the expression of the
cardiac transcription factors GATA-4 and Nkx2.5, although significantly depressed, was still detectable, and ANP was not significantly affected (Figure 8B). These data indicate that in unfractionated BMMNCs, Wnt11 activation of cardiomyogenic noncanonical Wnt signaling is completely dependent on PKC but only partially dependent on JNK, suggesting the existence of additional transducing elements in this pathway, likely upstream of JNK and downstream of PKC.

**Discussion**

Although unfractionated BMMNCs are being increasingly used for cell-based cardiac repair, little is known about whether these cells are capable of adopting a cardiac phenotype. In this study, we have developed a new method for inducing cardiomyogenic differentiation of unfractionated BMMNCs using the noncanonical morphogen Wnt11 as the only stimulus. Our results can be summarized as follows. First, Wnt11, in itself, is sufficient to induce activation of the cardiac gene program in cultured unfractionated BMMNCs, resulting in cardiomyocytic differentiation of ~30% of the cells within this compartment. Second, at the same time, Wnt11 reduces the expression of markers of pluripotency (Oct-4 and Nanog) in cultured BMMNCs. Third, in contrast, Wnt3a upregulates markers of pluripotency, induces proliferation, and promotes hematopoietic lineage commitment. Fourth, cardiomyogenic Wnt11/noncanonical signaling in BMMNCs is transduced by PKC, with JNK being a downstream mediator (but not the only one). Finally, although Wnt11 suppresses Wnt3a and β-catenin expression in unfractionated BMMNCs, inhibition of canonical signaling attenuates Wnt11/noncanonical cardiomyogenic potential, indicating cooperative synergistic cross-talk between both pathways in the determination of the differentiation fate of these cells.

Previous reports have demonstrated Wnt-mediated myogenic induction in BM-derived mesenchymal stem cells. However, to the best of our knowledge, this is the first study to demonstrate induction of a cardiac phenotype in unfractionated BMMNCs in vitro using a specific, well-defined, and nontoxic stimulus, without coculture with cardiomyocytes or other cell types, and without incubation with mutagens such as 5-azacytidine. The
clinical implications of our results stem from recent studies in which transplantation of BMMNCs has been shown to result in improved left ventricular function and perfusion without any major adverse effect.\textsuperscript{3,6} These studies support the efficacy of BMMNCs in promoting cardiac repair. Although various types of adult cells with more primitive features, including multipotent adult progenitor cells, very small embryonic-like stem cells, cardiospheres-derived stem cells, and cardiac stem cells, may be effective in organ repair, these cells are rare and often difficult to expand in vitro. In conjunction with our results indicating the ability of BMMNCs to undergo cardiac differentiation, the easy availability of large numbers of these cells, their beneficial effects in clinical trials, and their safety profile support the potential usefulness of BMMNCs for therapeutic cardiac repair.

The results of this study provide the first demonstration that Wnt11, in itself, can effectively induce cardiac differentiation of unfractionated BMMNCs. A previous study by Koyanagi et al\textsuperscript{16} found that Wnt11 enhanced cardiac differentiation of circulating progenitor cells cocultured with neonatal cardiomyocytes; however, Wnt11 was unable to induce cardiac differentiation by itself. In another study,\textsuperscript{15} Wnt-mediated myogenic differentiation was observed in a discrete subset of expanded mesenchymal...
stem cells referred to as murine adult multipotent stem cells. The authors reported expression of $\beta$-MyHC, GATA-4, and Nkx2.5 after treatment with Wnt11 and demonstrated expression of cTnT in a subset of murine adult multipotent stem cells.15 Interestingly, $\alpha$-MyHC and ANP were not expressed. Because mesenchymal stem cells constitute only ~0.01 to 0.001% of BMCs,12 our results suggest that Wnt11 can induce cardiac differentiation in other subsets of BMCs that are collectively isolate as BMMNCs. It is noteworthy that the Wnt11-induced expression of cardiac-specific contractile proteins and ANP in BMMNCs followed a pattern characteristic of the cardiac fetal gene program. Specifically, in addition to upregulation of ANP, BMMNCs exhibited a differential expression of $\beta$-MyHC, the isoform that predominates in fetal life, over the $\alpha$-isoform (Figure 6), similar to the pattern observed before the developmental isoform-specific switch that occurs later during fetal development. This phenomenon has been described by others in cultured embryoid bodies and mesenchymal stem cells.27-29

Our observations demonstrate a dichotomous effect of Wnt proteins on the expression of markers of pluripotency in unfiltered BMMNCs. The noncanonical Wnt11 reduced the expression of Oct-4 and Nanog, promoted differentiation, and had little effect on proliferation. In contrast, the canonical Wnt3a markedly increased the expression of Oct-4 and Nanog, promoted morphological homogeneity and hematopoietic potential, and enhanced proliferation. These results are consistent with previous findings on Wnt signaling in several other primitive cell populations, including hematopoietic stem cells.23,24,33

Although canonical signaling is distinct from the noncanonical pathways, crosstalk does occur, apparently in a bidirectional manner. Specifically, noncanonical Wnt5a can enhance $\beta$-catenin signaling to promote renewal of both umbilical cord blood–derived cells and hematopoietic stem cells,23,33 whereas Wnt11 is known to suppress Wnt3a signaling at the level of $\beta$-catenin, in favor of cardiogenic pathways.9,30,31 In addition, recent studies have demonstrated that Wnt-1 and Wnt3a are capable of activating both canonical and noncanonical Wnt signaling pathways in rodent mammary epithelial cell lines and explants from dorsal root ganglia, respectively.34,35 Our data suggest similar crosstalk in BMMNCs because ectopic overexpression of Wnt11 attenuated expression of both Wnt3a and $\beta$-catenin. Likewise, in P19 embryonal carcinoma cells, Wnt3a expression begins early (48 hours after induction) but is quelled later when Wnt11 signaling begins.12,31 Our finding that Wnt11 suppresses Wnt3a and $\beta$-catenin expression indicates that the repression mechanism observed in the temporal expression patterns present in vivo during cardiogenesis and in embryonic stem cells11,12 can be effectively recapitulated in adult BMMNCs. Nevertheless, the fact that Dkk-1 resulted in diminished Wnt11-induced cTnT expression in cultured BMMNCs, yet had no effect on these cells by itself, indicates a tightly regulated interdependent feedback system between these 2 pathways.

That Wnt11 uses PKC and JNK as signal transducers for cardiomyogenesis has been demonstrated, albeit somewhat inconclusively.9,15,17 Although Koyanagi et al16 found that JNK played no role in Wnt11-induced enhancement of cardiac differentiation in circulating progenitor cells cocultured with rat cardiomyocytes, data from other laboratories indicate that JNK is necessary for the cardiogenic activity of Wnt11.9,17 In murine adult multipotent stem cells, PKC inhibition did not completely block the activation of GATA-4 and Nkx2.5 by Wnt11.15 Our results demonstrate that in adult BMMNCs, PKC signaling is obligatorily required for Wnt11-mediated JNK activation and is indispensable for the Wnt11-dependent activation of this cardiac gene program. In contrast to previous studies,9,17 we found that a block in JNK signaling in the Wnt11-cardiomyogenic pathway can be partially overcome, as evidenced by decreased yet persistent expression of Nkx2.5, GATA-4, and ANP in BMMNCs (Figure 8). This suggests the presence of other elements responsible for Wnt11 signaling in the absence of JNK. Alternatively, the expression of GATA-4, ANP, or Nkx2.5 may not be exclusively cardiac specific. Together with our data on the effect of Dkk-1, these observations suggest that although canonical signaling alone is not able to induce cardiomyogenesis in BMMNCs, it is perhaps involved in the potentiation of this effect, at least in vitro. Further investigation is necessary to elucidate the regulation of Wntcanonical/noncanonical pathways in cardiac differentiation.

Conclusions
We have shown that adult unfiltered (density-gradient separated) BMMNCs, a cell population frequently used for cardiac repair in humans, are able to undergo cardiac differentiation in vitro in significant numbers without coculture with cardiomyocytes. Noncanonical Wnt11 signaling via PKC is sufficient to elicit a cardiomyocytic phenotype in these cells via the induction of the cardiac fetal gene program and expression of cardiac-specific proteins. In contrast, canonical signaling via Wnt3a enhances pluripotency, promotes hematopoietic potential, and induces proliferation. These results may have important implications for our understanding of Wnt signaling in adult cells and for therapeutic cardiac repair.

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Disclosures
None.

References


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SUPPLEMENTAL FIGURE 1A

BMMNC isolation

BMMNCs →

BMMNC attachment

21 days

Control
Wnt11-CM
Wnt3a

Proliferation

10 days

BMMNCs

21 days

Control
Wnt11-CM
Wnt3a

Morphology/Differentiation

0, 3, 7, and 21 days

Control
Wnt11-CM
Wnt3a

mRNA Analysis (baseline)

SUPPLEMENTAL FIGURE 1A
SUPPLEMENTAL FIGURE 1B

BMMNC isolation

BMMNCs →

Wnt11-CM ± BIM

3 h

Western blotting for JNK / p-JNK

10 days

BMMNC Attachment

Vector control

Wnt11-CM control

Wnt11-CM

+ BIM or SP600125

3 days

mRNA analysis for cardiac markers

SUPPLEMENTAL FIGURE 1B
BMMNC isolation

BMMNCs → BMMNC Attachment

10 days

Vector control

+Dkk-1

Wnt11-CM

21 days

Vector control

+Dkk-1

Wnt11-CM

Quantitative ELISA for cTnI

SUPPLEMENTAL FIGURE 1C