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Non-canonical Wnt Signaling Enhances Differentiation of Human Circulating Progenitor Cells to Cardiomyogenic Cells*

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Human endothelial circulating progenitor cells (CPCs) can differentiate to cardiomyogenic cells during co-culture with neonatal rat cardiomyocytes. Wnt proteins induce myogenic specification and cardiac myogenesis. Here, we elucidated the effect of Wnts on differentiation of CPCs to cardiomyogenic cells. CPCs from peripheral blood mononuclear cells were isolated from healthy volunteers and co-cultured with neonatal rat cardiomyocytes. 6–10 days after co-culture, cardiac differentiation was determined by α-sarcomeric actinin staining of human lymphocyte antigen-positive cells (fluorescence-activated cell-sorting analysis) and mRNA expression of human myosin heavy chain and atrial natriuretic peptide. Supplementation of co-cultures with Wnt11-conditioned medium significantly enhanced the differentiation of CPCs to cardiomyocytes (1.7 ± 0.3-fold), whereas Wnt3A-conditioned medium showed no effect. Cell fusion was not affected by Wnt11-conditioned medium. Because Wnts inhibit glycogen synthase kinase-3β, we further determined whether the glycogen synthase kinase-3β inhibitor LiCl also enhanced cardiac differentiation of CPCs. However, LiCl (10 mM) did not affect CPC differentiation. In contrast, Wnt11-conditioned medium time-dependently activated protein kinase C (PKC). Moreover, the PKC inhibitors bisindolylmaleimide I and III significantly blocked differentiation of CPCs to cardiomyocytes. PKC activation by phorbol 12-myristate 13-acetate significantly increased CPC differentiation to a similar extent as compared with Wnt11-conditioned medium. Our data demonstrate that Wnt11, but not Wnt3A, augments cardiomyogenic differentiation of human CPCs. Wnt11 promotes cardiac differentiation via the non-canonical PKC-dependent signaling pathway.

Cardiomyocytes have been generated from murine and human embryonic stem cells as well as from embryonic endothelial cells (1, 2). Meanwhile, increasing evidence suggests that adult stem and progenitor cells also have the capacity to differentiate to the cardiomyogenic lineage. Bone marrow-derived hematopoietic stem cells, mesenchymal stem cells, side population cells, and cardiac stem cells showed expression of cardiac marker proteins after infusion in animal models after myocardial infarction (3–5). More recently, tissue-resident cardiac progenitor cells have been identified (6, 7), which can differentiate to cardiomyocytes. However, very recent studies questioned whether c-kit$^+$/lin$^-$ bone marrow-derived stem cells can acquire a cardiac phenotype after injection in a mouse model after acute myocardial infarction (8, 9). Recently, cell fusion was proposed as an additional mechanism used by bone marrow-derived cells to acquire a cardiac phenotype (10). Interestingly, some studies showed that both fusion and differentiation occur *in vivo* after infusion of cardiac stem cells in a model of myocardial infarction (7).

The mechanisms underlying cardiomyogenic differentiation of adult stem or progenitor cells are mainly unclear. Recent studies provided evidence that the extracellular "niche" exerts a critical role for the developmental fate of stem and progenitor cells (11). Based on this concept, a co-culture system of neonatal rat cardiomyocytes was established to mimic the cardiac environment (12). Co-culture of embryonal endothelial cells (12), circulating progenitor cells isolated from human blood (13), or human CD34$^+$ cells (14) with neonatal cardiomyocytes triggered the expression of cardiac genes. In this co-culture assay, gap-junctional communication between neonatal cardiomyocytes and differentiating stem or progenitor cells was detected (12, 13). Functional activity of the differentiated circulating progenitor cells was further documented by showing oscillating calcium transients after pacing (13). These data suggest that the co-culture system might be useful as an experimental model to study cardiomyogenic differentiation of adult progenitor cells.

Proteins of the Wnt family are known as regulators of cardiomyogenesis. Wnt genes relate to *Drosophila* wingless and encode for secreted glycoproteins (15). Wnts can bind to Frizzled (Fz)[1] receptors on target cells to activate different signaling pathways. Activation of the so-called canonical pathway leads to stabilization of β-catenin through inactivation of glycogen synthase kinase (GSK)-3β, to β-catenin-dependent activation of T-cell factor/lymphocyte enhancer factor transcription factors, and to induction of Wnt-responsive

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[1] The abbreviations used are: Fz, Frizzled; CPC, circulating progenitor cell; HLA, human lymphocyte antigen; FACS, fluorescence-activated cell-sorting; RT, reverse transcription; PKC, protein kinase C; JNK, c-Jun NH_2-terminal kinase; GSK, glycogen synthase kinase; PLP, 2% paraformaldehyde, 0.01 M $NaIO_4$, 0.075 M lysine, and 0.037 M sodium phosphate, pH 7.2; GFP, green fluorescent protein; MHF, myosin heavy chain; ANF, atrial natriuretic factor; Dil-ac-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein.

genes (16). In contrast, Wnt11 was shown to signal through a β-catenin-independent non-canonical pathway involving protein kinase C (PKC) and c-Jun NH_2-terminal kinase (JNK) (17). Whereas initial studies considered Wnts as negative regulators of heart muscle formation in *Xenopus* and chicken embryos, more recent studies demonstrated that Wnts induced myogenic specification and mammalian cardiac myogenesis (17–19, 25). Wnt3A up-regulated early cardiac markers in mouse PC19C6 cells through β-catenin (19). Wnt11 induced cardiac differentiation in *Xenopus* and in murine embryonic cell lines (17, 25). Furthermore, combined signaling of Wnt5A, Wnt5B, and Wnt7A induced myogenic commitment of adult resident $CD45^+$ stem cells during muscle regeneration (18). Thus, Wnts are attractive candidates to mediate myogenic differentiation. Therefore, we investigated the effect of Wnt3A- and Wnt11-conditioned medium on cardiac differentiation of human progenitor cells and analyzed the underlying signaling mechanisms.

MATERIALS AND METHODS

Cell Culture Experiments—Neonatal ventricular cardiomyocytes were isolated from 0- to 1-day-old Wistar rats and cultivated as described previously (13). CPCs were isolated from peripheral blood of healthy volunteers by density gradient centrifugation followed by plating onto fibronectin-coated plates using serum (20% fetal calf serum)- and growth factor-supplemented endothelial cell culture medium (13, 20). After 3 days in culture, adherent CPCs were labeled with Dil-ac-LDL (2.5 μg/ml; Cell Systems, St. Katharinen, Germany) for 60 min at 37 °C followed by three washing steps with phosphate-buffered saline as described previously (13, 20). Expression of endothelial markers such as von Willbrandt factor, endothelial nitric-oxide synthase, and vascular endothelial-cadherin was routinely confirmed (13, 20). CPCs (1.5×10^5) were mixed with freshly isolated cardiomyocytes at a ratio of 1:3 (13). LacZ-, Wnt3A-, and Wnt11-conditioned media were generated and functionally tested as described previously (17, 21).

Immunostaining and Flow Cytometry Analysis—Co-cultured cells were stained with phycoerythrin-conjugated antibodies recognizing human HLA-DR and HLA class I (both from Caltag Laboratories, Burlingame, CA) followed by permeabilization using the Cytofix/Cytoperm kit (BD Pharmingen) and staining with directly fluorescein isothiocyanate-conjugated (Pierce) anti-α-sarcomeric actinin antibody (clone EA-53; Sigma) or fluorescein isothiocyanate-conjugated (Pierce) anti-troponin I antibody (Sigma). At least 20,000 cells were analyzed on a BD FACSCalibur cell sorter (BD Biosciences).

For immunostaining, cells were fixed with PLP buffer for 15 min on ice. After permeabilization with 0.2% saponin (Sigma), cells were incubated with α-sarcomeric actinin (1:600; Sigma), followed by staining with fluorescein isothiocyanate-conjugated anti-mouse IgG (1:100; Jackson Laboratories). Nuclei were counterstained with TO-PRO-3 iodide (Molecular Probes).

Detection of Cell Fusion—Adenoviruses coding for enhanced GFP were generated by using pAdTrack-cytomegalovirus and pShuttle-cytomegalovirus (provided by B. Vogelstein) (22). 1 day after isolation, cardiomyocytes were infected (multiplicity of infection = 20). After 3 days, 98.7 ± 1.1% of cardiomyocytes were GFP^+ and subsequently used for co-cultivation with Dil-ac-LDL-labeled CPCs for an additional 6 days. GFP^+/Dil-ac-LDL^+ fused cells were measured by FACS at the time point indicated. The data were confirmed by manual counting of double-positive cells.

RNA Isolation and RT-PCR Analyses—Total RNA was isolated by using TRIzol (Invitrogen). RNA was subjected to RT-PCR by using the SuperScript One-Step RT-PCR with platinium *Taq* (Invitrogen). Because RNAs were from different species (human and rat), we designed human-specific primers to specifically monitor expression of human genes. Primers are as follows: human ANF, 5′-CTAGGTCAGACCAGAGCT-3′ and 5′-CATTCGGCTCACTGAGCA-3′); human β-MHC, 5′-CACAGCCCTCAAAGAGGC-3′ and 5′-CAGGGTGGAAGAGCCAAC-3′); and human glyceraldehyde-3-phosphate dehydrogenase, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. Rat mRNA was controlled by PCR against rat ANF (data not shown).

Western Blot Analysis—CPCs were incubated in LacZ-conditioned medium for 3 h. Then, the medium was changed to LacZ- or Wnt11-conditioned medium as indicated. Cells were lysed with lysis buffer (Cell Signaling) containing 1 mM phenylmethanesulfonyl fluoride. After centrifugation, the supernatants were collected and subjected to SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and incubated with anti-phospho-PKC (pan) antibody (Cell Signaling) or anti-β-actin antibody (Sigma) overnight at 4 °C. Bound antibody was visualized by using horseradish peroxidase-conjugated sheep anti-mouse antibody or donkey anti-rabbit antibody (both from Amersham Biosciences).

Statistical Analysis—Data are expressed as means ± S.E. Unpaired, two-tailed Student's *t* test was used for the comparison between groups based on the original data.

RESULTS

Effect of Wnt3A- and Wnt11-conditioned Medium on Cardiac Differentiation—Co-culturing of human adult CPCs with rat neonatal cardiomyocytes induced cardiac differentiation of human CPCs, as demonstrated by RT-PCR directed against human MHC and ANF; immunostaining against MEF2, ANF, and α-sarcomeric actinin; and FACS analysis (Fig. 1, *A* and *B*) (13). A representative confocal image is shown in Fig. 1*B*. Moreover, human cells showed calcium transients (13) and contraction (data not shown). Human CPCs, which co-express cardiac marker proteins, may derive from fusion with cardiomyocytes. Therefore, we determined the incidence of fusion events. Fusion was evaluated by co-culture of GFP-infected cardiomyocytes with Dil-ac-LDL-labeled CPC. Cells positive for GFP and Dil-ac-LDL were considered as fused cells. To compare differentiation and fusion, we measured GFP^+/Dil-ac-LDL^+ fused cells and α-$actinin^+$ human cells time-dependently (Fig. 1*C*). Before day 4 of the co-culture, the percentage of GFP^+/Dil-ac-LDL^+ fused cells and α-$actinin^+$ human cells was comparable, suggesting that all cells, which acquired a cardiomyogenic phenotype, represent fused cells. However, after day 6 of co-culture, the percentage of α-$actinin^+$ human cells was significantly higher than that of GFP^+/Dil-ac-LDL^+ fused cells, suggesting that differentiation starts after day 4 of co-culture.

To test the functional effects of Wnts, Wnt3A- and Wnt11-conditioned medium was added to the co-culture system, and cardiac differentiation of CPCs was quantified by FACS analysis. Co-incubation of Wnt11-conditioned medium significantly increased the number of α-$actinin^+$ human cells (Fig. 2, *A* and *B*) as well as troponin I^+ human cells (Fig. 2*C*). In contrast, the addition of Wnt3A-conditioned medium did not alter the differentiation rate during co-culture (Fig. 2*A*). Although Wnt3A-conditioned medium did not affect differentiation, it activated the β-catenin target gene siamois in *Xenopus* animal cap cells, indicating proper function of the medium (21) (Fig. 2*D*). Control experiments confirmed that the Wnt11-conditioned medium did not affect survival of CPCs in the co-culture assay (Fig. 2*E*).

Moreover, the addition of Wnt11-conditioned medium did not affect the number of GFP^+/Dil-ac-LDL^+ cells (Fig. 2*F*), indicating that Wnt11 enhances differentiation rather than fusion.

In order to determine whether Wnt11 alone is sufficient to induce differentiation, CPCs were incubated with Wnt11-conditioned medium in the absence of cardiomyocytes. Although CPCs showed some morphological change (enlargement of cell size), no cardiac-specific proteins were detected at day 7 (data not shown). Taken together, these data indicate that Wnt11 enhances differentiation of CPCs induced by co-culture with rat cardiomyocytes but is not sufficient to induce differentiation *per se*.

Wnt Signaling Pathways Involved in Cardiomyogenic Differentiation—Most Wnts signal via a canonical GSK-3β-dependent pathway. However, inhibition of GSK-3β by LiCl had no effect on the differentiation of CPCs to cardiomyocytes (Fig. 3*A*) but significantly increased the number of adherent surviving CPCs in the co-culture system (Fig. 3*B*).

In order to test whether Wnt11 may act via a non-canonical

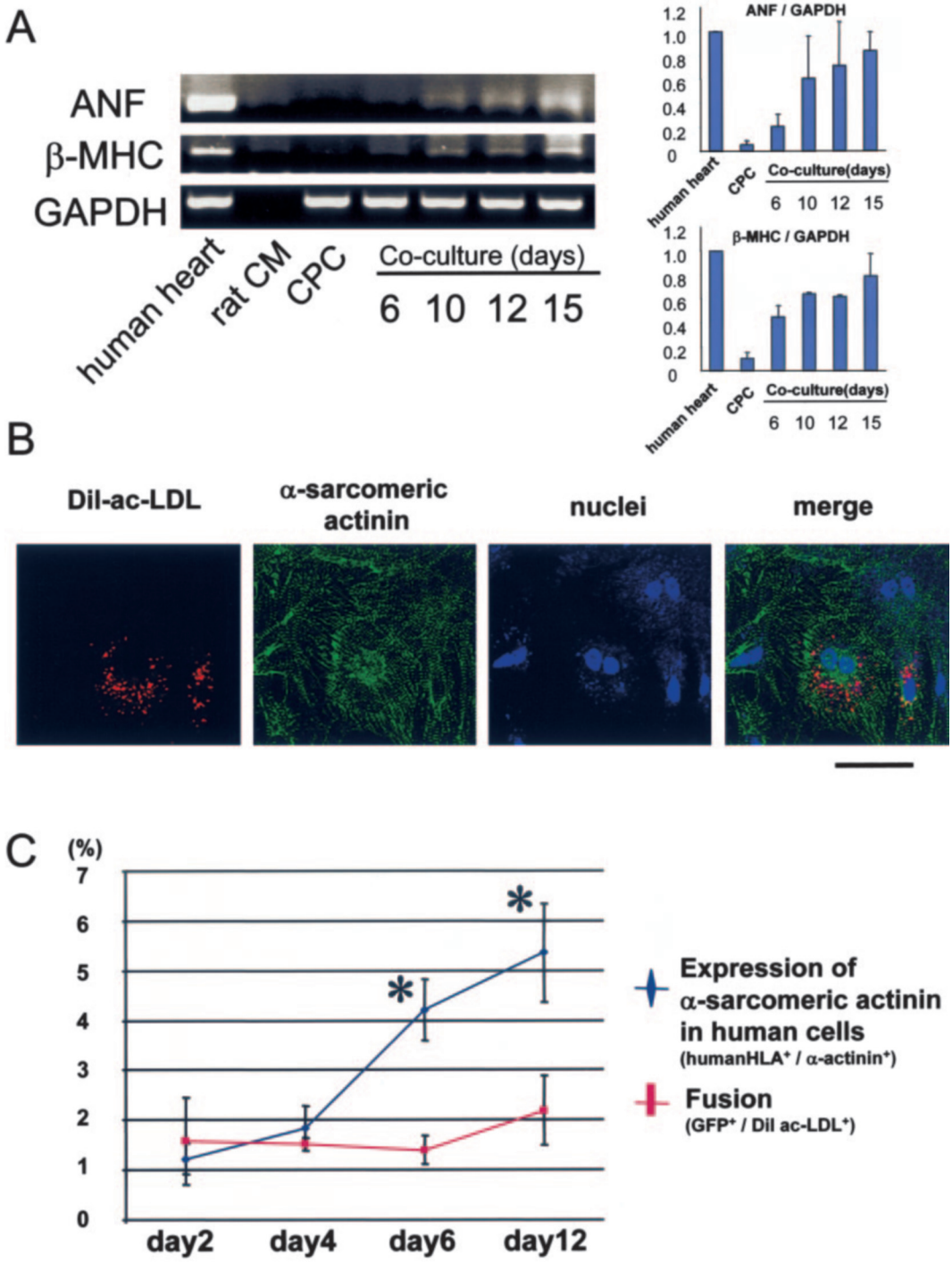

FIG. 1. *A*, expression of cardiac genes (ANF and β-MHC) by RT-PCR using human-specific primers. Human adult heart tissue and rat neonatal cardiac myocytes (*CM*) served as positive and negative controls, respectively. Gene expressions were controlled by human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Quantitative analysis is shown in the *right panels* ($n = 3$). *B*, representative confocal image 6 days after co-culture. Human CPCs were labeled by Dil-ac-LDL (*red*), and cardiomyocytes were detected by α-sarcomeric actinin (*green*). Nuclei are stained in *blue*. Representative confocal images from $n = 5$ experiments are shown. *Bar*, 50 μm. *C*, time course of fusion *versus* the expression of cardiac proteins. For detection of fusion events, GFP^+/Dil ac-LDL^+ cells were counted after incubation of GFP-transduced cardiac myocytes with Dil-ac-LDL-labeled CPCs. The expression of α-sarcomeric actinin in the human cells was determined by FACS analysis in non-transduced cells ($n = 3$–6). *, $p < 0.05$ *versus* fused cells at the same time point.

pathway, we investigated the effect of Wnt11-conditioned medium on PKC activation. Wnt11-conditioned medium time-dependently induced phosphorylation and thus activation of PKC in CPCs (Fig. 3, *C* and *D*). Moreover, inhibition of PKC by bisindolylmaleimide I and bisindolylmaleimide III significantly reduced differentiation of CPCs to cardiomyogenic cells (Fig. 3*A*) and PKC phosphorylation (Fig. 3*D*). The enhanced differentiation induced by Wnt11-conditioned medium was also inhibited by the PKC inhibitor (70.6 ± 11.7%, $p < 0.05$). Controls confirmed that the inhibition of cardiac differentiation by the PKC inhibitor used was not caused by a reduced survival (data not shown). In addition, PKC activation with phorbol 12-myristate 13-acetate significantly enhanced the CPC differentiation to 17.5 ± 4.6%. This increase was similar as compared with that of Wnt11-conditioned medium-treated cells (15.4 ± 2%).

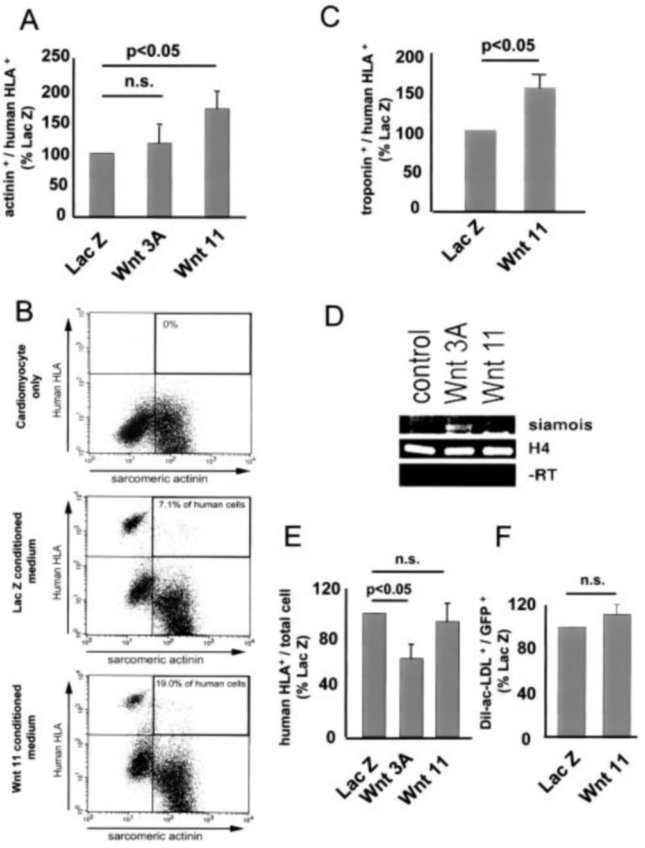

FIG. 2. *A*–*C*, the co-culture was incubated with Wnt3A- or Wnt11-conditioned medium for 6 days, and CPC differentiation was detected by FACS analysis by using α-sarcomeric actinin/human HLA (*A* and *B*) and troponin I/human HLA (*C*) double staining. LacZ-conditioned medium served as control. Negative controls were performed by using cardiomyocytes cultured for 6 days without CPCs ($n = 3$–10). *D*, Wnt3A, but not Wnt11, induces the TCF/β-catenin target gene siamois in *Xenopus* pluripotent precursor cells as monitored by RT-PCR. H4 served as loading control. *−RT*, negative control. *E*, CPC number (survival of CPCs) was detected by FACS analysis using the same method as described in *A*–*C*. LacZ-conditioned medium served as control ($n = 3$–10). *F*, cardiac myocytes were transduced with GFP adenovirus and co-cultured with Dil-ac-LDL-labeled CPCs. Fused cells were defined as cells positive for GFP and Dil-ac-LDL and detected 6 days after initiation of the co-culture. $n = 6$.

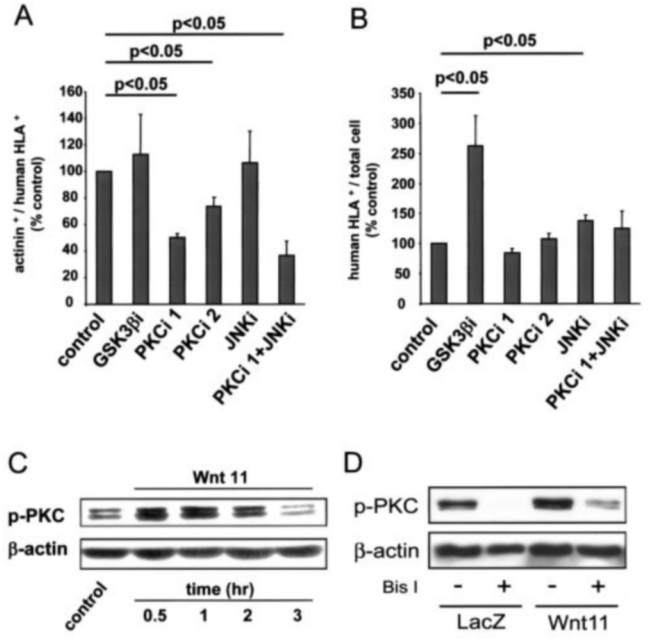

FIG. 3. *A* and *B*, effect of GSK-3β inhibitor (*GSK3βi*, 10 mM LiCl), PKC inhibitor (*PKCi 1*, 1 μM bisindolylmaleimide I), 2 μM bisindolylmaleimide III (*PKCi 2*), and JNK inhibitor (*JNKi*, 10 μM SP600125) on CPC differentiation (*A*) or survival (*B*) after incubation for 6 days in the co-culture assay ($n = 4$–7). Differentiation was defined as the percentage of cells expressing human HLA and α-sarcomeric actinin. Survival was defined as the percentage of human HLA^+ cells per total cell number. *C* and *D*, immunoblot of CPCs cultured in LacZ (control)- or Wnt11-conditioned medium for the indicated times with or without PKC inhibitor (bisindolylmaleimide I); 1-h incubation: 1.5 ± 0.15-fold increase, $p < 0.05$ compared with LacZ; $n = 4$.

Because Wnt11 also activates the mitogen-activated protein kinase JNK, we examined the effect of the JNK inhibitor SP600125. However, addition of the JNK inhibitor did not affect cardiac differentiation of CPCs (Fig. 3*A*). Moreover, the combination of a PKC inhibitor with a JNK inhibitor was not superior to the PKC inhibitor alone (Fig. 3*A*), suggesting that JNK does not play a major role in cardiac differentiation in the co-culture assay.

DISCUSSION

The data of the present study demonstrate that Wnt11 facilitates the differentiation of CPCs to cardiomyogenic cells. The addition of Wnt11 enhanced CPC differentiation. Interestingly, only Wnt11, but not Wnt3A, promoted cardiac differentiation. Wnt3A and related family members such as Wnt8a were recently shown to increase differentiation of PC19 cells to cardiomyocytes via the canonical GSK-3β signaling pathway (19). However, other studies demonstrated that activation of the canonical Wnt pathway does not promote differentiation and even markedly inhibited differentiation of embryonic stem cells (23). These conflicting reports in the literature regarding the responses of stem/progenitor cells toward Wnts may be due to the diversity of signaling cascades (canonical *versus* non-canonical) and possibly to the type and differentiation status of the stem/progenitor cell. Interestingly, in the present study, inhibition of GSK-3β did not affect differentiation but increased survival of CPCs, suggesting that the canonical Wnt pathway affects cell survival but not differentiation in this system. In line with these findings, Wnt11 activated the non-canonical PKC pathway in CPCs as shown previously in *Xenopus* embryonic pluripotent precursor cells (17).

A causal contribution of the non-canonical PKC-dependent Wnt pathway for cardiac differentiation is supported by the finding that inhibition of PKC blocked cardiac differentiation, whereas activation of PKC increased cardiac differentiation of CPCs to a similar extent as compared with Wnt11-conditioned medium. In contrast, inhibition of JNK did not significantly affect differentiation. Taken together, these data indicate that the non-canonical PKC-dependent pathway plays a predominant role for cardiac differentiation, whereas JNK appears to be of minor importance. The question remains which PKC isoforms are involved? Because differentiation of CPCs to cardiac myocytes in the co-culture system is calcium-dependent (13) and phorbol 12-myristate 13-acetate enhanced cardiac differentiation, it is tempting to speculate that the classical PKC isoforms, which require both calcium and lipids (α, β1, β2, and γ), are mediating the effect. Indeed, the PKC β1 and β2 isoforms are highly expressed in CPCs (data not shown).

Although our study demonstrates that the non-canonical PKC pathway contributes to cardiac differentiation of CPCs, the upstream signaling remains to be determined. Wnt11 is known to act via the frizzled receptors Fz2 and Fz7. Both receptors are expressed in CPCs (data not shown). Importantly, Wnt11 was not sufficient to induce cardiac differentiation of CPCs in the absence of co-culturing with neonatal rat cardiac myocytes. Thus, Wnt11 rather amplifies cardiac differentiation

induced by the physical interaction of CPCs with cardiomyocytes. Clearly, our data document that Wnt11 does not increase the number of human-derived cardiac myocytes by promoting cell fusion as assessed by co-cultivation of GFP-labeled cardiomyocytes with Dil-ac-LDL-labeled CPCs. Moreover, Wnt11 also promoted cardiac differentiation in a previously established experimental set-up (13), in which CPCs were co-cultured with fixed cardiomyocytes, which precludes fusion events (data not shown). Interestingly, previous studies showed that activation of Fz2 induces myocyte aggregation and adhesion in the presence of fibroblasts, resulting in activation and complex formation of cadherins (24). Thus, one may speculate that Wnt11 may amplify cell-to-cell aggregation and cadherin interactions, finally resulting in enhanced differentiation. Overall, the augmentation of cardiac differentiation of human adult progenitor cells may be well suited to promote cardiac regeneration by cell therapy.

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