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Increased Connexin 43 Expression Improves the Migratory and Proliferative Ability of H9c2 Cells by Wnt-3a Overexpression

Xiaoyu LIU[1#], Wen LIU[1#], Ling YANG[1#], Beili XIA[1], Jinyan LI[1], Ji ZUO[1]*, and Xiaotian LI[2]*

[1] *Department of Cellular and Genetic Medicine, Shanghai Medical College, Fudan University, Shanghai 200032, China;*
[2] *The Obstetrics and Gynecology Hospital of the Medical Center of Fudan University, Shanghai 200032, China*

Abstract The change of connexin 43 (Cx43) expression and the biological behaviors of Cx43 in rat heart cell line H9c2, expressing Wnt-3a (wingless-type MMTV integration site family, member 3A), were evaluated in the present study. Plasmid pcDNA3.1/Wnt-3a was constructed and transferred into H9c2 cells. The cell model Wnt-3a$^+$-H9c2 steadily expressing Wnt-3a was obtained. Compared with H9c2 and pcDNA3.1-H9c2 cells, the expression of Cx43 in Wnt-3a$^+$-H9c2 cells was clearly increased, the proliferation of Wnt-3a$^+$-H9c2 cells was significantly changed, and cell migration abilities were also improved (P<0.05). In comparison with H9c2 and pcDNA3.1-H9c2 cells, the G_2 phase of the cell cycle increased by 11% in Wnt-3a$^+$-H9c2 cells. Thus, Wnt-3a overexpression is associated with an increase in Cx43 expression and altered migratory and proliferative activity in H9c2 cells. Cx43 might be one of the downstream target genes regulated by Wnt-3a.

Keywords Wnt-3a; Cx43; H9c2 cell line; cell migration

Gap junctions are aggregates of intercellular channels that permit intercellular exchange of ions, small metabolites and low molecular mass signaling molecules between cells [1]. Studies over the last decade have revealed that gap junctions are encoded by a multigene family known as the connexins. They obtain selective permeability by the use of different connexin family members [2]. By regulating the direct exchange of ions and small molecules between cells, gap junction channels have been implicated in a diverse assortment of biologic processes including cellular differentiation and development, and metabolic homeostasis [3]. Impairment of gap junctional intercellular communication, caused by mutations or loss of function of connexins, especially connexin 43 (Cx43) which is expressed in several tissues and organs, like heart, gonads, lens and skin, is involved in a number of diseases [4].

One potential regulator of gap junction expression and function that might have important implications for developmental processes as well as for normal function in adult stages is the wingless-type MMTV integration site (Wnt) family of genes. Wnt genes encode a large family of secreted polypeptides that can influence cell-cell communication, both during embryonic development and in adult life [5]. In *Xenopus* embryos it was found that Wnt-1 and Wnt-8 induced an increase in intercellular communication mediated by gap junctions [6]. Interestingly, during mouse embryogenesis Cx43 is expressed as a gradient at the mid/hindbrain border which matches Wnt-1 expression at this stage in the same region [7]. Cx43 is by far the most abundant cardiovascular gap junction channel protein, found in both atrial and ventricular gap junctions in perinatal death in mice [8]. Although this congenital cardiac defect was unexpected and its occurrence remains unexplained, studies support the hypothesis that loss of function of Cx43 might account for this phenotype [9]. Gap junction communication mediated by Cx43 plays an important role in cardiac neural crest migration. The reducing gap junction communication in cardiac crest cells also resulted in a reduction in the rate

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# These authors contributed equally to this work
*Corresponding authors:
Ji ZUO: Tel, 86-21-54237091; E-mail, jzuo@shmu.edu.cn
Xiaotian LI: Tel, 86-21-63770161; E-mail, xiaotianli555@163.com

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of crest migration. Cardiac-restricted conditional Cx43-deficient mice show a similar effect in ventricular tissue [10]. Complete loss of Cx43 expression in the mouse leads to a number of developmental consequences, including obstruction of the right ventricular outflow tract [11]. Therefore the correct timing and amount of Cx43 expression appears to be essential for normal neural and cardiac development. Although several studies have suggested an important association between Wnt signaling and normal heart formation and function, the relationship between Wnt-3a proteins and Cx43 expression in the mammalian heart has not been investigated.

In this study, we tried to determine whether Wnt-3a proteins regulate Cx43 expression and function in H9c2 cells.

Materials and Methods

Plasmid construction

The *Rattus Wnt-3a* full-length coding sequence was amplified by polymerase chain reaction (PCR) using the primers containing *Eco*RI and *Bam*HI restriction sites: Wnt-3a-sense, 5′-CCGGAATTCATGGCTCCTCTCGGA-TACCT-3′; and Wnt-3a-antisense, 5′-CGCGGATCC-CTTGCAGGTGTGCACGTCATA-3′. The PCR product was subcloned into the pcDNA3.1 expression vector that contains the neomycin resistant gene. The recombinant plasmid construct, pcDNA3.1/Wnt-3a, was confirmed by *Bam*HI/*Eco*RI digestion (New England Biolabs, Beverly, USA) and DNA sequencing (Invitrogen, Carlsbad, USA).

Cell culture and stable cell line generation

Rat heart cell line H9c2 cells, purchased from American Type Culture Collection (Manassas, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.6% penicillin/streptomycin (Invitrogen) at 37 ºC with 5% CO_2 in a humidified atmosphere. Stable *Rattus Wnt-3a* expressing H9c2 cells were generated by transfection of a pcDNA3.1/Wnt-3a expression vector containing the entire *Rattus Wnt-3a* cDNA. Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with modification. Briefly, 1×10^5 cells in 1 ml of DMEM containing 10% FBS were seeded in 60-mm dishes and grew to 70%–75% confluence. pcDNA3.1/Wnt-3a DNA (8 μg) and Lipofectamine 2000 were added separately in tubes each containing 500 μl of DMEM, mixed well and allowed to stand for 5 min at room temperature. These two mixtures were then combined together and incubated for 20 min at room temperature. One milliliter of medium containing Lipofectamine packaged plasmid DNA was added to the 60-mm dish and mixed gently by rocking the plate back and forth. The medium was refreshed after 6 h of transfection and cells were incubated at 37 ºC with 5% CO_2 in a humidified atmosphere for 24 h. Then cells were passaged at 1:10 into fresh DMEM supplemented with 10% FBS. Stable clones were obtained by G418 selection (600 μg/ml) (Calbiocham, San Diego, USA) for 2–3 weeks. Individual clones were isolated and cultured further in the presence of 250 μg/ml G418. For the control, empty vector pcDNA3.1 was transfected and cells were harvested after selection.

Western blot analysis

H9c2 cells, stably transfected with Wnt-3a constructs, were washed twice in cold phosphate-buffered saline (PBS), lysed in radio-immunoprecipitation buffer [pH 8.0, 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 1 mM phenylmethyl sulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin] and harvested on ice by scraping. After sonication for 10 s and centrifugation at 4 ºC for 10 min at 13,000 *g*, the supernatants were denatured by heating at 95 ºC for 5 min in protein sample buffer (295 mM sucrose, 2% SDS, 2.5 mM EDTA, 62.5 mM Tris-HCl, pH 8.8, 0.05% bromophenol blue, 26 mM dithiothreitol). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Biocolors, Shanghai, China). A total of 30 μg protein of cellular lysates was then electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidine difluoride membranes by electroblotting. The membranes were incubated in 5% nonfat milk overnight, then incubated with mouse anti-Wnt-3a or anti-Cx43 (1:500; Zymed Laboratories, San Francisco, USA) antibody for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, USA) for 1 h. Immunoreactive proteins were visualized using a commercially available enhanced chemiluminescence kit (Pufei, Shanghai, China) with exposure of the transfer membrane to X-ray film (Eastman Kodak, Rochester, USA).

To normalize protein loading, the membranes were gently stripped of antibodies and immunoblotted for β-actin (1:20,000 dilution; Sigma-Aldrich, St. Louis, USA). The density of the bands on the membranes was scanned and results were quantified using Scion Image version 4.03

software (Scion, Frederick, USA).

Real-time PCR

Total RNA was isolated from H9c2, Wnt-3a$^+$-H9c2 and pcDNA3.1-H9c2 cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The first-stranded complementary DNA was synthesized from 2 μg of total RNA using a RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. Reverse transcription (RT) was carried out at 42 ºC for 30 min following incubation at 95 ºC for 5 min. cDNA amplification was carried out according to the following temperature profile: 94 ºC, 30 s; 55 ºC, 30 s; and 72 ºC, 1 min. At the end of 33 cycles, the reaction was prolonged for 10 min at 72 ºC, then 5 μl of product was analyzed on a 1.5% agarose gel. The following are the sequences of the primers: Cx43 sense, 5′-TTGTTTCTGTCACCAGTAAC-3′; Cx43 antisense, 5′-GATGAGGAAGGAAGAGAAGC-3′; β-actin sense, 5′-AGGCATCCTGACCCTGAAGTAC-3′; β-actin antisense, 5′-GAGGCATACAGGGACAACACAG-3′. The intensity of the bands was measured with Scion Image version 4.03 software.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was carried out on cDNA generated from 0.3 μg of total RNA using a RevertAid First Strand cDNA Synthesis Kit. A total of 30 ng of cDNA, 200 nM both sense and antisense primers, and iQ SYBR Green Supermix (Bio-Rad, Hercules, USA) in a final volume of 25 μl was used for PCR. Amplification and detection of specific products were carried out with the Mini Opticon Real-Time PCR System (Bio-Rad) with the following cycle profile: 1 cycle at 94 ºC for 2 min; 40 cycles at 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 85 s; and 1 cycle at 72 ºC for 5 min. The melting curve from 65 ºC to 90 ºC was read every 0.3 ºC. As an internal control, β-actin primers were used for RNA template normalization. Fluorescent signals were normalized to an internal reference (ΔRn), and the threshold cycle (Ct) was set within the exponential phase of the PCR. The relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the β-actin Ct value, which gives the ΔCt value. From this value, the relative expression level between treatments can be calculated using the following equation: relative gene expression=$2^{-[\Delta Ct(\text{Sample})-\Delta Ct(\text{Control})]}$.

Cell proliferation assay

Cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. H9c2, Wnt-3a$^+$-H9c2 and pcDNA3.1-H9c2 cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After different times of incubation, cells were used for experiment. Briefly, 25 μl of MTT per well (5 mg/ml, dissolved in D-Hanks' solution) was added to the 96-well plates and incubated at 37 ºC for 4 h, then 100 μl of DMSO was added. Two hours later, the absorbance was determined using a multiplate reader (Thermo, Anaheim, USA) at a wavelength of 490 nm (A_{490}).

Cell cycle analysis

A fluorescence-activated cell sorter (FACS) analysis was carried out for the cells after synchronizing for 72 h with a plating cell count of 1×10^4 in each flask. Cells were washed twice with PBS, fixed in 70% methanol, then kept at −20 ºC overnight. The suspension was centrifuged at 800–1000 rpm for 5 min and the pellet was gently washed twice with PBS. To the final pellet, 4 μl of RNase (1 mg/ml) and 100 μl of propidium iodide (500 μg/ml) (Sigma-Aldrich) were added and each sample was kept at 4 ºC for 30 min. The samples were then assessed on a Becton Dickinson FACSCalibur system (BD Biosciences, Franklin Lakes, USA). They were excited with an argon laser and the emission (emay) was collected at 615 nm. The data were analyzed by ModFit LT software (version 2.0; Verity Software House, Topsham, USA).

Cell migration

The migration ability of the cells was assessed by an *in vitro* model, transwell migration assay [12]. Cells (5×10^4 cells per well) were resuspended in DMEM containing 1% fetal serum and seeded into the upper wells of the chamber with 8 μm pores (Costar, Cambridge, USA), which was situated in a well of a 24-well culture plate and immersed in the 10% fetal serum culture medium. For the control group, 1% fetal serum culture medium was used. After incubation at 37 ºC for 48 h, the cells were fixed with 4% paraformaldehyde and stained with haematoxylin and eosin. After removing the cells attached on the upper side of the membrane by wiping with a wet cotton swab, those attached on the bottom side of the membrane were counted under a microscope. Invasiveness was evaluated by the number of cells penetrating through the membrane per field.

Statistical analysis

Data were expressed as mean±SE. Statistical significance was determined using the Newman-Keuls test. Differences with $P<0.05$ compared with the normal group

were considered significant.

Results

Construction and identification of the Wnt-3a expressing vector pcDNA3.1/Wnt-3a

The *Rattus Wnt-3a* gene (1.0 kb) was cloned into the multicloning site of the pcDNA3.1 vector (5.4 kb), designated as pcDNA3.1/Wnt-3a (6.4 kb), and verified by *Bam*HI and *Eco*RI restriction endonuclease digestion (**Fig. 1**). The complete sequence of *Wnt-3a* was further verified by sequencing (data not shown).

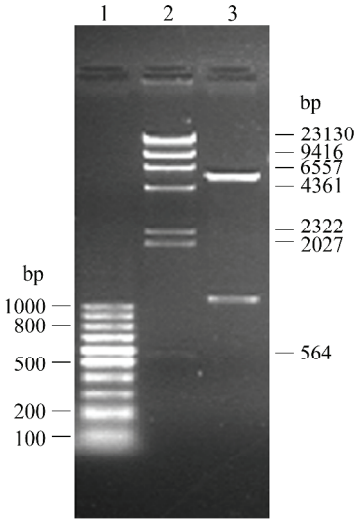

Fig. 1 Analysis of recombinant pcDNA3.1/Wnt-3a digested with restriction enzyme using 1% agarose gel electrophoresis
1, 100 bp DNA ladder; 2, Lambda DNA/*Hin*dIII marker; 3, pcDNA3.1/Wnt-3a digested with *Bam*HI and *Eco*RI.

Wnt-3a increases expression of Cx43 in H9c2 cells

H9c2 cells were transfected with *Rattus Wnt-3a* and a neomycin resistance gene and selected for stable transfectants. To check for Wnt-3a expression we carried out Western blot analysis on protein extracts of wild-type and Wnt-3a transfected clones. Compared to untransfected groups, an increased single band of the expected size (40 kDa) was observed in two independent Wnt-3a expressing clones, designated as Wnt-3a-1 and Wnt-3a-3 (**Fig. 2**, lanes 1 and 4). When using the anti-β-actin antibody, a band of the expected size was found in equivalent amounts in both Wnt-3a$^+$-H9c2 and untransfected H9c2 cell lines without change.

To compare Cx43 protein levels, cell lysates were prepared from H9c2 and Wnt-3a$^+$-H9c2 cell lines. Subsequently, Cx43 was immunoprecipitated from 1 mg of total protein for each lysate, and immunoprecipitates were analyzed for Cx43 levels by Western blot. As also shown in **Fig. 2**, Cx43 protein is expressed in non-transfected H9c2 cells and gave a single stained band of 43 kDa on SDS-polyacrylamide gel electrophoresis by immunoblotting using anti-Cx43 antibody (**Fig. 2**, lane 2). Cx43 protein levels were clearly increased in Wnt-3a expressing cell lines (**Fig. 2**, lanes 1 and 4). Here, the internal control, β-actin, was also almost equally expressed in different groups.

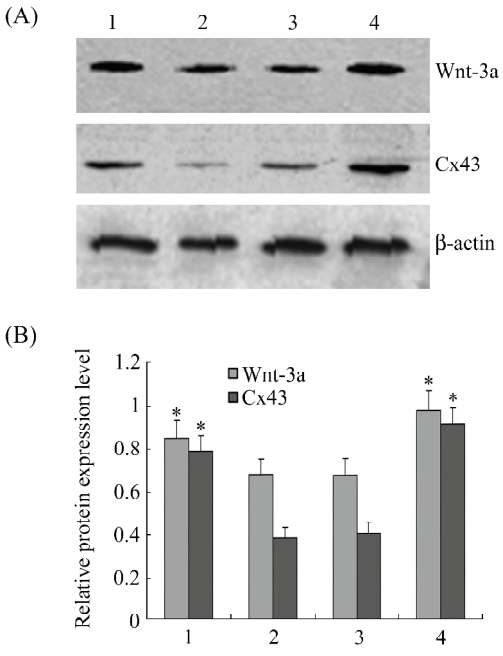

Fig. 2 Characterization of Wnt-3a and connexin 43 (Cx43) protein expression in wild-type and stably transfected H9c2 rat heart cell lines determined by Western blot (A) and densitometry (B) analysis
1 and 4, H9c2 cells transfected with pcDNA3.1/Wnt-3a (clones 1 and 3); 2, untransfected H9c2 cells; 3, H9c2 cells transfected with blank pcDNA3.1 vector. Data are expressed as mean±SE from three independent experiments. * P<0.05 versus H9c2 cells.

We then analyzed if the expression of the gap-junctional Cx43 gene was altered in the Wnt-3a$^+$-H9c2 expressing cells. Therefore, changes in Cx43 mRNA expression in the Wnt-3a cell lines were examined by RT-PCR. Interestingly, the amount of Cx43 PCR product yielded

from Wnt-3a$^+$-H9c2 cells, derived RNA preparations, appeared to be increased compared to wild-type H9c2 cells (**Fig. 3**). As an internal control, β-actin was almost equally expressed in different groups. These findings are consistent with previous report on PC12 cells [27].

These PCR findings were confirmed by real-time quantitative RT-PCR analysis, and are presented in **Fig. 4**. The relative gene expression level of Cx43 was significantly increased (2.38-fold) in Wnt-3a$^+$-H9c2 cells when compared with the normal control group (P<0.05, n=3) [**Fig. 4(C)**].

Cell proliferation

Proliferation of the cells was determined at 12, 24, 36, 48, 60 and 72 h of culture. The growth curves are shown in **Fig. 5**. The Wnt-3a$^+$-H9c2 cell proliferation increased compared to the control after 24 h of culture.

FACS

FACS analysis was applied to study the cell cycle distribution in H9c2 cells transfected with Wnt-3a. Using H9c2 cells untransfected and transfected with blank vector (pcDNA3.1) as controls, we found the percentage of Wnt-3a$^+$-H9c2 cells in the G_2 phase increased by 11% and that in the G_1 phase decreased by 11%, whereas the proportion of Wnt-3a$^+$-H9c2 in the S phase was almost unchanged (**Fig. 6**).

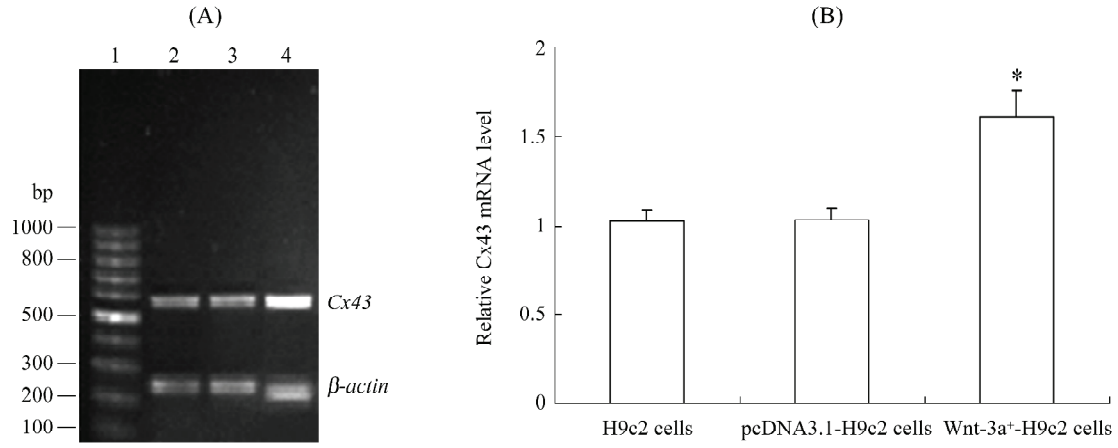

Fig. 3 Expression of connexin 43 (Cx43) mRNA in H9c2 rat heart cells determined by reverse transcription-polymerase chain reaction

(A) Lane 1, 100 bp DNA ladder; lane 2, bands corresponding to cDNA fragments amplified from untransfected H9c2 cells; lane 3, H9c2 cells transfected with blank pcDNA3.1 vector; lane 4, H9c2 cells transfected with pcDNA3.1/Wnt-3a. (B) Results were quantified by densitometry analysis as described in "Materials and Methods". Data are expressed as mean±SE from three independent experiments. * P<0.05 versus H9c2 cells.

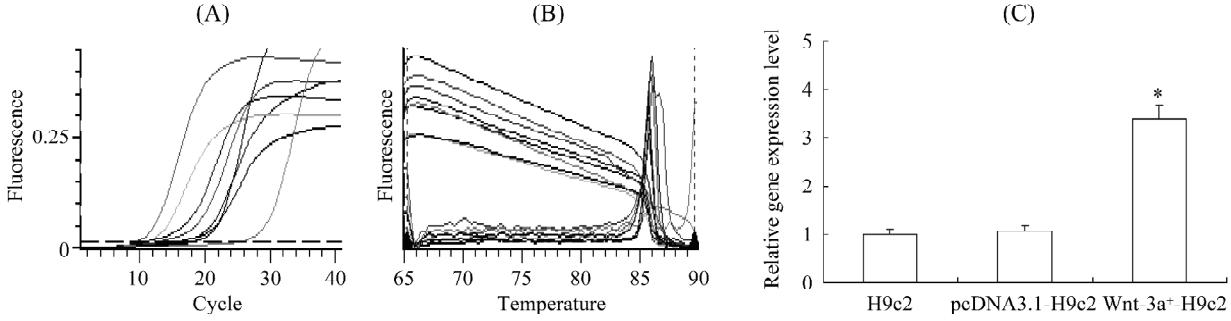

Fig. 4 Expression of connexin 43 (Cx43) mRNA in cells determined by real-time quantitative polymerase chain reaction analysis

(A) Increase in fluorescence intensity with the increase in polymerase chain reaction cycle number. (B) Melting curve analysis of RNA amplification products. (C) The relative gene expression levels are represented as the ratios of the values in H9c2 cells transfected with pcDNA3.1/Wnt-3a or H9c2 cells transfected with blank pcDNA3.1 vector versus the corresponding H9c2 cells group. Data are expressed as mean±SE of three independent experiments. * P<0.05 versus H9c2 cells.

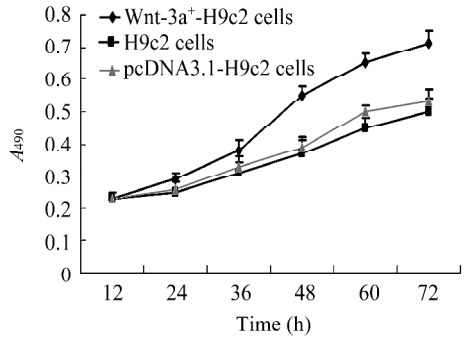

Fig. 5 Cell proliferation in H9c2, Wnt-3a$^+$-H9c2 and pcDNA3.1-H9c2 cells

All cells were seeded in 96-well plates, each well containing approximately 5×10^3 cells. After 12, 24, 36, 48, 60 or 72 h, cells were washed once with phosphate-buffered saline and cell viabilities were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The Wnt-3a$^+$-H9c2 cell proliferation increased compared to H9c2 cells and pcDNA3.1-H9c2 cells after 24 h of culture.

Improvement of Wnt-3a$^+$-H9c2 cell migration ability

The results of transwell migration assay demonstrated that H9c2 cells possessed the ability to migrate across the pored filter membrane. Five independent visual fields were selected to calculate cell numbers, and 60.3±4.1 seeded cells migrated to the bottom side of the filter membrane in 48 h. **Fig. 7(A)** shows representative fields of H9c2 cells that successfully migrated across the chamber filters. Furthermore, the cell migration of Wnt-3a$^+$-H9c2 cells was significantly enhanced in the presence of 10% fetal serum (101.1±6.2, $P<0.05$) [**Fig. 7(B)**], compared that of H9c2 cells in the presence of 1% fetal serum [**Fig.7 (C)**], when only a few H9c2 cells migrated through the pores (10.5±1.3).

Discussion

Changes in Wnt signaling and microtubule function are reported to affect the connexin level [13]. Wnt signaling has also been shown to be an important modulator of Cx43-dependent intercellular coupling in the heart [14]. However, the molecular nature of Wnt-induced Cx43 expression is still unclear. In the heart, three major connexin isotypes are expressed: Cx43, Cx45, and Cx40 [15]. Each of these connexins shows different channel properties and is regulated by different gating mechanisms [16]. Cx43 is the only connexin known to be expressed in the adult working myocardium, and might additionally facilitate coupling to the surrounding working myocytes [5]. There is general agreement that gap junction channels formed by the Cx43 protein most likely have important roles during heart development [17]. Slowing in the spread of activation was reported in an earlier study of the heterozygous Cx43 knockout mouse [18]. Studies in CMV43 transgenic mice showed that there were cell proliferation changes in the myocardium [19]. Thus, the regulation of Cx43 expression is very important in heart development and function.

In the present study, the effect of Wnt-3a on Cx43 expression and function in cardiac muscle cells was investigated. To this end, we used the clonal rat myocyte cell line H9c2 as a cell model of cardiac myocytes. This cell line is derived from embryonic rat heart tissue and has properties of skeletal muscle [20] as well as cardiac muscle [21,22]. As H9c2 cells possess properties of cardiac muscle, they are commonly used as a model system to

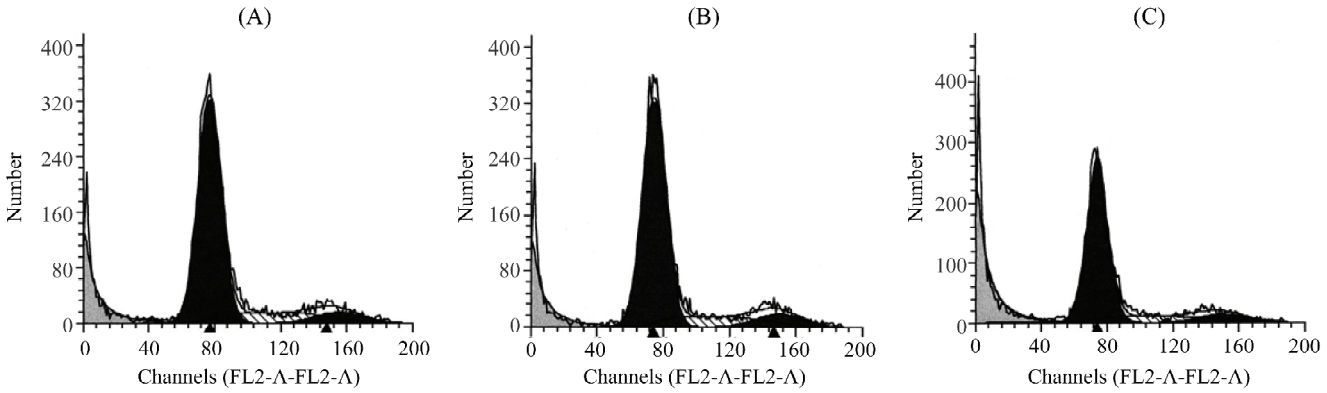

Fig. 6 Effect of Wnt-3a on cell cycle distribution analyzed by flow cytometry assay

(A) Untransfected H9c2 cells. G_1, 76.91%; G_2, 7.58%; S, 15.51%. (B) H9c2 cells transfected with blank pcDNA3.1 vector. G_1, 76.74%; G_2, 8.44%; S,14.82%. (C) H9c2 cells transfected with pcDNA3.1/Wnt-3a vector. G_1, 65.86%; G_2, 18.76%; S, 15.38%.

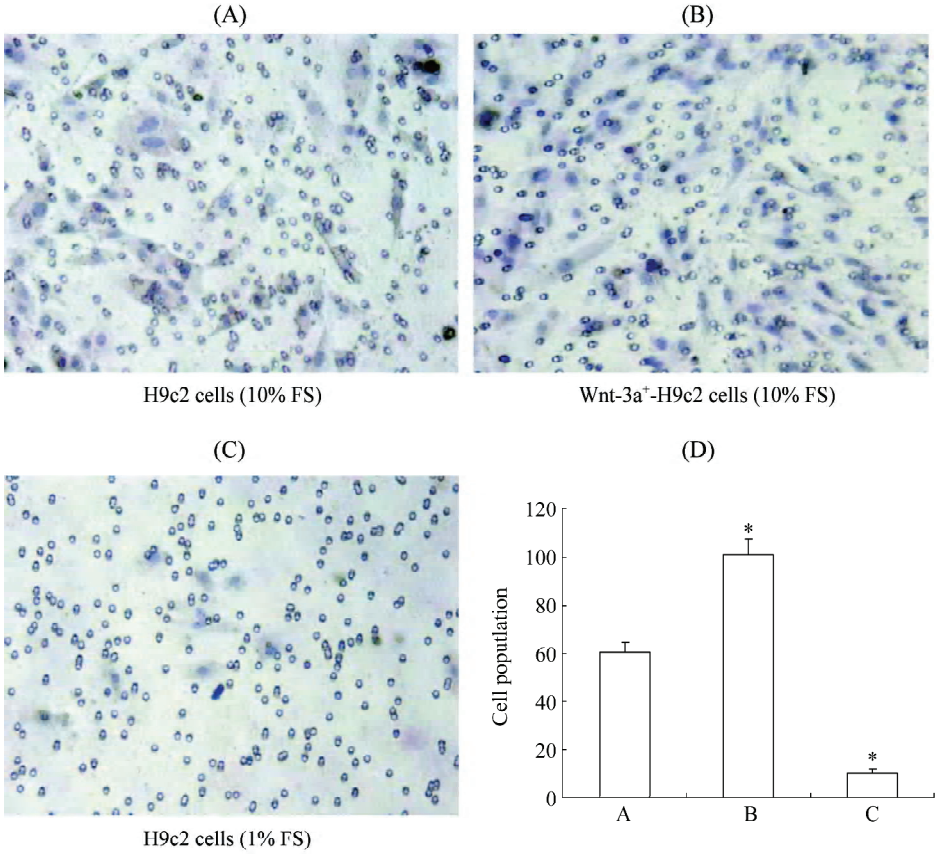

Fig. 7 Cell migration of H9c2 cells and Wnt-3a$^+$-H9c2 cells

Figures show the migrated cells on the bottom side of the polycarbonate membranes stained with hematoxylin and eosin. They were observed by light microscopy and captured by the CCD digital camera (Leica Leica Camera AG, Solms, Germany). (A–C) Photomicrographs of the bottom side of filters with 10% fetal serum (FS) (A,B) and with 1% FS (C). The pores of the filter membrane with some migrated cells was shown in (C). Magnification, 200×. (D) The quantitative summary of the experiment illustrated in figures (A–C). Data are expressed as mean±SE from five independent experiments. * P<0.05 versus H9c2 cells with 10% FS.

study metabolic properties of myocardial tissue [23–26]. Our results show that the overexpression of *Rattus Wnt-3a* in this cell line leads to similar changes as seen for murine Wnt-1 [27]. From the comparison of Cx43 expression as measured at the RNA and protein levels (**Figs. 3–5**), it is suggested that Wnt-3a signaling might also regulate the Cx43 protein level. Interestingly, an increase in Cx43 expression has also been observed by the overexpression of quail Wnt-11 in a mesodermal cell line [28]. This indicated that the increase in Cx43 expression observed by us might reflect a more basal function among the different Wnt family members [29].

In parallel with the increased Cx43 we found changes in the cell cycle and proliferation of H9c2 cells transfected with pcDNA3.1-Wnt-3a. One of the most profound changes of Wnt-3a expressing H9c2 cells in contrast to wild-type H9c2 was the improvement in migration ability, confirming that Cx43 plays an important role in cell migration. Earlier studies describing PC12 cells overexpressing murine Wnt-1 indicated that these PC12 cells displayed an increased cellular adhesion probably mediated by E-cadherin [30]. Here, the improvement of cell migration was positively related to the increase in Cx43 expression in H9c2 cells. These aspects are all critical for the cells to maintain their behavior, even gap junction communication.

Several converging lines of evidence have suggested that the effects of Wnt signaling might result in part through the modulation of gap junction channel activity. Here we found that Wnt-3a was also a specific and potent inducer of Cx43 expression in H9c2 cells and that this effect resulted in enhanced accumulation of Cx43 protein that might form functional gap junction channels.

In summary, our results suggest that Wnt-3a

overexpression in H9c2 cells increases Cx43 expression and results in increased proliferation and migration ability. Our current studies are directed toward more fully understanding of the potential contribution of regulated Wnt signaling to abnormal connexin gene expression and gap junctional remodeling, both during cardiac development and, in particular, in the setting of various forms of heart disease.

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Edited by
Minghua XU