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Inhibition of proliferation and differentiation and promotion of apoptosis by cyclin L2 in mouse embryonic carcinoma P19 cells

Lili Zhuo, Jie Gong, Rong Yang, Yanhui Sheng, Lei Zhou, Xiangqing Kong *, Kejiang Cao

Department of Cardiology, The First Affiliated Hospital of Nanjing Medical University, No. 300 Guangzhou Road, Nanjing 210029, PR China

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ABSTRACT

Cyclin L2 (CCNL2) is a novel member of the cyclin gene family. In a previous study, we demonstrated that CCNL2 expression was upregulated in ventricular septum tissues from patients with ventricular septal defect compared to healthy controls. In the present study, we established a stable CCNL2-overexpressing P19 cell line that can differentiate to myocardial cells when treated with 1% dimethyl sulfoxide (DMSO). Our data showed that stable CCNL2-overexpressing P19 cells were less differentiated after treatment with 1% DMSO and that expression of myocardial cell differentiation-related genes (such as cardiac actin, GATA4, Mef2C, Nkx2.5, and BNP) were reduced compared to vector-only transfected P19. Moreover, P19 cells overexpressing the CCNL2 gene had a reduced growth rate and a remarkably decreased S phase. We also found that these cells underwent apoptosis, as detected by two different apoptosis assays. The anti-apoptotic Bcl-2 protein was also downregulated in these cells. In addition, real-time PCR analysis revealed that expression of Wnt and β-catenin was suppressed and GSK3β was induced in the CCNL2-overexpressing P19 cells. These data suggest that overexpression of CCNL2 inhibited proliferation and differentiation of mouse embryonic carcinoma P19 cells and induced them to undergo apoptosis, possibly through the Wnt signal transduction pathway.

Introduction

Congenital heart defects (CHDs) are the most common birth defect, occurring in approximately 8 per 1000 live births or 40,000 cases each year [1]. A CHD is a defect in the structure of the heart and great vessels of a newborn, resulting in either obstruction of blood flow into the heart or vessels or an abnormal pattern of blood flow through the heart. CHDs are a significant cause of death in infancy [1,2], thus a better understanding of the causes of these relatively common developmental malformations is urgently needed. Although most CHD-responsible genes remain to be defined molecularly, significant advances have been made in recent years in identifying CHD-related genes and understanding how gene mutations cause specific heart defects [3,4]. For example, a number of genes (such as NKX2.5 [5,6], GATA4 [7,8], and TBX5 [9,10]) are known to be associated with atrial and ventricular septal defects.

In a previous study, we performed a human cDNA microarray analysis and found 1056 genes that were differentially expressed in the tissue specimens from patients with atrial septal defect (ASD) compared with normal controls [11]. More recently, we analyzed the gene expression profiles of ventricular septal defect (VSD) and identified 299 genes that are upregulated in VSD and 252 genes that are upregulated in normal hearts [12]. Notably, human cyclin L2 (CCNL2) was simultaneously upregulated in ASD and VSD, indicating that it may play a role in causing human CHDs.

In this study, we determined the effects of CCNL2 on proliferation, apoptosis, and differentiation of mouse embryonic carcinoma P19 cells in vitro. We used P19 cells because they can undergo myocardial cell differentiation after treatment with 1% dimethyl sulfoxide (DMSO). Under this condition, P19 cells may mimic some behaviors of myocardial cells. We also investigated the underlying molecular mechanisms responsible for CCNL2 activity.

Materials and methods

Cell culture and induction of cell differentiation. Mouse embryonic carcinoma P19 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in α-MEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C with 5% CO_2. COS-7 and HeLa cells from ATCC were maintained in DMEM with 10% FBS, 100 U/mL penicillin, and 50 μg/mL streptomycin. P19 cell differentiation was induced using 1% DMSO as described previously [13]. Briefly, P19 cells were grown in α-MEM supplemented with 7.5% FBS and 2.5% calf serum until confluence. Two days after completed cell confluence (counting as day 0), the cells were cultured in bacteriological dishes as aggregates in the presence of α-MEM

* Corresponding author. Fax: +86 25 83672050.
E-mail address: kong_xq2009@126.com (X. Kong).

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containing 10% FBS and 1% DMSO. At day 4, the cell aggregates were transferred to the cell culture flasks. The medium was then switched back to α-MEM containing 10% FBS for an additional period of time as determined by the experimental design.

Establishment of CCNL2-expressing sublines. The coding sequences of mouse CCNL2 were subcloned into the EcoRI and XbaI sites of a pcDNA 3.1/myc-His B expression vector to generate a plasmid expressing a CCNL2-6× His fusion protein. The primers used to clone the open-reading frame of CCNL2 cDNA were 5′-CGGAATTCGCCACCATGGCGGCGGCGGCG-3′ and 5′-GCTCTAGACTCCTCCGATGCCTGCTGTGT-3′. The pcDNA 3.1/myc-His B expression vector carrying the CCNL2 coding sequence or the vector alone was transfected into P19 cells using lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Two days after transfection, 600 μg/mL of neomycin (G418, Roche, Basel, Switzerland) was added to the growth medium for selection of stable CCNL2-expressing cells. Drug-resistant cells began to form small colonies after 2 weeks of G418 addition. Individual colonies were then isolated and propagated, and CCNL2-6× His fusion protein was identified by Western blot using an anti-His antibody (Clontech, Mountain View, CA, USA). Colonies expressing the highest levels of CCNL2 were selected for cell proliferation and differentiation studies.

Subcellular localization of the CCNL2 protein. A green fluorescent protein (GFP)-tagged CCNL2 fusion protein was expressed by using the pEGFP-N2-CCNL2 vector. The coding sequence of CCNL2 was amplified by PCR and inserted into the EcoRI and BamH1 sites of the pEGFP-N2 vector. For gene transfection, the cells were plated onto coverslips in six-well plates and cultured for 24 h and then transfected with pEGFP-N2-CCNL2 or pEGFP-N2 vector using lipofectamine 2000. Two days later, the transfected cells were examined under a confocal laser microscope (Zeiss, Göttingen, Germany) for GFP expression.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from P19 cells using TRIzol (Invitrogen, Carlsbad, CA, USA). One gram of the RNA was then converted to cDNA using 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA), and an aliquot (10%) of the resulting cDNA was amplified for PCR with the primers listed in Table 1. The number of cycles and reaction temperatures used in the semi-quantitative RT-PCR assay were optimized to provide a linear relationship between the amount of input template and PCR product.

Quantitative real-time PCR (qRT-PCR). Gene-specific primers (Table 1) were designed to flank exon junctions to ensure that all of the expected PCR products were generated from mRNA. For real-time PCR, 2× SYBR® Green PCR Master Mix (Applied Biosystems, Weiterstadt, Germany) was performed on an ABI 7300 (Applied Biosystems) for 40 cycles. Serial dilutions of standard cDNA prepared from P19 cells were used to generate a standard curve of PCR efficiency. Standard curves for Bax, bcl-2, Wnt, GSK3β, β-catenin, and GAPDH exhibited similar slopes, allowing GAPDH-normalized gene expression levels to be determined using the $\Delta\Delta C_t$ method (Applied Biosystems' manual). The expression ratio was calculated according to the $2^{-\Delta\Delta C_t}$ method [14,15]. All measurements were performed in triplicate.

Cell proliferation assay. The growth of stable CCNL2-transfected P19 cells and control cells were assessed using a cell viability CCK-8 assay. Briefly, the cells were seeded in 96-well culture plates at a density of 250 cells/well and cultured in α-MEM supplemented with 10% FBS and G418 (250 μg/mL). The cell viability was measured using a Cell Counting kit-8 (CCK-8) from Dojin Laboratories (Kumamoto, Japan) every 24 h for a period of 7 days. The absorbance of optical density (OD) was measured at 450 nm as recommended by the manufacturer.

Flow cytometry assay. The stable CCNL2-transfected P19 cells and the empty-vector control P19 cells were cultivated in α-MEM with 10% FBS overnight and the cells were then starved in serum-free α-MEM for 48 h to synchronize the cell cycles. The cells were then added back with serum-containing medium and subjected to the flow cytometer analysis to determine the cell cycle distribution at various time points (0, 12, 18, or 24 h). The cell cycle distributions were assessed using methods described previously [16]. The labeled cells were analyzed using a BD FACScan (New Jersey, USA) and data were analyzed using CellQuest software (BD, New Jersey, USA).

Table 1
Primers used for RT-PCR and quantitative RT-PCR.

Gene	Size (bp)	Primers	T_m (°C)	Cycles
GAPDH	450	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	60	26
Cardiac actin	291	5′-GTGGGTATGGGACAGAAGGA-3′ 5′-AGACAGCACTGCCTGAATGG-3′	61	26
GATA4	480	5′-CCAACTGCCAGACTACCAC-3′ 5′-GGACCAGGCTGTTCCAAGA-3′	58	32
Mef2C	610	5′-CAGCACTGACATGGATAAGG-3′ 5′-CTGCCAGGTGGGATAAGAACG-3′	60	32
BNP	455	5′-AAGGGAGAACACGGCATCA-3′ 5′-CAGGCAGAGTCAGAAACTGGA-3′	56	30
Wnt	216	5′-CTGGTGGTCCTTGGCTGT-3′ 5′-CTGTTGCTGACGGTGGTG-3′	60	40
β-Catenin	189	5′-CGTTTAGCAGTTTTGTCAGCTC-3′ 5′-TCCCTGAGACGCTAGATGAGG-3′	60	40
GSK3β	125	5′-ACCAGGTTAAGGTAGACCTCATC-3′ 5′-GACAAGCGATTTAAGAACCGAGA-3′	60	40
Bax	229	5′-CCAGCCCATGATGGTTCTGAT-3′ 5′-CCGGCGAATTGGAGATGAACT-3′	60	40
Bcl-2	284	5′-CAGACATGCACCTACCCAGC-3′ 5′-GTCGCTACCGTCGTGACTTC-3′	60	40
GAPDH-Q-RT- PCR	251	5′-GCCAGTAGAGGCAGGGATGATGTTC-3′ 5′-CCATGTTCGTCATGGGTGTGAACCA-3′	60	40

Note. Cardiac actin, cardiac muscle α-actin; GATA4, GATA binding protein 4; Mef2C, myocyte enhancer factor 2C; Nkx2.5, NK2 transcription factor related locus 5; BNP, natriuretic peptide precursor type B; T_m, annealing temperature.

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Hoechst 33258 staining. To detect apoptosis, the cells were grown and stained with Hoechst 33258 (Beyotime Biotechnology, Shanghai, China). Briefly, P19 cells were transiently transfected with pcDNA3.1-CCNL2 or pcDNA3.1 for 48 h and then fixed in 0.5 mL of methanol for 15 min. After rinsing twice in PBS, the cells were stained with 1 g/mL of Hoechst 33258 solution in the dark for 10 min at room temperature and then rinsed with PBS again. Analysis was conducted using a fluorescence microscope with UV excitation at 348 nm and emission at 480 nm. The apoptotic cells were characterized by pyknotic and fragmented nuclei emitting intense fluorescence.

Annexin-V assay. After transient transfection of pcDNA3.1-CCNL2 or pcDNA3.1 vector for 48 h, P19 cells were harvested and then stained with 10 μL of Annexin-V-FITC and 10 μL of PI at room temperature for 5 min (Biovision, CA, USA). The percentage of Annexin-V-positive apoptotic cells was determined using a BD FACScan (New Jersey, USA) and data were analyzed with Modfit software (BD, New Jersey, USA).

Measurement of caspase-3 activity. Cell lysates were prepared from the above-transfected cells and caspase-3 activity was measured using a commercial available kit from Sigma (St. Louis, MO, USA) according to the manufacturer's protocol. The values of OD at 405 nm were read by a Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). The activities of caspase-3 were calculated using the formula of ODt/ODc as % of control.

Results

Establishment of stable overexpressing CCNL2 P19 cells

Stable CCNL2-overexpressing P19 cells and the empty-vector controls were established and maintained in α-MEM containing 250 μg/mL of G418. Expression of CCNL2 protein was verified by Western blot analysis (Fig. 1A). We also localized the CCNL2 protein in P19 cells using transient transfection of pEGFP-N2-CCNL2 vector into P19, COS-7, and HeLa cells. Fluorescent imaging demonstrated that the fusion protein was localized primarily in the nucleus of the cells, whereas GFP in the empty vector was distributed throughout the cells without any specific compartmentalization (Fig. 1B). This result confirms that CCNL2 is a nuclear protein.

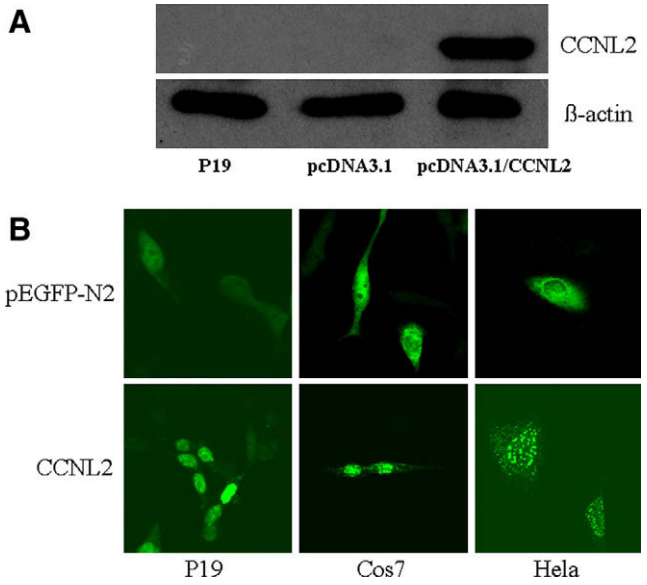

Fig. 1. Establishment of stable overexpressing CCNL2 P19 cells and subcellular localization of the CCNL2 protein. (A) Western blot analysis of CCNL2 expression. The pcDNA3.1-CCNL2 and pcDNA3.1 empty vectors were stably transfected into P19 cells, and total cellular protein was then extracted from the cells and subjected to Western blot analysis with a mouse anti-His antibody. The experiment was repeated three times, and similar results were obtained. (B) Subcellular localization of the CCNL2 protein. P19, COS-7, and HeLa cells were transiently transfected with pEGFP-N2/CCNL2 or pEGFP-N2 vector; 48 h later, the cells were observed under the confocal microscope and photographs were taken. The data indicate that the CCNL2 protein is in the nuclei of the cells.

Effect of CCNL2 on P19 cell differentiation

We next assessed the effects of CCNL2 on changed phenotypes of P19 cells. We first measured cell differentiation using 1% DMSO; this chemical can induce P19 cells to differentiate into cardiomyocytes. During cardiomyocytes differentiation of embryonic stem cells (such as P19), a number of cardiac-specific genes are expressed, such as cardiac actin, GATA4, Mef2C, and BNP. As shown in Fig. 2, the expression of these genes was suppressed by CCNL2 transfection compared to the empty-vector controls. The results indicate that CCNL2 inhibited myocardial cell differentiation of P19 cells.

Effect of CCNL2 on P19 cell proliferation

As shown in Fig. 3A, P19 cells overexpressing CCNL2 had a decreased growth rate compared with the control cells. At days 1, 2, and 3, the OD numbers were not much different between CCNL2 and empty vector-transfected P19 cells. However, beginning at day 4, the OD number of the cells overexpressing CCNL2 was much lower than that of the control cells.

We also performed flow cytometry analysis of the cell cycle distributions in these stable sublines. The data show that the proportion of CCNL2-transfected P19 cells in the S phase of the cell cycle was lower than that of the control cells (Fig. 3B).

Effect of CCNL2 on P19 apoptosis

We first determined the changes in cell morphology that occur when apoptosis takes place in P19 cells. Forty-eight hours after gene transfection, CCNL2-transfected P19 cells showed more cells with condensed and fragmented nuclei and apoptotic bodies than did the control cells (Fig. 3C). We next performed the Annexin-V assay to quantitatively evaluate apoptosis. The percentage of Annexin-V positive cells was higher in the CCNL2-overexpressing cells than that in the controls (Fig. 3D). We also examined the CCNL2-induced activation of caspase-3 and found that caspase-3 was more activated in CCNL2-overexpressing P19 cells than that in the controls (Fig. 3E).

Effect of CCNL2 on apoptosis-related gene expression

Using a quantitative real-time PCR analysis, we assessed expression of the pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) genes. CCNL2-transfected P19 cells inhibited Bcl-2 mRNA levels but did not change Bax mRNA levels compared to the controls (Fig. 4A).

Expression of Wnt signal transduction genes during P19 cell differentiation

We detected expression of Wnt signal transduction-related genes in the CCNL2-overexpressing and control cells using qRT-PCR analysis. Fig. 4B shows that the expression levels of Wnt and β-catenin mRNA were much lower in CCNL2-overexpressing P19 cells than that in the control cells, whereas the GSK3β expression

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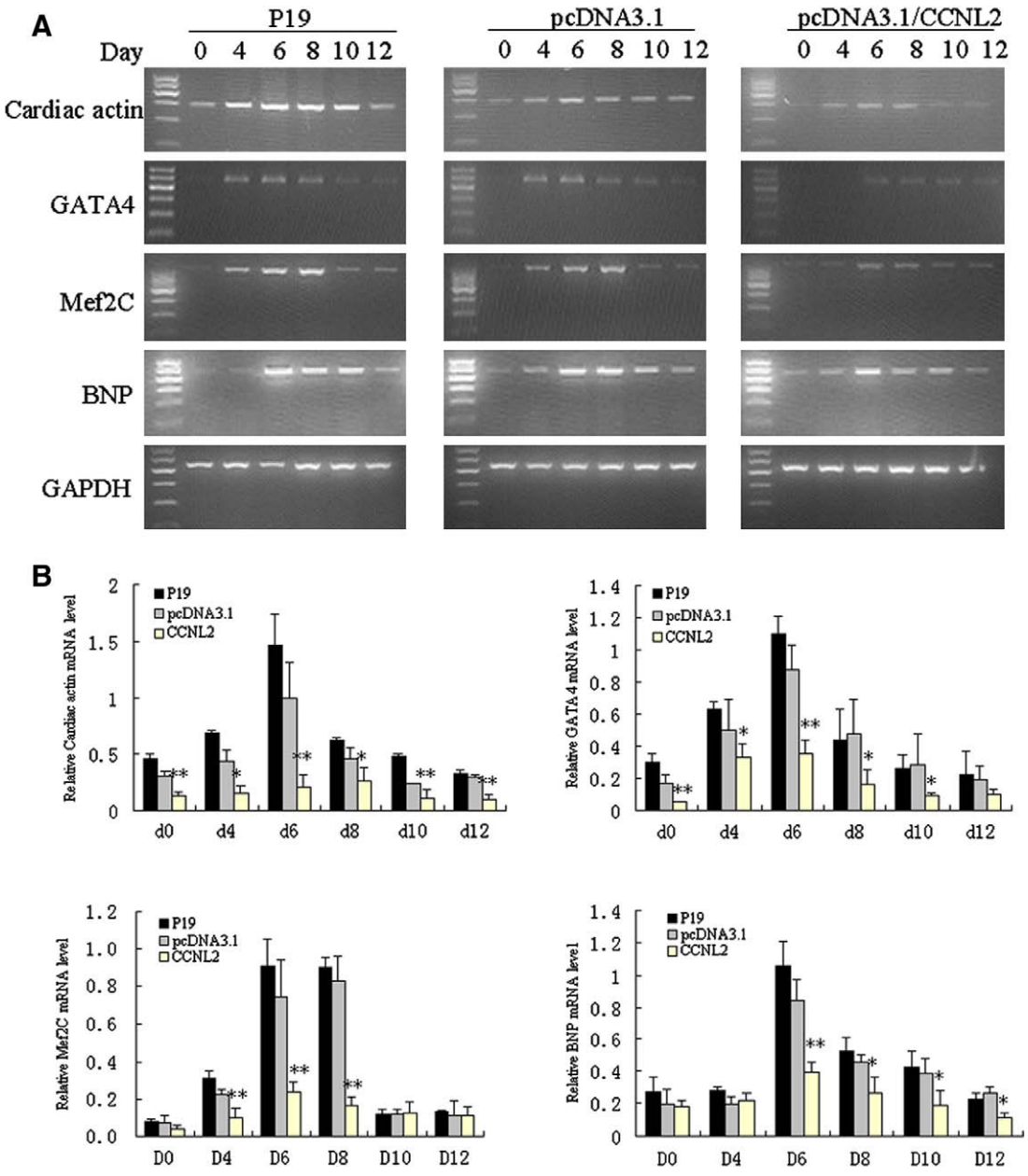

Fig. 2. Detection of gene expression after DMSO-induced P19 cell differentiation. The cells were grown and treated with 1% DMSO (see Methods for details). RNA from these cells was isolated and subjected to RT-PCR analyses. (A) The PCR products were electrophoresed in an ethidium bromide agarose gel and visualized under UV light. (B) Quantitative data (means ± SD) from the gels were normalized to GAPDH. These data are based on three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$.

level was significantly higher in CCNL2-overexpressing P19 cells than that in the controls.

Discussion

In this study, we determined the effects of the CCNL2 protein in regulating proliferation and differentiation of mouse embryonic carcinoma P19 cells. Our data showed that stable CCNL2-overexpressing P19 cells were less differentiated after treatment with 1% DMSO and that expression of myocardial cell-differentiation-related genes (such as cardiac actin, GATA4, Mef2C, Nkx2.5, and BNP) were reduced compared to vector-only transfected P19 cells. We further showed that P19 cells overexpressing CCNL2 have a reduced growth rate and a decreased S phase of the cell cycles. Moreover, these cells were undergoing apoptosis, and the anti-apoptotic Bcl-2 protein was downregulated. In addition, real-time PCR analysis showed that expression of Wnt and β-catenin was suppressed but expression of GSK3β was induced in the CCNL2-overexpressing P19 cells. Our data suggest that overexpression of CCNL2 inhibited proliferation and differentiation of mouse embryonic carcinoma P19 cells and induced them to undergo apoptosis. The Wnt signal transduction pathway may be responsible for the CCNL2 activity in P19 cells.

Cyclins are key regulatory proteins that complex with and activate cyclin-dependent kinase (CDK) subunits [17–20], which in turn play a pivotal role in regulation of the progression of the cell cycle [21–23]. The cyclin gene family includes more than 20 proteins. The cell cycle regulator cyclins, which include cyclins A, B, D1, D2, D3, E, and F, bind to their CDK partners to promote the cell cycle [24]. The transcription regulator cyclins, such as cyclins C, H, K, L1, T1, and T2, tend to contribute to transcriptional regulation of gene expression [25–28]. Other cyclins also have been identified,

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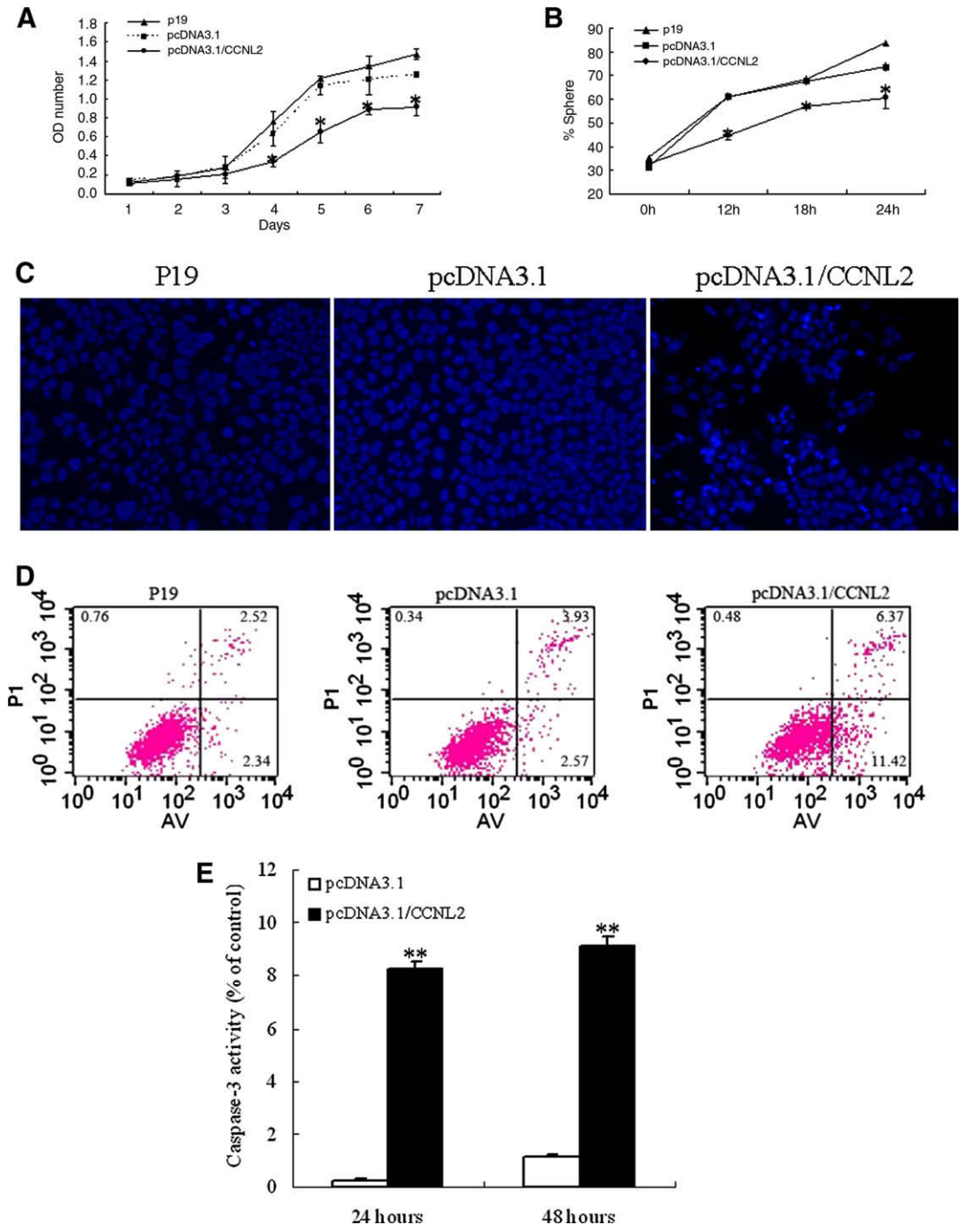

Fig. 3. Effect of CCNL2 on P19 cell proliferation and apoptosis. (A) Effect of CCNL2 on P19 cell proliferation. The stable pcDNA3.1-CCNL2- or empty vector-transfected P19 cells were grown in a monolayer and then subjected to a cell viability CCK-8 assay and (B) flow cytometry assay. The experiments were repeated three times. $^{*}p < 0.05$. (C) Effect of CCNL2 on apoptosis by Hoechst 33258 staining. P19 cells were grown in a monolayer and transiently transfected with pcDNA3.1-CCNL2 or empty vector for 48 h. The cells then were stained with Hoechst 33258 and viewed under a fluorescence microscope. Apoptotic cells are characterized by pyknotic and fragmented nuclei. (D) Annexin-V assay. The same transfected cells were subjected to an Annexin-V assay. (E) Caspase-3 activation assay. The same transfected cells were subjected to a caspase-3 activation assay. $^{**}p < 0.01$.

but their roles in cell cycle control and RNA transcription, if any, are unclear and remain to be studied. CCNL2 is a new member of the cyclin family. Our previous data revealed that CCNL2 was differentially expressed in ventricular septum tissue between patients with VSD and normal controls and that its expression was upregulated in ventricular septum tissue of VSD patients [12]. The current study showed that CCNL2 can regulate growth, differentiation, and apoptosis of P19 cells. Molecularly, CCNL2 reduced expression of Wnt and β-catenin mRNA but induced that of GSK3β mRNA in P19 cells. These data suggest that CCNL2 may participate in VSD during human development, although we cannot at this time provide direct evidence of its role in heart development.

It is well known that P19 cells are pluripotent when exposed to DMSO and that they can differentiate into embryonic myocardial cells. Thus, this cell line is a useful model system that reflects the in vivo biological features of myocardial cells [29,30]. In the present study, we demonstrated that expression of cardiac actin,

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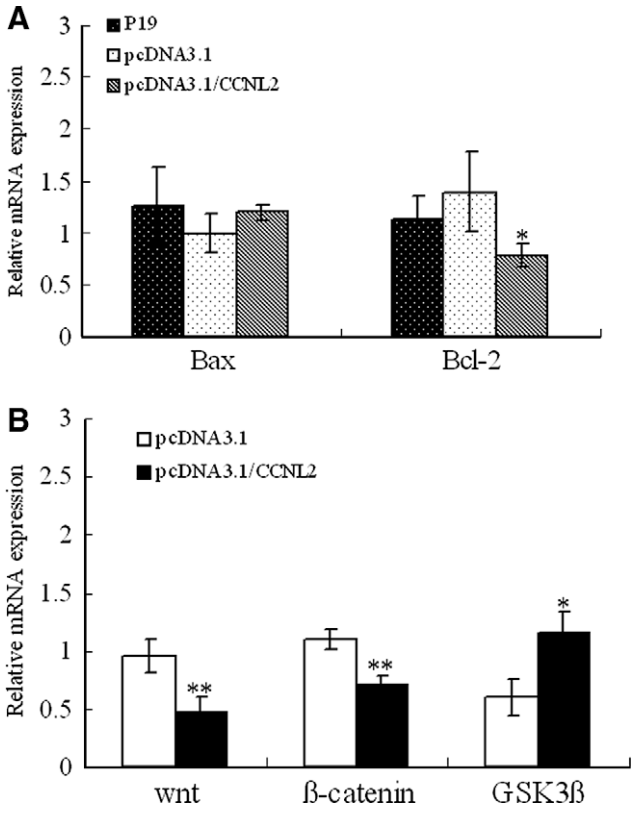

Fig. 4. Real-time PCR analyses of gene expression. (A) Effect of CCNL2 on apoptosis-related gene expression. P19 cells were grown in a monolayer and transiently transfected with pcDNA3.1-CCNL2 or empty vector for 48 h. The cells then were subjected to real-time RT-PCR analysis of gene expression. $^{*}p < 0.05$. These data are representative of three independent experiments. (B) Real-time PCR analyses of Wnt signal transduction gene expression. P19 cells were grown in a monolayer and transiently transfected with pcDNA3.1-CCNL2 or empty vector for 48 h. Total RNA then was isolated and then subjected to real-time PCR analyses of gene expression. The data were summarized as means ± SD after being normalized to GAPDH. The experiments were run in triplicate and repeated once with similar findings. $^{*}p < 0.05$; $^{**}p < 0.01$.

GATA4, Mef2C, Nkx2.5, and BNP was altered after treatment with 1% DMSO, but that CCNL2 expression prevented such changes in P19 cells; this finding suggests that CCNL2 inhibits P19 cell differentiation. Changes in GATA4 and cardiac actin expression occurred as early as day 0, suggesting that CCNL2 is important in the expression of these genes.

Cell proliferation and apoptosis are two aspects of cell growth and control, but they are inter-related events in the cells; factors that affect apoptosis usually also affect cell proliferation. Indeed, previous studies have demonstrated that CCNL2 was able to induce human hepatocyte carcinoma cells [31] and lung adenocarcinoma cells [32] to undergo apoptosis. In our current study, we found that CCNL2 induced apoptosis in P19 cells, as shown by Hoechst staining, Annexin-V-FITC analysis, and caspase-3 activity assays. In addition, the anti-apoptotic Bcl-2 gene was downregulated due to CCNL2 transfection.

It remains unclear how specific nondifferentiated mesoderm contributes to myocardial tissue and how CCNL2 directly anticipate in heart development; these are long-standing questions for developmental biologists. In recent years, Wnt protein and its signal transduction pathway have been implicated in cardiac development [33–35]. The Wnt gene family encodes for a conserved class of secreted signaling molecules and is considered to be one of the major gene families that are essential for proper embryonic patterning and organogenesis [36]. In our study, Wnt and ß-catenin expression levels were depressed but GSK3β was induced in CCNL2-overexpressing P19 cells. Thus, our data may have further confirmed the importance of Wnt proteins in heart development. In this context, altered expression of Wnt signaling pathway genes by CCNL2 may play a role in suppressing P19 cell differentiation into myocardial cells. However, further study of this process is warranted.

In summary, our data demonstrated that CCNL2 overexpression repressed differentiation of mouse embryonic carcinoma P19 cells into embryonic myocardial cells. P19 cell proliferation was also inhibited, but apoptosis was activated by CCNL2 gene transfection. These events may occur through the Wnt signal transduction pathway. However, the exact role of CCNL2 in P19 cells and heart development requires further investigation.

Acknowledgments

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