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CCN2 Promotes Cigarette Smoke-Induced Proliferation of Rat Pulmonary Artery Smooth Muscle Cells Through Upregulating Cyclin D1 Expression

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ABSTRACT

Cigarette smoke has been demonstrated to induce pulmonary vascular remodeling, which is characterized by medial thickening of the pulmonary arteries mainly resulting from the abnormal proliferation of pulmonary artery smooth muscle cells (PASMCs). However, the molecular mechanism underlying this process is still unclear. In the present study, we investigated whether CCN2 regulated rat PASMCs (rPASMCs) proliferation induced by cigarette smoke extract (CSE) and nicotine by upregulating cyclin D1 in vitro. CCN2 siRNA or cyclin D1 siRNA were transfected to rPASMCs which were then exposed to CSE and nicotine. Both mRNA and protein expressions of CCN2 were significantly increased in rPASMCs treated with 2% CSE or 1 μM nicotine, which markedly promoted the proliferation of rPASMCs. CCN2 siRNA inhibited the proliferation of rPASMCs induced by CSE or nicotine. Furthermore, CCN2 siRNA markedly suppressed the mRNA and protein expressions of cyclin D1 in rPASMCs and led to cell cycle arrest in G0/G1 phase resulting in reduced rPASMCs proliferation. These findings suggest that CCN2 contributes to the CSE and nicotine-induced proliferation of rPASMCs at least in part by upregulating cyclin D1 expression. J. Cell. Biochem. 113: 349–359, 2012.

KEY WORDS: CCN2; CYCLIN D1; PULMONARY HYPERTENSION; SMOKE; SMOOTH MUSCLE

Pulmonary arterial hypertension (PAH) is a complex disease of small pulmonary arteries, which is characterized by sustained vasoconstriction, thickening of pulmonary artery walls, vascular remodeling, and progressive increase in pulmonary vascular resistance, leading to right ventricular failure and finally death [Humbert et al., 2004]. Enhanced pulmonary artery smooth muscle cell (PASMC) proliferation is a major cause of medial hypertrophy, vascular remodeling and vascular lumen narrowing [Rabinovitch, 2004].

It is well known that cigarette smoke is one of the most important risk factors for chronic obstructive pulmonary disease (COPD) and pulmonary hypertension [Wright et al., 2008]. Studies from animals and humans have demonstrated that cigarette smoke might exert a direct effect on the vessel structure, resulting in pulmonary vascular remodeling and pulmonary hypertension [Barbera et al., 2003; Wright et al., 2005]. However, the precise mechanisms underlying this process remain unclear.

CCN2, a member of the CCN family, is a 38 kDa cysteine-rich protein and was first identified in the conditioned medium of human umbilical vein endothelia cells [Bradham et al., 1991]. It has been demonstrated that CCN2 can mediate cell adhesion, migration, proliferation, and extracellular matrix (ECM) synthesis in a variety of cell types, including vascular endothelial cells, fibroblasts, osteoblastic cells, chondrocytic cells, and smooth muscle cells [Grotendorst, 1997; Hishikawa et al., 1999; Shimo et al., 1999; Nishida et al., 2000; Takigawa, 2003; Bonniaud, 2004]. Previous studies have documented that CCN2 plays an important role in airway remodeling [Churg et al., 2006; Ou et al., 2009]. However, the evidence that CCN2 is involved in the pulmonary vascular remodeling remains limited. In our previous study [Tian et al., 2007], CCN2 was shown to be involved in the pulmonary vascular remodeling. However, the precise mechanism underlying the process remains to be clarified further.

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Moreover, in recent years, accumulating evidence shows that CCN2 is vital for the cell cycle progression [Kothapalli and Grotendorst, 2000; Wahab et al., 2002]. It has been widely accepted that cyclins, including D type cyclins, may act together with their Cdk partners to control the mammalian cell proliferation [Koffler et al., 2000]. CCN2 can activate the cyclin D1 in various cell types, such as human lung fibroblasts, human glomerular mesangial cells, and esophageal squamous cell carcinoma cells. Nevertheless, the relationship between CCN2 and cyclin D1 in the rat PASMCs (rPASMCs) is still not clear.

The present study aimed to examine the influence of cigarette smoke extract (CSE) and nicotine on the expression of CCN2 in the rPASMCs and investigate whether CCN2 regulates the CSE or nicotine-induced proliferation of rPASMCs by upregulating cyclin D1 in vitro. Our results indicate that CSE and nicotine significantly upregulate the CCN2 expression in the rPASMCs, induce rPASMCs proliferation and promote the cell cycle progression in a cyclin D1-dependent manner.

MATERIALS AND METHODS

MATERIALS

Mouse polyclonal antibody against β-actin and α smooth muscle actin (α-SMA), rabbit polyclonal antibodies against CCN2 and cyclin D1, and mouse monoclonal antibody against BrdU were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Cy3-, FITC-, or biotin-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Nicotine, 5-bromo-2-deoxyuridine (BrdU), propidium iodide (PI) and 4′,6-diamidino-2-phenylindole(DAPI) were purchased from Sigma–Aldrich (St. Louis, MO). Reverse transcription kit and PCR Master Mix were purchased from Toyobo Biotechnology Co. (Japan). Trizol and Lipofectamine2000 were bought from Invitrogen Biotechmology Co. (Carlsbad, CA). All siRNAs were synthesized by GenePharma Co., Ltd. (Shanghai, China). All primers were synthesized by Bio-engineering Co. Ltd. (Shanghai, China). The Bradford assay kit was bought from Laboratories Bio-Rad (Hercules, CA).

CELL CULTURE

rPASMCs was prepared from male Sprague–Dawley rats (200–250 g) as previously described [Golovina and Blaustein, 2006]. Briefly, the distal pulmonary arteries were incubated in Hanks' solution containing collagenase (1.5 mg/ml) for 20 min, and then a thin layer of adventitia was carefully stripped off with a fine forcep, and the endothelium was removed by gently scratching of the intimal surface with a surgical blade. The remaining smooth muscle was then digested with collagenase (2.0 mg/ml) and elastase (0.5 mg/ml) for 35–45 min at 37°C. Cells were grown in the Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 mg/ml), and cultured in a humidified incubator at 37°C. The cells were passaged by trypsinization with 0.05% trypsin-EDTA and those at passages 3–8 were used for experiments. Cells were identified by phase-contrast microscopy and immunochemistry for α-SMA. For all experiments, cells were made quiescent by incubation in serum-free media for 24 h before 24 h exposure to CSE or DMEM or nicotine. Care and use of the animals was in accordance with the Chinese Association for Laboratory Animal Science Policy. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

PREPARATION OF CSE SOLUTION

CSE was prepared freshly for each experiment, using Marlboro cigarettes. CSE was prepared as previously described [Oltmanns et al., 2005] with a few modifications. Briefly, cigarette smoke derived from one cigarette was drawn slowly into a 50 ml syringe and bubbled through 30 ml of DMEM. One cigarette yielded five draws of the syringe, with each individual draw taking approximately 10 s to complete. The resulting solution, which was considered "100%" strength, was then filtered before being diluted in DMEM to the required strength for application to rPASMCs.

siRNA TRANSFECTION

The siRNA sequences of rat CCN2, cyclin D1, and negative control were described in previous studies [George and Tsutsumi, 2007; Kundumani-Sridharan et al., 2007]. For transfection, cells were seeded into plates, incubated overnight, and then transfected using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions when cells were 40–60% confluent.

CELL COUNTING

Cells were seeded in 24-well plates (5,000 cells/well) and cultured overnight. Cells were then maintained in serum-free media for 24 h, followed by different treatments. At the end-point, cells were harvested and counted using a hemocytometer.

BrdU INCORPORATION ASSAYS

BrdU was added 2 h before culture termination. At the end of culture, cells were immunoblocked with goat serum, and incubated overnight at 4°C with the anti-BrdU antibody. Then, they were incubated for 60 min with biotinylated goat anti-mouse IgG (1:200), and stained with DAB. The percentage of stained cells was determined by counting the number of positively stained cells within a field and dividing by the total number of cells in that same field.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted by using Trizol (Invitrogen) according to the manufacturer's protocol. Then, first-strand cDNA was synthesized by using the First-Strand cDNA synthesis kit (ReverTra Ace-α-, FSK-100; Toyobo) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the SYBR Green Realtime PCR Master Mix (QPK-201, Toyobo) with the Light Cycler Instrument (Roche Diagnostics Corp., Basel, Suisse). The cDNA fragments were denatured at 95°C for 15 s, annealed at 58°C for 15 s, and extended at 72°C for 45 s for 40 cycles. Each sample was examined in triplicate and the amounts of the PCR products produced were normalized to that of β-actin which served as internal control. The primers used in the study were as follows: CCN2 (5′-CGTTTGTGCCTATTGTTCTTGTT-3′ and 5′-TGATCCATTGCTT-

TACCGTCTAC-3′), cyclin D1 (5′-TGTTCGTGGCCTCTAAGATGAAG-3′ and 5′-GGAAGTGTTCGATGAAATCGTG-3′), β-actin (5′-GAC-TACCTCATGAAGATCCTG-3′ and 5′-CATAGAGGTCTTTACG-GATGT-3′). Relative transcript levels of each gene were calculated using the $\Delta\Delta C_t$ method, using β-actin as the housekeeping gene [Winer et al., 1999].

WESTERN BLOTTING AND IMMUNOFLUORESCENCE STAINING

Protein was isolated from cultured rPASMCs, and total protein concentration was determined by the Bradford method [Bradford, 1976] using Bradford reagent. Protein samples (50 μg) were separated by 10% SDS–PAGE. CCN2 and cyclin D1 were detected by immunoblotting with primary antibodies, CCN2 (1:200) or cyclin D1 (1:500), respectively. Immunoblots were scanned and protein bands were quantitated with Quantity One software (Bio-Rad Laboratories, Hercules, CA). β-Actin expression was used as internal control for loading.

The primary polyclonal rabbit anti-CCN2 antibody or anti-cyclin D1 antibody was incubated overnight at 4°C and then a Cy3-coupled secondary antibody was incubated for 1 h at room temperature.

CELL CYCLE ANALYSIS

To estimate the proportions of cells in different phases of the cell cycle, cellular DNA contents were measured by flow cytometry. After treatments, cells were harvested, washed twice with cold PBS, and then fixed overnight at −20°C in 70% ethanol. Immediately before flow cytometry, the cells were resuspended in PBS containing PI (50 μg/ml) and DNase-free RNase (10 μg/ml). Flow cytometry was performed using a FACScalibur (Becton Dickinson, San Diego, CA) system with CELLquest software. The percentages of cells in different phases of the cell cycle were determined using the ModFit software.

STATISTICAL ANALYSIS

All data were expressed as means ± SEM. Statistical analysis was carried out using one-way ANOVA (for multiple-group comparison) followed by the least significant difference (LSD) test with the computer software SPSS 12.0 (Chicago, IL). For all tests, data were considered statistically significant when $P < 0.05$.

RESULTS

EFFECTS OF CSE AND NICOTINE ON THE rPASMCs PROLIFERATION

To investigate the effect of CSE and nicotine on the proliferation of rPASMCs in vitro, rPASMCs were challenged with CSE of 0%, 1%, 2%, 5%, and 10% and cell proliferation was evaluated. Additionally, cells were incubated with 0–10 μM nicotine to examine the effect of nicotine on cell proliferation. CSE at concentrations of 1% and 2% caused a significant increase in cell number compared with control group, and the peak increase in cell number was observed following treatment with 2% CSE. In contrast, CSE at higher concentrations (5% and 10%) did not induce cell proliferation, but even resulted in suppression of cell proliferation (Fig. 1A). A significant increase in cell number was also observed when rPASMCs were incubated with nicotine at 0.1–1 μM when compared with cells in the control group and the peak response was observed at 1 μM nicotine (Fig. 1B). Thus, the following experiments were performed with 2% CSE and 1 μM nicotine.

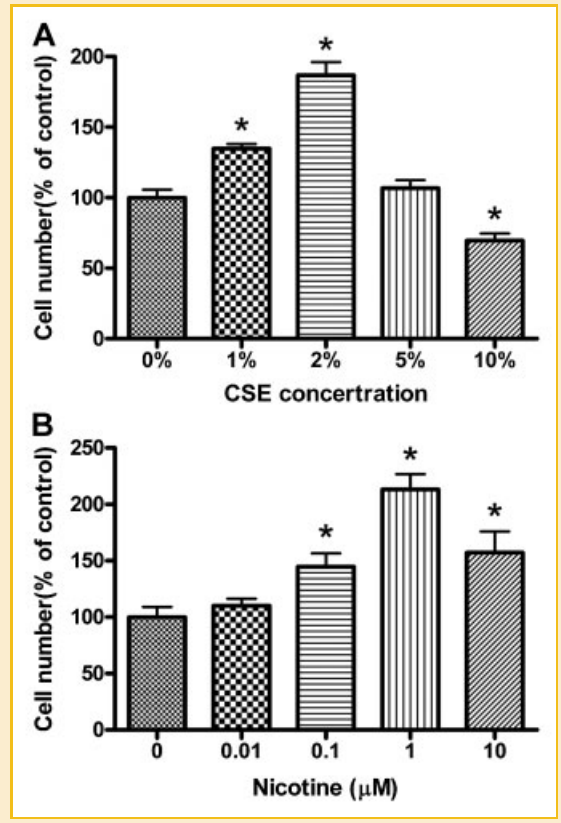

Fig. 1. Effects of CSE and nicotine on the rPASMCs proliferation were evaluated by cell counting. The cells were seeded in 24-well plates and incubated with CSE at different concentrations (1–10%) for 24 h. 0% group was considered as control group (A). The cells were seeded in 24-well plates and incubated with nicotine at various concentrations (0–10 μM) for 24 h (B). Data are presented as means ± SEM from six independent experiments. *$P < 0.05$ versus control.

CCN2 IS INVOLVED IN CSE- OR NICOTINE-INDUCED rPASMCs PROLIFERATION

To demonstrate the effect of cigarette smoke on CCN2 expression and its role in rPASMCs proliferation, we investigated the effect of CCN2 expression on CSE- and nicotine-induced rPASMCs proliferation.

Our results showed that rPASMCs exhibited a low expression of CCN2 in the control group. Treatment with 2% CSE significantly increased the mRNA and protein expressions of CCN2 in the rPASMCs. CCN2 siRNA substantially blocked the upregulation of CCN2 expression following CSE treatment at both mRNA and protein levels (Fig. 2A,B), while the negative control siRNA had no effect on the CCN2 expression. These findings show that CSE increases CCN2 expression, which can be significantly inhibited by CCN2 siRNA.

Moreover, 2% CSE significantly promoted the proliferation of rPASMCs as assessed by cell counting and BrdU incorporation assay,

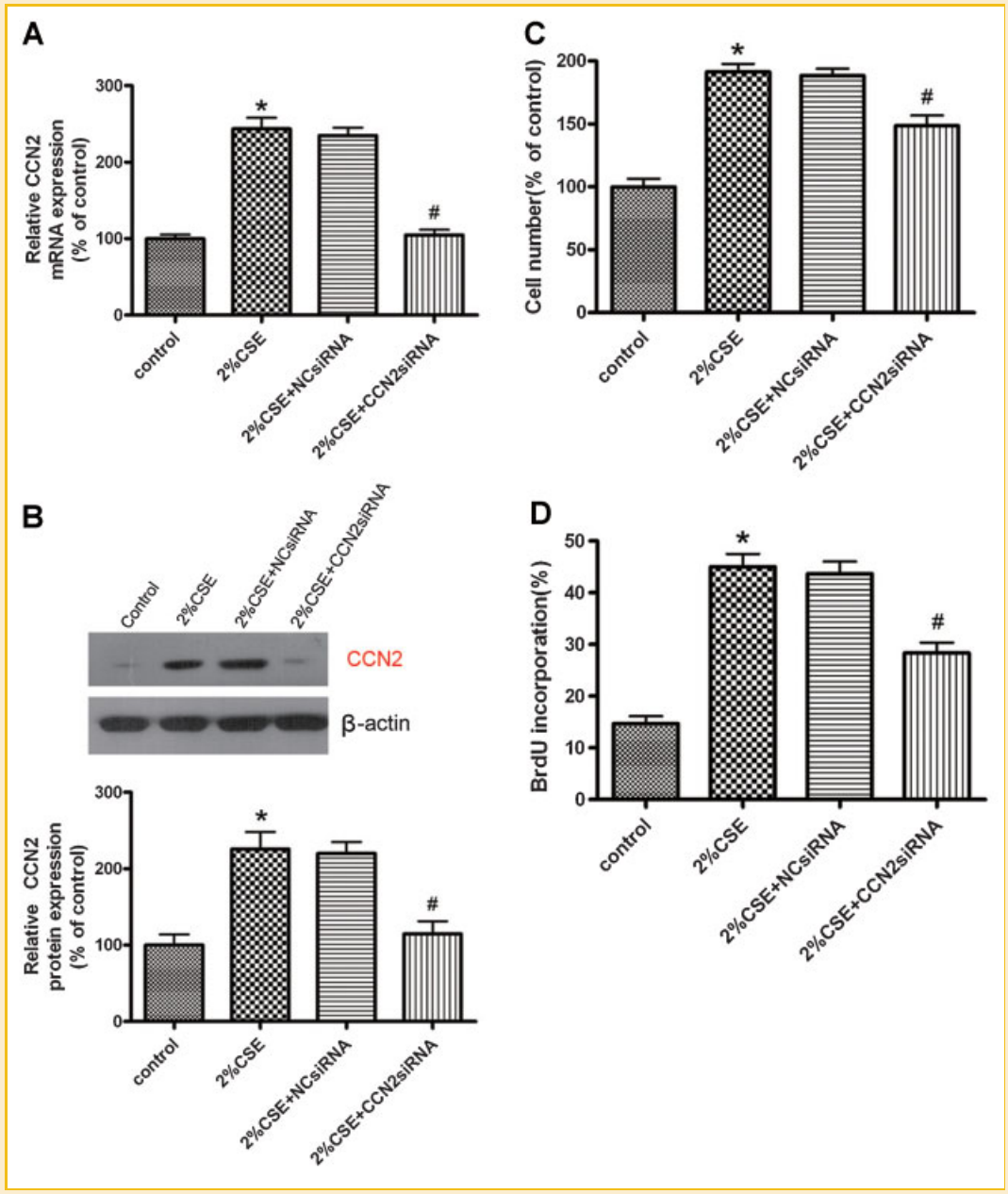

Fig. 2. CCN2 siRNA inhibited the upregulation of CCN2 expression induced by 2% CSE and suppressed the proliferation of rPASMCs induced by 2% CSE. The rPASMCs were transfected with CCN2 siRNA or negative control siRNA and then incubated with 2% CSE for 24 h. CCN2 mRNA levels were determined by quantitative real-time RT-PCR, normalized against β-actin. Data are presented as percentages of the control group (A). CCN2 protein levels were determined by Western blotting, normalized against β-actin. The graph shows the results of densitometry quantification of the Western blot, which are presented as percentages of the control group (B). Quantification of cell number after different treatments. Results are presented as percentages of the control group (C). Percentages of BrdU-positive cells after different treatments. Results are presented as percentages of the control group (D). Data are presented as means ± SEM from six independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus 2% CSE. NCsiRNA, negative control siRNA. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

but the increased proliferation was efficiently suppressed by CCN2 siRNA. The negative control siRNA had no effect on rPASMCs proliferation (Fig. 2C,D). These results reveal the critical role of CCN2 in the CSE-induced rPASMCs proliferation.

Furthermore, our results also displayed nicotine substantially increased the mRNA and protein levels of CCN2, which, however, was dramatically reduced by CCN2 siRNA (Fig. 3A,B). The negative control siRNA had no effect on the CCN2 expression. In addition, nicotine significantly induced the proliferation of rPASMCs as assessed by cell counting and BrdU incorporation assay, but this increases was efficiently reduced by CCN2 siRNA. However, the negative control siRNA had no effect on the proliferation of rPASMCs (Fig. 3C,D). These results demonstrate that CCN2 is involved in the nicotine-induced rPASMCs proliferation.

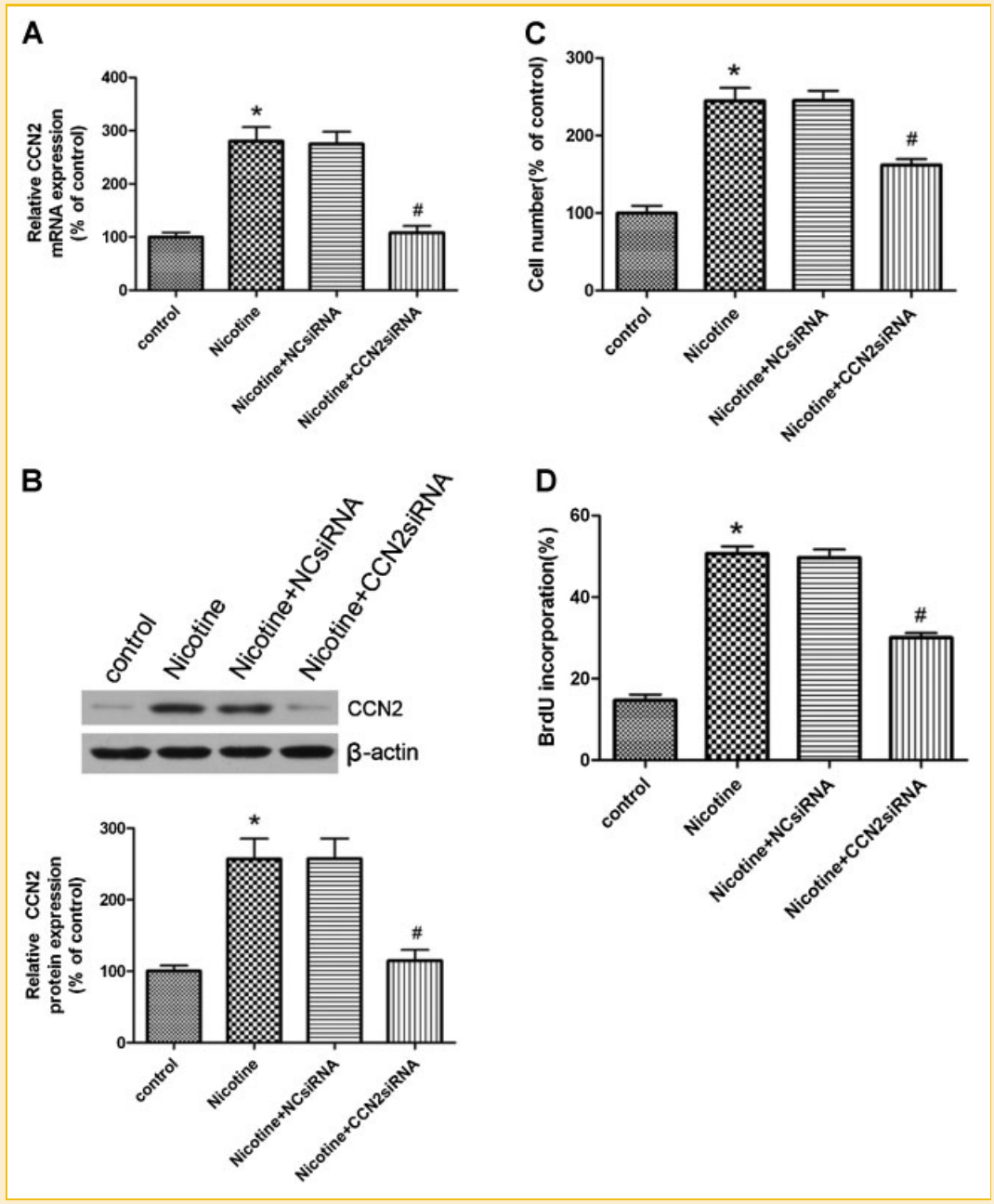

Fig. 3. CCN2 siRNA inhibited the upregulation of CCN2 expression and suppressed the proliferation of rPASMCs induced by 1 μM nicotine. The rPASMCs were transfected with CCN2 siRNA or negative control siRNA and then incubated with 1 μM nicotine for 24 h. CCN2 mRNA levels were determined by quantitative real-time RT-PCR, normalized against β-actin. Results are presented as percentages of the control group (A). CCN2 protein levels were determined by Western blotting, normalized against β-actin. The graph shows the results of densitometry quantification of the Western blot, which are presented as percentages of control group (B). Quantification of cell number after different treatments. Results are presented as percentages of the control group (C). Percentages of BrdU-positive cells after different treatments. Results are presented as percentages of the control group (D). Data are presented as means ± SEM from six independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus nicotine. NCsiRNA, negative control siRNA.

CCN2 siRNA INHIBITS THE UPREGULATION OF CYCLIN D1 mRNA AND PROTEIN EXPRESSION INDUCED BY CSE OR NICOTINE

We first examined the effect of CCN2 siRNA on the mRNA level of cyclin D1 in rPASMCs using real-time RT-PCR in order to determine the role of CCN2 in the regulation of cyclin D1 transcription. Our results showed that 2% CSE and nicotine significantly increased the mRNA and protein expressions of CCN2 and cyclin D1 as compared to control group (Figs. 4A,B,D,E and 5A–D). The CCN2 siRNA substantially reduced the mRNA and protein expressions of CCN2 (Figs. 4A,D and 5A,C) and cyclin D1 (Figs. 4B,E and 5B,D) following 2% CSE treatment or nicotine. However, cyclin D1 siRNA had no effect on the mRNA and protein expressions of CCN2 after both treatments. These results show that knockdown of CCN2 can reduce the CSE and nicotine induced increase of cyclin D1 expression in rPASMCs.

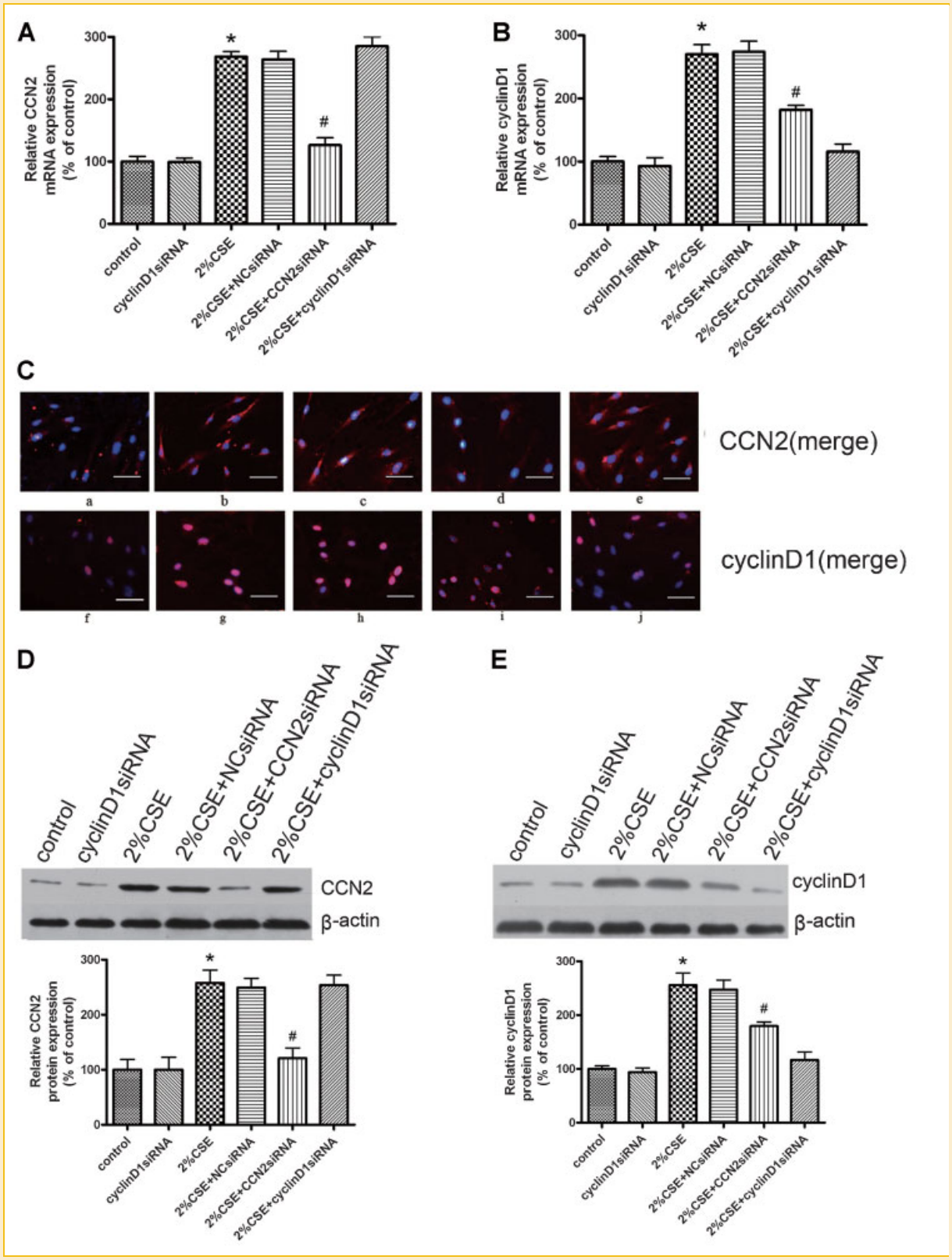

Fig. 4. CCN2 siRNA inhibited the mRNA and protein expressions of CCN2 and cyclin D1 induced by CSE in rPASMCs. The rPASMCs were transfected with CCN2 siRNA, cyclin D1 siRNA or negative control siRNA and then treated with 2% CSE for 24 h. The mRNA expressions of CCN2 and cyclin D1 in different groups were evaluated by quantitative real-time RT-PCR, normalized against β-actin, and expressed as percentages of the control group (A,B). The rPASMCs were divided into groups of control (a,f), 2% CSE (b,g), NCsiRNA (c,h), CCN2 siRNA (d,i), and cyclin D1 siRNA (e,j). Scale bars: 20 μm. Representative images from six independent experiments were selected (C). CCN2 and cyclin D1 protein expressions were determined by Western blotting, normalized against β-actin. The graphs shows the results of densitometry quantification of the Western blot, which are presented as percentages of control group (D,E). Data are presented as means ± SEM from six independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus 2% CSE. NCsiRNA, negative control siRNA. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

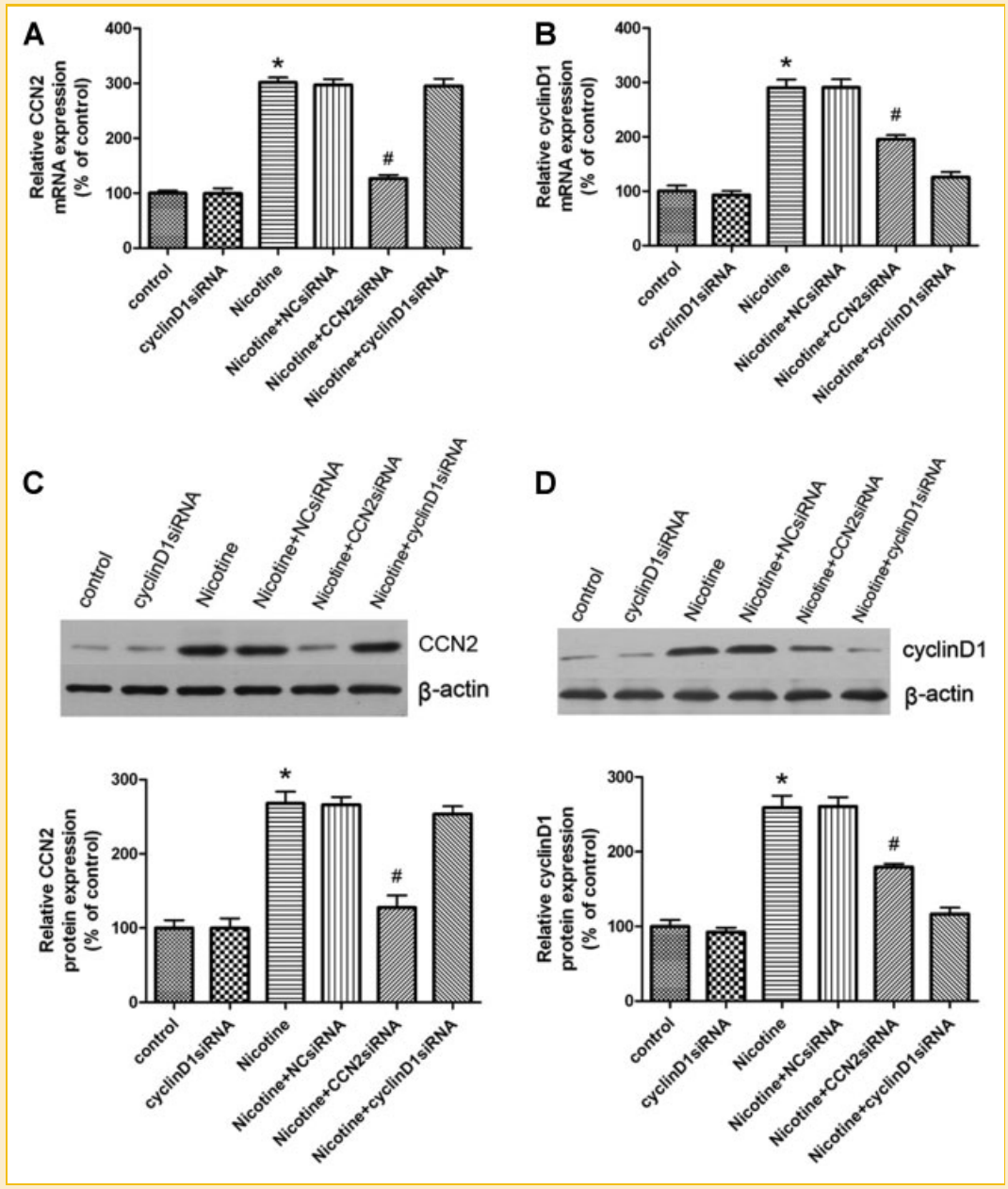

Fig. 5. CCN2 siRNA inhibited the mRNA and protein expression of CCN2 and cyclin D1 induced by nicotine in rPASMCs. The rPASMCs were transfected with CCN2 siRNA, cyclin D1 siRNA, or negative control siRNA and then treated with 1 μM nicotine for 24 h. The mRNA expressions of CCN2 and cyclin D1 in different groups were evaluated by quantitative real-time RT-PCR, normalized against β-actin, and expressed as percentages of the control group (A,B). CCN2 and cyclin D1 protein levels were determined by Western blotting, normalized against β-actin. The graph shows the results of densitometry quantification of the Western blot, which are presented as percentages of the control group (C,D). Data are presented as means ± SEM from six independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus nicotine. NCsiRNA, negative control siRNA.

Figure 4C depicts the results of immunofluorescence staining for CCN2 and cyclin D1 in rPASMCs after different treatments. The levels of CCN2 and cyclin D1 protein expression were low in the control group (Fig. 4C-a,f). CSE promoted CCN2 expression in rPASMCs (Fig. 4C-b), which was blocked by CCN2 siRNA (Fig. 4C-d), but not by the negative control siRNA (Fig. 4C-c) or cyclin D1 siRNA (Fig. 4C-e). Moreover, CCN2 siRNA induced a reduction of the CSE-induced upregulation of cyclin D1 protein expression (Fig. 4C-g) in rPASMCs (Fig. 4C-i) compared to the negative control siRNA (Fig. 4C-h). Notably, cyclin D1 siRNA suppressed the increased protein levels of cyclin D1 induced by 2% CSE (Fig. 4C-j), but it had no effect on CCN2 protein expression (Fig. 4C-e).

CCN2 siRNA AND CYCLIN D1 siRNA INHIBIT G1 TO S CELL CYCLE PROGRESSION IN rPASMCs

We further investigated the effect of CCN2 siRNA on cell cycle and cell proliferation. Flow cytometry showed that 2% CSE or 1 μM nicotine reduced the proportion of cells in the G0/G1 phase and simultaneously increased the proportion of cells in the S phase of the cell cycle (Figs. 6B and 7B). However, either CCN2 siRNA or

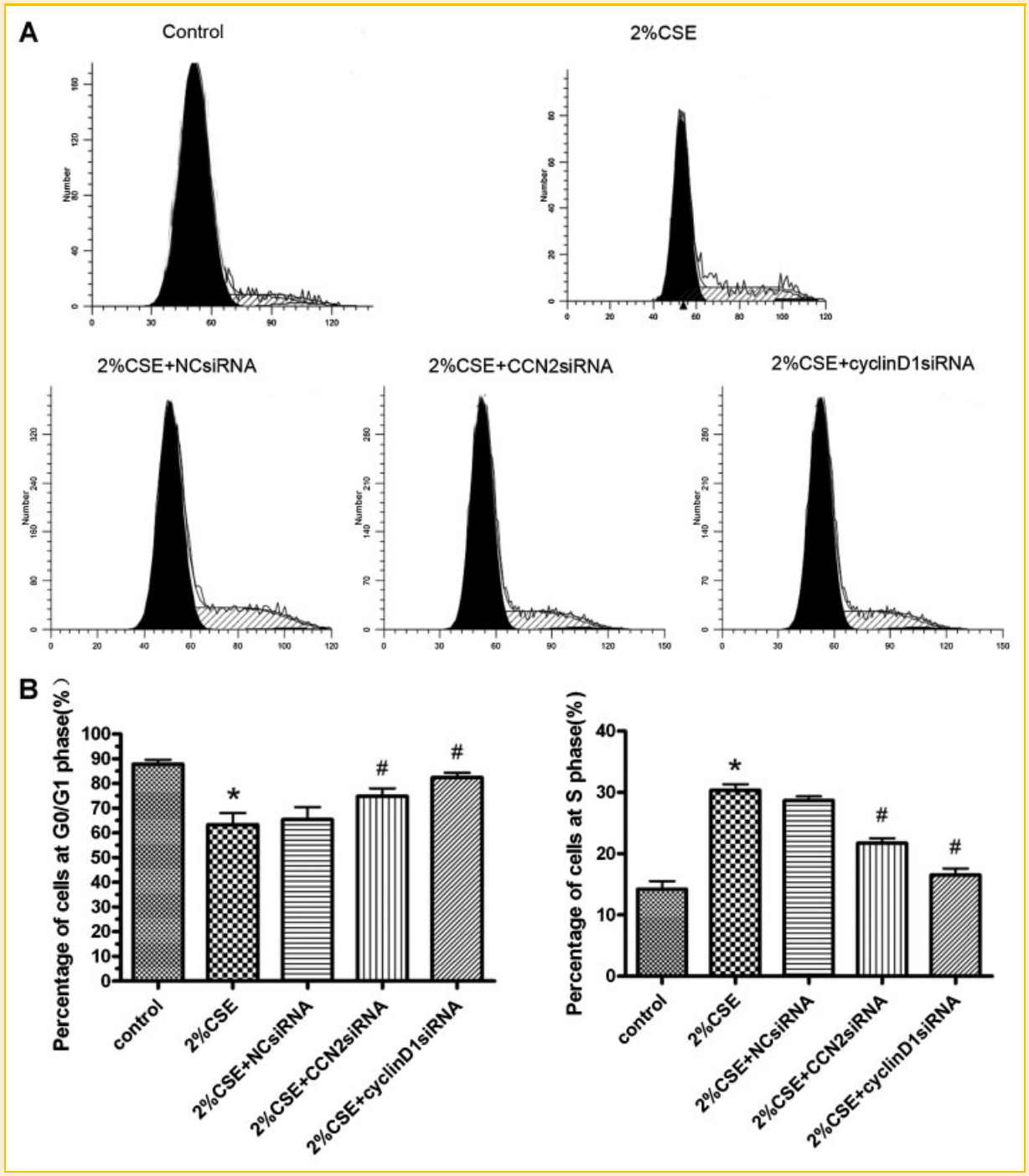

Fig. 6. CCN2 siRNA caused the G0/G1 cell cycle arrest and suppressed rPASMCs proliferation induced by CSE. The rPASMCs were transfected with CCN2 siRNA, cyclin D1 siRNA, or negative control siRNA, and then treated with 2% CSE for 24 h. Cells in different phases (G0/G1, S, and G2/M) were determined after different treatments (A). The percentages of cells in G0/G1 and S phase (B). Data are presented as means ± SEM from six independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus 2% CSE. NCsiRNA, negative control siRNA.

cyclin D1 siRNA resulted in G0/G1 cell cycle arrest in rPASMCs (Figs. 6B and 7B).

However, CCN2 siRNA could not completely suppress the cell cycle progression after both treatments by inhibiting cyclin D1 (Figs. 6B and 7B).

DISCUSSION

This study demonstrates that CSE and nicotine can induce the upregulation of CCN2 and cyclin D1 expression in the rPASMCs which promote the proliferation of rPASMCs. Moreover,

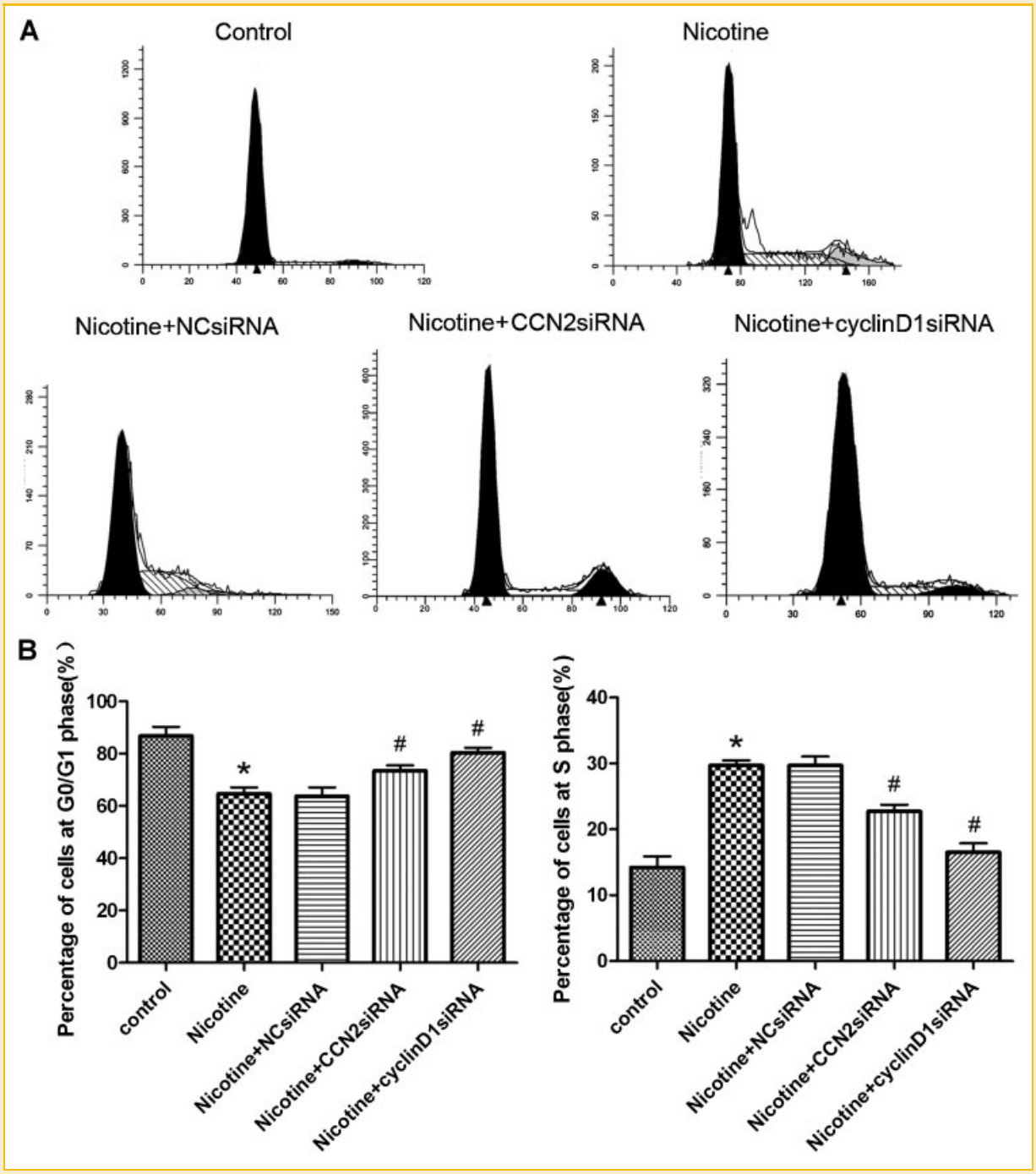

Fig. 7. CCN2 siRNA caused the G0/G1 cell cycle arrest and suppressed rPASMCs proliferation induced by nicotine. The rPASMCs were transfected with CCN2 siRNA, cyclin D1 siRNA, or negative control siRNA, and then treated with 1 μM nicotine for 24 h. Cells in different phases (G0/G1, S, and G2/M) were determined after different treatments (A). The percentages of cells in G0/G1 and S phase (B). Data are presented as means ± SEM from six independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus nicotine. NCsiRNA, negative control siRNA.

upregulation of CCN2 can promote the G1/S phase transition of rPASMCs and upregulate cyclin D1expression in rPASMCs. Conversely, downregulation of CCN2 with CCN2-specific siRNA inhibits the expressions of both CCN2 and cyclin D1 in the rPASMCs, which inhibits the cell cycle progression and rPASMCs proliferation induced by CSE or nicotine. These results demonstrate that cigarette smoke induces the proliferation of rPASMCs by CCN2-mediated upregulation of cyclin D1.

Cigarette smoke can promote the proliferation of several types of pulmonary cells including lung epithelial cells and pulmonary

endothelial cells [Villablanca, 1998; Luppi et al., 2005]. In the present study, CSE at low concentrations (such as 1% and 2%) significantly induced the proliferation of rPASMCs. In contrast, CSE at higher concentrations suppressed the cells growth. Cell necrosis was observed when cells were exposed to CSE at high concentrations. These results were consistent with previous studies [Carty et al., 1997; Nishio and Watanabe, 1998; Wang et al., 2011]. However, other investigations reported different effects of CSE [Ambalavanan et al., 2001; Kim et al., 2004]. The discrepancy is likely attributable to both different cell types and distinct processes for CSE preparation. Cigarette smoke contains thousands of chemical compounds, some of which such as nicotine are mitogenic for vascular smooth muscle cells. Nevertheless, other compounds such as acrolein and acetaldehyde can inhibit cell proliferation [Ambalavanan et al., 2001]. The relative quantities of all compounds may change as the concentration of CSE varies, resulting in different effects of CSE on the proliferation of rPASMCs. Our findings revealed that 0.1–1 μM nicotine significantly induced rPASMCs proliferation, while 10 μM nicotine suppressed the cells growth.

CCN2 exhibits a variety of biological effects including those on the cell proliferation, adhesion, and migration [Takigawa, 2003]. Furthermore, there is accumulating evidence that CCN2 is implicated in the regulation of proliferation in smooth muscle cells [Fan et al., 2000; Wang et al., 2005], but there is little evidence on its role in the rPASMCs proliferation. In this study, knockdown of CCN2 by CCN2 siRNA strongly reduced the elevated mRNA and protein levels of CCN2 induced by CSE or nicotine and then inhibited the rPASMCs proliferation. These results indicate that CSE and nicotine can induce CCN2 production, which is consistent with other studies [Takeuchi et al., 2010; Wang et al., 2011]. Furthermore, these results reveal that CCN2 plays an important role in the regulation of cigarette smoke-induced rPASMCs proliferation. Nonetheless, in contrast to our results, others revealed that CCN2 could suppress the cell proliferation or even cause apoptosis in some cell types [Hishikawa et al., 1999; Kubota et al., 2000; Chien et al., 2006]. This may be attributed to the different cell types and experimental designs may explain the differences between studies.

Accumulating evidence has demonstrated that CCN2 plays a key role in cell cycle G1/S transition [Kothapalli and Grotendorst, 2000; Wahab et al., 2002; Wu et al., 2006; Deng et al., 2007]. To address the role of CCN2 in the cell proliferation, we further analyzed the effect of CCN2 on the cell cycle distribution. Our results showed that CCN2 could promote the cell cycle progression from G1 to S phase by upregulating the cyclin D1 expression in rPASMCs exposed to CSE or nicotine, which was consistent with previous studies indicating that CCN2-activated cyclin D1 in other cell types [Wahab et al., 2002; Wu et al., 2006; Deng et al., 2007]. Cyclin D1 is a critical regulator in the progression of cell cycle, and plays a key role in controlling G1/S transition [Sherr, 1994]. Cyclin D1, together with its binding partners cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (Rb) [Morikawa-Futamatsu et al., 2006; Ouyang et al., 2007]. Taken together, CSE and nicotine induce the proliferation of rPASMCs and promote cell cycle progression by regulating cyclin D1 expression which is mediated by CCN2.

Furthermore, CCN2 siRNA did not completely inhibit the proliferation of rPASMCs induced by CSE and nicotine, although the specific siRNA markedly suppressed the CCN2 expression. It is indicated that other mediators are also involved in CSE/nicotine-induced rPASMCs proliferation, such as endothelin, vascular endothelial growth factor, protein kinase C, and neutrophil elastase [Wright et al., 2003, 2006; Hu et al., 2007]. Additionally, it is noteworthy that CCN2 siRNA reduced, but could not completely abrogate the cyclin D1 expression, suggesting that although CCN2 is clearly involved in cyclin D1-mediated rPASMCs proliferation induced by CSE and nicotine, other intracellular signals may be also involved in regulating the process, such as MAPK/β-catenin and PI3K/Akt [Wahab et al., 2002; Wu et al., 2006; Deng et al., 2007]. These signals are also implicated in regulating the expression of cyclin D1, consequently silencing CCN2 expression in rPASMCs resulted in a reduction, but not a complete inhibition of cyclin D1 expression and proliferation of rPASMCs.

Although the exact mechanism by which CCN2 regulates the cyclin D1 expression still needs to be further clarified, upregulation of cyclin D1 may at least in part delineate the stimulating effects of CCN2 on the cell proliferation and G1/S transition.

In conclusion, our findings strongly indicate that CSE and nicotine can increase the CCN2 expression in the rPASMCs which then promote the rPASMCs cell proliferation and G1/S transition at least in part by regulating cyclin D1. This process might play an important role in the pulmonary vascular remodeling induced by CSE and nicotine. Our results suggest that CCN2 may be a promising new target for the treatment of pulmonary vascular diseases.

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