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The Matricellular Protein CCN1 Suppresses Lung Cancer Cell Growth by Inducing Senescence via the p53/p21 Pathway

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ABSTRACT

CCN1, a secreted matrix-associated molecule, is involved in multiple cellular processes. Previous studies have indicated that expression of CCN1 correlates inversely with the aggressiveness of non-small-cell lung carcinoma (NSCLC); however, the underlying mechanisms remain elusive. Using three NSCLC cell line systems, here we show that long-term treatment of cells with the recombinant CCN1 protein led to a permanent cell cycle arrest in G1 phase; cells remained viable as judged by apoptotic assays. CCN1-treated NSCLC cells acquired a phenotype characteristic of senescent cells, including an enlarged and flattened cell shape and expression of the senescence-associated β-galactosidase. Immunoblot analysis showed that addition of CCN1 increased the abundance of hypo-phosphorylated Rb, as well as accumulation of p53 and p21. Silencing the expression of p53 or p21 by lentivirus-mediated shRNA production in cells blocked the CCN1-induced senescence. Furthermore, a CCN1 mutant defective for binding integrin α6β1 and co-receptor heparan sulfate proteoglycans was incapable of senescence induction. Our finding that direct addition of CCN1 induces senescence in NSCLC cells provides a potential novel strategy for therapeutic intervention of lung cancers. J. Cell. Biochem. 114: 2082–2093, 2013.

KEY WORDS: LUNG CANCER; CCN1; SENESCENCE; p53; p21

CCN1, originally named CYR61, is a matricellular regulator of the CCN protein family. CCN proteins typically contain four conserved structural domains: an insulin-like growth factor-binding protein-like domain I, a von Willebrand factor type C repeat domain II, a thrombospondin type I repeat domain III, and a cysteine knot-containing domain IV [Perbal, 2004; Leask and Abraham, 2006; Jun and Lau, 2011]. Like many other matricellular proteins, CCN1 is implicated in multiple cellular functions, including cell adhesion, migration and angiogenesis. Moreover, CCN1 has also been shown to be involved in determination of different cell fates, such as differentiation, apoptosis, and proliferation [Lau, 2011]. A report has demonstrated that CCN1 can induce and accelerate fibroblast senescence, thereby avoiding fibrosis in the process of wound healing [Jun and Lau, 2010]. CCN1 exerts its activities by interacting with multiple integrin receptors, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_6\beta_1$, $\alpha_M\beta_2$, and $\alpha_{IIb}\beta_3$ [Chen and Lau, 2009; Lau, 2011]. Accumulating evidence suggests that distinct integrin receptors are employed by CCN1 for unique functions in specific target cells. For example, in fibroblasts CCN1 stimulates cell migration through integrin $\alpha_v\beta_5$ but enhances growth factor-induced mitogenesis through integrin $\alpha_v\beta_3$, whereas in endothelial cells CCN1 promotes cell adhesion through integrin $\alpha_6\beta_1$ but increases both migration and mitogenesis via integrin $\alpha_v\beta_3$

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[Grzeszkiewicz et al., 2001; Leu et al., 2002]. In CCN1-induced apoptosis of primary fibroblasts, heparan sulfate proteoglycans (HSPGs) can act as the co-receptor for integrin $\alpha_6\beta_1$ [Todorovicc et al., 2005; Juric et al., 2009; Chen and Lau, 2010]. The region within CCN1 protein for interacting with integrin $\alpha_6\beta_1$ and HSPGs has been localized to the DM site of domain IV, which is important for CCN1-dependent apoptosis and senescence induction [Chen et al., 2000; Lau, 2011].

Cellular senescence is a state of irreversible cell cycle arrest. Senescent cells are metabolically active but remain arrested in the cell cycle even in the presence of mitogenic stimulation. Senescent cells undergo morphological changes; cells acquire an enlarged and flattened phenotype and express some characteristic biochemical markers such as senescence-associated-β-galactosidase (SA-β-gal) [Campisi and d′Adda di Fagagna F., 2007]. Activation of p53 promotes senescent arrest via the induction of its transcriptional target p21, a general Cdk inhibitor known to play a major role in p53-mediated G1 and G2 arrests [Ben-Porath and Weinberg, 2005; Vazquez et al., 2008]. Aside from p21, other Cdk inhibitors including p16, p27 and p15 have also been shown to be involved in the induction and maintenance of cellular senescence [Zhang, 2007]. Tumor cells can undergo senescence when exposed to radiation and chemotherapeutic agents. Directing tumor cells to a permanently growth-arrested senescence-like state has been proposed as a promising targeted therapeutic approach for cancers [Dimri, 2005; Acosta and Gil, 2012].

CCN1 is differentially expressed in cancers of various types. Overexpression of CCN1 has been found in breast, pancreas, oral, and prostate cancers [Jiang et al., 2004; Holloway et al., 2005; Kok et al., 2010; D′Antonio et al., 2010]. Up-regulation of CCN1 enhances the tumorigenicity of breast cancer cells [Xie et al., 2001; Tsai et al., 2002]. Conversely, expression of CCN1 is down-regulated in non-small-cell lung cancers (NSCLCs), and is inversely correlated with tumor stages and mortality rates among patients [Chen et al., 2007; Mori et al., 2007]. Overexpression of CCN1 inhibits the proliferation of NSCLC cells, suggesting a tumor suppressor role of CCN1 specifically in lung cancers [Tong et al., 2001].

In the present study, we investigate whether CCN1 exerts its growth suppressive effects on NSCLC cells via the induction of cellular senescence. To circumvent the problem of losing cells with growth disadvantages during the process of selecting cells stably over expressing CCN1 using conventional strategies, we directly applied the bioactive recombinant CCN1 proteins to the culture medium. Using three NSCLC cell systems, we have shown that senescence induction is responsible for the CCN1-dependent growth suppression via the p53/p21 pathways. We have further demonstrated that the DM site of CCN1 is important for the senescence-inducing function. As expression of CCN1 is inversely correlated with the aggressiveness of lung cancers, our findings suggest a possible novel therapeutic strategy by locally administering the CCN1 protein to induce permanent growth arrest in NSCLC cells.

MATERIALS AND METHODS

CELL CULTURE

The A549, NCI-H460, and NCI-H520 human non-small cell lung carcinoma cell lines were obtained from Bioresource Collection and Research Center, Taiwan. These cells were cultured in RPMI 1640 medium (Life Technologies, NY) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. SF-9 and high-five insect cells were purchased from Life Technologies (Grand Island, NY) and cultured in Grace′s and EX405 medium, respectively (JRH Biosciences, Kansas).

REAGENTS, CHEMICALS, AND ANTIBODIES

SP sepharose and StrepTactin agarose were obtained from Novagen (Rockland, MA). Antibodies against p53, p21, Erk1/2, phospho-Erk1/2, phospho-Rb (Ser780), phosphor-Rb (Ser 807/811), and Rb (4H1) were purchased from Cell Signaling Technology (Boston, MA). Anti-Rb (G3-245) was from BD Pharmigen (San Jose, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

RECOMBINANT CCN1 PROTEIN PREPARATION

The human CCN1 cDNA was cloned from human umbilical vein endothelial cells and subcloned into the pFastBac1 plasmid for recombinant protein production via the Bac-to-Bac baculovirus expression system (Life Technologies, NY). Baculovirus particles were prepared by SF-9 insect cells. Secreted CCN1 proteins were collected from serum-free media of baculovirus-infected High-Five cells, and further purified by ion-exchange chromatography as described previously [Leu et al., 2002, 2004]. This expression strategy allowed purification of 2–3 mg CCN1 proteins from one liter conditioned supernatant. To exclude the possibility of unwanted effects from contaminants acquired during purification, the empty pFastBac1 vector was used to prepare a mock-control baculovirus. Following the same procedure of purifying CCN1 protein, similar fractions were collected from conditioned media of mock-infected cells and used as a control protein reagent (Mock control). CCN1-DM, a CCN1 mutant deficient in integrin $\alpha_6\beta_1$- and HSPG-binding, was previously prepared in the context of full-length murine CCN1 by substituting the basic residues (K or R) with alanine mutations in the H1 and H2 sites [Chen et al., 2000; Leu et al., 2004]. The H1 and H2 peptide sequences are identical between human and murine CCN1 proteins. We disrupted the corresponding codons in human CCN1 cDNA for alanine substitutions using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). To overcome the difficulty of purifying human CCN1-DM by ion-exchange chromatography, an additional DNA sequence (5′-TGGTCCCATCCACAGTTCGAGAAGAGCGGAGGTCAC-CATCACCATCACCATCACCACTGA-3′), encoding the 8-amino acid StrepII tag (WSHPQFEK) and a $(His)_8$ tag, was engineered to fuse with the C-terminus of the wild-type or mutant CCN1. The StrepII-tagged CCN1 and CCN1-DM proteins were then purified by StrepTactin affinity chromatography according to the manufacturer′s protocols (IBA GmbH, Germany). This StrepII-tagged wild-type version of CCN1 was used in parallel with CCN1-DM in the experiments shown in Figure 4. In addition, CCN1-DM was also used as an alternative control reagent to verify the specificity of CCN1-induced growth suppression on NSCLC cells (Fig. S3 and S4). The purity and amounts of recombinant proteins were evaluated by SDS-PAGE followed by Coomassie Brilliant Blue and silver staining (Fig. 4B). Protein concentrations were further determined using the Micro BCA protein assay kit (Pierce, Rockford, IL). Purified proteins were concentrated and exchanged into a 50 mM phosphate buffer containing 500 mM

NaCl, pH 7.0 by an ultrafiltration column (GE Healthcare, NJ), sterile-filtered (0.22 μm), and then kept at −80°C (stock 0.5–1.0 mg/ml).

POPULATION DOUBLING LEVEL

Cells were seeded at 1.5×10^4 cell/cm^2 (approximately 10∼20% confluency) and split every 60 h. For treatment of cancer cells, CCN1 proteins (0.5–4 μg/ml) were directly added to the culture medium in a soluble form. Alternatively, cells were allowed to adhere onto dishes pre-coated with CCN1 (10 μg/ml) as an immobilized form. Cells were counted and replated at the same low densities for continuous passages. At each passage, cell growth was evaluated by population doubling level (PDL) using the formula: PDL = log(N1/N0)/log2, where N0 is the initial cell number seeded, and N1 is the number obtained at each passage. Cumulative PDLs were presented as results, and a downward curve indicates growth retardation.

CELL CYCLE ANALYSIS

Cells were cultured in 6 cm dishes to 40% confluency, and fixed in ice-cold 70% ethanol. The fixed cells were treated with RNase A for 5 min, and stained with propidium iodide (0.1 mg/ml, Sigma) for 60 min. Cells were analyzed by flow cytometry using the FACSCalibur; the data were processed with CellQuest software (Becton Dickinson).

CELL PROLIFERATION ASSAYS

Two BrdU incorporation assays were performed to evaluate cell proliferation. For immunohistochemistry staining, cells were plated onto glass slides and labeled with BrdU using the labeling kit from Roche diagnostics (Germany). The cells were reacted with an anti-BrdU antibody and counterstained by Mayer's Hematoxylin for visualization. Alternatively, a colorimetric ELISA-based kit for BrdU incorporation (Millipore, MA) was used for quantification of cell proliferation. BrdU incorporation was measured by absorbance at 450 nm after the samples reacted with the HRP-conjugated anti-BrdU.

SENESCENCE-ASSOCIATED β-GALACTOSIDASE ASSAY

The staining method for senescence biomarker was performed according to the protocol by Campisi and colleagues [Itahana et al., 2007; Debacq-Chainiaux et al., 2009]. Cells were washed with PBS and fixed in 3% formaldehyde. After washing, the cells were incubated with a freshly prepared X-gal staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 40 mM citric acid in sodium phosphate at pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$ for 8–16 h, and senescent cells were visualized under a microscope. For quantitative measurement of the β-galactosidase activity, an alternative protocol based on conversion rate of 4-methylumbelliferyl-β-D-galactopyranoside (MUG) substrate to hydrolysis product 4-methylumbelliferone (4-MU) was adopted [Gary and Kindell, 2005].

LENTIVIRUS PREPARATION AND INFECTION

The lentiviral pLKO.1 vectors expressing the control non-targeting shRNA as well as shRNAs against human p53 (NM_000546, TRCN0000003756) and human p21 (NM_000389) were obtained from the National RNAi core (Academia Sinica, Taiwan). Lentivirus particles were generated in HEK293T cells cotransfected with the pLKO.1-shRNA plasmid, pCMV-ΔR8.91, and pMD.G vectors. To obtain the lentiviral transduction particles for effective p21 knockdown, an equal mixture of three p21-shRNA plasmids (TRCN0000287021, TRCN0000287091, TRCN0000294421) were used to cotransfect 293T cells, using a protocol described previously [Kuwahara et al., 2010]. Cancer cells (10^5 cells/ml) were infected with lentivirus in the presence of protamine sulfate (8 μg/ml) according to the procedures recommended by the National RNAi core. After viral infection, shRNA-expressing cells were enriched by puromycin selection. Effectiveness of gene silencing was examined by immunoblotting using anti-p53 or anti-p21 antibodies. For forced expression of CCN1, cDNA of human CCN1 was subcloned into the pLKO-as3w-puro lentiviral vector, which allows CMV promoter-driven transgene overexpression (Academia Sinica, Taiwan). Lentivirus particles and the selected pool of CCN1-overexpressing cancer cells were prepared using similar procedures as above.

WESTERN BLOTTING

Cell lysates were prepared and proteins were separated on 8∼13% SDS-PAGE gels (60 μg/lane). The proteins were transferred to a nitrocellulose membrane (Millipore, MA) by electroblotting and probed with specific primary antibodies. After incubation with secondary antibodies, that is goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) or HRP-conjugated anti-rabbit IgG, an ECL kit (Millipore, MA) was used for signal detection.

COLONY FORMATION ASSAY

Cells were seeded as a single-cell suspension at low densities (200–2000 cells per 10 cm dish) for 3–4 weeks. The colonies formed were then fixed and stained with 0.1% crystal violet or analyzed by SA-β-gal staining. Plating efficiency represents the ratio of the number of resultant colonies to the number of cells seeded.

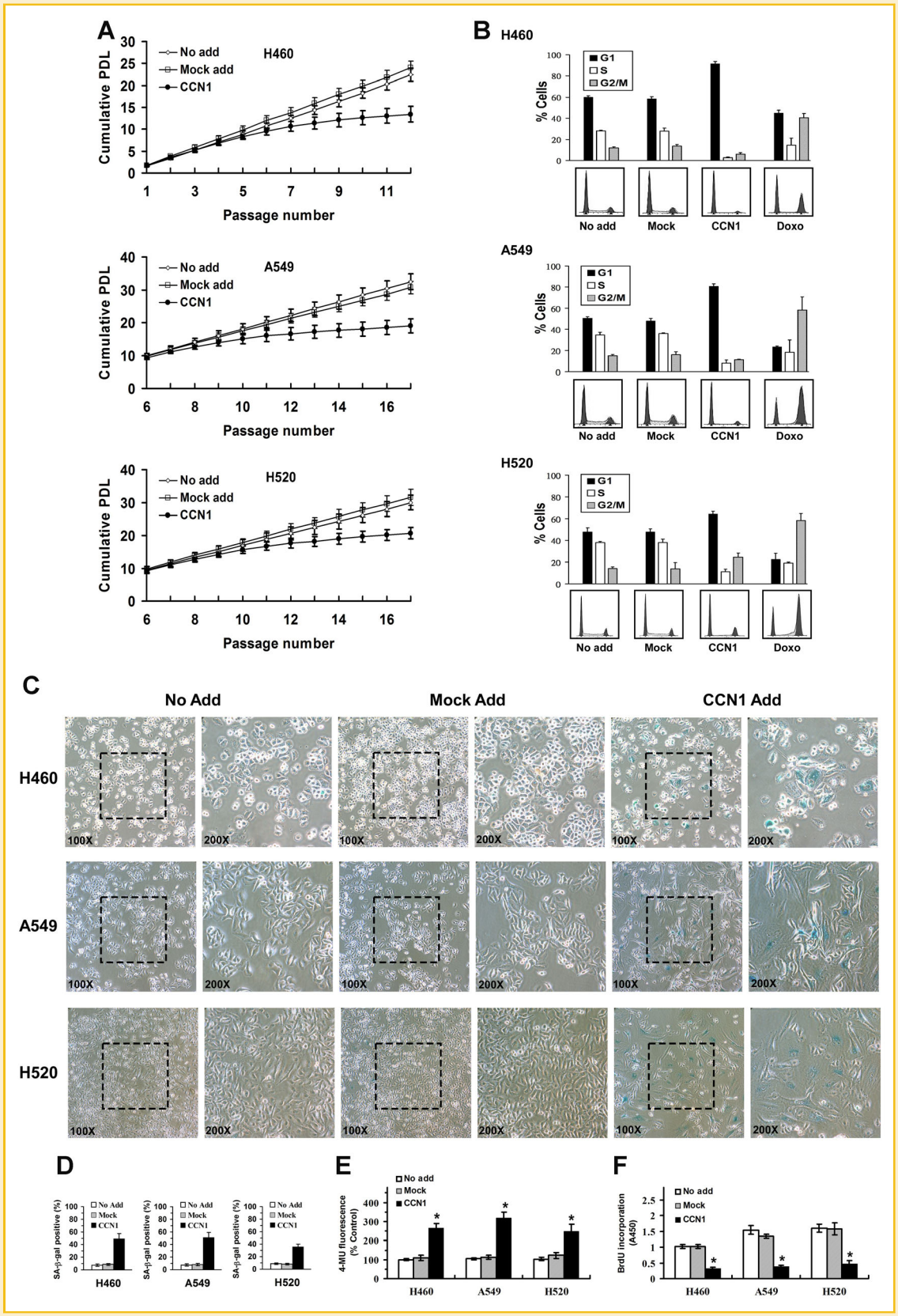
DATA ANALYSIS

All experiments were independently repeated for at least three times. The results were expressed as mean ± SD and statistical significance was determined by the Student's *t*-test.

RESULTS

ADDITION OF EXOGENOUS CCN1 INDUCES GROWTH ARREST AND SENESCENCE IN HUMAN NON-SMALL CELL LUNG CARCINOMA CELLS

It has previously been shown that overexpresison of CCN1 inhibits proliferation of NSCLC cells [Tong et al., 2001, 2004]; however, the underlying mechanism has not been fully elucidated. We investigated the effects of exogenous CCN1 addition on human NSCLC cells using three cell line models, including H460 (large cell carcinoma), A549 (adenocarcinoma), and H520 (squamous cell carcinoma). After continuous passage of these cells in the presence of added CCN1 proteins, all three cell lines showed significant cell growth suppression. The CCN1-treated H460 cells showed prominent growth


A
Cumulative PDL
No add
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CCN1
H460
A549
H520
Passage number
B
H460
A549
H520
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G1
S
G2/M
No add
Mock
CCN1
Doxo
C
No Add
Mock Add
CCN1 Add
H460
A549
H520
100X
200X
D
SA-β-gal positive (%)
No Add
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CCN1
H460
A549
H520
E
4-MU fluorescence (% Control)
F
BrdU incorporation (A450)

Fig. 1.

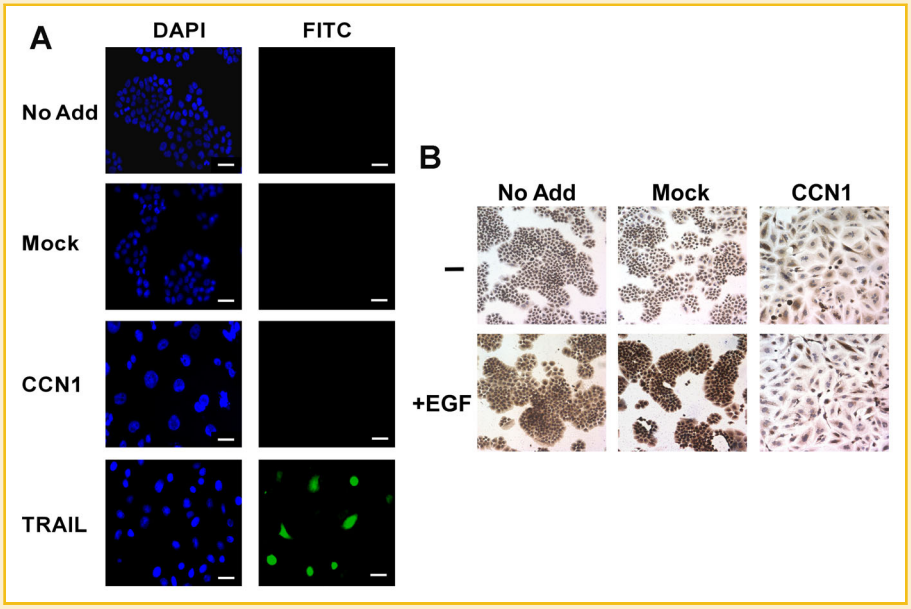

Fig. 2. CCN1-treated cells are not apoptotic and are less responsive to growth factor stimulation. A: H460 cells were treated as indicated. At passage 12, cells were plated on glass slides and analyzed by a TUNEL assay for apoptosis; total cell nuclei were shown by DAPI counterstaining. Cells treated with TRAIL (10 ng/ml, 12 h) were included as a positive control for TUNEL (FITC-positive nuclei). Bar, 10 μm. B. Cells were treated with or without CCN1 as indicated followed by incubation with EGF (10 ng/ml) for 24 h. Direct BrdU staining was performed as described in Section Materials and Methods to examine individual cells for active proliferation.

retardation starting at passage number 8. For A549 and H520 cells, significant growth suppression was also observed starting at passage number 11 and 13, respectively (Fig. 1A). In contrast, control cells with no CCN1 administration remained capable of proliferating (Fig. 1A). Analysis by flow cytometry for cell cycle distributions of H460, A549, and H520 cells at passage number 11, 14, and 16, respectively, indicated that CCN1 induced cell cycle arrest in the G1 phase in all three NSCLC cell lines. In contrast, the anti-cancer drug doxorubicin, used as a control, caused accumulation of cells in the G2/M phase (Fig. 1B). The CCN1-treated cells exhibited a flattened morphology with concomitant cell enlargement, the presence of vacuole-rich cytoplasm, and high expression of perinuclear senescence-associated-β-galactosidase (SA-β-gal), which are characteristic senescence-like morphologies (Fig. 1C). Assays by SA-β-gal staining showed that CCN1 significantly increased the percentages of SA-β-gal positive cells (40–60% vs. 5–10% positive cells in control groups) in all three NSCLC cell lines (Fig. 1D). Quantification of cytochemical staining confirmed that CCN1 treatment significantly increased SA-β-gal activities (2∼3 increase vs. control groups, Fig. 1E). The CCN1-treated cells were further analyzed for DNA replication by BrdU incorporation assays. As shown, addition of CCN1 reduced BrdU incorporation to ∼20% of that observed in control samples with no or mock treatment in all three cell lines, suggesting the cessation of DNA synthesis in the senescence-like cells (Fig. 1F).

We also examined the effects of lentivirus-based forced expression of CCN1 in the three NSCLC cell lines. After infection, cells were seeded at a low density and cultured continuously for 30 days. We observed significant reduction of the plating efficiency in colony formation assays in CCN1-overexpressing cells (5–10% versus

Fig. 1. Addition of exogenous CCN1 induces growth arrest and senescence in human NSCLC cells. A: Recombinant human CCN1 proteins produced in insect cells using a baculovirus expression system was prepared as described in Section Materials and Methods. Protein samples purified from insect cells undergone mock infection were used as the reagent control (Mock add). Human NSCLC cell lines H460 (large cell carcinoma), A549 (adenocarcinoma), and H520 (squamous cell carcinoma) were continuously treated with purified recombinant CCN1 proteins (4 μg/ml). Cells were initially plated at low densities and split every 60 h after plating. This procedure was repeated until indicated passages. At each passage, the cell number was counted for calculating the population doubling level (PDL). Data presented are cumulative PDLs from three independent experiments. B: H460, A549, and H520 cells were subjected to cell cycle analysis at passage number 11, 14, and 16, respectively. The percentage of cells in each phase of cell cycle was indicated and a representative profile of cell cycle was shown. CCN1-treated cells appeared to accumulate at the G1 phase. The doxorubicin-treated cells (Doxo, 1 μM, 24 h) were shown as controls for the G2/M phase arrest. C: Cells were fixed and stained for SA-β-gal. Representative micrographs were captured at 100× magnification. The central area marked by the box in each photo was further magnified at 200× at the right. D: Quantitative representation of results from C. Approximately 500 cells were counted from randomly selected fields for each condition. Bar graphs show percentages of cells stained positive for SA-β-gal (mean ± SD) from three independent experiments. E: The SA-β-gal activity was measured by the conversion of MUG to 4-MU, and compared to that obtained from the untreated cells (no add), which was set as 100%. F: Analysis of active replication of cells by BrdU incorporation. Cells were seeded for determination of BrdU incorporation as described in the Materials and Methods. Bars represent (mean ± SD) from three independent experiments. $^{*}P < 0.05$ versus the control group.

40–60% in control cells). Furthermore, the resultant colonies of CCN1-overexpressing cells, which were much smaller compared with the control, stained positive for SA-β-gal (Fig. S1). Together, the results suggest that CCN1 may suppress cell growth via the induction of cellular senescence-like phenotypes in NSCLC cells.

CCN1-TREATED CELLS ARE NOT APOPTOTIC

As indicated by trypan blue assays, addition of CCN1 did not cause cell death during the course of continuous passage (data not shown). TUNEL assays were further performed in H460 cells to exclude the possibility that the growth suppression exerted by CCN1 is due to apoptosis induction. As shown in Figure 2A, no TUNEL-positive cells were detected in mock treatment control or CCN1-treated cells, whereas about 30% of cells treated with the TNF-related apoptosis-inducing ligand (TRAIL) were apoptotic. As shown by direct BrdU staining, the CCN1-treated cells displayed a senescent morphology with cell enlargement and allowed little BrdU incorporation; when stimulated with EGF, which is a potent mitogen for NSCLC cells, the majority of the senescence-like cells showed little increases in BrdU incorporation compared to control cells (Fig. 2B). Proliferation of cells was also analyzed by the ELISA-based BrdU incorporation assay, and the results showed that the extent of BrdU incorporation was indeed significantly reduced by prior CCN1 treatment (Fig. S2); however, it should be noted that the fold changes of BrdU incorporation in response to EGF stimulation were comparable among cells without prior treatment, mock-treated cells, and CCN1-treated cells. Together, our results indicate that CCN1 can cause long-term growth arrest without affecting cell survival in NSCLC cells.

BOTH SOLUBLE AND IMMOBILIZED CCN1 CAN INDUCE SENESCENCE

We tested whether matricellular CCN1 can, in addition to acting in a soluble form, induce senescence in a substratum-bound form. The H460 cells were plated onto dishes pre-coated with CCN1 for continuous cell culture. As shown in Figure 3A, immobilized CCN1 suppressed cell growth to a comparable extent, compared with that obtained from direct addition of CCN1 to the culture media. In addition, analysis of SA-β-gal activities (Fig. 3B and C) or BrdU incorporation (quantified in 3D) indicated that CCN1 functioned equally well either as a soluble factor or in a substratum-bound form. To test the colony formation capacity of the senescent cells, the CCN1-treated cells (passage number 12) were replated onto the culture dishes, and grown in the absence of CCN1. As shown in Figure 3E, the senescence-like cells resulting from treatment with either soluble or immobilized CCN1 were unable to repopulate into colonies, indicating that the CCN1-induced senescence-like cells are in a state of permanent cell cycle arrest.

THE DM REGION OF CCN1 IS REQUIRED FOR SENESCENCE INDUCTION

It has been shown that the suppression effects of murine CCN1 require integrin $\alpha_6\beta_1$ and the co-receptor HSPG [Todorovicc et al., 2005; Juric et al., 2009; Jun and Lau, 2010]. We have established the human CCN1 mutant equivalent of the mouse CCN1 mutant (CCN1-DM) that harbors mutations in domain IV, rendering it incapable of binding to integrin $\alpha_6\beta_1$ or HSPG. Unlike the wild-type CCN1, addition of the CCN1-DM mutant protein failed to suppress cell growth (Fig. 4C), to induce morphological changes of characteristics of cellular senescence (Fig. 4D), to inhibit BrdU incorporation (Fig. 4F), or to induce SA-β-gal activities (Fig. 4E). These results indicate that the integrity of the DM region within the CCN1 protein is required for senescence induction in NSCLC cells. In addition, we also demonstrated that the exogenous addition of purified recombinant CCN1-DM could not reproduce the CCN1-mediated cell cycle arrest (Fig. S3A), suppression of BrdU incorporation (Fig. S3C), or inhibition of colony formation (Fig. S3D). These observations indicate that the above-mentioned effects of direct CCN1 addition are specific and not resulting from contaminants co-purified with CCN1.

THE CCN1-INDUCED NSCLC SENESCENCE IS P21 AND P53-DEPENDENT

We analyzed the CCN1-induced senescent cells for their expression of relevant cell-cycle regulatory proteins. CCN1 was either added to the culture media or coated on the culture dishes for senescence induction in H460 and A549 cells. As shown by immunoblot analysis (Fig. 5), treatment of CCN1 inhibited the phosphorylation of Rb; concomitant reduction in levels of phosphorylated Rb and increased abundance of hypo-phosphorylated Rb were observed in CCN1-treated cells. CCN1 treatment also resulted in increased levels of p53 and p21 in both H460 and A549 cells. We also found that the addition of recombinant CCN1-DM did not inhibit Rb phosphorylation or induce the accumulation of p21 and p53 (Fig. S4).

We next investigated the role of p21 and p53 in the CCN1-mediated cellular senescence by specific shRNAs targeting p21 and p53 expression (Fig. 6A,E). Addition of CCN1 to the p21-deficient cells failed to suppress cell growth (Fig. 6B), to induce senescence-like morphologies (6C), or to enhance SA-β-Gal activities (6D). Similar results were found in the p53-knockdown cells (Fig. 6F–H). Together, these results indicate that p21 and p53 are required for CCN1-mediated cellular senescence in NSCLC cells.

DISCUSSION

Lung cancer, of which NSCLC is the most common type, is one of the leading causes of cancer-related mortality worldwide [Bray et al., 2012]. The matricellular protein CCN1 has been shown to control the growth of human NSCLC [Tong et al., 2001, 2004]. Using three NSCLC model cell systems, we have found that CCN1 can inhibit the growth of NSCLC cells via the induction of cellular senescence. Long-term treatment of lung cancer cells with exogenous CCN1 induces growth suppression with characteristics of cellular senescence, including morphological changes, SA-β-gal expression, and cell cycle arrest at the G1 phase. Although a previous study has demonstrated that CCN1 can induce senescence in fibroblasts [Jun and Lau, 2010], our results have uncovered, for the first time to our best knowledge, a role of CCN1 in inducing senescence of cancer cells.

There are noticeable differences in CCN1-induced senescence in fibroblasts and in NSCLC cells. While fibroblasts enter cellular senescence within 3–6 days of CCN1 treatment [Jun and Lau, 2010], we could only observe senescence phenotypes in CCN1-treated NSCLC cells after at least 8 passages. Despite the difference, we demonstrate that, similar to fibroblasts, activation of the p53/p21

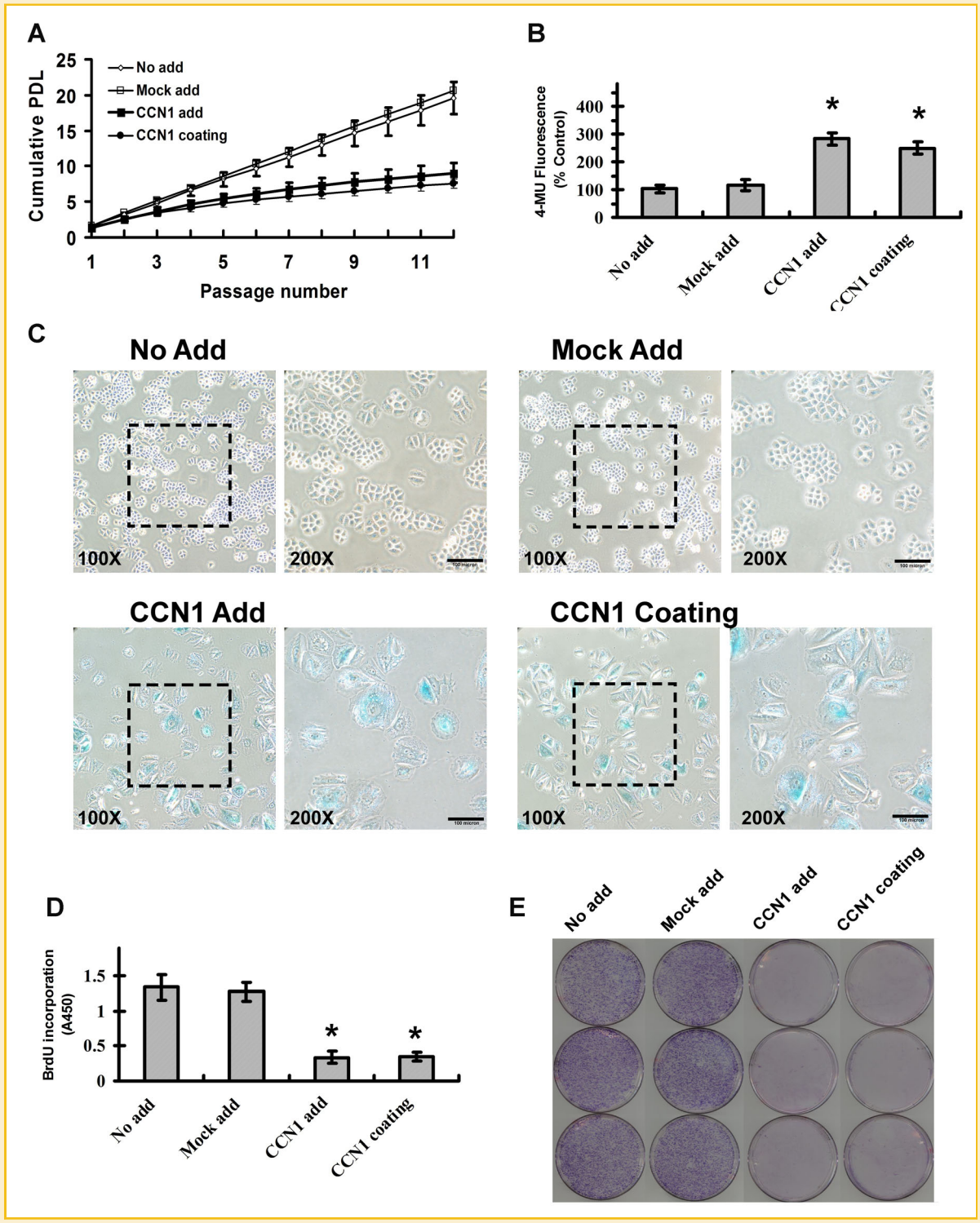

Fig. 3. Both soluble and immobilized CCN1 can induce cellular senescence. A: H460 cells were continuously supplied with soluble CCN1 (4 μg/ml) in the culture medium (CCN1 add) or plated onto CCN1-coated (10 μg/ml) dishes (CCN1 coating). As a control, cells were also continuously treated with the reagent control (prepared as described in Section Materials and Methods) (Mock add). Cells were counted at each passage for calculating the population doubling level (PDL), and cumulative values are shown. B: The SA-β-gal activity was determined at passage 12 by measuring the 4-MU production. Bars (mean ± SD) are from three independent experiments, each performed in triplicates. $^{*}P < 0.05$ versus the control group. C: Morphological changes induced by CCN1 treatment. Representative photos of direct SA-β-gal staining of cells at 100× magnification were shown, and boxed areas were further magnified at 200×. Bar, 100 μm. Senescent cells displayed an enlarged and flattened morphology, and are positive for SA-β-gal (blue). D: Determination of the cell proliferation index by the colorimetric BrdU incorporation assay. BrdU uptake was measured by absorbance at 450 nm as described. Triplicate data are expressed as mean ± SD from three independent experiments. $^{*}P < 0.05$ versus the control group. E: Colony formation assays. Cells pre-treated under indicated conditions were replated at passage 12 onto 10 cm dishes at a density of 2,000 cell per dish, and allowed to further grow for 3 weeks in the absence of CCN1 proteins.

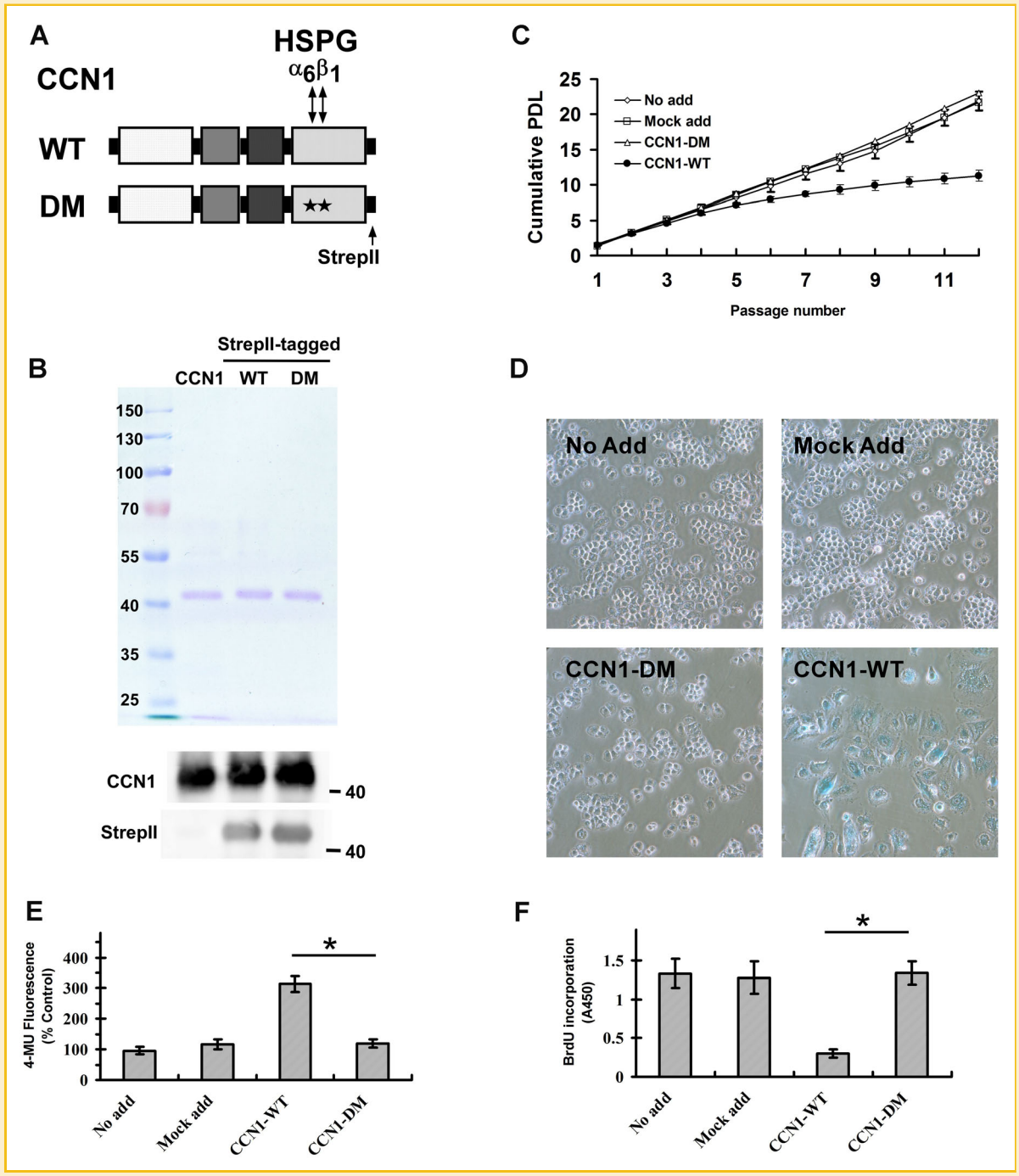

Fig. 4. The DM region of CCN1 is required for senescence induction. A: A schematic diagram of CCN1 domain structure. Asterisks, positions of the residues important for interacting with integrin $\alpha_6\beta_1$ and HSPG; these residues are mutated in CCN1-DM. B: Analysis of purified recombinant proteins. One μg each of recombinant human CCN1 (CCN1), wild-type (WT) and mutant (DM) versions of CCN1 with a StrepII tag was subjected to SDS-PAGE followed by Coomassie Brilliant blue staining (top) or Western analysis using anti-CCN1 or anti-StrepII antibodies (bottom). Molecular weight markers (kDa) are shown on the left. C: H460 cells were treated with successive addition of reagent control (Mock add), CCN1-WT, or CCN1-DM protein (4 μg/ml) into the culture medium. Proliferation of cells was monitored by the cumulative population doubling level (PDL). D: Representative results of SA-β-gal staining (magnification 200×). E: Analysis of the SA-β-gal activity by 4-MU fluorescent production. F: Analysis of cell proliferation by BrdU incorporation. All experiments were performed in triplicates; bar graphs represent (mean $\pm$ SD) from three independent experiments. $^*P < 0.05$ versus the CCN1-WT control.

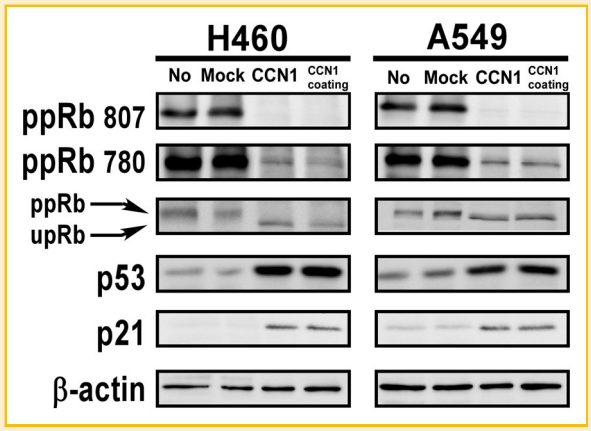

Fig. 5. Expression of cell cycle regulators in the CCN1-treated cells. The cells were treated with the indicated reagents as in Figure 3. Cell lysates were prepared after 12 consecutive passages, and analyzed by immunoblotting with specific antibodies recognizing Rb phosphorylated at Ser807/811 (ppRb807), Rb phosphorylated at Ser780 (ppRb780), total phosphorylated Rb (ppRb), and unphosphorylated Rb (upRb). β-actin was used as the sample loading control.

pathway is instrumental for CCN1 to induce senescence-like changes and permanently arrest the growth of NSCLC cells. This is not surprising because p53 is regarded as a master regulator of cell fate determination. As a transcription factor, p53 directly modulates the transcription of several hundred genes. The choice of a particular cell fate is a consequence of the combinatorial actions of the p53-regulated targets [Ben-Porath and Weinberg, 2005; Vazquez et al., 2008]. For example, high levels of p53 tend to elicit apoptosis, whereas low levels permit senescence. Due to the multitude of factors involved, the exact mechanisms downstream of p53/p21 responsible for CCN1-mediated senescence remain to be explored. We are setting up strategies including microarray and proteomic analyses to delineate the detailed signaling pathway of CCN1-induced cellular senescence.

Intriguingly, our results do suggest that the intracellular signaling pathway involved in CCN1-induced cellular senescence in NSCLC cells is distinct from that in primary fibroblasts. In primary fibroblasts, p53 and the Cdk inhibitor p16 are both important for CCN1-induced senescence [Jun and Lau, 2010]. However, all three NSCLC cell systems tested in this study are deficient for p16 expression (COSMIC database, www.sanger.ac.uk) [Jin et al., 1995; Okamoto et al., 1995; Shapiro et al., 1995; Kubo et al., 1999], which argues against the involvement of p16 in CCN1-induced senescence in these cells. In addition, while CCN1-induced senescence in fibroblasts is accompanied by MAPK activation and a sustained elevation of levels of reactive oxygen species (ROS) [Jun and Lau, 2010], our preliminary data indicate that administration of CCN1 to NSCLC cells results in reduced activation of the MAPK pathway and only transient increases of ROS levels in the senescent cancer cells (Leu, unpublished results). The molecular mechanism underlying this delay in CCN1-induced senescence in cancer cells is unclear at this point. Further investigation is required to determine whether the delayed onset of cellular senescence observed in CCN1-treated NSCLC cells can be attributed to the absence of a p16-linked pathway and/or unique mechanisms regulating MAPK signaling and cellular ROS levels. Consistent with the current view that CCN1 effects are cell context-dependent [Lau, 2011], these findings together suggest that CCN1 may employ different mechanisms to induce senescence in fibroblasts for curbing fibrosis and in NSCLC cells for suppressing tumor growth.

A previous study using CCN1-overexpressing stable clones of H460 and H520 NSCLC cells has also demonstrated that CCN1 can induce growth suppression in NSCLC cells [Tong et al., 2001]. Clones with CCN1 overexpression display nuclear accumulation of β-catenin, which induces expression of c-Myc and p53 for the CCN1-mediated growth suppression in these cells [Tong et al., 2004]. However, we were unable to detect any changes in cellular c-Myc and β-catenin levels when CCN1 was directly added to the medium of cultured NSCLC cells (Leu, unpublished results). The discrepancy may have arisen from the difference in the experimental set-up. It is conceivable that in the study using stable clones, those cells undergone CCN1-induced senescence may have been eliminated during the process of clonal selection, and the stable clones obtained may have accumulative adaptive changes that lead to increased expression of c-Myc and β-catenin; the mechanisms by which c-Myc inhibits the growth of these CCN1-overexpressing cells remain to be elucidated. Our observation of no activation of the c-Myc and β-catenin pathways in CCN1-treated NSCLC cells is in better agreement with the many reports in the literature indicating that activations of c-Myc and β-catenin signaling pathways contribute to the malignancy of lung cancers [Suzuki et al., 2007; Tseng et al., 2008; Rapp et al., 2009; Iwakawa et al., 2011].

It has been shown that CCN1 interacts with multiple receptors to attain particular functions in a cell type-specific manner. Therefore, the effects of CCN1 on growth suppression and cellular senescence may be context- and cell type-dependent. For example, in contrast to the tumor suppression effect in NSCLC, CCN1 is known to promote tumorigenesis in breast cancers primarily by specifically binding to the $\alpha_v\beta_3$ integrin [Menendez et al., 2005]. Our results show that the integrity of the DM site within domain IV of CCN1 is responsible for inducing senescence in NSCLC cells, suggesting the involvement of integrin $\alpha_6\beta_1$ and the co-receptor HSPG, which appears similar to CCN1 action mechanism in fibroblasts. However, because different proteoglycans may be engaged in CCN1-directed activities, it remains to be determined which type of HSPG mediates the CCN1-induced cellular senescence in NSCLC cells. In accordance with our findings that domain IV is responsible for senescence induction and growth suppression in NSCLC cells, the C-terminal region of CCN1 has been suggested to be involved in tumor suppression [Lau, 2011; Jun and Lau, 2011]. The production of CCN1 fragments that retain the senescence-inducing activity while devoid of unwanted tumor-promoting activities, may serve as potential NSCLC-targeting reagents.

There is emerging evidence suggesting that tumor microenvironment and extracellular matrix components play a significant role in regulating cell growth and survival [Joyce and Pollard, 2009; Hanahan and Weinberg, 2011; Lu et al., 2012]. Unlike the structural matrix molecules involved in tissue integrity, the matricellular proteins modulate cell-matrix interaction and cellular functions. As a matricellular protein, CCN1 expression is under contextual regulation

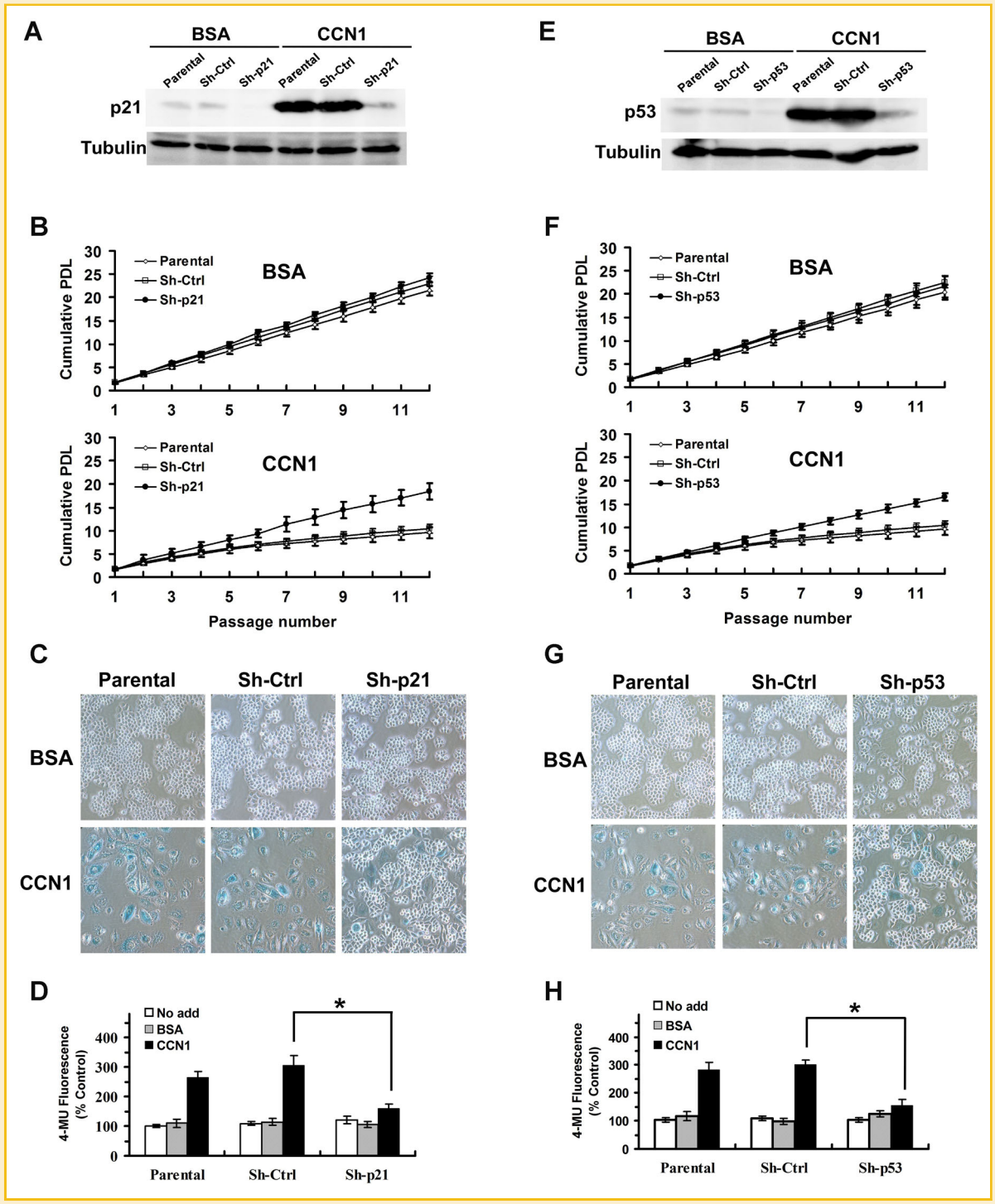

Fig. 6. The CCN1-induced senescence is p21- and p53-dependent. A: H460 cells were infected with lentivirus expressing shRNA against p21 (Sh-p21), and both basal and CCN1-induced p21 expression was effectively silenced. Non-targeting shRNA was used as the control (Sh-Ctrl). B: The control and p21-knockdown cells were continuously cultured in the presence of CCN1 or BSA. The cell number was counted at each passage and population doubling level (PDL) was calculated as in Figure 1. C: Senescent cells were monitored and photographed by SA-β-gal staining at 200× magnification. D: Quantitative assays for the SA-β-gal activity. Results from three independent experiments done in triplicates were analyzed and represented as mean ± SD. $^{*}P < 0.05$ compared with Sh-Ctrl. E–H: Similar analyses were carried out on p53-knockdown cells.

[Lau, 2011]. In congruence with our finding that addition of CCN1 induces senescence in lung cancer cells, upregulation of the CCN1 gene has been noted in the drug-induced cancer senescence [Chang et al., 2002], the replicative senescent dermal fibroblasts [Quan et al., 2012], and in senescent NSCLC cells (Leu, unpublished results). Compared with small cell lung carcinoma, NSCLC is less sensitive to radiation therapy and chemotherapy treatments [Goldstraw et al., 2011; van Meerbeeck et al., 2011]. Our finding that direct addition of CCN1 induces cellular senescence in NSCLC cells provides a potential novel strategy for therapeutic intervention of lung cancers.

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