Single nucleotide mutation changes the capability of CCN3 in osteosarcoma cell invasion and differentiation

Xuejing Yan [a], Wei Yan [b,c,**], Xin Fu [b], Yuqiao Xu [b], Ning Zhu [a], Chuan Qiu [a], Mengmeng Bu [a], Yan Shen [a], Meihong Chen [a,*]

[a] Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, China
[b] Department of Pathology, Xijing Hospital of Fourth Military Medical University, Xi'an, China
[c] Department of Surgery, The University of Michigan, MI, USA

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ABSTRACT

This study aimed to identify significant mutations in CCN3 gene in osteosarcoma, and to explore the influence of this gene on cell invasion and differentiation and the underlying mechanism. Sanger sequencing was used to identify CCN3 gene sequence in human osteosarcoma cell lines, peripheral blood mononuclear cells (PBMC), and osteosarcoma tissues. Wild-type and mutant CCN3 (mCCN3) were ectopically expressed by lentivirus in human osteosarcoma cell lines. Tumor cell invasion was measured by trans-well assay. Osteogenic differentiation was induced by osteogenic differentiating medium and evaluated based on alkaline phosphatase activity and collagen type I alpha 1 chain and osteocalcin expression. Western blotting was used to detect protein levels of CCN3 and mCCN3 in cytoplasmic, nuclear and secreted fractions of cells. A G-to-A single nucleotide mutation in the coding region of CCN3 was found in both osteosarcoma cells and tissues. The frequency of this mutation in osteosarcoma tissue was much higher than that in para-carcinoma tissue and PBMC of healthy people. This nucleotide mutation decreased nuclear glycosylated full length protein level of CCN3 and affected osteosarcoma cell invasion and differentiation. A lower nuclear ratio of glycosylated/non-glycosylated isoforms accounted for the different behavior of mCCN3 compared with CCN3. The G-to-A mutation identified in CCN3 resulted in differential glycosylated full-length protein levels and altered the functional role of CCN3 in osteosarcoma cell invasion and differentiation.

Introduction

Osteosarcoma is a malignant tumor that occurs mostly in children and adolescents. It has a strong tendency to chromosomal instability [1]. The current standard treatment is a combination of neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy based on doxorubicin, cisplatin, and high-dose methotrexate [2]. Although perioperative chemotherapy has greatly improved 5-year survival rates and limb salvage rates in osteosarcoma patients, with a 5-year survival rate of 60–70% in patients with limited-stage disease, there has been no breakthrough in the survival benefit resulting from chemotherapy in the past 40 years [3]. The 5-year survival rate remains approximately 20% [4], owing to high rates of relapse and metastasis. Lack of pathologic histology grading causes further difficulties in treating osteosarcoma

[5]. Currently there are multiple osteosarcoma cell lines available. However, their characteristics vary greatly. Comparison of the commonly used osteosarcoma cell lines has shown that Saos-2 cells exhibit the most mature osteoblastic profile, whereas MG-63 cells display features of the undifferentiated early osteoblast phenotype, that is, the least mature one [6,7].

CCN3, also known as nephroblastoma over-expressed (NOV), is a member of the CCN family of proteins. Like other members of this family, CCN3 contains a signal peptide followed by four distinct domains: insulin-like growth factor binding protein (IGFBP), von Willebrand type C repeat (VWC), thrombospondin type I domain (TSP-1), and cysteine-rich carboxyl terminal (CT). The predicted molecular weight of the human CCN3 protein is 39 kD, and it has two putative N-glycosylation sites at position 97 and 280. Post-translational glycosylation

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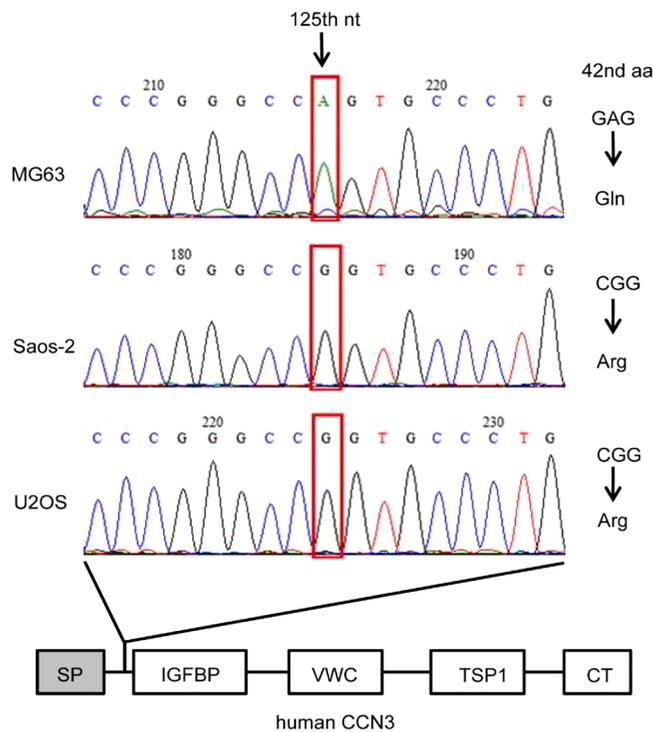
Fig. 1. Sequencing of CCN3 in human osteosarcoma cell lines and diagram of mutation.

Table 1
Genomic sequencing result of the 125th nucleotide in human CCN3 coding sequence in clinic samples.

125th nucleotide	cases/total	percentage	*P* value
PBMC of normal control			
G/G	20/30	66.67%	
A/G	6/30	20.00%	
A/A	4/30	13.33%	
para-carcinoma tissue[1]			
G/G	0/18	0.00%	<0.001
A/G	14/18	77.78%	
A/A	4/18	22.22%	
osteosarcoma tissue[1,2]			
G/G	3/35	8.57%	
A/G	1/35	2.86%	
A/A	31/35	88.57%	

[1] Compared with PBMC of normal control.
[2] Compared with para-carcinoma tissue
 Examined by Fisher's exact test.

results in different CCN3 isoforms [8]. The observed molecular weight of the human full-length CCN3 protein ranges from 46 to 55 kD, depending on the cell type [9–11]. Multiple smaller CCN3 bands have also been detected, these have been proposed to represent the amino-truncated forms following proteolysis or products of mRNA splicing [12–14]. CCN3 displays diverse subcellular localizations. It can be distributed in nucleus, cytoplasm, or cell membrane and can be secreted and associate with extracellular matrix [15,16]. These complicated properties of CCN3 make it difficult to clarify its function and the underlying mechanism.

CCN3 has important roles in various cellular functions, including proliferation, differentiation, apoptosis, and migration. The functions of CCN3 vary greatly across different cell types and are believed to be dependent on cell context. For example, CCN3 inhibits proliferation of choriocarcinoma Jeg3 cells [17], whereas it promotes proliferation of muscle skeletal cells [18]. CCN3 is involved in many types of tumors. However, to date, there has been no report of CCN3 mutation in tumors,

and there have been insufficient studies of CCN3 in osteoblasts and osteosarcoma. The few reports available indicate that CCN3 impairs normal osteoblast differentiation [19,20]. In osteosarcoma cells, CCN3 inhibits proliferation while promoting apoptosis and migration [21,22]. High expression of CCN3 is significantly correlated with worse prognosis in osteosarcoma [23].

In this study, we found a single nucleotide mutation of CCN3 gene that changed the amino acid at the corresponding position in the protein in human osteosarcoma cell line MG-63. We checked this mutation site in the genomic sequence of several osteosarcoma cell lines, as well as in clinical samples from osteosarcoma patients and healthy volunteers. Multiple assays showed differences in the behavior of the same osteosarcoma cell type depending on whether CCN3 or mutant CCN3 (mCCN3) was ectopically expressed. To explore the mechanism underlying this difference in behavior, the cellular localization and isoforms of both wild-type CCN3 and mCCN3 were investigated.

Materials and methods

Cell lines and clinical samples

MG-63, Saos-2, and U2OS human osteosarcoma cell lines were purchased from the Cell Bank of the Chinese Academy of Medical Sciences and Peking Union Medical College. MG-63 cells were cultured in MEM/EBSS medium (ThermoFisher). Saos-2 cells were cultured in Ham's/F12 medium (ThermoFisher). All the above media were supplemented with 10% fetal bovine serum (FBS). Peripheral blood mononuclear cells (PBMCs) of healthy volunteers from Shaanxi province in China were provided by Prof. Guangliang Shan of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Paraffin sections of tissues from osteosarcoma patients in Shaanxi province in China were obtained from Prof. Wei Yan of the Department of Pathology, Xijing Hospital, Fourth Military Medical University.

Lentiviral infection of cells

MG-63 or Saos-2 cells were subcultured in six-well plates the day before infection at a density of 1.5×10^5 cells and 2.0×10^5 cells per well, respectively. On the day of infection, the cell culture media were removed and discarded, polybrene (Sigma) was added to freshly prepared lentivirus medium to a final concentration of 6 µg/ml, and the mixed lentivirus medium was added to cells. Twenty-four hours later, the lentiviral medium was replaced with fresh culture medium for culture of MG-63 and Saos-2 cells. For stable selection, blasticidin S (Invitrogen) was added to the culture medium of cells 48 h after infection, to final concentrations of 7 µg/ml for MG-63 cells and 3 µg/ml for Saos-2 cells.

Polybrene was dissolved in dimethyl sulfoxide at a concentration of 100 mg/ml. Blasticidin S was dissolved in distilled water at a concentration of 10 mg/ml.

Trans-well invasion assay

Cell invasion assays were performed using a BioCoat™ Matrigel Tumor Invasion System (BD) according to the manufacturer's protocol. Briefly, 1×10^5 cells in FBS-free MEM/EBSS or Ham's/F-12 medium were seeded into the top chamber of the trans-well apparatus, while the bottom chamber was filled with MEM/EBSS or Ham's/F-12 medium containing 15% FBS. After 24 h of incubation at 37 ℃, the cells on the upper surface were gently removed with a cotton swab, and the membrane was fixed in 4% paraformaldehyde (Solarbio) for 15 min and stained with crystal violet solution (Beyotime) for a further 15 min. The cells that migrated to the lower side of the membrane were captured, and numbers of cells were counted. The invasion percentage was calculated as the ratio of the mean RFU of cells invading through the Matrigel-coated membrane to the mean RFU of cells migrating through
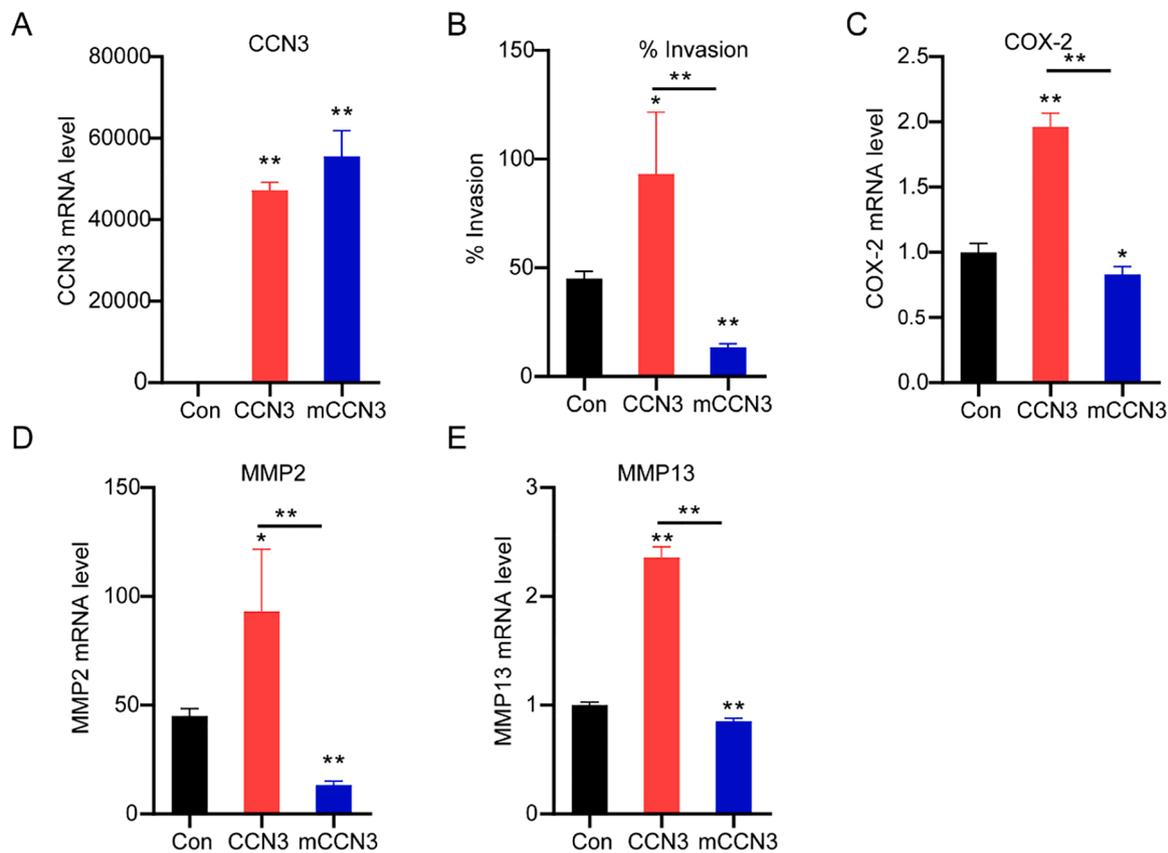
Fig. 2. Effects of overexpressed CCN3 and mCCN3 on Saos-2 cell invasion. (A–E) Saos-2 cells were infected with lentiviruses overexpressing CCN3 and mCCN3. The cells were subjected to qRT-PCR analysis of CCN3 (A), COX-2 (C), MMP2 (D), and MMP13 (E) and to trans-well assays (B). Con: pLenti6.3-V5 vector control; CCN3: pLenti6.3-CCN3 plasmid expressing human CCN3 coding sequence; mCCN3: pLenti6.3-mCCN3 plasmid expressing human mCCN3 coding sequence. *$p < 0.05$, **$p < 0.01$ compared with Con (t-test).

the uncoated membrane.

Induction of osteosarcoma cell differentiation

Cells were induced to differentiate toward maturation with differentiating medium: culture medium containing 2% FBS supplemented with 10 mM β-glycerophosphate (Sigma), 50 μg/ml L-ascorbic acid (Sigma), and 10 nM dexamethasone (Sigma). Cells were maintained in differentiation medium for up to 18 days. Media were renewed every 4 days. Cells are harvested at various time points as previously indicated [23]. Alkaline phosphatase (ALP) activity was measured with an ALP activity assay kit (Nanjing Jiancheng Bioengineering Institute).

Western blotting

Protein isolation and western blotting were carried out following the standard protocol. Protein was extracted by RIPA lysis buffer, and 40 μg total protein was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were then blocked in nonfat milk for 1 h. After incubation with primary antibodies at 4 °C overnight and with secondary antibodies at room temperature for 2 h, the membranes were washed with 0.1% Tris-buffered saline with Tween-20 and subjected to chemiluminescence analysis using an enhanced chemiluminescent reagent kit (Millipore). Nuclear and cytoplasmic fractions of cellular proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher). The primary antibodies used were as follows: anti-CCN3 (Abcam ab137677), anti-histone H3 (GeneTex GTX122148), anti-β-actin (Abcam ab8226), and anti-V5 (GeneTex, GTX117997). The secondary antibody was anti-rabbit IgG (Abcam). PageRuler™ Plus Prestained Protein Ladder (Fermentas) was used as a molecular weight marker. The density of each band was measured by AlphaEaseFC software (Alpha Innotech).

qRT-PCR

RNA was extracted using TRIzol reagent according to the manufacturer's instructions, and 1 μg of total RNA was reverse transcribed with transcriptase (Takara). qRT-PCR was performed using GoTaq® qPCR Master Mix (Promega) according to the manufacturer's instructions. The SYBR qRT-PCR reaction was performed on a CFX RT-PCR machine (BioRad) at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 40 s. Primers were designed by Primer Express 3.0 (ABI). The primer sequences (5′→3′) were as follows. Human β-actin: CTGGCACCCAGCACAATG and GCCGATCCA-CACGGAGTACT; human CCN3: ACGAGCTTTTGTCTCCGAAA and ACACCAGACAGCATGAGCAG; human cyclooxygenase-2 (COX-2): CTCCTGTGCCTGATGATTGC and AACTGATGCGTGAAGTGCTG; human matrix metalloproteinase 2 (MMP2): ATGACAGCTGCACCACTGAG and ATTTGTTGCCCAGGAAAGTG; human MMP9: TTGACAGCGGACAA-GAAGTGG and GCCATTCACGTCGTCCTTAT; human MMP13: TTCTGGTGGCTCAATCCCTT and CTGTGCGTCATTCTCAGCTC; and human collagen I (COL1A1): GCTACTACCGGGCTGATGAT and ACCAGTCTCCATGTTGCAGA. Primers were synthesized by Invitrogen. The expression of target genes are all normalized to human β-actin.

Statistical analysis

All the experiments were independently repeated in at least triplicate. Data are presented as mean±standard deviation. Differences
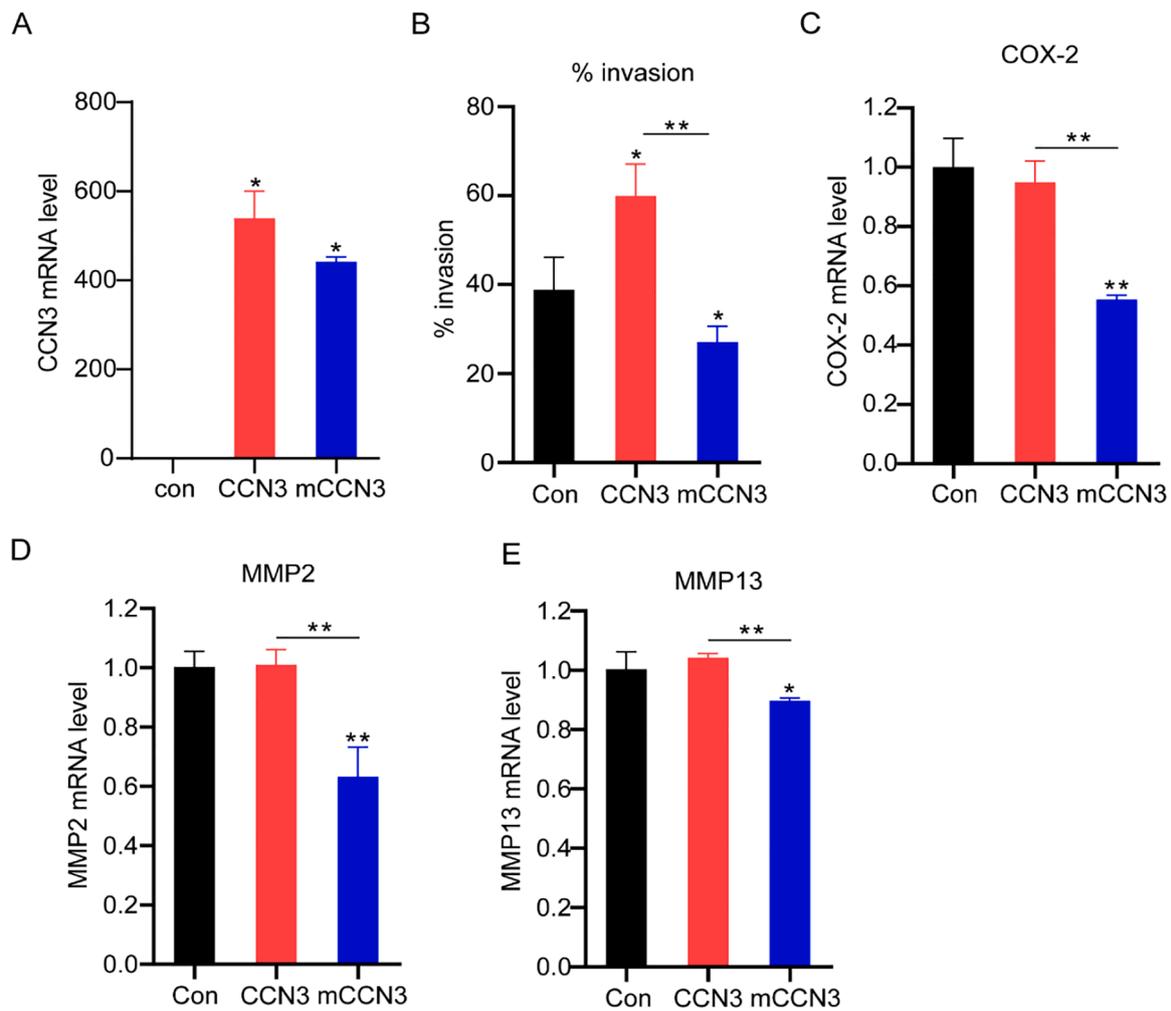
Fig. 3. Effect of overexpressed CCN3 and mCCN3 on MG-63 cell invasion. (A–E) MG-63 cells were infected with lentiviruses overexpressing CCN3 and mCCN3. The cells were subjected to qRT-PCR analysis of CCN3 (A), COX-2 (C), MMP2 (D), and MMP13 (E) and to trans-well assays (B). Con: pLenti6.3-V5 vector control; CCN3: pLenti6.3-CCN3 plasmid expressing human CCN3 coding sequence; mCCN3: pLenti6.3-mCCN3 plasmid expressing human mCCN3 coding sequence. *$p < 0.05$, **$p < 0.01$ compared with Con (*t*-test).

between two groups were analyzed by Student's *t*-test. Differences among more than two groups in Figs. 2–4, 6–8 were analyzed by one-way analysis of variance followed by Tukey's post hoc test. A difference was considered to be statistically significant when the *P*-value was < 0.05.

Results

Nucleotide mutation in osteosarcoma cells

By sequencing genomic DNA, we found that position 125 in CCN3 coding region was homozygous A/A in human osteosarcoma cell line MG-63. In the other two human osteosarcoma cell lines (Saos-2 and U2OS), the nucleotide at this position was homozygous G/G, consistent with that recorded for human CCN3 in GenBank. This G to A single nucleotide mutation resulted in substitution of Arg42 by Gln in the amino acid sequence of the CCN3 protein. The position of this mutation was between the signal peptide and the IGFBP domain, at the third amino acid upstream of the IGFBP domain (Fig. 1).

We then investigated whether this mutation could also be found in osteosarcoma patients. Genomic DNA was isolated from PBMC of healthy volunteers, and from paraffin sections of para-carcinoma tissues and osteosarcoma tissues of osteosarcoma patients. Sequencing showed that in the PBMC of healthy people, nucleotide 125 was homozygous G/

G in 66.67% of cases, heterozygous A/G in 20% of cases, and homozygous A/A in 13.33% of cases. In para-carcinoma tissue of osteosarcoma patients, this nucleotide was 77.78% heterozygous A/G and 22.22% homozygous A/A; no homozygous G/G was detected. In osteosarcoma tissue of osteosarcoma patients, it was 8.57% homozygous G/G, 2.86% heterozygous A/G, and 88.57% homozygous A/A. Therefore, the predominant state of nucleotide 125 was homozygous G/G in PBMC of healthy people, homozygous A/A in osteosarcoma tissue, and heterozygous A/G in para-carcinoma tissue (Table 1). As we could not exclude the possibility that some osteosarcoma cells might invade into para-carcinoma tissue, it was unclear whether the high frequency of A/G in para-carcinoma tissue was an original characteristic of this tissue or whether it resulted from a mixture of normal and malignant cells.

These results indicate that there are differences in nucleotide 125 of CCN3 between normal tissues and malignant osteosarcoma tissues. The frequency of mutated homozygous A/A at this position is much higher in osteosarcoma tissue than in either para-carcinoma tissue or normal controls.

CCN3 and mCCN3 function differently in osteosarcoma cell invasion

We then considered whether CCN3 and mCCN3 were functionally different. As CCN3 and mCCN3 differ only in one nucleotide, it was likely that they would have similar functions. If so, knockdown or
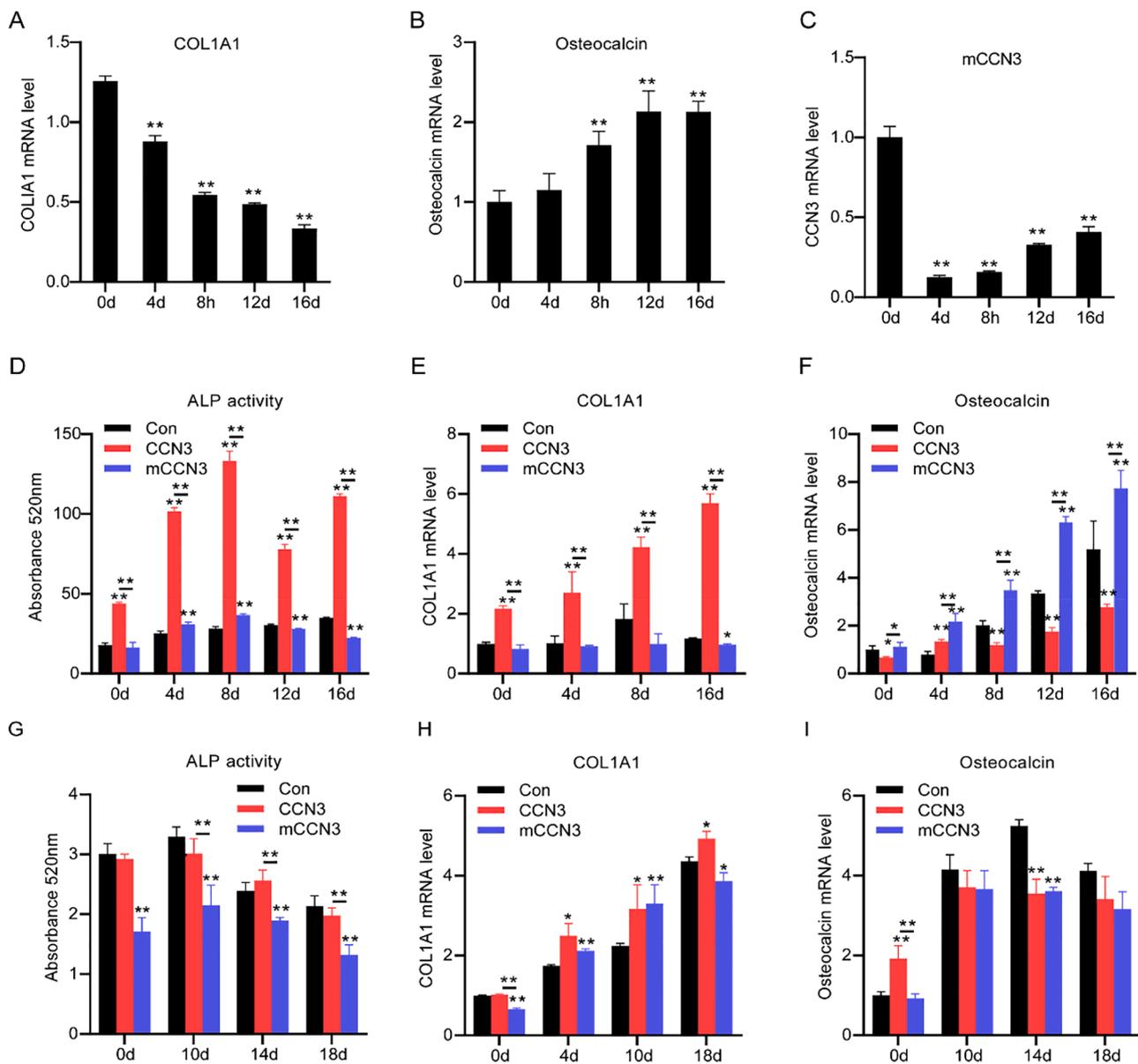
Fig. 4. Effects of overexpressed CCN3 and mCCN3 on osteosarcoma cell differentiation. COL1A1 mRNA (A), osteocalcin mRNA (B), and CCN3 mRNA (C) expression were detected by qRT-PCR in differentiating MG-63 cells. (D–F) Saos-2 cells were infected with lentiviruses overexpressing CCN3 and mCCN3. The cells were subjected to ALP activity detection (D) and qRT-PCR analysis of COL1A1 (E) and osteocalcin (F) mRNA levels. (G–I) MG-63 cells were infected with lentiviruses overexpressing CCN3 and mCCN3. The cells were subjected to ALP activity detection (G) and qRT-PCR analysis of COL1A1 (H) and osteocalcin (I) mRNA levels. Con: pLenti6.3-V5 vector control; CCN3: pLenti6.3-CCN3 plasmid expressing human CCN3 coding sequence; mCCN3: pLenti6.3-mCCN3 plasmid expressing human mCCN3 coding sequence. $*p < 0.05$, $**p < 0.01$ compared with day 0 (*t*-test).

knockout of CCN3 and mCCN3 might not be able to distinguish the functional difference between them. Therefore, we chose to ectopically overexpress the full-length coding sequence of CCN3 or mCCN3 by lentiviral infection into osteosarcoma cell lines, and compared the functional consequences in the same cell line overexpressing either CCN3 or mCCN3. Specifically, when CCN3 or mCCN3 was overexpressed in MG-63 cells possessing endogenous mCCN3, if CCN3 and mCCN3 had identical functions, MG-63 cells overexpressing CCN3 or mCCN3 would be expected to exhibit the same behaviors. By contrast, if CCN3 and mCCN3 were functionally different, the overexpressed CCN3 would act as a dominant-negative competitor for mCCN3, whereas overexpressed mCCN3 would not, thus, MG-63 cells overexpressing CCN3 or mCCN3 would show different behaviors. The same logic applies when CCN3 or mCCN3 is overexpressed in Saos-2 cells that possess endogenous CCN3.

Following this strategy, we first checked whether overexpressed

CCN3 and mCCN3 had different effects on osteosarcoma cell invasion. Stable overexpression of CCN3 (Fig. 2A) in Saos-2 cells promoted cell invasion (Fig. 2B) and increased the expression of metastasis-related genes cytochrome c oxidase subunit II (COX-2) (Fig. 2C), matrix metallopeptidase 2 (MMP2) (Fig. 2D), and matrix metallopeptidase 13 (MMP13) (Fig. 2E). Conversely, overexpression of mCCN3 (Fig. 2A) in Saos-2 cells inhibited cell invasion (Fig. 2B) and decreased the expression of COX-2 (Fig. 2C), MMP2 (Fig. 2D), and MMP13 (Fig. 2E). The difference between the abilities of CCN3 and mCCN3 to modulate cell invasion was significant. Similar results were obtained when CCN3 and mCCN3 were stably overexpressed in MG-63 cells (Fig. 3A–E).

These results demonstrate that CCN3 and mCCN3 function differently in osteosarcoma cell invasion, mCCN3 lacks the ability to promote cell invasion and acquires the ability to inhibit cell invasion.
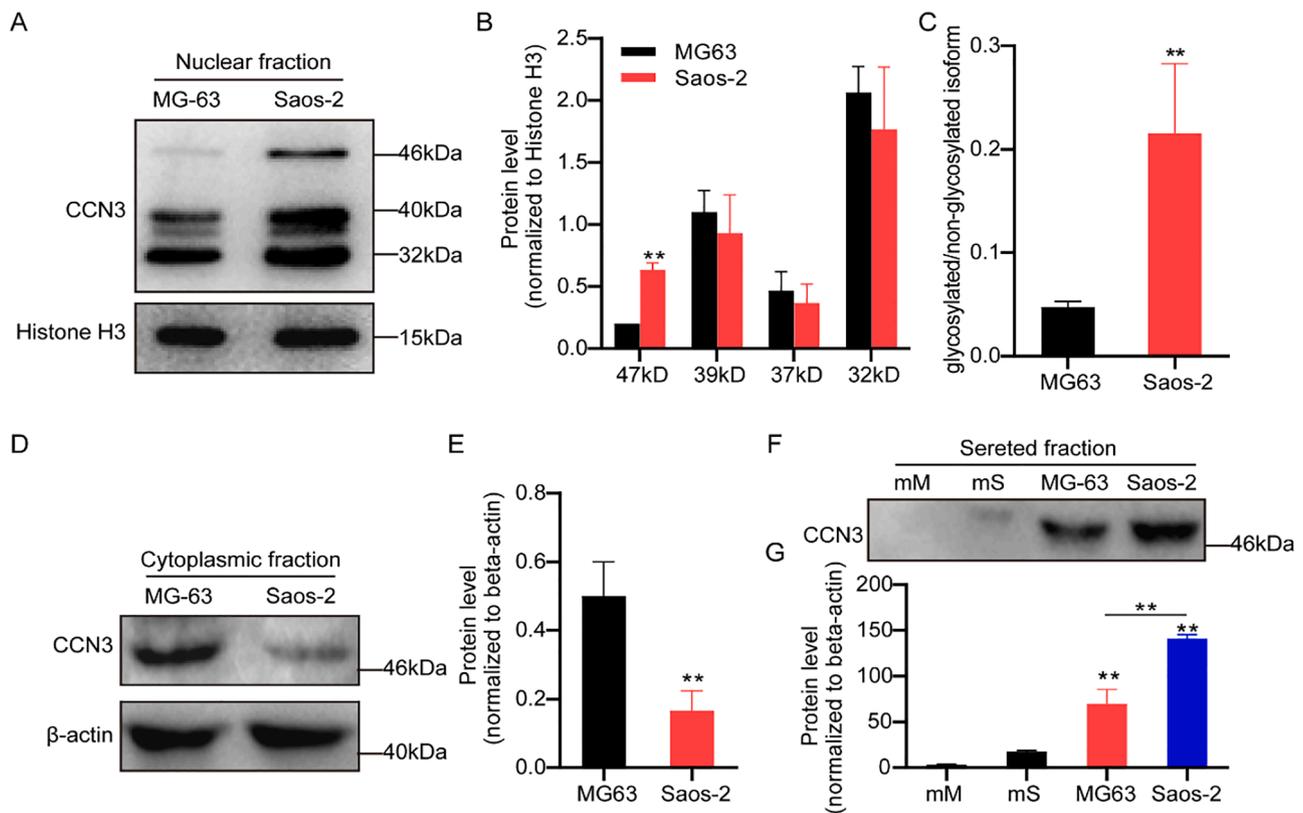
Fig. 5. Protein levels of CCN3 and mCCN3 isoforms in human osteosarcoma cell lines. Western blotting examination of CCN3 and mCCN3 isoforms and corresponding band intensity in nuclear fraction (A–C) and cytoplasmic fraction (D,E), compared with MG63 (t-test), and in secreted fraction (F,G) compared with mM or compared with mS (t-test). mM: culture medium for MG-63 cells; mS: culture medium for Saos-2 cells. **$p < 0.01$.

CCN3 and mCCN3 acted differently in osteosarcoma cell differentiation

Osteosarcoma cells have varying degrees of differentiation defects. However, they can still be induced to differentiate to a certain degree of maturation. As the expression of CCN3 has been reported to increase during induced Saos-2 cell differentiation [21], we detected the expression of mCCN3 during the process of induced MG-63 cell differentiation. As differentiation progressed, expression of COL1A1 decreased (Fig. 4A), whereas expression of osteocalcin increased (Fig. 4B). This was consistent with Perbal's observations in Saos-2 and OS-7 cells [23], indicating that differentiation had been successfully induced. The mRNA level of mCCN3 was the highest before induction of differentiation and dramatically decreased 4 days after induction. The mRNA level of mCCN3 steadily increased as differentiation progressed but was still much lower than that on day 0 (Fig. 4C). Perbal's group observed that the mRNA level of CCN3 in Saos-2 cells continuously increased with the progression of differentiation [23]. These findings indicate that both CCN3 and mCCN3 might be involved in osteosarcoma cell differentiation, but that they probably have different roles.

We then used the strategy described above in Section 3.2 for cell invasion to examine whether overexpression of CCN3 and mCCN3 showed any difference in terms of the effect on induced osteosarcoma cell differentiation. We set three comparisons: CCN3 versus Con, mCCN3 versus Con, mCCN3 versus CCN3. In Saos-2 cells, stable overexpression of CCN3 significantly enhanced ALP activity (Fig. 4D) and increased the expression of COL1A1 (Fig. 4E) but decreased the expression of osteocalcin (Fig. 4F). However, overexpressed mCCN3 in Saos-2 cells showed a significant lack of ability to enhance ALP activity and COL1A1 expression compared with overexpressed CCN3, but increased osteocalcin expression (Fig. 4D–F). That is, the roles of CCN3 and mCCN3 in Saos-2 cell differentiation varied greatly. In MG-63 cells, stable overexpression of CCN3 did not affect ALP activity (Fig. 4G) but increased

the expression of COL1A1 (Fig. 4H), whereas overexpression of mCCN3 in MG-63 cells inhibited ALP activity (Fig. 4G). Although there was no difference between overexpressed CCN3 and mCCN3 in terms of the effect on COL1A1 and osteocalcin expression during days 4–18 of MG-63 cell differentiation, it is worth noting that there were significant differences for both COL1A1 and osteocalcin expression before differentiation (day 0) (Fig. 4H,I).

The above results show that CCN3 and mCCN3 have different functions in the process of induced osteosarcoma cell differentiation, mCCN3 not only loses the functions of CCN3 but also acquires converse functions.

Contents of CCN3 and mCCN3 isoforms varied in osteosarcoma cells

As CCN3 had been reported to display diverse subcellular localizations, we considered whether CCN3 and mCCN3 might have different subcellular localizations. As MG-63 cells exclusively express mCCN3 and Saos-2 cells exclusively express CCN3, we first examined the subcellular levels of endogenous CCN3 protein in MG-63 and Saos-2 cells. We separated the nuclear and cytoplasmic fractions of cellular proteins of MG-63 and Saos-2 cells and collected the supernatants. CCN3 protein contents were checked in each fraction. In both MG-63 and Saos-2 cells, four bands of 47 kD, 39 kD, 37 kD, and 32 kD were detected in the nuclear fraction (Fig. 5A–C), a single band of 47 kD was detected in the cytoplasmic fraction (Fig. 5D,E), and only the 47 kD band was secreted into the supernatant (Fig. 5F,G). According to the predicted molecular weight of CCN3, the 39 kD band was the non-glycosylated full-length isoform, the 47 kD band was the glycosylated full-length isoform, and the two smaller bands were truncated isoforms. In the nuclear fraction and supernatant, the amounts of 47 kD glycosylated full-length mCCN3 in MG-63 cells was much lower than that of 47 kD glycosylated full-length CCN3 in Saos-2 cells (Fig. 5A,B, F,G). As different nuclear
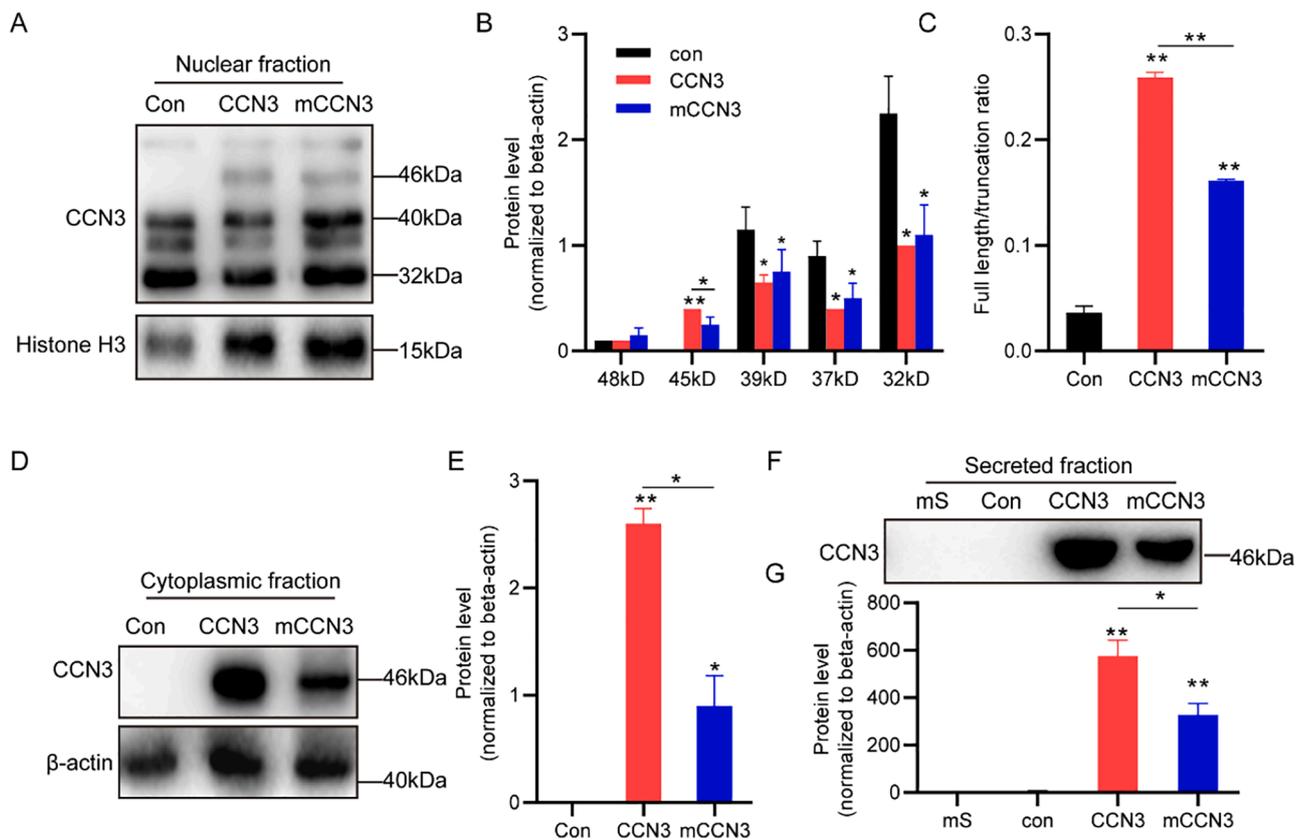
Fig. 6. Protein levels of overexpressed CCN3 and mCCN3 in Saos-2 cells. Saos-2 cells were infected with lentiviruses overexpressing CCN3 and mCCN3. The cells were subjected to western blotting analysis of CCN3 and mCCN3 isoforms and corresponding band intensity in the nuclear fraction (A–C), cytoplasmic fraction (D,E), and secreted fraction (F,G); mRNA levels of CCN3 and mCCN3 were quantified by qRT-PCR (H). Con: pLenti6.3-V5 vector control; CCN3: pLenti6.3-CCN3 plasmid expressing human CCN3 coding sequence; mCCN3: pLenti6.3-mCCN3 plasmid expressing human mCCN3 coding sequence. *$p < 0.05$, **$p < 0.01$ compared with Con (*t*-test).

isoforms of CCN3 exhibit different functions [13,23,24], we also calculated the ratio of glycosylated/non-glycosylated isoforms (glycosylated full length/the sum of all the other non-glycosylated isoforms) for CCN3 and mCCN3 in the nuclear fraction and found that the nuclear ratio of glycosylated/non-glycosylated isoforms was much lower for mCCN3 in MG-63 cells than for CCN3 in Saos-2 cells (Fig. 5C). Conversely, in the cytoplasmic fraction, the amount of glycosylated full-length mCCN3 in MG-63 cells was higher than the amount of glycosylated full-length CCN3 in Saos-2 cells (Fig. 5D). However, it should be noted that the amounts of CCN3 and mCCN3 protein were much lower in cytoplasm than in the nucleus. These results indicate that endogenous CCN3 and mCCN3 protein have different subcellular amounts of glycosylated full-length protein and a different nuclear ratio of glycosylated/non-glycosylated isoforms in osteosarcoma cells.

However, as MG-63 and Saos-2 cells have different properties, these results did not exclude the possibility that the different amounts of glycosylated full-length CCN3 and mCCN3 might have been due to the different cell contexts of the two cell types, rather than the properties of CCN3 and mCCN3 themselves. To explore this possibility, we overexpressed the full-length CCN3 and mCCN3 coding sequences in cells of the same type to determine whether the difference in isoforms still existed.

Overexpression of CCN3 and mCCN3 had similar results in MG-63 and Saos-2 cells. In Saos-2 cells, the amount of overexpressed glycosylated full-length mCCN3 protein was much lower than that of CCN3 protein in the nuclear (Fig. 6A and B), cytoplasmic (Fig. 6D and E), and secreted (Fig. 6F and G) fractions. The nuclear ratio of glycosylated/non-glycosylated isoforms of mCCN3 was lower than that of CCN3 (Fig. 6C). No difference was observed between the mRNA levels of CCN3 and

mCCN3 (Fig. 6H), indicating identical infection efficiency of lentiviral CCN3 and mCCN3 vectors. Overexpression of CCN3 and mCCN3 in MG-63 cells had similar results (Fig. 7A–H)

The above results show that cells with endogenous or ectopically overexpressed mCCN3 exhibited lower glycosylated full-length protein levels and lower nuclear ratios of glycosylated/non-glycosylated isoforms compared with those with CCN3. This indicates that nucleotide 125 in the CCN3 coding sequence is important for the production of the glycosylated full-length CCN3 protein.

Fusion of V5 tag at the C-terminus of CCN3 and mCCN3 abolished their protein level and functional difference

When the C-terminus of CCN3 and mCCN3 was fused with a V5 tag, the difference between the levels of glycosylated full-length CCN3 and mCCN3 disappeared (Fig. 8A–D), and their functional difference with respect to osteosarcoma cell invasion and differentiation was also abolished (Fig. 8E–J). We speculated that the fusion of V5 tag at the C-terminus of CCN3 and mCCN3 not only affected the functions of CCN3 and mCCN3 but also changed their protein levels via effects on the mRNA and/or protein configuration.

We deduced that the functional difference between CCN3 and mCCN3 could not be entirely caused by their different glycosylated full-length protein levels, as this would not explain why the protein levels of overexpressed CCN3 and mCCN3 were both much higher than those of the vector control group, but overexpressed CCN3 and mCCN3 exhibited contrary effects on osteosarcoma cell invasion and differentiation (Figs. 2–4Fand G). Therefore, we proposed that the change of amino acid 42 from Arg to Gln in the CCN3 protein, resulting from the mutation of
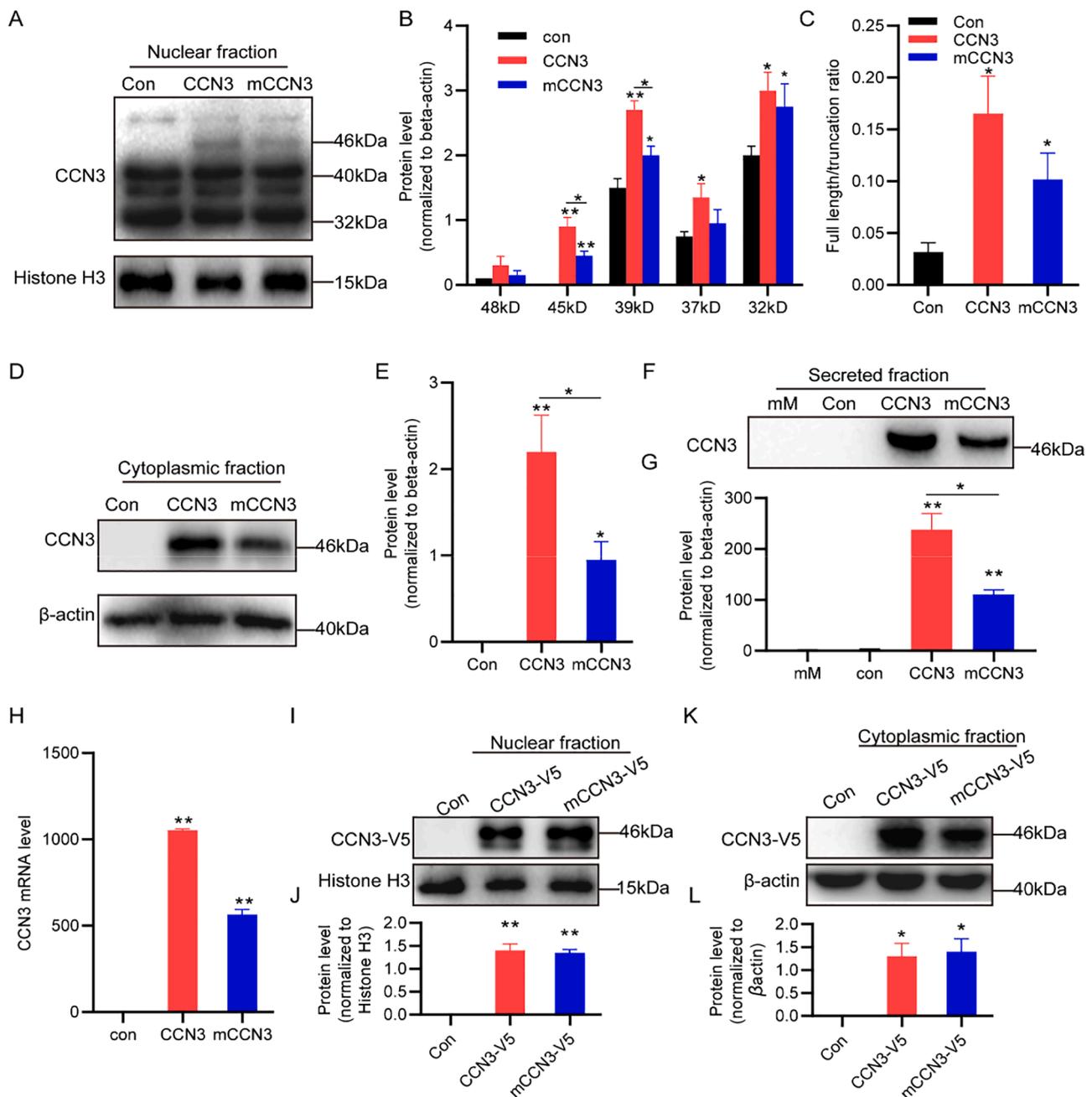
Fig. 7. Protein levels of overexpressed CCN3 and mCCN3 in MG-63 cells. MG-63 cells were infected with lentiviruses overexpressing CCN3 and mCCN3. The cells were subjected to western blotting analysis of CCN3 and mCCN3 isoforms and corresponding band intensity in the nuclear fraction (A–C), cytoplasmic fraction (D,E), and secreted fraction (F,G); mRNA levels of CCN3 and mCCN3 were quantified by qRT-PCR (H). Con: pLenti6.3-V5 vector control; CCN3: pLenti6.3-CCN3 plasmid expressing human CCN3 coding sequence; mCCN3: pLenti6.3-mCCN3 plasmid expressing human mCCN3 coding sequence. *$p < 0.05$, **$p < 0.01$ compared with Con (t-test).

nucleotide 125 in the CCN3 coding sequence, might contribute to the functional difference between CCN3 and mCCN3. As different nuclear isoforms of CCN3 exhibit different functions [13,23,24], the different nuclear ratio of glycosylated/non-glycosylated isoforms of CCN3 and mCCN3 might also contribute to their functional difference.

Collectively, we found that CCN3 and mCCN3 exhibited differential glycosylated full-length protein levels and a differential nuclear ratio of glycosylated/non-glycosylated isoforms, and that these two types of CCN3 protein were functionally different with respect to their roles in osteosarcoma cell invasion and differentiation. These results demonstrate that mutation of nucleotide 125 from G to A in the CCN3 coding sequence results in differential glycosylated full-length protein levels

and altered functions of CCN3 protein in osteosarcoma cells.

Discussion

Isoforms of different sizes have been reported for the CCN3 protein, with different cellular localizations. Several CCN3 isoforms may exist within one cell. The pattern of CCN3 isoform composition is cell type specific. CCN3 was originally believed to be a secreted protein. However, nuclear CCN3 has recently attracted attention and has been suggested to be involved in the regulation of transcription [24]. The function and source of each isoform has not been clearly elucidated. However, different isoforms have different functions. For example,
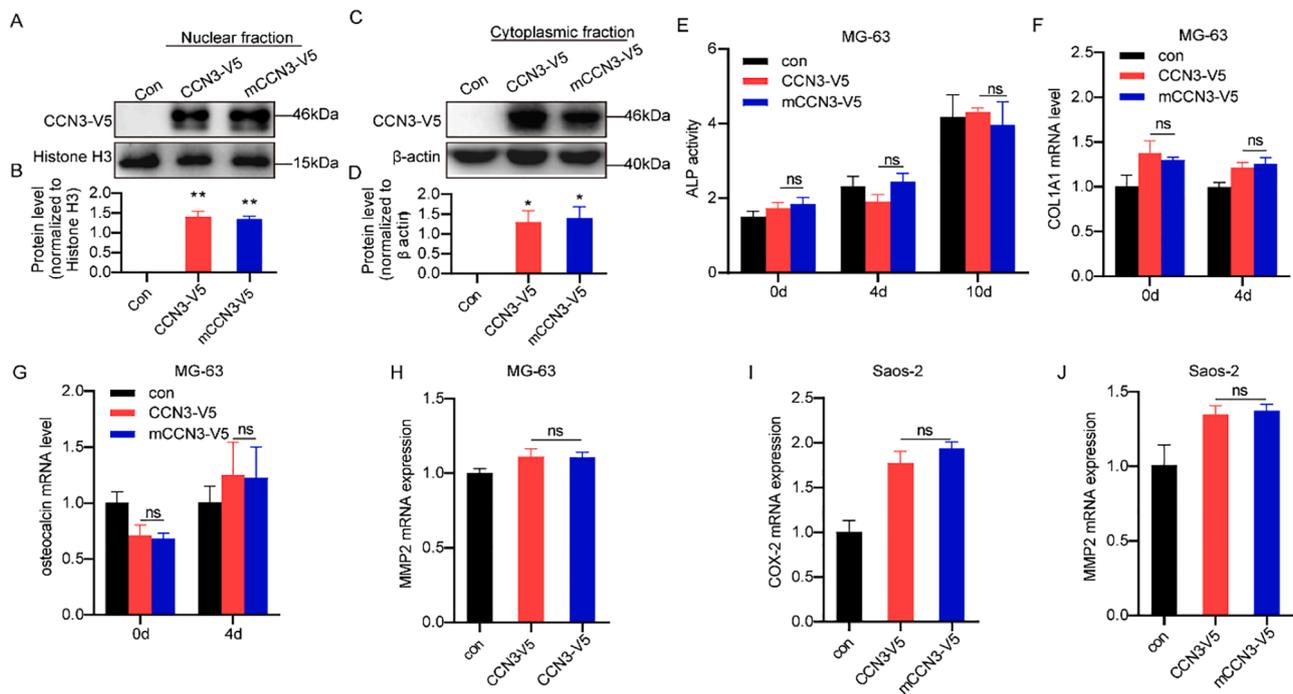
Fig. 8. Protein levels of overexpressed CCN3-V5 and mCCN3-V5 and their effects on osteosarcoma cell invasion and induced differentiation. MG-63 cells were infected with lentivirus overexpressing CCN3-V5 and mCCN3-V5. The cells were subjected to western blotting analysis of CCN3 and mCCN3 isoforms, the corresponding band intensity in the nuclear fraction (A, B) and cytoplasmic fraction (C, D) was shown. The cells were also subjected to ALP activity detection (E) and qRT-PCR analysis of COL1A1 (F), osteocalcin (G), and MMP2 mRNA levels (H). Saos-2 cells were infected with lentivirus overexpressing CCN3-V5 and mCCN3-V5. The cells were subjected to qRT-PCR analysis of COX-2 (I) and MMP2 (J) mRNA levels. CCN3-V5: pLenti6.3-CCN3-V5 plasmid expressing human CCN3 coding sequence fused with V5 tag at 3′-terminus; mCCN3: pLenti6.3-mCCN3-V5 plasmid expressing human mCCN3 coding sequence fused with V5 tag at 3′-terminus. $*p < 0.05$, $**p < 0.01$, ns, no significance (t-test as compared with Con).

Planque et al. reported that amino-truncated CCN3 exhibited transforming properties and acted as a tumorigenic agent, whereas the full-length CCN3 showed an anti-proliferative property [15,25]. The ratio between different isoforms may also be a factor that influences the effect of CCN3 on cellular functions. In the present study, we found that the mutation of nucleotide 125 from G to A in the CCN3 coding region resulted in differential glycosylated full-length protein levels, which contributed to functional change of CCN3 in osteosarcoma cells.

Various studies have reported that missense mutations may affect the biological function of proteins. A missense mutation in myocilin gene was found to inhibit protein secretion, and the resulting intracellular accumulation of myocilin protein caused damage to trabecular meshwork cells [26]. In addition, wild-type optineurin and E50K mutant optineurin were shown to behave differently in autophagy [27]. Here, we found that a missense mutation in the CCN3 gene led to functional change in CCN3 protein. Further investigation is needed to elucidate how the single nucleotide mutation in CCN3 affected its function and levels of glycosylated full-length protein. The mutation site is not located in either of the two putative N-glycosylation sites in human CCN3 protein. Interestingly, blocking the C-terminus of CCN3 and mCCN3 with a fused V5 tag abolished the difference in functions and protein levels between CCN3 and mCCN3. The 5′ and 3′ regions of mRNA interact with each other during protein translation, it is possible that nucleotide 125 is involved in this process and is thus important for CCN3 protein translation. Another possibility is that the mutation results in an amino acid change at position 42 of the CCN3 protein, which in turn may affect CCN3 protein stability.

MG-63 cells and Saos-2 cells vary greatly in many aspects including proliferation rate, invasion ability and gene expression profiles. They represent different osteoblastic differentiation stages. Therefore, it is unsurprising that these two cell types were not identical in terms of the differences in function and protein content between the CCN3 and

mCCN3 overexpression groups. A comparison study showed that Saos-2 cells exhibit much higher invasion ability than MG-63 cells [28]. This is consistent with our finding that mCCN3 inhibited whereas CCN3 promoted cell invasion.

The position of the nucleotide 125 mutation is also a single nucleotide polymorphism (SNP) site. According to population diversity analysis results from the SNP database (https://www.ncbi.nlm.nih.gov/snp), among 46 Asian individuals, 87% had G/G genotype at this position, 13% had A/G genotype, and the A/A genotype was not found. Among 46 European individuals, 56.5% had G/G genotype, 43.5% had A/G genotype, and again the A/A genotype was not found. Among 40 African American individuals, 20% had G/G genotype, 35% had A/G genotype, and 45% had A/A genotype (from GenBank database). The results for the Asian population were similar to our sequencing results based on 30 people from Shaanxi province in China, which showed that most of them had G/G genotype. According to our results, the frequency of homozygous A/A at this position was much higher in osteosarcoma tissue than in para-carcinoma tissue or PBMC of healthy people. This suggested that the A/A genotype at this position might be associated with osteosarcoma development. However, a large-scale genome-wide association study (GWAS) did not find any relationship between this SNP site and osteosarcoma [29]. One possible explanation for this discrepancy is that in GWAS, DNA was isolated from blood samples, which might not well reflect the situation in bone. Another explanation is that SNP variation alone at this site may be insufficient to induce osteosarcoma, whereas the combination of A/A genotype with other mutations may contribute to the development of osteosarcoma. It is also possible that CCN3 does not act as a driver of osteosarcoma tumorigenesis but is instead a consequence of tumor development. Although CCN3 gene mutation has not been reported, it is worth noting that according to the SNP database, as many as 45% of African Americans have the A/A genotype. Therefore, our results indicate that osteosarcoma

patients with differences in nucleotide 125 in CCN3 may display different osteosarcoma behaviors that are associated with tumor progression.

Conclusion

In summary, we found that the frequency of point mutation at position 125 of CCN3 was higher in osteosarcoma tissues than in para-carcinoma tissues or PBMC of healthy people. Nucleotide 125 in the CCN3 coding sequence is extremely important in determining the level and function of CCN3 protein. Mutation of this nucleotide from G to A in the CCN3 coding sequence resulted in differential glycosylated full-length CCN3 protein levels and altered the function of CCN3 protein in osteosarcoma cell invasion and differentiation.

Data availability

All the data during the current study are included in the article or uploaded as supplementary information.

Ethics approval and consent to participate

The experimental protocols were approved by the Ethics Committee of Basic Medical Sciences Institute of Chinese Academy of Medical Sciences (No. 028-2013). This paper has not been published elsewhere in whole or in part. All authors have read and approved the content, and agree to submit it for consideration for publication in your journal. Informed consent was obtained from all individual participants included in the study.

CRediT authorship contribution statement

Xuejing Yan: Writing – original draft, Writing – review & editing, Methodology. **Wei Yan:** Writing – original draft, Writing – review & editing, Methodology, Funding acquisition. **Xin Fu:** Software, Project administration. **Yuqiao Xu:** Software, Project administration. **Ning Zhu:** Data curation. **Chuan Qiu:** Conceptualization, Validation. **Mengmeng Bu:** Data curation. **Yan Shen:** Conceptualization, Funding acquisition, Validation, Supervision. **Meihong Chen:** Writing – original draft, Writing – review & editing, Methodology, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101485.

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