A Novel Drug, CC-122, Inhibits Tumor Growth in Hepatocellular Carcinoma through Downregulation of an Oncogenic TCF-4 Isoform☆,☆☆

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Abstract

Immunomodulatory drugs such as lenalidomide (LEN) have shown significant anti-tumor activity against hematologic malignancies and they may have similar actions on solid tumors as well. We studied the effect of a new analog of the immunomodulatory drugs (CC-122) on the growth of hepatocellular carcinoma (HCC) and explored mechanisms of anti-tumor activity by analyzing expression of a novel oncogenic T-cell factor (TCF)-4 J and its downstream gene activation. LEN and CC-122 significantly reduced the expression levels of TCF-4 J and its target genes (*SPP1, AXIN2, MMP7, ASPH, CD24, ANXA1,* and *CAMK2N1*); however, CC-122 was more potent. In a xenograft tumor model with a HAK-1A-TCF-4 J derived stable cells, tumor growth was significantly inhibited by CC-122, but not by LEN or vehicle control. The mice with HCC xenograft tumors treated with CC-122 exhibited decreased TCF-4 J expression compared to LEN and control. Furthermore, expression of TCF-4 J-responsive target genes (*SPP1, AXIN2, MMP7, ASPH, JAG1, CD24, ANXA1, and CAMK2N1*) was reduced by CC-122 and not by LEN or control. These results suggest that CC-122 inhibits HCC tumor growth through downregulation of the oncogenic TCF-4 J isoform.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death worldwide [1]. Although surgical resection plays a major role in the treatment of HCC, less than 30% of patients are surgical candidates owing to limiting clinical factors such as advanced cirrhosis, impairment of hepatic function and extrahepatic metastasis. Furthermore, following surgical resection, there is a recurrence rate of greater than 70% [2]. Recently, systemic chemotherapy have shown anti-tumor activity for HCC, but the effect remains limited; therefore, new therapeutic approaches are urgently needed [3–5].

Increasing evidence suggests a significant role for thalidomide-derive immunomodulatory drugs (IMiDs) in anti-cancer therapy [6–8]. For example, lenalidomide (LEN), which is one of the thalidomide analogues with both immunomodulatory and anti-angiogenic properties, has been employed for treatment of multiple myeloma and myelodysplastic syndromes associated with a cytogenetic 5q deletion abnormality. In addition, LEN has been shown to exhibit anti-tumor effects for solid tumors such as prostate, thyroid and renal malignancies in some clinical studies as well [9–11]. Indeed, the anti-tumor effect of LEN on HCC has been evaluated in a phase II study; preliminary results of the ongoing study demonstrate significant anti-tumor activity in some individuals with this disease [12]. Recently, cereblon (CRBN), a substrate binding component of the E3 ubiquitin ligase complex, which is highly conserved from
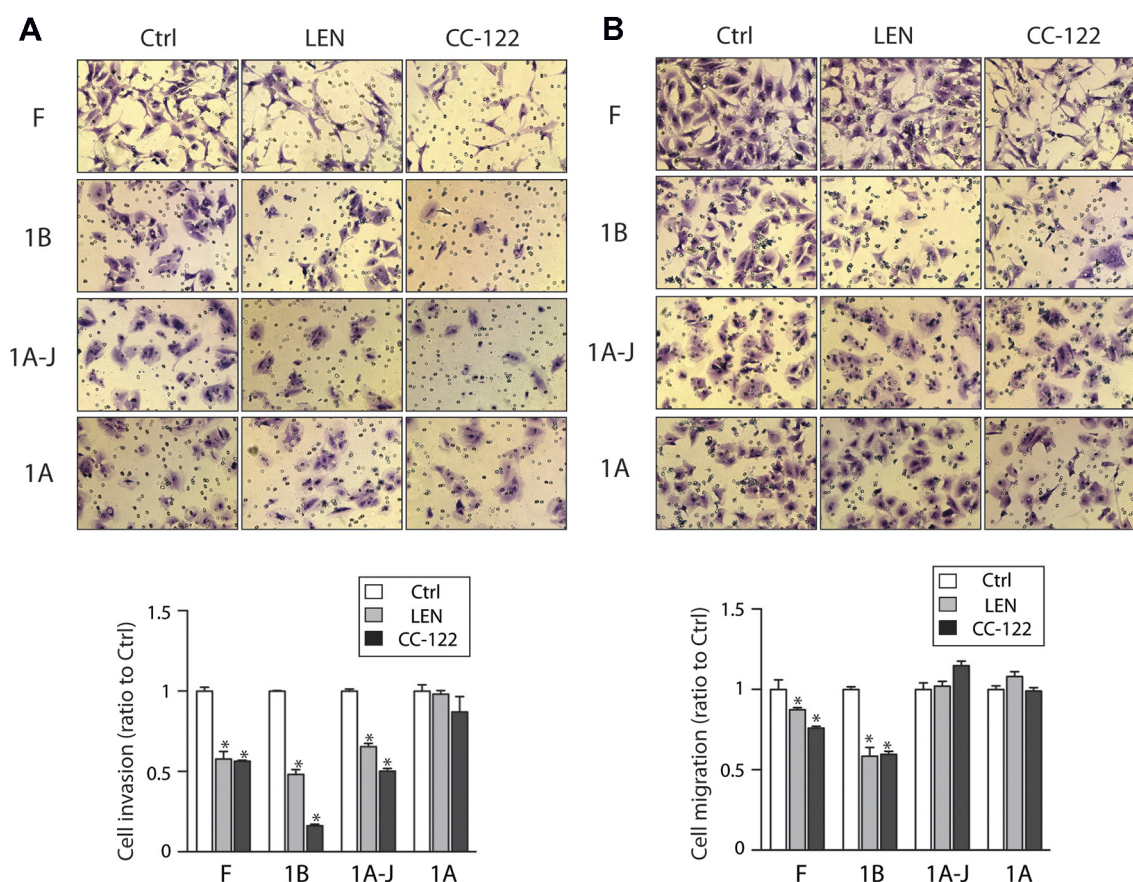
Figure 1. CC-122 and LEN inhibiting cell invasion and migration in HCC cell lines.(**A**) The effect of cell invasion was assessed using MATRIGEL™ invasion chambers. Invasive cells were reported as ratio to control (Ctrl) for FOCUS (F), HAK-1B (1B), HAK-1A (1A) and HAK-1A-J (1A-J) (bottom panel). Representative photographs of invasive cells treated by DMSO, LEN (10 μM), or CC-122 (10 μM) (100×) were shown (top panel). (**B**) The effect of cell migration was assessed using a transwell assay system. Migratory cells were depicted as ratio to control (Ctrl) for FOCUS (F), HAK-1B (1B), HAK-1A (1A) and HAK-1A-J (1A-J) (bottom panel). Representative photographs of migratory cells treated by DMSO (Ctrl), LEN (10 μM), or CC-122 (10 μM) (100×) were shown (top panel). The results are shown as mean ± SD. *$P < .05$ compared to DMSO-treated cells.

plants to mammals and ubiquitously expressed, has been identified as a cellular target for the anti-tumor activity of IMiDs [13,14]. This observation has stimulated the development of compounds related to LEN as a next generation of potential therapeutic agents; the CC-122 compound is a thalidomide analogue of the IMiDs and Cereblon E3 ligase modulating drug, which can bind to the CRBN-E3 ligase complex. The anti-proliferative activity of CC-122 for hematological malignancies has been shown to be more potent than other IMiDs including LEN [15], and clinical effects of CC-122 in the treatment of such diseases are currently investigated in ongoing clinical trials [16]. However, anti-tumor effect of HCC has not investigated well. These findings led us to explore the hypothesis that CC-122 may more strongly inhibit HCC tumor growth than LEN, and also to investigate possible molecular mechanisms of its growth-inhibitory effect on HCC.

One of the most common alterations in signaling pathways of HCC involve the aberrant activation of Wnt/β-catenin signaling cascade found in over 90% of the tumors described thus far [17,18]. T-cell factor (TCF)/lymphoid enhancer-binding factor proteins are transcription factors which can bind to its β-catenin partner to activate downstream Wnt-responsive target genes [19]. Previous studies have described and characterized 14 different TCF-4 isoforms generated by alternative splicing events, which exhibit differential

transcriptional activity and biologic properties in HCC cell lines [20]. It has also been noted that the TCF-4 J isoform promotes a robust malignant phenotype in HCC cell lines and the gene expression level has been found to be significantly upregulated in human HCC tumor(s) compared to normal liver and adjacent peritumor tissues [20,21]. Furthermore, TCF-4 J-responsive target genes involved in Wnt/β-catenin, insulin/IGF1/IRS1, and Notch signaling pathways which are important in hepatic oncogenesis have recently been identified as being upregulated [22]. These findings imply that TCF-4 J isoform expression is the one of the main drivers of growth factor signaling pathway activation and is a major contributor to HCC tumor formation and growth. In this context, we investigated the anti-tumor effects of LEN and CC-122 on TCF-4 J and downstream responsive target genes expression as well as characterized the functional outcome of enhanced activity using HCC cell lines and tumor growth in a subcutaneous xenograft murine model.

Materials and Methods

Reagents and HCC Cell Lines

For the *in vitro* experiments, LEN and CC-122 (Celgene Corporation, Summit, NJ) were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, MO) to prepare 50 mM stock
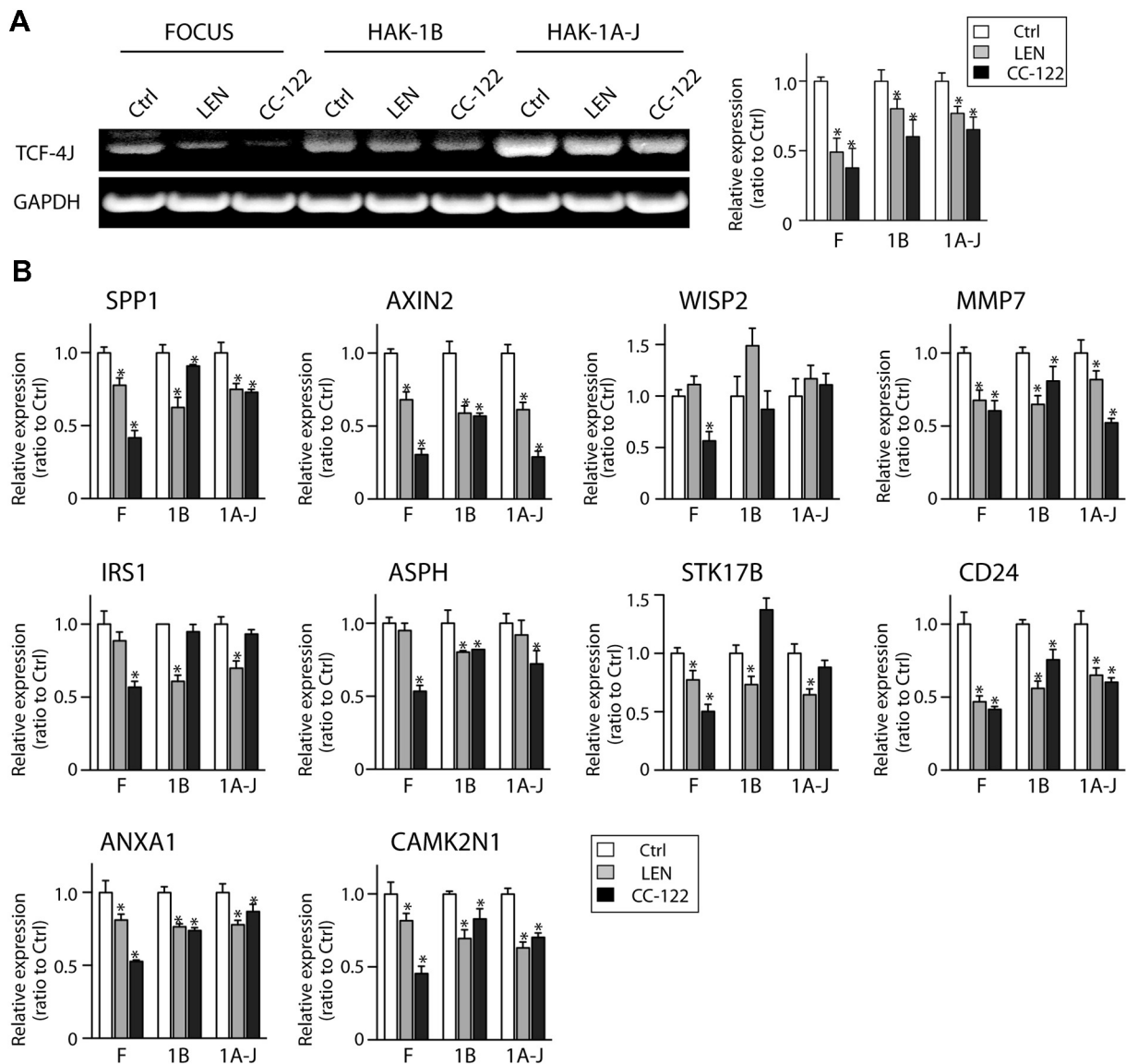
Figure 2. The anti-tumor agents CC-122 and LEN inhibiting TCF-4 J and downstream responsive target genes expression in HCC cell lines. Cells (FOCUS, HAK-1B, HAK-1A-J) were treated with LEN or CC-122 and RT-PCR for TCF-4 J expression was performed as previously described (15). (**A**) Agarose gel analysis of PCR product for FOCUS, HAK-1B, and HAK-1A-J, TCF-4 J expression was examined (Left panel). The expression was quantified with normalization to GAPDH (Right panel). The data were expressed as ratio to Ctrl-treated cells. (**B**) Expression levels of 10 TCF-4 J responsive target genes were examined in FOCUS (F), HAK-1B (1B), and HAK-1A-J (1A-J) treated with LEN, CC-122 or DMSO (Ctrl) assessed by qRT-PCR. The results are shown as mean ± SD. *$P < .05$ compared to Ctrl-treated cells.

solution. Final DMSO concentrations were 0.02%. HCC cell lines FOCUS [23], HAK-1B and HAK-1A were maintained in DMEM medium (Lonza, Basel, Switzerland) supplemented with L-glutamine (200 mM, Gibco, Carlsbad, CA), fetal bovine serum (FBS, 10%, Lonza) and penicillin/streptomycin (P/S, 100 µg/ml, Gibco). A stable clone overexpressing TCF-4 J (HAK-1A-J) derived from HAK-1A HCC cell line has been previously described [21]. For *in vivo* experiments, LEN and CC-122 were suspended in vehicle (0.5% carboxyl methylcellulose in water with 0.25% Tween 80).

Cell Migration and Invasion Assays

A wound healing assay was employed as follows; a scratch was made on a uniform layer of cells using a sterile micropipette tip and cells were washed to remove debris. Photographs of the same area of the wound

were taken after 4, 8, and 24 hours to measure the width of the wound. For the transwell migration assay, cells were resuspended in serum-free medium and seeded into the insert well of a 24-wells plate (8 µm pores, BD biosciences, Franklin Lakes, NJ) for 24 hours. The culture medium containing 10% FBS was used as a chemoattractant and placed in the bottom chamber. Cells were fixed in PFA (4%) and stained in crystal violet (0.5% in 20% methanol). Remaining cells in the upper chamber (non-migratory cells) were removed with a cotton swab. Adherent cells on the bottom of the membrane (migratory cells) were counted under a microscope (Olympus America Inc., Center Valley, PA) and Stereologer System (Dissector, Stereology Resource Center, Inc., Chester, MD). The invasion assay was performed under the same conditions using the BD BioCoat™ growth factor reduced MATRI-GEL™ invasion chamber (BD biosciences).
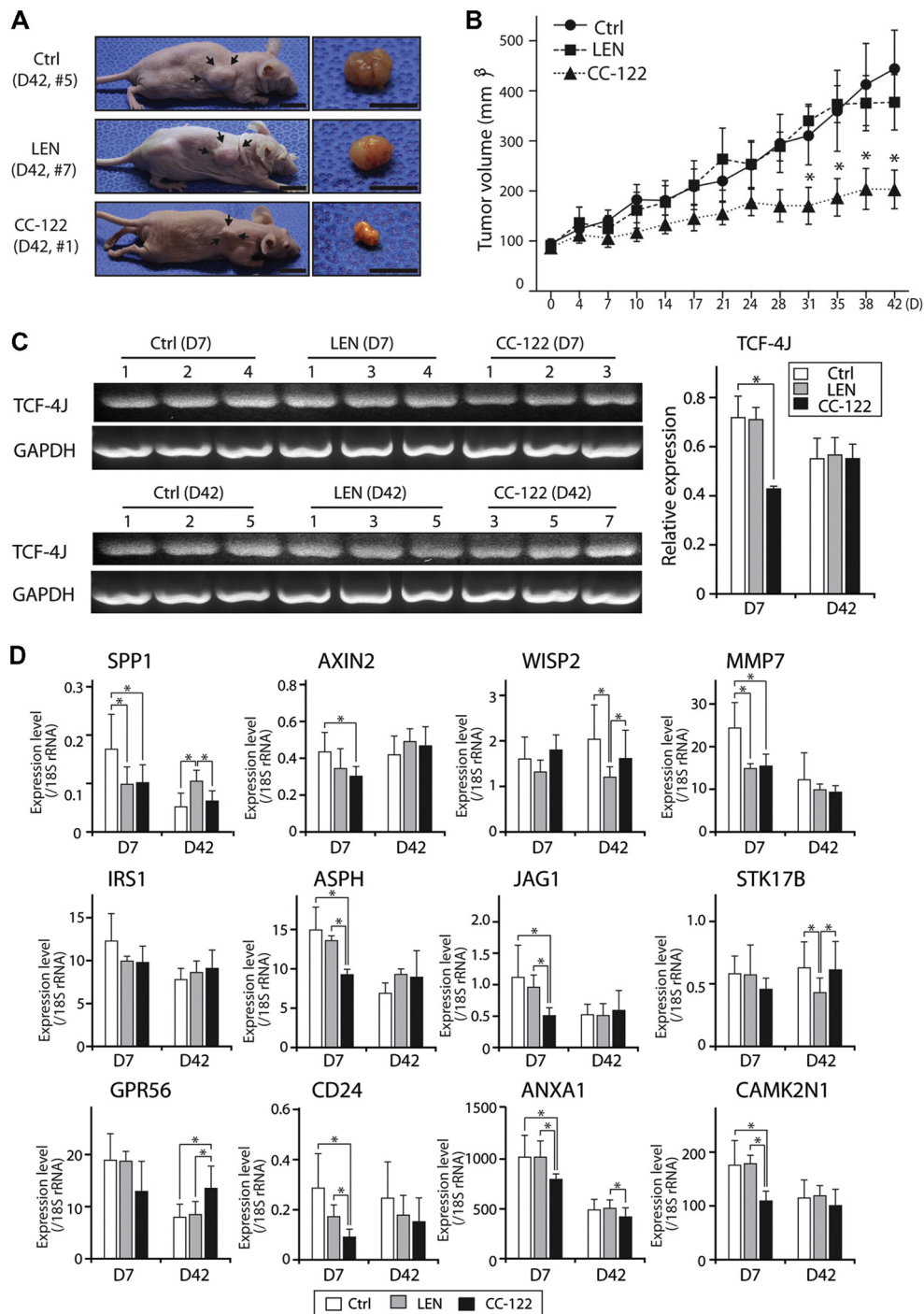
]**Figure 3.** *In vivo* **effects of CC-122 on human HCC growth in nude mice injected with HAK-1A-J cells.**(**A**) Representative photographs of the tumor-bearing mice and the excised tumors 42 days after starting the treatment (D42) were shown. Arrows indicate the tumors. Bar = 10 mm. (**B**) The graph showed *in vivo* tumor growth curve. Tumor-bearing mice were divided into 3 groups to receive LEN (30 mg/kg), CC-122 (30 mg/kg), or the control vehicle once a day for 42 days. The results are shown as mean ± SD. *$P < .05$ compared to the LEN and the control group. (**C**) TCF-4 J mRNA expression was examined in the xenograft tumors treated with LEN or CC-122 (left panel). Semi-quantitative RT-PCR analysis was performed as previously described (15), for evaluating expression levels of TCF-4 J and GAPDH in the tumors treated with LEN, CC-122, or the control vehicle for either 7 days (D7) (top panel) or 42 days (bottom panel). TCF-4 J expression level was quantified by densitometric analysis with normalization to GAPDH (right panel). The results are expressed as mean ± SD. *$P < .05$. (**D**) The expression levels of the 12 TCF-4 J-responsive genes in the tumors treated for 7 days (D7) or 42 days (D42) were measured by qRT PCR analysis and the values were normalized to 18S rRNA. The results are expressed as mean ± SD. *$P < .05$. (**E**) Protein expressions of TCF-4 J-responsive target genes (SPP1, AXIN2, MMP7, ASPH, and CD24) in the tumors treated with LEN, CC-122, or the control vehicle for either 7 days (D10) or 42 days (D42) were evaluated by Western blot analysis using antibodies against Myc-tag, SPP1, AXIN2, MMP7, ASPH, CD24, and actin. (**F**) The expression levels were quantified by densitometric analysis with normalization to actin. The results are shown as mean ± SD. *$P < .05$. **$P < .01$.
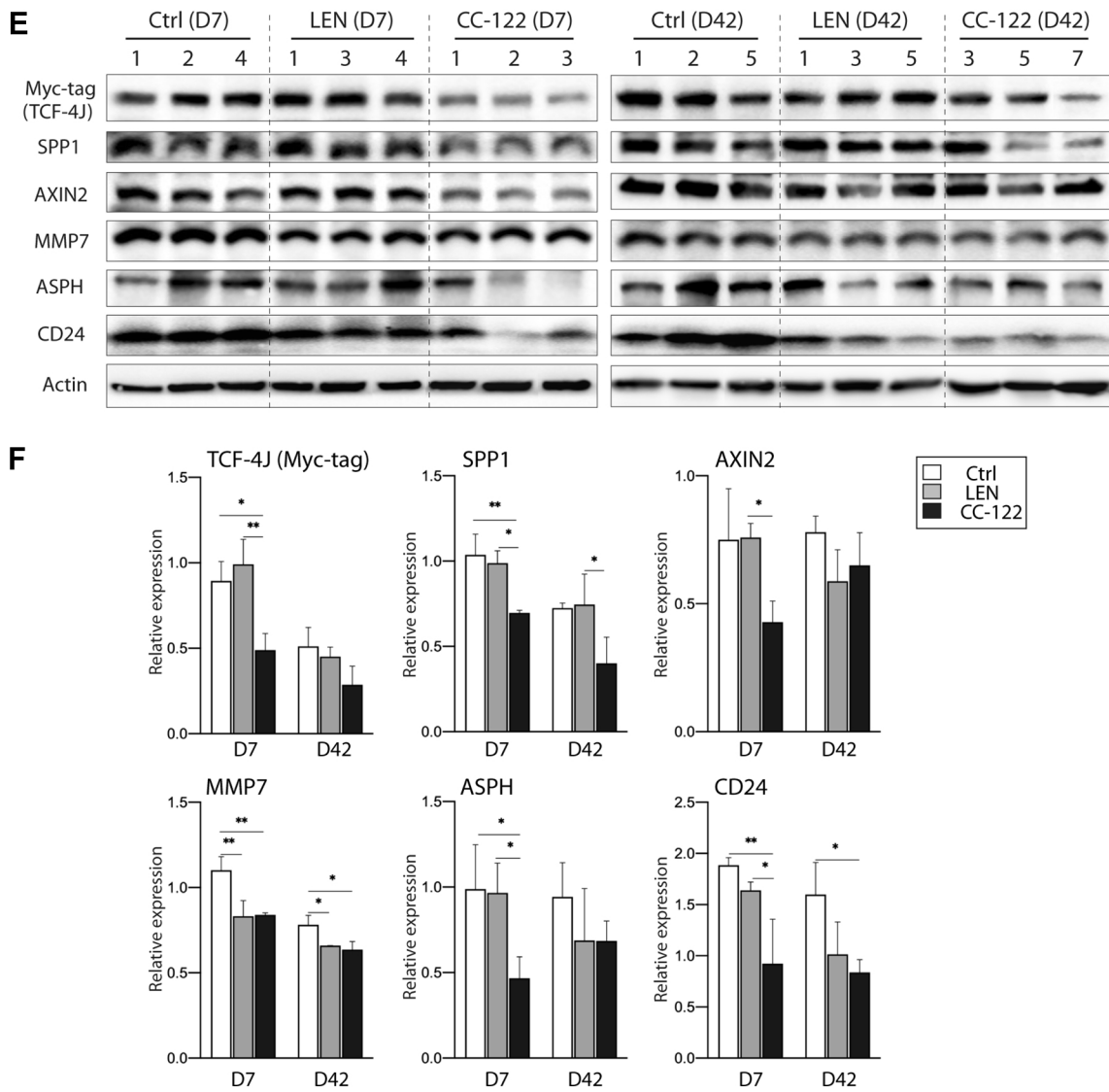
Figure 3. (*continued*)

Semi-Quantitative RT-PCR and Quantitative Real-Time PCR Analysis

Total RNA was extracted from cells or tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA), and reverse transcription was performed with First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Indianapolis, IN) according to the manufacturers' instruction. Semi-quantitative RT-PCR for TCF-4 J expression was performed as previously described [20]. The quantitative real-time PCR (qRT-PCR) analysis was carried out using the QuantiTect SYBR Green PCR Kit (Qiagen, Germantown, MD) and the Mastercycler ep Realplex system (Eppendorf AG, Hamburg, Germany) as described previously [22]. Relative quantification was performed using $\Delta\Delta$Ct method, normalizing to 18S rRNA. Dissociation curves were generated to evaluate PCR product specificity and purity. Primers were used in this study as previously described [22].

Western Blot Analysis

Western blot analysis was carried out as previously described [18] using primary antibody against Myc-tag (Cell Signaling Technology, Beverly, MA), Osteopontin (SPP1), MMP7, CD24 (Abcam, Cambridge, UK), Axin-2 (AXIN2) (Millipore, Billerica, MA), ASPH [24], and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Immunohistochemistry

Immunochemical staining was performed as previously described [21] using primary antibodies against Osteopontin (SPP1), Axin-2 (AXIN2), MMP7, ASPH, and CD24. Staining of these proteins was observed in cytoplasm, and the intensity of the staining for each protein was scored as negative (0+), weakly positive (1+), or strongly positive (2+), and independently assessed by two investigators.

Mice Xenograft Studies

Subcutaneous xenografts were established in flanks of 5-week-old male BALB/c nude mice (Taconic Farms, Cranbury, NJ) using HAK-1A-J cells (1×10^7) and FOCUS cells (1×10^7). In 44 mice out of 45 mice injected with HAK-1A-J cells, palpable tumors were confirmed 21 days after the injection, and the tumor-bearing mice were randomized into 3 groups to receive LEN (n = 15), CC-122 (n = 15), or the control vehicle (n = 14). LEN (30 mg/kg), CC-122

(30 mg/kg), or the control vehicle was administered by oral gavage to the mice once a day for 42 days. Seven and 42 days after the starting the administration, the 5 and 9 or 10 mice in each group, respectively, were sacrificed and the tumors were excised. In all mice injected with FOCUS cells, palpable tumors were confirmed 3 days after the injection, and the tumor-bearing mice were randomized into 2 groups to receive CC-122 (n = 20), or the control vehicle (n = 20). CC-122 (30 mg/kg), or the control vehicle was orally administered to the mice once a day for 19 days. Ten and 19 days after the starting the administration, mice were sacrificed and the tumors were excised. The tumor size was measured twice per week after starting the administration, and tumor volume was estimated using the following formula: tumor volume (mm^3) = (longer diameter) × (shorter diameter)^2 × 0.5. All animal experiments were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by Lifespan Animal Welfare Committee of Rhode Island Hospital, Providence, RI. Excised tumor samples fixed in 10% buffered formalin and embedded in paraffin were stained with hematoxylin and eosin, and subjected to immunohistochemistry. RNA and cell lysates were extracted from homogenized tumor samples, and used at semi-quantitative RT-PCR analysis, quantitative real-time PCR analysis, and Western blot analysis.

Statistical Analysis

Data are expressed as means ± SD. Differences between groups were assessed by the χ^2, Fisher's exact or Mann–Whitney U tests. P value <.05 was considered statistically significant. Data analyses were performed using Statview (version 5.0; SAS Institute Inc., Cary, NC).

Results

LEN and CC-122 Inhibit Cell Invasion and Migration of Human HCC Cells

Based on the observation that LEN reduces prostate cancer cell invasion without inhibition of cell proliferation [25], we evaluated if LEN and CC-122 altered on proliferation, as well as migration and invasion, in 4 different HCC cell lines. In this regard, FOCUS and HAK-1B cells were derived from poorly differentiated HCC tumors, whereas HAK-1A was established from a well-differentiated hepatitis C virus-related HCC [21]. The generation of a TCF-4 J isoform overexpressing HAK-1A-J stable clone has been described previously [21]. The activities of LEN and CC-122 on HCC cell invasion were evaluated using the BD BioCoat™ growth factor reduced MATRI-GEL™ invasion chamber assay. We observed a significant inhibition of cell invasion generated by 10 μM of both LEN and CC-122 exposure in FOCUS, HAK-1B and HAK-1A-J poorly differentiated HCC cell lines, but not in the well differentiated HAK-1A cells (Figure 1A). Reduced cell invasion was found to be 35–52% and 44–84% after LEN and CC-122 treatment, respectively, indicating that CC-122 was more potent in this regard. In addition, LEN and CC-122 treatment reduced HCC cell migration in FOCUS and HAK-1B, but not in HAK-1A or HAK-1A-J cells (Figure 1B). These observations were also confirmed by a wound-healing assay. At 24 hours after a scratch-wound, significant reduction of cell migration was observed in CC-122 treated FOCUS (S Figure 1A) and HAK-1B cells (S Figure 1B). There was no difference of cell migration in HAK-1A-J cells (S Figure 1C). In all HCC cell lines tested, there was no cellular toxicity or anti-proliferative effect induced by 10 μM of

LEN or CC-122 treatment as assessed by MTS, MTT or propidium iodide staining (data not shown). These results suggest that inhibition of HCC cell invasion by LEN and CC-122 may be effective in poorly differentiated endogenous TCF-4 J overexpressing cells such as FOCUS and HAK-1B but not in a well-differentiated HCC cell line such as HEK-1A or HAK-1A-J.

LEN and CC-122 Inhibit Expression of TCF-4 J and Its Downstream Target Genes in HCC Cell Lines

Previous investigations have demonstrated that TCF-4 J isoform expression generates a highly malignant phenotype *in vitro* as well as *in vivo* [20,21]. Furthermore, up-regulation of TCF-4 J expression has been found in poorly differentiated HCC tumors compared to normal liver and adjacent peritumor tissues [21]. Considering that the expression levels of TCF-4 J has been associated with poorly differentiated HCC tumors and suppression of HCC cell invasion was observed in poorly differentiated HCC cell lines or TCF-4 J overexpressing HAK-1A cells with LEN and CC-122, we explored the hypothesis that treatment of such cell lines with LEN or CC-122 may modulate TCF-4 J levels and this phenomenon was evaluated by RT-PCR as previously described [20]. As shown in Figure 2A, the level of TCF-4 J expression was significantly decreased by 20−51% and 35−62% with LEN and CC-122 treatment, respectively. Furthermore, the expression of recently identified TCF-4 J-responsive downstream target genes associated with Wnt/β-catenin, insulin/IGF1/IRS1, and Notch signaling pathway activation was also assessed since all three cascades play a key role during HCC development [22]. Expression levels of TCF-4 J-responsive target genes involved in these signaling pathways of the three human HCC cell lines were evaluated following treatment with LEN or CC-122 by quantitative real-time PCR analysis (qRT-PCR). Expression levels of *SPP1*, *AXIN2*, *MMP7, CD24, ANXA1, and CAMK2N1* were significantly decreased with both LEN and CC-122 treatments (Figure 2B). Intriguingly, the level of *ASPH* expression was decreased in all three cell lines treated with CC-122 but not with LEN. *JAG1* expression was unchanged (data not shown). These results suggest that inhibition of TCF-4 J isoform expression and downstream gene activation by LEN or CC-122 may contribute to reduced HCC cell invasion as shown in Figure 1A.

Anti-Tumor Effect of CC-122 in HCC Xenografts Model

The HAK-1A-J clone has aggressive tumorigenic potential in xenograft model [21]. To determine whether LEN or CC-122 affects tumor growth *in vivo*, we injected HAK-1A-J cells subcutaneously into nude mice, allowed the tumor to grow to 100 mm^3 followed by administration of LEN or CC-122 once a day orally for 42 days. As shown in Figure 3, A and B, the tumor growth in the group of mice treated with LEN was not significantly different from that of the vehicle administered to mice as a control. In contrast, significant regression and reduced growth rate of HAK-1A-J tumors was observed in the group that received CC-122 treatment compared with the control. The average tumor volume in the CC-122 group on day 42 was 45.8% of that in the control group. Mice receiving LEN or CC-122 showed no obvious systemic signs of toxicity compared to the untreated control and tolerated therapy well suggesting little adverse effects of the drugs at the administered dose under these experimental conditions. These results revealed that CC-122, but not LEN, significantly inhibited HCC tumor growth generated by the HAK-1A-J clone *in vivo*.
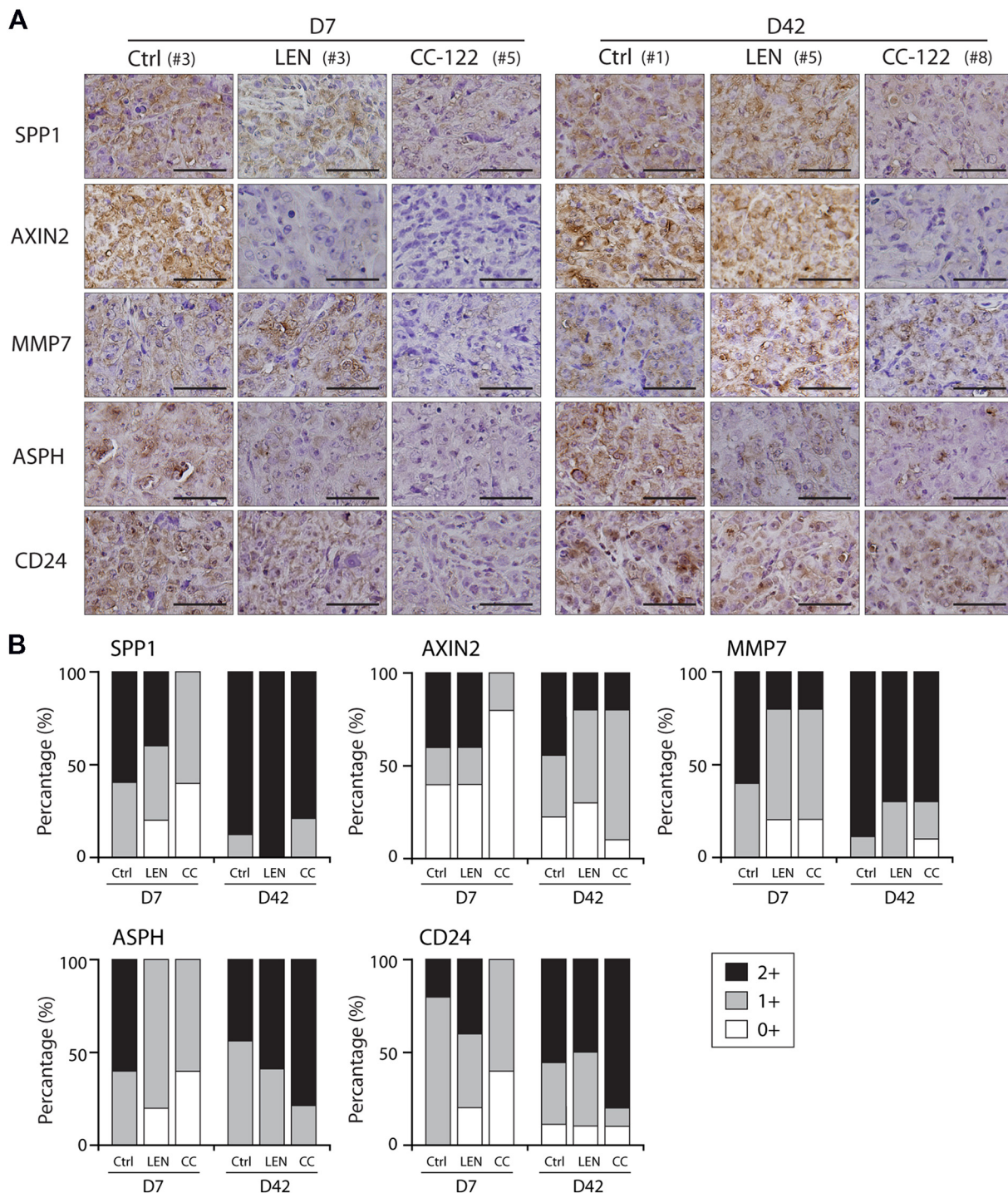
Figure 4. Immunohistochemical staining for TCF-4 J-responsive target gene expression in the xenograft tumors. The tumors treated with LEN, CC-122, or the control vehicle (Ctrl) for 7 days (D7) or 42 days (D42) were stained by using antibodies against SPP1, AXIN2, MMP7, ASPH, and CD24. (**A**) Photographs showed stained sections from a representative tumor sample in each group (400×). Bar = 50 μm. (**B**) Distribution of the score in all the excised tumor samples was investigated based on the staining intensity [negative (0+), weakly positive (1+), or strongly positive (2+)] of each protein.

Oncogenic TCF-4 J and Its Responsive Target Genes in Tumor Tissue Are Suppressed by CC-122 Treatment

As previously described above, CC-122 treatment suppressed TCF-4 J and its downstream target gene expression and subsequently inhibited migration and invasion of HCC cells. The level of expression of TCF-4 J was examined in the subcutaneous grown murine tumors since it is known to be an oncogenic TCF-4 isoform

in human HCC tumor samples [21]. Thus, we explored the possible underlying molecular mechanism(s) responsible for the anti-tumor effects observed with CC-122 treatment in this model. As shown in Figure 3C, the TCF-4 J expression level in the tumors treated with LEN for either 7 or 42 days was not significantly different from levels found in untreated control tumors. In contrast, the TCF-4 J expression level in the tumors treated with CC-122 for 7 days were
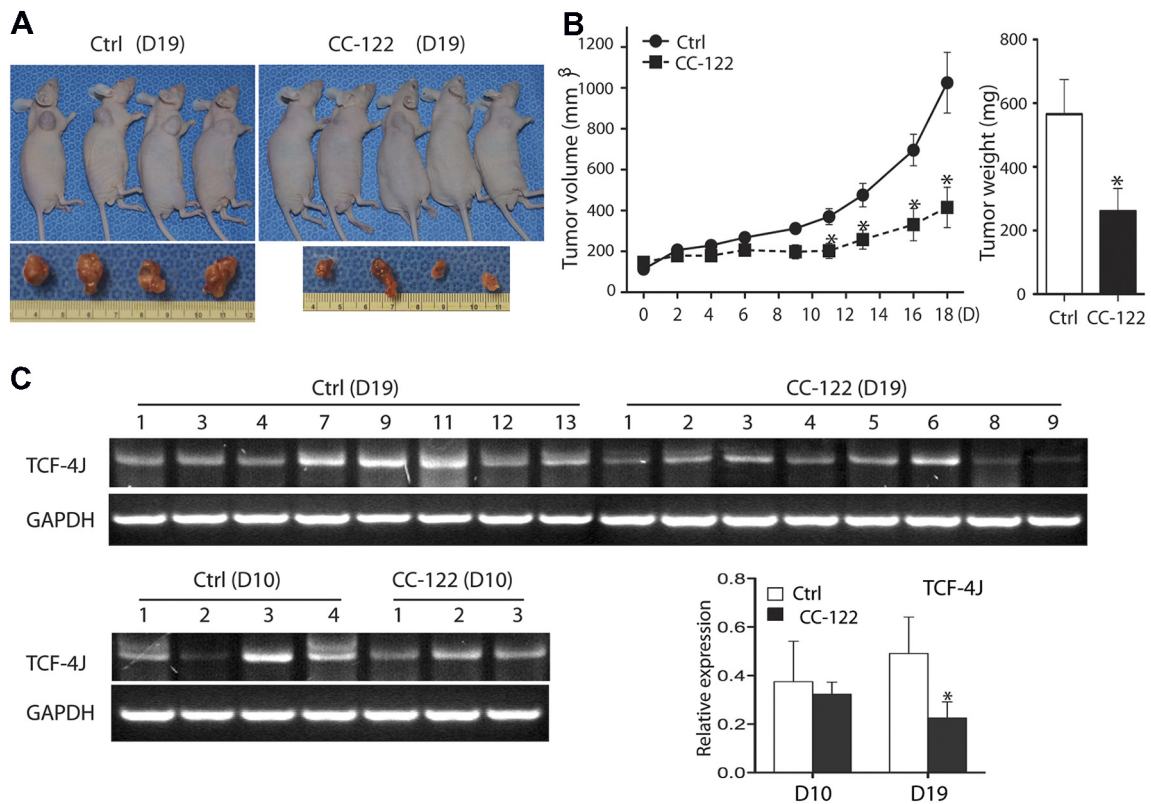
Figure 5. *In vivo* **effects of CC-122 on human HCC growth in nude mice injected with FOCUS cells.**(**A**) Representative photographs of the tumor-bearing mice and the excised tumors 19 days after starting the treatment (D19) were shown. (**B**) *In vivo* tumor growth curve and tumor weight were shown. The results are shown as mean ± SD. *P < .05 compared to the control group. (**C**) TCF-4 J mRNA expression in the xenograft tumors treated with CC-122. Semi-quantitative RT-PCR analysis was performed for evaluating expression levels of TCF-4 J and GAPDH in the tumors treated with CC-122 or the control for either 10 days (bottom panel) or 19 days (top panel). TCF-4 J expression level was quantified by densitometric analysis with normalization to GAPDH. The results are shown as mean ± SD. *P < .05.

significantly decreased compared to the tumors treated with the control vehicle. This reduction in TCF-4 J expression was not apparent in tumors treated with CC-122 after 42 days. Consistent with the result found with RT-PCR, Western blot analysis revealed that exogenous TCF-4 J protein expression, as revealed by a Myc-tag antibody, was also repressed in the tumors treated with CC-122 for 7 days compared to the tumors derived from both LEN-treated and the control groups (Figure 3, *E* and *F*). These results demonstrated that CC-122, but not LEN, inhibits TCF-4 J protein expression level in the human HAK-1A-J clone induced HCC tumors. Furthermore, we assessed expression levels of TCF-4 J-responsive target genes in HCC tumors treated with LEN, CC-122, or control vehicle by qRT-PCR analysis. As shown in Figure 3D, mRNA expression levels of 8 TCF-4 J-responsive target genes (*SPP1*, *AXIN2*, *MMP7*, *ASPH*, *JAG1*, *CD24*, *ANXA1*, and *CAMK2N1*) out of the 12 genes selected for study [22] were significantly decreased in the tumors treated with CC-122 for 7 days compared to the control tumors. These significant differences were not apparent after 42 days of treatment. In contrast, mRNA levels of only 2 genes (*SPP1* and *MMP7*) were significantly downregulated in the animals bearing HCC tumors and treated with LEN for 7 days as compared to tumors derived from controls. Western blot analysis revealed that protein expression levels of SPP1, AXIN2, MMP7, ASPH, and CD24 were significantly decreased in the tumors treated with CC-122 for 7 days compared to the tumors derived from both LEN-treated and control mice, which was

comparable and consistent with the mRNA expression results (Figure 3, *E* and *F*). With exception of AXIN2, the protein expression levels were decreased in tumors treated with CC-122 for 42 days (Figure 3, *E* and *F*). Whereas the expression levels of these genes in the LEN-treated tumors were not significantly different from the control tumors when analyzed at 7 and 42 days post treatment. These observations of protein expression levels of the TCF-4 J-responsive target genes were in general agreement with the downregulation of mRNA expression of the target genes observed in the tumors treated with CC-122 for 7 days.

Immunohistochemical staining was performed in all excised tumor samples to validate the protein levels of above genes. The distribution of the staining reactivity in the tumors was scored as shown in Figure 4B. In the staining for SPP1, MMP7, ASPH, and CD24 protein levels, any detectable staining (1+ or 2+) was present in all the tumors treated with the control vehicle for 7 days, while 20−40% of the samples were scored as 0+ in the tumors treated with CC-122 for 7 days. Difference of AXIN2 staining was the most evident. Eighty percent of the sample was scored as 0+ in the tumors treated with CC-122 for 7 days, while the percentage of samples scored as 2+ was higher in the control tumors (40%) than the tumors treated with CC-122 (0%) for 7 days. In contrast, comparison of protein expression among the tumor samples treated for 42 days revealed little if any difference except for ASPH and AXIN2. These observations support the hypothesis that the anti-tumor effect observed with
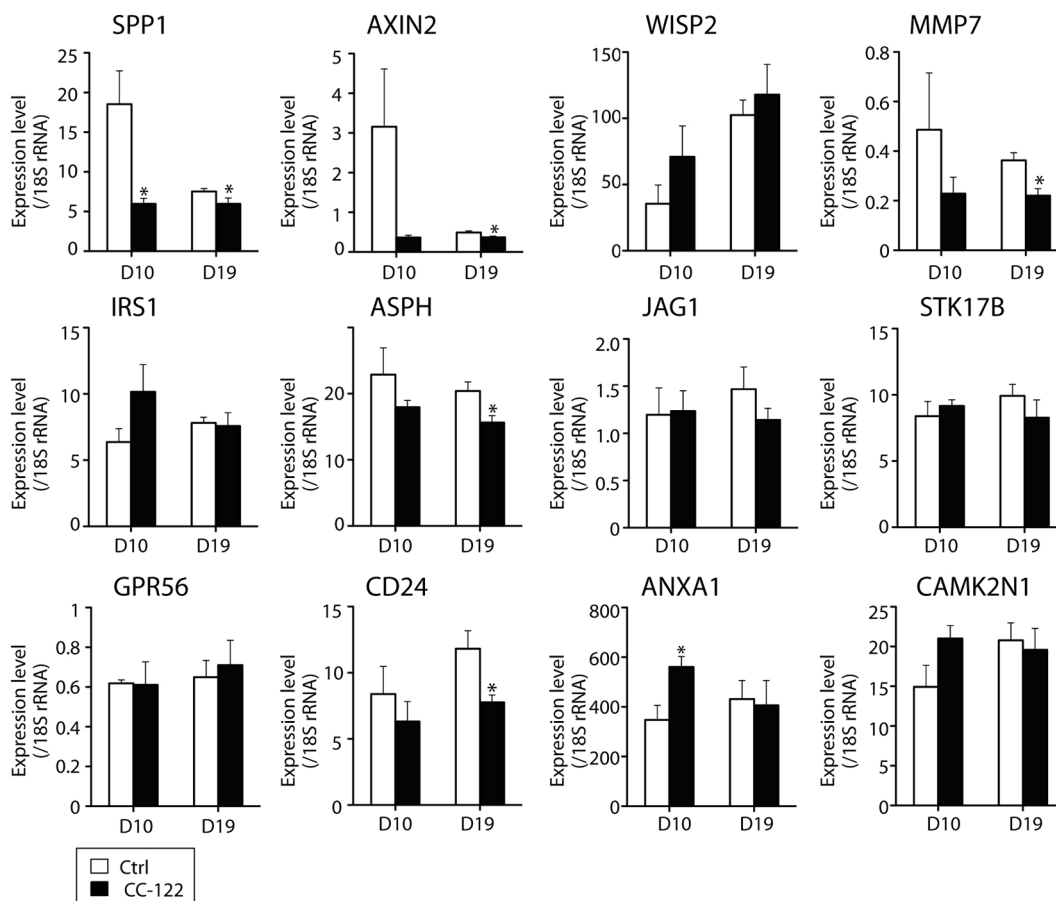
Figure 6. Expression levels of TCF-4 J-responsive target genes in the FOCUS xenograft tumors. The expression levels of the 12 TCF-4 J-responsive genes in the tumors treated for 10 days (D10) or 19 days (D19) were measured by quantitative real-time PCR analysis and the values were normalized to 18S rRNA. The results are expressed as mean ± SD. *$P < .05$.

CC-122 treatment may be, in part, mediated through the downregulation of this TCF-4 J isoform which subsequently reduces downstream gene activation central to tripartite growth factor signaling cascade regulation in HCC [21].

### CC-122 Inhibits Tumor Growth in a Xenograft Model Using FOCUS HCC Cells

To confirm antitumor effect of CC-122 linked to oncogenic TCF-4 J transcription factor protein *in vivo*, we treated CC-122 in nude mice injected with FOCUS HCC cells. Three days after implantation, when tumors are well established, the mice were administered with vehicle or CC-122 (30 mg/kg) once a day orally for 19 days. As anticipated, the tumor growth and tumor weight were significantly reduced in the group of mice treated with CC-122 compared with the control (Figure 5, *A* and *B*). The average tumor volume in the CC-122 group on day 19 was 40.5% of that in the control group. Mice receiving CC-122 showed no systemic signs of toxicity compared to the untreated control and tolerated therapy well suggesting little adverse effects of the drugs at the administered dose under these experimental conditions. These results demonstrated that CC-122 significantly inhibited HCC tumor growth generated by the FOCUS HCC cells *in vivo*. Consistent with results in HAK-1A-J derived tumor model, CC-122 treatment suppressed TCF-4 J and its downstream target gene expression. The TCF-4 J expression level in the tumors treated with CC-122 for 19 days were significantly

decreased compared to the tumors treated with the control vehicle (Figure 5*C*). This reduction in TCF-4 J expression was not apparent in tumors treated with CC-122 after 10 days. Expression levels of 5 TCF-4 J-responsive target genes (*SPP1*, *AXIN2*, *MMP7*, *ASPH*, and *CD24*) were significantly decreased in the tumors treated with CC-122 for 19 days compared to the control tumors (Figure 6). In contrast, mRNA level of only *SPP1* was significantly downregulated in the animals bearing HCC tumors and treated with CC-122 for 10 days as compared to controls. Comparable and consistent with these results, protein expression levels of SPP1, AXIN2, MMP7, ASPH, and CD24 were significantly decreased in the tumors treated with CC-122 for 19 days compared to the tumors derived from control mice (Figure 7). In addition, the protein expression levels were reduced in tumors treated CC-122 for 10 days.

Taken together, we are led to believe that CC-122 treatment represses TCF-4 J isoform expression as well as TCF-4 J-responsive target genes involved in Wnt/β-catenin, IN/IGF-1 and Notch signaling cascades and resulting in the significant inhibition of tumor growth.

Discussion

We have demonstrated that CC-122 treatment exhibits significantly more anti-tumor activity as compared to LEN in HCC tumor bearing mice. A recent study suggests that IMiDs exhibit anti-proliferative activity in hematological malignancies through binding and inhibiting the E3 ligase protein CRBN [14]. In the present investigation, our
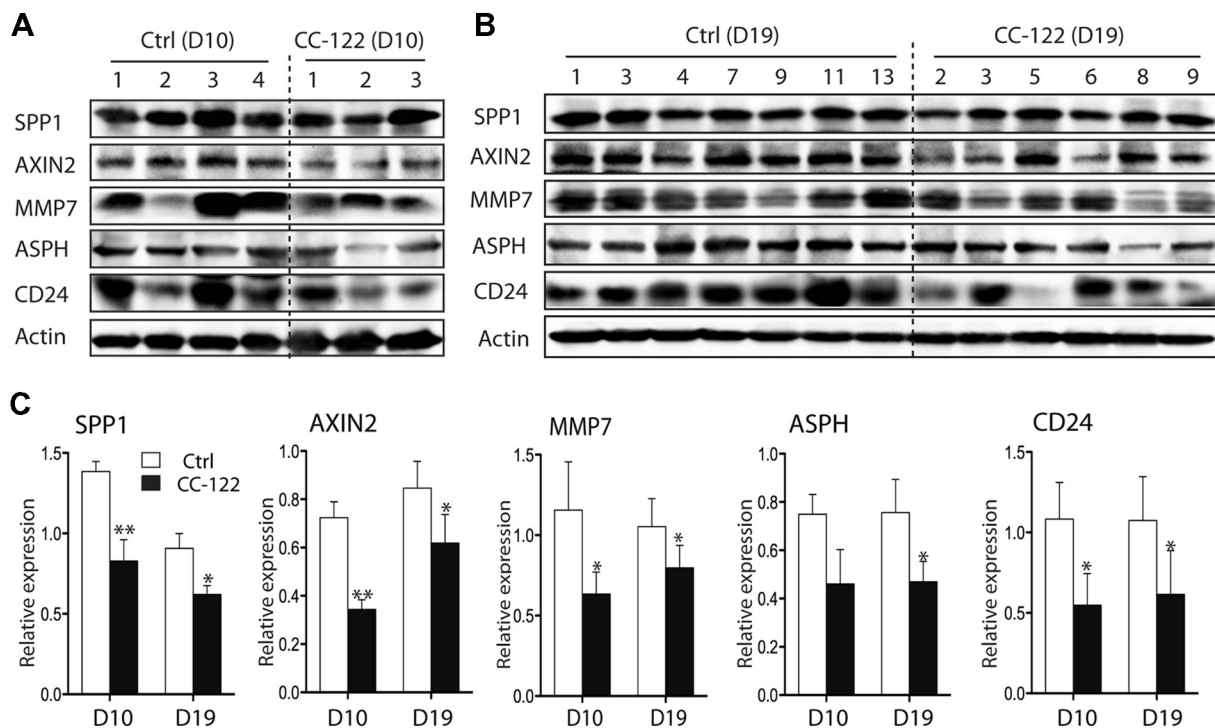
Figure 7. Protein expression levels of TCF-4 J-responsive target genes in the human FOCUS xenograft tumors treated with CC-122. (A, B) Protein expressions of TCF-4 J-responsive target genes (SPP1, AXIN2, MMP7, ASPH, and CD24) in the tumors treated with CC-122 or the control vehicle for either 10 days (D10) (**A**) or 19 days (D19) (**B**) were evaluated by Western blot analysis using antibodies against Myc-tag, SPP1, AXIN2, MMP7, ASPH, CD24, and actin. (**C**) The expression levels were quantified by densitometric analysis with normalization to actin. The results are shown as mean ± SD. *$P < .05$. **$P < .01$.

finding that CC-122 shows stronger anti-tumor effects than LEN led us to hypothesize that CC-122 may have different mechanisms of action independent of binding to CRBN relative to LEN. Although there have been no studies investigating the binding affinity of CC-122 and LEN to CRBN in HCC, a recent preliminary investigation using hematological cancer cell lines revealed that CC-122 binding to CRBN was significantly weaker than LEN; however, CC-122 exerted an unexplained stronger anti-proliferative effect [15]. Such observations suggest that the major anti-tumor activities of CC-122 may be mediated through other underlying cellular mechanisms rather than linked to CRBN biology and function.

We, therefore, explored the effect of CC-122 on modulation of expression level of the TCF-4 J isoform that is a key oncogenic regulator of the Wnt/β-catenin signaling cascade since it may represent one of the possible mechanisms responsible for the CC-122 anti-tumor activity in HCC where its activation is believed to be a major factor during hepatic oncogenesis [21]. TCF-4 J is one of the TCF-4 isoforms generated by alternative splicing events in HCC cell lines [20]. It has been observed that the TCF-4 J isoform induces a robust malignant phenotype in HCC cell lines and expression was significantly upregulated in human HCC tumors compared to normal liver and adjacent peritumor tissues as well [20,21]. Recent studies have identified TCF-4 J-responsive target genes to be key regulators and involved in Wnt/β-catenin, insulin/IGF1/IRS1, and Notch signaling pathways, which are important contributors to the pathogenesis of HCC [22]. The implications of these findings would be that suppression of TCF-4 J expression may lead to or contribute to inhibition of HCC tumor growth. To explore the hypothesis that the anti-tumor activity of CC-122 was mediated by inhibition TCF-4

J expression, we assessed the RNA and protein levels of the TCF-4 J isoform as well as TCF-4 J-responsive target genes on the CC-122-treated mice bearing human HCC tumors with over-expression of the TCF-4 J isoform. We found TCF-4 J and its target genes were significantly downregulated in the tumors treated with CC-122. This downregulation was not observed in tumors treated for longer period of time indicating the molecular effects of this drug are seen very early on at 7 days following initiation of therapy; however, the anti-tumor effects on growth of HCC persist for a much longer period of time as shown in Figure 3. Moreover, another *in vivo* experiment using poorly differentiated FOCUS HCC cells showed similar results. Accordingly, these findings support the idea that the anti-tumor effect of CC-122 is mediated by the downregulation of the tumorigenic TCF-4 J isoform and may represent a new class of chemotherapeutic agents that affect oncogenic transcription factors important in the regulation of signaling pathways key for hepatic oncogenesis. This hypothesis may be further supported by the recent observations that doxorubicin stimulates proteolytic cleavage and activation of the CREB3L1 transcription factor where it enters the nucleus and activates transcription of genes encoding inhibitors of the cell cycle to retard cell proliferation and tumor growth [26,27].

Results from the current study may have clinical applications. Recently, molecular-targeted drugs have been developed to treat various malignant tumors including HCC, based on alterations in gene expression, and such therapies are attractive for development of future treatment strategies [28,29]. The TCF-4 J isoform is found to activate multiple signal transduction pathways including Wnt/β-catenin, insulin/IGF1, and Notch signaling which have important roles in hepatocarcinogenesis [19], suggesting a potential direct target for a new therapeutic approach against HCC that affect multiple

growth factor signaling cascades involved in HCC cell proliferation, migration, invasion and transformation. In this regard, CC-122 is a promising drug for HCC treatment, since it can suppress tripartite signaling cascade activated in over 90% of HCC [19]. Furthermore, characterization of the TCF-4 J isoform as a key driver of the malignant phenotype raises the possibility that a transcriptional inhibitor such as CC-122 may give us a clue to predicting the biologic response to this drug in individual cases by measuring TCG-4 J and downstream target gene expression in tumor tissue. Indeed, it is a possibility that patients harboring HCC tumors associated with high expression level of TCF-4 J and/or its target genes would be more likely to clinically benefit from CC-122 treatment.

The present study demonstrated a significant anti-tumor effect of CC-122 in two human xenograft tumor models derived from the HAK-1A-J overexpressing HCC cell line and FOCUS which is poorly differentiated human HCC cell lines. Therefore, as previously shown, the TCF-4 J isoform has been found to be significantly upregulated in human tumors compared to peritumor tissues and associated with activation of major signaling pathways important in pathogenesis of these tumors and include similar evidence in other poorly differentiated HCC cell lines such as FOCUS (Figure 1 and Figure 2). These observations support the concept that the TCF-4 J isoform plays an oncogenic role in HCC development [18,19]. Another important observation derived from our study is that LEN did not significantly inhibit tumor growth in this model, which may be relevant to the finding that LEN only shows a limited clinical response in a small HCC patient population studied thus far and where the TCF-4 J and downstream gene activation and expression have not been characterized. Our results are consistent with the concept that TCF-4 J isoform mediates anti-tumor activity inhibited by treatment with CC-122; however, further study is required to define how CC-122 regulates TCF-4 J expression level in HCC.

There was an apparently arguable point in our results; although CC-122 did not have any effect on cell proliferation from *in vitro* assay, anti-tumor activity was significant in *in vivo* assay. It should not be ruled out the possibility that the experimental condition of cell proliferation may not be a proper setting *in vitro* assay. On the other hand, a previous study by Liu et al. [30] reported similar results to our data; certain IMiDs suppressed invasion, migration, and colony--formation capacity at 10 μM concentration, while there was no significant difference of anti-proliferative capacity at *in vitro* assay using the same concentration. Nevertheless, the drug exhibited significant anti-proliferative capacity at *in vivo* assay. Accordingly, it suggests that the anti-proliferative capacity by IMiDs may be difficult to be tested at conventional *in vitro* study.

In summary, CC-122 administration significantly inhibits tumor growth in the HCC xenograft models. The anti-tumor effect of CC-122 was mediated through down-regulation of TCF-4 J isoform as well as downstream target gene expression. These preclinical findings suggest that CC-122, as a regulator or modulator of a unique oncogenic TCF-4 J isoform, expression may be a promising and novel therapeutic approach for this devastating disease.

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Conflict of Interest Statement
The authors declare no competing financial interests.

Author's Contributions
Conceived and designed the experiments: YT, AA, JRW, MK.
Performing the experiments: YT, AA, MK.
Analysis and interpretation of the data: YT, AA, MK.
Writing, review and editing the paper: YT, JRW, CA, MK.

Appendix A. Supplementary Data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.07.002.

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