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Targeting CALM2 Inhibits Hepatocellular Carcinoma Growth and Metastasis by Suppressing E2F5-mediated Cell Cycle Progression

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Abstract. *Background/Aim: The aim of this study was to reveal the novel roles of calmodulin 2 (CALM2) in hepatocellular carcinoma (HCC) progression. Materials and Methods: The effects of knockdown of CALM2 expression by siRNA were investigated using various experimental approaches in both cellular and molecular levels. Results: Silencing of CALM2 inhibited HCC cell proliferation and colony formation through induction of apoptosis. At the molecular level, CALM2-specific knockdown led to the common dysregulation of 154 genes in HCC cells. Notably, E2F transcription factor 5 (E2F5), which is functionally associated with migration, invasion and proliferation, was generally down-regulated. These functional associations were confirmed in HCC clinical samples. Reflecting the molecular changes, CALM2 knockdown reduced the migration and invasion abilities of HCC cells and abrogated the potency of tumor formation in vivo. Conclusion: Targeting CALM2 may be a molecular strategy for both primary HCC treatment and prevention of metastasis or recurrence.*

Hepatocellular carcinoma (HCC) is the sixth most frequent malignant tumor and was the fourth highest cause of cancer death worldwide in 2018, with approximately 782,000 deaths and 841,000 new cases (1). Chronic hepatitis B virus infection is the leading cause of HCC. Other factors, such as alcohol abuse, obesity, hepatitis C virus infection, and type 2 diabetes have also been implicated; the prevalence of HCC varies geographically (2). Most HCCs are induced by chronic inflammation caused by viral infections, resulting in continuous hepatic inflammation and hepatocyte regeneration (3). Regarding the therapeutic efficacy, late diagnosis, presence of other hepatic complications, and the lack of possible therapeutic options limit HCC treatment. Conventional treatments, such as liver transplantation, surgical resection, and thermal ablation, are performed on HCC patients in the very early or early stages (4, 5). However, liver transplantation is limited by donor organ availability, while the disadvantage of surgical resection is that it does not eliminate parts of the liver that lack function and are at risk for malignant transformation (6). Currently, sorafenib, a potent small molecule inhibitor of multiple kinases, is the most recommended prescribed drug for advanced stage HCC (BCLC stage C) with invasive or extra-hepatic tumor tissues. However, this standard treatment can extend patient survival for only about 12 months (7) and it does not appear to be able to eliminate cancer stem cells (CSCs), as evidenced by frequent tumor relapse and resistance after therapy (8). Thus, there is an urgent need to identify other novel molecular targets that could be explored for treatment options to improve the therapeutic index. Since HCCs have shown certain common traits including genetic and epigenetic alterations (9), targeting any of the common dysregulated genes might provide a new HCC treatment (10).

Calmodulin (CALM), a Ca^{2+}-binding protein, is a relatively small and highly conserved protein. CALM is expressed in eukaryotic cells and contributes to signaling pathways regulating many important processes, such as cell growth, proliferation, and migration (11). CALM is a Ca^{2+}

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sensor that binds to proteins such as Ca^{2+}/calmodulin-dependent protein kinase (CaMK), cyclins, and Fas, which are functionally involved in cell proliferation, apoptosis, tumor progression, and autophagy. CALM is overexpressed in different types of tumor tissues, and its altered expression is significantly associated with malignant tumor pathogenesis and progression (12). For example, an increase in CALM expression enhanced cell proliferation in lung cancer cells by altering DNA synthesis (13). *CALM2* is a member of the *CALM* gene family (*CALM1, CALM2,* and *CALM3*) that encodes calmodulin (14, 15), and it is one of the upregulated genes in HCC tissues (16). Despite the functional significance of CALM2 and its genetic alteration in human HCC, its specific functional role in HCC progression remains to be clarified.

The purpose of this study was to explore and reveal the novel roles of CALM2 during HCC development. To address this, we first examined if inhibition of CALM2 expression by target-specific siRNA could change HCC phenotypes *in vitro* and *in vivo*. We then examined the molecular mechanisms underlying the therapeutic response induced by CAML2 silencing. Our findings indicate that CALM2 is functionally associated with E2F transcription factor 5 (E2F5)-mediated cell cycle progression and also suggest that molecular targeting of CALM2 can be an anti-HCC therapeutic strategy.

Materials and Methods

Cell culture and siRNA transfection. The human HCC cell lines Huh1 and PLC/PRF/5 were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan) and the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea), respectively. Huh1 and PLC/PRF/5 cells were cultured in Dulbecco's Modified (DMEM) and RPMI-1640 media, respectively, supplemented with 1% penicillin/streptomycin solution and 10% fetal bovine serum (FBS) (Welgene, Daegu, Republic of Korea). Prior to siRNA transfection, cells were plated at 30% density for 24 h. siRNAs (15 nM) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were mixed in Opti-MEM (Thermo Scientific, Rockford, IL, USA) and added to cells for 5 h. CALM2-siRNA (ID# s2343) and negative control siRNA (NC siRNA) duplexes were purchased from Ambion (Austin, TX, USA) and Bioneer (Daejeon, Republic of Korea), respectively. The sequences of NC siRNA were as follows: 5'-ACGUGACACGUUCGGAGAA(UU)-3' (sense) and 5'-UUCUCCGAACGUGUCAC GU-3' (antisense).

Cell proliferation and clonogenic assay. Cell proliferation was analyzed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent, as stated in the manufacturer's instructions (Duchefa Biochemie, Haarlem, Netherlands). Huh1 (1×10^3 cells) and PLC/PRF/5 (1×10^3 cells) were transfected with siRNAs as described above. At 96 h of cell culture, 90 μl of plain media and 10 μl of MTT were added into each well and cultured for 1-2 h in a 37°C humidified incubator. After the removal of MTT, 100 μl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) solution were added, and the OD value was evaluated at the wavelength of 540 nm using an Asys UVM 340 microplate reader (Biochrom, Cambridge, UK). For the clonogenic assay, a total of 1×10^3 siRNA transfected cells were seeded in 6-well plates and incubated in medium for 12 days until the viable cells propagated to sizable colonies for quantification. The colonies formed in each well were fixed with methanol and then stained with 0.5% crystal violet for 30 min. The colonies were counted under a microscope.

RNA purification and qRT-PCR. Gene expression alterations at the mRNA level were analyzed using qRT-PCR. Total RNA was isolated using TRIzol (Ambion) and synthesized into cDNA using a cDNA Synthesis Kit, as stated in the manufacturer's instructions (Takara Biotech, Kusatsu, Shiga, Japan). Then, cDNA was amplified by using a corresponding pair of primers (CALM2 forward, 5'-CAACAAAGGAATTGGGA ACTG-3'; CALM2 reverse, 5'-TGTCATCACATGGCGA AGTT-3' and GAPDH forward, 5'-ACATCAAGAAGGTGGTGA-3'; GAPDH reverse, 5'-GGTGTC GCTGTTGAAGTC-3', and E2F5 forward, 5'-ACCTGATGA CCTCACACAGCCTTC-3'; E2F5 reverse, 5'-GGGGTAGG AGAAAGCCGTAAAAG-3'), which were synthesized by Genotech (Daejeon, Republic of Korea). Relative mRNA expression was evaluated using LightCycler 96 (Roche, Basel, Switzerland) and quantified using LightCycler 96 software version 1.1. This was compared with the Ct (threshold cycle) values of each target gene, according to the instructions of the manufacturer, and the GAPDH mRNA levels were used for normalization.

Cell cycle analysis and detection of apoptosis. For cell cycle analysis and detection of apoptosis, cells were transfected with siRNA as described above, but were cultured in 100 mm petri dishes and harvested 72 h after siRNA transfection. To analyze the percentage of cells in each cell cycle phase, cells were washed with cold PBS and then fixed for 24 h with 70% cold ethanol. Cells were washed again and incubated for 30 min at room temperature in the dark in propidium iodide (PI) solution containing RNase A (BD Biosciences, San Diego, CA, USA). For the detection of apoptosis, cells were washed with cold PBS and stained with both Annexin V and PI using an FITC Annexin V Apoptosis Detection Kit I (BD Biosciences), according to the manufacturer's instructions. The cell death and cell cycle were analyzed using a FACS Canto II flow cytometer (BD Biosciences) and quantified using the FACSDiva software program.

RNA sequencing. The RNA sequencing method has been described in detail previously (17). The datasets generated and analyzed in this study are accessible at BioProject PRJNA594473 with a Sequence Read Archive (SRA) accession number SRP235450.

Western blot analysis. Equal amounts of total protein were fractionated by SDS-PAGE on a 10% gel and transferred to a PVDF membrane (Roche). The membrane was blocked with 5% milk/Tris-buffered saline plus Tween 20 (TBST) and incubated with primary antibodies against E2F5 (sc-271497), CDK2 (sc-6248) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and GAPDH (NB600-502, Novus Biological, Littlleton, CO, USA). Anti-mouse HPR (115-035-062, Jackson ImmunoResearch Lab, West Grove, PA, USA) was used as the secondary antibody. Immunoreactive bands were visualized using a FUSION SOLO S (Vilber Lourmat, Marne La Vallee, France).

Wound-healing Assay. Huh1 and PLC/PRF/5 cells were seeded in 100 mm dishes (3×10^5 cells per dish). Seventy-two h after siRNA transfection, the transfectants were then seeded in 24-well plates (3×10^5 cells per well). On the next day, three longitudinal scratch wounds were made using a sterile 10 μl pipette tip and floating cell debris were removed by washing two times with PBS. The cells were cultured in medium supplemented with 1% penicillin/streptomycin solution and FBS. Images were acquired after 2-3 days.

Invasion assay. Invasion assays were performed using a 24-well Matrigel invasion chamber (#354480, Corning, Corning, NY, USA). Approximately 5×10^4 NC siRNA- or CALM2 siRNA-transfected Huh1 and PLC/PRF/5 cells suspended in 200 μl of medium were plated in the upper chamber. The lower compartment was filled with 600 μl of medium containing FBS as an attractant. After 24 h, the cells were fixed and stained with 0.5% crystal violet. Images were obtained at 100× magnification using a phase-contrast microscope.

Tumorigenic assay in nude mice. PLC/PRF/5 cells were transfected with NC siRNA or CALM2 siRNA using Lipofectamine 2000. After 24 h of treatment, silenced cells were harvested and mixed with Matrigel (Corning). A total of 5×10^6 cells were injected subcutaneously into the left and right flanks of four-week-old male BALB/c nude mice (Orientbio, Seongnam, Republic of Korea). Tumor size was then measured at 2-3 day intervals for 31 days using a Vernier caliper. At the end of observation, the tumor weight of each mouse was evaluated. All animal experiments were performed under the approval number of KM-2018-20, according to the guidelines of Keimyung University Institutional Animal Care and Use Committee (KM-IACUC).

Statistical analysis. The SPSS statistical package, version 23.0 for Windows (IBM, Armonk, NY, USA) was used for all statistical analyses. Student's *t*-test was used to determine differences in means. All results are presented as the mean±standard error of mean (SEM). *p*-Values <0.05(*), <0.01(**), and <0.001(***) were considered statistically significant.

Results

Knockdown of CALM2 expression inhibits HCC cell growth. To investigate the role of CALM2 silencing on cell growth, two HCC cell lines, Huh1 and PLC/PRF/5, were transfected with a negative control (NC) siRNA or CALM2 siRNA. After 96 h, we found that the viability of CALM2-silenced cells was significantly reduced by microscopic observation (Figure 1A). Cell viability was also assessed in the Huh1 and PLC/PRF/5 cells using an MTT assay (Figure 1B). The proliferation of CALM2 siRNA-treated Huh1 and PLC/PRF/5 cells was reduced by approximately 88% and 50%, respectively, when compared with that of the control group. Consistent with the changes in cell growth, CALM2 siRNA significantly silenced the target gene expression in both Huh1 and PLC/PRF/5 cells (Figure 1C). Moreover, targeting CALM2 significantly reduced the long-term colony formation by Huh1 and PLC/PRF/5 cells, with a reduction of approximately 70% at day 12 (Figure 1D). These results suggest that CALM2 is involved in HCC cell proliferation.

Targeting CALM2 induces apoptosis in HCC cells. We then examined whether the inhibition of HCC cell growth by CALM2 knockdown was due to delay of cell cycle progression and/or induction of apoptotic cell death. In comparison with the control group, silencing of CALM2 increased the percentage of sub-G_1 phase cells by more than 2-fold in both Huh1 and PLC/PRF/5 cells (Figure 2A and B). Because the sub-G_1 population contains apoptotic cells, apoptosis was examined by staining HCC cells with Annexin V, an apoptotic marker. This showed that targeting CALM2 increased the number of apoptotic cells 72 h after transfection (Figure 2C and D). The percentages of late (Q2 portion) plus early apoptotic cell populations (Q4 portion) in Huh1 and PLC/PRF/5 cells increased by approximately 15% and 6%, respectively. These results show that the inhibition of HCC cell proliferation by CALM2 silencing was incurred by block of cell cycle progression and induction of apoptosis.

Knockdown of CALM2 down-regulates E2F5 expression. To elucidate the molecular mechanisms by which CALM2 silencing could induce the observed phenotypic changes, we performed RNA sequencing and compared the patterns of global gene expression in CALM2-deficient Huh1 and PLC/PRF/5 cells to that of NC siRNA-treated cells. When defined using a Bootstrap ANOVA and at least a 2-fold change ($p<0.001$), silencing of CALM2 disturbed the expression of 539 RNA transcripts in Huh1 and 512 in PLC/PRF/5 cells (Figure 3A). Overlapping of these two gene sets generated a commonly dysregulated list of 154 genes (44 upregulated and 110 down-regulated) (Figure 3B). Clue Go analysis revealed that the 154 mRNA transcripts were functionally associated with the regulation of various pathways such as nitric-oxide synthase biosynthetic process, fatty acid transport, TGF-β signaling pathway, DNA replication, and cell cycle (Figure 3C). Notably, we found that the expression levels of E2F5, which is involved in cell cycle and TGF-β signaling, were down-regulated by CALM2 knockdown. We validated this by real-time RT-PCR (Figure 3D and E). Consistent with the transcriptional profiling of the knockdown of CALM2 expression, the protein levels of E2F5 were reduced under the same conditions (Figure 4A). In addition, a concurrent decrease in cyclin-dependent kinase 2 (CDK2), which regulates cell cycle progression, was observed. These associations were confirmed in the TCGA data. CALM2 mRNA expression was positively correlated with E2F5 ($r=0.216$, $p<0.001$) and CDK2 ($r=0.134$, $p=0.011$) in human HCC tissues (Figure 4B and C). These results partially explain the molecular mechanisms by which targeting CALM2 could delay cell cycle progression and induce apoptotic cell death.

Targeting CALM2 inhibits HCC cell migration and invasion. It has been previously demonstrated that E2F5 silencing in

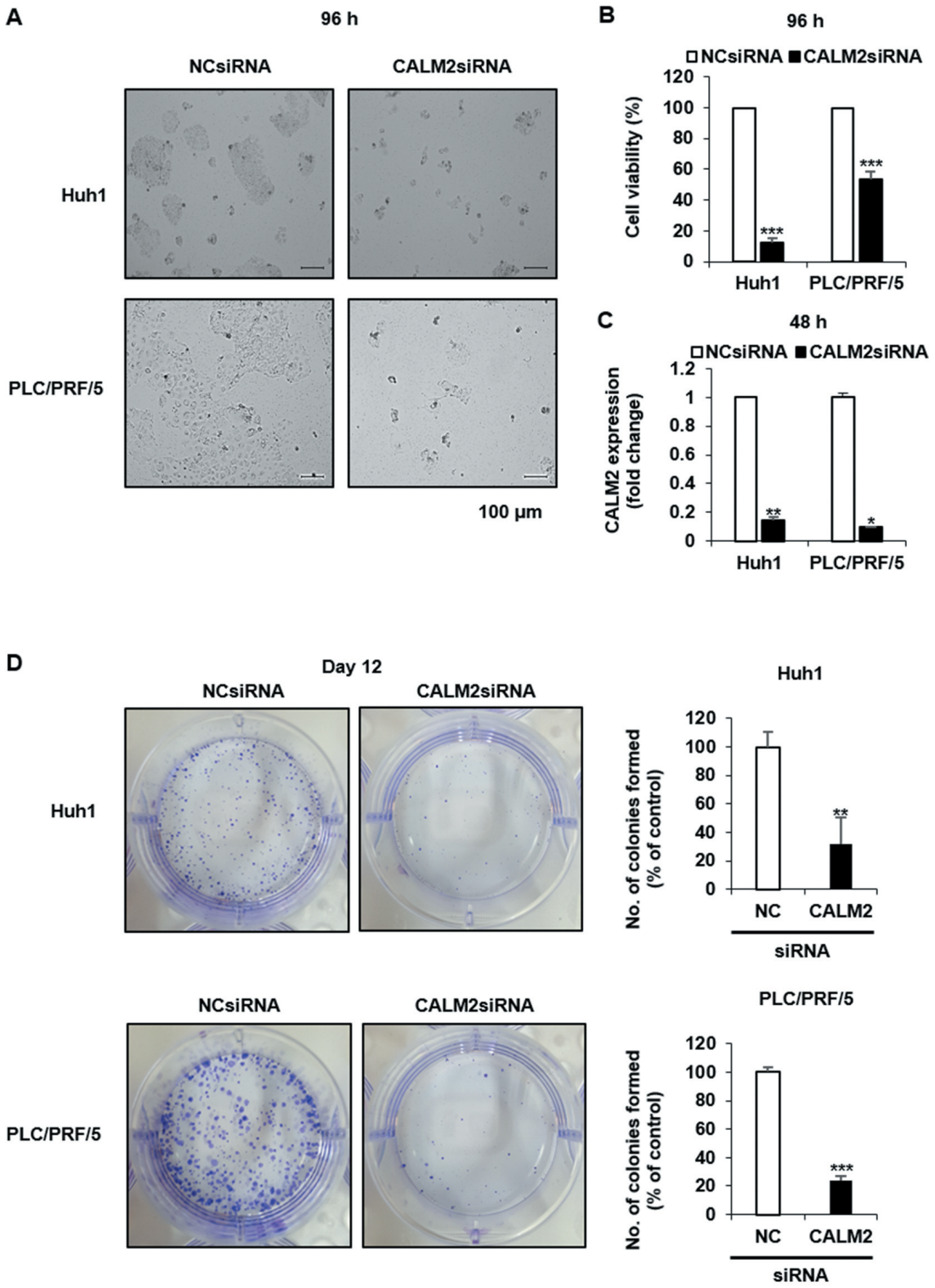

Figure 1. *Silencing CALM2 inhibits viability of HCC cells. (A) Representative Light microscopy images of Huh1 and PLC/PRF/5 cells 96 h after siRNA transfection. Scale bar, 100 µm. (B) Detection of cell viability after 96 h of siRNA transfection. It is expressed as fold change relative to that of NC siRNA. Data were obtained from four independent experiments. (C) Detection of CALM2 mRNA expression 48 h following siRNA transfection. The data are shown as the mean±SEM of triplicate experiments. (D) Observation of long-term colony formation at 12 days after transfection of Huh1 and PLC/PRF/5 cells. The number of colonies was counted from three independent experiments and graphed for effective comparison. NC siRNA, negative control siRNA, CALM2 siRNA, CALM2-specific siRNA. *p<0.05; **p<0.01; ***p<0.001 vs. control.*

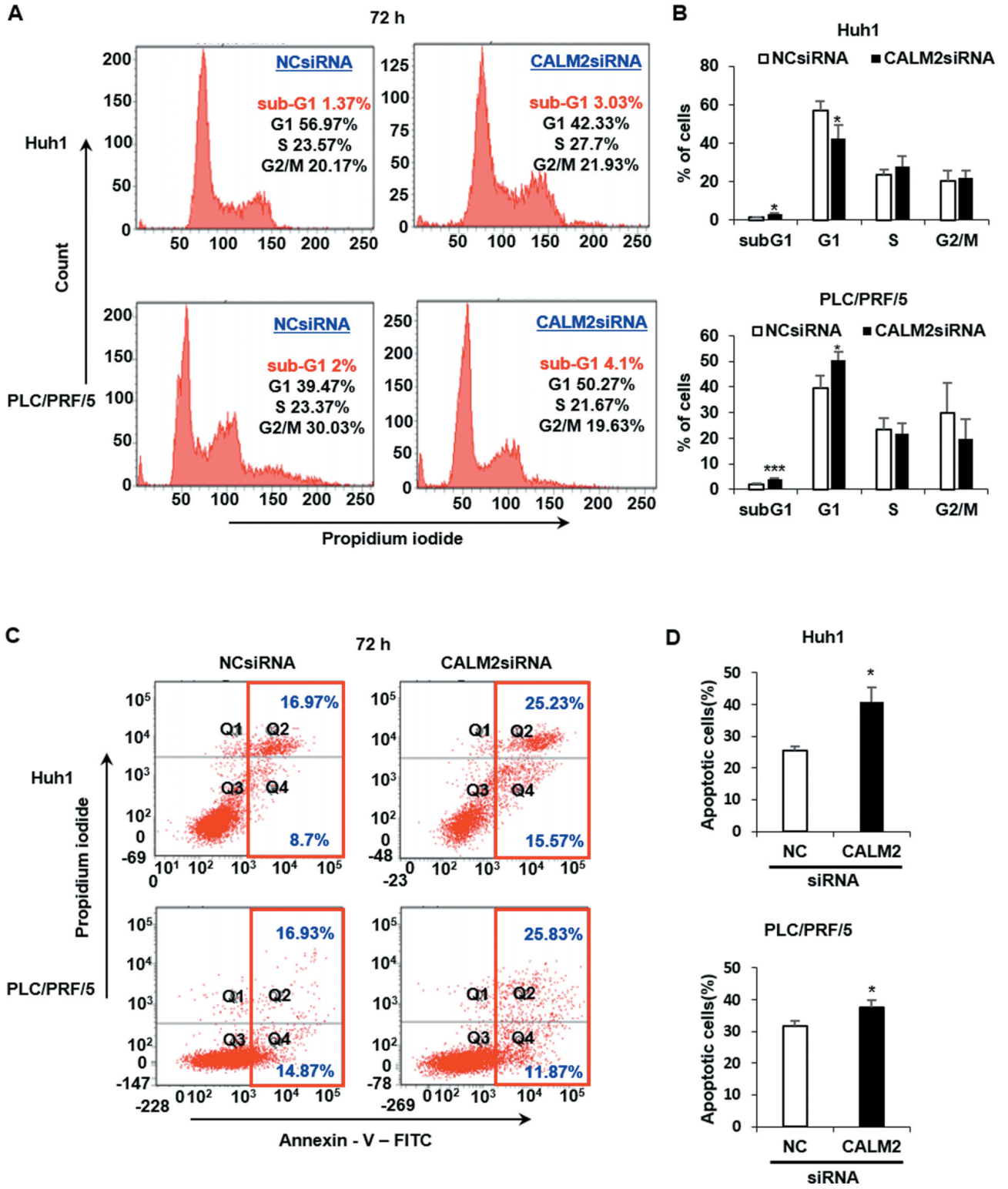

Figure 2. *Targeting CALM2 increases the sub-G1 population and induces apoptosis in both Huh1 and PLC/PRF/5 cells. (A) At 72 h after siRNA treatment, changes in cell cycle progression were measured by flow cytometry and shown in a histogram. (B) Percentage of cells in each phase is shown as a bar graph. (C) The fraction of apoptotic cells was measured by FACS analysis after 72 h of siRNA treatment. (D) The total percentage of apoptotic cells in the Q2+Q4 region is shown in a bar graph. All the data were obtained from three independent experiments. *p<0.05; ***p<0.001 vs. control.*

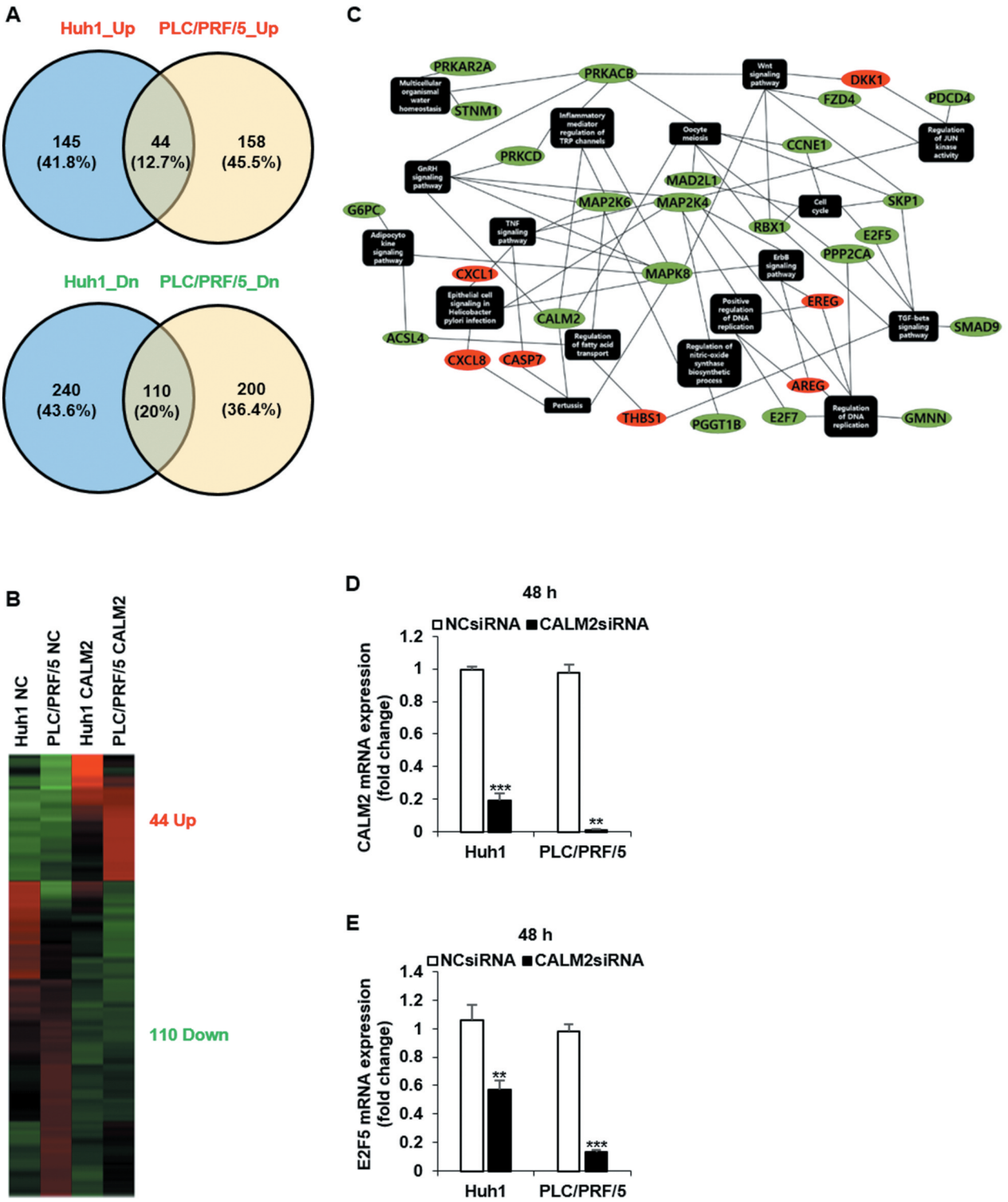

Figure 3. *Molecular mechanisms underlying HCC phenotypic changes induced by CALM2 silencing. (A) The number of genes dysregulated in Huh1 and/or PLC/PRF/5 cells 48 h after siRNA transfection. (B) A heat map of the 154 commonly up- or down-regulated genes in both Huh1 and PLC/PRF/5 cells after normalization to the corresponding NC siRNA-treated cells (Red, up-regulated; green, down-regulated). (C) A representative image of genes commonly dysregulated in Huh1 and PLC/PRF/5 cells following CALM2 down-regulation. (D-E) Detection of CALM2 (D) or E2F5 (E) mRNA expression 48 h after siRNA transfection of Huh1 and PLC/PRF/5 cells. Results are presented as the mean±SEM of triplicate experiments. **p<0.01; ***p<0.001 vs. control.*

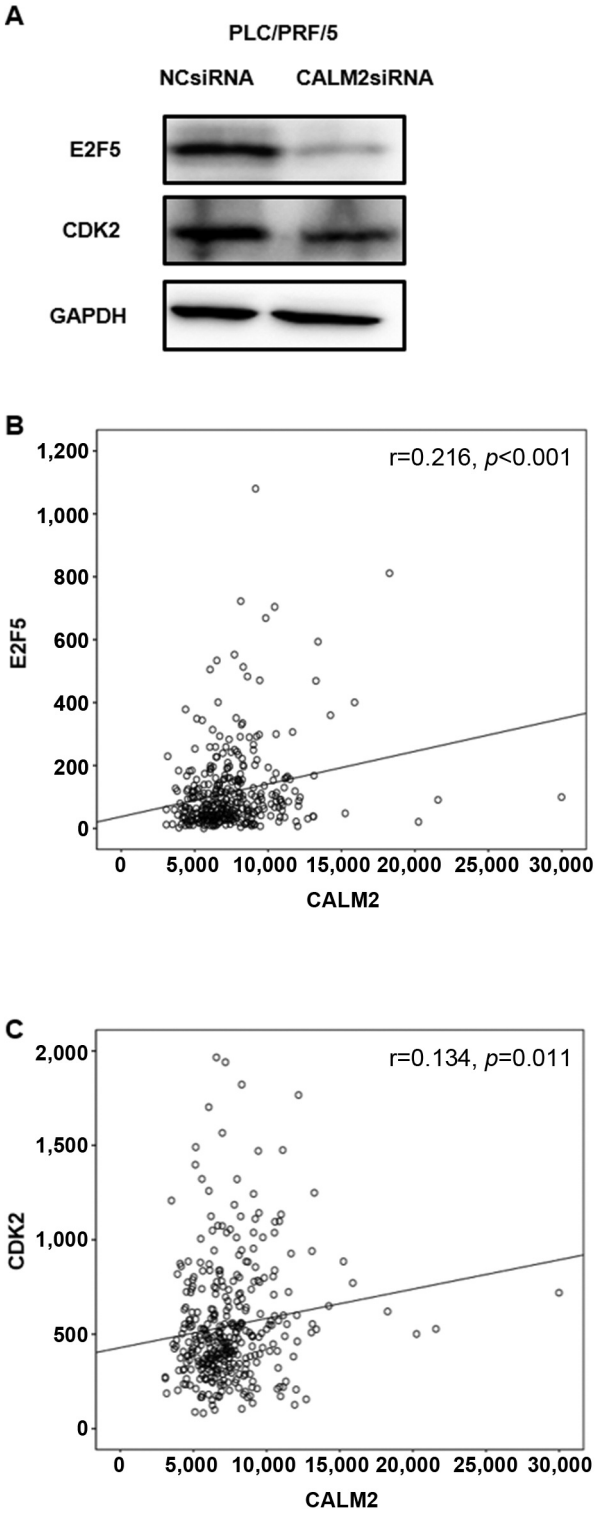

Figure 4. *The function of CALM2 is associated with E2F5 and CDK2. (A) The levels of E2F5 and CDK2 proteins in CALM2-silenced PLC/PRF/5 cells. GAPDH was detected as a loading control. (B-C) The correlation between the expression of CALM2, E2F5 and CDK2 in HCC tissues. TCGA data showed that CALM2 was positively correlated with E2F5 (B) and CDK2 (C).*

HCC cells leads to decreased proliferation, migration, and cell invasion (18). Given the functional significance of E2F5 in cancer cell phenotypes and the observation of E2F5 repression under CALM2 knockdown conditions, we examined whether targeting CALM2 could eliminate the metastatic potential of HCC cells by using wound healing and invasion assays. As expected, CALM2 knockdown effectively reduced the number of migrated (Figure 5A) and invaded cells (Figure 5B) in both Huh1 and PLC/PRF/5 cell populations. These data imply that targeting CALM2 may be a molecular strategy for both primary HCC treatment and prevention of metastasis or recurrence.

Silencing of CALM2 suppresses tumor growth in vivo. To confirm the suppressive effect of CALM2 silencing *in vivo*, a tumor formation assay was performed in BALB/c nude mice. PLC/PRF/5 cells transfected with NC siRNA or CALM2 siRNA for 24 h were subcutaneously inoculated into the left and right flank, respectively, and then the size of each tumor was measured for 31 days at 2-3-day intervals. Fourteen days after the inoculation, the tumor sizes of the control (NC siRNA) group were larger than those in the experimental (CALM2 siRNA) group (Figure 6A). On day 31, a significant difference was evident in the tumor size and weight of the control and CALM2 knockdown cells (Figure 6B). Tumor growth was not detected on the right flank where we inoculated CALM2 siRNA transfected PLC/PRF/5 cells. The results showed that, under our experimental conditions, targeting CALM2 completely abrogated the potency of tumor development *in vivo*.

Discussion

Recently, a variety of therapeutic strategies targeting calcium signaling modulators have been developed (19). There has also been evidence that CALM2, a Ca^{2+}-binding protein, is commonly upregulated in HCC (16) and calcium signaling is one of the major functional pathways promoting the development of HCC (20). Nevertheless, the function of CALM2 in HCC progression remains unclear. We demonstrated in this study that targeting CALM2 results in the suppression of HCC cell proliferation *via* the induction of apoptosis and delay of cell cycle progression. Additionally, an *in vivo* mouse model showed that CALM2 silencing suppresses tumor growth. The phenotypic changes were related to the dysregulation of 154 common transcripts in the CALM2-silenced condition, including the down-regulation of E2F5 (Figure 3B and C).

The E2F members are divided into inhibitor E2F, repressor E2F, and activator E2F. E2F5 is a repressor of E2F. In the G0 phase, it associates with pocket protein p130 and other co-repressors to maintain the repression of E2F-responsive genes and promote the entry of cells into the G1

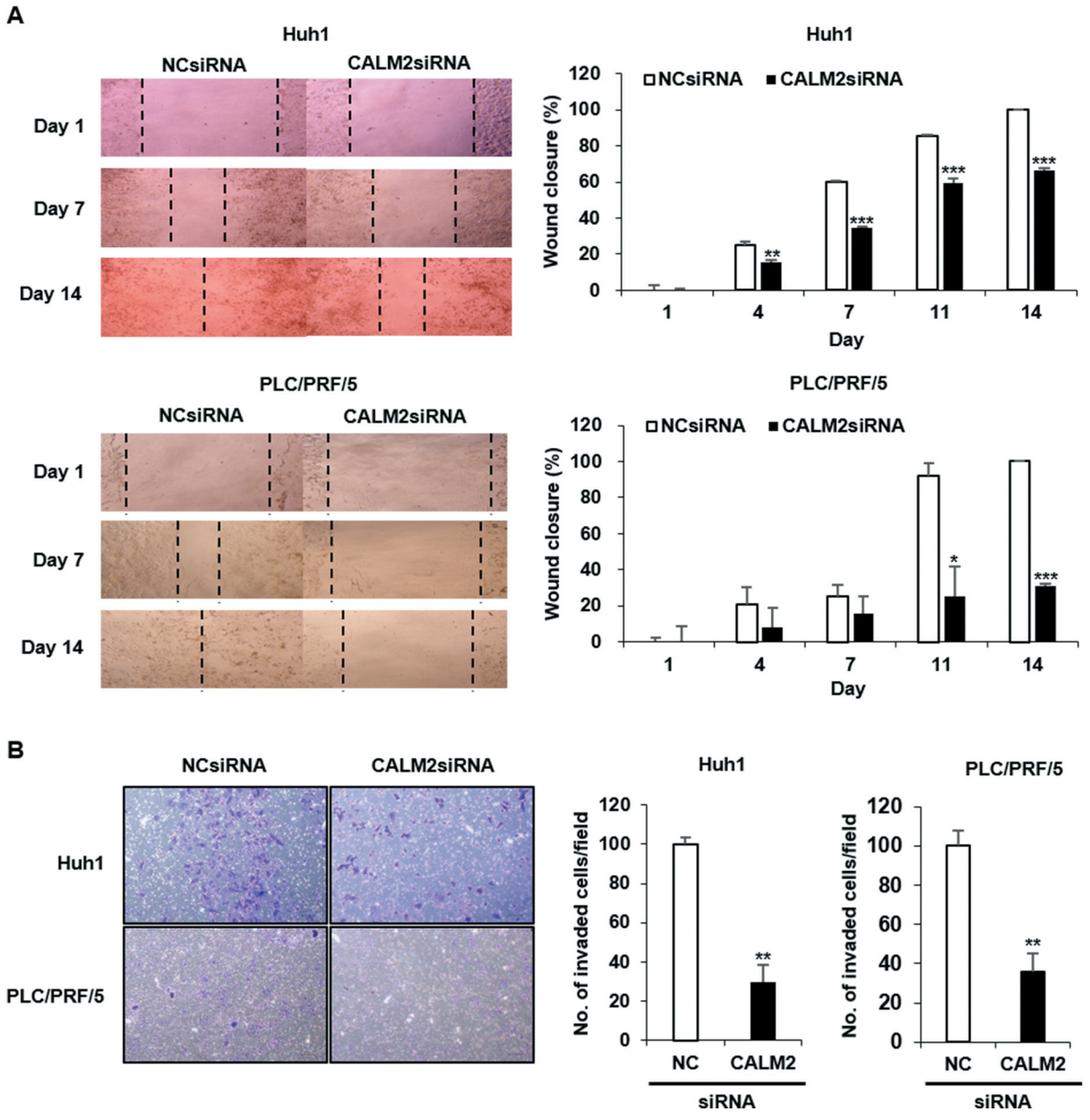

Figure 5. *Silencing of CALM2 reduces migration and invasion. (A) Light microscopy images of migratory NC siRNA- or CALM2 siRNA-transfected Huh1 and PLC/PRF/5 cells. The number of migrated cells was counted and graphed at the indicated days after siRNA treatment. (B) Representative light microscopy images of invasive NC siRNA or CALM2 siRNA transfected Huh1 and PLC/PRF/5 cells. The number of invaded cells was counted and graphed. *p<0.05; **p<0.01; ***p<0.001 vs. control.*

phase during cell cycle progression (21). It is well known that E2F5 has a significant role in the regulation of cell cycle progression, differentiation, proliferation, and apoptosis (18, 21). Furthermore, increased expression of E2F5 has been found in various types of human cancers, such as breast cancer (22), epithelial ovarian cancer (23), prostate cancer (24), and HCC (18). Thus, this study focused on defining a functional association between E2F5 and CALM2 among all genes that were deregulated because it was observed that the oncogenic E2F5 was generally down-regulated by silencing CALM2. In agreement with the RNA sequencing data, it was confirmed by using real-time PCR and western blot analysis that targeting CALM2 reduced the expression of E2F5 at both the mRNA (Figure 3E) and protein levels (Figure 4A).

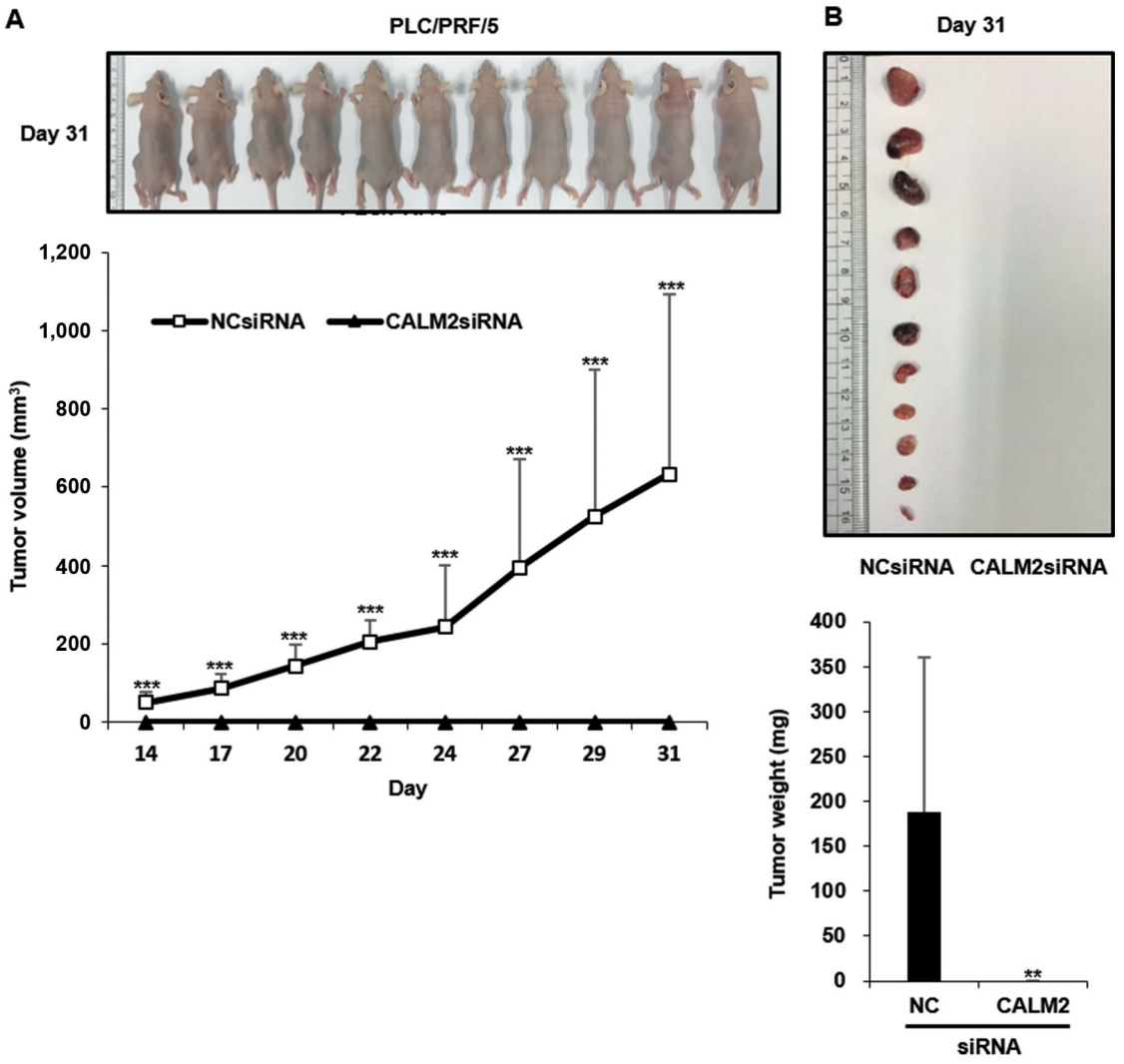

Figure 6. *Silencing of CALM2 suppresses tumor growth in vivo. (A) Kinetics of tumor growth: PLC/PRF/5 cells transfected with NC siRNA or CALM2 siRNA for 24 h were subcutaneously injected into nude mice. Tumor diameters were measured on the indicated days with digital calipers. The data were obtained from eleven independent experiments. (B) Measurement of the control and CALM2 knockdown tumor weight. Thirty-one days after the injection, the mice were sacrificed, and each tumor weight was measured. **p<0.01; ***p<0.001 vs. control.*

However, we were not able to show the reduction of CALM2 protein level itself as CALM2-specific antibodies are not commercially available yet. We proceeded in the subsequent experiments after assuring that *CALM2* mRNA levels were significantly down-regulated by CALM2-specific validated siRNA (Figure 1C or Figure 3D). We also found that the protein levels of the cell cycle regulator CDK2 were decreased under the same conditions (Figure 4A), and that the functional association between CALM2, E2F5, and CDK2 coincided with their expression pattern in clinical HCC specimens (Figure 4B and C). In addition to the suppression of E2F5 expression, it was also observed that silencing of CALM2 down-regulated the mRNA expression of other cell cycle-related genes, such as cyclin E1 (*CCNE1*) (25) and mitotic arrest deficient 2 like 1 (*MAD2L1*) (26), which was consistent with the suppression of mitogen-activated protein kinase 8 (*MAPK8*) (27) and mitogen-activated protein kinase kinase 4 (*MAP2K4*) (28) genes involved in cell proliferation. Conversely, the expression of dickkopf-related protein 1 (*DKK1*) (29) and caspase-7 (*CASP7*) (30) genes that are tumor suppressive or apoptosis-related were upregulated by CALM2 silencing (Figure 3C). These molecular alterations might explain how targeting CALM2 could inhibit HCC cell proliferation and induce cell cycle arrest or apoptosis and could be suggested as the mode of action of a future-developed CALM2-targeted inhibitor.

To affirm the growth suppressive effect of CALM2 silencing *in vivo*, we performed a tumorigenic assay in nude mice transplanted with PLC/PRF/5 cells transfected with control siRNA or CALM2 siRNA for 24 h. As shown in Figure 6, tumor growth in the CALM2 siRNA group was not detected until day 31, the end point observation. This could be due to the inhibition of cell proliferation by the transfection of CALM2-specific siRNA compared to the transfection with control siRNA.

Meanwhile, it is well known that a CSC subset is responsible for tumor initiation, migration, recurrence, chemo-resistance, and radio-resistance; therefore, targeting this subset in the tumor microenvironment is essential for the prevention of relapse. Previous evidence indicates that any hepatic lineage cells, such as hepatocytes/cholangiocytes, progenitor cells, and stem cells, can acquire CSC properties through accumulated genetic and epigenetic alterations in diverse signaling pathways (31). We have also found that the up-regulation of the CSC marker CD133 by STAT3 transcription factor promotes HCC development (32). Thus, the identification of target genes functionally associated with CSC biology can provide useful treatment strategies against HCC. The current standard-of-care sorafenib inhibits tumor angiogenesis and induces apoptosis in HCC by blocking the RAF/MEK/ERK pathway, one of the key functional pathways of HCC development (33). However, sorafenib treatment is associated with frequent tumor relapse and resistance after therapy (8). A previous study showed that E2F5 silencing in HCC cells decreased cell proliferation, migration, and invasion (18). Thus, we showed that the migration and invasion abilities were decreased in CALM2 knockdown Huh1 and PLC/PRF/5 cells compared to those in the control (Figure 5). However, further studies are warranted to examine whether the inhibition of metastatic potential in the parental HCC cell population derives from a substantial elimination of CSCs and reduction in sphere forming capacity of CSCs.

In conclusion, we showed here for the first time that CALM2 functionally interacts with E2F5 cell cycle regulator to promote HCC progression and that targeting CALM2 may be a molecular strategy for both primary HCC treatment and prevention of metastasis or recurrence.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

SY and YH designed the study and wrote the manuscript. SY, YR, MJ KO, JH, and MJ Kim conducted the experiments and acquired the data. YH, JH, KS, YK, and TJ interpreted and discussed the results and reviewed the manuscript. The study was supervised by YH.

Acknowledgements

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