Published in final edited form as:
Curr Med Chem. 2013 ; 20(33): 4131–4141.

1, 25(OH)$_2$D$_3$ Inhibits Hepatocellular Carcinoma Development Through Reducing Secretion of Inflammatory Cytokines from Immunocytes

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Abstract

Epidemiological and clinical studies have indicated that low vitamin D activity is not only associated with an increased cancer risk and a more aggressive tumor growth, but also connected with an aggravated liver damage caused by chronic inflammation. Meanwhile, increasing evidence has demonstrated that 1,25(OH)$_2$D$_3$ (the most biologically active metabolite of vitamin D) can inhibit inflammatory response in some chronic inflammatory associated cancer, which is considered to have the anti-tumor potency. However, the interaction between 1,25(OH)$_2$D$_3$ and inflammation during hepatocellular carcinoma (HCC) initiation and progression is not yet clear. Here, we report an anti-tumorigenesis effect of 1,25(OH)$_2$D$_3$ via decreasing inflammatory cytokine secretion in HCC and hypothesize the possible underlying mechanism. Firstly, we show that the enhanced tumor growth is associated with elevated inflammatory cytokine IL-6 and TNF-α in *1α(OH)ase* gene-knockout mice. Secondly, 1,25(OH)$_2$D$_3$ can inhibit vitamin D receptor (VDR) shRNA interfered tumor cell growth through decreasing inflammatory cytokine secretion *in vitro* and *in vivo*. Finally, using *p27^{kip1}* gene knock-out mouse model, we demonstrate that the effect of 1,25(OH)$_2$D$_3$ in inhibiting immune cell related inflammatory cytokine secretion, exerts in a *p27^{kip1}* gene dependent way. Collectively, 1,25(OH)$_2$D$_3$ inhibits HCC development through up-regulating the expression of *p27^{kip1}* in immune cell and reducing inflammatory cytokine production.

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CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

Keywords

HCC; chronic inflammation; 1,25$(OH)_2D_3$; *1α(OH)ase*; gene knockout; IL-6; TNF-α; STAT3 signaling; *p27kip1*; co-culture

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 90% of all primary liver malignancies, grows rapidly and is highly resistant to chemotherapy [1, 2]. The average survival time for HCC patients is less than 12 months after diagnosis. Only 10–20% of HCC cases are suitable for resection, with a 5-year recurrence-free survival of only 20–30% [3, 4]. Recent developments in new treatment modalities have led to an improved survival rate slightly [5]; however, current treatments are still not satisfactory, and thus, novel treatment strategies against HCC are in dire need.

Rapidly growing evidence reinforces the notion that tumors are promoted by inflammatory signals from the surrounding microenvironment [6,7]. Generally, in chronic inflammation, the inflammatory foci are dominated by infiltrated inflammatory cells which generate a great amount of growth factors, cytokines, reactive oxygen and nitrogen species that may cause DNA damage of normal cells [8]. A microenvironment comprehends the persistent inhabitation of activated inflammatory cells which may cause continued tissue damage and sustained cell proliferation, thus predisposes chronic inflammation to neoplasia [9]. The advancements in understanding the relationship between inflammation and hepatocarcinogenesis have highlighted that excessively and chronically produced pro-inflammatory cytokines contribute to HCC initiation and progression in virus hepatitis, non-alcoholic fatty liver disease (NAFLD) and chemical tumorigenesis of patients or mouse models [10, 11]. Previous studies have demonstrated that the tumor development in carcinogen-induced obesity mice is dependent on the elevated tumor-promoting cytokine IL-6 and TNF-α, which may augment the hepatic inflammatory response and the activation of oncogenic transcription factor STAT3, since inactivation of IL-6 or TNF-α inhibits the tumor promoting effects in these mice [12]. Meanwhile, a recent literature has also validated that, the reinforcement of STAT3 signaling in inflammatory cell, activated by inflammatory cytokine IL-6, accelerates HCC initiation and progression *in vitro* and *in vivo* [13]. Collectively, reducing the production of inflammatory cytokines, especially IL-6 and TNF-α, may serve as a new target for HCC therapy and prevention.

Epidemiological and clinical studies have indicated that the low circulating levels of vitamin D are associated with an increased risk of several types of cancer and a more aggressive tumor growth; while a high intake of 1,25$(OH)_2D_3$ reduces the risk of cancer [14,15]. Previous findings have already demonstrated that 1,25$(OH)_2D_3$ plays a major role in modulating calcium and skeletal homeostasis and exerts a significant influence on the growth and differentiation of a variety of tissues [16]. Likewise, existing evidence has also revealed that 1,25$(OH)_2D_3$ modulates the activity of various immune cells [17]. Therefore, except for reducing cell growth and inducing apoptosis, 1, 25$(OH)_2D_3$ has also the potency to inhibit inflammation, which is considered to exert the anti-tumor activity as well [18]. For

instance, a recent literature has demonstrated that 1, $25(OH)_2D_3$ interrupts the activating of Wnt signaling and the accelerating of cell proliferation by macrophage-derived IL-1β in colon cancer cells [19]. Furthermore, $1,25(OH)_2D_3$ also decreases the production of pro-inflammatory cytokine IL-6 through inactivating the p38 stress-induced kinase, which is considered valuable for prostate cancer prevention [20]. Moreover, $1,25(OH)_2D_3$ can inhibit ConA-induced mouse hepatitis [21], and a poor vitamin D status is considered to aggravate NAFLD [22]. Consequently, $1,25(OH)_2D_3$ may have the potency to inhibit HCC development since both hepatitis and NAFLD are the major causes of HCC initiation. Finally, through up-regulating the cyclin dependent kinase inhibitor (CKI) $p27^{kip1}$, $1,25(OH)_2D_3$ inhibits the proliferation of many types of immune cells and thus reduces the production of inflammatory cytokines, such as IL-6 and TNF-α, which may also contribute to the prevention of tumor. [23].

Despite the multiple anti-tumor effects $1,25(OH)_2D_3$ exerts an extraordinary high prevalence of vitamin D deficiency has been reported in patients with chronic liver disease [24, 25]. Furthermore, a recent clinical study has indicated the inverse correlation of vitamin D levels and liver dysfunction in several liver diseases [26]. Vitamin D deficiency of chronic liver disease patients is a consequence of impaired vitamin D synthesis and absorption, or vitamin D deficiency facilitates the pathogenesis of chronic liver diseases via decreasing the anti-inflammatory and anti-infectious effects, the fact that whether vitamin D deficiency is associated with HCC initiation and progression and the underlying mechanisms are still ambiguous. $1,25(OH)_2D_3$ is a prohormone that can be metabolically converted from 25-hydroxyvitamin D_3 by the enzyme 1α-hydroxylase [*1α(OH)ase*] to the active form of the vitamin, 1,25-dihydroxyvitamin D_3 [27]. In order to investigate whether $1,25(OH)_2D_3$ deficiency accelerates while exogenous $1,25(OH)_2D_3$ supply inhibits HCC development *in vivo*, respectively, we use the carcinogen-induced or transplanted tumor bearing *1α(OH)ase* and $p27^{kip1}$ gene knockout (*KO*) mouse models, and validate the positive correlation between elevated inflammatory cytokines and enhanced HCC development.

MATERIALS AND METHODS

Cell Lines and Cell Culture

H22 (China Center for Type Culture Collection, Wuhan, China), and Hepa1–6 (from a C57BL mouse hepatoma; American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM (Q&K Bio-Chemical Engineering Limited Company, Shanghai) supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 units/ml streptomycin. For the experiments, 3×10^6 cells were plated in 75-cm^2 tissue culture flasks and grown in a humidified atmosphere (37°C, 5% CO_2). Treatments with different concentration (1 nM, 10 nM, 100 nM, 1000 nM) $1,25(OH)_2D_3$ (Sigma-Aldrich, St. Louis, MO) or the vehicle were initiated the day after plating and the effects were observed 48 hours later.

Analyses of Proliferation and Apoptosis on HCC Cells

For proliferation analysis, we incubated HCC cells with $1,25(OH)_2D_3$ for 48 hours and added BrdU (1μM) to the culture medium after 28 hours. For BrdU detection, the cells were

incubated with an anti-BrdU antibody (1:100) overnight at 4°C. Secondary anti-rabbit antibody conjugated with FITC was used to label BrdU and allowed for the detection of proliferating cells. For apoptosis analysis, the phosphatidylserine (PS) exposure in HCC cells was detected with an annexin V–FITC/PI apoptosis detection kit (Beckman Coulter). HCC cell apoptosis was subsequently analyzed by flow cytometry (Epics XL Coulter).

Animals

1α(OH)ase KO Babl/c mice (*1α(OH)ase*$^{-/-}$) were generated by homologous recombination in embryonic stem cells as previously described. We fed *1α(OH)ase*$^{-/-}$ mice with a high-calcium diet (TD96348 Teklad, Madison, WI, USA) containing 2% calcium, 1.25% phosphorus, and 20% lactose for 3 months after weaning to rescue the abnormities and hypocalcemia of *1α(OH)ase*$^{-/-}$ mice as previously described. *p27 KO* C57BL/J mice (*p27*$^{-/-}$), first generated and described by Dr. Fero, were purchased from Jackson Laboratory (The Jackson Laboratory, Bar Harbor, USA. *1α(OH)ase*$^{+/+}$, *p27*$^{+/+}$ and *p27*$^{-/-}$ mice fed with normal diet. Both *1α(OH)ase*$^{-/-}$ and *p27*$^{-/-}$ mice were used along with corresponding age-matched *WT* (*1α(OH)ase*$^{+/+}$ or *p27*$^{+/+}$ mice). The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Genotyping of Mice

Tail fragment genomic DNA was isolated by standard phenol–chloroform extraction and isopropanol precipitation. Mouse genotype was determined by PCR of tail DNA using the following primers: for p27 knockout allele (5′ primer: CTCTCTATCGCCTTCTTG, 3′ primer: TGGAACCCTGTGCCATCTCTAT); for p27 wild type allele (5′ primer: GATGGACGCCAGACAAGC, 3′ primer: ACGGGCTTA TGATTCTGAAAGTCG). For the wild-type *1α(OH)ase* allele, the forward primer (5′-AGACTGCACTCCACT CTGAG-3′) and reverse primer (5′-GTT TCC TAC ACG GAT GTC TC-3′) were used. The neomycin gene was detected with the primers neo-F (5′-ACA ACA GAC AAT CGG CTG CTC-3′) and neo-R (5′-CCA TGG GTC ACG ACG AGA TC-3′). All PCR reactions were performed with 1 cycle of 95°C for 4 minutes and 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

Chemical Hepatocarcinogenesis

Since the susceptibility to liver tumorigenesis in response to DEN in mouse is strain-dependent, and the pure C57BL/6 strain is highly resistant DEN for liver tumorigenesis, for the chemical tumorigenesis we used a DEN and PB two stage carcinogenesis protocol as described before (Sun *et al.*, 2008). Briefly, male mice and their corresponding wild-type littermates were injected intraperitoneally with 100 mg/kg DEN (Sigma, St. Louis, MO). Four weeks after DEN injection, the mice received 0.07% phenol barbital (PB) (Sigma) in drinking water and randomly assigned to the treatment (0.1 μg/kg 1,25-$(OH)_2D_3$ per day), (5 mg/kg NSC 74859) or control group (propylene glycol) until their sacrifice at 9 month of age. After sacrifice, livers of mice were removed, and the tumor number and sizes were measured. Serum IL-6, and TNF-α were measured by ELISA (eBioscience). The *p27*$^{-/-}$ mice were used in addition to corresponding age-matched *p27*$^{+/+}$ mice. The Institutional

Animal Care and Use Committee of Nanjing Medical University approved the use of animals in this study.

Co-Culture of Immune Cells and HCC Cells

The mouse hepatocellular carcinoma cell line H22 or Hepa1–6 were grown in RPMI-1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. Monocytes were isolated by negative selection using Dynal Monocyte Negative Isolation Kit (Invitrogen) according to the manufacturer's instructions. T cells were isolated with the MACS pan T cell Isolation kit II by negative selection in MACS LD depletion magnetic columns (MiltenyiBiotec, Bergisch Gladbach, Germany). All *in vitro* experiments were performed in Ultra Low Attachment Plates (Corning) to prevent monocytes & T lymphocytes activation by adhesion to the plastic plate. For monocytes & T lymphocytes culture, the immune cells were isolated and transferred to a vial of chilled RPMI-1640 medium (Invitrogen) and supplemented with 10% FBS, 5000 IU penicillin and streptomycin. Viable cells were identified by a trypan blue dye exclusion assay, counted using a hemocytometer and plated at 5×10^6 cells/well in a 24-well tissue culture plate. In co-culture experiments, freshly isolated splenocytes (5×10^6) were added to the inserts separated by 0.4μm membrane (Costar; Corning) from HCC cells.

Silencing of VDR by sh-RNA Plasmid

To silence the expression of VDR in H22 and Hepa1–6 cells, we used sh-RNA plasmid containing the sequence rat VDR, 5′-CCTGTCCCTTCAATGGAGATT-3′, or a scrambled plasmid as a negative control containing the sequence 5′-GGAATCTCATTCGATGCATAC-3′ (SA Bioscience, Frederick, Maryland, USA). HCCs at 80% confluence were transfected with 2 mg of sh-RNA plasmids using the Fugene 6 reagent (Roche Diagnostics) according to the manufacturer's instructions.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay

Cells (1×10^4) were seeded in 24-well microplates in complete culture medium in the absence or presence of 1,25-$(OH)_2D_3$ (100nM), anti-IL-6 antibody (10μm/ml) or NSC 74859 (100μM). After 72 h of culturing, the number of viable cells was measured by adding 100 μl/well of 2 mg/ml MTT solution. The medium was removed 2 h later and the formazan crystals were dissolved by adding 100 μl dimethyl-sulfoxide per well. The absorbance was read at 590 nm with an enzyme-linked immunosorbent assay reader. Each treatment point was performed with an n=6.

HCC Orthotopic Transplantation Model

To generate an orthotopic *in vivo* HCC model, *1α(OH)ase*$^{-/-}$, *p27*$^{-/-}$ and *WT* mice were used. After anesthetizing the mice, the liver was exposed following an upper middle incision, and approximately $1x10^6$ HCC cells (H22 or Hepa1–6) suspended in 30 μL of PBS were implanted into the left lobe of the liver using a microsyringe. Twenty-four hours after inoculation, the animals were randomly assigned to either the treatment (0.1 μg/kg 1,25-$(OH)_2D_3$ per day) or control group (propylene glycol). Using vernier calipers, the tumor diameters (mm) were measured at 14 days after injection. Tumor volumes were calculated

using the formula $1/6\Pi d^3$. For determining serum inflammatory factor levels, 0.5 ml of blood were taken from mice under general anesthesia at the end of the experiment by cardiac puncture. Serum IL-6 and TNF-α levels were measured by ELISA (Biomedical Technologies, Inc., Stoughton, MA, USA), following the manufacturer's instructions.

Immunohistochemical Staining and TUNEL Assay

Phosphorylated STAT3 and proliferating cell nuclear antigen (PCNA) were detected by immunohistochemistry using the avidin-biotin-peroxidase complex technique with a mouse anti-p-STAT3(Y705) monoclonal antibody (Biomedical Technologies, Inc., Stoughton, MA, USA) and a mouse anti-PCNA monoclonal antibody (Medicorp Inc., Montreal, Canada). The *in situ* cell death detection assay kit was purchased from Roche and performed according to the manufacturer's instructions. The percentage of apoptotic cells was calculated by counting the number of TUNEL-positive nuclei out of 500 total cells for each sample. At least three randomly chosen fields were assessed. The percentage of TUNEL-positive nuclei was determined in three independent experiments.

Statistical Analysis

Data from image analyses are presented as the mean ± SEM. Statistical comparisons were made using a two-way ANOVA, with $P < 0.05$ considered significant.

RESULTS

$1,25(OH)_2D_3$ Inhibits Cell Proliferation and Induces Apoptosis in HCC Cells

Prior to addressing the anti-tumor effects of $1,25(OH)_2D_3$ in HCC-derived allografts, we first investigated its anti-proliferative and apoptosis-inductive effects in the mouse cell lines H22 and Hepa1–6. Both cell lines were either normally cultured or treated with different concentration of $1,25(OH)_2D_3$ for 48 hours, after which cell growth status was investigated. Consistent with previous studies, cell growth in the $1,25(OH)_2D_3$-treated group was inhibited in both cell lines with a dose dependant manner. In addition, the vitamin D receptor (VDR) RNAi in both H22 and Hepa1–6 cells effectually suppressed the proliferation inhibitory effect of $1,25(OH)_2D_3$ (Fig. 1A). The colony formation experiment showed the dramatically proliferation inhibitory effect of $1,25(OH)_2D_3$ with the dosage 100 nM (Fig. 1B). Analysis of BrdU incorporation revealed that the ratio of BrdU-positive cells decreased in the $1,25(OH)_2D_3$-treated H22 and Hepa1–6 cells (Fig. 1C). Analyses of Annexin V and PI double-staining indicated that the ratio of apoptosis in $1,25(OH)_2D_3$-treated H22 and Hepa1–6 cells evidently increased in comparison with the normally cultured group (Fig. 1D).

1α(OH)ase Ablation Promotes Tumor Development, and Conversely, Supplying $1,25(OH)_2D_3$ Inhibits Tumor Development

To further investigate the anti-tumor effects of $1,25(OH)_2D_3$ *in vivo*, we performed an intervention survival study on both $1\alpha(ase)^{+/+}$ (n=6) and $1\alpha(ase)^{-/-}$ (n=6) male mice that were orthotopically transplanted with HCC and monitored tumor volume biweekly. After operation, the average survival time of $1\alpha(ase)^{-/-}$ mice was shorter and the average tumor volume was larger in comparison with $1\alpha(ase)^{+/+}$ mice. In contrast, the average survival

time was extended and the average tumor volume was shrunk in both tumor bearing *1α(ase)*$^{+/+}$ and *1α(ase)*$^{-/-}$ mice when supplied with 1,25$(OH)_2D_3$ (0.2 μg/kg per day). Of note, there was no difference in the survival and tumor volume between tumor bearing *1α(ase)*$^{+/+}$ and *1α(ase)*$^{-/-}$ mice whice were supplied with 1,25$(OH)_2D_3$ (Fig. 2A and B). We next investigated the cell proliferation inhibition and apoptosis induction effects of 1,25$(OH)_2D_3$ by PCNA immunohistochemistry and TUNEL staining in tumor slices. In *1α(ase)*$^{-/-}$ mice, the 1,25$(OH)_2D_3$ deficiency specifically increased proliferation and decreased apoptosis, relative to in *1α(ase)*$^{+/+}$ mice, whereas the 1,25$(OH)_2D_3$ supplementation significantly decreased the ratio of cell proliferation and increased the ratio of cell apoptosis in both *1α(ase)*$^{+/+}$ and *1α(ase)*$^{-/-}$ mice (Fig. 2C and D). Immunoblotting results showed that 1,25$(OH)_2D_3$ deficiency raise the cell-cycle-related protein (Cyclin D, Cyclin E, Cdk2 and Cdk4) and anti-apoptotic protein Bcl-2 expression, and decrease the pro-apoptotic protein Bad in the tumor bearing *1α(ase)*$^{-/-}$ mice. While the 1,25$(OH)_2D_3$ supplementation inhibited the affection of 1,25$(OH)_2D_3$ deficiency in these cell-cycle-related and apoptotic related protein expression (Fig. 2E).

1,25$(OH)_2D_3$ Deficiency Results in Enhanced Inflammatory Cytokine Production and STAT3 Signaling Activation in Orthotopic Transplantation Mice

To further understand whether the 1,25$(OH)_2D_3$ deficiency influences the inflammatory response and thus affects the tumor growth in *1α(ase)*$^{-/-}$ mice, we detected the serum inflammatory cytokines TNF-α and IL-6 levels in orthotopic transplantation *1α(ase)*$^{+/+}$ and *1α(ase)*$^{-/-}$ mice. There were no apparent differences of serum TNF-α and IL-6 in normal *1α(ase)*$^{+/+}$ and *1α(ase)*$^{-/-}$ mice (data not show). However, after orthotopic tumor transplantation, these two inflammatory cytokines' levels were much higher in *1α(ase)*$^{-/-}$ mice relative to their wild-type counterparts. While exogenous 1,25$(OH)_2D_3$ supplementation decreased these inflammatory cytokines' levels in both *1α(ase)*$^{-/-}$ and *1α(ase)*$^{+/+}$ mice (Fig. 3A). Since the tumor-promoting effect of IL-6 in HCC is mainly exerted via STAT3 signaling, we assessed whether STAT3 activation was affected by the ablation of 1α(ase) in mice following orthotopic tumor transplantation. Immunochemistry results showed that, p-STAT3-positive cells were obviously increased in *1α(ase)*$^{-/-}$ tumor but dramatically decreased in both *1α(ase)*$^{-/-}$ and *1α(ase)*$^{+/+}$ tumor slices after 1,25$(OH)_2D_3$ supply. Meanwhile, immunoblotting results of STAT3 and p-STAT3 validated our observation above (Fig. 3B and C).

Since it was well documented that the vitamin D receptor (VDR) is existed in almost all immune cells, in order to understand whether 1,25$(OH)_2D_3$ will inhibit tumor growth via its anti-inflammation effect and elucidate the possible mechanism, we silenced VDR gene in tumor cell with VDR shRNA to rule out the direct anti-proliferation effect of vitamin D. After VDR gene silencing, 1,25$(OH)_2D_3$ treatment didn't affect the cell proliferation (Fig. 1A). Then we conducted the immune cell and VDR gene silenced tumor cell co-culture experiment using transwell chambers and treated with either vitamin D or STAT3 signaling pathway inhibitors. Results showed that the tumor cell growth was evidently increased when co-cultured with monocytes or lymphocytes from wild-type Babl/c mice. Intriguingly, although VDR gene was silenced in these tumor cells (Babl/c strain derived), 1,25$(OH)_2D_3$ treatment can also inhibit tumor cell growth in the co-cultures. Furthermore, IL-6 antibody

or STAT3 signaling inhibitor NSC74589 can also inhibit tumor cell growth when co-cultured with monocytes or lymphocytes (Fig. 4A and B). In order to demonstrate this effect of $1,25(OH)_2D_3$ *in vivo*, we then transplanted the VDR gene silenced tumor cell to *1α(ase)*$^{-/-}$ mice and treated with either $1,25(OH)_2D_3$ or NSC74589. Consistent with the *in vitro* results, both $1,25(OH)_2D_3$ and NSC74589 inhibited the transplanted tumor growth (Fig. 4C). Considering the well established cell cycle arrest effect of vitamin D, finally we detected cell cycle inhibitor p21, p27 and p53 in monocytes or lymphocytes treated with or without vitamin D. Results showed that after vitamin D treatment, only p27 protein was increased significantly in both monocytes and lymphocytes (Fig. 4D). These findings implicated that the elevated inflammatory cytokines of $1,25(OH)_2D_3$ deficient mice facilitates the progression of orthotopic transplanted HCC via activation of the STAT3 signaling pathway.

Lack of $p27^{kip1}$ Suppresses the Anti-Tumor Effect of $1,25(OH)_2D_3$

Since previous studies have demonstrated that $p27^{kip1}$ deficiency promotes carcinogen induced HCC initiation and progression in mice, and $p27^{kip1}$ gene knock-out mice spontaneously developed thymic hyperplasia with an increased T lymphocyte population. To decipher whether $p27^{kip1}$ deficiency suppresses the anti-inflammation and anti-tumor effects of $1,25(OH)_2D_3$, we examined the tumor development, serum inflammatory cytokines and vitamin D level in carcinogen induced *p27*$^{+/+}$ or *p27*$^{-/-}$ mice, supplemented with or without $1,25(OH)_2D_3$. Results showed that in consistent with previous studies, the tumor number and tumor volume were increased in *p27*$^{-/-}$ mice after 9 months chemical tumorigenesis. However, $1,25(OH)_2D_3$ treatment inhibited the tumor development in *p27*$^{+/+}$ mice, but not in *p27*$^{-/-}$ mice (Fig. 5A and B). Likewise, serum IL-6 level was increased *p27*$^{-/-}$ mice after chemical tumorgenesis, and $1,25(OH)_2D_3$ only decreased serum IL-6 in *p27*$^{+/+}$ mice (Fig. 5C). In addition, although serum $1,25(OH)_2D_3$ level was decreased in both *p27*$^{+/+}$ and *p27*$^{-/-}$ mice followed chemical tumorigenesis, the $1,25(OH)_2D_3$ level was much lower in *p27*$^{-/-}$ mice than that of in *p27*$^{+/+}$ mice (Fig. 5D). Then we used the VDR silenced tumor cell Hepa1–6 (C57BL strain derived) co-cultured with immune cell either from *p27*$^{+/+}$ and *p27*$^{-/-}$ mice. Results showed that the proliferation of tumor cell was increased when co-cultured with *p27*$^{-/-}$ mice derived monocytes or lymphocytes, and IL-6 antibody and STAT3 signaling inhibitor NSC74589 inhibited the tumor cell proliferation when co-cultured with both *p27*$^{+/+}$ and *p27*$^{-/-}$ mice derived immune cell, while $1,25(OH)_2D_3$ only suppressed the tumor cell proliferation when co-cultured with *p27*$^{+/+}$ derived immune cell (Fig. 5E and F).

Finally, we transplanted these VDR silenced Hepa1–6 cells to *p27*$^{+/+}$ and *p27*$^{-/-}$ mice (C56BL/J strain) and examined the tumor growth and the serum inflammatory cytokine, as well as the activation of STAT3 signaling. In consistent with prior results, the tumor growth, serum inflammatory cytokines, tumor cell proliferation, cell cycle related protein, and phosphorylation of STAT3 were increased while apoptosis was decreased in *p27*$^{-/-}$ orthotopic transplanted tumors. Nevertheless, $1,25(OH)_2D_3$ supplement only effected these elements above in *p27*$^{+/+}$ orthotopic transplanted tumors (Fig. 6A–E).

DISCUSSION

Nowadays, epidemiological studies indicate that reduced concentration of serum vitamin D is associated with an increased risk of many types of cancers [14, 15]. Although previous studies have indicated that $1,25(OH)_2D_3$ inhibits the proliferation of human liver cancer cell line HepG2 in a dose dependent manner [28], the usage of nude mice and lack of a $1,25(OH)_2D_3$ deficient animal model limits these research into the detailed mechanisms behind these observations. Since the *1α(ase)*$^{-/-}$ mice has a significantly reduced survival time thus not available for chemical hepatocarcinogenesis, in this study, we conduct the HCC homotransplantation to *1α(ase) KO* mice and their *WT* littermates. Despite the *1α(ase)*$^{-/-}$ mice develop hypocalcemia, hypophosphatemia, retarded growth, and skeletal abnormalities characteristic of rickets, when fed with a high calcium after weaning, the serum calcium and phosphorus levels, growth, development, and skeletal phenotype are normalized [29]. Therefore, the usage of the *1α(OH)ase KO* tumor bearing mouse enable us the first to demonstrate that the lack of $1,25(OH)_2D_3$ accelerates HCC development *in vivo*.

Rapidly growing evidence reinforces the notion that excessively and chronically produced pro-inflammatory cytokines contribute to HCC initiation and progression [30]. The excessive production of pro-inflammatory cytokine IL-6 and TNF-α, generated by macrophages and lymphocytes in the inflammatory tumor microenvironment, lead tumor cells to exert anti-apoptotic and pro-angiogenic effects [31]. Meanwhile, clinical data has indicated that the inflammatory conditions of liver correlates with high circulating IL-6 levels, which is further elevated in patients who develop HCC [32]. In addition, a recent literature has demonstrated that the enhanced tumor formation and progression in carcinogen-induced obesity mice is attribute to the elevated tumor-promoting cytokines IL-6 and TNF-3, which further accelerates the proliferation of tumor cell through activating STAT3 signaling [33].

Considerable evidence suggests that the capability to inhibit inflammatory response makes $1,25(OH)_2D_3$ has the potency to exert the anti-tumor activity as well [18]. For example, a resent literature has demonstrated that $1,25(OH)_2D_3$ interrupts the activating of Wnt signaling and the accelerating of cell proliferation by macrophage-derived IL-1β in colon cancer cells [19]. Furthermore, $1,25(OH)_2D_3$ also decreases the production of pro-inflammatory cytokine IL-6 through inactivating the p38 stress-induced kinase, which is considered valuable for prostate cancer prevention [20]. Moreover, $1,25(OH)_2D_3$ can inhibit ConA-induced mouse hepatitis [21], and a poor vitamin D status is considered to aggravate NAFLD [22]. Consequently, $1,25(OH)_2D_3$ has the potency to inhibit HCC development since both hepatitis and NAFLD are the major causes of HCC initiation. Paralleled with those findings above, our results show that the lack of $1,25(OH)_2D_3$ in *1α(OH)ase* ablation mice promotes tumor growth and serum inflammatory cytokines secretion, and conversely, $1,25(OH)_2D_3$ supplement diminishes the secretion of serum inflammatory cytokine and the tumor growth concurrently. In addition, the growth inhibitory effect of $1,25(OH)_2D_3$ in the VDR gene silenced tumor cells expels the direct cell cycle inhibitory effect of $1,25(OH)_2D_3$, further indicating the positive correlation between elevated inflammatory cytokines and enhanced tumor development. The elevated production of inflammatory cytokine IL-6 and sequential activation of IL-6/STAT3 signaling will affect the target gene and thus enhancing

cell cycle progression which is considered to be an important factor for HCC progression. And also, the elevated TNF-α can also promote HCC progression through inducing the expression of genes encoding NF-κB–dependent anti-apoptotic molecules, such as Bcl-2.

It has been generally accepted that an inflammatory microenvironment comprehending many innate immune cells and a variety of mediators such as cytokines and chemokines, will persistently activate the proliferation of the surrounding stroma cells such as hepatic stellate cells (HSCs) thus cause the tissue remodeling and liver fibrosis, and eventually predisposes chronic inflammation to neoplasia. Although a recent study has demonstrated that treatment with $1,25(OH)_2D_3$ significantly reduces extracellular matrix deposition and lowers the fibrotic score in TAA-induced liver fibrosis through inhibiting the proliferation of HSCs [34]. On the other hand, recent studies have also demonstrated the elevated inflammatory cytokine and consequently activated STAT3 signaling in monocytes can also accelerate liver cancer progression [13]. Meanwhile, clinical data have validated that some chronic liver disease patients developed HCC are without liver fibrosis. Therefore, our findings further indicate that the direct tumor-promoting effect of inflammation in HCC initiation and progression.

Among the various types of cell-cycle regulators, the $p27^{Kip1}$ protein is initially identified due to its ability to bind and inhibit cyclin/cdk2 complexes, thus leading to an arrest in the G1-phase of the cell cycle [35]. Previous studies have documented that loss of $p27^{Kip1}$ enhances tumor progression in chronic hepatocyte injury-induced liver tumorigenesis and promotes carcinogen-induced mouse liver tumorigenesis [36, 37]. Furthermore, our recent studies have indicated that $p27^{kip1}$ inactivation promotes DEN-PB induced liver hepatocarcinogenesis through enhancing inflammatory cytokine IL-6 and TNF-α secretion and STAT3 signaling activation *in vivo*. And we also demonstrate that p27 gene silencing in splenocytes will promote the proliferation of the co-cultured tumor cells [38]. Moreover, previous report has demonstrated that $1,25(OH)_2D_3$ inhibits cell proliferation in many types of immune cells and subsequently diminishes the production of inflammatory cytokines through up-regulating $p27^{kip1}$ [23]. In this study, we found that $1,25(OH)_2D_3$ supplement only inhibits tumor development and decreases inflammatory cytokine level in $p27^{kip1}$ *WT* mice, indicating the anti-inflammation effect of $1,25(OH)_2D_3$ exerts in a $p27^{kip1}$ dependent way.

CONCLUSION

Although $1,25(OH)_2D_3$ has the potency to inhibit inflammatory response in several liver diseases, the fact that whether $1,25(OH)_2D_3$ can inhibit HCC initiation and progression is still unclear. Our current study provides a novel finding that through up-regulating the cyclin dependent kinase inhibitor $p27^{kip1}$, $1,25(OH)_2D_3$ reduces the secretion of inflammatory cytokines and consequently inhibits the activation of STAT3 signaling, which eventually suppresses HCC development in tumor bearing mice. First, *1α(OH)ase* ablation promotes tumor development and enhances inflammatory response, and conversely, supplying $1,25(OH)_2D_3$ inhibits tumor development and diminishes inflammatory response. Second, the elevated serum inflammatory cytokine IL-6 and activated STAT3 signaling exists in the tumor bearing $1\alpha(OH)ase^{-/-}$ mice, while $1,25(OH)_2D_3$ supplement decreases serum IL-6

concentration and inactivates STAT3 signaling. Third, $1,25(OH)_2D_3$ and STAT3 signaling inhibitor NSC 74859 restrain the VDR gene silenced tumor cell proliferation through suppressing the inflammatory response and diminishing the secretion of inflammatory cytokines from immunocytes *in vitro* and *in vivo*. And finally, $1,25(OH)_2D_3$ can not inhibit tumor initiation and progression in $p27^{kip1}$ gene *KO* mice. In aggregate, although the precise mechanism of $1,25(OH)_2D_3$ in regulating inflammation remains ambiguous, further studies on the anti-tumor effects of $1,25(OH)_2D_3$ during hepatocarcinogenesis will provide better insights into the relationship between inflammation and cancer. In addition, the decreased vitamin D level in patients with chronic liver disease might be a consequence of impaired vitamin D synthesis and absorption, which in turn facilitates HCC initiation and progression. Concurrently, the use of $1,25(OH)_2D_3$ may provide us an effective preventive or therapeutic strategies for HCC.

Acknowledgments

This study was supported by a grant from National Natural Science Foundation of China (No.81301981), The New Drug Target Discovery Project (Grant No. 08143073) of the First Affiliated Hospital, Xi'an Jiaotong University, China. The Fundamental Research Funds for the Central Universities (08143014) and project grants from the NCRR (P20 RR020151) and the NIGMS (P20 GM103505 and P30 GM103332-01) from the NIH. The contents of this report are solely the responsibility of the authors and do not necessarily reflect the official views of the Xi'an Jiaotong University, NSFC, the NIH, National Center for Research Resources (NCRR), or National Institute of General Medical Sciences (NIGMS).

ABBREVIATIONS

HCC	Hepatocellular carcinoma
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D3
NAFLD	Non-alcoholic fatty liver disease
KO	Knockout
CDK	Cyclin-dependent kinase
TNF-α	Tumor necrosis factor-α
IL-6	Interleukin-6
DEN	Diethylnitrosamine
1α(OH)ase	1α-hydroxylase
BrdU	5-bromo-2′-deoxy-uridine
PB	Phenobarbital

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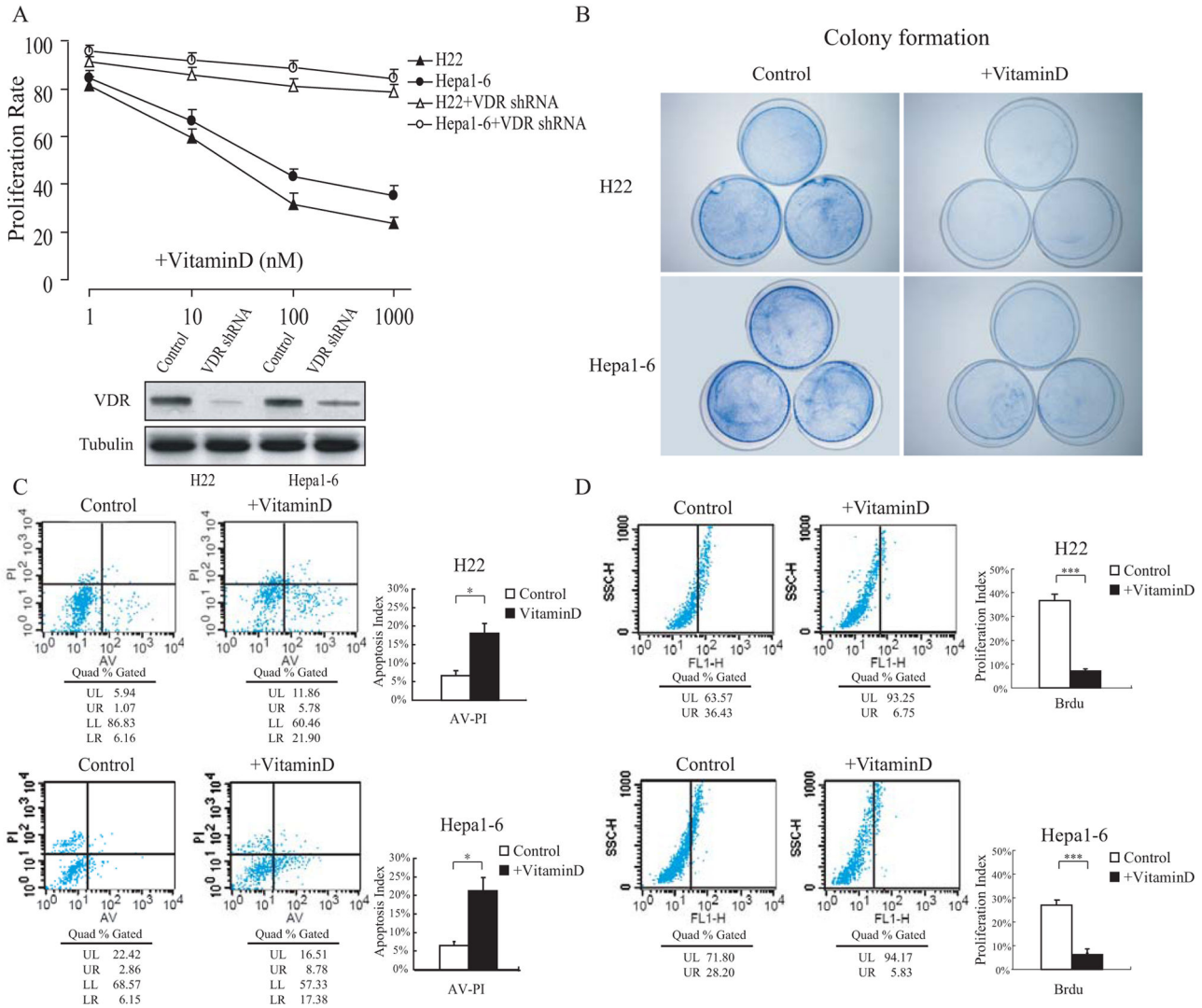

Fig. (1). 1,25(OH)$_2$D$_3$ inhibits cell proliferation and induces apoptosis in HCC cells

The mouse-derived HCC cell lines H22 (Babl/c background) and Hepa1–6 (C57BL background) were different concentrations of 1,25(OH)$_2$D$_3$ the day after plating, and the effects were observed 48 hours later. (**B**) Colony formation was performed to assess cell proliferation *in vitro*. (**C**) H22 and Hepa1–6 cells were incorporated with 1μM BrdU to the culture medium 28 hours after 1,25(OH)$_2$D$_3$ or vehicle treatment, and cell proliferation analysis was performed by FACS. (**D**) Apoptosis analysis by Annexin V and PI double staining was performed by FACS. The results are representative of at least 3 independent experiments. *$p < 0.05$, ***$p < 0.001$.

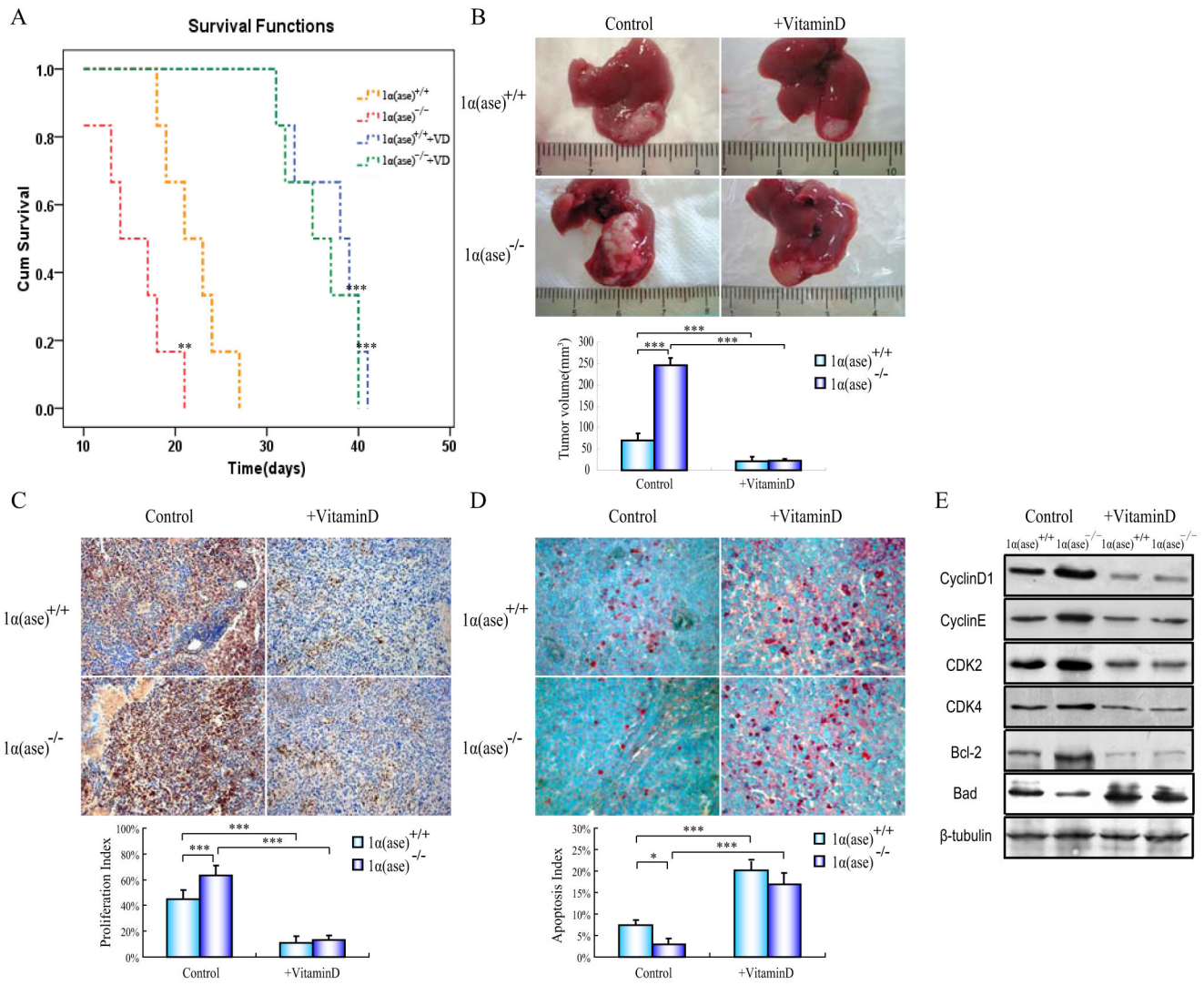

Fig. (2). *1α(OH)ase* ablation promotes tumor development in allograft transplanted transplanted mice

Babl/c background derived H22 cells ($1x10^6$) were implanted to the $1\alpha(ase)^{+/+}$ or $1\alpha(ase)^{-/-}$ mice, and twenty-four hours after inoculation, the animals were randomly assigned to either the treatment intraperitoneal injection 0.1 μg/kg 1,25-$(OH)_2D_3$ or propylene glycol (control group) per day. (**A**) Overall survival time analysis of HCC transplanted $1\alpha(ase)^{+/+}$ and $1\alpha(ase)^{-/-}$ mice. After H22 cell inoculation and 1,25-$(OH)_2D_3$ treatment, each mouse's survival time was documented and made up to the survival curve. Compared with the PBS-injected $1\alpha(OH)ase^{+/+}$ group (yellow dotted line): **p<0.01, ***p<0.001 (**B**) Gross appearances of representative livers with transplanted tumors in either normal or 1,25$(OH)_2D_3$-treated $1\alpha(ase)^{+/+}$ and $1\alpha(ase)^{-/-}$ mice (n=6). (**C**) PCNA immunohistochemistry staining of representative tumor slices from either normal or 1,25$(OH)_2D_3$-treated $1\alpha(ase)^{+/+}$ and $1\alpha(ase)^{-/-}$ mice (magnification 200x). (**D**) TUNEL staining of representative tumor slices from either 1,25$(OH)_2D_3$-untreated or -treated $1\alpha(ase)^{+/+}$ and $1\alpha(ase)^{-/-}$ mice (magnification 400x). (**E**) Immunoblotting results of cell

cycle and apoptosis-related proteins in transplanted tumors from either $1,25(OH)_2D_3$-untreated or -treated $1\alpha(ase)^{+/+}$ and $1\alpha(ase)^{-/-}$ mice. The results are representative of at least 3 independent samples. *p<0.05,***p<0.001.

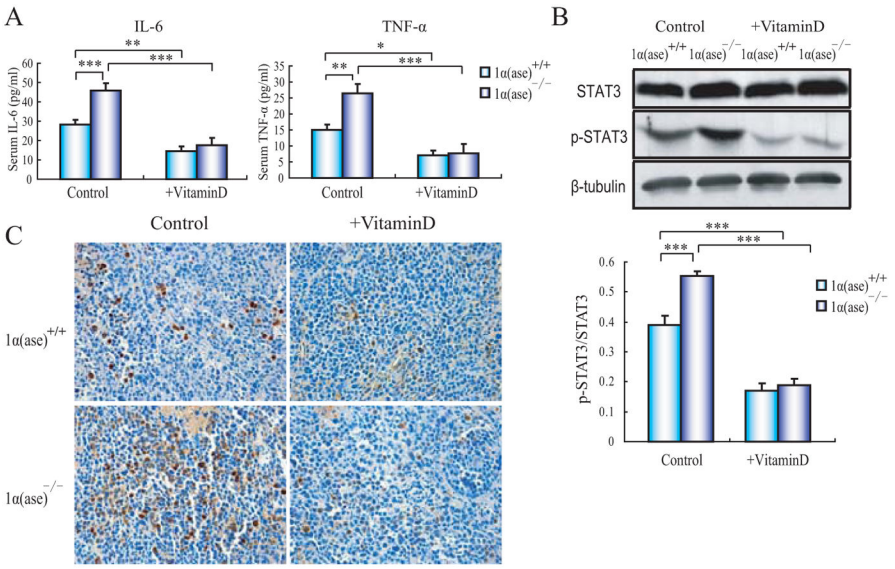

Fig. (3). $1,25(OH)_2D_3$ deficiency promotes enhanced inflammatory cytokine production and STAT3 phosphorylation

(**A**) Serum biochemistry results for IL-6 and TNF-α levels in either tumor-transplanted normal or $1,25(OH)_2D_3$-treated *1α(ase)*$^{+/+}$ and *1α(ase)*$^{-/-}$ mice. (**B**) Immunoblotting results of STAT3 phosphorylation levels in transplanted tumors in either normal or $1,25(OH)_2D_3$-treated *1α(ase)*$^{+/+}$ and *1α(ase)*$^{-/-}$ mice. (**C**) p-STAT3 immunohistochemistry staining results of representative tumor slices in either normal or *1α(ase)*$^{+/+}$ and *1α(ase)*$^{-/-}$ mice treated with or without $1,25(OH)_2D_3$ (magnification x400). The results are representative of at least 3 independent samples. *p<0.05; **p<0.01; ***p<0.001.

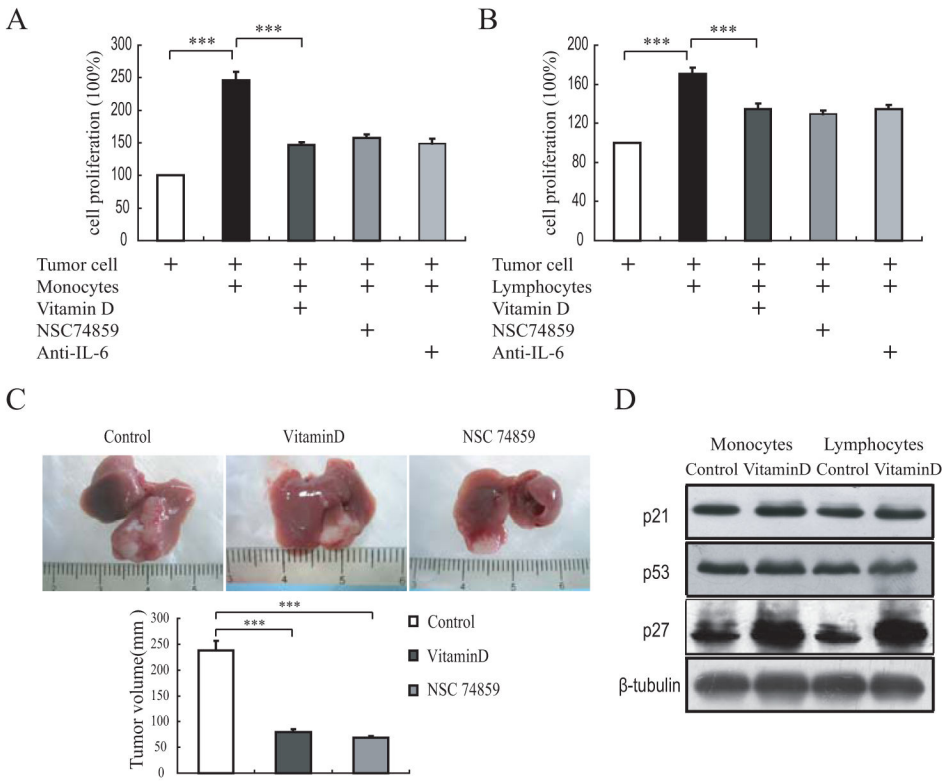

Fig. (4). $1,25(OH)_2D_3$ treatment inhibits VDR gene silenced tumor cell proliferation through IL-6-STAT3 signaling pathway during which co-cultured with immune cell or transplanted to *1α(ase)*$^{-/-}$ mice

The proliferation of H22 cell alone or after co-cultured with (**A**) monocytes or (**B**) lymphocytes, and treated with $1,25(OH)_2D_3$, IL-6 anti-body or NSC 74859, respectively. (**C**) Gross appearances of representative livers with VDR gene silenced tumor cell transplantation in vehicle, NSC74589 or $1,25(OH)_2D_3$ treated *1α(ase)*$^{-/-}$ mice (n=6). (**D**) Immunoblotting results of p21, p27 and p53 protein levels of monocytes or lymphocytes from *WT* mice when co-cultured with tumor cells and treated with or without $1,25(OH)_2D_3$. The results are representative of at least 3 independent samples. *p<0.05; **p<0.01; ***p<0.001.

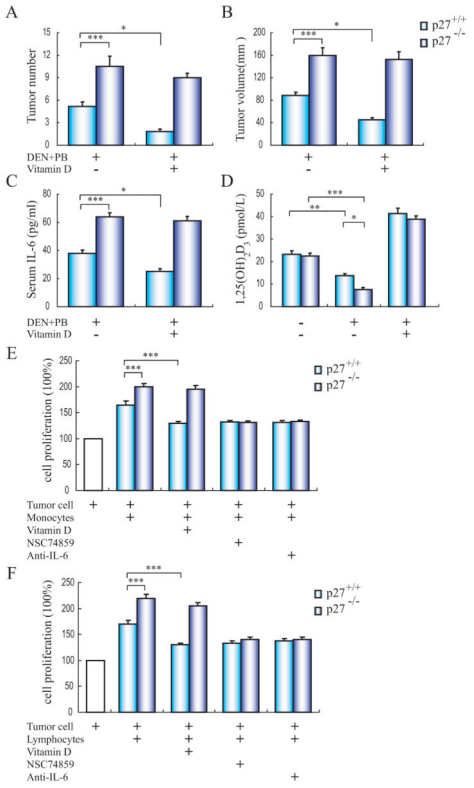

Fig. (5). $1,25(OH)_2D_3$ treatment inhibits chemical hepatocarcinogenesis through decreasing inflammatory cytokine IL-6 and through a *p27*^{kip1} gene dependent way

Male *p27*$^{-/-}$ mice (n=8) and their corresponding wild-type *p27*$^{+/+}$ littermates (n=8) at postnatal day 15 were treated with DEN+PB for six months, after which the $1,25(OH)_2D_3$ or vehicle were treated every other day after. (**A**) The number and (**B**) size of tumors on the liver surface were counted. (**C**) Serum biochemistry results for IL-6 and (**D**) $1,25(OH)_2D_3$ levels in *p27*$^{+/+}$ or *p27*$^{-/-}$ mice with vehicle or $1,25(OH)_2D_3$ for nine months. (**E**) The proliferation of VDR gene silenced Hepa1–6 cell alone or after co-cultured with monocytes or (**F**) lymphocytes from *p27*$^{+/+}$ or *p27*$^{-/-}$ mice, and treated with $1,25(OH)_2D_3$, IL-6 antibody or NSC 74859, respectively. The results are representative of at least 3 independent samples. *p<0.05; **p<0.01; ***p<0.001.

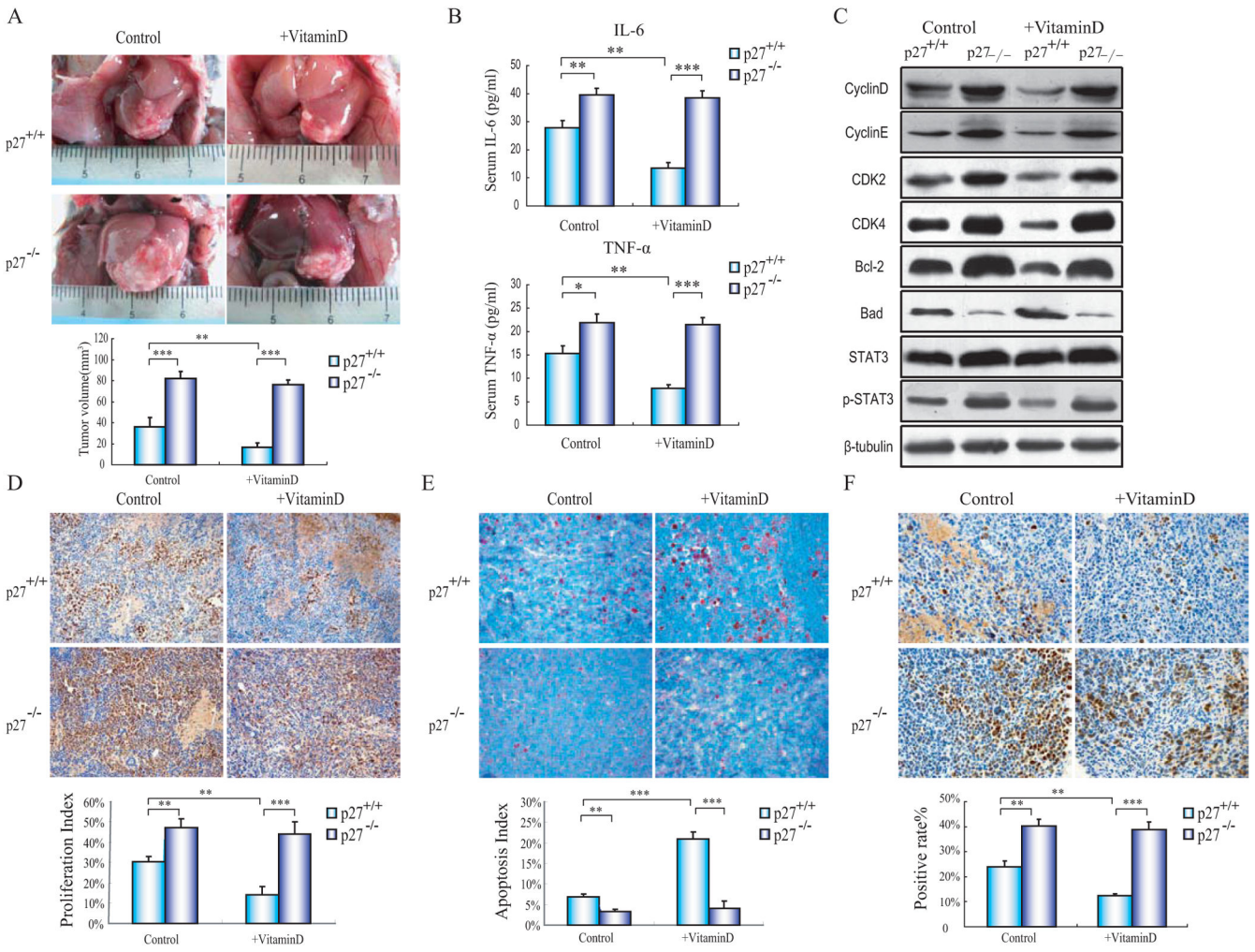

Fig. (6).

$1,25(OH)_2D_3$ inhibits VDR gene silenced tumor cell growth in wild-type mice, but not in $p27^{kip1}$ gene knock-out mice. C57BL background derived Hepa1–6 cells were pre-treated with VDR shRNA and implanted to the $p27^{+/+}$ or $p27^{-/-}$ mice, addition with $1,25(OH)_2D_3$ or PBS intraperitoneal injection every day. (**A**) Gross appearances of representative livers with transplanted tumors in either normal or $1,25(OH)_2D_3$-treated $p27^{+/+}$ and $p27^{-/-}$ mice (n=6). (**B**) Serum IL-6 and TNF-α level. (**C**) Immunoblotting results of cell cycle, apoptosis-related, and STAT3 phosphorylation proteins in transplanted tumors from either $1,25(OH)_2D_3$-untreated or -treated $p27^{+/+}$ and $p27^{-/-}$ mice. (**D**) PCNA immunohistochemistry of representative tumor slices (magnification 200x). (**E**) TUNEL staining of representative tumor slices (magnification 400x). (F) p-STAT3 immunohistochemistry of representative tumor slices from either vehicle or $1,25(OH)_2D_3$-treated $p27^{+/+}$ and $p27^{-/-}$ mice (magnification 400x). The results are representative of at least 3 independent samples. *p<0.05, **p<0.005, ***p<0.001.