Notch1 Signaling Inhibits Growth of Human Hepatocellular Carcinoma through Induction of Cell Cycle Arrest and Apoptosis

Runzi Qi,1 Huazhang An,1 Yizhi Yu,1 Minghui Zhang,1 Shuxun Liu,1 Hongmei Xu,1 Zhenghong Guo,1 Tao Cheng,2 and Xuetao Cao1

1Institute of Immunology, Second Military Medical University, Shanghai, People’s Republic of China, and 2Stern Cell Biology, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

ABSTRACT
Notch signaling plays a critical role in maintaining the balance between cell proliferation, differentiation, and apoptosis; hence, perturbed Notch signaling may contribute to tumorigenesis. Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in Africa and Asia. The mechanisms that orchestrate the multiple oncogenic insults required for initiation and progression of HCC are not clear. We constitutively overexpressed active Notch1 in human HCC to explore the effects of Notch1 signaling on HCC cell growth and to investigate the underlying molecular mechanisms. We show here that overexpression of Notch1 was able to inhibit the growth of HCC cells in vitro and in vivo. Biochemical analysis revealed the involvement of cell cycle regulated proteins in Notch1-mediated G1/G0 arrest of HCC cells. Compared with green fluorescent protein (GFP) control, transient transfection of Notch1 ICN decreased expression of cyclin A (3.5-fold), cyclin D1 (2-fold), cyclin E (4.5-fold), CDK2 (2.8-fold), and the phosphorylated form of retinoblastoma protein (3-fold). Up-regulation of p21WAF1/CIP1 protein expression was observed in SMMC7721-ICN cells stably expressing active Notch1 but not in SMMC7721-GFP cells, which only express GFP. Furthermore, a 12-fold increase in p53 expression and an increase (4.8-fold) in Jun-NH2-terminal kinase activation were induced in SMMC7721-ICN cells compared with SMMC7721-GFP cells. In contrast, expression of the antiapoptotic Bel-2 protein could not be detected in SMMC7721-ICN cells. These findings suggest that Notch1 signaling may participate in the development of HCC cells, affecting multiple pathways that control both cell proliferation and apoptosis.

INTRODUCTION
Since originally discovered in Drosophila as critical regulatory proteins in embryonic development, Notch genes have been found genetically conserved in many species including humans. Notch genes encode highly conserved type I transmembrane glycoproteins, which can be activated via direct interaction with transmembrane ligands expressed on the surface of neighboring cells (1, 2). Upon activation, Notch is cleaved, releasing an ICN,3 which then translocates into the nucleus. The ICN associates with transcriptional factors known as Su(H)/CBF1, regulating the expression of target genes, and successively modulating the development and growth of cells (3, 4). Constitutive expression of active ICN in targeted cells results in an “activated” Notch phenotype (5, 6).

Notch signaling is involved in a variety of cell specification, proliferation, and apoptosis that affect the development and function of many organs (7, 8). For example, in hematopoietic system, Notch is involved in T cell commitment and B cell development (9). Overexpression of Notch1 has been demonstrated to promote the self-renewal of hematopoietic stem cells in vivo and in vitro (10, 11). Pathophysiological alterations in Notch signaling have been associated with tumorigenesis. In human acute T-lymphoblastic leukemia and lymphomas, Notch1 transcripts encode a series of truncated Notch1 polypeptides containing at least the cytosolic domain of Notch1 (1). Similar truncations, caused by insertion of wild-type mouse mammary tumor virus within the Notch1 and Notch4 genes, are associated with mammary tumors (3, 12). These observations suggest that dysfunction of intracellular Notch prevents differentiation and predisposes differentiated cells to malignant transformation (13). On the other hand, constitutive activation of Notch1 signaling can cause a profound growth arrest in small cell lung cancer cells, associated with a G1 cell cycle block (14). Overexpression of active Notch1 inhibited the proliferation of various prostate cancer cells (15), suggesting that Notch activation can also induce growth arrest and apparently reduce the neoplastic potential of tumors.

HCC accounts for 80–90% of liver cancers and is one of the most prevalent carcinomas throughout the world. Many efforts have been made to understand the molecular mechanisms that underlie the cellular malignancy phenotype, and that are involved in the process of cancer invasion and metastasis. Investigations into the roles of apoptosis related factors (p53, Fas, and Fas ligand; Ref. 16), cell cycle regulators (cyclin A, cyclin D, cyclin E, cdc2, p27, and p73), as well as oncopgenes (ras, HGF, and c-myc) and their receptors (erb-B and c-met receptor family members; Refs. 17, 18) have been conducted. However, the mechanisms underlying the disruption of these critical pathways in the tumorigenesis of HCC are still not fully elucidated. There is compelling evidence that the Notch signaling pathway may play an important role in liver disease. Expression and localization of Notch receptors and their ligands have been observed in the adult human liver tissue and altered during liver pathogenesis (19, 20). Notch signaling pathway may be important both for normal bile duct formation and the abnormal neovascularization (19). These studies prompted us to additionally investigate the potential roles of Notch signaling in the development of HCC.

Up to now, there have been no reports on the relationship between Notch1 signaling and HCC. In the present study, we have demonstrated that Notch1 signaling can significantly inhibit the in vitro and in vivo growth of the HCC cell line SMMC7721. Notch1 signaling-induced growth suppression is at least partially due to G1/G0 cell cycle arrest. Consistent with cell cycle arrest, expression of cyclin A1, cyclin D1, cyclin E, CDK2, and phosphorylation of Rb protein decreased, whereas p21 expression increased. Notch1 signaling also induced apoptosis of SMMC7721 cells. Up-regulation of p53 expression, down-regulation of Bel-2, and activation of the stress-activated protein kinase/JNK pathway were observed, and may be related to Notch1 signaling-induced apoptosis. Therefore, Notch1 signaling can inhibit HCC growth through induction of cell cycle arrest and apoptosis.

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Requests for reprints: Xuetao Cao, Institute of Immunology, Second Military Medical University, 800 Xiangyang Road, Shanghai 200433, People’s Republic of China. Phone: 86-21-2507-0316; Fax: 86-21-6538-2502; E-mail: caoxut@public3.sta.net.cn.

The abbreviations used are: ICN, intracellular domain of Notch1; HCC, hepatocellular carcinoma; CDK, cyclin-dependent kinase; Rb, retinoblastoma; GFP, green fluorescent protein; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase.
MATERIALS AND METHODS

Cell Culture and Retroviral Infection. Human HCC cells SMMC7721 were maintained in RPMI 1640 (21) with 10% FBS (HyClone, Logan, UT). 293T cells (American Type Culture Collection) were grown in DMEM containing 10% FBS. Retroviral vector, MSCV-ICN/GFP (Ref. 22; ICN, cDNA encoding a constitutively active form of Notch1 consisting of the intracellular domain, bp 5308–7665), MSCV-GFP, pkan, encoding a retroviral packaging plasmid (23), and pCMV-BSV-G (24), encoding the vesicular stomatitis virus G-glycoprotein, were kindly provided by Dr. David T. Scadden (Partners AIDS Research Center and MGH Cancer Center, Boston, MA). To generate pseudotyped virus, pkan, pCMV-BSV-G, and MSCV-GFP vectors were cotransfected into subconfluent 293T cells using the calcium phosphate precipitation method (24, 25). The virus stocks were collected at 48 and 72 h after transfection, filtered through a 0.45 μm filter, and then frozen at −80°C. Human HCC SMMC7721 cells were infected with retrovirus stocks containing 8 μg/ml Polybrene (Sigma, St. Louis, MO) for 6–8 h, washed, and cultured in fresh complete medium. A second and a third infection were conducted on subsequent days, after an identical procedure (22).

Stable cell lines overexpressing ICN and/or GFP were generated from SMMC7721 cells infected with recombinant retrovirus, selected by limited dilution, and designated as SMMC7721-ICN and SMMC7721-GFP cells, respectively. SMMC7721-ICN subclones were initially generated in RPMI 1640 containing 15% FBS and then maintained in RPMI 1640 with 10% FBS for after experiments.

Analysis of Cell Growth in Vitro. The in vitro growth rate of SMMC7721 cells expressing Notch1 (ICN) either transiently, or stably, was measured using the MTT method (26). Briefly, cells were seeded into 96-well plates at 2000 or 1000 cells per well for SMMC7721 cells transiently or stably expressing Notch1 (ICN), respectively. On the day of harvest, 100 μl of spent medium was replaced with an equal volume of fresh medium containing 10% MTT 5 mg/ml stock. Plates were incubated at 37°C for 4 h, then 100 μl of DMSO (Sigma) was added to each well and plates shaken at room temperature for 10 min. The absorbance was measured at 570 nm. The proliferation of SMMC7721 cells stably expressing Notch1 (ICN) was measured by [3H]-thymidine incorporation assay (15). SMMC7721-ICN, SMMC7721-GFP, and parental SMMC7721 cells (4 × 10³/well) were seeded into 96-well plates and cultured for 96 h. For the final 18 h, each well was supplemented with 0.5 μCi of [3H]thymidine (Amersham Pharmacia Biotech). The cells were harvested onto glass fibers using a multiple cell harvester, and the proliferation of SMMC7721 cells was detected by [3H]thymidine incorporation using a liquid Scintillation Counter (Wallac).

Colony-Forming Assay. SMMC7721-ICN, SMMC7721-GFP, or parental SMMC7721 cells (5 × 10³/0.1 ml) were mixed with 1 ml MethoCult methylcellulose-based medium (StemCell, Vancouver, British Columbia, Canada) and plated in six-well plates according to the manufacturer’s instructions. After 7–10 days of incubation at 37°C in a humidified atmosphere containing 5% CO₂ in air, colonies (>50 cells) were counted using an inverted microscope.

Tumorigenicity of SMMC7721 Cells Constitutively Overexpressing Active Notch1. Female athymic nude mice, 5–6 weeks of age, were obtained from SIPPR-BK Experimental Animal Co. (Shanghai, China) and housed in a pathogen-free facility for all of the experiments. One × 10⁶ SMMC7721-ICN, SMMC7721-GFP, or parental SMMC7721 cells were s.c. injected into the nude male nude mice. Tumor growth was measured using caliper every 3 days, and the tumor volumes were calculated according to the formula: volume = length × (width)²/2 (27).

Primary HCC-Bearing Mice and Intratumoral Gene Transfer of Active Notch1. Nude mice were s.c. inoculated with 1 × 10⁶ SMMC7721 cells. When the tumors had grown to approximately 3–4 mm in diameter, mice were randomly divided into three groups. Each group contained 6 mice. In vivo electroporation was performed to deliver naked DNA into pre-established tumors (28, 29). Twenty μg of plasmid DNA (MSCV-GFP or MSCV-ICN/ GFP) in 30 μl of saline was injected into the tumors of HCC-bearing mice. One min after injection, the site where the plasmid was injected was sandwiched in an electrode (BTX 533 2-Needle array electrode) with poles 5 mm in diameter. Three electrical pulses of 20 ms duration at a voltage of 600 V were delivered using an ECM 830 Electro Square porator (BTX, San Diego, CA). After this procedure tumor growth, as well as survival of HCC-bearing mice, was monitored and recorded, on a 3-daily and daily basis, respectively.

Analysis of Cell Cycle. The cell cycle was analyzed by flow cytometry as described previously (30). Briefly, 1 × 10⁶ cells were washed and in PBS, then fixed in 75% alcohol for 30 min at 4°C. After washing in cold PBS, three times, cells were resuspended in 1 ml of PBS solution with 40 μg of propidium iodide (Sigma) and 100 μg of RNase A (Sigma) for 30 min at 37°C. Samples were then analyzed for their DNA content by FACScalibur (Becton Dickinson, Mountain View, CA).

Detection of Apoptosis. Five × 10⁴ SMMC7721-ICN, SMMC7721-GFP, or parental SMMC7721 cells were plated in 24-well plates in RPMI 1640 supplemented with 2% FBS and cultured for 24, 48, or 72 h. Cells were fixed with 5% paraformaldehyde and incubated with 5 μg/ml Hoechst 33342 (Sigma) solution for 15 min. The cells were then washed and analyzed under fluorescence microscopy. The extent of apoptosis was visualized and counted (magnification, ×200).

Reverse Transcription-PCR Analysis of Cycle-Regulatory Factors. Total RNA from transfected and nontransfected SMMC7721 cells was isolated using TRIzol reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer’s instructions. Reverse transcription was performed on 1 μg of total RNA from each sample using oligod(T)₁₆ primers and 200 units of SuperScript II (Life Technologies, Inc.) for extension. PCR amplification was performed with 1.25 units Ex Taq polymerase (TaKaRa, Dalian, China). All of the PCR products were resolved on a 1.8% agarose gel containing ethidium bromide.

Western Blot Analysis. Nontransfected and transfected SMMC7721 cells were lysed in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) containing protease inhibitors (CalBiochem, San Diego, CA). Cell extracts were collected and centrifuged at 12,000 × g for 5 min. The protein concentrations were determined using the BCA Protein Assay (Pierce), according to the manufacturer’s instructions. The proteins were analyzed as described previously (31). Twenty μg of total proteins from whole cell lysates were boiled for 5 min in 1× SDS buffer, resolved by 6% or 12% SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with 0.1M Tris (pH 7.5), 0.9% NaCl, and 0.05% Tween-20 (TBST) containing 10% nonfat milk powder and then incubated with appropriate primary antibody (cyclin A1, cyclin D1, cyclin E, CDK2, Rb, pRb, p21, p27, p33, caspase3, extracellular signal-regulated kinase, p38, JNK, pJNK, and Bcl-2; Santa Cruz Biotechnology, CA), followed by incubation with antibody (goat or mouse) horsedarish peroxidase-conjugated antibody (Santa Cruz Biotechnology). The probed proteins were detected using SuperSignal West Femto Maximum Sensitivity Substrate chemiluminescent Western blot reagents (Pierce). Quantification of band intensity was carried out using LabWorks 4.0 Image Acquisition and Analysis Software (UVP, Inc., Upland, CA).

Statistical Analysis. Experimental data were analyzed by ANOVA for the statistical significance of the data obtained and to compare the means between groups. The Kaplan-Meier survival curves were generated using SPSS11.0 statistical software. A P < 0.05 represented a statistically significant difference.

RESULTS

Notch1 Signaling Inhibits in Vitro Growth of Human HCCs. We harvested the VSV-G pseudotyped viruses at the maximally productive period occurring between 48 and 72 h after transfection (25, 32). We used MSCV-ICN/GFP or control MSCV-GFP retroviruses generated 48 h after transfection to infect HCC SMMC7721 cells. The efficiency of transduction was determined by flow cytometry (Fig. 1A). The mean transduction efficiency of MSCV-GFP retroviruses was ~65%; however, the mean transduction efficiency of MSCV-ICN/GFP was noticeably lower; after transient transfection, the percentage of SMMC7721 cells constitutively expressing active Notch (ICN/GFP) was ~44%. We also observed the transfection efficiency of MSCV-GFP and MSCV-ICN/GFP in the HCC cell line Hep3B, and found that the efficiency was 53.14% and 20.36%, respectively (data not shown), suggesting that the transfection efficiency of this retroviral system may vary between different HCC cell lines. Therefore, we selected SMMC7721 cells as the HCC model.
Materials and Methods.

Human HCC cells SMMC7721 were infected with recombinant viruses as described in “Materials and Methods.” GFP expression in the cells was analyzed by fluorescence-activated cell sorter 24 h after transduction. For our experiments. As shown in Fig. 1B, the in vitro growth of SMMC7721 cells transiently expressing ICN was inhibited significantly. To investigate whether stably expressed ICN displays the same inhibitory effects on the growth of SMMC7721 cells, we selected several subclones of SMMC7721-ICN cells stably expressing both ICN and GFP, as well as several subclones of SMMC7721-GFP cells stably expressing GFP. The growth of SMMC7721-ICN cells was inhibited significantly compared with that of SMMC7721-GFP cells (Fig. 1, C and D). We also performed methylcellulose colony-forming assays and found that clonal growth of SMMC7721-ICN cells was also significantly inhibited (Fig. 1E). These results demonstrated that transient and stable expression of the constitutively active Notch1 was able to substantially inhibit in vitro growth of the HCC cells.

**Notch1 Signaling Suppresses in Vivo Growth of Human HCC Cells.** We first examined the tumorigenicity of stably transfected SMMC7721-ICN, SMMC7721-GFP, or parental SMMC7721 cells in nude mice, and found that tumorigenicity of SMMC7721-ICN was profoundly decreased compared with both control groups (Fig. 2A), indicating that stable expression of active Notch1 may suppress HCC growth in vivo. Next, we investigated whether intratumoral gene transfer of constitutively active Notch1 could inhibit the growth of parental SMMC7721 cells in nude mice. pMSCV/GFP or pMSCV-ICN/GFP plasmid DNA was in vivo electroporatically transferred into pre-established SMMC7721 tumors as described previously (21, 28, 29). Introduction of pMSCV/GFP did not influence either the growth of tumors or the survival of tumor-bearing mice, all of which died within 7 weeks. Compared with control plasmid vector, intratumoral gene transfer of constitutively active Notch1 significantly inhibited the growth of SMMC7721 tumors and prolonged the survival of HCC SMMC7721-bearing nude mice significantly (Fig. 2, B and C).

**Notch1 Signaling Induces Cell Cycle Arrest and Apoptosis in Human HCCs.** As observed above, Notch1 signaling can inhibit the growth of SMMC7721 cells in vitro and in vivo. We then explored the mechanisms underlying the growth inhibition by Notch1 signaling.

Because cell proliferation and death are closely linked to progression of the cell cycle, we analyzed cell cycle kinetics in SMMC7721 cells transfected with either GFP or Notch1 (ICN). Representative cell cycle profiles of infected SMMC7721 cells are shown as histograms in Fig. 3, with data expressed as mean percentage of cells in each cell cycle phase, 24, 48, or 72 h after retrovirus infection, derived from three independent experiments. SMMC7721 cell populations transduced with MSCV-ICN/GFP showed a higher proportion of cells in G0/G1 phase (72.86%), compared with control SMMC7721 MSCV-GFP cells (56.58%), and a decrease in the proportion of cells in G2/M phase (13.48%) relative to that observed in controls (29.1%). Cell cycle distribution analysis showed that the increase in G0/G1 phase cells observed in SMMC7721 populations transiently expressing ICN was significant (P < 0.01), suggesting that Notch1 signaling induces G0/G1 cell cycle arrest in SMMC7721 cells.

Because cell proliferation and death are closely linked to cell cycle arrest and apoptosis, we investigated the mechanisms underlying the growth inhibition by Notch1 signaling. Using flow cytometry, we analyzed the proportion of cells in each phase of the cell cycle. As shown in Fig. 3, transient and stable expression of the constitutively active Notch1 was also significantly inhibited (Fig. 1, C and D). The growth of SMMC7721 cells transiently expressing ICN was inhibited significantly compared with that of SMMC7721-GFP cells (Fig. 1, C and D). We also performed methylcellulose colony-forming assays and found that clonal growth of SMMC7721-ICN cells was also significantly inhibited (Fig. 1E). These results demonstrated that transient and stable expression of the constitutively active Notch1 was able to substantially inhibit in vitro growth of the HCC cells.

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infected SMMC7721 cells had a decreased growth rate that was significantly different from the control virus (P < 0.01; experiment performed in quadruplicate; bars, ±SE). C, growth of SMMC7721 cells stably expressing Notch1 (ICN) was detected by MTT assay. SMMC7721-ICN cells had a significant decreased growth rate compared with the control cells (P < 0.01). D, effect of active Notch1 (ICN) stable expression on the DNA synthesis of SMMC7721 cells. SMMC7721-ICN, SMMC7721-GFP, and parental SMMC7721 cells (4 × 10⁶/well) were seeded into 96-well plates and cultured for 96 h, and the proliferation of SMMC7721 cells was detected by [³H]thymidine incorporation. ∗P < 0.01 compared with control cells. Data are means of three independent experiments; bars, ±SE. E, effects of Notch1 (ICN) stable expression on clonal proliferation of SMMC7721 cells. SMMC7721-ICN, SMMC7721-GFP, and parental SMMC7721 cells (5 × 10⁶/well) were seeded into MethoCult methylcellulose-based medium. Colonies (>50 cells) were evaluated after 7 days of incubation. Results are expressed as the percentage of clone number compared with that of SMMC7721 cells. ∗P < 0.01 compared with control cells. Data are means of three independent experiments; bars, ±SE.
During subcloning of SMMC7721-ICN cells that stably express ICN, we found that a portion of SMMC7721-ICN cells, which normally grow as a tightly adherent monolayer, became detached and exhibited a rounded shape. These morphological changes were not observed in control cell lines. Only after raising the FBS concentration in culture medium from 10% to 15%, did we succeed in generating the SMMC7721-ICN subclones. This change in morphology and difficulty in generating subclones lead us to speculate that the expression of ICN may lead to apoptosis of SMMC7721 cells. To detect apoptosis, SMMC7721-ICN, SMMC7721-GFP, and SMMC7721 cells were maintained in RPMI 1640 containing 2% FBS for 24, 48, or 72 h, then stained with Hoechst 33342. As shown in Fig. 4, the fraction of SMMC7721-ICN cells that were apoptotic was greater than that observed in SMMC7721-GFP and SMMC7721 cultures at all of the time points, demonstrating that Notch1 signaling could also induce apoptosis of SMMC7721 cells.

Notch1 Signaling Down-Regulates the Expression of Cyclin A1, Cyclin D1, CDK2, and the Hyperphosphorylated Form of the Rb Protein but Up-Regulates p21WAF1/CIP1 Expression. To understand the molecular basis for Notch1-induced cell cycle arrest in the cells, we assayed the protein expression of cyclin A1, cyclinD1, cyclin E, CDK2, and Rb with Western blotting. Western blot analysis showed that cyclin A and cyclin D1 expression was
decreased significantly 72 h after transient transfection with Notch1 ICN. Cyclin E expression became lower 24 h after infection, and CDK2 expression was significantly decreased at 24 h. Phosphorylation of Rb results in the activation of E2F1, which is thought to drive the G1 to S phase transition (33). We examined the expression of phosphorylated Rb and found that its expression was also decreased (Fig. 5A). Data from three independent experiments showed that SMMC7721 cells transduced with MSCV-ICN/GFP expressed significantly less cyclin A (3.5-fold), cyclin D (2-fold), cyclin E (4.5-fold), CDK2 (2.8-fold), and pRb (3-fold) when compared with control SMMC7721 cells or SMMC7721-GFP cells, respectively. The expression profile of these proteins was comparable in stable SMMC7721-ICN subclones (Fig. 5B). SMMC7721-ICN clones expressed significantly less cyclin A1 (1.8 or 2.2-fold), cyclin E (12 or 10-fold), CDK2 (8 or 5-fold), Rb (4.8 or 5-fold), and pRb (11 or 7.5-fold) when compared with control SMMC7721 cells or SMMC7721-GFP cells, respectively. Conversely, p21waf1/cip1 protein expression was observed in SMMC7721-ICN cells but not in control cells. We also detected mRNA expression of these proteins using reverse transcription-PCR, achieving results consistent with those obtained by Western blot (data not shown). These results demonstrate that activation of Notch1 signaling modulates the expression of critical cell cycle regulators, leading to cell cycle arrest.

Notch1 Signaling Up-Regulates the Expression of p53 and Activation of JNK. Notch1 signaling induced the apoptosis of SMMC7721 cells. Cellular apoptosis involves complex molecular cascades, and dysfunction of a variety of genes may lead to the onset and progression of apoptosis. We analyzed the expression of the apoptosis-related proteins p53, Bax, FADD, TRADD, caspase3, and the antiapoptotic protein Bcl-2. As shown in Fig. 6A, transient expression of constitutively active Notch1 induced 9-fold increase in p53 expression in SMMC7721 cells 72 h after infection. Significantly up-regulated p53 expression was also observed in SMMC7721 cells stably expressing active Notch1 (8-fold or 12-fold) compared with control SMMC7721 cells or SMMC7721-GFP cells, respectively (Fig. 6B). These results demonstrated that Notch1 signaling up-regulated p53 expression in SMMC7721 cells. Conversely, Bcl-2 expression was down-regulated in SMMC7721 cells stably expressing active Notch1. However, the other apoptosis-related proteins were not detected (data not shown).

The MAPK pathway plays important roles in controlling cell proliferation and apoptosis. Cross-talk between Notch1 signaling and the MAPK pathway has been reported (13). We investigated whether the MAPK pathway was activated by Notch1 signaling in SMMC7721 cells. Although constitutive expression of ICN did not induce detectable phosphorylation of extracellular signal-regulated kinase and p38 (data not shown), a 4.8-fold increase in phosphorylation of JNK was observed (Fig. 6B), indicating that the JNK-MAPK pathway may be involved in the apoptosis of SMMC7721 cells induced by Notch1 overexpression.
Perhaps due to the fundamental roles of Notch proteins in balancing cell proliferation versus differentiation, Notch signaling has been suggested to be involved in malignant transformation (9). Its actual impact appears to be tumor type-dependent (3, 12, 15). Expression of activated Notch1 causes growth inhibition of human papillomavirus-positive cervical carcinoma cells, prostate cancer cells, and apparent reduction in small lung cancer cells (14, 15, 34). More recently, in Notch1-deficient mice, Notch1 has been shown to function as a tumor suppressor in mouse skin through inhibition of β-catenin signaling (35). Our results in the current report provide a new line for the potential role of Notch signaling in modulating the state of human liver carcinoma.

Notch1 signaling-induced growth inhibition of SMMC7721 cells is related to G1/G0 cell cycle arrest. Protein levels and kinase activities of cyclin A, cyclin D1, cyclin E, and cdk4 are significantly elevated in HCC (36). In human HepG2 hepatoma cells, increased cellular levels of p21 (CIP1) and decreased levels of the hyperphosphorylated form of Rb and cyclinD1 were correlated with growth inhibition and G1/G0 cell cycle arrest (37). Activated Notch1 causes keratinocyte growth arrest through increased p21 expression (38, 39). In SMMC7721 cells expressing active Notch1, we observed up-regulation of expression of p21, down-regulation of cyclin A1, cyclin D1, cyclin E, and CDK2, and decreased levels of the hyperphosphorylated form of the Rb protein, which were correlated with the altered cell cycle distribution phenotype and growth suppression. We approached the question regarding how Notch1 signaling induced apoptosis in SMMC7721 cells by assessing the effect of Notch1 signaling on the expression of the apoptosis-related proteins p53, Bax, Bcl-2, and caspase3. After transient expression of active Notch1 in SMMC7721 cells, only p53 expression was observed to increase significantly. However, in SMMC7721 subclones stably expressing Notch1 (ICN), down-regulation of Bcl-2 expression could also be observed. Previous studies have demonstrated that overexpression of mutant or wild-type p53 can down-regulate Bcl-2 expression, resulting in apoptotic cell death (39). Combined with these observations, our results suggest that after cell cycle arrest Notch1 signaling induces apoptosis, possibly through a p53-dependent reduction in Bcl-2 pathway signaling. To the best of our knowledge, our study provides the first evidence that Notch1 signaling can up-regulate p53 expression. Because p53 also can cause cell cycle arrest by transcriptionally up-regulating p21 (40, 41), it would be interesting to know the molecular interface between cell cycle arrest and apoptosis upon activation of Notch signaling in the cells.

Notch1 signaling has been reported to induce apoptosis of B cells by up-regulating Hairy1 (42). Whether Hairy1 participates in active Notch-induced apoptosis of B cells remains unclear. In SMMC7721 cells, active Notch1 signaling induced significant decreases in Rb expression and an increase in phosphorylation of JNK. In cells expressing wild-type Rb, loss of Rb protein is associated with the induction of apoptosis, occurring through caspase-mediated cleavage (43). Recent studies have also linked activation of intracellular Notch1 signals to the onset of apoptosis (44, 45), and shown that Rb can inhibit JNK/stress-activated protein kinase activity and, thus, negatively regulate stress-activated cellular events, including cell death (46). The interactions among p53, Rb, and JNK in the scenario of Notch1 signaling-induced apoptosis require additional study.

In summary, our results demonstrate that Notch1 signaling results in significant growth inhibition of HCC cells both in vitro and in vivo, which is related to growth arrest and apoptosis induction. Taking these results into consideration, along with those arising from previous studies, our observations raise the possibility that dysregulation of Notch receptor activity may be one of the mechanisms of HCC tumorigenesis.

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