

The P2Y₂ Nucleotide Receptor Mediates the Proliferation and Migration of Human Hepatocellular Carcinoma Cells Induced by ATP*

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Background: Hepatocarcinogenesis is a complex process that involves various modifications to a number of molecular pathways.

Results: The P2Y₂ receptor (P2Y₂R) mediates the effect of ATP on the cellular behavior of hepatocellular carcinoma (HCC) cells through store-operated calcium channel (SOCs)-mediated Ca²⁺ signaling.

Conclusion: P2Y₂R is involved in the development and progression of HCC.

Significance: P2Y₂R may be a promising therapeutic target against human HCC.

ATP is an abundant biochemical component of the tumor microenvironment and a physiologic ligand for the P2Y₂ nucleotide receptor (P2Y₂R). In this study, we investigated the effect of ATP on the cellular behavior of human hepatocellular carcinoma (HCC) cells and the role of P2Y₂R in ATP action and aimed to find a new therapeutic target against HCC. The experiments were performed in native isolated human HCC cells, normal hepatocytes, human HCC cell lines, and nude mice. We found that the mRNA and protein expression levels of P2Y₂R in native human HCC cells and the human HCC cell lines HepG2 and BEL-7404 were enhanced markedly compared with human normal hepatocytes and the normal hepatocyte line LO2, respectively. ATP induced intracellular Ca²⁺ increases in HCC cells and promoted the proliferation and migration of HCC cells and the growth of HCC in nude mice. The P2Y receptor antagonist suramin, P2Y₂R-specific shRNA, the store-operated calcium channel inhibitors 2-aminoethoxydiphenyl borate (2-APB) and 1-(β-3-(4-methoxy-phenyl) propoxyl-4-methoxyphenethyl)1H-imidazole-hydrochloride (SKF96365), and stromal interaction molecule (STIM1)-specific shRNA inhibited the action of ATP on HCC cells. In conclusion, P2Y₂R mediated the action of ATP on the cellular behavior of HCC cells through store-operated calcium channel-mediated Ca²⁺ signaling, and targeting P2Y₂R may be a promising therapeutic strategy against human HCC.

Hepatocellular carcinoma (HCC)⁴ is a frequent malignant tumor worldwide, and its very poor prognosis makes it the third leading cause of cancer-related mortality. Recent studies demonstrated that the incidence of HCC has increased in the West and in Asia, and it is possible that HCC will be a growing health problem and burden in coming years (1). Surgical intervention is the most common treatment of HCC, but most patients are ineligible for surgery because of either advanced disease and distant metastases or underlying liver dysfunction (2). In addition, because the molecular pathogenesis in the promotion and progression of HCC is not clear, the mechanism-based therapies for HCC are rare, and HCC is refractory to nearly all currently available anticancer therapies. Therefore, HCC remains one of the deadliest human cancers, with less than a 5-year survival rate (3). Therefore, it is of critical importance to look for new therapeutic targets for this disease.

Purinergic receptors comprise a family of transmembrane receptors that are activated by extracellular nucleosides and nucleotides. This large family of receptors has been subdivided into two major classes, P1 and P2. They are distributed widely across animal species, are present ubiquitously on both excitable and nonexcitable mammalian cells, and serve a variety of biological functions, from acting as neurotransmitters to autocrine and paracrine signaling to mitogenesis, angiogenesis, chemotaxis, proliferation and migration of cells, and inflammation (4, 5). In recent years, most studies have concentrated on the effect of purinergic signaling on the development and progress of tumors. It has been shown that the expression of P2Y receptors is up-regulated in some cancer tissues (6, 7). Extracellular nucleotides regulate the proliferation of several cancer cell lines through P2Y receptors (8, 9). In addition to P2Y receptors, P2X

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⁴ The abbreviations used are: HCC, hepatocellular carcinoma; PCNA, proliferating cell nuclear antigen; 2-APB, 2-aminoethoxydiphenyl borate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SOC, store-operated calcium channel; STIM1, stromal interaction molecule 1.

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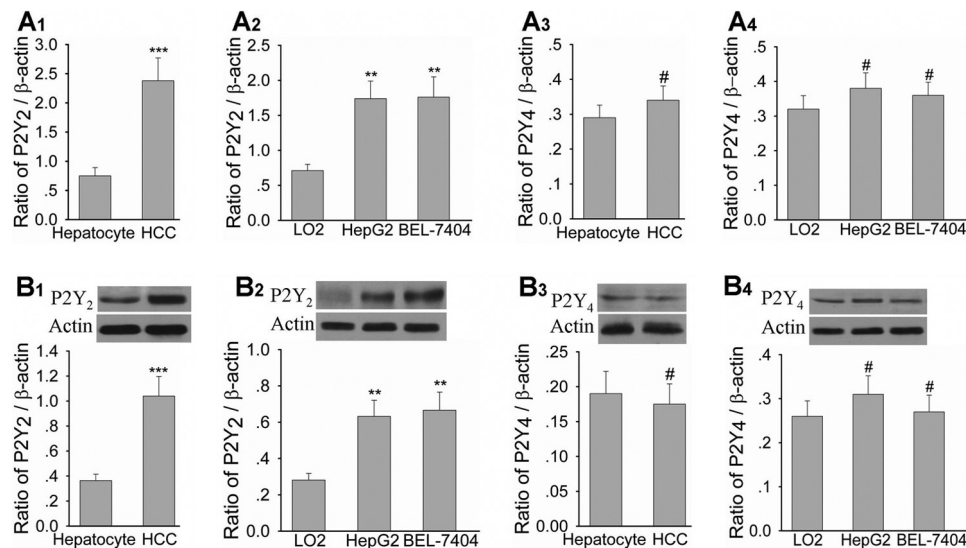


FIGURE 1. Expression of mRNA and protein of P2Y₂R and P2Y₄R in HCC cells. A1–A4, mRNA expression levels of P2Y₂R (A1 and A2) and P2Y₄R (A3 and A4) in native human normal hepatocytes and native human HCC cells, the normal hepatocyte line LO2, and the HCC cell lines HepG2 and BEL-7404 by real-time PCR analysis. B1–B4, protein expression levels of P2Y₂R (B1 and B2) and P2Y₄R (B3 and B4) in native human normal hepatocytes and native human HCC cells, the normal hepatocyte line LO2, and the HCC cell lines HepG2 and BEL-7404 by Western blot analysis. Top panels, representative blots for P2Y₂R, P2Y₄R, and β-actin. Bottom panels, protein expression levels of P2Y₂R and P2Y₄R. Data are mean ± S.E. of eight independent experiments. #, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with hepatocytes or LO2 cells.

and P1 receptors are also involved in tumor growth (10). These studies indicate that purinergic signaling might be a crucial player in the promotion and progression of tumors, which might open promising perspectives for the development of innovative therapies against tumors.

The P2Y₂ nucleotide receptor (P2Y₂R) is a member of the purinergic receptor family, belongs to the superfamily of G protein-coupled receptors, and mediates a variety of physiological and pathological responses through multiple and divergent intracellular signaling pathways (4, 11). P2Y₂R is expressed in hepatocytes of various species, including humans, and mediates ATP-stimulated intracellular Ca²⁺ ([Ca²⁺]_i) increases in hepatocytes (12, 13). However, the role of P2Y₂R in the promotion and progression of HCC is understood poorly. Therefore, in this study, we investigated the expression and functional role of P2Y₂R in human HCC and aimed to find a new therapeutic target against HCC.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies directed against P2Y₂R, P2Y₄R, proliferating cell nuclear antigen (PCNA), stromal interaction molecule 1 (STIM1), and β-actin were purchased from Santa Cruz Biotechnology. Fura-2 acetoxymethyl ester (Fura-2/AM) was purchased from Invitrogen. ATP, UTP, ADP, suramin, 2-aminoethoxydiphenyl borate (2-APB), 1-(β-3-(4-methoxy-phenyl)propoxyl-4-methoxyphenethyl)1H-imidazole-hydrochloride (SKF96365), and other routine chemicals were obtained from Sigma, except for those mentioned in the text.

Human Tissue Samples—Human HCC tissues were obtained from eight male patients, aged 40–50 years, with moderately differentiated primary HCC who underwent surgical resection in the Affiliated Hospital of Zunyi Medical College. The diagnosis of HCC was further confirmed by the histology of resected specimens. Histologically confirmed normal liver tissues were obtained from eight age-matched male patients who under-

went surgery for hepatic hemangioma. The tissue samples were collected immediately after surgical resection for cell isolations. This study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and the guidelines of the Ethics Committee of Zunyi Medical College and were approved by the Ethics Committee of Zunyi Medical College. An informed consent form was signed by each patient.

Isolations of Cancerous Cells of Human HCC Tissues and Hepatocytes of Human Normal Liver Tissues—Isolation of cancerous cells of native human HCC tissue and hepatocytes of native human normal liver tissues was performed as described previously (14). Briefly, freshly excised native human HCC tissues were cut into small fragments and incubated in RPMI 1640 medium containing 10 mg/ml collagenase and DNase (4 ml/g of tumor tissue) for 4 h. The single cell suspensions were prepared by pressing the digested tissues through a stainless 200-gauge mesh. After hypotonic lysis of red blood cells, the cells were further isolated by Percoll gradient centrifugation. For the isolation of normal hepatocytes, freshly excised human normal liver tissues were perfused with perfusion solution (Hanks' balanced salt solution, Invitrogen) containing 0.5 mM EGTA and 50 mM HEPES), followed by Williams' medium E (Invitrogen) containing collagenase type IV (0.05%) and 5 mM CaCl₂. After enzymatic dissociation, the hepatocytes were further separated from nonparenchymal cells through Percoll gradient centrifugation. Then, one portion of the isolated purified cancerous cells or normal hepatocytes was cultured in RPMI 1640 medium plus 10% fetal calf serum for 24 h and was then used for the measurement of [Ca²⁺]_i, and another was used for the examination of mRNA and protein expression levels of P2Y₂R and P2Y₄R.

Cell Lines and Cell Culture—The human HCC cell lines HepG2 and BEL-7404 and the human normal hepatocyte cell

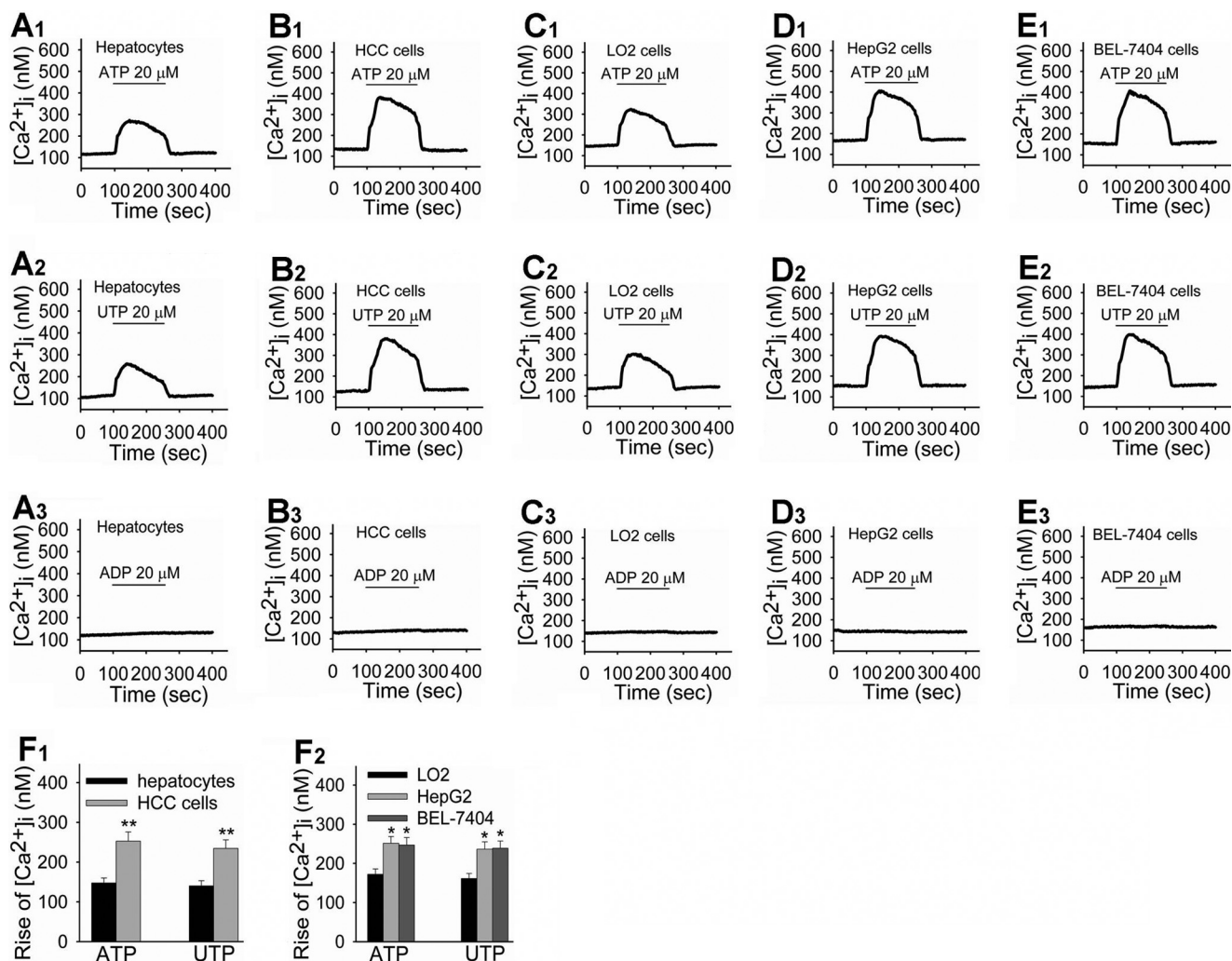


FIGURE 2. Nucleotides induced $[Ca^{2+}]_i$ increases in HCC cells. A1–E3, representative time courses of $[Ca^{2+}]_i$ changes in native human normal hepatocytes (A1–A3), native human HCC cells (B1–B3), LO2 cells (C1–C3), HepG2 cells (D1–D3), and BEL-7404 cells (E1–E3) induced by ATP, UTP, or ADP. ATP, UTP, or ADP was added at the indicated times. F1 and F2, comparisons of $[Ca^{2+}]_i$ rises between native human normal hepatocytes and native human HCC cells (F1) and the normal hepatocyte line LO2 and the HCC cell lines HepG2 and BEL-7404, induced (F2) by ATP or UTP. Data are mean \pm S.E. of eight independent experiments. Each representative time course of $[Ca^{2+}]_i$ change is from a typical cell. *, $p < 0.05$; **, $p < 0.01$ compared with hepatocytes or LO2 cells.

line LO2 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and maintained in an incubator at 37 °C and 5%CO₂.

RNA Extraction and Real-time RT-PCR—Total RNA from HepG2 cells, BEL-7404 cells, and LO2 cells, freshly isolated native human HCC cells, and normal hepatocytes was extracted using RNAiso Plus reagent (Takara Bio, Dalian, China) according to the instructions of the manufacturer. The concentration of all RNA samples was determined spectrophotometrically. The cDNA was produced from 2 μ g of total RNA using a PrimeScript[®] RT reagent kit (Takara Bio) according to the instructions of the manufacturer. Quantitative real-time RT-PCR was performed on a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad) using a SYBR[®] Premix Ex Taq[™] II kit (Takara Bio) following the instructions of the manufacturer. All samples were run in triplicate, and β -actin was used as an internal control. The expression level of P2Y₂R mRNA or P2Y₄R mRNA was normalized to that of β -actin and was expressed as a ratio relative to β -actin. The primers were as follows: P2Y₂R, CTGAG-

CATCCTGACCTGGAGA (forward) and CCAGGGCTTTC-ATTGCCATC (reverse); P2Y₄R, TGCCTGTGAGCTATGC-AGTTGTC (forward) and GGAACATGTAGGTGGCCGTTG (reverse); and β -actin, GGAAGGAAGGCTGGAAGAG (forward) and CCTTCCTTCCGACCTTCTC (reverse).

Western Blot Analysis—HepG2 cells, BEL-7404 cells, LO2 cells, freshly isolated native human HCC cells, and normal hepatocytes were homogenized in lysis buffer at 4 °C as described previously (14). The protein concentration of the supernatant was measured by BCA protein assay (Pierce). An equivalent amount of protein from each lysate was subjected to SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Blots were blocked with 5% nonfat milk and incubated with anti-P2Y₂R, anti-P2Y₄R, anti-PCNA, anti-STIM1, or anti- β -actin (which served as internal control) as the primary antibody. The membranes were washed and incubated with secondary antibody. After additional washing, the blots were developed with enhanced chemiluminescence reagents (Amersham Biosciences Life Science) and exposed to films in a dark room. Protein bands were analyzed with image analysis

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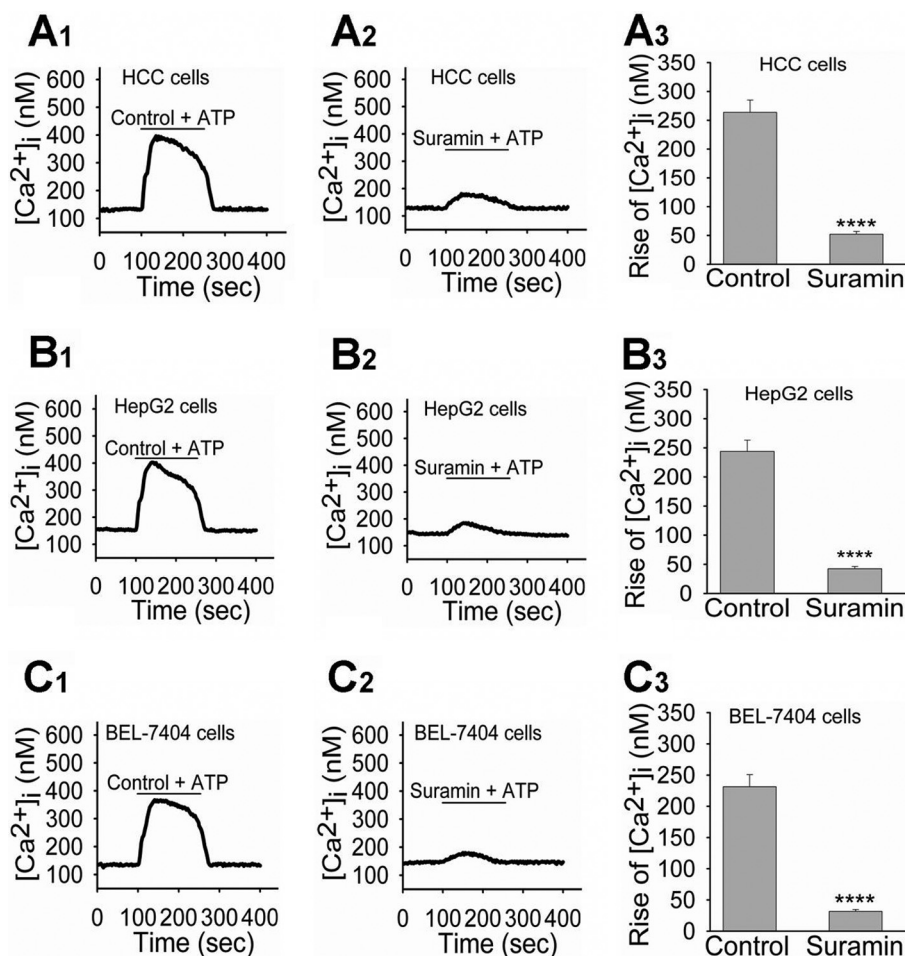


FIGURE 3. Effects of the P2Y₂ receptor antagonist suramin on ATP-induced [Ca²⁺]_i increases in HCC cells. A1, A2, B1, B2, C1, and C2, representative time courses of [Ca²⁺]_i changes in native human HCC cells (A1 and A2), HepG2 cells (B1 and B2), and BEL-7404 cells (C1 and C2). A3, B3, and C3, comparisons of [Ca²⁺]_i rises in native human HCC cells (A3), HepG2 cells (B3), and BEL-7404 cells (C3). ATP (20 μM) was added at the indicated times. Suramin (10 μM) was added 5 min before ATP. Data are mean ± S.E. of eight independent experiments. Each representative time course of [Ca²⁺]_i change is from a typical cell. ****, *p* < 0.0001 compared with control.

software. The results were expressed as the ratio relative to β-actin.

shRNA—To silence P2Y₂R or STIM1, lentivirus-based shRNA was used. SMARTchoice lentiviral human P2RY₂ shRNA (catalog no. SH-003688-01, NM_002564, TAAGTTACAGCCTCACT), human STIM1 shRNA (catalog no. SH-011785-02, NM_003156, TACTCGTCCTATCTCCCT), and SMARTvector 2.0 non-targeting control particles (catalog no. S-005000-01) were purchased from Dharmacon. HepG2 or BEL-7404 cells were transfected according to the protocol of the manufacturer. The protein expression of P2Y₂R or STIM1 was detected by Western blot analysis to demonstrate successful silencing.

Measurement of [Ca²⁺]_i—[Ca²⁺]_i levels in HepG2 cells, BEL-7404 cells, LO2 cells, isolated native human HCC cells, and normal hepatocytes were measured using the Ca²⁺-sensitive dye Fura-2/AM. The cells were grown on coverslips, loaded with 5 μM Fura-2/AM for 1 h at 37 °C in physiological saline solution before measurement and then washed in physiological saline solution for 20 min. The coverslips were mounted in an open perfusion chamber and perfused continuously using physiological saline solution with 2 mM Ca²⁺ or 0 mM Ca²⁺ (0 mM Ca²⁺ plus 0.5 mM EGTA). Real-time images were taken

using an epifluorescence Nikon Eclipse Ti microscope (×40 objective) and EasyRatioPro software (Photon Technology International). The 340/380 fluorescence ratio was measured from regions of interest within the cytosol. [Ca²⁺]_i concentration was quantified from the ratio of 340/380 fluorescence intensities using a method described previously (15). In each experiment, the [Ca²⁺]_i concentration of 10 cells was measured and averaged.

Cell Proliferation Assay—Cell proliferation was measured using both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and BrdU assays. HepG2 or BEL-7404 cells with specific shRNA, non-targeting shRNA, or without shRNA were seeded onto a 24-well plate at a density of 1 × 10⁴ cells/well. During the MTT assay, the cells were incubated in ATP, inhibitor plus ATP, or control for 0, 24, 48, or 72 h and then with MTT (0.5 mg/ml) for 4 h at 37 °C. The plates were then read at 570 nm using a microplate spectrophotometer. In the BrdU assay, the cells were incubated in ATP, inhibitor plus ATP, or control for 72 h. Cell proliferation was then estimated using a BrdU kit (Roche Diagnostics) following the protocol of the manufacturer. Each experiment was performed in triplicate. The results of the cell proliferation assays were expressed as percent of control.

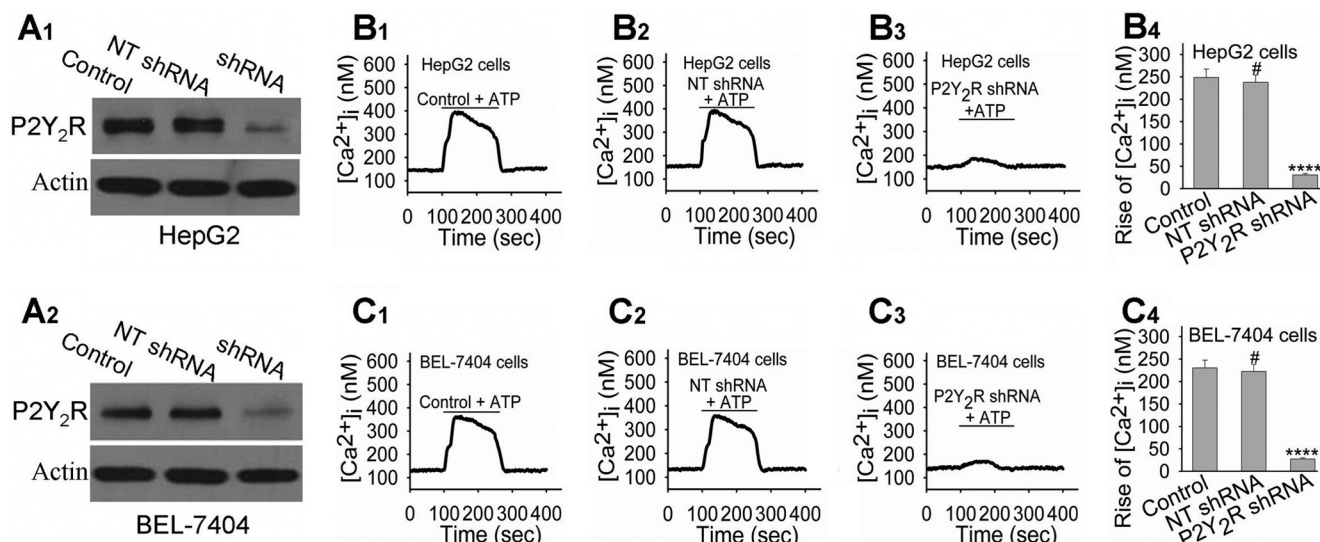


FIGURE 4. Effects of P2Y₂R-specific shRNA on ATP-induced [Ca²⁺]_i increases in HCC cells. A1 and A2, Western blot analysis of shRNA for P2Y₂R in HepG2 (A1) and BEL-7404 cells (A2). Results are representative blots. B1–B4, effects of P2Y₂R-specific shRNA on ATP-induced [Ca²⁺]_i increases in HCC cells. B1–B3 and C1–C3, representative time courses of [Ca²⁺]_i changes in HepG2 cells (B1–B3) and BEL-7404 cells (C1–C3). B4 and C4, comparisons of [Ca²⁺]_i rises in HepG2 cells (B4) and BEL-7404 cells (C4). ATP (20 μM) was added at the indicated times. Data are mean ± S.E. of eight independent experiments. Each representative time course of [Ca²⁺]_i change is from a typical cell. NT, non-targeting. #, $p > 0.05$; ****, $p < 0.0001$ compared with control.

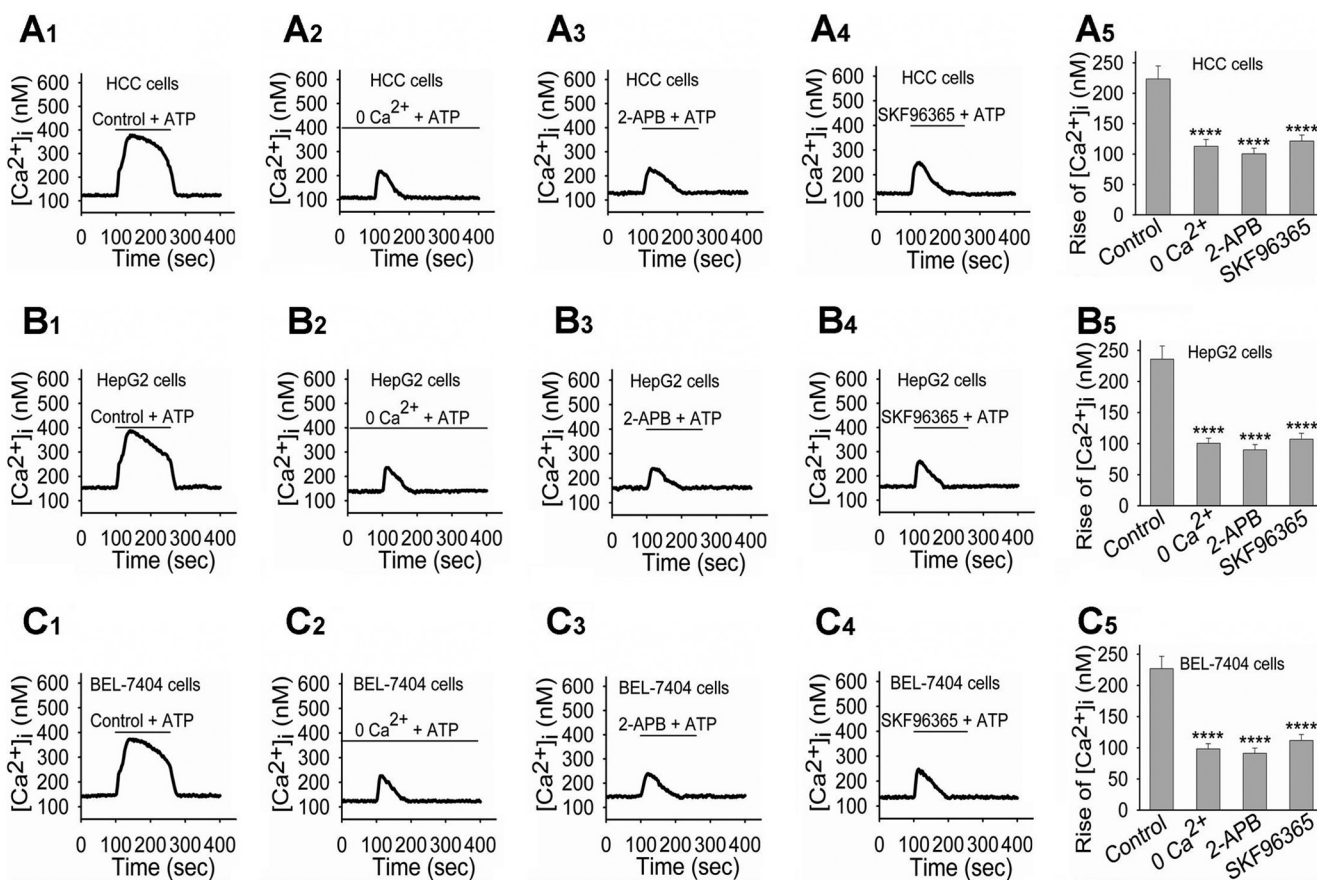


FIGURE 5. Effects of the extracellular Ca²⁺-free medium and the SOC inhibitors 2-APB and SKF96365 on ATP-induced [Ca²⁺]_i increases in HCC cells. A1–A4, B1–B4, and C1–C4, representative time courses of [Ca²⁺]_i changes in native human HCC cells (A1–A4), HepG2 cells (B1–B4), and BEL-7404 cells (C1–C4). A5, B5, and C5, comparisons of [Ca²⁺]_i rises in native human HCC cells (A5), HepG2 cells (B5), and BEL-7404 cells (C5). ATP (20 μM) was added at the indicated times. 2-APB (30 μM) or SKF96365 (10 μM) was added 5 min before ATP. Data are mean ± S.E. of eight independent experiments. Each representative time course of [Ca²⁺]_i change is from a typical cell. ****, $p < 0.0001$ compared with control.

Cell Migration Assay—Cell migration was estimated by using both scratch wound and Transwell migration assays. For the scratch wound migration assay, HepG2 or BEL-7404 cells with

specific shRNA, non-targeting shRNA, or without shRNA were cultured on a 24-well plate. The cells were treated with ATP, inhibitor plus ATP, or control. The cell monolayer was then

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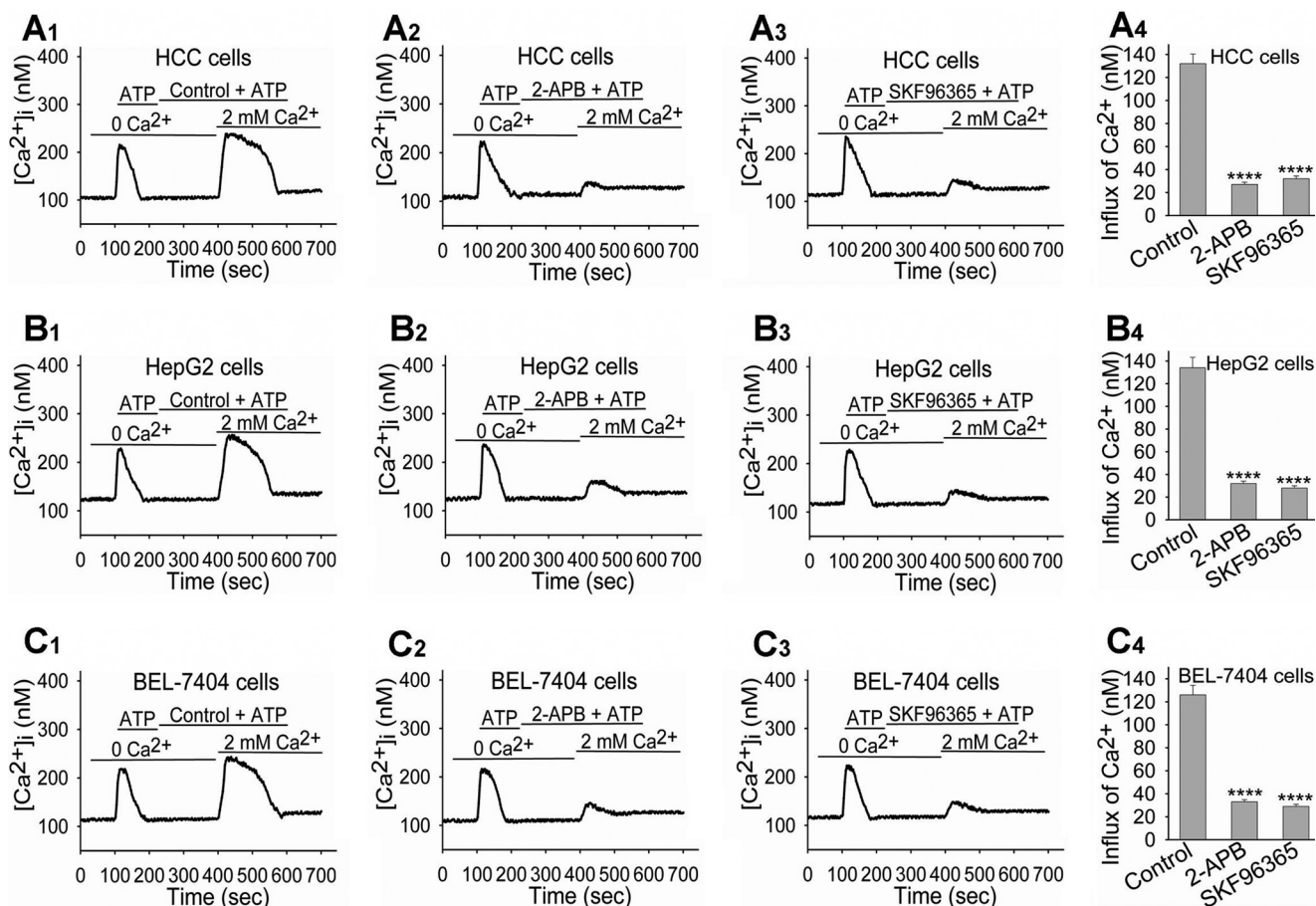


FIGURE 6. Effects of the SOC inhibitors 2-APB and SKF96365 on ATP-induced Ca^{2+} influxes in HCC cells. A1–A3, B1–B3, and C1–C3, representative time courses of $[Ca^{2+}]_i$ changes in native human HCC cells (A1–A3), HepG2 cells (B1–B3), and BEL-7404 cells (C1–C3) after extracellular Ca^{2+} restoration in extracellular Ca^{2+} -free medium. A4, B4, and C4, comparisons of Ca^{2+} influxes in native human HCC cells (A4), HepG2 cells (B4), and BEL-7404 cells (C4). ATP (20 μ M), 2-APB (30 μ M), or SKF96365 (10 μ M) was added at the indicated times. Data are mean \pm S.E. of eight independent experiments. Each representative time course of $[Ca^{2+}]_i$ change is from a typical cell. ****, $p < 0.0001$ compared with control.

scraped with a micropipette tip to generate a wound 1 mm in width. Images were captured 24 h after wounding using a Nikon Eclipse Ti microscope. Each experiment was performed in triplicate. The wound healing was quantified and averaged from digital images of five randomly selected fields with Image-Pro Plus image analysis software (Media Cybernetics). The results were expressed as the migration distance (in micrometers) by the leading edge of one side of the wound during 24 h. For the Transwell migration assay, the cells were replated onto the upper chamber of a Transwell filter with 8- μ m pores (Costar). The lower chamber was filled with medium containing ATP, inhibitor plus ATP, or control. The chamber was placed in serum-free DMEM. After 24 h, the cells were fixed with 4% paraformaldehyde in PBS. Each experiment was performed in triplicate, and the number of cells in five random fields on the underside of the filter was counted and averaged. The results were expressed as the migrated cell number.

Establishment of an HCC Xenograft Model—HCC xenografts were carried out with male BalB/c nude mice (4–6 weeks of age). The experimental protocol was approved by the Animal Care Committee of Zunyi Medical College in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985). Aliquots of 200 μ l of HepG2 cell suspension (1×10^6 cells) with specific shRNA,

non-targeting shRNA, or without shRNA were injected subcutaneously into the backs of the mice. The growth of established tumor xenografts was monitored. Five days later, when tumors were formed, the tumor-bearing mice were divided randomly into control, ATP, suramin plus ATP, and SKF96365 plus ATP groups. ATP (10 mg/kg), suramin (10 mg/kg), SKF96365 (10 mg/kg), or control vehicle was given intraperitoneally once daily. The mice were killed at 10, 15, 20, 25, or 30 days after implantation, and there were six mice in each series. The size of the local tumor at the implantation site was calculated by measuring the length, width, and thickness with a caliper. The result was expressed as tumor volume (tumor volume (mm^3) = length \times width \times thickness).

Statistical Analysis—Statistical analysis was processed using the SPSS PC statistics package. All results are expressed as means \pm S.E. Data were analyzed by one-way analysis of variance followed by Newman-Keul's post hoc test or, when appropriate, by two-tailed Student's t test. $p < 0.05$ was considered to be statistically significant.

RESULTS

Expression of P2Y₂R and P2Y₄R in Human HCC Cells—There are at least eight accepted human subtypes of P2Y receptors in which P2Y₂R and P2Y₄R are the major subtypes. It has been

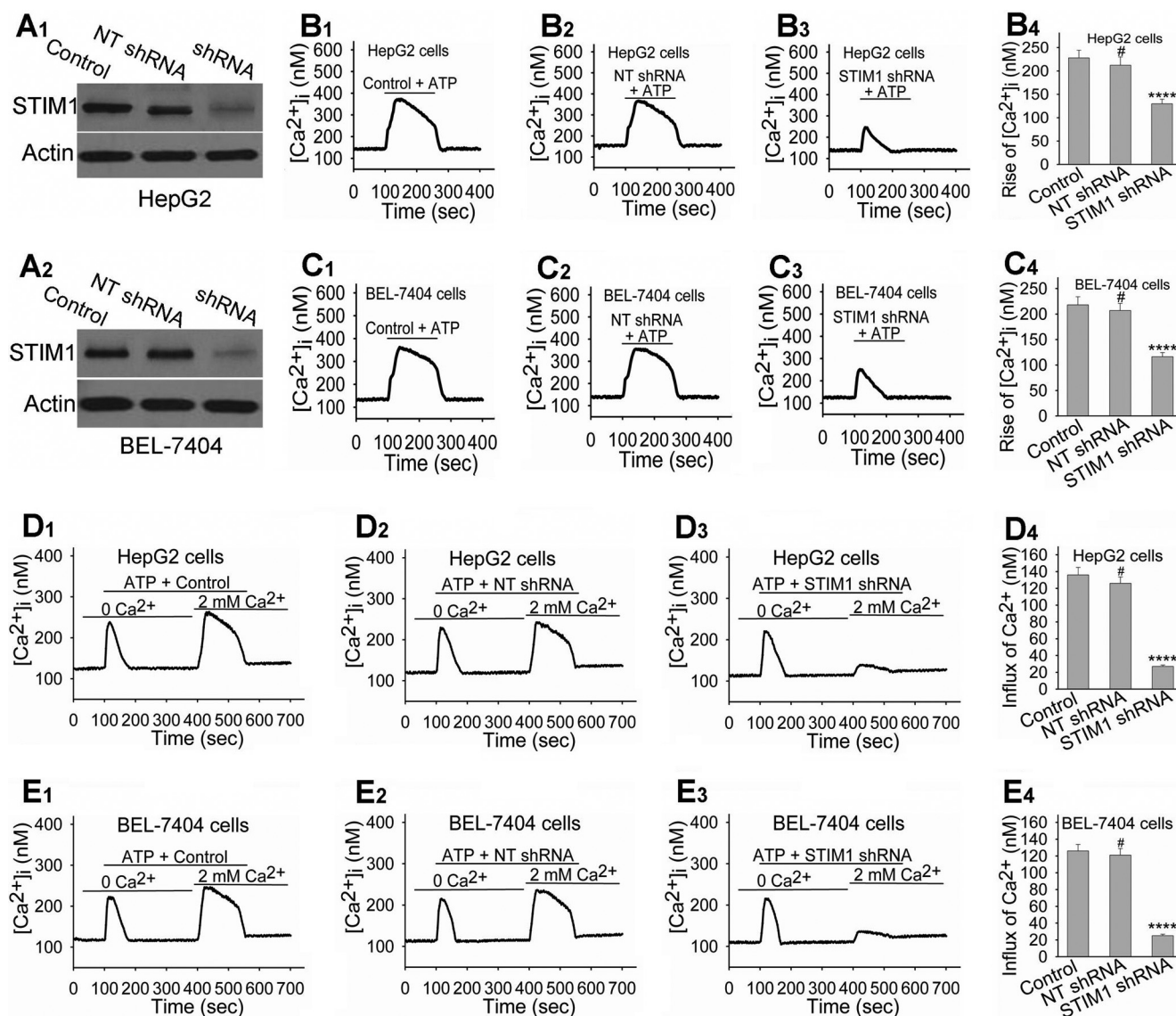


FIGURE 7. Effects of STIM1-specific shRNA on ATP-induced $[Ca^{2+}]_i$ increases and Ca^{2+} influxes in HCC cells. A1 and A2, Western blot analysis of shRNA for STIM1 in HepG2 (A1) and BEL-7404 cells (A2). Results are representative blots. B1–C4, effects of STIM1-specific shRNA on ATP-induced $[Ca^{2+}]_i$ increases in HCC cells. B1–B3 and C1–C3, representative time courses of $[Ca^{2+}]_i$ changes in HepG2 cells (B1–B3) and BEL-7404 cells (C1–C3). B4 and C4, comparisons of $[Ca^{2+}]_i$ rises in HepG2 cells (B4) and BEL-7404 cells (C4). D1–D4 and E1–E4, effects of STIM1-specific shRNA on ATP-induced Ca^{2+} influxes in HCC cells. D1–D3 and E1–E3, representative time courses of $[Ca^{2+}]_i$ changes in HepG2 cells (D1–D3) and BEL-7404 cells (E1–E3) after extracellular Ca^{2+} restoration in extracellular Ca^{2+} -free medium. D4 and E4, comparisons of Ca^{2+} influxes in HepG2 cells (D4) and BEL-7404 cells (E4). Data are mean \pm S.E. of eight independent experiments. Each representative time course of $[Ca^{2+}]_i$ change is from a typical cell. NT, non-targeting. #, $p > 0.05$; ****, $p < 0.0001$ compared with control.

shown that both P2Y₂R and P2Y₄R are expressed in human hepatocytes (12). Therefore, we first examined the mRNA and protein expression of P2Y₂R and P2Y₄R in native human HCC cells and normal hepatocytes. As shown in Fig. 1, both P2Y₂R and P2Y₄R were detected in native human HCC cells and normal hepatocytes, but the mRNA and protein expression levels of P2Y₂R in native human HCC cells were markedly higher than those in normal hepatocytes (Fig. 1, A1 and B1) ($p < 0.001$), whereas there were no significant differences in the mRNA and protein expression levels of P2Y₄R between native human HCC cells and normal hepatocytes (Fig. 1, A3 and B3) ($p > 0.05$). We further examined the expressions of P2Y₂R and P2Y₄R in the human HCC cell lines HepG2 and Bel-7404 and the normal hepatocyte line LO2. The results showed that the mRNA and protein expression levels of P2Y₂R in both HepG2 and BEL-

7404 cells were significantly higher than those in LO2 cells (Fig. 1, A2 and B2) ($p < 0.01$). Likewise, there were no significant differences in the mRNA and protein expression levels of P2Y₄R between the HCC cell lines and the normal hepatocyte cell line (Fig. 1, A4 and B4) ($p > 0.05$).

P2Y₂R Mediates ATP-induced $[Ca^{2+}]_i$ Increases in Human HCC Cells—Nucleotides are physiological ligands for P2Y receptors that couple to the inositol 1,4,5-triphosphate- Ca^{2+} signaling pathway. Our results showed that extracellular nucleotides, ATP and UTP, but not ADP, at a low concentration of 20 μ M, stimulated $[Ca^{2+}]_i$ increases in normal hepatocytes and native human HCC cells and human HCC cell lines and a normal hepatocyte line (Fig. 2). ATP and UTP induced a similar magnitude of $[Ca^{2+}]_i$ increase in the same cells. However, ATP- and UTP-induced $[Ca^{2+}]_i$ increases in native human HCC cells

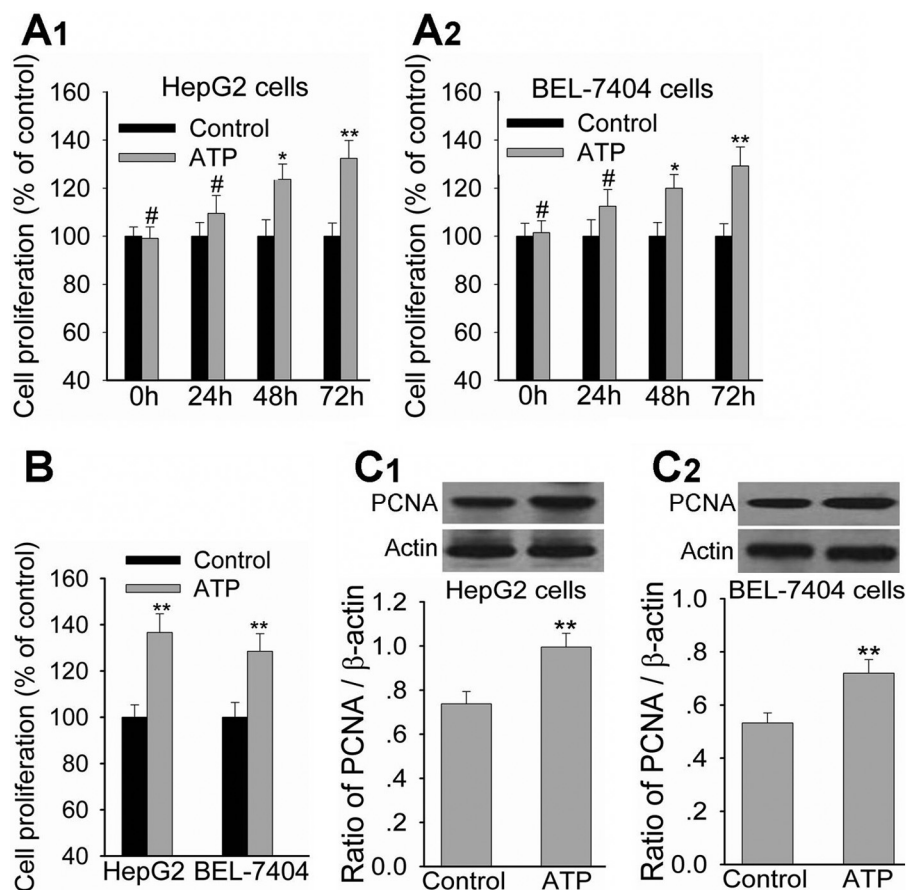


FIGURE 8. Effect of ATP on HCC cell proliferation. A1 and A2, effect of ATP on the proliferation of HepG2 cells (A1) and BEL-7404 cells (A2) by MTT assay. The cells were treated for 0, 24, 48, and 72 h with ATP (20 μ M) or control. B, effect of ATP on the proliferation of HepG2 cells and BEL-7404 cells by BrdU assay. The cells were treated for 72 h with ATP (20 μ M) or control. C1 and C2, effect of ATP on PCNA protein expression of HepG2 cells (C1) and BEL-7404 cells (C2) by Western blot analysis. The cells were treated for 72 h with ATP (20 μ M) or control. Data are mean \pm S.E. of eight independent experiments. #, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$ compared with control.

were markedly higher than those in normal hepatocytes (Fig. 2, F1) ($p < 0.01$), and, likewise, ATP- and UTP-induced $[Ca^{2+}]_i$ increases in HepG2 cells and BEL-7404 cells were also markedly higher than those in LO2 cells (Fig. 2, F2) ($p < 0.05$). Suramin, a P2Y receptor antagonist, inhibited ATP-induced $[Ca^{2+}]_i$ increases in native human HCC cells, HepG2 cells, and BEL-7404 cells (Fig. 3, A1–C3) ($p < 0.0001$), and P2Y₂R-specific shRNA of HepG2 cells and BEL-7404 cells inhibited ATP-induced $[Ca^{2+}]_i$ increases in HepG2 cells and BEL-7404 cells (Fig. 4, B1–B4 and C1–C4) ($p < 0.0001$). These results indicate that P2Y₂R mediates ATP-induced $[Ca^{2+}]_i$ increases in HCC cells.

Role of Store-operated Calcium Channels (SOCs) in ATP-induced $[Ca^{2+}]_i$ Increases in Human HCC Cells—As shown in Fig. 5, in extracellular Ca^{2+} -free medium, ATP-induced $[Ca^{2+}]_i$ increases in native human HCC cells, HepG2 cells, and BEL-7404 cells were reduced markedly compared with Ca^{2+} -containing medium ($p < 0.0001$), and ATP did not induce a further raised and sustained plateau of $[Ca^{2+}]_i$ increase. These results suggest that extracellular Ca^{2+} influx also contributes to ATP-induced $[Ca^{2+}]_i$ increases in HCC cells in addition to the release of Ca^{2+} in the intracellular stores. The initial $[Ca^{2+}]_i$ increase induced by ATP is caused by the release of Ca^{2+} in the intracellular stores, and the sustained plateau $[Ca^{2+}]_i$ increase requires Ca^{2+} influx from the extracellular space. Further

results showed that both 2-APB and SKF96365, SOC inhibitors, markedly decreased ATP-induced $[Ca^{2+}]_i$ increases in native human HCC cells, HepG2 cells, and BEL-7404 cells (Fig. 5, A1–C5) ($p < 0.0001$). ATP induced Ca^{2+} influxes in native human HCC cells, HepG2 cells, and BEL-7404 cells after the restoration of extracellular Ca^{2+} in extracellular Ca^{2+} -free medium, and both 2-APB and SKF96365 inhibited ATP-induced Ca^{2+} influxes after the restoration of extracellular Ca^{2+} (Fig. 6, A1–C4) ($p < 0.0001$). STIM1-specific shRNA of HepG2 cells and BEL-7404 cells decreased ATP-induced $[Ca^{2+}]_i$ increases (Fig. 7, B1–B4 and C1–C4) ($p < 0.0001$) and inhibited ATP-induced Ca^{2+} influxes after the restoration of extracellular Ca^{2+} in extracellular Ca^{2+} -free medium (Fig. 7, D1–D4 and E1–E4) ($p < 0.0001$). These results indicate that SOCs are involved in ATP-induced extracellular Ca^{2+} influx in HCC cells.

P2Y₂R Mediates ATP-induced Proliferation and Migration of HCC Cells through SOCs-mediated Ca^{2+} Signaling—We first examined the effects of ATP on the proliferation of HepG2 and BEL-7404 cells by MTT assay. The results showed that ATP promoted the proliferation of both HepG2 cells and BEL-7404 cells time-dependently and that the difference was significant 48 h after administration compared with controls ($p < 0.05$) (Fig. 8, A1 and A2). To further confirm this, BrdU incorporation

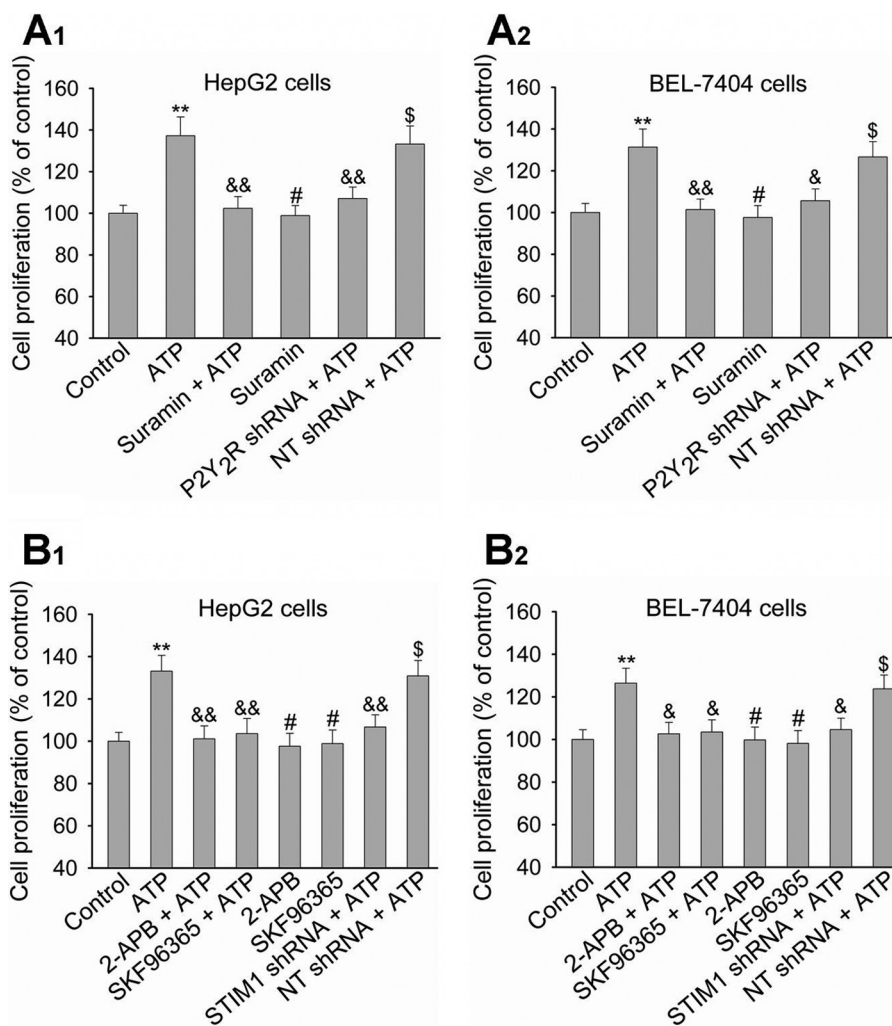


FIGURE 9. Effect of P2Y₂R and SOC inhibition on ATP-induced proliferation of HCC cells. A1 and A2, effect of the P2Y receptor antagonist suramin and P2Y₂R-specific shRNA on ATP-induced proliferation of HepG2 (A1) and BEL-7404 (A2) cells. B1 and B2, effect of the SOCs inhibitors 2-APB and SKF96365 and STIM1-specific shRNA on ATP-induced proliferation of HepG2 (B1) and BEL-7404 (B2) cells. Cells with shRNA, non-targeting shRNA (NT shRNA), or without shRNA were treated for 72 h with ATP (20 μ M), suramin (10 μ M), suramin (10 μ M) plus ATP (20 μ M), 2-APB (30 μ M), SKF96365 (10 μ M), 2-APB (30 μ M) plus ATP (20 μ M), SKF96365 (10 μ M) plus ATP (20 μ M), or control. Data are mean \pm S.E. of eight independent experiments. #, $p > 0.05$; **, $p < 0.01$ compared with control; \$, $p > 0.05$; &, $p < 0.05$; &&, $p < 0.01$ compared with ATP.

assay was performed, and the results also showed that ATP markedly promoted the proliferation of both HepG2 cells and BEL-7404 cells ($p < 0.01$) (Fig. 8B). ATP also markedly increased the expression of PCNA protein, a cell proliferation marker, in these cells ($p < 0.01$) (Fig. 8, C1 and C2). The P2Y receptor antagonist suramin and P2Y₂R-specific shRNA, but not non-targeting shRNA, significantly inhibited ATP-induced HepG2 and BEL-7404 cell proliferation ($p < 0.05$ and $p < 0.01$, respectively), and suramin had no significant effect on cell proliferation by itself (Fig. 9, A1 and A2). The SOC inhibitors 2-APB and SKF96365 and STIM1-specific shRNA, but not non-targeting shRNA, also significantly inhibited ATP-induced HepG2 and BEL-7404 cell proliferation ($p < 0.05$ and $p < 0.01$, respectively), and 2-APB or SKF96365 had no significant effect on cell proliferation by itself (Fig. 9, B1 and B2).

We further examined the effects of ATP on the migration of HepG2 and BEL-7404 cells. The results from scratch wound and Transwell migration assays showed that ATP promoted the migration of HepG2 cells and BEL-7404 cells. The P2Y receptor

antagonist suramin, the SOC inhibitors 2-APB and SKF96365, P2Y₂R-specific shRNA, and STIM1-specific shRNA, but not non-targeting shRNA, significantly inhibited ATP-induced HepG2 and BEL-7404 cell migration (Fig. 10 and Fig. 11) ($p < 0.001$ and $p < 0.0001$, respectively). Likewise, suramin, 2-APB, or SKF96365 had no significant effect on cell migration by itself (data not shown). Taken together, these results indicate that P2Y₂R mediates ATP-induced HCC cell proliferation and migration through SOC-mediated Ca²⁺ signaling.

P2Y₂R Mediates ATP-induced Growth of Human HCC through SOC-mediated Ca²⁺ Signaling—We further studied the effect of ATP on human HCC growth and the role of P2Y₂R. Heterotopic human HCC implantation in nude mice was performed, and the size of the implanted HCC was observed 10, 15, 20, 25, and 30 days after implantation. As shown in Fig. 12A, ATP markedly promoted the growth of human HCC in nude mice compared with controls ($p < 0.05$ and $p < 0.01$, respectively). The P2Y receptor antagonist suramin and the SOC inhibitor SKF-96365 markedly inhibited ATP-induced HCC

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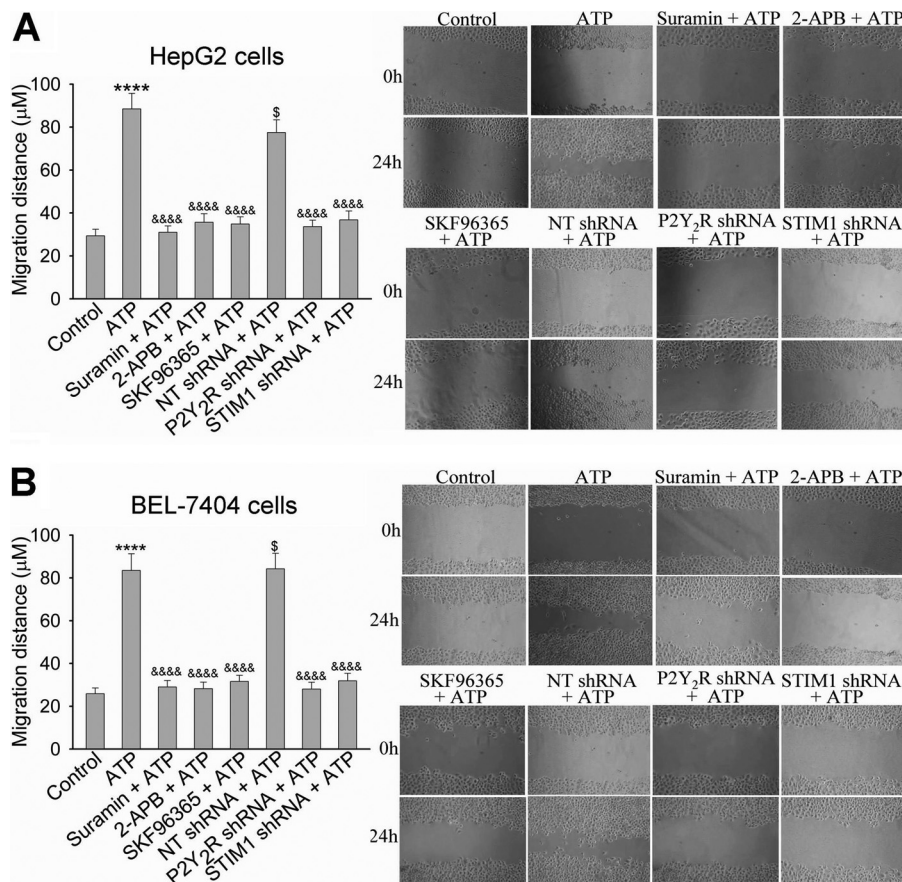


FIGURE 10. **Effect of P2Y₂R and SOC inhibition on ATP-induced migration of HepG2 (A) and BEL-7404 (B) cells by scratch wound assay.** Left panels, comparisons of cell migration distance between groups. Right panels, representative images of the scratch wound assay. Cells with shRNA, non-targeting shRNA (NT shRNA), or without shRNA were treated for 24 h with ATP (20 μM), suramin (10 μM) plus ATP (20 μM), 2-APB (30 μM) plus ATP (20 μM), SKF96365 (10 μM) plus ATP (20 μM), or control. Data are mean ± S.E. of eight independent experiments. ****, $p < 0.01$ compared with control; S, $p > 0.05$; #####, $p < 0.0001$ compared with ATP.

growth in nude mice ($p < 0.05$ and $p < 0.01$, respectively). Further results showed that P2Y₂R- and STIM1-specific shRNA, but not non-targeting shRNA, also markedly inhibited ATP-induced HCC growth in nude mice ($p < 0.05$ and $p < 0.01$, respectively). ATP increased the expression of PCNA protein in the implanted tumor tissue ($p < 0.05$), and P2Y₂R- and STIM1-specific shRNA inhibited an ATP-induced PCNA expression increase (Fig. 12C). These results indicate that ATP promotes the growth of human HCC through P2Y₂R- and SOC-mediated Ca²⁺ signaling.

DISCUSSION

In this study, we found that both P2Y₂R and P2Y₄R were expressed in native human HCC cells and normal hepatocytes, the human HCC cell lines HepG2 and BEL-7404, and the human normal hepatocyte line LO2 but that the expression levels of P2Y₂R rather than P2Y₄R in native human HCC cells and HCC cell lines were elevated markedly compared with human normal hepatocytes and a normal hepatocyte line, respectively.

Both P2Y₂R and P2Y₄R mediate the activation of phospholipase Cβ, leading to the production of inositol 1,4,5-trisphosphate and Ca²⁺ release from the intracellular stores. Nucleotides are ligands for P2Y₂R and P2Y₄R. Previous studies have demonstrated that both ATP and UTP have an equivalent

potency for P2Y₂R, whereas ADP is a much less effective agonist for P2Y₂R and P2Y₄R and UPT has a higher potency for P2Y₄R than ATP (4). In this study, our results showed that both ATP and UTP induced [Ca²⁺]_i increases in native human HCC cells and normal hepatocytes, the HCC cell lines HepG2 and BEL-7404, and the normal hepatocyte line LO2, whereas ADP did not induce significant [Ca²⁺]_i changes. However, ATP and UTP induced a similar magnitude of [Ca²⁺]_i increase in the same cells. In addition, both ATP and UTP induced higher [Ca²⁺]_i increases in native human HCC cells and HCC cell lines than in normal hepatocytes and a normal hepatocyte line. These results suggest that P2Y₂R, rather than P2Y₄R, is most likely involved in ATP-induced [Ca²⁺]_i increases in HCC cells. Further results showed that the P2Y receptor antagonist suramin and P2Y₂R-specific shRNA inhibited ATP-induced [Ca²⁺]_i increases in HCC cells, indicating that P2Y₂R mediates the effect of ATP on [Ca²⁺]_i in HCC cells. These results demonstrate that P2Y₂R is not only up-regulated in native human HCC cells and HCC cell lines but also mediates the ATP-induced [Ca²⁺]_i increase.

To determine the role of P2Y₂R in the promotion and progression of human HCC, we further studied the effects of ATP on the proliferation and migration of HCC cells and HCC growth and the role of P2Y₂R in ATP action. We found that

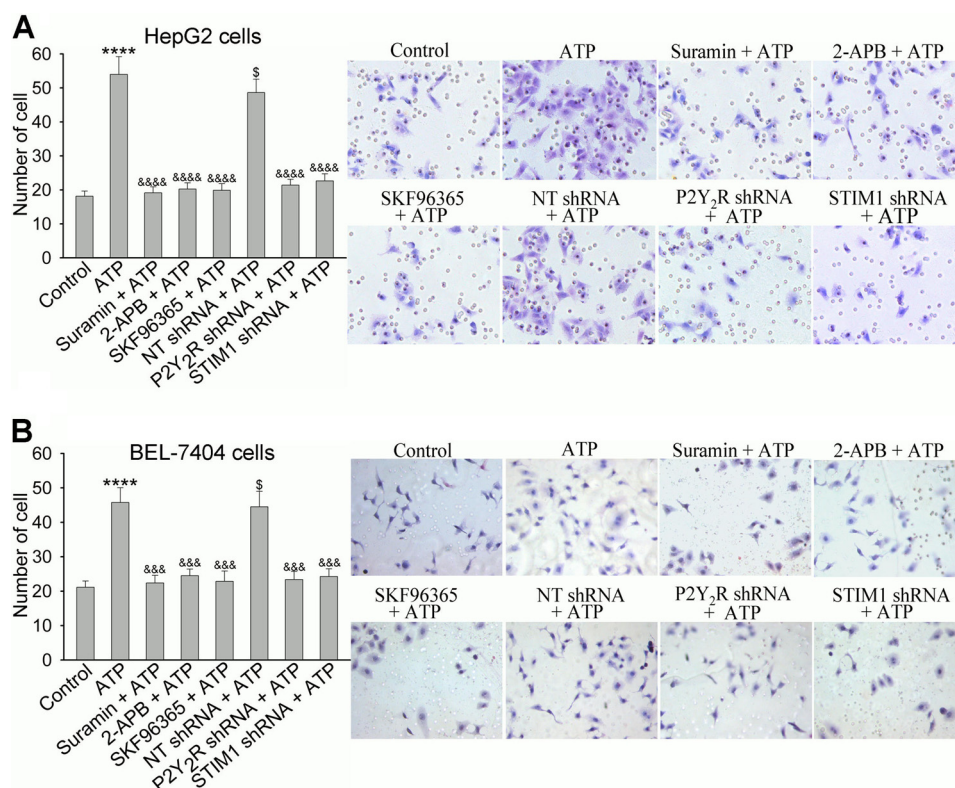


FIGURE 11. Effect of P2Y₂R and SOC inhibition on ATP-induced migration of HepG2 (A) and BEL-7404 (B) cells by Transwell assay. Left panels, comparisons of cell migration number between groups. Right panels, representative images of the Transwell assay. Cells with shRNA, non-targeting shRNA (NT shRNA), or without shRNA were treated for 24 h with ATP (20 μ M), suramin (10 μ M) plus ATP (20 μ M), 2-APB (30 μ M) plus ATP (20 μ M), SKF96365 (10 μ M) plus ATP (20 μ M), or control. Data are mean \pm S.E. of eight independent experiments. ****, $p < 0.01$ compared with control; \$, $p > 0.05$; &&&, $p < 0.001$; &&&&, $p < 0.0001$ compared with ATP.

ATP promoted the proliferation and migration of human HCC cells, both HepG2 and BEL-7404, and the growth of implanted human HCC in nude mice. The P2Y receptor antagonist suramin and P2Y₂R-specific shRNA inhibited ATP-induced human HCC cell proliferation and migration and the growth of implanted human HCC in nude mice. These results demonstrate that P2Y₂R mediates the action of ATP on HCC cells.

Extracellular nucleotides have emerged as regulators of tumor proliferation and possible effectors of neoplastic transformation. Although other nucleotides, such as UTP and UDP, have important extracellular signaling roles, the most recent studies have highlighted the involvement of ATP in host-tumor interaction. ATP is an abundant biochemical component of the tumor microenvironment and plays an important role in host-tumor interaction (10). Previous studies found that extracellular ATP concentration was in the 100 nM range in the tumor extracellular milieu, whereas it was undetectable (submicromolar) in healthy tissues (16). ATP promoted the proliferation of lung cancer cells and breast cancer cells (8, 9), facilitated the migration of lung cancer cells (17), and enhanced the invasion of prostate cancer cells (18). In addition, ATP could rapidly activate multiple components of the c-Jun NH₂-terminal kinase cascade, a central player in hepatocyte proliferation and liver regeneration (19). Deletion of *Cd39*, an ectonucleotidase that regulates extracellular nucleotide/nucleoside concentrations by scavenging nucleotides to ultimately generate adenosine, resulted in higher concentration of extracellular ATP and promoted the development of both induced and spontaneous

autochthonous liver cancer in mice (20). Direct intra-arterial delivery of 3-bromopyruvate, a potent inhibitor of cell ATP production, arrested the growth of liver cancer (21). These results demonstrate that ATP plays an important role in the development and progression of HCC. In this study, our results demonstrate that the expression of P2Y₂R is up-regulated in human HCC cells and that P2Y₂R mediates the action of ATP on the proliferation and migration of human HCC cells and the growth of human HCC. It is well known that inflammation plays an important role in the malignant transformation of tumor cells and the promotion and progression of tumors (22). Chronic hepatitis induced by hepatitis viruses B and C is a major cause of HCC (23, 24). In addition, malignant tumors can trigger a strong inflammatory response, and inflammation is a key component of the tumor microenvironment (25). ATP can be released from inflammatory cells and tumor cells and promotes the development and progression of tumors (10). Therefore, it is likely that P2Y₂R is a key player in the development and progression of chronic hepatitis-associated HCC.

P2Y₂R couples to G proteins to lead to Ca²⁺ release from the intracellular stores. Ca²⁺ is a ubiquitous cellular signal. The change of intracellular Ca²⁺ controls various cellular processes relevant to the development and progression of tumors, such as proliferation, apoptosis, motility, gene transcription, and angiogenesis. Ca²⁺ signaling proteins and organelles have been emerging as additional cellular targets of oncogenes and tumor therapeutic intervention (26). In this study, our results demonstrate that both extracellular Ca²⁺ influx and intracellular Ca²⁺

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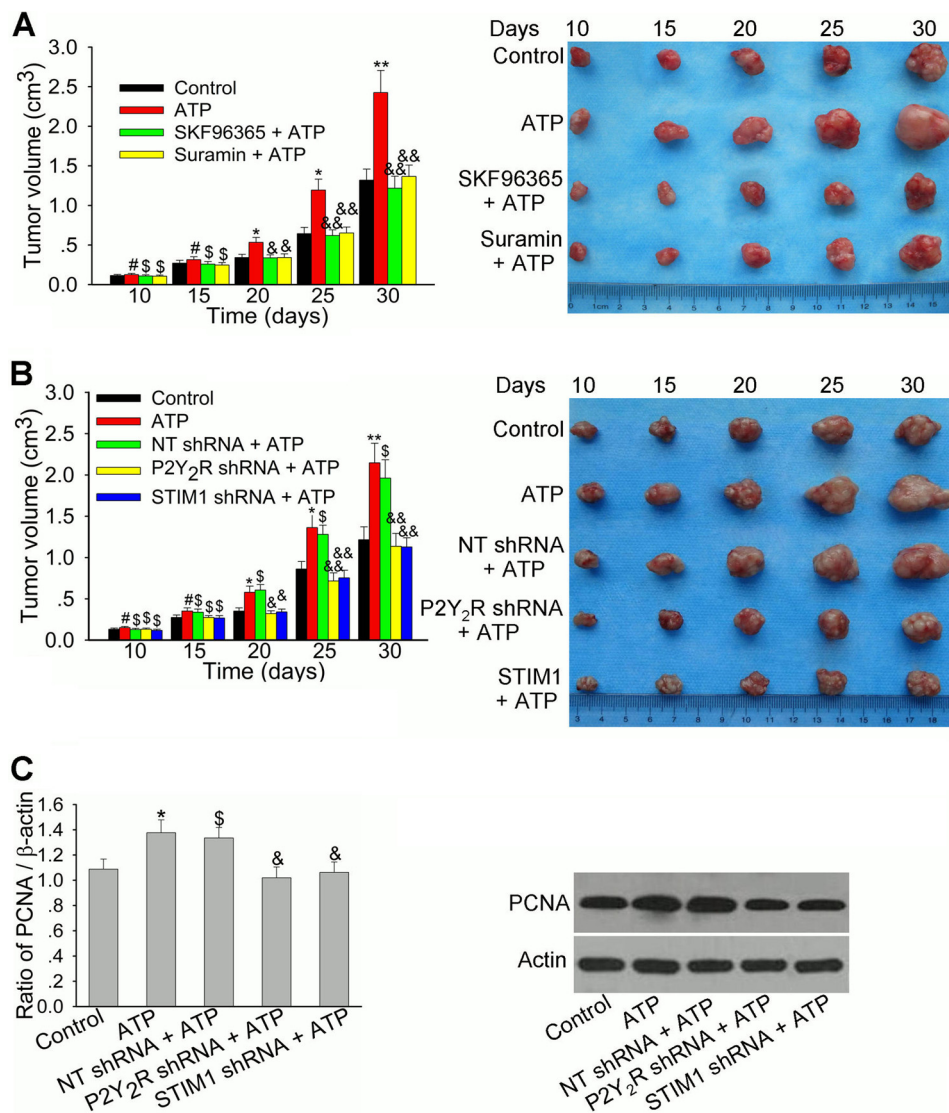


FIGURE 12. Effect of P2Y₂R and SOC inhibition on ATP-induced HCC growth. Human HCC xenografts were performed as described under “Experimental Procedures.” *A*, effect of the P2Y receptor suramin and the SOC inhibitor SKF96365 on ATP-induced HCC growth. *B*, effect of P2Y₂R-specific shRNA and STIM1-specific shRNA on ATP-induced HCC growth. *Left panels*, comparisons of tumor volume between groups at 10, 15, 20, 25, and 30 days after implantation. *Right panels*, representative images of tumors. *C*, protein expression of PCNA in implanted tumor tissues by Western blot analysis. The tumor tissue samples were from the implanted tumor tissues in the backs of nude mice at 20 days after implantation. *Left panel*, comparison of PCNA protein expression levels between groups 20 days after implantation. *Right panel*, representative blots of PCNA protein expression. #, $p > 0.05$; \$, $p < 0.05$; *, $p < 0.05$; **, $p < 0.01$ compared with control; #, $p > 0.05$; \$, $p < 0.05$; &, $p < 0.05$; &&, $p < 0.01$ compared with ATP.

release contribute to ATP-induced $[Ca^{2+}]_i$ increases through P2Y₂R and that an ATP-induced sustained plateau $[Ca^{2+}]_i$ increase requires Ca^{2+} influx from the extracellular space. SOCs are nearly ubiquitous Ca^{2+} entry pathways stimulated by numerous cell surface receptors via the reduction of Ca^{2+} concentration in the intracellular stores (27). 2-APB and SKF96365 are two commonly used SOC inhibitors (28, 29). In this study, both 2-APB and SKF96365, and specific STIM1 shRNA, a key regulator of SOCs, inhibited ATP-induced extracellular Ca^{2+} influx in HCC cells, ATP-induced HCC cell proliferation and migration, and HCC growth, demonstrating that P2Y₂R mediates the action of ATP through SOC-mediated Ca^{2+} signaling.

In summary, hepatocarcinogenesis is a complex process that involves various modifications to a number of molecular pathways as well as genetic alterations and, ultimately, leads to malignant transformation and HCC disease progression

(30). A number of studies have demonstrated a key role of the inflammatory tumor microenvironment in cancer development, progression, and metastasis (22, 31). ATP can be released from inflammatory cells and tumor cells and accumulates within the tumor microenvironment (10, 16). In this study, we demonstrate, for the first time, that P2Y₂R mediates ATP-induced human HCC cell proliferation and migration and HCC growth through SOC-mediated Ca^{2+} signaling. This means that P2Y₂R might be a key player in the development and progression of inflammation-associated HCC and that targeting P2Y₂R is a promising therapeutic strategy against human HCC.

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