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Original Article

Inhibition of IκB kinase β restrains oncogenic proliferation of pancreatic cancer cells

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Purpose: **Pancreatic cancer is characterized by an extremely poor prognosis due to the aggressive disease course and lack of effective therapeutic intervention. IκB kinase (IKK), a central kinase for nuclear factor-κB (NF-κB) activation, is often constitutively activated in pancreatic cancer cells, playing a crucial role in the malignant phenotype and resistance to anti-cancer agents. This study explored how specific inhibition of IKKβ suppresses oncogenic proliferation of pancreatic cancer cells.**

Experimental Design: **We employed two different approaches, RNA interference-mediated depletion of IKKβ (IKKβi) and use of a novel molecularly designed IKKβ inhibitor IMD-0354 to investigate the effects on the *in vitro* and *in vivo* growth and apoptotic response of pancreatic cancer cells.**

Results: **IKKβi and IMD-0354 efficiently suppressed constitutive NF-κB activity and the growth of pancreatic cancer cells in monolayer and soft agar. IMD-0354 induced Annexin V expression, a typical apoptotic cell response. Notably, daily administration of IMD-0354 significantly suppressed tumor growth in NOD/SCID/γc^{null} (NOG) mice without any deleterious side effect.**

Conclusions: These results identify IKKβ as an attractive molecular target for pancreatic cancer therapy.

Key words: nuclear factor-κB (NF-κB), IκB kinase β (IKKβ), pancreatic cancer, RNA interference, IMD-0354

Introduction

Pancreatic cancer is one of the most common cancers with an extremely poor prognosis around the world because of its aggressive invasion, early metastasis, resistance to existing chemotherapeutic agents and radiation therapy, lack of specific early symptoms and difficulty in diagnosis at an early stage. The majority of patients suffer from unresectable tumor(s) with systemic chemotherapy being largely ineffective. Even if the tumor was resectable, the prognosis of those patients is very poor compared with other gastrointestinal cancer patients[1-3]. To improve the prognosis of pancreatic cancer patients, it is necessary to develop a rational strategy for treatment that specifically targets molecules playing a critical role in the growth or survival of pancreatic cancer cells.

NF-κB was originally identified as a transcription factor regulating the expression of the light chain of immunoglobulins in B cells[4]. The NF-κB/Rel family is composed of p50, p52, RelA (p65), RelB and c-Rel

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subunits, which can form various homo- and hetero-dimers. The most studied is a hetero-dimer of the p50 and p65 subunits predominant in many types of cells. In most normal cells, NF-κB is inactive by its tight association with the cytoplasmic inhibitory proteins, called inhibitor of NF-κB (IκB)[5-8]. Following cell stimulation, IκB proteins become phosphorylated by IκB kinase (IKK), a large kinase complex consisting of two catalytic subunits, IKKα and IKKβ, and regulatory subunits, IKKγ /NEMO and ELKS[9-12]. Phosphorylation of IκB proteins targets them for ubiquitination and degradation, which results in the release and subsequent translocation of NF-κB to the nucleus to activate transcription of a variety of genes[13]. IKKβ is the main catalytic subunit of the IKK complex in the phosphorylation of IκBα at two conserved serine residues within the N-terminal regulatory domain[14]. Recently, a bulk of evidence has demonstrated that NF-κB plays a critical role in carcinogenesis as well as in resistance to anti-cancer therapies[15]. Constitutively high NF-κB activity has been demonstrated in a wide range of human hematopoietic cancer cells, such as adult T-cell leukemia (ATL)[16], Hodgkin lymphoma[17], and multiple myeloma cells[18] as well as in solid cancers including breast[19], prostate[20] and pancreatic cancer cells[21]. In these cells, inhibition of constitutive NF-κB activity often induces apoptotic cell death, suggesting that targeting a molecule critically involved in constitutive NF-κB activation will be an effective strategy to treatment of these cancer patients.

In the present study, we focused on IKKβ as a molecular target to suppress constitutive NF-κB activity in pancreatic cancer cells. We employed two different approaches, lentiviral expression of a small hairpin RNA that specifically suppresses the expression of endogenous IKKβ, and a novel IKKβ inhibitor IMD-0354 that docks with the ATP-binding site of the kinase, thereby inhibiting its activity.

Materials and methods

Cell culture

Human pancreatic cancer cell lines, Panc-1 and PK8 were kindly provided from Cell Resource Center for Biomedical Research, Tohoku University. Cells were maintained in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate under atmosphere of 5% CO_2 with humidity at 37°C.

Plasmids

Each shRNA expression vector was constructed based on the pSuperRetro vector (Oligoengine) according to the manufacturer's instructions. Targeting sequences inserted immediately downstream of the H1 promoter were as follows: human IKKβ, 5'-GTACAGCGAGCAAACCGAG-3'; unrelated control (specific to Renilla luciferase), 5'-GTAGCGCGGTGTATTATAC-3'. The resultant plasmids were referred to as pSR-IKKβi and pSR-Ctrli, respectively. To construct a lentivirus vector carrying the puromycin resistance gene expressed under the control of phosphoglycerate kinase (PGK) promoter, DNA containing the PGK promoter and puromycin resistance gene was amplified by PCR using the pSuperRetro vector as template and a primer containing *ApoI, XhoI, BamHI and EcoRI* restriction sites, 5'-ATCAATTGCTCGAGGGATCCGAATTCATTCTAC-CGGGTAGGGGA-3' and that containing a *Sal*I restriction site, 5'-TAGTCGACTCAGGCACCGGGCTT-3'. The PCR product was subcloned in pDrive (Qiagen) and sequenced. *A Bam*HI-*Sal*I fragment of the above plasmid and an *ApoI-BamHI* fragment of DNA obtained by annealing two oligonucleotides, 5'-AATTTGCGGCCGCCTCGAGG-3' and 5'- GATCC-CTCGAGGCGGCCGCA-3' were inserted between the *EcoRI* and *XhoI* sites of the pCS-CDF-CG-PRE vector, a kind gift from Dr. Miyoshi, H., generating pCS-puro-PRE. Lentivirus vectors capable of expressing shRNA were constructed by inserting an *EcoRI—XhoI* DNA fragment containing the H1 promoter and targeting sequence from pSR-IKKβi or pSR- Ctrli between the *EcoRI* and *XhoI* sites of pCS-puro-PRE, denoted pCS-puro-IKKβi and pCS-puro-Ctrli, respectively.

Reagents

Antibodies against phospho-IκBα (5A5) and rabbit IgG (H&L) were purchased from Cell Signaling Technology (Beverly, MA). Anti-IKKβ (H470) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse IgG secondary antibody was purchased from American Qualex (San Clemente, CA), and anti-α-Tubulin antibody was from Sigma-Aldrich (St. Louis, MO). IMD-0354, a novel IKKβ inhibitor, was from the Institute of Medicinal Molecular Design, Inc. (Tokyo, Japan). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified.

Preparation of cell extracts

For preparation of whole-cell extracts, cells were suspended in RIPA buffer (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate and 0.1% SDS), supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin and 1 μg/ml aprotinin. Extracts were cleared by centrifugation. Cytoplasmic and nuclear extracts were prepared as described previously[22].

Immunoblotting

Whole-cell extracts or cytoplasmic extracts were fractionated on 8-12% SDS-polyacrylamide gels and transferred onto Immobilon membranes (Millipore, MA, USA). Blots were revealed with an enhanced chemiluminescence detection system (ECL, Perkin Elmer, MA, USA). All the experiments were repeated at least three times.

Virus infection and transfection

293T cells were co-transfected with pCS-puro-IKKβi or pCS-puro-Ctrli together with pCMVΔR8.2 packaging construct and pHCMV-VSV-G (kind gifts from Dr. I.S.Y. Chen) using FuGENE 6 (Roche Applied Science). Culture supernatants were collected 60 hr after transfection and filtered. Panc-1 and PK8 cells were infected with these viruses for 6 hr in the presence of 10 mg/ml of polybrene. At 48 hr after infection, cells were cultured in medium containing 2 μg/ml of puromycin for additional 48 hr. IgκCona-luc[23] and EF1-LacZ[22] were used to determine NF-κB-dependent transcriptional activity in Panc-1 and PK8 cells. Cells were transfected using DMRIE-C reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Assays for luciferase and β-galactosidase were performed 36 hr after transfection in standard methods. Luciferase activity was normalized on the basis of β-galactosidase activity. Each experiment was repeated at least three times, and the results are expressed as an average with s.d.

Soft agar assay

Anchorage-independent cell growth was examined essentially as described previously[24]. The experiment was repeated at least three times, and the results are expressed as an average with s.d.

Annexin V analysis.

The apoptotic status was analyzed by using Annexin V-PE (BD Biosciences, San Jose, CA). Briefly, cells (1×10^6 cells/ml) were incubated for 48 hr in the presence or absence of IMD-0354. Cells were then incubated with a mixture of Annexin V-PE for 15 minutes at room temperature according to the manufacturer's instruction. Annexin V-positive cells were detected by FACScalibur analysis. The data were analyzed with the CellQuest program.

Animal experiments

The NOD/SCID/γc^{null} (NOG) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions in the Animal Center of Tokyo Medical and Dental University (Tokyo, Japan). The Ethical Review Committee of the institute approved the experimental protocol. Panc-1 cells were inoculated subcutaneously in the post-auricular region of NOG mice at a dose of 1×10^7 cells per mouse. IMD-0354 was suspended in saline and 20 mg/kg body weight of IMD-0354 (suspended in 200 μl/mouse) was given intraperitoneally to each mouse once a day for 28 days after tumor cell inoculation. Saline was injected in control mice. Each group consisted of five mice. Twenty eight days later, mice were sacrificed and tumors were measured. Estimated tumor volume (mm^3) was given as the following formula: tumor volume = $[(\text{width})^2\times\text{length}] / 2$[25]. All animal experiments complied with the standards in the guidelines of the University Animal Care and Use Committee of the Tokyo Medical and Dental University.

Statistical analysis.

Two-tailed Student's t test (Figure 1B, 2, 4C and 6A) and Dunnet test (Figure 3A, 5A and 5B) were done for statistical analysis of the data, and $P < 0.05$ was taken as the level of significance using StatView-J 5.0 software (SAS Institute, Cary, NC, USA).

Results

IKKβ knock-down suppresses constitutive NF-κB activity and growth of pancreatic cancer cells

To determine if pancreatic cancer cell proliferation is supported by the IKK/NF-κB signaling, we sought to suppress the expression of IKKβ, a central kinase that phosphorylates IκB proteins, through lentiviral expression of shRNA to IKKβ (shIKKβ) in Panc-1 and PK8 pancreatic cancer cells. Infection of these cells with the lentivirus capable of expressing shIKKβ, but

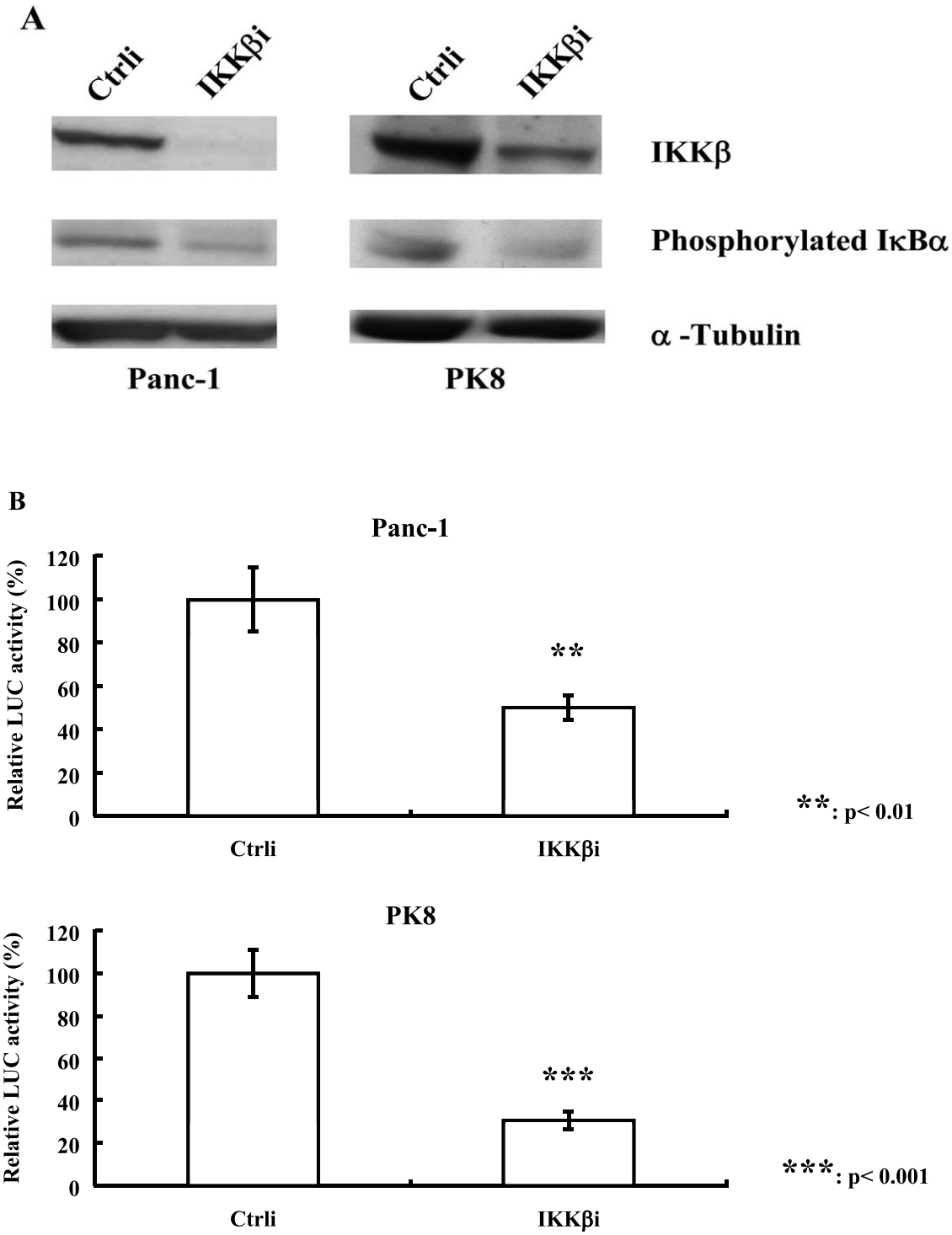

Fig. 1. Knock-down of IKKβ suppressed constitutive NF-κB activation in Panc-1 and PK8 cells. **(A)** Panc-1 and PK8 cells were infected with lentivirus capable of expressing shRNA to *IKKβ* (IKKβi) or *renilla luciferase* (Ctrli) and selected in the presence of 2 μg/ml of puromycin for 3 days. Whole-cell lysates (30 μg) were subjected to SDS-PAGE and immunoblotting with anti-IKKβ (top panel), anti-phosphorylated-IκBα (middle panel) or anti-α-tubulin (bottom panel) antibodies. **(B)** Cells were transiently transfected with 0.5 μg of IgκCona-luc, 0.5 μg of EF1-lacZ and 1.0 μg of CS-puro-Ctrli or CS-puro-IKKβi, and then harvested 72 hr after transfection. Luciferase activity was normalized on the basis of β-galactosidase activity. The results are expressed as an average with s.d. (N=3, Panc-1; P<0.01, PK8; P< 0.001)

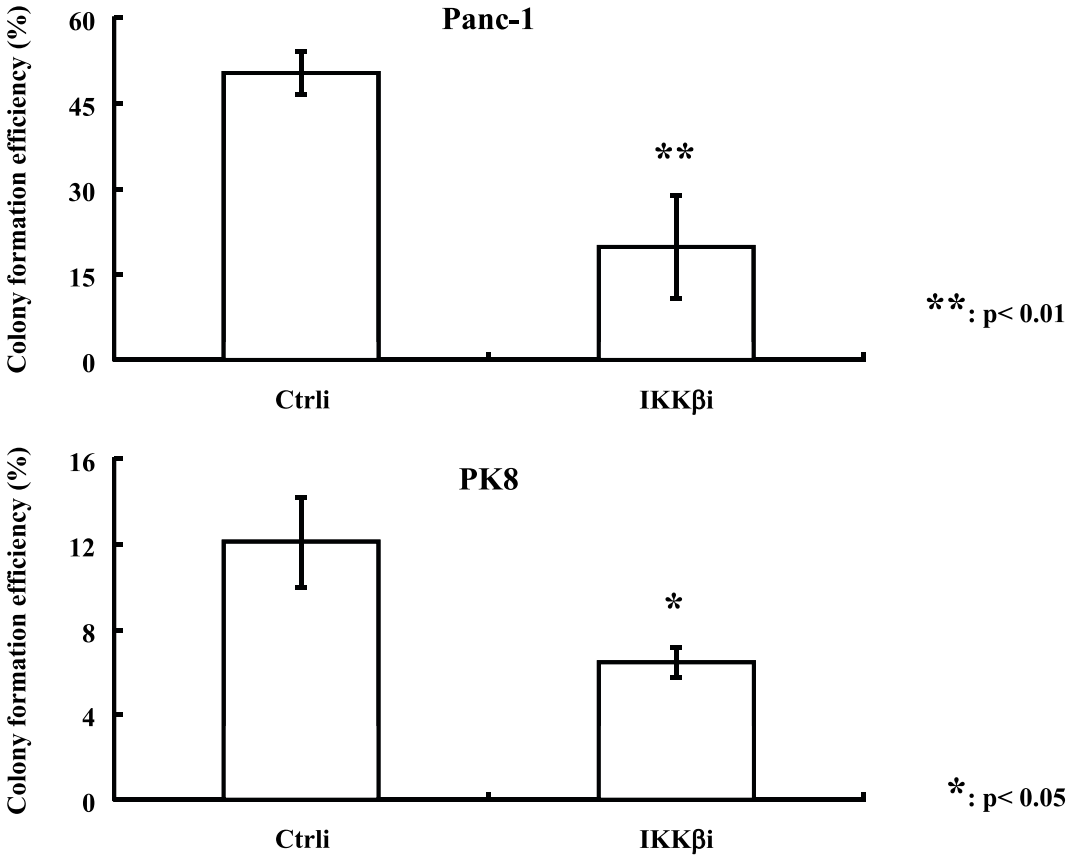

Fig. 2. Knock-down of IKKβ interfered with anchorage-independent growth of Panc-1 and PK8 cells in soft agar. Lentivirus-infected Panc-1 and PK8 cells were seeded in 0.33% soft agarose. Colonies larger than 60 μm were counted 3 weeks after inoculation. The results are expressed as an average with s.d. (N=3, Panc-1; P<0.01, PK8; P< 0.05)

not the control virus, efficiently suppressed IKKβ expression (Fig. 1A). As expected, the specifically phosphorylated form of IκBα was reduced in either cell lines, suggesting that shIKKβ expression successfully inhibited endogenous IKKβ activity. Consistently, assays of transcription with an NF-κB-dependent luciferase reporter gene revealed that transient expression of shIKKβ suppressed constitutive NF-κB activity in these pancreatic cancer cells (N=3, Panc-1; P < 0.01, PK8; P < 0.001) (Fig. 1B). To assess the biological impact of NF-κB suppression in pancreatic cancer cells, we examined efficiency of colony formation in soft agar. shIKKβ expression resulted in significant reduction in the number of colonies larger than 60 μm in diameter, compared with the control shRNA expression (N=3, Panc-1; P < 0.01, PK8; P < 0.05) (Fig. 2). These data suggest that IKKβ is a promising molecular target for reducing NF-κB activity in pancreatic cancer cells.

IMD-0354 inhibits constitutive NF-κB activity in pancreatic cancer cells

RNA interference-mediated IKKβ suppression efficiently suppressed NF-κB activity in pancreatic cancer cells, but is not ready for clinical application, because an effective gene delivery to pancreatic cancer cells has not been established. Instead, use of IMD-0354, a novel IKKβ inhibitor that has successfully been used to suppress neoplastic proliferation of human mast cells[26] and breast cancer cells[19] with constitutive NF-κB activity, could be an alternate choice. Panc-1 and PK8 cells were incubated in medium containing increasing concentrations of IMD-0354 for 24, 48, and 72 hr, and their viability was determined. As shown in Fig. 3A, IMD-0354 suppressed the proliferation of these cells compared to control cells. In Fig. 3B, the number of the cells undergoing apoptosis was measured. Panc-1 and PK8 cells incubated for 48 hr with increasing concentrations of IMD-0354 expressed phosphatidyl serine outside the cell membrane, a typical marker of apopto-

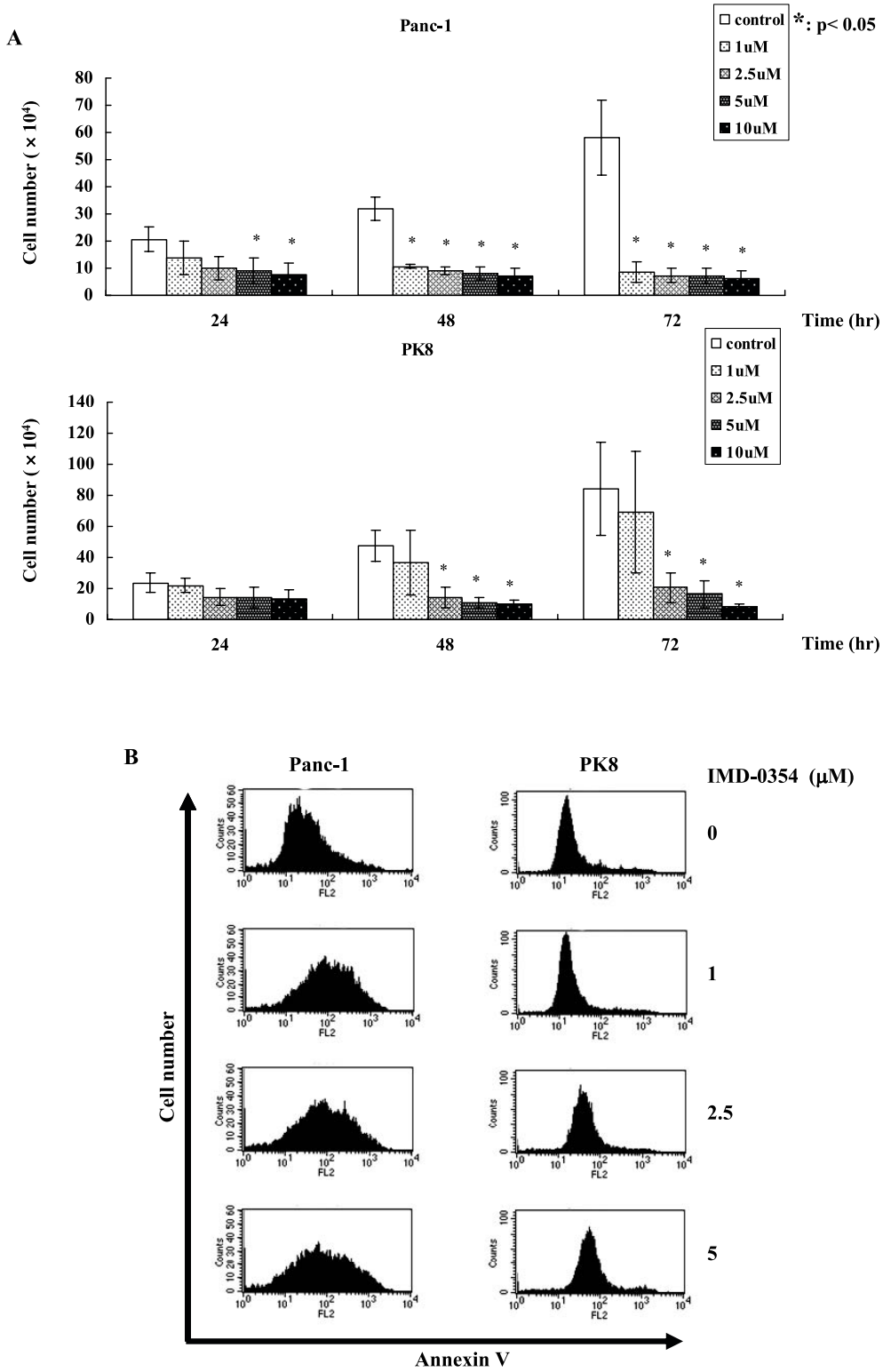

Fig. 3. IMD-0354 inhibited proliferation of Panc-1 and PK8 cells. **(A)** Approximately 1×10^5 Panc-1 and PK8 cells were inoculated with increasing concentrations of IMD-0354 for the indicated periods. Viable cells were counted by trypan-blue dye exclusion test. The results are expressed as an average with s.d.. The asterisks represent $p < 0.05$ by Dunnet test. **(B)** The apoptotic status was analyzed based on Annexin V-binding to cells. Panc-1 and PK8 cells (1×10^6 cells) were incubated for 48 hr in the presence of the indicated concentrations of IMD-0354.

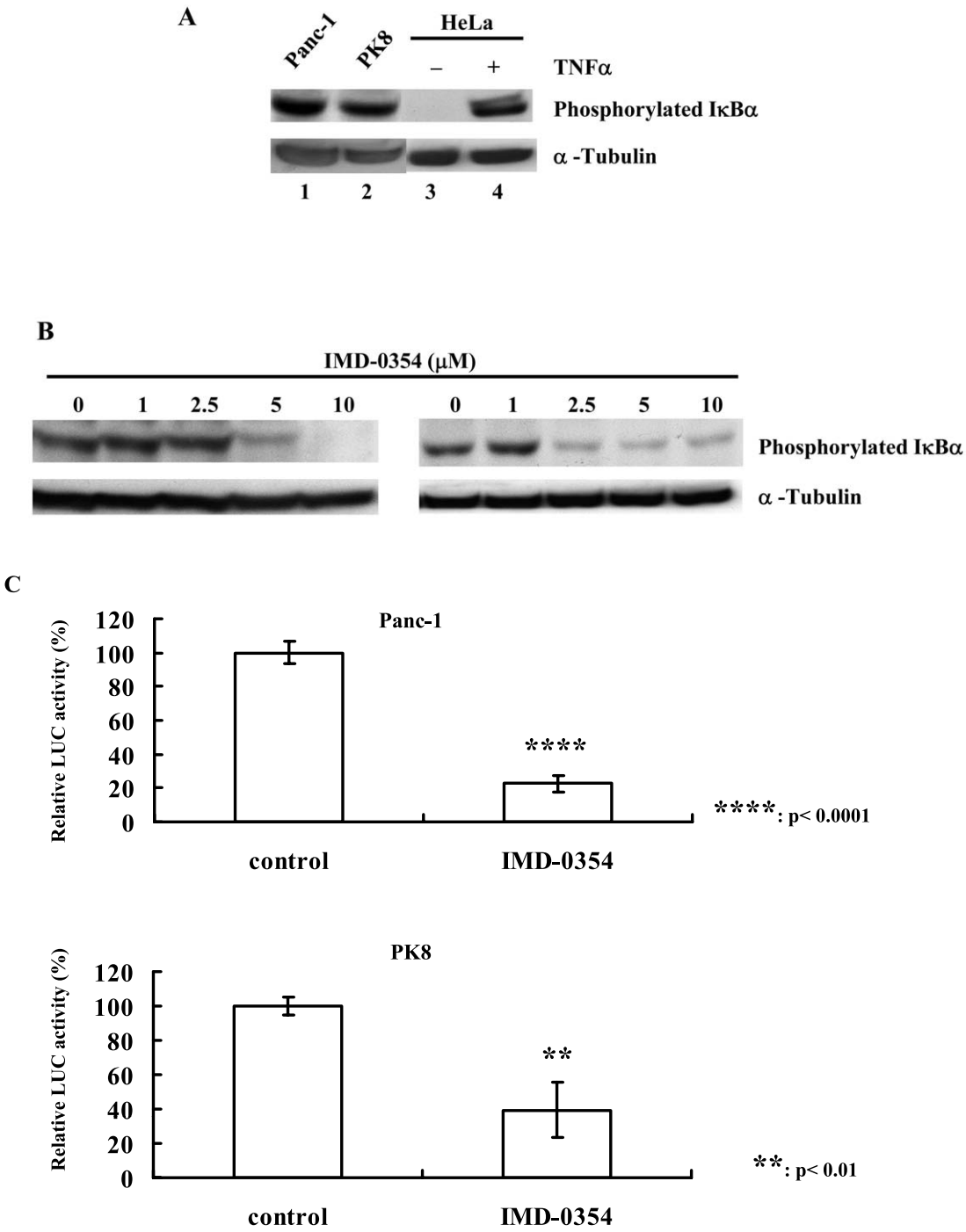

Fig. 4. IMD-0354 inhibited constitutive NF-κB activation in Panc-1 and PK8 cells. **(A)** Whole-cell lysates (30 μg) of Panc-1 (lane 1) and PK8 (lane 2) cells were subjected to SDS-PAGE and immunoblotting with anti-phospho-IκBα or anti-α-tubulin antibody. HeLa cells were treated with (lane 4) or without (lane 3) TNF-α for 5 min as positive and negative controls for detection of phosphorylated IκBα. **(B)** Panc-1 and PK8 cells were cultured in the presence of the indicated concentrations of IMD-0354 for 48 hr. Whole-cell lysates (30 μg) of Panc-1 and PK8 cells were subjected to SDS-PAGE and immunoblotting with anti-phospho-IκBα or anti-tubulin antibody. Alternatively, cells were cultured in the presence of IMD-0354. Whole-cell lysates (30 μg) were subjected to SDS-PAGE and immunoblotting with anti-phospho-IκBα or anti-tubulin antibody. **(C)** Cells were transfected with 0.5 μg of IgκCona-luc, 0.5 μg of EF1-lacZ, and treated 12 hr later with 0.1% DMSO or IMD-0354 (2.5 μM) for 36 hr. Luciferase activity was determined 48 hr after transfection and normalized on the basis of β-galactosidase activity. The results are expressed as an average with s.d. (N=3, Panc-1; P<0.0001, PK8; P< 0.01)

sis, which was detected by Annexin V. Immunoblot analyses showed that both Panc-1 and PK8 exhibited phosphorylation of IκBα (Fig. 4A) and that incubation with higher doses of IMD-0354 reduced this phosphorylation (Fig. 4B). Transient transfection of Panc-1 and PK8 cells with the NF-κB-dependent luciferase reporter gene revealed that constitutive NF-κB activity in pancreatic cancer cells was remarkably down-regulated when cells were treated with IMD-0354 for 36 hours (N=3, Panc-1; $P < 0.0001$, PK8; $P < 0.01$) (Fig. 4C). We next examined if IMD-0354 affects colony formation of pancreatic cancer cells in soft agar. Incubation of cells in soft agar with IMD-0354 for three weeks reduced colonies in number (Fig. 5A) and the size of colonies (Fig. 5B) compared to control cells.

IMD-0354 suppresses tumor growth in NOG mice

To evaluate the effect of IMD-0354 on tumor growth, Panc-1 cells were inoculated subcutaneously in NOG mice. Mice received 20 mg/kg of IMD-0354 intraperitoneally everyday for 4 weeks. The data showed a significant difference in tumor growth between the IMD-0354-treated and control mice. On day 28th, the average volume of tumor was 72.1±26.8 mm^3 in the mouse group receiving IMD-0354, which was significantly smaller than that in the control group (159.7±68.0mm^3; N=5, $P < 0.05$) (Fig. 6A, B). It is important to note that no toxic effects, such as body weight loss, vomiting, or dermatitis were observed in any of the IMD-0354-treated mice during the entire course of the experiment.

Discussion

Despite advances in our understanding of the molecular and genetics basis of pancreatic cancer, the disease remains a clinical challenge. It is because pancreatic cancer has often a highly invasive and metastatic phenotype, and is diagnosed mostly at an advanced state[27], limiting the possibility of surgical resection that is the only curative option. Current therapies for pancreatic cancer thus rely on traditional cytotoxic agents with only limited effects[28], because pancreatic cancer cells have profound resistance to anticancer drugs[29-32]. One of the important determinants of anti-apoptotic responses in pancreatic cancer cells is the constitutive activation of the transcription factor NF-κB[33,34]. NF-κB is constitutively activated in about 67% of pancreatic adenocarcinomas compared with normal pancreatic tissue[33,35]. Treatment with various NF-κB inhibitors or expression of a super-repressor form of IκBα strongly enhanced apoptotic effects of chemotherapeutic drugs on otherwise resistant pancreatic cancer cells[36,37].

We demonstrated for the first time that targeted depletion of IKKβ effectively suppressed NF-κB activity and anchorage-independent cell growth of pancreatic cancer cells, which strongly argues that IKKβ is a promising molecular target for cancer therapy. It may be further desirable to inhibit NF-κB activation in cancer cells through identifying a cancer-specific target(s), but the mechanisms of persistent NF-κB activation in many types of cancer, including pancreatic cancer, remain unknown. Thus, targeting a central signaling molecule such as IKKβ is likely to be a rational and realistic strategy for improving the prognosis of patients diagnosed with this deadly disease. In addition, pancreatic cancer is primarily located in the retro-peritoneum and often invades to the surrounding tissues and metastasizes to distant organs. This hampers successful delivery of anti-cancer agents to pancreatic cancer, and there is a dire need for developing an effective chemotherapeutic regimen.

For the above reasons, we focused on a novel inhibitor IMD-0354, which was molecularly designed to dock with the ATP-binding site of IKKβ, a central kinase for NF-κB activation. Recently, IMD-0354 was reported to suppress neoplastic proliferation of human mast cells[26] and breast cancer cell lines[19]. In these reports, IMD-0354 suppressed constitutive NF-κB activity and induced apoptosis by arresting the cell cycle progression. It is important to point out that growth inhibitory effects of pancreatic cancer cells were observed below the concentrations generated in humans through oral intake of the drug. Another important aspect of anti-cancer drugs is its toxicity to normal cells and tissues. In this regard, it should be emphasized that daily administration of 20 mg/kg of IMD-0354, which effectively suppressed tumor growth in mice, did not cause body weight loss or any other deleterious effect. Thus, this drug can be used safely *in vivo*, although its long-term effects on the host immune system remain to be studied in immune-competent mice. Additional experiments, beyond the scope of this report, are underway to clarify how IMD-0354 can be used in combination with other anti-cancer drugs.

Collectively, our results indicate that IKKβ could be an attractive molecular target for the treatment of pancreatic cancer. New strategies for combined chemotherapy of pancreatic cancer could be

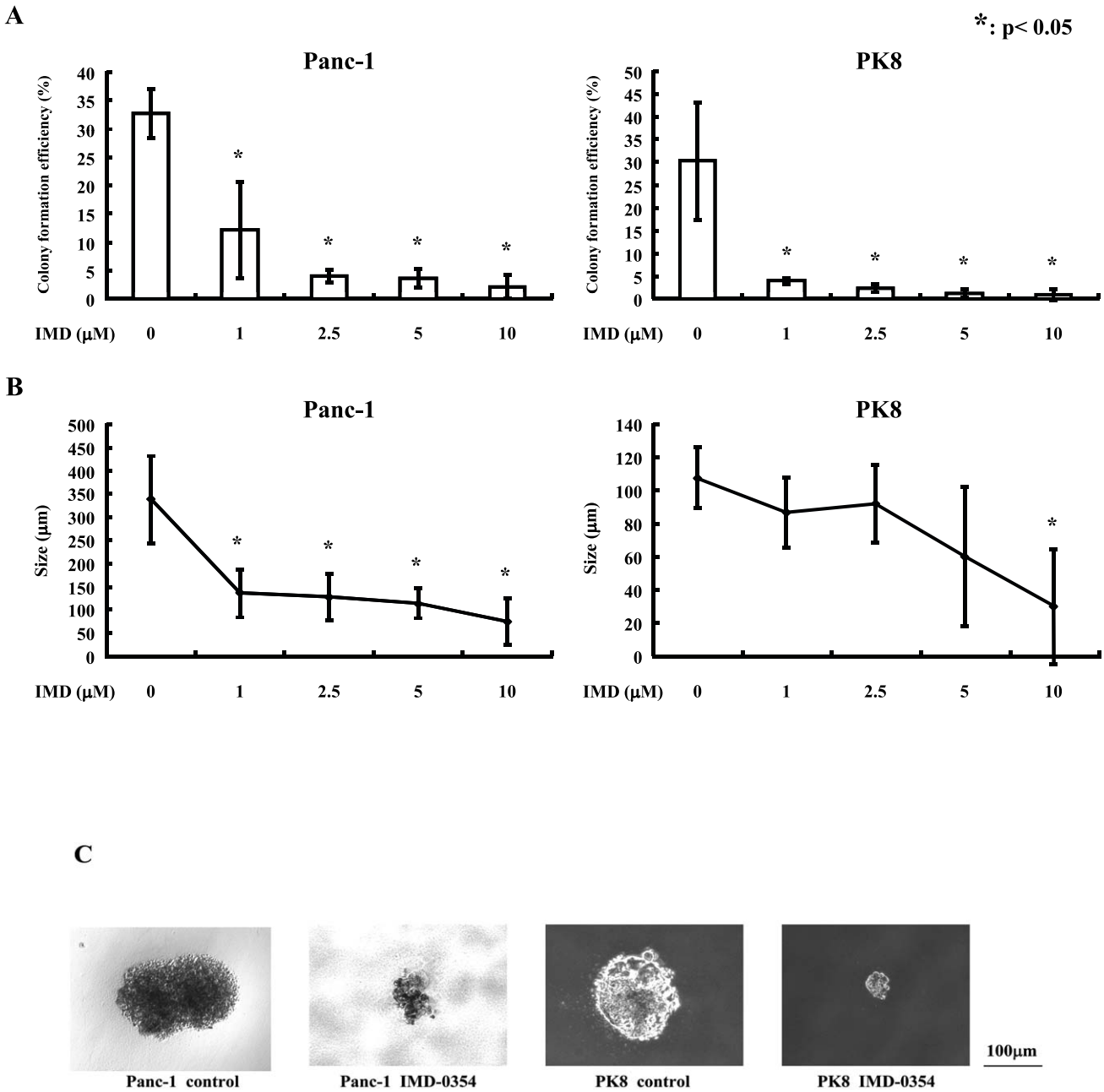

Fig. 5. IMD-0354 suppressed anchorage-independent growth of Panc-1 and PK8 cells. Approximately 1×10^4 Panc-1 and PK8 cells were inoculated in 0.33% soft agar containing 0.1% DMSO, 1, 2.5, 5 or 10 μmol/L of IMD-0534 and incubated for 3 weeks. **(A)** Colonies larger than 60 μm were microscopically counted as positive. The results are expressed as an average with s.d.. The asterisks represent p <0.05 by Dunnet test. **(B)** Average size of positive colonies was calculated and analyzed. . The asterisks represent p <0.05 by Dunnet test. **(C)** Phase-contrast micrographs of representative Panc-1 and PK8 cell colonies grown for 3 weeks in soft agar containing 0.1% DMSO or 2.5 μmol/L of IMD-0354.

designed, in which specific inhibition of IKKβ leads to effective NF-κB suppression, and thereby renders otherwise chemo-resistant cells sensitive to anti-cancer drugs.

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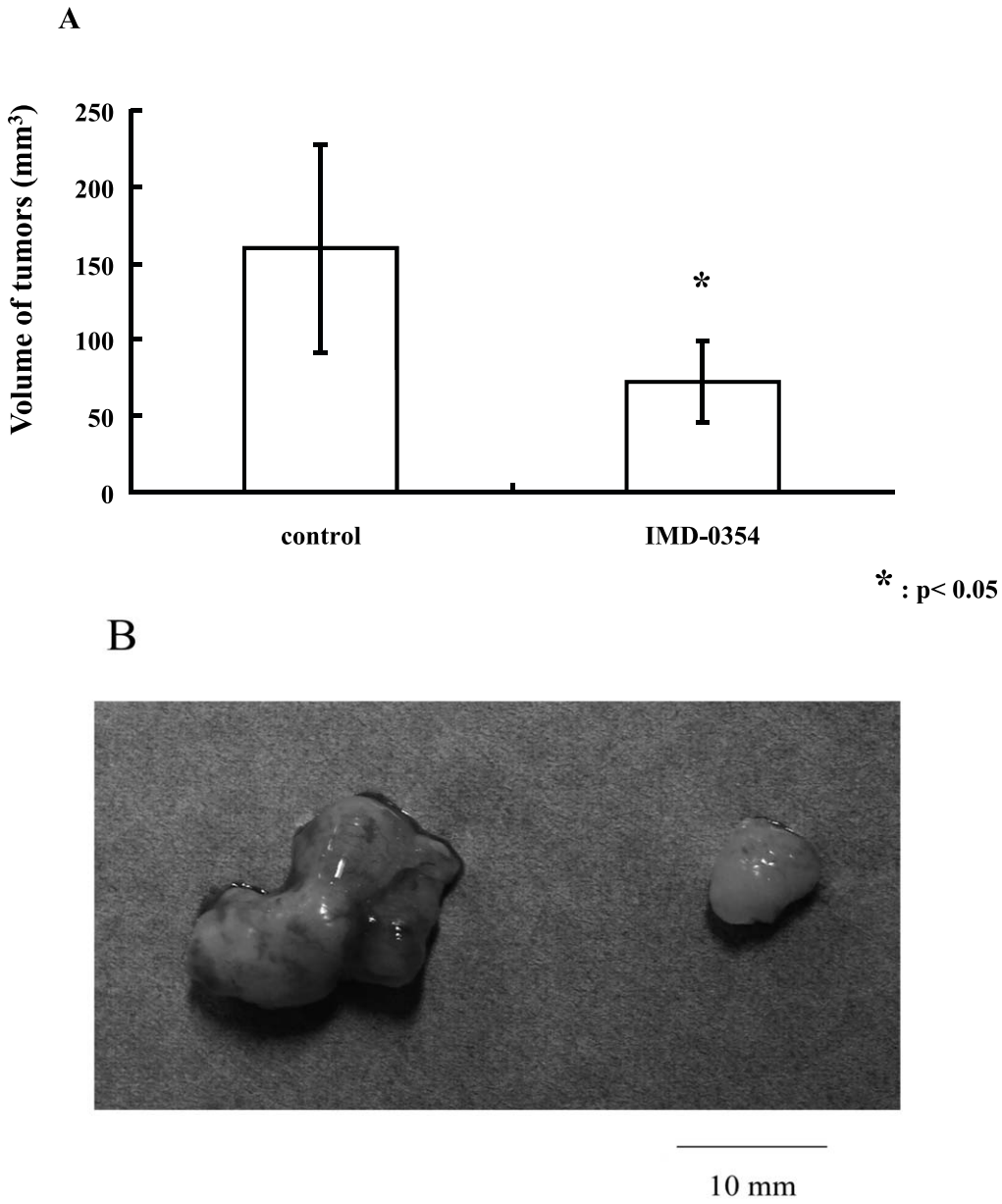

Fig. 6. IMD-0354 suppresses tumor formation in NOG mice. **(A)** Approximately 1×10^7 Panc-1 cells were inoculated subcutaneously in NOG mice. IMD-0354 (20mg/kg body weight) or saline was injected daily for 28 days intraperitoneally, and then the volume of tumors was calculated. The results shown (N=5, P<0.05) are the average±s.d.. **(B)** Tumors excised from control (left) and IMD-0354-treated (right) mice. Bar indicates 10 mm.

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