

# 4-Methylumbelliferone inhibits enhanced hyaluronan synthesis and cell migration in pancreatic cancer cells in response to tumor-stromal interactions

XIAO-BO CHENG<sup>1</sup>, NORIHIRO SATO<sup>2</sup>, SHIRO KOHI<sup>2</sup>, ATSUHIRO KOGA<sup>2</sup> and KEIJI HIRATA<sup>2</sup>

<sup>1</sup>Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, P.R. China; <sup>2</sup>Department of Surgery 1, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Fukuoka 807-8555, Japan

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**Abstract.** Hyaluronic acid (HA) in tumor stroma promotes tumor invasion and progression. 4-Methylumbelliferone (4-MU) is a potent HA synthesis inhibitor. In the present study, the effects of 4-MU on enhanced HA synthesis and cell migration in pancreatic ductal adenocarcinoma (PDAC) cells, in response to co-culture with stromal fibroblasts, was investigated. The HA concentration was determined using ELISA and a Transwell migration assay was used to analyze cell migratory capability. The mRNA expression levels of hyaluronan synthases (*HAS1*, *HAS2* and *HAS3*) were determined using the quantitative polymerase chain reaction. Co-culture between Panc-1 cells and stromal fibroblasts markedly increased cell migration in association with increasing HA production, which was markedly associated with an increase in *HAS3* mRNA expression. Treatment with 4-MU markedly decreased the HA production and cell migration of Panc-1 cells in the co-culture system. The results of the present study suggested that interactions between PDAC cells and stromal fibroblasts increased HA production, resulting in a marked increase in migration of PDAC cells, and 4-MU may be used

as a chemotherapeutic agent to inhibit the enhanced migration of PDAC cells in response to tumor-stromal interactions.

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a life-threatening malignant neoplasm which may invade and metastasize at an early stage (1). The deoxycytidine analogue gemcitabine [2',2'-difluorodeoxycytidine (dFdC)] remains the standard of care for disseminated PDAC, prolonging the survival time by >5 weeks in a minority of patients (2). Gemcitabine-based combination therapies may offer a survival benefit by decreasing the progression of PDAC (3). A previous study has identified that targeted inhibition of the epidermal growth factor receptor with erlotinib increases the median survival time by 2 weeks (4). PDAC exhibits a poor response to chemotherapy; therefore, the identification of an effective therapy for treating advanced PDAC is required.

Hyaluronan (HA) is synthesized by three types of HA synthase (HAS) termed HAS1, HAS2 and HAS3 (5). HA is synthesized in distinct amounts and sizes, depending on the type of synthase: HAS1 and HAS2 synthesize low and high (in the range of millions of Da) amounts of high-molecular-weight HA (HMW-HA), respectively, whereas HAS3 produces high amounts of low-molecular-weight HA (LMW-HA), in the range of several thousands of Da (6). The size of HA may vary between 20 kDa and 10 MDa, and, depending on the size, extracellular HA regulates a number of cellular biological functions, including cell motility, tumor viability, migration, metastasis, chemotherapeutic resistance and cytokine production, via direct interactions with cell-surface receptors (7-9). A number of human PDAC cell lines synthesize and secrete HA (10), and the highest distribution of HA between the tumor mass and the normal tissue is exhibited in human primary pancreatic carcinomas, suggesting that HA may promote tumor invasion and form a barrier for cancer cells against host immunocompetent cells and anticancer agents (11-14). A previous study revealed the association between increased expression of HA and poor prognosis in pancreatic cancer (15). Therefore, inhibiting HA synthesis to control tumor invasion and drug resistance, and subsequently to improve prognosis in patients with PDAC is required.

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*Correspondence to:* Dr Norihiro Sato, Department of Surgery 1, School of Medicine, University of Occupational and Environmental Health, 1-1, Iseigaoka, Kitakyushu, Fukuoka 807-8555, Japan  
E-mail: norisato@med.uoeh-u.ac.jp

Dr Xiao-Bo Cheng, Shanghai Public Health Clinical Center, Fudan University, 2901 Caolang Road, Jin Shan, Shanghai 201508, P.R. China  
E-mail: cl3840564883@163.com

*Abbreviations:* HA, hyaluronic acid; 4-MU, 4-methylumbelliferone; PDAC, pancreatic ductal adenocarcinoma; HAS, hyaluronan synthase; dFdC, 2',2'-difluorodeoxycytidine; HMW-HA, high-molecular-weight hyaluronan; LMW-HA, low-molecular-weight hyaluronan; UGT, uridine diphosphate-glucuronyltransferase; UDP-GlcUA, uridine diphosphate-glucuronic acid

*Key words:* pancreatic ductal adenocarcinoma, hyaluronan, hyaluronan synthase, migration, 4-methylumbelliferone

4-Methylumbelliferone (4-MU; 7-hydroxy-4-methylcoumarin) has been identified to be an inhibitor of HA synthesis in a number of previous studies (16-28). In particular, the inhibitory effect of 4-MU on HA synthesis demonstrates anticancer effects through decreasing cell viability, adhesion, migration and invasion (19,25-28), and increasing the efficacy of anticancer agents (23). The mechanisms that enable 4-MU to inhibit HA synthesis remain unclear. It has been hypothesized that 4-MU inhibits HA synthesis via glucuronidation by endogenous uridine phosphate (UDP)-glucuronosyltransferase (UGT), which results in the depletion of UDP-glucuronic acid (UDP-GlcUA) (20), and a decrease in HAS mRNA levels (24,25). In addition, previous studies have identified that 4-MU decreases the expression of a number of matrix metalloproteinases (29,30) and cell-adhesion molecules (31), alters phosphorylation of intracellular proteins (26,32,33) and increases levels of UGT1 enzymes, leading to decreased UDP-GlcUA levels (24).

The present study aimed at investigating whether the interaction between PDAC cells and fibroblasts may increase HA production and cell migration. Furthermore, whether 4-MU may decrease the migration of PDAC cells in co-culture with fibroblasts was investigated.

## Materials and methods

**Cell lines and reagents.** The Panc-1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Primary fibroblasts derived from PDAC tissues were a gift from Kyushu University (Fukuoka, Japan). All the cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (all from Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. 4-MU was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

**ELISA determination of HA concentrations.** HA concentrations in cell culture media were determined according to a previous protocol (15). The quantity of HA was expressed per ml.

**Migration assay.** Panc-1 cells (2x10<sup>5</sup> cells/ml) in 250 μl serum-free RPMI-1640 medium were seeded to the upper chamber (24-well insert, 8 μm pore size; BD Biosciences, Franklin Lakes, NJ, USA). A total of 750 μl serum-free RPMI-1640 medium was added to the lower chamber as a monoculture. Primary fibroblasts (ike-f3 cells) in 750 μl serum-free RPMI-1640 medium were seeded (1x10<sup>5</sup> cells/ml) in the lower chamber as a co-culture without or with various concentrations (10, 100 and 1,000 μM) of 4-MU for 72 h. Additionally, 7.5x10<sup>4</sup> cells/ml ike-f3 cells were seeded in serum-free RPMI-1640 medium into the lower chamber for 72 h as a fibroblast monoculture. The supernatant fractions of monoculture and co-culture were divided into aliquots and stored at -80°C until use. Non-migrating cells on the upper surface of the membrane were removed with a cotton swab. Migrating cells penetrated onto the lower surface of the membrane and were fixed with 70% methanol, stained with hematoxylin (at room temperature for 10 min) and eosin (at room temperature, between 5-10 min) and air-dried. The

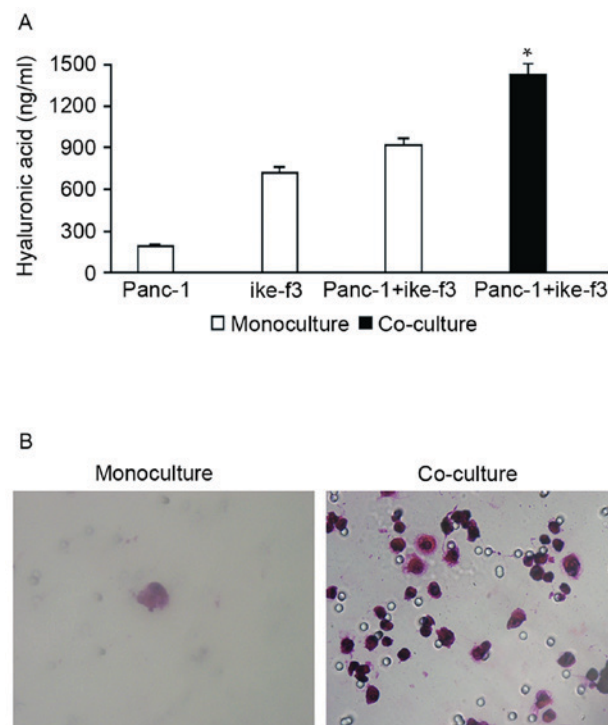


Figure 1. Alterations in HA production and pancreatic ductal adenocarcinoma cell migration in response to co-culture with fibroblasts. (A) ELISA demonstrating significantly increased HA production in co-culture system between Panc-1 cells and ike-f3 cells, compared with the sum of the incorporations by each cell line cultured alone ( $P < 0.05$  vs. monocultures, paired Student's t-test). Results are presented as the mean  $\pm$  standard deviation of three replicates. (B) Transwell migration assay (magnification, x400) revealed that the co-culture system markedly increased the number of migrating Panc-1 cells. Results are presented as the mean  $\pm$  standard deviation of six replicates. HA, hyaluronan.

number of migrating cells was determined in 6 randomly selected fields at x400 magnification by light microscope. Subsequently, the average number of cells per microscopic field was calculated as the extent of migration.

**Co-culture system.** Panc-1 cells (2x10<sup>5</sup> cells/ml) in serum-free medium were seeded in the upper chamber (High Density, Translucent PET Membrane 6-well insert, 0.4 μm pore size; BD Biosciences) and 3 ml serum-free medium was added into the lower chamber as a monoculture. Ike-f3 cells (1x10<sup>5</sup> cells/ml) in 3 ml serum-free medium were seeded in the lower chamber as a co-culture for 72 h. All samples were used to extract RNA.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** mRNA expression analysis of *HAS1* (Hs00758053\_m1), *HAS2* (Hs00193435\_m1), *HAS3* (Hs00193436\_m1) and *GAPDH* (Hs02758991\_g1), as a control (Applied Biosystems; Thermo Fisher Scientific, Inc.), were performed (monoculture and co-culture), according to a previously described protocol (34).

**Trypan blue dye-exclusion assay cellular viability test.** The effects of 4-MU (10, 100 and 1,000 μM) on cell viability were analyzed using trypan blue dye-exclusion (TBDE) assays as cytotoxic measurements. After 72 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, the untreated and treated

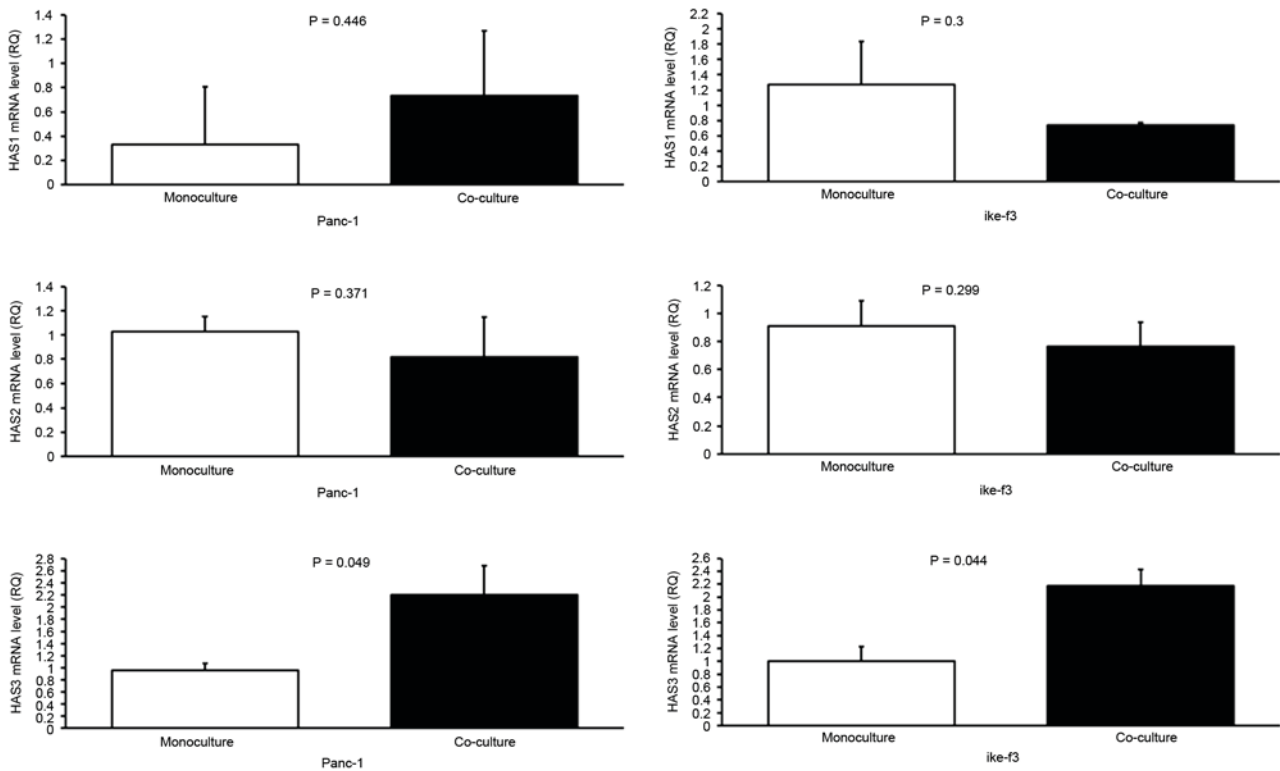


Figure 2. Alterations in *HAS1*, *HAS2* and *HAS3* mRNA expression levels in the co-culture system. *HAS3* mRNA expression was significantly increased in Panc-1 cells ( $P=0.049$ ) and ike-f3 cells ( $P=0.044$ ), compared with monoculture. Results are presented as the mean  $\pm$  standard deviation of three replicates. HAS, hyaluronan synthase; RQ, relative quality.

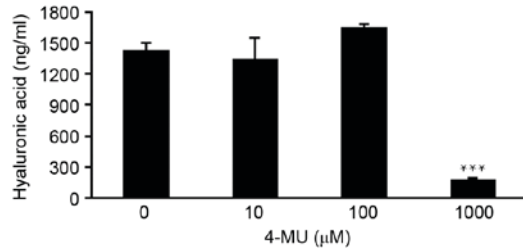


Figure 3. HA concentration in the co-culture system following treatment with 4-MU. HA synthesis was significantly decreased following treatment with 1,000  $\mu\text{M}$  4-MU, but was essentially unchanged at 10 and 100  $\mu\text{M}$  ( $^{***}P<0.001$ , one-way analysis of variance and Fisher's least significant difference test). Results are presented as the mean  $\pm$  standard deviation of three replicates. HA, hyaluronan; 4-MU, 4-methylumbelliferone.

cells were harvested and stained with 4% trypan blue at room temperature and then counted by the LUNA<sup>TM</sup> automated cell counter (Logos Biosystems, Annandale, VA, USA) according to the manufacturer's protocol. Cytotoxicity was determined from the number of viable cells (no color) in treated samples as a percentage of the untreated control.

**Statistical analysis.** Data were expressed as the mean  $\pm$  standard deviation. All statistical analyses were performed using SPSS software (version 21.0; IBM Corp., Armonk, NY, USA). Differences in HA concentration and *HAS1*, *HAS2* and *HAS3* mRNA levels between monoculture and co-culture were compared using a paired Student's t-test. Comparisons between HA concentration and the migrating cell number, in all subgroups with various concentrations of

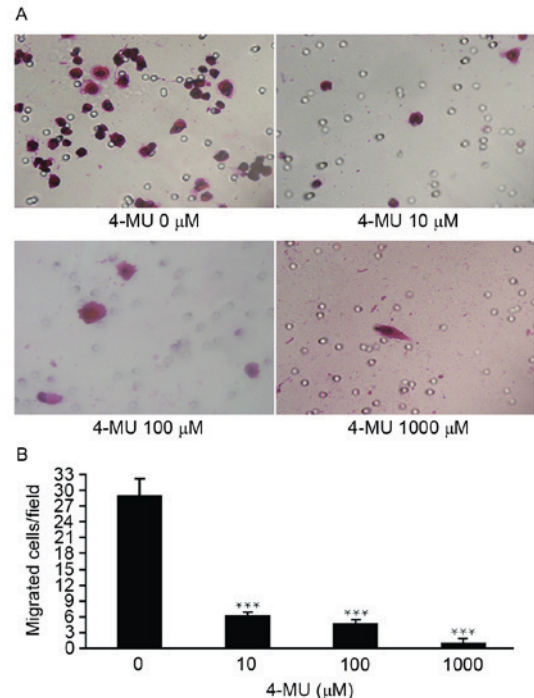


Figure 4. Alterations in the migration of pancreatic ductal adenocarcinoma cells in the co-culture system following treatment with 4-MU using a Transwell migration assay. (A) Photomicrographs of migrating cells on the underside of chambers (magnification,  $\times 400$ ). Migrating cells penetrated onto the lower surface of the membrane and were fixed with 70% methanol, stained with hematoxylin and eosin, and air-dried. (B) Panc-1 cell migration was inhibited by 4-MU in a dose-dependent manner ( $^{***}P<0.001$ , one-way analysis of variance and Fisher's least significant difference test). Results are presented as the mean  $\pm$  standard deviation of six replicates. HA, hyaluronan; 4-MU, 4-methylumbelliferone.

4-MU, were made using one-way analysis of variance and Fisher's least significant difference test.  $P < 0.05$  indicated a statistically significant difference. All  $P$ -values were two-tailed and all investigations were repeated three times independently.

## Results

*Stimulation of HA production and cell migration in the co-culture system between human PDAC cells and fibroblasts.* Co-culture of Panc-1 cells with fibroblasts resulted in a significant increase ( $P = 0.016$ ) in HA production, compared with those in monocultures (Fig. 1A). In addition, the Transwell migration assay revealed that co-culture with fibroblasts significantly increased the migration of Panc-1 cells (Fig. 1B).

To elucidate the mechanism of enhanced HA production by co-culture system, the mRNA expression levels of *HAS1*, *HAS2* and *HAS3* in Panc-1 cells and fibroblasts was investigated using RT-qPCR. The increased HA production was associated with a significantly increased mRNA expression of *HAS3*, but not *HAS1* and *HAS2* (Fig. 2).

*Effects of 4-MU on HA biosynthesis and cell migration in the co-culture system.* Panc-1 cells in the co-culture system were treated with various concentrations (10, 100 and 1,000  $\mu\text{M}$ ) of 4-MU. The results demonstrated that no marked effects on the cell viability were observed following treatment with the aforementioned range of 4-MU concentrations (data not shown). HA synthesis was inhibited by 88%, compared with the control, following treatment with 1,000  $\mu\text{M}$  4-MU; however, treatment with 10 and 100  $\mu\text{M}$  revealed almost no alterations in HA production (Fig. 3).

Panc-1 cell migration was evaluated using a Transwell migration assay (Fig. 4A), which revealed that 4-MU inhibited Panc-1 cell migration in co-culture with fibroblasts. Inhibition of cell migration was observed at 10  $\mu\text{M}$  and maximal inhibition was revealed to be at 1,000  $\mu\text{M}$  (Fig. 4B).

## Discussion

A previous study identified interactions between tumor cells and fibroblasts which stimulated HA synthesis and identified that HA is increased in tumors (35). In the present study, Panc-1 cells were co-cultured with fibroblasts which resulted in a marked increase in HA synthesis. Additionally, *HAS3* mRNA expression in Panc-1 cells and fibroblasts was significantly increased in this co-culture system. The results of the present study suggested that, for the first time, to the best of our knowledge, the increase in HA in a PDAC co-culture system was associated with markedly increased mRNA expression of *HAS3*. *HAS3* may produce increased amounts of LMW-HA (6) and this contributes to tumor progression by increasing the motility of cancer cells (36-38). These interactions may explain why the co-culture system markedly increased PDAC cell migration.

HA production may be decreased by 4-MU via the depletion of cellular UDP-GlcUA and the downregulation of *HAS2* and/or *HAS3*, and inhibit cell viability, migration and invasiveness (25). Therefore, PDAC cells in the co-culture system were treated with 4-MU. The results revealed a marked decrease in HA production and PDAC cell migration. This

drug is promising because the safety of 4-MU for humans has already been confirmed. Oral 4-MU has been used to treat hepatobiliary disease due to the cholagogic and spasmolytic actions exhibited on the sphincter of Oddi (39,40). The results of the present study demonstrated that the interaction between PDAC cells and fibroblasts stimulated HA production and PDAC cell migration, and 4-MU may inhibit HA synthesis and cell migration.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Author's contributions

NS, XC and KH conceived the experimental design. XC, SK and AK performed the experiments. XC analyzed the data. XC, NS and KH wrote the paper. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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