Establishment of a Three-dimensional Floating Cell Culture System for Screening Drugs Targeting KRAS-mediated Signaling Molecules

TOSHIYUKI TSUNODA[1,2*], SHUHEI ISHIKURA[1,2*], KEIKO DOI[1,2], YURI IWAIHARA[1], HIROMASA HIDESIMA[1], HAO LUO[1], YUMIKO HIROSE[1,2] and SENJI SHIRASAWA[1,2]

[1]*Department of Cell Biology, Faculty of Medicine Fukuoka University, Fukuoka, Japan;*
[2]*Central Research Institute for Advanced Molecular Medicine, Fukuoka University, Fukuoka, Japan*

Abstract. *Background/Aim: Oncogenic mutations in the KRAS gene are critically involved in many human tumors but drugs targeting oncogenic KRAS have not yet been clinically developed. Herein, we established a three-dimensional floating (3DF) culture system for screening drugs that target KRAS-mediated signaling molecules. Materials and Methods: HKe3 cells, derived from colorectal cancer HCT116 cells and disrupted at mutated (mt) KRAS gene, were infected with a retrovirus expressing wild-type (wt) KRAS or mtKRAS to establish HKe3-derived cells expressing wtKRAS or mtKRAS. Established cells were cultured in 96-well plates with an ultra-low attachment surface and round bottom for 3DF culture. Results: HKe3-wtKRAS and HKe3-mtKRAS cells in 3DF culture rapidly assembled into respective single spherical structures (spheroids). Furthermore, mtKRAS but not wtKRAS expression inhibited luminal apoptosis in spheroids indicating that the 3DF culture was compatible with the 3D matrigel culture. Conclusion: This 3DF culture system could be useful for screening drugs that target KRAS-mediated signaling molecules.*

Mutations in the *KRAS* gene are common in human colorectal cancer (CRC) (1, 2). KRAS is involved in the transduction of mitogenic signals in response to extracellular signals, including growth factors, cytokines and hormones (3). The KRAS protein is a small GTPase and functions as a molecular switch (4). The exchange of KRAS between the active GTP-bound form and the inactive GDP-bound form is tightly regulated by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (5). Oncogenic mutations in *KRAS* are invariably point-mutations that either interfere with GAP binding or directly disrupt the GTPase activity (6). Thus, mutated KRAS (mtKRAS) results in a constitutively active GTP-bound form, which further leads to the activation of the downstream pro-proliferative and anti-apoptotic signalling.

We have previously reported many types of critical roles of mtKRAS in tumorigenesis by comparing human CRC HCT116 cells with HKe3 cells, which are derived from HCT116 cells and are specifically disrupted at the mt*KRAS* gene (7-11). Indeed, HKe3 cells lose anchorage-independent growth and tumorigenecity in nude mice, whereas the parental HCT116 cells exhibit both these traits (7). Despite intensive efforts, no effective KRAS-targeting therapies have successfully made it to the clinic level (2). Therefore, the development of a screening system to identify drugs that target KRAS-mediated signaling molecules is required.

The three-dimensional (3D) culture of cells closely resembles the *in vivo* microenvironment of tissues. Therefore, a 3D culture system provides the ability to directly investigate the importance of cell-cell and cell-extracellular matrix interactions, which are frequently de-regulated in tumorigenesis (12, 13). We have previously compared HKe3 cells with HCT116 cells in the 3D matrigel culture and found that HKe3 cells formed a polarised luminal structure with concomitant luminal apoptosis, whereas HCT116 cells formed a structure without cellular polarity or luminal apoptosis (14), indicating mtKRAS involvement in the inhibition of luminal apoptosis and disruption of cellular polarity. Furthermore, phosphodiesterase 4B (PDE4B) expression in the 3D culture was upregulated in HCT116 cells compared with that of HKe3 cells. PDE4 inhibitors,

*These Authors contributed equally to this study.

Correspondence to: Senji Shirasawa, MD, Ph.D., Department of Cell Biology, Faculty of Medicine Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Tel: +81 928011011, Fax: +81 928643865, e-mail: sshirasa@fukuoka-u.ac.jp

Key Words: KRAS, colorectal cancer, 3-dimensional floating culture, drug screening.

including rolipram and resveratrol, induced luminal apoptosis in HCT116 spheroids in the 3D matrigel culture (15, 16) suggesting that these inhibitors were potential agents for mtKRAS-targeting therapies. However, the 3D matrigel culture is not appropriate for a drug screening system because of its complicated procedures.

In this study, to improve the 3D culture system, we developed a 3D floating (3DF) culture system by establishing HKe3-derived cells expressing wtKRAS or mtKRAS. Furthermore, we validated whether this system had the ability to identify drugs targeting KRAS-mediated signalling molecules.

Materials and Methods

Antibodies and reagents. The antibodies used were: anti-KRAS (RAS10) from EMD Millipore (Billerica, MA, USA), anti-HA (3F10) from Roche (Basel, Switzerland). Resveratrol (trans-3,4',5-trihydroxysilbene) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. Human CRC HCT116 cells were obtained from the American Type Culture Collection (Frederick, MD, USA). HCT116 and HKe3 cells were maintained as previously described (7, 14, 17).

Retroviral production and generation of stable cell line. cDNAs for *zoanthus sp*. green fluorescent protein (ZsGreen; Clontech, Mountain View, CA, USA), HA-tagged human wild-type KRAS (wtKRAS) and HA-tagged human mutated KRAS (G13D, mtKRAS) were inserted into a pMSCVpuro vector (Clontech) to generate retrovirus vectors, pMSCV-ZsGreen, pMSCV-wtKRAS and pMSCV-mtKRAS, respectively. Retroviruses were produced by the transfection of the retrovirus vectors together with pAmpho vector (Clontech) into GP2-293 packaging cells (Clontech) by standard calcium phosphate transfection in the presence of 25 μM chloroquine (Sigma-Aldrich). At 48 h after transfection, the viral supernatants were collected and supplemented with 8 μg/ml polybrene (Sigma-Aldrich). The HKe3 cells in six-well plates were infected with the viruses by being spun for 2 h at 32˚C and 2000 × *g*. At 48 h after infection, the cells were treated with 2 μg/ml puromycin (Sigma-Aldrich) for one week to establish HKe3-derived cells stably expressing ZsGreen, wtKRAS or mtKRAS. The cells were further maintained in the medium containing 2 μg/ml puromycin.

Three-dimensional floating cell culture. Cells were seeded in a 96-well plate with an ultra-low attachment surface and round bottom (Product Number 7007; Corning Inc., Corning, NY, USA,). Photomicrographs of cells were taken using a BIOREVO BZ9000 microscope (Keyence, Osaka, Japan) and the area of the spheroid was measured using a BZ Analyzer (Keyence) as previously described (14, 15, 18, 19).

Immunoblotting. Cells were lysed in the RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche)) and subjected to immunoblotting as previously described (15, 20).

Immunocytochemistry. Cells seeded on cover glasses were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at room temperature (RT), blocked for 30 min at RT (with 5% bovine serum, 0.1% Tx-100, 150 mM NaCl and 50 mM Tris-HCl, pH 7.5) and then subjected to immunostaining with anti-HA antibody (3F10; Roche). The primary antibodies were visualised with goat anti-rat IgG antibody conjugated with Alexa Fluor 488 (Life Technologies, Carlsbad, CA, USA). Fluorescence images were acquired by a confocal fluorescence microscope (TCS-SP5; Leica Microsystems, Wetzlar, Germany) that was assisted by Leica Application Suite Advanced Fluorescence 1.6.0 software (Leica Microsystems).

Detection of apoptotic cells. The cells were incubated with 10 μM CellEvent Caspase-3/7 green detection reagent (Life Technologies) according to the manufacturer's protocol. Stained cells were observed under the BIOREVO BZ9000 microscope.

Statistical analyses. The data were presented as the mean±standard deviation. The statistical analyses were performed using the unpaired two-tailed Student's *t*-test. All *p*-values of <0.05 were considered to be statistically significant.

Results

Establishment of HKe3-derived cells expressing wtKRAS or mtKRAS. HKe3 cells established by us was a sub-clone, which was disrupted at mtKRAS gene in HCT116 cells (7). To eliminate the clonal variations affecting cellular and/or molecular phenotypes, we have established HKe3-derived cells stably-expressing wtKRAS or mtKRAS using retrovirus-mediated protein expression without selecting clones. The expression of exogenous wtKRAS or mtKRAS in these cells was confirmed by immunoblotting using anti-KRAS antibody (Figure 1A). Furthermore, immunofluorescence staining using anti-HA antibody revealed that exogenous wtKRAS or mtKRAS was almost equally expressed in each cell between the individual cells (Figure 1B). HKe3 cells expressing exogenous wtKRAS (HKe3-wtKRAS) showed a cobblestone-like appearance in a two-dimensional (2D) cell culture, which was similar to the parental HKe3 cells and HKe3 cells expressing ZsGreen (HKe3-ZsGreen, Figure 1C). In contrast, the HKe3 cells expressing exogenous mtKRAS (HKe3-mtKRAS) showed a spindle-like appearance, which was similar to that of HCT116 cells (Figure 1C), suggesting that the expression of exogenous mtKRAS transforms HKe3 cells to HCT116-like cells. Taken together, these results suggested that analysing HKe3-wtKRAS and HKe3-mtKRAS cells simultaneously could eliminate clonal variations.

Establishment of 3DF culture using HKe3-wtKRAS and HKe3-mtKRAS cells. To establish a drug-screening system to identify drugs targeting mtKRAS-mediated signaling molecules, the cells were seeded in the 96-well plates with an ultra-low attachment surface and round bottom for the 3DF cell culture. HKe3-wtKRAS and HKe3-mtKRAS cells rapidly assembled into respective single spherical structures (spheroids) in the 3DF culture (Figure 2A). The areas of the

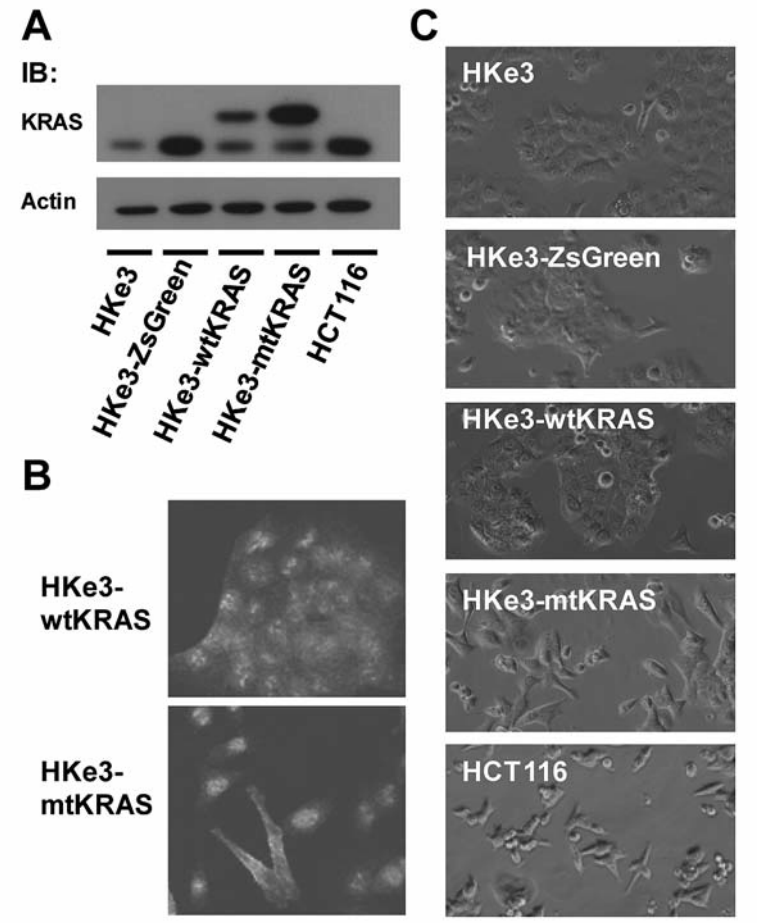

Figure 1. *Establishment of HKe3-derived cells stably expressing wtKRAS or mtKRAS. A: The expression of KRAS protein in HKe3, HKe3-ZsGreen, HKe3-wtKRAS, HKe3-mtKRAS and HCT116 cells. β-Actin was used as a loading control. B: Immunocytochemical staining for exogenous KRAS protein using anti-HA antibody in HKe3-wtKRAS and HKe3-mtKRAS cells. C: The morphologies of HKe3, HKe3-ZsGreen, HKe3-wtKRAS, HKe3-mtKRAS and HCT116 cells grown in 2D cell culture plates. Bright-field images of the cells were taken.*

spheroids at day 6 were significantly different for both cell types (Figure 2B). The spheroids formed by HKe3-mtKRAS cells were much larger than those formed by HKe3-wtKRAS cells. Furthermore, the relative-fold increase in the area of the spheroid formed at day 6 of the HKe3-wtKRAS cells was much higher than that of HKe3-mtKRAS cells when a lower number of cells were initially seeded at day 0 (Figure 2C). Based on these results, we determined that the starting cell number was 600 in the following experiments.

Next, we compared the area of spheroids of HKe3-derived cells at day 3 and day 6 after seeding. The area of the spheroids formed by the HKe3-wtKRAS cells was similar to those formed by the parental HKe3 and HKe3-ZsGreen cells (Figure 3) indicating that neither wtKRAS expression nor retrovirus infection affected the growth of spheroids in the 3DF culture. In contrast, the area of spheroids formed by the HKe3-mtKRAS cells was significantly larger than the areas of spheroids of the parental HKe3, HKe3-ZsGreen and HKe3-wtKRAS cells at both day 3 and day 6 (Figure 3). Furthermore, the area of the spheroids formed by the HKe3-wtKRAS cells, as measured at day 6, was marginally larger by 1.2-fold compared to that of day 3, whereas there was a 2.4-fold increase from day 3 to day 6 for the spheroid area of the HKe3-mtKRAS cells (Figure 3) suggesting that mtKRAS expression stimulated the growth of spheroids in the 3DF culture.

3DF culture of HKe3-wtKRAS and HKe3-mtKRAS cells is compatible with 3D matrigel culture of HKe3 and HCT116 cells. Previously, we reported that the proportion of apoptotic cells formed by HKe3 cells in the lumen of spheroids in the 3D matrigel culture was significantly higher than that of those formed by HCT116 cells (14). To compare the 3DF culture with the 3D matrigel culture, the proportion of apoptotic cells in the spheroids formed in the 3DF culture

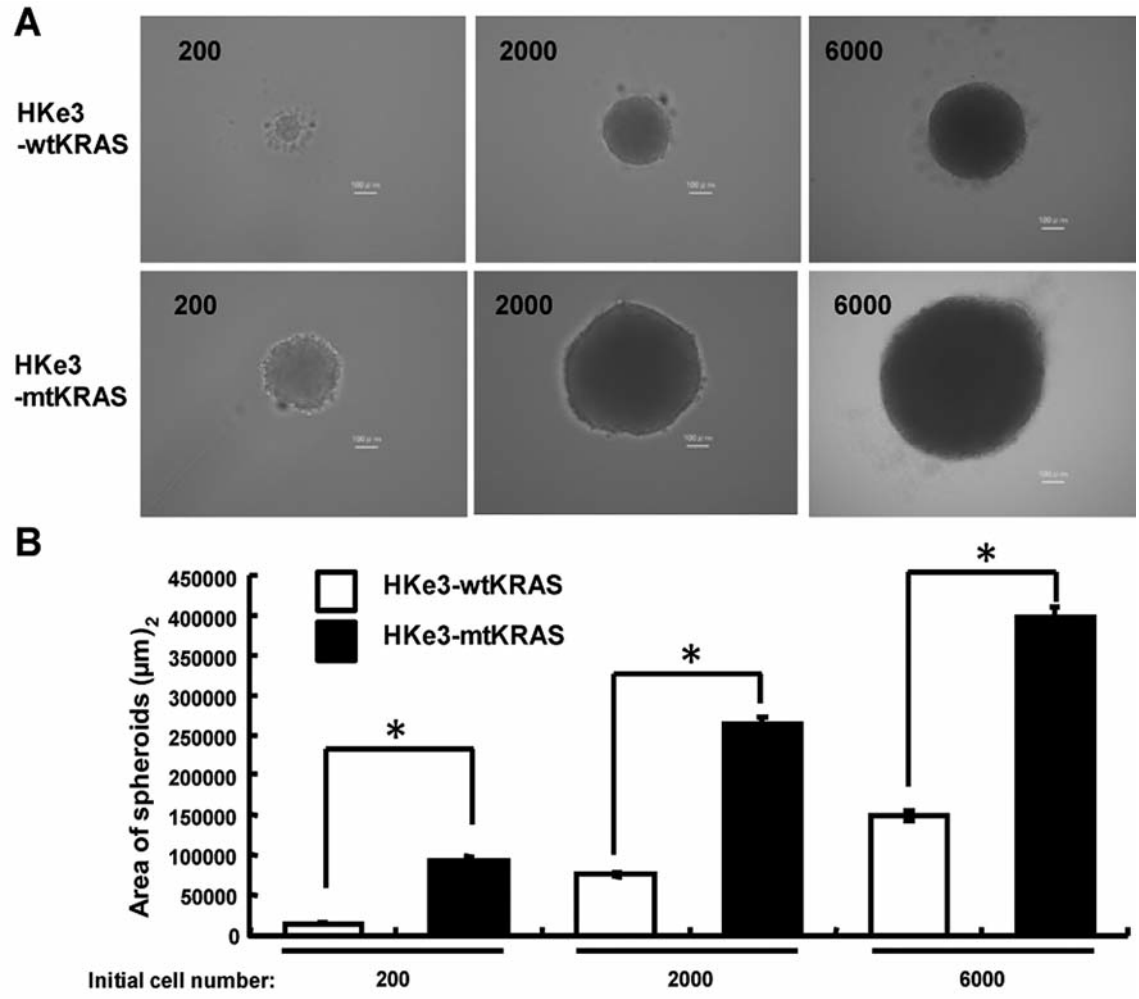

Figure 2. *Differences in the area between HKe3-wtKRAS and HKe3-mtKRAS spheroids formed in 3DF culture at day 6. A: The representative images of spheroids at day 6 formed by HKe3-wtKRAS and HKe3-mtKRAS cells. Numbers in the panels represent the number of cells seeded at day 0. Scale bar; 100 μm. B: The area of spheroids formed at day 6. *p<0.0001. C: The relationship between the number of cells seeded at day 0 and the relative-fold increase in the area of spheroids of HKe3-mtKRAS cells to that of HKe3-wtKRAS cells at day 6.*

was examined by detecting the activated caspases 3 and 7. Although most of the interior cells in HKe3-wtKRAS spheroids were positive for activated caspases 3 and 7 signals at day 3, the apoptotic cells were rarely observed in HKe3-mtKRAS spheroids (Figure 4) indicating that the expression of mtKRAS in HKe3 cells inhibited luminal apoptosis in the spheroids formed in the 3DF culture, which was comparable to the results previously obtained in the 3D matrigel culture.

Previous studies also showed that a PDE4 inhibitor, resveratrol, inhibited the growth of spheroids formed by the HCT116 cells in the 3D matrigel culture (16). Next, the effect of resveratrol was examined on the growth of spheroids formed by the HKe3-wtKRAS and HKe3-mtKRAS cells in the 3DF culture. Resveratrol did not affect the growth of HKe3-wtKRAS spheroids in the 3DF culture (Figure 5), which was similar to its effect on the HKe3 cells in the 3D matrigel culture. By contrast, resveratrol dramatically decreased the area of HKe3-mtKRAS spheroids in the 3DF culture (Figure 5). Taken together, these results suggested that resveratrol selectively inhibited the growth of spheroids dependent on the mtKRAS in the 3DF culture. All these results indicated that the 3DF culture using HKe3-wtKRAS and HKe3-mtKRAS cells was compatible with the 3D matrigel culture using HKe3 and HCT116 cells.

Discussion

In the present study, we developed 3DF culture system through the establishment of HKe3-derived cells expressing wtKRAS or mtKRAS. Although oncogenic mutations in

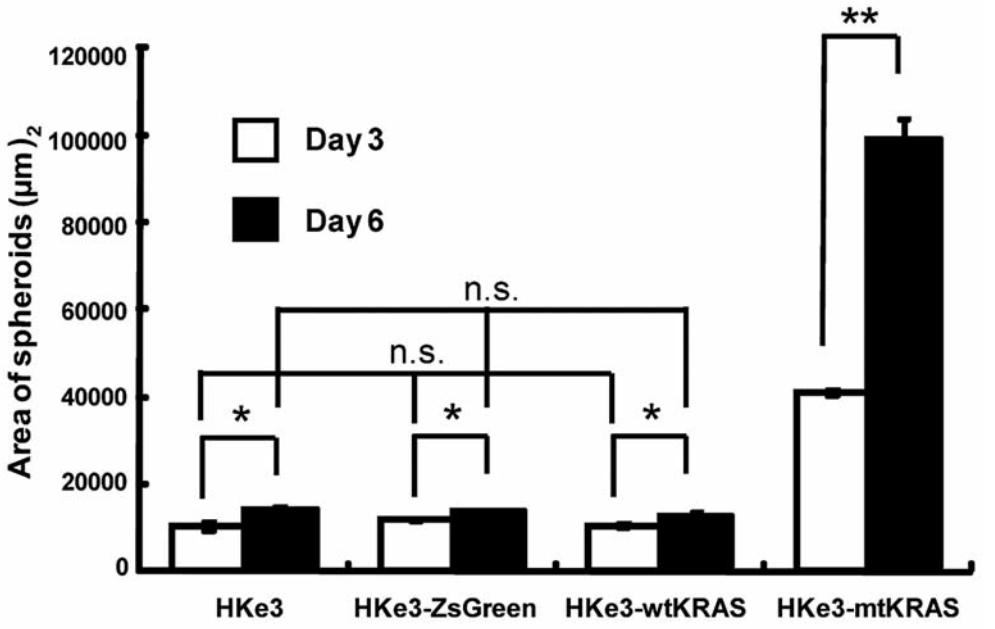

Figure 3. *The area of spheroids formed by HKe3-derived cells at day 3 and day 6 in 3DF culture. Six-hundred cells of the parental HKe3, HKe3-ZsGreen, HKe3-wtKRAS and HKe3-mtKRAS were seeded in the 3DF culture. The area of spheroids was measured at day 3 and day 6. *p<0.05; **p<0.005; n.s., not significant.*

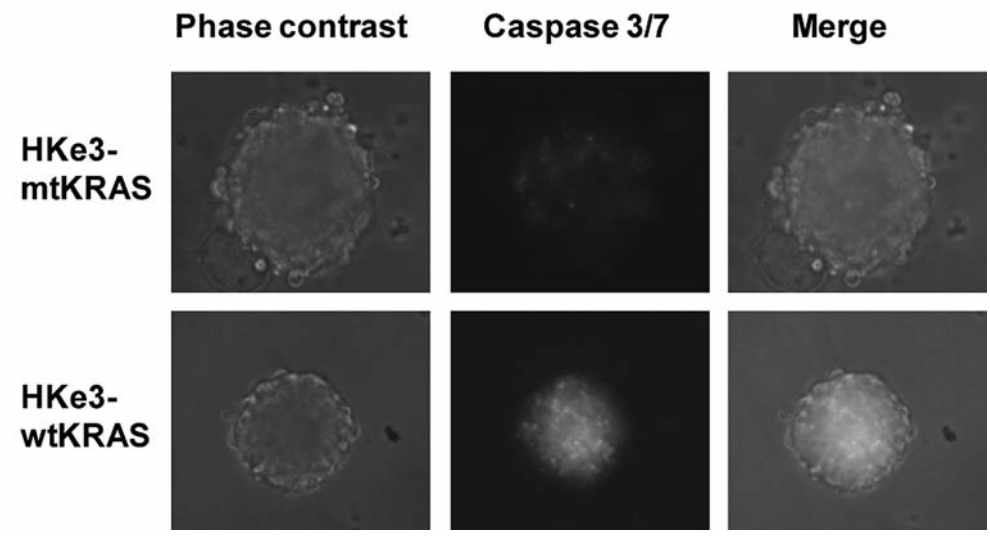

Figure 4. *Expression of mtKRAS in HKe3 cells inhibits luminal apoptosis in the spheroids formed in 3DF culture. The signals for activated caspases 3 and 7 in HKe3-wtKRAS and HKe3-mtKRAS spheroids at day 3 in the 3DF culture; Scale bar, 100 μm.*

KRAS underlie the pathogenesis and chemoresistance of many human tumors (2, 21), drugs specifically targeting the oncogenic *KRAS* have not yet been clinically developed. This 3DF culture system will be useful for screening of drugs that target KRAS-mediated signaling molecules critically involved in tumorigenesis.

The retrovirus-mediated expression of exogenous mtKRAS transformed the HKe3 cells into HCT116-like cells. HKe3 cells are one of the HCT116-derived sub-clones, which were specifically disrupted at the mtKRAS allele by homologous recombination (7). Hence, there could be a possibility that the differences in cellular and molecular

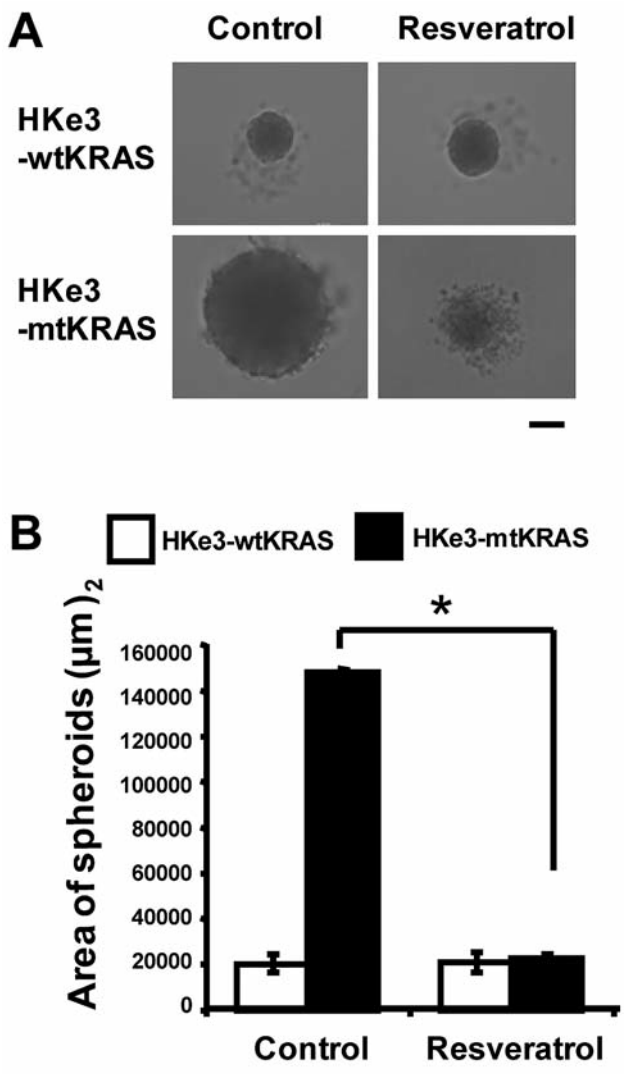

Figure 5. *Resveratrol suppresses the growth of HKe3-mtKRAS spheroids in the 3DF culture. A: Representative images of HKe3-wtKRAS and HKe3-mtKRAS spheroids grown in the presence or absence of 30 μg/ml resveratrol in the 3DF culture at day 6. B: The area of spheroids. *p<0.02; **p<0.00001.*

phenotypes observed between HCT116 and HKe3 cells may be caused by clonal variation. In contrast, HKe3-wtKRAS and HKe3-mtKRAS cells in this study were established without selecting clones; thus, clonal variation should not be considered in the data obtained using these cells.

Various 3D cell culture models have been developed in cancer research (22, 23). We previously reported on the 3D matrigel cell culture system using the HCT116 and HKe3 cells and showed that PDE4B inhibitors, rolipram and resveratrol, specifically inhibited mtKRAS-mediated signalling in 3D culture but not in 2D culture (15, 16). However, the 3D cell culture using matrigel is not appropriate for a high-throughput type screening system because of its complicated procedures. In the present study, we adopted the 3DF culture to develop drug-screening system that targets mtKRAS-mediated signaling molecules. The 3DF culture system possesses the following desirable advantages for high-throughput screening: a 96-well suspension culture, a single spheroid per well suited for optical imaging, high reproducibility, harvesting cells easily for further analysis and automated imaging and quantitative analysis (24). Indeed, HKe3-wtKRAS and HKe3-mtKRAS cells that were seeded in plates with ultra-low attachment surface and round bottom rapidly assembled into respective single spheroids in a well. Furthermore, in this 3DF culture, the expression of mtKRAS in HKe3 cells accelerated the growth of the spheroids and inhibited luminal apoptosis. Intriguingly, resveratrol specifically reduced the area of the spheroids of HKe3-mtKRAS cells, but not of HKe3-wtKRAS cells, suggesting that resveratrol specifically affected the molecules downstream of mtKRAS but not of wtKRAS. Taken together, these results indicated that our novel 3DF culture system using HKe3-wtKRAS and HKe3-mtKRAS cells would be compatible with the 3D matrigel culture using HKe3 and HCT116 cells. In summary, this 3DF culture system will be useful to screen drugs that target mtKRAS-mediated signaling molecules.

Acknowledgements

We thank Chiyo Isoyama and Takami Danno for technical assistance. This study was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Clinical Research Foundation and 'Fukuoka Foundation for Sound Health' Cancer Research Fund.

References

1 Sideris M and Papagrigoriadis S: Molecular biomarkers and classification models in the evaluation of the prognosis of colorectal cancer. Anticancer Res *34*: 2061-2068, 2014.

2 Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ and Zalcberg JR: *K-ras* mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med *359*: 1757-1765, 2008.

3 Adjei AA: Blocking oncogenic Ras signaling for cancer therapy. J Natl Cancer Inst *93*: 1062-1074, 2001.

4 Milburn MV, Tong L, deVos AM, Brunger A, Yamaizumi Z, Nishimura S and Kim SH: Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. Science *247*: 939-945, 1990.

5 Rodriguez-Viciana P, Sabatier C and McCormick F: Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. Mol Cell Biol *24*: 4943-4954, 2004.

6 Al-Mulla F, Milner-White EJ, Going JJ and Birnie GD: Structural differences between valine-12 and aspartate-12 Ras proteins may modify carcinoma aggression. J Pathol *187*: 433-438, 1999.

7 Shirasawa S, Furuse M, Yokoyama N and Sasazuki T: Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science *260*: 85-88, 1993.
8 Trobridge P, Knoblaugh S, Washington MK, Munoz NM, Tsuchiya KD, Rojas A, Song X, Ulrich CM, Sasazuki T, Shirasawa S and Grady WM: TGF-beta Receptor Inactivation and mutant *Kras* induce intestinal neoplasms in mice *via* a beta-catenin-independent pathway. Gastroenterology, 2009.
9 Vartanian S, Bentley C, Brauer MJ, Li L, Shirasawa S, Sasazuki T, Kim JS, Haverty P, Stawiski E, Modrusan Z, Waldman T and Stokoe D: Identification of mutant K-Ras-dependent phenotypes using a panel of isogenic cell lines. J Biol Chem *288*: 2403-2413, 2013.
10 Tsunoda T, Takashima Y, Yoshida Y, Doi K, Tanaka Y, Fujimoto T, Machida T, Ota T, Koyanagi M, Kuroki M, Sasazuki T and Shirasawa S: Oncogenic KRAS regulates miR-200c and miR-221/222 in a 3D-specific manner in colorectal cancer cells. Anticancer Res *31*: 2453-2459, 2011.
11 Ota T, Doi K, Fujimoto T, Tanaka Y, Ogawa M, Matsuzaki H, Kuroki M, Miyamoto S, Shirasawa S and Tsunoda T: KRAS up-regulates the expression of miR-181a, miR-200c and miR-210 in a three-dimensional-specific manner in DLD-1 colorectal cancer cells. Anticancer Res *32*: 2271-2275, 2012.
12 O'Brien LE, Zegers MM and Mostov KE: Opinion: Building epithelial architecture: insights from three-dimensional culture models. Nat Rev Mol Cell Biol *3*: 531-537, 2002.
13 Griffith LG and Swartz MA: Capturing complex 3D tissue physiology *in vitro*. Nat Rev Mol Cell Biol *7*: 211-224, 2006.
14 Tsunoda T, Takashima Y, Fujimoto T, Koyanagi M, Yoshida Y, Doi K, Tanaka Y, Kuroki M, Sasazuki T and Shirasawa S: Three-dimensionally specific inhibition of DNA repair-related genes by activated KRAS in colon crypt model. Neoplasia *12*: 397-404, 2010.
15 Tsunoda T, Ota T, Fujimoto T, Doi K, Tanaka Y, Yoshida Y, Ogawa M, Matsuzaki H, Hamabashiri M, Tyson DR, Kuroki M, Miyamoto S and Shirasawa S: Inhibition of phosphodiesterase-4 (PDE4) activity triggers luminal apoptosis and AKT dephosphorylation in a 3-D colonic-crypt model. Mol Cancer *11*: 46, 2012.
16 Tsunoda T, Ishikura S, Doi K, Matsuzaki H, Iwaihara Y and Shirasawa S: Resveratrol induces luminal apoptosis of human colorectal cancer HCT116 cells in three-dimensional culture. Anticancer Res *34*: 4551-4555, 2014.
17 Baba I, Shirasawa S, Iwamoto R, Okumura K, Tsunoda T, Nishioka M, Fukuyama K, Yamamoto K, Mekada E and Sasazuki T: Involvement of deregulated epiregulin expression in tumorigenesis *in vivo* through activated Ki-Ras signaling pathway in human colon cancer cells. Cancer Res *60*: 6886-6889, 2000.
18 Yoshida Y, Tsunoda T, Doi K, Tanaka Y, Fujimoto T, Machida T, Ota T, Koyanagi M, Takashima Y, Sasazuki T, Kuroki M, Iwasaki A and Shirasawa S: KRAS-mediated up-regulation of RRM2 expression is essential for the proliferation of colorectal cancer cell lines. Anticancer Res *31*: 2535-2539, 2011.
19 Yoshida Y, Tsunoda T, Doi K, Fujimoto T, Tanaka Y, Ota T, Ogawa M, Matsuzaki H, Kuroki M, Iwasaki A and Shirasawa S: ALPK2 is crucial for luminal apoptosis and DNA repair-related gene expression in a three-dimensional colonic-crypt model. Anticancer Res *32*: 2301-2308, 2012.
20 Tsunoda T, Inokuchi J, Baba I, Okumura K, Naito S, Sasazuki T and Shirasawa S: A novel mechanism of nuclear factor κB activation through the binding between inhibitor of nuclear factor-κBα and the processed NH2-terminal region of Mig-6. Cancer Res *62*: 5668-5671, 2002.
21 Irving J, Matheson E, Minto L, Blair H, Case M, Halsey C, Swidenbank I, Ponthan F, Kirschner-Schwabe R, Groeneveld-Krentz S, Hof J, Allan J, Harrison C, Vormoor J, von Stackelberg A and Eckert C: Ras pathway mutations are prevalent in relapsed childhood acute lymphoblastic leukemia and confer sensitivity to MEK inhibition. Blood *124*: 3420-3430, 2014.
22 Asthana A and Kisaalita WS: Microtissue size and hypoxia in HTS with 3D cultures. Drug Discov Today *17*: 810-817, 2012.
23 Kunz-Schughart LA, Freyer JP, Hofstaedter F and Ebner R: The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. J Biomol Screen *9*: 273-285, 2004.
24 Vinci M, Gowan S, Boxall F, Patterson L, Zimmermann M, Court W, Lomas C, Mendiola M, Hardisson D and Eccles SA: Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. BMC Biol *10*: 29, 2012.

Received April 3, 2015
Revised May 7, 2015
Accepted May 8, 2015