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

TAKEHARU OTA[1,2,3], KEIKO DOI[1,2], TAKAHIRO FUJIMOTO[1,2], YOKO TANAKA[1,2], MASAHIRO OGAWA[2], HIROSHI MATSUZAKI[1], MASAHIDE KUROKI[2], SHINGO MIYAMOTO[3], SENJI SHIRASAWA[1,2] and TOSHIYUKI TSUNODA[1,2]

Departments of [1]Cell Biology and [3]Obstetrics and Gynecology, Faculty of Medicine, and [2]Central Research Institute for Advanced Molecular Medicine, Fukuoka University, Fukuoka, Japan

Abstract. *Background: We previously found that oncogenic KRAS induces increased expression of microRNAs (miRNAs), such as miR-200c and miR-221/222, in human colorectal cancer (CRC) HCT116 cells in a three-dimensional (3D)-specific manner, however, the regulation of miRNA expression through oncogenic KRAS in other types of CRC remains unclear. Materials and Methods: The differential expression of 94 cancer-related miRNAs was examined in DLD-1 and DKO-4 cells (DLD-1 cells with a disrupted oncogenic KRAS) in 3D cultures. Results: Increased miR-15b, miR-16, miR-23a, miR-24, miR-103 and miR-222 expression was observed in 3D and in 2D cultures. Of note, increased miR-181a, miR-200c and miR-210 expression was only observed in 3D cultures. Furthermore, miR-181a and miR-210 were significantly overexpressed in DLD-1 cells in 3D culture compared with those in HCT116 cells, and were significantly overexpressed in human CRC specimens. Conclusion: Oncogenic KRAS regulates 3D-specific miRNAs that are possibly associated with CRC development in vivo.*

Cell–cell and cell–extracellular matrix interactions are important in developmental programs and in elucidating three-dimensional (3D) architectures *in vivo* (1, 2). De-regulation of these interactions is frequently observed in cancer (3). We previously established the HKe3 cell line, which derives from human colorectal cancer (CRC) HCT116 cells with a disruption in oncogenic *KRAS* (4). Analysis using these HKe3 cells has contributed to the understanding of tumor development through *in vitro* and *in vivo* oncogenic *KRAS* signaling (4-8). However, no treatments that target tumors with *KRAS* mutations have been developed yet. Systems for the elucidation of the detailed molecular mechanisms underlying the activities of oncogenic *KRAS* in the 3D microenvironment are essential for the design and the development of novel cancer therapies.

We previously investigated the behavior of HKe3 cells in 3D culture and reported that the cells form an organized structure resembling a colonic crypt (9). In this model, oncogenic *KRAS* was found to inhibit luminal apoptosis, to affect cell polarity in 3D culture and down-regulate DNA repair genes (including *TP53*) in a 3D-specific manner (4, 9). These results indicate that oncogenic *KRAS* plays crucial roles in the inhibition of organized structures and the accumulation of genetic alterations, resulting in the disruption of the barrier-to-tumor progression in the colonic crypt (4, 9).

Recently, we found that the oncogenic *KRAS* induces increased expression of microRNAs (miRNAs), such as miR-200c, miR-221 and miR-222, in HCT116 cells compared with that in HKe3 cells in 3D culture (10). These miRNAs were significantly overexpressed in human CRC specimens. Of note, protein expression of, phosphatase and tensine homolog (PTEN), which is a putative target of the miR-200c and miR-221/222 cluster, was reduced under the control of oncogenic *KRAS* in a 3D-specific manner, suggesting that these miRNAs are associated with the transition from normal colonic mucosa to carcinoma. However, details of the regulation of miRNA expression through oncogenic *KRAS* in other types of CRC remain unclear.

In this study, we examined 94 cancer-related miRNAs in colorectal cancer DLD-1 cells and DKO-4 cells (DLD-1 cells with a disrupted oncogenic *KRAS*) in 3D culture. Furthermore, we examined the biological relevance of 3D-specific miRNAs that are regulated by oncogenic *KRAS* in this model using public datasets of miRNA expression analysis of CRC.

Correspondence to: Toshiyuki Tsunoda, MD, Ph.D., Department of Cell Biology, Faculty of Medicine Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Tel: +81928011011, Fax: +81928643865, e-mail: tsunoda@fukuoka-u.ac.jp

Key Words: KRAS, microRNA, colorectal cancer, miR-200c, 3D culture, 2D culture.

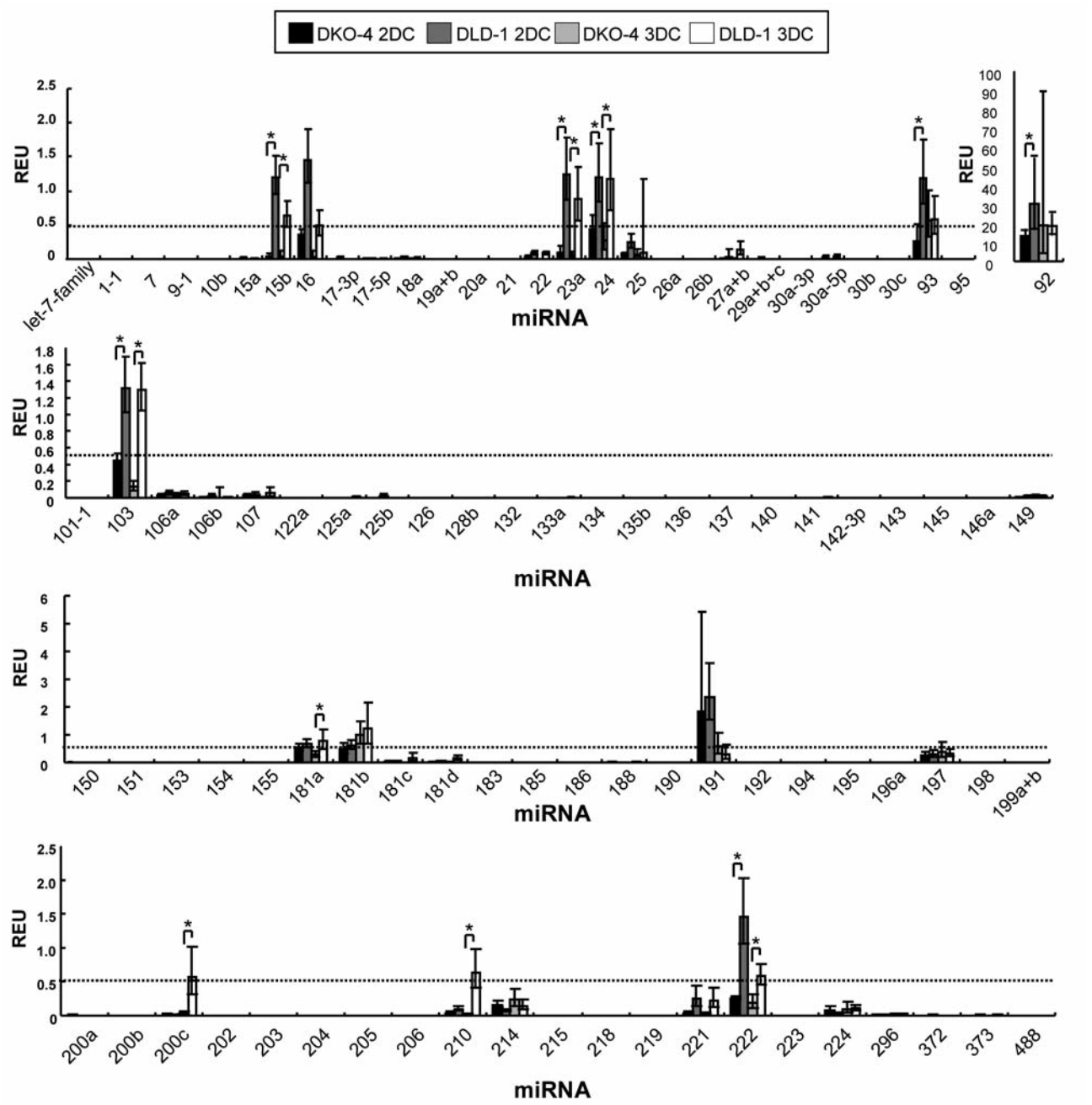

Figure 1. *Expression of 94 cancer-related miRNAs in two-dimensional and three-dimensional cultures (3DC and 2DC). The expression profiles of cancer-related miRNAs in DKO-4 cells in 2DC, DLD-1 cells in 2DC, DKO-4 cells in 3DC and DLD-1 cells in 3DC are shown. The expression (REU) of DLD-1 cells and DKO-4 cells was determined in relation to that of miR-181b in DKO-4 cells grown in 3DC, which was set as 1.0. $p<0.05$.*

Materials and Methods

Cell culture. DLD-1 (American Type Culture Collection, Manassas, VA, USA) and DKO-4 cells were grown in 2D or 3D cultures as described previously (4, 9, 11).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Real-time qRT-PCR was performed using Cancer microRNA qPCR Array with QuantiMir (System Biosciences, Mountain View, CA, USA) for miRNAs. miRNA expression was normalized to U6 snRNA expression in each cell. Data were analyzed by the ΔΔCt method, as previously described (12). The relative expression units (REUs) of DLD-1 cells were determined by the REU of miR-181b in DKO-4 cells in 3D culture, which was set as 1.0.

Dataset sources. The Arndt dataset, which comprises the miRNA profiles of human CRC specimens from 58 patients with CRC (five

with Dukes' A, 26 with Dukes' B, 24 with Dukes' C and three with Dukes' D) and colonic mucosa specimens from eight healthy controls (13), were obtained from the Gene Expression Omnibus (Series GSE10259), using the import module of GenePattern software (14). The differential expression of miRNAs between the two classes was ranked according to a signal-to-noise metric using GenePattern (14, 15). The statistical significance of the differentially expressed genes was determined by the comparative marker selection module of GenePattern (14).

Statistical analysis. The data are presented as the means±standard deviation. The statistical analyses were performed using the unpaired two-tailed Student's *t*-test. Differences at $p<0.05$ were considered to be statistically significant.

Results

miRNA expression in DLD-1 and DKO-4 cells in 2D and 3D cultures. In order to identify miRNAs showing differential expression levels in CRC cells, we performed qRT-PCR assays for 94 cancer-related miRNAs in DLD-1 and DKO-4 cells in 2D and 3D cultures (Figure 1). We selected miRNAs with an expression level of more than 0.5 (Figure 1). In 2D cultures, miR-92 and miR-93 expression in DLD-1 cells was higher than that in DKO-4 cells with a statistically significant difference (Figure 1). Furthermore, miR-15b, miR-16, miR-23a, miR-24, miR-103 and miR-222 expression in DLD-1 cells in 2D and 3D cultures was statistically significantly higher than that of DKO-4 cells (Figure 1). In 3D culture, miR-181a, miR-200c and miR-210 expression in DLD-1 cells was statistically significantly higher than that of DKO-4 cells (Figure 1).

Differential expression of miRNAs in HCT116 and DLD-1 cells in 3D culture. We previously showed the differential expression of miRNAs in HCT116 cells and HKe3 cells (10). We found that miR-23a, miR-125b and miR-191 expression in HCT116 cells in 2D and 3D cultures was higher than that in HKe3 cells (10). In addition, we found that miR-200c, miR-221 and miR-222 expression in HCT116 cells in 3D culture was higher than that in HKe3 cells in 3D culture (10). In order to identify miRNAs that are commonly up-regulated by oncogenic *KRAS* in both HCT116 and DLD-1 cells, we compared the expression profiles of miRNAs. Among miRNAs up-regulated in 2D and 3D cultures, miR-23a was commonly up-regulated in both HCT116 and DLD-4 cells. Among the miRNAs up-regulated in a 3D-specific manner, miR-200c was commonly up-regulated in both HCT116 and DLD-1 cells, and miR-181a and miR-210 were specifically up-regulated only in DLD-1 cells. These results suggest a common role of miR-200c in the 3D structure and a specific role of miR-181a and miR-210 in tumor progression in DLD-1 cells.

3D-Specific miRNAs were overexpressed in CRC. To examine if these miRNAs were also expressed in CRC, we analyzed the public microarray expression data for CRC using the GenePattern software (14). We previously showed that 3D-specific miRNAs, including miR-200c and miR-221/222, were overexpressed in CRC (10). In this study, up-regulation of 3D-specific miRNA expression (miR-181a and miR-210) and expression in both 2D and 3D cultures (miR-103, miR-15b, miR-16, miR-23a and miR24) in DLD-1 cells were examined using the Arndt dataset (13). The differential expression of miRNAs between healthy controls and tumor specimens in all Dukes' stage from patients with CRC are shown in Figure 2, suggesting that 3D-specific miRNA expression (miR-181a and miR-210) in CRC was higher than the expression in controls. These results suggest that 3D-specific miRNAs are potential candidates as diagnostic biomarkers.

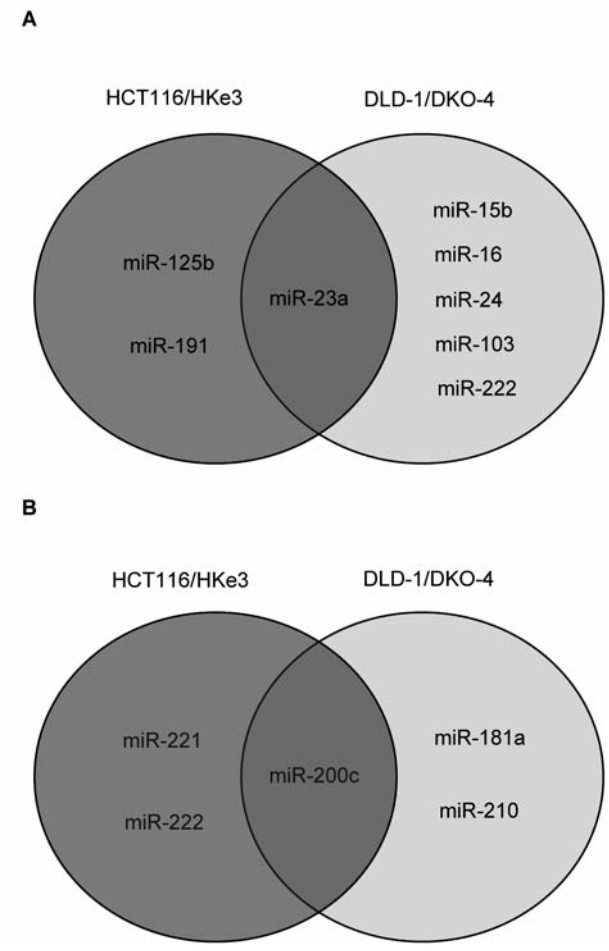

Figure 2. *Differential expression of miRNAs in HCT116 cells and DLD-1 cells in 3D culture (3DC). A: Venn diagram showing miRNA expression up-regulated in HCT116 cells compared with that in HKe3 cells in 2D culture (2DC) and 3DC and in DLD-1 cells compared with that in DKO-4 cells, both in 2DC and 3DC. B: Venn diagram showing up-regulated miRNA expression in HCT116 cells, compared with that in HKe3 cells in 3DC and in DLD-1 cells, compared with that in DKO-4 cells in 3DC.*

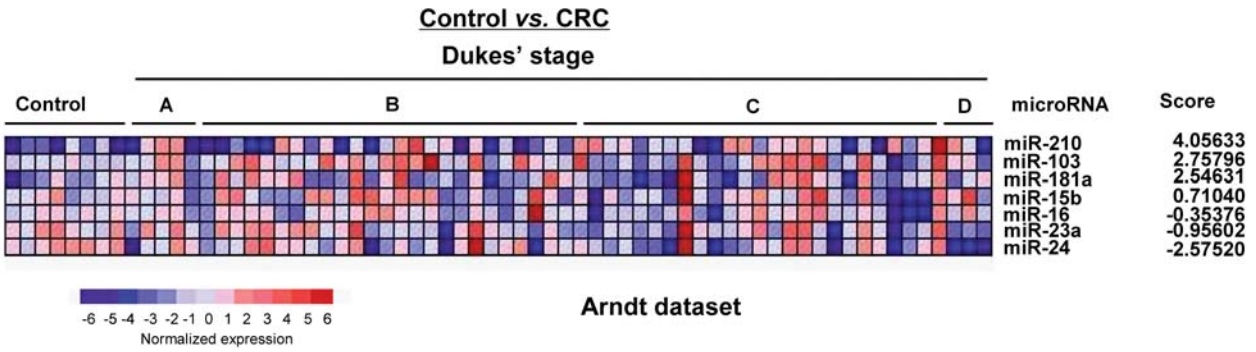

Figure 3. *Expression of the seven miRNAs 3D-specifically up-regulated in DLD-1 cells in human CRC specimens and control specimens in the Arndt dataset. Rows represent miRNAs and score. Columns represent normalized expression of the seven miRNAs selected from human CRC specimens from 58 patients with CRC compared with colonic mucosa specimens from eight healthy controls.*

Discussion

3D-Specific morphological alterations, including the inhibition of cellular polarity and luminal cavity formation with apoptosis, through oncogenic *KRAS* signaling (9) may be associated with triggering the 3D-specific miRNA expression. In this study, miR-15b, miR-16, miR-23a, miR-24, miR-103, miR-181a, miR-200c, miR-210 and miR-222 expression was up-regulated under the control of oncogenic *KRAS* in DLD-1 cells grown in 3D culture. Interestingly, miR-181a, miR-200c and miR-210 expression was dysregulated by oncogenic *KRAS* in a 3D-specific manner and these miRNAs were overexpressed in CRC specimens.

We previously showed that increased miR-200c, miR-221 and miR-222 expression was observed only in HCT116 cells in a 3D-specific manner, and these miRNAs were significantly overexpressed in human CRC specimens (10). Of note, miR-200c was up-regulated in both HCT116 and DLD-1 cells, suggesting the 3D-specific role of miR-200c in CRC with oncogenic *KRAS*. Indeed, recent studies have shown that miR-200c is abundantly expressed in clinical CRC specimens (16, 17), and furthermore it induces chemoresistance in esophageal cancer (18).

Interestingly, miR-181a and miR-210 were specifically up-regulated by oncogenic *KRAS* in DLD-1 cells in a 3D-specific manner. miR-181 is reported to be a biomarker for cancer stem cells (19) which are associated with aggressive and metastatic CRC (20). In addition, miR-210 is a sensor for hypoxic stress during tumorigenesis (21), suggesting that the expression of miR-210 represents the hypoxic state of the innermost region of the 3D structure. Thus, these results suggest that oncogenic *KRAS* promotes cancer development through 3D-specific miRNAs in DLD-1 cells.

Furthermore, analysis of a public dataset strongly indicated that all 3D-specific miRNAs, including miR-200c, miR-211/222 (10), miR-181 and miR-210, reflected the *in vivo* status of CRC and suggests a strong correlation between the miRNAs that are dysregulated by oncogenic *KRAS* in a 3D colonic-crypt model and in clinical CRC specimens.

In summary, we found increased miR-181a, miR-200c and miR-210 expression in DLD-1 cells grown in 3D culture and in human CRC specimens. Further elucidation of the precise molecular mechanisms of action of miRNAs that are regulated by oncogenic *KRAS* using this 3D colonic-crypt model will lead to a better understanding of CRC development *in vivo* and will provide a novel approach for cancer therapy.

Acknowledgements

We thank Takami Danno and Yumiko Hirose for their technical assistance. This study was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Clinical Research Foundation.

References

1 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.

2 Yamada KM and Cukierman E: Modeling tissue morphogenesis and cancer in 3D. Cell *130*: 601-610, 2007.

3 Griffith LG and Swartz MA: Capturing complex 3D tissue physiology *in vitro*. Nat Rev Mol Cell Biol *7*: 211-224, 2006.

4 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.

5 Yu JL, May L, Lhotak V, Shahrzad S, Shirasawa S, Weitz JI, Coomber BL, Mackman N and Rak JW: Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. Blood *105*: 1734-1741, 2005.

6 She QB, Halilovic E, Ye Q, Zhen W, Shirasawa S, Sasazuki T, Solit DB and Rosen N: 4E-BP1 is a key effector of the oncogenic activation of the AKT and ERK signaling pathways that integrates their function in tumors. Cancer Cell *18*: 39-51, 2010.

7 Di Nicolantonio F, Arena S, Tabernero J, Grosso S, Molinari F, Macarulla T, Russo M, Cancelliere C, Zecchin D, Mazzucchelli L, Sasazuki T, Shirasawa S, Geuna M, Frattini M, Baselga J, Gallicchio M, Biffo S and Bardelli A: Deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. J Clin Invest *120*: 2858-2866, 2010.

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-β receptor inactivation and mutant Kras induce intestinal neoplasms in mice *via* a β-catenin-independent pathway. Gastroenterology *136*: 1680-1688 e1687, 2009.

9 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.

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 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.

12 Tsunoda T, Takashima Y, Tanaka Y, Fujimoto T, Doi K, Hirose Y, Koyanagi M, Yoshida Y, Okamura T, Kuroki M, Sasazuki T and Shirasawa S: Immune-related zinc finger gene *ZFAT* is an essential transcriptional regulator for hematopoietic differentiation in blood islands. Proc Natl Acad Sci USA *107*: 14199-14204, 2010.

13 Arndt GM, Dossey L, Cullen LM, Lai A, Druker R, Eisbacher M, Zhang C, Tran N, Fan H, Retzlaff K, Bittner A and Raponi M: Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. BMC Cancer *9*: 374, 2009.

14 Reich M, Liefeld T, Gould J, Lerner J, Tamayo P and Mesirov JP: GenePattern 2.0. Nat Genet *38*: 500-501, 2006.

15 Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD and Lander ES: Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science *286*: 531-537, 1999.

16 Xi Y, Formentini A, Chien M, Weir DB, Russo JJ, Ju J, Kornmann M and Ju J: Prognostic values of microRNAs in colorectal cancer. Biomark Insights *2*: 113-121, 2006.

17 Monzo M, Navarro A, Bandres E, Artells R, Moreno I, Gel B, Ibeas R, Moreno J, Martinez F, Diaz T, Martinez A, Balague O and Garcia-Foncillas J: Overlapping expression of microRNAs in human embryonic colon and colorectal cancer. Cell Res *18*: 823-833, 2008.

18 Hamano R, Miyata H, Yamasaki M, Kurokawa Y, Hara J, Moon JH, Nakajima K, Takiguchi S, Fujiwara Y, Mori M and Doki Y: Overexpression of miR-200c induces chemoresistance in esophageal cancers mediated through activation of the AKT signaling pathway. Clin Cancer Res *17*: 3029-3038, 2011.

19 Ji J, Yamashita T, Budhu A, Forgues M, Jia HL, Li C, Deng C, Wauthier E, Reid LM, Ye QH, Qin LX, Yang W, Wang HY, Tang ZY, Croce CM and Wang XW: Identification of microRNA-181 by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. Hepatology *50*: 472-480, 2009.

20 O'Brien CA, Pollett A, Gallinger S and Dick JE: A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature *445*: 106-110, 2007.

21 Mathew LK and Simon MC: mir-210: a sensor for hypoxic stress during tumorigenesis. Mol Cell *35*: 737-738, 2009.

Received April 4, 2012
Revised May 14, 2012
Accepted May 15, 2012