Oncogenic KRAS Regulates miR-200c and miR-221/222 in a 3D-Specific Manner in Colorectal Cancer Cells

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Abstract. *Background: Oncogenic KRAS plays several key roles in a three-dimensional (3D) colonic-crypt model. However, miRNA expression regulated by oncogenic KRAS in this model is still elusive. Materials and Methods: The differential expression of 105 cancer-related microRNAs was examined and compared in HCT116 cells and HKe3 cells (HCT116 cells in which mutated KRAS allele was deleted) in 3D culture. HKe3 cells stably overexpressing oncogenic KRAS and the public datasets for microRNA expression analysis of colorectal cancer were further examined. Results: The increased expression of miR-200c, miR-221 and miR-222 were observed exclusively in 3D culture, but not in the two-dimensional culture. These microRNAs were regulated by oncogenic KRAS and were significantly overexpressed in human colorectal tumor specimens. Of note, the protein expression level of Phosphatase and tensin homolog (PTEN), a putative target of miR-221/222 cluster, was reduced under the control of oncogenic KRAS in a 3D-specific manner. Conclusion: Oncogenic KRAS regulates 3D-specific molecules, possibly being associated with colorectal tumor development in vivo.*

Both cell-cell and cell-extracellular matrix interactions are critically involved in cell developmental programs and provide three-dimensional (3D) architectures *in vivo* (1, 2). The deregulation of these interactions is frequently observed in cancer (3). We established HKe3 cells: human colorectal cancer (CRC) HCT116 cells with a disruption in oncogenic *KRAS* (4), and the analysis using these HKe3 cells has contributed to the understanding of tumor development through the oncogenic KRAS signaling *in vitro* and *in vivo* (4-8). However, no treatment targeting tumors with *KRAS* mutations has been developed. Systems for elucidation of the detailed molecular mechanisms underlying the activities of oncogenic KRAS in the 3D microenvironment are essential for the design and development of novel cancer therapies.

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

Recent studies also indicate the involvement of microRNA (miRNA) regulation in cancer development and tissue morphology (10). Notably, the expression of KRAS is reported to be regulated by the let-7 family (11, 12). CRC patients with *KRAS* mutation (13, 14) or with decreased expression of let-7 do not benefit from therapy with cetuximab (15-17), a monoclonal antibody against the epidermal growth factor receptor, suggesting a close correlation between KRAS and miRNAs.

As miRNA expressions regulated by oncogenic KRAS *in vivo* remain still elusive, we examined 105 cancer-related miRNAs in 3D culture of the isogenic CRC cell lines with or without oncogenic KRAS. We further tested the biological relevance of 3D-specific miRNAs regulated by oncogenic KRAS in this model using the public datasets for miRNA expression analysis of CRC.

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Materials and Methods

Cell culture. Human CRC HCT116 cells were obtained from the American Type Culture Collection. HCT116 cells, HKe3 cells and e3-MKRas#14 cells were grown in two-dimensional (2D) or three-dimensional (3D) cultures as described previously (4, 9, 18).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Real-time qRT-PCR was carried out using Cancer microRNA qPCR Array with QuantiMir (System Biosciences, Mountain View, CA, USA) for miRNAs. Expression levels of miRNAs were normalized by the expression level of U6 snRNA being taken as 1×10^6 units in each cell. Data were analyzed by the ΔΔCt method as previously described (19). Relative expression units (REUs) of HCT116 cells and e3-MKRas#14 cells were determined relative to the REU of HKe3 cells set as 1.0.

Dataset sources. The Arndt datasets, consisting of the miRNA profiles of human colorectal tumor specimens from 58 CRC patients (5 Dukes' A, 26 Dukes' B, 24 Dukes' C and 3 Dukes' D) and colonic mucosa specimens from eight healthy controls (20), were obtained from Gene Expression Omnibus (GEO; Series GSE10259) with the use of the import module in the GenePattern software package (21). The differentially expressed miRNAs between two classes were ranked according to signal-to-noise metric with the GenePattern (21, 22). The statistical significance of the differentially expressed genes was determined by the comparative marker selection module in GenePattern (21).

Western blotting analysis. Western blotting analysis was carried out as described previously (9) using anti-PTEN antibody (#9552; Cell Signaling Technology, Beverly, MA, USA) and anti-ERK1 antibody (sc-94; Santa Cruz Biotechnology, Santa Cruz, CA, USA). ERK1 intensity was used as a control in the Western blotting analysis for PTEN, and the relative intensity of the signal (PTEN/ERK1) was normalized by taking the signal intensity in HKe3 in 3D culture as 1.0.

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

Results

Expression levels of miRNAs in 3D and 2D culture for HCT116 and HKe3 cells. To identify miRNAs showing the differential expression levels in CRC cells, we performed qRT-PCR assays on HCT116 and HKe3 cells in 3D and 2D cultures for 105 selected miRNAs associated with cancer (Figure 1). We then selected six miRNAs including miR-23a, miR-125b, miR-191, miR-200c, miR-221 and miR-222, of which expression levels in HCT116 cells in 3D culture were higher than those of HKe3 cells in 3D culture, both with a statistically significant difference and with an expression level of more than 5,000 (Figure 1). Similarly, we selected two miRNAs, let-7b and let-7i, of which expression levels in HCT116 cells in 3D culture were lower than those of HKe3 cells in 3D culture, both with a statistically significant difference (Figure 1).

3D-Specific miRNAs up-regulated by oncogenic KRAS. Expression levels of miR-200c, miR-221 and miR-222 in 2D and 3D cultures are shown in Figure 2A. Increased expression levels of miR-200c, miR-221 and miR-222 in HCT116 cells compared with those in HKe3 cells were observed in the 3D culture, but not in the 2D culture (Figure 2A; $*p<0.05$). To determine whether or not oncogenic KRAS regulates these miRNAs, qRT-PCR assay was carried out in e3-MKRas#14 cells, HKe3-derived stable transfectants expressing oncogenic KRAS (18). Similarly, increased REUs of miR-200c, miR-221 and miR-222 in e3-MKRas#14 cells compared with those in HKe3 cells were observed in the 3D culture, but not in the 2D culture (Figure 2B; $*p<0.05$). These results suggest that these miRNAs are controlled by oncogenic KRAS in a 3D-specific manner.

Expression levels of miR-23a, miR-125b and miR-191 in 2D and 3D cultures are shown in Figure 2C. Increased expression of miR-23a, miR-125b and miR-191 in HCT116 cells compared with those in HKe3 cells was observed in both 2D and 3D cultures (Figure 2C; $*p<0.001$). Similarly, increased REUs of miR-23a, miR-125b and miR-191 in e3-MKRas#14 cells compared with those in HKe3 cells were observed in both 2D and 3D cultures (Figure 2D; $*p<0.001$). These results suggest that miR-23a, miR-125b and miR-191 are regulated by oncogenic KRAS in both 2D and 3D cultures.

3D-Specific miRNAs down-regulated by oncogenic KRAS. Expression levels of let-7b and let-7i in 2D and 3D cultures are shown in Figure 3A. Decreased expression of let-7b in HCT116 cells compared with that in HKe3 cells was observed in the 3D culture, but not in the 2D culture (Figure 3A; $*p<0.01$), whereas decreased expression of let-7i in HCT116 cells compared with that in HKe3 cells was observed in both 2D and 3D cultures (Figure 3B; $*p<0.05$). Similarly, decreased REUs of let-7b in e3-MKRas#14 cells compared with those in HKe3 cells were observed in the 3D culture, but not in the 2D culture (Figure 3B; $*p<0.05$), whereas decreased REUs of let-7i in e3-MKRas#14 cells compared with those in HKe3 cells were observed in both 2D and 3D cultures. These results suggest that oncogenic KRAS down-regulates let-7b in a 3D-specific manner and down-regulates let-7i in both 2D and 3D culture.

3D-Specific miRNAs were overexpressed in colorectal tumors. To explore whether these miRNAs were also expressed in CRC, we analyzed the public microarray expression data for CRC using the GenePattern software package (21). The miRNA expression levels of the eight miRNAs were examined in the Arndt dataset consisting of the miRNA profiles of human colorectal tumor specimens from 58 CRC patients and colonic mucosa specimens from

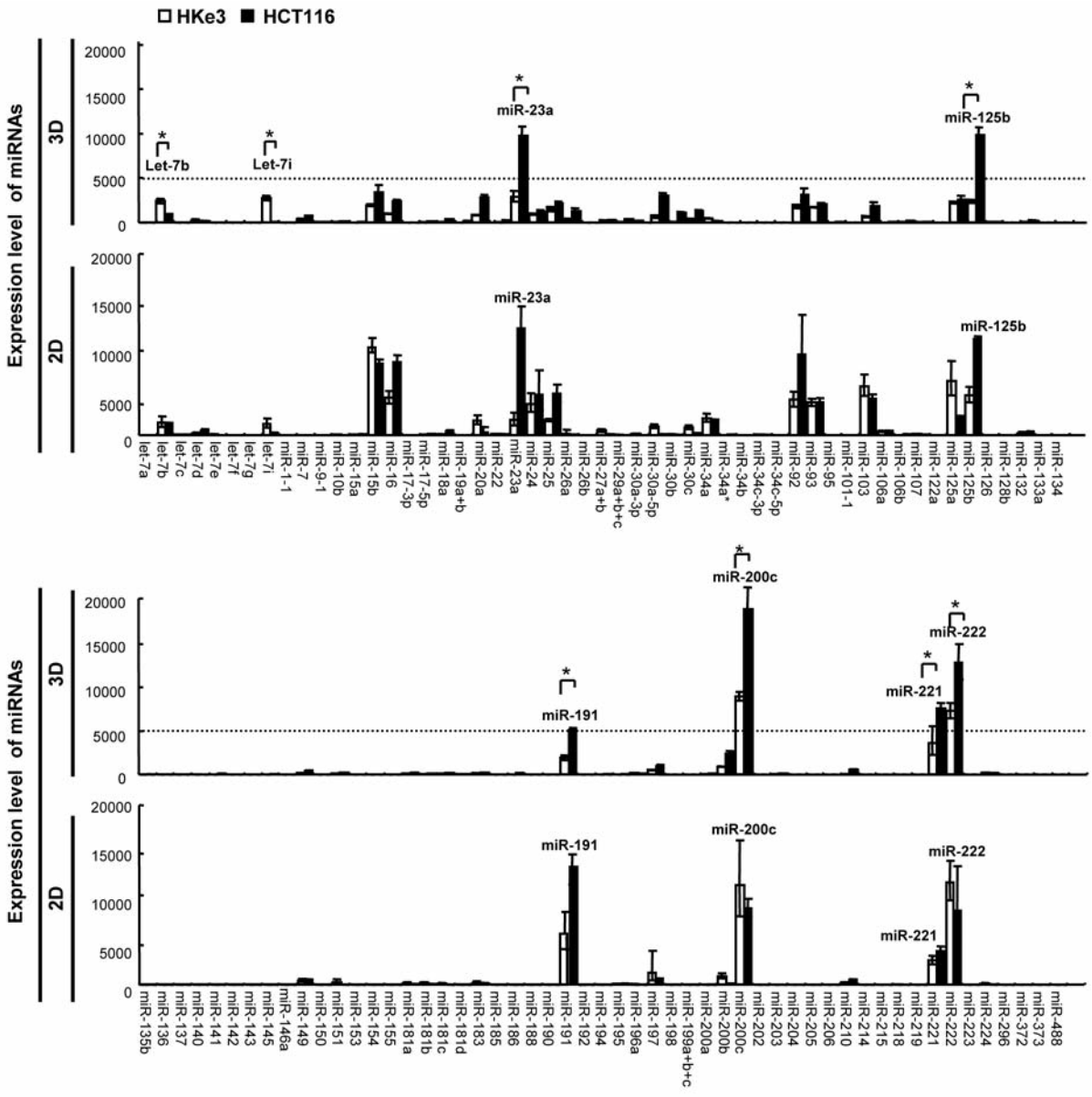

Figure 1. *Expression levels of 105 cancer-associated miRNAs in 3D and 2D cultures of HCT116 cells and HKe3 cells. *p<0.05.*

eight healthy controls (20). The differentially expressed miRNAs between healthy controls and tumor specimens in Dukes' A patients are shown in Figure 4A, suggesting that the increased expression of 3D-specific miRNAs including miR-200c, miR-221 and miR-222 correlate with the early stage of CRC. The differentially expressed miRNAs between healthy controls and tumor specimens from all Dukes' stage of CRC patients are shown in Figure 4B, suggesting that the expression levels of these 3D-specific miRNAs (miR-200c, miR-221 and miR-222) in CRC are significantly higher compared with those in controls.

3D-Specific reduction of PTEN expression. Interestingly, a lower protein level of tumor suppressor PTEN, a putative target of miR-221 and miR-222 (23, 24), in HCT116 and e3-MKRas#14 cells compared with that of HKe3 cells was observed in the 3D culture, but not in the 2D culture (Figure 5A), whereas expression of *PTEN* mRNAs did not differ among HCT116, HKe3 and e3-MKRas#14 cells (Figure 5B), suggesting that PTEN expression was reduced possibly by post-transcriptional modifications by these miRNA targeting PTEN under the control of oncogenic KRAS exclusively in the 3D culture only.

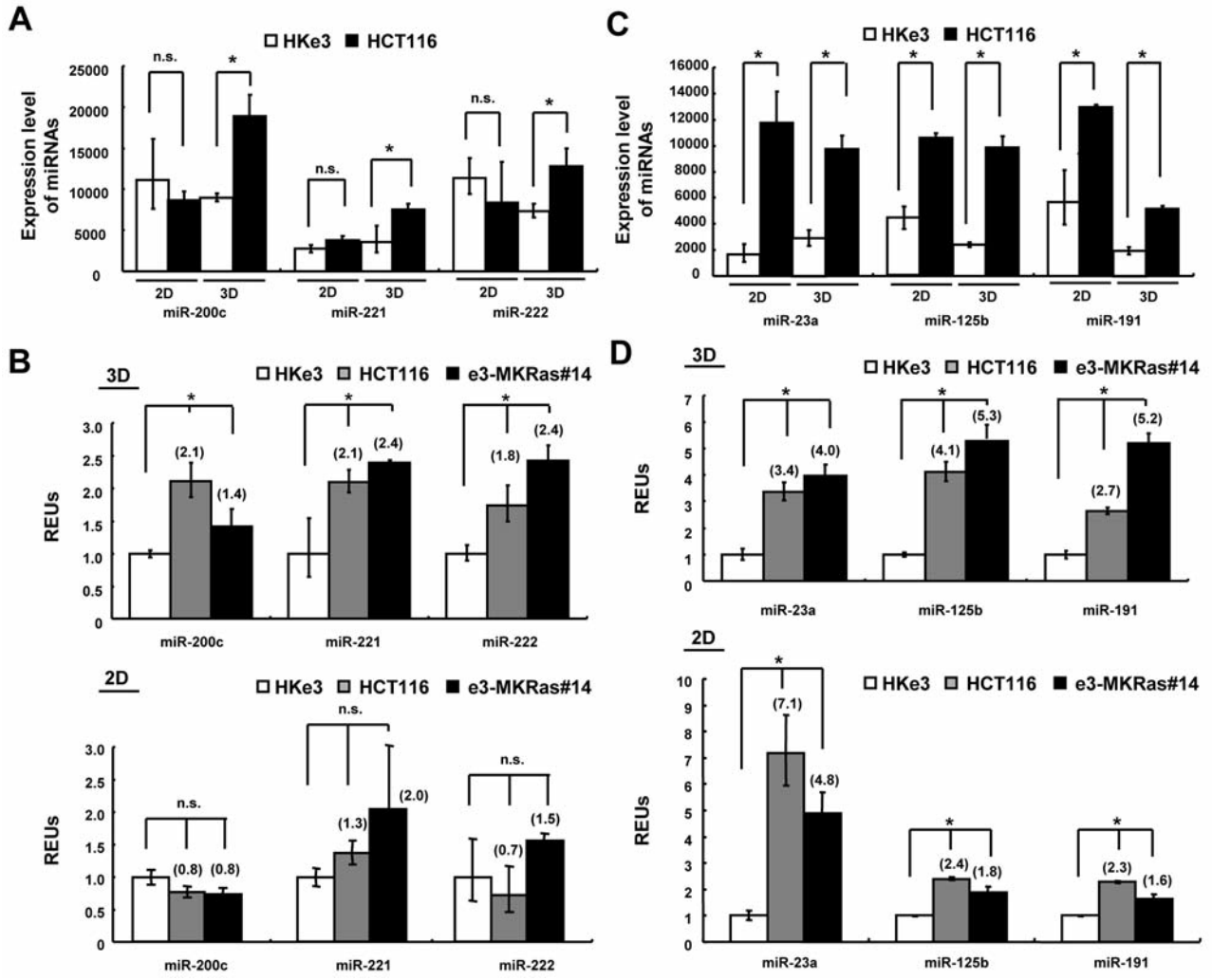

Figure 2. *3D-Specific miRNAs up-regulated by oncogenic KRAS. A: Expression levels for miR-200c, miR-221 and miR-222 in HKe3 cells and HCT116 cells grown in 2D and 3D cultures. *p<0.05. B: Up-regulation of miR-200c, miR-221 and miR-222 by oncogenic KRAS in a 3D-specific manner. *p<0.05. C: Expression levels for miR-23a, miR-125b and miR-191 in HKe3 cells and HCT116 cells grown in 2D and 3D cultures. *p<0.05. D: Up-regulation of miR-23a, miR-125b and miR-191 by oncogenic KRAS in both 2D and 3D culture. *p<0.05. Relative expression units (REUs) of HCT116 cells and e3-MKRas#14 cells were determined by setting the REU of HKe3 cells as 1.0. The number in brackets represents the fold change of HCT116 cells or e3-MKRas#14 cells compared with that of HKe3 cells grown in 3D culture.*

Discussion

In this study, we found six miRNAs to be up-regulated, namely miR-23a, miR-125b, miR-191, miR-200c, miR-221 and miR-222, and two to be down-regulated let-7b and let-7i, under the control of oncogenic KRAS in 3D culture. Interestingly, miR-200c, miR-221, miR-222 and let-7b, and the PTEN expression were dysregulated by oncogenic KRAS in a 3D-specific manner. Of these, 3D-specific miRNAs including miR-200c and the PTEN-targeting miR-221/222 cluster (25) were overexpressed in clinical samples of CRC. Although numerous mechanisms regulating expression of these miRNAs in a 3D culture may be involved, 3D-specific morphological alterations, including inhibition of cellular polarity and luminal cavity formation with apoptosis through oncogenic KRAS signaling (9), may be associated with triggering the 3D-specific expression of miRNAs. For example, the inhibition of apoptosis is reported to be associated with increased expression of miR-221 and miR-222 through targeting PTEN (23, 24), and disruption of acinar formation with luminal apoptosis is reported to be critically associated with down-regulation of PTEN (26, 27). Together these reports and our results suggest a strong correlation among oncogenic KRAS signaling, miR-221, miR-222 and PTEN signals in luminal apoptosis observed in a 3D colonic-crypt model.

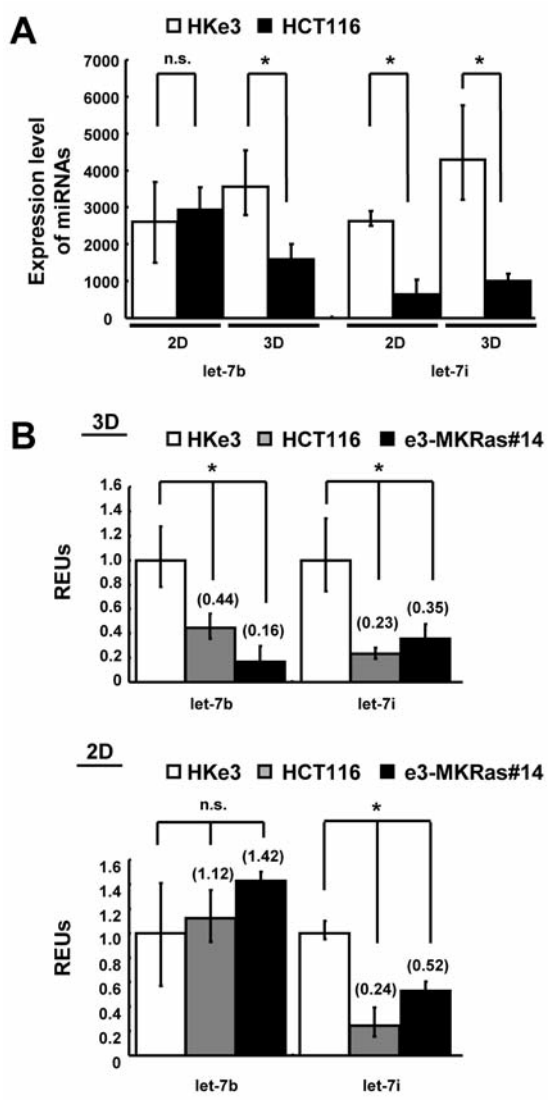

Figure 3. *3D-Specific miRNAs down-regulated by oncogenic KRAS. A: Expression levels for let-7b and let-7i in HKe3 cells and HCT116 cells. *p<0.01. B: Down-regulation of let-7b and let-7i by oncogenic KRAS. Relative expression units (REUs) of HCT116 cells and e3-MKRas#14 cells were determined by setting the REU of HKe3 cells as 1.0. n.s., No significant difference. The number in brackets represents the fold change of HCT116 cells or e3-MKRas#14 cells compared with that of HKe3 cells grown in 3D culture. *p<0.05.*

Of these miRNAs detected in our study, all the up-regulated miRNAs in 3D culture are reported to be abundantly expressed in clinical CRC samples (28-30). Furthermore, lower expression levels of let-7b (30) are also observed in clinical CRC samples. Notably, the analysis of a public dataset strongly indicates that 3D-specific miRNAs reflect the *in vivo* status of CRC. Together these reports and our data suggest a correlation between the miRNAs dysregulated by oncogenic KRAS in a 3D colonic-crypt model and expression patterns of miRNAs in clinical CRC samples.

In summary, we found that increased expression of miR-200c, miR-221 and miR-222 and decreased expression of let-7b and PTEN protein are regulated by oncogenic KRAS in a 3D-specific manner. The increased expression of 3D-specific miRNAs, including miR-200c, miR-221 and miR-222, were also observed in human colorectal tumor specimens. Further elucidation of the precise molecular mechanisms of miRNAs regulated by oncogenic KRAS with the use of this 3D colonic-crypt model will lead to a better understanding of colorectal tumor development *in vivo* and may provide a novel approach for cancer therapy.

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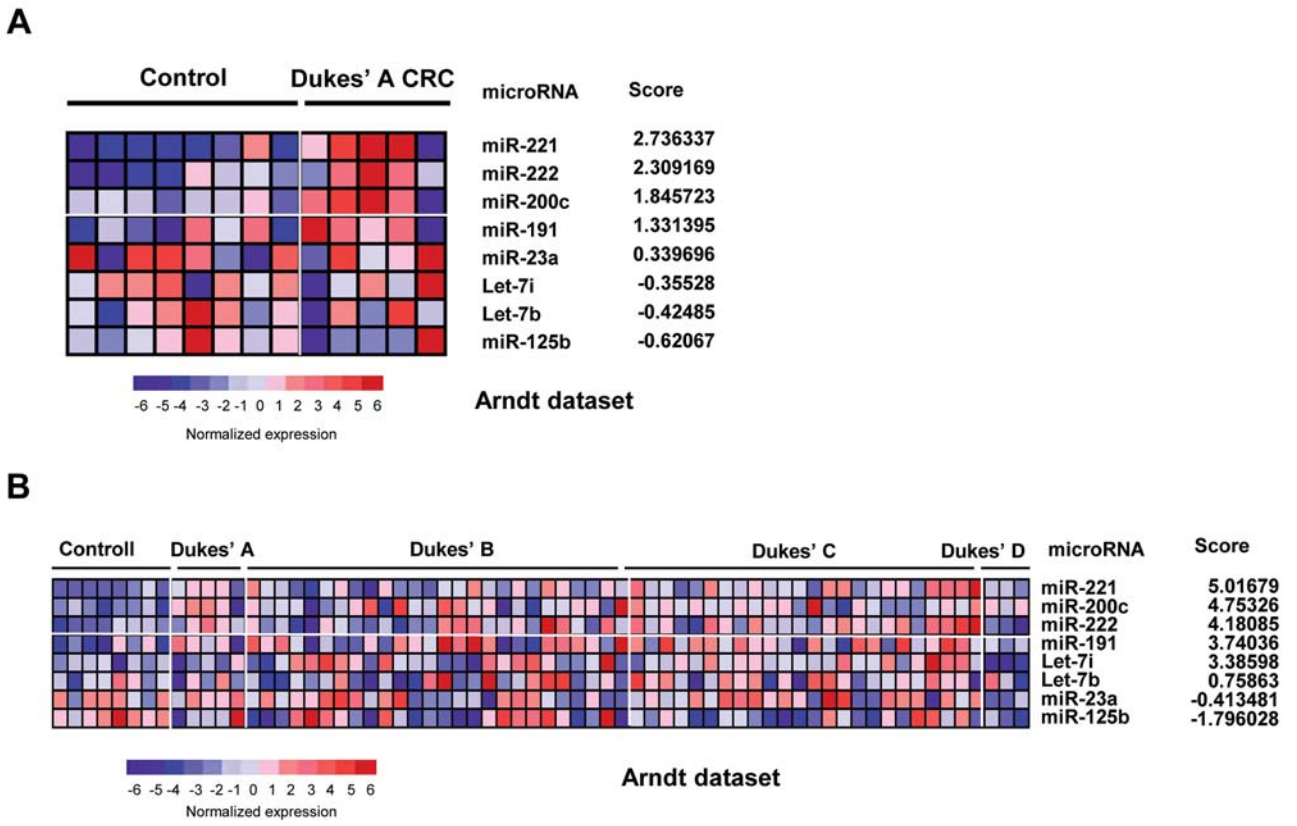

Figure 4. *The expression in human colorectal tumor specimens and colonic mucosa specimens in the Arndt dataset of the eight miRNAs regulated by oncogenic KRAS in 3D culture. Rows represent miRNAs and score. Columns represent normalized expression levels of the eight miRNAs selected in human colorectal tumors from five Dukes' A CRC (A) and 58 CRC patients (B) compared with colonic mucosa specimens from eight healthy controls.*

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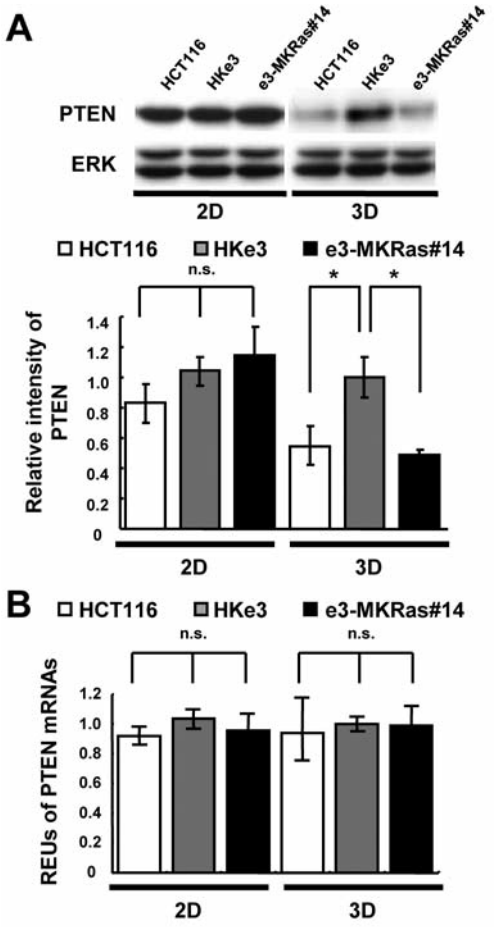

Figure 5. *3D-Specific reduction of PTEN expression. A: Western blot analysis for PTEN (upper panel). Quantitative analysis of PTEN protein (lower panel). *p<0.005. B: Quantitative analysis of PTEN mRNA. Relative expression levels of PTEN mRNAs were determined by setting PTEN mRNA expression in HKe3 cells in 3D culture at day 6 as 1.0. n.s., No significant difference.*

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