MicroRNA-124 inhibits cancer cell growth through PTB1/PKM1/PKM2 feedback cascade in colorectal cancer

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A B S T R A C T

Altered levels and functions of microRNAs (miRs) have been associated with carcinogenesis. In this study, we investigated the role of miR-124 in colorectal adenoma (CRA) and cancer (CRC). The expression levels of miR-124 were decreased in CRA (81.8%) and CRC (57.6%) in 55 clinical samples. The ectopic expression of miR-124 induced apoptosis and autophagy in colon cancer cells. Also, miR-124 targeted polyuridyrimidine tract-binding protein 1 (PTB1), which is a splicer of pyruvate kinase muscles 1 and 2 (PKM1 and PKM2) and induced the switching of PKM isoform expression from PKM2 to PKM1. Also, siR-PTB1 induced drastic apoptosis in colon cancer cells. Furthermore, we found that the ectopic expression of miR-124 enhanced oxidative stress and the miR-124/PTB1/PKM1/PKM2 axis constituted a feedback cascade. Finally, we showed that intratumor injection of miR-124 and siR-PTB1 induced a tumor-suppressive effect in xenografted mice. The axis was established by both in vitro and in vivo experiments to function in human colorectal cancer cells. These findings suggest that miR-124 acts as a tumor-suppressor and a modulator of energy metabolism through a PTB1/PKM1/PKM2 feedback cascade in human colorectal tumor cells.
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Introduction

Colorectal cancer (CRC) is the third leading cause of death among all human malignancies, causing over 600,000 deaths per year all over the world [1]. MicroRNAs (miRNAs) are single-stranded non-coding small RNAs that repress translation or induce degradation of target messenger RNAs (mRNAs) through binding to specific complementary sites within the 3’ untranslated region (3’UTR) of mRNAs [2]. A growing body of evidence indicates that dysregulation of miRNA expression contributes to various human cancers, including CRC [3]. Genes that are regulated by these cancer-associated miRNAs, such as APC/β catenin, Cox-2, K-Ras, and p53, are important for both the initiation and progression of CRC [4].

MiRNA-124 (miR-124) is a brain-enriched miRNA that has been broadly investigated with respect to physiological neural development [5,6]. MiR-124 has a tumor-suppressive effect especially in brain tumors [7] and is also down-regulated in various cancers, including CRC [8,9]. It regulates some proliferation-related genes such as signal transducer and activator of transcription 3 (STAT3) and others [8,10,11].

We previously found that DEAD-box protein RNA helicase (DDX6) is highly expressed in colorectal adenoma (CRA) and CRC [12,13]. We examined which miRNAs target DDX6 and found that miR-124 efficiently regulates DDX6. However, the functions of miR-124 in the adenoma–carcinoma sequence and the detailed molecular mechanisms have not been elucidated yet. In the current study, therefore, we focused on the role of miR-124 in carcinogenesis in both CRA and CRC.

Cancer cell growth entails numerous metabolic changes. Cancer cells exhibit a metabolic phenotype characterized by increased glycolysis, regardless of oxygen availability – a phenomenon termed the Warburg effect [14]. In mammals, this is partly achieved through control of the expression of pyruvate kinase muscle (PKM) isoforms [15]. PKM has 2 isoforms, i.e., PKM1 and PKM2, which are produced by alternative splicing of transcripts of the PKM gene. PKM is alternatively spliced to produce either the PKM1 or PKM2 isoform,
which contains exon 9 or exon 10, respectively [16]. PKM2 is exclusively expressed in embryonic, proliferating, and cancer cells, promoting glycolysis even in an aerobic environment. PKM1 is expressed in some normal differentiated tissues and promotes oxidative phosphorylation [15]. Recent studies suggest that heightened expression of PKM2 is critical for the maintenance of cancer cell growth [17,18].

Three heterogeneous nuclear ribonucleoproteins (hnRNPs) proteins, polypyrimidine tract-binding protein 1 (PTB1, also known as hnRNP1), hnRNP A1, and hnRNP A2, bind repressively to sequences flanking exon 9. In the presence of these PKM alternative splicing proteins, exon 10 is included in the PKM transcript [19,20]. Other studies demonstrated that miR-124 regulates PTB1 genes and promotes neuronal differentiation [5] or controls the phenotype of pulmonary vascular fibroblasts [23]. However, the function and relationship among miR-124, PTB1, PKM1, and PKM2 in cancer cells have not been elucidated yet.

In this study, we showed that miR-124 was down-regulated in CRA and CRC patients. In addition, the ectopic expression of miR-124 induced apoptosis and autophagy by regulating the PKM1/PKM2 ratio through the targeting of PTB1 in colon cancer cells. Our data suggest that the miR-124/PTB1/PKM1/PKM2 cascade plays critical roles in cancer energy metabolism and that this cascade is a possible target for the treatment of CRC.

Materials and methods

Patients and samples

All human samples were obtained from patients who had undergone biopsy or surgery for resection at Fujita Health University Hospital (Toyoake, Aichi, Japan), Osaka Medical College Hospital (Takatsuki, Osaka, Japan) or Saiseikai Ibaraki Hospital (Ibaraki, Osaka, Japan). Informed consent in writing was obtained from each patient. Collection and distribution of the samples were approved by each of the appropriate institutional review boards in accordance with the Declaration of Helsinki. Thirty-three patients with previously untreated (or recently diagnosed) colorectal cancer and 22 with adenomas were selected. The distribution according to other clinical parameters is shown in Table 1. Under a pathologist’s supervision, all tissue sample pairs were collected from surgically or endoscopically resected tissues, with these paired samples being from the primary tumor and its adjacent non-tumor mucosal tissue in the same patient.

Cell culture and cell viability

All cell lines were obtained from JCRB [Japanese Collection of Research Bioresources] Cell Bank. All cell lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS (Sigma-Aldrich Co, St. Louis, MO, USA) and 2 mM L-glutamine under an atmosphere of 95% air and 5% CO2 at 37 °C. The number of viable cells was determined by performing the trypan-blue dye exclusion test.

Transfection experiments

DLD-1 cells or WiDr cells were seeded in 6-well plates at a concentration of 0.5 × 10^5 per well (10–30% confluence) on the day before the transfection. The mature type of miR-124 (miVana™ miRNA mimic; Ambion, FosterCity,CA,USA), antagoniR-124 (miVana™ miRNA inhibitor; Ambion) or siRNAs for PTB1 and PKM2 (si-PTB1 and siR-PKM2; Invitrogen, Carlsbad, CA) was used for the transfection of the cells, which was achieved by using cationic liposomes, Lipofectamine™ RNAiMAX (Invitrogen), according to the manufacturer’s Lipofection protocol. The nonspecific control miRNA (HSS, Hokkaido, Japan) sequence was 5′-GUAGGAGUGUAGGAGCC-3′, which was used as a control for nonspecific effects [24]. The sequence of the mature type of miR-124 used in this study was 5′-UAAGGCAACCGGUGAUAAGCC-3′; that of siR-PTB1 for the 3′UTR region, 5′-AUCCUGCUGCGGUGAUAAGCGC-3′, which was used as a control for nonspecific effects [24]. The sequence of the mature

<table>
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<th>Characteristic</th>
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<th>Expression of miR-124 ( % )</th>
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<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
<td>36</td>
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<tr>
<td>Female</td>
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<tr>
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<tr>
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<td>Adenoma</td>
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<td>9 (60)</td>
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<td>Left colon</td>
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<td>10 (62.5)</td>
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<td>&gt;45</td>
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<tr>
<td>C</td>
<td>14</td>
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<td>Tumor diameter in adenoma (mm)</td>
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Table 1: Characteristics of study population and expression of miR-124 in colorectal tumors.

Inhibitor agents

We used pan-caspase inhibitor Z-VAD-fmk (zVal-Ala-Asp-fluoromethyl ketone), which was purchased from MBL (Nagoya, Japan); an autophagy inhibitor, 3-methyladenine (3-MA), from Calbiochem (San Diego, CA, USA); and free radical scavenger N-acetyl-L-cystine (NAC), from Sigma Aldrich (St. Louis, MO, USA). DLD-1 cells were pretreated with 3-MA (0.5 mM) and Z-VAD (20, 50 μM) before the transfection with miR-124. DLD-1 cells were treated with NAC (1, 5 mM) 24 h after transfection with miR-124 or siR-PTB1.

Western blotting

Protein extraction and Western blotting experiments were performed as described in our previous reports [25,26]. Primary antibodies were used as follow: anti-EZ1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-PTB1, PARP, LC3B, pAkt, Akt, pErk1/2, Erk1/2, c-Myc, STAT3 or mTOR (Cell Signaling Technology, Inc., Danvers, MA, USA; anti-PKM1 or PKM2 (Novus Biologicals, USA), and anti-j-actin antibody (Sigma-Aldrich Co.). HRP-conjugated goat anti-rabbit and horse anti-mouse IgG (Cell Signaling Technology) were used as secondary antibodies. β-actin was used as an internal control.

Real-time reverse transcription-PCR

RNA extraction and RT-PCR experiments were performed as described in our previous reports [25,26]. The primers for PTB1, PKM1, PKM2, and GAPDH were the following: PTB1-sense, 5′-ATC AGG CCT TCA TCG AGA TGC ACA-3′; PTB1 antisense, 5′-TGT CTT CAG CTC CTT GTG GGT GTA-3′; PKM1-sense, 5′-CCA CCC TCA ACT CAC TCC AC-3′; PKM1-antisense, 5′-GTC ACC AGA CTT GCC AGA CT-3′; PKM2-sense, 5′-ATT ATT TCA GGA CCC CCG CTT-3′; PKM2-antisense, 5′-ATT CGG GCT AAC AAT GAT GGC-3′ [18]; GAPDH-sense, 5′-CTC AGA CCG CAG TTC AGC ACC-3′; GAPDH-antisense, 5′-CCA CCC ATT GCA AAT TCC ATG GCA-3′; RN68B and GAPDH were used as an internal control. The relative expression levels were calculated by the ΔΔCt method.

Luciferase reporter assay

Searching the Target Scan 6.2 database (http://www.targetscan.org/) to find algorithms-based binding sites of miR-124, we found the predicted binding sites to be at positions 329–336 in the 3′UTR of PTB1 mRNA. The sequence regions 1861–2293, containing the putative binding sequence of miR-124, were inserted into a pMIR-REPORT™ Luciferase miRNA Expression Reporter Vector (Applied Biosystems) according the manufacturer’s protocol. Moreover, we made another pmiR construct encompassing a mutated seed sequence for miR-124 (Wild type; GTGCGCCA, mutant; GTAATTCA) by using a Primestar® Mutagenesis Basal Kit (TaKaRa). The mutation of the vector was confirmed by sequence analysis. pRL-TK Renilla Luciferase Reporter vector (Promega, Madison, WI, USA) was used as an internal control vector. DLD-1 were seeded into 96-well plates at a concentration of 0.1 × 10^3 well per well on the day before the transfection. DLD-1 cells were co-transfected with either reporter
demonstrated that the down-regulation of L1 is the long axis and L2 is the short axis of the tumor. This formula was described in the manufacturer’s protocol. Luciferase activities were reported as the firefly luciferase/Renilla luciferase ratio.

Electron microscopic study

DLD-1 cells treated or not with miR-124 (40 nM) were harvested and rinsed with PBS. The cells were then fixed for 2 h with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4; PB), rinsed in PB, and postfixed in 2% osmium tetroxide for 2 h. After having been washed with PB, the cells were progressively dehydrated by passage through a 10% graded series of 30–100% ethanol and then cleared in Quetol-812 (Nissin EM, Tokyo, Japan). Thereafter, they were embedded in Epon 812 resin (TAAB Laboratories Equipment, Reading, UK) and thin sections (70-nm thickness) were prepared. Finally, the sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy with a Hitachi-7650 (Hitachi, Tokyo, Japan) operating at 80 kV.

Electron spin resonance spectroscopy (ESR)

Productions of free radicals were determined using the ESR trapping technique in combination with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Tokyo Chemical Industry Co.). ESR spectra of free radicals were measured using a quartz capillary tube (i.d. 0.75 mm; JES-LC01) and JEOl JES-FA2000E free radical monitor (JEOl Ltd., Akishima, Japan). The measurement conditions were as follows: magnetic field, 336 ± 5 mT; power, 8.98 mW; sweep time, 4 min; modulation, 100 kHz, 0.08 mT; amplitude, 4000; time constant, 0.3 s. DLD-1 cells treated with control miRNA and miR-124 (20 nM). Signal intensity was compared with control and miR-124 at 24 h after the transfection.

Lactate assay

DLD-1 cells were collected at 48 h after the transfection. Lactate was measured with L-Lactate Assay kit according to the manufacturer’s instructions (Cayman Chemical Company, Michigan, USA). Lactate production was normalized to the number of cells.

Hoechst33342 staining

DLD-1 cells were collected at 72 h after the transfection. The cells were stained with Hoechst33342 (5 μg/ml) at 37°C for 1 h, washed once with phosphate-buffered saline, resuspended, pipetted dropwise onto a glass slide, and examined by fluorescence microscopy using an Olympus microscope (Tokyo, Japan) equipped with an epifluorescence and appropriate filters. The cells with condensed and/or fragmented nuclei stained with Hoechst33342 were assessed to be apoptotic.

Immunofluorescence study

We transfected DLD-1 cells with nonspecific control miRNA or miR-124. At 48 h after the transfection, the cells were fixed for 15 min with 4% paraformaldehyde. They were then transferred to PBS and kept in it for 15 min, followed by exposure to 15% Block Ace (Dainippon Sumitomo Pharma Co., Ltd., Tokyo, Japan) for 20 min to block nonspecific antibody binding. Next, the cells were incubated with primary antibody against PKM1 or PKM2 (Novus Biologicals, USA) for 2 h at room temperature (RT). After a rinse in PBS for 15 min, the cells were then incubated with avidin-antibody (Dako, Carpentaria, CA, USA) for 60 min, followed by streptavidin-conjugated Alexa Fluor 555 (Molecular Probes, Eugene, OR, USA) for 30 min at RT. Finally, the samples were counterstained with DAPI for 15 min and observed under a fluorescence microscope.

Human tumor xenograft model

Animal experimental protocols were approved by the Committee for Animal Research and Welfare of Gifu University. BALB/cSCID-nu/nu (nude) mice were obtained from Japan SLC (Hamamatsu, Japan). Human colon cancer DLD-1 or WiDr cells were inoculated at 2.0 × 10⁶ cells/100 μl or 5.0 × 10⁶ cells/100 μl per site into the back of each mouse. The inoculation day was set as day 0. At 10 days after inoculation, we confirmed the engraftment of the tumors. After miR-124, siR-PTB1 or control miRNA (0.2 nmol per 1 administration) in 50 μl of Opti-MEM had been incubated with 1 μl of Lipofectamine RNAiMAX, the mixture was injected into the tumor 2 (miR-124) or 3 (siR-PTB1) times per week. MiR-124 group contained 3 and PTB-1 group contained 5 mice. In the cases in which the tumors had disappeared, we stopped the injections. The tumor volume was calculated by the formula: $V = \frac{1}{2}L_2 \times L_3^2$, where $L_1$ is the long axis and $L_2$ is the short axis of the tumor. This formula was described in a previous report [35,27].

Statistics

Each examination was performed in triplicate. In experiments on clinical samples, the expression levels >1.5 were designated as up-regulation and those <0.67 as down-regulation, which fold changes were obtained from the results of linear discriminant analysis of miR-124 expression patterns from 55 pairs of colon tumors and non-tumorous tissues. Statistical differences between clinicopathologic parameters and the miR-124 level of tumor samples were evaluated by using Pearson’s χ² test or Fisher’s exact test, unless otherwise specified. For in vitro experiments, statistical significances of differences were evaluated by performing the two-sided Student’s t-test. In the case of the volume of the tumors xenografted into mice, the data were compared by using the Mann–Whitney U-test. The values were presented as the mean ± standard deviation. A P value < 0.05 was considered to be statistically significant.

Results

MiR-124 was down-regulated in clinical samples from CRA and CRC

Firstly, we examined the expression profile of miR-124 in normal tissues. As a result, the expression levels of miR-124 were preferentially high in the brain, hematopoietic systems, muscle, and heart, compared with those in the gastrointestinal tract such as the stomach and colon (Fig. 1A). Next, we extensively examined the expression profiles of miR-124 in the tumor samples from the patients with adenoma or cancer. Table 1 demonstrated that the down-regulation of miR-124 was frequent in both adenoma and cancer samples; however, no significant positive finding in clinical parameters was found. It should be noted that the down-regulation of miR-124 occurred more frequently in adenomas (81.8%) than in cancerous tissues (57.6%). In the human colon cancer cell lines tested, the expression of miR-124 was also down-regulated (data not shown). These findings imply that miR-124 likely functioned as a tumor suppressor, especially in the initiation of the adenoma–cancerine sequence.

MiR-124 induced apoptosis and/or autophagic survival in human colon cancer cells

In order to examine the effect of miR-124 on the growth of human colon cancer cell lines, we transfected DLD-1 and WiDr cells with miR-124. As shown in Fig. 1B, the growth of either cell line was significantly inhibited even at 20 nM. Western blot analysis indicated that the level of the cleaved form of PARP-1 and the transition of LC3I to II were remarkable after the transfection of both cell lines. Also, PI3K/Akt signaling-related pAkt, Akt and mTOR, and MAPK Erk1/2 were down-regulated in both treated cells. On the other hand, the phosphorylated levels of Erk1/2 were activated in DLD-1 cells; whereas those in WiDr cells were inactivated (Fig. 1C). The morphological study by electron microscopy indicated apoptosis combined with autophagy in the transfected cells (Fig. 1D). Therefore, we validated this cell death by apoptosis combined with autophagy by using inhibitors, 3MA for autophagy and Z-VAD for apoptosis. As a result, 3MA significantly promoted cell death; however, Z-VAD partially canceled the apoptotic cell death elicited by miR-124 (Fig. 1E and F). These findings strongly suggest that miR-124 functioned as a tumor-suppressor and that the ectopic expression of miR-124 induced apoptosis and/orautophagic survival in both cell lines.

PTB1 is a direct target of miR-124

Then, we searched for candidate target genes associated with such programmed cell death, and finally focused on PTB1, which modulates the expression of PKM1 and PKM2 by splicing the PKM mRNA. As shown in Fig. 2A and B, the mRNA and the protein expression of PTB1 were significantly down-regulated in miR-124-transfected cells, as estimated by RT-PCR and Western blot analysis, respectively.
Fig. 1. (A) Relative expression levels of miR-124 in various human normal tissues. (B and C) Effects of ectopic expression of miR-124 on cell viability (B) and expression of various proteins estimated by Western blot analysis (C) at 72 h after transfection of DLD-1 and WiDr cells with miR-124 at a concentration of 20 or 40 nM. (D) Morphological study by using electron microscopy. DLD-1 cells were treated with control miRNA or miR-124 (40 nM) for 72 h. N: fragmented nuclei, V: vacuole, M: mitophagy. (E) Inhibition of autophagy by using 3-MA and (F) inhibition of apoptosis by using pan-caspase inhibitor Z-VAD-fmk in DLD-1 cells. DLD-1 cells were pretreated with 3-MA (0.5 mM) or Z-VAD (20, 50 μM) and then transfected with miR-124 (40 nM) for 72 h. NT: not treated. To validate appropriate effects of these inhibitors, Western blot analysis was performed. Densitometric values were calculated for the cleaved form of PARP-1 and for LC3I. Results are presented as mean ± SD; *P < 0.05; **P < 0.01; ***P < 0.001.
Interestingly, the expression levels of E2F1, STAT3 and c-Myc, which are upstream of hnRNPs including PTB1 [15], were also downregulated. As shown in Fig. 2C, the results of the luciferase reporter assay indicated PTB1 to be a direct target of miR-124. On the other hand, the treatment with antagomiR-124 significantly reversed the growth inhibition elicited by miR-124 and increased the expression level of PTB1 (Fig. 2D). Based on these results, we concluded that miR-124 targeted PTB1.
MiR-124 increased the PKM1/PKM2 ratio in human colon cancer cells

PTB1 is a splicer of the splicing complex that modulates the expression of PKM1 and PKM2 mRNAs. So next we examined the mRNA expression levels of PKM1 and PKM2 after the transfection with miR-124. As a result, the PKM1/PKM2 ratio was remarkably elevated from 7 up to 8 fold compared with that for the control in both cell lines (Fig. 3A). Fig. 3B gives the results on the protein expression levels of PKM1 and PKM2. The PKM1/PKM2 ratio almost paralleled the ratio in mRNA levels. In other words, PKM isomorph expression was shifted from PKM2 to PKM1 after transfection with miR-124. Moreover, to validate whether this finding would hold at the single-cell level, we performed immunofluorescence (IFC) using DLD-1 cells that had been transfected with miR-124. As a result, immunostaining for PKM1 showed a significantly increased intensity in the treated cells at the single-cell level. On the other hand, PKM2 expression was slightly decreased (Fig. 3C). Therefore, the PKM1/PKM2 ratio was also remarkably elevated at the single-cell level. These findings suggest that miR-124 regulated the switching PKM isoforms expression from PKM2 to PKM1 through the targeting PTB1.

Gene silencing of PTB1 increased the PKM1/PKM2 ratio and induced apoptosis and/or autophagy

To test the function of PTB1, we silenced PTB1 expression by using the siRNA for PTB1 (Fig. 4). We silenced both the open reading frame (ORF) of PTB1 and its 3′UTR. The gene silencing of PTB1 resulted in a marked growth inhibition in both cell lines, and siR-PTB1 for the 3′UTR exhibited a greater suppressive effect on cell growth than did the siR-PTB1 for the ORF (Fig. 4A and Supplementary Fig. S1). Based on this result, we used siR-PTB1 for 3′UTR to examine the effect of gene silencing of PTB1. Interestingly, the cleaved form of PARP and the LC3I to II transition were remarkable in both cell lines by Western blot analysis. The inactivation of P38/Akt signaling was found when we treated with siR-PTB1 in both cells (Fig. 4B). Also, Hoechst33342 nuclear staining indicated a typical apoptotic appearance such as chromatin condensation and nuclear fragmentation in the siR-PTB1-treated DLD-1 cells (Fig. 4C). These data altogether demonstrated the induction of apoptosis and/or autophagy by the silencing of PTB1. Furthermore, this silencing enhanced PKM1 and PKM2 expression levels, resulting in an extremely elevated the PKM1/2 ratio, by approximately 5–6 times, compared with that for the control (Fig. 4D). These results were very similar to those observed for the ectopic expression of miR-124. The PKM1 and PKM2 mRNA levels also positively correlated with those of protein expression (Fig. 4E). Finally, we examined the lactate production after the transfection with miR-124 and siR-PTB1 respectively. As a result, lactate production was remarkably decreased in both cases (Fig. 4F). These findings altogether indicate that miR-124 functioned on growth inhibition and modulation of the Warburg effect in part through targeting PTB1 in colon cancer cells.

MiR-124, PTB1, PKM1, and PKM2 were involved in the feedback cascade

To clarify the relationship between the expression of PKM1 and PKM2, we explored the gene silencing of PKM2, which is dominantly expressed in cancer cells [17,18]. The silencing of PKM2 induced a significant growth inhibition in both cell lines, even at 5 nM (Fig. 5A). Interestingly, the PKM1 level, estimated by Western blot analysis, was elevated in a dose-dependent manner; in contrast, the splicer PTB1 levels were decreased in both cells (Fig. 5B). Moreover, the levels of miR-124 were significantly elevated dose-dependently in both cells (Fig. 5C). Furthermore, in other kinds of cell lines, such as human monocytic leukemia cells (THP1) and human gastric cancer cells (MKN45), the same results were observed as in the case of colon cancer cells (Supplementary Fig.S2). Thus, the expression levels of PKM1 and miR-124 were inversely related to that level of PKM2. It seemed that the expressions of miR-124, PKM1, and PKM2 harmonized with each other for the maintenance of cell proliferation.

Fig. 3. (A) mRNA expression of PKM1 and PKM2 at 72 h after the transfection with miR-124 (20, 40 nM) in DLD-1 and WiDr cells. The PKM1/PKM2 ratio was calculated based on their relative mRNA levels. (B) Protein expression of PKM1 and PKM2 at 72 h after the transfection with miR-124 (20, 40 nM) in DLD-1 and WiDr cells. (C) Immunofluorescence of PKM1 (upper panels) and PKM2 (lower panels) at 48 h after transfection of DLD-1 cells with miR-124 (20 nM). Left panels, treatment with control miRNA; Right panels, treatment with miR-124. PKM1 or PKM2 is stained red, and nuclei are stained blue. Results presented as mean ± SD; ***P < 0.001.
Oxidative stress was partly involved in growth inhibition and cell death induced by miR-124

Since the ectopic expression of miR-124 elevated the PMK1/PKM2 ratio, we examined the contribution of free radicals by ESR; because of the ROS including free radicals such as superoxide ion (O$_2^-$) and other species elicited by the dominantly activated TCA cycle loop, and induction of apoptosis and autophagy [28]. As a result, O$_2^-$ was almost unchanged, but other species of free radical were remarkably increased after the transfection with miR-124 (Fig. 5D). In addition, we considered that free radical scavenger, NAC would cancel the apoptosis with autophagy induced by transfection with miR-124. As expected, NAC significantly blocked the apoptotic cell death and possibly weakened autophagic cell survival, based on the results of the reduced proform of PARP-1 and transition of LC3-I to II (Fig. 5E). In this case, it is likely that the oxidative stress was closely associated with apoptosis and/or autophagy induced by ectopic expression of miR-124. These findings induced by NAC were also observed in the case of PTB1 knock out (Supplementary Fig. S3). These data altogether suggest that the apoptotic cell death and/or autophagy was induced by the common pathway through the PTB1/PMK1/PKM2 cascade and that the miR-124/PTB1/PMK1/PKM2 feedback loop certainly contributed to the maintenance of cell proliferation and the Warburg effect.

Anti-tumor effect of miR-124 or siR-PTB1 on DLD-1 cell-xenografted tumor in nude mice

In order to examine the anti-tumor effect of miR-124 in vivo, DLD-1 and WiDr cells were inoculated subcutaneously into nude mice. At 10 days after the inoculation, we injected control or miR-124 into the developed tumor. As a result, a significant suppression of tumor growth was observed in the group injected with miR-124 (Fig. 6A). In addition, we examined the anti-tumor effect of siR-PTB1 in vivo in DLD-1 cells because PTB1 was shown to be an important target gene of miR-124. Surprisingly, in 3 of the 5 cases tested, the tumors disappeared. As a result, from the second administration, a significant suppression of tumor growth was observed in the group injected with siR-PTB1. In addition, Western blot analysis of the tumor samples indicated the similar results to those in vitro (Fig. 6B). These findings indicate that miR-124 induced growth inhibition in part through targeting PTB1 even in vivo.
Fig. 5. Effects of PKM2 knockdown on cell viability of (A) and related protein expression examined by Western blot analysis in DLD-1 and WiDr cells at 72 h after siR-PKM2 transfection at a concentration of 5 or 10 nM (B). (C) Expression levels of miR-124 at 72 h after the transfection of DLD-1 and WiDr cells with siR-PKM2 (5, 10 nM). (D) Free radicals were evaluated by ESR at 24 h after the transfection of DLD-1 cells with miR-124 (20 nM). The left panels show the representative ESR spectra. O$_2^-$: superoxide ion, Other OPs: other oxidative products. (E) Effects of NAC on the growth and cell death of DLD-1 cells after the transfection with miR-124. DLD-1 cells were treated with NAC (1, 5 mM) at 24 h after the transfection with miR-124 (20 nM). Cell viability and Western blot analysis at 48 h after the transfection of DLD-1 cells with miR-124. Densitometric values are shown for proform of PARP-1 and LC3II.
Down-regulation of miR-124 increased the ratio of PKM2/PKM1 in clinical CRA and CRC samples

Finally, we investigated the correlation between miR-124 and the expression profile of PKM1 and PKM2 in clinical CRA and CRC samples. Clinical samples were divided into 2 groups, one with the down-regulation of miR-124 (miR-124 D; N = 15) and the other without the down-regulation (miR-124 ND; N = 10). We examined the mRNA expression levels of PKM1 and PKM2 in tumors and adjacent normal mucosas by RT-PCR. Then we calculated the PKM2/PKM1 ratio for tumors divided by that for the normal mucosas in each sample and compared it between the miR-124 D group and the ND group. The PKM2/PKM1 ratio for the miR-124 D group was remarkably elevated compared with that for the ND group (Fig. 6C). These data suggest that miR-124 may also regulate PKM isoforms expression through PTB1 even in clinical samples.

Discussion

In the current study, we firstly suggest that miR-124 contributed to the initiation of the adenoma–carcinoma sequence. It was shown in a study based on 96 samples that the down-regulation of miR-124 is an independent prognostic factor in patients with CRC [29]. In our study, the down-regulation of miR-124 was not correlated with any parameters according to the progression of CRA or CRC (Table 1). However, we showed that approximately 80% of the CRA cases showed decreased expression of miR-124 and the frequency was higher than that for the CRC cases. Previously, we showed that miR-145 targeted catenin δ-1 (CTNND1) to regulate APC/β catenin signaling in human colon cancer cells [30]. Similarly, miR-124 down-regulated CTNND1 (data not shown). These findings suggest that miR-124 plays a role in the development of adenoma rather than in that of malignant transformation on cancer pathogenesis.
Autophagy has long been known for its roles in cancer metabolism and tumor growth [17,18] and various functions such as protein kinase in cancer cells [32,33]. Also, it has been reported that SRSF3 (serine/arginine-rich splicing factor 3) is another PKM alternative splicing protein and acts as an oncogene by up-regulation of PKM2 in cancer cells [34]. Altogether, these data indicate that PTB1 and PKM2 could function as an oncogenic cascade and that a reduction in PTB1 expression may be a novel strategy for the development of anti-cancer drugs.

The reason why PKM2 is dominant in cancer cells is not entirely understood; however, it is accepted that the aerobic glycolysis increase metabolic intermediates that are required for synthesis of biological macromolecules and to avoid cell death induced by ROS [35]. In this present study, we indicated that ectopic expression of miR-124 enhanced the free radical activities evaluated by ESR (Fig. 5D) and the cell death, dubbed apoptosis and/or autophagy, was partly suppressed by a using free radical scavenger, NAC (Fig. 5E). These results support the above findings. However, the cancellation of cell death by NAC was not complete, which indicates that another machinery was involved in the cell death.

It was earlier shown that hypoxia-inducible factor 1 (HIF-1) mediates PKM2 gene transcription and metabolic reprogramming in cancer cells [36]. HIF-1 also has a critical role in the regulation of angiogenesis growth factors. HIF-1 has the potential for therapeutic intervention in ischemic cardiovascular diseases and cancer by promoting the expression of vascular endothelial growth factor (VEGF) [37]. Likely, energy metabolism and tumor angiogenesis are closely associated with each other. It would be plausible that the expression profiles of miR-124, PKM1, PKM2, and PTB1 could be therapeutic markers for the appropriate use of anti-VEGF agents.

Secondly, we showed that miR-124 induced apoptosis and/or autophagy, which was confirmed by electron microscopy and biochemical studies (Fig. 1C and D). Autophagy has long been known for its roles in protecting cells against stresses such as starvation [31]. Cells can then recycle the resulting degradation products and thus provide the energy and cellular re-building necessary for their survival. We showed that miR-124 induced autophagic survival, as evaluated by using 3-MA, an autophagy inhibitor (Fig. 1E). As cause of autophagy by ectopic expression of miR-124, we found that miR-124 targeted PTB1 and the silencing of PTB1 had a critical effect on energy metabolism in cancer cells (Figs. 2 and 4). It has been reported that the simultaneous depletion of PKM alternative splicing proteins (PTB1/hnRNPA1/hnRNPA2) causes a substantial change in the PKM1/PKM2 ratio [19] and miR-124, miR-137, and miR-340 regulate these PKM alternative splicing proteins [21]. Although we investigated the effects of miR-124 on hnRNPA1 and A2 splicers, we couldn't find any change in their mRNA expression levels (Supplementary Fig. S4). Therefore, we concluded that the PTB1 could be one of the important target genes of miR-124 in colon cancer cells. Moreover, the expression levels of E2F1, STAT3 and c-Myc, which are upstream of PTB1, were down-regulated by miR-124 (Fig. 2B). Based on these results, we concluded that miR-124 closely contribute to the modulation of Warburg effect (Fig. 7).

PTB1 is known as a suppressor of PKM1, so far considered to act by repressing the exon 9 included in this splice variant in embryonic and cancer cells [19,20]. In this study we showed that the ectopic expression of miR-124 down-regulated PTB1 and repressed the expression of PKM2 and increased the expression of PKM1, even in a single-cell level by IFC (Figs. 2 and 3). Interestingly, the knockdown of PTB1 induced drastically apoptotic cell death, which strongly indicates that PTB1 acted as an oncogene (Fig. 4A–C). Furthermore, the local injection of siR-PTB1 into xenografted-tumors exhibited a marked tumor growth-suppressing effect (Fig. 6B). Emerging evidence indicates that PKM2 has an important role in cancer metabolism and tumor growth [17,18] and various functions such as protein kinase in cancer cells [32,33]. Also, it has been reported that 3RSF3 (serine/arginine-rich splicing factor 3) is another PKM alternative splicing protein and acts as an oncogene by up-regulation of PKM2 in cancer cells [34]. Altogether, these data indicate that PTB1 and PKM2 could function as an oncogenic cascade and that a reduction in PTB1 expression may be a novel strategy for the development of anti-cancer drugs.
study will be needed to elucidate the molecular mechanism inducing by miR-124 and the reason why PKM2 is dominant in cancer cells from various aspects.

Finally, the existing molecular target drugs such as tyrosine kinase inhibitors are apt to reprogram the tumor growth environment by other activating compensatory cascades. However, the results of the present study strongly suggest that miR-124 and PTB1 have the potential to be target molecules for the development of anti-cancer drugs, because they are able to affect negatively the entire energy metabolism that specifically works in cancer cells.

Authors' contributions

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Conflict of interest

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.03.026.

References
