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Positive feedback of DDX6/c-Myc/PTB1 regulated by miR-124 contributes to maintenance of the Warburg effect in colon cancer cells

Kohei Taniguchi [a,b,*], Nobuhiko Sugito [a], Minami Kumazaki [a], Haruka Shinohara [a], Nami Yamada [a], Nobuhisa Matsuhashi [c], Manabu Futamura [c], Yuko Ito [d], Yoshinori Otsuki [d], Kazuhiro Yoshida [c], Kazuhisa Uchiyama [b], Yukihiro Akao [a]

[a] United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] Department of General and Gastroenterological Surgery, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki, Osaka 569-8686, Japan
[c] Department of Oncological Surgery, Gifu University School of medicine, 1-1 Yanagido, Gifu 501-1193, Japan
[d] Department of Anatomy and Cell Biology, Division of Life Sciences, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki, Osaka 569-8686, Japan

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ABSTRACT

The human DEAD/H-box RNA helicase gene DDX6 is a target of the t(11;14)(q23;q32) chromosomal translocation observed in human B-cell lymphoma, and the overexpression of its protein has been shown to cause malignant transformation. DDX6 has a variety of functions such as translation initiation, pre-mRNA splicing, ribosome assembly, and more. However, details of the regulatory mechanism of DDX6 and functions of DDX6 in cancer cells are largely unknown. On the other hand, the Warburg effect is a well-known feature of cancer cells. Pyruvate kinase in muscle (PKM), which is a rate-limiting glycolytic enzyme, has 2 isoforms, PKM1 and PKM2. It has been frequently reported that PKM2 is a tumor-specific isoform and promotes the Warburg effect. However, the functions of the PKM1 gene have been hardly mentioned. Here, we showed that DDX6 was overexpressed in colorectal cancer specimens and regulated by microRNA (miR)-124 in colon cancer cells. Also, a DDX6/c-Myc/PTB1 positive feedback circuit regulated by miR-124 was shown to be established and to contribute to maintenance of the Warburg effect. Moreover, we showed that knockdown of DDX6 induced mainly apoptosis through an imbalance of PKM gene expression, especially causing down-regulation of PKM1 in colon cancer cells. These results suggest that miR-124 is a fine tuner of the Warburg effect and that DDX6 is one of the key molecules in Warburg effect-related miR-124 targeting various genes.

1. Introduction

The human DEAD/H-box RNA helicase gene DDX6 (also termed RCK/p54) was previously identified through chromosomal breakpoint studies on a t(11;14)(q23;q32) translocation in a B-cell lymphoma cell line, RC-K8 [1–3]. Previously, we reported that the oncogenic RNA helicase DDX6 is highly overexpressed in most malignant cell lines and clinical colorectal tumors [4,5]. Also, we showed that DDX6 expression is linked to the regulation of cell growth and differentiation of cancer cells through internal ribosomal entry site (IRES)-dependent translation of c-Myc [6–8]. Furthermore, DDX6 protein interacts with the 5-cap structure-binding protein eIF4E, which is a rate-limiting factor in the initiation of translation and acts as a translational regulator [6,7]. Recently, we found that DDX6 negatively controls non-coding RNA gene (NCR) 143/145 RNA stability in P-bodies and regulates miR-143/145 expression post-transcriptionally in cancer cells [9]. However, much is still largely unknown about the functions of DDX6 in cancer cell growth.

MiRNAs are single-stranded non-coding small RNAs that repress translation or induce degradation of target mRNAs through binding to specific complementary sites within the 3′ untranslated region (3′UTR) of mRNAs [10]. A great deal of evidence indicates that dysregulation of miRNA expression contributes to the development of various human cancers, including colorectal cancer (CRC) [11,12]. Therefore, we investigated which miRNAs targeted DDX6 and finally focused on miR-124.

MiR-124 is known as a brain-enriched miRNA and related with physiological neural development [13,14]. Also, miR-124 play a tumor-suppressor, especially against brain tumors [15] and various cancers, including CRC [16,17]. Previously, we found that miR-124 targets polypyrimidine tract-binding protein 1 (PTB1, also known as hnRNPI or PTBP1) and modulates cancer energy metabolism in

Abbreviations: ACSL1, long-chain acyl-CoA synthetase; CPT1, carnitine palmitoyltransferase-1; DDX6, DEAD-box RNA helicase 6; eIF4E, eukaryotic translation initiation factor 4E; FAO, fatty acid oxidation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hnRNPs, heterogeneous nuclear ribonucleoproteins; IRES, internal ribosomal entry site; miRNA, microRNA; PKM, pyruvate kinase in muscle; PTB1, polypyrimidine tract-binding protein 1; ROS, Reactive Oxygen Species; RNU6B, U6 small nuclear B RNA

* Corresponding author at: United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan.
E-mail address: sur144@poh.osaka-med.ac.jp (K. Taniguchi).

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colon cancer cells through the regulation of pyruvate kinase in muscle (PKM) isoforms, which are rate-limiting glycolytic enzymes [17,18].

PKM has 2 isoforms, PKM1 and PKM2, which are produced by alternative splicing of transcripts in the PKM gene. PKM1 contains exon 9 and lacks exon 10, whereas PKM2 contains exon 10 and lacks exon 9 [19]. It has been frequently reported that PKM2 is exclusively expressed in embryonic, proliferating, and cancer cells, and promotes the Warburg effect [20,21]. On the other hand, it was previously thought that PKM1 is expressed in normal differentiated tissues and promotes oxidative phosphorylation [22]. However, the functions of PKM1 hardly have been elucidated yet.

In the current study, we sought to clarify the biological significance of DDX6 from the view point of the Warburg effect. We concluded that DDX6 was partly regulated by miR-124. In addition, DDX6/c-Myc/PTB1 was shown to constitute a positive feedback circuit regulated by miR-124, which affects the Warburg effect. Moreover, we propose that PKM1 played essential roles in the growth of cancer cells, as revealed by the down-regulation of PKM1 by siR-DDX6.

2. Materials and methods

2.1. Patients and samples

The study was reviewed and approved by the institutional review board of Gifu University Hospital (Gifu, Gifu, Japan) in accordance with the Declaration of Helsinki. All patients were informed of the investigational nature of the study and provided written informed consent before enrollment in the study. All human samples were obtained from patients who had undergone surgery for resection during 2014. Twenty patients with previously untreated (or recently diagnosed) colorectal cancer were selected. The characteristic of these patients are shown in Table 1. Under a pathologist's supervision, all tissue samples pairs were collected from surgically resected tissues, with these paired samples being from the primary tumor and its adjacent non-tumor mucosal tissue in the same patient.

Table 1
Clinicopathological features in colorectal cancer.

Case	Age	Sex[a]	Site[b]	Size[c]	Depth[d]	Stage[e]	DDX6[f]	miR-124[g]
1	60	M	R	52 × 68	SS	C	+	D
2	62	M	S	55 × 40	SS	D	+	D
3	38	F	R	50 × 95	MP	A	+	D
4	52	M	S	20 × 25	MP	A	+	ND
5	67	M	S	55 × 50	MP	A	+	D
6	56	F	S	27 × 24	SS	C	+	D
7	59	F	S	60 × 50	SE	D	+	D
8	42	M	R	30 × 20	SS	B	+	D
9	53	F	F	45 × 40	SS	B	+	D
10	72	F	A	35 × 32	SS	B	+	D
11	81	M	R	50 × 40	MP	C	+	D
12	49	M	R	50 × 40	SS	B	+	D
13	68	F	R	35 × 35	SS	B	+	D
14	62	M	R	39 × 38	MP	A	+	D
15	82	F	A	33 × 26	MP	A	−	D
16	64	M	R	28 × 22	MP	A	−	D
17	73	M	R	40 × 40	MP	A	−	D
18	78	F	C	21 × 25	SS	B	−	ND
19	41	M	R	70 × 45	SS	B	−	ND
20	73	F	T	55 × 25	SS	D	−	ND

[a] M, male; F, female.
[b] Location of tumor; C, cecum; A, ascending colon; T, transverse colon; S, sigmoid colon; R, rectum.
[c] Diameter in mm.
[d] MP, Mucosa propria; SS, Subserosa; SE, Serosa exposure; SI, Serosa invasion.
[e] Dukes' system.
[f] +, overexpression; −, no overexpression.
[g] D, down-regulation of miR-124 relative ratio (tumor tissue/normal adjacent tissue <0.67).
ND, no down-regulation of miR-124 relative ratio (tumor tissue/normal adjacent tissue ≧0.67).

2.2. Cell culture and cell viability

All cell lines were obtained from JCRB (Japanese Collection of Research Bioresources) Cell Bank. The medium used for the cultures was RPMI-1640. The details of the cell culture method were described in our previous reports [17,18]. The number of viable cells was determined by performing the trypan-blue dye-exclusion test.

2.3. Transfection experiments

All 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 (mirVana™ miRNA mimic; Ambion, Foster City, CA, USA), antagomiR-124 (mirVana™ miRNA inhibitor; Ambion) and siRNAs for DDX6, PTB1, PKM1, PKM2, and c-Myc (Invitrogen Carlsbad, CA) were 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′-GUAGGAGUAGUGAAAGGCC-3′, which was used as a control for nonspecific effects [23]. The sequence of the mature type of miR-124 used in this study was 5′-UAAGGCACGCGGUGAAUGCC-3′; that of siR-DDX6, 5′-GGAUAUUAUUCUCACGCUACCUAAA-3; that of siR-PTB1, 5′-AUCUCUGGUCUGCUAAGGUCACUUC-3′; that of siR-c-Myc, 5′-UUUGUGUUUCAACUGUUCUCGUCGU-3′; that of siR-PKM1, 5′-AAGUUCUUCAAACAGCUUGCGGUGG-3; and that of siR-PKM2, 5′-CAGACUUGGUGAGGACGAUUAUGGC-3′. The effects manifested by the introduction of miRs and siRNAs into the cells were assessed at 48 or 72 h after the transfection.

2.4. Western blotting

Whole cells were homogenized in chilled lysis buffer comprising 10 mM Tris–HCl (pH 7.4), 1% NP-40, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1% Protease Inhibitor Cocktail (Sigma-Aldrich Co.) and stood for 20 min on ice. After centrifugation at 13,000 rpm for 20 min at 4 °C, the supernatants were collected as whole-cell protein samples. Protein contents were measured with a DC Protein assay kit (Biorad, Hercules, CA, USA). Ten micrograms of lysate protein was separated by SDS-PAGE using 10.0 or 12.5% polyacrylamide gels, and electroblotted onto a PVDF membrane (PerkinElmer Life Sciences, Inc., Boston, MA, USA). After blockage of nonspecific binding sites for 1 h with 5% nonfat milk in PBS containing 0.1% Tween 20 (TBS-T), the membrane was incubated overnight at 4 °C with primary antibodies. The next day, the membrane was then washed 3 times with TBS-T, incubated further with secondary antibodies at room temperature for 1 h, and then washed 3 times with TBS-T. The immunoblots were visualized by use of Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Primary antibodies used were as follows: anti-PTB1, -PARP, -LC3B, -c-Myc, -ACSL1, -CPT1A (Cell Signaling Technology, Inc., Danvers, MA, USA); anti-CPT1C (Santa Cruz Biotechnology, CA, USA); anti-PKM1 and -PKM2 (Novus Biologicals, USA); anti-caspase 9 (MBL, Nagoya Japan); anti-DDX6 [6], and anti-β-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.

2.5. Real-time reverse transcription-PCR

Total RNA was isolated from cultured cells or tumor tissues by using a Nucleo Spin microRNA isolation kit (TaKaRa, Otsu, Japan). To determine the expression levels of miR-124, we conducted quantitative RT-PCR (qRT-PCR) by using TaqMan MicroRNA Assays (Applied Biosystems) and THUNDERBIRD Probe qPCR Mix (TOYOBO Co., LTD., Osaka Japan) according to the manufacturer's protocol. *RNU6B* was

used as an internal control. For determination of the expression levels of *DDX6* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs, total RNA was reverse-transcribed with a PrimeScript® RT reagent Kit (TaKaRa). Real-time PCR was then performed with primers specific for them by using THUNDERBIRD SYBR qPCR Mix (TOYOBO). The primers for *DDX6 and GAPDH* were the following: *DDX6*-sense, 5′-GGC TGG GAA AAG CCA TCT-3′, and *DDX6*-antisense, 5′-ACC TGA TCT TCC AAT ACG-3′; *GAPDH*-sense, 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′, and *GAPDH*-antisense, 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′. *GAPDH* was used as an internal control. The relative expression levels were calculated by the ΔΔCt method.

2.6. Luciferase reporter assay

Searching the Target Scan 6.2 database (http://www.targetscan.org/) to find algorithm-based binding sites of miR-124, we found the predicted binding sites to be at position 455–462 in the 3′UTR of DDX6 mRNA. The region 2270–2366 of DDX6, containing the putative binding sequence of miR-124, was 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, GTGC CTT; mutant, GTATTTT) 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 0.5×10^4 per well on the day before the transfection. The cells were co-transfected with either reporter vector (0.01 μg/well each) and 20 nM miR-124 or nonspecific non-coding siRNA (Dharmacon, Tokyo, Japan), which co-transfection was achieved by using Lipofectamine™ RNAiMAX. Luciferase activities were measured at 24 h after co-transfection by using a Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol. Luciferase activities were reported as the firefly luciferase/*Renilla* luciferase ratio.

2.7. Electron microscopic study

DLD-1 cells treated or not with miR-124 (40 nM) or siR-DDX6 (10 nM). The details of the method were described in our previous reports [17,24].

2.8. Hoechst 33342 staining

DLD-1 cells were collected at 48 or 72 h after the transfection. The cells were stained with Hoechst 33342 (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 epi-illuminator and appropriate filters. The cells with condensed and/or fragmented nuclei stained with Hoechst 33342 were assessed to be apoptotic, and the number of apoptotic cells among 200 cells was counted.

2.9. Lactate assay

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

2.10. Statistics

Each examination was performed in triplicate. In experiments on clinical samples, we calculated the relative miR-124 ratio of tumor/adjacent normal tissues. We defined the expression levels >1.5 as up-regulation and those <0.67 as down-regulation, which fold changes were obtained from the results of linear discriminant analysis of the miRNA expression patterns from many of our previous reports [25,26]. Statistical significances of differences were evaluated by performing the two-sided Student's *t*-test. The values were presented as the mean ± standard deviation. A P value <0.05 was considered to be statistically significant.

3. Results

3.1. Overexpression of DDX6 and down-regulation of miR-124 in clinical colorectal tumors

Previously, we found that DDX6 is highly overexpressed in colorectal tumor [4,5]. Again, we confirmed the overexpression of DDX6 in clinical colorectal tumor samples by performing Western blot analysis. As a result, 14 out of the 20 cases examined showed up-regulated DDX6 (Fig. 1A and Table 1). In order to clarify the role of miRNAs in the overexpression of DDX6, we searched the database of Target Scan 6.2 (http://www.targetscan.org/), with the results indicating that several miRNAs targeted DDX6. Among them, only miR-124 had a higher probability of preferential targeting of the conserved sites in the 3′UTR of DDX6. In addition, the DDX6 expression level after the transfection of DLD-1 cells with miR-124 was more down-regulated than that after transfection with other miRs that targeted DDX6, such as miR-19 (Supplementary Fig. S1). Based on these findings, we focused on the relationship between DDX6 and miR-124 as a negative regulator of it. As to the expression level of miR-124 in the clinical samples, 16 of the 20 cases showed a significant down-regulation of miR-124 (Fig. 1B). Moreover, 13 of the 14 cases with overexpressed DDX6 also showed a significantly low expression of miR-124 (Fig. 1A, B and Table 1). These findings implied that the overexpression of DDX6 in colorectal tumor was regulated at least in part by miR-124.

3.2. DDX6 is a direct target of miR-124 in colon cancer cells

To further clarify the relationship between DDX6 and miR-124, we firstly examined the mRNA and protein expression of DDX6 after we had transfected human colon cancer DLD-1 and WiDr cells with miR-124. As shown in Fig. 1C and D, the mRNA and protein expression of DDX6 were remarkably down-regulated in both cells. Also, we found that the ectopic expression of miR-124 significantly down-regulated DDX6 in other human colon cancer COLO201, too. However, we couldn't find the same effect in human colon cancer SW480 cells (Supplementary Fig. S2). Secondly, we performed a luciferase activity assay for DDX6 and found that the activity of wild-type pMIR-DDX6 was significantly inhibited after the introduction of miR-124 into DLD-1 cells. On the other hand, mutation of the DDX6 3′-UTR-binding site markedly abolished this ability of miR-124 (Fig. 1E). Thirdly, treatment with antagomir-124 reversed the growth inhibition elicited by miR-124 and the increased expression level of DDX6 in both cells (Fig. 1F). These findings altogether suggested that miR-124 targeted DDX6, thus contributing to the down-regulation of DDX6 at the translational level in colon cancer cells. Actually, the down-regulated miR-124 could cause the overexpression of DDX6 at least in part in colon cancer cells.

3.3. Knockdown of DDX6 induces apoptosis in colon cancer cells

To validate the function of DDX6, we examined the effects of gene silencing of DDX6 in human colon cancer DLD-1 and WiDr cells. As shown in Fig. 2A, this gene silencing induced a significant growth inhibition in both cells. Western blotting analysis clearly showed the cleaved form of PARP-1 and the active forms of caspase 9; whereas a significant transition of LC3I to LC3II was not found in either cell type (Fig. 2B). Moreover, Hoechst 33342 nuclear staining indicated a typical

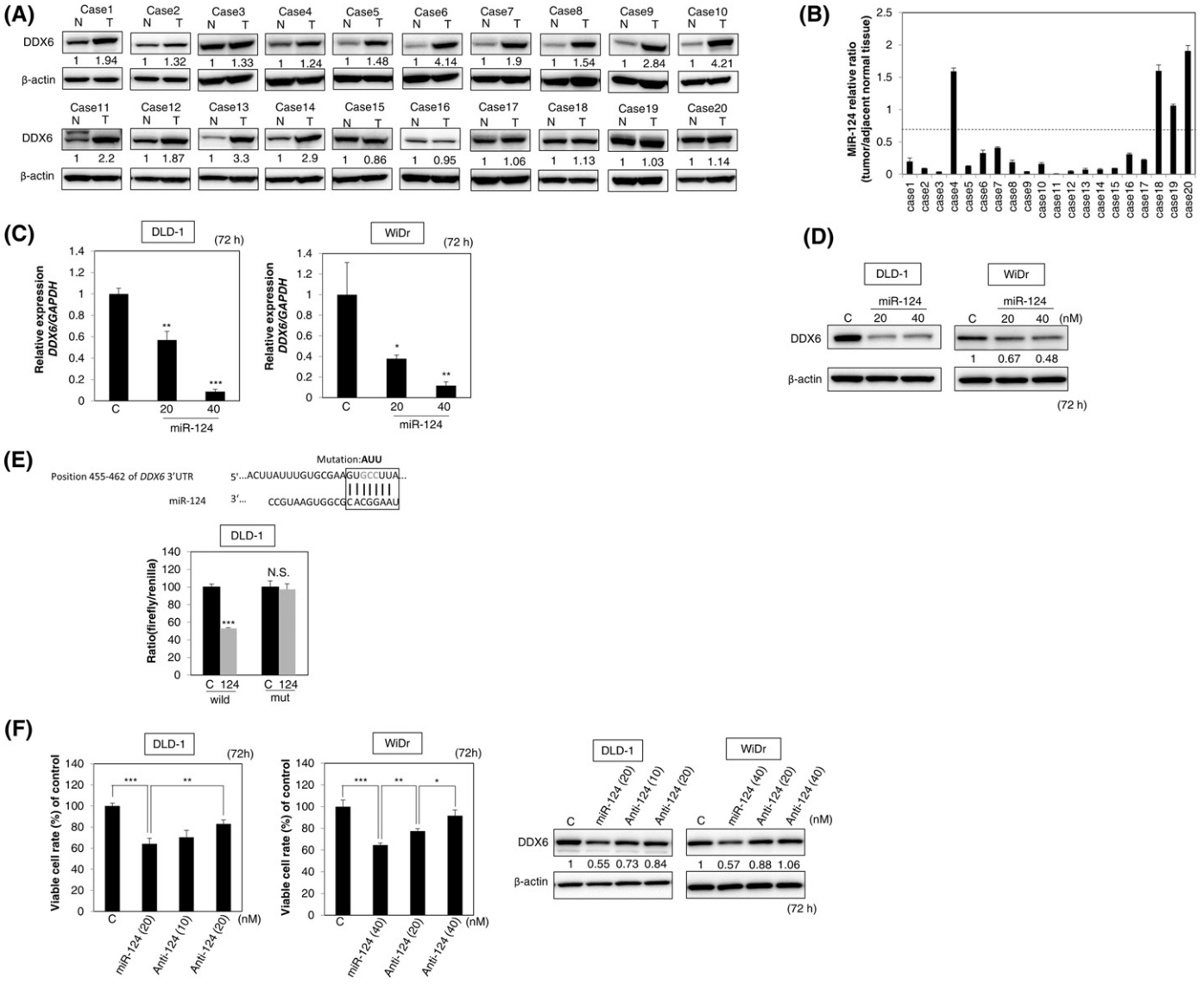

Fig. 1. (A, B) Overexpression of DDX6 and down-regulation of miR-124 in clinical colorectal tumors. (A) The protein expression of DDX6 in the 20 clinical samples. Densitometric values are shown for DDX6 in each sample (B). Expression of miR-124 in 20 colorectal tumor clinical samples. The value of each sample was expressed as the ratio of the expression in tumor sample to that in normal adjacent tumor sample. The lower cases (ratio under 0.67) and the higher cases (ratio above 1.5) are significant. Dotted line in the graph shows the ratio of 0.67. (C)–(F) MiR-124 targeted DDX6 and contributed to the expression of DDX6 in colon cancer cells. The mRNA (C) and protein expression (D) of DDX6 in DLD-1 and WiDr cells at 72 h after the transfection with miR-124 (20, 40 nM) is shown. Densitometric values are shown for DDX6 in WiDr cells after the transfection with miR-124. (E) Luciferase activities after co-transfection with control or miR-124 and wild-type or mutant-type pMIR vectors having the predictive miR-124 binding site in the 3′UTR of DDX6 are also given. The upper panel shows the region of the 3′-UTR of human DDX6 mRNA complementary to the mature miR-124. The box indicates the predicted binding sites for miR-124. (F) Also, the effect of combined treatment of DLD-1 cells with antagomiR-124 and miR-124 is indicated. DLD-1 cells were transfected with non-specific control, miR-124 (20 nM), miR-124 (20 nM) + antagomiR-124 (10 nM) or miR-124 (20 nM) + antagomiR-124 (20 nM). WiDr cells were transfected with non-specific control, miR-124 (40 nM), miR-124 (40 nM) + antagomiR-124 (20 nM) or miR-124 (40 nM) + antagomiR-124 (40 nM). Left panel: viable cell rate; Right panel: the expression level of DDX6 was assessed at 72 h after the transfection. Densitometric values are shown for DDX6. Results are presented as mean ± SD; $^{*}P < 0.05$ $^{**}P < 0.01$; $^{***}P < 0.001$. N.S., not statistically significant.

apoptotic appearance such as condensed chromatin and nuclear fragmentation in the siR-DDX6-treated DLD-1 cells (Fig. 2C). These findings suggested that knockdown of DDX6 induced apoptosis in colon cancer cells.

3.4. MiR-124 regulates the Warburg effect through the PTB1/PKM axis by targeting PTB1

Next, we examined the effects of ectopic expression of miR-124 in colon cancer cells. As a result, the growth of either cell line was significantly inhibited, even at 20 nM (Fig. 3A). Previously, we found that miR-124 targets PTB1, which is a splicer of the PKM gene and modulates the Warburg effect through the switching of PKM isoform expression from PKM2, which is dominant in proliferating cells, to PKM1 [18]. The ectopic expression of miR-124 or gene silencing of PTB1 induced the switching of PKM isoform expression from PKM2 to PKM1 in both cells, as reported before (Fig. 3B and C). These results indicated that the miR-124/PTB1/PKM axis contributed to the Warburg effect. Furthermore, Western blot analysis indicated that the cleaved form of PARP-1 and the active forms of caspase-9 were remarkably evident (Fig. 3D), and Hoechst 33342 nuclear staining revealed the typical apoptotic appearance (Fig. 3E), in the miR-124-treated DLD-1 cells. Also, the ectopic expression of miR-124 induced a significant transition of LC3I to LC3II in both kinds of cells (Fig. 3D). Moreover, morphologically, we compared the electron microscopic findings made after transfection with miR-124 or siR-DDX6. The results showed that the ectopic expression of miR-124 induced apoptosis combined with autophagy;

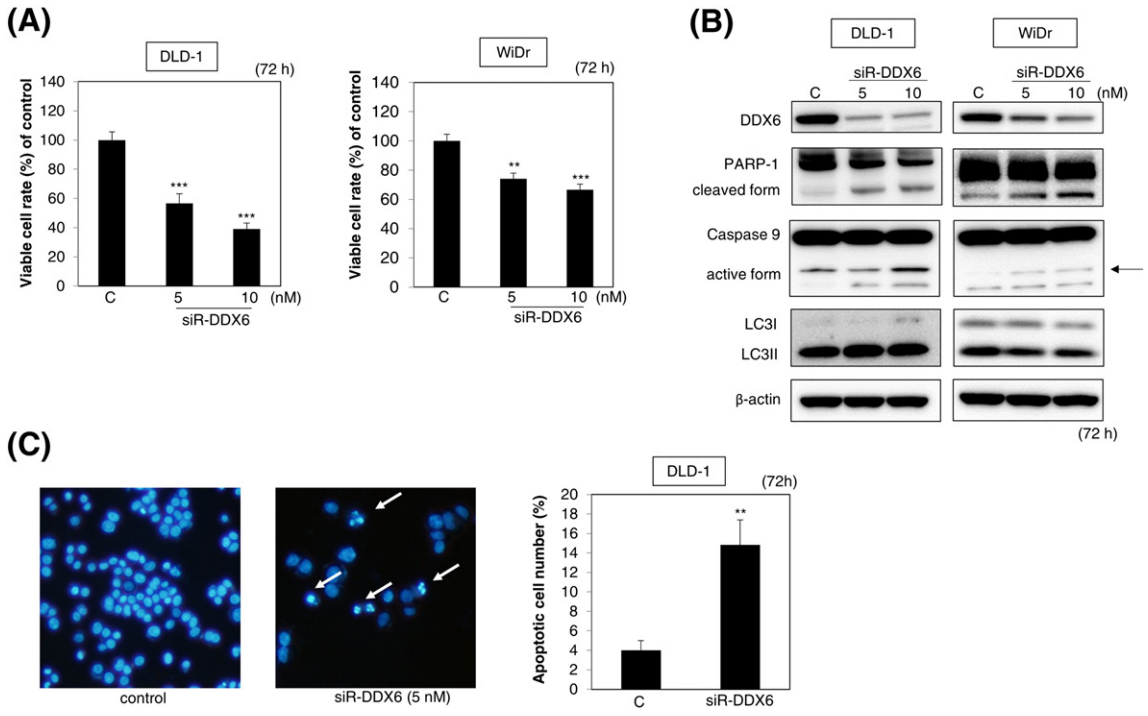

Fig. 2. Knockdown of DDX6 induced mainly apoptosis in colon cancer cells. (A) Effects of DDX6 knockdown on cell growth of colon cancer cells and (B) on the protein expression of apoptosis and autophagy-related genes when knockdown of DDX6 in colon cancer cells was performed. We transfected DLD-1 and WiDr cells with siR-DDX6 at a concentration of 5 or 10 nM. The effects were evaluated at 72 h after the transfection. The active form of caspase 9 in WiDr cells is indicated by the black arrow. (C) Hoechst 33342 staining at 72 h after knockdown of DDX6 in DLD-1 cells at a siR-DDX6 concentration of 5 nM. Apoptotic cells are indicated by the white arrows. Results are presented as the mean ± SD;$^{**}P < 0.01$; $^{***}P < 0.001$.

whereas siR-DDX6 induced mainly apoptosis (Fig. 3F), which results were consistent with those obtained from the biological chemical analyses. This phenotypic difference, especially in silencing DDX6, may reflect the differences in the expression profile of PKM isoforms.

3.5. DDX6/c-Myc/PTB1 constitutes a circuit regulated by miR-124 and affects the Warburg effect

C-Myc is known as one of the molecules strongly contributing to glycolysis in cancer cells [27]; and it up-regulates transcription of PTB1, ensuring a high PKM2/PKM1 ratio [28]. Previously, we found that DDX6 exhibits RNA unwinding activity toward c-Myc RNAs [29] and promotes translation of c-Myc mRNA by unfolding the IRES structure of its 5′UTR [5,7,8]. Based on these findings, we tried to clarify the mechanism by which DDX6 contributed to the Warburg effect through the regulation of c-Myc and PTB1. Firstly, we performed the knockdown of c-Myc in both cells. Expectedly, gene silencing of c-Myc up-regulated the PKM1/PKM2 ratio through the down-regulation of PTB1 as seen in ectopic expression miR-124 or gene silencing of PTB1 (Fig. 4A). Also, gene silencing of DDX6 down-regulated PTB1 through the repression of c-Myc in both cells (Fig. 4B). Furthermore, the ectopic expression of miR-124 down-regulated c-Myc in both cells (Fig. 4C). Therefore, we focused on the expression of PKM isoforms and production of lactate, which is the final product of the glycolysis pathway, after the gene silencing of DDX6. As a result, the knockdown of DDX6 decreased the expression of PKM2 and lactate production (Fig. 4B and D). Of course, the ectopic expression of miR-124 decreased lactate production (Fig. 4D). These findings suggested that DDX6 contributed to the Warburg effect through the regulation of the c-Myc/PTB1/PKM2 pathway and that miR-124 regulated this pathway. Unexpectedly, gene silencing of DDX6 down-regulated PKM1 expression in both cells. In addition, the expression levels of miR-124 were significantly up-regulated and the protein expression of DDX6 and c-Myc were significantly down-regulated after the transfection with siR-PTB1 (Fig. 4E and F). These findings taken together suggested that DDX6, c-Myc, and PTB1 formed a positive feedback-like circuit and that miR-124 regulated this circuit through the modulation of DDX6 and PTB1 expression.

3.6. Knockdown of DDX6 induces apoptosis through the down-regulation of PKM1 in colon cancer cells

Based on the results shown in Fig. 4B, we hypothesized that the down-regulation of PKM1 was likely related to the induction of apoptosis caused by the knockdown of DDX6. Therefore, we examined the effects of gene silencing PKM1 on the growth of both kinds of cells. As a result, the gene silencing of PKM1 induced a significant growth inhibition in both cell lines (Fig. 5A). In addition, Western blot analysis indicated that the levels of the cleaved form of PARP-1 and the active form of caspase-9 were remarkably elevated after the transfection of either cell type with siR-PKM1, even when used at 2 nM; whereas the transition LC3I to LC3II was not seen (Fig. 5B), as was also the case for siR-DDX6 (Fig. 2B). Moreover, Hoechst 33342 nuclear staining indicated typical apoptotic findings such as condensed chromatin and nuclear fragmentation in the siR-PKM1-treated DLD-1 cells (Fig. 5C). All of these findings taken together suggested that PKM1 was closely associated with the induction of apoptosis, which was also observed through the down-regulation of PKM1 after the transfection with siR-DDX6.

3.7. PKM1 is one of the molecules responsible for the induction of apoptosis by acting through the miR-124/PTB1/PKM axis

Finally, to further validate the functions and importance of PKM1, which was down-regulated only in DDX6-silenced cells, resulting

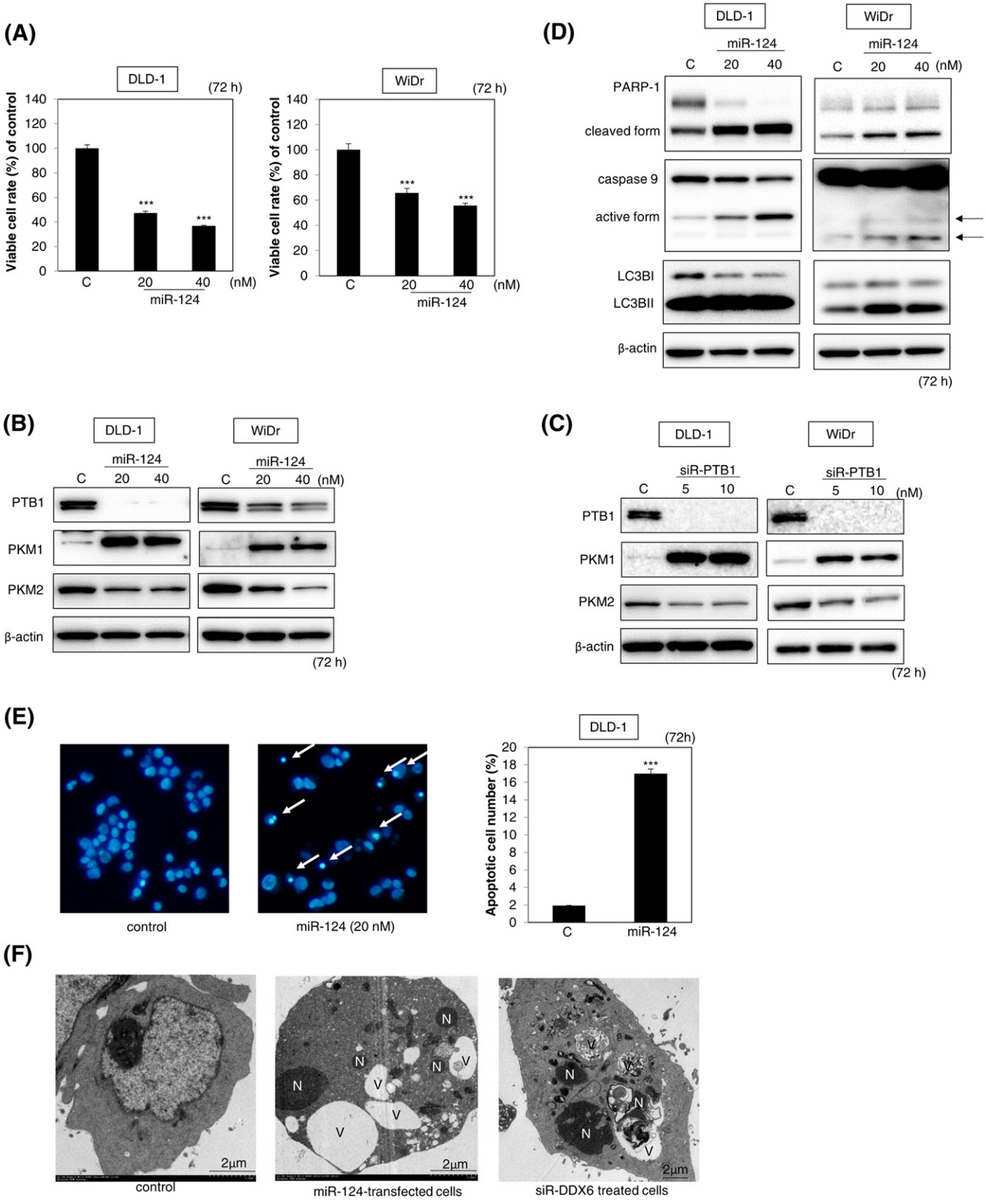

Fig. 3. MiR-124 regulated PTB1/PKM axis and induced apoptosis and autophagy in colon cancer cells. (A) Effects of ectopic expression of miR-124 on cell growth of colon cancer cells. (B and C) The protein expression of Warburg effect-associated genes after the transfection of colon cancer cells with miR-124 (B) or siR-PTB1 (C). The concentration of miR-124 used was 20 or 40 nM. That of siR-PTB1 used was 5 or 10 nM. The effects were evaluated at 72 h after the transfection. (D) Protein expression of apoptosis- and autophagy-related genes when ectopic expression of miR-124 in colon cancer cells was performed. The concentration of miR-124 used was 20 or 40 nM. The effects were evaluated at 72 h after the transfection. The active form of caspase 9 in WiDr cells is indicated by the black arrow. (E) Results of Hoechst 33342 staining at 72 h after treatment with miR-124 in DLD-1 cells at a miR-124 concentration of 20 nM. Apoptotic cells are indicated by the white arrows. (F) Morphological study by using electron microscopy. DLD-1 cells were treated with control miRNA, miR-124 (40 nM) or siR-DDX6 (10 nM) for 72 h. *N*: fragmented nuclei, *V*: vacuole. Results are presented as the mean ± SD; $^{***}P < 0.001$.

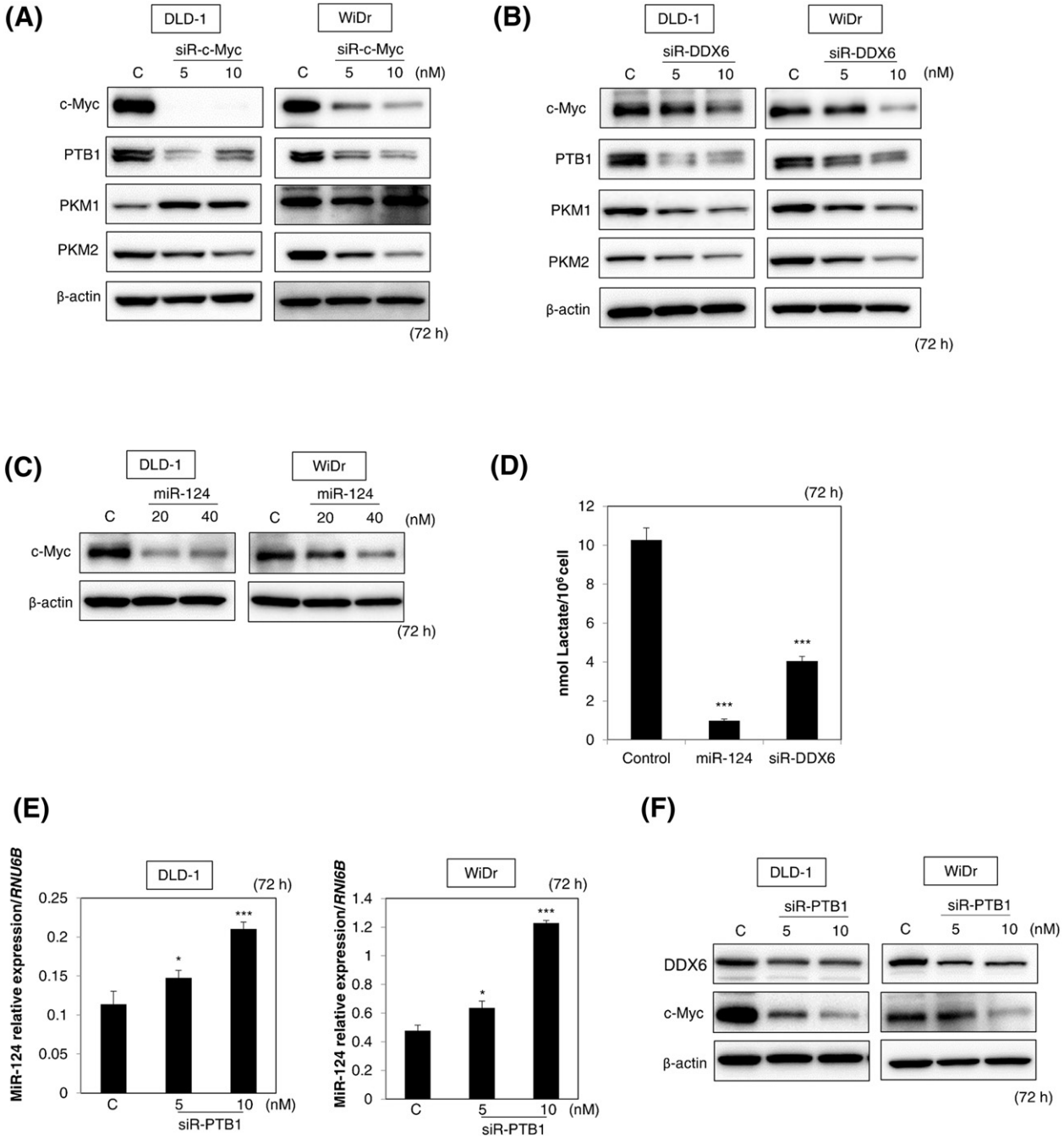

Fig. 4. DDX6, c-Myc, and PTB1 constituted a circuit regulated by miR-124 and affected the Warburg effect. The protein expression of Warburg effect-associated genes after the transfection of colon cancer cells with siR-c-Myc (A) or siR-DDX6 (B) is shown. The expression of these proteins was assessed at 72 h after the transfection. The concentrations of siR-c-Myc and siR-DDX6 were 5 and 10 nM, respectively. (C) The protein expression of c-Myc after the transfection of colon cancer cells with miR-124 is shown. The expression of these proteins was assessed at 72 h after the transfection. The concentration of miR-124 was 20 or 40 nM. (D) Lactate production was measured at 48 h after the transfection of DLD-1 cells with miR-124 (20 nM) or siR-DDX6 (5 nM). (E and F) The expression levels of miR-124 (E) and the protein expression of miR-124-related genes (F) after the transfection of colon cancer cells with siR-PTB1 are shown. The effects were detected at 72 h after the transfection with siR-PTB1. The concentration of siR-PTB1 used was 5 or 10 nM. Results are presented as the mean ± SD; $^{*}P < 0.05$;$^{***}P < 0.001$.

in cancer cell death, we compared the effects of knockdown of PKM1, PKM2 or both and made two interesting findings. Firstly, as shown in Fig. 5D, the strength of growth inhibition after the transfection with siR-PKM1 was almost the same as that with siR-PKM2, which is the dominant isoform in cancer cells [18]. Secondly, the cleaved forms of PARP-1 were observed only in the case of knockdown of PKM1 (siR-PKM1 or siR-PKM1 + PKM2) by Western blot analysis (Fig. 5E). These findings suggested that PKM1 played important roles in promoting cancer cell growth, comparable to those of PKM2, which is dominant in the Warburg effect in cancer cells (Fig. 6).

4. Discussion

In the current study, we firstly demonstrated that the up-regulated expression of DDX6 in colon cancer cells was partly associated with the RNA-interference effect by miR-124 that targets PTB1 (Figs. 1, 4E and F). Of course, we should consider other machineries of DDX6 over-expression, too. For example, it has been reported that the miR-130 family regulates the hypoxia response signal through the targeting of DDX6 [30].

Earlier, we proved that many kinds of miRs, such as miR-143 and -145, are down-regulated during colorectal tumor development

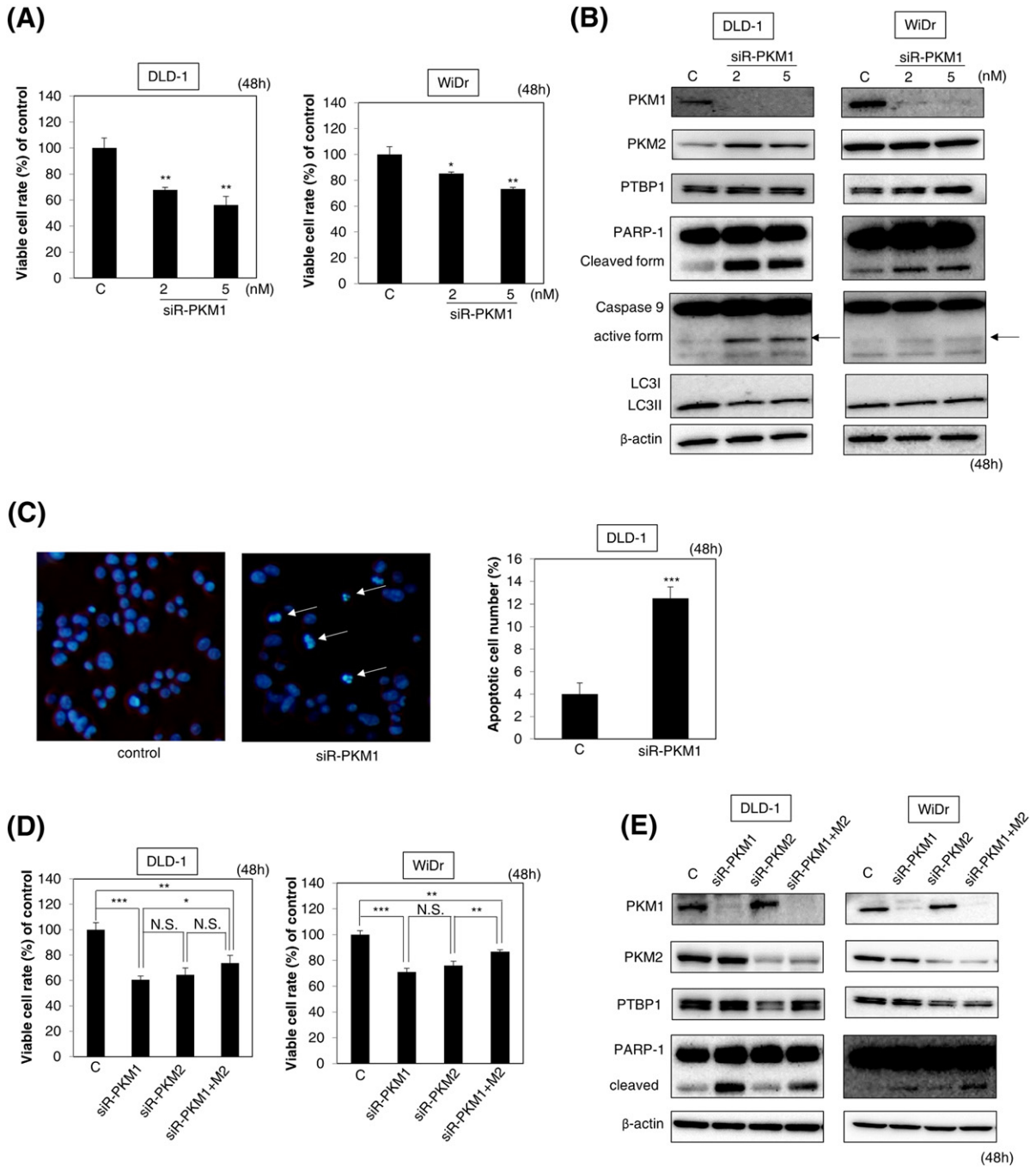

Fig. 5. PKM1 is one of the molecules responsible for cancer cell growth. (A) Effects of PKM1 knockdown with its siR on cell growth of colon cancer cells and (B) protein expression of apoptosis, autophagy, and Warburg effect-related genes when knockdown of PKM1 in colon cancer cells was performed. We transfected DLD-1 and WiDr cells with siR-PKM1 at a concentration of 2 or 5 nM. The effects were evaluated at 48 h after the transfection. (C) Hoechst 33342 staining at 48 h after knockdown of PKM1 in DLD-1 cells at a siR-PKM1 concentration of 5 nM. Apoptotic cells are indicated by the white arrows. (D) Effects of knockdown of PKM1, PKM2 or PKM1 + PKM2 on cell growth in colon cancer cells and (E) the protein expression of apoptosis and Warburg effect-related genes when knockdown of PKM1, PKM2 or PKM1 + PKM2 in colon cancer cells was done. We transfected DLD-1 and WiDr cells with siR-PKM1, siR-PKM2 or siR-PKM1 + siR-PKM2 at a concentration of 5, 5 or 2.5 + 2.5 nM. The effects were evaluated at 48 h after the transfection. Results presented as the mean ± SD; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. N.S., not statistically significant.

[31,32]; and recently, we reported that the expression of such onco-related miRNAs was closely associated with the morphological appearance of colorectal tumors [26]. Therefore, we consider that the down-regulation and loss of functions of these anti-oncomiRs caused gain of functions of targeting oncogenes during carcinogenesis. It has been reported that dysregulation of anti-oncomiRs such as miR-124 is caused by epigenetic abnormalities such as methylation [11,33]. However, the mechanisms and effects of such dysregulation are still largely unknown.

There are some mechanisms known to be involved in the growth inhibition induced by knockdown of DDX6. Previously, we found that siR-DDX6 induced cell-cycle arrest at the S phase in HeLa cells [6]. In addition, we found that DDX6 protein interacts with the 5′-cap structure-binding protein eIF4E, which is well known as a rate-limiting factor in the initiation of translation. Therefore, many genes related to cancer cell growth such as those of the Wnt signaling pathway, as well as phosphorylation of Akt, are down-regulated by knockdown of DDX6 [6,34]. One of the interesting findings made

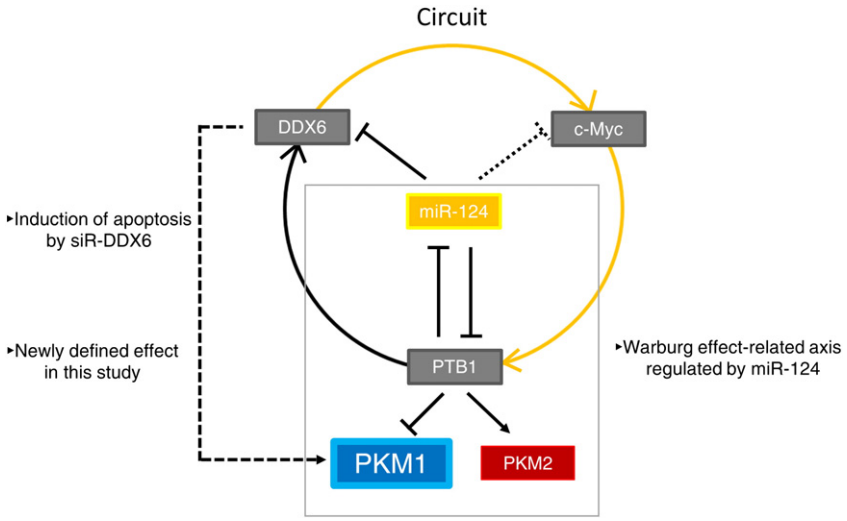

Fig. 6. Schematic diagram of DDX6/c-Myc/PTB1 circuit regulated by miR-124. DDX6 is highly overexpressed in colorectal tumors, and miR-124 contributes to that expression. MiR-124 directly targets DDX6 in colon cancer cells. DDX6 regulates the expression level of c-Myc and c-Myc regulated the expression of PTB1 directly. Also, miR-124 regulates the expression of c-Myc indirectly. Furthermore, siR-PTB1 up-regulated the expression level of miR-124 and down-regulated the expression of DDX6 and c-Myc. Therefore, DDX6/c-Myc/PTB1 constitutes a circuit regulated by miR-124 and affects the Warburg effect. On the other hand, knockdown of DDX6 induced apoptosis through an imbalance of PKM expression, especially by that caused by down-regulation of PKM1 in colon cancer cells. Knockdown of PKM1 induced typical apoptosis in colon cancer cells. PKM1 is one of the essential molecules in cancer cell proliferation.

in this study was that knockdown of DDX6 down-regulated the expression of Warburg effect-related genes such as PTB1 and PKM2 and affected production of lactate, which is the final product of the glycolysis pathway (Fig. 4B and D). Previously, we found that DDX6 acts as a translational promoter of c-Myc mRNA [5,7,29]. Also, c-Myc is well known as one of the molecules strongly contributing to glycolysis in cancer cells [27,28]. Therefore we hypothesized that knockdown of DDX6 affected the Warburg effect through down-regulation of c-Myc. Based on this hypothesis, in this study, we showed that DDX6, c-Myc, and PTB1 constitute a positive- feedback network regulated by miR-124 (Figs. 4 and 6).

Next, we want to emphasize especially that our present study revealed that PKM1 played an important role in cancer cell growth, even though PKM1 was shown to be expressed in very low amount in cancer cells. Previously, we found that the expression level of PKM1 is much lower than that of PKM2 in all cancer cell lines and even in control cell lines and in cells in primary culture tested [18]. It has been well established that PKM2 has important functions in cancer cell growth [20]. Recently, we reported that the PKM2/PKM1 ratio was up-regulated during tumor development induced by down-regulation of PTB1-associated miRs including miR-124 in colorectal tumor [18]. Also, it has been reported that PKM2 has various other functions in cancer cells. For example, PKM2 regulates gene transcription by acting as a protein kinase and directly phosphorylating *STAT3* [35]. In addition, nuclear PKM2 regulates β-catenin transactivation upon EGFR activation [36]. However, the functions of PKM1 have been hardly known until now. In this study, we found this kinase to be one of the essential molecules in cancer cell growth, based on the results of PKM1 silencing (Fig. 5). As stated above, PTB1 is regulated by c-Myc (Fig. 4A). Therefore, at first, we hypothesized that the knockdown of DDX6 induces switching of PKM isoforms from PKM2 to PKM1 as seen in the transfection with miR-124, siR-PTB1 or -c-Myc (Figs. 3B, C and 4A). However, PKM1 was down-regulated as well as PKM2 (Fig. 4C). To clarify the mechanism of this result, we considered the possibility of other gluconeogenesis such as β-oxidation. Therefore, we examined the expression levels of several enzymes which are rate-limiting factors of β-oxidation. The expression level of long-chain acyl-CoA synthetase (ACSL1) was almost unchanged in siR-DDX6-treated cells. However, the expression level of carnitine palmitoyltransferase-1A (CPT1A) was up-regulated in siR-DDX6-treated DLD-1 cells and that of carnitine palmitoyltransferase-1C (CPT1C) was up-regulated in both cells (Supplementary Fig. S3A). Furthermore, the sensitivity to Hydrogen peroxide (H_2O_2) was significantly increased in siR-DDX6-treated cells (Supplementary Fig. S3B). Also, it has been reported that fatty acid oxidation (FAO) cancels the accumulation of Reactive Oxygen Species (ROS) in conditions of metabolic stress through the generation of NADPH [37,38]. By considering these findings, it is possible that siR-DDX6-treated cells activate compensatory FAO to produce energy for survival and avoid fatal situation caused by accumulation of ROS through acceleration of TCA-cycle by up-regulated PKM1. Of course, further investigation will be needed to clarify the mechanism of down-regulation of PKM1 in siR-DDX6-treated cells. On the other hand, the database Target Scan 6.2 shows that 3′UTR of PKM is targeted by only miR-122. We found that the ectopic expression of miR-122 down-regulated both PKM1 and PKM2 in DLD-1 cells because the 3′UTR of PKM1 and PKM2 is common in both. Also, the cleaved form of PARP-1 was significantly up-regulated after the treatment with miR-122 in DLD-1 cells (Supplementary Fig. S4). We consider that these results support the notion that down-regulation of PKM1 induced apoptosis. It is known that PKM1 promotes oxidative phosphorylation [22]. In our results, knockdown of PKM1 activated the intrinsic apoptosis pathway (Fig. 5B and C). This finding may be evidence that PKM1 was closely related to mitochondrial functions. To elucidate the molecular functions of PKM1 in cancer cells, further study will be needed and is now under way. These findings taken together suggest that the oncogene RNA helicase DDX6 is extremely important not only to maintain a stable onco-related gene expression, but also to maintain the Warburg effect through the machineries of gene translation and energy metabolism mediated by miR-124.

5. Conclusions

The important finding in this study was that DDX6 affected the Warburg effect through the DDX6/c-Myc/PTB1 positive-feedback circuit regulated partly by miR-124. Another important finding was that the balance of PKM1/PKM2 was crucial to the growth of cancer cells.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.06.022.

Conflicts of interest statement

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.

Authors' contributors

Conception and design of the study or acquisition of data: K. Taniguchi, N. Sugito, Y. Ito, Y. Otsuki, and Y. Akao.

Analysis and interpretation of data: K. Taniguchi, N. Sugito, Y. Ito, M. Kumazaki, H. Haruka, N. Yamada, Y. Otsuki, K. Uchiyama, and Y. Akao.

Writing, review and/or revision of the manuscript: K. Taniguchi and Y. Akao.

Technical or material support: Y. Ito, N. Matsuhashi, M. Futamura, K. Yoshida, and K. Uchiyama.

Study supervision: Y. Akao.

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References

[1] Y. Akao, M. Seto, T. Takahashi, M. Saito, K.R. Utsumi, S. Nakazawa, R. Ueda, Rearrangements on chromosome 11q23 in hematopoietic tumor-associated t(11;14) and t(11;19) translocations, Cancer Res. 51 (1991) 6708–6711.
[2] Y. Akao, M. Seto, K. Yamamoto, S. Iida, S. Nakazawa, J. Inazawa, T. Abe, T. Takahashi, R. Ueda, The RCK gene associated with t(11;14) translocation is distinct from the MLL/ALL-1 gene with t(4;11) and t(11;19) translocations, Cancer Res. 52 (1992) 6083–6087.
[3] Y. Akao, O. Marukawa, H. Morikawa, K. Nakao, M. Kamei, T. Hachiya, Y. Tsujimoto, The rck/p54 candidate proto-oncogene product is a 54-kilodalton D-E-A-D box protein differentially expressed in human and mouse tissues, Cancer Res. 55 (1995) 3444–3449.
[4] Y. Nakagawa, H. Morikawa, I. Hirata, M. Shiozaki, A. Matsumoto, K. Maemura, T. Nishikawa, M. Niki, N. Tanigawa, M. Ikegami, K. Katsu, Y. Akao, Overexpression of rck/p54, a DEAD box protein, in human colorectal tumours, Br. J. Cancer 80 (1999) 914–917.
[5] K. Hashimoto, Y. Nakagawa, H. Morikawa, M. Niki, Y. Egashira, I. Hirata, K. Katsu, Y. Akao, Co-overexpression of DEAD box protein rck/p54 and c-myc protein in human colorectal adenomas and the relevance of their expression in cultured cell lines, Carcinogenesis 22 (2001) 1965–1970.
[6] Y. Akao, K. Matsumoto, K. Ohguchi, Y. Nakagawa, H. Yoshida, Human DEAD-box/RNA unwindase rck/p54 contributes to maintenance of cell growth by affecting cell cycle in cultured cells, Int. J. Oncol. 29 (2006) 41–48.
[7] Y. Akao, A role of DEAD-Box RNA helicase rck/p54 in cancer cells, Curr. Drug Ther. 4 (2009) 29–37.
[8] T. Matsui, K. Hogetsu, J. Usukura, T. Sato, T. Kumasaka, Y. Akao, N. Tanaka, Structural insight of human DEAD-box protein rck/p54 into its substrate recognition with conformational changes, Genes Cells 11 (2006) 439–452.
[9] A. Iio, T. Takagi, K. Miki, T. Naoe, A. Nakayama, Y. Akao, DDX6 post-transcriptionally down-regulates miR-143/145 expression through host gene NCR143/145 in cancer cells, Biochim. Biophys. Acta 1829 (2013) 1102–1110.
[10] Y.S. Lee, A. Dutta, MicroRNAs in cancer, Annu. Rev. Pathol. 4 (2009) 199–227.
[11] A. Goel, C.R. Boland, Epigenetics of colorectal cancer, Gastroenterology 143 (2012) 1442–1460 (e1441).
[12] C.M. Croce, Causes and consequences of microRNA dysregulation in cancer, Nat. Rev. Genet. 10 (2009) 704–714.
[13] E.V. Makeyev, J. Zhang, M.A. Carrasco, T. Maniatis, The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing, Mol. Cell 27 (2007) 435–448.
[14] N.A. Maiorano, A. Mallamaci, The pro-differentiating role of miR-124: indicating the road to become a neuron, RNA Biol. 7 (2010) 528–533.
[15] H. Xia, W.K. Cheung, S.S. Ng, X. Jiang, S. Jiang, J. Sze, G.K. Leung, G. Lu, D.T. Chan, X.W. Bian, H.F. Kung, W.S. Poon, M.C. Lin, Loss of brain-enriched miR-124 microRNA enhances stem-like traits and invasiveness of glioma cells, J. Biol. Chem. 287 (2012) 9962–9971.
[16] M. Furuta, K.I. Kozaki, S. Tanaka, S. Arii, I. Imoto, J. Inazawa, miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma, Carcinogenesis 31 (2010) 766–776.
[17] K. Taniguchi, N. Sugito, M. Kumazaki, H. Shinohara, N. Yamada, Y. Nakagawa, Y. Ito, Y. Otsuki, B. Uno, K. Uchiyama, Y. Akao, MicroRNA-124 inhibits cancer cell growth through PTB1/PKM1/PKM2 feedback cascade in colorectal cancer, Cancer Lett. 363 (2015) 17–27.
[18] K. Taniguchi, Y. Ito, N. Sugito, M. Kumazaki, H. Shinohara, N. Yamada, Y. Nakagawa, T. Sugiyama, M. Futamura, Y. Otsuki, K. Yoshida, K. Uchiyama, Y. Akao, Organ-specific PTB1-associated microRNAs determine expression of pyruvate kinase isoforms, Sci. Rep. 5 (2015) 8647.
[19] T. Noguchi, H. Inoue, T. Tanaka, The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing, J. Biol. Chem. 261 (1986) 13807–13812.
[20] H.R. Christofk, M.G. Vander Heiden, M.H. Harris, A. Ramanathan, R.E. Gerszten, R. Wei, M.D. Fleming, S.L. Schreiber, L.C. Cantley, The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth, Nature 452 (2008) 230–233.
[21] J.Y. Lim, S.O. Yoon, S.Y. Seol, S.W. Hong, J.W. Kim, S.H. Choi, J.Y. Cho, Overexpression of the M2 isoform of pyruvate kinase is an adverse prognostic factor for signet ring cell gastric cancer, World J. Gastroenterol. 18 (2012) 4037–4043.
[22] M. Chen, J. Zhang, J.L. Manley, Turning on a fuel switch of cancer: hnRNP proteins regulate alternative splicing of pyruvate kinase mRNA, Cancer Res. 70 (2010) 8977–8980.
[23] Y. Akao, Y. Nakagawa, I. Hirata, A. Iio, T. Itoh, K. Kojima, R. Nakashima, Y. Kitade, T. Naoe, Role of anti-oncomirs miR-143 and -145 in human colorectal tumors, Cancer Gene Ther. 17 (2010) 398–408.
[24] H. Shinohara, K. Taniguchi, M. Kumazaki, N. Yamada, Y. Ito, Y. Otsuki, B. Uno, F. Hayakawa, Y. Minami, T. Naoe, Y. Akao, Anti-cancer fatty-acid derivative induces autophagic cell death through modulation of PKM isoform expression profile mediated by bcr-abl in chronic myeloid leukemia, Cancer Lett. 360 (2015) 28–38.
[25] T. Takagi, A. Iio, Y. Nakagawa, T. Naoe, N. Tanigawa, Y. Akao, Decreased expression of microRNA-143 and -145 in human gastric cancers, Oncology 77 (2009) 12–21.
[26] Y. Nakagawa, Y. Akao, K. Taniguchi, A. Kamatani, T. Tahara, T. Kamano, N. Nakano, N. Komura, H. Ikuno, T. Ohmori, Y. Jodai, M. Miyata, M. Nagasaka, T. Shibata, N. Ohmiya, I. Hirata, Relationship between expression of onco-related miRNAs and the endoscopic appearance of colorectal tumors, Int. J. Mol. Sci. 16 (2015) 1526–1543.
[27] S.J. Yeung, J. Pan, M.H. Lee, Roles of p53, MYC and HIF-1 in regulating glycolysis — the seventh hallmark of cancer, Cell. Mol. Life Sci. 65 (2008) 3981–3999.
[28] C.J. David, M. Chen, M. Assanah, P. Canoll, J.L. Manley, HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer, Nature 463 (2010) 364–368.
[29] Y. Akao, H. Yoshida, K. Matsumoto, T. Matsui, K. Hogetu, N. Tanaka, J. Usukura, A tumour-associated DEAD-box protein, rck/p54 exhibits RNA unwinding activity toward c-myc RNAs in vitro, Genes Cells 8 (2003) 671–676.
[30] K. Saito, E. Kondo, M. Matsushita, MicroRNA 130 family regulates the hypoxia response signal through the P-body protein DDX6, Nucleic Acids Res. 39 (2011) 6086–6099.
[31] Y. Akao, Y. Nakagawa, T. Naoe, MicroRNA-143 and -145 in colon cancer, DNA Cell Biol. 26 (2007) 311–320.
[32] A. Kamatani, Y. Nakagawa, Y. Akao, N. Maruyama, M. Nagasaka, T. Shibata, T. Tahara, I. Hirata, Downregulation of anti-oncomirs miR-143/145 cluster occurs before APC gene aberration in the development of colorectal tumors, Med. Mol. Morphol. 46 (2013) 166–171.
[33] T. Ando, T. Yoshida, S. Enomoto, K. Asada, M. Tatematsu, M. Ichinose, T. Sugiyama, T. Ushijima, DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect, Int. J. Cancer 124 (2009) 2367–2374.
[34] F. Lin, R. Wang, J.J. Shen, X. Wang, P. Gao, K. Dong, H.Z. Zhang, Knockdown of RCK/p54 expression by RNAi inhibits proliferation of human colorectal cancer cells in vitro and in vivo, Cancer Biol. Ther. 7 (2008) 1669–1676.
[35] X. Gao, H. Wang, J.J. Yang, X. Liu, Z.R. Liu, Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase, Mol. Cell 45 (2012) 598–609.
[36] W. Yang, Y. Xia, H. Ji, Y. Zheng, J. Liang, W. Huang, X. Gao, K. Aldape, Z. Lu, Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation, Nature 480 (2011) 118–122.
[37] S.M. Jeon, N.S. Chandel, N. Hay, AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress, Nature 485 (2012) 661–665.
[38] A. Carracedo, L.C. Cantley, P.P. Pandolfi, Cancer metabolism: fatty acid oxidation in the limelight, Nat. Rev. Cancer 13 (2013) 227–232.