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# Effects of miR-34a on cell growth and chemoresistance in prostate cancer PC3 cells

Yasunori Fujita [a,1], Keitaro Kojima [b,1], Nanako Hamada [a], Riyako Ohhashi [a], Yukihiro Akao [a], Yoshinori Nozawa [a], Takashi Deguchi [b], Masafumi Ito [a,*]

[a] Department of Longevity and Aging Research, Gifu International Institute of Biotechnology, 1-1 Naka-fudogaoka, Kakamigahara, Gifu 504-0838, Japan
[b] Department of Urology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan

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ABSTRACT

Tumor suppressor p53 transcriptionally regulates expression of microRNA-34a, which confers translational inhibition and mRNA degradation of genes involved in cell cycle control and apoptosis. In various cancers, miR-34a expression is lost or reduced. Here, we investigated the role of miR-34a in prostate cancer cell lines. MiR-34a expression was markedly reduced in p53-null PC3 cells and p53-mutated DU145 cells compared with LNCaP cells expressing wild-type p53. In PC3 cell, ectopic expression of miR-34a decreased the SIRT1 mRNA and protein levels as well as protein levels of known direct target genes. Reporter assays revealed that miR-34a-induced SIRT1 inhibition occurred at the transcriptional but not post-transcriptional level despite the presence of a potential miR-34a binding site within its 3′-UTR. Ectopic miR-34a expression resulted in cell cycle arrest and growth inhibition and attenuated chemoresistance to anticancer drug camptothecin by inducing apoptosis, suggesting a potential role of miR-34a for the treatment of p53-defective prostate cancer.



Mutations in the tumor suppressor p53 have been found in most of cancers and are often associated with clinically aggressive features [1]. p53 can act as a transcriptional activator and induce cell cycle arrest, cell senescence, and apoptosis in response to DNA damage, oxidative stress or oncogene activation through transactivation of a variety of genes. On the other hand, p53 represses gene expression either directly or indirectly. However, the precise mechanism by which p53 down-regulates gene expression remained elusive.

microRNAs (miRNAs) are small, non-coding RNAs that post-transcriptionally regulate gene expression through translational repression and mRNA degradation [2]. Primary miRNAs transcribed from genomic regions are processed in the nucleus, giving rise to pre-miRNAs, which are then exported to the cytoplasm where they are processed to yield miRNA duplexes. Mature miRNAs derived from one strand of the duplex are incorporated into miRNA-induced silencing complexes and guides them to the partially complementary binding sites located within the 3′-untranslated region (UTR) of target mRNAs. MiRNAs participate in the regulation of many cellular processes including proliferation, differentiation and apoptosis. Accumulating evidence has suggested that miRNAs are involved in tumor formation [3].

Recently, several groups have reported that p53 binds to and transactivates the miR-34a promoter, thereby increases its expression and regulates cellular processes including cell cycle arrest, cell senescence, and apoptosis [4]. miR-34a induces cell cycle arrest and apoptosis by down-regulating cell cycle and apoptosis related proteins such as CDK4, CDK6, Cyclin D1, Cyclin E2, E2F3, and BCL2. Furthermore, loss or reduced expression of miR-34a has been detected in a variety of tumors and cancer cell lines.

SIRT1 (Silent mating type information regulation 2 homolog 1), which is an NAD-dependent histone/protein deacetylase, is implicated in diverse cellular processes including apoptosis [5]. SIRT1 deacetylates pro-apoptotic proteins such as p53 and promotes cell survival under genotoxic and oxidative stresses. The anti-apoptotic activity of SIRT1 is implicated in tumorigenesis, since SIRT1 expression is elevated in a variety of cancer cell lines and tumors [6]. Furthermore, SIRT1 is suggested to be involved resistance to anticancer drug [7]. We have recently reported that SIRT1 expression is markedly up-regulated in androgen-refractory PC3 and DU145 cells compared with androgen-sensitive LNCaP cells and that increased expression of SIRT1 may play an important role in promoting cell growth and chemoresistance in these cells [8].

* Corresponding author. Fax: +81 58 371 4412.
*E-mail address:* mito@giib.or.jp (M. Ito).
[1] These authors equally contributed to this work.

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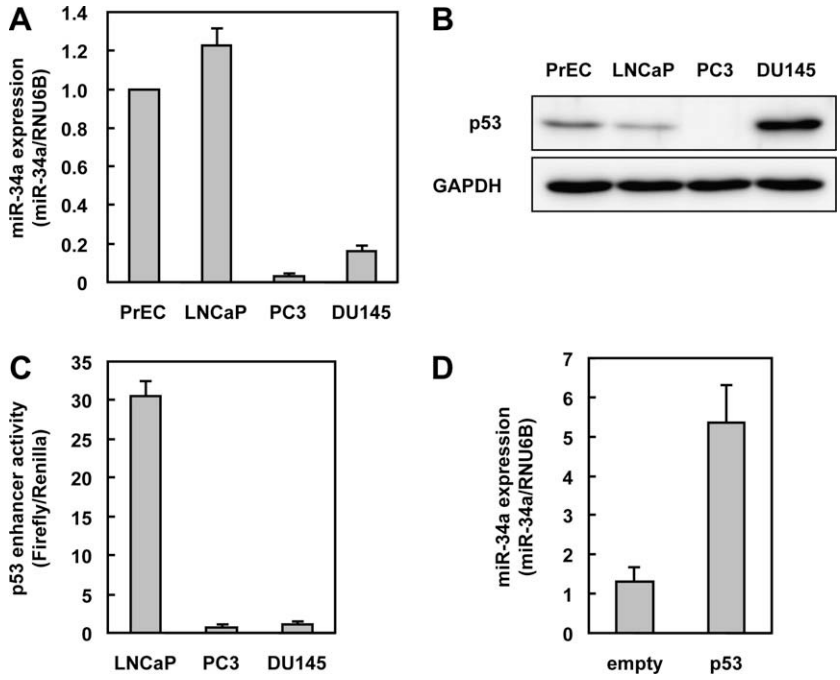


**Fig. 1.** Expression of miR-34a and p53 in normal and cancerous human prostate cell lines. (A) Total RNA containing miRNA was extracted from PrEC, LNCaP, PC3, and DU145 cells and subjected to real-time RT-PCR for miR-34a. Data are expressed as means ± SD ($n = 3$). (B) Cell lysates obtained from PrEC, LNCaP, PC3, and DU145 cells were subjected to Western blot analysis using anti-p53 and -GAPDH antibodies. (C) The p53 enhancer reporter gene and phRL-TK control vector were co-transfected into LNCaP, PC3, and DU145 cells followed by luciferase assays. The p53 enhancer activity was calculated as a ratio of firefly to Renilla luciferase activity. Data are expressed as means ± SD ($n = 3$). (D) Thirty-six hours after transfection into PC3 cells with either an empty or the p53 expression vector, real-time RT-PCR for miR-34a was performed. Data are expressed as means ± SD ($n = 3$).

In this study, we determined the miR-34a expression levels in normal and cancerous human prostate cell lines and investigated effects of ectopic expression of miR-34a on the expression levels of SIRT1 as well as cell cycle and apoptosis related proteins and also on cell growth and chemoresistance in p53-null PC3 cells.

## Materials and methods

*Reagents and antibodies.* Camptothecin and nocodazole were purchased from Sigma. Antibodies against p53, CDK6, E2F3, and E2F1 were from Santa Cruz Biotechnology. Anti-SIRT1 and -β-actin antibodies were obtained from Abcam and Sigma, respectively. Antibodies recognizing Cyclin D1 and GAPDH were from Cell Signaling Technology.

*Cell culture.* PrEC normal human prostate epithelial cells obtained from Clonetics were cultured as recommended by the supplier. LNCaP, PC3, and DU145 human prostate cancer cells obtained from ATCC were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

*Total RNA extraction and real-time RT-PCR.* For quantitation of miR-34a, total RNA containing miRNA was extracted using miRNeasy Mini Kit (Qiagen) and cDNA was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Real-time RT-PCR was performed using TaqMan MicroRNA Assay kit (Applied Biosystems). The expression level of miR-34a was normalized to RNU6B. For quantitation of SIRT1 mRNA, total RNA was extracted using RNeasy Mini Kit (Qiagen) and cDNA was synthesized using SuperScriptIII Reverse Transcriptase (Invitrogen). Real-time RT-PCR was carried out using LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I (Roche). The expression level of SIRT1 was normalized to β-actin. Primers for SIRT1 and β-actin were described previously [8].

*Western blot analysis.* Whole cell lysates were separated by SDS–PAGE and transferred onto PVDF membranes. After blocking in 5% skim milk, membranes were incubated with a primary antibody and then incubated with a horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were detected using the ECL Plus Western Blotting Detection System (GE Healthcare).

*Plasmid construction.* For construction of the p53 enhancer reporter gene, the p53 enhancer elements were placed upstream of the thymidine kinase minimal promoter linked to a firefly luciferase gene. For construction of the human SIRT1 3′-UTR reporter gene,

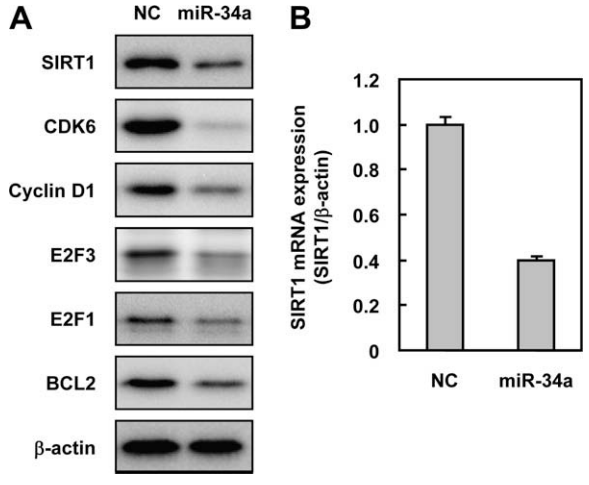


**Fig. 2.** Effects of ectopic expression of miR-34a on SIRT1 expression in PC3 cells. (A) Forty-eight hours after transfection with either negative control miRNA precursor or miR-34a precursor at 30 nM, cell lysates were prepared from PC3 cells and subjected to Western blot analysis. (B) Total RNA isolated from PC3 cells transfected with either control or miR-34a precursor were subjected to real-time RT-PCR for SIRT1 mRNA. Data are expressed as means ± SD ($n = 3$).

the 1.7 kb SIRT1 3′-UTR was amplified by PCR using the following primers: forward; 5′-GATCGAGCTCTGTAATAATTGTGCAGGTACA GG-3′, reverse; 5′-GATCACGCGTCAGAAAAAAGTCAAATGACAA-3′). Primers were designed to introduce SacI and MluI sites (underlined) at the end of PCR products. After restriction digestion, amplified DNA was cloned into the corresponding sites of pMIR-REPORT miRNA Expression Reporter Vector (Ambion). A potential miR-34a binding site within the SIRT1 3′-UTR (Fig. 3A) was deleted by overlap extension PCR. Nucleotide sequences of the inserts were confirmed by DNA sequencing for all constructs. The −2487 human SIRT1 promoter reporter gene was described previously [8]. The phRL-TK control vector expressing Renilla luciferase (Promega) and pMIR-REPORT Beta-gal control vector expressing β-galactosidase (Ambion) were used for normalization of cell number and transfection efficiency. The p53 expression vector was a gift from Dr. Mitsutoshi Setou.

*Transfection and reporter assays.* Transfection of miRNA precursors, reporter genes, control vectors and expression vectors were carried out using Lipofectamine 2000 (Invitrogen) unless otherwise noted. MicroRNA precursors used are Pre-miR miRNA Precursor Molecule (hsa-miR-34a) and Pre-miR Negative Control #1 (Ambion). Firefly and Renilla luciferase activities were determined by Pikkagene Dual Luciferase Assay System (Toyo-B-Net). Firefly luciferase and β-galactosidase activities were measured using Luciferase Assay Systems (Promega) and β-Galactosidase Enzyme Assay System (Promega), respectively.

*Cell cycle analysis.* Cells were treated with *or without* 100 ng/ml nocodazole for 12 h and then fixed in the presence of 70% ethanol at −20 °C. After washing, fixed cells were incubated in PBS containing 20 μg/ml propidium iodide, 200 μg/ml RNaseA and 0.1% Triton X-100 at 37 °C for 20 min. Cell cycle distribution was determined using FACSCalibur (Becton Dickinson).

*Cell viability determination and morphological evaluation of apoptosis.* Viable cell numbers were determined by trypan blue exclusion assay. To morphologically evaluate apoptosis (e.g. condensed or fragmented nuclei), cells were stained with Hoechst 33342 (0.5 μg/ml) at 37 °C for 30 min and visualized by fluorescence microscopy.

## Results

### *Down-regulation of miR-34a expression in PC3 and DU145 cells*

Real-time RT-PCR revealed that the expression levels of miR-34a were markedly decreased in androgen-refractory PC3 and DU145 cells compared to androgen-sensitive LNCaP and normal epithelial PrEC cells (Fig. 1A). Especially in PC3 cells, little or no miR-34a was detected. Consistent with a previous report showing that PC3 cells lack p53 expression and DU145 cells express mutated p53, while LNCaP cells express wild-type p53 proteins [9], Western blot analysis showed that p53 expression was completely lost in PC3 cells (Fig. 1B). In DU145 cells, the p53 expression level appeared to be much higher than in PrEC and LNCaP cells. As revealed by luciferase reporter assays, however, the p53 enhancer activity was lost in PC3 cells and almost totally abolished in DU145 cells compared with LNCaP cells expressing wild-type p53, suggesting that mutated p53 in DU145 cells is defective in terms of activation of the p53 enhancer (Fig. 1C). As shown in Fig. 1D, introduction of p53 into PC3

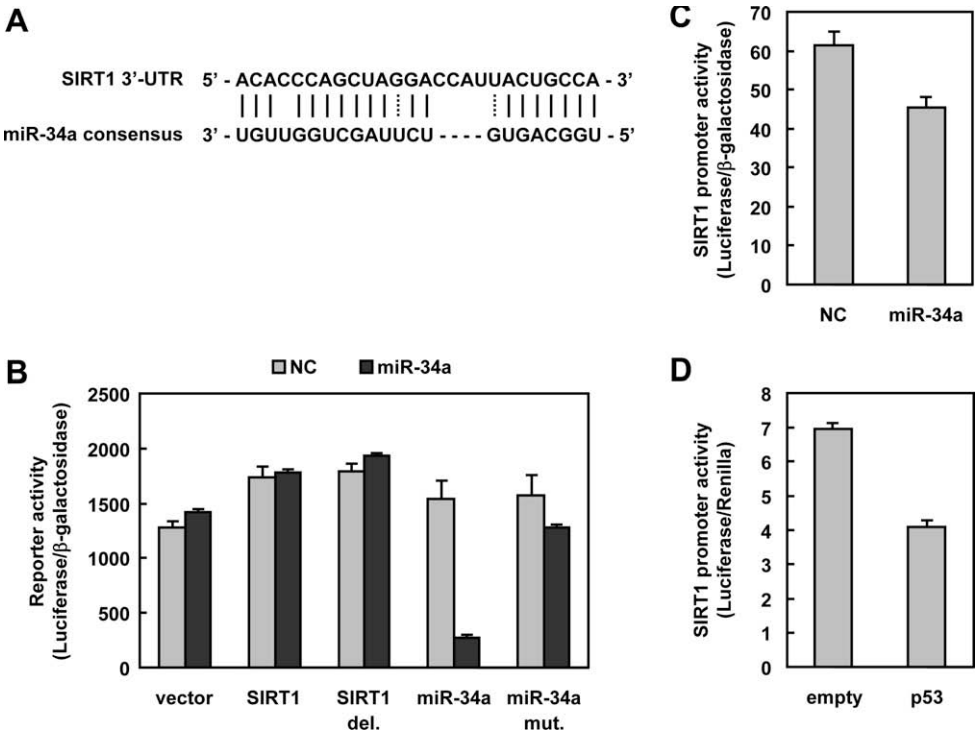


**Fig. 3.** Mechanism of inhibition of SIRT1 expression by miR-34a in PC3 cells. (A) Schematic representation of a potential miR-34a binding site within the SIRT1 3′-UTR. (B) PC3 cells were transfected with a reporter gene and β-galactosidase control vector along with either negative control miRNA precursor or miR-34a precursor. The reporter genes include an empty vector and vectors with the following DNA fragments inserted immediately downstream of the firefly luciferase coding region; the SIRT1 3′-UTR with or without deletion of a potential miR-34a binding site and the miR-34a consensus binding site with or without a mutation. Twenty-eight hours after transfection, firefly luciferase and β-galactosidase activities were measured. Reporter activity was expressed as a ratio of luciferase to β-galactosidase activity. Data are expressed as means ± SD ($n = 3$). (C) PC3 cells were first transfected with either control or miR-34a precursor for 8 h. After incubation in fresh medium for 16 h, cells were then transfected with the SIRT1 promoter reporter gene and β-galactosidase control vector using TransIT-LT1. Twenty-four hours later, luciferase and β-galactosidase assays were performed. The SIRT1 promoter activity was expressed as a ratio of luciferase to β-galactosidase activity. Data are expressed as mean ± SD ($n = 3$). (D) The SIRT1 promoter reporter gene and phRL-TK control vector were transfected into PC3 cells along with either an empty or the p53 expression vector. Twenty eight hours later, firefly and Renilla luciferase activities were measured. The SIRT1 promoter activity was calculated as a ratio of firefly to Renilla luciferase activity. Data are expressed as means ± SD ($n = 3$).

cells resulted in increased expression of miR-34a. Taken all these data together, it is suggested that miR-34a expression is dependent on the p53 activity in prostate cancer cell lines and is completely absent in p53-null PC3 cells. In the subsequent experiments, we used PC3 cells as a representative of androgen-refractory, p53-defective human prostate cancer cell lines.

*Down-regulation of SIRT1 protein and mRNA expressions by ectopic expression of miR-34a in PC3 cells*

We have recently reported that the expression level of SIRT1 is up-regulated in p53-defective PC3 and DU145 cells compared to p53 wild-type PrEC and LNCaP cells [8]. On the other hand, it has been previously shown that p53 normally represses the SIRT1 promoter and that SIRT1 expression was increased in certain tissues of $p53^{-/-}$ mice [6,10]. These findings led us to speculate that increased expression of SIRT1 might be the consequences of lack of miR-34a caused by loss of p53 in PC3 cells. To determine whether SIRT1 expression is regulated by miR-34a, we investigated effects of ectopic miR-34a expression on SIRT1 expression in PC3 cells. Introduction of miR-34a precursor into PC3 cells resulted in a decrease of the SIRT1 protein level compared to those transfected with negative control miRNA precursor (Fig. 2A). In addition, the expression levels of proteins involved in cell cycle control and apoptosis such as CDK6, Cyclin D1, E2F3, E2F1, and BCL2 were decreased in PC3 cells as a result of ectopic expression of miR-34a, all of which except for E2F1 were identified to be its direct targets. On the other hand, the SIRT1 mRNA level was decreased by introduction of miR-34a precursor into PC3 cells (Fig. 2B). These results indicate that both the SIRT1 mRNA and protein levels are down-regulated by ectopic miR-34a expression in PC3 cells.

*Down-regulation of SIRT1 expression through transcriptional, but not post-transcriptional mechanisms in PC3 cells*

Inspection of nucleotide sequences of the SIRT1 3′-UTR using TargetScan revealed a potential miR-34a binding site (Fig. 3A). We therefore constructed pMIR-REPORT miRNA Expression Reporter Vector to which a miR-34a consensus binding site or the 1.7 kb SIRT1 3′-UTR were introduced. We also constructed reporter genes having a mutation within the consensus binding site or a deletion of the potential miR-34a binding site within the 3′-UTR. The relative luciferase activity from the reporter gene containing the consensus binding site was significantly decreased by transfection of miR-34a precursor (Fig. 3B). This effect was abolished when a mutation was introduced into the consensus binding site. Contrary to our prediction, the relative luciferase activity from the reporter gene containing the SIRT1 3′-UTR was not significantly altered by miR-34a. Deletion of the potential miR-34a binding site from the 3′-UTR did not affect the reporter activity. On the other hand, the SIRT1 promoter activity was decreased by ectopic expression of miR-34a (Fig. 3C) and by overexpression of p53 (Fig. 3D). It is therefore suggested that miR-34a-induced SIRT1 inhibition is mediated at the transcriptional level but not at the post-transcriptional level in PC3 cells.

*Cell growth inhibition and G1 arrest by ectopic expression of miR-34a in PC3 cells*

Here, we examined effects of ectopic expression of miR-34a on cell growth in PC3 cells. Transfection of miR-34a precursor resulted in inhibition of cell growth and appearance of enlarged senescence like cells (data not shown), when compared with cells transfected with negative control miRNA precursor (Fig. 4A). Furthermore, we

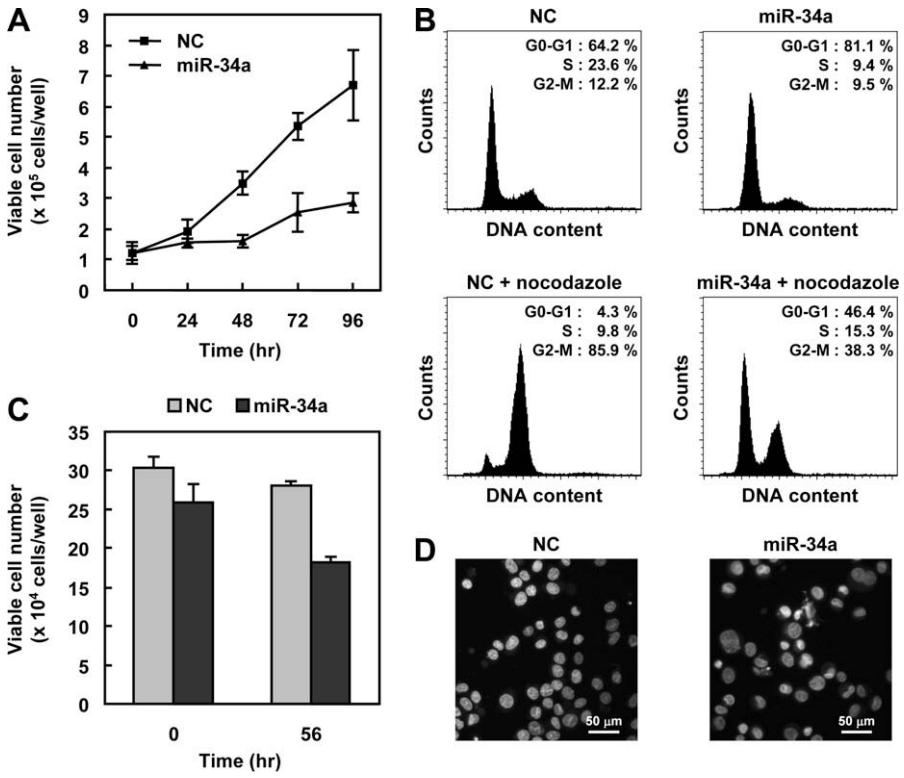


**Fig. 4.** Effects of ectopic expression of miR-34a on cell growth and chemoresistance in PC3 cells. (A) PC3 cells were transfected with either negative control miRNA precursor or miR-34a precursor. Viable cell numbers were determined at indicated times by trypan blue exclusion assay. The beginning of transfection was set as time zero. Data are expressed as mean ± SD ($n = 3$). (B) PC3 cells transfected with either control or miR-34a precursor were treated with or without nocodazole. After fixation and staining with propidium iodide, cells were subjected to cell cycle analysis using FACSCalibur. (C,D) PC3 cells were transfected with either control or miR-34a precursor for 8 h. After incubation in fresh medium for 16 hr, cells were replated onto 6-well plates and cultured for 24 h. Cell were then treated with camptothecin (5 μM) for 56 h. Cell viability was determined by trypan blue staining at the beginning and end of treatment. Data are expressed as means ± SD ($n = 3$). At the end of treatment, Hoechst 33342 nuclear staining was performed.

assessed cell cycle distribution profiles after transfection of either miR-34a or control precursor (Fig. 4B). Ectopic expression of miR-34a resulted in a significant increase in fraction of cells arrested at G1 phase and a concomitant decrease in fraction of cells arrested at G2-M compared with those transfected with control precursor. These effects were markedly enhanced when cells were pretreated with nocodazole, which blocks cell cycle progression in G2-M. Thus, it is shown that cell growth inhibition and G1 arrest can be induced by ectopic expression of miR-34a in PC3 cells.

*Attenuation of the resistance to camptothecin by ectopic expression of miR-34a in PC3 cells*

We studied if the resistance to camptothecin (topoisomerase inhibitor) could be attenuated by miR-34a in PC3 cells. Cells transfected with either miR-34a or negative control miRNA precursor were treated with camptothecin (5 μM). Tranfection with miR-34a precursor resulted in a reduced cell viability (Fig. 4C) and increased apoptotic cell death (Fig. 4D) compared with cells transfected with control precursor, suggesting that ectopic miR-34a expression can enhance the sensitivity to camptothecin by inducing apoptosis in PC3 cells that are highly resistant to the anticancer drug.

## Discussion

Prostate cancers are generally androgen-sensitive at the initial diagnosis and thus most of the patients are treated with anti-androgen therapy. However, patients eventually develop androgen-refractory tumors that will inevitably result in metastasis and death. Mutations in p53 have been identified in a variety of cancers and are usually a poor prognostic indicator [1]. Even in prostate cancer, it has been suggested that loss of p53 function plays a critical role in the cancer development [11]. Among human prostate cancer cell lines, wild-type p53 is expressed in androgen-sensitive LNCaP cells, while p53 is deleted and mutated in androgen-refractory PC3 and DU145 cells, respectively [9]. In both cell lines, the p53 enhancer activity is defective as confirmed in the present study (Fig. 1C).

Microarray-based miRNA profiling of prostate cancer and cell lines have indicated that miR-34a expression was reduced in tumor tissues than in normal tissues and was also decreased in PC3 cells than in LNCaP cells [12,13]. However, reduced expression of miR-34a has not been validated by quantitative RT-PCR nor its consequences have not yet been evaluated. As shown in Fig. 1A, the miR-34a expression was much higher in p53 wild-type PrEC and LNCaP cells than in p53-defective PC3 and DU145 cells. Thus, the expression levels of miR-34a were positively correlated with the p53 enhancer activities among LNCaP, PC3 and DU145 cells (Fig. 1C). In PC3 cells, ectopic expression of p53 significantly increased miR-34a expression (Fig. 1D). Taken all these data together, it is suggested that miR-34a expression is markedly decreased mainly due to defects in p53 function in PC3 and DU145 cells.

Several proteins involved in cell cycle control and apoptosis have been identified as direct targets of miR-34a [4]. In the present study, down-regulation by miR-34a of SIRT1 and E2F1 as well as CDK6, Cyclin D1, E2F3, and BCL2 was detected in PC3 cells (Fig. 2A). In addition, the SIRT1 mRNA level was also decreased in response to ectopic expression of miR-34a (Fig. 2B). It is therefore suggested that SIRT1 expression may be regulated by miR-34a at the transcriptional and/or post-transcriptional levels. A computational search using TargetScan algorithm for the miRNA complementary sites predicted a potential miR-34a binding site within the SIRT1 3′-UTR (Fig. 3A). Reporter assays revealed that the firefly luciferase activity from cells transfected with the reporter gene containing the SIRT1 3′-UTR with or without a deletion of the predicted site was not altered by miR-34a (Fig. 3B). Essentially the same results were obtained even when different experimental designs were employed, in which the reporter gene was changed to pGL3 luciferase reporter vector, different mutations were introduced to the potential miR-34a binding site and the miR-34a expression vector (Takara) was used instead of miR-34a precursor (data not shown). On the other hand, the SIRT1 promoter activity was decreased by ectopic expression of miR-34a (Fig. 3C) and p53 (Fig. 3D). Based on all of these findings, we have drawn a conclusion that SIRT1 is unlikely to be a direct target of miR-34a and that SIRT1 inhibition is mediated at the transcriptional level but not at the post-transcriptional level in PC3 cells. While preparing this manuscript, Yamakuchi et al. has reported that miR-34a targets the same potential binding site within the SIRT1 3′-UTR in HCT116 human colon carcinoma cells, in which miR-34a down-regulated SIRT1 protein expression, but did not affect its mRNA level [14]. This is in sharp contrast to our results, suggesting a possibility that binding characteristics of miR-34a to the target sequence and its effects on gene regulation may vary depending on the cellular context.

We have recently demonstrated that the SIRT1 promoter activity as well as its mRNA and protein expression levels was up-regulated in PC3 and DU145 cells compared with LNCaP cells [8]. It has been previously reported that p53 and E2F1 bind to the SIRT1 promoter and negatively and positively regulate SIRT1 transcription, respectively [10,15]. In p53-null PC3 cells, introduction of p53 increased miR-34a expression, ectopic expression of which in turn reduced SIRT1 expression through transcriptional mechanisms, suggesting that increased expression of SIRT1 in PC3 cells is in part due to loss of miR-34a expression caused by lack of p53. E2F3, one of known direct targets of miR-34a, has been shown to regulate E2F1 expression at the transcriptional level [16]. In the present study, expression of both E2F1 and E2F3 was decreased by miR-34a transfection into PC3 cells. Thus, it would be possible that increased expression of SIRT1 in the cell line could be explained partially by reduced E2F1 expression resulted from down-regulation of E2F3 expression at the post-transcriptional level by miR-34a.

Consistent with its known roles [4], introduction of miR-34a caused cell growth inhibition and cell cycle arrest at G1 phase in PC3 cells (Fig. 4A and 4B). Furthermore, ectopic expression of miR-34a attenuated chemoresistance to camptothecin through inducing apoptosis (Fig. 4CD). Our recent report has shown that inhibition of the SIRT1 activity or expression resulted in attenuation of cell proliferation and chemoresistance in PC3 and DU145 cells [8]. It is therefore suggested that miR-34a can inhibit cell growth and enhance chemosensitivity through directly or indirectly down-regulating expression of SIRT1 as well as cell cycle and apoptosis regulators in p53-null PC3 cells. It would be of great importance to determine whether other anticancer drugs than camptothecin could also sensitize PC3 cells to apoptosis, which is under current investigation. Modulation of miR-34a activity may represent a novel approach for treating malignant cancers with aberrant p53 function.

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