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MiR-34a targets GAS1 to promote cell proliferation and inhibit apoptosis in papillary thyroid carcinoma via PI3K/Akt/Bad pathway

Yanfei Ma [a], Huadong Qin [a], Yunfu Cui [b,*]

[a] Department of Fourth Surgery, the Second Affiliated Hospital of Harbin Medical University, 148 Xuefu Road, Nangang District, Harbin 150086, PR China
[b] Department of First Surgery, the Second Affiliated Hospital of Harbin Medical University, 148 Xuefu Road, Nangang District, Harbin 150086, PR China

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ABSTRACT

MicroRNAs (miRNAs) are fundamental regulators of cell proliferation, differentiation, and apoptosis, and are implicated in tumorigenesis of many cancers. MiR-34a is best known as a tumor suppressor through repression of growth factors and oncogenes. Growth arrest specific1 (GAS1) protein is a tumor suppressor that inhibits cancer cell proliferation and induces apoptosis through inhibition of RET receptor tyrosine kinase. Both miR-34a and GAS1 are frequently down-regulated in various tumors. However, it has been reported that while GAS1 is down-regulated in papillary thyroid carcinoma (PTC), miR-34a is up-regulated in this specific type of cancer, although their potential roles in PTC tumorigenesis have not been examined to date. A computational search revealed that miR-34a putatively binds to the 3′-UTR of GAS1 gene. In the present study, we confirmed previous findings that miR-34a is up-regulated and GAS1 down-regulated in PTC tissues. Further studies indicated that GAS1 is directly targeted by miR-34a. Overexpression of miR-34a promoted PTC cell proliferation and colony formation and inhibited apoptosis, whereas knockdown of miR-34a showed the opposite effects. Silencing of GAS1 had similar growth-promoting effects as overexpression of miR-34a. Furthermore, miR-34a overexpression led to activation of PI3K/Akt/Bad signaling pathway in PTC cells, and depletion of Akt reversed the pro-growth, anti-apoptotic effects of miR-34a. Taken together, our results demonstrate that miR-34a regulates GAS1 expression to promote proliferation and suppress apoptosis in PTC cells via PI3K/Akt/Bad pathway. MiR-34a functions as an oncogene in PTC.

1. Introduction

Growth arrest specific1 (GAS1) is a glycosylphosphatidylinositol (GPI)-anchored protein that plays a role in cell growth suppression. Induction of GAS1 inhibits cell proliferation and induces apoptosis in a variety of cell types [1–3], and suppression of GAS1 has opposite effects [1]. Ectopic expression of GAS1 in tumor cells also suppresses cell proliferation *in vitro* and inhibits tumor growth *in vivo* [4,5]. Recently, an increasing number of reports have highlighted the clinical significance of GAS1 in cancer diagnosis and prognosis. GAS1 is down-regulated in a number of cancers such as gastric cancer [6], prostate cancer [7], metastatic melanoma [8], colorectal cancer [9], and papillary thyroid carcinoma (PTC) [10,11], indicating that it is a potential biomarker for cancer diagnosis and prognosis. In addition, emerging evidence suggests that GAS1 plays an important role in pathophysiology of certain types of cancer. GAS1 has been shown to act as a tumor suppressor in gastric cancer [6] and inhibits metastasis in melanoma [8]. The role of GAS1 in tumorigenesis of other types of cancer such, however, waits to be elucidated.

RET is a receptor tyrosine kinase for members of the glial cell-derived neurotrophic factor (GDNF) family of ligands and mediates GDNF-dependent survival signals [12]. Gain of function mutations of RET are associated with several human cancers of neuroendocrine origin including PTC [13]. RET-activated PI3K/Akt and MAPK pathways play fundamental roles in the tumorigenesis and progression of thyroid cancer [14]. GAS1 has been shown to directly bind to RET and thereby block RET-mediated survival signals including the PI3K/Akt pathway [15]. Given that GAS1 is down-regulated in human PTC tissues, we speculated that GAS1 might play a role in tumorigenesis of PTC through inhibition of RET signaling. MicroRNAs (miRNAs) are endogenous single-stranded noncoding RNAs of about 22 nucleotides that suppress post-transcriptional expression of target genes by binding to the complementary 3′-untranslated region (3′-UTR) of messenger RNAs (mRNAs). MiRNAs may regulate thousands of human genes and

Abbreviations: GAS1, growth arrest specific1; miR-34a, microRNA-34a; PTC, papillary thyroid carcinoma; GPI, glycosylphosphatidylinositol; GDNF, glial cell-derived neurotrophic factor; UTR, untranslated region.

* Corresponding author. Fax: +86 0451 86605113.
E-mail addresses: yfma77@126.com, Cuiyunfuhm@163.com (Y. Cui).

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have fundamental importance in biological processes such as cell differentiation, proliferation, and survival [16], and in human diseases such as cancer [17]. MiR-34a, a member of the miRNA34 family, is a transcriptional target of p53 and is frequently inactivated in various types of human cancers [18,19]. A growing body of literature has shown that miR-34a acts as a tumor suppressor in a considerable number of cancers through repression of an assemblage of genes that promote cell proliferation [20–27]. Interestingly, in contrast to its frequent down-regulation in numerous cancers, miR-34a has been reported to be up-regulated in PTC tissues and cell lines [28,29], suggesting that miR-34a might have a pro-tumor effect in this specific type of cancer. To investigate potential underlying molecular mechanisms, we conducted a computational search for tumor suppressor genes that are potential targets of miR-34a, and identified GAS1 mRNA as such a putative target. In the present study, we first confirmed that miR-34a is up-regulated and GAS1 down-regulated in human PTC tissues. We then demonstrated that GAS1 is a direct target of miR-34a in TPC-1 cells, a human PTC cell line. Finally we investigated the regulatory role of miR-34a in PTC cell growth and apoptosis through regulation of GAS1 and the downstream RET/PI3K/Akt/Bad pathway.

2. Materials and methods

2.1. Human tissues and cell line

25 Pairs of human PTC and adjacent normal tissues were provided by Shanghai 10th People's Hospital (Shanghai, China). All human tissues were obtained from patients who provided informed consent. Human TPC-1 cell line was purchased from Shanghai Cancer Institute (Shanghai, China). TPC-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY, USA) at 37 °C, 5% CO_2 in a humidified incubator.

2.2. RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies, Inc.) according to manufacturer's instructions. RT and PCR primers for miR-34a and U6 snRNA were purchased from RiBoBio (Guangzhou, China). For miR-34a expression, total RNA was reverse-transcribed using the One Step PrimeScript® miRNA cDNA Synthesis Kit (Takara, Otus, Shiga, Japan) according to manufacturer's instructions. For GAS1 mRNA expression, total RNA was reverse-transcribed using PrimeScript™ RT reagent Kit (TaKaRa) and real-time PCR was carried out using SYBR green PCR master mix (TaKaRa) on an 7300 Real-time PCR system (Applied Biosystems, Foster City, CA). Primers for GAS1 expression analysis were: forward, GGACGAGAACTGCAAGTCCA; reverse, GCCATGTTCTCCTTGACCGA. U6 snRNA or GAPDH expression was determined and used as an endogenous control. Relative gene expression levels were calculated and normalized to U6 or GAPDH using the $2^{-\Delta\Delta Ct}$ method. All real-time PCR reactions were run in triplicate.

2.3. Western blot analysis

Western blot analysis was performed as previously described [2,3]. Briefly, samples of 50 μg total protein were separated on 12 or 6% SDS–PAGE gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After being blocked in 5% non-fat milk in TBST (0.05% Tween-20, TBS) for 1 h, membranes were incubated overnight at 4 °C with primary antibodies against p-RET (1:500), p-PI3K (Tyr508 of the p85 subunit, 1:1000), p-Akt (1:500), p-Bad (1:500), p-caspase 3 (1:1000), and GAPDH (1:1000), respectively. p-RET and p-Akt antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and p-PI3K, p-Bad, p-caspase 3, and GAPDH antibodies were from Cell Signaling Technology (Danvers, MA, USA). Membranes were subsequently incubated with goat anti-rabbit IgG-HRP (1:2000) (Santa Cruz Biotechnology) at room temperature for 1 h and visualized by ECL-PLUS reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein expression was normalized to GAPDH.

2.4. Generation of stable TPC-1 cell line

To obtain stable expression of miR-34a in TPC-1 cell line, primers used for pre-miR-34a DNA amplification were: forward, 5′-CACGGATCCTCGGGGGCATTTGGAGATTTT-3′; reverse, 5′-CTGTCTAGATCGCTTCATCTTCCCTCTTGG-3′. The PCR product was inserted into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). TPC-1 cells were either transfected with pre-miRNA-34a plasmid DNA or control vector using Lipofectamine 2000 (Invitrogen). After 48 h, cells were subcultured in selective medium containing neomycin (Gibco) at pre-determined concentration. The medium was changed every 2–3 days until neomycin-resistant TPC-1-miR clones were identified. Expression of miR-34a was confirmed by qRT-PCR as described above.

2.5. Vector construction and luciferase reporter assay

A fragment of 3′-UTR of human GAS1 (161bp) containing the putative miR-34a binding site (GAS1-wt) was amplified by PCR using the following primers: forward, 5′-GTCCTCTAGACTAAGGGGTTGCCATGGTGT-3′; reverse, 5′- GTCCGGCCGGCCAGAGGCAATATACATTCTCCGCA-3′. A mutant 3′-UTR fragment of GAS1 with a total of six mutations at predicted miR-34a binding site (GAS1-mut) was created by site-directed mutagenesis, in which four consecutive bases at one site and two consecutive bases at another were replaced to disrupt miR-34a binding. GAS1-wt and GAS1-mut were subcloned into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA) downstream of luciferase gene sequence and transfected into TPC-1 cells using Lipofectamine 2000. Luciferase activity was determined 48 h after transfection using a Dual-Glo Luciferase Assay System (Promega) according to manufacturer's instructions.

2.6. Oligonucleotide transfection

TPC-1 cells were transfected with anti-miR-34a (Life Technologies, Inc.), GAS1 siRNA (Santa Cruz Biotechnology), Akt siRNA (Cell Signaling Technology), or their respective non-specific control using Lipofectamine 2000. Cells were collected 48 h after transfection.

2.7. Cell viability assay

Cells were plated in 96-well plates at 2×10^3 per well 24 h post-transfection. MTT (20 μl, 5 mg/ml) (Sigma) was added to each well and cells were incubated for another 4 h at 37 °C. The reaction was stopped by addition of 150 μl DMSO and optical density at 570 nm was determined on a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.8. Colony formation assay

TPC-1 cells stably expressing miR-34a were transfected with anti-miR-34a, GAS1 siRNA, Akt siRNA, or their respective nonspecific control. After 24 h, cells were seeded into six-well plates at 300 cells per well and grown for 14 days. Cells were subsequently

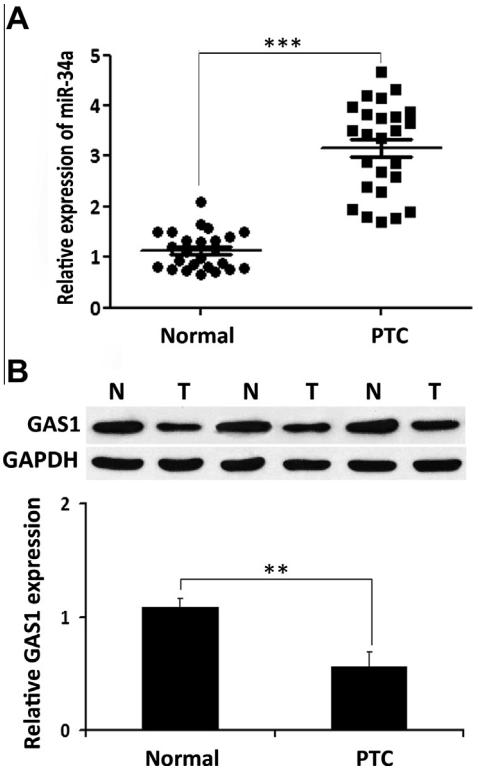

Fig. 1. Relative levels of miR-34a and GAS1 expression in PTC vs. adjacent normal tissues. (A) miR-34a mRNA expression by qRT-PCR normalized to U6 snRNA ($n = 25$) (***$P < 0.001$). (B) GAS1 protein expression by western blot normalized to GAPDH ($n = 25$) (**$P < 0.01$). Results represent mean ± SD.

washed twice with PBS, fixed with 10% methanol/10% acetic acid, and stained with 1% crystal violet. The number of colonies formed was counted in four different field visions and the mean value was calculated.

2.9. Apoptosis assay

TPC-1 cells stably expressing miR-34a and transfected with anti-miR-34a, GAS1 siRNA, Akt siRNA, or their respective nonspecific control were seeded in six-well plates at 3×10^4 per well and cultured for 72 h. Cells were subsequently double-stained with fluorescein (FITC)-conjugated Annexin V and propidium iodide (FITC-Annexin V/PI) (BD Biosciences, San Diego, CA, USA), and analyzed on a FACSCalibur flow cytometer (BD Biosciences) to determine rate of apoptosis.

2.10. Statistical analysis

All results were expressed as mean ± S.D. Data analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was analyzed using Student's t test or one-way ANOVA. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. MiR-34a is up-regulated and GAS1 is down-regulated in human PTC tissues

We assessed miR-34a mRNA and GAS1 protein expression by qRT-PCR and Western blot analysis, respectively, in the 25 PTC tissues vs. matched adjacent normal tissues. The relative expression of miR-34a mRNA in PTC tissues was significantly higher than that of their matched adjacent normal tissues ($P < 0.001$) (Fig. 1A). Meanwhile, the relative expression of GAS1 protein in PTC tissues was significantly lower than that of adjacent normal tissues ($P < 0.01$) (Fig. 1B). Our results confirmed previous findings that miR-34a is up-regulated [28,29] and GAS1 is down-regulated [10,11] in PTC.

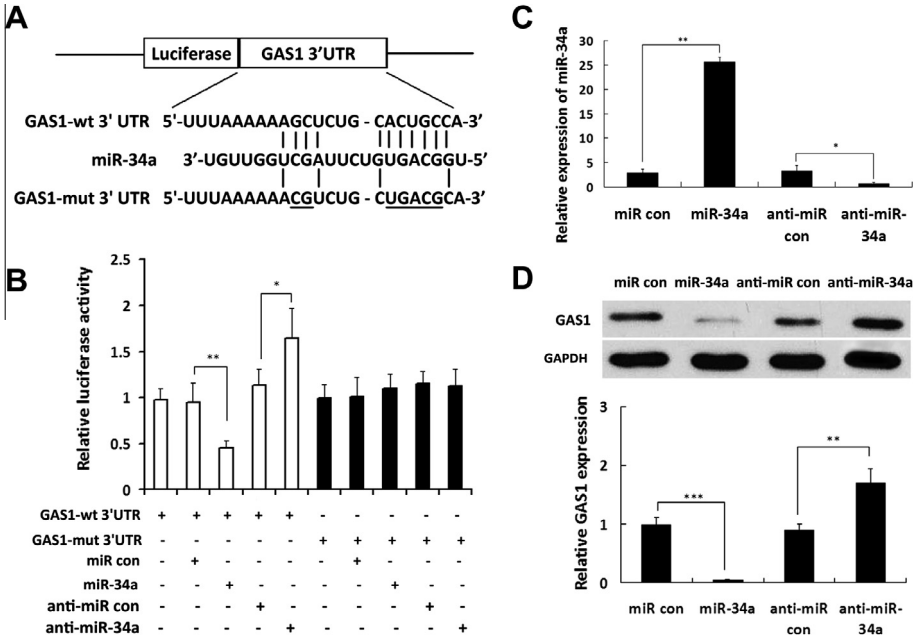

Fig. 2. GAS1 is a direct target of miR-34a. (A) Diagram of GAS1 3′-UTR-containing reporter constructs. (B) Luciferase reporter assay in TPC-1 cells transfected with reporter vectors containing wildtype or mutant GAS1 3′-UTR. Relative luciferase expression was normalized to Renilla activity. (C) miR-34a mRNA expression by qRT–PCR in TPC-1 cells transfected with miR-34a, anti-miR-34a, or their respective non-specific control. (D) GAS1 protein expression by western blot in TPC-1 cells transfected with miR-34a, anti-miR-34a, or their respective nonspecific control. qRT-PCR and western blot data were normalized to U6 snRNA and GAPDH, respectively. Results represent mean ± SD (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3.2. GAS1 is a direct target of miR-34a

To test whether GAS1 mRNA is a direct target of miR-34a, we prepared a luciferase reporter expression system using a fragment of 3′-UTR of human GAS1 (161bp) containing the putative miR-34a binding site (GAS1-wt 3′-UTR). We also created expression vectors containing a mutant 3′-UTR fragment with mutations at the putative miR-34a binding site to disrupt miR-34a binding (GAS1-mut 3′-UTR) (Fig. 2A). Co-transfection of GAS1-wt 3′-UTR reporter system with miR-34a in TPC-1 cells resulted in a 50% loss of luciferase reporter expression ($P < 0.01$) while no significant change was observed with miRNA control (Fig. 2B). MiRNA control does not have a predicted target site within the GAS1 3′-UTR and therefore served as a negative control. In addition, co-transfection of GAS1-wt 3′-UTR reporter system with anti-miR-34a led to a significant increase in luciferase expression ($P < 0.05$) compared with anti-miRNA control, presumably attributed to silencing of endogenous miR-34a. However, luciferase expression was not affected by miR-34a or anti-miR-34a transfection when GAS1-wt 3′-UTR was replaced with GAS1-mut 3′-UTR. Therefore, our reporter assay results indicated that GAS1-wt 3′UTR is directly targeted by miR-34a with high specificity.

We also examined effects of overexpression of miR-34a and anti-miR-34a on GAS1 protein expression in TPC-1 cells. Compared with cells transfected with miRNA control, cells transfected with miR-34a showed significantly increased miR-34a mRNA expression ($P < 0.01$) (Fig. 2C) accompanied by greatly reduced GAS1 protein expression ($P < 0.001$) (Fig. 2D). In contrast, transfection with anti-miR-34a significantly decreased miR-34a mRNA expression ($P < 0.05$) (Fig. 2C) and increased GAS1 protein expression ($P < 0.01$) (Fig. 2D) when compared with control. Collectively, our results demonstrated that GAS1 is a direct target of miR-34a.

3.3. MiR-34a promotes growth and inhibits apoptosis in TPC-1 cells

We studied the role of miR-34a in TPC-1 cell proliferation and apoptosis using cells transfected with miR-34a or anti-miR-34a. We found that miR-34a overexpression significantly promoted cell growth ($P < 0.05$) (Fig. 3A) and suppressed apoptosis ($P < 0.01$) (Fig. 3C) when compared with control. In contrast, overexpression of anti-miR-34a significantly inhibited cell growth ($P < 0.01$) and induced apoptosis ($P < 0.001$). Similarly, miR-34a overexpression significantly stimulated TPC-1 colony formation ($P < 0.001$) (Fig. 3B) while anti-miR-34a overexpression had the opposite effect. We also found that silencing of GAS1 had similar pro-growth, anti-apoptotic effects as overexpression of miR-34a (Fig. 3A, B, C), suggesting that miR-34a promotes cell proliferation and prevents apoptosis through repression of GAS1 expression.

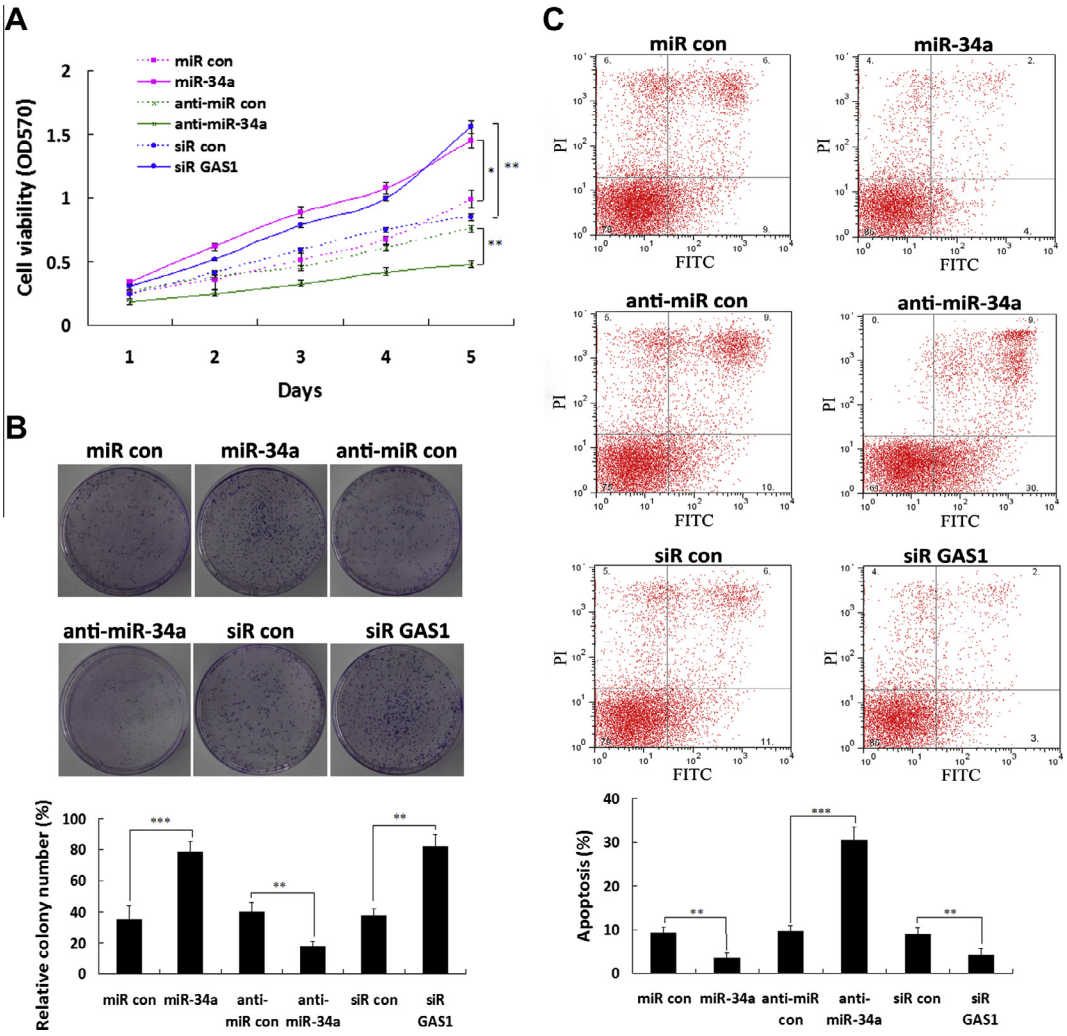

Fig. 3. Effects of transfection with miR-34a, anti-miR-34a, GAS1 siRNA, or their respective nonspecific control on TPC-1 cell proliferation (A), colony formation (B), and apoptosis (C). Results represent mean ± SD (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3.4. MiR-34a promotes TPC-1 cell proliferation and inhibits apoptosis through activating PI3K/Akt/Bad pathway

RET-activated PI3K/Akt pathway plays an important role in pathology of thyroid cancers including PTC [14]. Since GAS1 has been shown to block RET signaling [15], we speculated that repression of GAS1 by miR-34a leads to activation of RET and downstream PI3K/Akt pathway, which drives cell proliferation. To investigate the role of RET and PI3K/Akt pathway in miR-34a-induced TPC-1 cell proliferation, we assessed activation of this pathway by western blot. Our results showed that miR-34a transfection significantly increased levels of p-RET, p-PI3K, p-Akt, and p-Bad in TPC-1 cells while decreasing levels of GAS1 and cleaved caspase 3, while transfection with anti-miR-34a showed the opposite effects (Fig. 4A, B). Bad is a pro-apoptotic member of the Bcl-2-family and its phosphorylation by Akt inactivates its pro-apoptotic activity [30]. These results suggested that miR-34a-induced TPC-1 cell proliferation is mediated by activation of RET and downstream PI3K/Akt/Bad pathway. To further investigate the role of Akt pathway in the pro-growth effect of miR-34a, we studied TPC-1 cells in which Akt was knocked down by transfection with Akt siRNA. We found that silencing of Akt significantly inhibited cell proliferation and colony formation and induced apoptosis in TPC-1 cells (Fig. 4C), in accordance with the crucial role of Akt-dependent pathway in PTC tumorigenesis. Importantly, while miR-34a transfection promoted cell proliferation, stimulated colony formation, and inhibited apoptosis, these effects were blocked in cells co-transfected with Akt siRNA, indicating that the pro-growth, anti-apoptotic effects of miR-34a are Akt-dependent.

4. Discussion

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, representing 75 to 85 percent of all thyroid cancer cases [31]. The most common etiologic factor is radiation, but genetic susceptibility also contributes to the development of PTC. The most common mutations include chromosomal translocations involving RET proto-oncogene and NTRK1, and point mutations in BRAF oncogene. Chromosomal translocation at RET proto-oncogene occurs in approximately a fifth of PTCs. The fusion oncoproteins generated are termed RET/PTC proteins, and constitutively activate downstream MAPK/Erk and PI3K/Akt pathways, which play fundamental roles in PTC tumorigenesis [14]. MiRNAs regulate fundamental cellular processes including proliferation, differentiation, and apoptosis. MiRNAs are differentially expressed in tumor tissues and implicated in tumorigenesis in many cancers. A considerable number of miRNAs have been shown to be deregulated in PTC and potentially contribute to PTC initiation and development [32]. Studies have shown that miR-34a is up-regulated in PTC tissues and cell lines [28,29], although its biological role in this disease has not been revealed. As an effector of p53, miR-34a is often down-regulated in cancer tissues and acts as a tumor suppressor in *in vitro* and *in vivo* models through negative regulation of growth factors and oncogenes. However, miR-34a is capable of regulating hundreds of genes [33], and its functions in cancer development may depend on the type of tumor, the clinical stage, and other conditions. Considering that miR-34a is up-regulated in PTC tissues, we speculated that miR-34a might promote PTC cell proliferation through repression of tumor suppressor genes. A computational search identified GAS1, a tumor suppressor that is down-regulated in PTC tissues, as such a putative target for miR-34a.

In the present study, we first confirmed that miR-34a is up- and GAS1 is down-regulated in PTC tissues. We then demonstrated that GAS1 is a direct target for miR-34a using several approaches. In TPC-1 cells transfected with GAS1 3′-UTR-containing luciferase reporter system, luciferase expression was reduced by miR-34a overexpression and enhanced by anti-miR-34a overexpression. However, these effects were lost when wildtype GAS1 3′-UTR in the reporter system was replaced with a mutant GAS1 3′-UTR, which contains six mutations within the predicted miR-34a binding site. In addition, GAS1 protein expression in TPC-1 cells was decreased by miR-34a overexpression and increased by anti-miR-34a overexpression. Taken together, these results indicate that miR-34a represses GAS1 expression via directly binding to GAS1 3′-UTR.

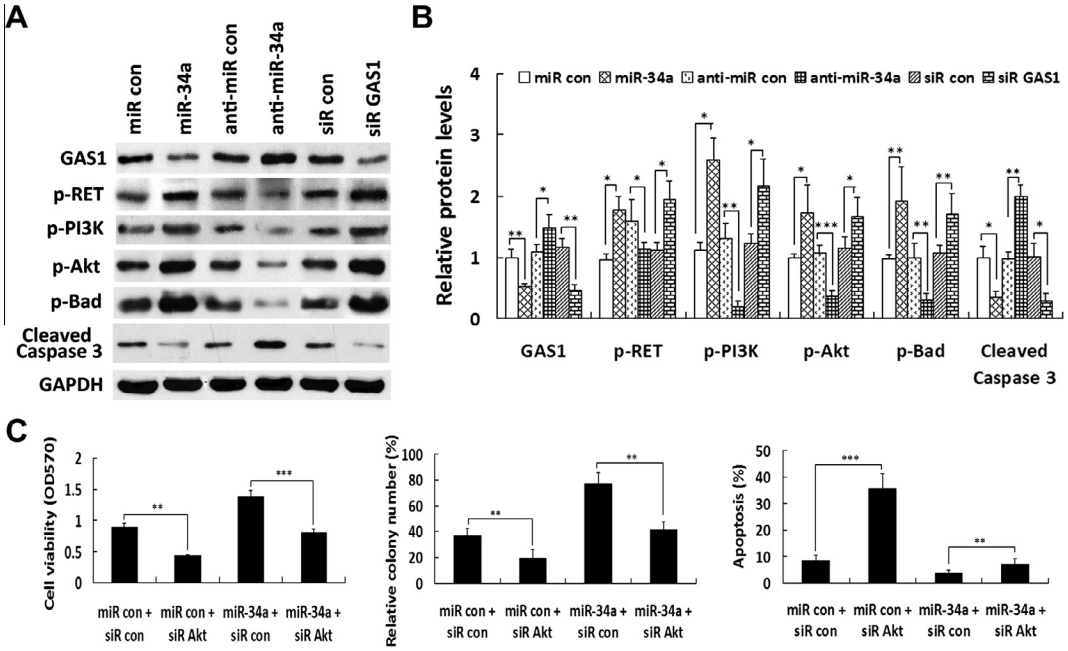

Fig. 4. MiR-34a promotes TPC-1 cell proliferation and prevents apoptosis via activation of PI3K/Akt/Bad pathway. (A) Protein levels by western blot. Cells were transfected with miR-34a, anti-miR-34a, GAS1 siRNA, or their respective nonspecific control. (B) Quantification of band intensity in A normalized to GAPDH. (C) Effects of Akt silencing on cell growth, colony formation, and apoptosis. Cells were transfected with miR-34a, Akt siRNA, or their respective nonspecific control as indicated. Results represent mean ± SD (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

We then studied functions of miR-34a in TPC-1 cell growth, colony formation, and apoptosis. We found that miR-34a overexpression promoted cell proliferation, stimulated colony formation, and inhibited apoptosis while anti-miR-34a overexpression had the opposite effects. Moreover, silencing of GAS1 had similar pro-growth, anti-apoptotic effects as overexpression of miR-34a. Collectively, these results suggest that miR-34a promotes cell growth and prevents apoptosis in TPC-1 cells through repression of GAS1 expression.

GAS1 has been shown to inhibit RET activation. Since RET-activated PI3K/Akt pathway plays an important role in PTC tumorigenesis, we speculated that miR-34a-induced TPC-1 cell proliferation is mediated by activation of RET and downstream PI3K/Akt pathway attributed to GAS1 repression. Indeed, we found that miR-34a overexpression led to increased levels of p-RET, p-PI3K, p-Akt, and p-Bad and the opposite was observed with anti-miR-34a overexpression. Moreover, silencing of GAS1 activated RET and downstream PI3K/Akt/Bad pathway similarly to overexpression of miR-34a. Finally, we examined the effect of Akt silencing on TPC-1 cell growth and apoptosis. Silencing of Akt in TPC-1 cells inhibited cell proliferation, reduced colony formation, and induced apoptosis, in accordance with the fundamental role of Akt in PTC tumorigenesis. Importantly, silencing of Akt reserved miR-34a overexpression-induced cell growth and inhibition of apoptosis. Collectively, our results demonstrate that miR-34a promotes TPC-1 cell growth and prevents apoptosis by repressing GSA1 expression and thereby activating RET and downstream PI3K/Akt/Bad pathway.

MiRNAs are actively exploited as biomarkers for cancer diagnosis and prognosis as well as therapeutic targets and agents for cancer treatment [17]. In this study, we demonstrate that miR-34a regulates novel target GAS1 to promote PTC cancer cell proliferation and inhibit apoptosis via PI3K/Akt/Bad pathway. MiR-34a functions as an oncogene in PTC. Pharmaceutical interventions targeting miR-34a or GAS1 may provide novel therapy for PTC treatment.

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