

RESEARCH ARTICLE

miR-186 Regulates Glycolysis through Glut1 During the Formation of Cancer-associated Fibroblasts

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Abstract

Emerging evidence has suggested that glycolysis is enhanced in cancer-associated fibroblasts (CAF), and miR-186 is downregulated during the CAF formation. However, it is not clear whether miR-186 is involved in the regulation of glycolysis and what the role of miR-186 plays during the CAF formation. In this study, quantitative PCR analyses show miR-186 is downregulated during the CAF formation. Moreover, miR-186 targets the 3' UTR of Glut1, and its overexpression results in the degradation of Glut1 mRNA, which eventually reduces the level of Glut1 protein. On the other hand, knockdown of miR-186 increased the expression of Glut1. Both time course and dose response experiments also demonstrated that the protein and mRNA levels of Glut1 increase during CAF formation, according to Western blot and quantitative PCR analyses, respectively. Most importantly, besides the regulation on cell cycle progression, miR-186 regulates glucose uptake and lactate production which is mediated by Glut1. These observations suggest that miR-186 plays important roles in glycolysis regulation as well as cell cycle checkpoint activation.

Keywords: miR-186 - Glut1 - cancer-associated fibroblast - CDK2

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Introduction

Cancer-associated fibroblasts (CAFs) are activated fibroblasts and a major component of the tumor stroma. Through specific communication with cancer cells, CAFs can directly promote tumor initiation, progression and metastasis (Dimanche-Boitrel et al., 1994; Olasso et al., 1997; Olumi et al., 1999; Bhowmick et al., 2004; Kuperwasser et al., 2004; Grum-Schwensen et al., 2005; Orimo et al., 2005). CAFs secrete growth factors and ECM-degrading proteases to promote tumor growth and invasiveness. In addition to the important role of TGF- β and VEGF in tumor progression, CAF-secreted SDF1 (stromal cell-derived factor 1) also mediates the recruitment of bone marrow-derived endothelial cells and directly increases cancer cell proliferation (Orimo et al., 2005). CAF-secreted MMPs and other proteases also directly affect the motility and invasiveness of the cancer cells (Lochter et al., 1997; Boire et al., 2005) and help the cancer cells cross tissue boundaries and escape the primary tumor site (Stetler-Stevenson et al., 1993; Sternlicht et al., 1999; Boire et al., 2005).

Aerobic glycolysis, which is also known as "Warburg effect" described the unique metabolic phenomenon that conversion of glucose into lactic acid is enhanced even in the presence of oxygen (Warburg 1956, Mathupala et al., 2009). Compared with normal somatic cells, tumor

cells preferentially utilize this far less-efficient glycolytic process for energy production and for biosynthesis. In cancer cells glucose uptake increase, the oxygen consumption decrease, and lactate secretion increase. The dependence of cancers on glycolysis partially results from the induction of glucose transporter 1 (GLUT1). Recent studies suggested that glycolysis is enhanced in the CAF cells.

Glut1, also known as SLC2A1 (the solute carrier 2A family member 1), is a rate-limiting transporter for glucose uptake. The expression level of Glut1 usually correlates with the rate of cellular glucose metabolism, and highly expressed in erythrocytes and in the blood-tissue barriers, in particular in the endothelial cells forming the blood-brain barrier. Moreover, Glut1 is often overexpressed in a variety of malignant neoplasms, which suggests that this increase is required for increased glucose uptake and accelerated metabolism (Amann et al., 2009). However, it is not clear whether and how the expression of Glut1 is upregulated during the CAF formation.

MicroRNAs (miRNAs) are abundant small noncoding RNAs, approximately 22 nucleotides in length. miRNA binds imperfectly to miRNA response elements in target mRNAs and forms a RNA-induced silencing complex (RISC), which inhibits translation and results in degradation of miRNA (Olsen et al., 1999; Bagga et al., 2005; Lim et al., 2005). Base-pairing between the miRNA-

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response elements and the first 2-7 nt of the miRNA, the "seed region", are critical for effective repression (Lai 2004; Brennecke et al., 2005; Lewis et al., 2005; Xie et al., 2005). MiRNAs play key roles in diverse physiological and pathological processes, such as cell differentiation, apoptosis and cancer etiology (Farh et al., 2005; Lu et al., 2005; Calin et al., 2006a; 2006b; Wang et al., 2007). The data from Li group demonstrated that miR-186 inhibited cell-cycle progression through targeting cyclin D1 (CCND1), cyclin-dependent kinase 2 (CDK2), and CDK6; and the expression levels of miR-186 were correlated with the survival of patients bearing with NSCLC (non-small cell lung carcinoma) (Cai et al., 2013). Moreover, miR-186 stimulates degradation of CKIIa mRNA by targeting its 3' untranslated regions (UTRs). Downregulation of protein kinase CKII induces cellular senescence in human colon cancer HCT116 cells (Kim et al., 2012). The miR-186 mimics increased senescent-associated β -galactosidase (SA- β -gal) staining, p53 and p21Cip1/WAF1 expression, and reactive oxygen species (ROS) production. In contrast, knockdown of the miR-186 increased the CKIIa protein level. However, it is unknown about the role of miR-186 in glycolysis.

In this study, we have demonstrated that Glut1 protein level increases during CAF formation, and miR-186 directly targets Glut1 mRNA; in turn, miR-186 regulates glycolysis through affecting Glut1 protein level.

Materials and Methods

Lentiviral packaging and stable cell line establishment

Two short-harpin RNA (shRNA) fragments specifically targeting human GLUT1 (NM_006516.2) were synthesised (Sangon Biotech, Shanghai, China) and inserted into pGIPZ lentiviral shRNAmir vector (Open Biosystems, Lafayette, CO). Lentiviruses were generated in 293T cells by the co-transfection of vector containing favorite gene or gene fragment with psPAX2 and PMD2G for 72 hours. Viral stocks collected from the supernatant of transduced 293T cells were used to infect the human fibroblast. MiR-186 over-expression plasmid was constructed by the same way as GLUT1 knockdown. The miR-186 knockdown lentivirus was generated with a 4 plasmids packaging system, including pMirzip-miR-186, pVSVg, pRSV-Rev, pMDL (g/p).

Real-time PCR

Total RNAs were extracted from cultured cells using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instruction. For the reverse transcription of GLUT1 mRNA, 2 μ g of total RNA per sample were transcribed to cDNA using the PrimerScript RT Reagent Kit (Takara, Dalian, China). For realtime PCR, the forward primer: 5'-CTTTGTGGCCTTCTTTGAAGT-3', reverse primer: 5'-CCACACAGTTGCTCCACAT-3'. The reverse transcription of miR-186 was done by the TaqMan[®] MicroRNA RT Kit (Invitrogen, CA, USA). The primer for its cDNA synthesis was GTCGTATCCAGTGCAGGGTCCGAGG TATTGCACTGGATACGACAGCCCA. The reactions were incubated at 16°C for 30 min, at 42°C for 30

min, at 85°C for 5 min and then held at 4 °C. For the quantification PCR of miR-186, the forward primer: 5'-GCGGCGCAAAGAATTCTCCT-3', the reverse primer: 5'-GTGCAGGGTCCCAGGT-3'. The PCR was performed by the real-time PCR system (ABI 7500 Fast, CA, USA) according to the protocol. The β -actin and RNU48 were chosen as an internal control respectively. The forward primer for β -actin: 5'-GCGGGAAATCGTGCGTGACATT-3', the reverse forward: 5'-GATGGAGTTGAAGGTAGTTTTCG-3'. The forward primer for RNU48: 5'-TGATGATGACCCCAGGTAAGT-3', the reverse primer for RNU48: 5'-GAGCGCTGCGGTGATG-3'.

Western Blot

Cells were harvested and lysed on ice for 10 min in lysis buffer containing 150 mM NaCl, 0.1% SDS, 1% NaMoO₄, 1% NP40, 50 mM Tris-HCL (PH 7.5), 0.02% Na₃N, supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF). Protein concentration was detected by using the BCA assay kit (Ding guo changsheng Biotechnology, Shanghai, China). Then protein was separated on polyacrylamide gels, transferred to a nitrocellulose membrane and incubated with antibodies against GLUT1 (Epitomics, CA, USA); antibody against β -Actin (Santa Cruz Biotechnology, CA, USA) as an internal control.

Luciferase reporter assay

The full length 3'UTR of the human GLUT1 gene was amplified by PCR using human genomic DNA as a template. The sense primer was: 5'-TTTGAGCTCCTCTTCCTACCCAACCACTCAA-3', the antisense primer was: 5'-TTTCTCGAGGAACC AAATCGGCATCTTCTCAT-3'. Site-directed mutagenesis was performed to generate GLUT1 3'UTR mutants containing mutations in the conserved miR186-binding site. In the 3'UTR mutants, the nucleotide sequence complementary to nt 2-5 of miR-186 was mutated to the sequence as that in miR-186 (from ATTCTTT to AATGTAT). 293T cells were seeded at 2×10^5 cells in 12-well plates and co-transfected with 200 ng of pmirGLO-GLUT1 (wide type or mutant), 3 μ g of miR186 over-expression plasmid, using Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. After 48 hours transfection, the luciferase activity was measured using the Dual-luciferase reporter assay system (Promega, Beijing, China).

Lactate and glucose uptake Assay

Cells were cultured at 2×10^4 in 60mm dishes in DMEM supplemented with 15% FBS for 48 hours. To measure the secretion of lactate, the supernatant were removed and replaced with DMEM without FBS for 2 hours, then the supernatant were collected and stored at -80°C until measurement. Lactate assay was performed according to manufacturer's protocol and normalized to cell number (BioVison, Shanghai, China). 2×10^4 cells were cultured and glucose uptake experiment was conducted according to the instruction of manufacture's introduction (BioVison, Shanghai, China).

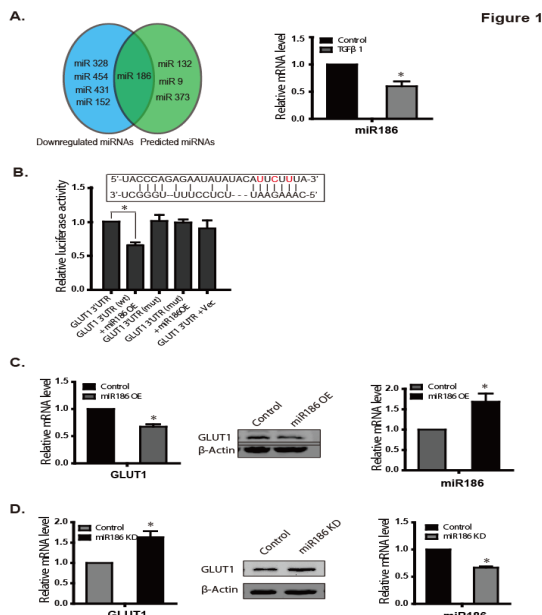


Figure 1. Downregulation of miR-186 Increases *Glut1* Protein level During the CAF Formation. **A)** miR-186 expression decreases in fibroblasts 12 hours after TGF-1 treatment. The miR-186 level is assessed by Realtime PCR, ($*p<0.05$). **B)** *Glut1* is a downstream target of miR-186. The schematic drawing represents the miRNAs downregulated during CAF formation and predicted to target 3'UTR of *Glut1* gene; the histogram represents the results for the 3' UTR luciferase assay (the red nucleotides are mutated to cytosine), ($*p<0.05$). **C)** Overexpression of miR-186 decreases the expression of *Glut1*. The protein and mRNA level of *Glut1* are assessed by western blot and realtime PCR respectively, ($*p<0.05$). **D)** *Glut1* expression increases in miR-186 knockdown fibroblasts, ($*p<0.05$). data are expressed as means \pm SD

Cell proliferation assay

Cells were seeded in 60-mm dishes at a density of 2×10^5 cells/dish and were cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO₂. The cell number was counted using the Countless cell counter (Invitrogen, Grand Island, NY, USA) at different time points. For the cell proliferation assay, cells were seeded in 96-well plates at a density of 5×10^3 cells/well. Relative cell growth was analyzed at the indicated time points using a CCK8 kit (Dojindo Laboratories, Japan) according to the manufacturer's instructions.

Cell cycle assay

Cells were cultured overnight in 60-mm dishes at a density of 2×10^6 cells/plate. After trypsinization, the cells were fixed with 70% ethanol overnight at 4°C. The fixed cells were re-hydrated in PBS. Following PI/RNase treatment, the cells were analyzed using a FACS (Fluorescence-Activated Cell Sorter Scan) flow cytometer (BD Inc., Franklin Lakes, NJ, USA). The percentage of cells in each phase of cell cycle was calculated using the provided software.

Results

Downregulation of miR-186 increases *Glut1* protein level during the CAF formation

Through deep sequencing, miRNA profile showed

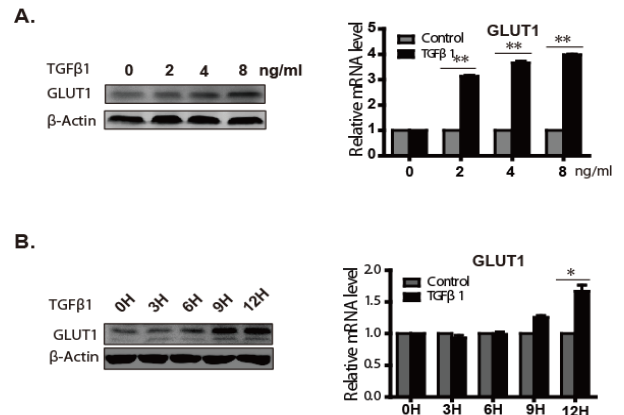


Figure 2. The expression level of *Glut1* increases during the CAF formation. **A)** The dose response experiment shows that *Glut1* expression reaches a peak at the concentration of 8ng/ml TGF β 1, 12 hours after treatment, ($**p<0.01$); **B)** The time course experiment demonstrates that *Glut1* expression reaches a peak at 12 hours with 8ng/ml TGF- β 1 treatment, ($*p<0.05$), data are expressed as means \pm SD

that miR-186 was downregulated during CAF formation, of which the model was established previously (Li et al., 2013b). In the mean time, the protein level of *Glut1* was upregulated. Moreover, miR-186 was predicted to target the 3'UTR of *Glut1* mRNA using web-based software analysis (<http://starbase.sysu.edu.cn>) (Yang et al., 2011). Thus, we proposed that miR-186 regulated *Glut1* expression during CAF formation.

Quantitative PCR has first shown the level of miR-186 decreased 12 hours after TGF- β 1 treatment in the fibroblast (Figure 1A), which was consistent with the deep sequencing data. Computation analysis has shown that only miR-186 was predicted to target *Glut1* and downregulated in fibroblasts. The 3' UTR luciferase assay was performed to verify whether miR-186 regulates the expression of *Glut1*. Our data show that miR-186 reduced the activity of firefly luciferase in the 3' UTR luciferase assay; after the three nucleotides (indicated in red in vc 1B) in the *Glut1* 3' UTR that were predicted to bind to the miR-186 seed sequence were mutated, miR-186 could no longer affect the expression of firefly luciferase. These data suggested that miR-186 is involved in the regulation of *Glut1* expression in fibroblasts.

To verify the regulation of miR-186 on endogenous *Glut1* expression, miR-186 was overexpressed in fibroblasts via lentivirus, the expression of endogenous *Glut1* was assessed by Western blot or Real time PCR. Ectopic expression of miR-186 in human fibroblasts did reduce both the protein level and mRNA level of *Glut1* gene by approximately 50% (Figure 1C). To further confirm the miR-186 regulation of *Glut1*, miR-186 was knocked down in fibroblasts. Figure 1D showed that both *Glut1* mRNA and protein levels increased more than 50% in fibroblasts with miR-186 knockdown, which is consistent with the data shown in Figure 1C. These data have together demonstrated that miR-186 regulates the expression of *Glut1*.

The expression level of *Glut1* increases during the CAF formation

To further verify the expression of *Glut1* in fibroblasts

during CAF formation, the dose response experiment was performed, the expression of Glut1 was assessed by Western blot and quantitative PCR analysis. Twelve hours after treatment, the expression level of Glut1 in fibroblasts was gradually elevated with an increase in the concentration of TGFβ1 from 2 to 8 ng/ml, with its highest expression observed after treatment with 8 ng/ml TGFβ1 (Figure 2A). Additionally, time course response experiment was also conducted. Western blot result revealed that the expression of Glut1 increased gradually and reached the peak after 12 hours treatment with 8 ng/ml TGFβ1; and the real-time PCR data are consistent with the results of the western blot analysis (Figure 2B).

MiR-186 regulates glycolysis in fibroblast through Glut1

Glut1 is a rate-limiting transporter for glucose uptake. To determine whether miR-186 regulates glycolysis through the mediation of Glut1, lactate production and glucose uptake rate was analyzed in fibroblasts that were depleted of miR-186 and/or Glut1.

The histogram in Figure 3A shows that the knockdown of miR-186 increases lactate production and glucose uptake in fibroblast, The graphic in Figure 3B shows that the overexpression of miR-186 decreases lactate production, and Glut1 knockdown decreases glucose uptake in fibroblast. In brief, these data have demonstrated that miR-186 regulates glycolysis in fibroblast through Glut1.

MiR-186 regulates cell cycle progression independent of Glut1

MiR-186 regulated cell cycle progression in NSCLC

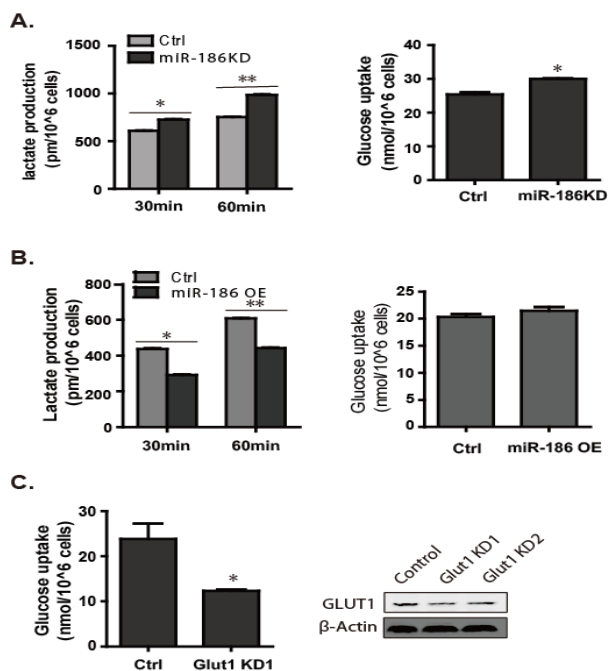


Figure 3. MiR-186 Regulates Glycolysis in Fibroblast through Glut1. A) miR-186 decreases lactate production and glucose uptake during CAF formation. Lactate production and Glucose uptake is measured by the assay kit in miR-186 knockdown fibroblast, (** $p < 0.01$, * $p < 0.05$). B) Lactate production and Glucose uptake is measured by the assay kit in miR-186 overexpression fibroblast, (** $p < 0.01$, * $p < 0.05$). C) Glucose uptake is measured by the assay kit in glut1 knockdown fibroblast, (* $p < 0.05$), data are expressed as means±SD.

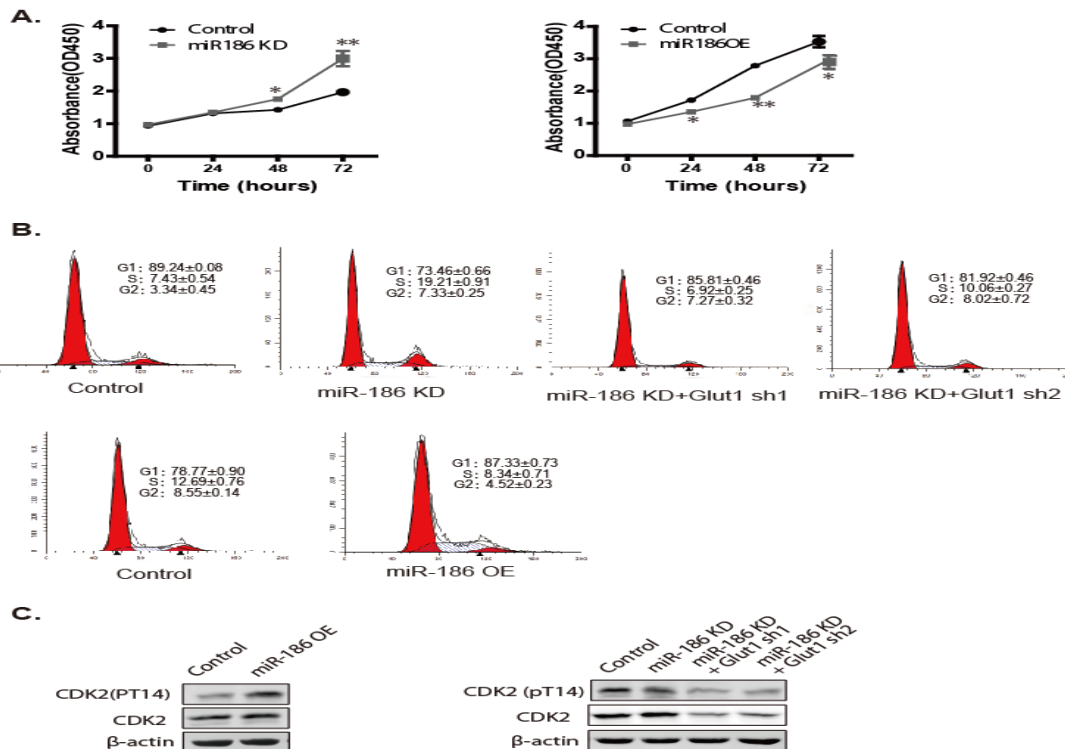


Figure 4. MiR-186 Regulates Cell Cycle Progression Independent of Glut1 in Fibroblasts. A) miR-186 inhibits cell proliferation of fibroblasts. The graphics show the cell proliferation rate at different time points using CCK8 assay, (** $p < 0.01$, * $p < 0.05$). B) Upregulation of miR-186 results in cell cycle arrest. The phase of cell cycle is analyzed by flow cytometry. The numbers in the table represent the mean value of three independent experiments. C) Upregulation of miR-186 enhanced the protein levels of CDK2 phosphorylation at Thr14 and reduced its total protein level independent of Glut1. Phosphorylated CDK2 and total CDK2 were detected by Western blot. β-actin was used as the internal control, data are expressed as means±SD

cells through targeting cyclin-dependent kinases, such as CDK2, CDK6. To determine whether miR-186 regulates cell cycle progression in fibroblasts and whether Glut1 is involved in this regulation, cell cycle and CDK2 were analyzed. The cell proliferation rate was first assessed by the CCK8 Method in fibroblasts that were depleted of miR-186 and/or Glut1. The graphic in Figure 4A shows that miR-186 overexpression inhibits the proliferation of fibroblasts ($*p < 0.05$), and downregulation of Glut1 has no significant effects on cell proliferation compared to the control cells ($\#p > 0.05$). In contrast, miR-186 knockdown promotes the proliferation of fibroblasts ($*p < 0.05$).

To determine whether miR-186 inhibits cell proliferation through regulating cell cycle progression, the fibroblasts in different phases of cell cycle were analyzed by flow cytometry. The graphic in Figure 4B shows that downregulation of miR-186 reduces the cell number in G0/G1 phase of cell cycle, and increases the cell number in S and G2 phase, Glut1 knockdown doesn't influence this changes (Figure 4B). In contrast, upregulation of miR-186 increases the cell number in G0/G1 phase of cell cycle, and declines the cell number in S and G2 phase (Figure 4B). These data suggests miR-186 regulates cell cycle progression.

To further determine how miR-186 regulates cell cycle progression, the protein level of CDK2 were analyzed according to the previous study. The Western blot analysis has demonstrated that miR-186 knockdown increases the protein level of CDK2 while the overexpression of miR-186 decreases the protein level of CDK2; and Glut1 doesn't affect the protein level of CDK2. These data suggest that miR-186 regulates cell cycle progression which is independent of Glut1.

Discussion

MiRNAs play important roles in tumor progression. Ting Guan' lab obtained a total of 18 key miRNAs which may play important regulatory roles in ovarian cancer (Wan et al., 2012). MicroRNA-101 Inhibits Cell Proliferation, Invasion, and Promotes Apoptosis by Regulating Cyclooxygenase-2 in Hela Cervical Carcinoma Cells (Huang et al., 2013). MiR-186 was downregulated in tumor cells (Cai et al., 2013; Li et al., 2013a), and correlated with poor survival of patients bearing with lung adenocarcinoma (Cai et al., 2013). Cyclin D1 (CCND1), cyclin-dependent kinase 2 (CDK2), and CDK6 are direct target of miR-186 (Cai et al., 2013). The overexpression of miR-186 in NSCLC cells inhibited proliferation by inducing G1-S checkpoint arrest. Our data also show that miR-186 regulates cell cycle progression in fibroblasts through targeting CDK2. This observation further confirms miR-186 is an important regulator of cell cycle progression. Moreover, miR-186 downregulates the expression of CKIIa, of which downregulation induces cellular senescence in human colon cancer HCT116 cells (Kim et al., 2012). These observations suggest that miR-186 negatively regulates cell growth through cell cycle arrest and apoptosis, at least in tumor cells and fibroblasts. However, it's not clear how miR-186 is regulated in tumor cell and fibroblast.

Glut1 is a rate-limiting transporter for glucose uptake, and often overexpressed in malignant tumor cells and contribute to glycolysis, and this increase is required for increased glucose uptake and accelerated metabolism in cancer cell (Amann and Hellerbrand 2009). Previous study showed Glut1 expression was regulated by hypoxia, and HIF-1 α might be involved in this regulation (Airley et al., 2007). Our data have demonstrated that the expression of Glut1 is regulated by miR-186, and downregulation of miR-186 increases glucose uptake and lactate production through upregulation of Glut1 during CAF formation.

These observations suggest miR-186 not only regulates cell cycle progression, but also promotes glycolysis, at least in fibroblasts. Although miR-186 regulating cell cycle arrest is independent of Glut1, but miR-186-regulated glycolysis is mediated by Glut1, miR-186 finely tunes the biological function via the different pathways in response to environmental alteration.

In brief, our study has demonstrated that miR-186 not only regulates cell cycle progression, but also modulates glycolysis via Glut1.

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