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MicroRNA-125a inhibits cell growth by targeting glypican-4

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Abstract Heparan sulfate proteoglycan (HSPG), such as glypican, plays a role as a co-receptor for growth factor to influence cells proliferation. However the mechanism is still vague. Micro-RNAs (miRNAs) regulate cell proliferation. Their capacity to direct the translation and stability of targeted transcripts can dramatically influence cellular physiological function. To explore how the function of glypican is regulated involved in cell proliferation, glypican-4 was chosen with a bioinformatics search identifying targeting seed sequences for *miR-125a* within the 3′-untranslated regions (3′UTR). Indeed, luciferase constructs containing the 3′UTR of glypican-4 demonstrated around 54 % less activity in *miR-125a e*xpressing cells relative to the controls. The expression of glypican-4 at both the transcript and protein level was down-regulated by transition trasfection of *miR-125a* in the human embryonic kidney cell line 293T (HEK293T). Although cell proliferation of HEK293T was not influenced by the silence of glypican-4, DNA synthesis in response to FGF2 in the cells was attenuated by knockdown of glypican-4 using siRNA technique. Further study showed that phosphorylation of $ERK_{1/2}$ and AKT was suppressed by overexpressing *miR-125a, whereas the suppressed MAPK and AKT signaling could be recovered by anti-miR-125a* treatment. Both DNA synthesis and cell proliferation were impaired by the inhibitor of $ERK_{1/2}$ signaling. MTT assay demonstrated that the cell proliferation was impaired by *miR-125a* overexpression, however, rescued by anti-*miR-125a* in HEK293T cells. These results disclosed new function of miR-125a by targeting gene glypican-4 in cell growth process and illustrated the feasibility of using miRNAs as a therapeutic strategy to suppress cells proliferation.

Keywords miR-125a · glypican-4 · $ERK_{1/2}$ · AKT

Abbreviations

HSPG	Heparan sulfate proteoglycan
miRNAs	Micro-RNAs
3′UTR	3′-untranslated regions

Introduction

Heparan sulfate proteoglycans (HSPGs) are ubiquitously expressed molecules composed of a core protein to which heparan sulfate (HS) glycosaminoglycan chains are covalently O-linked. They are distributed on the cell surface, extracellular matrix, and basement membranes of a wide range of cells. HSPGs family includes syndecan, glypican, agrin and perlecan. Their core protein diversity, structural heterogeneity and high negative charge ensure that HSPGs play multiple important roles in many biological processes [1], such as cell migration, adhesion, embryonic morphogenesis, angiogenesis, metastasis, inflammation, neurite outgrowth, tissue repair, etc.. HS on HSPGs acts as co-receptors for growth factors, such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) to influence cell behavior including differentiation [2, 3]. Glypicans are a family of HSPGs that are linked to the exocytoplasmic surface of the plasma membrane via a glycosyl-

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phosphatidyl-inositol (GPI) anchor [4]. The size of the core proteins of glypicans is similar (60 to 70 kDa). As expected, they all contain an N-terminal secretory signal peptide and a hydrophobic domain in the C-terminal region required for the insertion of the GPI anchor [5]. To date, six distinct glypican gene products have been shown in mammals and two in Drosophila [6]. Glypican-4 is a member of glypican family of HSPGs which are characterized by the GPI-anchorage to cell surfaces and strictly conserved cysteine residues in their core proteins. During the last few years it has been clearly established that cell-surface HSPGs are required for the optimal activity of heparin-binding growth factors, such as FGFs and Wnts. In the case of FGFs, a model has been proposed in which the HS chains interact with both the ligand and the high-affinity FGF receptor. This interaction increases FGF2-FGF receptor binding and also promotes FGF receptor dimerization [7]. Recent study has shown that glypican-4 plays a critical role in the regulation of basic fibroblast growth factor 2 ($bFGF_2$) action during cortical neurogenesis [8]. Interestingly, overexpressing Glypican-4 but not Glypican-3 or Glypican-1 induces sustained hepatocyte growth factor (HGF)-stimulated ERK activation and rescues the renal tubulogenic response in these cells. This suggests that HGF-mediated morphogenesis may specifically require cell surface expression of glypican-4 [9]. Although glypican-4 plays the role in neurogenesis and morphogenesis involved in growth factors action, how this gene is regulated is still unknown.

MicroRNAs (miRNA) are a class of non-coding RNAs that have recently emerged as important regulators of gene expression. Recent research has demonstrated that although these molecules are small, they are involved in some crucial biological functions as well as a broad spectrum of human diseases. Experimental evidence suggests that miRNAs regulate the expression of more than 30 % of protein-coding genes. MiRNA mediates posttranscriptional gene control by depending upon the extent of sequence homology between miRNA and target RNA [10, 11]. Based on this function, miRNAs have been implicated in a wide range of cellular processes including cellular growth, differentiation and disease [12] through binding to the 3′ untranslated region (3′UTR) of target mRNAs and down-regulating their translation to protein or degrading the mRNAs [13–15]. By either blocking translation or inducing target mRNA degradation, miRNA not only participates in regular biological processes within cells and tissues but is also involved in pathological processes. Many human malignancies have been linked to specific miRNA expression patterns, while such human disease-related mechanisms have been discussed and reviewed. It is now clear that the biogenesis and function of miRNAs are implicated in the molecular mechanisms of various clinical diseases. They can potentially regulate every aspect of cellular activity, including proliferation, metabolism, apoptotic cell death and viral infection. Some papers clarified that miR-125a inhibited cell growth via a dramatic suppression of cell proliferation and promotion of apoptosis in MCF-7 breast cancer cells [16]. Expression of miR-125a has also been demonstrated in a number of malignancies including ovarian cancer cells [17]. Although validated targets of miR-125a include $ERBB_{2/3}$ and BAK_1, numerous reports indicate that a single miRNA may have hundreds if not thousands of targets [18, 19]. This means miR-125a may target other genes except $ERBB_{2/3}$ and BAK_1.

Here, we extend these findings and report that miR-125a targets the glypican-4 gene. MiR-125a appears to reduce glypican-4 protein levels by translationally arresting glypican-4 mRNA via binding the 3′UTR of glypican-4 to influence HEK293T cells proliferation. Our findings also disclosure how glypican-4 function implicated in cell growth is regulated by miR-125a.

Materials and methods

Reagents and chemicals

DMEM medium was purchased from GIBCO BRL/Invitrogen (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was from Sijiqing Co. Ltd. (Hangzhou, China) and GIBCO-BRL (Grand Island, NY, USA). Human recombinant bFGF was purchased from Shanghai PrimeGene Bio-Tech Co., Ltd (Shanghai, China). Dual-Luciferase Reporter Assay system was from Promega Biotech Co., Ltd (Madison, WI, USA). Antibodies used in this study included the rabbit polyclonal antibodies against total ERK1/2, phospho-Ser-ERK1/2, total AKT and phospho-Ser-AKT from Cell Signaling Technology Inc. (Beverly, MA, USA.), and mouse monoclonal antibody against β-actin from Sigma (St. Louis, MO, USA), Glypican 4 from Abcam (Cambridge, UK). Heparitinase was from Seikagaku Corporation (Tokyo, Japan). All other reagents unless otherwise indicated were all from Sigma (St. Louis, MO, USA).

Cell culture, plasmid constructs and transfection

HEK 293T cell lines (Cell Bank, Type Culture Collection of Chinese Academy of Sciences, China) were cultured in DMEM medium supplemented with 10 % (56 °C) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. 5×10^6 cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. HEK 293T cells were transfected with plasmid DNA by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction. Brifely, after cells reach at 70 %–90 % confluent in 24 well plate, 0.8 μg plasmid was added into 50 μl Opti-MEM while 2 μl Lipofectamine™ 2000 was added into another 50 μl Opti-MEM. After standing for 5 min at room temperature,

both media were mixed and incubated for 20 min. The cells were then transfected with the mixture media.

A fragment containing human miR-125a was PCR-amplified from normal genomic DNA using primers sense: 5′-CACAACTCGAGCTAGGTCTCTGCCCCTCCC GATA-3′,antisense: 5′- AAG GATCCGGGGGAAGG GCCAGTGGTCTG-3′) and cloned into the pll3.7 vector. The fragments of the 3′UTR of glypican-4 was subcloned into the psiCHECK-2 dual luciferase reporter plasmid with following primers: Glypican-4 sense: 5′-CACAACTCG AGCCACTGGTTTAAGAAGTGCTGAC-3′, anti-sense: 5′-TGAAGATC TAGTTGAATGAAATGTAGACCCTGA-3′. The resultant vectors were confirmed by DNA sequencing. Anti-miR-125a and siRNA of Glypican-4 were from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequence of 2′-O-Me anti-miR-125a is 5′-UCACAGGU UAAAGGGUCUCAGGGA-3′. Scrambled 2′-O-Me modified RNA (5′-CAGUACUUUUGU GUAGUACAA-3′) was used as negative control. Three siRNA oligo for knocking down Glypican-4 were identified. The best effect sequence is 5′-CGUAUUUCUGAAAUAUU AATT-3′. Transfection was carried out using lipofectamine™ 2000 (Invitrogen).

RNA isolation, semi-quantitative RT-PCR analysis

Total RNA was isolated from control and treated cells using TRIZOL reagent (Invitrogen) following the manufacture's instructions. Single-strand cDNA was synthesized from 1 μg total RNA using AMV reverse transcriptase (TaKaRa, Japan), while 18S rRNA was used as control. The primers used are as following: Glypican-4, 5′-AGTGTGGTCAGC GAACAGTG-3′ (sense) and 5′-GCGAGCCCAGAAGTC ATTTA-3′ (anti-sense); 18S rRNA, 5′-GATATGCTC ATGTGGTGTTG-3′ (sense) and 5′-AATCTTCTTC AGTCGCTCCA-3′ (anti-sense). PCR amplification was performed with Ex Taq (TaKaRa, Japan) using the following conditions: denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. The thermal cycling was 25 cycles for 18S rRNA, 32 cycles for Glypican-4. The RT-PCR products were analyzed and visualized on 1.5 % agarose gel containing ethidium bromide. Images were acquired by Tanon 3500 digital gel imaging system (Tanon Science & Technology, China) and quantified using Image J densitometry analysis software (NIH, USA).

Measurement of cell viability and proliferation

Cell viability was measured by MTT assay. In brief, cells were seeded in 96-well plate at a cell density of 1×10^4 per well and were cultured for 24 h. The cells were transfected with or without an vector control (EV), siRNA of glypican-4, anti-miRNA-125a and miRNA-125a, respetively. After the transfection for 48 h, 0.5 mg/ml MTT was added and incubated with cells for 4 h in an incubator at 37 °C. The formazan was dissolved in DMSO after the medium was removed. Finally the optical density was measured using a spectrophotometer (Thermo Multiskan MK3, German) at an absorption wavelength of 570 nm.

BrdU proliferation assay was carried out (Chemicon, Millipore, USA.) following the manufacturer's instructions. Brifely, cells were seeded in a sterile 96-well tissue culture plate at cell density of 2×10^5 cell/ml in 100 μl media per well and incubated overnight. BrdU was added into the cell and maintained for 24 h. After fixation and denaturation, the cell was washed using buffer provided by the company. 100 μl/well of anti-BrdU antibody was added and incubated for 1 h at room temperature followed by exposure to the goat anti-mouse IgG and incubation for 30 min at room temperature. After washing and aspirating the media from the cell wells, 100 μl/well of TMB peroxidase substrate was added and maintained for 30 min at room temperature in dark. The reaction was stopped by the stop solution followed by reading using spectrometer.

Dual-luciferase reporter assays

Luciferase reporter assays were performed using the psiCHECK2-3′UTR vector. 3×10^5 of HEK293 cells were seeded at a 12 well plate and maintained overnight. When cells were grown to approximately 80 % confluent in 48-well plates, psiCHECK2-3′UTR, pll3.7-miR-125a, and empty vector were co-transfected as described previously [3]. Cells were incubated with transfection reagent/DNA complex for 48 h followed by luciferase reporter assay using the Dual Luciferase Assay System. Cells lysates were subjected to luciferase activity measurement according to the manufacturer's instructions. Renilla luciferase activity was normalized to firefly luciferase activity.

Western blot

5×10^5 293T cells were seeded in 6 well plate and cultured overnight before the transfection. Cells treated for certain time were washed with cold PBS and lysed with RIPA buffer for 30 min on ice. For ERK, Akt and their phophorylation protein detection, total protein content was measured by Protein Assay Reagent (Bio-Rad, USA). Equivalent amount of protein of each sample was subjected to SDS-PAGE and transferred to PVDF membranes (Bio-Rad, USA). The membranes were blocked with 5 % nonfat milk in TBST buffer [20 mM Tris (pH 8.0), 150 mM NaCl and 0.1 % Tween-20] and incubated overnight at 4 °C with the primary antibodies then probed using horseradish peroxidase conjugated secondary antibody (Jackson, USA). Visualization of bands by enhanced chemiluminescence kit (PIERCE, USA) was performed according to manufacturer's instructions.

The protein extract of glypican-4 was performed as described in reference [20]. Briefly, the expression level of glypican-4 was evaluated by immunostaining using anti-glypican-4 antibody and by immunoblotting of detergent extractable proteoglycans. For the latter measurements, cells were extracted with proteoglycans extraction buffer (2 % Triton X-100, 0.15 M NaCl, 10 mM EDTA, 10 mM KH_2PO_4, pH 7.5, along with 5 μg/ml BSA, 100 μg/ml PMSF, and 25 μg/ml *N*-ethylmaleimide) for 10 min at 4 °C. After centrifugation, the supernatant was subjected to DEAE Sephacel purification and were eluted with 150, 250, and 750 mM NaCl in 50 mM Tris–HCl, pH 8.0, at 4 °C. The pool eluted by 750 mM NaCl was desalted on PD-10 column followed by lyophilization. The resulting material was digested with 10 mU/ml heparinase III (37 °C overnight in digestion buffer (3 mM Ca(OAc), 50 mM NaOAc, 50 mM Hepes, and 10 mM EDTA, pH 6.5) and chondroitinase ABC (20 mU/ml in 0.1 M Tris–HCl, 10 mM EDTA, pH 7.3) and subjected to 4–20 % gradient SDS-PAGE and immunoblotting using anti-glypican-4 antibody.

Statistical analysis

Data are from at least three independent experiments±standard deviation (SD). Student's *t*-test was used to determine

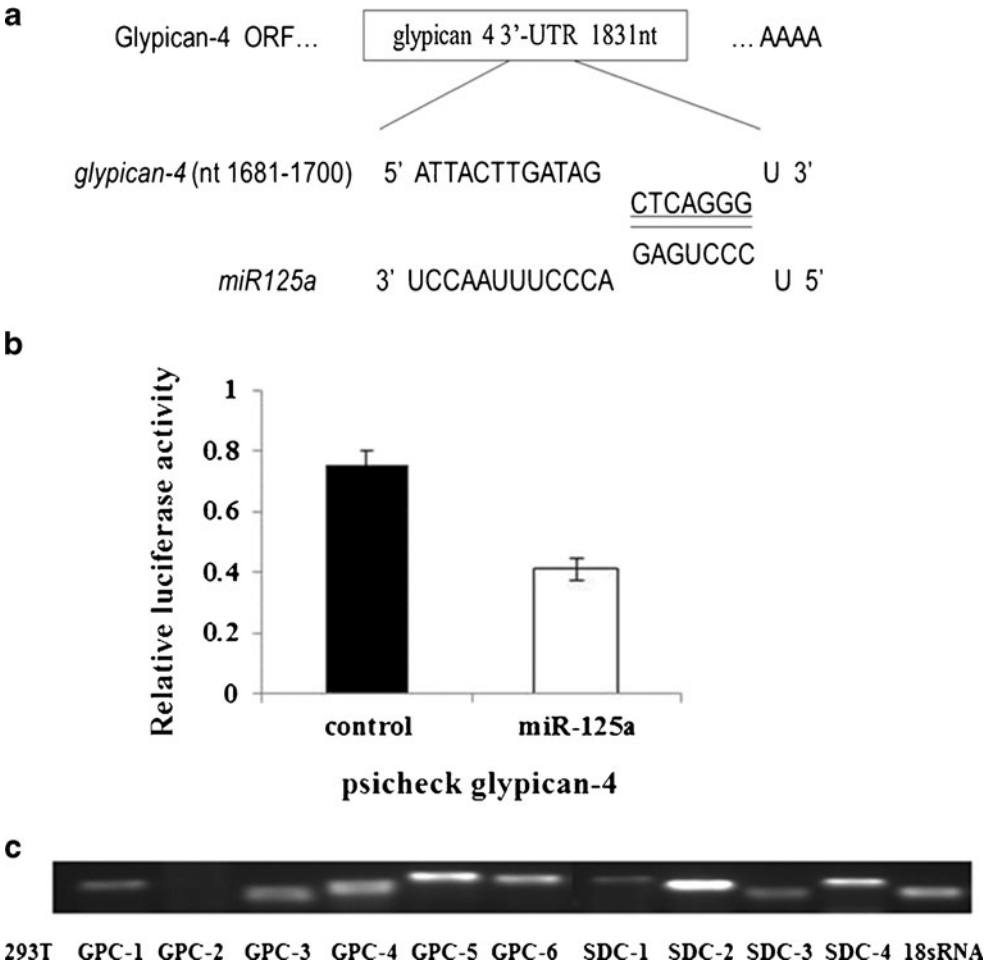

Fig. 1 Glypican-4 is a functional target of miR-125a in 293T cells. **a** Alignment of potential miR-125a-binding sites in the 3′UTR of the glypican-4. Paired sequence of miR-125a and the 3′UTR of glypican-4 was predicated with pattern based microRNA target identification algorithm (Pictar) online software. Activity of miRNA-125a on the 3′ UTR of glypican-4 was initially assessed by luciferase based reporter assays. The glypican-4 3′UTR was incorporated into the firefly luciferase gene and run off a single promoter. All constructs were introduced into HEK 293T cells with miRNA-125a or an empty control vector (EV) and luminescence was measured at 48 h. **b** MiR-125a reduced luciferase levels by 54 %. MiR-125a reduced the activity of a luciferase reporter gene fused to the wild-type 3′UTR of glypican-4. HEK293 cells were transfected with miR-125a or vehicle with psicheck2-glypican-4 3′UTR. Luciferase activities were measured as described in "Dual luciferase reporter assays" in "Materials and methods". Relative luciferase activity was normalized to firefly luciferase activity ($P < 0.01$, t test). Data are presented as mean fold reduction±S.D. All experiments were performed in triplicate. **c** The genes expression level of syndecans and glypicans in 293T cell. 293T cells were harvested and total RNA was extracted followed by cDNA synthesis, Glypican 1–6 (GPC-1~6) and Sdecan 1–4 (SDC-1~4) genes expression level were detected by RT-PCR in 293T cells. 18S rRNA was employed as a control

the significances of differences in multiple comparisons. Values of $P<0.05$ were considered statistically significant.

Results

miRNA-125a targets the 3′UTR of Glypican-4

MiR-125a inhibited cell growth by down-regulating expression of HuR at protein level in breast cancer cells [16]. This suggested that miR-125a might influence cell growth by targeting other genes. Indeed, we found that the 3′UTR of glypican-4 harbored a sequence motif that was paired with the seed sequence (nucleotides 2–7 from the 5′-end) of miR-125a (Fig.1a) with several public algorithms among several potential targets of miR-125a. We hypothesized that miR-125a might down-regulate glypican-4 gene by direct binding to the sites within the 3′UTR of its mRNA. To test this hypothesis, we constructed luciferase reporter vector in which encoded the complete 3′UTR of glypican-4 mRNA followed by its co-transfection with miR-125a into HEK 293T cells. Briefly, 1831 nt long of 3′UTR of glypican-4 from human genomic DNA was amplified by RT-PCR. The resulting amplicon was inserted into a luciferase reporter vector (see in Fig. 1b). Transfection of miRNA-125a and luciferase vectors into HEK 293T cells led to the 54 % reduction in normalized luciferase activity compared to relevant controls including an empty miRNA expression plasmid (see in Fig.1). The binding test clearly indicated that miR-125a reduced the activity of the luciferase reporter gene fused to the wild-type 3′UTR of glypican-4.

MiR-125a decreases expression of glypican-4 at mRNA and protein level

To understand whether miR-125a down-regulates the targeting gene after its binding to 3′UTR of glypican-4, firstly, the expression level of miR-125a and glypican-4 was measured by RT-PCR in HEK 293 cells. Indeed, both miR-125a (Fig. 2a) and glypican-4 (Fig. 2b) were expressed in HEK 293T cells. These data were confirmed by the fact that expression of miR-125a and glypican-4 was specifically silenced by anti-miR-125a (Fig. 2a) and siRNA of glypican-4 (Fig. 2b), respectively. Next, we explore whether glypican-4 expression is substantially down-regulated by miR-125a. HEK 293T cells were transfected by miRNA-125a expression vector followed

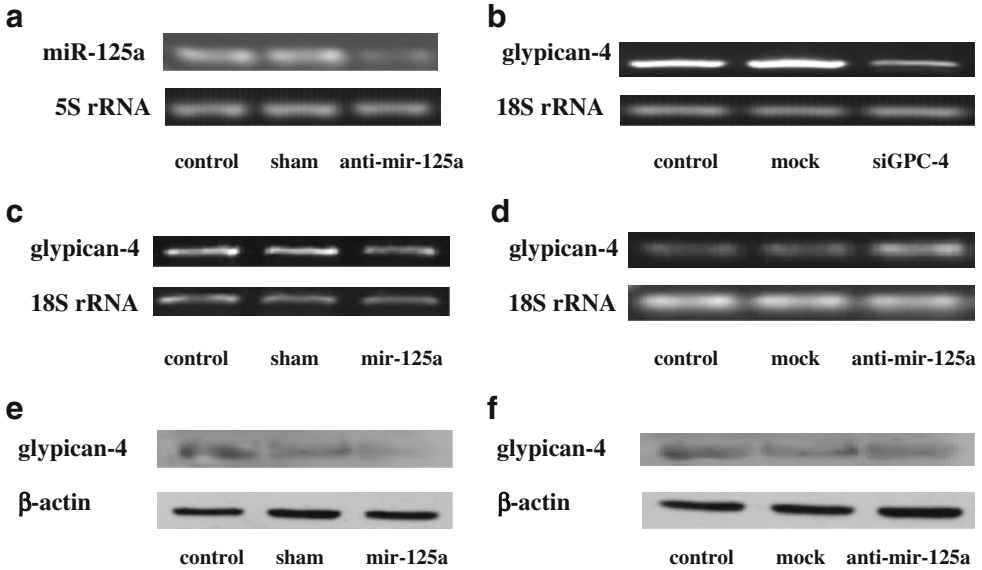

Fig. 2 MiR-125a down-regulates expression of glypican-4 while anti-miR-125a up-regulates mRNA expression of glypican-4 and recover the glypican-4 protein expression suppressed by miR-125a. Glypican-4 protein levels were quantified by Western blot in HEK 293T cells that had been transfected with either miRNA-125a or miRNA-125a specific siRNA (anti-miRNA-125a). **a** MiR-125a expression was decreased by anti-miR-125a and, respectively, 293T cells transfected without (blank) or sham or anti-miR-125a for 48 h. MiR-125a expression was measured by RT-PCR in 293T cells, 5 S rRNA serves as control. **b** HEK 293T cells were transfected without or with random RNA sequence (mock) or siRNA of glypican-4 for 48 h followed by glypican-4 mRNA expression detection suing RT-PCR. 18S rRNA was served as a control. The knockdown of glypican-4 expression in 293T cells was induced by glypican-4 gene silence. **c** and **e** MiR-125a down-regulates expression of glypican-4 at mRNA (**c**) and protein level (**e**). 293T cells were transfected with either vector control or miR-125a and then incubated for 48 h. Total RNA and protein were extracted followed by RT-PCR analysis probed with glypican-4 primers and Western blotting measurement probed with glypican-4 antibody. 18S rRNA and β-actin were used as control for RT-PCR and immunoblotting assay, respectively. **d** and **f** Anti-miR-125a up-regulates expression of glypican-4 at mRNA and protein level. The 293T cells were transfected with miR-125a for 24 h followed by anti-miR-125a or modified RNA (mock) transfection for 48 h. Glypican-4 expression analysis at mRNA (**d**) and protein level (**f**) was detected by RT-PCR and Western blotting. Data are presented as mean fold reduction±S.D. All experiments were performed in triplicate

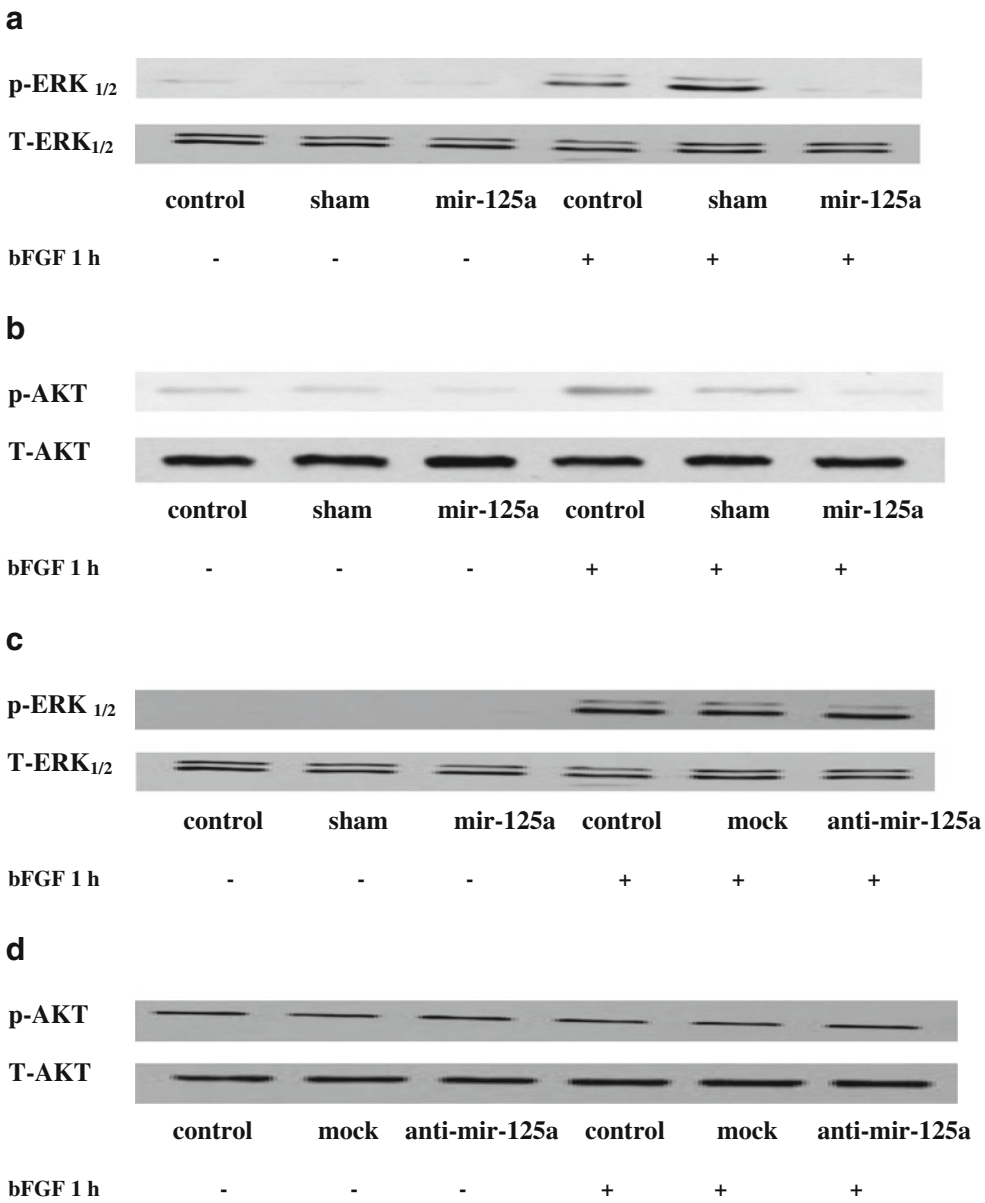

Fig. 3 ERK1/2 and AKT phosphorylation induced by bFGF miRNA-125a was blocked while their suppressed phosphorylation was recovered by anti-miRNA-125a in 293T cells. 293T cells were transfected with miRNA-125a for 48 h or followed by anti-miR-125a transfection for 48 h. After protein extract, the ERK1/2 and AKT phosphorylation expression was detected by Western blot. T-ERK1/2 and T-AKT levels were used to normalize protein input. **a** and **b** 293T cells were seeded at a density of 1×106 per well into 6 well plates and then transfect with sham (the empty vector plasmid) and miR-125a for 48 h, followed by exposure to bFGF (10 ng/ml) for 1 h. The proteins were extracted and the MAPK and AKT activation were detected by western blotting probed with p-ERK1/2 and p-AKT antibody. Mir-125a reduced the phosphorylation levels of ERK1/2 (**a**) and AKT in 293T cell (**b**). **c** and **d** 293T cells were plated at a density of 1×106 per well into 6 well plates and transfected with miR-125a for 48 h. Then the cells were transfected with sham (scrambled modified RNA) and anti-miR-125a for 48 h. The cells were then incubated in the presence of bFGF (10 ng/ml) for 1 h following protein extract. The phosphorylation levels of ERK1/2 (**c**) and AKT (**d**) were measured by western blotting

by glypican-4 expression detection. The results indicated that expression of glypican-4 at mRNA (Fig. 2c) and protein (Fig. 2e) level was significantly decreased by miRNA-125a in the cells but not in the cells that had been transfected by an empty vector. The specificity was confirmed by data that the expression of glypican-4 at mRNA (Fig. 2d) and protein level (Fig. 2f) were recovered by anti-miR-125a treatment.

MiR-125a inhibits ERK1/2 and Akt phosphorylation induced by bFGF

As a co-receptor, heparan sulfate on HSPGs binds bFGF2 and its receptor tyrosine kinase (FGFR) [21] and triggers mitogen-activated protein kinase (MAPK) signaling [22]. Since glypican-4 induces sustained ERK activation stimulated by

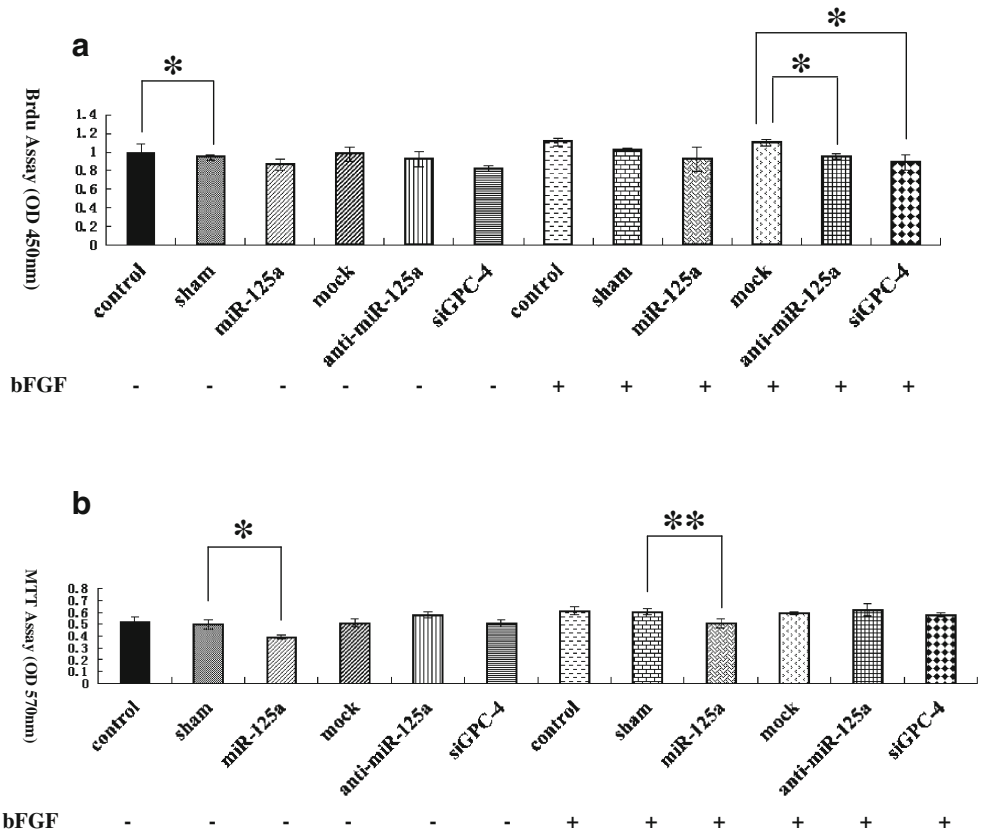

Fig. 4 DNA synthesis and cells proliferation is arrested by miRNA-125a independent of cell responses to bFGF. **a** The 293T cells were transfected with miR-125a, siRNA of glypican-4 and their controls with or without bFGF. **b** The 293T cells were transfected with miR-125a, anti-miR-125a, and siRNA of glypican-4 and their controls in the presence or absence of bFGF (10 ng/ml). Cell DNA synthesis (**a**) and proliferation (**b**) were assessed by the BrdU incorporated DNA assay and MTT method, respectively, after 293T cells were transfected and treated with or without bFGF for 24 h. 293T cells were plated at a density of 1×104 per well into 96-well plates and then transfect with miRNA-125a, siRNA of glypican-4, anti-miR-125a and their corresponding control EV, mock and sham, respectively, for 48 h. All experiments were performed in triplicate and the average and standard deviation of viable cells is shown. Data were means±SD for triplicate experiments ($p<0.01$, *t*-test)

hepatocyte growth factor[8], while glypican-4 expression is down-regulated by miR-125a. Hence, theoretically, MAPK signaling might be regulated by miR-125a. To address this hypothesis, HEK 293T cells were transfected with miR-125a followed by ERK activation detection. Indeed, ERK phosphorylation induced by bFGF was suppressed by miR-125a (Fig. 3a), while this action could be rescued by anti-miR-125a treatment (Fig. 3b).

It's reported that heparin-binding epidermal growth factor (HB-EGF) augmented phosphatidylinositol 3'-kinase/Akt (PI3K/Akt) signaling in cell proliferation[23]. This suggested that the action of glypican-4 in response to bFGF mediated by miR-125a might involve in Akt signaling pathway. HEK 293T cells were treated with miR-125a as described above followed by Akt phosphorylation detection. The results demonstrated that Akt phosphorylation was disrupted by miR-125a (Fig. 3c). However, the Akt signaling could be rescued by anti-miR-125a treatment (Fig. 3d).

MiR-125a inhibits DNA replication by targeting glypican-4

Given that miR-125a is a regulator of the phosphorylation of ERK1/2 and Akt, the cells DNA synthesis, viability and proliferation might be mediated by miR-125a. To test the consequence of suppression of ERK and Akt phosphorylation, HEK 293T cells were transfected by miR-125a for 48 h followed by BrdU incorporation DNA synthesis and MTT assay. The results revealed that BrdU incorporated DNA replication in the cells was attenuated by miR-125a (Fig. 4a). The cells DNA synthesis inhibition was indeed caused by miR-125a since the suppression could be recovered by anti-miR-125a treatment (Fig. 4a). In addition, BrdU incorporated DNA synthesis was also inhibited by siRNA of glypican-4 (Fig. 4a). Thus, based on the fact that 3′UTR of glypican-4 binds to miR-125a (Fig. 1b) and expression of glypican-4 was down-regulated by miR-125a (Fig. 2c and e), we deduced that the suppression of DNA synthesis induced by miR-125a in HEK 293T cells was through targeting glypican-4. To test whether the cells DNA synthesis inhibition could induce cells proliferation arresting, HEK 293T cells were transfected by miR-125a solely or then treated by anti-miR-125a followed by bFGF treatment and MTT assay. The results showed that cell proliferation induced with or without bFGF was impaired by miR-125a (Fig. 4b). This proliferation arresting was specifically due to the miR-125a overexpression since the cell proliferation suppression was rescued by anti-miR-125a treatment (Fig. 4b). Surprisingly, although cells' DNA synthesis arresting was induced by the silence of glypican-4 expression (Fig. 4a), this effect did not interfere cells proliferation (Fig. 4b)

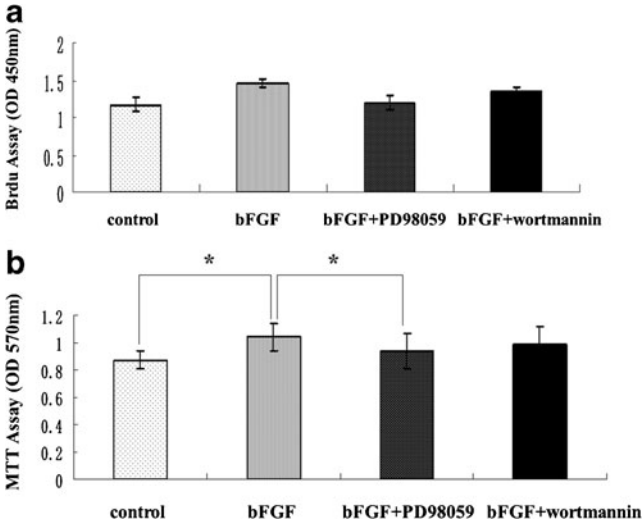

Fig. 5 BrdU incorporated DNA synthesis and cell proliferation induced by bFGF were blocked by the inhibitors (PD98059) of ERK1/2 phosphorylation. 293T cells were cultured at a density of 1×10^4 per well into 96-well plates and then treated with or without bFGF in presence of the inhibitors of ERK1/2 and AKT phosphorylation. **a** 293T cells were treated by bFGF (40 ng/ml), PD98059 (2 μM) and wortmannin (50 nM) for 24 h followed by BrdU assay using BrdU Proliferation Assay kit. **b** 293T cells were exposed to bFGF (40 ng/ml), PD98059 (2 μM) and wortmannin (50 nM) for 24 h. Then 0.5 mg/ml of MTT was added and incubated with cells for 4 h in an incubator. The optical density was measured using a spectrophotometer at an absorption wavelength of 570 nm. All experiments were performed in triplicate and the average and standard deviation of viable cells is shown. Data were means±SD for triplicate experiments (*$p<0.01$, *t*-test)

Cells proliferation induced by bFGF was disrupted by inhibitors of ERK and Akt phosphorylation

Although both ERK and Akt signaling activated by bFGF were blocked by miR-125a (Fig. 3a and b), while the cells DNA synthesis and proliferation induced by bFGF were also attenuated by miR-125a (Fig. 4a and b), whether the impairing of ERK or/and Akt signaling renders cells DNA synthesis or/and proliferation arresting in HEK 293T cells were not sure. To address this question, HEK 293T cells were treated with or without bFGF combined with the inhibitors of ERK and Akt phosphorylation, respectively. The results indicated that the inhibitor of ERK phosphorylation not only attenuated the cells DNA synthesis (Fig. 5a) but also impaired cells proliferation in response to bFGF2 (Fig. 5b). However, it seems that the inhibitor of Akt phosphorylation has no effect on HEK 293T cells DNA synthesis or proliferation (Fig. 5a and b).

Discussion

One of the most recognized functions of HSPGs is the ability to modulate different growth factor activities. Glypican-4 binds bFGF through its heparan sulfate chains [8]. Overexpression of glypican-4 renders sustained ERK phosphorylation stimulated by HGF [9]. However, little is known about how this molecule in response to bFGF is regulated. MicroRNA-125a represses cell growth by targeting HuR in breast cancer. Besides, it also down-regulates the epidermal growth factor receptor to repress mesenchymal morphology in ovarian cancer cells [17]. However, since miRNAs have multiple targets, miR-125a could also influence other target gene to play important roles in other biological function processes. These targets include bFGF-binding heparan sulfate proteoglycan, hepatocyte growth factor-mediated and HuR [8, 9, 16]. However, it is not yet clear whether miR-125a has effect on 293T cells. In this report, we demonstrated that miR-125a might not only bind 3′UTR of glypican-4 to suppress its expression in HEK 293T cells, but also render such cells insensitive to FGF2 treatment (Fig. 4a and b). The consequence of the down-regulation of glypican-4 expression was the arresting of cells DNA synthesis. Although Akt phosphorylation was blocked by miR-125a, such inactivation of the signaling pathway was not enough to stop HEK 293T cells proliferation. However, miR-125a made the cells DNA synthesis and proliferation arrested by inactivating ERK signaling. Our results suggested that DNA synthesis of HEK 293T cell was impaired by miR-125a through targeting glypican-4 to block ERK phosphorylation.

As mentioned above, a number of miRNAs have been implicated in the natural history of malignant disease and have the potential of serving as clinically useful biomarkers [24]. Since the data indicated that miR-125a down-regulated glypican-4 expression to inhibit cells DNA synthesis, we also wondered if the phenomenon had wide relevance. To address this question, miR-125a was transfected into glioma U87 cells followed by MTT assay. However, miR-125a could not inhibit U87 cell growth or DNA synthesis (data not Shown). Firstly, perhaps the reason might be due to the expression of glypican-4 in U87 cells was very lower than that in HEK 293T cells (data not shown), while glypican-4 might do not play a key role in U87 cells proliferation. Secondly, miR-125a could regulate multiple target genes among which have contradictory effects on cells proliferation or DNA synthesis. Besides, the expression patern of six members in glypican family and/or four members in syndecan family is different in HEK 293T cells and U87 cells (data not shown). Thus the compensatory effects from other HSPGs as co-receptor in response to FGF2 in these two cell lines could also be different.

Although miR-125a impaired HEK 293T cells proliferation, it seems this effect was not due to the down-regulation of glypican-4 expression, since the cells proliferation was not arrested by the silence of glyican-4 expression (Fig. 4b). This suggested that miR-125a inhibited HEK 293T cells proliferation might be by targeting other genes. In fact,

except glypican-4, other HSPGs, for example glypican-5, glypican-6, syndecan-2 and syndecan-4 also highly expressed in HEK 293T cells (Fig. 1c). These HSPGs might compensate the influence from glypican-4 silence in response to FGF2 in the MAPK signaling pathway. However, the cells DNA synthesis was indeed lessened by glypican-4 expression depression or miR-125a transfection. Thus cells DNA synthesis arrested by miR-125a could be the contribution of glypican-4 function inactivated by this miRNA, since miR-125a bound to 3′UTR of glypican-4 (Fig. 1a and b) and decreased its expression.

Hagihara reported that recombinant glypican-4 bound FGF2 through its heparan sulfate chains and suppressed the mitogenic effect of FGF2 on E13 rat cortical precursor cells [8]. In this case, glypican-4 functions as suppressor gene in cells proliferation. However, we found that DNA synthesis induced by FGF2 was reduced by the silence of glypican-4 expression in HEK 293T cells (Fig. 4a). Although the silence of glypican-4 gene expression had no effect on HEK 293T cells proliferation (Fig. 4b), the role of glypican-4 in response to FGF2 treatment was contradictory to that in rat cortical precursor cells. This suggested that glypican-4 might play different roles in different cells.

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