miR-27a regulates the self renewal of the H446 small cell lung cancer cell line *in vitro*

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Abstract. Cancer growth is driven by cancer stem-like cells within a tumor, called cancer stem cells (CSCs). Since miRNAs can regulate cell-fate decisions, we compared miRNA expression in stem-like cells and differentiated cells from small cell lung cancer (SCLC) cell lines to develop further understanding of the molecular mechanisms involved in the pathogenesis of SCLC. First, SCLC stem-like cells were enriched by isolating sphere-forming cells using a defined serum-free medium. Further, microRNA microarrays were used to measure the expression of 1212 miRNAs in sphere-forming cells and parental cells. We found 86 miRNAs that were differentially expressed, including 48 upregulated miRNAs and 38 downregulated miRNAs between sphere-forming cells and parental cells. Among them, five downregulated miRNAs (let-7, miR-20, 21, 27a and 30b) and one upregulated miRNA (miR-149*) were selected for validation in 3 sets of SCLC cell lines by qRT-RCR. The qRT-PCR analysis confirmed that all six miRNAs were indeed differentially expressed. However, only miR-27a was consistently downregulated in sphere-forming cells of all 3 cell lines. Antagonizing miR-27a by inhibitor in parental cells enhanced proliferation, self renewal, and the proportion of undifferentiated cells *in vitro*. The candidate miRNA and some miRNAs with same seed sequence are predicted to have several target genes related to apoptosis, cell proliferation and cell cycle. Our results suggest that downregulation of miR-27a enhanced the stem-like properties of SCLC cells *in vitro* and may be critical to maintaining a stem cell function in SCLC.

Introduction

Small cell lung cancer (SCLC) is an aggressive malignancy representing ~13% of 220,000 new lung cancer cases projected for 2009 (1-3). Despite moderate progress achieved in the past two decades, the survival rate of SCLC patients is still very poor (3,4). The poor prognosis of SCLC patients is due to its high metastatic potential and chemoresistance upon relapse. Cancer stem cells (CSCs) are thought to be responsible for tumor initiation, therapy resistance, progression, relapse, and metastasis. Therefore, targeting of CSCs may be one of approaches to eradicate cancerous tumors early. However, lack of understanding the regulatory mechanisms underlying CSCs of SCLC is an obstacle in the progress of targeting of CSCs to improve SCLC therapy.

microRNAs (miRNAs) are small noncoding regulatory RNAs that regulate the translation of mRNAs by inhibiting ribosome function, decapping the 5'Cap structure, deadenylating the poly(A) tail, and degrading the target mRNA (5). They can regulate a variety of cell functions, including stem cell maintenance and differentiation, and play roles in controlling cancer initiation and progression (6,7). Previous studies have shown an aberrant miRNA expression in a various types of cancer stem cells, and changes in specific miRNAs have been associated with stem cell self-renewal and differentiation (6,8,9). For example, the expression of let-7 reduces tumor sphere formation in breast cancer cell lines and inhibits tumorigenicity in an *in vivo* xenograft tumor assay (8). miR-130b promotes $CD133^+$ liver tumor-initiating cell growth and self-renewal (9). The downregulation of miR-200 family miRNAs suggests that breast cancer stem cells and normal stem cells share common molecular mechanisms that regulate stem cell functions, such as self-renewal, proliferation, and the epithelial-mesenchymal transition (6). These findings demonstrate that miRNAs are critical regulators of self-renewal and differentiation.

Currently, miRNA dysregulation in many CSCs, including breast (6,8), prostate (10), non-small cell lung cancer (11), liver (9), pancreas (12), and glioblastoma (13) stem cells have been identified. However, the dysregulation of miRNAs in CSCs of SCLC remains unclear. Understanding the miRNA expression of CSCs may provide insight into the origin of and new therapeutics for SCLC. Therefore, we looked at whether differences in miRNA expression might distinguish CSCs from their more differentiated progeny.

Previous work from our laboratory showed that tumor spheres from SCLC cell lines were characterized by stem-like properties, and stem-like cells were enriched after consecutively passaging in a defined serum-free medium. Furthermore, the

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stem-like cell population may be enriched in cells expressing urokinase plasminogen activator receptor (uPAR) cell surface marker (14). In this study, we undertook a systematic comparison of the miRNAs in SCLC stem/progenitor cell populations and their differentiated progeny. Our results showed that SCLC stem/progenitor cell populations expressed a limited set of miRNAs compared to differentiated cells. Moreover, miR-27a was consistently downregulated in stem/progenitor cells of all 3 SCLC cell lines. By antagonizing miR-27a in parental cells, we found that miR-27a regulates the key feature of SCLC stem cells - self renewal *in vitro*. Therefore, lack of miR-27a may be critical to maintaining a stem cell function in SCLC.

Materials and methods

Cell lines and cell culture. SCLC cell lines H446, H209, and H69 were obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in humidified air at 37˚C with 5% CO_2. The procedure for obtaining tumor sphere cell culture has been described in our recent study (14).

microRNA microarray. Microarray assay was performed using a service provider (LC Sciences). The assay started from 5 μg total RNA, which was size-fractionated using a YM-100 Microcon centrifugal filter (from Millipore). The small RNAs (<300 nt) were isolated and labeled with Cy5 fluorescent dyes. Hybridization was performed overnight on a μParaflo microfluidic chip containing 1212 mature human microRNA (miRNA) probes (Sanger miRBase, release 16.0). Each detection probe consisted of a chemically modified nucleotide coding segment complementary to target miRNA (from miRBase, http://microrna.sanger.ac.uk/sequences/). After hybridization detection used fluorescence labeling using tag-specific Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Raw data were normalized the signals using a LOESS filter (15) (locally-weighted regression) after first subtracting the background. A Student's t-test was performed to analyze the statistical significance of the signal differences between the fourth passage sphere cells and parental cells, and differentially detected signals were those with P<0.01.

qRT-PCR analysis of miRNAs expression. The total RNA (2 μg) extracted separately from cell samples was used to generate cDNA by using SuperScript II reverse transcriptase (Invitrogen) with special stem-loop primer for miRNA. The primers for the analysis of miRNAs expression were designed according to Chen *et al* (16). Human small nuclear U6 RNA was amplified as an internal control. Real-time qPCR was performed on a Bio-Rad iQ5 real-time PCR detection system using SYBR® Premix Ex Taq™ (Takara, RR041A). The PCR reaction contained 1 μl RT product, 10 μl 2X SYBR Premix Ex Taq, 1 μl forward primer and 1 μl reverse primer (5 μmol/l each), and nuclease-free water in a final volume of 20 μl. Standard PCR samples were analyzed with a Bio-Rad iQ5 thermal cycler. Melting curves were generated for each real-time RT-PCR

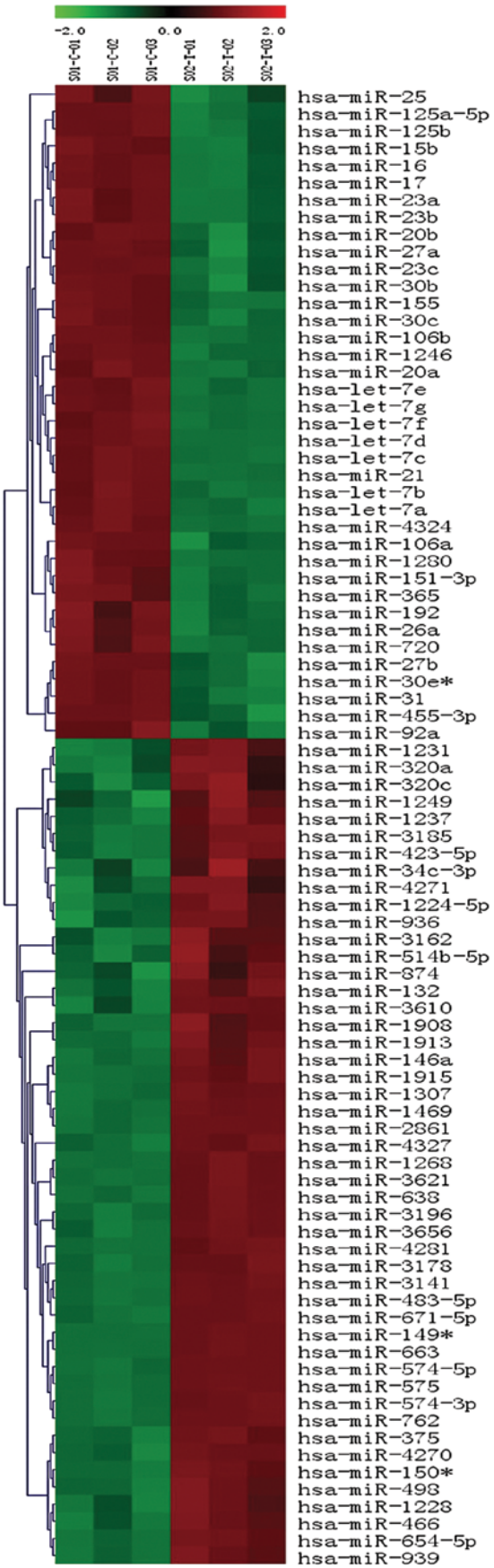

Figure 1. Cluster analysis of miRNA differentially expressed in the fourth passage sphere cells and parental cells. Green represents decreased expression and red represents increased expression. Top row: cell line samples. S01 and S02 stand for parental cells and the fourth passage sphere cells. Right column: miRNAs that were significantly upregulated or downregulated (P<0.01) in the fourth passage sphere cells relative to parental cells.

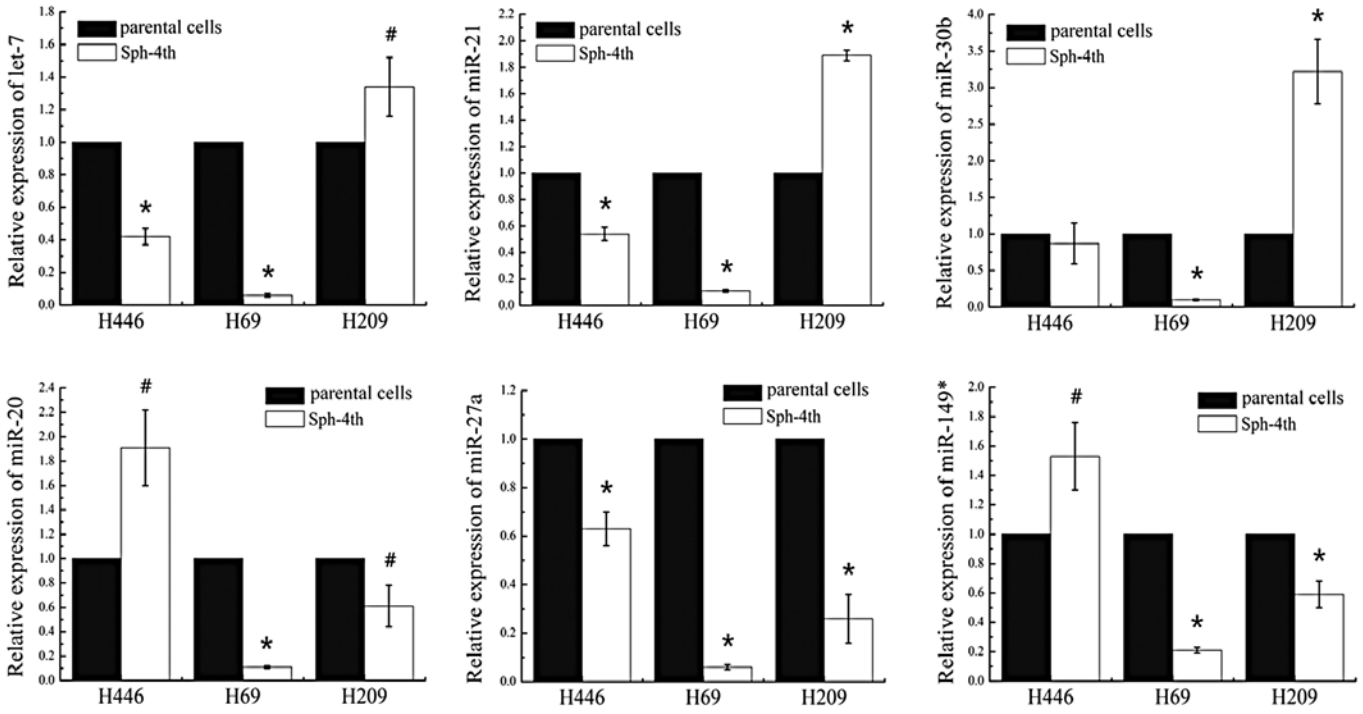

Figure 2. Validation of microarray analysis data by qRT-PCR. The expression levels of six selected miRNAs were determined in the fourth passage sphere cells (Sph-4th) and parental cells from H446, H69 and H209 cell lines by qRT-PCR. Each qRT-PCR assay was performed at least three times, #P<0.05, *P<0.01.

to verify the specificity of each PCR reaction. All quantitative PCR reactions were performed in triplicate. Expression levels of each miRNA were evaluated using the comparative threshold cycle (Ct) method as normalized to that of U6 ($2^{-\Delta Ct}$). Relative fold changes were calculated by the equation $2^{-\Delta\Delta Ct}$.

Cell transfection. Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen) following the manufacturer's protocol. Briefly, cells were trypsinized, counted and seeded in plates the day before transfection to ensure a suitable cell confluence on the day of transfection. miR-27a inhibitor or scrambled oligonucleotides as the negative control (GenePharma, Shanghai, China) were transfected into H446 cells at a final concentration of 40 nM in antibiotic free Opti-MEM medium (Invitrogen). Transfection efficiency was monitored by FAM-oligonucleotides.

MTT assay. H446 cells were seeded in 96-well plate at 3000 cells per well in 100 μl cell culture medium and incubated at 37˚C for 24 h. Then, they were transfected with miR-27a inhibitor or scrambled oligonucleotides performed as mentioned above. After incubation for 72 h, the cells were incubated with 10 μl MTT (at a final concentration of 0.5 mg/ml) at 37˚C for 4 h. The medium was removed and the precipitated formazan was dissolved in 100 μl DMSO. After shaking for 10 min, the absorbance at 570 nm was detected using TECAN GENios Pro (BioTek Instruments). This procedure was repeated at 24, 48 and 72 h after transfection.

Tumor sphere formation assay and colony formation assay. After transfection with 40 nM of miR-27a inhibitor, 40 nM of scrambled oligonucleotides, cells were trypsinized, counted, and seeded for tumor sphere formation assay and colony formation assay in 24-well ultra low attachment plates at 500 cells per well. The tumor sphere culture performed as mentioned above. The total number of tumor spheres was counted after 5-14 days of culture. During colony growth, the culture medium was replaced every 3 days. The colony was counted only if it contained >50 cells, and the number of colonies was counted the 5th day after seeding. Each experiment was carried out in triplicate.

Immunofluorescence. For immunofluorescence studies, H446 cells were treated with miR-27a inhibitor or scrambled oligonucleotides for 72 h in 96-well plate and then fixed with 4% paraformaldehyde for 15 min at 37˚C, permeabilized with 0.1% Triton X-100/PBS for 15 min at room temperature and then incubated with the following monoclonal antibodies: uPAR (American Diagnostica), pan-cytokeratin (CK, Zymed). FITC-conjugated rabbit anti-mouse IgG (Dako) was used as the secondary antibody. Cell nuclei were counterstained with DAPI. Images were recorded on a Nikon Eclipse Ti.

Statistical analysis. All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed with Student's t-test and P<0.05 was considered statistically significant.

Results

miRNA expression profiles of sphere-forming cells and parental cells. Previous work from our laboratory showed that tumor spheres from SCLC cell lines were characterized by stem-like properties, and stem-like cells were enriched after consecutively

Table I. Significantly differentially expressed miRNAs in the fourth passage sphere cells relative to parental cells (P<0.01, fold changes ≥4).

Downregulated miRNAs			Upregulated miRNAs		
miRNA name	Fold changes	Location	miRNA name	Fold changes	Location
hsa-miR-125a-5p	4.2	19q13.41	hsa-miR-3185	8.49	17
hsa-miR-125b	6.88	11q24.1	hsa-miR-146a	7.07	5q34
		21q21.1	hsa-miR-1915	12.23	10
hsa-miR-15b	5.43	3q25.33	hsa-miR-1469	76.24	15q26.2
hsa-miR-16	8.85	13q14.2	hsa-miR-2861	54.21	9
		3q25.33	hsa-miR-3621	31.91	9
hsa-miR-23b	4.39	9q22.32	hsa-miR-4281	20.16	5
hsa-miR-20b	28.77	Xq26.2	hsa-miR-3656	4.09	11
hsa-miR-27a	12.48	19p13.13	hsa-miR-3178	17.28	16
hsa-miR-23c	14.08	X	hsa-miR-3141	41.71	5
hsa-miR-30b	25.21	8q24.22	hsa-miR-483-5p	15.11	11p15.5
hsa-miR-30c	12.5	1p34.2	hsa-miR-149*	112.56	2q37.3
hsa-miR-1246	6.86	2q31.1	hsa-miR-663	31.53	20p11.1
hsa-miR-20a	4.77	13q31.3	hsa-miR-574-5p	16.78	4
hsa-let-7g	7.17	3p21.1	hsa-miR-575	531.92	4q21.22
hsa-let-7f	8.96	9q22.32	hsa-miR-574-3p	13.31	4
		Xp11.22	hsa-miR-762	28.68	16
hsa-let-7d	6.49	9q22.32	hsa-miR-763[a]	28.68	-
hsa-let-7c	6.36	21q21.1			
hsa-miR-21	8.97	17q23.1			
hsa-let-7a	7.1	9q22.32			
		11q24.1			
		22q13.31			
hsa-miR-1280	6.04	3			
hsa-miR-27b	10.29	9q22.32			

[a]The corresponding chromosome location was not found in http://www.genenames.org.

passaging (14). We further studied the underlying mechanism that regulates these cells. As miRNAs are critical regulators of self-renewal and differentiation in both normal and cancer stem cells, we compared the miRNA expression profiles of the fourth passage sphere cells and parental cells from H446 using miRNA array containing 1212 mature miRNAs. In the array 86 miRNAs were found to be differentially expressed (P<0.01), including 48 upregulated miRNAs and 38 downregulated miRNAs in the fourth passage sphere cells (Fig. 1). Among 86 differentially expressed miRNAs, 18 of the 48 upregulated miRNAs and 20 of the 38 downregulated miRNAs (Table I) showed at least a 4-fold changes in the fourth passage sphere cells relative to parental cells. Of the upregulated miRNAs, several miRNAs were not previously implicated in lung cancer, such as miR-1469, miR-2861, miR-3141, miR-3621, miR-663, miR-762, miR-763 and miR-4281.

Validation of miRNA expression profiles with qRT-PCR. To confirm the miRNA microarray data and filter out the common miRNAs, 6 tumor-related miRNAs with same seed sequence and significant fold changes (TableI; downregulated: let-7, miR-20, 21, 27a and 30b; upregulated: miR-149*) were selected for validation in 3 sets of human SCLC fourth passage sphere cells and parental cells by qRT-PCR. For each sample, the expression values were normalized to the U6 gene. As Fig. 2 shown, qRT-PCR analysis confirmed that these 6 miRNAs were indeed differentially expressed. The trend of expression change of five miRNAs (let-7, miR-20, 21, 30b and 149*) were aberrant in the fourth passage sphere cells of 3 cell lines. let-7, miR-21 and miR-30b were downregulated in H446 and H69, but upregulated in H209. Similarly, miR-149* and miR-20 were downregulated in H69 and H209, but upregulated in H446. However, miR-27a was consistently downregulated in the fourth passage sphere cells of all 3 cell lines (Fig. 2). Therefore, we focused on miR-27a in the following studies.

Reduced miR-27a increases tumor sphere formation and colony formation. To evaluate miR-27a function, we used a 'loss-of-function' approach by transfecting H446 with a specific inhibitor

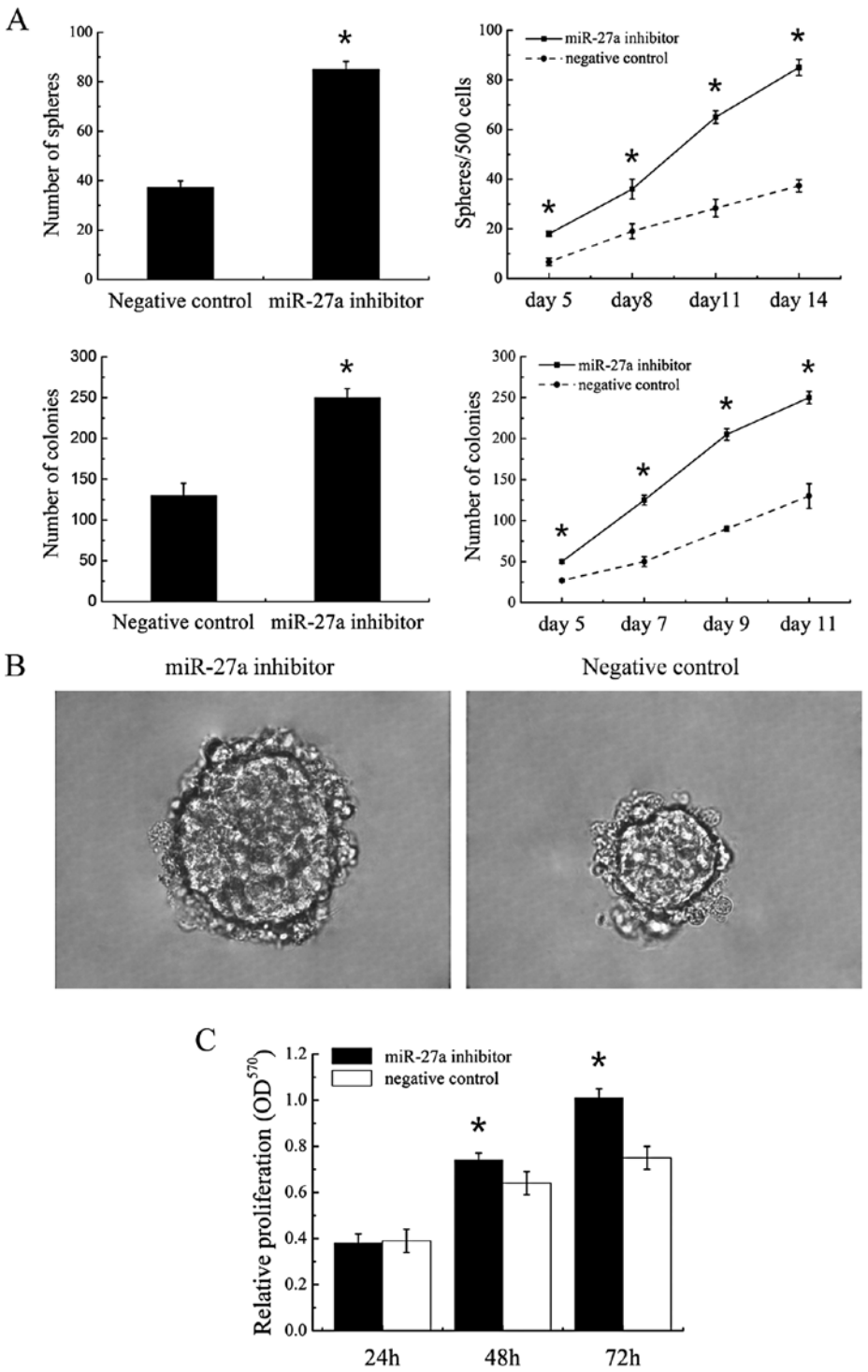

Figure 3. The role of miR-27a in regulating self renewal and proliferation in H446 cells is shown. (A) The effect of miR-27a on self renewal was evaluated by tumor sphere formation assay (upper panels) and colony formation assay (lower panels). The column graphs show the amount of spheres (upper panel, on day 14) and colonies (lower panel, on day 11) formed from 500 cells. The line graphs show the amount of spheres (upper panel) and colonies (lower panel) on the indicated day. *P<0.05. Data are presented as the mean ± SD of three independent experiments. (B) Phase-contrast micrographs of spheres generated from cells transfected with miR-27 inhibitor and negative control. (C) The cell vitality of transfected with miR-27a inhibitor or negative control was measured using MTT assay at 24, 48 and 72 h. *P<0.05. Values are the means ± SD of five duplications.

to miR-27a. Tumor sphere formation assay and colony formation assay was performed to investigate the effect of miR-27a on self renewal. The cells transfected with miR-27a inhibitor formed significantly more tumor spheres and more clones than those transfected with negative control (Fig. 3A). Meanwhile, the tumor spheres that formed were larger in transfected with miR-27a-inhibitor cells than negative control cells (Fig. 3B). Therefore miR-27a inhibitor enhanced self-renewal capacity of parental cells *in vitro*.

Reduced miR-27a increases cell proliferation but inhibits differentiation. The effect of miR-27a on the proliferation and

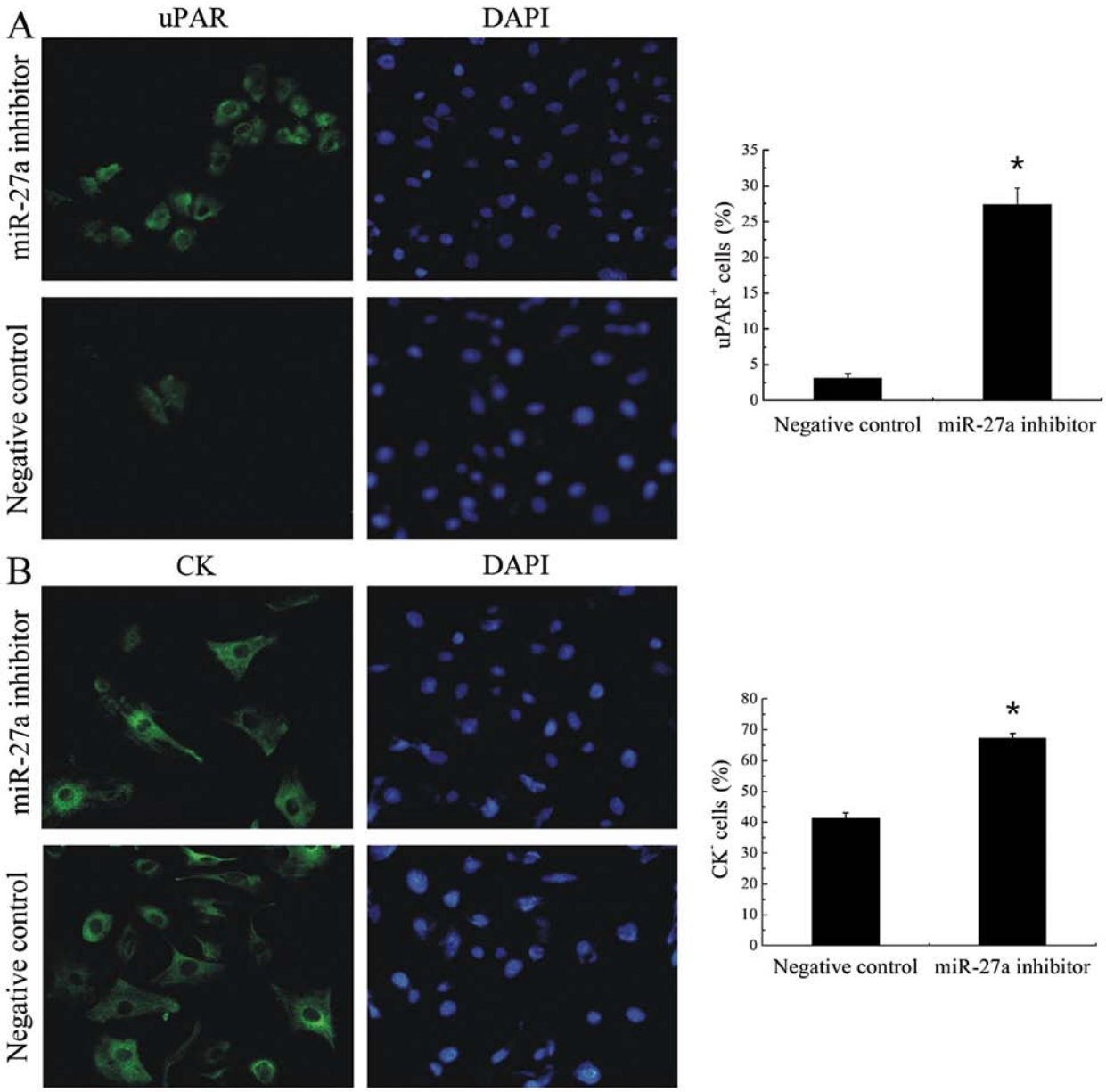

Figure 4. Immunofluorescence staining for uPAR (A) and CK (B) in transfected with miR-27a inhibitor or negative control cells. Green color represents expression of uPAR or CK and blue color is the nuclear counter-stain. The summarized graphs (right panels) show enrichment of $uPAR^+$ (upper panel) and CK^- (lower panel) cells in cells transfected with miR-27a inhibitor compared with negative control cells. Magnification, x100. *P<0.001. Data are presented as the mean ± SD of three independent experiments.

differentiation was investigated in cell line. At different time points post-transfection, the vitality of cells was tested by the MTT assay. The cells transfected with miR-27a inhibitor showed enhanced proliferation at 48 and 72 h compared with cells transfected with negative control (Fig. 3C). Another hallmarker of stem-like cells is their undifferentiated state. Previous work from our laboratory showed that stem-like cell population may be enriched in the $uPAR^+$ fraction of tumor cells in the H446 cell line. To test the effect of miR-27a on the differentiation, we further detected the expression of uPAR and cell differentiation marker CK in cell line. miR-27a inhibitor significantly (P<0.001) increased the proportion of $uPAR^+$ cells from 3.13±0.6% to 27.37±2.32% (Fig. 4A) and the proportion of CK^- cells from 41.33±1.72% to 67.17±1.66% (Fig. 4B) compared with negative control inhibitor in H446 cells. Therefore, low miR-27a enhanced the proliferative potential and increased the population of undifferentiated cells.

Prediction of miRNA targets. miRNAs participate in various physiological and pathological processes by regulating the expression of their target genes. Identification of miRNAs target genes is very important to understand the mechanisms involved in the malignant biological behavior of SCLC. So the candidate miRNA and some miRNAs with same seed sequence were picked for target prediction and analyzed with TargetScan 5.1 (http://www.targetscan.org/). By analysis of the database, human genes which were known to be involved in cell proliferation, apoptosis and cell cycle were selected from the TargetScan 5.1. Then, those genes predicted to be targets of the miRNAs were chosen and listed in Table II. All data might

Table II. Target prediction of miRNAs.

miRNA name	Putative targets of miRNAs and their functions	
	Apoptosis and proliferation	Cell cycle
miR-27a	BCLAF1, BAK1	CCNG1
miR-27b	BAK1	CCNG1
let-7g[a]	DAPK	-
let-7f[a]	-	CCND2
let-7d	TP53, FASLG, TNFSF9, BCL2L11, TP53INP1	CCNF, E2F2, TP53
miR-30b	BCLAF1, KIPK5, CASP3, TANK	CCNE2, CCNK, TFDP1, E2F7
miR-30c[a]	-	CCNT2
miR-23a	APAF1, SATB1, FAS, BNIP2, CASP7	CCNH, CCND1, RBL2
miR-23b[a]	-	CCNT2

[a]The corresponding chromosome location was not found in http://www.targetscan.org/.

provide the foundation for further analysis of mechanisms of self-renewal and tumor growth in SCLC.

Discussion

There is growing evidence that cancers are initiated and maintained by cancer stem cells. miRNAs regulate both normal stem cells and CSCs (6,8,17-19), and alterations in the expression of miRNA genes contribute to the pathogenesis of most human malignancies (10,11,20). miRNA dysregulation in many CSCs has been identified, but how miRNAs regulate CSCs in SCLC remains unclear. In this study, we obtained a large number of self-renewing cells to study changes in miRNA expression compared to parental cells. The microarray data showed that 86 miRNAs were differentially expressed in the fourth passage sphere cells versus parental cells of H446 cell line. The upregulation and downregulation miRNAs in sphere-forming cells suggest a role for small RNA molecules in the maintenance of stem cell properties in SCLC cells. Among them, we selected 6 tumor-related miRNAs for validation and further screened the miRNAs of steady expression trend in 3 SCLC cell lines by qRT-PCR. However, only miR-27a was consistently downregulated in the fourth passage sphere cells of all 3 cell lines and others were downregulated in two cell lines.

Furthermore, we used the 'loss-of-funtion' approach to study the role of miR-27a in the H446 SCLC cell line. Our results showed that antagonization of miR-27a in parental cells enhanced proliferation, self renewal, and increased the proportion of undifferentiated cells. Downregulation of miR-27a enhanced the stem-like properties of SCLC cells *in vitro*, and converted less malignant cells into highly malignant cells. These results suggest that reduced miR-27a may be critical to maintaining a stem cell function in SCLC. This statement is also supported by previous studies. For example, miR-27a plays important roles in normal stem cells and poorly differentiated cells. In the hematopoietic cells, reduced miR-27a can antagonize the colony-stimulating factor-mediated granulocyte differentiation (21). In muscle cells, miR-27 can modify muscle stem cell behavior by regulating the Pax3, which downregulation ensures rapid and robust entry into the myogenic differentiation program (22). miR-27a was also reported to be downregulated in SP cells of human lung adenocarcinoma A-549 cell (11). Therefore, we speculate that correction of miR-27a expression represents a good candidate strategy for targeted therapy.

Among the other five miRNAs, let-7 and miR-21 are well studied in other cancers. In breast cancer, there are absent expression of let-7 in cancer stem cells. Moreover, lack of let-7 is required for self-renewal *in vitro* and tumorigenicity *in vivo* (8). This family is also downregulated in SP cells of human lung adenocarcinoma A-549 cell(11) and associated with poor lung cancer prognosis (23). However, in our study, let-7 shows different expression patterns in SCLC cell lines, which is downregulated in the fourth passage sphere cells of H446 and H69 cell lines, yet upregulated in H209 cell line. These results indicate that the expression level of let-7 is different in different classes of cancer, and the underlying association mechanisms also might be different.

miR-21 was reported by many studies and was overexpressed in a wide variety of cancers, such as breast (24,25), colorectal (26), gastric (27), and non-small cell lung cancer (28), which causally linked to poor prognosis, invasion and metastases (25,26,28-32). But in mouse embryonic stem cells, miR-21 suppressed the self-renewal (33) and increased dramatically upon differentiation (34). In our study, miR-21 was downregulated in tumor sphere cells of two cell lines. The same miRNA exhibiting diverse expression and functions in different cellular contexts, may depend on the specific microenvironment and the combination of its direct target genes, but the specific function of miRNAs need to be further verified through function studies.

In summary, these results established global expression profiles for SCLC stem/progenitor cells and differentiated cells, which provide new knowledge on gene regulation during the SCLC developmental progress, and are valuable in further studies of SCLC tumorigenic mechanism. Moreover, reduced miR-27a is a potential intrinsic property of SCLC stem/progenitor cells. Downregulation of miR-27a enhanced the cell

stem-like properties of SCLC cells *in vitro* and may be critical in maintaining the stem cell function in SCLC.

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