THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Differentially expressed microRNAs and the antiproliferative role of miR-126 in small cell lung cancer

by

Edit Miko

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

DEBRECEN, 2011

ABBREVIATIONS

AC	- adenocarcinoma
Ago	- argonaute protein
Akt	- protein kinase B
ALL	- acute lymphoblastic leukemia
AML	- acute myeloid leukemia
ARF-BP1	- ADP-ribosylation factor binding protein 1
ASH1/GFI1	- ash1 (absent, small, or homeotic)-like (Drosophila)/ growth factor independent 1 transcription repressor
BCL-2	- B-cell CLL/lymphoma 2
BIC	- B-cell integration cluster
BRCA1	- breast cancer 1, early onset
CAR	- cancer-associated retinopathy
CAT-1	- calcium transport protein 1
CCND2	- cyclin D2
CDC25	- cell division cycle 25
CDK	- cyclin-dependent kinase
CDKI	- cyclin-dependent kinase inhibitor
CDS	- coding sequence
CF	- cystic fibrosis
C-KIT	- v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CLL	- chronic lymphocytic leukemia
C-MET	- met proto-oncogene (hepatocyte growth factor receptor)
COPD	- chronic obstructive pulmonary disease
CRK	- v-crk sarcoma virus CT10 oncogene homolog

CT	- computed tomography
CTGF	- connective tissue growth factor
CXCL12	- chemokine (C-X-C motif) ligand 12
CXCR4	- chemokine (C-X-C motif) receptor 4
CXR	- chest radiography
DLBCL	- diffuse large B cell lymphoma
DLEU2	- deleted in lymphocytic leukemia 2
DND1	- dead end 1 protein
dsRNA	- double stranded RNA
E2F1	- E2F transcription factor 1
ECM	- extracellular matrix
EGFR	- epidermal growth factor receptor
EGFR-TKI	- EGFR-tyrosine kinase inhibitor
eIF4E	- eukaryotic translation initiation factor 4E
EMT	- epithelial-mesenchymal transition
ER	- estrogen receptor
ERK	- extracellular signal-regulated kinase
ERF	- Ets2 repressor factor
EtBr	- ethidium bromide
ETS2	- v-ets erythroblastosis virus E26 oncogene homolog 2
FBXO33	- F-box protein 33
FDG-PET	- F-18-fluorodeoxyglucose /Positron emission tomography
FFPE	- formalin fixed, paraffin embedded
FHIT	- fragile histidine triad gene
FITC	- fluorescein isothiocyanate
FOXO3	- forkhead box O3

FXR1 - fragile X mental retardation, autosomal homolog 1

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GPCR - G protein-coupled receptor

GRP - gastrin-releasing peptide

H19 - H19, imprinted maternally expressed transcript

HBEC - human bronchial epithelial cell

HDAC4 - histone deacetylase 4

HE - hematoxylin-eosin

HGNT - high-grade neuroendocrine tumor

HIF-1α - hypoxia-inducible factor-1α

HPRT1 - hypoxanthine phosphoribosyltransferase 1

HRP - horse-radish peroxidase

HuR - ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)

HUVEC - human umbilical vein endothelial cells

IHC - immunohistochemistry

INK4 - cyclin-dependent kinase inhibitor 2A

IP - immunoprecipitation

IRS - insulin receptor substrate

JNK - c-jun N-terminal kinase

LCNEC - large cell neuroendocrine cancer

LDSCT - low-dose spiral computed tomography

LEMS - Lambert-Eaton myasthenic syndrome

LNA - locked nucleic acid

LOH - loss of heterozygosity

Maspin - serpin peptidase inhibitor

MDR	- multidrug resistance
MEST	- mesoderm specific transcript
miRISC	- miRNA-induced silencing complex
miRNA	- microRNA
MMP	- matrix metalloproteinase
MRP-1	- MDR-associated protein
mTOR	- mammalian target of rapamycin
MTPN	- myotrophin
MYC	- v-myc myelocytomatosis viral oncogene homolog
MUC1	- mucin 1, cell surface associated
Ncam1	- neural cell adhesion molecule
NSCLC	- non small cell lung cancer
NSE	- neuron-specific enolase
NUDT1	- nudix (nucleoside diphosphate linked moiety X)-type motif 1
P bodies	- proccessing bodies
p16INK4	- cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
PAK1	- p21/CDC42/RAC1-activated kinase 1
Pax	- paired box transcription factor
PBMC	- peripherial blood mononuclear cells
PBS	- phosphate buffered saline
PDCD4	- programmed cell death 4
PEM/SN	- paraneoplastic encephalomyelitis/sensory neuronopathy
PGP	- P-glycoprotein
PI3K	- phosphatidylinositol 3-kinase
PIK3R2	- phosphoinositide-3-kinase, regulatory subunit 2 (beta)
piRNA	- PIWI interacting RNA

PKC	- protein kinase C
PLC	- phospholipase C
PLK2	- polo-like kinase 2
PNS	- paraneoplastic neurologic syndrome
PP1A	- cyclophilin A
PPARgamma	- peroxisome proliferator-activated receptor gamma
pri-miRNA	- primary microRNA
ProGRP	- progastrin-releasing peptide
PTEN	- phosphatase and tensin homolog
PTPN9	- protein tyrosine phosphatase, non-receptor type 9
qPCR	- real time quantitative polymerase chain reaction
qRT-PCR	- real-time quantitative reverse transcriptase–polymerase chain reaction
RAB14	- RAB14, member RAS oncogene family
RAS	- v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
rasiRNA	- repeat-associated RNAs
RB1	- retinoblastoma 1
RBP	- RNA binding protein
RGS3	- regulator of G-protein signaling 3
RIP-ChIP	- immunoprecipitation of RNA-binding protein
RISC	- RNA-induced silencing complex
RNAi	- RNA interference
ROC	- receptor-operated non-selective cation channels
RT	- reverse transcription
S6K	- ribosomal protein S6 kinase, 70kDa, polypeptide
SCC	- squamous cell lung cancer
SCF	- stem cell factor

SCLC	- small cell lung cancer
SDF1α	- stromal cell-derived factor 1
SDS	- sodium dodecyl sulphate
siRNA	- small interfering RNA
SLC7A5	- solute carrier family 7, member 5
SNAI	- snail homolog
SOX	- SRY (sex determining region Y)-box
SPRED1	- sprouty-related, EVH1 domain containing 1
SRF	- serum response factor
tasiRNA	- trans-acting siRNAs
TBP	- TATA box binding protein
TIMP3	- TIMP metallopeptidase inhibitor 3
TLX	- T-cell leukemia homeobox
TNF	- tumor necrosis factor
TPM1	- tropomyosin 1
TP53	- tumor protein p53
TRAIL	- TNF-related apoptosis-inducing ligand
TSP1	- thrombospondin-1
TWIST1	- twist homolog 1 (Drosophila)
TTF1	- thyroid-transcription factor-1
UTR	- untranslated region
VEGF	- vascular endothelial growth factor
VGCC	- voltage-gated calcium channel
ZEB	- zinc finger E-box binding homeobox

CONTENTS

1. INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality in the world. Neuroendocrine tumors of the lung can be divided into 4 subtypes: typical and atypical carcinoid tumors, large cell neuroendocrine cancers (LCNECs), and small cell lung cancer (SCLC). The 4 subtypes can be differentiated by morphology, immunohistochemistry, and specific patterns of chromosomal aberrations [1–4]. Global gene expression profiling identifies only 2 prognostically different subtypes: carcinoid tumors and high-grade neuroendocrine tumors (HGNTs), the latter including SCLC and LCNEC [5, 6]. SCLC accounts for close 20 % of all lung cancer and is characterized by its rapid progression and frequent metastasis. Its proliferation is fuelled in part by autocrine/paracrine growth stimulatory neuropeptides and by several aberrantly activated pro-proliferative signalling pathways. The predominant risk factor for SCLC is cigarette smoking with smokers facing a 20–30-fold higher incidence of developing SCLC than non-smokers. SCLC is sensitive to initial chemotherapy, but most patients relapse rapidly and become resistant to any therapy. Even with combination chemotherapy and radiotherapy treatments, the 5-year survival rate is only about 5% [7, 8]. Treatment is complicated by the fact that SCLC is almost always diagnosed as a late-stage disease.

Improving the survival rate of patients with SCLC requires a better understanding of tumor biology and development of novel therapeutic and diagnostic strategies. Molecular abnormalities in SCLC have been used as targets for new therapies, such as those that focus on cell signalling and other pathways involved in tumorigenesis. Clinical research in this field is still in progress and several targeted agents, such as mTOR (mammalian target of rapamycin) inhibitors and antiangiogenic agents, offer a promise of improved outcomes. However, despite intensive research efforts and several clinical trials, none of the new experimental therapeutics have resulted in significantly improved survival of SCLC patients. Therefore, novel approaches to decipher SCLC biology are critical.

A rapidly developing field of cancer research is the role of microRNAs (miRNAs) in neoplastic transformation, and their potential to interfere with cancer progression. MiRNAs are small, 20-25 nucleotide long non-coding RNAs. MiRNAs can regulate gene expression by binding to the 3' untranslated region (3' UTR) of target mRNAs, inducing their degradation or translation repression [9]. Approximately one-third of protein-coding genes in humans are

regulated by miRNAs and a single miRNA may regulate over a hundred target mRNAs [10]. They are involved in the control of various biological processes, such as normal development, cell proliferation, apoptosis and differentiation. Several miRNA genes are located in chromosomal regions with frequent amplification or deletion in cancers, resulting in deregulated expression of miRNAs [11]. Tumor suppressor genes and oncogenes with a role in lung tumorigenesis have been demonstrated to be targets of miRNAs and manipulation of miRNA levels has been used to control lung cancer cell survival and proliferation [12,13]. In addition, since miRNA expression profiles are well suited to differentiate between cancer subtypes, overexpressed miRNAs may be utilized in the future both as early diagnostic tools and for differential diagnostics.

The purpose of our first study was to characterize the miRNA expression profile of primary SCLC tumors. We compared the expression of 319 miRNAs in 3 SCLC cell lines and in normal lung tissue by microarray. Next, 62 miRNAs expressed differentially were selected for validation with real-time quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR), and their expression levels were compared between SCLC cell lines, normal lung tissue, and a small panel of primary SCLC tumors. Lastly, the expression of 15 prominently deregulated miRNAs was analyzed in a larger panel of primary SCLC tumors. Among the differentially expressed miRNAs in SCLC, miR-126 appears to have a complex role in regulating cellular proliferation. Since our work has shown that miR-126 is a uniformly downregulated miRNA in primary SCLC tumors, in our second study we investigated its role in regulating the proliferation of SCLC cells.

2. THEORETICAL BACKGROUND

2.1. Early diagnostics in lung cancer

SCLC is very aggressive tumor due to its rapid progression and frequent metastases. Prognosis of SCLC depends especially on early detection and immediate treatment prior to metastatic spread.

The well known screening methods for lung cancers are sputum cytology, low-dose computed tomography and chest radiography (CXR). There is also intensive research focusing on novel diagnostic biomarkers, such as autoantibodies specific to the tumors, or miRNAs and aberrant

proteins produced by the tumors, which may be detected with highly sensitive molecular biology methods.

2.1.1. Sputum cytology

This method is a non-invasive and relatively simple test in the diagnosis of lung cancer and suitable for detection of adenocarcinomas and squamous carcinomas of the lung. Positive cytology results are highly specific for lung cancer, although various problems may influence the success of this technique such as sensitivity, handling of sputum material etc. Sputum cytology in combination with CXRs has been used as a screening tool in large studies. These studies showed that adding traditional sputum cytology screening to CXRs offers no further long-term survival advantage for the screened population [14,15].

The discovery of new molecular markers in sputum, such as miRNAs, promises to increase sensitivity of sputum-based diagnostics. Therefore, sputum cytology in combination with other screening methods could be effective in the early detection of lung cancer.

2.1.2. Computed tomography (CT)

For stage I lung tumors the detection rate by computed tomography studies was relatively high. Using a CT method, it is possible to detect peripheral squamous carcinomas or adenocarcinomas of the lung. CT is a good method for detection of slowly growing cancers in the periphery of the lung, but is not a suitable method for detection of fast growing tumors such as SCLC and centrally localised tumors. In addition, since SCLC can metastasize when the primary lesion is still very small, early detection of SCLC through imaging is a real challenge.

Low-dose spiral computed tomography (LDSCT) is a promising method for early detection of lung cancer, because it can detect tumors at a smaller size and earlier stage than chest radiography and sputum cytology. Positron emission tomography (PET) with the glucose analog F-18-fluorodeoxyglucose (FDG) is an imaging test for diagnosis of pulmonary nodules. Early detection of lung cancer with LDSCT- based protocol with the addition of FDG-PET for smaller (>7 mm) nodules is possible. Although CT can noninvasively detect lung cancer earlier, at smaller size, the sensitivity is associated with overdiagnosis [16-20].

Unfortunately, FDG-PET scanning as an early diagnostic tool has not decreased mortality in SCLC patients. On the other hand, FDG-PET can be useful for early assessment of response to chemotherapy in patients with SCLC, and it is more sensitive than CT. However, this application is still limited; therefore, additional data is needed to determine the exact role of FDG-PET in the early assessment of therapeutic response in patients with SCLC [21].

In summary, it appears that sensitive imaging-based screening alone is not enough for early detection of SCLC - however, the development of additional methods such as detection of molecular markers may increase the efficacy of diagnosis.

2.1.3. Autoantibodies

SCLC-associated autoantibodies are relatively new and promising molecular markers for early detection and diagnosis of SCLC. These autoantibodies are directed against neuronal antigens that are abnormally expressed by the tumor, but are normally expressed by neurons. The autoantibodies cause autoimmune disease with severe, but highly variable neurological dysfunctions, also called as paraneoplastic neurologic syndromes (PNS). PNS affect a small percentage of SCLC patients, but the antibodies can be found in a substantial fraction of SCLC patients without neurological symptoms [22,35]. Therefore, these autoantibodies could be used for early detection and diagnosis of SCLC, especially if they appear before the tumor starts to metastatize.

There are three different types of SCLC-associated PNS and their target autoantigens: 1) paraneoplastic encephalomyelitis/sensory neuronopathy (PEM/SN), targeting Hu proteins, 2) Lambert-Eaton myasthenic syndrome (LEMS), targeting voltage-gated calcium channels (VGCC) and SOX proteins, and 3) cancer-associated retinopathy (CAR), targeting recoverin [23].

2.1.4. MicroRNAs

MiRNAs represent a new field for molecular diagnosis of lung cancer. Circulating miRNAs are present in blood and can be used as potential biomarkers for different types of cancers. Shen and colleagues identified miR-21, miR-486-5p and miR-210 as potential plasma marker for NSCLC [24]. Another study demonstrated that different miRNAs are deregulated in plasma before and at the time of disease, suggesting that genes involved in the

early phase of disesase are different from those required for the progression of tumor [25]. Chen and colleagues identified ten serum miRNAs from a genome-wide serum miRNA expression profiling as novel non-invasive biomarkers for NSCLC diagnosis [26].
MiRNAs are also present in sputum and can be used as highly sensitive and specific sputum markers for early detection of lung adenocarcinoma (AC) and squamous cell lung cancer (SCC). In this regard, in SCC patients the sensitivity and specificity of 3 miRNAs (miR-205, miR-210, miR-708) was 73 % and 96 % [27], while in lung AC 4 miRNAs (miR-21, miR-486, miR-375, miR-200b) had a sensitivity of 81% and a specificity of 92% [28].

2.1.5. Protein markers

Protein markers are also suitable for differential diagnosis, monitoring therapy response and the early detection of recurrent disease.

Neuron-specific enolase (NSE) is well known tumor marker in SCLC, which produced in neurons and malignant tumors of neuroectodermal origin, including SCLC. Elevated NSE levels in SCLC were found in 60 to 80 of the cases. In lung cancer, NSE can be used for differential diagnosis, particularly for the diagnosis of SCLC . NSE could potentially be used for therapy monitoring in SCLC, and for the detection of recurrent disease of SCLC after primary therapy [29,31].

Progastrin-releasing peptide (ProGRP) is precursor of the gut hormone, gastrin releasing peptide, and its levels are frequently elevated in SCLC, while only rarely in NSCLC and other types of malignancy. The sensitivity for detecting SCLC improved when ProGRP was used in combination with NSE. ProGRP is in clinical use for differential diagnosis, treatment monitoring, and detection of recurrent disease of SCLC after primary therapy [30,31].

2.2. Gene mutations and aberrant signal transduction in small cell lung cancer

SCLC cells display many neuroendocrine characteristics and express neuroendocrine markers, like neuroendocrine cells like neural cell adhesion molecule (NCAM1), neuronspecific enzymes (e.g. L-DOPA decarboxylase, NSE) and neuropeptide hormones like bombesin/gastrin-releasing peptide (GRP). These neuroendocrine markers are highly expressed in SCLC tumors (80–100%) compared with NSCLC (0–20%). Therefore, the

current hypothesis is that SCLC arises from neoplastic transformation of neuroendocrine cells in the pulmonary epithelium [32]. There are several genetic abnormalities that are found characteristically in SCLC, or occur at significantly higher rates in SCLC than in NSCLC. Although loss of cell cycle control is a common characteristic in both lung cancer types, the mechanism by which this genetic change affects the process of neoplastic transformation differs significantly.

2.2.1. Inactivation of the retinoblastoma (RB) gene

RB is located in 13q1 and functions as a tumor-suppressor gene regulating the G1/S phase transition of the cell cycle. Reduced RB expression is found in 80–100% of SCLC but is much less frequent in NSCLC (15–35%). SCLC expresses normal levels of cyclin D1 and the cyclin-dependent kinase inhibitor (CDKI) p16INK4 while cyclin D1 is overexpressed, and p16INK4 is inactivated in more than 70% of NSCLC cases [33,34].

2.2.2. p53 mutations

P53 inactivating mutations are the most frequent mutations in lung cancer, found in over 70% of SCLC cases, and in up to 50% of NSCLC cases [33,34].

2.2.3. FHIT tumor suppressor mutations

Loss of heterozygosity (LOH) in chromosome 3p is detected at high rates in lung cancer [33,34]. A tumor-suppressor gene, FHIT is located in this chromosomal region and abnormalities in FHIT gene were found in 80 % of SCLC tumors and in 42 % of NSCLC.

2.2.4. Other mutations

Many other genetic alterations occur in SCLC, including increased expression of the anti-apoptotic gene BCL-2, activation of autocrine pathways, increased telomerase function, reduced expression of matrix metalloproteinase inhibitors, and increased expression of vascular growth factors. C-MYC gene amplification and hypermethylation of the anti-apoptotic caspase-8 gene are seen exclusively in SCLC [33,34].

2.2.5. Neuropeptide-dependent regulation of proliferation in SCLC

Multiple neuropeptides and polypeptides promote SCLC cells growth in an autocrine/paracrine fashion [35]. Receptors for neuropeptide hormones belong to the G

protein-coupled receptors (GPCRs). Neuropeptide receptors are stimulated by neuropeptide hormones, then activate either $G\alpha_{12/13}$ or $G\alpha_{q/11}$ proteins. $G\alpha_{12/13}$ transfer the signals to RhoGTPases, which, among other effects, can alter the dynamics of the actin cytoskeleton. $G\alpha_{q/11}$ proteins signal via the PLCß (phospholipase C) pathway, activate protein kinase C (PKC) and increase intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). PKC has been implicated in cell cycle control via the Ras/Raf/extracellular signal-regulated kinase (ERK) pathway. Discordant signalling of neuropeptide hormone receptors inhibits SCLC cell proliferation and leads to apoptosis through activation of the c-jun N-terminal kinase (JNK) cascade (Figure1) [36].

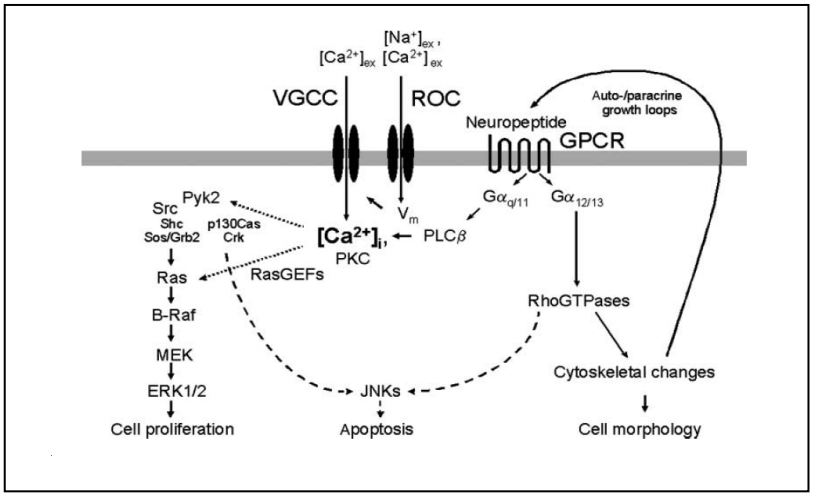


Figure 1. Ca^{2+}-dependent signaling in SCLC cells

G protein-coupled receptors (GPCRs); receptor-operated non-selective cation channels (ROC); voltage-gated Ca^{2+} channels (VGCCs)

Gudermann T, Roelle S.,: Calcium-dependent growth regulation of SCLC cells by neuropeptides. Endocr Relat Cancer. 2006 Dec;13(4):1069-84.

2.2.6. The role of extracellular matrix in SCLC survival

SCLC cells exist in a rich extracellular matrix (ECM) environment, and interact with ECM component via integrins. The integrin-ECM interaction stimulates signal transduction pathways that regulate cellular processes important in cancer growth, including cell cycle transition and protection from apoptosis. Chemotherapeutic agents such as etoposide and adriamycin act by inhibiting of topoisomerase II and induce multiple breaks in the DNA, which leads to activation of caspase 3 and apoptosis (Figure 2) [37,38]. Adhesion of SCLC

cells to ECM components via ß1-intregins confers resistance to these chemotherapeutic agents, by blocking chemotherapy-induced activation of the caspase pathway and apoptosis. CXCR4 is the major chemokine receptor in SCLC and its ligand CXCL12 (SDF1α) mediates ß1-integrin-dependent adhesion to ECM components and chemoresistance, and also cooperates with SCF (c-kit receptor ligand) to induce downstream signaling targets, such as protein kinase B (Akt) [39].

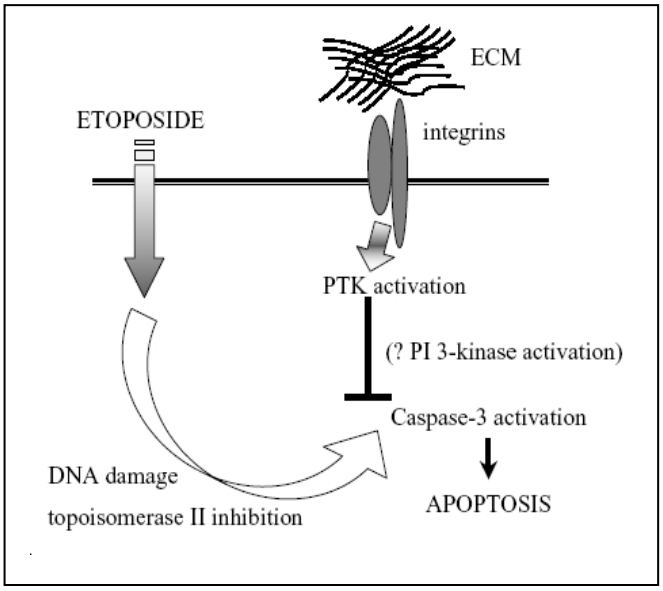


Figure 2. Proposed mechanism by which SCLC cell adhesion to ECM proteins can promote chemoresistance
Buttery RC, Rintoul RC, Sethi T., Small cell lung cancer: the importance of the extracellular matrix, Int J Biochem Cell Biol. 2004 Jul;36(7):1154-60. Review.

Targeted inhibition of cell-surface growth-factor receptors and their downstream signaling pathways has been a major focus of anticancer drug development. Several receptor tyrosine kinases, including c-Kit, c-Met and GRP receptor are highly expressed in SCLC. Binding of these receptors to their ligands results in the activation of multiple proliferative signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)–Akt and mTOR pathway. Activation of PI3K–Akt–mTOR pathway promotes tumorigenesis and therapeutic resistance, whereas its inhibition can lead to chemotherapy sensitization of SCLC cell lines [40].

2.3. Novel therapeutic approaches to SCLC

Based on the increased knowledge about SCLC biology, several therapeutic targets were under investigation [reviewed in ref. 41, 42]. For examples:

Vascular endothelial growth factor (VEGF): in a phase II trial, humanized monoclonal antibody directed against VEGF, Bevacizumab was tested in combination with irinotecan and radiotherapy. In the first study bevacizumab increased the risk for tracheoesophageal fistula (TE) development in SCLC. In further trials the combination of chemotherapy with bevacizumab (carboplatin/etoposide/ bevacizumab) showed a promising toxicity profile.

The effect of bevacizumab on the outcome of SCLC is currently unclear, therefore, further studies are required.

Thalidomide: inhibitor of angiogenesis, modulates the expression of VEGF. The efficacy of thalidomide was tested in combination with carboplatin and etoposide in SCLC. Patients treated with thalidomide did not have longer survival, and neuropathy and thrombotic events occured more frequently than in patients treated with placebo. Therefore, this agent was eliminated from further trials.

BCL-2 and p53: BCL-2 is overexpressed in SCLC and is associated with chemotherapy resistance. Antisense oligonucleotide against BCL-2 (oblimersen) have shown antitumor activity. Unfortunately, the use of oblimersen did not improve the outcome of SCLC, which may be because of the insufficient suppression of BCL-2 in vivo.

Patients with p53 mutation are more likely to be resistant to chemotherapy; therefore, gene therapies targeting p53 are explored. A vaccine composed of dendritic cells that are transduced with an adenoviral vector expressing p53 (DC-Ad-p53) has been used in combination with chemotherapy in SCLC patients. The responses to the vaccination alone were not satisfactory, suggesting that vaccines could serve as an adjunct to chemotherapy.

DNA topoisomerase II: Several drugs used in the treatment of SCLC, such as doxorubicin or etoposide, act by inhibiting DNA topoisomerase II. Amrubicin represent a newer generation of potent topoisomerase II inhibitors. The results are reassuring but there are several question that have to be answered with the use of amrubicin in the treatment of SCLC.

CD56: CD56 is one of the NCAM gene isoform which is expressed in SCLC. A humanized anti-CD56 monoclonal antibody conjugated to a cytotoxic maytansinoid drug, DM-1, (BB10901) has been developed. In phase II clinical trial BB10901 was well tolerated and clinical activity was observed in several patients.

Matrix metalloproteinases (MMPs): MMPs are negative predictor of survival in SCLC, synthetic MMP inhibitors, such as Marimastat and Tenomastat, have been tested in clinical trials to determine whether these drugs could prolong survival of SCLC patients. Unfortunately, none of these agents show any advantage in term of survival. One of the explanation of these negative results could be the lack of our understanding about how the complex MMP system is involved in tumor progression.

Epidermal growth factor receptor (EGFR) : Gefinitib is an active inhibitor selective for the EGFR tyrosine kinase. SCLC expresses very low level of EGFR, but preclinical data suggest that gefinitib is effective against cancers with low EGFR expression, including SCLC. In clinical trials gefitinib treatment showed limited antitumor activity and quality of life did not improve.

Farnesyltransferase: Tipifarnib is a farnesyltransferase inhibitor which blocks the activity of farnesylated proteins involved in signaling pathways critical for cell survival. In clinical trials there were no responses to tipifarnib treatment. Tipifarnib showed no activity as a single-agent therapy in SCLC.

Multidrug resistance (MDR): MDR is associated with the overexpression of two membrane-bound glycoproteins, P-glycoprotein (Pgp) and MDR-associated protein (MRP-1). These proteins are able to expel the drugs from the cells. Tumors with MDR frequently upregulate MRP-1 and Pgp proteins. Biricodar is an agent that binds to Pgp and MRP-1 and inhibits their pump efflux activity and increases intracellular accumulation of cytotoxic agents. The treatment of SCLC patients with biricodar was not successful because the agent did not enhance antitumor activity or survival.

mTOR Inhibitors : mTOR is a downstream mediator of PI3K/AKT signaling pathway, which play a role in regulating cell proliferation. Inhibition of this pathway can lead

to chemotherapy sensitization of SCLC cell lines. mTOR inhibitor rapamycin and its analogues (temsirolimus, everolimus, deforolimus) were tested in clinical studies. Results reported very low activity and no responses to the treatments.

c-Kit Receptor Tyrosine Kinase Inhibitors: SCLC cells overexpress c-Kit and its ligand SCF. After binding to its ligand, c-Kit initiates intracellular signaling cascades. Imatinib (STI-571) is a small-molecule inhibitor of c-Kit. This drug has been shown to inhibit proliferation of SCLC cells. The inhibitory effect of imatinib appears to be related to the presence of KIT expression. In clinical study imatinib treatment was well tolerated, but did not show antitumor activity against SCLC. Therefore, imatinib should probably be tested in combination with other drugs.

2.4. RNA interference (RNAi)

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism which is triggered by double-stranded RNA (dsRNA), resulting mRNA degradation or inhibition of mRNA translation. The RNAi methology was first recognized by Fire and colleagues, who observed that the response to dsRNA in the nematode Caenorhabditis elegans (C.elegans) resulted in potent sequence-specific gene silencing at the posttanscriptional level [43]. In 2006, Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine for their work on RNA interference in C. elegans. The key players in RNA silencing are small regulatory RNAs, including small interfering RNAs (siRNAs), processed from longer dsRNAs and miRNAs, generated in a multistep process from endogenous primary transcripts (pri-miRNA). MiRNAs play an important role in many biological processes such as development, differentiation, proliferation and apoptosis. Deregulated expression of miRNAs has been implicated in a wide range of pathologies, especially in cancer and several number of genes, including tumor suppressors and oncogenes are regulated by these small RNAs. The use of RNAi for therapies has been widely studied, expecially in viral infections, cancers, and inherited genetic disorders. RNAi technology is a potentially useful method to develop highly specific dsRNA-based gene-silencing therapeutics.

2.5. Small RNAs

Small non-coding RNAs can be classified into different groups based on their origin and exact function. These small RNAs include miRNAs, siRNAs, trans-acting siRNAs (tasiRNAs), repeat-associated RNAs (rasiRNAs) and PIWI interacting RNAs (piRNAs). Summary of the characteristics of these small RNAs are listed in Table 1.

Table1.

Small RNAs	Characteristics
miRNAs	small RNAs ~22 nt long; derived from hairpin-structured precursors; associate with Argonaute (Ago) proteins and are primary involved in post-transcriptional gene regulation (facilitate translation repression)
siRNAs	small RNAs ~21-22 nt long; are the products of long double-stranded RNAs; silence genes by cleaving their target mRNAs; widely used as an exogenous tool to study gene function by targeting mRNAs in mammalian cells
tasiRNAs	endogenous siRNAs; trigger mRNA degradation; repress the expression of genes that have little overall resemblance to the genes from which they originate; present in plants
rasiRNAs	longer small RNAs than siRNAs, silence repetitive and mobile genetic elements in yeast, plants and flies; fly rasiRNAs are similar to the mammalian piRNAs
piRNAs	typically 24-32 nt long; associate with germline-specific Piwi proteins; processed from single stranded precursors; control mobile genetic elements; play a crucial role during germline development and gametogenesis

2.6. MicroRNA biogenesis

Transcription of miRNA genes is mediated by the RNA polymerase II, although RNA polymerase III is also reported to generate a subset of miRNAs. MiRNA maturation begins in the nucleus. The pri-miRNAs are usually several kilobases long and contain 5' 7-methyl guanosine cap and 3' poly-A tail [44,45]. The pri-miRNAs are processed in the nucleus by the RNase III enzyme Drosha and its binding protein Pasha (also known as DGCR8), into ~60-70-nucleotide pre-miRNAs, which contain a 5' phosphate group, a 2-nucleotide 3'overhang and a stem-loop hairpin stucture. Drosha, together with DGCR8, forms the Microprocessor protein complex [46]. A Drosha/DGCR8-independent processing pathway can also produce pre-miRNAs. In this pathway, the nuclear splicing machinery provides pre-miRNA from introns. MiRNAs produced by this pathway called mirtrons [47].

Pre-miRNAs are transported from the nucleus to the cytoplasm by Exportin-5, in a Ran GTP-dependent manner [48]. In the cytoplasm pre-miRNAs are processed by another RNase III enzyme, Dicer. Dicer cleaves pre-miRNAs into 21–25 nt long miRNA/miRNA* duplexes (Figure 3). Each strand bears a 5' monophosphate group, a 3'hydroxyl group and a 3' 2-nt overhang. The duplex is then loaded into the RNA-induced silencing complex (RISC), which includes the Ago proteins. One strand of the duplex (guide strand) becomes the mature miRNA; the other one (passenger strand) is degraded. Strand selection depends on the balance of at least three properties of a miRNA/miRNA* duplex: the structure; the 5' nucleotide identity; and the thermodynamic asymmetry [49,50].

The core component of RISC is a member of Ago proteins [51,52], which has four paralogs (Ago1–4) in humans. These proteins contain four domains: the N-terminal, PAZ, middle and Piwi domains. The PAZ domain binds to the 3' end of guide miRNA, while other domains are responsible for creating grooves for target and guide RNA interactions. The mature miRNA associates with the Ago2-containing RISC and RISC-loaded miRNAs are guided to the 3'UTR of target mRNAs by a seed region, which is a specific complementary region between nucleotides 2-7 of the guide strand. The miRNA-induced silencing complex (miRISC) interferes with gene expression by repressing protein translation, by accelerating mRNA degradation and by sequestrating mRNA to storage compartments.

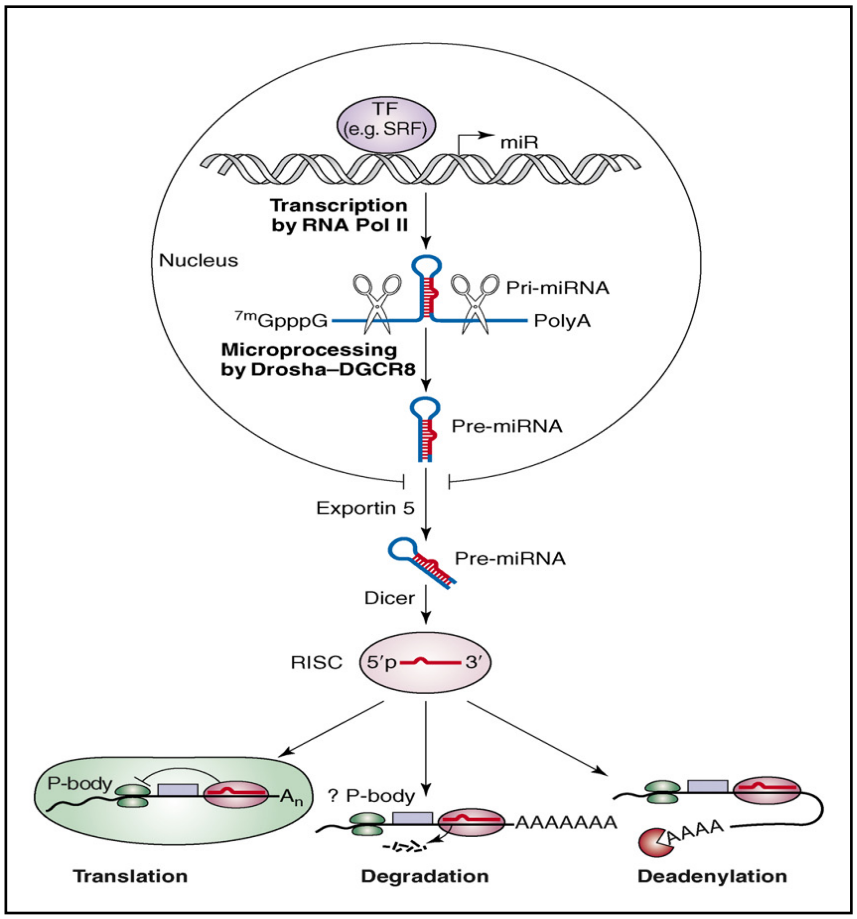


Figure 3. **miRNA biogenesis and function**

Zhao Y, Srivastava D.: A developmental view of microRNA function. Trends Biochem Sci. 2007 Apr;32(4):189-97.

2.6.1. Translational repression

The mechanisms by which miRNAs repress protein synthesis are obscure. There are several studies dealing with the molecular mechanisms of miRNA-mediated translational repression but the problem is that the results obtained in different systems are often quite contradictory. There are at least five models of translational repression (Figure 4):
(A) mRNA undergoing translation in the absence of a bound miRNA. (B) Inhibition of translation initiation; there is a competition between RISC and eukaryotic translation initiation factor 4E (eIF4E) for cap binding [53], (C) Inhibition of translation initiation after cap recognition, such as by inhibiting the association of the small and large ribosomal subunits [54]. (D) Inhibition of post-initiation step such as elongation and⁄or ribosome drop-off [55].

(E) Degradation of nascent polypeptides during translation [56]. RISC delivers target mRNAs to processing bodies (P bodies), in which mRNA is degraded and⁄or stored in a translationaly inactive state. Further research is required to clarify the exact molecular mechanisms of translational repression.

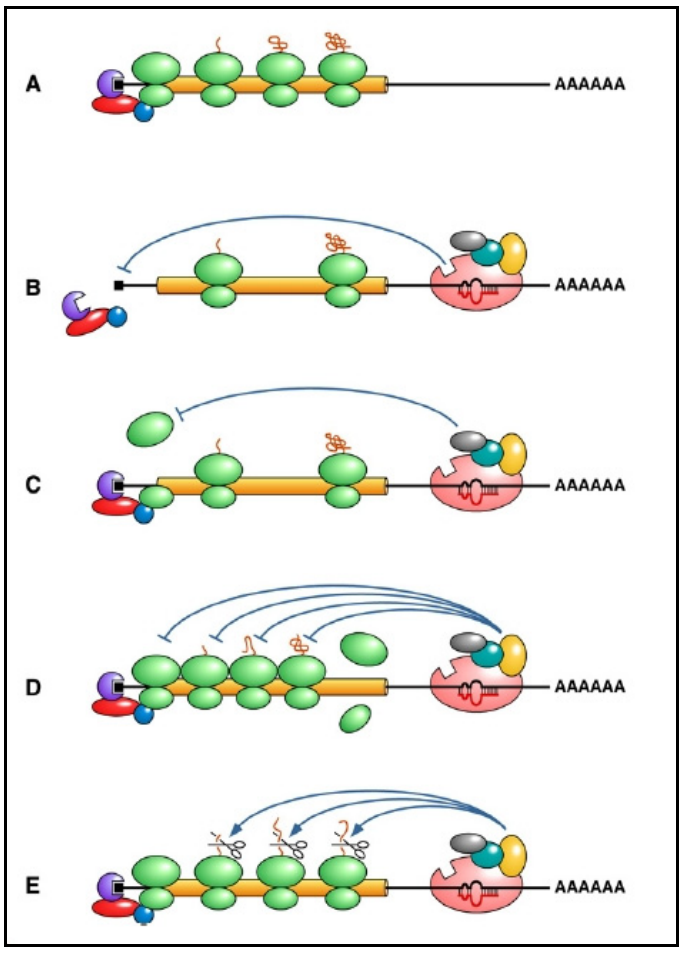


Figure 4. Hypothetical mechanism of translational repression by miRNAs

black square: m7G cap
amber cylinder: protein-coding region
AAAAAA: poly(A) tail

colors:
ribosomes: green
nascent polypeptides: brown
eIF4E subunit of the cap- binding complex: violet
miRNA in RISC complex: red
Ago protein: pink

(A) mRNA undergoing translation in the absence of miRNA.
(B) Inhibition of translation initiation by competition between RISC and eIF4E for cap binding
(C) Inhibition of translation initiation at a step after cap recognition
(D) Inhibition of translation elongation
(E) Degradation of nascent polypeptides

Wu L, Belasco JG.: Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. Mol Cell. 2008 Jan 18;29(1):1-7

2.6.2. P bodies

P bodies are large ribonucleoprotein aggregates, which contain high concentration of miRNAs, RISC-associated proteins (Ago1-4, GW182 etc.) and RNA degradative enzymes. There is evidence pointing to the possibility that P body localization might be important for silencing by miRNAs. First, P bodies are devoid of ribosomes [57]; second, mRNAs that are targeted for translational repression by miRNAs become concentrated in P-bodies in a miRNA-dependent manner. These results provide a link between miRNA function and

mammalian P-bodies and suggest that translation repression by RISC delivers mRNAs to P-bodies, either as a cause or as a consequence of inhibiting protein synthesis [58]. Third, depleting cells of RISC-associated proteins such as GW182 disaggregates P bodies and impairs miRNA fuction [59]. The simple model is that miRNA bound to an Ago protein recognizes its mRNA targets by base pairing. Ago protein interacts with GW182 protein and the miRNA-mRNA-Ago complex is delivered to P bodies. When it reaches the P body, the targeted mRNA is deadenylated, then either decapped and degraded.

2.6.3. Translational activation

Some data indicate that miRNAs can stimulate translation under specific conditions. Two proteins are known to be important for the ability of miRNAs to activate translation in growth-arrested mammalian cells. One is *Ago2*, the other one is *FXR1*, an RNA-binding protein homologous to the fragile X mental retardation protein FMR1/FMRP. Surprisingly, it was found that human Ago2 activates translation of target mRNAs on cell cycle arrest caused by serum starvation or contact inhibition, while it normally represses translation of the same target mRNAs in proliferating cells. FXR1 associates with Ago2 and helps to mediate the positive influence of miRNAs on the translation [60,61]. Like translational repression, such activation requires base pairing between mRNA and the seed region of miRNAs. The mechanism by which the efficiency of translation increases has not yet been determined. These exciting findings made it even more difficult to explain how miRNAs regulate protein synthesis.

2.6.4. mRNA degradation

There are two mechanism for mRNA degradation, the first being when mRNAs undergo endonucleolytic cleavage by Ago2, as guided by a fully complementary miRNA (Figure 5A) [62]; the second is when mRNAs undergo poly(A) removal by deadenylases, guided by a partially complementary miRNA (Figure 5B) [63].

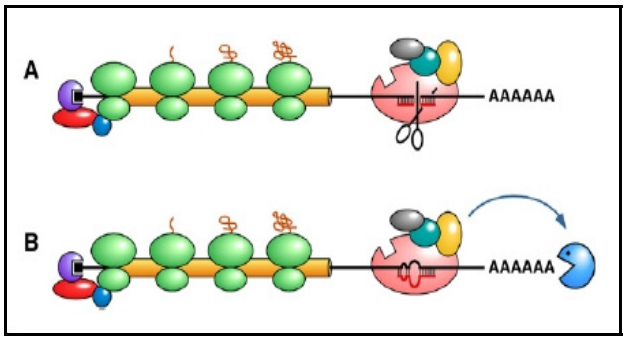


Figure 5. Mechanism by which miRNA trigger mRNA degradation

black square: m7G cap
amber cylinder: protein-coding region
AAAAAA: poly(A) tail

colors:
ribosomes: green
nascent polypeptides: brown
eIF4E subunit of the cap- binding complex: violet
miRNA in RISC complex: red
Ago protein: pink

(A) mRNA undergoing cleavage by Ago2 (perfect complementarity)
(B) mRNA undergoing poly(A) removal by deadenylases (partial complementarity)

Wu L, Belasco JG.: Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. Mol Cell. 2008 Jan 18;29(1):1-7.

2.7. Interplay between microRNAs and RNA binding proteins

MiRNA activity can be affected by RNA-binding proteins (RBPs). For example, the repression of CAT-1 mRNA by miR-122 in hepatocarcinoma cells is relieved in stress conditions by binding of HuR, an AU- rich element binding protein, to the 3'UTR of CAT-1 mRNA [64]. Dead end (Dnd1) is an other RNA-binding protein which mediates germ-cell viability and suppresses the formation of germ-cell tumors. Agami and colleagues shows that Dnd1 binds to the U-rich region on the mRNA and without these regions Dnd1 cannot affect the miRNA action. There are two possible explanation about how Dnd1 prevents the interaction between miRNA and target mRNA. First, Dnd1 may block the access to a miRNA target site (Figure 6B). Second, Dnd1 may change the subcellular localization of a mRNA, taking it out of reach of miRNAs. (Figure 6C) [65].
In a similar manner, other proteins interacting with the 3'UTR of the mRNAs may also act as modifiers altering the potential of miRNAs to repress gene expression.

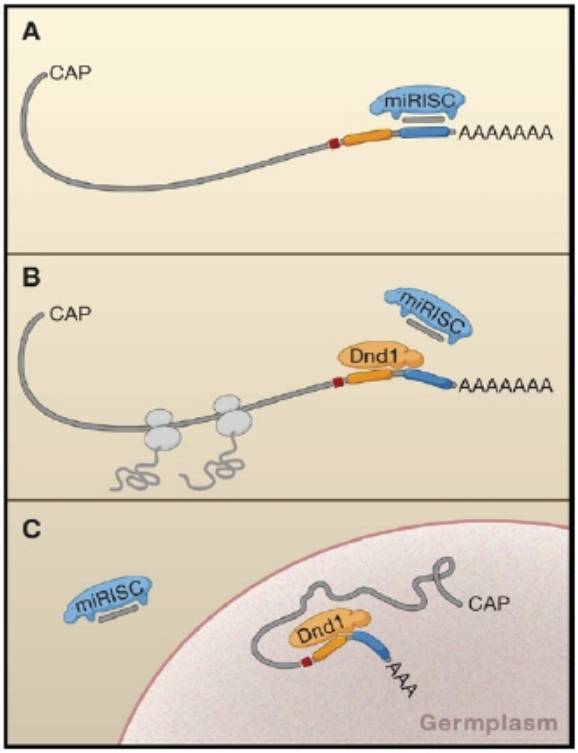


Figure 6. Action of Dnd1 protein

(A) MiRNAs are part of the miRISC complex. This complex can bind to mRNAs when a region in the mRNA (blue box) is homologue with miRNA. MiRNAs can inhibit translation and decrease the stability of target mRNAs.

(B) Dnd1 protein binds to a U-rich region in the mRNA (orange box) and block recognition of the mRNA by miRISC.

(C) Dnd1 may translocate the bound mRNA to structures that are inaccessible to miRISC, such as the germplasm of germ cells.

Ketting RF.: A dead end for microRNAs. Cell. 2007 Dec 28;131(7):1226-7.

2.8. Nomenclature and genomic organization of microRNAs

miRBase is the central online repository for miRNA nomenclature, sequence data, annotation and target prediction. miRBase provides a range of data to facilitate studies of miRNA genomics. miRBase is available at http://microrna.sanger.ac.uk/. MiRNA names have a prefix indicative of the species, like **hsa**-miR-21 refers to the mature miR-21 miRNA of humans and **mmu**-miR-21 refers to the corresponding murine ortholog.

MiRNAs whose sequences differ at only one or two nucleotide positions are given lettered suffixes, like mature miRNAs hsa-miR-10**a** and hsa-miR-10**b**. In many cases, mature miRNAs from both 5' and 3' arms of the hairpin precursor are identified, suggesting that both may be functional, like miR-140-**5p** and miR-140-**3p**. Mature miRNAs that have an identical sequence but arise from different genes are given additional numerical suffices, like hsa-miR-1-**1** and hsa-miR-1-**2**, whose genomic loci are on chromosomes 20 and 18. **miR*** sequences are biogenesis by-products that are often detected at very low levels and are likely non-functional. Mature miR and miR* sequences are better distinguished in the database and distributed in separate files.

MiRNAs can be grouped into several categories according to the genomic localization: intronic miRNAs in protein-coding genes; exonic miRNAs in non-coding genes; intronic miRNAs in non-coding genes. Many mammalian miRNAs lie within introns of protein-coding genes and have the same transcription pattern as the protein-coding genes in which they reside. In some cases, miRNA genes contain their own independent promoters and enhancers. Such miRNAs might still be located in introns but have an antisense orientation. In humans, miRNA genes are found in all chromosomes, with the exception of the Y chromosome. Several miRNAs are found in clusters and transcribed as polycistronic transcripts. MiRNAs in one cluster are coordinately regulated, indicating that they are members of the same regulatory unit.

2.9. MicroRNA targets

Determining the exact function of miRNAs requires identification of its target mRNAs. Target identification in plants is based on the perfect complementarity between miRNAs and target mRNAs. In C. elegans, Drosophila and mammals most miRNAs are not perfectly complementary to their target mRNAs, which makes target identification quite complicated. The list of known miRNAs is large and the official miRNA database, miRBase, lists approximately 750 human miRNAs. Relatively few experimentally validated miRNA target genes are known; therefore, validating new miRNA targets is one of the most important steps in miRNA biology to understand the function of a miRNA. Functional analyses are further complicated by the fact that a miRNA may target over a hundred of different mRNAs, and a mRNA may be targeted by several different miRNAs.

2.9.1. MicroRNA target features

1, *miRNA:mRNA pairing*

miRNA targets have at least one stretch of nucleotides with perfect complementerity to the 5' end of the miRNA. This is known as'**seed**', located at positions 2-7 from the 5' end of miRNA (Figure 7). The corresponding site(s) in the mRNA are referred to as **'seed sites**'.

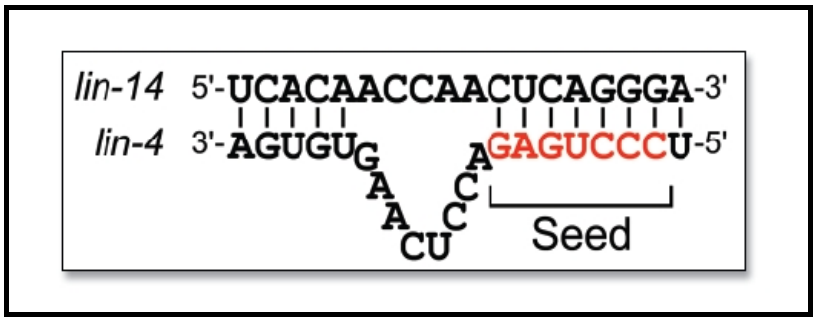


Figure 7. Typical example of miRNA:mRNA interactions.
Lin-4 binding site in the 3'UTR region of lin-14 mRNA.

2, *Site location*

Most miRNA target sites can be found in the 3'UTR of the target mRNAs (Figure 7). MiRNA target genes tend to have longer 3'UTRs than ubiquitously expressed genes. Although functional miRNA sites are located in the 3'UTR, seed sites in the coding sequence (CDS) and 5'UTR regions can also occur, but 3'UTR is thought to be more accessible for the miRISC complex than the two other mRNA regions.

3, *Conservation*

MiRNAs and their target genes are conserved among related species but there are some exceptions where studies reported species-specific miRNAs and targets.

4, *Site accessibility*

The mRNA secondary structure is very important for miRNA-target recognition. MiRNAs need an open, single stranded structure of the target site on the mRNA to be able to hybridize.

5, *Multiple sites*

Strong miRNA targets have multiple target sites instead of one single site. The number of putative miRNA sites per mRNA can enhance target prediction. Two target sites within optimal distance enhance target site efficacy, the optimal length being between 17 and 35 nucleotides. Multiple sites involving more than two sites can also contribute to enhanced recognition.

6, *Expression profile*

If negatively correlated expression levels of a miRNA:mRNA pair are detected across different tissue profiles, the mRNA of the pair is probably targeted by the miRNA. MiRNA targets appears to be regulated both at the mRNA and protein level, but some targets only show an effect at the protein level.

Several computational programs exist to identify miRNA targets, but the number of targets predicted for a given miRNA ranges from 100 to 500, making validation studies difficult. Two popular computational program are TargetScan (www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de/). To verify target genes through direct experimentation is critical for understanding miRNA function and improving target prediction algorithms. Two main approaches are used to verify targets. The first is luciferase reporter assay, a method showing miRNA 3'UTR binding. This assay measures the ability of a miRNA to decrease luciferase activity in cells when its potential binding site is cloned downstream of the luciferase gene. Paired constructs are generated with mutations in the miRNA binding sites. In the second experiment is imperative to show that miRNA regulates the expression of the target mRNA or protein. This validation can be assessed by transiently transfecting cells with mimic and inhibitor of the miRNA of interest or by stably overexpressing the miRNA of interest in a cell line. When the miRNA levels are increased in the cell, miRNA regulation should result in decreased mRNA and/or protein expression, whereas decreased levels of miRNA should result in increased mRNA and/or protein expression. High-throughput approaches for genome-wide identification of target mRNAs, such as Ago-IP and RIP-ChIP are being actively developed.

2.10. The role of microRNAs in normal development and differentiation

MiRNAs play an important role in normal development and differentiation. One method to test the global importance of miRNAs was to knock out the miRNA-processing enzyme Dicer, which inhibits the production of all miRNAs. Targeted deletion of Dicer in mice causes embryonic lethality before embryonic day 7.5, suggesting an important role for miRNAs in development. Moreover, Dicer-deficient embryonic stem cells are defective in differentiation both in vitro and in vivo [66].

Tissue specific inactivation of Dicer by using a Cre/lox gene knock-out system shows further importance of miRNAs in specific tissues. For example, in the epidermal Dicer knockout mice, several anatomic and molecular hair follicle defects were observed. Newly-forming hair germs, instead of invaginating downward into the dermis, evaginated upward into the epidermis and several days latter formed cyst-like pearls in the epidermis. This phenotype suggest that miRNA may be important to guide proper anatomic location and orientation in hair follicle development [67].

Many studies demonstrate that **miR-1, miR-133 and miR-206** are necessary for skeletal and cardiac muscle development and function. MiR-1 promotes myogenesis by targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle gene expression, while miR-133 enhances myoblast proliferation by repressing serum response factor (SRF) [68]. In addition, the myogenic factors Myogenin and MyoD bind to regions upstream of these miRNAs and can regulate their expression [69]. Skeletal muscle satellite cells are adult stem cells which are responsible for postnatal skeletal muscle growth and regeneration. MiR-1 and miR-206 are up-regulated miRNAs during satellite cell differentiation and facilitate differentiation by restricting their proliferative potential. Paired-box transcription factor Pax7 plays a central role in satellite cell survival, self-renewal and proliferation, and was identified as one of the direct regulatory targets of miR-1 and miR-206 [70].

Several lines of evidence suggest that miRNAs have an important role in normal hematopoiesis. **MiR-155** and **miR-150** play an essential role in the differentiation of B and T lymphocytes**, miR-221** and **miR-222** have a suppressive role in erythroid differentiation, **miR-181** has an inhibitory effect on hematopoietic differentiation and **miR-223** enhances myeloid differentiation [71,72].

MiR-143 play a crucial role in adipocyte differentiation and miR-143 expression levels were closely correlated with expression levels of adipocyte differentiation markers such as PPARgamma and aP2, as well as plasma levels of leptin [73,74].

miR-375 was identified as an islet-specific miRNA and it is highly expressed during human pancreatic islet development. Overexpression of miR-375 suppressed glucose-induced insulin secretion, and conversely, inhibition of endogenous miR-375 function enhanced insulin secretion. Myotrophin (Mtpn) is a valid target of miR-375 which mimicked the effects of miR-375 on glucose-stimulated insulin secretion and exocytosis [75].

2.11. The role of microRNAs in cancer

A miRNA that is downregulated in cancer and targets an oncogene might normally function as a tumor suppressor, whereas an upregulated miRNA that targets a tumor suppressor might act as an oncogene. Calin and collegues reported in 2004 [76] that approximately 50 % of miRNA genes are in cancer-associated genomic regions or in fragile sites, as well as in minimal regions of loss of heterozygosity, minimal regions of amplification or common breakpoint regions.

2.11. 1. Tumor suppressor miRNAs: miR-15a and miR-16-1

The first reported tumor suppressor miRNAs were miR-15a and miR-16-1. The miR-15a–16‑1 cluster is located in the chromosome 13q14 region. Loss of this region [del(13q)] is the most common genomic aberration in chronic lymphocytic leukemia (CLL), occurring in more than 50% of patients [77,78]. Interestingly, the putative tumor suppressor deleted in lymphocytic leukemia 2 (DLEU2) is the host gene of miR-15a and miR-16-1. DLEU2 negatively regulates Cyclin E and Cyclin D through miR-15a/miR-16-1 and DLEU2 overexpression blocks cellular proliferation and inhibits the colony-forming ability of tumor cell lines in a miR-15a/miR-16-dependent way [79].
Another effector of miR-15a/16-1 is BCL2, since BCL2 repression by these miRNAs induces apoptosis in CLL [80].

2.11. 2. Tumor suppressor miRNAs: Let-7 and its role in lung cancer

Let-7 miRNA is located at a chromosome region that is usually deleted in human cancers [76]. To identify the mechanism by which let-7 regulate proliferation pathways, Johnson and colleagues overexpressed let-7 family members in liver cancer cells and they observed an accumulation of cells in the G0 and G1 cell-cycle phase. They identified several genes involved in promoting the G1-to-S and G2-to-M phase, including CDK6, CDC25 and CCND2, which are direct targets of let-7 [81]. In addition to the cancer-related genes, let-7 directly affects Dicer expression, which is a key player in miRNA processing. Overexpression of let-7 significantly reduced the expression of Dicer at both the protein and mRNA levels, as well as the expression of many other mature miRNAs. Conversely, antisense-mediated

reduction of let-7 expression increased Dicer at both levels with upregulation of mature miRNAs. Therefore, let-7 appears to be a constituent of another regulatory loop within the miRNA processing steps [82]. The involvement of reduced Dicer expression in the development of lung cancer, associated with shorter postoperative survival, was described in 2005 [83]. Furthermore, conditional deletion of Dicer1 enhanced tumor development in a K-Ras–induced mouse model of lung cancer, suggesting that abrogation of global miRNA processing promotes tumorigenesis [84].

Let-7 is poorly expressed in lung cancers and similar to Dicer, reduced let-7 expression was significantly associated with shortened postoperative survival, independent of disease stage. Overexpression of let-7 in lung adenocarcinoma cell lines inhibited lung cancer cell growth in vitro [85]. In addition to the cell cycle regulatory proteins, let-7 negatively regulates RAS expression by pairing at the 3' UTR of RAS mRNA for translation repression. The correlation between reduced let-7 expression and increased RAS protein expression in the lung tumor samples relative to normal lung tissue suggests that let-7 functions as a tumor suppressor gene in lung oncogenesis [86]. In mouse NSCLC xenograft and orthotopic models, ectopic let-7g expression reduces tumor burden [87] and intranasal *let-7* administration reduces tumor formation in vivo in the lungs of animals harboring the G12D activating mutation for the *K-ras* oncogene [88]. Interestingly, a SNP in the 3'UTR of the KRAS gene alters binding of let-7 miRNA. This variant, which affects the 6^{th} let-7 complementary site in the KRAS 3'UTR (LCS6), results in up-regulation of the KRAS gene and concomitant downregulation of let-7. In addition, this polymorphism is associated with an increase in lung cancer risk, particularly among low-dose smokers [89,90].

MiRNA expression profiles may be utilized in the future as diagnostic and prognostic markers of lung cancer. A five-miRNA signature was identified that correlated with disease-free survival in a cohort of 122 NSCLC patients; reduced let-7 and miR-221 expression, and increased levels of miR-137, miR-372 and miR-182* correlated with poorer prognosis. In another study, elevated miR-155 expression also correlated with poor overall survival in lung adenocarcinoma patients [91,92].

2.11. 3. MicroRNAs involved in the regulation of apotosis: the p53-miR-34 network

Cancer cells can alter the balance between pro- and anti-apoptotic factors to allow cell survival even in the presence of strong apoptotic inducers. There is ample evidence that

miRNAs are critical apoptotic regulators in tumorigenesis. P53 is a tumor suppressor protein which plays a role in senescence, apoptosis, cell-cycle arrest, DNA damage and other cellular responses. MiR-34 family members miR-34a, miR-34b, miR-34c are downregulated in several types of cancers and are direct transcriptional targets of p53. Ectopic expression of miR-34 induces cell cycle arrest and leads to apoptosis or cellular senescence, whereas reduction of miR-34 function attenuates p53-mediated cell death. MiR-34 may act in concert with other effectors to inhibit inappropriate cell proliferation [93,94]. Cell cycle effectors (including CDK4, CDK6, CYCLIN E2) are also direct targets of miR-34 [95]. Taken together, miR-34a/b/c can involved in cell cycle control, apoptosis and may participate in tumor suppression as part of the p53 network. MiR-34b/c expression is dramatically reduced in NSCLC and restoration of miR-34 expression inhibits growth of NSCLC cells [96].

MiR-34a is also a novel prognostic marker in NSCLC patients. The miR-34 family was significantly downregulated in lung tumors compared with normal tissue, and low levels of miR-34a were correlated with a high rate of relapse [97]. Patients with both p53 mutations and low miR-34a expression had a very poor prognosis, indicating a potential synergism for these two factors. In addition to p53 mutations, epigenetic gene inactivation can also decrease miR-34 expression. Promoter hypermethylation of miR-34b/c is a relatively common event in NSCLC and the correlation between gene methylation status and miR-34a expression levels raise the possibility that miR-34a expression could be restored by demethylation therapy [98].

2.11.4. Oncogenic miRNAs: the miR-17-92 cluster and its role in lung cancer

The miR-17-92 cluster is a good example for oncogenic miRNAs. The cluster is located in the third intron of the C13orf 25 gene at the 13q31.3 chromosomal region, and produces seven miRNAs: miR-17-5p/3p, miR-18, miR-19a, miR-20a, miR-19b-1 and miR-92. The cluster is markedly overexpressed in lung cancers, especially in SCLC [99]. Suppression of miR-17-5p and miR-20a with antisense oligonucleotides can induce apoptosis selectively in lung cancer cells overexpressing miR-17-92 and overexpression of the miR-17-92 cluster enhanced cell growth [100]. All these suggest that marked overexpression of the miR-17-92 cluster may play a role in the development of lung cancers, especially in the most aggressive form, small-cell lung cancer. Studies about the role of miR-17-92 cluster in lung development showed that this cluster is normally required for the high proliferation and undifferentiated phenotype of lung cancer cells [101].

The miR-17-92 cluster regulates cell proliferation through a network including c-myc and E2F. C-Myc is a proto-oncogene, which regulates cell proliferation, growth and apoptosis; and the E2F family of transcription factors are essential in the regulation of the cell cycle and apoptosis. C-Myc can activate the expression of E2F and the miR-17-92 miRNA cluster, while miR-17-5p and miR-20a negatively regulate E2F translation [102]. In the context of SCLC, the connection between the miR-17-92 cluster and the c-myc oncogene is interesting, because the members of myc gene family have been shown to be frequently amplified and/or overexpressed in small-cell lung cancers.

Another interesting aspect of miR-17-92 function is its effect on the production of reactive oxygen species and the resulting DNA damage. The majority of SCLC tumors carry inactivated RB and p53, and as a result, there is evidence for continuing DNA damage. Overexpression of the miR-17-92 cluster, however, inhibits ROS production resulting from RB inactivation, which may explain in part why SCLC tumors depend on the overexpression of this cluster [103].

2.11.5. Oncogenic miRNAs: other miRNAs regulating the cell cycle in cancer

In addition to let-7 and the miR-17-92 cluster, a number of other miRNAs have been reported to interfere with cell cycle regulation, by targeting cyclin dependent kinases (CDK) or cyclin-dependent kinase inhibitors (CKI) (Figure 8). The Cip/Kip CDKI proteins, including p21, p27 and p57, inhibit CDK2 activity, thereby inhibiting cell cycle progression. Several studies demonstrate that the human miR-221/miR222 cluster is a direct regulator of p27. Enforced expression of miR-221/miR222 accelerated proliferation of glioblastoma cells, whereas the suppression of this cluster induced G1 arrest [104,105]. The INK4 cyclin-dependent kinase inhibitors, including p16, p15, p18 and p19 function in early G1 phase by competing with cyclin D for binding to partner CDKs. MiR-24 can regulate the expression of p16 in senescing cells, and miR-124 regulates CDK6 expression in medulloblastoma cells [106,107].

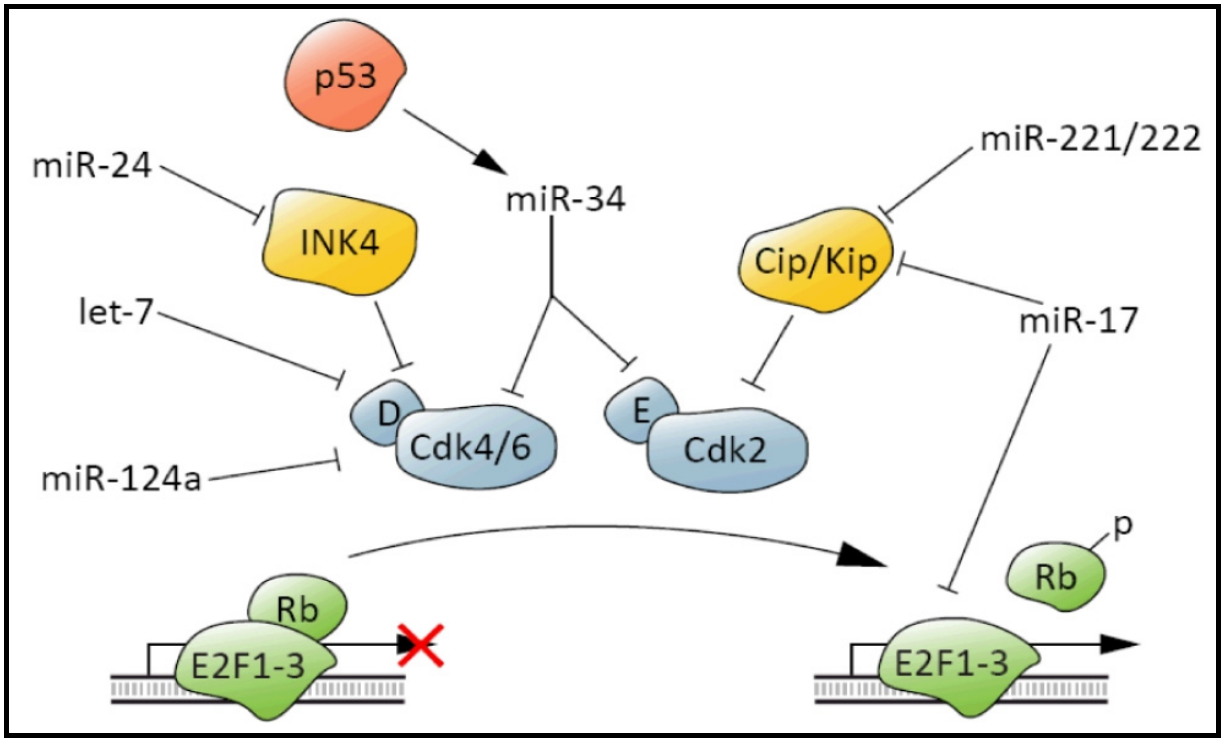


Figure 8. MiRNAs that regulate the G1 to S transition

In G1/S transition one of the most important events is the regulation of Rb phosphorylation. Rb protein (green) phosphorylation results the liberation of E2Fs (green) to activate gene expression required for S phase entry. Rb phosphorylation, in turn, depends on the coordinate regulation of cyclins (blue), cyclin-dependent kinases (blue), and Cdk inhibitors (yellow). Numerous miRNAs have been implicated in the direct or indirect regulation of this cellular pathway.

Chivukula RR, Mendell JT.: Circular reasoning: microRNAs and cell-cycle control. Trends Biochem Sci. 2008 Oct;33(10):474-81.

2.11.6. Oncogenic miRNAs: miR-155

MiR-155 is overexpressed in several types of lymphomas, including Burkitt's lymphomas and especially in aggresive B cell neoplasm, such as diffuse large B cell lymphoma (DLBCL). MiR-155 is located in the phylogenetically conserved region of the non-coding gene B-cell integration cluster (BIC), suggesting that miR-155 may be responsible for the activity of the BIC gene [108].

2.11.7. Oncogenic miRNAs: miR-21

Mir-21 is frequently overexpressed in many type of tumors, including glioblastoma, colorectal, lung, breast and pancreatic cancer. MiR-21 was first implicated as an anti-

apoptotic factor by the observation that knock-down of miR-21 increased apoptotic cell death in glioblastoma cells because of upregulation of its targets, the tumor suppressors phophatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4) [109,110]. Downregulation of miR-21 in breast cancer also inhibited both tumor growth in vivo and cell growth in vitro, as a result of increased expression of its target, BCL-2 [111,112]. In addition, miR-21 has been shown to promote metastasis by suppressing PDCD4, tropomyosin 1 (TPM1) and Maspin, which are negative regulators of migration and invasion [113,114].

2.11.8. MicroRNAs involved in the regulation of tumor invasion and metastasis

The development of metastases is the main reason for cancer-related death in many tumor types, inlcuding NSCLC and SCLC. Epithelial-mesenchymal transition is a critical step in tumor invasion and metastasis, and refers to the molecular reprogramming and phenotypic changes involved in the conversion of stationary epithelial cells to mobile mesenchymal cells. E-cadherin is a central component of the adherens junction complex responsible for cell-cell adhesion and maintainance of cytoskeletal organisation. Loss of E-cadherin expression has been identified as a causal factor in cancer progression. Several transcription factors, such as zinc finger E-box binding homeobox 1, 2 (ZEB1, ZEB2) and snail homolog (SNAI), also play a crucial role in EMT by binding to the E-cadherin promoter and repressing its transcription.

The miR-200 family was identified as a master regulator of EMT. The miR-200 family is organized in two clusters, miR-200b/a/429 and miR-200c/141. The main role of these miRNAs in EMT is the regulation of expression of ZEB1 and ZEB2, the transcriptional repressors of E-cadherin. Under normal conditions, miR-200 suppresses the expression of ZEB1, ZEB2, thereby favoring an epithelial phenotype. In response to appropriate signals, like transforming growth factor beta (TGFß), ZEB1 and ZEB2 expression is activated, leading to decreased expression of miR-200, thereby promoting EMT, invasion and metastasis (Figure 9). In line with this, ectopic expression of miR-200 caused up-regulation of E-cadherin in cancer cells and reduced their motility. Conversely, inhibition of miR-200 reduced E-cadherin expression and induced EMT [115,116]. Lastly, recent studies show that the loss of miR-200c expression induces an aggressive, invasive, and chemoresistant phenotype in NSCLC and that assessment of its expression could aid the better clinicopathologic definition of patients with NSCLC [117].

Several other miRNAs have also been identified as regulators of metastasis. MiR-125a-3p and miR-125a-5p mature miRNAs are processed from the complementary arms of the same precursor miRNA, and are both downregulated in NSCLC tumors. Interestingly, miR-125a-3p suppresses NSCLC cell migration and invasion, whereas 125a-5p enhances these cellular functions [118]. MiR-10b is a pro-metastatic miRNA and positively regulates cell migration and invasion in breast cancer [119]. MiR-373, miR-520c and miR-10b are all metastasis-promoting miRNAs [120], whereas miR-126 and miR-335 have been identified as metastasis suppressor miRNAs [121]. V-crk sarcoma virus CT10 oncogene homolog (CRK) proteins are considered to be the main mediators of the effect of miR-126 on metastasis, since CRK is a valid target gene of miR-126 in NSCLC, and CRK proteins are involved in the signalling pathways regulating cell adhesion, proliferation, and migration [122].

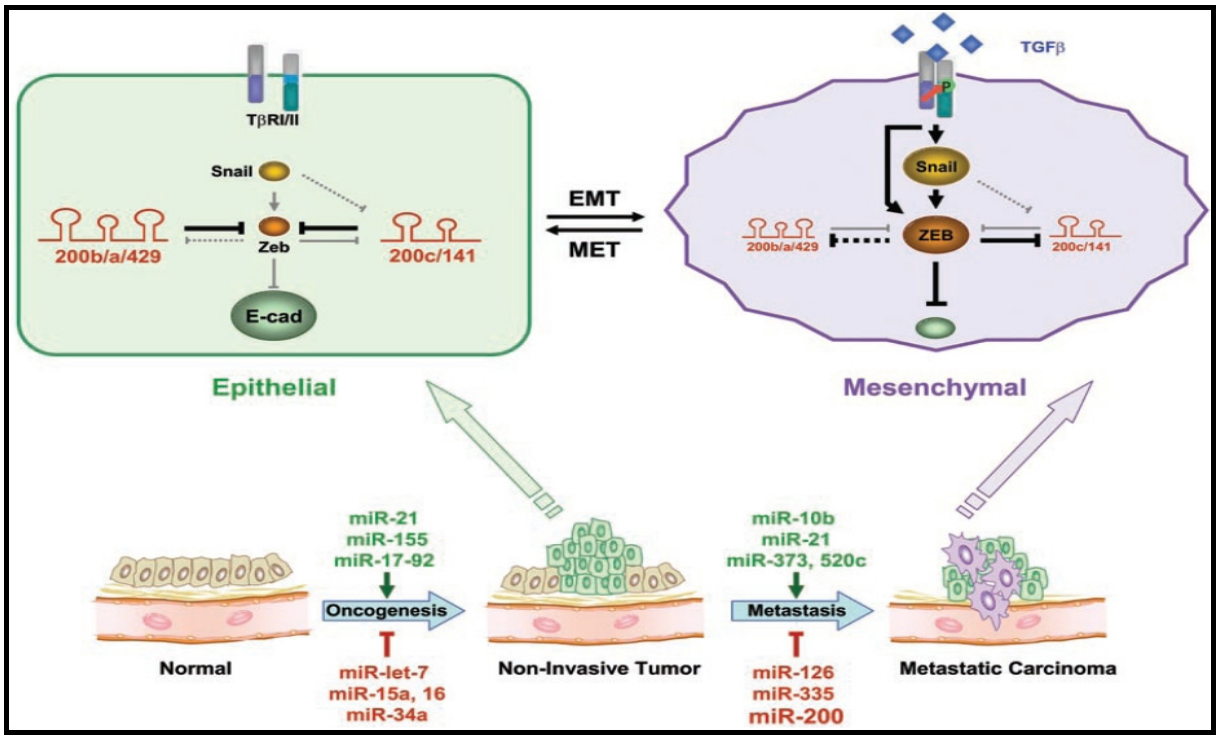


Figure 9 . MiRNAs involved in tumor progression

MiRNAs that play a role in tumor promotion are miR-21, miR-155, and the miR-17-92 cluster; miRNAs implicated in tumor suppression are miR-let-7, miR-15a, miR-16 and miR-34a. Several miRNAs have also been characterized as metastasis promoters such as miR-10b, miR-21, miR-373 and miR-520c, or metastasis suppressors such as miR-126 and miR-335.

Epithelial cells (left, green outline); mesenchymal cells (right, purple outline)

Solid lines show confirmed regulations and dashed lines indicate putative regulations.

Korpal M, Kang Y.: The emerging role of miR-200 family of microRNAs in epithelial-mesenchymal transition and cancer metastasis. RNA Biol. 2008 Jul-Sep;5(3):115-9. Review.

2.11.9. MicroRNAs involved in the regulation of angiogenesis in cancer

Angiogenesis is pivotal in tumor development. Vascular endothelial growth factor-A (VEGF-A) is one of the most important angiogenic factor and several miRNAs have been associated with vascular development. In the treatment of NSCLC, VEGF and its receptors can be targeted by agents such as bevacizumab, a monoclonal antibody that binds to VEGF and blocks interaction with its cell surface receptor.

Earlier knock-out studies in zebrafish and mice suggested a major role of miR-126 in angiogenesis and vascular integrity, which is mediated by the repression of inhibitors of VEGF-induced proliferation in endothelial cells [123-125]. Functional role of VEGF and miRNAs in the context of lung cancer was reported in one study where was shown that the expression level of miR-126 is low in various lung cancer cell lines and overexpression of miR-126 inhibits the growth of lung cancer in vitro and in vivo by downregulating VEGF [126]. Recent studies investigated the prognostic impact of miR-126 and its co-expression with VEGF-A in NSCLC patients, which identified miR-126 as a strong and independent negative prognostic factor for NSCLC [127].

The miR-17-92 cluster is also involved in the induction of angiogenesis. C-Myc activation, which is a potent inducer of angiogenesis, results in downregulation of anti-angiogenic factors, such as thrombospondin-1 (TSP1), connective tissue growth factor (CTGF) which are negatively regulated by miR-17-92 cluster [128].

MiR-9 is a MYC/MYCN-activated miRNA, which participates in the regulation of E-cadherin expression. MiR-9-mediated E-cadherin downregulation activates beta-catenin signalling, which contributes to upregulation of VEGF and leads to increased tumor angiogenesis [129]. Hypoxia-inducible factor-1α (HIF-1α) is one of the key regulators of tumor angiogenesis. HIF-1α expression is regulated indirectly by miR-17-92 (through c-myc) and directly by miR-519 under normoxic conditions, although these miRNAs do not affect the massive upregulation of HIF-1α under hypoxia [130,131].

In summary, regulation of angiogenesis by miRNAs suggests that anti-angiogenic therapy of cancer may be possible through novel miRNA-based therapies.

2.11.10. MicroRNAs as potential biomarkers and therapeutic targets in cancer

The previous paragraphs listed examples of miRNAs involved in cancer development and tumor progression. Compared to mRNAs, miRNAs are relatively stable in solid tissue samples, or in the serum and other body fluids. Therefore, the profiles of circulating miRNAs have been explored in a variety of studies aiming at the identification of novel non-invasive biomarkers [132,133]. The use of miRNAs as tumor markers for diagnosis and prognosis is under intensive research: if altered expression of a miRNA correlates with a malignant phenotype, it might be useful as diagnostic marker, and if altered expression of a miRNA predicts disease progress, such as future metastatic relapse, it may be utilized as a prognostic biomarker.

MiRNAs may also be used as therapeutic agents. If the expression of certain miRNAs is consistently reduced in a disease, miRNA-mimetics may be used. MiRNA mimetics are synthetic, double-stranded small RNAs containing the exact sequence of the endogenous miRNA. On the other hand, if the expression of certain miRNAs is increased in a disease, antagomiRs can be used to block miRNA expression specifically. Elmén and colleagues demostrated that uptake of the LNA-antimiR-122 in African green monkeys silences liver-specific miR-122 expression, and decreases total plasma cholesterol without hepatotoxicity [134]. LNA-antimiR antisense oligonucleotides are quite stable in vivo, and due to their low toxicity, they may be well-suited for the development of novel therapeutic approaches targeting cancer-associated miRNAs.

Delivery of RNA-mimetics or antagomiRs is a critical issue for effective therapy. The various chemical modifications in oligonucleotides may improve the delivery of oligonucleotides to tissues, but this is often associated with impaired biological activity and increased toxicity. Other strategies, such as nanoparticles, have recently become popular, in an attempt to enhance the cellular uptake and the pharmacological effectiveness of antisense oligonucleotides. Nanoparticle strategies provide improved delivery and stability with minimal toxicity.

MiRNAs regulate many genes, and the potential off-target effect of miRNA therapeutics is a major problems. These problems could be alleviated by engineering effective systems that deliver the synthetic miRNA oligonucleotides specifically to the diseased tissue and to cancer cells.

2.11.11. MicroRNAs and targeted therapies in lung cancer

Lung cancer is one of the leading causes of mortality in the world, indicating the need for innovative therapies for the disease. Numerous studies have documented that miRNAs participate in almost every step of carcinogenesis in lung cancer, including tumor development, apoptosis, invasion and metastasis, as well as anti-cancer drug resistance, making these tumor-specific miRNAs ideal target candidates for developing novel therapeutic approaches.

Deregulated expression of EGFR and abnormal EGFR signalling promotes tumor cell proliferation and survival in different types of cancer. EGFR is frequently overexpressed in lung cancer [135] and is an important therapeutic target; in fact, EGFR-tyrosine kinase inhibitors (EGFR-TKIs) are in clinical use for the treatment of lung cancer.

Several studies demostrated a relationship between the EGFR signalling pathway and miRNAs in lung cancer. MiR-128b is a regulator of EGFR in NSCLC. Loss of 3p22.3, which harbors miR-128b, is a frequent event in lung cancer and correlates significantly with clinical response and survival following gefinitib treatment (small–molecule tyrosine kinase inhibitor against EGFR) [136]. Interestingly, EGFR gene mutations are frequent even in lung tumors of never-smokers. MiR-21 expression is aberrantly increased in lung tumors of both never-smokers and smokers, and there is clearly a link between the activated EGFR signalling pathway and the aberrant upregulation of miR-21.
[137]. Aberrant EGFR signalling in lung cancer also induces miR-7 expression through the Ras/ERK/Myc pathway, and miR-7 in turn suppresses the level of ERF, a transcriptional repressor of Ets2. MiR-7 is overexpressed in human primary lung cancers and negatively correlates with disease free survival, showing the important role of miR-7 in cancer progression [138]. Therefore, it may be argued that antisense oligonucleotide-mediated knockdown of miR-21 or miR-7 as a combination therapy with EGFR-TKI has a good chance to improve clinical outcomes.

TNF-related apoptosis-inducing ligand (TRAIL) is also a promising anticancer agent with high specificity for cancer cells. In regard to miRNAs, in TRAIL-resistant NSCLC cells the levels of miR-221/222 are increased, and in line with that, overexpression of miR-221/222 induces TRAIL resistance and enhances cellular migration through targeting the PTEN and TIMP metallopeptidase inhibitor 3 (TIMP3) tumor suppressors [139,140]. Thus, suppression of miR-21 combined with TRAIL-therapy may also improve clinical outcomes.

3. AIM OF THE STUDIES

The aim of our first study was to characterize the miRNA expression profile of SCLC cell lines and primary SCLC tumors using miRNA microarray and qRT-PCR.
Many miRNA genes are found in chromosomal regions with frequent genetic aberrations in tumors. Some of the downregulated miRNAs are found in regions with frequent loss of heterozygosity in SCLC, but the overexpressed miRNAs are not embedded in chromosomal regions frequently amplified in SCLC. Therefore, we also analysed DNA copy number changes in SCLC cell lines and primary SCLC tumors for 5 genomic regions harboring prominently overexpressed miRNAs. Zsolt Czimmerer contributed equally to our first study.

The second aim of our work was to study the function of downregulated miRNAs using gain-of-function experiments. MiR-126 was uniformly downregulated miRNA in all SCLC samples, therefore, we studied the effect of miR-126 overexpression on SCLC cell proliferation and cell cycle progression.

MiRNAs can regulate gene expression by binding to the 3' UTR of target mRNAs, inducing mRNA degradation or translation repression. We aimed to identified a novel target of miR-126 contributing to its effects on SCLC cell proliferation.

4. MATERIALS AND METHODS

4.1. Cell lines and human tissue samples

SCLC cell lines were purchased from the American Type Culture Collection (HTB-172, HTB-173, HTB-184, HTB-119=H69). The cell lines were maintened in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C with 5% CO_2. Normal human lung tissue samples were obtained from the 2nd Department of Surgery at the University of Debrecen. Patients were heavy smokers, tumor-free, with an average age of 45 (range, 40-50), and had COPD. Peripheral blood samples were collected at the Pulmonology Clinic of University of Debrecen from tumor-free individuals. 23 archived formalin fixed, paraffin embedded (FFPE) SCLC tumor samples were obtained from the Department of Pathology at the University of Debrecen.
Eszter Csánky from the Pulmonology Clinic and Balázs Dezső from the Department of Pathology participated in clinical sample collection and characterization.

4.2. Microdissection of FFPE tumors

Blocks of tumors were cut to yield minimum 12 serial sections. The first and last sections (4 μm) were used for hematoxylin-eosin (HE) staining. 5 sections, cut at 20 μm to prevent loss of small RNA species during isolation, were used for microdissection. These sections were microdissected by an experienced pathologist to separate tumor and normal tissue, under direct observation with a microscope, guided by the HE-stained sections, using a fine needle. The remaining 4 μm sections were used for immunohistochemical staining. Microdissection of tumors were performed by Balázs Dezső in the Department of Pathology.

4.3. Nucleic acid extraction

Total RNA was isolated from surgical lung samples and SCLC cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA was extracted from SCLC cell lines and peripherial blood mononuclear cells (PBMC) using the Qiagen Blood and Cell Culture kit, according to the instructions of the manufacturer. Total RNA and DNA was isolated from FFPE samples with RecoverAll Total Nucleic Acid

Isolation Kit (Ambion) according to the manufacturer's protocol. Nucleic acid isolations were performed by Gábor Boros and Júlia Buslig.

4.4. Experimental design

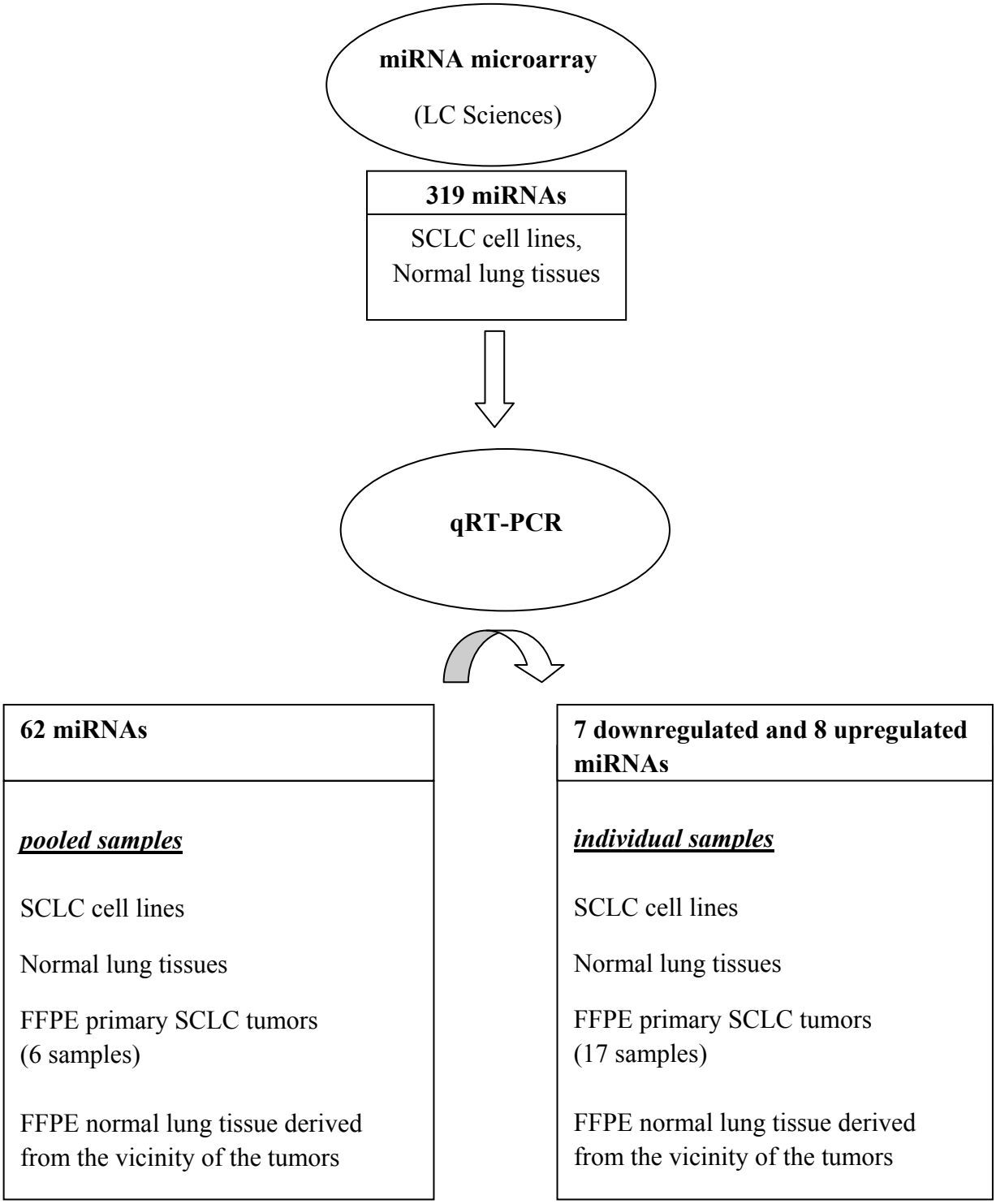


Figure 10. Sematic representation of the experimental design

4.5. Microarray analysis.

MiRNA profiling of normal lung and SCLC cell lines was performed using a service provider (LC Sciences, Houston, TX, http://www.lcsciences.com, miRNA dual-color microarray platform, Human V7.1). Briefly, the assay started from 2 to 5 ug total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter (from Millipore) and the small RNAs (< 300 nt) isolated were 3'- extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a uParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target miRNA (from miRBase, http://microrna.sanger.ac.uk/sequences/) or other RNA (control sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using PGR (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 uL 6xSSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34 °C. After hybridization detection used fluorescence labeling using tagspecific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics).

The microarray interrogated 319 mature miRNAs, each represented by 4 individual probes on the chip. RNA from 3 SCLC cell lines (H69, HTB-172, HTB-184) was used for the microarray screen, compared to RNA from fresh surgical samples of lung tissue as normal control. RNA from primary SCLC tumors was available only from FFPE samples, since SCLC patients are rarely operated for their cancer. However, RNA isolated from FFPE tumor samples could not be used in the microarray experiment, mainly because the FFPE sections did not provide enough material for the microarray platform. Normal lung RNA was pooled from 6 samples isolated from surgical material, and the three SCLC cell lines were assayed individually on 3 chips, each chip comparing the expression profile of one cell line to the common normal lung reference.

MiRNAs with raw signals above the averaged microarray background +5 STDEV were selected for further analysis. Data was normalized to hsa-miR-342, and analyzed by the GeneSpring 7.3 software. Relative miRNA expression values (SCLC cell lines relative to normal lung) were derived after normalization from averaged values of 3 SCLC cell line microarrays, and averaged values of 3 normal lung microarrays. MiRNAs showing statistically significant 5-fold higher or lower relative expression were selected for further analysis by qRT-PCR (paired t-test, two-tailed p value, CI 95%).

4.6. MiRNA real-time quantitative RT-PCR analysis.

Expression of selected miRNAs were tested by qRT-PCR analysis either by using the TaqMan MicroRNA Assays Early Access Kit or by individual miRNA qRT-PCR assays, according to the manufacturer's protocol, with minor modifications (Applied Biosystems). In the first screen we assayed 63 miRNAs, on pooled RNA samples isolated from 6 FFPE SCLC tumor samples. Pooling of RNA in this experiment was necessary due to the limiting amount of RNA obtained from the FFPE sections. In subsequent experiments pooling of the RNA samples was no longer necessary, because we assayed fewer miRNAs per sample (7 downregulated and 8 overexpressed miRNAs in 17 SCLC tumors). qPCR was performed on the ABI 7900 HT Sequence Detection System. Relative miRNA expression values (tumor vs normal) were calculated using the 2-ddCt method [141]. MiRNA expression levels in FFPE SCLC primary tumor samples were compared to their own FFPE normal lung tissue counterparts, isolated from the vicinity from the tumor. MiRNA expression levels in SCLC cell lines were compared to fresh surgical normal lung tissue. The normalizing gene was hsa-miR-342, which showed the smallest variation between normal lung, SCLC cell lines, and primary tumors (Figure 13)

4.6.1. Multiplex and Singleplex reverse transcription.

In the pooled samples expression of 43 miRNAs was determined with multiplex RT followed by qPCR. Since some miRNA assays performed poorly after multiplex RT priming, but worked with singleplex RT, expression of 20 other miRNAs were analyzed with singleplex RT using the protocol suggested by the manufacturer, except for using 50 nM

stem-loop RT primer and 50 ng RNA. The primary SCLC tumors analyzed individually were all reverse transcribed in singleplex RT reaction.

For multiplex RT, 4 different RT primer mixes were prepared, each containing 15 to 20 different stem-loop RT primers. Reverse transcriptase reactions contained 500 ng of RNA samples, 5 nM of each of stem-loop RT primer (Primer mixes I-IV), 1X RT buffer, 1 mM each of 4 dNTPs, 3.33 U/µl MultiScribe reverse transcriptase (all from the High Capacity cDNA Archive Kit, Applied Biosystems) and 0.25 U/µl RNasine Inhibitor (Invitrogen), in a 15 µl final volume. Reactions were incubated in a thermocycler (DNA Engine DYADTM) for 30 min at 16°C, followed by 60 cycles of 30 sec at 20°C, 30 sec at 42°C, 1 sec at 50°C, and final incubation of 5 min at 85°C.

4.6.1.1. RT primer mixes for multiplex reverse transcription.

Primer mix I.
For miRNAs hsa-mir-100, 125b, 145, 200b, 200c, 221, 222, 26a, 30c, 30d, 320, 324-5p, 335, 342, 374, 9, 9*, 92, 95, 98.
Primer mix II.
For miRNAs hsa-mir-106a, 150, 17-5p, 181c, 182, 186, 191, 199a, 19a, 200a, 21, 210, 25, 26b, 27a, 27b, 29a, 301, 30b
Primer mix III.
For miRNAs hsa-mir-103, 132, 141, 148a, 151, 15a, 15b, 16, 181a, 181b, 183, 185, 195, 214, 223, 23a, 23b
Primer mix IV.
For miRNAs hsa-mir-105, 107, 10a, 125a, 126, 128a, 128b, 130a, 130b, 194, and hsa-let-7a, let-7b, let-7d, let-7e, let-7g.

4.6.1.2. Real-time quantitative PCR with the TaqMan MicroRNA Assays Early Access Kit.

The 20 µl PCR reaction mixture contained 2 µl RT product (singleplex RT) or 0.15 ul RT product (multiplex RT), 1X PCR buffer, 2.5 mM MgCl2, 0.1 mM each of 4 dNTPs, 0.5 µl 0.025 U/µl Taq polymerase (Invitrogen), 1X ROX (Invitrogen) and 2 µl of primers and probe mix of the TaqMan microRNA assays (Applied Biosystems). The reactions were incubated in

a 96-well optical plate at 95 °C for 10 min, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C.

4.6.1.3. Real-time quantitative RT-PCR with the individual TaqMan MicroRNA Assays.

cDNA was synthesized from total RNA using miRNA-specific stem-loop RT primers supplied by Applied Biosystems. RT reactions contained 10 ng of RNA samples, 25 nM stem-loop RT primer, 1X RT buffer, 0.25 mM each of 4dNTPs, 1 µl of 3.3 U/µl MultiScribe reverse transcriptase and 1 µl of 0.25 U/µl RNase Inhibitor (cDNA Archive Kit, Applied Biosystems), in a 10 µl final volume. The reaction mixture was incubated in a thermocycler for 30 min at 16 °C, 2 h at 42 °C, and 5 min at 85 °C. qPCR was carried out as previously described with the following modification: 2 µl RT product and 0.5 µl of primers and probe mix of the TaqMan MicroRNA assays (Applied Biosystems) were used.

4.7. Copy number analysis.

Five primer sets were designed to measure the genomic copy number of five miRNAs (miR-183, miR-182, miR-95, miR-301 and the miR-17-92 cluster) in SCLC cell lines (H69, HTB-172, HTB-173, HTB-184), in four FFPE SCLC tumors and their normal tissue counterparts isolated by microdissection. PBMC genomic DNA was mixed from the samples of 5 tumor-free individuals. Relative gene copy numbers were calculated using the 2-ddCt method. Since tumor cell lines and primary tumors harbor many amplified chromosomal regions, we tested several candidate normalizing genes. Out of these, cyclophilin A (PP1A), TATA box binding protein (TBP) and H19, imprinted maternally expressed transcript (H19) proved to be suitable candidates to normalize miRNA copy number in our samples. Copy numbers of the five miRNA genomic regions, and the TBP and H19 normalization genes were measured using SYBR Green I qPCR, and TaqMan assay was used to quantify PP1A (Table 2, Table 3). Ct values for miRNAs and the normalizer genes were the average of 3 or 5 independent PCR measurements, each sample tested in duplicate in each PCR. Significance of differences for the means was assessed with paired t-test, using two tailed p value, CI 95%.

Table 2. **Primer sequences, positions and amplicon lengths of the genomic qPCR assays for five miRNAs**

Symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
hsa-miR-183	GCCATAAACAGAGCAGAGAC AGAT	AGCGGAGAGACACAAGCAAAAAT	210
hsa-miR-182	CCATCCTAACTGTCTCTGTCTC TTCC	GACCTGAGTCCCCTCCTTCCTC	248
hsa-miR-301	AAGATGCTTTTGTGTTCCTT	ATCCTGCTTTCAGATGCTTTGACA	119
hsa-miR-95	TCACCAAGTAGCCCCCAGAC	CAACAGACATTTATTGAGTGCCCA	185
hsa-miR-17-92	AGTTGATTCCAGGCTTATTTG ACTT	TCAGGTATCCCACATTATTCTACACA	155

Table 3. **Primer sequences, positions and amplicon lengths of the genomic qPCR assays for the three reference genes**

Symbol	Ensembl ID	Forward primer (5'-3')	Size (bp)
TBP	ENSG00000112592	AGAGCTTCGCCCCTCCTTACCTATG	274
		Reverse primer (5'-3')	
		GTACTCTTTTCTGACGGTTCGGGCG	

Symbol	Ensembl ID	Forward primer (5'-3')	Size (bp)
H19	ENSG00000130600	ACAGGGTCTCTGGCAGGCACAGAAA	222
		Reverse primer (5'-3')	
		CGTCCTGGGTCACCCAAGCCAGGTG	

Symbol	Ensembl ID	Forward primer (5'-3')	Size (bp)
PPIA	ENSG00000196262	ACGGCGAGCCCTTGG	154
		Reverse primer (5'-3')	
		CCCTGACACATAAACCCTGGAAT	
		Probe (5'-3')	
		FAM-CGCGTCTCCTTTGAGCTGTTTGCA-TAMRA	

4.7.1. SYBR Green I qPCR

The reaction mixture contained 1X SYBR Green I buffer [10 mM Tris-Cl (pH 8.3), 50mM KCl, 0.01 % TWEEN 20, 0.8% glycerol], 2.5 mM MgCl2, 0.1 mM 4dNTP (Fermentas), 0.3 μM of forward and reverse primers (Sigma Genosys), 1X ROX Reference Dye (Invitrogen), 0.0125 U Brazilian Taq DNA polymerase (Invitrogen), 1X SYBR Green I (Molecular Probes) and 50 ng genomic DNA in a total volume of 25 μl. Real-time quantitative PCR was performed on an ABI PRISM 7900 HT Sequence Detection System in 96-well optical plates (Applied Biosystems). The following PCR program was used for amplification: 95°C for 30 sec, followed by 40 cycles of 95°C for 30 sec, 65-68°C for 30 sec, 72°C for 45 sec. To avoid detection of primer-dimers, the fluorescence signal acquisition was set as indicated in Table 4. A melting curve was generated by heating the sample to 95°C for 15 sec followed by cooling down to 60°C for 15 sec and slowly heating the sample to 99°C for 15 sec, while the fluorescence was measured continuously.

Table 4. Characteristics of the SYBR Green I qPCR programs

	annealing temperatures	signal acquisition
miR-183	68 ºC	80 ºC
miR-182	68 ºC	80 ºC
miR-95	68 ºC	80 ºC
miR-301	65 ºC	80 ºC
miR-17-92	65 ºC	72 ºC
TBP	68 ºC	91 ºC
H19	65 ºC	80 ºC

4.7.2. TaqMan qPCR

The reaction mixture contained 1X PCR buffer (Invitrogen), 2.5 mM MgCl2 (Invitrogen), 0.1 mM 4dNTP (Fermentas), 0.3 μM forward and reverse primers, 0.16 μM TaqMan probe, 1X ROX Reference Dye (Invitrogen), 0.0125 U Brazilian Taq DNA

polymerase (Invitrogen), and 5 µl of 1:10 diluted template cDNA in a total volume of 25 µl. Amplification was performed starting with a 1 min denaturation step at 94 °C, followed by 40 cycles of 94 °C for 12 sec and 60 °C for 1 min.

4.8. QRT-PCR and semi-quantitative PCR

For quantification of polo-like kinase 2 (PLK2), solute carrier family 7, member 5 (SLC7A5) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNAs, mature miR-126 and RNU38B, reverse transcription was performed by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT primers for mature miR-126 and RNU38B were supplied by Applied Biosystems. qPCR was performed with FastStart SYBR Green Master Mix (Roche) with 0.3 µM of forward and reverse primers on an ABI 7900 HT Sequence Detection System. The PCR program used for amplification was: 95°C 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec. HPRT1 was used for normalization. Primer sequences are listed in Table 5. The same primers were used for semiquantitative RT-PCR of mature miR-126 and RNU38B. The PCR program used for semi-quantitative amplification was: 95°C for 10 min, followed by 15 cycles of 15 sec at 95°C and 60 sec at 60°C. PCR products were separated by agarose gel electrophoresis and visualized by EtBr staining. Semi-quantitative RT-PCR analysis was performed by Zsolt Czimmerer.

Table 5. qRT-PCR Primer sequences (5'-3')

PLK2 (FW)	AGA TCT CGC GGA TTA TCG TC
PLK2 (REV)	TGT CAA ATC TGT CAT CTC GTA ACA
SLC7A5 (FW)	ACG GCC GTG AAC TGC TAC
SLC7A5 (REV)	GGA TCT AGA TTG GAC ACA TCA CC
HPRT1(FW)	TGA CCT TGA TTT ATT TTG CAT ACC
HPRT1(REV)	CGA GCA AGA CGT TCA GTC CT

4.9. Reagents and transfection

MiRNA Precursor Molecule hsa-miR-126 (PM12841) and Pre-miR Negative Control ≠1(AM17110) were obtained from Ambion. siRNAs targeting SLC7A5 (s15653), PLK2 (s64) and Silencer Select Negative Control≠1 siRNA (4390843) were obtained from Applied Biosystems. Cells were transfected with pre-miR-126, SLC7A5, PLK2 siRNAs and controls at a final concentration of 50 nM in all experiments, using Lipofectamine 2000 (Invitrogen). Pre-miR Negative Control 1 miRNA precursor is a nontargeting sequence that bears no homology to the sequences of human, mouse or rat transcript. Cells were incubated with the transfection complexes for 6 h before replacing the medium. Cells were refed daily with fresh growth medium.

4.10. Establishing the growth curves

H69 cells: 1.2 x 10^6 cell/well were plated in 6-well plates and transfected with miRNA precursors. 48 hours post-transfection 1/10^{th} of the cells were replated in 24-well plates, and grown further. Cell numbers were determined by trypan blue exclusion at different time points (48h, 72h, 96h) post-transfection. Results represent the mean of three independent experiments.

HTB-172 cells: 1.2 x 10^6 cell/well were plated in 6-well plates and transfected with pre-miR-126 or NegMiR (negative control). 6 hours post-transfection cells were replated in 96-well plates (5000 cell/well), and grown further. Cell numbers were determined by the EZ4U Cell Proliferation Assay (Biomedica) at 48 hours and 72 hours post-transfection.

4.11. Cell cycle analysis

1.2 x 10^6 cell/well were plated in 6-well plates and transfected with pre-miRs and siRNAs. 48 hours post-transfection 1/5^{th} of the cells from each transfection were replated and grown further. Cells were harvested at 72 and 96 hours post-transfection, washed in PBS and fixed in ice-cold 95% methanol at -20 °C. Fixed cells were washed twice in PBS, then resuspended in 0.5 ml PBS containing propidium iodide (50 ug/ml) and RNase A (200 ug/ml) and were stained overnight at 4 °C.

Measurements were made on a FACSArray 96-well plate flow cytometric bioanalyzer (Becton Dickinson). The DNA dye was excited with the 532 nm laser line and emission was collected in the yellow channel in linear mode with a 585/42 nm bandpass filter. Cell clusters were gated out using FSC-A/SSC-A (Area) and FSC-W/SSC-W (Width) 2D-histograms. Fluorescence intensity data were fit with the automatic one cycle diploid model of the *Modfit LT 3.0* software (Verity Software House) with the AutoDebris Compensation, AutoAggregate Compensation and Apoptosis Model turned on. In the measurements the G1-G2 linearity ratio was around 1.92 and the R.C.S. of the fit (reduced chi-square, a measure of goodness of fit) was less than 5. All samples were prepared in triplicates and generally 50,000 cells were collected from each well. FACSArray analysis was performed by our collaborators Zsolt Bacsó and Zoltán Margitai.

4.12. Western blot

H69 cells were washed with PBS and lysed in 2X SDS loading buffer. Proteins were separated on 10% SDS polyacrylamide gels and transferred onto nitrocellulose membrane by electroblotting. Membranes were blocked with 2.5 % nonfat milk, and probed with primary antibody against human SLC7A5 (3157-1, 1:1000, Epitomics), PLK2 (Snk/H90, sc-25421, 1:100, Santa Cruz Biotechnology) and GAPDH (6C5, sc-32233, 1:1000, Santa Cruz Biotechnology). The membranes were further probed with horseradish peroxidase-conjugated secondary antibodies (1:10,000; anti-mouse or anti-rabbit; Amersham) and proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Pierce). I performed the Western blot experiments in Árpád Lányi's laboratory in the Institute of Immunology.

4.13. SLC7A5 3' UTR cloning and luciferase reporter assay

For luciferase reporter assays, 331 bp from 3'UTR of SLC7A5 gene, including the miR-126 target site, was amplified by PCR using F1 and R1 primers with XhoI and NotI sites. PCR was performed on H69 cDNA created by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The XhoI/NotI-digested PCR product was cloned into the XhoI/NotI digested psiCHECK2 dual luciferase vector (Promega). F1/R2 and F2/R1 primers were used to delete the miR-126 target site from the 3'UTR. After mixing the two PCR products, and digestion with XhoI and NotI, the 3'UTR fragment with a deleted miR-126

binding site was cloned into XhoI/NotI-digested psiCHECK2 vector. I performed the cloning and mutagenesis, under the supervision of Á. Lányi. Primer sequences are listed in Table 6. H69 cells were cotransfected with 500 ng psiCHECK2 constructs (WT-UTR or DEL-UTR) and 50 nM pre-miR-126 or pre-miR-negative control in 6-well plates. 48 hours post-transfection, firefly and renilla luciferase activities were measured using Dual-Glo Luciferase assay system (Promega) in a Perkin Elmer Victor3 V Multilabel Plate Reader. The ratio of the luminescent signals from renilla versus firefly was used to determine the target specificity of miR-126. All experiments were done in triplicate.

Table 6. Primer sequences for SLC7A5 3'UTR subcloning and mutagenesis (5'-3')

F1/ with XhoI site	TAA TCT CGA GCA TGC GCA GAG GCC AGT T
R1/ with NotI site	CTT GGC GGC CGC AAA TCA TCT TAG TGG TGT C
R2	GTT GAG GGA TGA GAT GAG TTT TCA CAG CAG
F2	CTG CTG TGA AAA CTC ATC TCA TCC CTC AAC

4.14. Statistical analyis

Statistical analyis was done using GraphPad Prism IV software. P values were calculated by paired t test. P values < 0.05 were considered significant.

4.15. Immunohistochemistry

Tissue microarrays of formalin-fixed paraffin-embedded surgical specimens representing primary SCLC tumors were constructed. These tumor specimens were characterised in our study as chromogranin-A/synaptophysin and thyroid-transcription factor-1 (TTF1) positive tumors (>70%). Following hematoxylin-eosin staining, serial sections and antigen retrieving were made for immunohistochemistry (IHC) labeling using rabbit monoclonal antibody to SLC7A5 (1:300 dilution; Epitomics) for 1 hour at room temperature. Envision (biotin-free) peroxidase-based detection kit (Dako, Glostrup, Denmark) for mouse/rabbit antibodies was then used with the red AEC or brown DAB substrate-chromogen

(Vector Labs) followed by hematoxylin nuclear counterstaining. Alternatively, we performed double immunofluorescent (IF) staining where SLC7A5 antibody was visualized with the use of horse-radish peroxidase (HRP)-coupled anti-rabbit IgG$(Fab)_2$ and tyramide-FITC for green fluorescence followed by mAB to TTF1 and biotinylated secondary antibody treatments (all from Dako), developed with streptavidin-texas red for red fluorescence (Vector). Finally, tissue-reactivities for the antibodies (percentage of positive cells) were evaluated for each case by using a 3D-Histotech-Zeiss slide-scanner and Mirax-viewer software program (3D-Histotech, Budapest, Hungary). Immunohistochemistry was performed by our collaborators Balázs Dezső and Ildikó Várkonyi.

5. RESULTS

5.1. MiRNA microarray expression profiling of SCLC cell lines and normal lung.

The LC Sciences miRNA microarray platform, interrogating 319 mature miRNAs was performed to investigate the differential expression of miRNAs in SCLC. The miRNA expression profile of normal lung and three SCLC cell lines: HTB-184, HTB-172, and H69 was characterized. The reference normal lung RNA was pooled from 6 RNA samples isolated from surgically removed lung tissue. Since the patients providing the samples were tumor-free, but middle-aged and heavy smokers, the miRNA expression profile of "normal" lung probably reflects the effects of age, smoking, and chronic inflammation evident in smoker's lung as well.

142 miRNAs were detected above background levels in both sample types, 32 miRNAs in normal lung only, and 57 miRNAs in SCLC cell lines only – in contrast, signals for 88 miRNAs were below background in both sample types. The three replica arrays for normal lung demonstrated that the individual arrays have low variability.

We identified 19 significantly overexpressed miRNAs, with relative expression levels of at least 10-fold in SCLC cell lines compared to normal lung (Table 7). MiR-9 and miR-7 showed the highest overexpression (> 50-fold) in SCLC cell lines, and miR-183, miR-182, miR-206, miR-95, miR-375 were expressed over 30-fold higher in SCLC cell lines than in normal lung tissue. We also identified 34 miRNAs that are downregulated in SCLC cell lines compared to normal lung, and a large region in 19q13.41 containing more than 30 downregulated miRNAs (miR-512 to miR-373) (Table 7).

Several differentially expressed miRNAs are organized in clusters, such as the miR-17-92 cluster, the miR-200b-429 cluster, or the miR-145/143 cluster and are transcribed as polycistronic transcripts. MiRNAs in one cluster are usually up- or downregulated in concert, indicating that they are members of the same regulatory unit. It is also evident that miRNAs present in multiple copies on different chromosomes (miR-29b, miR-199a), or closely related miRNAs (miR-199a/b, miR-200a/b/c, miR-181- c/d, miR-106a-363 and miR-17-92 clusters) are often coordinately regulated.

miRNA	Chr	Rel. expression	p value
miR-200b	1	12.11	<.01
miR-200a	1	6.99	.04
miR-429	1	12.03	.04
miR-10b	2	13.58	.04
miR-128a	2	5.43	.01
miR-375	2	39.45	<.01
miR-128b	3	6.45	.01
miR-95	4	40.64	<.01
miR-206	6	39.17	<.01
miR-25	7	12.49	<.01
miR-93	7	10.81	<.01
miR-106b	7	7.42	<.01
miR-182	7	30.92	.01
miR-96	7	2.37	.02
miR-183	7	33.55	.01
miR-335	7	13.48	.04
miR-200c	12	4.91	<.01
miR-141	12	1.47	<.01
miR-17-5p	13	12.35	<.01
miR-18a	13	6.4	<.01
miR-19a	13	6.86	<.01
miR-20a	13	12.49	<.01
miR-19b-1,2	13,X	3.68	<.01
miR-92-1,2	13,X	6.40	<.01
miR-494	14	7.88	<.01
miR-301	17	7.18	<.01
miR-181c	19	0.75	<.01
miR-181d	19	9.15	<.01
miR-130b	22	14.06	<.01
miR-374	X	22.35	<.01
miR-363	X	0.47	.20
miR-92-2,1	X,13	6.40	<.01
miR-19b-2,1	X,13	3.68	<.01
miR-20b	X	20.70	<.01
miR-18b	X	4.15	<.01
miR-106a	X	15.18	<.01
miR-9-1,2	1,5	58.64	<.01
miR-7-1,2,3	9,15,19	54.35	<.01

miRNA	Chr	Rel. expression	p value
miR-34a	1	.046	.09
miR-214	1	.009	<.01
miR-199a-2	1	.004	.01
miR-135b	1	.028	.01
miR-29c	1	.035	<.01
miR-29b-2,1	1,7	.018	<.01
miR-302c	4	.012	.01
miR-143	5	.001	<.01
miR-145	5	<.001	<.01
miR-146a	5	.005	<.01
miR-133b	6	.029	.03
miR-490	7	.025	.03
miR-29a	7	.063	<.01
miR-29b-1,2	7,1	.018	<.01
miR-32	9	.040	.03
miR-199b	9	.020	.03
miR-126	9	.005	.02
miR-511-1,2	10	.010	<.01
miR-125b	11,21	.046	<.01
miR-100	11	.019	<.01
miR-190	15	.017	.02
miR-497	17	.014	.02
miR-451	17	<.001	<.01
miR-142-3p	17	.015	<.01
miR-142-5p	17	.025	<.01
miR-338	17	.059	.05
miR-150	19	.002	<.01
miR-512/373 (over 30 miRNAs)	19	Ave:0.028	<.01
miR-296	20	.019	.02
miR-155	21	.005	<.01
miR-33	22	.030	.03
miR-502	X	.053	.03
miR-223	X	.003	.01
miR-222	X	.186	.02
miR-325	X	.021	<.01
miR-506	X	.014	<.01
miR-514-1,2,3	X	.010	<.01
miR-224	X	.037	<.01

Table 7. MiRNA microarray profiling of SCLC cell lines H69, HTB-184 and HTB-172 , compared to normal lung

Clustered miRNAs are boxed. MiRNAs quantified by qRT-PCR as well are bold-faced.

Chr=Chromosomal localization. Significance was assessed by paired t-test, two-tailed p value, CI95%.

5.2. Validation of miRNA expression profile with qRT-PCR.

Microarray technique has lower sensitivity compared with real-time qRT-PCR, therefore, validation of the microarray results by qRT-PCR is critical. Based on the microarray experiment we selected 21 significantly up- or downregulated miRNAs for further validation; in addition, we also selected 41 miRNAs because of their potential role in cancer biology, based on literature data.

Expression levels of the 62 (+1 normalizer) miRNAs were validated by qRT-PCR in four sample types:

a) three SCLC cell lines (pooled RNA from H69, HTB-184 and HTB-172)

b) normal lung tissue samples (pooled RNA from 6 samples)

c) FFPE primary SCLC tumor samples (pooled RNA, isolated from 6 samples)

d) FFPE normal lung tissue derived from the vicinity of the tumors (pooled RNA, isolated from 6 samples).

The pooled RNA from 6 normal lung samples was the same that was used for the microarray experiment. Sections from FFPE tumor samples were microdissected under the microscope and RNA was isolated separately from the normal lung tissue and the tumor tissue (Figure 11). Although the microdissected tumor tissue samples still contained some normal cells, they can be considered significantly enriched for tumor cells. Successful separation of normal tissue from tumor tissue was verified by the barely detectable NSE mRNA levels in the FFPE normal lung samples, versus the high expression of NSE in the enriched SCLC tumor samples, as determined by qRT-PCR (Figure 12).

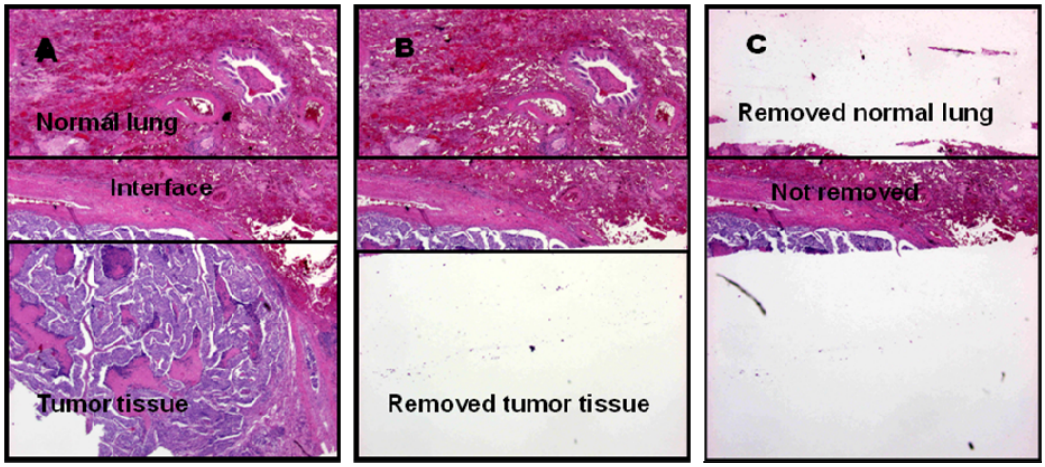


Figure 11. Stepwise microdissection

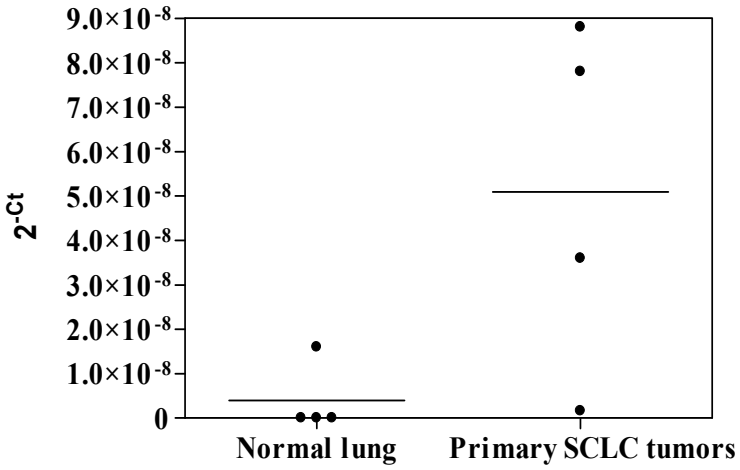


Figure 12. **qRT-PCR of NSE in the FFPE normal lung samples and SCLC tumor samples**

In the qRT-PCR validation we identified 16 miRNAs that are overexpressed in primary SCLC tumors, as well as in SCLC cell lines (Table 8, rows 1-16). MiR-105, miR-301 and miR-17-5p were expressed over 40-fold higher in SCLC cell lines than in normal lung tissue, and miR-17-5p showed the highest overexpression (16.67-fold) in primary SCLC tumor samples. It was previously described that miR-17-92 cluster is markedly overexpressed in SCLC and may play a role in the development of lung cancers [99].

qRT-PCR analysis also confirmed downregulation of 8 miRNAs in primary SCLC tumors and in SCLC cell lines (Table 8, rows 17-24). To detect miRNA downregulation in primary SCLC tumor samples was more difficult than in SCLC cell lines. This occured probably because of the presence of normal cells in the tumor samples, which probably decreased the difference between tumor and normal for several miRNA. The average relative expression level changes were 0.07-fold in SCLC cell lines, compared to 0.23-fold in pimary SCLC tumor samples for the same 8 miRNAs. The normalizing gene was hsa-miR-342, which showed the smallest variation between normal lung, SCLC cell lines, and primary tumors (Figure 13).

		Primary SCLC tumors	SCLC cell lines
		S1-6, pooled	Pooled
1	miR-17-5p	16,67	41,53
2	**miR-301**	13,09	51,23
3	miR-98	13,08	18,26
4	**miR-106a**	12,42	34,08
5	miR-335	11,52	15,61
6	**miR-95**	10,93	17,66
7	miR-19a	6,94	6,38
8	**miR-374**	6,79	4,16
9	miR-96	6,31	5,28
10	miR-200a	5,96	3,56
11	miR-182	5,85	12,63
12	**miR-25**	4,45	5,33
13	miR-210	4,17	12,67
14	**miR-183**	3,02	17,34
15	**miR-105**	2,68	53,93
16	miR-200c	2,21	4,80
17	**miR-29a**	0,44	6,07E-02
18	miR-125a	0,44	0,42
19	**miR-199a**	0,38	6,86E-03
20	**miR-214**	0,19	1,43E-02
21	**miR-223**	0,16	1,34E-04
22	**miR-126**	0,11	2,13E-03
23	**miR-150**	0,10	3,83E-04
24	miR-27a	0,04	1,67E-02

		Primary SCLC tumors	SCLC cell lines
		S1-6, pooled	Pooled
25	miR-324-5p	4,44	2,16E-08
26	miR-15a	2,35	0,21
27	miR-130b	2,31	1,63
28	miR-10a	2,12	1,26
29	miR-125b	1,90	9,21E-02
30	miR-186	1,78	3,51
31	miR-194	1,53	0,30
32	miR-27b	1,53	0,81
33	let-7e	1,50	1,16
34	miR-21	1,48	0,72
35	miR-191	1,42	0,38
36	miR-15b	1,38	0,55
37	miR-141	1,33	0,40
38	**miR-185**	1,27	0,30
39	let-7a	1,27	1,11
40	miR-148a	1,26	0,20
41	let-7d	1,24	1,27
42	let-7b	1,24	0,70
43	miR-181c	1,12	0,72
44	miR-30c	1,08	0,78
45	miR-132	1,05	0,29
46	miR-103	1,03	0,39
47	*miR-342*	*1,00*	*1,00*
48	miR-26a	0,99	0,29

		Primary SCLC tumors	SCLC cell lines
		S1-6, pooled	Pooled
50	miR-26b	0,97	0,49
51	miR-16	0,94	0,16
52	miR-151	0,93	0,42
53	let-7g	0,92	0,44
49	miR-320	0,91	1,22
54	miR-23a	0,74	5,85E-02
55	miR-100	0,74	0,06
56	**miR-222**	0,73	1,93
57	miR-30d	0,64	0,21
58	miR-30b	0,63	0,15
59	miR-23b	0,63	0,11
60	miR-130a	0,56	0,16
61	miR-195	0,55	0,14
62	miR-181a	0,55	0,09
63	miR-181b	0,43	1,43

Table 8. Relative expression values of miRNAs measured by qRT-PCR

RNA isolated from 6 microdissected, primary SCLC tumors (S1-6) and H69, HTB-184, and HTB-172 SCLC cell lines were pooled before RT-qPCR. miR-342 is the normalizing miRNA. miRNAs in rows 1–24 (left side of figure) show at least two-fold change in both primary tumors and in SCLC cell lines when compared to normal lung. miRNAs in bold were also quantified in additional primary SCLC tumor samples (see results in Figure 14 A, B).

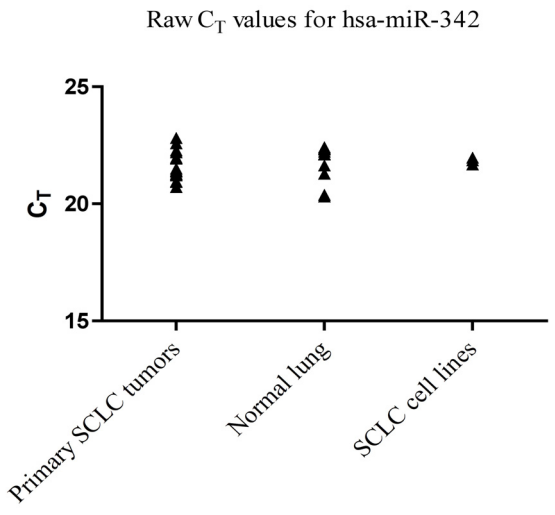


Figure 13. Ct values of miR-342 measured by RT-qPCR

MiR-342 was used as a normalizing gene in our experimental system, because this gene showed the smallest variation between normal lung, SCLC cell lines, and primary tumors.

5.3. Expression patterns of selected miRNAs in SCLC cell lines and primary SCLC tumors.

7 downregulated and 8 overexpressed miRNAs were selected for further qRT-PCR analysis in 17 individual SCLC tumors. Clinical data for the patients is summarized in Table 9.

Code	Age/gender	Surgery date	Therapy before surgery	Origin of tumor tissue	Metastasis found	Location of met.
S1	49/F	2004	none	Lung, T2N3Mx	yes	Ly, B, Li
S2	68/M	2004	none	Lung, T2N1Mx	yes	Ly
S3	68/M	2004	none	Lung, T2N0Mx	yes	Ly
S4	49/M	2004	chemotherapy	Lung, T3N1Mx	yes	Ly, Br
S5	63/M	2005	none	Lung, T2N0M0	no	n/a
S6	68/F	2004	none	Lung, T1N0Mx	yes	B
S13	56/M	2000	none	Lung, T2N0M0	no	n/a
S16	60/M	2000	none	Lung, T2NxMx	yes	Ly
S17	57/M	2001	none	Lung, T1N0M0	no	n/a
S18	52/M	2001	none	Lung, T2N0M0	no	n/a
S19	46/M	2002	none	Lung, T2N2Mx	yes	Ly, Li
S20	56/M	2000	chemotherapy	Lung, T1N0M0	no	n/a
S21	72/M	2001	none	Lung, T1N0M0	no	n/a
S22	54/F	2002	none	Lung, T1N1Mx	yes	Ly
S23	65/M	2001	none	Lung,T1N2Mx	yes	Ly, Li
S24	48/F	2002	chemotherapy	Lung, T2N1Mx	yes	Ly, B, Br, Li
S10M	54/M	2000	none	Mediastinum, lymph node met.	yes	Ly, B, Li, A
S11M	62/M	2001	none	Liver met.	yes	Li
S12M	49/M	2001	none	Mediastinum, lymph node met.	yes	Ly
S14M	53/M	2001	none	Submandibular saliv. gland met.	yes	Ly, S, B, Br, Li, A
S15M	61/M	2001	none	Mediastinum, lymph node met.	yes	Ly
S23M	65/M	2001	none	Mediastinum, lymph node met.	yes	Ly, Li
S24M	48/F	2002	chemotherapy	Lung, lymph node met.	yes	Ly, B, Br, Li

Table 9. Clinical data for primary SCLC and lung carcinoid tumors.

Tumor grading determined by the pathologists is shown in "Origin of tumor tissue" column. Other metastases were found (if indicated) by imaging diagnostics. Ly=lymph node, Li=liver, B=bone, Br=brain, A=adrenal gland, S=salivary gland, C=colon.

QRT-PCR analysis verified that miR-126 is uniformly downregulated in SCLC cell lines, as well as in primary tumors (Table 10, lower panel; Figure 14B). MiR-150, miR-223, miR-214 and miR-199a are also downregulated miRNAs in all SCLC cell lines, while miR-222 expression value is unchanged in two SCLC cell lines (Table 10, lower panel). MiR-150, miR-222 and miR-223 are downregulated in the majority of the primary tumor samples, whereas miR-29a, miR-214 and miR-199a showed a more variable expression pattern (Figure 14B).

Four miRNAs (miR-301, miR-183, miR-106a, miR-105) were overexpressed in all SCLC

cell lines (Table 10, upper panel), from these miRNAs miR-301 and miR-183 were uniformly overexpressed in SCLC tumors, and miR-106a, miR-25, and miR-95 were upregulated in the majority of the tumor samples. MiR-105, miR-374, miR185 showed a variable expression pattern in these samples (Figure 14A).

We did not find any evidence of the tumor location (lung or metastatic site) influencing the expression of the miRNAs studied in these samples.

miRNA / Tumor	miR-301	miR-183	miR-374	miR-106a	miR-25	miR-105	miR-95	miR-185
H69	10.86	43.85	0.54	7.93	3.47	3.05	3.31	0.65
HTB-172	24.10	144.64	2.82	4.45	0.43	14.44	9.17	6.28
HTB-184	18.90	71.93	16.01	6.54	1.40	43.44	0.51	4.71

miRNA / Tumor	mir-126	mir-222	mir-150	mir-223	mir-29a	mir-214	mir-199a
H69	4.09E-03	0.99	0.06	0.02	3.44	0.07	0.03
HTB-172	3.79E-03	0.01	0.10	0.05	0.21	0.09	0.02
HTB-184	8.47E-02	1.38	0.01	0.13	0.42	0.06	0.01

Table 10. Overexpressed and down-regulated miRNAs in SCLC cell lines.

Relative miRNA expression was determined in SCLC cell lines relative to normal lung tissues by stem-loop qRT-PCR. Threshold value 2 indicate overexpression (upper panel) and 0.5 indicate downregulation (lower panel). Values below or above the threshold are marked in the table.

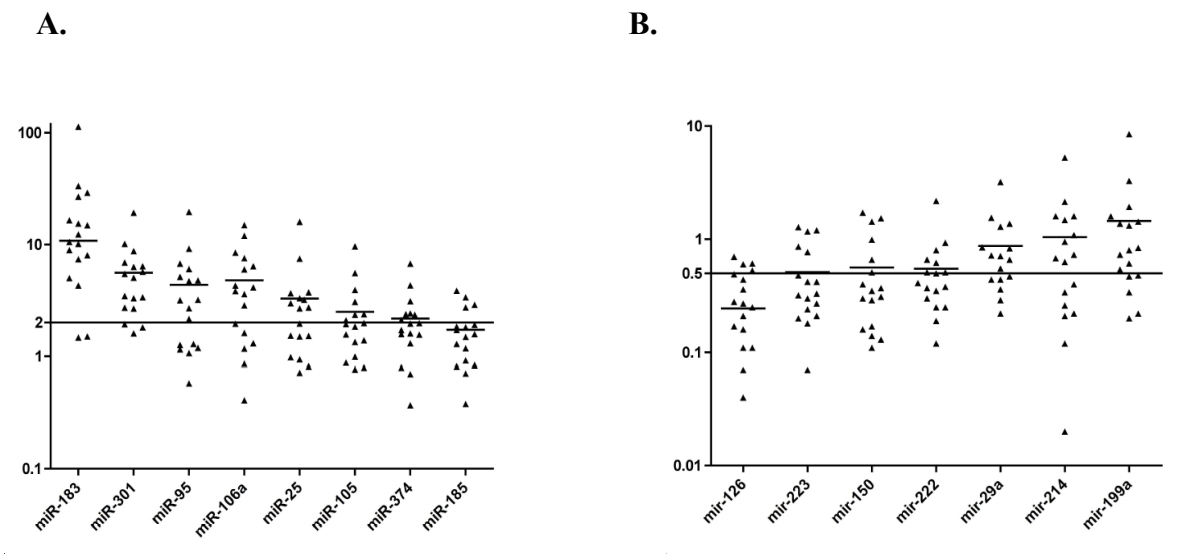


Figure 14. Overexpressed and down-regulated miRNAs in primary SCLC tumors.

Relative miRNA expression was determined in FFPE primary SCLC tumors relative to FFPE normal lung tissue, by stem-loop qRT-PCR. The solid horizontal lines at 2 indicate overexpression (*A*) or at 0.5 indicate downregulation (*B*).

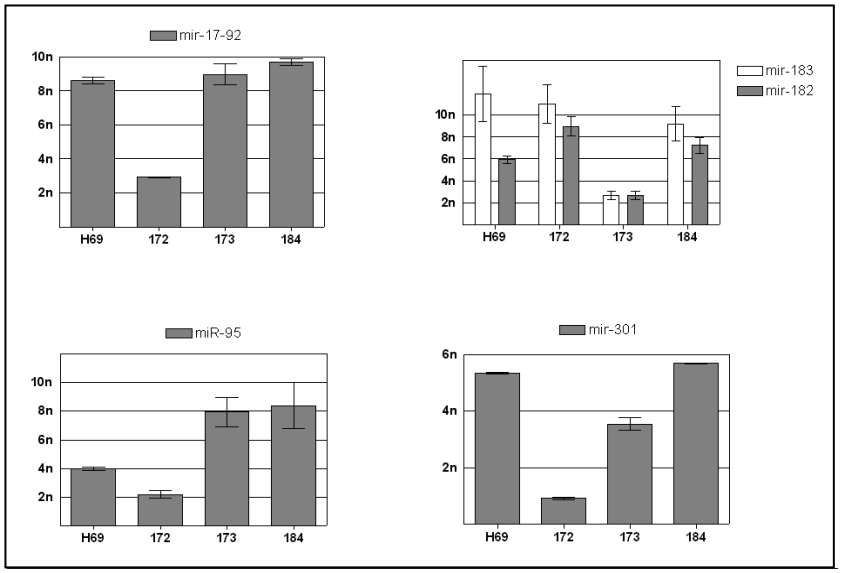
Significance for the differences of the mean expression values (tumor vs normal lung) was determined by paired *t* test, 95% CI. miR-214, miR-199a, and miR-29a relative expressions were not significantly different.

5.4. Gene amplification is not the general mechanism causing overexpression of several miRNAs in SCLC tumors.

Many miRNA genes are found in cancer-associated genomic regions, such as in minimal regions of loss of heterozygosity or minimal regions of amplification in tumors. We used q-PCR to determine the copy numbers for 5 genomic regions harboring prominently overexpressed miRNAs: miR-17-92, miR-183/96, miR-182, miR-95 and miR-301 in SCLC cell lines and in primary SCLC tumors. Gene amplification was clearly detected for all 5 miRNA genomic regions in the SCLC cell lines (Figure 15A) including for the miR-17-92 cluster, which is in accordance with previous observations [99]. However, in primary SCLC tumors only the miR-183/96/182 genomic region was characteristically amplified, in 3 out of 4 samples (Figure 15B). Although miR-301 was uniformly and highly overexpressed in SCLC tumors, gene copy number gain was detected in only 1 sample (S6), and another

sample in fact lost one allele (S5). Amplification was not detected for miR-95 or the miR-17-92 cluster in SCLC tumors.

A.



B.

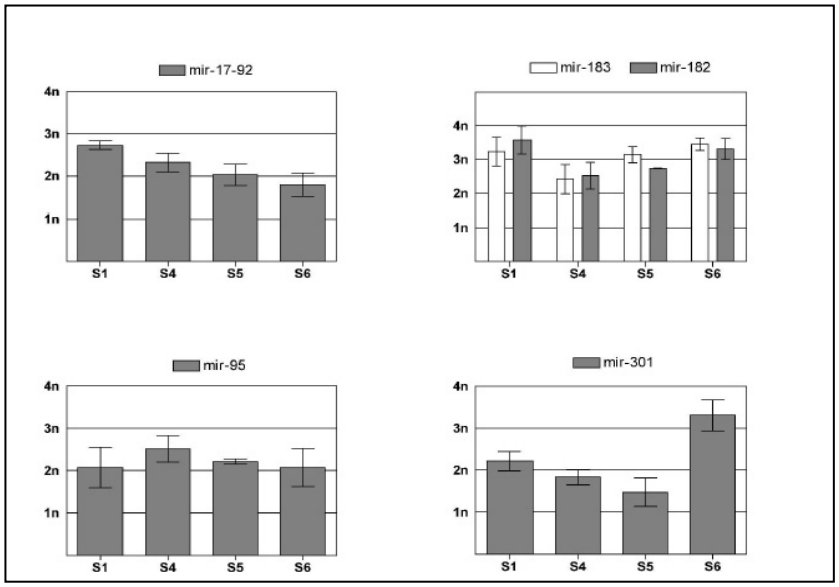


Figure 15. Gene copy numbers of 5 overexpressed miRNAs in SCLC tumors

Genomic DNA isolated from 4 SCLC cell lines (A) and 4 microdissected primary SCLC tumors (B: S1, S4, S5 and S6) was analyzed by qPCR for copy number changes. 2n represents the value for normal, diploid genome, whereas 3n indicates one gained copy, and 1n indicates loss of heterozygosity. Data is shown as mean ±SEM.

5.5. Overexpression of miR-126 inhibits proliferation of SCLC cells by causing delay in the G1 phase

Since our previous work has shown that miR-126 is uniformly under-expressed in primary SCLC tumors, in the next phase of our studies we investigated its role in regulating the proliferation of SCLC cells. H69 cells were transiently transfected with miR-126 precursor (pre-miR-126) or the negative control miRNA (NegmiR) using lipofectamine 2000 transfection reagent, and viable cell numbers were monitored for 96 hours using cell counting and trypan blue exclusion. As expected, transfection of pre-miR-126 into H69 cells resulted in increased miR-126 expression compared to non-transfected or NegMiR control-transfected cells (Figure 16C). Overexpression of miR-126 resulted in a significantly decreased proliferation of H69 cells, evident from 72 hours post-transfection (Figure 16A). On the other hand, overexpression of miR-199a, which is also down-regulated in H69 cells, had no effect on cell proliferation (Figure 16B).

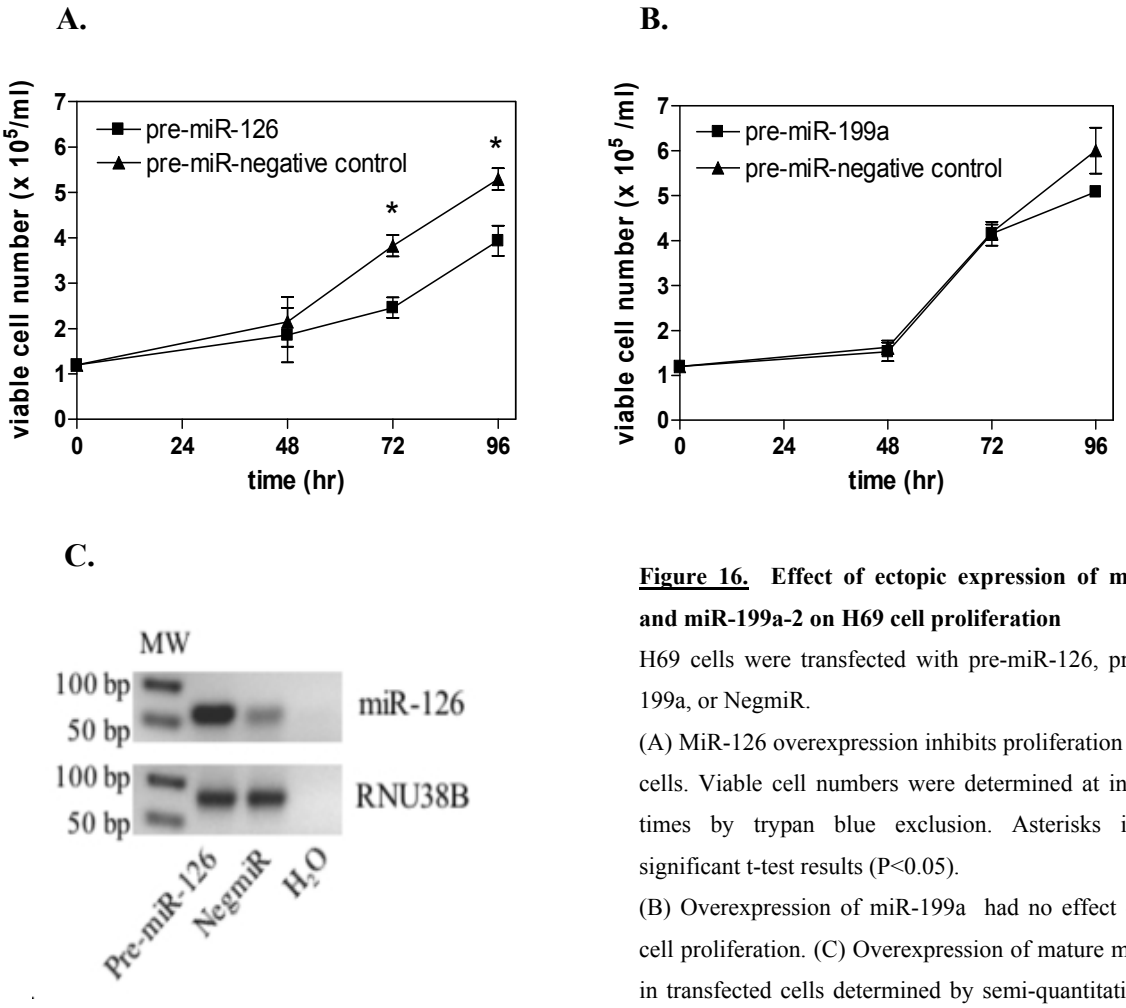


Figure 16. Effect of ectopic expression of miR-126 and miR-199a-2 on H69 cell proliferation

H69 cells were transfected with pre-miR-126, pre-miR-199a, or NegmiR.

(A) MiR-126 overexpression inhibits proliferation of H69 cells. Viable cell numbers were determined at indicated times by trypan blue exclusion. Asterisks indicate significant t-test results ($P<0.05$).

(B) Overexpression of miR-199a had no effect of H69 cell proliferation. (C) Overexpression of mature miR-126 in transfected cells determined by semi-quantitative RT-PCR. PCR products were visualized after electrophoresis in an EtBr-stained agarose gel. RNU38B was used as a reference small RNA.

We verified the effect of miR-126 on SCLC cell proliferation in another SCLC cell line, HTB-172. HTB-172 cells grow in compact aggregates, making cell counting difficult - therefore, the EZ4U proliferation assay was used to determine the proliferation rates for this cell line. We found that miR-126 overexpression inhibits the proliferation of HTB-172 cells similar to the H69 cell line (Figure 17).

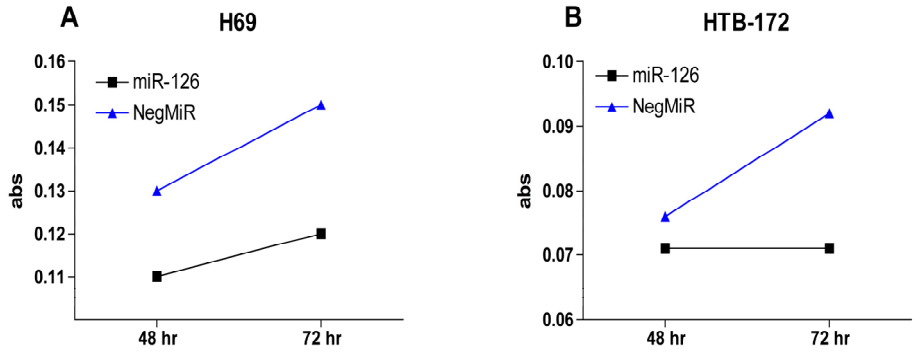
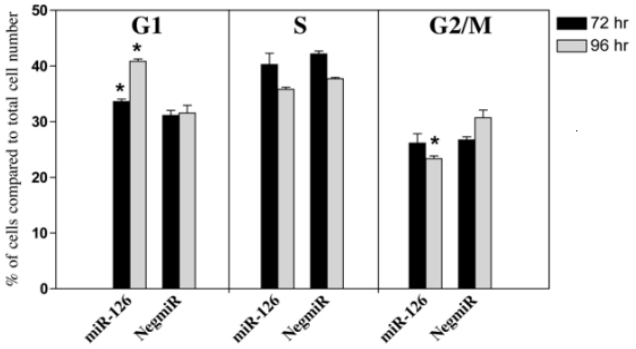


Figure 17. MiR-126 overexpression inhibits the proliferation of HTB-172 cells.
(A) H69 (positive control) and (B) HTB-172 cells were transfected with pre-miR-126 or NegmiR. Cell numbers were determined by the EZ4U Cell Proliferation Assay (Biomedica) at 48 hours and 72 hours post-transfection.

Flow cytometric cell cycle analysis at two time points (72 and 96 hrs post-transfection) revealed an increasing percentage of miR-126-transfected H69 cells in the G1 phase over time, and a concomitant decrease in the percentage of cells in the G2/M phase, when compared to the negative control (Figure 18 A,B). This suggests that overexpression of miR-126 delays H69 cells in the G1 phase of the cell cycle.

A.



B.

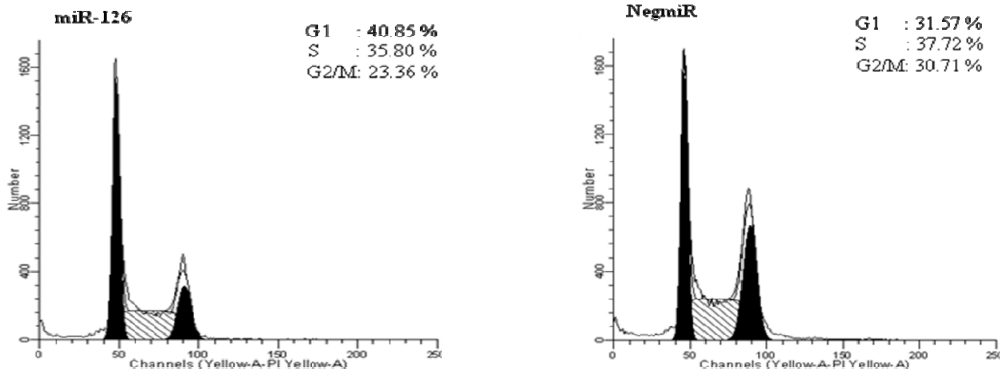


Figure 18. Effect of ectopic expression of miR-126 on H69 cell cycle progression.

H69 cells were transfected with pre-miR-126 or NegmiR.

(A) MiR-126 overexpression delays H69 cells in G1 phase of the cell cycle. Cells were analyzed on a FACSArray bioanalyzer at 72 hours and 96 hours post-transfection. (B) Representative cell cycle analyis of H69 cells at 96 hours post-transfection. Asterisks indicate significant t-test results ($P<0.05$).

5.6. Overexpression of miR-126 suppresses SLC7A5 expression at both the RNA and the protein level

To identify potential targets for miR-126 that might play a role in regulating proliferation of SCLC cells, we first performed an in silico analysis using the miRNA target prediction databases TargetScan and PicTar. Six of the predicted targets were identified by

both algorithms (CRK, PLK2, SLC7A5, PTPN9, FBXO33 and RGS3), and in fact, CRK and PLK2 are bona fide targets of miR-126 as proven by functional studies (Table 11) [122,232]. However, with the exception of SLC7A5, none of the validated or doubly-predicted target genes are known to be overexpressed in SCLC cell lines or tumors [236]. SLC7A5 protein overexpression in SCLC is in accordance with the previously described downregulation of miR-126 expression. Therefore, we selected SLC7A5 for further studies to analyse the role of miR-126 in the cell cycle regulation of SCLC.

We next investigated the effect of miR-126 overexpression on SLC7A5 and PLK2 expression. miR-126 overexpression in H69 cells caused more than a 50% reduction in SLC7A5 mRNA levels, and also a slight suppression of PLK2 mRNA expression, as determined by qRT-PCR (Figure 19C). Subsequent western blot analysis of SLC7A5 and PLK2 demonstrated that while miR-126 overexpression resulted in decreased SLC7A5 protein levels, PLK2 protein levels did not change significantly (Figure 19A,B).

Table 11. Possible miR-126 target genes

Gene symbol	Ensembl ID	TargetScan	PicTar	Experimentally validated
CRK	NM_016823	+	+	+
PLK2	NM_006622	+	+	+
SLC7A5	NM_003486	+	+	
PTPN9	NM_002833	+	+	
FBXO33	NM_203301	+	+	
RGS3	NM_021106	+	+	
SPRED1	NM_152594		+	+
TOM1	NM_005488		+	+
IRS1	NM_005544		+	+
HOXA9	NM_002142		+	+
VCAM1	NM_001078			+
PIK3R2	NM_005027			+
SOX2	NM_003106			+

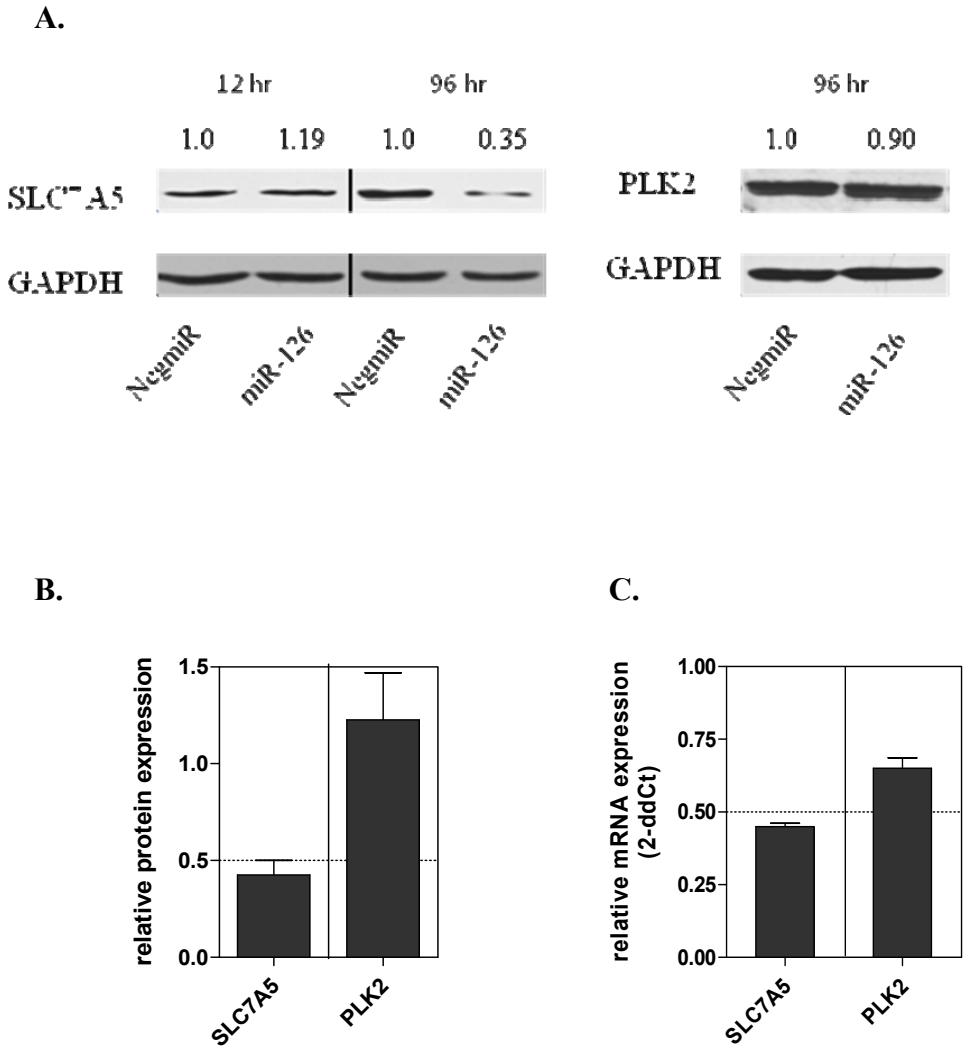


Figure 19. miR-126 suppresses SLC7A5 protein and RNA expression.

H69 cells were transfected with pre-miR-126 or NegmiR. Cell lysates and total RNA were prepared at 96 hours post-transfection. (A) Representative western blot for SLC7A5 and PLK2 in miR-126 transfected H69 cells. GAPDH protein levels were used for normalization. The numbers above the blot indicate normalized protein amounts relative to the negative control, as determined by densitometry. (B) Effect of miR-126 overexpression on SLC7A5 and PLK2 protein levels, as determined by western blot analyses. SCL7A5, but not PLK2 protein production is suppressed by miR-126. (C) Effect of miR-126 overexpression on SLC7A5 and PLK2 mRNA levels, as determined by qRT-PCR. The results are means of three independent expreriments.

SLC7A5 expression was also suppressed in pre-miR-126 transfected HTB-172 cells (Figure 20).

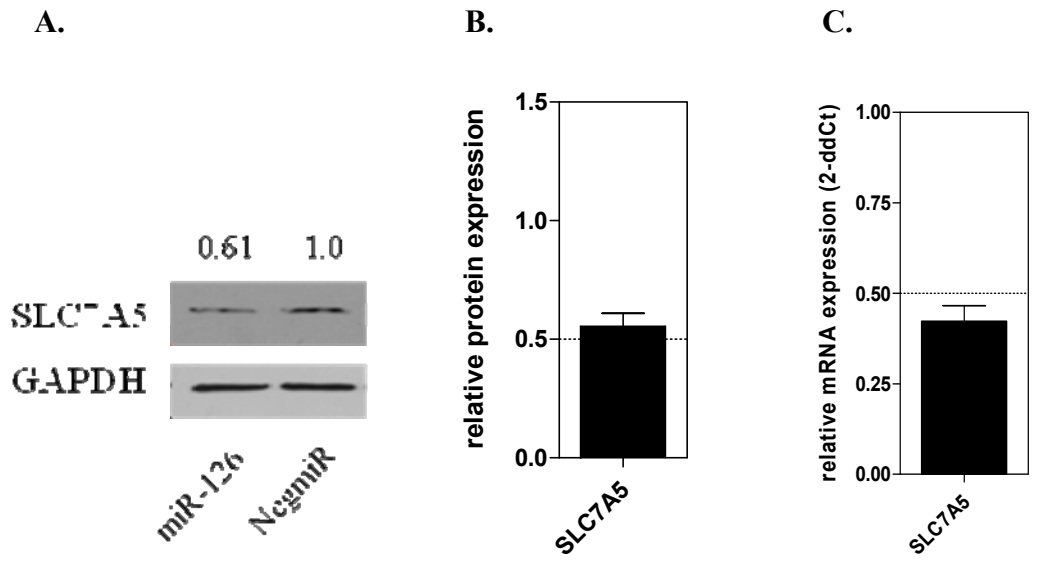


Figure 20. **MiR-126 suppresses SLC7A5 protein production in HTB-172 cells.**

HTB-172 cells were transfected with pre-miR-126 or NegmiR. Cell lysates and total RNA were prepared at 72 hours post-transfection.

(A) Representative western blot for SLC7A5 in transfected HTB-172 cells. GAPDH protein levels were used for normalization. The numbers above the blot indicate normalized protein amounts relative to the negative control, as determined by densitometry. (B) SCL7A5 protein production is suppressed by miR-126, as determined by western analysis. (C) Effect of miR-126 overexpression on SLC7A5 mRNA levels, as determined by qRT-PCR.

5.7. Suppression of SLC7A5 by RNAi delays SCLC cells in the G1 phase

To better understand the effect of SLC7A5 in SCLC cell cycle control, we utilized RNA interference to specifically suppress SLC7A5 production in H69 cells, and performed cell cycle analysis by flow cytometry at 72 and 96 hours post-transfection. Transfection of specific siRNA into H69 cells resulted in significantly lower SLC7A5 expression when compared to the negative control siRNA (Figure 21A). Similarly to the effect of miR-126 overexpression, suppression of SLC7A5 resulted in an increasing percentage of transfected cells in the G1 phase over time, and a concomitant decrease in the percentage of cells in the G2/M phase, when compared to the negative control siRNA (Figure 21 B,C). In contrast, specific suppression of PLK2 expression by RNAi had no such effect on the cell cycle

distribution of transfected H69 cells (Figure 21 B,C). These results support the hypothesis that miR-126 inhibits proliferation and cause cell cycle delay of H69 SCLC cells by negatively regulating SLC7A5.

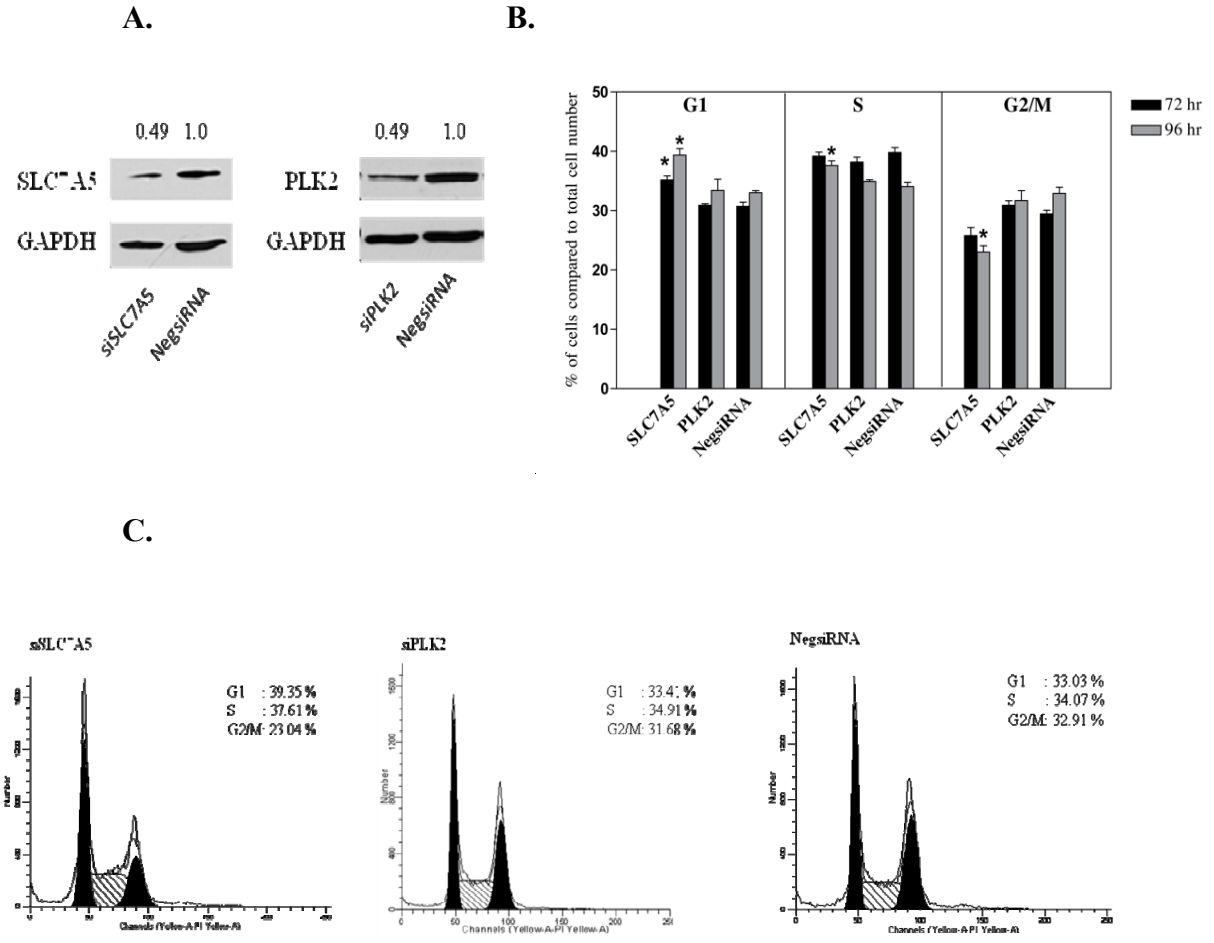


Figure 21. Suppression of SLC7A5 production by RNAi delays H69 cells in the G1 phase.

H69 cells were transfected with siRNAs specific to SLC7A5 (siSLC7A5) or PLK2 (siPLK2), or with the negative control siRNA. (A) Representative western blot for SLC7A5 and PLK2 in siRNA-transfected H69 cells. GAPDH protein levels were used for normalization. The numbers above the blot indicate normalized protein amounts relative to the negative control, as determined by densitometry. (B) Suppression of SLC7A5 production by RNAi delays H69 cells in the G1 phase at 96 hrs post-transfection. Suppression of PLK2 had no effect on cell cycle distrubution. Cells were analyzed on a FACSArray bioanalyzer at 72 hours or 96 hours post-transfection. Asterisks indicate significant t-test results ($P<0.05$).

(C) Representative cell cycle analyis of siRNA-transfected H69 cells at 96 hours post-transfection analyzed on a FACSArray bioanalyzer.

5.8. SLC7A5 is a direct target of miR-126.

To validate that SLC7A5 is a molecular target of miR-126, as suggested by the previous experiments, we constructed a luciferase reporter vector containing 331 bp of the SLC7A5 3' UTR, including the predicted miR-126 binding site (WT-UTR). We also constructed a control luciferase vector with the miR-126 binding site deleted from the SLC7A5 3' UTR (DEL-UTR) (Figure 22A). The sequenced plasmids showed 100% identity with the SLC7A5 3' UTR, and the intended deletion in the control vector (data not shown). H69 cells were transiently transfected with the WT-UTR-luciferase or the DEL-UTR-luciferase vector and with pre-miR-126. Co-transfection of WT-UTR with pre-miR-126 resulted in a significant decrease of reporter activity as compared with the control; however, deletion of the miR-126 binding site from the SLC7A5 3' UTR abolished this effect of miR-126 (Figure 22B). These data suggest that SLC7A5 is a direct functional target of miR-126 in H69-SCLC cell line.

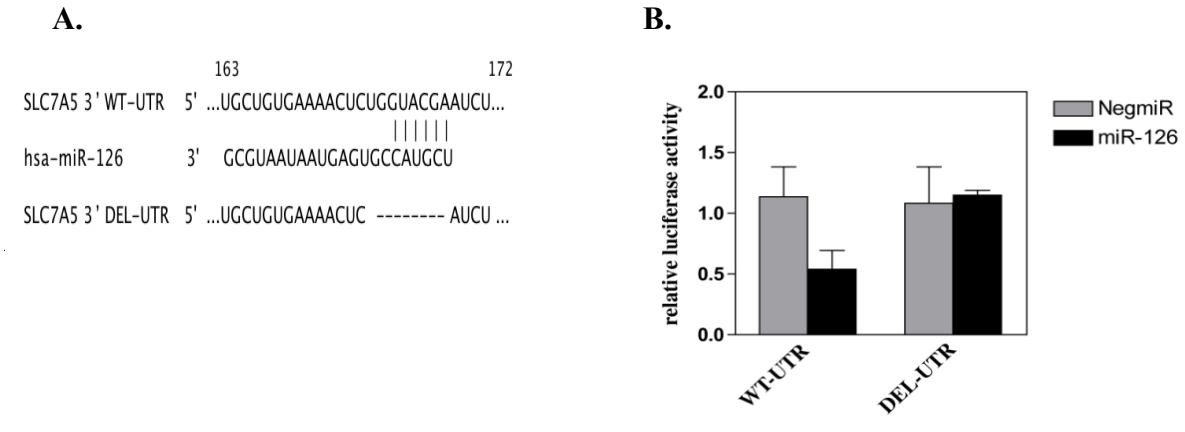


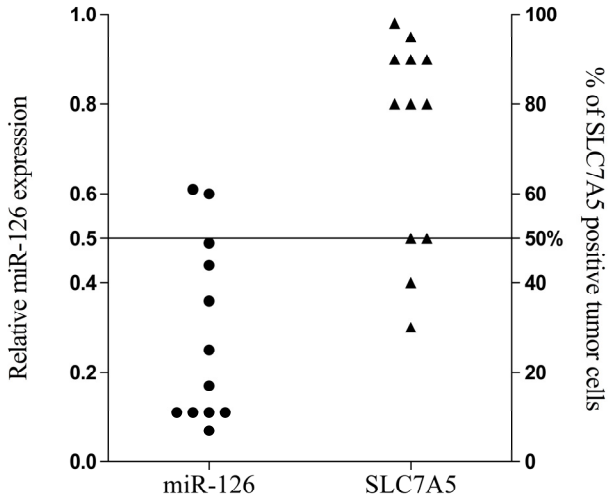
Figure 22. SLC7A5 is a direct target of miR-126.

(A) The predicted miR-126 binding site in the wild type SLC7A5 3'UTR (WT-UTR), and in the deleted construct (DEL-UTR). (B) Relative luciferase activity of the SLC7A5 WT-UTR and the DEL-UTR luciferase constructs in H69 cells transfected with miR-126 or NegmiR.

5.9. SLC7A5 and miR-126 expression levels are inversely correlated in primary SCLC tumors.

The correlation of miR-126 and SLC7A5 expression was also investigated in 12 primary SCLC tumor samples, using immunohistochemistry (IHC) with SLC7A5-specific antibody (Figure 23A). SLC7A5 expression was not detectable in normal lung tissue, which is in accordance with previous observations (Figure 23B, inset, and [12]). In contrast, the SCLC tumors tested positive for SLC7A5 protein expression - in fact, 8 tumors contained more than 70% SLC7A5-positive cells. As demonstrated by the double IF stained specimens, the majority of tumor cells exhibited nuclear staining for TTF1 (typical feature for SCLC) with SLC7A5 co-expression (Figure 23B). The tumor samples analysed with IHC were the same samples analysed before for aberrant miR-126 expression (chapter 5.3). Since all 12 SCLC tumors overexpressed SLC7A5 and under-expressed miR-126, the inverse correlation between the expression levels of miR-126 and its target could be corroborated in primary tumors (Figure 23A).

A.



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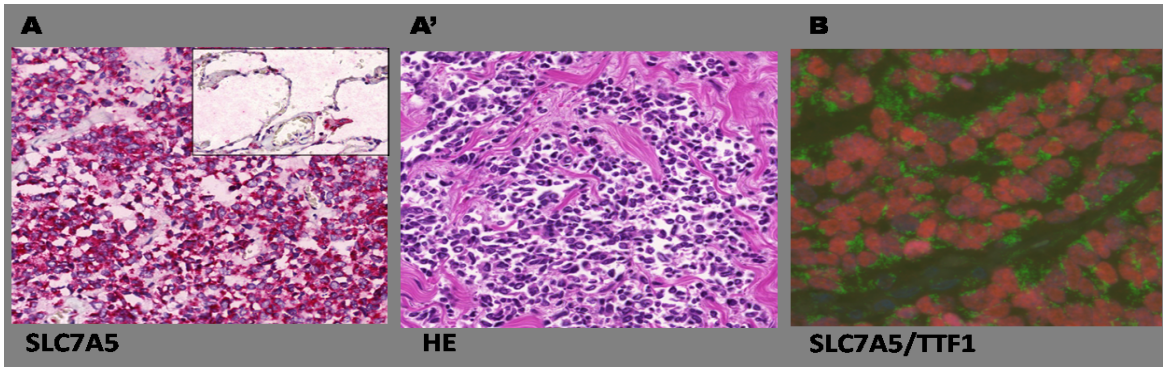

Figure 23. SLC7A5 and miR-126 expression levels are inversely correlated in primary SCLC tumors.

(A) Relative expression levels of mature miR-126 (left Y axis) were determined in primary SCLC tumor specimens by qRT-PCR (chapter 5.3). Overexpression of SLC7A5 in the same panel of primary SCLC tumors was determined by immunohistochemistry, using an SLC7A5-specific monoclonal antibody. Percentage of SLC7A5-positive neoplastic cells was determined for each tumor specimen (right Y axis).

(B) A: SLC7A5 expression was detected with immunohistochemistry using a monoclonal antibody specific to SCL7A5 (red staining). The representative SCLC tumor specimen contains >95% SLC7A5-positive neoplastic cells. Normal lung tissue exhibited no SLC7A5-specific staining. (inset) A': Hematoxylin-eosin (HE)-stained section of the same specimen. Original magnifications for A-A' images: 20x.

B: SLC7A5 and TTF1 co-expression was detected with double immunofluorescence (IF) staining using SLC7A5 and TTF1-specific antibodies. The representative SCLC tumor specimen exhibits 100% TTF1 nuclear staining (red fluorescence) and ~80% SLC7A5-positivite neoplastic cells (green fluorescence). SLC7A5 staining is mainly along the cell membrane. The blue fluorescence represents DAPI nuclear counterstaining. Original magnification: 40x.

6. DISCUSSION

6.1. Differentialy expressed microRNAs in small cell lung cancer, and their biological functions in normal or neoplastic cells.

We combined microarray and qRT-PCR to identify miRNAs aberrantly expressed in SCLC. The microarray approach alone identified 19 overexpressed miRNAs, and more than 35 downregulated miRNAs in SCLC cell lines compared to normal lung. Out of these, 7 downregulated and 8 overexpressed miRNAs were selected for further qRT-PCR analysis in 17 individual SCLC tumors and 3 SCLC cell lines. The qRT-PCR analyses verified that miR-126 is a uniformly downregulated miRNA, while miR-301 and miR-183 are uniformly overexpressed miRNAs in all SCLC sample types. As described previously, miR-17-5p showed the highest expression level in primary SCLC tumors [99].

The microarray results showed for the first time that miR-374 and miR-506 can be deregulated in cancer, and, thus, may contribute to the tumorigenic phenotype. In addition, several miRNAs were not implicated before in lung cancer, such as overexpressed miR-429, miR-128a/b and miR-130b, and downregulated miR-135b, miR-511, miR-190, miR-296, miR-33, miR-502, and miR-325. The role of these miRNAs in neoplastic transformation is well documented, but quite variable depending on the actual tumor type. For example, miR-128a/b are significantly overexpressed in acute lymphoblastic leukemia (ALL), but the same miRNAs are downregulated in glioblastoma, presumably because of their antiproliferative effects in this tumor type [142,143].

A growing body of evidence supports that the overexpressed miRNAs are involved in normal development, differentiation and cancer, although little is known about their exact function in the development of lung cancer. Members of the miR-182/miR-183/miR-96 cluster were shown to be upregulated in several malignancies [144-147]. MiR-183 was significantly up-regulated in hepatocellular carcinoma, inhibited apoptosis by repressing PDCD4 gene expression [148], and, interestingly, could inhibit cancer metastasis [149,150]. MiR-182 was shown to be strongly overexpressed in primary tumors of the lung [151], and regulated lung tumor growth [152]. MiR-182 is frequently overexpressed in melanoma as well, and promotes melanoma metastasis [153,154]. Relatively new findings show that miR-

182 may play a role in DNA repair by regulating breast cancer 1, early onset (BRCA1) protein levels [155]. Less is known about miR-301; it is a crucial oncogene mediating proliferation and invasion in breast cancer [156], and, together with miR-95, it was shown to be overexpressed in pancreatic cancer [157-158].

Three of the highly overexpressed miRNAs, miR-9, miR-7 and miR-375 are expressed at high levels during human pancreatic islet development as well [159-161]. It is conceivable that miR-9, miR-7 and miR-375 regulate key pathways of normal neuroendocrine cell development, and the same pathways become aberrantly activated in the undifferentiated cells of neuroendocrine tumors, such as SCLC. This is in fact a recurrent theme in tumor biology; there are several examples of pathways and regulatory networks involved both in embryonic development and neoplastic transformation, including the ASH1/GFI1 network and the hedgehog signalling pathway in SCLC [162-163].

In addition to its role in pancreatic islet development, miR-9 is also important for brain development. In the developing brain, miR-9 controls the balance between neural stem cell proliferation and differentiation by forming a negative regulatory loop with nuclear receptor TLX [164-166]. Recent studies suggest that miR-9 also functions as a regulator of metastasis by regulating E-cadherin expression, which is a key player in the epithelial-mesenchymal transition during metastasis [167-169]. Interestingly, miR-9 is also involved in regulating cell proliferation in human ovarian cancer and gastric adenocarcinoma, through targeting NF-kappaB [170,171].

Similar to miR-128a/b, miR-375 and miR-7 can apparently function either as oncogenes or tumor suppressors, depending on the type of cancer. In this regard, mir-375 is overexpressed in lung adenocarcinoma, but it is downregulated and functions as a tumor suppressor in gastric cancer [172,173]. MiR-7 is also an oncogenic miRNA in lung cancer, whereas it acts as a tumor suppressor in several human cancers and targets a number of proto-oncogenes, such as insulin receptor substrate 1/2 (IRS1/2), and PAK1 (p21/CDC42/RAC1-activated kinase 1) [174-176]. MiR-7 has a complex role in EGFR signalling, by participating in different regulatory loops and networks, resulting in different signalling outcomes depending on the cell type [177,178].

MiR-206 was overexpressed close to 40-fold in SCLC cell lines. MiR-206 is a muscle-specific miRNA [179] regulating myoblast differentiation through targeting the Pax7 transcription factor, which is required for muscle satellite cell biogenesis [180]. miR-206 is also a key regulator of osteoblast differentiation by targeting the gap junction protein

connexin 43 in osteoblasts [181]. MiR-206 is a potential serum biomarker of rhabdomyosarcoma [182], suppressor of breast cancer metastasis [183] and could be a novel target for endocrine therapy targeting ERalpha in breast cancer [184]. Lastly, a recent study suggests that mir-206 expression may be inversely correlated with the metastatic capability of NSCLC cell lines [185].

Both our results and previous observations showed that closely related miRNAs, or miRNAs with multiple copies on different chromosomes are often co-regulated, suggesting that aberrant transcriptional regulation plays an important role in the overexpression or downregulation of many miRNAs in SCLC.

Most of the aberrantly expressed miRNAs in SCLC are intergenic, but some genes harbor overexpressed miRNAs in their introns. For example, miR-335 is embedded in the mesoderm specific transcript (MEST), which is overexpressed as a result of loss of imprinting or promoter switch in lung or breast cancer [186,187]. The miR-106b/93/25 cluster is harbored by minichromosome maintenance complex component 7 (MCM7), a positive regulator of cell cycle progression, which is amplified and/or overexpressed in many tumor type [188,189]. A close relative, miR-106a is overexpressed SCLC, as well as in breast tumor and gastric cancer [190-192]. The miR-98 host gene, ARF-BP1 is an important mediator of p53/ARF-dependent tumor suppression. However, SCLC tumors frequently have p53-defects, and interestingly, in p53-null cells ARF-BP1 appears to promote cell [193,194]. It should be emphasized that for many miRNAs the amount of primary transcripts do not correlate well with the amount of mature miRNAs, since the steady state levels of mature miRNAs are determined by several steps of the processing. Given that in SCLC the expression levels of these miRNAs and their host genes is well correlated, they may coordinately regulate the same biological pathways, or may be part of regulatory feedback loops for each other. Thus, it would be of great interest to study the functional relationship between these miRNAs and the proteins coded by their host genes.

The microarray analyses identified 35 miRNAs that are downregulated in SCLC cell lines compared to normal lung, and a large region in 19q13.41 containing more than 30 downregulated miRNAs (miR-512 to miR-373). Of the downregulated miRNAs, miR-126 appears to have a complex role in regulating cellular proliferation. It has an anti-proliferative

effect in several tumor types including SCLC, NSCLC, and colon cancer through targeting different members of the PI3K/Akt pathway [195-197], or in breast cancer cells, by targeting insulin receptor substrate 1 (IRS1) [198]. In addition, miR-126 is a key regulator of vessel development, by targeting sprouty-related, EVH1 domen containing 1 (SPRED1) and phosphoinositide-3-kinase, regulatory subunit 2 (PIK3R2) in endothelial cells [124,125] and may be involved in the metastatic process, as evidenced by its effects on mammary or gastric carcinoma cell migration [198-201]. The potential role of miR-126 in SCLC will be discussed in detail in the next chapter.

MiR-145/143 were shown before to be downregulated in lung cancer [202-204]. MiR-145 functions as a tumor suppressor, capable of inhibiting tumor cell growth, invasion and metastasis by targeting c-Myc, mucin 1 (MUC1), EGFR and nudix-type motif 1 (NUDT1) [205-207]. Downregulation of miR-143 correlates with the lung metastasis of human osteosarcoma cells by promoting cell invasion through MMP-13 [208].

We also detected downregulation of the apoptosis regulators miR-34a and miR-214 [209]. MiR-199a and miR-214 are regulated as a cluster located within the human Dnm3os gene. It was found that the EMT transcription factor, twist homolog 1 (Twist1), is a key regulator of this miRNA cluster and 'stemness' in epithelial ovarian cancer cells [210]. Accordingly, reduced level of miR-214 was found in ovarian carcinoma and cervical cancer compared with normal tissue [211, 212], and low levels of miR-199a and miR-214 was found in epithelial ovarian cancer stem cells as well.

Interestingly, although miR-223 is downregulated in SCLC, it is actually upregulated in many cancer types, such as in sera of patients with hepatocellular carcinoma [213], esophageal squamous cell carcinoma [214], or gastric and bladder cancer [215,216]. Similar to SCLC, miR-223 is downregulated in acute myeloid leukemia (AML), and the resulting deregulation of its targets, C/EBPalpha and E2F1, contribute to the aberrant granulocyte differentiation program in AML [217,218].

The significance of low-level miR-451 expression in SCLC samples is less clear. On one hand, miR-451 was shown to be downregulated in NSCLC, and regulates survival of NSCLC cells through RAB14, member of RAS oncogene family [219]. On the other hand, expression of miR-451 is increased during erythroid maturation [220], and it is possible that high signals for this miRNA in normal lung simply reflects the presence of blood in our surgical samples.

Several studies investigating chromosomal aberrations in SCLC identified characteristically lost genomic regions. Some of the downregulated miRNAs are found in regions with frequent loss of heterozygosity in SCLC: the 5q32-ter region, containing the miR-143/145 cluster (5q33.1), is lost in 79% of SCLC tumors; the 9q22-32 region, containing miR-32 (9q31.3), is lost in 64% of SCLC tumors, and the region 17p12-ter, containing miR-497, as well as the p53 gene (17p13.1), is deleted in 93% of SCLCs [221-225,4]. Interestingly, the overexpressed miRNAs are not embedded in chromosomal regions frequently amplified in SCLC, with the exception of the miR-200b/200a/429 cluster, which is located in 1p36.33 in the vicinity of the antiapoptotic gene TNFRSF4, frequently amplified in SCLC [226]. Therefore, we analyzed DNA copy number changes in primary SCLC tumors for 5 genomic regions with overexpressed miRNAs: miR-17-92, miR-183/96, miR-182, miR-95 and miR-301. We identified one novel amplified region in SCLC: 7q32.2 contains the miR-183/96/182 cluster, and copy number gains were clearly detected in 3 of 4 SCLC tumors. In contrast, 3 other genomic regions studied showed no or infrequent amplification (miR-17-92, miR-95, and miR-301, respectively).

Some miRNAs have similar expression patterns in SCLC and NSCLC: miR-182, miR-375, miR-210, miR-200b and miR-301 are overexpressed, while miR-143, miR-145 and miR-126 are downregulated miRNAs in both lung cancer types [202-204,227]. However, there are several miRNAs expressed differently between SCLC and NSCLC. For example, miR-98, miR-17-5p and miR-106a are overexpressed in SCLC cells compared to human bronchial epithelial cells (HBECs), but not in NSCLC cells [227,228]. Similarly, MiR-27a and miR-29a/b/c expression was downregulated in SCLC cells compared to HBECs, but not in NSCLC cells [203,227]. These distinct miRNA expression pattern suggests the possibility of developing miRNA profiling as a diagnostic tool for distinguishing SCLCs from NSCLC and normal lung. In this regard, miR-21 is an oncogenic miRNA upregulated in many solid tumors, and its detection in sputum samples of lung adenocarcinoma patients indicates that it may be utilized as a biomarker for the early detection of lung cancer [203,204]. Interestingly, our study and others' have shown that miR-21 in not overexpressed in SCLC [227].

6.2. MiR-126 inhibits proliferation of small cell lung cancer cells by targeting SLC7A5.

In our previous work we identified miR-126 as a downregulated miRNA in all examined SCLC samples. Several studies investigated the function of miR-126 in other types of cancer, but prior to our work, little was known about its role in SCLC. Previous studies have drawn a complex picture about the potential roles of miR-126 in cancer biology.

In several tumor types, miR-126 has a tumor-suppressor and pro-apoptotic effect, and is down-regulated accordingly in the tumor cells. Specifically, in NSCLC cells miR-126 can inhibit proliferation in vitro and in vivo by targeting VEGF or EGFL7 [126,229], promotes apoptosis [196], and inhibits tumor cell adhesion, migration and invasion through regulating Crk function [122]. Similarly, miR-126 also function as tumor suppressor in gastric cancer by affecting the proliferation and metastatic potential of gastric cancer cells [200], suppresses breast cancer metastasis and, importantly, the loss of miR-126 expression is associated with poor distal metastasis-free survival of breast cancer patients [230].

On the other hand, miR-126 is overexpressed in acute myeloid leukemias [231,232] and has an anti-apoptotic effect in this tumor type. Thus, mir-126 is not an obligatory anti-proliferative miRNA; rather, it appears to have multiple functions depending on the cell type and the actual cellular environment. Based on the decreased expression of miR-126 in SCLC, we expected that it will have an anti-proliferative effect in this tumor type, which was confirmed by the proliferation assays and cell cycle analyses of miR-126-overexpressing H69 and HTB-172 cells.

The complexity of miR-126 functions (in tumor cells, and presumably in normal cells as well) is underscored by the observations, that not all validated miR-126 target mRNAs are affected by miR-126 in every cell type. For example, in SCLC cells miR-126 overexpression does not suppress PLK2 expression, even though PLK2 was shown to be a bona fide target for miR-126 in CBF AML cells, and plays a role in cell cycle and DNA damage checkpoint regulation [232-234]. A similar observation can be made for TOM1, which is targeted by miR-126 in CF airway epithelium cells, but not in MCF7 cells cells [235,198]; or for SPRED1, which is targeted in HUVEC cells, but not in AML cell lines lines [124,232].

It is presently unclear how certain target mRNAs are presented to, and others are protected from miR-126 in these experimental setups, but the resulting target selectivity could contribute to the varied functions of miR-126.

In this regard, it is important to mention that miRNA activity can be affected by RNA-binding proteins [64,65]. These RBPs can bind to the 3'UTR of the mRNAs and block the interaction between specific miRNAs and mRNAs, thereby protecting the mRNA from miRNA-mediated repression. The presence of RBPs may well explain the target selectivity of a miRNA depending on the cell type or the actual proteome of the cell. Therefore, it is important to validate the predicted target genes for a given miRNA in all novel exprerimental setups.

Importantly, we identified SLC7A5 as a novel target of miR-126 in SCLC cells. This is of special interest, since with the exception of SLC7A5, none of the validated or doubly-predicted target genes for miR-126 (Table 11) are known to be overexpressed in SCLC cell lines or tumors. SLC7A5 (LAT1) is an L-type amino acid transporter, transporting large neutral amino acids through the plasma membrane. SLC7A5 requires a covalent association with the heavy chain of the 4F2 cell surface antigen (CD98) for its functional expression in the plasma membrane. SLC7A5 is widely expressed in many cancer types, including SCLC, and was shown to play essential roles in cancer growth and survival [236,237]. SLC7A5 expression levels are usually correlated to cancer progression and aggressiveness, and was shown that SLC7A5 expression may be utilized as a prognostic factor for patients with NSCLC [238-241].

We demonstrated that in SCLC cells, similarly to other tumor types, suppression of SLC7A5 expression has an anti-proliferative effect [242-244]. SCL7A5 suppression or miR-126 overexpression both delay SCLC cells in the G1 phase, suggesting that the effect of miR-126 on the cell cycle is at least in part mediated through SLC7A5. Consequently, high level of SLC7A5 expression in SCLC cells may be maintained partly by decreased miR-126 expression, and, thus, may ensure efficient transport of essential amino acids in the rapidly proliferating tumor cells.

On the other hand, miR-126 may also be involved in a more direct regulation of the cell cycle in SCLC. For instance, several observations suggest that SLC7A5 may affect cell proliferation not just by providing nutrients, but also by activating nutrient-dependent growth regulatory pathways, such as the PI3K/Akt/mTOR pathway, which was shown before to be regulated by miR-126 in several experimental models. The PI3K/Akt/mTOR pathway can be activated by mitogens, growth factors, nutrients and other extracellular molecules. Mitogens

activate receptor tyrosine kinases and through several steps activate PI3K. Class IA PI3Ks are the most studied class of PI3K family widely implicated in cancers. These kinases function as heterodimers composed of the regulatory subunit p85 (α,ß,p55γ) and the catalytic subunit p110(α,ß,δ). p85 ß is a bona fide target of miR-126 in breast cancer [245], in colon cancer [195], and in endothelial cells [124]. MiR-126 also regulates the PI3K/Akt pathway in NSCLC [196, 197]. A class III PI3K, vps34 is another member of PI3K family which plays a role in the regulation of mTOR activity in response to amino acid availability. Akt is a downstream target of PI3K which controls several key molecules participating in cell cycle progression, growth, angiogenesis and survival. Overexpression or mutation of PI3Ks or Akt lead to constitutive activation of mTOR pathway and promotes tumorigenesis. mTOR has two different forms, mTORC1 and mTORC2. mTORC1 play a significant role in cell cycle progression and protein synthesis, while mTORC2 play a role in cell survival and actin cytoskeleton organization. Leucine, transported by SLC7A5, activates mTOR, which in turn phosphorylates ribosomal protein S6 kinase1 (p70 S6 kinase1/ S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), leading to the production of growth promoting proteins [237, 246-248].

SCLC represent ~20% of all cases of lung cancer. It is a very aggressive form of lung cancer, is highly metastatic and responsive to chemotherapy. Several studies suggest that different signaling pathways involved in cell proliferation and survival are upregulated in lung tumors, such as components of the PI3K/Akt/mTOR pathway. Aberrant activation of this pathway participate in tumor growth and progression.

Activated PI3K pathway promotes SCLC cell growth, metastasis and resistance of SCLC cells to various therapies [249-252]. High level of phosphorylated Akt is detected in SCLC tumors and the level of mTOR effectors, S6K1 and S6K2 are also overexpressed in SCLC and correlate with chemoresistance and promote survival of SCLC tumors. SCLC cells exist in rich extracellular matrix environment which activates the PI3K/Akt pathway through ß1-integrin activation leading to chemoresistance and protection of apoptosis. Blocking PI3K activity or inhibition of mTOR with rapamycin promote apoptosis and decrease SCLC cell growth suggesting that blocking the mTOR pathway could be a very promising approach for SCLC treatment. AKT can also be activated by EGFR representing the connection between mTOR and EGFR pathways. Both pathways are active in SCLC and dual inhibition of these pathways could be a novel strategy to treat SCLC.

In summary, our work has identified miR-126 as an important negative regulator of the growth and proliferation of SCLC cells, which probably fine-tunes the activity of the PI3K/Akt/mTOR network through multiple targets, including SLC7A5. Removal of miR-126 from the regulatory network may enhance the existing positive feedback between SLC7A5 and mTOR, and can contribute significantly to the proliferative potential of the tumor cells. In addition, miR-126 may regulate PI3K/Akt pathway by targeting PIK3R2/p85ß in SCLC cells as well. However, mir-126 may have additional functions in the tumor stroma: in normal endothelial cells it is a positive regulator of angiogenesis, and it is likely to have interesting functions in regulating the immune response. Therefore, more research is needed to understand the complex role of miR-126 in the growth, survival and progression of SCLC tumors *in vivo*, and to determine how miR-126 may potentially be exploited as an anti-tumor agent.

7. SUMMARY

MiRNAs are small, non-coding RNAs that regulate gene expression by binding to the 3' untranslated region (3'UTR) of target mRNAs, inducing mRNA degradation or translation repression. Approximately 50% of miRNA genes are in cancer-associated genomic regions, suggesting that miRNAs play a significant role in tumor biology. SCLC is a high-grade neuroendocrine tumor characterized by rapid progression and frequent metastasis.

We combined microarray and qRT-PCR analyses to identify miRNAs aberrantly expressed in SCLC. The microarray approach alone identified 19 miRNAs that are significantly overexpressed, by at least 10-fold in SCLC cell lines compared to normal lung. Although a number of these miRNAs were implicated before in other tumor types, our results show the first time that miR-374 and miR-506 are deregulated in cancer, and, thus, may contribute to the tumorigenic phenotype. We also identified 35 miRNAs that are downregulated in SCLC cell lines compared to normal lung, and a large region in 19q13.41 containing more than 30 downregulated miRNAs (miR-512 to miR-373).

RNA samples from SCLC cell lines and a small number of microdissected primary SCLC tumors were analyzed with qRT-PCR as well. At first we identified 16 overexpressed and 8 downregulated miRNAs in primary SCLC tumor samples, as well as in SCLC cell line samples. MiR-17-5p showed the highest expression level (more than 15-fold) in primary SCLC tumors and the expression level of miR-17-5p was more than 30-fold in SCLC cell lines compared to normal lung, in line with the results of others.

7 downregulated and 8 overexpressed miRNAs were selected for further analysis in individual SCLC tumors and SCLC cell lines. qRT-PCR analysis verified that miR-126 is a uniformly downregulated miRNA, while miR-301 and miR-183 are uniformly overexpressed miRNAs in all SCLC sample types.

Some of the downregulated miRNAs are found in regions with frequent loss of heterozygosity in SCLC. Interestingly, the overexpressed miRNAs are not embedded in chromosomal regions frequently amplified in SCLC. Therefore, we analyzed DNA copy number changes in primary SCLC tumors for 5 genomic regions with overexpressed miRNAs. We identified one novel amplified region in SCLC: 7q32.2 contains the miR-183/96/182 cluster.

In our further work we demonstrated that miR-126 overexpression has a negative effect on SCLC cell proliferation, by delaying cells in the G1 phase of the cell cycle. Importantly, we identified SLC7A5 as a novel target of mir-126 in SCLC cells. miR-126

downregulates the expression of SLC7A5 at the translation level, and reduces mRNA stability simultaneously.

SLC7A5 (LAT1) is an L-type amino acid transporter widely expressed in many cancer types and was shown to play essential roles in cancer cell growth and survival. We demonstrated that in SCLC cells, similarly to other tumor types, suppression of SLC7A5 expression has an anti-proliferative effect. SCL7A5 suppression or miR-126 overexpression both delay SCLC cells in the G1 phase, suggesting that the effect of mir-126 on the cell cycle is, at least in part, mediated through SLC7A5. SLC7A5 provides the essential amino acids that act as signal to enhance growth of cancer cells through mammalian target-of-rapamycin (mTOR)-stimulated translation. Through different targets, miR-126 can negatively regulate PI3K/Akt pathway, which is aberrantly active in a large percentage of SCLC tumors. Therefore, miR-126 is an important negative regulator of the growth and proliferation of SCLC cells, which probably fine-tunes the activity of the PI3K/Akt/mTOR network through multiple targets, including SLC7A5.

ÖSSZEFOGLALÁS

A mikroRNS-ek rövid, nemkódoló RNS molekulák, melyek a génexpresszió szabályozásában vesznek részt azáltal, hogy a target mRNS-hez kapcsolódva gátolják azok transzlációját, illetve bizonyos esetekben az mRNS degradációját váltják ki. A tumorbiológiában betöltött szerepüket alátámasztja az a tény is, hogy az ismert mikroRNS-ek 50%-a olyan genomi régiókban található, melyek tumorokban amplifikálódnak vagy deletálódnak. Az általunk vizsgált tumorttípus a kissejtes tüdőrák (SCLC) ami egy neuroendokrin eredetű, gyorsan metasztatizáló tumor.

Kísérleteinkben microarray és qRT-PCR technikák kombinálásával sikerült meghatározni a kissejtes tüdőrákra jellemző mikroRNS-ek expressziós mintázatát. MikroRNS microarray technikával azonosítottunk 19 magasan expresszálódó mikroRNS-t (legalább 10-szer magasabb) a kissejtes tüdőrák sejtvonalakban a normál tüdőszövethez viszonyítva. Sikerült azonosítani olyan microRNS-eket, mint a miR-374 és miR-506, melyeket még nem hoztak kapcsolatba rákos folyamatokkal illetve olyanokat, melyek lehetséges funkcióját már leírták egyes ráktípusokban. Ezenkívül azonosítottunk 35 alacsonyan expresszálódó mikroRNS-t, és a 19q13.41 régiót, mely több mint 30 alacsonyan expresszálódó mikroRNS-t tartalmaz (miR-512-miR-373).

A továbbiakban qRT-PCR technikával vizsgáltuk a mikroRNS-ek expressziós szintjét SCLC sejtvonalakból származó RNS mintában, és kisszámú, mikrodisszektált primer SCLC tumorból származó RNS mintában. A microarray adatoknak megfelelően sikerült igazolni 16 magas és 8 alacsony expressziós szintet mutató mikroRNS-t az összes SCLC mintában a normál mintákhoz képest. A miR-17-5p mutatta a legmagasabb expressziós szintet a primer SCLC tumorokban (15-ször magasabb), míg az SCLC sejtekben 30x magasabb volt a miR-17-5p expressziója a normál tüdőszövethez képest.

A továbbiakban már nagyobb számú primer SCLC tumorban és SCLC sejtvonalakban vizsgáltuk a különbözően expresszálódó mikroRNS-eket. A qRT-PCR egyöntetűen igazolta a miR-126 alacsony, míg a miR-301 és a miR-183 magas expresszióját az összes SCLC mintában, sejtvonalakban és primer tumorokban egyaránt.

Néhány alacsonyan expresszálódó mikroRNS olyan kromoszóma régióban található, melyeknél SCLC-ben gyakori a heterozigóta deléció. A magas expressziós szintet mutató mikroRNS-ek viszont általában nem fednek át olyan kromoszóma régiókkal, melyeknél gyakori az amplifikáció SCLC-ben. Kísérleteinkben ezért 5 magas expressziós szintet mutató mikroRNS kópiaszám változását vizsgáltuk primer SCLC tumorokban, és azonosítottunk egy új SCLC-ben amplifikálódó régiót, a 7q32.2-t, mely tartalmazza a miR-183/96/182 klasztert.

További kísérleteinkben az alacsony szinten expresszálódó miR-126 microRNS szerepét vizsgáltuk a kissejtes tüdőrák sejtekben. Kimutattuk hogy a miR-126 az SCLC sejtek proliferációját gátolja, a sejtciklus G1 fázisból S fázisba való átmenetét lassítva. Azonosítottunk egy új miR-126 célpontot a kissejtes tüdőrákban, az SLC7A5 proteint.

SLC7A5 egy L-típusú aminosav transzporter, amely magasan expresszálódik a különböző ráktípusokban, így a kissejtes tüdőrákban is, és szerepet játszik a tumor sejtek növekedésében és túlélésében. Az SCLC sejtekben, más tumorokhoz hasonlóan, az SLC7A5 expressziójának gátlása anti-proliferatív hatással bírt. Mind az SLC7A5 expressziójának gátlása, mind a miR-126 expressziójának növelése késleltette az SCLC sejtek kilépését a sejtciklus G1 fázisából, azt mutatva, hogy a miR-126 sejtciklusra kifejtett hatása részben az SLC7A5 fehérje expressziójának gátlásán keresztül megy végbe. Az SLC7A5 animosav transzporter gondoskodik az esszenciális aminosavak felvételéről, melyek az mTOR szignál útvonal aktiválásán keresztül szignál molekulaként szolgálnak a tumor sejtek proliferációjához. A miR-126 több fehérjén keresztül vesz részt a PI3K/Akt útvonal szabályozásában, mely az SCLC tumorok nagy részében aberránsan aktív. A miR-126 mikroRNS tehát az SCLC sejtek

növekedésének egy fontos negatív regulátora, részben a PI3K/Akt/mTOR szignál útvonalra gyakorolt hatása révén, melyet olyan target géneken keresztül fejthet ki, mint az SLC7A5.

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8.1. PUBLICATION LIST (prepared by the Kenézy Life Sciences Library)

Register Number: DEENKÉTK /149/2011.
Item Number:
Subject: Ph.D. List of Publications

Candidate: Edit Miko
Neptun ID: QXTP9E
Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **Miko, E.**, Margitai, Z., Czimmerer, Z., Várkonyi, I., Dezső, B., Lányi, Á., Bacsó, Z., Scholtz, B.: miR-126 inhibits proliferation of small cell lung cancer cells by targeting SLC7A5. *FEBS Lett. 585* (8), 1191-1196, 2011.
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2. **Miko, E.**, Czimmerer, Z., Csánky, E., Boros, G., Buslig, J., Dezső, B., Scholtz, B.: Differentially expressed microRNAs in small cell lung cancer.
   Exp. Lung Res. 35 (8), 646-664, 2009.
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List of other publications

3. Varga, I., Hutóczki, G., Petrás, M., Scholtz, B., **Miko, E.**, Kenyeres, A., Tóth, J., Zahuczky, G., Bognár, L., Hanzély, Z., Klekner, Á.: Expression of Invasion-Related Extracellular Matrix Molecules in Human Glioblastoma Versus Intracerebral Lung Adenocarcinoma Metastasis.
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4. Balogh, A., Paragh jr., G., Juhász, A., Köbling, T., Törőcsik, D., **Miko, E.**, Varga, V., Emri, G., Horkay, I., Scholtz, B., Remenyik, É.: Reference genes for quantitative real time PCR in UVB irradiated keratinocytes.
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IF:1.838

9. KEYWORDS

microRNA, small cell lung cancer, miR-126, SLC7A5, G1 delay

TÁRGYSZAVAK

microRNS, kissejtes tüdőrák, miR-126, SLC7A5, G1 késés

10. ACKNOWLEDGEMENTS

First of all I would like to thank my supervisor Beáta Scholtz for the permanent support during my PhD, for the training and scientific discussions, her ambitions for motivation and for the solution of problems occurring during my work.

I am thankful to Árpád Lányi for his help, useful scientific discussions and for the possibility that I could work in his laboratory.

I also want to thank Professor László Fésüs, the head of the Department of Biochemistry and Molecular Biology, for giving me the chance to work in the department.

I am grateful to Zsolt Czimmerer for his help, support and advices.

I would like to thank our collaborators Zsolt Bacsó and Zoltán Margitai for performing the FACSArray analysis and Balázs Dezső for the FFPE sample preparations and immunohistochemistry.

Thanks to the collegues with whom I worked during these years: Júlia Buslig, Gábor Boros and Mária Balogh.