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Interplay between microRNA-21 and Sprouty2 in non small cell lung cancer-derived cell lines

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KURZFASSUNG

Einer einzelnen microRNA ist es möglich über die Bindung an den 3' untranslatierten Bereich hunderte mRNAs zu regulieren. Deswegen sind sie maßgeblich an der Ausbildung von spezifischen Expressionsmustern beteiligt, die die Entwicklungsprogramme festlegen und Krankheiten verursachen. Von einigen microRNAs ist bekannt, dass sie in Krebs dereguliert sind, was eine wichtige Rolle bei Entstehung und Entwicklung von Tumoren nahelegt. Zu dieser Gruppe von microRNAs gehört auch microRNA-21(miR-21); sie agiert als sogenanntes oncomiR und ist in einer Vielzahl verschiedener soliden Tumoren überexpremiert. Bekannte Substrate von miR-21 sind Tumorsupressoren wie z.B. Programmed Cell Death4 (PDCD4), Phosphatase and tensin homolog (PTEN), Sprouty1 (Spry1) und Sprouty2 (Spry2).

In dieser Studie haben wir die miR-21 Expression im Lungenkrebs untersucht und herausgefunden, dass in kultivierten Lungenkrebszelllinien die Levels von miR-21 kaum von jenen in normalen Lungenfibroblasten abweichen. Zusätzlich war die Korrelation miR-21 Expression und Proteinlevel von PDCD4, PTEN, Spry1 und Spry2 insignifikant. Auch die Proliferationsrate der Zelllinien stand in keinem Zusammenhang zu den gemessenen miR-21 Levels. Ebenso insignifikant war der Unterschied von miR-21 Expression in Adenokarzinomen und Plattenzellkarzinomen.

Auf der Suche nach endogenen Verursachern der miR-21 Expression in Tumoren fanden wir, dass Zelllinien mit einer Mutation im EGFR Gen annähernd dieselben miR-21 Levels wie Zelllinien, die die Wildtypversion dieses Gens tragen, zeigen.

Zelllinien, die eine onkogene Mutation im Ras Gen haben im Mittel zwar geringfügig höhere miR-21 Werte, aber die Abweichung war nicht signifikant. Da auch eine ektopische Expression von onkogen mutierten H-, K- und N-Ras zu keiner Veränderung der miR-21 Expression führte, liegt nahe dass Ras Mutationen keinen Einfluss auf die Regulation von miR-21 Expression haben.

Während Überexpression von Spry1 keinen Effekt auf miR-21 hat, führen hohe Levels von Spry2 zu einer signifikanten Reduktion der miR-21 Expression. Möglicherweise gibt es eine Rückkopplungsschleife zwischen miR-21 und Spry2.

Im zweiten Teil der Studie wurden verschiedene Strategien zur Modulierung der miR-21 Expression verglichen. Überexpression von verkürzten primiR 21 RNA durch ein virales System zeigte in einer der getesteten Zelllinien die gewünschte Wirkung. Um miR-21 zu inhibieren wurden verschiedene Ansätze ausprobiert. Während die adenovirale Produktion von antisense Oligonukleotiden und von siRNA durch einen RNA Polymerase III Promotor gegen primiR 21 die miR-21 Levels und die Expression der untersuchten Substrate nicht nennenswert veränderte, zeigen Zellen der Zelllinie U373, die mit dem sponge eraser Konstrukt infiziert wurden, im Vergleich zu Kontrollzellen signifikant reduzierte miR-21 Expression. Diese Daten führen zu der Annahme, dass die Produktion von sponge eraser Konstrukten die beste Strategie ist um mit der miR-21 Funktion in Tumor- Zelllinien zu interferieren.

ABSTRACT

MicroRNAs can regulate hundreds of mRNA by binding at the 3' untranslated regions and are therefore involved in regulation of gene expression during development and disease. Some microRNAs are known to be deregulated in cancer and play an important role in governing initiation and progression of tumors. MicroRNA-21(miR-21) is one of the microRNA known to act as an oncomiR. It is overexpressed in diverse solid tumors. Identified targets of miR-21 are tumor suppressors like Programmed Cell Death4 (PDCD4), Phosphatase and tensin homolog (PTEN), Sprouty1 (Spry1) and Sprouty2 (Spry2).

In this study we investigated miR-21 expression in non small cell lung cancer (NSCLC) derived cell lines and discovered that in cultured NSCLC derived cell lines miR-21 expression is comparable to the levels in normal lung fibroblasts. MiR-21 levels failed to show significant inverse correlation to the protein expression of PDCD4, PTEN, Spry1 and Spry2. Furthermore we were unable to detect a correlation between expressions of miR-21and proliferation rate. MiR-21 expression in cell lines derived from adenocarcinoma and squamous cell carcinoma were analyzed but no connection between histological type of lung cancer and miR-21 levels can be found.

Cell lines with a mutation in EGFR gene have almost the same levels of miR-21 compared to cell lines harboring the wild type gene.

Although cell lines harboring an oncogenic mutation in the Ras gene show on average higher levels, overexpression of oncogenic H-, K- and N-Ras does not lead to an alteration of miR-21 expression. These results indicate that Ras has no influence on the regulation of miR-21 expression.

While overexpression of Spry1 has no effect on miR-21 levels, high levels of Spry2 lead to significant reduced miR-21 expression. Maybe there is a feedback loop between miR-21 and Spry2.

In the second part of the study we compared different strategies to modulated miR-21 levels. Overexpressing of a shortened primiR 21 RNA by a viral system worked at least in one of the chosen cell lines. In order to achieve inhibition of miR-21 we tested different strategies. While adenoviral production of antisense oligonucleotides by an

RNA polymerase III promoter as well as expression of an siRNA against primiR 21 failed to reduce miR-21 levels and increase substrate levels, cells which are infected with a sponge eraser, show significant reduced miR-21 levels in U373 cell line compared to control cells. These data indicate that production of sponge eraser constructs is the most powerful way to interfere with miR-21 function in tumor-derived cell lines.

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1 INTRODUCTION

1.1 Cancer

Tumorigenesis is a multistep process, whereby cells are subject to various alterations, which are the result of an accumulation of genetic and epigenetic changes (1) (2). Alterations in three types of genes are involved in tumorigenesis: oncogenes, tumor suppressor genes and stability genes. Oncogenes are altered by mutations which lead to an activate gain of function, while mutations in tumor suppressor genes result in reduce of activity of these gene products. Stability genes are involved in DNA damage repair (e.g. miss match repair, nucleotide excision repair and base excision repair genes) and therefore these genes reduce mutations in other genes. When stability genes are altered, mutation rate in other genes increases (3).

In tissue it is necessary that cell proliferation, arrest and cell death are in equilibrium. On that account cell growth is regulated by several signaling pathways. Six essential alterations involved in the transformation from normal to malignant cells are shown in Figure 1. These so called hallmarks of cancer are sustaining proliferative signaling, evading growth suppressors, resisting cell death, inducing angiogenesis, enabling replicative immortality and activating invasion and metastasis (1).

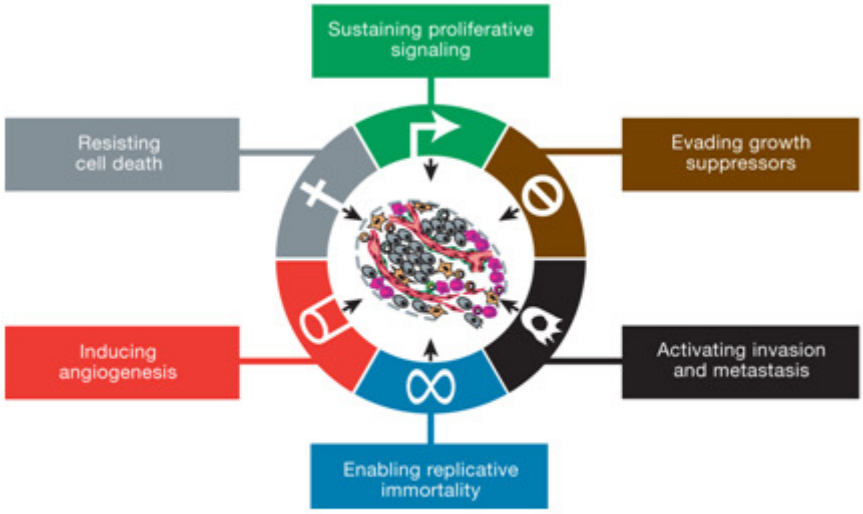

Figure 1: *Acquired Capabilities of Cancer (1)*

Cancer cells do not respond to growth promoting or growth suppressing signals, like other normal cells do. The independence of these factors can be acquired for example from alterations of receptors or changes in components of downstream signaling pathways like Ras.

Programmed cell death is a natural limitation for tumor development. Through apoptosis, cells with DNA damage can be removed and it is an important program for growth homeostasis. In tumor tissue, antiapoptopic factors (e.g. Bcl-2) or survival signals (e.g. Igf 1/2) are increased, while proapoptotic factors are downregulated.

Another feature which is unique for tumor cells is enabling replicative immortality. Normal cells can only pass a limited number of cell doublings before they undergo senescence or crisis. Cancer cells get immortalized, so that they have the potential for unlimited replication, which can be achieved e.g. by activation of telomerase.

Additionally, tumor tissue has to induce angiogenesis because it is essential for cells to get nutrients and oxygen. The so called angiogenic switch leads to new vessels, which help to provide the tumor. Proangiogenic factors are signal proteins which are increased in malignant cells.

Another hallmark of cancer is activation of invasion and metastasis. Patients die much more often due to metastasis, than on the primary tumor. Formation of the secondary tumors results from cells which leave the primary tumor and travel in blood or lymphatic vessels to other parts of the body where they begin to grow and form a new tumor. Invasion and metastasis is an effect of increased migratory and invasive abilities. Alterations of gene expression, including modification of signals of some receptor tyrosine kinases and changed binding specify of cadherins and integrins are essential for activating metastasis and invasion (4) (5).

Additionally to these 6 hallmarks of cancer, microenvironment plays an important role in cancer development. Tumor cells do not grow isolated; they are in contact with their environment. Tumor stroma, the microenvironment of malignant cells communicates with the tumor via paracrine signals and leads to cancer initiation and growth. Tumor cells are in touch with the components of the stromal parts, e.g. endothelial cells, pericytes, fibroblasts and immune cells (6).

1.1.1 Lung cancer:

In 2008 lung cancer was the most frequent diagnosed cancer worldwide. 18% of all cancer related deaths were arisen from lung cancer, which makes it to the leading cause of cancer deaths (7).

Lung cancer is strongly associated with tobacco smoking. The main risk factor for lung cancer is smoking. Cancerogens in the smoke lead to an accumulation of genetic changes. Additionally, environment factors (e.g. exposure to cancerogens like asbestos, arsenic and radon) and genetic factors are involved in the development of lung cancer (8) (9).

Histological there are two subtypes of lung cancer:

Small cell lung cancer (SCLC) counts for 20% of all lung cancer cases and consists of small and round cells, which grow very aggressive with distant metastasis. This form of lung cancer is strongly associated with smoking; 98% of all cases have a history of cancer (10). Several genetic and epigenetic alterations are representative for SCLC. For example loss of heterozygosity of Chromosome 3p and Fragile histidine triade (FHIT) are common and also inactivation of Retinoblastoma (Rb) and p53 is very frequent in SCLC (8).

Non small cell lung cancer (NSCLC) represents about 80% of all lung cancers and can be categorized in non squamous carcinomas, including adenocarcinomas, large cell carcinomas and other types of cells and squamous cell (epidermoid) carcinomas. The most common form of NSCLC is adenocarcinomas, which are also the most frequent lung cancer in non-smokers. Squamous cell carcinoma counts for 20% of all lung cancer cases and is the subtype with the best prognosis (9) (10).

Activation of oncogenic K-Ras occurs to 20% in NSCLC, while in SCLC it's very rare. Other genes which are overexpressed in lung cancer are e.g. ERBB1 (which encodes for EGFR), Cyclin D1 and B1, Bcl-2 and the myc proteins (8).

1.2 MicroRNA

MicroRNAs (miRNAs) are 19-24 nucleotides (nt) long, non coding RNAs, which are involved in the regulation of gene expression by targeting messenger RNAs (mRNAs) posttranscriptional. The first miRNA was discovered 1993 in *C. elegans* and was called lin-4. Since that time thousands more were explored. MiRNAs are involved in nearly every cellular process, including development, differentiation, cell proliferation and apoptosis. About 30% of all protein coding genes in humans are regulated by miRNAs, wherein a single miRNA can regulate up to 200 different targets. Because of these facts, it is hardly surprising that changes in the expression pattern of miRNAs leads to human pathologies, e.g. cancer (11) (12).

1.2.1 Biogenesis and mechanisms of post-transcriptional regulation of microRNAs

Processing of miRNA starts with a primary transcript, termed primiR, which is 100 base pairs (bp) to several kilo bp long. PrimiR is a transcript from independent miRNA genes and is available as a hairpin structure, containing a polyA tail and a 5' cap (11) (13).

PrimiRs get cleaved by a nuclear RNase III enzyme, called Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) into a 60-70 nt long precursor miRNA (termed premiR) (11). Cleavage by Drosha is not indispensable: PremiR can also be a result from splicing, which is called mitron (13). Afterwards premiR is transported into the cytoplasm by Exportin 5, a nuclear export protein (11).

Maturation takes place in cytoplasm by Dicer, a cytoplasmic RNase III and its binding partner human immunodeficiency virus transactivating response RNA-binding protein (TRBP), which results in a 19-29 nt mature miRNA duplex. One of these strands is loaded to ribonucleoprotein complexes (microRNPs) or miRNA-induced silencing complexes (miRISCs), the other is removed for degradation. Important components of the microRNPs are the Argonaute protein family (AGO). In mammalians 4 AGO proteins can be found, which are all involved in miRNA-mediated translational repression (12). Figure 2 shows a schematic illustration of the biogenesis of miRNA.

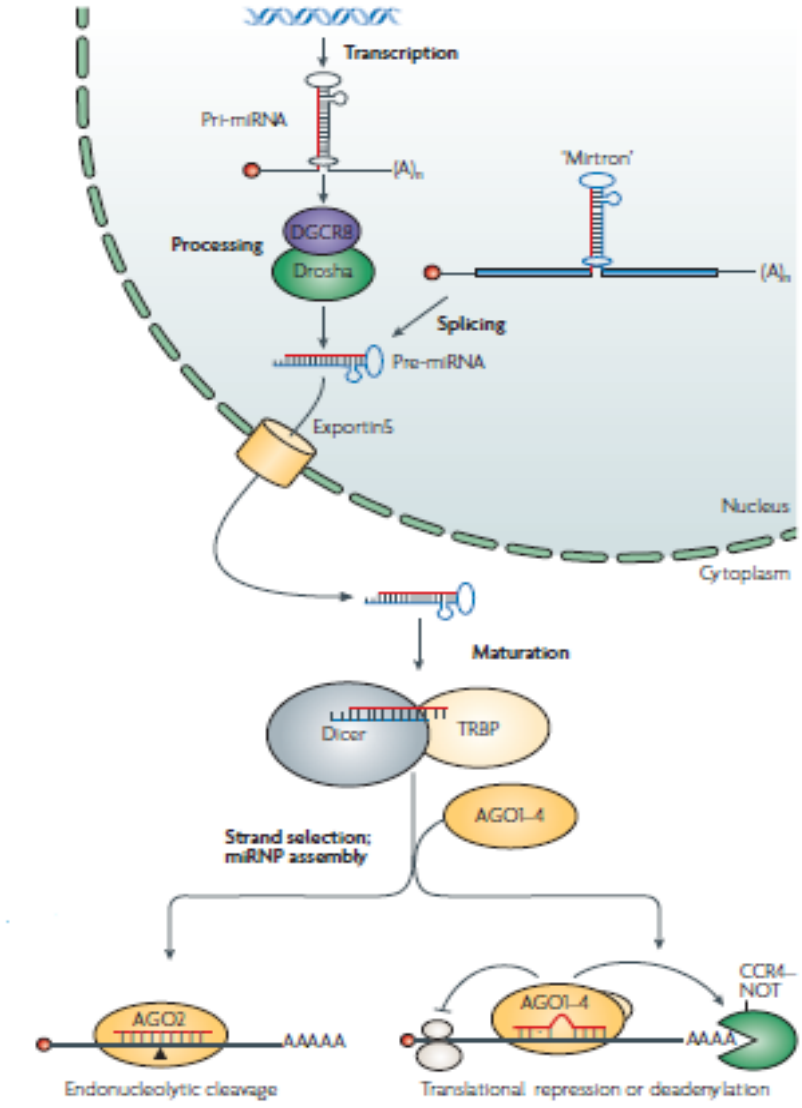

Figure 2: *Biogenesis of microRNAs* (12)

MiRNAs find their substrates by sequences which are complementary to the nucleotide 2 to 8, the so called seed region. The target region can mainly be found at the 3'untranslated regions (UTR) of target mRNAs, but sometimes there are binding sites for miRNAs at the 5'UTR or in coding regions too (12).

MiRNA base pairing with target mRNA influences stability and translation of this specific mRNA. An important factor in this connection is the base pairing between miRNA and mRNA: nearly perfect complementary results in mRNA degradation by endonucleolytic cleavage of the target mRNA at the miRNA binding site by AGO

proteins. After cleavage RISC gets released and can bind to another mRNA. Thereby one miRNA can regulate several mRNAs. Imperfect base pairing leads to translational silencing, blocking of target mRNAs and mRNA destabilization (11). Silenced mRNA moves to cytoplasmatic processing bodies, the so called p-bodies, where they get stored. Finally degradation of these mRNA takes place in the p-bodies by proteins like Dcp1/ Dcp2 (mRNA decapping enzymes) and Xrn 1 (exonuclease) (14) (15).

1.2.2 MicroRNAs and cancer

50% of human miRNAs are located at regions of the genome which are associated with cancer, e.g. on fragile sites. This finding indicates that miRNAs play an important role in tumorigenesis. Characterization of miRNA expression profiles shows that the expression correlates with the development of cancer. MiRNAs can function either as tumor suppressors or as oncogenes ("oncomiRs") (16) (17).

The reduction or deletion of miRNAs, which act as a tumor suppressor leads to the development of tumors and results in uncontrolled expression of target oncogenes. Consequences of this are increased proliferation, invasion and angiogenesis and reduced cell death. If miRNAs have an oncogenic role, they are called oncomiRs. OncomiRs are overexpressed in cancer and so the functions of target tumor suppressors are inhibited (18).

Examples for miRNAs which are involved in cancer development and progression are shown in Table 1 and Table 2.

The first connection between aberrant miRNA expression and cancer was shown in B-cell chronic lymphocytic leukemia (CLL). Deletion or downregulation of miR-15a and miR-16-1 were found in 68% of all CLL cases (19). These miRNAs act as tumor suppressor genes and their loss or downregulation results in changed expression of oncogenes. For example miR-15a and miR-16-1 negative regulate Bcl-2 expression, which is known as an anti-apoptotic gene (20).

miRNA gene	Localization	miRNA target	Dysregulation in cancer
miR-155	21q21.3	Ship c/EBPβ Pu.1 Cutl1 Pcalm	Upregulated in pediatric Burkitt's lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma, and CLL, as well as breast, lung, colon, and pancreatic cancer
miR-21	17q23.1	PTEN PDCD4	Overexpressed in breast, lung, prostate, gastric, cervical, head and neck, and colorectal cancer, as well as glioblastoma
miR-221&222	Xp11.3	PTEN TIMP3 p27 p57 Bim DDIT4 FOXO3A	Upregulated in hepatocarcinoma, CLL, melanoma, and glioblastoma, as well as lung, breast, thyroid, and prostate cancer
miR-106b-93-25 cluster	7q22.1	P21/Cip1 Bim	Overexpressed in gastric, prostate, colon, and pancreatic cancer, as well as neuroblastoma and multiple myeloma
miR-17-92 cluster	13q31.3	p63 E2F1 P21 Bim	Upregulated in lung and colon cancer, as well as lymphoma, medulloblastoma, and multiple myeloma

Table 1: *Overview of common upregulated miRNAs in cancer (oncomiRs) (21)*

miRNA gene	Localization	miRNA target	Dysregulation in cancer
miR-15/16	21q21.3	Bcl-2 CCND1 WNT3A	Downregulated in CLL, DLBCL, multiple myeloma, pituitary adenoma, prostate cancer, and pancreatic cancer
let-7	17q23.1	Ras Myc HMGA2	Downmodulated in liposarcoma and CLL, as well as gastric, lung, prostate, breast, ovarian, and colon tumors
miR-34a/b/c	Xp11.3	CCNE2 MET Bcl2 MycN Notch1/Notch2 CDK4/6	Downregulated in pancreatic cancer and Burkitt's lymphoma without Myc translocation Hypermethylated in colon tumors
miR-29	7q22.1	Mcl-1 CDC42	Downmodulated in CLL and cholangiocarcinoma, as well as colon, breast, and lung cancer
miR-122	13q31.3	ADAM17	Downregulated in HCC

Table 2: *Overview of common miRNAs, which act as tumor suppressors (21)*

MiRNAs, which are often aberrant expressed in cancers, are miR-143, miR-145, let-7 and miR-21.

MiRNAs, which are often downregulated in tumors, are miR-143 and miR-145. Interestingly in colorectal tumors only the mature form was found to be downregulated compared to normal tissue. The precursor transcript was found to be expressed similar in normal and tumor tissue, indicating that processing of miR-143 and miR-145 is disrupted in cancer (22).

MiRNAs which are encoded by the let-7 family often regulate oncogenes, e.g. Ras, and therefore are involved in cell proliferation and differentiation. Decreased expression of these miRNAs is associated with lung cancer (23).

MiR-21 is an example for miRNAs, which are overexpressed in several types of cancer.

1.2.3 Modulation of microRNAs in cancer

MiRNAs show an aberrant expression in cancer, thus they are a target anticancer drugs. With one therapeutic there is the possibility to target multiple genes. For this reason several strategies for modulation of miRNAs were explored (24) (25).

The first strategy to inhibit miRNA was antisense oligonucleotides (as oligonucleotides), which anneal to the mature miRNA strand and lead to degradation or duplex formation. This results in inactivation of the function of miRNAs and a derepression of their targets. Oligonucleotides, which were induced in *D. melanogaster* inhibit successful their target miRNA but in *C. elegans* this strategy fails. The reason therefore is that chemical structure modulations were necessary to increase stability, specify and binding affinity to the target miRNA. One of these modulations is the introduction of 2'-O-methyl groups which leads to a higher binding affinity to RNA. Further experiments show that such oligonucleotides are effective in several human cancer cell lines (Figure 3B) (24) (21).

MiRNA sponges carry multiple binding sites in tandem to a target miRNA. Sponges contend with the miRNA targets for binding with the mature miRNA. Sponge activity is under control of strong promoters like Human cytomegalovirus (CMV) and U6 and often carries a reporter gene like green fluorescence protein (GFP) or luciferase. Sponges which are totally complementary to the seed sequence show moderate efficiency of inhibiting miRNA, while base mispairing at position 9-12 leads to more efficiency by a more stable interaction with target miRNA (Figure 3D) (24) (25) (26).

MiR-masking antisense oligonucleotides technology (miR-mask) are single strand oligonucleotides, modified by a 2'-O-methyl-group and show fully complementary to the binding sites of miRNA at the 3' UTR of the target genes. MiR-masks cover up this binding sites and lead to the suppression of miRNA activity (Figure 3C) (24).

Another strategy to inhibit miRNA is with RNA interference (RNAi) methods by small interfering RNA (siRNAs). This strategy was formerly thought to be useless because processing of miRNA take place in the nucleus whereas siRNAs are mainly active in the cytoplasm. But if siRNA is designed to target the promoter of miRNA gene as well as the sequence of the primiR, RNAi is a successful tool to inhibit miRNAs. MiRNA processing was reduced by expressing hairpin siRNA constructs, which are complementary to the precursors of miRNAs, resulting in a decrease of active mature miRNA (Figure 3A) (25).

Not all miRNAs act as oncomiRs, some of them show activity as tumor suppressors and therefore approaches for targeting miRNA by overexpressing them were done. Synthetic oligonucleotides were introduced into the cells. These so called miRNA mimics are identical to endogenous miRNAs and are small double stranded and chemical modified oligonucleotides. Often longer mimics which include sequences of precursors of miRNA (premiR or primiR) which should be overexpressed, are used. MiRNA mimics are shown to induce cell death and decrease cell proliferation (Figure 3E) (24) (27).

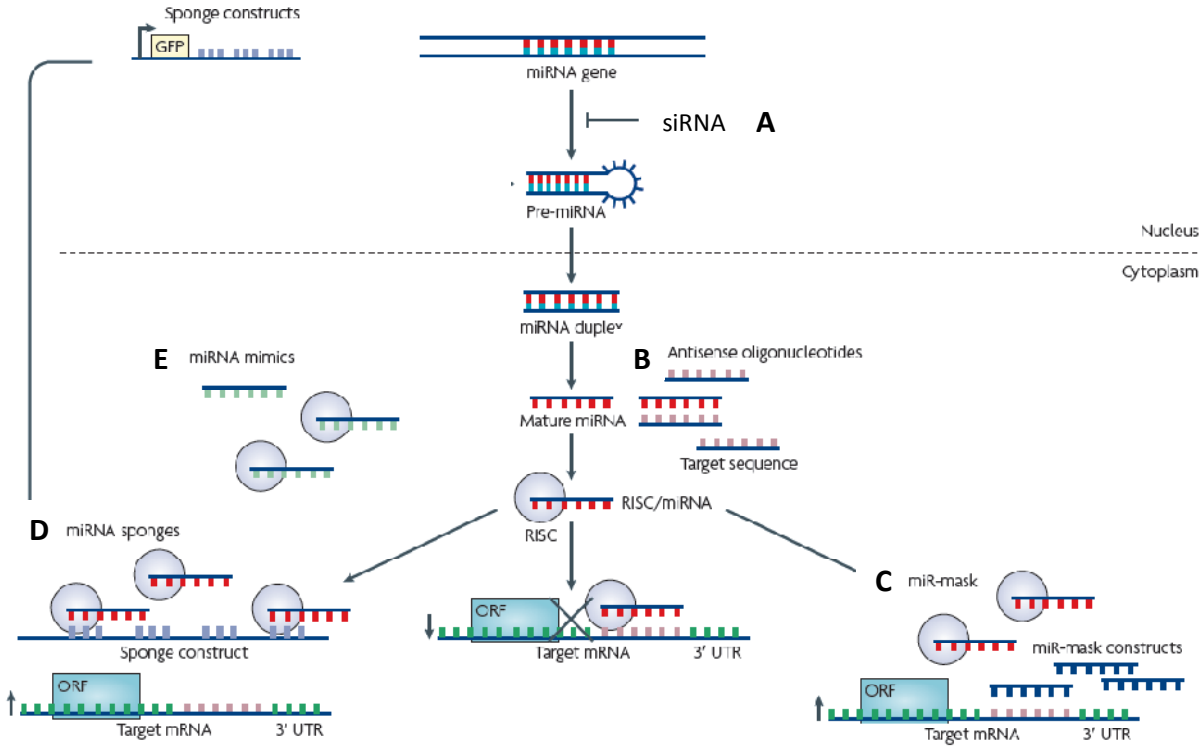

Figure 3: *Modulation of miRNAs in cancer;* ***A:*** *Inhibition of miRNA by complementary siRNAs to miRNA precursors;* ***B:*** *As oligonucleotides with complementary to mature miRNA;* ***C:*** *miR-mask are complementary to the 3'UTR by covering up the binding site for miRNAs;* ***D:*** *miRNA sponges carried multiple tandem sequences and compete with targets of miRNA;* ***E****: miRNA mimics are identical with endogenous miRNAs and were introduced into the cell (adapted from* Garzon et al. (24)).

1.2.4 MicroRNA-21

MiR-21 plays a role in cancer development und diagnoses and is downregulated in cardiovascular diseases (28) (29).

MiR-21 is a highly conserved miRNA among species. The miR-21 gene is located at Chromosome 17, on the 10th intron of the Transmembrane protein 49 gene (TMEM49). Expression of the miRNA is under control of an own promoter and not driven by the promoter of the TMEM49 gene (28) (29).

The primary transcript, the 3433 nt long primiR 21 is processed to the 72 nt long premiR21. This precursor gets cleaved to a 22 nt mature miR-21 (28) (29).

Overexpression of miR-21 was found in many different tumor types, e.g. glioblastoma (30), breast (31) (32), colorectal (33) (34), liver (35), head and neck (36), esophageal (37), and lung cancer (38) (39). According to the function of miR-21 as an oncomiR, most targets are genes which act as tumor suppressors. An overview of the activity of miR-21 in cancer cells is depicted in Figure 4.

The regulation of expression of miR-21 is very complex and multiple transcription factors like activator protein1 (AP-1) (40), Transforming growth factor (TGF)-β1 (41) and signal transducer and activator of transcription 3 (STAT3) (42) are important for regulation of transcription. An auto regulatory loop between AP-1, miR-21 and Programmed cell death 4 (PDCD4) is known: After induction of miR-21 expression by AP-1 the PDCD4 levels, a target of miR-21 decrease. PDCD4 is important for stimulation of AP-1 in response to Ras (43).

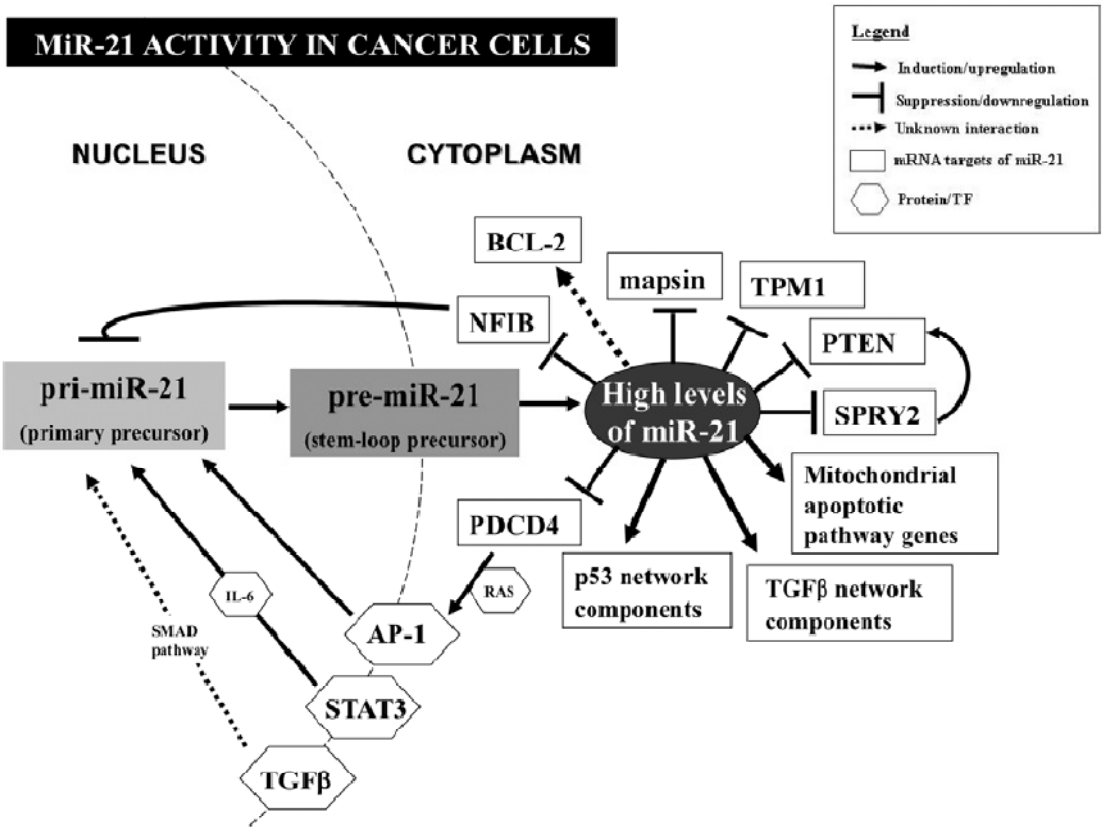

Figure 4: *miR-21 activity in cancer* (28)

The best studied target of miR-21 is PDCD4 (44) (45), which acts as tumor and metastasis suppressor and is upregulated during apoptosis.

Another gene which is negatively regulated by miR-21 is Phosphatase and tensin homolog (PTEN) (35), which inhibits the phosphoinositide-3-kinase (PI3K) pathway. This pathway is often activated in many malignant cells and is involved in the regulation of cell survival. PTEN is repressed by miR-21, which leads to increased cell migration and metastasis.

Sprouty1 (Spry1) (46) and Sprouty2 (Spry2) (47) were identified as target of miR-21. Both are regulators of the receptor tyrosine kinase (RTK) signaling and act as repressors of mitogen-activated protein kinase (MAPK) pathway. Spry2 is also known to induce the upregulation of PTEN (48).

miR-21 target	Validated by	Function
PTEN	Luciferase reporter assay, real-time PCR, western blot, immunocytochemistry; cell proliferation, migration and invasion assays	Tumor suppressor, decreases tumor cell proliferation, migration, and invasion.
PDCD4	Computational analysis, luciferase reporter assay, western blot; cell invasion, intravasation and distal metastasis formation	Tumor suppressor, reduces cancer cell invasion, reduce intra-vasation and lung metastasis in chicken embryo metastasis assay.
Tropomyosin	Two- dimentional protein electrophoresis of breast tumor samples, luciferase reporter assay	Actin- binding protein, suppresses anchorage-independent cell growth
Sprouty 1	Western blot following miR-21 modulation, luciferase reporter gene assay, protein modulation in the heart following miR-21 silencing *in vivo*	Negative regulator of ERK-MAP kinase activity
Sprouty 2	luciferase reporter assay, immunocytochemistry following miR-21 expression modulation, migration assay	Inhibitor of branching morphogenesis and neurite outgrowths
RECK	Computational analysis, western blot, cell migration and invasion assays, clinical studies	Tumor suppressor, negative regulator of MMP9
Bcl2	Luciferase reporter assay, western blot	Apoptosis inhibitor
MARCKS	Reported RISC-coimmunoprecipitation, western blot, cell motility, apoptosis and proliferation assays	PKC substrate, an actin filament crosslinking protein
HNRPK	Computational analysis, real- time PCR, luciferase reporter gene assay	One of the major pre-mRNA-binding proteins, regulation of cell cycle
IL-12p35	Computational analysis, luciferase reporter gene expression	Is invovled in allergic airway inflammation
JAG1	Computational analysis, luciferase reporter gene assay, cell differentiation assay	Ligand for the receptor Notch 1, mediates Notch signalling, is involved in cardiovascular development, enhances FGF- induced angiogenesis
BTG2	Computational analysis, RT-PCR in laryngeal carcinoma samples, EGFP reporter gene assay, cell proliferation assay	Antiproliferative protein
LRRFIP1	EGFP reporter gene assay, RT-PCR, western blot	Inhibitor of NF-κB signalling
BMPRII	Computational analysis, luciferase reporter gene assay	Receptor for BMP2 and BMP7 and less to BMP4, mutation in BMPRII causes primary pulmonary hypertension
TGFBR2	Real-time PCR, western blot, luciferase reporter gene assay	Ser/Thr protein kinase, a member of TGFβ receptor subfamily
Cdc25A	MicroRNA analysis, computational analysis, RT-PCR, western blot, cell proliferation assay, colony formation assay	Tyrosine protein phosphatase, oncogene
TAp63	Luciferase reporter assay, real-time PCR, apoptosis and cell growth assays, cycle analysis	A homolog of p53, mediates cell arrest and apoptosis

***Table 3**: Overview of miR-21 targets (29)*

1.3 Sprouty proteins

1.3.1 Structure of Sprouty proteins

Sprouty (Spry) was first discovered in *D. melanogaster*, it is involved in development of the trachea by antagonizing Fibroblast Growth factor (FGF) signaling (49). Subsequent, Spry proteins were shown to inhibit RTK signaling. Further analyses show that Spry had homolog proteins in e.g. mouse (50), chicken (51), zebrafish (52), frog (53) and humans (54). In mammals there are four different Spry proteins known, named Spry1-4 (55). dSpry contains 501 amino acids and has a molecular mass of 63kDa, while hSpry (human Spry) are smaller and show a molecular mass of 32-35kDa (56) (57).

The whole Spry family is characterized by highly conserved cystein rich domain at the C-terminus and shows divergence in the N-terminus of the protein (58) (59). In Figure 5 schematic illustration of the structure of Spry proteins is shown. The cystein-rich region (termed Spry Domain) harbors a highly conserved Raf-1 binding domain (RBD) (57). This domain is necessary for the localization of Spry at the plasma membrane by binding to phosphatidylinositol-4,5-bisphosphate (56) (58).

The N-terminus shows more variance between the different Spry proteins, but includes a short motif, with a central tyrosine residue (Y) (57) and a serine rich domain (55). Phosphorylation of tyrosine is for each Spry protein specific and the function as inhibitor depends on it (56).

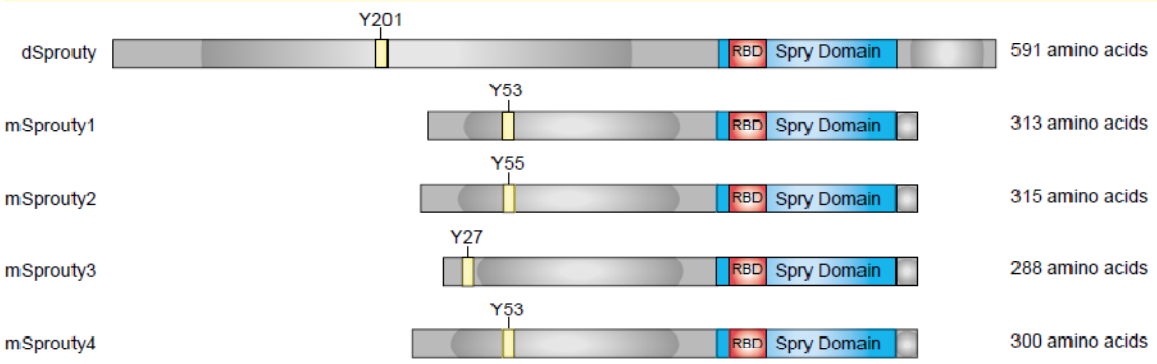

Figure 5: *Structure of Spry proteins. Tyrosine residue is shown in yellow and the serine rich region in green. Additionally Raf1- binding domain is labeled red, while Spry domain is represented in blue (57).*

1.3.2 Expression of Sprouty proteins

Spry1, 2 and 4 are expressed widespread over adult and embryonic tissue, while Spry3 seems to be restricted to adult brain and testes.

Expression of Spry depends on tissue specific factors and differs in different cell types. Spry expression is induced by growth factor stimuli such as Epidermal Growth Factor (EGF), FGF, Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), Nerve Growth Factor (NGF), Hepatocyte Growth Factor (HGF) and other factors like insulin, stem cell factor/kit ligand (SCF), Glial cell line-Derived Neurotrophic Factor (GDNF), Brain-Derived Neurotrophic Factor (BDNF) and T-cell receptor activation. Spry expression is dependent on RTK signaling, which indicates that Spry is included into a negative feedback loop of signaling (60).

After the induction of expression of Spry by several stimuli, Spry translocates to the plasma membrane, where MAPK pathway modulation takes place. There are different mechanisms, how Spry modulate RTK signals. Figure 6 depicts action of Spry under FGF, VEGF (Figure 6C) and EGF-mediated signaling (Figure 6D) (58) (60).

1.3.3 Function of Sprouty proteins

Spry are modulators of RTK signaling by interacting with extracellular signal-regulated kinase (Erk)/ MAPK pathway.
Figure 6A shows Ras/Raf/MAPK pathway and PLC-γ pathway in the absence of Spry. Signal transduction is activated by the binding of the growth factor to the RTK, which leads to autophosphorylation of the target receptor. Thereby adaptor proteins like Grb2 can bind by Src-homolog2 (SH2) or phosphotyrosine-binding (PTB) domain. Accordingly Sos bind to Grb2 and activate Ras- GTPase through the change of GDP to GTP. Ras, which have bound GTP activate Raf (MAPK kinase kinase) by phosphorylation, which activates MEK (MAPK kinase). MEK in turn activates ERK1/2 (MAPK), which results in phosphorylation of target proteins. The point where Spry interact with the pathway varied (58).
In phosphatidylinositol-specific phospholipase C (PLC)-γ pathway, the downstream effector PLC-δ (a Raf kinase) is able to activate MAPK in a Ras independent way by phosphorylating and activating Raf.

If c-Cbl ubiquitin ligase is present, it can bind to the phosphorylated receptor, which results in ubiquitination and later on in degradation of RTK (Figure 6B) (58).
In case of FGF, Spry1 or Spry2 gets phosphorylated at its tyrosine residue after the activation of RTK. This generates binding sites molecules, which harboring SH2 or PTB domains, such as Grb2. Binding of Spry to Grb2 leads to in inhibition of Ras/Raf/MAPK pathway upstream of Ras (61). FGF stimulation shows that Spry proteins are able to act as homo- and heterooligomers (62). RTK stimulation of VEGF results in direct binding of Raf on the RBM domain of Spry 4, which prevents Raf from phosphorylation through PLC-δ kinase (63). Based on the fact, that all Spry homologues harboring a RBM, it is supposed that all members of the Spry protein family are able to inhibit MAPK pathway in this way (Figure 6C) (58).

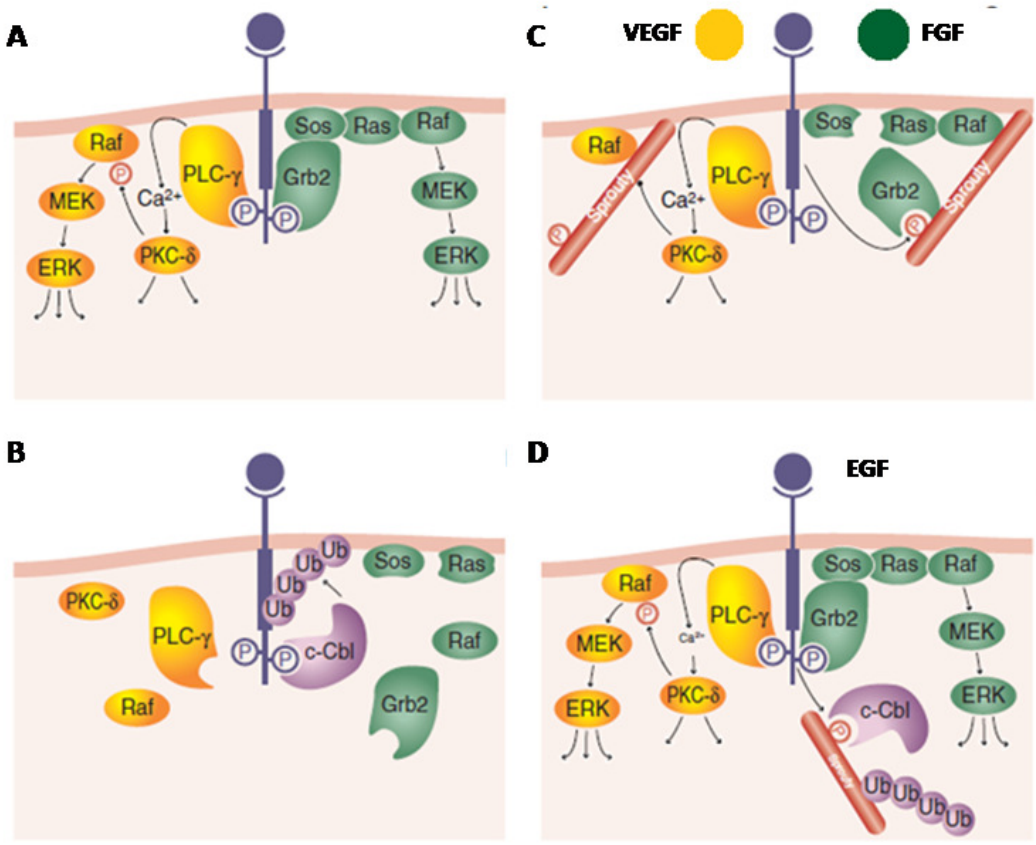

Figure 6: *Modulation of RTK signaling by Spry proteins;* ***A:*** *Ras/Raf/MAPK pathway (green) and PLC-γ pathway (yellow) when no Spry is available;* ***B:*** *Ubiquitination and resulting degradation of growth factor receptor by c-Cbl;* ***C:*** *Repression of RTK by Spry when either VEGF (yellow) or FGF (green) bind to the receptor;* ***D:*** *Enhancing of EGFR signaling through Spry (adapted from Christofori et al.(58))*

In contrast to inhibition of MAPK pathway through Spry, EGF mediated-signaling is enhanced by Spry2. EGF Receptor (EGFR) is normally degraded by ubiquitination through c-Cbl. In presence of Spry2, c-Cbl prefers to bind to it than to EGFR. Interaction of Spry2 with c-Cbl, CIN85 an endocytic adapter, which is needed for clustering of c-Cbl and Hepatocyte growth factor-regulated tyrosine substrate (Hrs) which is involved in EGFRs trafficking to late endosomes, leads to sustained EGF-mediated signaling as well as to multiubiquitination and degradation of Spry2 (Figure 6D) (64) (65) (66).

Spry proteins are also known to interact with PLC-γ and therefore leads to decreased PLC-γ signaling (67).

1.3.4 Sprouty proteins in cancer

In cancer RTK signaling is often activated due to aberrant expression of their modifiers. Aberrant expression of Spry proteins, such a modifier of RTK signaling, is associated with the malignant phenotype (57) (68) (69) (70). Downregulation of Spry2 was shown in breast (71), prostate (72), liver (73), lung cancer (74) and melanomas (75). Results of Spry2 expression in colorectal cancer are not that clear: Holgren et al. shows that Spry2 expression is upregulated in colonic adenocarcinomas (76), while Feng et al. detected a decrease of Spry2 expression in colon cancer, especially in advanced stage tumors (77).

The first evidence that Spry proteins act as tumor suppressor was found in an osteosarcoma cell line, in which overexpression of Spry2 inhibit tumor growth and metastasis (78). Later on it was shown that overexpression of Spry1 in prostate cancer cell lines leads to decrease in cell proliferation and inhibits colony formation (79). In addition, inhibition of anchorage-independent growth and migration can be observed (80).

In prostate cancer overexpression of Spry1 in cell lines shows a tumor suppressing effect by inhibiting cell proliferation and inducing apoptosis (79). Spry4 shows no influence on proliferation but inhibits cell migration (81).

Increased expression of Spry2 in NSCLC leads to inhibition of cell proliferation in K-Ras mutated cell lines and tumor formation in vivo (74).

Studies with a dominant negative Spry2 form, which is mutated at the tyrosine residue show larger tumors compared to the control in breast (71) and increased phosphorylated Erk levels and neoplastic transformation in liver cancer (82).

Spry is involved in other cellular functions, which are involved in development of cancer. In Spry4 knock down mice the effect of Spry4 on angiogenesis was investigated and showed that downregulation of Spry4 results in increased growth and vascularization (83).

2 AIM OF THE STUDY

Cancer is the result of an accumulation of genetic and epigenetic changes, which results in changes in cellular features like increased proliferation and migration as well as inhibition of apoptosis (1) (2). Altered expression and functions of oncogenes and tumor suppressor genes involved in cancerogenesis are not only results of changes in the coding sequences but may also result from altered functions of small non coding RNAs. Especially miRNAs were found deregulated in tumors and seem to play an important role in initialization and progression of cancer (16) (17). The most frequently overexpressed endogenous miRNA in solid tumors is miR-21 (38). MiR-21 acts as an oncomiR and represses translation by binding to the 3'UTR of the mRNA of tumor suppressors, such as PTEN, Spry and PDCD4 (29). Four different Spry proteins exist in mammalians (Spry1, 2, 3 and 4), whereby only Spry1 and Spry2 are confirmed to be targets of miR-21. Spry proteins regulates MAPK pathway mainly by repressing RTK signaling, with exception of EGFR-mediated signaling where Spry shows an enhancing effect (56) (58).
MiR-21 was found to be upregulated in lung cancer tissue (38) (39). In this study it should be determined if the same observation which was made in tumor can also be found in NSCLC derived cell lines compared to normal lung fibroblasts (WI-38). Therefore cell lines harboring mutations in Ras or EGFR gene as well as cell lines without mutations are used and endogenous levels of miR-21 in different NSCLC cell lines will be detected by Northern blotting to explore if miR-21 is overexpressed in cell lines too. Additionally the endogenous expression of target proteins of miR-21 (PDCD4, PTEN, Spry1 and Spry2) will be detected by Western blotting. Protein levels will be compared with miR-21 expression. Furthermore expression of miR-21 in cell lines derived from adenocarcinomas will be compared to levels in squamous cell carcinoma cell lines.

Due to the fact that miR-21 expression is regulated by a feedback loop with its target PDCD4 (43), we will explore if there is a feedback loop between miR-21 and Spry1 or Spry2.

Within this study methods for overexpression and inhibition of miR-21 by viral vectors should be establish. For overexpression a vector which express primiR 21 will be used. To figure out which is the best strategy to inhibit miR-21, three different methods will be used: The plasmid pAdlox sponge 21 containing a CMV promoter, a luciferase reporter gene and the antisense sequence against miR-21 in tandem is already available in the labor. Construction of pAdlox oligo eraser 21(plasmid containing an U6 promoter and one antisense sequence for miR-21) and pAdlox simiR 21 (plasmid producing siRNA against primiR 21) will be constructed.

3 MATERIAL AND METHODS

3.1 Cell culture

3.1.1 Used cell lines

All used cell lines are derived from human origin and are split in an adequate ratio with Trypsin/ EDTA (T/E). All used cell lines are listed in Table 4.

Cell line Source / Alias	*Origin Site*	*Type*	*Medium*	*Characteristics*
U373 ATCC-HTB17	Brain	Glioblastoma	DMEM	Split ratio: 1:9
WI 38 ATCC-CCL75	Lung	Diploid human fibroblasts, derived from normal embryonic lung tissue	DMEM	Split ratio: 1:2- 1:3
A427 ATCC-HTB53	Lung	Adenocarcinoma	DMEM	Split ratio: 1:4 K- Ras mut Glycin 12
A549 ATCC-CCL185	Lung	Adenocarcinoma	DMEM	Split ratio: 1:10 K- Ras mut Glycin 12
Calu-6 ATCC-HTB56	Lung	Adenocarcinoma	DMEM	Split ratio: 1:6 K- Ras mut Arg 61
SKLU-1 ATCC-HTB57	Lung	Adenocarcinoma	DMEM	Split ratio: 1:4 K- Ras mut Glycin 12
CRL5883 ATCC	Lung	Adenocarcinoma	RPMI	Split ratio: 1:3 EGFR mut
CRL2868 ATCC	Lung	Adenocarcinoma	RPMI	Split ratio: 1:5 EGFR mut

VL-2 **ICR**	Lung	Large cell carcinoma	DMEM	Split ratio: 1:2 K- Ras mut
VL-4 **ICR**	Lung	Large cell carcinoma	DMEM	Split ratio: 1:5 K- Ras mut
VL-10 **ICR**	Lung	Squamous cell carcinoma	RPMI	Split ratio: 1:3 K- Ras wt
VL-3 **ICR**	Lung	Squamous cell carcinoma	DMEM	Split ratio: 1:4 K- Ras wt
VL-6 **ICR**	Lung metastasis in lymphnode	Squamous cell carcinoma	DMEM	Split ratio: 1:4 K- Ras wt
VL-8 **ICR**	Lung metastasis in lymphnode	Squamous cell carcinoma	DMEM	Split ratio: 1:3 K- Ras wt
VL-5 **ICR**	Lung metastasis in lymphnode	Squamous cell carcinoma	DMEM	Split ratio: 1:5 K- Ras wt
VL-7 **ICR**	Lung metastasis in lymphnode	Squamous cell carcinoma	DMEM	Split ratio: 1:2 K- Ras wt
293 **ATCC-CRL1573**	Kidney	Embryonic	DMEM	Split ratio: 1:6 constantly expressing parts of adenoviral genome (see *Construction of adenoviruses*)

Cre-8	Kidney	DMEM	Split ratio: 1:9 Derived from 293 cells and were stably transfected with a cre recombinase gene

Table 4: *Overview of used cell lines with detailed information of origin, types and adequate medium for cultivation of cells*

ATCC American Type Culture Collection
ICR Institute of Cancer Research, Vienna
VL Vienna lung
All cells are cultured in respective medium with 10% FCS in a humidified incubator at 37°C with 7.5% CO_2.

Medium replacement and splitting of the cell are carried out according to the needs of the different cell lines.

FCS Fetal Calf Serum, heat inactivated (PAA)
DMEM Dulbecco's Modified Eagle Medium (Sigma)
RPMI RPMI-1640 medium (Sigma)

Trypsin/EDTA
0.1% trypsin
0.01% EDTA
0.001% phenol red

3.1.2 Storage of cells

For freezing 50-60% confluent cells are washed with PBS and are trypsinized. Afterward cells get resuspended in 20-30ml medium and are pelleted by centrifugation at 1500rpm for 5 minutes (min). Medium is sucked of and cells are resuspended in 1ml serum containing 10%DMSO. Subsequently, cell suspension is transferred into a cryopreservation tubes (Nalgene, Cryoware). Cells are stored at -80°C for 2-3 days and afterwards finally put in liquid nitrogen for long term storage.

3.1.3 Growth curve

Cells are infected with adequate amount of adenovirus and after 2 days equal amounts of cells were split into several 60mm dishes and are cultured in their appropriate medium with 10% FCS. Cells will be counted by using a microscope counting chamber every 1-3 days. For each time point 3 plates of cells were counted Counting chamber consist of 4 panels, which are divided in 4x4 squares. For cell counting, cells get trypsinized and cell suspension gets properly diluted. 10µl of this dilution get peptized between cover slip and counting chamber. Cells in each panel are counted separately and mean gets generated. To calculate the original concentration in cells per ml mean gets multiplied with the factor 10^4.

Absolute cell number is calculated with following formula:

$$absolute\ cellnumer = mean\ of number of counted\ cells * 10^4 * volume\ of\ dilution$$

For calculating the doubling time of the cells following formula was used:

$$doubling\ time = t * \frac{ln\ (2)}{ln(\frac{N2}{N1})}$$

t… time in hours (h) between time point of counting 1 and time point of counting 2
N1… number of cells at time point of counting 1
N2… number of cells at time point of counting 2

3.1.4 Construction of adenovirus

Construction of an adenovirus was based on the Cre-lox recombination between ψ5 virus and pAdlox vector containing the insert of interest.
ψ5 virus is a version of Ad 5, which means that E1 and E3 genes are deleted. This virus contains loxP sites in the same orientation, flanking the packaging site (ψ). The packaging site can be removed by recombination between the loxP sites. The pAdlox shuttle plasmid is a derivate of pCMV Ad and contains the following sequences a left ITR, a packaging site (ψ), a CMV promoter, multiple cloning sites (MCS), loxP site for recombination and a right ITR. On both sites these sequences were flanked by Sfi I restriction sites. For selective propagation in bacteria these shuttle plasmid contains an ampicillin resistance cassette (Figure 7) (84).

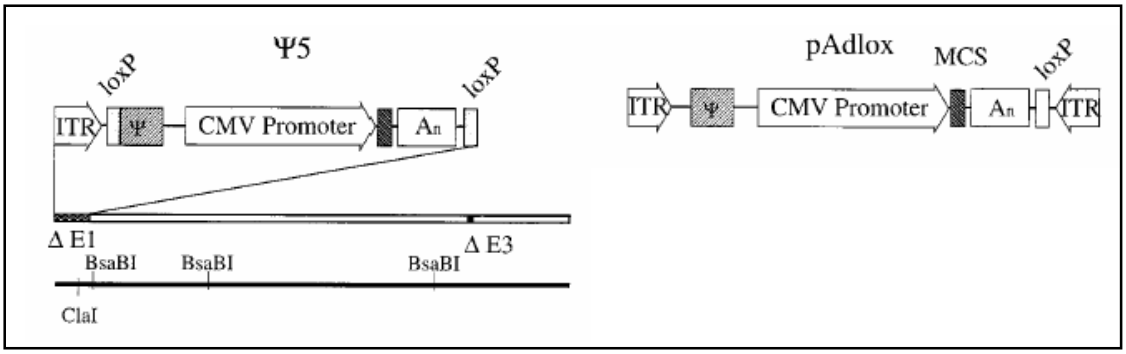

Figure 7: *Functional groups of the ψ5 adenovirus and the pAdlox plasmid (84)*

Selective Cre-8 cell line contains a β Actin based expression cassette driving the Cre recombinase from phage P1, with a N-terminal nuclear localization signal, which are integrated in 293 cells. 293 cells in general contain an integrated viral E1 region. This region contains all genes that are necessary for viral replication and therefore it is possible to propagate E1-deleted viruses.

The DNA sequences which are inserted in the MCS of the pAdlox shuttle plasmid are included in recombinant virus in a Cre recombinase-catalyzed recombination event between pAdlox and ψ5.

First a recombination between the two loxP sites in ψ5 was catalyzed by Cre recombinase. This leads to the removal of the cis-acting packaging sequence. The resulting viral genome is now unable to be encapsulated (ψ-). Then the pAdlox vector was digested by Sfi I restriction enzyme. This produces a linear DNA fragment containing 2 ITRs, a ψ signal, a loxP site and the target insert. Linear pAdlox fragments lead to higher yields of recombinant viruses compared to the circular plasmid. Next Cre recombinase catalyses recombination between ψ5 (ψ-) and the pAdlox fragment. The result is a recombinant virus with only one loxP site. Reinfection cycles lead to the loss of all ψ- viruses because of the wasting of their package signals, while the number of recombinant viruses will be increased (Figure 8) (84).

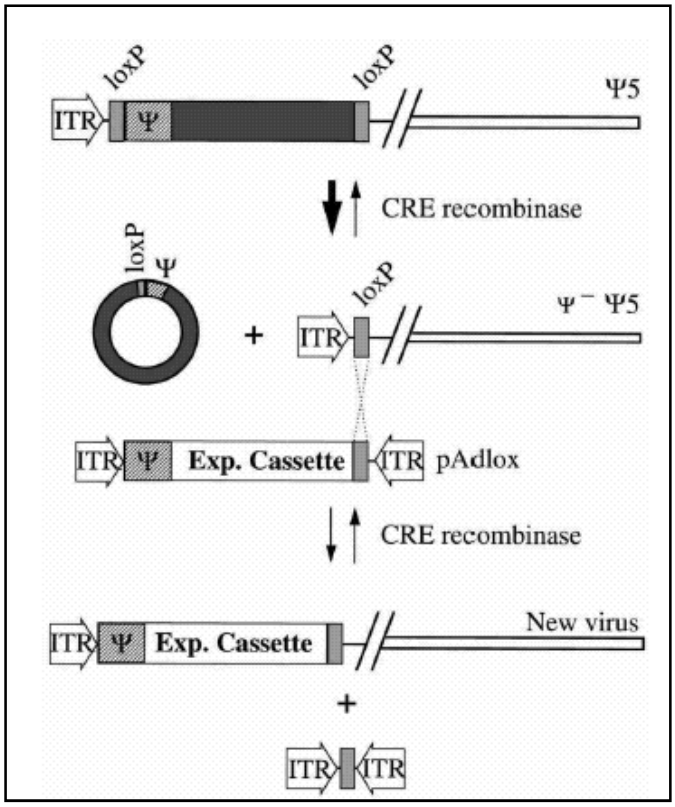

Figure 8: *The Cre recombinase-driven generation of adenoviruses (84)*

Creation of the target recombinant virus

A PCR product or a DNA fragment derived from another vector, are ligated into the pAdlox backbone vector. 6µl of the plasmid are digested by Sfi I (see *Restriction digestion & ligation*) for 2 h at 50°C. For control of the restriction digestion an agarose gel is performed.

3µg of the digested pAdlox shuttle plasmid are co-transfected with 1µg ψ5 virus DNA in Cre-8 cells (shuttle plasmid: virus DNA ratio 1:3).

Fugene Transfection:

Cre-8 cells are splited the day before in the ratio 1:5 so that the confluence on the next day was about 50%. Next day medium is reduced to the minimal necessary amount. In an eppendorf tube add 6µl of FuGene6 transfection reagent (Roche, #1815091) to 200µl medium (avoid even traces of serum) without touching the walls and incubate the mixture for 5 min at room temperature (RT). In a separate tube pAdlox shuttle plasmid and the ψ5 virus DNA are mixed together and FuGene medium solution can be added after and incubation time of 15 to 45 min at RT. Reagent solution should be added drop wise directly on the cells.

Propagation of the target recombinant virus

After transfection and the first reinfection cycle cells are cultured 3 to 4 days, until they start to lyse at later reinfection cycles. Then they are scraped of and collected by centrifugation for 3 min at 1500rpm. The supernatant (SN) will be reduced to 2ml or discard and 2ml of 10mM Tris pH=8.8 is added. Afterwards virus is extracted by four to five rounds of freeze in N_2 and thaw at 37°C in the water bath out of the cells. The sample will be centrifuged again for 3 min at 1500rpm before new Cre-8 cells, splitted one day prior to infection, are re-infected with less amount of virus-containing SN than before.

After about 5 reinfections (rule: virus titer is than high enough, when 10µl of the SN of the re-infection before are enough to lyse the cells into 2 days), 15x 15cm cell culture dishes of 293 cells are infected with an adequate amount of virus derived from the last Cre-8 infection. When cells start to lyse, after about 2 to 3 days they will be scraped off and collected by centrifugation for 5 min at 1500rpm. SN was discarded and pellet is resuspended on 8ml 10mM Tris pH=8. For release of the virus cells are lysed by 5 cycles of freeze and thaw. After centrifugation (5 min, 1500rpm) the virus-containing SN is collected and the virus is purified by density-gradient ultracentrifugation. Therefore SN is mixed with 5g CsCl, filled into an appropriate ultracentrifugal tube and is covered with a drop of mineral oil. Centrifugation is carried out in an ultracentrifuge at 32000rpm for 18-24 h at 10°C without brake. The purified virus is collected from the gradient using an 18G needle and is mixed with 2x storage buffer. The virus is stored in 200µl aliquots at -20°C.

2x Storage buffer

10mM Tris, pH=8
100mM NaCl
0.1% BSA
50% glycerol

Measuring viral titer

Viral titers are determined by measuring the optical density at 260nm (OD260). 15µl of the virus is mixed with 100µl 1x TE/0.1% SDS, vortexed rigorously for at least 30 seconds (sec) and centrifugated 5 min at 13000rpm. For blank calibration 15µl of the blank solution is mixed with 100µl 1x TE/0.1% SDS and is treated as described above. Afterwards virus is measured at Thermoscientific NanoDrop microspectrophotometer.

1 unit at 260nm ~ 10^{12} viral particles

Blank solution

0.5g/ml CsCl in 10mM Tris, pH=8; 2x Storage buffer = 1:1

3.1.5 Adenoviral infection of cultured cells

Cells are infected with an adequate dilution of the adenovirus, when they have reached a confluence of 50-70% and incubated 48 hours before harvesting.

3.1.6 Infectivity test by using CFP expressing adenovirus

For testing infectivity, the cells are infected with the recombinant virus expressing CFP (cyan fluorescent protein) and the infection efficiency is determined. Therefore 3x 10cm dishes with equal numbers of cells are infected with 3 different CFP virus concentrations (low: 0.1µl, mid: 0.5µl, high: 1.5µl of the virus). After 48 hours blue cells are visualized under fluorescence microscope and infection efficiency is determined by percentage of blue fluorescence cells. Cell lines are categorized in 3 classes: cells with low, middle and high infectivity rate.

3.2 Methods for working with DNA

3.2.1 Polymerase chain reaction (PCR)

PCR is used to amplify specific sequences of DNA to get higher amounts of DNA if they are necessary for further analysis.

PCR components:

xµl DNA
5µl 10x Buffer
1.25µl dNTPs (10mM)
2µl Primer A (10µM)
2µl Primer B (10µM)
0.15µl AmpliTaq- Polymerase (Appplied Biosystems)
xµl ddH_2O to a total volume of 50µl

PCR reaction

Initial denaturation 1 min at 95°C
Denaturation 30 sec at 95°C
Annealing 30 sec at 59°C } 32 cycles
Extension 30 sec at 72°C
Final extension 10 min at 72°C

3.2.2 Phosphorylation of oligonucleotides

For better annealing, oligonucleotides (oligos) were phosphorylated with ATP. 1µl oligonucleotides (100µM) and 14µl ddH_20 are incubated at 70°C for 10 min. Afterwards oligos are placed on ice and
2µl 10x PNK buffer
2µl Polynucleotidekinase (AppliChem) (PNK)
1µl ATP (1mM)
are added.

The mixture has to be incubated at 37°C for 1 hour. After this the PNK has to be inactivated (10 min at 65°C).

3.2.3 Annealing

Annealing is the pairing of complementary of single stranded nucleic acids, resulting in a double stranded molecule. For the “pAdlox simiR 21” it was necessary to anneal two specific oligonucleotides to get the insert for the plasmid.
For the annealing, 100µl of annealing buffer was added and the mixture was heated 5 min at 98°C. To cool down slowly we switch the thermoblock of and wait until it reaches a temperature about 37°C. This step was repeated once.

Annealing buffer:

10mM Tris pH=8
50mM NaCl
1mM EDTA

3.2.4 Agarose gel electrophoresis

To check quality and quantity of PCR Products and plasmid DNA fragments agarose gel electrophoresis is performed.

Preparation of the agarose gel

Depending on the size of the analyzed DNA fragments 0.8 to 2% agarose gels are performed. Therefore the adequate amount of agarose is dissolved in ddH_2O by boiling it in the microwave. Afterwards 50x TAE is added, so that it results in a final concentration of 1x TAE. After this the agarose solution is filled into a gel slide and a comb is inserted. After gel solidified the comb is removed and the electrophoresis tank is filled with 1x TAE.

Gel electrophoresis

DNA samples are mixed with an adequate amount of 6x sample loading buffer and are loaded into the slots. A λ marker or a low range DNA ladder (dependent on the size of the DNA fragment which should be detected) is loaded to evaluate DNA size by comparison. After electrophoresis is run by 70-100 V, DNA can be visualized by staining with an ethidium bromide solution (0.25µg/ml) for about 10 min. Then the DNA bands are detected under UV-screen.

1xTAE

0.04M Tris acetate

1mM EDTA

in ddH_2O

pH=8.3

6x sample loading buffer

40% saccharose

24% urea

0.25% xylene blue

0.25% bromphenol blue

in 1x TAE

λ marker

λ DNA (Fermentas), digested by Hind III/ EcoR I

For production of λ-marker 200µl λ DNA (0.3µg/µl), 42µl Buffer B (Roche, 10mM Tris-HCl, 5mM $MgCl_2$, 100mM NaCl, 1mM 2-Mercaptoethanol, pH=8.0), 4µl Hind III, 4µl EcoR I and 167µl ddH_2O are mixed together and are incubated at 37°C over night (o/n). On the next day 83µl of 6x loading dye are added. Marker is stored at 4°C. Before loading, marker gets heated for a few minutes at 65°C.

3.2.5 DNA extraction from agarose gel and DNA purification

The DNA fragments of interest are cut out under the UV-screen. Then the agarose is dissolved and the DNA was obtained by purification with a column. For this Zymoclean Gel DNA Recovery Kit (Zymo Research) is used according to the manufacturer's manual.

Desalting is done with the use of Sephadex G50 or G25 (Mini Quick Spin DNA Columns and Mini Quick Spin OLIGO columns (Roche)). First the matrix of the columns is resuspended then it gets packed by centrifugation 1 min with 2000rpm. After this step the sample is added to the column bed and the column is again centrifuged for 3 min at 2000rpm. The eluate, which contains the DNA is collected in a tube.

3.2.6 Restriction digestion and ligation

Enzyme restriction digestion is performed according to the conditions which are defined by the manufacturer company. For each restriction enzyme the adequate 10x reaction buffer (for buffer components see Table 5 and Table 6) is used. The reaction volume can differ between 20 to 50µl, dependent on the amounts of DNA.

Buffer components	Buffer A	Buffer B	Buffer L	Buffer M	Buffer H
Tris-Acetate	33mM				
Tris- HCl		10mM	19mM	10mM	10mM
MgCl2		5mM	10mM	10mM	10mM
NaCl		100mM		50mM	100mM
Mg Acetate	10mM				
K Acetate	66mM				
Dithioerythritol			1mM	1mM	1mM
Dithiothreitol	0,5mM				
2-Mercaptoethanol		1mM			

Table 5: *Composition of restriction digestion buffers (Roche); final concentration are given (10x buffers were diluted 1:10 in the final reaction)*

Buffer components	Buffer 1	Buffer 2	Buffer 3	Buffer 4
Bis-Tris-Propane-HCl	10mM			
Tris-HCl		10mM	50mM	
Tris-Acetate				20mM
MgCl2	10mM	10Mm	10mM	
NaCl		50mM	100mM	
Mg Acetate				10mM
K Acetate				50mM
Dithiothreitol	1mM	1mM	1mM	1mM

Table 6*: Composition of restriction digestion buffers (New England Biolabs NEB); final concentrations are given (10xbuffers were diluted 1:10 in the final reaction*

Used restriction enzymes

Figure 7 gives an overview of used restriction enzymes in this study.

Restriction enzyme	Recognition sequence	Cuts at	Heat inactivation	Company	Buffer
Bgl II (***Bacillus globigii***)	A GATCT	37°C	No	Roche	A: 100% B: 100% L: 25-50% M: 100% H: 100%
Hind III ***(Haemophilus influenzae Rd com-10)***	A AGCTT	37°C	65°C; 15 min	Roche	A: 50-75% B: 100% L: 25-50% M: 100% H: 50-75%
Nde I ***(Neisseria denitrificans)***	CA TATG	37°C	65°C; 20 min	NEB	1: 75% 2: 100% 3: 75% 4: 100%
Sal I ***(Streptomyces albus G)***	G TCGAC	37°C	65°C; 20 min	NEB	1: 0% 2: 0% 3: 100% 4: 0%
Sfi I ***(Streptomyces fimbriatus)***	GGCCNNNN NGGCC	50°C	No	Roche	A: 25-50% B: 25-50% L: 75-100% M: 100% H: 25-50%
Xho I **(Xanthomonas campestris)**	C TCGAG	37°C	No	Roche	A: 25-50% B: 75-100% L: 10-25% M: 25-50% H: 100%

Table 7*: Overview of used restriction enzymes and detailed information about them*

Generally reaction (for reaction volume of 50µl):

1-3µg DNA

5µl 10x reaction buffer

1µl restriction enzyme A

1µl restriction enzyme B

1µl BSA (if it was necessary for the activity of one of the restriction enzymes)

xµl ddH_2O to a final volume of 50µl

Incubation for at least 1 h at the appropriate temperature (usually 37°C)

Reaction for a control restriction digestion (to check if the correct plasmid was isolated):

5µl of the STETL- Mini preparation (see DNA preparation)

2µl 10x reaction buffer

0.3µl restriction enzyme 1

0.3µl restriction enzyme 2

2,4µl ddH_20

Incubation between 30 min and 1 h at 37°C (except of restriction enzymes which need other temperature for their optimal activity)

Sfi I digestion

6µg DNA

1µl Sfi I

5µl 10x buffer M (Roche)

xµl ddH_2O to a final volume of 50µl

Incubation at 50°C for 2 h

Inactivation of restriction enzymes by phenol-chloroform-isoamylalcohol extraction

Phenol-chloroform-isoamylalcohol (PCI) extraction was done to inactivate restriction enzymes, if they are not deactivatable by incubation at heat.

Fill up the reaction to 100µl with ddH_2O, add equal volume of PCI (49.5:49.5:1) and vortex rigorously. Afterwards centrifugation was carried out with 10000g at 4°C for 15 min. The upper phase was collected and 2 volumes (V) of 100% cooled EtOH and 0.1V 3M NaAc was added. The mixture was vortexted and incubated at -80°C for 30 min or at -20°C o/n. After this centrifugation was carried out with 10000g at 4°C for 15 min. SN was discarded and the pellet washed with 70% chilled EtOH.

Centrifugation was carried out as described before. SN was discarded and the pellet was dried for about 3 min at 65°C. Afterwards the DNA was dissolved in 25µl ddH2O.

Dephosphorylation

To minimize relegation of plasmid vectors the ends have to be dephosphorylated, using a Shrimp Alkaline Phosphatase (SAP)

250ng Vector
2µl SAP (Roche)
2µl 10x reaction buffer
xµl ddH_2O to a final volume of 20µl

Incubation at 37°C is carried for 15 min. Afterwards incubation at 65°C for 15 min is required to inactivate and remove the enzyme. For storage put the reaction directly to -20°C.

Ligation

Ligation of DNA fragments is achieved by usage of a T4-ligase at 16°C o/n.
Different ligation approaches are done, using different DNA amounts and different ratios of insert to vector.

The appropriate insert mass was calculated using the followed formula:

$$Insertmass(ng) = \frac{x * insert\ length\ (bp)}{vector\ length\ (bp)} * vector\ mass\ (ng)$$

x … ratio vector: insert
bp… basepairs

Generally reaction

25-50ng Vector
xng Insert (dependent on the vector: insert ratio)
2µl 10xbuffer
1µl T4 Ligase (Roche)
xµl ddH_2O to a final volume of 20µl

For standard ligations we use a 3:1 ratio of insert to plasmid.

3.2.7 Competent bacteria

XL-1 bacteria are plated on LB Agar without Ampicillin (amp) and are incubated o/n at 37°C. Next day a single colony is inoculated in 2.5ml LB medium and under constant shaking of 180rpm at 37°C incubated o/n. On the next day bacteria are subcultivated in 250ml LB medium supplemented with 20mM $MgSO_4$ and are left at 37°C and constant shaking until the culture has reached a optical density of 0.4-0.6 at 600nm. Then bacteria are pelleted (4500g, 5 min) and the SN is discarded. Pellet gets resuspended in cold LB medium and repelleted. The pellet is resuspended in chilled TFB1 buffer and incubated on ice for 5 min. Then the bacteria are centrifugated at 4500g for 5 min at 4°C to get a pellet again. This pellet is resuspended in 10ml of chilled TFB2. After an incubation of 30 min on ice, aliquots of 300µl are stored at -80°C.

TFB1

30mM Kaliumacetate
10mM $CaCl_2$
50mM $MnCl_2$
100mM RbCl
15% glycerol
in ddH_2O
adjust to pH=5.8 with 1M CH_3COOH
sterilize with a 0.45µm filter

TFB2

10mM N-morpholino propanesulfonic acid (MOPS)
75mM $CaCl_2$
10mM RbCl
15% glycerol
in ddH_2O
adjust to pH=6.5 with 1M KOH
sterilize with a 0.45µm filter

Transformation

100µl XL-1 bacteria are added to maximal 20µl DNA and are incubated on ice for 20 min. Transformation is done by heat shock: Bacteria and DNA are incubated for 90 sec at 42°C, followed by incubation for 2 min on ice. Optional, transformation efficiency can be increased by performing a recovery time for the bacteria. Thus, 500µl LB medium is added and bacteria are incubated for minimal 30 min at 37°C under constant shacking. Afterwards bacteria are centrifugated at 5000rpm for 2 min and 500µl of the SN is discarded. The pellet is resuspended in the remaining LB Medium. The bacteria are plated on selective LB agar containing 0.2 mg/ml amp and incubated o/n at 37°C.

LB medium

10g NaCl

10g trypton

5g yeast extract

in 1l ddH_2O

Selective LB plates

Dilute 15g Agar to 1l ddH_2O and autoclave it. Afterwards let it cool down to 40-50°C and add 1ml antibiotics-stock [100mg/ml] (Ampicillin)

3.2.8 DNA preparation

STETL (Sucrose–Triton–EDTA–Tris-HCl–Lysozyme) MINI preparation

A single colony of XL-1 bacteria is inoculated in 3ml LB Medium. Bacteria are incubated o/n at 37°C by constant shacking. Next day 1.5ml of this culture are pelleted by centrifugation at 10000g for 3 min at RT and the SN is discarded. Then the bacteria pellet is resuspended in 110µl STETL-buffer and incubated for 1 min at 95°C. Afterwards bacteria are centrifugated by 10000g at 4°C for 10 min. The pellet is removed with an autoclaved toothpick, which is dipped in chilled RNase A before. Accordingly 110µl isopropanol are added and rigorously vortexed, following of centrifugation at 10000g for 15 min at 4°C. The SN is discarded and the pellet is washed with 200µl chilled 70% Ethanol by resuspension and centrifugation at 10000g for 10 min at 4°C. After this the pellet is tried for 10 min at RT and resuspended in 30µl ddH_2O. The DNA solution is stored at -20°C.

STETL buffer

8% sucrose

0.5% Triton X-100

50mM Tris, pH=8

50mM EDTA

0.05% lysozyme (Roche), added freshly

in ddH_2O

RNase A

10mM Tris

15mM NaCl

5% Ribonuclease A (Sigma)

in ddH_2O

Adjust to pH=7.55

Heat for 15 minutes at 100°C

MIDI preparation

Bacteria are inoculated in 50 to 100ml selective LB Medium. This culture is incubated o/n at 37°C under constant shacking. On the next day MIDI preparation is carried out using Plus Midi kit (Quiagen) according to the manufactures manual.

The concentration is determined by measuring with Thermoscientific NanoDrop microspectrophotometer by 260nm (1OD= 50 µg/ml).

To confirm correct cloning, an aliquot of the plasmid is sent to Microsynth for sequencing.

3.3 Methods for working with RNA

For RNA work it is always necessary to wear gloves and use only Diethylpyrocarbonate (depC)-H_20 to protect the RNA from degradation through RNAses. Additionally every working utensil is treated with 0.2M NaOH.

DepC ddH_2O

DepC solution 1:1000 in ddH2O

DepC ddH_2O get incubated for 2 h at 37°C under constant shacking and get autoclaved twice.

3.3.1 Collection and storage of cultured mammalian cells for RNA isolation

For RNA isolation a 15cm dish of cells is usually needed. The dishes are washed with 10ml chilled 1x PBS. After washing 1 ml 1x PBS is added, and the cells are scratched off with a scraper. The cell suspension is transferred into an eppendorf tube. Cells are then pelleted by centrifugation at 1000g for 5 min. After removing PBS cell pellets are immediately shock frozen in liquid nitrogen and can be further stored at -80°C.

1xPBS

137mM NaCl

2.7mM KCl

4.3mM $Na_2HPO_4.2H_2O$

1.4mM KH_2PO_4

pH=7.4 in ddH_2O

3.3.2 RNA isolation

500µl lysis buffer are added to the defrozen cell pellet. After resuspending the cells, lysis is achieved during an incubation period of 10 to 30 min at RT. 50µl 2M Na Acetate (pH=4) is added and the sample was mixed by vortexing. The same volume of chloroform: isoamylalcohol (24:1) is added and the sample is rigorously vortexed and incubated 5 min at ice. Afterwards separation of the phases is accelerated by a centrifugation at 12000g for 15 min at 4°C. The upper phase is transferred into a new tube and the same volume of PCI (49.5:49.5:1) is added. The sample is vortexed again and incubated for minimal 15 min on ice. The phases are separated by centrifugation at 12000g for 15 min at 4°C. Only the upper phase is carefully removed and collected into a new eppendorf tube. The chloroform: isoamlyalcohol extraction is repeated, followed by a second extraction with PCI.

The RNA is precipitated by adding 1V of isopropanol. After the mixture is vortexed rigorously, incubation for 30 min at -20°C is carried out. The RNA is pelleted by centrifugation at 12000g for 15 min at 4°C. Afterwards the SN is carefully removed and the pellet is washed with 70% Ethanol. After an incubation of a few minutes at RT, RNA is again pelleted by centrifugation at 12000g for 10 min and SN is removed. The pellet is dissolved in 50µl depC ddH_2O containing 1µl RNase inhibitor (RiboLock,

Fermentas) by incubation at 65°C for about 30 min under constant shacking of 650rpm.

Lysis buffer

4M guanidinthiocyanat

42mM sodium citrate

2.5% polyvinylpyrolidone

5% beta-mercaptoethanol, is added right before use

10mM magnesium chloride, is added right before use

3.3.3 MOPS gel electrophoresis

Isolated RNA is visualized in a MOPS gel electrophoreses for controlling the quality of the RNA to exclude degradation on one hand, and to equalize the amounts of RNA for the Northern blot on the other hand.

0.84g agarose are dissolved in 48ml depC-H_2O and 6ml 10x MOPS by heating up the solution at a heating plate. After agarose is dissolved completely and solution is cooled down until it reaches a temperature about 60°C, 6ml formaldehyde is added and the solution is filled into a gel slide and a comb is inserted. After gel is harden the comb is removed and the gel is put into an electrophoresis tank, filled with 1x MOPS. Meanwhile RNA samples are prepared: 5µl RNA is mixed with 20µl RNA loading buffer and 1µl ethidium bromide solution (1:50 from stock solution of 1% EtBr (c=10mg/ml)). Before samples are loaded, they are heated for 1 minute at 65°C. Gel is run with a constant voltage of 70V. Meanwhile concentration is measured by Thermoscientific NanoDrop microspectrophotometer (1 OD= 40µg/ml). After the run of the gel is ready, the RNA is visualized under the UV screen and the equal amounts of RNA are determined by comparing their 28s and 18srRNA bands. Results are compared to the measurement of the Thermoscientific NanoDrop microspectrophotometer.

If it is necessary, RNA can be enriched by extraction with 1-butanol. Therefore 1 volume 1-butanol is added to the RNA and vortexed. To separate the phases centrifugation for 1 min at 10 000g and the upper phase (butanol phase) is discarded. This step can be repeated. Each time the water volume decreases. To finally remove the butanol rests a diethylether extraction is carried out. Therefore 500µl diethylether are added. Mixture is vortexed and centrifugated again. The upper phase is removed

and RNA is incubated in open eppendorf tube for a few minutes at 50°C until diethylether is evaporated.
Afterwards RNA is aliquoted so that every sample contained the same amounts of total RNA (10-15µg) and equal volume of 2x loading buffer is added. Afterwards RNA is stored at -80°C until RNA separation gel is performed, but not longer than 2 to 4 days.

10xMOPS

200mM MOPS
50mM sodium acetate
10mM EDTA
in depC H_2O

RNA loading buffer

50% formamide
5.9% formaldehyde
6.7% glycerol
bromphenolblue until the solution is stained
in 1xMOPS

Ethidium bromide stock solution [10mg/ml] in ddH2O

3.3.4 RNA polyacrylamide gel electrophoresis (PAGE) for miRNAs

For miRNA size separation an 18% RNA PAGE gel containing 7.6M urea is performed. Therefore 4.5g urea (or 2.35g when acrylamide already contains urea (c=500g/l)) is dissolved in 4.5ml acrylamide (40%, 19:1) and 2ml 5xTBE by heating. While dissolving urea the gel equipment is prepared. Therefore two glass plates with spacer in between are assembled in a cassette and are stacked into a casting stand. After urea is completely dissolved the solution is filled up to 10ml with depC ddH_2O and when it is cooled down 100µl 10% APS and 4µl TEMED are added. Then immediately the solution is poured between the glass plates and a comb is inserted. After polymerization the gel is put into a tank filled with 1xTBE running buffer. Comb is removed and the slots are rinsed with running buffer using a string. The pre-run of the gel is carried out in the constant ampere modus (25mA) until buffer has reached about 50°C, which took about one hour. Meanwhile the RNA samples are prepared

by thawing them on ice and putting them on 65°C until they are completely in solution. A tRNA sample is prepared as a positive control, too.
The prepared RNA samples are incubated for 5 min at 70°C. Before loading the samples the slots are rinsed again with running buffer. The electrophoresis is carried out at constant 80V.

2x loading buffer
10ml formamide
200µl 0.5M EDTA pH=8
1mg Bromphenolblue

5xTBE
1.1M Tris
900mM Borate
25mM EDTA
in depC ddH_2O

Tris	Tris-(hydromethyl)-aminomethane
APS	Ammoniumpersulfate
TEMED	N,N,N‘,N‘-Tetramethylethylenediamine

3.3.5 Northern blotting

RNA Transfer

For transferring the RNA from the gel to the nylon membrane a sandwich consisting of a sponge, 2 layers of 3MM whatman paper, PAGE gel, nylone membrane, again 2 layers of 3MM whatman paper and sponge are assembled. All components expect of the PAGE gel are wetted with DepC ddH_2O before assembling. For elimination of disturbing air bubbles, a plastic pipette is rolled over the blot.
The blotting cassette is put into the blotting tank, which is filled with 0.5x TBE. Blotting was carried out o/n at 150mA at 4°C under constant stirring. The RNA was transferred to the nylon membrane from the negative cathode to the positive anode.

Nylon membrane	GeneScreen Plus® Hybridization Transfer Membrane, pore size 45µm

Crosslinking and Methylenblue staining

On the next day transfer is disassembled and the membrane is put on a dry filter paper and RNA is cosslinked to the membrane (Autocrosslink 1200+100µJ energy) in a Stratagene Crosslinker.

For control of the transfer and exclusion of degradation of the RNA while electrophoresis the membrane is stained with methylenblue until rRNA bands are visible and washed with ddH_2O. Photography is taken to document the amounts and quality of RNA. Afterwards membrane is washed again with ddH_2O and is stored in foil at -20°C for further procedure.

Methylene blue

0.04% methylene blue

in 0.5M sodium acetate (pH=5.2)

Pre-hybridization

Hybridization oven is heated to 42°C and hybridization buffer is preheated.

A freezer bag is cut open and one 1MM whatman paper is placed on it. The 1MM whatman paper is soaked with hybridization buffer and the nylon membrane with the RNA side up is put on the paper. Another 1MM paper is placed on the membrane and soaked with hybridization buffer containing t-RNA (0.1mg/ml). The next membrane can be put on the 1MM whatman paper with RNA side down. Then another 1MM whatman paper can be put on the second membrane. The sandwich can contain up to 10 membranes spaced by 1MM whatman paper soaked with hybridization buffer containing tRNA, but the outer layer of the sandwich must be 1MM whatman paper. Finally the sandwich is heat sealed in the freezer bag and airbubbles get eliminated by crop out, followed by putting the sandwich into another plastic bag. The sandwich was placed into the hybridization oven and was incubated at 42°C 2 to 20 hours.

Hybridization solution

5x SSPE

0.1M Natriumphosphate pH=6.5

50% Formamide

5x Denhardt's

10mM EDTA pH=8

1% Sarkosyl (Sigma)

20x SSPE

3.6M NaCl

200mM NaH_2PO_4

20mM EDTA pH=8

50x Denhardt's

1% BSA

1% Polyvinylpyrrolidan

1% Ficoll

Probe labeling and hybridization

2µl DNA probe (10µM) are mixed with 10µl ddH_2O and incubated for 10 min at 70°C. Afterwards probe is immediately placed on ice and 2µl 10xPNK buffer is added. At a space certified for working with radioactive material 5µl gamma ^{32}P-ATP (3000 to 6000 Ci/mmole) and 1µl T4 Polynukleotidkinase (PNK) are added. Probe is incubated at 37°C for 1 hour, to inactivate PNK subsequently the sample is incubated at 65°C for 10 min. Afterwards 30µl ddH_2O are added and probe is cleaned by using a G25 sepharose column. Incubation is carried at 95°C for 5 minutes to denature probe. After this, probe is placed on ice. Dependent on the expected signal intensity, one probe is enough for hybridization of 2 to 10 membranes.

Hybridization is carried out as described in pre-hybridization but the hybridization buffer contains additionally the labeled probe. The sealed sandwich is incubated o/n at 42°C in the hybridization oven.

Washing

All washing steps are carried out at 50°C. Sealed sandwich is cut open and membrane is put into glass tubes. First non stringent washing buffer is added to rinse the membrane. Then the washing buffer is discarded and new non stringent buffer is added and the membrane is incubated for about 15 min in the hybridization oven by

rotating. Repeat this step twice and wash membrane about 30 min each time. Afterwards the membrane is washed with stringent washing buffer for about 20 to 60 min.

Non stringent wash buffer

3x SSC

25mM NaH_2PO_4 pH=7.5

5% SDS

In ddH_2O

Stringent wash buffer

1xSSC (15mM NaCl, 1,5mM sodium citrate)

1% SDS

in ddH_2O

Detection of Northern blot

For detection the washed membranes are heat sealed into foil and are incubated for 6 hours (for U6) to 4 days (for miR-21) in a storage phosphor screen (Amersham Biosciences). The RNA bands are detected with a phosphor imager screen (Typhoon).

Quantification

Quantification of RNA bands are done with Image Quant 5.0 software by background correction.

3.4 Methods working with proteins

3.4.1 Isolation of total protein and determination of protein concentration

Cells are harvest as described before, but the cell amount growing in a 10cm dish is sufficient (*see harvesting cells for RNA isolation)*. Cell pellet is resuspended in chilled protein lyses buffer (TGH buffer) and incubated for 10 to 30 minutes on ice. Afterwards cell lysate is treated for 3 minutes by sonication in water bath. Cell debris is then removed by centrifugation at 14000rpm at 4°C for 10 min. SN, containing the proteins, is transferred into a new tube and an aliquot is used for determination of protein concentration.

The concentration of protein is measured using Micro BCA Protein Assay Reagent Kit (Pierce). 2.5µl of the protein lysate are mixed with the reagents of the kit according to the manufactures manual. After incubation of 2 hours at 37°C, protein concentration is determined by absorbance measurement at 562nm and is compared with a BSA protein standard curve containing 0µg/ml to 200µg/ml BSA.

TGH protein lysis buffer

50mM Hepes
150mM NaCl
1mM EDTA
10mM NaF
1.5mM $MgCl_2$
10% glycerol
1% Triton X-100
0.5mM Sodium-vandate (added freshly)
2.5% Complete (Roche) (added freshly)
1µM PMSF (added freshly)
in ddH_2O

3.4.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

In SDS PAGE protein are separated by their molecular weight.

Preparation of polyacrylamide gel

For coomassie-staining small SDS PAGE gels (7.2x10.2cm) are done, while for western blotting 11-13% gels with the size of 16x18cm are performed. Therefore two glass plates with plastic spacer in between are assembled in a cassette and were stacked into a casting stand. The separation gel is poured between the two glass plates and immediately overlaid with isopropanol to avoid the gel from exposure to O_2 and produce even surface. After polymerization isopropanol is removed completely, the stacking gel is poured on the separation gel and a comb is inserted. After polymerization of the stacking gel comb is removed and electrophoresis is carried out.

15x18cm	
Separation Gel: 11%	***stacking gel: 5%***
8.25ml Aa (37:1)	1.275ml Aa (37:1)
7.5ml Tris pH= 8.8	1.2ml Tris pH= 8.8
150µl 20% SDS	50µl 20% SDS
300µl 10% APS	100µl 10% APS
12µl TEMED	10µl TEMED
13.8µl ddH2O	7.3ml ddH2O
Volume=30ml	*Volume=10ml*

7.2x10.2cm	
Separation Gel: 11%	***stacking gel: 5%***
2.89ml Aa (37:1)	446µl Aa (37:1)
2.68 ml Tris pH= 8.8	420µll Tris pH= 8.8
52.5µl 20% SDS	17.5µl 20% SDS
105µl 10% APS	35µl 10% APS
4.2µl TEMED	3.5µl TEMED
4.83µl ddH2O	2.56µl ddH2O
Volume=10.5ml	*Volume=3.5ml*

Aa	37.1% Acrylamide, 1% Bis-acrylamide
SDS	Sodium dodecylsulfate
Tris	Tris-(hydromethyl)-aminomethane
APS	Ammoniumpersulfate
TEMED	N,N,N‘,N‘-Tetramethylethylenediamine

Electrophoresis

Gel is put into an electrophoresis chamber and the inner space of this as well as half of the container is filled with 1x electrophoresis buffer. Equal amounts of total protein are mixed with adequate volume of 4x sample loading buffer. Proteins are denaturated by incubation for 10 min at 95°C. The samples and the marker (Presicion Plus ProteinTM Standards–All Blue, BioRad) are loaded into the slots. Electrophoresis is carried out maximal 150V in the constant voltage mode from the cathode (-) to the anode (+).

1x Electrophoresis buffer

50mM Tris

0.19M Glycine

3.5mM SDS

in ddH_2O

4x Sample loading buffer

40% Glycerine

5% 2-Mercaptoethanol

9.2% SDS

250mM Tris, pH=6.8

Bromphenolblue (until the solution was stained)

in ddH_2O

3.4.3 Coomassie-staining

Coomassie-staining allows visualization of small amounts of proteins. It can be used to verify measured protein concentrations and check quality of the preparation.

After electrophoresis gel is put into a glass container and staining solution was added. For accelerating the staining process the solution can be heated for a few sec in the microwave and then is incubated for 10 to 30 min. After incubation staining solution is discarded and gel is incubated o/n in destaining solution. This process can be accelerated by warming up the solution. On the next day a photograph is taken and protein concentrations are adjusted. Gel can be tried between a foil.

Staining solution

30% Methanol

10% acetic acid

Coomassie blue until the solution is stained

Destaining solution

30% Methanol

10% Ethanol

3.4.4 Western blotting

Protein transfer

For protein transfer from PAGE to nitrocellulose membrane a sandwich consisting of a sponge, 2 layers of 3MM whatman paper, PAGE gel, nitrocellulose membrane, again 2 layers of 3MM whatman paper and sponge are assembled. All components expect of the PAGE gel are wetted with 1x transfer buffer before assembling. For elimination of disturbing air bubbles, a plastic pipette is rolled over the blot.
The blotting cassette is put into the blotting tank, which is filled with 1x transfer buffer. Under constant stirring blotting is carried out at 400mA in constant ampere mode at 4°C for at least 7 hours (better o/n). The protein is transferred to the nitrocellulose membrane from the negative cathode (-) to the positive anode (+).

1x Transfer buffer

48mM Tris
39mM Glycine
0.037% SDS
20% Methanol
in ddH_2O

Nitrocellulose membrane PROTAN original nitrocellulose ROLLS, BA83, pore size 0,2µm

Ponceau staining

After transfer membrane is washed to remove the transfer buffer and is stained with Ponceau-red solution until the protein bands get visible. Afterwards membrane gets de-stained with 1xTBST until all of the Ponceau-red solution was removed.

10xTBS

250mM Tris
1.37 M NaCl
27mM KCl
pH=7.6
in ddH_2O

1xTBST

10% 10xTBS

0.1% Tween20 (BioRad)

in ddH_2O

Ponceau-red solution

0.1% Ponceau-red

5% acetic acid

In ddH_2O

Solution should be stored at 4°C and can be used several times.

Immunostaining

To minimize unspecific signals membrane is incubated in blocking solution for at least 1 hour. After washing 2 times with 1xTBST, membrane is incubated in primary antibody with an adequate dilution of the primary antibody o/n at 4°C. Afterwards membrane is washed for 2-3 times with 1xTBST, each at least 10 min, followed by incubation with secondary antibody (dilution: 1:5000) for about 1 hour at RT. Finally membrane is washed again 2-3 times with 1xTBST. If there is a lot of background signal, membrane is washed once with TNN buffer for at least 15 min and twice with 1xTBST, each time for at least 20 min.

Blocking solution

5% non fat dry milk powder

in 1xTBST

TNN buffer

50mM Tris, pH=7.5

250mM NaCl

5mM EDTA

50mM NaF

0.5% NP-40

Used Antibodies

rabbit anti-Spry1, established by group of Dr. Sutterlüty (dilution 1:100)

rabbit anti-Spry2, established by group of Dr. Sutterlüty (dilution 1:250)

rabbit anti-Erk1/2, Upstate (dilution 1:5000)

mouse anti-β Actin, Novus Biological (dilution 1:20000)

rabbit anti-PTEN, Epitomics (dilution 1: 1000)

rabbit anti-PDCD4, Epitomics (dilution 1:5000)

rabbit anti- Au5, Bethyl Laboratories, TX, USA (1:6000)

anti-mouse IgG, Pierce (#31450)

anti-rabbit IgG, Pierce (#31463)

Primary antibody dilution

Primary antibody in adequate dilution

1% BSA

0.1% NaN_3

in 1xPBS

is stored at 4°C and used several times for different membranes

Secondary antibody dilution

1:5 000 anti mouse IgG or anti rabbit IgG

2% milk

in 1xTBST

is made freshly

Analysis

The secondary antibody is conjugated to horseradish peroxidase (HRP) to visualize proteins by chemiluminescence. Therefore 10ml of ECL solution 1 is mixed with 30µl ECL solution 2 and membrane is incubated in this detection solution for 1 min. Membrane is placed between two plastic foils and air bubbles are removed. The x-ray film is put on the top for an adequate period of time which mainly depends on the primary antibody to detect emitted light. After detection membrane is washed 2-3 times with 1x TBST to remove the detection solution completely.

ECL solution 1

200mM p-Coumaric acid

1.25mM Luminol

in 0.1M Tris, pH=8.8

ECL solution 2

3% H_2O_2

Further treatment

Membranes are incubated several times in different antibodies. Each time the procedure is done as described in *Immunostaining*.

If membrane is not used for a longer time, it get dried and stored at RT. For reactivation, membrane is incubated in 1xPBS for 5 min.

Quantification

Quantification of RNA bands are done with Image Quant 5.0 software by background correction.

4 RESULTS

4.1 Endogenous miR-21 levels in NSCLC-derived cell lines

As an initial experiment miR-21 expression in 14 NSCLC-derived cell lines were analyzed and compared to miR-21 levels in normal lung fibroblasts (WI-38).Therefore logarithmical growing cells with a confluence of about 90% were harvested, RNA was isolated and Northern blot was performed as described in *Material and Methods*. Equal loading was verified by 5srRNA (Figure 9A).

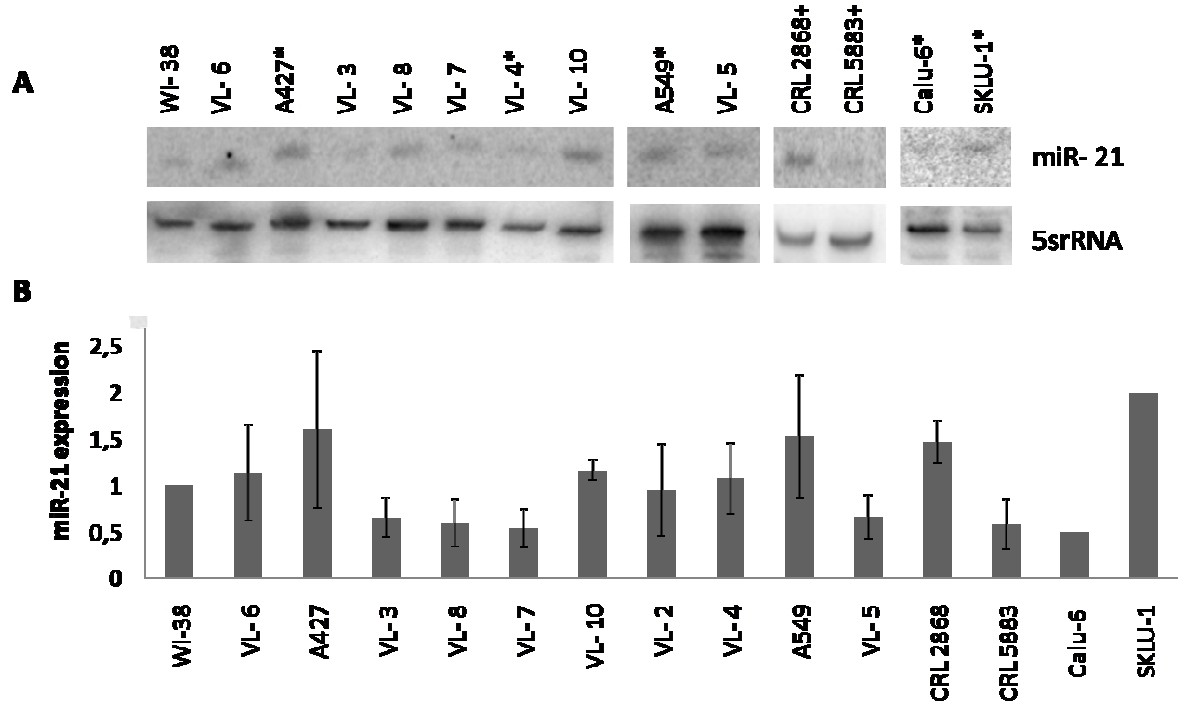

Figure 9: *MiR-21 expression in cell lines derived from NSCLC.* ***A:*** *Detection of miR-21 by Northern blot in 14 different NSCLC-derived cell lines and in WI-38. Cells harboring K-Ras mutations are labeled with *, cells with an activating EGFR-receptor mutation with +. One representative Northern blot is shown.* ***B:*** *Intensity of the bands was quantified with Image Quant 5.0 software and expressions normalized to 5srRNA and WI-38 are depicted. Data from 1-5 Northern blots are summarized.*

We observe only modest variation in miR-21 expression. All together there was only a 0.5 fold to 2 fold difference in the expression level of miR-21 in tumor cell lines

compared to normal fibroblast indicating that none of the cell lines derived from NSCLC shows clearly overexpression compared to normal fibroblasts (WI-38).

6 cell lines VL-6, A427, VL-10, A549, CRL2868 and SKLU-1 showed a slightly increased miR-21 expression (less than 2 fold) compared to WI-38 cells. All other used cell lines have less or the same expression of miR-21 as WI-38 (Figure 9B).

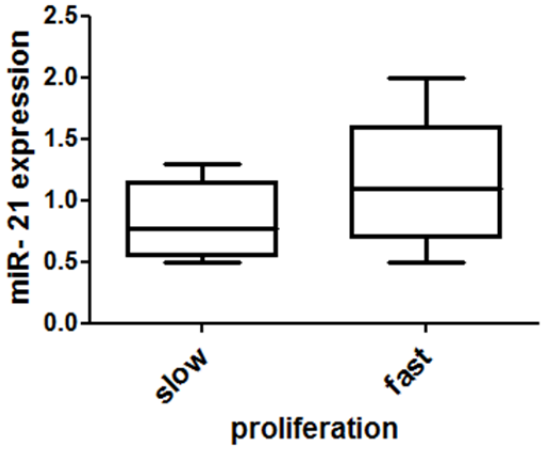

Figure 10: *Rate of proliferation compared to miR-21 expression.*

Cell lines were categorized in slow and fast proliferating cells according to their doubling time. All cells which need more than two days for doubling are determined as slow proliferating. Fast growing cell lines are A427, VL-3, A549, CRL2868, VL-6, VL-5, VL-4, Calu-6 and SKLU-1 while WI-38, VL-7, VL-2, VL-10, VL-8 and CRL5883 proliferate much slower. A box blot diagram was performed to examine the connection between miR-21 expression and the proliferation rate of the cells. Detected miR-21 levels in cells which grow faster are on average 1.2 fold increased compared to miR-21 expression in WI-38, while slow growing cells have a mean of 0.83 (Figure 10). Despite this observation, statistical analysis revealed that there is no significant difference in miR-21 expression of fast and slow growing cells.

Concerning tumor entities, miR-21 expression of cell lines derived from adenocarcinoma and squamous cell carcinoma were analyzed and compared by performing a box blot diagram. Figure 11 shows that on average cell lines derived from adenocarcinoma have higher levels of miR-21 (on average 1.3 fold increased compared to WI-38) than cell lines derived from squamous cell carcinomas (on average 0.82 fold decreased compared to WI-38), although no statistical significance can be observed (p=0.1134).

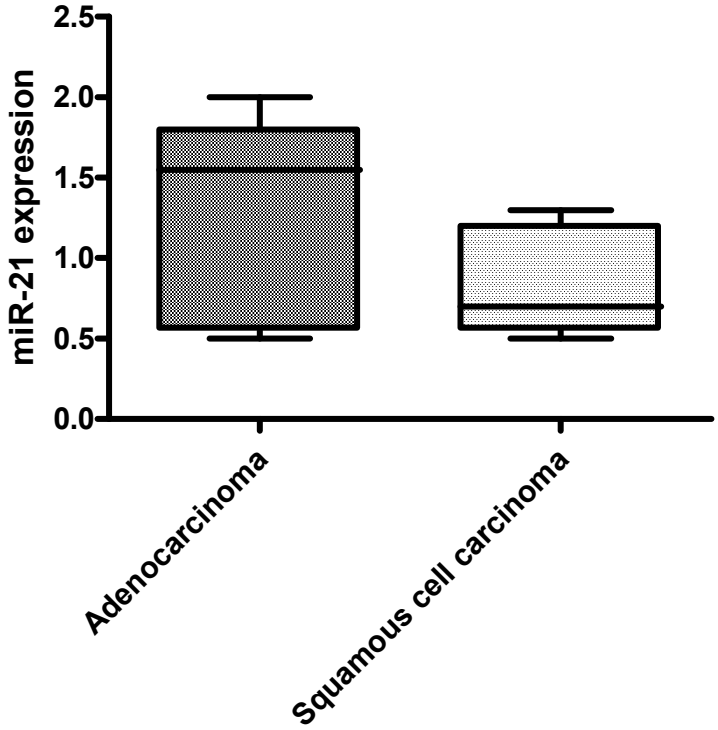

Figure 11: *MiR-21 expression of cell lines derived from adenocarcinoma or squamous cell carcinoma. Expression data observed from Northern blots were grouped into miR-21 expression from adenocarcinoma and squamous cell carcinoma.*

4.2 Influence of oncogenic mutations on miR-21 expression

To analyze the influence of intrinsic factors on miR-21 expression, cell lines with oncogenic mutations in EGFR where compared with their wt counterparts. MiR-21 levels of two cell lines (CRL5883 and CRL2868) with an EGF-receptor mutation were detected (in Figure 9A labeled with +). Expression of these cell lines and cell lines

without an EGFR mutation were depicted in a box blot diagram. As shown in Figure 12 no difference in miR-21 levels can be observed. MiR-21 levels of cell lines harboring an EGFR mutation are on average 1.05 fold increased compared to WI-38, while cells with the wt of this gene show a mean of 1.06.

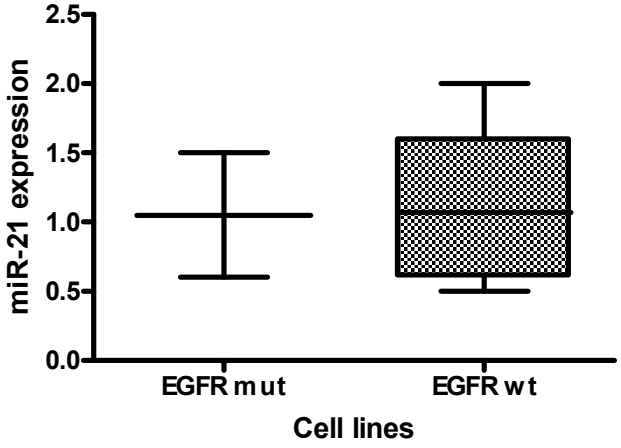

Figure 12: *MiR-21 expression of cells with EGFR mutation compared to cells with the wt form of this gene*

Additionally, cell lines were classified into cells carrying a K-Ras mutation (A427, VL-2, VL-4, A549, Calu-6, SKLU-1) and cells which harbor the wt of the gene. Detection of miR-21 shows that cells which carry the mutation have on average higher miR-21 levels (mean±SEM= 1.30±0.17) than these with the wt gene (mean±SEM= 0.87±0.13) although the difference in miR-21 expression does not show significance (p=0.0853), possibly due to the small sample size (Figure 13A).

To investigate if oncogenic Ras have an influence on miR-21 expression, oncogenic H-Ras V12, K-Ras V12 and N-Ras N61 viruses, with an Au5 tag were expressed in WI-38, cells were harvest 48 hours after infection and miR-21 expression was detected by Northern blot. For equal loading, Northern blot for U6 was performed too (Figure 13B).

To verify the expression of oncogenic Ras, Western blot was performed. Since the ectopic proteins were epitop tagged, Au5 antibody was used to verify their expression. As loading control β Actin was used (Figure 13D).

None of the oncogenic Ras isoforms influences the expression of miR-21 in WI-38 cells, which indicates that Ras is not an important determinant of miR-21 expression (Figure 13C).

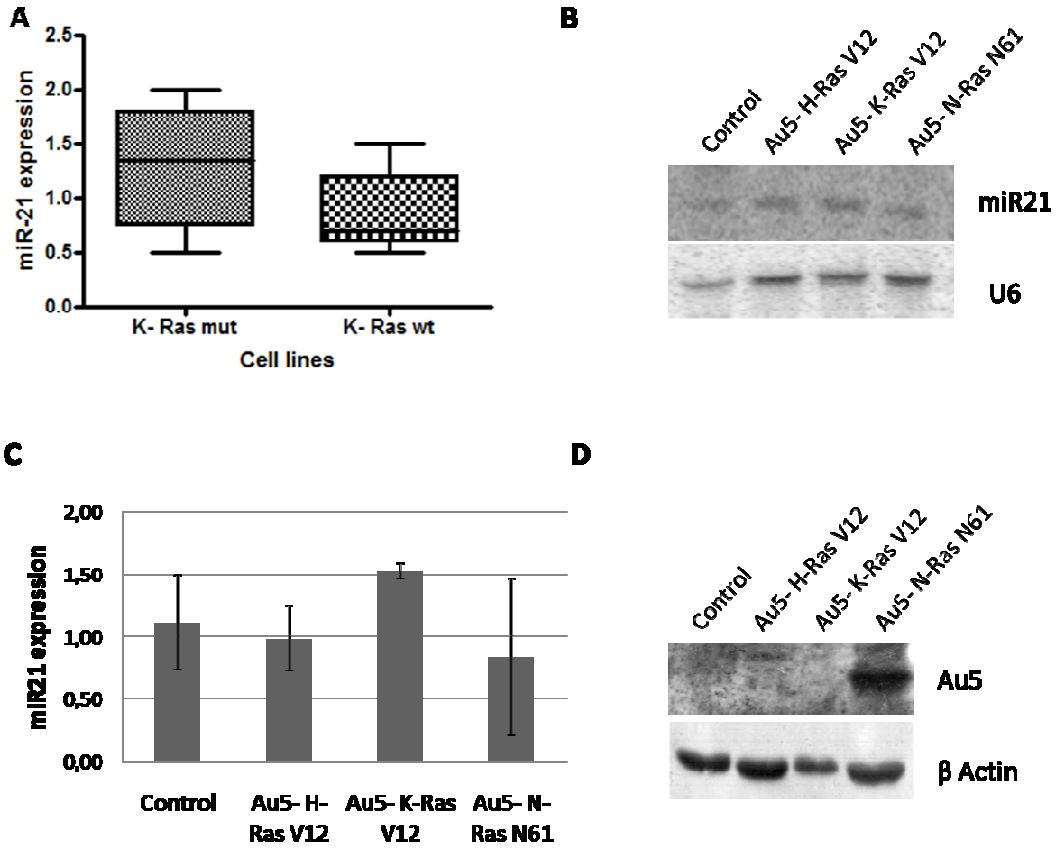

Figure 13: *Influence of oncogenic Ras on miR-21 expression in WI-38.* ***A:*** *MiR-21 expression of cells with K-Ras mutation (K-Ras mut) compared to cells harboring the wt allele of this gene (K-Ras wt).* ***B:*** *One representative of 2 Northern blot of miR-21 in WI-38 cells, harvest 2 days after infection with oncogenic H-Ras V12, K-Ras V12 and N-Ras N61.* ***C:*** *Quantification of miR-21 expression in WI38 expressing of oncogenic Ras was done with Image Quant 5.0 software. Expression was normalized to U6 and to the expression of control WI-38 cells (cells infected with a control virus and uninfected WI-38 cells).* ***D:*** *Expression control of oncogenic Ras by Western blot- All three oncogenic Ras carries an Au5 tag for easier detection. β Actin was performed for control of equal loading.*

4.3 Correlation of miR-21 levels with its substrates

PTEN, PDCD4 and Spry1 and Spry2 were found to be targets of miR-21 (29). To figure out if there is a correlation between the expression of these proteins and miR-21 levels in NSCLC-derived cell lines, Western blots were performed.

4.3.1 Endogenous expression of PTEN and PDCD4 and its correlation with miR-21 levels in NSCLC-derived cell lines:

To determine PTEN and PDCD4 amounts in cell lines, cells were harvest at 90% confluence, total protein was isolated and the protein concentrations were measured. 150µg of protein was loaded on SDS gel for analysis by Western blot. For control of equal loading, Ponceau red staining was performed, following by incubation of the membrane first in PTEN and afterwards in PDCD4 antibody (Figure 14A).

While in all cell lines detectable amounts of PTEN are observed, only a few cell lines express PDCD4.

Due to the fact, that not all cell lines have detectable amounts of PDCD4, they were grouped in two classes: in used cell lines either express PDCD4 like VL-6, VL-7, VL-10, CRL5883 and CRL2868 and cell lines which do not. No correlation between expression of PDCD4 and miR-21 levels was found (Figure 14B). Both groups show on average the same amounts of miR-21.

To correlate PTEN levels with the expression of miR-21, cell lines were classified into two classes: low and high expressers. Cell lines with low PTEN levels are A427, VL-3, VL-8, CRL5883 and SKLU-1, while VL-6, VL-7, A549, VL-5, CRL2868, Calu-6, VL-10 and VL-2 express more PTEN (Figure 14A).

On average cell lines with a lower expression of PTEN show nearly the same miR-21 levels (mean±SEM= 1.08±0.23) than cells with higher PTEN levels (mean±SEM= 1.03±0.15). No connection between PTEN expression and miR-21 levels can be observed (Figure 14C).

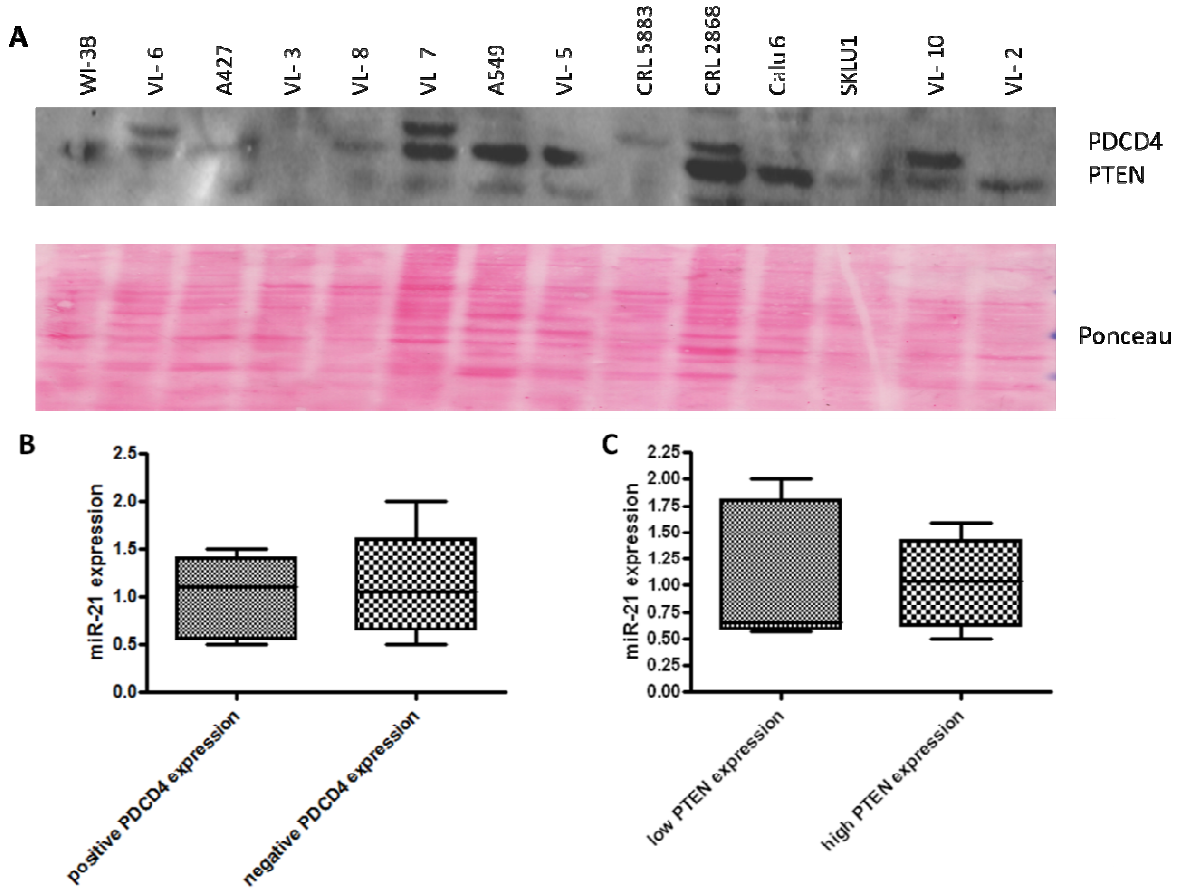

Figure 14: *Correlation between expression of PTEN and PDCD4 and levels of miR-21.* ***A:*** *One representative of 2 blots analyzing endogenous levels of PTEN and PDCD4 in different NSCLC-derived cell lines is shown, Ponceau staining of the blot is depicted as loading control.* ***B:*** *Box blot summarizing miR-21 expression in connection with PDCD4; Data were generated from 1 to 2 Western and 1 to 5 Northern blots per cell line, respectively.* ***C:*** *Box blot summarizing miR-21 expression in connection with PTEN; Data were generated from 1 to 2 Western and 1 to 5 Northern blots per cell line, respectively.*

4.3.2 Endogenous expression of Spry1 and its correlation with miR-21 levels in NSCLC-derived cell lines

To determine Spry1 amounts in cell lines, cells were harvest at 90% confluence, total protein was isolated and the protein concentrations were measured. 150µg of protein was loaded on SDS gel for analysis by Western blot. For control of equal loading, Ponceau red staining was performed, followed by incubation of the membrane in Spry1 antibody (Figure 15A).

To correlate the Spry1 expression with miR-21 levels, cell lines were classified in high, middle and low Spry1 expressers (compared to detected levels in WI-38).
Cell lines with a high Spry1 expression are A427, VL-6 and Calu-6, while VL-6, VL-8, VL-7, VL-10 and A549 showed only less amounts of Spry1.
To show if there is an influence of miR-21 on Spry1, box blots were performed in Graph Pad Prism. Low (mean±SEM= 1.02±0.21), middle (mean±SEM= 1.08±0.23) and high expresser (mean±SEM= 1.07±0.32) of Spry1 show on average nearly the same miR-21 expression so that no correlation between Spry1 protein levels and miR-21 expression can be observed (Figure 15B).

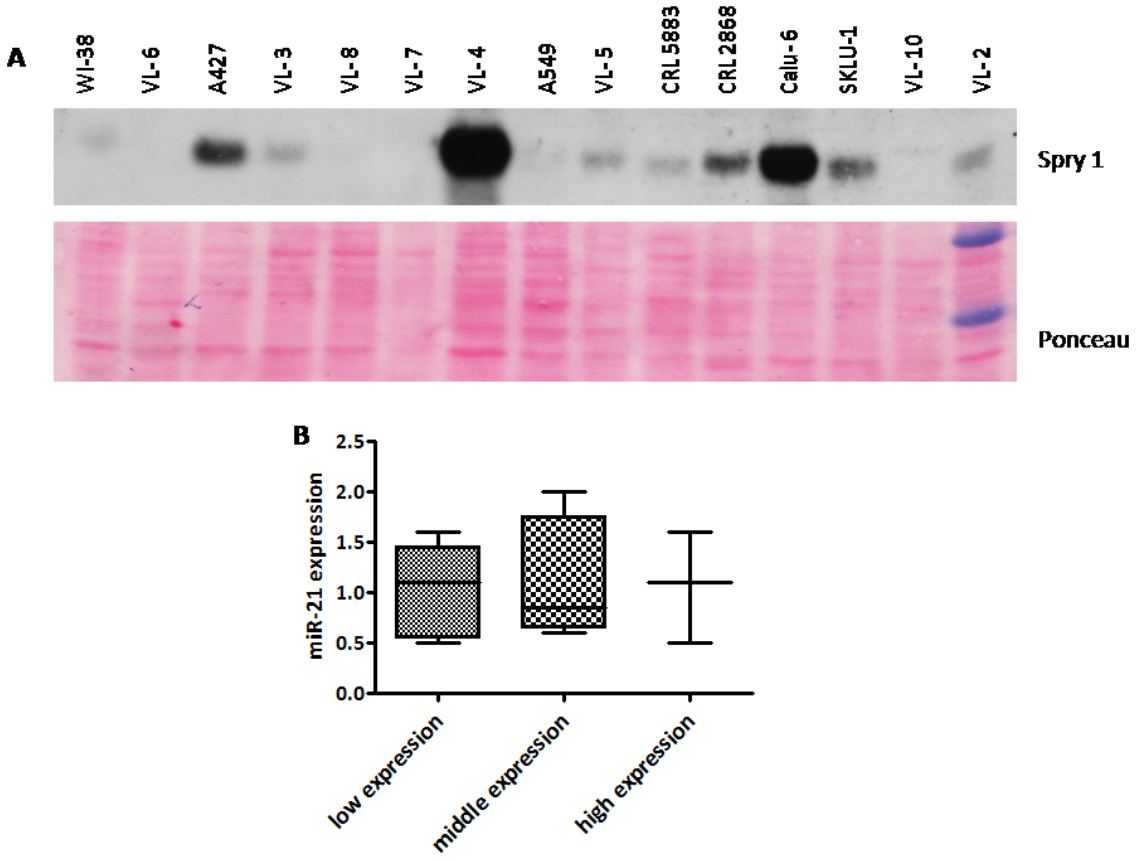

Figure 15: *Correlation between expression of Spry1 and levels of miR-21.* ***A:*** *A representative blot analyzing endogenous levels of Spry1 in different NSCLC-derived cell lines is shown; Ponceau staining of the blot is depicted as loading control.* ***B:*** *Classification of low, mid and high Spry1 expressers compared to WI-38 were correlated with their miR-21 expression; data observed from 1 to 2 Western and 1 to 5 Northern blots.*

4.3.3 Endogenous expression of Spry2 and its correlation with miR-21 levels in NSCLC-derived cell lines

To detect the amounts of Spry2, cells were treated similar as described for the Western Blot of Spry1, but membranes were incubated in Spry2 antibody (Figure 16A).

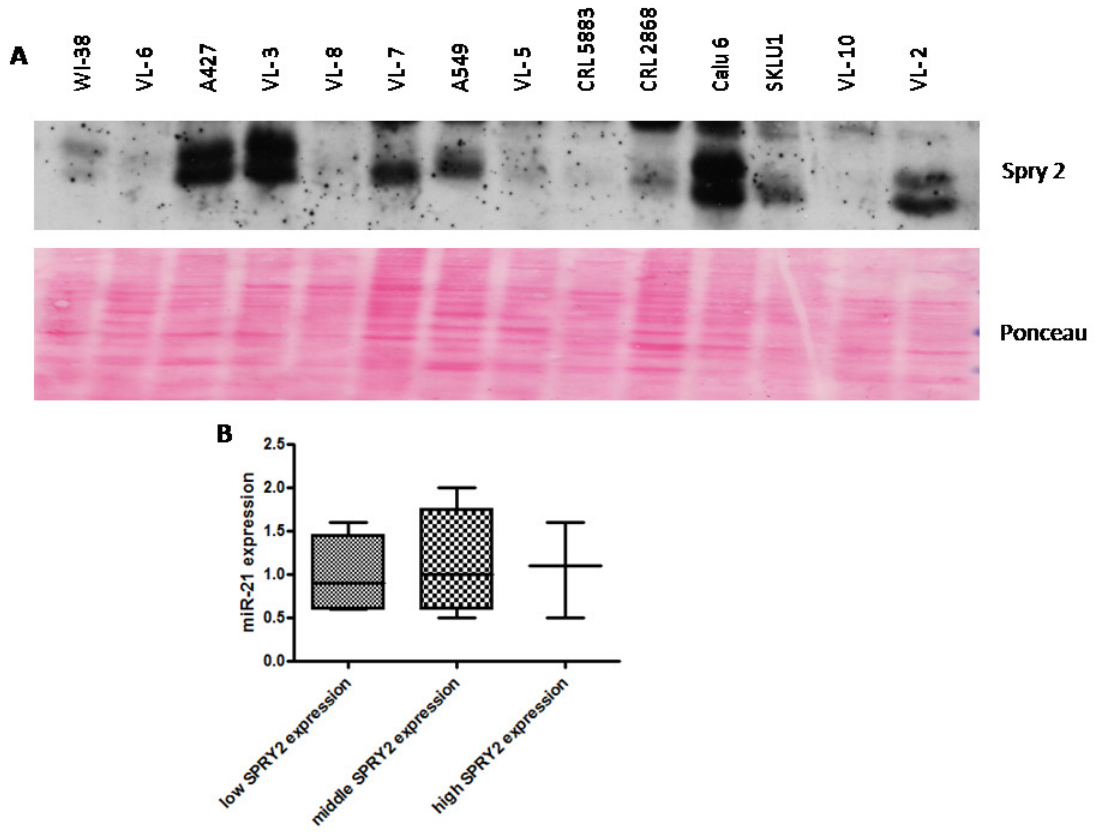

Figure 16: *Spry2 expression in correlation with miR-21 levels.* ***A:*** *A representative Western blot showing endogenous levels of Spry2 in different NSCLC-derived cell lines is shown; Ponceau staining was used as loading control* ***B:*** *After quantification of 1-2 Western and 1-5 Northern blots low, middle and high Spry2 expressers were grouped and their average miR-21 expressions depicted.*

The highest amounts of Spry2 were detected in A427, VL-4 and Calu-6, whereas VL-4, VL-10, A549, VL-5, CRL5883 and VL-8 show less expression (Figure 16B). VL-3 was classified as middle expresser of Spry2 because this cell line is highly variable and dependent on cell preparation Spry2 levels fluctuate enormously.

After correlation the expression of Spry2 with their miR-21 levels, no connection can be found. Low expressers of Spry2 show on average the same miR-21 levels than normal fibroblasts (mean±SEM= 0.98±0.17), while miR-21 expression of cells with

middle expression of Spry2 are 1.14 fold increased. High expressers show again a mean±SEM of 1.07±0.32.

4.4 Influence of Sprouty overexpression on miR-21 expression level

As described before Spry1 (46) and Spry2 (47) are negative regulated by miR-21. To investigate if there is a feedback loop between miR-21 and Spry proteins, Spry1 and Spry2 were overexpressed by adenoviral system. Therefore cells were infected with 3 concentrations of pAdlox Spry1 and pAdlox Spry2. Two days postinfection cells were harvest and RNA was isolated, followed by Northern blot to detect miR-21 expression. For control of equal loading U6 levels were measured (Figure 17A).

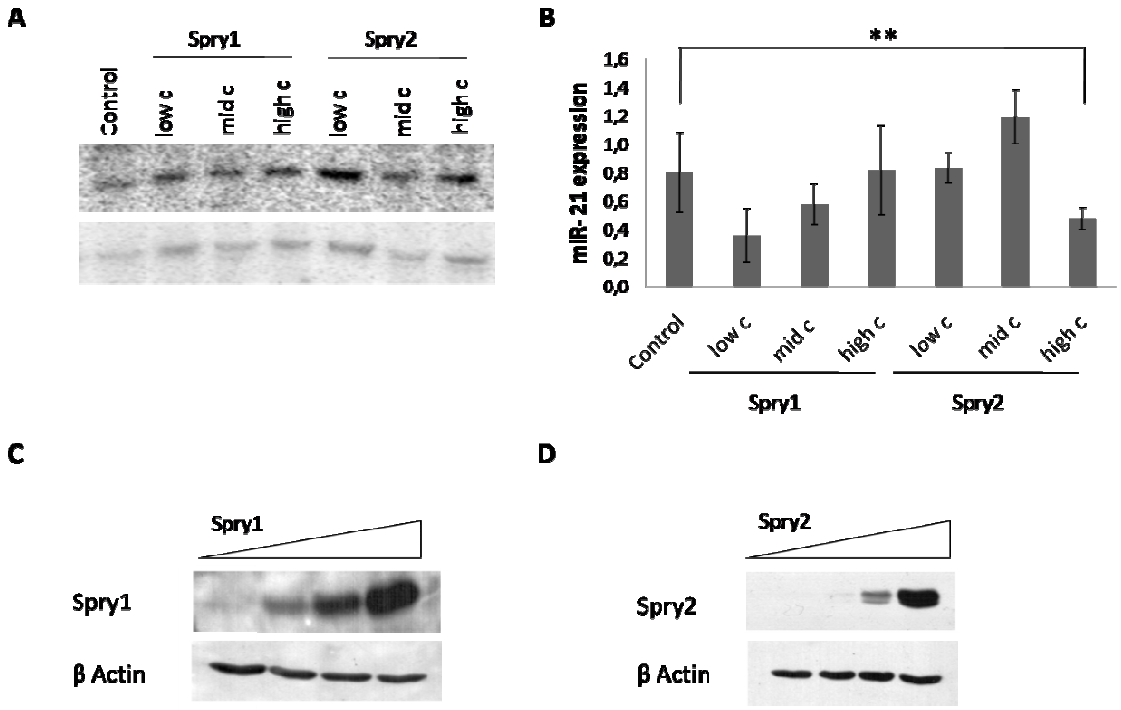

Figure 17: *Influence of Spry proteins on miR-21 expression.* ***A:*** *One representative Northern blot measuring miR-21 expression after viral overexpression of Spry1 and Spry2 in 3 different concentrations is shown. U6 is used as loading control.* ***B:*** *Quantification of miR-21/ U6 ratio was carried out with Image Quant 5.0 software; a graphic summarizing two experiments is shown. Significance was tested using unpaired t-test in Graph Pad Prism (* p<0.05; ** p<0.01; *** p<0.001). Successful overexpression of* ***C*** *Spry1 and* ***D*** *Spry2 was verified by Western blot.*

In addition Western blots were carried out to confirm the overexpression of Spry proteins (Figure 17C+ D).

Although overexpression of Spry1 is obvious (Figure 17C), detection of miR-21/ U6 ratio shows that there is no difference in miR-21 levels after overexpression of Spry1 (Figure 17B).

Overexpression of Spry2 with fewer amounts does not lead to an alteration in miR-21 expression. Only overexpression of Spry2 with the highest concentration show a significant ($p=0.008$) decrease of miR-21 expression compared to control cells (Figure 17B). Overexpression of Spry2 can be confirmed via Western blot (Figure 17D).

4.5 Modulation of miR-21 levels

4.5.1 Preparatory experiments

4.5.1.1 Infectivity assay by using CFP expressing adenovirus

For further experiments it was necessary to construct a control virus to exclude general viral effects.

To construct the control virus an existing pAdlox CFP plasmid was digested with Sfi I and the virus was constructed like it is described in *material and methods*. Before the cells were harvested for viral reinfection, photographs with the fluorescent microscope were taken.

After transfection there are only a few transfected cells, which incorporated and expressed the transfected pAdlox CFP plasmid. Due to the low titer in the first round of virus generation, 3 days after infection with the crude lysate no blue fluorescent cells were visible. Nonetheless cells were harvested and after the second reinfection there were a lot of fluorescent cells, indicating that most cells were infected with the generated recombinant virus. In the consecutive rounds of infections nearly all cells were infected. To assure that there are no original ψ5 viruses contaminating the viral preparation, in total five reinfection cycles were performed (Figure 18). Each time the viral titer of recombinant CFP viruses increased as concluded from a clearly more effective.

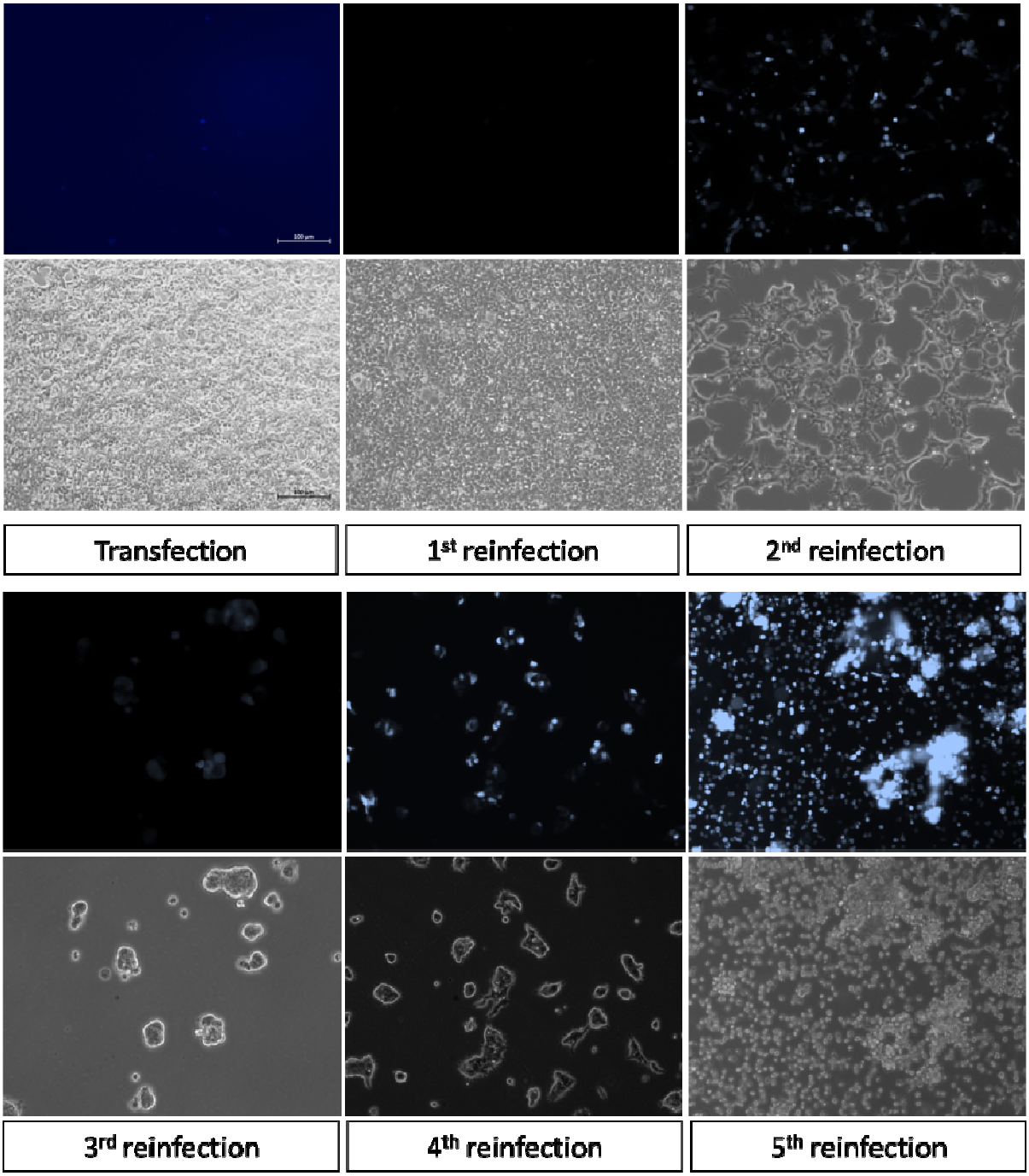

Figure 18: *Generation of recombinant adenoviruses is monitored by picturing CFP expressing cells with a fluorescence microscope. Cells were pictured 48 h after transfection or infection with a crude cell extract harboring the viruses. Reinfection cycle is indicated. To visualize the number of monitored cells pictures with bright field were taken.*

For evaluation infection efficiency of the cell lines planned to use for further analysis, and as initial experiment adenovirus infectivity test was performed Therefore WI-38 and NSCLC-derived cell lines were infected with three different concentrations of the recombinant CFP virus and 48 hours later photographs were taken with a fluorescent microscope and infection rate was determined as percentage of blue fluorescent cells. Afterwards cell lines were classified into 4 categories: high, middle, low and non infection efficiency (Table 8).

Cell lines	*Infection efficiency*
WI-38	high
A427	middle
A549	middle
Calu-6	middle
VL-2	low
VL-3	low
VL-6	middle
VL-7	no
VL-10	low
VL-5	middle
VL-8	high
VL-4	middle
SKLU-1	no

Table 8: *Infection efficiency of CFP virus in different NSCLC derived cell lines*

Examples of all these categories are shown in Figure 19. As example for cells which are low infection rate VL-3, for middle infection efficiency A427 and for high VL-8 were chosen.

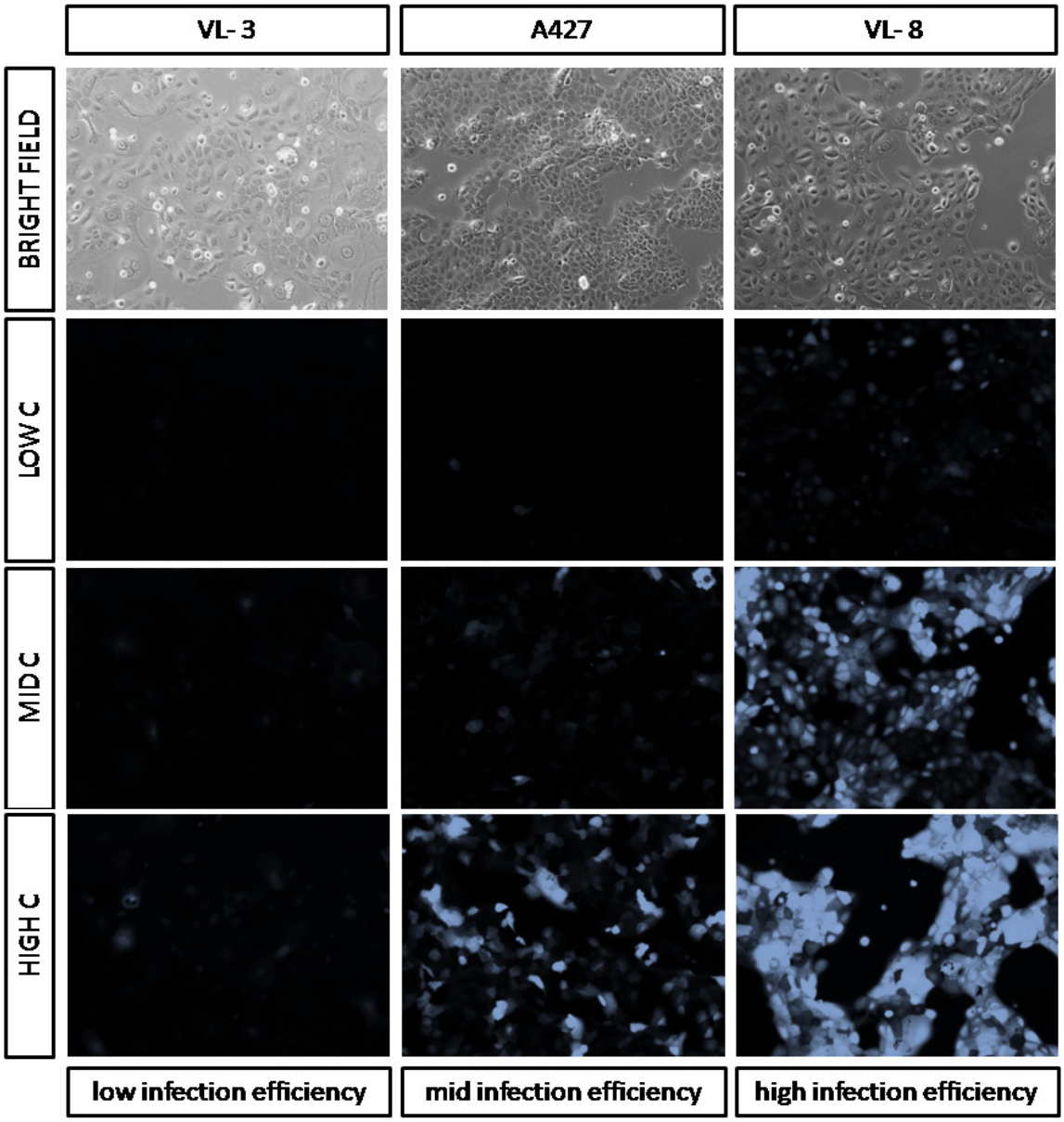

Figure 19: *Examples for cell lines defined as high (VL-8), middle (A427), and low (VL-3) susceptible are depicted. Images of cells infected with the low, middle and high CFP virus concentration are shown.*

4.5.1.2 Adenoviral influence on expression of miR-21

Since adenoviruses are lytic viruses, cells show sometimes a general viral effect, which can influence the results of the experiments and can lead to problems with experimental interpretation. To exclude a general viral effect at the chosen titer, it was examined if there is a difference in miR-21 expression between uninfected cells and those infected with the control virus.

Therefore one plate of cells was infected with the control virus while another one was left untreated, 48 hours postinfection cells were harvest and Northern blot for detection of miR-21 was performed. Afterwards miR-21/ U6 ratio was analyzed via Image Quant 5.0 software to compare miR-21 expression in uninfected cells and cells which are infected with the control virus.

Although the difference between miR-21 levels in this two cell populations varied due to experimental differences between 0.4 and 1.8, on average no effect of the control virus in miR-21 expression compared to uninfected cells can be observed (Figure 20).

Due to the result, in further experiments expression of uninfected and cells infected with a control virus were combined to one control value.

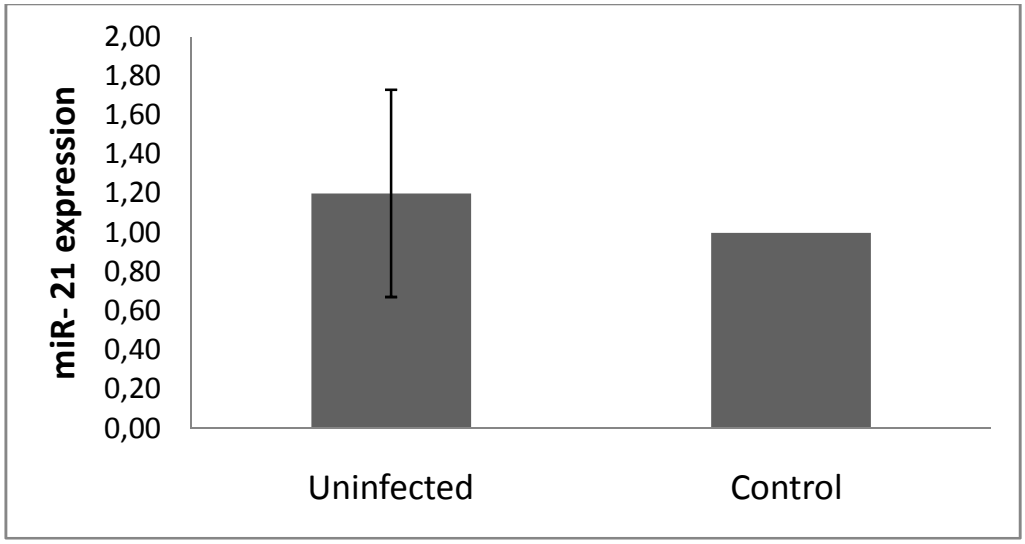

Figure 20: *Quantification of miR-21/ U6 ratio from 10 Northern blots. Analysis was done with Image Quant 5.0 software. Expression of cells infected with the control virus is randomly set to 1.*

4.5.1.3 Preparation of the constructs for modulation of miR-21 expression

For overexpression of miR-21, a pAdlox plasmid containing a fragment of the primiR 21 was already available in the labor. The fragment of the primiR 21 used to overexpress miR-21 includes premiR-21 and about 200 nt flanking sequences 3' as well as 5' of the loop. Vector map can be found in the appendix.

For inhibition of miR-21 three different strategies were used:

pAdlox sponge eraser 21 was already available at the begin of the study and is a so called sponge. The plasmid contains a CMV promoter, where RNA polymerase II can bind. As substrate, luciferase reporter followed by a tandem of an antisense sequence for miR-21 is included. Vector map of the construct can be found in appendix.

pAdlox oligo eraser 21 contains a U6 promoter where RNA polymerase III can bind. In contrast to pAdlox sponge eraser 21 the new plasmid contains no luciferase reporter and only one antisense sequence for miR-21.
In order to clone pAdlox oligo eraser 21, U6 promoter was amplified via PCR from another plasmid (pSilencer). After successful amplification, insert (=U6 promoter) was digested with Xho I and Sal I to generate compatible ends to the vector for ligation (Figure 21A).
The vector pAdlox sponge eraser 21 was digested with Xho I to remove the CMV promoter. The resulting fragments with the size of 2333 bp and 3592 bp are shown. DNA from vector fragment of size 3592 bp was eluted from the gel (Figure 21B). To disable religation of the vector, the ends were dephosphorylated. For the control of restriction digestion and gel elution, agarose gel electrophoresis was performed for the insert and the vector.
Afterwards ligation was performed, following by transformation in competent E. coli. To purify cloned plasmid, STETL preparation of the plasmid DNA was performed and control digestions were done with Bgl II and Nde I. Agarose gel electrophoresis was carried out to verify correct cloning and correct direction of the insert. Digestion of pAdlox oligo eraser 21 results in 2 fragments with the size of 3495 bp and 369 bp, while digestion of pAdlox sponge eraser 21 leads to fragments of 2371 bp and 3554 bp. Gel electrophoreses shows clones (1-3) which were not correct. Clone 4 shows

the two fragments which were expected and identify correct clones (Figure 21C). To verify the sequence, MIDI preparation was performed and an aliquot of DNA was sent to Microsynth for sequencing.

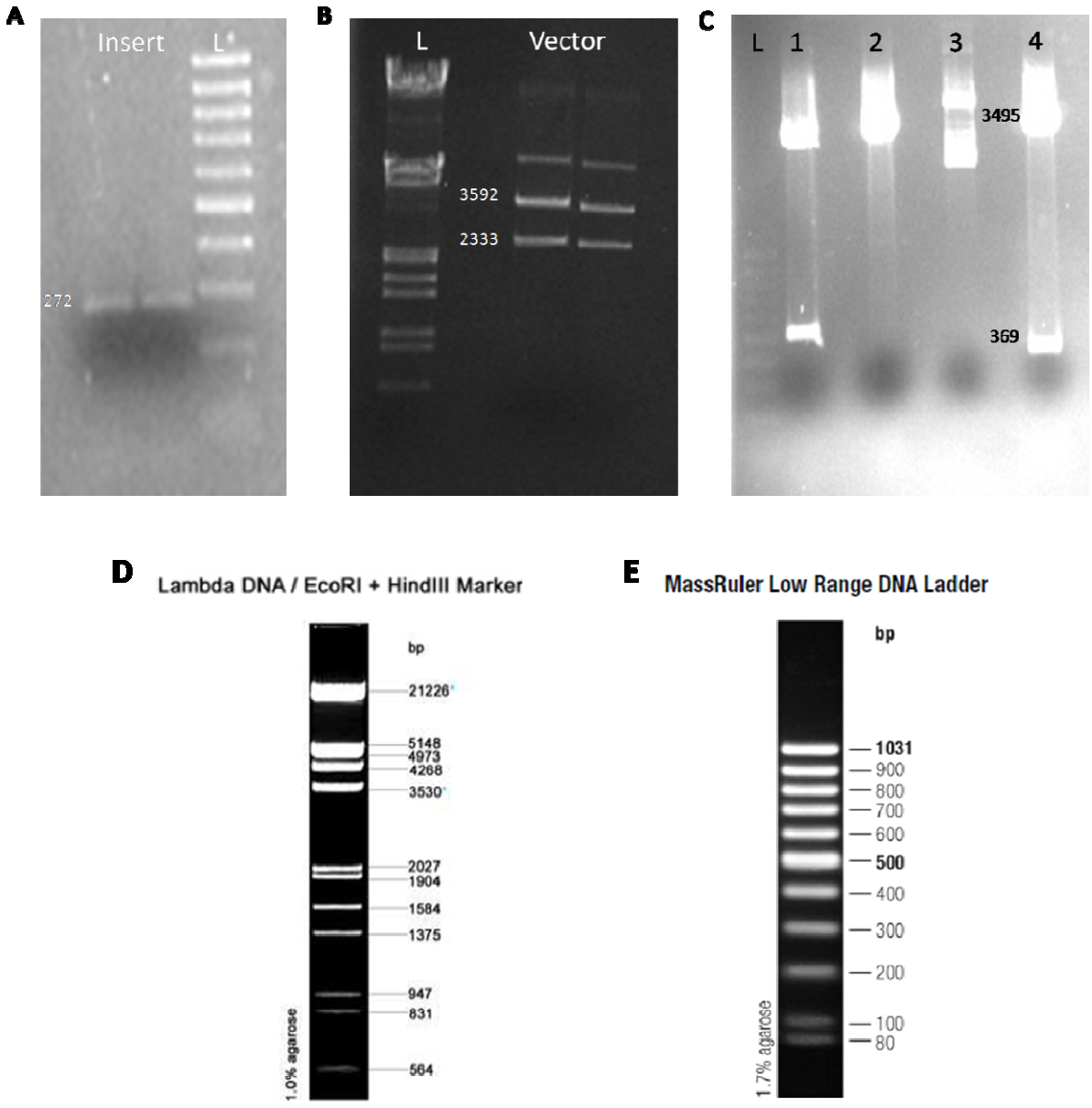

Figure 21: *Control of pAdlox oligo eraser 21 cloning steps.* ***A:*** *PCR fragment of U6 promoter (insert) digested with Xho I and Sal I has compared with Mass ruler DNA ladder (L= ladder is MassRuler Low Range DNA Ladder)* ***B:*** *Gel electrophoresis of the vector (pAdlox* sponge eraser 21*) was digested with Xho I. (L= ladder is Lambda DNA Ladder)* ***C:*** *Different clones after a control digestion with Bgl II and Nde I are shown* ***D:*** *λ DNA ladder* ***E:*** *MassRuler low range DNA ladder (Fermentas)*

pAdlox simiR 21 should inhibit miR-21 expression by producing siRNA against primiR 21, which should lower the level of mature miR-21.

pAdlox simiR 21 was cloned using pAdlox oligo eraser 21 as backbone. Therefore pAdlox oligo eraser 21 (= vector) was digested with Xho I and Bgl II, resulting in two fragments with the size of 3567 bp and 297 bp (Figure 22A). The removed 297 bp contains the eraser and part of the polyadenylation signal. Afterwards restriction enzymes were deactivated by PCI extraction and agarose gel electrophoresis was performed. The band with the size of 3567 bp was eluted from the gel by DNA gel recovery kit.

Meanwhile two complementary primers were annealed to create the simiR 21 insert with compatible overhangs to the digested vector. To facilitate ligation primers were phosphorylated and annealing was carried out. Fragments were purified by Sephadex column to elude all ingredients which can annoy the ligation.

Ligation, followed by transformation with recovery time for bacteria in competent E. coli was performed. Different concentrations were used and a 9:1 insert/ vector ratio using 25ng vector was most successful. 3 clones compared to 0 clones with the control ligation were obtained. STETL preparation of 3 clones was carried out and control digestion first with Hind III and afterwards with Sfi I was performed. All picked clones showed 3 fragments at a size comparable to the calculated fragments sizes of 2528 bp, 865 bp and 253 bp (Figure 22B). The original backbone vector (pAdlox oligo eraser 21) was included as control and yielded two fragments (2528 bp and 1336 bp) as visible at the agarose gel.

For verification the plasmid, MIDI preparation was performed and an aliquot of the plasmid was sent for sequencing.

Preparation of adenovirus of the pAdlox oligo eraser 21 and pAdlox simiR 21 construct was done as described in *material and methods*.

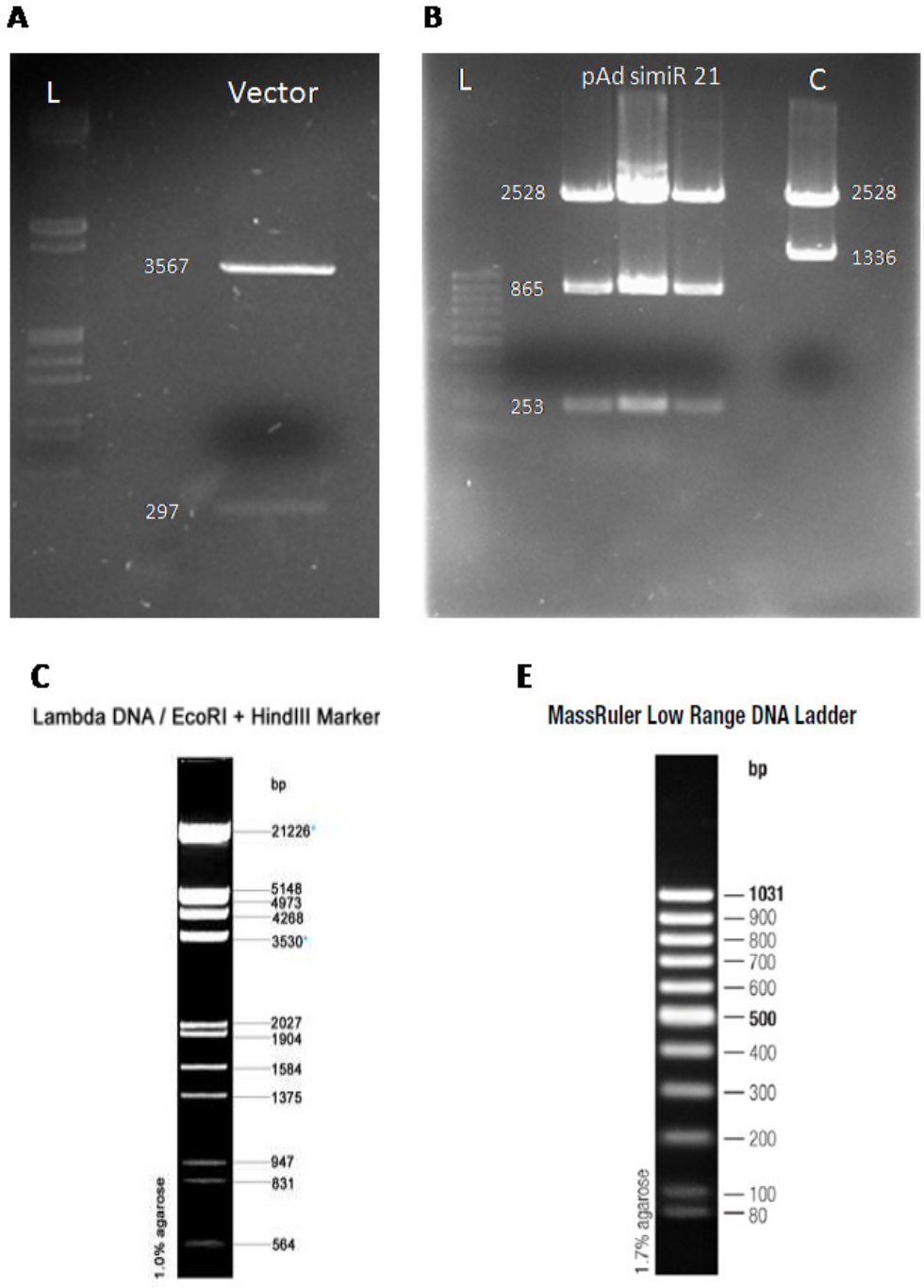

Figure 22: *Construction of pAdlox simiR 21* ***A:*** *Gel electrophoresis of the vector (pAdlox* oligo eraser 21*) was digested with Xho I and Bgl II. (L= ladder is Mass Ruler Low Range DNA ladder)* ***B:*** *Different clones after a control digestion with Hind III and Sfi I are shown (C= control, original vector; L= ladder is λ DNA ladder)* ***C:*** *λ DNA ladder* ***D:*** *MassRuler low range DNA ladder (Fermentas)*

4.5.2 Influence of overexpression of miR-21 on proliferation

First a growth curve in A459 was performed to examine the influence of miR-21 expression on proliferation. Therefore cells were infected with adenoviruses generated through recombination with pAdlox primiR 21 and with a control virus. 2 days afterwards, cells were counted and equal amounts of cells were split into 6cm dishes. For a week, every day triplicates of these cells were counted to follow their proliferation.

Results were analyzed and are shown in Figure 23A. Between the cells, infected with the control virus and cells which should have higher expression of miR-21, no difference in proliferation can be found.

To control if the pAdlox primiR 21 virus works correct, cells were infected with the virus and after 2 days they were harvest and RNA was isolated. After performing Northern blot, it was shown that there was no increase of miR-21 expression in cells infected with pAdlox primiR 21 virus (Figure 23B). In contrast to the supposed results miR-21 in the analyzed cells infected with the highest concentration of the virus decrease 0.3 fold compared to control cells. To exclude that the construct only works on protein level, Western blot of cells infected with pAdlox primiR 21 was performed. Spry1 and Spry2 expressions both targets of miR-21 were examined. The results were similar to the results maintained from the Northern blot before, which means that also at protein level no difference between cells which were infected with the primiR 21 virus and control cells can be found (Figure 23C+D). Neither Spry1 nor Spry2 is decreased compared to control cells, no matter which concentration of pAdlox primiR 21 virus was used.

Therefore we considered that the viral recombination was not as efficient as expected and we decided to repeat the production. Cre8 cells were transfected with the primiR 21 plasmid and empty virus. Construction of adenovirus was carried out, as described in *material and methods*. After purification U373 cells were infected with 3 different concentrations of the newly prepared virus, and 48 hours postinfected cells were harvested. Afterwards RNA was isolated and miR-21 and U6 levels were detected by Northern blot.

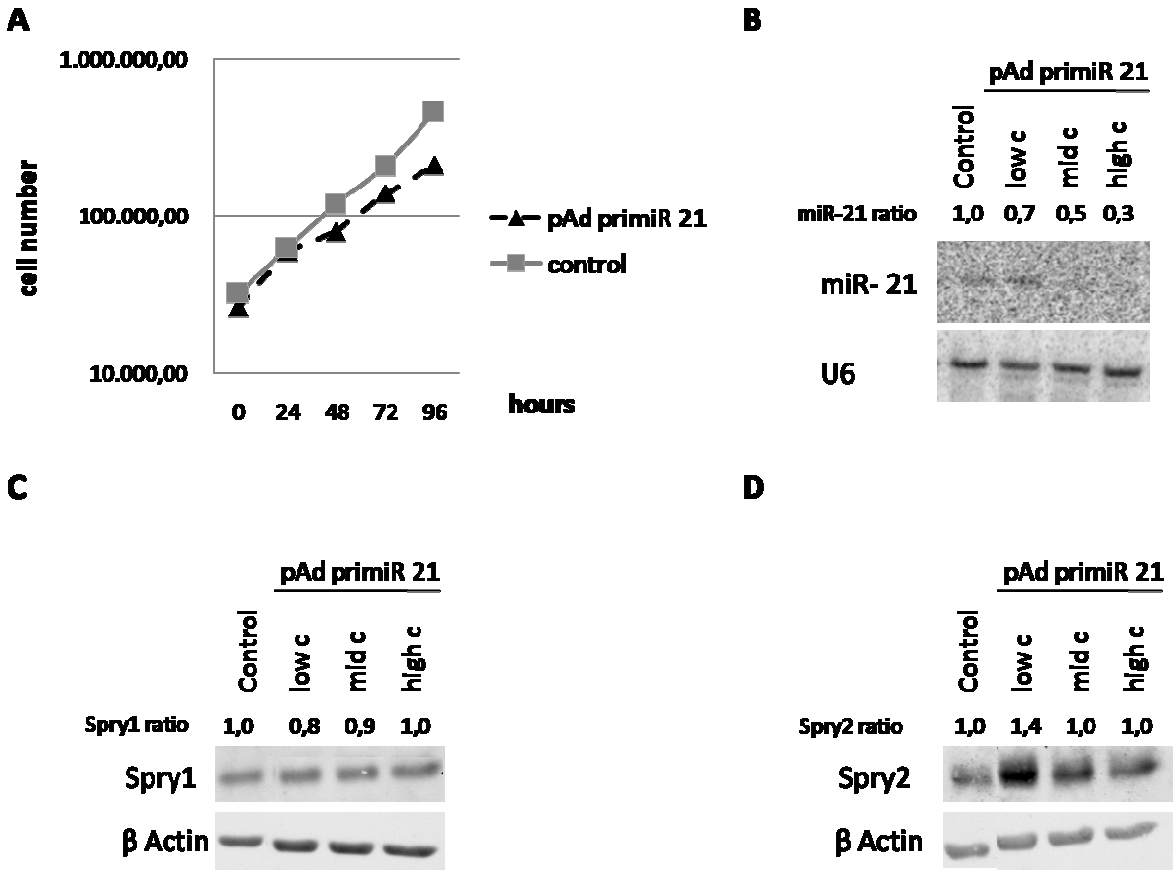

Figure 23: *Infection of A549 cells with adenoviruses expressing a primiR 21;* ***A:*** *Cells were infected with pAdlox primiR 21 adenovirus and proliferation was examined in a proliferation curve by counting cells;* ***B:*** *Cells were infected with pAdlox primiR 21 and mature miR-21 was measured by Northern blot. Quantification of miR-21/ U6 ratio was detected with Image Quant 5.0 software. Corresponding Western blots to B for* ***C:*** *Spry1 and* ***D:*** *Spry2 were performed. Quantification of miR-21 and Spry1 and Spry2 was done with Image Quant 5.0 software.*

As seen in Figure 24 mature miR-21 levels increase when infected with the adenovirus expressing primiR 21. Dependent on the concentration of the virus miR-21 increases in cells which were infected with the high concentration of the pAdlox primiR 21 virus up to 2.8 fold higher compared to cells which were infected with a control virus, which shows that overexpression of miR-21 in U373 works.

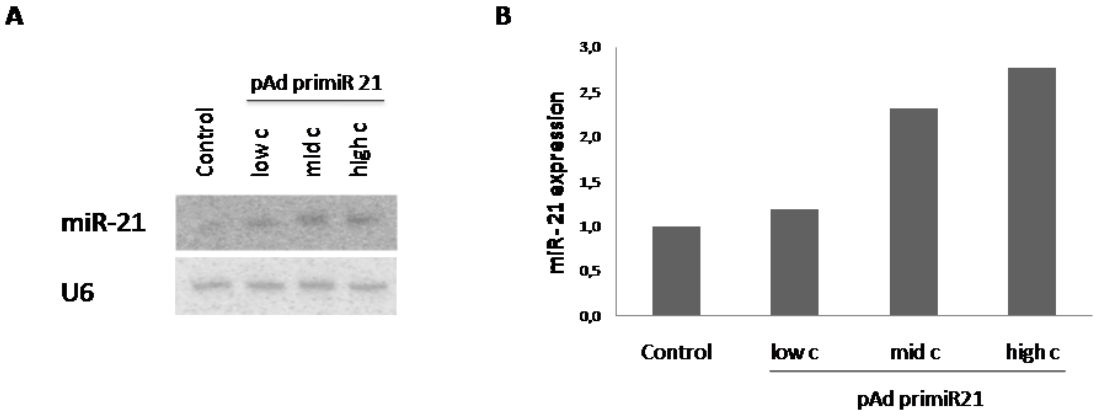

Figure 24:*U373 cells were infected with newly synthesized pAdlox primiR 21 virus* ***A:*** *MiR-21 expression was determined by Northern blot. Equal loading was controlled by U6 Northern blot;* ***B:*** *Quantification of miR-21 was done with Image Quant 5.0 software. MiR-21 expression was normalized to U6 and the expression of the control cells.*

4.5.3 Comparison of different constructs for miR-21 inhibition

4.5.3.1 Inhibition of miR-21 by pAdlox sponge eraser 21

The influence of pAdlox sponge eraser 21 on miR-21 levels was tested in the cell lines U373 and A427. Therefore cells were infected with 3 different concentrations of the adenovirus, 48 hours after infection cells were harvest and RNA was isolated. For detection of miR-21 and U6, which was used for the control of equal loading, Northern blot was performed (Figure 25A).

To investigate if miR-21 function was inhibited by expressing the sponge eraser, Spry1 and Spry2 protein levels, both verified targets of miR-21, were determined by Western blot. Cells were infected with 3 different concentrations of the virus and were harvest 48 hours post infection. Total proteins were isolated, page gel and Western blot were performed. Membranes were incubated in Spry1 and Spry2 primary antibody, followed by an incubation in total Erk or β Actin antibody to verify equal loading (Figure 25C+D upper panel).

In U373, inhibition of miR-21 expression is shown to be significant, when cells were infected with the middle ($p<0.001$) or the high concentration ($p=0.0043$) of the virus. Interestingly the decrease in miR-21 level is improved when cells were infected with the middle concentration of the virus (mean±STD= 0.4±0.1) compared to the decrease in cells which were infected with higher amounts (mean±STD= 0.7±0.2). Cell infected with low levels of the sponge eraser 21 virus do not show any effect on miR-21 expression (mean±STD= 1.0±0.4) (Figure 25B).

In contrast to the results of the Northern blot, no significant difference on the protein level of Spry1 and Spry2 can be found.

Spry1 level shows a high standard deviation when cells were infected with low (mean±STD=1.08±0.81) and middle concentration (mean±STD=1.02±0.88) of the virus. Only cells infected with the highest level of pAdlox sponge eraser 21 show increased Spry1 level (mean±STD=1.67±0.33), but no statistical significance can be observed ($p=0.096$) (Figure 25C lower panel).

Also quantification of Spry2/ total Erk ratio show high standard deviation, so that no increased of Spry2 levels can be observed (Figure 25D lower panel).

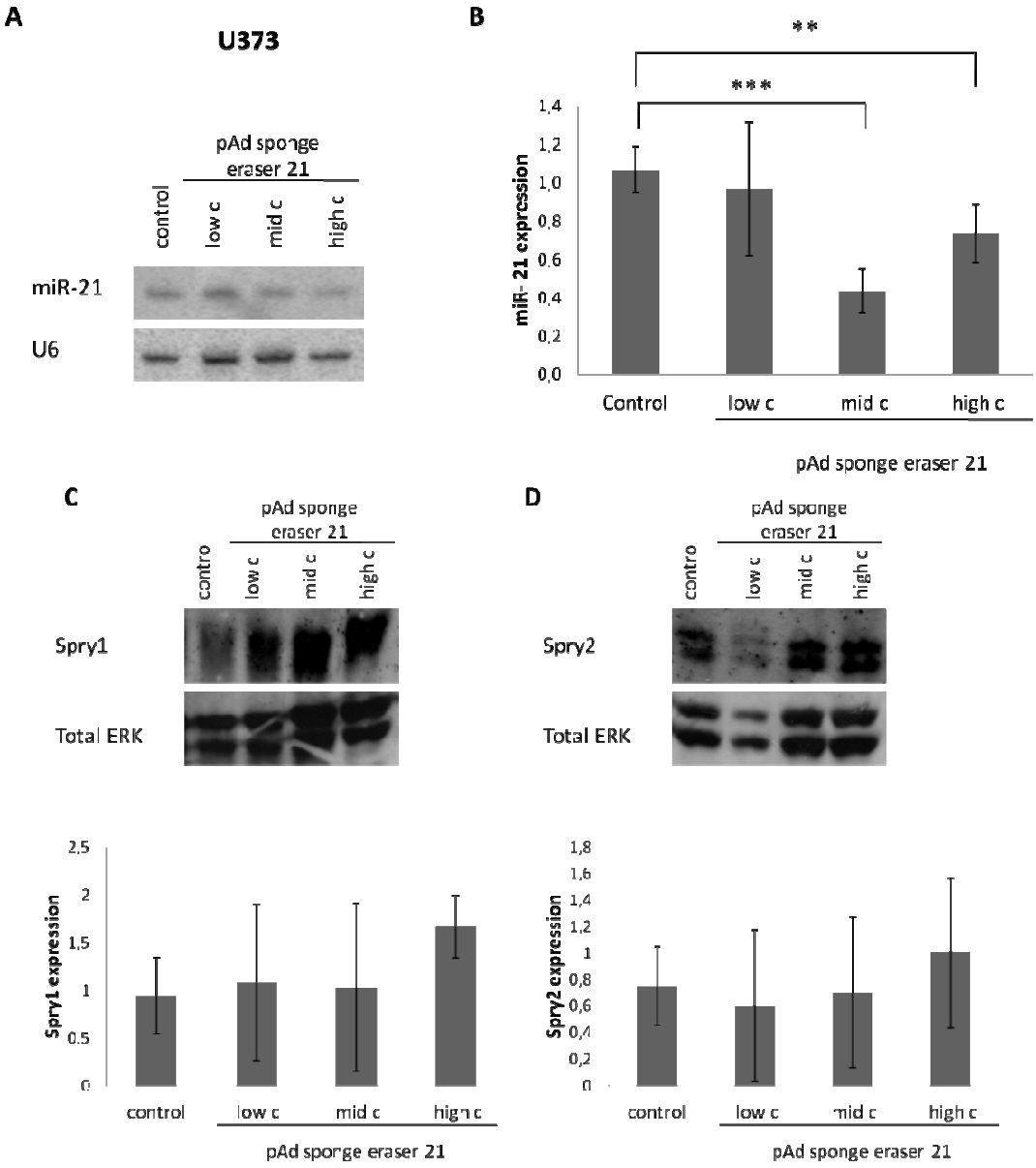

Figure 25: *Influence of pAdlox sponge eraser 21 infection on miR-21 expression and function in U373* ***A:*** *A representative Northern blots of miR-21 expression is shown. Equal loading was determined by U6 loading control.* ***B:*** *Summary of the 3 Northern blots after quantification of miR-21/ U6 using Image Quant 5.0 software. MiR-21 levels were normalized to U6 and to the expression of miR-21 in control cells. Significance was analyzed with t-test in Graph Pad Prism (* p<0.05; ** p<0.01; *** p<0.001) Levels of* ***C:*** *Spry1 and* ***D:*** *Spry2 of U373 after infection with pAdlox sponge eraser 21 were detected by Western blotting (upper panel). One representative of 2 blots is shown. Quantification was carried out with Image Quant 5.0 program. Expression levels as ratio to β Actin or total Erk were normalized to control cells (lower panel).*

In A427 all 3 concentrations of the pAdlox sponge eraser 21 virus causes almost the same miR-21 levels compared to the control cells.

Although in Western blot no increase of Spry1 and Spry2 can be found (Figure 26C+ D upper panel). Quantification of Spry1 levels show no big fluctuation between cells infected with a low, middle and high concentration of the virus (Figure 26C lower panel), while Spry2 levels are changed. Cells infected with low and high concentration show a trend of increased Spry2 expression (Figure 26D lower panel). Nonetheless we assumed that this construct does not work in this specific cell line.

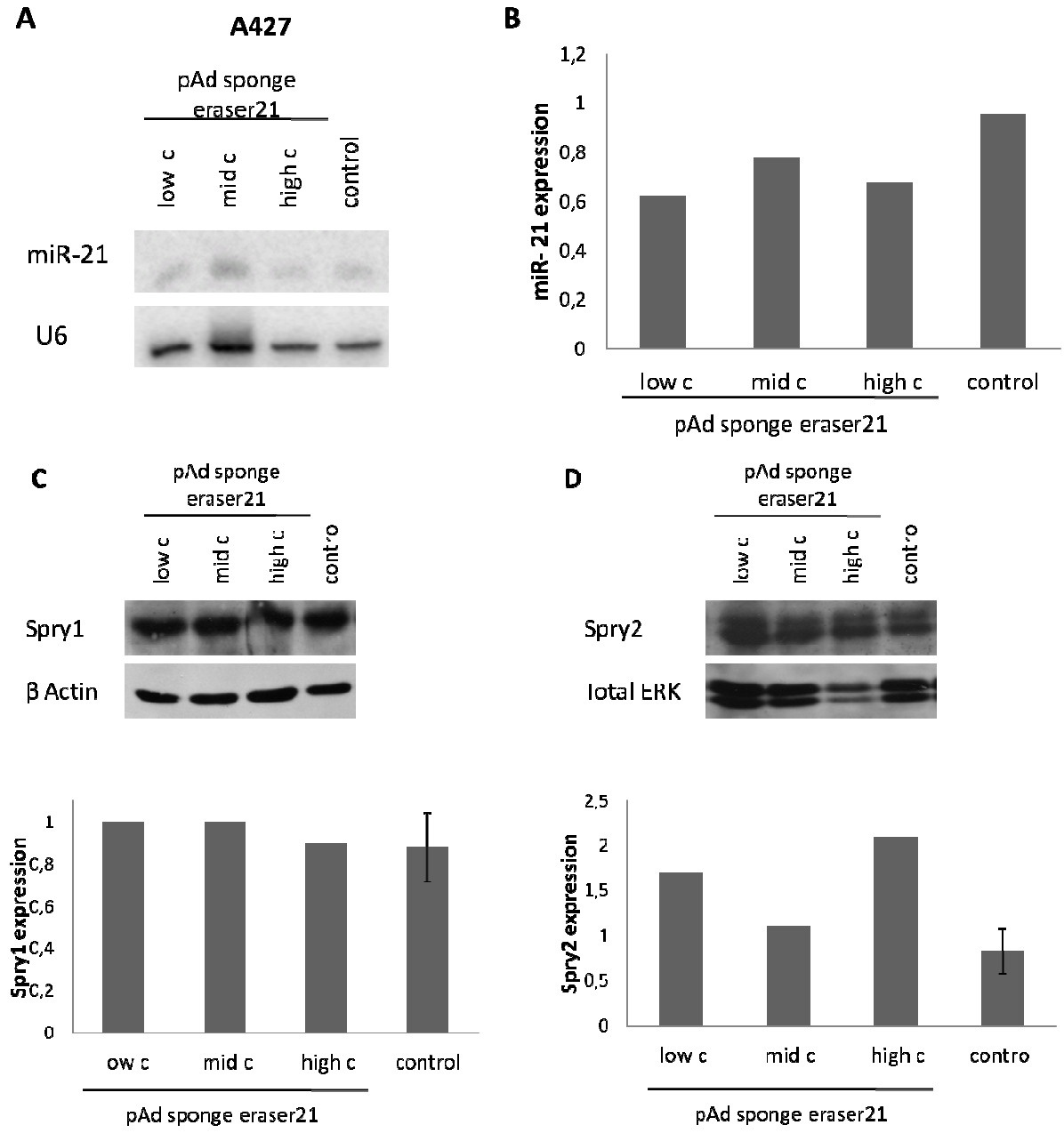

Figure 26: *Influence of pAdlox sponge eraser 21 infection on miR-21 expression and function in A427* ***A:*** *Northern blot of miR-21 expression is shown. Equal loading was determined by U6 loading control.* ***B:*** *For quantification of miR-21/ U6 Image Quant 5.0 software was used. MiR-21 levels were normalized to U6 and to the expression of miR-21 in cells infected with a control virus. Levels of* ***C:*** *Spry1 and* ***D:*** *Spry2 of A427 after infection with pAdlox sponge eraser 21 were detected by Western blotting (upper panel). Quantification was carried out with Image Quant 5.0 program. Expression levels as ratio to β Actin or total Erk were normalized to control cells (lower panel).*

4.5.3.2 Inhibition of miR-21 by pAdlox oligo eraser 21

To examine if pAdlox oligo eraser 21 has the excepted influence on miR-21 expression, the cell lines U373 were infected with 3 different ratios of the adenovirus. 48 hours after infection cells were harvest and total protein and total RNA were isolated. For detection of miR-21 expression Northern blot was performed. Detection of U6 was used as loading control (Figure 27A).

In U373 cell line no difference in miR-21 expression of cells infected with the pAdlox oligo eraser 21 virus compared to control cells can be observed. Quantification of miR-21 levels of control cells leads to a mean of 1.4. Cells infected with the lowest level of the virus show on average a slightly increased expression of miR-21 (mean±STD= 1.73±0.02), while infection with the middle lead to no decrease (mean±STD= 1.29±0.37). Cells infected with the highest level show nearly the same miR-21 expression like the control cells (mean±STD=1.09±0.45). MiR-21 levels are not influenced by this eraser construct (Figure 27B).

Since it is possible that the construct has only an influence on the protein level, Western blot for Spry1 and Spry2 was performed, too. Equal loading of samples were controlled with incubation of the membrane in β Actin or total Erk antibody (Figure 27C+D upper panel).

In U373 cells no difference between infected cells and control cells at the protein level of Spry1 and Spry2 can be observed. Spry1 levels detected from cells infected with pAdlox oligo eraser 21 show high standard deviation. On average cells infected with the middle (mean±STD= 1.60±1.04) and the highest concentration (mean±STD= 1.44±0.93) of the virus show increased Spry1 levels, but due to the huge standard deviation no statistical significance can be observed (Figure 27C upper panel).

Expression of Spry2 cells infected with the low and the high level of the virus is nearly the same. Only middle concentration had an effect and leads on average to an increase of Spry2 expression (mean±STD= 2.03±0.42), but no statistical significance can be observed (p=0.075) (Figure 27D upper panel).

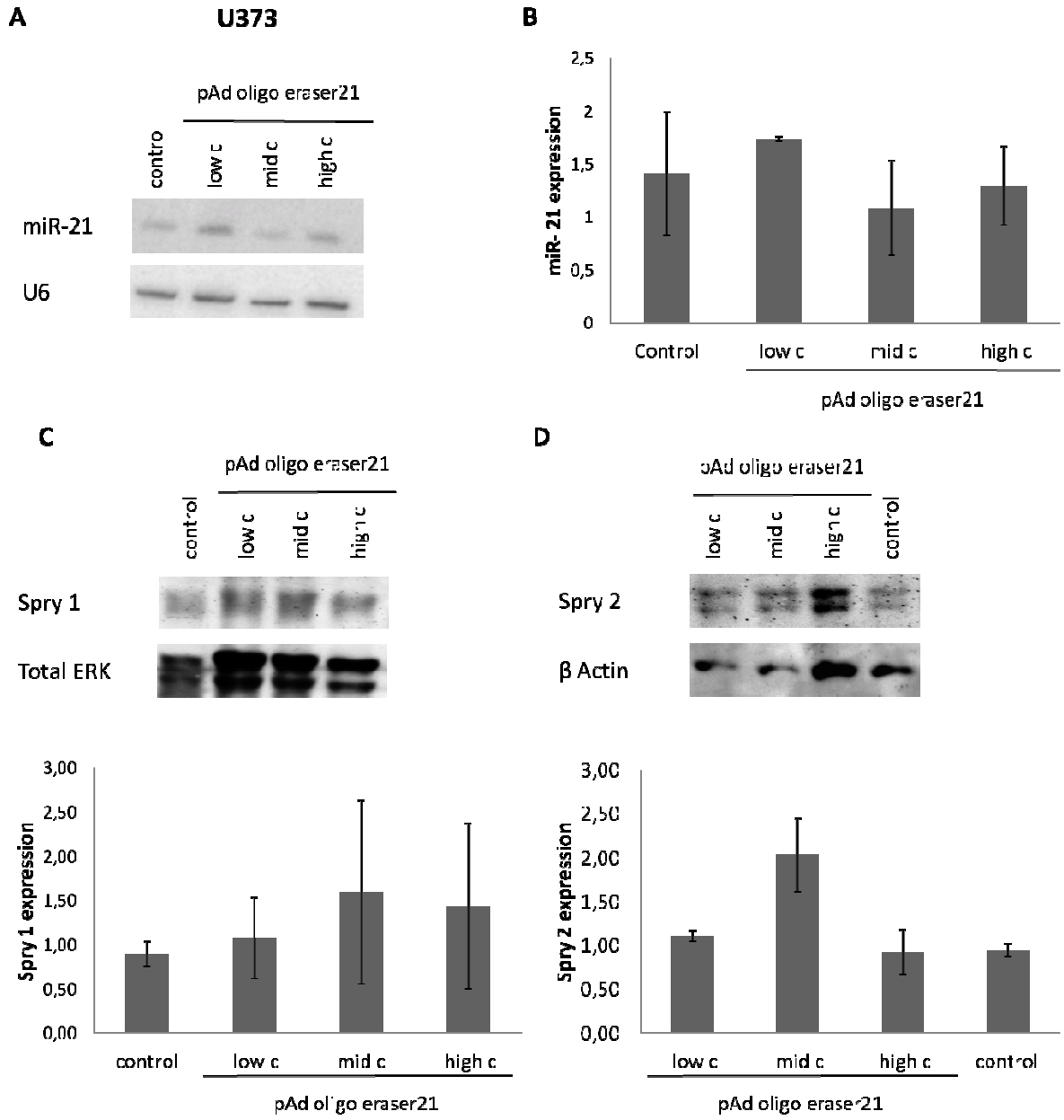

Figure 27: *Influence of pAdlox oligo eraser 21 infection on miR-21 expression and function in U373* ***A:*** *A representative Northern blots of miR-21 expression is shown. Equal loading was determined by U6 loading control.* ***B:*** *Summary of the 3 Northern blots after quantification of miR-21/ U6 using Image Quant 5.0 program. MiR-21 levels were normalized to U6 and to the expression of miR-21 in cells infected with a control virus. Levels of* ***C:*** *Spry1 and* ***D:*** *Spry2 of U373 after infection with pAdlox oligo eraser 21 were detected by Western blotting (upper panel). One representative of 2 blots is shown. Quantification was carried out with Image Quant 5.0 program. Expression levels as ratio to β Actin or total Erk were normalized to control cells (lower panel).*

To examine if pAdlox oligo eraser 21 has the excepted influence on miR-21 expression, the cell line A427 were infected with 2 different ratios of the adenovirus. 48 hours after infection cells were harvest and total protein and total RNA were isolated. MiR-21 expression was detected by Northern blot. For loading control, Northern blot of U6 was used (Figure 28A). Additionally Western blots for Spry1 and Spry2 were performed, to examine the influence of the construct on protein level (Figure 28C+D upper panel).

In A427 no difference between infected cells and control cells at the miR-21 can be observed (Figure 28B).

Spry1 and Spry2 levels are increased in cells infected with low ratio of the virus, while at the high concentration expression of these proteins decreases in comparison to the expression in control cells (Figure 28C+D lower panel).

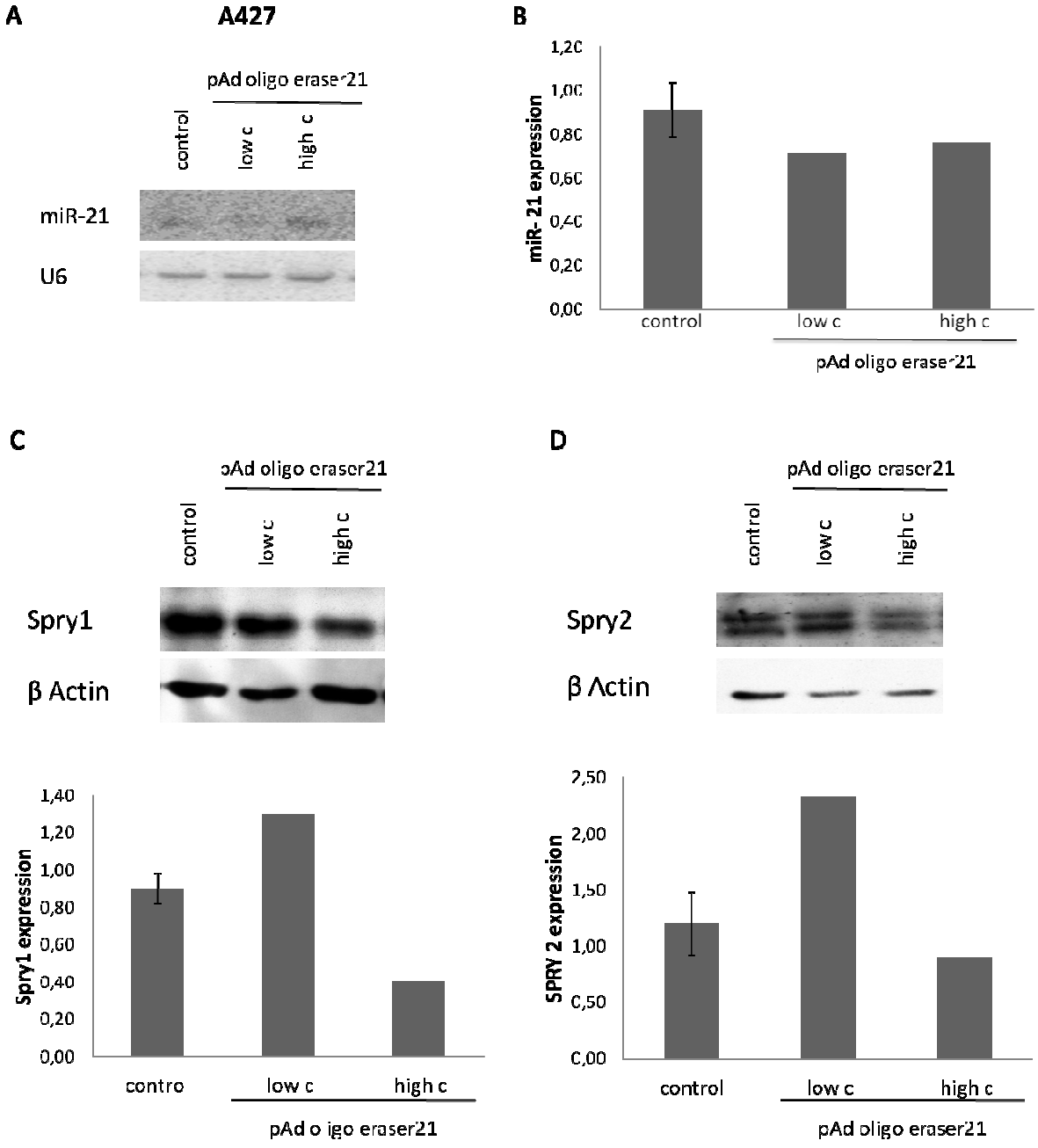

Figure 28: *Influence of pAdlox oligo eraser 21 infection on miR-21 expression and function in A427* ***A:*** *Northern blot of miR-21 expression is shown. Equal loading was determined by U6 loading control.* ***B:*** *For quantification of miR-21/ U6 Image Quant 5.0 software was used. MiR-21 levels were normalized to U6 and to the expression of miR-21 in cells infected with a control virus. Levels of* ***C:*** *Spry1 and* ***D:*** *Spry2 of A427 after infection with pAdlox oligo eraser 21 were detected by Western blotting (upper panel). Quantification was carried out with Image Quant 5.0 program. Expression levels as ratio to β Actin were normalized to control cells (lower panel).*

4.5.3.3 Inhibition of miR-21 by pAdlox simiR 21

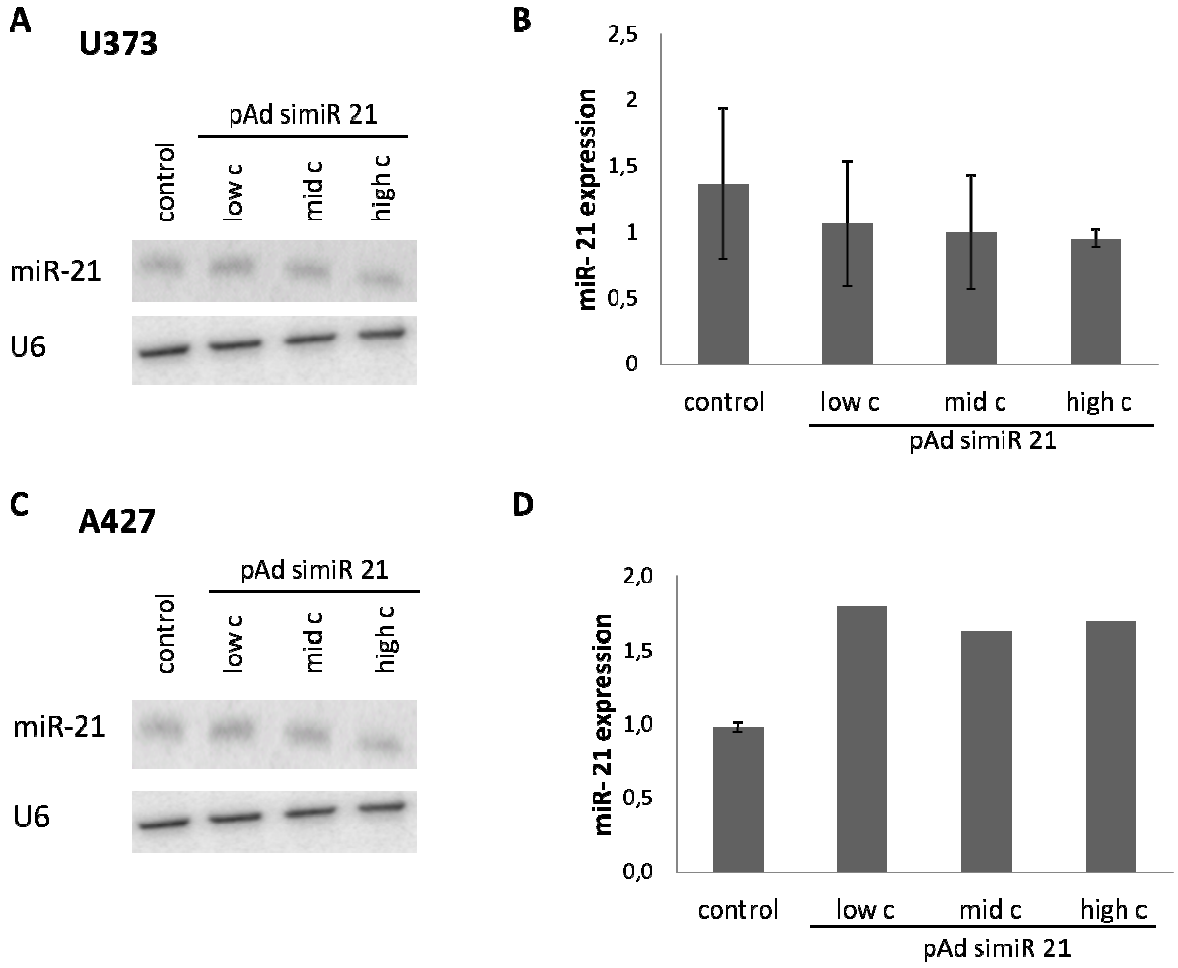

Figure 29: *Influence of pAdlox simiR 21 infection on miR-21 expression and function in U373 and A427* ***A:*** *A representative Northern blot of miR-21 expression in U373 is shown. Equal loading was determined by U6 loading control.* ***B:*** *Summary of the 2 Northern blots of U373 after quantification of miR-21/ U6 using Image Quant 5.0 program. MiR-21 levels were normalized to U6 and to the expression of miR-21 in control cells.* ***C:*** *Northern blot of miR-21 expression in A427 is shown. Equal loading was determined by U6 loading control.* ***D:*** *For quantification of miR-21/ U6 Image Quant 5.0 software was used. MiR-21 levels were normalized to U6 and to the expression of miR-21 in cells infected with a control virus.*

To examine if pAdlox simiR 21 virus will be successful in inhibition of miR-21 expression, U373 and A427 cells were infected with 3 different concentrations. 48 hours post infection cells were harvest and RNA was isolated and miR-21 levels were detected by Northern blotting. Equal loading of the samples was verified by hybridization with U6 (Figure 29A+C).

In both cell lines no decrease of miR-21 expression can be observed. In U373 no difference in the miR-21 levels to the expression in control cells can be found, no matter which infection ratio was used (Figure 29B).

In A427 inhibition of miR-21 by pAdlox simiR 21 does not works, too. In contrast to the supposed results, miR-21 is slightly increased after infection with pAdlox simiR 21 compared to the control cells (Figure 29D).

5 DISCUSSION

MiR-21 is shown to be upregulated in lung tumor compared to normal tissue (38) (39), while Spry2 is downregulated in NSCLC (74). In this study endogenous levels of miR-21 from 14 NSCLC-derived cell lines were analyzed and the expression was compared to normal fibroblast cells (WI-38). In contrast to the results observed from tissue (38) (39), no NSCLC-derived cell line shows a more than 2-fold increase of miR-21 expression by comparison with the levels in WI-38 and there were also cell lines which expressed lower levels of miR-21 than normal lung fibroblasts. Conform with these results, it is demonstrated, that miR-21 expression in colon cancer-derived cell lines fails to be increased (85) or only shows rare changes (86), while in colorectal tumor tissue levels are usually elevated (33) (34). Overexpression of miR-21 in cancer derived-cell lines which were cultured under standardized conditions is only observed in glioblastoma (30). By comparison of the proliferation rate of the cell lines, we cannot show a significant difference between miR-21 levels of slow and fast cell lines.

Cell lines, which harbor a K-Ras mutation, have on average higher levels of miR-21 than cells with the wt of this gene. All cell lines with a K-Ras mutation are adenocarcinomas, which is not surprising because this mutation was mostly found in adenocarcinomas and is very uncommon in squamous cell carcinomas (87). In literature it is shown in tissue, that adenocarcinomas have on average a higher miR-21 expression than tumors staged as squamous cell carcinoma (88). In this study miR-21 levels in cell lines harboring an activating mutation in K-Ras and EGFR gene show no significant difference compared with cell lines with the wt gene. Literature shows that EGFR (89) and K-Ras mutations (90) (91) influence the expression of miR-21. This is not in contrast to our findings, because in these studies the influence of changed pathways was analyzed in tissue. Seike et al. show that the level of phosphorylated EGFR correlates with the expression of miR-21 in tissue of never smokers (89). Frezetti et al. and Hatley et al. analyze the influence of oncogenic Ras on miR-21 levels by comparing tissue from adenocarcinomas, which were activated by Ras expression with normal tissue (90) (91). Our data show that overexpression of ongenic K-, H- and N-Ras via adenoviral system in WI-38, does not lead to an increase in miR-21 levels.

These results indicate that miR-21 in NSCLC-derived cell lines is not increased by intrinsic alteration of cancer, but maybe by external factors. As a consequence of this study, further experiments will be necessary to investigate why miR-21 is highly overexpressed in tumor tissue while no upregulation in NSCLC-derived cell lines can be found.

The investigated miR-21 substrates show no correlation with miR-21 expression. This can be due to the fact, that no huge difference in miR-21 levels between the cell lines can be found. Additionally proteins are not only regulated by one factor and it is possible that other regulatory signaling have more influence on protein expression than miR-21. In the case of Spry2 there are several possibilities how it can be regulated, like ubiquitination through c-Cbl (64) or by the influence of PTEN (48). Additionally Spry1 and Spry2 are regulated by a negative feedback loop in MAPK pathway (56). PDCD4 is not only regulated by miR-21, but also for example phosphorylation by S6K1 and/ or Akt influence its stability (92) (93). PTEN is known to be regulated by various molecular mechanisms at mRNA and protein level (94). Due to this knowledge, miR-21 is only one of many ways to influence regulation of PDCD4, PTEN, Spry1 and Spry2.

Spry1 and 2 are known to be targets of miR-21 and are negatively regulated by this miRNA (46) (47). Overexpression of human Spry1 in WI-38 fails to change miR-21 expression, while miR-21 is decreased when Spry2 is highly overexpressed. Lower concentrations of Spry2 do not show an effect on miR-21 levels. MiR-21 expression is known to be regulated via a negative feedback-loop involving PDCD4 and AP-1. PDCD4 was also found to be a negative AP-1 regulator. Downregulation of PDCD4 by miR-21 is necessary for activation of AP-1 in response to Ras (43). According to the results of our study, Spry1 is no regulator of miR-21 expression, while high levels of Spry2 seem to have an influence on miR-21 levels by a positive and not as supposed by a negative feedback loop. This result must be confirmed by further experiments, where adequate levels of Spry2 are expressed.

Due to the fact that miRNAs are involved in nearly every cellular process, like cell differentiation (95), proliferation and apoptosis (96), a lot of research of miRNAs as anticancer therapeutic was done. Modulation of miRNA by overexpressing or

inhibiting them, are important tools for therapeutic as well for more detailed understanding of the influence of miRNAs on proliferation and migration. Chan et al. show that miRNA-21 knock down leads to an activation of caspases and induces apoptosis in glioblastoma cells (30), while overexpression of miR-21 in myeloma cells inhibits programmed cell death (42). MiR-21 was also found to promote cell invasion and metastasis in colorectal cancer (45) and inhibition of miR-21 leads to decreased cell growth in vivo and in vitro (97).

For the overexpression of miR-21 there are three strategies: MiR-21 can be overexpressed as its mature form or as one of its precursors (pre- or primiR 21) (24). For this study, it was decided to overexpress primiR 21 constructs. Overexpression of miR-21 works in our study, even when we can only observe a 2.8 fold increase. In literature there is a study, which uses the expression of a part of the primiR 21 precursor loop, including the sequence of premiR 21 and variable flanking sites. Yue et al. utilize primiR 21 constructs with 400 bp upstream and 135 bp downstream or 160 bp upstream and 35 bp downstream flanking sequence and after transfection in 293FT cells mature miR-21 and primiR 21 levels were detected. They explored, that if premiR 21 has a short flanking sequence the expression of miR-21 is more efficient than it has a long flanking sequence (98).The reason why precursors are preferred to be used for overexpression is, that during miRNA biogenesis diverse factors are active which are important for the function of mature miRNA. For example microprocessors, which includes Drosha and DGCR8 and which are active at the first cleavage step of processing of primiR to premiR. They are integrated in signaling events from different pathways which results in changes of miRNA expression. There are also RNA-binding proteins which directly bind to the primiR and are so involved in the expression of miRNAs (99).

In our study for inhibition of miR-21 three different constructs were used: The first strategy are antisense oligonucleotides (pAdlox oligo eraser 21 under control of U6 promoter, a RNA polymerase III promoter), the second a miRNA sponge (pAdlox sponge eraser 21 under control of a CMV promoter, a RNA polymerase II promoter) and the third a shRNA against primiR 21 (pAdlox simiR 21). All constructs are expressed via the adenoviral system in glioblastoma cell line (U373) and in NSCLC-derived cell line (A427) and the efficiency of the constructs in both cell lines was

compared. No modulation of miR-21 in A427 can be found, with none of the three erasers. In U373 cells the sponge construct leads to a significant decrease of miR-21 levels.

Antisense oligonucleotides were the first tool to modulate miRNA expression and their functionality was proven in several studies. Studies demonstrated that 2'-O-methyl oligonucleotides silence the activity of miRNAs in vivo as well as in vitro (100) (101). LNA-modified oligonucleotides are able to inhibit endogenous miRNA levels in Drosophila cells as well as exogenous introduced miRNAs (102). Krützfeld et al. used so called "antagomiRs", oligonucleotides with a phosphorothioate backbone and modification at the 3'end as well as 2'-O-methyl modification to inactivate miRNA functions in vivo (103). Modification leads to induced and increased efficiency of inhibition, but is maybe not necessary to inhibit miRNAs. In our study pAdlox oligo eraser-21 fails to inhibit miR-21 in both cell lines. Additionally Spry1 and Spry2, as examples for miR-21 substrates are unaffected, indicating that either we looked at the wrong substrates or this method does not work in our experiments.

Usage of miRNA sponges, a new strategy to modulate miR-21 expression, shows several advantages compared to antisense oligonucleotides. Sponges are often associated with reporter genes like GFP or luciferase, so it is easy to select or screen for cells which express sponge (104). Furthermore, regulatory elements can be integrated in the promoter of the sponge, so that the constructs are drug inducible and tissue specific (104), while antisense oligonucleotides which are injected into mice, are not expressed constantly through tissue and mostly accumulate in the liver (103). Sponges are shown by Ebert *et al.* to inhibit all tested miRNAs (miR-15, miR-18, miR-20, miR-21 and miR-30) more effective in comparison to antisense oligonucleotides which were transfected in the same concentration in 293T cells (26). The sponge construct, used in our study works only in glioblastoma cell line U373, cells which have high endogenous miR-21 expression. The experiment in A427 fails, maybe because this cell line expresses only basal levels of miR-21 and for this reason sponge eraser cannot work.

As mentioned before sponges have multiple binding sites for their target miRNA. These sites can show perfect basepairing or some mismatches, so called buggle sites at position 9-12. The sponges with buggle sites are demonstrated in several

studies to be more effective than sponges with perfectly match (26) (105) (106). Kluiver et al. performed GFP competition assays which show that miR-19 sponges with bugled binding sites inhibit the target miRNA more effective compared to sponges without mismatch (107). This can be caused by a more stable interaction with the target miRNA or by the degradation of sponge as a result of the cleavage activity of AGO2 (108) (109). Nevertheless there are examples in literature which show that sponges with total complementary work too (47) (110) (111).

Additional to the factors described before, the number of binding sites is important for the function of a sponge eraser. More binding sites can maximize the inhibitory function of the eraser and can increase the degradation of the sponge transcript (112). But there are also limited effects as shown from Kluvier et al. (107). MiR-19 sponges with 6 repeating binding sites in murine cells inhibit miRNA at the same grade as a sponge with more repeats. This indicates that there is a sufficient level of sponges for maximal inhibition, which can be dependent on the level of endogenous miRNA and their binding activity to sponges. We detected also a dosage effect in our study: the maximum decrease of miR-21 due to the inhibitory effect of the sponge construct can be found in the middle concentration of the virus. When cells get infected with the highest amounts of virus, miR-21 expression is increased compared to levels at the middle expression but still significantly decreased in comparison with control cells.
We used sponge constructs with a total complementary and two binding sites in this study. For further experiments, to improve the function of pAdlox sponge eraser 21 it will be necessary to construct a new eraser construct, with more than two binding sites and sequences which shows not totally complementary to miR-21.

With exception of the study from Sayed et al. who introduce miR-21 sponges via viral vectors into cells (47), all studies do not show decrease of the target miRNA, they only investigate the expression of reporter genes or of target genes of the miRNA of interest. Due to this fact, at the start of our experiments we cannot exclude, that sponge erasers only work at the protein level of cells. Therefore we performed, beside Northern blots to detect miR-21 levels, Western blots of Spry1 and Spry2, confirmed targets of miR-21. Interestingly we can observe a decrease in miR-21 expression after infection of cells with pAdlox sponge eraser 21, but Spry1 and Spry2 levels are unaffected.

Another strategy to inhibit miRNAs that we used is a RNAi method, so called siRNAs or shRNAs. SimiR includes the sequence of primiR of the target miRNA, so that they can work in the nucleus, where miRNA processing takes place. A long time period it was thought that RNAi methods are useless to inhibit miRNAs, due to the fact that siRNAs are mainly active in the cytoplasm. But since hairpin siRNA can be expressed, RNAi can be a successful strategy to modulate miRNA levels by influence the processing of the precursors to mature miRNA (25). In the experiments shown in this study, siRNA-21with the sequence we choose does not work. Possibly inhibition of miR-21 by RNAi tools which are active in the nucleus, does not work so far, but there is also the chance that another sequence can lead to inhibition of miR-21 expression.

Further investigations of the influence of modulation of miR-21 on proliferation will be helpful to understand the oncogenic action of this specific oncomiR and to evaluate the potential of miR-21 as an anticancer- therapeutic.

6 ABBREVIATIONS

AGO	Argonaut protein
Amp	Ampicilin
AP-1	Activator protein1
APS	Ammoniumpersulfate
ATP	Adenosin-5‘-triphosphate
Bcl-2	B-cell lymphoma 2
BDNF	Brain-Derived Neurotrophic Factor
bp	basepair(s)
c-Cbl	Casitas B-lineage-lymphoma
CFP	cyan fluorescent protein
CMV	cytomegalovirus
Dcp1	mRNA-decapping enzyme 1
ddH2O	double-distilled water
DepC- ddH2O	Diethylpyrocarbonate double-distilled water
DGCR8	DiGeorge syndrome critical region gene 8
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTPs	deoxy-nucleotide tri-phosphates
EGF/EGFR	Epidermal Growth Factor/ Epidermal Growth Factor Receptor
Erk	extracellular-signal-regulated kinase
FCS	Fetal calf serum
FGF	Fibroblast Growth Factor
FHIT	Fragile histidine triade
GDNF	Glial cell line-Derived Neurotrophic Factor
GFP	green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine-5'-triphosphate
HGF	Hepatocyte Growth Factor
HRP	horseradish peroxidase
Hrs	Hepatocyte growth factor-regulated tyrosine substrate
Igf	Insulin-like growth factor

LB	Lysogeny Broth
MAPK	Mitogen-activated protein kinase
MCS	multiple cloning sites
min	minute(s)
miR-21	microRNA-21
miRISCs	miRNA-induced silencing complexes
miR-mask	miR-masking antisense oligonucleotides technology
miRNA	microRNA
MOPS	N-morpholino propanesulfonic acid
mRNA	messanger RNA
NGF	Nerve Growth Factor
NSCLC	Non small cell lung cancer
nt	nucleotides
o/n	over night
OD	Optical density
PAGE	polyacrylamide gel electrophorese
PBS	Phosphate buffered saline
PCI	Phenol-Chloroform-Isoamylalkohol
PCR	Polymerase chain reaction
PDCD4	Programmed Cell Death4
PDGF	Platelet-Derived Growth Factor
PLC	Phospholipase C
PNK	Polynucleotidkinase
premiR	precursor miRNA
primiR	primary transcript miRNA
PTB	phosphotyrosine-binding domain
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
RBD	Raf-1 binding domain
RNAi	RNA interference
RNP	Ribonucleoprotein complexes
rpm	rounds per minute
RTK	Receptor tyrosine kinase
SAP	Shrimp Alkaline Phosphatase

SCF	Stem cell factor
SCLC	Small cell lung cancer
SDS	Sodium dodecylsulfate
sec	second(s)
SH2	Src homology 2
siRNA	small interfering RNA
SN	Supernatant
Sos	son of sevenless
Spry	Sprouty proteins
STAT3	Signal transducers and activator of transcription3
STETL	Sucrose –Triton–EDTA –Tris–HCl–Lysozyme
T/E	Trypsin/ EDTA
TEMED	Tetramethylethylenediamine
TGF-β1	Transforming growth factor-β1
TMEM49	Transmembrane protein 49
TRBP	human immunodeficiency virus transactivating response RNA-binding protein
UTR	untranslated region
VEGF	vascular endothelial growth factor
wt	wildetype
Xrn 1	5'-3' exoribonuclease 1

7 LIST OF FIGURES

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9 REFERENCES

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10 APPENDIX

10.1 Primer sequences

U6 promotor

NAME	SEQUENCE 5'- 3'
U6 promotor sense	AGCTCTCGAGCGGTGTTTCGTCCTTT
U6 promotor antisense	AGCTGTCGACAAGGTCGGGCAGGAAGAGGG

simiR 21

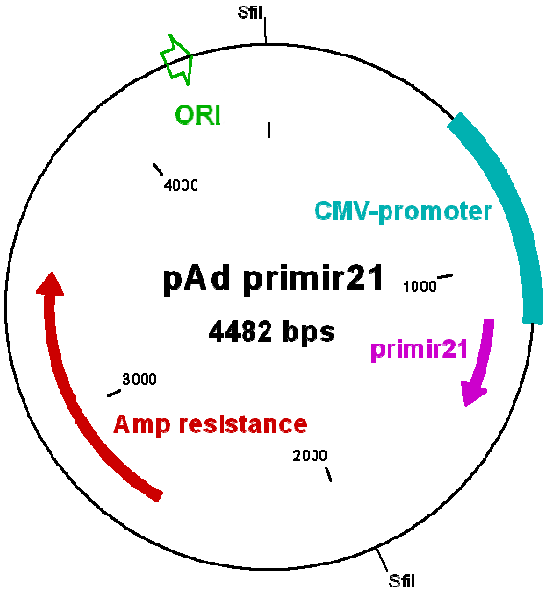
NAME	SEQUENCE 5'- 3'
simiR 21 sense	GATCAAAAAAAAGTTCAGCTATGGTAAGAGCCTTGGTTTAT CAAGCTTCATAAACCAAGGCTCTTACCATAGCTGAACC
simiR 21 antisense	TCGAGTTCAGCTATGGTAAGAGCCTTGGTTTATGAAGCTTG ATAAACCAAGGCTCTTACCATAGCTGAACTTTTTTTTG

10.2 Probes

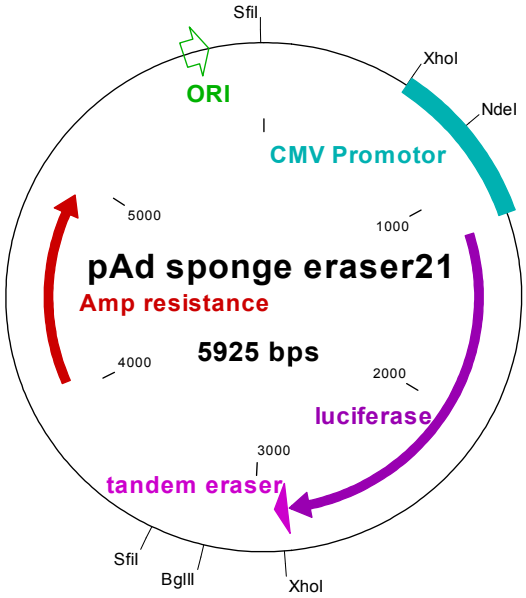
NAME	SEQUENCE 3'-5'
miR-21	TCAACATCAGTCTGATAAGCTA
U6 (probe for HomosapiensU6smallnuclear2 RNA (RNU6-2)):	CACGAATTTGCGTGTCATCCTT
5srRNA	ATTCCCAGGCGGTCTCCCATCC

10.3 Vector maps

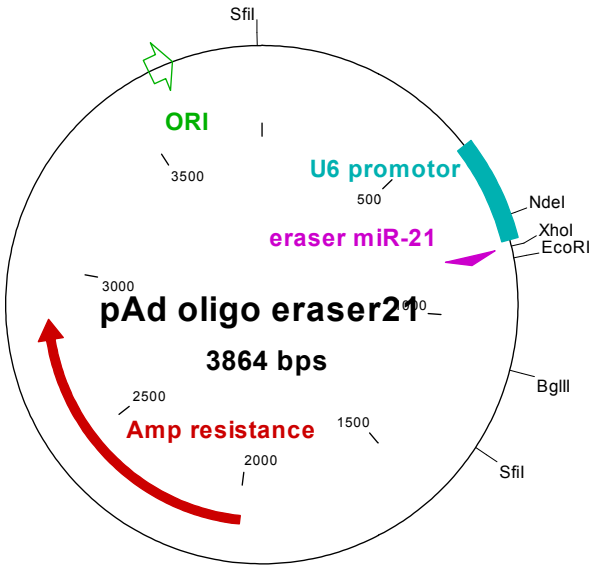
pAdlox primiR 21



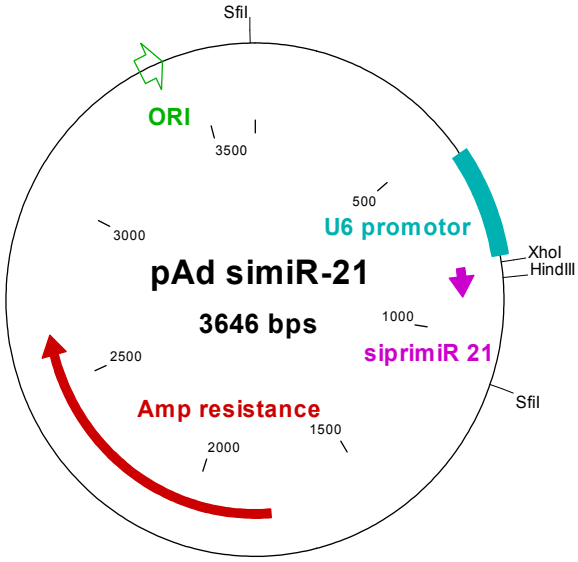
pAdlox sponge eraser 21



pAdlox oligo eraser 21



pAdlox simiR 21



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