Experimental evaluation of gene silencing as potential therapeutic option in the treatment of multiresistant Non-small-cell lung cancer

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät der
Eberhard Karls Universität
zu Tübingen

vorgelegt von
Mircea-Gabriel Stoleriu

2014
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Mircea-Gabriel Stoleriu

2014
Dekan: Professor Dr. I. B. Autenrieth

1. Berichterstatter: Privatdozent Dr. T. Walker
2. Berichterstatter: Professor Dr. J. Meier
„I have no special talents. I am only passionately curious.”

A. Einstein
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A-549</td>
<td>adenocarcinomic human alveolar basal epithelial cells</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADH</td>
<td>antidiuretic hormone</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>AKT/PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase (ALK) also known as ALK tyrosine kinase receptor or CD246</td>
</tr>
<tr>
<td>Anti Hu</td>
<td>antibodies against intracellular neuronal antigens, associated with small-cell carcinoma of the lung</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>BIRC5</td>
<td>baculoviral inhibitor of apoptosis repeat-containing 5</td>
</tr>
<tr>
<td>BRAF</td>
<td>murine sarcoma viral oncogene homolog B1</td>
</tr>
<tr>
<td>c-fos</td>
<td>c-Fos is a protein encoded by the FOS gene</td>
</tr>
<tr>
<td>c-myc</td>
<td>(myelocytomatosis) regulator gene that codes for a transcription factor</td>
</tr>
<tr>
<td>c-MET</td>
<td>proto-oncogene that encodes a protein known as hepatocyte growth factor receptor (HGFR)</td>
</tr>
<tr>
<td>CCNI</td>
<td>cyclin-I</td>
</tr>
<tr>
<td>CDK1</td>
<td>cyclin-dependent kinase 1</td>
</tr>
<tr>
<td>Chk1</td>
<td>serine/threonine-protein kinase Chk1, an enzyme that in humans is encoded by the CHEK1 gene</td>
</tr>
<tr>
<td>CK 2 alpha</td>
<td>casein kinase 2 alpha</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin/ Doxil</td>
</tr>
<tr>
<td>DDP /CDDP</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco PBS (phosphate buffered saline)</td>
</tr>
<tr>
<td>DSMZ</td>
<td>German Collection of Microorganisms and Cell Cultures</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded ribonucleic acid</td>
</tr>
</tbody>
</table>
E2F1 transcription factor E2F1 is a protein that in humans is encoded by the E2F1 gene

EDTA Ethylenediaminetetraacetic acid

EGFR epidermal growth factor receptor

EGFR/HER 2 epidermal growth factor receptor/ Human Epidermal Growth Factor Receptor 2

EIF3B subunit B of eukaryotic translation initiation Factor 3

EML4ALK echinoderm microtubule-associated protein-like 4 (EML4) gene fused to the anaplastic lymphoma kinase (ALK) gene

ERBB2 receptor tyrosine-protein kinase erbB-2

ERBB3 receptor tyrosine-protein kinase erbB-3

ERCC1 expression of the DNA repair enzymes: excision repair cross-complementation group 1

ESCs embryonic stem cells

FACS® fluorescence activated cell sorting

FCS conditioned fetal calf serum

FHIT bis(5’-adenosyl)-triphosphatase/ fragile histidine triad Protein

FITC Fluorescein isothiocyanate

FoxM1 mammalian forkhead box transcription factor 1

FRS2 fibroblast growth factor receptor substrate 2

FUS1 fused in sarcoma (RNA-binding protein)

5FU 5-fluorouracil

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

HCC hepato-carcinoma

Her-2/neu human epidermal growth factor receptor 2

HGF tumor-derived hepatocyte growth factor

HIF 1 hypoxia-inducible factor 1

HIF 2 hypoxia-inducible factor 2

HIV human immunodeficiency virus

HIV-PR 1 HIV-1 protease

HKI 272 (Neratinib) irreversible tyrosine kinase inhibitor for HER2 and EGFR, respectively
HMGB1-RAGE  high-mobility group protein B1- receptor for advanced glycation endproducts
HSP 90  heat shock protein HSP 90
IAP  inhibitor of apoptosis
IGF  insulin-like growth factor
K-ras  Kirsten rat sarcoma viral oncogene homolog
KEAP1  Kelch-like ECH-associated protein 1
LKB1 (STK11)  liver kinase B1 (serine/threonine kinase 11)
LRF  leukemia/lymphoma-related factor
LRRK2  leucine-rich repeat kinase 2
LZTS1  leucine zipper putative tumor suppressor 1
MADS  box superfamily of transcription factors MCM1, Agamous, Deficiens, and SRF (serum response factor)
MAPK  mitogen-activated protein kinase pathway
MDR1 / ABCB1  human multi-drug-resistant gene, or ATP-binding cassette sub-family B member 1 (ABCB1) or cluster of differentiation 243
MDX-214  Anti-EGFr/CD89 Antibody
MEK 1  dual specificity mitogen-activated protein kinase kinase 1
MEK 2  dual specificity mitogen-activated protein kinase kinase 2
mRNA  messenger RNA
MRP1  human multidrug-resistance protein 1
mTOR  mammalian target of rapamycin (mTOR) also known as mechanistic target of rapamycin
MT-ND2  mitochondrial complex-I subunit ND2 (NADH-ubiquinone oxidoreductase chain 2)
NF-κB  nuclear factor-κB
NOTCH1  notch homolog 1, translocation-associated (Drosophila)
NRF2  nuclear factor (erythroid-derived 2)-like 2
NSCLC  Non–small-cell lung cancer
Oct-4  octamer-binding transcription factor 4
pAkt / Pk B  protein kinase B
P16/ CDKN2A, p16\textsuperscript{\textsubscript{ink4A}}  cyclin-dependent kinase inhibitor 2A
P  (GW) Bodies  processing bodies
PBS phosphate buffered saline
PCR polymerase chain reaction
PDX-1 pancreatic and duodenal homeobox-1
PET CT positron emission tomography - computed tomography
PFA Paraformaldehyde
P-gp / MDR1 P-glycoprotein / permeability glycoprotein
PI3K phosphatidylinositol 3-kinase
PIK3CA class I PI 3-kinase / phosphatidylinositol 3-kinase
PKC alpha protein kinase C alpha
PLGA poly(lactic-co-glycolic acid)
PTEN phostatase and tensin homolog (PTEN) is a protein encoded by the PTEN gene
PTHrP parathyroid hormone-related protein
qRT-PCR Real Time quantitative Reverse Transcription PCR
R2D2 partner protein for Dicer enzyme
RAC 1 ras-related C3 botulinum toxin substrate 1
RAGE receptor for Advanced Glycation Endproducts
RARß retinoic acid receptor beta (RAR-beta)
RASSF1A ras association (RalGDS/AF-6) domain family member 1
RB1 retinoblastoma protein
RdRP RNA-dependent RNA polymerase
RISC RNA-induced silencing complex
RNAi RNA interference
RNR ribonucleotide reductase
ROS 1 proto-oncogene tyrosine-protein kinase encoded by ROS1 gene
RRM1 ribonucleotide reductase regulatory subunit M1
SCLC small cell lung cancer
SCR scrambled siRNA
SEMA3B semaphorin-3B
SEMA3F semaphorin-3F
shRNA small hairpin RNA or short hairpin RNA
shSTAT3 small hairpin signal transducer and activator of transcription 3
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SIADH</td>
<td>syndrome of inappropriate antidiuretic hormone secretion</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA /short interfering RNA/ silencing RNA</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppression of cytokine signaling</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor (c-fos serum response element-binding transcription factor)</td>
</tr>
<tr>
<td>SRFDelta5</td>
<td>serum response factor Delta5 protein</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded ribonucleic acid</td>
</tr>
<tr>
<td>STAT-3</td>
<td>signal transducer and activator of transcription/ signal transduction and transcription</td>
</tr>
<tr>
<td>Survivin/ BIRC5</td>
<td>baculoviral inhibitor of apoptosis repeat-containing 5</td>
</tr>
<tr>
<td>Tau</td>
<td>family of microtubule stabilizing proteins</td>
</tr>
<tr>
<td>TC21/R-Ras2</td>
<td>ras-related protein R-Ras2</td>
</tr>
<tr>
<td>TGF-beta1</td>
<td>tumor growth factor beta1</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine-kinase inhibitor</td>
</tr>
<tr>
<td>TLR7</td>
<td>Toll-like receptor 7</td>
</tr>
<tr>
<td>TLR8</td>
<td>Toll-like receptor 8</td>
</tr>
<tr>
<td>TNF alpha</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNM</td>
<td>tumor-node-metastasis</td>
</tr>
<tr>
<td>TNS</td>
<td>Trypsin Neutralization Solution</td>
</tr>
<tr>
<td>TP 53</td>
<td>tumor protein 53</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
1. Introduction

According to observational epidemiological studies, cancer is the second leading cause of death in developed countries after cardiovascular diseases [1], [2] and represents a major public health problem worldwide.

In terms of incidence and mortality, lung cancer is the most common cancer worldwide, in both men and women [3].

Because of the poor prognosis of this type of cancer (only 15.7% of patients survive more than five years) [3], it is very important to understand the risk factors, pathogenesis and treatment of this disease.

In most cases, lung cancer develops silently and asymptptomatically. Therefore only in rare cases can it be treated at an early stage. Consequently, the tumor mass continues to progress until it interferes with vital processes and functions in the lungs and in metastasized organs. In these situations, most therapeutic options are ineffective and treatment remains strictly palliative [4],[5].

Therefore, early diagnosis, effective treatment and adherence to the treatment are the most important goals to prevent lung cancer evolution in the advanced stages.

Because most cases are diagnosed late, research efforts in this area of cancer are divided not only into prevention and early detection, but also into new therapeutic strategies for the advanced stages.

According to histological classification, there are two forms of lung cancer. The first form, Small-cell lung cancer (SCLC), includes only 12.95% of all patients and is associated with the worst prognosis of all types of lung cancer [6], [7].

The second category, Non-small-cell lung cancer (NSCLC), comprising 85% of all cases [8], includes various histological subtypes (squamous cell carcinoma, large cell carcinoma and adenocarcinoma).

Due to the high incidence of this type of lung cancer, we proposed in our study to present a role for siRNA as a potential therapeutic option for the treatment of advanced NSCLC with chemoresistant cell lines.

Generally, treatment can be curative when the disease is diagnosed at an early stage [9], but most patients benefit from a multimodal therapy based on chemotherapy, radiotherapy and surgery, even if their initial surgery is potentially curative [10], [11].

For patients diagnosed late, chemotherapy and radiotherapy remain the main therapeutic options. The response of the tumor cells to different therapeutic agents is a widely studied topic. Some authors consider that the resistance to current
chemotherapeutics (e.g. Gefitinib) represents one of the most significant barriers to improving long-term outcomes for this vulnerable patient group [12].

1.1. Aim
Due to the poor prognosis of lung cancer, we consider it absolutely essential to discover and study new drugs with beneficial effects in cancer treatment. On the other hand, the increased chemoresistance encountered in the last decade and the impact of siRNA in the treatment of lung cancer are certainly two topics still insufficiently researched. Therefore, the aim of our work is to emphasize the effect of siRNA silencing of six crucial molecules involved in the pathogenesis of lung cancer (Survivin, E2F1, HIF 1, HIF 2, STAT 3 and SRF) and demonstrate that siRNA is a promising alternative in the treatment of chemoresistant NSCLC. To understand the pathways involved and the effect of siRNA on the tumor cells, we will first emphasize some information about the pathogenesis, epidemiology and risk factors associated with this type of cancer.

1.2. Lung cancer
1.2.1. Definition
According to the formal definition of the National Cancer Institute, lung cancer represents a disease characterized by uncontrolled cell growth in tissues of the lung, usually in the cells lining air passages (bronchi, bronchioles and alveoli).

1.2.2. Risk factors
Risk factors include cigarette smoking, occupational exposure to various toxic agents (Arsenic, Nickel, Polycyclic aromatic hydrocarbons, Radon), radiation therapy, chronic obstructive pulmonary disease and a positive family history. Cigarette smoking is the most important cause of lung cancer, accounting for about 85% of cases. Smoking-associated lung cancer risk differs according to age, smoking intensity and smoking duration [13], [3]. Interestingly, even nonsmokers can develop lung cancer; several mutations detected in the epidermal growth factor gene (EGFR) [14] appear to be the responsible agents in these situations.
1.2.3. Etiology
The hypothesis that lung cancer has a genetic component is supported by two mechanisms. These two mechanisms are the activation of dominant oncogenes, on the one hand, and the inactivation of tumor suppressor or recessive oncogenes, on the other.

For example, mutation of the K-ras gene has a determinant role in adenocarcinoma of the lung [15], whereas overexpression of the EGFR protein or amplification of the EGFR gene has been found with an incidence rate of 26.3% [16]. Other oncogenes involved in this process are BRAF, PIK3CA, c-myc, bcl-2, Her-2/neu and ERBB3 [17], [18]. In addition, the inactivation of several tumor-suppressor genes such as TP53, RB1, RASSF1A, SEMA3B, SEMA3F, FUS1, p16, LKB1 (STK11), CDKN2A/B, RARß, and FHIT appear to be involved in lung cancer pathogenesis [19].

1.2.4. Epidemiology
According to international epidemiological data, lung cancer (15%) is the second most common type of cancer in men after prostate cancer (25%). In women, lung cancer is the second most common cause of cancer after breast cancer (26%). In both men and women, lung cancer represents the leading cause of cancer mortality worldwide [20], [21].

According to the German Cancer Research Institute, in 2008 lung cancer affected 34,000 men and 15,500 women, and approximately 29,500 men and 13,000 women died from it. Thus, lung cancer certainly represents the most common cause of cancer death in men (26%) and the third leading cause of cancer death among women (13%) in Germany.

Since 1990, the incidence of lung cancer has increased among women (by 30%), whereas the rate among men has steadily declined. In 2012 a very slight decrease in the incidence of lung cancer among men was noted (from 33,960 patents in 2008 to 33,700 patients in 2012). On the other hand, the number of lung cancer cases among women increased from 15570 to 17700 [21].

1.2.5. Classification
There are two methods of classifying lung cancer. The first is based on the histology of the tumor cells and the second is based on a mutational analysis.
1.2.5.1. Histological classification of lung cancers

According to the classification proposed by the National Cancer Institute, there are two types of lung cancer: Small-cell Lung Cancer (13–15%) and Non–small-cell Lung Cancer (85–87%) [3]. The second category includes Squamous cell or Epidermoid carcinoma (30–35%), Adenocarcinoma or Bronchoalveolar carcinoma (25–35%) and Large cell or Anaplastic carcinoma (10–15%).

I) Small-cell lung cancer is often centrally located (submucosa of airways/ primary and secondary bronchi, perihilar mass). The main risk factor for this type of cancer is smoking. This cancer has a rapid growth and is associated with a very early metastasis. In most cases (70–80%), the cancer already has peripheral filiae at presentation and thus treatment options are very limited (palliative intent). Therefore, when diagnosed late, Small-cell lung cancer has the worst prognosis of all types of lung cancer [6], [7]. According to histopathological data, the cancer cells contain different molecules (neuroendocrine hormones such as ACTH, Calcitonin, and ADH, and antibodies against muscle cells or neurons: Anti-Hu onconeural antibodies), which may explain some of the early signs and symptoms of Small-cell lung cancer [22].

When diagnosed early, Small-cell lung cancer is sensitive to chemotherapy (Etoposid/ Irinotecan/ Topotecan plus Carboplatin/ Cisplatin) and radiation therapy [23]. However, many studies find that radiotherapy and chemotherapy are effective only in the first years due to a rapid increase in chemoresistance, whereas surgery is ineffective or only slightly effective in the context of multimodal therapy [24].

II) Adenocarcinoma (25-35%) is commonly located peripherally. It is the most common form of cancer in nonsmokers and in women due to a mutation in the Epidermal growth factor receptor (EGFR) [3], [25]. Although adenocarcinoma evolves slowly compared to Small-cell lung cancer, it tends to form metastases widely at an early stage.

In early stages, the treatment options include surgery (pneumonectomy or lobectomy) with or without adjuvant chemotherapy [26], [27]. In late stages surgery is ineffective and therefore replaced with palliative chemotherapy or radiation therapy [27].
In our study, we demonstrate that siRNA silencing of various proteins involved in lung carcinogenesis (in particular in lung adenocarcinoma) can induce a decrease in tumor cell proliferation in chemoresistant adenocarcinoma cell lines treated previously with Gemcitabine, Vinflunine, Vinorelbine and Methotrexate.

III) Squamous cell carcinoma (30–35% of all lung cancers) is centrally located and commonly associated with different paraneoplastic syndromes. Most frequently, it can be identified by an ectopic production of parathyroid hormone-related protein (PTHrP), resulting in hypercalcemia. Major risk factors are smoking (85%) as well as environmental and occupational exposures to asbestos, radiation and radon (15%). Therapeutic strategies include surgery in the early stages and radiotherapy or chemotherapy in advanced stages [28].

IV) Large cell carcinoma is also located peripherally and represents 10–15% of all types of lung cancer. This form of carcinoma is associated with tobacco smoking over 30–40 years. This tumor is very aggressive and metastasizes in its early stages. Therefore, most epidemiological studies consider that the prognosis of large cell carcinoma is very poor, even in the early stages of the disease. The five year Survival Rate (all stages) is estimated at 11%. Because most therapeutic options including surgery, chemotherapy and radiation therapy are ineffective, novel therapeutic approaches in this area need to be established [29].

1.2.5.2 Molecular classification of lung cancer
Recently, a new classification scheme for lung cancer based on molecular heterogeneity and responsiveness to treatment has been proposed [30]. Each subtype is characterized by individual aberrations in various oncogenes/ tumor suppressor genes, and thereby each subtype has personalized treatment guidelines. There are nine molecular subtypes, including three primary and six secondary subtypes.

In the first category the most common pathway is the EGFR pathway. The three most important mutations are EGFR sensitizing mutations/ EGFR resistance mutations, K-ras mutations and EML4-ALK mutations. All these aberrations are common in adenocarcinoma cell lines. Treatment is represented by a combination of various chemotherapeutic agents (dual EGFR/ HER 2 TKI, c-MET inhibitors +/- 1st or 2nd
generation EGFR TKIs, Hsp90 inhibitors, dual MET/VEGFR 2 inhibitors or Chk1 inhibitors) [30].

The second category includes mutations in other six important oncogenes/tumor suppressor genes involved in lung carcinogenesis (c-MET, PI3KCA, PTEN, VEGFR, Bcl-2, ROS1, IGF). Each of these subtypes has an individualized treatment with c-MET inhibitors, dual Met/VEGFR2 inhibitors, ALK/MET inhibitors, PI3K, AKT, mTOR inhibitors, VEGFR inhibitors, BCL-2 inhibitors, ROS1 inhibitors, and IGF1R monoclonal antibodies, respectively [30].

1.2.6. Signs and symptoms
In 75% of all cases, lung cancer expresses a very aggressive clinical behavior. The most common symptoms include cough, dyspnea, wheezing, chest pain, hemoptysis, shoulder pain, dysphagia and recurrent infections. In advanced stages, neurological symptoms due to brain metastasis, pathologic fractures due to bone metastasis, as well as jaundice due to liver metastasis may occur. Furthermore, some endocrine paraneoplastic syndromes may occur due to the inappropriate secretion of various hormones (e.g. hypercalcemia, syndrome of inappropriate antidiuretic hormone secretion, hypertrophic pulmonary osteoarthropathy, migratory superficial thrombophlebitis, myasthenia).

1.2.7. Diagnosis and Staging
Because of the poor prognosis and aggressive clinical behavior of lung cancer, early diagnosis is crucial. According to the diagnostic scheme proposed by the German Cancer Society (S3 Guidelines, 2010), an accurate diagnosis includes the initial evaluation of the patient (patient history, physical examination) and laboratory testing (complete blood count, electrolytes, calcium, hepatic transaminases and alkaline phosphatase). In the follow-up, the diagnosis must be completed via chest x-ray, CT or PET–CT. If a mediastinal spread is suspected, cytopathology of pleural fluid or sputum, bronchoscopy-guided biopsy, fine-needle aspiration or open lung biopsy via thoracoscopy or mediastinoscopy are required. If the mediastinum is normal configured, thoracoscopy or mediastinoscopy are usually needed to confirm the diagnosis. Through these procedures a very accurate staging of lung cancer can be obtained. The actual edition of TNM classification is based on the AJCC Cancer
Staging Manual published in 2010 [31] and includes new criteria based on the prognosis and the efficiency of the treatment. Details are shown in the following table.


<table>
<thead>
<tr>
<th>TNM staging system for lung cancer 2010</th>
<th>Stage IA</th>
<th>Stage IB</th>
<th>Stage IIA</th>
<th>Stage IIIB</th>
<th>T4</th>
<th>Primary tumor (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1a</td>
<td>T1b</td>
<td>T2a</td>
<td>T2b</td>
<td>T3</td>
<td>T4</td>
<td></td>
</tr>
<tr>
<td>&lt;2cm / &gt;2cm, &lt;3cm</td>
<td>&gt;3cm / &lt;5cm</td>
<td>&gt;5cm / &lt;7cm</td>
<td>&gt;7cm</td>
<td>Any</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No invasion proximal to lobar bronchus</td>
<td>Main bronchus (&gt;2cm, distal to the carina)</td>
<td>Main bronchus (&lt;2cm, distal to the carina)</td>
<td>-</td>
<td>b. Endobronchial Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surrounded by lung or visceral pleura</td>
<td>Visceral pleura</td>
<td>Chest wall/ diaphragm/ mediastinal pleura/ parietal pericardium</td>
<td>Mediastinum/ trachea/ Heart / great vessels / esophagus/ vertebral body / carina</td>
<td>c. Local invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atelectasis/ obstructive pneumonitis that extends to the hilar region but does not involve the entire lung</td>
<td>Atelectasis/ obstructive pneumonitis of entire lung; separate tumor nodule(s) in ipsilateral primary tumor lobe</td>
<td>Separate tumor nodule(s) within the ipsilateral lung but different lobe as the primary mass</td>
<td>d. Other</td>
<td></td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>TNM Staging 2010</th>
<th>Supraclavicular</th>
<th>Scalene</th>
<th>Contra mediastinal</th>
<th>Lpsi mediastinal</th>
<th>Sub Carinal</th>
<th>Contra hilar</th>
<th>Ipsi hilar</th>
<th>Peri Bronchial (ipsilateral)</th>
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<tbody>
<tr>
<td>N0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>N1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Metastatic (M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1a:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>local intrathoracic spread</td>
</tr>
<tr>
<td>M1b:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Disseminated (extrathoracic) disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Malignant pleural/ pericardial effusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Separate tumor nodule(s) in the contralateral lung</td>
</tr>
</tbody>
</table>

7
1.2.8. Therapy

1.2.8.1. Algorithm for Therapy of Non-small-cell lung cancer

The therapeutic strategies for Non-small-cell lung cancer depend on the histological and molecular type of cancer. According to the S3 Guidelines for NSCLC, surgery with or without adjuvant chemotherapy is the first-line therapy in early stages (Stage I and II). For stage IIIA there are many therapeutic options (surgery with or without adjuvant therapy or concurrent chemotherapy/ radiation therapy, surgery with chemotherapy plus radiation therapy, chemotherapy with surgery, or chemotherapy plus radiation therapy). For stage IIIB, surgery remains ineffective. In this situation, radiation therapy with or without chemotherapy represents the first line therapy. For stage IV, platinum derivatives in combination with the best supportive care remain the recommended therapy [32].

Table 2: Algorithm for Therapy of Non-small-cell lung cancer (modified after S3 Guidelines for Lung cancer published by German Respiratory Society and German Cancer Society) [33].

<table>
<thead>
<tr>
<th>Stage</th>
<th>Standard Management</th>
<th>Therapeutic alternatives</th>
<th>Survival rate</th>
</tr>
</thead>
</table>
| IA and IB | Surgical Resection | Adjuvant therapy (chemotherapy/ radiation or Chemoradiotherapy) | Stage I A: 75% 
Stage I B: 55% |
| II A and IIB | Surgical Resection | Adjuvant therapy (chemotherapy/ radiation or Chemoradiotherapy) | Stage II A: 50% 
Stage II B: 40% |
| IIIA | Chemoradiotherapy (and) Surgical Resection in selected patients | Neoadjuvant combined modality: Therapy to downstage primary tumor | Stage III A:10-35% |
| IIIB | Chemoradiotherapy | Neoadjuvant combined modality: Therapy to downstage primary tumor | Stage III B: 5% |
| IV | Chemotherapy (Cisplatin) Surgical resection if solitary metastatic lesion with resectable primary tumor | More efficacious single-agent and combination chemotherapy | Stage IV: <5% |

As presented in this table, radiotherapy can be given before and after surgery with curative intent or in advanced stages with palliative intent. The actual regimens must be given for a period of six weeks. Nowadays, the common procedures include External beam radiation therapy (2D, 3D, Intensity Modulated Radiation Therapy or stereotactic radiation therapy in clinical trials) as well
internal radiation (brachytherapy) [34],[35]. The same study showed that the proportion of NSCLC cases that ever require radiotherapy is stage dependent (Stage I: 41.0% +/- 5.5%; Stage II: 54.5% +/- 6.5%; Stage III: 83.5% +/- 10.6%; Stage IV 65.7% +/- 7.6%) [34].

A recent study demonstrated the beneficial role of adjuvant radiotherapy after radical surgical treatment for metastatic lung cancer (N2), leading to a statistically significant increase in 5-year overall survival from 14.7 to 19.7% [36].

Another crucial component in the treatment of NSCLC is chemotherapy. The drugs commonly used are platinum derivatives (Cisplatin, Carboplatin), anthracycline antibiotics (Adriamycine), taxanes (Paclitaxel, Docetaxel), vinca alkaloids (Vinorelbine, Vincristine), nitrogen alkylating agents (Cyclophosphamide), glycosid derivatives (Etoposid) or semisynthetic analogues (Irinotecan, Topotecan) [37].

1.2.8.2. Novel regimens for the management of advanced NSCLC

Recent studies emphasize the beneficial role of monoclonal antibodies as personalized treatment according to the classification of NSCLC into nine molecular subtypes. The most common monoclonal antibodies used are Cetuximab (EGFR), Panitumumab (EGFR), Matuzumab (EGFR), Pertuzumab (EGFR-ERB B2), MDX 214 (EGFR), Trastuzumab (Her 2), Bevacizumab (VEGF), Erlotinib (EGFR-TK), Gefitinib (EGFR-TK), Lapatinib (EGFR, ERB B2 -TK), Canertinib (EGFR, ERB B2, ERB B3 -TK), and HKI 272 (EGFR, ERB B2 -TK).

Table 3: Novel agents/ regimens under development for the management of advanced NSCLC.

<p>| Novel agents/ regimens under development for the management of advanced NSCLC (2013) |</p>
<table>
<thead>
<tr>
<th>Agent</th>
<th>Class</th>
<th>Mechanism</th>
<th>Phase of study</th>
<th>References/ study identifiers</th>
<th>Publication Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amrubicin</td>
<td>Synthetic anthracycline</td>
<td>inhibitor of topoisomerase II</td>
<td>II</td>
<td>Harada T. et al [38]</td>
<td>2013</td>
</tr>
<tr>
<td>Calcitriol</td>
<td>Vitamin D</td>
<td>Calcium metabolism</td>
<td>I/II</td>
<td>Ramnath N. et al [39]</td>
<td>2013</td>
</tr>
</tbody>
</table>
1.2.8.3. Chemoresistance in NSCLC

As presented in the S3 Guidelines of NSCLC, the current therapeutic regimen is nowadays an individualized, multidisciplinary concept. In addition, chemotherapy represents an important component of treatment for all stages of the disease [37].

According to epidemiological studies, more than half of patients (55%) have stage IV at diagnosis [37].

According to the S3 Guidelines, for most of these patients, chemotherapy becomes the gold standard. As presented in the following table, for the majority of the patients diagnosed in advanced stages, chemoresistance might represent the most important obstacle to an effective treatment.

Table 4: Prevalence of extreme chemotherapy resistance in vitro in resected NSCLC (Modified after D’Amato TA et al, Ann Thorac Surg 2006, [45])

<table>
<thead>
<tr>
<th>Chemotherapy agent</th>
<th>Prevalence of extreme chemoresistance of resected NSCLC in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboplatin</td>
<td>68%</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>63%</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>75%</td>
</tr>
<tr>
<td>Chemotherapeutic</td>
<td>Percentage</td>
</tr>
<tr>
<td>------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Etoposide</td>
<td>63%</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>72%</td>
</tr>
<tr>
<td>Vinorelbine (Navelbine)</td>
<td>42%</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>40%</td>
</tr>
<tr>
<td>Docetaxel (Taxotere)</td>
<td>52%</td>
</tr>
<tr>
<td>Topotecan</td>
<td>31%</td>
</tr>
</tbody>
</table>

A recent study shows that the chemotherapeutics utilized in the treatment of NSCLC could only modestly increase the overall survival (2 months per decade) and quality of life of the investigated patients because of an increase in chemoresistance [46]. The resistance can be acquired via alterations in drug influx or efflux, detoxification through glutathione conjugation, alterations in DNA repair capacity and cell cycle control [37], [47].

In addition, some studies consider that chemoresistance may be genetically conditioned; the major contributors are the multi-drug-resistant- MDR1 gene and multidrug-resistance protein 1- MRP1 [48]. According to the same author, P-glycoprotein (P-gp) in peripheral CD56+ cells can represent a predictive biomarker for the identification of chemoresistance in Non-small-cell lung cancer [48].

Mutations in mitochondrial Complex-I subunit ND2 (MT-ND2) as well as mutations in the NRF2 protein or Kelch-like ECH-associated protein 1 (KEAP1) were also found to be responsible for adaptive chemoresistance in the A549 Non-small-cell lung cancer cell line [49],[50]. There are four possible mechanisms involved in chemoresistance to cisplatin in NSCLC:

1. pathways preceding the binding of cisplatin to DNA (pre-target resistance),
2. pathways that directly relate to DNA-cisplatin adducts (on-target resistance),
3. mechanisms concerning the lethal signaling pathway(s) elicited by cisplatin-mediated DNA damage (post-target resistance)
4. mechanisms affecting molecular circuits that do not present obvious links with cisplatin-elicited signals (off-target resistance) [51].

As observed above, the causes of chemoresistance are multifactorial. To prevent chemoresistance it is particularly important to explore and propose new therapeutic alternatives (e.g. gene therapy, molecular therapy, radiotherapy), all of which should be embedded in an individualized treatment according to the molecular and genetic variant of the subtype of cancer on the one hand, and the clinical severity of the disease on the other.
The current trend is to promote a range of tumor biomarkers (beta III-tubulin, ERCC1, K-ras, RRM1, Tau) that can predict the tumor stage early and accurately [37], to identify new targets in the pathogenesis of lung cancer and to develop individual therapies based on the genetic pathways of chemoresistance (e.g. siRNA).

Due to the increased chemoresistance and consequent decrease in treatment effectiveness, it is absolutely necessary to find and study new therapeutic alternatives (e.g. siRNA). Recent studies emphasize the role of siRNA as a promising concept in the chemosensitization of various chemoresistant tumor cell lines (hepatocellular carcinoma [52], breast cancer [53], lung cancer [54], colorectal cancer [55]).

According to this concept, we described a potential therapeutic strategy for the treatment of chemoresistant lung cancer via siRNA silencing of six crucial molecules involved in lung carcinogenesis.

1.3. siRNA

1.3.1. Definition

Small interfering RNAs (siRNA), also referred to as short interfering RNA or silencing RNA, are double-stranded RNA molecules containing 20–25 nucleotides with a very important role in the RNA interference (RNAi) pathway [56]. RNA interference (RNAi) represents a process found in many eukaryotes that regulates the expression of specific genes with complementary nucleotide sequences. Its two most important roles are in innate immunity (against parasitic nucleotide sequences or various viruses) and in regulating gene expression [56], [57].

The discovery of its mechanism, structure and some possible therapeutic implications brought the researchers Andrew Fire (Professor of Pathology and Genetics at the Stanford University School of Medicine) and Craig C. Mello (Professor of Molecular Medicine at University of Massachusetts Medical School in Worcester) the Nobel Prize in Medicine in 2006 [58], [59].

1.3.2. Pathways

In eukaryotes, double-stranded RNA (dsRNA) molecules are cleaved into short fragments of ~20 nucleotides (siRNAs) by an endoribonuclease called Dicer [60]. The resulting siRNAs are composed of two single-stranded (ss) RNAs. Each ssRNA contains a passenger strand and a guide strand.
The passenger strand, also known as the anti-guide strand, has a thermodynamically stable 5' end, whereas the guide strand has a less thermodynamically stable 5' end. While the passenger strand is recognized by R2D2 protein and subsequently degraded, the guide strand plays a crucial role in post-transcriptional gene silencing [61].

After degradation of the passenger strand, the guide strand forms the RNA-induced silencing complex (RISC) [60]. The less thermodynamically stable 5' end of the guide strand is recognized by a catalytic protein called argonaute. This protein is located in specific regions of the cytoplasm called P-bodies or GW bodies and plays a critical role in transcriptional silencing [62], [63]. The guide strand incorporated in the RISC complex has the ability to recognize, bind (strong divalent cationic binding) and catalyze different complementary messenger RNA (mRNA) molecules via argonaute protein. As a result, a considerable reduction in protein translation and gene expression occurs. This mechanism is called the dsRNA cleavage pathway.

Figure 1: siRNA pathway.
1.3.3. Technological applications
siRNA is frequently used in experimental biology to knockdown a target gene and consequently to inhibit synthesis of the protein encoded by the target gene and its function. The RNA interference pathway may only knockdown the target genes (decrease the expression of the genes) and not knockout the genes, which indicates gene destruction and complete elimination of their functions [64].
More recently, due to advanced genetic engineering processes, siRNA molecules can be produced with a high affinity for the target gene, thereby reducing cross reactivity and off target effects [64].

1.3.4. Therapeutic strategies
I) Inhibition of oncogenesis in different types of cancer, e.g. lung cancer [65], hepatocellular carcinoma [66], colon cancer [67], pancreatic cancer [2], and mantle cell lymphoma [68]).
Experiments conducted in our laboratory generated promising results regarding the effect of siRNA in lung tumors using chemoresistant cell lines. The targets (SRF, E2F1, Survivin, STAT 3, HIF 1 and HIF 2) as well the primary mechanisms involved (specific siRNA gene silencing) will be discussed in the next Chapter (Material and Methods).

II) Innate immunity
One of the most important roles of siRNA is in antiviral defense. siRNA molecules can induce knockdown of different classes of viruses (Influenza A virus [69], Herpesvirus [70], Rotavirus [71], Hepatitis B and C virus, HIV, Dengue Virus, Coxsackievirus B3, and Metapneumovirus [72]). The inhibition of this wide range of viruses can be achieved both in vitro and in vivo [73]. Generally, the primary mechanism consists of precise identification of the target genes, followed by specific binding to the target receptor and strong suppression of key genes in viral replication.

III) Suppression of atherosclerosis in vein grafts and prevention of intravascular obliteration via siRNA eluting stents
Recent studies demonstrate that siRNA silencing of c-myc can inhibit endothelial cell proliferation [74], whereas the silencing of STAT 3 may attenuate neointimal formation in both in vivo and in vitro models [75], suggesting that early administration
of siRNA nanoparticles may be an effective approach to prevent vein graft restenosis and neointimal formation.

On the other side, recent studies have emphasized a role for siRNA eluting stents in preventing myocardial infarction. With an efficiency of up to 70% in vitro, this new siRNA stent model may be a very important platform for developing new minimally invasive technologies [76].

All the studies mentioned above emphasize the importance of target molecule selection. Once the target molecule is established, a combination of PCR, Flow-cytometry or Western Blotting can be used to determine the efficiency of the covalent binding between the siRNA and the target molecule, on the one hand, and the efficiency of siRNA silencing of the target gene, on the other.

In our study we will discuss the importance of six target molecules (SRF, E2F1, Survivin, HIF 1, HIF 2 and STAT 3) in lung adenocarcinoma using various chemoresistant cell lines.

1.4. Critical molecules involved in the carcinogenesis of NSCLC

1.4.1. Serum response factor (SRF)

SRF is a transcription factor that belongs to the MADS (MCM1, Agamous, Deficiens and SRF) superfamily and regulates apoptosis, cell growth and cell differentiation. The gene is located on chromosome 6 (Location: 6p21.1) and represents an important target for many pathways (e.g. the mitogen-activated protein kinase pathway/ MAPK). Therefore, SRF plays a critical role in cell cycle regulation. During embryogenesis, it plays a crucial role in developing mesoderm and thereby in the formation of the skeletal and muscle systems [77], [78].

SRF is also a very accurate nuclear repressor of tumor growth factor beta1 (TGF-beta1) and consequently an inhibitor of cell proliferation in different types of cancer: breast cancer [79], prostate cancer [80], lung cancer [81], hepatocellular carcinoma [82] and ovarian cancer [83].

1.4.2. E2F1

E2F1 transcription factor plays an important role in cell proliferation and regulation of the cell cycle as well as in the functioning of some tumor suppressor proteins [84]. E2F1 is a strong inducer of apoptosis in response to DNA damage [85], through its capacity to activate p53/p73 death pathways. New studies demonstrate that aberrant
E2F1 expression may be associated with carcinoma recurrence, metastasis and resistance to anti-neoplastic agents [86]. The same study demonstrates that the E2F1-p73/DNp73-miR-205 axis is a crucial mechanism for chemoresistance and, thus, a target for metastasis prevention.

Related to the involvement of E2F1 in the pathogenesis of lung cancer, Duan HY et al. demonstrated that this transcription factor may induce G2/ M Arrest and Apoptosis in the A549 and H1299 lung cell lines [87]. On the other side, E2F1 is a very potent promoter of cell differentiation and tumor growth in lung cancer; therefore, siRNA silencing of this gene has been related to effective suppression of tumor growth in NSCLC [81].

In addition, our study using various chemoresistant adenocarcinoma cell lines demonstrates that E2F1 might play a potential role in suppression of gene expression in multiresistant NSCLC.

1.4.3. Survivin

Survivin is a protein encoded by the BIRC 5 gene (baculoviral inhibitor of apoptosis repeat-containing 5) and is a member of the inhibitor of apoptosis (IAP) family. Inhibiting Survivin may suppress caspase activation and consequently decrease tumor cell differentiation and proliferation in the G2-M phase. There have been multiple demonstrations implicating this molecule in the inhibition of carcinogenesis by different mechanisms. As an oncogene, Survivin has been identified in 60 different human tumor lines [88]. Furthermore, downregulation of Survivin has been shown to play a crucial role in suppressing human hepatocellular carcinoma cells [89], breast cancer [90], hormone refractory prostate cancer [91], different oral cancer cell lines [92] and NSCLC [93], [81].

Most studies have emphasized that aberrant overexpression of Survivin may facilitate the acquisition of resistance to chemotherapeutic drugs. Thus, Survivin plays a crucial role in lung tumors in different chemoresistant cell lines. Recent experiments show that the silencing of Survivin sensitized H292 lung cancer cells in combination with Cisplatin therapy can efficiently inhibit angiogenesis, suppress tumor cell proliferation and consequently reduce tumor volume by approximately 83.13% [94]. Moreover, by knocking down AKT, CREB, Bcl-xL, Survivin and Bcl-2 molecules, a prompt reduction in NSCLC colony formation and an increase in the chemosensitivity of NSCLC can be achieved [95].
1.4.4. HIF1 and HIF 2 (Hypoxia-inducible factor)

HIF 1 and HIF 2 are two heterodimers that respond to changes in available oxygen in the cellular environment (e.g. hypoxia/ hyperoxia). As a response to hypoxia, the HIF family promotes the formation of blood vessels and consequently the formation of the vascular system in embryos and different types of tumors.

On the other side, HIF1 stimulates collagen production and bone development and thereby allows chondrocytes to maintain their function as professional secretory cells in the hypoxic growth plate [96]. Recent studies demonstrate that chronic intermittent hypoxia disrupts the balance between HIF-1-dependent pro-oxidant and HIF-2-dependent anti-oxidant activities, and this loss of redox homeostasis can facilitate the pathogenesis of autonomic morbidities [97].

Moreover, HIF may play an important role in hypoxia-regulated control of macrophages and, thus, in various inflammatory processes and tumor cell development.

Some clinical studies have demonstrated that downregulation of HIF 1 may stimulate TGF-β1 gene expression, thereby promoting crucial pathways in carcinogenesis (e.g. angiogenesis, recruitment of mesenchymal stem cells into hypoxic area of solid tumors and metastasis) [98],[99].

The role of HIF 1 and HIF 2 in carcinogenesis is discussed by various authors in different types of cancer: neuroblastoma [100], esophageal carcinoma [101], breast cancer [102], laryngeal carcinoma [103] and NSCLC [104].

1.4.5. STAT 3

STAT 3 (Signal transducer and activator of transcription 3) belongs to the STAT protein family and is an important transcription factor that plays a crucial role in cell growth and apoptosis. Interestingly, STAT 3 protein appears in early stages of cell development and may modulate the differentiation and growth of embryonic stem cells (ESCs).

Recently, the role of STAT 3 was demonstrated in inflammation [105]. The pathways invoked include new proinflammatory molecules (e.g. Resistin) that play an important role in the suppression of cytokine signaling (SOCS). Thus, siRNA silencing of STAT 3 might modulate the action of Resistin-induced SOCS, and consequently pro-inflammatory processes [106]. Recent studies demonstrate the role of STAT 3 in carcinogenesis. siRNA silencing of STAT 3 may induce G0/ G1 arrest and
subsequently an effective reduction in cell differentiation and cell proliferation in bladder cancer [107], hepato-carcinoma [108] and lung adenocarcinoma [109].

In our study, we demonstrate that siRNA specific silencing of STAT 3 can induce inhibition of gene expression in various A549 chemoresistant cell lines in vitro in a concentration-dependent manner.

Taken together, the aim of our study is to point out that silencing SRF, E2F1, Survivin, HIF 1, HIF 2 and STAT 3 can significantly decrease cell differentiation and tumor growth in multiresistant adenocarcinoma cell lines, thereby inducing effective results in the treatment of NSCLC. The mechanisms and pathways responsible for these effects will be presented in the next chapter (Material and Methods).
2. Materials and Methods
2.1. Cell culture and primary cell isolation

In order to reproduce the siRNA transfectability in vitro and to facilitate a similarity to in vivo situations, we examined the chemoresistance of four different chemotherapy agents on the A 549 cell lines (adenocarcinomic alveolar basal epithelial cells). This cell line, first described in 1972 by D. J. Giard, has many practical advantages. The cells grow as monolayer cells, may easily be suspended in a solution in vitro and contain high level of desaturated fatty acids, with a crucial role in the membrane stability [110]. The A 549 cell lines belonging to the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany) were cultured under standard conditions, at 37 °C, in a humidified atmosphere containing 5% CO₂.

Table 5: Description of A 549 cell line

<table>
<thead>
<tr>
<th>Name of cell line</th>
<th>Tissue-type</th>
<th>Manufacturer</th>
<th>Basal medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 549</td>
<td>Lung adenocarcinoma (adenocarcinomic alveolar basal epithelial cells)</td>
<td>University Medical Centre, Hematology Department, Tübingen, Germany DSMZ (Braunschweig, Germany)</td>
<td>DMEM-high glucose</td>
</tr>
</tbody>
</table>

In order to support the growth of adenocarcinoma cells, the cells were cultured in DMEM-high glucose medium (PAA, Cölbe, Germany).

Table 6: Description of culture medium

<table>
<thead>
<tr>
<th>Description of culture medium</th>
<th>Product code</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-high glucose medium</td>
<td>PAA E15-009</td>
<td>PAA Laboratories, Pasching, Austria PAA, Cölbe, Germany</td>
</tr>
</tbody>
</table>

The advantage of this medium is that it contains four times the concentration of amino acids and vitamins in comparison to other media. To ensure an optimal growth of the cells, the selected medium was supplemented with 100 U Penicillin (PAA, Cölbe, Germany), 20 µg Streptomycin (PAA, Cölbe, Germany), 2 nM L-Glutamine (PAA, Cölbe, Germany) and 10 % conditioned fetal bovine serum/FBS (PAA, Cölbe, Germany).
In order to develop the chemoresistance similar to in vivo situations, the A549 cells were treated repeatedly with low doses of the desired chemotherapeutics (Methotrexate 50 ng, Gemcitabine 100 ng, Vinflunine 500 ng, Vinorelbine 200 ng). Dead cells that responded to chemotherapy were removed from the medium, whereas the surviving cells were treated gradually with repeated low doses of the same agent, until they developed the desired chemoresistance against the agent. These chemoresistant cells were obtained with the courtesy of Mr. Prof. Martin Michaelis (University of Kent, Canterbury, UK) and Mr. Prof. Dr. Jindrich Cinatl (Department of Pediatric Cancer and Virus Research, Frankfurt am Main, Germany).

Table 7: Description of used materials

<table>
<thead>
<tr>
<th>Description of consumables</th>
<th>Product code</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,5 ml DNA LoBind Tube</td>
<td>0030 108.035</td>
<td>Eppendorf AG, Hamburg, Germany</td>
</tr>
<tr>
<td>1,5 ml DNA LoBind Tube</td>
<td>0030 108.051</td>
<td>Eppendorf AG, Hamburg, Germany</td>
</tr>
<tr>
<td>12 Well Plate</td>
<td>3512</td>
<td>Corning Incorporated Costar, New York, USA</td>
</tr>
<tr>
<td>15 ml Cellstar Tube (Falcon)</td>
<td>188271</td>
<td>Greiner Bio-One International AG, Kremsmünster, Germany</td>
</tr>
<tr>
<td>50 ml BD Falcon</td>
<td>352070</td>
<td>BD Biosciences, Heidelberg, Germany</td>
</tr>
<tr>
<td>Multiply pro Gefäß 0,2 ml</td>
<td>72.737.002</td>
<td>Sarsted AG&amp;Co, Nümbrecht, Germany</td>
</tr>
<tr>
<td>PCR microseal 'B'</td>
<td>MSB 1001</td>
<td>Bio Rad Laboratories, Munich, Germany</td>
</tr>
<tr>
<td>Safe Lock Tubes 1,5 ml</td>
<td>0030120.086</td>
<td>Eppendorf AG, Hamburg, Germany</td>
</tr>
<tr>
<td>Pipette tips Dualfilter, (PCR clean, steril)</td>
<td>0030077.512 0030077.539 0030077.547 0030077.571</td>
<td>Eppendorf AG, Hamburg, Germany</td>
</tr>
<tr>
<td>Twin.tec real time PCR plate 96</td>
<td>0030132.718</td>
<td>Eppendorf AG, Hamburg, Germany</td>
</tr>
</tbody>
</table>
2.2. Transfection of siRNAs

In order to demonstrate the effectiveness of siRNA treatment in comparison to the control group, 100,000 A549 cells were seeded 24 hours before transfection with specific siRNA in 12 well plates for qRT-PCR. Another 20,000 A549 cells were seeded 24 hours prior transfection to facilitate the CASY analysis.

Table 8: Recommended number of cells to seed for different culture formats 24 hours before transfection

<table>
<thead>
<tr>
<th>Culture format</th>
<th>Suggested number of adherent cells to seed (day before transfection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-well plate</td>
<td>4.0 – 8.0 x 10^4</td>
</tr>
<tr>
<td>12-well plate</td>
<td>0.8 – 2.0 x 10^5</td>
</tr>
<tr>
<td>6-well plate</td>
<td>1.5 – 4.0 x 10^5</td>
</tr>
</tbody>
</table>

Before transfection, media containing basal medium (PAA, Cölbe, Germany) interferin™ (Polyplus, Illkirch, France) and different concentrations of siRNA were prepared. For experiments using qRT PCR, the cells were treated with 300 µl of transfection medium containing 25 nM or 100 nM siRNA. For CASY analysis a siRNA concentration of 50 nM was used.

The interferin™ served as lipid cation in order to form the transfection complexes. This transfection method is based on lipofection, a highly efficient, lipid-mediated DNA-transfection procedure, which uses lipid cations as transporter molecules for the siRNA.

Table 9: Standard Concentrations for the production of siRNA transfection approach

<table>
<thead>
<tr>
<th>Concentration</th>
<th>siRNA (20 µM)</th>
<th>Interferin™</th>
<th>Basal medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 nM</td>
<td>0,4 µl</td>
<td>1,17 µl</td>
<td>318,0 µl</td>
</tr>
<tr>
<td>50 nM</td>
<td>0,8 µl</td>
<td>1,75 µl</td>
<td>317,5 µl</td>
</tr>
<tr>
<td>100 nM</td>
<td>1,6 µl</td>
<td>1,75 µl</td>
<td>316,7 µl</td>
</tr>
</tbody>
</table>
To facilitate the formation of transfection complexes, all reagents were incubated for 20 minutes at room temperature. Cells were incubated with the respective transfection medium for 2 hours at 37°C. Afterwards, the complexes were replaced by 1 ml fresh cell culture medium containing 100 U Penicillin (PAA, Cölbe, Germany), 20 µg Streptomycin (PAA, Cölbe, Germany), 2 mM L-Glutamine (PAA, Cölbe, Germany) and 10 % conditioned fetal bovine serum/ FBS (PAA, Cölbe, Germany).

To analyze the results, we have isolated and studied for each target molecule three different groups of cells:

Group I: non-siRNA or control nonsense-siRNA represented by chemoresistant A549 cells treated previously with Gemcitabine, Vinflunine, Vinorelbine and Methotrexate and transfected with nonsense-siRNA. Nonsense-siRNA was purchased from Qiagen (Hilden, Germany).

Figure 2: Clinical implementation of transfection procedure in our laboratory
According to the transfection protocol proposed by Qiagen, the transfection reagent enables a high transfection efficiency with low cytotoxicity in the presence of serum. On the other hand, Qiagen does not provide the sequence of their nonsilencing siRNAs, but ensures that they have no homology to any known mammalian gene. These nonsilencing siRNAs are validated by using Affymetrix GeneChip arrays as well a variety of cell-based assays and have been shown to ensure minimal nonspecific effects on gene expression and phenotype.

Group II: the siRNA control group, entitled also SCR-siRNA (scrambled), represented by chemoresistant A549 cell lines treated with nonspecific siRNA. In order to demonstrate that the transfection is concentration dependent, we have used two different concentrations (25 nM and 100 nM SCR-siRNA). The SCR-siRNA was provided from Qiagen (Hilden, Germany).

Group III: specific siRNA group, represented by chemoresistant A549 adenocarcinoma cells treated previously with Gemcitabine, Vinflunine, Vinorelbine or Methotrexate and transfected with specific siRNA targeting SRF, E2F1, Survivin, HIF 1, HIF 2 or STAT 3.

Table 10: Description of used reagents

<table>
<thead>
<tr>
<th>Description of used reagents</th>
<th>Product code</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000 U Penicillin/10000 µg Streptomycin (Pen/Strep)</td>
<td>P11-010</td>
<td>PAA Laboratories, Pasching, Austria; PAA, Cölbe, Germany</td>
</tr>
<tr>
<td>CASYton®</td>
<td>05651808001</td>
<td>Roche Diagnostics GmbH, Mannheim, Germany</td>
</tr>
<tr>
<td>FCS</td>
<td>10500-064</td>
<td>Gibco Life Technologies</td>
</tr>
<tr>
<td>Interferin/Interferin™</td>
<td>04INF1309F8</td>
<td>PEQ LAB, Erlangen, Germany; Polyplus, Illkirch, France</td>
</tr>
<tr>
<td>L-Glutamine (200 mM)</td>
<td>M11-004</td>
<td>PAA Laboratories, Pasching, Austria; PAA, Cölbe, Germany</td>
</tr>
<tr>
<td>Dulbecco’s PBS, 1x, with Ca²⁺/Mg²⁺</td>
<td>H15-001</td>
<td>PAA Laboratories, Pasching, Austria</td>
</tr>
<tr>
<td>TNS (0,05% Trypsin Inhibitor, 0,1% BSA)</td>
<td>C-41120</td>
<td>Promo Cell, Heidelberg, Germany</td>
</tr>
<tr>
<td>0,04% Trypsin / 0,03% EDTA</td>
<td>C-41020</td>
<td>Promo Cell, Heidelberg, Germany</td>
</tr>
</tbody>
</table>
2.3. siRNA sequences

In order to facilitate a high transfection efficiency, the specific targets were characterized by a sense sequence and an antisense sequence, as it follows:

I) E2F1-siRNA (validated by Eurofins): sense 5’-GACGUGUCAGGACCUCGU-3’; antisense 5’-ACGAAGGUCCUGACACGUC-3’.

II) SRF-siRNA: sense 5’-GAUGGAGUAUCGACAAACA-3’; antisense 5’-GUUGUCGAGAUCUAUCU-3’ [111];

III) Survivin-siRNA (BIRC5): sense 5’-GGACCACCGCAUCUCUACA-3’; antisense 5’-UGUAGAGCGGUGGUCC-3’;

IV) HIF1- siRNA: sense 5’-AGAGGUGGUAUGUGUGGG-3’; antisense 5’-CCCACACAUACCUCUCU-3’;

V) HIF2- siRNA: sense 5’-AGAUUCCUCGUUAUGUG-3’; antisense 5’-CAACAAUACGAGAUCU-3’;

VI) STAT3-siRNA: sense 5’-GCCUCUCUGCAGAAUCAA-3’; antisense 5’-UUGAAUUCUGCAGAGGC-3’;

Both SRF-siRNA sense and antisense sequences were previously characterized by Werth et al [111]. The E2F1-siRNA sense and antisense sequences were validated by Eurofins. All these siRNA specific sequences were synthesized by Eurofins MWG Operon, Ebersberg, Germany.

Table 11: Characterization of the sense and antisense sequences for the targeted molecules

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Target gene</th>
<th>Provider</th>
</tr>
</thead>
</table>
| E2F1   | Sense: 5’GACGUGUCAGGACCUCGU3’  
Antisense: 5’ACGAAGGUCCUGACACGUC3’ | E2F1 gene, Chromosome 20 (human) | Eurofins MWG Operon, Ebersberg, Germany |
| Survivin | Sense: 5’GGACCACCGCAUCUCUACA3’ 
Antisense: 5’UGUAGAGAUGCGGUGGUCC3’ | BIRC5 gene, Chromosome 17 (human) | Eurofins MWG Operon, Ebersberg, Germany |
| STAT3  | Sense: 5’GCCUCUCUGCAGAAUCAA3’  
Antisense: 5’UUGAAUUCUGCAGAGGC3’ | STAT3 gene, Chromosome 17 (human) | Eurofins MWG Operon, Ebersberg, Germany |
| HIF 1A | Sense: 5’AGAGGUGGUAUGUGUGGG3’  
Antisense: 5’CCCACACAUACCUCUCU3’ | Chromosome 14, q21-q24 (human) | Eurofins MWG Operon, Ebersberg, Germany |
2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

This technique is used to amplify and simultaneously quantify a targeted DNA molecule. In order to identify and quantify different levels of messenger RNA (mRNA) and non-coding RNA in cells, qRT-PCR was combined with reverse transcription. Therefore, 24 hours after transfection, total RNA from cells was extracted using the Aurum™ total RNA mini-kit delivered from Bio-Rad (Hercules, CA, USA).

Table 12: Description of the used kits

<table>
<thead>
<tr>
<th>Kit description</th>
<th>Product code</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aurum™ Total RNA Mini Kit</td>
<td>732-6820</td>
<td>Bio-Rad Laboratories GmbH, Munich, Germany</td>
</tr>
<tr>
<td>iQ™ SYBR® green</td>
<td>172-5006CUST</td>
<td>Bio Rad Laboratories GmbH, Munich, Germany</td>
</tr>
<tr>
<td>iScript cDNA Synthesis Kit</td>
<td>170-8891</td>
<td>Bio Rad Laboratories GmbH, Munich, Germany</td>
</tr>
</tbody>
</table>

Afterwards, 200 ng RNA of each sample was reverse transcribed by using iSkript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The primer sequences were design using a special software developed by Premier Biosoft International. The synthesized primers are necessary for the elongation process and serve as a starting point for the polymerase chain reaction. The elongation process occurs at about 72°C and is repeated up to 40 times until a detectable amount of DNA is present and can be evaluated. In order to facilitate the qRT-PCR, a green fluorescent substrate (SYBR ® Green) was used. This substrate can be identified during the
elongation phase in the double-stranded DNA, thus producing an increase in fluorescence. Therefore, the fluorescence can increase proportional to the amount of DNA produced.

At a certain value (C_t value), the fluorescence level was exceeded, and this obtained value can be used for the quantification of gene expression. To compare the efficiency of gene expression, the comparative delta Ct method was used. According to these measurements, the average value was calculated and the standard deviation was determined.

Calculation of relative expression using C_t values:

\[ \text{relative expression} = 2^{\Delta \text{Ct}} \]

\[ \Delta \text{Ct} = \text{C}_{t,q} - \text{C}_{t,cb} \]

whereas \( C_{t,q} \) is value for the target molecule and \( C_{t,cb} \) is the endogenous reference relative to a calibrator [112].

After reverse transcription of the RNA samples via iSkript™ Bio Rad, individual primer sequences for each target molecule were synthesized by Eurofins MWG Operon (Ebersberg, Germany). The sequences targeted by the siRNAs are as follows:

I) SRF forward 5'-AGTGCAGGCCATTCAAGT-3'; reverse 5'-ACGGATGACGTCATGATGGTG-3';

II) Survivin (BIRC5) forward 5'-CTTTCTTGGGAGGGCTGC-3'; reverse 5'-TGGGGTTCATCTGCTGC-3';

III) E2F1 forward 5'-ACCATCAGTACCTGGCCGAGAGC-3'; reverse 5'-ATAGCGTGACTTCTCCCCCGGG-3';

IV) HIF1 forward 5'-TGCAAGATGCTCAGAGAAAGCGAA-3'; reverse 5'-GCTGCATGATCTGGCTGGCTGCT-3'.

V) HIF2 forward 5'-TGTCAGGCATGGCAAGCCGG-3'; reverse 5'-GCACGGGCACGTACCTCA-3';

VI) STAT3 forward 5'-CGGAGAAACAGTTGGGACCCCT-3'; reverse 5'-GCACGGGCACGTACCTCA-3';

VII) GAPDH forward 5'-TCAACAGCGACACCCACTCC-3'; reverse 5'-TGAGGTCCACCACC-CTGTG-3';
In all PCR reactions, a standardized mixture containing IQ™ SYBR® Green Supermix (Bio Rad, Hercules, CA, USA), 400 nM forward and reverse primer and 2 ng of cDNA in a total volume of 15 µL was used.

For the evaluation of qRT-PCR, cells were cultured and transfected in duplicates. For the validation of PCR reactions, GAPDH (Glycerinaldehyd-3-phosphat-Dehydrogenase) was used as reference gene. This gene was found to be the most suitable housekeeping gene for expression studies in reticulocytes. It encodes the GAPDH glycolysis enzyme and is equally expressed under different environmental conditions in every mammalian cell [113].

Table 13: Characterization of the forward and reverse sequences for the target molecules

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (forward)</th>
<th>Sequence (reverse)</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2F1</td>
<td>5'-ACCATCAGTACCTGGCCGAGAGC-3'</td>
<td>5'-ATAGCGTGACTTCTCCCCCCGGG-3'</td>
<td>Eurofins MWG Operon (Ebersberg, Germany).</td>
</tr>
<tr>
<td>Survivin</td>
<td>5'-CTTTCTTGAGGGCTGC-3'</td>
<td>5'-TGGGGGTCGTCATCTGGC-3'</td>
<td>Eurofins MWG Operon (Ebersberg, Germany).</td>
</tr>
<tr>
<td>STAT 3</td>
<td>5'-CGGAGAAACAGTTGGGACCCT-3'</td>
<td>5'-GAGCTGCTCCAGGTACCCTGT-3'</td>
<td>Eurofins MWG Operon (Ebersberg, Germany).</td>
</tr>
<tr>
<td>HIF 1</td>
<td>5'-TGAGAATGTGCTCAGAGCAGGGAAGC-3'</td>
<td>5'-GCTGCATGATCGTCTGGCTG-3'</td>
<td>Eurofins MWG Operon (Ebersberg, Germany).</td>
</tr>
<tr>
<td>HIF 2</td>
<td>5'-TGTCAGGCATGGCAAGCCGTG-3'</td>
<td>5'-GCACGGGCACGTTCACCTCA-3'</td>
<td>Eurofins MWG Operon (Ebersberg, Germany).</td>
</tr>
<tr>
<td>SRF</td>
<td>5'-AGTGCAGGCGCAATTCAAGT-3'; 5'-ACGGATGACGTCATGATGTTG-3';</td>
<td></td>
<td>Eurofins MWG Operon (Ebersberg, Germany).</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TCAACAGCGACACCACCTCC-3'</td>
<td>5'-TGAGGTCCACCACC-CGTTG-3'</td>
<td>Eurofins MWG Operon (Ebersberg, Germany).</td>
</tr>
</tbody>
</table>

In order to demonstrate the efficiency of siRNA delivery, no significant sequence homology with other human genes was found for any of these target molecules.
2.5. CASY cell confirmation

The remaining cells after specific transfection with siRNA targeting E2F1, Survivin, STAT 3, HIF 1, HIF 2 and SRF were counted with a CASY® Cell Counter System (Schärfe System, Reutlingen, Germany). The measurements were made three days after transfection. The principle of CASY technology is based on electric current exclusion and pulse area analysis. The difference between dead cells and viable cells is given by the lower resistance and broken cellular membrane of dead cells, whereas living cells have intact membranes and do not conduct the electric current, under the influence of an electric field. To facilitate an accurate differentiation between dead and viable cell populations, the studied cells were detached by using 500 μl Trypsin/EDTA (PromoCell, Heidelberg, Germany), subsequently inhibited with 500 μl TNS (PromoCell, Heidelberg, Germany) and afterwards counted by CASY. Practically, 50 μl suspension treated with 10 ml CASYton® was placed in the analyzer. The program no. 2 for Human Embryonic Kidney Cells was used for the practical implementation (Capillary: 150 μm, sample volume: 400 μl, X-axis: 50 μm, cycles: 3, dilution: 1:200, evaluation cursor: 11.25 μm-50 μm, normalization cursor: 7.5 μm-50 μm). The number of non-transfected cells was set to 100% and the number of living cells per ml was used for further calculation.

2.6. Statistical analysis

All data were expressed as mean±standard error of mean (S.E.M.). Experiments were carried out three-six times independently with different A 549 chemoresistant cell populations. Each single experimental approach was executed in duplicates for quantitative real time PCR and quadruplicates for CASY cell confirmation. A special statistical software (GraphPad Prism, La Jolla, USA) was used.
3. Results
As presented in the previous chapter, we studied the response of different chemoresistant adenocarcinoma cell lines to Gemcitabine, Vinflunine, Vinorelbine and Methotrexate after siRNA mediated silencing of six crucial molecules implicated in cell differentiation, cell proliferation and tumor growth (SRF, E2F1, Survivin, STAT 3, HIF 1 and HIF 2).

To analyze the results, we isolated and studied three different groups of cells for each target molecule:

Group I: the non-siRNA control group, represented by chemoresistant A 549 cells treated with Gemcitabine, Vinflunine, Vinorelbine or Methotrexate.

Group II: the siRNA control group, also called SCR-siRNA (scrambled-siRNA), represented by A 549 cell lines treated with nonspecific siRNA. In order to demonstrate that the response after specific silencing is concentration dependent, we used two different concentrations (25 nM and 100 nM SCR-siRNA).

Group III: the specific siRNA group represented by A 549 adenocarcinoma cells resistant to Gemcitabine, Vinflunine, Vinorelbine or Methotrexate and transfected with specific siRNA targeting SRF, E2F1, Survivin, HIF 1, HIF 2 or STAT 3.
The A549 cell lines were cultured under standard conditions, whereas the transfection of all six different target molecules was performed in a non-viral manner. In order to demonstrate that the response was concentration dependent, we used two different concentrations (25 nM and 100 nM of specific siRNA). In all these groups we then measured reductions in intracellular mRNA levels via qRT-PCR. The remaining A549 cells after transfection and the proliferation of A549 cells in the control groups (Group I and II) were counted with a CASY cell counter 3 days after transfection.

3.1. Quantitative Real Time - PCR (qRT-PCR)

3.1.1. Gemcitabine-chemoresistant A549 cell lines

In the Gemcitabine-resistant A549 cell line, transfection with E2F1 siRNA led to a 33–37% reduction in gene expression. As seen in Figure 4, the concentration of the siRNA (25 nM and 100 nM) played only a minor role in the siRNA silencing of E2F1. In this situation, our experiments demonstrate that transfection with 25 nM siRNA was slightly more effective than with 100 nM siRNA (37% with 25 nM and 33% with 100 nM, respectively). Furthermore, in the siRNA control group, SCR-siRNA transfection did not decrease the expression of E2F1. The expression of E2F1 was increased 1.19 fold with 25 nM SCR-siRNA and 1.14 fold with 100 nM SCR-siRNA. Taken together, due to siRNA silencing of E2F1, a moderate suppression of Gemcitabine-resistant A549 cells can be achieved, with an efficiency of up to 37%.

Transfection of A549 cells with HIF 1 siRNA led to a 3–9.4% reduction in gene expression. In this experiment, the results were concentration dependent (3% reduction in gene expression at 25 nM siRNA compared with a 9.4% reduction in gene expression at 100 nM siRNA). Compared with the control group, the results proved very similar. With 25 nM, the SCR-siRNA transfection facilitated a 6% reduction in gene expression in Gemcitabine-resistant tumor cells. In conclusion, siRNA silencing of HIF 1 in A549 Gemcitabine-resistant cells plays only a minor role in reducing gene expression and consequently in inhibiting tumor growth.

Transfection of A549 cells with HIF 2 siRNA led to a 35–73% reduction in gene expression. In this experiment, we achieved better results at a lower siRNA concentration (a 73% reduction in gene expression with 25 nM siRNA, whereas with 100 nM siRNA we achieved only a 35% reduction in gene expression). These results
were significantly better in comparison to the control group. The SCR-siRNA transfection did not result in decreased gene expression of HIF 2. Furthermore, the expression of HIF 2 increased 1.05 fold with 25 nM SCR-siRNA and 1.15 fold with 100 nM SCR-siRNA. According to these results, we can postulate that siRNA silencing of HIF 2 can induce a reduction in the proliferation of Gemcitabine-resistant A549 cell lines, with an efficiency of 35–73%.

Similar results were found in the SRF group. Transfection of A549 cells with SRF led to a 47–69% reduction in gene expression. The efficiency of the gene inhibition achieved was also concentration-dependent. With 25 nM we obtained a 47% reduction in gene expression, whereas with 100 nM we obtained significantly better results (69% suppression of gene expression). In the control group we found that SCR siRNA did not play a significant role in modulating gene expression (2–7%).

Transfection of A549 cells with STAT 3 siRNA led to a 38–53% reduction in gene expression. In this experiment, we achieved better results at a lower concentration of siRNA (a 53% reduction in gene expression with 25 nM siRNA but only a 38% reduction with 100 nM siRNA). Compared with the control group, these results were significantly better. As shown in Figure 4 E, SCR-siRNA transfection did not result in a decrease in STAT 3 gene expression.

We found that the expression of STAT 3 increased 1.62 fold with 25 nM SCR-siRNA and 1.09 fold with 100 nM SCR-siRNA. Also, in this experiment we demonstrated that siRNA silencing of STAT 3 could induce a 38–53% reduction of gene expression in Gemcitabine-resistant A549 cell line.

Transfection of A549 cells with Survivin led to a 53–62% reduction in gene expression. Similar to the results obtained in the SRF group, the efficiency of the gene knockdown was concentration-dependent. With 25 nM siRNA we obtained a 53% reduction in gene expression, whereas with 100 nM siRNA a 62% reduction was noted.

In the control group we found that SCR siRNA plays only a minor role in modulating gene expression (3–8%). Taken together, similar to the results obtained with the SRF group (Figure 4D, 4F), the silencing of Survivin leads to a significant reduction in gene’s level of expression in the Gemcitabine-resistant A549 cell line in a concentration-dependent manner (53–62%).
3.1.2. Vinflunine-chemoresistant A 549 cell lines

The transfection of Vinflunine-resistant A549 cells (Figure 5) with E2F1 led to a 43-80% reduction in gene expression in a concentration-dependent manner. In this experiment we achieved better results with a higher siRNA concentration: 43% reduction of gene expression with 25 nM siRNA and significantly better results with 100 nM (80% reduction in gene expression).

Compared to the results obtained in the control group, SCR-siRNA transfection of E2F1 did not result in a decrease in gene expression. Interestingly, the expression of E2F1 increased 1.21 fold after transfection with 25 nM SCR-siRNA.

According to these results, we can postulate that siRNA silencing of E2F1 induced a reduction in the proliferation of Vinflunine-resistant A549 cell lines up to 80% in a concentration-dependent manner.

Transfection of Vinflunine-resistant A549 cells with HIF 1 led to a 31% reduction in gene expression only with 25 nM siRNA. Contrary to the results obtained with 25 nM siRNA-HIF1, we found that the gene expression increased 1.30 fold after siRNA silencing of HIF 1 using 100 nM siRNA. The artifacts obtained with 100 nM siRNA targeting HIF 1 were possibly caused by transfection toxicity induced by the transfection reagent.

In the control group we achieved a 3% reduction in gene expression with 25 nM SCR siRNA and a 7% reduction in gene expression with 100 nM SCR siRNA.

In conclusion, siRNA silencing of HIF 1 in A549 Vinflunine-resistant cells induced a moderate (31%) reduction in gene expression with 25 nM siRNA.

Transfection of A549 cells with siRNA-HIF 2 led to a significant reduction in gene expression (up to 74% with 25 nM and 76% with 100 nM). In this experiment, we achieved similar results using different siRNA concentrations.

Furthermore, SCR-siRNA transfection did not result in decreased gene expression of HIF 2. Similar to the HIF 2 group treated with Gemcitabine, the expression of HIF 2 increased 1.17 fold with 25 nM SCR-siRNA and 1.08 fold with 100 nM SCR-siRNA.

According to these results, we can postulate that siRNA silencing of HIF 2 was able to induce a reduction in the gene expression in Vinflunine-resistant A549 cell lines with an effectiveness up to 76% at both siRNA concentrations.

Transfection of Vinflunine-resistant A549 cells with siRNA-SRF led to a significant reduction in gene expression (74–85%) in a concentration-dependent manner. In this experiment we obtained slightly better results at the higher siRNA concentration: a
74% reduction in gene expression with 25 nM siRNA and an 85% reduction in gene expression with 100 nM siRNA. In comparison to these results, SCR-siRNA transfection of SRF did not decrease the gene expression significantly (only 3% with 100 nM SCR siRNA).

Taken together, siRNA silencing of SRF induced an effective reduction in the proliferation of Vinflunine-resistant A549 cells (up to 85%) in a concentration-dependent manner.

Because of the similar results achieved in the A549 resistant cell lines treated with Gemcitabine, we postulate that SRF is a highly predictive transcription factor in the process of lung carcinogenesis.

Transfection of A549 cells with siRNA-STAT 3 led to an effective reduction in gene expression in a concentration-dependent manner (62% with 25 nM siRNA-STAT 3 and 85% with 100 nM siRNA-STAT 3), with significantly better results in comparison to the control group. As shown in Figure 5 E, SCR-siRNA transfection did not result in decreased gene expression of STAT 3.

Similar to A549 Gemcitabine group transfected with siRNA STAT 3, we found that the expression of STAT 3 increased 1.65 fold at 25 nM SCR-siRNA and 1.01 fold at 100 nM SCR-siRNA.

According to these results, we demonstrated that the siRNA silencing of STAT 3 can reduce the gene expression in Vinflunine-resistant A 549 cell lines with an efficiency of 62-85%, in a concentration-dependent manner.

The transfection of Vinflunine-resistant A 549 cells with siRNA-Survivin leads to a significant reduction of gene expression with similar results at both 25 nM siRNA and 100 nM siRNA (84% and 85%, respectively). We found that SCR siRNA plays only a minor role in modulation of gene expression (5-23%). Taken together, similar to the results obtained in the HIF 2 group (Figure 5 C), the silencing of Survivin leads to a significant reduction of Vinflunine resistant A 549 cell lines in a concentration-independent manner.

3.1.3. Vinorelbine-chemoresistant A 549 cell lines

In Vinorelbine-resistant A 549 cell lines (Figure 6), the transfection of A 549 cells with E2F1 leads to a moderate reduction of gene expression up to 58% at 25 nM. In the siRNA control group SCR-siRNA transfection does not decrease the gene expression
of E2F1. Furthermore, the expression of E2F1 increased 1.32 fold at 25 nM SCR-siRNA and 1.39 fold at 100 nM SCR-siRNA.

The transfection of A 549 cells with HIF 1 leads to a minimal reduction of gene expression (15%) at 25 nM. In conclusion, the siRNA silencing of HIF 1 in A 549 Vinorelbine-resistant cells, plays only a minor role in reduction of gene expression and consequently in inhibition of tumor growth.

The transfection of A 549 cells with HIF 2 leads to a 45-63% reduction of gene expression. In this experiment, we achieved better results at a lower siRNA concentration (63% reduction of gene expression at 25 nM siRNA in comparison to 45% at 100 nM siRNA). These results are significantly better than those obtained in the control group.

Furthermore, the expression of HIF 2 increased 1.03 fold at 100 nM SCR-siRNA. According to these results, we can postulate that the siRNA silencing of HIF 2 might induce a moderate reduction of gene expression in Vinorelbine-resistant A549 cell lines with an efficiency of 45-63%.

We found similar results in the SRF group. The transfection of A 549 cells with SRF leads to a 48-71% reduction of gene expression. The efficiency of gene inhibition is achieved in a concentration-dependent manner. At 25 nM we obtained a 48% reduction of gene expression, whereas at 100 nM we obtained significantly better results (71% inhibition of gene expression).

The transfection of A 549 cells with STAT 3 leads to a 57-88% reduction of gene expression. In this experiment, the results are also concentration dependent (57% reduction of gene expression at 25 nM siRNA and 88 % reduction of gene expression at 100 nM siRNA). Compared to the control group, these results are significantly better. As shown in the figure 6 E, the SCR-siRNA transfection did not lead to a decrease of gene expression. We found that the expression of STAT 3 increased 0.99 fold at 25 nM SCR-siRNA and 1.27 fold at 100 nM SCR-siRNA. Taken together, we can postulate that siRNA silencing of STAT 3 might induce an effective reduction of gene expression in Vinorelbine-resistant A549 cell lines with an efficiency by up to 88%.

The transfection of A549 cells with Survivin leads to a 77-83% reduction of gene expression. We obtained better results at 25 nM siRNA (83% reduction of gene expression), whereas at 100 nM SiRNA we obtained a 77% reduction of gene expression. In the control group we found that SCR siRNA plays only a minor role in
modulation of gene expression (1-6%). Taken together, similar to the results obtained in the SRF group (Figure 6 D, 6 F), the silencing of Survivin leads to a significant reduction in gene expression in Vinorelbine-resistant A 549 cell lines with slightly better results at 25 nM siRNA.

3.1.4. Methotrexate-chemoresistant A 549 cell lines
The transfection of A549 cells with HIF 2 leads to a 63% reduction of gene expression. In this experiment, we achieved significantly better results at 25 nM siRNA (Figure 7).
These results obtained at 25 nM are significantly better in comparison to the control group. The SCR-siRNA transfection did not result in a decreased gene expression of HIF 2.
Similar to the HIF 2 group treated with Gemcitabine, the expression of HIF 2 increased 1.74 fold at 25 nM SCR-siRNA and 1.56 fold at 100 nM SCR-siRNA.
According to these results, we can postulate that the siRNA silencing of HIF 2 might induce at 25 nM a significant reduction in the viability of Methotrexate resistant A549 cell lines with an efficiency of exactly 63%. The marginal results at 100 nM siRNA-E2F1, 100 nM siRNA-HIF 1 and 100 nM siRNA-HIF 2 could be caused due to the possible toxicity induced by the high concentration of transfection reagent.
The transfection of Methotrexate resistant A 549 cells with siRNA-SRF leads to a significant reduction of gene expression (70-84%). In this experiment, we obtained slightly better results at a lower siRNA concentration: 84% reduction of gene expression at 25 nM siRNA and 70% reduction of gene expression at 100 nM siRNA.
In comparison to these results, the SCR-siRNA transfection of SRF did not result in a decrease of gene expression. We observed that the expression of SRF increased 1.37 fold at 25 nM SCR-siRNA and 1.39 fold at 100 nM SCR-siRNA.
According to these results, we demonstrated that SRF is a very predictive transcription factor, whose inhibition may effectively regulate the process of lung carcinogenesis.
The transfection of A 549 cells with STAT 3 leads to a 68-74% reduction of gene expression, with slightly better results at lower concentration. As shown in the figure 7 E, the expression of STAT 3 increased 1.44 fold at 25 nM SCR-siRNA and 1.83 fold at 100 nM SCR-siRNA. According to these results, we demonstrated that the
siRNA silencing of STAT 3 might reduce the viability of Methotrexate-resistant A 549 cell lines with an efficiency up to 74% in a concentration-independent manner. The transfection of Methotrexate-resistant A 549 cells with Survivin leads to a moderate reduction of gene expression with slightly better results at lower concentration (at 25 nM siRNA 53% and at 100 nM siRNA 47%). In comparison to these results, the expression of Survivin increased 2.37 fold at 25 nM SCR-siRNA and 2.68 fold at 100 nM SCR-siRNA. Taken together, the siRNA silencing of Survivin might induce a moderate reduction of gene expression up to 53% in Methotrexate-resistant A549 cells in a concentration-independent manner.

3.2. Cell quantification
The proliferation potential of A 549 adenocarcinoma cell lines treated with Vinflunine Gencitabine, Vinorelbine or Methotrexate was analyzed with a CASY cell counter 3 days after transfection. As shown in the Figures 8 and 9, we compared the control group (non siRNA) and siRNA nonspecific group (SCR-siRNA) with siRNA specific group (siRNA-SRF, E2F1, Survivin, STAT 3, HIF 1 and HIF 2).

3.2.1. Gencitabine-chemoresistant A 549 cell lines
In Gencitabine group (Figure 8 A) we obtained a moderate reduction of A 549 tumor cells (cells remaining in the control group 100% cells, SCR group 61%, SRF group 65%, E2F1 group 48.3%, Survivin group 59%, STAT 3 group 49%, HIF 1 group 42.6% and HIF 2 group 39.3%, respectively).

3.2.2. Vinflunine-chemoresistant A 549 cell lines
In the A 549 cell lines treated with Vinflunine (Figure 8 B) we identified different results compared to the Gencitabine-resistant A549 cells (cells remaining after transfection, analyzed with the CASY counter: control group 100%, SCR group 77.33%, SRF group 57.33%, E2F1 group 50.33%, Survivin group 55.33%, STAT 3 group 28.33%, HIF 1 group 41.33%, and HIF 2 group 51.66%).
3.2.3. Vinorelbine-chemoresistant A 549 cell lines
In Vinorelbine group (Figure 9 A) we found a very potent reduction of A 549 tumor cells (cells remaining in the control group 100%, SCR group 70%, SRF group 34%, E2F1 group 37%, Survivin group 23%, STAT 3 group 36%, HIF 1 group 28%, and HIF 2 group 39%).

3.2.4. Methotrexate-chemoresistant A 549 cell lines
In Methotrexate group (Figure 9 B) we achieved a very potent reduction of A 549 tumor cells (cells remaining in the control group 100%, SCR group 95%, SRF group 43%, E2F1 group 46%, Survivin group 24%, STAT 3 group 40%, HIF 1 group 18%, and HIF 2 group 43%).

Taken together, in Gemcitabine group we obtained a moderate reduction of adenocarcinoma chemoresistant cell lines, whereas in the Vinflunine, Vinorelbine and Methotrexate group we achieved a very effective suppression of cell lines with a consecutive reduction of tumors cells by up to 82%.
Figure 4: Relative E2F1 (A), HIF1 (B), HIF 2 (C), SRF (D), STAT 3 (E) and Survivin (F) expression of Gemcitabine-resistant A 549 adenocarcinoma cells 24 hours after transfection with corresponding siRNAs. The X-axis describes the different concentrations of the used siRNAs, whereas the Y-axis represents the relative expression of the silenced genes with untransfected cells set to one.
Figure 5: Relative E2F1 (A), HIF1 (B), HIF 2 (C), SRF (D), STAT 3 (E) and Survivin (F) expression of Vinflunine-resistant A 549 adenocarcinoma cells 24 hours after transfection with corresponding siRNAs. The X-axis describes the different concentrations of the used siRNAs, whereas the Y-axis represents the relative expression of the silenced genes with untransfected cells set to one.
Figure 6: Relative E2F1 (A), HIF 1 (B), HIF 2 (C), SRF (D), STAT 3 (E) and Survivin (F) expression of Vinorelbine-resistant A549 adenocarcinoma cells 24 hours after transfection with corresponding siRNAs. The X-axis describes the different concentrations of the used siRNAs, whereas the Y-axis represents the relative expression of the silenced genes with untransfected cells set to one.
Figure 7: Relative E2F1 (A), HIF 1 (B), HIF 2 (C), SRF (D), STAT 3 (E) and Survivin (F) expression of Methotrexate-resistant A 549 adenocarcinoma cells 24 hours after transfection with corresponding siRNAs. The X-axis describes the different concentrations of the used siRNAs, whereas the Y-axis represents the relative expression of the silenced genes with untransfected cells set to one.
Figure 8: Percentage of cells 3 days after transfection with specific siRNA targeting E2F1, HIF 1, HIF 2, SRF, STAT 3 and Survivin, compared with untransfected cells, set to 100% in A549 cell lines treated with Gemcitabine (A) and with Vinflunine (B).
Figure 9: Percentage of cells 3 days after transfection with specific siRNA targeting E2F1, HIF 1, HIF 2, SRF, STAT 3 and Survivin, compared with untransfected cells, set to 100% in A 549 cell lines treated with Vinorelbine (A) and with Methotrexate (B)
To summarize all our in vitro results regarding the relative expression of the studied molecules after specific siRNA transfection, we create a diagram (Figure 10) with the molecules with the strongest effect after transfection.

<table>
<thead>
<tr>
<th>Specific siRNA</th>
<th>E2F1</th>
<th>HIF 1</th>
<th>HIF 2</th>
<th>SRF</th>
<th>STAT 3</th>
<th>Survivin</th>
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<tr>
<td>Gemcitabine resistant cell line</td>
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<td>Vinflunine resistant cell line</td>
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<td>Vinorelbine resistant cell line</td>
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<td>Methotrexate resistant cell line</td>
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Figure 10: Relative expression of specific siRNA targeting E2F1, HIF 1, HIF 2, SRF, STAT 3, and Survivin 24h after transfection.

(-) modest reduction of gene expression (0-33%),
(-- ) moderate reduction of gene expression (34-66%),
(---) very potent reduction of gene expression (67-100%).
4. Discussion

4.1. Clinical implications

Lung cancer is the second leading cause of death worldwide after cardiovascular diseases and the leading cause of death among all types of cancers. NSCLC accounts for 85% of all lung cancer cases in the United States and, due to its poor prognosis, it represents a major public health problem [8].

According to epidemiological studies, the overall 5-year survival rate for stage I is 60–80%, 40–50% for stage II, 23% for stage IIIA, 10% for stage IIIB and less than 10% for Stage IV. Furthermore, the median survival rate of metastatic NSCLC is about eight months [8]. More than 40% of patients with NSCLC show metastases in another part of the body at presentation [114],[37]. For all these patients, the recommended therapy according to the S3 guidelines for lung cancer is an individualized multimodal concept based on radiation, chemotherapy and surgery. Thus, chemotherapy represents an important component of treatment for all stages of the disease.

Therefore, the effectiveness of the selected chemotherapeutic agents is decisive for the success of treatment. Unfortunately, in recent years an increased chemoresistance to commonly used drugs has been observed (up to 60% for platinum derivatives, up to 70% for Gemcitabine and Doxorubicine and up to 40% for Paclitaxel and Docetaxel) with negative consequences on the effectiveness of treatment and consequently on the survival rates [115].

Due to the development of chemoresistance, chemotherapeutics are only able to modestly increase overall survival (2 months per decade) and the quality of life [46]. Therefore, the multifactorial etiology of chemoresistance (modification of the drug target, mutations in mitotic checkpoints, drug sequestration, detoxification of cytotoxic agents) represents the most important obstacle to effective treatment [37].

For this reason, it is particularly important to explore and suggest new therapeutic multimodal concepts (e.g. gene therapy, molecular therapy, radiotherapy) according to the molecular subtype of NSCLC, on the one hand, and the clinical severity of the disease on the other.

One of the most intensively studied recent treatment alternatives in the last few years is based on siRNA posttranscriptional silencing.

Recent studies have emphasized the role of siRNA as a multimodal concept in radiosensitizing various carcinomas (e.g. colon cancer [27], prostate cancer [116]).
and chemosensitizing various chemoresistant tumor cell lines (hepatocellular carcinoma [52], breast cancer [53], lung cancer [54]).

According to this concept, we proposed a new in vitro model for the treatment of chemoresistant lung cancer via siRNA silencing of six crucial molecules involved in lung carcinogenesis.

Our in vitro model is based on the A549 adenocarcinoma cell line. According to histological data, these squamous alveolar cells allow the diffusion of water and electrolytes through the membrane due to their high permeability. Therefore, medium supplementation with Glucose, L-Glutamine and FCS (fetal calf serum) favors cell growth under very similar conditions to the in vivo situation. Growing in a single layer, these squamous cells may easily be suspended in an in vitro solution. On the other hand, A549 cells exhibit good membrane stability in vitro due to their increased levels of desaturated fatty acids [110]. In order to facilitate optimal tumor growth, the A549 cell lines were cultured on DMEM-high glucose medium. The advantage of this medium is that it contains 4-fold higher levels of amino acids and vitamins in comparison to other media. These differences favor tumor growth similar to in vivo conditions.

A second important point is the transfection protocol and the reagents used. Basically, transfection protocols are frequently used to introduce foreign DNA, RNA, or protein molecules into eukaryotic cells. In our laboratory, we used a transient transfection protocol and not a long-term protocol in order to facilitate short-term modifications in the relative expression of targeted molecules. To improve transfection effectiveness and create optimized in vitro conditions as close as possible to the in vivo situation, we adopted a special transfection protocol according to the original publications of T. Walker, A. Nolte and H.P. Wendel [117],[81]. This transfection method is based on lipofection, a highly efficient, lipid-mediated DNA-transfection procedure that uses lipid cations as transporter molecules for the siRNA via cellular membrane. As result, no cellular toxicity to the neighboring healthy cells was observed [117], [81].

According to the actual therapy regimens used for lung cancer (S3 Guidelines), a multimodal concept including chemotherapy, radiation therapy and surgery in selected patients may increase the effectiveness and survival rate in the treatment of NSCLC. In order to study the effectiveness of siRNA-mediated interference, we
examined the chemoresistance of A549 cells against four different chemotherapeutics (Methotrexate, Gemcitabine, Vinflunine and Vinorelbine). The chemoresistance was artificially induced in vitro. In order to develop the chemoresistance similar to in vivo situations, the A549 cells were treated repeatedly with low doses of specific chemotherapy agents until they developed the desired chemoresistance against the agent.

The first chemotherapy agent used in our experiments was Methotrexate. It is an antimetabolite and acts by inhibiting the metabolism of folic acid. It competitively inhibits the synthesis of DNA, RNA, thymidylates and various proteins involved in carcinogenesis. Furthermore, Methotrexate is a very cytotoxic drug during the S-phase of the cell cycle and, therefore, it is a very potent agent against rapidly dividing malignant cells. Because of its apoptotic effect, it can be used for chemotherapy alone or in combination with various chemotherapeutics.

In our study, we obtained a very strong suppression of gene expression in Methotrexate resistant A549 cells after transfection with specific siRNA targeting SRF (up to 84% at 25 nM) and STAT 3 (up to 74% at 25 nM). These results demonstrate that the application of siRNA in combination with Methotrexate might have a beneficial role for suppressing tumor growth in NSCLC.

The second drug examined in our study was Gemcitabine. It is a nucleoside analog. During DNA replication, Gemcitabine replaces a very important nucleoside in synthesized RNA, thus inducing apoptosis and consequently suppression of tumor growth. Gemcitabine also irreversibly inhibits the enzyme ribonucleotide reductase (RNR), implicated in DNA replication and DNA repair. Due to its apoptotic effect, it can be used as a potent drug either alone or in combination with platinum derivatives in various types of cancer (NSCLC, bladder cancer, breast cancer).

The development of chemoresistance against Gemcitabine in NSCLC might induce a decrease in the effectiveness of treatment. In this situation, transfection of A549 resistant cells with specific siRNA targeting HIF 2, SRF, STAT 3 and Survivin was able to induce a moderate suppression of tumor proliferation (73% with 25 nM siRNA-HIF 2, 69% with 100 nM siRNA-SRF, 53% with 25 nM siRNA-STAT 3 and 62% with 100 nM siRNA-Survivin). These results demonstrate the beneficial role of specific siRNA silencing in the A549 Gemcitabine resistant cell line.

The third drug analyzed was Vinorelbine. It is a semi-synthetic vinca alkaloid that binds to tubulin, thereby inhibiting tubulin polymerization into microtubules and
resulting in apoptosis of susceptible cancer cells. It is a very effective anti-mitotic chemotherapy drug, used in breast cancer, ovarian cancer and non-small-cell lung cancer.

In our experiments we obtained very strong suppression of Vinorelbine-resistant cells in a concentration dependent manner (up to 88% after transfection with siRNA specifically targeting STAT 3). According to our in-vitro results, we can postulate that siRNA might represent a very potent platform for new drugs in Vinorelbine-resistant NSCLC cell lines.

The fourth drug examined in our study was Vinflunine, a novel fluorinated Vinca alkaloid synthesized from Vinorelbine. Vinflunine induces G2 and M arrest and consequently interferes with the microtubular network in interphase cells in a concentration-dependent manner. In comparison to vinorelbine, it is characterized by superior in vivo activity in preclinical carcinoma models and inferior tubulin binding properties [118]. It can suppress tumor growth in bladder cancer and lung cancer.

Our experiments demonstrated that transfection of Vinflunine-resistant A549 cells with specific siRNA targeting HIF 2, SRF, STAT 3 and Survivin may induce a very strong suppression in gene expression, with slightly better results in comparison to the Vinorelbine group (76% with 100 nM siRNA-HIF 2, 85% with 100 nM siRNA-SRF, 85% with 100 nM siRNA-STAT 3 and 85% with 100 nM siRNA-Survivin). These results demonstrate the beneficial role of specific siRNA in Vinflunine-resistant cell lines.

Therefore, we can postulate according to our results that siRNA nanoparticles might play a beneficial role in advanced stages of lung adenocarcinoma treated previously with Vinflunine, Vinorelbine, Gemcitabine or Methotrexate.

Second, our experiments show for the first time the beneficial role of siRNA in a novel in vitro cell model with A549 multiresistant cell lines. Similar studies have demonstrated the role of siRNA in different multiresistant cancer lines (e.g. multidrug-resistant human breast cancer cells [119], multidrug-resistant hepatocellular carcinoma cells [120],[66], multidrug-resistant colorectal carcinoma [121]).

Another approach is focused on a combination therapy between various chemotherapy agents and siRNA nanoparticles in order to improve the sensitivity of the chemotherapy agents (chemosensitization). For example, recent studies have emphasized the beneficial role of siRNA in chemosensitizing pancreatic cancer cells.
previously treated with Gemcitabine, 5-FU, and Oxaliplatin [122]. Another study suggested an optimal siRNA combination with Doxorubicine in order to overcome doxorubicin (Dox) resistance in a multidrug resistant (MDR) human breast cancer xenograft by co-delivering Dox and siRNA [123]. Preliminary studies have also emphasized siRNA mediated FoxM1 inhibition as a potential strategy in the chemosensitization of NSCLC cells previously treated with Cisplatin [124].

Our study postulates a role for specific siRNA targeting E2F1, STAT 3, Survivin, HIF 1, HIF 2 and SRF in chemosensitizing multiresistant adenocarcinoma cell lines. According to the S3 Guidelines for lung cancer, most patients have to be treated with an intensive combined-modality therapy (surgery, radiotherapy and chemotherapy) according to the histology, molecular subtype and clinical stage of the disease. In the last few years, the personalized, molecular targeted cancer therapy has become the gold standard in lung medicine [125], [126]. For this reason, we not only studied four different chemotherapy agents in our laboratory, but also six important molecules involved in lung carcinogenesis (E2F1, HIF 1, HIF 2, SRF, STAT 3 and Survivin). In order to facilitate an accurate molecular diagnostic, identify predictive biomarkers and overcome drug resistance, we examined the effectiveness of specific siRNA transfection against all six target molecules for each chemoresistant cell line. Interestingly, each of these molecules had individual pathways and mechanisms of action, thereby ensuring a very effective multi-targeted knockdown of resistant adenocarcinoma cell lines through multiple mechanisms of action.

The first molecule examined was E2F1, a transcription factor that plays an important role in cell proliferation and regulation of the cell cycle [84]. It also acts as a strong inducer of apoptosis [85] in response to DNA damage, through its capacity to activate p53/p73 death pathways. Recent studies have indicated that specific siRNA targeting E2F1 may facilitate an effective suppression of tumor growth in NSCLC [81]. In addition, we examined the potential role of specific E2F1-siRNA in multiresistant A549 cell lines. We obtained a very potent reduction in gene expression in a concentration-dependent manner in the Vinflunine group (80% with 100 nM siRNA) and a moderate reduction in gene expression in the Gemcitabine group (63–67% with both 25 nM and 100 nM siRNA). In terms of cells remaining after transfection, we observed significantly better results in comparison to the nonspecific siRNA group or the
control group (48.3% in the Gemcitabine group, 50.33% in the Vinflunine group, 37% in the Vinorelbine group, and 46% in the Methotrexate group, respectively).

The next two molecules examined were HIF 1 and HIF 2. These two heterodimers respond to changes in available oxygen in the cellular environment and promote angiogenesis in embryos as well as neoangiogenesis in different types of cancer (neuroblastoma [100], esophageal carcinoma [101], breast carcinoma [102], laryngeal carcinoma [103] and NSCLC [104]). The mechanism of action involves aberrant expression of TGF-β1 as a response to hypoxia and subsequent induction of carcinogenesis and metastasis into the hypoxic area [98],[99].

In our experiments, HIF 1 induced only a modest reduction in gene expression in the Vinflunine (31% with 25 nM siRNA) and Gemcitabine groups (9.4% with 100 nM siRNA). In comparison to the control group, these results were significantly better in the Vinflunine group and slightly better in the Gemcitabine group.

CASY confirmation of cell viability demonstrated significant survival of chemoresistant A549 cells treated with nonspecific siRNA, in comparison with specific siRNA targeting HIF 1 (42.6% of cells remaining in the Gemcitabine group, 41.33% in the Vinflunine group, 18% in the Methotrexate group and 28% in the Vinorelbine group, respectively). These results show that HIF 1 might be a very important target in order to induce cell death in NSCLC.

Preliminary data referring to HIF induced chemoresistance in NSCLC cell lines treated previously with Gefitinib were published by Minakata K. et al [127]. According to this paper, downregulation of HIF might induce an increase in chemosensitivity to Gefitinib in NSCLC. In comparison with our experiments, this study examined three mutant NSCLC cell lines, HCC 827, PC 9, and HCC 2935 and two target molecules (TGF beta and HIF).

The next molecule examined in our experiments was HIF 2. We obtained a moderately efficient suppression with the siRNA targeting HIF 2 in the Methotrexate and Vinorelbine groups (63% with 25 nM siRNA-HIF 2 in the Vinorelbine group), and a strong reduction in gene expression in the Gemcitabine and Vinflunine groups (73% and 76%, respectively). These results were significantly better in comparison to the control group. In terms of cells remaining after transfection, we obtained a significant reduction in the number of tumor cells in all four groups (51.66% of cells remaining in the Vinflunine group, 39.3% in the Gemcitabine group, 39% in the
Vinorelbine group and 43% in the Methotrexate group). These results demonstrate that HIF 2 might be a very potent target for chemoresistant NSCLC.

The fourth molecule studied was SRF, a transcription factor that regulates apoptosis and cell growth as well cell differentiation via the mitogen-activated protein kinase pathway (MAPK). It acts as a nuclear suppressor of TGF-beta 1 and consequently as an inhibitor of cell proliferation in different types of cancer: breast cancer [79], prostate cancer [80], hepatocellular carcinoma [82], and lung cancer [81].

We observed that SRF is a very potent suppressor of gene expression in multiresistant A549 cell lines (69% with 100 nM siRNA-SRF in the Gemcitabine group, 71% with 100 nM siRNA-SRF in the Vinorelbine group, 84% with 25 nM siRNA-SRF in the Methotrexate group and 85% with 100 nM siRNA-SRF in the Vinflunine group).

After specific transfection, we observed a moderate survival of A549 tumor cells (65% of cells remaining in the Gemcitabine group, 57.33% in the Vinflunine group, 43% in the Methotrexate group and 34% in the Vinorelbine group). These results also suggest that SRF might be a potent target for the treatment of multiresistant NSCLC.

Another molecule examined was STAT 3. STAT 3 is an important transcription factor that plays a crucial role in early stages of cell development (G0/G1) in various cancer lines (bladder cancer [107], hepato-carcinoma [108] and lung adenocarcinoma [109]).

Our study emphasizes the beneficial role of specific siRNA targeting STAT 3 in multiresistant cell lines derived from lung adenocarcinoma.

We obtained a moderate efficiency of suppression in all examined groups (53% in the Gemcitabine group, 74% in the Methotrexate group, 85% in the Vinflunine group and 88% in the Vinorelbine group). The survival rate yielded significantly better results in comparison to the control group (100% of cells remaining in the control group, 49% in the Gemcitabine group, 40% in the Methotrexate group, 36% in the Vinorelbine group and 28.33% in the Vinflunine group). These results suggest that knockdown of STAT 3 might be a potent trigger of cell death in multiresistant NSCLC.

The last target examined was Survivin, a potent suppressor of caspase activity and consequently of tumor cell differentiation and proliferation in the G2-M phase. As a potent oncogene identified in among 60 different human tumor lines [88], Survivin also plays an important role in the development of resistance against various chemotherapy drugs.
Recent studies have demonstrated the beneficial role of specific siRNA targeting Survivin in combination with Cisplatin in the treatment of H 292 lung cancer cells. The transfection efficiency was 83.13% [94]. The target molecules responsible for the chemosensitization of the chemoresistant H 292 lung cancer cells were AKT, CREB, Bcl-xL, Survivin and Bcl-2. In comparison to these studies we cultured different cell lines, four different chemotherapy agents and examined six different target molecules involved in lung carcinogenesis. Interestingly, we obtained results comparable to those published by Tian H. et al [94] (83% transfection efficiency in the Vinorelbine group and 85% in the Vinflunine group). These comparable data suggest that the transfectability of NSCLC cells by siRNA is reliable and reproducible.

In terms of survival rate after specific siRNA transfection, we also obtained significantly better results in comparison to the control group (100% of cells remaining in the control group, 59% in the Gemcitabine group, 55.33% in the Vinflunine group, 24% in the Methotrexate group and 23% in the Vinorelbine group 23%).

Comparable results regarding the downregulation of survivin expression by RNA interference were published by Chen XQ et al [128] and Okamoto K et al in Erlotinib-resistant NSCLC [129].

Taken together, the knockdown of all six molecules might induce an effective suppression of tumor growth in the selected chemoresistant adenocarcinoma cell lines. According to our promising in vitro results, we suppose that the concomitant suppression of these six target molecules, involved in different pathways of lung carcinogenesis, might be a complex method of preventing the further development of chemoresistance.

Interestingly, the analyzed cell lines belong to a heterogeneous group of tumor cells, with variable chemoresistance to different pharmacological agents. Furthermore, for each molecule tested we obtained individual responses and variable effects in a concentration-dependent manner. This is another argument in support of the concept of individualized multimodal therapy, according to the molecular subtype involved in lung carcinogenesis, the degree of chemoresistance, and the severity of the disease.

A diagram illustrating all the results regarding the relative expression of the studied molecules after specific siRNA transfection (Figure 10) might be an important tool in identifying the most effective target molecules in multiresistant NSCLC. The same concept was published by Raparia K. et al, who suggested molecular profiling in
NSCLC as a crucial step toward personalized medicine. The same study emphasized that the driver mutations responsible for lung carcinogenesis may serve as "drugable" therapeutic targets [130].

Another important aspect to discuss is the in vitro half-life of the siRNA. According to the published data, the half-life of mRNA may vary from minutes to days, while the half-lives of their protein products can range from a few minutes to several days. Therefore, the used nanotechnologies allow sufficient time for the siRNA to associate with RISC and deplete mRNA/protein concentrations to desired levels. The recommended time course ranges are 12 to 72 hours (in order to efficiently suppress the target mRNA) and 24 to 96 hours to adequately knockdown the target proteins and assess phenotypic outcomes. In our study we examined the relative expression of the target molecules 72 hours after transfection in order to achieve the maximum efficiency possible.

A critical point of the study is that after a single-shot transfection with specific siRNA, some residual activity of each target molecule was still observed. The CASY cell counter also showed numbers of surviving chemoresistant adenocarcinoma cells (39–65% in the Gemcitabine group, 28–57% in the Vinflunine group, 23–39% in the Vinorelbine group and 18–46% in the Methotrexate group). According to these results we deduce that this therapy alone cannot totally destroy all tumor cells, suggesting that this therapy might prove more successful in terms of a multimodal concept (siRNA therapy as neoadjuvant/ adjuvant active therapy with chemotherapy, radiation therapy and surgery).

One interesting approach in the future could be a multi-shot transfection strategy in the surviving cells. A comparison study between multi-shot versus single-shot specific siRNA transfection could clarify the therapeutic efficiency of siRNA even more in the future. According to these preliminary in vitro data, new strategies have to be established in order to improve siRNA half-life as well as the potency of the siRNA in the surviving chemoresistant cells. On the other hand, the short half-life of these molecules can be a considerable advantage in terms of cellular toxicity and adverse effects. Due to its short half-life and fast elimination by kidney filtration [131], siRNA interference can be considered a transient process on the one hand and a very protective process with fewer adverse effects for the body on the other.
Taken together, these preliminary in vitro results obtained in our laboratory suggest that siRNA might represent an important platform for new alternative regimens in the treatment of NSCLC.

According to our promising results, we believe that siRNA-based nanotechnology might prove a very accurate individualized transient therapy. Similar to the experimental data obtained in other carcinoma cell lines, a new concept concerning a multimodal therapy in lung cancer that includes siRNA, chemotherapy and radiotherapy should be established in order to improve the effectiveness of the treatment, patient adherence to the treatment, patient quality of life and the survival rate of patients undergoing this severe disease.

4.2. Limitations

Despite of these promising results obtained in our laboratory, siRNA mediated interference has various limitations.

First of all, the results obtained in vitro cannot be directly extrapolated in vivo. The most important obstacle limiting the in vivo transfectability of siRNA is delivery of the siRNA to its intracellular target. Its large molecular weight, size and plasmatic instability (plasma half-life of about 10 minutes) are the main contributors [132], [133], [134], [135]. For this reason, current efforts are directed not only at increasing the plasma stability of the siRNA by changing the structure of the RNA chain, but also toward various multifunctional coating processes.

Second, its low in vivo stability and rapid renal elimination are two other important factors limiting the effectiveness of siRNA transfection in vivo [131].

Third, lysosomal endocytosis may diminish the activity of siRNA agents [135], suggesting that new coating processes and transfection protocols have to be established in order to improve the plasmatic half life and survival rate of siRNA nanoparticles.

Fourth, an RNA interference mechanism may only knockdown the target genes (decrease the expression of the genes) and not knockout the genes, which would mean completely eliminating their functions.

Fifth, conventional siRNA have different repetitive sequences, which can potentially produce cross-reactivity and consequently substantial off-target effects. As a result of advanced genetic engineering processes, siRNA molecules can now be produced
with very high-affinity for the target gene, thereby reducing cross reactivity and off
target effects.
Sixth, at present a single shot transfection with a specific siRNA does not lead to the
death of all chemoresistant cells, suggesting that a multi shot setting in the context of
a multimodal therapy (adjuvant chemotherapy, radiation and surgery) should be
evaluated in order to increase the effectiveness of the siRNA.
5. Zusammenfassung

Nach den interdisziplinären S3-Leitlinien für Prävention, Diagnostik, Therapie und Nachsorge des nicht-kleinzelligen Bronchialkarzinoms, ist die vielversprechendste Behandlungsstrategie aktuell ein individualisiertes und multidisziplinäres Konzept (Chemotherapie, Strahlentherapie sowie chirurgische Resektion). Darüber hinaus stellt die Chemotherapie eine wichtige Komponente der Behandlung aller Stadien der Erkrankung dar. Die Entwicklung der Chemoresistenz (bis zu 60% für Platin Derivate, bis zu 70% für Gemcitabine und Doxorubicine Derivate, sowie bis zu 40% für Paclitaxel und Docetaxel) ist aktuell das wichtigste Hindernis für eine effiziente Behandlung, insbesondere bei Zweit- und Drittlinientherapien.

Im Rahmen der vorliegenden Arbeit wurde ein mögliches neues Therapieverfahren in der Behandlung der chemoresistenten Adenokarzinom-Zelllinien via siRNA Silencing von sechs spezifischen Molekülen (SRF, E2F1, Survivin, HIF 1, HIF 2 und STAT 3) auf Zellkulturniveau bearbeitet.

Um die in vivo Bedingungen zu simulieren, wurde die Chemoresistenz gegen vier verschiedenen Chemotherapeutika (Gemcitabine, Vinflunine, Vinorelbine und Methotrexate) nach wiederholter lokaler Anwendung der oben genannten Mittel künstlich induziert. Die resistenten A 549 Adenokarzinom-Zelllinien wurden sodann unter Standardbedingungen bei 37°C und 5% CO₂ kultiviert. Die Zellen wurden zwei Stunden bei 37°C mit spezifischen siRNA gegen SRF, E2F1, Survivin, HIF 1, HIF 2 und STAT 3 transfiziert. Die Effizienz des siRNA silencing sowie die suppressionsabhängige Reduktion des spezifischen intrazytoplasmatischen mRNA-Levels wurden über quantitative Echtzeit-PCR ausgewertet. Die residuellen Tumorzellen nach spezifischer siRNA Transfektion sowie das Tumorwachstum wurden mit einem CASY Zellzählsystem drei Tage nach der Transfektion analysiert.

Es konnte gezeigt werden, dass mit siRNA Transfektionen eine konzentrationsabhängige Wirkung auf die Viabilität der chemoresistenten Zelllinien induziert werden konnte. Sowohl bei 25 nM als auch bei 100 nM siRNA, war die Unterdrückung der Tumorzellen in allen vier Gruppen wesentlich effizienter als in der Kontrollgruppe. Auf der anderen Seite zeigte die CASY Zellanalyse eine moderate Reduktion der chemoresistenten Adenokarzinom-Zelllinien in der Gemcitabine Gruppe, sowie eine sehr effiziente Unterdrückung der Zelllinien mit einer
konsekutiven Reduktion der Tumormasse in der Vinorelbine, Vinflunine und Methotrexate Gruppe.

Vor dem Hintergrund dieser ermutigenden in vitro Ergebnisse kann man zusammenfassend sagen, dass siRNA eine neue adjuvante Alternative in der Behandlung von chemoresistenten Adenokarzinomen sein könnte, durch eine spezifische siRNA Transfektion könnte eine sehr effiziente Unterdrückung von multiresistenten Zelllinien erreicht werden.

Diese individualisierte temporäre Therapie (keine Gentherapie) eröffnet möglicherweise neue therapeutische Strategien für die Zukunft (z.B. siRNA Transfektion in Kombination mit verschiedenen thoraxchirurgischen Eingriffe unter Einsatz der Herz-Lungen-Maschine über eine isolierte Lungenperfusion) sowie ggf. neue Perspektiven in Bezug auf eine multimodale Therapie des Bronchialkarzinoms.
6. Summary
According to the S3 Guidelines for the prevention, diagnosis and therapy of NSCLC, the most promising therapeutic regimen is currently an individualized, multidisciplinary concept. In addition, chemotherapy represents an important component of treatment at all stages of the disease. The development of chemoresistance (up to 60% for platinum derivatives, up to 70% for Gemcitabine and Doxorubicine) represents the most important obstacle to an effective treatment, especially with second-and third-line therapies.

In the present work, a potential new therapeutic strategy for the treatment of chemoresistant adenocarcinoma cell lines via siRNA silencing of six specific molecules (SRF, E2F1, Survivin, HIF 1, HIF 2 and STAT 3) was examined in a cell culture model.

In order to accurately simulate in vivo conditions, chemoresistance against four different chemotherapeutic agents (Gemcitabine, Vinflunine, Vinorelbine and Methotrexate) was artificially induced after repeated application of the above mentioned agents. Afterwards, the chemoresistant A549 adenocarcinoma cell lines were cultured under standard conditions at 37°C and 5% CO₂. These cells were then transfected two hours at 37°C with specific siRNA targeting SRF, E2F1, Survivin, HIF 1, HIF 2 and STAT 3 in a non-viral manner. The efficiency of the siRNA silencing and the suppression of specific intracytoplasmic mRNA levels were evaluated via quantitative real time-PCR.

The tumor cells remaining after specific siRNA silencing were analyzed with a CASY cell counter system three days after transfection as an accurate predictor of tumor growth.

We demonstrated that transfection with specific siRNA had a concentration-dependent effect on the viability of the chemoresistant cell lines. At both 25 nM and 100 nM, the suppression of the tumor cells in all four groups was significantly more efficient in comparison to the control group.

The CASY System demonstrated a moderate reduction of adenocarcinoma chemoresistant cells in the Gemcitabine group and very effective suppression of adenocarcinoma cell lines in the Vinorelbine, Vinflunine and Methotrexate groups.
According to these promising in vitro results, we conclude that siRNA delivered by specific transfection could be a new alternate adjuvant in the treatment of chemoresistant adenocarcinomas. Given the specific posttranscriptional gene silencing, potent suppression of various chemoresistant cell lines can be achieved.

Therefore, this individualized temporary therapy (not a gene therapy) might open new therapeutic strategies for the future (i.e. siRNA transfection in combination with various thoracic surgical procedures using a heart-lung machine on an isolated perfused lung) and possibly open new perspectives in terms of a multimodal therapy for bronchial carcinoma.
7. Outlook and future perspectives
The evolution of technological and therapeutic applications of siRNA since the initial description of the interference process published by the two Nobel Prize winners (A. Z. Fire and C.C. Melo) in 2006 has been extremely rapid and very productive. Over about 8 years, different scientists described many pathways and mechanisms of this molecule. The first suggestions of a role for siRNA in immune processes and tumor processes were succeeded by further extensive research. Currently, at least 15 systemic infections and 10 tumor entities have been described in which siRNA mediated interference can play an auspicious role.

In the last few years, promising data regarding the transfection of specific siRNA have been published in the medical literature. Research into the involvement of siRNA in genitourinary, neurological (e.g. Parkinson’s disease), hematological and cardiovascular diseases (siRNA eluting stents, siRNA interference in venous grafts during coronary artery bypass graft operations) has recently produced multiple productive platforms for new therapeutic options.

In particular in lung pathology, current efforts are focused on the establishment and application of siRNA nanoparticles (e.g. liposomes, aptamers) during various thoracic surgical procedures utilizing isolated lung perfusion.

The discovery and clinical implementation of some siRNA specific tumor biomarkers might also play a crucial role in cancer screening with major therapeutic implications. The interesting preliminary data regarding siRNA mediated chemosensitization and radiosensitization of (multi)resistant lung cancer also require extensive research in the future.

Given the difficulties concerning the reproducibility of all these experiments, new coatings and plasmatic stabilization processes should be further intensively studied, in order to increase the effectiveness of siRNA-mediated specific transfection in vivo.
8. References


9. Authors’ contributions

M. Stoleriu contributed to the study design, performed and interpreted the in vitro experiments, analyzed the statistical data, designed the figures and tables and wrote the complete thesis.

PD Dr. med T. Walker (Main supervisor of the doctoral thesis, Department of Thoracic, Cardiac and Vascular Surgery, Tuebingen University Hospital, Germany) supervised and coordinated the project, participated in the study design, provided scientific support and corrected the manuscript.

Prof. Dr. H.-P. Wendel (Head of the Research Institute of Eberhard Karls University, Department of Thoracic, Cardiac and Vascular Surgery, Tuebingen, Germany) provided technical support and supervised the experiments.

Dr. rer. nat. A. Nolte-Karayel and J. Kurz (Research Institute of Eberhard Karls University, Tuebingen, Germany) supported the laboratory experiments (cell isolation, cell culture, siRNA transfection, CASY confirmation, qRT PCR).

Prof. M. Michaelis (Department of Cell Biology, University of Kent, United Kingdom) and Prof. Dr. J.Cinatl (Department of Pediatric Tumor and Virus Research, Frankfurt, Germany) supervised the manufacture and delivery of the chemoresistant cell lines.
10. Acknowledgements

I started working on my doctoral thesis entitled "Experimental evaluation of gene silencing as potential therapeutic option in the treatment of multiresistant Non-small-cell lung cancer" in July 2012 with the premise to explore a complex theme, extensively studied, but still poorly understood.

The topic of the paper, suggested by my main supervisor, Mr. PD Dr. med. T. Walker (Department of Thoracic and Cardiovascular Surgery, University Medical Center Tuebingen, Germany) was the best opportunity for me to try to answer the many questions that this interesting subject proposes.

The current paper raises many difficult questions concerning tumor pathology with a very poor prognosis (Non-small-cell lung cancer with chemoresistant cell lines). Based on the idea that „In the middle of difficulty lies opportunity“ (A. Einstein), I found the subject a way to discover and study new and innovative data for the benefit of patients suffering from this disease.

I started reading the literature and formulated my first working hypotheses according to the existing papers published in this domain.

Guided by Mr. PD. Dr. med. T. Walker and Mr. Prof. H. P. Wendel (Head of Research Laboratory, Department of Thoracic and Cardiovascular Surgery, University Medical Center Tuebingen, Germany), I found the first answers regarding the topic discussed.

I personally believe this work is only a summation of current opinions, selected from the latest international publications, all embedded in a personal format.

First of all, I would like to express my very great appreciation to Mr. Prof. Dr. Dr. h.c. Ch. Schlensak (Medical Director of the Department of Thoracic and Cardiovascular Surgery, University Medical Center Tuebingen, Germany) which gave me the opportunity to interact with many critically ill patients in our hospital, to understand their complexity and simultaneously to work intensively at this work in our research laboratory.

Equally, I would like to offer my special thanks to Mr. PD Dr. med. T. Walker, (Department of Thoracic and Cardiovascular Surgery, University Medical Center Tuebingen, Germany) my main supervisor for his very good cooperation and scientific guidance and for being available to answer in detail and at any time the many questions that appeared in the context of the latest bibliographic references.
I am particularly grateful to Mr. Prof. H. P. Wendel (Head of Research Laboratory, Department of Thoracic and Cardiovascular Surgery, University Medical Center Tuebingen, Germany) for the technical and scientific support that made this work possible and for all the additional information required to complete the laboratory experiments.

To complete this project in the laboratory, I was sustained by many colleagues with a great deal of experience and excellent research capabilities (My sincerest gratitude to Dr. rer. nat. A. Nolte-Karayel and J. Kurz).

I would also especially like to thank Mr. Prof. M. Michaelis (Department of Cell Biology, University of Kent, United Kingdom) and Mr. Prof. Dr. J. Cinatl (Department of Pediatric Tumor and Virus Research, Frankfurt am Main, Germany) for their assistance regarding the manufacturing and delivery of the chemoresistant cell lines.

I would like to thank God, who blessed me with great parents, who always encouraged and supported me in my social and professional formation.

I would also like to remember and thank my wife, Cosmina for her never-ending optimism as well for her sincere support during all these years spent together.

Finally, the idea I want to express at the end of this work is that everything is possible... together.

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Coming together is a beginning,
staying together is progress,
and
working together is success.

(Henry Ford)
11. Curriculum Vitae

Personal dates:
Name: Stoleriu Mircea-Gabriel
Date of birth: 06. March 1985, Iasi, Romania
Nationality: Romanian
Status: married

School education:
09.2000-06.2004 „Mihai Eminescu“ high school (profile mathematics), Iasi, Romania
06.2004 Secondary school Diploma (Abitur), Iasi, Romania

University Education:
10.2004 Enrollment at the University of Medicine and Pharmacy „Gr. T. Popa“ (General Medicine), Iasi, Romania.
10.2007 Erasmus Grant and enrollment at the Medical University „Albert Ludwigs Universität“, Freiburg, 15.10.2007-15.08.2008
09.2010 Final medical Examination („Examen de licenta”), Iasi, Romania (16.09.2010)
11.2010 Approbation als Arzt, Regierungspräsidium Stuttgart
since 12.2010 Assistant at „Eberhard Karls Universität Tübingen“, Department of Cardiothoracic and Vascular Surgery.
since 06.2012 Doctoral studies in alternative strategies in the treatment of Non small cell lung cancer

Scientific activities and publications
10.2007 Second Prize as author of the Paper „Anatomofunctional study of glaserian fissure“ at the 18th European Students Conference, Berlin, 7-11th October 2007
10.2007 First Prize as co-author of the Paper „Anatomical Landmarks of sphenoidal Sinus endoscopical surgery“ at the 18th European Students Conference, Berlin, 7-11th October 2007
11.2011 Presentation of the Paper ,,Aptamer-Based Isolation and Subsequent Imaging of Mesenchymal Stem Cells in Ischemic Myocardium by Magnetic Resonance Tomography” at 3. Fokustagung Herz der Deutschen Gesellschaft für Thorax-, Herz- und Gefäßchirurgie und Deutschen Gesellschaft für Kardiotechnik, Weimar

02.2012 Presentation of the Paper ,,Quartz sensor based approach towards online haemostasis monitoring” at 41. Jahrestagung der Deutschen Gesellschaft für Thorax-, Herz- und Gefäßchirurgie, Freiburg, Germany


02.2013 Presentation of the Paper ,,siRNA silencing of SRF, Survivin and E2F1- in chemoresistant NSCLC cell lines - a new option ?” at 42. Jahrestagung der Deutschen Gesellschaft für Thorax-, Herz- und Gefäßchirurgie, Freiburg, Germany.

10.2013 Presentation of the paper ,,A new strategy in the treatment of chemoresistant lung adenocarcinoma via siRNA silencing of SRF, E2F1, Survivin, HIF, and STAT 3 ” at 27th EACTS, Annual Meeting, Vienna, Austria

11.2013 Presentation of the Paper ,,Detection of platelet GP IIb/IIIa receptors on artificial surfaces used for extracorporeal circulation” at 42. Jahrestagung der Deutschen Gesellschaft für Thorax-, Herz- und Gefäßchirurgie and 5. Fokustagung Herz, Nürnberg, Germany.

Scholarships, training stages, seminars:

08.2005 General Surgery fellowship at Alexandria Main Hospital, Alexandria, Egypt

09.2006 DAAD Grant: German language course at „Ludwig-Maximilians Universität“ München (C1 Advanced Certificate)

10.2007 Medical terminology course (Fachsprachenkurs Medizin) at „Albert Ludwigs Universität“ Freiburg, Germany.

10.2007 Erasmus Grant and enrollment at the Medical University „Albert Ludwigs Universität“, Freiburg, 15.10.2007-15.08.2008

02.2008 Practical clinical elective (Dermatology) at „Albert-Ludwigs Universität“, Freiburg, Germany

03.2008 Advanced german language certificate DSH 3 (Deutsche Sprachprüfung für den Hochschulzugang), Sprachlehrinstitut Freiburg.

04.2009 Graduation of online Harvard CME Course “Clinical Challenges in Electrocardiography“, Harvard Medical School, Department of Continuing Education.

08.2009 Practical clinical elective (Nephrology) at „Albert-Ludwigs Universität“, Freiburg, Germany.

08.2010 Practical clinical elective (Cardiology) at Deutsches Herzzentrum München, an der Technischen Universität München.

09.2010 Practical clinical elective (Cardiology) at Deutsches Herzzentrum München, an der Technischen Universität München

01.2012 Training seminar: Anticoagulation Management Service, München

05.2012 Practical echocardiography course at „Albert-Ludwigs Universität“, (Universitäts Freiburg–Bad Krotzingen Herzzentrum).

02.2013 Interdisciplinary basic course of Doppler and Duplex sonography of the vessels (DEGUM), Landesaerztekammer Hessen, Academy of doctor's education and training