HELLENIC REPUBLIC
National and Kapodistrian University of Athens

"ALEXANDER FLEMING"
Biomedical Sciences Research Center

Exploring the role of nuclear receptor NR5A2 in lung cancer

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Athens, 2021

Dissertation Thesis in MSc "Molecular Biomedicine:

Mechanisms Of Disease, Molecular And Cellular Therapies, And Bioinnovation"

Performed in the Center for Basic Research, Biomedical Research Foundation of the Academy of Athens (BRFAA)

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Acknowledgements

I would like to thank my supervisor Dr. Panagiotis Politis, who gave me the opportunity to perform my master's thesis in his lab at the Center for Basic Research of the Biomedical Research Foundation of the Academy of Athens. I am grateful for his major advice, his encouragement, and excellent guidance in the shaping of my research. His constructive reviews on my work and the conversations on different methods and approaches have been very helpful from the beginning of our collaboration until the completion of this study.

I also express my gratitude to both members of my committee, Dr. Constantin Tamvakopoulos and Dr. Maria Roubelakis, for their willingness to help and support me at this stage of my academic course and for honouring me by participating in the three-member committee for the evaluation of this study.

In addition, I wish to extend my special thanks to PhD candidate Dimitrios Gkikas, for his treasured support in implementing the experiment methods and evaluating the results of this study. His technical assistance, insightful comments, and consistent belief in me played an important role in the completion of this work. Moreover, I am deeply grateful to all the past and present members of the lab for their help and for providing me a friendly working environment. Finally, I would like to express my gratitude to my family and friends for their tremendous understanding and encouragement during the past few months.

Abstract

Lung cancer is the leading cause of cancer-related deaths worldwide with a poor overall prognosis and lower survival rates than most cancers. Non-small cell lung cancers (NSCLCs) account for about 80% of all lung cancers, with adenocarcinomas comprising up to 50% of NSCLCs. These clinical observations underscore the need for novel therapeutic insights and pharmacological targets. To this end, here we identify the orphan nuclear receptor NR5A2/LRH-1 as a negative regulator of epithelial cancer cell proliferation and promising pharmacological target for lung adenocarcinoma. In particular, publicly available clinical data from the Oncomine database reveal a downregulation of *NR5A2* in lung adenocarcinoma patients. Consistently, we experimentally show that *NR5A2* overexpression is able to suppress the proliferation of four different lung cancer cell lines representing either adenocarcinoma or large cell carcinoma. Notably, its anti-proliferative effect can be observed *in vivo* as well, since *NR5A2* overexpression inhibits tumor growth in a mouse xenograft model. Cancer cell migration is also inhibited due to *NR5A2* overexpression *in vitro*. Moreover, shRNA-mediated knockdown of the basal expression levels of *NR5A2* in lung adenocarcinoma cells promotes cell cycle progression. The anti-proliferative effect of NR5A2 is possibly mediated by the transcriptional induction of negative cell cycle regulators, *CDKN1A* (encoding for $p21^{cip1}$) and *CDKN1B* (encoding for $p27^{kip1}$), and the simultaneous downregulation of a G1-S transition inducer, *CCND1* (encoding for cyclin D1). Interestingly, a well-established agonist of NR5A2, dilauroyl phosphatidylcholine (DLPC) is able to mimic the anti-proliferative action of NR5A2 in human NSCLC cell lines, without however affecting cell migration. These data suggest a tumor suppressor role of NR5A2 in NSCLC and render this nuclear receptor a potential pharmacological target for the treatment of human lung adenocarcinoma.

1. Introduction

1.1 Lung cancer

Lung cancer is the leading cause of cancer-related deaths worldwide and is one of the most common cancers. In the U.S., lung cancer has surpassed breast cancer as the most common cause of cancer-related deaths in women. The overall prognosis for lung cancer is poor when compared with some other cancers. Survival rates for lung cancer are generally lower than those for most cancers, with an overall five-year survival rate for lung cancer of about 17% compared to 65% for colon cancer, 91% for breast cancer, 81% for bladder cancer, and over 99% for prostate cancer[1].

Lung cancer was not common prior to the 1930s but increased dramatically over the following decades as tobacco smoking increased. In many developing countries, the incidence of lung cancer is beginning to fall owing to public education about the dangers of cigarette smoking and the introduction of effective smoking-cessation programs. Nevertheless, lung cancer remains among the most common types of cancers in both men and women worldwide.

The principal function of the lungs is to exchange gases between the air and the blood. The right lung has three lobes, while the left lung has two lobes and a small structure called the lingula that is the equivalent of the middle lobe on the right. The major airways entering the lungs are the bronchi arising from the trachea, which is outside the lungs (**Figure 1**). The bronchi branch into progressively smaller airways called bronchioles that end in tiny sacs known as alveoli where gas exchange occurs (**Figure 2**). A thin layer of tissue called the pleura covers the lungs and chest wall.

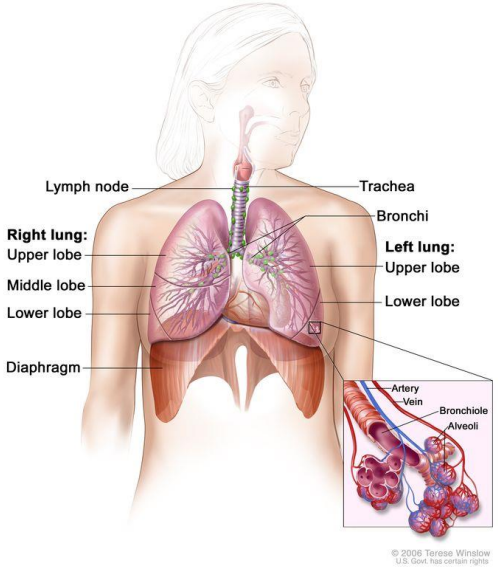

Figure 1: Anatomy of the respiratory system, showing the trachea, both lungs and their lobes and airways. Lymph nodes and the diaphragm are also shown[3].

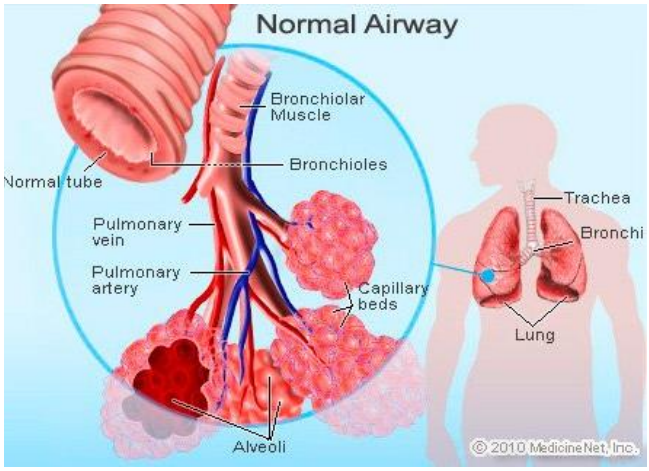

Figure 2: Bronchiole, a tiny tube in the air conduit system within the lungs that is a continuation of the bronchi and connects to the alveoli (the air sacs) where oxygen exchange occurs[1].

Lung cancers can arise in any part of the lung, but 90%-95% arise from epithelial cells, the cells lining the larger and smaller airways (bronchi and bronchioles). Therefore, lung cancers are sometimes called bronchogenic cancers or bronchogenic carcinomas. Cancers can also arise from the pleura (called mesotheliomas) or rarely from supporting tissues within the lungs, for example, the blood vessels[1].

CAUSES

The main causes of lung cancer can be categorized as follows:

a. Smoking

Tobacco use contributes to 80% and 90% of lung cancer deaths in women and men, respectively. Men who smoke are 23 times more likely to develop lung cancer, while women are 13 times more likely, compared to never smokers[5]. The risk of lung cancer increases with the number of cigarettes smoked and the time over which smoking has occurred; doctors refer to this risk in terms of pack-years of smoking history (the number of packs of cigarettes smoked per day multiplied by the number of years smoked). For example, a person who has smoked two packs of cigarettes per day for 10 years has a 20 pack-year smoking history. While the risk of lung cancer increases with even a 10-pack-year smoking history, those with 30-pack-year histories or more are considered to have the greatest risk for the development of lung cancer. Among those who smoke two or more packs of cigarettes per day, one in seven will die of lung cancer[1].

Pipe and cigar smoking also can cause lung cancer, although the risk is not as high as with cigarette smoking. Tobacco smoke contains over 4,000 chemical compounds, many of which have been shown to be carcinogenic. The two primary carcinogens among them are nitrosamines and polycyclic aromatic hydrocarbons. The risk of developing lung cancer decreases each year following smoking cessation as normal cells grow and replace damaged cells in the lung. In former smokers, the risk of developing lung cancer begins to approach that of a nonsmoker about 15 years after smoking cessation.

Passive smoking or the inhalation of tobacco smoke by non-smokers who share living or working quarters with smokers, is another established risk factor for the development of lung cancer. Research has shown that nonsmokers who reside with a smoker have a 24% increase in risk for developing lung cancer when compared with nonsmokers who do not reside with a smoker. The risk appears to increase with the degree of exposure (number of years exposed and number of cigarettes smoked by the household partner) to secondhand smoke. Over 7,000 lung cancer deaths occur each year in the U.S. that are attributable to passive smoking[1].

b. Exposure to asbestos fibers

Asbestos fibers are silicate fibers that can persist for a lifetime in lung tissue following exposure to asbestos. In the past, asbestos was widely used as both thermal and acoustic

insulation, thus the workplace was a common source of exposure to asbestos fibers for construction workers. Today, asbestos use is limited or banned in many countries, including the U.S. Both lung cancer and mesothelioma (cancer of the pleura of the lung as well as of the lining of the abdominal cavity called the peritoneum) are associated with exposure to asbestos. Cigarette smoking drastically increases the chance of developing an asbestos-related lung cancer as it impairs the lungs' ability to remove asbestos fibers[6]. In fact, asbestos workers who do not smoke have a fivefold greater risk of developing lung cancer than nonsmokers, but asbestos workers who smoke have a risk that is 50- to 90-fold greater than nonsmokers[1].

c. Exposure to radon gas

Radon is a colorless, odorless, radioactive gas that forms naturally from the decay of radioactive elements, such as uranium. Radon breaks down into solid radioactive elements called radon progeny, which can attach to dust and other particles and can be breathed into the lungs. As radon and radon progeny in the air break down, they emit a type of ionizing radiation that can damage the DNA inside the body's cells. Radon gas can travel up through the soil and enter homes through gaps in the foundation, pipes, drains, or other openings. The U.S. Environmental Protection Agency estimates that one out of every 15 homes in the U.S. contains dangerous levels of radon gas and lists radon as the second leading cause of lung cancer and the number one cause of lung cancer among non-smokers, estimating it is responsible for about 20,000 lung cancer deaths every year. Though radon gas is invisible and odorless, simple test kits can detect it. As with asbestos exposure, concomitant smoking greatly increases the risk of lung cancer with radon exposure[7].

d. Familial predisposition

While the majority of lung cancers are associated with tobacco smoking, the fact that not all smokers eventually develop lung cancer suggests that other factors, such as individual genetic susceptibility, may play a role in the causation of lung cancer.

Familial lung cancer is more complex than other familial cancers, and can be caused by shared environmental factors or shared genetic factors among family members. The role of genetic factors in lung cancer development is poorly understood, as they are masked by the influence of environmental factors, including smoking, air pollution, and coal burning. The development of lung cancer in never-smokers is a complex clinical problem, and a large number of studies have indicated that individuals with a family history of lung cancer are two- to three-fold more susceptible to lung cancer development than those without such a history; this association is strongest for those whose siblings have been affected by lung cancer. The risk of lung cancer in individuals with a family history of cancer among first-degree relatives is increased by ~50% compared with those without a family history, and this association is not affected by gender, ethnicity, histological types and other known lung cancer risk factors. Approximately 8% of lung cancers are inherited or occur as a result of a genetic predisposition [8].

The most frequently mutated genes associated with lung cancer include EGFR, KRAS, ALK, and BRCA2. Although targeted therapy against epidermal growth factor receptor (EGFR) mutations and echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) rearrangements improved the prognosis in the last decade, mutations in EGFR are only present in 10–26% of NSCLC, and EML4-ALK rearrangements are only found in 4–5% of NSCLC [9-11].

e. Lung diseases

The presence of certain diseases of the lung, notably chronic obstructive pulmonary disease (COPD), pneumonia and tuberculosis, is associated with an increased risk for the development of lung cancer even after the effects of concomitant cigarette smoking are excluded. COPD is characterized by airflow obstruction in the lungs and the related symptoms that impede the normal expiratory volume of the lungs. COPD most commonly refers to patients with emphysema (the enlargement and destruction of the alveoli) and/or chronic bronchitis (chronic inflammation and scarring of bronchi). Pulmonary fibrosis (scarring of the lung) appears to increase lung cancer risk about sevenfold, and this risk is not related to smoking. The incidence rate of COPD among never smokers increases with age to approximately 10–12% by age 75 in males and to approximately 20% by age 75 in females. Pneumonia is an infection of the lungs and respiratory tract most often caused by viruses, bacteria and other organisms. Infection is quite common among adults and pneumonia incidence is highest in the elderly and very young where the immune system is compromised. Tuberculosis, another type of infection affecting the lungs is caused by mycobacteria, predominantly Mycobacterium tuberculosis. Although mortality due to tuberculosis is low in industrialized countries, inflammation and ensuing lung remodeling has been hypothesized to lead to lung cancer development[12].

f. Prior history of lung cancer

Survivors of lung cancer have a greater risk of developing a second lung cancer than the general population has of developing a first lung cancer. Survivors of non-small-cell lung cancers (NSCLCs) have an additive risk of 1%-2% per year for developing a second lung cancer. In survivors of small-cell lung cancers (SCLCs), the risk for development of second lung cancers approaches 6% per year. Moreover, the risks of a second cancer are especially high among lung cancer survivors who continue to smoke [1].

g. Air pollution

Air pollution from vehicles, industry, and power plants can raise the likelihood of developing lung cancer in exposed individuals. Up to 1%-2% of lung cancer deaths are attributable to breathing polluted air, and it is believed that prolonged exposure to highly polluted air can carry a risk for the development of lung cancer similar to that of passive smoking.

TYPES OF LUNG CANCER

Lung cancers are classified into two types: small-cell lung cancers (SCLC) and non-small-cell lung cancers (NSCLC). This classification depends upon the microscopic appearance of the tumor cells, specifically their size. These two types of cancers grow and spread in different ways and may have different treatment options, thus, a distinction between them is important (**Figure 3**).

SCLCs comprise about 20% of lung cancers and are the most aggressive and rapidly growing of all lung cancers. SCLC is related to cigarette smoking, with only 1% of these tumors occurring in nonsmokers. SCLCs metastasize rapidly to many sites within the body and are most often discovered after they have spread extensively. Referring to a specific cell appearance often seen when examining samples of SCLC under the microscope, these cancers are sometimes called oat cell carcinomas (**Figure 4**). SCLC has the most aggressive growth of all lung cancers, with a median survival time of only two to four months after diagnosis when untreated. However, SCLC is also the type of lung cancer most responsive to radiation therapy and chemotherapy. Because SCLC spreads rapidly and is usually disseminated at the time of diagnosis, methods such as surgical removal or localized radiation therapy are less effective. When chemotherapy is used alone or in combination with other methods, survival time can be prolonged four- to fivefold; however, of all patients with SCLC, only 5%-10% are still alive five years after diagnosis. Most of those who survive have limited-stage SCLC before treatment.

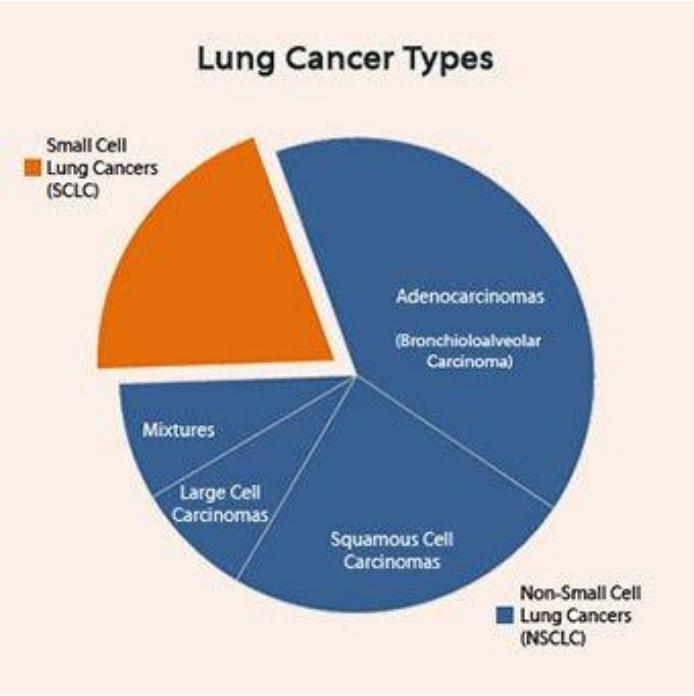

Figure 3: Distribution of lung cancer cases among different tumor types [1].

NSCLCs are the most common lung cancers, accounting for about 80% of all lung cancers. NSCLC can be divided into several main types that are named based upon the type of cells found in the tumor:

- Adenocarcinomas constitute the most commonly seen type of NSCLC in the U.S. and comprise up to 50% of NSCLC (**Figure 5**). While adenocarcinomas are associated with smoking like other lung cancers, physicians see this type in nonsmokers who develop lung cancer, as well. Most adenocarcinomas arise in the outer, or peripheral, areas of the lungs and in many cases, may be found in scars or areas of chronic inflammation. Resultant genetic mutations in the p53 gene are the most frequent cause of tumorigenesis in NSCLC in 52% of cases [13].
- Squamous cell carcinomas were formerly more common than adenocarcinomas; at present, they account for about 30% of NSCLC. Also known as epidermoid carcinomas, squamous cell cancers arise most frequently in the central chest area in the bronchi (**Figure 6**).
- Large cell carcinomas, sometimes referred to as undifferentiated carcinomas, are the least common type of NSCLC (**Figure 7**).
- Mixtures of different types of NSCLC are also seen.

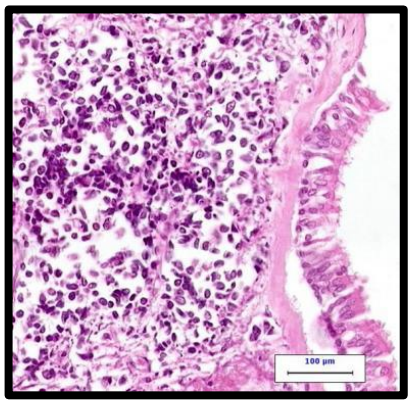

Figure 4: High-power photomicrograph of small cell carcinoma on the left side of the image with normal ciliated respiratory epithelium on the right side of the image. https://www.medscape.com/answers/280104-37743/what-are-the-histologic-findings-of-small-cell-lung-cancer-sclc

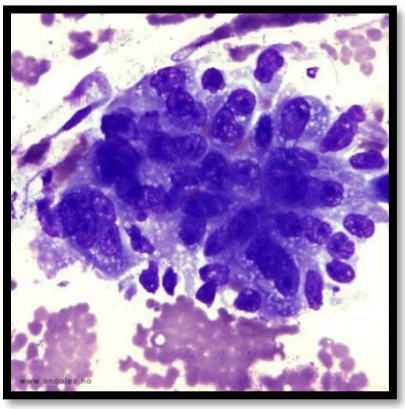

Figure 5: Photomicrograph demonstrating lung adenocarcinoma in a cytological specimen after a Giemsa staining. http://oncolex.org/Lung-cancer/Background/Histology

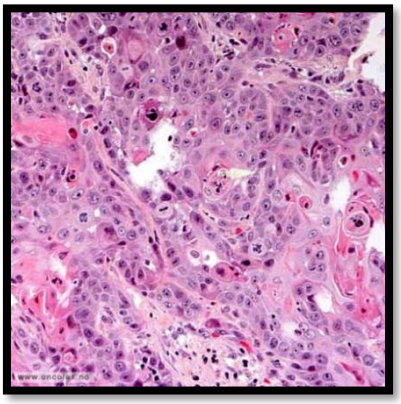

Figure 6: Photomicrograph showing a squamous cell carcinoma with keratinization. http://oncolex.org/Lung-cancer/Background/Histology

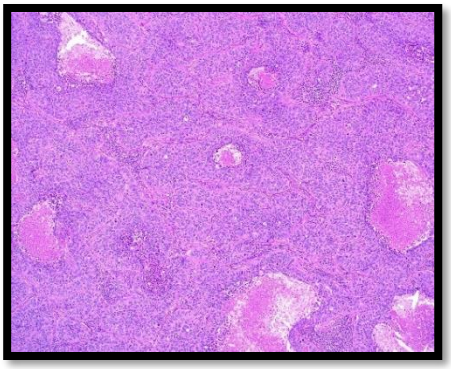

Figure 7: Histological image of a large cell lung carcinoma after hematoxylin and eosin (H&E) staining, 50x magnification. http://www.pathologyoutlines.com/topic/lungtumorlargecell.html

In non-small-cell lung cancer (NSCLC), the most important prognostic factor is the stage (extent of spread) of the tumor at the time of diagnosis. Results of standard treatment are generally poor in all but the smallest of cancers that can be surgically removed. However, in stage I cancers that can be completely removed surgically, five-year survival approaches 75%. Radiation therapy can produce a cure in a small minority of patients with NSCLC and leads to relief of symptoms in most patients. In advanced-stage disease, chemotherapy offers modest improvements in survival although rates of overall survival are poor.

Nevertheless, other types of cancers can arise in the lung; these types are much less common than NSCLC and SCLC and together comprise only 5%-10% of lung cancers:

- Bronchial carcinoids account for up to 5% of lung cancers. Physicians sometimes refer to these tumors as lung neuroendocrine tumors. They are generally small (3 cm-4 cm or less) when diagnosed and occur most commonly in people under 40 years of age. Unrelated to cigarette smoking, carcinoid tumors can metastasize, and a small proportion of them secrete hormone-like substances that may cause specific symptoms related to the hormone produced. Carcinoids generally grow and spread more slowly than bronchogenic cancers, and medical professionals detect many early enough to be amenable to surgical resection.
- Cancers of supporting lung tissue such as smooth muscle, blood vessels, or cells involved in the immune response can rarely occur in the lung.

As discussed previously, metastatic cancers from other primary tumors in the body often appear in the lung. Tumors from anywhere in the body may spread to the lungs either through the bloodstream, through the lymphatic system, or directly from nearby organs. Metastatic tumors are most often multiple, scattered throughout the lung, and concentrated in the peripheral rather than central areas of the lung [1].

LUNG CANCER STAGING

The stage of a cancer is a measure of the extent to which a cancer has spread in the body. Staging involves evaluation of a cancer's size and its penetration into surrounding tissue as well as the presence or absence of metastases in the lymph nodes or other organs. Staging is important for determining the treatment of a particular cancer, since lung cancer therapies are geared toward specific stages. Staging of a cancer also is critical in estimating the prognosis of the patient, with higher-stage cancers generally having a worse prognosis than lower-stage cancers[1].

Doctors assign a stage to NSCLC from I to IV in order of severity:

- In stage I, the cancer is confined to the lung.
- In stages II and III, the cancer is in the lung and nearby lymph nodes (with larger and more invasive tumors classified as stage III).
- Stage IV cancer has spread from the chest to other parts of the body.

Most doctors use a two-tiered system to determine treatment for SCLC:

- Limited-stage (LS) SCLC refers to cancer that is confined to its area of origin in the chest.
- In extensive-stage (ES) SCLC, the cancer has spread beyond the chest to other parts of the body[14].

LUNG CANCER TREATMENT

Treatment for lung cancer primarily involves surgical removal of the cancer, chemotherapy, or radiation therapy, as well as combinations of these treatments. Targeted therapies and immunotherapy treatments are becoming more common, as well. The decision about which treatments will be appropriate for a given individual depends on the location and extent of the tumor, as well as the overall health status of the patient.

As with other cancers, doctors may prescribe therapy intended to be curative (removal or eradication of a cancer) or palliative (measures that are unable to cure a cancer but can reduce pain and suffering). Doctors may prescribe more than one type of therapy. In such cases, the therapy that is added to enhance the effects of the primary therapy is referred to as adjuvant

therapy. An example of adjuvant therapy is chemotherapy or radiotherapy administered after surgical removal of a tumor in an attempt to kill any tumor cells that remain following surgery.

- Surgery: Doctors generally perform surgical removal of the tumor for limited-stage (stage I or sometimes stage II) NSCLC and is the treatment of choice for cancer that has not spread beyond the lung. About 10%-35% of lung cancers can be removed surgically, but removal does not always result in a cure, since the tumors may already have spread and can recur later. Among people who have an isolated, slow-growing lung cancer removed, 25%-40% survive five years after diagnosis. However, although a tumor may be anatomically suitable for resection, surgery may not be possible if the person has other serious conditions (such as severe heart or lung disease) that would limit their ability to survive an operation. Surgery is performed less often with SCLC than with NSCLC because these tumors are less likely to be localized to one area that can be removed.

- Radiation: Radiation therapy treats both NSCLC and SCLC using high-energy X-rays or other types of radiation to kill dividing cancer cells. Radiation therapy may be given as curative, palliative (using lower doses of radiation than with curative therapy), or as adjuvant therapy in combination with surgery or chemotherapy. Medical professionals deliver the radiation either externally by using a machine that directs radiation toward the cancer, or internally through placement of radioactive substances in sealed containers within the area of the body where the tumor is localized. Brachytherapy is a term that describes the use of a small pellet of radioactive material placed directly into the cancer or into the airway next to the cancer.

  Radiation therapy can be given if a person refuses surgery, if a tumor has spread to areas such as the lymph nodes or trachea making surgical removal impossible, or if a person has other conditions that make them too ill to undergo major surgery. Radiation therapy generally only shrinks a tumor or limits its growth when given as a sole therapy, yet in 10%-15% of people it leads to long-term remission and palliation of the cancer. Combining radiation therapy with chemotherapy can further prolong survival. A person with severe lung disease in addition to a lung cancer may not be able to receive radiotherapy since radiation can further decrease the function of the lungs.
  Radiation therapy does not carry the risks of major surgery, but it can have unpleasant side effects, including fatigue and lack of energy. A reduced white blood cell count (rendering a person more susceptible to infection) and low blood platelet levels (making blood clotting more difficult and resulting in excessive bleeding) can occur with radiation therapy. If the digestive organs are in the field exposed to radiation, patients may experience nausea, vomiting, or diarrhea.

- Chemotherapy: Both NSCLC and SCLC may be treated with chemotherapy. Chemotherapy refers to the administration of drugs that stop the growth of cancer cells by killing them or preventing them from dividing. Chemotherapy may be given alone, as an adjuvant to

surgical therapy, or in combination with radiotherapy. While a number of chemotherapeutic drugs have been developed, the platinum-based drugs have been the most effective in treating lung cancers.

Chemotherapy is the treatment of choice for most SCLC, since these tumors are generally widespread in the body when they are diagnosed. Only half of people with SCLC survive for four months without chemotherapy. With chemotherapy, their survival time is increased up to four- to five-fold. Chemotherapy alone is not particularly effective in treating NSCLC, but when NSCLC has metastasized, it can prolong survival in many cases.

Chemotherapy may be given as pills, as an intravenous infusion, or as a combination of the two. Unfortunately, the drugs used in chemotherapy also kill normally dividing cells in the body, resulting in unpleasant side effects. Damage to blood cells can result in increased susceptibility to infections and difficulties with blood clotting (bleeding or bruising easily). Other side effects include fatigue, weight loss, hair loss, nausea, vomiting, diarrhea, and mouth sores. The side effects of chemotherapy vary according to the dosage and combination of drugs used and may also vary among individuals.

- Targeted therapy: Molecular targeted therapy involves the administration of drugs that have been identified to work in subsets of patients whose tumors have specific genetic changes (driver mutations) that promote tumor growth.

  The drugs erlotinib (Tarceva), afatinib (Gilotrif), and gefitinib (Iressa) are examples of targeted drugs that more specifically target cancer cells, resulting in less damage to normal cells than general chemotherapeutic agents. Erlotinib, gefitinib, and afatinib target the epidermal growth factor receptor (EGFR) that is important in promoting cell division. The *EGFR* gene is mutated in many cases of non-small-cell lung cancer, promoting tumor growth. Mutations in the *EGFR* gene are more common in cancers in women and in people who have never smoked. Drugs that target the EGFR receptor sometimes stop working after some time, which is known as resistance to the drug. Resistance often occurs because the cancer has developed a new mutation in the same gene, and a common example of this is the EGFR T790M mutation. Some newer EGFR-targeted drugs also work against cells with the T790M mutation, including osimertinib (Tagrisso). Necitumumab (Portrazza) is another drug that targets EGFR. It can be used along with chemotherapy as the first treatment in people with advanced NSCLC of the squamous cell type.

  ALK tyrosine kinase inhibitor drugs crizotinib (Xalkori), alectinib (Alecensa), brigatinib (Alunbrig), and ceritinib (Zykadia) are used in patients whose tumors have an abnormality of the *ALK* gene as a driver mutation. Some of these drugs may also be helpful for people whose cancers have an abnormality of the *ROS1* gene.

The *BRAF* gene can also be abnormal in lung cancers causing the production of BRAF protein that promotes cancer growth. Dabrafenib (Tafinlar) is a direct BRAF protein inhibitor. Trametinib (Mekinist) is a MEK inhibitor attacking MEK proteins, which are related to BRAF proteins. These may be used for patients with tumors that have abnormal *BRAF* genes.

Other attempts of targeted therapy include antiangiogenic drugs, which block the development of new blood vessels within a cancer. Without adequate blood vessels to supply oxygen-carrying blood, the cancer cells will die. The antiangiogenic drug bevacizumab (Avastin) has also been found to prolong survival in advanced lung cancer when it is added to the standard chemotherapy regimen. Bevacizumab is given intravenously every two to three weeks. However, since this drug may cause bleeding, it is not appropriate for use in lung cancer patients who are coughing up blood, if the lung cancer has spread to the brain, or in people who are receiving anticoagulation therapy. Furthermore, bevacizumab is not used in cases of squamous cell cancer because it leads to bleeding from this type of cancer cells. Ramucirumab (Cyramza) is another angiogenesis inhibitor that can be used to treat advanced non-small-cell lung cancer.

- Immunotherapy drugs work by strengthening the activity of the immune system against tumor cells. The drugs nivolumab (Opdivo) and pembrolizumab (Keytruda) are checkpoint inhibitors targeting molecules that control the immune response. These two drugs target the PD-1 protein, which strengthens the immune response against cancers. Atezolizumab (Tecentriq) and durvalumab (Imfinzi) are examples of drugs that target PD-L1, a protein related to PD-1 that is found on some tumor cells and immune cells.

- Radiofrequency ablation (RFA) is sometimes used for small tumors located near the outside of the lungs as an alternative to surgery, particularly in cases of early stage lung cancer. In this type of treatment, a needle is inserted through the skin into the cancer, usually under guidance by CT scanning. Radiofrequency (electrical) energy is then transmitted to the tip of the needle where it produces heat in the tissues, killing the cancerous tissue and closing small blood vessels that supply the cancer. Studies have shown that this treatment can prolong survival similarly to surgery when used to treat early stages of lung cancer but without the risks of major surgery and the prolonged recovery time associated with major surgical procedures.

- Experimental therapies: Since no therapy is currently available that is absolutely effective in treating lung cancer, patients may be offered a number of new therapies that are still in the experimental stage. New drugs or new combinations of drugs are tested in clinical trials as well as lung cancer treatment vaccines. Newer types of immunotherapy are also being studied that involve the use of vaccine-related therapies attempting to utilize the body's immune system to directly fight cancer cells.

- Photodynamic therapy: Photodynamic therapy (PDT) is a procedure that treats precancerous cells, in addition to other types of cancer cells. The medical treatment does this with the help of a photosensitizing drug and a light source that activates the applied drug, destroying cancer cells. For patients with early-stage non-small cell lung cancer, photodynamic therapy is primarily employed as an endobronchial therapy to definitely treat endobronchial, roentgenographically occult (non-visible in X-rays), or synchronous primary carcinomas. For patients with advanced-stage non-small cell lung cancer, photodynamic therapy can be used to palliate obstructing endobronchial lesions, as a component of definitive multi-modality therapy, or to increase operability or reduce the extent of operation required [15].

1.2 Molecular alterations associated with lung cancer

Neoplasms are characterized by a rapid and uncontrolled cellular division, with increased survival due to dedifferentiation phenomena and accumulation of genetic alterations. The scientific approach to investigation into cancer pathogenesis requires a suitable knowledge of cell division's control mechanisms. Three levels can be individualized in cell proliferation's control mechanism: 1) activation of growth factor receptors from their ligands; 2) signal transduction from activated nuclear receptors; and 3) activation of cell division. Every alteration of this chain may cause loss of cell division control, independently of all the other regulative events. Most of the identified oncogenes can be considered components of this model: growth factors, growth factor receptors, transduction signals, or transcription nuclear activators [16]. **Table 1** summarizes the most commonly observed somatic alterations in lung cancer.

Table 1 : Major somatic alterations in lung cancer [2]. AC, adenocarcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung cancer

Gene	PATHWAY	ABERRATION	%AC	%SCC	%SCLC	Drugs, approved and investigational
EGFR	RTK	Mutation Amplification	20-30% >20%	rare 7%		Erlotinib, Gefitinib, Afatinib (approved), Dacomitinib, Cetuximab, Necitumumab, Neratinib
ALK		Fusion with EML4 and other rare partners	3-13%			Crizotinib (approved), X-396, LDK378; Ganetispib, AUY922, AT13387
MET		Mutation, amplification post-treatment with EGFR inhibitor	5% 20%			Tivantinib, Cabozantinib, Ornatuzumab, Tivantinib
ERBB2		Mutation, Amplification	2-4% 5-10%			Trastuzumab, Afatinib, Neratinib, MGHA22
ERBB3		Mutation		2%		MM-121
ROS		Mutation	1.5 %			Crizotinib, AT13387 (HSP90)
RET		Translocation with KIF5B and other genes	1-2%			Vandetanib, Cabozantinib ?
FGFR1		Amplification	1-3%	22%	6%	AZD4547, BGJ 398, BIBF 1120/nintedanib, dovitinib, HGS1036
DDR2		Mutation		3.8%		Dasatinib
IGF1R		Overexpression	ND	ND	95%	AXL1717, OSI-906
KRAS	RAS	Mutation	30%	5%		Selumetinib, Trametinib, MEK162, and BKM120, everolimus,sirolimus AUY922, BYL719,Reolysin MEK162
NF1			8-10%	11%		
HRAS				3%		
NRAS			<1%	<1%		
RASA1				4%		
BRAF	RAF	Mutations	6%	4%		Vemurafenib (only for V600E)
PIK3CA	PI3K	Mutation	rare	16%		BKM120, PX-866, GDC-0941
PTEN		Deletion	rare	8%		BKM120, PX-866, GDC-0941 (PI3K), MK-2206 (AKT)
AKT1,2,3			rare	16%(AKT3) 20% all		MK-2206
TSC1,2				6%		Everolimus, sirolimus, temsirolimus
LKB1	LKB1/AMPK	Mutation	15-30%	2%		Biguanide compounds
TP53	TP53	Mutation Copy number loss	50% 20%	80%	70%	
MDM2		Amplification	20%			Inhibitors of TP53 – MDM2 interaction
CDKNA2 /p16INK4	RB1/ CDK	Deletions, silencing, mutation	>20%	72%		CDK inhibitors PD0332991, BAY1000394
RB1		Mutation	rare	7%	100%	
CCNE1		Copy gain	12%			
MYC MYCN MYCL	Transcriptional regulators	Amplification	31%	rare	16%	Aurora kinase inhibitors, BH3 mimetics
SMARCA4	Epigenetic regulation	Mutation	10%			
ARID1A		Mutation	8%			
SETD2		Mutation	5%			
MLL2		Mutation		19%		
EP300		Mutation			9%	
CREBBP		Mutation			9%	
MLL		Mutation			10%	
KEAP1	Oxidative stress response	Mutation	11%	12%		
NRF2		Mutation		19%		
CUL3		Mutation		7%		
NKX2.1/TTF1	Developmental pathways	Amplification	20%			
SOX2		Amplification, overexpression		21%		
TP63		Amplification, overexpression		16%		
NOTCH1, NOTCH 2		Mutation		8 and 5%		
ASCL4		Mutation		3%		

Cyclins-CDKIs-PCNA

A key role in cell cycle control is played by cell cycle kinases (CDKs), relatively small proteins with an apparent molecular mass between 33 kDa and 43 kDa. Their activity is regulated by their arrangement in a multimeric complex with cyclins. Different cyclin–CDK complexes, formed with precise timing throughout the cell cycle, together with their phosphorylation/dephosphorylation, efficiently regulate the activity of the multimeric holoenzyme. Conversely, CDK–cyclin complexes are negatively modulated by the binding of CDK inhibitors (CDKIs), namely the CIP (p21, p27 and p57) and INK (INK4a/p16; INK4b/p15; INK4c/p18 and INK4d/p19) families. The p21 family (also known as the CIP/KIP family) includes three related proteins: p21, p27 (also known as CDKN1B or KIP1), and p57 (also known as CDKN1C or KIP2). The CIP CDKIs bind to the cyclin–CDK complex by contacting both subunits via different motifs to block kinase activity and substrate binding. They are localized predominantly in the nucleus in most tissues, but they have also been frequently observed in the cytoplasm, where they have been linked to CDK-independent functions and tumor development. In particular, reduced nuclear p27 and accumulation of cytoplasmic p27 have been observed in multiple types of human cancers and are associated with poor prognosis of breast cancer. p27 shares homology sequence regions with p21 and its product is able to stop E cycline /cdk2, A cycline /cdk2 and D cycline/cdk4 complexes' activity [16]. p21 (also known as CDKN1A, CIP1, or WAF1), binds to and inhibits the activity of multiple CDK–cyclin complexes, such as E cycline /cdk2, A cycline /cdk2, D1, D2, D3 cyclines/cdk4. p21 is one of the most important mediators of the p53 pathway. In fact, the p21 promoter contains two recognizable elements by p53, through which p21 expression is upregulated. p21 is able to inhibit cdk4/D cyclin and cdk2/E cyclin complexes' kinase activities, which are necessary for the beginning of S phase. In the case of DNA damage, during the replication process, p21 is able to inhibit A cycline / cdk2 complex, a necessary event for cell cycle progression through the S phase. In NCSLC, p21 plays a critical role in drug resistance, as it is required for cancer cells to become sensitive to Gefitinib, an FDA-approved EGFR inhibitor. p21 can also stop DNA replication by binding PCNA and forming a quaternary complex with cdk2 and A cycline (**Figure 8**). Proliferating cell nuclear antigen (PCNA) is involved in the activation of DNA polymerase d, which is required for DNA replication and repair. PCNA inhibition through the p53–p21 pathway can disrupt DNA replication without affecting the DNA repair function of PCNA [17]. PCNA plays an important role in tumor proliferation and its high protein and mRNA levels have been associated with the occurrence, development, and prognosis of NSCLC in recent clinical studies [18].

P53

The P53 tumor suppressor gene encodes a protein that acts as a transcription factor for several cell cycle regulatory proteins, including the CDKN1A ($p21^{CIP1}$) gene. Inactivating mutations in P53 are the most common alterations affecting any specific gene in human cancer. Inactivating mutations in P53 disrupt its key function in controlling cellular proliferation and apoptosis. Some mutations in P53 confer "gain of function" and the resulting mutant p53 protein contributes to different stages of tumorigenesis or to drug resistance. Mutations in P53 in lung cancer are strongly associated with smoking, and are also the most common somatic alterations in NSCLC, occurring in over half cases of adenocarcinoma, 80% of squamous cell carcinoma and 70% of small cell lung cancer. The P53 locus is also frequently affected by copy losses. Mutations in P53 are associated with poor prognosis and resistance to chemotherapy [2].

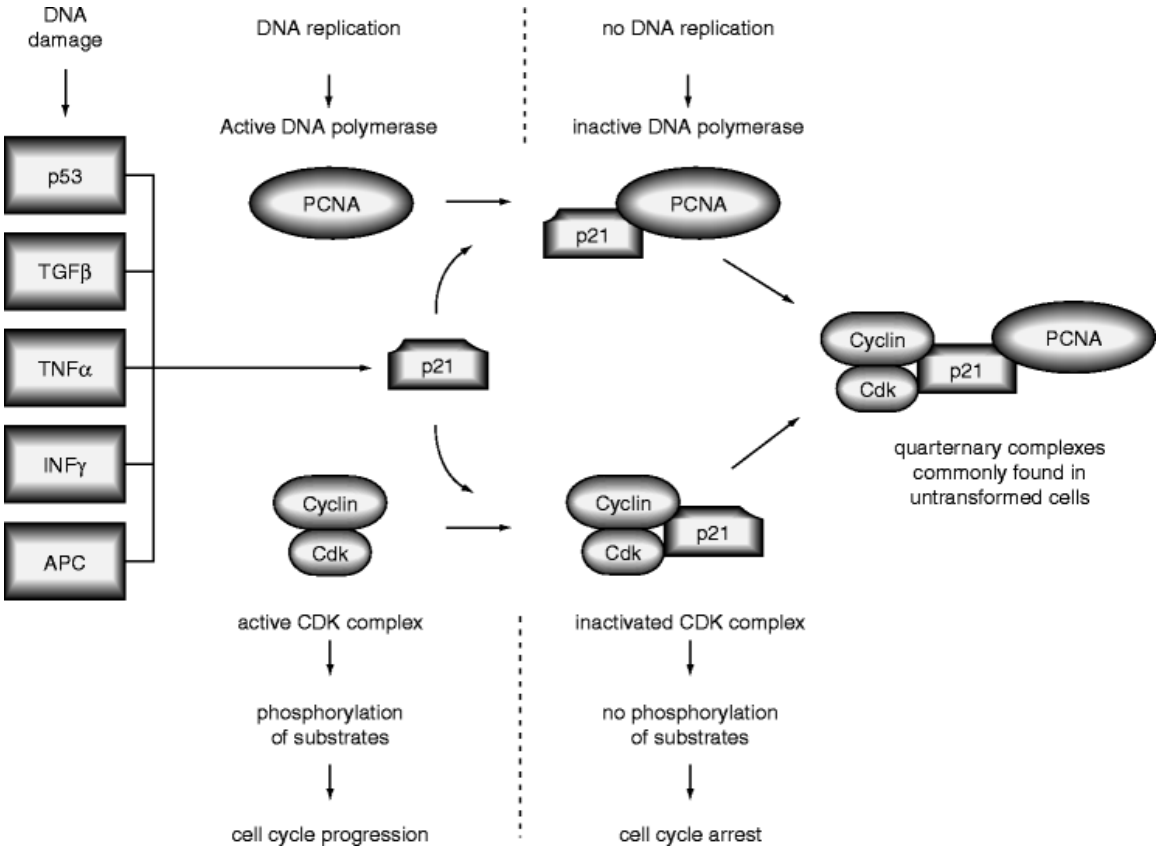

Figure 8: p21 binds to the DNA polymerase subunit PCNA and inactivates its DNA-replicative function. Consequently, DNA replication, but not DNA repair, is blocked. p21 also binds to cyclin/cdk complexes and inhibits their enzymatic activity. As a result, the substrates of the cyclin/cdk complexes are not being phosphorylated, and the cell cycle is arrested. Thus, through the increase of p21 levels, cells are able to stop proliferation. This process can be reversed: a reduction in the amount of p21 protein releases the inhibition of PCNA and of cyclin/cdk, and cell growth resumes. *Schönthal A.H. (2011) p21. In: Schwab M. (eds) Encyclopedia of Cancer. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-16483-5_4327*

EP300

p300 (*EP300* or *KAT3B* gene) is a transcriptional co-activator with intrinsic acetyltransferase activity. Being a large adaptorprotein, p300 bridges the basal transcription machinery to DNA sequence-specific transcription factors, thereby stabilizing the transcription complex. This protein is often recruited to assembled regulatory complexes, where it acetylates histone tails in the vicinity. In this way, p300 promotes localized chromatin accessibility and, subsequently, selective transcriptional initiation. p300 is a co-activator of the tumor suppressor p53 and acetylates it on several lysine residues, mainly on its C-terminal domain (**Figure 9**). *EP300* has been qualified as a classical tumor-suppressor gene, but with a low detected mutation rate in cell lines. Its growth-suppressing effect might be well explained by its ability to augment p53-mediated transcription. Following DNA damage, p53 is activated by kinase-mediated phosphorylation as well as by acetylation at specific residues by CBP/p300, resulting in increased stability of the p53-CBP/p300-DNA complex [19]. However, recent studies revealed that p300 promotes proliferation, migration, and invasion of NSCLC cells, through the induction of epithelial-mesenchymal transition, while low p300 levels constituted an independent prognostic marker of better survival in operable NSCLC patients [20].

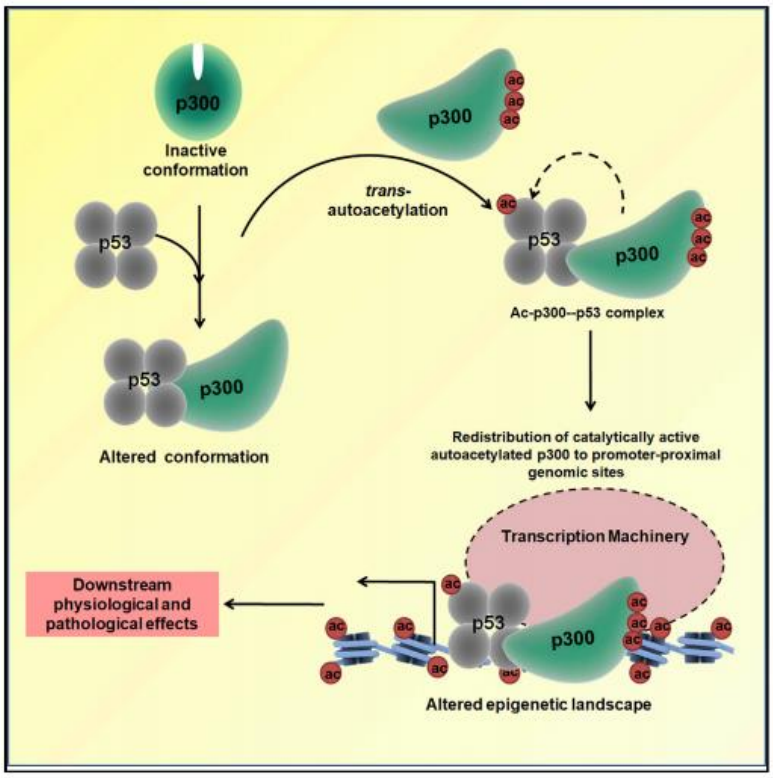

Figure 9: p53 activates p300 catalytic activity by altering its structural conformation. Induction of p300 autoacetylation possibly enhances p53-targeted gene expression.

https://doi.org/10.1016/j.isci.2018.06.002

EGFR-PI3K-AKT

Of the ~90 known tyrosine kinases (TKs), the receptor TKs (RTKs) form a group of 58 cell-surface growth factor receptors with ligand-mediated TK activity. Whereas RTK activity in normal resting cells is tightly regulated, mutations or deregulated expression might cause them to function as potent oncogenes. Epidermal growth factor receptor is the prototypical member of a family of four RTKs, EGFR (ERBB1, HER1), ERBB2 (HER2, Neu), ERBB3 (HER3) and ERBB4 (HER4). The greatest sequence homologies among the four genes are in the TK domain (59–81% identity). Multiple ligands activate epidermal growth factor receptor (EGFR), including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, heregulin, and heparin binding-EGF 3, leading to the activation of Class I phosphoinositide – 3 kinases (PI3K). Once activated, PI3K phosphorylates phosphotatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2] generating phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3]. PtdIns(3,4,5)P3 initiates Akt activation by its translocation to the plasma membrane, leading to a conformational change in Akt. Subsequently, Akt is phosphorylated at three regulatory sites and in turn phosphorylates multiple downstream targets involved in key cellular processes including apoptosis, metabolism, cell proliferation, and cell growth. EGFR deregulation has been observed in multiple tumor types, including NSCLCs, due to either overexpression, amplification, or mutation. EGFR overexpression is often associated with adverse prognosis [18]. Although several EGFR inhibitors are currently available (FDA-approved drugs include erlotinib-Tarceva, gefitinib-Iressa, and afatinib-Gilotrif), nearly all patients with EGFR-mutant-adenocarcinoma develop resistance to the first-generation inhibitors erlotinib and gefitinib. Tyrosine kinase domain (TKD) mutation and particularly exon 20 insertion mutations of *ERBB2* have been extensively reported in NSCLC as drivers of tumorigenesis. Moreover, rearrangements and copy number amplification have been reported as oncogenic mutations, rendering ERBB2 a poor prognostic factor of overall survival in NSCLC as well as a resistance mechanism to EGFR tyrosine kinase inhibitors [21]. Acquired amplification or mutation of PIK3CA (the gene encoding the p110a subunit of class I PI3K) result in a marked upregulation of the PI3K signaling itself and have been shown to cause resistance to selective PI3K inhibitors [22]. Akt promotes cell survival by blocking the function of pro-apoptotic proteins and promoting the induction of cell survival proteins. *AKT* is a proto-oncogene and its expression and activation is increased in many cancers, including NSCLC, while it is involved in the resistance of cancer cells to chemotherapy and radiation treatment [23].

Mammalian target of rapamycin (mTOR)

PI3K/AKT/mTOR signaling is one of the most important intracellular pathways, which can be considered as a master regulator for cancer (**Figure 10**). Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that functions as a key regulatory protein in normal cell growth, survival, metabolism, development, and angiogenic pathways. Activation of mTORC1 is initiated by Akt-mediated phosphorylation and sequential inactivation of the tuberous sclerosis complex. Once phosphorylated, the tuberous sclerosis complex loses its ability to suppress Rheb1, which subsequently activates mTORC1 [23]. Aberrant activation of the Akt/mTOR pathway is commonly observed in lung cancer. mTOR has been reported activated in 90% of adenocarcinomas, 40% of squamous cell carcinomas, and 60% of large cell carcinomas. Data suggest that mTOR activation is associated with both KRAS and epidermal growth factor receptor (EGFR) mutation and may be a mechanism of resistance to treatment with EGFR inhibitors. There are also data demonstrating that mTOR activation correlates with lymph node metastasis [4].

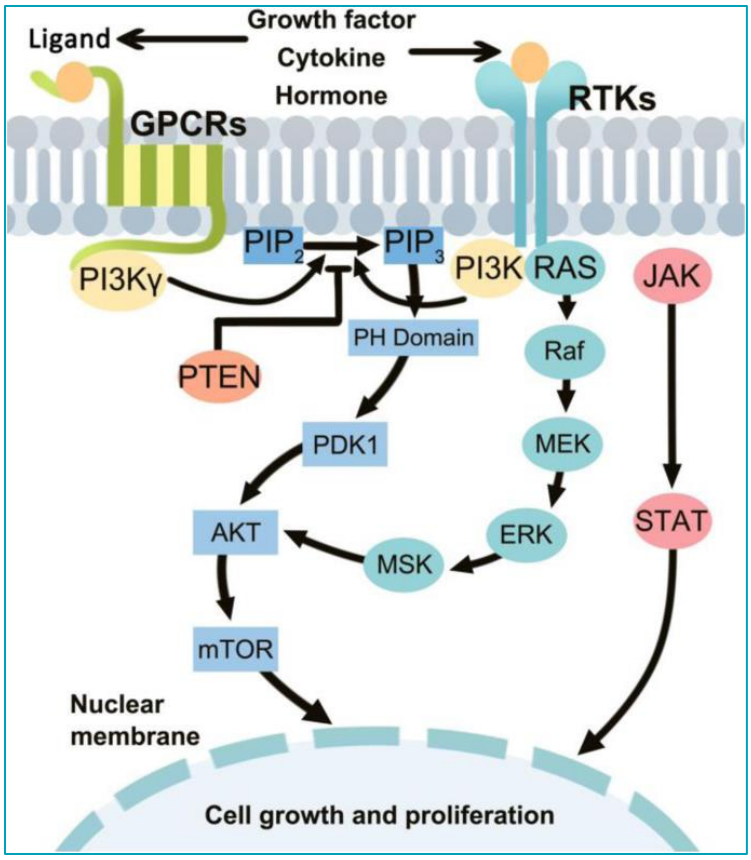

Figure 10: Overview of the PI3K/AKT/mTOR signaling pathway[4].

C-MYC

The proto-oncogene and master regulator of transcription, *MYC*, controls transcription networks governing proliferation, metabolism, ribosome biogenesis, and protein synthesis. However, when overexpressed, *MYC* induces expression of pro-apoptotic protein BAX, which induces mitochondrially-mediated apoptosis. *MYC* is amplified in >30% of adenocarcinomas and in 16% to 30% of SCLC patients. MYC-family genes (*C-MYC, N-MYC, L-MYC*) are amplified in a mutually exclusive manner in SCLC, as observed in other tumors of neuroendocrine origin [2]. However, the overexpression of *C-MYC* without its amplification has been found in more than 50% of NSCLC samples. *C-MYC* overexpression has been identified as a delayed event in LC pathogenesis in the majority of SCLC patients. Cell lines derived from metastatic tumors showed a high incidence of *C-MYC* amplification and that finding may explain the relationship between *C-MYC* amplification and poor clinical prognosis [24].

Notch

The Notch family consists of transmembrane proteins that have dual function, as membrane receptors and as transcription factors. In mammals, the Notch family has 4 receptors (Notch 1, 2, 3, and 4) and 5 ligands (Delta 1, 3, 4 and Jagged 1 and 2). When a ligand binds to its receptor, the pathway is activated and TACE metalloproteinase (tumor necrosis factor-A converting enzyme) and γ-secretase complex degrade the receptor by proteolysis. The intracellular part of Notch (NICD) is then released into the cytoplasm and transferred to the nucleus, where it activates the transcription of target genes (**Figure 11**). The main target genes of that signaling pathway are two families of transcription factors, Hes (HES1-7) and Herp, as well as the genes encoding Cyclin D1, p21, NF-kB, preTa, GATA3, NRAPP, c-Myc and Deltex1 [24]. The abnormal expression of Notch signaling pathway members, including Notch receptors, ligands and downstream genes, is relatively frequent in NSCLC studies [25]. In fact, Notch3 has been found highly expressed in 51.1% of patients with NSCLC, while gain-of-function mutations of Notch1 were present in 10% of patients with NSCLC. Notch signaling ligand DLL4 and its target gene, Hes family bHLH transcription factor 1 (Hes1), were inversely associated with the overall survival rate in patients with NSCLC. However, the expression and function of Notch1 are distinct in different phases of tumor development. Notch1 stimulates NSCLC tumor growth and survival through direct upregulation of IGF-1R and survivin in hypoxia. Notch3 promotes cell proliferation and inhibits apoptosis of NSCLC cells [25].

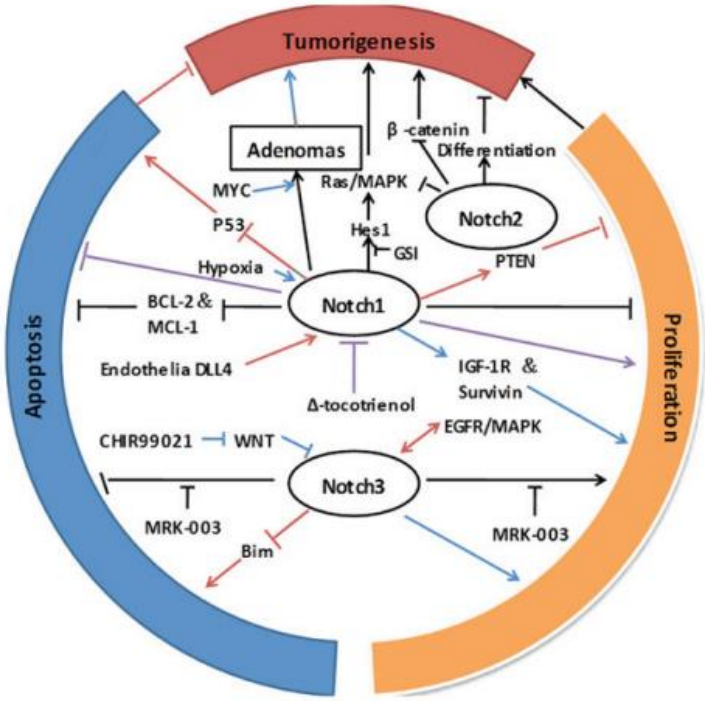

Figure 11: Roles of Notch signaling in tumorigenesis, proliferation, and apoptosis of NSCLC cells.

BMI-1

BMI-1 (Moloney murine leukemia virus integration site 1) is an epigenetic chromatin modifier that acts as a key component of the PRC1 complex to mediate transcriptional repression. It was initially identified as an oncogene that cooperates with c-myc in generation of B-cell lymphomas. The oncogenic potential of BMI-1 is, in part, because of negative regulation of the Ink4a/Arf locus that encodes two proteins, p16/INK4a and p19/ARF that suppress proliferation and promote apoptosis. *BMI-1* is highly expressed in a variety of human tumors, and promotes rapid proliferation of cancer stem cells leading to tumor metastasis and recurrence. Moreover, knocking down the expression of BMI-1 can induce senescence and apoptosis in some tumor cells, while rendering them sensitive to radiation and chemotherapy [26]. BMI-1 is overexpressed in non-small-cell lung cancer (NSCLC) and is correlated with poor prognosis for lung cancer patients [27].

1.3 Nuclear receptors

Nuclear receptors (NRs) are transcription factors capable of directly binding to DNA sequences (DNA response elements) and have cell-type and tissue-specific target genes. NRs, upon ligand-dependent or -independent activation, can up- or down-regulate the expression of a variety of genes in several tissues [28]. To achieve this genetic control, NRs share a common canonical structure of four functional domains. From N-terminus to C-terminus, these are: the domain required for the receptor to activate the promoters of the target genes (ligand-independent AF-1 transactivation domain, A/B domain), the zinc- finger domain needed for binding to the DNA response elements (DNA-binding domain-DBD, C domain),the domain responsible for ligand or hormone binding (ligand-binding domain-LBD, ligand-dependent AF-2 transactivation domain, E/F domain) and the domain D which functions as a flexible hinge between DBD and LBD, and includes the nuclear localization signal (NLS)[29] **(Figure 12)**.

Figure 12: Nuclear receptors contain four major functional domains: the N-terminal domain (A/B domain), the DNA-binding domain-DBD (C domain), a hinge region (D domain), and the ligand-binding domain-LBD (E/F domain). AF-1, ligand-independent transactivation domain; H, hinge; AF-2, ligand-dependent transactivation domain [28].

The nuclear receptor (NR) superfamily is composed of 49 members in humans and includes receptors for steroid hormones, thyroid hormones, various lipids, and oxysterols that act as transcription factors to regulate development, homeostatic physiology, and cellular metabolism. Approximately half of the superfamily has well characterized natural ligands whereas the remaining receptors are considered orphan receptors and remain a focus of several investigators assessing their ability to be regulated by ligands. The vast majority of receptors that have identified natural ligands are also validated targets for clinical drugs. This superfamily has been a rich source of drug targets for myriad diseases including inflammation, cancer, and metabolic disorders. Thus, there remains significant interest in the identification of ligands that regulate orphan members of the NR superfamily, due to their potential for utilization as prospective drugs to treat human disease [29].

1.3.1 NR5A2 nuclear receptor

The orphan nuclear receptor NR5A2 (Nuclear Receptor 5A2), or LRH1 (Liver Receptor Homolog 1) belongs to the subfamily 5A of nuclear receptors that also includes the close homolog, steroidogenic factor-1 (SF-1), and is encoded by the 1q32.11 genetic locus. The human NR5A2 gene consists of 8 exons extending to over 150 kb of chromosome 1, while at least three LRH-1 isoforms derived from alternative splicing exist in humans. Mouse LRH-1 was initially identified because of its homology to the Drosophila Fushi tarazu factor 1 (Ftz-F1; NR5A3). Three binding sites for GATA transcription factors and one motif that recognizes Nkx homeodomain proteins are conserved between the mouse and human LRH-1 promoter. The *NR5A2* gene is conserved in human, chimpanzee, Rhesus monkey, dog, mouse, rat, chicken, zebrafish, mosquito, and frog, while there are currently 328 organisms known to harbor *NR5A2* orthologues [30].

Members of the NR5A subfamily bind DNA with high affinity as monomers to the Ftz-F1-consensus binding site YCA AGG YCR (where Y is any pyrimidine and R is any purine). In the NR5A subfamily, specificity of DNA binding is dictated by the Ftz-F1 box, which is a stretch of 26 amino acids at the C terminus of the DBD functional domain. The LBD of most NRs consists of twelve conserved a-helical regions, numbered H1–H12, which are folded into a three-layered, antiparallel helical sandwich with a conserved b-turn between H5 and H6 (**Figure 13)**. The LBD also contains the ligand-binding pocket, the size of which varies among NRs. Ligand binding induces a reversible conformational change that repositions H12 in the LBD, enabling coactivator recruitment and subsequent transcriptional activation. Orphan members of the Ftz-F1 subfamily are constitutively active. The N-terminal region of the LBD, including H1, H2 and H3, is conserved among members of the NR5A subgroup and shares limited sequence similarity with other NR subfamilies. In contrast to ligand activated NRs, interaction of this N-terminal region with the remaining part of the LBD is ligand-independent in NR5A NRs [30].

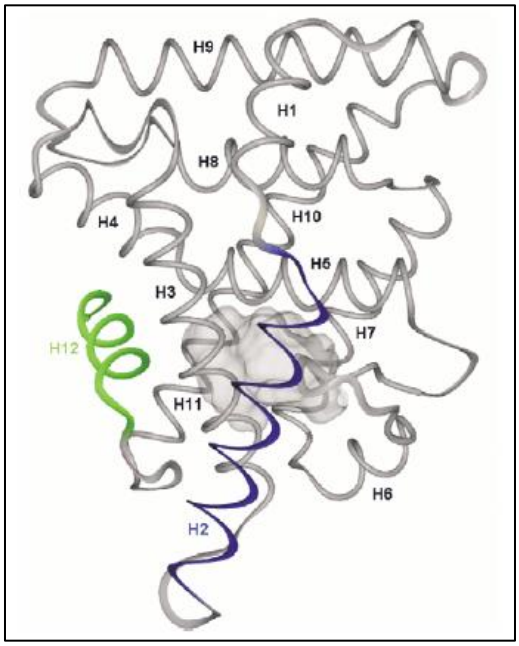

Figure 13: 3D structure of the ligand-binding domain (LBD) of mouse liver receptor homolog-1 (LRH-1). Helix (H) 12, the position of which determines the active state of a nuclear receptor, is highlighted in green. The extended NR5A-specific H2 (blue) provides an additional layer to the canonical LBD fold and stabilizes the active position of H12 in the LRH-1 crystal structure. The empty ligand-binding pocket is shown in gray.

In adult mammals, *LRH-1* expression is confined principally to tissues of endodermal origin, such as the liver, pancreas, and intestine – justifying its functional classification as an enterohepatic NR. *LRH-1* is also expressed in the ovary, pre-adipocyte and, at lower levels, in the placenta. In the adrenal and testis, LRH-1 expression seems to be species-specific. Moreover, LRH-1 has been shown to drive early embryogenesis, since knockout mice die between E6.5-E7.5 showing morphological characteristics of unsuccessful gastrulation and endoderm dysfunction [30-32]. Several studies have demonstrated the role of NR5A2 during development. There are reports on its ability to replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells (iPSCs - induced pluripotent stem cells), while other studies have elucidated its essential role in the maintenance of pluripotency in embryonic stem cells at the epiblast stage, where it colocalizes with Nanog and Sox2 [33, 34].

Later on during organogenesis, NR5A2 is involved in the proper function of diverse organs, as well as metabolism homeostasis, renal fibrosis, circadian rhythms and steroidogenesis [35, 36]. It is well established that LRH-1 regulates critical enzymes involved in cholesterol homeostasis and bile-acid biosynthesis in the liver as it is involved in reverse cholesterol transport, bile acid synthesis, and enterohepatic bile acid circulation. In the liver, LRH-1 regulates the expression of cholesteryl-ester-transfer protein (CETP), which remodels the high-density lipoprotein (HDL) particles that transport cholesterol from peripheral tissues back to the liver. LRH-1 also induces scavenger receptor type BI (*SR-BI*) expression, thereby facilitating cholesterol uptake from HDL by the liver. Once in the liver, cholesterol is converted to bile acids, a step also controlled by LRH-1 through the regulation of cholesterol 7a-hydroxylase (CYP7A1) and sterol 12a-hydroxylase (CYP8B1). NR5A2 interacts with the orphan nuclear receptor SHP-1 and the latter inhibits LRH-1-mediated transactivation of the CYP7A1 promoter. This repression is also attributable to Prox1 (Prospero homeobox protein 1) transcription factor; Prox1, as identified through a yeast two-hybrid screening, can directly interact with human LRH-1 and suppress LRH-1-mediated transcriptional activation of human CYP7A1 gene [30].

Most importantly, NR5A2 appears to be involved in tumorigenesis by controlling proliferation of progenitors and cancer cells from various tissues.

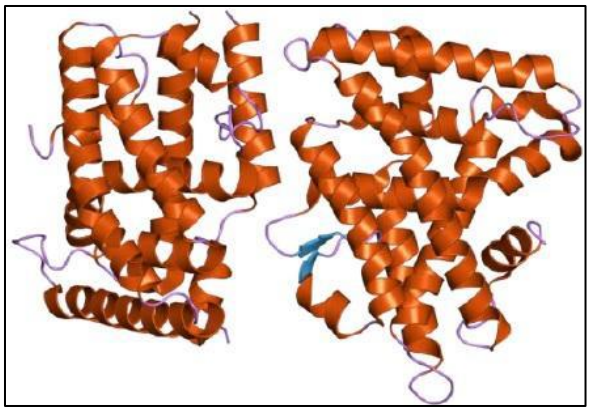

Figure 13: 3D illustration of NR5A2 protein structure.

1.3.2 The role of NR5A2 in cancer

In general, nuclear receptors constitute critical cell cycle regulators and define the progression of various cancer types. Different studies have shown that nuclear receptors can act either as oncogenes or as tumor suppressors, favoring or inhibiting tumor formation respectively.

Given the availability of high quality genomic data for multiple tumors it is possible to investigate NR expression and function individually and in gene networks across different tumor and tissue controls. In most cancer types, nuclear receptors are found downregulated; however, in contrast to other transcription factor families, rarely is this attributed to copy number variation (CNV) or genetic mutations. Therefore, an interesting implication of this observation is the idea that epigenomic, rather than genomic, mechanisms may be the drivers for this phenomenon, and possibly that while NRs are downregulated in cancer, they may remain functional [37].

The role of NR5A2 in cancer has not yet been clearly defined. Several studies have linked its expression with increased cell proliferation, tumor growth and progression, as well as epithelial-mesenchymal transition (EMT), and cancer cell resistance to chemotherapy in different human cancers [38, 39]. It has been shown that when NR5A2 is paired with β-catenin, cell cycle genes *CCND1, CCNE1*, and *MYC* are affected.

COLON CANCER

Recent studies showed that LRH-1 and β-catenin interact and contribute to tumorigenesis of the gastrointestinal system. Specifically, it was revealed that LRH-1 promotes cell cycle progression by two mechanisms; first, LRH-1 induces cyclin D1 expression in a DNA binding-independent manner by acting as a coactivator for b-catenin/Tcf4. Second, LRH-1 binds directly to the promoter of cyclin E1. Thus, the combination of both DNA-dependent and -independent transcriptional events leads to accelerated cell cycle progression in intestinal cells [36]. Moreover, LRH-1 enhanced the expression of key steroidogenic enzymes Cyp11A1 and Cy11B1, increasing immune-regulating corticosteroid levels and allowing tumor cells to escape the host's natural immune response. Furthermore, haploinsufficiency of LRH-1 was shown to reduce intestinal tumorigenesis in two independent murine models that are commonly used to study intestinal tumorigenesis. Alterations in both the expression and subcellular localization of LRH-1 were observed and further underscored the critical role of LRH-1 in intestinal tumorigenesis [40, 41].

PANCREATIC CANCER

Genome-wide association studies linked mutations in the *LRH-1* gene and its upstream regulatory regions to development of pancreatic cancer. *LRH-1* transcription was activated up to 30-fold in human pancreatic cancer cells compared to normal pancreatic ductal epithelium. This activation correlated with markedly increased LRH-1 protein expression in human pancreatic ductal adenocarcinomas in vivo [42]. In the adult pancreas, NR5A2 cooperates with the acinar-specific transcription factor complex pancreas-specific transcription factor 1 (PTF1) to directly bind and activate several acinar-specific genes. Loss of acinar gene expression has been suggested to represent a first step of pancreatic ductal adenocarcinoma (PDAC) development. Recent studies have shown that acinar cells transiently downregulate NR5A2 during recovery from pancreatitis and this downregulation persists during PDAC formation. Moreover, NR5A2 was not required for the development of the pancreatic acinar lineage but was important for maintenance of acinar identity. NR5A2 deletion lead to destabilization of the mature acinar differentiation state, acinar to ductal metaplasia and loss of regenerative capacity following acute pancreatitis. Loss of NR5A2 also dramatically accelerated the development of oncogenic Kras-driven acinar to ductal metaplasia and PDAC precursor lesions [43]. Accordingly, another study revealed that reduced or eliminated NR5A2 dosage dramatically sensitized the murine pancreas to Kras-induced pancreatic intraepithelial neoplasia progression [44]. Together, these findings suggest that NR5A2 inhibits the ductal transformation of adult acinar cells by mutant *KRAS*, providing functional evidence supporting a potential role of *NR5A2* as a susceptibility gene for human PDAC.

BREAST CANCER

LRH-1 regulates estrogen receptor α (ERα) expression and controls ERα target genes by cooperative binding with ERα at estrogen response elements. *LRH-1* is also expressed in adipose tissue and cancer-associated fibroblasts surrounding the tumor microenvironment where it controls cytochrome P450 aromatase expression (*CYP19A1*), the essential enzyme required for estrogen synthesis, thereby acting in a paracrine manner on neighboring tumor cells. Nevertheless, *LRH-1* is also expressed in ERα-negative breast cancer cells. The cyclin-dependent kinase inhibitor CDKN1A ($p21^{CIP1/WAF1}$) was found to be overexpressed in breast cancer cell lines following *LRH-1* depletion through a p53-independent manner, while LRH-1 was associated with poor prognosis [45]. Additionally, LRH-1 levels have been found elevated in primary breast cancer tissues in patients who developed early recurrence. Similarly, adriamycin (ADR)-resistant breast cancer cell lines exerted high *LRH-1* expression, while overexpression of *LRH1* attenuated cytotoxicity of chemotherapeutic drugs ADR and cisplatin (DDP) in breast cancer cells *in vitro* and in a nude mice tumor model [39]. Thus, LRH-1 is considered to enhance breast cancer cell chemoresistance by transcriptionally activating the mediator of DNA damage checkpoint 1 (MDC1) to attenuate drug-induced DNA damage [39].

As we can conclude by the cases described above, NR5A2 cannot be defined as a tumor-inducing or –suppressing nuclear factor, since its expression and function appear diverse among different tissue types.

However, recent clinical data from the Oncomine database, on several human cancer types connect the downregulation of NR5A2 with disease progression in various tissues, such as the prostate, bladder, liver, kidney, lung, and brain **(Figure 15)**. This emphasizes the important role of this receptor in a wide range of cancer and tissue types and demonstrates its possible tumor-suppressing activity.

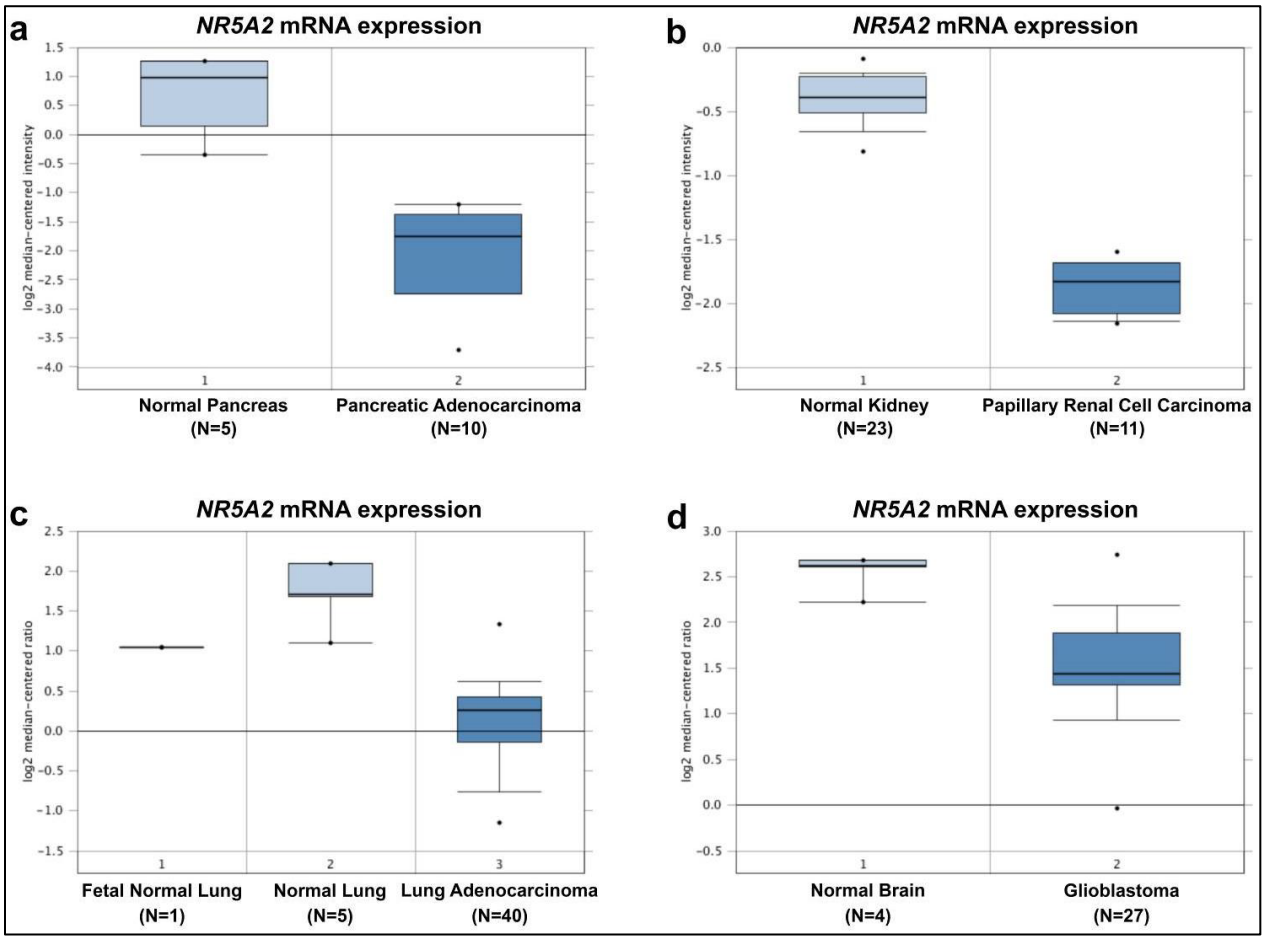

Figure 15: Meta-analysis of NR5A2 expression data, based on transcriptome sequencing studies from the Oncomine database, across multiple cancer types: a) pancreas, b) kidney, c) lung and d) brain. N, number of human samples.

1.3.3 Synthetic Agonists of NR5A2

Several studies revealed that different phospholipid species, including phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidyl choline, as well as the second messengers phosphatidyl inositols, can bind to the large ligand binding pocket of human LRH-1, but much less to mouse LRH-1. Thus, a cluster of non-conserved residues in the human LBD seems to be essential for proper binding of phospholipids [46].

In particular, NR5A2 can be targeted by lipophilic compounds, which are acting as pharmacological agonists. For example, dilauroyl-PC (DLPC) and diundecanoyl-PC (DUPC) have been shown to act as direct and potent ligands of NR5A2 that induce its transcriptional activity [47]. Both phospholipids were able to induce activation of LRH-1-specific promoters such as SHP and OCT-4, whereas closely related phospholipids such as dipalmitoyl PC (DPPC; C16:0/C16:0) did not. In addition, a previous study demonstrated that these phospholipids were specific to NR5A family members and did not cross-react with other families of nuclear receptors such as PPARa. Mammalian two-hybrid assays confirmed the ability of these two phospholipids to bind to LRH-1 and selectively recruit important coactivators such as SRC-3 [48].

Therefore, although NR5A2 is considered an orphan receptor, a number of reports have identified the presence of bacterial phospholipids in its binding pocket [29], and remains to be determined whether these represent merely structural or fortuitous ligands or if they can modulate receptor activity.

1.4 Aims - Experimental Hypothesis

As supported by a variety of experimental studies, NR5A2 constitutes a multifunctional nuclear factor, exerting a pivotal role in embryonic development and participating in cell proliferation-related processes, among others. In fact, several studies have identified NR5A2 as an important oncogene in different cancer types, as is commonly observed for other nuclear factors, with the exception of pancreatic cancer where it appears to have an anti-proliferative effect. Nevertheless, very little is currently known regarding its role in the development and progression of lung cancer, and non-small cell lung cancer (NSCLC) in particular, while the exact mechanisms through which cancer cell proliferation is regulated remain unmapped. Although recent studies reported that NR5A2 could be considered a driver of tumorigenesis and predictor of metastasis in NSCLC, clinical data from the Oncomine database indicate a trend of *NR5A2* downregulation in lung adenocarcinoma tissues compared to normal lung tissues. Thus, conflicting results render the role of NR5A2 in human NSCLC still unclarified. Based on these observations we attempted to study the role of NR5A2 in human NSCLC cell lines. Specifically we used three cell lines for lung adenocarcinoma, and one for large cell lung carcinoma to study the effect of *NR5A2* overexpression on cell proliferation using adenoviral vectors, while one adenocarcinomic cell line was also used for knockdown studies as well (stable shRNA-mediated degradation of endogenous NR5A2 mRNA). Moreover, DLPC, a synthetic lipophilic compound was administered in the aforementioned cell lines with the aim of studying its function as an NR5A2 agonist in human NSCLC *in vitro*. Furthermore, one adenocarcinomic cell line was employed for mouse xenografts generation to study the effect of NR5A2 overexpression *in vivo*.

2. Materials and Methods

2.1 Culture of human lung cancer cell lines

Human lung cancer cell lines were cultured in either Dulbecco's Modified Eagles Medium (DMEM, Biosera, France) or RPMI 1640 (Biosera, France), supplemented with 1% L-glutamine (200 mM, Gibco, Thermo Scientific, USA), 1% Penicillin-Streptomycin (5,000 U/mL, Gibco, Thermo Scientific, USA) and 10% Fetal bovine serum (FBS, Gibco, Thermo Scientific, USA). Proliferative cultures were incubated at 37°C in a humidified 5% CO_2 incubator and subculture was carried out by washing the cell monolayers with calcium and magnesium-free phosphate buffered saline (PBS) followed by addition of 1x Trypsin-EDTA solution (Trypsin-EDTA 10X, Biosera, France) and incubation at 37°C until the cells detached. Trypsin was inactivated by addition of growth medium before seeding into fresh flasks or plates. The cell lines used in this study were the following:

- A549 (ATCC® CCL-185™, USA) adenocarcinomic alveolar basal epithelial cells
- A549 cells stably expressing shNR5A2, puromycin-resistant (produced in-house)
- EKVX adenocarcinomic lung epithelial cells
- NCI-H460 large cell lung cancer cells derived from pleural effusion
- NCI-H1944 adenocarcinomic lung cancer cells (stage 3B) derived from metastatic site

EKVX, NCI-H460, and NCI-H1944 were kindly provided by Dr G. Stathopoulos, Faculty of Medicine, University of Patras.

2.2 Adenoviral transduction of human lung cancer cell lines

During transduction assays, recombinant adenoviruses (constructed in-house) were introduced in proliferative cell cultures. The control cultures received Adeno-GFP viral particles (expressing the eGFP gene), while the experimental ones received Adeno-NR5A2 viral particles (expressing the human NR5A2 gene and eGFP gene). Viruses were added in the culture medium and cells were cultured for 6 hours, according to the standard protocol. The exogenous expression of NR5A2 was confirmed by RT–qPCR.

2.3 DLPC treatment in human lung cancer cells

Phospholipids (dilauroyl-phosphatidylcholine, DLPC, Avanti Polar Lipids, USA) dissolved in ethanol were added in human lung cancer cell cultures in DMEM or RPMI and incubated for 24 h or 48 h in two different concentrations (150 uM, 200 uM). The quantities used per well in a 24-well plate are shown in **Table 2.**

Table 2: Reagents used during DLPC treatment on lung cancer cell cultures in three different conditions.

	EtOH	150 uM DLPC	200 uM DLPC
DLPC	-	7.5 ul	10 ul
100% EtOH	7.5 ul	-	-
Growth medium	42.5 ul	42.5 ul	40 ul
Total	50 ul	50 ul	50ul

2.4 2D Wound healing assay

During 2D wound healing assays, a confluent cell monolayer is deliberately destructed to create a cell-free region, which is then available for cells to bridge and repair. The method applied in this study for 2D wound healing experiments was the scratch assay or mechanical wounding. A549 cells were seeded in 12-well plates and cultured until confluent. When confluency was achieved, a (yellow) pipette tip was used to make a straight scratch across the entire diameter of each well, simulating a wound in the monolayer. After scratching, the medium was removed, and the wells were washed twice in PBS solution to remove all cellular debris. Fresh medium and treatments (adenoviral particles or DLPC) were added to each well, and cells were grown for up to 92 hours. Images were obtained from the same fields immediately after scratching (t_0) and every 24 hours thereafter using an inverted microscope and a LEICA camera at x10 total magnification. Data were analyzed using the ImageJ software. The percentage of wound closure was calculated using the following formula:

$$[100 - (\text{Wound area } t/\text{Wound area } t_0)] \times 100$$

2.5 Immunofluorescence

70–80% confluent cells were replated on coverslips into 24-well plates in a density of 3 x 10^4 cells/well, 24 h before adenoviral transduction or DLPC treatment. For BrdU (5-bromo-2'-deoxyuridine) staining, 4.5 ul BrdU 1 mM were added per well, followed by incubation at 37°C for 2 hours. Subsequently, cells were fixed using 4% paraformaldehyde (PFA) solution and labelled with the following primary antibodies:

- anti-BrdU antibody (rat, Abcam, 1:400 dilution)
- anti-GFP antibody (chicken, Abcam, 1:400 dilution)
- anti-phospho-Histone 3 (rabbit, Abcam, 1:600 dilution)
- anti-Ki67 (mouse, BD Pharmingen, 1:1000 dilution)

The secondary antibodies used for signal development were conjugated with AlexaFluor 488 (green, 1:200 dilution), 555 (red, 1:400 dilution) or 647 (far red, 1:400 dilution), all purchased from Molecular Probes. All antibodies were detected with the standard immunofluorescence experimental protocol [49]. Cell nuclei were labelled with DAPI (1:2,000 dilution, Molecular Probes). Specimens were viewed and analyzed with fluorescent microscopy (Leica DM RXA2 upright microscope). Statistical analysis was performed using the two-tailed paired Student's t-test.

2.6 RNA extraction and real-time RT–qPCR

Total RNA was isolated by TRI reagent solution (TRI Reagent, T9424, Sigma-Aldrich) according to manufacturer's instructions. RNA concentration and purity were measured by Nanodrop 2000c (Thermo Scientific, United States), and 1 mg was used for cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, USA) together with random hexamer primers. Quantitative Real time PCR was performed in a LightCycler 96 Instrument (Roche, Switzerland). Measured values were normalized using ACTB (β-actin) and RPL13A mRNA levels as internal references. Primer sequences used in RT–qPCR assays were as follows:

Target genes	**Direction**	**Primer sequence (5'-3')**
human NR5A2	forward	AGCACCTTTGGGCTTATGTG
	reverse	GCAGCTTCATTTGGTCATCA
human ACTB (β-actin)	forward	CTCTTCCAGCCTTCCTTCCT
	reverse	AGCACTGTGTTGGCGTACAG
human RPL13A	forward	GCGGACCGTGCGAGGTAT
	reverse	CACCATCCGCTTTTTCTTGTC
human CCND1 (Cyclin D1)	forward	CCCTCGGTGTCCTACTTCAA
	reverse	AGGAAGCGGTCCAGGTAGTT
human CCNE1 (Cyclin E1)	forward	ATCCTCCAAAGTTGCACCAG
	reverse	AGGGGACTTAAACGCCACTT
human CDKN1A (p21^{CIP1})	forward	GGAAGACCATGTGGACCTGT
	reverse	GGCGTTTGGAGTGGTAGAAA
human CDKN1B (p27^{Kip1})	forward	AGAGTTAACCCGGGACTTGG
	reverse	GCCCTCTAGGGGTTTGTGAT
human STAT3	forward	CAACTTCAGACCCGTCAACA
	reverse	CGATTCTCTCCTCCAGCATC
human EP300	forward	AACCACATGACACACTGCCA
	reverse	GGGGAGACACACAGGACAAT

human EGR1	forward	GAGATGCAGCTGATGTCCCC
	reverse	GCTCAGCAGCATCATCTCCT
human DLL3	forward	GTCTACACCGAGCAGCCC
	reverse	TGAAAGAGAAGGTGCCAGGC
human HES1	forward	ATGACAGTGAAGCACCTCCG
	reverse	CGTTCATGCACTCGCTGAAG
human HEY2	forward	ACTCGGGGCAAAGTACTAGC
	reverse	ATTTATCCGATCCCGACGCC
human Notch3	forward	GTGGACGAGTGCTCTATCGG
	reverse	CCGACAGACACTCGTTGACA
human EGFR	forward	ATGTGGTGACAGATCACGGC
	reverse	AGGCCCTTCGCACTTCTTAC
human PIK3CA	forward	CCCAGGTGGAATGAATGGCT
	reverse	AGCACCCTTTCGGCCTTTAA
human mTORC	forward	AACGAGCTGGTCCGAATCAG
	reverse	AGGTTTTGTTCCGAAGCCCA
human c-MYC	forward	CGTCCTCGGATTCTCTGCTC
	reverse	GCCTGCCTCTTTTCCACAGA
human ERBB2	forward	TGCTGGGGAGAGAGTTCTGA
	reverse	GGGATTGGGCATGGACTCAA
human AKT1	forward	ACTGTCATCGAACGCACCTT
	reverse	CTCCTCCTCCTCCTGCTTCT
human CCNG1	forward	TGAAGGTACAGCCCAAGCAC
	reverse	TCGGATCAAGTCAGTTGCCA

The ΔΔCt method was used to quantify all RT-qPCR results. All samples were tested in triplicates.

2.7 Western blot analysis

Total protein extracts were obtained from human lung cancer cells using radioimmunoprecipitation assay (RIPA) buffer. Cells were suspended in RIPA buffer mixed with a cocktail of protease inhibitors, and lysed using an ultrasonic sonicator. Clear supernatants containing the total solubilized proteins were subjected to immunoblot analysis with:

- mouse antibody raised against the human p27 peptide (BD biosciences, USA)
- rabbit antibody raised against the human p21 peptide (Santa Cruz Biotechnology, USA)
- rabbit antibody raised against the human NR5A2 peptide (kindly donated by Dr. I. Talianidis)
- mouse antibody raised against the human b-actin peptide (Abcam, United Kingdom)

Protein electrophoresis, transfer and western blotting were performed with the standard protocol [49]. Protein loads were verified with b-actin as reference protein.

2.8 Generation of cell line-derived mouse xenografts

A549 cells were cultured in 10-mm plates in DMEM supplemented with 1% L-glutamine, 1% Penicillin-Streptomycin and 10% Fetal bovine serum. Cells were transduced with either adeno-GFP or adeno-NR5A2 for 6 h three subsequent times. When confluency was achieved the culture medium was removed, cells were detached with Trypsin-EDTA, centrifuged and the pellet was resuspended in 1x PBS. Centrifugation and pellet resuspension was repeated two more times and the suspended cells were counted in a Neubauer hemocytometer. According to the desired cell concentration, both the control (ad-GFP) and experimental (ad-NR5A2) cultures were transferred to new tubes diluted in 1x PBS. Cells were injected in NOD/SCID mice (100 uL cell suspension/mouse) intraperitoneally and were allowed to form tumors.

2.9 Statistical analysis

For statistical analysis, the measurements and experimental values from independent experiments were estimated with the two-tailed paired Student's t-test, or ordinary one-way ANOVA. All the results are shown as mean ±SD values. P values lower than 0.05 ($P<0.05$) were considered statistically significant. All analyses were done using Microsoft Excel 2013 or GraphPad Prism 6.05 softwares.

3. Results

3.1 NR5A2 overexpression reduces proliferation in human lung cancer cells *in vitro*

In order to study the effect of NR5A2 overexpression *in vitro*, human lung adenocarcinoma A549 cells were cultured on coverslips in 24-well plates and were transduced with either adeno-GFP (control cultures) or adeno-NR5A2 viral particles. Two hours prior to cell fixation, BrdU was added in all wells. BrdU (Bromo-deoxy-uridine) is a synthetic nucleoside, an analog of thymidine, which is commonly used in the detection of proliferating cells as it is incorporated in the DNA during the S phase. Cell fixation was followed by immunofluorescence experiments with a view to detect the GFP, BrdU, and pH3 proteins in both control and experimental samples. pH3 (phosphorylated histone 3 or Phospho-Histone 3) is a specific marker of mitotic figures, as it emerges in pericentromeric chromatin in late G2 phase, it spreads in a systematic, non-random fashion throughout the condensing chromatin during prophase, persists through anaphase, and it is typically no longer detectable when mitosis is completed. Cell nuclei were stained with DAPI. In both control and experimental samples, the transgenic GFP protein was detected in the nucleus as well as the cytoplasm, as normally expected.

Immunofluorescence experiments were followed by counting of transduced cells and subsequent quantification of the BrdU and pH3 signals. The measurements revealed that the percentage of BrdU incorporation in adeno-GFP cells was 41.4%, while in the adeno-NR5A2 it only reached 6.6% (**Figure 16 a-b**). Similar results were observed regarding pH3-positive transduced cells, with adeno-GFP cells at 12.5% and adeno-NR5A2 cells at 0.48% (**Figure 16 c-d**).

Therefore, NR5A2 overexpression inhibited incorporation of BrdU in the nuclei of A549 cells, and reduced the number of pH3 positive cells in a statistically significant manner (**Figure 16**).

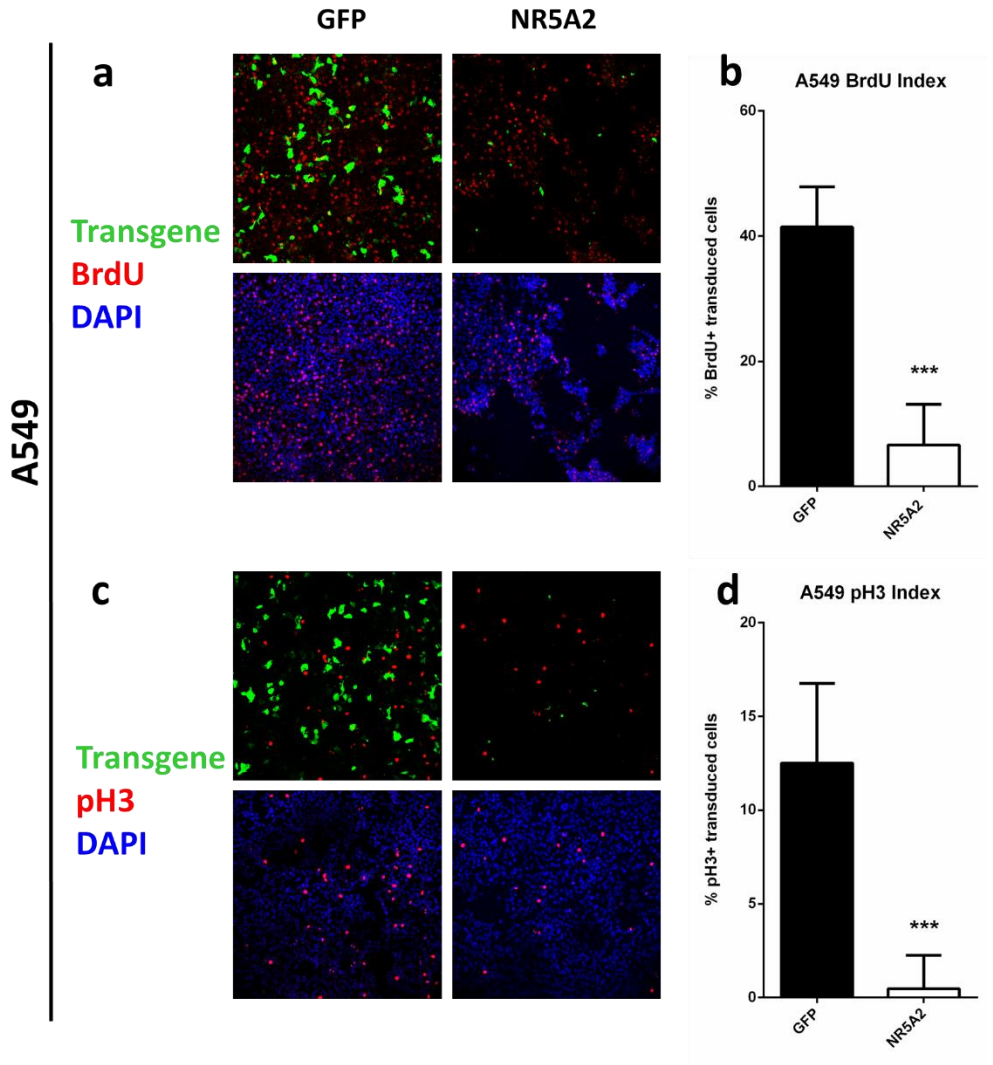

Figure 16: Immunofluorescence results of transduced A549 cells stained for BrdU **(a, b)** and pH3 **(c, d)**. Images were obtained at 20x magnification. Statistical analysis on the quantitative variables (number of BrdU+ and pH3+ transduced cells) was performed using the two-tailed paired Student's t-test (non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P< 0.0001).

Since a phenotypic change was observed in A549 cells, three additional human lung cancer cell lines were employed to examine whether the effect of NR5A2 overexpression was context-specific. Thus, the same experimental protocol was followed for EKVX (lung adenocarcinoma), NCI-H460 (large cell lung cancer), and NCI-H1944 (lung adenocarcinoma stage 3B) cells. Immunofluorescent staining and subsequent quantification of the BrdU and pH3 indices revealed a similarly decreasing trend in cell proliferation after NR5A2 overexpression (**Figures 17-19**).

In EKVX cells, the percentage of BrdU incorporation decreased 1.3 times (10.25% in adeno-GFP- compared to 7.53% in adeno-NR5A2-transduced cells), while pH3-positive cells were 5.2 times less after NR5A2 overexpression (4.27% in the control versus 0.82% in adeno-NR5A2-transduced cells) **(Figure 17**).

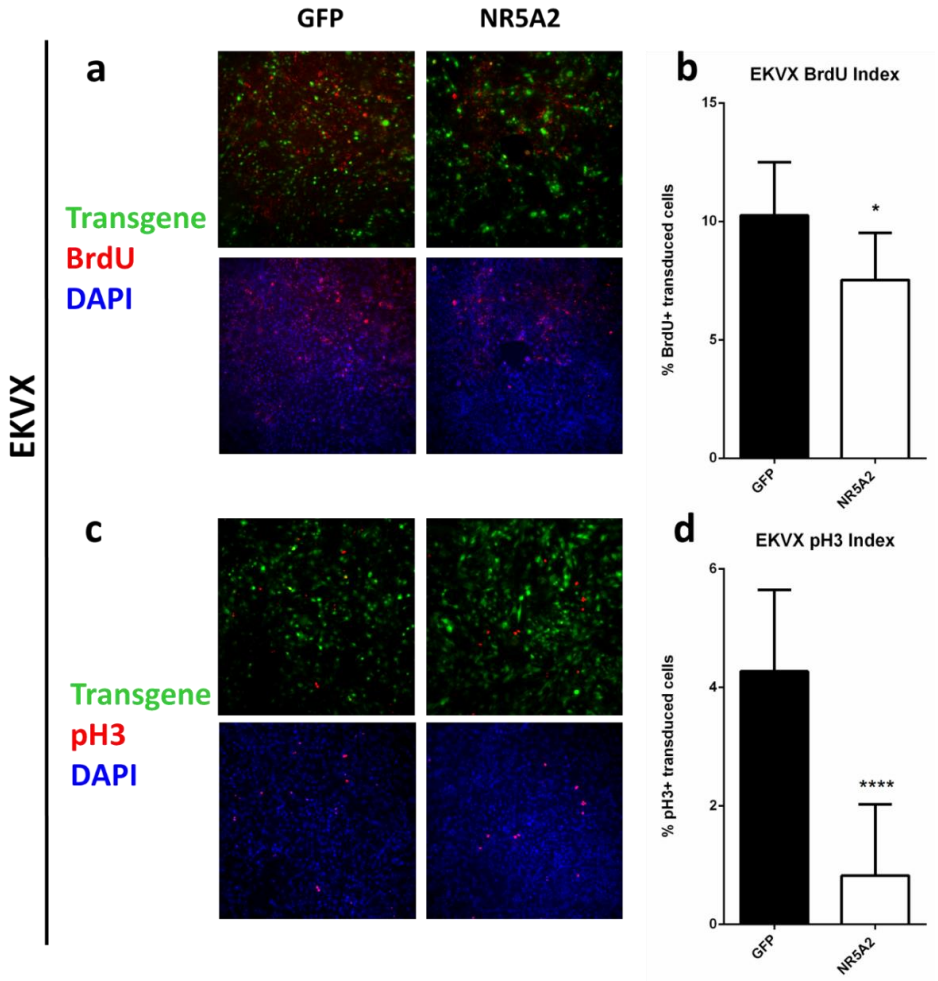

Figure 17: Immunofluorescence results of transduced EKVX cells stained for BrdU **(a, b)** and pH3 **(c, d)**. Fluorescent images were obtained at 20x magnification. Statistical analysis on the quantitative variables (number of BrdU+ and pH3+ transduced cells) was performed using the two-tailed paired Student's t-test (*P<0.05, **P<0.01, ***P<0.001, ****P< 0.0001).

Accordingly, in NCI-H460 cells, BrdU incorporation was reduced 1.3 times (20.47% in adeno-GFP- versus 15.59% in adeno-NR5A2-transduced cells), while the number of pH3-positive cells dropped 3.2 times (3.5% in the control versus 1.08% in adeno-NR5A2-transduced cells) (**Figure 18**).

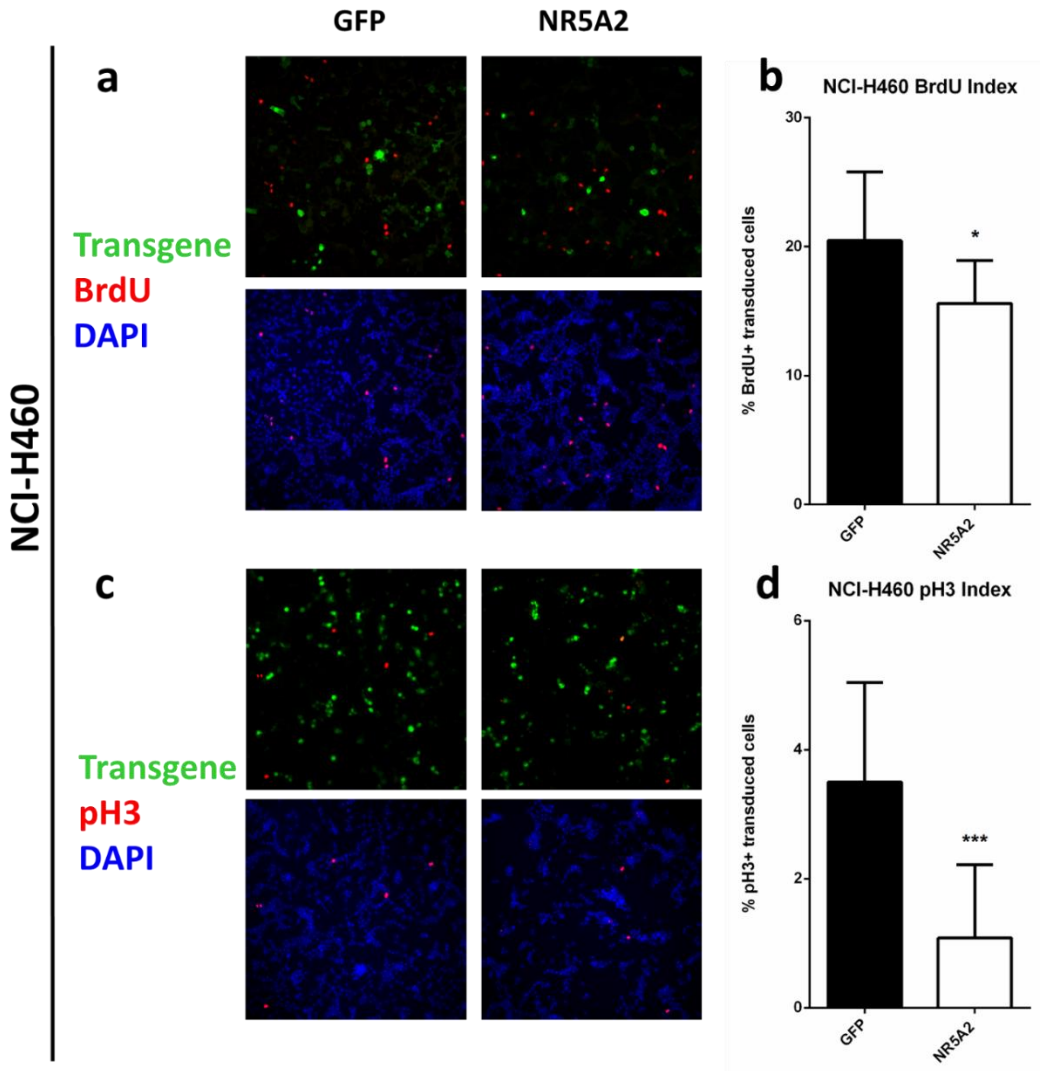

Figure 18: Immunofluorescence results of transduced NCI-H460 cells stained for BrdU **(a, b)** and pH3 **(c, d)**. All stained images were obtained at 20x magnification. Statistical analysis on the quantitative variables (number of BrdU+ and pH3+ transduced cells) was performed using the two-tailed paired Student's t-test (non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P< 0.0001).

Moreover, in NCI-H1944 cells the percentage of BrdU incorporation was 24.31% in adeno-GFP- contrary to 17.88% in adeno-NR5A2- transduced cells, while pH3-positive cells dropped from 4% to 0.97% after NR5A2 overexpression (**Figure 19**). In all cases, the observed differences were statistically significant and a greater decrease in the pH3 index was noted, compared to the BrdU index.

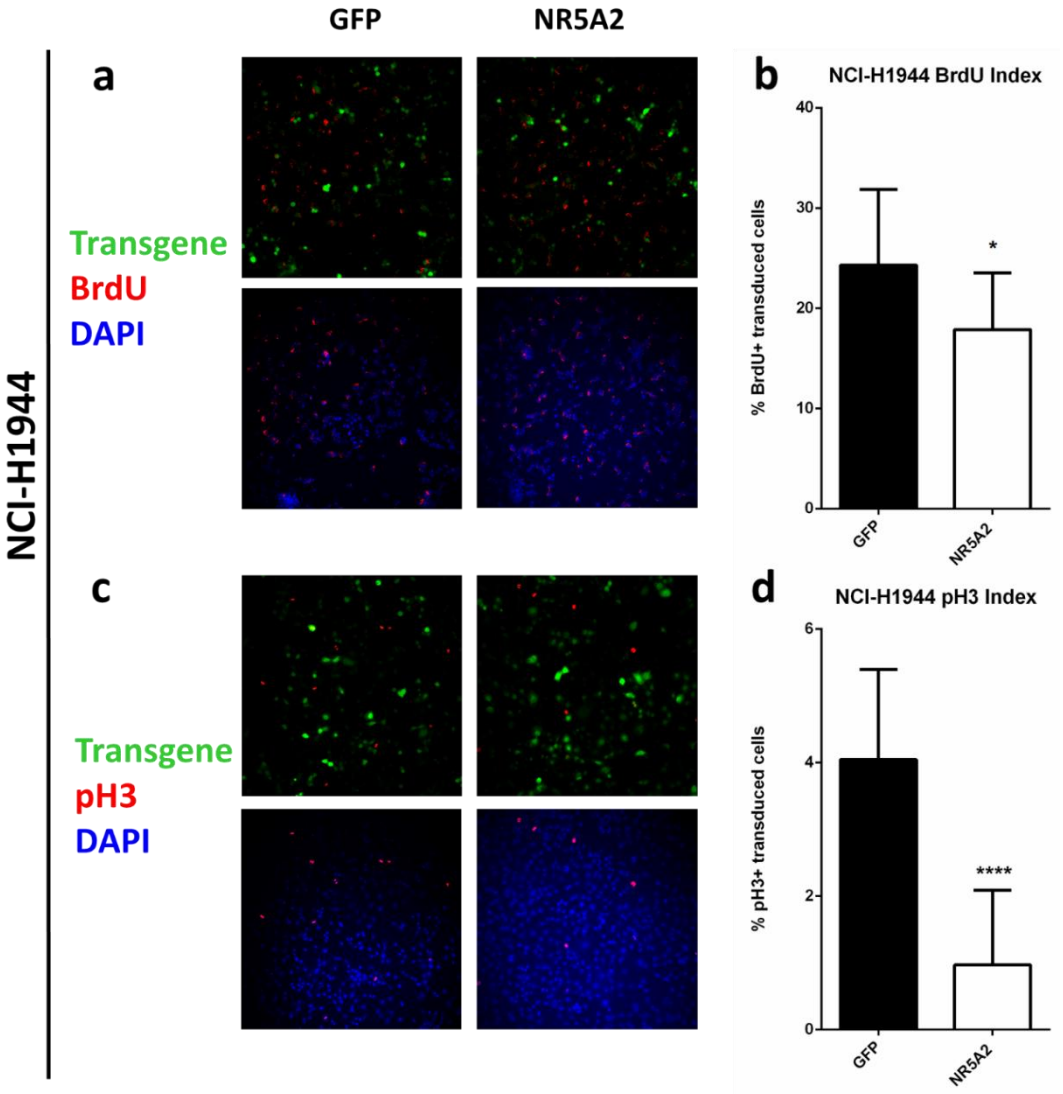

Figure 19: Immunofluorescence results of transduced NCI-H1944 cells stained for BrdU **(a, b)** and pH3 **(c, d**). Fluorescent images were obtained at 20x magnification. Statistical analysis on the quantitative variables (number of BrdU+ and pH3+ transduced cells) was performed using the two-tailed paired Student's t-test (*P<0.05, **P<0.01, ***P<0.001, ****P< 0.0001).

3.2 NR5A2 overexpression upregulates cell cycle inhibitors

After confirming that NR5A2 overexpression reduced the proliferation of A549, EKVX, NCI-H460, and NCI-H1944 cells by immunofluorescence experiments, we performed RT-qPCR screenings to detect differential expression of proliferation-related genes. All the employed cell lines were cultured for 48 hours post-transduction, when RNA extraction and cDNA synthesis were performed.

In A549 cells we tested the expression of:

- *P53*, encoding a transcriptional activator of cell cycle inhibitors
- *EP300*, encoding a transcriptional co-activator of p53
- *CDKN1A* (p21), encoding a cell cycle inhibitor controlling the G1-S transition
- *CDKN1B* (p27), encoding a cell cycle inhibitor controlling progression through the G1 phase
- *CCND1* (Cyclin D1), and *CCNE1* (Cyclin E1), encoding inducers of the G1-S transition
- *CCNG1* (Cyclin G1), encoding a driver of the G0-G1 transition in quiescent stem cells
- *PCNA*, encoding an activator of DNA replication and repair.

Real time qPCR results indicated increased expression of *CDKN1B* ($p27^{kip1}$) and downregulation of *CCND1* (Cyclin D1) after overexpression of NR5A2. *CCNE1* (Cyclin E1) mRNA levels were found elevated in NR5A2-overexpressing A549 cells, as well. *P53, PCNA*, which encode for cooperating molecules, and *CDKN1A* ($p21^{cip1}$) exhibited a trend of upregulation after NR5A2 overexpression. However, NR5A2 overexpression did not alter the expression of *CCNG1* and *EP300* (**Figure 20**).

Moreover, western blot analysis showed a trend of increasing $p27^{kip1}$ protein levels in NR5A2 overexpressing cells, supporting the qPCR results. In contrast, the protein levels of $p21^{cip1}$ appeared similar between the samples (**Figure 21**). NR5A2 overexpression was confirmed with both real time RT-qPCR and western blot analysis, while *ACTB* (b-actin) and *RPL13A* were the housekeeping genes used for normalization.

A549

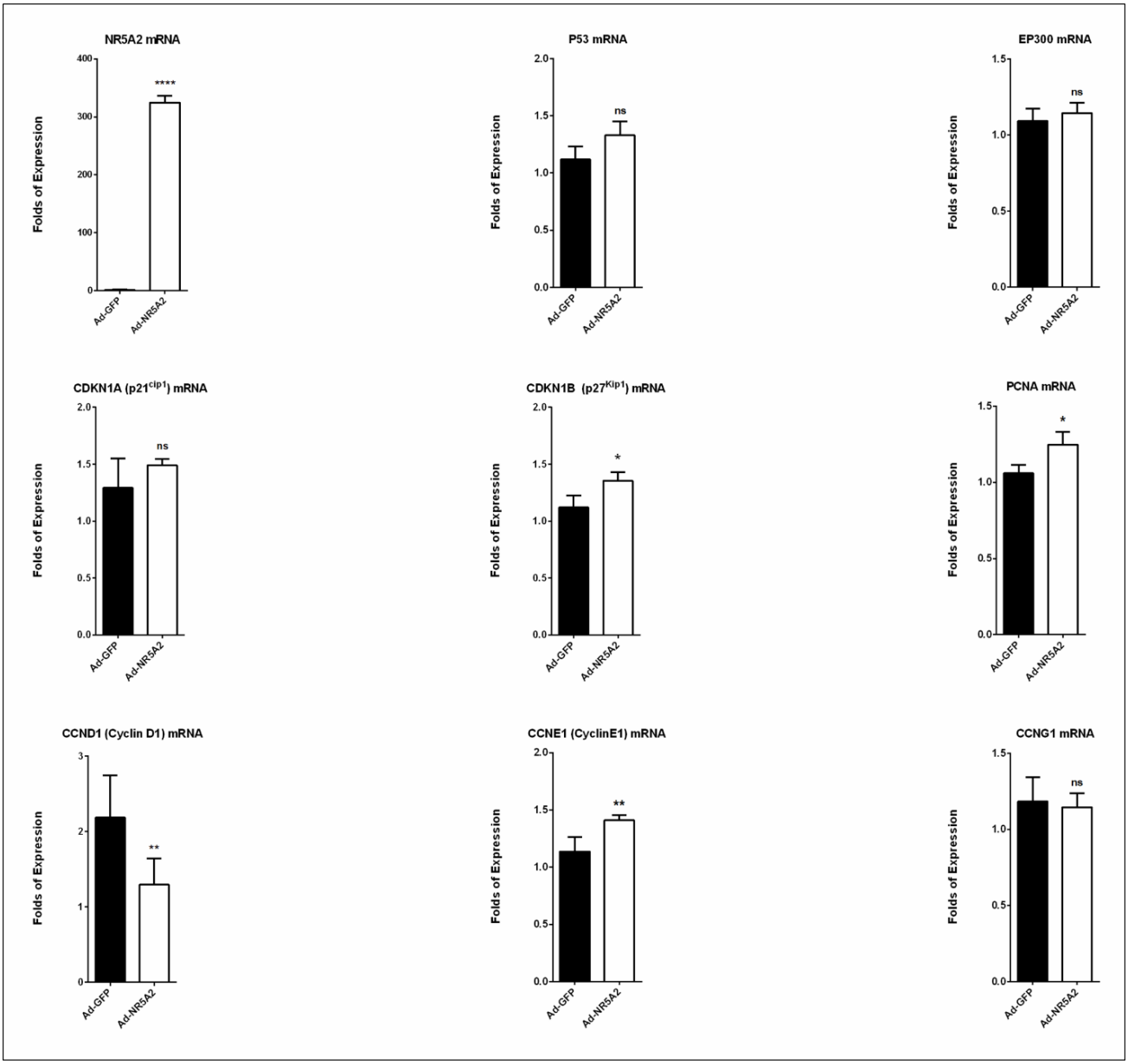

Figure 20: RT-qPCR analysis of the anti-proliferative effect of NR5A2 overexpression in A549 lung adenocarcinoma cells. The differential expression of *NR5A2, P53, EP300, CDK1A, CDK1B, CCND1, CCNE1, CCNG1*, and *PCNA* was analyzed using the ΔΔCt method. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

A549

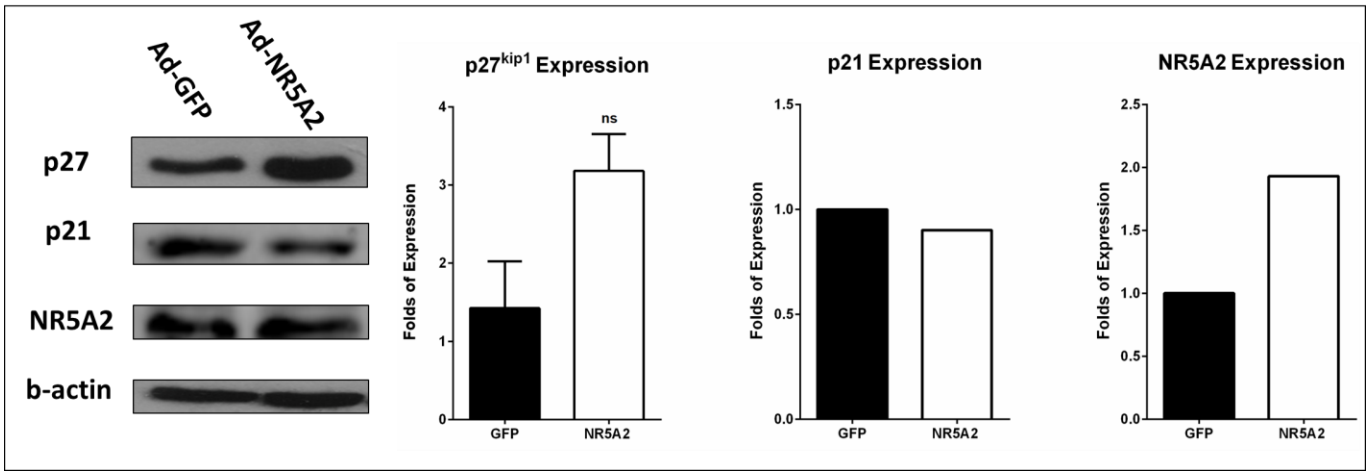

Figure 21: Comparison of the protein levels of cell cycle inhibitors p21 and p27 in A549 cells with and without NR5A2 overexpression. Western blot results were quantified using the ImageJ software. NR5A2 overexpression was depicted at the protein level as well. B-actin was used for normalization.

Subsequently, the expression of genes encoding components of the EGFR-PI3K-AKT and Notch signalling pathways, as well as the expression of several nuclear receptors was examined in A549 cells with and without NR5A2 overexpression. In particular, we tested the expression of:

- *EGFR*, encoding the epidermal growth factor receptor
- *ERBB2*, encoding a tyrosine kinase receptor of the EGFR family
- *PIK3CA*, encoding the PI3K kinase, downstream target of EGFR
- *AKT1*, encoding a serine/threonine-specific protein kinase that is activated by PI3K
- *mTOR*, encoding a kinase, target of the AKT1 protein
- *DLL3*, encoding an inhibitory Notch ligand
- *HES1, HEY2* encoding transcriptional repressors, downstream effectors of the Notch signaling pathway
- *STAT3*, encoding a signaling molecule and transcription factor
- *C-MYC*, encoding a transcription factor activating the expression of genes involved in cell growth and apoptosis
- *EGR1*, encoding a transcription factor
- *BMI-1*, encoding an epigenetic chromatin modifier.

Real time RT-qPCR results indicated a statistically significant decrease in the expression of *HES1* after overexpression of *NR5A2*. Conversely, the expression levels of *HEY2*, another downstream effector of the Notch pathway, were similar in A549 cells with and without *NR5A2* overexpression. *DLL3* also exhibited reduced mRNA levels but not in a statistically significant manner. *AKT1* and *mTOR*, which encode for interacting molecules, *STAT3*, and *BMI-1*, exhibited a trend of upregulation after *NR5A2* overexpression, which however could not be considered statistically significant. The mRNA levels of *EGFR, ERBB2, PIK3CA, C-MYC*, and *EGR1* appeared unaffected by NR5A2 overexpression (**Figure 22)**. All mRNA levels were normalized using the *ACTB* and *RPL13A* expression levels.

A549

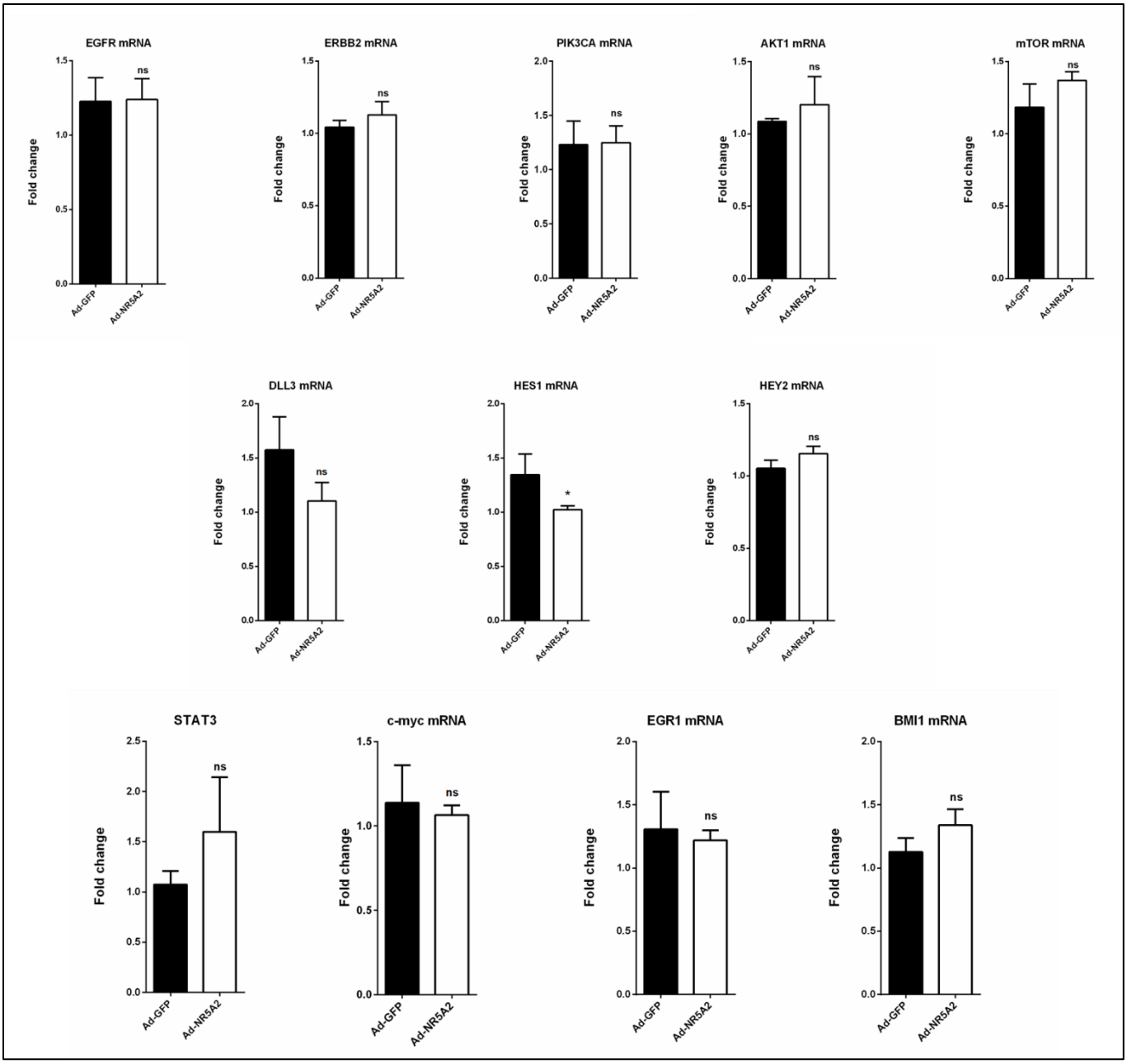

Figure 22: RT-qPCR screening of genes encoding key molecules in different pathways signaling cell proliferation and tumor growth in lung cancer, to study the effect of NR5A2 overexpression in A549 lung adenocarcinoma cells. The differential expression of EGFR, ERBB2, PIK3CA, AKT1, mTOR, DLL3, HES1, HEY2, STAT3, C-MYC, EGR1, and BMI-1 was analyzed using the ΔΔCt method. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

Following the same protocol for RNA extraction and cDNA synthesis after NR5A2 overexpression, RT-qPCR was performed for EKVX, NCI-H460, and NCI-H1944 cells as well.

In EKVX cells, we tested the expression of:

- *CDKN1A* (p21)
- *CDKN1B* (p27)
- *CCND1* (Cyclin D1)
- *CCNE1* (Cyclin E1)
- *HES1*
- *NOTCH1*, encoding the Notch1 transmembrane receptor
- *NOTCH3*, encoding the Notch 3 transmembrane receptor.

Real time RT-qPCR results revealed a 200-fold increase in NR5A2 mRNA levels confirming the effectiveness of the overexpression process. However, none of the investigated genes exhibited statistically significant differential expression between ad-GFP and ad-NR5A2 samples. In particular, the expression levels of *NOTCH3, HES1, CCNE1*, and *CDKN1B* were similar in EKVX cells with and without *NR5A2* overexpression. *NOTCH1* and *CDKN1A* exhibited increased mRNA levels but not statistically significant due to high standard deviation. Moreover, *CCND1* mRNA levels exhibited a trend of downregulation after *NR5A2* overexpression, which however could not be considered statistically significant (**Figure 23)**. All mRNA levels were normalized using the *ACTB* expression levels.

EKVX

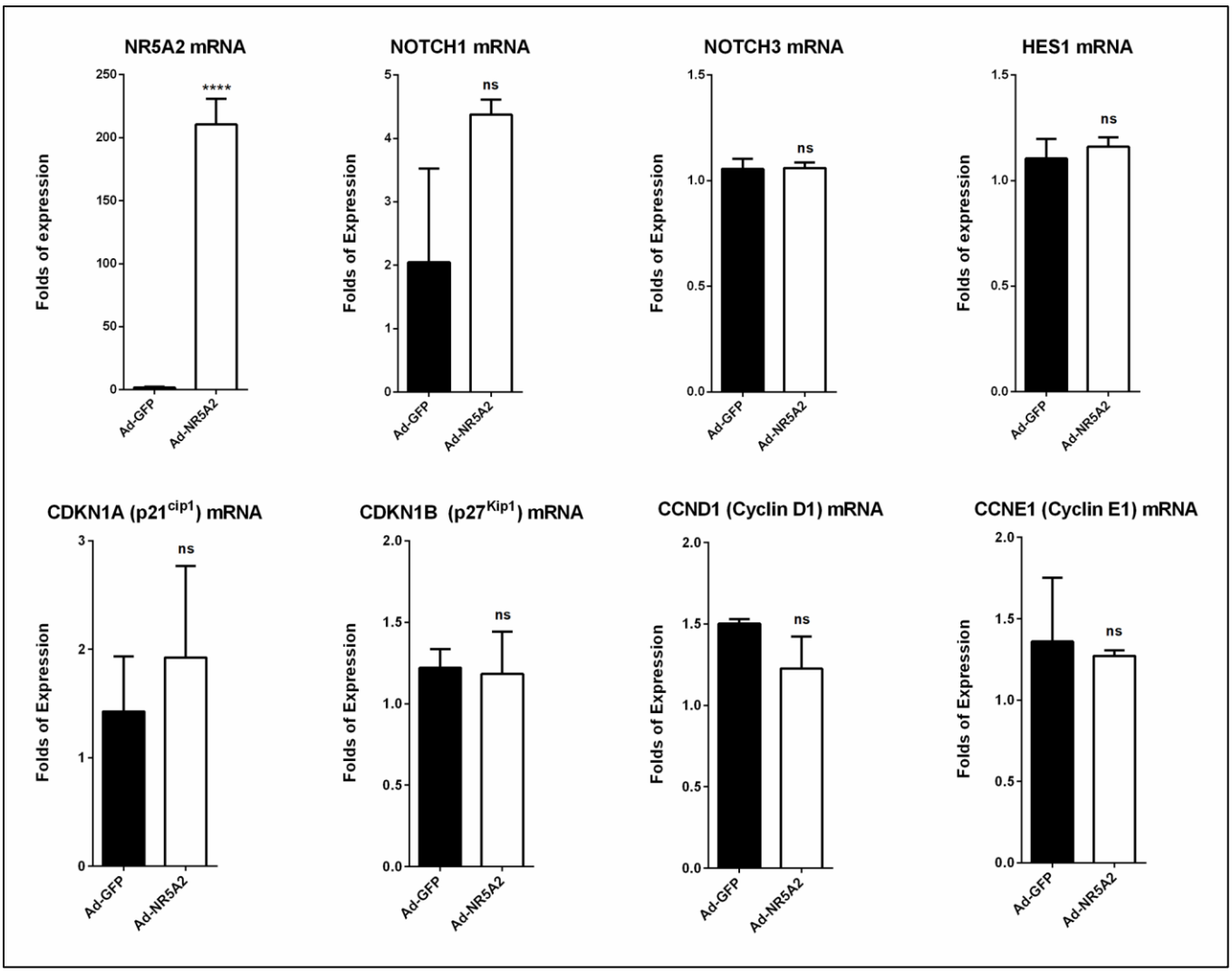

Figure 23: RT-qPCR **a**nalysis of the anti-proliferative effect of NR5A2 overexpression in EKVX lung adenocarcinoma cells. The differential expression of *NR5A2, NOTCH1, NOTCH3, HES1, CDKN1A, CDKN1B, CCND1*, and *CCNE1* was analyzed using the ΔΔCt method. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

In NCI-H460 cells, we tested the expression of:

- *PCNA*
- *HES1*
- *CDK1A*
- *CDK1B*
- *CCND1*
- *CCNE1*

Real time qPCR results revealed a 30-fold increase in NR5A2 mRNA levels. Moreover, some of the results were the opposite than expected regarding the differential expression of cell cycle-associated genes between ad-GFP and ad-NR5A2 samples. In particular, the expression levels of *PCNA, HES1, CDKN1A*, and *CDKN1B* were significantly downregulated in NCI-H460 cells after *NR5A2* overexpression. The expression levels of *CCND1* were significantly decreased after *NR5A2* overexpression, as observed in A549 and EKVX cells as well. *CCNE1* mRNA levels exhibited a trend of downregulation in ad-NR5A2 samples, which however could not be considered statistically significant (**Figure 24 a**). All mRNA levels were normalized using the *ACTB* expression levels.

Western blot analysis showed a trend of increasing $p27^{kip1}$ protein levels in *NR5A2* overexpressing cells, despite the reduced mRNA levels observed in the qPCR results. In contrast, the protein levels of $p21^{cip1}$ appeared decreased after NR5A2 overexpression. *NR5A2* overexpression was not confirmed with western blot analysis demonstrating a relatively low effectiveness of the overexpression process compared to A549 and EKVX cells. B-actin levels were used for normalization in western blot analysis (**Figure 24 b).**

NCI-H460

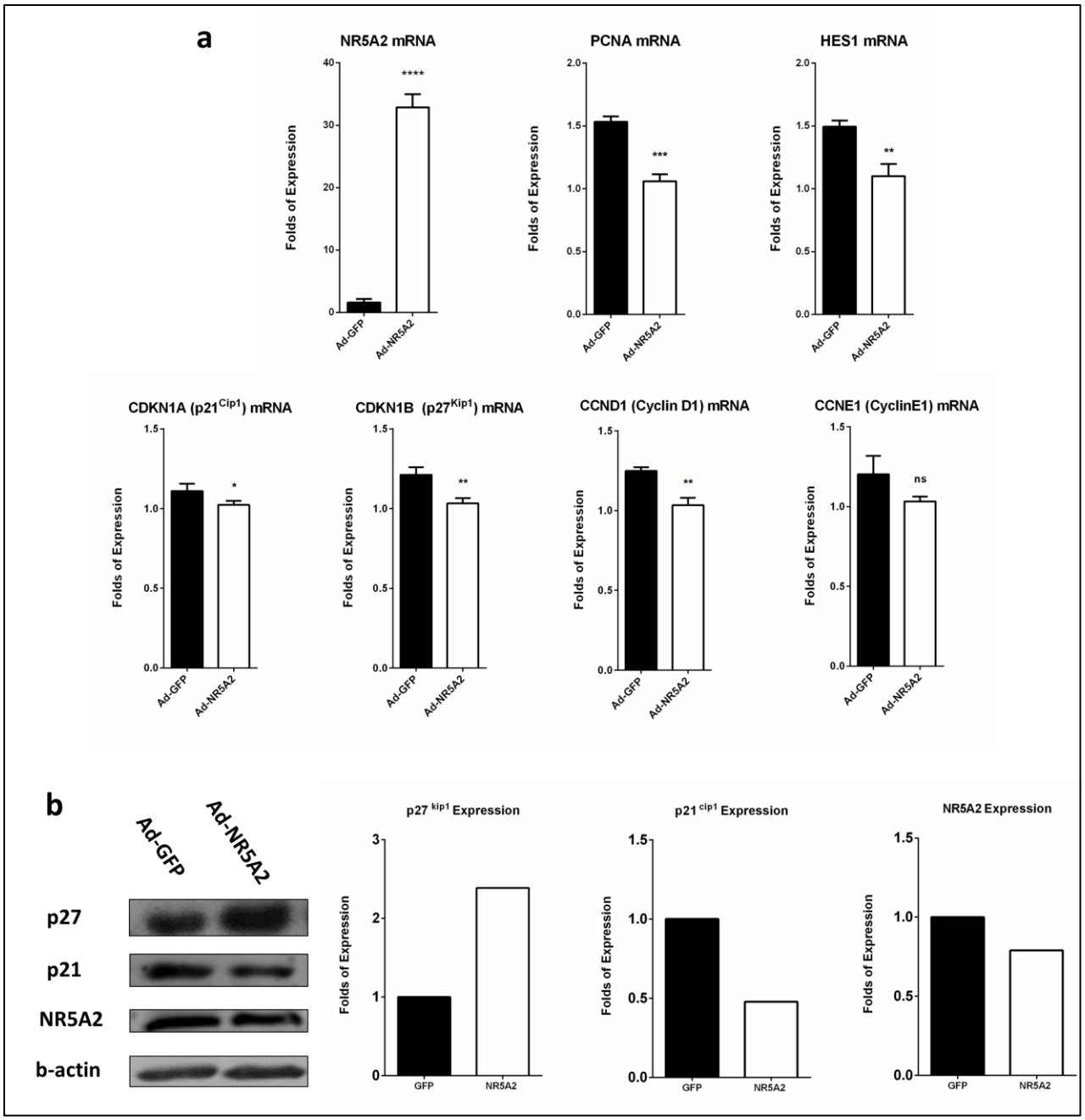

Figure 24: (a) RT-qPCR analysis of the anti-proliferative effect of NR5A2 overexpression in NCI-H460 large cell lung cancer cells. The differential expression of PCNA, HES1, CDK1A, CDK1B, CCND1 and CCNE1 was analyzed using the ΔΔCt method. **(b)** Comparison of the protein levels of cell cycle inhibitors p21 and p27 in NCI-H460 cells with and without NR5A2 overexpression. Western blot results were quantified using the ImageJ software. NR5A2 overexpression was not depicted at the protein level. B-actin was used for normalization. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

In NCI-H1944 cells, we tested the expression of:

- *CDKN1A*
- *CDKN1B*
- *CCND1*
- *CCNE1*
- *NOTCH3*
- *HES1*

NCI-H1944

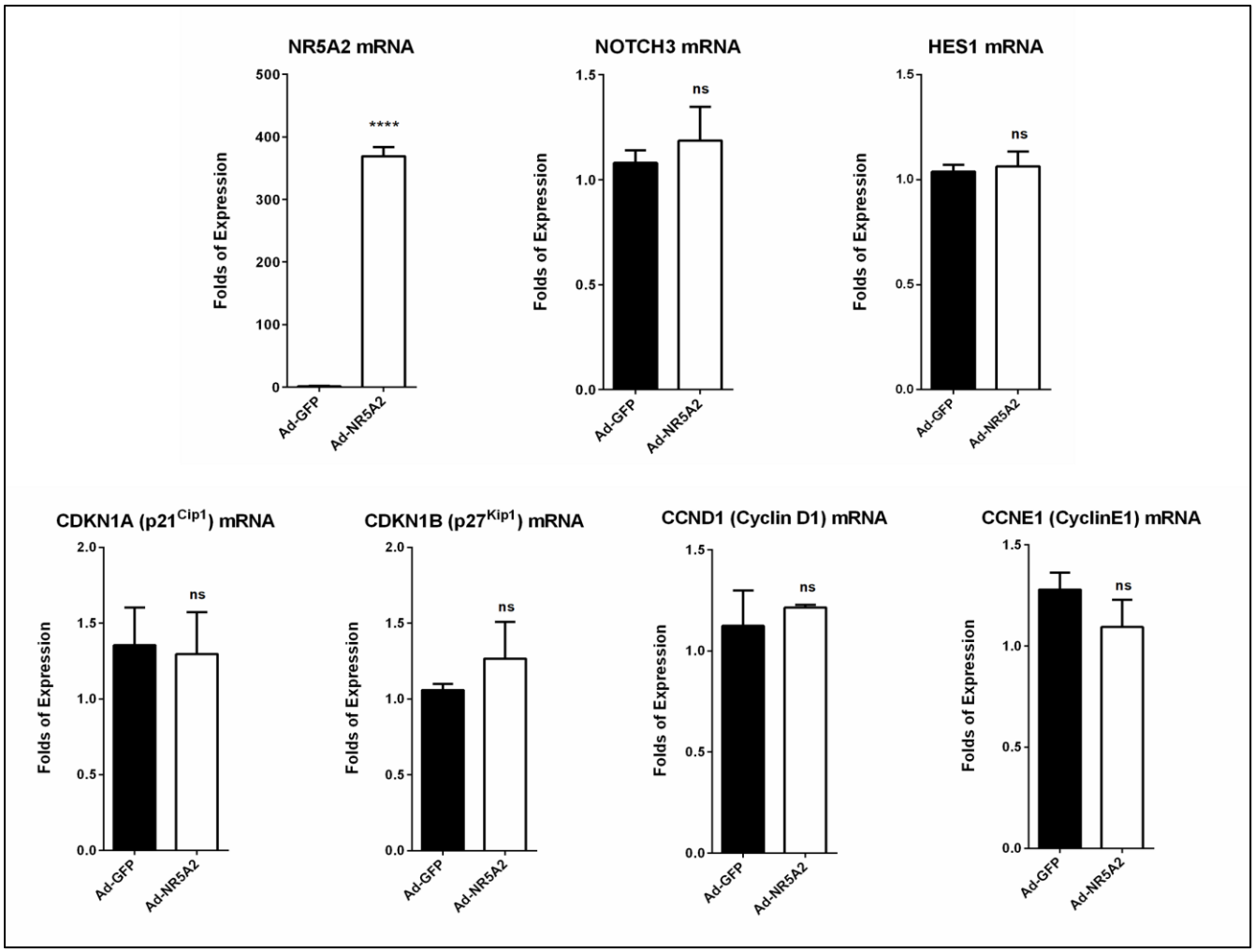

Figure 25: Analysis of the anti-proliferative effect of NR5A2 overexpression in NCI-H1944 lung adenocarcinoma cells. The differential expression of *NR5A2, NOTCH3, HES1, CDKN1A, CDKN1B, CCND1*, and *CCNE1* was analyzed using the ΔΔCt method. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

Real time qPCR results revealed a 200-fold increase in *NR5A2* mRNA levels confirming the effectiveness of the overexpression process. However, none of the investigated genes exhibited statistically significant differential expression between ad-GFP and ad-NR5A2 samples. In particular, the expression levels of *NOTCH3, HES1, CCND1*, and *CDKN1A* were similar in NCI-H1944 cells with and without NR5A2 overexpression. *CDKN1B* exhibited increased mRNA levels but not statistically significant due to high standard deviation. Moreover, *CCNE1* mRNA levels exhibited a trend of downregulation after *NR5A2* overexpression, which however could not be considered statistically significant (**Figure 25)**. All mRNA levels were normalized using the *ACTB* expression levels.

3.3 NR5A2 overexpression decelerates wound healing in A549 cell monolayers

To assess the effect of NR5A2 overexpression on cell migration, wound-healing assay was applied using A549 cells. Cells were seeded and cultured in 6-well plates and clear lines were created with a sterile 200 µl pipette tip after 24 h of adenoviral transduction (for ad-GFP and ad-NR5A2 samples respectively). Then the cells were continuously cultured in the medium for 24 hours. The wound areas were recorded at 0, 24, 48, 72, and 96 hours using an inverted microscope and a LEICA camera at x10 total magnification. Data were analyzed using the ImageJ software. The experiment was performed in tetraplicates.

At 0 and 24 h time points, the wound area was similar between ad-GFP and ad-NR5A2 samples. However, at 48 h wound closure became visible in the ad-GFP samples and rapidly advanced until the complete formation of cell monolayers at 96 h. In contrast, the wound healing process was much slower in NR5A2-overexpressing A549 cells, so that even at 96 h the scratch was still visible (**Figure 26**).

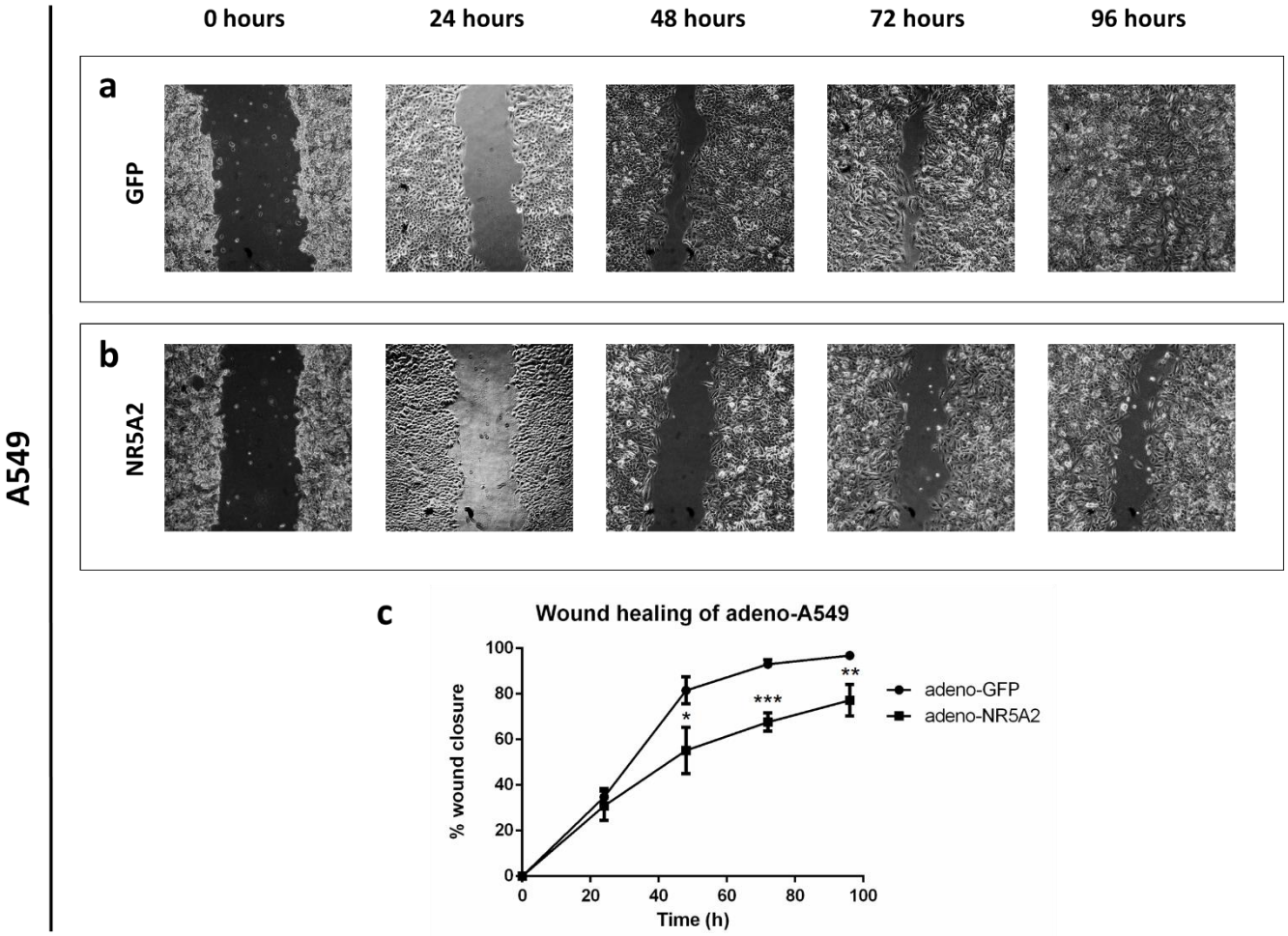

Figure 26: Microscope images representing the *in vitro* wound healing potential of A549 lung cancer cells after NR5A2 overexpression (10x magnification). A549 cell monolayers were incubated after transduction with either **(a)** adeno-GFP or **(b)** adeno-NR5A2 and images were captured at 0, 24, 48, 72, and 96 hours. **(c)** Wound closure was measured by calculating the wound area in each time point and expressed as a percentage of the initial wound area at time zero. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01,

3.4 Administration of NR5A2 agonist (DLPC) in human lung cancer cells *in vitro*

In the previous experiments we observed that NR5A2 constitutes a limiting factor of cellular growth in different human lung cancer cell lines. Thus, our next step was to investigate the effect of a lipophilic compound that according to the literature acts as an NR5A2 agonist, enhancing its function. The compound used in our experiments was dilauroyl-phosphatidylcholine (DLPC, Avanti® Polar Lipids).

24hours prior to DLPC addition in the culture medium, cells were seeded in a 24-well plate on coverslips coated with L-polylysine. NR5A2 agonist, DLPC, was preserved in 10 mM 100% ethanol. To determine the optimal concentration and incubation time for the treatment of lung cancer cell lines with DLPC, A549 cells were cultured in six groups under the following conditions:

- 1st group: Control (200 uM Ethanol), 24 h-treatment
- 2nd group: 150 uM DLPC, 24 h-treatment
- 3rd group: 200 uM DLPC, 24 h-treatment
- 4th group: Control (200 uM Ethanol), 48 h-treatment
- 5th group: 150 uM DLPC, 48 h-treatment
- 6th group: 200 uM DLPC, 48 h-treatment

This cell line has a doubling time of approximately 22 hours; thus we set the lower treatment time threshold at 24h to detect potential differences in proliferation markers. In addition, DLPC was administered in the cell cultures in two different concentrations to determine cell tolerance. The control cultures were treated with 200 uM ethanol (DLPC dissolver). Immunofluorescent staining for BrdU and pH3 markers was followed by signal quantification and subsequent statistical analysis using ordinary one-way ANOVA. DAPI was used for visualization of the nuclei.

When cells were incubated for 24 h with DLPC no differences were observed in proliferation markers (groups 2 and 3 versus group 1). Similar results were obtained after 48 h-incubation with 150 uM DLPC (group 5 versus group 4). However, the experiment revealed a significant reduction in both BrdU and pH3 indices after 48h-treatment of A549 cells with 200 uM DLPC (**Table 3** , **Figure 27**).

24 h-treatment			48 h-treatment		
Groups	%BrdU Index	%pH3 Index	Groups	%BrdU Index	%pH3 Index
Control	47.13	31.13	Control	61	46
150 uM DLPC	44.38	34	150 uM DLPC	49.8	39
200 uM DLPC	42.11	27.5	200 uM DLPC	44.1	20.4

Table 3: Calculation of BrdU and pH3 indices for A549 cells after different conditions of treatment with DLPC.

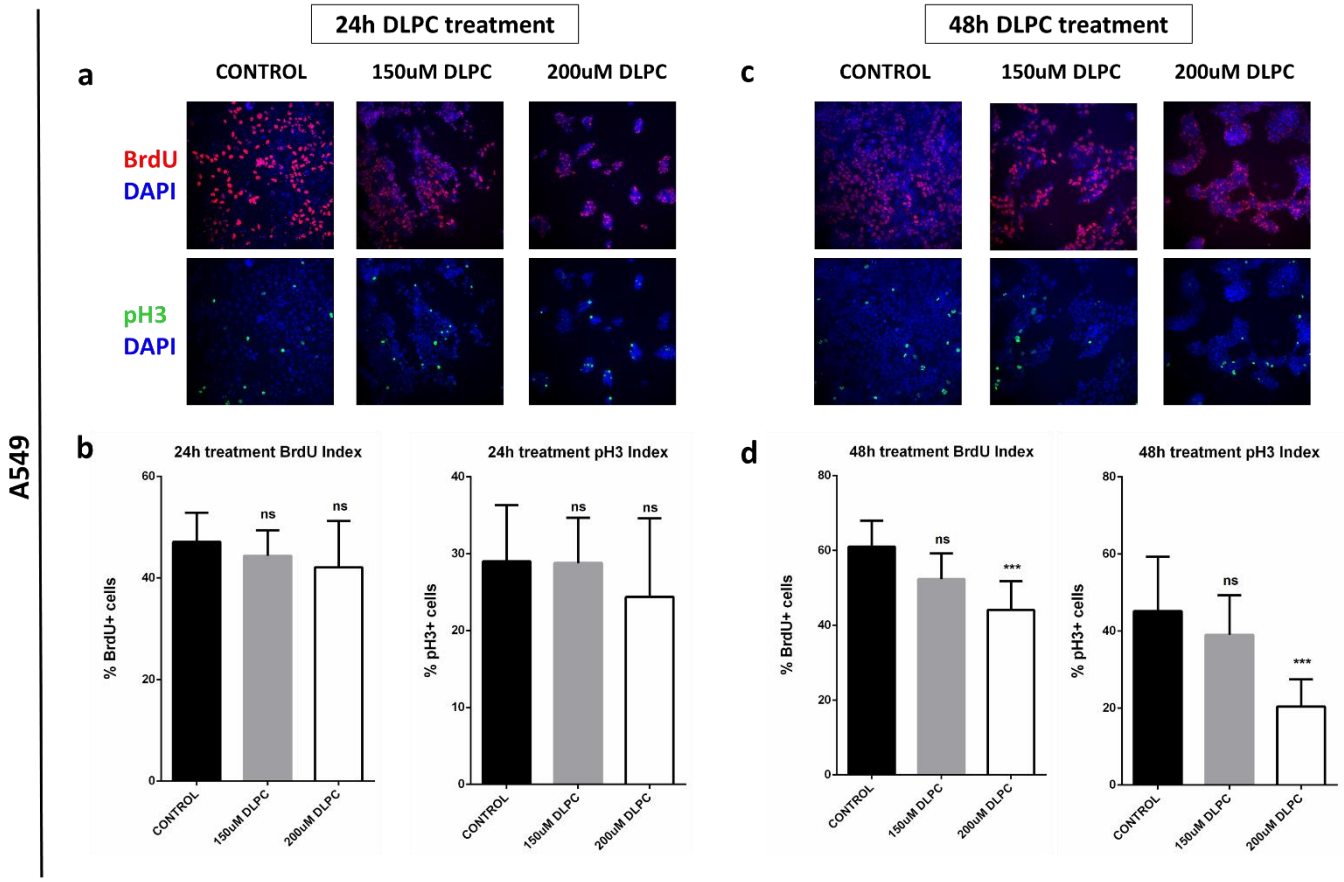

Figure 27: Double immunostainings of A549 cells with BrdU (red) and pH3 (green) after treatment with DLPC for 24 **(a)** or 48 **(c)** hours. Quantifications of the indices of the above markers are shown in **b**, and **d**, respectively (% of BrdU positive cells, % of pH3 positive cells).

3.5 DLPC reduces proliferation in different human lung cancer cell lines

After determining the optimal treatment conditions, the same protocol was applied in EKVX and NCI-H460 cells as well. NCI-H1944 cells were not tolerant of DLPC even in small concentrations, thus the specific experiment resulted in cell death and cell proliferation indices could not be determined.

In accordance with the results for A549 cells, double immunostaining on EKVX and NCI-H460 cells revealed that treatment with 200 uM of NR5A2 agonist, DLPC, significantly inhibited incorporation of Ki67 (proliferation marker) and reduced the percentage of pH3 positive cells. Interestingly though, the administration of 150 uM DLPC was also able to significantly alter the index of Ki67 in both cell lines and the index of pH3 in NCI-H460 cells (**Figures 28-29**).

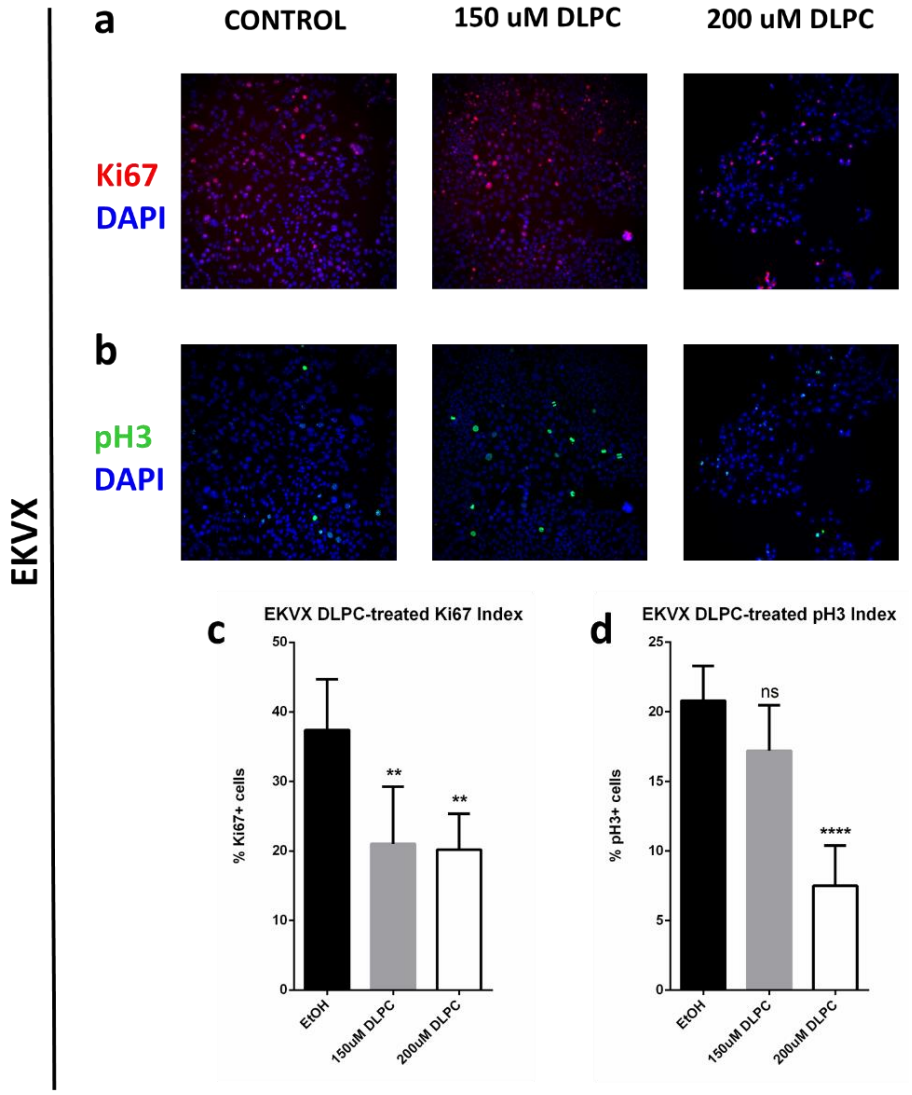

Figure 28: Immunofluorescence results of DLPC- and Ethanol- treated EKVX cells stained for Ki67 (**a**) and pH3 (**b**). Statistical analysis on the quantitative variables (number of Ki67+ and pH3+ cells) was performed using ordinary one-way ANOVA (**c, d**). Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001.

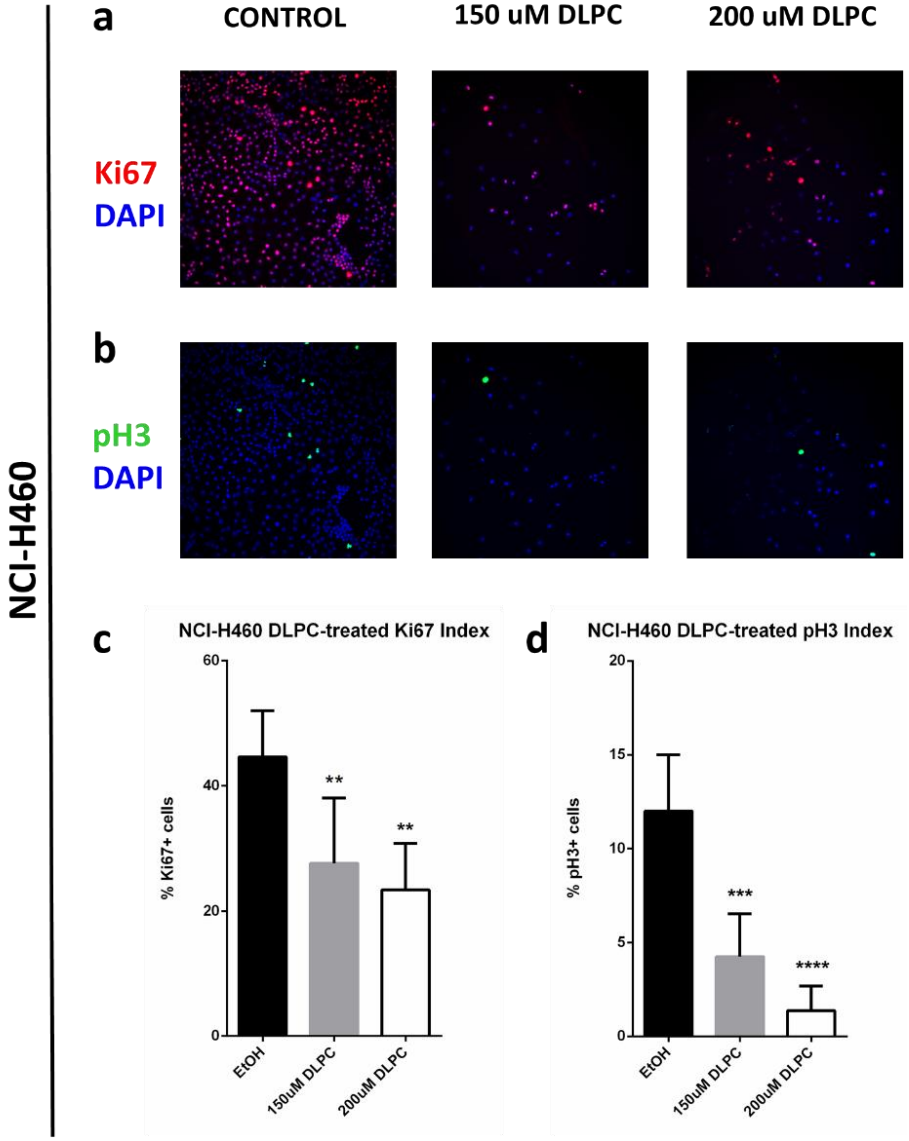

Figure 29: Immunofluorescence results of DLPC- and Ethanol- treated NCI-H460 cells stained for Ki67 (**a**) and pH3 (**b**). Statistical analysis on the quantitative variables (number of Ki67+ and pH3+ cells) was performed using the two-tailed paired Student's t-test (**c, d**) (non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001).

3.6 The effect of DLPC on the expression of cell cycle regulators

Immunofluorescent staining with proliferation markers BrdU and pH3 revealed a significant reduction in cell proliferation of A549 lung cancer cells after treatment with 200 uM DLPC. Therefore, we aimed to investigate whether this finding was also depicted on the cellular mRNA profile.

A549 cells were cultured in growth medium and were separated in two groups. In one group, cells were cultured in the presence of 200 uM DLPC, while in the second group DLPC was replaced with Ethanol, the DLPC dissolver (control group). Cells were cultured for 48 h after DLPC or Ethanol addition, when RNA extraction was performed followed by cDNA synthesis. We then used RT-qPCR to test the expression of *NR5A2, CDKN1A, CDKN1B, CCND1*, and *CCNE1*.

The results revealed a significant reduction in the mRNA levels of *CDKN1B* (p27) after DLPC treatment of A549 cells. In contrast, *NR5A2, CDKN1A*, and *CCNE1* exhibited a trend of downregulation in DLPC-treated cells, which however could not be considered statistically significant. Moreover, the mRNA levels of *CCND1* appeared unaffected by DLPC treatment. All mRNA levels were normalized using the *ACTB* expression levels (**Figure 30)**.

A549

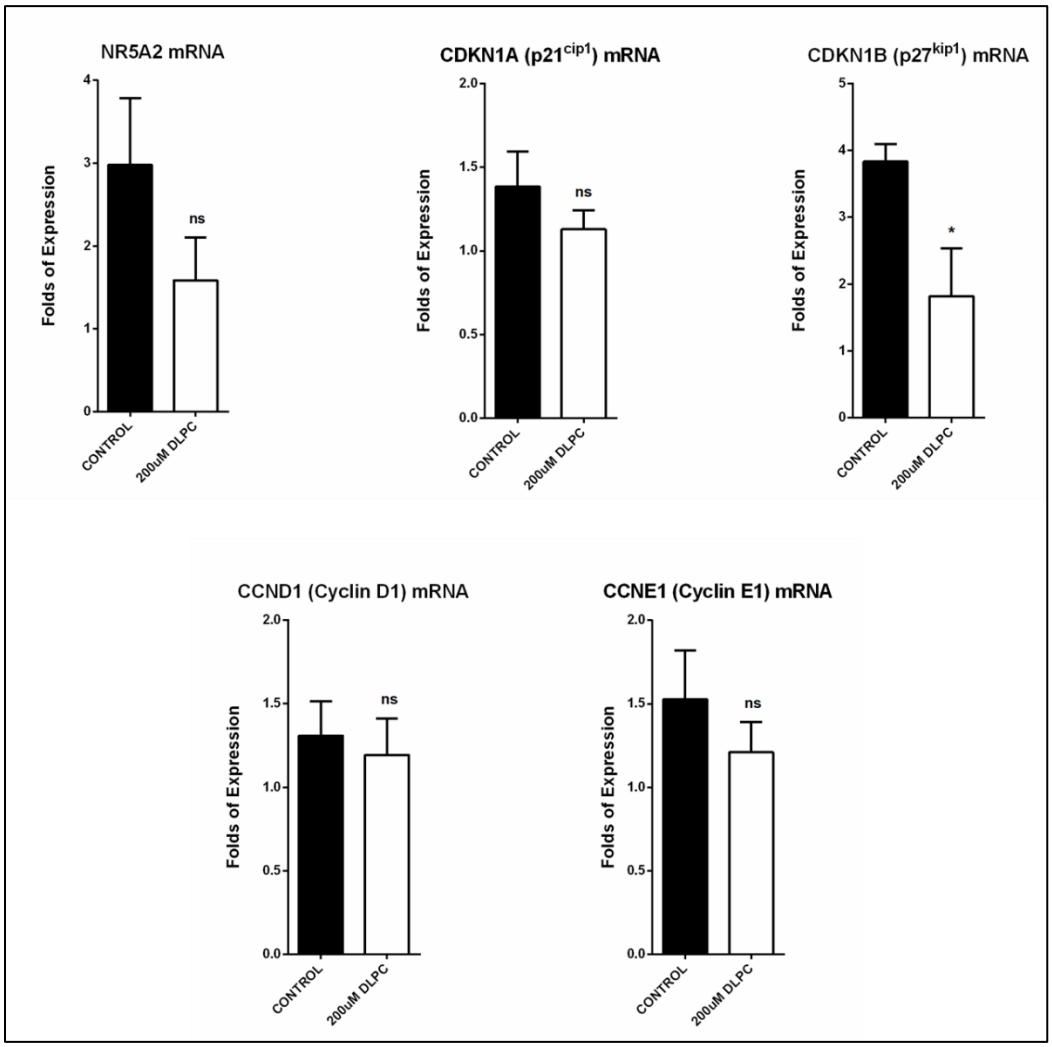

Figure 304: Real time qPCR analysis on A549 cells, after treatment with 200 uM DLPC. RNA was extracted from A549 cells treated with either Ethanol (control culture) or DLPC (experimental culture), and was used for cDNA synthesis and qPCR. RT-qPCR results were analyzed using the ΔΔCt method. Statistical analysis was performed using the two-tailed paired Student's t-test. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001.

3.7 DLPC does not affect wound healing in A549 cell monolayers

To assess the effect of NR5A2 agonist, DLPC, on cell migration, wound-healing assay was applied using A549 cells. Cells were seeded and cultured in 6-well plates and clear lines were created with a sterile 200 µl pipette tip just before the addition of either 200 uM DLPC or EtOH (experimental and control samples respectively). Then the cells were continuously cultured in the medium for 48 hours. The wound areas were recorded at 0, 24, and 48 hours using an inverted microscope and a LEICA camera at x10 total magnification. Data were analyzed using the ImageJ software. The experiment was performed in tetraplicates.

At 24 h, the wound area was slightly but significantly wider in the control samples, while cell migration was visible in DLPC-treated wells. However, at 48 h wound closure had equally advanced in both samples towards the complete formation of cell monolayers. Ultimately, the wound healing process did not exhibit differential progression rate due to treatment of A549 cells with 200 uM DLPC (**Figure 31**).

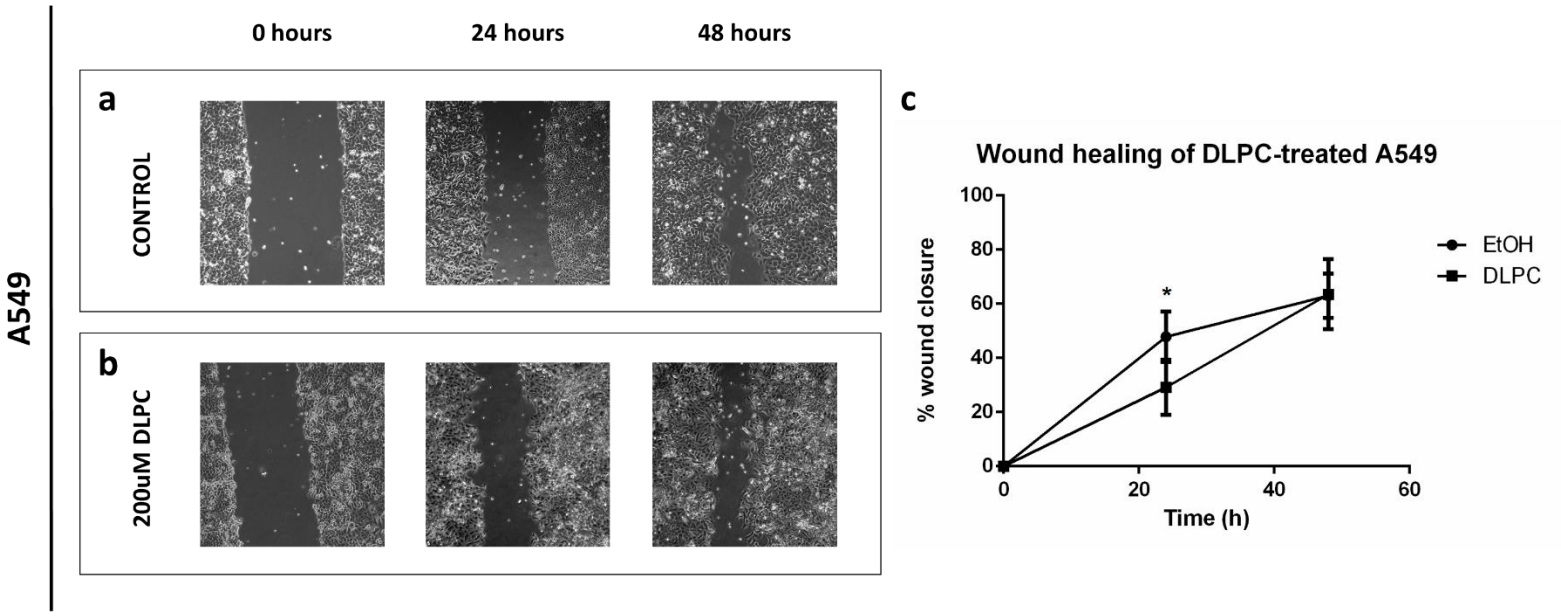

Figure 31: Microscope images representing the *in vitro* wound healing potential of A549 lung cancer cells after treatment with 200uM DLPC (10x magnification). A549 cell monolayers were incubated after treatment with either **(a)** EtOH or **(b)** DLPC and images were captured at 0, 24, and 48 hours. **(c)** Wound closure was measured by calculating the wound area in each time point and expressed as a percentage of the initial wound area at time zero. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001.

3.8 Stable NR5A2 knock down induces proliferation of human lung adenocarcinoma cells

After observing the anti-proliferative effect of NR5A2 overexpression in different human lung cancer cell lines, we aimed to investigate whether NR5A2 knockdown could produce the opposite results. We used A549 cells that were developed to stably express either a random (scrambled) or an shNR5A2 sequence, thus establishing constant NR5A2 knockdown. To acquire genetically homogenous cultures, the stably transfected cells were selected based on their survival and expansion after addition of rapamycin in the culture medium.

The generated cultures were stained for Ki67 and pH3 proliferation markers. Immunofluorescence experiments and subsequent signal quantification revealed a significant increase in both Ki67- and pH3- positive cells in shNR5A2- expressing A549 samples (**Figure 32**). The experiment was performed in triplicates and two-tailed paired Student's t-test was used for statistical analysis.

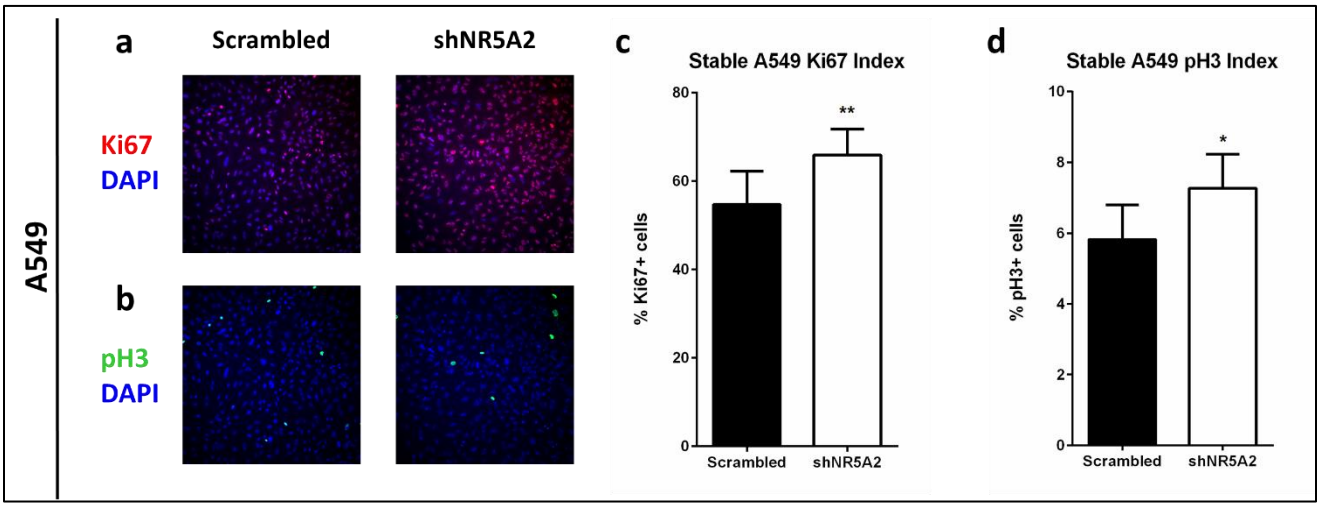

Figure 325: Immunofluorescence results of A549 cells stably expressing the scrambled or shNR5A2 sequence stained for Ki67 **(a, c)** and pH3 **(b, d)**. Images were obtained at 20x magnification. Statistical analysis on the quantitative variables (number of Ki67+ and pH3+ cells) was performed using the two-tailed paired Student's t-test (non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P< 0.0001).

NR5A2 knockdown was confirmed by RT-qPCR as depicted in the endogenous *NR5A2* mRNA levels between the scrambled and shNR5A2 samples. Furthermore, the levels of *CDKN1B* (p27) appeared significantly decreased, in contrast to *CDKN1A* (p21) levels that were elevated after NR5A2 knockdown. A trend of downregulation was also observed in *CCNE1* expression, while the levels of *CCND1* did not exhibit significant differences between the control and shNR5A2 samples (**Figure 33**). *ACTB* was the housekeeping gene used for normalization.

A549

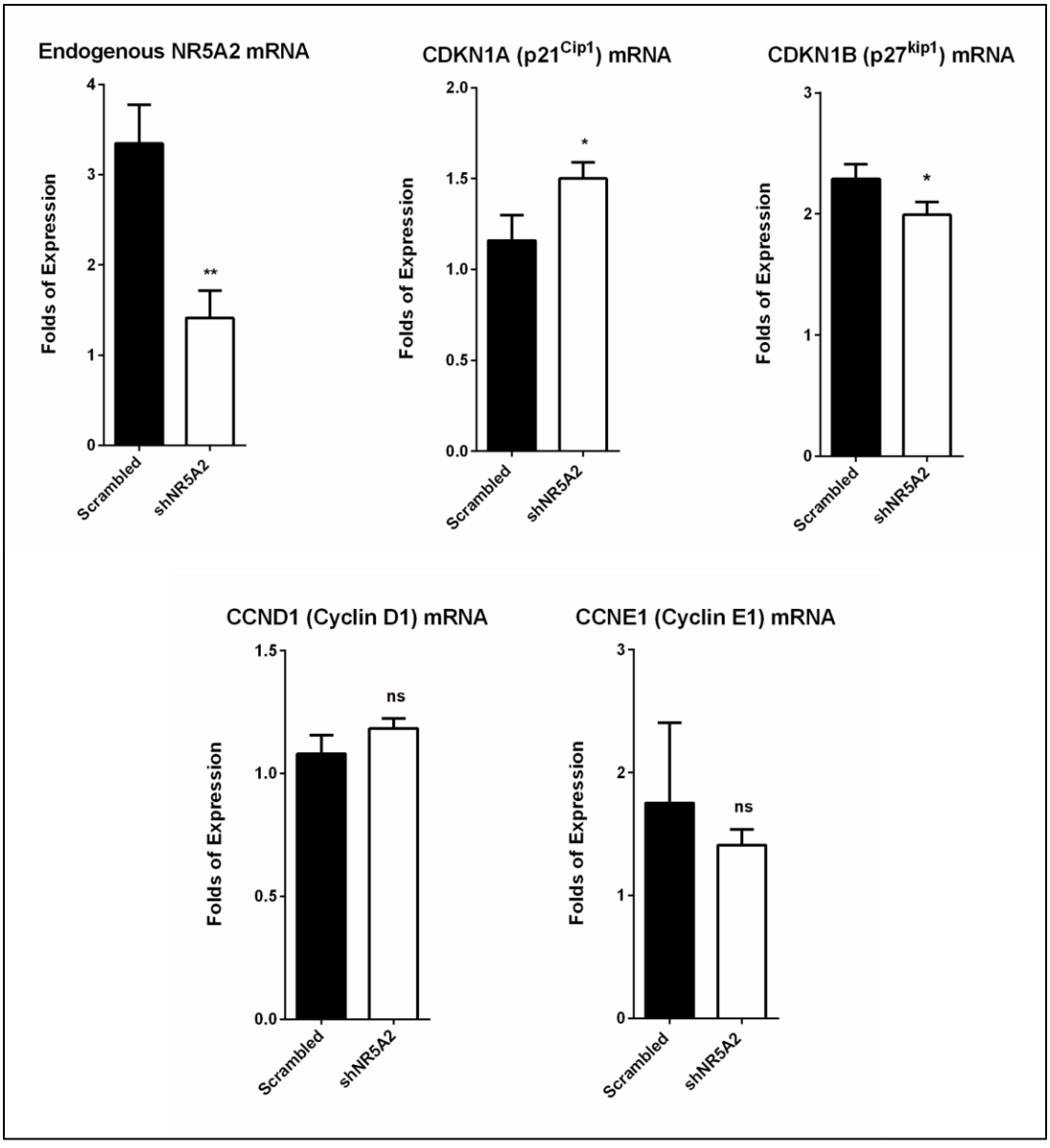

Figure 33: RT-qPCR analysis of cell cycle regulating genes in A549 cells stably expressing the scrambled or shNR5A2 sequence. The differential expression of endogenous *NR5A2, CCND1*, *CCNE1, CDKN1A*, and *CDKN1B* was analyzed using the ΔΔCt method. Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

3.9 NR5A2 overexpression reduces tumor volume in mouse xenografts

NR5A2 overexpression as well as administration of its lipid agonist (DLPC) suppressed the proliferation of A549 human lung adenocarcinoma cells. Therefore, we generated cell line-derived mouse xenografts to investigate whether the anti-proliferative effect of NR5A2 could be observed *in vivo* as well. A549 cells were transduced in culture with adenoviral particles (adeno-GFP or adeno-NR5A2) and were injected in NOD/SCID mice (1 million cells/mouse). The mice were divided in two groups: in the control group, 4 mice received intraperitoneal injection of ad-GFP A549 cells, while in the experimental group 5 mice were intraperitoneally injected with ad-NR5A2 A549 cells. Mice were maintained in optimal conditions while the injected cells created tumors. Several weeks after the injections, we began to measure tumor volume regularly. Specifically, tumor size was measured four times, during which the length and width of the tumor in each mouse was estimated using a caliper. Tumor volume was calculated using the equation

$$\text{Volume} = (\text{length} \times \text{width}^2) / 2,$$

and was used to compare tumor development between the control and NR5A2 groups.

Statistical analysis using the two-tailed paired Student's t-test revealed significantly decreased tumor volume when ad-NR5A2 rather than ad-GFP A549 cells were injected in the mice. In fact, tumor volume in the control mice was 5 times higher than in the experimental group at 21 days post-injection. At 25 days post-injection, tumor volume was 8 times smaller in the NR5A2 group, while the difference at 29 days was 5.9 times. At 40 days post-injection, when the last measurement was performed, tumor volume was twice as high in the control group as in the NR5A2 group (**Figure 34**).

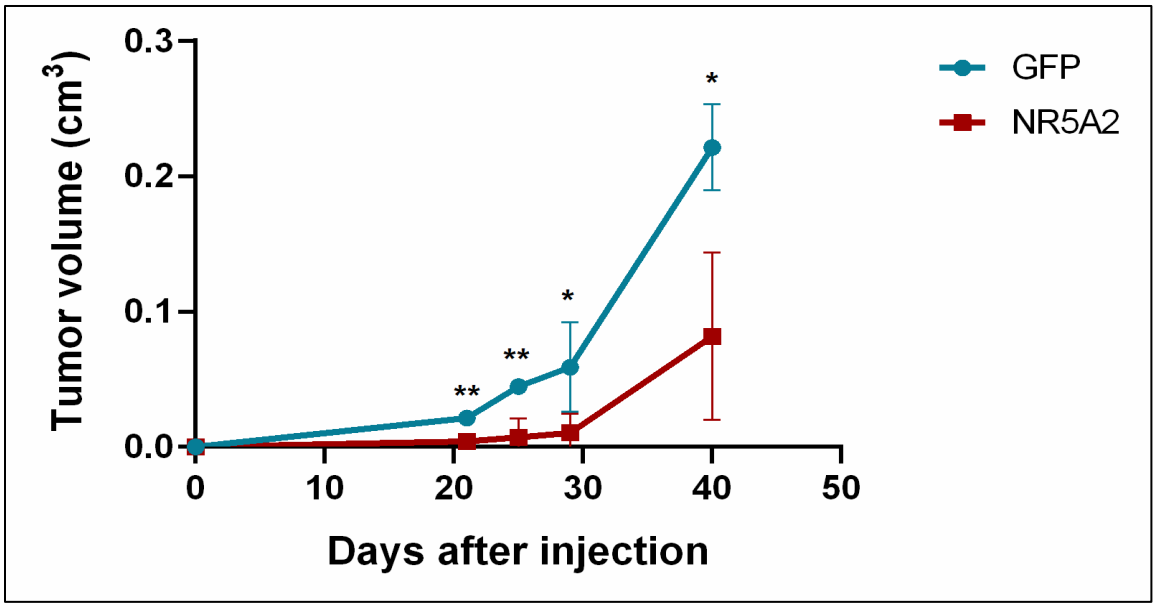

Figure 34: Tumor volume measurement in NOD/SCID mice injected with either ad-GFP or ad-NR5A2 A549 cells. Volume was calculated as (length x width ^{2}) / 2, in 9 mice (4 in the control and 5 in the experimental group). Data are shown as mean ± SD. Non-significant (ns) P>0.05, *P<0.05, **P<0.01, ***P<0.001.

4. Discussion

Clinical data available from the Oncomine database suggested that in different types of non-small cell lung cancer *NR5A2* exhibited reduced expression compared to normal lung tissues. In the current study, we suggest a previously undefined role of NR5A2 in human non-small cell lung cancer. First, we investigated the expression profile and phenotypic effect of nuclear receptor NR5A2 in human NSCLC cell lines, driven by clinical data indicating reduced *NR5A2* expression in patients with lung adenocarcinoma. To this end, we examined the role of NR5A2 in human cell lines representing two types of non-small cell lung cancer: adenocarcinoma and large cell lung cancer. Specifically, we employed the A549, EKVX, NCI-H460, and NCI-H1944 cell lines for our experiments. Among them, NCI-460 cells derived from human large cell lung cancer, while the rest represented different stages or tissues of lung adenocarcinoma. The results revealed an impressive reduction in cancer cell proliferation *in vitro* after *NR5A2* overexpression in all four cell lines, as indicated by immunofluorescence experiments evaluating the BrdU, Ki67, and pH3 proliferative indices. The most intense phenotypic alteration was observed in A549 cells, while large cell lung cancer NCI-H460 cells exhibited the slightest, yet statistically significant, differences in proliferative indices. This outcome may be attributable to their distinct histological identity, as NCI-H460 cells constitute lung cancer metastasis isolated from the pleural fluid and thus may exhibit a more aggressive behavior. Notably, NR5A2 seemed to exert its anti-proliferative function *in vivo* as well through the inhibition of tumor growth in mice. After the generation of A549-derived mouse xenografts, *NR5A2* overexpression resulted in significantly smaller tumor volumes compared to the control A549-injected NOD/SCID mice. Although this is an ongoing experiment, this preliminary finding is the first preclinical evidence that positive regulation of NR5A2 could be a novel therapeutic approach to inhibit lung adenocarcinoma progression. Conversely, stable shRNA-mediated knockdown of *NR5A2* in lung adenocarcinoma cells was able to induce a significant increase in proliferation indices, including the number of Ki67+ and pH3+ cells. Therefore, alterations in *NR5A2* mRNA levels seem to affect cancer cell proliferation in a bidirectional manner, supporting its central role in calibrating the expression of cell cycle regulators.

Apart from controlling proliferation, NR5A2 also seemed to inhibit migration in A549 lung adenocarcinoma cells. The 2D wound healing assay revealed that at 96 h post-scratch formation NR5A2-overexpressing cultures exhibited significantly reduced migration as opposed to the control cultures that rapidly recreated cell monolayers. This result further supports the tumor-suppressive role of NR5A2 in lung adenocarcinoma, as malignant cell invasion and migration in the surrounding tissues is a very common complication of lung cancer.

Furthermore, NR5A2 is a druggable nuclear receptor as it can be targeted by lipophilic compounds acting as pharmacological agonists [45, 56]. In particular, DLPC is an FDA approved phospholipid (FDA UNII: 31NE1DVE91) with good oral bioavailability and minimal toxicity, as well as validated NR5A2 agonist function [57]. The anti-proliferative action of

NR5A2 overexpression in lung adenocarcinoma cells raised the question of whether an NR5A2 agonist could recapitulate this effect *in vitro*. Immunofluorescence experiments confirmed that DLPC was able to suppress proliferation in all four non-small cell lung cancer cell lines, mimicking the effect of NR5A2. However, DLPC treatment could not inhibit migration of lung adenocarcinoma cells *in vitro*, as revealed by the 2D wound healing assay. This result could be attributable to the fact that DLPC functions as an NR5A2 agonist by inducing its action, yet not strongly enough to efficiently reproduce the effects of NR5A2 overexpression regarding cell migration. These observations render this lipid NR5A2 agonist an interesting molecule to be investigated in other tumor types in the context of cancer cell proliferation. Due to its structure and lipophilic nature, it has already been used for administration of paclitaxel in a murine renal carcinoma model, where it successfully inhibited pulmonary metastases [58]. However, its function as an NR5A2 agonist and its anti-proliferative potential has never been studied before in primary cancer sites. Thus, DLPC along with other small molecule agonists of NR5A2 should be investigated as potential components in novel therapies for non-small cell lung cancer.

To unravel the downstream target genes mediating the anti-proliferative effect of NR5A2, we focused on key cell cycle regulators. Interestingly, *NR5A2* overexpression affected the expression of both inducers and inhibitors of cell proliferation. Specifically, a 300-fold increase in *NR5A2* mRNA of lung adenocarcinoma cells resulted in significant upregulation of *CDKN1B* (p27) and *CCNE1*, while the levels of *CDKN1A* (p21), and *P53* also appeared elevated. *CDKN1A* and *CDKN1B* encode cell cycle inhibitors, thus their upregulation is in line with immunofluorescence results observed after NR5A2 overexpression. *P53* is a well-defined tumor suppressor with growth control function encoding a transcriptional activator of *CDKN1A* (p21). Furthermore, the *CCNE1* gene is a known NR5A2 target that encodes Cyclin E1, which induces G1-S transition. Increased *CCNE1* mRNA could represent a cellular attempt to counterbalance the significant NR5A2-induced reduction in *CCND1* mRNA levels. In addition, *NR5A2* overexpression did not alter the expression of genes encoding components of the EGFR-PI3K-AKT-MTOR pathway. However, *HES1* mRNA levels exhibited a decreasing trend in response to *NR5A2* overexpression. Interestingly, this finding is in accordance with the observed inhibition in proliferation as Hes1 is a transcriptional repressor able to promote cancer cell stemness, metastasis, and multidrug resistance [50]. *HES1* levels can be modulated by different signaling pathways, one of which is the Notch pathway. The expression levels of genes encoding the Notch 1 and Notch 3 receptors were examined but found unaffected by *NR5A2* overexpression in non-small cell lung cancer cell lines, suggesting that NR5A2 may affect *HES1* expression in a Notch-independent manner. Correspondingly, stable *NR5A2* knockdown in lung adenocarcinoma cells significantly downregulated *CDKN1B* (p27), while *CCND1* mRNA levels were found slightly increased, corroborating the observed increase in cell proliferation. On the other hand, DLPC treatment appeared to lower *NR5A2* mRNA levels although in a statistically insignificant manner. The same decreasing trend was observed in the mRNA levels of *CDKN1B* (p27) after DLPC treatment. Thus, we could hypothesize that DLPC may exert its anti-proliferative

action and affect the expression of NR5A2 target genes through a still unclear indirect mechanism.

Despite the aforementioned findings suggesting a tumor preventing role of NR5A2, a number of reports have indicated that this orphan nuclear receptor should be considered a potential oncogene in breast [45], colorectal [40], and gastric carcinoma [51]. Specifically, in colon and breast cancer NR5A2 promotes cell cycle progression by inducing cyclin D1 (*CCND1* gene) and cyclin E1 (*CCNE1* gene) expression. Of note, despite the strong anti-proliferative effect of NR5A2 on lung adenocarcinoma cells in our study, it also appeared to enhance the expression of *CCNE1* gene. Nevertheless, in lung adenocarcinoma cells, NR5A2 exerted additional actions by enhancing the expression of $p21^{Cip1}$ and $p27^{Kip1}$ that probably managed to surmount the effect of cyclin E1 on proliferation and further block cell cycle progression. This hypothesis is supported by the fact that $p21^{Cip1}$ and $p27^{Kip1}$ negatively regulate cell cycle progression via their ability to directly inactivate the CDKs/Cyclin E1 complexes. In a gastric cancer cell line, knockdown of *NR5A2* inhibited cell proliferation via arresting the cell cycle in the G2/M phase and suppressing cell mobility by blocking the Wnt/beta-catenin pathway, while preventing the EMT process [51]. In the literature, very little information can be found regarding NR5A2 in lung cancer. Recently, two research groups provided evidence that NR5A2 promoted lung cancer stem cell properties through positively regulating *NANOG* [52], and that its upregulation could be considered a predictor of lymph node metastasis [53]. As can be deduced by its multifaceted functions, it is possible that NR5A2 cannot control cellular fate (e.g. transition from normal to malignant state) and tumor progression by itself. The divergent experimental evidence on the role of NR5A2 in cancer highlights the complexity and context-dependent actions of this nuclear receptor in cancer pathogenesis and cell cycle modulation, and emphasizes the undefined interrelating pathways orchestrating its regulation.

Most nuclear receptors often control the expression of genes leading to contradictory cellular outcomes through their interaction with multiple molecular partners. In agreement, NR5A2 has been previously reported to interact with many co-activators, co-repressors or other transcription factors in a cell-type specific manner, including CTNNB (Beta catenin), PGC-1a, CBP, CREB1, FXR, MBF1, SRC1/3, NRIP1, SMARCD3, SMRT, SHP, DAX1 and Prox1 [46, 54]. Thus, NR5A2 may be able to affect cell cycle genes and either promote or inhibit them depending on the cellular and/or developmental context. Indeed, NR5A2 can simultaneously exert a tumor-suppressive role, as in the case of pancreatic carcinoma. Genome-wide association studies (GWAS) recently identified several genomic regions containing potential common risk single-nucleotide polymorphisms (SNPs) for pancreatic adenocarcinoma localized either over or in close proximity to the *NR5A2* gene [55, 56], suggesting that genetic variations in the *NR5A2* locus may constitute pancreatic carcinoma risk factors. Accordingly, heterozygosity or pancreas-specific deletion of *NR5A2* was associated with an exacerbated inflammatory response and delayed recovery from pancreatitis, as well as with acceleration of KRas-driven tumorigenesis in mice [57]. Moreover, unpublished data from

our lab have revealed that NR5A2 has an anti-proliferative effect in mouse and human glioblastoma and neuroblastoma cells.

Ultimately, we hypothesize that in lung adenocarcinoma epithelial cells NR5A2 could exert a tumor-suppressive function by activating the transcription of both *CDKN1A* and *CDKN1B* cell cycle inhibitors, and by simultaneously repressing the transcription of *CCND1*, thus inhibiting G1-S transition (**Figure 35**). In other cancer types or tissues where NR5A2 demonstrates an oncogenic role, it is possible that this mechanism does not suffice to control proliferation, as the transcription of cell cycle inducers may be more intensely activated, e.g. *CCNE1* upregulation. Additionally, NR5A2 antagonists may bind and effectively suppress its action, thus preventing the transcription of genes encoding cell cycle inhibitors and enabling cancer progression.

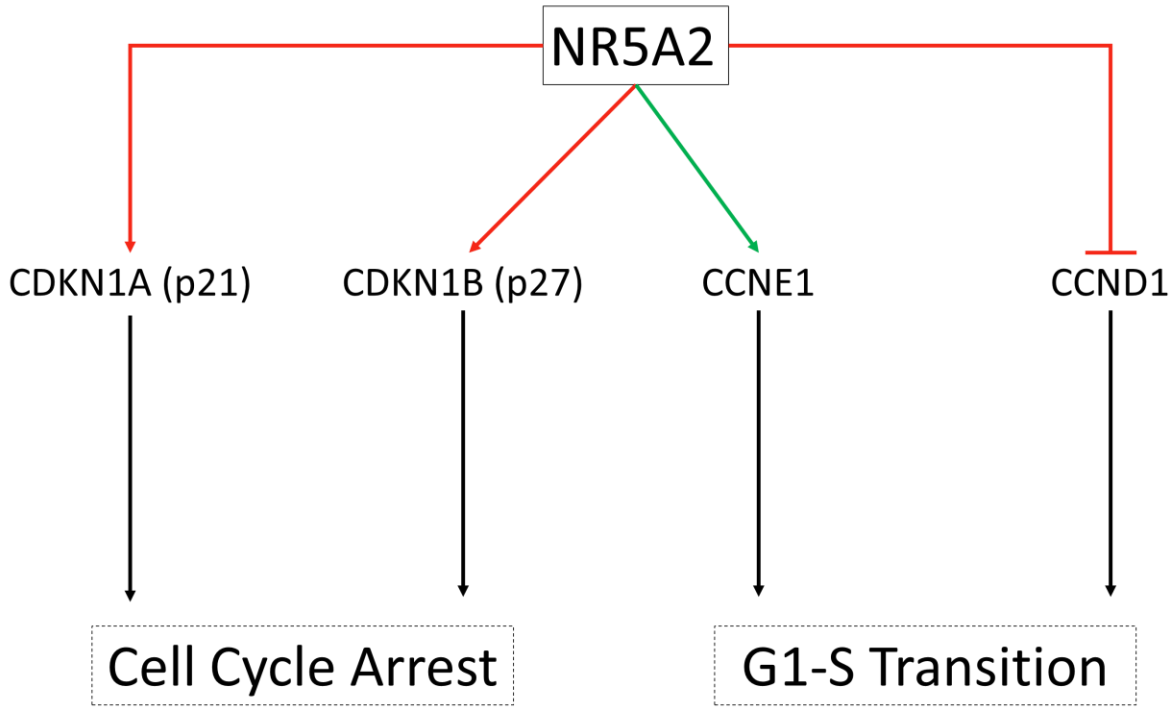

Figure 35: Possible mechanism through which NR5A2 exerts a tumor-suppressive function in human lung adenocarcinoma. Red arrows represent anti-proliferative signals while the green arrow signifies the transcriptional activation of a cell cycle inducer.

5. References

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