AGRICULTURAL UNIVERSITY OF ATHENS

DEPARTMENT OF BIOTECHNOLOGY

BIOMEDICAL RESEARCH FOUNDATION ACADEMY OF ATHENS

LABORATORY OF GENE REGULATION

POSTGRADUATE PROGRAM

SYSTEMS BIOLOGY

Master Thesis

Investigating the role of NR5A2 in non-small cell lung cancer

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«Διερεύνηση του ρόλου του μεταγραφικού παράγοντα NR5A2 στον μη μικροκυτταρικό καρκίνο του πνεύμονα»

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Biomedical Research Foundation Academy of Athens
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Abstract

Lung cancer is one of the most frequently diagnosed malignancies with poor overall prognosis and high mortality rates worldwide. About 85% of incidences exhibit non-small cell lung cancer (NSCLC), mainly comprising the adenocarcinoma (ADC) subtype. These clinical findings highlight the need for new insights into pharmaceutical targets and combination therapies. To this end, here, we identify NR5A2/LRH-1, a druggable nuclear receptor, as a negative regulator of lung cancer progression. In particular, our metanalysis of clinical data from publicly available databases supports a correlation between high NR5A2 expression levels and the survival of lung cancer patients. Consistently, we experimentally show that NR5A2 is sufficient to impair the proliferation of NSCLC cell lines *in vitro* and *in vivo*. The antiproliferative effect is possibly mediated by the transcriptional induction of the negative cell cycle regulators *CDKN1A* (encoding for $p21^{Cip1}$) and *CDKN1B* (encoding for $p27^{Kip1}$) and the simultaneous downregulation of G1-S transition inducer *CCND1* (encoding for Cyclin D1). Moreover, NR5A2 overexpression also inhibits cancer cell migration *in vitro*. Most importantly, a well-established agonist of NR5A2, dilauroyl phosphatidylcholine (DLPC), is able to recapitulate the antiproliferative action of NR5A2 in NSCLC cell lines. These observations suggest a tumour-suppressor function of NR5A2 in NSCLC. They also provide a preclinical proof of concept for its use as a potential pharmaceutical target for lung cancer treatment.

Scientific area: Cancer biology

Keywords: cancer treatment, non-small cell lung cancer, nuclear receptor NR5A2, DLPC, cell cycle, CDKIs

Διερεύνηση του ρόλου του μεταγραφικού παράγοντα NR5A2 στον μη μικροκυτταρικό καρκίνο του πνεύμονα

Ίδρυμα Ιατροβιολογικών Ερευνών Ακαδημίας Αθηνών
Εργαστήριο Γονιδιακής ρύθμισης

Περίληψη

Ο καρκίνος του πνεύμονα αποτελεί μία από τις συχνότερα διαγνωσθείσες κακοήθειες με κακή πρόγνωση και υψηλά ποσοστά θνησιμότητας παγκοσμίως. Περίπου το 85% των περιστατικών παρουσιάζουν μη μικροκυτταρικό καρκίνο του πνεύμονα (ΜΜΚΠ), που αντιστοιχεί κυρίως στον υπότυπο του αδενοκαρκινώματος. Το σύνολο των κλινικών ευρημάτων καθιστά αναγκαία την εύρεση νέων φαρμακευτικών στόχων και συνδυαστικών θεραπειών. Για τον σκοπό αυτό, στην παρούσα διπλωματική εργασία, ο πυρηνικός υποδοχέας, NR5A2/LRH-1, που αποτελεί φαρμακευτικό στόχο, χαρακτηρίζεται ως αρνητικός ρυθμιστής της έκβασης του καρκίνου του πνεύμονα. Ειδικότερα, η μετα-ανάλυση κλινικών δεδομένων από δημόσια διαθέσιμες βάσεις δεδομένων υποστηρίζει τη συσχέτιση μεταξύ των υψηλών επιπέδων έκφρασης του NR5A2 και της επιβίωσης ασθενών με καρκίνο του πνεύμονα. Κατά συνέπεια, αποδεικνύεται πειραματικά ότι ο NR5A2 μπορεί να οδηγήσει σε μείωση του κυτταρικού πολλαπλασιασμό του μη μικροκυτταρικού καρκίνου του πνεύμονα *in vitro* και *in vivo*. Η δράση έναντι του πολλαπλασιασμού πιθανά οφείλεται στη μεταγραφική επαγωγή των αρνητικών ρυθμιστών του κυτταρικού κύκλου *CDKN1A* (που κωδικοποιεί την $p21^{Cip1}$ πρωτεΐνη) και *CDKN1B* (που κωδικοποιεί την $p27^{Kip1}$ πρωτεΐνη), καθώς και την ταυτόχρονη μείωση της έκφρασης του γονιδίου *CCND1* (που κωδικοποιεί την κυκλίνη D1) και επάγει τη μετάβαση από το G1 στάδιο στο S. Επιπλέον, η υπερέκφραση του NR5A2 αναστέλλει τη μετανάστευση των καρκινικών κυττάρων *in vitro*. Μία σημαντική παρατήρηση είναι ότι το DLPC (διλαουροϋλοφωσφατιδυλοχολίνη), ως αγωνιστής του NR5A2, μπορεί να μιμηθεί την δράση του NR5A2 έναντι του πολλαπλασιασμού, σε κυτταρικές σειρές μη μικροκυτταρικού καρκίνου του πνεύμονα. Τα παραπάνω δεδομένα υποδηλώνουν την ογκοκατασταλτική λειτουργία του NR5A2 στον μη μικροκυτταρικό καρκίνο του πνεύμονα. Ακόμα, παρέχουν αρχική ένδειξη της χρήσης του ως δυνητικό φαρμακευτικό στόχο για τη θεραπεία του καρκίνου του πνεύμονα σε προκλινικό επίπεδο.

Επιστημονική περιοχή: Βιολογία καρκίνου

Λέξεις κλειδιά: Αντικαρκινική θεραπεία, μη μικροκυτταρικός καρκίνος πνεύμονα, μεταγραφικός παράγοντας NR5A2, DLPC, κυτταρικός κύκλος, αναστολείς κυκλινοεξαρτώμενων κινασών

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Contents

List of abbreviations

ALK	Anaplastic lymphoma kinase
CDKs	Cyclin-dependent kinases
CDKIs	Cyclin-dependent kinase inhibitors
CIN	Chromosomal instability
CMV	Copy number variation
DBD	DNA-binding domain
DMSO	Dimethyl sulfoxide
DLPC	1,2-dilauroyl-sn-glycero-3-phosphocholine
ERα	Estrogen receptor α
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
FOXO	Forkhead box class O family
LBD	Ligand-binding domain
MASI	Mutant allele specific imbalance
NRs	Nuclear receptors
NSCLC	Non-small cell lung cancer
PBS	Phosphate-buffered saline
PML	Promyelocytic leukemia protein
Rb	Retinoblastoma protein
SCLC	Small cell lung cancer
TNBC	Triple-negative breast cancer

Introduction

1. Lung cancer

1.1. Epidemiology and risk factors

Lung cancer is the cancer type with one of the highest incidence and mortality rates worldwide. It is the second most common cancer in men and women, after prostate and breast cancer, respectively. However, it is the leading cause of cancer mortality among the sexes globally, with 1.8 million deaths per year [1], [2]. The areas where lung cancer is mainly detected are developing countries and, more frequently, those of moderate and low incomes [1], [3] (**Figure 1**). The main difference between these regions is related to the morbidity rate of cancer patients due to the limited infrastructure and resources required for early and effective detection, diagnosis and treatment [3].

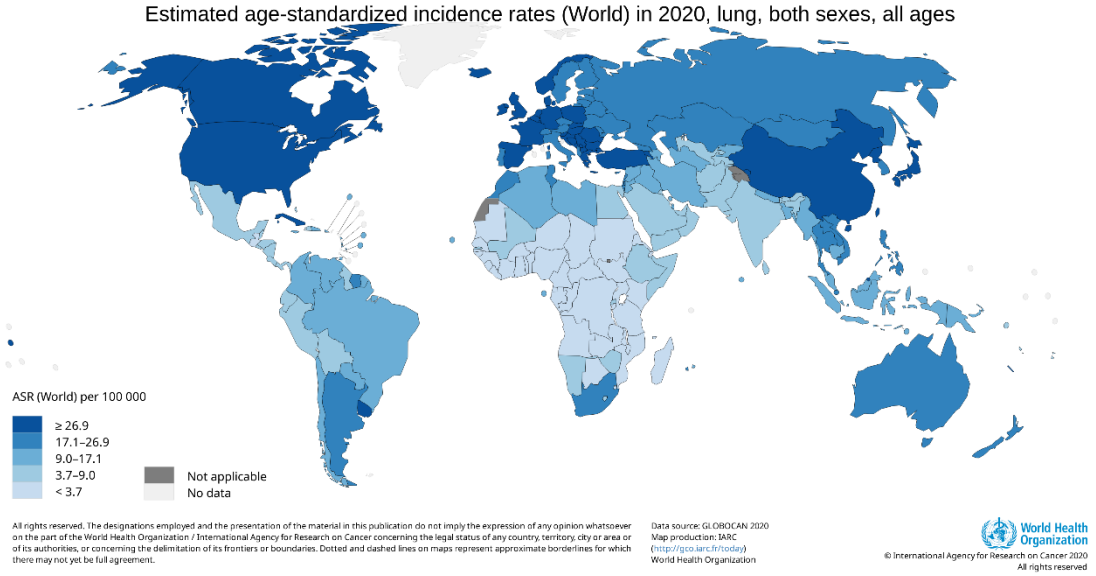

Figure 1: *Illustration of estimated age-standardised global lung cancer incidence rates in 2020 [4]*

Although the disease occurs worldwide, the number of cases, mortality and survival varies from place to place owing to a myriad of factors that cause its occurrence, such as population structure, lifestyle, genetic and environmental factors. In 2020, lung cancer accounted for an estimated 1,796,144 deaths, 18% of all cancer-related deaths around the globe, with the highest rates in Asia (58.3%) (**Figure 2**). Unlike men, the number of affected females continues to rise as industrialisation and access to tobacco are increasing [4]. Unfortunately, survival rates of lung cancer are low since it becomes clinically apparent at an advanced stage. Remarkably, more than 75% of the incidences are diagnosed when cancer is advanced or metastatic [2]. Analysis of survival data reports that the average 5-year survival rate is 17%, with some fluctuations depending on the subtype of lung cancer. However, improvements in survival are likely due to earlier diagnosis and effective treatment modalities with the introduction of targeted therapies [1].

In general, the epidemiology of lung cancer is constantly changing over the years as there is an increase in the number of affected people. Its incidence has been limited

or reduced in the United States due to decades of public education and tobacco control policies. Nevertheless, increasing numbers of incidences are observed in developing countries related to the commencement of the tobacco epidemic [3]. In the United States, the age groups with the highest risk are those aged 55-74 years (53% of the cases) and >75 years (37% of the cases). Younger people can also be affected, presenting 10% of the cases, mainly at 20-46 years, with the highest proportion of female patients [1].

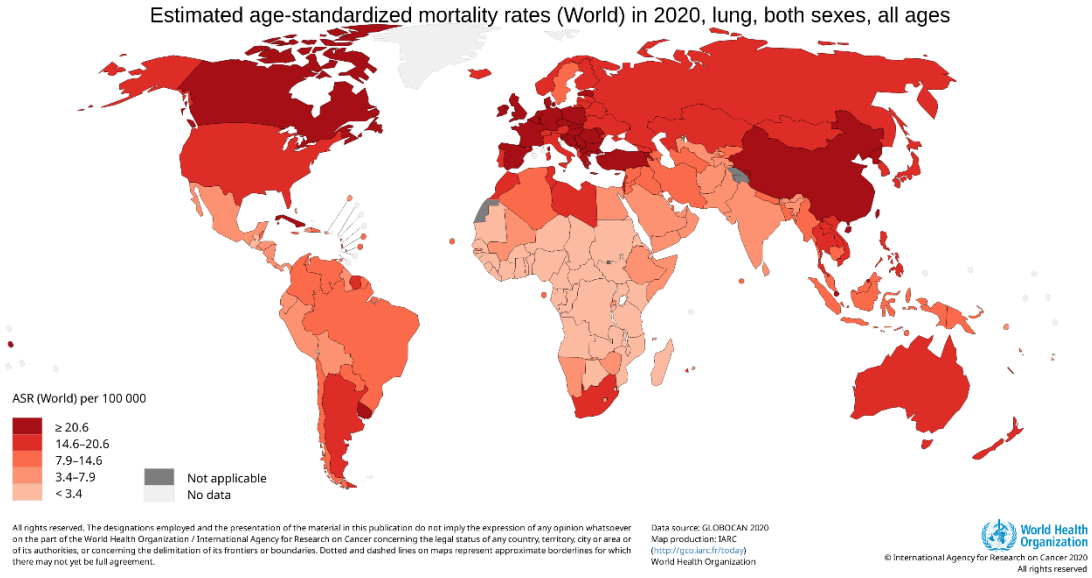

Figure 2: *Illustration of estimated age-standardised global lung cancer mortality rates in 2020 [4]*

Although it is one of the most commonly diagnosed cancer types, early diagnosis and innovations in treatment can be considered effective tools to maintain the disease at stable levels as well as future reduction.

Various risk factors, including demographic and hereditary factors, lifestyle, and living environment, can lead to lung cancer.

Behavioural risk factors

Smoking is considered as the leading preventable cause of developing lung cancer. Tobacco products, such as cigarettes, snuffs, pipes and cigars, contribute to more than 80% of lung cancer cases. Nicotine itself, as the addictive ingredient of tobacco, is not carcinogenic, but according to the International Agency of Research on Cancer (IARC), the combustion of tobacco produces substances deemed carcinogens, including poly-cyclic aromatic hydrocarbons (PAH) and N-nitrosamines. These compounds induce DNA damage and mutations that promote cancer development even decades after use [3], [5]. The risk of lung cancer for smokers varies from 10- to 30-fold, as it depends on the number of cigarettes smoked on a daily basis and years of smoking history. Although continuous efforts since the 1960s to reduce tobacco consumption have led to decreasing numbers of smokers in the United States, measures need to be taken to achieve the same goal in other countries as well, as the numbers are increasing every year [1], [2].

The legalisation of marijuana use in several states of the United States (in 11 states for recreational use and 33 states for medical use) has already increased the research on the effects of marijuana on health and mainly on the risk of developing lung

cancer. It is known that cannabis combustion produces carcinogenic substances like those in tobacco. Moreover, studies have revealed that pre-malignant histological changes in bronchial epithelium, similar to those caused by tobacco smoking, are detected among people who regularly smoke marijuana [1], [3]. However, more studies are required to understand further both the risk and the medical use of cannabis.

Electronic nicotine delivery systems (ENDS), commonly known as e-cigarettes, have rapidly evolved and gained a foothold. Despite their promotion as a stimulating tobacco smoking without carcinogenic compounds, there are studies supporting that e-cigarettes contain PAH, N-nitrosamines and metal traces in various concentrations, but their long-term effect has not been yet determined. Moreover, scientific data reveal that formaldehyde, acetaldehyde and reactive oxygen species are components of e-cigarette vapour in concentrations that can cause inflammatory damage to the respiratory system. Since safety data regarding ENDS are lacking, their use is not considered safe for adolescents, pregnant women and non-smokers [1], [3].

Passive smoking contributes in a dose-dependent manner to lung cancer risk. Side-stream smoke contains carcinogens in higher amounts than filtered tobacco smoke, with adverse effects on health, mainly on pregnant and children. Non-smoking spouses of smokers have a 20-30% increased risk of becoming lung cancer incidence. As many deaths have been attributed to second-hand smoke, the US Surgeon General supports that there is no safe level of exposure to this [1]–[3], [5].

Environmental risk factors

- ***Chemical exposures***

Radon

Radon is a naturally colourless gas produced from uranium decay in the ground. This mutagenic chemical emits alpha particles, whose decay produces polonium and bismuth. Residential radon exposure is deemed the second risk factor for developing lung cancer since it is associated with 10% of cases in the Western world. In the United States, the Environmental Protection Agency recommends air circulation systems to reduce radon exposure, as it can be accumulated in basements and lower building levels. Moreover, there are data supporting that radon might act synergistically with tobacco smoking in developing lung cancer [1]–[3].

Asbestos

Asbestos is a naturally produced mineral with ongoing use in construction since the 1800s. Lung pathologies, such as pneumoconiosis, bronchogenic lung cancer and mesothelioma, have the greatest association with asbestos fibres in the lungs. These fibres can capture tobacco particulates; this explains their involvement in lung cancer, as increased risk has been reported under their coexistence [1]–[3], [5].

Arsenic

According to the IARC, arsenic is a heavy metal with mutagenic properties associated with various cancer types, such as skin, lung, prostate, liver, kidney and bladder. Leakage of inorganic arsenic in groundwater is considered the main exposure source. Although its involvement in lung cancer development is unclear, epidemiologic data reveal the dramatic increase in lung cancer mortality in Chile over the last fifty years after people exposure to high levels of this chemical through water consumption [1].

- ***Chronic obstructive pulmonary disease and other infections of the respiratory system***

Chronic obstructive pulmonary disease (COPD) patients often develop lung cancer, with prevalence varying from 30% to 70%. This might be triggered by DNA damage and inflammatory response, which induce cell proliferation and impair DNA repair mechanisms [1]. Likewise, alterations caused by other respiratory system infections, such as tuberculosis (TB) and HIV, can increase the risk of lung cancer regardless of smoking status. Clinical studies reveal lung cancer as the leading cause of death in the HIV population in the United States. Furthermore, COVID-19 patients exposed to pulmonary inflammation and cytokine release might face an increased risk of developing lung cancer as a long-term effect [1], [3].

- ***Air pollution***

Outdoor and indoor air quality plays a crucial role in developing lung cancer. The concentration of carcinogens, like PAH, sulfur dioxide and trace metals, is crucial mainly in occupations with prolonged exposure, as these chemicals can increase the risk of lung cancer. Additionally, particulate matter, as a Group I carcinogen by IARC, contributes to lung cancer development, as increased numbers of incidence have been detected in cities of the United States with the highest levels of it. Moreover, the combustion of unprocessed fossil fuels, such as soft coal and biomass fuels, leads to indoor air pollution, mainly in developing countries. To avoid the increased risk of lung cancer, ventilation in indoor places is recommended [1], [3], [5].

Genetic risk factors

Considering that not all non-smokers develop lung cancer, the research community has focused on examining a possible connection between genetic susceptibility and lung cancer development. Studies reveal that first-degree relatives of lung cancer patients are prone to develop lung cancer with increased risk 2 to 4 times, regardless of their smoking history [3]. Chromosomal regions, such as 5p15, 15q25-26, and 6p21, are strongly associated with higher lung cancer risk due to their involvement in cell replication and other signaling pathways that control the susceptibility to cancer development [1]. Furthermore, mutations targeting *KRAS*, *EGFR*, and tumour suppressor genes, such as *p53*, *p16*, and *PTEN*, are identified and linked with an increased predisposition [3]. Family history might improve early diagnosis and prognosis, especially among high-risk individuals.

1.2. Classification

Lungs are the main organs of the human respiratory system. Their main task is the external respiration, as oxygen passes into the bloodstream and carbon dioxide from the human body leaves it (gas exchange). The right lung is divided into three sections, called lobes, whereas the left lung consists only of two and has a small structure called lingula with an equivalent size of the middle lobe of the right lung [6], [7]. The air travels through the trachea (windpipe), divided into bronchi, which branch into thousands of inner tubes called bronchioles inside the lungs. Clusters of tiny air sacs called alveoli are found at the end of the bronchioles, where gas exchange occurs. The

pleura, a thin layer, surrounds the lungs to protect them during breathing [7] (**Figure 3**).

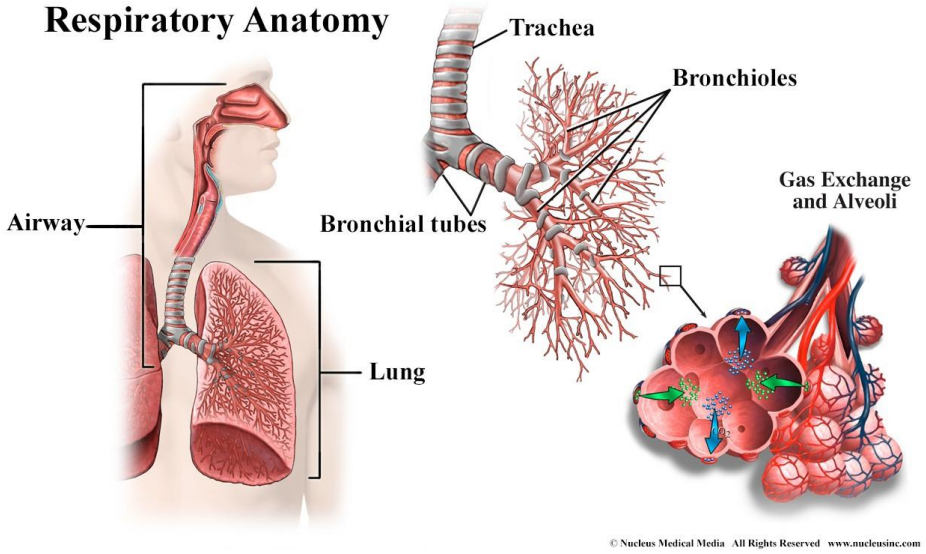

Figure 3: *Respiratory system overview, depicting trachea, bronchial tubes and bronchioles, where gas exchange occurs. Blue arrows represent oxygen in inhaled air passing into the blood circulation, and green arrows represent the carbon dioxide from the human body leaving the blood circulation [7]*

Although lung cancer can develop in any part of the lung, most cases are derived from epithelial cells found in the bronchi and bronchioles. Thus, bronchogenic malignancy or carcinoma are different names for lung cancer. Moreover, other parts where cancer may also arise are the pleura and, rarely, some tissue types that support the lungs, such as the blood vessels [7]–[9]. Overall, there are two anatomic types of lung cancer, central and peripheral [10].

Lung tumours are classified via histopathology into (i) *non-small cell lung cancer* (NSCLC), presenting 80-85% of the cases, and (ii) *small cell lung cancer* (SCLC), comprising 15-20% of the incidences. Considering the complexity of these malignancies pathophysiology, the World Health Organization (WHO) is constantly updating the classification system of lung tumours to improve the diagnosis and management of this cancer type, as their differentiation is crucial before starting treatment [10]–[13] (**Figure 4**).

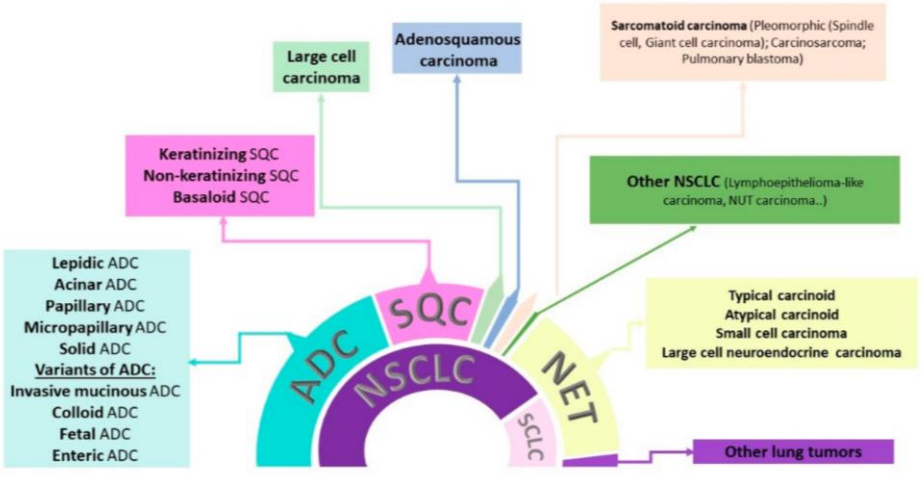

Figure 4: *Histological classification of lung tumours [13]*

Although NSCLC is not present in the histological classification, in general terms, it mainly consists of adenocarcinoma, squamous cell carcinoma, and large cell carcinoma groups.

- *Adenocarcinoma* is the most commonly detected lung cancer type, comprising 50% of NSCLC cases, with its incidence rate increasing among non-smokers and young women in recent decades. It is a malignant epithelial neoplasm with glandular differentiation or mucin production. Its spread occurs in the same or contralateral lung; it frequently metastasises to the liver, bones, brain and suprarenal glands. Its association with multiple gene alterations, such as *ALK* gene rearrangements, *EGFR* and *KRAS* mutations, may pave the way for targeted molecular therapies to improve patient survival [10]–[12], [14] (**Figure 5A**).
- *Squamous cell carcinoma* accounts for 40% of NSCLC incidences, and it is developed in the central chest area and can form cavities. Its incidence rate has decreased due to changes in smoking prevalence in the last few years. The patient survival rate is significantly higher than those affected by adenocarcinoma, as it is characterised by the slowest growth rate among lung tumours. Although these types of tumours are excluded from molecular testing, identifying mutations in squamous cell carcinoma may also have applications in personalised therapy [10]–[12], [14] (**Figure 5B**).
- *Large cell carcinoma* is the minority of NSCLC incidences (less than 10%) and is usually formed peripherally. It usually spreads to the mediastinum and hilar nodes. Most cases have a poor prognosis, particularly those with null immunophenotype [10]–[12], [14].

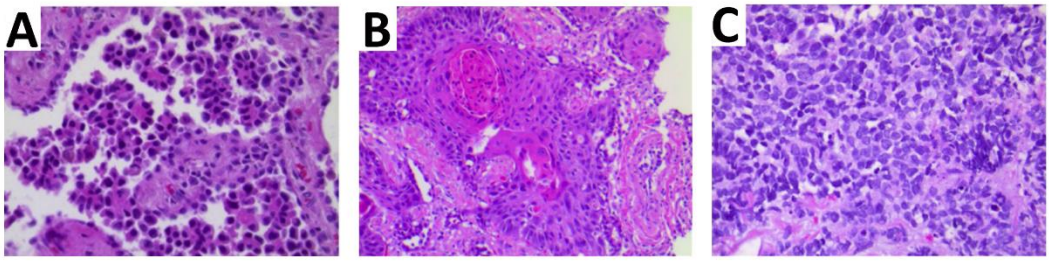

Figure 5: (A) *Micropapillary adenocarcinoma (H&E staining, original magnification x200).* ***(B)*** *Keratinizing Squamous Cell Carcinoma with keratin pearl formation (H&E staining, original magnification x200).* ***(C)*** *Small cell lung carcinoma with small sized nuclei and scanty cytoplasm (H&E staining, original magnification x400) [12]*

Small cell lung carcinoma (SCLC) derives from neuroendocrine cells of the basal bronchial epithelium and is strongly correlated with the patient's smoking history (**Figure 5C**). Unfortunately, SCLC is detected at a metastatic stage since it is highly aggressive and can rapidly spread beyond the lungs. Survival rates are increasing with the application of chemotherapy and radiation therapy, but the majority of patients relapse within the first two years after treatment completion. The five-year survival rate ranges from 5% to 10% [9]–[12].

Besides the aforementioned types of lung cancer, other tumour types can arise from lungs, such as precursor glandular lesions, sarcomatoid carcinomas, and others [11].

1.3. Lung Cancer Progression

Cancer staging refers to establishing the extent of (i) the primary tumour and (ii) the tumour spread within the human body. It is crucial for the diagnosis and treatment processes, as it contributes to [14]:

- The design of a treatment plan
- The indications of prognosis
- The evaluation of the treatment results
- The exchange of knowledge among the treatment centres
- The ongoing investigation of human cancer

The American Joint Committee on Cancer (AJCC) developed the TNM staging system to determine the stage of cancer by using three descriptors regarding the size of the primary tumours (**T**), the tumours spread to lymph nodes (**N**), and the absence/ presence of metastasis (**M**). The system classification consists of the different groups of tumour characteristics (T1 to T4), the type of the involved lymph nodes (N0 to N3), and the absence (M0) or presence (M1) of metastasis (**Figure 6**). The combination of these three descriptors provides an overall stage (I-IV) for the tumour to categorise the patients into stages with similar prognoses and treatment approaches [15], [16] (**Figure 7**).

<table>
<tr><th colspan="2">Tumour size (cm)</th><th colspan="2">Lymph Nodes</th><th colspan="2">Metastasis</th></tr>
<tr><td>T1</td><td><3</td><td>N0</td><td>No lymph nodes</td><td rowspan="3">M0</td><td rowspan="3">Absent</td></tr>
<tr><td rowspan="2">T2</td><td>T2a 3-5</td><td rowspan="2">N1</td><td rowspan="2">Ipsilateral bronchopulmonar/hilar lymph nodes</td></tr>
<tr><td>T2b 5-7</td></tr>
<tr><td>T3</td><td>>7</td><td rowspan="3">N2</td><td rowspan="2">Ipsilateral mediastinal/subcarinal lymph nodes</td><td rowspan="3">M1</td><td rowspan="3">Present</td></tr>
<tr><td rowspan="3">T4</td><td>Invasion</td></tr>
<tr><td>Mediastinal organs</td><td rowspan="2">N3</td><td rowspan="2">Contralateral/ hilar/ mediastinal supraclavicular lymph nodes</td></tr>
<tr><td>Vertebral bodies</td></tr>
</table>

Figure 6: *TNM system of malignant lung tumour classification [16]*

<table>
<tr><th colspan="7">TNM Stage grouping</th></tr>
<tr><td>Any T
Any N</td><td colspan="5">IV</td><td>M1</td></tr>
<tr><td>N3</td><td colspan="5">IIIB</td><td rowspan="6">M0</td></tr>
<tr><td>N2</td><td colspan="4">IIIA</td><td>IIIB</td></tr>
<tr><td>N1</td><td>IIA</td><td>IIB</td><td>IIA</td><td>IIB</td><td>IIIA</td><td rowspan="2">IIIA</td></tr>
<tr><td>N0</td><td colspan="2">IA</td><td>IB</td><td>IIA</td><td>IIB</td></tr>
<tr><td></td><td colspan="2">T1</td><td colspan="2">T2</td><td>T3</td><td>T4</td></tr>
</table>

Figure 7: *TNM stage grouping [14]*

1.4. Lung Cancer Prognosis, Diagnosis and Treatment

The poor prognosis of lung cancer remains a major challenge, as the majority of the patients, approximately more than 75%, are diagnosed with lung cancer at an

advanced stage (stage III or IV). Most regional lymph node involvement cases or even a distant disease are detected. Typical symptoms include chest pain, cough, dyspnea, weight loss, loss of appetite and hemoptysis. Therefore, diagnosis at the earliest stage is imperative, as it is associated with early prognosis [16], [17].

Radiography

Radiographic screening is currently recommended for early detection at a treatable stage, mainly in high-risk groups. Computed tomography (CT) can quickly provide an image of the whole chest while effectively detecting peripheral lung lesions. However, there are restrictions to its implementation due to its high cost and limited accessibility. Patient exposure to multiple CT scans, even under low-dose radiation, can lead to the development of cancer types, such as breast, thyroid, or lung cancer. Moreover, its high rate of false positives triggers the need for invasive methods, which might be applied to subjects free of lung cancer [13], [16], [17].

Sputum examination

Another diagnostic tool is the cytological examination of sputa, which detects central tumours from the larger bronchi, such as squamous and small cell carcinomas. Its sensitivity to detect lung cancer at an earlier stage ranges from 20% to 30%, depending on the number and type of cells. Considering its low accuracy, studies support that immunostaining can provide more sufficient and favourable data compared to sputum cytology and chest X-ray, as it has a 2-to 3-fold higher sensitivity for early detection [13], [16].

Bronchoscopy

White light bronchoscopy (WLB) is commonly used to obtain a definite histological lung cancer diagnosis. Since the diagnosis of pre-malignant lesions is limited, new approaches are developed, with LIFE-lung Fluorescence Endoscopy gaining the field. Many studies report increased diagnostic sensitivity, though its specificity needs improvement [13], [16], [17].

Lung Tissue Biopsy

Tissue biopsies comprise the benchmark for cancer confirmation. The initial biopsy should be applied carefully to avoid repetition and confirm an early diagnosis without delay in treatment initiation [13], [16].

Biomarkers

Limitations to early lung cancer screening and diagnosis by the aforementioned methods could be addressed by an accurate, reproducible, and inexpensive test, which can be used as a general screening tool every year. Several biomarkers are promising candidates for detecting lung cancer at an early stage. Tissue samples from the respiratory tract, including sputum, saliva, nasal/bronchial airway epithelial cells, exhaled breath condensate, and liquid biopsies, can be analysed and provide data to identify a broad spectrum of biomarkers. Although quite invasive procedures are required, their evaluation is repeatable and inexpensive compared to imaging methods. Most established biomarkers can be detected by PCR, metabolomics, or other molecular biology techniques with fast data acquisition. Some examples of these biomarkers are the following:

- Circulating free DNA (cfDNA)
- Circulating Tumour Cells (CTCs)
- Blood Circulating Antigens (CYFRA 21-1, NSE, etc.)
- Mutations in genes (*p16*, *p53*, *KRAS*, etc.)
- microRNAs (miRNAs) (mir-155, mir-197, etc.)
- Volatile Organic Compounds (VOCs)

It is commonly believed that more reliable tests could derive from biomarker discovery for early diagnosis and prediction to alleviate patient discomfort and lead to targeted therapies with better outcomes [13], [16], [18], [19].

Treatment

The main goal of a lung cancer treatment regimen is to maintain the quality of life while prolonging the patient's life expectancy. The most frequently applied treatment approaches are surgical resection, radiotherapy, adjuvant and neoadjuvant chemotherapy, and immunotherapy. The administered drugs can help relieve a wide range of symptoms, increase survival rate, and occasionally treat the disease. Treatment regimens usually have multiple targets to increase their efficiency, as there is a significant likelihood of disease recurrence and death due to metastasis, mainly in the brain. A successful treatment approach depends on the following:

I. The stage at diagnosis (tumour size, regional lymph nodes involvement, presence of metastasis)
II. The histologic subtype
III. The molecular characterisation

A treatment regimen consists of one of the above-mentioned methods or their combination to increase effectiveness. Although several targeted therapies have been approved, such as epidermal growth factor receptor (EGFR) inhibitors, anaplastic lymphoma kinase (ALK) inhibitors, and others, research is continuously needed to identify the underlying molecular alterations and predispositions affecting clinical outcomes. Reliable biomarkers may contribute to the field of precision medicine by selecting the appropriate treatment and improving the early diagnosis and prognosis of lung cancer [14], [16], [17].

2. Molecular Profile of Lung Cancer

2.1. Molecular alterations

Tumourigenesis is the outcome of the accumulation of various molecular alterations and is characterised by extensive heterogeneity, which is associated with critical issues in the diagnosis and treatment of cancer patients [20]. In lung cancer, tumour heterogeneity has been detected in different levels, such as interpatient, intratumour, and intertumour. Several genetic, epigenetic, and non-genetic mechanisms could contribute to forming subpopulations of cells with genetic, epigenetic, and phenotypic differences, resulting in heterogeneity. Chromosomal instability (CIN), as a type of genomic instability, depicts the numerical and structural variations of part(s) or whole chromosomes. It is correlated with poor prognosis in lung cancer patients and might stimulate drug resistance. Another genetic mechanism is mutant allele specific imbalance (MASI), which promotes heterogeneity and triggers tumourigenesis,

progression, metastasis, prognosis, and therapeutic response in cancer. Additionally, epigenetic mechanisms, including DNA methylation, chromatic remodelling, and post-translational histone modifications, play an essential role in this molecular variability. Apart from the genetic and epigenetic mechanisms, stem cell populations and the immune microenvironment, as non-genetic sources, could result in tumour heterogeneity. Cancer stem cell characteristics, such as self-renewal, multipotency, ability to initiate tumour *in vivo*, and increased proliferation and differentiation capacity, could lead to distinct phenotypes and genotypes of tumours. Furthermore, the immune microenvironment can determine tumour context by inducing the formation of specific molecules to support responses to environmental alterations. Overall, lung cancer heterogeneity could contribute to target- and immuno- therapy response [21].

Next-generation sequencing contributes to distinguishing the genomic profiles among the different lung cancer histotypes (**Figure 8**). It allows a better understanding of their molecular characterisation and might provide information regarding identifying prognostic and predictive factors [21]. Some of the most commonly altered and clinically significant oncogenes and tumour suppressor genes are described below.

Histotype		Type of genomic aberrations		Gene	Frequency (%)	Currently available target therapy
Non-small cell lung carcinoma (NSCLC)	Adenocarcinoma	Fusions		ALK	3-7	A
				ROS1	2-3	A
				RET	1-2	NA
				NTRK1	1-2	NA
		Mutations		EGFR	30-40	A
				BRAF	0.5-5	NA
				KRAS	20-30	NA
				MET	3-4	NA
				PTEN	1.7	NA
				PDGFRA	6-7	NA
				PIK3CA	5	NA
				TP53	52	NA
		Copy number gene alterations	Gains	ERBB2	2-5	NA
				EGFR	10	NA
				MET	2-5	NA
				TERT	75	NA
			Losses	CDKN2A	7	NA
	Squamous cell carcinoma	Fusion Mutations		FGFRs	23	NA
				TP53	79	NA
				NF1	10	NA
				FGFR1	20	NA
				FGFR2	3	NA
				DDR2	2-3	NA
				BRAF	4-5	NA

	Squamous cell carcinoma	Fusion Mutations		KRAS	1-2	NA
				PDGFRA	4	NA
				PIK3CA	15	NA
				PTEN	10	NA
		Copy number gene alterations	Gains	SOX2	65	NA
				PIK3CA	15	NA
				TP53	79	NA
			Losses	CDKN2A	15	NA
				PTEN	8	NA
Small cell lung carcinoma (SCLC)		Mutations		TP53	90	NA
				RB1	90	NA
				EP300	4-6	NA
				CREBBP	4-6	NA
				PTEN	10-18	NA
		Copy number gene alterations	Gains	MYC	20-30	NA
				MYCN	20-30	NA
				MYCL1	20-30	NA
				SOX2	27	NA
				FGFR1	5-6	NA

Figure 8: *Molecular profile of lung cancer histotypes [21]*

- ***ALK***

The *ALK* gene is located on chromosome 2 (2p23.3-p23.1) and encodes for a transmembrane protein, which is a receptor tyrosine kinase and a member of the insulin receptor superfamily. Genetic alterations, such as fusions, amplifications, and mutations, are found in non-small cell lung cancer, with most alterations related to chromosomal rearrangements. They are mainly detected in adenocarcinomas from young patients who are never or light smokers. Although crizotinib is efficient in treating gene amplifications, mutations in *ALK* induce resistance to current tyrosine kinase inhibitors [22]–[24].

- ***EGFR***

EGFR is a gene located on chromosome 7 (7p11.2) and encodes for a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for epidermal growth factor family members. It is involved in several cellular processes, such as cell proliferation, invasion and metastasis. Mutations in this gene are associated with developing various cancer types, including lung cancer. All *EGFR* mutations are correlated with adenocarcinoma histotype and are mainly detected in younger female patients with no smoking history. Tyrosine kinase inhibitors (TKIs), mainly gefitinib and erlotinib, are efficient in *EGFR* amplifications, though *EGFR* mutations, particularly the variant T790M, might trigger drug resistance. The later generation of TKIs target this mutation and comprise a promising practice in non-small lung cancer treatment [22]–[24].

- ***KRAS***

Kirsten ras oncogene is located on chromosome 12 (12p12) and encodes for a protein member of the GTPase superfamily with a vital role in cell proliferation, differentiation, and survival. A single amino acid substitution induces mutations resulting in several malignancies, including lung cancer. Male smokers in the Western world are frequently prone to obtain these genomic abbreviations. *KRAS* mutations in lung cancer are associated with poor prognosis and reduced or no response to EGFR TKIs. Efforts to directly target the *KRAS* gene or its upstream or downstream proteins are needed to develop targeted therapies [22]–[24].

- ***BRAF***

This gene is mapped on chromosome 7 (7q34) and encodes for a protein belonging to the RAF serine/threonine kinase family. This protein regulates the MAPK/ERK signaling pathway and affects cell differentiation, mobility, and survival. Mutations in this gene are detected in melanoma and non-small cell lung cancer. V600E, the most common genomic alteration, is detected in female patients with no smoking history. *BRAF* mutations are classified depending on their resistance to inhibitors and differ in their application for therapeutic or prognostic purposes [22]–[24].

- ***PTEN***

PTEN is a tumour suppressor gene located on chromosome 10 (10q23.31) and encodes for a protein with lipid and protein phosphatase activities. It is a key modulator of the PI3K/AKT/mTOR pathway and is rarely mutated in non-small cell lung cancer, mainly in squamous cell carcinoma. The application of miRNAs targeting *PTEN* is under investigation, as mTOR inhibitors seem to be ineffective in treating lung cancer patients [22], [24].

- ***TP53***

TP53 is a gene mapped on chromosome 17 (17q13) and encodes for a phosphoprotein that identifies and binds to DNA-damaged regions and regulates gene expression. It is essential in cellular processes, such as cell cycle arrest, DNA repair, and others. Specifically, DNA damage or carcinogenic stress induce *TP53* by activating cyclin kinase inhibitors, which supress cell cycle progression and initiate DNA repair or apoptosis. Inactivation of this gene, due to genomic alterations, occurs in lung cancer, with higher frequency in small cell carcinomas, and is correlated with tobacco smoking and passive smoking. *TP53* mutations are detected in the tetramer responsible for its transcription factor activity and might provoke drug resistance. P53 loss protein function is associated with poor prognosis in lung cancer patients and often coexists with *EGFR* and *KRAS* mutations [22], [24].

2.2. Cell cycle in cancer

The cell cycle is a vital process that controls cell division and proliferation. Its control is mainly focused on genomic DNA replication and its subsequent segregation between the daughter cells. A network of regulatory mechanisms checks and ensures that the cell can continue its division to the next phase without having been mistakes. However, cancer cells undergo uncontrolled cell cycle progression with several

defective cell cycle checkpoints. Notably, they can escape these checkpoints to enter and progress through the cell cycle resulting in their division even under adverse conditions [25].

There are two critical periods in cell cycle control, the former is during the G1 phase by checking the integrity of DNA to decide whether the cell will enter the cell cycle, and the latter is during the G2 phase by ensuring the completion of DNA replication to initiate the procedure for chromosome segregation. These processes are regulated by Cyclin-dependent kinases (CDKs), with the accumulation of specific cyclins at different cell cycle phases leading to cell cycle progression through transcriptional regulation and inhibition of protein degradation (**Figure 9**). Their activity is regulated by cyclin-dependent kinase inhibitors (CDKIs), such as p21 and p27, leading to cell cycle arrest. Cancer cells are commonly characterised by dysregulation of CDKs, comprising an attractive therapeutic target [25], [26].

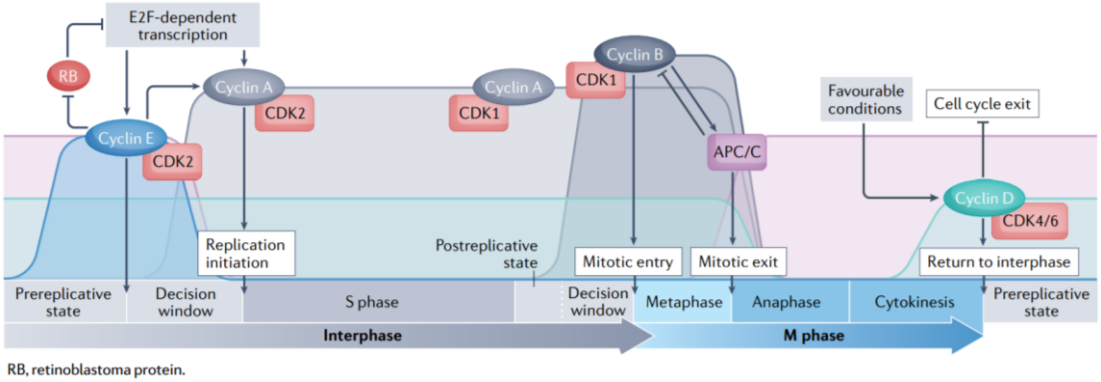

Figure 9: *CDK activity during cell cycle progression [25]*

Investigating the mechanisms by which the cell cycle is regulated might provide more data to develop effective therapeutic approaches, either by improving the current ones or by extending the range of therapeutic methods for designing a treatment regimen with better outcomes for cancer patients [25].

2.2.1. $p21^{Cip1}$

The *CDKN1A* gene encodes for a cyclin-dependent kinase inhibitor (CDKI), a member of the Cip/Kip family of CDKIs. It binds to and inhibits the activity of cyclin-CDK2 and cyclin-CDK4 complexes and modulates cell cycle progression in G1 phase [24], [27]. Although the tumour suppressor protein P53 tightly regulates $p21^{Cip1}$ expression, it can also be induced by p53-independent pathways, such as *MYC* involvement in regulating $p21^{Cip1}$ at RNA level. Moreover, post-translational modifications impact the protein function, as phosphorylation of Thr145 triggers the localisation of $p21^{Cip1}$ to cytoplasm resulting in its inactivation [27], [28].

This CDKI seems to have a dual role in tumourigenesis and tumour progression, which might be affected by its localisation in the cytoplasm or the nucleus. Somatic mutations in the *CDKN1A* gene are rare, but in some cases, epigenetic silencing has been detected [28]. In non-small cell lung cancer, increased protein levels are associated with a favourable prognosis for patients, while it might lead to inhibiting tumour growth and enhancing chemosensitivity [29], [30]. In further agreement, some studies support that functional loss of $p21^{Cip1}$ could stimulate the drug resistance phenotype in cancer

therapy [29]. However, recent scientific data reveal that high levels of $p21^{Cip1}$ are correlated with poor prognosis in small cell carcinoma, the opposite effect observed in NSCLC, which denotes a context-dependent function of $p21^{Cip1}$ in lung cancer. These data indicate $p21^{Cip1}$ as a promising prognostic and predictive marker, but further investigations are needed to understand how it regulates tumour progression and determines the response to therapy regimens [28]–[30].

2.2.2. $p27^{Kip1}$

The *CDKN1B* gene encodes for a cyclin-dependent kinase inhibitor (CDKI), a member of the Cip/Kip family of CDKIs. It interacts, via its N-terminal domain, with cyclin D-, E-, A-, and B-CDK complexes and inhibits their catalytic activity [24], [28]. $p27^{Kip1}$ regulated cell cycle progression and migration. A broad spectrum of independent phosphorylation events regulates $p27^{Kip1}$ by inducing its proteolysis after its localisation to the cytoplasm. Moreover, Forkhead box class O family (FOXO) proteins activate transcription of *CDKNIB* gene as a response to cytokines or promyelocytic leukemia protein (PML) and nuclear Akt signaling. Its activity depends on its concentration, subcellular localisation, and phosphorylation status [28], [31].

Reduced $p27^{Kip1}$ nuclear concentration is detected in several human cancer types and is associated with poor prognosis, mainly in NSCLC [32], [33]. In lung cancer, in 30% of the incidences, loss of $p27^{Kip1}$ expression is detected, notably in adenocarcinoma is higher compared to squamous cell carcinoma [32]. However, in SCLC patients, high expression levels of $p27^{Kip1}$ are observed and possibly induced by genetic alterations, such as *MYC* amplification and retinoblastoma protein (*Rb*) activation, and extracellular microenvironment changes, such as nutrient insufficiency and low oxygen concentration, which lead to a non-functional state of $p27^{Kip1}$ allowing SCLC cells to escape cell cycle arrest [33]. Furthermore, restoration of *CDKN1B* protein levels and/ or nuclear localisation might be useful as a predictive marker for molecular therapies targeting EGFR and IGFR families. Interestingly, slightly more than 60% of NSCLC cases are characterised by *EGFR* overexpression, whose activation triggers $p27^{Kip1}$ proteolysis. Therefore, in treated cancer cases, EGFR inhibitors should increase $p27^{Kip1}$ levels. Response to EGFR inhibitors, such as gefitinib and tipifarnib, in NSCLC could be predicted depending on the observed $p27^{Kip1}$ levels in tumours or post-treatment conditions [31]. These observations increase the need for further studies to identify the biological factors involved in $p27^{Kip1}$ role in cancer and its application for patient prognosis.

3. Nuclear Receptor NR5A2

Nuclear receptors (NRs) comprise an extensive superfamily of transcription factors that bind directly to DNA sequences and evoke diverse functions in several biological processes, including development, differentiation, and metabolism. It comprises 48 functional members, classified into seven subfamilies (N0-N6) with three classes (I-III) depending on their structural similarities and DNA-binding characteristics. NRs share a conserved modular structure that comprises [34], [35]:

- the modulatory **A/B domain** (NH_2-terminal) often containing a ligand-dependent activation function-1 (AF-1)
- the highly conserved **C domain** (DNA-binding domain or **DBD**) consisting of two zinc fingers and being responsible for the NR ability to target specific DNA sequences, known as hormone response elements (HREs)
- the **E domain** (C-terminal, ligand-binding domain or **LBD**) containing a ligand-dependent activation function-2 (AF-2) involved in co-activator interactions
- the **D domain** serving as a flexible hinge between DBD and LBD

Various lipophilic molecules, such as steroid hormones, metabolites, and endo/xenobiotics, bind to numerous NRs, causing conformational changes and regulating their activity. Specifically, upon ligand binding, activation of NRs induces the dissociation of co-suppressors or the recruitment of co-activators, selectively inhibiting or facilitating the transcription of downstream target genes. Ligands for several members of the NR superfamily have been established and validated as targets of clinical drugs with applications in cancer, inflammation, and metabolic diseases. Thus, it is denoted that NRs are potential drug targets to treat human diseases, increasing the interest in identifying more ligands, mainly for orphan NRs [35], [36].

3.1. NR5A2: Structure, Regulation and Role in Health

NR5A2, also known as liver receptor homolog-1 or LRH-1, is a member of the NR5A subfamily of nuclear receptors, which consists of four members (NR5A1-NR5A4). It was initially identified in *Drosophila* called *fushi tarazou factor-1* (*ftz-f1*), and its orthologs have been identified in several species, including Xenopus, horse, mouse, rate, zebrafish, horse, and human [34], [35], [37].

The human gene encoding for NR5A2 is located on chromosome 1 (1q32.11). It consists of 8 exons extending to more than 150 kilobases (kb) while having three isoforms as products of alternative splicing occurring in humans. It displays the typical structure of the NR superfamily with some alterations. An additional 30 amino acid sequence, called **FTZ-F1 box** (or A box), is located at the C-terminal of the DBD, adjacent to the second zinc finger. It interacts with the P box (in the first zinc finger of the DBD), allowing NR5A2 to bind, as a monomer, to its DNA-targeted sequences with high affinity. Specifically, NR5A2 binds to response elements that contain the consensus binding site of the FTZ-F1 box (YCAAGGYCR, Y=pyrimidine, R=purine). The LBD domain is responsible not only for the AF-2 but also facilitating co-modulator association, as ligand binding induces conformational changes allowing co-activator recruitment and subsequent transcriptional activation. However, orphan NRs, whose natural ligands remain elusive, are constitutively active. NR5A subfamily members, so as NR5A2, are classified into this group. Moreover, the A/B domain of NR5A2 interacts with the remaining part of LBD in a ligand-independent manner [34]–[38]. The detailed three-dimensional structures of NR5A2 and its LBD are illustrated in (**Figure 10**).

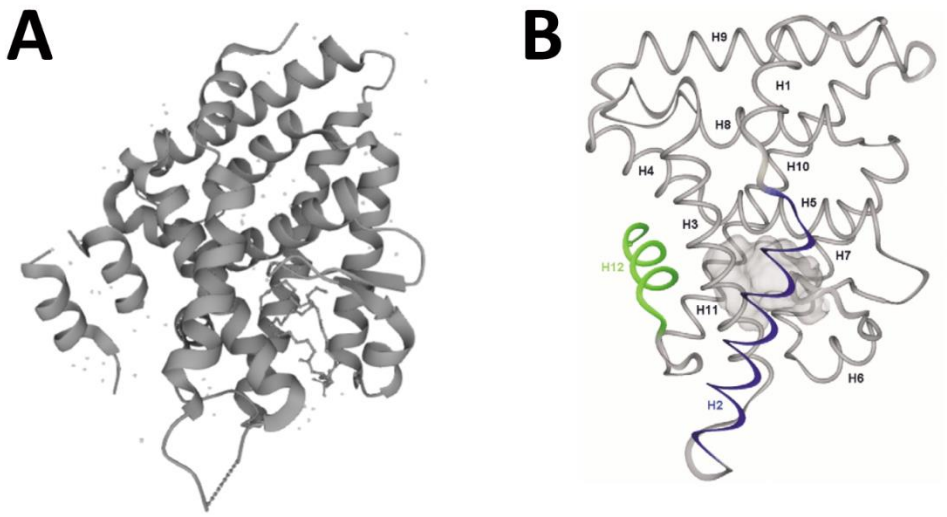

Figure 10: (A) *3D structure of human NR5A2 [39]* ***(B)*** *3D structure of the LBD of mouse NR5A2. Helix (H) 12 (green) is the active site containing the AF-2. H2 (blue) provides an additional layer to the canonical LBD fold stabilising the active position. The grey area depicts the empty ligand-binding pocket. [35]*

In adults, NR5A2 is distributed in endodermal tissues, such as intestine, liver, and pancreas, but it can also be detected in other tissues, including lung, ovary and pre-adipocyte. Its expression in adrenal gland and testis is species-specific, and it is present at low levels in endometrium and placenta [34], [36] (**Figure 11**).

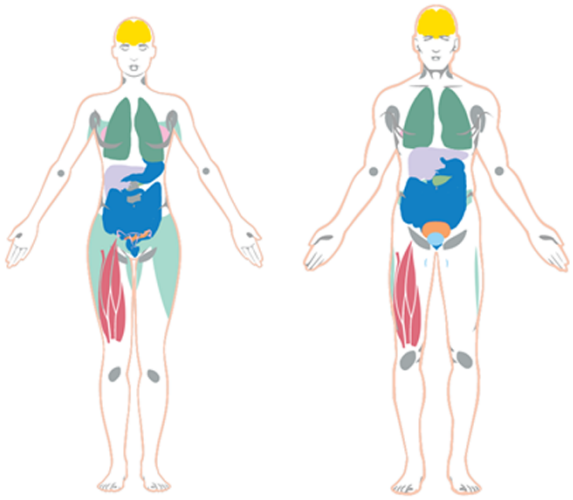

Figure 11: *Tissue distribution of NR5A2 [40]*

NR5A2 plays a significant role in multiple physiological processes aiming to maintain cellular homeostasis (**Figure 12**).

Figure 12: *Role of NR5A2 in normal homeostasis [36]*

In early embryonic development, it is involved in the differentiation of liver, pancreas, and intestine since loss-of-function studies support that NR5A2 deficient mice die between E6 and E7.5 due to defective endoderm development. Moreover, it is vital to maintain stem cell pluripotency, as it regulates the expression of Oct4 and Nanog through the Wnt signaling pathway in early embryonic stages. In adults, it is a key regulator of steroidogenesis and cholesterol/bile acid homeostasis. Cholesterol is essential for various cellular processes, including membrane biogenesis, steroid hormones and bile acid biosynthesis. NR5A2 regulates pathways that control intracellular and circulating cholesterol levels, including reverse cholesterol transport, bile acid synthesis, and enterohepatic bile acid circulation. Mainly, NR5A2 induces the expression of the cholesterol-ester-transfer protein (CETP), facilitating the transport of the accumulated cholesterol from non-hepatic peripheral tissues to liver. Subsequently, the upregulation of scavenger receptor class B type I (SR-B1) initiates the cholesterol uptake from high-density lipoproteins (HDLs). NR5A2 controls the conversion of cholesterol to bile acid by inducing cholesterol 7a-hydroxylase (*CYP7A1*) and sterol 12a-hydroxylase (*CYP8B1*) genes. Dysregulation of NR5A2 is associated with several metabolic disorders, such as type 2 diabetes and non-alcoholic fatty liver disease [34], [35], [37], [41].

Treatment of many human diseases, including cancer, might benefit from NR5A2 activity modulation. Its activity can be regulated at multiple levels. At the transcriptional level, several signaling pathways, such as the Wnt/β-catenin, MAPK/ERK, and PI3K/Akt pathways, can regulate NR5A2 expression and activity. Additionally, the interaction between NR5A2 and its co-regulators may induce its

activity in the case of co-activators (like SRC and β-catenin) or suppress it in the case of co-repressors (like Prox1 and NCoR1). At the post-transcriptional level, microRNAs (miRNAs) can regulate this nuclear receptor by targeting its mRNA for degradation or translational repression. Studies support that miR-30d and miR-1275 inhibit NR5A2 expression in many tissues and cells. Moreover, alternative splicing can lead to different isoforms with distinct functions. Several post-translational modifications, including SUMOylation, phosphorylation, ubiquitination, and acetylation, can affect NR5A2 activity. SUMOylation of NR5A2 can determine its subcellular localisation and stability, while phosphorylation and acetylation can enhance or inhibit its activity. Various ligands can also modulate the activity of NR5A2 by binding to its LBD domain, resulting in conformational changes that facilitate the interaction with co-regulators and regulatory proteins. Overall, these mechanisms can precisely regulate NR5A2, allowing its response to cellular and environmental alterations and enhancing its function in various biological processes [34], [36].

3.1.1. NR5A2 ligands as a therapeutic approach

Although NR5A2 natural ligands remain elusive, several phospholipid derivatives, including phosphatidylglycerol, and second messengers like phosphatidylinositol bind to the ligand-binding pocket, denoting that phospholipids act as endogenous ligands. NR5A2 is constitutively active, but its transcriptional activity can also be enhanced by phospholipid binding to its ligand-binding pocket. DLPC (**Figure 13A**) and DUPC, two phospholipids, are well-established NR5A2 ligands, and they modulate its activity via specific binding to NR5A2 promoters [36].

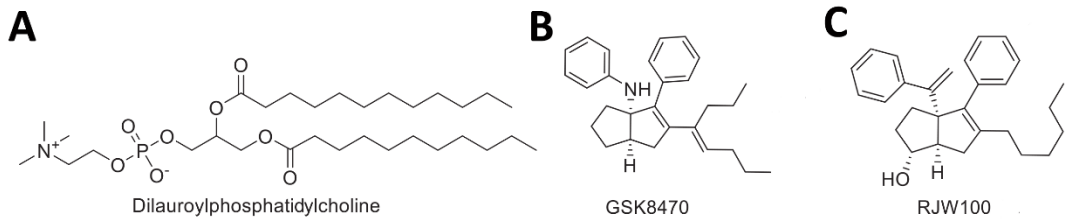

Figure 13: *Structure of NR5A2 ligands* ***(A)*** *1,2-dilauroyl-sn-glycero-3-phosphocholine,* ***(B)*** *GSK8470,* ***(C)*** *RJW100 [42]*

Studies support that DLPC regulates bile acid metabolism and glucose homeostasis [42]. In recent decades, several synthetic agonists and antagonists of NR5A2, such as GSK8470 (**Figure 13B**) and RJW100 (**Figure 13C**), have been developed to modulate its function for therapeutic approaches [36]. They can also be used to understand the various biological functions of NR5A2 and identify analogues with improved properties [43], [44].

3.2. NR5A2: Role in Cancer

Nuclear receptors (NRs) significantly impact various physiological processes, while their implication in the development and progression of many cancer types has been reported. Aberrant expression or activation of NRs is associated with tumour

formation. Scientific data support that NRs are downregulated in most cancer types, although, unlike other transcription factor families, copy number variation (CMV) or genetic alterations are rarely detected. However, epigenetic alterations might affect these events resulting in the downregulation of NRs but with the maintenance of their function [34], [45].

NR5A2 is implicated in the development and progression of several cancer types, as it regulates the expression of genes involved in cell proliferation and differentiation and tumour growth and metastasis. An interaction between NR5A2 and β-catenin, *KRAS*, *MYC*, *CCND1*, and *CCNE1* has been reported in different studies [41], [45], [46].

- ***Pancreatic cancer***

In pancreatic cancer, NR5A2 seems to have a dual role. On the one hand, increased NR5A2 levels are associated with cancer progression, metastasis, and poor prognosis in patients. Genome-wide association studies provide data regarding the link between single nucleotide polymorphisms in its gene and high pancreatic cancer risk. Overexpression of NR5A2 induces tumour formation and confers to a more aggressive malignant phenotype, as it promotes cell migration and invasion. Additionally, the interaction between cancer cells and the tumour microenvironment can be affected by the pro-inflammatory environment created through the induction of chemokine and cytokine expression by NR5A2. Recent studies manage to block the activity of NR5A2 using siRNA, inhibiting cell proliferation and differentiation *in vitro*. Conversely, recent studies reveal the tumour-suppressor function of NR5A2, as it inhibits the development of KRAS (G12V)-driven mouse pancreatic intraepithelial tumours. However, future studies investigating the effect of the individual SNPs on the protein function of NR5A2 and the underlying mechanisms of its involvement in every stage of pancreatic cancer could help understand its role [36], [41], [45]–[47].

- ***Breast cancer***

Estrogens promote breast cancer since nuclear estrogen receptor α (ERα) is expressed in most breast cancer cases. NR5A2 is also expressed in slightly less than half of breast carcinomas and is positively correlated with tumour ER status. It stimulates aromatic expression in adipose stromal cells, promoting the development of ER-positive tumours. NR5A2 induces cell cycle progression and suppresses apoptosis by activating *CCND1* and inhibiting *Bax*, respectively. Its association with cancer cell increased invasive and metastatic properties is based on its interaction with genes involved in epithelial-mesenchymal transition (EMT). Although in triple-negative breast cancer (TNBC) ERα, progesterone receptor, and HER2 expression are absent, NR5A2 is expressed and promotes cell invasion and metastasis. Moreover, scientific data suggest that NR5A2 induces cell proliferation by regulating *CDKN1A* gene expression regardless of ERα and p53 status. Although NR5A2 is an attractive potential biomarker in breast cancer prognosis, further investigation into its role is needed [45], [48]–[50].

- ***Colorectal cancer***

In colorectal cancer, NR5A2 is correlated with tumourigenesis, as it is revealed to promote cell proliferation by two mechanisms, characterised by independent and

dependent DNA-binding manner, respectively. The former comprises the induction of *CCND1* acting as a co-activator of β-catenin, while the latter is the direct binding of this nuclear receptor to the promoter of *CCNE1*. Moreover, increased expression of steroidogenic enzymes, including Cyp11A1 and Cy11B1, by NR5A2 stimulates cancer cells to escape immune responses. Recent studies support that subcellular localisation of NR5A2 might affect its activity in colorectal cancer. These data indicate that detecting NR5A2 expression might be implicated in diagnosing colorectal cancer [45], [51].

- ***Liver cancer***

In liver cancer, NR5A2 induces the metabolic activity of cancer cells by regulating genes involved in the mitochondrial glutamine catabolism and creating the pro-tumorigenic status of hepatocytes. However, the exact mechanism underlying this effect is not fully understood, making further investigations pivoting [52].

- ***Nervous system malignancies***

NR5A2 seems to have a tumour-suppressor function in nervous system malignancies, as its increased levels are associated with favourable prognosis in glioblastoma and neuroblastoma patients. Furthermore, it inhibits cancer cell tumorigenic properties by inducing the cyclin-dependent kinase inhibitors, *CDKN1A* and *CDKN1B*, leading to cell cycle arrest. DLPC and DUPC, as NR5A2 agonists, can recapitulate its antiproliferative effect on glioblastoma *in vivo* and *in vitro*. Although these data support the implementation of NR5A2 in glioblastoma and neuroblastoma prognosis and treatment, identifying the molecular mechanism related to its function might shed light on further development of future approaches [53].

- ***Head and neck squamous cell carcinoma (HNSCC)***

In HNSCC, NR5A2 downregulation is associated with poor prognosis in patients. Interestingly, it is reported to regulate tumour development in a TP53 status-dependent manner since it comprises a critical tumour suppressor in this cancer type. On the one hand, NR5A2 inhibits cell proliferation under normal or higher expression levels of TP53, whereas it induces cell proliferation under low TP53 expression by upregulating glycolytic enzymes. These data indicate NR5A2 as a promising therapeutic target in HNSCC cases with loss-of-function *TP53* mutations [47].

4. Scope of the study

NR5A2 is a nuclear receptor with a significant role in several biological processes, although its involvement in tumourigenesis is reported. Scientific data reveal a dual role of this nuclear receptor in different cancer types. Still, there is limited knowledge regarding its role in lung cancer, mainly non-small cell lung cancer (NSCLC). Although recent studies supported that NR5A2 promotes oncogenesis, enhancing its diagnostic and prognostic value, clinical data analysis from databases indicates a negative association between NR5A2 expression levels and lung cancer progression. Therefore, the exact role of NR5A2 in human NSCLC is still unclear. To this end, we provide evidence suggesting that NR5A2 inhibits cell proliferation in two subtypes of NCSLC, the lung adenocarcinoma and the large cell adenocarcinoma. To further agreement, the antiproliferative action of NR5A2 is also detected *in vivo*. Gain-

of-function and loss-of-function studies reveal NR5A2 involvement in promoting cell cycle arrest by the induction of two cell cycle inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$. Furthermore, DLPC, a well-established NR5A2 agonist, can mimic the antiproliferative effect of NR5A2 in lung adenocarcinoma by the induction of the aforementioned genes.

Materials and Methods

1. Cell lines and Culture Conditions

The human NSCLC cell line A549 was obtained from ATCC (American Type Culture Collection, USA). NCI-H460, EKVX and NCI-H1944 cells were kindly provided by Dr G. Stathopoulos, Faculty of Medicine, University of Patras. All cell lines were cultured at 37 ºC in a humidified incubator with 5% CO_2. A549 and EKVX cells were maintained in DMEM high glucose medium (Biosera, UK) supplemented with 10% heat-inactivated FBS (Biosera, UK) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Invitrogen, USA). NCI-H460 and NCI-H1944 cells were cultured in RPMI 1640 medium (Biosera, UK) supplemented with 10% heat-inactivated FBS (Biosera, UK), 1% L-glutamine (200 mM, Gibco, Thermo Scientific, USA) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Invitrogen, USA). All cell lines were harvested and passaged when an 80-90% confluence was reached.

2. Gain-of-function study

- ***Adenoviral Transduction***

The recombinant control (Ad-GFP) and NR5A2 (Ad-NR5A2-GFP #1, #2)-overexpressing adenoviruses were constructed using the pAd/PL-DEST Gateway vector (ViralPower Adenoviral Expression System, Invitrogen, USA) in accordance with the manufacturer's instructions. Concisely, the cDNAs encoding wild-type *NR5A2* and *eGFP* were cloned into a modified version of the pENTR.GD entry vector and introduced into the Destination vector (kindly provided by Dr M. Xilouri and Dr A. Klinakis, BRFAA, Athens, Greece). The production of recombinant adenoviral particles was accomplished using homologous recombination into the HEK-293A cell line. Viral titers were determined by the plaque assay method. Viral transductions were performed for 19 h at a multiplicity of infection (MOI) of 1. The exogenous expression of *NR5A2* was confirmed by RT-qPCR 24 h post-transduction.

3. Loss-of-function study

Regarding shRNA knockdown studies, lentiviral vectors with the phosphoglycerate kinase (*hPGK*) promoter driving *eGFP* expression were produced in HEK-293T cells according to the supplier's instructions (Sigma, TRC lentiviral Library). Viral transductions with lentiviruses expressing shSCR or shNR5A2 constructs were performed overnight at a MOI of 10 in A549 cells, and cultured in serum-free DMEM medium (high glucose, Biosera, UK) supplemented with 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Invitrogen, USA). A549 cells incorporating the lentiviral genome were selected by puromycin (2 μg/mL). The reference number and corresponding sequence for each shRNA construct used in this study can be found on Sigma's TRC library webpage.

4. DLPC treatment

The effect of DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine, #8850335P, Avanti, USA) on non-small cell lung cancer was estimated at three different concentrations (150 uM, 170 uM and 200 uM). After diluting phospholipids in ethanol, all cell lines were treated with DLPC for 48h. An equal volume of ethanol was used for the control conditions. The cells were seeded either in a 24-well plate ($2x10^4$ cells/well) for immunofluorescence assay or in a 6-well plate ($6x10^5$ cells/well) for RNA and protein extraction. The amount of chemicals was determined by the final volume added per well.

5. Wound healing assay

Regarding the wound healing experiments, the scratch assay was applied. A549 cells expressing the aforementioned shRNA constructs (shSCR and shNR5A2) and A549 cells for transduction studies were seeded in 24-well plates. When confluence was achieved, a scratch across the entire diameter of each well was made using a pipette tip. After removing the culture medium, 1X Phosphate-buffered Saline (PBS) was used to wash the wells to remove cell debris. Subsequently, a fresh medium or medium containing adenoviral particles was added. Wound healing and cell migration were evaluated every 24 h, using an inverted microscope with a LEICA camera (10X magnification).

6. RNA extraction and Real-Time RT-qPCR analysis

Total RNA was isolated by cells using RNeasy Protect Mini Kit (74124, Qiagen, Germany) in accordance with the manufacturer's instructions. RNA concentration and purity were measured by Nanodrop 2000c (Thermo Scientific, USA), and 1 mg was used for cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen, USA) together with random hexamer primers. Quantitative Real-time RT-PCR analysis was performed in a LightCycler 96 Instrument (Roche, Switzerland). Measured values were normalized using beta-actin mRNA levels as internal references.

Genes		Sequence
hNR5A2	Forward	AGCACCTTTGGGCTTATGTG
	Reverse	GCAGCTTCATTTGGTCATCA
hbeta Actin	Forward	CTCTTCCAGCCTTCCTTCCT
	Reverse	AGCACTGTGTTGGCGTACAG
hCDKN1B ($p27^{Kip1}$)	Forward	AGAGTTAACCCGGGACTTGG
	Reverse	GCCCTCTAGGGGTTTGTGAT
hCDKN1A ($p21^{Cip1}$)	Forward	GGAAGACCATGTGGACCTGT
	Reverse	GGCGTTTGGAGTGGTAGAAA
hCCND1 (cyclin D1)	Forward	CCCTCGGTGTCCTACTTCAA
	Reverse	AGGAAGCGGTCCAGGTAGTT
hCCNE1 (cyclin E1)	Forward	ATCCTCCAAAGTTGCACCAG
	Reverse	AGGGGACTTAAACGCCACTT
hPTEN	Forward	TGAAGGCGTATACAGGAACAAT
	Reverse	CGGTGTCATAATGTCTTTCAGC

hMYC	Forward	CGTCCTCGGATTCTCTGCTC
	Reverse	GCCTGCCTCTTTTCCACAGA
hHRAS	Forward	TATAAGCTGGTGGTGGTGGG
	Reverse	CCCATCAATGACCACCTGCT
hRB1	Forward	AGTGCTGAAGGAAGCAACCC
	Reverse	CATTCGTGTTCGAGTAGAAGTCAT
hE2F1	Forward	CCTGAGGAGACCGTAGGTGG
	Reverse	GACAACAGCGGTTCTTGCTCC
hPKM	Forward	AGAAAGGTGCCGACTTCCTG
	Reverse	GCTCGACCCCAAACTTCAGA
hLDHA	Forward	AGAGGTTCACAAGCAGGTGG
	Reverse	GTGCACCCGCCTAAGATTCT
hSLC2A3	Forward	TGGCCCAGATCTTTGGTCTG
	Reverse	ATGGAAGGGCTGCACTTTGT
hPDK1	Forward	TGCTGTATGGCCTGCAAGAT
	Reverse	ACATTCTGGCTGGTGACAGG
hFOXO3	Forward	TGTCCCAGATCTACGAGTGG
	Reverse	TATGCAGTGACAGGTTGTGC
hTP53	Forward	CCTGAGGTTTGGCTCTGACTG
	Reverse	TCAAAGCTGTTCCGTCCCAG

7. Western blot analysis

Total protein was isolated from cells with RIPA lysis buffer mixed with a cocktail of protein inhibitors. Cells were completely dispersed by sonication (80% amplitude, three cycles of 10 s with 10 s ice incubation), and the homogenates were centrifuged at 13000 rpm for 15 min at 4 ^{o}C. The clear supernatants containing the total solubilized proteins were collected. Protein concentration was measured with Bradford protein assay (Bio-Rad protein assay), and 30 µg of the samples were subjected to immunoblot analysis each time. Proteins were transferred to nitrocellulose membranes (Amersham, UK) using the semi-dry transfer system (Bio-Rad, USA). The membrane blocking was attained by a 1-hour incubation with a blocking solution consisting of 5% BSA (A1391, Applichem, USA) dissolved in Tris-buffered Saline (1x) containing 0.1% Tween-20 at room temperature (RT). The membranes were incubated with primary antibodies at 4 ^{o}C overnight, followed by secondary antibodies for 2 h at RT. Protein loads were verified with beta-actin or beta-tubulin as reference proteins using a primary mouse monoclonal anti-beta-actin (1:20000 dilution) (A5441, Sigma, USA) or rabbit polyclonal anti-beta-tubulin (1:10000 dilution) (Ab6046, Abcam, UK) antibody. The primary antibodies used in the Western blot were mouse monoclonal anti-p27-kip1 (1:2000 dilution) (610241, BD Transduction Laboratories, USA), mouse monoclonal anti-p21-cip1 (1:2000 dilution) (DCS60, Cell signaling, USA) and rabbit custom-made polyclonal antibody (1:1500 dilution) raised against the mouse peptide of NR5A2 (produced and purchased from Davids Biotechnologie GmbH-Custom Antibodies, Regensburg, Germany; amino acid sequence: HSASKGLPLSHVALPPTDYDR; HPLC-purified peptide). The secondary antibodies were rabbit polyclonal anti-mouse IgG (1:10000 and 1:40000 dilutions) (A9044, Sigma, USA) and goat polyclonal anti-rabbit IgG (1:10000 dilution) (A6154, Sigma, USA).

8. Immunofluorescence

Regarding the immunostaining experiments, cell lines were seeded onto poly-L-lysine (Sigma, USA) coated coverslips in 24-well plates. Cells derived from stable cell lines, but also cells after transduction and treatment with DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine, #8850335P, Avanti, USA) were fixed on the coverslips with 4% PFA (paraformaldehyde) and blocked with 5% FBS diluted in (1X) Phosphate-buffered Saline (PBS) containing 0.1% Triton X-100 for 1 h at room temperature (RT). Subsequently, the coverslips were incubated with primary antibodies at 4 ^{o}C overnight, followed by secondary antibodies for 1 h at RT. At the final stage, an incubation with DAPI diluted in 1X PBS for 5 min at RT was required before mounting the coverslips with Dako Fluorescent Mounting Medium (CA93013, Dako North America Inc., USA). NR5A2 was detected using a rabbit polyclonal anti-NR5A2 antibody (1:600 dilution) (kindly donated by Dr I. Talianidis, BSRC Alexander Fleming, Athens, Greece). Mouse monoclonal anti-Ki67 (1:1000 dilution) (9449) and rabbit polyclonal anti-cleaved caspase 3 (1:600 dilution) (9661) were purchased from Cell Signaling. Chicken polyclonal anti-GFP (1:400 dilution) (Ab13970) and rabbit polyclonal anti-phospho-Histone 3 (1:600 dilution) (Ab5176) were obtained from Abcam. Secondary antibodies conjugated with AlexaFluor 488 (green), 568 (red) or 647 (far red) were from Invitrogen (1:400 dilutions).

9. Heterotopic xenografts

The heterotopic allotransplantation experiment was established using 8-week-old male NOD-SCID mice. A549 cells were transduced with the appropriate adenoviruses (Ad-GFP for the control and Ad-NR5A2-GFP for the treatment) and subcutaneously injected in the flanks ($1x10^6$ A549 cells/injection). Mice were housed under aseptic conditions in individually ventilated cages for every experimental group at 24 ^{o}C and a light-controlled (12 h per day) environment. Tumour growth was measured every 3-4 days using a calliper. After 46 days, mice were sacrificed to isolate the tumours from the two experimental groups.

10. Experimental design and statistical analysis

Each experimental design is described in the corresponding part of the section "materials and methods". The normal distribution of values was verified with the Shapiro-Wilk normality test using IBM SPSS Statistics for Windows, Version 26.0. All experiments were conducted independently three to five times to ensure the reproducibility of results. All measurements and experimental values from independent experiments were estimated with two-tailed Student's t-test, or ANOVA test, for statistical analysis. All the results are represented as mean ± SD. Each Figure legend describes precisely the *P* values. P values < 0.05 are considered statistically significant. All analyses were performed using GraphPad 8 and Microsoft Excel 2013.

1. NR5A2 expression is negatively correlated with tumour progression and patient survival in non-small cell lung cancer

At the outset of investigating the involvement of NR5A2 in lung cancer, we examined publicly available clinical data from data databases such as Oncomine (www.oncomine.org), TNMplot (www.tnmplot.com), and the Kaplan-Meier plotter (www.kmplot.com) for clinical associations between *NR5A2* expression and lung cancer progression. Therefore, data analysis indicated a negative association between NR5A2 expression levels and lung cancer progression. Data analysis from different studies from the Oncomine database revealed a significant downregulation of *NR5A2* in two subtypes of NSCLC, the squamous cell lung carcinoma and the lung adenocarcinoma, in comparison to healthy samples (**Figure 14**) [54]–[56].

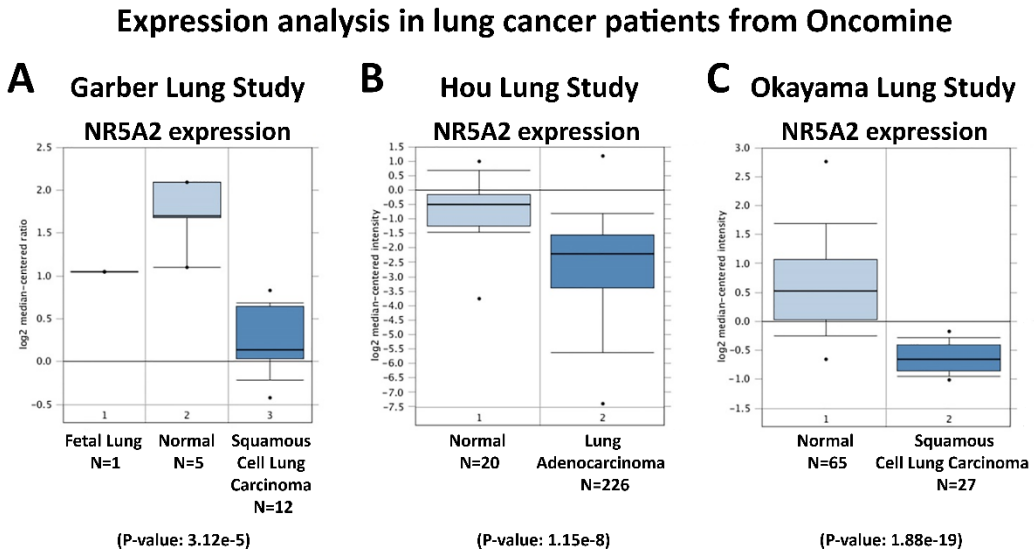

Figure 14: *Expression analysis in lung cancer patients from the Oncomine database* ***(A)*** *Clinical data from Garber Lung Study,* ***(B)*** *Clinical data from Hou Lung Study,* ***(C)*** *Clinical data from Okayama Lung Study*

In further agreement, similar analysis from different sources using the TNMplot web tool confirms that reduced expression of *NR5A2* is detected in tumour specimens compared to non-paired normal tissues and adjacent healthy ones (**Figure 15A-15B**) [57]. Consistently, survival analysis of patients' data from the Kaplan-Meier plot database suggests a positive correlation between *NR5A2* expression and increased survival rates of lung cancer patients (**Figure 15C**) [58]. These observations raise the hypothesis that NR5A2 may exert a tumour-suppressor function in lung cancer.

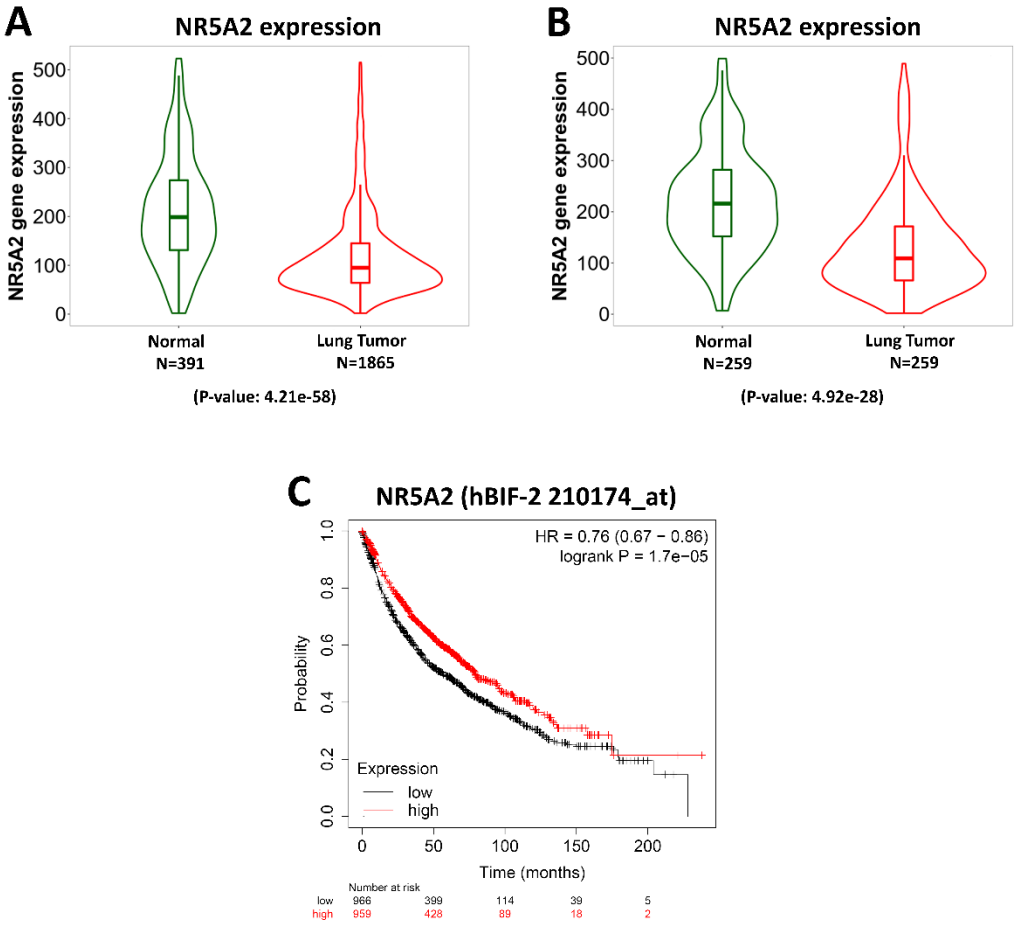

Figure 15: (A) *Graphical representation (violin plot) of NR5A2 expression in lung tumours (N=1865) and non-paired healthy lung tissues (N=391) p<0.001,* ***(B)*** *Graphical representation (violin plot) of NR5A2 expression in lung tumours (N=259) and adjacent healthy lung tissues (N=259) p<0.001,* ***(C)*** *Survival curve (Kaplan-Meier) of lung cancer patients with relative high and low expression of NR5A2 from the KM-plotter, p<0.001. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

2. NR5A2 overexpression inhibits cell proliferation in NSCLC cells

To investigate the effect of *NR5A2* overexpression *in vitro*, four NSCLC cell lines, specifically A549, NCI-H460, EKVX, and NCI-H1944, were chosen for this study. The overexpression of NR5A2 was attained by a previously constructed and tested adenoviral-based overexpression system [59], and its overexpression capacity was confirmed by Real-time qPCR and Western Blot analysis (**Figure 16**).

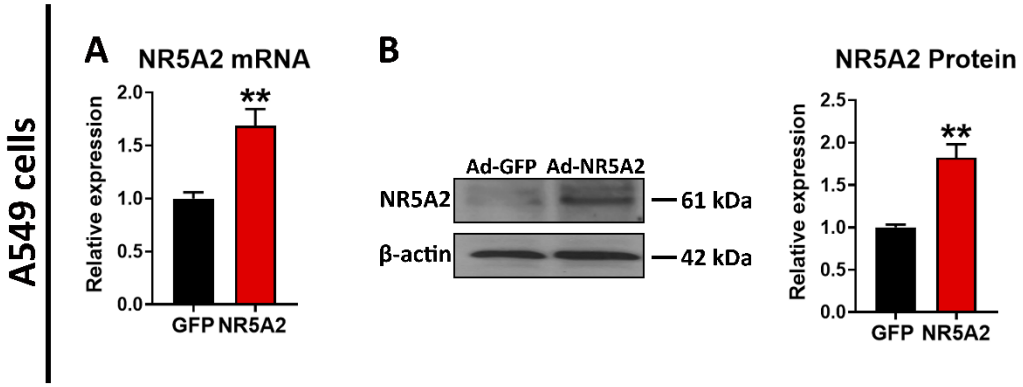

Figure 16: ***(A)*** *Relative expression of NR5A2 mRNA in A549 cells overexpressing GFP or NR5A2 conditions, measured with quantitative Real-time RT-PCR, $p<0.01$.* ***(B)*** *Western blot analysis of NR5A2 and β-actin in A549 cells overexpressing GFP or NR5A2 conditions. Quantification of protein expression levels of NR5A2 in A549 cells overexpressing GFP or NR5A2 conditions, $p<0.01$.*

After seeding cells on coverslips in a 24-well plate, they were treated for 19-20 hours with the appropriate adenoviruses (Ad-GFP for the control or Ad-NR5A2-GFP for the treatment). The adenoviral withdrawal was followed by a 24-h rest of cells. The effect on cell proliferation was evaluated with immunofluorescence assays using Ki67 and phosphorylated histone H3 (pH3) as proliferation markers. Ki67 is a DNA-binding protein expressed in actively proliferating cells, while pH3 marks cells undergoing mitosis. As theoretically expected, the transgenic GFP protein was detected in the nucleus and the cytoplasm in both experimental conditions.

The collected data were analysed by counting transduced cells and subsequently quantifying the Ki67 and pH3 signals. Regarding A549 cells, reduced numbers of transduced cells that are Ki67+ or pH3+ were observed when NR5A2 was overexpressed. Indeed, Ki67 was detected in 89.27% of Ad-GFP cells, whereas in Ad-NR5A2 cells, it only reached 32.48% (**Figure 17A-17B**). Similar observations were attained by pH3 immunostainings, as the number of Ad-NR5A2 pH3+ cells decreased approximately 11.5 times, with percentages reaching 13.7% for the control condition and 1.2% for the treatment (**Figure 17C-17D**). Therefore, these results indicate that NR5A2 overexpression impairs A549 cell proliferation in a statistically significant manner (**Figure 17**).

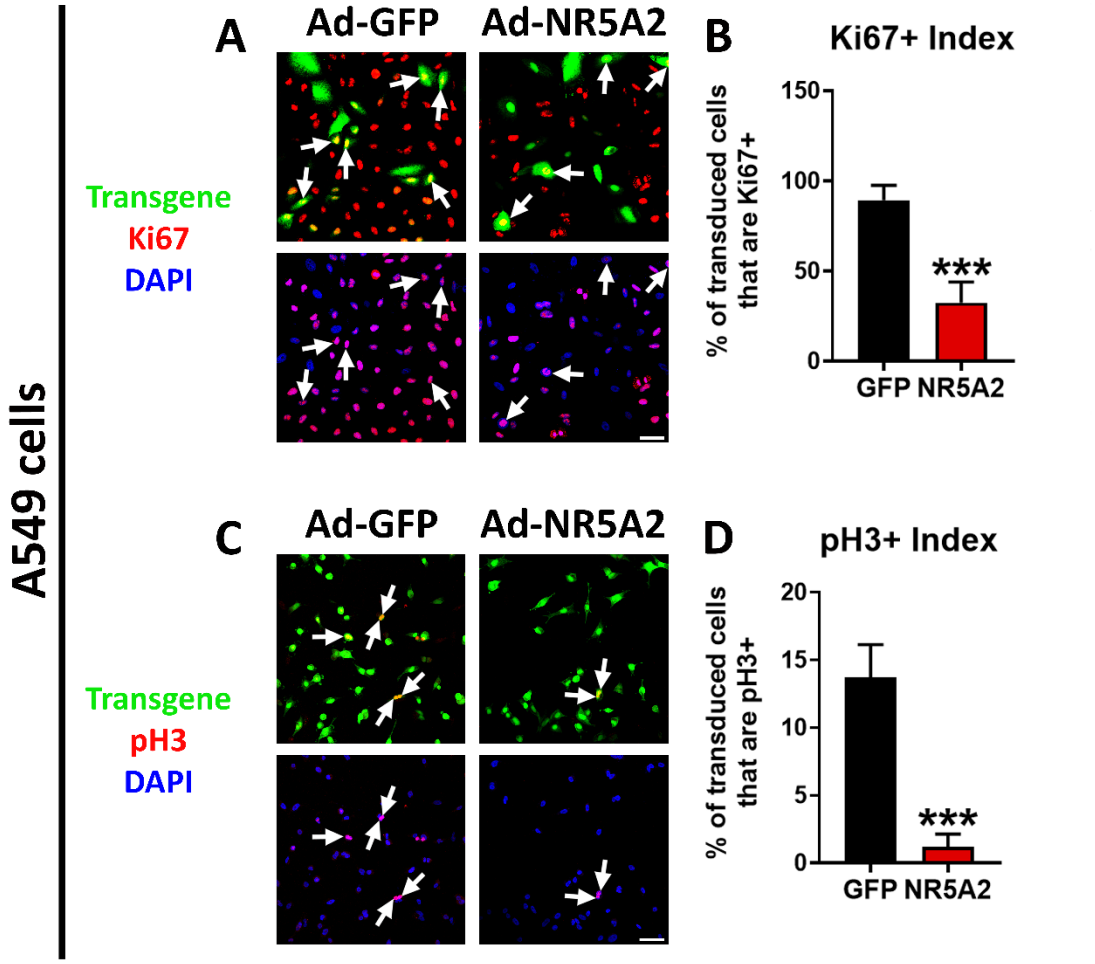

Figure 17: *Immunofluorescence results of transduced A549 cells stained for Ki67* ***(A, B)*** *and pH3* ***(C, D)****. Arrows indicate representative double-positive cells (GFP positive and Ki67 or pH3 positive, respectively). Scale bar: 50 um. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

To further examine whether the antiproliferative effect of NR5A2 is context-dependent, three additional NSCLC cell lines were employed for this scope. The same experimental protocol was followed for NCI-H460 (large cell lung cancer), EKVX (lung adenocarcinoma), and NCI-H1944 (lung adenocarcinoma) cell lines. Interestingly, a similar decreasing trend was observed in cell proliferation when NR5A2 was overexpressed, as the same phenotypic changes were detected in all three cell lines (**Figure 18-19**).

Regarding NCI-H460 cells, the number of transduced Ki67+ cells after NR5A2 overexpression compared to the control condition is significantly reduced, with numbers having stood at 28% and 38.36%, respectively (**Figure 18A-18B**). Furthermore, a 3.4-fold decrease in the percentage of transduced pH3 cells was stimulated after NR5A2 overexpression (2.6% in Ad-GFP cells versus 0.76% in Ad-NR5A2 cells) (**Figure 18C-18D**).

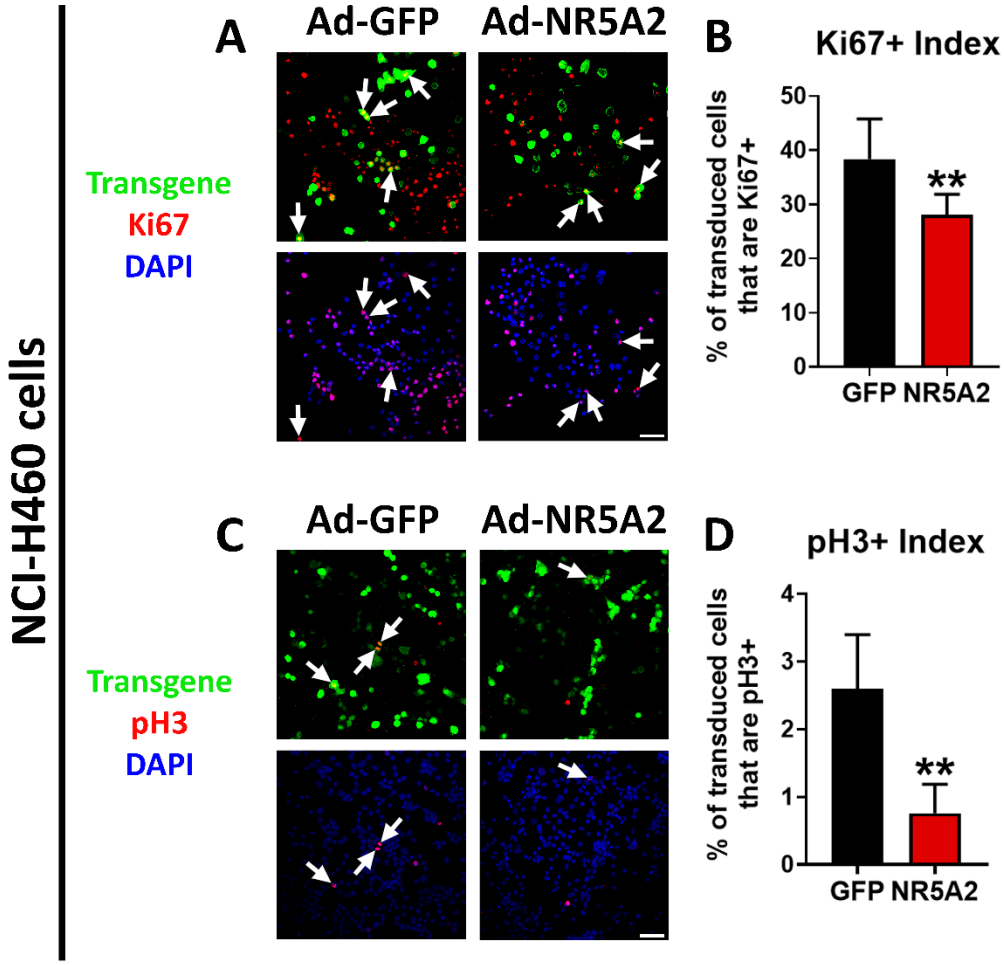

Figure 18: *Immunofluorescence results of transduced NCI-H460 cells stained for Ki67* ***(A, B)*** *and pH3* ***(C, D)****. Arrows indicate representative double-positive cells (GFP positive and Ki67 or pH3 positive, respectively). Scale bar: 50 um. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

Accordingly, in EKVX cells, the percentage of Ad-GFP Ki67+ cells was 45.23% in contrast to 28.52% of Ad-NR5A2 Ki67+ cells (**Figure 19A**), while the number of pH3+ cells plunged from 4.39%, in control, to 1.19%, after NR5A2 overexpression (**Figure 19B**).

Similar results obtained from NCI-H1944 cell lines revealed that the number of transduced Ki67+ cells decreased 1.25 times (32.58% in Ad-GFP cells versus 25.97% in Ad-NR5A2 cells) (**Figure 19C**), while the number of pH3+ cells dropped from 4.03% to 0.60% after NR5A2 overexpression (**Figure 19D**). In all cases, the observed differences were statistically significant, and a greater decrease in the pH3 index was reported compared to the Ki67 index.

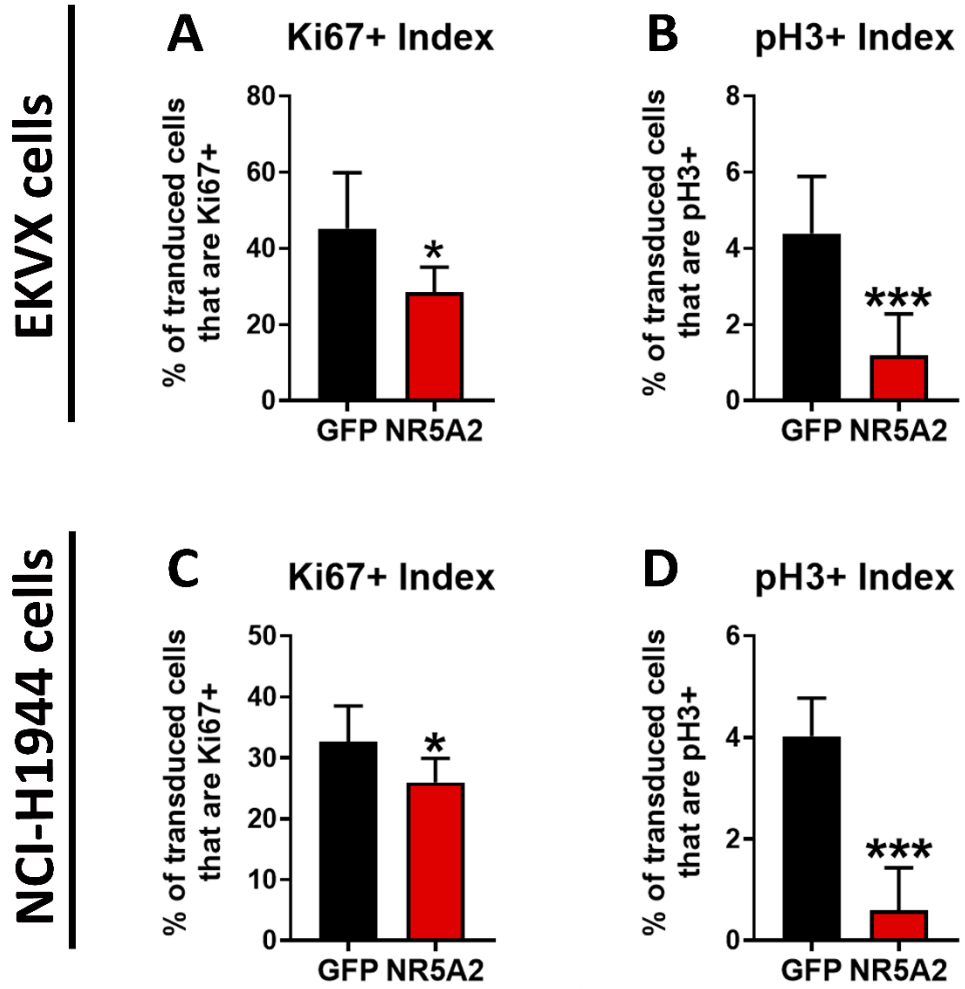

Figure 19: *Quantification of immunofluorescence results of transduced EKVX cells stained for Ki67* ***(A)*** *and pH3* ***(B)*** *and NCI-H1944 stained for Ki67* ***(C)*** *and pH3* ***(D)****. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

Subsequently, to exclude the possibility of low proliferation rates of transduced A549 cells when NR5A2 is overexpressed, caused by cell apoptosis, an immunofluorescence assay with an antibody against cleaved caspase3 was carried out. Data analysis indicated that no significant induction of apoptosis is observed when NR5A2 is overexpressed compared to the control, with the number of Ad-GFP cas3+ cells and that of Ad-NR5A2 cas3+ cells having been 1.73% and 1.19%, respectively (**Figure 20**). Together, these findings support that NR5A2 can effectively inhibit the proliferation of NSCLC cells without inducing apoptosis.

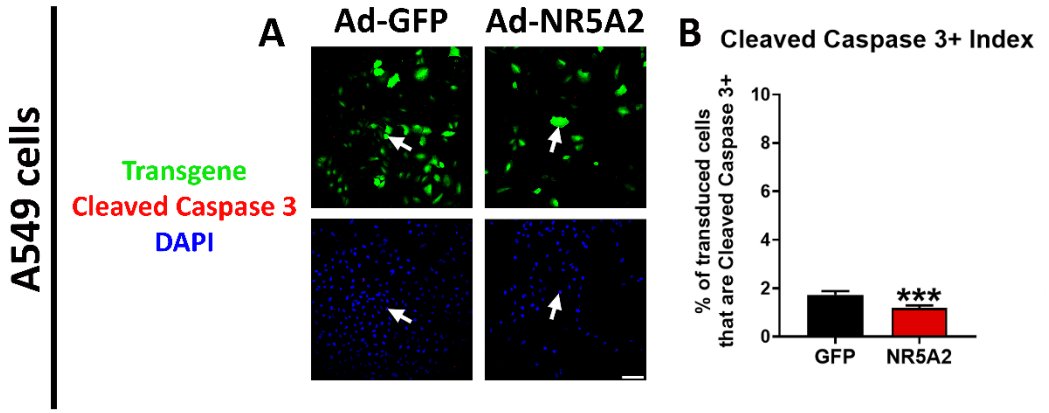

Figure 20: ***(A)*** *Immunofluorescence results of transduced A549 cells stained for cleaved caspase3 Arrows indicate representative double-positive cells (GFP positive and cleaved caspase3 positive). Scale bar: 50 um.* ***(B)*** *Quantification of immunofluorescence results of transduced A549 cells stained for cleaved caspase3. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

3. NR5A2 overexpression decelerates cell migration *in vitro*

To study the potential involvement of NR5A2 in cell migration, wound healing assay on A549 monolayers was carried out. After seeding cells in a 24-well plate, they were treated for 19-20 hours with the appropriate adenoviruses (Ad-GFP for the control or Ad-NR5A2-GFP for the treatment). The adenoviral withdrawal was followed by creating one scratch across the entire diameter of each well using a pipette tip. Wound healing and cell migration were evaluated every 24 h, throughout 72 h, using an inverted microscope. The experiment was performed in quintuplicates.

Data analysis indicates that NR5A2 overexpression decelerates cell migration *in vitro*, as transduced A549 cells that overexpressed NR5A2 seemed to have a delayed wound healing rate compared to the control. Notably, the wound areas between the two conditions were comparable at 0 and 24 h time points. However, wound closure was noticed in Ad-GFP A549 experimental group at 48 hours, and it progressed rapidly as the wound area approached its complete healing at 72 h time point. Conversely, the scratch was still discernible in Ad-NR5A2 A549 experimental group at 72 hours (**Figure 21**).

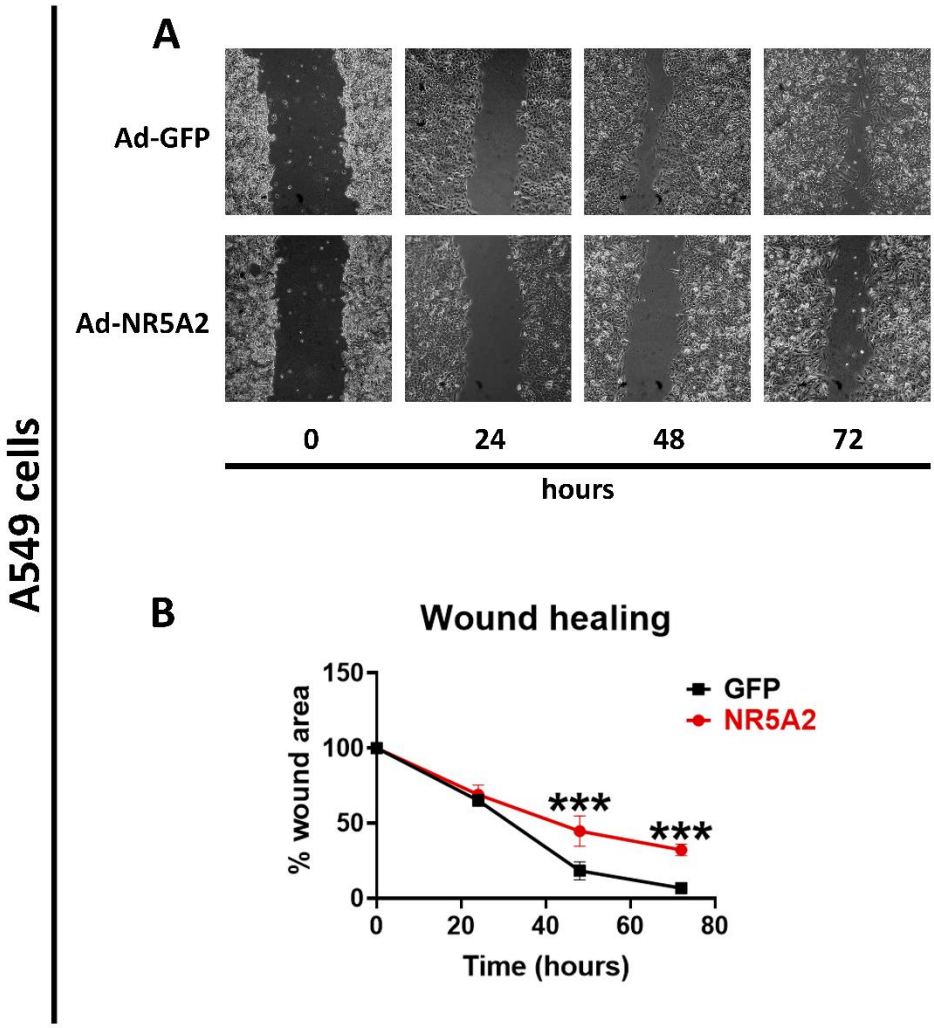

Figure 21: ***(A)*** *NR5A2 and GFP infected A549 cells were measured for their migration capacity using the wound healing assay at 24, 48, and 72 hours.* ***(B)*** *Quantification of the % average wound area at 24, 48, and 72 hours in A549 cells in GFP and NR5A2 over-expression conditions. Statistical analysis of the quantitative variables was performed using the two-way ANOVA. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

4. Knockdown of *NR5A2* promotes cell proliferation in NSCLC cells

Considering the observations that indicated the antiproliferative effect of NR5A2 overexpression on NSCLC cell lines, we aimed to investigate the involvement of basal expression levels of NR5A2 in regulating cell proliferation. To tackle this question, two validated shRNA constructs were developed based on the lentiviral TRC library (pLKO.1-puro lentiviral vector, Sigma, USA), one control scrambled shRNA construct and one targeting the human NR5A2 homolog. A549 cell line was selected for the knockdown studies due to its highest expression of *NR5A2* at mRNA level among the studied cell lines (**Figure 22**).

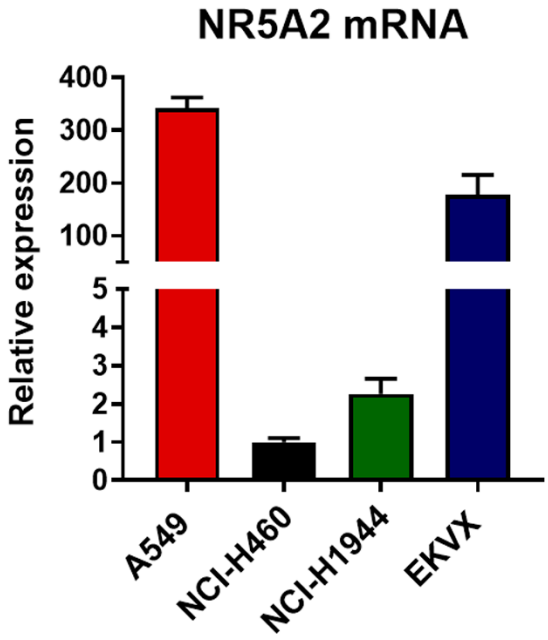

***Figure 22**: mRNA expression analysis of NR5A2 in A549, NCI-H460, EKVX, and NCI-H1944 cells over-expressing GFP*

The indicated lentiviral particles were used for the infection of A549 cell line, and the acquisition of genetically homologous cultures was attained by puromycin selection. Real-time qPCR and Western Blot analysis confirmed that the shRNA against NR5A2 could strongly downregulate the basal expression of endogenous NR5A2 both at RNA and protein levels, as reduced mRNA and protein levels of NR5A2 were detected as compared to the control (**Figure 23**).

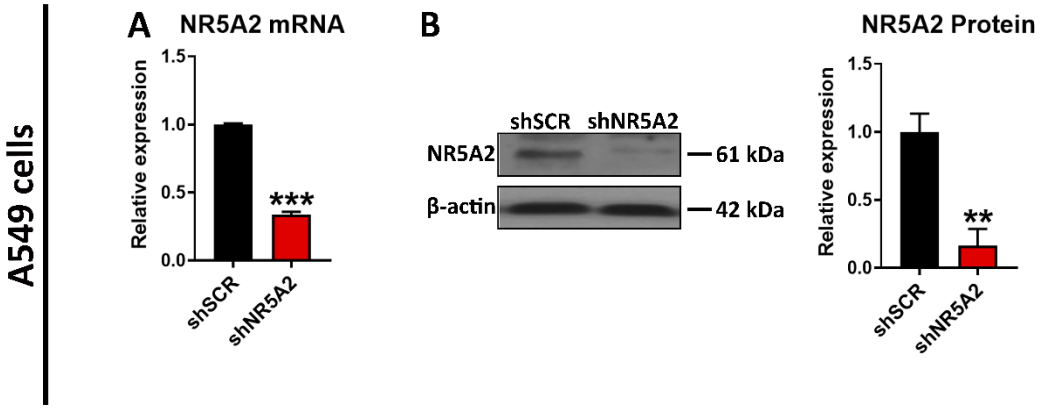

***Figure 23:** **(A)** Relative expression of NR5A2 mRNA in A549 cells in control and NR5A2 knockdown conditions, measured with quantitative Real-time RT-PCR, $p<0.001$. **(B)** Western blot analysis of NR5A2 and β-actin in A549 cells in control and NR5A2 knockdown conditions. Quantification of protein expression levels of NR5A2 in A549 cells in control and NR5A2 knockdown conditions, $p<0.01$.*

The proliferation properties of these stable cell lines were evaluated with immunofluorescence experiments using Ki67 and pH3 as proliferation markers.

Immunostaining analysis revealed a significant increase in the numbers of Ki67+ and pH3+ cells after NR5A2 knockdown. In particular, the number of Ki67+ cells expressing shNR5A2 increased 1.2 times, with figures having stood at 54.86% in control and 66.14% in NR5A2 knockdown condition (**Figure 24A-24B**). Similar observations were obtained by pH3 immunostainings comparing control and knockdown conditions, as the percentage of pH3+ cells rose from 5.58% to 6.98%, respectively (**Figure 24C-24D**).

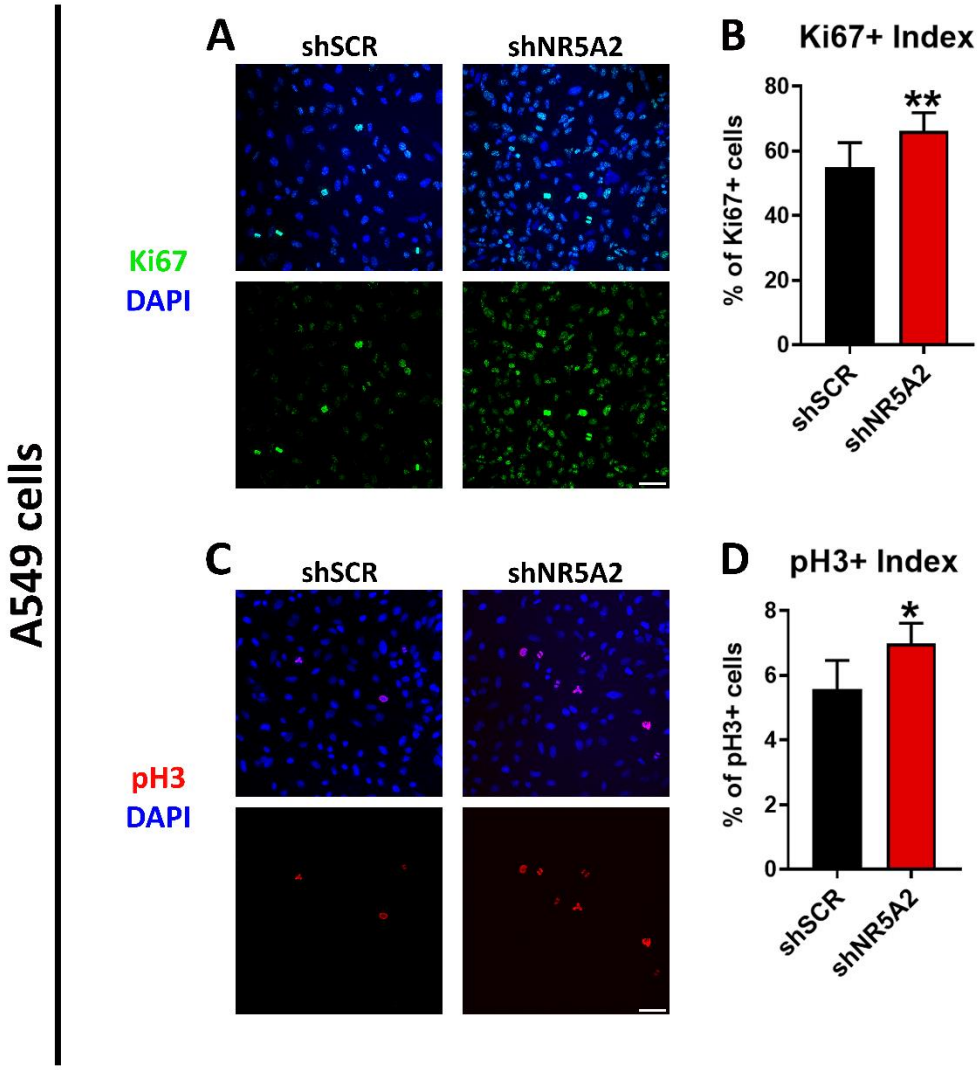

Figure 24: *Immunofluorescence results of A549 cells stained for Ki67* ***(A, B)*** *and pH3* ***(C, D)*** *in control and NR5A2 knockdown conditions. Scale bar: 50 um. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

5. Knockdown of *NR5A2* accelerates cell migration *in vitro*

To examine the potential impact of *NR5A2* knockdown in cell migration, wound healing assay on A549 monolayers derived from stable knockdown cell lines was

carried out. After seeding cells in a 24-well plate, when confluence was reached, one scratch was created across the entire diameter of each well using a pipette tip. Wound healing and cell migration were evaluated every 24 h, throughout 48 h, using an inverted microscope. The experiment was performed in quintuplicates.

Data analysis indicates that NR5A2 knockdown promotes cell migration *in vitro*, as A549 cells that expressed shNR5A2 seemed to have an accelerated wound healing rate compared to the control condition. Notably, wound closure was noticed in both experimental groups at 24 hours; however, it progressed rapidly in *NR5A2* knockdown condition as the wound area started to approach its complete healing at 48 h time point. Conversely, the scratch was still discernible in the control experimental group at 48 hours (**Figure 25**).

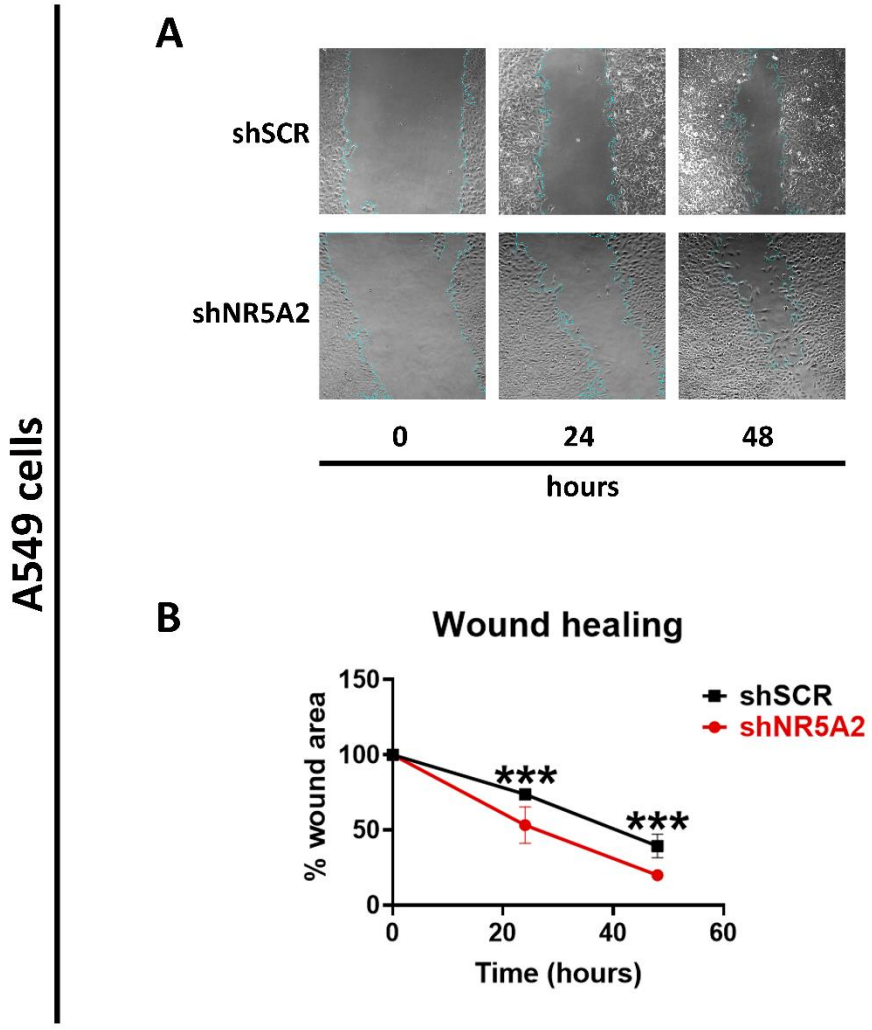

Figure 25: ***(A)*** *A549 cells, in control and NR5A2 knockdown conditions, measured for their migration capacity using the wound healing assay at 24 and 48 hours.* ***(B)*** *Quantification of the % average wound area at 24 and 48 hours in A549 cells in control and NR5A2 knockdown conditions. Statistical analysis of the quantitative variables was performed using the two-way ANOVA. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

6. NR5A2 induces the expression of cell cycle inhibitors

As a next step in this study, the aim was to investigate further the underlying molecular mechanism of the antiproliferative action of NR5A2. We focused on examining the expression of several genes involved in regulating vital cellular processes, such as cell cycle progression, metabolism, and signalling, in transduced A549 cells. A previously constructed and tested adenoviral-based overexpression system was used to obtain NR5A2 overexpression in A549 cell line [59]. RNA and protein extraction, as well as cDNA synthesis, were performed 24 hours post-transduction. In transduced A549 cells, we analysed the expression patterns of the following genes [24], [60]:

- *PTEN*: identified as a tumour suppressor, and the encoded protein negatively regulates the AKT/PKB signaling pathway
- *FOXO3*: a member of a transcription factors family characterised as tumour suppressors with a significant role in regulating cellular homeostasis [61], [62]
- *E2F1*: a member of a transcription factors family with a crucial role in regulating the cell cycle and with tumour suppressor function
- *RB1*: a tumour suppressor gene, which encoded protein negatively regulates the cell cycle
- *TP53*: the encoded protein as a tumour suppressor responds to diverse cellular stresses and induces cell cycle arrest, apoptosis, DNA repair, or alterations in metabolism
- *MYC* (c-Myc): known as a proto-oncogene, and the encoded protein is involved in cell cycle progression, apoptosis, and cellular transformation
- *HRAS*: a member of the Ras oncogene family, and the encoded protein is involved in signal transduction pathways
- *CCND1* (Cyclin D1): the encoded protein forms a complex with CDK4 or CDK6, whose activity is required for cell cycle G1/S transition
- *CCNE1* (Cyclin E1): the encoded protein forms a complex with CDK2, whose activity is required for cell cycle G1/S transition
- *CDKN1A* ($p21^{Cip1}$): the encoded protein regulates the cell cycle progression at the G1 phase
- *CDKN1B* ($p27^{Kip1}$): the encoded protein controls the cell cycle progression at the G1 phase
- *PKM*: the encoded protein is a pyruvate kinase with a crucial role in glycolysis, as it catalyses the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, generating ATP and pyruvate
- *LDHA*: the encoded protein catalyses the conversion of L-lactate and NAD to pyruvate and NADH in the final step of anaerobic glycolysis
- *SLC2A3*: the encoded protein is a facilitative glucose transporter that can also mediate the uptake of various other monosaccharides across the cell membrane
- *PDK1*: the encoded kinase regulates glucose and fatty acid metabolism and homeostasis via phosphorylation of the pyruvate dehydrogenase subunits PDHA1 and PDHA2. It is also involved in cellular responses to hypoxia, especially in cell proliferation apoptosis

The results revealed that NR5A2 overexpression did not impact the majority of the aforementioned genes, with some exceptions (**Figure 26**). The expression of *E2F1*, *CCNE1*, *LDHA*, and *PDK1* followed an opposite pattern to the stimulated changes during cell cycle arrest (**Figure 26H, 26N, 26J, 26L**). This might be indicated as cell response through a feedback mechanism aiming to induce cell cycle progression and survival. However, *FOXO3*, *CDKN1A* ($p21^{Cip1}$), and *CDKN1B* ($p27^{Kip1}$) seemed to have been upregulated (**Figure 26C, 26A, 26B**), whereas *PKM* and *CCND1* were downregulated after NR5A2 overexpression (**Figure 26I, 26M**).

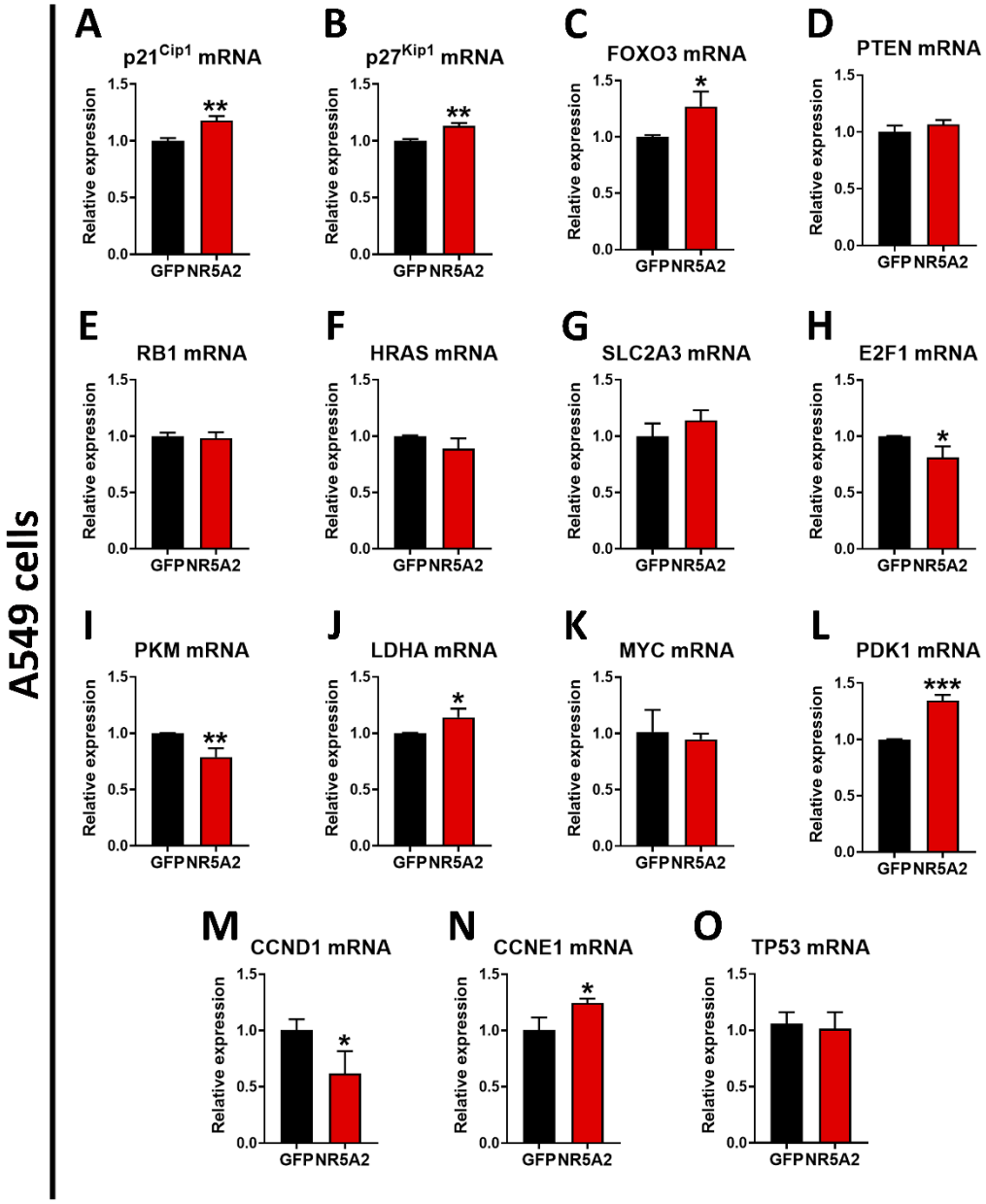

Figure 26: *mRNA expression analysis of genes involved in several biological processes in A549 cells over-expressing GFP or NR5A2. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

Our previous studies supported that NR5A2 overexpression in glioblastoma tumours is not only potent to induce the expression of $p21^{Cip1}$ and $p27^{Kip1}$ both at mRNA and protein levels, but this NR5A2-mediated transcriptional regulation may also be achieved through direct interactions with regulatory sequences of these genes [53]. Considering these data, we focused on examining whether NR5A2 overexpression also promotes the expression of the aforementioned genes in non-small cell lung cancer. Western blot analysis indicated that NR5A2 overexpression promotes the expression of these two negative cell cycle regulators, as significantly increased protein levels of $p21^{Cip1}$ and $p27^{Kip1}$ were detected compared to the control (**Figure 27**).

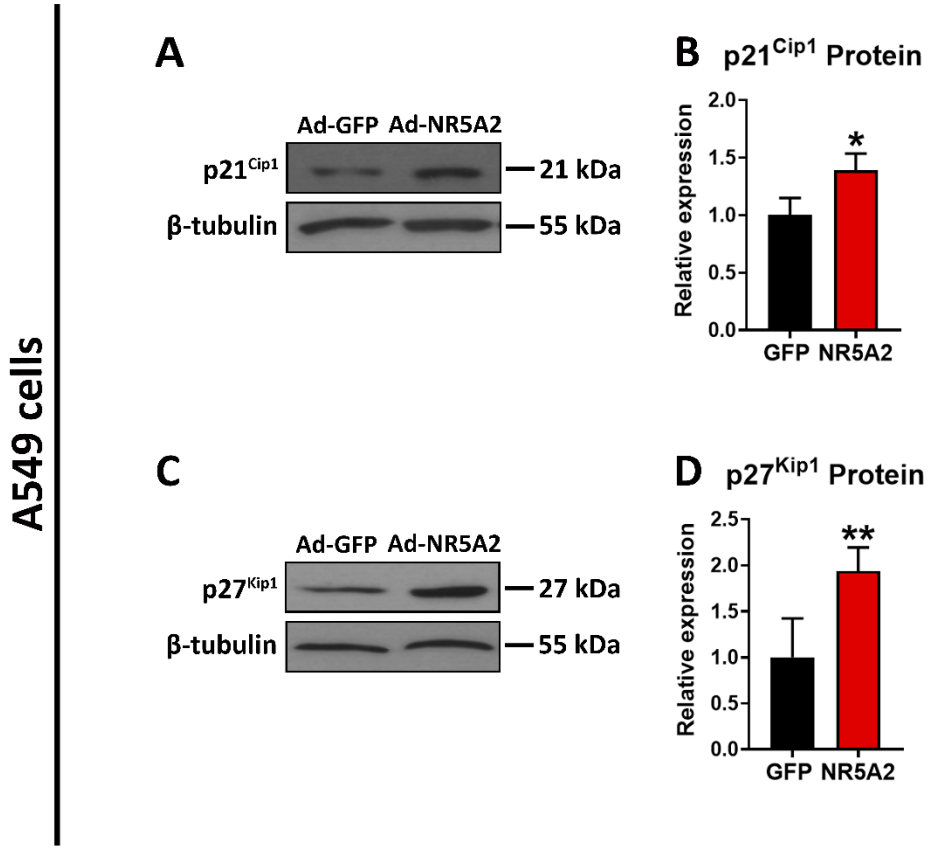

Figure 27: *(A) Western blot analysis of $p21^{Cip1}$ and β-actin in A549 cells over-expressing GFP or NR5A2.* ***(B)*** *Quantification of protein expression levels of $p21^{Cip1}$ in A549 cells over-expressing GFP or NR5A2, $p<0.05$.* ***(C)*** *Western blot analysis of $p27^{Kip1}$ and β-actin in A549 cells over-expressing GFP or NR5A2.* ***(D)*** *Quantification of protein expression levels of $p27^{Kip1}$ in A549 cells over-expressing GFP or NR5A2, $p<0.01$.*

To further confirm this hypothesis, we investigated whether *NR5A2* knockdown might affect the expression of *CDKN1A* ($p21^{Cip1}$) and *CDKN1B* ($p27^{Kip1}$). Protein extracts from stable A549 cells expressing scrambled shRNA (shSCR) or shNR5A2 were analysed. Interestingly, $p21^{Cip1}$ and $p27^{Kip1}$ were downregulated, as significantly reduced protein levels were detected after *NR5A2* knockdown (**Figure 28**).

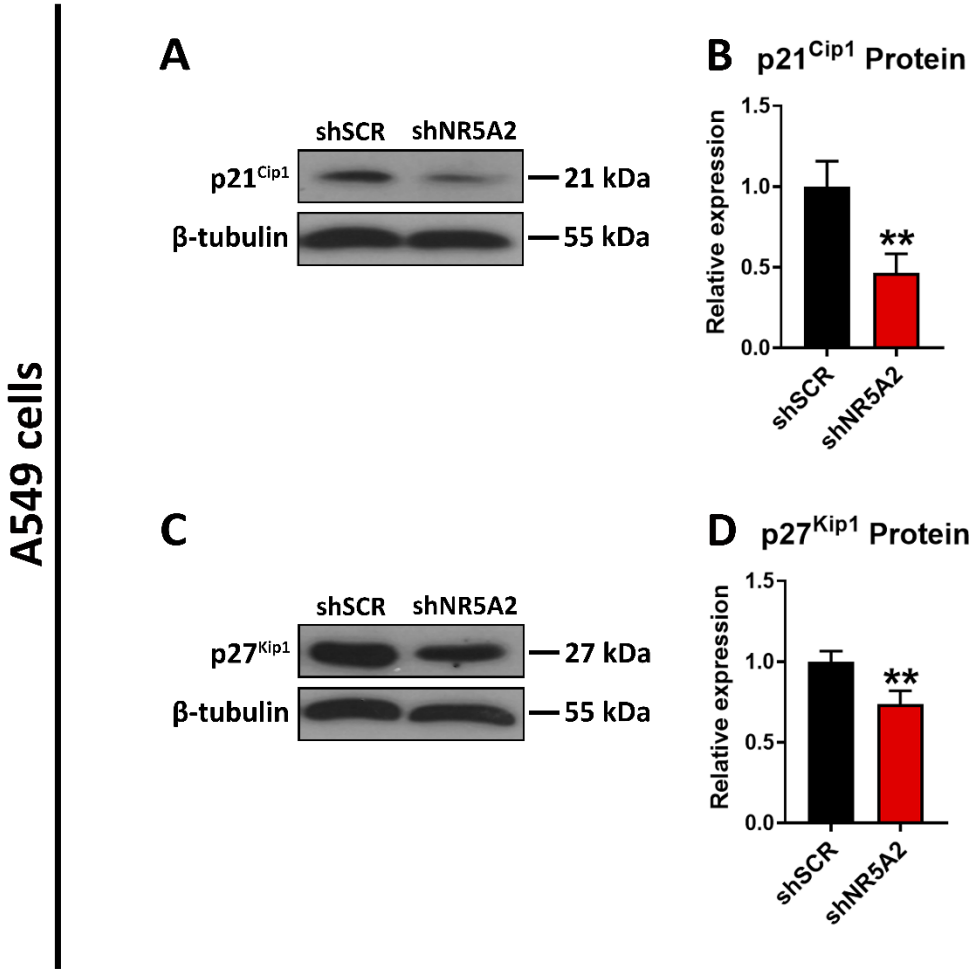

Figure 28: ***(A)*** *Western blot analysis of $p21^{Cip1}$ and β-actin in A549 cells in control and NR5A2 knockdown conditions.* ***(B)*** *Quantification of protein expression levels of $p21^{Cip1}$ in A549 cells in control and NR5A2 knockdown conditions, $p<0.01$.* ***(C)*** *Western blot analysis of $p27^{Kip1}$ and β-actin in A549 cells in control and NR5A2 knockdown conditions.* ***(D)*** *Quantification of protein expression levels of $p27^{Kip1}$ in A549 cells in control and NR5A2 knockdown conditions, $p<0.01$.*

7. DLPC as an NR5A2 agonist can mimic its antiproliferative effect on NSCLC cells

Although discovering NR5A2 natural ligands remains challenging, several synthetic agonists and antagonists have been developed in recent years. These small molecule ligands can bind to its LBD domain and modulate its transcriptional activity [43]. The scientific interest in developing these chemical compounds was mainly focused on further enhancing this nuclear receptor's anti-lipidemic and anti-diabetic capacity [42]. Considering that there is no scientific data related to their involvement in lung cancer progression, we first investigated whether these molecules can impair non-small cell lung cancer development. For this scope, DLPC, a well-established agonist of NR5A2, was selected to study whether it can mimic the anti-proliferative effect of NR5A2 on NSCLC cells. A549 cells were treated with three different

concentrations of DLPC (150 uM, 170 uM, and 200 uM) for 48 h, whereas as the control condition, cells were treated with ethanol, which is the DLPC dissolvent. The effect on cell proliferation was evaluated with immunofluorescence assays using Ki67 and phosphorylated histone H3 (pH3) as proliferation markers.

The collected data were analysed by counting the cells and subsequently quantifying the Ki67 and pH3 signals. Ordinary one-way ANOVA was implemented for the statistical analysis. The treatment with 150 uM DLPC resulted only in a significantly decreased number of pH3+ cells, with numbers having stood at 1.61% and 1.04% for the control and the treatment conditions, respectively (**Figure 30**). Interestingly, the administration of 170 uM and 200 uM DLPC significantly reduced the percentages of both Ki67+ and pH3+ A549 cells. The number of Ki67+ cells after 170 uM and 200 uM DLPC treatment compared to the control condition was significantly reduced, with numbers having been at 87.06% and 71.67%, respectively (**Figure 29**).

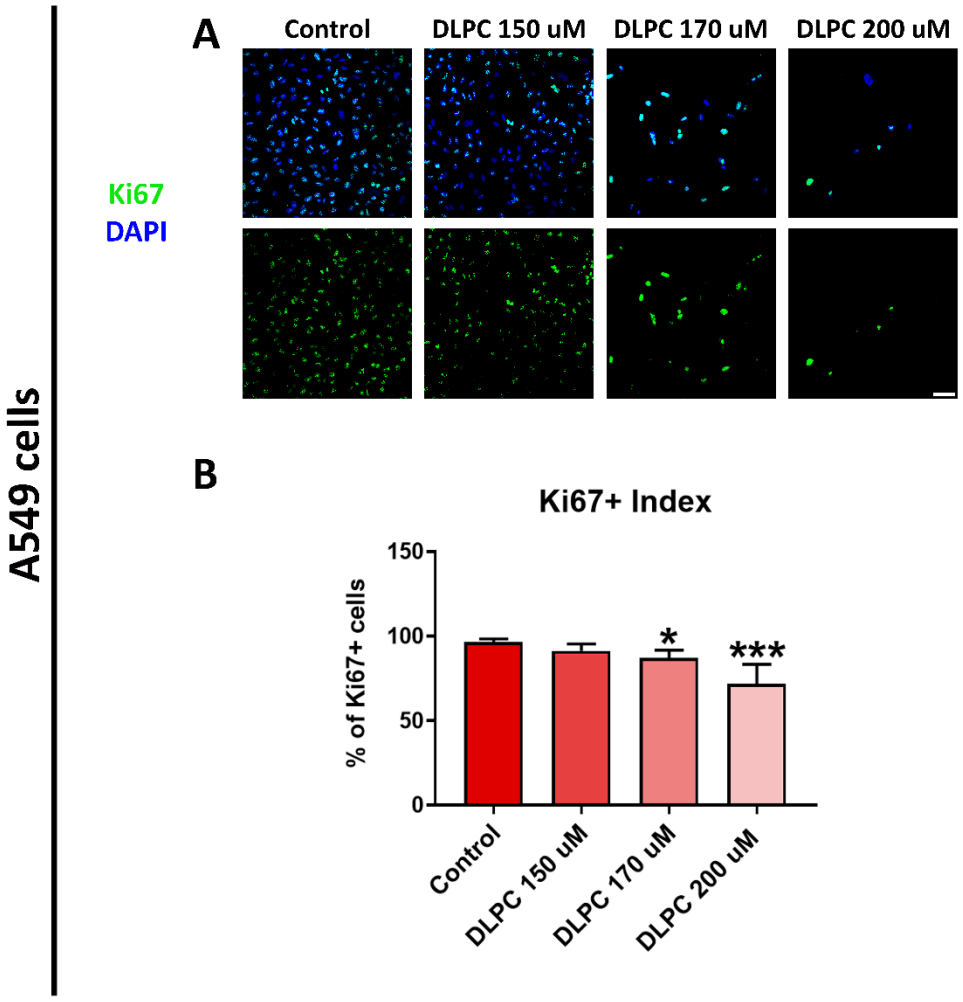

Figure 29: ***(A)*** *Immunofluorescence results of A549 cells stained for Ki67 in control and DLPC treatment conditions. Scale bar: 50 um.* ***(B)*** *Quantification of immunofluorescence results of A549 cells stained for Ki67 in control and DLPC treatment conditions. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

Furthermore, a 2.56-fold decrease in the percentage of pH3+ cells was stimulated after DLPC treatment at 170 uM (0.63% in DLPC treated A549 cells). Interestingly, when cells were treated with 200 uM DLPC, no pH3+ cells were detected (**Figure 30**). Together, the results support that DLPC reduces cell proliferation in a dose-dependent manner (**Figure 29, 30**).

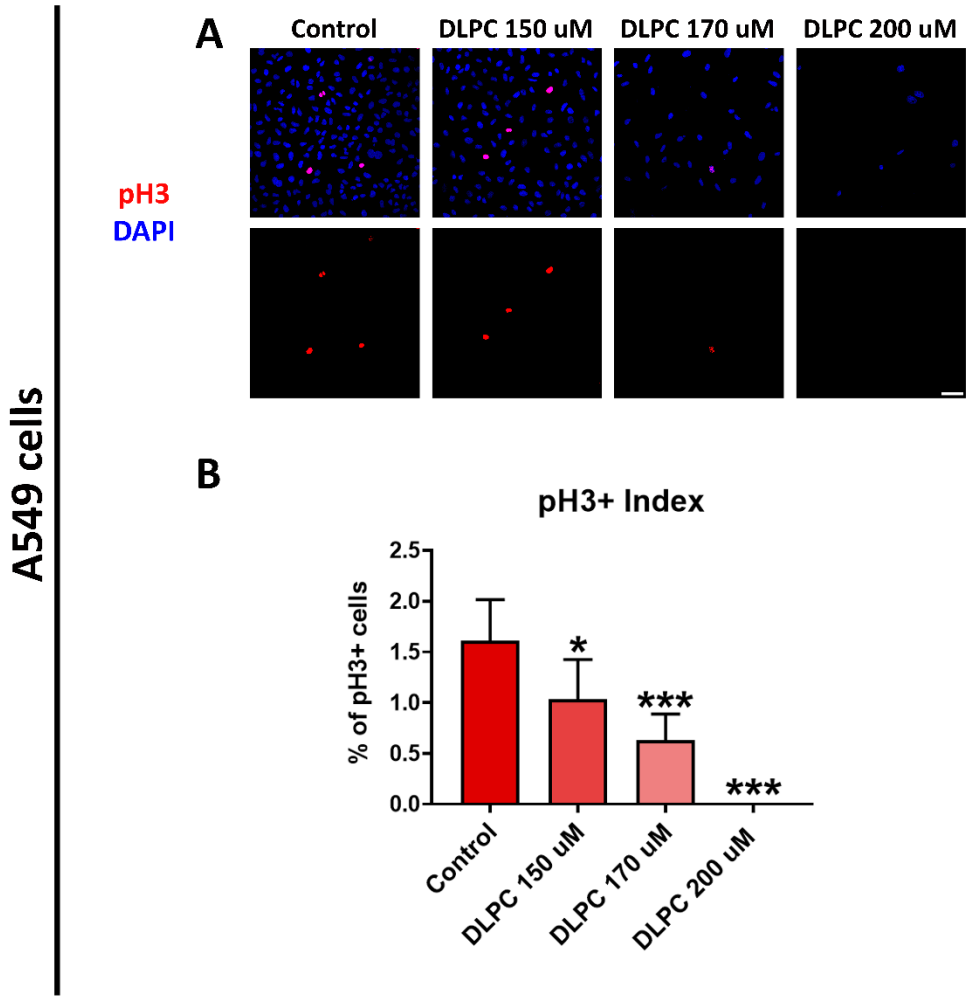

Figure 30: ***(A)*** *Immunofluorescence results of A549 cells stained for pH3 in control and DLPC treatment conditions. Scale bar: 50 um.* ***(B)*** *Quantification of immunofluorescence results of A549 cells stained for pH3 in control and DLPC treatment conditions. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

After determining 200 uM as the optimal concentration of DLPC, three additional NSCLC cell lines (NCI-H460, EKVX and NCI-H1944) were treated with NR5A2 agonist, and immunofluorescence protocol was followed. NCI-H1944 cells were not tolerant of DLPC even in low concentrations; thus cell proliferation indices could not be determined as cell death was induced.

In both cell lines, significantly reduced numbers of Ki67+ and pH3+ cells were observed when treated with DLPC. Indeed, Ki67 was detected in 23.37% of NCI-H460 cells and 20.17% of EKVX cells, whereas in control, it reached 40.5% and 35% in each cell line, respectively (**Figure 31A-31B, 32A**). Similar observations were attained by

pH3 immunostainings, as the number of NCI-H460 pH3+ cells decreased approximately 7.97 times, with percentages reaching 5.5% for the control and 0.69% for the treatment conditions (**Figure 31C-31D**).

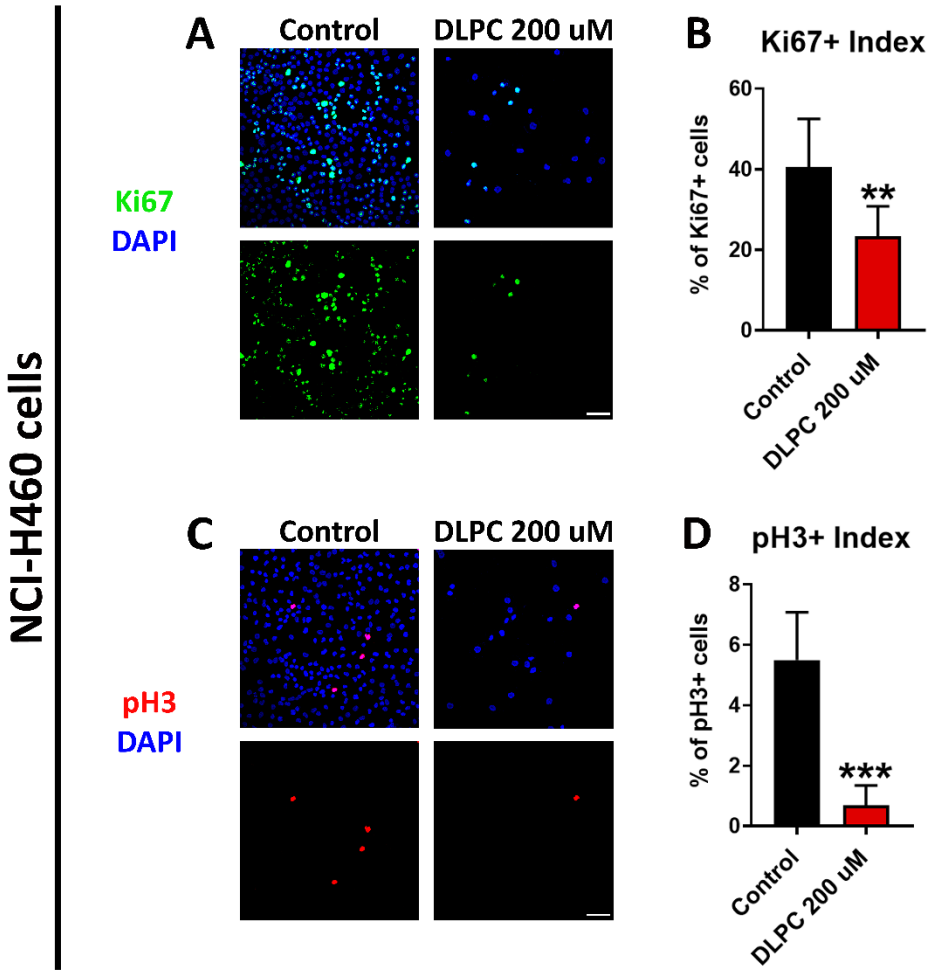

Figure 31: *Immunofluorescence results of NCI-H460 cells stained for Ki67* ***(A, B)*** *and pH3* ***(C, D)*** *in control and DLPC treatment conditions. Scale bar: 50 um. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

Regarding EKVX cells, the number of pH3+ cells dropped from 6.17% to 2.5% after DLPC treatment (**Figure 32B**). Together, these findings support that DLPC can effectively inhibit the proliferation of NSCLC cells, as in all cases, the observed differences were statistically significant, and a greater decrease in the pH3 index was reported compared to the Ki67 index (**Figure 31, 32**).

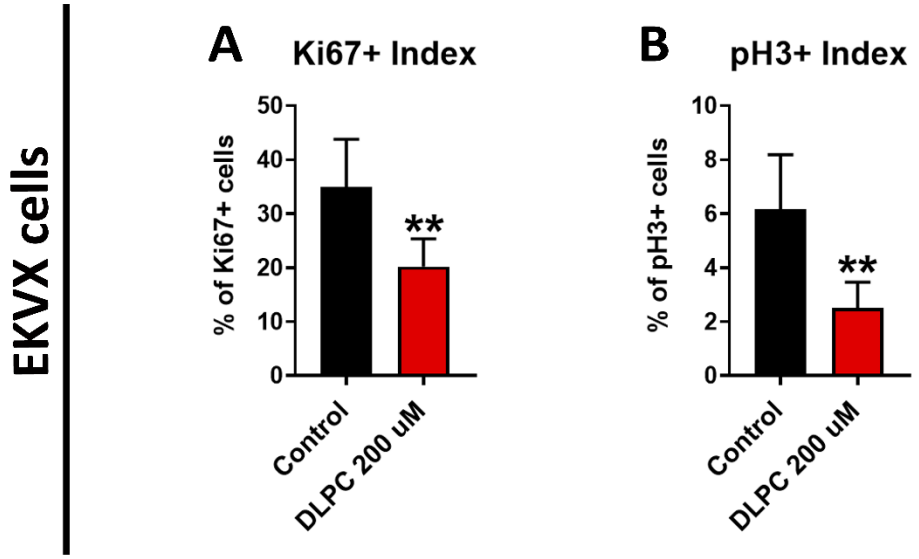

Figure 32: *Quantification of immunofluorescence results of EKVX cells stained for Ki67* ***(A)*** *and pH3* ***(B)*** *in control and DLPC treatment conditions. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001.*

8. DLPC induces the expression of cell cycle inhibitors

To further investigate the potential involvement of DLPC in cell cycle regulation, we isolated total protein from A549 cells after DLPC treatment and tested the expression of $p21^{Cip1}$ and $p27^{Kip1}$. Cells were incubated with three different concentrations of DLPC (150 uM, 170 uM, and 200 uM) (treatment group) or EtOH (control group) for 48 h, and then protein extraction was performed.

Western blot analysis indicates that DLPC, as an NR5A2 agonist, promotes the expression of these two negative cell cycle regulators, as significantly increased protein levels of $p21^{Cip1}$ and $p27^{Kip1}$ were detected as compared to the control conditions (**Figure 33, 34**). When cells were treated with 150 uM DLPC, no significant change was noticed at the protein levels of these two cell cycle inhibitors (**Figure 33A-B, 34A-B**). However, the administration of 170 uM DLPC caused a significant increase only at the protein levels of $p21^{Cip1}$ (**Figure 33C-D**), whereas no difference was observed regarding $p27^{Kip1}$ expression (**Figure 34C-D**). Only the treatment with 200 uM DLPC resulted in the upregulation of $p27^{Kip1}$, as significantly increased protein levels were detected after DLPC addition (**Figure 34E-F**). Moreover, the aforementioned concentration triggered the highest increase at the protein levels of $p21^{Cip1}$ (**Figure 33E-F**).

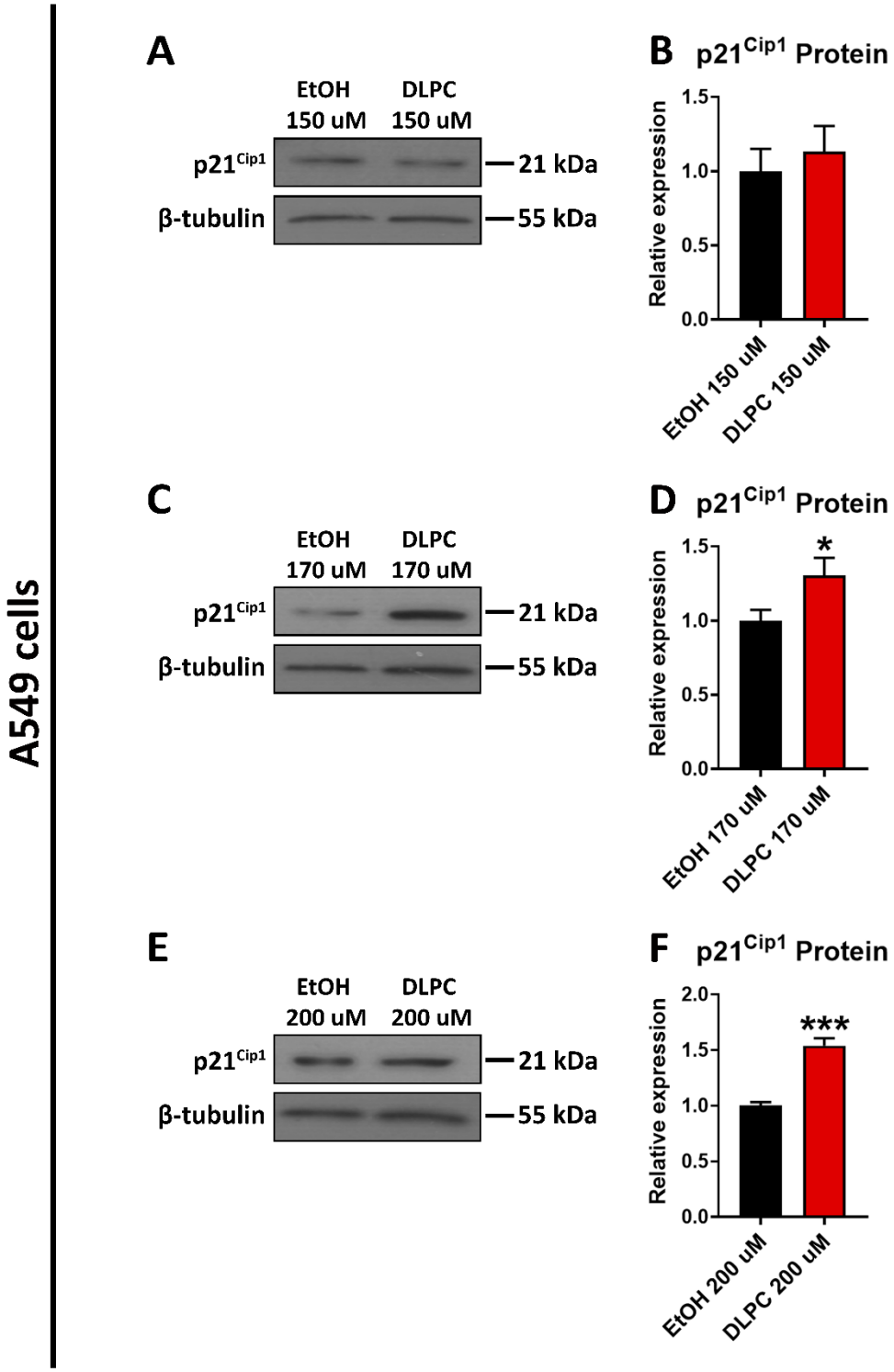

Figure 33: *Western blot analysis of $p21^{Cip1}$ and β-actin in A549 cells in control and 150 uM* ***(A)****, 170 uM* ***(C)****, and 200 uM* ***(E)*** *DLPC treatment conditions. Quantification of protein expression levels of $p21^{Cip1}$ in A549 cells in control and 150 uM, $p>0.05$* ***(B)****, 170 uM, $p<0.05$* ***(D)****, and 200 uM, $p<0.001$* ***(F)*** *DLPC treatment conditions.*

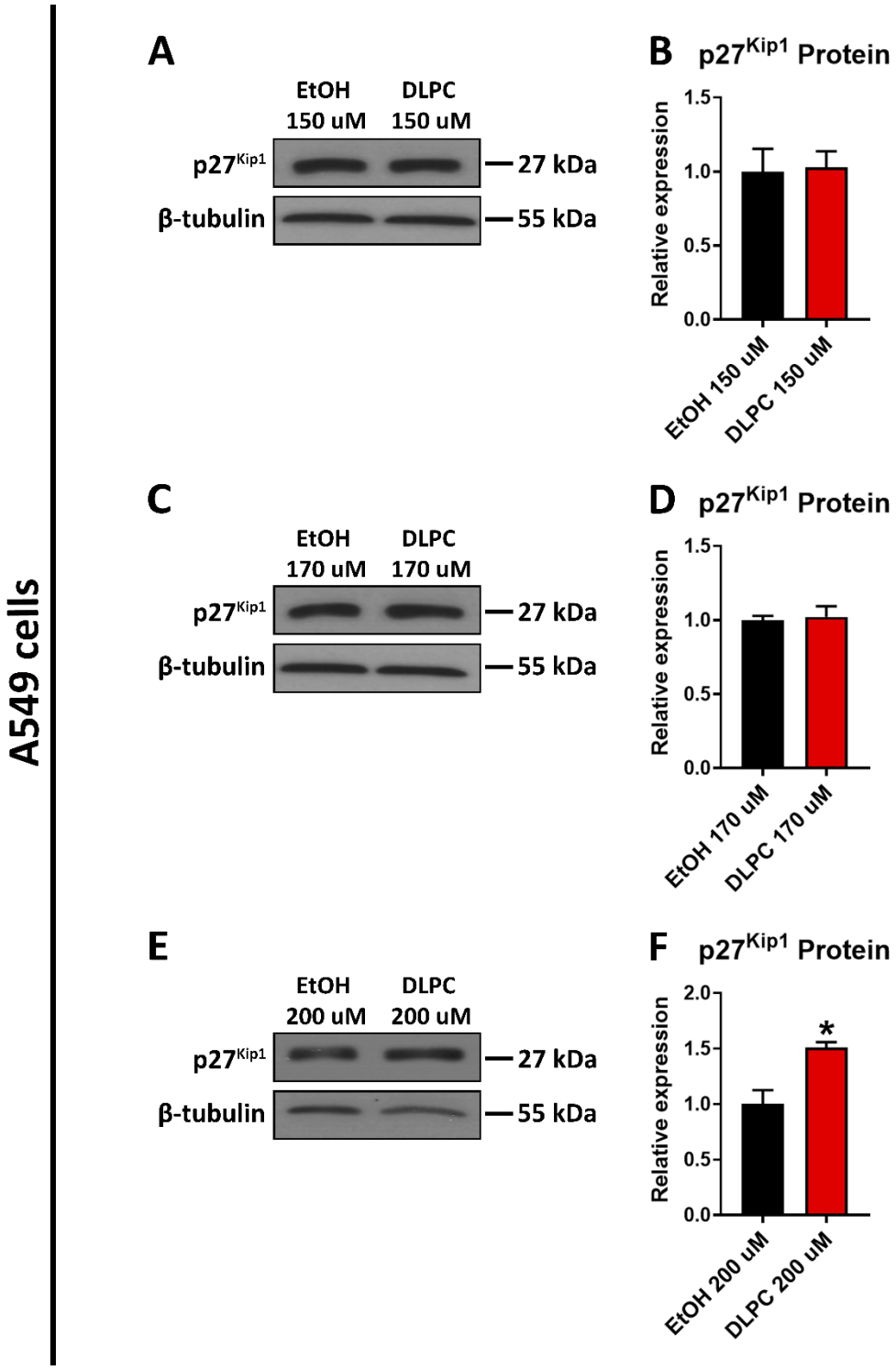

Figure 34: *Western blot analysis of $p27^{Kip1}$ and β-actin in A549 cells in control and 150 uM* ***(A)****, 170 uM* ***(C)****, and 200 uM* ***(E)*** *DLPC treatment conditions. Quantification of protein expression levels of $p27^{Kip1}$ in A549 cells in control and 150 uM, p>0.05* ***(B)****, 170 uM, p>0.05* ***(D)****, and 200 uM, p<0.05* ***(F)*** *DLPC treatment conditions.*

9. NR5A2 overexpression suppresses tumour growth in heterotopic xenografts

A heterotopic allotransplantation experiment was conducted using NOD-SCID mice to examine further whether the antiproliferative effect of NR5A2 overexpression might also be detected in vivo. A previously constructed and tested adenoviral-based overexpression system was used to obtain NR5A2 overexpression in A549 cell line [59]. Mice were subcutaneously injected in the flanks and divided into the control group comprising four mice and the treatment group consisting of four mice. Mice were housed under aseptic conditions in individually ventilated cages for every experimental group at 24 ºC and a light-controlled (12 h per day) environment. Tumour size was monitored and measured with a calliper every 3-4 days. Tumour volume was calculated using the equation: volume (cm^3) = (length x $width^2$)/2. After the mice sacrifice, tumours from the two experimental groups were isolated and weighed (**Figure 35A**). Interestingly, tumour volume in the control condition was 6 times higher than in the experimental group at 32 days post-injection. At 36 days post-injection, tumour volume was 6.14 times smaller in the NR5A2 group, while the difference at 43 days was 3.25 times. At 46 days post-injection, when the last measurement was performed, tumour volume was 2.3 times as high in the control group as in the NR5A2 group (**Figure 35B**). Moreover, the tumour weight in the control condition is 1.73 times higher than in the experimental group (**Figure 35C**). Therefore, these results indicate that NR5A2 overexpression impairs tumour growth in a statistically significant manner (**Figure 35**).

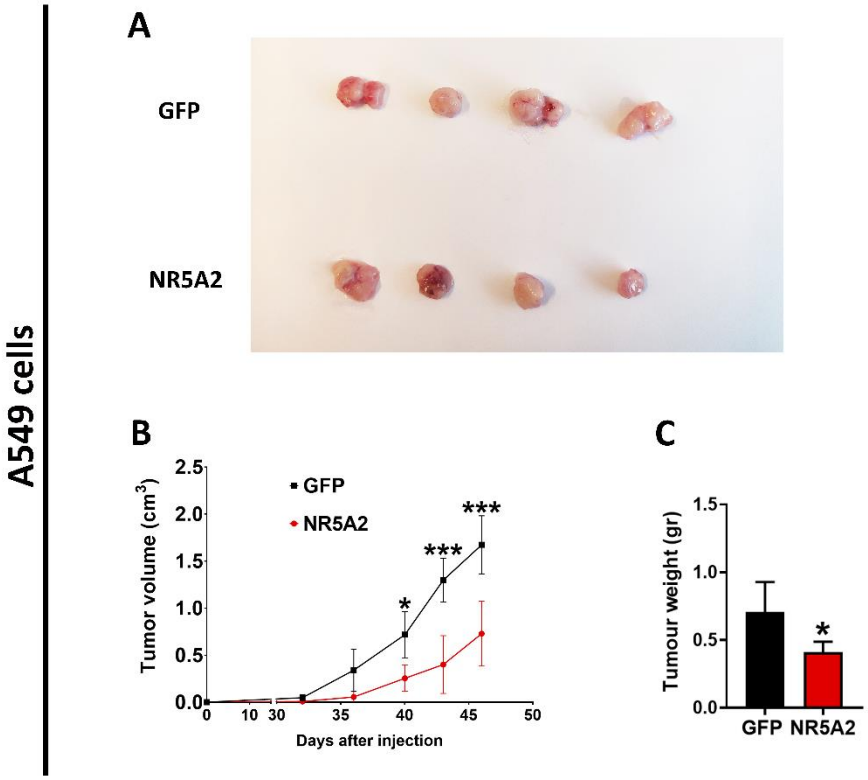

Figure 35: ***(A)*** *Representative images of whole tumours that were grown in NOD/SCID animals after A549 transduced cell injections. The tumour images were taken at the end of the experiment, e.g., 46 days after the initiation of treatment.* ***(B)*** *Quantification of the tumour volume every 3 to 4 days after the initiation of treatment (day 0).* ***(C)*** *Quantification of the tumour weight at the end of the experiment. Statistical analysis of the quantitative variables was performed using the two-tailed paired Student's t-test. For all cases, ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001*

Discussion

Analysis of publicly available clinical data from databases, including the Oncomine, the TNM plot, and the Kaplan-Meier plot web tools, indicates a negative association between NR5A2 expression levels and lung cancer progression. Interestingly, our previous studies suggested that NR5A2 may inhibit tumour progression in glioblastoma and neuroblastoma patients [53]. These observations raised the hypothesis that NR5A2 may exert a tumour-suppressor function in lung cancer. In this study, we investigate whether NR5A2 is only a marker or whether it also has a functional effect on two subtypes of NSCLC, lung adenocarcinoma and large cell carcinoma. A549, EKVX, and NCI-H1944 cell lines for the first subtype, and NCI-H460 cells for the second, are utilised for the experimental workflow. First, we examine the phenotypic effect of NR5A2 in NSCLC *in vitro* with immunofluorescence assays using Ki67 and pH3 as proliferation markers. The results support that NR5A2 overexpression can effectively inhibit the proliferation of all four cell lines, with the most distinct phenotypic changes observed in A549 cells. The NCI-H460 cell line exhibits slight phenotypic alterations, possibly due to its aggressive histological properties. This antiproliferative effect of NR5A2 is independent of cell apoptosis, as indicated by immunofluorescence experiments in A549 cells evaluating the cleaved caspase3 index. Most importantly, NR5A2 overexpression also suppresses the tumour growth *in vivo*, providing sufficient data to enhance the hypothesis regarding the antiproliferative function of NR5A2. In further agreement, loss-of-function studies reveal that the stable knockdown of *NR5A2* results in increased proliferation rates in the A549 cell line. Therefore, alterations in *NR5A2* mRNA expression might oppositely impact cell proliferation, indicating its crucial role in regulating cell cycle progression.

An additional effect of NR5A2 on cell migration was observed through wound healing assays in A549 lung adenocarcinoma cells. Notably, the wound area was still discernible in the NR5A2-overexpressing experimental group at 72 hours, compared to the control condition, where it approached its complete healing at the same time. Conversely, in *NR5A2* knockdown condition, accelerated migration rates for recreating cell monolayers were detected at 48 h time point. These observations raise the hypothesis that NR5A2 may exert a tumour-suppressor function in lung adenocarcinoma since cell migration and invasion in the adjacent healthy tissues are hallmarks of this cancer type.

To identify the downstream targets mediating the antiproliferative effect of this nuclear receptor, we examine the expression of several genes involved in cellular processes, including cell cycle progression, metabolism, and signaling. NR5A2 overexpression does not alter the expression of tumour suppressor genes, including *PTEN*, *TP53*, and *RB1*, and oncogenes, including *MYC* and *HRAS*. However, the expression of *E2F1*, *CCNE1*, *LDHA*, and *PDK1* follows an opposite pattern to the stimulated changes during cell cycle arrest. This might be indicated as cell response through a feedback mechanism aiming to induce cell cycle progression and survival. Downregulation of *CCND1* is consistent with the reduced cell proliferation rates. Although NR5A2 overexpression does not affect the expression of *SLC2A3*, and results in the upregulation of *LDHA*, and *PDK1*, decreased mRNA levels of *PKM* denote that NR5A2 might be involved in regulating cancer metabolism through modulation of

critical genes like *PKM*, which is also not further investigated in the present study. Moreover, increased *FOXO3* mRNA expression is detected after NR5A2 overexpression suggesting a potential regulatory relationship between NR5A2 and FOXO3 in cellular processes, which is not investigated in the present study. We focused our investigation on two negative cell cycle regulators, *CDKN1A* ($p21^{Cip1}$) and *CDKN1B* ($p27^{Kip1}$), based on our previous reports supporting a potential direct interaction between NR5A2 and regulatory sequences of these genes [53]. Interestingly, NR5A2 overexpression promotes the expression of these two cell cycle inhibitors, as significantly increased mRNA and protein levels of $p21^{Cip1}$ and $p27^{Kip1}$ were detected. In further agreement, knockdown studies reveal a significant downregulation of $p21^{Cip1}$ and $p27^{Kip1}$. These data further enhance the hypothesis that the antiproliferative effect of NR5A2 might be mediated due to explicitly targeting these two cyclin-dependent kinase inhibitors (CDKIs).

Considering that several synthetic ligands of NR5A2 are included in studies for unravelling or improving its therapeutic properties [42]–[44], it would be interesting to examine their impact in the context of lung tumours. For this scope, DLPC, a well-established agonist of NR5A2, was selected to study whether it can mimic the antiproliferative effect of NR5A2 in NSCLC cells. Results from immunofluorescence experiments support that DLPC reduces cell proliferation in a dose-dependent manner in A549 lung adenocarcinoma, determining 200 uM comprising as the optimal concentration for further investigation. Additional experiments in the EKVX and NCI-H460 cell lines indicate that DLPC can effectively inhibit the proliferation of NSCLC cells, as in all cases, except for NCI-H1944 cells which were not tolerant of DLPC even in low concentrations. Furthermore, our studies regarding the potential involvement of DLPC in cell cycle regulation reveal that it induces the expression of the aforementioned negative cell cycle regulators, as increased protein levels of $p21^{Cip1}$ and $p27^{Kip1}$ were detected. These observations denote that DLPC, as an NR5A2 agonist, may recapitulate its antiproliferative effect by inducing these CDKIs, but the underlying mechanism needs further investigation to be elucidated.

Scientific evidence supports the dual role of NR5A2 in cancer development. Although several studies reveal the tumour-promoting function of NR5A2 in breast [48]–[50], colorectal [51], and liver cancer [52], there is a variety of scientific reports indicating the tumour-suppressing function of NR5A2 in nervous system malignancies [53], HNSC [47], and pancreatic carcinomas [46], [47]. Specifically, NR5A2 promotes cell proliferation in breast cancer cells by the induction of the *CCND1* gene. Also, some data indicate the potential regulation of the *CDKN1A* gene (encoding for $p21^{Cip1}$) by NR5A2 regardless of the p53 cellular status. Additionally, in colorectal cancer, NR5A2 impact on cell cycle progression through the induction of *CCND1* and *CCNE1* expression. Conversely, NR5A2 in glioblastoma tumours is potent to induce the expression of $p21^{Cip1}$ and $p27^{Kip1}$ both at mRNA and protein levels, and this NR5A2-mediated transcriptional regulation may also be achieved through direct interactions with regulatory sequences of these genes. Similarly, in HNSCC, NR5A2 affects cell cycle progression depending on TP53, stimulating cell cycle progression at low TP53 expression levels. Interestingly, in pancreatic cancer, NR5A2 can act both by promoting and inhibiting cell proliferation and migration, while the SNPs might determine its

effect over the NR5A2 gene or close to it [41], [46], [47]. These observations highlight the complexity of NR5A2 in tumour development.

Limited information regarding the role of NR5A2 in lung cancer is available. Two recently published studies support that NR5A2 promotes lung cancer stem cell properties by positively regulating *NANOG* expression [63] and that its upregulation may serve as a diagnostic marker and can predict lymph node metastasis [64]. However, the present study supports that NR5A2 has an antiproliferative effect on non-small cell lung cancer, specifically lung adenocarcinoma. Although the underlying mechanism is not completely understood, NR5A2 seems to downregulate the *CCND1* gene and counteract the effect of the *CCNE1* gene on cell cycle progression by inducing the expression of $p21^{Cip1}$ and $p27^{Kip1}$ in lung adenocarcinoma cells. These two negative cell cycle regulators can directly inactivate the complexes between CDKs and Cyclin D1 or Cyclin E1, resulting in cell cycle arrest. The effect of NR5A2 on $p21^{Cip1}$ and $p27^{Kip1}$ might be facilitated through potential interactions with their regulatory sequences, as it is indicated in glioblastoma (U87-MG cell line) [53] and breast cancer (MCF-7 cell line) [50]. Additionally, the context-dependent tumour-promoting role of NR5A2 in other organs might be modulated through its inability to effectively induce $p21^{Cip1}$ and $p27^{Kip1}$, allowing Cyclin E1 to promote cell cycle progression. Moreover, considering the several functions of NR5A2, it is more likely to control cell fate and tumour progression by cooperating with other molecules rather than acting independently. The divergent experimental evidence regarding the role of NR5A2 in cancer underscores the complexity and context-dependent activity of this nuclear receptor in tumour development and cell cycle regulation and highlights that further investigation about the interrelated pathways that regulate its expression is pivoting.

Nuclear receptor diverse interactions with a broad spectrum of molecular partners can differentially regulate their downstream target genes and might modulate the molecular mechanisms through which they exert multiple regulatory effects on different cell types. Consistently, NR5A2 interactions with co-regulators and other transcription factors, including CTNNB (β-catenin), PGC-1a, CBP, CREB1, FXR, MBF1, SRC1/3, NRIP1, SMARCD3, SMRT, SHP, DAX1 and Prox1, have been observed to take place in a cell-type dependent manner [45], [65]. Therefore, NR5A2 is potent in regulating genes involved in the cell cycle by either enhancing or inhibiting their expression depending on the cellular and/or developmental context.

Overall, the present study denotes a tumour-suppressor function of NR5A2 and its agonist in non-small cell lung cancer, suggesting NR5A2 as a potential therapeutic target gene.

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