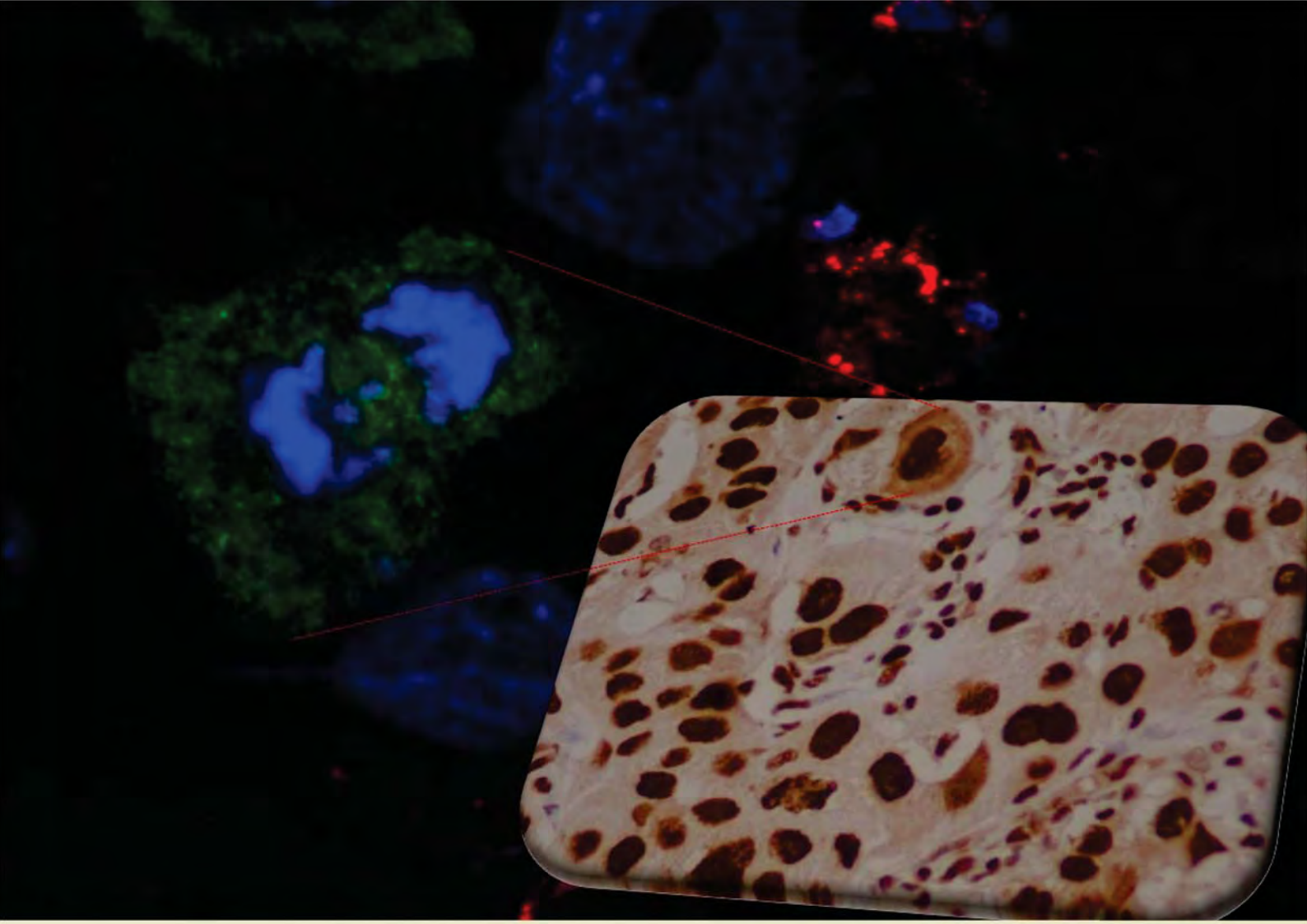

Ângela Filipa Valério Fernandes

TARGETING NUCLEOLIN IN LUNG CANCER: TOWARDS A PERSONALIZED THERAPY

Tese de Doutoramento em Biologia Experimental e Biomedicina, ramo de Oncobiologia, orientada pelo Professor Doutor João Nuno Moreira e Professor Doutor Sérgio Simões, apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra.

Setembro 2015

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UNIVERSIDADE DE COIMBRA

TARGETING NUCLEOLIN IN LUNG CANCER: TOWARDS A PERSONALIZED THERAPY

Thesis submitted to the Institute for Interdisciplinary Research of the University of Coimbra to apply for the degree of Doctor in Philosophy in the area of Experimental Biology and Biomedicine, specialization in Oncobiology.

Ângela Filipa Valério Fernandes

2015

The work described in this thesis was conducted at the Center for Neuroscience and Cell Biology (CNC) from the University of Coimbra, under the scientific supervision of Professor João Nuno Moreira and Professor Sérgio Simões.

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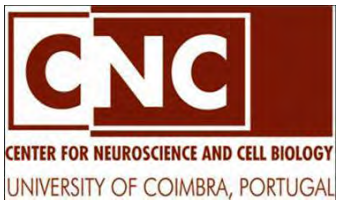

UNIVERSIDADE DE COIMBRA

"Às vezes ouço passar o vento;
e só de ouvir o vento passar,
vale a pena ter nascido"

Alberto Caeiro in *Poemas Inconjuntos*
Heterónimo de Fernando Pessoa

*To my family**
&
My modest tribute to all those who have fought and those who are fighting against lung cancer,
in special to a person who touched my life and fought until the end.

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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis." *Fernando Pessoa*

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E, à minha querida e doce avó Di.

To my *other half*, my sister, Calinho.

To my, since ever and forever, Joana.

And, what would I do without love? To my, João Daniel.

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Thesis abstract

Lung cancer is a dramatic public health problem, being the leading cause of cancer-related mortality both in men and women, worldwide. Most of the patients are diagnosed with locally advanced inoperable or metastatic stage, which makes the disease often incurable.

Research in the field of tumor angiogenesis has provided the foundation for a radical change in the management and treatment of human cancer, including lung cancer. Bevacizumab was the first anti-angiogenic agent approved for the first-line treatment of non-squamous lung cancer and has become a standard treatment option for these patients. Despite these important advances, while many patients do not respond to this therapy, responders are short-lived, and resistance arises in the majority of patients. Accordingly, further research is needed to develop more efficacious therapeutic strategies that overcome these limitations.

Targeting the tumor vasculature can potentially overcome several problems in cancer treatment, such as drug resistance of cancer cells or insufficient drug penetration into the tumor. Furthermore, endothelial cells of the tumor-associated vasculature are easily accessible to drugs administered intravenously, and have greater genetic stability than neoplastic cells, thus evidencing lower risks of relapse and resistance to therapy. In this respect, the identification of novel molecular targets is of high relevance.

Cell surface nucleolin has been reported as a highly attractive therapeutic target for anti-cancer strategies targeting the tumor microenvironment in breast cancer, as it is overexpressed in cancer cells as well as endothelial cells from tumor blood vessels. Accordingly, in a model of breast cancer, lipid-based nanoparticles functionalized with the nucleolin-binding F3 peptide, and containing a chemotherapeutic drug (doxorubicin) enabled a significant decrease of the viable rim tumor area, as well as vascular density, with suppression of tumor invasion. Based on these developments, potential benefits are expected in the clinic. A promising challenge is to investigate, in the pre-clinical setting, the potential anti-tumor impact of targeting nucleolin in the lung tumor microenvironment using the new developed nanoparticle.

One of the goals of this project was to validate nucleolin as a therapeutic target in bevacizumab-resistant human lung cancer cell lines and in human pulmonary carcinoma specimens, using this nucleolin-based targeting strategy. Binding and internalization of the F3 peptide-targeted liposomes by lung cancer cells, suggested a ligand-specific interaction, in an extent of association that, depending on the cell line, was 34- to 170-fold higher than the

one observed with the tested controls. Improved association and intracellular delivery of the encapsulated doxorubicin enabled by F3 peptide-targeted liposomes, supported a maximum of 19-fold increase of doxorubicin cytotoxicity relative to the activity of doxorubicin delivered by non-targeted liposomes. Significant extent of association of radiolabeled F3 peptide-targeted liposomes with human pulmonary carcinoma tissues, was demonstrated by *ex vivo* autoradiography.

The potential of nucleolin as a therapeutic target in lung cancer was further supported by its distinct subcellular overexpression (nuclear and extra-nuclear, *i.e.*, cytoplasm-membrane) in several cell populations of the tumor microenvironment of patient-derived pulmonary carcinomas, including endothelial cells, in a tumor-specific manner. Nevertheless, nucleolin expression and vascular density in subcutaneous pulmonary adenocarcinoma xenografts, derived from the tested bevacizumab-resistant cell lines, were incomparably lower than the one observed in human pulmonary carcinomas.

The subcellular distribution of extra-nuclear nucleolin in the tumor microenvironment of patient-derived pulmonary carcinomas was further characterized. Extra-nuclear nucleolin was expressed among pulmonary carcinoma samples analyzed, in cancer and endothelial cells, independently of both clinicopathological features (including histopathological type) and other angiogenic markers studied (PDGFRα, HIF-1α, IL-17, VEGFR2, FGFR2 and CD133). These findings further suggested that nucleolin could be a hallmark of lung carcinogenesis and angiogenesis, regardless the clinicopathological features and expression of other angiogenic markers.

To gain further insights on the relevance of nucleolin in different stages of lung cancer, its expression on bone metastasis from lung cancer patients, and also from others primary origins, was assessed. Interestingly, a similar pattern of nucleolin expression between pulmonary primary carcinomas and bone metastases derived from lung primary cancer was identified.

Overall, these results support the therapeutic relevance of both nucleolin as a molecular target in lung cancer, including in the tumor microenvironment of primary lung cancer and metastatic sites in the bone, as well as of nucleolin-based targeting strategies.

Keywords: Lung cancer; Tumor microenvironment; Nanomedicine; Nucleolin; Targeting.

Resumo da tese

O cancro do pulmão é um grave problema de saúde pública a nível mundial, sendo a principal causa de morte relacionada com o cancro, tanto em homens como em mulheres. A maioria dos doentes são diagnosticados na fase localmente avançada ou metastática da doença, o que reduz as possibilidades de sucesso terapêutico.

A investigação na área da angiogénese tumoral tem fornecido as bases para uma mudança no tratamento do cancro humano. O bevacizumab foi o primeiro agente terapêutico anti-angiogénico aprovado para o tratamento de cancro de pulmão não escamoso, passando a constar nas opções terapêuticas de primeira linha para estes doentes. No entanto, apesar dos enormes avanços alcançados, muitos doentes não respondem à terapia, e para aqueles que o fazem, as respostas são de curta duração e a maioria desenvolve resistência à terapia. Desta forma, é necessária mais investigação no sentido do desenvolvimento de estratégias que superem estes problemas encontrados na clínica.

Estratégias terapêuticas que visam o direcionamento específico de quimioterapia para a vasculatura do tumor podem, potencialmente, superar diversos problemas no tratamento do cancro. A resistência das células tumorais aos agentes terapêuticos, ou a penetração insuficiente de fármacos no interior da massa tumoral devido aos elevados gradientes de pressão intersticial nos tumores, bem como a toxicidade nos tecidos normais, podem potencialmente ser diminuídos. Para além disso, as células endoteliais da vasculatura tumoral representam um alvo de fácil acesso para terapias administradas por via intravenosa, sendo também geneticamente mais estáveis do que as células neoplásicas, evidenciando assim menor risco de recorrências e desenvolvimento de resistências. A identificação de novos alvos terapêuticos é determinante neste processo de desenvolvimento de novas estratégias terapêuticas especificamente dirigidas.

A nucleolina de superfície celular tem sido apontada como um alvo terapêutico muito atrativo para o direcionamento de terapias para o microambiente tumoral em cancro da mama, sendo que esta proteína está sobre-expressa tanto em células neoplásicas, como em células endoteliais da vasculatura tumoral. Recentemente, uma nova nanopartícula (encapsulando doxorrubicina), funcionalizada com o peptídeo F3, específico para a nucleolina, induziu um significativo impacto terapêutico contra modelos de cancro da mama.

Um desafio promissor é investigar, no contexto pré-clínico, o potencial anti-tumoral desta abordagem terapêutica em cancro do pulmão.

Neste trabalho foi proposto investigar o impacto da terapia especificamente dirigida

à nucleolina do microambiente tumoral, em modelos de cancro do pulmão resistentes ao bevacizumab, usando esta nova estratégia terapêutica. Neste sentido, primeiramente fomos validar a nucleolina como potencial alvo terapêutico em modelos celulares de cancro do pulmão e, no microambiente tumoral de amostras de carcinomas do pulmão de doentes. Os resultados demonstraram ligação e internalização da nanopartícula, especificamente dirigida para a nucleolina, pelas células humanas de cancro do pulmão. A extensão da interação promovida pelo ligando F3 foi 34 a 170 vezes superior à observada com as nanopartículas controlo. A elevada extensão de associação celular e consequente libertação intracelular da doxorrubicina, justifica o aumento significativo (19 vezes) dos efeitos citotóxicos do fármaco libertado por esta nanopartícula, relativamente às formulações controlo. Para além disso, estes resultados foram reforçados pela elevada extensão de associação da nanopartícula dirigida pelo peptídeo F3 ao tecido tumoral humano, demonstrada através de estudos *ex vivo* de autoradiografia. O potencial da nucleolina como alvo terapêutico em cancro do pulmão foi ainda reforçado pela sobre-expressão da proteína em diferentes localizações celulares (nuclear e extra-nuclear – citoplasma-membrana), bem como em diferentes populações do microambiente tumoral (incluindo as células endoteliais), de forma específica. Como consequência da caracterização do microambiente tumoral de modelos subcutâneos de cancro do pulmão (gerados com as linhas celulares resistentes ao bevacizumab), verificamos que, tanto a expressão da nucleolina, como a densidade vascular são incomparavelmente inferiores nestes modelos tumorais, e por isso não representativos dos carcinomas pulmonares humanos analisados.

Sendo o nosso objetivo final validar o potencial translacional desta estratégia terapêutica para o tratamento de doentes com cancro do pulmão, de seguida propusemos investigar com maior detalhe a distribuição sub-celular da nucleolina extra-nuclear em amostras de carcinomas pulmonares de doentes. Esta, revelou estar expressa nas amostras analisadas, tanto nas células neoplásicas, como nas células endoteliais, de forma independente tanto dos dados clínicos, como de outros marcadores angiogénicos aqui analisados (VEGFR2, PDGFRα, FGFR2, HIF-1α, IL-17 e CD133). Estes resultados sugerem que a nucleolina pode ser um fator importante na carcinogénese pulmonar, bem como na angiogénese nestes carcinomas, independentemente de outros fatores. No sentido de investigar o potencial da nucleolina em diferentes fases da doença, exploramos a expressão da nucleolina em amostras de tumores metastáticos ósseos, derivados de doentes de cancro com diferentes origens primárias, incluindo pulmão. Resultados demonstraram que a nucleolina se encontra sobre-expressa em todas as amostras em estudo, com um padrão de distribuição de expressão

semelhante ao encontrado nos carcinomas pulmonares primários. Estes resultados são uma primeira indicação de que a nucleolina poderá ser um alvo terapêutico relevante não só em tumores primários, mas também em metástases, possibilitando o acesso também ao microambiente metastático.

Globalmente, os resultados produzidos neste trabalho validam a relevância da nucleolina como alvo terapêutico no microambiente de carcinomas do pulmão, incluindo carcinomas primários e metástases no osso. Desta forma, enaltece-se igualmente o potencial de estratégias terapêuticas dirigidas à nucleolina em cancro do pulmão.

Keywords: Cancro do pulmão; Microambiente tumoral; Nanomedicina; Nucleolina; Direcionamento.

ABBREAVIATIONS LIST

US FDA	United States Food and Drug Administration
US	United States
EU	European Union
AIDS	Acquired Immune Deficiency Syndrome
ECM	Extracellular Matrix
BM	Basement Membrane
VEGF-A	Vascular Endothelial Growth Factor A
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2
HIF-1α or -2α	Hypoxia-inducible Transcription Factor 1α or 2α
FGF-1 or -2	Fibroblast Growth Factor 1 or 2
PDGF-B or –C	Platelet-derived Growth Factor B or C
EGF	Epidermal Growth Factor
TGF-β	Transforming Growth Factor β
MMP's	Matrix Metalloproteinase's
TNF	Tumor Necrosis Factor
Ang-2	Angiopoietin 2
TSP-1	Thrombospondin 1
IFN-α or –β	Interferon α or β
BMDCs	Bone Morrow-derived Cells
ECs	Endothelial Cells
PlGF	Placental Growth Factor
TAMs	Tumor-associated Macrophages
TANs	Tumor-associated Neutrophils
CAFs	Cancer-associated Fibroblasts
MDSCs	Myeloid-Derived Suppressor Cells
CXCL	CXC-Chemokine Ligand
CXCR2	CXC-Chemokine Receptor 2
PD1/PDL1	Programmed cell Death 1/Ligand 1
CSCs	Cancer Stem Cells
IL-17	Interleukin 17
T_H17	Tumor-infiltrating T helper Lymphocytes type 17
G-CSF	Granulocyte Colony-stimulating Factor

NF-kB	Nuclear Factor kB
ERK	Extracellular-related Kinase
NSCLC	Non-small Cell Lung Cancer
SCLC	Small-cell Lung Cancer
ADC	Adenocarcinoma
SQC	Squamous Cell Carcinoma
ADSQC	Adenosquamous Cell Carcinoma
PLMC	Pleomorphic Carcinoma
LCLC	Large Cell Carcinoma
EGF	Endothelial Growth Factor
p-EGFR	Phosphorylated-Endothelial Growth Factor Receptor
MVD	Microvessel Density
ASD	Angiogenic Squamous Dysplasia
KRAS	Kirsten Rat Sarcoma viral oncogene homolog
TTF1	Thyroid Transcription Factor 1
CK or KRT5, 6	Cytokeratin or Keratin 5, 6 or 7
SOX2	Transcription Factors SRY-box 2
GEMMs	Genetically Engineered Mouse Models
SPC	Surfactant Protein C
AcTUB	Acetylated Tubulin
AT	Alveolar epithelial Type
CCSP	Club Cell Secretory Protein
CGRP	Calcitonin Gene-Related Peptide
BASCs	Bronchioalveolar Stem Cells
Lkb1	Liver Kinase B1
IKKα	Inhibitor of nuclear factor-κB Kinase subunit-α
ALK	Anaplastic Lymphoma Kinase
PFS	Progression-Free Survival
mTOR	Mechanistic Target of Rapamycin
Dox	Doxorubicin hydrochloride
EPR	Enhanced Permeability and Retention effect
MPS	Mononuclear Phagocytic System
PEG	Poly(ethylene)glycol
HMGN2	High Mobility Group Protein 2

NCL	Nucleolin
TNM	Tumor-Node-Metastasis
WHO	World Health Organization
IASCL	International Association for the Study of Lung Cancer
ATS	American Thoracic Society
ERS	European Respiratory Society
UICC	International Union Against Cancer
DAB	3,3-diaminobenzidine tetrahydrochloride
LSAB	Labeled Streptavidin-biotin staining method
IHC	Imnnunohistochemistry
ICC	Immunocytochemistry
H&E	Hematoxylin & Eosin
GSH	Glutathione
HMPAO	Hexamethyl-propylene-amine oxime
^{99m}Tc	Technetium-99m
BSA	Bovine Serum Albumin
PBS	Phosphate-buffered Saline
TL	Total Lipid
DOPE	2-dioleoyl-sn-glycero-3-phosphoethanolamine
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG$_{2k}$	1,2-distearoyl-sn-glycero-3 phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
RhoD-PE	Rhodamine-PE
DSPE-PEG$_{2k}$-MAL	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]
2-IT	2-Iminothiolane
PDI	Polidispersion Index
FBS	Fetal Bovine Serum
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
MES	2-(N-Morpholino) ethanesulfonic acid
EDTA	Disodium ethylenediaminetetraacetate dehydrate
CHEMS	3β-hydroxy-5-cholestene-3-hemisuccinate
CHOL	Cholesterol
F3-L[Dox]	F3-peptide targeted liposomes, encapsulating doxorubicin

NS-L[Dox]	Non-specific peptide targeted liposomes, encapsulating doxorubicin
L[Dox]	Non-targeted liposomes, encapsulating doxorubicin
F3-L-RhoD	Rhodamine-labeled F3-peptide targeted liposomes
NS-L-RhoD	Rhodamine-labeled non-specific peptide targeted liposomes
L-RhoD	Rhodamine-labeled non-targeted liposomes

Preface

The PhD thesis here presented describes the potential of nucleolin as emergent therapeutic target for multi-targeting the tumor microenvironment of lung cancer patients using a new developed nanotherapeutic strategy, F3 peptide-targeted pH-sensitive liposomes. The thesis is organized in four chapters according to the established publication strategy.

The first chapter: assesses a new vision about cancer in general, and lung cancer in particular, in which the tumor microenvironment plays a critical role in lung tumorigenesis. Particularly, it describes the relevance of the tumor angiogenesis as hallmark of cancer development, from which new molecular and cellular targets have been identified and, a new generation of anti-cancer therapies have emerged – the targeted therapies, such as anti-angiogenic therapy. These have changed the treatment paradigm of lung cancer, specially advanced lung cancer. Special attention is given to recent advancements in nanomedicine as a strategy to overcome some of the biological barriers posed by tumor heterogeneity and plasticity. In this respect, nucleolin has gained relevance as an attractive therapeutic target for drug delivery nanosystems in cancer targeted therapies development.

The second chapter: addresses the potential application of F3 peptide-targeted pH-sensitive liposomes, targeting nucleolin, in bevacizumab-resistant lung cancer models. Particularly, addresses the specificity and efficacy of this nanoparticle for the intracellular trigger doxorubicin release in lung cancer cells. In this respect, cellular cytotoxicity studies and cell viability assessment were performed to evaluate anti-cancer efficacy against those cells. Furthermore, the potential of nucleolin as a valuable therapeutic target in resistant lung cancer cells and in patient-derived lung carcinomas was explored.

The third chapter: a detailed analysis of extra-nuclear nucleolin expression in the tumor microenvironment, as well as its correlation with clinicopathological features of patients with lung cancer, and with several other angiogenic factors expression, including VEGFR2, PDGFRα, FGFR2, HIF-1α, IL-17 and CD133, in human pulmonary carcinoma samples. Since metastatic disease is actually the major cause of lung cancer-related mortality, the nucleolin expression in bone metastasis microenvironment was also discriminated to validate its potential as a therapeutic target in different stages of the disease. The role of nucleolin in lung carcinogenesis, and angiogenesis, was also investigated.

***The fourth chapter*:** highlights the major achievements presented in the preceding chapters, and contextualizes them relatively to translational potential towards a personalized therapy in lung cancer.

Chapter 1

PERSONALIZING LUNG CANCER THERAPY IN THE ERA OF NANOMEDICINE

1.1. Creating a new vision about Cancer

Cancer is a major human health problem worldwide. Currently, one in four deaths in men and one in five deaths in women are caused by cancer, overtaking the acquired immune deficiency syndrome (AIDS), tuberculosis, and malaria combined death rate [1].

Over the past 30 years, significant progress has been achieved in understanding the molecular basis of cancer [2]. The accumulation of this thorough understanding has established cancer as a variety of distinct diseases arising from a diversity of defective genes and causing diverse mechanisms of carcinogenesis [3]. As such, almost every human tissue can develop malignant tumors with distinct features. Nevertheless, the basic events that give rise to different types of tumors are similar [4].

Cancer is characterized by the uncontrolled growth and spread of abnormal cells that arise from one single cell that initiated a program for inappropriate cell-division. The transformation from a normal cell into a cancer cell is a multistage process with progression from a pre-cancerous lesion/condition to a malignant tumor. Even so, the most overwhelming property of malignant cells is the ability to migrate from the original primary tumor, disrupting nearby tissues and forming malignant masses at distant sites in the body [3,4]. This metastatic capability leads to a spread of the disease, resulting in the death of the individual [4].

Research over the past decades has revealed a set of basic principles - acquired capabilities – that govern the development of most and perhaps all types of human cancer. These traits that control the transformation of normal human cells into cancer cells were designated by Hanahan and Weinberg as *the hallmarks of cancer* [3,5]. Owing to the emergent advances in cancer biology these capabilities were recently updated from six to ten: (1) sustained proliferative signaling, (2) evading growth suppressors, (3) enabling replicative immortality, (4) resisting cell death, (5) angiogenesis induction, (6) activation of invasion and metastasis, (7) immune system evasion, (8) deregulation of cellular energetics, and also enabling characteristics as (9) genome instability and mutation and (10) tumor-promoting inflammation [5].

The discovery of the aforementioned cancer capabilities changed the reductionist vision of cancer implemented until then. Tumors were increasingly recognized as complex tissues composed of several specialized cell types, interconnected with one another, forming a heterogeneous and complex organ (Figure 1.1) [5].

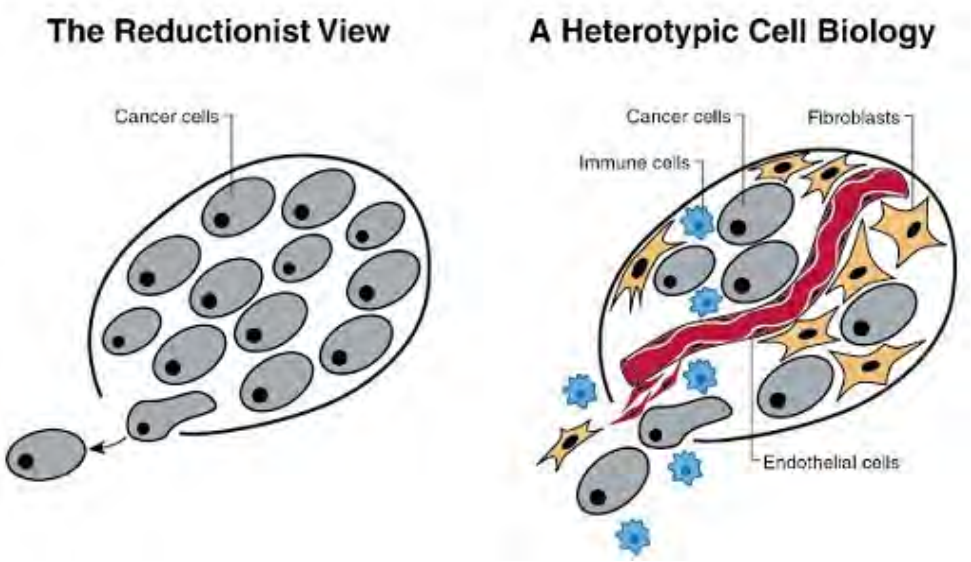


Figure 1.1| Tumor as a complex and heterogeneous organ.
In past decades, the biology of cancer was mainly focused in a reductionist vision that cancer cells and corresponding genes (left panel) were the core of tumor development and progression. Nowadays, tumors are recognized as complex tissues in which mutated cancer cells have may recruit distinct normal cells to be enrolled as active collaborators in tumorigenesis (right panel). The study of the interactions between cancer cells and the surrounding stroma proved to be critical to better understand cancer pathogenesis and to the development of novel therapies (*adapted from Hanahan and Weinberg, 2000* [3]).

1.1.1. Tumor Microenvironment: cancer–stromal cell interactions in tumorigenesis

Tumor stroma is formed by the recruited normal reprogrammed cells. It has been demonstrated that these cells play an essential role in tumorigenesis and actively contribute to the development and expression of certain cancer capabilities [3,6]. Indeed, several hallmarks of cancer are provided by various stromal components, including endothelial cells, pericytes, cancer-associated fibroblasts and different types of inflammatory cells (Figure 1.2).

In physiological conditions, the stroma in healthy individuals is a physical barrier against tumorigenesis. However, mutated cancer cells trigger a cascade of events that substitute and remodel the adjacent microenvironment into a pathological entity. The organization of such transformation implicates migration of stromal cells, remodeling of matrix and expansion of vasculature [7,8].

Alterations in the tumor microenvironment under selective pressures (e.g., acidity and hypoxia) drastically influence its progression, through mutations that ensure survival and proliferation of cancer cells, eventually creating tumor heterogeneity. Moreover, the tumor stroma provides a physical/biological barrier that prevents the effective delivery of anti-cancer

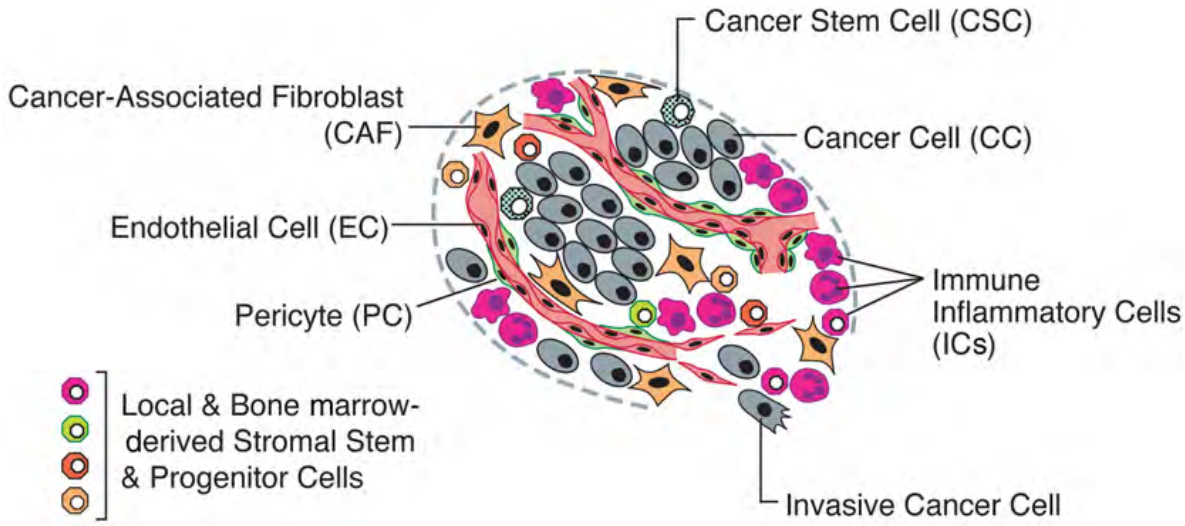


Figure 1.2| The cellular components of the tumor microenvironment.
Several specialized cell types constitute solid tumors. Both the parenchyma (cancer cells) and the stroma contain distinct cell types that compose the tumor microenvironment. These cells actively participate in tumorigenesis, enabling tumor growth, survival and progression (*adapted from Hanahan and Weinberg, 2011* [5]).

drugs to the tumor. Such protection represents a favorable condition to cancer cells escape from programmed cell death and eventually develop acquired resistance, as a preliminary step towards more malignant phenotypes [9,10].

The stroma actively contributes to tumor growth, survival and metastasis by constructing a milieu that supports angiogenesis, migration and invasion [5,6,11-14]. Because of their fundamental role in tumor development and growth, components of the tumor stroma are relevant targets for therapeutic intervention [6,14].

1.1.2. Lung cancer: a dramatic scenario

1.1.2.1. A heterogeneous set of diseases

In 1912, William Osler stated that *primary tumors of the lung are rare* [15]. In 2015, lung cancer accounts for higher mortality rates than any other cancer in both men and women in the United States and worldwide, with over one million deaths occurring yearly [1].

Lung carcinogenesis is a complex multistage process. It starts with morphological changes in the lung epithelium that include hyperplasia, metaplasia, dysplasia and carcinoma *in situ*. All lung carcinomas are aggressive, locally invasive and metastasize for different organs. Genetically, these carcinomas are initiated by amplification/activation of proto-oncogenes, which turn into oncogenes if exposed to carcinogens, and/or the inactivation of tumor suppressor genes [26]. Unfortunately, the majority of lung cancer cases are diagnosed in

advanced stage, when surgery becomes an unviable option.

Lung cancer consists in a set of different and heterogeneous histological types [16-19]. Based on different biological and histological characteristics, lung cancer is subclassified in small-cell lung cancer (SCLC), comprising 15% of cases; adenocarcinoma (ADC) that comprises about 50% of all lung cancers and is actually the most common type; followed by squamous cell carcinoma (SQC), large cell carcinoma (LCLC) and pleomorphic carcinoma (PLMC) [20,21].

Patients with adenocarcinoma often express biomarkers that are consistent with an origin in the terminal respiratory unit, including thyroid transcription factor 1 (TTF1) and cytokeratin 7 (CK7). Contrariwise, squamous cell carcinomas are characterized by metaplasia of the pseudostratified columnar epithelium that lines the trachea and upper airways, followed by squamous differentiation [22]. In the clinic, adenocarcinoma and squamous cell carcinoma are distinguished by immunostaining for cytokeratin 5.6 (CK5.6), transcription factors SRY-box 2 (SOX2) and p63 [21-24]. Large cell carcinoma is diagnosed by absence of adenocarcinoma and squamous morphology, but could express its biomarkers, although with unclear genetic relation with those types [21,22]. Pleomorphic carcinoma is a rare (0.1-0.4% of all pulmonary carcinomas) subtype of sarcomatoid carcinomas of the lung. The diagnostic of this subset of undifferentiated lung cancer is challenging due to its uncommon histopathological features [20,21,25]. Small cell lung cancer corresponds to the more aggressive type of pulmonary carcinomas [26]. Cellular and molecular heterogeneity in lung cancer underlie, at least in part, the different treatment responses in patients with similar clinical stage and histological type.

Despite decades of research, treatment options remain limited in terms of providing a cure or a significant survival benefit. The average 5-year survival rate has not improved greatly over the last 40 years, being cited currently at 17% [27]. This highlights the need for improved therapeutic options, including the identification of novel therapeutic targets as well as predictive markers of treatment response.

1.1.2.2. Lung tumor microenvironment

Neoplastic cells, in the lung as in other organs, are closely associated with the extracellular matrix (ECM), mesenchymal and fusiform cells such as fibroblasts, infiltrating immune cells and tumor blood vessels (Figure 1.3). In lung tumorigenesis, formation of new blood and lymphatic vessels supplies necessary nutrients for tumor growth and allows for an influx of immune cells of the myeloid and lymphoid lineages. The myeloid cells that

are implicated in this process include tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs) [28-30]. Production of pro-angiogenic factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) by TAMs in lung cancer supports the involvement of these cells with increased microvessel formation [31]. In the tumor microenvironment, accumulated myeloid-derived suppressor cells (MDSCs) are thought to promote tumor progression by increasing matrix degradation, tumor cell proliferation, metastasis and angiogenesis [18,28].

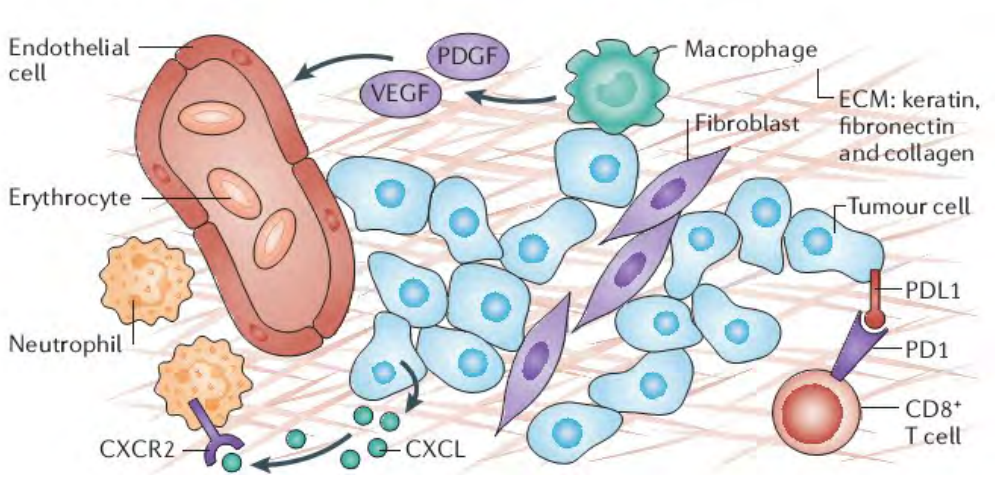


Figure 1.3| Lung tumor microenvironment heterogeneity.
In the microenvironment of lung tumors, extracellular matrix (ECM) is associated with fusiform cells, including cancer-associated fibroblasts (CAFs), giving structural support to tumor cells. Formation of tumor blood vessels depends on endothelial cells recruitment via platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) stimuli. During angiogenesis, several blood cells, including macrophages, neutrophils, T and B lymphocytes, infiltrate into the tumors. Tumors can recruit neutrophils through secretion of CXC-chemokine ligand (CXCL) family members, which bind to the neutrophil receptor CXCR2. Moreover, tumor cells often express immune molecules, such as programmed cell death 1 (PD1) ligand 1 (PDL1), to attenuate a cytotoxic response from T cells against tumor cells (*adapted from Chen et al. 2014* [18]).

1.1.2.3. Changing the paradigm of lung cancer treatment

The treatment of lung cancer involves a multifaceted approach, comprising surgery, chemotherapy and radiotherapy, whose choice is based on the type and stage of the disease (histopathology, disease progression/invasion and patient clinical status). Notwithstanding the limitations of chemotherapy in terms of efficacy and safety, it has been the best option until the approach to cancer treatment changed drastically with the discovery of a new phenomenon: *oncogene addiction* [32]. Despite the diversity of genetic alterations typically observed in cancer, some tumors depend on one single dominant oncogene for growth and survival. Accordingly, the loss of even a single mutated protein cancer cells have come to rely on, can induce massive cell death and prevent disease progression [32,33]. Thus, based on a better understanding of

the biology of lung cancer, targeted therapies have become available to patients, changing the paradigm of lung cancer treatment [34].

Molecular profiling of lung cancer, led to the characterization of some genetic adaptations that could be therapeutically targeted [35]. Mutations on the epidermal growth factor receptor (*EGFR)* and anaplastic lymphoma kinase (*ALK*) gene rearrangements are some of the most characterized ones [36,37]. Consequently, targeted inhibition of EGFR or ALK signaling pathways has been clinically validated for advanced lung cancer, with a number of currently approved drugs, including the tyrosine kinase inhibitors (TKIs) erlotinib (*Tarceva*®) [38], afatinib (*Giotrif*®) [39], gefitinib (*Iressa*®) [40] and crizotinib (*Xalkori*®) [41]. These therapies have improved the progression-free survival and quality of life of patients, as compared with conventional chemotherapy. Despite these relevant advancements, acquired resistance to TKIs may arise after 1 or 2 years [37].

1.1.3. Tumor angiogenesis: an opportunity for targeted therapeutic intervention

Angiogenesis is a multistep process resulting in the generation of new blood vessels from pre-existing ones, being a hallmark of tumor progression [42-45]. The relevance of angiogenesis in tumor growth was initially proposed four decades ago by Judah Folkman. It was hypothesized that tumor growth and survival depended on the formation of a vascular network and its inhibition could be a new form of therapeutic intervention [46].

Tumors arise as an avascular small cluster of host-derived cells that proliferate atypically as they have lost the ability to control their growth [2]. Initially, tumors survive and thrive on vasculature in the surrounding environment of the host to grow until around 1-2 mm in diameter [47]. At that point, hypoxia and nutrient deprivation within the tumor microenvironment, trigger the requirement for development of a tumor vascular network to supply their needs, in a mechanism that closely resembles normal angiogenesis [48,49].

In adults, as part of physiologic normal processes such as female reproductive cycling or wound healing, angiogenesis is activated, but just transiently. Instead, during tumorigenesis some cells within the tumor change to a persistent angiogenic phenotype - *angiogenic switch* [49]. Tumors recruit normal surrounding vasculature to continually sprout new blood vessels, thus satisfying the nutrient needs arising from tumor progression [49,50]. Cancer cells exploit their microenvironment by releasing cytokines and growth factors to activate normal/mesenchymal, quiescent cells around them and initiate a cascade of events (*e.g.,* the released of

VEGF that stimulates the sprouting and proliferation of endothelial cells) that quickly becomes dysregulated – *tumor angiogenesis* [5,42,47,51] (Figure 1.4).

The switch to an angiogenic phenotype is maintained by a change in the equilibrium

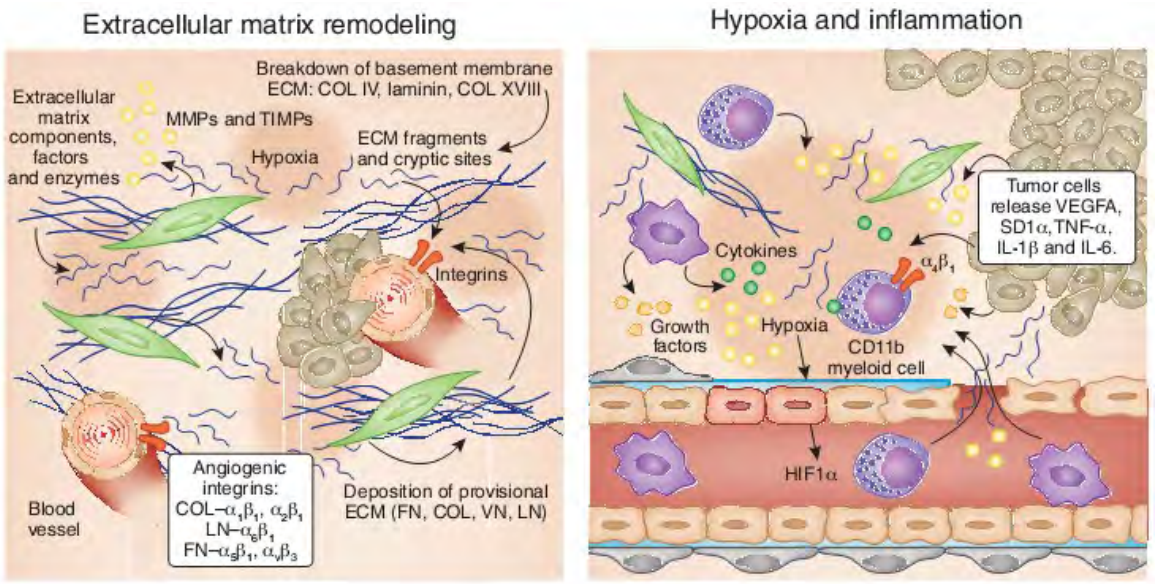


Figure 1.4| Tumor microenvironment as an active driver in angiogenesis.
Once a tumor lesion exceeds a 1-2 mm in diameter, hypoxia and nutrient deprivation triggers an angiogenic switch to allow tumor growth. Cancer cells exploit their microenvironment by releasing several growth factors that initiate the sprouting and proliferation of formerly quiescent endothelial cells. Tumor angiogenesis initiate with the extracellular matrix (ECM) and basement membrane breakdown, followed by endothelial cells proliferation, migration and finally formation of a new blood vessel. Most tumors elicit an inflammatory response that attracts myeloid cells into the tumor microenvironment to increase the angiogenic response. (*adapted from Weis & Cherech, 2011* [47]).

between pro- and anti-angiogenic regulators secreted either by cancer cells or by stromal cells of the tumor microenvironment [49,50]. Some of these angiogenic factors are signaling proteins that bind to stimulatory or inhibitory surface receptors displayed by vascular endothelial cells.

The major mediator of physiological and pathological angiogenesis is the vascular endothelial growth factor A (VEGF-A or VEGF). VEGF signals mainly through VEGF receptor 2 (VEGFR-2), which is overexpressed by endothelial cells engaged in tumor angiogenesis and by circulating bone marrow–derived endothelial progenitor cells. The majority of human cancer cells express VEGF and is often associated with an unfavorable prognosis [52]. This is likely the consequence of VEGF activation, upon activation of oncogenes or inactivation of tumor-suppressor genes, hypoxia and acidic pH at the tumor level, or the presence of specific pro-inflammatory cytokines and/or growth factors [53-55]. Moreover, it is well known that VEGF can be secreted, in levels sufficient to drive tumor angiogenesis, from several host cells

such as platelets and muscle cells, as well as tumor-associated stromal cells [56,57]. This could explain, in part, the reason why circulating VEGF is not a good biomarker for efficacy, within the context of an anti-angiogenic therapy [58,59].

Additionally to VEGF, fibroblast growth factor (FGF)-1 and -2 [60], platelet-derived growth factor (PDGF)-B and C [61,62], epidermal growth factor (EGF) [63], transforming growth factor (TGF)-β [64], matrix metalloproteinases (MMPs) [65], tumor necrosis factor (TNF) [66] and angiopoietin (Ang)-2 [11] are also implicated in sustaining tumor angiogenesis [43].

In contrast to the activities of pro-angiogenic factors, thrombospondin (TSP)-1, an extracellular matrix glycoprotein, is a well-recognized endogenous angiogenesis inhibitor. It also binds transmembrane receptors displayed by endothelial cells thereby triggering suppressive signals that can counterbalance the angiogenic stimuli [67]. Other examples of anti-angiogenic factors are: endostatin [68], a proteolytic cleavage product of collagen XVII, canstatin [69] and tumstatin [70], two proteolytic fragments of collagen IV, and soluble factors like interferon-α and -β (IFN-α and -β) [71] and angiostatin [72], a cleavage product of plasmin. Inhibitors and angiogenic factors can be derived from both cancer cells themselves and from tumor-stromal cells. Thus, the *angiogenic switch*, as a change in the balance between pro- and anti-angiogenic factors, is an intrinsic event of multistage tumorigenesis.

An important parameter that could be determinant of the overall efficacy of anti-angiogenic therapy is the typically aberrant nature of tumor blood vessels [73]. They are distinct in several aspects relative to normal vasculature, as they present an irregular, disorganized and tortuous architecture, sprout chaotically and their spatial distribution is significantly heterogeneous, resulting in an uneven tumor drug distribution [74,75]. Functional pericytes are often absent from the tumor blood vessels or they are abnormal since they may not be in close association with endothelial cells, leaving an incomplete basement membrane. This promotes the formation of leaky and dilated vessels [76,77].

The tumor vessel abnormalities create a microenvironment characterized by hypoxia, low pH and high fluid pressure, which can select for more malignant cancer cells as well as enable their escape. [73,78]. The abnormal structure and function of the tumor vasculature underlie, in part, some of the fundamental hallmarks of cancer [5] and even challenges of anti-cancer therapies [79-81].

1.1.3.1. Angiogenesis in lung cancer

Some angiogenic factors are directly correlated with the development and prognosis of lung cancer [16,82,83].

Decades of research on the anti- and pro-angiogenic proteins at the tumor level has validated the hypothesis of tumor angiogenesis inhibition as a valuable cancer therapy [43,84]. The development of new blood vessels is a hallmark of cancer development that has long been considered an attractive therapeutic target. The signaling molecule VEGF plays a central role in angiogenesis and is frequently highly expressed in human cancers. Thus, clinical efforts to develop anti-angiogenic therapies have largely focused on inhibiting VEGF/Rs pathways. VEGF has gain further relevance as a therapeutic target in lung cancer with the clinical approval of bevacizumab, an anti-VEGF monoclonal antibody [92].

1.1.3.2. Blocking the tumor needs: targeting angiogenesis

The discovery of several hallmark principles in tumorigenesis has changed the paradigm of cancer treatment towards a more personalized medicine.

Targeted therapies rely on the inhibition of molecular targets that impair tumor growth and progression.

Since angiogenesis is a phenomenon critical to primary tumor growth and metastization, it was hypothesized that inhibition of tumor angiogenesis would be a highly promising tumor-specific therapeutic strategy to treat human cancer [46,55]. Endothelial cells of the tumor-associated vasculature are easily accessible to drugs administered intravenously, and have higher genetic stability than cancer cells, thus presenting lower risks of relapse and resistance to therapy [43,85]. From that time on, thorough research on this subject has led to the identification of specific therapeutic targets and development of a novel class of cancer treatment – *anti-angiogenic therapy* [84,86-91]. This therapy offers a great promise and have been often used to treat different types of cancer, either alone or in combination with chemotherapy or with other targeted therapies [87].

Owing to its central role in promoting angiogenesis, VEGF has become the major anti-angiogenic target, with approval by the Food and Drug Administration (FDA) of several VEGF inhibitors for human use [87].

In 2004, FDA approved the first generation of anti-angiogenic drug for human use – bevacizumab (*Avastin*®) [89], a humanized anti-VEGF monoclonal antibody, for the treatment

of metastatic colorectal cancer in combination with chemotherapy regimens [92]. After that, bevacizumab was also approved as a first-line treatment for other cancer types such as metastatic breast cancer (which has been suspended in the meantime) [93,94], advanced or metastatic non-squamous lung cancer [95,96], metastatic renal cell carcinoma [97] and, more recently, as a second-line therapy in glioblastoma multiforme [98].

In 2012, an engineered recombinant fusion protein (VEGF-trap), aflibercept (*Zaltrap*®) that binds VEGF as well as anti-placental growth factor (PlGF), was also approved in combination with chemotherapy for the treatment of patients with metastatic colorectal cancer [99].

Monotherapy with second generation anti-angiogenic broad-spectrum small-molecule receptor TKIs, was also approved for several types of cancer. This included sunitinib (*Sutent*®) [100], sorafenib (*Nexavar*®) [101,102], pazopanib (*Votrient*®) [103,104] and vandetanib (*Zactima*®) [105].

Anti-VEGF drugs inhibit tumor blood vessel sprouting by blocking vascular branching or inhibiting homing of bone morrow-derived cells (BMDCs). These agents also induce regression of pre-existing tumor blood vessels and sensitize endothelial cells to the effects of chemo- and radiotherapy by depriving them of VEGF survival activity. Moreover, these drugs enable the normalization of abnormal tumor blood vessels by pruning immature pericyte-uncovered vessels and by providing maturation into more functional tumor vascular network [85,106,107]. This results in a better tumor penetration of chemotherapy thus justifying its improved efficacy when with the combined administration of bevacizumab [73].

1.1.3.3. From bench to bedside: lessons from translating anti-angiogenic therapies into the clinic

Currently, hundreds of thousands of cancer patients worldwide are being treated with anti-angiogenic therapies. Nevertheless, translating the preclinical successes into clinical practice, identifying the optimal clinical indications, and maximizing the efficacy of anti-angiogenic therapy for cancer patients have been more challenging than anticipated. Although effective at reducing angiogenesis, these drugs have not generated enduring clinical benefits in many patients and clinical experience shows that most advanced cancers can escape from therapy, in particular through intrinsic refractoriness and acquired evasive escape [108,109].

Anti-angiogenic therapy prolongs the survival of patients with certain types of tumors by months, but it fails to induce a survival benefit in others [110]. In the clinic, bevacizumab has demonstrated better efficacy when combined with chemotherapy or cytokine therapy,

while TKIs has revealed to be more effective as monotherapy. A large number of trials have demonstrated a transient stabilization of the disease with tumor regression and an extended progression-free survival (PFS), but failed in improving overall survival (OS) [93,109,111].

The relative inefficacy of anti-angiogenic therapies in oncological practice has led to an intense research into the mechanisms underlying resistance [112-118]. Patients can be intrinsically refractory and never respond to therapy, or develop evasive resistance during the course of treatment [108]. Several mechanisms have been proposed to explain these phenomena, which are related to changes in cancer cells, endothelial cells or other stromal cells [119,120] (Figure 1.5), summarized as follows:

a) Tumor angiogenesis can become VEGF-independent at a more advanced stage due to the production of other pro-angiogenic molecules (activating redundant signaling pathways), and thus become less sensitive to VEGF blockade [53]. Hypoxia induced by vessel regression after anti-VEGF treatment can also switch to a more invasive and metastatic program, whereas in other cases, cancer cells or, a special set of cancer cells – cancer stem cells (CSCs), can become hypoxia-resistant and survive in a poor oxygen environment [121].

b) VEGF blockade inhibits sprouting angiogenesis, but may not be as efficient in suppressing other modes of tumor vascularization, relying on the recruitment of BMDCs, such as vessel co-option, vasculogenic mimicry or vessel splitting. Vessel pruning by VEGF blockade can increase hypoxia, resulting in the upregulation of other angiogenic factors such as PlGF, FGFs and chemokines, and this can ensure tumor vascularization [108]. Evidences demonstrated that some tumor endothelial cells present cytogenetic abnormalities and stem cell potential [122], which would ultimately impair the inhibition of VEGF pathway.

c) In addition, tumor blood vessels often do not present pericytes-coverage, but upon anti-VEGF therapy some microvessels in the tumor acquire a mature morphology with a complete pericytes-coverage and a thick basement membrane. Such mature vessels are usually less sensitive to VEGF inhibition [123].

d) Other stromal cells contribute to the resistance to VEGF inhibition. For example, hypoxia triggers the recruitment of BMDCs, including TAMs, CAFs, TANs, mast cells and $CD11b^{+}GR\text{-}1^{+}$ myeloid-derived suppressor cells, which release pro-angiogenic signals such as VEGF and MMPs [53] that can revascularize tumors - *vasculogenic rebound* [85,124].

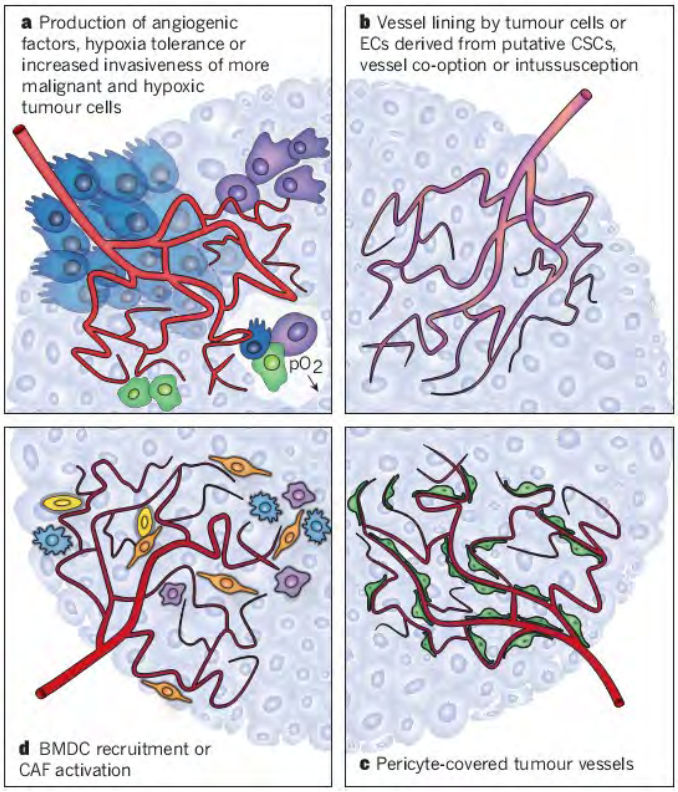


Figure 1.5| Different mechanisms underlying resistance associated with VEGF inhibition in cancer.
a. VEGF inhibition could promote: hypoxia, which upregulates the secretion of additional angiogenic factors to continue stimulating angiogenesis, tumor cell invasiveness, or even, selection of hypoxia tolerant cancer (stem) cells. This could ultimately increase tumor recurrence and aggressiveness. **b.** Activation of additional modes of tumor vascularization as intussusception, vasculogenic mimicry, differentiation of putative cancer stem cells (CSCs) into endothelial cells (ECs) or vasculogenic vessel growth and vessel co-option, might give rise to tumor blood vessels less sensitive to VEGF blockade. **c.** Tumor vessels covered by pericytes (green) are more resistant to anti-VEGF therapy. **d.** Recruited pro-angiogenic BMDCs (yellow), macrophages (blue and purple) or activated cancer-associated fibroblasts (CAFs, orange) secret pro-angiogenic factors, which rescue tumor angiogenesis (*adapted from Carmeliet and Jain, 2011* [43]).

These are only a few examples of several mechanisms of resistance associated with the use of this novel class of anti-cancer drugs, which can vary even among different types of cancer. Notwithstanding these clinical setbacks, anti-angiogenic therapy remains a major class of cancer drugs. Accordingly, it become evident that identification of alternative targets within the tumor vasculature could be highly relevant to the improvement of these therapies.

1.1.3.4. Stroma-mediated angiogenic resistance in lung cancer

It is well established that tumors present an enormous plasticity and adaptive capabilities to conventional chemo- or radiotherapy, and these features also contribute to resistance against targeted therapies, such as anti-angiogenic therapies [108,125].

Cascone *et al.* [116] investigated the stromal adaptation and resistance to bevacizumab therapy in xenograft models of lung adenocarcinoma representing different types of resistance. In animals bearing A549 tumors (a model of intrinsic resistance), while therapy with bevacizumab did not alter tumor growth, it induced a disorganized sprouting revascularization, leading to a more tortuous vasculature with lower pericytes coverage. In this model, the

endothelial upregulation of p-EGFR suggested that a possible mechanism of intrinsic resistance could be associated with a switch to EGFR-mediated endothelial proliferation and angiogenesis (Figure 1.6).

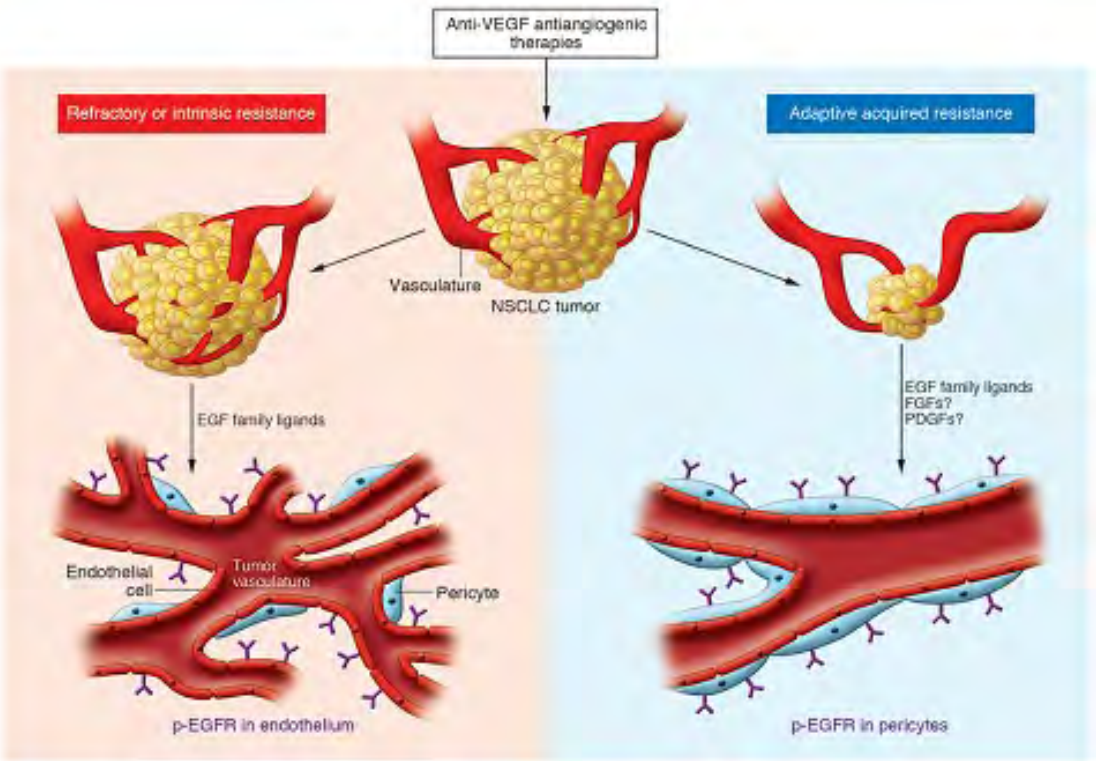


Figure 1.6| Mechanisms of stroma-mediated resistance in non-small cell lung cancer (NSCLC). This diagram represents the mechanisms of refractory (intrinsic; left side of the figure) or acquired (right side of the figure) resistance to bevacizumab therapy in NSCLC models. In the intrinsic model, anti-VEGF therapy does not alter tumor growth, but induces morphologic alterations, such as disorganized sprouting revascularization that leads to a more tortuous vasculature with lower pericyte coverage, as well as endothelial upregulation of p-EGFR in treated tumors. In the acquired resistance models, tumors initially respond to anti-VEGF therapy, but quickly acquire resistance with efficient revascularization following a pattern of pericyte-covered normalized vasculature and, also an increase in p-EGFR on pericyte cells. Differences in the repertoire of pro-angiogenic factors expressed in each of these models drive the mechanisms of stromal resistance to anti-angiogenic therapies (*adapted from Casanovas et al., 2011* [120]).

On the other hand, in animals bearing H1975 or H441 tumors (models of acquired resistance), after an initial period of response to bevacizumab therapy, with decrease of tumor size, tumors became insensitive. During the resistance period, an intense revascularization with a pattern of normalized vessels covered by pericytes and increased expression of activated EGFR on perivascular cells was observed (Figure 1.6). Moreover, combined inhibition of both VEGFR and EGFR pathways extended progression-free survival, as compared with monotherapy in both models.

These findings supported the hypothesis that EGFR signaling in pericytes was implicated in the resistance to VEGF/R blockade in lung adenocarcinoma, thus suggesting that dual targeting may delay resistance and improve therapeutic efficacy. In addition, it has also reinforced the idea that targeting multiple stromal pathways in lung cancer patients would translate into more durable therapeutic benefits in clinic [116,120].

Despite important recent advances on the understanding of anti-angiogenic resistance mechanisms, it is evident that angiogenesis inhibitors currently approved for clinical use are not providing long-term efficacy. They represent an important class of anti-cancer agents, but the current treatment response is transient, and more research is therefore required to improve the clinical outcome of lung cancer patients.

1.2. Nanomedicine: improving cancer therapy

1.2.1. Advanced drug delivery for cancer therapy: from tumor microenvironment heterogeneity to nanotechnology

1.2.1.1. PEGylated Liposomes

From years of research on nanotechnology-based drug delivery, it has been recognized that prolonged blood circulation times translate to an increased tumor accumulation of the encapsulated drug [126]. This has been achieved by the engraftment of poly(ethylene) glycol (PEG) on the surface of nanoparticles, thus decreasing the extent of opsonisation and subsequent blood clearance by the mononuclear phagocyte system (MPS) [127,128]. PEGylated liposomal doxorubicin (*Doxil*®, or *Caelyx*® in Europe [129]), is the first example of a pegylated nanotechnology approved for clinical use in oncology. It is currently approved for the treatment of AIDS-related Kaposi's sarcoma, multiple myeloma and recurrent ovarian cancer in North America, Europe, and other countries, and for metastatic breast cancer in Europe. PEGylated liposomal doxorubicin has significantly decreased the cardiotoxicity associated with the free drug, both in pre-clinical [127] and in clinical studies [130,131].

1.2.1.1.1. Enhanced Permeability and Retention Effect: enabling passive tumor targeting

The dysfunctional and hyperpermeable tumor vasculature is a key factor for the activity demonstrated by pegylated liposomal formulations of chemotherapeutic agents in animal models. The fenestrations of the leaky tumor blood vessels is estimated to have an average pore size of 100–600 nm [132] that, combined with poor tumor lymphatic drainage, originates the *enhanced permeability and retention* (EPR) effect (Figure 1.7). Liposomes with a mean size of approximately 65–90 nm [133,134] are small enough to passively infiltrate the tumor endothelium, but large enough to be excluded from normal endothelium. As such the described features of the EPR effect tend to favour the tumor extravasation and retention of nanoparticles with prolonged blood circulation half-lives.

1.2.1.1.2. EPR-mediated passive targeting limitations

Nano-therapies based on the EPR effect have represented significant benefits for patients, mainly by reducing free drug-associated side effects. However, passively targeted liposomal drug delivery systems have also presented their own toxicity profile (*e.g.*, the dose-dependent Palmar-Plantar Erythrodysthesia syndrome) [135].

In addition, the heterogeneity of the tumor microenvironment, and thus the extent at which the EPR takes place between tumors of different histological types, or even within the same type, along with several other factors influencing it, namely tumor size and stage, represent a major barrier to the activity of nanomedicines [136].

1.2.1.2. Controlling drug release: improving intracellular bioavailability

The development of strategies to increase pH-dependent intracellular delivery of biologically active compounds-containing liposomes has concentrated many efforts [137,138]. pH-sensitive liposomes have been engineered in such way that they are stable at physiological pH (7.4), but undergo destabilization and acquire fusogenic properties under acidic conditions, following receptor-mediated endocytosis, thus releasing the encapsulated payload [139]. Several studies have reported applications of pH-sensitive liposomes for the transport and intracellular delivery of agents for cancer treatment [137,140].

Notwithstanding, to date no pH-sensitive formulation has reached clinical trials.

Behind this, could be the fact that pH-sensitive liposomes present inferior blood circulation profiles as well as lower drug retention capacity relative to non-pH sensitive liposomes [141]. Nonetheless, the hidden potential of this type of liposomes could be revealed in case further modifications were introduced, namely upon covalent attachment, at the end of PEG-grafted pH-sensitive liposomes, of internalizing ligands targeting molecular markers overexpressed on the readily accessible tumor vasculature (besides cancer cells) [133,134,140,142] (Figure 1.7).

1.2.1.3. Receptor-mediated targeting: improving specificity

Ligand-mediated targeting of liposomes can significantly increase the amount of drug delivered into the target cell relative, to free drug or non- targeted liposomes. The selective cellular binding and receptor-mediated endocytosis enabled by this strategy can result in improved therapeutic outcomes with enhanced efficacy and reduced systemic toxicity [126,143-145]. In this type of strategy, the selection of the target receptor (or antigen) is one of the most relevant. Ideally, it should be expressed homogenously and at high levels by the tumor cell population, along with low expression in healthy tissues, besides being internalizing upon binding of a specific ligand [143,145].

In this targeting approach, at the tumor microenvironment level, various cellular targets can be distinguished, including cancer cells, tumor endothelial cells and other cellular components of the tumor stroma (Figure 1.7) [146,147]. Tumors are complex organs and their progression and survival depends on the interaction of cancer cells with the surrounding microenvironment, namely on the support of different processes, such as the development of a vascular network [148]. Therefore, selecting more than one target from the pool of tumor-stroma interactions can increase the benefit of therapeutic approaches [140,147,149,150].

1.2.2. Nanoparticle-based drug delivery in lung cancer

Cisplatin has been the first treatment option for patients with lung cancer (ADC, SQC and LCLC) for the last two decades, being associated with the adverse side-effects in 20% of patients receiving high doses [151]. In 2004, Boulikas developed a cisplatin-loaded liposome designed *Lipoplatin*™, to reduce systemic toxicity of cisplatin [152]. *Lipoplatin*™ has successfully completed a Phase III clinical trial for lung cancer. The study compared response rates and toxicities of patients treated with *Lipoplatin*™ plus paclitaxel versus cisplatin plus paclitaxel, as a first line treatment for non-squamous lung cancer. The results established the

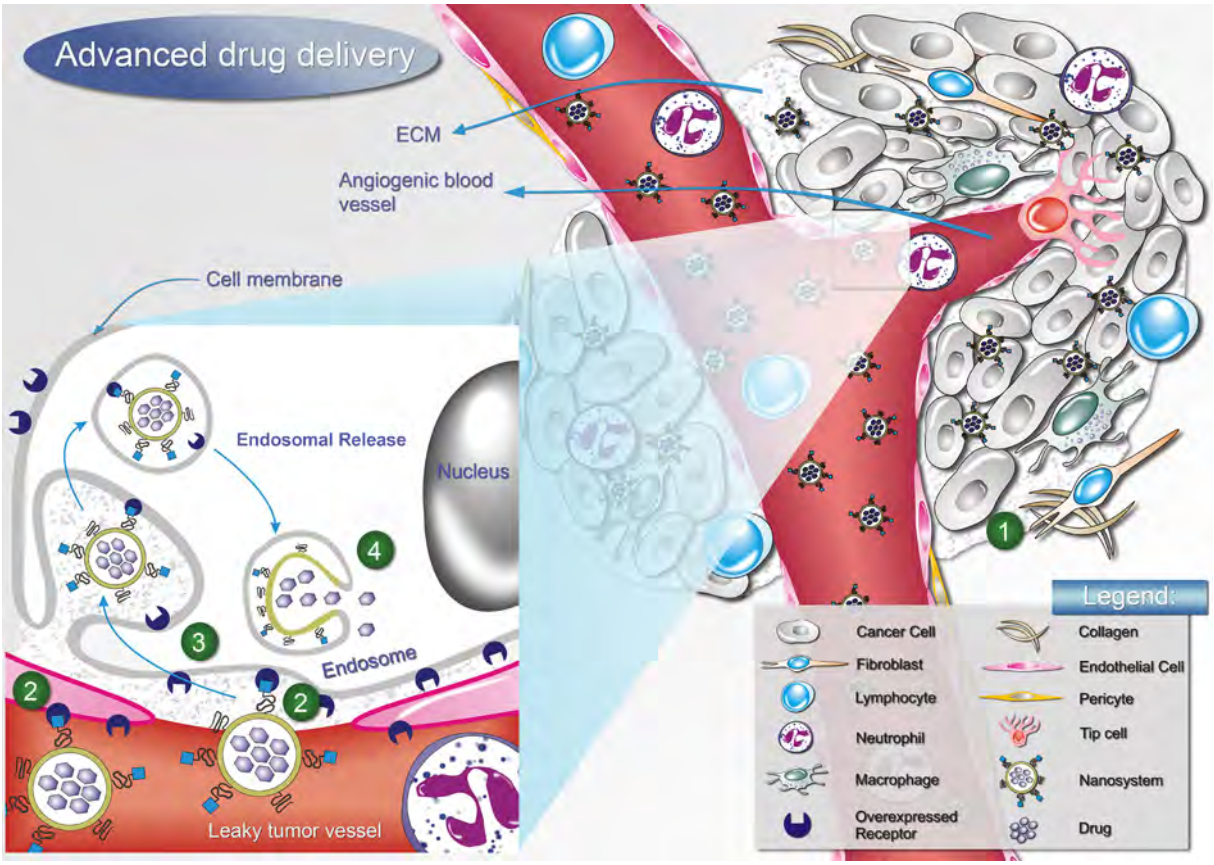


Figure 1.7| Exploiting the tumor microenvironment: taking advantage of advanced drug delivery systems.

Passive Targeting (1). Upon systemic administration, the long circulating nanoparticles accumulate at the tumor site mainly due to the enhanced permeability and retention (EPR) effect. **Receptor-mediated Targeting (2).** This type of targeting that use different moieties to recognize overexpressed receptors on the surface of tumor cells represents an approach with great potential to increase specificity and efficacy while decrease off-target toxicity. **Nano-system internalization (3).** To allow a successful intracellular delivery of the payload, nano-systems should be functionalized with a targeting moiety able to promote ligand-specific internalization in order to increase the payload delivered into the target cells. **Endosomal escape (4).** Cell internalization per se, does not guarantee the increased efficacy. The system should be engineered to enable drug release from the endosomal compartment, therefore increasing drug intracellular bioavailability, while avoiding or limiting **drug degradation in the lysosomes (5)** (*adapted from Fonseca et al., 2014* [146]).

increased efficacy and safety of *Lipoplatin*™ compared to cisplatin [153].

Paclitaxel, another chemotherapeutic drug widely used in the treatment of lung cancer, was formulated with *Cremophore EL*® to enhance its solubility in physiological fluids. However, side-effects associated with paclitaxel, such as hypersensitivity reactions, myelosuppression and peripheral neuropathy were worsen by *Cremophor EL*® [154]. Nanoparticle albumin-bound paclitaxel ([nab-paclitaxel] *Abraxane*® ABI-007) is a new formulation of paclitaxel that obviates the need for *Cremophor EL*®, resulting in a safer and faster infusion without requiring the use of pre-medication with corticosteroids to avoid hypersensitivity.

Abraxane® was the first nano-drug approved by the FDA for the first-line treatment of

locally advanced or metastatic lung cancer, in combination with carboplatin, in patients who are not candidates for curative surgery or radiation therapy. The approval was based upon the results of CA-031, a phase III, multi-center, randomized open-label study where patients with advanced lung cancer were weekly treated, every three weeks, either with *Abraxane*® plus carboplatin or paclitaxel plus carboplatin. The study met its primary end-point, demonstrating a statistically significantly higher overall response rate for patients in the *Abraxane*® arm compared to those in the paclitaxel arm (33% vs 25%). In respect to the activity as a function of the type of lung cancer, *Abraxane*® demonstrated a higher overall response rate as compared to paclitaxel in squamous cell carcinoma (41% *vs* 24%) and large cell carcinoma (33% *vs* 15%), and a similar overall response rate in patients with adenocarcinoma (26% *vs* 27%) [155].

Doxil® has been also widely investigated for the treatment of lung cancer [156-158]. A phase I study demonstrated *Doxil*®´s activity as a single agent in advanced or metastatic lung cancer in patients who progressed after a platinum-based first-line chemotherapy. Seventeen patients were enrolled in this study and *Doxil*® was administered at the dose of 35-45 mg/m^2 every 21 days. One partial response was confirmed (5.8%), five patients (29.4%) had stable disease and nine (52.9%) had disease progression. Median time to progression was 9.5 weeks and median survival was of 18.6 weeks. Data indicated that *Doxil*®, at the doses used in this study, could be safely administered and demonstrated activity in platinum pre-treated lung cancer patients [156].

1.2.3. Nucleolin: the pursuit of personalized therapy in lung cancer

The identification and validation of targets for cancer therapy is a relevant step in drug development. Nucleolin is an ubiquitous non-histone protein, comprising three structural domains. The N-terminal domain contains both acidic regions and multiple phosphorylation sites and regulates transcription and the maturation of ribosomal RNA; the central domain contains four RNA-binding domains and associates with mRNA or pre-ribosomal RNA to regulate their stability; the C-terminal is rich in glycine, arginine and phenylalanine residues and interacts with ribosomal proteins to regulate RNA translation [159].

Initially, nucleolin was thought to be simply a RNA-binding protein involved in the organization of nucleolar chromatin, packaging of pre-RNA, rDNA transcription and ribosome assembly [159]. Recent findings have revealed that nucleolin is also implicated in modulating transcriptional process, cytokinesis, nucleogenesis, signal transduction, apoptosis, induction of chromatin decondensation, and replication [160]. Accumulating evidence indicates that

nucleolin in the nucleolus can shuttle to the cytoplasm and membrane, in a temperature-dependent manner [161,162]. Although more than 90% of nucleolin is present in the nucleus, it has been identified at the cell surface of various cell types in its phosphorylated form [163]. The half-life of nuclear nucleolin is more than 8 h, while that of cell surface nucleolin is approximately 45 min [164].

Recently, nucleolin expression was observed on the surface of endothelial cells in angiogenic tumor blood vessels[165]. In the absence of cell surface nucleolin, the endothelial cells partially lost their motility and failed to form tubule structures, thus suggesting its involvement in tumor angiogenesis [166]. Importantly, expression of surface nucleolin is constantly induced in cancer and endothelial cells, independently of nuclear expression [164]. As important, no mutated forms of nucleolin nor the its involvement in any mechanism of drug resistance have been revealed so far.

Nucleolin is the receptor for the 31-aminoacid F3 peptide, a fragment of a high mobility group protein 2, HMGN2 [167]. This peptide was discovered in a screening procedure that used a phage-display cDNA library and combined *ex vivo* screening on cell suspensions prepared from mouse bone marrow and *in vivo* screening for tumor homing. The potential of the F3 peptide as a targeting ligand was reinforced by its internalization upon binding to nucleolin-overexpression cells [165].

1.2.3.1. A new nanotechnology-based strategy targeting nucleolin in the tumor microenvironment: *Pegasemp* ™

In our laboratory, Moura *et al.* [140] evaluated the therapeutic impact of targeting the nucleolin receptor overexpressed on the surface of two different populations within the tumor microenvironment (cancer cells and endothelial cells of tumor blood vessels) of breast cancer models, using F3 peptide-targeted sterically-stabilized pH-sensitive liposomes containing doxorubicin (Patent n°. US 8,231,895 B2, 2012). It demonstrated that the nanoparticle targeted the nucleolin receptor on a cell- and ligand-specific manner, with the ability to promote intracellular triggered drug release.

Their findings demonstrated that the combined targeting of cancer cells and tumor vasculature, along with intracellular triggered release of the encapsulated drug was crucial for the successful therapeutic outcome. This was the first study, according to the authors' knowledge, in which a pH-sensitive nanoparticle was able to combine stability in the blood stream, increased retention of the encapsulated drug and dual targeting to a solid tumor, with

proven therapeutic efficacy [140]. One of the future challenges will be to investigate, in a preclinical setting, the pharmacodynamics of this strategy against tumors of diverse histological origin, in particular lung cancer.

1.3. Objectives of the work

Tumor vasculature represents a readily-accessible entry into solid tumors.

However, available anti-angiogenic therapies are still associated with limited impact on enhancing overall survival, whether by lack of specificity or by development of resistance mechanisms, arising from tumor heterogeneity and plasticity. Such therapeutic limitations claim for novel molecular markers in the tumor vasculature. In this respect, nucleolin could be one of such alternative targets, with the advantage of being overexpressed on cancer and endothelial cells (as well as cancer stem cells) of the tumor microenvironment. So, nucleolin provides a relevant opportunity of targeting solid tumors at different cellular levels.

Accordingly, nanotechnology-based strategies can be easily tailored according to the biology of the disease/tumor to be targeted. The covalent coupling of internalizing ligands at the end of PEG-grafted liposomes is a strategy that, upon targeting a receptor overexpressed in different cellular components of the tumor microenvironment, could be a good match to the challenges posed by the aforementioned tumor heterogeneity and plasticity.

In the context of the current state-of-the-art, and taking advantage of the versatility of nanomedicine-based approaches, this work aimed at:

- assessing the potential of nucleolin as a therapeutic target in bevacizumab-resistant human lung cancer cell lines and in human pulmonary carcinoma specimens, using nucleolin-binding F3 peptide-targeted liposomes, containing doxorubicin;
- characterizing the sub- and intercellular expression of nucleolin in the tumor microenvironment (including stromal vasculature) in patient-derived pulmonary carcinomas, as well as in lung cancer subcutaneous xenografts;
- investigating whether expression of extra-nuclear nucleolin, in cancer and endothelial cells, correlated with clinicopathological features, as well as with the expression of relevant angiogenic markers (VEGFR2, PDGFRα, FGFR2, HIF-1α, IL-17 and CD133) implicated in lung carcinogenesis;
- and ultimately, characterizing the expression of nucleolin in the tumor microenvironment of human bone metastatic specimens with different primary origins, including lung.

Chapter 2

NUCLEOLIN MEDIATES TARGETED INTRACELLULAR DELIVERY IN LUNG CANCER CELLS AND PRESENTS A MISMATCHED OVEREXPRESSION BETWEEN PATIENT-DERIVED CARCINOMAS AND SUBCUTANEOUS XENOGRAFTS

ABSTRACT

Life expectancy of lung cancer patients remains poor with a 5-year survival rate around 17%, thus highlighting the need for novel therapeutic strategies. Anti-angiogenic approaches, as bevacizumab, have become a standard treatment option in some histological types of lung cancer. However, a substantial number of patients are intrinsically refractory to anti-VEGF therapies or become refractory and relapse. Accordingly, the identification of alternative targets within the tumor vasculature is highly relevant. This work aims at validating nucleolin as a therapeutic target in bevacizumab-resistant lung cancer cells and in the human lung tumor microenvironment of patients-derived samples, including vasculature.

Nucleolin demonstrated to be a functionally active target in lung cancer cells, as it enabled binding and internalization of liposomes targeted by the nucleolin-binding F3 peptide, in a ligand-specific manner and in an extent 34- to 170-fold higher than the one observed for the non-targeted counterparts. This was followed by the cytosolic delivery of the encapsulated payload, thus preventing drug co-localization with lysosomes. This led to a maximum of 19-fold increase in doxorubicin cytotoxicity delivered by F3 peptide-targeted liposomes, for incubation times as short as 1 h, relative to the one delivered by the tested control liposomes, and regardless the nature of bevacizumab resistance. The potential of nucleolin as a therapeutic target in lung cancer has been further supported by its overexpression in several cell populations of the tumor microenvironment of patient-derived pulmonary carcinomas, in a tumor-specific manner. This was reinforced by the significant extent of association of F3 peptide-targeted liposomes with human pulmonary carcinoma tissues as demonstrated by autoradiography. Nevertheless, nucleolin expression and vascular density in subcutaneous pulmonary adenocarcinoma xenografts, derived from the tested bevacizumab-resistant cell lines, were markedly lower than the one observed in human pulmonary carcinomas.

Overall, data herein presented reinforced the translational potential of nucleolin-based targeted strategies in lung cancer.

1.1. Introduction

Lung cancer is the most frequent cause of cancer-related death worldwide for both men and women [168,169]. Adenocarcinoma is the most common histological type of all pulmonary carcinomas, followed by squamous cell carcinoma, large cell carcinoma, adenosquamous carcinoma and pleomorphic carcinoma [20,21]. The average 5-year survival rate has not improved greatly over the last 40 years, hovering at 17% [27], highlighting the need for novel therapeutic strategies. The limitations associated with chemotherapy, such as high levels of systemic toxicity related with poor specificity to the tumor site [170], have urged the development of targeted therapies, as those against vascular endothelial growth factor (VEGF) [34,171,172]. As a consequence, anti-angiogenic therapy has gained an enormous attention [91]. Endothelial cells of the tumor-associated vasculature are easily accessible to drugs administered intravenously, and have greater genetic stability than neoplastic cells, thus evidencing lower risks of relapse and resistance to therapy [43,85]. Drugs that inhibit the VEGF signaling pathway enabled the clinical development of several angiogenesis inhibitors (e.g., the humanized monoclonal antibody bevacizumab or, small molecules like sorafenib or sunitinib) for the treatment of tumors of diverse histological origins, including lung cancer [85,95,96,173].

To date, bevacizumab is the only anti-angiogenic agent approved for the first-line treatment of non-squamous lung cancer and has become a standard treatment option [96]. Notwithstanding some clinical successes with VEGF inhibitors, a substantial number of cancer patients are intrinsically refractory to anti-VEGF therapies or, following initial response, they ultimately become refractory and relapse [81,108,174]. In this respect, several host factors and stromal components play an important role in resistance to angiogenesis inhibitors [125,174,175]. Cascone and colleagues [174] using different models of human lung adenocarcinoma with varying de novo responsiveness to bevacizumab demonstrated that changes of gene expression associated with resistance occurred primarily in stromal cells, highlighting different modes of vascular remodeling that might sustain the emergence of resistance. Accordingly, the identification of alternative targets, and therapeutic strategies, within the tumor vasculature are of great interest.

Accumulating evidence validates cell surface nucleolin as a highly attractive protein for anti-cancer therapies targeting the tumor microenvironment, as it is overexpressed in cancer cells as well as endothelial cells from tumor blood vessels [176]. In an orthotopic model of triple-negative breast cancer, lipid-based nanoparticles functionalized with the nucleolin-

binding F3 peptide [167], and containing a chemotherapeutic drug (doxorubicin) with anti-angiogenic properties [177], enabled a significant decrease of the viable rim tumor area as well as vascular density, with suppression of tumor invasion [140].

Building on the current state-of-the-art, this work aims at validating nucleolin as a therapeutic target for F3 peptide-targeted liposomes, containing doxorubicin, in bevacizumab-resistant human lung cancer cell lines, as well as characterize the protein expression in the corresponding subcutaneous xenografts and in patient-derived pulmonary carcinomas.

1.2. Results

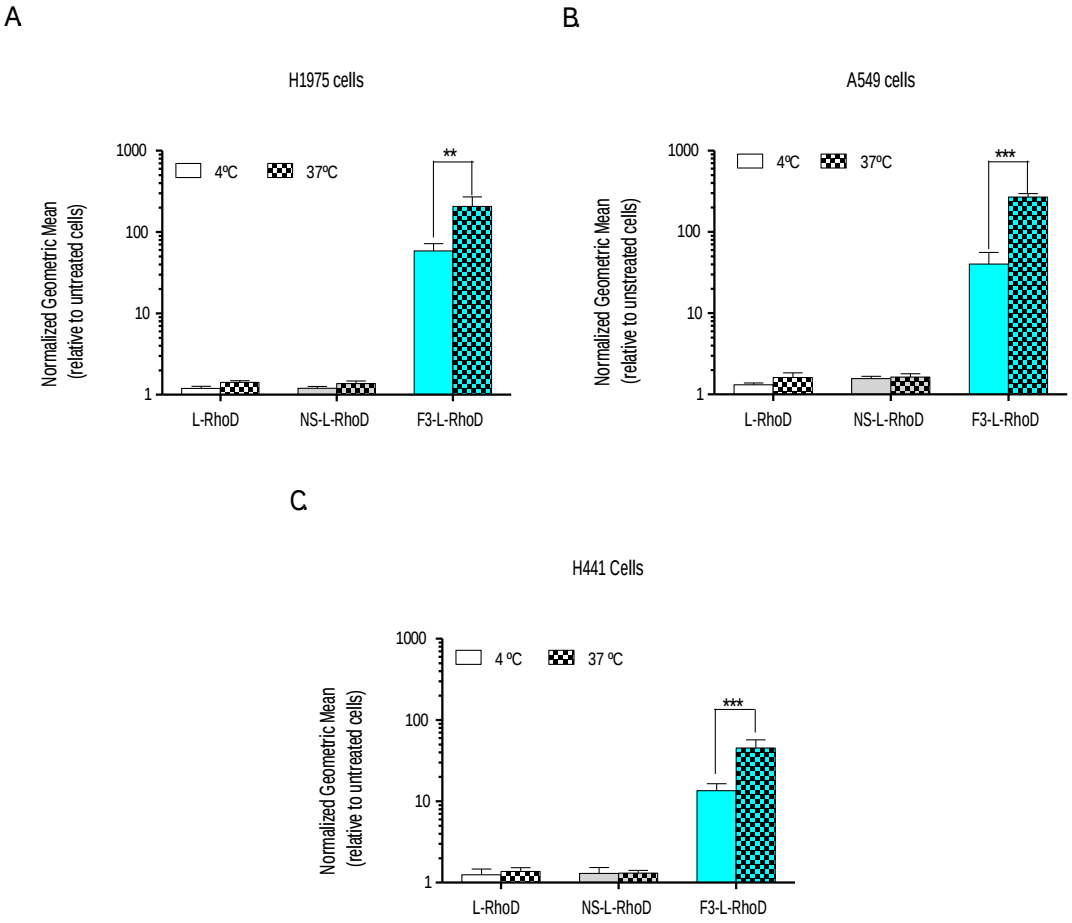
1.2.1. Significant association and internalization of F3 peptide-targeted liposomes by bevacizumab-resistant lung cancer cell lines

It has been previously demonstrated that F3 peptide-targeted pH-sensitive liposomes associated with cellular models of breast cancer and endothelial cells from tumor blood vessels to a higher extent than the non-targeted counterparts, and were internalized through receptor-mediated endocytosis [140]. In order to confirm the specificity and potential application of the previously developed nanoparticle in lung cancer, cellular association studies were performed, either at 4°C (temperature non-permissive to endocytosis) or 37°C, for 1 h, with lung cancer cell lines presenting intrinsic resistance (A549) or acquired resistance (H1975 and H441) to bevacizumab [174]. The extent of cellular association of fluorescently-labeled F3 peptide-targeted liposomes (F3-L-RhoD) at 37°C was significantly higher than for the non-targeted (L-RhoD) counterparts. A maximum of 150- or 170-fold improvement was observed for H1975 cells (Figure 2.1A) or A549 cells (Figure 2.1B), respectively, while for H441 cells it decreased to 34-fold (Figure 2.1C). The difference relative to the experiments performed at 4°C, strongly suggested a relevant component of internalization in all cell lines tested (Figure 2.1A-C).

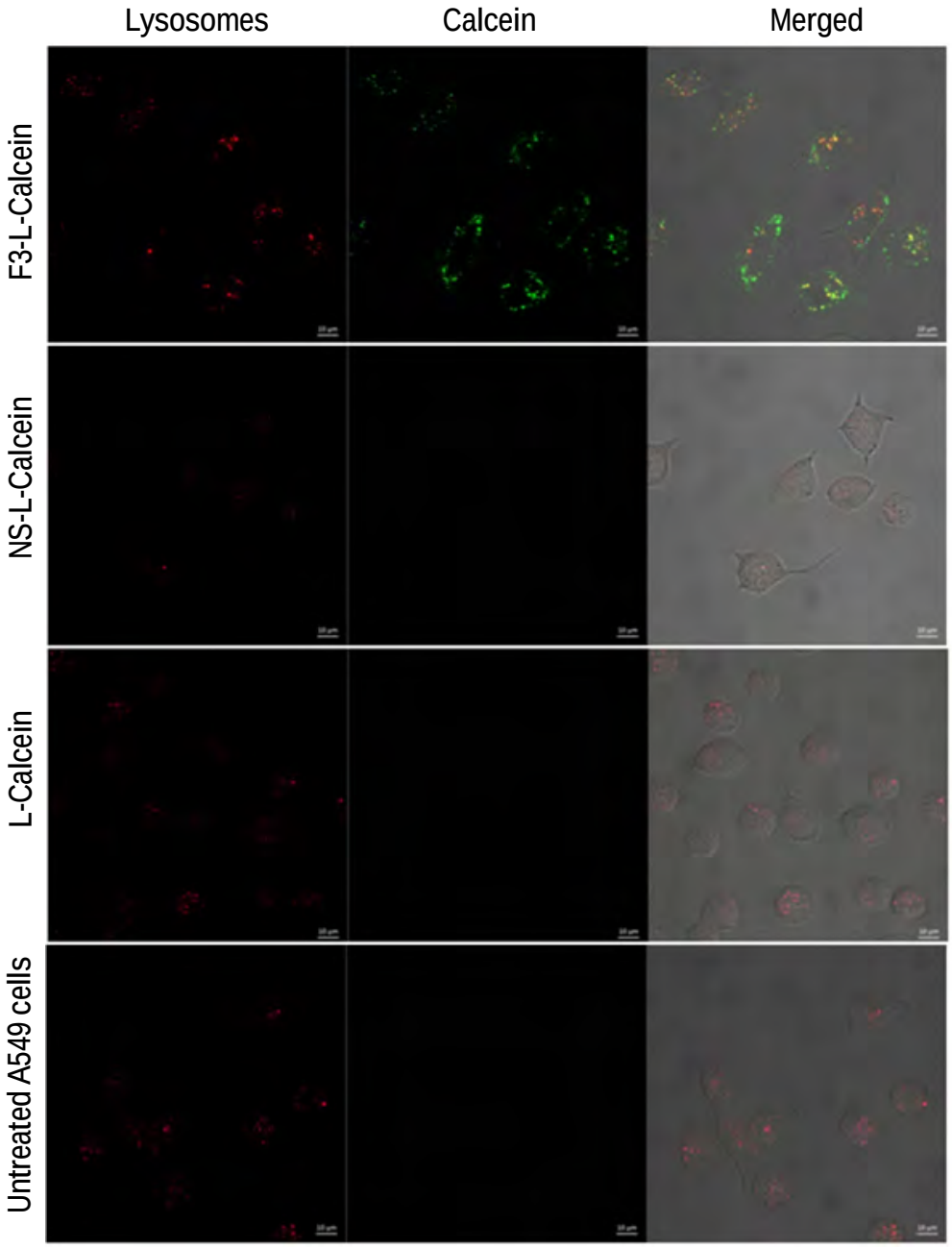
Cellular internalization of F3 peptide-targeted liposomes was further confirmed by confocal microscopy, with the cell lines that presented the highest extent of cellular association (A549 and H1975). Co-localization studies between liposomal and lysosome markers (calcein and *Lysotracker Red*, respectively) were performed to assess the endosomal escape and thus evaluate the effectiveness of the intracellular-enabled drug delivery system. As shown in Figures 2.1D and E, the intracellular green staining observed after incubation with F3 peptide-targeted liposomes loaded with calcein (F3-L-Calcein) supported a higher

extent of binding and intracellular delivery of the encapsulated payload into both cell lines, relative to non-targeted liposomes (L-Calcein) or targeted by a non-specific peptide (NS-L-Calcein). The low prevalence of yellow staining suggested a reduced extent of co-localization between *Lysotracker Red* (red) and calcein (green), ultimately indicating that the liposomal payload efficiently escaped from the endosomal route into the cytoplasm, owing to an acidic destabilization of the F3 peptide-targeted pH-sensitive liposomes (Figures 2.1D and E).

A competitive inhibition study was also performed upon 0.5 h pre-incubation with non-labeled F3 peptide-targeted liposomes (F3-L), to saturate nucleolin receptors and further decrease the extent of binding. Lung cancer cells were then incubated with fluorescently-labeled F3 peptide-targeted liposomes (F3-L-RhoD). The significant decrease on the cellular association, ranging between 6- and 13-fold (Figure 2.2) suggested that cellular internalization of F3 peptide-targeted liposomes was ruled by a relevant component of receptor (i.e. nucleolin)-mediated endocytosis. The involvement of nucleolin in this internalization process, might correlate with its high expression in all lung cancer cell models tested (in a lower extent in H441), including the cell membrane (Figure 2.3).



D.


Lysosomes
Calcein
Merged
F3-L-Calcein
NS-L-Calcein
L-Calcein
Untreated A549 cells

E.

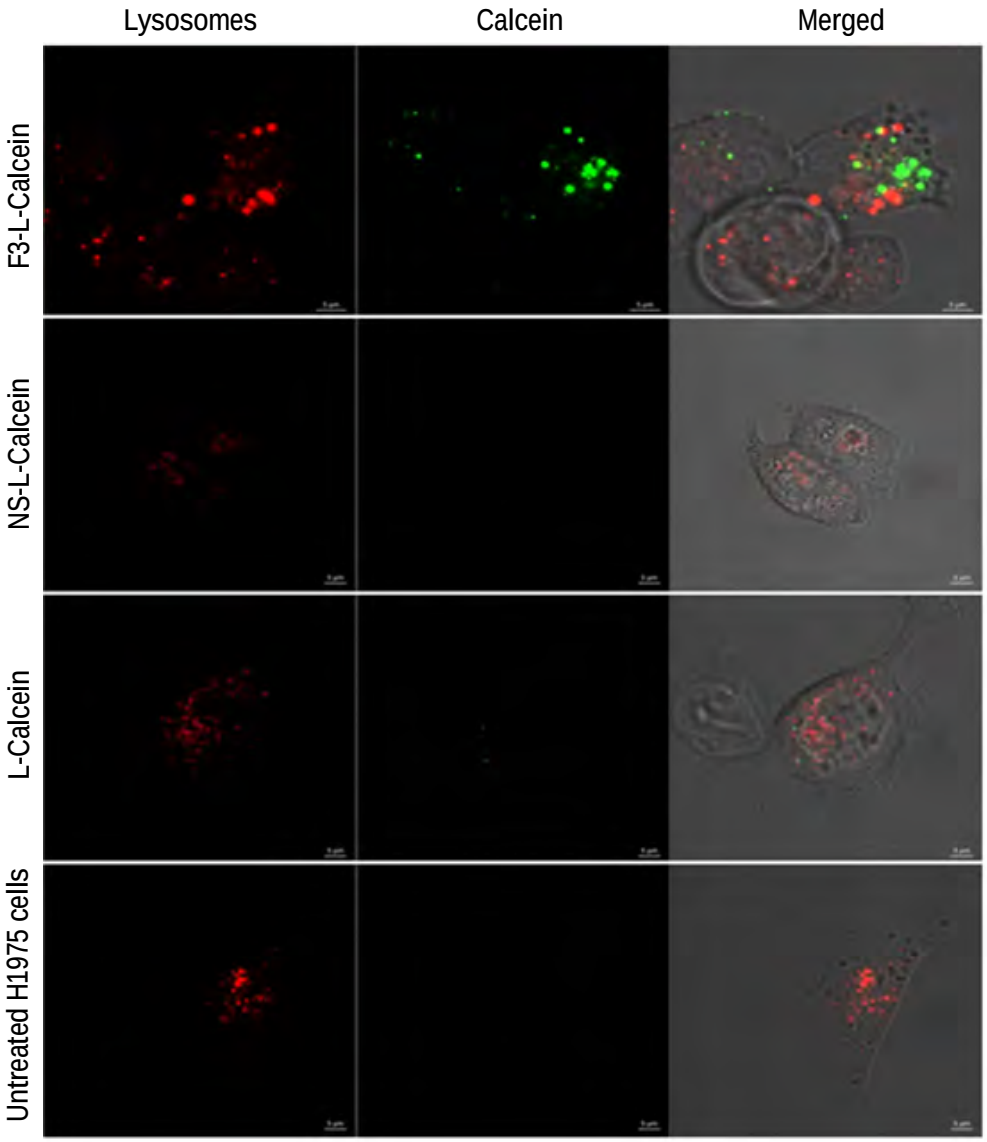


Figure 2.1| In vitro cellular association of different liposomal formulations with human lung cancer cell lines.

Two-hundred thousand A549 **(A)**, H1975 **(B)** and H441 **(C)** cells were incubated with rhodamine (Rhod)-labeled non-targeted (L-RhoD), targeted by a non-specific peptide (NS-L-RhoD) or F3 peptide-targeted (F3-L-RhoD) liposomes, at 0.6 mM total lipid/well, for 1 h, at 4 and 37°C. Untreated cells were used as control. Results were presented as the geometric mean value (normalized for the value of untreated cells) of three independent experiments ± SEM, depicted from the cell-associated fluorescence analyzed by flow cytometry. In **A – C**, levels of significance with respect to the control groups were analyzed by *2-way ANOVA and Bonferroni's* post-test (***p*<0.01 and ****p*<0.001) for 95% CI. In confocal experiments, twenty thousand A549 **(D)** and H1975 **(E)** cells were incubated with liposomes loaded with calcein (green label of the aqueous core) at 0.6 mM TL/well, for 1 h at 37°C. Afterwards, lysosomes were labeled with *LysoTracker Red* (red) and live cells were visualized in a point scanning confocal microscope.

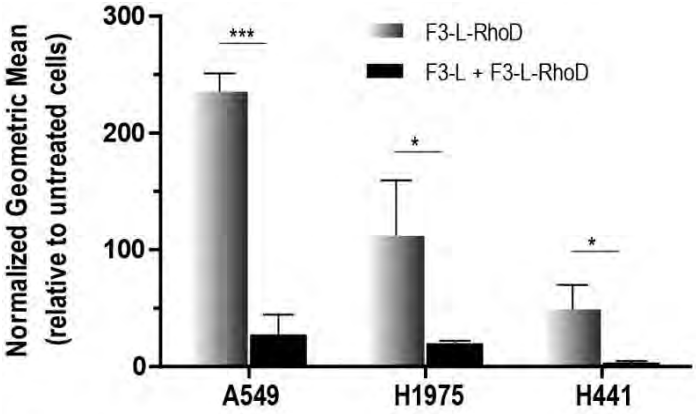


Figure 2.2| Competitive inhibition of F3 peptide-targeted liposomes association by lung cancer cell lines.

Cells were pre-incubated with (black bars) or without (grey bars, control) non-labeled F3 peptide-targeted liposomes (F3-L), at 2 mM TL/well, for 0.5 h at 37°C, followed by incubation with rhodamine-labeled F3 peptide-targeted liposomes (F3-L-RhoD) as previously described. Results were presented as the geometric mean value (normalized for the value of untreated cells) of three independent experiments ± SEM, depicted from the cell-associated fluorescence analyzed by flow cytometry. Levels of significance with respect to the control groups were analyzed by *Unpaired t test (one-tailed)* with *p<0.05 and ***p<0.001, for 95% CI.

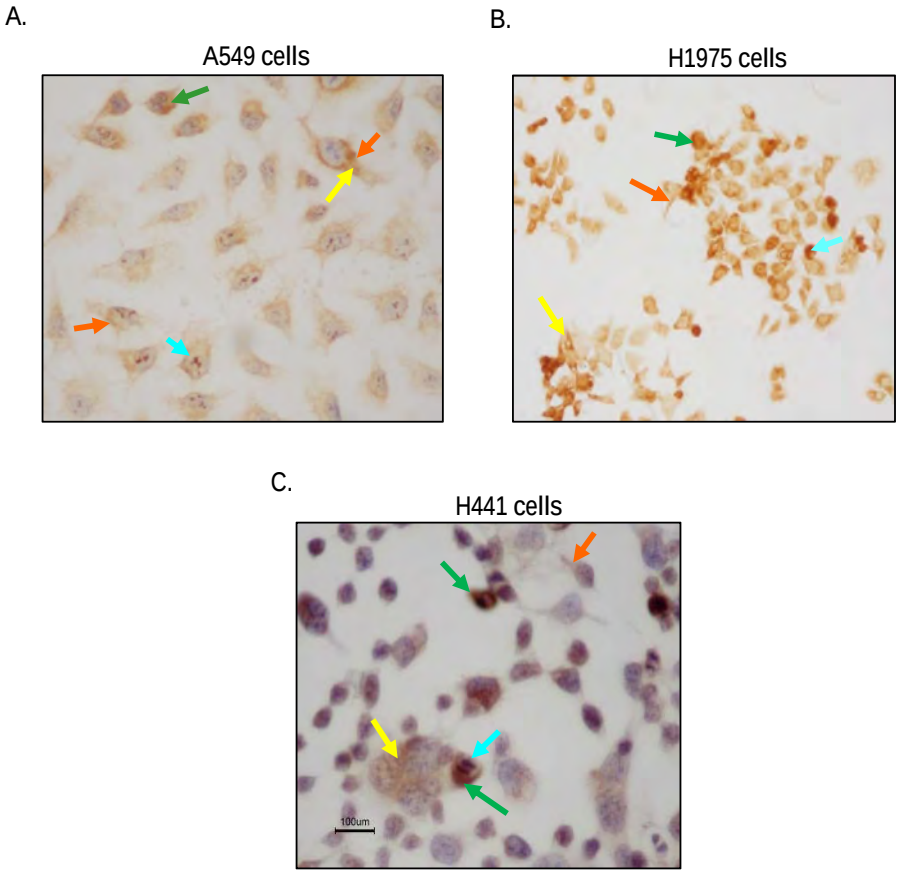


Figure 2.3| Nucleolin expression in human lung cancer cell lines.

Immunocytochemical analysis was performed using the *Labeled Streptavidin-Biotin* staining method. High positive (+++) nucleolin expression was observed in A549 (**A,** x400) and H1975 cells (**B,** x200), whereas positive expression (++) was observed in H441 cells (**C**, x400). Different intracellular localizations of nucleolin expression were observed in the cytoplasm (yellow arrows), cell membrane (orange arrows) nucleolus (light-blue arrows), and nucleus (green arrows).

1.2.2. In vitro cytotoxicity of doxorubicin delivered by F3 peptide-targeted liposomes against human lung cancer cells

Upon the significant nucleolin-mediated association of F3 peptide-targeted liposomes to the tested lung cancer cell lines, the activity of the encapsulated drug (doxorubicin, Dox), used as a model of an anti-cancer drug, was assessed as a function of drug exposure time (Table 2.1). The unique combination of active and specific targeting to nucleolin and intracellular triggered release of the payload (arising from the pH-sensitive liposomal formulation), justified a marked increase in the cytotoxic activity of F3-L[Dox] liposomes for short incubation times (1 and 4 h) in all the tested cell lines. After 1 h incubation, H1975, A549 and H441 cell lines evidenced a maximum of 19-, 9- and 2.7-fold decrease of IC_{50}, respectively, relative to the non-targeted (L[Dox]) and non-specific targeted (NS-L[Dox]) counterparts (Table 2.1).

Table 2.1 | Cytotoxicity of different formulations of liposomal doxorubicin against bevacizumab-resistant human lung cancer cells.

Drug exposure time (h)	IC_{50} (μM) Mean ± SEM			
A549	free-Dox	L[Dox]	NS-L[Dox]	F3-L[Dox]
1	1.4 ± 0.18	19.1 ± 2.01	20.3 ± 5.49	2.1 ± 1.46
4	0.1 ± 0.18	10.2 ± 5.06	10.5 ± 3.96	0.6 ± 0.55
24	<0.01	<0.39	<0.39	<0.39
H441				
1	0.1 ± 0.03	4.3 ± 0.93	4.6 ± 1.36	1.7 ± 0.90
4	0.1 ± 0.04	1.1 ± 0.49	0.7 ± 0.34	<0.39
24	<0.01	<0.39	<0.39	<0.39
H1975				
1	0.3 ± 0.25	6.1 ± 2.67	3.6 ± 2.65	0.4 ± 0.26
4	0.1 ± 0.01	2.4 ± 0.18	1.3 ± 0.02	0.2 ± 0.14
24	<0.01	<0.39	1.2±1.1	<0.39

Dox, doxorubicin; L[Dox], non-targeted liposomes; NS-L[Dox], non-specific targeted liposomes; F3-L[Dox], F3 peptide-targeted liposomes. Data represent the IC_{50} values from the mean dose-response curves of either free or liposomal doxorubicin, using *The Median Effect Equation: fa/fu [D/Dm]^m* [179].

With all formulations, when the incubation time increased from 1 to 4 and 24 h, the IC_{50} decreased. As expected in an *in vitro* cytotoxicity study, free doxorubicin elicited some of the highest levels of cell death against all cancer cell lines, which does not take into account the known unfavorable pharmacokinetics and biodistribution presented by the free drug *in vivo* [178]. Notwithstanding, it is important to emphasize that for the A549 and H1975 cell lines, doxorubicin-containing F3 peptide-targeted liposomes presented IC_{50} values within the same

range of the free drug, thus correlating with the high extent of cellular association previously observed (Figure 2.1A and B). Ultimately, these results suggested that the use of liposomes targeted by the nucleolin-binding F3 peptide might significantly improve the therapeutic index, either relative to the free drug or doxorubicin-containing non-targeted liposomes.

The *in vitro* data generated so far suggested a marked nucleolin expression in bevacizumab-resistant human lung cancer cell lines, thus justifying the high extent of cellular association and the improved cytotoxicity of doxorubicin encapsulated in F3 peptide-targeted liposomes. In order to assess the clinical potential of the developed strategy, identification of nucleolin in human pulmonary carcinomas surgically staged was further performed.

1.2.3. High nucleolin expression in the tumor microenvironment of human pulmonary carcinomas

1.2.3.1. Characterization of human samples

Nucleolin has been reported to be overexpressed not only in the nucleus but, most importantly, in the cytoplasm and surface of cells with high proliferation status, which is the case of tumor cells, such as cancer and endothelial cells from tumor blood vessels [176]. However, the expression of the different subcellular localization of nucleolin in the tumor microenvironment of lung cancer patients, has not been well characterized yet. Aiming at investigating the expression status of nucleolin in human lung cancer, 58 patient-derived carcinomas (from 19 females and 39 males), with a mean age of 66 years old, were analyzed by immunohistochemistry. Nuclear and extra-nuclear (cytoplasm-membrane) nucleolin expression in cancer cells, endothelial cells, tumor-infiltrating lymphocytes and cancer-associated fusiform cells (which could include cancer-associated fibroblasts) of the tumor stroma were analyzed in the four different histological types of pulmonary carcinoma, using the semi-quantitative score criteria described in the Material and Methods section.

1.2.3.2. Frequency and pattern of nucleolin expression in human pulmonary carcinomas

In an overall assessment of the 58 carcinoma samples, a significant nuclear staining of cancer cells was observed in 97% of the cases (Table 2.2 and Figure 2.4, blue head-arrows), and 33% evidenced a positive cytoplasm-membrane immunostaining (Table 2.2 and Figure 2.4, orange head-arrows). In the supportive stroma, nucleolin expression was observed in

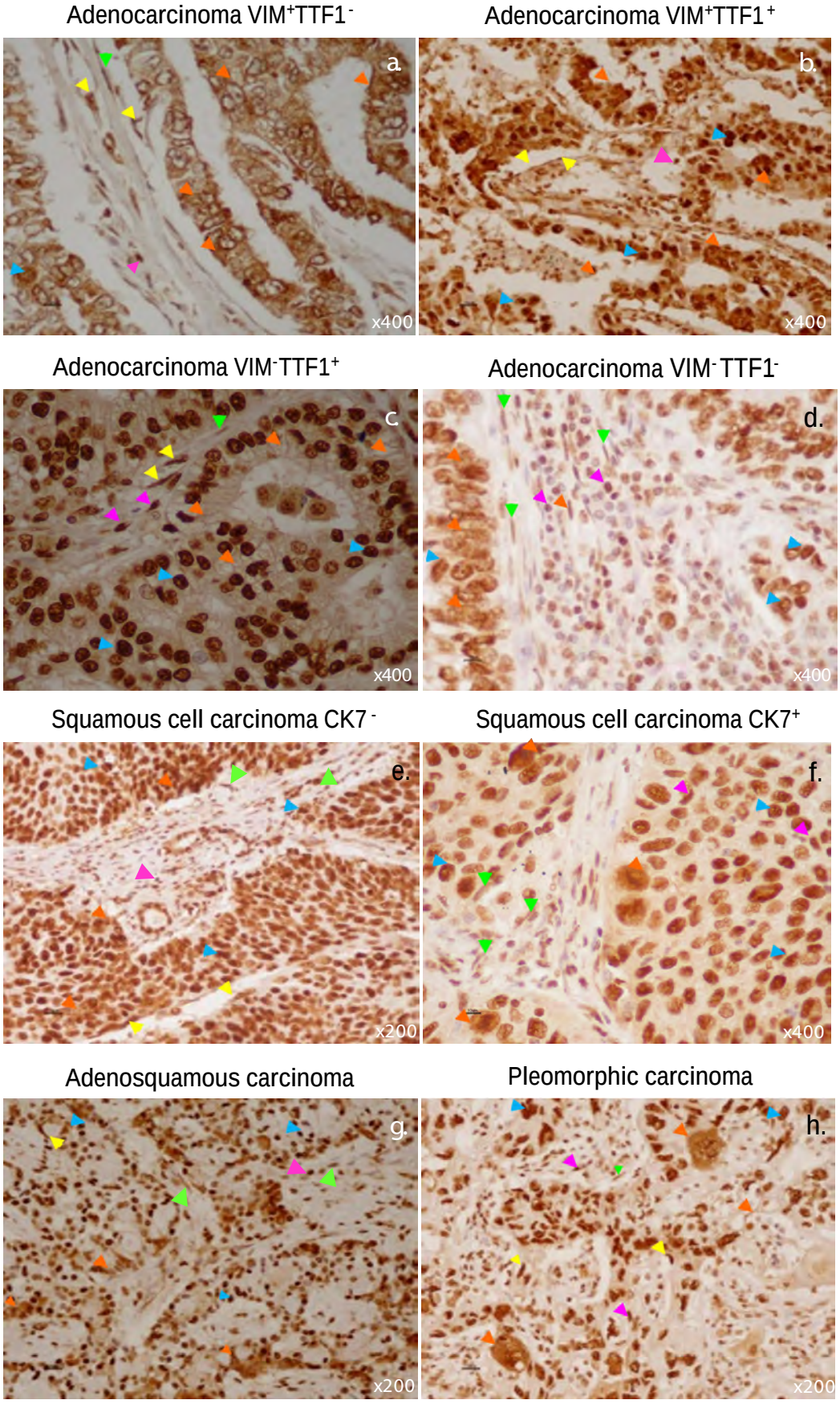

Adenocarcinoma VIM+TTF1-
Adenocarcinoma VIM+TTF1+
a.
x400
b.
x400
Adenocarcinoma VIM-TTF1+
Adenocarcinoma VIM-TTF1-
c
x400
d.
x400
Squamous cell carcinoma CK7 -
Squamous cell carcinoma CK7+
e.
x200
f.
x400
Adenosquamous carcinoma
Pleomorphic carcinoma
g.
x200
h.
x200
NUCLEOLIN EXPRESSION

Figure 2.4| Nucleolin expression in different histological subtypes of human pulmonary carcinomas. Representative immunohistochemistry images of nucleolin expression **(a-h)** assessed in: Adenocarcinomas (ADC; **a – d**), either vimentin positive (VIM^+; **a, b**) or negative (VIM^-; **c, d**), and thyroid transcription factor-1, either negative (TTF1-; **a, d**) or positive ($TTF1^+$; **b, c**); Squamous cell carcinoma, cytokeratin 7 negative (SQC, $CK7^-$; **e**) or positive ($CK7^+$; **f**); Adenosquamous carcinoma (ADSQC; **g**) and Pleomorphic carcinoma (PLMC; **h**). Distinct subcellular nucleolin expression is presented: in cancer cells (nuclear, blue head-arrows; and extra-nuclear, i.e. cytoplasm-membrane, orange head-arrows); endothelial cells (ECs; yellow head-arrows); tumor-infiltrated lymphocytes (TILs; pink head-arrows); and cancer-associated fusiform cells (could include fibroblasts) (green head-arrows).

endothelial cells (95%, Figure 2.4, yellow head-arrows; endothelial cells were defined by CD31, data not shown), tumor-infiltrating lymphocytes (83%, Figure 2.4, pink head-arrows) and in cancer-associated fusiform cells, including fibroblasts (57%, Figure 2.4, green head-arrows; fibroblasts were defined by vimentin positive and fusiform cells by vimentin negative, data not shown) (Table 2.2). In the non-neoplastic adjacent pulmonary tissue, a discrete nucleolin nuclear staining was observed in 41% of the analyzed samples and there was no evidence of cytoplasm-membrane staining (Figure 2.5A and B).

1.2.3.3. Association of frequency and tumor cell distribution of nucleolin expression with clinicopathological data

To determine the importance of nucleolin in lung cancer, it was further analyzed whether results of nucleolin expression were related with clinicopathological data (Table 2.2). Nucleolin expression in cancer cells, either in the nucleus or in the cytoplasm-membrane (extra-nuclear), was independent of clinical data, as disease pTNM staging, presence of metastases, gender, age, smoking status and histopathological type. The expression of nucleolin in the endothelial cells of tumor blood vessels depended on the histological type, with adenocarcinoma and pleomorphic carcinomas presenting the highest frequency of expression (100%) (Table 2.2). The same dependency applied to the expression of nucleolin in stromal tumor-infiltrating lymphocytes, with the highest prevalence observed for adenocarcinoma (93%) and pleomorphic (90%) carcinomas (Table 2.2).

1.2.4. Nucleolin expression in subcutaneous tumor models of lung adenocarcinoma

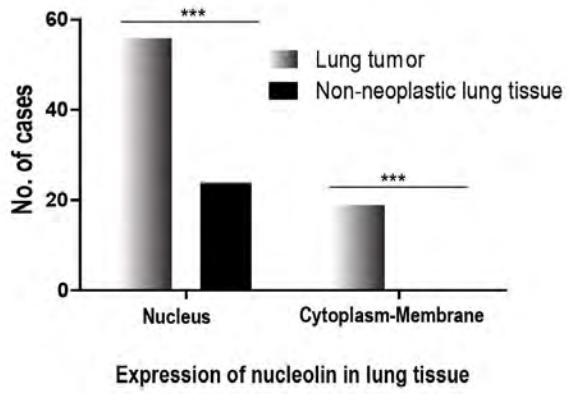
The successful preclinical proof-of-concept of targeted strategies in oncology depends on the establishment and characterization of animal models that closely mimic the human disease in terms of its microenvironment [180]. Prior to any further assessment regarding the

Table 2.2 | Clinicopathological data and nucleolin expression in human pulmonary carcinomas.

CLINICOPATHOLOGICAL STATUS		NUCLEOLIN EXPRESSION FREQUENCY IN THE TUMOR MICROENVIRONMENT									
Variables	Cases	Cancer cells					Tumor Stroma				
Gender	n=58	Nucleus (n,%)	*p* value	Cytoplasm-Membrane (n,%)	*p* value	Nuc+Cyt-Memb (n,%)	*p* value	ECs (n,%)	*p* value	TILs (n,%)	*p* value	Fusiform cells/CAFs (n,%)	*p* value
Female	19	19 (100.00)	*p* =1.000	5 (26.31)	*p* =0.559	5 (26.31)	*p* =0.764	19 (100.00)	*p* =0.696	16 (84.21)	*p* =1.000	15 (78.95)	*p* =0.05
Male	39	37 (94.87)		14 (35.89)		13 (33.33)		36 (92.31)		32 (82.05)		18 (46.15)	
All	58	56 (96.55)		19 (32.75)		18 (31.03)		55 (94.83)		48 (82.76)		33 (56.89)	
Age (years)													
≤55	9	9 (100.00)	*p* =1.000	3 (33.33)	*p* =1.000	3 (33.33)	*p* =1.000	8 (88.89)	*p* =0.403	7 (77.78)	*p* =0.787	6 (66.67)	*p* =0.695
>55	49	47 (95.92)		16 (32.65)		15 (30.61)		47 (95.92)		41 (83.67)		27 (55.10)	
All	58	56 (96.55)		19 (32.76)		18 (31.03)		55 (94.83)		48 (82.76)		33 (56.89)	
Smoking Status													
Never	18	18 (100.00)	*p* =0.497	6 (33.33)	*p* =1.000	6 (33.33)	*p* =1.000	18 (100.00)	*p* =0.078	16 (88.89)	*p* =0.120	14 (77.78)	*p* =0.098
Current	14	14 (100.00)		4 (28.57)		4 (28.57)		12 (85.71)		10 (71.43)		5 (35.71)	
Former	26	24 (92.31)		9 (34.62)		8 (30.77)		25 (96.15)		22 (84.62)		14 (53.85)	
All	58	56 (96.55)		19 (32.76)		18 (31.03)		55 (94.83)		48 (82.76)		33 (56.89)	
Histological type													
ADC	28	27 (96.43)	*p* =0.399	9 (32.14)	*p* =0.953	8 (28.57)	*p* =0.907	28 (100.00)	*p* =0.022	26 (92.86)	*p* =0.002	15 (53.57)	*p* =0.736
SQC	12	12 (100.00)		4 (33.33)		4 (33.33)		10 (83.33)		8 (66.67)		6 (50.00)	
PLMC	10	10 (100.00)		4 (40.00)		4 (40.00)		10 (100.00)		9 (90.00)		7 (70.00)	
ADSQC	8	7 (87.50)		2 (25.00)		2 (25.00)		7 (87.50)		5 (62.50)		5 (62.50)	
All	58	54 (93.10)		19 (32.76)		18 (31.03)		55 (94.83)		48 (82.76)		33 (56.89)	
pTNM Stage													
I-IIB	47	45 (95.74)	*p* =1.000	15 (31.91)	*p* =1.000	14 (29.79)	*p* =0.724	44 (93.62)	*p* =1.000	38 (80.85)	*p* =0.651	26 (55.32)	*p* =0.903
III-IV	11	11 (100.00)		4 (36.36)		4 (36.36)		11 (100.00)		10 (90.91)		7 (63.64)	
All	58	56 (96.55)		19 (32.76)		18 (31.03)		55 (94.83)		48 (82.76)		33 (56.89)	
Metastasis													
Yes	20	20 (100.00)	*p* =0.540	6 (30.00)	*p* =1.000	6 (30.00)	*p* =1.000	19 (95.00)	*p* =1.000	16 (80.00)	*p* =0.752	11 (55.00)	*p* =1.000
No	38	36 (94.74)		13 (34.21)		12 (31.58)		36 (94.74)		32 (84.21)		22 (57.89)	
All	58	56 (96.55)		19 (32.76)		18 (31.03)		55 (94.83)		48 (82.76)		33 (56.89)	

Nuc+Cyt-Memb (Nucleus plus Cytoplasm-Membrane); ECs (Endothelial cells); TILs (Tumor Infiltrating Lymphocytes); Cancer Associated Fusiform cells/Fibroblasts(CAFs). Differences were considered statistically significant when $p<0.05$, *Fisher's Exact Test.*

A.



B.

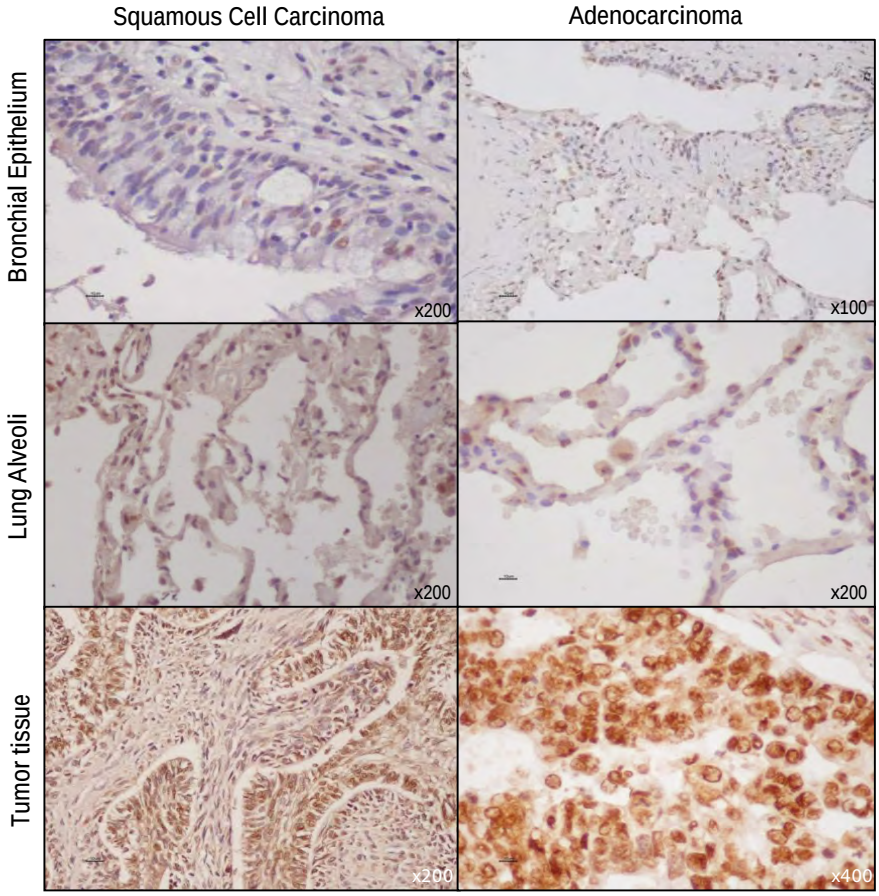


Figure 2.5| Nucleolin expression in the nucleus and cytoplasm-membrane of cancer cells in pulmonary carcinomas and in non-neoplastic adjacent lung tissue.

(A) Each case was analyzed by immunohistochemistry for nucleolin expression in the tumor (*grey bars*) and in the normal (surrounding non-neoplastic tissue, *black bars*) tissue of each case. Significance at level of $p<0.05$ was considered, with ***$p<0.0001$, calculated by *Fisher's Exact test*. **(B)** Representative images of immunohistochemistry expression of nucleolin in squamous cell carcinoma and adenocarcinoma and in non-neoplastic lung tissue (bronchial epithelium and alveolar pneumocytes).

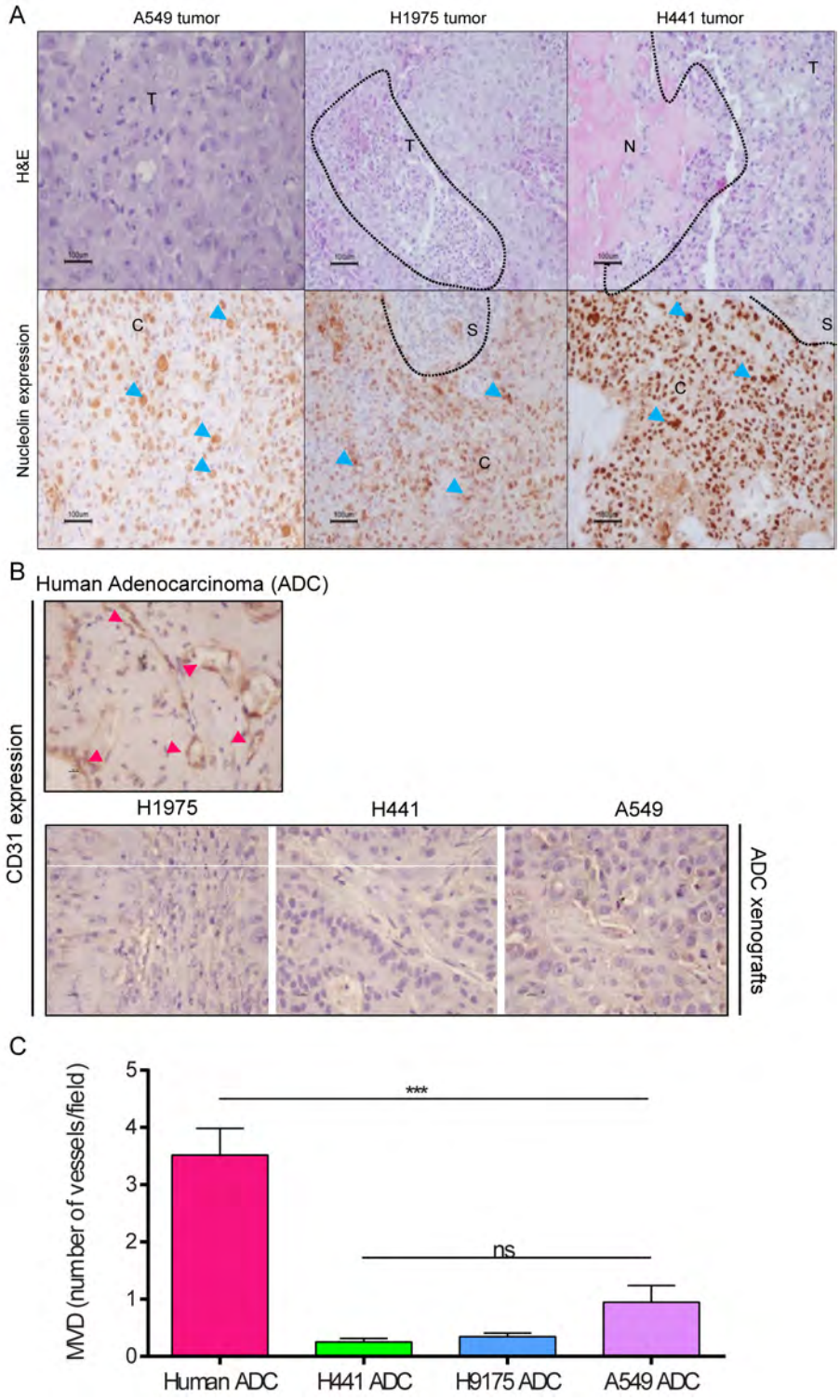


Figure 2.6| Characterization of nucleolin expression and microvessel density in subcutaneous xenografts of lung adenocarcinoma.

(A) Representative images of histopathological analysis by H&E staining and immunohistochemical analysis of nucleolin expression (blue head-arrows represent nuclear expression in cancer cells) in xenograft models of lung adenocarcinoma (A549, H1975 and H441 models, x200). T, tumor area; N, necrotic area; S, stroma area; C, cancer

cells. **(B)** Representative images of CD31 immunostaining (exemplified as pink head-arrows, x400) in human lung adenocarcinoma (ADC) samples and lung ADC xenografts. (C) Microvessel density (MVD; number of vessels/field) in human lung ADC (n=12) versus ADC xenografts (n=3, each model). Data represent mean ± SEM, bars. *One-way ANOVA* and *Kruskal-Wallis* test with ***$p<0.0001$, $^{ns}p>0.05$.

in vivo activity, a histopathological characterization of tumor xenografts derived from human lung adenocarcinoma cell lines (A549, H1975 and H441) was performed, and compared to the previous analysis of patient-derived pulmonary carcinoma samples. This analysis was mainly focused on the complexity/heterogeneity of the generated tumor microenvironment, particularly the microvessel density and nucleolin expression. Representative images presented in Figure 2.6A (H&E staining) indicated the formation of less than 5% of supportive stroma relative to the overall neoplastic volume, along with the absence of an effective vasculature (Figure 2.6B, pink head-arrows, and 2.6C), as compared with the human pulmonary adenocarcinomas. Although the frequency of nuclear expression of nucleolin was similar in all the analyzed samples (Table 2.3), the intensity of expression was generally lower in subcutaneous xenografts models (Figure 2.6A, blue head-arrows) than in human carcinomas (Figure 2.4, blue head-arrows). Extra-nuclear expression was not detected (Figure 2.6A and Table 2.3). Overall, these results suggested that the tumor microenvironment of these lung cancer xenograft models was significantly different than their human counterpart, namely in the vascular density and nucleolin expression, which strongly limits their use to test nucleolin-targeted strategies.

Table 2.3 | Frequency of nucleolin expression in human and mouse xenografts of lung adenocarcinoma.

	Lung Adenocarcinoma (ADC) xenografts					
	A549 model	H1975 model	H441 model		Human ADC	
	n=5	n=5	n=6		n=12	
	Cancer cells					
Markers	n, %	n, %	n, %	*p*	n, %	*p*
nuclear NCL	5 (100.00)	5 (100.00)	6 (100.00)	nd	12 (100.00)	nd
extra-nuclear NCL	0 (0.00)	0 (0.00)	0 (0.00)	nd	5 (41.67)	*0.0437

Differences were considered statistically significant when $p<0.05$, *Fisher's Exact Test.* *$p<0.05$; **nd**, not defined

Based on aforementioned limitations of lung cancer xenografts, resected human pulmonary carcinomas were further used to evaluate the potential application of nucleolin-based targeted strategies. Using autoradiography, technetium-99m (^{99m}Tc)-labeled F3 peptide-targeted liposomes (^{99m}Tc-F3-L) [181] presented a significant extent of association (Figure 2.7A and B), in contrast with the non-targeted counterpart (^{99m}Tc-L) (Figure 2.7A). The marked extent of F3 peptide-targeted nanoparticle association by human pulmonary carcinoma tissues rendered an important indication of the therapeutic potential of targeting nucleolin in lung cancer patients.

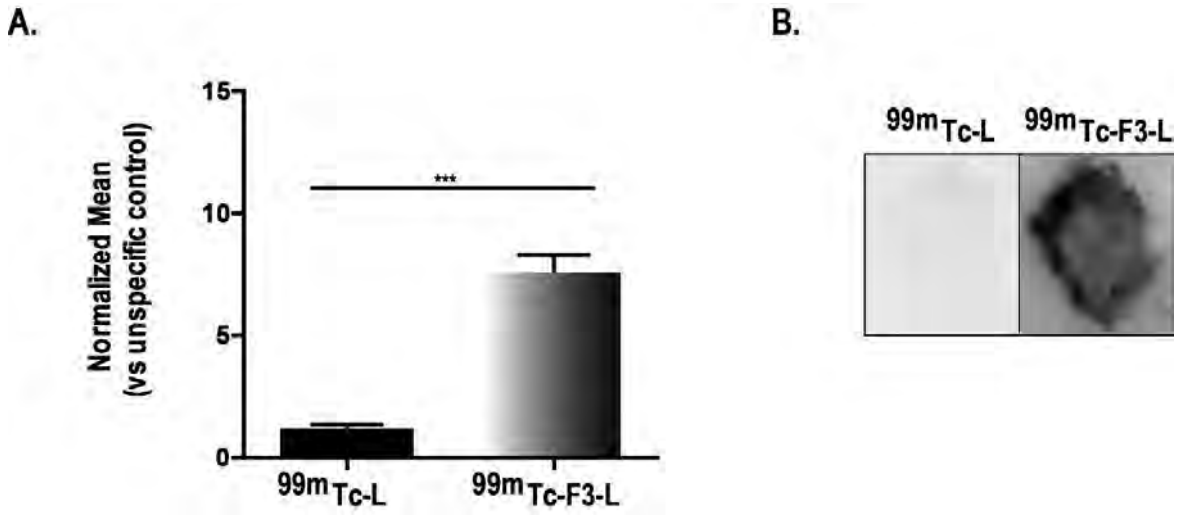


Figure 2.7| Association of different ^{99m}Tc-labeled liposomal formulations to sections of patient-derived pulmonary carcinomas.

Tumor sections were incubated with liposomes, either targeted (^{99m}Tc-F3-L) or non-targeted (^{99m}Tc-F3-L) for 1 h, at room temperature, after which, following several washing steps, they were air-dried and exposed to phosphor imaging screen overnight and further revealed in a biomolecular imager. **(A)** Extent of binding to human pulmonary carcinomas and **(B)** representative autoradiograph image are presented. Data represent the mean ± SEM, bars (n=7). *Unpaired t* test with ***p<0.0001, for a 95% CI.

1.3. Discussion

The paradigm of cancer treatment has undergone many changes since the awareness that tumors are independent entities formed by a complex relation between neoplastic cells and their supportive stroma, which supports tumor survival, progression and dissemination [5]. Treatment options for lung cancer have expanded beyond classic therapies (e.g., chemotherapy, radiotherapy and surgical resection) to include targeted therapies that act specifically against several hallmarks of cancer, including tumor angiogenesis [91,182,183].

The anti-angiogenic agent bevacizumab has become a standard treatment option in lung

cancer [96]. However, disappointing results have been reported with a substantial number of patients being or becoming resistant to anti-VEGF therapies. Some mechanisms of resistance are due, at least in part, to the activation of redundant pro-angiogenic signaling pathways, thus bypassing the inhibition of a single pathway [43,175]. Development of resistance mechanisms to these drugs involves a complex set of interactions between neoplastic cells and stromal components of the tumor microenvironment, which evidence that there is still room for improvement in the development of strategies targeting the tumor vasculature [91].

Nucleolin has been identified in different cell populations in the tumor microenvironment and in different subcellular localizations related with the distinct functions of the protein. In the nucleus, nucleolin is involved in essential processes, such as DNA metabolism and transcription. In the cytoplasm, nucleolin participates in RNA regulatory mechanisms, mRNA stability and translation, and also microRNA processing. In the cellular membrane, nucleolin acts as a shuttle to the intracellular compartment [184].

In an oncological context, nucleolin has been recognized as an angiogenic marker due to its overexpression in endothelial cells of tumor blood vessels, besides neoplastic cells [166,176,185].

In the present work, the potential of nucleolin as a therapeutic target in pulmonary carcinomas was studied by assessing the in vitro activity of PEGylated liposomes containing a cytostatic drug (doxorubicin) and functionalized by the nucleolin-binding F3 peptide [140], and by evaluating the levels of overexpression in human pulmonary carcinoma samples of different histological types.

The extent of cellular association of F3 peptide-targeted liposomes with three lung cancer cell lines, representing different types of resistance to bevacizumab, reached a maximum of ten times higher relative to the one previously observed with breast cancer cells [140].

The positive immunostaining in all lung cancer cell models, the marked decrease of cellular association in the competitive inhibition study, along with the energy-dependent cellular association, supported the original hypothesis of a specific receptor-mediated internalization of F3 peptide-targeted liposomes by nucleolin-overexpressing cells, in agreement with observations in breast cancer cells [140]. The improved cellular association supported increase of doxorubicin cytotoxicity relative to the activity of the same drug encapsulated in the non-targeted counterparts. Importantly, a significant reduction of cell viability was observed for incubation times as short as 1 h for all cell lines and regardless the nature of resistance to bevacizumab therapy. For longer incubation times (24 h), it is likely that the amount of drug released from extracellular liposomes will increase, and thus the released (free) drug will

make a larger contribution to the overall cytotoxicity. Doxorubicin concentrations needed to induce 50% of cell death, upon delivery by F3 peptide-targeted liposomes, were 280- to 1500-fold lower than the ones observed for breast carcinoma model [140]. Overall, these results could be supported by the higher levels of nucleolin surface density and drug sensitivity of lung cancer cells, as compared to the breast carcinoma model [140].

Notwithstanding the *in vitro* results, the formation of poor supportive stroma in the H1975, H441 and A549 tumor xenografts revealed that these models did not represent the complex and heterogeneous microenvironment observed in pulmonary carcinomas derived from patients, nor the extent of nucleolin expression. This poses a new challenge in terms of generating a predictive lung cancer model for therapeutic evaluation of nucleolin-based targeting strategies.

Others have demonstrated the relevance of cell surface nucleolin in lung cancer. In a CL1-5 lung adenocarcinoma xenograft murine model (NOD-SCID mice), lung cancer tumor growth inhibition, along with angiogenesis impairment were reported, upon targeting nucleolin with two aptamer-siRNA chimeras (aptNCL-SLUGsiR and aptNCL-NRP1siR), inhibiting snail family zinc finger 2 (SLUG) and neuropilin 1 (NRP1) [186]. In this work, nucleolin overexpression in CL1-5 lung cancer cells was demonstrated. However, in their specific tumor model, treatments have started seven days following tumor cells inoculation, when non-treated tumors only presented a significant level of vascularization 49 days post-inoculation. Notwithstanding the observed tumor growth inhibition by the developed targeted aptamer-siRNA chimeras, this work lacked the in vivo demonstration of the nucleolin-mediated delivery mechanism.

While the work by Lai *et al.* [186], overall, emphasized the relevance of nucleolin as a therapeutic target, the clinical report of Zhao *et al.* [187], pointed out the importance of nucleolin as a prognostic factor. Zhao *et al.* have identified the combined score of endothelial expression of nucleolin and CD31, as a possible new prognostic factor of clinical outcome for surgically resected lung cancer patients [187]. Herein, a 97% frequency of nucleolin overexpression was observed, with different subcellular localization (nucleus and cytoplasm-membrane). Moreover, it was detected in different cell populations of the tumor stroma (endothelial cells, tumor-infiltrating lymphocytes and cancer-associated fusiform cells/fibroblasts) and neoplastic cells. A higher extent of expression of nucleolin was observed in the tumor in contrast with the normal adjacent epithelium. In addition, a strong nuclear and extra-nuclear (cytoplasm-membrane) nucleolin staining was observed in pulmonary carcinoma tissues, in contrast with the diffuse nuclear expression in non-neoplastic tissue.

Such differentiated expression at the subcellular level, between neoplasic and non-neoplasic tissues, has also been reported for hepatocellular carcinomas [188] and gastric cancer [189]. These differences suggested that the subcellular localization of the protein could potentially change during lung carcinogenesis, highlighting its relevance in this process. The higher extent of association of targeted liposomes, relative to non-targeted, evidenced by autoradiography in patient-derived tumor sections, further supported the potential of nucleolin as a therapeutic target in pulmonary carcinomas.

To the best of our knowledge, this is the first time that nucleolin overexpression has been reported in several cell populations of the tumor stroma (besides endothelial cells) in adenocarcinomas, squamous cell, adenosquamous and pleomorphic human pulmonary carcinomas. In gastric and hepatocellular carcinomas, Qiu *et al.* [189] and Guo *et al.* [188] have correlated the subcellular expression of nucleolin with prognosis and survival rates in patients. The former suggested that a high level of nucleolar expression of nucleolin was an independent prognostic marker for better survival, while high cytoplasmic staining was closely associated with worse prognosis for gastric cancer patients [189]. In the latter, nucleolin was suggested as an independent prognostic marker for poor clinical outcome. They also detected nuclear, cytoplasmic and cytoplasmic-membrane expression of nucleolin in carcinoma tissues, while in non-neoplastic tissues expression was only restricted to the nucleus. This could implying that the subcellular localization of nucleolin may change during the carcinogenesis of hepatocellular carcinoma [188]. This differentiated pattern of expression was also observed by Qiu *et al.* in gastric cancer [189] and was in accordance with our observations in lung cancer.

Overall, the results generated with human samples suggested that the protein could have a relevant role in lung carcinogenesis from early to advanced stages of disease, regardless the clinicopathological features of the patients or the clinical outcomes. This could ultimately represent an indication of the applicability of nucleolin-based targeted strategies in different stages of the disease in a clinical setting [125,174].

1.4. Conclusion

In the present work it was demonstrated that nucleolin was highly expressed in different cell populations within the tumor microenvironment of lung cancer patients, in a tumor-specific manner, with a particular subcellular pattern and with an association to distinct types of pulmonary carcinoma. The potential of nucleolin as a target in lung cancer was further supported by the high extent of cytosolic delivery enabled by liposomes functionalized

with the nucleolin-binding F3 peptide, resulting in significant cytotoxicity towards lung cancer cells associated with diverse bevacizumab-resistant mechanisms. Not less important from a translational perspective, was the demonstration that existing subcutaneous xenograft models of lung cancer, did not represent the complex and heterogeneous microenvironment observed in carcinomas derived from lung cancer patients, nor the extension of nucleolin expression. This emphasized the importance of selecting the right animal model when addressing the proof-of-concept of targeted medicines. Despite that, the marked extent of F3 peptide-targeted nanoparticle association by human pulmonary carcinoma tissues rendered an important indication of the therapeutic potential of targeting nucleolin in lung cancer.

Chapter 3

EXPRESSION OF EXTRA-NUCLEAR NUCLEOLIN IN PATIENT-DERIVED PULMONARY CARCINOMAS IS INDEPENDENT OF CLINICOPATHOLOGICAL FEATURES AND OTHER ANGIOGENIC TARGETS EXPRESSION

ABSTRACT

Anti-angiogenic targeted therapies, such as bevacizumab, have changed the paradigm of lung cancer care, however their clinical benefit has been partially limited by the development of stroma-mediated drug resistance. Nevertheless, targeting the tumor vasculature remains a promising anti-cancer strategy. These aspects support the need to identify alternative targets within the tumor vasculature. In this respect, nucleolin has been considered an angiogenic marker and has become a relevant therapeutic target, owing to its overexpression in different cell populations in the tumor microenvironment, including cancer cells and endothelial cells from tumor blood vessels. This work aims at investigating whether extra-nuclear nucleolin expression correlates with several other angiogenic markers implicated in human lung carcinogenesis and, also with patient clinicopathological features. In addition, the expression of nucleolin in patient-derived bone metastatic specimens from lung, and other primary origins, was also evaluated.

Extra-nuclear nucleolin was expressed among pulmonary carcinoma samples analyzed, in cancer and endothelial cells, independently of both clinicopathological features (including histopathological type) and other angiogenic markers studied (PDGFRα, HIF-1α, IL-17, VEGFR2, FGFR2 and CD133). These findings further suggested that nucleolin could be a hallmark of lung carcinogenesis and angiogenesis, regardless the clinicopathological features and expression of other angiogenic markers. Interestingly, a similar pattern of nucleolin expression between pulmonary primary carcinomas and bone metastases with lung primary origin was identified.

In conclusion, our findings reinforced extra-nuclear nucleolin as a valuable therapeutic target in primary and metastatic lung cancer.

3.1. Introduction

At present, multiple histopathological types and subtypes of lung cancer are recognized [20]. Adenocarcinoma is the histological type with the highest incidence, followed by squamous cell carcinoma, large cell carcinoma, adenosquamoous carcinoma and pleomorphic carcinoma [20,21]. Each type presents distinct biological, genomic and histological features, thus making lung cancer as a heterogeneous set of diseases [18]. These features determine treatment options and prognosis, and render it the first cause of cancer-related death worldwide [18,20,21,169]. The high mortality and prevalence of drug resistance in lung cancer, mainly in advanced stages, have urged the development of targeted therapeutic approaches [190].

Vascular endothelial growth factor (VEGF) is overexpressed in several tumors of diverse histological origin, including colorectal cancer, breast, lung and others [85,87,88,191]. Given its central role in tumor angiogenesis and the correlation with tumor growth, VEGF has emerged as a promising therapeutic target for angiogenesis inhibition [88,192,193]. Bevacizumab [194] is an example of a targeted anti-angiogenic (VEGF) therapy, clinically approved for different types of cancer, including lung cancer [95], which has contributed to change the paradigm of lung cancer standard care [96,195]. However, its overall clinical benefit has been limited by the development of resistance [108,174].

Despite transient disease stabilization or tumor regression following treatment with anti-VEGF therapy, reflected by an increment of progression-free survival, overall survival is often not prolonged in these individuals [81,116]. Strategies that combine the inhibition of several angiogenic factors to overcome resistance [182,196], have also failed in improving overall survival, as tumors become resistant upon activation of alternative pathways [37,197]. These drawbacks prompted the identification of novel molecular targets within the tumor vasculature, a readily accessible path of entry into solid tumors for treatments administered intravenously.

Nucleolin has been identified has a relevant therapeutic target for cancer therapy [140,147,198]. Based on information emerging mainly from breast tumors, nucleolin has shown to provide a unique opportunity to target multiple cellular components of the tumor microenvironment, as it is overexpressed at the surface of cancer cells, cancer stem cells and endothelial cells of tumor blood vessels [147,165]. It has been reported that nucleolin has an increased expression in tumors of diverse histological origin, including breast [140], hepatocellular carcinoma [188], esophageal squamous cell carcinoma [199], colorectal carcinoma [200] and gastric [189]. In respect to lung cancer, nucleolin expression has been

associated with poor prognosis [187,201]. However, information available on nucleolin expression is mainly limited to the tumor vasculature [187], without addressing other cellular components of the tumor microenvironment.

Recently, nucleolin revealed to be functionally active in bevacizumab-resistant lung cancer cell lines, as it enabled significant binding, internalization and cytotoxicity of a nanoparticle containing doxorubicin and functionalized by the nucleolin-binding F3 peptide, relative to the non-targeted counterparts (described in chapter 2). Importantly, we have demonstrated in human surgically staged pulmonary carcinomas that nucleolin was highly expressed in different types of cells within the tumor microenvironment, in a tumor-specific manner, and regardless the type and subtype of pulmonary carcinoma. The relevance of these results was further reinforced by the high extent of ex vivo association of the F3 peptide-targeted nanoparticle to pulmonary carcinomas sections (described in chapter 2). Overall, these previous results supported the study of nucleolin-based strategies for the treatment of lung cancer.

To reinforce the potential of nucleolin as a cell surface therapeutic target in lung cancer patients, the present work aimed at correlating its extra-nuclear expression, in cancer and endothelial cells, with clinicopathological features, as well as with the expression of relevant angiogenic markers (VEGFR2 [202,203], PDGFRα [204], FGFR2 [205], HIF-1α [206,207], IL-17 [208] and CD133 [209,210]), which have been implicated in lung carcinogenesis. Furthermore, we also aimed at addressing the pattern of nucleolin expression in the tumor microenvironment of bone metastatic-patient samples with different primary origins, including lung.

3.2. Results and discussion

3.2.1. Frequency and pattern of expression of extra-nuclear nucleolin and several angiogenic regulators in pulmonary carcinomas

Zhao *et al.* [33] and Hsu *et al.* [34] have associated high levels of nucleolin expression with poor prognosis in lung cancer patients. However, the different subcellular distribution of nucleolin and its potential implication in lung carcinogenesis are still poorly investigated. In a previous study in human pulmonary carcinoma samples, we demonstrated that nucleolin presented different subcellular localizations in neoplastic cells. Moreover, extra-nuclear nucleolin was suggested to be involved in lung carcinogenesis (data detailed in chapter 2). Herein, we aimed at clarifying the relative expression between extra-nuclear nucleolin and

other relevant angiogenic factors in lung carcinogenesis.

All cases were scored as highly positive for nuclear nucleolin (Figure 3.1A.a-d, blue head-arrows), 36% of which were extra-nuclear positive, i.e., cytoplasm and membrane (Figure 3.1A.a-d, orange head-arrows and Table 3.1). As presented in Table 3.1, extra-nuclear expression was detected among all pulmonary carcinoma histological types studied, including 42% in adenocarcinoma, ADC (Figure 3.1A.a), 33% in squamous cell carcinoma, SQC (Figure 3.1A.b) and adenosquamous carcinoma, ADSQC (Figure 3.1A.c), and 25% in pleomorphic carcinoma, PLMC (Figure 3.1A.d). Additional analysis of the tumor microenvironment reinforced that different cell populations of the supportive tumor stroma also stained for nucleolin, including endothelial cells (Figure 3.1A.a-d, yellow head-arrows), regardless the type of pulmonary carcinoma.

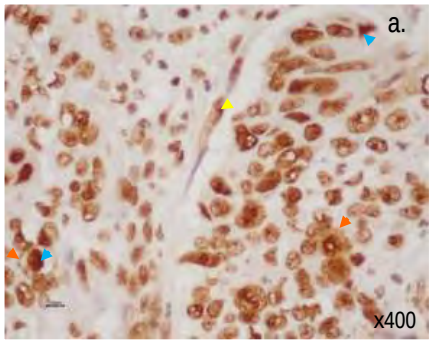
As for nucleolin, the other angiogenic regulators analyzed [202,210-218], presented three different levels of subcellular expression in cancer cells. Expression of platelet-derived growth factor receptor alpha (PDGFRα; Figure 3.1B.a-d) was observed in the nucleus (blue head arrows), cytoplasm (purple head arrows) and membrane (black head arrows), while expression of hypoxia-inducible factor alpha (HIF-1α; Figure 3.1C.a-d) and interleukin 17 (IL-17; Figure 3.1D.a-d) was limited to the first two subcellular compartments. Expression of the remaining angiogenic markers analyzed was exclusive to the cytoplasm (vascular endothelial growth factor receptor 2, VEGFR2, Figure 3.1E.a-d; fibroblast growth factor receptor 2, FGFR2, Figure 1F.a-d; and CD133, Figure 3.1G.a, b and d), excepting CD133 expression that was negative in ADSQC (Figure 3.1G.c). As shown in Table 3.1, VEGFR2, PDGFRα and FGFR2 were expressed in all histological types, whereas IL-17, HIF-1α and CD133 were expressed in 92%, 76% and 71% of all cases, respectively.

The expression of these angiogenic markers did not correlate with the expression of extra-nuclear nucleolin, neither in neoplastic cells nor in endothelial cells ($p>0.05$, *Sperman's* test), of the pulmonary carcinomas herein analyzed. Overall, these data suggested that extra-nuclear nucleolin could be an independent hallmark of carcinogenesis and angiogenesis in these patients.

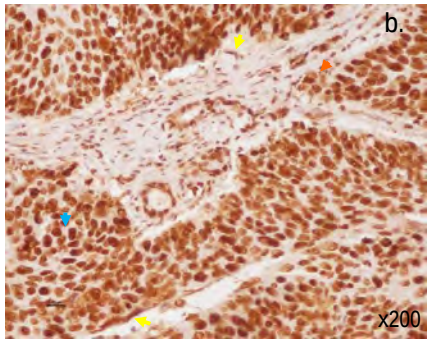
A.

NUCLEOLIN (NCL) EXPRESSION

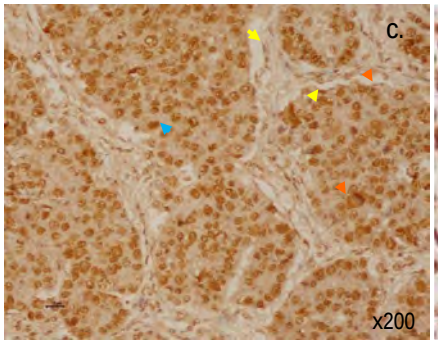
Adenocarcinoma



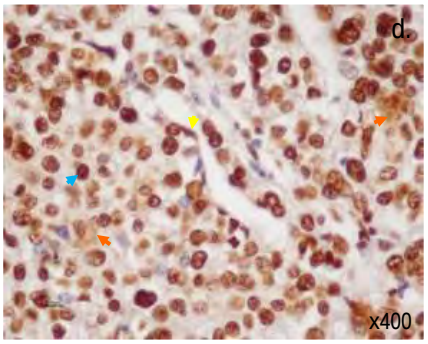
Squamous cell carcinoma



Adenosquamous carcinoma



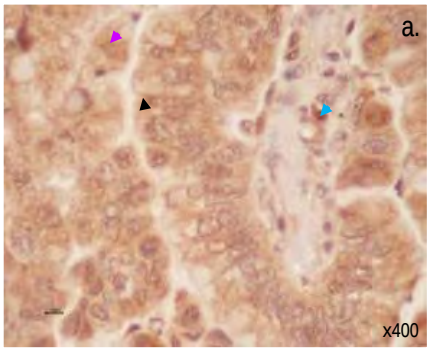
Pleomorphic carcinoma



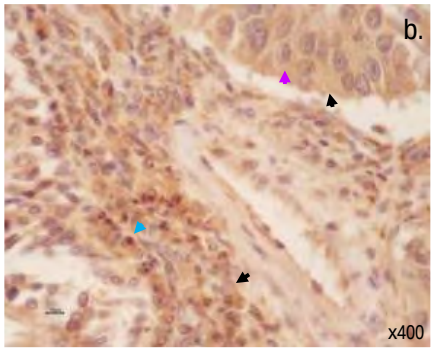
B.

PDGFRα EXPRESSION

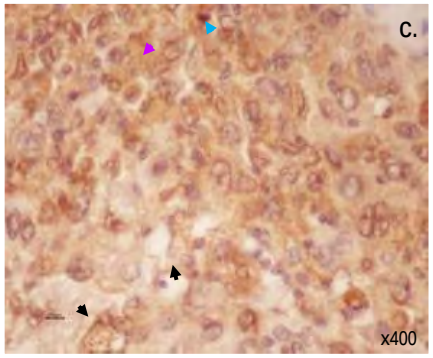
Adenocarcinoma



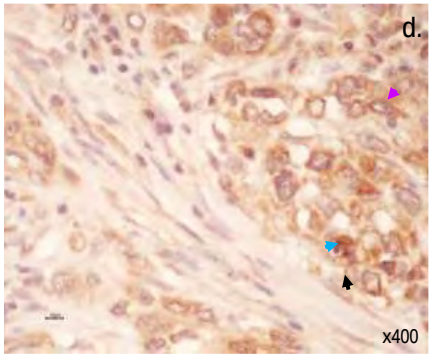
Squamous cell carcinoma



Adenosquamous carcinoma



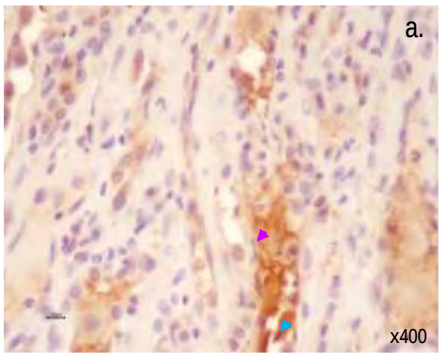
Pleomorphic carcinoma



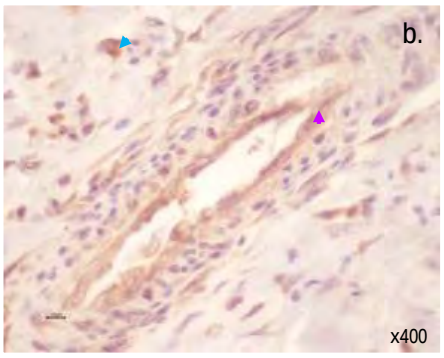
C.

HIF1-α EXPRESSION

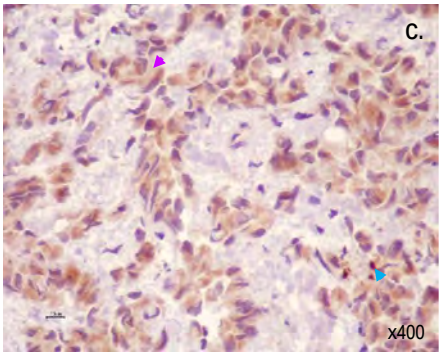
Adenocarcinoma



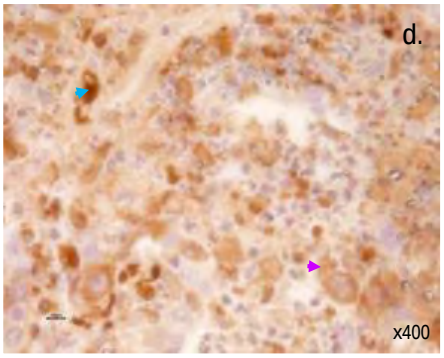
Squamous cell carcinoma



Adenosquamous carcinoma



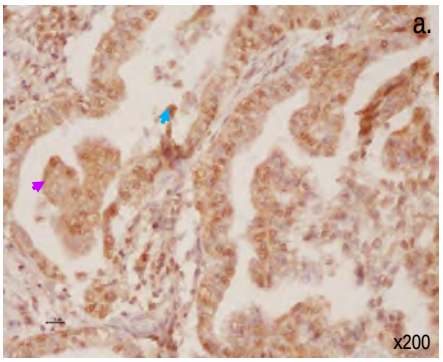
Pleomorphic carcinoma



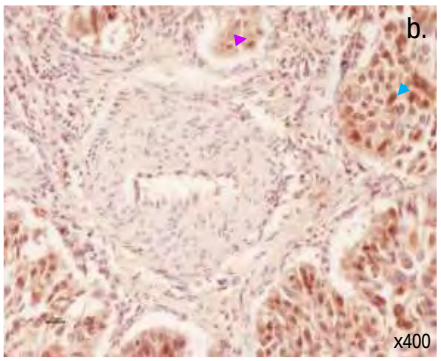
D.

IL-17 EXPRESSION

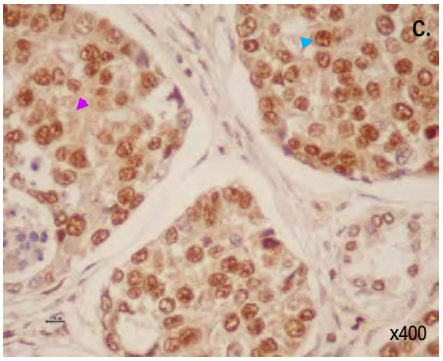
Adenocarcinoma



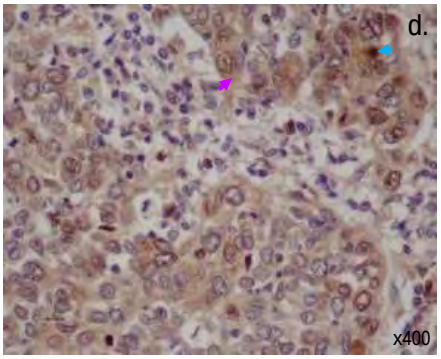
Squamous cell carcinoma



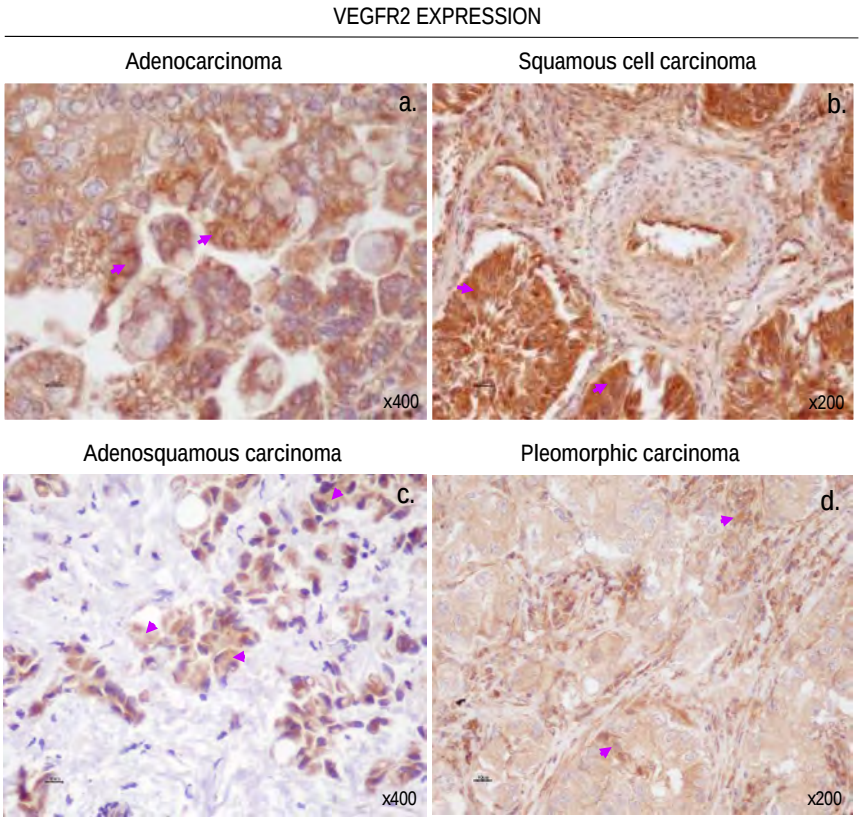
Adenosquamous carcinoma



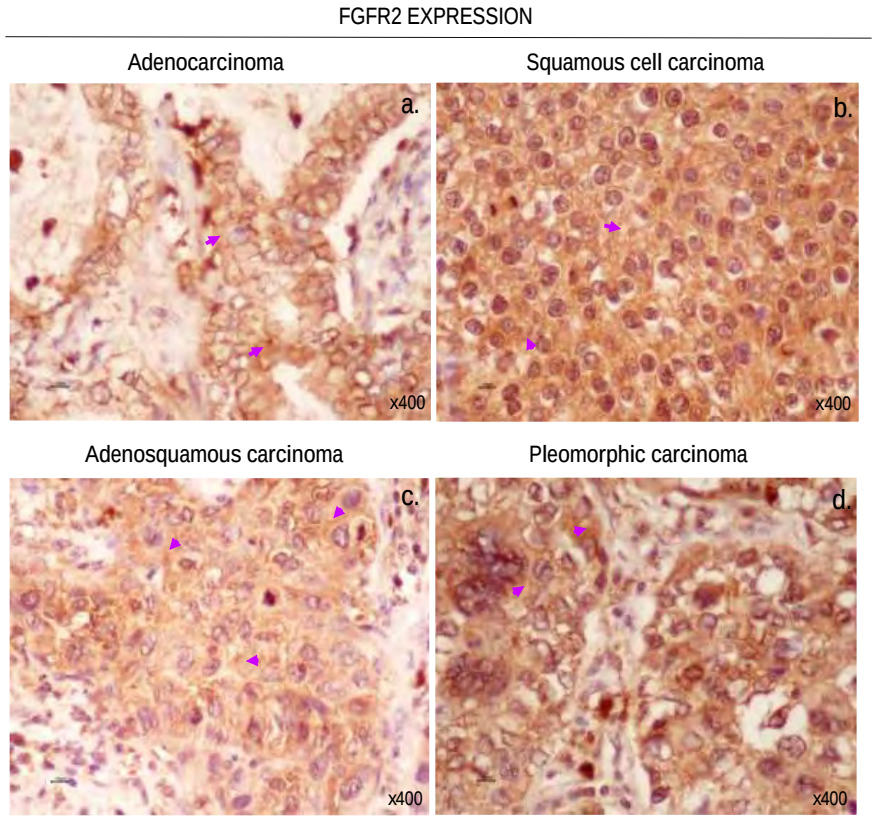
Pleomorphic carcinoma



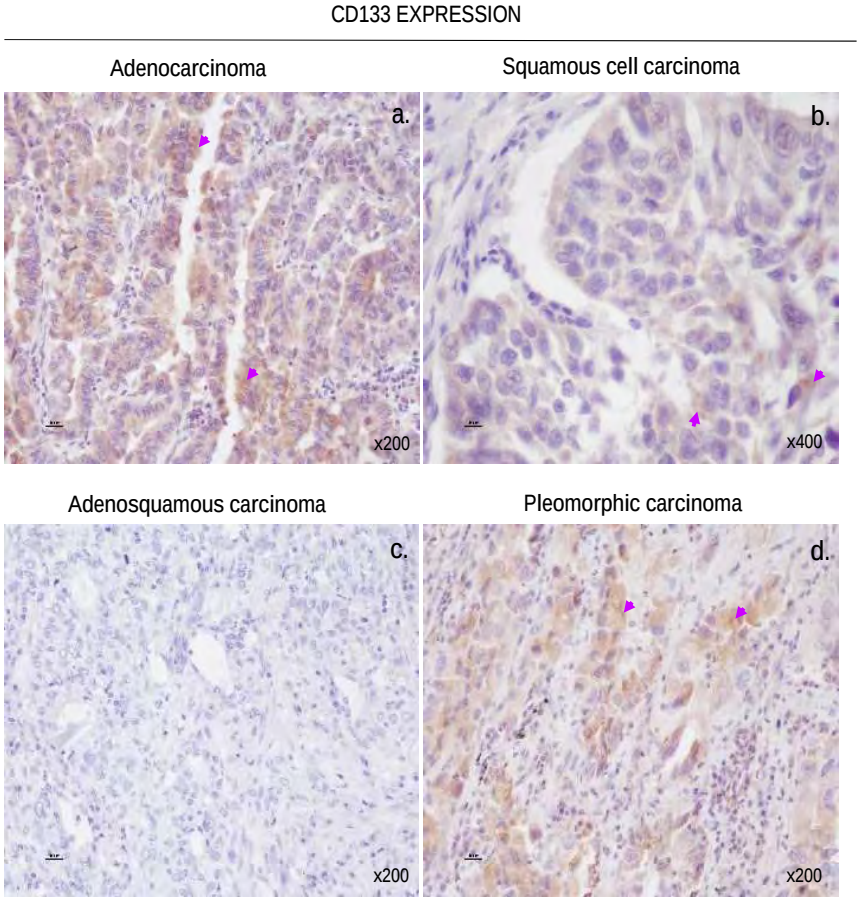
E.


VEGFR2 EXPRESSION
Adenocarcinoma
Squamous cell carcinoma
a.
x400
b.
x200
Adenosquamous carcinoma
Pleomorphic carcinoma
c.
x400
d.
x200

F.


FGFR2 EXPRESSION
Adenocarcinoma
Squamous cell carcinoma
a.
x400
b.
x400
Adenosquamous carcinoma
Pleomorphic carcinoma
c.
x400
d.
x400

G.



NCL, nucleolin; **PDGFRα,** platelet-derived growth factor receptor alfa; **HIF-1α**, hypoxia-inducible factor 1 alpha; **IL-17**, interleukine-17; **VEGFR2**, vascular endothelial growth factor receptor 2; **FGFR2**, fibroblast growth factor receptor 2; **CD133**, prominin-1.

Figure 3.1| Expression of nucleolin and several angiogenic markers in tumor cells of human pulmonary carcinomas.
Representative immunohistochemistry images of **(A)** nucleolin, **(B)** PDGFRα, **(C)** HIF-1α, **(D)** IL-17, **(E)** VEGFR2, **(F)** FGFR2 (**a-d**) and **(G)** CD133 (**a-d,** with negative expression in **c**) expression in different histological types of lung cancer: adenocarcinoma (ADC; **a**), squamous cell carcinoma (SQC; **b**), adenosquamous carcinoma (ADSQC; **c**) and pleomorphic carcinoma (PLMC; **d**). Nucleolin expression was further broken down at the cellular and subcellular level: cancer cells (nuclear, blue head-arrows; and cytoplasm-membrane, orange head-arrows) and stromal endothelial cells (yellow head-arrows). The same applied to the expression of the previous mentioned angiogenic regulators in cancer cells: PDGFRα, **(B)** presented nuclear (blue head-arrows), cytoplasmic (purple head-arrows), and membrane (black head-arrows) expression, while HIF-1α **(C)** and IL-17 **(D)** expression was limited to the first two subcellular compartments. Expression of the remaining angiogenic regulators analyzed was exclusive to the cytoplasm (VEGFR2 **(E)**, FGFR2 **(F)** and CD133 **(G)**).

Table 3.1| Association of extra-nuclear nucleolin and other angiogenic markers with clinicopathological data in pulmonary carcinomas.

CLINICOPATHOLOGICAL STATUS		EXPRESSION FREQUENCY IN HUMAN PULMONARY CARCINOMAS													
Variables	Cases	Extra-nuclear Nucleolin		Angiogenic markers								Hypoxia marker		Stemness marker	
				VEGFR2 (n, %)	*p* value	PDGFR**α** (n, %)	*p* value	FGFR2 (n, %)	*p* value	IL-17 (n, %)	*p* value	HIF-1**α** (n, %)	*p* value	CD133 (n, %)	*p* value
Gender	n=25	NCL (n, %)	*p* value												
Female	7	2 (28.57)	*p* =0.378	7	nd	7	nd	7	nd	6 (85.71)	*p* =0.235	4 (57.14)	*p* =0.084	2 (28.57)	*p* =0.217
Male	18	7 (38.89)		18		18		18		17 (94.44)		15 (83.33)		10 (55.56)	
All	25	9 (36.00)		25 (100.00)		25 (100.00)		25 (100.00)		23 (92.00)		19 (76.00)		12 (70.59)	
Age (years)															
≤55	4	2 (50.00)	*p* =1.000	4	nd	4	nd	4	nd	4 (100.00)	*p* =1.000	3 (75.00)	*p* =1.000	1 (25.00)	*p* =0.593
>55	21	7 (33.33)		21		21		21		19 (90.48)		16 (76.19)		11 (52.38)	
All	25	9 (36.00)		25 (100.00)		25 (100.00)		25 (100.00)		23 (92.00)		19 (76.00)		12 (70.59)	
Smoking Status															
Never	5	2 (40.00)		5		5		5		4 (80.00)		4 (80.00)		3 (60.00)	
Current	9	2 (22.22)	*p* =0.548	9	nd	9	nd	9	nd	9 (100.00)	*p* =0.411	6 (66.67)	*p* =0.712	6 (66.67)	*p* =0.1792
Former	11	5 (45.45)		11		11		11		10 (90.91)		9 (81.82)		3 (27.27)	
All	25	9 (36.00)		25 (100.00)		25 (100.00)		25 (100.00)		23 (92.00)		19 (76.00)		12 (70.59)	
Histologic type															
ADC	12	5 (41.67)		12		12		12		11 (91.67)		10 (83.33)		9 (75.00)	
SQC	6	2 (33.33)	*p* =0.939	6	nd	6	nd	6	nd	6 (100.00)	*p* =0.322	6 (100.00)	*p* =0.001	1 (16.67)	*p* =0.034
PLMC	4	1 (25.00)		4		4		4		4 (100.00)		0 (0.00)		2 (50.00)	
ADSQC	3	1 (33.33)		3		3		3		2 (66.67)		3 (100.00)		0 (0.00)	
All	25	9 (36.00)		25 (100.00)		25 (100.00)		25 (100.00)		23 (92.00)		19 (76.00)		12 (70.59)	
pTNM Stage															
I-IIB	22	8 (36.36)	*p* =0.593	22	nd	22	nd	22	nd	20 (90.91)	*p* =1.000	17 (77.27)	*p* =1.000	12 (54.55)	*p* =0.220
III-IV	3	1 (33.33)		3		3		3		3 (100.00)		2 (66.67)		0 (0.00)	
All	25	9 (36.00)		25 (100.00)		25 (100.00)		25 (100.00)		23 (92.00)		19 (76.00)		12 (70.59)	
Metastasis															
Yes	8	2 (25.00)	*p* =0.081	8	nd	8	nd	8	nd	8 (100.00)	*p* =1.000	7 (87.50)	*p* =0.624	4 (50.00)	*p* =1.000
No	17	7 (41.18)		17		17		17		15 (88.24)		12 (70.59)		8 (47.06)	
All	25	9 (36.00)		25 (100.00)		25 (100.00)		25 (100.00)		23 (92.00)		19 (76.00)		12 (70.59)	

Tumor specimens from patients with different histological types of pulmonar carcinomas: ADC, adenocarcinoma; SQC, squamous cell carcinoma; ADSQC, adenosquamous carcinoma and PLMC, pleomorphic carcinoma. NCL, nucleolin; VEGFR2, vascular endothelial growth factor receptor 2; PDGFRα, platelet-derived growth factor receptor alpha, HIF-1α, hypoxia-inducible factor 1 alpha; IL-17, interleukin-17. Differences were considered statistically significant when $p<0.05$ and nd, not defined, *Fisher's Exact* Test.

3.2.2. Association between extra-nuclear nucleolin and other angiogenic markers expression with clinicopathological data in cancer cells of pulmonary carcinomas

Overexpression of cell surface nucleolin has been indicated as a common feature between tumor development and angiogenesis [176,189,199,219-221]. However, the pattern and importance of extra-nuclear nucleolin expression in pulmonary carcinomas remains poorly understood. Therefore, the association between frequency of extra-nuclear (cytoplasmic-membrane) nucleolin expression and angiogenic markers assessed in the previous section, with clinicopathological data was further analyzed.

As shown in Table 3.1, neither extra-nuclear nucleolin nor VEGFR2, FGFR2, PDGFRα and IL-17 expression frequency related with clinicopathological features, including histopathological type ($p>0.05$, *Fisher's Exact* test). In respect to extra-nuclear nucleolin, this observation was in agreement with previous observations in esophageal squamous carcinoma [30] and in stage II pancreatic ductal adenocarcinoma [73]. Such data further supported nucleolin relevance in lung carcinogenesis and its potential as a valuable therapeutic target. In contrast, the expression frequency of the hypoxia marker, HIF-1α ($p=0.001$), and the cancer stem cell marker, CD133 ($p=0.034$), were dependent on the histological type. HIF-1α presented a high expression frequency in adenosquamous and squamous cell carcinoma samples (100%), as well as in adenocarcinoma (83%). CD133 expression was relevant in adenocarcinoma (75%) and pleomorphic carcinoma (50%).

3.2.3. Extra-nuclear nucleolin, CD31 and VEGFR2 expression in stromal endothelial cells of human pulmonary carcinomas is independent of the histopathological type

Cell surface nucleolin has been validated as a valuable marker for anti-cancer therapies targeting the tumor microenvironment [140,147,150,186]. Being overexpressed in endothelial cells from tumor blood vessels, nucleolin has been suggested as an angiogenic marker in cancer [166,176]. However, only few reports have addressed this issue in patient-derived lung cancer samples [187]. Herein, the relative frequency of expression between nucleolin (Figure 3.1A; yellow head-arrows) and other important players in tumor angiogenesis, as VEGFR2 (Figure 3.2A, green arrows) and CD31 (Figure 3.2B; dark blue head-arrows), including microvessel density formation, in endothelial cells of different human pulmonary carcinoma types was

further analyzed.

As shown in Table 3.2, all three markers presented a high frequency of expression in the tumor endothelial cells of all types of pulmonary carcinoma, with no statistical significant differences ($p>0.05$, *Fisher's Exact* test), thus suggesting to be independent of this clinical parameter (Table 3.2).

Notwithstanding stromal endothelial cells presented a high frequency of expression of CD31 in all samples analyzed, the tumor microvessel density (MVD, based on CD31 expression) revealed differences between the different histological types. A five-fold difference was observed between pleomorphic and adenosquamous carcinomas, presenting the highest and lowest MVD values (Figure 3.2B), respectively, similarly with the trend observed for nucleolin expression in the corresponding endothelial cells (Table 3.2).

Table 3.2 | Frequency of nucleolin, VEGFR2 and CD31 expression in stromal endothelial cells of pulmonary carcinomas.

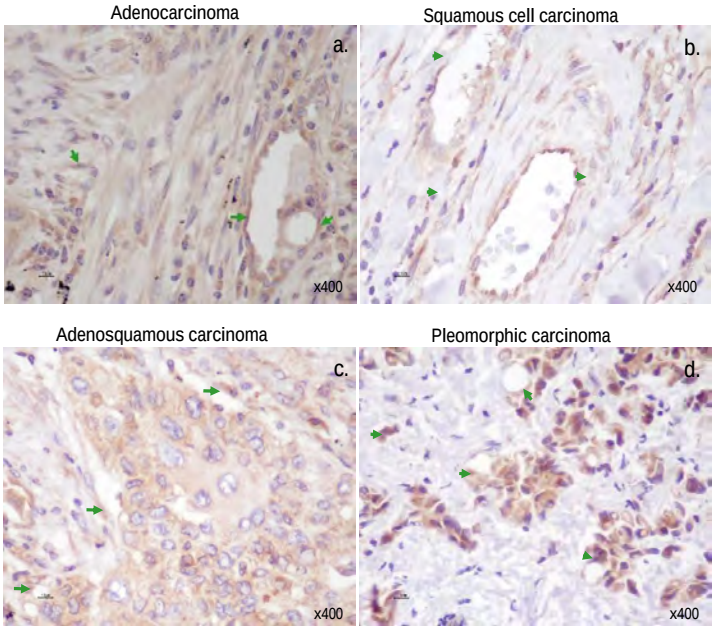
Variable	Cases	NCL		VEGFR2		CD31	
Histological type	n=25	n, %	*p*	n, %	*p*	n, %	*p*
ADC	12	12 (100.00)		11 (92.00)		12 (100.00)	
SQC	6	5 (83.00)	0.202	6 (100.00)	0.770	6 (100.00)	nd
ADSQC	3	2 (67.00)		3 (100.00)		3 (100.00)	
PLMC	4	4 (100.00)		4 (100.00)		4 (100.00)	

Tumor specimens from patients with different histological types of pulmonar carcinomas: **ADC**, adenocarcinoma; SQC, squamous cell carcinoma; **ADSQC**, adenosquamous carcinoma and **PLMC**, pleomorphic carcinoma. **NCL,** Nucleolin; **VEGFR2**, vascular endothelial growth factor receptor 2; **CD31**, cluster of differentiation 31, is a specific endotelial marker. Differences were considered statistically significant when $p<0.05$ and *nd*, not defined, *Fisher's Exact* Test.

Several studies have associated the angiogenic activity of nucleolin with VEGF/R signaling pathway. The autocrine VEGF/VEGFR2 signaling loop that was validated as an essential trait of highly angiogenic lung tumors [35]. VEGF-dependent activation of nucleolin translocation from the nucleus to the cell surface has been observed both in colon cancer cells [31] and endothelial cells [58], being essential in the latter to promote the migration and tubule formation. In the present study, a similar level of nucleolin and VEGFR2 expression was observed among the different histological types, particularly those with the highest microvessel density (adenocarcinoma, squamous cell and pleomorphic carcinomas). These data all together, suggested an association between these two markers that, along with the concomitant expression of CD31, could suggested an involvement of nucleolin (together with VEGFR2) in lung cancer angiogenesis. Moreover, these results indicated different extents

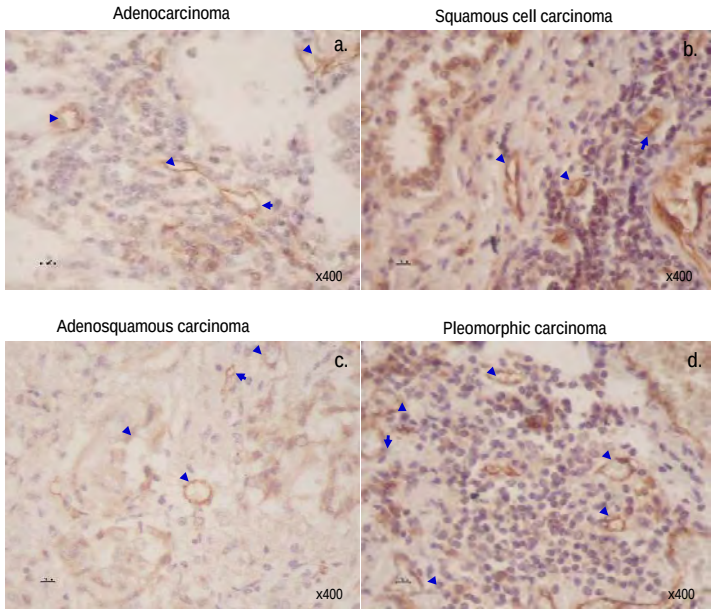
A.

VEGFR2 expression in endotelial cells from tumor blood vessels



B.

CD31 expression in endotelial cells from tumor blood vessels



C.

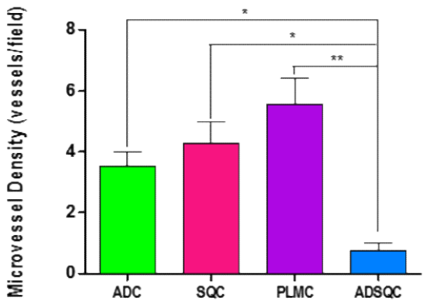


Figure 3.2| VEGFR2 and CD31 expression in stromal endothelial cells and microvessel density in different types of human pulmonary carcinomas.
Immunohistochemical analysis of **(A)** VEGFR2 expression (green arrows), **(B)** CD31 (dark blue head-arrows), and **(C)** extent of microvessel density, MVD (based on CD31 staining), in different pulmonary carcinoma histological types: **a.** Adenocarcinoma (ADC); **b.** Squamous cell carcinoma (SQC); **c.** Adenosquamous carcinoma (ADSQC) and **d.** Pleomorphic carcinoma (PLMC) (*Unpaired t* test, $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$).

of tumor angiogenesis between different types of pulmonary carcinoma, which might have implications in therapeutic decisions, mainly concerning to therapies targeted to the tumor vasculature.

3.2.4. High nucleolin expression in bone metastases

It is well recognized that the majority of pulmonary carcinoma patients are diagnosed at advanced stages, including metastatic disease stage [222], which limits treatment options and efficiency [183,223]. Accordingly, the expression of nucleolin in bone metastatic specimens from lung primary carcinomas was evaluated. As metastatic disease is responsible for more than 90% of cancer-associated deaths in different types of cancer [224], the analysis was extended to the microenvironment of bone metastases arising from primary tumors of diverse histological origins, including lung, breast, colon and thyroid.

All the 20 bone metastatic specimens analyzed showed high immunoreactivity to nucleolin, regardless the histological origin (Figure 3.3.a-d). Staining was observed in different subcellular locations of cancer cells (nuclear, blue arrows; and extra-nuclear, *i.e.*, cytoplasmic-membrane, orange arrows; Figure 3.3.a-d) and in different cell populations (endothelial cells, yellow arrows, Figure 3.3.a and d; inflammatory cells, pink arrows, Figure 3.3.c and d; and cancer-associated fusiform cells; green arrows, Figure 3.3.a, c and d). This pattern was similar with the one observed in the lung primary carcinomas previously studied (Figure 3.1A.a-d). This similarity reinforced the potential of nucleolin as a therapeutic target in lung cancer. Such observations were distinct from observations in esophageal squamous cell carcinomas [30] and in colorectal carcinomas [31]. In these tumors, nucleolin expression in metastases was mainly extra-nuclear, in contrast with the predominant nuclear expression observed in primary tumors. The therapeutic impact of this different pattern of expression remains to be clarified.

Overall, the presence of nucleolin in metastases from different primary origins (lung, breast, colon and thyroid), suggested the potential role of nucleolin in carcinogenesis/ metastization of tumors with different histological origins, thus highlighting its potential as a therapeutic target, besides lung cancer.

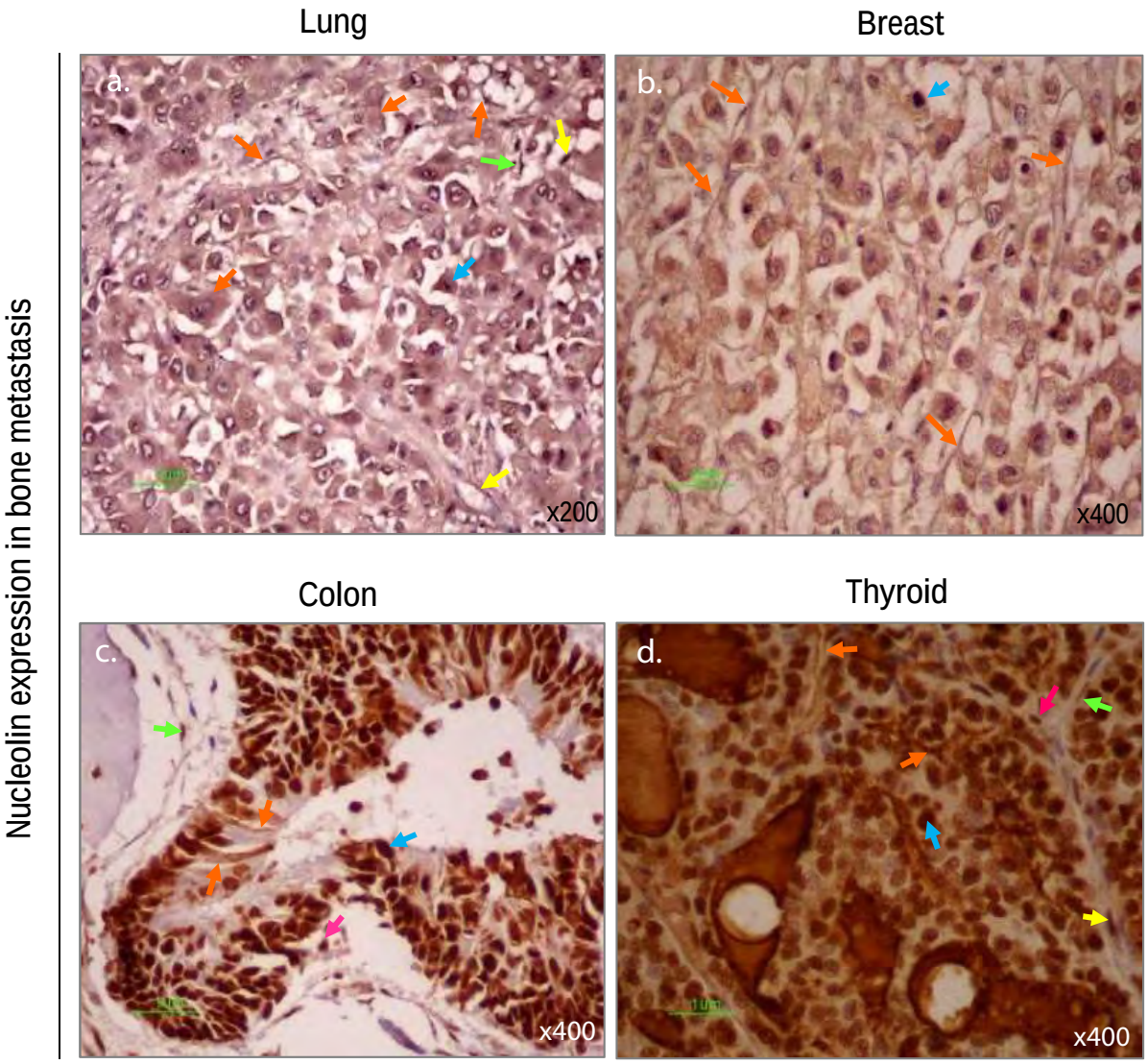


Figure 3.3| Nucleolin expression in bone metastases from human primary tumors of diverse histological origins.
Immunohistochemical analysis of nucleolin expression in bone metastases from diverse human primary tumors: lung **(a)**; breast **(b)**; colon **(c)**; and thyroid **(d)** carcinomas. Distinct subcellular distribution of nucleolin expression was presented in: cancer cells (nuclear, blue arrows; and extra-nuclear, orange arrows; Figure 3.3.a-d). Nucleolin was also identified in endothelial cells (yellow arrows; Figure 3.3.a and d); tumor-infiltrated lymphocytes (pink arrows; Figure 3.3.c and d); and cancer-associated fusiform cells (green arrows; Figure 3.3.a, c and d).

3.3. Conclusion

Lung cancer is a heterogeneous and complex disease, which justifies in part the failure of novel targeted therapies [18]. The tumor heterogeneity reinforces the importance to improve the knowledge on the molecular characteristics of the different types of pulmonary carcinomas, thus allowing the design of targeted therapies with a broad activity. Nucleolin

is consistently overexpressed in different populations in the tumor microenvironment of different histological types of human pulmonary carcinoma samples. The overexpression of nucleolin in stromal endothelial cells, further suggested its involvement in tumor angiogenesis in pulmonary carcinomas.

Importantly, extra-nuclear nucleolin expression appeared to be independent of both the clinicopathological features (including histopathological type) of lung cancer patients, and of the expression of other angiogenic markers described to be implicated in the disease. Accordingly, cell surface nucleolin could be an independent hallmark of lung carcinogenesis.

The potential of nucleolin as a therapeutic target in lung cancer was further reinforced by its similar pattern of expression (both nuclear and extra-nuclear) between primary pulmonary carcinomas and bone metastases with lung primary origin. Such observation was extended to bone metastases with breast, colon and thyroid primary origins.

In conclusion, our findings reinforced extra-nuclear nucleolin as a valuable therapeutic target in primary and metastatic lung cancer.

Chapter 4

CONCLUDING REMARKS & TRANSLATIONAL SIGNIFICANCE

Lung cancer is the leading cause of morbidity and mortality among all cancers. Over the past four decades, the knowledge on the pathogenesis of lung cancer has improved significantly and the treatment of advanced disease has witnessed two major breakthroughs that have changed patient care. The first was the recognition that tumor angiogenesis is a hallmark of cancer, crucial for tumor growth, survival and spread, leading to the development of anti-angiogenic therapies. The second was the recognition that distinct somatic molecular aberrations in tumor genes, correlated with dramatic and durable clinical benefit upon treatment with tyrosine kinase inhibitors.

Unfortunately, this progress has not yet resulted in major changes in lung cancer mortality rates. The clinical use of bevacizumab and other targeted therapies, has made remarkable breakthroughs in the treatment of many types of cancer, including lung cancer. However, clinical experience with anti-angiogenic therapy has shown, even in combination with chemotherapy, limited impact on enhancing overall survival or cancer remission, arising from tumor heterogeneity and plasticity with subsequent drug resistance. This scenario claims for better therapeutic strategies against lung cancer.

Nanomedicine represents an innovative field with potential for improving cancer treatment, with a first generation of nanoparticles of diverse nature, including liposomes, already approved for clinical use. Nanotechnology-based strategies can be easily tailored according to the biology/barriers of the tumor to be targeted. The covalent coupling of internalizing ligands at the end of PEG-grafted liposomes is a strategy that upon targeting a similar receptor within the tumor microenvironment, could be a good match to the new challenges posed by the aforementioned tumor heterogeneity and plasticity.

One of the goals of this project was to validate nucleolin as a therapeutic target for F3 peptide-targeted liposomes, containing doxorubicin, in bevacizumab-resistant human lung cancer cell lines (A549, H1975 and H441). **Nucleolin demonstrated to be a functionally active target in lung cancer cells**, as it enabled binding and internalization of liposomes targeted by the nucleolin-binding F3 peptide, in a ligand-specific manner and in an extent 34- to 170-fold higher than the one observed for the non-targeted counterparts. The nucleolin-mediated specific targeting led to a maximum of 19-fold increase in doxorubicin cytotoxicity delivered by F3 peptide-targeted liposomes, for incubation times as short as 1 h, relative to the one delivered by the tested control liposomes, and regardless the nature of bevacizumab resistance.

Subcutaneous adenocarcinoma mouse models of the previously tested bevacizumab-resistant cell lines were further generated. Surprisingly, a tumor stroma that was 5% lower

than the total tumor volume was observed, lacking, particularly, a tumor vascular network. This was in strong contrast with the rich stroma observed in patient-derived pulmonary carcinomas samples. Moreover, the extent of nucleolin expression was significantly lower and restricted to the nucleus, relative to the nuclear and extra-nuclear (cytoplasm-membrane) expression observed in human pulmonary carcinomas. Overall, these data suggested that such animal models were not appropriate for testing nucleolin-based targeting strategies.

Before the absence of an adequate animal model, the demonstration of the therapeutic potential of nucleolin-based targeting strategy was performed at two different levels. E*x vivo* autoradiography-binding studies, in patient-derived pulmonary carcinoma specimens, revealed a higher extent of binding of radiolabeled (^{99m}Tc)-F3 peptide-targeted liposomes, relative to non-targeted liposomes. In addition, the immunohistochemical analysis of different histopathological types (adenocarcinoma, squamous cell, adenosquamous and pleomorphic carcinomas) of patient-derived pulmonary carcinoma specimens, demonstrated a **97% frequency of nucleolin overexpression**. The protein **was expressed at different subcellular localizations** (**nuclear and extra-nuclear - cytoplasm-membrane**) and, importantly, it **was overexpressed by different cell populations within the tumor microenvironment, besides cancer cells**, such as cancer-associated fusiform cells (which could include a special set of fibroblasts), tumor-infiltrating lymphocytes and endothelial cells. This could imply that **the subcellular distribution of nucleolin potentially changes during lung carcinogenesis**. As the protein is enrolled in several essential physiological processes, it was not surprising its expression at the nuclear level, being diffuse in non-neoplastic lung tissue. Nonetheless, the extent of expression and the subcellular localization of nucleolin differed significantly between normal lung epithelium and tumor tissue. These findings supported the hypothesis that **extra-nuclear nucleolin is essential for lung cancer development, survival and progression**.

To gain further insights on the importance of nucleolin in lung cancer, its extra-nuclear expression was studied in more detail. Data demonstrated that extra-nuclear expression of nucleolin in cancer cells was independent of both clinicopathological features (including histological stype) and expression of other angiogenic factors. Besides cancer cells, expression of extra-nuclear was identified in several cell populations of the tumor stroma, including endothelial cells, which is consistent with its angiogenic role described by several authors. These data suggested that extra-nuclear **nucleolin could be an independent angiogenic marker in these patients**.

As the majority of lung cancer patients is diagnosed at advanced stage of disease, which

highly compromises treatment efficacy, the expression of nucleolin in bone metastases from lung cancer patients as well as from other primary origins, was further investigated. A similar pattern of nucleolin expression between pulmonary primary carcinomas and bone metastases with lung primary origin was identified. This result could support **the potential use of a nucleolin-specific targeted strategy against metastases, besides the primary tumor.**

Overall, these results support the therapeutic relevance of both nucleolin as a molecular target in lung cancer, including in the tumor microenvironment of primary pulmonary carcinomas and bone metastatic sites, as well as of nucleolin-based targeting strategies.

Notwithstanding the promising results, **future *in vivo* evidence and validation** of the targeting strategy is demanded. Accordingly, as future work it will be mandatory the development of an *in vivo* model that mimics the lung tumor microenvironment, being patient-derived xenografts an attractive option.

Chapter 5

MATERIALS & METHODS

5.1. Materials

Doxorubicin hydrochloride (Dox) was purchased from IdisPharma (UK). Calcein, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 2-(N-Morpholino) ethanesulfonic acid (MES), Disodium ethylenediaminetetraacetate dihydrate (EDTA), *Trizma®Base*, Sephadex G-50, ammonium sulfate, sodium chloride, 3β-hydroxy-5-cholestene-3-hemisuccinate (CHEMS), cholesterol (CHOL) and Resazurin sodium salt were purchased from Sigma-Aldrich (USA). The lipids 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3 phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2k}), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG_{2k}-maleimide), L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium Salt) (Egg-Transphosphatidylated, Chicken) (Egg Liss Rhod PE) were purchased to Avanti Polar Lipids (USA). F3 (KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK) and the non-specific (NS) (ARALPSQRSR) [167] peptides were custom synthesized by Genecust (Luxembourg).

5.1.1. Cell lines

A549 (ATCC® CCL-185™), H1975 (ATCC® CRL-5908™) and H441 (ATCC® HTB-174™) human lung cancer cell lines (ATCC, USA), were cultured in RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% (v/v) of heat-inactivated Fetal Bovine Serum (FBS) (Invitrogen, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Lonza, Switzerland) and maintained at 37°C in a 5% CO_2 atmosphere.

5.1.2. Patients and surgical samples

In this study, a series of 58 human pulmonary carcinomas, classified according with the World Health Organization (WHO) 2015 histological classification and the new International Association for the Study of Lung Cancer, the American Thoracic Society and the European Respiratory Society (IASLC/ATS/ERS) classification, were selected from the archive of the Pathology Service of the Coimbra University Hospital (CHUC) according to the following criteria: newly-diagnosed cancer of the lung without previous treatment and histologically-confirmed primary tumor after the local immunohistochemical panel (CK7, TTF1, CK5.6, CD56, vimentin, Ki67). Adjacent non-neoplastic lung tissue was also obtained for each case.

The histological grade and clinical stage of the tumors were defined according to the latest Tumor-Node-Metastasis (TNM) classification of the International Union Against Cancer (UICC). Four different histological types [20] were considered, including: adenocarcinomas (ADC), which were subtyped in - vimentin positive and thyroid transcription factor-1 negative (ADC $VIM^{+}TTF1^{-}$), vimentin and thyroid transcription factor-1 positive (ADC $VIM^{+}TTF1^{+}$), vimentin negative and thyroid transcription factor-1 positive (ADC $VIM^{-}TTF1^{+}$), and vimentin and thyroid transcription factor-1 negative (ADC $VIM^{-}TTF1^{-}$); squamous cell carcinomas (SQC), which were subtyped in - cytokeratin 7 negative (SQC $CK7^{-}$) and positive (SQC CK7); adenosquamous carcinomas (ADSQC) and pleomorphic carcinomas (PLMC).

Clinical data such as age, gender, smoking status, node metastases and disease staging were also registered (Table 2). The principles of Helsinki Declaration were respected and the study was performed according to the rules of the Ethics Committee of Faculty of Medicine of the University of Coimbra.

5.2. Methods

5.2.1. Preparation of liposomes

PEGylated pH-sensitive liposomes, with or without doxorubicin, were composed of DOPE:CHEMS:DSPC:CHOL:DSPE-PEG_{2k} at 4:2:2:2:0.8 molar ratio, as well as in some experiments with 1 mol% RhoD-PE lipid, relative to total lipid. Dried lipid films were hydrated at 60°C with ammonium sulfate (pH 8.5) and the resulting liposomes were extruded through 80 nm pore size polycarbonate membranes using a LiposoFast Basic mini extruder (Avestin, Canada). The buffer was exchanged in a Sephadex G-50 gel column equilibrated with Trizma®Base sucrose (10%, w/v, buffered at pH 9.0). Remote encapsulation of doxorubicin (18 mol% of doxorubicin relatively to total lipid) was carried out through the ammonium sulphate gradient method, upon incubation with liposomes for 1.5 h at 60°C [225]. Non-encapsulated doxorubicin was removed using a Sephadex G-50 gel column equilibrated with 25 mM HEPES, 140 mM NaCl buffer (HBS, pH 7.4). To further prepare F3 peptide-targeted liposomes, DSPE-PEG_{2k}-F3 conjugate was produced. Briefly, thiolated derivative of F3 peptide was generated by reaction with 2-iminothiolane (Sigma-Aldrich, USA) in 25 mM HEPES, 140 mM NaCl, 1 mM EDTA buffer (pH 8.0), for 1 h at room temperature, in an inert N_2 atmosphere. Thiolated derivatives were then incubated overnight at room temperature with DSPE-PEG_{2k}-maleimide micelles in 25 mM HEPES, 25

mM MES, 140 mM NaCl, 1 mM EDTA (pH 7.0). The resulting micelles of DSPE-PEG_{2k}-peptide conjugates were postinserted [226] onto the liposomal membrane at 2 mol% relative to total lipid (TL), upon incubation with pre-formed liposomes, for 1 h at 50°C.

To prepare calcein-loaded liposomes, the lipid film was hydrated with a 40 mM isosmotic calcein solution in 25 mM HEPES, 140 mM NaCl buffer (pH 7.4), and extruded as described above. Following removal of the excess of calcein, through a Sephadex-G50 column equilibrated with 25 mM HEPES, 140 mM NaCl buffer (pH 7.4), liposomes were immediately submitted to the post-insertion procedure as previously described.

For the autoradiography studies, F3 peptide-targeted or non-targeted liposomes were labelled with ^{99m}Tc-HMPAO as previously described [181].

5.2.2. Characterization of liposomes

Liposomal mean size and polydispersion index (PDI) were measured by light scattering with a N5 particle size analyzer (Beckman Coulter, USA). Final total lipid concentrations were determined upon quantification of cholesterol using Infinity® Cholesterol kit (ThermoScientific, USA). Encapsulated doxorubicin was assayed at 492 nm from a standard curve, after liposomal solubilization with 96% absolute ethanol, and the loading efficiency (%) was further calculated.

5.2.3. *In vitro* cellular association studies by flow cytometry

Different human lung cancer cells (2×10^5) were seeded in 24-well culture plates and cultured in RPMI 1640 overnight. Cells were then incubated with different formulations of rhodamine-labeled liposomes at 4 or 37°C, for 1 or 4 h, washed with phosphate buffer saline pH 7.4 (PBS), detached with dissociation buffer and immediately run in a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA) for detecting cell-associated rhodamine (FL2-H). A total of 20,000 events were collected and data were analyzed with the *CellQuest™ Pro* software.

5.2.4. Assessment of co-localization between liposomal and lysosomal markers

Twenty four hours after seeding twenty-thousand H1975 or A549 cancer cells on a µ-slide 8-well ibiTreat plates (Ibidi, Germany), incubation with targeted and non-targeted

pH-sensitive liposomes, loaded with a self-quenched concentration of calcein (40 mM), took place at 37°C, for 1 h. Hereafter, cells were gently washed with HBSS and lysosomes further stained upon incubation with 100 nM *LysoTracker* Red (Invitrogen, USA) for 30 min, at 37°C. Following washing with HBSS at 37°C, live cells were immediately visualized with a plan Apochromat 63/1.4× oil immersion objective, upon excitation at 488 (with argon/2 laser), 561 (with DPSS laser) and 633 nm (with helium–neon laser) for calcein, *Lysotracker Red* and differential interference contrast (DIC), respectively. Images were acquired and analyzed using the LSM 510 Meta software (Zeiss, Germany). All instrumental parameters pertaining to fluorescence detection and images analyses were held constant to allow sample comparison.

5.2.5. Competitive inhibition study

Following adherence of 2×10^5 lung cancer cells *per* well, in a 24-well flat bottom plate, incubation with 2 mM (total lipid, TL) of non-fluorescently labeled F3 peptide-targeted liposomes for 1 h, at 4 or 37°C was performed. A control experiment was carried out in parallel without pre-incubation with F3 peptide-targeted liposomes. Afterwards, rhodamine-labeled F3 peptide-targeted liposomes (at 0.6 mM TL/well) were added and further incubated for 1 h at the mentioned temperatures. Cellular association was assessed by measuring the levels of cell-associated rhodamine (RhoD) fluorescence signal by flow cytometry. Results were shown as the mean value of duplicates from three independent experiments.

5.2.6. *In vitro* cytotoxicity studies

A549, H1975 and H441 cells were seeded in 96-well plates at 6, 7 or 8 $\times10^3$ cells *per* well, respectively, and further incubated with serially diluted concentrations of doxorubicin (at a maximal concentration of 100 μM), free or encapsulated in liposomes either non-targeted (L[Dox]), or targeted by a non-specific peptide (NS-L[Dox]) or the F3 peptide (F3-L[Dox]), for 1, 4 and 24 h at 37°C, in an atmosphere of 95% humidity and 5% CO_2. After the corresponding incubation times, cell culture medium was exchanged for fresh one and the experiment was prolonged for a total of 96, 120 or 144 h, for A549, H1975 or H441 cells, respectively. Cell viability was then evaluated by *Resazurin Reduction* test [227], at 570 (reduced form) - 610 (oxidized form) nm in a Spectrophotometer SPECTRA max PLUS 384, as previously described [228]. Data represented the mean ± SEM for each concentration

tested, of three independent experiments, each done in triplicate. IC_{50} was determined from dose-response curves, using *The Median Effect Equation* [179].

5.2.7. Assessing nucleolin expression by the Labeled Streptavidin-Biotin (LSAB) staining method

5.2.7.1. Immunohistochemistry

Representative sections of the carcinomas and their patterns were submitted to immunohistochemical analysis. For histological preparations, the tumors were fixed in 10% formalin, processed by standard methods, and then embedded in paraffin. Tumor samples were sectioned in 3 μm thickness slices, placed on coated slides (SuperFrost Ultra Plus® microscope slides) and dried overnight for posterior histopathological examination by Hematoxylin & Eosin (H&E) and for immunostaining of nucleolin, VEGFR2, FGFR2, PDGFRα, HIF-1α (Thermo Fisher Scientific Inc; UK), IL-17 (Abcam; Cambridge; UK) and CD133 (Miltenyi Biotec Ltd; UK) analysis. After deparaffinization and rehydration, heat-induced antigen retrieval was performed, according to antibody specific requirements, before immunostaining protocol. Endogenous peroxidase activity was quenched upon incubation in 3% diluted hydrogen peroxide for 15 min. For blocking nonspecific binding with primary antibodies, Ultra V Block (Ultra Vision Kit; TP-125-UB; Lab Vision Corporation; Fremont CA; USA) was used. Tested tissues were incubated with primary anti-nucleolin monoclonal antibody (Thermo Scientific, at a 1:50 dilution) for 30 min at room temperature, and further washed with phosphate-buffered saline (PBS) (Ultra Vision; TP-125- PB; Lab Vision Corporation; Fremont CA; USA). Slides were then incubated with biotin-labeled secondary antibody (Ultra Vision Kit; TP-125-BN; Lab Vision Corporation; Fremont CA; USA) for 15 min. Primary antibody binding was localized in tissues using peroxidase-conjugated streptavidin (Ultra Vision Kit; TP-125-HR; Lab Vision Corporation; Fremont CA; USA). 3,3-diaminobenzidine tetrahydrochloride (DAB) (RE7190-K; Novocastra Laboratories Ltd, Newcastle, United Kingdom) was used as chromogen, according to manufacturer's instructions. Haematoxylin of Gill was used to counterstain the slides, which were then dehydrated and mounted. In parallel, known positive (nucleolin-positive human breast carcinoma tissue) and negative (nucleolin-positive human lung carcinoma tissue without primary antibody incubation) controls were used.

Analysis of stained tumor sections was performed by an experienced pathologist using

a microscope Nikon H600L with Digital Camera DXM 1200F (Nikon, Germany). Expression of nucleolin was quantified based on the extent of staining (percentage of positive cells: 0-100%) and the intensity of staining (graded on a scale of 0-3: 0, no staining; 1, weak staining; 2, moderate staining and 3, strong staining). A semi-quantitative global score was obtained by multiplying the grades of both extent and intensity of staining. This global score was translated in a semi-quantitative final score, in which: 0-10% = 0 (-, negative); 11-100% = 1 (+, low positive); 101-200% = 2 (++, positive) and 201-300% = 3 (+++, high positive).

5.2.7.2. Immunocytochemistry

Cancer cells were washed with PBS and fixed with 95% ethanol and further incubated for 15 min with 3% diluted hydrogen peroxide to neutralize endogenous peroxidase activity. Non-specific binding of primary antibodies was blocked with Ultra V Block (Ultra Vision Kit, Lab Vision Corporation, Thermo Scientific). Cells were incubated with monoclonal mouse anti-human antibody against nucleolin (at 1:50 dilution, Thermo Scientific), in a humidified chamber at room temperature. After washing with PBS, slides were incubated with a biotin-labeled secondary antibody for 15 min. Primary antibody binding was revealed with peroxidase-conjugated streptavidin and 3,3-diaminobenzidine tetrahydrochloride (DAB), according to manufacturer's instructions. Slides were counterstained with hematoxylin of Gill and then dehydrated and mounted. Adequate positive and negative controls were used throughout. The stained sections were observed with a microscope Nikon H600L with Digital Camera DXM 1200F (Nikon, Germany) and analyzed by an experienced pathologist.

5.2.8. Immunohistochemical staining of CD31 and determination of microvessel density (MVD)

A CD31 antibody (polyclonal; Abcam) diluted at 1:200 was used for immunohistochemical staining in tissue by the LSAB method. MVD was expressed as the number of microvessels *per* field. A single microvessel was defined as any CD31 immunostained endothelial cell that was separated from adjacent tumor cells and other connective tissue elements. Large vessels with thick muscular walls were not counted, and the presence of a lumen was not necessary for scoring as a microvessel. In each sample, four areas were randomly identified and microvessels were counted at high power (magnification, x400). Average microvessel counts of the four selected fields were recorded.

Table 5.1 | Antibody optimization for immunostaining experiments.

Antibody	Company	Function	Clonality	Host	Specie Reactivity	Concentration/Dilution	Incubation time	Pre-treatment (Heat-mediated Method)
NCL	ThermoScientific	angiogenic marker	monoclonal	mouse	Human	20 µg/ml (1:50)	30min	citrate pH 6, 20 min
					Mouse	20 µg/ml (1:50)	30min	citrate pH 6, 20 min
IL-17	Abcam	angiogenesis/resistance	polyclonal	rabbit	Human	10 µg/ml (1:100)	1h	citrate pH 6, 20 min
					Mouse	10 µg/ml (1:100)	30min	citrate pH 6, 20 min
CD31	Abcam	vasculature/MVD	polyclonal	rabbit	Human	5 µg/ml (1:200)	30min	citrate pH 6, 20 min
					Mouse	5 µg/ml (1:200)	30min	citrate pH 6, 20 min
CD133	Miltenyi Biotec	stemness marker	monoclonal	mouse	Human	20 µg/ml (1:50)	30min	EDTA pH 8, 20 min
					Mouse	20 µg/ml (1:50)	30min	EDTA pH 8, 20 min
VEGFR2	ThermoScientific	angiogenic marker	polyclonal	rabbit	Human	20-10 µg/ml (1:50-1:100)	30min-1h	EDTA pH 8, 20 min
					Mouse	10 µg/ml (1:100)	1h	EDTA pH 8, 20 min
FGFR2	ThermoScientific	angiogenic marker	polyclonal	rabbit	Human	10 µg/ml (1:100)	30min-1h	EDTA pH 8, 20 min
					Mouse	10 µg/ml (1:100)	30min-1h	EDTA pH 8, 20 min
HIF-1α	ThermoScientific	hypoxic factor	monoclonal	mouse	Human	10 µg/ml (1:100)	1h	EDTA pH 8, 20 min
					Mouse	10 µg/ml (1:100)	1h	EDTA pH 8, 20 min
PDGFRα	ThermoScientific	angiogenesis/resistance	polyclonal	rabbit	Human	10 µg/ml (1:100)	1h	EDTA pH 8, 20 min
					Mouse	10 µg/ml (1:100)	1h	EDTA pH 8, 20 min

5.2.9. Lung cancer subcutaneous tumor xenografts

Lung cancer subcutaneous tumors were generated upon subcutaneously inoculation of 1 million of human lung adenocarcinoma cells (A549, H1975 or H441) in the flank of 8-weeks old male swiss nude (Crl:NU(Ico)-Foxn1^{nu} strain, Charles River Laboratories International, Inc) mice. Tumors with a mean volume of 200 to 300 mm^3 were further processed by standard methods, aiming at staining for nucleolin and tumor vessels and for autoradiography studies. All animal experiments were conducted according to accepted standards of animal care (2010/63/EU directive and Portuguese Act 113/2013).

5.2.10. Association of different ^{99m}Tc-labeled liposomal formulations to sections of patient-derived pulmonary carcinomas

Tumor specimens from lung cancer patients were rapidly removed and rinsed in ice-cold saline and immediately snap-frozen in liquid nitrogen and stored at -80°C until sectioning. Tumor slices (12-15 µm thickness) were cut (two adjacent slices *per* condition - one for autoradiography and the other for H&E analysis) at -20°C, thaw-mounted onto SuperFrost Plus Microscope slides (Inopat), dried at 4°C under negative pressure for 2 h and further analysed.

The binding assay with different liposomal formulations was carried out according to the method of Przedborski *et al.* [229], with slight modifications. Tumor sections were pre-incubated in 170 mM Tris-HCl buffer (pH 7.6, with 5 mM $MgCl_2$), 0.25% (w/v) bovine serum albumin (BSA) for 10 min at room temperature. Then, the sections were incubated with a solution of ^{99m}Tc-F3-L or ^{99m}Tc-L (100 µCi in Tris-HCl buffer with 1% BSA) for 1 h, at room

temperature. After incubation, the sections were rinsed twice in cold Tris-HCl buffer (with 25% BSA) and pure Tris-HCl buffer for 5 min, and finally rinsed with cold distilled water. The sections were air-dried and exposed to phosphor imaging screen (Kodak Phosphor Screen BAS-IP MS, GE Healthcare - Life Sciences) overnight. Images were revealed in a Typhoon FLA 9500 biomolecular imager (GE Healthcare - Life Sciences).

5.2.11. Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was performed using *one-way ANOVA* and *Kruskal-Wallis* test or *two-way ANOVA*, following by *Bonferroni's* post-test. In intergroup comparisons, *Unpaired t* test was used to determine statistical significance. To compare nucleolin expression with various clinicopathological parameters, statistical analysis was performed using the *Fisher's Exact* test. Correlations between different markers were analyzed through *Sperman's* test. Differences were considered statistically significant when *p* value was less than 0.05.

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