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Utilisation de nanoghosts dérivés de cellules souches mésenchymateuses pour le ciblage de cellules tumorales du cancer du poumon résistantes aux inhibiteurs de tyrosine kinases

Sandra Pagano

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THÈSE

En vue de l'obtention du
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE
Délivré par l'Université Toulouse 3 - Paul Sabatier

Présentée et soutenue par
Sandra PAGANO

Le 21 mars 2022

**Utilisation de nanoghosts dérivés de cellules souches
mésenchymateuses pour le ciblage de cellules tumorales du
cancer du poumon résistantes aux inhibiteurs de tyrosine kinases**

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Thèse dirigée par

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Jury

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M. Olivier CALVAYRAC, Co-directeur de thèse

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List of abbreviations

ABL	Abelson
AGK	Acylglycerol Kinase
ALDH1A1	Aldehyde Dehydrogenase 1 family member A1
ALK	Anaplastic Lymphoma Kinase
AML	Acute myelogenous leukemia
ASC	Adipose-derived MSC
ATF2	Activating Transcription Factor 2
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine TriPhosphate
BAMBI	BMP And Activin Membrane Bound Inhibitor
BCR	Breakpoint Cluster Region protein
BMF	Bcl2 Modifying Factor
BM-MSC	Bone-Marrow derived MSC
BMP	Bone Morphogenetic Protein
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B
CAA	Carcinogenic Associated Adipocyte
CAF	Carcinogenic Associated Fibroblast
CCDC6	Coiled-Coil Domain Containing 6
CCL	C-C Motif Chemokine Ligand
CCR	C-C chemokine receptor
CD	Cluster of Differentiation
CFU-F	Colony-Forming Unit Fibroblasts
CML	Chronic myeloid Leukemia
CM-MSC	MSC Conditioned Medium
CO2	Carbon Dioxide
CoxII	Cytochrome c OXidase subunit II
CXCL	C-X-C motif Chemokine Ligand
CXCR	C-X-C Chemokine Receptor
DNA	DeoxyriboNucleic Acid
DNA-PK	DNA-dependent Protein Kinase
DSB	Double Strand Breaks
DTC	Drug Tolerant Cells
DTEC	Drug Tolerant Expanded Cells
DTP	Drug Tolerant Persisters
E-Caherin	Epithelial Cadherin
EGF	Epidermal Growth Factor
EGFR	EGF Receptor
EML	Echinoderm Microtubule-associated protein-Like
EMT	Epithelial-to-Mesenchymal Transition
ERCC	Excision Repair Cross-Complementation
ERK	Extracellular signal-Regulated Kinases
FABP4	Fatty Acid Binding Protein 4
FAK	Focal Adhesion Kinase

FAS	FAS cell surface death receptor
FASL	FAS Ligand
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FOXS1	Forkhead Box S1
F-RHOB	Farnesylated RHOB
FTI	FarnesylTransferase Inhibitors
GAP	GTPase Activating Protein
GDI	Guanosine nucleotide Dissociation Inhibitors
GDP	Guanosine DiPhosphate
GEF	Guanine Nucleotide Exchange Factor
GG-RHOB	GeranylGeranylated RHOB
GJIC	Gap Junctional Intercellular Communication
GOPC	Golgi Associated PDZ And Coiled-Coil Motif Containing
GTP	Guanosine Triphosphate
H2O2	Hydrogen Peroxid
HER	Human Epidermal Growth Factor Receptor
HG	Hedgehog
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-Inducible Factor
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	InterCellular Adhesion Molecule
IDO	Indoleamine 2,3-Dioxygenase
IGF	Insulin-like Growth Factor-1
IGF1R	Insulin-like Growth Factor-1 Receptor
IL	InterLeukin
ISCT	International Society of Cellular Therapy
JAK	JANus Kinase
JNK	c-Jun N-terminal Kinases
KDM5A	Lysine (K)-specific DeMethylase 5A
KRAS	Kirsten Ras Sarcoma 2 viral oncogene homolog
MAPK	Mitogen-Activated Protein Kinase
miRNA	micro Ribonucleic Acid
MMP	Matrix MetalloPeptidase
mRNA	messenger Ribonucleic Acid
MSC	Mesenchymal Stem/Stromal Cells
N-Cadherin	Neural Cadherin
NCOA4	Nuclear receptor COActivator 4
NG	Nanoghosts
NG2	Neural/Glial antigen 2
NG-C3	C3-exoenzyme encapsulated in NG
NRAS	Neuroblastoma RAS viral oncogene homolog
NSCLC	Non Small Cell Lung Cancer
NTRK	Neurotrophic Tyrosine Receptor Kinase
OGG1	8-OxoGuanine DNA Glycosylase 1
p53BP1	phospho-53 Binding Protein 1

PDGF	Platelet-Derived Growth Factor
PDGFR	PDGF Receptor
pDNA	plasmidDNA
PEAK1	Pseudopodium-Enriched Atypical Kinase 1
PEX	PEroXin
PFS	Progression Free Survival
PI3K	phosphatidylinositol 3-kinase
PI3KCA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
pPEX	PEX pDNA
PTEN	Phosphatase and TEnsiN homolog
RB	RetinoBlastoma
RB1	RB transcriptional corepressor 1
RHO	Ras Homolog Family
RHOB	RHO Member B
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinases
SCLC	Small Cell Lung Cancer
SDF1 α	Stromal cell-Derived Factor 1 α
STAT	Signal Transducer And Activator Of Transcription
sTRAIL	soluble Tumour-necrosis-factor Related Apoptosis Inducing Ligand
TACC3	Transforming Acidic Coiled-Coil containing protein 3
TEAD	TEA Domain transcription factor
TGF	Transforming Growth Factor
TIC	Tumour Initiating Cells
TIMP	TIMP Metallopeptidase Inhibitor 1
TKI	Tyrosine Kinase Inhibitor
TNF	Tumour Necrosis Factor
TNM	Tumour, Node and Metastasis
TPM	TroPoMyosin
VCAM	Vascular Cell Adhesion Molecule
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF Receptor
VLA-4	Very Late Antigen-4
YAP	Yes-Associated Protein
ZEB	Zinc finger E-Box binding homeobox
α SMA	α -Smooth Muscle Actin

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INTRODUCTION

1 Lung cancer

1.1 Epidemiology

1.1.1 Worldwide

According to the GLOBOCAN 2020 study (Sung et al., 2021) conducted on 36 cancers in 185 countries, lung cancer is the second most frequently diagnosed cancer, after breast cancer in women, accounting for 19.3 million new cases (11.4% of all cancers) and is the leading cause of cancer-related deaths with 9.9 million deaths (18.0%), all sexes combined (Figure 1).

In men, it is the most frequently diagnosed cancer with 10.1 million new cases (14.3%) but also the deadliest, with 5.5 million deaths (21.5%) (Figure 1). It is the most detected cancer (in 36 of the 185 countries surveyed) in many Asian countries, such as Russia, China or Indonesia, but also in Eastern European countries, such as Turkey, Ukraine or Poland, and in North Africa (Libya, Morocco, Algeria and Tunisia). It is the leading cause of cancer deaths in 93 of the 185 countries surveyed. This is the case for all countries in Europe (except Sweden) and North America, and for most countries in Asia, South America and North Africa.

In women, it is the third most frequently diagnosed cancer, after breast and colorectal cancer, with 9.2 million new cases (8.4%) and the second deadliest, after breast cancer, with 4.4 million deaths (13.7%) (Figure 1). It is the most frequently diagnosed cancer in North Korea. It is the leading cause of cancer deaths in 25 of the 185 countries surveyed. This is particularly the case for the majority of North American countries, for Northern European countries, for Australia and China.

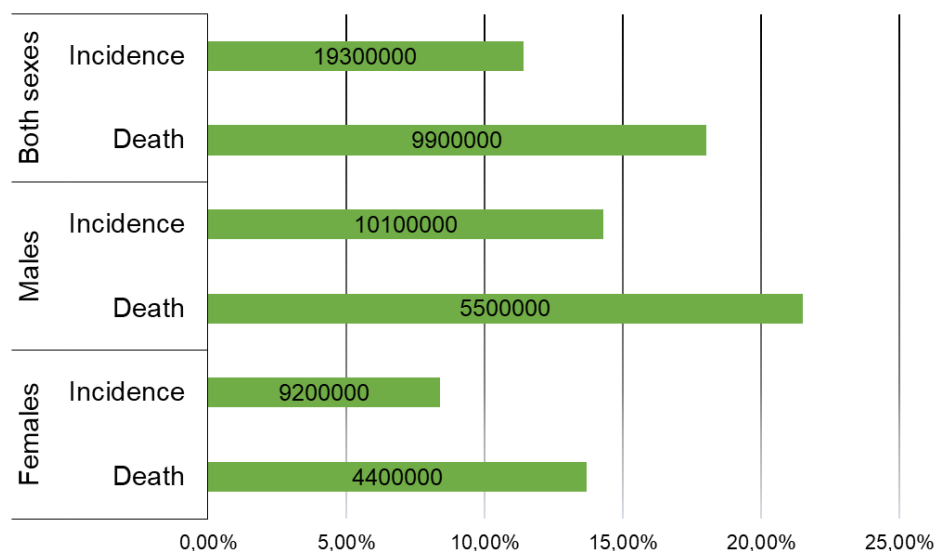


Figure 1 : Worldwide new cases and deaths for lung cancer. GLOBOCAN 2020 data on global lung cancer incidence and mortality in the general population, in males and females. Each histogram shows the number of cases and the length depends on the percentage of all cancers combined.

1.1.2 In Europe

A study conducted in 40 European countries (Ferlay et al., 2018) showed that lung cancer is the second most diagnosed cancer after colorectal cancer with 470,000 new cases (12%). It is the leading cause of cancer deaths with 387,900 deaths (20.0%) (Figure 2).

In men, it is the second most frequently diagnosed cancer, after prostate cancer, with 311 800 new cases (15.1%) (Figure 2). Lung cancer is the most diagnosed cancer in 13 of the 40 European countries surveyed. It is the first cause of cancer deaths, with 267 300 deaths (24.8%) and is the leading cause of cancer deaths in 39 of the 40 European countries surveyed, Sweden being the exception.

In women, it is the third most frequently diagnosed cancer, after breast and colorectal cancer, with 158 200 new cases (8.5%) and the second cause of cancer deaths, after breast cancer, with 120 600 deaths (14.2%) (Figure 2). Lung cancer is the leading cause of cancer deaths in 15 of the 40 European countries surveyed

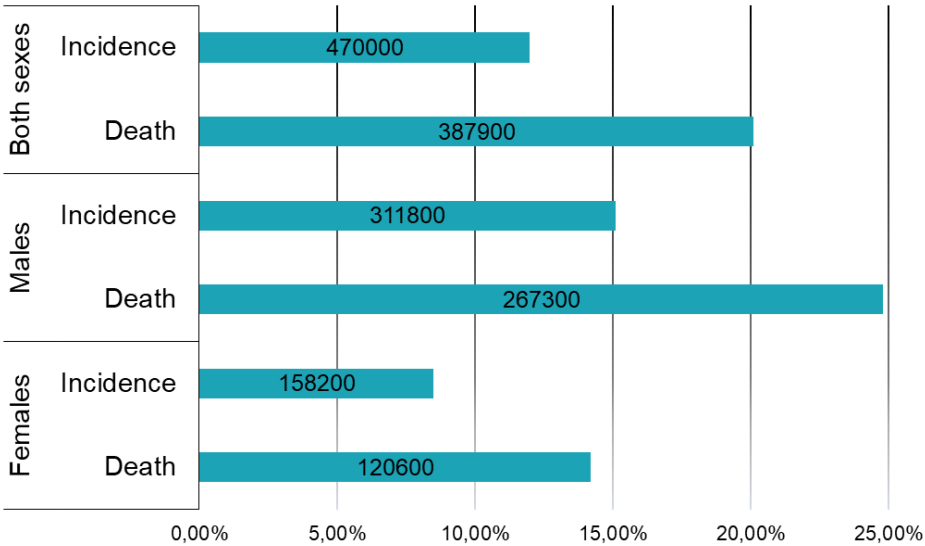


Figure 2 : New cases and deaths for lung cancer in Europe. Data on global lung cancer incidence and mortality in the general population, in males and females. Each histogram shows the number of cases and the length depends on the percentage of all cancers combined (adapted from Ferlay et al., 2018)

1.1.3 In France

According to the “Institut National du Cancer”, lung cancer is the third most frequently diagnosed cancer, after breast cancer in women and prostate cancer in men, accounting for 46 300 new cases (12.1% of all cancers) and is the leading cause of cancer deaths with 33 100 deaths (20.0%), all sexes combined (Figure 3). It is one of the cancers whose incidence rate has increased the most between 2010 and 2018 with a 5% increase. The standardised 5-year net survival rate is 20%. It is the fourth worst prognostic cancer after pancreatic, oesophageal and liver cancer, but its standardised 5-year net survival rate increased by 11 percentage points between 1990 and 2015.

In men, it is the second most frequently diagnosed cancer, after prostate cancer, with 31 200 new cases (15%) and also second cause of cancer deaths, with 22 800 deaths (25%) (Figure 3). The incidence of lung cancer is rather stable in men with a recession of 0.3% between 2011 and 2018. The standardised 5-year net survival rate is 18%.

In women, it is the third most frequently diagnosed cancer, after breast and colorectal cancer, with 15 100 new cases (8.5%) and the second deadliest, after breast cancer, with 10 300 deaths (15%) (Figure 3). Its incidence in women has dramatically increased between 2011 and 2018 with a 5% increase. The standardised 5-year net survival rate is 24%.

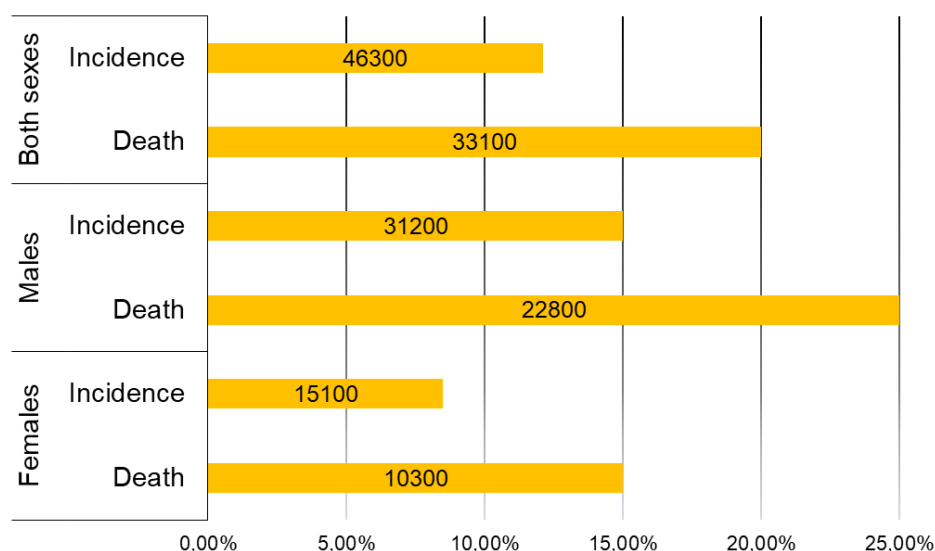


Figure 3 : New cases and deaths for lung cancer in France. "Institut National du Cancer" data on global lung cancer incidence and mortality in the general population, in males and females. Each histogram shows the number of cases and the length depends on the percentage of all cancers combined (adapted from Ferlay et al., 2018)

1.2 Risk factors

1.2.1 Intrinsic factors

1.2.1.1 Gender

As shown above, the incidence and mortality of lung cancer is lower in women (Bade and Dela Cruz, 2020; Donington and Colson, 2011). This can be explained by lifestyle factors that expose them less to carcinogens but also by genetic and biological protective factors (Bade and Dela Cruz, 2020; Donington and Colson, 2011; Mederos et al., 2020; Tanoue, 2021).. However, due to the trend towards gender equalisation, a projection shows that women's mortality will exceed that of men in 2045 (Jeon et al., 2018). Lung cancer affects women at a younger age than men and the proportion of non-smoking related cancers is higher, exposing them more to adenocarcinomas (Jeon et al., 2018).

1.2.1.2 Age

Lung cancer mainly affects older populations with the average age at diagnosis being 70 (Siegel et al., 2019). However, it is a cancer that can affect younger populations and is the leading cancer death for men in their 40s and for both sexes from the age of 60. The median age at death is 72 and increases from 80-85 (Siegel et al., 2019).

1.2.1.3 Genetic factors

Studies tend to point to genetic factors that predispose to the development of lung cancer and influence the response to therapy (Jiang et al., 2012; Kim et al., 2006; Paz-Elizur et al., 2020). It has been shown that the risk of developing lung cancer in smokers doubles if they have a family history of lung cancer. Familial predisposition also increases this risk in non-smokers (Jiang et al., 2012; Kim et al., 2006; Paz-Elizur et al., 2020).

Familial predisposition includes hypersensitivity to mutagenic factors with a link between DNA repair and lung cancer risk, with a polymorphism of repair factors involved in the various known repair mechanisms (base excision, double strand breaks, etc.) such as 8-oxoguanine DNA glycosylase 1

(OGG1), Excision Repair Cross-Complementation group 1 (ERCC1) and 2 or Ataxia Telangiectasia Mutated (ATM) (Jiang et al., 2012; Kim et al., 2006; Paz-Elizur et al., 2020). Susceptibility to tumour development is also influenced by the expression of tumour suppressor genes involved in the cell cycle such as RB transcriptional corepressor 1 (RB1), the tumour genes P53 or P73 or apoptosis genes such as FAS Cell Surface Death Receptor (FAS) and FAS Ligand (FASL) (Li et al., 2004; Offin et al., 2019; Zhang, 2005). It can also be influenced by DNA methylation, histone acetylation or phosphorylation (Langevin et al., 2015).

1.2.1.4 Geography

Lung cancer incidence and morbidity depend on where people live and on their socio-economic status. Socio-economic status includes smoking habits, access to care, early detection of the disease and access to appropriate treatment (surgery, chemotherapy, targeted therapy) (Siegel et al., 2011, 2019). They are highest for Africans and Americans and lowest for Hispanic women (Siegel et al., 2011, 2019).

1.2.2 *Extrinsic factors*

1.2.2.1 Smokers, non-smokers and second-hand smokers

Smoking is the first cause of lung cancer, it has been shown to be involved in 80-90% of diagnosed lung cancers (Jemal et al., 2011). It is a carcinogenic factor even after quitting smoking for 10 years or more. This is partly due to the content of over 4000 chemical constituents (Hoffmann, 1997). Many factors are known to be pro-oncogenic, including gaseous, particulate, organic, inorganic and radioactive components. Nicotine, the best known addiction factor, produces a particulate compound, the tobacco-specific N-nitrosamine, which is one of the main carcinogens of cigarettes (Hoffmann, 1997; Smith et al., 2000). Tobacco is responsible for DNA adducts, production of metabolites that can damage the cells. It is responsible for lung epithelial damage and the establishment of mutations that drive lung oncogenesis (Akopyan and Bonavida, 2006). Thus 20% of lung cancer deaths could be prevented by stopping smoking (Jemal et al., 2011). The probability of lung cancer due to smoking depends on the number of packs, the age of onset, the composition of the cigarette and the presence or absence of a filter.

Lung cancer also occurs in non-smokers who are defined as people who have consumed less than 100 cigarettes in their lifetime. Worldwide, 19% of lung cancers in men and 42% in women are not attributed to smoking, supporting a gender bias (Whiteman and Wilson, 2016).

Tobacco is also a risk for people who do not smoke but are exposed to cigarette smoke becoming passive smokers. Indeed, the risk of lung cancer is increased by 17% in people who were regularly exposed to cigarette smoke during childhood and adolescence. In the case of living with a smoker, this risk is increased by 24% (Bade and Dela Cruz, 2020).

1.2.2.2 E-cigarette.

The use of e-cigarette has grown significantly over the last 10 years, particularly among young people. It is an electronic box that delivers doses of nicotine while limiting the harmful chemical components found in conventional cigarettes (Bracken-Clarke et al., 2021). But the maintenance of the gesture and the various existing perfumes maintain the dependence and certain components such as formaldehyde found in e-cigarettes could be harmful due to the production of reactive oxygen species that can cause inflammation of the broncho-pulmonary epithelium (Bracken-Clarke et al., 2021; Glyns et al., 2018).

A few studies carried out on cell or mouse models tend to show pro-carcinogenesis effect of e-cigarette, in particular by induction of epithelial-to-mesenchymal transition of tumour cells and the development of adenocarcinoma associated with an accumulation of DNA damage (Bracken-Clarke et al., 2021; Tang et al., 2019; Zahedi et al., 2018). Although we do not yet have sufficient hindsight to judge the effects of e-cigarettes on health, the use of e-cigarettes among young people in particular is alarming and may encourage them to switch to traditional cigarettes.

1.2.2.3 Wood burning

In some countries, the use of wood and charcoal for cooking and heating is a major risk factor for lung cancer (Mumford et al., 1990). This is particularly true in southern China, where this method of heating is widely used and where 83% of lung cancers in women are not attributable to smoking (Mumford et al., 1990).

1.2.2.4 Air pollution

Global warming, industrialisation and an increase in the urban population at the expense of rural life are increasing exposure to particulate matter, CO₂ and smoke (Cohen et al., 2017). The biggest risk is lung cancer, both in terms of incidence and mortality. Indeed, fine particles increase the risk of lung cancer by 6-8% (Yang et al., 2020a). Diesel, commonly used as fuel, is also a potent carcinogen (Tsoi and Tse, 2012).

1.2.2.5 Uranium, radium and radon

Uranium and its decay product radium are found in soils, rocks and mines. Therefore, miners and populations living in granite regions are particularly at risk. α -radiation from these compounds can cause damage to the epithelium of the respiratory tract. Exposure to these two compounds for 40 years doubles the risk of lung cancer (Bade and Dela Cruz, 2020; Hubaux et al., 2012).

Radon, on the other hand, is found in houses as a gas and emanates from the soil and building stones. It is the second most common cause of lung cancer after smoking, being involved in 10% of cases (Ajrouche et al., 2017).

1.2.2.6 Occupational exposure

Truck drivers, miners and construction workers are particularly exposed. The first is particularly exposed to exhaust fumes and especially to diesel, which increases the risk of developing lung cancer by 30 to 50%. In the building industry, employees are exposed to two different carcinogenic compounds such as asbestos, nickel or silica (Malhotra et al., 2015).

Other compounds that can be found in the workplace, such as arsenic, beryllium, cadmium, chromium, are considered carcinogens (Bade and Dela Cruz, 2020). The risk depends on the material, the exposure time and the dose. In men, exposure to these compounds increases the risk of developing lung cancer by an average of 10%, compared with 5% in women (Bade and Dela Cruz, 2020).

1.2.2.7 Pathological background

Chronic diseases and certain infections have been shown to sensitise to lung cancer (Corrales et al., 2020).

In the first case the most predisposing disease contexts for lung cancer are chronic obstructive pulmonary disease (Eapen et al., 2018; Wang et al., 2017) and other diseases such as fibrosis (Kinoshita and Goto, 2019). This is due to a chronic inflammatory environment, hyperplastic epithelium and associated infections (Eapen et al., 2018; Kinoshita and Goto, 2019; Wang et al., 2017).

In the second case, infection with various viruses has been shown to predispose to lung cancer. This is the case for example with the *Human Papillomavirus*, which depending on the serotype may contain oncogenic sequences in its DNA (Hoppe-Seyler et al., 2018; Hu et al., 2020). *Chlamydia pneumonia* is also a cause of respiratory infection, particularly in smokers. It causes DNA damage and leads to cellular fragility which confers a tumorigenic advantage (Laurila et al., 1997). The intracellular pathogen, *Mycobacterium Tuberculosis* has also been shown to be a sensitising factor in lung cancer (Abudurehman et al., 2019; Cao et al., 2019b). Many other bacteria and infectious agents have been shown to be involved in the lung tumour process (Sheweita and Alsamghan, 2020).

1.2.2.8 Diet

Diet is a key factor in the field of cancer. In the case of lung cancer, over-consumption of red meat, salt, processed food, saturated fats and fatty acid is a predisposing factor. Fruit and vegetables, because of their content in vitamins, β -carotene and retinol, reduce the risk of cancer (Vieira et al., 2016). This is also the case with foods rich in minerals, magnesium, zinc, copper and iron. In relation to diet, obesity, which is predominantly found in developed countries, is a risk factor for lung cancer (Whiteman and Wilson, 2016).

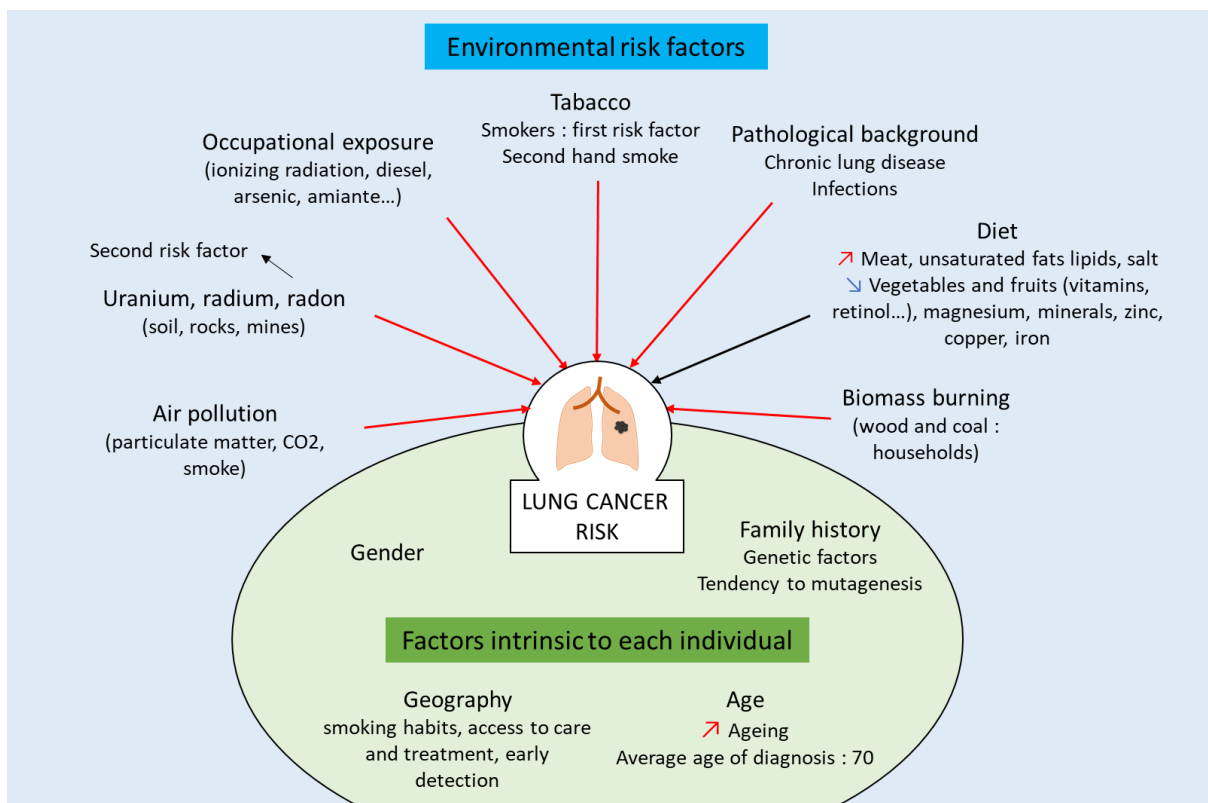


Figure 4 : Intrinsic and extrinsic factors that may influence lung carcinogenesis. The red arrows show the risk-increasing factors, while the blue arrow shows the risk-decreasing factor.

1.3 Histological classification

Lung cancer is a heterogeneous group of malignancies that most often developed from bronchial cells but can also emerge, more rarely, from cells in the lung alveoli. The cancer can then spread to nearby tissues such as the pleura or the pericardium. In advanced stages, lung cancer can spread through the bloodstream and colonise more distant tissues, mainly the liver, brain and bones.

This cancer is divided into two histological sub-categories:

-Small Cell Lung Cancer (SCLC) accounts for 15% of lung cancer cases. There are different histological types but overall it is an aggressive malignancy.

-Non Small Cell Lung Cancer (NSCLC) accounts for 85% of diagnosed lung cancers. It includes different histological subtypes The best known are lung adenocarcinomas, squamous cell carcinomas and large cell carcinomas. Other less common types exist and some patients also have histological atypia. Adenocarcinoma of the lung is the form that is least correlated with smoking. It is a cancer that is predominantly associated with women and even more so with the Asian community.

Table 1: TNM classification of tumours. (Goldstraw et al., 2016).

T: Primary tumor	
Tx	Primary tumor cannot be assessed or tumor proven by presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor ≤3 cm in greatest dimension surrounded by lung or visceral pleura without bronchoscopic evidence of invasion more proximal than the lobar bronchus (i.e., not in the main bronchus) <ul style="list-style-type: none"> • T1a(mi): Minimally invasive adenocarcinoma • T1a: Tumor ≤1 cm in greatest dimension • T1b: Tumor >1 cm but ≤2 cm in greatest dimension • T1c: Tumor >2 cm but ≤3 cm in greatest dimension
T2	Tumor >3 cm but ≤5 cm or tumor with any of the following features: <ul style="list-style-type: none"> - Involves main bronchus regardless of distance from the carina but without involvement of the carina - Invades visceral pleura - Associated with atelectasis or obstructive pneumonitis that extends to the hilar region, involving part or all of the lung <ul style="list-style-type: none"> ▪ T2a: Tumor >3 cm but ≤4 cm in greatest dimension ▪ T2b: Tumor >4 cm but ≤5 cm in greatest dimension
T3	Tumor >5 cm but ≤7 cm in greatest dimension or associated with separate tumor nodule(s) in the same lobe as the primary tumor or directly invades any of the following structures: chest wall (including the parietal pleura and superior sulcus tumors), phrenic nerve, parietal pericardium
T4	Tumor >7 cm in greatest dimension or associated with separate tumor nodule(s) in a different ipsilateral lobe than that of the primary tumor or invades any of the following structures: diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, and carina
N: Regional lymph node involvement	
Nx	Nx : Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
M: Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis present: <ul style="list-style-type: none"> ▪ M1a: Separate tumor nodule(s) in a contralateral lobe; tumor with pleural or pericardial nodule(s) or malignant pleural or pericardial effusion ▪ M1b: Single extrathoracic metastasis ▪ M1c: Multiple extrathoracic metastases in one or more organs

To determine the appropriate treatment histological identification of the type of cancer is essential but not sufficient. The stage of the disease must also be determined to choose the optimal treatment. To assess the stage of the disease and the appropriate treatment, the tumour, node and metastasis (TNM) classification is evaluated. This takes into account the size of the tumour (T), the involvement of the lymph nodes (N) and the presence or absence of metastases (M) (Goldstraw et al., 2016) (Table 1).

The TNM status of the tumour can be used to determine the stage of the disease and to determine the best treatment regimen for the patient (Table 2).

Table 2 : Stage classification of tumours. (Goldstraw et al., 2016)

	Characteristics
Stage I/II	<ul style="list-style-type: none"> ▪ Cancers located in the thoracic cavity. ▪ No lymph nodes are involved, or one or more in the vicinity of the tumour. ▪ No distant metastasis
Stage III	<ul style="list-style-type: none"> ▪ Locally advanced cancers. ▪ In the majority of cases, nodes in the area close to the lungs are affected. ▪ No distant metastasis
Stage IV	<ul style="list-style-type: none"> ▪ Metastatic cancers: distant metastases in organs other than the lung where the tumour is located.

Table 3 : Treatment regimen according to tumour stage. (Goldstraw et al., 2016)

	Treatment
Stage I	Operable patient <ul style="list-style-type: none"> ▪ Excisional surgery ▪ Optional: neoadjuvant and/or adjuvant conventional chemotherapy
	Inoperable patient <ul style="list-style-type: none"> ▪ Radiotherapy ▪ Optional: neoadjuvant and/or adjuvant conventional chemotherapy thermal ablation for tumours < 3 cm
Stage II	Operable patient <ul style="list-style-type: none"> ▪ Excisional surgery ▪ Conventional chemotherapy usually adjuvant, sometimes neoadjuvant
	Inoperable patient <ul style="list-style-type: none"> ▪ Radiotherapy ▪ Optional: neoadjuvant and/or adjuvant conventional chemotherapy thermal ablation for tumours < 3 cm
Stage III	Operable patient and resectable tumour <ul style="list-style-type: none"> ▪ Excisional surgery ▪ Conventional chemotherapy usually adjuvant, sometimes neoadjuvant ▪ Option: radiotherapy in case of incomplete resection
	Stage IIIA unresectable tumour, stage IIIB, inoperable patient <ul style="list-style-type: none"> ▪ Concurrent chemoradiotherapy ▪ or sequential chemotherapy-radiotherapy ▪ If radiotherapy is contraindicated and EGFR mutation or EGFR mutation or ALK translocation: targeted therapy
Stage IV	<ul style="list-style-type: none"> ▪ If EGFR mutation or ALK translocation identified: targeted therapy alone ▪ If no EGFR mutation or ALK translocation identified : conventional chemotherapy +/- antiangiogenic therapy ▪ If limited number of metastases: discussion of treatment with surgery or radiotherapy +/-conventional chemotherapy

In the early stages (stage I and II) of the disease, excisional surgery is prescribed with or without chemotherapy. If the tumour is not operable, radiotherapy is prescribed with or without chemotherapy. For stage III tumours, if the patient is operable, there is a resection of the tumour combined with chemotherapy and sometimes radiotherapy. For non-operable stage III tumours, chemotherapy alone or sequential treatment with radiotherapy is prescribed. For stage IV tumours, the treatment is less intuitive and depends on the individual cancer, depending on the molecular status of the tumour. In the absence of a driver mutation, chemotherapy with or without antiangiogenic drugs is preferred. The number of metastases will also determine the treatment. For stage III and IV tumours, the identification of driver mutations such as *Epidermal Growth Factor Receptor (EGFR)* or

Anaplastic Lymphoma Kinase (ALK) allows the prescription of a therapy targeting these mutations as described hereafter (Table 3).

The identification of specific gene driver mutations in tumours is essential for a better treatment of patients.

1.4 Molecular classification of NSCLC

The use of current sequencing tools makes it possible to identify oncogenic driver mutations that allow the development of targeted therapies for advanced disease (Figure 5).

The main mutation found is the *Kirsten RAS 2 viral oncogene homolog (KRAS)* gene mutation (20-25% of cases of NSCLC). This is followed by the *EGFR* mutation, found in 10-15% of cases. The *ALK* mutation is found in 5% of cases. Mutations in *ROS1*, *MET*, *BRAF*, *Phosphatidylinositol 3-Kinase (PI3K)/AKT*, *RET* and *Human Epidermal Growth Factor Receptor-2 (HER2)* are also found. In 48% of cases, no oncogenic gene mutation was found (Calvayrac et al., 2017; Fois et al., 2021) (Figure 5).

Research into the development of targeted therapies for *KRAS* mutations has so far been unsuccessful. Indeed, for a long time *KRAS* mutation was considered as "undruggable" but recent studies have allowed the development of subtype inhibitors of the receptor allowing in particular the development of Sotorasib, an inhibitor of *KRAS G12C*, which shows an anti-tumour activity *in vitro*, *in vivo* and in patients (Hong et al., 2020; Zhang and Nagasaka, 2021). Sotorasib is currently in clinical trials in combination with chemotherapy or immunotherapy (Canon et al., 2019; Hong et al., 2020; Skoulidis et al., 2021). However, the emergence of resistance to this treatment has been highlighted, notably by the epithelial-to-mesenchymal transition (EMT) of the cells and is currently being studied (Adachi et al., 2020; Dunnett-Kane et al., 2021; Liu et al., 2021b). In the case of *EGFR* and *ALK* mutations, targeted therapies are used as first line treatment for advanced cancers.

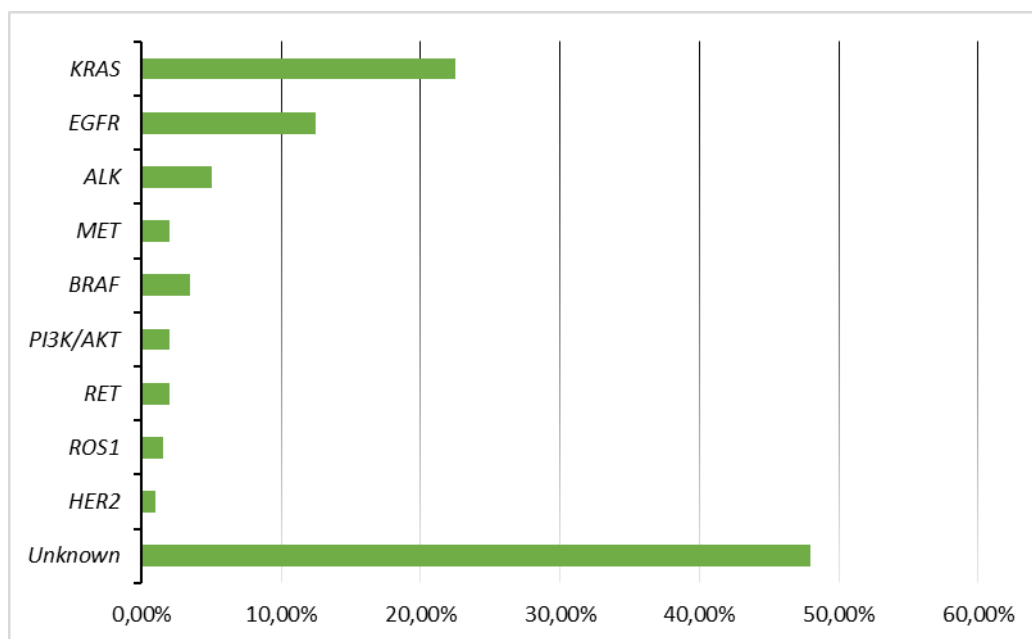


Figure 5 : NSCLC driver mutations (based on (Calvayrac et al., 2017))

The work in this thesis focused on the targeted treatment of *EGFR*-mutated NSCLC with tyrosine kinase inhibitors, *EGFR*-TKIs.

1.5 *EGFR* mutations

The discovery of activating mutations in *EGFR* gene and the development of *EGFR*-TKIs has led to improved management of patients with *EGFR*-mutated NSCLC. *EGFR* mutations predominantly

affect women, non-smokers and the Asian population. Indeed, in the Caucasian population EGFR mutation is found in 10-20% of NSCLC compared to 30-40% in the Asian population.

1.5.1 EGFR

1.5.1.1 EGFR structure

EGFR is a member of the ERBB family which consists of 4 members, *EGFR*, *HER2*, *HER3* (*ERBB3*), and *HER4* (*ERBB4*). In normal physiological conditions, it is a transmembrane receptor that plays a role in many biological processes.

The gene coding for *EGFR* is located on chromosome 7 and codes for a 170kDa transmembrane glycoprotein. The protein has an extracellular domain of 621 amino acids composed of 4 subdomains, L1, CR1, L2 and CR2. The L1 and L2 domains are the ligand binding sites while the CR1 site allows dimerization with another receptor (Normanno et al., 2006; Wang, 2017). A hydrophobic transmembrane domain of 22 amino acids, from residues 622 to 644, allows anchoring to the membrane. The transmembrane part is associated with a juxta-membrane domain that regulates the activity of the receptor by mediating its internalisation after ligand binding. Finally, the receptor contains an intracellular domain mainly composed of a tyrosine kinase and carboxy terminal domain which allows the activation of the receptor by auto-phosphorylation (Normanno et al., 2006; Wang, 2017) (Figure 6).

The ligand binding sites (L1 and L2) are encoded by exons 5-7 and 13-16 respectively, the transmembrane domain is encoded by exon 17, the tyrosine kinase domain by exons 18-24 and the carboxy-terminal part by exons 25-28 (Normanno et al., 2006; Wang, 2017).

1.5.1.2 EGFR activity

Its ligands such as the Epidermal Growth Factor (EGF), bind to the extracellular part of the receptor, resulting in a conformational change of the extracellular domain and in the homo- or hetero-dimerization (with HER-2, HER-3, HER-4) of the receptor leading to increase the binding affinity for adenosine triphosphate (ATP). This increases the binding affinity for adenosine triphosphate (ATP) and allows auto-phosphorylation on its tyrosine residues through its kinase activity. The phosphorylated tyrosine residues recruit phosphotyrosine-binding proteins that activate signalling cascades such as Mitogen-Activated Protein Kinases (MAPK), Signal Transducer and Activator of Transcription 3 (STAT3) or PI3K/AKT pathways, leading to genes expression involved in many biological processes such as cell survival, angiogenesis and proliferation (Normanno et al., 2006; Wang, 2017) (Figure 6).

When its activity is no longer necessary, the receptor and its ligand are ubiquitinated and endocytosed via clathrin-dependent endocytosis. One of the roles of this internalisation is to moderate the signal following ligand-receptor binding. Endocytosed EGFR has two main fates: recycling to the plasma membrane by exocytosis vesicles or degradation into lysosomes. Alternatively, the receptor can also have other roles after its internalization. It can be translocated into the nucleus where it can play a role in modulating transcription and deoxyribonucleic acid (DNA) repair through its interaction with DNA-dependent Protein Kinase (DNA-PK) (Dittmann et al., 2008, 2010). It can also have a pro-mitotic effect by influencing the expression of cell cycle genes such as *cyclin D1* (Wang, 2017). It can also be transported to the mitochondria where it can play its role as a modulator of apoptosis by phosphorylating cytochrome c oxidase subunit II (CoxII) (Boerner et al., 2004). *EGFR* has been shown to be mutated in many oncogenic contexts, such as lung cancers, and is a target for the development of anti-cancer drugs. Establishing the best treatment is highly dependent on the identification of the mutation.

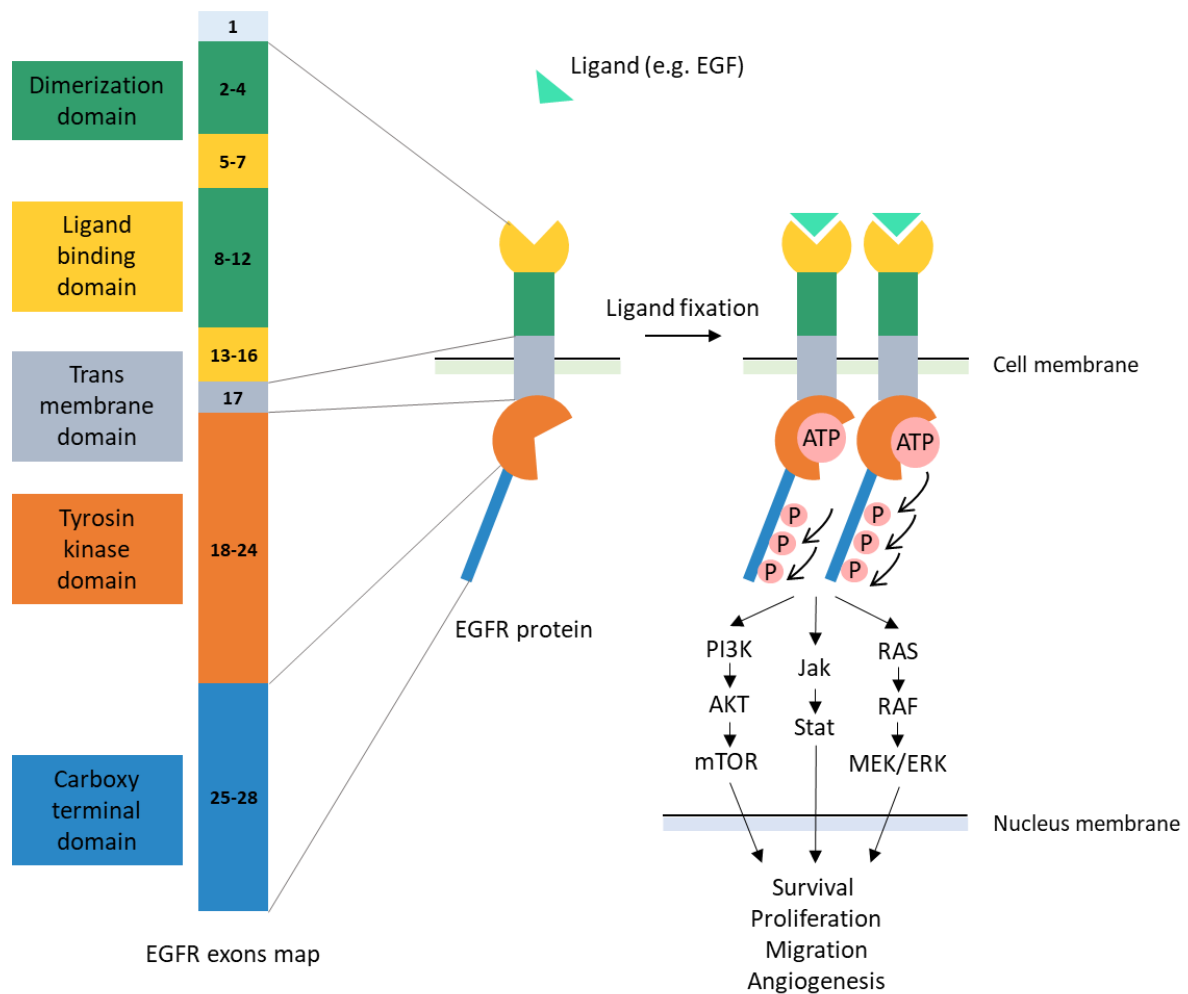


Figure 6 : EGFR exons map, structure of EGFR protein and activation after ligand fixation (based on (Yoneda et al., 2019))

1.5.2 EGFR mutations in lung cancer

EGFR mutation were first described in 2004 (Lynch et al., 2004). The majority of mutations are found in exons 18 to 21 of the kinase domain of the receptor and more precisely in the ATP binding pocket. These mutations increase the affinity of ATP for the receptor binding pocket resulting in a constitutive activity of the receptor independently of the binding of its ligand and therefore the pathways located downstream are permanently activated. This leads to uncontrolled survival and proliferation of cells, resulting in the formation of a tumour.

This activating mutations occur predominantly in women and non-smokers and are highly prevalent in the Asian (30-40% of lung cancers are EGFR mutated) and Caucasian (10-20% of cases) populations. Before treatment, the main mutations detected are the deletion in exon 19 ($\Delta E746-A750$) in 45-60% of cases and the substitution of lysine for arginine in position 858 of exon 21 (L858R) in 30-40% of cases (Sharma et al., 2007; Tartarone and Lerosé, 2015). The remaining 15% are rare mutations including insertions at exons 19 (1%) and 20 (6%), point mutations at exon 16 (G719X:3%) and exon 21 (L861X:1%) (Arrieta et al., 2015; Beau-Faller et al., 2014; Brandão et al., 2012).

1.5.3 EGFR-TKIs

EGFR-TKIs were used in the treatment of NSCLC before the identification of EGFR mutations and their roles as oncogenic drivers in lung cancer. Indeed, EGFR-TKIs have been developed with the rationale that NSCLC patients displayed an overexpression of EGFR, however EGFR-TKI were mostly

ineffective in these patients. It was only in 2004 that the *EGFR* mutation and their involvement in the response to EGFR-TKIs were identified (Lynch et al., 2004). EGFR-TKIs are targeted therapies that target and limit the activity of the receptor. Their anti-tumour efficacy is superior to that offered by chemotherapy (Ke and Wu, 2016).

1.5.3.1 Mechanism of action of EGFR-TKI

All EGFR-TKIs are competitive ATP inhibitors. Due to their high affinity for the EGFR ATP binding pocket, EGFR-TKIs prevent ATP binding. Since ATP is essential for the autophosphorylation of the receptor, the latter cannot be activated and the survival and proliferation pathways associated with the receptor are no longer able to feed the tumour. Furthermore, since EGFR activation is anti-apoptotic, its inhibition by EGFR-TKIs leads to cell death (Yoneda et al., 2019).

1.5.3.2 First and second generations of EGFR-TKI

Gefitinib and Erlotinib are two inhibitors of 1st generation that bind reversibly to the ATP pocket of the receptor. They recognise both the wild type and the L858R mutated form of the receptor. The Progression Free Survival (PFS) for under treatment with Gefitinib is 9.2-10.9 months (WJTOG3405, NEJ002, LUX-Lung 7, ARCHER 1050 trials) and 10.4-13.3 months for Erlotinib (EURTAC, OPTIMAL, NEJ026 trials) (Ke and Wu, 2016). EGFR-TKIs are used as monotherapy in patients with *EGFR*-mutated NSCLC. However, these 1st generation EGFR-TKIs lose their efficacy when resistance mechanisms appear. Mutations responsible for this resistance, as will be discussed below, can be of different types but the most common is the T790M secondary mutation in exon 20 of *EGFR*, found in 50% of cases. (Takeda and Nakagawa, 2019).

The second generation EGFR-TKIs, Afatinib and Dacotinib, are inhibitors that bind irreversibly to the ATP pocket of the receptor. Unlike the 1st generation inhibitors, they are not specific to EGFR and also target other receptors of the ErbB family. The PFS is 11.0-11.1 months (LUX-Lung 3, LUX-Lung 6, LUX-Lung 7 trials) and 14.7 months for the Afatinib and Dacotinib respectively (ARCHER 1050 trial) (Ke and Wu, 2016; Yoneda et al., 2019). They have a very low efficacy on resistance mutations such as T790M (Masood et al., 2019; Takeda and Nakagawa, 2019). To overcome the lack of efficacy against T790M mutated forms and the high toxicity due to the non-specificity of the inhibitor, third generation EGFR-TKIs have been developed.

1.5.3.3 Third generation of EGFR-TKI

Osimertinib and Rocicetinib, like the second generation inhibitors, are irreversible (Table 4). However, they specifically recognise the mutated form of the receptor (L858R) and display less affinity for the wild type EGFR. They have also shown efficacy in inhibiting the receptor with the T790M mutation (Cross et al., 2014; Jänne et al., 2015). Since 2015 and until recently, Osimertinib was used as second line treatment, after the appearance of the resistance mutation. Due to superior efficacy and comparable side effects, Osimertinib has been used as first-line treatment for patients with EGFR-mutated NSCLC since 2019 (Ramalingam et al., 2020). Indeed, the FLAURA clinical trial started in 2014 (Clinicaltrials.com, NCT02296125), compares Osimertinib to first generation EGFR-TKIs. It was conducted on 556 patients with locally advanced or metastatic untreated *EGFR*-mutated NSCLC (exon 19 deletion or L858R) (Soria et al., 2018). The first arm included patients treated with Osimertinib and the second arm included patients treated with 1st generation EGFR-TKIs, Gefitinib or Erlotinib. Osimertinib was shown to increase overall survival to 38.5 months (95% confidence interval [CI], 34.5-41.8) compared to 31.8 (95% CI 26.6-36.0) for the 1st generation EGFR-TKI group. Patients in both groups had comparable side effects resulting in comparable compliance (Ramalingam et al., 2020).

Despite their efficacy, resistance mechanisms invariably emerge, such as C797S third mutation on the *EGFR*, which appears at the inhibitor binding site (Ercan et al., 2015; Niederst et al., 2015a; Thress et al., 2015). The development of new EGFR-TKI is underway to try to circumvent these resistances.

Table 4 : Summary of targets of different generations of EGFR-TKIs (based on (Ke and Wu, 2016))

	EGFR-TKI		
	First-Generation	Second-Generation	Third-Generation
EGFR binding	Reversible	Irreversible	Irreversible
EGFR ^{WT}	+	+	–
EGFR ^{Del19/L858R}	+	+ +	+ +
EGFR ^{T790M}	–	+	+ + +
ErbB2	–	+	–
ErbB4	–	+	–
Agent	Gefitinib, erlotinib, icotinib	Afatinib, dacomitinib	Osimertinib, rociletinib, HM61713, EGF816, ASP8273

1.5.4 Resistance mechanisms to EGFR-TKI

EGFR mutations are found in 15-20% of cases (Calvayrac et al., 2017; Fois et al., 2021). The first line therapy in this case, and particularly in inoperable cancer, are tyrosine kinase inhibitors (TKIs). They increase patients' PFS and overall survival (Ramalingam et al., 2020). However, the systematic emergence of resistance prevents a lasting effect and complete remission, with inevitable relapse. The understanding of these resistance mechanisms, both at the level of the tumour cells and their microenvironment, must absolutely be deciphered for an optimal management of the patients.

The resistance mechanisms can be of different kinds. "On-target" mutations are found, i.e. on the oncogenic driver itself, with either the appearance of a new mutation (Kobayashi et al., 2005; Pao et al., 2005; Sharma et al., 2007) or the amplification of the receptor (Ercan et al., 2010; Nukaga et al., 2017; Piotrowska et al., 2015). There may also be activation of compensatory pathways to bypass the inactivation of *EGFR* (Nishiyama et al., 2020). This includes the amplification and activation of other receptor tyrosine kinases such as MET (Nishiyama et al., 2020) or HER2 (Papadimitrakopoulou et al., 2018; Ramalingam et al., 2020), the emergence of fusion proteins such as Echinoderm Microtubule-associated protein-Like 4 (EML4)-ALK (Capelletti et al., 2014; Piotrowska et al., 2018; Soda et al., 2007; Vojnic et al., 2019), or the activation of intracellular kinases without extracellular stimuli (Eng et al., 2015; Engelman et al., 2006; La Monica et al., 2019; Leonetti et al., 2018; Meng et al., 2020b). Finally, phenotypic changes also allow therapeutic escape, such as the NSCLC-to-SCLC switch (Ferrer et al., 2019; Ham et al., 2016; Liu, 2018; Marcoux et al., 2019; Taniguchi et al., 2018) or the transition from an epithelial to a mesenchymal phenotype by the establishment of the EMT (Lamouille et al., 2014).

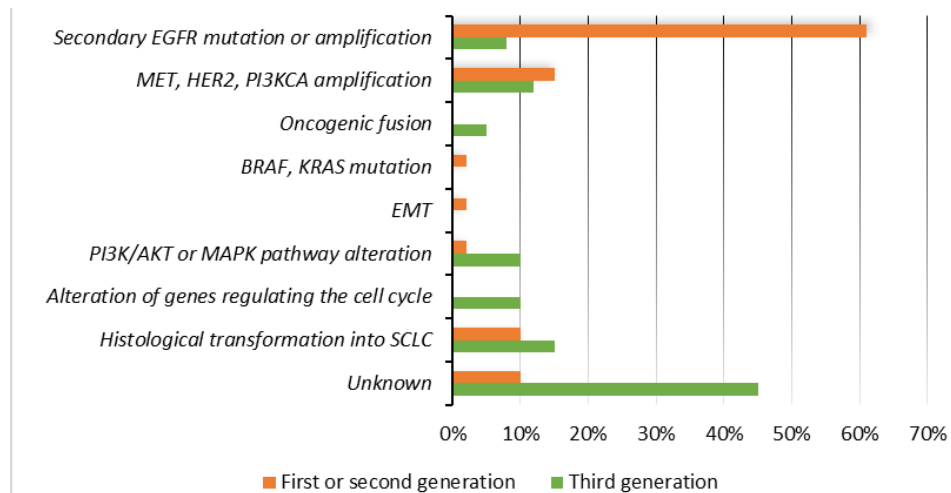


Figure 7 : The mechanisms of acquired resistance identified in EGFR-mutated NSCLC patients treated with EGFR-TKI. Those found for the first and second generations are represented by the orange histograms while those observed for the third generation are in green.

1.5.4.1 On-target resistance

This is a genetic alteration of the oncogenic driver *EGFR*. This occurs either through a mutation of the receptor or through its amplification

1.5.4.1.1 Secondary mutation of the *EGFR*

The establishment of secondary mutations on the *EGFR* gene limits the fixation of the TKI on its target. In the case of the 1st generation inhibitors, Erlotinib and Gefitinib, 50% of treated patients developed a mutation in exon 20, coding for the kinase domain of *EGFR*, resulting in the substitution of a threonine for a methionine in position 790 (T790M). This mutation alters the ATP binding site, which is also the TKI binding site (Kobayashi et al., 2005; Pao et al., 2005; Sharma et al., 2007). T790M increases the affinity of the receptor for ATP at the expense of the TKI (Yun et al., 2008).

To counteract this mutation, second generation TKIs, Afatinib and Dacotinib, and third generation TKIs, Osimertinib, were developed. Second generation *EGFR*-TKIs have little efficacy on the T790M mutation and secondary mutations of resistance appear. For the third generation TKIs the most common mutation found is the substitution of a cysteine for a serine (C797S or Cys797Ser) at position 797 on exon 20, which prevents the TKI from binding to its site of action by modification of the conformation of the receptor (Capelletti et al., 2014). Other less frequent mutations include L792H, G796R/S, L718Q, M766Q or G724S (Castellano et al., 2019; Ercan et al., 2015; Oztan et al., 2017; Uchibori et al., 2018; Zhang et al., 2018).

1.5.4.1.2 *EGFR* amplification

EGFR amplification was observed in 10% of *EGFR*-TKI treated patients (Ercan et al., 2010). For resistance with 1st and 2nd generation inhibitors, only amplification of the T790M mutated receptor was shown understood. Amplifications of the *EGFR* were also shown for the third generation *EGFR*-TKIs. (Ercan et al., 2010; Nukaga et al., 2017; Piotrowska et al., 2015).

1.5.4.2 Bypass pathway activation

1.5.4.2.1 *MET* amplification

Bypass pathway activation is the activation of other Receptor Tyrosine Kinases (RTKs) to compensate for the loss of the receptor targeted by the TKIs. In the case of mutated *EGFR*, the most

common compensation, in 10-20% of cases, is through *MET* gene amplification (Nishiyama et al., 2020). This circumvents the lack of EGFR activation under EGFR-TKI. MET receptor is found amplified whatever the EGFR-TKI used (Engelman et al., 2007; Shi et al., 2016). In particular, studies conducted on the *EGFR*-mutated NSCLC line HCC827 showed that *MET* amplification and protein overactivity under Erlotinib led to resistance to third generation EGFR-TKIs. Similarly, MET compensation under Osimertinib treatment desensitizes cells to other EGFR-TKIs. Inhibition of MET allows cells to be resensitized to EGFR-TKIs (Le et al., 2018; Shi et al., 2016). A clinical trial in patients with MET amplification combining the MET inhibitor Capmatinib with Gefitinib doubled the overall response compared to Gefitinib alone (Wu et al., 2018).

1.5.4.2.2 *HER2* amplification

The ErbB2 protein, encoded by the *HER2* gene, is a receptor tyrosine kinase of the same family as EGFR. They share common roles in the cell through the MAPK, STAT and PI3K/AKT signalling cascades. *HER2* amplification is found in 10 to 15% of patients treated with 1st and 2nd generation EGFR-TKI (Takezawa et al., 2012). This amplification was found in 5% and 2% of patients treated with Osimertinib as 2nd and 1st line therapy respectively (Chabon et al., 2016; Ortiz-Cuaran et al., 2016; Papadimitrakopoulou et al., 2018; Ramalingam et al., 2020). It has been shown *in vitro* that resistance is directly correlated to the amplification of the receptor, with overexpression leading to resistance to EGFR-TKI and decreased expression leading to sensitization to treatment (Takezawa et al., 2012). The combination of EGFR blocking antibody Cetuximab and of the inhibitor of all ErbB family members, Afatinib, conferred robust and durable clinical responses irrespective of T790M status (Janjigian et al., 2014; Regales et al., 2009).

1.5.4.2.3 PI3K and MAPK signalling activation

Activation of signaling pathways downstream of EGFR can bypass the action of EGFR-TKIs. This is achieved through the constitutive activation of kinases whose activity is physiologically dependent on the receptor activation. This allows the anti-proliferative and pro-apoptotic effect of TKIs to be circumvented (Eng et al., 2015; Ercan et al., 2012; Ramalingam et al., 2020).

Compensation by over-activation of the PI3K pathway is achieved by deletion of *Phosphatase and TENSin homolog (PTEN)* or by mutation and or amplification of Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit α (*PI3KCA*). Mutations in *PI3KCA* are found in 4-11% of cases. The main known mutations are E545K, E542K, R88Q, N345K and E418K (Eng et al., 2015; Engelman et al., 2006; Oxnard et al., 2018; Papadimitrakopoulou et al., 2018; Westover et al., 2018).

Compensation by activation of MAPK pathways occurs in 1-2% of cases. It can be the result of different genetic changes such as *MAPK1* amplification (Ercan et al., 2012), *BRAF* mutations of which the best known are V600E and G469A (La Monica et al., 2019; Leonetti et al., 2018; Meng et al., 2020b), *KRAS* amplifications or mutations such as G12A/D/S, G13D, Q61H/R/K or A146T (Ortiz-Cuaran et al., 2016; Oxnard et al., 2018; Ramalingam et al., 2020) or *Neuroblastoma RAS viral oncogene homolog (NRAS)* mutations such as the E63K mutation observed in Osimertinib resistant cells (Eberlein et al., 2015).

1.5.4.2.4 AXL receptor tyrosine kinase activation

AXL is a receptor tyrosine kinase that has been shown to be overexpressed, along with its ligand GAS6, in 20% of patient resistant to Erlotinib or gefitinib. AXL is notably involved in the EMT process. AXL compensates for EGFR inactivation by affecting the PI3K/AKT, MAPK or JAK/STAT signalling pathways (Byers et al., 2013; Ishikawa et al., 2013; Zhang et al., 2012). AXL inhibition has been shown to resensitize to EGFR-TKIs and the combination of EGFR-TKIs with an AXL inhibitor has

been shown to delay the emergence of resistance *in vitro* and *in vivo* (Jimbo et al., 2019; Okura et al., 2020).

1.5.4.2.5 Insulin Like Growth Factor 1 Receptor (IGF1R) activation

Over activation of the IGF1R has also been shown to be a bypass pathway. Indeed, it allows the activation of the PI3K/AKT pathway and its inactivation allows the restoration of sensitivity to EGFR-TKIs (Hayakawa et al., 2020; Lee et al., 2016b; Makimoto et al., 2021).

1.5.4.2.6 Fibroblast Growth Factor Receptor (FGFR) activation

Activation of the Fibroblast Growth Factor 2 (FGF2)/FGFR1 signalling pathway will also bypass EGFR inhibition through activation of the PI3K/AKT, JAK/STAT and MAPK pathways. This has been shown in treatment with all three generations. Inhibition of FGFR2 allows cells to be resensitized to EGFR-TKI *in vitro* and *in vivo*. (Raouf et al., 2019; Terai et al., 2013; Ware et al., 2013)

1.5.4.2.7 Activation of signalling pathways by fusion proteins

In the case of 1st and 2nd generation TKIs, the most common fusion genes are *Coiled-Coil Domain Containing 6 (CCDC6)-RET*, *Nuclear Receptor Coactivator 4 (NCOA4)-RE*, *Tropomyosin 3 (TPM3)-NTRK1*, *FGFR3-Transforming Acidic Coiled-Coil Containing Protein 3 (TACC3)* and various fusions of the *BRAF* gene (Capelletti et al., 2014; Piotrowska et al., 2018; Soda et al., 2007; Vojnic et al., 2019). Under 3rd generation TKIs, fusions of the *BRAF*, *NTRK1*, *FGFR3* and *Golgi Associated PDZ And Coiled-Coil Motif Containing (GOPC)-ROS1*, and *Echinoderm microtubule-associated protein-like (EML4)-ALK* genes are found (Capelletti et al., 2014; Piotrowska et al., 2018; Soda et al., 2007; Vojnic et al., 2019). Combination therapies have been tested to resensitize cells to TKIs. In the case of *Acylglycerol Kinase (AGK)-BRAF* fusion, Osimertinib can be combined with the MEK inhibitor Trametinib (Vojnic et al., 2019) and in the case of *CCDC6-RET* fusion with the RET inhibitor Cabozantinib (Piotrowska et al., 2018).

1.5.4.3 Phenotypic switch

The main role of this phenotypic modification is the overcoming of the initial oncogenic dependence and consequently the obsolete treatment. The phenotypic change may include the transformation of NSCLC into SCLC (Leonetti et al., 2021; Offin et al., 2019) or the switch from an epithelial to a mesenchymal phenotype by the establishment of the EMT (Lamouille et al., 2014).

1.5.4.3.1 Phenotypic switch from NSCLC to SCLC

SCLC is a more aggressive cancer than NSCLC and this phenotypic switch is responsible for resistance to EGFR-TKIs in 5-10% of cases and predominantly in *P53* and *RB1* mutated tumours (Leonetti et al., 2021; Offin et al., 2019). This switch is defined by a decrease in EGFR expression, a loss of RB expression and an increase in the expression of neuroendocrine factors (Niederst et al., 2015; Oser et al., 2015). The mechanism of this switch is not known yet. Clinical cases of histological transformation have been reported (Ferrer et al., 2019; Ham et al., 2016; Liu, 2018; Marcoux et al., 2019; Taniguchi et al., 2018). Patients then become sensitive to platinum-based or taxane-based chemotherapies (Marcoux et al., 2019). The maintenance of the EGFR mutation shows the transformation of the original cancer and not the emergence of a new tumour (Marcoux et al., 2019).

1.5.4.3.2 EMT

EMT is a phenotypic and physiological change in cells. They lose epithelial markers, such as Epithelial (E)-cadherin, and acquire mesenchymal markers, such as Neural (N)-cadherin or Vimentin. The cells also lose their polarity and cell junctions with the loss of adherent and tight junctions accompanied by a reshuffling of the cytoskeleton, particularly actin, and the establishment of stress

fibres and adhesion focal points (Lamouille et al., 2014). EMT allows cells in a tissue to become motile and is involved in metastatic dissemination (Mittal, 2018; Ribatti et al., 2020) but also in resistance to therapies.

The development of EMT under EGFR-TKI has been shown to exist regardless of generation of the inhibitor. Under Gefitinib treatment, the NSCLC cells lines PC9 and HCC827 were shown to lose epithelial markers and display a mesenchymal phenotype upon becoming resistant to treatment (Chung et al., 2011; Lee et al., 2017; Suda et al., 2011). The overexpression of SNAIL and SLUG, two mesenchymal markers, which leads to cell resistance to EGFR-TKI, supported the role of EMT in EGFR resistance (Lee et al., 2017). Resistance to Afatinib can also be mediated by the establishment of EMT as shown in the EGFR-mutated NSCLC lines NCI-H1650 and NCI-H1975 (Coco et al., 2015). Similar data have been collected for third generation EGFR TKIs shown in the EGFR-mutated NSCLC lines H1975 (Ji et al., 2020; Jiang et al., 2021). The involvement of EMT in EGFR-TKI resistance is supported by the fact that overexpression of key EMT factors or the use of EMT-inducing molecules, such as Transforming Growth Factor (TGF)- β , induces resistance to treatment (Lee et al., 2017). On the other hand, inhibition of pro-EMT factors or re-expression of E-cadherin in the cells allows them to be resensitized to EGFR-TKI treatment (Suda et al., 2011; Weng et al., 2019; Yoshida et al., 2016).

The establishment of EMT in EGFR-TKI treated cells can be explained by the activation of different intracellular signalling pathways. However, the exact molecular mechanism has not been defined yet. TGF- β appears to be involved in the acquisition of the mesenchymal phenotype (Lee et al., 2017; Suda et al., 2011). In particular, it influences the expression of the zinc finger E-box binding homeobox 1 (ZEB1) transcription factor by activation of the SMAD pathway in EGFR-TKI resistant cells (Suda et al., 2011; Yoshida et al., 2016). Furthermore, loss of TGF- β 1 and TGF- β 2 expression in cells will reverse EMT, restore an epithelial phenotype, and resensitize cells to EGFR-TKI treatment (Buonato and Lazzara, 2014; Suda et al., 2011; Yao et al., 2010; Yoshida et al., 2016). FGFRs have also been shown to be involved in resistance to EGFR-TKIs (Raouf et al., 2019). Pharmacological inhibition of FGFR1 allows resensitization of cells that have become mesenchymal under treatment (Raouf et al., 2019). FGFR3 in particular is overexpressed in resistant cells that have undergone EMT and allows their survival (Raouf et al., 2019). FGFRs inhibitor association with EGFR-TKIs inhibit, both *in vitro* and *in vivo*, the development of resistance to EGFR-TKIs (Raouf et al., 2019). AXL, shown as a bypass pathway in EGFR-TKI resistance, has been shown to be involved in EMT of tumour cells under treatment (Byers et al., 2013; Du et al., 2020; Zhang et al., 2012). Studies have shown that overexpression of AXL correlates with increases in factors involved in EMT, such as vimentin. Inhibition of AXL restores sensitivity to EGFR-TKIs both *in vitro* and *in vivo* (Byers et al., 2013; Du et al., 2020; Zhang et al., 2012). Other factors that have been identified as influencing EMT in resistant cells that include Notch-1 (Xie et al., 2012), Focal Adhesion Kinase (FAK) (Wilson et al., 2014), Interleukine (IL) 6 (Li et al., 2014) and Hedgehog (Hh) (Della Corte et al., 2017).

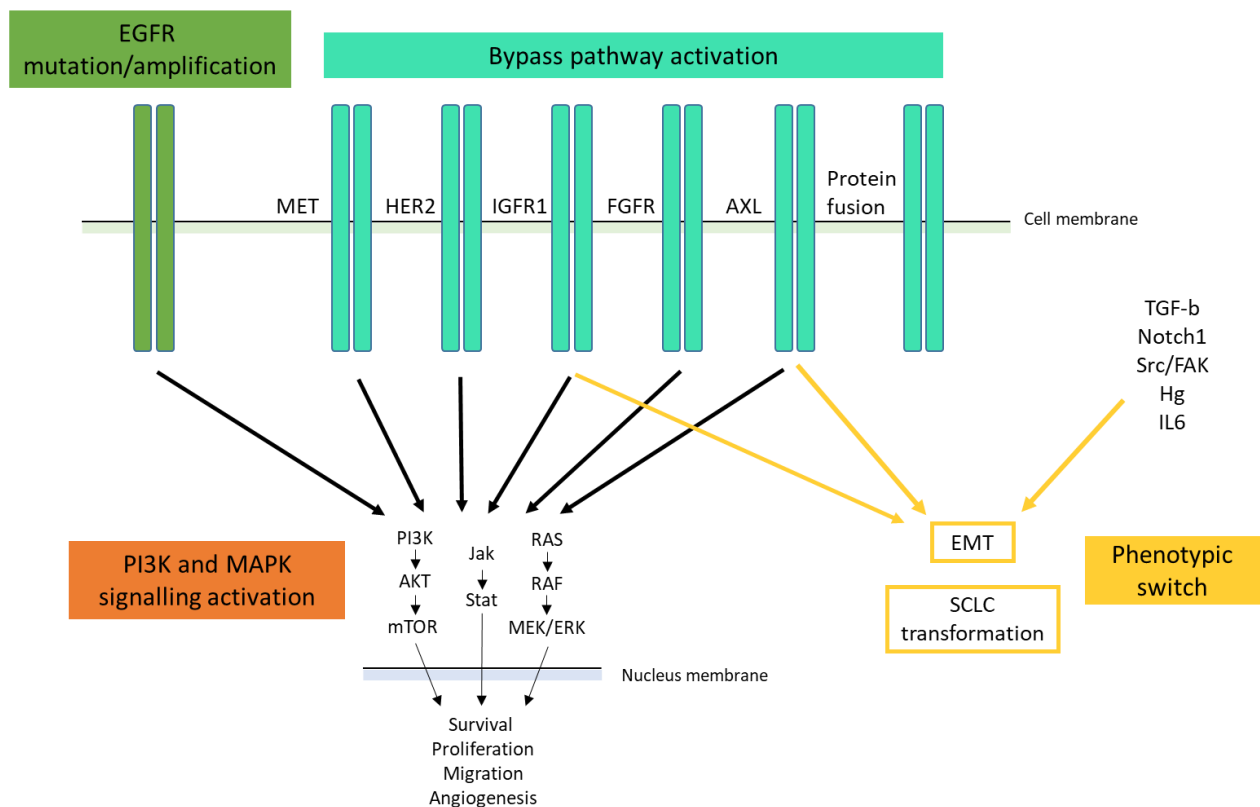


Figure 8 : Known mechanisms involved in EGFR-TKI resistances (based on ((Leonetti et al., 2019))

1.5.4.4 Drug-Tolerant Cells (DTC)

The mechanisms of resistance are of interest to many studies and are becoming better characterized. However, one of the current challenges is to understand how these resistances responsible for systematic relapse under EGFR-TKI emerge. Two theories are put forward:

-The first hypothesis suggests that inside the initial tumour there are cells that are sensitive to the treatment but also a resistant subpopulation that will be selected in a Darwinian way after the sensitive cells have died. The cells that are insensitive to treatment have an innate driver mutation that will allow re-proliferation and tumour resurgence. This would be a very small number of cells that cannot be observed and quantified with current technologies (Hata et al., 2016; Leonce et al., 2021; Marine et al., 2020) (Figure 9).

-The second hypothesis is based on Lamarck's law. A small population of cells that do not have a mutation at the outset would be able to resist the treatment by acquiring *de novo* resistance mechanisms. These cells, the drug tolerant cells or DTCs, would re-proliferate under treatment and cause the proliferation of tumour cells under treatment (Hata et al., 2016; Marine et al., 2020; Ramirez et al., 2016) (Figure 9).

Some *in vitro* studies have shown the coexistence of both theories in the tumour population (Hata et al., 2016). However, the study and validation of these theories *in vivo* is very much limited by current investigative techniques.

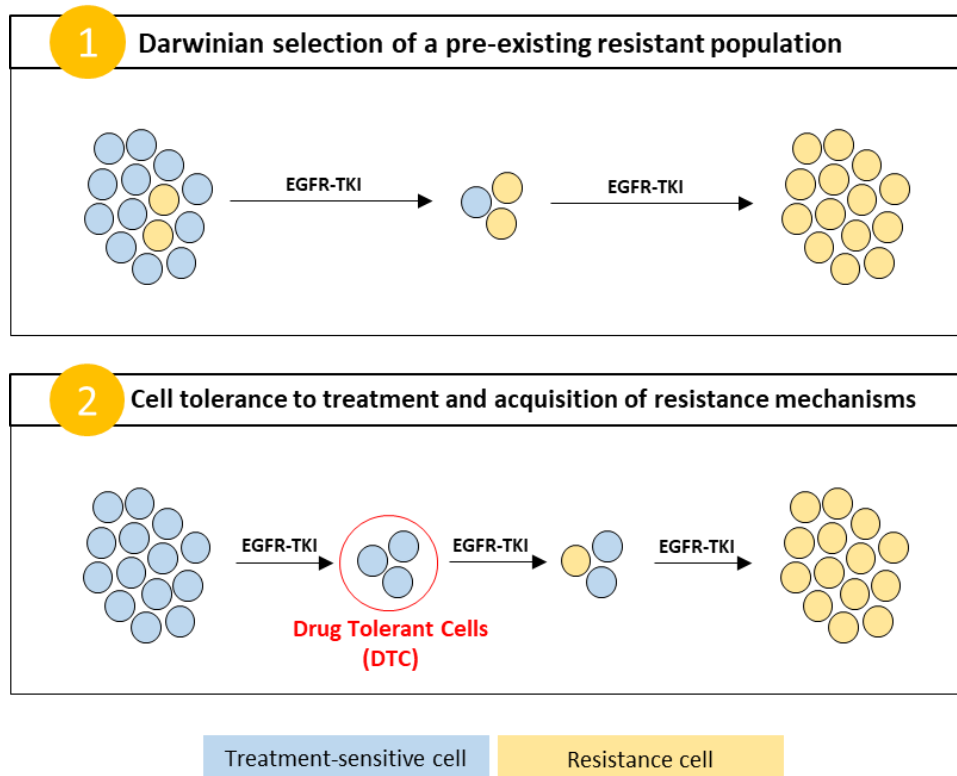


Figure 9 : Illustration of the two theories seeking to explain the emergence of EGFR resistance mechanisms

One of the first studies to define DTCs concept was that of Sharma et al. which showed the presence of a population, representing 0.3% of the initial population, resistant to treatment and responsible for cell re-proliferation under EGFR-TKI. Reproliferative cells are known as Drug Tolerant Expanded Cells (DTECs). Since that time, DTC, also named Drug Tolerant Persisters (DTP) has been described in many tumour models (Marine et al., 2020; Swayden et al., 2020) including lung cancer (Hata et al., 2016; Sharma et al., 2010), melanoma (Marin-Bejar et al., 2021), glioblastoma (Liau et al., 2017) and colon cancer (Shaffer et al., 2017). This population of DTCs has been shown in targeted therapies, for example, under EGFR-TKI treatment in lung cancer (Hata et al., 2016; Sharma et al., 2010), under Breakpoint Cluster Region Protein (BCR)-Abelson (ABL) kinase inhibitor treatment in glioblastoma (Liau et al., 2017) or under BRAF and MEK inhibitor treatment in melanoma (Marin-Bejar et al., 2021). Similar phenomena have also been highlighted as a mechanism of resistance to chemotherapies (Larrue et al., 2021).

The resistance of cells to treatment prior to the establishment of genetic modifications may be explained in these many oncogenic contexts by non-genetic mechanisms, defined as mechanisms that can modify gene expression without changing the DNA sequence. These are mainly chemical modifications superimposed on the genotype and are responsible for gene transcription states. This includes DNA methylation or histone modification by methylation, acetylation or phosphorylation (Guler et al., 2017; Sharma et al., 2010).

One of the arguments in favour of non-genetic changes is the reversibility of the DTC phenotype upon discontinuation of treatment (Hodgkinson et al., 2019). Indeed, it has been shown that cessation of treatment allows for the re-sensitisation of cells and the reconstitution of the initial cell pool (Hodgkinson et al., 2019). Studies on DTCs have shown a high degree of cellular heterogeneity associated with the emergence of genetic and non-genetic modifications (Marine et al., 2020; Sharma et al., 2010).

1.5.4.4.1 DTC and transcriptional repression

Transcriptional repression with modulation of histone acetylation and methylation has been shown to be involved in DTC resistance. In particular, histone H3 methylation on lysines 9 and 27 has been shown to *EGFR*-mutated NSCLC DTCs treated with EGFR-TKI and that the presence of the H3K9 methyl transferase was essential for phenotype establishment (Guler et al., 2017). It was also shown in *EGFR*-mutated NSCLC DTCs treated with an EGFR-TKI, a chromatin modification with demethylation of lysine 4 of histone H3, resulting in a change in the transcriptome of the cells (Sharma et al., 2010). This demethylation is mediated by the Lysine (K)-specific demethylase 5A (KDM5A) and its loss prevents the development of resistant cells *in vitro*. The study also shows that DTC survival and associated chromatid remodelling is dependent on IGF1R (Sharma et al., 2010).

This modification of gene accessibility and of the cell transcriptome has also been demonstrated in other oncogenic models, such as in ovarian cancer treated with chemotherapy (Gallon et al., 2021).

1.5.4.4.2 DTC and protective senescence

Recent work has shown that cell latency and accumulation in G1 of the cell cycle is linked to the entry of cells into a transient and reversible protective senescence state. A study by Kurppa et al. investigated the resistance observed in three *EGFR*-mutated NSCLC lines, PC9, HCC4006 and HCC827, treated with EGFR-TKI and MEK inhibitors. The survival of cells under treatment is explained by the entry of the cells into a pseudo-senescent state (β gal labelling positive cells) associated with an over activity of the Yes-Associated Protein (YAP)/ TEA Domain Transcription Factor (TEAD) pathway. This signalling pathway leads to the expression of the EMT-associated factor, SLUG, which inhibits the pro-apoptotic factor BCL2 Modifying Factor (BMF) and protects against cell death. Discontinuation of treatment leads to a loss of the senescent phenotype of the cells and to re-proliferation, showing that this is a reversible senescence. (Kurppa et al., 2020). The establishment of protective cell senescence under treatment has also been shown in other oncogenic and therapeutic contexts, such as in chemotherapy resistance in AML (Duy et al., 2021).

1.5.4.4.3 DTC and phenotypic switch

DTCs have been shown to exhibit high cellular plasticity, including the ability to change phenotype. For example, it has been shown that under treatment pressure the cells can dedifferentiate and acquire stem cell markers. Aldehyde Dehydrogenase 1 family member A1 (ALDH1A1) is an enzyme found associated with cancer stem cells (Ciccione et al., 2018). It has been shown to be expressed by DTCs of *EGFR*-mutated NSCLC treated with EGFR-TKI and essential for their maintenance (Raha et al., 2014). It has also been shown that DTCs increase the expression of stem cell markers such as CD133 and CD34 in the same oncogenic context (Sharma et al., 2010). This phenotypic change is also found in treatment tolerance in other oncogenic contexts treated with targeted therapies or chemotherapy.

1.6 RAS Homolog Family Member B (RHOB)

1.6.1 *RHO GTPases functions and classification*

The RAS Homolog family (RHO) family is a member of the RAS superfamily of guanine nucleotide-binding proteins, called small GTPases. These small GTPases and their regulators represent 1% of the human genome and are involved in many cellular processes. They play a role in gene expression, cytoskeleton remodelling, morphogenesis, cell polarity, cell cycle and cell division (Coleman et al., 2004; David et al., 2012; Haga and Ridley, 2016; Hall, 1998; Lawson and Ridley, 2018; Zegers and Friedl, 2014). They have an important role in many biological processes such as cell trafficking, tissue repair, embryonic development (Fernandez-Borja et al., 2005; Gutierrez et al., 2019;

Liu et al., 2001). Their deregulation is linked to cognitive disorders, immune and cardiovascular diseases but also to oncogenesis and metastatic processes (Jiang et al., 2004; Kusama et al., 2006; Zaoui et al., 2019).

The 20 RHO GTPases are divided into 8 subfamilies according to their sequence homology and mode of regulation: Rnd (Rnd1 to 3), RHO (RHOA, B and C), RHOV/RHOH, Rac (Rac 1 to 3 and RHOG), Cdc42/RHOJ/RHOQ, RHOV/RHOV, RHOH and RHOBTB (1 and 2) (Haga and Ridley, 2016) (Figure 10).

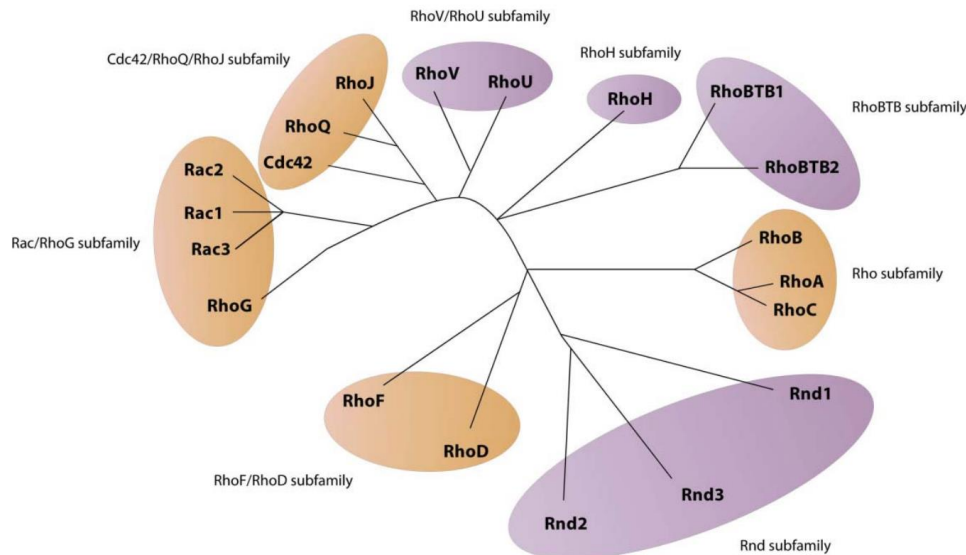


Figure 10 : Phylogeny of RHO GTPases from (Haga and Ridley, 2016).

In orange are the typical GTPases and in purple the atypical ones.

The RHO subfamily includes RHOA, RHOB and RHOC. Their sequences are 85% homologous and their three-dimensional structures are very similar (Wheeler and Ridley, 2004). Their major cellular function is the control of the actin cytoskeleton which will vary depending on the effector associated with the RHO GTPase (Fernandez-Borja et al., 2005; Tillement et al., 2008; Vega et al., 2012).

1.6.2 RHOB structure

The structure of the different RHO GTPases is very similar and evolutionarily conserved. RHOB, like all GTPases, consists of a guanosine triphosphate (GTP) and guanosine diphosphate (GDP) binding site, a GTPase domain with weak intrinsic hydrolysis activity and two short N- and C-terminal extensions. The N-terminal part includes the Switch 1 and 2 domains for binding to the regulators and effectors. These domains undergo a conformational change upon GTP binding to enable the effectors binding. In the carboxy-terminal part is a so-called "hypervariable" region, consisting of 25 amino acids, where the differences between the sequences of the different GTPases are concentrated. This region ends with a sequence of 4 amino acids or "CAAX" box, C for cysteine, A for aliphatic amino acid and X for any amino acid (i.e. CKVL for RHOB). The presence of this sequence is essential for post-translational modifications allowing the covalent attachment of a prenyl to the cysteine, which will be carboxylated after removal of the AAX sequence (Haga and Ridley, 2016; Wheeler and Ridley, 2004).

1.6.3 RHOB activity modulation

1.6.3.1 RHOB activation cycle

RHOB functions in a cyclic manner, alternating between an inactive state by binding to GDP and an active state by binding to GTP. This switch is enabled by two families of proteins. Firstly, the Guanine Nucleotide Exchange Factors (GEFs) which allow the dissociation of the GDP to favour the

fixation of GTP and therefore the activation of the GTPase. Secondly, the GTPase Activating Proteins (GAPs) hydrolyse the GTP into GDP to inactivate the GTPase (Haga and Ridley, 2016) (Figure 11).

Various GEFs were identified as potentially activating RHO, including Vav2 (Gampel and Mellor, 2002; Srougi and Burridge, 2011), GEF-H1 (Vega et al., 2011), Ect2, Net1 (Srougi and Burridge, 2011), XPLN (Arthur et al., 2002), p115-RhoGEF (Srougi and Burridge, 2011), PRG, p190-RhoGEF (Jaiswal et al., 2011) and RhoGEF12 (Arthur et al., 2002; Jaiswal et al., 2011).

The inactivation of RHO was shown *in vitro* to be catalysed by p190GAP (Kusama et al., 2006), DLC-1 and DLC-3 (Braun and Olayioye, 2015).

The regulation of RHO activity is tightly controlled by GEFs and GAP. This is achieved by extracellular signals (cytokines), by regulation of their subcellular localisation (phosphorylation and interactions with other players) and by interaction with guanosine nucleotide dissociation inhibitors (GDI) (Mosaddeghzadeh and Ahmadian, 2021). GDIs can prevent GDP dissociation or prevent GTP hydrolysis by competing with GEFs and GAPs (Haga and Ridley, 2016; Hodge and Ridley, 2016).

So far, three GDIs have been identified: RhoGDI1, RhoGDI2 and RhoGDI3. The first one is ubiquitous, the second one is mainly present in lymphocytic hematopoietic cells and the third one is found in brain, lung, testis and kidney of mice. The role of GDI in the regulation of RHO is still poorly understood (Ahmad Mokhtar et al., 2021; Mosaddeghzadeh and Ahmadian, 2021). So far only RhoGDI3 has been shown to interact with RHO (Ahmad Mokhtar et al., 2021; de León-Bautista et al., 2016).

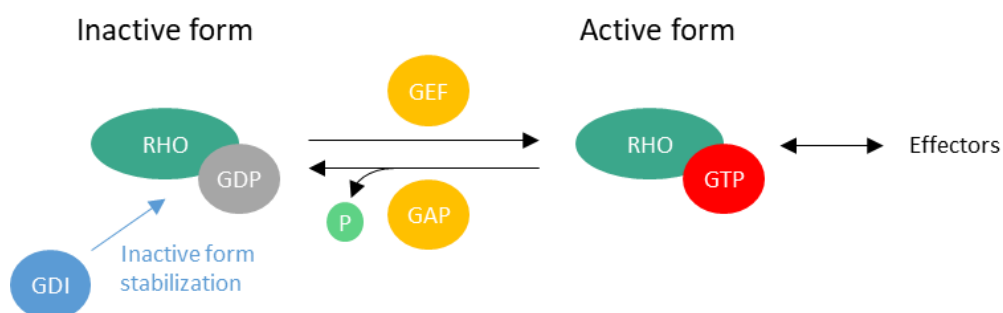


Figure 11 : Schematics of the GTPase activation cycle.

GEF: Guanine Exchange Factor; GAP: GTPase activating protein; GDI: guanosine nucleotide dissociation inhibitors

1.6.3.2 Regulation of RHO transcription

RHO gene is located on chromosome 2 and consists of only one exon (Gutierrez et al., 2019). Its transcription depends on different stimuli such as growth factors or drugs such as ionizing radiation (Monferran et al., 2008), chemotherapies (Fritz et al., 1995) or targeted therapies, such as BRAF (Delmas et al., 2015) or EGFR inhibitors (Calvayrac et al., 2017). Its expression depends in particular on epigenetic mechanisms. In the context of lung cancer, it has been shown that histone deacetylation by histone deacetylases (HDAC) decreases RHO expression (Gutierrez et al., 2019; Mazières et al., 2007; Wang et al., 2003).

1.6.3.3 Post-transcriptional modifications of RHO

The expression of RHO can be negatively regulated by miRNAs, which by pairing on the 3'UTR region of RHO messenger ribonucleic acid (mRNA) will cause either its degradation or the repression of its translation (Glorian et al., 2011; Shakeri et al., 2021; Wei et al., 2018). This is notably the case of miR-21, overexpressed in many cancers, which will have a pro-oncogenic role in particular by repressing RHO (Gutierrez et al., 2019; Lv et al., 2019; Shakeri et al., 2021).

1.6.3.4 Post-translational modifications of RHOB.

RHOB can undergo different types of post-translational modifications. Notably, RHOB can be prenylated, by adding a prenyl group to its CAAX box. It can be either geranylgeranylated (GG-RHOB) by the geranyltransferase I or farnesylated (F-RHOB) by farnesyltransferase (Adamson et al., 1992; Hodge and Ridley, 2016). The prenylation of the protein will greatly condition its localization. F-RHOB will be found at the plasma membrane while GG-RHOB will be preferentially found at the endosomes (Pérez-Sala et al., 2009; Wherlock et al., 2004). RHOB can also be palmitoylated by adding palmitic acid on two of its cysteines, at position 189 and 192 (Adamson et al., 1992). This modification allows the anchoring of RHOB to the plasma membrane.

RHOB can be inactivated by phosphorylation, notably at position 185 by serine/threonine casein kinase 1. Phosphorylation can induce the translocation of the RHO proteins to different cellular compartments (Hodge and Ridley, 2016)

Finally, RHOB can be sumoylated on its lysines 7, 135 and 194 which will lead to its addressing to the lysosome (Liu et al., 2018).

1.6.3.5 Regulation of RHOB degradation

RHOB has in its C-terminal region an addressing sequence to the lysosome where it will be degraded (Pérez-Sala et al., 2009). The prenylation and palmitoylation of the protein in C-terminal allows the stabilization of the protein and limits its degradation (Pérez-Sala et al., 2009).

1.6.4 *Biological roles of RHOB*

1.6.4.1 Regulation of the actin cytoskeleton

One study showed that expression of an active mutant of RHOB led to an increase in the formation of stress fibers and focal adhesion points (Allal et al., 2002; Tillement et al., 2008), whereas expression of an inactive mutant of RHOB decreased stress fibers (Papadopoulou et al., 2008; Vardouli et al., 2005). Inhibition of RHOB by siRNA in rat adrenal glands prevents reorganization of the actin cytoskeleton after treatment of the cells with lysophosphatidic acid (Ishida et al., 2004).

RHOB also modulates endosomal transport by promoting actin assembly via Dia1 (Fernandez-Borja et al., 2005).

1.6.4.2 Intracellular trafficking regulation

RHOB is involved in addressing different proteins to subcellular compartments where they can play their biological role (Huang and Prendergast, 2005). The role of RHOB is to polymerize actin to allow the trafficking of endocytosis vesicles (Huang et al., 2007; Sandilands et al., 2004).

This is notably the case for EGFR (Lajoie-Mazenc et al., 2008). Indeed, RHOB prevents the degradation of EGFR by inhibiting its addressing to the lysosome and by promoting its recycling to the plasma membrane where it can be activated (Gampel et al., 1999). This would be due to GG-RHOB, because following treatment of cells with a farnesyltransferase inhibitor (FTI), the GG-RHOB fraction increases and leads to a slowing down of EGFR addressing to lysosomes and increases its addressing to the plasma membrane (Wherlock et al., 2004).

RHOB has been shown to be involved in the trafficking of many other proteins such as Platelet-Derived Growth Factor Receptor (PDGFR) (Huang et al., 2007), RAC1, CDC42 (Huang et al., 2011), AKT (Adini et al., 2003) amongst others.

1.6.4.3 RHOB and cell migration and adhesion

The role of RHOB in adhesion and migration depends on the cell type and the biological context and RHOB expression can be associated to either pro- or anti-migratory properties (Vega et al., 2012).

When it is pro-migratory, RHOB will allow the formation of plasma membrane protrusions necessary for migration (Vega et al., 2012) by modulating the activity of RAC1 and CDC42 (Huang et al., 2011). It has been shown in prostate cancer cells that RHOB depletion inhibits cell migration and invasion (Alfano et al., 2012) while its overexpression leads to cell migration and invasion by inducing the expression of the metalloprotease MMP1 (Yoneda et al., 2010).

When it is anti-migratory, RHOB allows the maintenance of junctions between cells and E-cadherin, as shown in prostate tumour cells (Vega et al., 2015). In bronchial cell lines, inhibition of RHOB results in cell migration and invasion (Bousquet et al., 2009). Overexpression of RHOB in liver, breast (Connolly et al., 2010) or gastric (Zhou et al., 2011) tumour cells decreases cell migration and invasion.

1.6.5 *RHOB and cancer*

RHOB expression, unlike its homologues RHOA and RHOC, is decreased in many oncogenic contexts. This is notably the case in breast cancer (Médale-Giamarchi et al., 2013), brain cancer (Forget et al., 2002), melanoma (Wen et al., 2017) and lung cancer (Bousquet et al., 2009; Calvayrac et al., 2014; Mazieres, 2004; Sato et al., 2007). In this case, the loss of RHOB is correlated with the stage of the disease and its aggressiveness. The decrease of RHOB expression can be explained by an allelic loss at the gene locus on chromosome 2p24 as shown in NSCLC cell lines (Sato et al., 2007).

However, in rare literature, RHOB expression has been shown to be increased in breast (Fritz et al., 2002; Médale-Giamarchi et al., 2013) and bronchial cancer (Luis-Ravelo et al., 2014). Indeed, the role of RHOB is controversial and it is described, depending on the stage and nature of the cancer, as being either anti- or pro-oncogenic.

1.6.5.1 RHOB as pro oncogenic factor

A study showed that RHOB expression was increased in metastatic lung cancer (Luis-Ravelo et al., 2014). RHOB-depleted cells injected intracardially in mouse models decreased the size and number of bone metastases while RHOB overexpression promoted extra-pulmonary metastasis by promoting tumour cell invasion and intravasation (Luis-Ravelo et al., 2014).

Overexpression of RHOB in breast cancer has a poor prognosis (Fritz et al., 2002). (Fritz et al., 2002). Indeed, breast tumours overexpress RHOB and this correlates with the tumour grade, with overexpression in advanced grades (Fritz et al., 2002). The pro-oncogenic role is explained by its impact on the expression of oestrogen receptor α and progesterone receptor α , which impacts on cell proliferation (Médale-Giamarchi et al., 2013).

For example, in glioblastoma, the loss of RHOB expression limits cell proliferation by blocking the cycle and leads to their death by apoptosis (Ma et al., 2015). RHOB is also thought to promote cell adaptation to environmental stresses such as hypoxia (Skuli et al., 2006).

RHOB has been also shown to promote tumour cell migration prostate (Yoneda et al., 2010) and liver (Jin et al., 2017) cancer models, and to protect cells from apoptosis in melanoma and breast cancer models.

1.6.5.2 RHOB as tumour suppressor

RHOB and tumour progression

As previously mentioned, oncogenesis has been shown to correlate with loss of RHOB expression in many oncogenic contexts. This is notably the case in lung cancer where a decrease in RHOB expression has been shown to correlate with disease progression and tumour cell proliferation (Calvayrac et al., 2014; Mazieres, 2004). Ectopic expression of RHOB inhibited cell proliferation of lung cancer cells and decreased tumour growth in mouse models of xenografts (Mazieres, 2004).

RHOB expression was also shown to be decreased in melanoma cells. (Wen et al., 2017) Ectopic expression of RHOB decreases RAS oncogene-induced fibroblast transformation as well as their migration and invasion (Jiang et al., 2004). A study has also shown that inhibition of RHOB increases the likelihood of developing chemically induced tumours (Liu et al., 2001). Another study showed, on the contrary, that the inhibition of RHOB decreased the appearance of skin tumours induced by UV but also that the tumours developed were more undifferentiated and proliferative (Meyer et al., 2014).

RHOB, despite its pro-oncogenic role described above, has also anti-tumour potential in brain tumours (Forget et al., 2002). Indeed, RHOB expression is inversely correlated with tumour aggressiveness. RHOB appears to be involved in tumour invasion. Indeed, its re-expression limits the invasion and motility of glioblastoma cells (Baldwin et al., 2008; Forget et al., 2002).

In ovarian cancer, a loss of RHOB expression is correlated to the grade of the disease (Liu et al., 2013). Overexpression of RHOB in tumour cells limits cell proliferation and induces cell death (Liu et al., 2013). This is supported by the fact that re-expression of RHOB in tumour cells inhibits their migration and invasion (Liu et al., 2013). It has also been shown that overexpression of RHOB in ovarian cancer cell models results in decreased cell proliferation and increased apoptosis (Couderc et al., 2008). *In vivo*, this overexpression of RHOB inhibits tumour growth in xenografts (Couderc et al., 2008).

Similar data are available for many other cancers such as bladder cancer (Kamai et al., 2003), head and neck cancer (Adnane et al., 2002), kidney cancer (Chen et al., 2016) and thyroid cancer (Ichijo et al., 2014). In these oncogenic contexts, loss of RHOB is a marker of malignancy and its use as a biomarker is being considered in the clinic.

RHOB and maintenance of genome integrity

RHOB is thought to help maintain genome integrity by limiting DNA double strand breaks (DSBs). It has been shown in melanoma cells that loss of RHOB increases DSB markers such as γ H2AX or phospho-53 Binding Protein 1 (p-53BP1) (Meyer et al., 2014). Similarly, in murine fibroblasts depleted for RHOB was shown the accumulation of γ H2AX foci and chromosomal abnormalities (Mamouni et al., 2014).

1.6.6 RHOB and response to therapies

Radioresistance

RHOB has been shown to influence the response to radiotherapy. Notably in colon cancer, where RHOB expression has been shown to be proportional to radioresistance (Liu et al., 2019a). Depletion of RHOB sensitised SW480 colorectal cancer cells to radiotherapy (Liu et al., 2019a). In patients, RHOB can be overexpressed during this treatment, resulting in a poor prognosis, worsening of the disease, distant dissemination and poor survival of patients (Liu et al., 2019a).

In lung cancer, RHOB expression is also associated with radioresistance. However, overexpression of RHOB in lung tumour cells has no effect on radioresistance. It seems that it is the

overexpression of RHOB in the microenvironment and more precisely in the stromal cells that leads to the resistance (Luis-Ravelo et al., 2014).

Chemoresistance

The study that identified the role of RHOB in resistance to radiotherapy in lung cancer also identified its involvement in resistance to taxane-chemotherapy. Indeed, inhibition of RHOB increases the chemosensitivity of cells, while its overexpression decreases this sensitivity. (Luis-Ravelo et al., 2014).

However, RHOB is also involved, in some oncogenic contexts, in chemo sensitization. This is the case for laryngeal cancer where overexpression of ROHB sensitises cells to cisplatin and increases apoptosis, whereas inhibition of RHOB reduces this sensitivity by decreasing the accumulation of the drug in the cells (Čimborá-Zovko et al., 2010). Similar observations have been made in gastric cancer, where RHOB sensitizes cells to chemotherapy (Zhou et al., 2011).

Resistance to targeted therapies

In *BRAF*-mutated melanoma treated with Vemurafenib, activation of the JUN/RHOB/AKT pathway has been shown to allow the emergence of resistance to treatment and lead to cell survival. Indeed, BRAF inhibition leads to C-JUN transcription factor-dependent overexpression of RHOB correlating with resistance. Indeed, depletion of RHOB increases cell apoptosis and sensitises the cells to treatment. (Delmas et al., 2015).

RHOB also plays a role in resistance to EGFR-TKIs in *EGFR*-mutated NSCLC (Calvayrac et al., 2017b). *In vitro*, modulation of RHOB expression in bronchial tumour lines with EGFR mutations determines the level of response to EGFR-TKIs. Indeed, knockdown of RhoB by siRNAs sensitizes cells to EGFR-TKIs and re-expression of RhoB by adenoviral transduction reverses this effect (Calvayrac et al., 2017). In addition, the gradual increase in RHOB overexpression of RHOB, is associated to a proportional increase in the 50% inhibitory concentration of Erlotinib, suggesting a decrease in sensitivity to this EGFR-TKI. *In vivo* study performed in a transgenic mouse model with tumours carrying the *EGFR* L858R mutation showed that RHOB-proficient mice were resistant to erlotinib while RhoB-deficient mice were not. (Calvayrac et al., 2017). In NSCLC patients with activating mutations in EGFR, tumour expression of RHOB at diagnosis is a predictive marker of response to EGFR-TKIs. Indeed, at the time of diagnosis, patients with high tumour expression of RhoB have a significantly shorter progression-free survival than patients with low RHOB tumour expression. In addition, between diagnosis and the relapse under EGFR-TKIs, RHOB expression increased in 8 out of 11 patients, suggesting a role of RhoB in adaptive resistance to EGFR-TKIs (Calvayrac et al., 2017).

Therefore RHOB is, in many oncogenic contexts, a tumour suppressor but in targeted therapy treatments RHOB becomes predictive of a poorer response to treatment. Hence RHOB is proposed as a biomarker of response to targeted therapy. In addition, this also makes RHOB a potential target for inhibition to improve the response to targeted therapies and avoid the emergence of resistance.

1.6.7 *RHOB* inhibitors

1.6.7.1 FTI

As presented above, the localisation of RHOB greatly conditions these functions its localisation is conditioned by the prenylation of the protein (Pérez-Sala et al., 2009; Wherlock et al., 2004). Indeed, when the protein is farnesylated, it is found at the plasma membrane and when it is geranyl-

geranylated, it is found at the endosomes (Pérez-Sala et al., 2009; Wherlock et al., 2004). The addition of these two prenylation motifs is catalysed by FTase and GGTase I, respectively.

One of the best known farnesylated protein is RAS which is found mutated in many cancers. Farnesyltransferase inhibitors (FTI) have therefore been developed (Lebowitz et al., 1997a; Sebti, 2005). FTIs inhibit the function of H-RAS protein by blocking its post-translational farnesylation, which is essential for proper membrane localization and oncogenic activity (Huang and Prendergast, 2005; Rane and Prendergast, 2001). To present, four FTIs have been tested: Tipifarnib, Ionafranid, L-778123 and BMS-214662. The use of FTI as a single agent in *HRAS*-mutated cancers has been shown to be effective in thyroid tumours, breast cancer, head and neck and salivary gland cancer (Gilardi et al., 2020; Hanna et al., 2020; Ho et al., 2021; Lee et al., 2016a; Untch et al., 2018). FTIs were unsuccessful to treat mutant K/NRAS tumours because these were shifting towards a GG form to evade the FTI (Lerner et al., 1997; Whyte et al., 1997). However FTIs will have an inhibitory effect on other farnesylated proteins such as RHO GTPases (Lebowitz and Prendergast, 1998; Lebowitz et al., 1997a). Indeed, the apoptosis of *HRAS*-mutated tumour cells treated with FTI would be promoted by the inhibition of RHO GTPases and their role in cell adhesion (Lebowitz et al., 1997b).

FTI has been tested as a single agent in various tumour settings, with poor objective responses. A phase II trial in pancreatic cancer showed no effect of FTI on tumour (Macdonald et al., 2005). The same results were obtained in the context of lung cancer (Adjei et al., 2003; Heymach et al., 2004), colorectal cancer (Hata et al., 2016) and brain cancer (Fouladi et al., 2007). However, FTIs have shown some interesting results in the context of liquid cancers (Fenaux et al., 2007; Harousseau et al., 2007) and breast cancer (Johnston et al., 2003).

FTIs have also been tested in combination with other therapies in many oncogenic settings, including chemotherapy and radiotherapy. For example, a phase II clinical trial combining tipifarnib with gemcitabine showed high toxicity in patients and did not improve the results obtained with gemcitabine alone (Yam et al., 2018). In some oncogenic settings, FTI in combination with other therapies has shown minimal or no improvement in baseline therapy (Burnett et al., 2012; Dalenc et al., 2010; Jabbour et al., 2011; Li et al., 2012).

Phase II clinical trial shows FTI enhances the clinical efficacy of doxorubicin-cyclophosphamide in both early and advanced tumours (stage IIB-IIIC) (Sparano et al., 2009).

In NSCLC, treatment with Tipifarnib as a single agent was the subject of a phase II clinical trial which, although convincing in its lack of significant side effects, showed only minimal clinical activity. The same FTI was also tested in combination with the EGFR-TKI Erlotinib. This study showed 2 tumour regressions, 10 disease stabilisation and 11 tumour progressions. These poor results could be attributed to the lack of selection of patients on their EGFR mutation status (Adjei et al., 2003).

1.6.7.2 C3-exoenzyme

C3-exoenzyme is an exoenzyme from *Clostridium botulinum*. It is a specific inhibitor of RHOA, RHOB and RHOC (Quilliam et al., 1989). In particular, C3-exoenzyme has made it possible to better study and understand the biological functions of these GTPases in cells. It causes ADP ribosylation of its targets, which can no longer bind to GTP and lose the possibility of being activated (Aktories and Frevert, 1987).

The C3-exoenzyme destabilises the actin cytoskeleton associated with increased DNA damage in the cell (Magalhaes et al., 2020). Treatment of melanoma cells with C3-exoenzyme decreases cell survival and increases their mutability when exposed to UV light. The DNA damage repair defects can

be explained by constitutive activation of ERK1/2 and defective phosphorylation of proteins involved in damage repair, such as p53, γ H2AX and CHK1 (Magalhaes et al., 2020). Therefore, the use of C3-exoenzyme highlighted, in this context, the involvement of the cytoskeleton in the maintenance of genome stability (Magalhaes et al., 2020). Other studies have shown that C3-exoenzyme, through its inhibition of RHO GTPases, regulates the transcription of several genes, such as *SP1* and *Activating transcription factor 2 (ATF2)*, which are important for the regulation of cell targeting and cell survival (von Elsner et al., 2016, 2017). Consequently, C3-exoenzyme decreases the survival and proliferation of these cells (von Elsner et al., 2016, 2017). The C3-exoenzyme has also been used to study the involvement of the cell cytoskeleton in cell migration, particularly in macrophages or tumour cells (Rotsch et al., 2012; Yao et al., 2016; Zhang et al., 2011).

C3-exoenzyme is known to enter dendritic cells notably by endocytosis (Fellermann et al., 2020), or to be more easily taken up by cells strongly expressing vimentin (Rohrbeck et al., 2014). However, few cells are permeable to it. To enter cells, C3-exoenzyme is usually associated with a cell-permeable peptide (CPP): TAT. In this case, the molecule is called TAT-C3. The addition of this CPP favours C3-exoenzyme to enter into all cells. Nevertheless, because RHO GTPases are important in the majority of the cells of the human body, its systemic use is not possible. For the use of this RHO GTPase inhibitor, it is essential to find a therapeutic vector that allows its specific addressing to the tumour.

We established a collaboration with Prof. Marcelle Machluf's team (Technion, Israel) who is developing MSC-derived nanoghosts (NG), which have the particularity of having a specific addressing to tumour cells (Timaner et al., 2018; Kaneti et al., 2016). Their use as therapeutic vectors have been validated in numerous *in vitro* models and prostate cancer *in vivo* model (Timaner et al. 2018; Furman et al, 2013).

MSCs, from which NGs are derived, have been shown to be of therapeutic benefit and as a therapeutic vector in various tissue regeneration (Perry et al., 2021; Vieujean et al., 2021) and oncogenic contexts (Krueger et al., 2018; Li et al., 2018). However, their use for therapeutic purposes is limited by their pro-tumour potential, with a role in enhancing tumour growth (Quante et al., 2011; Yu et al., 2021a) and involvement in metastatic dissemination (Cortini et al., 2016; Mele et al., 2014; Xue et al., 2015). MSCs are also implicated in resistance to therapies such as chemotherapy (Pillozzi et al., 2018; Schelker et al., 2018) or targeted therapies (Wang et al., 2019a).

2 Mesenchymal stem cells (MSC)

2.1 Description

MSCs are multipotent adult stem cells derived for different tissues. The MSCs were first isolated and described in the bone marrow in 1968 by the Friedenstein's team (Friedenstein et al., 1968). The MSCs derived from this tissue are named bone-marrow derived MSC (BM-MSC). Since then, these cells have been isolated from many other tissues such as adipose tissues (named adipose-derived MSCs or ASCs), dental pulp, thymus, synovial fluid, cartilage, muscle tendon, spleen, dermis, lung, placenta, umbilical cord, Wharton's jelly... (Figure 12).

Because of the cell heterogeneity mainly due to this intertissular origin, one of the main difficulty to work with MSCs is the lack of specific proteins or transcriptional signature. The bulk of cells can't be identified by cell surface markers which limited *in vivo* studies.

To homogenize MSC studies, the International Society of Cellular Therapy (ISCT) has defined the minimal criteria to identify MSCs *in vitro*. The MSCs have to adhere to plastic under standard culture condition. After seeding, they acquire a spindle-shaped, fibroblast-like morphology and the

ability to create colony-forming unit fibroblasts (CFU-F). They have to present the surface cell markers CD105, CD73 and CD90 and lack the hematopoietic and immunological markers such as Cd11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR. Finally, the MSCs retain the ability to differentiate into three specific lineages: adipogenic, chondrogenic and osteoblastic lineages upon inductive medium (Viswanathan et al., 2019; Dominici et al., 2006; Horwitz et al., 2005) (Figure 12)..

Despite these criteria aimed to homogenise studies on MSCs, the capacities of the MSCs vary according to their tissue of origin. This is why we are going to focus only in two human tissue origins:

-the adipose tissue, the ASCs, being the most described in literature, due to the accessibility of the tissue notably thank to abdominal dermolipectomy.

-the bone marrow, the BM-MSCs, well described too, due to the possibility of isolating them from the femoral head.

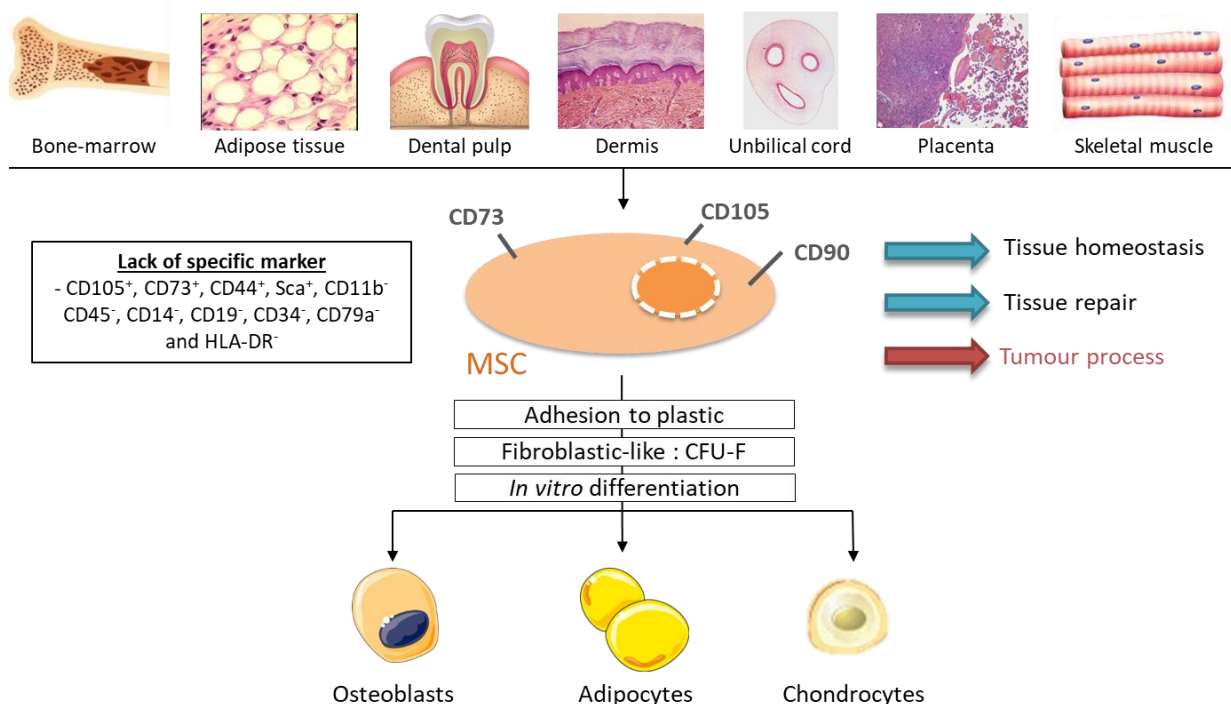


Figure 12 : MSCs characteristics

MSCs can be isolated from different tissues such as bone marrow, adipose tissue, dental pulp. Despite the absence of specific markers, MSCs must have certain markers on their surface and must be free of others. The minimum criteria for cell characterization are adherence to plastic, formation of CFU-F in vitro and differentiation capacity. MSCs, physiologically associated with the maintenance of homeostasis and tissue repair, will play a role in the tumour process.

2.2 Physiological role: wound healing and tissue homeostasis

MSCs present remarkable biological capabilities that make them a key player in tissue repair and homeostasis maintenance. The cells located in tissular niches would be recruited to the wound where they can enhance the repair notably by virtue of their differentiation, secretion and immunodulation capabilities.

The enhancement of tissue repair by MSCs has been demonstrated in different models of injury after MSC injection. They improve healing by limiting the formation of scar tissue, which corresponds to the replacement of the original tissue by a neoformed fibrous connective tissue, and

promoting the tissue regeneration, which restores the original tissue with preservation of its form and function. This has been demonstrated in many lesion models, such as the healing of a skin wound (Zhou et al., 2021), of lesions in various organs (nervous system (Volkman and Offen, 2017), liver (Jiao et al., 2021), lung (Wick et al., 2021), etc.), bones (Thomas and Jaganathan, 2021) and joints (Satué et al., 2019).

2.2.1 Homing to the injured tissue

After wound, the cells of the injured tissue secrete into the bloodstream some factors that can attract the MSCs located in the surrounding tissues (Becker and Riet, 2016). This attraction is mainly facilitated by the location of the MSCs in niches near to blood vessels. This allows the rapid recruitment of MSCs to the injured site.

The migration of MSCs presents similar steps to those observed during the migration of lymphocytes. MSCs leave their original niche and enter the bloodstream guided by gradients of chemokines and growth factors secreted by the injured cells. Once at the site of injury, MSCs slow down by attaching to surface molecules of vascular endothelial cells. Then, the MSCs adhere to the vascular endothelium, which leads to their immobilization. They flatten and finally leave the bloodstream by trans endothelial migration, allowing them to reach the injured tissue.

Many different soluble factors have been described as potential attractors for the MSCs. Cytokines and growth factors secreted by cells from the injured site into the bloodstream bind to their receptor on the surface of the MSCs causing MSC migration (Becker and Riet, 2016).

2.2.1.1 Molecules stimulating the migration of MSCs

Chemokines

CXCL12-CXCR4/7 axis

One of the most well documented factors is C-X-C motif chemokine ligand (CXCL)12, also known as Stromal cell-Derived Factor 1 α (SDF1 α). It is an important chemokine in physiological and pathological processes involved in the migration of haematopoietic precursors, endothelial cells and leukocytes. Its action involves the binding to C-X-C chemokine receptor (CXCR)4 and CXCR7 (Janssens et al., 2018). Physiologically, CXCR4 is essential for the retention of MSCs in their niche, for example in the bone marrow (Singh et al., 2020) and it plays a critical role in MSC mobilization and fate in injury context. The recruitment of MSCs depends on the plasma concentration of CXCL12. In a context of tissue repair, an important concentration of CXCL12 allows the passage of MSCs to the bloodstream. This chemokine is upregulated in some damaged organs, including notably in acute injury and fibrosis of skin (Cao et al., 2019a) and liver pathology (Chen et al., 2021). The role of this factor on MSCs is even the more important as the MSCs have and also can overexpressed the corresponding membrane receptor CXCR4 (Bobis-Wozowicz et al., 2011) and CXCR7. Multiple studies show that the action of CXCL12 on MSC homing is mediated by binding to the CXCR4 (Cao et al., 2019a; Hervás-Salcedo et al., 2021; Singh et al., 2020). In some lesion contexts, few in number, the role of CXCL12 does not involve binding to CXCR4 but to CXCR7 (Wang et al., 2014). The role of the axis CXCL12/CXCR4 and/or CXCR7 has been demonstrated for BM-MSCs (Liu et al., 2015; Wang et al., 2014) and ASCs (Bobis-Wozowicz et al., 2011; Li et al., 2013) in various lesion contexts. *In vitro* studies on CXCL12 gradient have confirmed the importance of this chemokine for the directional migration of MSCs (Park et al., 2017) and that it acts through both the JANUS KINASE (JAK)/STAT3 and Extracellular signal Regulated Kinases (ERK)/MEK pathways (Gao et al., 2009).

Apart from this direct role of CXCL12 on the cells, this chemokine, by its action on other cells of the lesion environment, leads to the secretion of other factors attracting MSCs. Indeed, CXCL12 will also activate the endothelial cells at the site of injury and have an indirect role on the recruitment of MSCs to the lesion. Indeed, in response to this stimulus, these cells secrete Platelet-Derived Growth Factor (PDGF), which can drive the migration of MSCs to the wound. PDGF binds to the PDGFRA receptor and activates various downstream signalling pathways, such as JAK2/STAT, PI3K/AKT or MAPK/Growth factor Receptor Bound protein 2 (GRB2), leading to directed motility of MSCs (Popielarczyk et al., 2019).

Other chemokines

Other chemokines whose corresponding receptors are present on the surface of MSCs may play a role in the MSC recruitment. For example, the couple C-C Motif Chemokine Ligand (CCL2)7- C-C chemokine Receptor (CCR)10 has been shown to allow the recruitment of MSCs to cutaneous tissue (Alexeev et al., 2013). The couple CCL21-CCR7 seems also to be involved in the mobilization of MSCs (Sasaki et al., 2008). In the context of cerebral ischemia, the recruitment of MSCs is facilitated by the expression on their surface of CCR2, one of whose ligands is CCL2 (Huang et al., 2018). CCL2 is also involved in MSCs recruitment to glioblastoma treated by radiotherapy (Thomas et al., 2018).

Growth factors

In addition to chemokines, MSCs can be recruited to the lesion site by growth factors, such as the Vascular Endothelial Growth Factor (VEGF) (Ishii et al., 2019), the Insulin-like Growth Factor-1 (IGF) (Ponte et al., 2007), the PDGF (Tyurin-Kuzmin et al., 2016) and TGF (Belotti et al., 2016; Nam et al., 2020; Ponte et al., 2007)

VEGF

In vitro studies have shown that the VEGF secreted by endothelial cells plays a role in the recruitment of MSCs to the injured site. It has been shown *in vitro* that VEGF-C, secreted notably by endothelial cells, allows the migration of MSCs, via its binding to VEGF Receptor (VEGFR)2/3 (Ishii et al., 2019). This involves the activation of the protein kinases ERK and FAK (Ishii et al., 2019). *In vivo*, VEGF-A is essential for recruitment of MSCs in the course of endochondral bone formation or remodelling, which is not the case for -E and -C isoforms. (Fiedler et al., 2005).

IGF

IGF increases MSC migration in a dose-dependent manner (Ponte et al., 2007). However, this doesn't seem to be a direct effect. Indeed, exposure of MSCs to IGF leads to over-expression of the CXCL12 receptor, CXCR4, on their surface, which, as previously described, is strongly involved in MSC migration (Huang et al., 2012). In particular, it enhances MSCs migration in myocardial infarction via the activation of AKT/SFRP2 (for secreted frizzled-related protein 2)/ β -catenin pathway (Lin et al., 2020).

PDGF

PDGF stimulates MSC migration *in vitro* (Tyurin-Kuzmin et al., 2016). The signalling pathways PI3K-Akt, MAPK, and Jak/Stat are involved when migration is activated by PDGF stimulation (Popielarczyk et al., 2019; Tyurin-Kuzmin et al., 2016). PDGF-AB and -BB seem to be pro-migration (Phipps et al., 2012; Ponte et al., 2007). Thrombospondin-1 enhances PDGF-mediated migration of MSCs by decreasing PDGF degradation and by activating integrin signalling pathway critical in determining the biological response of MSC to PDGF (Belotti et al., 2016).

2.2.1.2 Adhesion to endothelial cells and transmigration

Receptors and ligands expressed at the membrane of the MSCs permit their adhesion to endothelial cells and their slowing down in the neighbourhood of the injured site. MSCs express the integrin VLA-4 (for very late antigen-4), also expressed by leukocytes, which bind the receptor VCAM-1 present at the surface of the inflamed vascular endothelium. Pro-inflammatory cytokines, such as TNF-alpha, activate endothelial cells in the vicinity of the leaked site resulting in the expression of factors that allow the attachment of MSCs, such as VCAM-1 or ICAM-1 (Steingen et al., 2008; Teo et al., 2012). After MSC adhesion to endothelial cells, the rolling and diffusion steps are notably facilitated by CXCL9. This factor, and others (such as CXCL16, CCL20 and CCL25), also enhance the transmigration of MSCs (Chamberlain et al., 2011). It permits the immobilization of MSCs on the vascular wall allowing their transmigration and arrival to the injured site. VCAM-1 is also essential for the transmigration of MSCs between endothelial cells constituting blood vessels. The released homing factors form a chemical gradient from the injury site to the surrounding area, which initiates the transmigration of stem cells through the endothelium and directional migration into the stromal tissue. Transmigration can be either paracellular or transcellular. In the first case, the tight junctions between the endothelial cells are broken to allow the passage of MSCs. In the second case, the MSCs pass through pores that form in the endothelial cell (Teo et al., 2012).

MSC transmigration between endothelial cells is notably enabled by the expression of metalloproteinases (MMPs) such as MMP-1, MMP-2, MMP-13, MT1-MMP, as well as regulators of MMP activity as TIMP-1 and TIMP-2 (Tondreau et al., 2009). The expression of MMPs is strongly increased in an inflammatory environment which facilitates the recruitment of MSCs to the injured site (Ries et al., 2007; Steingen et al., 2008).

Since the majority of MSC homing studies are performed after cell injection, recruitment is mainly studied in blood tissue. However, some studies suggest a possible migration of MSCs into the lymphatic tissue or by transmigration within the tissue.

2.2.2 Role at the site of injury

Once recruited to the damaged site, MSC have several roles. They can enhance the healing by various process notably by their differentiation, pro-angiogenic, anti-inflammatory and immunomodulatory capacities.

2.2.2.1 MSCs differentiation

To be defined as MSCs *in vitro*, the cells have to be multipotent by their ability to differentiate in cells of mesodermal lineage such as adipocytes, chondrocytes and osteoblasts. But in an injury context, and in response to specific stimuli, they could differentiate in other cell types by virtue of their transdifferentiation abilities. Unfortunately, MSC differentiation is very complicated to study *in vivo*, due to the lack of specific markers. Transdifferentiation is considered following differentiation into non-mesodermal lineages *in vitro*, in the presence of cytokine cocktails and other factors.

Endothelial cells

One of the main role of the MSCs in injured site is to support the vascularization. For this purpose, they can differentiate into endothelial-like cells. BM-MSc, in presence of VEGF, acquire endothelial specific markers, with an overexpression of the receptor of the VEGF2 and of the Vascular Endothelial (VE)-cadherin. This differentiated cells have the ability to form specific capillary-like structures (Oswald et al., 2004; Wang et al., 2018).

Pericytes

It has been also shown that the ASCs can differentiate into pericyte-like cells *in vitro*. These cells are located in the basal lamina of the capillary endothelium and are essential for maintaining the integrity, function and exchange of the vessels. The pericyte-differentiated ASCs co-express the α -smooth muscle actin (α -SMA) and the neural/glial antigen 2 (NG2) after culture in pericyte medium (Mannino et al., 2020; Mendel et al., 2013). This differentiation allows to enhance protective and regenerative properties of ASCs in some pathologic contexts such as retinal vasculopathy (Mendel et al., 2013),

Tissue specific cells

Depending on the site of the lesion, MSCs can differentiate into tissue specific cells. MSCs have shown to express plasticity and transdifferentiation capability and can be differentiated into non-mesenchymal lineages, such as neurons, astrocytes, Schwann-cell like cells and myelinating cells of the peripheral nervous system to enhance nerve regeneration (Tohill et al., 2004; Fairbairn et al., 2015; Jiang et al., 2017; De la Rosa et al., 2018) (Darabi et al., 2017; Mathot et al., 2020). In the context of pancreatic disease, MSCs differentiate into islet-like cells, essential for the maintenance of blood glucose levels (Marappagounder et al., 2013; Zanini et al., 2011). MSCs also differentiate into lung-like cells. BM-MSCs and ASCs differentiate into type 2 pneumocytes expressing the specific factor surfactant protein C, while only ASCs can differentiate into lung-like cells (Mendez et al., 2014).

2.2.2.2 MSCs secretion of chemokines and exosomes

Despite their differentiation abilities, one of the major effects of MSCs on tissue repair is due to their secretions of soluble factors (chemokines and growth factors) and of exosomes and microvesicles. Exosomes and microvesicles are vesicles containing some constituents from the plasma membrane and from the cytoplasm of the cell. They contain membrane markers, lipids, proteins and different types of genetic material such as DNAs, mRNAs and miRNAs. They can be differentiated by their size, 30-100nm for the exosomes and 100-1000nm for the microvesicles. Some proteomics studies have allowed the identification of the various proteins present on and into the vesicles secreted by the MSCs. One of these studies, performed on BM-MSCs, identified 730 proteins, including MSC markers (CD13, CD29, CD44, CD73, CD90 and CD105), many surface receptors and signalling and cell adhesion molecules (Kim et al., 2012), which suggests all their possible effects. Similar studies conducted on ASCs allowed the characterization of the secreted exosomes (Huang et al., 2021). In particular, exosomes allow re-epithelialisation, stimulating fibroblast proliferation and migration and promoting vascularisation from endothelial cells through activation of the MEK/ERK pathway (Ren et al., 2019a).

Angiogenesis

After injury, the neo vascularization is essential to regenerate the tissue and limit scar formation. Angiogenesis is defined as the process of generation of new blood capillaries from pre-existing blood vessels. This allows a better perfusion of damaged tissues, enabling the recruitment of cells that promote healing process and the supply of nutrients essential to this healing process.

MSCs seem to be an important actor of angiogenesis by secreting some pro-angiogenic factors. Studies of the MSC secretome have revealed the secretion of many angiogenic factors such as VEGF-A, ANGPTs, IGF-1, and HGF (Oskowitz et al., 2011). VEGF secreted by MSCs, permits the differentiation of endothelial progenitor cells into endothelial cells, the main component of vessels (Ge et al., 2017). The *in vitro* treatment of HUVEC cells with MSC-conditioned medium enhances tube formation and this effect is reverted by VEGF inhibition, confirming VEGF involvement in the formation of new vessels (Katagiri et al., 2017). IGF secretion by MSCs permits the proliferation of endothelial progenitors,

essential for the repair of injured vascular endothelium and angiogenesis (Hou et al., 2017). Exosome secretion by ASCs, stimulated by PDGF, promotes angiogenesis (Ball et al., 2010)

Once at the site of the injury, the MSCs can secrete exosomes and chemokines which enhance the survival of cells essential for tissue integrity and regeneration. In addition to their ability to differentiate into pericytes, some studies have shown the importance of exosome secretion by MSCs to sustain pericytes in injured tissues. Exosomes from BM-MSCs permit to reduce pyroptosis of the pericytes and increase their survival in a context of spinal cord injury (Zhou et al., 2022).

2.3 MSCs and cancer

Tumours are considering as “wounds that do not heal” with many common actors between the wound healing process and the tumorigenesis (Flier et al., 1986). Indeed, growing literature supports the idea that tumours may exploit MSC’s tissue repair properties to promote tumorigenesis and resistance to conventional therapies. MSC-mediated events and mechanisms of tissue regeneration and repair, such as MSC activation, mobilisation, migration, homing to the stromal microenvironment, differentiation, transdifferentiation, as well as secretion of cytokines, growth factors, and other soluble factors modulating local immune responses, may support a pro-oncogenic role of MSCs. Despite this tumour-supportive role, MSCs also display some anti-tumour capacities as shown on some cancers. Therefore, the ability of MSCs to suppress or promote tumour development remains controversial (Ilmer et al., 2014; Rhee et al., 2015; Timaner et al., 2019). The supportive or suppressive role of MSCs on tumours depends on the type and the stage of cancer but also on the MSC origin. Indeed, MSCs are very heterogeneous not only according to their tissue origin but also within the same tissue. However, to date, the majority of studies support a pro-oncogenic role for MSCs.

2.3.1 MSCs homing to the tumour

Several studies have demonstrated the recruitment of MSCs into the tumour microenvironment in many tumour models. The process is the same of that observed in wound healing. MSCs can leave their niches to join the bloodstream and be recruited to the tumour microenvironment. This process is permitted by the tumour cell-mediated secretion of some attractive chemokines and the presence of the corresponding receptor at the surface of the MSCs. Due to the lack of a specific marker, the study of MSC recruitment *in situ* remains very difficult. The observation of MSC migration and homing is mainly realized after intra venous or intra peritoneal injection of labelled MSCs (e.g. bioluminescent MSCs) followed by intravital imaging (Kalimuthu et al., 2018; Kidd et al., 2009).

Numerous factors secreted by the tumour cells can lead to the migration of the tumour cells and then to invasion at the tumour site.

Attractive secreted factors

Chemokines

Many chemokines are involved in the recruitment of MSCs to the tumour microenvironment. They seem to be dependent on the oncogenic context, but there are common players in different tumour types.

As in the case of tissue repair, the involvement of CXCL12 binding to CXCR4 or/and CXCR7 at the surface of the MSCs has been described as allowing their recruitment to the tumour microenvironment. The involvement of CXCL12 was demonstrated in different cancers such as gastric cancer (Quante et al., 2011) or glioma (Pavon et al., 2018; Xu et al., 2010).

It has been also shown that the chemokines CXCL1 and CXCL8 allow the recruitment of ASCs to the prostate cancer microenvironment through their binding to CXCR1 and CXCR2, respectively (Zhang et al., 2016). The involvement of the axis CXCL8/CXCR2 has been also demonstrated for homing of BM-MSCs in oral squamous carcinoma (Meng et al., 2020a).

CCL2, through its binding to CCR2, leads to the recruitment of MSCs to glioma (Xu et al., 2010), breast tumour ((Dwyer et al., 2007) and hepatocellular carcinoma microenvironment (Bayo et al., 2017).

CXCL16, secreted by prostate tumour cells, via its binding to CXCR6, facilitates the recruitment of MSCs to the tumour microenvironment (Jung et al., 2013).

Growth factors

As for chemokines, several growth factors are involved in the recruitment of MSCs to the tumour and this is highly dependent on the oncogenic context.

VEGFA, secreted by certain tumours, can induce homing of MSCs by binding to VEGFR2 on their surface. This molecule has both a migratory and pro-invasive effect, especially in glioblastoma (Schichor et al., 2006).

TFG- β plays an important role in homing of MSCs to the tumour, notably in glioma (Shinojima et al., 2013), prostate cancer (Barcellos-de-Souza et al., 2016) and gastric cancer (Quante et al., 2011).

The different isoforms of PDGF are important for the MSC transmigration, especially in the context of breast cancer. PDGF action is mediated by its binding to different PDGFR isoforms and by the activation of the PI3K pathway (Salha et al., 2019). Not all PDGF isoforms have an effect on MSC migration. PDGF-AA has been shown to be involved in the recruitment of MSCs to the tumour in a head and neck squamous cells carcinoma context (Watts et al., 2016).

2.3.2 *MSCs as tumour-supportive cells*

2.3.2.1 Proliferation and survival of the tumour cells

Some studies show the involvement of MSCs on the proliferation of the tumour cells mainly by their secreted molecules and exosome.

The secretion of CXCL12 by the ASCs and then its binding to CXCR4 at the surface of the tumour cells, is involved in tumour cell survival and proliferation, in particular in prostate adenocarcinoma (Su et al., 2021). CXCL12 knock-down results in reduced tumour growth. The involvement of CXCL12 has been also highlighted in other cancers, such as breast cancer, via its binding to CXCR7 and/or CXCR4 (Al-toub et al., 2019; Rhodes et al., 2010) and osteosarcoma via CXCR4 (Yu et al., 2015).

MSCs secrete TGF- β , which can promote tumour growth. It has been shown, in particular in Acute Myelogenous Leukemia (AML), to have a positive role on the proliferation of tumour cells (Schelker et al., 2018). MSCs allow the maintenance or establishment of stem cells in the tumour, by constituting a favourable microenvironment. MSCs promote tumour growth or resistance to treatment, as discussed further below (indicate paragraph). TGF- β and Wnt pathways play a key role in the acquisition of the stem phenotype (Nishimura et al., 2012)

Other studies show the involvement of MSC secreted exosomes in promoting tumour growth in osteosarcoma and gastric cancer *in vivo* (Zhu et al., 2012). The inhibition of Hedgehog pathway suppresses this pro-tumour effect which suggests the importance of this axis on tumour progression (Qi et al., 2017).

2.3.2.2 Metastases

The development of metastases is due to the detachment of tumour cells from the initial cancer site, following the epithelial-mesenchymal transition that allows the acquisition of a migratory phenotype. These new mesenchymal tumour cells enter the bloodstream, which allows them to colonise other tissues and form secondary tumour sites.

The MSCs are known to secrete or to express on their surface some factors responsive or promoting the epithelial-to-mesenchymal transition of the tumour cells. Indeed, co-culture of colorectal tumour cells and BM-MSCs favours this transition which depends on the contact of the cells and the involvement of TGF- β expressed at the surface of the MSCs (Mele et al., 2014). The role of MSCs in the acquisition of migratory phenotype, via the EMT, of tumour cell have been shows in numerous tumour models, such as in melanoma by the activation of STAT1 and STAT3 by the secretome of the MSCs (de Souza et al., 2021) or in breast cancer (Xu et al., 2012).

CXCL12, in addition to its many other roles, is known to be involved in the establishment of metastases (Yang et al., 2020b). CXCL12 is secreted by the ASCs and promote the EMT by binding to CXCR4 at the surface of the tumour cells, notably in prostate adenocarcinoma. The depletion of CXCL12 suppresses the EMT of the tumour cells (Su et al., 2021). This chemokine is also involved in EMT of ovarian tumour cells (Zheng et al., 2019a) or colorectal cancer cells (Yu et al., 2017).

In hepatocellular carcinoma, IL6 secreted by BM-MSCs shows an invasion promoting effect via the stimulation of STAT3. The use of an anti-IL6 antibody, which inhibits the BM-MSCs-mediated secretion of this chemokine, diminishes the pro-migratory phenotype of the tumour cells by repressing the IL-6/STAT3 pathway (Mi and Gong, 2017). IL6 is also involved in gynaecologic cancer cell EMT (So et al., 2015).

MSCs also enhance the EMT by causing tumour cell overexpression of MMPs which permit the detachment of tumour cells of the extracellular matrix. Some examples of this overexpression are given by MMP11 in breast cancer (Martin et al., 2010), MMP9 in lung cancer (Gu et al., 2020) or MMP16 on gastric cancer (Xue et al., 2015).

2.3.2.3 Angiogenesis

As in the context of tissue repair, the effect of MSCs on angiogenesis is twofold. They differentiate into cells constituting or supporting vessels, but also supporting vessel formation through their secretory activity. The pro-angiogenic effect of MSCs has been shown in many tumour types such as colon, breast or prostate cancer (Batlle et al., 2019; Zhang et al., 2013).

Co-injection of ovarian tumour cells and fluorescent MSCs showed the perivascular localisation of MSC which supports the neo-vascularisation with the acquisition of a more epithelial phenotype demonstrated by the expression of specific markers (Suzuki et al., 2011). The differentiation of MSCs into endothelial cells has been shown in different oncogenic contexts. Notably, TGF promotes the differentiation of MSCs into endothelial cells by activation of the JNK (Batlle et al., 2019). MSCs also promote angiogenesis by secretion of Tie-2, endoglin, angiotensin and VEGF (Mihai et al., 2021).

2.3.2.4 Crosstalk between tumour cells and MSCs

Recruited MSCs are important for tumour progression, both in the context of tumour growth and metastatic spread (Yang et al., 2020b; Yu et al., 2015). This MSC role does not seem to be exclusively dependent to the MSC capacities linked to their tissue of origin. Indeed, a dialogue takes place between the tumour cells and the MSCs which leads to the modification of some MSC characteristics (Mishra et al., 2008). These modifications can involve the modification of the cell's

secretome but also the acquisition of a new phenotype, given that these cells are considered as being at the origin of the fibroblasts associated with carcinomas. Notably, the comparison between naïve MSCs and tumour-educated MSCs revealed that the latest display accentuated pro-tumour features in certain types of cancer.

In particular, in the context of glioblastoma, the tumour secretome was shown to allow increased proliferation and migration of MSCs but also their differentiation into pericytes and their ability to differentiate into cells essential for tumour vascularisation. Indeed, MSCs derived from the tumour have a stronger angiogenic potential (Yi et al., 2018).

MSCs derived from ovarian cancer promote tumour growth by enriching tumour stem cells, maintaining the "stemness" of the tumour, which is associated with a poorer prognosis. In particular, they show an increase in proteins associated with epithelial-to-mesenchymal transition and cell adhesion, such as proteins of the Bone Morphogenic Protein (BMP) family (McLean et al., 2011).

Prostate tumour-educated MSCs enhance tumour cell migration compared to naïve MSCs, as has been shown in prostate cancer. This is due in particular to an overexpression of interleukins such as IL-8 and other pro-migratory factors, such as FGF.

Carcinoma-Associated Fibroblasts (CAFs)

CAFs have been described, in many tumour contexts, as having a pro-oncogenic role. Indeed, through their secretome but also their interaction with the different cells composing the tumour, they notably regulate and support tumour cell survival and proliferation, angiogenesis, immunity and metastatic dissemination. CAFs are characterized by the expression of myofibroblast markers and the consensual marker is α -Smooth Muscle Actin (α -SMA).

According to the current literature, the origin of CAFs is multiple depending on the tumour but also within the same tumour. A possibility is that the CAFs derived from the modification of resident fibroblasts of the healthy tissue in the neighbourhood of the tumour. These healthy fibroblasts would be used by the tumour to sustain tumour progression, notably after their activation by the TGF β . CAFs could also be the results of transdifferentiation of others cells, such as endothelial cells, adipocytes, stellate cells, endothelial cells or macrophages (Bu et al., 2019). The cell type from which CAFs are generated, depends very strongly on the tissue nature of the tumour. Another theory is that MSCs, from local or distant sources, are transdifferentiate into CAFs thanks to factors secreted by the tumour microenvironment (Jung et al., 2013; Kidd et al., 2012). Finally, another hypothesis, based on the intra-tissue heterogeneity of MSCs, would be that CAFs correspond to a sub-population of MSCs expressing notably α -SMA factor (Kidd et al., 2012). Once again, the absence of a specific marker for MSCs does not allow us to definitively confirm these different theories. Studies of the fate of labelled-MSCs after injection or after engraftment of labelled-bone marrow or labelled-adipose tissue into mice seems to show that the different hypotheses coexist in the tumour (Kidd et al., 2012). The transdifferentiation of MSCs in CAFs has been shown in several tumour models such as breast cancer (Mishra et al., 2008) or colorectal carcinoma (Tan et al., 2020).

One of the factor responsive of the transdifferentiation of MSCs in CAFs is the TGF- β 1, secreted by cancer cells to evade immune surveillance, as has been shown in colorectal carcinoma (Tan et al., 2020; Zheng et al., 2019b) and in prostate cancer (Barcellos-de-Souza et al., 2016). Indeed, inhibition of TGF β /Smad signalling by BAMBI receptor blocks the differentiation of BM-MSCs into CAFs and decreases the pro-oncogenic capacity of BM-MSCs (Shangguan et al., 2012). Many other factors, secreted by neoplastic cells or by the tumour microenvironment, have been involved in the differentiation process such as PDGF, bFGF, IL6, IL1, hypoxia, Reactive Oxygen Species (ROS), H₂O₂,

proteins, mRNA and microRNA derived from tumour exosomes. Similarly, to mesenchymal cells, CAFs have an impact on the establishment of a microenvironment favourable to tumour growth and metastatic dissemination. This is mainly due their secretory activity.

As previously explained, the secretion of CXCL16 by prostatic tumour cells facilitate the recruitment of MSCs to tumour microenvironment via the binding of this chemokine on CXCR6. This fixation also allows the transdifferentiation of MSCs in CAFs which secrete CXCL12 promoting the EMT of the tumour cells and the metastatic dissemination (Jung et al., 2013). In a lung cancer model, it has been shown that tumour-derived MSCs show changes in the expression of certain genes including α SMA, CXCL12, VEGF or HIF-1 α . These CAFs have a modified secretion compared to that of naive MSCs, with in particular an increase in the pro-inflammatory cytokine IL6 or TNF α (Arena et al., 2018).

Cancer-Associated Adipocytes (CAA)

As with CAFs, the origin of CAA is still unclear. Some studies have shown that CAAs are derived from the modification of resident adipocytes in the tissue for integration into the tumour microenvironment. Other studies have reported that the differentiation of MSCs into adipocytes is potentiated by tumour secretions, suggesting their possible differentiation into the tumour microenvironment. CAAs differentiated from MSCs have been shown to have pro-oncogenic characteristics. Indeed, CAA exosomes promote tumour progression, particularly in breast cancer, by increasing the proliferation and migration of tumour cells. This is achieved in particular by activating these same cells of the Hippo pathway (Wang et al., 2019b). CAAs secrete adipokines, such as IGFBP-2, which can induce epithelial-mesenchymal transition of tumour cells and promote their metastatic dissemination, as reported in breast cancer. CAAs secrete pro-inflammatory proteins, MMPs and interleukins, which also promote a migratory phenotype, notably, MPP11 or IL-6 and IL-1 β in breast cancer (Dirat et al., 2011) or IL-8 and FABP4 in ovarian cancer (Nieman et al., 2011). CAAs also enable tumour growth by providing a source of fatty acids that can be used by the malignant cells which have high lipolysis associated with strong β -oxidation (Nieman et al., 2011).

2.3.3 *MSCs and resistance to therapies*

A growing literature is focusing on the involvement of MSCs in the resistance of cells to cancer therapies. These studies can be conducted by placing MSCs in the presence of the drug *in vitro* and studying the impact of this MSC pre-treatment on tumour cells *in vitro* and *in vivo*, but also by comparing native MSCs with MSCs derived directly from the treated tumour. Some studies have validated the involvement of MSCs on the resistance of cancer cells to treatment, notably to chemotherapy and targeted therapy. This effect is mainly mediated by MSC secretome, either of soluble particles or exosomes.

2.3.3.1 Resistance to chemotherapies

CXCL12, through its receptors CXCR4 and CXCR5, is a central cytokine in mediate the pro-tumour role of MSCs. In addition to its involvement in tumour cell proliferation, survival, dissemination and differentiation, CXCL12 also plays a key role in chemoresistance. In acute leukemia, treated with cytarabine, the CXCL12/CXCR4 axis has been shown as involved in chemoresistance. Inactivation of this pathway by a two-pronged approach triggers the death of chemotherapy-resistant cells by apoptosis (Pillozzi et al., 2018; Schelker et al., 2018). CXCL12/CXCR4 interaction is also implicated in the resistance to hyperthermic intraperitoneal chemotherapy used for the treatment of ovarian cancer. CXCL12 leads to the thermotolerance and the resistance of the tumour cells. The inhibition of the CXCL12 restores the cytotoxicity of the treatment (Lis et al., 2011). The involvement of CXCL12 in resistance has also been demonstrated in the context of multiple myeloma. CXCL12 activates IL-6

secretion responsible for activation of the P13K/AKT signalling pathway, thereby reducing tumour cell apoptosis (Liu et al., 2019b). IL-6 secretion by MSCs is also associated with resistance to paclitaxel, related to epigenetic changes in head and neck squamous cell carcinoma cells (Liu et al., 2021a).

Other factors secreted by tumour cells are the subject of less consensus in literature and are more specific to certain types of cancer. In triple negative breast cancer, IL-8 secretion by MSCs allows the resistance to doxorubicin (Chen et al., 2014). It has been shown that ovarian tumour-derived MSCs secrete BMP4, which is responsible for tumour stem cell enrichment, through activation of hedgehog pathway which promotes tumour growth under chemotherapy (Coffman et al., 2016). In the same oncogenic context, PDGF secretion has a similar effect than BMP4. Through activation of the Hedgehog pathway, it allows enrichment of tumour stem cells associated with cisplatin resistance (Raghavan et al., 2020). PDGF secreted by MSCs also reduces the sensitivity of oral squamous cell carcinoma cells to cisplatin. Through its binding to PDGFR- α , it activates AKT in particular, leading to resistance to treatment by promoting cell survival by limiting apoptosis (Wang et al., 2020). A specific subpopulation of cancer associated MSCs, with low CD90 expression, has also been shown as involved in the resistance to temozolomide in glioma. This resistance is mediated by the FOXS1-driven activation of the EMT following IL6 secretion (Xue et al., 2021). In acute leukemia treated with cytarabine, the blockade of TGF- β 1 sensitises tumour cells to chemotherapy (Schelker et al., 2018). AML chemotherapy resistance is associated with the emergence of tumour stem cells under the action of TGF- β (Yuan et al., 2020). In prostate cancer, it has been shown that TGF- β 1 secretion by MSCs activates protective autophagy, leading to cell survival in response to docetaxel treatment. Inhibition of autophagy or TGF- β 1 restores the sensitivity of tumour cells to chemotherapeutic treatment (Yu et al., 2021b).

In addition to cytokines and growth factors, MSCs also secrete miRNAs that are involved in chemotherapy resistance in various cancers. Notably, MiR-1180 has been shown to participate in cisplatin resistance in ovarian cancer (Gu et al., 2019). In the context of breast cancer, MiR-21-5p is involved in doxorubicin resistance (Luo et al., 2020). MiR-373-3p is, as for him, involved in the resistance of glioma to temozolomide treatment (Li et al., 2021).

It has been also shown in the context of pancreatic cancer that BM-MSCs pre-treated with gemcitabine, a pyrimidine antimetabolite used to treat many cancers, secrete the cytokine CXCL10, which, by binding to the CXCR3 receptor, allows the enrichment of tumour initiating cells. In this way, therapy-educated MSCs allow the enrichment of cells that will lead to chemotherapy resistance and cause tumour growth under treatment, as has been shown *in vivo* (Timaner et al., 2018).

2.3.3.2 Resistance to targeted therapies

Resistance to TKI

Several studies have shown an effect of TKIs on the proliferation of MSCs or on their capacities to differentiate. In particular, even at low doses of the the PDGFR β inhibitor Imatinib or Sunitinib, which inhibits ERK and AKT pathways, BM-MSCs have decreased proliferation, survival and clonogenicity as well as increased osteogenic differentiation capacity (Fierro et al., 2007; Jönsson et al., 2012; Nicolay et al., 2014; O'Sullivan et al., 2007). However, some studies show the effect of MSCs on TKI resistance, especially in the context of leukaemia (Mallampati et al., 2015; Zhang et al., 2019).

TKIs are used for the treatment of various types of leukaemia. Studies have shown that MSCs can participate to the development of resistance to this treatment. For example, in the case of Chronic Myeloid Leukaemia (CML) with a BCR-ABL mutation, BM-MSCs are associated with resistance to the TKIs Imatinib and Nilotinib (a BCR-ABL TKI), by decreasing tumour cell apoptosis and promoting cell

survival (Zhang et al., 2019b). Under the effect of these two EGFR-TKIs, BM-MSCs produce more IL7 which activates the JAK1/STAT5 pathway in tumour cells *in vitro* and *in vivo*. Inhibition of IL7 reverses this resistance mechanism (Zhang et al., 2019b). Similar results were obtained in a BCR-ABL+ acute lymphoblastic leukemia model. MSCs promote resistance to Imatinib by increasing IL7 secretion upon treatment (Mallampati et al., 2015). The involvement of MSCs on the resistance to Nilotinib treatment is notably explained by the role of CXCL12, expressed by MSCs, in the quiescent and resistant state of cancer stem cells. Depletion of CXCL12 in MSCs triggers the cell cycle re-entry of these cells and restore sensitivity to treatment (Agarwal et al., 2019). In the context of CML, BMP4, shown to be highly secreted by MSCs under treatment, promotes cell escape from TKI treatment, in particular by maintaining a population of tumour stem cells expressing TWIST-1 which is strongly associated with resistance (Grockowiak et al., 2017). In double negative breast cancer, MSCs have been shown to promote resistance to Lapatinib, by secreting of Pseudopodium-Enriched Atypical Kinase One (PEAK1) which modifies actin cytoskeleton and promotes cell plasticity and EMT (Hamalian et al., 2021), and Trastuzumab, by promoting cancer cell stemness (Han et al., 2021).

Only one study has investigated the impact of MSCs on EGFR-TKI resistance in NSCLC (Wang et al., 2019a). BM-MSCs have been shown to promote Erlotinib resistance under hypoxic conditions. Hypoxia observed in tumours, and in particular in lung cancer, is associated with a more aggressive phenotype and higher probability to resist to different therapies such as chemotherapy (Cosse and Michiels, 2008), radiotherapy (Milosevic et al., 2012), immunotherapy (Kopecka et al., 2021; Zandberg et al., 2021) and targeted therapies (Geng et al., 2018). This study shows that hypoxia modifies the tumour microenvironment and in particular BM-MSCs which secrete leptin (a molecule associated with adipocytes) and IGFBP2. These molecules are taken up by IGF1R on the surface of the tumour cells, leading to the activation of the AKT/ERK survival pathway, which allows the survival of the cells under treatment (Wang et al., 2019a).

2.3.4 *MSCs and MSC-derived vesicles as therapy or therapeutic vectors*

Due to their homing and healing capacities, MSCs are good candidates as therapeutic vectors in the fields of wound healing and tissue regeneration. The therapeutic potential of MSCs has been demonstrated in numerous pathologies in both pre-clinical and clinical studies. Numerous clinical trials are currently underway and in multiple lesion models. More than 1,200 trials have been identified to date (<https://www.clinicaltrials.gov/>). The trials include musculoskeletal, central nervous system, immune, respiratory, vascular and wound healing diseases. MSCs can be used as such for their own properties (detailed above). They can also be used as a therapeutic vectors as a vehicle to target the injured area. In this way, their migration and wound-specific addressing capabilities are particularly exploited. This makes it possible to combine the chosen drug with the healing capacities of MSCs. Exosomes and microvesicles derived from MSCs are also used as therapeutic vectors. MSCs are used in both lesion and tumour contexts.

2.3.4.1 *MSCs as therapy or as therapeutic vector*

MSCs are used either as autogenous or allogeneic. In the first case, the cells are recovered from the patient, cultivated in large quantities before being reinjected. This avoids any risks of graft-versus-host rejection and also the use of immunosuppressive treatment. In the second case, the MSCs are derived from a donor and are transplanted into a non-donor patient. This allows the treatment of several patients with cells from the same donor. This type of transplantation is facilitated by the easy genotyping of MSCs, enabling to determine compatible immunogenicity profiles between donor and recipient. In addition, the use of the least immunogenic MSCs, for example those from the umbilical

cord, is favoured. In certain pathologies, one of the main limitations of MSC treatment is their strong elimination and the limited number of cells that arrive and remain at the injured site. Therefore such a treatment requires a high dose of MSCs injected repeatedly. However, the use of MSCs as a treatment has been shown to be beneficial in various pathological contexts.

MSCs are used notably for their anti-inflammatory and anti-fibrotic properties. They are being considered as a treatment for Crohn's disease, with clinical trials underway (Vieujean et al., 2021; Wang et al., 2021b). The anti-inflammatory properties are also used in the treatment of systemic lupus erythematosus (Tang et al., 2021; Yang et al., 2021). MSCs can be used to treat joint pathologies, including knee joints injuries (Perry et al., 2021) and neural injuries (Sykova et al., 2021). Indeed, MSCs reduce inflammation, allow the survival of cells from the injured site and favour re-functionalization after tissue repair (Perry et al., 2021; Sykova et al., 2021). This is achieved by the secretion of neuroprotective growth factors which allow the protection of the tissue cells but also the emergence of new specialized cells. In particular, during neural lesions, MSCs secrete neurotrophins, growth factors and other supportive substances which may produce therapeutic benefits in the ischemic brain (Chen et al., 2002; Qu et al., 2007).

2.3.4.2 MSC derived-exosomes and microvesicles as therapy or as therapeutic vector

Exosomes are small (50-150 nm) extracellular vesicles that are released by almost all cell types, including stem cells and tumour cells. Exosomes provide intercellular communication. They can transport proteins and RNAs present in the cell of origin and perform a variety of physiological and pathological functions in adjacent or distant cells by transferring their contents into the recipient cells. Exosomes can be used for their intrinsic properties, for example, for their pro-angiogenic properties. In a model of hind limb ischaemia, VEGF and the angiomiR miR-210-3p contained in MSC-derived extracellular vesicles allow revascularisation of the injured area (Gangadaran et al., 2017). Exosomes also present, like MSCs themselves, immunomodulatory properties, which could be exploited for the treatment of autoimmune diseases such as systemic lupus erythematosus. Exosomes allow the polarization of M1 macrophages, specialized in pathogen degradation, into M2 macrophages, promoting tissue repair (Dou et al., 2021). MSC exosomes also limit senescence and death by apoptosis of injured cells in the wound, as shown in particular in the case of osteoarthritis (Jin et al., 2021). MSC exosomes are also being considered for the treatment of neurodegenerative diseases, such as Alzheimer's disease, by restoring the morphology and function of neuronal regions (Wang et al., 2021a) notably by virtue of their secretory abilities.

The administration of exosomes can be combined with the injection of MSCs, increasing the efficacy compared to the effects of single therapy. In particular, it can improve tissue repair by promoting re-epithelialisation, tissue regeneration over scarring, angiogenesis and collagen synthesis (Zhou et al., 2021).

Exosomes derived from MSCs are used to deliver chemotherapies directly to tumour cells. The drugs are internalised by the MSCs and the exosomes from these cells are then brought into contact with the tumour cells. The efficacy of this approach has been proven in a model of pancreatic tumour cells treated with paclitaxel, which results in decreased tumour cell growth (Pascucci et al., 2014).

2.3.4.3 MSC derived nanoghosts as therapeutic vector

However, the involvement of MSCs in the tumour process calls into question their use, particularly as a therapeutic vector for cancer. Prof. Marcelle Machluf's team has developed a new technology called NGs, which allows MSCs to retain their affinity for tumour cells while limiting their

pro-tumour activity (Kaneti et al., 2016; Toledano Furman et al., 2013). NGs retain some of the membrane characteristics of the MSCs from which they are derived. In particular, the NGs express the specific stem cell markers CD29, CD44, CD105, and CD90 (Oieni et al., 2019; Toledano Furman et al., 2013). They also exhibit low immunogenicity. They are strongly taken up by tumour cells as shown for prostate (Kaneti et al., 2016; Toledano Furman et al., 2013), pancreas (Timaner et al., 2018) and breast cells (Toledano Furman et al., 2013). This strong internalization of NGs seems to be specific to vesicles derived from MSCs, indeed vesicles produced by the same process but from muscle cells do not show the same tropism for tumour cells (Toledano Furman et al., 2013).

The NGs are produced from BM-MSCs. A hypotonic treatment step of homogenisation and serial centrifugations allows the removal of the stem cells content and to obtain cell ghosts. Then sonication or extrusion will reduce the size of the MSCs ghosts and obtain NGs which are isolated by ultracentrifugation. The desired encapsulated treatment can be added during this production step by sonication or extrusion or afterwards by electroporation. The NGs can then be modified for better storage or stability by PEGylation (Oieni et al., 2019) (Figure 13).

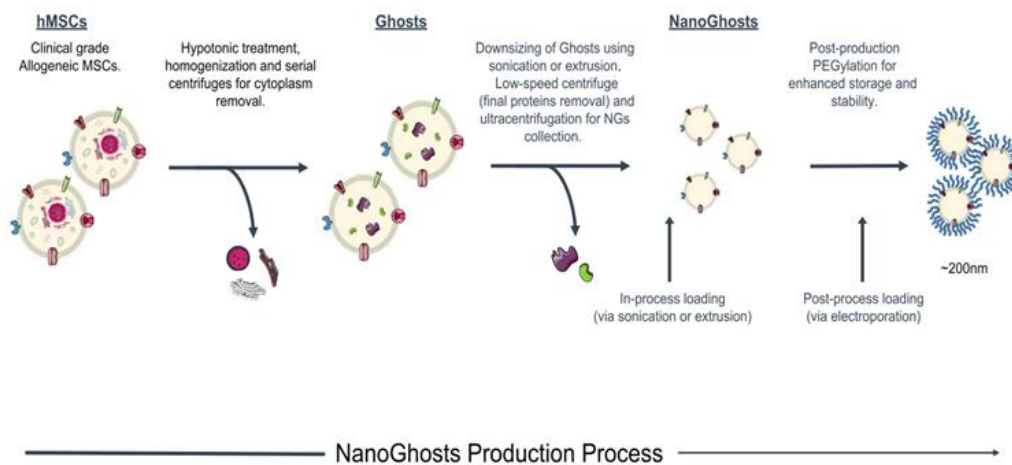


Figure 13 : NGs production process (from (Oieni et al., 2019))

One of the advantages of this technology is the ease of labeling NGs, both with fluorescent, notably par the incorporation of lipophilic tracer into the source cells membrane, and radioactive markers, which will allow to follow NG trafficking both in *vitro* and *in vivo* (Oieni et al., 2019). It is also possible to transduce MSCs with a lentivirus allowing the expression of GFP, which will result in NGs expressing GFP (Oieni et al., 2019).

NGs have been shown to encapsulate different types of molecules, including soluble ligands such as Tumour-necrosis-factor related Apoptosis Inducing Ligand (sTRAIL), (Toledano Furman et al., 2013), plasmid DNA such as Peroxin plasmid DNA (pPEX) (Kaneti et al., 2016; Oieni et al., 2019) and antisense oligonucleotides like miRNAs (Oieni et al., 2021). NGs can either encapsulate drugs at the time of production or MSCs can be made to express molecules, e.g. a ligand, which will remain embedded in the membrane after NGs formation (Oieni et al., 2019).

One of the main interests of this technology is to address directly to the tumour molecules that would be toxic systemically. This is the case, for example, with sTRAIL, which has a short half-life and high liver toxicity. The encapsulation of sTRAIL in NGs makes it possible to kill the treated prostate tumour cells *in vitro* and to reduce the tumour size *in vivo* by induction of apoptosis and a reduction in cell proliferation and tumour vascularisation greater than that observed for the ligand alone (Toledano

Furman et al., 2013). The same study confirmed the specificity of the NGs in addressing the tumour with little internalisation by the filtering organs and the low immunogenicity of the NGs due to the absence of an immune response in the mice up to three weeks after administration (Toledano Furman et al., 2013).

NGs can also be used as therapeutic carriers for other types of molecules. In particular, they can deliver plasmid DNA (pDNA) coding for an anti-tumour protein to the tumour, as was shown with the protein peroxin (PEX) in a prostate cancer model (Kaneti et al., 2016). NGs containing pPEX allow, *in vitro*, to increase the death by apoptosis of tumour cells, to limit their proliferation. These NGs also limit the migration of endothelial cells *in vitro* associated with a decrease in tumour vascularisation *in vivo* (Kaneti et al., 2016). They allow the slowing of tumour growth *in vivo* by reducing proliferation and increasing cell apoptosis. Finally, this study confirms the specific addressing of the NGs to the tumour and their low immunogenicity. Indeed, no immune response is observed either just after the injection of the NGs or one week later. (Kaneti et al., 2016). Injection of NG-pPEX into mice xenografted with the A549 NSCLC cell line reduces the number and size of liver metastases (Kaneti et al., 2016).

NGs can also encapsulate antisense oligonucleotides. Indeed, an *in vitro* study has shown that NGs encapsulating the anti-miR221, allowed the silencing of miR-221 in MSCs in the presence of NGs (Oieni et al., 2021). This study was completed by an *in vivo* study which showed that NGs loaded with the anti-miR221 could deliver the molecule inside endogenous cells (Oieni et al., 2021).

As presented previously, MSCs in the presence of cancer therapies, notably chemotherapy, favour the appearance of resistance mechanisms, notably through the enrichment of tumour initiating cells (TICs). NGs, on the other hand, even when manufactured from MSCs treated with chemotherapy, have a potentiated effect and do not promote the development of resistance to the therapy. This makes NGs an ideal candidate for delivering treatments to patients undergoing chemotherapy (Timaner et al., 2018). It has also been shown that treatment of the MSCs from which the NGs are derived can modulate their effect. Indeed, pre-treatment of MSCs with a conditioned medium of pancreatic tumour cells allows the NGs to be addressed by the immune system and pre-treatment with pro-inflammatory cytokines allows them to be homed to the tumour cells. This different addressing is notably due to the proteins expressed by the MSCs in each condition and which are conserved by the NGs. For example, in the pre-treatment condition with tumour cells, the ICAM-1 protein, a macrophage recognition protein, is over-expressed, which may explain the homing to immune cells (Lupu-Haber et al., 2019).

OBJECTIVES

The team identified an inhibitor of RHOB, the C3-exoenzyme, that prevents the development of resistance to EGFR-TKI therapy in the tumour setting of EGFR-mutated NSCLC. However, this inhibitor is not clinically applicable due to its high toxicity. For the following work we have associated with the team of Professor Marcelle Machluf, to use nanoghosts derived from mesenchymal stem cells (MSCs) to specifically release the C3-exoenzyme to tumour cells.

Thus, the objective of this thesis was to validate MSCs-derived nanoghosts as a therapeutic vehicle to specifically address C3-exoenzyme to *EGFR*-mutated NSCLC cells.

To this end, we have first of all studied the tropism of MSCs for our *EGFR*-mutated NSCLC cell models and then their implication in the resistance to EGFR-TKIs.

Then, we verified by different methods of fluorescence microscopy and cytometry, the capacity of EGFR-mutated NSCLC tumour cells to capture nanoghosts, *in vitro* and *in vivo*.

We then studied *in vitro* the ability of nanoghosts to deliver an active C3-exoenzyme to *EGFR*-mutated NSCLC tumour cells.

RESULTS

MATERIALS AND METHODS

3 *In vitro* experiments

3.1 Cell culture

3.1.1 *EGFR-mutated NSCLC cell lines*

Cell line	Reference	Tissue Origin	Genetic background
PC9	ECACC 90071810	Tumour tissue	EGFR Δ E746-A750
HCC4006	CRL-2871	Pleural effusion	EGFR Δ L747-E749, A750P
HCC827	CRL-2868	Tumour Tissue	EGFR Δ E746-A750, A750E, E746K, E746A
H3255	CRL-2882	Pleural effusion	EGFR L858R
HCC2935	CRL-2869	Pleural effusion	EGFR Δ E746-T751, S752I

The human NSCLC cell lines HCC4006, HCC827 and HCC2935 (CRL-2869, EGFR del E746-T751, S752I) were obtained from the American Type Culture Collection (Manassas, VA, USA). The H3255 NSCLC cell line (EGFR L858R) were a kind gift from Helene Blons (APHP, Paris, France). PC9 NSCLC cell line was a kind gift from Antonio Maraver (IRCM, Montpellier, France). NSCLC cell-lines were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum (FBS) (v/v) and were maintained at 37°C in a humidified chamber containing 5% CO₂.

3.1.2 *GFP transduced cell lines*

Tumour cells lines were transduced with a lentiviral vector designed by the vectorology platform of the Cancer Research Center of Toulouse (CRCT) (pLenti CMV GFP DEST, 9216bp) to obtain a constitutive expression of GFP protein. Then GFP-positives cells were selected by cell sorting by cytometry platform of the CRCT using FACS Melody (BD Biosciences).

3.1.3 *DTC generation*

Before each experiment the DTCs are generated in P140 before being reseeded in the appropriate support for the experiment. The treatment for PC9 DTCs is 7 days and 14 days for HCC4006 and HCC827 DTCs.

3.1.4 *DTEC generation*

DTEC "cell lines" were generated by treating the cells for 3 months with Osimertinib for each line. The DTECs, maintained in culture and passed under Osimertinib treatment, are seeded in a support adapted to each experiment.

3.1.5 *BM-MSC*

MSCs were a kind gift from Isabelle Ader (Restore, Toulouse, France). This is a primary culture of MSCs isolated from patient femoral heads. MSCs were cultured in α -MEM (Minimum Essential Medium Eagle - α modification) medium supplemented with 10% fetal bovine serum (FBS) (v/v) and were maintained at 37°C in a humidified chamber containing 5% CO₂.

3.1.6 *mCherry MSC*

MSCs were transduced with a lentiviral vector designed by the vectorology platform of the Cancer Research Center of Toulouse (CRCT) (pTRIP-CMV-mCherry, 10460bp) to obtain a constitutive expression of mCherry protein. Then mCherry-positives cells were selected by cell sorting by cytometry platform of the CRCT using FACS Melody (BD Biosciences).

3.1.7 MSC conditioned medium (CM-MSC)

CM-MSC is obtained by culturing the cells in α -MEM medium for 48h. The medium is then centrifuged and the supernatant is used for tumour cell culture. GFP-tumour cells were seeded in 96 wells plate in RPMI medium. 48h after seeding, the medium was replaced by the CM-MSC added or not of Osimertinib. Tumour cells were quantified as previously explain (cf. Cell growth and viability assays)

3.2 Inhibitors

Name	Targets	Concentration	Origin/Reference
Erlotinib (OSI-744, CP-358774, NSC718781)	EGFR	1 μ M	LC Laboratories/E4997
Osimertinib (AZD9291)			LC Laboratories/O-7200
TatC3	RhoA, RhoB, RhoC	2,5 to 10 μ g/ml	C3-exoenzyme coupled to permeant peptide TAT was produced and purified in our laboratory using an Akta purifier (GE Healthcare) as previously described (Sahai and Olson, 2006).
C3			Cytoskeleton/CT03

3.3 NG

The NGs are produced at The Lab for Cancer Drug Delivery & Cell Based Technologies (Technion, Haifa, Israel) as previously published (Oieni et al., 2019). The transport is done at 4°C. Empty NGs, NG-C3 and C3-exoenzyme (whose origin is specified above (session 3.2. Inhibitors)) underwent the same production process and are sonicated and vortexed every day after receipt. For the *in vitro* and *in vivo* experiments we used NGs labelled with three different lipophilic fluorescent markers: either with DiI (orange fluorescence : $\lambda_{ex}/\lambda_{em}$: 561/593), DiR (red deep fluorescence : $\lambda_{ex}/\lambda_{em}$: 748/780), or DiD (red fluorescence : $\lambda_{ex}/\lambda_{em}$: 644/663).

3.4 Cell growth and viability assays

In cells treated or not with Erlotinib or Osimertinib and in combination with TatC3, fluorescence intensity in each well of 96-well plate was evaluated twice a week using Synergy™ 2 Multi-Detection Microplate Reader. Relative cell survival in the presence of inhibitors which is proportional to fluorescence intensity was normalized to untreated cells after background corrections. $\lambda_{GFP_{ex/em}}$: 488/525 and $\lambda_{mCherry_{ex/em}}$: 587/610

3.5 Boyden chamber assay

150.000 tumour cells are seeded in 48 well plate. 48h after seeding the medium is changing added or not with Osimertinib. 24h after, 13.000 MSCs was seeded in the boyden chamber (Falcon® Permeable Support for 24-well Plate with 8.0 μ m Transparent PET Membrane). After 48h of migration, unmigrated cells are removed mechanically and the MSCs on the lower part of the chamber were incubated with NucBlue Live ReadyProbes™ (ThermoFisher Scientific, #R37605) according to manufacturer's instructions. 20 minutes after incubation, the cells were fixed with PFA 3,7% and each

well was imaged with Zeiss Axiovert microscope (6 images by chamber). Nuclei quantification was performed with ImageJ Software.

3.6 Western blot

The cells were lysate with RIPA buffer (Tris-HCl 50 mM (pH=8), NaCl 150 mM, Triton X100 à 1%, sodium désoxycholate 1%, SDS 0,1%, EDTA 5 mM and DTT (10 mM)) complemented with proteases- and phosphatases-inhibitors to obtain protein lysates. After sonication, protein content was quantified using Bradford method. Protein extracts were separated on SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. Blots were probed with primary RHOA (Santa Cruz Biotechnology, sc-418), RHOB (Proteintech, 14326-1-AP-1) or RHOC antibody (Cell Signaling, #3430). Detection was performed using peroxydase-conjugated secondary antibodies and chemilluminescence detection kit (Clarity™ Western ECL, Bio-Rad) with a ChemiDoc™ MP Imaging system (Bio-Rad).

3.7 Flow cytometry

500.0000 tumour cells were seeded 6 wells plate. 2 days after seeding, the cells are incubated with nanoghosts. After trypsinization and washings the cells were kept in PBS. For least 10,000 cells are processed on the MACSquant VYB cytometer (Miltenyi Biotec) : λ GFP_{ex/em}: 488/525 and λ DiI_{ex/em}: 561/593. The results were analysed with FlowLogic software. Cells are first characterized by their size and granularity, and then cellular doublets are excluded. Finally, the cells showing the DiI/GFP double labelling are quantified.

3.8 Inverted microscope

The tumour cells were imaged under an inverted fluorescence microscope Eclipse 90i microscope and Camera Control Unit DS-2 (Nikon). λ GFP_{ex/em}: 488/525 and λ mCherry_{ex/em}: 587/610.

3.9 Confocal microscopy

Tumour cells were seeded in 8 wells chamber slide (Ibidi) and imaged with confocal LSM880 Fast Airyscan microscope (Zeiss). 3D representation, orthogonal projection and fluorescence histograms are generated with Zen Black Software (Zeiss).

3.10 Operetta system

15.0000 tumour cells were seeded in CellCarrier-96 ultra (black, clear bottom) microplate (PerkinElmer). 2 days after seeding, the cells are incubated with nanoghosts. The plate was imaged by Operetta CLS - high content imaging system (PerkinElmer). Using the Columbus software, masks are used to delimited and quantify cells and NGs.

4 *In vivo* and *ex vivo* experiments

4.1 MSC injection on PC9 xenograft mice

8.000.000 of PC9 cells were injected in the flank of nude mice. Tumours size was measured twice a week. When tumour volume was close to 300 mm³, 1.000.000 of MSCs were injected in IV. 24h after injection mice were humanely killed by cervical dislocation at the end of the experiment and filtering organs and tumours harvested for dissociation.

4.2 Filtering organs and tumours dissociation

The filtering organs and tumours are dissociated enzymatically with Tumour Dissociation Kit, human (Miltenyi Biotec) and mechanically with gentleMACS™ Dissociator (Miltenyi Biotec) according to manufacturer's instructions. Red blood cells were removed with Red Blood Cell Lysis Solution (Miltenyi Biotec). The cells were fixed with PFA3,7% during 20 minutes and stored at 4°C in Phosphate Buffered Saline (PBS).

4.3 Flow cytometry

At least 10,000 cells are processed on the MACSquant VYB cytometer (Miltenyi Biotec) : $\lambda_{\text{GFP}_{\text{ex/em}}}$: 488/525 and $\lambda_{\text{mCherry}_{\text{ex/em}}}$: 561/615. The results were analysed with FlowLogic software. Cells are first characterized by their size and granularity, and then cellular doublets are excluded. Finally, the cells showing the mCherry/GFP double labelling are quantified.

4.4 NG injection on Patient-Derived Xenograft Mice Model

4.4.1 *Tumour model*

Patient tumour harboring EGFR790M/L858R mutation was obtained in collaboration with CNIO (Centro Nacional de Investigaciones Oncológicas – Madrid – Spain) and previously characterize (Quintanal-Villalonga et al., 2019). Tumour sections (0.5 to 1 mm³) were inserted subcutaneously into the right flanks of 6 to 8-weeks-old, female NSG mice. For the experiment conducted on treated PDX, when the tumour size was close to 300mm³ mice were randomized and treated during 20 days with Osimertinib 10mg/kg or vehicle, once daily, by oral gavage. Tumours size was measured twice a week.

4.4.2 *NG injection*

12,5µg are injected in IV or intraperitoneal (IP).

4.4.3 *IVIS acquisition*

The IVIS Spectrum *In Vivo* Imaging System (PerkinElmer), an imaging chamber coupled at a camera that contains a charged-coupled device (CCD), was used to visualize fluorescence associated with NGs in intravital or isolated mouse organs. In the first case, mice are sedated with isoflurane and placed in the imaging chamber. In the second case, the mice are sacrificed, the organs dissected and imaged in the IVIS spectrum. $\lambda_{\text{DiD}_{\text{ex/em}}}$: 644/663 and $\lambda_{\text{DiR}_{\text{ex/em}}}$: 748/780. The images are then analyzed on the IVIS spectrum software. First of all, Regions Of Interest (ROIs) are delimited around what we want to know the fluorescence. ROIs are also taken in the non-fluorescent areas corresponding to the background. The fluorescence is quantified in "radiant efficiency" to avoid exposure time and are expressed in "average radiance" which allows to avoid the size of each element studied by considering the fluorescence per pixel.

RESULTS

PART I

MSC and NSCLC

MSC involvement in NSCLC cells growth and response to EGFR-TKI

The role of MSCs in the oncogenic process has been demonstrated in many tumour contexts (Ridge et al., 2017; Timaner et al., 2019) but very rare studies have evaluated their implication in the tumorigenesis of *EGFR*-mutated NSCLC and in their response to EGFR-TKI treatment (Gottschling et al., 2013; Wang et al., 2019a; Zhang et al., 2019a). Our first objective was to study the effect of MSCs on the growth of *EGFR*-mutated NSCLC cell lines.

To discriminate tumour cells from MSCs during co-cultures we transduced tumour cells with a vector allowing constitutive expression of GFP and MSCs with a vector allowing constitutive expression of mCherry (Figure 14). Tumour cells could be followed by their green fluorescence and MSCs by their red fluorescence. We confirmed by flow cytometry and fluorescence microscopy that mCherry labelling was not detected at the GFP wavelength.

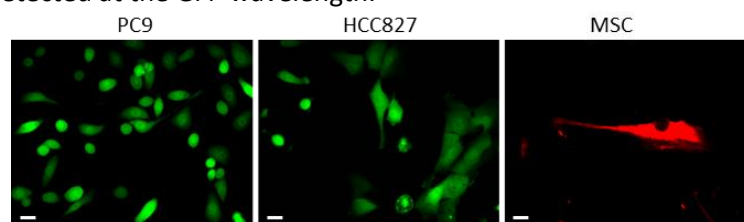


Figure 14 : Generation of stable GFP expressing tumour cell lines and stable mCherry expressing-MSC
Representative images of fluorescent microscopy of GFP transduced PC9 and HCC827 NSCLC EGFR-mutated cell lines and of mCherry transduced MSC. Scale: 30µm

To study the influence of MSCs on the response to EGFR-TKIs, we first aimed to evaluate whether EGFR-TKIs could have cytotoxic or cytostatic activity on MSCs. For this purpose, we quantified MSCs treated for 3 days with increasing concentrations of Erlotinib, (which was used at first line treatment at the beginning of this thesis), or Osimertinib, (which was used as second line therapy at the beginning of this thesis and is now used as first line treatment for *EGFR*-mutated NSCLC) (Figure 15A). We quantified MSC by detection of red fluorescence intensity.

We found that MSC were insensitive to neither of the EGFR-TKI at doses up to 1µM, which corresponds to the maximal concentration achieved in patients, although high dose Osimertinib (10 µM) but not Erlotinib slightly decreased MSC population (Figure 15B). The dose of 1 µM was selected for further experiments.

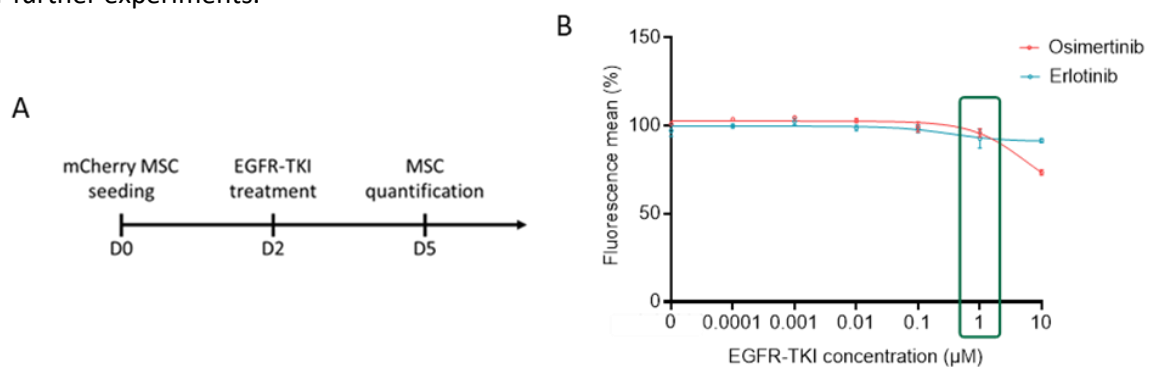


Figure 15: Treatment of MSCs with EGFR-TKI at the in vitro used concentration (1µM) does not impact MSC survival.

(A) Experimental procedure

(B) Fluorimeter quantification of mCherry-MSCs in response of 72h treatment of increasing concentrations of EGFR-TKI, Erlotinib (blue curve) or Osimertinib (red curve). The green rectangle shows the standard concentration of 1µM. n=2

Many biological effects of MSCs on tumour cells are mediated by their secretome (Ge et al., 2017; Meng et al., 2020a; Schelker et al., 2018). We studied whether the medium conditioned for 48 hours with MSCs could modify the survival and proliferation of tumour cells. To do this we cultured our tumour cells for 3 days in the presence of MSC-derived conditioned-medium (CM-MSC). Determination of green fluorescence intensity was used to quantify the number of tumour cells (Figure 16A).

We demonstrated that the factors secreted by the MSCs and found in CM-MSC did not modify the number of tumour cells, except for HCC4006 whose cell number increased when the cells were cultured in CM-MSC. The CM-MSC did not influence the survival and/or proliferation of tumour cells of PC9 and HCC827 but increased them for HCC4006 (Figure 16B).

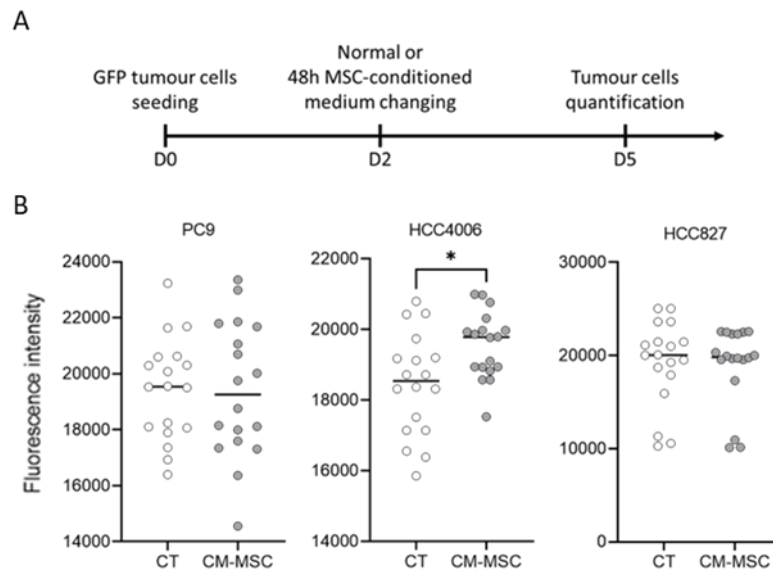


Figure 16 : Conditioned-medium of MSCs does not influence PC9 and HCC827 cell number and increased the number of HCC4006 cells

(A) Experimental procedure

(B) Fluorimeter quantification of GFP tumour cells after 72h of culture in control (CT) or MSC conditioned-medium (CM-MSC).

* $p < 0.05$ $n = 3$

Then, we investigated whether a CM-MSC influences the response of tumour cells to EGFR-TKI.

To this end, we cultured tumour cells for 3 days in the presence or absence of medium conditioned for 48 hours with MSCs and with increasing concentrations of Erlotinib or Osimertinib. The cells were quantified by measuring the green fluorescence associated with the GFP expressed by the tumour cells (Figure 17A). We observed that CM-MSC did not affect the response to Erlotinib nor Osimertinib (Figure 17B).

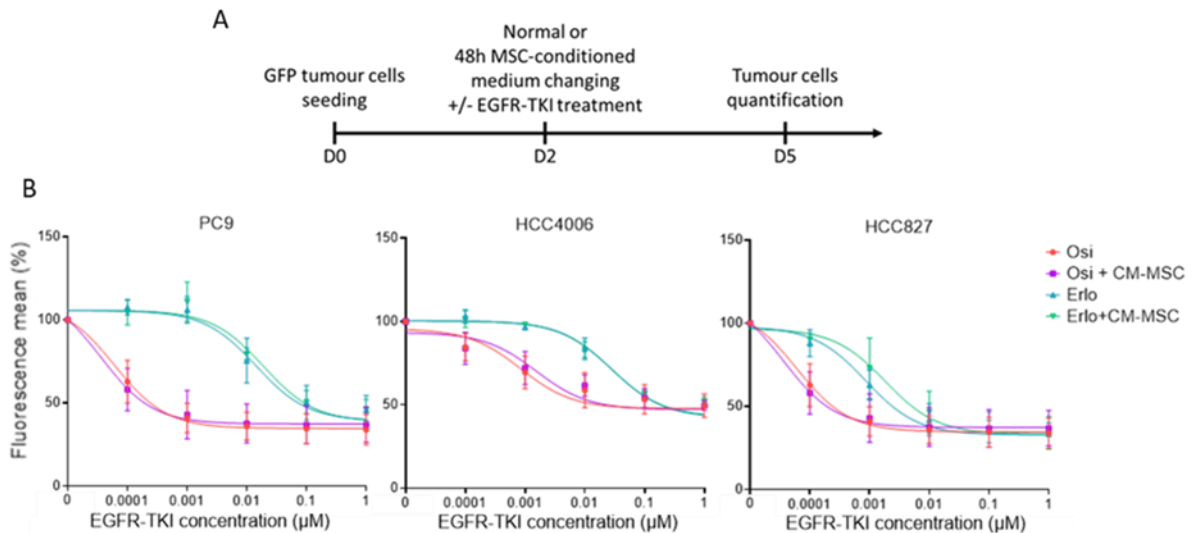


Figure 17 : MSC conditioned medium does not modify tumour cell response to EGFR-TKI

(A) Experimental procedure

(B) Fluorimeter quantification of GFP tumour cells in response of 72h treatment of increasing concentrations of EGFR-TKI, Erlotinib (blue and green curves) or Osimertinib (red and violet curves) in control medium (red and blue curves) or in MSC conditioned-medium (CM-MSC; green and violet curves) n=3

We next aimed to determine whether direct contact between MSC and tumour cells could affect tumour growth or response to EGFR-TKI by performing co-culture experiments.

Quantification of tumour cell and MSC was performed by fluorescence analysis using the specific fluorophores of each cell (red for MSC and green for tumour cells). We cultured tumour cells in the presence of MSC in a 10:1 ratio for 5 days and we quantified the green and red fluorescence signals (Figure 18A). For PC9 and HCC827, MSCs did not influence tumour cell growth while for HCC4006, MSCs decreased cell growth. The influence of MSCs on cell growth seemed to depend on the cell line studied (Figure 18B).

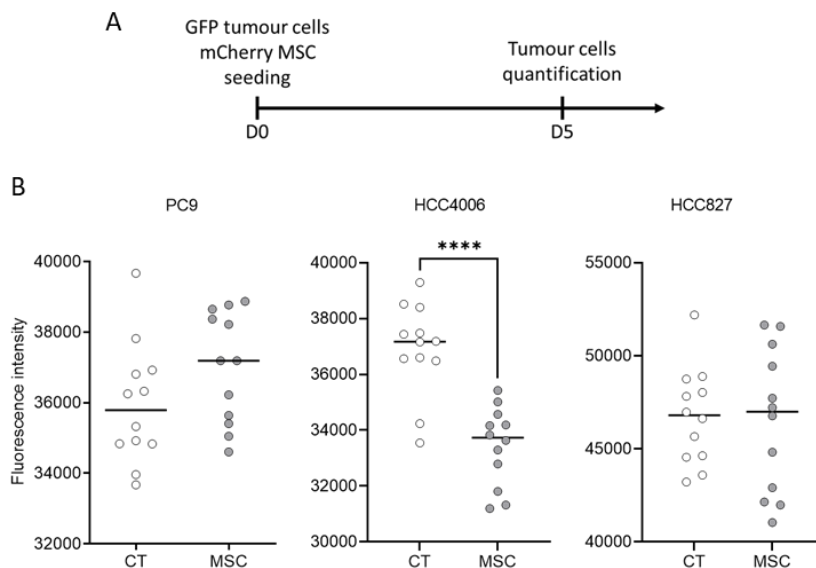


Figure 18 : Co-culture of MSC and tumour cells does not influence the number of PC9 and HCC827 cells and decreased the number of HCC4006 cells

(A) Experimental procedure

(B) Fluorimeter quantification of GFP tumour cells after 5 days of culture alone (CT) or in co-culture with MSC (MSC). **** p<0.0001 n=3

Next, we sought to determine the effect of MSCs on the response of tumour cells to EGFR-TKIs. For this purpose, MSCs and tumour cells were seeded at a ratio of 1:10, 2 days later the cells were treated or not with increasing concentrations of EGFR-TKI (0.0001 to 1 μ M). 3 days after treatment, the green fluorescence associated with GFP tumour cells was quantified (Figure 19A,B). We demonstrated the absence of influence of co-culture on the response to EGFR-TKI (Figure 19C). The presence of MSCs did not modify the response of tumour cells to EGFR-TKI in the short term.

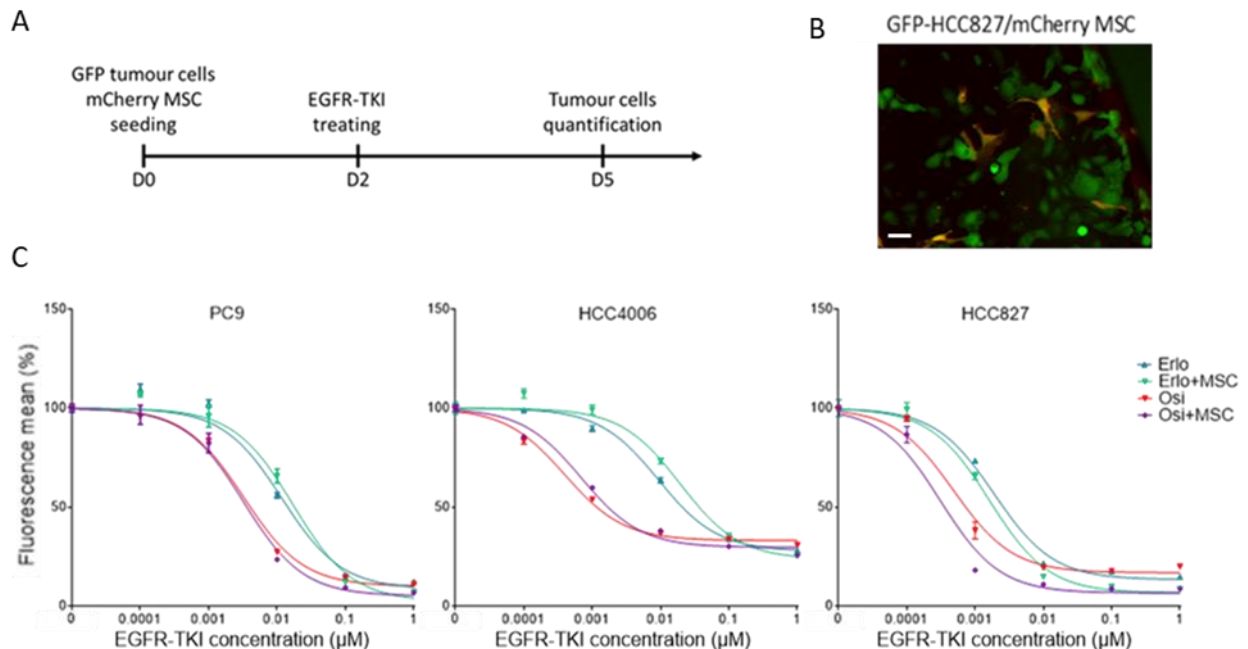


Figure 19 : MSCs do not impact tumour cells response to EGFR-TKI

(A) Experimental procedure

(B) Image of fluorescent microscopy of GFP-HCC827 (green) and mCherry-MSC (MSC). Scale: 30 μ m

(C) Fluorimeter quantification of GFP tumour cells in response of 72h treatment of increasing concentrations of EGFR-TKI, Erlotinib (blue and green curves) or Osimertinib (red and violet curves) in absence (red and blue curves) or in co-culture with BM-MSC (MSC; green and violet curves) $n=3$

As there was no impact of MSCs on the response of tumour cells to EGFR-TKIs in a short term, we wanted to evaluate the involvement of MSCs in a longer term response and development of resistance. As this experiment required a one-month follow-up, we needed to limit the proliferation of MSCs to avoid them colonising all the free space in the well. For this, we decided to irradiate the MSCs at 100Gy, the minimum ionizing radiation power to prevent MSCs proliferation. Although we were aware that irradiation could modify the phenotypic characteristics of the MSC, we estimated that the bias induced by MSC proliferation would be more detrimental for this experiment.

Tumour cells and irradiated-MSCs were seeded at a 10:1 ratio and then treated with Osimertinib (1 μ M) 2 days later. The treatment was repeated twice a week until relapse was observed. The green fluorescence of the tumour cells was monitored over time to assess the response of the cells to EGFR-TKI treatment (Figure 20).

The DTC ("Drug Tolerant Cells") state corresponds to the period during which the number of cells is the lowest and remains stable during treatment. The DTEC (Drug Expanded Tolerant Cells) state follows the DTC state and corresponds to the proliferation of the cells under treatment. The DTC state is, for both PC9 and HCC4006, about 14 days in the absence and presence of MSCs, with the proliferation of the cells under treatment occurring after 18 days of treatment (Figure 20). Moreover,

the number of tumour cells at DTC and DTEC states was not affected by the presence of irradiated-MSCs for both cell lines.

Irradiated-MSCs did not modify the response of tumour cells to EGFR-TKI treatment, having no effect on the kinetics of the response nor on the number of cells at each state (Figure 20).

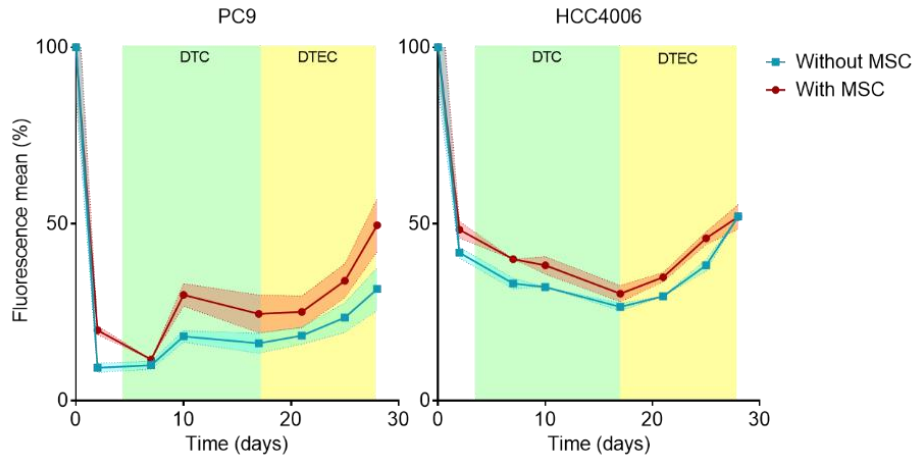


Figure 20 : MSCs do not have an effect on the response to EGFR-TKI treatment

Fluorimeter quantification of GFP tumour cells in response to treatment Osimertinib ($1\mu\text{m}$) overtime in absence (blue curve) or in co-culture with MSCs (red curve). The state of Drug Tolerant Cell (DTC) is in green and the state of Drug Tolerant Expanded Cell (DTEC) is in yellow. 100% refers to fluorescence intensity of GFP cells at the beginning of the treatment n=1

MSC tropism for NSCLC cells

The second objective to study the link between MSCs and *EGFR*-mutated NSCLC tumour cells was to investigate the tropism of MSCs for tumour cells. This study was used as proof-of-concept to evaluate the relevance of using MSC-derived nanoghosts as a therapeutic vector in this oncogenic context.

For this purpose, we first evaluated the attraction of MSCs by 5 *EGFR*-mutated NSCLC cell lines using a Boyden chamber assay (Figure 21A). Preliminary results of the lab indicate that 3 of these cell lines, PC9, HCC4006 and HCC827 form resistant proliferative clones (DTEC) under *EGFR*-TKI treatment after a period of drug-tolerance (DTC), while DTC generated from HCC2935 and H3255 failed to form DTEC and progressively died. MSCs were seeded in the upper part of the Boyden chamber of the migration chamber 2 days after tumour cells seeding in the lower part. After a migration time of 48h, the MSCs remaining in the upper chamber were removed and the migrated MSCs were quantified after the labelling of their nuclei with a fluorescent vital dye (Figure 21A,B). Active migration of MSCs towards tumour cells was assessed in comparison with passive migration of MSCs in the culture medium in the absence of tumour cells.

We observed little-to-no migration of MSCs in the absence of tumour cells, whereas migration was increased up to 10 to 15 times in the presence of tumour cells. This experiment showed that MSCs were attracted to all 5 *EGFR*-mutated NSCLC cell lines tested (Figure 21C).

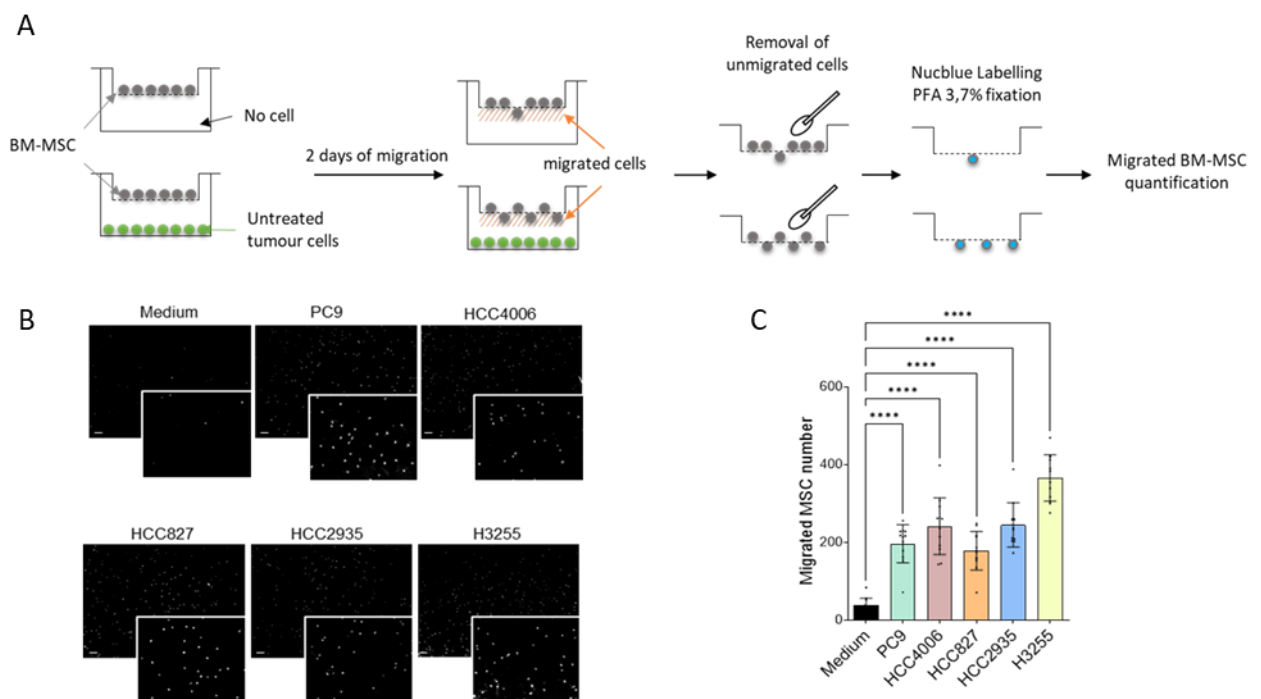


Figure 21 : MSC are attracted by *EGFR*-TKI resistant and sensitive NSCLC *EGFR*-mutates cell lines

(A) Experimental procedure

(B) Representative images of fluorescent microscopy of migrated MSC after NucBlue labelling and PFA fixation. Scale: 50µm. The smaller widow is a zoom of the big one

(C) Quantification of MSCs that have migrated in the absence of tumour cells (medium) or in the presence of different *EGFR*-mutated NSCLC tumour lines (PC9, HCC4006, HCC827, HCC2935, H3255) **** $p < 0.0001$ $n = 3$

We then investigated whether EGFR-TKI treatments could affect the capacity of tumour cells to attract MSCs. We treated *EGFR*-mutated lung cancer cell lines with Osimertinib at 1 μ M at different times: early response (24h), DTC (7 days) and DTEC (3 months) (Figure 22A). Since EGFR-TKI treatment induced a drastic decrease in tumour cell number, we generated DTC in larger culture plates before seeding the cells in the lower part of the Boyden chambers to have a sufficient amount of cells susceptible to attract MSC. DTEC were generated by cultivating cells in the presence of Osimertinib for 3 months before seeding the cells in the Boyden wells. Importantly, we used the same number of cells for each treatment to specifically address the effect of Osimertinib on MSC migration.

We first observed that the presence of Osimertinib in the medium did not affect MSC passive migration (Figure 22B). We also determined that Osimertinib treatment of tumour cells significantly increased MSC migration, with a maximum migration observed at the DTC state (Figure 22B).

This experiment demonstrated that MSCs tropism for tumour cells is enhanced by Osimertinib treatment and particularly when the cells are in the DTC state (Figure 22B).

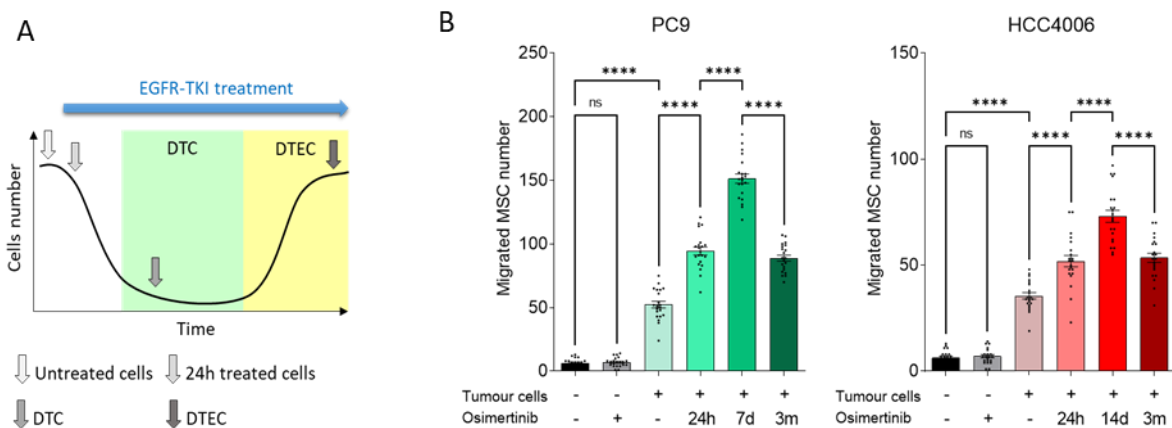


Figure 22 : MSC tropism is increased for Osimertinib treated tumour cells and is maximal for DTC

(A) Schematic of the treatment response kinetics of tumour cells developing resistance to EGFR-TKIs. The grey arrows show the timing of the study of MSC migration to untreated tumour cells, 24h Osimertinib treated cells, DTC and DTEC

(B) Quantification of MSCs that have migrated in the absence of tumour cells with or without Osimertinib, or in the presence of tumour cells (PC9 or HCC4006) under different conditions of treatment with Osimertinib **** $p < 0.0001$ $n = 3$

Finally, we wanted to study the tropism of MSCs for *EGFR*-mutated tumour cells *in vivo*. For this purpose, we xenografted nude mice with PC9 cells, and after one month of tumour growth we injected mCherry transduced BM-MSCs intravenously. 24 hours after injection we collected the tumour and filter organs (liver, lungs, spleen, kidneys), and we dissociated the tissues by enzymatic digestion. The presence of mCherry-labelled was determined by FACS (Figure 23A).

As expected, we observed a fraction of MSCs in filtering organs such as the liver (4,3% of the cells in the liver fraction are mCherry for mouse 1 and 7,3% for mouse 2) or the kidney (8,9% for mouse 1 and 1,4% for mouse 2). MSCs were also found in tumours for both mice with 3,6% and 2,2% of mCherry cells in the tumour fractions respectively (Figure 23B).

This experiment demonstrated the attraction of MSCs *in vivo* in a mouse model of *EGFR*-mutated NSCLC.

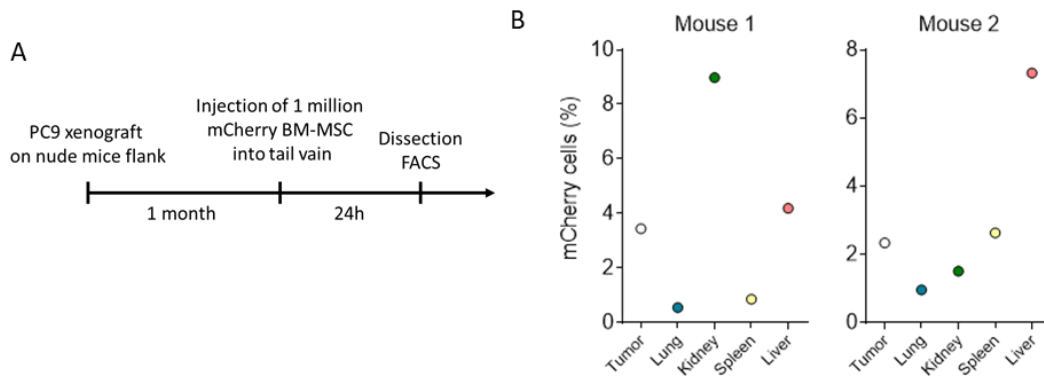


Figure 23 : MSC are attracted by the EGFR-mutated tumour in vivo

(A) Experimental procedure

(B) FACS quantification of the percentage of mCherry cells in tumour and filtering organs (lung, kidney, spleen, liver) $n=1$

Altogether, our results suggested that MSCs did not significantly influence *EGFR*-mutated tumour cell proliferation *in vitro*, nor the response to *EGFR*-TKI. However, we observed that MSCs displayed a tropism toward *EGFR*-mutated NSCLC tumour cells that was increased by Osimertinib treatment with a maximal attraction during the drug-tolerant phase.

These migration studies provided a proof of concept to consider the use of MSC-derived nanoghosts as a therapeutic vector to target *EGFR*-TKI-resistant tumour cells. The second part of this thesis focused on the use of nanoghosts in the context of *EGFR*-mutated NSCLC.

PART II

NG and NSCLC

We first investigated whether the NGs had the ability to interact with *EGFR*-mutated NSCLC tumour cells. To do so, we incubated green fluorescent tumour cells, the cell model presented in the first part of this thesis, with red fluorescent NGs labelled with a lipid marker, DiI (Figure 24A).

After overnight incubation, the tumour cells were observed by confocal microscopy. The NGs appeared as red dots within the green fluorescent tumour cells. The internalization of the NGs in the tumour cells was confirmed by the orthogonal projection and the superposition histograms of the fluorochromes associated with the tumour cells and the NGs. NG internalization occurred in untreated tumour cells but also in cells previously treated for 24h or 3 months with 1 μ M Osimertinib, although NG internalization appeared to be slightly reduced in 24h-treated cells Osimertinib (Figure 24B,C).

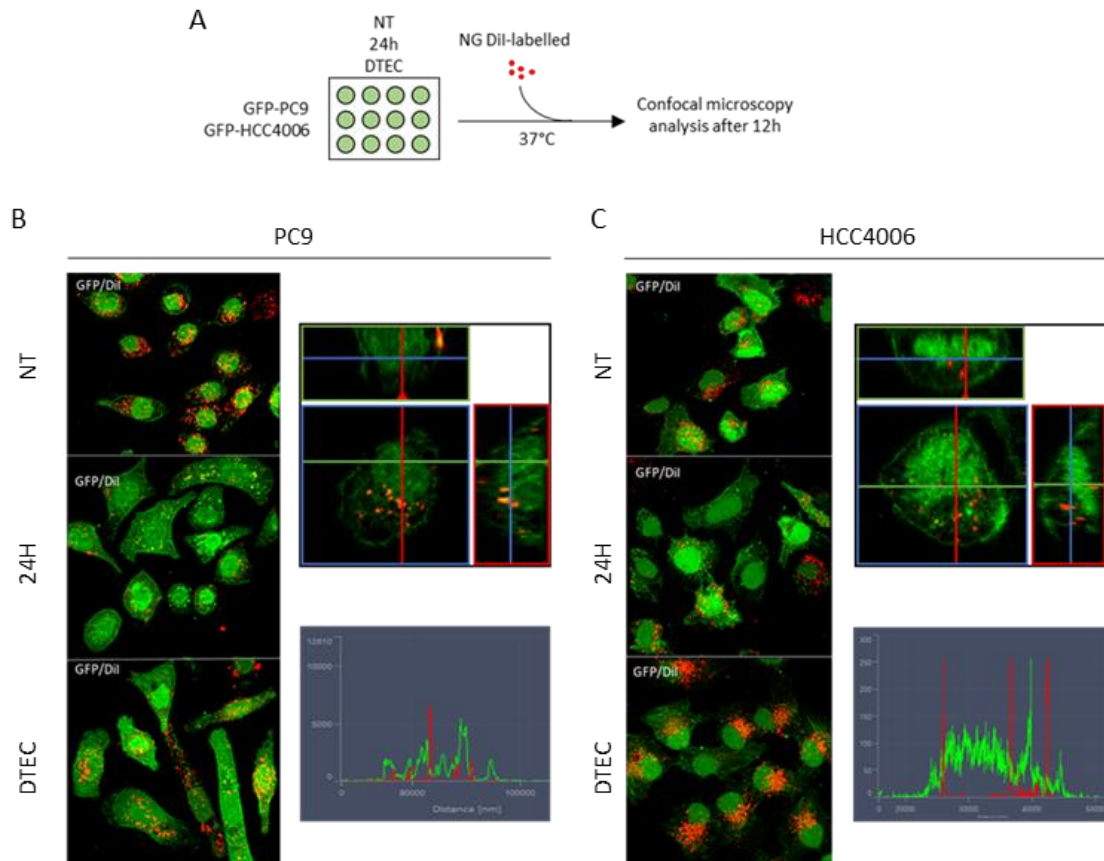


Figure 24 : NGs are internalized by NSCLC cell lines treated or not by Osimertinib

(A) Experimental procedure. The experiment is performed at 37°C.

(B) Left: Representative 3D images of confocal microscopy of the interaction of the NG (red) with untreated (NT), Osimertinib 24h treated (24h), or Osimertinib resistant (DTEC) PC9 cells (green). Top right: Representative orthogonal projection of untreated PC9 cells (green) to localized the NGs (red). For each panel: on the bottom left a 2D slide of the cell at the blue line in each orthogonal projection image, on the top the orthogonal projection at the green line and on the right the orthogonal projection at the red line Bottom right: histogram of the fluorescence of PC9 cells (green) and of the NGs (red) at the red line of the bottom left image of the orthogonal projection n=1

(C) Same representation as panel B but for the HCC4006 cell line n=1

To determine whether NG internalization occurred through a passive or an active uptake process, we performed the same experiment by incubating the cells at 4°C to inhibit membrane fluidity and blocking active internalization of the NGs (Figure 25A).

We observed that under these culture conditions, no red fluorescence associated with NGs was observed within the cells, as can be seen on the 3D representation of the cells as well as on the

orthogonal representation. The representation of the superposition of the histograms of the green fluorescence, of the tumour cells, and red fluorescence, of the NGs, shows that the fluorescence of the NGs was found at the periphery of the tumour cells and no longer inside. This results suggest that NG internalization occurs through an active membrane process of the tumour cells (Figure 25B,C).

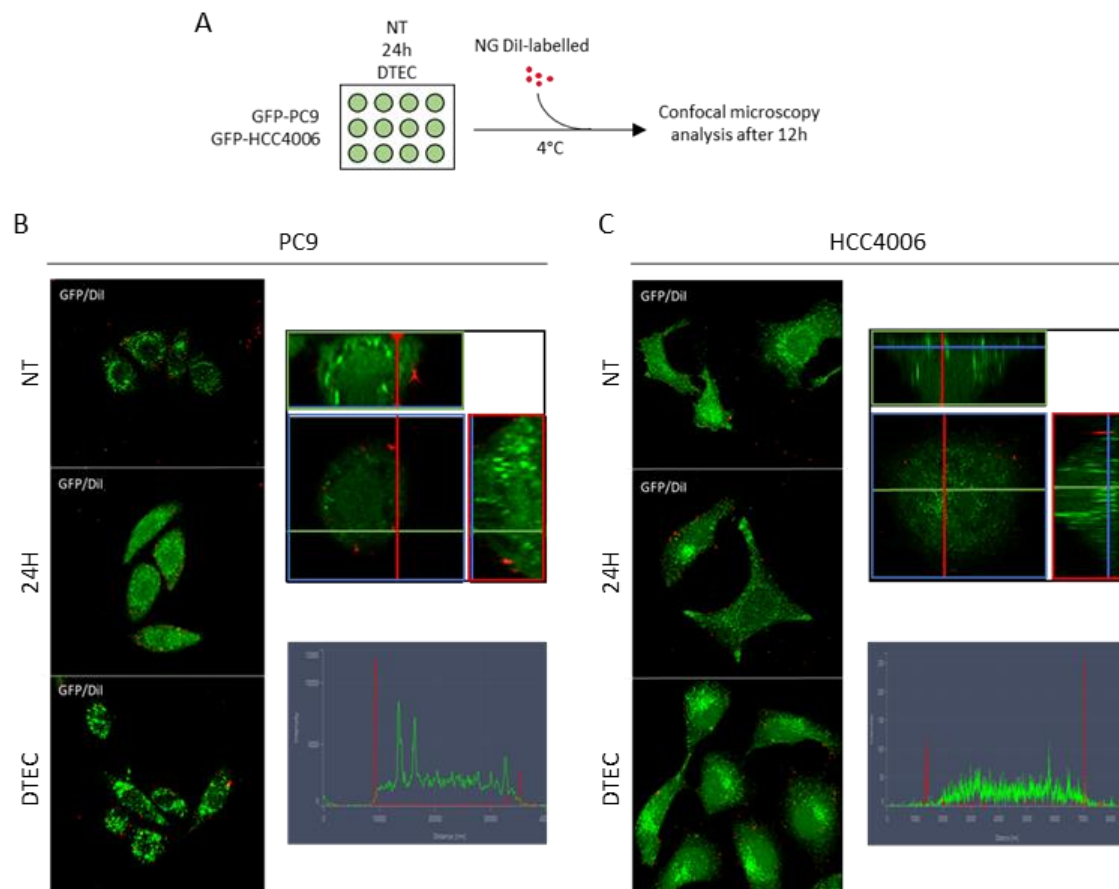


Figure 25 : NGs are internalized by an active process

(A) Experimental procedure. The experiment is performed at 4°C

(B) Left: Representative 3D images of confocal microscopy of the interaction of the NG (red) with untreated (NT), 1µM Osimertinib 24h treated (24h), or Osimertinib resistant (DTEC) PC9 cells (green). Top right: Representative orthogonal projection of untreated PC9 cells (green) to localized the NGs (red). For each panel: on the bottom left a 2D slide of the cell at the blue line in each orthogonal projection image, on the top the orthogonal projection at the green line and on the right the orthogonal projection at the red line Bottom right: histogram of the fluorescence of PC9 cells (green) and of the NGs (red) at the red line of the bottom left image of the orthogonal projection n=1

(C) Same representation as panel B but for the HCC4006 cell line n=1

To quantify NG internalization, we incubated GFP tumour cells with DiI-labelled NGs during 10, 30 and 60 minutes. Cells with both green and red fluorescence were quantified by flow cytometry. NG internalization was quantifying in untreated tumour cells, cells treated 24h with 1 µM Osimertinib or in proliferative resistant cells (DTEC).

Consistent with confocal observation, for the PC9 cell line, the uptake of NGs after 5 minutes of incubation was lower for cells treated for 24h with Osimertinib (17% of tumour cells show a red label) than for untreated cells (53%) or at the DTEC stage (68%). However, after a longer incubation the difference decreased and after 60 minutes of incubation the majority of tumour cells shown red fluorescence, whatever the treatment condition (between 87% for 24h treated cells and 92% for untreated cells) (Figure 26A,B).

For the HCC4006 cell line, the uptake was very dependent on the treatment condition. After 5 minutes of incubation, a small number of tumour cells showed red fluorescence in the case of untreated cells and cells treated for 24h with Osimertinib (respectively 28 and 17% of cells showing red labelling). For the DTEC condition, uptake after 5 minutes of incubation was much higher (93%). After 60 minutes of incubation, about 35% of the untreated cells and those treated for 24 hours shown a red labelling and 98% for DTEC. The internalization of NGs by HCC4006 seems to be dependent on the Osimertinib treatment conditions, being favoured by cells at the DTEC state (Figure 26C,D).

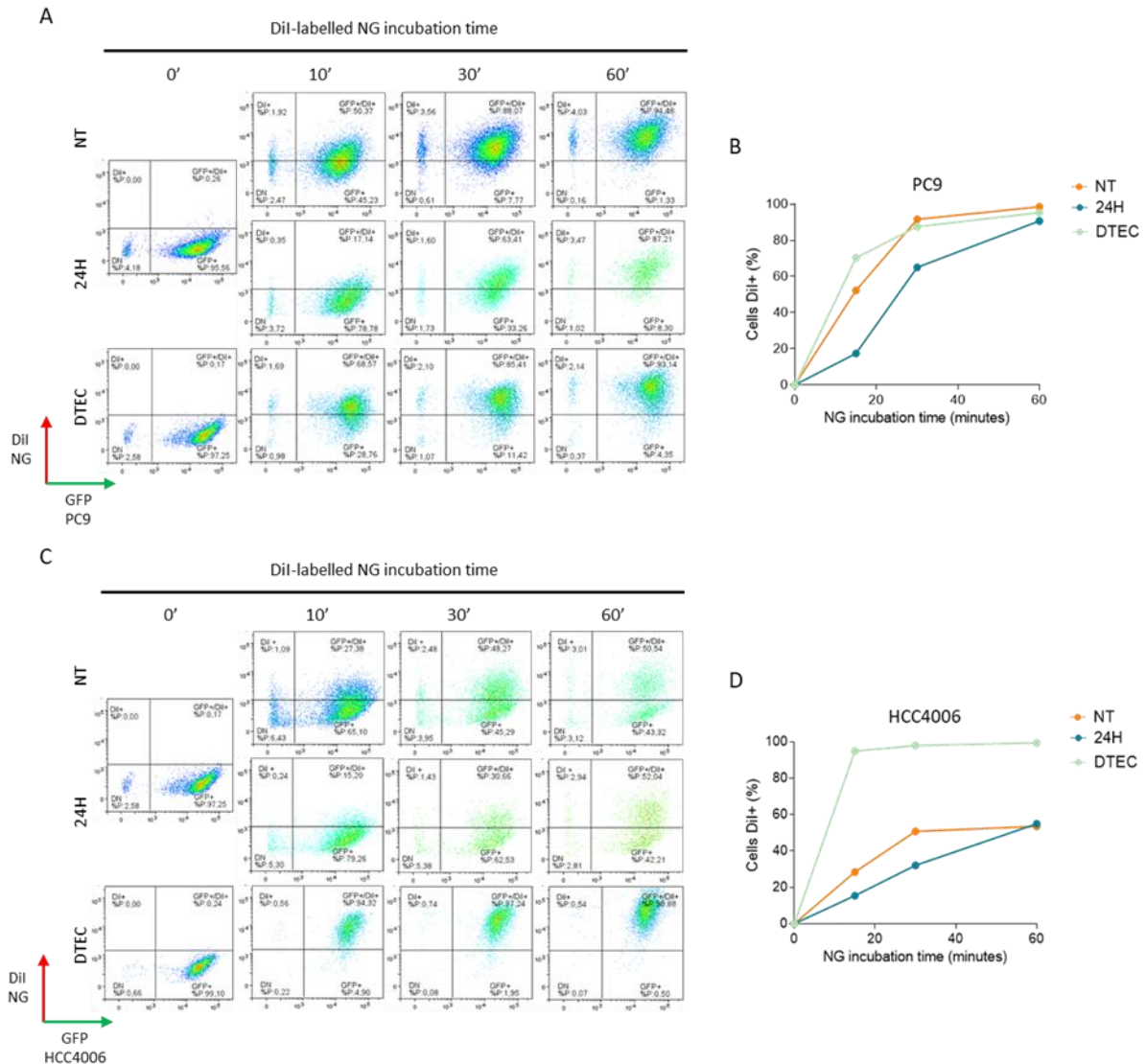


Figure 26 : NGs are internalized by tumour cells treated or not with Osimertinib

Flow cytometry quantification over time of the percentage of untreated (NT), Osimertinib 24h treated (24h) or Osimertinib resistant (DTEC) tumour cells which internalized NG=2

(A) Plot of cytometry data for PC9 cells. The green fluorescence of the tumour cells is shown on the abscissa and the red fluorescence associated to the NG on the ordinate.

(B) Graphic representation of results of flow cytometry analysis for PC9 cells

(C) Plot of cytometry data for HCC4006 cells. The green fluorescence of the tumour cells is shown on the abscissa and the red fluorescence associated to the NG on the ordinate

(D) Graphic representation of results of flow cytometry analysis for HCC4006 cells

We also used a technique to quantify NGs while visualizing the cells, the Operetta system. We incubated GFP tumour cells with DiI-labelled NGs. We imaged untreated tumour cells, Osimertinib-

treated for 24h, resistant cells (DTC) and proliferative-resistant cells (DTEC) after 5, 10, 15, 30 and 60 minutes of incubation of tumour cells with NGs (Figure 27A). To quantify the cells having internalized NGs and the number of NGs per cell we defined masks using Columbus software to delineate tumour cells and NGs (Figure 27B).

For the PC9 cell line, the kinetics of internalization were the same for untreated cells and those treated for 24h with Osimertinib, with an internalization by 95% of the cells after 5 minutes and 90% after 60 minutes of incubation. Internalization was 100% for DTC and DTEC from 5 minutes of incubation and the number remained constant throughout the experiment. (Figure 27C, top panel). For the HCC4006 line, cells treated for 24h and untreated cells were the treatment conditions internalizing the least NGs and the DTC and DTEC stages internalized the most NGs after 5 minutes of incubation. However, after 60 minutes of incubation, the vast majority of cells had internalized NGs (95% for cells treated during 24h and between 98% and 100% of cells for the three other conditions) (Figure 27C, central panel). For the HCC827 line, the 24h treated cells internalized less NGs than in the other treatment conditions, with between 80 and 85% of cells had internalized NGs during the experiment. The internalization of untreated cells was slower than for the DTC and DTEC conditions with 82% of cells having internalized NGs for untreated cells, 96% for DTC and 99% for DTEC after 5 minutes of incubation. However, at the end of the experiment, in all three conditions, 96% of the cells internalized NGs (Figure 27C, bottom panel).

Whatever the cell line, the cells at the DTC and DTEC states were more permissive to NGs than cells untreated or treated for 24 hours with Osimertinib. DTC and DTEC cells internalized NGs faster and in larger numbers.

With this Operetta experiment we also quantified the number of NGs per cell. For the three cell lines, untreated or 24 hours treated cells internalized NGs in lower numbers than DTC or DTEC over time (Figure 27D).

We demonstrated that NGs are internalized by Osimertinib-treated and untreated tumour cells. However, more cells at the DTC or DTEC stage internalized NGs, in greater numbers and more rapidly than cells untreated or treated for 24 hours with Osimertinib. (Figure 27C,D).

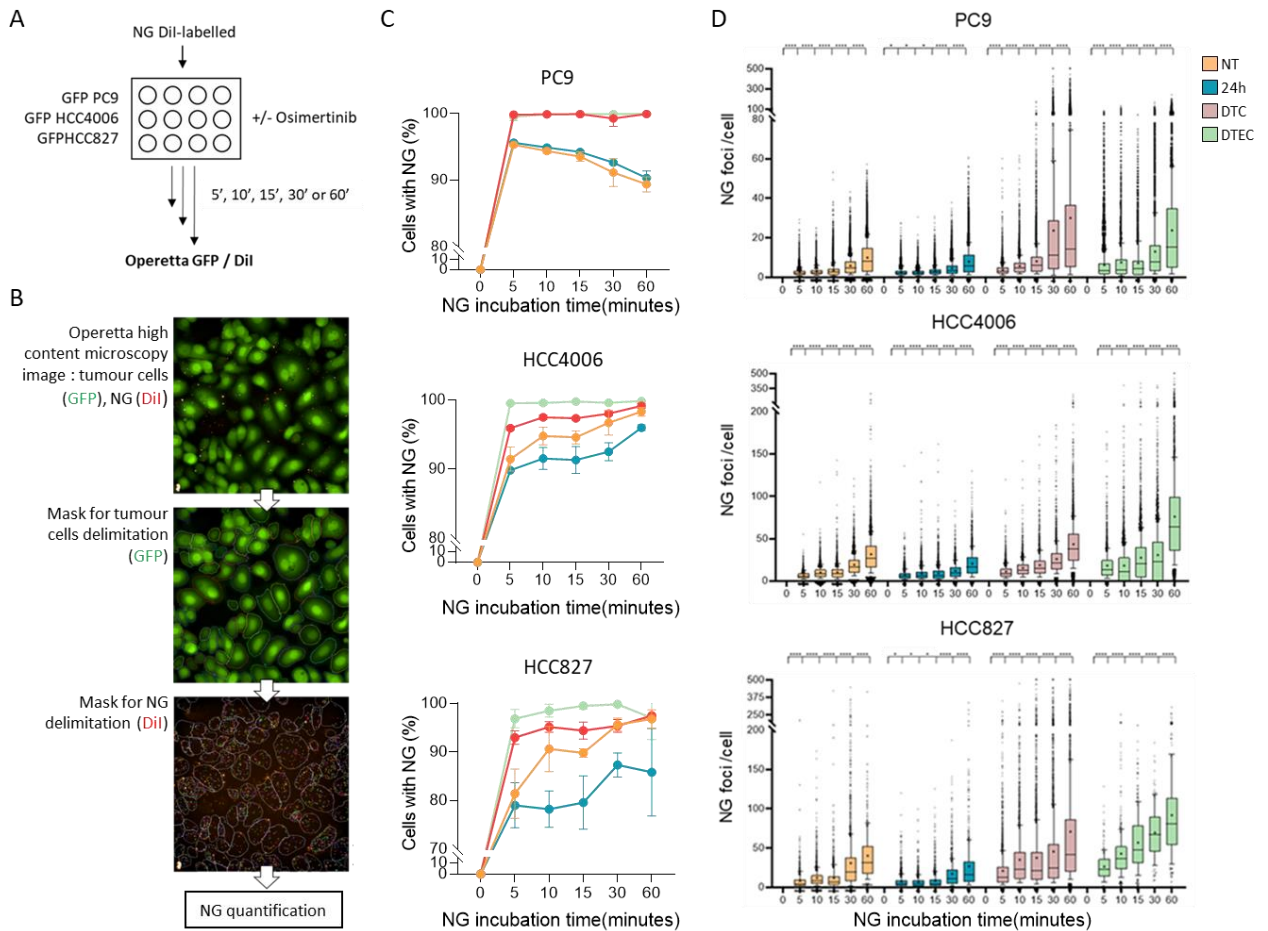


Figure 27 : Quantification over time of NG internalization by Osimertinib treated and untreated tumour cells

(A) Experimental procedure

(B) The top image is the one obtained at Operetta. The tumour cells fluoresce in green (GFP) and the NGs fluoresce in red (Dil). The middle image shows the mask used to delimit the tumour cells on the Columbus software. The bottom image shows the mask used to delimit the NGs on the same software. These masks were used to quantify the cells that internalized NGs and the number of NGs per cell $n=3$

(C) Columbus quantification of the cells that internalized NGs

(D) Columbus quantification of the number of NGs per cell

We then studied the capacity of NG uptake *in vivo*. For this purpose, we used a Patient-Derived Xenograft (PDX) model of *EGFR*-mutated NSCLC harbouring a double mutation of the *EGFR* gene (L858R and T790M) that was available in the lab. PDX were grafted into the flank of NSG mice and allowed to grow for several weeks until reaching an average tumour volume of of 200 mm³.

To study the uptake of NGs in the mouse organs and in their tumour, we chose to use the IVIS spectrum system to visualize intravital fluorescence.

First, we followed for 10 days the uptake of DiR-labelled NGs by the liver and the tumour in whole mice after intravenous (IV) NGs injection (Figure 28A,B). We observed a fluorescence associated with the liver and the tumour during the whole experiment, with a peak of fluorescence 3-4 days and a slow decrease of the fluorescence until the end of the experiment. The fluorescence was more prominent in the liver than in the tumour (Figure 28B,C).

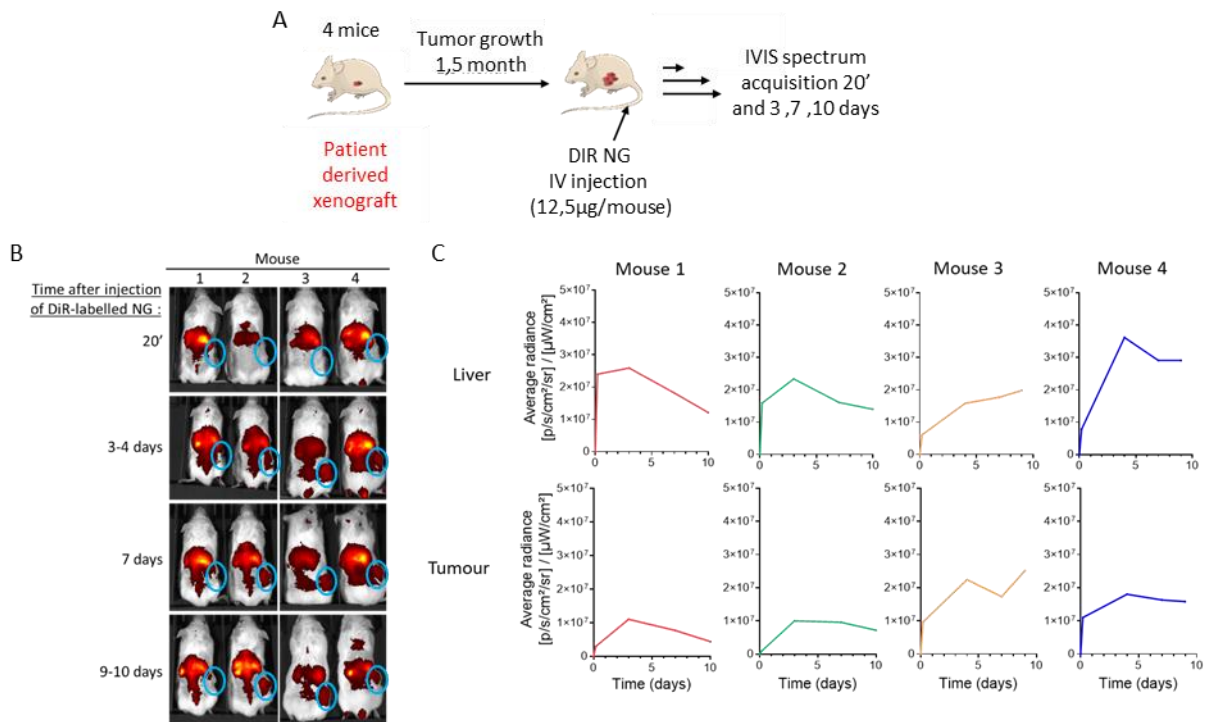


Figure 28 : After IV injection, the NGs can be visualized in the mouse, in the liver and in the tumour

(A) Experimental procedure

(B) DiR-NG distribution was assessed using the IVIS imaging system. Blue ovals show the site of the tumour.

(C) DiR fluorescence quantification by IVIS spectrum analysis in liver and tumour n=1

Therefore, on the mice monitored at IVIS spectrum and presented in the previous paragraph, we collected the filtering organs and tumour after sacrifice at 10 days after NG injection (Figure 29A). We imaged each organ with the IVIS spectrum and quantified the fluorescence associated with each of them.

We demonstrated that NGs uptake by the tumour was equal to that of the lungs, kidneys and spleen. However, the uptake was significantly higher in the liver (Figure 29B,C).

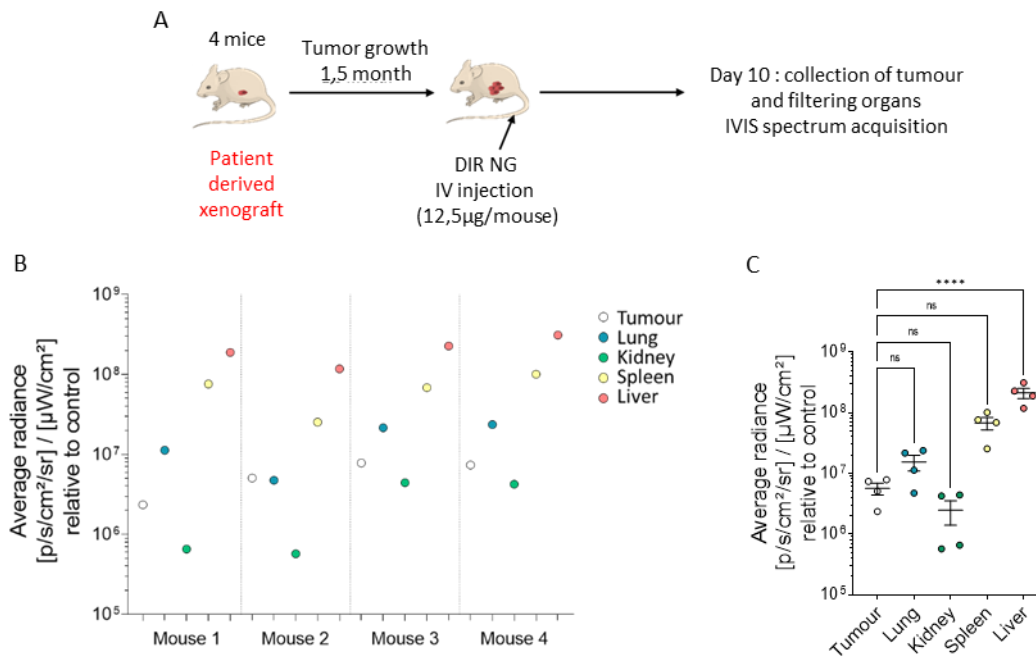


Figure 29 : After IV injection, the fluorescence associated to NGs is located into the tumour and filtering organs, especially the liver, after dissection and dissociation

(A) Experimental procedure n=1

(B) DiR fluorescence quantification by IVIS spectrum analysis by mouse in tumour and filtering organs

(C) Mean of DiR fluorescence quantification by IVIS spectrum analysis in tumour and filtering organs

Since the objective of this thesis was to use NGs to target EGFR-TKI resistant cells, we investigated whether NG uptake was modified by Osimertinib treatment.

For this purpose, we treated the same mouse models of PDX as above with Osimertinib for 20 days before injecting NGs in IV. 5 days after injection we recovered the filtering organs and the tumour from each mouse, and imaged them with IVIS spectrum before quantifying the fluorescence associated with each organ (Figure 30A). Under Osimertinib treatment, we observed exactly the same bio distribution of NGs in the filtering organs and in the tumour then previously, in untreated mice (Figure 30B,C).

Thanks to this experiment, we have demonstrated that the treatment with Osimertinib does not modify the uptake of NGs by the tumour and the filtering organs of the mouse.

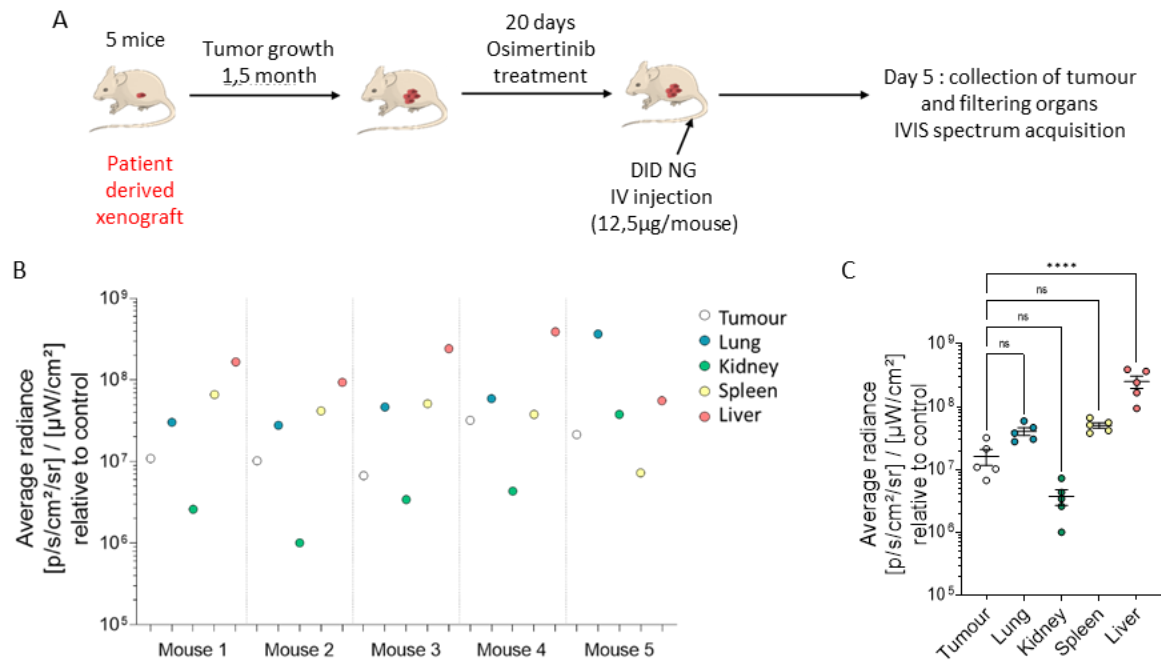


Figure 30 : Osimertinib treatment does not modify NGs repartition into the tumour and the filtering organs

(A) Experimental procedure n=1

(B) DiD fluorescence quantification by IVIS spectrum analysis by mouse in tumour and filtering organs

(C) Mean of DiD fluorescence quantification by IVIS spectrum analysis in tumour and filtering organs

In a prostate tumour model, it was shown that NGs were taken up in larger quantities by the tumour after an IP injection (Toledano Furman et al., 2013). We therefore evaluated the biodistribution of NGs after IP injection in the same mouse model of PDX treated with Osimertinib (Figure 31A). This experiment decreased the fluorescence in the liver without changing the fluorescence associated with the other filtering organs or the tumour (Figure 31B,C).

The proportion of NGs in the tumour was increased compared to that found in the liver after injection in IP. These three *in vivo* experiments allowed us to demonstrate that NGs are captured in our patient-derived xenograft model.

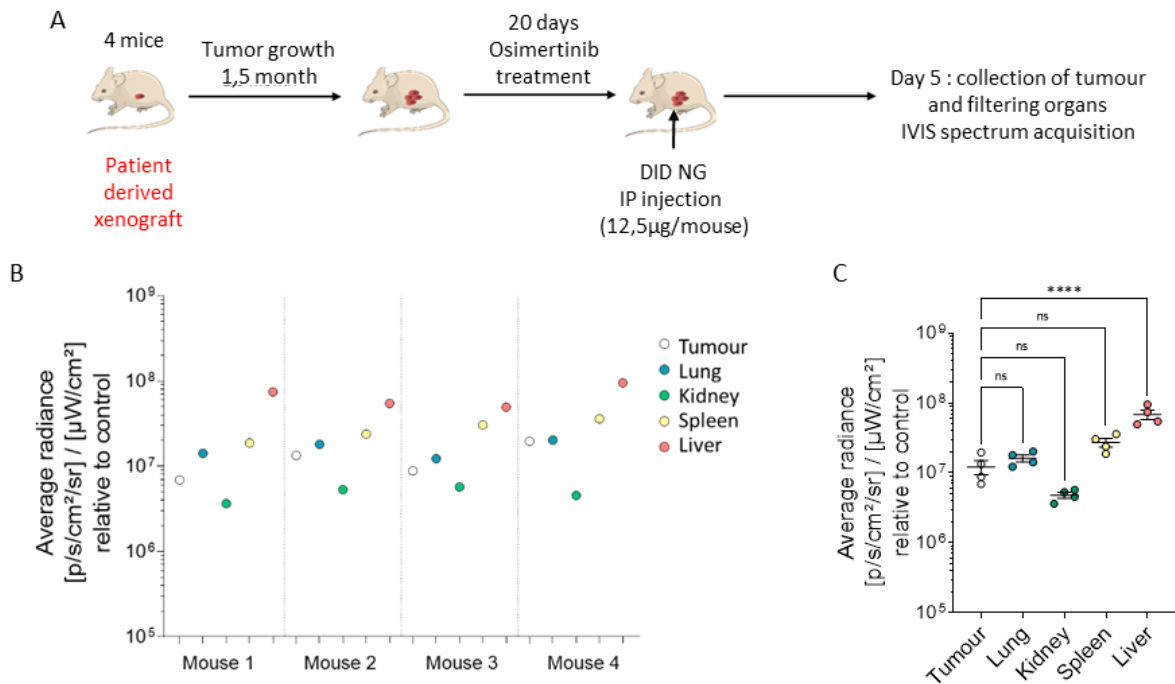


Figure 31 : IP injection decreases the quantity of NGs into the liver without modification of the quantity in the tumour

(A) Experimental procedure n=1

(B) DiD fluorescence quantification by IVIS spectrum analysis by mouse in tumour and filtering organs

(C) Mean of DiD fluorescence quantification by IVIS spectrum analysis in tumour and filtering organs

The next step was to validate the NGs as a therapeutic vector for the C3-exoenzyme, the treatment we wanted to combine with Osimertinib to limit the emergence of resistance. To this end, we validated the efficacy of C3-exoenzyme to limit the emergence of resistance *in vitro* in NSCLC cell lines.

We quantified over time GFP PC9 and HCC4006 cells fluorescence, treated with Osimertinib and TatC3, the C3-exoenzyme associated with the permeant peptide, the Tat fragment (Figure 32).

The number of PC9 cells treated with Osimertinib decreased during the first 48h, remains stable and increased after 18 days of treatment. However, the combination of Osimertinib and TatC3 removed all the cells during the first 48 hours of treatment. The number of HCC4006 cells treated with Osimertinib decreased by 30% during the first 18 days of treatment and then increased. Under Osimertinib and TatC3 treatment, the number of cells decreased by 90% during the first 18 days of treatment and remained stable before the number of cells increased again from the 43rd day of treatment.

The efficacy of the combination therapy depends on the cell line. It prevents resistance to Osimertinib treatment for the PC9 cell line and delays it for the HCC4006 cell line (Figure 32).

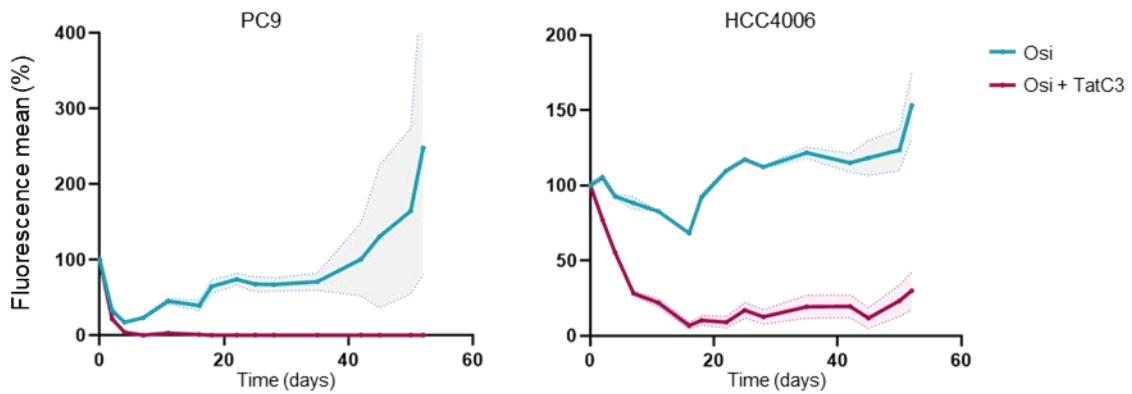


Figure 32 : TatC3 delays the resistance to Osimertinib treatment

Fluorimeter quantification of GFP tumour cells in response to treatment Osimertinib (1 μ m) overtime in absence or in combination with TatC3 (2 μ g/mL). 100% refers to fluorescence intensity of GFP cells at the beginning of the treatment n=2

We then validated that the C3-exoenzyme contained in the NGs entered the cells and was active. We treated PC9 and HCC4006 cells for 48h with C3-exoenzyme not permeable to cells (negative control), TatC3 (positive control) and C3 encapsulated in NGs (NG-C3). We next studied ADP-ribosylation of RHO GTPases that was catalyzed by C3-exoenzyme by western blot. The addition of the inhibitory ADP to the RHO GTPases is visualized by a band just above the total RHO GTPases band visualized by western blot.

Our positive and negative controls were validated for RHOB and RHOC but not for RHOA. NG-C3 allowed the ADP-ribosylation of RHOB and RHOC. Therefore, the C3-exoenzyme encapsulated in the NGs entered the cells and is effective for ADP-ribosylation of RHOB and RHOC (Figure 33).

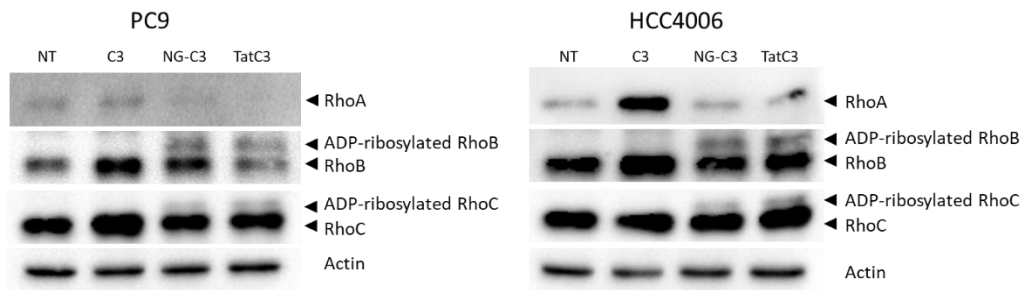


Figure 33 : NG-C3 are efficient to ADP-ribosylate the RHO GTPases RHOB and RHOC

Western blot analysis of RHOA, RHOB and RHOC in PC9 and HCC4006 cells alone (NT) or after 48h of treatment with C3-exoenzyme (C3), C3-encapsulated in NGs (NG-C3) or TatC3 n=1

DISCUSSION

EGFR-TKI, such as Erlotinib or Osimertinib, are effective therapies for *EGFR*-mutated NSCLC, but the majority of patients relapse due to the development of resistance mechanisms. Recent work suggests that these resistances may develop from a DTC population that enter a pseudo-dormant state before developing *de novo* resistance mechanisms responsible for cell re-proliferation (Hata et al., 2016; Sharma et al., 2010). Although therapeutic targeting of DTCs appears to represent a promising strategy in preventing relapse in patients, to date very little data is available on the nature of these DTCs. Previous results from the team showed that high expression of the small GTPase RHOB caused EGFR-TKI resistance *in vitro*, *in vivo* and in patients (Calvayrac et al., 2017), and recent work suggests that this RHOB-mediated resistance would be mediated through the maintenance of DTC survival. Indeed, our preliminary data showed that RHOB expression was increased in DTCs in response to EGFR-TKI, in contrast to its closer counterparts RHOA and RHOC, and that siRNA-mediated inhibition of RHOB strongly increased DTC susceptibility in several cell models (*unpublished data*). Although inhibition of RHOB may represent an effective therapeutic strategy to eradicate the reservoir of DTCs potentially responsible for patient relapse, no specific inhibitor of this GTPase is currently available for clinical use.

The C3-exoenzyme derived from *Clostridium Botulinum* is known to specifically inhibit RHOA, RHOB, and RHOC by ADP-ribosylation (Sahai et al., 2006). We were interested in determining whether inhibition of Rho-GTPases by C3-exoenzyme could sensitize DTC and prevent relapse *in vitro*. Our results showed that co-treatment with C3-exoenzyme fused to a permeant peptide (Tat) prevents or delays the emergence of EGFR-TKI resistance in a panel of *EGFR*-mutated NSCLC cell lines (PC9, HCC4006). Since the use of TatC3 is not feasible for systemic treatment *in vivo* and in patients due to the toxicity it would cause, we sought a solution to specifically address C3 to tumours and allow its therapeutic use. We established a collaboration with Prof. Marcelle Machluf's team (Technion, Israel) who is developing MSC-derived NGs, which have the particularity of having a specific addressing to tumour cells (Timaner et al., 2018; Kaneti et al., 2016). Their use as therapeutic vectors have been validated in numerous *in vitro* models and prostate cancer *in vivo* model (Timaner et al. 2018; Furman et al, 2013).

MSCs, from which NGs are derived, have been shown to be of therapeutic benefit and as a therapeutic vector in various tissue regeneration (Perry et al., 2021; Vieujean et al., 2021) and oncogenic contexts (Krueger et al., 2018; Li et al., 2018). However, their use for therapeutic purposes is limited by their pro-tumour potential, with a role in enhancing tumour growth (Quante et al., 2011; Yu et al., 2021a) and involvement in metastatic dissemination (Cortini et al., 2016; Mele et al., 2014; Xue et al., 2015). MSCs are also implicated in resistance to therapies such as chemotherapy (Pillozzi et al., 2018; Schelker et al., 2018) or targeted therapies (Wang et al., 2019a). However, very few studies have focused on the involvement of MSCs in tumour progression and response to therapies in NSCLC (Gottschling et al., 2013; Wang et al., 2019a; Zhang et al., 2019a).

In the first part of this thesis, we were interested in evaluating the involvement of MSCs in the tumour cells proliferation of *EGFR*-mutated NSCLC and in resistance to EGFR-TKI. The pro-tumour effect of MSCs is mainly due to their secretory activity (Adamo et al., 2018; Ren et al., 2019b; Schelker et al., 2018). We evaluated the effect of MSCs secretions in CM-MSC culture experiments, which we complemented by co-culture experiments of MSCs with tumour cells.

To perform these experiments and to discriminate the different cell populations, we transduced PC9, HCC4006 and HCC827 tumour cells with GFP lentivirus and MSCs with mCherry lentivirus. The respective green and red fluorescence of each cell type allows to discriminate tumour cells of MSCs during co-culture experiments but also to follow the number of cells by following the

fluorescence intensity, considering that the fluorescence is stable in the cell whatever the culture conditions.

We also verified that EGFR-TKIs did not alter the proliferation of tumour cells. We showed that the culture of MSCs in the presence of Erlotinib or Osimertinib at the commonly used concentration of 1 μ M did not modify MSC proliferation. The co-culture conditions of MSCs and tumour cells in the presence or absence of EGFR-TKI could be compared, the MSC proliferation rate being the same in both conditions.

We first studied cell proliferation and the response of cells to EGFR-TKIs, Erlotinib and Osimertinib, in the presence of CM-MSC. We observed an increase in cell proliferation for the HCC4006 line but not for the two other cell lines studied, PC9 and HCC827, and we did not observe any influence of CM-MSC on the response to EGFR-TKI of tumour cells whatever the line studied. MSCs secrete factors promoting the proliferation of HCC4006 which have no effect on the cells of the other lines studied nor on the response of the cells to the EGFR-TKIs studied. The study of cytokines presents in CM-MSC coupled with a comparative study of receptors present on the surface of the different cell lines would allow the identification of factors that could promote the proliferation of HCC4006.

The use of CM-MSC allows to study the effect of MSC cytokines in tumour cells but does not allow to appreciate the effect of the contact of MSC with tumour cells. Therefore, we co-cultured GFP tumour cells and mCherry MSCs and studied the proliferation and EGFR-TKI response of tumour cells. We observed a decrease in cell proliferation for the HCC4006 line but no effect of co-culture on the other two lines studied, PC9 and HCC827 and did not observe any influence of the presence of MSC on the response to EGFR-TKI of tumour cells whatever the line studied.

The results shown that the proliferation of HCC4006 is increased when tumour cells are cultured in CM-MSC and decreased when they are co-cultured with MSCs. This may be explained by the secretion by MSCs of antiproliferative cytokines that have a short half-life or are not stable in CM-MSC, favouring proproliferative cytokines. Indeed, MSCs secrete anti-proliferative cytokines, such as the IL4 or the IL10, which has been observed in ovarian cancer (Khalil et al., 2019), and pro-proliferative cytokines such as the CXCL12 (Rhodes et al., 2010) or the TGF- β (Schelker et al., 2018). Another hypothesis would be that the contact between tumour cells and MSCs would decrease the proliferation of tumour cells despite the pro-proliferative effect of the MSCs secretome. 3D culture on spheroid or organoid models would allow co-cultivation of tumour cells and MSCs to study the effect of continuous rather than end-point secretions of MSCs on tumour cell proliferation.

An effect of MSCs on the proliferation of tumour cells being observed in only one of the three lines studied, it would seem that tumour cells are not sensitive to the same signals that can influence their proliferation. The study of receptors differentially expressed on the surface of different cell lines would allow to identify to which factors HCC4006 cells are more sensitive than other cell lines. For example, the CXCR4 and 5 receptors define the sensitivity of tumour cells to the pro-tumour effect of CXCL12 secreted by MSCs (Rhodes et al., 2010; Schelker et al., 2018). It is conceivable that receptors for pro-proliferative chemokines such as CXCR4 and 5 are present on the surface of HCC4006 cells and not on the surface of cells of the other lines studied.

The studies conducted to investigate the response of tumour cells to EGFR-TKI in CM-MSCs and in co-culture only allowed to study a short-term response of the cells and not to evaluate the longer term influence of MSCs on the emergence of resistance to treatment. To study this, we conducted a co-culture experiment of tumour cells and MSCs over one month of treatment to evaluate the impact MSCs on the DTC state of the tumour cells as well as their resumption of proliferation at

the DTEC state. The proliferation of MSCs being a limiting factor in this study, we chose, while knowing the limits of this practice, to irradiate the MSCs at the minimal power limiting their proliferation which we evaluated at 100Gy. After irradiation, the MSCs presented the same morphology as the non-irradiated cells. This co-culture experiment of irradiated MSCs and treated tumour cells showed that MSCs had no effect on the resistance of PC9 and HCC4006 cells under Osimertinib treatment or on their resumption of proliferation. However, we cannot conclude that there is no effect of MSCs on the response of tumour cells to EGFR-TKI. Indeed, this lack of effect may be due to the modification of MSCs by irradiation. To avoid this bias, it would be possible to reproduce the same studies in 3D models, such as spheroids or organoids. Spheroids and organoids would allow to get rid of the bias that can represent the proliferation of MSCs in a 2D culture well.

The data from this first part of the thesis have shown that MSCs do not have an effect on tumour cell proliferation or on their response to the 1st and 3rd generation EGFR-TKIs, Erlotinib and Osimertinib. However, our studies do not allow us to conclude on the effect of MSCs in the tumour progression of EGFR-mutated NSCLC. Indeed, we only studied the direct or indirect effect of MSCs on tumour cells but the role of MSCs in the tumour process is strongly linked to their capacity to modify the microenvironment (Babazadeh et al., 2021; Oskowitz et al., 2011; Oswald et al., 2004). A study of the effect of MSCs on angiogenesis (Oskowitz et al., 2011; Suzuki et al., 2011; Vartanian et al., 2016), on the immune environment (Babazadeh et al., 2021; Thin Luu et al., 2013) or on the differentiation of MSCs in specialized cells (Oswald et al., 2004; Wang et al., 2019b) in the microenvironment of NSCLC would complete the data acquired.

The objective of this thesis being to use NGs as a therapeutic vector in *EGFR*-mutated NSCLC, we studied the tropism of MSCs for tumour cells as a proof of concept. This allowed us to demonstrate that MSCs are attracted to cells of the 5 cell lines studied PC9, HCC4006, HCC827, H3255 and HCC2935. The first 3 being lines developing resistance to the treatment and the other 2 being lines not developing resistance (unpublished data), we showed that MSCs are attracted to *EGFR*-mutated NSCLC cells regardless of their sensitivity to EGFR-TKI. A study of cytokines secreted by tumour cells coupled with a comparative study of the receptors present on the surface of MSCs would allow us to identify the factors promoting the migration of MSCs. This would allow us to evaluate the response of MSCs to chemoattractant known to promote the recruitment of MSCs in other cancerous contexts such as CXCL12 (Park et al., 2017; Pavon et al., 2018) or CXCL1 (Zhang et al., 2016). Wishing to use NGs to target treatment-tolerant cells, DTCs, we studied the tropism of MSCs for tumour cells at different stages of response to Osimertinib. As the tropism of MSCs was not modified by the addition of Osimertinib in the culture medium in absence of tumour cells, the modification of migration ability of MSCs to treated tumour cells observed were due to the effect of the treatment on the tumour cells and the effect of the treatment itself. We had demonstrated that a short treatment of 24 hours with Osimertinib increased the tropism of MSCs for tumour cells. The attraction of MSCs is maximal by DTC stage cells while the repopulative cells, DTECs, attract MSCs like the tumour cells treated 24 hours with Osimertinib. EGFR-TKI treatment modifies the tumour cells that will favour the recruitment of MSCs. This may be through a modification of the tumour cell secretome, which we could explore by chemoattractant cytokines differentially expressed in the presence or absence of treatment. A study performed by Kurppa et al. on Osimertinib and Trametinib-treated cells (Kurppa et al., 2020), as well as preliminary data from the laboratory on Osimertinib-treated cells, showed that cells of the PC9, HCC4006 and HCC827 cell lines at the DTC state acquired a senescent phenotype. This phenotype is associated with a Senescence-associated secretory phenotype (SASP). The senescent cells overexpress GM-CSF or CCL5, both known to promote MSC migration (Kim et al., 2019; Vainieri et al., 2020).

These migration experiments allowed us to demonstrate the tropism of MSCs for different *EGFR*-mutated NSCLC cell lines and the beneficial influence of *EGFR*-TKI treatment of the tumour cells on the migration abilities of MSCs on two cell lines. DTEC state cells attract more MSCs, which is very encouraging for the use of NGs to target *EGFR*-TKI resistant cells. But even if our objective was to target treatment tolerant cells, the data obtained also allow us to consider their use during early treatment, as MSCs are attracted to cells treated with Osimertinib since 24 hours, or during relapse, when cells are at DTEC state.

We then completed these data by an *in vivo* experiment of MSC mCherry migration injected in IV in two mice xenografted with cells of the PC9 lineage. MSCs were found in the filtering organs, notably in the liver for both mice and in the kidney for one of the two mice. Both mice shown fluorescence in the tumour. Finding MSCs in the filtering organs was expected, being the organs allowing blood purification and the fluorescence associated with the tumour was encouraging for the continuation of our studies. Even if these data seem to show a tropism of the MSCs for the tumour, the experiment being conducted on only two mice, it should be reproduced on more individuals. We could also reproduce this experiment on mice transplanted with PDX to have a model closer to the patient's tumour and recapitulating the tumour heterogeneity. Histological studies of tumours would also allow us to visualize the distribution of MSCs in the tumour and to study the fate of MSCs in the tumour. For this, we could use specific markers of blood vessels, adipose or endothelial cells or other cell types.

NGs are MSC-derived vesicles emptied of any nuclear and cytoplasmic components (Oieni et al., 2019, 2021). Their internalization by tumour cells has been demonstrated in different cell lines (Kaneti et al., 2016; Timaner et al., 2018; Toledano Furman et al., 2013) and *in vivo* in mouse models xenografted with PC3 prostate cancer cells (Kaneti et al., 2016; Toledano Furman et al., 2013). *In vivo* studies have shown a specificity of NG uptake by the tumour compared to the filtering organs (Kaneti et al., 2016; Toledano Furman et al., 2013). NGs being vesicles derived from MSCs membranes emptied of intracellular components (Oieni et al., 2019, 2021), their mechanism of arrival and interaction with tumour cells should not be mediated by the stimulation of migration by cytokines, as for MSCs migration, but rather on the presence on the surface of NGs allowing their specific attachment to the tumour, even if, for the time being, no study has been conducted to elucidate this. A proteomic study of NGs would allow the identification of proteins found on the surface of NGs that would allow their attachment to the surface of tumour cells. In *in vitro* breast cancer model, is known that MSCs interact with tumour cells by forming Gap junctional intercellular communication (GJIC) junctions (Mandel et al., 2013; Melzer et al., 2016). The presence of connexin proteins on the surface of MGs would allow to consider this mode of binding. In *in vitro* breast cancer model, MSCs also interact with tumour cells through the Notch ligand-receptor complex (Mandel et al., 2013; Melzer et al., 2016). The identification of Notch ligand and/or receptor on the surface of NGs would allow to consider this type of interaction.

The second objective of this thesis was to evaluate the relevance of NGs as a therapeutic vector to target *EGFR*-mutated NSCLC cells. To this end, we first evaluated the uptake of NGs by PC9, HCC4006 and HCC827 cell lines. We saw that tumour cells not treated with Osimertinib, treated for 24 hours or at DTEC state, had the ability to internalize the NGs. The orthogonal representation of the cells as well as the superposition of the GFP and Dil fluorescence histograms confirm that the NGs are inside the tumour cells and not on the membrane of the cells.

As this internalization could be either due to active internalization of NGs by tumour cells or to entry of NGs by passive diffusion, we performed the same experiment at 4°C to block membrane

fluidity and inhibit active internalization phenomena of the cell. In this case, there is no more NG found inside the tumour cells. The orthogonal representation and the histogram of the DiI fluorescence located outside of the GFP fluorescence confirm that the only NGs visualized are located at the periphery of the cells and not inside. NGs are internalized by cells treated or not with Osimertinib and this, thanks to an active phenomenon of the cells.

A study of proteins and other membrane determinants on the NG surface would allow us to understand how NGs can enter into contact with tumour cells while an electron microscopy study of the internalization mode with radiolabelled NGs (Oieni et al., 2019) would allow us to characterize the entry pathway of NGs into our cellular models.

We then quantified the internalization of NG by tumour cells. For this, we first considered the use of flow cytometry. For the PC9 line, orange fluorescence is found in about 90% of the tumour cells after 60 minutes of experiment, whatever the treatment conditions, even if the internalization by the cells treated for 24h with Osimertinib is a little slower. For HCC4006, almost all DTECs take up NG within the first 15 minutes of treatment. About 50% of the untreated cells or of treated for 24h with Osimertinib captured NGs with a slower internalization kinetics. For HCC4006, DTECs would capture NGs more or more rapidly. A longer term study would allow us to know if this is a difference of kinetic of internalization or of cell capacity of internalization.

We wanted to reproduce these results with a batch of NGs, produced from another batch of MSCs, but the number of cells showing dual GFP and DiI labelling were very low in all treatment conditions (data not shown). This could be due to a lack of internalization or a threshold effect of the technique. Indeed, the NGs being too small to be studied alone in cytometry, we cannot discriminate one or the other theory. We decided to quantify the internalized NG over time by a technique that also allows us to visualize the NGs inside the tumour cells, the Operetta. We quantified the proportion of cells having internalized NGs as well as the number of internalized NGs. For the three lines studied, PC9, HCC4006 and HCC827, almost all DTCs and DTECs internalized NGs. For PC9, the internalization by untreated cells or cells treated for 24h with Osimertinib was lower than for DTC and DTEC, whereas for HCC4006 and HCC827, only cells treated for 24h internalized less than DTC and DTEC. But for all the lines and whatever the treatment conditions, the percentage of cells having internalized NG is between 85 and 100%. The majority of tumour cells internalize NGs from the earliest stages of the experiment. We also quantified the number of NGs per cell over time. We were show that NGs are internalized in greater quantity by cells at the DTC and DTEC stages for the three cell lines studied.

One mechanism of resistance to EGFR-TKIs is the establishment of EMT in treated tumour cells (Chung et al., 2011; Lee et al., 2017; Suda et al., 2011). Resistant cells lose the expression of E-cadherin in favour of mesenchymal markers such as SNAIL and SLUG (Lee et al., 2017). One hypothesis is that the increased affinity of NGs for EGRF-TKI resistant cells could be due to a higher similarity between the membranes of NG and mesenchymal-like resistant tumour cells, thus favouring their interaction. To investigate this hypothesis, we could study the affinity of NGs for tumour cells whose resistance to EGFR-TKI is enabled by another resistance mechanism than this phenotypic switch. For example, cells of the PC9 cell line develop different resistance mechanisms from one cell to another. The study of the internalization of NGs by DTECs having resisted to the treatment by EMT or by another mechanism would allow us to explore this hypothesis.

These Operetta experiments have shown that NG are internalized by the majority of cells whether they are treated with EGFR-TKI or not. The rate of uptake is higher for cells at the DTC and DTEC stages and experiments over a longer period of time would allow us to know if this is a difference in the kinetics of internalization or in the capacity of the cells to take up the NG. These results could

be completed by a study of the capacity of endocytosis of the cells in particular experiments allowing to quantify the end-point endocytosis or of electronic microscopy, allowing in particular to visualize the endocytosis dependent on clathrin.

The homogeneity of the results obtained in three independent experiments with batches of NGs from different MSCs batches supported our idea that the heterogeneity observed in cytometry could be due to a bias of the fluorescence detection threshold. This is why for future experiments we have favoured imaging techniques.

Following these *in vitro* results, we studied the bio-distribution of NG *in vivo* in mice transplanted with an EGFR-mutated NSCLC PDX possessing the L858R/T790M double mutation. For this purpose, we injected IV NGs following what has been published for prostate tumour cell xenografts (Timaner et al., 2018; Toledano Furman et al., 2013). Monitoring of the mice with IVIS spectrum allowed us to visualize intravital distribution of red fluorescence associated with NGs. Immediately after injection, we observed an accumulation of fluorescence in the area where the liver is located, then in the following days, even if the fluorescence remains mainly in the liver area, we observed the appearance of a fluorescent area at the level of the PDX graft site. This fluorescence is not found on the contralateral paw, so the fluorescence is well associated with the tumour. Quantification of this fluorescence indeed confirms the presence of NG in the liver and in the tumour. However, intravital microscopy has limitations. First of all, the measurement of fluorescence does not consider the depth of the organs. The fluorescence associated with the liver is therefore underestimated compared to that of the subcutaneous tumour. Moreover, the observed fluorescence is not precisely localized and does not allow us to discriminate the fluorescence directly associated with the tumour from that of the adjacent tissue.

To overcome these biases, we have recovered the filtering organs (liver, kidney, spleen, lungs) that we have imaged with the IVIS spectrum. We find the fluorescence associated with the liver as in the study on whole mice, as well as the fluorescence, weaker, associated with the tumour but we also visualize a fluorescence similar to that of the tumour in the kidney, the spleen and the lungs of the mouse. We do not reproduce the results published in prostate cancer, showing a specific addressing of NG to the tumour and their absence from the filtering organs (Timaner et al., 2018; Toledano Furman et al., 2013). One of the hypotheses to explain this would be that there would be fewer molecules on the surface of the PDX used to capture the NGs or that their capacity for endocytosis would be diminished compared to the PC3 cells that were used in xenotransplantation in the existing data (Timaner et al., 2018; Toledano Furman et al., 2013). It is envisaged to reproduce these migration experiments in other *EGFR*-mutated NSCLC PDX models being characterized in the laboratory. Another hypothesis is that the lipid markers used to visualize NGs would alter the bio distribution of NGs. To overcome this, it would be possible to use radiolabelled NGs as it has been done (Lupu-Haber et al., 2019), or to use unlabelled NGs and to detect them in immunohistology and flow cytometry with a label of human CD90, which is a marker of MSCs found on the surface of NGs (Toledano Furman et al., 2013).

A proteomic study of NG surface molecules and PDX cells would allow us to determine which proteins might be involved in the NG uptake process. It has been shown that it is possible to genetically modify the membrane markers of MSCs which will then be found on the surface of NGs derived from these MSCs (Oieni et al., 2019). Even if this has not been done yet by transduction of MSCs with a vector allowing the expression of GFP, it is envisaged to use this technique to improve the uptake of NG by the tumour (Oieni et al., 2019). A comparative proteomic study in tumours and liver would allow the identification of tumour-specific markers that could potentially improve NG binding to the tumour

surface. Finally, a study showed that the culture of MSC in tumour cell conditioned medium or in a medium containing pro-inflammatory cytokines improved the uptake of NG by the tumour (Haber et al., 2005). We could try to improve the uptake of NGs by pre-treating the MSCs from which the NGs will be derived with conditioned medium of EGFR-mutated NSCLC tumour cells.

We then wanted to study whether Osimertinib treatment modified NG uptake by the tumour. We assessed NG uptake as in the previous experiment but after treating the mouse with Osimertinib. Quantification of fluorescence associated with each organ and tumour isolated from the mouse body yielded similar results to those obtained for mice not treated with Osimertinib. Osimertinib treatment does not alter the distribution of NGs in the tumour and filtering organs.

For these two *in vivo* experiments, we have favoured IV injection of NGs because this is the route of administration envisaged for the use of NGs in the clinic. However, the uptake of MSC by the tumour has been shown to be favoured by an IP injection (Toledano Furman et al., 2013). We have therefore, as in the previous experiment, studied the uptake of NG by tumours treated with Osimertinib but injecting the same quantity intraperitoneally. This did not change the fluorescence associated with NG found in the tumour but decreased the fluorescence in the liver. The differences between IP and IV injection have already been shown in other models of nanovesicles and are attributed to the retention of the vesicles in the peritoneum and their prolonged diffusion in the blood (Dadashzadeh et al., 2010). The injection in IP allows to maintain the capture of NG by the tumour while limiting their capture by the liver.

In vivo experiments have shown that NGs are vesicles that can be captured by the tumour in our study model. This study must be validated and completed by experiments on other PDX models. The IV injection allows the capture of NG by the tumour but also by the filtering organs, in particular the liver, an organ very involved in blood purification and this capture by the liver can be decreased by an injection in IP.

The next step consisted in validating the nanoghosts as a therapeutic vector of the exoenzyme C3, the treatment that we wish to combine with Osimertinib to limit the emergence of resistance. For the PC9 cell line, preliminary results indicate that the combination of TatC3 and Osimertinib kills all tumour cells while for the HCC4006 cell line, the combination of TatC3 and Osimertinib increases cell death but does not eradicate all cells (unpublished data). This study suggests that the RHO GTPases RHOA, RHOB and/or RHOC are essential for the tolerance and resistance of the PC9 cell line under treatment, while for the HCC4006 cell line, this inhibition is not sufficient to fully prevent resistance to treatment. This could be explained by the heterogeneity of the tumour cells as well as the heterogeneity of the resistance mechanisms within the same tumour line. PC9 and HCC4006 cells resist EGFR-TKI treatment by EMT (Fustaino et al., 2017; Suda et al., 2011), a process strongly associated with cell cytoskeletal remodeling, and *preliminary data* from the lab showed that the establishment of stress fibers, reverted by treatment of the cells with siRHOB, was associated with DTC state preliminary to EMT process. *Preliminary data* from the laboratory also showed that TatC3 prevented the establishment of these stress fibers under EGFR-TKI. It is envisaged that under treatment with EGFR-TKI and TatC3, PC9 cells cannot survive and some cells of the HCC4006 cells resist by another mechanism of resistance, such as cell dedifferentiation associated with the acquisition of stem markers (Sharma et al., 2010).

The next step was to validate NG as a therapeutic vector capable of delivering C3-exoenzyme to tumour cells and to verify its efficacy in ADP-ribosylating RHOA, RHOB and RHOC (Aktories and Frevert, 1987; Quilliam et al., 1989). We were unable to demonstrate ADP-ribosylation of RHOA in both the TatC3 positive treatment control and the NG-C3. However, we did demonstrate ADP-ribosylation

of RHOB and RHOC when the cells were in the presence of TatC3 or NG-C3 but not when the C3-exoenzyme was alone. The detection of those of RHOB and RHOC is sufficient to conclude on the efficiency of NG-C3. C3, in the same way as TatC3, allows the delivery of a C3-exoenzyme into tumour cells.

Thus, the work of this thesis has shown that *EGFR*-mutated NSCLC tumour cells are permeable to NGs and that *in vivo*, NGs are found in the tumour as well as in the filtering organs. C3-loaded NGs allow the delivery of active C3 to tumour cells. The next step would be to study in the long term whether C3-loaded NGs are able to recapitulate the effect of TatC3 in combination with EGFR-TKIs. We also want to study *in vivo* the efficacy of C3-loaded NGs on the response to EGFR-TKIs on cell death and relapse. Due to the addressing of NGs to the liver, a study of the cytotoxicity associated with TatC3 is essential, in particular by injecting NG-C3 alone into mice and evaluating the toxicity. We also propose to use NGs loaded with nanobodies, which have been developed in the laboratory, which specifically target GTP-Bound Conformation (Bery et al., 2019). This would allow us to evaluate the encapsulation capacity of nanobodies in NGs, as this has never been done before.

It would also be interesting to compare this NGs to other existing nanotechnologies. In particular, we could consider comparing in our oncogenic context the efficacy of NGs versus liposomes (Toledano Furman et al., 2013), as it has already been done in a prostate cancer model or exosomes derived from MSCs, used as drug delivery system (Weng et al., 2021). We could also compare NGs derived from MSCs to NGs derived from other cell types such as monocytes (Krishnamurthy et al., 2016).

In the long term, the goal would be to use patients' own NGs, but NGs are derived from BM-MSCs, which are mostly obtained from femoral heads. However, this is not a readily available tissue, especially for clinical use. It is possible to obtain NGs from MSCs derived from other tissues, notably umbilical cord (Kluth et al., 2012). This tissue is easily harvested and frozen, as is already done in some countries. Thus, a study of the use of NGs derived from umbilical cord MSCs could be interesting for personalized medicine.

The best mode of administration is also to be evaluated. For the moment, the least restrictive route of administration for the patient is IV injection. However, in the context of lung cancer, it would be possible to test the bio-distribution of NGs after inhalation, as has been tested for COVID-19 vaccines. This would allow a local diffusion of NGs by limiting its capture by the filtering organs (Wu et al., 2021).

In conclusion, this thesis works did not allow us to conclude on the effect of MSCs on the proliferation of *EGFR*-mutated NSCLC cells. However, we have shown the tropism of MSCs for tumour cells *in vitro* and *in vivo* and the potentiation of this tropism when tumour cells are treated with EGFR-TKI *in vitro*. The treatment condition that attracted MSCs the most was the DTC state. These results encouraged us to use MSC-derived NGs to target tumour cells tolerant to EGFR-TKI treatment.

In a second step, we demonstrated *in vitro* and *in vivo* the internalization capacity of NGs by tumour cells and evaluated their bio distribution in filtering organs in EGFR-TKI treated and untreated mouse models. *In vitro*, we showed that the internalization of NGs was maximal by tumour cells at the DTC and DTEC state, which validates *in vitro* the relevance of this therapeutic vector to target EGFR-TKI resistant cells.

Finally, we validated the NG-C3 as being able to deliver an active C3-exoenzyme to tumour cells *in vitro*. These results encourage the study of the effect of NG-C3 on cell survival under EGFR-TKI *in vitro* and *in vivo*.

Conclusion

The laboratory has identified the small GTPase RHOB as a predictive biomarker of response in *EGFR*-mutated NSCLC patients treated with EGFR-TKIs. However, there is no therapy yet available that specifically targets RHOB. We have identified TatC3, an inhibitor of RHOA, RHOB and RHOC, as preventing resistance to EGFR-TKIs. To overcome its systemic toxicity, we used MSC-derived NGs to specifically target tumour cells. This work allowed us to show (i) the absence of effect of MSCs on the proliferation of tumour cells or on their response to EGFR-TKIs (ii) the tropism of MSCs for tumour cells and their stronger tropism for cells treated with EGFR-TKIs and (iii) the relevance of NGs to deliver an active C3-exoenzyme and to specifically target EGFR-TKI resistant tumour cells.

These results encourage further investigation of the involvement of MSCs in the tumour process of *EGFR*-mutated NSCLC and a study of the effect of NG-C3 on the response of tumour cells to EGFR-TKI.

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Résumé : Les inhibiteurs de tyrosine kinase de l'EGFR (EGFR-ITK) sont des thérapies efficaces pour les cancers du poumon non à petites cellules présentant de mutation de l'EGFR (CPNPC EGFR-muté) mais la majorité des patients rechutent à cause de l'apparition de mécanismes de résistance. Une des principales hypothèses suggère que les résistances pourraient se développer à partir d'une population de cellules tolérantes au traitement (ou DTC pour Drug Tolerant Cells) qui développerait des mécanismes de résistance *de novo*. Des résultats de l'équipe ont montré qu'une forte expression de la petite GTPase RHOB dans les tumeurs prédispose à la rechute des patients traités par EGFR-ITK. L'expression de RHOB est augmentée dans les DTC en réponse aux EGFR-ITK et son invalidation génique par siARN augmente fortement la sensibilité aux EGFR-ITK *in vitro* et *in vivo*. Cependant, il n'existe à ce jour aucun inhibiteur spécifique de cette GTPase utilisable en clinique. Néanmoins, il existe des stratégies pour inhiber les Rho-GTPases *in vitro*, et l'une d'entre elles est l'utilisation de l'exoenzyme-C3 (C3) de Clostridium Botulinum, inhibiteur spécifique de RHOA, RHOB et RHOC par ADP-ribosylation. Nous avons montré qu'un co-traitement EGFR-TKI et C3 fusionnée à un peptide perméant (tat) était capable de prévenir l'émergence de résistances aux EGFR-ITK dans les lignées cellulaires testées (PC9, HCC4006 et HCC827), induisant la mort de toute la population de DTC. Cependant, l'utilisation de la tat-C3 n'étant pas envisageable pour un traitement systémique chez les patients de par la toxicité qu'elle provoquerait. Nous avons cherché une solution pour adresser spécifiquement la C3 aux tumeurs et permettre ainsi son utilisation thérapeutique. Nous avons établi une collaboration avec l'équipe du Pr. Marcelle Machluf (Technion, Israël) qui développe des vésicules dérivées de cellules souches mésenchymateuses (CSM), les nanoghosts (NG). Afin de valider une potentielle utilisation des NG en tant que véhicule pour la C3, des expériences de migration ont montré une attractivité des CSM dont sont issues les NG pour nos différentes lignées tumorales, indépendamment de la présence d'EGFR-ITK. Nous avons ensuite utilisé dans NG fluorescents qui nous ont permis de montrer une entrée active des NG dans nos modèles cellulaires, indépendamment de la présence d'EGFR-ITK. Nous avons également validé *in vivo*, dans des souris greffées de PDX de CPNPC EGFR-muté, l'arrivée des NG au niveau du site tumoral. Finalement nous avons également montré que la C3 encapsulée dans les NG entre dans les cellules et est active de par sa capacité à ADP-ribosyler les Rho-GTPases.

Mots clés : cellules souches mésenchymateuses, inhibiteurs de tyrosine kinase, cancer du poumon, résistance thérapeutique, C3-exoenzyme, nanoghosts

Abstract: EGFR tyrosine kinase inhibitors (EGFR-ITKs) are effective therapies for EGFR-mutated non-small cell lung cancer (EGFR-mutated NSCLC), but the majority of patients relapse due to the development of resistance mechanisms. One of the main hypotheses suggests that resistance may develop from a population of Drug Tolerant Cells (DTCs) that develop *de novo* resistance mechanisms. Results from the team showed that high expression of the small GTPase RHOB in tumours predisposes patients treated with EGFR-ITK to relapse. RHOB expression is increased in DTC in response to EGFR-ITK and its gene inactivation by siRNA strongly increases sensitivity to EGFR-ITK *in vitro* and *in vivo*. However, to date, there is no specific inhibitor of this GTPase that can be used in the clinic. Nevertheless, there are strategies to inhibit Rho-GTPases *in vitro*, and one of them is the use of exoenzyme-C3 (C3) from Clostridium Botulinum, a specific inhibitor of RHOA, RHOB and RHOC by ADP-ribosylation. We showed that co-treatment of EGFR-TKI and C3 fused to a permeant peptide (tat) was able to prevent the emergence of EGFR-ITK resistance in the tested cell lines (PC9, HCC4006 and HCC827), inducing the death of the entire DTC population. However, the use of tat-C3 is not feasible for systemic treatment in patients due to the toxicity it would cause. We sought a solution to specifically address C3 to tumours and thus allow its therapeutic use. We have established a collaboration with the team of Prof. Marcelle Machluf (Technion, Israel) who is developing vesicles derived from mesenchymal stem cells (MSC), the nanoghosts (NG). In order to validate a potential use of NG as a vehicle for C3, migration experiments showed an attractiveness of the MSCs from which NG are derived for our different tumour cell lines, independently of the presence of EGFR-ITK. We then used fluorescent NGs which allowed us to show an active entry of NGs in our cell models, independently of the presence of EGFR-ITK. We also validated *in vivo*, in EGFR-mutated NSCLC PDX transplanted mice, NG entry at the tumour site. Finally, we also showed that C3 encapsulated in NGs enters the cells and is active through its ability to ADP-ribosylate Rho-GTPases.

Key words: mesenchymal stem cells, tyrosine kinase inhibitors, lung cancer, therapeutic resistance, C3-exoenzyme, nanoghosts