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# NON-VIRAL VECTORIZATION OF THERAPEUTIC MOLECULES FOR LUNG CANCER THERAPY

Erh-Hsuan Lin

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UNIVERSITE JOSEPH FOURIER GRENOBLE 1

# THÈSE

Pour le grade de  
Docteur de l'Université Joseph Fourier

Discipline : **BIOLOGIE**

Présentée et soutenue publiquement par

**Erh-Hsuan LIN**

le 9 Octobre 2008

## **VECTORISATION NON-VIRALE DE MOLECULES THERAPEUTIQUES POUR LA THERAPIE DES CANCERS DU POUMON**

Equipe Cibles Diagnostiques ou Thérapeutiques et Vectorisation de  
Drogues dans le Cancer du Poumon

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## Résumé de la Thèse

L'utilisation de la thérapie génique du cancer est limitée actuellement par la faible efficacité de transfection, la durée d'expression du gène et la toxicité des vecteurs. Ces difficultés ont guidé l'orientation de mes travaux dans 3 directions:

1/ Utilisation de gènes codant pour des glycoprotéines fusogéniques (FMG) comme gènes suicides à fort effet bystander.

2/ La vectorisation de siRNA *in vivo* par le vecteur polycationiques : polyéthylèneimine (PEI).

3/ La stabilisation de l'expression du transgène à long terme *in vivo* à l'aide du transposon *Sleeping Beauty* (SB).

Les résultats de ces travaux montrent que :

1/ La thérapie génique basée sur l'utilisation de FMG montre un fort intérêt thérapeutique sur des cellules de cancer du poumon humain *in vitro* et *in vivo*. En effet, ces protéines FMG ont i/ un fort effet cytotoxique qui passe essentiellement par la fusion entre la cellule transfectée et de nombreuses cellules voisines non-transfectées, ii/ la capacité d'induire une immunité antitumorale induite par la libération des vésicules immunogènes au cours de la mort des cellules fusionnées. Trois FMG ont été testées: GALV, HERV-W et RD. Dans les 3 cas nous avons montré que la transfection de ~1% des cellules *in vitro* conduit à la formation de large syncytia et à la mort de 25 à 80% des cellules en culture en moins de 5 jours. Le traitement des tumeurs sous-cutanées implantées chez des souris nues induit une réduction du poids des tumeurs pouvant aller jusqu'à 70% alors que l'efficacité de transfection par injection directe des plasmides dans la tumeur est extrêmement faible ( $\leq 1\%$ ). Ces résultats démontrent que ces protéines FMG possèdent un potentiel intéressant pour la thérapie génique du cancer. Néanmoins, notre modèle de souris immunodéficient ne nous a pas permis de mesurer l'impact supplémentaire que nous pouvions attendre de la stimulation de la réponse antitumorale activée par la production de syncytiosomes. Cette étude est encore en cours.

2/ La vectorisation de polyplexes PEI/siRNA *in vivo* par voie intraveineuse, intrapéritonéale ou sous-cutané avec différentes formulations a montré des résultats faiblement positifs au mieux et souvent peu reproductibles. Nous avons étudié la biodistribution en temps réel de ces complexes en imagerie de fluorescence et mesuré leur capacité à inhiber l'expression d'un gène reporter ou d'un oncogène dans les poumons et/ou les tumeurs des souris. Globalement ces résultats démontrent que le PEI n'est pas un vecteur efficace pour les siRNA dans une approche systémique et que des modifications chimiques sur le PEI et/ou les siRNA devront être envisagées pour augmenter la stabilité et la performance de ces particules.

3/ L'insertion du transposon SB dans le plasmide vectorisé, complexé à du PEI et injecté en intraveineux, permet de stabiliser l'expression du transgène pendant plus de 4 mois dans les poumons. La mesure en cinétique à long terme du gène reporter dans les poumons montre en effet une forte expression du gène reporter codant pour la luciférase 1 jour après la transfection. Cette expression disparaît rapidement durant les 2 semaines suivantes jusqu'à devenir indétectable. De façon intéressante, le signal luciférase se rétablit ensuite progressivement pour atteindre un plateau 2 mois après la transfection. Le niveau d'intensité du signal de luciférase est alors d'environ 15% de celui mesuré le premier jour. Ces résultats suggèrent que le transposon SB permet une insertion stable du transgène dans un nombre très restreint de cellules pulmonaires ayant la capacité de se multiplier. Ce résultat est prometteur et offrira une plate-forme d'intérêt qui permettra de vectoriser des gènes codant pour des protéines biologiquement actives, telles que celle codée par le gène CFTR (cystic fibrosis transmembrane conductance regulator) pour la thérapie de la mucoviscidose, ou le gène K-Ras pour l'analyse de l'oncogénèse ras-dépendante dans le cancer du poumon. Enfin, les cellules touchées par l'insertion stable du transposon ayant un pouvoir de régénération du poumon important, il semble que nous ayons un moyen de modifier génétiquement des cellules souches pulmonaires. Nous souhaitons donc maintenant les caractériser précisément car cela ouvre des perspectives thérapeutiques importantes.



## Summary of the Thesis

Current cancer gene therapy protocols are strongly limited by several factors such as the low transduction efficiency, transient gene expression, or toxicity of the vector. To approach these problems, my work was concentrated on the non-viral delivery of biological molecules for the treatment of lung cancer with 3 main aspects:

1/ Study of the antitumoral effect mediated by several viral fusogenic membrane glycoproteins (FMG) gene transfer.

2/ Vectorization of siRNAs *in vivo* by the cationic polymer : polyethylenimine (PEI).

3/ Long-term expression of the transgene *in vivo* by non-viral delivery of the DNA vector containing *Sleeping-Beauty* (SB) transposon.

Our results showed that :

1/ The FMG-based gene therapy was found to produce a highly efficient antitumor effect toward human lung cancer cells *in vitro* and *in vivo*. FMG expression is known to a/ induce the fusion of a single transfected cell to multiple neighboring untransfected cells, leading to the formation of large syncytia committed to death in 5 days b/ to induce a specific antitumor T-cell immunity in the host, through the release of immunogenic vesicles during the death of the syncytia. These 2 properties are cumulative and participate in the very strong bystander effect related to the use of FMG. Using FMGs of different origins (GALV, HERVW and RD) *in vitro*, we showed that the transfection of ~1% cells led to the death of up to 80% of the cultured cells. *In vivo*, treatment of human xenografts of lung cancer in nude mice by direct repeated intratumoral injections of the naked plasmids encoding these FMG showed a 60-70% reduction in tumor weight. This antitumor effect is thus very strong, especially in regard to the very poor efficiency of the transfection method ( $\leq 1\%$  tumor cells are transfected). Furthermore, these results were obtained in immunodeficient mice. It is thus reasonable to assume that this FMG-based cancer therapy will be even more interesting in an immuno-competent animal. This study is currently going on in the laboratory.

2/ *In vivo* delivery of several formulations of PEI/siRNA polyplexes using the intravenous, intraperitoneal or subcutaneous administration routes showed partially positive, but usually transient and weak silencing effects against target genes (reporter gene or oncogene) in mouse lung or tumor xenografts. These results combined to the studies that we performed to measure the biodistribution of these complexes using *in vivo* fluorescent imaging, confirmed that the PEI is not as adapted for the delivery of siRNA as it is for plasmids. This suggests that additional chemical modifications of the PEI or siRNA would be necessary to augment the stability of these complexes *in vivo*.

3/ The systemic administration in the tail vein of nude mice of PEI-complexed plasmids containing a luciferase reporter gene inserted in SB transposon showed that a strong luciferase expression can be detected in the lung of mice for more than 4 months. As usual, the luciferase signal was very strong 1 day after transfection in the lung but rapidly disappeared in the following 2 weeks. However, because of the presence of the SB transposon, this signal was then progressively restored in the lung of these animals and reached a plateau 2 months after transfection. At this step the intensity of the luciferase signal was stable and represented around 15% of its maximum value measured at day 1. This pattern suggested that a stable transfection of the SB transposon into a small population of cells capable of lung regeneration was obtained. This result is promising and provides a platform for the delivery of active genes, such as CFTR (cystic fibrosis transmembrane conductance regulator) gene for therapeutic purposes, or the K-Ras gene for studying the Ras-dependant oncogenesis of lung cancer. It is thus of great importance to further characterize the nature of the stably transfected cells, and this will open a new field of investigation in the laboratory.

## **List of abbreviations:**

AAV: Adeno-associated virus

AdV: adenoviral vector

AMLV: amphotropic murine leukemia virus

Ang: Angiopoietin

ASO: antisense oligonucleotide

BAC: bacterial artificial chromosome

BLI: Bioluminescence optical Imaging

CAR: coxsackie- and adenovirus- receptor

CDK: cyclin-dependent kinases

CF: Cystic fibrosis

c-FLIP: cellular FLICE-inhibitory protein

CFTR: Cystic fibrosis transmembrane conductance regulator

CNS: central nervous system

CPP: cell-penetrating peptide

CTL: cytotoxic T lymphocyte

DAP: death-associated proteins

DC: dendritic cell

DIABLO: direct IAP-binding protein with low pI, also known as SMAC

DISC: death-inducing signaling complex

ds: double-stranded

E(1): early region (1) of adenovirus genome

EBV: Epstein–Barr virus

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: EGF receptor

FADD: Fas-associated death domain

FasL: Fas ligand

FLICE: FADD-like IL-1 $\beta$  converting enzyme

FMG: fusogenic membrane glycoprotein

FRI: Fluorescence Reflectance Imaging

GALV: Gibbon Ape Leukemia Virus

GM-CSF: granulocyte-macrophage colony-stimulating factor

GMP: glomeruloid microvascular proliferation

GVHD: graft-versus-host disease

HAC: human artificial chromosome

HD: helper-dependent

HERV-W: Human Endogenous Retrovirus-W

HES: haematoxylin - eosin - saffron

HIF: hypoxia-inducible transcription factor

HIV: human immunodeficiency virus

HSV-TK: Herpes Simplex virus thymidine kinase

IAP: inhibitor of apoptosis protein

IE: immediate-early

IF: Immunofluorescent or Immunofluorescence

IFN- $\alpha$ : interferon- $\alpha$

IGF: insulin-like growth factor

IGF-IR: IGF-I receptor

IL: interleukin

IP: intraperitoneal(ly)

IP3: inositol-1,4,5-trisphosphate

IR/DR: inverted/directed repeat

IRES: internal ribosome entry site

ITR: inverted terminal repeat

IV: intravenous(ly)

LAT: latency-associated transcript

LNA: locked nucleic acids

LTR: long terminal repeat

luc: luciferase

mHag: minor histocompatibility antigen

MAPK: mitogen-activated protein kinase

MHC: major histocompatibility complex

MLV: murine leukemia virus

MMP: matrix metalloproteinase

MoMLV: Moloney murine leukemia virus

mTOR: mammalian target of rapamycin

MVD: microvessel density

NLS: nuclear localization signal

NSCLC: non-small cell lung cancer

ORF: open-reading frame

p70<sup>S6K</sup>: p70 ribosomal protein-6 kinase

PAMAM: polyamidoamine

PDGF: platelet-derived growth factor

PDGFR: PDGF receptor

PEG: polyethylene glycol

PEI: polyethylenimine

PI3K: phosphoinositide 3-kinase

PIP2: phosphatidylinositol4,5-bisphosphate

PIP3: phosphatidylinositol3,4,5-trisphosphate

PKB, C: protein kinase B, protein kinase C

PLC- $\gamma$ : phospholipase C- $\gamma$

PLL: Poly-L-lysine

PMO: phosphorodiamidate morpholino oligomers

PNA: peptide nucleic acids

PTD: protein transduction domain

PTEN: phosphatase and tensin homolog

Rb: Retinoblastoma protein

RD: feline endogenous virus RD-114

RNAi: RNA interference

RT: room temperature

RTK: receptor tyrosine kinase

SB: Sleeping Beauty

SC: subcutaneous(ly)

SCID: severe combined immunodeficiency

SCLC: small cell lung cancer

SD: Standard Deviation

SEM: Standard Error of Mean

SH-2: Src homology-2

SIN: self-inactivating

siRNA: Small (or short) interfering RNA

SMAC: second mitochondria-derived activator of caspase, also known as DIABLO

S/MAR: scaffold/matrix attachment region

Sos: Son of sevenless

ss: single-stranded

SSR: site-specific recombinase

SV40: simian virus 40

TAA: tumor-associated antigen

TCR: T cell receptor

TGF: transforming growth factor

TIL: tumor-infiltrating lymphocyte

TKI: tyrosine kinase inhibitor

TNF: tumor-necrosis factor

TRAIL: TNF-related apoptosis-inducing ligand

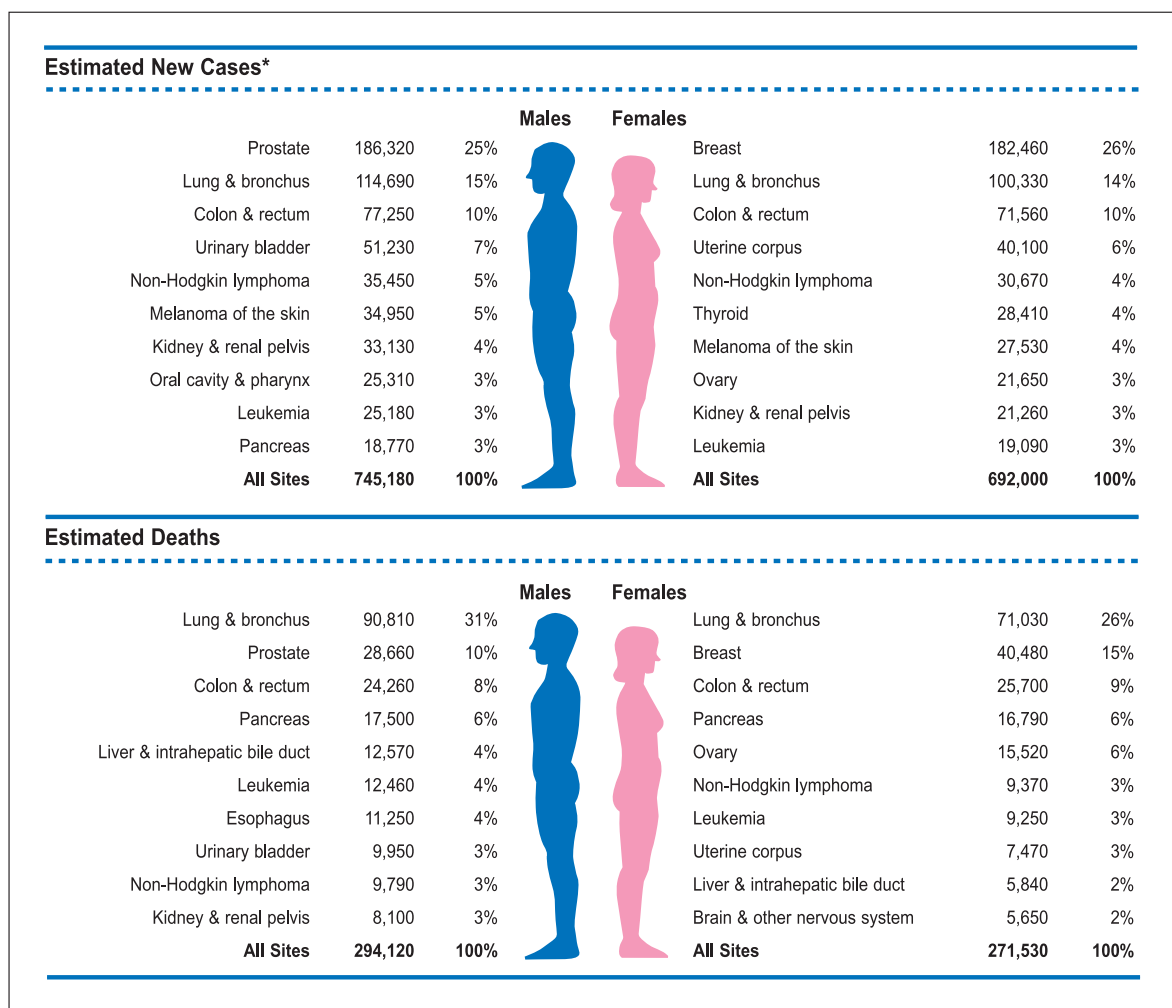
VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

wt: wild-type

YAC: yeast artificial chromosome

Figure 1. Estimated 10 leading types of new cancer cases and deaths by sex in US, 2008



\*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder. Estimates are rounded to the nearest 10. (Figure from Jemal et al., 2008)



# INTRODUCTION

## I. Lung Cancer and Gene Therapy

### I.1. Lung Cancer

Lung cancer is the leading type of cancer in term of incidence or mortality, in the whole world. Statistics obtained in the year of 2000 represented approximately 1.2 million cases diagnosed and 1.1 million deaths recorded worldwide (Parkin et al., 2001). In United States, although not being the commonest cancer type, lung cancer is by far the leading cause of cancer-related deaths in both sexes (Fig. 1), with an estimated mortality of more than 160,000 for 2008 (Jemal et al., 2008).

Lung cancer has a high morbidity because it is difficult to detect early and is frequently resistant to available chemotherapy and radiotherapy. Current standard therapies include surgical resection, platinum-based chemotherapy, and radiation therapy alone or in combination. However, lung cancer is still rarely cured, with an overall 5-year survival rate of 13% in 1975-1977 and 16% in 1996-2003 (Jemal et al., 2008). Large randomized clinical trials using the best available chemotherapy regimens have reported similar and limited activity, with 1 year survival rates of 31-36% and the overall median survival of only 8-11 months, suggesting that chemotherapy in lung cancer has reached a therapeutic plateau (Fossella et al., 2003; Scagliotti et al., 2002; Schiller et al., 2002). Developments of novel therapeutic strategies, such as gene therapy, with lower toxicity and better adapted to molecular phenotypes of lung cancer, as well as the more efficient early-detection methods are obviously necessary for this malignancy.

### **I.1a. Etiology**

The major risk factor for lung cancer is the exposure to tobacco smoke. Tobacco smoke contains more than 60 carcinogens, among which more than 20 are strongly associated with lung cancer development (Hecht, 2003). Other risk factors include occupational or environmental exposure to secondhand smoke, radon asbestos (particularly among smokers), certain metals (chromium, cadmium, arsenic), some organic chemicals, radiation, air pollution, and a history of tuberculosis (American Cancer Society, 2008).

Although it is generally accepted that smoking is strongly related to lung cancer and the risk increases with quantity of cigarette consumption and years of smoking duration, not everyone who smokes develops lung cancer. Epidemiological studies showed that smokers are 14 times more likely to develop lung cancer than nonsmokers, but only about 11% of heavy smokers develop lung cancer in their lifetime (Amos et al., 1999). Therefore, more common genetic variants or polymorphisms are hypothesized to affect lung cancer risk.

Inherited genetic susceptibility plays a contributing role in the development of lung cancer, especially in those who develop the disease at a younger age. Epidemiological studies showed a 2.5-fold increased risk attributable to the family history of lung cancer after controlling for tobacco smoke, suggesting that genetic factors other than those related to metabolizing carcinogens from tobacco smoke may influence a person's susceptibility to lung cancer (Amos et al., 1999). Acquired genomic instability (usually coincident with cigarette smoking) plays a significant role in lung cancer. It has been shown that most cancers are genetically unstable at two distinct levels: the instability observed at the nucleotide level that results in base substitutions, deletions or insertions of a few nucleotides; and that observed at the chromosome level that brings about losses and gains of whole or large portions of chromosomes (Lengauer et al., 1998). These chromosomal instability, loss of heterozygosity, mini- and microsatellite instabilities have been variously detected in lung tumor samples or plasma DNA of patients, providing the prospects in early detection and diagnosis by screening these altered DNA (Chen et al., 1996; Ninomiya et al, 2006; Sozzi et al., 1999; Wistuba et al., 2000).

All these instabilities finally lead to genetic or epigenetic abnormalities in “oncogenes”, “tumor-suppressor genes”, and the pathways they involve, which contribute to tumor initiation, growth, maintenance, and invasion (McCormick, 2001; Sato et al., 2007). Understanding these molecular events are major topics for the development of cancer gene therapy.

Table 1. 2004 WHO Classification of Lung Tumors

<b>Squamous cell carcinoma</b>	
Variants	
Papillary	
Clear cell	
Small cell	
Basaloid	
<b>Small cell carcinoma</b>	
Variant	
Combined small cell carcinoma	
<b>Adenocarcinoma</b>	
Adenocarcinoma, mixed subtype	
Acinar adenocarcinoma	
Papillary adenocarcinoma	
<b>Bronchioloalveolar carcinoma</b>	
Nonmucinous	
Mucinous	
Mixed nonmucinous and mucinous or indeterminate	
<b>Solid adenocarcinoma with mucin production</b>	
Variants	
Fetal adenocarcinoma	
Mucinous ("colloid") carcinoma	
Mucinous cystadenocarcinoma	
Signet ring adenocarcinoma	
Clear cell adenocarcinoma	
	<b>Large cell carcinoma</b>
	Variants
	Large cell neuroendocrine carcinoma
	Combined large cell neuroendocrine carcinoma
	Basaloid carcinoma
	Lymphoepithelioma-like carcinoma
	Clear cell carcinoma
	Large cell carcinoma with rhabdoid phenotype
	<b>Adenosquamous carcinoma</b>
	<b>Sarcomatoid carcinoma</b>
	Pleomorphic carcinoma
	Spindle cell carcinoma
	Giant cell carcinoma
	Carcinosarcoma
	Pulmonary blastoma
	<b>Carcinoid Tumor</b>
	Typical carcinoid
	Atypical carcinoid
	<b>Salivary Gland Tumors</b>
	Mucoepidermoid carcinoma
	Adenoid cystic carcinoma
	Epithelial-myoepithelial carcinoma

(Table from Beasley et al., 2005.)

### **I.1b. Classification**

From the clinical standpoint, lung cancers can be broadly divided into non-small cell lung cancer (NSCLC) and small cell carcinoma (SCLC) for treatment purposes, which account for about 80 and 20% of cases, respectively (Beasley et al., 2005). SCLC is neuroendocrine tumor, almost entirely related to smoking and is the most aggressive lung cancer type. Although SCLC is extremely sensitive to chemotherapy and radiotherapy, relapse is common and there has been almost no progress in survivorship since 1980s (Cooper and Spiro, 2006). NSCLCs traditionally include squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, but in the broadest sense may include any epithelial tumor that lacks a small cell component (Beasley et al., 2005). Adenocarcinoma is the most common NSCLC subtype observed, even in women or never smokers (Travis et al., 1995). NSCLCs are less sensitive to chemotherapy. Surgical resection remains the primary treatment modality for these tumors, whereas radiation therapy is preferred for locoregionally advanced ones (Yano et al., 2006).

A detailed histological classification of lung cancer (Tab. 1) was established by World Health Organization and International Association for the Study of Lung Cancer (WHO/IASLC, Histological Classification of Lung and Pleural Tumors). This classification system is primarily based on the histological characteristics of tumor sample seen in surgical or needle biopsy under normal light microscopy, simple and practical for all surgical laboratories. The systemic classification helps in tumor diagnosis and therapy, and provides a standard basis for epidemiological and clinical studies (Beasley et al., 2005; Travis et al., 1999).

Table 2. Examples of oncogenes and tumor-suppressor genes frequently observed to be altered in SCLC and NSCLC tumors.

Oncogenes	
Non-small cell lung cancer	Small cell lung cancer
K-Ras *	Raf
Raf	EGFR
EGFR *	Fms
HER-2	Rlf
IGF-1	Myc *
c-Fes	Myb
c-Sis	
c-Fur	
Myc	
Bcl-1, 2	

Tumor Suppressor Genes	
Non-small cell lung cancer	Small cell lung cancer
p53 *	p53 *
Rb	Rb *
p16 <sup>INK4a</sup>	p16 <sup>INK4a</sup>
p21 <sup>CIP1</sup>	
FHIT	

\* Most frequently mutated genes in lung tumor samples or cell lines evaluated.  
(Table according to Ferreira et al., 2002; Toloza et al., 2006)

## **I.2. Molecular Basis of Lung Cancer**

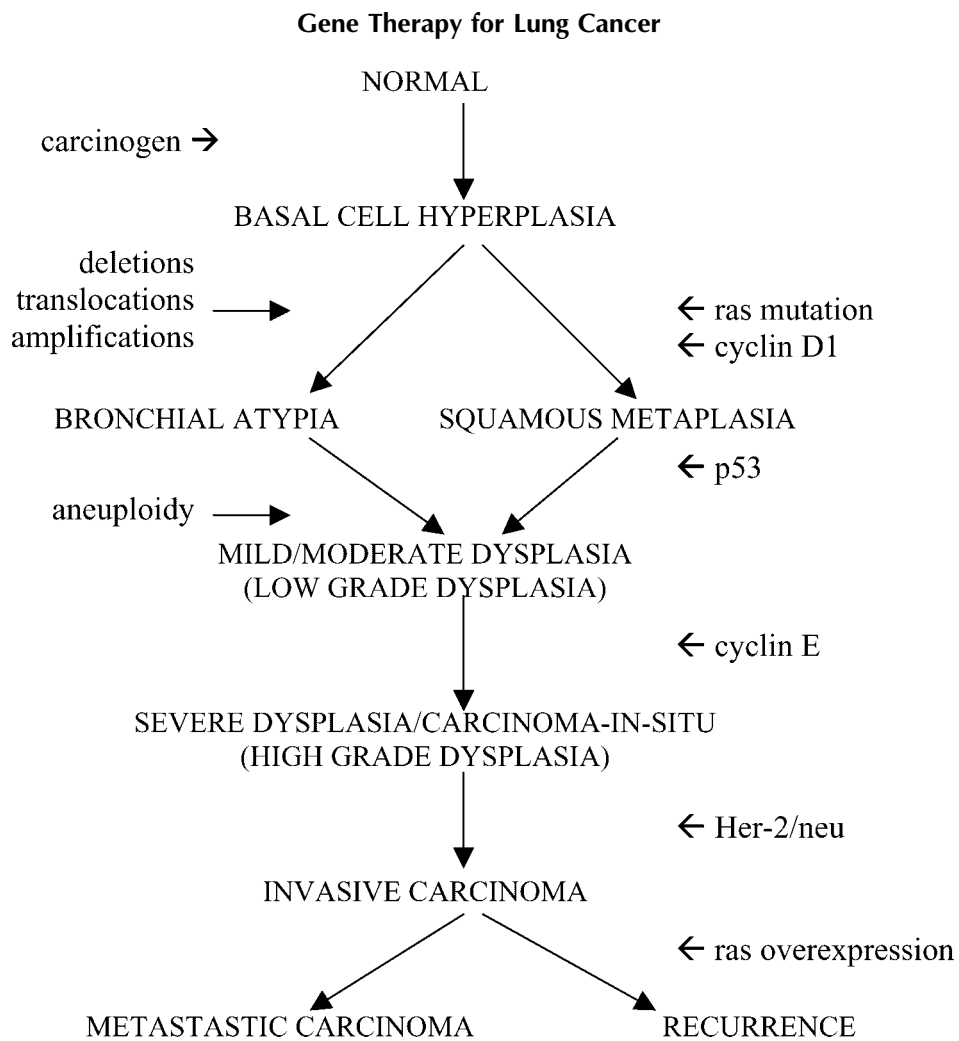
### **I.2a. Oncogenes and Tumor Suppressor-genes**

Molecular genetic studies of lung cancer have revealed that clinically evident lung cancers have multiple genetic and epigenetic abnormalities, such as DNA sequence alterations (mutation or chromosomal translocation), copy number changes, or aberrant promoter hypermethylation (Sato et al., 2007). These abnormalities result in the activation of oncogenes and inactivation of tumor-suppressor genes. Oncogenes are altered in ways that render the gene constitutively active or active under conditions in which the wild-type (wt) gene is not, potentiating a cell toward uncontrolled multiplication. Tumor-suppressor genes are targeted in the opposite way that the alterations reduce or abolish the activity of the gene product, predisposing a cell to oncogenesis (Vogelstein and Kinzler 2004). An activating somatic mutation in one allele of an oncogene is generally sufficient to confer a selective growth advantage to the cell; on the other hand, mutations in both alleles of a tumor-suppressor gene are generally required for a selective advantage to the cell in oncogenesis (Vogelstein and Kinzler 2004).

Oncogenes and tumor suppressor genes are key regulators of cell proliferation, differentiation, apoptosis, senescence or others. They are involved in important cellular pathways such as receptor tyrosine kinase (RTK), mitogen-activated protein kinase (MAPK), cell-cycle regulation or apoptosis pathways (Vogelstein and Kinzler 2004). Otherwise, a class of genes called stability or caretaker genes plays a role in preventing genomic instability and keeps genetic alterations to the minimum in a cell. Although not directly associated with cell proliferating, their dysfunctions lead to the higher rate of mutations in other genes and can accelerate the conversion in the neoplastic process (Friedberg, 2003; Vogelstein and Kinzler 2004).

In different cancer types, the pattern of mutated oncogenes and tumor suppressor genes is generally different. [Table 2](#) lists the genes frequently altered in SCLC and NSCLC tumor samples or cell lines in literatures. Most of them will be introduced in following chapters, according to the roles they play and the molecular pathways they belongs to.

Figure 2. Epidermoid bronchial multistep carcinogenesis.



(Figure from Toloza et al., 2000)

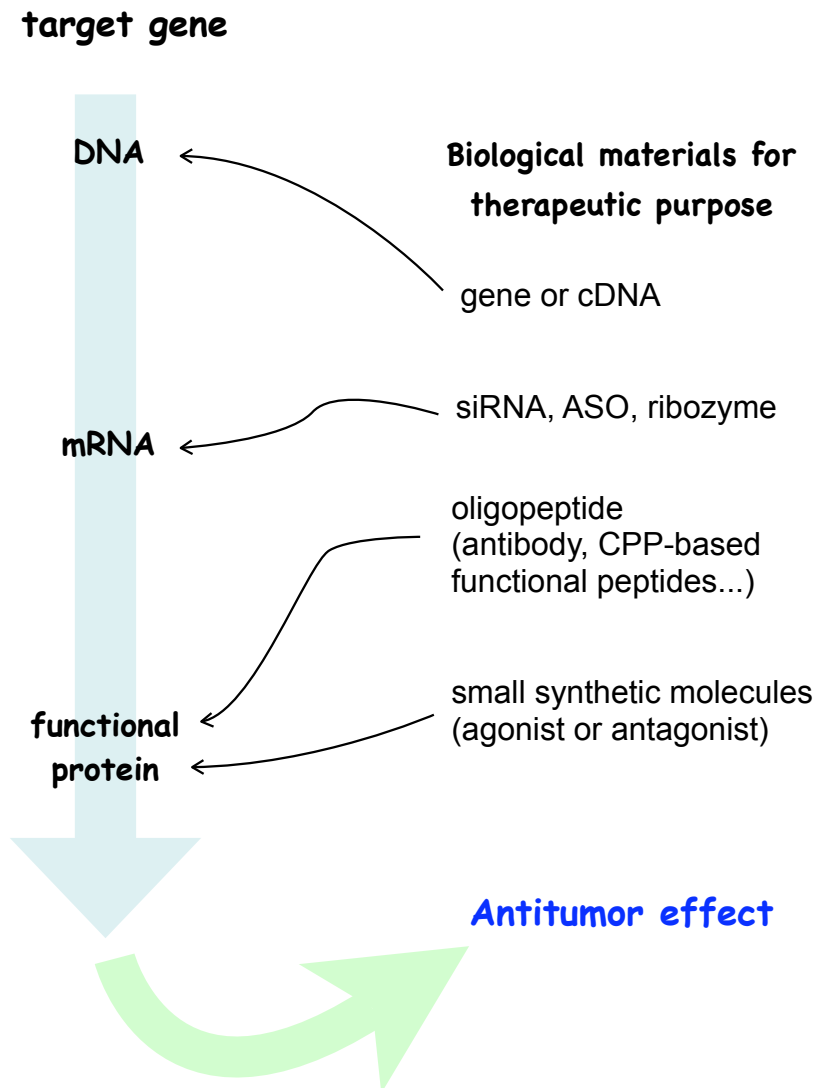


## **I.2b. Multistep Tumorigenesis**

Tumorigenesis is a “multistep” process, result of a series of genetic or epigenetic abnormalities, driving the progressive transformation of normal cells into highly malignant derivatives (Toloza et al., 2000). In humans, at least 4 to 6 mutations are required to reach malignant transformation, while fewer seem to be required in mice. (Hahn and Weinberg 2002). In lung cancer, it has been estimated that between 10 and 20 genetic alterations occur during tumorigenesis, including inactivation of 2 tumor suppressor genes (Ihde and Minna, 1991).

The study of colon carcinoma pathogenesis has well implicated at least 4-6 distinct histopathological stages in cancer development (Kinzler and Vogelstein, 1996). Although genetic biographies of other cancer types have not been described in comparable details, a model of epidermoid bronchial carcinogenesis was proposed in which it is suggested that this cancer progress histologically from normal to basal cell hyperplasia, stratification, regular metaplasia, then to mild, moderate, and sever atypical transitional and squamous dysplasia, and eventually to squamous cell carcinoma (Fig. 2) (Toloza et al., 2000).

Figure 3. Schematic diagram of therapeutic materials that can be applied to target a gene for the antitumor purpose.



The target gene can be a tumor-suppressor gene or oncogene for example, and various biological materials may be applied for a promoting or inhibiting effect that finally realizes the antitumor purpose (see the text for more detail description). ASO, antisense oligonucleotide; CPP, cell-penetrating peptide.

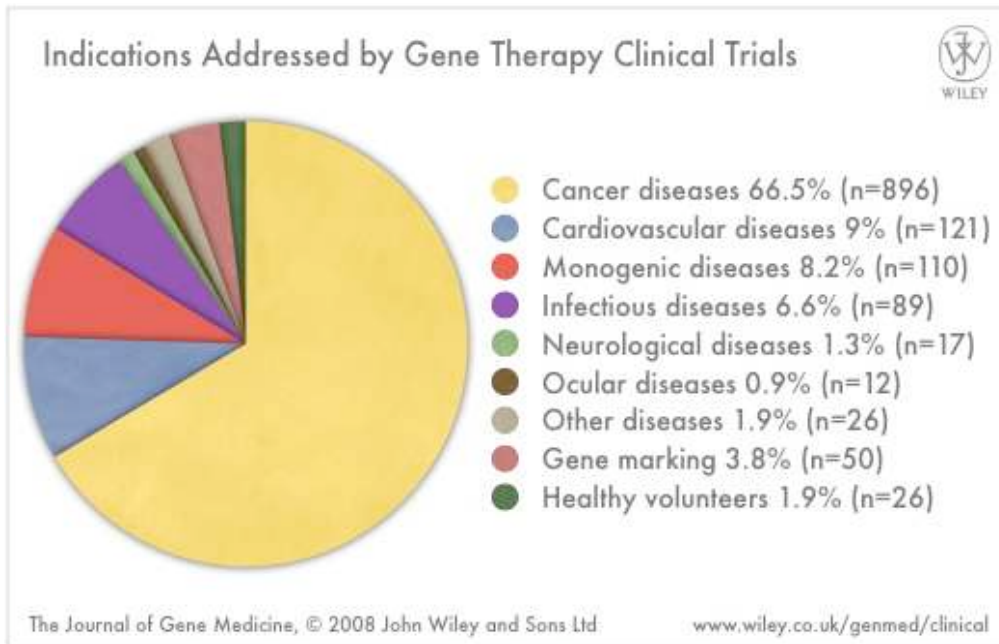
### **I.3. The Concept of Gene Therapy for Cancer**

Gene therapy has been previously described as the deliberate administration of genetic material to certain cells in a patient with the intention to correct a specific defect (Anderson, 1984; 1992). Gene therapy has been a promising approach for preventive and therapeutic purposes in a wide range of diseases including tumor, infectious, auto-immune, or hereditary diseases (Lundstrom and Boulikas 2003). Based on the growing understanding of molecular events in tumorigenesis, gene therapy has been applied to cancer treatments for destroying tumor cells or inhibiting their proliferation and spreading capacities (Boulaiz et al., 2005).

To design a successful gene therapy, a/ the gene (or pathway) to be targeted, b/ the genetic material to be used, and c/ the proper vector for delivery are critical factors to be considered. In some pathologies such as cystic fibrosis or severe combined immunodeficiency (SCID), the choice of gene is straightforward because the defective gene has been clearly identified. But in the case of cancer, it becomes much more complicated since tumorigenesis is a multistep and complex process, and no single gene defect is known to be sufficient to cause a malignancy.

Several DNA-based molecules can be used, according to the expected activity (Fig. 3). Introducing a cDNA (or gene) sequence to cells leads to the expression of the expected therapeutic protein. Delivery of the small interfering RNA (siRNA) or the antisense oligonucleotide (ASO) can block the translation of targeted mRNA and thus reduce the endogenous gene product level. Importantly, the delivery of these molecules requires the use of a vector, responsible for maintaining their stability and for crossing various barriers in a complex physiological environment (see the Thesis INTRODUCTION III. Vectorization).

Figure 4.



The distribution of gene therapy clinical trials in human diseases. (Figure from Wiley Database, 2008)

To date, clinical trials against cancer diseases account for more than 65% of gene therapy clinical trial cases (Fig. 4). It indicates that safe and efficient treatments against this malignant disease are still missing.

## **II. Current Lung Cancer Gene Therapy**

Despite of the complexity of tumorigenic pathways, malignant phenotypes observed in human cancers can be summarized as some abnormal capabilities that contribute to their immortalization, growth, and spread in the host (Hanahan and Weinberg, 2000). In the following sections, the molecular basis of these abnormalities and corresponding gene therapy strategies in lung cancer will be introduced.

## II.1. Growth Signaling

Cells receive information from the microenvironment that affects their growth, motility, differentiation, and death. Signals can be generated from direct cell-to-cell or cell-extra cellular matrix (ECM) interactions, or through soluble factors (e.g. hormone, cytokines). A number of different types of cell surface receptor subsequently activate the intracellular signaling pathways for responding. Among these pathways, cell growth-related signaling are generally modified in cancer cells (Heldin, 2001).

### II.1a. Growth factors and receptors

Cell membrane receptors can be classified into distinct families according to their ligands, biological response, or primary structures. Many growth factor receptors contain intrinsic tyrosine-kinase domain, and they belong to the receptor tyrosine kinase (RTK) family (Fantl et al., 1993; Heldin, 2001). Ligand binding induces RTKs' homodimerization and autophosphorylation of specific intracellular residues. They then become docking sites for proteins containing Src homology-2 (SH-2) domains (Reinmuth et al., 2004). This process then initiates a variety of signaling cascades toward the cytosol and nucleus (see also the following **II.1b.**).

In lung cancer, it is suggested that the ErbB receptor family plays an important role in tumor development and progression. This family includes the epidermal growth factor receptor (EGFR, also known as ErbB-1), HER-2 (also known as *neu* or ErbB-2), ErbB-3 and ErbB-4. They respond to several well-known growth ligands such as the epidermal growth factor (EGF) and transforming growth factor- $\alpha$  (TGF- $\alpha$ ), which act as potent mitogens for epithelial cell types including lung (Ferreira et al., 2002). Overexpression of EGFR is frequent in NSCLC (50-80%), and it is correlated with lymph node metastasis, more advanced stage and bad prognosis (Fujino et al., 1996; Ohsaki et al., 2000; Salomon et al., 1995). Although the rate of HER-2 overexpression is lower in lung cancer (about 15-30%), it is frequently seen in adenocarcinoma subtype and is associated with worse prognosis (Ferreira et al., 2002).

Other types of growth factor receptor found to be associated with lung cancer

include insulin-like growth factor (IGF)-I receptor (IGF-IR), platelet-derived growth factor (PDGF) receptor (PDGFR), and integrins (for review, see Reinmuth et al., 2004).

Pharmacological approaches against cancer growth factor/receptor activities can be divided into 2 orientations: 1/ one is the use of monoclonal antibodies, immunotoxins or ligand-binding agents that block the extracellular part of receptor; 2/ the other is the development of small molecule tyrosine kinase inhibitors (TKIs) that interfere with intracellular kinase activity of the receptor (Raymond et al., 2000).

1/ For example, the development of Cetuximab (C225), a monoclonal antibody targeting EGFR extracellular domain, has progressed to clinical trial. Phase II/III studies showed Cetuximab as a promising agent in conjunction with existing therapies for the treatment of a spectrum of solid tumors including NSCLC (Harding and Burtness, 2005). Trastuzumab is a monoclonal antibody against HER-2; but it did not present a significant clinical activity against NSCLC in the recent phase II trial (Clamon et al., 2005; Gatzemeier et al., 2004).

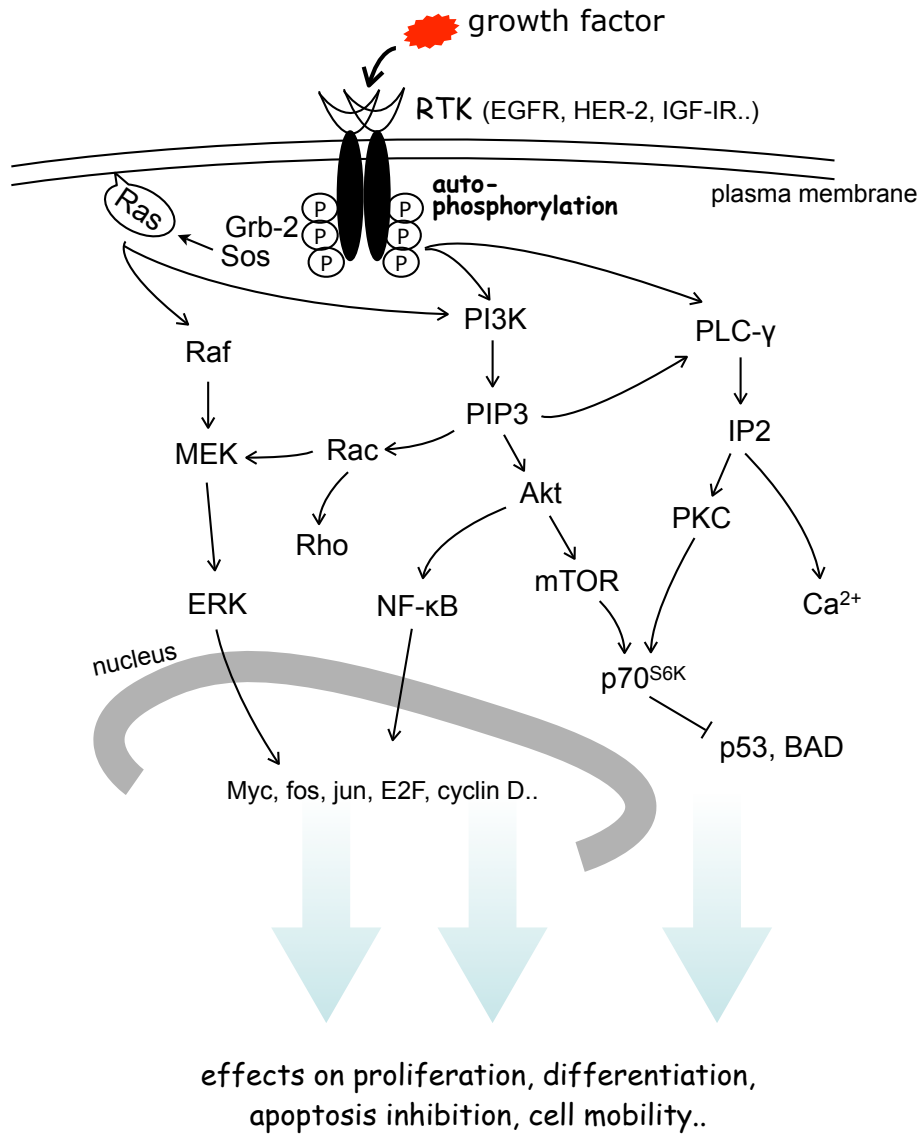
2/ TKIs targeting EGFR such as gefitinib (ZD1839, Iressa) and erlotinib have potent anti-tumoral activity in phase I clinical trials for NSCLC treatment after previous chemotherapy (Hidalgo et al., 2001; Ranson et al., 2002), but gefitinib failed to improve the overall survival benefit in later phase II/III trials in an unselected populations (Giaccone et al., 2004; Jubelirer et al., 2006; Kris et al., 2003; Thatcher et al., 2005). Clinical characteristics associated with good response to EGFR inhibitors include Asian origin, females, non-smokers, and adenocarcinoma histology. Gefitinib is currently marketed in several countries in eastern Asia but not available in United States or European Union. Unlike gefitinib, erlotinib showed prolonged survival benefits in NSCLC patients in phase II/III trials (Perez-Soler et al., 2004; Shepherd et al., 2005). Increased responses were significantly associated with adenocarcinoma, never smokers, and EGFR expression, but the significant survival advantage was observed in all patient subgroups. A later phase III trial also confirmed that erlotinib improved not only the survival in NSCLC patients who had progressed after prior chemotherapy, but also the tumor-related symptoms and



important aspects of quality of life (QOL) (Bezjak et al., 2006). Based on these results, erlotinib was approved by EMEA (European Medicines Evaluation Agency) in 2005 as second- and third- line treatment agent for chemotherapy-resistant, advanced NSCLC. However, results of a phase III trial still suggested that the addition of erlotinib to carboplatin and paclitaxel prolonged survival only in the subgroup of NSCLC patients who had never smoked (Herbst et al., 2005). Otherwise, somatic mutations in the EGFR gene have been found associated with response to EGFR-TKI (Lynch et al., 2004). These mutations activate the EGFR TK and are mainly associated with adenocarcinoma, never-smokers, female gender, and asian ascent (Sharma et al., 2007).

Inhibiting the expression of these growth factors/receptors is also a feasible way. SiRNA-induced EGFR silencing in several types, including head and neck squamous cell carcinoma, NSCLC and glioma *in vitro* and *in vivo*, elicited growth inhibition effects and made them more sensitive to chemotherapy drugs such as cisplatin (Zhang et al., 2005; Kang et al., 2006; Nozawa et al., 2006). Single mutant EGFRs can be frequently found in NSCLC patients (especially those with dramatic clinical response or resistance to TKIs treatments), and siRNA is able to target specifically these mutants (but not wt) and induce extensive apoptosis (Sordella et al., 2004). SiRNA or ASO may be seen as highly specific anticancer drugs if successfully delivered *in vivo*.

Figure 5. A simplified diagram of RTK associated intracellular signaling.



(Figure according to Adjei and Hidalgo, 2005; Heldin, 2001; Reinmuth et al., 2004)

## II.1b. Intermediate signaling molecules

Signal transduction is the process by which, information from a stimulus outside the cell is transmitted into the cell and stimulates a cellular response. This process involves a series of interactions between different signaling molecules, in which the general event is the alteration of phosphorylation state of tyrosine, serine, or threonine residues in special domains of targeted molecules. In principle, 2 different kinds of intracellular signaling molecules exist. One includes those with enzymatic activities such as protein kinases or lipid kinases; the other acts as adaptors that bring other signaling molecules together (for review, see Bode and Dong, 2005; Heldin, 2001).

### Ras-MAPK pathway

Among a number of signal transduction pathways known to date, an important and extensively studied one is the Ras-MAPK (mitogen-activated protein kinase) pathway. It is primarily activated in response to extracellular growth factors and the RTK signaling, as described above. Phosphorylated tyrosine residues of receptors become docking sites of the adaptor molecule Grb-2 through its SH-2 domains. By association with Grb-2, the nucleotide exchange molecule Sos (Son of sevenless) can be brought to its substrate Ras, which is a GTP/GDP binding protein residing at inner leaflet of cell membrane via post-translational farnesylation. Ras is activated by binding to GTP (the process catalysed by Sos) and then recruits and phosphorylates the serine/threonine protein kinase Raf at cell membrane. Activated Raf subsequently activates MEKs (MAPK/ERK kinases), which are dual-specificity kinases and can phosphorylate both serine/threonine and tyrosine residues of ERKs (extracellular signal-related kinase). Activated ERKs in turn regulate a diverse array of transcription factors such as *fos*, *jun*, AP-1, *myc*, as well as the cell cycle regulators cyclins D and E. Depending on the cellular context, these signals result in different cellular responses such as cell survival, proliferation, differentiation, or cytoskeletal rearrangements (Fig. 5) (for review, see Heldin, 2001; Reinmuth et al., 2004; Adjei and Hidalgo, 2005).

### Other signaling pathways

There are other Ras-independent pathways mediated by RTK signaling such as phosphatidylinositol 3'-kinase (PI3K) and phospholipase C- $\gamma$  (PLC- $\gamma$ ) pathways. Since PI3K and PLC- $\gamma$  are SH-2 domain containing molecules, they can bind to phosphorylated receptor by themselves and become activated. Activated PI3K phosphorylates and converts PIP2 (phosphatidylinositol-4,5-bisphosphate) to PIP3 (phosphatidylinositol-3,4,5-trisphosphate), which can then activate several serine/threonine kinases such as Akt (also known as protein kinase B, PKB), and the GTP-binding proteins such as Rac, Rho and Cdc-42. PTEN (Phosphatase and tensin homolog) works to dephosphorylate PIP3, acting as a negative regulator on PI3K signaling.

Activated PLC- $\gamma$  uses also PIP2 as substrate, but releases the products diacylglycerol and inositol-1,4,5-trisphosphate (IP3), which in turn activate the members of protein kinase C (PKC) family and mobilize  $\text{Ca}^{2+}$  ions from intracellular stores. These pathways contribute to cellular responses of growth, proliferation, differentiation, adhesion, and cell motility, depending on the cell types and the micro-environment (for review, see Heldin, 2001; Reinmuth et al., 2004; Adjei and Hidalgo, 2005).

Figure 5 illustrates a summary diagram of pathways described above. One important feature of these intracellular signal transductions is the extensive crosstalk among them. For example, PI3K and Ras, as both being major components downstream several receptor types, can interact and activate each other. PLC- $\gamma$  is a SH2 domain-containing molecule that can be activated by binding to phosphorylated receptors by itself, but it can be also activated via PIP3, the enzymatic product of PI3K. Thus the intracellular signaling is better to be considered as a "network" of interacting components, instead of a number of parallel pathways (Heldin, 2001).

### Perturbation of signaling molecules in lung cancer

In human, Ras mutations have been identified in approximately 30% of cancer types. The Ras family includes distinct members such as Ras (H-, K-, M-, N-, and R-) and Rap (1- and 2-) that share at least 50% sequence identity; among which K-Ras

is most frequently observed one to be mutated in human cancers. The K-Ras gene generates 2 alternatively spliced products 4A and 4B, and their mutations (mostly 4B subtype) are present in about 30% of NSCLC and are related to poor clinical outcome. Besides mutations, overexpression of wt Ras is also frequent in tumors (Adjei, 2001; Ferreira et al., 2002; Mitsudomi et al., 1991; Salgia and Skarin, 1998). Because of its significant role in lung cancer, a series of elegant experiments have been performed on transgenic mice and demonstrated the oncogenic potential of K-Ras *in vivo*, from the earlier design that relied on the spontaneous recombination to activate the oncogenic K-Ras expression (Johnson et al., 2001), to the later transgenic strains that can conditionally “turn on” the K-Ras expression at defined time point and tissues, based on the Cre-loxP recombination system (Collado et al., 2005; Guerra et al., 2003; Tuveson et al., 2004). These results together confirmed that the expression of oncogenic K-Ras is sufficient to initiate the transformation of lung cells. A small percentage of bronchiolo-alveolar cells underwent malignant transformation leading to the formation of both multiple adenomas (pre-malignant tumor) and adenocarcinomas (malignant tumor), among which a substantial number of adenomas cells were restricted, presumably by the effect of p16<sup>INK4a</sup> or p53, in the oncogene-induced senescence (Collado et al., 2005) (for K-Ras associated oncogenesis, see also Thesis RESULT and DISCUSSION, III.1.). Similarly, transgenic mice expressing oncogenically activated B/C-Raf developed benign lung tumors that only rarely progressed to adenocarcinoma, whereas loss of function of tumor-suppressor genes such as p16<sup>INK4a</sup>, p14<sup>ARF</sup>, or p53 accelerated tumor development and induced the phenotypic transformation (Dankort et al., 2007; Fedorov et al., 2003; Ji et al., 2007; Kerkhoff et al., 2000).

For PI3K, a study testing 80 primary lung carcinomas showed that the p85 and p110 subunits were overexpressed at protein level in 77% and 59% cases, respectively; whereas no overexpression was observed in normal lung tissue and benign lung tumors (Lin et al., 2001). PKC family comprises at least 12 related isoforms. NSCLC cell lines have been reported to show enhanced phosphorylation and altered expression of specific PKC isoforms compared with normal lung epithelial cells, and the addition of PKC $\delta$  inhibitor potentiated chemotherapy-induced apoptosis in these cells (Basu et al., 1996). Otherwise, amphiregulin and IGF-I cooperate to protect

NSCLC cell lines from serum-starved apoptosis through a specific PKC-p90(rsk)-dependent pathway, which leads to Bad and Bax inactivation; and the PKC inhibitors can remove this apoptosis protection (Hurbin et al., 2002; 2005). In SCLC, a specific pattern of PKC isoforms expression has been proposed to be associated with cisplatin-resistance (Basu et al., 1996).

### Current therapeutic strategies

To date, therapeutic approaches targeting these intermediate signaling molecules include the developments of ASO, siRNA, farnesyl transferase inhibitor, and kinase activity inhibitor, to block the oncogene expression or protein activity. Some of them are now tested in clinical trials.

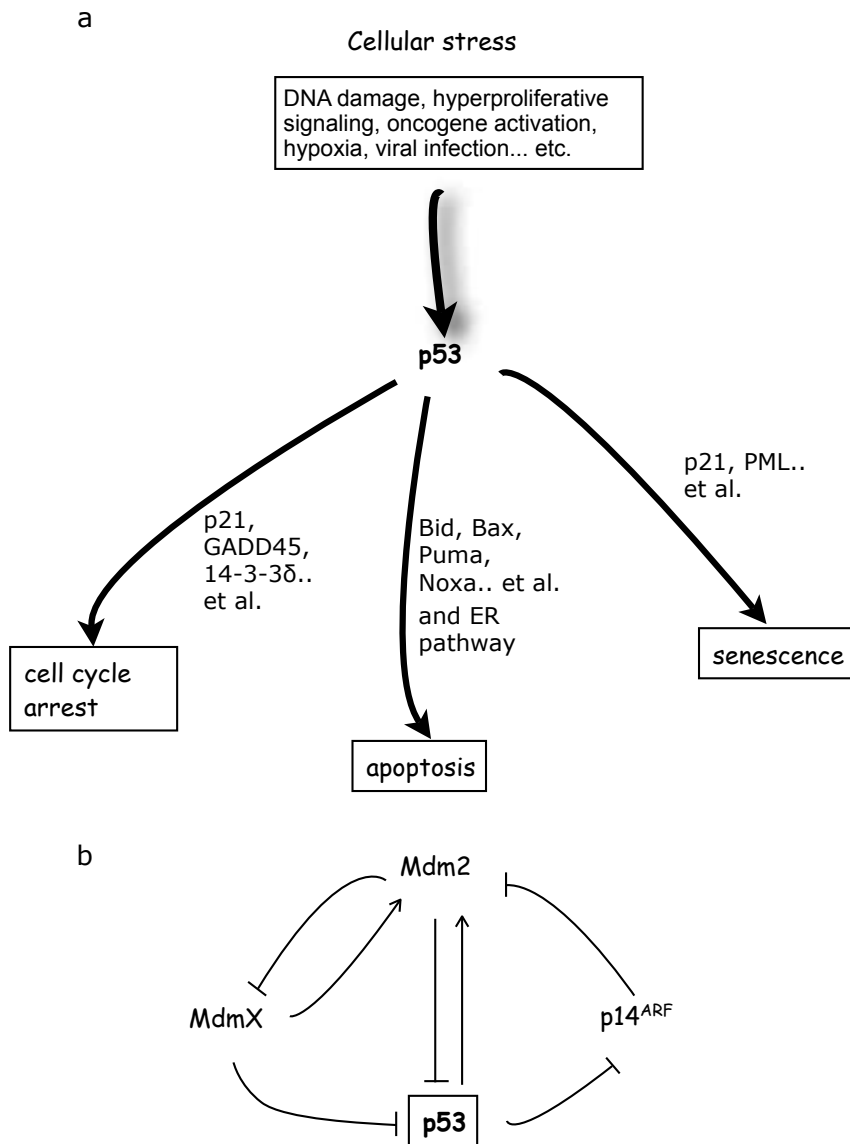
Farnesyl transferase inhibitors were designed to target Ras because the post-translational farnesylation is a critical step for its localization to the inner surface of the plasma membrane. Among several inhibitors, R115777 (Zarnestra) is the most advanced one. However, single-agent R115777 treatment in patients with advanced NSCLC or sensitive-relapse SCLC demonstrated minimal or no clinical activity (Adjei et al., 2003b; Heymach et al., 2004); while combining with gemcitabine and cisplatin was well tolerated and showed evidence of antitumor activity in patients with various advanced solid tumors (Adjei et al., 2003a). Future studies of this agent for lung cancer may focused on combination with systemic chemotherapy. ISIS-5132, an ASO against C-Raf, has been assessed in clinical trials. Two multi-centered phase II trials including 22 patients (18 NSCLC and 4 SCLC) were performed, but showed no objective responses (Coudert et al., 2001). The oral MEK inhibitor CI-1040 has also entered clinical trials. Although being a highly potent and selective inhibitor to both MEK isoforms (MEK-1 and 2), multi-center phase II study in patients with different advanced cancer types did not show sufficient antitumor activity to warrant further development (Rinehart et al., 2004).

For the PI3K pathway, although inhibitors of Akt and PI3K have been exploited, they represented a narrow therapeutic window because of the metabolic toxicity and the lack of selectivity, respectively (Reinmuth et al., 2004). A downstream therapeutic target is mTOR (mammalian target of rapamycin), which is mainly regulated by Akt

and functions to activate p70<sup>S6K</sup> (p70 ribosomal protein-6 kinase) for inducing the expression of certain genes associated with G1/S phase entry (Adjei and Hidalgo, 2005; Fesik, 2005). CCI-779 for example, a derivative of rapamycin, has shown antiproliferative activity by targeting mTOR in various tumor types including NSCLC (Raymond et al., 2004). For PKC, ISIS 3521 (ASO anti-PKC) and PKC412 (a oral PKC inhibitor, analogue of staurosporine) were developed and assessed in phase I/II clinical trial in combination with chemotherapy (Monnerat et al., 2004; Villalona-Calero et al., 2004), showing preliminary positive results.

Nonetheless, most of these single-target agents evaluated in clinical trial did not show significant impacts (especially when administered alone). A main reason for the failure may be the existence of multilevel crosstalk among signaling pathways (Adjei and Hidalgo, 2005). Blocking one of them may allow others to act as salvage or escape mechanisms in cancer cells. Some new drugs such as Sorafenib (BAY 43-9006) and Sunitinib were developed, which are the so-called 2<sup>nd</sup>-generation drugs with multi-target kinase inhibitor activity (Adjei and Hidalgo, 2005). Sorafenib is an inhibitor of kinase activities of B-, C-Raf, vascular endothelial growth factor receptor (VEGFR), PDGFR, as well as the stem cell factor receptor KIT. Sunitinib is the inhibitor of PDGFR, KIT, fms-like tyrosine kinase 3, and VEGFR. These targeted kinases directly and indirectly regulate tumor growth, survival, and angiogenesis, thus inhibitions to them are expected to result in a broader antitumor efficacy (Adjei and Hidalgo, 2005). The preclinical and phase I/II studies already suggested the therapeutic utility of these agents in several cancer types including lung cancer (Carter et al., 2007; Gridelli et al., 2007).

Figure 6.



a/ A simplified schematic representation of biological roles of p53 in cells.  
 (figure according to Artandi and Attardi, 2005; Wiman, 2006)

b/ The core regulation loop of p53 in cells.  
 (figure according to Levine et al., 2006)



## II.2. Cell-Cycle Regulation

### II.2a. p53

The p53 tumor suppressor protein has been discovered early in 1979 and immediately drew a lot of attention in tumor therapy. It plays a pivotal role in signaling pathway complex, and is able to sense a broad range of cellular stresses that could alter normal cell cycle progression or induce mutations of the genome, such as DNA damage, hyper-proliferative signaling, oncogene activation, hypoxia, matrix detachment, viral infection and ribonucleotide depletion (Bourdon et al., 2003). The p53 network is normally “switched off”, maintained at a low steady-state level, but is activated rapidly when a cellular stress appears, reflected in elevated p53 protein level as well as augmented biochemical capabilities (Bourdon et al., 2003). The p53 protein acts as the guardian of genome, prevents the multiplication of stressed cells containing mutations or exhibiting abnormal cellular growth. Activated p53 can act as a transcriptional regulator to induce or repress the expression of specific genes, or function through protein-protein interaction. As a consequence of p53 activation, cells can undergo marked phenotypic changes including increased DNA repair activity, cell cycle arrest, senescence or, in many cases, apoptosis that forces damaged cells to commit suicide (Fig. 6a) (Bourdon et al., 2003; Wiman, 2006). Loss of wild type p53 function allows apoptosis evasion and further selection of more malignant variants during tumor progression. Mutant p53-carrying tumors are found to show increased resistance to commonly used chemotherapeutic agents and radiotherapy (Wiman, 2006).

As playing a central role in cell cycle regulation, p53 itself is regulated in a complicated network (Fig. 6b). Mdm2 is an important factor for down-regulating p53 activities in cells, through either repression of p53-mediated transcription in the nucleus or ubiquitination of p53 protein in the cytoplasm. Interestingly, Mdm2 is also a transcribing target of p53 since its transcription can be induced by p53. MdmX (or Mdm4), a homolog of Mdm2, negatively regulate p53 directly and positively regulates Mdm2. The protein p14<sup>ARF</sup> is an endogenous inhibitor of Mdm2, but high levels of p53 repress the transcription of p14<sup>ARF</sup> (for review, see Levine et al., 2006). Besides the p53 regulation network, several studies have recently provided evidence that p14<sup>ARF</sup>

also interferes with the RB signaling pathway to mediate its antiproliferative activity (see the following **II.2b.**).

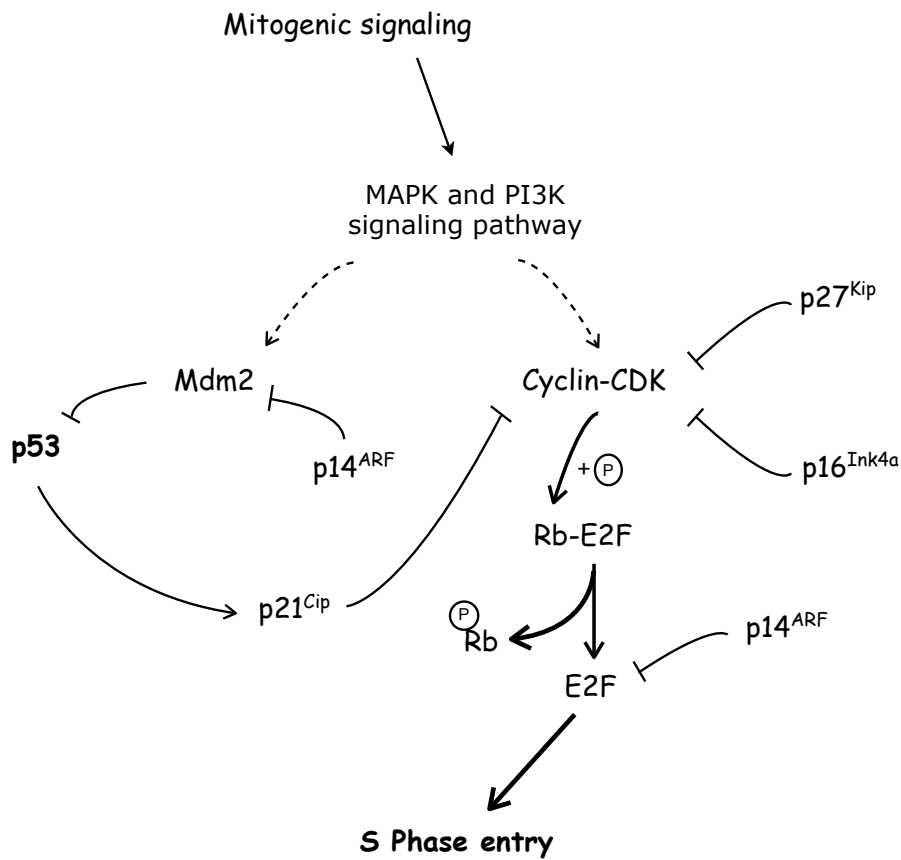
Mutations within p53 are one of the most common genetic alterations present in human cancers. In more than 50% of human tumors an inactivated p53 can be observed. In lung cancer, p53 is inactivated by mutation in approximately 80% of SCLCs and up to 50% in NSCLCs (Gazzeri et al., 1994; Hainaut et al., 1998; Salgia and Skarin, 1998; Takahashi et al., 1989), and p53 mutant pre-neoplastic lesions have a higher rate of progression to invasion (Brambilla et al., 1998). After immunohistochemistry examination, the expression of p14<sup>ARF</sup> was found to be lost in 65% of SCLC and 25% of NSCLC tumors (Gazzeri et al., 1998a). Mdm2 was overexpressed as compared to the normal lung tissue in 31% of primary human lung tumors analysed (Eymin et al., 2002). Interestingly, a highly significant inverse relationship was detected between the p14<sup>ARF</sup> loss and the Mdm2 overexpression, suggesting that the 2 events are mutually exclusive in human lung cancer (Eymin et al., 2002).

Despite of the presence of multiple genetic defects in lung cancer, the expression of wt p53 in cells containing mutant or deleted p53 is sufficient to cause apoptosis or growth arrest (Coll et al., 1998; Dubrez et al., 2001; Fujiwara et al., 1993; Takahashi et al., 1992). Growth of human lung cancer cells with defective p53 can be dominantly inhibited by p53 delivered through various vectors, alone or combined with other anticancer drugs (e.g. cisplatin) in animal model (Nguyen et al., 1996; Nguyen et al., 1997; Ramesh et al., 2001; Zhang et al., 1994). Interestingly, it was found that wt p53 transduced lung cancer cells can slow down the growth of non-transduced ones when coincubated (Cai et al., 1993), suggesting that a bystander effect of p53 exists. It was later proven that p53 transduced NSCLC cells can inhibit the *in vivo* tumor growth of adjacent non-transduced cells through antiangiogenesis (Nishizaki et al., 1999). These findings underline the value of wt p53 in cancer therapy.

Several clinical trials in advanced NSCLC have been performed. The first trial was carried out in 1996 in the United States. Retroviral vector expressing wt p53 was

directly injected to endobronchial tumors. Three of the 7 evaluable patients showed evidence of tumor regression and one showed no viable tumor 3 months after injection. Wt p53 and apoptotic cells were demonstrated in post-treatment tumor biopsies (Roth, 1996a). A later phase I trial was performed on 28 NSCLC patients with the tumors progressed to conventional treatments. Monthly injections (up to 6 months) of the adenoviral vector (AdV) expressing wt p53 led to clinical responses in 25 evaluable patients, including partial responses in two patients and the disease stabilization in 16 patients (64%), durable for 2-14 months (Roth, 1996b; Swisher et al., 1999). Clinical trials were also performed in different countries (Fujiwara et al., 2006; Schuler et al., 1998). In summary, these studies showed that p53-based gene therapy is feasible, biologically effective and well tolerated for patients with advanced NSCLC; although a result showing no additional benefit was also reported when combined with effective first-line chemotherapy (Schuler et al., 2001).

Figure 7.



The central role of Rb-E2F pathway in G1/S phase entry of cell cycle regulation, and a simplified representation of its linkage to p53 pathway, and growth factor-induced MAPK and PI3K signaling. p27<sup>Kip</sup>, p21<sup>Cip</sup>, p16<sup>Ink4a</sup> are the 3 major inhibitory factors to cyclin-CDK, while p14<sup>ARF</sup> directly inhibits the transcriptional activity of the E2F protein (Figure modified from Sherr and McCormick, 2002).

## II.2b. Rb (Retinoblastoma protein)-associated pathway

Like p53, Rb is one of the best-studied tumor-suppressor genes with known key functions in controlling cell proliferation and differentiation. The interactions among Rb protein, cyclin and cyclin-dependent kinase (CDK), and CDK inhibitors represent the core regulatory mechanism of G1/S phase entry in cell-cycle (Fig. 7). Hypophosphorylated forms of Rb family members in cytosol bind and block E2F proteins (a class of transcription factor). The complex is dissociated when phosphorylation on Rb is triggered by CDK. The release of E2Fs thus results in E2F-dependent gene expressions, which are mostly the enzymes required for DNA metabolism and synthesis, and the entry into S phase. Four INK4 proteins (including p16<sup>INK4a</sup>) specifically inhibit the activity of CDKs to prevent the phosphorylation of Rb proteins. The Cip/Kip family, including the well-studied p27<sup>Kip</sup> and p21<sup>Cip</sup>, is another class of CDK inhibitors (for review, see Sherr and McCormick, 2002; Wikman and Kettunen, 2006).

Besides being a regulator of p53 regulating network, p14<sup>ARF</sup> (see also II.2a. above) also interferes with Rb-associated pathway. P14<sup>ARF</sup> physically interacts with E2F1 and inhibits its transcriptional activity, and it also induces Rb accumulation through preventing the Tip60 (a histone acetyl transferase)-mediated Rb acetylation and proteasomal degradation (Eymin et al., 2001; Leduc et al., 2006). Thus, p14<sup>ARF</sup> can be regarded as a dual-acting tumour suppressor protein in both the p53 and RB pathways.

Table 3. The perturbations of RB pathway in human cancer.

Cancer type	p16 <sup>INK4a</sup> loss	Cyclin D1 or CDK4 overexpression	Rb loss
SCLC	15%	5% Cyclin D1	80%
NSCLC	58%		20% - 30%
Pancreatic cancer	80%		
Breast cancer	30%	>50% Cyclin D1	
Glioblastoma multiforme	60%	40% Cdk4	
T cell all	75%		
Mantle cell lymphoma		90% Cyclin D1	

The summarized frequencies of p16<sup>INK4a</sup> loss (by mutation, deletion, or gene silencing), RB mutation or deletion, and cyclin D1 or Cdk4 overexpression in different forms of cancer. (Table from Sherr and McCormick, 2002)

As the Rb pathway governs the G1/S progression of a cell, most members participating in this regulator machinery act as tumor suppressors or protooncogenes, and are frequently mutated in various types of cancer (Tab. 3). It is interesting to note that mutations affecting Rb pathway generally occur in a “mutually exclusive” fashion (Sherr and McCormick, 2002): one “hit” (e.g. p16<sup>INK4a</sup> mutation) is not accompanied by others (e.g. RB mutation or cyclin-CDK overexpression). A study investigating preinvasive bronchial lesions showed that Rb protein function was invalidated before invasion mainly through p16<sup>INK4a</sup> inhibition and/or by cyclin D1 overexpression (Brambilla et al., 1999). In SCLC, Rb is inactivated in up to 80% of the tumors, whereas 50-60% of NSCLC tumors are associated with the p16<sup>INK4a</sup> loss of function (Gazzeri et al., 1998b; Gouyer et al., 1994, 1998; Salgia and Skarin, 1998) (Tab. 3). Otherwise, p14<sup>ARF</sup> expression was lost in 65% of SCLC and 25% of NSCLC tumors (Gazzeri et al., 1998a).

Rb restoration-based gene therapy would take advantage of selectively killing tumor cells without (or minimally) adverse side effects to normal somatic cells. But to date, the efficacy of its therapeutic effect has only been demonstrated in vitro and in mouse models for NSCLC or pituitary melanotroph tumors (Riley et al., 1996; Xu et al., 1996). Like p53 or other single gene transfer strategies, the clinical success of Rb gene therapy to cancer may require more innovation in vector development. Meanwhile, the involvement of Rb gene family in tumor angiogenesis has recently been addressed, increasing its interest as a cancer therapy target (Gabellini et al., 2006). Another therapeutic strategy to target Rb-associated pathway is the development of small synthetic CDK inhibitors, such as E7070. This drug causes a blockade in the G1/S transition through inhibition of the activation of both CDK2 and cyclin E to sustain Rb hypophosphorylation. Preliminary results of phase II studies however, demonstrated limited antitumor activity as single agent in heavily pretreated patients with NSCLC and colon cancer (Van Kesteren et al., 2002).

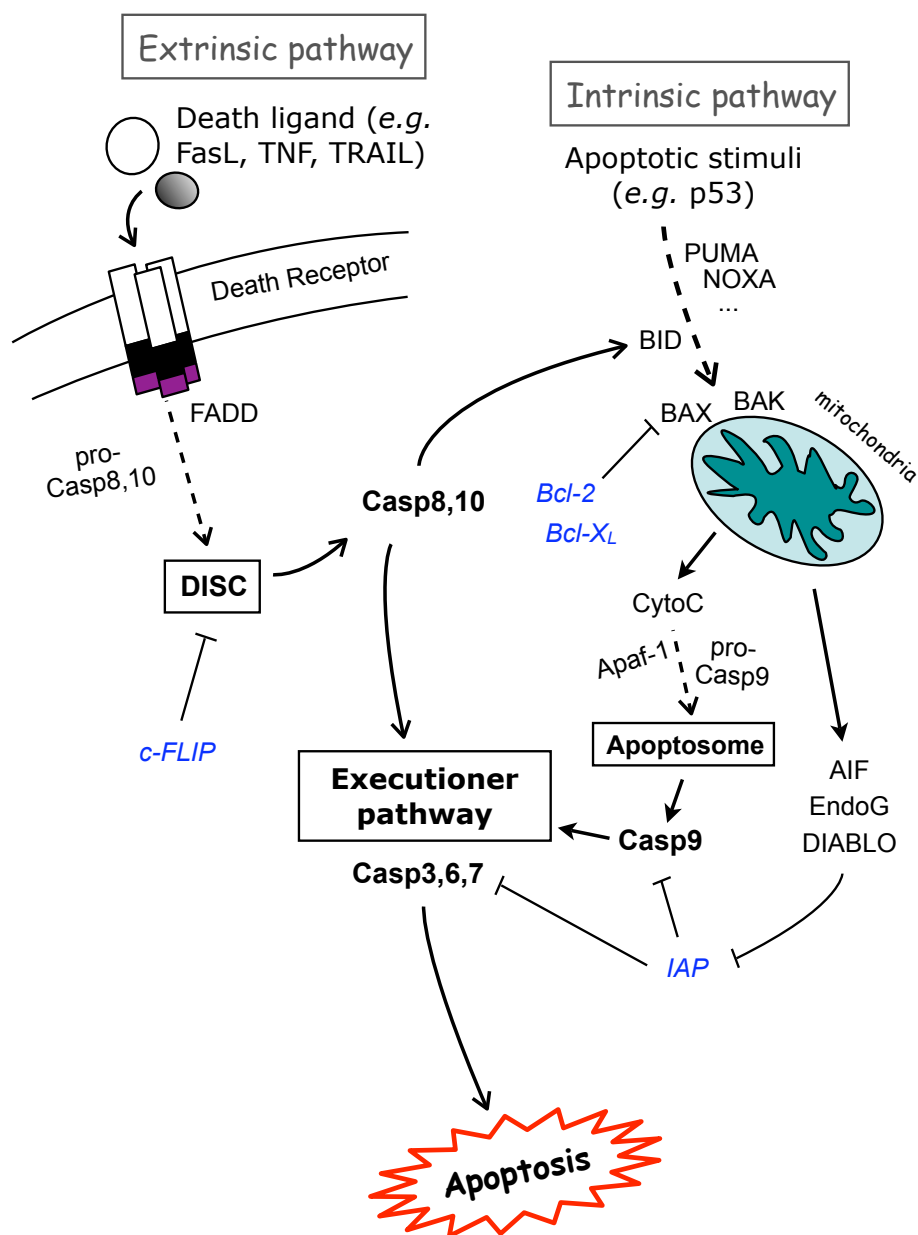
Tumor-suppressor genes involved in this pathway (e.g. INK4 proteins and Cip/Kip family) are naturally regarded as candidate therapeutic genes. Some studies compared the effect of different tumor-suppressor genes. As p16<sup>INK4a</sup>, p18<sup>INK4c</sup>, p19<sup>INK4d</sup>, p21<sup>CIP1</sup> and p27<sup>KIP1</sup> were tested in vitro, only p16<sup>INK4a</sup>, p18<sup>INK4c</sup>, and p27<sup>KIP1</sup>

were found to induce apoptotic death in transduced HeLa and A549 cells; but when these genes were delivered to tumors by AdV through intratumoral injection, only p16<sup>INK4a</sup> resulted in a delayed tumor growth (Schreiber et al., 1999). In another study, the same 5 CDK inhibitors were tested on malignant glioma cells. Although p27<sup>KIP1</sup> showed the best capacity to suppress tumor cell growth, the effect came mainly from autophagic cell death instead of apoptosis (Komata et al., 2003). A study compared the efficacy of p53 or p16<sup>INK4a</sup>-encoding AdVs in inhibiting the growth of ovarian carcinoma. Although the ability to induce apoptosis or growth arrest did not differ significantly between the 2 genes in single cells (through Flow Cytometry identification), p16<sup>INK4a</sup> was shown to be a much better growth suppressor for growing cells *in vitro* in all cell lines tested, and provided a longer survival in nude mice with ovarian carcinoma xenografts (Modesitt et al., 2001). These combined data suggested that p16<sup>INK4a</sup> may be more efficient for ovarian carcinoma treatment; however, it's interesting to be aware of the fact that while p53 mutation is an usual event in ovarian carcinoma, p16<sup>INK4a</sup> gene is rarely mutated (although decreased level of p16<sup>INK4a</sup> protein can be detected in a low percentage of tumor samples). The stronger efficiency of p16<sup>INK4a</sup> can be due to the mutational inactivation of p53 downstream pathway in tumor cells, or the existence of an un-clarified growth-suppressing activity of p16<sup>INK4a</sup> independent of Rb, such as the antiangiogenic activity (Harada et al., 1999; Murphy, 2001; Skilling et al., 1996).

These results underline the complexity of the crosstalks among molecular pathways in a cell. For designing an effective gene therapy, not only more basic researches have to be carried out, but molecular characteristics of different tumor types should be delineated.



Figure 8. Schematic diagram representing major apoptosis pathways and the important negative regulators inside.



Casp: caspase, EndoG: endonuclease G, CytoC: cytochrome C, AIF: apoptosis-inducing factor.  
 (Figure designed according to Shivapurkar et al., 2003; Wang, 2001)

## II.3. Apoptosis Regulation

### II.3a. Apoptosis mechanisms

Apoptosis, also named programmed cell death, is the natural process for removing unwanted cells such as those with potentially harmful genome damage or aberrant cell-cycle control. Deregulation of apoptosis can disrupt the delicate balance between cell proliferation and cell death, and lead to diseases such as cancer or neurodegenerative pathologies. Apoptotic cell death is immuno-suppressive and presents characteristics of DNA fragmentation, chromatin condensation, cell shrinkage and membrane blebbing. Normally, apoptosis is under fine and narrow control of a complex network utilizing over 150 known proteins. Most (and perhaps all) types of cancer cells however, develop highly efficient and usually multiple mechanisms to escape apoptosis (for review, see Fesik, 2005).

The mechanism of apoptosis can be classified as 2 distinct, but overlapped pathways: one is the extrinsic pathway (also known as the death receptor pathway) and the other is the intrinsic pathway centered on mitochondria. In the death receptor (DR) pathway, ligands such as tumor-necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), or Fas ligand (FasL, also known as the CD95 ligand) interact with their respective death receptors and trigger an intracellular recruitment of Fas-associated death domain (FADD) and caspase-8 and 10, forming the death-inducing signaling complex (DISC). The activated caspase-8 is then released from DISC to cytosol, activates the downstream effector caspases (e.g. caspase-3, 6) directly or indirectly through Bid - cytochrome C release (Fig. 8). The intrinsic pathway is generally initiated in response to some internal stimuli such as growth factor withdrawal, osmotic stress, or hypoxia. The activation of Bid leads to oligomerizations of Bak and Bax on the the mitochondrial membrane and the release of cytochrome C from the intermembrane space. Cytochrome C then induces the formation of a multiprotein complex called “apoptosome” in the cytosol, from where the activated caspase-9 is liberated and leads to downstream effector caspases activation. Thus the 2 pathways lead to a common “executioner pathway”, in which effector caspases-3, 6, 7 are activated, execute the proteolytic cascade and give rise to the apoptotic cell death (Fig. 8) (for review, see Fesik, 2005).

The c-FLIP (cellular FADD-like IL-1 $\beta$ -converting enzyme (FLICE)-inhibitory protein) is a negative regulator of the DR pathway, which prevents the activation of procaspase-8 by interfering with the DISC complex. Bcl-2 and Bcl-X<sub>L</sub> are 2 mostly studied anti-apoptotic Bcl-2 family members, which inhibit cytochrome C release by blocking the activation of Bax and Bak. Inhibitor of apoptosis protein (IAP) is a family of proteins which can bind and inhibit the caspases and block apoptotic signaling. IAPs are regulated by DIABLO (direct IAP-binding protein with low pI; also known as SMAC), another protein released from mitochondria, which can bind to IAPs and antagonize their anti-apoptotic activity (Fig. 8) (for review, see Fesik, 2005; Shivapurkar et al., 2003).

## II.3b. Lung cancer-associated apoptosis evasion

### Apoptosis evasion

Most types of cancer cells develop their ways to escape apoptosis. In lung cancers, the DR pathway seems to be differently inactivated. Some studies pointed out that caspase-8 gene expression was lost in 79% of SCLC cell lines, but retained in all 22 NSCLC lines tested. Methylation of the promoter is responsible for more than half cases losing caspase-8 expression (Shivapurkar et al., 2002b). Loss of expressions of other DISC components such as caspase-10, DR4, DR5, Fas and FasL were also shown in significantly higher rates in SCLC cells comparing to NSCLC (Hopkins-Donaldson et al., 2003; Shivapurkar et al., 2002a). These suggest a reason why SCLC is highly resistant to DR-induced apoptosis.

Another example of apoptosis evasion is the epigenetic silencing of death-associated proteins (DAP) kinase genes. DAP kinase is a novel pro-apoptotic serine/threonine kinase, which plays an important role in IFN- $\gamma$ , TNF or FasL induced apoptosis (Tang et al., 2004). Hypermethylation of the DAP kinase promoter can be detected in ~44% NSCLC tumor samples or cell lines, and patients (NSCLC stage I) whose tumors exhibited such hypermethylation had a statistically significantly poorer probability of overall 5-year survival after surgery than those without such hypermethylation (Tang et al., 2000; Toyooka et al., 2003). An *in vivo* murine model of cigarette smoke-induced lung cancer presented the similar frequency (43%) of methylation-inactivation on DAP kinase promoter in induced tumor samples (Pulling et al., 2004). NSCLC cells with hypermethylation on DAP kinase promoter were resistant to TRAIL-induced apoptosis, whereas those without that were sensitive to TRAIL treatment (Tang et al., 2004). Adding the demethylating reagent to cells with hypermethylation recovered the DAP kinase expression and made them sensitive to TRAIL.

Bcl-2 family includes a number of proteins sharing at least one BH domain. They are crucial effectors, either pro-apoptotic or anti-apoptotic, in the intrinsic apoptosis pathway (Fesik, 2005). Two best-studied anti-apoptotic members are Bcl-2 and Bcl-X<sub>L</sub>. Bcl-2 is overexpressed in many cancers including lung cancer (Higashiyama et al., 1995), and is more frequent in SCLC than in NSCLC (Joseph et al., 2000). There

is an inverse correlation between the scores of Bax and Bcl-2 expressions in neuroendocrine lung tumors; whereas Bcl-2 overexpression, Bax down-regulation, and Bcl-2/Bax ratio $>1$  correlated with lower apoptotic index in SCLC tumors (Brambilla et al., 1996). Otherwise, Bcl-2 or Bcl-X<sub>L</sub> overexpression is associated with resistance to numerous cytotoxic agents, chemotherapy and radiotherapy (Fesik, 2005). However, the role of Bcl-2 in lung cancer remains controversial since a systematic review of the literature with meta-analysis showed that Bcl-2 expression seemed to be associated with a better prognosis for survival in NSCLC, while the data were insufficient to assess the prognostic value in SCLC (Martin et al., 2003).

### Lung cancer therapy based on apoptosis induction

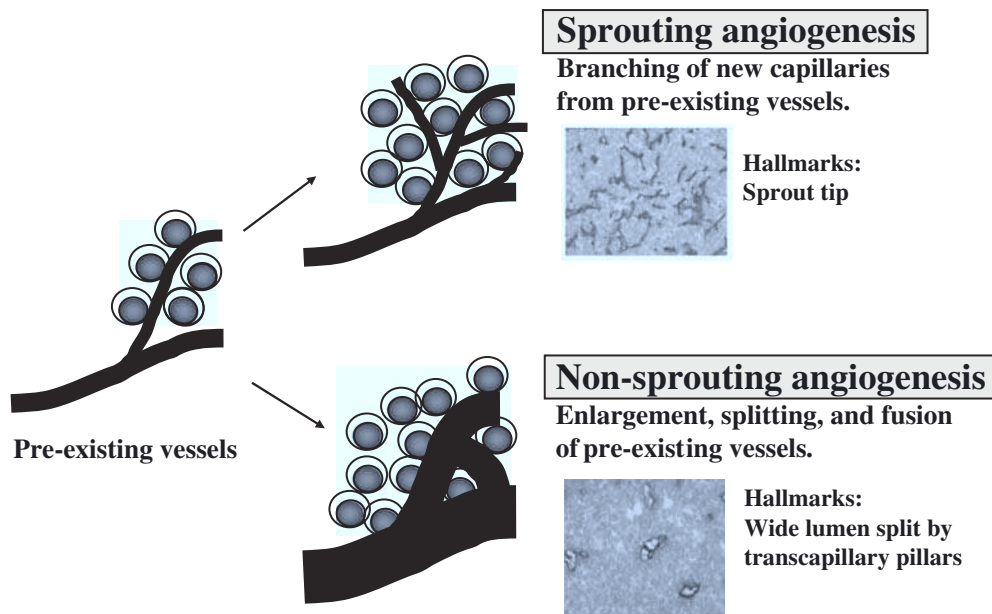
An attempt to develop effective apoptosis-inducing agents for cancer treatment is focused on TRAIL receptors. Advantages of this strategy include that the TRAIL-induced apoptosis is through DR4 or DR5 (also known as TRAIL receptor 1 or TRAIL receptor 2, respectively) independent of p53 signaling (which is mutated in many cancer types), and is effective in a wide variety of human cancer cells; whereas most normal human cell types are resistant to it (Ashkenazi, 2002). Agonistic antibodies against DR4 or DR5 have been developed. Soluble truncated TRAILs exhibited potent antitumor effect in subcutaneous (SC) or orthotopic human NSCLC xenograft in animal models, either alone or combined with chemotherapy (Jin et al., 2004; Shi et al., 2005). These approaches targeting TRAIL receptors are currently progressing in clinic trials (for review, see Fesik, 2005).

Another approach is to target anti-apoptotic proteins such as Bcl-2 and Bcl-X<sub>L</sub>. ASO against Bcl-2 has progressed to phase III clinical trial for treatments in several kinds of tumors, but there seemed to be no clinical benefit observed on melanoma or multiple myeloma, although it did improve the overall response rate in patients with chronic lymphocytic leukaemia (for review, see Fesik, 2005). A bispecific ASO targeting both Bcl-2 and Bcl-X<sub>L</sub> has also been described. These oligonucleotides were able to induce apoptosis in SCLC and NSCLC cell lines expressing different levels of Bcl-2 and Bcl-x<sub>L</sub>, alone or in combination with other anticancer agents (Simoes-Wust et al., 2004; Zangemeister-Wittke et al., 2000). Besides ASO, synthetic

BH3 peptides or small organic molecules that interact directly with Bcl-2 or Bcl-X<sub>L</sub> proteins can also elicit an inhibitory effect. ABT-737 is a potent small molecule inhibitor of Bcl-2, Bcl-X<sub>L</sub> and Bcl-w. It performed synergistic cytotoxicity with chemotherapeutics and radiation, but also exhibits single-agent-mechanism-based killing to lymphoma and SCLC cells, *in vitro* and *in vivo* (Oltersdorf et al., 2005).

Conversely, apoptosis can be induced by directly administrating pro-apoptotic factors such as tBid (truncated- or activated- Bid), Bax or Bak in cancer cells. Bax was shown to be a potent apoptosis inducer and presented a stronger antitumor activity than p53 when treating human NSCLC xenograft in a mouse model (Coll et al., 1998). Using viral vectors for delivering these pro-apoptotic genes is difficult in vector amplification and safety control because of the high toxicity. A binary system has allowed a large-scale propagation of Bax-encoding AdV. In this virus the human Bax cDNA was placed under control of a synthetic promoter consisting of GAL4-binding sites and a TATA box. This AdV can express Bax and induce extensive apoptosis only when the other AdV expressing the GAL4/VP16 fusion protein is co-introduced into cancer cells (Kagawa et al., 2000). In another study, the AdV expressing Bax under the transcriptional control of human VEGF promoter performed a strong cytotoxicity in human lung carcinoma cells, but not in normal human bronchial epithelial cells (BEAS-2B) that do not overexpress VEGF (Kaliberov et al., 2002).

Figure 9. Types of angiogenesis processes.



Angiogenesis consists of sprouting and nonsprouting processes. Sprouting angiogenesis involves the branching (true sprouting) of new capillaries from preexisting vessels, with the hallmark of sprout tips. The nonsprouting angiogenesis results from the enlargement, splitting, and fusion of preexisting vessels produced by the proliferation of endothelial cells at the wall of the vessel, and the transvascular bridge can be sometimes observed. Both types of angiogenesis can occur concurrently in the development of tissues and in tumorigenesis. (Figure from Yano et al., 2006)

## II.4. Tumor-Host Interaction

### II.4a. Angiogenesis

#### *Angiogenesis and cancer development*

Angiogenesis is the neovascularization from preexisting vasculature, which plays an important role in the development of organs or tissues, as well as the tumors. Angiogenesis consists of sprouting and nonsprouting processes, and the 2 ones can occur concurrently (Fig. 9) (Yano et al., 2003). Angiogenesis supplies oxygen and nutrition for tumor growth, and is absolutely required for the tumor beyond the volume of 1-2 cubic millimeters (Folkman, 1990). Otherwise, angiogenesis is a critical factor for tumor invasion and metastasis. Although a low ratio of NSCLC tumors (< 20%) was found to be able to grow without neovascularization if a suitable vascular bed is available (Pezzella et al., 1997), the growth of NSCLC is generally dependent on angiogenesis. Vascularization is commonly evaluated by immunohistochemistry to several endothelial markers such as CD31, CD34, CD105, and factor VIII; among which the CD105, a proliferative endothelial marker, seems to be a better predictive factor in NSCLC patients (Tanaka et al., 2001). Although angiogenesis is essential for tumour growth, whether intratumoral microvessel density (MVD) can serve as a prognostic indicator of NSCLC is controversial (Decaussin et al., 1999; Fontanini et al., 1997; Meert et al., 2002). A later report showed that glomeruloid microvascular proliferation (GMP), a focal proliferative budding of endothelial cells resembling a renal glomerulus, represented an aggressive angiogenic phenotype and was a better prognostic factor than MVD in NSCLC (Tanaka et al., 2003).



Table 4. Endogenous factors involved in the control of angiogenesis

Proangiogenic factors	Antiangiogenic factors
Growth factors VEGF, FGF-2, EGF, TGF- $\alpha$ , PDGF-AA, PDGF-BB	Matricellular glycoproteins Thrombospondin-1 and -2
Proteases Cathepsin, MMP-2, -7, -9, uPA	Collagen fragments Angiostatin, endostatin, tumstatin, Canstatin, malignostatin
Cytokines IL-1, IL-6, IL-8, MCP-1, TNF- $\alpha$	Cytokines IFN- $\alpha$ , IFN- $\beta$
Others Ang-1, Ang-2, integrins, hypoglycemia inhibitor Low levels of PO and pH, NOS, COX-2	Others Vasohibin, vascular endothelial growth, Pigment epithelium-derived factor

FGF, fibroblast growth factor; TGF- $\alpha$ , transforming growth factor- $\alpha$ ; uPA, urokinase type plasminogen activator; IL, interleukin; NOS, nitric oxide synthase; COX, cyclooxygenase. (Table from Yano et al., 2006)

### Angiogenic and anti-angiogenic factors in lung cancer

Angiogenesis is regulated by the balance of various angiogenic and antiangiogenic factors (Tab. 4). Vascular endothelial growth factor (VEGF, also known as vascular permeability factor, VPF) is one of the most potent angiogenic mediators and can be induced by multiple tumor-relevant stimuli. VEGF family consists of VEGF, VEGF-B, VEGF-C, VEGF-D, and VEGF-E, among which VEGF is the prototype and contains at least 6 isoforms that bind to two tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1, also known as Flt-1) and VEGFR-2 (also known as Flk-1/KDR). VEGF is not only a highly potent and specific growth factor for endothelial cells, but also found to regulate angiogenesis and vascular permeability in many tumor types including NSCLC (Dvorak, 2002; Vokes et al., 2006; Yano et al., 2006). An experiment establishing the brain metastasis by inducing tumor cells into the internal carotid artery of nude mice showed that the levels of VEGF production in NSCLC cells directly correlated with the potential of brain metastasis, and the inhibition of VEGF significantly decreased the incidence (Yano et al., 2000). VEGF-C expression was reported to correlate with lymph node metastasis, lymphatic invasion, and a poor prognosis in NSCLC (Arinaga et al., 2003). These results suggest that VEGF could represent an therapeutic target in NSCLC.

Angiopoietin (Ang)-1 and -2 are ligands for Tie-2, a receptor tyrosine kinase specifically expressed on endothelial cells, and play a role in angiogenesis in concert with VEGF. Ang-1 binds to Tie-2 and induces the stabilization of mature vessels by promoting the interaction between endothelial cells and surrounding extracellular matrix; while Ang-2 competitively binds to Tie-2 and antagonizes the stabilizing action of Ang-1, thus resulting in the destabilization of vessels (Holash et al., 1999). In NSCLC however, a later report indicated that the positive Ang-2 expression was significantly correlated with aggressive angiogenesis and a poor prognosis in NSCLC patients, and the correlations were further enhanced in the presence of VEGF expression (Tanaka et al., 2002). This result suggested the complex network of angiogenesis regulation in tumors. A newly identified antiangiogenic factor vasohibin was largely detected in tumor-associated endothelial cells in NSCLC patients. Vasohibin can be selectively induced in endothelial cells by proangiogenic factors such as VEGF, and appears to act as an intrinsic feedback inhibitor of angiogenesis.

Transfection of vasohibin in Lewis lung carcinoma resulted in tumor growth suppression via inhibition of angiogenesis (Watanabe et al., 2004).

### Anti-angiogenic therapy in lung cancer

Antiangiogenic therapy can be performed by blocking the expression or activity of angiogenic factors, or by introducing antiangiogenic factors into tumor cells. The orally available ZD6474 (vandetanib), a potent TKI of VEGFR-2 and EGFR, showed objective tumor regression activity in several solid tumor types including NSCLC, in a phase I trial (Tamura et al., 2006). AZD2171 is a TKI of VEGFR-1, 2, and 3, showed also the clinical activity in NSCLC patients (Hanrahan and Heymach, 2007). The anti-VEGF monoclonal antibody bevacizumab has demonstrated a significant clinical benefit in NSCLC patients in phase II and III trials (de Gramont and Van Cutsem, 2005; Vokes et al. 2006). Administration of siRNA targeting VEGF, VEGFR, or Raf-1 were also capable of, directly or indirectly, suppressing tumor angiogenesis and growth in preclinical studies (Filleur et al., 2003; Schiffelers et al., 2004; Takei et al., 2004; Culmsee et al., 2006). Angiostatin and endostatin are proteolytic products of plasminogen and collagen-XVIII respectively, which present antiangiogenic activities (O'Reilly et al. 1994, 1997); and the introduction of recombinant human angiostatin or endostatin showed clinical benefits in phase I and II studies in advanced NSCLC patients (Hansma et al., 2005; Kurup et al., 2006). In NSCLC, it was found that an imbalance of the expression of ELR+ (angiogenic) and ELR- (angiostatic) CXC chemokines exists, which favors angiogenesis and progressive tumor growth. The expression of ELR-CXC chemokines MIG (monokine induced by interferon gamma) or PF-4var (platelet factor-4 variant) was able to inhibit NSCLC tumor growth and metastasis development via a decrease of angiogenesis in preclinical studies (Addison et al. 2000; Struyf et al., 2007). Otherwise, an antiangiogenic approach is to introduce the tumor-suppressor gene p53, which has been suggested to play an antiangiogenic role through inhibiting VEGF expression and stimulating the brain-specific angiogenesis inhibitor-1 expression (Nishizaki et al., 1999).

## **II.4b. Matrix metalloproteinase (MMP)**

MMPs are a family of zinc endopeptidases capable of breaking down components of the extracellular membrane, thus degrading physical barriers and promoting the invasion and entry of cancer cells, into or out of blood and lymphatic vessels. It was also suggested that MMPs act as key regulators on tumor growth and angiogenesis, maintaining the environment that supports the initiation and development of tumor, both at primary and metastatic sites (Chambers and Matrisian, 1997; Nelson et al., 2000).

Development of MMP inhibition-based tumor therapy has been long, mostly using synthetic chemicals such as BMS-275291, Prinomastat, or Marimastat, and have all progressed to phase III trials of NSCLC patients. However, it seems that this class of agents did not produce the expected effect as suggested by preclinical studies (for review, see Ferreira et al., 2002).

## II.4c. Immunotherapy

### Immune-evasion of cancer cells

Despite of the highly developed immune system in mammalian, and the existence of tumor-specific antigens as well as the tumor-specific immune cells, effective antitumor immunity frequently fails. Although the reasons explaining the failure are still controversial, some of them can be summarized as:

1. Impaired tumor recognition by immune cells. Loss of expression of major histocompatibility complex (MHC) class I on tumor cell surface results in T cells failing to recognize tumors.

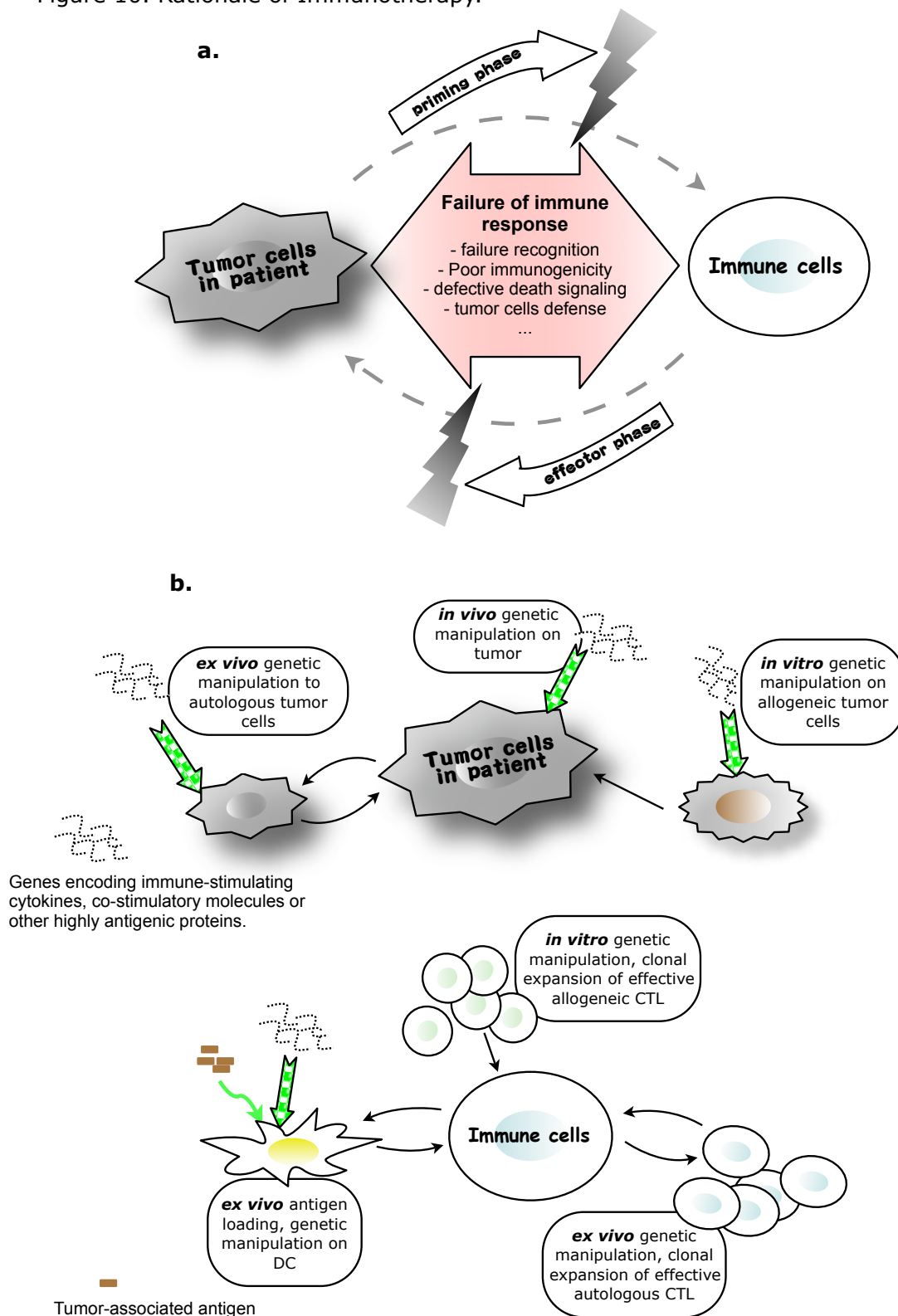
2. Poor tumor immunogenicity. Tumor cells are derived from host cells. Some antigens such as viral antigens and mutated gene products (*e.g.* p53, bcr/abl) are tumor-specific; but most known ones are “tumor-associated antigens” (TAAs), which are self-antigens and expressed also to some degree on normal tissues. This close relationship between tumor and self- antigen is a obstacle to the breaking of immune tolerance to cancer. Otherwise, some tumors may lack costimulatory molecules and result in failure of T cell priming. Finally, long-term avoidance from immune system may lead to evolution of tumor variants that do not express antigens.

3. Defective death receptor signaling. Two death receptors that play a role in immune surveillance against tumor are Fas and TRAILR. Down-regulation or loss of function/expression of these receptors as well as their downstream molecules (*e.g.* FADD, caspase 8, 10) in cancer cells can contribute to the resistance of cytotoxic T lymphocyte (CTL)-mediated apoptosis.

4. Defensive systems developed by tumors. Some tumor cells secrete immunosuppressive cytokines such as transforming growth factor  $\beta$  (TGF- $\beta$ ) or interleukin-10 (IL-10). A variety of cancer cells even express functional FasL ligand on the cell surface that induces apoptosis of lymphocyte.

(for review, see Armstrong et al., 2001; Khong and Restifo, 2002)

Figure 10. Rationale of Immunotherapy.



a/ The failure of immune response against cancer cells.

b/ Rounded rectangles indicate the strategies to re-boost immune system to target tumor cells. Genetic manipulations can be applied to tumor cells or immune cells, respectively. For a detail description, see the text.

Immunotherapies are strategies trying to re-boost the immune system to recognize and destroy cancer cells. Development of cancer immunotherapy has been long. Early attempts were focused on non-specific immune stimulants such as thymosin and BCG (Bacille Calmette-Guerin) during 1970s (Lipson et al., 1979; Issell et al., 1978), and the IL-2 and interferon- $\alpha$  (IFN- $\alpha$ ) until 1990s (Schiller et al., 1995). Despite of some positive results, overall benefit was weak. Later immunotherapy has combined genetic engineering technique to manipulate cancer or immune cells, to generate more powerful tumor- or dendritic cell (DC)- based vaccines, as well as effective CTLs (Fig. 10).

### Immunotherapy strategies- manipulating cancer cells

In order to produce a cancer vaccine, cancer cells are genetically engineered to be more properly detectable by the immune system. This is obtained by introducing genes that encode immune-stimulating cytokines, co-stimulatory molecules or other highly antigenic proteins. The transfection can be performed directly *in vivo*, or *ex vivo*. The latter uses cancer cells harvested from the patient himself (autologous), which are genetically modified, expanded, killed (e.g. by irradiation), and then re-injected to the patient. But the production of autologous tumor vaccines is expensive and time-consuming, and not all tumor cells can be efficiently expanded. Thus an alternative strategy is the use of other established cell lines (allogeneic) bearing common antigens to the patient's tumor type. More recently, the vaccine composed of autologous and allogeneic cancer was also explored (for review, see Dessureault et al., 2005; 2007), which showed to be feasible and can activate DCs and tumor-specific CTL responses in the phase I clinical trial. The phase II trial is underway.

### Immunotherapy strategies- manipulating immune cells

Genetic manipulations of immune cells for cancer therapy purpose are mostly focused on DC and CTL. DCs play a key role in human immunity. They are professional antigen presenting cells (APCs), able to capture and process antigens and to prime specific T-cell response. Coupled with well established technologies to generate autologous DCs from patient's peripheral blood monocytes or CD34<sup>+</sup>

haemopoietic stem cells into large number *in vitro*, vaccine development based on DC led to great interest in cancer immunotherapy. *Ex vivo* DC vaccine can be developed by loading with tumor antigen, either through direct incubation with a cocktail of TAAs or tumor lysate, or through gene transfer expressing antigens. Immunogenicity of the vaccine could be enhanced by transferring other genes such as those encoding co-stimulatory molecules, cytokines, or chemokines (for review, see Kikuchi, 2006).

Among different species of T lymphocytes, CD8<sup>+</sup> CTLs are particularly attractive for immunotherapy because they are direct effector cells. The use of allogeneic CD8<sup>+</sup> CTL started from allogeneic bone marrow transplantation, a common treatment of hematologic malignancies, in which donor-derived CTLs specific for patients' minor histocompatibility antigens (mHags) play an important role in both graft-versus-host disease (GVHD) and graft-versus-leukemia reactivities. This treatment was greatly improved by selective generations of mHags specific CTLs by antigen-pulsed DCs *ex vivo*, which provided an efficient way to produce large amount of specific CTLs against leukemia cells with low risk of GVHD (Mutis et al., 1999).

Usage of autologous CTL is evidently safer, but more expensive and time-consuming. The development of autologous CTL has been pioneered by Rosenberg and his co-workers in 1994, in which followed by IL-2 treatment, tumor-infiltrating lymphocytes (TILs) were isolated from patients and expanded *ex vivo*, then infused back to patients (Rosenberg et al., 1994). It then appeared that these TILs were rich in MHC class I-restricted CTLs specific to TAAs such as MART-1 and gp100 (Kawakami et al., 1996). Another strategy is the use of "chimeric antigen receptor", which is composed of the antigen recognition domain of a specific antitumoral antibody and the intracellular T cell receptor (TCR) -signaling chain. By expressing the chimeric antigen receptor on cell surface, these modified CTLs can be activated on contact to the specific tumor antigen without the need of MHC class I expression on tumor cell surface, -which is often lost or poorly expressed in tumor cells (Hwu et al., 1995). Later, to overcome the problem of poor expression of costimulatory molecules in tumor cells, the chimeric antigen receptor was improved to present a antigen-specific costimulation activity as well (Alvarez-Vallina and Hawkins, 1996; Finney et al., 1998). These manipulated autologous CTLs can be maintained in



culture by coculturing with antigen-expressing tumor cells, which leads to rapid tumor cell death and CTLs expansion, and can serve as resources of allogeneic CTLs as well for therapeutic and research use (Maher et al., 2002). A similar strategy is to introduce an “artificial TCR” specific of a defined TAA into autologous CTLs. Using artificial TCR allows the access to a greater repertoire of protein antigens as compared to chimeric antigen receptor, which can target only antigens expressed on tumor cell surface. However, artificial TCR is not effective to tumors expressing little or no MHC class I and costimulatory molecules (Maher and Davies, 2004).

#### Other immunotherapies- peptide vaccines, exosome and syncytiosome

One approach of immunotherapy to cancer is the vaccination with peptides derived from TAAs. Direct injection of peptide vaccine into human body avoids the extensive labor of pulsing autologous DC *ex vivo*. Recent improvements of this strategy include the addition of various adjuvants, the use of helper peptides or multipeptide vaccinations, and the delivery through mini-genes (Brinkman et al., 2004). Peptide-based cancer vaccines have demonstrated its feasibility in lung cancer treatment in clinic trials through inducing CD4<sup>+</sup> or CD8<sup>+</sup> T cell response (see examples below).

Exosome is a population of membrane vesicles with an average diameter of 60-90 nm, secreted after fusion of multivesicular endosomes with plasma membrane. Different cell types naturally produce exosomes, among which those secreted by professional APCs (especially DC) are highly immunogenic, containing MHC class I and II and costimulatory molecules (Zitvogel et al., 1998). Interestingly, tumor cells cultured *in vitro* were also found to secrete exosomes. The tumor-derived exosomes present MHC-I and are able to transfer autologous TAAs to DC, inducing potent CTL-dependent antitumor effects (Wolfers et al., 2001). This represents a novel source of cancer vaccine alternative to conventional whole cell (tumor or DC)-based material, and bears advantages in amplification scale, storage, and qualification processes (Chaput et al., 2004). The first Phase I trial in melanoma patients based on DC-exosome showed the feasibility and safety of this strategy (Escudier et al., 2005).

Recently described syncytiosomes are exosome-like small vesicles derived from dying syncytia. Syncytia formation can be simply induced by transfecting fusogenic

membrane glycoproteins (FMGs), a class of viral envelope proteins which recognize specific cell membrane receptors and play a role in viral entry into target cells. FMGs induce extensive cell-to-cell fusion, leading to the formation of giant multinucleated cells (the so-called syncytia) that eventually die after 2-5 days of formation (Bateman et al., 2000; Higuchi et al., 2000). Death of syncytia is mainly through necrosis pathway rather than apoptosis, and is accompanied by the release of abundant syncytiosomes that can also load DCs with TAA for cross-presentation and T-cell priming (Bateman et al., 2002). The study based on melanoma in murine model showed that when syncytia were formed by allogeneic cells fusion *in vivo*, an immune priming against specific TAA was observed, which protected the mice against a second challenge with the same tumor cells (Errington et al., 2006; Linardakis et al., 2002).

The study of FMG induced antitumor activity is also one of our research projects. For the description about it, see the Thesis RESULT and DISCUSSION, I.

### Current lung cancer immunotherapy in clinical trial

Several clinical trials have indicated initial data showing preliminary evidence of induction of immune responses and their clinical activities against lung cancer. These reports are introduced as below:

#### *Autologous tumor cell vaccine*

The tumor vaccine GVAX is composed of autologous tumor cells genetically modified to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), which demonstrated clinical activity in advanced-stage NSCLC. Tumors were harvested from 83 patients and vaccines were successfully manufactured for 67 patients, and 43 patients were vaccinated. Survival advantage was correlated with the doses of GM-CSF: the longer median survival (17 months) was observed in patients receiving vaccines secreting high levels of GM-CSF than in those receiving vaccines secreting low levels of GM-CSF (7 months) (Nemunaitis et al., 2004). For bypassing the extensive labor of genetic manipulations on each tumor, a “bystander” GVAX platform was then developed, which composed of autologous tumor cells mixed with an allogeneic GM-CSF-secreting cell line. However, it did not show a

more favorable outcome of the patients (Nemunaitis et al., 2006).

In another phase I clinical trial, an autologous cancer vaccine was generated by infecting tumor cells harvested from patients with a non-replicating canarypoxvirus (ALVAC) encoding both human carcinoembryonic antigen (CEA) and the B7.1 costimulatory molecule. Three of 6 patients presented a clinically stable disease correlated with increased CEA-specific precursor T-cells. This preliminary study demonstrated the feasibility of delivering a costimulatory molecule with a tumor antigen in order to improve the immune response (Ertl, 2002; Horig et al., 2000).

#### *Autologous dendritic cell vaccine*

A DC vaccine generated from autologous CD14<sup>+</sup> precursors, pulsed with apoptotic bodies derived from an allogeneic NSCLC cell line expressing HER-2, CEA, WT1, Mage2, and survivin was used in a phase I study. Although the vaccines were well tolerated, only 6 of 16 patients showed an antigen specific response, while 5 did not have any response and the other 5 showed a tumor-antigen independent response. Favorable and unfavorable clinical outcomes were independent of the specific immunologic responses (Hirschowitz et al., 2004; Yannelli et al., 2005).

#### *Allogeneic tumor cell vaccine*

An allogeneic tumor vaccine for NSCLC was established by transfecting an adenocarcinoma line AD100 with B7.1 (CD80) and HLA A1 or A2. In a phase I trial, 18 of 19 patients had measurable CD8 responses after three immunizations. Median survival of all patients was 18 months, suggesting a clinical benefit of the vaccine (Raez et al., 2004).

#### *Peptide-based vaccine*

The liposome-encapsulated peptide vaccine BLP25 contains a synthetic peptide derived from the mucinous carcinoma-associated glycoprotein MUC-1 antigen, which is a transmembrane protein associated with metastases overexpressed on many tumor cells. In a phase I clinical trial, no significant antitumor response was measured (Palmer et al., 2001). In a randomised phase II trial, the median survival was 17.2 months in the BLP25 vaccine arm versus 13 months in the best supportive care arm. Although the difference did not reach statistical significance, a much greater response was observed in a subgroup of patients (stage IIIB locoregional) treated by

the vaccine for whom the median survival time has not yet been reached on the date of publication (with a trend of >2 years) (Butts et al., 2005). These results suggest that BLP25 may have a great effect on survival in a selected subgroup of lung cancer patients.

Wilms' tumor gene WT1 is expressed at a high level in leukemias and several solid tumor including lung cancer. In a phase I trial, 26 patients with lung or other cancer types were intradermally injected with HLA-A\*2402-restricted 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant. Twelve of the 20 patients for whom the efficacy of WT1 vaccination could be assessed showed clinical responses such as reduction of tumor sizes or tumor markers. A clear correlation between the increase in the frequencies of WT1-specific cytotoxic T lymphocytes and clinical responses was observed (Oka et al., 2004).

Melanoma-associated antigen (MAGE)-3 is an antigen originally identified in melanoma but is also found to be expressed in lung tumors. In a phase II trial, vaccination of MAGE-3 recombinant protein without or with adjuvant AS02B to NSCLC patients successfully induced antibody, CD8<sup>+</sup>, and CD4<sup>+</sup> T cells responses. Seven of 8 patients received MAGE-3 with adjuvant developed high-titer antibodies against MAGE-3, and 4 of them had a strong concomitant CD4<sup>+</sup> T cell response to HLA-DP4-restricted peptide. One patient simultaneously developed CD8<sup>+</sup> T cells to HLA-A1-restricted peptide (Atanackovic et al., 2004).

(For a comprehensive review, see Raez et al., 2005, 2006)

## II.5. Other Strategies

Some strategies develop efficient ways to kill tumor cells, but do not target specifically any tumorigenesis pathway. These strategies could be applied universally to most kinds of tumors including lung cancer.

### II.5a. Suicide gene therapy

Suicide gene therapy is based on the use of a gene encoding an enzyme (suicide gene) that catalyses conversion of a normally nontoxic “prodrug” into a toxic substance (activated drug), resulting in selective killing of tumor cells. Although the suicide gene expression is limited to the transfected cells, the bystander effect -the passage of the activated drug to neighboring non-transduced tumor cells, enhances the toxic effect. The most commonly used suicide gene system so far is Herpes Simplex virus thymidine kinase (HSV-TK) gene in combination with the prodrug Ganciclovir (GCV). The activated phosphorylated form of GCV blocks DNA synthesis and causes cell death (Fillat et al., 2003).

Despite of very promising results obtained in animal models, a recent large HSV-TK phase III trial on 248 patients with primary brain tumors showed no clinical benefit. This could be due to the poor efficiency of the vector used (Rainov, 2000; Sandmair et al., 2000). A more recent phase I study involving glioblastoma patients using AdV delivered HSV-TK showed an improved median survival from 39 to 70.6 weeks (Immonen et al., 2004). This was also the first glioblastoma gene therapy trial to show any measurable improvement in survival. A phase III trial on resectable glioma using AdV was then scheduled (Palmer et al., 2006).

Different studies using lung cancer cell models have suggested the feasibility of the HSV-TK/GCV strategy *in vitro* and *in vivo* (Morimoto et al., 2001; Maatta et al., 2004), and a phase I trial based on AdV has been proposed in advanced NSCLC patients (Tolozza et al., 2006). Another suicide gene approach that has shown preliminary results in NSCLC cells was based on sodium iodide symporter and thyroperoxidase genes. The gene transfer resulted in rapid iodide uptake and retention and enhanced tumor cell apoptosis (Huang et al., 2001).

## II.5b. Oncolytic virus therapy

Oncolytic virus therapy is included in this section instead of in the “viral vector” paragraph, because the viruses used here are not designed for transfecting therapeutic genes, but for its capability to kill tumor cells directly. Oncolytic viruses are genetically engineered viruses able to target and destroy cancer cells, but are not cytotoxic to normal cells. Several viruses have been used, including vaccinia, adenovirus, herpes simplex virus I, vesicular stomatitis virus reovirus and Newcastle disease virus. They are engineered based on the rationale of breaking a viral gene that is normally required for virus replication, and whose function might be complemented by the cellular mutations in cancer cells. Thus oncolytic virus replication in normal cells is abrogated by the mutation of key viral replicative genes, or through normal cellular antiviral responses. Loss of cell cycle regulatory checkpoints such as p53 or Rb antiviral responses in cancer cells also facilitates virus replication and cell lysis.

Once more, despite of very promising results in preclinical models, no significant activity has been seen in clinical trials when these viruses were used as a single agent. As a technology not yet fully mature, there are still some aspects to be improved. Unique obstacles blocking oncolytic virotherapy in clinic including the host immune clearance to viral agents before they affect, and the expensive and cumbersome safety precautions because of using replicative viral particles. Otherwise, for a successful therapy, viral particle production/cell killing rates in the infected cancer cells should outstrip the growth rate of uninfected cancer cells, but this is difficult to achieve especially when dealing with large established tumors, thus combining with other therapy such as surgery in the initial treatment is necessary. But once these factors can be surmounted, oncolytic therapy approach holds great promise due to the selectivity and powerful killing nature, while retaining the flexibility to be ameliorated by introducing additional genes for synergistic effects (for review, see Cross and Burmester, 2006; Palmer et al., 2006; Liu and Kirn, 2007).

In lung cancer therapy, this approach is still in relatively preliminary stages of *in vitro* and preclinical part. The results obtained with oncolytic herpes vector G207 expressing secretable endostatin-angiostatin under control of a tumor-specific

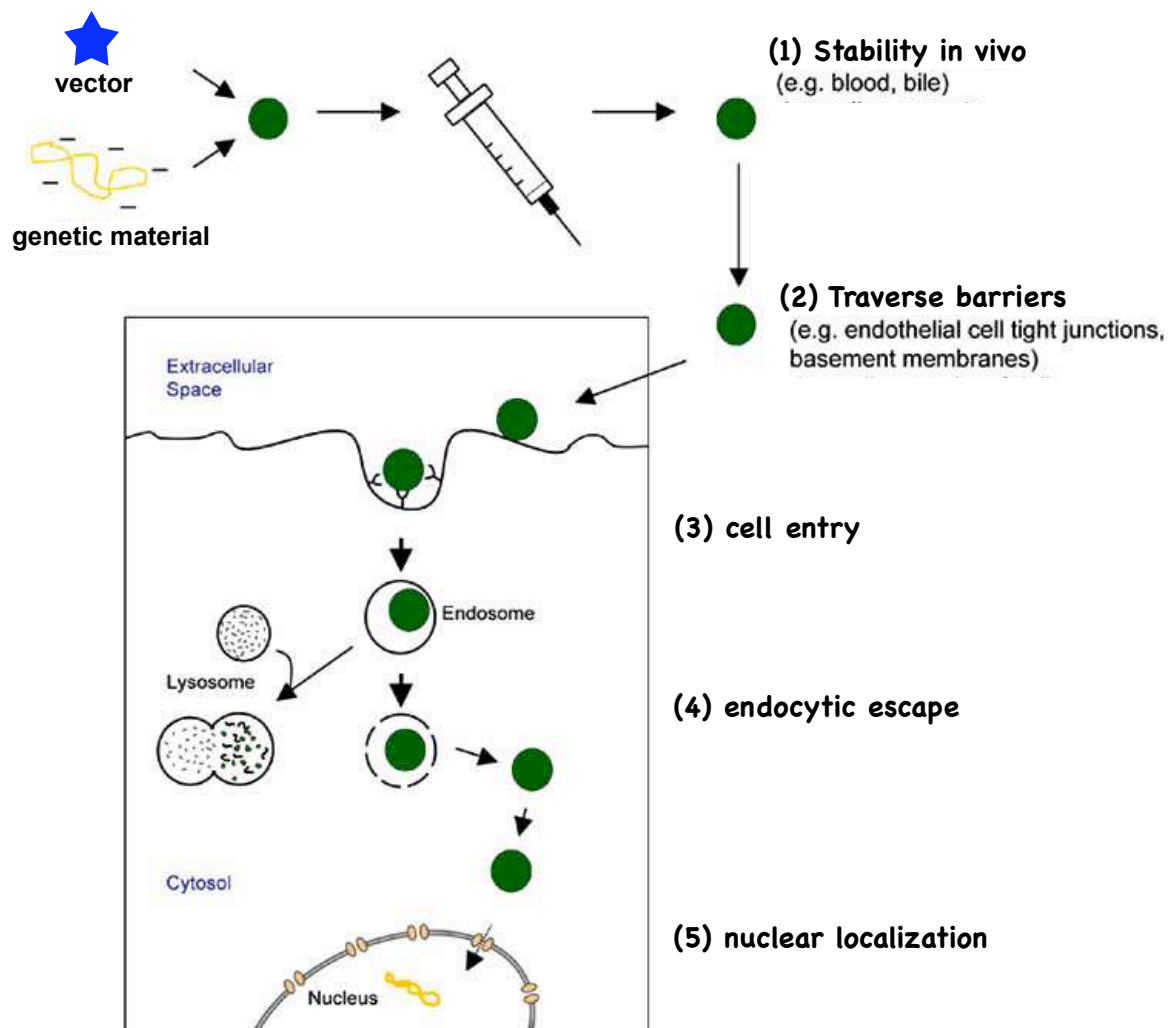
promoters ICP34.5, or survivin-mediated oncolytic adenovirus on NSCLC cells *in vitro* and *in vivo* have demonstrated tumor regression activity and shed light of this approach in lung cancer therapy (Kanai et al., 2006; Li et al., 2006; Yang et al., 2005).

### **II.5c. Fusogenic membrane glycoprotein (FMG)**

FMGs are able to induce syncytia formation and tumor cell death with a very strong bystander effect. Using FMGs as tumor therapy agent is one of our research projects. For the results of this part, see the Thesis RESULT and DISCUSSION, I.



Figure 11. The major barriers of vectorization in vivo.

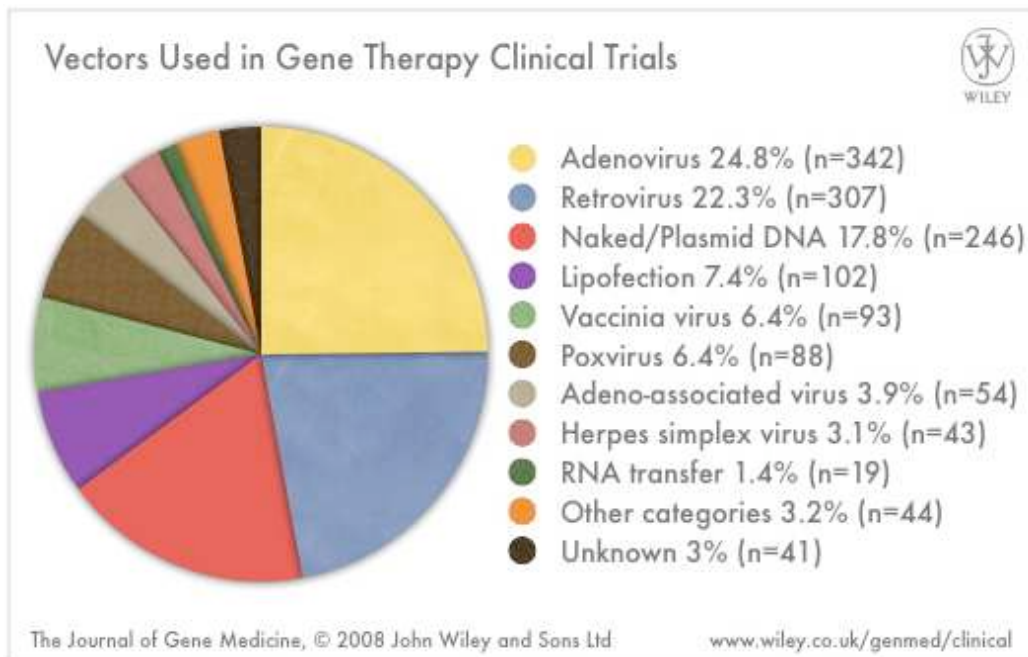


The in vivo vectorization of genetic material need to concern 1/ the stability in physiological environment, 2/ the ability to traverse multiple tissue barriers and to reach target cells, 3/ the mechanism of cell entry, 4/ the endosomal escape, 5/ (for DNA cargo) the arrival at the nucleus for gene expression. (Figure modified from Fabre and Collins, 2006)

### **III. Vectorization**

Several natural barriers have to be crossed by the therapeutic agents injected into body. For example, the genetic material will have to remain stable in the physiological environment (e.g. blood) in the presence of immune cells and enzymes. It needs to traverse multiple tissue barriers such as blood vessel endothelial wall, to have efficient cellular uptake (generally endocytosis or pinocytosis), and to escape from the endocytic pathway. Finally, the DNA material will have to reach the nucleus to be expressed (Fig. 11). Besides these obstacles, the bio-safety, duration, and cell-specific targeting are important issues for a successful vectorization (Pack et al., 2005).

Figure 12.

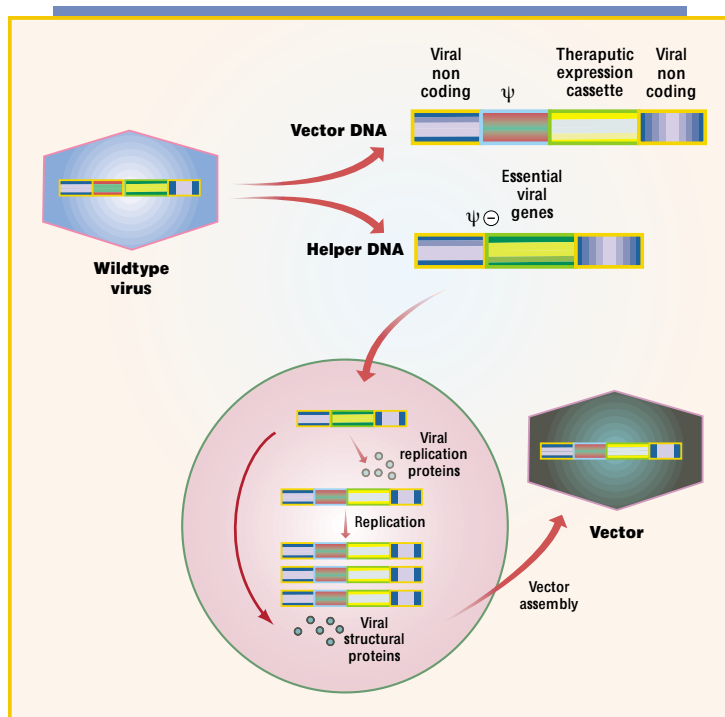


(Figure from Wiley Database, 2008)

Viruses have long evolved versatile functions to address these obstacles and therefore viral vectors offer relatively high transduction efficiency and stable gene expression. However, they are generally associated with problems of bio-safety and restricted target cell specificity (Thomas et al., 2003). Actually within 9 years, 2 patients died and 3 developed leukaemia related to the treatment with the different viral vectors. These accidents have severely hampered the usage of viral vector in human gene therapy. Non-viral vectors, on the other hand, are scarcely comparable to viral vectors in the transduction efficiency, but guarantee higher bio-safety and are amenable to chemical modifications that improve their stability and site-specificity (Boulaiz et al., 2005). Otherwise, therapeutic agents other than genetic material (e.g. antibody, antigen, enzyme or imaging molecules) can be delivered or included in some non-viral vectors such as new generation liposome or cell-penetrating peptides (CPP) (Pack et al., 2005). Currently, viral vectors are used in 67% of the gene therapy clinic trials (Fig. 12) (Wiley-Database, 2007).

The viral and non-viral vectors commonly applied in gene therapy will be introduced in following sections.

Figure 13. The art of turning infectious agents into vehicles of therapeutics.



For viral vector constructions, the wild-type virion is generally separated into 2 parts:  
 1/ the “helper DNA” that is placed in a heterologous DNA context such as a plasmid or helper virus, or is stably inserted in the genome of packaging cells. It contains viral genes essential for viral replication and packaging.  
 2/ the “vector DNA” that contains the therapeutic expression cassette and non-coding viral cis-acting elements essential for viral packaging.  $\psi$ , packaging domain.  
 As being transduced to the same cell, the viral vector containing the therapeutic gene can be produced, packaged, and released as a functional particle. (Figure from Kay et al., 2001)

Table 5. Main groups of viral vectors commonly applied in gene therapy

Vector	Genetic material	Packaging capacity	Tropism	Inflammatory potential	Vector genome forms	Main limitations	Main advantages
<b>Enveloped</b>							
Retrovirus	RNA	8 kb	Dividing cells only	Low	Integrated	Only transduces dividing cells; integration might induce oncogenesis in some applications	Persistent gene transfer in dividing cells
Lentivirus	RNA	8 kb	Broad	Low	Integrated	Integration might induce oncogenesis in some applications	Persistent gene transfer in most tissues
HSV-1	dsDNA	40 kb* 150 kb <sup>†</sup>	Strong for neurons	High	Episomal	Inflammatory; transient transgene expression in cells other than neurons	Large packaging capacity; strong tropism for neurons
<b>Non-enveloped</b>							
AAV	ssDNA	<5 kb	Broad, with the possible exception of haematopoietic cells	Low	Episomal (>90%) Integrated (<10%)	Small packaging capacity	Non-inflammatory; non-pathogenic
Adenovirus	dsDNA	8 kb* 30 kb <sup>§</sup>	Broad	High	Episomal	Capsid mediates a potent inflammatory response	Extremely efficient transduction of most tissues

\*Replication defective. †Amplicon. ‡Helper dependent. AAV, adeno-associated viral vector; dsDNA, double-stranded DNA; HSV-1, herpes simplex virus-1; ssDNA, single-stranded DNA.

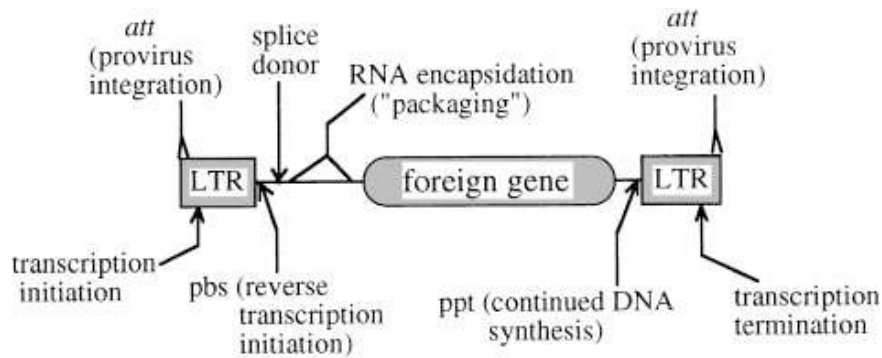
(Table from Thomas et al., 2003)

### III.1. Viral Vectors

Viral vectors are modified viruses that cannot replicate or cause diseases, while remaining capable of delivering and expressing exogenous (therapeutic) genes to target cells. To achieve this, the general strategy is to separate the viral genes and *cis*-elements of viral genome into distinct reservoirs to prevent their reconstitution into replicative viral particles. Viral genes are provided *in trans* as “helper DNA”, which can be expressed heterologously (by plasmid or helper virus) or incorporated in the chromatin of producer cells; whereas viral *cis*-acting elements are linked to the therapeutic gene expression cassette. Thus replication-defective viral particles encoding therapeutic gene can be produced when the 2 parts are transduced to the same producer cells (Fig. 13) (Kay et al., 2001).

Viral vectors derived from different species have their respective features and benefits (Tab. 5). The 5 viral vectors most commonly applied in human gene therapy will be introduced in the following sections.

Figure 14. Schematic illustration of the retroviral vector design.



The figure illustrates the construct of a retroviral vector. All viral genes are deleted and replaced by the foreign gene(s), of which the expression can be driven by the viral LTR directly, or from another internal heterologous promoter. Cis-acting elements necessary for vector replication and package are on the LTRs and neighboring regions. (Figure from Buchschacher and Wong-Staal, 2000)

### III.1a. Retrovirus

Retrovirus comprises the lipid-enveloped particle and a homodimer of linear, positive-sense, single-stranded (ss) RNA genome ranging from 7-11 kb. Three subgroups are included in this family: oncoretrovirus (e.g. mammalian and avian leukaemia viruses), lentivirus (e.g. HIV or other immunodeficiency viruses), and spumavirus (also known as foamy virus). Most recombinant retroviral vectors are derived from the murine leukemia virus (MLV), which were also the earliest viral vectors developed for gene therapy and are still the most widely used one in clinical trials to date (Sinn et al., 2005).

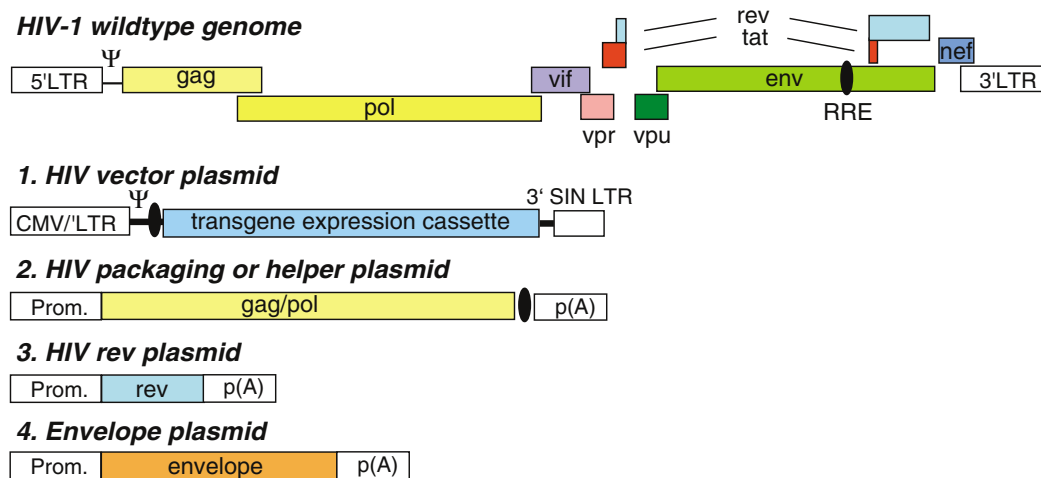
All retroviral genomes contain the long terminal repeats (LTRs) at both ends and at least the 3 genes *gag*, *pol*, and *env*, which encode the structural (or core) protein, nucleic acid polymerase/integrases and envelope glycoprotein, respectively. The LTRs and neighboring sequences contain *cis*-acting informations necessary for virus replication (Fig. 14). For vector design, all viral genes are removed and replaced by the foreign (therapeutic) gene(s) with the maximum size up to ~8 kb (Fig. 14). Viral vectors can be amplified and packaged when the viral genes are supplied *in trans* in packaging cell lines (Kay et al., 2001; Sinn et al., 2005).

Retroviral vectors offer a means to permanently correct genetic diseases by integration and stably expressing a transgene in renewing tissues. Nonetheless, their application is limited only in dividing cells because the disruption of the nuclear membrane is required for the viral pre-integration complex to access to the chromatin (Kay et al., 2001). Moreover, the integrating nature permits retroviral vector to carry the risk of insertional mutagenesis. The retroviral ITRs contain strong enhancer/promoter activity and their integration may transactivate a cellular gene adjacent to the insertion site, which probably represents the most significant risk since a single integration can result in the dominant phenotype (Sinn et al., 2005). Actually the retroviral vector mediated insertional mutagenesis has become a serious safety concern after 3 of the SCID-X1 patients cured by the *ex vivo* transduction of  $\gamma$ -c chain cytokine receptor gene into bone marrow stem cells developed leukaemia in a clinical trial, due to the aberrant activation of LMO2 oncogene through the vector integration (Cavazzana-Calvo et al., 2000; Check, 2005; Hacein-Bey-Abina et al., 2003). The



later genome-wide mapping study showed that the integration of MLV in the human genome prefers regions near the start of transcriptional units (Wu et al., 2003). These findings indeed hampered the application of retroviral vector in human gene therapy.

Figure 15. Schematic illustration of the wild-type HIV-1 genome, and the constructs developed for lentiviral vector generation.



Lentiviruses have a more complex genome than retrovirus. Besides *gag*, *pol*, and *env*, 2 regulatory genes *tat* and *rev* are essential for viral replication. A variable set of accessory genes (e.g. *vif*, *vpr*) exists depending on species.

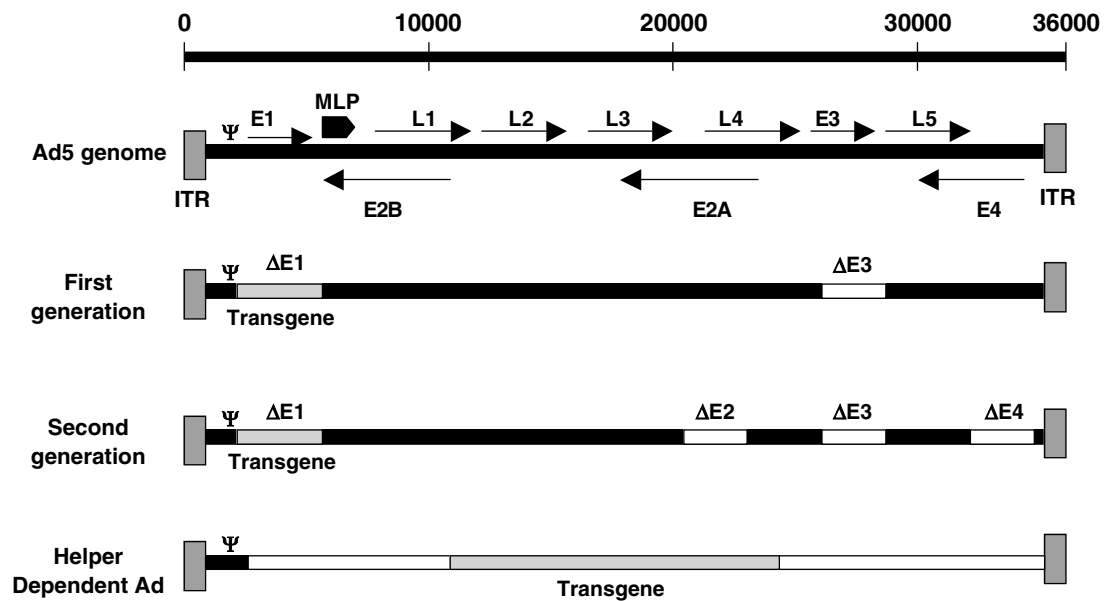
On the “minimal HIV vector construct” (1.) all viral genes are removed. It consists of the CMV/HIV LTR hybrid promoter followed by the packaging signal ( $\Psi$ ), the transgene expression cassette, and the 3' self-inactivating (SIN) LTR. Viral genes necessary for vector amplification and package are encoded on a “packaging plasmid” (2.), a “*rev* expressing plasmid” (3.), and an “envelope expressing plasmid” (4.). The expression of all 4 plasmids in a packaging cell is needed for producing the lentiviral vector, while lowering the risk of generating the replication competent virus (typically caused by homologous recombination) as well. (Figure from Sinn et al., 2005)

### III.1b. Lentivirus

Lentivirus is a subtype of retrovirus. The major advantage of lentiviral vectors over MLV is that they perform active transport of the preintegration complex through the nucleopore, thus are amenable to infect non-dividing cells. Lentivirus has a more complex genome: besides *gag*, *pol*, and *env*, 2 regulatory genes *tat* and *rev* are essential for viral replication, whereas a variable set of other accessory genes exist. Lentiviral vectors are originally derived from human immunodeficiency virus (HIV)-1. Other non-human lentivirus (e.g. simian, feline, or equine) have also been exploited for gene therapy approaches based on the safety rationale that their parental viruses don't infect human (Buchsacher and Wong-Staal, 2000; Sinn et al., 2005).

Because of the natural pathogenicity of HIV, biosafety is a major concern when using lentiviral vectors. Extensive efforts have been made to establish the “minimal” vector construct and the conditional packaging system (Fig. 15) (Dull et al., 1998). Self-inactivating (SIN) vector system further improves the safety profiles, in which the U3 region of the viral LTR is selectively deleted to diminish the LTR enhancer/promoter activity and the risk of vector mobilization and recombination (Logan et al., 2004; Miyoshi et al., 1998). However, the risk of insertional mutagenesis as mentioned in the previous section is still a significant safety concern. The genome-wide mapping has revealed that active genes in the human genome were preferential integration targets for HIV-1 (Schroder et al., 2002; Wu et al., 2003). More researches for dissecting the integration process and related factors behind are necessary to improve the safety of using these integrating vectors in human gene therapy applications (Kay et al., 2001; Sinn et al., 2005).

Figure 16. The maps of adenovirus serotype 5 genome and different generations of AdVs



E1–E4, early transcripts regions 1–4; L1–L5, late transcripts regions 1–5; MLP, major late promoter; Ψ, packaging signal. (Figure from Alba et al., 2005)

### III.1c. Adenovirus

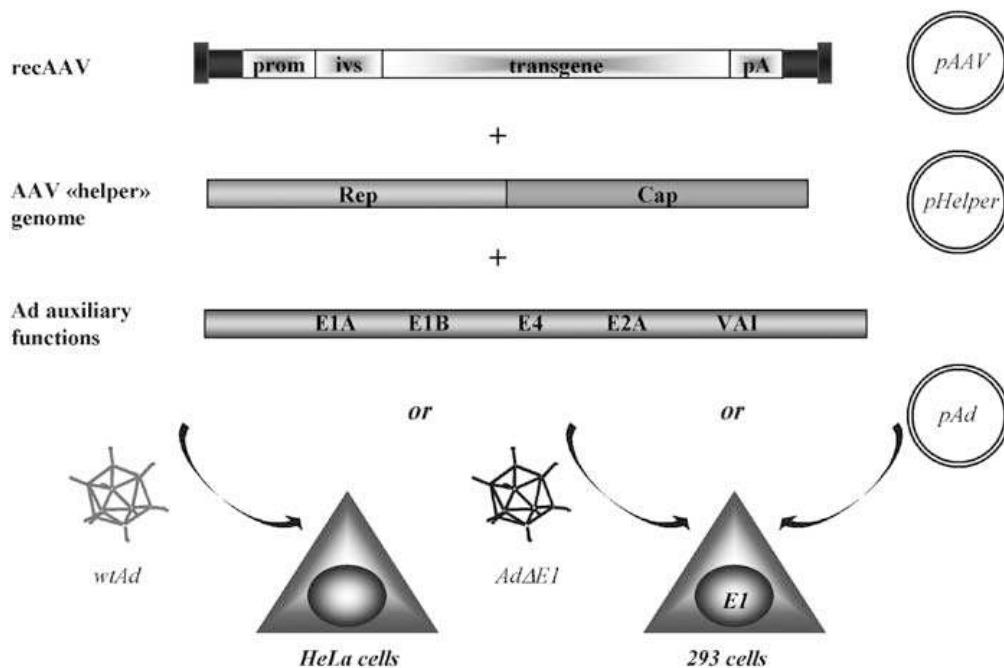
Adenovirus is a non-enveloped virus with the icosahedral capsid 60-90 nm in diameter and a linear double-stranded (ds) DNA genome. It causes benign respiratory tract infections in humans and most adults have already been exposed. Adenoviral vector (AdV) is one of the most popularly used viral vectors, mainly because of the availability of high-titer propagation and the broad range of host cell infections, including both proliferating and quiescent cell. After infecting cells, the core protein-coated viral genome is efficiently delivered to the nucleus through microtubuli-assisted transport and persists episomally. Since it does not integrate into the host genome, AdV expresses the transgene transiently and the risk for insertional mutation is very low (Kay et al., 2001; Thomas et al., 2003).

The natural virion contains approximately 36 kb of DNA with overlapping transcriptional units encoding over 50 polypeptides, among which the early region 1 (E1), E2 and E4 genes are required for viral genome replication. The deletion of E1 allows the generation of replication-deficient AdV ( the 1<sup>st</sup>-generation vector), which provides a space of 7-8 kb available for foreign gene expression cassette (Fig. 16) (Bangari and Mittal, 2006). This AdV can be amplified in the complementing producer cell line such as HEK293, which provides E1 functions *in trans*. The potent immunogenicity of 1<sup>st</sup>-generation AdV is a major obstacle for its application in human . Even in absence of E1 gene products, low-level transcription of other viral genes is sufficient to induce innate cytokine responses, followed by strong cytotoxic T-cell priming and the elimination of transduced cells (Bangari and Mittal, 2006). To approach this problem, a 2<sup>nd</sup>-generation AdVs contain additional deletions on E2 and/or E4 genes and showed reduced toxicity in animal models (Fig. 16). An important advance was the generation of helper-dependent (HD, also known as “gutless” or “guttled”) AdV, which was stripped for all viral genes but contains only viral inverted terminal repeats (ITRs) and the packaging recognition signal (Mitani et al., 1995). HD AdV has substantially reduced immunogenicity and performed long-term transgene expression (>1 year in liver cells in the mouse model) with negligible toxicity, except the unavoidable neutralizing antibody generation and cytokine-mediated inflammatory responses (Bangari and Mittal, 2006; Volpers and Kochanek, 2004). The carrying capacity of foreign DNA was greatly enhanced to 28-32 kb in HD AdV

because of the deletion of all viral genes (Fig. 16). The difficulty to scale up and the contamination of unwanted helper virus are major limits for the application of HD AdV in clinic. A special packaging system based on Cre/loxP site-specific recombination is able to excise the packaging signal on helper virus in packaging cells, thus efficiently enhances the HD AdV production and reduces the contamination of helper virus (Parks et al., 1996).

In clinic application, the use of AdV however, has been severely hampered. In September 1999, a 18-year-old youth was dead in a gene-therapy clinical trial at the University of Pennsylvania after receiving a 2<sup>nd</sup>-generation (E1- and E4-deleted) AdV to deliver the gene for OTC (ornithine transcarbamylase, a liver enzyme that is required for the safe removal of excessive nitrogen from amino acids and proteins) to the liver. Although the other female patient who received a similar vector dose ( $3.6 \times 10^{13}$  particles) experienced no unexpected side effects, the young patient developed a high fever and displayed symptoms of liver injury, and died from multiorgan failure within 4 days after the treatment. The following verification showed that systemic delivery of the vector triggered a massive inflammatory response that led to disseminated intravascular coagulation, acute respiratory distress and multiorgan failure. Subsequent researches in monkeys have indicated that the adenovirus capsid proteins, rather than the genetic cargo, might elicit an early inflammatory cytokine cascade. Although this fatal accident might be only a single special case, it has raised a high safety control to AdV in gene delivery manipulations. Especially, the vector dose and the interactions between viral vectors and human immune system should be tightly followed and investigated (Marshall, 1999; Thomas et al., 2003).

Figure 17. The rationale of AAV vector design and production



In the AAV vector, the viral ITRs at both terminals are retained but viral ORFs inside are replaced by the transgene expression cassette (pAAV). The AAV rep/cap genes are removed to another vector (pHelper) and must be supported in trans during vector production. The other part necessary for AAV vector propagation is the helper virus activity, which can be provided by wtAd or the E1-deleted AdV (AdΔE1, the 1<sup>st</sup>-generation AdV; in case that 293 cells are used) infection to producer cells. An alternative protocol is using a plasmid expressing essential helper genes (pAd) instead of living virus, which can avoid the unwanted contamination of helper virus.

Prom: promoter, IVS: intervening sequence (e.g. intron), wtAd: wild-type adenovirus. (Figure from Merten et al., 2005)

### III.1d. Adeno-associated virus

The Adeno-associated virus (AAV) was initially discovered as a contaminant of adenovirus preparations. It belongs to Parvoviridae family, consists of a small nonenveloped capsid ~18-25 nm and a ~4.7 kb ssDNA genome, either plus or minus sense. While 80-90% of adults are sero-positive for antibodies against AAV, there is no known disease associated with AAV infection (Lehtonen and Tenenbaum, 2003). AAV genome contains 2 ITRs at both ends flanking 2 open-reading frames (ORFs) *rep* and *cap*, which produce multiple polypeptides required for viral replication and packaging, respectively. After entering cells, the viral genome is transported into the nucleus and converted to dsDNA. In the absence of helper virus such as adenovirus, AAV integrates into the host genome and latently persists. Instead, AAV is actively replicated, packaged, and released when the helper virus is present (Lehtonen and Tenenbaum, 2003; Merten et al., 2005).

For the vector design, the 2 viral ORFs are removed and replaced by the transgene expression cassette, and the viral ITRs at both terminals are retained, which include *cis*-elements necessary for vector replication, packaging, and host genome integration. To produce AAV vectors, besides the *rep/cap* genes supported *in trans*, the helper virus activity is obligatory -which can be provided by virus infection or transfecting a plasmid expressing essential helper genes (Fig. 17) (Merten et al., 2005). Wt AAV has the unique property of integration at a specific locus (AAVS1 or q13.3-ter) on chromosome 19 of human genome without pathogenicity, but this property is not maintained in the recombinant AAV vector because of the absence of the *rep* gene (Lehtonen and Tenenbaum). AAV vectors persist in cells largely in the episomal form, or could also randomly integrate into the host genome at chromosomal breaks (Duan et al., 1998; Miller et al., 2004).

AAV vector bears several advantages for gene therapy such as its non-pathogenic nature, low immunogenicity, long-term transgene expression potential, ability to transduce both dividing and non-dividing cells, and broad host tropism including highly differentiated post-mitotic tissues such as skeletal muscle and central nervous system (CNS) (Buning et al., 2004; Merten et al., 2005). The lack of an efficient procedure for large-scale production and the limited package size (<5 kb) are major



restrictions for AAV vector's application (Merten et al., 2005). Based on the knowledge of vector genome linkage, some groups have demonstrated an enlarged coding capacity by splitting the expression cassette into 2 vectors and co-administrating them into the same cells (Nakai et al., 2000; Sun et al., 2000).

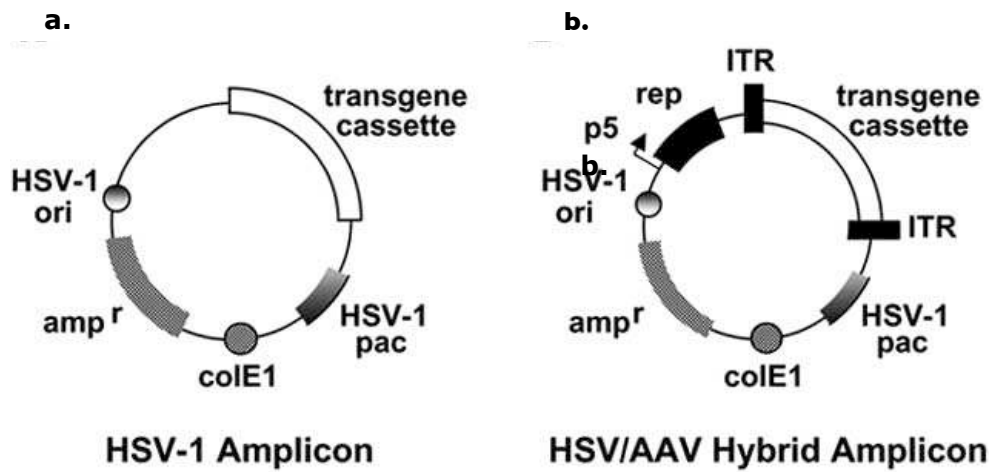
The major safety concern of AAV vector still comes from its integrating nature. It has been reported that in mouse hepatocytes transduced by AAV vector the integrations preferentially targeted active genes, and chromosomal deletions or other rearrangements at insertion sites were frequent (Miller et al., 2002; Nakai et al., 2003; Russell, 2003). However, it was a late fatal accident that severely questioned the safety issue of AAV vector's use in clinic. Like AdV, a patient in USA died after being treated with an AAV vector. It was a clinical trial sponsored by Targeted Genetics Corp. in Seattle in 2007, in which an AAV vector was used to transfer a gene encoding Enbrel (an inhibitor a pro-inflammatory cytokine TNF- $\alpha$ ) into joint cells of rheumatoid arthritis patients. The joint cells can then give patients a "localized depot" of Enbrel that should work on the long-term. The trial had enrolled 127 patients without any serious side effects, and 74 of them had received a second dose. However, one patient developed a severe adverse event after a second injection, and died 4 days later from the histoplasmosis and a large blood clot. This is a surprising accident since more than 500 patients have safely received AAV vector since 1992. The later reports from the company and the Recombinant DNA Advisory Committee suggested that the therapy and the viral vector should not be the cause of the patient's death. The Food and Drug Administration had also lifted its hold on the trial later. The company now plans to resume the safety trial of 127 patients, but a second dose will not be administrated to the patients if a fever or other signs of infection is shown. Otherwise, this AAV-based gene therapy has restored the health of about 20 children, yet with severe combined immunodeficiency (Kaiser, 2007a, 2007b). This is another case to show that, applying a viral vector may still present some risks that we do not fully understand or controlled, such as the interactions between the viral vector and the host.

### III.1e. Herpes Simplex virus

Herpes Simplex virus (HSV) contains a large dsDNA genome (~152 kb), an icosadeltahedral capsid, and a trilaminar lipid envelope. As latently persisting in cells, the viral genome maintains as a circular episome within nuclei and almost completely silences all viral transcription except the latency-associated transcripts (LATs), which is a set of untranslated RNA species of unclear functions. In lytic cycle, more than 80 genes are expressed in a cascade fashion, which will lead to host cell death and some mild illness symptoms such as cold sores in human (Burton et al., 2002; Epstein et al., 2005). Replication-defective HSV-1 vector is constructed by deleting a single (1<sup>st</sup>-generation) or a combination (2<sup>ed</sup>-generation) of the 5 immediate-early (IE) genes (ICP0, ICP4, ICP22, ICP27 and ICP47), which are essential for viral lytic infection and the expression of all viral proteins. HSV vector can be amplified and packaged in producer cells supplying the corresponding IE gene(s) *in trans* (Burton et al., 2002). Deletion of all five IE genes silences almost all viral gene expression and viral toxicity, but these vectors grow poorly and express transgenes also at very low levels due to the absence of the ICP0 transactivator, which is cytotoxic but required for sustaining the transgene expression (Burton et al., 2002; Samaniego et al., 1998). A protein encoded by the LAT was discovered to be able to complement ICP0 and overcome the repression of transgene expression on HSV vector. Substitution of this protein for ICP0 might facilitate efficient transgene expression without cytotoxicity in non-neuronal cells (Thomas et al., 1999, 2002b).

HSV vector is highly infectious and have a broad host range including non-dividing cells. Since about half of viral genes are not essential for viral replication, large capacity (>30 kb) is available by replacing dispensable viral genes with various therapeutic genes. HSV vector has the potential for long-term transgene expression without integrating into the host genome. Specially for nervous cells, it is possible to insert transgenes within LAT loci for chronic and stable expression (Burton et al., 2002).

Figure 18. HSV amplicons.



- a/ The standard HSV-1 amplicon is composed of the bacterial origin of replication (colE1), the antibiotic resistance gene (amp<sup>r</sup>), the HSV viral origin replication (HSV-1 ori), and the DNA packaging/cleavage signal (pac). The capacity of transgene expression cassette can be up to 150 kb.
- b/ HSV/AAV hybrid amplicon contains additionally the transgene cassette flanked by AAV-ITRs, along with the AAV rep gene under the control of its native p5 promoter.
- (figure from Glauser et al., 2006)

HSV amplicon is an alternative to replication-defective vectors. Amplicon is a bacterial plasmid carrying only two HSV-1 *cis*-elements: the origin of DNA replication and the DNA packaging/cleavage signal (Fig. 18a). Thus it is able to be amplified in *E. coli* and then packaged into HSV vector in mammalian cells by transfecting amplicon along with infecting the producer cells by the helper HSV (generally a replication-defective mutant) virus (Fraefel et al., 2000; Glauser et al., 2006). The advantages of amplicon includes being fully non-toxic for infected cells and the huge capacity for transgenes of >100 kb. In contrast, the low propagation rate, the contamination of helper virus, and the transient transgene expression are the drawbacks (Fraefel et al., 2000; Glauser et al., 2006). An approach is using the chimeric HSV/AAV hybrid amplicon (Fig. 18b). In addition to the *cis*-elements of the basic HSV amplicon, HSV/AAV amplicon contains a AAV-ITRs flanked transgene cassette and the AAV *rep* gene under the control of its native p5 promoter. HSV/AAV amplicon surmounts the capacity limit of AAV vector and gives rise to the stable transgene expression in the AAV site-specific manner (Glauser et al., 2006; Wang et al., 2002).

## III.2. Non-Viral Vectors

Non-viral vectors deliver genes or other therapeutic agents into cells through physical or chemical means. Generally they are non-infectious, less toxic and immunogenic. Moreover, they are easy to prepare, scale-up, and allow the delivery of large DNA fragments. But the low transduction efficiency and the transient expressing duration are still obstacles to their application in clinic (Boulaiz et al., 2005).

### III.2a. Physical means

#### Microinjection

Microinjection is the direct transduction of genetic materials into cells by a micromanipulator. Although it's non-toxic and straightforward for getting transgene expression, the manipulation is slow (transducing one cell at a time) and laborious. Otherwise, the gene expression is generally low and transient. Microinjection is therefore limited to some special applications such as in vaccination procedures or *ex vivo* delivery of artificial chromosomes. (Davis et al., 1993; Telenius et al., 1999)

#### Particle bombardment

Particle bombardment, also known as ballistic delivery, is a way to transfer DNA across cell membrane into cells by the physical force of impact. The DNA should be covered with metal (e.g. gold or tungsten) microparticles so that it can be accelerated to high velocity by electrical discharge or gas jet (Boulaiz et al., 2005). This method is simple and safe, applied in gene transfer to protozoa or *ex vivo* gene therapy purpose (Wittig et al., 2001). Current *in vivo* applications are limited to cutaneous targets in animals.

#### Naked DNA injection and hydrodynamic-based transfection

Naked DNA can be transfected *in vivo* simply by intramuscular or portal vein

injections (Herweijer and Wolff, 2003). It was demonstrated that a hydrodynamic-based transfection procedure, by injecting rapidly the DNA solution in large volume through tail vein, was able to induce high levels of transgene expression in mouse hepatocytes (Liu et al., 1999; Zhang et al., 1999). This method was then found applicable to transfer siRNA, or large size (>150 kb) bacterial artificial chromosome (BAC) to mouse liver (Lewis et al., 2002a; Magin-Lachmann et al., 2004; McCaffrey et al., 2002). Hydrodynamics-based transfection thus provides a convenient, economic, and non-toxic means for *in vivo* research.

The intramuscular transfection can be used in human by local administration with temporary increase in blood pressure, as already illustrated in nonhuman primates (Zhang et al., 2001).

### Electroporation

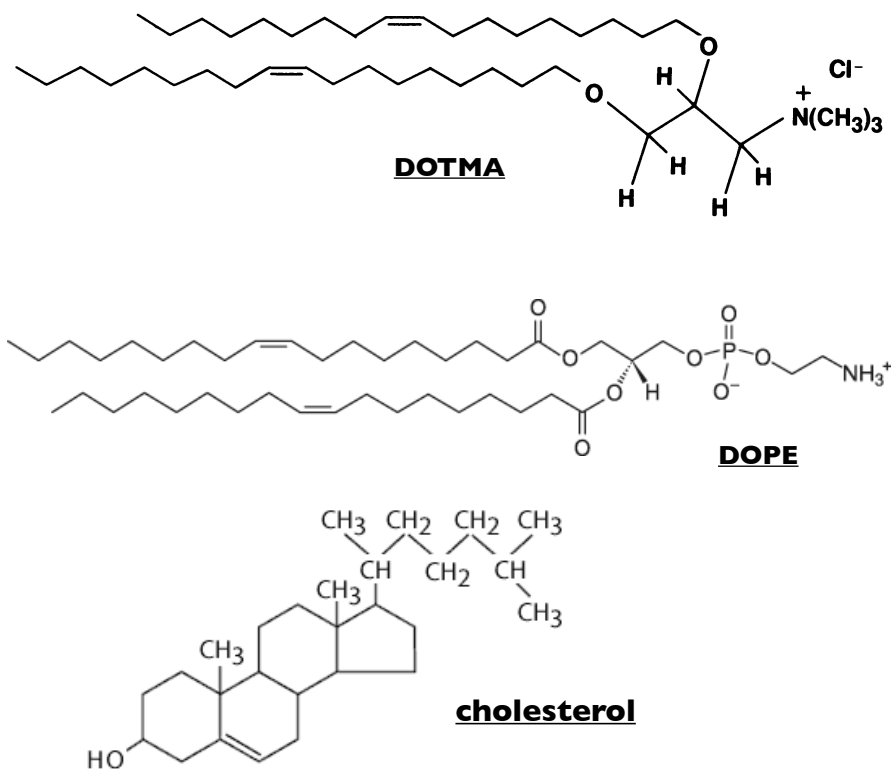
Electroporation is a technology to administer a controlled electric pulse to cell surface, which produces nanometric pores on the membrane and allows the transfer of negatively-charged DNA into the cell (Andre and Mir, 2004). Although it was initially restricted to *in vitro* for research purpose, development of new electrodes have allowed this technique to be applied *in vivo*, with appropriate pulses in contained areas such as tumor, muscle or liver (Andre and Mir, 2004; Herweijer and Wolff, 2003). Electroporation was successful for the delivery of the dystrophin gene in skeletal muscles in animal models of muscular dystrophies (Vilquin et al., 2001). Delivery of large size (>150 kb) BAC by electroporation was achieved both *in vitro* and *in vivo* (Magin-Lachmann et al., 2004).

### Others novel methodologies

Some novel physical approaches such as ultrasound and mechanical massage were exploited. The ultrasound irradiation with echo contrast microbubble induces the transient formation of holes (<5  $\mu\text{M}$ ) in the cell surface, and is able to transfect naked DNA *in vitro* and *in vivo* in skeletal muscles (Taniyama et al., 2002). Mouse liver can

be transfected by mechanical massage after intravenous (IV) injection of naked DNA (Liu et al., 2004). The mechanism behind is suggested to be associated with blood pressure.

Figure 19. Some lipids commonly used in gene delivery



Structures of the first developed cationic lipid that delivered DNA efficiently into cells (DOTMA), and the commonly used neutral helper lipid (DOPE and cholesterol).



## Chemical Means

The synthetic non-viral vectors can be mainly separated into 2 types: 1/ the cationic lipids, 2/ cationic polymers/proteins, such as polylysine, protamine or polyethylenimine (PEI) (Scherman et al., 1998). Nonetheless, later developments of combined vectors including both kinds of material were also reported. They will be introduced in following sections.

### III.2b. Lipid-based Vectorization

#### Cationic Lipid

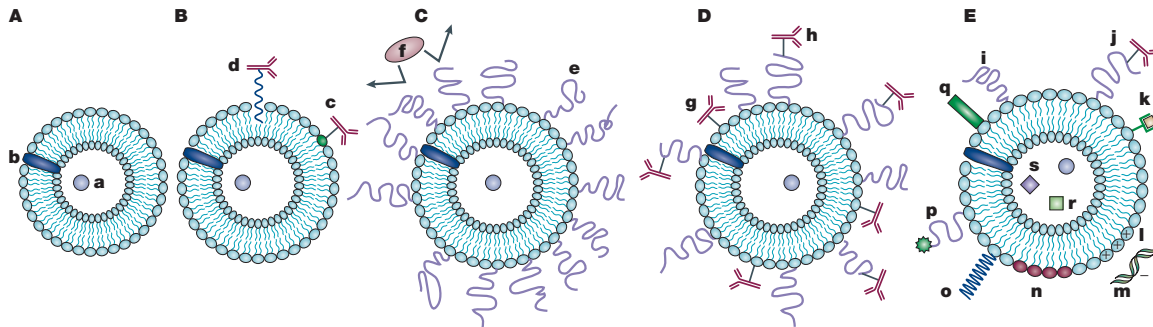
Cationic lipids are amphiphilic molecules which can entrap DNA and condense it into particles (lipid/DNA complex, or termed lipoplex). These lipoplexes are able to interact with the cellular plasma membrane and to efficiently promote plasmid entry into the cells (Scherman et al., 1998). The first efficient lipoplex transfection was described by the use of synthetic cationic lipid, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) (Fig. 19) (Felgner et al., 1987; Felgner and Ringold, 1989). Later on, several different kinds of cationic lipids suited for DNA delivery were developed.

It was later shown that either cationic liposome or lipid/DNA lipoplex enters cells mainly through endocytosis pathway (Friend et al., 1996; Wrobel and Collins, 1995). The addition of some neutrally charged “helper” lipid, such as dioleoyl-phosphatidylethanolamine (DOPE), cholesterol (Fig. 19) to liposome improved the transfection efficiency of DNA or oligonucleotide, presumably due to the promotions of endosomal membrane disruption and cytoplasmic release of encapsulated DNA after endocytosis (Hafez et al., 2001; Smisterova et al., 2001; Zuhorn et al., 2005). Other endosomal escape strategies were also applied to cationic lipid to enhance transfection efficiency, such as grafting a histidine residue or incorporating a pH-sensitive surfactant (Kumar et al., 2003; Liang and Hughes, 1998; Singh et al., 2004).

Until today, 7.4% of clinical trials use the lipofection as delivery method (Figure 12). In cancer therapy, lipoplex-mediated gene transfer has been applied in phase I and II trials for melanoma and breast and head and neck cancer treatment (Stopeck et al.,

2001; Yoo et al., 2001), and showed its feasibility and safety. The major advantages of cationic lipids are the ease to prepare and their non-immunogenicity. The relatively insufficient transfection efficiency and their interactions with serum proteins, which can reduce their *in vivo* efficiency and lead to deposition in the capillary, are their drawbacks (Dass and Choong, 2006). Development of new or modified cationic lipids better suited for *in vivo* transfection is still necessary.

Figure 20. Evolution of liposomes.



A/ Early traditional phospholipids ‘plain’ liposomes with water soluble drug (a) entrapped in the aqueous liposome interior, and the hydrophobic drug (b) incorporated in the liposomal membrane.

B/ The “immuno-liposome” with antibodies covalently coupled (c) to the phospholipid or anchored (d) in the liposomal membrane through a hydrophobic linker.

C/ Long-circulating liposome are grafted with a protective polymer (e) (e.g. polyethylene glycol) that shields the liposome surface from the interaction with opsonizing proteins (f).

D/ Long-circulating immuno-liposome simultaneously bearing both protective polymer and antibody. The antibody can be attached to the liposome surface (g) or to the distal end of the grafted polymeric chain (h).

E/ New-generation liposome have divers modifications separately or simultaneously: the attachment of protective polymer along (i) or combining with targeting ligand (j) (e.g. antibody); the incorporation of diagnostic labels (k) (e.g. imaging agents); the incorporation of positively charged lipids (l) allowing the complexation with DNA (m); the incorporation of stimuli-sensitive (e.g. pH sensitive) lipids (n) or polymer (o); the attachment of cell-penetrating peptide (p) or viral components (q). The magnetic particles (r) can be added to liposome for magnetic targeting, and colloidal gold or silver particles (s) can be applied for electron microscopy.

(Figure from Torchilin, 2005)

### Liposome

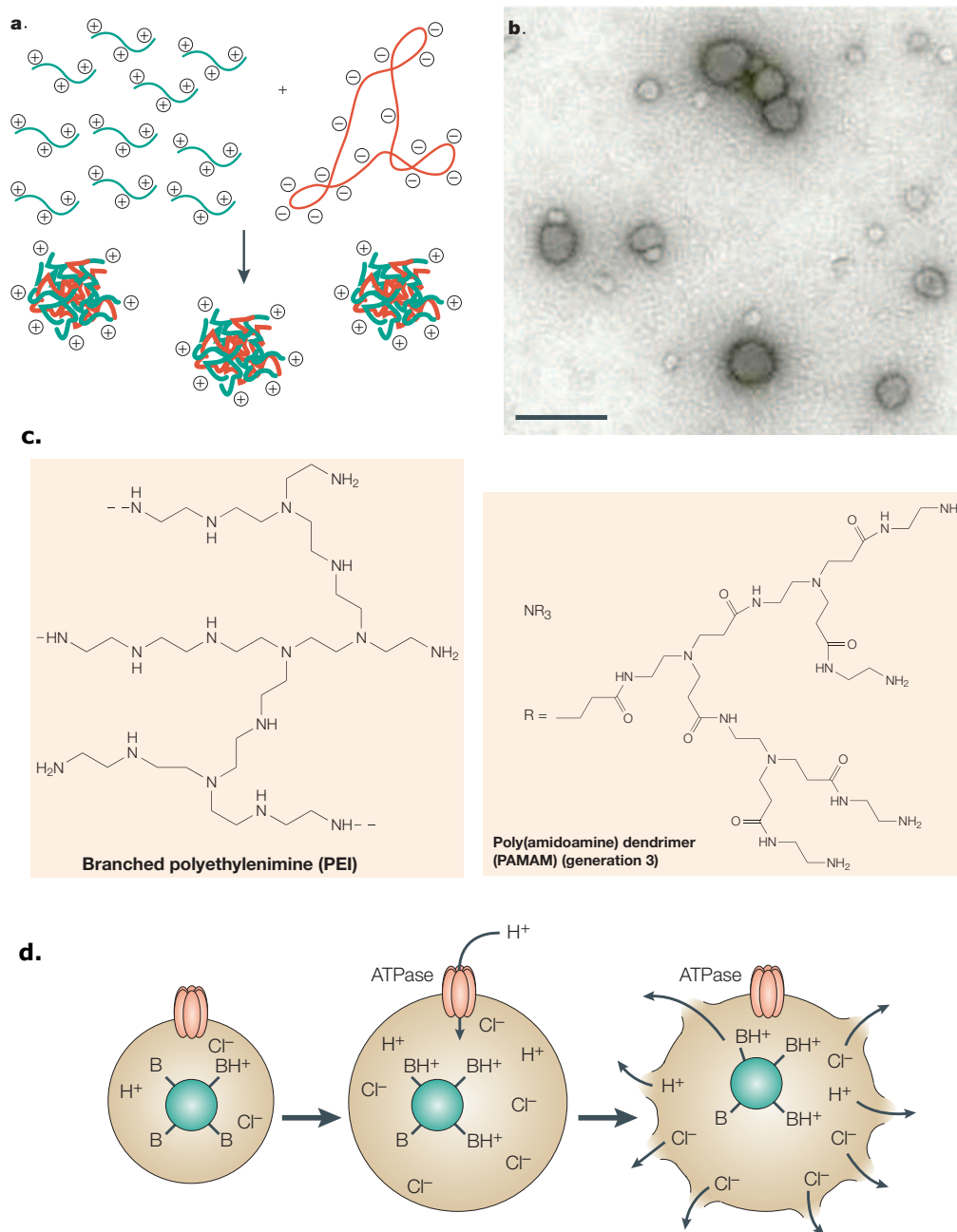
Besides forming lipoplex for DNA delivery, liposome itself, because of the structural nature, is also an versatile carrier for various biological molecules (Fig. 20). For example, ligands or antibodies can be attached to liposome by covalent binding or hydrophobic insertion for specific targeting (e.g. tumor) (Hatakeyama et al., 2004; Park et al., 2001; Schiffelers et al., 2003). Coating the liposome surface with inert polymers (e.g. polyethylene glycol (PEG)) provides a protective layer that shields liposomes from interacting with opsonizing proteins and prolongs its circulation period in blood (Klibanov et al., 1990). Contrast agents for medical imaging purpose can be also incorporated in liposome (Torchilin, 1996). Therapeutic enzyme such as SOD (superoxide dismutase, the protein that reduces ischemia/reperfusion oxidative stress) can be directly delivered by liposome *in vivo* (Stanimirovic et al., 1994; Lo et al., 2004). “Immuno-enzymosome” is a tumor-targeted immuno-liposome carrying prodrug-converting enzyme. It performed specific targeting to tumor cells *in vivo*, and also induced cytotoxicity with bystander effect (Fonseca et al., 2003). Otherwise, chemically stabilized siRNA encapsulated in specialized lipid bilayers (termed stable nucleic-acid-lipid particle, SNALP) has also been exploited recently for *in vivo* systemic delivery of RNAi (Morrissey et al., 2005).

For a special use, liposome can be a vector for protein for peptide while behaving as the immunological adjuvant for vaccinations against tumors or infectious diseases. “Virosome” is a unilamellar liposome vaccine carrying the viral component on the surface. Influenza virosome for example, which carries the spike protein of influenza, is able to elicit high titers of influenza-specific antibodies (Huckriede et al., 2003). A peptide vaccine “BLP25” is composed of the liposome and a encapsulated peptide derived from the mucinous carcinoma-associated glycoprotein MUC-1 antigen (a transmembrane protein associated with metastases, overexpressed on many tumor cells). This tumor vaccine has showed a clinical benefit in the treatment of a subgroup of NSCLC patients in a phase II trial (Butts et al., 2005). These researches figured out the versatility and feasibility of “pharmaceutical” liposomes in *in vivo* applications.

Besides these, when combining with the use of other non-viral vectors such as polymer and CPP, liposome performed additive effects in DNA delivery. These will be

described in following sections.

Figure 21. Cationic polymers as DNA delivery vector



a/ Polyplexes are formed by electrostatic interactions between polycations and DNA, when aqueous solutions of each are mixed. For gene delivery, an excess of polycation is typically used for generating particles with positive surface charge. A polyplex particle is generally 100–200 nm in diameter.

b/ Transmission electron micrograph of polyplexes formed by plasmid DNA and the cyclodextrin-modified branched PEI. Scale bar = 200 nm.

c/ The 2 “proton-sponge polymers” PEI and PAMAM dendrimer represent the most widely studied polyplex gene delivery vehicles in 1990s.

d/ Schematic of the proton-sponge mechanism. Protonation of the proton-sponge polymer (green) leads to the sustained influx of proton (as well as the counter-ion) into endocytic vesicles. Increased osmotic pressure causes the vesicle to swell and rupture.

(Figure from Pack et al., 2005)

### III.2c. Cationic Polymers

Cationic polymers are synthetic materials that can electrostatically bind DNA or RNA, condense them into particles in a few tens to hundreds nanometers of diameter, named “polyplex” (Fig. 21a and b). These particles can enter cells through endocytic pathway. Cationic polymers have great potential in human gene therapy because of their low price, facile manipulation, safety and efficiency, as well as their flexibility in physical/chemical modifications for improved properties (Pack et al., 2005).

#### Poly-L-Lysine (PLL)

PLL is one of the first cationic polymer that attracted extensive studies. This was firstly performed in hepatoma cell lines that PLL/DNA complex was delivered into cells by a receptor-mediated endocytosis (Wu and Wu, 1987). This technique was rapidly applied to *in vivo* transfection. The PLL covalently linked to AsOR (asialoorosomuroid, a asialoglycoprotein) permitted the DNA delivery specifically to mouse liver cells for foreign gene expression (Wu and Wu, 1988). But PLL-based transfection in *in vivo* applications were always limited because of their relatively low efficiency. This is generally accepted to be the cause of their insufficient endosomal escape.

The PLL transfection efficiency was improved when membrane-disrupting agents such as chloroquine, fusogenic peptides, or histidine residue are incorporated, which help disrupting endosomal membranes and favor the delivery of plasmid into the cytosol (Erbacher et al., 1996; Fajac et al., 2000; Midoux and Monsigny, 1999; Wagner et al., 1992). Nonetheless, the overall PLL-based transfection efficiency is still not comparable to PEI, an excellent cationic polymer vector that is widely used to date (Menesson et al., 2005).

#### Polyethylenimine (PEI)

PEI (Fig. 21c) is an efficient DNA delivery vector because it contains both strong DNA compaction capacity and excellent endosomolytic activity. The escape of PEI

complex from endosome is suggested by the “proton-sponge” hypothesis (Behr, 1997) (Fig. 21d). PEI can be linear or branched, contains large numbers of secondary and tertiary amines, which make the polymeric network an effective proton sponge at the pH between physiological and lysosomal environments. The ATPase proton-pump is an enzyme normally transporting  $H^+$  from cytosol into the endosome to lower the pH. Since PEI contains a lot of polyamines, it is able to titrate out the incoming protons. The ATPase then is forced to continue transporting more and more  $H^+$  to reach the desired pH. Accumulations of  $H^+$  and of the balancing ions ( $Cl^-$  in this case) finally cause osmotic swelling and the rupture of the endosomes, and the release of polyplexes to the cytosol (Behr, 1997; Sonawane et al., 2003).

PEI is so far one of the most efficient non-viral vectors for gene transfer *in vivo*. It is able to transfect DNA to mouse tissues including lung, CNS, and kidney (Boussif et al., 1995; Coll et al., 1999; Goula et al., 1998). The aerosol delivery of 25-kD branched PEI/DNA polyplex was able to transfect melanoma (B16-F10) lung metastasis and inhibit tumor growth when the p53 gene is harbored (Gautam et al., 2000; 2002). PEI can be also a vector for siRNA delivery *in vivo*. Previous report showed that PEI/siRNA polyplex can transfect mouse lung through IV injection, which then conferred the protection against influenza virus infection (Ge et al., 2004). PEI/siRNA delivery through retroorbital and intrathecal administrations were also reported (Tan et al., 2005; Thomas et al., 2005). In cancer therapy, PEI/siRNA was shown to mediate gene silencing and growth inhibition in mouse xenografts through SC, intraperitoneal (IP), or intracerebral deliveries (Urban-Klein et al., 2005; Grzelinski et al., 2006). Nonetheless, these are so far the 2 unique publications coming from the same team that described the successful tumor treatment by PEI/siRNA delivery. *In vivo* delivery of siRNA based on PEI vectorization is also one of our research projects. In this study, we showed inefficient siRNA delivery to either mouse lung or mouse xenografts. These results will be described in detail in the thesis RESULT and DISCUSSION, II.

Toxicity can be a problem of systemic PEI/DNA delivery *in vivo*. The positively charged polyplexes form aggregates with plasma proteins and erythrocytes (Ogris et al., 1999). Increasing the quantity of PEI/DNA polyplexes in the injection augmented



the gene expression in mouse lung, but also activated the lung endothelium, the formation of small aggregates, and combined with liver necrosis, shock, and death (Chollet et al., 2002). Otherwise, Although PEI/DNA can transfected mouse xenografts by intratumoral injection, systemic delivery can rarely target tumor cells in mouse (Coll et al., 1999).

A number of modifications have been applied to improve the biomedical efficacies of PEI. A centrifugation-based ultrafiltration can remove redundant PEI in crude polyplexes. The purified PEI/DNA polyplexes are more efficient for transfection, and showed also greatly reduced toxicity *in vitro* and *in vivo* (Erbacher et al., 2004). To address the poor tumor targeting in systemic administration, PEI is conjugated with antibodies or cell-binding ligand (e.g. galactose, transferrin, or EGF). These modifications were able to rendered the polyplexes, to some extent, to target preferentially hepatocytes or tumors *in vivo* (Kircheis et al., 2001; Moffatt et al., 2006; Ogris et al., 2003; Zanta et al., 1997).

Other PEI derivatives were also developed with improved activities. Partially acetylated PEI, although showing a lower H<sup>+</sup> buffering capacity, performed much more efficient gene delivery (up to >50-fold) *in vitro*, which may result from the weakened PEI/DNA interactions and thus the higher unpackaging degree of polyplexes in cells (Forrest et al., 2004; Gabrielson and Pack, 2006). A degradable PEI derivative showed similar DNA-binding property to 25 kD linear PEI, but mediate more efficient gene expression and are less toxic (Forrest et al., 2003). Dodecylation of primary amino groups on a 2 kD PEI yields a PEI derivative, which is less toxic and more efficient for gene delivery in the presence of serum (Thomas and Klibanov, 2002a). The “polycationic liposome” is the liposome anchored by PEI/DNA polyplex through a cetyl group linked on the PEI. The polycationic liposome showed better DNA loading capacity, and more efficient gene delivery as compared to PEI alone *in vivo* (Matsuura et al., 2003).

These improvements reaffirmed the advantage of the polymer vector, which bears the flexibility in physical/chemical modifications for a better activity.

### Polyamidoamine (PAMAM)

PAMAM dendrimers are spheroidal, cascade polymers (Fig. 21c). Depending on various number of “generation” in the synthesis, their size and surface charge are different. Because of bearing large number of secondary and tertiary amines, PAMAM dendrimers are also thought to be proton sponges. The first report describing PAMAM as efficient gene delivery vector found that the sixth-generation dendrimer was more efficient than higher and lower generations by ~10-fold (Haensler and Szoka, 1993). Then it was found that partially degraded PAMAM dendrimers was more effective than the intact polymer. Heat treatment degrades the polymer at the amide linkages, resulting in a hetero-disperse population of fractured dendrimers that show >50-fold enhanced transfection activity (Tang et al., 1996). PAMAM has been applied in *in vivo* delivery of a Epstein-Barr virus-based plasmid harboring a suicide gene for cancer therapy (Maruyama-Tabata et al., 2000).

There are still several other cationic polymers developed for gene delivery purpose, but they cannot be all introduced here. Nonetheless, it should be noticed that none of the polymers to date can be comparable to viral vectors in delivery efficiency.

Table 6. Selective examples of known CPPs.

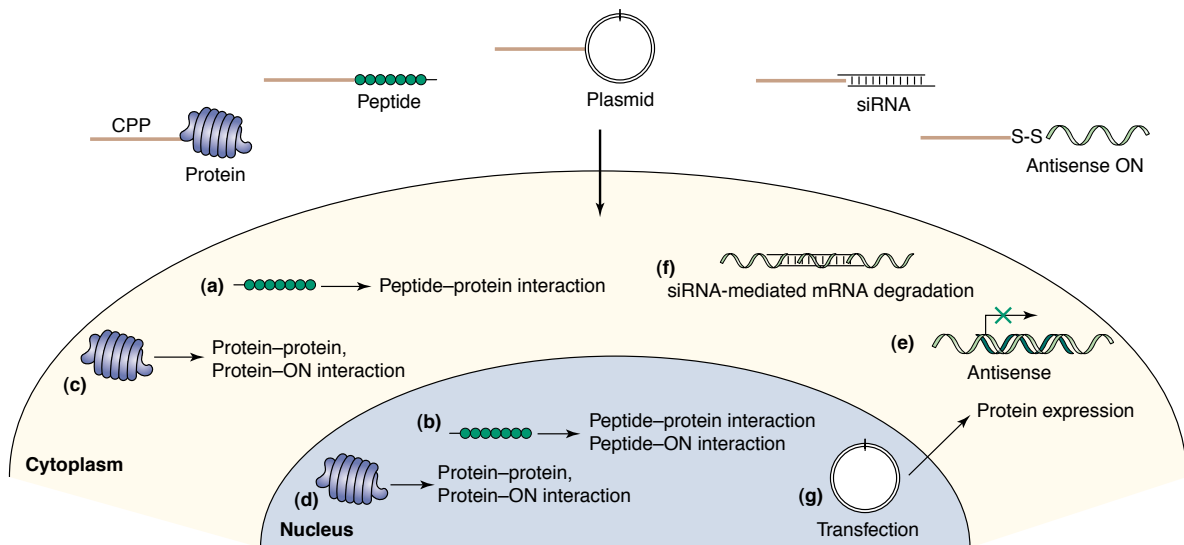
Peptides deriving from protein transduction domains and assimilated		
Names	Sequence	Reference
Penetratin	RQIKIWFQNRRMKWKK	Derossi et al., 1994
Tat(49–57)	RKKRRQRRR	Wender et al., 2000
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Pooga et al., 1998b
VP22	DAATATRGRSAASRPTERPRAPARSASRRRPVD	Elliott and O'Hare, 1997
Amphipathic peptides (secondary and primary)		
Names	Sequence	Reference
MAP	KLALKLALKALKAALKLA	Oehlke et al., 1998
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	Wyman et al., 1997
ppTG20	GLFRALLRLLRSLWRLLLRA	Rittner et al., 2002
Trimer	(VRLPPP) <sub>3</sub>	Fernandez-Carneado et al., 2004
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Morris et al., 1997
Pep-1	KETWWETWWTEWSQPKKKRKV	Morris et al., 2001
Others		
Names	Sequence	Reference
l oligomer	Branched Polylysine + NLS	Sheldon et al., 1995
hCT(9–32)	LGTYTQDFNKFHTFPQTAIGVGAP	Schmidt et al., 1998

(Table from Deshayes et al., 2005)

### III.2d. Cell-penetrating peptide (CPP)

Although no unambiguous definition of CPPs (also known as protein transduction domains, PTDs) has been proposed, they are generally oligopeptide less than 30 amino acids with a net positive charge. They have the ability to translocate the plasma membrane and transport into the cellular cytoplasm and nucleus in a seemingly energy-independent manner (Jarver and Langel, 2004). CPPs can be derived from native proteins of various species or artificially engineered (Tab. 6). The exact mechanisms by which CPP translocates across cell membrane remain unclear. Although CPP translocation was initially thought to be energy-independent because it was not affected by various endocytosis inhibitors or low temperatures (e.g. +4°C) (Derossi et al., 1994; Vives et al., 1997), later studies showed the results that the endocytosis pathway was involved (Console et al., 2003; Richard et al., 2003; Vives, 2003). However, since these studies were usually performed with different CPPs, carrying different cargos, and on various kinds of target cells, it is so far not possible to present a single mode of cell entry of these peptides. These studies need to be performed on a case by case basis and it is now commonly accepted that functional proofs of the activity of the delivered cargo have to be presented (Jarver and Langel, 2004; Kerkis et al., 2006; Silhol et al., 2002). In addition, some studies showed that the observed “uptake” was an artifact resulting from the membrane disruption during harsh fixation process, which allowed the penetration of the peptides attached on the outer cell membrane surface (Lundberg and Johansson, 2001; Richard et al., 2003).

Figure 22. Applications for CPP-mediated translocation.



CPP delivery as vectors for: (a) peptides that function in the cytosol or (b) in the nucleus; (c) protein that function in the cytoplasm or (d) in the nucleus; (e) antisense oligonucleotide for mRNA hybridization; (f) siRNA to mediate mRNA degradation; (g) plasmid for gene expression. ON, oligonucleotide.

(Figure from Jarver and Langel, 2004)

Despite of all these open questions, CPP-mediated deliveries of pharmaceutical molecules *in vitro* or *in vivo* have been widely documented. CPP performs the versatility in delivering a variety of biological molecules including peptides, proteins, plasmid, ASO, as well as siRNA (Fig. 22). Cargo molecules can be linked to the CPP through different means (Gupta et al., 2005; Jarver and Langel, 2004; Temsamani and Vidal, 2004):

1/ short peptide cargos can be synthesized in tandem with CPP in a peptide synthesiser.

2/ oligopeptide or intact protein cargos can be genetically fused to a CPP and produced as fusion proteins in bacteria or eukaryotic expression systems.

3/ dsDNA or siRNA cargos can be vectored simply by a CPP via electrostatic interactions.

4/ ASO cargo can be vectored by a CPP through a cleavable chemical linker such as disulfide bond. In this case, because of the ss-structure and its instability in physiological conditions, ASO is usually prepared in special forms such as phosphorothioate, peptide nucleic acids (PNA), locked nucleic acids (LNA), and phosphorodiamidate morpholino oligomers (PMO).

5/ in addition, imaging agents such as oxotechnetium, oxorhenium complexes, paramagnetic, and superparamagnetic iron oxide particles can be also ferried into cells by CPP.

Some CPPs that have been widely studied and documented will be introduced as follow:

### Penetratin

It was originally described that Antp, a 60-amino acid polypeptide corresponding to the homeodomain of *Drosophila antennapedia*, can be internalized by mammalian nerve cells and accumulated in their nuclei modifying the morphology of the neurons (Joliot et al., 1991). Later on, a minimal 16-mer peptide on Antp was identified to be involved in the translocation process. This peptide was termed “penetratin”, and it was shown to be internalized into cells even at 4 °C, suggesting an energy-independent mechanism of translocation not involving classical endocytosis (Derossi et al., 1994). The cellular uptake mechanism of penetratin was then a matter of debate. Two models were proposed, 1/ the first concerned the formation of inverted micelles (Derossi et al., 1996; Thoren et al., 2000), 2/ the second was based on the existence of a local electroporation-like membrane permeation (Binder and Lindblom, 2003). However, its membrane translocation process has been questioned. An article showed that internalization of penetratin was temperature- and energy-dependent, and an adsorptive-mediated endocytosis played a key role in the entry process (Drin et al., 2003).

Nonetheless, penetratin has performed intracellular deliveries of a variety of molecules. Through the PNA conjugation, penetratin vectored a 21-mer ASO against the human galanin receptor type 1 mRNA into cells *in vitro* and *in vivo*. The delivery suppressed the galanin receptor expression, and modified the pain transmission *in vivo* (Pooga et al., 1998a). The fusion proteins composed of penetratin and mdm-2 binding domain of p53 (peptides ranging from 9-15 mers) were able to enter cells and induced potent cytotoxicity in cultured cancer cells, but not in normal cells (Kanovsky et al., 2001). Nonetheless, penetratin showed poor efficiency in delivering larger proteins or dsDNA (Derossi et al., 1998). Recently, penetratin was also utilized as a component of tumor vaccine. Fusion peptides containing penetratin and CTL epitope of TAAs (e.g. ovalbumin or MUC1) can be efficiently taken up and presented by macrophages and DCs. The mice immunized by these vaccines generated T-cell response and protected against growths of the TAA-positive tumor cells (Apostolopoulos et al., 2006; Pouniotis et al., 2006).

## Tat

The transcription-transactivating (Tat) protein of HIV-1 is a protein of 101 residues. It was firstly described in 1988 for the ability to cross the plasma membrane of neighbouring cells (Frankel and Pabo, 1988). Exogenous proteins fused with the first 72 amino-acids of Tat were successfully delivered into mice tissues, including heart, liver, and spleen (high level), and to lung and skeletal muscles (low-to-moderate levels), 20 min after IV injection (Fawell et al., 1994). Later on, it was identified that the minimal peptide promoting membrane translocation activity of Tat was the 49-57 basic domain (Wender et al., 2000; Vives et al., 1997). Likewise, the mechanism involved in the cellular internalization process of Tat is still controversial (Richard et al., 2003). It appears now that 2 pathways are involved, one is a lipid raft-dependent macropinocytosis process (Kaplan et al., 2005; Wadia et al., 2004), the other is through the clathrin-dependent endocytosis (Richard et al., 2005).

Tat has been shown to be a delivery vehicle for a broad spectrum of cargo molecules including DNA, ASO, oligopeptides or full-length proteins (Wadia and Dowdy, 2005). Tat is able to form complexes with plasmid DNA by electrostatic interactions. These Tat/DNA complexes can be internalized into mammalian cells through different endocytosis-mediated pathways, depending on the cell lines used (Ignatovich et al., 2003). However, *in vivo* delivery of Tat/DNA showed very low efficiency, presumably because of the inactivation of positively charged Tat/DNA complexes in the bloodstream by the serum albumin (Ignatovich et al., 2003). Tat-mediated DNA transfection can be improved by the use of Tat di- or tri-mers (Rudolph et al., 2003).

Tat can also play an assistant role to improve the DNA delivery by other vectors. For example, when DNA was pre-compacted by Tat before complexing with PEI, the transfection efficiency was greatly enhanced, and the initiation of reporter gene expression was much earlier as compared to using PEI only. These enhancements were proposed to come from the nuclear localization function of Tat (Rudolph et al., 2003). A novel vector was made by covalently coupling the PEI with Tat through PEG spacer (Tat-PEG-PEI). Interestingly, this vector showed a lower DNA transfection efficiency *in vitro*, but performed significantly higher (>5×) gene delivery to mouse lung *in vivo* as compared to PEI only. The improved *in vivo* transfection efficiency was



suggested to result from the enhanced complex stability in serum, as well as the Tat-mediated cellular uptake and nuclear localization of DNA. Otherwise, the Tat-PEG-PEI mediated transfection was distributed through epithelial cells on bronchial and alveolar tissue, showing a broader tropism than PEI-mediated one (Kleemann et al., 2005). Similar improvement was also demonstrated when liposomes were modified by Tat on their surfaces. Complexes of Tat-liposome/DNA showed a fast and efficient translocation into the cell cytoplasm, and the subsequent migration to the perinuclear zone. Tat-liposome/DNA can transfect tumor cells *in vitro* and *in vivo*, via intratumoral injections (Torchilin et al., 2001, 2003).

Tat is also active for protein delivery. In cancer therapy studies, purified fusion protein of Tat and p53 was able to be delivered into cytoplasm and nucleus of cells, and was transcriptionally active to induce apoptosis in both p53 positive and negative cancer cell lines (Ryu et al., 2004). A therapeutic protein was made by fusing Caspase-3 with a Tat vector containing a ODD domain (Oxygen-Dependent Degradation domain derived from hypoxia-inducible factor-1 $\alpha$ ). This fusion protein (Tat-ODD-caspase3) was selectively stabilized in hypoxic tumor cells but degraded rapidly in tissues with normal oxygen tension. IP injection of purified Tat-ODD-caspase3 to tumor-bearing mice reduced the tumor sizes without obvious side effects to the mice (Harada et al., 2002). A Tat-survivin fusion peptide has recently shown to be an effective cancer vaccine, and performed promising results in the treatment of glioma in combination with chemotherapy in pre-clinical models (Cho et al., 2007; Kim et al., 2007).

Tat is also capable of ASO delivery. The phosphorothioate ASO against P-glycoprotein (a membrane ATPase associated with multi-drug resistance in tumor cells) was conjugated with Tat, and its delivery to cancer cells induced substantial inhibition of cell surface expression of P-glycoprotein (Astria-Fisher et al., 2000). The delivery of siRNA by Tat however, is relatively inefficient (Moschos et al., 2007a). Tat/siRNA showed a limited reduction of target gene expression *in vitro*, and showed no effect when delivered to the mouse lungs by intratracheal administration (Moschos et al., 2007a). The Tat-conjugated PAMAM was also poorly effective for siRNA delivery (Kang et al., 2005).

## VP22

The VP22 protein is a tegument protein of herpes simplex virus-type 1 (HSV-1). This protein was described to be expressed in one infected (or transfected) cell, but can then penetrate into numerous neighboring cells and enters their nuclei (Elliott and O'Hare, 1997). This unusual “intercellular trafficking” property remains functional when fused to other peptides or proteins *in vitro* and *in vivo*, thus making VP22 a promising tool for improving gene therapy efficiency (Dilber et al., 1999; Phelan et al., 1998; Zavaglia et al., 2003a). A weakness of this application is that the final localization of the cargo will be only in nucleus, thereby reducing the number of therapeutic proteins that can be applied (Deshayes et al., 2005).

Nevertheless, like other CPPs, numerous controversial results then appeared (for selective references: Fang et al., 1998; Phelan et al., 1998; Dilber et al., 1999; Elliott and O'Hare, 1999; Zavaglia et al., 2003; Roy et al., 2005; Zavaglia et al., 2005). There is so far no answer for these debates, especially when opposite results were described even when the same protein cargos were used (e.g. p53 or GFP). But since the enhanced antitumor activity can be observed when VP22 was fused to a tumor-suppressor gene (e.g. Zavaglia et al., 2003), and the diffusion of a fluorescent VP22 fusion protein was directly confirmed in living cells (Lemken et al., 2007), it should be true that VP22 contains a intercellular trafficking activity. But once more, this activity can be only studied on a “case by case” basis: depending on the cargo proteins, linker peptides, cell lines targeted, and the methodologies applied, the results can vary.

VP22's CPP activity was also questioned (Lundberg and Johansson, 2001). Nonetheless, the performance of “vectosome” seemed to provide a direct evidence for the CPP activity of VP22. Vectosome is a particle formed by purified VP22 protein and the fluorescein-labeled oligonucleotide. Vectosomes are actively internalized into cells, and can then be disrupted by the excitation with a light beam, which leads to the release of oligonucleotides (Normand et al., 2001). The vectosome containing an ASO anti-C-Raf1 has been shown to elicit an antitumor effect to human NSCLC in mouse xenografts, through intratumoral injections and light illumination (Zavaglia et al., 2003b). The intravitreal injection of vectosomes followed by the transscleral illumination also allowed the delivery of free oligonucleotides to retinal and retinal

pigment epithelial cells (Normand et al., 2005).

On the other hand, the vectosome composed of an oligonucleotide and VP22-BH3 (a fusion protein containing VP22 and the apoptotic BH3 domain of Bak) was reported to induce extensive apoptosis *in vitro*, after entering cells and followed by a light-activated dissociation (Brewis et al., 2003). Nevertheless, the use of VP22-BH3 was always in the form of vectosome, yet the cellular uptake and the biological activity of purified VP22-BH3 fusion protein alone was never described in this article. Otherwise, this is so far the only paper that we can find describing a biologically functional peptide cargo delivered by VP22 into cells.

Table 7. Examples of molecules that are delivered by CPP.

Cargo	Example	CPP	Conjugate	Reference
Peptide	c-Myc helix-1	Penetratin	peptide bond	Giorello et al., 1998
	p53 mdm-2 BD	Penetratin		Kanovsky et al., 2001
	Polo-box	Penetratin		Yuan et al., 2002
	JNK-binding motif	TAT		Borsello et al., 2003
Protein	p16 <sup>INK4A</sup>	TAT	peptide bond	Ezhevsky et al., 2001
	Caspase 3	TAT		Harada et al., 2002
	p53	TAT		Ryu et al., 2004
	Cre recombinase	FGF-4		Jo et al., 2001
Plasmids	Luciferase	Tat	Charge interaction	Rudolph et al., 2003
	Luciferase	Stearylated Arg-8		Futaki et al., 2001
	Luciferase	SV40 NLS loligomer		Brokx et al., 2002
	β-Gal	Tat		Ignatovich et al., 2003
	GFP	Tat		Torchilin et al., 2003
siRNA	GADPH	MPG peptide	Charge interaction	Simeoni et al., 2003
ASO (PNA)	GalR-1	Penetratin,Transportan	Disulfide bridge	Pooga et al., 1998
	PTPσ	Transportan	Disulfide bridge	Ostenson et al., 2002
	bcl-2	PTD-4	DOTA conjugate	Lewis et al., 2002b
(PMO)	c-Myc	TAT	PMO conjugate	Moulton et al., 2003
(Phosphorothioate)	P-glycoprotein	Penetratin, TAT	Disulfide bridge	Astriab-Fisher et al., 2000

ASO, antisense oligonucleotide; β-Gal, β-galactosidase; BD, binding domain; CPP, cell-penetrating peptide; DOTA, 1,4,7,10-tetraazacyclododecane-N,N,N,N-tetraacetic acid; FGF, fibroblast growth factor; GADPH, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; NLS, nuclear localization signal; PMO, phosphorodiamidate morpholino oligomers; PNA, peptide nucleic acids; PTD, protein transduction domain; PTP, protein-tyrosine phosphatase; TAT, transactivating regulatory protein.

(Table according to Jarver and Langel, 2004)

### MPG

The short peptide vector MPG (27-mers) was artificially generated. It contains 2 domains, derived from the hydrophobic fusion peptide of HIV-1 gp41 and the hydrophilic nuclear localization signal (NLS) of SV40 large T antigen, respectively. MPG was efficiently used for gene delivery (Morris et al., 1997, 1999). The cellular uptake mechanism of MPG/DNA is essentially independent of the endosomal pathway, and is functionally active at 4°C (Simeoni et al., 2003).

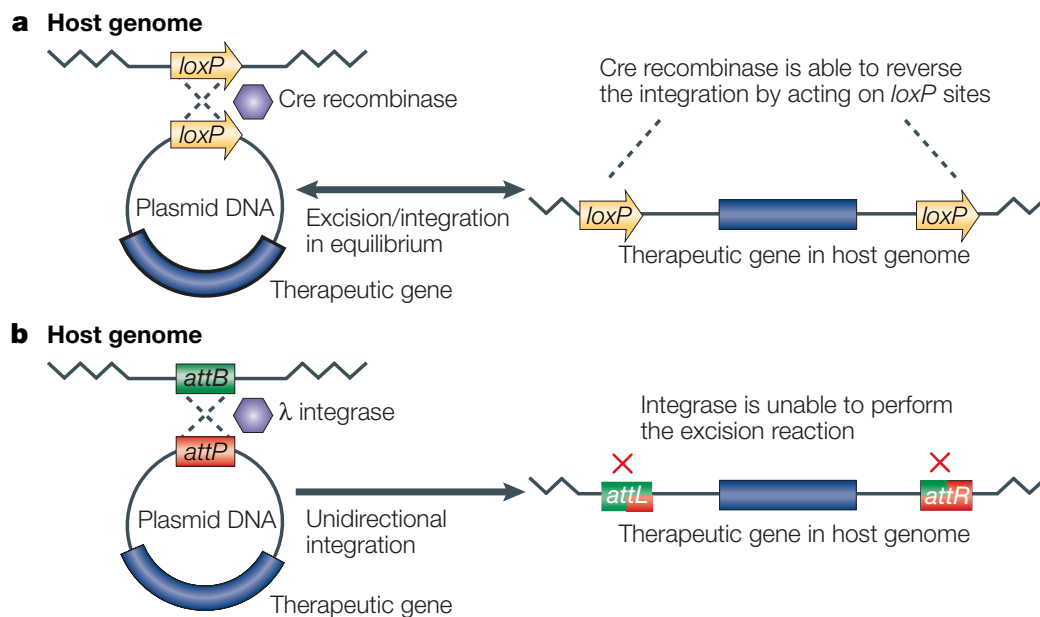
Nevertheless, a variant of MPG (MPG<sup>ΔNLS</sup>) containing a single mutation on the NLS motif, which was originally created for determining the importance of NLS in MPG-mediated DNA delivery, was found to be a powerful tool for siRNA delivery. Although MPG/siRNA and MPG<sup>ΔNLS</sup>/siRNA complexes were both sufficient in introducing siRNA into ~90% of the cells, MPG<sup>ΔNLS</sup>/siRNA showed an intense cytoplasmic localization and the rapid release of siRNA, and yielded a stronger decrease of luciferase activity than MPG/siRNA (~95% vs. ~80%) (Simeoni et al., 2003). Although the versatility of this peptide delivery system has not been validated *in vivo*, its potent silencing effect in cultured cells at relatively low concentrations (25 to 100 nM) showed promising results. This is also one of the few examples so far showing effective siRNA deliveries into various cell lines, through a simple CPP/siRNA electrostatic interaction (Meade and Dowdy, 2007). This case also suggests that specific variations in the peptide sequence may yield novel carriers with distinct targeting features.

Many other CPPs that demonstrate a vector activity *in vitro* and *in vivo* were developed and reported. It is not possible to introduce each of them. [Table 7](#) illustrates some examples that performed successful delivery of various biological molecules. Overall, CPP is a class of vectors that performs versatile delivery activities, and is still under rapid and novel developments.

### **III.3. Long-Term Expression through Non-Viral Delivery**

As mentioned above, viral vectors are currently the preferred delivery system in gene therapy clinical trials. Non-viral vectors are easier to engineer and manufacture, but their delivery efficiency is a major challenge, and the lack of chromosomal integration activity precludes long-term therapeutic effect. Extensive studies have intended to improve their *in vivo* stability, transfection efficiency, and the cell-specificity through chemical modifications (as introduced in distinct parts above). To address the problem of transient expression of non-viral gene delivery, current technologies include the use of genomic integration, extra-chromosomal maintenance (as episomes), or of large artificial chromosomes.

Figure 23. SSR mediated site-specific recombination mechanisms.



Two different types of SSR mediated recombination are illustrated.

a/ Cre recombinase mediates recombination between *loxP* recognition elements on the host genome and the introduced plasmid to generate a hybrid molecule. However, the recombination is reversible and the DNA between two direct *loxP* sites on the resultant hybrid molecule can be excised again into a circular form. The equilibrium of the recombination reaction generally favours the excision direction (Thyagarajan et al., 2000).

b/ Some integrases such as  $\gamma$  integrase mediate the recombination between *attB* and *attP* sites to create hybrid *attL* and *attR* sites that are no longer recognized by the enzyme (Christ et al., 2002). The reverse recombination reaction can occur only in the presence of the integrase and another enzyme, the excisionase.

(Figure from Glover et al., 2005)

### III.3a. Genomic integration

#### Site-specific integration

Site-specific integration of a transgene into the host genome inside of a “safe genomic locus” can avoid the dangerous insertional mutagenesis. Although homologous recombination offers safe and high sequence specificity, the efficiency is generally quite low (<0.1%) (Goncz et al., 2002; Kapsa et al., 2001). Nevertheless, several site-specific integration systems evolved by viruses are applicable in non-viral human gene therapy (see below).

#### *Site-specific recombinase (SSR) from bacteriophages*

Some viruses infecting prokaryotes are able to integrate into specific sequences of the host genome by the SSR, an enzyme that recognizes unique sequences within virus and host genomes and mediates recombination between them (Fig. 23) (Voziyanov et al., 1999). This activity is functional under appropriate conditions in eukaryotic cells, albeit with variable efficiencies. Although these SSRs work in lower organisms and the required specific recombination sites are generally absent in the human genome, certain SSRs can catalyze the integration in foreign hosts to the “pseudo sites” that are similar to the defined native recombination site (Thyagarajan et al., 2000, 2001). These discoveries represent an effective site-specific integration system for higher cells.

The *Streptomyces* phage  $\phi$ C31 integrase is currently the most efficient SSR. It preferentially integrates transgenes at a number of sites (e.g. psA) in safe locations of the human and mouse genomes, with efficiencies of 24-56% (Groth et al., 2000; Ortiz-Urda et al., 2002; Thyagarajan et al., 2001). The  $\phi$ C31 integrase mediates an unidirectional integration (Fig. 23b), it is thus suitable for treating several recessive genetic disorders, by stably introducing therapeutic genes into cells. The human coagulation factor IX (F9 or FIX) gene was stably transfected to mouse liver cells *in vivo*, expressed over 8 months at a significant level thought to be sufficient to overcome the deficiency in human patients (Olivares et al., 2002). With an *ex vivo*



model, the laminin and type VII collagen genes (COL7A1) were transfected to primary cells derived from severe laminin 5-deficient junctional epidermolysis bullosa (JEB) and recessive dystrophic epidermolysis bullosa (RDEB) patients, respectively. The  $\phi$ C31 integrase was able to stably correct the genetic deficiency, and the regeneration of human skin with normal phenotype was achieved on immune-deficient mice using these cells (Ortiz-Urda et al., 2002; Ortiz-Urda et al., 2003b). The full-length dystrophin gene accounting for Duchenne muscular dystrophy was also successfully integrated into the genome of muscle-derived mouse stem cells and normal human myoblasts *in vitro* with the help of  $\phi$ C31 integrase (Quenneville et al., 2004). Among these examples, COL7A1 cDNA and full-length dystrophin are large genes (~9 and 11 kb respectively) that are normally difficult to package into viral vectors. However, although the safety analysis of the integration sites in terms of proximity to cancer genes suggested minimal cancer risk, a number of aberrant events associated with  $\phi$ C31-mediated integration were recently described, including deletions and chromosome rearrangements at the target site (Chalberg et al., 2006; Ehrhardt et al., 2005). Furthermore, numerous chromosomal abnormalities including translocations were found in primary human cells stably expressing  $\phi$ C31, suggesting that the enzyme itself could be a mutagen (Liu et al., 2006a).

The bacteriophage P1 recombinase Cre is another SSR used in biology. It recognizes loxP elements and mediates a homologous recombination. Pseudo loxP elements also exist in the human genome and the recombination between wt and pseudo loxPs can occur, although with an efficiency about 4-fold lower (Thyagarajan et al., 2000). However, unlike the unidirectional integration induced by the  $\phi$ C31 integrase, Cre mediated recombination is reversible and the equilibrium of the recombination reaction generally favors the excision direction (Fig. 23a) (Thyagarajan et al., 2000). Hence, the Cre-loxP recombination system is commonly used for the targeting inducible gene, such as in transgenic mice, rather than the stable expression of therapeutic gene (Sauer, 1998).

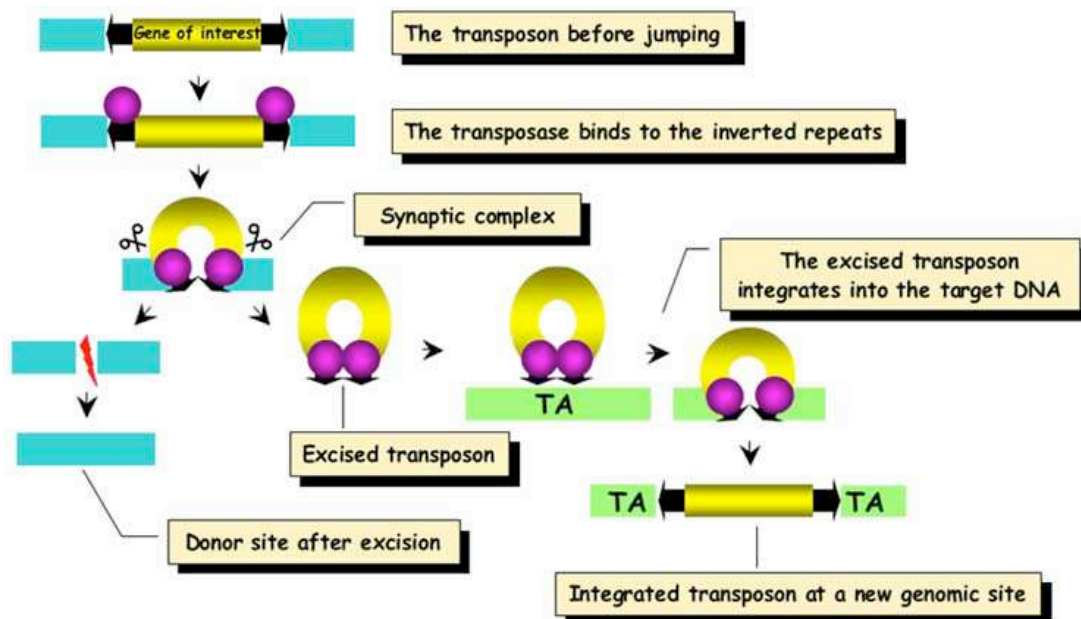
Recombinase variants were also generated by directed molecular evolution technique. They present improved or novel properties, such as thermolability,

modified integration frequency and sequence specificity, or a capability to recognize novel recombination sites (Buchholz et al., 1998; Buchholz and Stewart, 2001; Santoro and Schultz, 2002; Scimmenti et al., 2001).

#### *AAV-viral Rep proteins*

The AAV is so far the only known mammalian virus that is capable of site-specific integration into the human genome. Unlike SSRs described above, the AAV viral Rep proteins that mediate the integration are not recombinase and the integration occurs through a non-homologous recombination into the AAVS1 locus of human chromosome 19 (Young et al., 2000). The recombinant AAV vector used in gene therapy does not present this property because of the deletion of *rep* gene (see the Thesis INTRODUCTION III.1d.). Non-viral approaches by delivering plasmids carrying AAV-ITRs with the support of Rep gene *in cis* or *in trans* were capable of inducing site-specific integration and long-term expression of a transgene flanked by ITRs (Balague et al., 1997; Pieroni et al., 1998). Nonetheless, overexpression of Rep is cytotoxic and cytostatic, and not all stably transfected cells harbored the transgene on AAVS1 locus. It was found that excess Rep expression led to abortive rearrangement of AAVS1 without transgene integration (Urabe et al., 2003), and that constitutive expression of Rep excised the transgene from the AAVS1 locus and resulted in a reduction of transgene expression over time (Philpott et al., 2004). To address these problems, a purified recombinant Rep protein was delivered to the cells by polycationic liposome and was functionally active to promote site-specific integration of ITR-flanked cassette (Lamartina et al., 1998). Satoh *et al.* used a single plasmid system in which the Rep expression was mediated only after the Cre-loxP recombination and would be switched off once integrated into the host genome (Satoh et al., 2000). A ligand-dependent form of the Rep protein was generated by fusing the N-terminal Rep with the truncated hormone binding domain of the human progesterone receptor. The activity of this chimeric protein was induced only when synthetic antagonist RU486, but not progesterone, was added (Rinaudo et al., 2000).

Another strategy to engender site-specific integration is to fuse the retroviral integrase with a sequence-specific DNA-binding domain protein, such as phage  $\gamma$  repressor or *E. coli* LexA repressor (Bushman, 1994; Holmes-Son and Chow, 2002).

Figure 24. Mechanism of *Sleeping Beauty* transposition.

The transposable element carrying a gene of interest is maintained and delivered as part of a DNA vector. In the first step of the transposition reaction, the transposase binds to the sites within the transposon IRs. Excision of the element probably takes place in the context of a synaptic complex that is formed by transposase interactions that hold the ends of the transposon together. Excision physically separates the transposon from the donor DNA, and the double-strand DNA breaks that are generated during this process are repaired by host factors. The excised element integrates into a new TA site in the target DNA that will be duplicated and will be flanking the newly integrated transposon.

(Figure from Ivics and Izsvak, 2006)

### Non-specific integration

Stable transgene expression can be also achieved by using ubiquitous natural transposable elements capable of genomic integrations in eukaryotic cells. Unlike SSRs, these elements transpose through a “cut-and-paste” mechanism: the element-encoded transposase recognizes the transposon inverted/directed repeats (IRs/DRs) and catalyzes its excision and reintegration elsewhere in the genome (Plasterk, 1996). Several transposable elements have been described in vertebrate genomes, but few of them have been demonstrated to be active because they accumulated several mutations during evolution.

### *Transposon systems currently used in gene therapy*

A transposon called “Sleeping Beauty” (SB) was discovered in 1997. SB is a *Tc1/mariner*-like transposable element, artificially reconstructed by site-directed mutagenesis from inactive salmonid transposable element (Ivics et al., 1997). The SB system contains 2 parts: the transgene expression cassette flanked by SB IRs and the SB transposase expression source. They can be maintained either on 2 separate vectors or one single cis-vector. SB can be delivered to target cells as plasmids or incorporated into viral vectors. After its expression in the cell, SB transposase binds to SB-IRs in a substrate-specific manner, excises and inserts the transposon into a new DNA location within TA dinucleotides (Fig. 24) (Ivics et al., 1997; Izsvak and Ivics, 2004). SB transposition was shown to be active in a wide range of cultured vertebrate cells (Izsvak et al., 2000), as well as in embryo or germline cells of fish, frogs and mice (Davidson et al., 2003; Dupuy et al., 2002; Fischer et al., 2001; Sinzelle et al., 2006). It can thus serve as a useful tool in functional genomics research. SB-mediated genomic integration in somatic cells also provides the basis for long-term transgene expression for therapeutic purposes. In the past years, SB system has been successfully used for non-viral delivery to stably integrate a transgene into cells for the treatment of a number of inherited or acquired diseases. These include haemophilia A and B (Liu et al., 2006b; Yant et al., 2000), type I diabetes (He et al., 2004), tyrosinemia I (Montini et al., 2002), glioblastoma (Ohlfest et al., 2005), and Huntington disease (Chen et al., 2005) in mouse model, and the JEB in human patients *ex vivo* (Ortiz-Urda et al., 2003a). SB transposon was also

used as a cancer gene discovery tool, by generating transgenic strains with the active mutagenic transposon inducing both loss-of-function and gain-of-function mutations (Collier et al., 2005; Dupuy et al., 2005). Induction of tumor development in liver in adult mice by somatic integration of oncogenic Ras-harboring SB transposon was also demonstrated (Carlson et al., 2005).

Another artificial transposon named *Frog Prince* was reconstructed from an inactive transposon in the genome of the amphibian *Rana pipiens*, which shows approximately 50% sequence similarity to SB. *Frog Prince* transposition is efficient in fish, amphibian and mammalian cell lines, and shows especially a much higher activity in zebrafish cells than SB (Miskey et al., 2003).

The *Tol-2* transposon isolated from the medakafish *Oryzias latipes* is so far the only known naturally occurring and active transposable element of vertebrate origin (Koga and Hori, 2001; Koga et al., 1996). The minimal sequences necessary for transposition were characterized in 2006, and the stable transfection of fumarylacetoacetate hydrolase gene mediated by *Tol-2* transposon in liver cells of mouse with hereditary tyrosinemia type I was able to correct the metabolic deficiency (Balciunas et al., 2006; Urasaki et al., 2006). *Tol-2* transposon exhibits a high cargo-capacity up to 10 kb without decreasing its efficiency, and transposition activity is not inhibited under high transposase concentration (Balciunas et al., 2006; Kawakami and Noda, 2004). These are advantages of *Tol-2* over SB because SB shows a reduced transposition activity when the transposon is >6 kb or when the transposase is overexpressed (Geurts et al., 2003; Mikkelsen et al., 2003).

Recently, a transposon called *piggyBac* was derived from the cabbage looper moth *Trichoplusia ni*. It showed an efficient transposition activity in mammalian cells and mice (Ding et al., 2005; Fraser et al., 1996). However, the study also demonstrated that *piggyBac* preferably transposes into transcription units in the mouse genome, raising safety issues concerning its use in human gene therapy. A more recent study demonstrated that *piggyBac* present a significantly higher transposition activity than SB or *Tol-2* in mammalian cell lines. Furthermore, *piggyBac* transposase retained its

transposition activity when genetically coupled to the GAL4 DNA-binding domain (whereas *Tol-2* and SB did not in this report), suggesting the potential of *piggyBac* to be used as a site-specific transposase (Wu et al., 2006).

#### *Safety issues of the transposon-based gene therapy*

All vectors that integrate randomly into chromosome present a risk of insertional mutation. SB does not integrate into DNA in a totally random manner. A palindromic AT-repeat consensus sequence associated with a bendable structure and hydrogen bonding potential was found to affect SB target selection, with the central TA as the canonical target site. However, the primary sequence (the AT repeat) is clearly not the unique determining factor for target selection. It was shown that a unique deformation inherent to the sequence may be the recognition signal for SB target selection (Liu et al., 2005; Vigdal et al., 2002). A mathematical description of DNA-deformability called  $V(\text{step})$  was initially constructed to distinguish SB preferential integration sites. Later on, an automated method “ProTIS” was developed for this analysis to accommodate large sequences within chromosomal DNA, which is able to generate profiles of predicted integration events (Geurts et al., 2006). These researches allowed to assess theoretical risks associated with transposon insertions in particular genomic regions, and can be useful in delineating the safety profile of SB-based human gene therapy.

Despite of this integrating specificity at the actual DNA sequence level, an early analysis of a data set of 138 unique SB insertions in cultured human cells showed that SB integration can be considered fairly random at the genomic level (Vigdal et al., 2002). A subsequent study analysed a significant data set of >1300 SB insertions in primary and cultured mammalian cells, and the results supported that physical properties may be the major determining factor in SB target site selection. However, SB showed a small but significant (39%) bias toward genes and their upstream regulatory sequences in this report, much less pronounced than the risk mentioned with integrating viruses. Nonetheless, there was not a significant correlation between SB integration and the transcriptional status of the targeted genes. A strong bias toward microsatellite repeats was also observed, which are predominantly enriched in non-coding DNA (Yant et al., 2005). These properties suggest that SB might be a

safer vector than integrating viruses currently used in terms of stable therapeutic gene delivery. It's also notable that no dominant adverse effect associated with SB integration has been reported so far in experimental animals (Ivics and Izsvak, 2006).

The genomic stability is another important safety issue. As described above, AAV vector and the  $\phi$ C31-mediated integrations are frequently associated with various chromosomal abnormalities, including deletions and translocations (see the Thesis INTRODUCTION III.1d., and the section above, "*SSR from bacteriophages*"). No alteration of target chromosomes other than the 2-bp TA duplication was observed thus far during SB integration (Vigdal et al., 2002; Yant et al., 2005). Unlike  $\phi$ C31, which reacts with several endogenous pseudo *attP* recognition sites in the human genome, SB transposase was not reported to have pseudo binding sites. Although 2 sequences on the human chromosome 8 and 15 were identified to show significant similarity to SB transposase binding sites, the engineered transposons comprising these sequences were not transposable (Walisko et al., 2006). Therefore, it is likely that SB transposase mediates integrations without compromising the integrity of the cellular genome.

Finally, cytotoxic effect or immune response associated with SB system was not observed so far in experimental animals receiving SB plasmids (for review, see Ivics and Izsvak, 2006). This mainly comes from the fact that the SB-transposase is expressed only during the initial transient transfection.

Non-viral delivery of SB-mediated long-term transgene expression in mouse lung is one of our research projects. For the results of this part, see the Thesis RESULT and DISCUSSION, III.

### III.3b. Episomal maintenance

The stable maintenance of introduced genes can be also achieved by using an episome, which is a stable DNA molecule that persists in the cellular nucleus and can be replicated and segregated to daughter cells autonomously. Since episome does not integrate into the cellular genome, it avoids the risk of insertional mutagenesis. DNA viruses such as SV40 and Epstein–Barr virus (EBV) normally replicate and persist episomally in mammalian cells through functions of viral proteins large T antigen (TAg) and EBV Nuclear Antigen 1 (EBNA1), respectively. By including the viral-ori and the requisite viral gene, episomal plasmid vectors have been generated that can sustain the expression of a transgene (Cooper et al., 1997; Sclimenti et al., 2003; Wade-Martins et al., 2000). However, the safety concern exists since these viral proteins are associated with transforming pathways (Ali et al., 2004; Humme et al., 2003).

The episomal plasmid vector that contains a SV40-ori but does not require viral gene products for maintenance has been exploited. The function of TAg was replaced by the scaffold/matrix attachment region (S/MAR) from the human  $\beta$ -interferon gene cluster. S/MAR is the chromosomal region known to be attached to the nuclear scaffold/matrix, and the plasmid containing it can be propagated episomally for several hundreds of divisions in cultured cells, without the need of other viral proteins or selective pressure (Baiker et al., 2000; Piechaczek et al., 1999).



### III.3c. Artificial chromosome

Human artificial chromosome (HAC) is another option for non-viral long-term transgene expression. HACs are large DNA molecules that resemble natural chromosomes. They are capable of replication and retention at low, defined copy number within host cells. The yeast artificial chromosome (YAC) containing replicator, centromere, telomere, and the cloned transgenes has been exploited early (Murray and Szostak, 1983). Construction of HAC, was difficult because of the complex structure of mammalian chromosome. In 1997 the first prototype HAC was generated. It was a linear microchromosome of 6-10 megabases in size containing telomeric, genomic, and exogenous alpha-satellite DNA. They were mitotically and cytogenetically stable in the absence of selection for up to six months in cultured cells (Harrington et al., 1997). Another approach concerns the use of neocentromere-based chromosomes. Neocentromere is a functional variant form of human centromeres, but is devoided of centromeric alpha-satellite repeats and derived from a non-centromeric region of the chromosome (du Sart et al., 1997). Truncations of neocentromere-associated chromosome allowed production of neocentromere-based HAC "minichromosomes" that are approximately 0.7 to 1.8 Mb in size, showing stability in both structure and mitotic transmission in the absence of drug selection (Saffery et al., 2001; Wong et al., 2002).

The major advantage of using HAC is that due to its exceptionally high cargo capacity, HAC can tolerate insertions of many large genes as well as regulatory elements; however, the enormous size also becomes a major problem for its delivery. As viral vectors are impossible to package HAC, the non-viral techniques such as microcell-mediated chromosome transfer (Shen et al., 2000) and microinjection (Telenius et al., 1999) were applied for vectorization. Liposome-based delivery has been used to transfer YAC of up to 650 kb in size containing the entire 400 kb human  $\beta$ -amyloid precursor protein gene into mouse embryonic stem cells (Lamb et al., 1993). Lipofectamine in combination with either PLL or PEI has been used to deliver YAC. PLL-condensed YACs remained intact in 36% of positive clones after transfection, whereas PEI-condensed ones were intact in 100%, showing that PEI is a good condensing reagent, able to protect YAC from shearing and endosomal degradation even when YACs contained up to 2.3 Mb in size (Marschall et al., 1999).

For in vivo delivery, IV injection of 150 kb DNA complexed with PEI showed very poor efficiency. In contrast, delivery by electroporation or hydrodynamic injection of pure DNA was very efficient in muscle and liver, respectively (Magin-Lachmann et al., 2004). Recently, a novel method using liposome in conjunction with ultrasound demonstrated efficient HAC delivery to cultured cells. Evidence supported a mechanism that the integration of lipids to cell membrane created unstable domains particularly prone to the ultrasound, which then induced transient pore formations and allowed HAC entry into cells (Oberle et al., 2004).

## RESULT and DISCUSSION

The general frame of my work is centered around the use of large molecules to treat lung pathologies. These large molecules can be siRNA or plasmid DNA, which are often fragile, bio-degradable and too large to enter cells without the help of a delivery vector. Thus the choice, quality and property of the therapeutic molecules, as well as their bio-distribution, efficiency, and duration after vectorization are critical parameters to be carefully optimized to address each pathology.

In this work I focused most my efforts on the delivery of siRNA and therapeutic DNA (FMG encoding plasmids), and on the stabilization of gene expression (SB transposon), through the non-viral vectorization *in vivo*.

# I. The FMG-mediated Cancer Therapy in Vitro and in Vivo

## I.1. Introduction

Fusogenic Membrane Glycoproteins (FMG) are viral envelope proteins, playing a role in the virus interaction with the cell membrane and inducing its entry into target cells. FMG are promising tools for gene therapy of cancer because, 1/ they induce cancer cells death with a very strong bystander effect and, 2/ they induce an important antitumor immunogenic response which also add its own contribution to the bystander effect.

The aim of this work was to apply FMG-based gene therapy to lung cancer and to study the importance of these 2 properties.

FMG coming from viruses of different animal species have been characterized. In this study, we selected 5 different FMG-expression vectors, as presented in this table:

Expression Vector	Name of FMG	Virus Origin	Cell Lines Applied
phCMV-GALVfus	GALV-FMG	Gibbon Ape Leukemia Virus (GALV)	Human NSCLCs
phCMV-HERV-W	HERV-W-FMG	Human Endogenous Retrovirus-W (HERV-W)	Human NSCLCs
phCMV-RD-Rless	RD-FMG	Feline Endogenous Virus RD-114 (RD)	Human NSCLCs
pFBASALF	AMLV-FMG	Amphotropic Murine Leukemia Virus (AMLV)	Mouse Adenocarcinoma
pFBMoSALF	MoMLV-FMG	Ecotropic Moloney Murine Leukemia Virus (MoMLV)	Mouse Adenocarcinoma

All these vectors are generous gifts from Dr. Cosset (ENS Lyon, France)

Since the FMG must recognize a cell receptor in order to induce the membrane fusion, some of them have a large range of inter-species recognition, while others are more specific. Actually, it has been shown previously in the literature that GALV-FMG does not fuse murine cells (Bateman et al., 2002), and we have also confirmed that GALV-, HERV-W- and RD- FMGs are not able to induce syncytia formation in mouse cells (data not shown).

We tested the capacity of FMG to form syncytia and to induce cancer cell death in human NSCLC xenografts in nude mice. But this model was not satisfying for the examination of the induction of FMG-mediated antitumor immunity. We thus split this study in 2 parts:

1/ The experiments performed with GALV-, HERV-W- and RD- FMGs in human NSCLC cells *in vitro* and xenografts in nude mice.

2/ The experiments concerning the use of AMLV and MoMLV in the TS/Apc BABLC murine breast cancer cell line, which has been characterized for its immunogenic properties about exosome production (Wolfers et al., 2001).

## **I.2. Results and Discussions**

### **I.2a. The FMG-mediated cancer therapy to human lung cancer cells in vitro and in vivo**

*(The manuscript of this part has been submitted to the Journal of Gene Medicine)*

**Title :** Fusogenic membrane glycoprotein induces syncytia formation and cell death in vitro and in vivo: a potential tumor therapy agent for lung cancer

**Short title :** FMG-mediated tumor therapy

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## **Abstract**

**Background** Fusogenic membrane glycoproteins (FMGs) are viral envelope proteins, which bind surface receptors and induce its fusion with the cell membrane. A single FMG-transfected cell can induce the formation of a large multinucleated syncytium with neighbor cells, which will dye within 5 days. Because of this bystander effect, FMG-gene therapy is thus a promising anti-cancer treatment.

**Methods** : Plasmids encoding for FMGs from *Human Endogenous Retrovirus-W* (HERV-W), *Gibbon Ape Leukemia Virus* (GALV) and *feline endogenous virus RD-114* (RD) were transfected in human non-small cell lung cancer (NSCLC) cells in vitro or directly injected into subcutaneous tumors in nude mice. Syncytia formation, cell viability and tumor growth were then measured.

**Results** : All FMGs induced syncytia formations in vitro and more than 50 cells/ syncytia were counted. Transfection of HERV-W or GALV FMGs strongly decreased cell viability (up to 80%), despite of low transfection efficiencies, and also inhibited tumor growth (60 to 70% reduction of tumor mass ( $P < 0.01$ ) at the end of the treatment). In contrast, RD FMG was not efficient in vitro or in vivo.

**Conclusions** : Our results demonstrate that FMG can induce syncytia formation and cell death in human lung cancer cells. Even with a very low transfection efficiency, the anti-tumor activity of FMG, including a direct and an indirect bystander effect, is effective in vitro and in vivo. We conclude that FMG-based gene therapy is a promising approach for the treatment of human lung tumors.

**Keywords** : Fusogenic membrane glycoprotein (FMG), non-small cell lung cancer (NSCLC), syncytia, bystander effect

## Introduction

Fusogenic membrane glycoproteins (FMGs) are constituent of the viral envelope. They recognize specific cell membrane receptors and play a role in viral entry into target cells by inducing a fusion of the virus envelope with the membrane of its target cell.

Interestingly, after being transiently transfected, an FMG positive cell can also fuse with its neighbors, leading to the formation of multinucleated syncytia. Because these syncytia are not viable and will eventually die within a few days (Bateman et al., 2000; Fielding et al., 2000; Higuchi et al., 2000), FMG transfection has rapidly been identified as a potential anti-tumor treatment. This recruitment of FMG-negative neighboring cells is responsible of a powerful bystander effect superior to that of suicide genes such as herpes simplex virus thymidine kinase (HSV-TK) (Bateman et al., 2000; Diaz et al., 2000). This has been demonstrated after viral delivery of the *Gibbon Ape Leukemia Virus* (GALV) FMG or of the measles virus proteins F and H (MV-F and MV-H) in human tumor xenografts in vivo (Diaz et al., 2000; Galanis et al., 2001; Allen et al., 2004; Zhang et al., 2004). In addition to this syncytia-mediated toxicity, FMG has also been shown to enhance the anti-tumor therapeutic activity of replicating adenovirus, oncolytic herpes simplex virus and *vesicular stomatitis virus* (VSV) (Ahmed et al., 2003; Fu et al., 2003; Ebert et al., 2004; Hoffmann and Wildner, 2006; Simpson et al., 2006).

FMG induced cytotoxicity is not immediate. Multinucleated syncytia develop within 24 hr after transfection and gradually die in 5 days (Higuchi et al., 2000). The exact mechanism by which syncytia die remains unclear, but necrosis may play a major role since signs of nuclear fusion, mitochondrial failure, ATP depletion, and autophagic degeneration were observed. Procaspase-3 activation and PARP cleavage plays a minimal role in FMG-mediated cytotoxicity and caspase inhibitor Z-VAD-fmk failed to prevent syncytium death. Instead, addition of fructose partially prevented syncytia from ATP depletion and death (Higuchi et al., 2000; Bateman et al., 2002). However, it has also been described that syncytia-death can show the hallmark of apoptosis in cultured glioma cell lines, suggesting that the underlying mechanism of FMG-mediated toxicity could be cell-line dependent (Galanis et al., 2001).

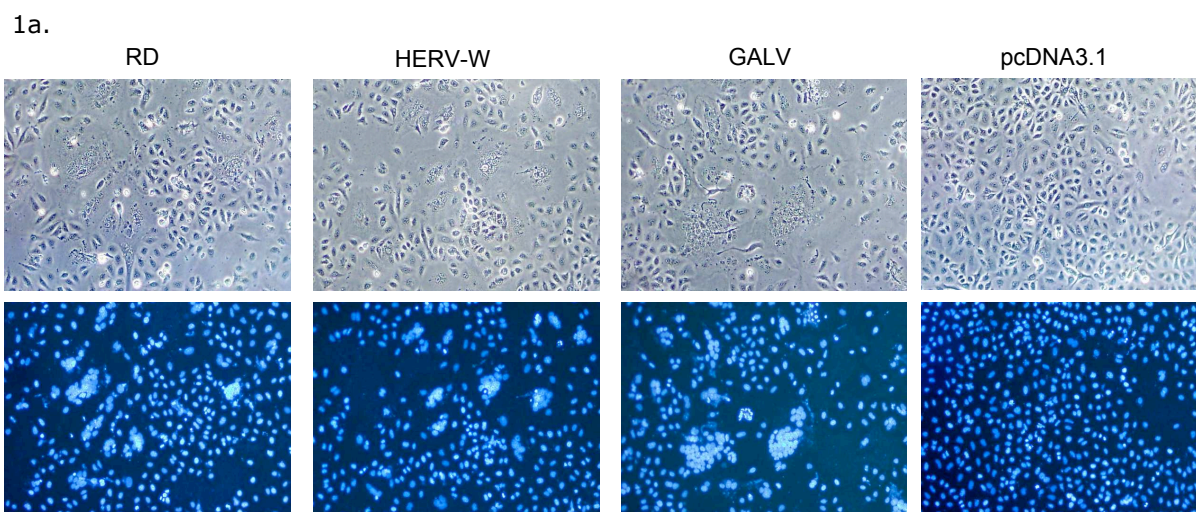


In addition to this toxicity, the interest of FMG-based gene therapy arose also from their ability to stimulate an anti-tumor immunity, because a dying syncytium releases exosome-like vesicles, named syncytiosomes. These vesicles can load dendritic cells (DCs) with tumor-associated antigen (TAA) and then mediate T-cell priming (Bateman et al., 2002). *In vivo* study based on VSV-FMG and murine melanoma cells demonstrated that allogeneic fusing cells can induce a specific immune priming against TAA and protect mice against a second challenge with the same tumor (Linardakis et al., 2002; Errington et al., 2006). (For the introduction about exosome/syncytiosome in more detail, see the Introduction part of **II.2b**.)

In our study, FMGs from different origins were used: HERV-W, GALV and RD. The *Human Endogenous Retrovirus-W* (HERV-W) is a D-type-related retrovirus. Its envelope glycoprotein EnvERVWE1, also called syncytin, fuses human cells that express the type D mammalian retrovirus receptor. Because of this fusogenic activity, this retrovirus is known to play a role in human placental morphogenesis (Blond et al., 1999; Mi et al., 2000; Cheynet et al., 2005). GALV and *feline endogenous virus RD-114* (RD) are C-type mammalian retroviruses. GALV enter cells after binding with type III sodium-dependent phosphate transporters PiT-1 (Kavanaugh et al., 1994; Olah et al., 1994; van Zeijl et al., 1994). RD envelope protein shares the common cell surface receptor as HERV-W (Rasko et al., 1999; Tailor et al., 1999). It has been demonstrated that in D- and C-type mammalian retroviruses, the 16 carboxy-terminal residues of the intracytoplasmic tail of these viruses' envelope proteins, called the "R-peptide", inhibit their fusion activities (Yang and Compans, 1996; Yang and Compans, 1997). Thus R-less versions of these envelope proteins were used in this study, except for the HERV-W, which doesn't rely on the cleavage of the R-like peptide for its fusion (Cheynet et al., 2005).

In this report, FMGs from GALV, HERV-W and RD were tested in human lung cancer cell lines in vitro, as well as in subcutaneous human lung tumors in nude mice in vivo for their anti-cancer activity.

**Figure 1. Syncytia formation in human NSCLC cells mediated by FMGs**



1b.

	RD	HERV-W	GALV
number of nuclei	23.4±10	36.8±15.8	51.7±13.7

Figure 1

a/ A549 cells were transfected by vectors expressing FMGs of different origins or pcDNA3.1 as negative control. Cells were fixed 2 days later and observed using phase contrast microscopy (top panel) or epifluorescence after Hoechst 33342 coloration of the nuclei (original magnification  $\times 40$ ).

b/ Number of nuclei per syncytium counted from 2 independent experiments (average  $\pm$  Standard Deviation (SD)), totally 10 syncytia of each group were counted.

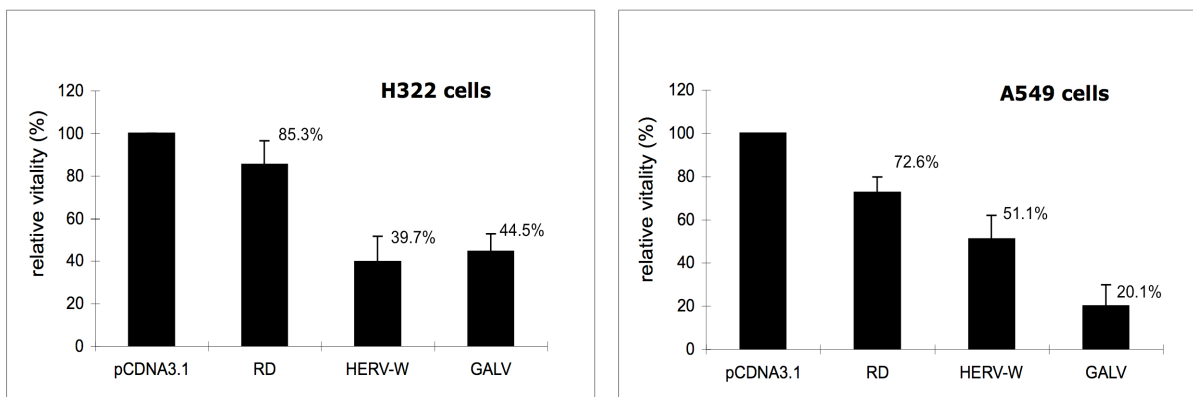
## **Results**

### **Syncytia formation induced by the different FMGs in vitro**

FMGs-mediated formation of syncytia was evaluated on cultured cells. One  $\mu\text{g}$  of plasmid expressing RD, HERV-W or GALV FMGs were transiently transfected into the human NSCLC cell line A549. Two days after transfection, syncytia formation was visible either using phase contrast microscopy on live cells or under UV light after Hoerscht 33342 staining of the nuclei (Fig 1a). The number of nuclei per syncytia was quantified as shown in Figure 1b. GALV FMG-mediated syncytia contained an average of >50 nuclei, while RD FMG-induced ones contained around 25 nuclei only. HERV-W FMG induced an average  $\pm 40$  nuclei per syncytium.

## Figure 2. FMGs-mediated toxicity

2a.



2b.

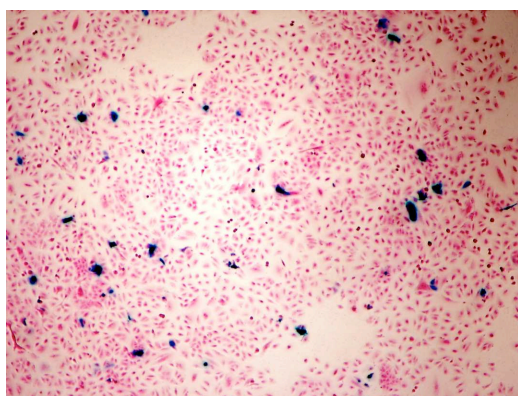


Figure 2

a/ H322 and A549 cells were transiently transfected by FMGs and cell viabilities were measured using MTT assay after 5 or 4 days, respectively. In both cell lines pCDNA3.1 was used as a negative control plasmid (100%). All results were averaged from triplicates and coming from 3 independent experiments (n=9). Error bars indicate the SD.

b/ Transfection efficiency of A549 cells was determined by X-Gal staining and nuclear red counter-staining 2 days after pCMV $\beta$  transfection.

### **FMG-induced syncytia triggered tumor cell death in vitro**

The cytotoxic activities of the different FMGs were measured on A549 and H322 cells, 4 or 5 days after transfection respectively (Fig. 2). The results showed that transfection of HERV-W and GALV FMGs into H322 cells was associated with a  $\pm$  60% decrease of cell viability (Fig. 2a). Similar results were obtained when A549 cells were used: GALV FMG showed an even stronger killing effect (80% loss of viability), while HERV-W FMG killed 50% of the cells. RD FMG was not as efficient as the other envelopes since it was toxic for only 15% and 28% of the H322 and A549 cells, respectively (Fig. 2a).

To check transfection efficiency, H322 and A549 cell lines were transfected by the  $\beta$ -galactosidase expression vector pCMV $\beta$  in the same conditions. We observed that the transfection efficiency was only  $1.16 \pm 0.11\%$  and  $1.1 \pm 0.1\%$  in H322 and A549 cells respectively, as measured after X-gal staining 2 days after transfection (Fig. 2b).

Thus taken together, these results indicated that formation of syncytia efficiently induced tumor cell death in vitro, and that a strong bystander effect was involved. Indeed, up to 80% cell death resulted from only  $\pm$  1% of cell transfection.

**Figure 3. Antitumor activities in vivo**

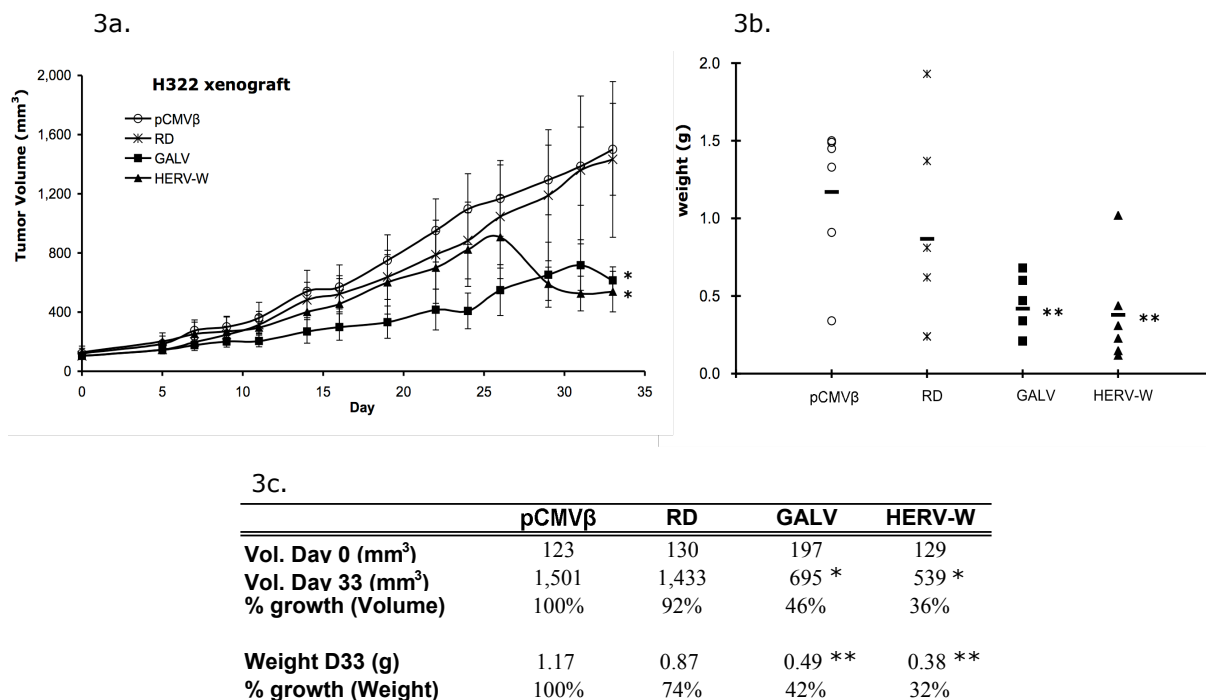


Figure 3

a/ Subcutaneous H322 tumors in nude mice were treated by repeated intratumoral injections of 10 µg of naked plasmids expressing different FMGs every 3 days. β-galactosidase expressing vector (pCMVβ) was used as negative control. Tumor volumes were measured before every DNA injection.

b/ At the end of experiment, mice were sacrificed and the weight of each tumor was measured.

c/ Summary of the results. The percentage of growth was obtained as compared to the pCMVβ control, arbitrarily set at a value of 100%

n=6 in all groups. Error bar represents the Standard Error of Mean (SEM); P values on both figures were estimated by Student's T Test, unpaired and two-tails (\*, P<0.05; \*\*, P<0.01).

### **FMG inhibits tumor growth in vivo**

Human H322 subcutaneous tumors were grafted in nude mice and transfected by direct intratumoral injection of naked plasmids expressing the FMGs, every 3 days. The plasmid pCMV $\beta$  was used as negative control (Fig. 3). At the end of the experiment, no significant growth inhibition effect was observed on tumors treated by RD FMG as compared to pCMV $\beta$ . In contrast, transfection of GALV FMG rapidly and significantly slowed down the tumor progression. HERV-W FMG treated tumors were not affected during the first 25 days. Surprisingly, they then started to shrink and finally reached the same size than those treated by GALV FMG (Fig. 3a).

The weight of the tumors was measured at the end of the experiment (Fig. 3b and c). Tumors coming from GALV or HERV-W FMG treated groups were confirmed to be significantly smaller. The weight of the tumors corresponded with the measurement of their volumes. Indeed, GALV or HERV-W treated tumors were weighting only 42 and 32% in mass to the control group, respectively ( $P < 0.01$ ).

**Figure 4. Syncytia formation in vivo**

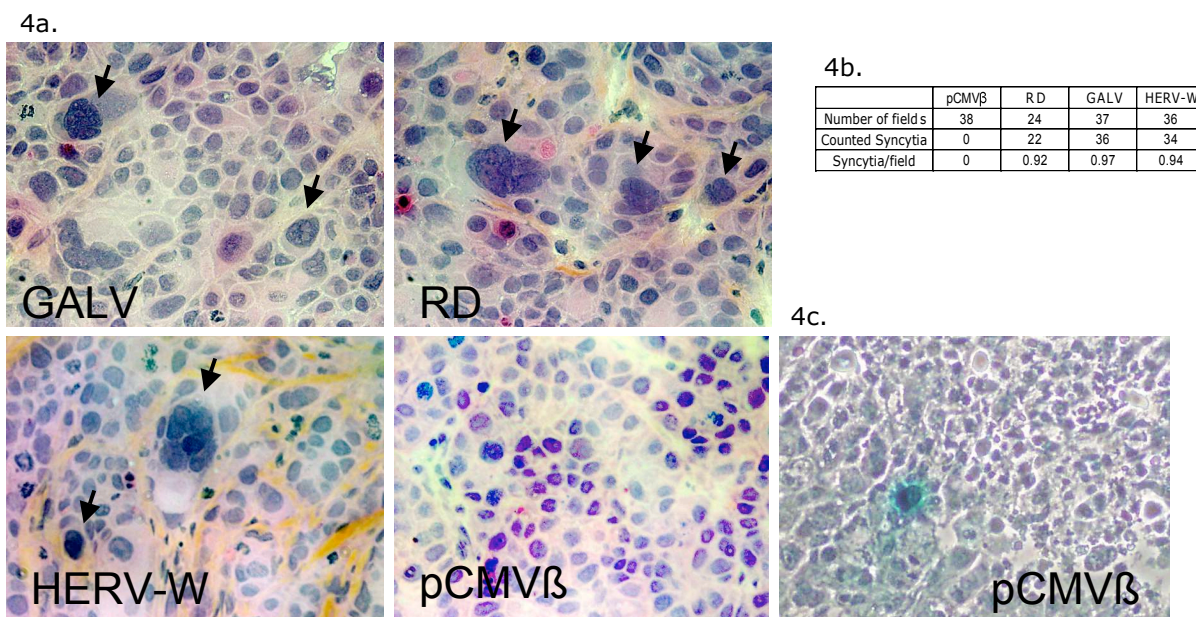


Figure 4

a/ H322 tumors obtained at the end of the experiment were frozen and sliced before being colored with HE and observation (original magnification  $\times 400$ ).

b/ The number of syncytia (indicated by arrows in 4a) were counted from lower magnification photos taken randomly ( $\times 200$ ).

c/ pCMV $\beta$  treated tumor sections were also stained with X-Gal and haematoxylin for verifying the  $\beta$ -galactosidase expression as an indicator of the transfection efficiency (original magnification  $\times 40$ ).



We then counted the number of syncytia formed in these tumors as shown in [Figure 4](#). The presence of multinucleated cells was verified in each sample and no statistical difference was noticed after counting the syncytia in the samples coming from the different FMGs treated groups ([Fig. 4b](#)). Staining of the control sample with X-gal ([Fig. 4c](#)) allowed us to confirm the very low transfection efficiency due to the direct injection of the naked plasmids intratumorally, with the presence of individualized transfected cells only.

Altogether, these results confirmed those obtained in vitro, showing that GALV and HERV-W FMGs efficiently induced tumor cell death via a strong bystander effect. In these assays, RD FMG did not show a sufficient activity to be usable in gene therapy protocols relying only on syncytia formation.

## **Discussion**

Since year 2000, FMGs have been used for tumor therapy because of their cytotoxic effect on cancer cells, their ability to induce an antitumor immunization in vivo and also in combination therapy strategies with oncolytic adenovirus, herpes simplex virus, VSV, or chemotherapy [1-4, 7-15]. In this study, we demonstrate that their cytotoxicity resulting from the formation of gigantic cells is sufficient to induce tumor regression with a very strong bystander effect after non-viral intratumoral gene delivery.

FMG-mediated antitumor activity relies strongly on the existence of a powerful bystander effect. Indeed, we show here, in agreement with other reports, that FMG-expressing cells can fuse with 50 to 100 neighboring cells, form multinucleated syncytia which will efficiently die (Higuchi et al., 2000). This bystander effect is an important issue for gene therapy on tumor since efficient delivery of DNA remains the major problem in vivo. This bystander effect could be also amplified by the use of VP22-mediated delivery as well as by the use of suicide genes like thymidine kinase. VP22 is a herpes simplex virus tegument protein, with unusual intercellular trafficking properties allowing it to spread from one producing (transfected) cell to numerous surrounding cells (Elliott and O'Hare, 1997). Fusion-proteins between VP22 and a therapeutic polypeptide were thus successfully used for in vivo tumor therapy although conflicting results have also been reported (Fang et al., 1998; Phelan et al., 1998; Dilber et al., 1999; Elliott and O'Hare, 1999; Zavaglia et al., 2003; Roy et al., 2005; Zavaglia et al., 2005; Lemken et al., 2007). Positive gene therapy results using suicide genes like the Herpes simplex type 1 thymidine kinase (HSV-TK) associated with the injection of a prodrug (ganciclovir) were also largely documented. However, despite numerous and encouraging preclinical results, this strategy has not been very successful so far in clinical trials, in part because of the poor efficiency of the transfection methods, but also because the bystander effect was not as strong in human beings than the one usually measured in rodents (Harsh et al., 2000; Rainov, 2000). As compared with VP22 or HSV-TK, FMG presents advantages because once expressed on the cell surface, it simply recruits a large number of neighbor cells into one single growing syncytium committed to die. However, a possible limit could come from a poor fusogenic activity if the adequate cell surface receptor is not present on

the tumor cells. Nonetheless, commonly used FMGs such as GALV FMG or MV F and H seem to be able to fuse all the tested human cancer cell lines so far, and previous reports demonstrated a 10 times more stronger bystander effect using GALV FMG than HSV-TK (Bateman et al., 2000).

Previous studies established successful treatments of human glioma or fibrosarcoma xenografts on mouse model after intratumoral delivery of GALV FMG or MV F and H genes (Bateman et al., 2000; Diaz et al., 2000; Galanis et al., 2001; Zhang et al., 2004). However, these results were mostly based on repeated treatments with high-doses of the vectors injected at very early stage of tumor development (3 days after SC implantation; 0.1-0.4 cm in diameter). In our study, the antitumor effect of FMG was measured on human lung tumors after repeated intratumoral injections of naked DNA in relatively large size tumors ( $\geq 0.6$  cm in diameter). Despite of the very low transduction efficiency of naked DNA in H322 tumors in vivo, significant reduction of tumor growth was observed with GALV and HERV-W but not with RD FMG. Interestingly, the growth of HERV-W FMG treated tumors did not seem to be affected during the first 25 days of treatment, but then rapidly decreased in size. The volume of HERV-W FMG treated tumors finally became significantly smaller than that of the control tumors, and was actually even smaller than that of GALV FMG treated ones. This in vivo evaluation of the respective antitumor activities was somehow in direct agreement with their *in vitro* cytotoxicities despite the delayed response of the tumors treated with HERV-W FMG. This phenomenon could be related to a retarded or less efficient death of the syncytia in vivo, which could indicate that RD FMG treated tumors could have shrunk after the end of the experiment. Because of ethical issues, we do not accept to let the tumor grow too large and this question was not addressed.

Since the death of these syncytia occurs via the release of highly immunogenic exosome-like vesicles also called syncytiosomes (Bateman et al., 2002), we can expect that FMG delivery in tumors in an immuno-competent animal or patient would be even more efficient than the one we measured in this study using human xenografts in nude mice. In this study, despite the absence of immune antitumor activity and of the very inefficient transfection method (Coll et al., 1998), the strong antitumor responses suggest that FMG genes are very potent and promising tools for

the treatment of human lung tumors using gene therapy.

### **Acknowledgements**

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## **I.2b. FMG-mediated antitumor immunity**

### **Introduction**

As mentioned earlier, FMG-mediated cell death is known to induce a potent antitumor immunity. The use of human xenografts in immunodeficient nude mice was obviously not adapted to this part of the study. Unfortunately, these GALV-, HERV-W- and RD- FMGs were not able to induce syncytia formation and cell death in murine cell lines.

We thus used 2 other FMGs derived from murine leukemia viruses (MLVs) in this part of our study. Besides verifying their syncytia-inducing activities and cytotoxic effects in mouse cells, we also measured the capacity of these 2 FMGs to stimulate the production of syncytiosomes, a kind of exosome-like vesicles derived from dying syncytia. These vesicles have been described to be largely responsible for the immunogenic antitumor response triggered by FMG expression.

Exosomes are small vesicles, resembling apoptotic bodies. They were initially described as exfoliated microvesicles (40 nm) containing ecto-enzyme activity released from neoplastic cells (Trams et al., 1981). Electron microscopy studies demonstrated that through fusion with plasma membrane, multivesicular endosomes release their internal "intraluminal vesicles" to the extracellular environment. These released microvesicles were termed exosomes, which were proposed to represent a mechanism of recycling or shedding of cell surface proteins during reticulocyte maturation (Harding et al., 1983; Pan et al., 1985). Exosomes can be purified by centrifugation of cell culture medium, and they showed distinct characteristics in composition and enzymatic activities (Johnstone et al., 1987). Later on, exosome productions were confirmed in various cell types including CTLs, EBV-transformed B cells, mastocytes, platelets, and DCs (for review, see Thery et al., 2002). Among all of them, exosomes derived from DCs are highly specific, which express antigens, functional MHC class I, II and T-cell costimulatory molecules (Zitvogel et al., 1998). These DC-derived exosomes are highly immunogenic and once pulsed with TAA, they are able to prime specific CTLs *in vivo* and eradicate or suppress the growth of established murine tumors in a T cell-dependent manner (Zitvogel et al., 1998).

Interestingly, tumor cells in culture were also found to secrete exosomes. These tumor-derived exosomes present MHC-I and can transfer some autologous TAAs to DC, inducing potent CTL-dependent antitumor effects (Wolfers et al., 2001).

More recently, syncytiosomes derived from dying syncytia were identified, which are exosome-like vesicles that can also load DC with TAA of fusing tumor cells and induce specific T-cell priming (Bateman et al., 2002). *In vivo* studies based on VSV-FMG and murine melanoma cells have demonstrated that allogeneic fusing cells can induce a specific immune priming against TAAs and protect mice from a second challenge of the same tumor (Linardakis et al., 2002; Errington et al., 2006).

Although similar in size and properties, the protein composition differs between syncytiosomes and exosomes (Bateman et al., 2002). Some of their biological properties also differ. Purified DC-derived exosomes presenting TAA are capable of inducing T-cell priming directly (Zitvogel et al., 1998); while purified tumor-derived exosome, although bearing MHC-1 molecules on the membrane, cannot directly promote CTL activation but need to be delivered through DC (Wolfers et al., 2001). Purified syncytiosome however, can neither prime T-cell nor pulse DC; the incubation of whole dying syncytia with DC is necessary to transfer TAA to DC and induce the DC maturation (Bateman et al., 2002). These differences suggest that although a common mechanism may be behind the exosome/syncytiosome generation, these microvesicles are not totally identical in composition and function.

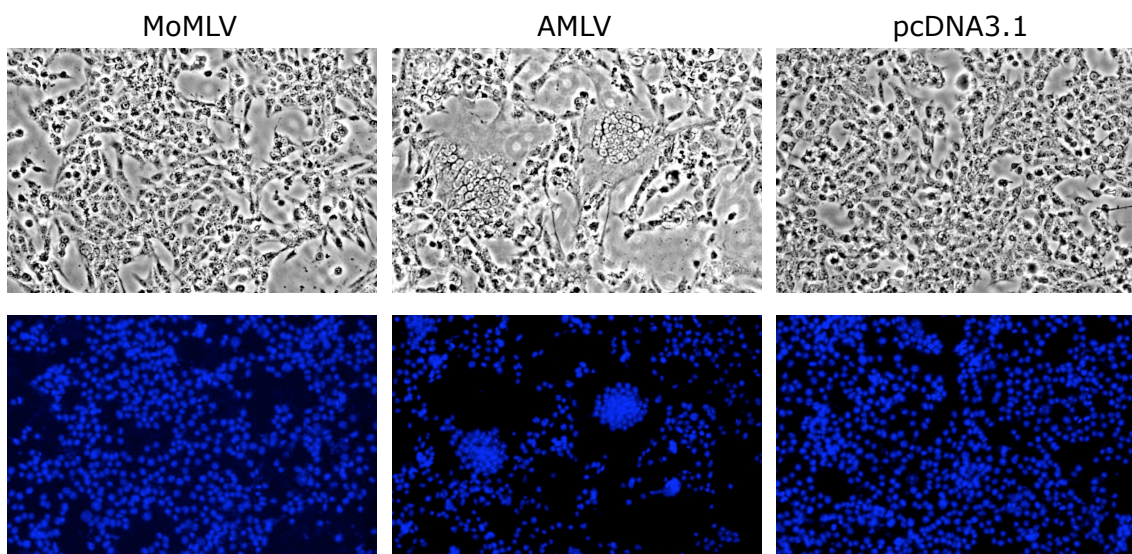
Systematic proteomic assay based on DC-derived exosome established an extensive protein map, in which different populations of proteins were identified such as those related to cytoskeleton, membrane fusion, signal transduction, apoptosis, and antigen presentation (They et al., 2001). Alix (apoptosis-linked gene 2 (ALG-2)-interacting protein X; also named ALG-2 interacting protein 1, AIP1) is a protein found to be abundant in exosomes. Alix was initially characterized as a cytoplasmic protein binding to the pro-apoptosis factor ALG-2 in the presence of  $Ca^{2+}$  (Missotten et al., 1999; Vito et al., 1999), and was later described to be also an important regulator in the formation and organization of multivesicular body (Katoh et al., 2003; Matsuo et al., 2004). Alix was shown to connect ESCRT (Endosomal Sorting Complexes Required for Transport)-II and -III in the multivesicular body biogenesis pathway, and to act as a component of HIV viral budding machinery (von Schwedler et al., 2003;

Strack et al., 2003). Although its role in exosome/syncytiosome secretion has not been documented, its involvement in multivesicular body biogenesis makes us interested in knowing if its overexpression can promote exosome/syncytiosome secretion.

In collaboration with Dr. Chatellard-Causse (INSERM EMI 0108, Grenoble, France), we analyzed the productions of exosome/syncytiosome in cultured TSA cells with or without the overexpression of Alix, and verified their compositions by western blot.

**Figure 5. Syncytia formation in mouse cell line mediated by FMG**

5a.



5b.

	AMLV	MoMLV
Number of nuclei	41.1±6.5	0

Figure 5

a/ TSA cells were transfected by vectors expressing FMGs of different virus origins, or pcDNA3.1 as negative control. Cells were fixed 2 days later and investigated using phase contrast microscopy (top panel) or epifluorescence after Hoechst 33342 coloration of the nuclei (original magnification  $\times 40$ ).

b/ The number of nuclei per syncytium counted from 2 independent experiments (average  $\pm$  SD); totally 19 syncytia were counted.



## Results

### FMG-mediated syncytia formation in the murine model

Expression vectors pFBASALF and pFBMoSALF encode the FMGs derived from *amphotropic murine leukemia virus* (AMLV) and *ecotropic Moloney murine leukemia virus* (MoMLV), respectively (Lavillette et al., 1998). The 2 MLV-FMGs cannot induce syncytia in human NSCLC cells tested in our laboratory (data not shown), thus mouse cell lines including TSA (mouse adenocarcinoma), 3LL (murine Lewis lung carcinoma cell line), and B16 (mouse melanoma cell line) were then tested. However, no syncytium was observed in 3LL cells after transfection, and only a few ones formed in B16 cells with very limited size (data not shown). In TSA cells, extensive syncytia formations were observed after the transfection of AMLV-FMG, but not by MoMLV-FMG (Fig. 5a). The counting of nuclei numbers showed that an average of >40 cells can be fused in one single syncytium triggered by AMLV-FMG (Fig. 5b). The absence of syncytia in the 3LL and B16 cell lines may come from the lack of expression of proper cell surface receptor.

**Figure 6. FMG-mediated toxicity in mouse cell line**

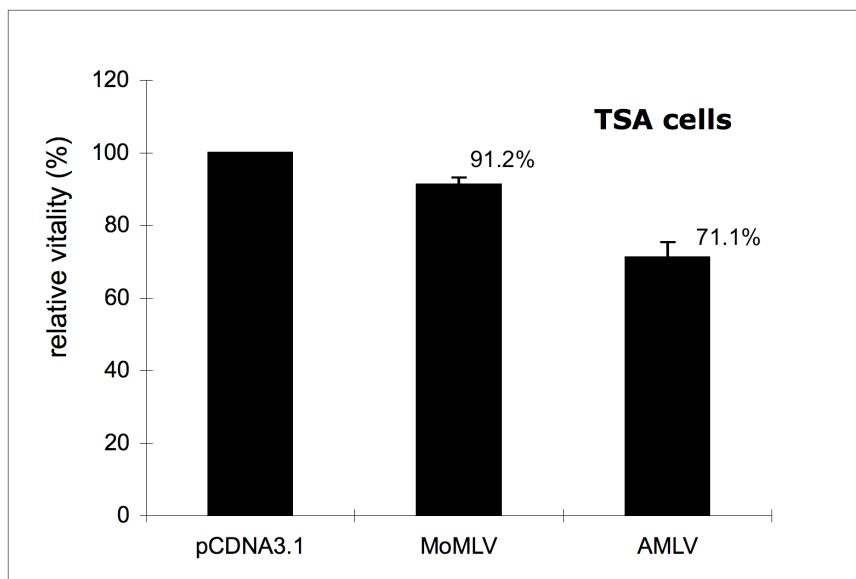


Figure 6

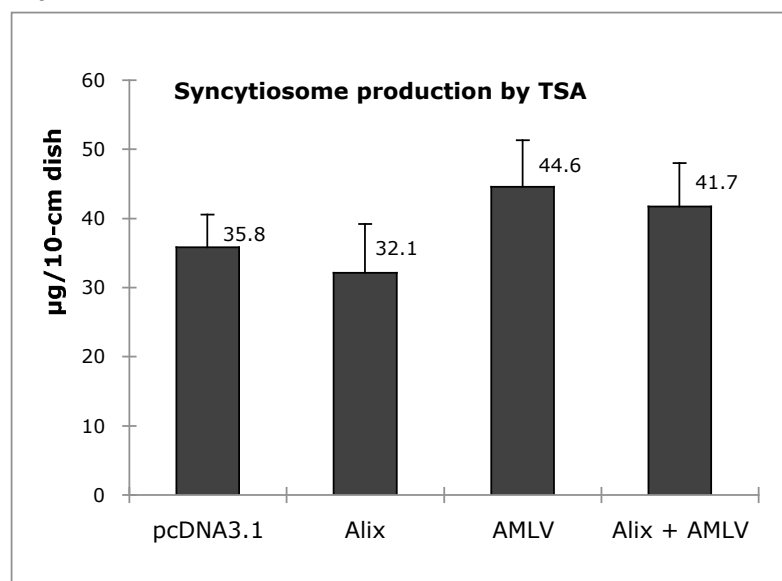
TSA cells were transfected by FMG-expressing vectors or pcDNA3.1. Cell viabilities were measured by MTT assay 5 days post-transfection. The pcDNA3.1 group was set as negative control (100%). All results were averaged from triplicates and coming from 3 independent experiments (n=9). Error bars indicate the SD.

### **FMG-mediated toxicity in the TSA cell line**

Despite the efficient induction of syncytia by AMLV-FMG in TSA cells, a loss of only ~30% of cell viability was observed after transfection (Fig. 6). MoMLV-FMG, which did not induce any syncytia in TSA cells, showed a very weak cytotoxicity (Fig. 6). These results suggested that although induction of syncytia is a critical first step toward FMG-mediated cytotoxicity, it is not always sufficient. In this case, AMLV-FMG allowed the formation of large syncytia (>40 cells), but the observed cytotoxicity *in vitro* was not as elevated than expected.

### Figure 7. Exosome/Syncytiosome production and identification

7a.



7b.

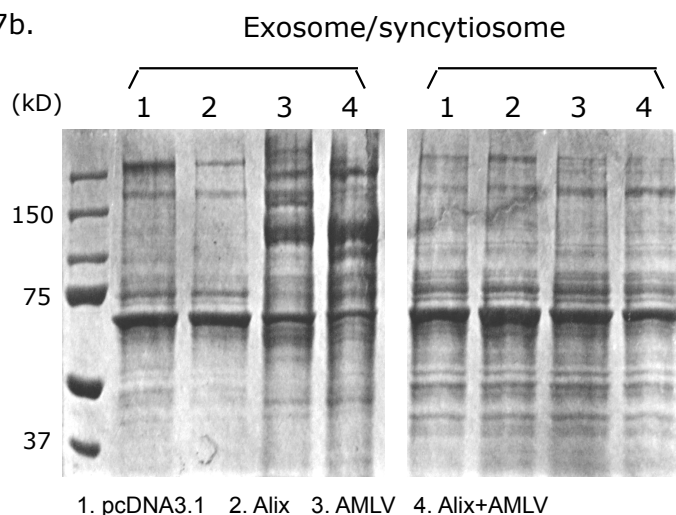


Figure 7

a/ TSA cells were transfected by different combinations of plasmids (as indicated on the figure), and the secreted microvesicles (exosome/syncytiosome) were collected from cell culture medium. The amount of microvesicle production per 10-cm dish was determined by the total protein quantity ( $\mu\text{g}$ ) present in the final solution resuspending exosome/syncytiosome. Results were averaged from 8 independent experiments. Error bars indicate the SEM.

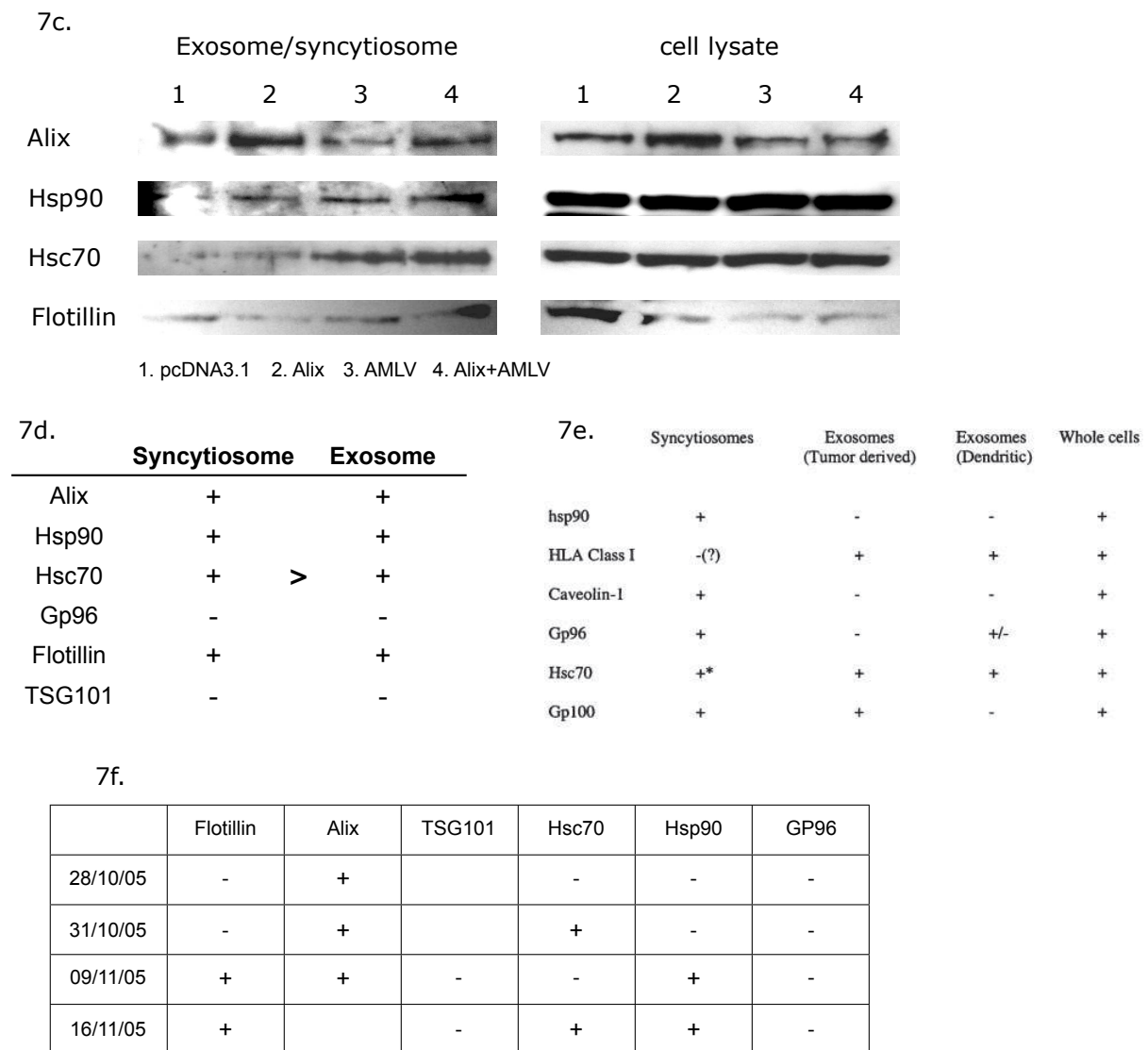
b/ Proteins ( $10 \mu\text{g}$ ) in the collected microvesicles were analyzed on 7% SDS-PAGE gels and visualized by coomassie blue staining.

### **Exosome/Syncytiosome production and identification**

We then analyzed the AMLV-FMG mediated syncytiosome production in TSA cells. Alix was also transfected alone or in combination with AMLV-FMG to TSA cells to examine its ability to enhance the exosome/syncytiosome production. The results coming from 8 independent experiments showed that the transfection of Alix did not increase the production of exosome/syncytiosome, since the total quantities of proteins in collected microvesicles were similar either with or without Alix (Fig. 7a). On the other hand, AMLV-FMG did augment the production of microvesicles to a certain extent, but not statistically significant ( $P>0.3$ ).

We then analyzed the protein contents of these vesicles. Coomassie blue staining of the SDS-PAGE gels demonstrated that the exosome/syncytiosome vesicles purified from AMLV-FMG transfected cells can have a different pattern of protein content than the exosomes only (without AMLV-FMG transfection) (Fig. 7b). This could demonstrate the different natures of these vesicles; but as observed in the right panel of this figure, the result did not always confirm the similar protein content from independent experiments.

**Figure 7. (continue)**



c/ The western blot analysis of the proteins incorporated in exosomes/syncytiosomes and corresponding cell lysates.

d/ A summary of proteins detected in exosomes/syncytiosomes. +/- indicate the protein was detected/undetected in western blots. Specifically for Hsc70, its quantity in syncytiosome was much more than that in exosome.

e/ The table from a publication article (Bateman et al, 2002), in which the human melanoma cell line Mel888 was used for exosome/syncytiosome productions. (?), only one weak signal relative to levels in the whole cell lysates detected from four experiments; +, consistently positive in Western blot analysis. \* is not explained in the original paper.

f/ For the western blot result described on figure 7c and d, totally 4 independent protein batches were collected and analyzed, and their respective results are summarized here. +/- indicate the protein was detected/undetected on the film, and blank indicates the detection was no performed.

Then we used several antibodies to examine some proteins previously described to be incorporated in exosomes and/or syncytiosomes. The results confirmed the presence of Alix, Hsp90, Hsc70, and Flotillin, while Gp96 and TSG101 cannot be detected in at least 2 independent experiments (Fig. 7c and d). Interestingly, the slight increase of Alix can be found in exosomes/syncytiosomes collected from cells transfected by Alix (Fig. 7c, left, lane 2 and 4); while in cell lysates, only cells transfected by Alix alone (but not Alix+AMLV-FMG) showed an increased in Alix pattern (Fig. 7c, right, lane 2). This result may indicate that the overexpressed Alix was incorporated in the secretion of exosome/syncytiosome, although this does not increase the total microvesicle production. Otherwise, the cell lysate coming from cells co-transfected by Alix+AMLV-FMG (Fig. 7c, right, lane 4) did not show an increase in Alix, which may be because that most transfected cells were dead at day 5 due to AMLV-FMG.

Hsc70 content was augmented in the AMLV-FMG induced exosome/syncytiosome fractions as compared to exosome only, but not in the corresponding cell lysates, suggesting that Hsc70 could be incorporated more importantly in the syncytiosome production mechanism. Hsp90 was present in all the microvesicle fractions including exosomes only, suggesting an opposite result to the previous (Fig. 7e).

In summary, AMLV-FMG mediated induction of syncytia slightly enhanced the microvesicle production in TSA tumor cells. These vesicles contained the proteins previously described in exosomes and/or syncytiosomes (Bateman et al., 2002; They et al., 2001). Specially, an enhancement of Hsc70 was observed in microvesicles collected from AMLV-FMG treated cells, suggesting that this protein is associated more importantly in syncytiosome production than in “naturally” produced exosome in cultured tumor cells. However, it should be noticed that the reproducibility of these results is not perfect and that variations in the protein contents were noticed among independent experiments (Fig. 7b and f).

## Discussion

The only FMG used for immune-priming study so far *in vivo* is VSV-FMG (Linardakis et al., 2002; Errington et al., 2006), because of the lack of fusing capacity in rodent cells of other commonly used ones such as GALV-FMG or MV-F and H. We showed in this report that AMLV-FMG is also able to induce syncytia formation in the mouse breast adenocarcinoma cell line TSA (Fig. 5a). MoMLV-FMG has been documented to induce extensive syncytia formation in NIH3T3 and XC cells (Ragheb and Anderson, 1994; Kubo et al., 2003). However, no (or few) cell fusion was observed in TSA, B16, or 3LL cells tested in our laboratory followed by MoMLV-FMG transfection. The absence of syncytia induction might come from the lack of proper cell surface receptor in these cells, although we did not analyzed this point.

The number of nuclei that can be included in a single syncytium induced by AMLV-FMG is around 41 (Fig. 5b). By comparing this number to others induced by FMGs shown in figure 1 (although different cells were used), AMLV-FMG showed a fusing capacity close to HERV-W-FMG (AMLV: 41 vs. HERV-W: 37). However, the levels of cytotoxicity induced by these 2 FMGs were quite different: AMLV-FMG killed ~30% of the treated cells, while HERV-W-FMG killed around 50-60%. This suggests that the size of the induced syncytia is not sufficient to estimate the cytotoxicity of a FMG and that other factors may be of importance.

The microvesicles collected from TSA cells with syncytia (“AMLV” and “Alix+AMLV” groups in figure 7) should contain both exosomes and syncytiosomes, since not all cells were fusing and tumor cells in culture naturally produce exosomes. Comparing the amounts of microvesicles collected, it showed that the presence of syncytia was correlated with an augmented production of microvesicles by ~1.25-fold. But this augmentation was not statistically different ( $P>0.3$ ) despite a large number of independent experiments were performed ( $n=8$ ). However, the augmented production is in agreement with published results, which showed a 2.4- to 4.5-fold increase of microvesicles in GALV-FMG transfected human melanoma cells (Bateman et al., 2002). The variation in efficiency could be the result of different FMGs, transfection efficiencies, and basal levels of exosome production in cell lines used. Indeed, when



using a cell line already producing high levels of exosomes, the augmentation due to the presence of syncytiosome can be weakened, especially if the transfection efficiency is low (TSA cells perform regularly ~1% of transfection efficiency in our laboratory, data not shown).

The other purpose of this experiment was to determine if the overexpression of Alix, an important regulator in protein sorting and multivesicular body biogenesis, could improve the production of exosome/syncytiosome. However, Alix apparently did not enhance the generation of these microvesicles, either alone or in the presence of AMLV-FMG (Fig. 7a).

The presence of proteins Alix, Hsp90, Hsc70, and Flotillin in collected exosomes/syncytiosomes confirmed the published results (Fig. 7d and e). Hsp90, Hsc70, and gp96 belong to heat-shock protein family; TSG101, like Alix, is an ESCRT protein; Flotillin is a Raft-associated protein (for review, see Fevrier and Raposo, 2004; They et al., 2002). Although TSG101 and gp96 were not detected in our system, showing conflictive results to published data (Bateman et al, 2002; They et al., 2001), the difficulty to obtain reproducible results may also have to be taken into account (Fig. 7f).

Hsc70 appears to be more elevated in syncytiosome than in exosomes (Fig 7c and d). This result agrees with the hypothesis that exosome and syncytiosome produced from the same cell line can have different protein compositions. As previously reported, proteins Hsp90, Caveolin-1, and gp96 were detected in syncytiosomes but not in exosomes (Fig. 7e).

However, it should be noticed that the reproducibility of our results concerning protein contents was not perfect and that variations existed among independent experiments (Fig. 7b and f). We do not have a definite explanation of these variations, but we suggest that the conditions of cell growth and transfection efficiency may account for. At the same time, it may indicate that these results should be further analyzed with caution.

## I.3. Material and method

### Plasmid and construction

The FMG coding vectors phCMV-GALVfus, phCMV-RD-Rless, phCMV-HERV-W, pFBASALF, and pFBMoSALF are kindly provided by Dr. Francois Loic Cosset (ENS Lyon, France). phCMV-GALVfus (Fielding et al., 2000), phCMV-RD-Rless (Lavillette et al., 2002), pFBASALF, and pFBMoSALF (Lavillette et al., 1998) express the “R-less” version of the envelope proteins derived from GALV, RD, AMLV, and MoMLV, respectively. phCMV-HERV-W (Blond et al., 2000) expresses the full length HERV-W envelope protein (*for all FMGs used and their host viruses, see also the list presented on I.1. -Introduction*). The expression vector of Alix (Alix) is a generous gift of Dr. Chatellard-Causse (INSERM EMI 0108, Grenoble, France). pCMV $\beta$  (Ozyme, Saint Quentin Yvelines, France) is the mammalian reporter vector which expresses the  $\beta$ -galactosidase under the transcription control of a CMV promoter. pcDNA3.1 (Invitrogen, Cergy Pontoise, France) served as the negative control in *in vitro* transfections.

### Cell culture and transfection

A549 (ATCC, CCL-185) and H322 (ATCC, CRL5806) are both human NSCLC cell lines. TS/Apc (TSA) is a mouse adenocarcinoma cell line. All cells were cultured at 37°C in a humidified CO<sub>2</sub>-controlled (5%) incubator, in RPMI 1640 (Invitrogen, Cergy Pontoise, France) supplied with 10% (v/v) fetal bovine serum (Invitrogen, Cergy Pontoise, France) and 0.5% (v/v) PEN-STREP (Cambrex, Verviers, Belgium). The medium for TSA cells was supplied additionally with 25  $\mu$ M  $\beta$ -mercaptoethanol (Invitrogen, Cergy Pontoise, France).

For syncytia formation and cytotoxicity assays, A549 and TSA cells were seeded on cover slips in 24-well plates at the density of 10<sup>5</sup> and 5 $\times$ 10<sup>4</sup> cells/well, respectively, and were transfected 24 h later with 1  $\mu$ g of each FMG expressing vector, pcDNA3.1, or pCMV $\beta$  by jetPEI (PolyPlus Transfection, Illkirch, France) according to the manufacturer's recommendation. For H322 cells, shortly before transfection, cells

were trypsinized and resuspended in growth medium by  $2 \times 10^5$  cells/ml. One hundred  $\mu$ l of transfection solution (according to the manufacturer's recommendation) was mixed gently with 1 ml of H322 cells and added to the well of a 24-well plate.

For the exosome/syncytiosome production,  $5 \times 10^5$  TSA cells were seeded in 10-cm dish the day before transfection. A total of 8  $\mu$ g of DNA was transfected to each dish by jetPEI, which included 4  $\mu$ g of each plasmid indicated (fig. 7a, b), and 4  $\mu$ g of pcDNA3.1 for complement (in the case if only one plasmid was indicated on the figure).

### **Exosome/syncytiosome isolation**

To collect the exosome/syncytiosome in culture medium, 3 days and 5 days after transfection (the medium needs to be changed on day 3 to keep cells alive; the medium collect on day 3 was temporally stored at  $-20^\circ\text{C}$ ), the culture medium together with the PBS used for washing the cells were collected in a 50-ml tube, and the sequential centrifugations (at  $4^\circ\text{C}$ ) were performed as follow (as described in Wolfers et al., 2001): 300 g for 10 min to remove cells, 800 g twice for 15 min, 10,000 g for 30 min to remove cellular debris (J2-HC centrifuge, Beckman Instruments, CA, USA). Supernatant was collected after every centrifugation, and microvesicles were finally pelleted-down by 100,000 g for 1 hr, washed in 10 ml of PBS, and pelleted-down again by 100,000 g for 1 hr (SW-28 rotor and LE-80K ultracentrifuge, Beckman Instruments, CA, USA), and resuspended in 50-100  $\mu$ l of PBS. The protein concentrations in microvesicles were measured by the kit DC Protein Assay (Bio-Rad, Hercules CA, USA) and the Model 680 Microplate Reader (Bio-Rad, Hercules CA, USA).

### **SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot**

Exosomal (5 or 10  $\mu$ g) or cell lysate (20  $\mu$ g) proteins were boiled at  $95^\circ\text{C}$  for 5 min in reducing sample buffer (final condition: 80 mM Tris-HCl pH 6.8, 10% (w/v) glycerol, 5% (v/v)  $\beta$ -mercaptoethanol, 2% SDS, and 0.002% bromphenol blue), fractionated in

7% SDS-PAGE, and then stained by Coomassie Blue or transferred to an Hybond-P PVDF membrane (Amersham, Tokyo, Japan). For western blotting, the non-specific sites of the PVDF membrane were blocked in 5% nonfat dried milk in PBS containing 0.05% Tween. The membrane was then incubated with various primary and peroxidase labeled secondary antibodies (see below) at suppliers' recommended conditions. Chemiluminescent signals were visualized by autoradiography on Hyperfilm (Amersham, Tokyo, Japan) after incubating with Pierce ECL Western Blotting Substrate (Thermo Scientific, MA, USA). The exposure time is typically 1-2 min for cell lysates and >30 min for exosomal proteins, depending on the signal intensities.

The primary antibodies used in these experiments include Rabbit anti-Hsp90 (StressGen, Victoria, Canada), Rat anti-Hsc70 (StressGen), Rabbit anti-gp96 (Zymed Laboratories Inc., CA, USA), and anti-Flotillin-1 (BD Biosciences, Le Pont-de-Claix, France). Mouse monoclonal anti-TSG101 (Abcam, Cambridge, UK). Rabbit polyclonal antibody anti-Alix is a generous gift of Dr. Chatellard-Causse. Secondary antibodies conjugated with horseradish peroxidase (HRP) include Goat anti-mouse IgG (Molecular Probes, Cergy Pontoise, France), Goat anti-Rabbit (DakoCytomation, Denmark), and Goat anti-Rat IgG (Immunotech, Marseille, France).

### **Cell fixation and staining**

Two days after transfection of FMG expression vectors, cells were washed by PBS and fixed in methanol for 5 min at room temperature (RT), then washed twice by PBS for 5 min. DNA was stained by 1 µg/ml Hoechst 33342 in PBS for 5 min at RT, and then washed again by PBS. Cover-slips were mounted in Moewiol (Sigma-Aldrich, St Quentin Fallavier, France) and examined under a fluorescent microscope (BX 41, Olympus France).

For the transfection efficiency assay, A549 or H322 cells transfected with pCMVβ were fixed 2 days after transfection in PBS containing 0.5% glutaraldehyde and 1% formaldehyde for 5 min at RT, and then stained by incubating for 2 hr at 37 °C in the solution containing 4 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 4 mM K<sub>4</sub>Fe(CN)<sub>6</sub> · 3H<sub>2</sub>O, 0.02% NP40, 2 mM MgCl<sub>2</sub>, and 0.4 mg/ml X-gal in PBS. Percentages of blue cells were counted under a

light transmission microscope using a Glasgow Cell-Counting Graticule (Datasights Ltd, Middlesex, UK).

### **MTT cell viability assay**

Four or five days after transfection, for A549 or H322 cells respectively, cells were washed by PBS and incubated in 180  $\mu$ l of RPMI without phenol red (Invitrogen, Cergy Pontoise, France) containing 5 mg/ml of 3-(4,5-Dimethyl-2-thiazolyl) 2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Quentin Fallavier, France) at 37°C for 3 hr. Cells and the converted formazan inside were solubilized by adding 1 ml of isopropyl alcohol-0.1 N HCl and vibrated for 20 min. Viability was then determined by measuring the difference in absorbance at 570 and 655 nm (OD 570-655).

### **In vivo tumor treatments**

Five-week-old female Swiss nude mice (Janvier CERT, Le Genest St Isle, France) were injected subcutaneously with  $2 \times 10^7$  H322 cells. Eight weeks later when all mice had palpable tumors (140 mm<sup>3</sup> averaged size of total 25 mice), intratumoral injections of 10  $\mu$ g of DNA (diluted in 100  $\mu$ l of PBS) were performed every 3 days using U-100 insulin syringes (Becton Dickinson, Pont De Claix, France). Tumor volumes were measured before every DNA injection, using the formula  $\text{Volume} = 0.4 \times a \times b^2$ , in which a and b represent the larger and smaller diameters, respectively.

### **Tumor section and histochemistry staining**

For histological analysis, tumor samples were embedded in optimal cutting temperature (O.C.T.) compound (Sakura Finetek France, Villeneuve d'Ascq, France) and stored at -80°C. Ten  $\mu$ m-thick sections were stained with Haematoxylin-eosin (HE) for microscopic examination. For verification of *in vivo* transfection efficiency,

sections were fixed by 0.5% glutaraldehyde and 1% formaldehyde and then stained by X-Gal (following the same protocol as described in “Cell fixation and staining” above) and Haematoxylin.

## **II. PEI-mediated siRNA Delivery in Vivo**

### **II.1. Introduction**

RNA interference (RNAi) is an evolutionarily conserved response to double-stranded RNA (dsRNA) that leads to sequence specific post-transcriptional gene silencing (Fire et al., 1998; Hamilton and Baulcombe, 1999; Tuschl et al., 1999; Hannon, 2002). dsRNAs present in the cytosol are processed by the RNase III enzyme Dicer into short 21-23 nucleotide duplexes, termed short-interfering RNA (siRNA). siRNA can then associate with the multiprotein RNA-inducing silencing complex (RISC), target the homologous mRNA and degrade it (Hammond et al., 2000; Bernstein et al., 2001; Hammond et al., 2001; Hannon, 2002; Meister and Tuschl, 2004). The fact that chemically synthesized 21-nucleotide long siRNA can also function to silence the expression of specific endogenous or heterologous genes in mammalian cells opened its great promises in biological applications (Elbashir et al., 2001). RNAi has been quickly adopted as a powerful tool to selectively turn off genes, applied to functional genomics research, drug target discovery, as well as therapy of human diseases including cancer (Dykxhoorn and Lieberman, 2005; Pai et al., 2006; Kim and Rossi, 2007). The RNAi mediated therapeutic modality takes advantage of high specificity, efficiency and versatility since synthetic siRNA can be designed against virtually any gene (Li et al., 2006). In addition, the combination of multiple siRNAs targeting a set of genes is feasible and could improve the net effect of antitumor or antiviral treatments (Song et al., 2003; Kim et al., 2004).

Commonly used RNAi molecules are chemically synthesized siRNA and the short-hairpin RNA (shRNA). Synthetic 21-nt siRNA duplex containing 2 nucleotides overhangs in 3' of both strands are extensively used. This allows large-scale synthesis and a uniform production and is also amenable to chemical modifications for increasing the stability (Kim and Rossi, 2007). Longer synthetic siRNA (27-nt) were recently reported to elicit a more potent gene silencing effect, presumably because they are substrate of Dicer. This directly links the siRNA production to its incorporation in RISC (Kim et al., 2005). Although shRNA can be also chemically

synthesized, a DNA vector under the transcriptional control of RNA Pol II or III promoters is usually producing them. An advantage of using shRNA is that the silencing effect can last as long as the DNA vector exists (Brummelkamp et al., 2002; Paddison et al., 2002); instead, the effect of synthetic siRNA is generally transient, and can be rapidly diluted after cell division (Bartlett and Davis, 2006; Kim and Rossi, 2007).

So far, the efficient *in vivo* delivery of siRNA remains a major hurdle that prevents their use in clinical studies. *In vivo*, siRNA is not stable and will have to cross tissue and cell barriers (Xie et al., 2006). Fortunately, since siRNA functions in the cytoplasm, they will not have to pass through the very selective nuclear membrane.

To date, several non-viral vectors have been shown to allow systemic delivery of synthetic siRNA *in vivo*, including the polyethylenimine (PEI). PEI is a linear (L-PEI) or branched (B-PEI) cationic polymer that has been widely applied in gene delivery *in vitro* and *in vivo*, taking advantage of combining strong DNA compaction capacity and an excellent endosomolytic activity (Boussif et al., 1995; Behr, 1997; Lungwitz et al., 2005). After an intravenous (IV) injection in the tail vein of an adult mouse, PEI delivers DNA preferentially in epithelial lung alveolar cells (Goula et al., 1998) but not in subcutaneous tumor cells or lung metastases (Coll et al., 1999). However, PEI/DNA transfection to tumors can be achieved by intratumoral administration.

PEI can also deliver siRNA and transfect mouse lung after an IV injection. This can lead to a protection against influenza virus infection (Ge et al., 2004). For cancer therapy, PEI/siRNA was shown to mediate gene silencing and growth inhibition in mouse xenografts after SC, intraperitoneal (IP), or intracerebral injections (Urban-Klein et al., 2005; Grzelinski et al., 2006). Thus, L-PEI seemed to be an interesting siRNA delivery vector and justified our investigations.

Concerning the target genes, we focused our attention on the luciferase reporter system to quantify the *in vivo* inhibitory effect of siRNA and on the inactivation of C-Raf for inducing a therapeutic activity.

The Raf protein family comprises 3 members, A-, B-, and C-Raf (or Raf-1), among which C-Raf is the most ubiquitously expressed isoform. Raf proteins are attractive therapeutic targets because they are important signal transducers involving several



pathways associated with cancer cell proliferation, angiogenesis and metastasis (Beeram et al., 2003). The mitogen-activated protein kinase (MAPK) pathway is the most highly characterized pathway that Raf proteins involve. Upon signaling from activated Ras, Raf mediates downstream activations of MEKs and ERKs, leading to the subsequent transcription of a number of factors promoting cell growth, survival or cytoskeletal rearrangements (Howe et al., 1992; Macdonald et al., 1993; Moodie et al., 1993; Beeram et al., 2003). In addition to the MAPK pathway, C-Raf can bind to BAG-1 (Bcl-2-associated athanogene-1) and functionally cooperate with Bcl-2 in suppressing apoptosis (Wang et al., 1996a; Wang et al., 1996b). C-Raf can physically interact with Rb after mitogen stimulation in proliferating cells, and efficiently inactivate Rb function and reverse the Rb-mediated repression of E2F1 transcription and cell proliferation (Wang et al., 1998). C-Raf also promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 (ASK1, a stress-activated proapoptotic protein kinase) through protein-protein interactions, independent of the kinase activity (Chen et al., 2001). In endothelial cells, activated Raf is associated with increased levels of the proangiogenic factor VEGF (vascular endothelial growth factor) (Grugel et al., 1995), and the inhibition of C-Raf can result in the reduction of tumor angiogenesis (Leng and Mixson, 2005; Culmsee et al., 2006). Through differential phosphorylation at separate sites by VEGF or bFGF signaling respectively, C-Raf can be activated in response to distinct angiogenic pathways and shields endothelial cells from apoptosis (Alavi et al., 2003; Hood et al., 2003). These combined results suggest an important role of C-Raf in angiogenesis. Finally, inhibition of Raf activity was also found to suppress tumor invasion and metastasis *in vitro* and *in vivo* (Khatib et al., 2002; Fu et al., 2003; Campbell et al., 2007).

Our laboratory has previously demonstrated the antitumor effect of an antisense oligonucleotide (ASO) against C-Raf delivered by intratumoral injection of VP22-based “vectosomes” in mouse xenografts of human NSCLC tumor (Zavaglia et al., 2003b). The delivery of siRNA anti-C-Raf has also demonstrated anticancer effects in various human cancer cells including myeloid, cerebral, and gastric cell lines *in vitro* (Cioca et al., 2003; Meng et al., 2005; Culmsee et al., 2006), and in malignant breast and prostate xenografts *in vivo* through the delivery of cationic peptide or liposome

vectors (Leng and Mixson, 2005; Pal et al., 2005).

The aim of my work was to study how the L-PEI vector could be used for the systemic *in vivo* delivery of synthetic siRNA against the luciferase reporter gene or the C-Raf oncogene.

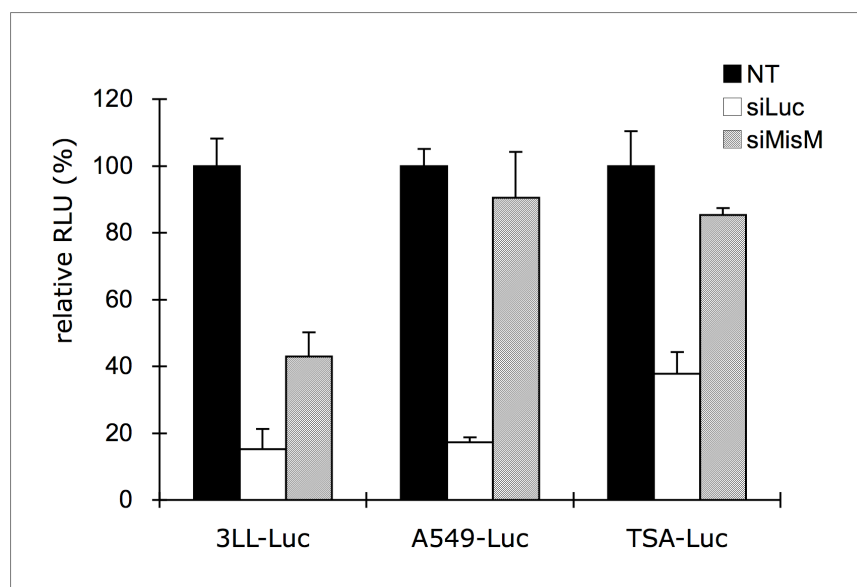
**Figure 1. siRNA silencing to luciferase endogenously expressed in cells**

Figure 1.

Cells stably expressing luciferase were transfected by 0.2  $\mu$ M siRNA anti-luciferase (siLuc) or mismatched (siMisM) in 24-well plates. Four days after transfection, cells were lysed and quantified for luciferase expression. RLUs expressed on the figure were normalized by the corresponding protein concentration. The average RLU values of non-treated (NT) cells were set as 100% for respective cell lines, and those of treated cells were expressed as relative percentages. Error bars indicate standard deviations (SD).

## II.2. Results

### II.2a. siRNA-mediated silencing of the luciferase reporter gene in vitro

In order to verify the efficacy of the selected anti-luc siRNA, 3 cell lines stably containing the pGL3 plasmid from Promega and thus positive for luciferase expression (3LL-luc, TSA-luc, A549-luc) were transfected with 0.2  $\mu$ M siRNA anti-luciferase (siLuc) or siRNA mis-matched anti-luciferase (siMisM) using Oligofectamine in 24-well plate. The enzymatic activity of luciferase present in the cell extracts was measured using a commercial kit (Promega) that contained at least the luciferin substrate, coenzyme A and ATP. The production of photons during 10s accompanying the oxidation of luciferin can be counted by a luminometer (Berthold), expressed as Relative Light Units (RLU) and normalized according to the protein concentration present in the samples (measured using another commercial kit from BioRad (DC compatible)). As shown in [figure 1](#), transfection of siLuc potently inhibited luciferase expression in all cell lines ( $\pm$  80% inhibition) while transfection of siMisM had almost no effect on A549-luc or TSA-luc cells and a small inhibition of luciferase expression in 3LL-luc cells.

These results confirmed that the selected sequences for the 2 siRNAs were efficient.

**Figure 2. in vivo biodistribution of siRNA by 2D-FRI**

2a.

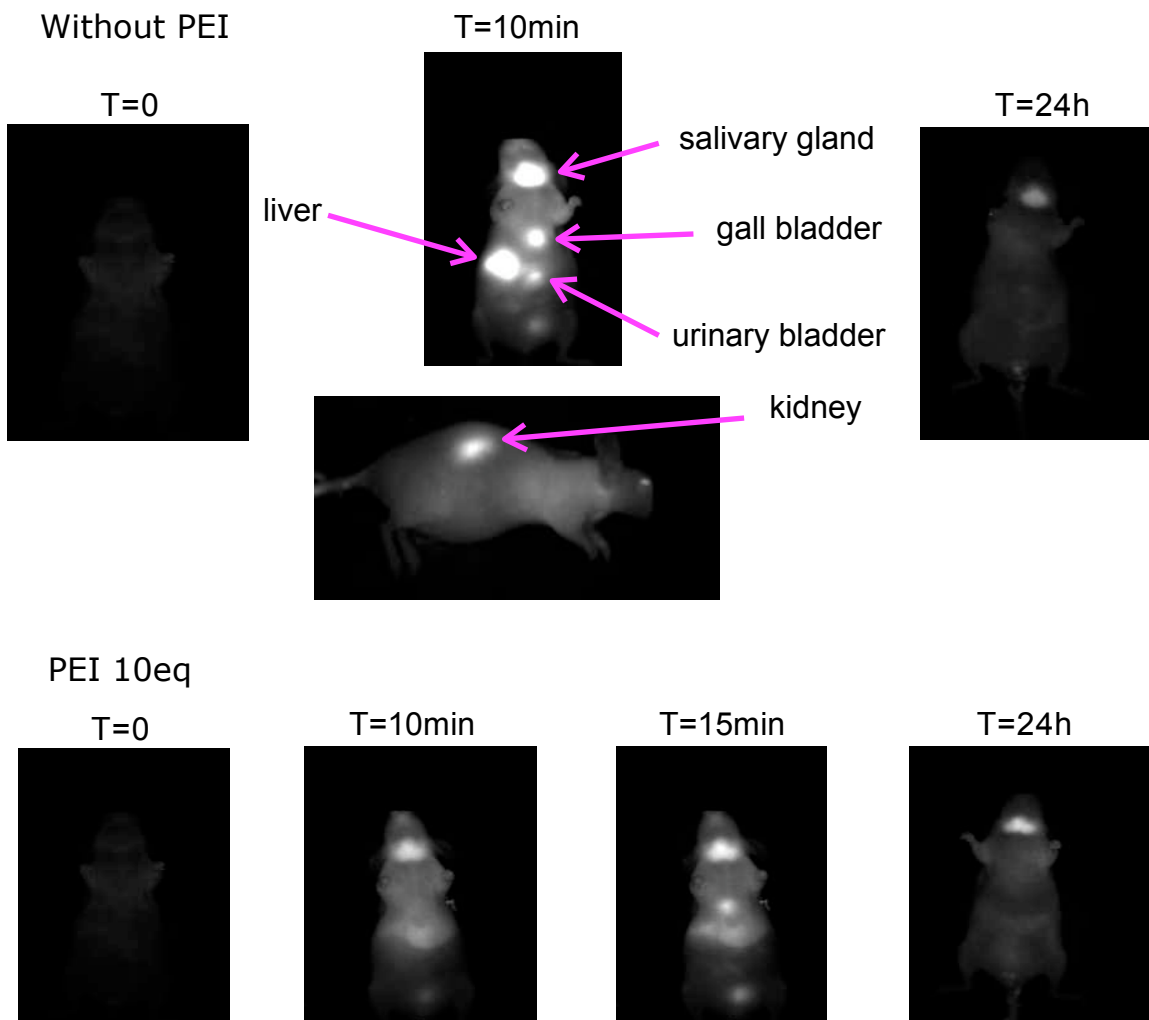


Figure 2.

a/ The mice were IV injected with 200  $\mu$ l of 5% glucose containing PEI/DNA/siRNA complex, and imaged at time points as indicated on the figure. The DNA/siRNA mixture contained 30  $\mu$ g of siLuc-Cy5 + 20  $\mu$ g of pcDNA3.1, with or without the PEI compaction.

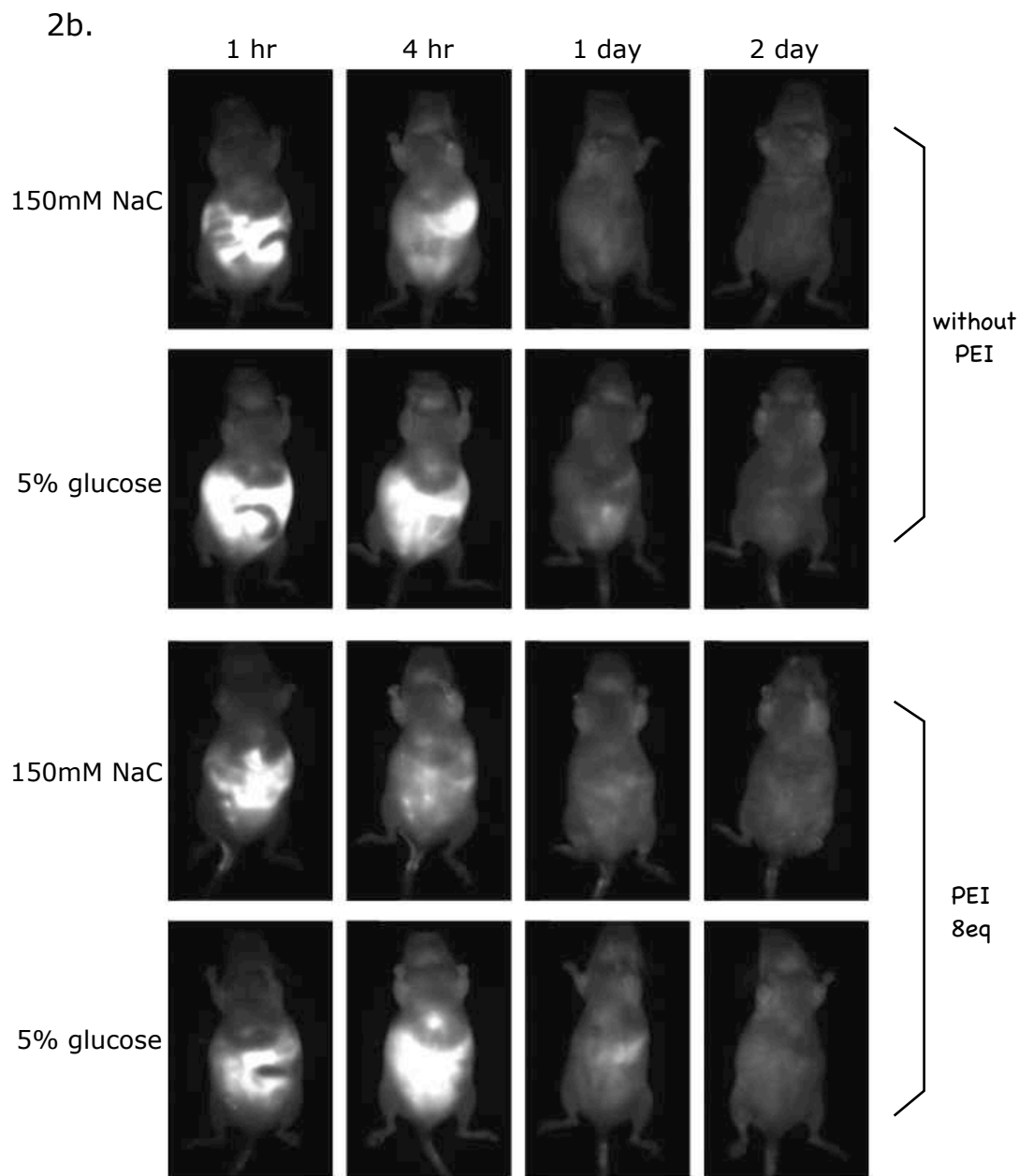
## II.2b. The biodistribution of PEI/siRNA in mouse

To understand the biodistribution of siRNA after delivery into the mouse body, siRNA was labeled with Cyanine-5 (Cy5) and compacted by L-PEI in different conditions and injected. The mixtures of siLuc-Cy5 and a plasmid pcDNA3.1 were solved in the solution of 5% glucose or 150mM NaCl, with or without PEI compaction, and injected IV or IP into the mice. Different ratios of positively charged amine groups on L-PEI over negatively charged phosphate groups on DNA/siRNA were used for polyplex compaction (this ratio is called the “N/P ratio”, and is indicated as “eq” in the following text and figure). DNA was mixed with siRNA in order to complement the total negative charge for the PEI-complexing condition that is known to be efficient in *in vivo* gene delivery by IV injection (Goula et al., 1998).

The biodistribution of siRNA was followed using a non-invasive 2D Fluorescent Reflectance Imaging system (2D-FRI). The images obtained after IV injection showed that either with or without the PEI compaction, the major part of the injected siRNA was accumulated in the salivary gland, liver, gall bladder, urinary bladder, and kidney. These signal distributions can be detected clearly in 10 min after injection, and then decayed gradually without significant change in the distribution pattern, in the following 24 hr (Fig. 2a). The presence of siRNA in liver, kidney, and urinary bladder is usual and should reflect the normal capture and excretion of these charged molecules via urinary system. Oppositely, the presence of the siRNA in the salivary glands was surprising and not anticipated. This is a hallmark of siRNA distribution, and was verified several times in independent experiments using siRNA with various specificities, ruling out the possibility that this accumulation in salivary glands could be sequence specific.

The biodistributions of siRNA were identical either with or without PEI compaction, suggesting that the PEI/siRNA complex was not stable *in vivo* and that the siRNA was detached from PEI rapidly after its injection into the vein. The weaker signal in the mouse injected by PEI/siRNA complex may come from the interference of PEI to the Cy5 signal on siRNA, as compared to siRNA only.

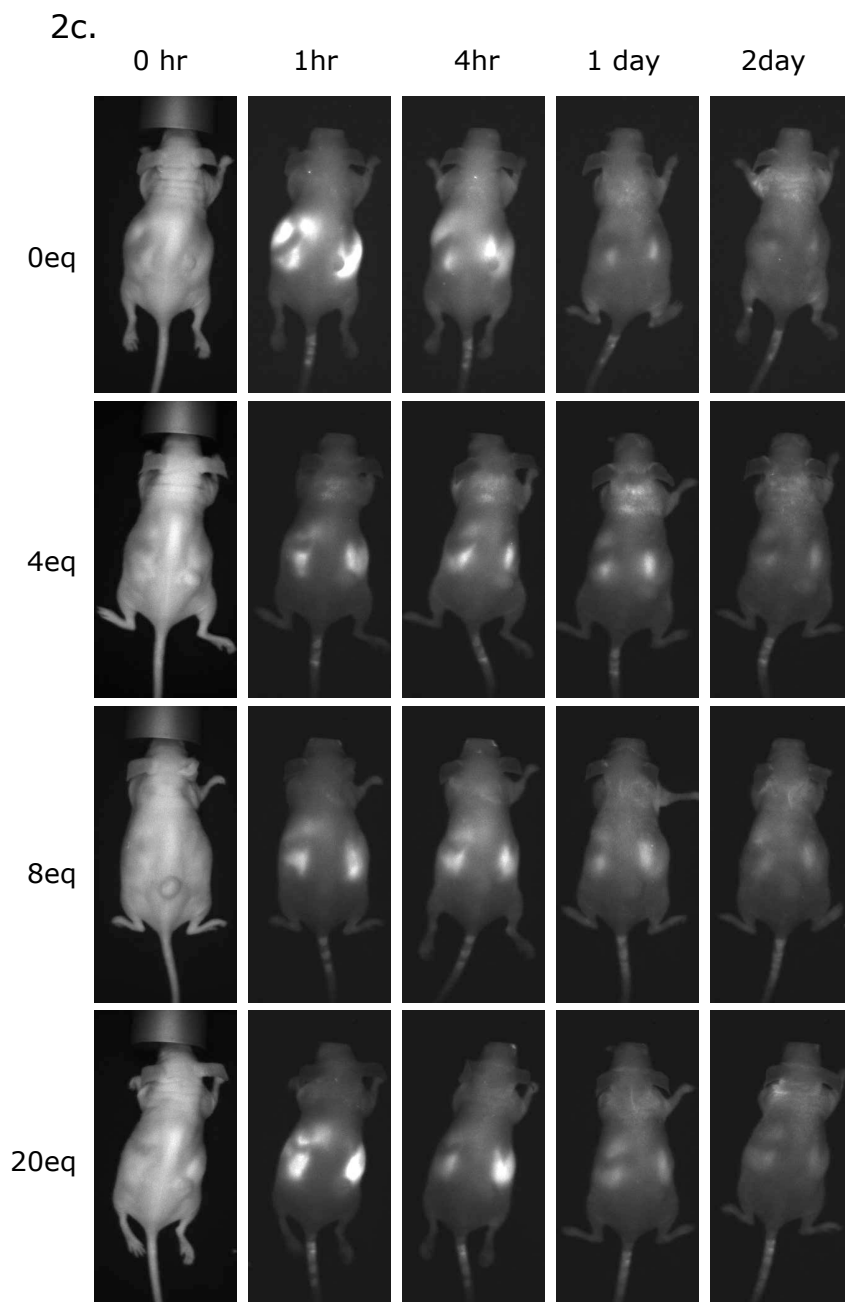
**Figure 2. (continue)**



b/ The mice were injected IP with PEI/DNA/siRNA complex and imaged at the time points as indicated. The DNA/siRNA mixture contained 15  $\mu$ g of siLuc-Cy5 and 10  $\mu$ g of pcDNA3.1, which was solved in 1 ml of 150mM NaCl or 5% glucose solution, with or without the PEI compaction, as indicated on the figure.

Then we tried to deliver siRNA through IP administration. Similarly, siRNA with or without PEI compaction was prepared, but 2 different solutions (NaCl or glucose) for preparing the complexes were used in this experiment (Fig. 2b). However, in each condition most of the siRNA signal simply distributed in the whole abdominal cavity and gradually decayed during the following 48 hr after injection, except the partial accumulations in the liver and kidney (see also Fig. 2c). The common biodistribution pattern of siRNA with or without PEI compaction suggested again the instability of PEI/siRNA complex once injected *in vivo*.



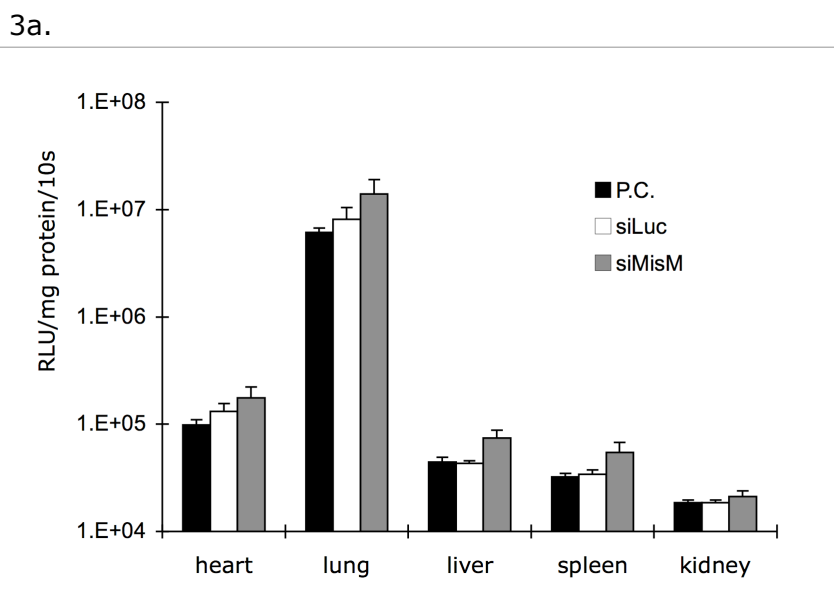
**Figure 2. (continue)**

c/ All mice with A549-luc xenografts have received PEI/DNA/siRNA complex by IP injection, and were imaged at time points as indicated on the figure. The DNA/siRNA mixtures contained 15  $\mu\text{g}$  of siLuc-Cy5 + 10  $\mu\text{g}$  of pCDNA3.1, and were compacted by PEI in 0, 4, 8, or 20eq (0eq indicates without PEI) in 1 ml of 150mM NaCl solution.

The images obtained on 0 hr were before PEI/DNA/siRNA injection and were taken under normal light to visualize the position of tumor.

To determine if PEI/siRNA complex is able to reach tumor cells *in vivo*, we injected IP the complex into the mice carrying SC A549-luc xenografts. The IP administration was selected in order to correlate with the following experiments in which we tried to evaluate the antitumor activity of siRNA in tumor xenografts. DNA/siRNA mixtures were compacted by PEI in 4 different equilibrium ratios (0, 4, 8, or 20eq) in this experiment (Fig. 2c). The positions of the tumor were visualized by imaging under normal light ("0 hr" on Fig. 2c). However, no siRNA signal was observed in xenografts. Once more, siRNA was present universally in the liver and kidney, in all conditions.

**Figure 3. Delivery of PEI/siLuc to mouse lung via IV injection**



3b.

condition	i	ii	iii
siRNA (µg)	30	30	20 or 40
DNA (µg)	40	40	40
eq	10	8	8

Figure 3

a/ Forty µg of pGL3-Promoter (pGL3) was mixed with 30 µg of siLuc or siMisM, compacted by PEI in 10eq and injected to mice through tail vein. Mice were sacrificed one day after injection, and RLUs of homogenates from different organs were measured and normalized by the corresponding protein concentrations. P.C.: positive control, pGL3 (40 µg) + pcDNA3.1 (30 µg). siLuc: pGL3 (40 µg) + siLuc (30 µg). siMisM: pGL3 (40 µg) + siMisM (30 µg). N=5 for P.C. and siLuc groups, n=6 for siMisM group. Error bars indicate standard error of mean (SEM).

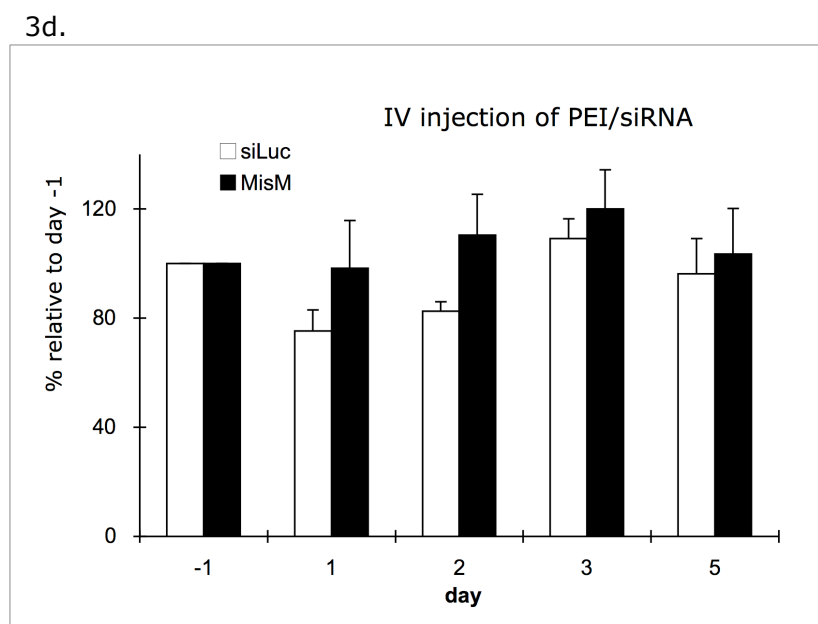
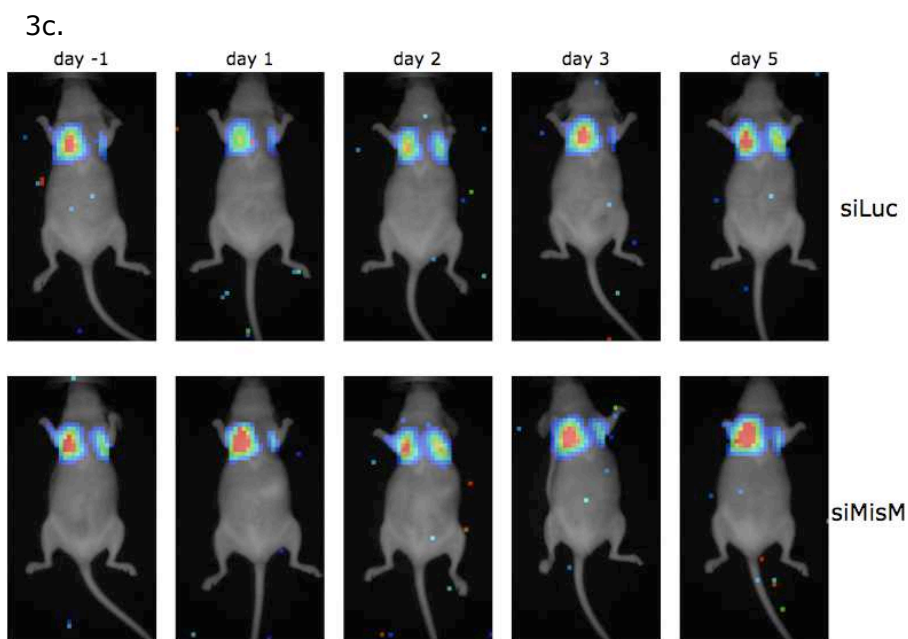
b/ A summary of the conditions that have been tested in the experiments as described on figure 3a. The solution was 5% glucose in all conditions. N=3 for condition i, n=1 for condition ii or iii.

### **II.2c. In vivo delivery of PEI/siRNA to mouse lung via IV administration**

L-PEI is a good DNA transfection reagent in vivo, leading mainly to the transfection of 1-5% epithelial lung alveolar cells after IV administration of PEI/DNA complexes (Goula et al., 1998). In order to demonstrate that L-PEI could also deliver siRNA to these lung cells, we firstly co-transfected a luciferase-expressing plasmid with the siRNA anti-luc or mismatch. Forty  $\mu\text{g}$  of the pGL3 plasmid was mixed with 30  $\mu\text{g}$  of siLuc or siMisM and complexed by PEI in 10eq before tail vein injection. In the positive control (no siRNA) the final DNA concentration was adjusted to 70  $\mu\text{g}$  by adding 30  $\mu\text{g}$  of the pcDNA3.1 “empty” plasmid DNA. One day after transfection, mice were sacrificed and pieces of  $\pm$  200 mg of each organ of interest were collected, blended in 1ml of passive lysis buffer (Promega) and frozen at  $-20^{\circ}\text{C}$  O/N. The level of luciferase and the protein concentrations were then determined as described previously.

The results presented in [figure 3a](#) showed that the expected strong transfection of lung cells was obtained when the plasmid was transfected alone. However, no statistically significant silencing effect was measured in the presence of siLuc. We then modified the transfection conditions by changing the ratio of plasmid versus siRNA, or the equilibrium ratio ([Fig. 3b](#)). However, no reliable luciferase gene silencing was observed whatever the transfection condition used. These negative results could signify either that L-PEI is not able to deliver siRNA efficiently to the lung and/or that the co-transfection method we used was not adapted to the measurement of a silencing effect.

**Figure 3. (continue)**



c/ Fifty  $\mu\text{g}$  of siLuc or siMisM was compacted by PEI in 8eq and injected IV to mice stably expressing luciferase in lung. The changes of RLU levels were monitored by the non-invasive optical imaging system at time points indicated, and photos were processed on the software Wasabi. Red color indicates more intensive luminescence, while blue means weaker. Day -1 indicates the day before the transfection

d/ RLU levels in lungs of all mice were quantified from the images by the software Wasabi. For each mouse, the RLU intensity on day -1 was set as 100% and RLUs in following days were expressed as relative percentages to day -1. N=5 for siLuc group, n=4 for siMisM group. Error bars indicate SEM.

We thus used another mouse model, which expresses stable levels of luciferase in the lung after L-PEI mediated transfection of the *Sleeping-Beauty* (SB) transposon-luciferase (luc) plasmids. L-PEI/SB-luc transfected mice present a stable and detectable level of luciferase expression in lung approximately 1 month after the intravenous transfection (see the Thesis RESULT and DISCUSSION III for a detailed description). Since this plasmid was initially transfected using L-PEI, we can expect that the cells expressing stably luc will be favorite targets for another transfection with L-PEI/siRNA performed one month later.

We prepared L-PEI/siRNA complexes by mixing 50 µg of siLuc or siMisM with L-PEI in 8eq in 200 µl of 5% glucose and injected them IV in these mice. Luciferase expression levels were monitored initially the day before siRNA injection and 1, 2, 3, or 5 days after, using the non-invasive bioluminescence optical imaging (BLI). In this case, 150 mg/kg of Beetle Luciferin (Promega) was IP injected 5 minute before exposing the mice to the ultrasensitive pre-cooled (-80°C) CCD camera (Hamamatsu Orca BTL), in a dark box under Isoflurane gaseous anesthesia (Fig. 3c). Digital images are then processed using the Wasabi software. This allows us to quantify the amount of photons emitted by the lung and to compare the variation of signal in each mouse (Fig. 3d).

When comparing to the RLU level before transfection (day -1), a transient and significant decrease of 25% and 18% of luc expression was measured in lung, 1 and 2 days respectively, after the transfection of siLuc ( $P < 0.05$ ). In contrast, no significant variation of RLU was noticed in the lung of mice transfected by siMisM ( $P > 0.9$ ). This result suggests that a significant inhibition of luc expression in lungs was associated with the PEI/siLuc injection, but not with PEI/siMisM.

If we compare now siLuc versus siMisM treated groups, a 23% and 28% of decrease in the siLuc group was observed on day 1 and 2, respectively. However, these values were not statistically different ( $P > 0.19$ ).

These results may suggest that PEI-mediated delivery of siRNA has a weakly specific effect but also a certain toxicity. Thus it is possible that the siLuc transfection combined these two effects, while the siMisM only destroyed some of the luc-expressing cells.

Thus L-PEI is poorly able to deliver efficiently an siRNA into alveolar epithelial cells of the lung after systemic administration. Nonetheless, a moderate diminution is obtained in these lung cells normally sensitive to L-PEI transfection, which suggests however a weak and transient silencing of an “endogenous” gene.

**Figure 4. Delivery of PEI/siLuc to mouse xenografts via IV injection**

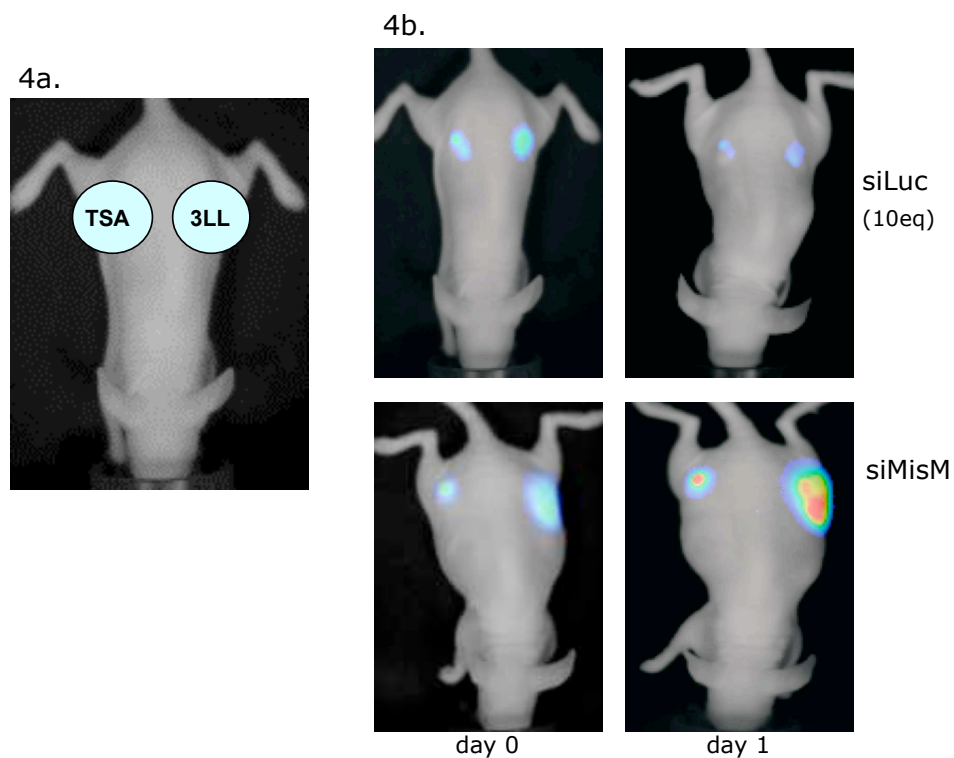


Figure 4

a/ The schematic representation of TSA-luc and 3LL-luc tumor implantations on mouse.

b/ Mice transfected by siRNA through IV injection were monitored for luc expression in tumors by the CCD camera. One example coming from siLuc or siMisM group was illustrated for the change of RLU in tumors between day 0 (the day of transfection) and day 1.

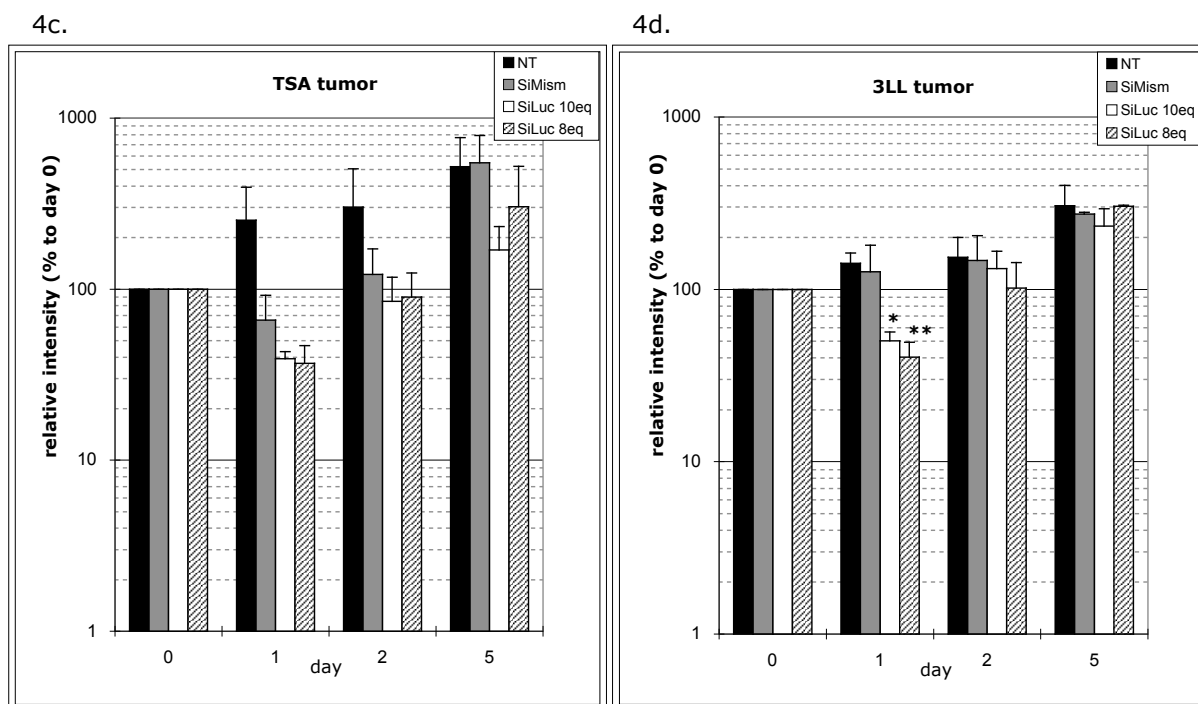


### **II.2d. In vivo delivery of PEI/siRNA to mouse xenograft via IV administration**

We then evaluated the capacity of PEI to transfer siRNA to tumors *in vivo*. TSA-luc and 3LL-luc cells were implanted SC on the 2 flanks of nude mice (Fig. 4a). Seven to 10 days later when tumors were detectable, mice were injected IV with PEI/siRNA polyplexes. We used 2 different formulations (8 or 10eq) for complexing 60 µg of siLuc or siMisM. Luc expression was monitored longitudinally over time by the CCD non-invasive BLI system.

One day after injection of PEI/siLuc complexes, luc expression in both tumors was potently reduced. At the same time, luc expression in siMisM treated mice weakly increased, presumably due to the growth of the tumor (Fig. 4b).

Figure 4. (continue)



Quantification of RLU intensity in c/ TSA-luc or d/ 3LL-luc tumors. The RLU of each tumor on day 0 was set as 100% and the relative intensities in following days were expressed. For siLuc treatment, the results of 2 equilibrium ratios (10 and 8eq) of PEI compaction were expressed separately. N=4, 6, 5, 6 for NT, siMisM, siLuc 10eq, and siLuc 8eq groups, respectively. In siMisM group, n=3 for 10eq and n=3 for 8eq were combined as one result. These data include the results from 2 independent experiments. Error bars indicate SEM.

Treatment with siLuc reduced luciferase expression in both tumors at day 1 (Fig. 4c and d). Up to 63% of inhibition was calculated, in comparison with the RLU before injection. Changing the equilibrium ratio (8 or 10eq) had no significant influence. In TSA-luc tumors this inhibition lasted for 5 days, whereas this effect was observed at day 1 only within 3LL-luc tumors. However, the silencing effect was not sufficient or reproducible enough to be statistically significant in TSA-luc tumors (  $P > 0.2$  when siLuc is compared to NT or siMisM groups). This was not the case within 3LL-luc tumors, in which the RLU between siLuc and NT groups on day 1 was found significantly different ( $P < 0.05$  and  $0.01$  for 10 and 8eq, respectively), but this was not true anymore when the siLuc group was opposed to the siMisM ( $P > 0.17$ ).

These results demonstrated that the IV injection of L-PEI/siRNA polyplexes could induce a transient inhibition of luc expression in SC tumors, in agreement with our previous results obtained in the lung. Once more however, this effect was weak and often lost its statistical impact, possibly because of large variations between animals, the inefficiency of PEI-mediated siRNA delivery, and also the non-negligible interfering effect of the siMisM.

**Figure 5. Delivery of PEI/siLuc to A549-luc xenograft via IP injection**

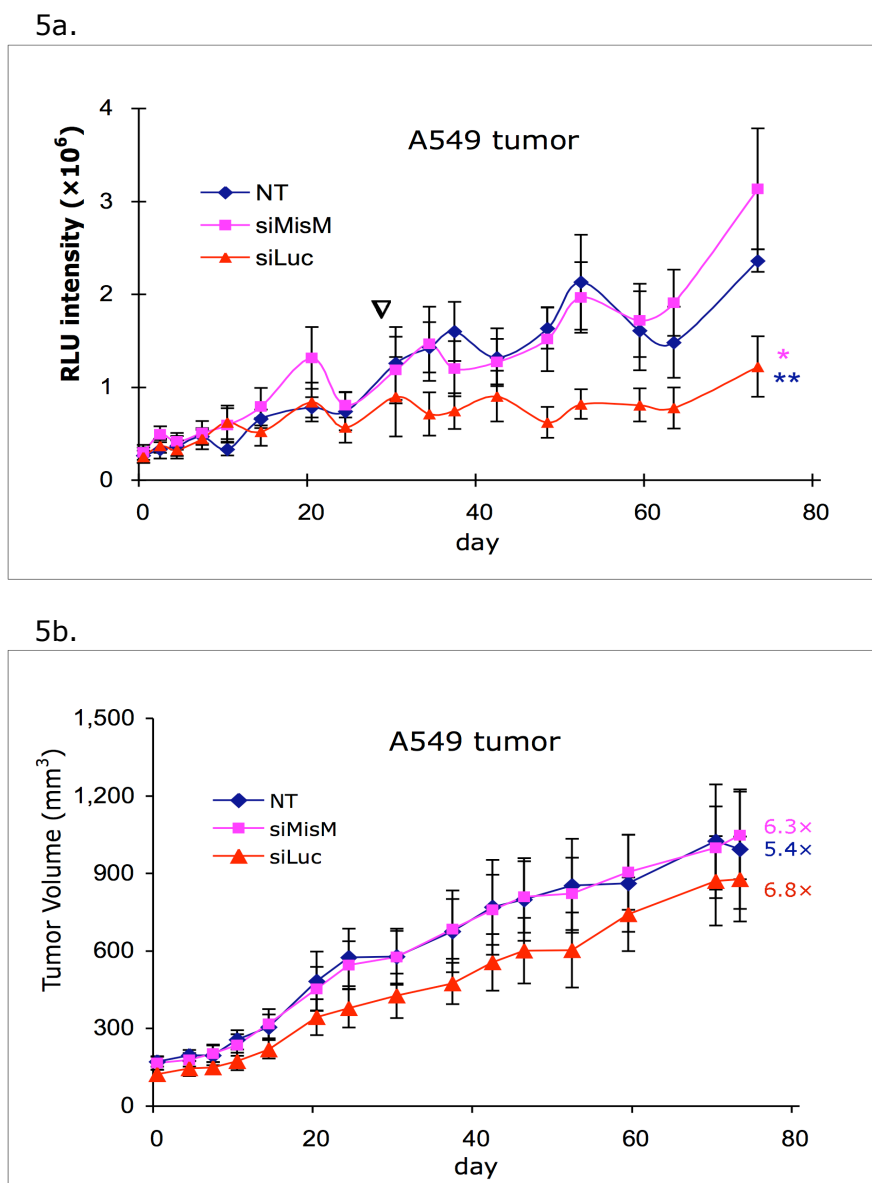


Figure 5

A549-luc xenografts were implanted by SC injection of  $2 \times 10^7$  cells in nude mice. Ten days later when solid and palpable tumors formed, IP injections of PEI/siRNA compacted in 8eq were administrated 2-3 times a week.

a/ RLU intensities of A549-luc tumors were quantified from BLI photos. ▽ indicates the day 29, and since that the quantity of siRNA for each transfection was increased from 5 to 10  $\mu$ g.

b/ The corresponding tumor volumes were recorded.

Error bars indicate SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$

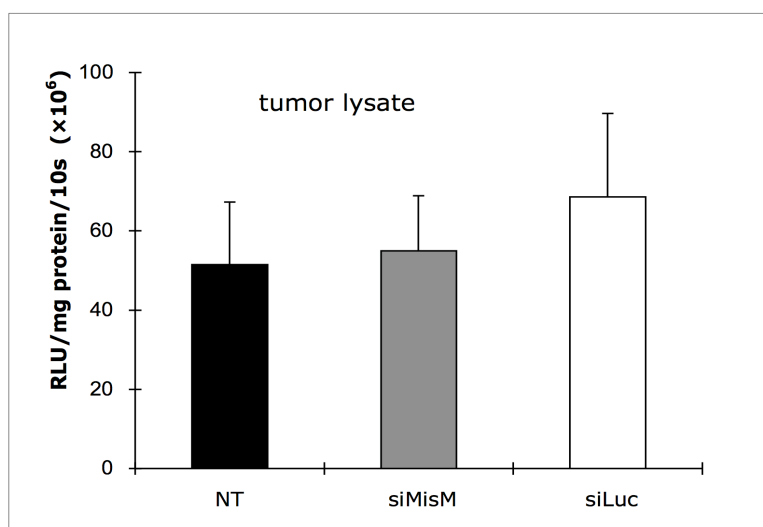
## II.2e. In vivo delivery of PEI/siRNA to mice xenografts via IP and SC administrations

Published results suggested that IP administration of PEI/siRNA anti-HER-2 was able to deliver siRNA to tumors and led to tumor growth suppression (Urban-Klein et al., 2005). IP injection can be repeated every few days in mice, while this is not possible using the IV route. My major objective in this research is to measure a potential therapeutic activity of siRNA in mouse xenografts. Former results of our laboratory have demonstrated that the C-Raf downregulation using ASO efficiently prevented the growth of A549 human NSCLC xenograft in mouse model, due to the induction of apoptosis (Zavaglia et al., 2003b). Thus we have decided to use the A549 xenograft model for the treatment of PEI/siRNA anti-C-Raf by IP injection. Nevertheless, in regard to the low efficiency of siRNA delivery, we thus measured in advance the effect of IP injected PEI/siLuc on A549-luc SC tumors, before administrating the “therapeutic” anti-C-Raf treatment.

IP injections were repeated every 3 days, and the RLU levels of tumor were followed using BLI 1-3 times per week (Fig. 5a). A dose of 5 µg of siRNA per injection was initially used till day 27. As shown in figure 5a, only a slight decrease of luc expression was noticed during this period in the siLuc group. We thus decided to increase the dose of siRNA to 10 µg/injection since day 29. This had an important effect and the level of RLU in the siLuc group started to be different than those measured in the 2 other groups. When the variation of RLU levels between day 73 and day 0 were calculated, we observed that in NT and siMisM groups they were 10.5 and 8.7 times more elevated, respectively. On the other hand, tumors from the siLuc group showed a elevated ratio of 4.9 only (statistical significance between siLuc and siMisM or NT group of  $P < 0.05$  and  $0.01$  respectively). Importantly, as shown in figure 5b, the growth of the tumors were comparable in the 3 groups since the ratios of tumor volumes at Day 73/Day 0 were 5.4, 6.3, and 6.8, in NT, siMisM, and siLuc groups, respectively. Thus the reduction of RLU in the siLuc group was not due to a lower tumor growth rate.

**Figure 5. (continue)**

5c.



c/ At the end of the experiment, mice were sacrificed and the RLUs of tumor lysates were measured using a luminometer and normalized by the corresponding protein concentrations.

N=7 for siLuc and siMisM groups, and n=4 for NT group. Error bars indicate SEM.

At this step, the animals were sacrificed and the luciferase enzymatic activities in the tumor extracts were measured directly by a luminometer (Fig. 5c). Surprisingly, these results did not confirm the former ones obtained using BLI quantification in living mice, and no inhibition of luciferase expression could be measured.

**Figure 6. Delivery of PEI/siC-Raf to A549-luc xenograft via IP injection.**

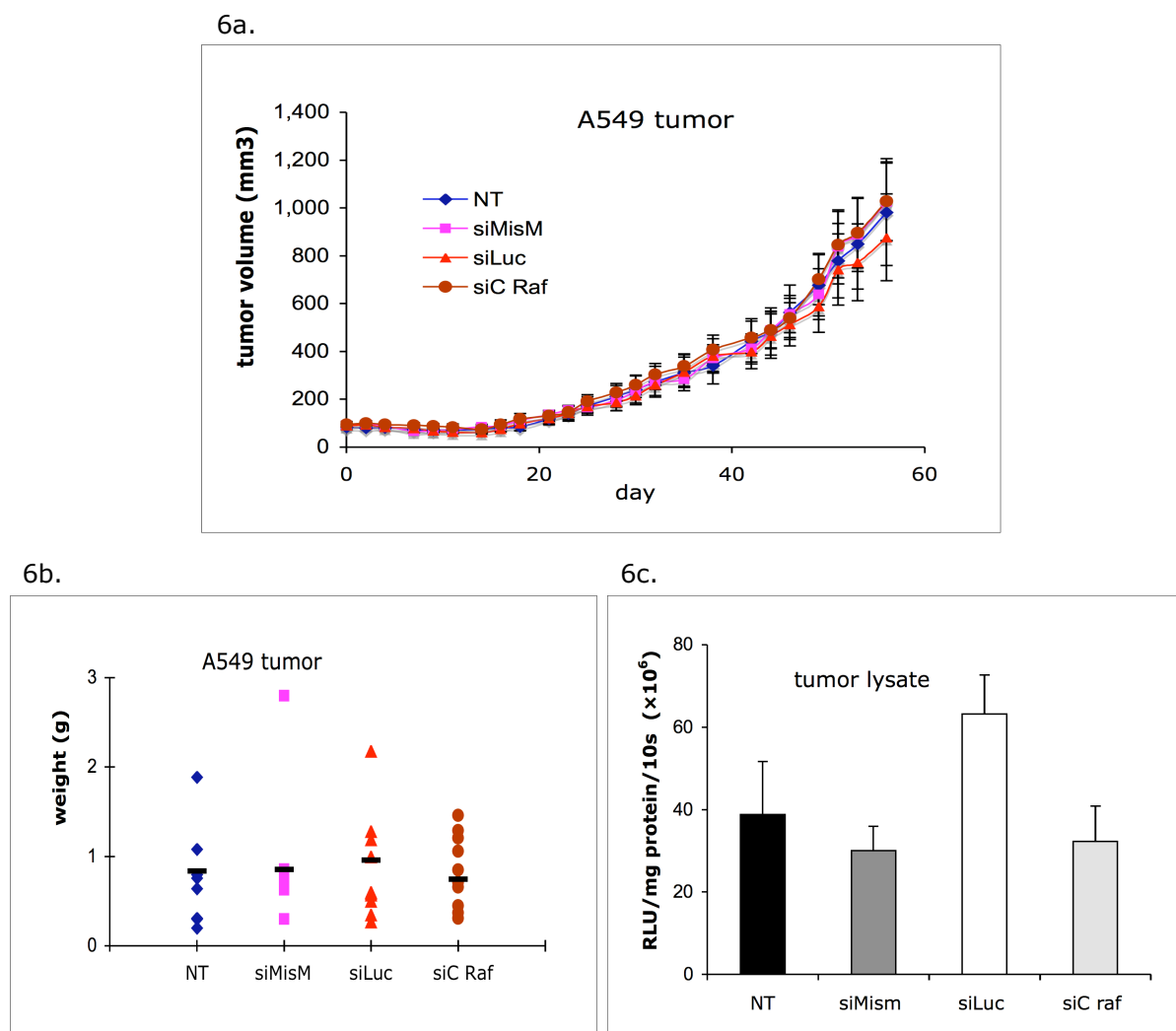


Figure 6.

One week after implantation of A549-luc xenografts, PEI/siRNA was IP injected 3 times per week. The PEI/siRNA was complexed by 10  $\mu$ g of siRNA and PEI in 8eq.

a/ Tumor volumes were measured 2-3 times per week.

b/ At the end of the experiment, tumors were removed and weighted after sacrifice of the mice.

c/ RLUs of tumor lysates were measured using the luminometer and normalized by the corresponding protein concentrations.

N=10 for siC-Raf and siLuc groups, and n=8 for siMisM and NT groups. Error bars indicate SEM.



We thus repeated this experiment using the same protocol, except that the siRNA injected dose was set up at 10 µg/injection from the beginning. In addition, a group treated with the siRNA anti-C-Raf (siC-Raf) was added. Tumor volumes and RLU levels using BLI were measured twice a week. At the end of the experiment tumors were weighted and lysed, and their luc activities were measured using the luminometer. However, none of the siRNA treatment modified the tumor growth, the final tumor weight, or the luciferase expression measured by both methods (Fig. 6a, b and c).

**Figure 7. Delivery of PEI/siLuc to mouse xenografts via IP and SC injections**

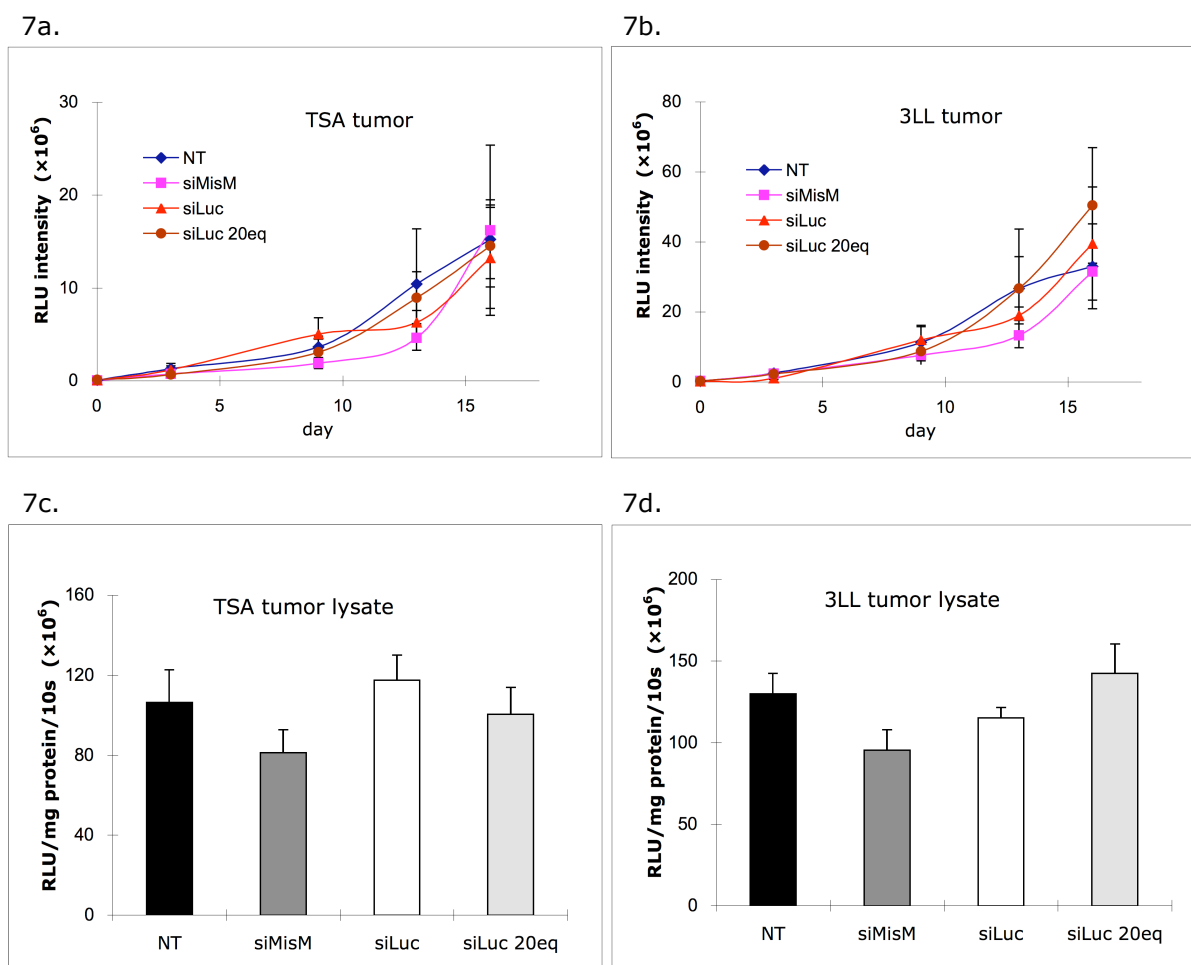


Figure 7.

TSA-luc and 3LL-luc SC tumors were established as described previously (Fig. 4a). Three days after implantation, mice were transfected by PEI/siRNA polyplex injections IP and SC. Each injection type was performed twice a week, so each mouse received 4 doses/week. For each injection, the quantity of siRNA was 16  $\mu$ g compacted by PEI in 20eq in 150mM NaCl containing 10 mM HEPES pH 7.4 (siLuc 20eq group), or in 8eq in 5% glucose as before (the other 3 groups).

a/ RLU intensity of TSA-luc tumors quantified from BLI system.

b/ RLU intensity of 3LL-luc tumors quantified from BLI system.

c/ RLU intensity of TSA-luc tumor lysates measured by luminometer and normalized by protein concentration at the end of experiment.

d/ RLU intensity of 3LL-luc tumor lysates measured by luminometer and normalized by protein concentration at the end of experiment.

N=8 for siLuc, siMisM, and siLuc 20eq groups, and n=4 for NT group. Error bars indicate SEM.

A final experiment was then performed, in which we came back to the TSA-Luc and 3-LL-luc SC tumors. This time, and in order to augment the chances to measure an inhibition, nude mice were transfected by PEI/siRNA twice by IP and twice by SC injections per week, and the quantity of siRNA in each transfection was raised to 16  $\mu\text{g}$ . Two formulas for PEI/siLuc polyplexes (8eq and 20eq) were evaluated in this experiment, while PEI/siMisM was tested at 8eq only. BLI images were taken once or twice a week. Once again, no significant difference in RLU level among different groups was noticed, for both TSA-luc and 3LL-luc tumors (Fig. 7a and b). In addition, the measurement of luc activities with the luminometer at the end of the experiment was not in favor of an inhibitory effect of siLuc at 8 or 20 eq (Fig. 7c and d).

## II.3. Discussion

The aim of our research was to exploit a convenient and safe modality of systemic siRNA delivery that is able to target special tissue (e.g. lung) or tumors *in vivo*. In this report, we used 22 kDa L-PEI as a vector for systemic siRNA delivery. Initial *in vitro* transfection of siLuc to lung cancer cells stably expressing luciferase showed the ability of these siRNA to silence their target gene (Fig. 1).

Based on the well-studied properties of L-PEI to deliver a plasmid to the lung epithelial alveolar cells in mouse after an IV injection, we evaluated its capacity to deliver also the siRNA molecule. This was performed either in co-transfection experiments, where the plasmid and siRNA were mixed and complexed by PEI, or by transfection of the PEI/siRNA only to mice stably expressing the luciferase gene in epithelial alveolar cells. Our results usually suggested a weak silencing effect to luc expression after PEI/siLuc treatment. But this effect was more a tendency than a measurable effect and in most of the case, no statistically significant difference in luc expression was found between the siLuc and siMisM groups (Fig. 3).

Concerning the ability of PEI to deliver siRNA to tumor cells *in vivo*, it has been previously demonstrated that PEI can compact and efficiently deliver siRNAs against HER-2 and PTN to ovarian carcinoma and glioblastoma xenografts. This is accompanied by an antitumoral effect and this happens either after an IP or SC administration of the polyplexes (Urban-Klein et al., 2005; Grzelinski et al., 2006). To better understand the efficiency and kinetics of PEI/siRNA transfection in growing tumors, we chose the BLI live animal imaging system to monitor the siRNA-mediated reporter gene silencing and the 2D-FRI to follow the distribution of the siRNA in a living mouse. After an IV administration, PEI/siRNA prepared with 8 or 10eq showed a transient inhibition of luc expression in the tumors 1 day after transfection (Fig 4). This could inhibit up to 60% of luciferase expression (Fig. 4c and d). An interference effect induced by siMisM treatment was also observed in TSA tumors, suggesting a possible non-specific silencing of the luc gene but could also be due to a certain toxicity of the transfection. This result indicated that PEI can deliver siRNA and transfect tumor xenografts transiently *in vivo* via IV administration, although an overall statistical significance cannot be reached (see also II.2c. above).

In order to be able to repeat several times a week the siRNA administration, we then evaluated the efficiency of PEI/siRNA transfection by IP injection. We also raised the dose of siRNA injected from 5 to 10 and 16 µg/injection and varied the ratio of PEI for siRNA compaction (8, 10 or 20 eq). After several weeks of treatment, with injections repeated every 3 days and even when IP injection was combined with an SC administration, our results did not show a significant activity of siRNA-mediated silencing, and altogether our results were negative (Fig. 5, 6, and 7).

In addition, results obtained in FRI system indicated that the presence of PEI did not modify the distribution of the labeled siRNA (Fig. 2). This thus suggested that the stability of these polyplexes was not satisfying *in vivo* and that the siRNA was liberated rapidly from its vector. However the distribution and accumulation of siRNA in the salivary gland are very interesting and should be investigated in more details.

Concerning the biological activity, we got conflicting results in 1 out of 3 independent experiments. Indeed in the first experiment, the BLI longitudinal follow-up of luc expression suggested an inhibitory effect after PEI/siLuc treatments (Fig. 5a). However, this result was not confirmed by the “standard” measurement of luciferase activity in the A549 tumor lysates after sacrifice of the mice (Fig. 5c and 6c). We emitted 3 hypothesis to explain this phenomenon:

1/ the siRNA inhibition works mainly on the fast growing tumor cells preferentially located at the periphery of the tumor. Since these cells are directly exposed to the camera (at least for the part of the tumor in contact with the dorsal skin), it is possible that a bias was introduced in the BLI measurement. In whole tumor extracts, this effect would be diluted because the majority of the tumor cells were untouched.

2/ Repeated administrations of PEI/siRNA could plug the tumor vasculature and reduce the distribution of luciferin in this tissue.

3/ Repeated administration of the luciferin injected IP could affect the measure. Indeed, the oxyluciferin (intracellular product of luciferin oxidation by luciferase) could progressively accumulate in luc-positive cells and inhibit competitively the luc enzymatic reaction.

Among these 3 possibilities, the first one is our favorite. Indeed, according to the 2 others, it is difficult to explain why the siLuc would react differently than the siMisM.

We do not have a final explanation, but these results suggest that the measurement of a reporter gene expression can be subject to different artifacts and should always be considered with caution. Eventually, these results must always be sustained by a measure of the “true” biological effect.

L-PEI-based delivery of siRNA has been described *in vitro* and *in vivo*. Performed by the same group, 10 pmol of siRNA compacted by L-PEI (10eq) showed *in vitro* at least 50% reduction of targeted gene in SKOV-3 cells (ovarian carcinoma) and U87 (glioblastoma) cells, and 50-70% reduction of targeted oncogenes in established SC tumors by IP or SC injection of 0.6 nmol of siRNAs every 2 to 3 days for at least 2 weeks (Urban-Klein et al., 2005; Grzelinski et al., 2006). Additionally, an intratracheal transfection of PEI/DNA expressing luciferase followed by an IV injection of 60 µg siRNA compacted by L-PEI in 5eq resulted in 17-fold lower luciferase expression in mouse lung homogenates one day after transfection, in a small number of animals (n=3) (Ge et al., 2004). A separate study however, reported that L-PEI was not efficient for siRNA delivery to newborn mouse brain within their dose range tested. In this study, co-transfection of 0.1 µg plasmid expressing luciferase with 0.2 pmol of siRNA anti-luc showed no inhibition to luc activity, while only 20% reduction of RLU was obtained when siRNA was increased to 1 pmol (Hassani et al., 2005).

Our results after co-transfection of siRNA and pGL3 with L-PEI did not indicate that the siRNA was efficient (Fig. 3a and b). However, transfection of PEI/siLuc to mice stably expressing luciferase in lung showed a reduction of 25% at day 1, but without statistical significance (Fig. 3c and d). This thus indicates that the transfection efficiency is weak. This is also sustained by the result of a study using guinea pigs. In this study, it is shown that IP injections of PEI/siRNA (5eq) at a dose of 8 mg siRNA/kg before and after Ebola virus infection protected only 1 of the 5 mice from death, despite of the reduced plasma viremia levels. In contrast, 5 out of 5 mice treated with the same siRNA formulated in SNALPs (stable nucleic acid-lipid particles) at a lower dose (0.75 mg siRNA/kg by IP injection) survived without evidence of illness

(Geisbert et al., 2006). These results are in agreement with ours and suggest that PEI-based siRNA delivery is not sufficient to be reproducibly detected and/or measured.

In another work, the biophysical and structural properties of polyplexes were studied, which showed that the capacity of PEI for transferring functionally active siRNA to cells was restricted to a narrow window of conditions. Only 25 kDa B-PEI at 6 and 8eq was efficient, while the 22 kD L-PEI failed to transfer siRNA to HR5-CL11 cells (HeLa derivative) *in vitro*. This inefficiency was suggested by the relatively lower binding affinity between L-PEI and siRNA that made the polyplex unstable during transfection (Grayson et al., 2006). This may also explain our partially positive but not reproducible results.

Interestingly, the diameter of particles formed by siRNA (in 250 nM) and PEI in 10eq in the solution containing 10 mM HEPES and 150 mM NaCl during 1h was measured at 41.5 nm (Grzelinski et al., 2006), while particles formed by siRNA (in 200 nM) and PEI in 8eq in the solution containing only 10 mM HEPES during 20 min had a diameter of 155 nm (Grayson et al., 2006). As the smaller particles showed successful *in vitro* and *in vivo* siRNA transfection, the larger were not efficient even when the N/P ratio was enhanced to 10. Thus, it should be interesting to repeat our experiments using different buffers or N/P ratios for preparing the complexes. In our case, we prepared mostly PEI/siRNA in 5% glucose, a condition according to the fixed DNA transfection. In [figure 3 and 4](#) the siLuc was compacted in 8 or 10eq, and in [figure 7](#) we also tried to prepare PEI/siRNA complex in 20eq in saline. Nevertheless, none of these modifications illustrated an augmentation of the siRNA efficiency.

Although the failure of siC-Raf mediated tumor growth inhibition can be strongly related to the poor efficiency of siRNA delivery, we can also question the choice of C-Raf as a therapeutic target. The involvement of Raf proteins in tumor cell growth and survival has been extensively described. The antitumor effect associated with the inhibition of C-Raf expression has been demonstrated by using ASO *in vivo* either alone (Monia et al., 1996) or combined with other therapy (Kasid and Dritschilo,

2003; Mewani et al., 2004; Pei et al., 2004). Anti C-Raf siRNA were able to suppress tumor growth in various mouse models of xenografts (Leng and Mixson, 2005; Meng et al., 2005; Pal et al., 2005; Culmsee et al., 2006). Our group also contributed to establish that delivery of ASO against C-Raf by VP22 vectosomes led to growth inhibition in the same A549 NSCLC tumor used in the present work (Zavaglia et al., 2003b). Thus it is reasonable to assume that the negative results we obtained here are certainly due to the poor transfection efficiency rather than to the choice of C-Raf as target. However, recent data suggest that B-Raf could be a better target than the other Raf family members (Wellbrock et al., 2004). This was established in melanoma, where it was found that B-Raf, but not A- or C-Raf, was accounting for the ERK signaling (Brose et al., 2002; Davies et al., 2002; Karasarides et al., 2004), but the relevance to lung cancer cells has not been described.

Nevertheless, another part of our group have used the same L-PEI-mediated transfection strategy to deliver 2 different siRNAs against Amphiregulin. In this case, repeated IP and SC injections of PEI polyplexes containing a combination of the 2 siRNAs were associated with a significant tumor growth suppression on H358 xenografts, another human NSCLC cells (A Hurbin and B Busser, personal communication). It is thus likely that the efficacy of L-PEI-mediated siRNA delivery is variable according to the tumor model and/or the choice of target oncogene. In addition, it may also be necessary to introduce several different sequences of siRNA directed against the target mRNA to elicit a more efficient silencing effect. These parameters would certainly benefit to be addressed in future experiments.

In conclusion, we propose that *in vivo* siRNA delivery based on L-PEI would still hold promise, but its efficacy may vary a lot according to different cell types, the protocols or formulations to be used, and the target gene. Meanwhile, we believe that modifications to the currently used L-PEI are necessary. It has been reported that the ligand-targeted PEI (PEI-PEG-RGD) showed a tumor-selective delivery of siRNA and tumor growth inhibition through IV administration (Schiffelers et al., 2004). Additionally, deacylated L-PEI had an enhanced DNA delivery efficiency and mouse lung specificity, and was also effective to deliver siRNA. It showed a suppression of up to 90% luciferase activity in mouse lung and conferred the protection against



influenza virus by inhibiting the viral nucleocapsid protein (Thomas et al., 2005). These examples clearly illustrate that there is still a strong potential to improve L-PEI “intrinsic” siRNA-transfection properties.

## II.4. Material and method

### Cell culture

Cell lines 3LL-luc, TSA-luc, and A549-luc were derived from murine Lewis lung carcinoma cell line 3LL (ATCC, CRL1642), mouse adenocarcinoma cell line TS/Apc (TSA), and human NSCLC cell line A549, respectively, stably transfected by the luciferase expression vector pGL3-Promoter (pGL3) (Promega, Charbonnières, France). A549-luc and TSA-luc were cultured in RPMI 1640, and 3LL-luc in D-MEM medium (Invitrogen, Cergy Pontoise, France). Mediums were supplied with 10% (v/v) fetal bovine serum, 0.5% (v/v) PEN-STREP, and 400 µg/ml of G418 (Life Technologies, Cergy, France) for the selective condition of pGL3. The medium for TSA-luc cells was supplied additionally with 25 µM β-mercaptoethanol (Invitrogen, Cergy Pontoise, France). All cells were cultured at 37°C in the humidified CO<sub>2</sub>-controlled (5%) incubator.

### Tumor implantation and measurement

For tumor implantation, cultured cells were trypsinized and suspended in 200 µl of PBS, SC injected into five-week old female Swiss nude mice. For the experiment in [figure 4](#), 10<sup>7</sup> and 5×10<sup>6</sup> of 3LL-luc and TSA-luc cells, respectively, were used for each injection; in [figure 7](#), 10<sup>6</sup> and 5×10<sup>5</sup> of 3LL-luc and TSA-luc cells, respectively, were used per injection. For the experiments illustrated on [figure 5 and 6](#), 2×10<sup>7</sup> of A549-luc cells were injected for each implantation. Tumor volume was calculated as described in [I.3. -In vivo tumor treatments.](#)

### siRNA

The siRNAs used in our experiments were purchased from Eurogentec (Belgium) in their annealed form at a concentration of 100 µM. The siRNA targeting luciferase gene (siLuc) and its mis-matched form (siMisM) are designed according to the previous report (Elbashir et al., 2001), siLuc: 5'- CUUACGCUGAGUACUUCGATT -3',

siMisM (with 3 nucleotide mutated): 5'- CUUACGCUCACUACUGCGATT -3'. The siRNA anti-C-Raf (siC-Raf) is identical to that reported in the reference (Cioca et al., 2003): 5'- UAGUUCAGCAGUUUGGCUATT -3'.

For the siRNA used in biodistribution assay, the sense-strand of siLuc labeled with Cyanine-5 (Cy5) on the 5' and its antisense-strand (without label) were bought separately (Eurogentec, Belgium). The siLuc-Cy5 was annealed by mixing equal amount of each strand in nuclease-free water (supported by Eurogentec), heating at 95°C for 2 minutes and then cooling at RT for >20 min. The concentration of mixed RNA was 5-10 µg/µl, in a volume of < 50 µl per tube for annealing. Annealed siLuc-Cy5 was stored at -20°C before use.

### **Transfection in vitro**

For *in vitro* siRNA transfection, cells were seeded in 24-well plate by  $5 \times 10^4$  cells/well the day before transfection. For the transfection in each well, 5 µl of 20 µM siRNA was diluted in 87.5 µl medium without serum and mixed with 7.5 µl medium containing 1.5 µl of Oligofectamine (Invitrogen, Cergy Pontoise, France) gently, then incubated at RT for 15-20 min. Cells to be transfected were washed once by PBS and incubated in 400 µl of medium without serum. The complex of siRNA and Oligofectamine was gently added to the well, and then the cells were incubated at 37 °C for 4 hr for transfection. At the end of transfection, 250 µl of medium containing 30% of fetal bovine serum was added to the well and the cells were put back to the incubator until the luc measurement.

### **Transfection in vivo**

*In vivo*-JetPEI (Polyplus Transfection, Illkirch, France) was used as *in vivo* transfection reagent, and the Sterilized Water for Injections (Laboratoire Aguetant, Lyon, France) was used for preparing all solutions described below. The final volume of the polyplex solution was always 200 µl (by adding 100 µl of solution containing *In vivo*-JetPEI to 100 µl of solution containing DNA/siRNA) for each injection in all

experiments.

For the co-transfection of DNA/siRNA to lung (Fig. 3a), for each mouse, 14  $\mu\text{l}$  of *In vivo*-JetPEI was diluted in 100  $\mu\text{l}$  (final volume) of 5% glucose solution, which was then added to 100  $\mu\text{l}$  of 5% glucose solution containing 40  $\mu\text{g}$  of pGL3 and 30  $\mu\text{g}$  of siRNA, vortex-mixed and incubated at RT for 15 min before injection. This composition of PEI and DNA/siRNA mixture equals to the N/P ratio (the number of nitrogen residues on PEI versus per phosphate on DNA/siRNA) of 10 (10eq). IV injection was administrated through tail vein, by the 29G U-100 insulin syringes (Becton Dickinson, Pont-de-Claix, France). For the experiment described in figure 3c and d, PEI/siRNA polyplex was complexed in 8eq by 8  $\mu\text{l}$  of *In vivo*-JetPEI and 50  $\mu\text{g}$  of siRNA for each injection, according to the same process as described above.

For the siRNA transfection targeting SC tumors, PEI/DNA/siRNA polyplex for IV injection (Fig. 4) in each mouse was complexed by 16  $\mu\text{l}$  (for 8eq) or 20  $\mu\text{l}$  (for 10eq) of *In vivo*-JetPEI and the DNA/siRNA mixture containing 40  $\mu\text{g}$  of pcDNA3.1 (Invitrogen, Cergy Pontoise, France) and 60  $\mu\text{g}$  of siRNA, according to the same process as described above. For the tumor transfection performed by IP injection (Fig. 5), PEI/siRNA polyplex was complexed by 0.8  $\mu\text{l}$  of *In vivo*-JetPEI and 5  $\mu\text{g}$  of siRNA (8eq) for each mouse, and the injection was performed 2 or 3 times per week. The quantity of siRNA for each injection was increased from 5  $\mu\text{g}$  to 10  $\mu\text{g}$  (without changing N/P ratio) since day 29. The same protocol was used for the experiment illustrated on figure 6, but regularly with 10  $\mu\text{g}$  of siRNA per injection, and 3 times of injection per week.

For the experiment showed on figure 7, 2 formulas for PEI/siRNA polyplex were used: either in 8eq in 5% glucose as described above, or in 20eq in 150 mM NaCl containing 10 mM HEPES pH 7.4 (labeled as siLuc 20eq on the figure). The PEI/siRNA polyplex for each injection was complexed by 2.56  $\mu\text{l}$  (for 8eq) or 6.4  $\mu\text{l}$  (for 20eq) of *In vivo*-JetPEI and 16  $\mu\text{g}$  of siRNA, prepared according to the same process as described above. The solution was administrated twice by IP and twice by SC peritumoral injections (totally 4 injections) per week.

## Luciferase assay in vitro

For the quantification of luciferase expression *in vitro*, transfected cells in 24-well plates were lysed in 1× Passive Lysis Buffer (Promega, Charbonnières, France) by 100 µl/well 4 days after transfection. Luminescent intensity was measured by adding 100 µl of Luciferase Assay Substrate (Promega, Charbonnières, France) to 10 µl of cell lysate, and reading for 10 sec in the luminometer Lumat LB9501 (Berthold, Germany). The obtained value was expressed as Relative Light Units (RLU) and was normalized by the corresponding protein concentration.

For the quantification of luciferase in homogenates of mouse organ or tumor, samples were taken off after sacrifice of the mouse, washed in PBS, chopped finely, and vortex-mixed for 10 sec in proper volume of 1× Passive Lysis Buffer, incubated at RT for 20 min, and then stored at -20 °C. Before measurement, the lysates were unfrozen at RT, vortex-mixed for 2 sec and centrifuged for 5 min at 13,000× g at 4 °C. Ten µl of the supernatant was taken and diluted 100× in PBS, and then 10 µl of the diluted supernatant was used for luminescent measurement and 5 µl for protein concentration determination, as described above.

### **Live imaging and quantification of luciferase expression in vivo**

For analyzing the siRNA biodistribution *in vivo*, the siRNA labeled with Cy5 (see “*siRNA*” above) was used. The PEI/DNA/siRNA polyplexes were prepared by the same process as described (see “*Transfection in vivo*” above). Briefly, 100 µl of 5% glucose containing 0 or 10 µl of *In vivo*-JetPEI was added to 100 µl of 5% glucose containing 30 µg of siLuc-Cy5 and 20 µg of pcDNA3.1 to form the polyplex for IV injection (Fig 2a). On figure 2b, the PEI/DNA/siRNA polyplex was formed by 500 µl of solution (150mM NaCl or 5% glucose, as indicated on the figure) containing 0 or 4 µl of *In vivo*-JetPEI and 500 µl of solution containing 15 µg of siLuc-Cy5 and 10 µg of pcDNA3.1, for IP injection. On figure 2c, the PEI/DNA/siRNA polyplex was formed by 500 µl of 150mM NaCl containing 0, 2, 4, or 10 µl of *In vivo*-JetPEI (corresponding to 0, 4, 8, 20eq, respectively) and 500 µl of 150mM NaCl containing 15 µg of siLuc-Cy5 and 10 µg of pcDNA3.1, for IP injection.

The Fluorescence Reflectance Imaging (FRI) system was used for visualizing the biodistribution of siRNA *in vivo*. In brief, the mouse to be imaged was placed in a

plexiglass chamber supplied with 4% isoflurane (Baxter S.A.S., Maurepas, France) for anesthesia. Imaging was carried out in a dark box, and the anesthetized animal was illuminated with a monochromatic 633 nm light ( $50 \mu\text{W cm}^{-2}$ ). The re-emitted fluorescence was filtered using a colored glass filter RG 665 (optical density  $>5$  at the excitation wavelength 633 nm) and collected with a cooled ( $-70^\circ\text{C}$ ) digital charge-coupled device (CCD) camera (Hamamatsu digital camera C4742-98-26LWGS, Hamamatsu Photonics K.K., Japan). Image acquisition parameters were kept constant throughout the experiment ( $1\times 1$  binning, Medium Gain, and 100 ms of exposure). Images were acquired as 16-bit TIFF files which can provide a dynamic of up to 65535 grey levels. Image processing used in this study such as setting LUT (look-up-table) range was performed using the Wasabi software (Hamamatsu). It is also important to note that all the images in this study are presented without background subtraction.

For *in vivo* imaging of luciferase expression, each mouse was IP injected with 300  $\mu\text{l}$  of PBS containing Beetle Luciferin (Promega, Charbonnières, France) at a concentration of 10 mg/ml (except for mice with A549-luc tumors, see below) before anesthesia. The images were taken under the same CCD camera (but  $-80^\circ\text{C}$  cooled) as described above. For mice stably expressing luciferase in lung (Fig. 3c and d), images were taken 5 min after luciferin injection by the condition of  $8\times 8$  Binning, High Gain, and 5 min of exposure. For mice with 3LL-luc and TSA-luc SC tumors (Fig. 4 and 7), images were taken between 15-20 minutes after luciferin injection, with a  $4\times 4$  Binning, High Gain, and 20 sec of exposure. For mice carrying A549-luc tumors (Fig. 5), Beetle Luciferin was used in the concentration of 5 mg/ml, and was SC injected to mice beside the tumor. Images were taken between 15-20 min after luciferin injection, with a  $4\times 4$  Binning, High Gain, and 20 sec of exposure.

Images were processed using the Wasabi software. For quantification, the luciferase intensity (indicated as relative light unit (RLU) in the figures) was determined by the value of total photon number obtained in a region of interest (ROI) enclosing the lungs or tumor, subtracting that obtained in an equal ROI enclosing the background.

### **III. Stable in Vivo Transfection Using the Sleeping-Beauty Transposon**

PEI mediated gene therapy after an IV injection of the polyplexes is not translated in clinical trials for several reasons. One of them is the short duration of the transient expression. This blocks the possibility to use this approach for the correction on inherited diseases such as cystic fibrosis. Another one comes from the fact that only normal cells but not tumor cells are transfected as delivered systemically (even if the tumors cells are located in the lung alveoli). It has thus limited use for cancer therapy either.

The aim of this part of my work was to investigate the interest of using a transposon that stably integrates into the chromosome of the transfected cells after the delivery to mouse lung by PEI. Again, our strategy was to establish a protocol using the luciferase reporter gene, and then to demonstrate that we could use this system to deliver an active gene like the *Ras* protooncogene for the induction of tumor.

### III.1. Introduction

#### Sleeping Beauty

*Sleeping-Beauty* (SB) transposon is a Tc1/mariner-like transposon system, artificially reconstructed by site-directed mutagenesis from an inactive salmonid transposable element (Ivics et al., 1997). The SB system is composed of 2 parts: the transgene expression cassette flanked by SB inverted/directed repeats (IR/DR) (the “transposon”) and the SB transposase expression source (Fig. 1a), which can be maintained either on 2 separate vectors or one single cis-vector. The SB transposases bind to SB-IRs in a substrate-specific manner, excise the transposon and insert it into a new DNA location within TA dinucleotides (Ivics et al., 1997; Izsvak and Ivics, 2004) (see also the Thesis INTRODUCTION III.3a. *-Non-specific integration*). SB transposition can function in a wide range of vertebrate cell lines *in vitro* (Izsvak et al., 2000), and its genomic integration activity can provide the basis for long-term (or possibly permanent) transgene expression for therapeutic purposes. The SB system has been successfully used as a non-viral means of transgene integration for treatments of inherited or acquired diseases including haemophilia A and B (Yant et al., 2000; Liu et al., 2006), type I diabetes (He et al., 2004), tyrosinemia I (Montini et al., 2002), glioblastoma (Ohlfest et al., 2005), and Huntington disease (Chen et al., 2005) in animal models, as well as the blistering skin disease junctional epidermolysis bullosa (JEB) *ex vivo* in human patients (Ortiz-Urda et al., 2003). SB transposon was also used as a cancer gene discovery tool. For this aim, transgenic strains of mice were generated in which the transposon insertion induced loss- and gain-of-function mutations (Collier et al., 2005; Dupuy et al., 2005). Induction of liver tumors in adult mice was also reported after a somatic integration of an oncogenic Ras-harboring SB transposon (Carlson et al., 2005) (see also below).

#### PEI-mediated gene delivery and lung targeting *in vivo*

As described earlier, PEI is a good non-viral vector for delivery of a gene preferentially to lung cells including squamous and alveolar cells (type I and II



pneumocytes) (Goula et al., 1998). Aerosol delivery of PEI/DNA polyplexes also permitted DNA transfection in epithelial cells in conducting and peripheral airways (Gautam et al., 2001). In this study, we used the linear PEI for *in vivo* delivery of plasmids harboring SB transposon system to mouse lung.

(for more introduction about PEI, see also the Thesis INTRODUCTION III.2c. - *Cationic polymers*, and the Thesis RESULT and DISCUSSION II.1.)

### **Ras and oncogenesis**

Ras plays a critical role in the signal transduction between a tyrosine kinase receptor (RTK) and downstream partners such as the mitogen-activated protein kinases (MAPK) (see also the Thesis INTRODUCTION II.1b. -*Ras-MAPK pathway*). Ras proteins are small guanine triphosphatases (GTPases) located in the inner cell membrane, cycling between guanosine diphosphate (GDP)- and guanosine triphosphate (GTP)- bound forms, which represent inactive and active states, respectively (Kamata and Feramisco, 1984). Upon binding to extracellular ligands (e.g. epidermal growth factor (EGF)), RTKs undergo autophosphorylation and create intracellular docking sites for adaptor proteins (e.g. Grb-2) and guanine nucleotide-exchange factors (e.g. SOS), which induce the GTP-bound state of Ras (Boguski and McCormick, 1993; Buday and Downward, 1993; Kamata and Feramisco, 1984). Activated Ras then initiates several downstream signaling cascades such as MAPK through Raf kinases (Moodie et al., 1993; Vojtek et al., 1993), phosphoinositide 3-kinase (PI3K) (Rodriguez-Viciano et al., 1994), and phospholipase C (PLC) (Kelley et al., 2001) pathways that, depending on the cellular context, result in diverse biological responses including cell-cycle progression, cell proliferation, differentiation, survival, or cytoskeletal rearrangements. Normally, the Ras signaling is transient because the wild-type Ras has a mild intrinsic GTPase activity, and the existence of cytoplasmic GTPase-activating proteins (GAPs), which stimulate the hydrolysis of bound GTP and inactivate Ras. Oncogenic mutations in Ras however, generate GTP-bound form of Ras that is constitutively active in signaling downstream effectors, resulting in possible malignant transforming processes (Trahey and McCormick, 1987; Downward, 2003).

The Ras family includes distinct members of Ras (H-, K-, M-, N-, and R-) and Rap (1- and 2-) that share at least 50% sequence identity. In human, K-, N-, and H-Ras genes are potentially oncogenic and their mutations have been identified in approximately 30% of cancer types, among which K-Ras mutation is the most frequent (Adjei, 2001). In human NSCLC, K-Ras mutations are present in up to 30% adenocarcinomas and related to poor clinical outcome (Mitsudomi et al., 1991; Salgia and Skarin, 1998).

*In vitro* study showed that the expression of oncogenic Ras in primary human or rodent cells resulted in the growth arrest or senescence accompanied by accumulation of p53 and p16; while inactivation of either p53 or p16 led to cell transformation, supporting the role of Ras in multistep tumorigenesis (Serrano et al., 1997). A series of elegant experiments using transgenic mouse model demonstrated the *in vivo* tumorigenic potential of Ras, from the earlier design that relied on the spontaneous recombination for activating the oncogenic K-Ras expression (Johnson et al., 2001), to the later transgenic strains that can conditionally “turn on” the expression at the defined time point and tissue based on the Cre-loxP recombination system (Guerra et al., 2003; Tuveson et al., 2004; Collado et al., 2005). These results confirmed that the expression of oncogenic K-Ras is sufficient to initiate transformations in lung: a small percentage of bronchiolo-alveolar cells underwent malignant transformation leading to formations of both multiple adenomas (pre-malignant tumor) and adenocarcinomas (malignant tumor), among which a substantial number of adenomas cells were restricted in oncogene-induced senescence, presumably by the effect of p16 or p53 (Collado et al., 2005). Similar experiments using tissue-specific Cre mouse strains led to the induction of pancreatic cancer precursor lesions and some of them progressed to invasive carcinomas with prolonged latency (Aguirre et al., 2003; Hingorani et al., 2003).

However, these transgenic mice models are labor intensive and expensive. Recently, a model using an oncogenic N-Ras-harboring SB transposon successfully induced multifocal liver cancer in p19<sup>Arf</sup>-null mice, although similar attempt failed in wild-type mice (Carlson et al., 2005). This method provides a convenient way to obtain spontaneous sporadic tumors by a defined somatic mutation without requiring germline transgenesis or knockout manipulations. In our study, oncogenic H- and K-

Ras-harboring SB transposons were constructed and via PEI-mediated gene delivery, their capacity to induce senescence or lung tumors was evaluated in nude mice.

**Figure 1. Plasmid constructs used in experiments**

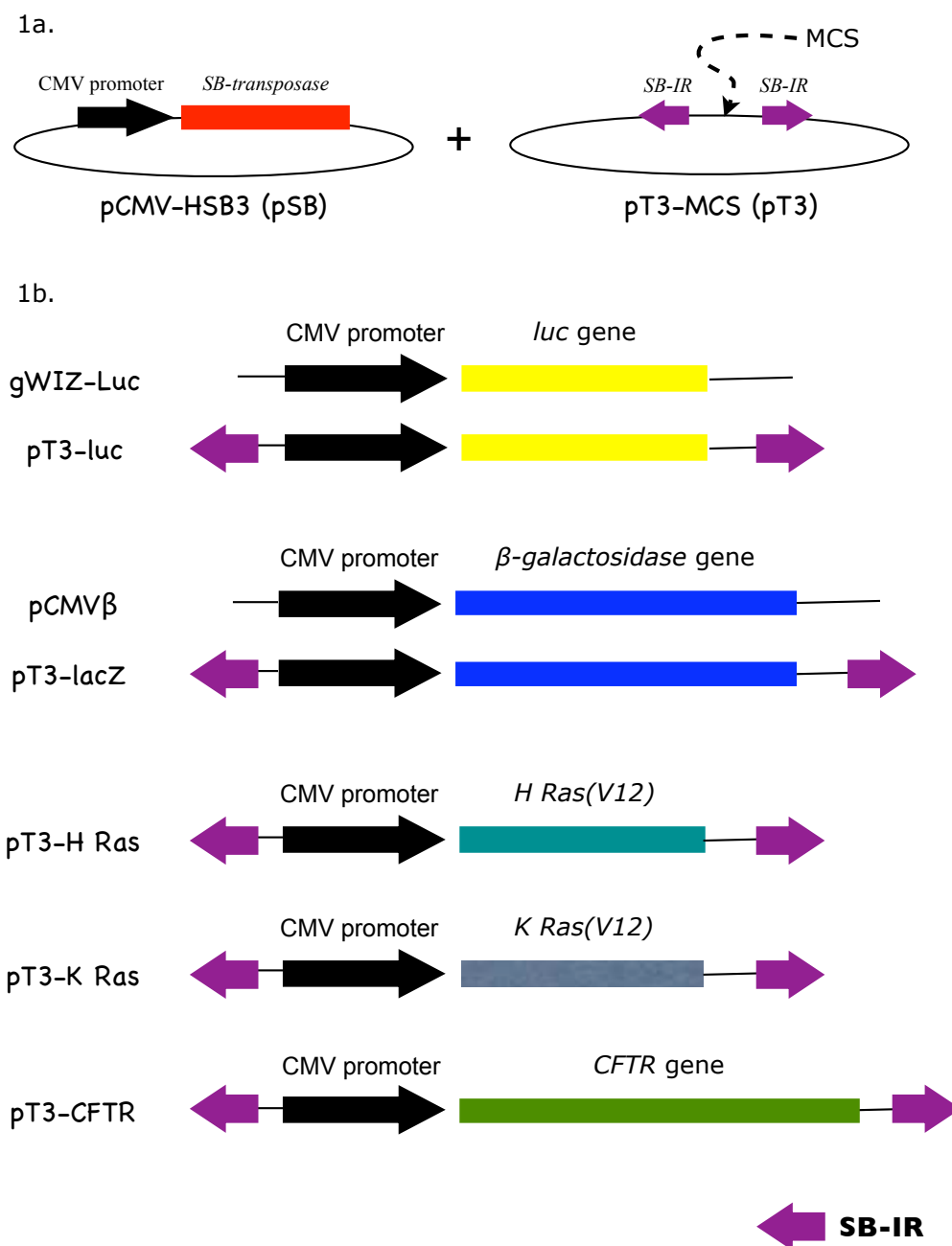


Figure 1

a/ The SB-transposon system contains 2 plasmids : One encodes for the SB-transposase and the other contains the “empty” transposon, in which the transgene expression cassette should be introduced between the 2 SB-IRs via the multiple cloning site (MCS).

b/ Schematic representation of the different expression vectors used in this report with or without SB incorporation.

## III.2. Results

### III.2a. SB transposon mediated Long-term luciferase expression in vitro

In our study, 2 plasmids comprising the SB transposon system were used: the SB transposase expression vector pCMV-HSB3 (pSB), and the transposon vector pT3-MCS (pT3) that contains the 2 SB-IRs flanking the transgene expression cassette (Fig 1). To identify their stable transfection activity *in vitro*, plasmids pSB and pT3-gWiz-Luc (pT3-luc) were co-transfected to B16 cells (mouse melanoma) by PEI in 6-well plate. As a negative control, the gWiz-Luc plasmid without transposon structure was used to replace pT3-luc for co-transfection. No antibiotic selection was applied. When transfected cells reached >90% confluence, cells were trypsinized, counted, measured for the luc activity, and the  $10^5$  of remaining cells were seeded in a fresh culture plate. The same manipulations were repeated during several weeks.

**Figure 2. SB-mediated long-term transgene expression in vitro**

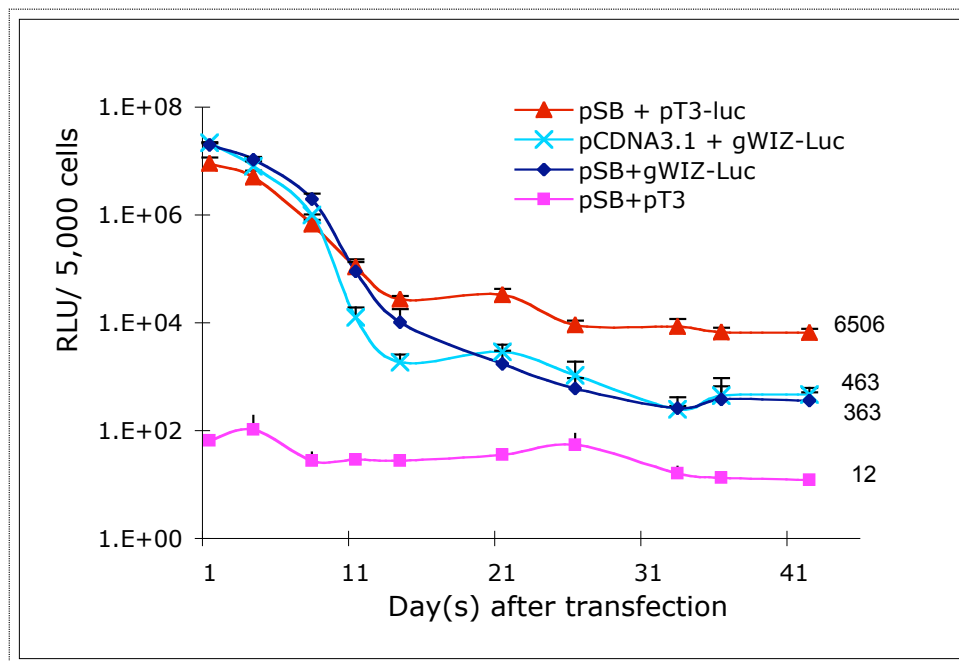


Figure 2

The different combinations of plasmids (1  $\mu$ g + 1  $\mu$ g) indicated in the figure were cotransfected into B16 cells in triplicate, and this experiment was repeated twice independently. The cells were not submitted to a selection of antibiotic, and were harvested after trypsin treatment and reimplanted to a fresh plate 2-3 times a week. The result of the first experiment is presented here and similar results were obtained in the second one. Error bars indicate the Standard Deviation (SD).

The results (Fig. 2) showed that a strong and similar transfection efficiency was obtained at day 1 with the 3 luciferase expression groups (pSB + pT3-luc, pSB + gWIZ-Luc, and pcDNA3.1 + gWIZ-Luc). Luciferase activity then rapidly dropped down to almost baseline negative levels (measured with the pSB+pT3 transfection). In the absence of transposon system (pSB + gWIZ-Luc or pcDNA3.1 + gWIZ-Luc; indicated as “non-SB-luc” transfection in the following text), very low but detectable levels of luciferase ( $\pm 400$  RLU/5000 cells) were conserved in the cell culture till the end of the experiment (day 41). When the SB transposon system was introduced (pSB + pT3-luc; indicated as “SB-luc” transfection), the final stable level of luc expression was >10 times more elevated (>6,000 RLU/5000 cells).

**Figure 3. Efficiency of SB-mediated stable transfection in vitro**

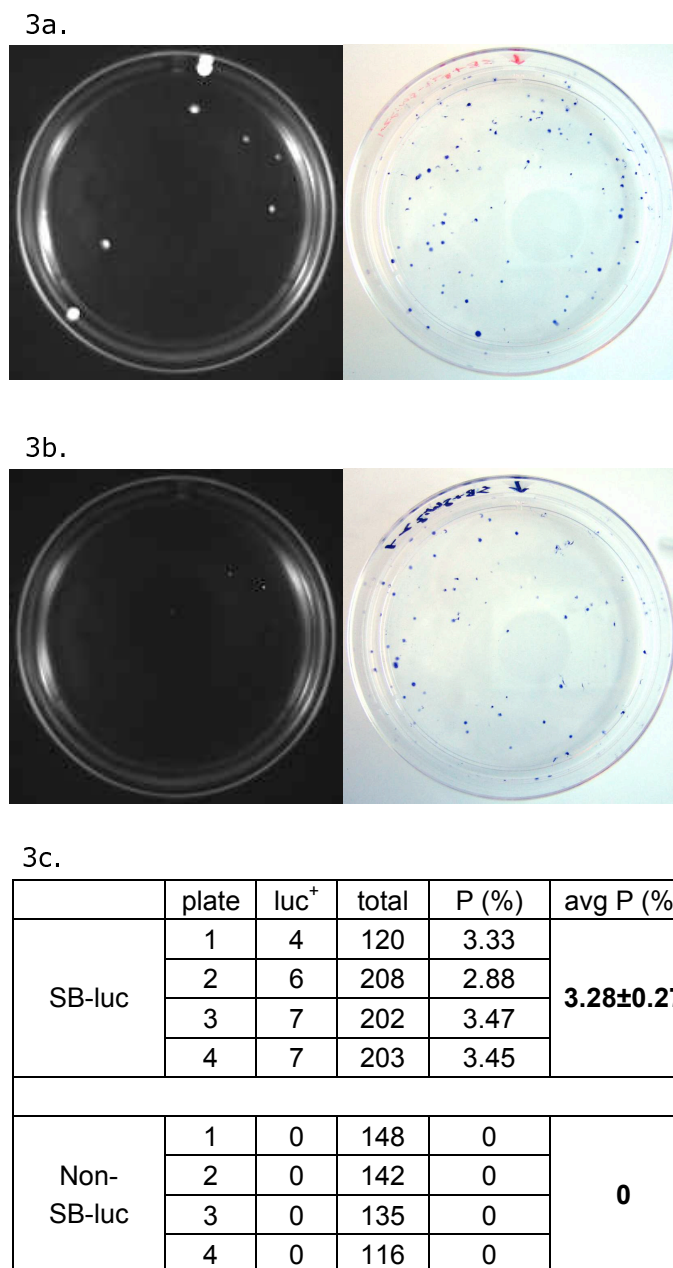


Figure 3

B16 cells co-transfected with pSB and a/ pT3-luc or b/ gWIZ-Luc were maintained for more than 1 month (as described on figure 2), and plated on 10-cm dishes by 500 cells/dish, 4 dish/group. Luciferase expression in the colonies was detected using the BLI system after incubating the cells with Beetle Luciferin (left panel). The cells were then fixed and stained with methylene blue (right panel) for counting the total number of colonies.

c/ Frequency of stable transfection. The number of luciferase positive (luc<sup>+</sup>) versus total colonies were counted in each dish. P (%), percentage of luc<sup>+</sup> colonies. Avg P (%), mean of the P (%) ± SD.



To quantify the frequency of apparition of these stable clones, the same experiment was reproduced but, one month after transfection, 500 cells of each group were diluted in 10 ml medium and plated on 10-cm dishes. One week later when the cell colonies were visible (about 2-3 mm in diameter), luc positive (luc<sup>+</sup>) clones were detected using the BLI system after incubating the cells with PBS containing Beetle Luciferin (Fig. 3a and b, left panel). Cell colonies were then fixed and stained with methylene blue (Fig. 3a and b, right panel) for counting. As shown in figure 3c, the frequency of luc<sup>+</sup> colonies was ~3.28% in the SB-luc group, while no positive clone out of 541 colonies was found in non-SB-luc group (<0.2%). We also verified the initial transfection efficiency by transfecting a  $\beta$ -galactosidase expression vector (pSB + pCMV $\beta$ ) into B16 cells using the identical protocol. After staining the cells with X-Gal 1 day after transfection (data not shown), we determined that the average transfection efficiency was 10.93%. Thus taken together, these results indicated that without any selection, statistically ~30% of the transiently transfected B16 cells stably expressed the SB-delivered transgene.

These results also confirmed that stable integration of a non-integrative plasmid still occurs after transient transfection, although at a very low frequency (less than 0.2%). However, this phenomenon suggests that the risk of random insertional mutation is not negligible even when using non-viral and non-integrative gene delivery systems, since the events can be detected using a sensitive method. This also confirmed that SB augments the chance of integration of a transgene. Without pressure of selection, the level of stable expression is augmented for more than 10 times. X-gal staining indicated a frequency of initial transfection ~11%, while luciferase detection using BLI shows that ~3.3% of the cells are stably transfected after on month. Thus, assuming both methods have the same sensibility, this would suggest that SB-mediated integration occurs in near to 1/3 of the transiently transfected cells. Nevertheless this should be certainly overestimated, because X-Gal is not very sensitive and that the frequency of transient transfection is underestimated using this method.

### Figure 4. SB-mediated long-term luc expression in vivo

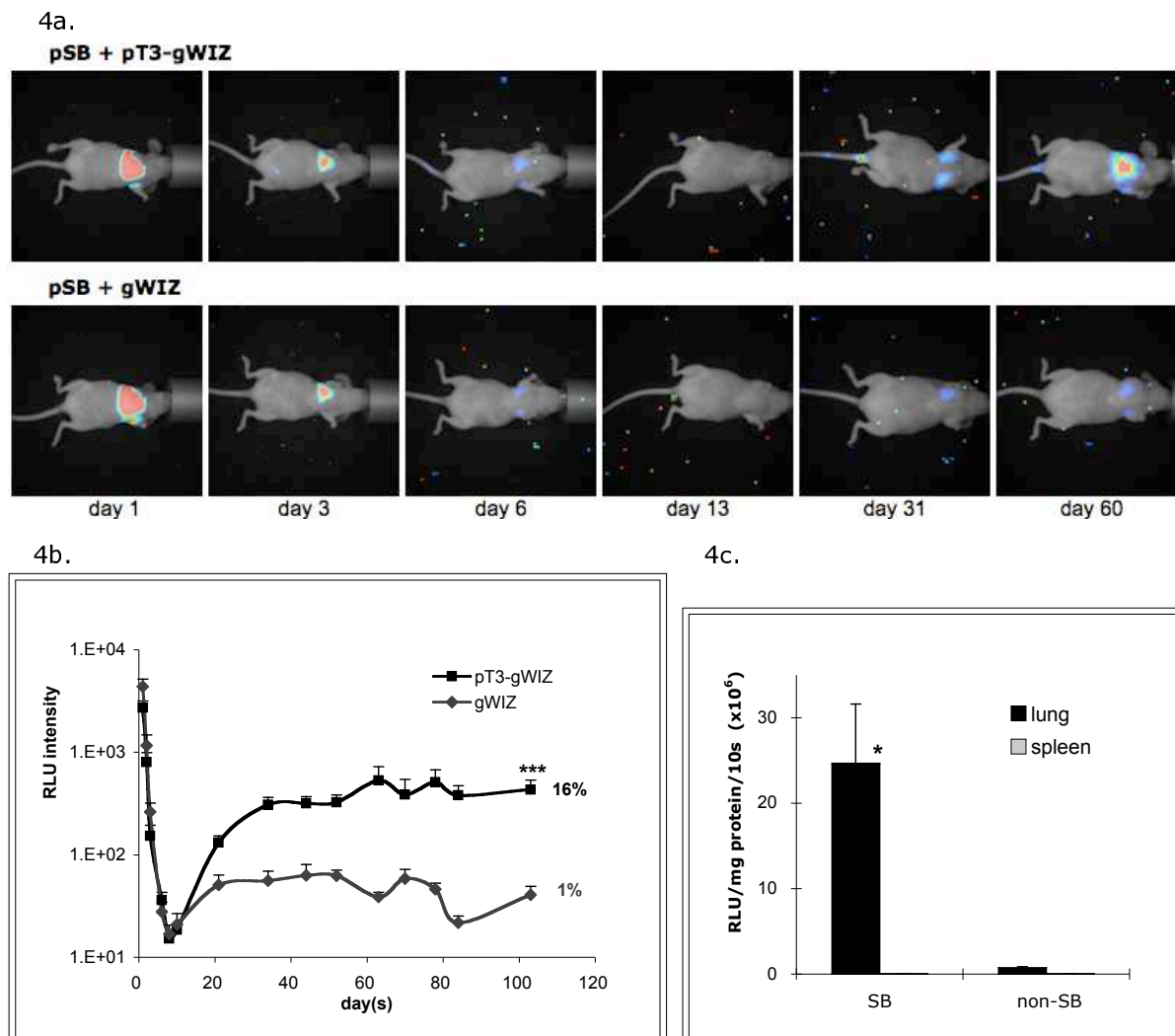


Figure 4

a/ BLI-acquired images of one of the mice transfected by SB-luc or non-SB-luc at different time points. The transient luc expression faded out in 2 weeks in both groups, but luc signal re-appeared 1 month later more importantly in SB-luc mice. However, it's notable that the non-SB-luc transfection also provided detectable long-term expression.

b/ Quantification of luc signals in the lung of these mice. The percentage of final intensities relative to day 1 are indicated on the right of the curves. N=11 for the SB-luc group and n=6 for non-SB-luc.

c/ Luc enzymatic activity in homogenates of lung and spleen of these mice were measured using the luminometer at the end of the experiment. N=7 for SB-luc group and n=6 for non-SB-luc.

Error bars indicate SEM; P value is estimated by the Student's T Test, unpaired and two-tails (\*, P<0.05; \*\*\*, P<0.005).

### **III.2b. SB-efficiency in vivo**

Nude mice were injected IV with a solution of 200  $\mu$ l of 5% glucose containing 60  $\mu$ g of DNA compacted with L-PEI in 8eq. The transfected DNA contained the pSB (30  $\mu$ g) and the pT3-luc or gWIZ-Luc (30  $\mu$ g) plasmids. The luc expression in mouse lung was followed using BLI system (Fig. 4a, b). All transfected mice presented intensive luc signals in lung one day post-transfection. The signal faded out rapidly during the following 2 weeks, and there was almost no detectable signal at day 13 in both groups. One month later however, SB-luc mice showed a significantly elevated luc signal, which increased regularly and reached a plateau 2 months after transfection. At this step, the luciferase signal reached 16% of the strongest transient level initially measured at day 1. Mice transfected with non-SB-luc also presented a detectable signal in lung 1 month later, but the plateau was stabilized at only 1% of the original level. The difference between both groups was statistically significant ( $P < 0.005$ ).

At the end of the experiment, the stable expression of luciferase in lungs was confirmed by directly measuring the lung homogenates using the luminometer after sacrifice of the mice. Homogenates of spleen from the same mice were used as the negative control. The results showed that lung tissues from SB-luc mice contained ~20-fold higher luc activity than those from non-SB-luc mice ( $p < 0.05$ ), while the luc activity was undetectable in spleen of all mice (Fig. 4c).

### Figure 5. SB-mediated stable expression of $\beta$ -gal in lung cells

5a.



5b.

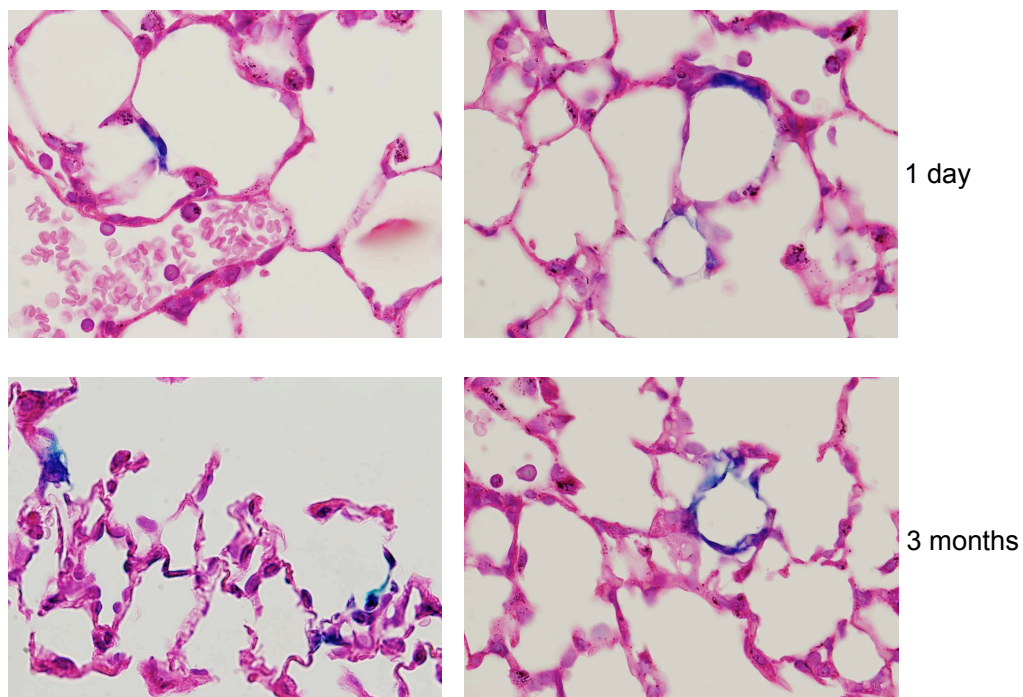


Figure 5.

a/ The lungs of SB-lacZ (left) and non-SB-lacZ (right) transfected mice 3 months post-transfection, fixed and stained *in toto* with X-Gal.

b/ Sections of lungs from the mice transfected by SB-lacZ, 1 day (upper panel) or 3 months (lower panel) post-transfection, stained with X-Gal and HES.

The shape of the curve presented in [figure 4b](#) could suggest that 2 weeks after transfection, the level of luc was below the range of sensitivity of BLI system. But this level increased slowly between day 13 and day 30, suggesting that the stably transfected cells were multiplying. In order to visualize this phenomenon, we constructed the vector pT3-lacZ, which contains a  $\beta$ -galactosidase expression cassette flanked by the SB-IRs, and co-transfected it with pSB (SB-lacZ) as previously. One day or 3 months later, the lungs were stained with X-gal and removed after sacrifice of the mice. The level of X-gal staining was much more elevated in the SB-lacZ transfected one at 3 months ([Fig 5a](#)). The lungs were also embedded in paraffin, sliced and counterstained with HES ([Fig 5b](#)). We can observe that only single alveolar cells were positively stained at day 1, while the “colonies” of blue cells were frequently present at 3 months ([Fig 5b](#)). These results suggested that one original cell stably transfected by  $\beta$ -galactosidase gave birth to its progeny. This phenomenon was observed only in SB-lacZ transfected mice. In contrast, no blue cells were found in a certain number of lung sections of non-SB-lacZ transfected mice at 3 months (data not shown). Note that even with the help of expert anatomopathologists, we were not able to discriminate with certitude the percentage of transfected endothelial cells among a vast majority of pneumocytes.

These combined results of *in vivo* transfection confirmed those obtained *in vitro*, which demonstrate that the SB transposon system augments the frequency of stable integration of a transgene expression cassette. Once more, this also draws attention on the risk of random integration of a transfected plasmid, even without SB.

The fact that luc signal decreases and then reappears suggests that a small population of progenitor cells is stably transfected. Among the transiently transfected cells, some of them are stably integrated by the transposon. If all these cells were terminally differentiated, the signal would have been expected to fade out gradually and disappear eventually. This is not what we observe.

Actually, the shape of the curve mainly suggests that the percentage of stably transfected cells is very low and below the level of detection during 1-2 week post-transfection. In the following month, some of the luc<sup>+</sup> clones may multiply, associated with the process of nature turnover of terminally differentiated cells (e.g. P-I pneumocytes) and the simultaneous compensation by a regular multiplication of the “reservoir cells”. It is thus tempting to propose that the luc<sup>+</sup> stable clones, which gave rise to the augmented luc signal after 2 weeks, are progenitor cells.

However, since these cells are very rare, it is quite difficult to identify them. Indeed, in the experiment of SB-lacZ transfection described in [figure 5](#), we were not able find any X-Gal stained cells in lung sections from the mice sacrificed 1 week post-transfection (data not shown).

**Figure 6. Repeated PEI/DNA transfection of mice lungs**

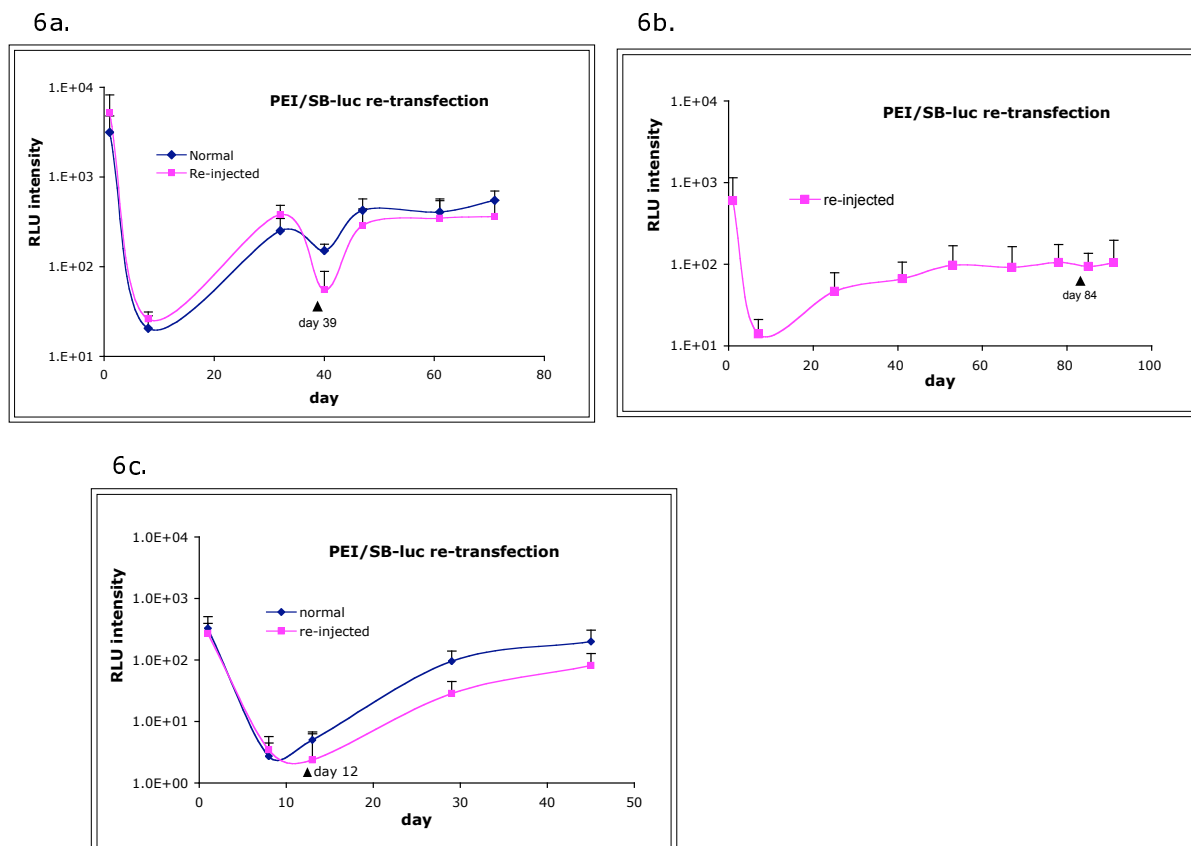


Figure 6

Mice were transfected by an IV injection of PEI/SB-luc on day 1 and followed using BLI for luc expression. In 3 independent experiments, a second injection was performed at day 39, 84 or 12 (indicated with ▲ on the figure), respectively.

a/ n=4 and 3 for normal and re-injected groups, respectively.

b/ n=8.

c/ n=6 for both groups.

Normal group : only injected at day 1, without second transfection. Error bars indicate SEM.

### **III.2c. Can we transfect a SB-plasmid repeatedly using PEI?**

We have estimated that the long-term expression level of luc mediated by PEI/SB transfection is ~16% of that obtained at day 1 (Fig. 4b) in mouse lung. The PEI/DNA mediated transient transfection is regularly strongest at day 1, and usually concerns a level of 1-5% of epithelial alveolar cells transfected (Goula et al., 1998). This would suggest that only less than 1% of lung cells could stably express the PEI/SB-delivered therapeutic gene. To enhance this low percentage, we thus tried to repeat the transfection.

Three independent experiments were performed to evaluate the effect of a second transfection. Mice were transfected by PEI/SB-luc at day 1 as described before. A second transfection was then performed 12, 39 or 84 days later using the same protocol. These time points are chosen because they are associated with different stages (Fig. 4):

1/ day 12 : the transient luc signal disappears (no luc detectable), and a later luc signal is going to appear and increase.

2/ day 39 : the level of reappeared luc signal reaches the plateau.

3/ day 84 : the plateau has been reached and it has left enough time for the lung reconstruction after the first injection.

Nonetheless, results of the 3 experiments indicated that the second transfection did not improve luciferase expression in the lung (or elsewhere) (Fig. 6). Thus it appeared that the lung is resistant to a second transfection for a still unknown reason.

In addition, when the re-injection was performed at day 39, a transient decreased of luc signal was noticed (Fig. 6a). But the luc level rapidly returned to its original values without the enhancing effect that we expected for the second injection. Since this phenomenon was also observed in the control group (without second transfection), it may be an artifact or error in manipulations of BLI at day 40. In addition, this problem did not occur in the 2 other experiments, which confirmed that it should be an error.



### **III.2d. H-Ras, K-Ras and CFTR gene transfer**

In order to demonstrate a biological interest of SB-mediated stable gene delivery, we constructed 3 transposon plasmids encoding for oncogenic H- and K-Ras, and the CFTR protein (Fig. 1b).

Long-term expression of H-Ras mutant was expected to induce senescence in the transfected cells. The K-Ras mutant was expected to induce a hyper-proliferation of the stably transfected cells and eventually lung tumors. The SB-CFTR was designed to correct cystic fibrosis in a mouse model. All 3 constructs have been generated successfully.

A first transfection of PEI/SB-H-Ras in nude mice has been performed and there was no sign of toxicity or lung pathology in these mice for 2 months (n=4) after DNA injection. After sacrifice, the lung of these animals were extracted, sliced and observed histologically. No sign of particular defects were observed and the samples are currently under analysis to demonstrate the presence of senescent cells using a modified X-gal staining method.

Transfection of PEI/SB-K-Ras were also performed. The presence of possible lung tumors was addressed regularly using an IV injection of RAFT-(cRGD)4-Alexa700 to mice (Koenig et al., 2008), which were then followed using our non-invasive 3D imaging technology, based on fluorescence diffuse optical tomography (fDOT). So far the 2 groups of mice (n=5 and 4, respectively) transfected with SB-K-Ras (in Mars and May 2008) did not show any sign of lung tumors for more than 4 months, but this experiment is still ongoing.

Cystic fibrosis (CF) is an inherited disease resulting from the presence of defective Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, which encodes a chloride channel protein (Rich et al., 1990). Restoring wt-CFTR expression in a small amount of cells should improve the deadly outcome of this disease in a rodent model of CFTR<sup>-/-</sup> mice. In collaboration with Dr. B Pitard (INSERM U533, Nantes, France), we plan to study the survival of these mutant mice by IV injection of PEI/SB-CFTR. However, since PEI-mediated transfection does not

touch epithelial cells of the bronchi, our therapy may not be very effective. But Dr Pitard developed his own transfection reagent that is known to deliver the DNA to these cells (Desigaux et al., 2005). We will compare both transfection methods.

### III.3. Discussion

In this project, the SB-transposon mediated stable transfection was evaluated *in vitro* and *in vivo* via a reporter gene assay. Unsurprisingly, the initial *in vitro* test showed the positive stable transfection activity in B16 cells. In repeated passages without any selection for >1 month, the SB-luc transfection group showed a 10-fold more elevated RLU level than that of the non-SB-luc group (Fig. 2). This result should reflect the difference in probabilities between random integration and SB-mediated transposition inside the living cell since no selection was applied. The BLI system allowed us to define that ~3% cells were stably expressing luc. Regarding the initial transient transfection efficiency in this cell line (~11%), this result suggests that ~30% cells receiving SB-transposon plasmids could be stably transfected. This number can provide us a basic estimation, although we believe it is overestimated because of the different sensibilities between X-Gal and luc assay (luc > X-Gal).

Interestingly, when applied *in vivo* by IV injection with SB-luc into nude mice, the curve of luc signal showed a different pattern from *in vitro*. Indeed, the expression of transgene simply diminished over time *in vitro* and finally stabilized at a level reflecting the probability of SB-transposition occurrence. *In vivo* luc signal however, rapidly faded out in 2 weeks, showing an usual transient property of PEI/DNA-mediated transfection, but then re-augmented regularly until reaching a plateau 2 months later (Fig. 4b).

We do not have a definite explanation for this *in vivo* curve. Our hypothesis is that the stable signal comes from the multiplication of a small population of stably transfected cells, which could be pneumocyte type II (P-II) cells, functioning like progenitor cells, because:

- 1/ Earlier reports have demonstrated that PEI-mediated gene delivery preferentially targets lung squamous and alveolar cells including P-I and P-II (Goula et al., 1998).
- 2/ P-II pneumocytes can replicate in the alveoli and are responsible for the replacement of damaged P-I cells. This natural physiological process has a kinetic, which would fit with our data.

In addition, our results obtained in the lung of SB-lacZ transfected mice also support this hypothesis. Samples obtained one day after transfection showed randomly dispersed single alveolar cells positively stained by X-Gal. This typical transient transfection pattern was observed either in the presence or absence of SB system. In the samples obtained 3 months later however, “colonies” of positive cells were observed (Fig. 5b). This suggested that the multiplication of one original cell gave birth to its progeny. Unfortunately, we can never found positively stained cells in the lung samples 1 week post-transfection, presumably because of the very low number of positive cells. We are thus unable to obtain the information about the morphology of stably transfected cells at this stage, for confirming our hypothesis. Thus more direct evidences are still needed to clarify this point.

L-PEI mediated transient transfection in lung touches usually 1-5% of alveolar cells (Goula et al., 1998). According to our estimation of SB-mediated integration frequency, stably transfected cells should represent only less than 1% of lung alveolar cells. Re-transfecting these mice with the same PEI/SB-reporter gene was not efficient and thus no additive effect could be obtained. This is very surprising since we carried out the re-injection 2, 5 or 12 weeks after the original transfection in 3 independent experiments. The mice are thus resistant to the second PEI/DNA injection for a still unknown reason.

### III.4. Material and method

#### Plasmid construction

The plasmid gWIZ-Luc (Genlantis Inc., San Diego, USA) is an expression vector of luciferase driven by a Human Cytomegalovirus (CMV) immediate early gene (IE) promoter/enhancer. pCMV $\beta$  is the expression vector of  $\beta$ -galactosidase (see above I. 3. -Plasmid and construction). Plasmids pCMV-HSB3 (pSB) and pT3-MCS (pT3) are generous gifts of Dr. Mark Kay (Stanford University School of Medicine, Stanford, CA, USA). pCMV-HSB3 contains a hyperactive SB transposase mutant under the transcriptional control of a CMV promoter. pT3 is the transposon vector that contains the 2 SB specific inverted repeats (IRs) flanking a multiple cloning site.

(All enzymes used for cloning the plasmids as described below were bought from New England BioLabs (Ozyme, St. Quentin Yvelines, France))

To generate the plasmid pT3-gWIZ-Luc (pT3-luc), the luciferase expression cassette (containing the CMV promoter, luciferase gene, and a downstream poly-A signal) on gWIZ-Luc was amplified by PCR. An *SpeI* site and an *XhoI* site were generated on the 5' and 3' primers by point mutations, respectively. The amplified fragment was then digested and cloned into pT3 between the 2 SB-IRs via *SpeI* and *XhoI* sites.

To generate pT3-lacZ, pCMV $\beta$  was digested by *NarI* and *Sall*, and the released lacZ expression cassette was cloned into pT3 between the 2 SB-IRs through *Clal* and *XhoI* sites.

Ras protein expression vectors pBabe(hyg)-K-Ras(V12) and pBck-H-Ras(V12) are generous gifts of Dr. R Pedoux (INSERM U823, Grenoble, France). These vectors encode the oncogenic form of K- or H-Ras, which contains a point mutation at the position 12 of the amino-acid sequence (where the Glycine is substituted by a Valine), resulting in a constitutively active form of Ras protein. The 2 vectors both express Ras proteins under the transcription control of a retroviral LTR. To match our purpose that these oncogenes should be expressed strongly and constitutively in

mammalian cells, we firstly cloned the Ras genes to a expression vector driven by a CMV promoter, and then cloned these Ras expression cassettes to the pT3 vector. These processes are described as below:

For H-Ras, the pBck-H-Ras(V12) was digested by *BamHI*, treated by the DNA Polymerase I Large Fragment (or named “*Klenow*” which results in a blunt end at the DNA digested site), and then digested by *EcoRI*. These processes released the H-Ras gene fragment containing a blunt end and a *EcoRI* digested end. The commercial VP22 expression vector pVP22/MycHis1 (Invitrogen, Cergy Pontoise, France) was digested by *HindIII*, treated by *Klenow*, and then digested by *EcoRI*. These processes released the VP22 gene, which was then removed. The linearized “empty” vector was then ligated with the H-Ras fragment described above. The H-Ras expression cassette on this new vector was then released by digesting with *DraIII*, treating with *Klenow*, and digesting by *BglII*. The pT3 vector was digested by *NotI*, treated by *Klenow*, digested by *BglII*, and then ligated with the H-Ras expression cassette described above, giving rise to the plasmid pT3-H-Ras.

For K-Ras, the gene encoding for oncogenic K-Ras(V12) on the plasmid pBabe (hyg)-K-Ras(V12) was amplified by PCR. An *XbaI* site was generated by point mutations on the 3' primer. The amplified K-Ras gene fragment was then digested by *BamHI* and *XbaI*. The commercial vector pVP22/MycHis2 (Invitrogen) was digested by *BamHI* and *XbaI*, which resulted in the release of VP22 gene that was then removed. The linearized “empty” vector was then ligated with the K-Ras fragment described above. The K-Ras expression cassette on this new vector was then released by digesting with *DraIII*, treating with *Klenow*, and digesting by *BglII*. The pT3 vector was digested by *NotI*, treated by *Klenow*, digested by *BglII*, and then ligated with the K-Ras expression cassette described above, giving rise to the plasmid pT3-K-Ras.

For CFTR, the DNA fragment containing a eGFP-CFTR fusion gene was amplified from the plasmid pTRE-eGFP-CFTR (a generous gift of Dr. B Pitard) by PCR. An *EagI* site was generated on both primers by point mutations. The amplified fragment was then digested by *EagI*. The plasmid pT3-lacZ (see above) was digested by *EagI*,

and the released  $\beta$ -galactosidase gene was removed. The linearized “empty” vector was then ligated with the eGFP-CFTR fragment described above, giving rise to the plasmid pT3-G-CFTR. The pT3-CFTR was then generated by digesting pT3-G-CFTR with *Xho*I, removing the released eGFP gene fragment, and the self-ligation of the remaining vector.

## **Cell culture and transient transfection**

B16 mouse melanoma cells (ATCC, CRL-6475) were cultured in D-MEM medium supplied with 10% (v/v) fetal bovine serum and 0.5% (v/v) PEN-STREP. Cells are cultured at 37°C in a humidified CO<sub>2</sub>-controlled (5%) incubator.

For the transient transfection, B16 cells were seeded in 6-well plate by  $3 \times 10^5$  cells/well the day before transfection. To each well 1  $\mu$ g of pCMV-HSB3 plus 1  $\mu$ g of pT3, gWIZ-Luc, pT3-luc or pCMV $\beta$ , or 1  $\mu$ g of pcDNA3.1 plus 1  $\mu$ g of gWIZ-Luc, were cotransfected to cells by jetPEI according to the manufacturer’s recommendation.

## **Reporter gene assays and transfection efficiency estimation**

For the luciferase activity assay *in vitro*,  $10^5$  cells trypsinized from each well were collected, washed, and lysed in 100  $\mu$ l of 1 $\times$  Passive Lysis Buffer, and 5  $\mu$ l of the lysate was taken for the RLU measurement by luminometer as described (see above **II.4. -Luciferase assay in vitro**).

RLU of homogenates of mouse lung and spleen was measured as described (see above **II.4. -Luciferase assay in vitro**). The values expressed on [figure 4c](#) were normalized by the corresponding protein concentration.

For estimating the transfection efficiency, 1 day after transfection, B16 cells transfected by pSB + pCMV $\beta$  were fixed, stained and counted as described (see above **I.3. -Cell fixation and staining**).

## **In vivo transfection and optical imaging**

For the *in vivo* transfection of a mouse, 30 µg of pSB plus 30 µg of pT3-luc or gWIZ-Luc were compacted by *in vivo*-jetPEI in 8eq and injected in the tail vein as described (see above **II.4. -Transfection *in vivo***).

For the non-invasive optical imaging, each mouse was injected IP with 300 µl of PBS containing 10 mg/ml Beetle Luciferin, anesthetized, imaged, and quantified for RLU as described (see above **II.4. -Live imaging and quantification of luciferase expression *in vivo***). Because of the great difference in RLU intensity in mouse lung at different time points (**Fig. 4b**), the imaging condition was High Gain, 5 min of exposure, but with different Binning ranges (from 1×1 to 8×8) depending on the RLU intensity at the moment of measurement. For expressing on **figure 4b**, all RLU values were normalized by the formula :  $(L-B)/(\text{pixel units} \times \text{Binning value})$ , among which L and B indicate the total photons received in an equal ROI enclosing the lungs or background, respectively.

For imaging the luciferase positive (luc<sup>+</sup>) B16 colonies on 10-cm dishes, cells were washed once by PBS and incubated in 7 ml PBS containing 150 µg/ml of Beetle Luciferin for 1 min, and then imaged under the cooled CCD camera by the condition of High Gain, 2 min exposure, and the Binning 2×2. Images were processed with the Wasabi software.

### **Histochemistry staining of lung**

Following the lethal and irreversible anesthesia by an IP injection of Ketamine/Valium, the mice were perfused with 30 ml of PBS. The lungs were then inflated, fixed and stained by slowly infusing the following solutions from trachea: 1/ PBS containing 0.5% glutaraldehyde and 1% formaldehyde for 5 min, 2/ 5 ml PBS for rinsing, 3/ X-Gal solution (composition as described above “Reporter gene assays and transfection efficiency estimation”) for 5-10 min. The lungs were then taken off, washed in PBS, fixed again by incubating in PBS containing 0.5% glutaraldehyde and 1% formaldehyde for 5 min, washed with PBS, and stained again by incubating in X-Gal solution at 37 °C for 5 hr. At the end of staining, lungs were washed with PBS and incubated in 4% paraformaldehyde (PFA) at 4 °C for 4 hr, and then embedded in paraffin. Ten µm-thick sections were stained with Haematoxylin-eosin



(HE) for histochemical examination.

## CONCLUSION and PERSPECTIVE

The aim of my doctoral study was to develop new delivery systems for the transfer of large molecules including siRNA and DNA for the treatment of lung pathologies through non-viral vectorization. I focused my studies more specially on the methods allowing effective delivery of siRNA, and on the delivery of the genes for antitumor activity or long-term expression.

In the siRNA delivery, I analyzed the potential of PEI for siRNA vectorization *in vivo*, since PEI is so far the best synthetic molecule described for the systemic delivery of plasmid DNA.

In the gene delivery, I studied the antitumor potential of the viral protein FMG, particularly its capacity to induce an important bystander effect. On the other hand, the SB-transposon mediated long-term expression of the transfected gene in lung tissue is another interest of my study.

In the following chapters I will summarize the results that we obtained and some possible perspectives of these work.

# I. The FMG-mediated Cancer Therapy in Vitro and in Vivo

## I.1. Conclusion

FMG-based cancer therapy was attractive because of the expected bystander effect associated with the formation and death of syncytia and of the possible induction of an antitumor immune response.

The FMG-mediated induction of the bystander effect was very strong. Basically, one cell transfected by FMG can fuse >50 non-transfected surrounding cells and commit them to death. In terms of toxicity, ~1% of transient transfection in cultured cells can lead to up to 80% of cell-death. *In vivo*, FMG encoding plasmids directly injected into human NSCLC SC tumors also showed an impressive outcome. Some 60-70% reductions of final tumor weights were noticed despite the very poor transfection efficiency. These *in vivo* results are particularly encouraging, especially because it is well known that most current cancer gene therapies in clinic encounter the problem of poor transfection efficiency. These FMGs proved to be active even at very low transfection efficiency and in immuno-deficient animals.

The production of immunogenic microvesicles (exosomes/syncytiosomes) should augment its already strong bystander effect. The presence of syncytiosomes was confirmed in our system. Microvesicle production was more pronounced after the transfection of FMG to cultured tumor cells. This was associated with the augmented presence of some secreted proteins in the culture medium, including heat-shock, ESCRT, or Raft-associated proteins, previously described to be incorporated in exosomes. However, these results were largely variable in independent experiments. This may probably be due to the low transfection efficiency.

## **I.2. Perspectives concerning the use of FMG in human NSCLC treatment**

Our perspective concerning this project are :

1. To verify the mechanism associated with the syncytia death in our model.
2. Repeat the *in vivo* tumor treatment by using a murine syngeneic tumor model in which we will be able also to measure the importance of the immune response.

As mentioned before, the death of the syncytia is known to involve predominantly a necrotic pathway since nuclear fusion, mitochondrial failure and ATP depletion were observed. In addition, other cellular events like procaspase-3 activation and PARP cleavage were not strongly associated with FMG-mediated cytotoxicity, and caspase inhibitor Z-VAD-fmk did not prevent the death of the syncytia (Higuchi et al., 2000; Bateman et al., 2002). However, conflictive result was also reported. It has also been described that syncytia death can show the hallmark of apoptosis in cultured glioma cell lines, suggesting that the underlying mechanism of toxicity could be cell-line dependent (Galanis et al., 2001).

Future experiments will have to address this issue in our systems. We will try to detect the apoptosis signals such as (pro-)caspase-3 activation during syncytia death (2-5 days after transfection) by western blot. In the meantime, comparing the syncytia death in the absence/presence of caspase inhibitors can provide a direct evidence that apoptosis is involved.

Besides apoptosis and necrosis, we are also interested in knowing if senescence can be observed in late syncytia. Senescence is a signal transduction program leading to irreversible cell-cycle arrest. It can be triggered by alterations of telomeres or different forms of stress. Although the oncogenic process allows cancer cells to bypass the senescence program, they retain the capacity to undergo senescence. Overexpression of tumor-suppressor genes or inhibition of oncogenes is able to induce rapid senescence in tumor cells. Many conventional anticancer therapies have also been confirmed to induce not only apoptosis in some cells, but also senescence in many others. Thus treatment-induced senescence is now one of the

key determinants of tumor response to therapy (for review, see Roninson et al., 2003; Shay et al., 2004).

As the exact mechanism of syncytia death remains to be clarified, we are interested to know if senescence is one of the participating mechanism. Especially, although non-dividing, senescent cells remain metabolically active and secret several factors that can affect the growth of their neighboring cells as well as tissue organization. Senescence could thus be involved in the death of syncytia, since they do not divide anymore but contain an organized cytoskeleton structure and remain metabolically active like senescent cells. It is thus important to measure the possible induction of senescence after FMG expression. This can be addressed by measuring the presence of senescence-associated (SA)- $\beta$ -galactosidase expression using X-gal staining at pH 6.0 (Dimri et al., 1995).

Finally, necrosis can be also involved. This will be evaluated by different assays, which are focused on the detection of mitochondrial dysfunction, ATP depletion, proteolysis by calpains and cathepsins, and early plasma membrane rupture.

**Figure 1. The proposed protocol to evaluate FMG-based immunotherapy in immuno-competent mice**

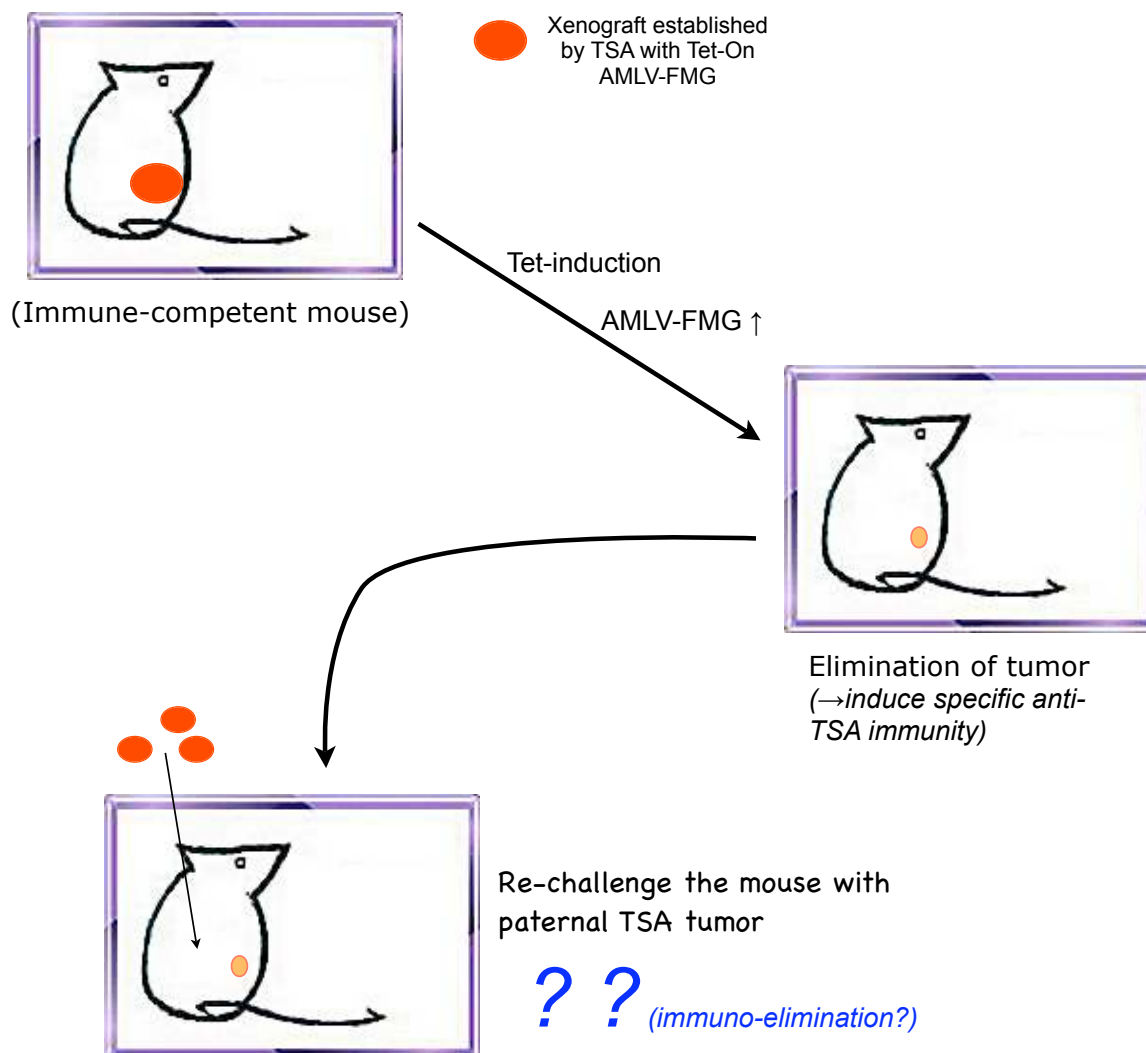


Figure 1

Our proposed model to evaluate the FMG-based immunogenic potential in immune-competent mice. A TSA stable clone with tetracyclin-inducible (Tet-On) AMLV-FMG was expected to be generated. The mouse xenograft established by this clone (red ellipse) would be eliminated following the Tet induction, during which the specific anti-TSA immunity would be expected to occur. This immune response can be evaluated by a second challenge with the same tumor cell line.

The second axes concerns the evaluation of antitumor immune response generated after syncytiosome production. Our first idea to address this issue was to study the antitumor activity of AMLV-FMG in TSA tumors in immuno-competent BalbC mice. However for an unknown reason, the transfection efficiency obtained after naked DNA injection is at least 1000-fold less efficient in TSA tumor as compared to H322 human NSCLC tumor, which is already very weakly transfected (JLC, personal communication). A direct intratumoral injection of AMLV-FMG is thus not possible in the TSA model.

We thus tried to establish a TSA stable clone with a tetracycline-inducible AMLV-FMG (Tet-On). This stable clone would allow us to establish syngeneic graft in BalbC immuno-competent mice, and to measure the antitumor activity of AMLV-FMG after its induction *in vivo* by adding Doxycycline in the drinking water. The possible vaccinal effect induced by AMLV-FMG could then be analyzed by performing the challenge with a second SC injection of the same tumor cell line (Fig. 1). Unfortunately, despite of repeated attempts, this stable clone was not established. This may possibly come from a leaky basal expression of AMLV-FMG, sufficient to kill the transfected cells. Thus this project was not finished and should be continued using a better Tetracycline repressor-expressing cell line, or using another inducible systems rather than the Tet-On. An alternative is also to use a virus encoding for the AMLV-FMG and to inject it directly into the tumor. We are currently working on this issue in collaboration with the laboratory of FL Cosset (ENS Lyon).

## II. PEI-mediated siRNA Delivery in Vivo

### II.1. Conclusion

So far, our results concerning PEI-mediated delivery of siRNA to lung or tumor cells showed a very poor efficacy. Among the large panel of parameters to be tested, we tried different modes of *in vivo* administration (IV, IP, and SC injections), different tumor cell types (A549, 3LL, and TSA), and also different formulas for PEI/siRNA complexing including N/P ratios (8, 10, or 20eq) and solutions (5% glucose or 150 mM NaCl).

The results of PEI/siRNA delivery *in vivo* generally showed a weak (of any) activity, which was not statistically significant in the measurement of luciferase reporter gene. Otherwise, PEI/siC-Raf injection was not associated with an anti-tumor effect in established A549 xenografts.

In regard of the transient and weak silencing effect frequently observed, it may suggest the instability of this polyplex in physiological conditions. Indeed, complex formation and interaction between the cationic polymer and a plasmid or a siRNA differ by nature and strength. A rigid two-turn double-helical siRNA is difficult to be “condensed” compactly, and the PEI/siRNA complex formation lacks cooperativity. The major consequence of these conditions may be the unwanted exchange of siRNA with large polyanions found outside cells (Bolcato-Bellemin et al., 2007). In the current stage, we suppose that the physical/chemical modifications to the materials (PEI, or siRNA) are required for an improved *in vivo* siRNA delivery.



## II.2. Perspective in PEI-delivered siRNA transfection in vivo

A new strategy discovered by PolyPlus Transfection is the use of sticky siRNA (ssiRNA). In their studies, non-viral vectors (especially polymers) form looser complexes with siRNA than with plasmid DNA. As a consequence, exchange of siRNA for larger polymeric anions such as proteoglycans on the cell surface may occur and lower the delivery efficiency. Using long dsRNAs instead of the typical 21-mer siRNA may enhance the delivery, but the use of them is associated with nonspecific gene shutdown and the interferon response.

Based on these concerns, PolyPlus Transfection developed the sticky siRNA (ssiRNA). Without any chemical modification, this strategy only adds short A/T (5-8 mers) overhangs at 3'-end of single strand RNA. The complementary overhangs make ssiRNA to form "gene-like" concatemers and complex with PEI more stably, enhancing the gene silencing effect *in vitro* up to 10-fold. After cell-entry and decomplexation in the cytoplasm, ssiRNA concatemers fall apart and therefore do not induce antiviral responses, as examined by the absence of IFN production. More importantly, PEI/ssiRNA also showed an enhanced silencing effect in mouse lung as compared to PEI/siRNA (Bolcato-Bellemin et al., 2007). Thus ssiRNA may serve as a convenient and low-priced modification suited to gene silencing with L-PEI *in vivo*.

In collaboration with PolyPlus Transfection, we will start again the evaluation of its *in vivo* delivery efficiency. The biodistribution of PEI/ssiRNA polyplex in mouse will be the first step, and the following delivery to target mouse lung or xenograft, against a reporter gene or an oncogene expression will be carried out successively.

## **III. Stable in Vivo Transfection Using the Sleeping-Beauty Transposon**

### **III.1. Conclusion**

In this project we evaluated the capacity of PEI to deliver a gene that can be expressed for long-term due to the presence of SB transposon system.

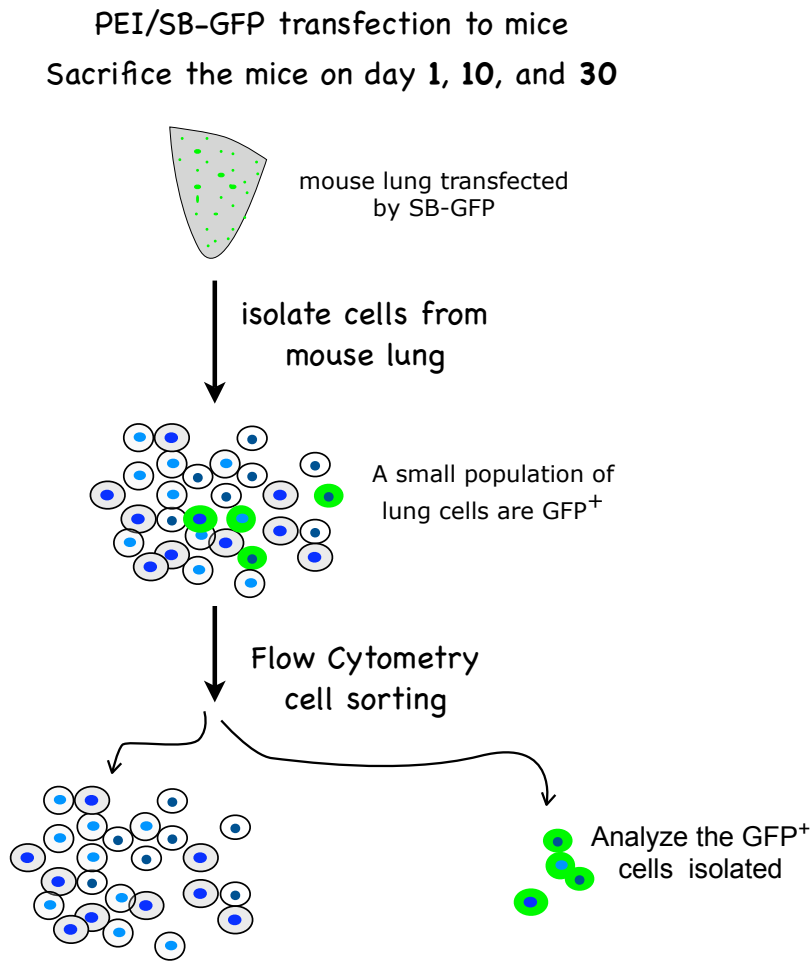
Non-invasive, longitudinal measurements of luciferase reporter gene expression in mouse by BLI system allowed us to evaluate the capacity of the SB-transposon to induce the stable expression of a transgene. In the PEI-delivered SB-luc transfection in mouse lung, we observed that the transient expression of the reporter gene rapidly decreased in the first 2 weeks and became almost undetectable. This result was as expected and reflected the usual transient nature of PEI-mediated DNA transfection. Later on however, the luc signal appeared again, which augmented regularly and reached eventually a plateau 2 months after transfection. The final Luc signal was around 16% of that measured on day 1.

We do not have a definite explanation for this bi-phasic curve, but we suppose that this phenomenon is associated with the multiplication of a small population of undifferentiated cells (lung stem cell, precursor of P-II, or P-II cells?) stably transfected by the SB-luc. Nevertheless, we do not have a direct evidence to prove this hypothesis.

We then tried to augment this effect by a second transfection. However, our results showed that a second transfection is not effective, even it is performed 12 weeks after the first. Although the reasons underlying this phenomenon are unclear, it raises an important issue about using PEI in the future somatic gene therapy protocol. Indeed, we imagine that the PEI polyplexes will have to be repeatedly injected during the patients' life if we expect to treat a genetic disease. Thus PEI will have to be modified if we want to use it in future gene therapy trials.

Although the experiments are still in progress and a definite conclusion has not been made, no sign of tumor development was noticed 4 months after the PEI/SB-K-Ras transfection in mice. Unlike previously described models, which used inducible transgenic mice that express K-Ras in the majority of lung cells after induction, our system transfects only 1-5% of the lung cells. Among these cells, a small population of multiplying cells will be stably transfected, which finally results in <1% cells harboring the SB-K-Ras in lung. This relatively low percentage of K-Ras positive cells may not be sufficient to induce tumor formation in such a short period of observation of the mice.

**Figure 2. Our proposal to isolate the PEI/SB-transposon transfected cells.**



### III.2. Perspectives

To understand which type of cells are stably transfected by the SB-transposon in mouse lung, we transfected the SB-lacZ and analyzed the cells stained by X-Gal. Unfortunately, presumably because of the very low number of positive cells in the lung between 1 to 2 weeks after transfection, we were not able to identify which type of cells were transfected at this stage.

To address this problem, we must firstly collect enough transfected lung cells 1-2 weeks after transfection. We will use the FACSaria Cell Sorting System (BD Biosciences, CA, USA), which is able to sort cells according to their fluorescent marker. The vector pT3-GFP will be constructed and co-transfected with pSB by the same protocol as PEI/SB-luc and -lacZ. The lungs will be removed from the transfected mice 1, 10 or 30 day(s) post-transfection, and lung cells will be isolated and suspended in PBS solution for flow cytometry analysis. The FACSaria will then separate the population of cells in 2 groups according to the presence/absence of GFP signal (Fig. 2).

We did not observe any tumors after the PEI/SB-K-Ras transfection so far. P53 and p16 should very likely play an important role in blocking K-Ras mediated oncogenesis. Indeed, these 2 genes are known to induce growth arrest or senescence in lung cells, as already illustrated in primary cells *in vitro* and the transgenic mice model *in vivo* (Collado et al., 2005; Serrano et al., 1997).

It would be of interest to inject PEI/SB-K-Ras in p53<sup>-/-</sup> or p16<sup>-/-</sup> knockout mice. Indeed, a similar model using the SB-transposon encoding for N-Ras was shown to induce liver cancer after hydrodynamic transfection in p19<sup>Arf</sup>-null mice, but not in wt ones (Carlson et al., 2005). This project would thus rely on the possibility to get the mouse strain knock-out for p53 or p16. This hypothesis is under investigation.

Additionally, we also constructed a SB-CFTR transposon. In collaboration with Dr B Pitard, we scheduled the injection of CFTR<sup>-/-</sup> mice (B6; 129-CFTR<sup>tm1Unc</sup>) with PEI/SB-CFTR polyplexes. These mice normally die rapidly because of the absence of functional CFTR. We will thus evaluate whether the re-introduction of a wt-CFTR

gene by PEI/SB-transposon will improve the outcome of these animals.

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## VECTORISATION NON-VIRALE DE MOLECULES THERAPEUTIQUES POUR LA THERAPIE DES CANCERS DU POUMON

L'utilisation de la thérapie génique du cancer est limitée actuellement par la faible efficacité de transfection, la durée d'expression du gène et la toxicité des vecteurs. Ces difficultés ont guidé l'orientation de mes travaux dans 3 directions:

- 1/ Utilisation de gènes codant pour des glycoprotéines fusogéniques (FMG) comme gènes suicides à fort effet bystander.
- 2/ La vectorisation de siRNA *in vivo* par le vecteur polycationiques : polyéthylèneimine (PEI).
- 3/ La stabilisation de l'expression du transgène à long terme *in vivo* à l'aide du transposon *Sleeping Beauty* (SB).

Les résultats de ces travaux montrent que :

- 1/ La thérapie génique basée sur l'utilisation de FMG montre un fort intérêt thérapeutique sur des cellules de cancer du poumon humain *in vitro* et *in vivo*. En effet, ces protéines FMG ont i/ un fort effet cytotoxique qui passe essentiellement par la fusion entre la cellule transfectée et de nombreuses cellules voisines non-transfectées, ii/ la capacité d'induire une immunité antitumorale induite par la libération des vésicules immunogènes au cours de la mort des cellules fusionnées. Trois FMG ont été testées: GALV, HERV-W et RD. Dans les 3 cas nous avons montré que la transfection de ~1% des cellules *in vitro* conduit à la formation de large syncytia et à la mort de 25 à 80% des cellules en culture en moins de 5 jours. Le traitement des tumeurs sous-cutanées implantées chez des souris nues induit une réduction du poids des tumeurs pouvant aller jusqu'à 70% alors que l'efficacité de transfection par injection directe des plasmides dans la tumeur est extrêmement faible ( $\leq 1\%$ ). Ces résultats démontrent que ces protéines FMG possèdent un potentiel intéressant pour la thérapie génique du cancer. Néanmoins, notre modèle de souris immunodéficient ne nous a pas permis de mesurer l'impact supplémentaire que nous pouvions attendre de la stimulation de la réponse antitumorale activée par la production de syncytiosomes. Cette étude est encore en cours.
- 2/ La vectorisation de polyplexes PEI/siRNA *in vivo* par voie intraveineuse, intrapéritonéale ou sous-cutané avec différentes formulations a montré des résultats faiblement positifs au mieux et souvent peu reproductibles. Nous avons étudié la biodistribution en temps réel de ces complexes en imagerie de fluorescence et mesuré leur capacité à inhiber l'expression d'un gène reporter ou d'un oncogène dans les poumons et/ou les tumeurs des souris. Globalement ces résultats démontrent que le PEI n'est pas un vecteur efficace pour les siRNA dans une approche systémique et que des modifications chimiques sur le PEI et/ou les siRNA devront être envisagées pour augmenter la stabilité et la performance de ces particules.
- 3/ L'insertion du transposon SB dans le plasmide vectorisé, complexé à du PEI et injecté en intraveineux, permet de stabiliser l'expression du transgène pendant plus de 4 mois dans les poumons. La mesure en cinétique à long terme du gène reporter dans les poumons montre en effet une forte expression du gène reporter codant pour la luciférase 1 jour après la transfection. Cette expression disparaît rapidement durant les 2 semaines suivantes jusqu'à devenir indétectable. De façon intéressante, le signal luciférase se rétablit ensuite progressivement pour atteindre un plateau 2 mois après la transfection. Le niveau d'intensité du signal de luciférase est alors d'environ 15% de celui mesuré le premier jour. Ces résultats suggèrent que le transposon SB permet une insertion stable du transgène dans un nombre très restreint de cellules pulmonaires ayant la capacité de se multiplier. Ce résultat est prometteur et offrira une plate-forme d'intérêt qui permettra de vectoriser des gènes codant pour des protéines biologiquement actives, telles que celle codée par le gène CFTR (cystic fibrosis transmembrane conductance regulator) pour la thérapie de la mucoviscidose, ou le gène K-Ras pour l'analyse de l'oncogénèse ras-dépendante dans le cancer du poumon. Enfin, les cellules touchées par l'insertion stable du transposon ayant un pouvoir de régénération du poumon important, il semble que nous ayons un moyen de modifier génétiquement des cellules souches pulmonaires. Nous souhaitons donc maintenant les caractériser précisément car cela ouvre des perspectives thérapeutiques importantes.

**Mots clés :** Thérapie génique, cancer, tumeur, poumon, siRNA, FMG, transposon

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## NON-VIRAL VECTORIZATION OF THERAPEUTIC MOLECULES FOR LUNG CANCER THERAPY

Current cancer gene therapy protocols are strongly limited by several factors such as the low transduction efficiency, transient gene expression, or toxicity of the vector. To approach these problems, my work was concentrated on the non-viral delivery of biological molecules for the treatment of lung cancer with 3 main aspects:

- 1/ Study of the antitumoral effect mediated by several viral fusogenic membrane glycoproteins (FMG) gene transfer.
- 2/ Vectorization of siRNAs *in vivo* by the cationic polymer : polyethylenimine (PEI).
- 3/ Long-term expression of the transgene *in vivo* by non-viral delivery of the DNA vector containing *Sleeping-Beauty* (SB) transposon.

Our results showed that :

- 1/ The FMG-based gene therapy was found to produce a highly efficient antitumor effect toward human lung cancer cells *in vitro* and *in vivo*. FMG expression is known to a/ induce the fusion of a single transfected cell to multiple neighboring untransfected cells, leading to the formation of large syncytia committed to death in 5 days b/ to induce a specific antitumor T-cell immunity in the host, through the release of immunogenic vesicles during the death of the syncytia. These 2 properties are cumulative and participate in the very strong bystander effect related to the use of FMG. Using FMGs of different origins (GALV, HERVW and RD) *in vitro*, we showed that the transfection of ~1% cells led to the death of up to 80% of the cultured cells. *In vivo*, treatment of human xenografts of lung cancer in nude mice by direct repeated intratumoral injections of the naked plasmids encoding these FMG showed a 60-70% reduction in tumor weight. This antitumor effect is thus very strong, especially in regard to the very poor efficiency of the transfection method ( $\leq 1\%$  tumor cells are transfected). Furthermore, these results were obtained in immunodeficient mice. It is thus reasonable to assume that this FMG-based cancer therapy will be even more interesting in an immuno-competent animal. This study is currently going on in the laboratory.
- 2/ *In vivo* delivery of several formulations of PEI/siRNA polyplexes using the intravenous, intraperitoneal or subcutaneous administration routes showed partially positive, but usually transient and weak silencing effects against target genes (reporter gene or oncogene) in mouse lung or tumor xenografts. These results combined to the studies that we performed to measure the biodistribution of these complexes using *in vivo* fluorescent imaging, confirmed that the PEI is not as adapted for the delivery of siRNA as it is for plasmids. This suggests that additional chemical modifications of the PEI or siRNA would be necessary to augment the stability of these complexes *in vivo*.
- 3/ The systemic administration in the tail vein of nude mice of PEI-complexed plasmids containing a luciferase reporter gene inserted in SB transposon showed that a strong luciferase expression can be detected in the lung of mice for more than 4 months. As usual, the luciferase signal was very strong 1 day after transfection in the lung but rapidly disappeared in the following 2 weeks. However, because of the presence of the SB transposon, this signal was then progressively restored in the lung of these animals and reached a plateau 2 months after transfection. At this step the intensity of the luciferase signal was stable and represented around 15% of its maximum value measured at day 1. This pattern suggested that a stable transfection of the SB transposon into a small population of cells capable of lung regeneration was obtained. This result is promising and provides a platform for the delivery of active genes, such as CFTR (cystic fibrosis transmembrane conductance regulator) gene for therapeutic purposes, or the K-Ras gene for studying the Ras-dependant oncogenesis of lung cancer. It is thus of great importance to further characterize the nature of the stably transfected cells, and this will open a new field of investigation in the laboratory.

**Key words :** Gene therapy, cancer, tumor, lung, siRNA, FMG, transposon