Crosstalks between SOX2 and the Wnt/β-catenin signaling pathways in lung carcinogenesis

UE Projet de recherche collaboratif interdisciplinaire

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Abbreviations

APC : Adenomatous polyposes coli
CCND1 : Cyclin D1
CBP : CREB Binding protein
CD : Cluster of differenciation
CK : Casein Kinase
DKK : Dickkopf
Dv : Disheveled
BCL9 : B-cell CLL/lymphoma
BRN : Brainiac
EF3 : Elongation Factor
Fra : Fos-related Antigene
Fzd : Frizzled
GSK : Glycogen Synthase Kinase
HDAC : Histone Deacetylase
HMG : High Mobility Group
LEF : Lymphoid enhancer factor
LRP : Low density lipoprotein receptor related protein
OCT : Octamer
NSCLC : Non-Small Cell Lung Carcinoma
MMP : Matrix Metalloprotéinase
Myc : Myelocytomatosis
Pygo : Pygopus
SCC : Squamous Cell Carcinoma
SCLC : Small Cell Lung Carcinoma
sFRP : Secreted, Frizzled Related Proteins
shRNA : short hairpin RNA
SOX : SRY-related-HMG-box
SRY : Sex-determining Region Y
Rb : Retinoblastoma
TCF : T cell factor
TP53 : Tumor Protein 53
Ub : Ubiquitin
VEGFR : Vascular Endothelial Growth Factor Receptor
Wnt : Wingless-Type MMTV integration site
WIF : Wnt Inhibitor Factor
I. Introduction

Lung cancer is the first cause of mortality by cancer in the world, with 1.2 million deaths per year [1]. Lung cancer is divided into small cell lung carcinoma (SCLC; 20% of the cases) and non-small cell lung carcinoma (NSCLC) which represents 80% [1]. This study concerns the histological sub-type squamous cell carcinoma (SCC, 49% of NSCLC) [2] (Fig. 1). Smoking is by far the main etiologic factor to lung SCC causing almost 90% of lung cancers [1]. Lung SCC develops in the large conducting airways from the pseudostratified epithelium of the bronchial tree by a sequential process involving abnormal differentiation (epidermoid/squamous metaplasia) and a typical progression sequence of hyperplasia-dysplasia-carcinoma stages [1]. Tumor establishment and progression imply specific genetic alterations (Fig. 2) through loss of tumor suppressor genes (TP53, Rb) or activation of oncogenic signals (c-Myc, Cyclin D1) [1, 3].

Mapping of gene amplifications in lung SCC has pinpointed the SOX2 transcription factor as a novel and major oncogene [3]. Interestingly, SOX2 is also involved in breast cancer, where it interacts with β-catenin to activate some target genes [4]. Target genes regulated by SOX2 in lung SCC are still largely unknown but are likely cell-specific and may overlap with target genes that SOX2 controls during lung development and homeostasis. We will therefore summarize the scientific literature about the respective implications of SOX2 and β-catenin in the development of the bronchial epithelium and in lung SCC since similar mechanisms are at play (proliferation, migration and differentiation). Finally, we will explain the rationale and specific objectives of this study.

1. The SOX2 transcription factor

1.1. Introduction

The SOX (for SRY-related-HMG-box) genes were discovered shortly after the identification of Sry. In the vertebrate genome, there are approximately 20 different SOX genes that are classified into 8 subfamilies (SOX A-H) according to sequence homologies in their HMG domains [5]. This domain enables SOX proteins to bind to specific sequences (C(T/A)TTG(T/A(T/A)) located in the promoter of target genes to exert their transcriptional regulatory role [5]. Importantly, the activity of SOX proteins is exerted through cell type-specific associations with several partners (such as OCT3/4, BRN2, Nanog, δEF3 and β-catenin) and downstream transcriptional regulation of specific target genes [5]. This enables SOX proteins to tightly control wide transcriptional programs and associated cellular mechanisms that ensure embryonic/organ development and adult tissue homeostasis. As examples, SOX2 is one of the master controller of pluripotency in Embryonic Stem Cells and Neural Stem cells [6, 7].
1.2. SOX2 in lung development and homeostasis

Lung morphogenesis is the result of a complex multi-step branching process that is highly controlled through instructive epithelial-mesenchyme interactions [8, 9]. These allow to form the adult lung epithelium that is composed of several different cell types (ciliated, clara, basal and alveolar cells, etc.; Fig. 3).

Several SOX genes, including SOX2, are expressed and important during lung morphogenesis. Among the different epithelial cell types forming the lung epithelium, SOX2 is broadly expressed in the large conducting airways (trachea, bronchi and bronchioli), but more abundantly in ciliated epithelial cells, and not detected distally in the alveolar pneumocytes [8-10]. SOX2 over-expression in murine lung epithelium revealed its role for correct secondary branching and epithelial cell differentiation [10]. Epithelial dysplasias and accumulation of committed progenitors (pre-basal cells, which represent candidates as the cell-of-origin of lung SCC) were observed in the transgenic animals [10]. Finally, during repair of the injured lung epithelium, the expression of SOX2 is increased (together with β-catenin) in the squamous cells lining the airways [8].

1.3. SOX2 is a novel and major oncogene in lung SCC

Our laboratory has recently identified SOX2 as a novel and major oncogene in lung SCC [3]. We found high-level amplifications of the SOX2 locus in about 20% of lung SCC (and additionally this locus is gained in about 80%). These high occurrences of amplifications likely reflect a critical cellular selective advantage conferred by the activated oncogene. We demonstrated that SOX2 is capable of transforming bronchial epithelial cells that are initially non-tumorigenic (Fig 4A). Moreover, the SOX2 protein is activated in a wide majority of lung SCC (67%, Fig. 4B) and can induce a poorly differentiated state that may favor cellular plasticity and help cells to adapt during tumor progression. Finally, using a knockdown approach, we showed that SOX2 expression is necessary for lung squamous cell viability [3]. Importantly, our results are supported by a similar report from independent research groups [11], altogether unambiguously designating SOX2 as a novel and major oncogene in lung SCC.

2. The Wnt/β-catenin signaling pathway

2.1. Description of the canonical Wnt/β-catenin signaling pathway

This project will focus on the canonical Wnt/β-catenin signaling pathway (Fig. 5), but not on the non-canonical Wnt pathways [12 and references therein]. It should be noted that β-catenin has a dual role in epithelial cells. The protein is very stably associated with cadherins as a structural component of adherens junctions mediating epithelial cell-cell interactions. However, a dynamic pool of β-catenin is also available in the cytoplasm as an essential component of Wnt signaling.
In the absence of Wnt ligand (Fig. 5A), β-catenin is captured by a « destruction complex » made from the adenomatous polyposes coli (APC), axin and glycogen synthase kinase 3 (GSK3) proteins. GSK3 phosphorylates serine residues located at the N-terminus of β-catenin which leads to its degradation by the proteasome [12]. In the presence of extracellular Wnts (Fig. 5B), they bind to their receptors (frizzled and LRP) to activate a cascade of intracellular signaling events ultimately leading to β-catenin stabilization, translocation to the nucleus and binding to its partners (TCF/LEF transcription factors). This displaces co-repressors and recruits co-activators to stimulate the expression of target genes that include matrix metalloproteinases (MMP 2, 3, 7 and 9), CCND1, c-myc, c-jun, Fra-1, VEGFR and others [12].

Through its target genes, the Wnt/β-catenin signaling pathway is implicated in the regulation of embryogenesis by controlling the processes of cell fate/differentiation, migration and proliferation during embryo patterning and development of several tissues [12]. It is also crucial for the homeostasis of several adult tissues, including the skin, intestine and lung. Mis-regulation of Wnt signalling cause severe defects and diseases during embryonic development while in the adult it can lead to tumor formation [12].

2.2. Wnt/β-catenin signaling pathway in lung development and homeostasis

During lung development, Wnt/β-catenin signaling plays a critical role in establishing the proximal-distal cell fate in the epithelium and is necessary for appropriate branching morphogenesis (but not for establishment of the primary branching pattern) [13]. Some Wnts are implicated in the pulmonary development [12].

Deletion or inhibition of β-catenin in respiratory epithelial cells impaired branching morphogenesis and inhibited peripheral airway cell differentiation in vivo [12, 13]. Conversely, activated β-catenin signaling during lung formation in transgenic mice disrupts branching morphogenesis and induces both aberrant differentiation and hyperplasia of the airway epithelium [12, 13].

2.3. Wnt/β-catenin signaling in lung SCC

Using mice models, it was shown that the expression of a constitutively active form of β-catenin in murine lung epithelial cells induces several abnormal tissular processes, notably including squamous metaplasia and dysplasia [13]: as noted earlier, these represent precursor lesions of lung SCC.

Studies of primary lung cancers have demonstrated that the pathway is frequently activated (50% in NSCLC) as revealed by an increase of nuclear β-catenin [12]. However, mutations in pathway members such as APC or β-catenin (which are both very common in colorectal cancer) are very rare in lung cancer [12]. In these tumors, alternative mechanisms
are used to activate the pathway such as the overexpression of activators (including Wnts and Dvl), or the down-regulation of antagonists (Table 1) [14-22].

3. Rationale for SOX2 / β-catenin crosstalks in lung SCC

SOX proteins and Wnt/β-Catenin signaling regulate many of the same biological processes including organ development and tissue specification, stem cell homeostasis and cancer [5]. Accordingly, several specific crosstalks between SOX proteins and the Wnt/β-Catenin pathway have been shown to rely on different molecular mechanisms in various cellular and tissular contexts [5]. Concerning SOX2, a few reports only have highlighted an interaction with β-catenin so far.

Interestingly, the expression of both SOX2 and β-Catenin is induced in murine lung epithelial cells in vivo after naphtalene injury, which mimics tobacco smoke effects [8]. Additionally, SOX2 functions directly downstream of canonical Wnt signaling to control cell fate choices along alternate lineages in taste bud sensory cells [6] or retinal cells [23] in vivo. In vitro, cellular models have demonstrated that SOX2 can directly bind to β-Catenin and either function as a repressor or activator of Wnt/β-Catenin signaling, in osteoblasts [24] or in breast cancer cells [4], respectively. Moreover, the latter study showed that SOX2 is frequently over-expressed in aggressive human breast carcinomas where its association with β-catenin activates the transcription of the CCND1 oncogene [4].

All these results indicate that the output of SOX2 on Wnt/β-catenin signaling (and vice et versa) is strongly dependent on the cellular context, which is a known characteristic of SOX transcription factors [5]. This further reinforces the importance of addressing the question of their potential crosstalks specifically in lung squamous cells, which has not been previously assessed. Overall, our working hypothesis is that similar mechanisms of SOX2 and β-catenin interactions could be implicated in lung SCC (Fig. 6).

II. Objectives of the project

The overall objective of this study is to highlight a possibly cooperative interaction between β-catenin and SOX2 as a possible ground underlying the tumor phenotype in lung SCC. We will first test if the 2 proteins are co-expressed and if they are capable of physical interaction. Then, we will determine the Wnt/β-catenin signaling activity in our lung SCC models and its contribution to the tumor phenotype. Lastly, we will determine the reciprocal interactions between SOX2 and β-catenin signaling and assess their possible functional cooperation in lung SCC. Overall, our objectives will be achieved using genetic tools or chemical agents, to overexpress or inhibit SOX2 and/or β-catenin signaling. Importantly, the project has been designed so that direct perspectives will reside in the translation of the in vitro findings obtained during the masters training period to the in vivo situation. A long-term perspective would be to treat SCC with pharmacological agents inhibiting the Wnt and/or SOX2 pathways.
III. Description of the project

We will first describe cellular models and *in vitro* assays (point 1) and then the experimental design (points 2 and 3) to address specific questions.

1. **Molecular and cellular tools: analyzing tumor traits in vitro**

1.1. Lung squamous cell lines

We will use four different cell lines as *in vitro* cellular models of normal or cancer lung squamous cells. Characteristics of these models are thereafter detailed with the longer-term perspective of reimplanting these cells into mice models of cancer. All these cell lines were previously obtained from the American Type Culture Collection (ATCC, a bioresource repository, http://www.lgcstandards-atcc.org/) and are grown under recommended conditions [3].

**The BEAS-2B** cell line (CRL-9609) was originally derived from normal (non cancerous) human lung bronchial epithelial cells immortalized by Adenovirus-SV40 hybrid virus. However they are non-transformed and do not lead to tumor formation when implanted in immunocompromized mice *in vivo*. These cells represent a simple *in vitro* model of lung bronchial epithelial cells and can become tumorigenic upon oncogene over-expression as we showed for SOX2 ([3], cf Fig. 4A).

**The NCI-H226** (CRL-5826) and **Calu-1** (HTB-54) tumor cell lines were derived from human lung Squamous Cell Carcinomas (SCC) and are fully tumorigenic and invasive when xenografted in immunocompromized mice.

**The KLN205** (CRL-1453) cell line was derived from a spontaneous murine lung squamous cell carcinoma. A major interest of this model resides in the capacity of the cells to form poorly differentiated SCC tumors when implanted subcutaneously or in the lung of syngeneic (DBA/2 mouse strain) fully immunocompetent mice.

Of note, additional cell lines are available in the laboratory in the case(s) where none of the above mentioned cell lines would allow to address a specific question of the project.

1.2. Lentiviral preparations for transductions

Third-generation lentivirus vectors are efficient tools to produce stably expressing cellular models from cycling and non cycling cells. In this vector, the HIV-derived packaging component was reduced to the minimum to improve its biosafety [25]. The process is divided in two steps: i) The production of genetically modified lentiviral particles: this is obtained by HEK-293T cells transitory transfections with three plasmids necessary for viral production (Fig. 7A), together with a construct for insert transfert (Fig. 7B) using the CaPO4 tranfection
method (Fig. 7C). ii) In a second step, these lentiviruses particles are incubated with target cells in presence of polybrene (to enhance transduction efficiency) and are capable of transferring the insert into the DNA of infected cells (Fig. 7D) but not further replicate in infected cells (self-inactivating vectors). An example of transduced cells expressing GFP is shown (Fig. 8A). Using these tools, lung squamous cells stably overexpressing SOX2 were previously established and SOX2 expression verified by Western Blot (Fig. 8B; [3]). A construct allowing for active β-catenin expression (Fig. 8D) has been built and is available to similarly produce lentiviruses and transduce lung squamous cells.

For transductions, cells are incubated prior to adding the viruses into a minimal volume of cell culture medium for 24h. Typically 5.10⁴ to 2.10⁶ cells (depending on the cells) are transduced in formats ranging from 24-well plates to 10cm dishes.

1.3. In vitro assays to assess tumor phenotypical traits

Classical assays to assess cell proliferation (MTT assay), anchorage independent growth ("soft-agar"), migration ("wound healing" assay), invasion ("transwell" assay) and resistance to apoptosis will be carry out as we previously described [3]. These assays are presented in details in Figure 9.

1.4. Reporter assays to measure Wnt/β-catenin signaling activity

We will use the “Super 8x TopFlash” reporter system [26]. This synthetic reporter system relies on a plasmid derived from the pTA-luc backbone (Promega) which contains eight copies of a consensus TCF/LEF DNA-binding site (5’-AGATCAAAGG-3’) upstream of a minimal TA promoter driving the expression of the firefly luciferase (Fig. 10A). A control experiment with the “FOP-flash” plasmid is carried out in parallel: it contains eight mutated copies of the DNA-binding site (5’-AGATCAAAGG-3’) that do not bind TCF/LEF transcription factors. Finally, the Dual-Luciferase™ Reporter Assay is employed for the readouts, following manufacturer’s instructions (Promega). In brief, the cells are lysed, the luciferin (substrate of the firefly luciferase) is added and will be convert into oxyluciferin. This conversion permits the emission of light which can be quantified using a luminometer.

2. Experimental design : β-Catenin and SOX2 expression/interaction

Specific goals: To determine SOX2/β-catenin expression patterns and their potential interaction in lung squamous cells.

2.1. SOX2 and β-catenin expression patterns in lung squamous cells.

Our primary goal is to determine if β-catenin and SOX2 cooperate in lung SCC which implies that they are co-expressed in the same cells. First, we will evaluate if both proteins
are expressed and their subcellular distribution (nuclear or cytoplasmic) in our different cell lines by immunofluorescence. After cell fixation, we will co-stain cells for both SOX2 and β-Catenin specifically revealed with secondary antibodies coupled to different fluorochromes. Primary antibodies namely: Anti SOX2 (R&D Systems AF2018); Anti β-catenin (mouse IgG1 clone 14, BD Biosciences); Anti Actin (control; sc-1615, Santa Cruz Biotechnology Inc.). Secondly, to confirm these results Western Blot experiments will be done to evaluate the levels of β-catenin and SOX2.

**Expected Results:** A SOX2 nuclear staining should be observed in a majority of cancer cell lines. Based on published results in tumors, some cancers cell lines should be positive for nuclear β-catenin. We will determine the cell lines with the highest expression levels of both SOX2 and β-catenin which will be the best candidates for IP studies (point 2.2). After definitive demonstration of the status of the Wnt/β-catenin signaling pathway by reporter assay (Fig. 10), stimulation or inhibition of this pathway will be done in Wnt/β-catenin inactive or active cell lines (see point 3 below).

2.2. SOX2 and β-catenin physical interaction in lung squamous cells

To investigate the hypothesis that β-catenin and SOX2 physically interact, we will realize co-immunoprecipitation assays in cell line(s) with the highest endogeneous protein expression. In the case, where these IP on endogenous material would not give conclusive results, we will carry out transient overexpression.

**Expected result:** to determine if SOX2-β-catenin physical interactions can be detected in lung squamous cells.

3. **Experimental design: Importance of Wnt/β-catenin signaling and crosstalks with SOX2 in lung SCC**

**Specific goals:** To determine Wnt/β-catenin signaling level in lung squamous cell lines, and the reciprocal interactions between the SOX2 and the Wnt/β-catenin pathway.

3.1. Wnt pathway activity in lung SCC

Using the Wnt reporters, we will measure the activity of Wnt/β-catenin signaling by the quantification of the luciferase activity in TOPFlash vs FOPFlash transfected lung squamous cells (Fig. 10B).

**Expected results:** these experiments will allow to assess quantitatively which lung squamous cell lines undergo active Wnt/β-catenin signaling and which do not.
3.2. Functional consequences of Wnt/β-catenin signaling modulation in lung squamous cells

i) Inhibition of Wnt/β-catenin signaling (Fig. 10C)

We will investigate if Wnt signaling can be blocked in the adequate cell lines and the effects of blocking it. To do that, we will use several genetic tools blocking the pathway at different levels: available molecular tools include constructs encoding secreted inhibitors of Wnt signaling (sFRPs or DKKs), Dominant Negative (DN) LRP6 or LEF, shRNA targeting β-catenin. We will evaluate the effect on different tumor traits with classical assays in vitro as previously detailed (Fig. 9).

ii) Activation of Wnt/β-catenin signaling (Fig. 10C).

We will activate Wnt/β-catenin signaling in cells in which this pathway is inactive, by chemical activators (LiCl, an inhibitor of GSK-3b or others small molecules) or with genetic tools. Cell lines overexpressing constitutively active β-catenin will be established upon lentiviral transductions (Fig. 9D). We will then measure the effect of this activation on tumor traits in vitro.

iii) Effect of Wnt signaling inhibition/activation on SOX2 (Fig. 10D)

We will analyze SOX2 gene expression by quantitative RT-PCR and Western Blot (as previously, [3]) upon Wnt/β-catenin inhibition or activation in appropriate cells (Fig 10C). Accordingly, we will determine whether SOX2 is a direct β-catenin target in lung SCC or not.

**Expected results:** to determine whether SOX2 is a direct target of Wnt/β-catenin signaling in lung SCC.

3.3. Impact of SOX2 on Wnt/β-Catenin signaling in lung squamous cells (Fig. 10E)

The Wnt signaling reporter will be transfected into SOX2-overexpressing or SOX2-depleted cells to measure the effect of SOX2 protein level on the Wnt pathway. We will make use of already established cell lines that stably overexpress SOX2 (Fig. 9B & D). Conversely, we will knockdown SOX2 with validated shRNAs using transient transfections (since we found that SOX2 stable knockdown is lethal for lung squamous cells [3]).

**Expected results:** to assess whether SOX2 acts as a repressor or an activator of Wnt/β-catenin signaling in lung SCC.
IV. Estimated planning

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V. Conclusion

Several modes of interactions between SOX2 and Wnt/β-catenin pathway have been described in different physiological and pathological conditions (see introduction). Our planned experiments will help to determine which of the SOX2 and Wnt/β-catenin modes of interaction occur in lung SCC (direct physical interaction, indirect interaction, SOX2 upstream of Wnt/β-catenin or the reverse…) and affect main cancer phenotypical traits as cell proliferation, migration and invasion. Long term perspectives will reside in the translation of the in vitro findings to the in vivo situation by analyzing i) mice models in a pre-clinical setting ii) primary lung SCC tumors. This project will possibly lead to the identification of new potential pharmacological targets implicated in an aggressive tumor phenotype that may improve the treatments for lung SCC patients.


Le cancer du poumon est la première cause de mortalité par cancer dans le monde. Parmi les cancers du poumon, on distingue plusieurs types histologiques dont les carcinomes épidermoides (SCC). Des résultats récents indiquent que les facteurs de transcription SOX2 et la β-Caténine pourraient participer à la tumorigénèse pulmonaire.

SOX2 et la β-Caténine induisent tous deux la transcription de gènes impliqués dans le développement et l'homéostase du poumon. Notre laboratoire a récemment mis en évidence le pouvoir oncogénique et l'activation récurrente de SOX2 dans les SCC bronchiques. Par ailleurs, la voie de signalisation Wnt/β-Caténine est également activée de manière récurrente dans ces tumeurs. Finalement, une interaction synergique entre SOX2 et la β-Caténine a été démontrée dans le cancer du sein.

Nous allons tester l'hypothèse d'une interaction entre SOX2 et β-caténine dans les SCC du poumon en utilisant des modèles cellulaires in vitro de ces tumeurs. Ainsi, cette étude permettra de disséquer fonctionnellement l'importance de cette interaction pour le phénotype tumoral et participera à une meilleure compréhension des mécanismes moléculaires. A long terme, il est envisagé de confirmer ces résultats in vivo, par l'analyse de modèles animaux et de tumeurs primaires, ce qui pourrait aboutir à l'identification de nouvelles cibles thérapeutiques.
Figure 1: Schematic view of the histo-pathogenesis of main Non-Small Cell Lung Carcinoma subtypes (adapted from [1]).

This model shows filiation between the different cell types of the bronchopulmonary epithelium and carcinomas. The formation of squamous cell carcinoma, which is the scope of this project, implies a multi-step tumorigenic process. An epithelial stem cell will be transformed into a squamous cell which will undergo metaplasia. This squamous metaplasia can then further evolve into a carcinoma. The red arrow indicates the type of cancer (lung SCC) and the two main molecular players involved in this project.

Figure 2. Molecular alterations leading to squamous cell carcinoma (adapted from [1]).

This diagram shows the recurrent genetic alterations during the establishment and progression of lung SCC. Bronchial epithelial cells under the influence of oncogenic factors overexpressed and/or inactivation of tumor suppressor factors to give rise to squamous metaplasia and dysplasia. Activation of oncogenic signals such as CCND1 (activation of the G1/S cell cycle transition) can lead to primary squamous cell carcinoma which can finally become invasive through further gene losses. SOX2 activation could be implicated in the initial driving process of metaplasia/dysplasia [8] and in the progression to primary squamous cell carcinoma [3].
The lung is composed of a continuous epithelium involving several different cellular types. Cell types vary according to the proximo-distal position.

Lung SCC essentially arise from the proximal epithelium as indicated. This proximal tracheobronchial region is made of a pseudostratified epithelium composed of ciliated, Clara, and basal cells.

**Figure 3. Simplified view of the bronchopulmonary epithelium** (adapted from Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons, Ltd).

**Figure 4. SOX2 is a novel transforming oncogene recurrently activated in lung SCC (from [3]).**

A. Overexpression of SOX2 in BEAS-2B bronchial epithelial cells leads to the formation of poorly differentiated SCC in immunocompromized mice. Hematoxylin/Eosin stainings of representative areas from subcutaneous tumors (magnification 100x). The tumors have typical histological traits of poorly differentiated SCC.

B. Immunohistochemical stainings of SOX2 in human primary lung SCC. SOX2 is strongly expressed in the nucleus of nearly all tumor cells (>80%) as shown for 5 tumors. Analysis of a series of 51 lung SCC revealed SOX2 high level and nuclear expression in 67% of tumors.
Figure 5. Simplified view of the canonical Wnt/β-catenin signaling pathway (adapted from Cell signaling technology).

A. In the absence of Wnt stimulation, β-catenin is constitutively recruited to a “destruction complex” that contains the Axin and Adenomatous Polyposis Coli proteins. This destruction complex facilitates the phosphorylation of β-catenin by GSK-3. Phosphorylated β-catenin is then recognized as a substrate by the E3 ubiquitin ligase that targets it for proteasome-mediated degradation. In the nucleus, LEF and TCF transcription factors of the Wnt/β-catenin pathway bind co-repressors (Groucho and HDAC) to repress the expression of Wnt target genes.

B. Wnt signalling is activated by the binding of Wnts to their receptor Fzd (seven-pass transmembrane receptors) and LRP co-receptors (low density lipoprotein receptor related protein LRP5 or LRP6, single-pass transmembrane molecules). This activation leads to the phosphorylation and activation of Dvl which will lead to GSK-3 phosphorylation and consequent inhibition. This allows the cytosolic accumulation of β-catenin and then its translocation to the nucleus (mediated by BCL9 and Pygopus), where it forms an active transcription complex with members of the TCF/LEF (LEF1, TCF1, TCF3 or TCF4) and transcription co-activators such as CBP/p300. Target genes of the Wnt/β-catenin pathway include c-myc, cyclinD1, TCF-1, PPARδ, matrix metalloproteinases (MMP2, MMP3, MMP7, and MMP9), CD44 and numerous other genes.
### Table 1: Dysregulation of Wnt pathway in lung cancer.

(↗; increased and ↘; decreased)

<table>
<thead>
<tr>
<th>Pathway component</th>
<th>Expression change</th>
<th>Pathology</th>
<th>Presence in lung SCC (In %)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt1</td>
<td>↗</td>
<td>Lung NSCLC</td>
<td>57.8% (in SCC and NSCLC)</td>
<td>[15]</td>
</tr>
<tr>
<td>Dvl1</td>
<td>↗</td>
<td>Lung SCC</td>
<td>75%</td>
<td>[16]</td>
</tr>
<tr>
<td>Axin2</td>
<td>↘</td>
<td>Lung SCC (NSCLC)</td>
<td>52%</td>
<td>[17]</td>
</tr>
<tr>
<td>TCF 4</td>
<td>↗</td>
<td>Lung SCC</td>
<td>75%</td>
<td>[18]</td>
</tr>
<tr>
<td>sFRP</td>
<td>↘</td>
<td>Lung Cancer</td>
<td>1 SCC cell line</td>
<td>[19]</td>
</tr>
<tr>
<td>WiF-1</td>
<td>↘</td>
<td>Lung NSCLC</td>
<td>83%</td>
<td>[20]</td>
</tr>
<tr>
<td>DKK-3</td>
<td>↘</td>
<td>Lung SCC (NSCLC)</td>
<td>63% (in SCC and NSCLC)</td>
<td>[21,22]</td>
</tr>
</tbody>
</table>

### Figure 6. Model of synergistic interaction between SOX2 and β-catenin.

The Wnt pathway activates nuclear translocation of β-catenin. In the nucleus, β-catenin interacts with SOX2 that binds DNA at TCF/LEF binding sites. This complex recruits co-activators such as CBP/p300 to activate target genes transcription. Target genes may include oncogenic factors, as for example *CyclinD1* [4]. Our project will permit to determine the mode of interaction between SOX2 and β-catenin and its consequences in lung SCC.
Figure 7. Lentiviruses production: tools and simplified protocol.

The three plasmids (A), essential for viral production, are transfected with the construct for insert transfer (B; from [25]), into HEK-293T cells by CaPO4 precipitate method (C, note the numerous thin CaPO4 precipitates). Transfected cells will produce viruses (D) which are then concentrated by ultracentrifugation and either stored frozen at -80°C or used immediately to infect target cells.
Figure 8. Lentiviruses as tools to efficiently transduce cells.

**A.** Example of GFP transduced cells *in vitro*. Fluorescent markers are co-expressed from the lentiviral constructs to allow for FACS sorting of transduced cells.

**B.** WB analysis of SOX2 expression level in stably transduced cells (from [3]). Cells transduced with control or SOX2/GFP viruses (constructs in panels C and D) were FACS sorted (GFP+ cells) and protein extracts made to assess SOX2 expression levels. Cells transduced with SOX2 viruses indeed overexpress the SOX2 protein.

**C and D.** Simplified linear maps of the pRRLs lentiviral constructs used. (C) Luciferase (Control plasmid) and (D) SOX2 and active β-catenin are overexpressed from PGK promoters together with a fluorescent marker (GFP or DsRed) allowing for the FACS-mediated selection of transduced cells. The position of the HMG-box of SOX2 and the mutated residues in the N-ter of β-catenin are represented on their respective map. The SOX2 and active β-catenin constructs have been engineered with different fluorescent markers (GFP and DsRed respectively) on purpose, allowing for transduction of cells with both viruses and sorting of co-infected cells (GFP+ and DsRed+).
Figure 9. Classical *in vitro* assays to characterize oncogenic properties of cell lines.

**A. Determination of proliferation rate.** 5000 cells are initially seeded into 96-well plates (1 plate/day, 3 replicates/condition/day) and followed for 6 days. Everyday for 6 days, the experiment is arrested for one plate: cell number are estimated using MTT labelling. Cells are finally lysed in DMSO to resolubilize formazan crystals and measure absorbance (OD at 576 nm). At the end of the experiment, OD values are plotted and control and test conditions compared: in the example, the TEST condition strongly inhibits cell proliferation compared to control.

**B. Growth in soft-agar** (from [3]). 5000 cells are plated/well of a 6-well plate in 0.3% agar containing medium over a 0.6% agar basis. After three weeks, macroscopic and microscopic images are acquired to count colony number (histogram upper part) and size (histogram lower part) of the colonies.

**C. Wound healing assay** (from [3]). Cells are plated so that they will form a monolayer in a 6 well plate 48h after (numbers vary from cell line to cell line). A wound is made using a 200µl tip and an image acquired (t=0, wound size =100%). After 24h, the same region is imaged to requantify wound size using ImageJ and calculate wound closure for control and test (SOX2 in the example) cells and compare them (plot).

**D. Transwell assays** for the determination of invasion capacities. Cells are placed in the upper chamber. After an appropriate incubation time, we determine the number of cells that have degraded the matrigel and migrated to the lower side of the microporous membrane.

**E. Resistance to apoptosis.** Apoptosis is initially induced in culture by an external stimulus (chemotherapeutic agents for example). Cell viability is assessed after a few days for control and test situations.
Figure 10. Wnt/β-Catenin signaling pathways activity using a reporter system.

A. Super 8x TopFlash reporter system. This construct (and the FOPflash control construct, non represented) have been established and validated previously [26], and were obtained from Addgene (a non-profit plasmid repository, http://www.addgene.org/).

B. Simulation of the relative luciferase activity in the different cell lines.

C. Representation of the different tools to block or activate the Wnt pathway. Positive and negative control conditions are included in the reporter assays by co-transfecting plasmids encoding stimulating (such as active β-Catenin) or inhibitory (DominantNegative LRP6 or Axin) molecules, respectively.

D. Determination of the impact of the blocking or activation of Wnt/β-catenin signaling on tumoral traits in vitro and SOX2 expression level

E. Simulation of a stimulatory effect of SOX2 on Wnt/β-catenin signaling.