# UNIVERSITAT POLITÈCNICA DE VALÈNCIA

## DEPARTAMENTO DE BIOTECNOLOGÍA


# *CHARACTERIZATION OF CANCER STEM CELLS FROM NON-SMALL CELL LUNG CANCER*

**TRABAJO FIN DE MÁSTER EN BIOTECNOLOGÍA BIOMÉDICA**

**ALUMNO:** ALEJANDRO HERREROS POMARES

**TUTORA**: ELOISA JANTUS LEWINTRE

**COTUTORA**: SILVIA CALABUIG FARIÑAS

***Curso Académico: 2014/2016***

**VALENCIA, 4 DE MARZO DE 2016**

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

**DEPARTAMENTO DE BIOTECNOLOGÍA**

# INFORME TUTOR TRABAJO FIN DE MÁSTER (para cualquiera de las modalidades)

Informe del tutor (per a qualsevol de les modalitats del treball fin de master)

***TUTOR's REPORT (for any type of final Master's degree thesis)***

**CURSO - Curs - *Academic year*** **2015-2016**

**DATOS DEL ALUMNO/A - Dades de l'alumne/a - Student's identification**

Máster (Màster –Master's degree): Biotecnología Biomédica

Apellidos (Cognoms - *Surname(s)*): Herreros Pomares
Nombre (Nom – *Name*): Alejandro
DNI (*ID card nr.*): 21010490J
Teléfono (Telèfon - *Phone nr.*): 633704095
Correo electr. (Correu electr. – *email*): alherpo@etsiamn.upv.es

**TÍTULO DEL TRABAJO FIN DE MÁSTER - Títol del Treball Fi de Màster - Title of the Final Master's degree thesis**

**CARACTERIZACIÓN DE CÉLULAS MADRE TUMORALES EN CÁNCER DE PULMÓN NO MICROCÍTICO**

Tutor/a (*Tutor*): Eloísa Jantus Lewintre
Cotutor/a (*Co-Tutor*): Silvia Calabuig Fariñas

**Considero que el TFM está finalizado y se puede aceptar para la defensa del mismo** **SI**
*Considere que el TFM està finalitzat i es pot aceptar la defensa d'aquest*
*I consider that the TFM is completed and can accept for the defense*

**Evaluación de la calidad del TFM y la labor del estudiante**.
*Avaluació de la qualitat del TFM i la labor del estudiant.*
*Evaluation of the quality of the Final Master's degree thesis and the work of the student*

En este trabajo se han caracterizado tumoresferas obtenidas a partir de líneas celulares y tumores primarios de pacientes con cáncer de pulmón, las cuales expresan mayores niveles de marcadores específicos de CSC. Además, genes pertenecientes a las rutas de señalización de Notch y Wnt están mayoritariamente expresados en los esferoides, sugiriendo estas vías de señalización como dianas potenciales contra las CSC en cáncer de pulmón. La novedad de este proyecto radica en trabajar con muestras de tumores primarios, lo que hace posible que algunos de los marcadores analizados puedan ser usados como biomarcadores en el contexto clínico.

| El tutor/a – *Tutor:* | El cotutor/a – *Co-Tutor:* |
|---|---|
| Eloísa Jantus Lewintre | Silvia Calabuig Fariñas |
| Fdo. – *Signed* | Fdo. – *Signed* |

**FECHA** (Data) ***Date***: 01 de Marzo de 2016

Universitat Politècnica de València
Departamento de Biotecnología
Edificis 3J bajo. Camí de Vera, s/n, 46022 València
Tel. +34 96 387 74 20 • Fax +34 96 387 74 29
depbtc@upv.es

DEPARTAMENTO DE BIOTECNOLOGÍA
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
AGRONÓMICA Y DEL MEDIO NATURAL

**AUTORIZACIÓN DEL COTUTOR PARA PRESENTACIÓN DEL TRABAJO FIN DE MÁSTER DE BIOTECNOLOGÍA BIOMÉDICA**

LA COTUTORA:

**D.ª** SILVIA CALABUIG FARIÑAS Adscrita al organismo FUNDACIÓN PARA LA INVESTIGACIÓN DEL HOSPITAL GENERAL UNIVERSITARIO DE VALENCIA (FIHGUV) **donde se ha realizado el Trabajo Fin de Máster** titulado:

CARACTERIZACIÓN DE CÉLULAS MADRE TUMORALES EN CÁNCER DE PULMÓN NO MICROCÍTICO

Del que es **AUTOR:**

**D.** ALEJANDRO HERREROS POMARES

**AUTORIZA** la presentación del TRABAJO FIN DE MÁSTER para su defensa.

Valencia, Marzo 2016

LA COTUTORA

Fdo.: D./D.ª SILVIA CALABUIG FARIÑAS

DEPARTAMENTO DE BIOTECNOLOGÍA
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
AGRONÓMICA Y DEL MEDIO NATURAL

**RESUMEN PARA PRESENTACIÓN DEL TRABAJO FIN DE MÁSTER DE BIOTECNOLOGÍA BIOMÉDICA**

**EL ALUMNO: D. Alejandro Herreros Pomares**

Autor Del **Trabajo Fin de Máster** titulado:

**CARACTERIZACIÓN DE CÉLULAS MADRE TUMORALES EN CÁNCER DE PULMÓN NO MICROCÍTICO**

Aporta el siguiente **RESUMEN**:

**Introducción**: A pesar de los avances en la caracterización molecular del cáncer de pulmón, la resistencia a la quimioterapia, la progresión tumoral y la metástasis hacen del mismo la primera causa de muerte debida a cáncer a nivel mundial. Las células madre tumorales (CSC) son pequeñas subpoblaciones de células con capacidad de autorenovación, diferenciación y tumorigenicidad que constituyen una diana terapéutica prometedora, pero cuya caracterización es aún un campo poco explorado. El objetivo de este trabajo es aislar y analizar la expresión génica de CSCs procedentes de líneas celulares de cáncer de pulmón y de tejido tumoral de pacientes con cáncer de pulmón no microcítico (CPNM) en estadios resecables.

**Material y Métodos**: El estudio se realizó en líneas celulares (H1650, H1993, A549 and PC9) y en muestras tumorales de pacientes resecados con CPNM crecidas en monocapa y en placas de baja adherencia con medio sin suero (tumoresferas). La expresión de marcadores de CSC (*CD133, EPCAM1*, *ALDH1A1*, *CD166*, *ABCG2*, *CD44, MUC1, BMI1*), genes de pluripotencia (*KLF4*, *OCT4*, *NANOG*, *SOX2*, *MYC*, *CCND1*), genes reguladores del ciclo celular (*CDKN1A*, *CDKN2A*, *MDM2*, *WEE1*), genes asociados a metástasis (*CDH1*, *VIM*, *SNAI1, MMP2*, *MMP9, CEACAM5*); y genes de las vías de señalización Notch (*NOTCH1*, *NOTCH2, NOTCH3*, *DLL1, DLL4, HEY1, HES1*); Wnt (*WNT1, WNT2, WNT3, WNT5A*, *CTNBB1, DKK1*, *FZD7*) y Hedgehog (*SMO*, *PTCH1*, *SHH, GLI1*) fue analizada mediante PCR cuantitativa a tiempo real (qPCR), normalizándose frente a la expresión de tres genes controles seleccionados: *ACTB*, *CDKN1B* y *GUSB* , utilizados para el cálculo de la expresión relativa.

**Resultados**: Las tumoresferas de pulmón presentan una expresión incrementada de *EPCAM1, CD44, ALDH1A1* y *CDKN1A* (p= 0.028, p= 0.021, p= 0.043 and p= 0.021, respectivamente)

comparadas con sus correspondientes células crecidas en adherencia. Además, el inductor de la transición epitelio-mesenquimal (EMT), *SNAI1*, está sobreexpresado (p= 0.011) en tumoresferas. Los genes de la vía Notch: *DLL4*, *NOTCH1* y *NOTCH2* también muestran mayor expresión en esferoides (p= 0.028, p= 0.038 and p= 0.036, respectivamente) que en células crecidas en monocapas. En cuanto a los genes de la ruta de Wnt, se observan mayores niveles de expresión de *WNT3*, *CTNBB1* and *GSK3B* (p= 0.021, p= 0.008 and p= 0.021, respectivamente) en esferoides, mientras que el activador de la vía no canónica de Wnt, *WNT5A*, tiende a estar menos expresado en las células cultivadas en suspensión frente a las células cultivadas en adherencia. No se encontraron diferencias significativas en el resto de genes analizados.

**Conclusiones**: Las tumoresferas de pulmón obtenidas a partir de líneas celulares y tumores primarios de pacientes muestran mayores niveles de marcadores de CSC. Además, genes pertenecientes a las rutas de señalización de Notch y Wnt están mayoritariamente expresados en tumoresferas, sugiriendo estas vías de señalización como dianas potenciales contra las CSC en cáncer de pulmón.

**Palabras Clave**: Células Madre Tumorales, Cáncer de Pulmón No Microcítico, Expresión génica, Ruta Notch, Ruta Wnt, Ruta Hedgehog.

**Valencia, 4 de Marzo de 2016**

Fdo.: D. Alejandro Herreros Pomares

DEPARTAMENTO DE BIOTECNOLOGÍA. E.T.S.I.A.M.N
Camino de Vera, s/nº.46022VALENCIA●Tel.+34963877420●Fax+34963877429

DEPARTAMENTO DE BIOTECNOLOGÍA
ESCUELA TÉCNICA SUPERIOR DE
INGENIERÍA AGRONÓMICA Y DEL MEDIO
NATURAL

**SUMMARY FOR MASTER'S DEGREE IN BIOMEDICAL BIOTECHNOLOGY FINAL PROJECT PRESENTATION**

**STUDENT: Mr., Alejandro Herreros Pomares**

Author of the Master's Degree Project entitled:

**CHARACTERIZATION OF CANCER STEM CELLS FROM NON-SMALL CELL LUNG CANCER**

Presents the following **SUMMARY**:

**Background**: Despite the advances in the molecular characterization of lung cancer, chemoresistance, tumor progression and metastasis make of lung cancer the first cause of death cancer-related worldwide. Cancer stem cells (CSCs) are small subpopulations of stem-like cells with self-renewal, differentiation and tumorigenic properties that constitute a promising target, but remain largely unknown. The aim of this study was to isolate and analyze gene expression of CSCs from lung cancer cell-lines and tumor-tissue from resectable NSCLC patients.

**Methods**: This study was performed on cells from NSCLC tumor samples and cell lines (H1650, H1993, A549 and PC9) grown in monolayer and as spheroids. The expression of: CSC-markers (*CD133, EPCAM1*, *ALDH1A1*, *CD166*, *ABCG2*, *CD44, MUC1, BMI1*); pluripotency (*KLF4*, *OCT4*, *NANOG*, *SOX2*, *MYC*, *CCND1*); cell cycle (*CDKN1A*, *CDKN2A*, *MDM2*, *WEE1*); invasiveness (*CDH1*, *VIM*, *SNAI1, MMP2*, *MMP9, CEACAM5*); Notch pathway (*NOTCH1*, *NOTCH2, NOTCH3*, *DLL1, DLL4, HEY1, HES1*); Wnt pathway (*WNT1, WNT2, WNT3, WNT5A*, *CTNBB1, DKK1*, *FZD7*) and Hedgehog pathway (*SMO*, *PTCH1*, *SHH, GLI1*) were analyzed by quantitative real-time PCR (qPCR). Housekeeping genes *ACTB*, *CDKN1B* and *GUSB* were used as endogenous controls for relative expression calculation.

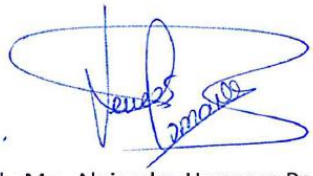
**Results**: Lung tumorspheres had increased expression of *EPCAM1, CD44, ALDH1A1* and *CDKN1A* (p= 0.028, p= 0.021, p= 0.043 and p= 0.021, respectively) when compared to their paired-adherent cells. In addition, epithelial to mesenquimal transition (EMT) inducer *SNAI1* was overexpressed (p= 0.011) in tumorspheres. Regarding Notch pathway, *DLL4*, *NOTCH1* and *NOTCH2* showed higher expression in spheroids (p= 0.028, p= 0.038 and p= 0.036, respectively). In Wnt pathway, we found higher expression levels of *WNT3*, *CTNBB1* and *GSK3B*

(p= 0.021, p= 0.008 and p= 0.021, respectively) in lungspheres, whereas the activator of the non-canonical Wnt pathway, *WNT5A*, tended to be less expressed in spheroids compared to adherent-cultured cells. No significant differences were found in other analyzed genes.

**Conclusions**: Lung spheroids from cancer cell lines and primary tumors showed increased levels of CSC-markers. Genes related to Notch and Wnt were found to be more expressed in tumorspheres, suggesting these pathways as interesting lung-CSC targets.

**Key Words**: Cancer Stem Cells, Non-Small Cell Lung Cancer, Relative Gene Expression, Notch Pathway, Wnt Pathway, Hedgehog Pathway.

**Valencia, March 4th, 2016**



Sgd.: Mr., Alejandro Herreros Pomares

DEPARTAMENTO DE BIOTECNOLOGÍA. E.T.S.I.A.M.N
Camino de Vera, s/nº.46022 VALENCIA●Tel.+34963877420●Fax+34963877429

# INDEX

## ABBREVIATIONS

ABCG2: ATP-binding cassette, sub-family G

ACTB: Actin, Beta

ADC: Adenocarcinoma

ALDH1A1: Aldehyde dehydrogenase 1 family, member A1

BMI1: BMI1 proto-oncogene, polycomb ring finger

CCND1: Cyclin D1

CD45: Protein tyrosine phosphatase, receptor type, C

CDH1: Cadherin 1, type 1

CDKN1A: Cyclin-dependent kinase inhibitor 1A

CDKN1B: Cyclin-dependent kinase inhibitor

CDKN2A: Cyclin-dependent kinase inhibitor 2A

CEACAM5: Carcinoembryonic antigen-related cell adhesion molecule 5

CSC: Cancer Stem Cell

CTNBB1: Catenin beta 1

DKK1: Dickkopf WNT signaling pathway inhibitor 1

DLL: Delta-like

EGFR: Epidermal growth factor

EMT: Epithelial-mesenchymal transition

EPCAM1: Epithelial cell adhesion molecule

FZD7: Frizzled class receptor 7

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GLI1: GLI family zinc finger 1

GSK3B: Glycogen synthase kinase 3 beta

GUSB: Glucuronidase, beta

HES1: Hes family bHLH transcription factor 1

HEY1: Hairy ears, Y-linked

HPRT1: Hypoxanthine phosphoribosyltransferase 1

KLF4: Kruppel-like factor 4 (gut)

LGALS-2: Lectin, galactoside-binding, soluble, 2

LIN28B: Lin-28 homolog A

MDM2: MDM2 proto-oncogene, E3 ubiquitin protein ligase

MMP: Matrix metallopeptidase

MUC1: Mucin 1, cell surface associated

MYC: V-myc avian myelocytomatosis viral oncogene homolog

NANOG: Nanog homeobox

NSCLC: Non-small cell lung carcinoma

OCT4: POU class 5 homeobox 1

PTCH1: Patched 1

RT: Reverse Transcription

RTqPCR: Quantitative real time PCR

SCC: Squamous cell carcinoma

SCLC: Small cell lung carcinoma

SHH: Sonic hedgehog

SMO: Smoothened, frizzled class receptor

SNAI1: Snail family zinc finger 1

SOX2: SRY (sex determining region Y)-box 2

SP: Side Population

TGFB: Transforming growth factor, beta 1

TNM: Tumor/Nodules/Metastasis

VIM: Vimentin

WEE1: WEE1 G2 checkpoint kinase

WNT: Wingless-type MMTV integration site family

WT: Wild type

# 1. INTRODUCTION

## 1.1.CONCEPT OF CANCER

According to the World Health Organization (WHO) and the National Cancer Institute (NIH), cancer is a generic term that defines a large group of diseases that can affect almost any part of the body. Indeed, there are more than 100 types of cancer, and subtypes of tumors can be found in each specific organ as well. In cancer, abnormal cells have defects in their regulatory mechanisms that control normal cells, making them able to grow uncontrolledly and spread into surrounding tissues. In addition to this reductionist view of cancer, there are two other dimensions of complexity. Firstly, tumors are complex tissues in which cancer cells can recruited normal cells types to serve as active collaborators, creating 'tumor microenvironments' in which proliferation and invasion are favored (Hanahan & Weinberg, 2000). Secondly, the genetic diversity in populations of tumors cells is an unavoidable consequence of the genome instability, which is caused by defects affecting components of the DNA-maintenance machinery in combination with the large numbers of cell divisions required for the formation of macroscopic tumors (Marusyk *et al*. 2012).

### 1.1.1 MOLECULAR BIOLOGY OF CANCER

In the past decade, Hanahan and Weinberg suggested that all cancers have in common six characteristics, which are acquired during multistep tumorigenesis: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2000). However, the manner of acquisition of these features as well as the order in which they are acquired varies significantly across cancer types and subtypes. Several years later, in 2011, Hanahan and Weinberg proposed two enabling characteristics that make possible the acquisition of the six acquired capabilities of cancer described before: the genome instability and the tumor-promoting inflammation. Moreover, two emerging hallmarks of cancer consequence of new research facts were defined: the deregulation of cellular energetics and the breakout of the immune destruction, constituting the ten hallmarks of cancer (Figure 1, Hanahan & Weinberg, 2011).

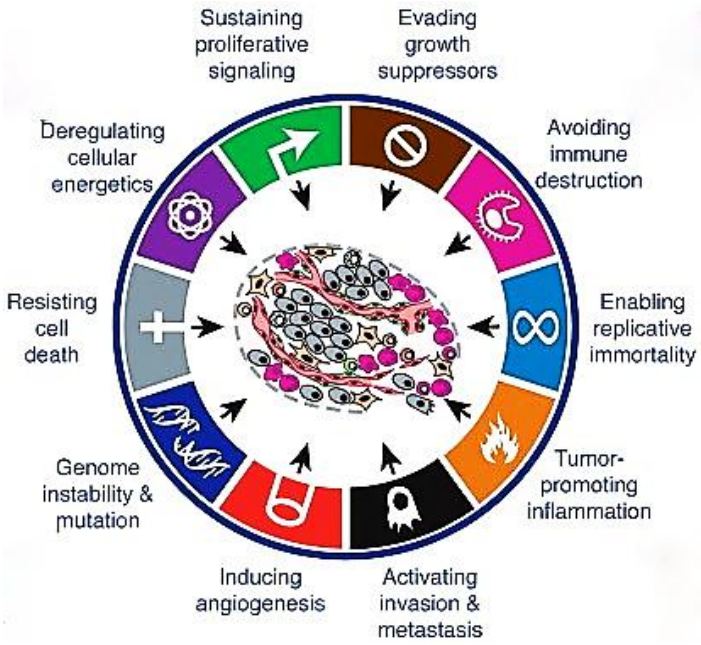



**Figure 1. The ten Hallmarks of Cancer. Adapted from (Hanahan & Weinberg 2011).**

## 1.2 LUNG CANCER

### 1.2.1 EPIDEMIOLOGY

Cancer is the second leading cause of death worldwide with 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 and it is expected to exceed heart diseases as the principal cause of death in the next few years (Torre *et al.* 2015; Siegel *et al.* 2016). Currently, lung cancer is the most frequently diagnosed and is the leading cause of cancer-related death around the world, with more than 1.8 million estimated new cases and more than 1.5 million estimated deaths in 2012 (Figure 2, Torre *et al.* 2015). Furthermore, although cancer mortality has moderately declined across Europe in the last decade, female lung cancer is an exception in most European countries, including Spain (Bosetti *et al.* 2013). This persistent increase in lung cancer mortality reflects the women's pattern of tobacco consumption in countries of western Europe (Bosetti *et al.* 2013; Ferlay *et al.* 2015) .

In addition, despite the advances in biomedical research and the improvements in diagnosis and therapies of the past decades, five-year survival for lung cancer after diagnosis remains 9.5% (de Groot & Munden 2012; Bosetti *et al.* 2013; Malvezzi *et al.* 2014; Lortet-Tieulent *et al.* 2014). The main reason why five-year survival has such a low percentage is that lung cancer is mainly diagnosed in advanced states, when patients have developed symptomatic manifestations and curative surgery is no longer possible.

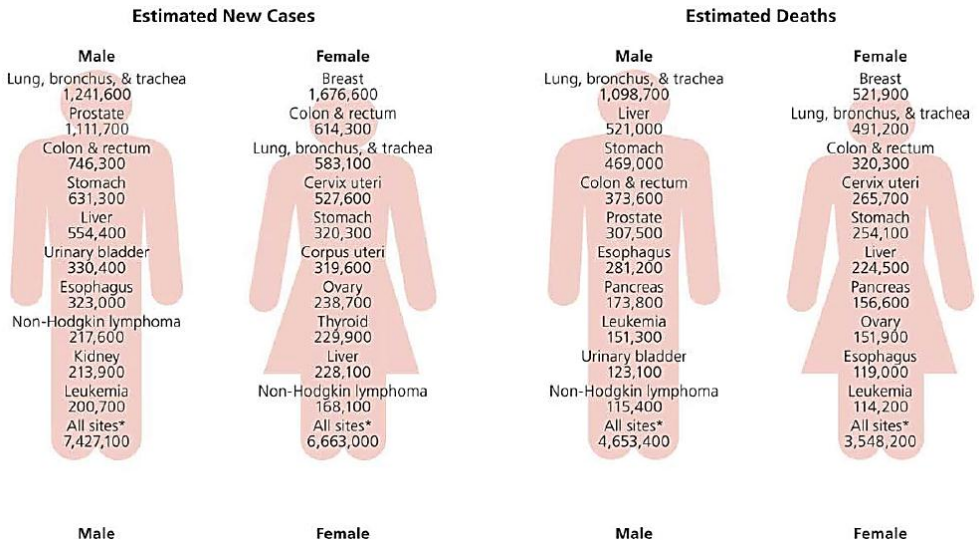



**Figure 2. Estimated new cancer cases and deaths worldwide by sex in 2012. Adapted from (Torre *et al.* 2015).**

### 1.2.2 RISK FACTORS

Tobacco smoking is the principal cause of lung cancer due to toxic compounds contained in its smoke and is responsible for 85-90% of these tumors (Freedman *et al*. 2008; de Groot & Munden 2012). About 4000 chemical substances, 60 of which are carcinogenic, are present in cigarette smoke, triggering the accumulation of a large number of mutations. It has been observed that in lung cancer, an average of 200 mutations are accumulated per tumor, while in other frequent tumors, such as breast or prostate cancer, this number goes from 25 to 50 mutations on average (Fry *et al*. 2013).

Other factors associated with lung cancer development are environmental or occupational exposures to carcinogenic pollutants such as radon gas, arsenic and polycyclic hydrocarbons, diet type, alcohol consumption, infectious agents and other diseases such as diffuse cystic fibrosis (Sawyers *et al*., 2013).

### 1.2.3 CLASSIFICATION

Based on clinical expression, lung cancer is classified into two major groups: small cell lung carcinoma (SCLC: 15% of cases) and non-small cell lung carcinoma (NSCLC: 85% of cases). Nevertheless, histological and pathological techniques allow dividing the latter into three subtypes: squamous cell carcinoma (SCC, 30% of cases), originated in the squamous epithelium of the lung or bronchi and highly related to tobacco smoking, adenocarcinoma (ADC, 40% of cases), originated in broncho-alveolar cells and classified into different patterns (lepidic, acinar, papillary, micropapillart and solid), and large carcinoma (LCC, 10% of cases), with a neuroendocrine origin (Travis 2002; Travis *et al*. 2013; Gridelli *et al*. 2015).

In the last decades, due to the development of a new generation of sequencing techniques, substantial advances in the knowledge of cancer genomes has been made. These advances have allowed to develop new treatment strategies based on molecular targets and to renew the classical histological classification for a molecular one. Indeed, NSCLC is one of the most genomically diverse tumors and there are a great variety of molecularly defined subsets of patients characterized by specific driver mutations, such as EGFR, ALK or KRAS (Ladanyi 2008; Hirsch *et al.* 2010; Pao & Girard 2011).

EGFR (Epidermal Growth Factor Receptor) encodes a transmembrane tyrosine kinase with an extracellular binding domain and an intracellular component with a tyrosine kinase domain. Binding to its ligand leads to receptor homo- or heterodimerization with other members of the EGFR family and activation of the tyrosine kinase domain (Scagliotti *et al.* 2004). EGFR alterations are implicated in many cancer types, including lung cancer, where overexpression or aberrant activation is present in approximately 60% of cases (Hirsch *et al.* 2003; Li *et al.* 2008).

KRAS (Kirsten Rat Sarcoma viral oncogene homolog) is a proto-oncogene from RAS family (KRAS, NRAS and HRAS in humans), that encodes a G-protein with a key role in controlling signal transduction pathways involved in cell proliferation, differentiation and survival. It plays a crucial role in downstream signal transduction induced by several growth factor receptors, included EGFR. KRAS-activating mutations in codons 12 and 13 are the most frequent oncogenic alteration identified in lung ADCs, occurring in about 25-40% of cases (Downward 2003; Karnoub & Weinberg 2008).

ALK (Anaplastic lymphoma kinase) can also be oncogenic by forming a fusion gene with any of several other genes. In NSCLC, EML4 (Echinoderm microtubule-associated protein-like 4)-ALK fusion gene is responsible for approximately 3-5% of cases. This rearrangement occurs in chromosome 2p, among intron 13 of EML4 and intron 19 of ALK, which results in the fusion of the intracellular kinase domains, causing a constitutive oligomerization that produces a continuous mitogenic signal and, as a last resort, a malignant transformation (Soda *et al.* 2007). More recently, different partner genes have been identified in a small subset of ALK rearrangements with a low frequency in NSCLC (less than 1% of cases), including KIF5B (kinesin family member 5b), TFG (TRK-fused gene) and KLC-1 (kinesin light chain 1; Peters *et al.* 2013).

In addition to the driver mutations mentioned before, there are many somatic mutations in all types of lung cancers, such as chromosomal rearrangements or copy-number alterations, compared with other tumor types, being lung cancer among the most mutated forms of

cancers (Gridelli *et al.* 2015). This diversity and complexity of the somatic mutational processes underlying carcinogenesis are currently being revealed and will be further detailed later due to their implications in cancer therapy.

### 1.2.4 DIAGNOSIS AND PROGNOSIS

Routine screening for lung cancer presents several limitations and risks, including a high-rate of false-positive results, cumulative radiation exposure from multiple CT scans, and unnecessary lung biopsy and surgery. These potential harms together with economic difficulties trigger that the development of risk models or biomarkers for predicting became a necessity (Torre *et al.* 2015). Nowadays, lung cancer is commonly detected because symptomatic manifestations such as pain, hemoptysis, dyspnea or weight loss, are developed by patients. 70-75% of patients with NSCLC present advanced disease at the time of diagnosis, with no curative surgery possible and a 40% of patients with distant metastases (Morgensztern *et al.* 2010).

Diverse technologies are now available for locating the primary tumor and further staging, including chest radiograph, low-dose chest computed tomography (CT) or fluorodeoxyglucosa (18F-FDG)-positron emission tomography (PET) as well as for biopsying the tumor (bronchoscopic techniques or endoscopic ultrasound, among others). The variables that have been associated with prognosis can be grouped into three different categories: a) tumor-related, such as primary site, cell type and disease extension; b) patient-related, such as performance status (PS), comorbidity, and sex; and c) environmental factors, such as nutrition. The anatomical extent of disease, as described by the TNM (Tumor, Nodes, Metastasis) classification, shown in Table 1, is one of the most important prognostic factors in lung cancer (Goldstraw *et al.* 2011; Jantus-Lewintre *et al.* 2012).

### 1.2.5 TREATMENT

Lung cancer treatment essentially depends on anatomopathological classification, tumor stage and PS. Although surgery is still the standard treatment for early-stage patients with a good PS (25-30% of diagnosed NSCLC patients), stereotactic body radiotherapy (SBRT) has emerged as an alternative treatment for stage I-II patients with a borderline medical indication for surgery (Robinson *et al.* 2013). In addition, adjuvant chemotherapy with platinum salts in combination with vinorelbine, etoposide or docetaxel in patients who underwent surgery increases survival rates significantly (Felip *et al.* 2010). On the other hand, advanced NSCLC is considered incurable and the therapy aims to extend survival of patients and alleviate symptoms as well as possible. In these cases, standard chemotherapy is the combination of platinum compound with a third-generation cytotoxic agent, such as gemcitabine, vinorelbine

or paclitaxel. In addition, although radiotherapy can be indicated in patients with a good PS and non-resected disease, palliative intention is still its main use (Goldstraw *et al.* 2011).

**Table 1. Seventh edition of the Tumor, Node, Metastasis classification for lung cancer (Mirsadraee 2012).**

| | |
|---|---|
| T: Tumour | |
| TX | Primary tumour cannot be assessed, or tumour proven by the presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy |
| T0 | No evidence of primary tumour |
| Tis | Carcinoma *in situ* |
| T1 | Tumour < 3 cm in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus (i.e., not in the main bronchus) |
| T1a | Tumour < 2 cm in greatest dimension |
| T1b | Tumour > 2 cm but < 3 cm in greatest dimension |
| T2 | Tumour > 3 cm but < 7 cm or tumour with any of the following features (T2 tumours with these features are classified T2a if < 5 cm):<br>Involves main bronchus, > 2 cm distal to the carina<br>Invades visceral pleura<br>Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung |
| T2a | Tumour > 3 cm but < 5 cm in greatest dimension |
| T2b | Tumour > 5 cm but < 7 cm in greatest dimension |
| T3 | Tumour > 7 cm or one that directly invades any of the following:<br>Chest wall (including superior sulcus tumours), diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium<br>Tumour in the main bronchus < 2 cm distal to the carina but without involvement of the carina<br>Associated atelectasis or obstructive pneumonitis of the entire lung<br>Separate tumour nodule(s) in the same lobe |
| T4 | Tumour of any size that invades any of the following:<br>Mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina<br>Separate tumour nodule(s) in a different ipsilateral lobe |
| N: Nodes | |
| NX | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension |
| N2 | Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s) |
| N3 | Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s) |
| M: Metastases | |
| MX | Distant metastasis cannot be assessed |
| M0 | No distant metastasis |
| M1 | Distant metastasis |
| M1a | Separate tumour nodule(s) in a contralateral lobe<br>tumour with pleural nodules or malignant pleural/ pericardial effusion |
| M1b | Distant metastasis |

Over the last years, specific anti-target therapies have appeared, increasing NSCLC patients' survival and decreasing conventional chemotherapies toxicity. Some examples of immunotherapeutic drugs are: tyrosine-kinase inhibitors (TKIs) such as gefitinib or erlotinib, which are specific treatments for patients bearing mutations in the EGFR gene; crizotinib, a small-molecule TKI that inhibits mesenchymal epithelial transition factor proto-oncogene, receptor tyrosine kinase (cMET), reactive oxygen species proto-oncogene 1, receptor tyrosine kinase (ROS1) and ALK; or bevacizumab, a recombinant, humanized, monoclonal vascular endothelial growth factor (VEGF) antibody (Soria *et al.* 2013). However, there is an important group of patients where targeted mutations are not detected and whose treatment is based on conventional chemotherapy.

Currently, even having a targeted therapy available, treatment resistance to chemotherapy and immunotherapy is the main cause of death in lung cancer. In that sense, it is known that cancer cells are heterogeneous and there is strong evidence pointing out that

chemoresistance, tumor progression and metastasis are linked to a subpopulation of stem-like cells present in tumors, named cancer stem cells (CSCs, Han *et al.* 2013).

## 1.3 HETEROGENEITY IN CANCER

Cancers exist in an extraordinary variety of types and subtypes, making each cancer individually unique. Genetic and phenotypic variations are observed among tumors of different tissue and cell types, as well as between individuals with the same tumor type (inter-tumor heterogeneity). In addition, cancers evolve over time in every particular patient in terms of clonal structure, genotype and phenotype, complicating diagnosis, prognosis and treatment (Greaves & Maley, 2012; Burrell *et al.* 2013). In this sense, traditional diagnostic classification of tumors by pathologists relies on phenotypic traits such as histological subtypes, treatment sensitivity profiles and clinical outcomes among different patients (Marusyk *et al.* 2012; Zardavas *et al.* 2015). However, genetic and phenotypic diversity exists not only between tumors, but also within populations of cells in single tumors (intra-tumor heterogeneity). Similar to inter-tumor heterogeneity, intra-tumor heterogeneity of cellular phenotypes, resulted from genetic and non-genetic influences, can make difficult definitive diagnostics and obstruct therapeutic decision-making. Firstly, spatial phenotypic heterogeneity could trigger that a biopsy did not supply an adequate reflection of the whole tumor. Secondly, decision-making based on scoring the dominant phenotype in a given specimen might be biased if they do not account for minor subpopulations with clinically and biologically important distinguishing features (Figure 3, Marusyk *et al.* 2012; Burrell *et al.* 2013).

Currently, the major frameworks to explain cancer cell heterogeneity are two (Shackleton *et al.* 2009; Marusyk *et al.* 2012):

The clonal evolution model, also known as the stochastic model, was proposed by Nowell in 1976 and holds that neoplasms arise from a single cell of origin, and tumor progression results from acquired genetic variability within the original clone (Nowell, 1976). As a result, the genetic and epigenetic changes that occur over time in individual cancer cells can confer a selective advantage in a Darwinian-like way, allowing individual clones to generate other clones and leading to genetic heterogeneity and phenotypic and functional differences among the cancer cells within a single patient (Greaves & Maley 2012; Wang *et al.* 2014; Landau *et al.* 2014). In this model, the frequency of cancer cells with tumorigenic potential is high, the tumor organization is not necessarily hierarchical and the rational approach to therapy has been to target most or all cells.

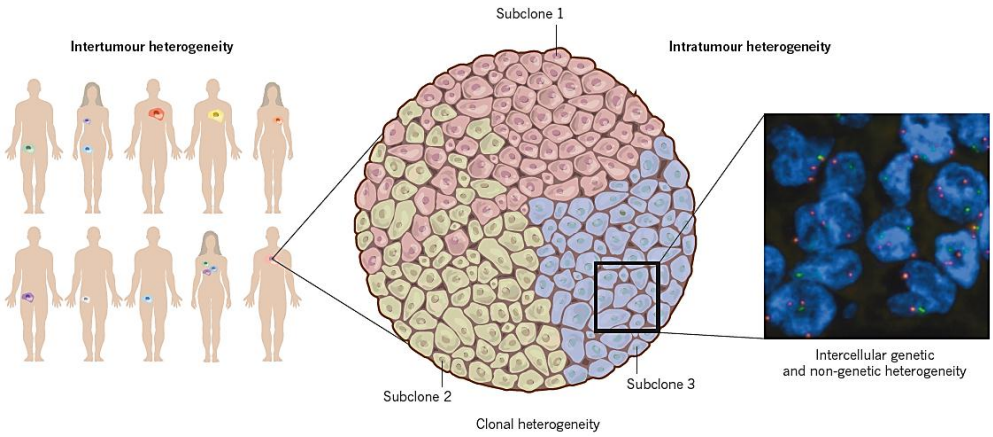



**Figure 3. Inter-tumor and intra-tumor heterogeneity (Burrel *et al.* 2013).**

The cancer stem cell model, also known as the deterministic model, proposes that the growth and progression of many cancers are driven by small subpopulations of stem-like cells with self-renewal and differentiation properties, named cancer stem cells (CSCs). It was developed in the late 1990s, when researchers began to address the possible relationship between hematopoietic stem cells and human leukaemias via transplantation experiments of hematological tumor cells into immunodeficient mice (Dick 2008; Greaves & Maley 2012; Sourisseau *et al.* 2014). The CSC concept was coined in 1997, when Bonnet and Dick demonstrated that human acute myeloid leukaemia is organized as a hierarchy that originates from a primitive hematopoietic cell (Bonnet & Dick, 1997). Since then, CSCs have been identified in several solid tumors, including brain (Singh *et al.* 2003), breast (Smalley & Ashworth, 2003), lung (Kim *et al.* 2005), colon (Ricci-Vitiani *et al.* 2007) and pancreas (Li *et al.* 2007). In this model, the frequency of cancer cells with tumorigenic potential varies from rare to moderate, the tumor organization is always hierarchical and the therapy approach enables to target only tumorigenic cells.

Even at first, these two models were considered mutually exclusive (Cheng *et al.* 2009), nowadays clonal evolution and CSC models are proposed as a unified model by some authors (Figure 4; Meacham & Morrison, 2013; Kreso & Dick, 2014). In the integrated model, the acquisition of favorable mutations can result in clonal expansion of the founder cell. In parallel, another cell may gain a different mutation that allows it to form a new subclone. Over time, genetic mutations accumulate and subclones evolve in parallel. CSCs are considered as not static entities, they can evolve over their lifetime and genetic changes can influence the CSC frequency. Some subclones may contain a steep developmental hierarchy, where only few self-renewing CSCs exist among a large number of non-CSCs. Other subclones may contain an intermediate hierarchy, where the number of CSCs is relatively high but a hierarchy still exists.

Some subclones may have the genetic alterations that confer high-renewal potential, where most cells are tumorigenic (Kreso & Dick, 2014).

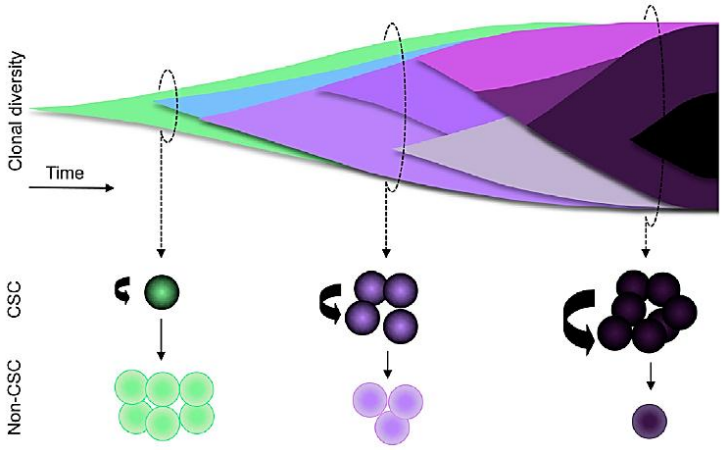



**Figure 4. Unified model of clonal evolution and Cancer Stem Cells (CSCs).**

## 1.4 CANCER STEM CELLS

### 1.4.1 PROPERTIES

CSCs and normal stem cells share many similarities in terms of self-renewal, production of differentiated progeny, expression of specific surface markers and oncogenes, utilization of common signaling pathways or the importance of the stem cell niche. However, CSCs differ significantly from normal stem cells in their tumorigenic activity, given that CSCs can form tumor when transplanted into animals (tumor initiating cells, TIC), but normal stem cells cannot. In general, CSCs are defined through four key features:

A) Self-renewal: The CSCs can be serially transplanted through multiple generations, indicating the self-renewal capacity.

B) Asymmetric division/ Differentiation: Pluripotent CSCs can not only form tumorigenic daughter CSCs by symmetrical cell division but also generate bulk populations of non-tumorigenic cells by asymmetrical cell division.

C) Tumorigenicity: A small subpopulation of CSCs has tumorigenic potential when transplanted into animals.

D) Specific surface markers and signaling pathways, by which the CSCs subpopulation can be identified and isolated from the non-stem tumor cells and can be functionally differentiated from tumor cells (McCaffrey & Macara 2011; Peitzsch *et al.* 2013; Zhang *et al.* 2015).

In addition, CSCs possess most of the normal stem cell properties, such as the sphere forming ability in non-adherent medium, dye exclusion ability because of the over-expression of efflux transporters or exclusive intracellular enzyme activity (Dou & Gu 2010; Alison *et al.* 2011; Ghani *et al.* 2011; Zhang *et al.* 2011; Han *et al.* 2013).

As mentioned before, one important characteristic of CSCs is to form spheres or grow into colonies in serum-free medium or in soft agar medium. To show aggregation and proliferation of stem cells *in vitro*, cells are harvested from tumor specimens and suspended at a low density in serum-free medium supplemented with minimum growing requirements. It has been found that spheroids derived from many solid tumors, including lung cancer, had increased *in vitro* drug-resistance properties and *in vivo* tumorigenic potentials. Even though, there are several limitations in the selection of cells with CSC's features by the spheres-forming assays (Kitamura *et al.* 2009). Firstly, cells are selected under a pressure exerted by the culture conditions, leading to an enrichment of cell populations that are able to survive and proliferate under such specific conditions. Secondly, *in vitro* assays determine *ex vivo* proliferation instead of their true self-renewal capacity. The third point to be addressed is the ability of CSC to initiate tumors, which cannot be performed in vitro (Han *et al.* 2013).

To overcome these drawbacks, the results of *in vitro* assays must be confirmed by *in vivo* assay. For this purpose, selected populations of tumor cells are transplanted into immunocompromised (frequently NOD/SCID) mice to confirm the TIC capacity of these cells. It has been reported that a large number of cancer cells, in the order of millions of cells, are required to initiate tumor growth when xenotransplanted into animal models (Wicha *et al.* 2006; Koch *et al.* 2010; Alison *et al.* 2011). However, only miles of cancer cells sorted based on specific CSC-markers are able to give rise to a whole tumor (Han *et al.* 2013).

### 1.4.2 SPECIFIC MARKERS

The main markers used for identification and isolation of CSCs include surface cell-adhesion molecules, as CD133 or CD44, cytoprotective enzymes, such as aldehyde dehydrogenase (ALDH), and transcription factors and drug-efflux pumps, for instance, ATP binding cassette (ABC) drug transporters or multidrug resistance transporter1, MDR1 (Mannelli & Gallo 2012; Qiu *et al.* 2012; Dou *et al.* 2007; Takaishi *et al.* 2009; Chen *et al.* 2009). The most widely used method for identifying CSCs is based on specific cell surface markers, being the most widely used CD133, CD24 or CD44. However, the surface markers of CSCs in one organ/tissue are frequently not completely shared with those markers in other organs/tissues. The most important markers used to identify CSCs in solid tumors are listed in Table 2.

It has been proposed a set of markers for the characterization of lung CSCs, which includes: surface cell molecules CD133, CD90, EpCAM, CD44, ALCAM and BMI1, the cytoprotective enzyme ALDH1A1 and the expression of the drug-efflux pump ABCG2 protein (Eramo *et al.* 2008; Zhang *et al.* 2012; Wang *et al.* 2013; Yuan *et al.* 2014a; Zakaria *et al.* 2015). The search of lung-CSC markers is still under development and there are controversial data regarding the markers listed in Table 2.

**Table 2. Examples of proposed CSCs markers in solid tumors (Medema 2013).**

| Breast | Colon | Glioma | Liver | Lung | Melanoma | Ovarian | Pancreatic | Prostate |
|---|---|---|---|---|---|---|---|---|
| ALDH1 | ABCB5 | CD15 | CD13 | ABCG2 | ABCB5 | CD24 | ABCG2 | ALDH1 |
| CD24 | ALDH1 | CD90 | CD24 | ALDH1 | ALDH1 | CD44 | ALDH1 | CD44 |
| CD44 | β-catenin activity | CD133 | CD44 | CD90 | CD20 | CD117 | CD24 | CD133 |
| CD90 | CD24 | $\alpha_6$-integrin | CD90 | CD117 | CD133 | CD133 | CD44 | CD166 |
| CD133 | CD26 | nestin | CD133 | CD133 | CD271 | | CD133 | $\alpha_2\beta_1$-integrin |
| Hedgehog-Gli activity | CD29 | | | | | | c-Met | $\alpha_6$-integrin |
| $\alpha_6$-integrin | CD44 | | | | | | CXCR4 | Trop2 |
| | CD133 | | | | | | Nestin | |
| | CD166 | | | | | | Nodal-Activin | |
| | LGR5 | | | | | | | |

CD133 (prominin-1, PROM1) is a pentaspan transmembrane glycoprotein overexpressed in both humans and mice tumors (Mizrak *et al*. 2008). Some evidence has suggested that $CD133^+$ CSCs display strong resistance to chemotherapy and radiotherapy. Several studies showed that $CD133^+$ stem-like cells survived standard chemotherapeutic treatment with oxaliplatin and 5-fluorouracil (5-FU). In addition, downregulation of CD133 using short hairpin RNAs has been associated with slower cell growth, reduced cell motility and decreased ability to form spheroids and metastasize (Liu *et al*. 2006; Todaro *et al*. 2007; Baumann *et al*. 2009; Mizrak *et al*. 2008). Furthermore, monoclonal antibodies directed against CD133 have been used, finding inhibition of the cells growth and cytotoxic effects *in vitro* (Rappa *et al*. 2008; Smith *et al*. 2008). This way, it is believed that CD133 is not only a potential CSC marker but might also be an important therapeutic target for many CD133-expressing cancer types (Han *et al*. 2013).

EpCAM (epithelial cell adhesion molecule; CD326) is a transmembrane glycoprotein that plays a role in balancing cell proliferation and differentiation. In healthy tissue, high EpCAM levels are associated with proliferation during morphogenesis, tissue regeneration and stem cell maintenance (Schnell *et al.* 2013). High EpCAM expression has been found to promote tumor progression and because of its tumor-specific overexpression, it has been explored as a prognostic/diagnostic marker and as an anti-cancer target. In CSCs, EpCAM overexpression has

also been found, corresponding to a poor prognosis and more aggressive cancers (Trzpis *et al*. 2007; Patriarca *et al*. 2012).

ALCAM (activated leukocyte cell adhesion molecule (CD166) is a highly preserved transmembrane protein that belongs to the immunoglobulin superfamily. ALCAM is expressed in several tissues, including neural, epithelial and hematopoietic stem cells. Recently, CD166 has been identified as a CSCs marker for NSCLC, although few data exist regarding the clinical relevance of CD166 expression for now (Zhang *et al*. 2015). Moreover, immunohistochemistry evaluation on a tissue microarray basis revealed no significant survival benefit of CD166+ NSCLC patients, so that further studies are required to investigate the functional role of CD166 in NSCLC (Tachezy *et al*. 2014).

ALDH are a group of NAD(P)+-dependent enzymes that catalyze the oxidization of aldehydes into carboxylic acids. ALDH1 is an isoenzyme of ALDH superfamily that not only acts as a marker for both normal and CSCs, but may also play important functional roles in self-protection, differentiation and expansion. It is thought that ALDH can act as drug-detoxifying enzymes and be responsible for therapeutic resistance (Sun & Wang 2010; Ma & Allan 2010; Marcato *et al*. 2011). In the Aldefluor assay, ALDH-activated fluorescent substrate is used as a marker for measuring and isolating normal and CSCs with high ALDH activity. In addition, a subpopulation of stem-like $ALDH^{hi}CD44^{+}$ cells has been identified in human breast cancer (Croker & Allan 2011). It is demonstrated that $ALDH^{hi}CD44^{+}$ cells are more resistant to standard cancer therapy, and that inhibiting ALDH activity of cell populations through specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) or all-trans retinoic acid (ATRA) sensitized these cells to treatment (Croker & Allan 2011).

ABC drug transporters are overexpressed in both normal and CSCs as efflux pumps to protect stem cells from xenobiotic toxins. ABCG2 (also known as BCRP), an important member of ABC transporter family, is regarded as a potential marker of CSCs as well as a mechanism in multidrug resistance. It is also a determinant of the side-population (SP) phenotype. SP cells show many features of CSCs with regard to self-renewal, lineage capacity and tumorigenicity (Han *et al*. 2013). Xia *et al*. 2010 identified 12 potent high drug efflux cancer cell inhibitors from 1280 pharmacologically active compounds. Using *in vitro* and *in vivo* assays, they showed that these inhibitors were able to overcome MDR by inhibiting SP, increase the efficacy of chemotherapy and reduce the tumorigenicity of lung cancer cells, possibly by affecting CSCs (Xia *et al*. 2010).

Despite these findings, it is necessary to clarify the marker profile of lung CSCs since well-defined CSC markers are not available in NSCLC. Further studies are needed to elucidate prognostic and diagnostic markers and discover anti-cancer therapy targets. In that sense, some signaling pathways showed increased activity in CSCs, making them interesting cascades to be analyzed.

### 1.4.3 SIGNALING PATHWAYS

The major difference between normal stem cells and cancer stem cells lies in their ability to regulate self-renewal and differentiation pathways. In normal stem cells, stemness pathways, including Notch, Wnt/β-catenin, Hedgehog, JAK/STAT, TGF-β and Hippo, are tightly controlled with intact genetics or epigenetics. In CSCs, deregulation of these pathways along with improper interactions between them may represent key events for CSC propagation and pathogenesis. From all of them, abnormal activity of Notch, Wnt and Hedgehog pathways are probably the most crucial to the tumorigenicity of CSCs, making these developmental pathways important therapeutic targets for blockade of CSC self-renewal and proliferation, and tumor progression (Takebe *et al.* 2015).

#### *Notch Signaling Pathway*

The Notch pathway is an evolutionarily conserved signaling pathway that constitutes a critical component in the molecular circuits that regulate a broad range of events during embryonic and post-natal development, including border formation, cell fate decisions, differentiation, migration, proliferation and apoptosis (Sjölund *et al.* 2005; Chiba 2006). The role of Notch in human cancer has been highlighted recently by the presence of activating mutations and amplifications of Notch genes in human cancer and by the demonstration that genes in the Notch signaling pathway could be potential therapeutic targets (Shih & Wang 2007). Furthermore, it is suggested that Notch pathway function is context dependent, since different Notch receptors or ligands could induce different gene expression programs, explaining the different and even opposite outcomes that have been observed in this signaling pathway (Figure 5, Wilson & Radtke 2006).

The core components of the Notch pathway comprises four transmembrane receptors (Notch1-Notch4) and five structurally similar ligands (Delta-Like1, -3, -4 and Jagged1 and Jagged2), although there is very little evidence that Delta-like3 physically binds to the Notch receptors or that it truly functions as a notch ligand (Ladi *et al.* 2005; Chiba 2006). The Notch signaling cascade is initiated by ligand-receptor interaction between two neighboring cells resulting in two successive proteolytic events as part of the activation mechanism (Figure 6,

Brou *et al.* 2000; Mumm & Kopan 2000; Sjölund *et al.* 2005; Wilson & Radtke 2006). The first cleavage is mediated by a metalloprotease of the ADAM family (TACE, tumor necrosis factor-a-converting enzyme) in close proximity to the extracellular side of the plasma membrane. The release extracellular domain is then transendocytosed by the ligand-expressing cell.

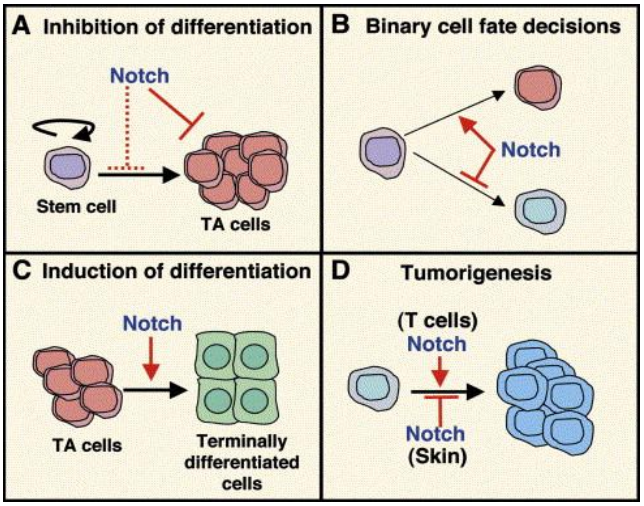



**Figure 5. The four major pleiotropic effects of Notch cascade that are relevant within self-renewing tissues or during tumorigenesis.** (A) Gate-keeper function. Notch maintains stem cells in an undifferentiated state. In the intestine for example, Notch prevents crypt progenitor cells from differentiating. (B) Binary cell fate decisions: In the lymphoid system, it specifies the T cell lineage at the expense of the B cell lineage from a bi-potent early thymocyte progenitor. (C) Induction of differentiation. In the skin, Notch induces terminal differentiation events, and during thymocyte differentiation, *NOTCH1* promotes differentiation of pro-T-cells into pre-T-cells. (D) Tumorigenesis: overexpression of Notch within hematopoietic bone marrow cells or in T cell progenitors results in T cell leukaemias and as such, Notch functions as an oncogene. However, in the skin Notch functions as a tumor repressor since loss of Notch signaling results in the development of basal cell carcinoma-like tumors (Wilson & Radtke 2006).

The second one occurs within the transmembrane domain, mediated by a multi-protein complex, γ-secretase, consisting of presenilin, nicastrin, APH1 and PEN2, which leads to the release of the Notch intracellular domain (NICD, Sjölund *et al.* 2005; Wilson & Radtke 2006; Shih & Wang 2007; Fortini 2009). Upon cleavage, NICD translocates to the nucleus where it forms a complex with the ubiquitously expressed transcription factor CSL (CBF1 in humans). The translocation of NICD is counteracted by Numb, through a mechanism that is not completely understood (Roegiers & Jan 2004; Gonczy 2008; Westhoff *et al.* 2009). In the absence of NICD, CSL is a transcriptional repressor due to its association with co-repressors. When NICD associates with CSL, a number of co-activators are recruited, including mastermind-like (MAML1, -2 and -3), resulting in a multiprotein complex, which acts as a potent transcriptional activator. The most well-defined targets of the NICD-CSL complex are the hairy enhancer of split (HES) family, the Hes-related repressor protein (HERP, also called HEY) family, cell cycle regulators, such as CDKN1A, p21 and CCND1 and apoptosis regulators (Iso *et al.* 2003; Kageyama *et al.* 2005; Egloff & Grandis 2012).

The first link between Notch and human tumors was made in the late 1980s and early 1990s in a small number of patients suffering from T cell acute lymphoblastic leukemia (Reynolds *et al*. 1987). More recently deregulated expression of members of Notch signaling pathway has also been reported in solid tumors including breast (Reedijk *et al*. 2005) and lung cancers (Allen *et al*. 2011).

The involvement of Notch on lung cancer was experimentally proved in transgenic mouse model by the alveolar epithelium specific expression of activated Notch. The mice developed alveolar hyperplasia as early as 7 days' after *NOTCH1* induction with a Dox system and when crossed with mice conditionally overexpressing *MYC* in the alveolar epithelium, mice developed adenocarcinomas (Allen *et al*. 2011). Furthermore, using a model of lung adenocarcinoma with expression of oncogenic Kras and deletion of *NOTCH1*, it was found that *NOTCH1* function was required for tumor initiation via suppression of p53-mediated apoptosis through the regulation of p53 stability (Licciulli *et al*. 2013). Molecular analyses defined a subpopulation of $CD24^{+}$ $ITGB4^{+}$ $Notch^{hi}$ cells that were capable of propagating tumor growth in both clonogenic assays and in serial orthotopic transplantation assays (Zheng *et al*. 2013). These data supported a strong and direct role of Notch signaling in NSCLC initiation and proliferation. Other studies had shown that under hypoxic conditions, *NOTCH1* stimulated NSCLC tumor growth through direct upregulation of IGF1-R and survivin, both of which enhanced cell proliferation and survival (Eliasz *et al*. 2010; Chen *et al*. 2011; Yuan *et al*. 2014b).

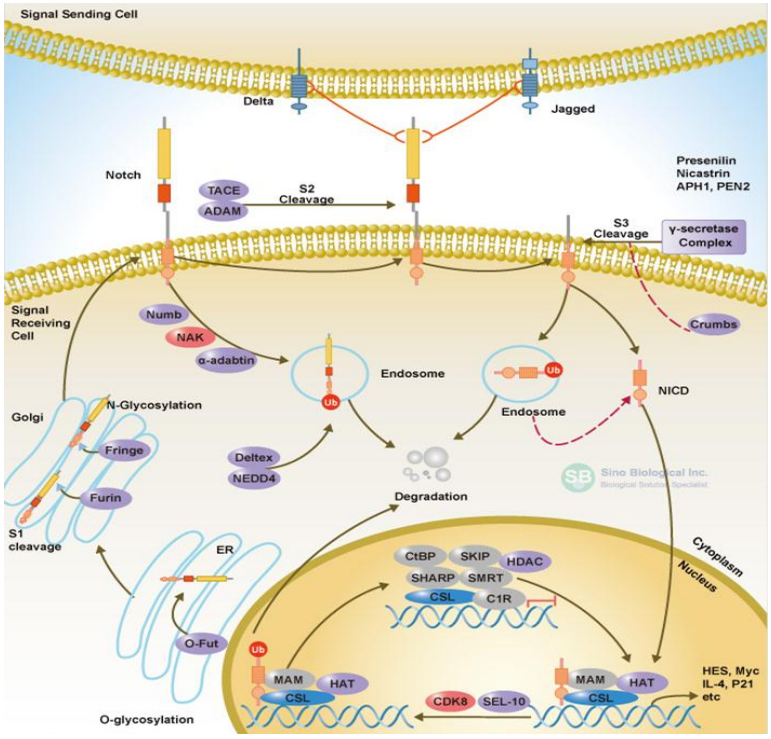



**Figure 6. Illustration of the Notch signaling pathway.**

However, Notch pathway expression is not only tissue-dependent, but cell-dependent as well. While in SCLC Notch signaling is not active, in NSCLC it was found that it is active, possibly due to loss of Numb inhibitor expression or to the presence of gain-of-function mutations in Notch receptors (Westhoff *et al.* 2009), leading to high expression levels of Notch target genes, and making this tumor type susceptible to therapies based on Notch inhibition (Ball *et al.* 1993; Chen *et al.* 1997; Sriuranpong *et al.* 2001; Sjölund *et al.* 2005).

### *Wnt Signaling Pathway*

The evolutionarily conserved Wingless-type protein (Wnt) signaling pathway is involved in a multitude of developmental processes and the maintenance of adult tissue homeostasis by regulating cell proliferation, survival, differentiation, migration and polarity, genetic stability and self-renewal in stem cells (Clevers & Nusse 2012). Not surprisingly, aberrant Wnt signaling underlies a wide range of diseases, including cancer (Porfiri *et al.* 1997; de La Coste *et al.* 1998), fibrosis (Dees & Distler 2013; Chilosi *et al.* 2003) and neurodegenerative disorders (Inestrosa *et al.* 2012; Berwick & Harvey 2012; Okerlund & Cheyette 2011).

The WNT signaling cascade is extremely complex. Firstly, there are 19 WNT ligands, which are glycoproteins of 40kDA in size that contain lipid modifications with many conserved cysteines, and more than 15 receptors and co-receptors distributed over seven protein families in mammals (Niehrs 2012; Franch-Marro *et al.* 2008; Anastas & Moon 2012a). Furthermore, WNT proteins can trigger a variety of responses, often gathered at two groups: the canonical WNT signaling pathway for the classical WNT-induced activation of β-catenin-TCF (T-cell factor) transcriptional complexes, and the non-canonical WNT signaling pathway, which includes the planar cell polarity (PCP) signaling pathway (Takahashi-Yanaga & Kahn 2010), the Wnt/$Ca^{++}$ flux pathway (Mazieres *et al.* 2005; Takahashi-Yanaga & Kahn 2010) and the protein kinase A pathway (Takahashi-Yanaga & Kahn 2010) and cJun N-terminal kinase (JNK) and small GTPase Rho, Rac and Cdc 42 signaling networks (Mazieres *et al.* 2005; Wang 2009). Moreover, crosstalk from various non-WNT factors has also been reported to modulate nuclear β-catenin accumulation (Kahn 2014).

In the absence of Wnt proteins, canonical Wnt signaling is inhibited due to a β-catenin degradation complex consisting of Axis inhibition protein (AXIN), adenomatous polyposis coli (APC), casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK-3β) that phosphorylates β-catenin, resulting in ubiquination and proteosomal destruction of β-catenin, which is unavailable for interaction with other factors (Takahashi-Yanaga & Kahn 2010; Teng *et al.* 2010; Stewart 2014). If Wnt -1, -2 or -3 is present, porcupine contributes to its secretion,

making it available to binds to members of the Frizzled (FZD) family of receptors (Figure 7). Binding of Wnt to FZD results in the formation of a stable receptor complex between Wnt, FZD, lipoprotein receptor-related protein 5/6 (LRP), Disheveled (Dvl) and the β-catenin degradation complex. This new complex phosphorylates Dvl, inactivating GSK-3β as a result and reducing the proteolytic destruction of β-catenin. Hence, cytoplasmic levels of β-catenin rise, from where it can migrate to the nucleus to bind with members of the TCF/lymphoid enhancer-binding factor (LEF1) family of transcription factors. Basal transcription machinery and transcriptional coactivators are then recruited, including cAMP response element-binding protein (CREB)-binding protein/E1A binding protein p300 (CBP/p300) and Pygopus 2, initiating transcription of multiple factors that promote cell growth and resistance to chemotherapy and radiotherapy, including cyclin D1 and c-Myc. The β-catenin protein can also interact with E-cadherin at the cell membrane to enhance cellular adhesion. Although the impact of increased β-catenin on transcription may promote tumor cell growth and resistance, the interaction of β-catenin with E-cadherin could potentially decrease malignant characteristics by increasing cell adhesion (Stewart 2014; Clevers & Nusse 2012; Anastas & Moon 2012a; Kahn 2014).

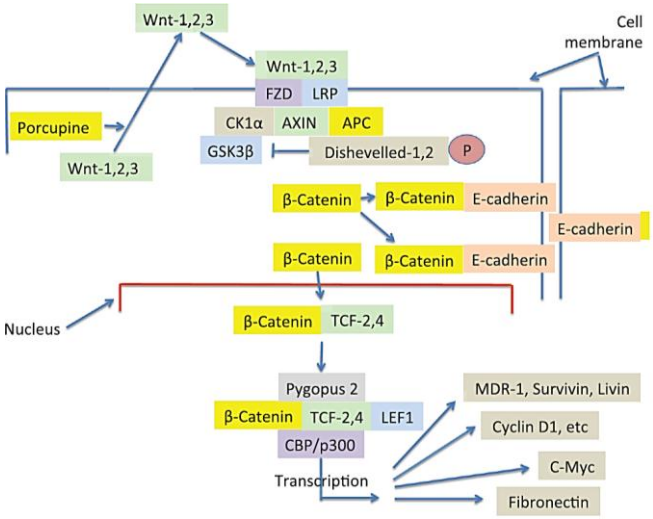



**Figure 7. Simplified representation of the canonical WNT–β-catenin signaling cascade (modified figure from Stewart 2014).**

In addition to inhibition of the pathway by β-catenin complex, canonical Wnt pathway can also be inhibited or promoted in other ways (Figure 8). On the one hand, secreted frizzled-related proteins (sFRPs) compete with Wnt for binding to FZD, Wnt inhibitory factor 1 (WIF-1) and Cerberus bind secreted Wnt, Disabled 2 (Dab2) and the Dickkopf (Dkk) family inhibit Wnt signaling by binding to the LRP5/6 component of the Wnt receptor complex and the human homolog of Dapper (HDPR1) and Idax antagonist Dvl. On the other hand, tankyrases-1 and -2

promote Wnt signaling by destabilizing AXIN and EGFR promote signaling by phosphorylating and consequently inactivating GSK-3β.

Given the importance of WNT signaling for adult stem cell biology, it is not surprising that WNT pathway mutations are frequently observed in cancer. A role for WNT pathway in cancer was first described in the 1980s in mouse models of mammary cancer and in human and mouse colon cancer (Anastas & Moon 2012b). Researches showed that aberrant overexpression of *WNT1* in mice induced by a proviral insertion at the *WNT1* locus or by transgenesis triggers spontaneous mammary hyperplasia and tumor in mice (Nusse & Varmus 1982; Tsukamoto *et al.* 1988). Other studies pointed to a crucial role for hyperactivated WNT-CTNNB1 signaling in colorectal cancer (Korinek *et al.* 1997; Morin *et al.* 1997). Germline inactivating mutations in the adenomatous poluposis coli (APC) – which is a negative regulator of CTNNB1 stability – are found in patients with a hereditary cancer syndrome termed familiar adenomatous polyposis (FAP), which can progress to colorectal carcinomas following concomitant activating mutations in KRAS and inactivating mutations in TP53. Both APC and CTNNB1 are also frequently mutated in colorectal cancers of non-FAP patients (Kinzler & Vogelstein 1996; Segditsas & Tomlinson n.d.), and overexpression of constitutively active *CTNBB1* or loss of APC function can result in colorectal tumorigenesis.

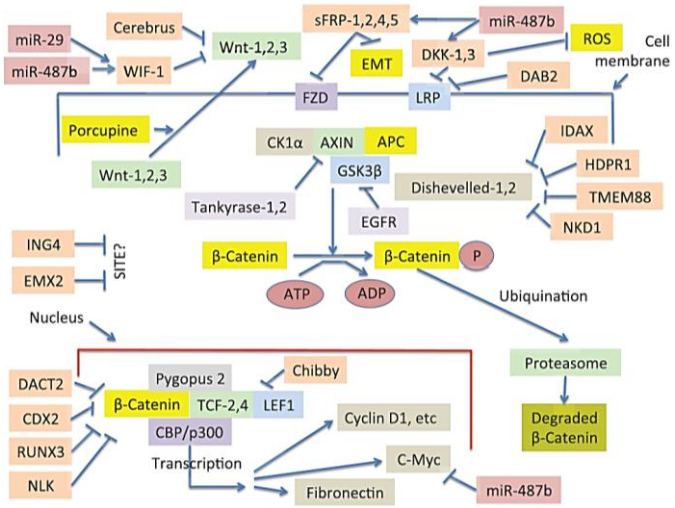



**Figure 8. Inhibition molecules of canonical Wingless-type protein (Wnt) signaling (Stewart 2014).**

There is growing evidence that the Wnt pathway is important in the development of NSCLC. However, most of the researches have been developed in mice and *in vitro* models (Stewart 2014). In murine models, activation of Wnt signaling is associated with increasing tumor initiation potential (Vaughan *et al.* 2012). In cultured respiratory epithelium, cigarette

smoke components upregulated Wnt and Hedgehog signaling (Lemjabbar-Alaoui *et al.* 2006; Liu *et al.* 2010). A majority of NSCLC cell lines have active Wnt signaling (He *et al.* 2004; Licchesi *et al.* 2008; Teng *et al.* 2010). Downregulation of Wnt signaling by anti-Wnt-1 monoclonal antibody or small interfering RNA (siRNA) induced apoptosis in cancer cells (He *et al.* 2004), inhibited NSCLC cell line proliferation (Akiri *et al.* 2009), blocked xenograft growth (Lee *et al.* 2012; He *et al.* 2004) and reduced cell motility and invasion (Lee *et al.* 2012).

Wnt pathway activation and overexpression of Wnt-1, -2 or -3 are also associated with poor prognosis clinically. Of resected NSCLCs, 37% to 63% stained positively for Wnt ligands by immunohistochemistry (Nakashima *et al.* 2008; Xu *et al.* 2011; He *et al.* 2004), which has been associated with a low apoptotic index, aberrant β-catenin expression, increased expression of c-Myc, Cyclin D1, VEGF-A , MMP-7, Ki-67, survivin and bigger intratumoral microvessel density (Nakashima *et al.* 2010; Nakashima *et al.* 2008; Xu *et al.* 2011). On the other hand, it has been reported that the Wnt signaling pathway helps to maintain CSCs since putative stem cell markers such as LGR57GRP49, CD44, CD24, EpCAM or OCT4 are Wnt targets (Takahashi-Yanaga & Kahn 2010; Teng *et al.* 2010). Thus, the Wnt pathway seems to be a promising target in NSCLC tumorigenesis, making it worthwhile to explore Wnt signaling pathway expression as a therapeutical option for NSCLC patients (Stewart 2014).

### *Hh Signaling Pathway*

The hedgehog (Hh) signaling pathway regulates proliferation and differentiation in a time- and position-dependent fashion during embryonic development (Velcheti & Govindan 2007). In adult tissues, Hh pathway plays a central role in tissue repair and regeneration. It has been reported that both mutations and deregulations of genes related to Hh pathway can contribute to the onset of cancer or to accelerate the rate of tumor growth (Rubin & de Sauvage 2006).

Mammalian Hh signaling pathway is mainly constituted by three Hh ligands homologues with different spatial and temporal distribution patterns: Sonic hedgehog (*SHH*), Indian hedgehog (IHH) and Desert hedgehog (DHH), transmembrane receptor-patched homolog 1 and 2 (*PTCH1*, *-2*), a G protein-coupled receptor, smoothened (*SMO*) and a cytoplasmatic complex that regulates the glioma-associated oncogene homolog (GLI) family. *GLI1* is a transcription activator, and *GLI2* and *GLI3* are both activators and repressors of transcription.

The Hh signaling cascade is initiated by Hh binding to the *PTCH1* protein on the target cell (Figure 9, Kalderon 2000). In the absence of the Hh ligand, *PTCH1* represses the activity of *SMO*, preventing its localization to the cell surface from intracellular endosomes, where *SMO* is

predominantly located (Denef *et al.* 2000). Under these circumstances, different kinases phosphorylate and activate repressor forms of GLI transcription factors. The active form of GLI is prevented from transactivating Hh-responsive genes by the serine-threonine protein kinase suppressor of fused (SUFU) and the atypical kinesin-like protein Costa (COS) in a manner that is still not completely understood. Upon binding an Hh ligand, *PTCH1* is internalized, and apparently destabilized, so that it can no longer transport the endogenous agonist molecules outwards. This allows them to accumulate intracellularly and activate *SMO*, which sequestrate COS and SUFU, releasing the GLI transcription factors to exert their effects in nucleus. KIF3A and β-arrestin are required for *SMO* activation (Rubin & de Sauvage 2006; Takebe *et al.* 2015).

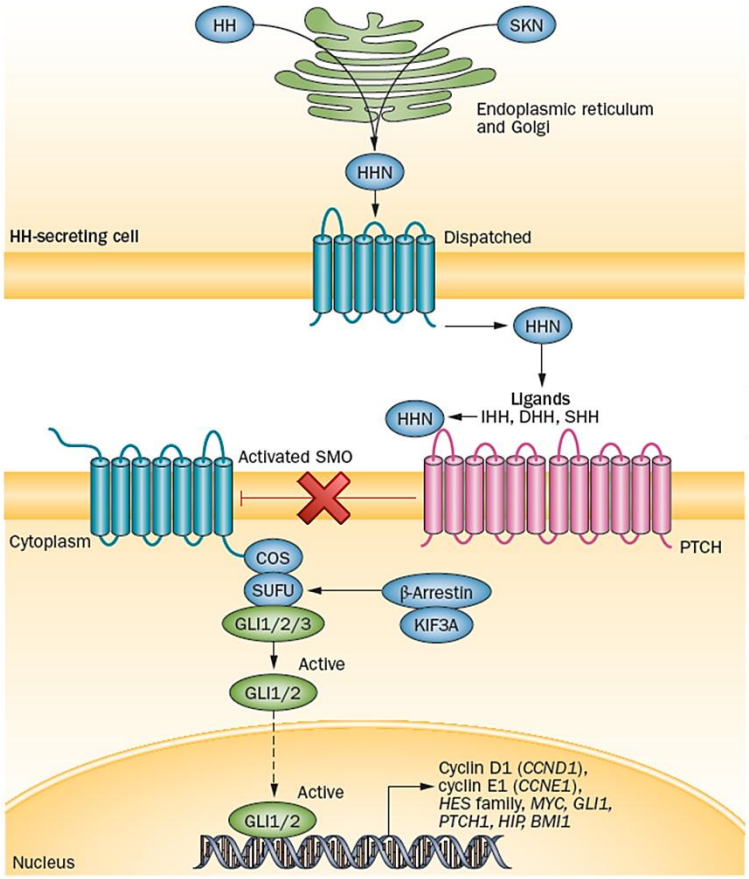



**Figure 9. Representation of the Hedgehog signaling pathway. Adapted from (Takebe *et al.* 2015).**

The first connection between aberrant Hh signaling and cancer was the discovery that the rare condition Gorlin syndrome is caused by a mutation in the transmembrane receptor-patched homolog 1 (*PTCH1*), a key component of Hh signaling (Hahn *et al.* 1996; Johnson *et al.* 1996). Gorlin patients develop several basal cell carcinomas during their lifetimes and are predisposed to other kinds of cancer. In addition, one-third or more of all human

medulloblastoma cases have been shown to involve increased Hh signaling, often due to *PTCH1* and some SUFU mutations. In all these cases, it is believed that deregulated Hh signaling leads to increased cell proliferation and tumor formation (Rubin & de Sauvage 2006; Velcheti & Govindan 2007).

Regarding lung cancer, Hh signaling is possibly inactive in the human adult lung epithelium except in the epithelial progenitor (stem) cells. This persistence of Hh signaling in the epithelial progenitor (stem) cells could help maintain these cells and play a critical role in the response to airway epithelial injury. Studies on animal lung airway epithelial injury/regeneration model suggest that persistent injury to the airway is a potent stimulus for the activation of the Hh signaling, and this helps the expansion of airway epithelial progenitor cells (Watkins *et al.* 2003; Watkins & Peacock 2004; Velcheti & Govindan 2007). Studies on cultured cells showed that SCLC and NSCLC cell lines tend to express *SHH* protein. In fact, 70% of SCLC cell lines expressed both *SHH* and *GLI1* in contrast to NSCLC, which expressed exclusively *GLI1* (Watkins *et al.* 2003). Analysis of clinical samples of human lung cancer tissue demonstrated 50% of SCLC expressed both *SHH* and *GLI1* compared to only 10% of NSCLC. However, it is still necessary to clarify the role of activation of Hh pathway in the process of carcinogenesis and progression in lung cancer. There have been virtually no significant advances in the systemic therapy of lung cancer. With the current trend toward developing targeted therapies, the Hh pathway modulators offer a potential new avenue in the treatment of lung cancer (Velcheti & Govindan 2007).

Unfortunately, progression and treatment resistance are frequent in NSCLC. As it was mentioned above, CSCs population seems to be related with these facts, but there are no specific biomarkers available for lung CSCs, which could be used for their isolation from tumor tissue (Zakaria *et al.* 2015; Swarts *et al.* 2013). Therefore, it is necessary to look for lung CSCs-markers, which could discriminate CSCs from cancer cells and deeply characterize CSCs in order to develop specific therapies against this population.

In this study, we analyze the gene expression of Notch, Wnt and Hedgehog signaling pathways, which are known to control CSC characteristics, as well as genes that have been previously reported as potential lung CSCs biomarkers in early stages. The validation of these genes as specific CSC markers would facilitate information about disease prognosis and/or discover potential therapeutic targets.

# 2. OBJECTIVES

In the current context of lung cancer research, cancer stem cells (CSCs) have been proposed as promising targets, but this cell population remains largely unknown. Some studies have been done in cancer cell-lines, but few data is available from patients' samples. The main objective of this study is to analyze and characterize gene expression patterns of CSCs, using paired samples of tumorspheres and adherent cultured cells derived from lung cancer cell-lines and tumor-tissue from resectable NSCLC patients.

The specific aims of this study are:

1. To establish NSCLC primary cultures, from surgical resections specimens, under adherent and non-adherent, serum-free conditions (CSCs possess the ability to form spheroids in serum-free medium).

2. To isolate and assess the quality of RNA from cells grown under standard and non-adherent culture conditions.

3. To analyze relative gene expression of CSCs markers (*EPCAM1, ALDH1A1, CD133, ALCAM, ABCG2, CD44, BMI1, MUC1*); pluripotency genes (*KLF4, OCT4, NANOG, SOX2, MYC, CCND1*); cell cycle (*CDKN1A, CDKN2A, MDM2, WEE1*); metastasis-related genes (*CDH1, CEACAM5, VIM, SNAI1, MMP2, MMP9*); Notch pathway (*NOTCH1, NOTCH2, NOTCH3, DLL1, DLL4, HEY1, HES1*); Wnt pathway (*CTNBB1, WNT1, WNT2, WNT3, WNT5A, DKK1, FZD7, GSK3B*) and Hedgehog pathway (*SMO, PTCH1, SHH, GLI1*).

4. To evaluate gene expression profiles of tumorspheres in order to find out a set of genes that better characterize them and could act as biomarkers or therapeutic targets of lung CSCs.

# 3. MATERIALS & METHODS

## 3.1.SAMPLES INCLUDED IN THE STUDY

In this study, tumor cells from resected NSCLC patients from Consorcio Hospital General Universitario de Valencia were collected. Patients that met the eligibility criteria (resected non-pre-treated stage I to stage IIIA cancer, according to the American Joint Committee on Cancer staging system) with a histological diagnosis of NSCLC were informed and invited to participate in the study. The most relevant demographic and clinicopathological characteristics of the cohort are shown in Table 3.

**Table 3. Clinicopathological characteristics of the patients included in the study.**

| Primary culture | Age | Gender | Stage | Histology | Smoking status | Mutation Status | Progression | Growing |
|---|---|---|---|---|---|---|---|---|
| **301** | 71 | Male | IIB | SCC | Former | Wt. EGFR, ALK | NO | NO |
| **302** | 74 | Female | IB | ADC | Never | Wt. EGFR, KRAS, ALK | NO | YES |
| **303** | 57 | Male | IB | ADC | Current | Wt. EGFR, KRAS, ALK | YES | YES |
| **304** | 48 | Male | IIA | SCC | Current | Wt. EGFR, ALK | NO | NO |
| **305** | 52 | Male | IIB | ADC | Current | Wt. EGFR, ALK | NO | NO |
| **306** | 50 | Female | IIA | ADC | Current | Wt. EGFR, ALK | NO | NO |
| **307** | 62 | Male | IB | ADC | Former | Wt. EGFR, ALK | NO | NO |
| **308** | 73 | Male | IIB | ADC | Former | Wt. EGFR, ALK | NO | NO |
| **310** | 68 | Male | IB | ADC | Current | Wt. EGFR, ALK | NO | NO |
| **315** | 65 | Female | IA | ADC | Never | Wt. EGFR, KRAS, ALK | NO | YES |
| **316** | 43 | Female | IIIB | ADC | Current | Wt. EGFR, ALK | YES | NO |
| **317** | 76 | Male | IIB | SCC | Current | Wt. EGFR, KRAS, ALK | NO | YES |
| **320** | 65 | Male | IIIB | ADC | Current | Wt. EGFR, KRAS, ALK | NO | YES |
| **321** | 83 | Male | IB | SCC | Former | Wt. EGFR, ALK | NO | NO |
| **323** | 68 | Male | Fibrous Nodule | NA | Current | Wt. EGFR, ALK | NA | YES |

**ADC, adenocarcinoma; SCC, squamous cell carcinoma; Wt., Wildtype; NA, not applicable.**

Four commercial lung cancer cell lines (A549, H1650, H1993 and PC9) were also analyzed in this project. All selected cell lines were obtained from the American Type Culture Collection (ATCC), except for PC9 that was obtained from the European Collection of Authenticated Cell

Cultures (ECACC). The study was approved by the institutional ethical review board, and conducted in accordance with the Declaration of Helsinki, and Spanish regulatory guidelines. Table 4 shows the general properties of cell lines included in this study.

**Table 4. Characteristics of the lung cancer cell lines analyzed in the study.**

| Cell Line | Tissue | Age | Gender | Stage | Histology | Smoking Status | Mutational Status |
|---|---|---|---|---|---|---|---|
| **A549** | Lung | 58 | Male | Early stage | ADC | NS | KRAS p.G12S |
| **H1650** | Derived from metastatic pleural effusion | 27 | Male | IIIB | ADC | Light Smoker | EGFR p.E746_750del |
| **PC9** | Lung | NS | Female | NS | ADC | Never Smoker | EGFR p.E746_750del |
| **H1993** | Derived from metastatic lymph node | 47 | Female | IIIA | ADC | Smoker | TP53 p.C24W |

**ADC, adenocarcinoma; NS, not specified.**

## 3.2.PRIMARY CELL CULTURE ESTABLISHMENT

Fresh tumor tissue from NSCLC patients (n=15) who underwent lobectomy or pnemonectomy was washed in PBS containing penicillin-streptomycin before dissociation using mechanical, and enzyme-based methods. Briefly, each tumor piece was minced into small pieces using a scalpel and incubated in 7 mL of DMEM/F12 (Gibco, Paisley, UK) with 0,001% DNAse (Sigma-Aldrich, St Louis, USA), 1 mg/mL collagen (Gibco, Paisley, UK), 1 mg/mL dispase (Gibco, Paisley, UK), 200 U/mL penicillin and 200 µg/mL streptomycin (2% antibiotics, Gibco, Paisley, UK) at 37$^{o}$C for 3 hours in a water bath with intermittent shaking. Then, samples were centrifuged at 290 g for 5 minutes and the resulting suspension was sequentially passed through 70 µm and 40 µm cell strainers (BD Falcon, San Jose, USA) and centrifuged for a second time at 200 g for 5 minutes. Then, cells were resuspended in 500 µL of serum-free medium and 10 mL of red blood cell lysis buffer 10x (eBioscience, San Diego, USA) and incubated at 37ºC with intermittent shaking for 20 minutes. After erythrocytes lysis, cells were centrifuged at 130 g for 5 minutes and cell viability was evaluated by trypan blue (Gibco, Paisley, UK).

Half of the live cells were transferred into standard 25 cm$^2$ coated flasks and cultured in Defined Keratinocyte-Serum Free Medium to inhibit fibroblasts growing (Gibco, Paisley, UK). Once the primary cultures were established, the maintenance was performed in DMEM/F12

supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), 2% penicillin-streptomycin and 1% of L-glutamine (Gibco, Paisley, UK). For spheroids formation, the rest of disaggregated cells were grown in serum free culture medium DMEM/F12 supplemented with 50 µg/mL epidermal growth factor (EGF, Gibco, Paisley, UK), 20 µg/mL basic fibroblast growth factor (bFGF, Gibco, Paisley, UK), insulin-transferrin-selenium (ITS, BD Biosciences), 0.4% Bovine Serum Albumin (BSA, Gibco, Paisley, UK) and 2% B27 (Gibco, Paisley, UK). Cells were plated in 6-well ultra-low attachment plates (Corning, Lowell, MA, USA) at a density of 5000 cells/mL for 7 to 10 days. Cultures were expanded by mechanical dissociation of spheroids, followed by re-plating of both single cells and residual small aggregates in complete fresh medium. All cultured cells were maintained at 37ºC in 5% $CO_2$ atmosphere and medium was replaced twice a week.

## 3.3. CELL LINES CULTURE

Commercial cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2% penicillin-streptomycin and 0.001% non-essential amino acids (Gibco, Paisley, UK), at 37ºC and 5% $CO_2$ atmosphere.

To obtain sphere cultures, 70-80% of confluence monolayer cells were enzymatically dissociated into a single cell suspension using 0.5% Trypsin-EDTA 1x (Gibco, Paisley, UK). Cells were seeded at desired density into 75 $cm^2$ ultra-low attachment flasks (Corning, Lowell, MA, USA) under serum-free medium conditions supplemented with 50 µg/mL EGF, 20 µg/mL bFGF, ITS, 0.4% BSA and 2% B27. Cultures were expanded by enzymatical dissociation of spheroids, followed by re-plating of both single cells and residual small aggregates in complete fresh medium. Culture medium was replaced twice a week.

## 3.4. RNA/DNA ISOLATION

For RNA/DNA isolation, cells were centrifuged at 290 g for monolayer cells and 200 g for tumorspheres during 5 minutes. Cell pellets were washed twice with PBS and stored at -80ºC until further analysis.

RNA and DNA were isolated using a TRZol based method (Tri Reagent®, Invitrogen, USA) according to the manufacturer's instructions. Briefly, 1 mL of Tri Reagent® and 200 µL of chloroform were added to each cell pellet in order to separate the aqueous phase containing the RNA. Isopropanol was used to precipitate the nucleic acids and ethanol was used for washing. Isolated messenger RNA (mRNA) was dissolved in nuclease free water and stored at -80ºC until further analysis. The DNA interphase was recollected in absolute ethanol and

washed, first with 10% ethanol/0.1 M sodium citrate buffer and then with 75% ethanol. At the end, it was dissolved in nuclease free water and stored at -80ºC until further analysis. RNA and DNA quantity and quality were assessed using a nanospectrophotometer (Nano Drop 2000C, Thermo Fisher Scientific, USA).

## 3.5. REVERSE TRANSCRIPTION

Reverse transcription was performed in order to transform mRNA into complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit® (Applied Biosystems). Each reaction comprised 2 µL of reverse transcription (RT) buffer, 0.8 µL of dNTPs mix, 2 µL of RT random primers, 1 µL of MultiScribe™ Reverse Transcriptase, 1 µL of RNase inhibitor and a different volume of RNA depending on sample concentration (1000 ng of RNA per reaction), made up to a total of 20 µL with nuclease free water. The reaction took place in a MasterCycler® thermocycler (Eppendorf) following the conditions described in Table 5. Resulting cDNA was stored at -80ºC until further analysis.

**Table 5. Cycling program for reverse transcription reaction.**

| Phase | Time | Temperature |
|---|---|---|
| 1 | 10 minutes | 25° C |
| 2 | 2 hours | 37° C |
| 3 | 5 seconds | 85° C |
| 4 | ∞ | 4° C |

## 3.6. QUANTITATIVE REAL TIME PCR

Target gene quantification was performed by RTqPCR using hydrolysis probes labeled with a reporter dye linked to the 5' end of the probe (TaqMan®, Applied Biosystems). This system also includes a non-fluorescent quencher (NFQ) at the 3' end of the probe and a Minor Grove Binder (MGB) attached to the NFQ, which increases the melting temperature (Tm) without increasing the length of the probe (Figure 10).

In this study, a total of 43 genes, selected according to their implications in the biology of CSCs, were analyzed (Table 6). The relevance of these genes was established from a PubMed database search, which revealed published information demonstrating or suggesting a role of these genes in CSCs maintenance, self-renewal and proliferation.

Gene expression levels were assessed using TaqMan® Gene Expression Assays (Applied Biosystems) listed in Table 6. Different endogenous gene controls were tested in samples in order to evaluate the best internal control using GeNorm software. This software

automatically calculates the gene-stability measurement 'M' for all control genes and allows the worst-scoring housekeeping genes to be eliminated (Vandesompele *et al.* 2002a).

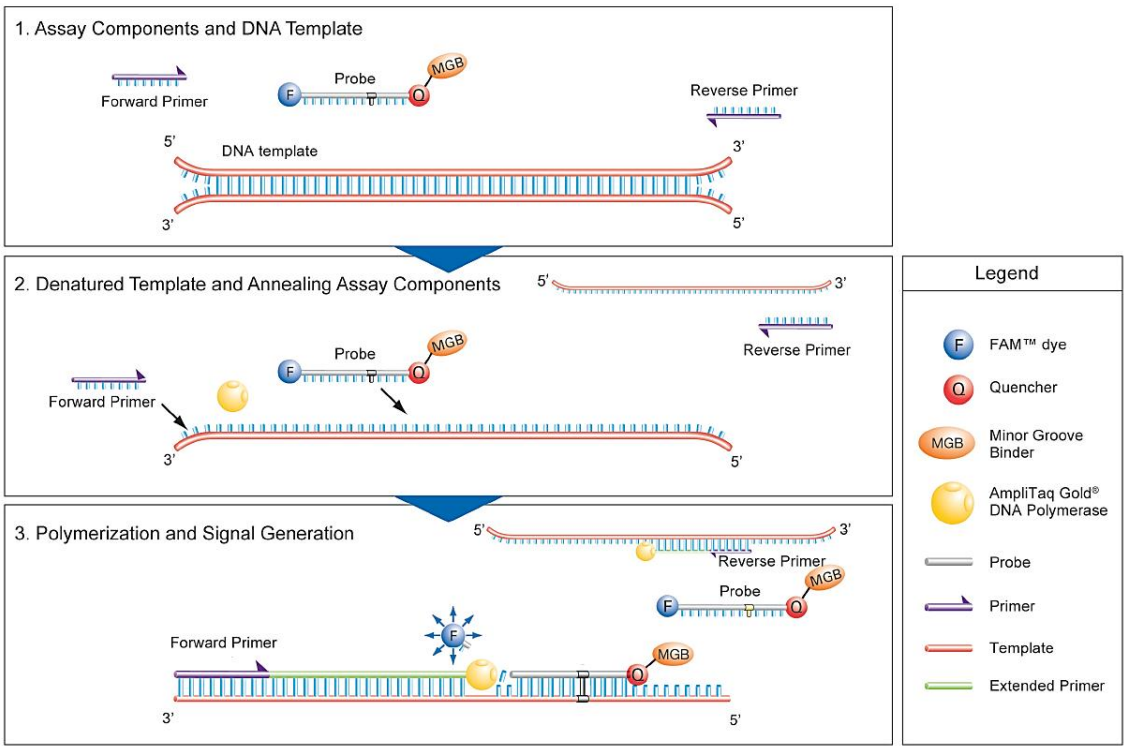



**Figure 10. TaqMan® qPCR reaction steps (Life Technologies).**

Each reaction was performed twice in 384-well plates with a final volume of 5 µL comprising: 2.5 µL of TaqMan® Gene Expression Master Mix (Applied Biosystems), 1.25 µL of nuclease free water, 0.25 µL of TaqMan® Gene Expression Assay mix (Applied Biosystems) and 1 µL of cDNA. Non-template controls (NTCs) were included in each run, as well as positive reference controls: Jurkat cell line, and a commercially available reference cDNA (Clonetech). The reactions took place in a Light Cycler 480 thermocycler system (Roche) following the cycling conditions described in Table 7.

**Table 6. Genes analyzed and amplicon length of the TaqMan® Gene Expression Assays used for QPCR.**

| Gene | Full Name | Assay | Amplicon Length |
|---|---|---|---|
| *ACTB* | Actin, Beta | Hs99999903_m1 | 171 |
| *CDKN1B* | Cyclin-dependent kinase inhibitor | Hs00153277_m1 | 71 |
| *GUSB* | Glucuronidase, beta | Hs01558067_m1 | 71 |
| HPRT1 | Hypoxanthine phosphoribosyltransferase 1 | Hs01003267_m1 | 72 |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | Hs99999905_m1 | 122 |
| ABCG2 | ATP-binding cassette, sub-family G | Hs01053790_m1 | 83 |
| ALDH1A1 | Aldehyde dehydrogenase 1 family, member A1 | Hs00946916_m1 | 61 |
| BMI1 | BMI1 proto-oncogene, polycomb ring finger | Hs00180411_m1 | 105 |

| | | | |
|---|---|---|---|
| CD44 | CD44 molecule | Hs01075861_m1 | 70 |
| CD133 | CD133 molecule | Hs01009257_m1<br>Hs01009250_m1<br>Hs00195682_m1 | 80<br>75<br>107 |
| CD166 | CD166 molecule | Hs00233455_m1 | 70 |
| EPCAM1 | Epithelial cell adhesion molecule | Hs00158980_m1 | 64 |
| MUC1 | Mucin 1, cell surface associated | Hs00159357_m1 | 84 |
| OCT4 | POU class 5 homeobox 1 | Hs01895061_u1 | 130 |
| NANOG | Nanog homeobox | Hs02387400_g1 | 109 |
| SOX2 | SRY (sex determining region Y)-box 2 | Hs01053049_s1 | 91 |
| KLF4 | Kruppel-like factor 4 (gut) | Hs00358836_m1 | 110 |
| MYC | V-myc avian myelocytomatosis viral oncogene homolog | Hs00153408_m1 | 107 |
| CCND1 | Cyclin D1 | Hs00765553_m1 | 57 |
| CDKN1A | Cyclin-dependent kinase inhibitor 1A | Hs99999142_m1 | 99 |
| CDKN2A | Cyclin-dependent kinase inhibitor 2A | Hs00923894_m1 | 115 |
| MDM2 | MDM2 proto-oncogene, E3 ubiquitin protein ligase | Hs01066930_m1 | 99 |
| WEE1 | WEE1 G2 checkpoint kinase | Hs00268721_m1 | 66 |
| CDH1 | Cadherin 1, type 1 | Hs01023894_m1 | 61 |
| VIM | Vimentin | Hs00185584_m1 | 73 |
| SNAI1 | Snail family zinc finger 1 | Hs00195591_m1 | 66 |
| MMP2 | Matrix metallopeptidase 2 | Hs01548727_m1 | 65 |
| MMP9 | Matrix metallopeptidase 9 | Hs00234579_m1 | 54 |
| CEACAM5 | Carcinoembryonic antigen-related cell adhesion molecule 5 | Hs00944025_m1 | 71 |
| *NOTCH1* | *NOTCH1* | Hs01062014_m1 | 80 |
| *NOTCH2* | *NOTCH2* | Hs01050702_m1 | 60 |
| *NOTCH3* | *NOTCH3* | Hs01128541_m1 | 81 |
| *DLL1* | Delta-like 1 | Hs00194509_m1 | 74 |
| *DLL4* | Delta-like 4 | Hs00184092_m1 | 78 |
| *HEY1* | Hairy ears, Y-linked | Hs01114113_m1 | 82 |
| *HES1* | Hes family bHLH transcription factor 1 | Hs00172878_m1 | 78 |
| *WNT1* | Wingless-type MMTV integration site family, member 1 | Hs01011247_m1<br>Hs00180529_m1 | 108<br>77 |
| *WNT2* | Wingless-type MMTV integration site family, member 2 | Hs00608224_m1 | 119 |
| *WNT3* | Wingless-type MMTV integration site family, member 3 | Hs00902257_m1 | 76 |
| *WNT5A* | Wingless-type MMTV integration site family, member 5A | Hs00998437_m1 | 61 |
| *CTNBB1* | Catenin beta 1 | Hs00355049_m1 | 67 |
| GSK3B | Glycogen synthase kinase 3 beta | Hs01047719_m1 | 65 |
| *DKK1* | Dickkopf WNT signaling pathway inhibitor 1 | Hs00183740_m1 | 68 |
| *FZD7* | Frizzled class receptor 7 | Hs00275833_s1 | 70 |
| *SMO* | Smoothened, frizzled class receptor | Hs01090242_m1 | 54 |
| *PTCH1* | Patched 1 | Hs00181117_m1 | 72 |
| *SHH* | Sonic hedgehog | Hs00179843_m1 | 70 |
| *GLI1* | GLI family zinc finger 1 | Hs01110766_m1 | 83 |
| LGALS-2 | Lectin, galactoside-binding, soluble, 2 | Hs00197810_m1 | 73 |
| CD45 | Protein tyrosine phosphatase, receptor type, C | Hs00898488_m1 | 61 |
| JUNB | Jun B proto-oncogene | Hs00357891_s1 | 89 |
| TGFB | Transforming growth factor, beta 1 | Hs00998133_m1 | 57 |
| LIN28B | Lin-28 homolog A | Hs01013729_m1 | 130 |
| STAT3 | Signal transducer and activator of transcription 3 | Hs01047580_m1 | 87 |

The step at 50° C is required for optimal UNG enzyme activity. The step at 95° C is required to activate the AmpliTaq Gold enzyme.

The efficiency of each TaqMan® assay was evaluated by carrying out serial dilutions (50 ng/μL, 5 ng/μL, 0.05 ng/μL, 0.005 ng/μL and 0.0005 ng/μL) using the commercial reference cDNA mentioned above and calculated by using $E=10^{-1/slope}$ equation. Relative gene expression levels were expressed as the ratio of target gene expression to reference gene expression by using the Pfaffl formula (Pfaffl 2001). Herein, relative quantification determines the changes in steady-state mRNA levels of a target gene across multiple samples and expresses it relative to the levels of control RNA. The expression is normalized against a reference gene, which is often a housekeeping gene.

**Table 7. Cycling program for RTqPCR.**

| | Step | Time | Temperature |
|---|---|---|---|
| **Pre-PCR** | UNG incubation | 2 min | 50° C |
| | Taq activation | 10 min | 95° C |
| **PCR (40 cycles)** | Denature | 15 sec | 95° C |
| | Anneal/Extend | 1min | 60° C |

## 3.7. DATA ANALYSIS

Before statistical analyses, expression data were carefully reviewed and those values considered as outliers were excluded.

The first statistical analysis was to evaluate if the analytical variables followed a normal distribution by using the Kolmogorov-Smirnov test. In those cases were the variables did not follow a normal distribution, statistical analyses were conducted by non-parametric tests. Continuous variables were compared using non-parametric Mann Whitney U and Kruskall Wallis tests. Spearman's rank was used to test for correlations between continuous variables, and associations between dichotomized variables were evaluated using the Chi-square test.

For each case, adherent cells and tumorspheres gene expression was paired and analyzed using Wilcoxon test. For relative gene expression comparison, commercial reference cDNA was used for normalization. For categorical analysis, gene expression values higher than 2 or lower than 0.5 were considered over- and underexpressed, respectively. Median value was used instead of mean because median is less affected by data variability and provides statistical robustness to the analysis. All the statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 (Chicago, IL), considering statistically significant those analyzes were $p< 0.05$.

# 4. RESULTS AND DISCUSSION

## 4.1. TUMORSPHERES FORMATION ASSAY

The sphere-formation *in vitro* assay is a well-described method of CSCs isolation, identification and enrichment (Han *et al*. 2013). Cancer cells that lack stem cells properties have limited sphere-forming potential due to telomere loss and cellular senescence (Patel & Rameshwar 2013).

We have processed 15 biological specimens from surgical lung cancer resection (lobectomies or pneumonectomies) through this study. In six samples (40%), we were able to establish a primary culture, being possible to grow them in adherent and non-adherent conditions. There were several causes that influence the successful rate of primary cultures establishment such as excessive necrosis of tumor samples, deficient preservation of tumor samples before culture, fibroblast overgrown, and lack of cell viability, among others. In addition, one tissue sample was considered tumor-free and discarded after anatomical pathology evaluation. As it was mentioned before, four lung cancer cell lines (A549, H1650, H1993 and PC9) were included in the study. Figure 11 shows some examples of cell lines and primary cultures (labeled as 302, 303, 315 and 320) grown as monolayer and as spheroids.

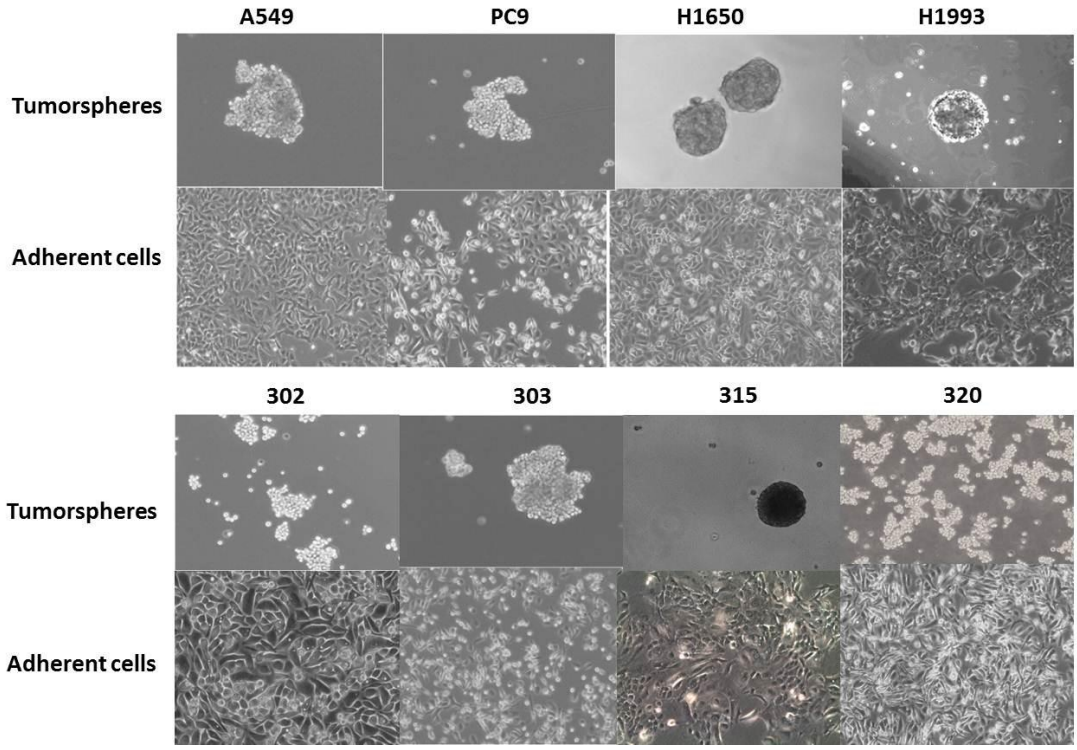



**Figure 11. Cell lines and NSCLC patient's primary cultures: adherent cultures and tumorspheres.**

## 4.2. RNA QUANTIFICATION AND QUALITY ASSESSMENT

For relative gene expression analysis, RNA from cellular pellets was isolated using standard trizol extraction procedure. RNA concentration and quality was assessed using a nanospectrophotometer. Only the samples with an optimal concentration (≥ 40 ng/μL) and quality were included in the study. The mean RNA concentration for adherent-cultured cells was 738.6 [108.9-2681.8] ng/μL and 221.7 [97.2-565.8] ng/μL for tumorspheres. Regarding the quality of the RNA obtained, the ratios $A_{260/280}$ and $A_{260/230}$ showed values from 1.8 to 2.0 and from 2.0 to 2.2, respectively, meaning that all samples included in this study could be considered optimal for further analysis. Therefore, this RNA extraction protocol from cellular pellets allows obtaining enough quantity of good quality RNA for gene expression analysis by qPCR.

## 4.3. TAQMAN ASSAYS EFFIENCIENCY CALCULATION

As mentioned before, efficiency for each TaqMan assay was evaluated using the Ct slope method. Ct values for a serial dilution of the target template (triplicates of each dilution) were obtained. After that, a plot of Ct values versus log of target DNA concentration was constructed, followed by the calculation of the slope, which should be near to -3.33, value that represent a 100% of amplification efficiency. In Table 8, a list of the slope and efficiency values for all of the genes included in the qPCR expression analysis is shown.

**Table 8. Efficiency results for the gene's assays performed in this study.**

| Gene | Slope | Efficiency | Percentage Efficiency |
|---|---|---|---|
| *ACTB* | -3,322 | 2,000 | 100 |
| *CDKN1B* | -3,704 | 1,862 | 93 |
| *GUSB* | -3,322 | 2,000 | 100 |
| HPRT1 | -3,570 | 1,906 | 95 |
| GAPDH | -3,322 | 2,000 | 100 |
| ABCG2 | -3,396 | 1,970 | 99 |
| ALDH1A1 | -3,165 | 2,070 | 104 |
| BMI1 | -3,322 | 2,000 | 100 |
| CD44 | -3,644 | 1,881 | 94 |
| CD133 | -3,322 | 2,000 | 100 |
| CD166 | -3,422 | 1,960 | 98 |
| EPCAM1 | -3,165 | 2,070 | 104 |
| MUC1 | -3,322 | 2,000 | 100 |
| OCT4 | -3,322 | 2,000 | 100 |
| NANOG | -3,541 | 2,000 | 100 |
| SOX2 | -4,001 | 1,778 | 89 |
| KLF4 | -3,541 | 1,916 | 96 |
| MYC | -3,743 | 1,850 | 93 |
| CCND1 | -3,993 | 1,780 | 89 |
| CDKN1A | -3,623 | 1,888 | 94 |
| CDKN2A | -3,475 | 1,940 | 97 |

| | | | |
|---|---|---|---|
| MDM2 | -3,617 | 1,890 | 95 |
| WEE1 | -4,073 | 1,760 | 88 |
| CDH1 | -3,322 | 2,000 | 100 |
| VIM | -3,393 | 1,971 | 99 |
| SNAI1 | -3,322 | 2,000 | 100 |
| MMP2 | -3,899 | 1,805 | 90 |
| MMP9 | -3,947 | 1,792 | 90 |
| CEACAM5 | -3,322 | 2,000 | 100 |
| *NOTCH1* | -3,881 | 1,810 | 91 |
| *NOTCH2* | -3,932 | 1,796 | 90 |
| *NOTCH3* | -3,396 | 1,970 | 99 |
| *DLL1* | -3,322 | 2,000 | 100 |
| *DLL4* | -3,322 | 2,000 | 100 |
| *HEY1* | -3,743 | 1,850 | 93 |
| *HES1* | -3,322 | 2,000 | 100 |
| *WNT1* | -3,814 | 1,829 | 91 |
| *WNT2* | -3,322 | 2,000 | 100 |
| *WNT3* | -3,682 | 1,869 | 93 |
| *WNT5A* | -3,710 | 1,860 | 93 |
| *CTNBB1* | -3,932 | 1,796 | 90 |
| GSK3B | -3,322 | 2,000 | 100 |
| *DKK1* | -3,186 | 2,060 | 103 |
| *FZD7* | -3,845 | 1,820 | 91 |
| *SMO* | -2,946 | 2,185 | 109 |
| *PTCH1* | -3,711 | 1,860 | 93 |
| *SHH* | -3,388 | 1,973 | 99 |
| *GLI1* | -3,805 | 1,831 | 92 |
| LGALS-2 | -3,464 | 1,944 | 97 |
| CD45 | -3,654 | 1,878 | 94 |
| JUNB | -3,810 | 1,830 | 92 |
| TGFB1 | -3,617 | 1,890 | 95 |
| LIN28B | -3,831 | 1,824 | 91 |
| STAT3 | -3,322 | 2,000 | 100 |

## 4.4. RELATIVE GENE EXPRESSION ANALYSIS

We analyzed the expression of 43 genes related to CSC maintenance, proliferation and self-renewal grouped in the following categories: CSCs markers (*EPCAM1, ALDH1A1, CD133, ALCAM, ABCG2, CD44, BMI1, MUC1*), pluripotency genes (*KLF4, OCT4, NANOG, SOX2, MYC, CCND1*); cell cycle (*CDKN1A, CDKN2A, MDM2, WEE1*), metastasis-related genes (*CDH1, CEACAM5, VIM, SNAI1, MMP2, MMP9*); Notch pathway (*NOTCH1, NOTCH2, NOTCH3, DLL1, DLL4, HEY1, HES1*); Wnt pathway (*CTNBB1, WNT1, WNT2, WNT3, WNT5A, DKK1, FZD7, GSK3B*) and Hedgehog pathway (*SMO, PTCH1, SHH, GLI1*).

The expression of five endogenous genes (*ACTB, GAPDH, GUSB, HPRT1 and CDKN1B*) was also tested in all samples in order to establish the more stable internal control. For this purpose, we used GeNorm software (see *Materials and Methods*), which indicated that the combination of *ACTB*, *GUSB* and *CDKN1B* was the most reliable option. Following the procedure proposed by Vandesompele *et al*., a normalization factor based on the expression of these three endogenous genes was calculated using the geometrical mean (Vandesompele *et*

*al.* 2002b). Results for CSCs-markers and signaling pathways are presented and discussed below.

### 4.4.1 CSC MARKERS EXPRESSION

For CSC markers study, *EPCAM1, ALDH1A1, CD133, ALCAM, ABCG2, CD44, BMI1* and *MUC1* were analyzed, as these genes were pointed out as possible markers for identifying CSCs in several tumors. Relative gene expression results for these genes with respect to the commercial reference cDNA are shown in Figure 12.

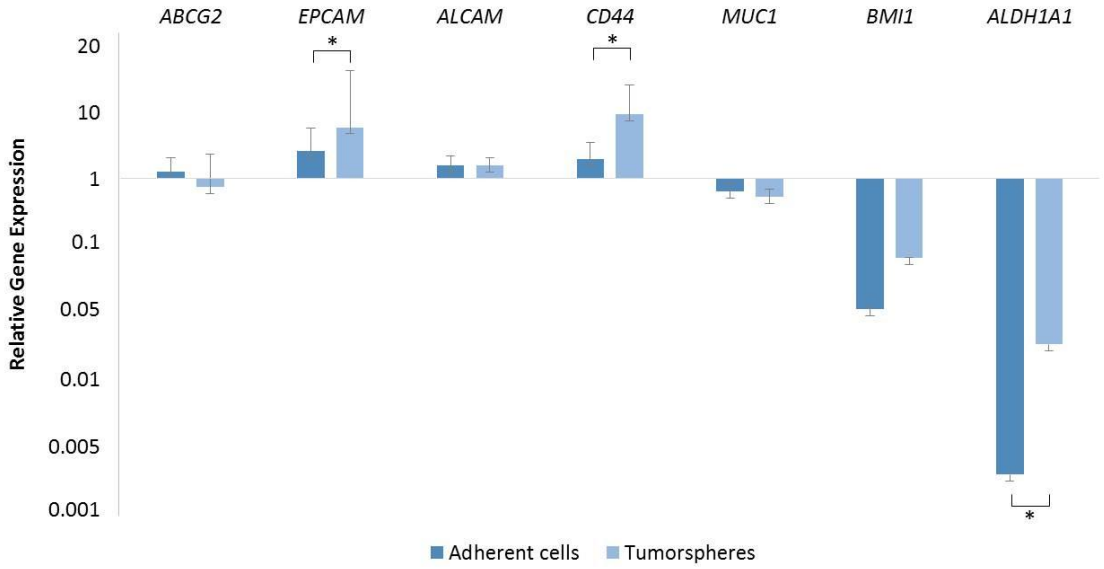



**Figure 12. Relative mRNA expression of CSC markers in adherent-cultured cells and tumorspheres.** Bars represent mean expression values ± standard deviation. *p<0.05.

Compared with the reference cDNA, both, adherent tumor cells and tumorspheres, showed overexpression of *EPCAM, ALCAM* and *CD44* (> 2x), whereas *ALDH1A1*, *MUC1* and *BMI1* expression values were underexpressed (<0.5x).

CSC markers gene expression was significant higher (Wilcoxon test) in tumorspheres when compared to adherent cells for *EPCAM* (7.72 *vs.* 4.10, p= 0.028), *CD44* (9.67 *vs.* 2.87, p= 0.021), and *ALDH1A1* (0.028 *vs.* 0.002, p= 0.043).

*EPCAM1, ALDH1A1* and *CD44* are three proposed biomarkers of CSCs in different solid tumors. Karimi-Busheri *et al.* proposed the overexpression of *EPCAM1* and *ALDH1A1* as a signature of enriched CSCs in H460 NSCLC cell line (Karimi-Busheri *et al.* 2011). Isolated $ALDH^+$ lung cancer cells were observed to be highly tumorigenic and clonogenic as well as capable of self-renewal compared with their $ALDH^-$ counterparts (Sullivan *et al.* 2010). Regarding the prognostic impact of this marker, a recent study on a cohort of stage I NSCLC patients, show

that high *ALDH1* expression is associated with poor survival (Huo *et al*. 2015). Regarding *EPCAM1* (also known as *CD326*) and *CD44*, our group has identified three compounds that alter CSCs expression of *EPCAM1*, *CD44* and *ALCAM* and trigger differentiation and cell death in A549 and patients-derived CSCs (Soto-Cerrato *et al.* 2015). Previous *in vivo* and *in vitro* experiments suggested that $CD133^{+}/CD326^{+}$ and $CD34^{+}/CD326^{+}$ subpopulations represent CSCs in lung primary tumors and cell lines, including A549 (Tirino *et al.* 2009; Lin *et al.* 2012). *CD44* was evaluated as a CSC marker in colon (Sahlberg *et al*. 2014), breast (de Beca *et al*. 2013), prostate (Liu *et al*. 2011) and gastric (Takaishi *et al*. 2009) cancers. In NSCLC patients, *CD44* expression was significantly higher in squamous cells carcinomas (SCC), and the increased expression of this gene was significantly correlated with higher grade tumors and poor prognosis (Roudi Raheleh *et al*. 2014).

We found no significant differences in the rest of the analyzed genes: *ALCAM*, *MUC1*, *BMI1*, and *ABCG2*, having similar levels of expression in anchorage-independent cells and in monolayer-cultured cells.

Even *ALCAM* (also known as *CD166*) has been proposed as a specific CSC marker for NSCLC (Zakaria *et al*. 2015), there are few data concerning their clinical implications in this pathology. In fact, a recent evaluation of this gene expression using immunohistochemistry in a large cohort of NSCLC patients (n= 1910) on a tissue microarray basis found an inverse association between its expression and tumor size and lymph node status (Tachezy *et al*. 2014). Our results are in concordance with these findings, indicating the doubts regarding the functional role of *ALCAM* in NSCLC-CSC biology. *MUC1* is another proposed marker for lung CSC. The detection of EpCAM/MUC1 mRNA-positive circulating tumor cells (CTCs) in blood before and after surgery is useful for predicting a poor prognosis in NSCLC patients who undergo surgery (Zhu *et al*. 2014). *MUC1* has also been associated with EMT and self-renewal through LIN28B-LET-7 pathway in NSCLC (Alam *et al*. 2015) and, current *in vitro* and *in vivo* studies have linked *MUC1* expression to chemoresistance in A549 cell line (Ham *et al*. 2016). Regarding *BMI1* and its transcriptional target, *ABCG2*, contradictory information have been reported about their involvement in CSCs biology and further investigation is required (Han *et al.* 2013; Su *et al.* 2015; Liang *et al.* 2015; Koren *et al.* 2016).

We have used three different TaqMan Gene Expression Assays for mRNA quantification of CD133, but the levels of expression of this gene were below the detection limit of the assays. There are a number of publications that focused on *CD133* as a potential lung CSC marker (Liu *et al.* 2006; Todaro *et al.* 2007; Eramo *et al.* 2008; Mizrak *et al.* 2008; Rappa *et al.* 2008; Smith

*et al.* 2008; Baumann *et al.* 2009). Nevertheless, some authors reported that the use of *CD133* expression to discriminate lung CSC is overstated (Zakaria *et al*. 2015). For example, some *CD133*$^-$ lung cancer cells also possess the ability to self-renew and generate the formation of xenograft when transplanted into recipient mice (Meng *et al*. 2009). In addition, CD133 expression in lung cancer is not associated with patient prognosis (Howard & Boockvar 2008; Tirino *et al.* 2009; Salnikov *et al.* 2010) and in many lung cancer samples, it could not be detected (Tirino *et al.* 2009; Bertolini *et al.* 2009; Salnikov *et al.* 2010), as it is reported in this study.

This is the first study that analysis CSC markers expression from early-stage NSCLC patient-derived CSCs by RTqPCR, which is a highly reliable and objective method, compared to other methods, such as immunohistochemistry or flow cytometry. The significant overexpression of *EPCAM1*, *CD44* and *ALDH1A1* CSC markers observed in lungspheres confirms the success of the tumorspheres formation assay for CSCs enrichment. Our group has confirmed that EpCAM and CD44 are highly expressed in tumorspheres obtained from cell lines and primary tumors by flow cytometry, and EpCAM$^+$/CD90$^-$ subpopulation are the ones able to induce tumor in xenotransplanted mouse model, demonstrating tumor-initiating capacity *in vivo.*

### 4.4.2 PLURIPOTENCY AND CELL CYCLE REGULATION GENE EXPRESSION

The genes selected in order to study pluripotency and cell cycle regulation in lungspheres and monolayer-cultured cells were *KLF4, OCT4, NANOG, SOX2, MYC* and *CCND1,* for pluripotency, and *CDKN1A, CDKN2A, MDM2* and *WEE1* for cell cycle control. A summary of the relative gene expression levels for these genes is shown in Figure 13.

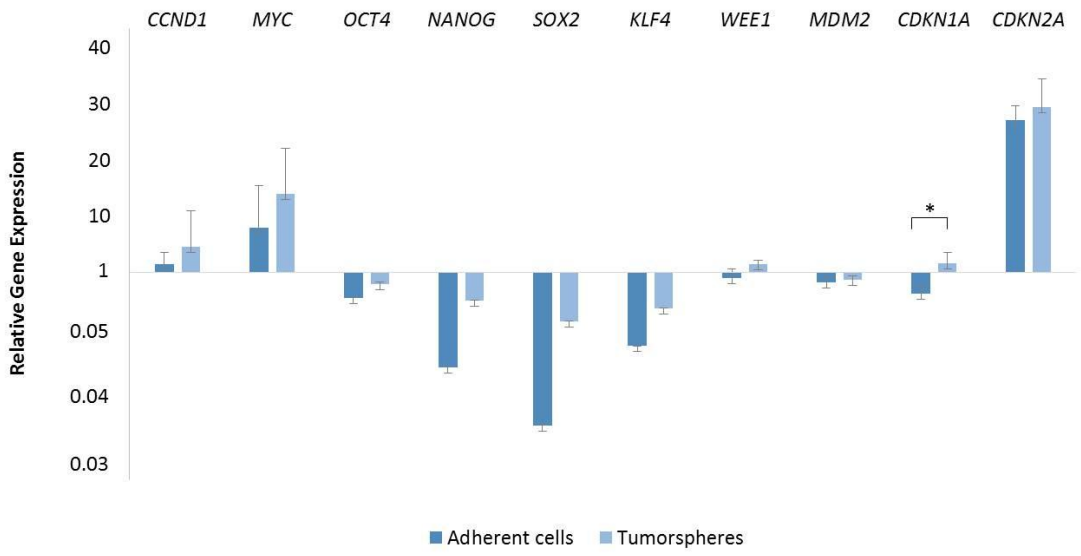



**Figure 13. Relative gene expression for analyzed pluripotency and cell cycle regulation genes.**
Bars represent mean expression values ± standard deviation. *p<0.05.

In general, expression values for classical pluripotency genes were lower in both tumorspheres and adherent cells compared with the reference cDNA used for normalization. On the other hand, *CCND1* and *MYC*, show higher levels of expression when compared with the reference sample. Regarding cell cycle related-genes, it is remarkable the high expression of *CDKN2A* in adherent cells as well as in spheroids.

As expected, all analyze genes had higher expression in lung oncospheres compared to monolayer cells. Wilcoxon analysis showed significant increased expression of *CDKN1A* (1.60 *vs.* 0.26, p = 0.021), and the same tendency was observed for *CCND1* (4.48 *vs.* 1.50, p = 0.051) and *SOX2* (0.11 *vs.* 0.04, p = 0.066).

In concordance with our results, elevated gene expression levels of pluripotency genes *CCND1, MYC, OCT4, NANOG, SOX2* and *KLF4* have been widely associated with CSC biology maintenance, tumor initiation, EMT induction, drug resistance and metastasis in many tumors, including lung cancer (Chiou *et al.* 2010; Tian *et al.* 2012; Liu *et al.* 2013; Wang *et al.* 2014; Slawek *et al.* 2015).

Pointing the focus on cell cycle regulators, our findings of *CDKN1A* overexpression in lungspheres is in concordance with previously published data. It has been reported that a quiescent state is necessary for preserving self-renewal of stem cells and is also a critical factor in CSC resistance to chemotherapy and targeted therapies (Abbas & Dutta 2009; Li & Bhatia 2011). Essers and Trumpp data revealed cytokines to be efficient agents for promoting cycling of leukemic stem cells and, most interestingly, such cell cycle activated stem cells become sensitive to killing by different chemotherapeutic agents (Essers & Trumpp 2010). In that sense, *CDKN1A* is a negative regulator of the cell cycle and it has been reported that DNA damage in stem cells activates *CDKN1A*, inhibits p53 and induces symmetric self-renewing divisions (Insinga *et al*. 2013).

Another interesting cell cycle regulator studied, *WEE1,* is an inhibitor of checkpoint kinases currently being tested in preclinical and clinical trials for NSCLC treatment (Syljuåsen *et al.* 2015). Glioblastoma stem cells studies indicate that high levels of *WEE1* may be required to maintain a stem-like state of CSCs (Forte *et al.* 2013), making this gene an attractive target for the development of new therapeutic strategies against lung-CSCs. *MDM2* oncogene has been reported to enhance stemness-promoting, being required for the efficient generation of induced pluripotent stem cells (Wienken *et al.* 2015). In that sense, *MDM2* inhibition promotes cell apoptosis and differentiation of CSCs (Daniele *et al.* 2015) and increased *MDM2* expression has been associated with poor clinical outcome of NSCLC patients (Javid *et al*. 2015). In

addition, several *MDM2* interactions and p53 mutations have been found in lung CSCs, making MDM2 a potential target in order to develop new therapies in lung cancer (Gadepalli *et al.* 2014). Finally, it has been found that loss of *CDKN2A* (p16) expression reduces the response of estrogen receptor-negative breast cancer chemotherapy and confers CSC properties (Arima *et al.* 2012). In addition, *CDKN2A* inactivation is common in NSCLC, and hypermethylation of *CDKN2A* was associated with a worse outcome in NSCLC patients with age at diagnosis of 60 years or younger (Bradly *et al.* 2012).

Our group has obtained promising expression results for genes related to pluripotency and cell cycle regulation by RTqPCR in concordance with some previous reported results by other methods. However, these results should be confirmed in a larger cohort of patients in order to obtain robust statistical results.

### 4.4.3 CELL ADHESION AND METASTASIS GENE EXPRESSION

Expression of genes related with cell adhesion and metastasis process were evaluated analyzing the following genes: *CDH1, CEACAM5, VIM, SNAI1, MMP2* and *MMP9.* Gene expression results for these genes are represented in Figure 14.

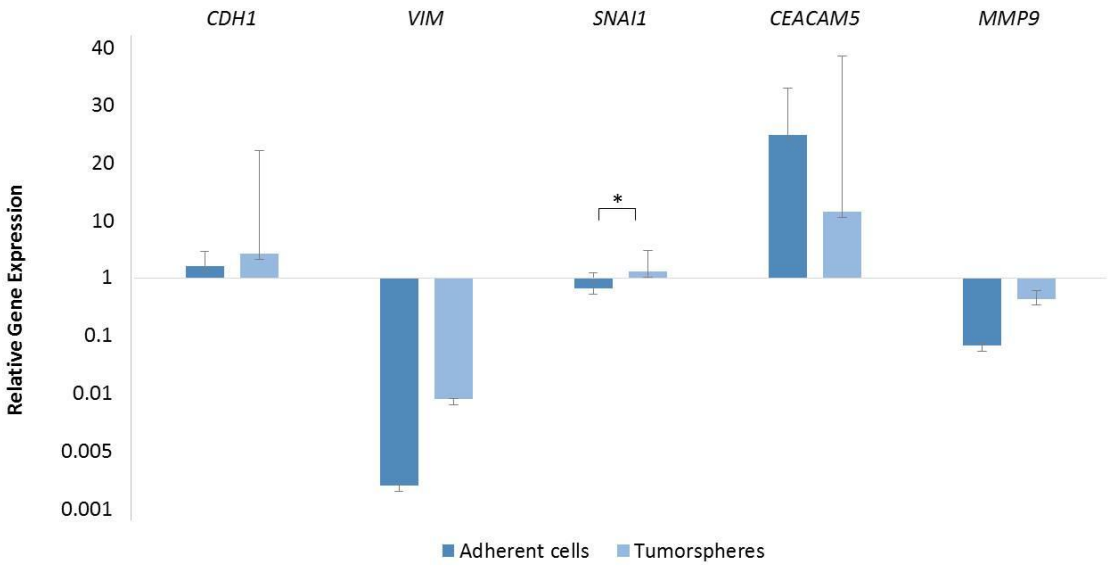



**Figure 14. Relative gene expression results for cell adhesion and metastasis-related genes.** Bars represent mean expression values ± standard deviation. *p<0.05.

*CDH1* (also known as *E-CADHERIN)* and *CEACAM5* expressions were higher than reference sample, whereas *VIM* and *MMP9* genes were underexpressed in the analyzed samples. There was a tendency to higher levels of expression in tumorspheres in comparison with their paired adherent culture cells in genes like *VIM* (0.008 *vs.* 0.004), and *MMP9* (0.27 *vs.* 0.08). We found a great degree of variation in *CDH1* and *CEACAM5* expression among analyzed samples.

Interestingly, we found that *SNAI1* expression, one of the major inducer of epithelial mesenchymal transition (EMT) expression was significantly increased in lungspheres compared to monolayer-cultured cells (1.16 *vs*. 0.58, p= 0.011).

In line with our results, the overexpression of *SNAI1* was related with CSC-like properties in a number of solid tumors such as thyroid (Yasui *et al*. 2013), colorectal (Fan *et al*. 2012), head and neck (Ota *et al*. 2016), pancreatic (Zhou *et al*. 2014), and lung (Wang *et al*. 2014) cancer. *SNAI1* is a known transcriptional repressor of *CDH1* and one of the key regulators of EMT. Particularly, in A549 cell line, it was reported that *SNAI1* expression directly correlates with *NANOG* expression, induction of EMT, and increasing of malignancy in mice models (Liu *et al.* 2014). To the best of our knowledge, this is the first study to illustrate the increased mRNA expression of *SNAI1* in lungspheres derived from primary NSCLC tumors, underlying its potential as a driver of EMT and metastasis and also as a very attractive therapeutic target in NSCLC.

No significant differences were obtained for the rest of genes analyzed, although a tendency to higher expression in lungspheres was observed for *VIM* and *MMP9*.

*MMP9* is a matrix metalloproteinase involved in extracellular matrix degradation and, particularly in lung cancer, induces both tumor growth and metastasis (Li *et al*. 2015). Increased expression of *MMP9* have been found in resected NSCLC patients (Liu *et al*. 2014), in A549 cell line, and in CSC-like cells with migratory capacity (Tirino *et al*. 2013). Regarding VIM expression, it has been described that cells that undergo EMT are characterized by a decrease in *E-CADHERIN* expression whereas the levels of *VIM* are increased (Chaw *et al.* 2012; Richardson *et al.* 2012; Zhang *et al.* 2015).

### 4.4.4 NOTCH SIGNALING PATHWAY EXPRESSION

Notch signaling pathway involvement in lung CSC was evaluated through the following key cascade components: *DLL1* and *DLL4* ligands, *NOTCH1, NOTCH2* and *NOTCH3* receptors and *HEY1* and *HES1* effector genes. Relative gene expression results for these genes are shown in Figure 15. Unfortunately, the levels of *DLL1* gene expression were below the limit of detection of the qPCR.

Comparisons between spheroids and monolayer cultures show that lungspheres had higher expression levels compared to adherent cells for the following genes: *DLL4* (0.009 *vs.* 0.001, p= 0.028), *NOTCH1* (1.16 *vs.* 0.41, p= 0.028), and *NOTCH2* (1.05 *vs.* 0.66, p= 0.036).

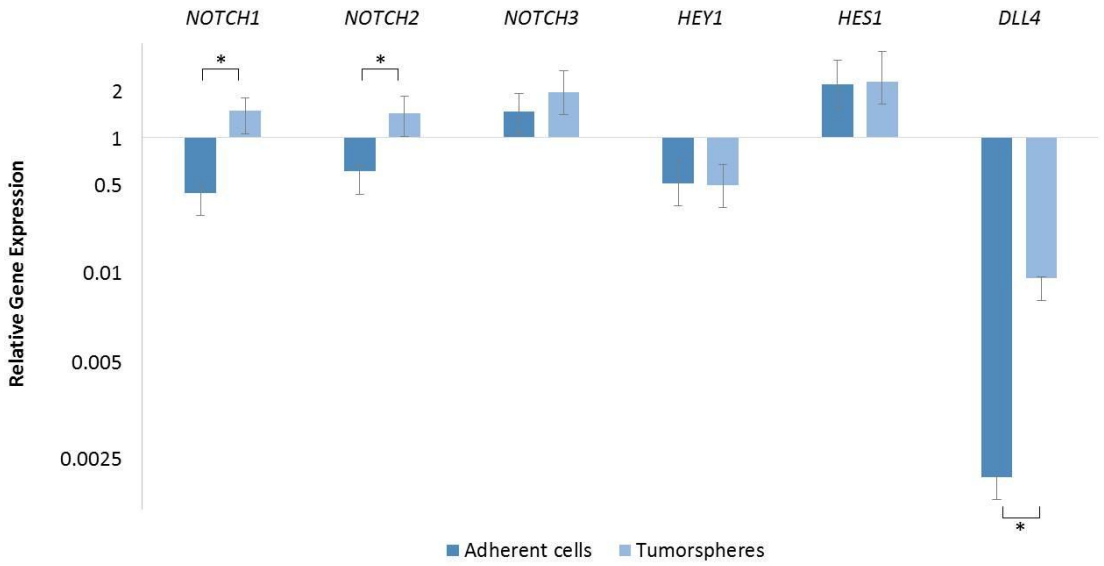



**Figure 15. Relative gene expression results for analyzed Notch signaling pathway genes.** Bars represent mean expression values ± standard deviation. *p<0.05.

These results suggest a possible activation of Notch signaling pathway in lung CSCs (Figure 16). Notch cascade activation in tumorspheres correlates with the *CDKN1A* and *SNAI1* overexpression (*CDKN1A-DLL4*, p =0.004; *SNAI1-DLL4*, p = 0.005) and would explain the CSC phenotype seen in anchorage independent cells. The role of Notch signaling in lung cancer was suggested when overexpression of Notch components was first detected in NSCLC, correlating with poor prognosis (Dang *et al.* 2000; Westhoff *et al.* 2009). Regarding CSCs in NSCLC, Hassan *et al.* reported the decisive role of Notch signaling for sphere formation and self-renewal *in vitro* and for tumor initiation and tumor heterogeneity formation *in vivo* (Hassan *et al.* 2013). *NOTCH1* overexpression has been significantly correlated with disease progression, metastasis and poorer prognosis of NSCLC patients (Zhou *et al.* 2015). Upregulation of *NOTCH2* has been associated with progression of early-stage lung adenocarcinoma and aggressive phenotype at advanced stages (Mimae *et al.* 2012). Moreover, targeting Notch signaling pathway with *NOTCH2*/*NOTCH3* antagonist Tarextumab inhibits tumor growth and decreases tumor-initiating cell frequency in patient-derived xenografts (Yen *et al.* 2015). Furthermore, *HES1* has been seen to enhance CSC phenotype, promoting cell proliferation, and migration by activating *BMI1* in colorectal cancer patients (Yuan *et al.* 2015; Gao *et al.* 2015).

There is accumulating evidence showing the importance of Notch signaling in the regulation of CSCs in numerous malignancies. Our results reinforce the implication of Notch signaling pathway in lung CSCs and highlight the potential of therapeutic targeting of this pathway as a strategy to abrogate the tumor initiation and metastatic capacity of lung-CSC.

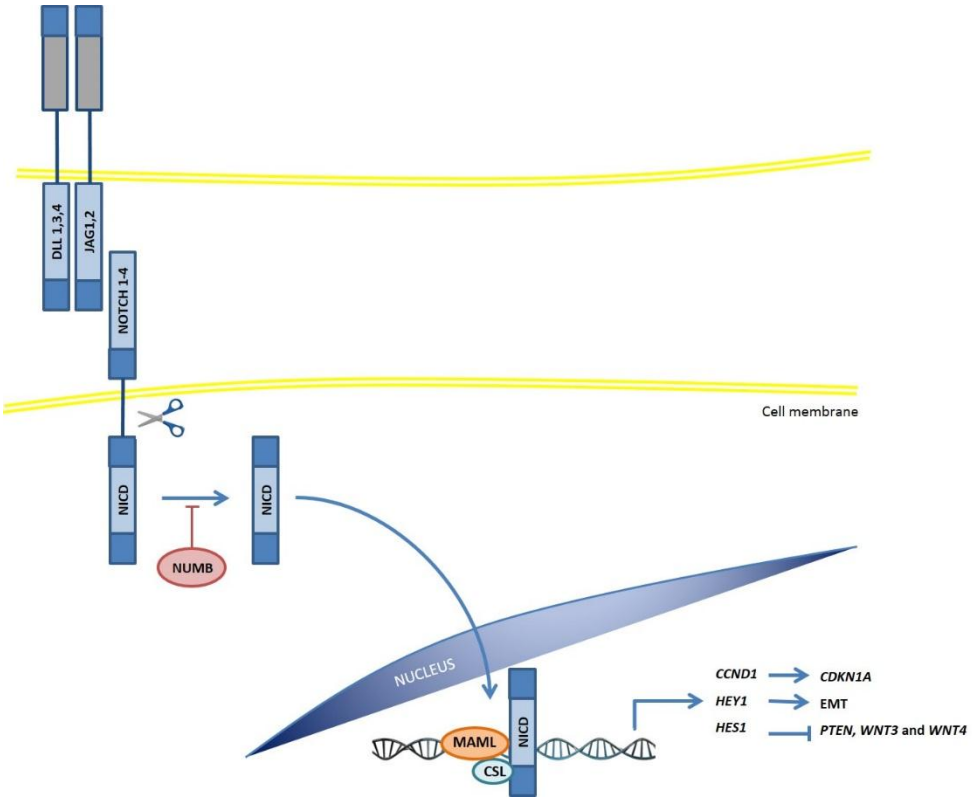



**Figure 16. Schematic representation of the main Notch signaling pathway components.**

### 4.4.5 WNT SIGNALING PATHWAY EXPRESSION

*CTNBB1, WNT1, WNT2, WNT3, WNT5A, DKK1, FZD7, GSK3B* gene expression was determined in order to analyze the activation status of the canonical and non-canonical Wnt signaling pathways. Figure 17 shows the relative gene expression results for these genes.

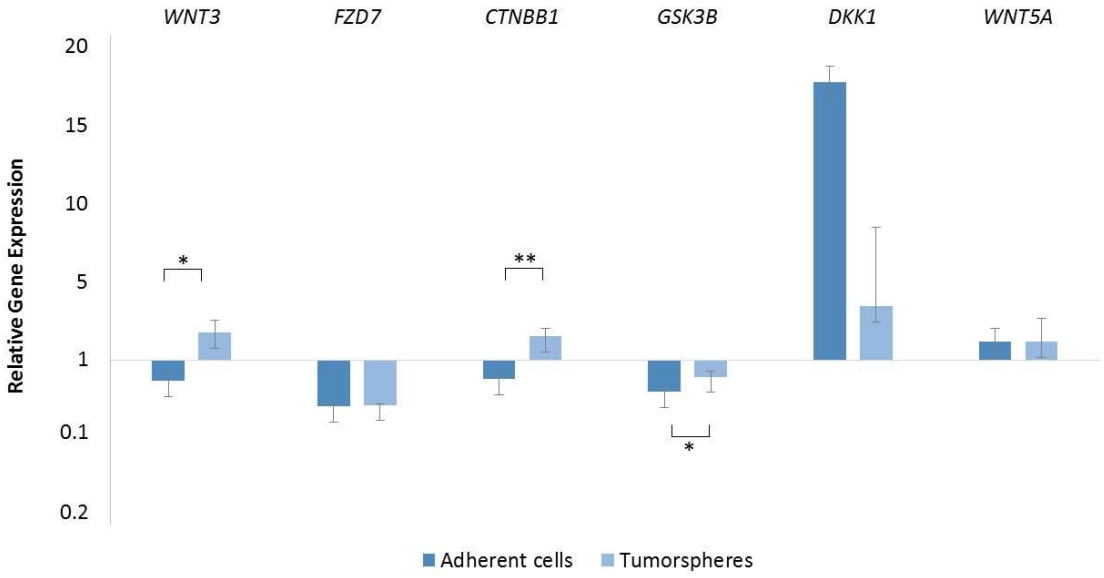



**Figure 17. Relative gene expression results for analyzed genes from Wnt signaling pathway.**
Bars represent mean expression values ± standard deviation. *p<0.05. **p<0.01.

Compared to the commercial reference cDNA, *DKK1* and *WNT5A* were highly expressed in both, non-adherent and adherent-cultured cells. Instead, *FZD7* receptor and *GSK3B* were less expressed in both of them whereas *WNT3* and *CTNBB1* gene expression was higher in lungspheres and lower in adherent cells.

Suppressors of the canonical Wnt signaling pathway, *DKK1* and *WNT5A*, were less expressed in tumorspheres than in monolayer cells, although the differences were not significant [3.46 *vs.* 17.73 and 1.19 *vs.* 1.21, respectively]. All the other genes showed higher expression in spheroids in relation to monolayer-cultured cells: barely higher for *FZD7* (0.35 *vs.* 0.33) and statistically significant for *WNT3* (1.76 *vs.* 0.76, p= 0.021), *CTNBB1* (1.53 *vs.* 0.85, p= 0.008) and *GSK3B* (0.95 *vs.* 0.50, p= 0.021). Independently of the set of primers selected for amplification (2 or 3 different TaqMan® Gene Expression Assays were tested), *WNT1* and *WNT2* genes had expression levels below the limit of detection of qPCR.

The overexpression detected in key genes of the WNT pathway suggests an activation of this signaling network in lung CSC and correlates with some finding already reported. Nakashima *et al*. found that *WNT3* promotes tumor progression in a study including 128 resected NSCLC patients (Nakashima *et al*. 2012). Additionally, an in vitro study correlates *WNT3* expression to metastasis, cell invasion, anchorage-independent growth, EMT-like morphological changes and F-actin reorganization in NSCLC cells (Li *et al*. 2015). On the other hand, increased expression of *CTNBB1* has been associated with *OCT4* and *CCND1* overexpression and resistance to a number of chemotherapeutic drugs in sorted lung CSCs (Jiang *et al*. 2015). Furthermore, knockdown of *CTNBB1* suppresses the metastatic potential of lung tumor xenografts (Chen *et al*. 2015). Regarding *GSK3B*, in the absence of Wnt proteins, GSK-3B phosphorylates β-catenin, resulting in ubiquitination and proteosomal degradation of β-catenin (Takahashi-Yanaga & Kahn 2010; Teng *et al*. 2010; Stewart 2014). However, when Wnt proteins are present, *GSK3B* functions remain unclear. Tivantinib has been found to target *GSK3B* and that pharmacological inhibition caused apoptosis in NSCLC cells (Remsing *et al*. 2014). Additionally, in a lung xenograft model, astrocyte elevated gene-1 (AEG-1) behaved as a critical protein in the activation of EMT by directly targeting *GSK3B* (He *et al*. 2015).

About inhibitors of the canonical Wnt pathway, the role of *DKK1* and *WNT5A* in NSCLC are not fully understood. Firstly, recombinants *DKK1* and *WNT5A* inhibited and increased, respectively, mice mammospheres formation (Many & Brown 2014). However, differential expression of *DKK1* has been found among cancer cells from 98 NSCLC patients (Xiang *et al*. 2015), and the overexpression of *DKK1* promoted migratory and invasive activity of in lung

cancer cell lines, suggesting an oncogenic role of *DKK1* in lung cancer (Li *et al*. 2013). Remarkably, loss of *WNT5A* in hepatocellular carcinoma has been associated with poor prognosis (Geng *et al*. 2012), but overexpression has also been correlated with unfavorable prognosis and angiogenesis promotion in NSCLC patients (Yao *et al*. 2014; Lu *et al*. 2015).

On our behalf, *DKK1* has proved tendency to overexpression in anchored cells compared to tumorspheres. The high variability between lungspheres would explain the lack of statistical significance. In contrast, *WNT5A* did not present appreciable differences between cell cultures. As it was mentioned above (see *Introduction*), canonical Wnt pathway can be inhibited in multiple ways. For instance, sFRPs compete with Wnt for binding to FZD, WIF-1 and Cerberus bind secreted Wnt, Dab2 and Dkk family inhibit Wnt signaling by binding to the LRP5/6 component of the Wnt receptor complex and HDPR1 and Idax antagonist Dvl. Our results suggest other pathway(s) as the main controller(s) of canonical Wnt pathway in NSCLC and agree with authors pointing out alternative roles of *WNT5A* in non-canonical Wnt pathways for NSCLC.

### 4.4.6 HEDGEHOG SIGNALING PATHWAY EXPRESSION

Activity of Hedgehog pathway was evaluated through core components of this signaling cascade: *SHH, SMO, PTCH1, GLI1* genes. Relative gene expression results for hedgehog pathway genes are shown in Figure 18.

Relative gene expression analysis revealed a reduced expression of *SMO* and *PTCH1* in both tumorspheres and adherent cells. In addition, both genes were less expressed in lungspheres than in monolayer-cultured cells: 0.11 *vs.* 0.21, for *SMO* and 0.15 *vs.* 0.19, for *PTCH1,* but differences were not significant. As it was observed with other genes included in the present study, the expression of *SHH* and *GLI1* were below the limit of detection of our RTqPCR assays. These results are in concordance with previous results in which NSCLC cell lines were negative for *SHH* expression but positive in the case of *GLI1* (Watkins *et al.* 2003). In addition, analysis of clinical samples of human lung cancer tissue demonstrated that 50% of SCLC expressed both *SHH* and *GLI1* compared to only the 10% found in NSCLC (Velcheti & Govindan 2007). In the same line, less than 9% of lung cancers have been found to have at least 2 hedgehog signaling-related genes expressed and immunohistochemistry in NSCLC metastases identified only a 10% of *PTCH1* protein positive staining, suggesting that activation of hedgehog pathway is not specifically associated with NSCLC (Chi *et al*. 2006).

Many cancer types have been linked to aberrant hedgehog signaling in CSCs, including oral cancer, esophageal cancer and SCLC (Varjosalo & Taipale 2008; Cochrane *et al*. 2015), but in the case of lung CSC, the role of HH pathways remains unclear (Giroux Leprieur *et al*. 2015).

Thus, further studies are required in order to determine the real potential of targeting this pathway in NSCLC.

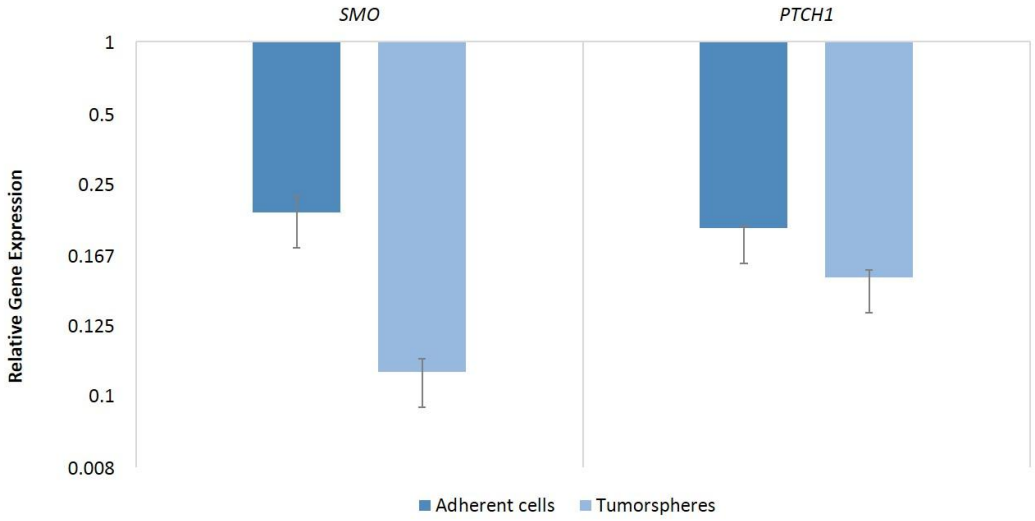



**Figure 18. Relative gene expression results for analyzed genes from Hedgehog signaling pathway.** Bars represent mean expression values ± standard deviation.

In summary, spheroids are excellent models for the study of CSC biology. The possibility to isolate oncospheres from patients' samples is particularly valuable due to the possible clinical implications in the generation of new therapeutic and personalized approaches. The results of this study are in concordance with our data for flow cytometry and *in vivo* assays (Soto-Cerrato *et al.* 2015), and reveal potential genes for CSCs targeting. Our results propose three specific lung CSC markers and add important data for a major role of Notch and Wnt pathways in regulating proliferation, survival, self-renewal and tumorigenicity of CSCs from NSCLC (Figure 19). To our knowledge, this is the first time that isolated CSCs from early-stage NSCLC patients have been analyzed by a highly reliable and objective method like RTqPCR.

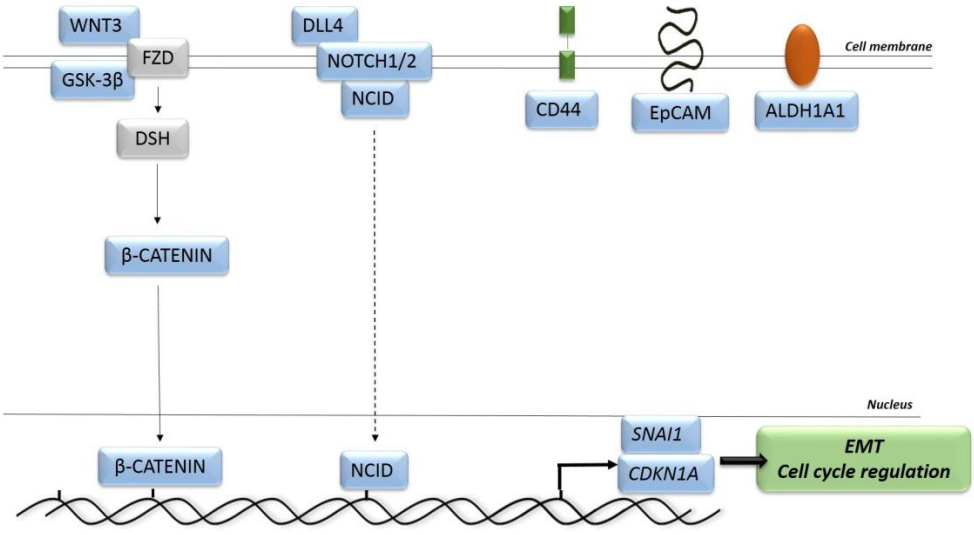



**Figure 19. Schematic representation of the main results obtained in this study.**

# 5. CONCLUSIONS

1. The non-adherent, serum-free *in vitro* culture was a satisfactory method for CSCs isolation and enrichment, permitting to obtain oncospheres from lung cancer cell lines and from resected NSCLC tumors as well.

2. Spheroids are suitable *in vitro* models for CSC analysis, that allow isolating RNA samples of excellent quality to perform extensive expression analysis and profiling.

3. Characterization of oncospheres derived from lung cancer cell lines and patients' samples reveals a significant overexpression of the CSC markers *EPCAM1*, *ALDHA1* and *CD44* in these cells compared with their paired monolayer cultures.

4. Lung tumorspheres had significant overexpression of genes related to WNT (*WNT3, CTNBB1* and *GSK3B*), NOTCH (*DLL4, NOTCH1* and *NOTCH2*) pathways, quiescent state (*CDKN1A*) and EMT (*SNAI1*) when compared to adherent cells, suggesting the involvement of them in lung CSC biology and maintenance. In consequence, these genes would be interesting therapeutic targets for CSCs and biomarkers in NSCLC.

# 6. REFERENCES


Abbas, T. & Dutta, A., 2009. p21 in cancer: intricate networks and multiple activities. *Nature reviews. Cancer*, 9(6), pp.400–414.

Akiri, G. *et al.*, 2009. Wnt pathway aberrations including autocrine Wnt activation occur at high frequency in human non-small-cell lung carcinoma. *Oncogene*, 28(21), pp.2163–2172.

Alam, M. *et al.*, 2015. MUC1-C Induces the LIN28B-->LET-7-->HMGA2 Axis to Regulate Self-Renewal in NSCLC. *Molecular cancer research : MCR*, 13(3), pp.449–460.

Alison, M.R., Lim, S.M.L. & Nicholson, L.J., 2011. Cancer stem cells: problems for therapy? *The Journal of Pathology*, 223(2), pp.148–162.

Allen, T.D. *et al.*, 2011. Activated NOTCH1 induces lung adenomas in mice and cooperates with MYC in the generation of lung adenocarcinoma. *Cancer research*, 71(18), pp.6010–6018. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3174331/.

Anastas, J.N. & Moon, R.T., 2012a. WNT signaling pathways as therapeutic targets in cancer. *Nature Reviews Cancer*, 13(1), pp.11–26.

Anastas, J.N. & Moon, R.T., 2012b. WNT signaling pathways as therapeutic targets in cancer. *Nature Reviews Cancer*, 13(1), pp.11–26.

Arima, Y. *et al.*, 2012. Loss of p16 expression is associated with the stem cell characteristics of surface markers and therapeutic resistance in estrogen receptor-negative breast cancer. *International Journal of Cancer*, 130(11), pp.2568–2579.

Ball, D.W. *et al.*, 1993. Identification of a human achaete-scute homolog highly expressed in neuroendocrine tumors. *Proceedings of the National Academy of Sciences* , 90 (12 ), pp.5648–5652.

Baumann, M. *et al.*, 2009. Cancer stem cells and radiotherapy. *International Journal of Radiation Biology*, 85(5), pp.391–402.

de Beca, F.F. *et al.*, 2013. Cancer stem cells markers CD44, CD24 and ALDH1 in breast cancer special histological types. *Journal of clinical pathology*, 66(3), pp.187–191.

Bertolini, G. *et al.*, 2009. Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proc.Natl.Acad.Sci.U.S.A*, 106(38), pp.16281–16286.

Berwick, D.C. & Harvey, K., 2012. The importance of Wnt signaling for neurodegeneration in Parkinson's disease. *Biochemical Society transactions*, 40(5), pp.1123–8.

Bonnet, D. & Dick, J.E., 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine*, 3(7), pp.730–737.

Bosetti, C. *et al.*, 2013. Cancer mortality in Europe, 2005-2009, and an overview of trends since 1980. *Annals of Oncology*, 24(10), pp.2657–2671.

Bradly, D.P. *et al.*, 2012. CDKN2A (p16) Promoter Hypermethylation Influences the Outcome in Young Lung Cancer Patients. *Diagnostic Molecular Pathology*, 21(4).

Brou, C. *et al.*, 2000. A Novel Proteolytic Cleavage Involved in Notch Signaling. *Molecular Cell*,

5(2), pp.207–216.

Burrell, R.A. *et al.*, 2013. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature*, 501, pp.338–345.

Chaw, S.Y. *et al.*, 2012. Epithelial to mesenchymal transition (EMT) biomarkers--E-cadherin, beta-catenin, APC and Vimentin--in oral squamous cell carcinogenesis and transformation. *Oral oncology*, 48(10), pp.997–1006.

Chen, H. *et al.*, 1997. Conservation of the Drosophila lateral inhibition pathway in human lung cancer: A hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. *Proceedings of the National Academy of Sciences* , 94 (10 ), pp.5355–5360.

Chen, X. *et al.*, 2015. Fibulin-5 inhibits Wnt/beta-catenin signaling in lung cancer. *Oncotarget*, 6(17), pp.15022–15034.

Chen, Y. *et al.*, 2011. Notch-1 signaling facilitates survivin expression in human non-small cell lung cancer cells. *Cancer Biol Ther*, 11.

Chen, Z. *et al.*, 2009. Oct4, a novel marker for human gastric cancer. *Journal of Surgical Oncology*, 99(7), pp.414–419. Available at: http://dx.doi.org/10.1002/jso.21270.

Cheng, L. *et al.*, 2009. Implications of Cancer Stem Cells for Cancer Therapy. *Cancer Drug Discovery and Development*, (1), pp.255–262.

Chi, S. *et al.*, 2006. Activation of the hedgehog pathway in a subset of lung cancers. *Cancer Letters*, 244(1), pp.53–60.

Chiba, S., 2006. Concise Review: Notch Signaling in Stem Cell Systems. *STEM CELLS*, 24(11), pp.2437–2447.

Chilosi, M. *et al.*, 2003. Aberrant Wnt/β-Catenin Pathway Activation in Idiopathic Pulmonary Fibrosis. *The American Journal of Pathology*, 162(5), pp.1495–1502.

Chiou, S.H. *et al.*, 2010. Coexpression of Oct4 and Nanog enhances malignancy in lung adenocarcinoma by inducing cancer stem cell-like properties and epithelial-mesenchymal transdifferentiation. *Cancer Res.*, 70(24), pp.10433–10444.

Clevers, H. & Nusse, R., 2012. Wnt/??-catenin signaling and disease. *Cell*, 149(6), pp.1192–1205.

Cochrane, C. *et al.*, 2015. Hedgehog Signaling in the Maintenance of Cancer Stem Cells. *Cancers*, 7(3), pp.1554–1585. Available at: http://www.mdpi.com/2072-6694/7/3/0851/.

Croker, A.K. & Allan, A.L., 2011. Inhibition of aldehyde dehydrogenase (ALDH) activity reduces chemotherapy and radiation resistance of stem-like ALDHhiCD44+ human breast cancer cells. *Breast Cancer Research and Treatment*, 133(1), pp.75–87.

Dang, T.P. *et al.*, 2000. Chromosome 19 translocation, overexpression of Notch3, and human lung cancer. *J Natl Cancer Inst*, 92.

Daniele, S. *et al.*, 2015. Combined inhibition of AKT/mTOR and MDM2 enhances Glioblastoma Multiforme cell apoptosis and differentiation of cancer stem cells. *Scientific Reports*, 5, p.9956.

Dees, C. & Distler, J.H.W., 2013. Canonical Wnt signaling as a key regulator of fibrogenesis –

implications for targeted therapies? *Experimental Dermatology*, 22(11), pp.710–713. Available at: http://dx.doi.org/10.1111/exd.12255.

Denef, N. *et al.*, 2000. Hedgehog Induces Opposite Changes in Turnover and Subcellular Localization of Patched and Smoothened. *Cell*, 102(4), pp.521–531.

Dick, J.E., 2008. Stem cell concepts renew cancer research. *Blood*, 112(13), pp.4793–4807.

Dou, J. *et al.*, 2007. Isolation and identification of cancer stem-like cells from murine melanoma cell lines. *Cellular & molecular immunology*, 4(6), pp.467–472.

Dou, J. & Gu, N., 2010. Emerging strategies for the identification and targeting of cancer stem cells. *Tumor Biology*, 31(4), pp.243–253.

Downward, J., Targeting RAS signaling pathways in cancer therapy. *Nat.Rev.Cancer*, 3(1), pp.11–22.

Egloff, A.M. & Grandis, J.R., 2012. Molecular Pathways: Context-dependent approaches to Notch targeting as cancer therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 18(19), pp.5188–5195.

Eliasz, S. *et al.*, 2010. Notch-1 stimulates survival of lung adenocarcinoma cells during hypoxia by activating the IGF-1R pathway. *Oncogene*, 29.

Eramo, A. *et al.*, 2008. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death.Differ.*, 15(3), pp.504–514.

Essers, M.A.G. & Trumpp, A., 2010. Targeting leukemic stem cells by breaking their dormancy. *Molecular oncology*, 4(5), pp.443–450.

Fan, F. *et al.*, 2012. Overexpression of snail induces epithelial-mesenchymal transition and a cancer stem cell-like phenotype in human colorectal cancer cells. *Cancer medicine*, 1(1), pp.5–16.

Felip, E. *et al.*, 2010. Preoperative chemotherapy plus surgery versus surgery plus adjuvant chemotherapy versus surgery alone in early-stage non-small-cell lung cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 28(19), pp.3138–3145.

Ferlay, J. *et al.*, 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int.J.Cancer*, 136(5), pp.E359–86.

Forte, S. *et al.*, 2013. Gene expression analysis of PTEN positive glioblastoma stem cells identifies DUB3 and Wee1 modulation in a cell differentiation model. *PloS one*, 8(12), p.e81432.

Fortini, M.E., 2009. Notch signaling: the core pathway and its posttranslational regulation. *Developmental cell*, 16(5), pp.633–47.

Franch-Marro, X. *et al.*, 2008. Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. *Nat Cell Biol*, 10(2), pp.170–177.

Freedman, N.D. *et al.*, 2008. Cigarette smoking and subsequent risk of lung cancer in men and women: analysis of a prospective cohort study. *Lancet Oncol.*, 9(7), pp.649–656.

Fry, J.S. *et al.*, 2013. How rapidly does the excess risk of lung cancer decline following quitting

smoking? A quantitative review using the negative exponential model. *Regulatory Toxicology and Pharmacology*, 67(1), pp.13–26.

Gadepalli, V.S. *et al.*, 2014. Lung Cancer Stem Cells, p53 Mutations and MDM2 BT - Mutant p53 and MDM2 in Cancer. In P. S. Deb & S. Deb, eds. Dordrecht: Springer Netherlands, pp. 359–370.

Gao, F. *et al.*, 2015. Hes1 promotes cell proliferation and migration by activating Bmi-1 and PTEN/Akt/GSK3beta pathway in human colon cancer. *Oncotarget*, 6(36), pp.38667–38680.

Geng, M. *et al.*, 2012. Loss of Wnt5a and Ror2 protein in hepatocellular carcinoma associated with poor prognosis. *World journal of gastroenterology*, 18(12), pp.1328–1338.

Ghani, F.I. *et al.*, 2011. Identification of cancer stem cell markers in human malignant mesothelioma cells. *Biochemical and biophysical research communications*, 404(2), pp.735–42.

Giroux Leprieur, E. *et al.*, 2015. [Role of the Sonic Hedgehog pathway in thoracic cancers]. *Revue des maladies respiratoires*, 32(8), pp.800–808.

Goldstraw, P. *et al.*, The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumors. *J.Thorac.Oncol.*, 2(8), pp.706–714.

Gonczy, P., 2008. Mechanisms of asymmetric cell division: flies and worms pave the way. *Nat Rev Mol Cell Biol*, 9(5), pp.355–366.

Greaves, M. & Maley, C.C., 2012. Clonal Evolution in Cancer. *Nature*, 481(7381), pp.306–313.

Gridelli, C. *et al.*, 2015. Non-small-cell lung cancer. *Nature Reviews Disease Primers*, p.15009. Available at: http://dx.doi.org/10.1038/nrdp.2015.9.

de Groot, P. & Munden, R.F., 2012. Lung Cancer Epidemiology, Risk Factors, and Prevention. *Radiologic Clinics of North America*, 50(5), pp.863–876.

Hahn, H. *et al.*, 1996. Mutations of the Human Homolog of Drosophila patched in the Nevoid Basal Cell Carcinoma Syndrome. *Cell*, 85(6), pp.841–851.

Ham, S.Y. *et al.*, 2016. Mucin 1-mediated chemo-resistance in lung cancer cells. *Oncogenesis*, 5(1), p.e185. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4728677/.

Han, L. *et al.*, 2013. Cancer stem cells: therapeutic implications and perspectives in cancer therapy. *Acta Pharmaceutica Sinica B*, 3(2), pp.65–75.

Hanahan, D. & Weinberg, R., 2000. The Hallmarks of Cancer. *Cell*, 100, pp.57–70.

Hanahan, D. & Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell*, 144(5), pp.646–74.

Hanahan, D. & Weinberg, R.A., 2000. The hallmarks of cancer. *Cell*, 100(1), pp.57–70.

Hassan, K.A. *et al.*, 2013. Notch pathway activity identifies cells with cancer stem cell-like properties and correlates with worse survival in lung adenocarcinoma. *Clinical Cancer Research*, 19(8), pp.1972–1980.

He, B. *et al.*, 2004. A Monoclonal Antibody against Wnt-1 Induces Apoptosis in Human Cancer Cells. *Neoplasia (New York, N.Y.)*, 6(1), pp.7–14.

He, W. *et al.*, 2015. Astrocyte elevated gene-1(AEG-1) induces epithelial-mesenchymal transition in lung cancer through activating Wnt/beta-catenin signaling. *BMC cancer*, 15, p.107.

Hirsch, F.R. *et al.*, Increased epidermal growth factor receptor gene copy number detected by fluorescence in situ hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a Southwest Oncology Group Study. *J.Clin.Oncol.*, 23(28), pp.6838–6845.

Hirsch, F.R. *et al.*, 2010. The tissue is the issue: personalized medicine for non-small cell lung cancer. *Clin.Cancer Res.*, 16(20), pp.4909–4911.

Howard, B. & Boockvar, J., 2008. Stem cell marker CD133 expression predicts outcome in glioma patients. *Neurosurgery*, 62(6), p.N6.

Huo, W. *et al.*, 2015. Prognostic value of ALDH1 expression in lung cancer: a meta-analysis. *International Journal of Clinical and Experimental Medicine*, 8(2), pp.2045–2051.

Inestrosa, N.C., Montecinos-Oliva, C. & Fuenzalida, M., 2012. Wnt Signaling: Role in Alzheimer Disease and Schizophrenia. *Journal of Neuroimmune Pharmacology*, 7(4), pp.788–807.

Insinga, A. *et al.*, 2013. DNA damage in stem cells activates p21, inhibits p53, and induces symmetric self-renewing divisions. *Proceedings of the National Academy of Sciences of the United States of America*, 110(10), pp.3931–3936.

Iso, T., Kedes, L. & Hamamori, Y., 2003. HES and HERP families: Multiple effectors of the notch signaling pathway. *Journal of Cellular Physiology*, 194(3), pp.237–255. Available at: http://dx.doi.org/10.1002/jcp.10208.

Jantus-Lewintre, E. *et al.*, 2012. Update on biomarkers for the detection of lung cancer. *Lung Cancer: Targets and Therapy.*, 3(-), pp.21–29.

Javid, J. *et al.*, 2015. Association of p53 and mdm2 in the development and progression of non-small cell lung cancer. *Tumor biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, 36(7), pp.5425–5432.

Jiang, H.-L., Jiang, L.-M. & Han, W.-D., 2015. Wnt/beta-catenin signaling pathway in lung cancer stem cells is a potential target for the development of novel anticancer drugs. *Journal of B.U.ON. : official journal of the Balkan Union of Oncology*, 20(4), pp.1094–1100.

Johnson, R.L. *et al.*, 1996. Human Homolog of patched, a Candidate Gene for the Basal Cell Nevus Syndrome. *Science*, 272(5268), pp.1668–1671.

Kageyama, R. *et al.*, 2005. Roles of bHLH genes in neural stem cell differentiation. *Experimental cell research*, 306(2), pp.343–8.

Kahn, M., 2014. Can we safely target the WNT pathway? *Nature Reviews Drug Discovery*, 13(2), pp.513–532.

Kalderon, D., 2000. Transducing the Hedgehog Signal. *Cell*, 103(3), pp.371–374.

Karimi-Busheri, F. *et al.*, 2011. Pivotal role of CD38 biomarker in combination with CD24, EpCAM, and ALDH for identification of H460 derived lung cancer stem cells. *Journal of*

*stem cells*, 6(1), pp.9–20.

Karnoub, A.E. & Weinberg, R.A., 2008. Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol*, 9(7), pp.517–531.

Kim, C.F.B. *et al.*, 2005. Identification of Bronchioalveolar Stem Cells in Normal Lung and Lung Cancer. *Cell*, 121(6), pp.823–835.

Kinzler, K.W. & Vogelstein, B., 1996. Lessons from Hereditary Colorectal Cancer. *Cell*, 87(2), pp.159–170.

Kitamura, H. *et al.*, 2009. Cancer stem cell: implications in cancer biology and therapy with special reference to lung cancer. *Lung Cancer*, 66(3), pp.275–281.

Koch, U., Krause, M. & Baumann, M., 2010. Cancer stem cells at the crossroads of current cancer therapy failures--radiation oncology perspective. *Seminars in cancer biology*, 20(2), pp.116–24.

Koren, A. *et al.*, 2016. BMI1, ALDH1A1, and CD133 Transcripts Connect Epithelial-Mesenchymal Transition to Cancer Stem Cells in Lung Carcinoma. *Stem Cells International*, 2016, p.9714315.

Korinek, V. *et al.*, 1997. Constitutive Transcriptional Activation by a β-Catenin-Tcf Complex in APC−/− Colon Carcinoma. *Science*, 275(5307), pp.1784–1787.

Kreso, A. & Dick, J.E., 2014. Evolution of the Cancer Stem Cell Model. *Cell Stem Cell*, 14(3), pp.275–291.

de La Coste, A. *et al.*, 1998. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proceedings of the National Academy of Sciences of the United States of America*, 95(15), pp.8847–51.

Ladanyi, M., 2008. Targeted therapy of cancer: new roles for pathologists. *Mod.Pathol.*, 21 Suppl 2, p.S1–.

Ladi, E. *et al.*, 2005. The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. *The Journal of Cell Biology*, 170(6), pp.983–992.

Landau, D.-A. *et al.*, 2014. Clonal evolution in hematologic malignancies and therapeutic implications. *Leukemia*, 28(1), pp.34–43.

Lee, J.S. *et al.*, 2012. A novel sLRP6E1E2 inhibits canonical Wnt signaling, epithelial-to-mesenchymal transition, and induces mitochondria-dependent apoptosis in lung cancer. *PLoS ONE*, 7(5).

Lemjabbar-Alaoui, H. *et al.*, 2006. Wnt and Hedgehog Are Critical Mediators of Cigarette Smoke-Induced Lung Cancer C.-P. Heisenberg, ed. *PLoS ONE*, 1(1), p.e93.

Li, A.R. *et al.*, 2008. EGFR mutations in lung adenocarcinomas: clinical testing experience and relationship to EGFR gene copy number and immunohistochemical expression. *J.Mol.Diagn.*, 10(3), pp.242–248.

Li, C. *et al.*, 2007. Identification of pancreatic cancer stem cells. *Cancer Res.*, 67(3), pp.1030–1037.

Li, C. *et al.*, 2015. Wnt3a increases the metastatic potential of non-small cell lung cancer cells in vitro in part via its upregulation of Notch3. *Oncology reports*, 33(3), pp.1207–1214.

Li, J. *et al.*, 2015. MiR-129 regulates MMP9 to control metastasis of non-small cell lung cancer. *Tumor biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, 36(8), pp.5785–5790.

Li, L. & Bhatia, R., 2011. Stem cell quiescence. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 17(15), pp.4936–4941.

Li, S. *et al.*, 2013. Dickkopf-1 Is Oncogenic and Involved in Invasive Growth in Non Small Cell Lung Cancer S. Deb, ed. *PLoS ONE*, 8(12), p.e84944.

Liang, S.-C. *et al.*, 2015. ABCG2 localizes to the nucleus and modulates CDH1 expression in lung cancer cells. *Neoplasia (New York, N.Y.)*, 17(3), pp.265–278.

Licchesi, J.D.F. *et al.*, 2008. Epigenetic alteration of Wnt pathway antagonists in progressive glandular neoplasia of the lung. *Carcinogenesis*, 29(5), pp.895–904.

Licciulli, S. *et al.*, 2013. Notch1 is required for Kras-induced lung adenocarcinoma and controls tumor cell survival via p53. *Cancer Res*, 73.

Lin, S. *et al.*, 2012. Aberrant microRNAs Expression in CD133(+)/CD326(+) Human Lung Adenocarcinoma Initiating Cells from A549. *Molecules and Cells*, 33(3), pp.277–283.

Liu, C. *et al.*, 2011. Identification of miR-34a as a potent inhibitor of prostate cancer progenitor cells and metastasis by directly repressing CD44. *Nature medicine*, 17(2), pp.211–215.

Liu, C.-W. *et al.*, 2014. Snail regulates Nanog status during the epithelial–mesenchymal transition via the Smad1/Akt/GSK3β signaling pathway in non-small-cell lung cancer. *Oncotarget*, 5(11), pp.3880–3894.

Liu, F. *et al.*, 2010. Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. *Oncogene*, 29(25), pp.3650–3664. Available at: http://dx.doi.org/10.1038/onc.2010.129.

Liu, G. *et al.*, 2006. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol.Cancer*, 5, p.67–.

Liu, J. *et al.*, 2014. Correlations of lysyl oxidase with MMP2/MMP9 expression and its prognostic value in non-small cell lung cancer. *International journal of clinical and experimental pathology*, 7(9), pp.6040–6047.

Liu, J. *et al.*, 2013. Lung cancer tumorigenicity and drug resistance are maintained through ALDH(hi)CD44(hi) tumor initiating cells. *Oncotarget*, 4.

Lortet-Tieulent, J. *et al.*, 2014. International trends in lung cancer incidence by histological subtype: Adenocarcinoma stabilizing in men but still increasing in women. *Lung Cancer*, 84(1), pp.13–22.

Lu, C. *et al.*, 2015. Over-expression of ROR2 and Wnt5a cooperatively correlates with unfavorable prognosis in patients with non-small cell lung cancer. *Oncotarget*, 6(28), pp.24912–24921.

Ma, I. & Allan, A.L., 2010. The Role of Human Aldehyde Dehydrogenase in Normal and Cancer Stem Cells. *Stem Cell Reviews and Reports*, 7(2), pp.292–306.

Malvezzi, M. *et al.*, 2014. European cancer mortality predictions for the year 2014. *Annals of Oncology*, 25(8), pp.1650–1656.

Mannelli, G. & Gallo, O., 2012. Cancer stem cells hypothesis and stem cells in head and neck cancers. *Cancer Treatment Reviews*, 38(5), pp.515–539.

Many, A.M. & Brown, A.M.C., 2014. Both canonical and non-canonical Wnt signaling independently promote stem cell growth in mammospheres. *PloS one*, 9(7), p.e101800.

Marcato, P. *et al.*, 2011. Aldehyde dehydrogenase: Its role as a cancer stem cell marker comes down to the specific isoform. *Cell Cycle*, 10(9), pp.1378–1384. Available at: http://dx.doi.org/10.4161/cc.10.9.15486.

Marusyk, A., Almendro, V. & Polyak, K., 2012. Intra-tumor heterogeneity: a looking glass for cancer? *Nature Reviews Cancer*, 12(5), pp.323–334. Available at: http://dx.doi.org/10.1038/nrc3261.

Mazieres, J. *et al.*, 2005. Wnt signaling in lung cancer. *Cancer letters*, 222(1), pp.1–10. McCaffrey, L.M. & Macara, I.G., 2011. Epithelial organization, cell polarity and tumorigenesis. *Trends in cell biology*, 21(12), pp.727–35.

Meacham, C.E. & Morrison, S.J., 2013. Tumor heterogeneity and cancer cell plasticity. *Nature*, 501(7467), pp.328–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24048065.

Medema, J.P., 2013. Cancer stem cells: the challenges ahead. *Nature cell biology*, 15(4), pp.338–44.

Meng, X. *et al.*, 2009. Both CD133+ and CD133− subpopulations of A549 and H446 cells contain cancer-initiating cells. *Cancer Science*, 100(6), pp.1040–1046.

Mimae, T. *et al.*, 2012. Upregulation of notch2 and six1 is associated with progression of early-stage lung adenocarcinoma and a more aggressive phenotype at advanced stages. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 18(4), pp.945–955.

Mirsadraee, S., 2012. The 7th lung cancer TNM classification and staging system: Review of the changes and implications. *World Journal of Radiology*, 4(4), p.0.

Mizrak, D., Brittan, M. & Alison, M.R., 2008. CD133: molecule of the moment. *The Journal of Pathology*, 214(1), pp.3–9. Available at: http://dx.doi.org/10.1002/path.2283.

Morgensztern, D. *et al.*, Trends in stage distribution for patients with non-small cell lung cancer: a National Cancer Database survey. *J.Thorac.Oncol.*, 5(1), pp.29–33.

Morin, P.J. *et al.*, 1997. Activation of β-Catenin-Tcf Signaling in Colon Cancer by Mutations in β-Catenin or APC. *Science*, 275(5307), pp.1787–1790.

Mumm, J.S. & Kopan, R., 2000. Notch signaling: from the outside in. *Developmental biology*, 228(2), pp.151–65.

Nakashima, N. *et al.*, 2010. Intratumoral Wnt1 expression affects survivin gene expression in non-small cell lung cancer. *International journal of oncology*, 37(5), pp.687–694.

Nakashima, N. *et al.*, 2012. Wnt3 gene expression promotes tumor progression in non-small cell lung cancer. *Lung cancer (Amsterdam, Netherlands)*, 76(2), pp.228–234.

Nakashima, T. *et al.*, 2008. Wnt1 overexpression associated with tumor proliferation and a poor prognosis in non-small cell lung cancer patients. *Oncology Reports*, 19, pp.203–209.

Niehrs, C., 2012. The complex world of WNT receptor signaling. *Nat Rev Mol Cell Biol*, 13(12), pp.767–779.

Nowell, P.C., 1976. The clonal evolution of tumor cell populations. *Science*, 194(4260), pp.23–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/959840.

Nusse, R. & Varmus, H.E., 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell*, 31(1), pp.99–109.

Okerlund, N.D. & Cheyette, B.N.R., 2011. Synaptic Wnt signaling—a contributor to major psychiatric disorders? *Journal of Neurodevelopmental Disorders*, 3(2), pp.162–174.

Ota, I. *et al.*, 2016. Snail-induced EMT promotes cancer stem cell-like properties in head and neck cancer cells. *Oncology reports*, 35(1), pp.261–266.

Pao, W. & Girard, N., 2011. New driver mutations in non-small-cell lung cancer. *Lancet Oncol.*, 12(2), pp.175–180.

Patel, S. & Rameshwar, P., 2013. Tumorsphere Passage for Breast Cancer Stem Cells. Available at: http://dx.doi.org/10.1038/protex.2013.023.

Patriarca, C. *et al.*, 2012. Epithelial cell adhesion molecule expression (CD326) in cancer: a short review. *Cancer treatment reviews*, 38(1), pp.68–75.

Peitzsch, C. *et al.*, 2013. Discovery of the cancer stem cell related determinants of radioresistance. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, 108(3), pp.378–87.

Peters, S. *et al.*, 2013. Treatment and detection of ALK-rearranged NSCLC. *Lung Cancer*, 81(2), pp.145–154.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.*, 29(9), p.e45.

Porfiri, E. *et al.*, 1997. Induction of a beta-catenin-LEF-1 complex by wnt-1 and transforming mutants of beta-catenin. *Oncogene*, 15(23), pp.2833–2839.

Qiu, X. *et al.*, 2012. Characterization of sphere-forming cells with stem-like properties from the small cell lung cancer cell line H446. *Cancer Letters*, 323(2), pp.161–170.

Rappa, G., Fodstad, O. & Lorico, A., 2008. The Stem Cell-Associated Antigen CD133 (Prominin-1) Is a Molecular Therapeutic Target for Metastatic Melanoma. *Stem cells (Dayton, Ohio)*, 26(12), pp.3008–3017.

Reedijk, M. *et al.*, 2005. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Research*, 65(18), pp.8530–8537.

Remsing Rix, L.L. *et al.*, 2014. GSK3 alpha and beta are new functionally relevant targets of tivantinib in lung cancer cells. *ACS chemical biology*, 9(2), pp.353–358.

Reynolds, T.C., Smith, S.D. & Sklar, J., 1987. Analysis of DNA surrounding the breakpoints of

chromosomal translocations involving the beta T cell receptor gene in human lymphoblastic neoplasms. *Cell*, 50(1), pp.107–117.

Ricci-Vitiani, L. *et al.*, 2007. Identification and expansion of human colon-cancer-initiating cells. *Nature*, 445(7123), pp.111–115.

Richardson, F. *et al.*, 2012. The evaluation of E-Cadherin and vimentin as biomarkers of clinical outcomes among patients with non-small cell lung cancer treated with erlotinib as second- or third-line therapy. *Anticancer research*, 32(2), pp.537–552.

Robinson, L.A. *et al.*, Treatment of non-small cell lung cancer-stage IIIA: ACCP evidence-based clinical practice guidelines (2nd edition). *Chest*, 132(3 Suppl), p.243S–265S.

Roegiers, F. & Jan, Y.N., 2004. Asymmetric cell division. *Current opinion in cell biology*, 16(2), pp.195–205.

Roudi Raheleh *et al.*, 2014. CD44 and CD24 cannot act as cancer stem cell markers in human lung adenocarcinoma cell line A549. *Cellular and Molecular Biology Letters*, 19, p.23.

Rubin, L.L. & de Sauvage, F.J., 2006. Targeting the Hedgehog pathway in cancer. *Nature reviews Drug discovery*, 5(12), pp.1026–1033.

Sahlberg, S.H. *et al.*, 2014. Evaluation of cancer stem cell markers CD133, CD44, CD24: Association with AKT isoforms and radiation resistance in colon cancer cells. *PLoS ONE*, 9(4).

Salnikov, A. V *et al.*, 2010. CD133 is indicative for a resistance phenotype but does not represent a prognostic marker for survival of non-small cell lung cancer patients. *International Journal of Cancer*, 126(4), pp.950–958.

Sawyers, C.L., 2013. AACR Cancer Progress Report 2013. *Clin Cancer Res.*, 19(Supplement 1), pp.1–98.

Scagliotti, G. V *et al.*, 2004. The biology of epidermal growth factor receptor in lung cancer. *Clin.Cancer Res.*, 10(12 Pt 2), p.4227s–4232s.

Schnell, U., Kuipers, J. & Giepmans, B.N.G., 2013. EpCAM proteolysis: new fragments with distinct functions? *Bioscience reports*, 33(2), p.e00030.

Segditsas, S. & Tomlinson, I., Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene*, 25(57), pp.7531–7537.

Shackleton, M. *et al.*, 2009. Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell*, 138(5), pp.822–829.

Shih, I.-M. & Wang, T.-L., 2007. Notch Signaling,  -Secretase Inhibitors, and Cancer Therapy. *Cancer Research*, 67(5), pp.1879–1882.

Siegel, R.L., Miller, K. & Jemal, A., 2016. Cancer Statistics, 2016. *CA Cancer J Clin*, 66(1), pp.7–30.

Singh, S.K. *et al.*, 2003. Identification of a cancer stem cell in human brain tumors. *Cancer Res.*, 63(18), pp.5821–5828.

Sjölund, J. *et al.*, 2005. The Notch pathway in cancer: differentiation gone awry. *European journal of cancer (Oxford, England : 1990)*, 41(17), pp.2620–9.

Slawek, S. *et al.*, 2015. Pluripotency transcription factors in lung cancer-a review. *Tumor biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*.

Smalley, M. & Ashworth, A., 2003. Stem cells and breast cancer: A field in transit. *Nat Rev Cancer*, 3(11), pp.832–844. Available at: http://dx.doi.org/10.1038/nrc1212.

Smith, L.M. *et al.*, 2008. CD133/prominin-1 is a potential therapeutic target for antibody-drug conjugates in hepatocellular and gastric cancers. *British Journal of Cancer*, 99(1), pp.100–109.

Soda, M. *et al.*, Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*, 448(7153), pp.561–566.

Soria, J.-C. *et al.*, 2013. Systematic review and meta-analysis of randomised, phase II/III trials adding bevacizumab to platinum-based chemotherapy as first-line treatment in patients with advanced non-small-cell lung cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, 24(1), pp.20–30.

Soto-Cerrato, V. *et al.*, 2015. Facilitated Anion Transport Induces Hyperpolarization of the Cell Membrane That Triggers Differentiation and Cell Death in Cancer Stem Cells. *Journal of the American Chemical Society*, 137(50), pp.15892–8.

Sourisseau, T. *et al.*, 2014. Lung cancer stem cell: fancy conceptual model of tumor biology or cornerstone of a forthcoming therapeutic breakthrough? *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*, 9(1), pp.7–17.

Sriuranpong, V. *et al.*, 2001. Notch Signaling Induces Cell Cycle Arrest in Small Cell Lung Cancer Cells 1. , 1640(17), pp.3200–3205.

Stewart, D.J., 2014. Wnt signaling pathway in non-small cell lung cancer. *Journal of the National Cancer Institute*, 106(1), pp.1–11.

Su, J. *et al.*, 2015. Reduced SLC27A2 induces cisplatin resistance in lung cancer stem cells by negatively regulating Bmi1-ABCG2 signaling. *Molecular Carcinogenesis*, p.n/a–n/a.

Sullivan, J.P. *et al.*, 2010. Aldehyde dehydrogenase activity selects for lung adenocarcinoma stem cells dependent on Notch signaling. *Cancer research*, 70(23), pp.9937–9948.

Sun, S. & Wang, Z., 2010. ALDH high adenoid cystic carcinoma cells display cancer stem cell properties and are responsible for mediating metastasis. *Biochemical and biophysical research communications*, 396(4), pp.843–8.

Swarts, D.R.A. *et al.*, 2013. CD44 and OTP are strong prognostic markers for pulmonary carcinoids. *Clinical Cancer Research*, 19(8), pp.2197–2207.

Syljuåsen, R.G. *et al.*, 2015. Targeting lung cancer through inhibition of checkpoint kinases. *Frontiers in Genetics*, 6, p.70.

Tachezy, M. *et al.*, 2014. Activated Leukocyte Cell Adhesion Molecule (CD166): An “Inert” Cancer Stem Cell Marker for Non-Small Cell Lung Cancer? *STEM CELLS*, 32(6), pp.1429–1436.

Takahashi-Yanaga, F. & Kahn, M., 2010. Targeting Wnt signaling: Can we safely eradicate

cancer stem cells? *Clinical Cancer Research*, 16(12), pp.3153–3162.

Takaishi, S. *et al.*, 2009. Identification of Gastric Cancer Stem Cells Using the Cell Surface Marker CD44. *Stem cells (Dayton, Ohio)*, 27(5), pp.1006–1020. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2746367/.

Takebe, N. *et al.*, 2015. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nature Reviews Clinical Oncology*, 12(8), pp.1–20. Available at: http://www.nature.com/doifinder/10.1038/nrclinonc.2015.61.

Teng, Y. *et al.*, 2010. Wnt/beta-catenin signaling regulates cancer stem cells in lung cancer A549 cells. *Biochemical and biophysical research communications*, 392(3), pp.373–9.

Tian, T. *et al.*, 2012. Sox2 enhances the tumorigenicity and chemoresistance of cancer stem-like cells derived from gastric cancer. *Journal of biomedical research*, 26(5), pp.336–345.

Tirino, V. *et al.*, 2013. TGF-beta1 exposure induces epithelial to mesenchymal transition both in CSCs and non-CSCs of the A549 cell line, leading to an increase of migration ability in the CD133+ A549 cell fraction. *Cell Death Dis*, 4.

Tirino, V. *et al.*, 2009. The role of CD133 in the identification and characterisation of tumor-initiating cells in non-small-cell lung cancer. *Eur.J.Cardiothorac.Surg.*, 36(3), pp.446–453.

Todaro, M. *et al.*, 2007. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell stem cell*, 1(4), pp.389–402.

Torre, L.A. *et al.*, 2015. Global Cancer Statistics, 2012. *CA: a cancer journal of clinicians.*, 65(2), pp.87–108.

Travis, W.D., 2002. Pathology of lung cancer. *Clin.Chest Med.*, 23(1), pp.65–81, viii.

Travis, W.D., Brambilla, E. & Riely, G.J., 2013. New pathologic classification of lung cancer: Relevance for clinical practice and clinical trials. *Journal of Clinical Oncology*, 31(8), pp.992–1001.

Trzpis, M. *et al.*, 2007. Epithelial Cell Adhesion Molecule: More than a Carcinoma Marker and Adhesion Molecule. *The American Journal of Pathology*, 171(2), pp.386–395. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1934518/.

Tsukamoto, A.S. *et al.*, 1988. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell*, 55(4), pp.619–625.

Vandesompele, J. *et al.*, 2002a. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3(7), pp.research0034.1–research0034.11.

Vandesompele, J. *et al.*, 2002b. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3(7), pp.research0034.1–research0034.11.

Varjosalo, M. & Taipale, J., 2008. Hedgehog: functions and mechanisms. *Genes & development*, 22(18), pp.2454–2472.

Vaughan, A.E., Halbert, C.L. & Wootton, S.K., 2012. Lung Cancer in Mice Induced by the Jaagsiekte Sheep Retrovirus Envelope Protein Is Not Maintained by Rare Cancer Stem

Cells , but Tumorigenicity Does Correlate with Wnt Pathway Activation Lung Cancer in Mice Induced by the Jaagsiekte Sheep. , 10(January), pp.86–95.

Velcheti, V. & Govindan, R., 2007. Hedgehog signaling pathway and lung cancer. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*, 2(1), pp.7–10.

Wang, H. *et al.*, 2014. Acquisition of epithelial-mesenchymal transition phenotype and cancer stem cell-like properties in cisplatin-resistant lung cancer cells through AKT/β-catenin/Snail signaling pathway. *European journal of pharmacology*, 723, pp.156–66.

Wang, P. *et al.*, 2013. Identification and Characterization of Cells with Cancer Stem Cell Properties in Human Primary Lung Cancer Cell Lines. *PLoS ONE*, 8(3).

Wang, Y., 2009. Wnt/Planar cell polarity signaling: a new paradigm for cancer therapy. *Molecular cancer therapeutics*, 8(8), pp.2103–2109.

Watkins, D.N. *et al.*, 2003. Hedgehog signaling within airway epithelial progenitors and in small-cell lung cancer. *Nature*, 422(6929), pp.313–317.

Watkins, D.N. & Peacock, C.D., 2004. Hedgehog signaling in foregut malignancy. *Biochemical pharmacology*, 68(6), pp.1055–60.

Westhoff, B. *et al.*, 2009. Alterations of the Notch pathway in lung cancer. *Proceedings of the National Academy of Sciences* , 106 (52 ), pp.22293–22298.

Wicha, M.S., Liu, S. & Dontu, G., 2006. Cancer stem cells: An old idea - A paradigm shift. *Cancer Research*, 66(4), pp.1883–1890.

Wienken, M. *et al.*, 2015. MDM2 Associates with Polycomb Repressor Complex 2 and Enhances Stemness-Promoting Chromatin Modifications Independent of p53. *Molecular Cell*, 61(1), pp.68–83.

Wilson, A. & Radtke, F., 2006. Multiple functions of Notch signaling in self-renewing organs and cancer. *FEBS letters*, 580(12), pp.2860–8.

Xia, X. *et al.*, 2010. Image-Based Chemical Screening Identifies Drug Efflux Inhibitors In Lung Cancer Cells. *Cancer research*, 70(19), pp.7723–7733.

Xiang, X.J. *et al.*, 2015. Differential expression of Dickkopf-1 among non-small cell lung cancer cells. *Molecular medicine reports*, 12(2), pp.1935–1940.

Xu, X. *et al.*, 2011. Aberrant Wnt1/β-catenin expression is an independent poor prognostic marker of non-small cell lung cancer after surgery. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*, 6(4), pp.716–24.

Yao, L. *et al.*, 2014. Overexpression of Wnt5a promotes angiogenesis in NSCLC. *BioMed research international*, 2014, p.832562.

Yasui, K. *et al.*, 2013. SNAIL induces epithelial-to-mesenchymal transition and cancer stem cell-like properties in aldehyde dehydroghenase-negative thyroid cancer cells. *Thyroid : official journal of the American Thyroid Association*, 23(8), pp.989–996.

Yen, W.-C. *et al.*, 2015. Targeting Notch signaling with a Notch2/Notch3 antagonist (tarextumab) inhibits tumor growth and decreases tumor-initiating cell frequency. *Clinical cancer research : an official journal of the American Association for Cancer*

*Research*, 21(9), pp.2084–2095.

Yong Wang, Jill Water, Marco L. Leung, Anna Unruh, Whijae Roh, Xiuqing Shi1, K., Chen, Paul Scheet, Selina Vattathil, Han Liang, Asha Multani, Hong Zhang, R. & Zhao, Franziska Michor, Funda Meric-Bernstam, and N.E.N., 2014. Clonal Evolution in Breast Cancer Revealed by Single Nucleus Genome Sequencing. *Nature*, 512(7513), pp.155–160.

Yuan, R. *et al.*, 2015. HES1 promotes metastasis and predicts poor survival in patients with colorectal cancer. *Clinical & experimental metastasis*, 32(2), pp.169–179.

Yuan, X. *et al.*, 2014a. Notch signaling and EMT in non-small cell lung cancer : biological significance and therapeutic application. , pp.1–10.

Yuan, X. *et al.*, 2014b. Notch signaling and EMT in non-small cell lung cancer: biological significance and therapeutic application. *Journal of Hematology & Oncology*, 7(1), pp.1–10.

Zakaria, N. *et al.*, 2015. Human non-small cell lung cancer expresses putative cancer stem cell markers and exhibits the transcriptomic profile of multipotent cells. *BMC cancer*, 15, p.84.

Zardavas, D. *et al.*, 2015. Clinical management of breast cancer heterogeneity. *Nat Rev Clin Oncol*, 12(7), pp.381–394.

Zhang, C. *et al.*, 2011. Identification of CD44+CD24+ gastric cancer stem cells. *Journal of Cancer Research and Clinical Oncology*, 137(11), pp.1679–1686.

Zhang, D.-G. *et al.*, 2015. Isolation, cultivation and identification of human lung adenocarcinoma stem cells. *Oncology letters*, 9(1), pp.47–54.

Zhang, W.C. *et al.*, 2012. Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell*, 148(1-2), pp.259–72.

Zhang, X. *et al.*, 2015. C6orf106 enhances NSCLC cell invasion by upregulating vimentin, and downregulating E-cadherin and P120ctn. *Tumor biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, 36(8), pp.5979–5985.

Zheng, Y. *et al.*, 2013. A rare population of CD24(+)ITGB4(+)Notch(hi) cells drives tumor propagation in NSCLC and requires Notch3 for self-renewal. *Cancer Cell*, 24.

Zhou, L. *et al.*, 2015. [Expression of CD133 and Notch1 in non-small cell lung cancer and the clinicopathological significance]. *Nan fang yi ke da xue xue bao = Journal of Southern Medical University*, 35(2), pp.196–201.

Zhou, W. *et al.*, 2014. Snail contributes to the maintenance of stem cell-like phenotype cells in human pancreatic cancer. *PloS one*, 9(1), p.e87409.

Zhu, W.-F. *et al.*, 2014. Prognostic value of EpCAM/MUC1 mRNA-positive cells in non-small cell lung cancer patients. *Tumor biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, 35(2), pp.1211–1219.

# 7. APPENDICES

## 7.1. COMMUNICATIONS DERIVED FROM THIS STUDY

### 1. V Meeting of Young Researchers (RTICC). Pamplona, Spain 2015.

**Comparison of gene expression profile between lung tumorspheres and adherent cells in non-small cell lung cancer (NSCLC)**

**Alejandro Herreros-Pomares**, Silvia Calabuig-Fariñas, Ester Munera, Andrea Palomar, Ricardo Guijarro, Eloísa Jantus-Lewintre, Carlos Camps

**Background**: Chemoresistance, tumor progression and metastasis have made of lung cancer the first cause of mortality cancer-related worldwide. These characteristics seem to be linked to a subpopulation of stem-like cells, cancer stem cells (CSCs). Thus, it is essential to understand the molecular mechanisms that regulate CSCs self-renewal and differentiation properties and the alterations in gene expression involved in pathways that participate in tumor growth. Here, we have analysed genes of Notch and Wnt signaling pathways and several CSCs and pluripotency markers in turmorspheres and adherent cells.

**Methods:** RNA from monolayer cells and tumorspheres from 6 patients and 4 cell lines (A549, H1650, H1993 and PC9) was isolated. QPCR was performed to analyze the expression of *NOTCH1*, *NOTCH3*, *WNT5A*, *DKK1*, *FZD7*, *MYC*, *CCND1*, *ALDH1A1*, *EPCAM*, *CD44*, *KLF4*, *OCT4* and *NANOG* genes. Relative expression was normalized by *ACTB* and *CDKN1B* endogenous genes using Pfaffl formulae.

**Results:** Gene expression analysis revealed an activation of the canonical Wnt pathway in tumorspheres when compared to monolayer cultured cells. High expression levels of effector genes *CCND1* and *MYC*, along with a strong repression of *DKK1* inhibitor was found. Moreover, the non-canonical Wnt pathway, which inhibits the canonical Wnt pathway, was more inactivated, since *WNT5A* activator ligand had lower expression in spheroids. Likewise, Notch signaling pathway, associated with maintenance and proliferation of CSCs, seemed to be more activated in tumorspheres than in adherent cells. *NOTCH1* and *NOTCH3* showed high gene expression levels, which correlates with the elevated levels of *CCND1* and *MYC* mentioned above. Lastly, CSCs markers, *ALDH1A1*, *EPCAM* and *CD44* as well as pluripotency genes, *KLF4*, *OCT4* and *NANOG* exhibited increased expression levels in lung tumorspheres.

**Conclusions:** Lung tumorspheres, like CSCs, possess stem-like properties, making them a potential platform to test targeted CSCs therapies.

## 2. American Association for Cancer Research (AACR). Louisiana, USA 2016.

**Characterization of lung-tumorspheres by gene expression and flow cytometry. Differential expression in CSC-related markers and signaling pathways.**

**Alejandro Herreros Pomares**, Ester Munera Maravilla, Alicia Martínez Romero, Sandra Tejedor, Silvia Calabuig, Eloisa Jantus Lewintre, Rut Lucas, Eva Escorihuela, Rosa Farràs, Carlos Camps.

Chemoresistance, progression and metastasis have made of lung cancer the first cause of cancer mortality. These features were linked to a subpopulation of cells, named cancer stem cells (CSCs), which remain largely unknown. The aim of this study was to isolate and characterize CSCs from lung cancer cell-lines and tumor-tissue from resectable non-small cell lung cancer (NSCLC).

**Methods:** Tumor cells from resected NSCLC and cell lines (H1650, H1993, A549, and PC9) were grown in monolayer and as spheroids. QPCR was performed to analyze the mRNA expression of CSCs-related genes: CSC-markers (*EPCAM1, ALDH1A1, CD166, ABCG2, CD44, CD133)*; pluripotency genes (*KLF4, OCT4, NANOG, SOX2, MYC, CCND1*); Notch pathway (*NOTCH1, NOTCH3, HEY1*); Wnt pathway (*WNT1, WNT5A, DKK1, FZD7*) and Hedgehog pathway (*SMO, PTCH1, SHH, GLI1*). *ACTB* and *CDKN1B* were used as endogenous controls for relative expression calculation. The expression of lung stem cell markers EpCAM, CD166, E-cadherin, CD90, CD44, CD34, CD133 and ABCG2 was assessed by flow cytometry. The tumor-initiating cell capacity of selected lung-spheres was tested *in vivo* to confirm tumorigenicity.

**Results:** Lung-tumorspheres had increased expression of *EPCAM, CD44* and *ALDH1A1* (p= 0.028, p= 0.021 and p= 0.043, respectively) when compared to cells grown in adherence. Likewise, *NANOG*, *KLF4* and *OCT4* tended to be more expressed in tumorspheres. Relative gene expression of *NOTCH1* was also higher in spheroids than in monolayer cells (p= 0.028), in concordance with the same tendency observed in *NOTCH3*. Similarly, QPCR analysis revealed a possible activation of the canonical Wnt pathway in tumorspheres, with high expression levels of the downstream effector gene *CCND1* (p = 0.05), along with a repression of *DKK1* inhibitor. Regarding to the non-canonical Wnt pathway, its activator *WNT5A* showed lower expression levels in spheroids compared to monolayer-culture cells. Concerning the expression levels of Hedgehog pathway`s genes, we found that *SMO* and *PTCH1* were underexpressed in lung-tumorspheres compared with their paired adherent-cultured cells (p = 0.028 and p=0.069). Flow cytometry revealed that EpCAM and CD44 were highly expressed in lungspheres obtained

from cell lines and primary tumors. The expression of CD166 differed among the cell lines. Furthermore, EpCAM+/CD90- subpopulation were the ones able to induce tumor in xenotransplanted mouse model demonstrating tumor-initiating capacity *in vivo.*

**Conclusions:** Lung-tumorspheres derived from cancer cell lines and primary tumor tissues show increased levels of EpCAM and others CSC markers. Genes related to Notch and Wnt signaling pathways were more expressed in spheroids compared to the cells grown in adherence, suggesting both pathways as interesting lung-CSC targets.

## 3. European Lung Cancer Congress (ELCC). Geneva, Switzerland 2016.

**Expression analysis of tumorspheres from non-small cell lung cancer show significant differences in CSC-markers and signaling pathways**

**A. Herreros-Pomares**, E. Munera, S. Calabuig-Fariñas, B. Lafuente, A. Blasco, R. Guijarro, R. Farrás, E. Jantus, C. Camps

**Background**: Despite the advances in the molecular characterization of lung cancer it remains as the leading cause of cancer death worldwide. Cancer stem cells (CSCs) are small subpopulations of stem-like cells with self-renewal and differentiation properties that constitute a promising target, but remain largely unknown. The aim of this study was to isolate and characterize gene expression of CSCs from lung cancer cell-lines and tumor-tissue obtained from resectable NSCLC patients.

**Methods**: This study was performed on cells from NSCLC tumor samples and cell lines (H1650, H1993, A549 and PC9) grown in monolayer and as spheroids. The expression of: CSC-markers (*EPCAM1*, *ALDH1A1*, *CD166*, *ABCG2*, *CD44*); pluripotency (*KLF4*, *OCT4*, *NANOG*, *SOX2*, *MYC*, *CCND1*); cell cycle (*CDKN1A*, *CDKN2A*, *MDM2*, *WEE1*); invasiveness (*CDH1*, *CEACAM5*, *VIM*, *MMP2*, *MMP9*); Notch pathway (*NOTCH1*, *NOTCH2, NOTCH3*, *HEY1*); Wnt pathway (*CTNBB1*, *WNT1, WNT5A*, *DKK1*, *FZD7*) and Hedgehog pathway (*SMO*, *PTCH1*, *SHH, GLI1*) were analyzed by QPCR. *ACTB*, *CDKN1B* and *GUSB* were used as endogenous controls for relative expression calculation.

**Results**: Lung tumorspheres had increased expression of *EPCAM1, CD44, ALDH1A1* and *CDKN1A* (p= 0.028, p=0.021, p= 0.043 and p=0.021, respectively) when compared to their paired-adherent cells. Regarding the expression of Notch-pathway genes, *NOTCH1* and *NOTCH2* showed higher expression in tumorspheres (p= 0.028 and p = 0.038, respectively) and *NOTCH3* also showed the same tendency. We found higher expression levels of *CTNBB1* (Wnt pathway) (p=0.008) in lungspheres whereas the activator of the non-canonical Wnt pathway, *WNT5A,* tended to be less expressed in spheroids compared to adherent culture cells. Our results show that *SMO* was underexpressed (p = 0.028) in tumorspheres, whereas no significant differences were found in other analyzed genes.

**Conclusions**: Lung spheroids from cancer cell lines and primary tumors showed increased levels of CSC-markers. Genes related to Notch and Wnt were found to be more expressed in tumorspheres, suggesting that these pathways as interesting lung-CSC targets.