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Drug Resistance and Cancer Stem Cells

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Maria Carmen Alpoim (Universidade de Coimbra).

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Aos meus pais e avôs

“Standing on the shoulders of giants”

Sir Isaac Newton

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List of Symbols and Abbreviations

1. **ABC –** adenosine triphosphate-binding cassette
2. **ALDH –** aldehyde dehydrogenases
3. **AML** – acute myeloid leukemia
4. **APC** - Adenomatous polyposis coli
5. **ara-C –** arabinoside
6. **ASR** - age-standardised rates
7. **AT1** – alveolar type 1
8. **AT2** – alveolar type 2
9. **ATP** – adenosine triphosphate
10. **Bax** - Bcl-2–associated X protein
11. **Bcl2** – B- cell lymphoma 2
12. **BER** - base excision repair
13. **bFGF** – basic fibroblast growth-factor
14. **BRCA1** - breast cancer type 1 susceptibility protein
15. **BSA –** bovine serum albumin
16. **cDDP** – cisplatin
17. **Cr (VI) –** hexavalent chromium
18. **CSCs** – cancer stem cells
19. **Cyclin D1 -** G1/S-specific cyclin-D1
20. **DAB -** 3,3-diaminobenzidine tetrahydrochloride
21. **DAPI** - 4',6-diamidino-2-phenylindole

22. **DMEM -** dulbecco's Modified Eagle Medium
23. **DMSO** – dimethyl sulphoxide
24. **DNA** – desoxyribonucleic acid
25. **ECM** – extracellular matrix
26. **EGF** – epidermal growth factor
27. **EGFR** – epidermal growth factor receptor
28. **EMT** – epithelial-to-mesenchymal transition
29. **ES** – embryonic stem cells
30. **FBS** – fetal bovine serum
31. **FDA** – food and drug administration
32. **FISH** – fluorescent *in situ* hybridization
33. **GEM** – gemcitabine
34. **H_2O_2 –** hydrogen peroxide
35. **HBSS -** hank's balanced salt solution
36. **HCl –** hydrogen chloride
37. **HGF** – hepatocyte growth factor
38. **Hh** – hedgeh
39. **HIF** – hypoxia-inducible factor
40. **HSCs** – hematopoietic stem cells
41. **IARC** - International Agency for Research on Cancer
42. **IGF –** insulin growth factor

43. **IGFBP –** insulin growth factor-binding protein

44. **KCl –** potassium chloride

45. **KH_2PO_4 –** monopotassium phosphate

46. **LC** – lung cancer

47. **LCC** – large-cell lung carcinoma

48. **LOH** – loss of heterozigosity

49. **MDR** – multidrug resistance

50. **MDR1** – multidrug resistance transporter 1

51. **MMR** - mismatch repair

52. **MET** – mesenchymal-to-epithelial transition

53. **MLH1** - MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)

54. **miRNAs** - microRNAs

55. **mRNA** – messenger RNA

56. **MRP** – MDR-associated proteins

57. **MTT** - 3-[4, 5-dimethylthiazol-2-yl]2, 5-diphenyltetrazolium bromide

58. **MW –** multiwall plates

59. **Na_2HPO_4 –** sodium phosphate dibasic

60. **NaOH –** sodium hydroxide

61. **NCC** – neurocysticercosis

62. **NHEJ** - Non-homologous end joining

63. **NSCLC** – non-small cell lung cancer

64. **Oct4** - octamer-binding transcription factor 4

65. **OGG1** - 8-oxoguanine DNA glycosylase

66. **PBS** – phosphate buffer saline

67. **PCD** – programmed cell death

68. **PDGF** – platelet-derived growth factor

69. **P-gp** – p-glycoprotein

70. **PTEN** - Phosphatase and tensin homolog

71. **RAD51** - RAD51 homolog

72. **Rb** – retinoblastoma protein

73. **RNA** – ribonucleic acid

74. **SCs** – stem cells

75. **SCC** – squamous cell lung carcinoma

76. **SCLC** – small cell lung cancer

77. **SHH** – sonic hedgehog

78. **Sox2/SRY** – sex determining region Y – box 2

79. **SP** – side population

80. **SSC** – sodium chloride/sodium citrate

81. **SV40** – simian virus

82. **TGF-α** – transforming factor alpha

83. **TGF-β** – transforming factor beta

84. **TICs** – tumour initiating cells

85. **TKI** – tyrosine cynase inhibitor

86. **TP53** – tumour protein 53

87. **UV** – ultraviolet light

88. **vCE** - variant Clara cell secretory protein-expressing cells

89. **XRCC1** - X-ray repair complementing defective repair in Chinese hamster cells 1

90. **XRCC3** - X-ray repair complementing defective repair in Chinese hamster cells 3

91. **XRCC5** - X-ray repair complementing defective repair in Chinese hamster cells 5

Abstract

Lung cancer is the most common cause of cancer death worldwide. Despite the increasing knowledge on the mechanisms underlying the tumorigenic process, there isn't a significant increase on lung cancer patients' survival. Due to this, it is urgent to develop new approaches that surpass the chemotherapeutic resistance, both intrinsic as acquired, observed with the current available therapeutic options.

Tumours are characterized by their cellular heterogeneity due to the co-existence of different cellular sub-populations, whose hierarchic organization in certain cancers lead to the hypothesis that the target cells of transforming mutations are stem-like cells. However, in other tumours, restricted progenitors or even differentiated cells may be the cell of origin. Cancer stem cells (CSCs) are consequently, stem-like cells with self-renewal and multipotent differentiation characteristics which can originate all cell types found in a tumour.

Recently, following prolonged treatment of differentiated non-tumorigenic bronchial epithelial cells (BEAS-2B) with hexavalent chormium, a known lung carcinogenic agent, a malignant cell line RenG2 was established and, subsequently, two more malignant cell lines, DRenG2 and DDRenG2, were implemented out of tumors induced in nude mice. Unexpectedly, we demonstrated that CSCs could be obtained by dedifferentiation of the malignant bronchial epithelial cells DRenG2, DDRenG2 and/or their precursor RenG2.

In the present work the proliferation rate of Cont-1, RenG2, DRenG2 and DDRenG2 cell lines, and their non-malignant precursor BEAS-2B was assessed by the trypan blue method. The cytogenetic evolution from BEAS-2B to DDRenG2 was evaluated, and the epithelial/mesenchymal phenotype of the cell lines was assessed by immunocytochemistry (MNF116, Vimentin and Oct3/4). Finally, the chemoresistance of

the cells lines to gemcitabine (2'2'-diflourodeoxycytidine) and cisplatin [cis-$Pt(NH_3)_2Cl_2$], the most common drugs used to treat lung cancer, was evaluated and correlated to the presence of the efflux pump P- Glycoprotein. The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was used to assess the ability of gemcitabine and cisplatin to reduce cells' viability.

The cytogenetic analysis of the more malignant and more proliferative DRenG2 and DDRenG2 cell lines revealed a common structural difference relative to progenitor BEAS-2B cells in chromosome 7 ($7p^-$). However, while the predominant structural alterations in DRenG2 were the $7q^-$ and $iso9q^+$, DDRenG2 revealed the predominance of t(7:14) and $17q^+$. All the malignant cell lines, RenG2, DRenG2 and DDRenG2, predominant ploidy was 75/76 chromosomes in contrast, to their non-malignant progenitor BEAS-2B and non-malignant control Cont-1.

Immunocytochemistry analysis revealed that all cell lines were MNF116- and Vimentin-positive but, Otc3/4- and P-Glycoprotein-negative. The MNF116- and Vimentin staining levels distribution revealed that all the cell lines were composed of different cellular sub- populations that account for their transition phenotypes, with BEAS-2B more epithelial while the more malignant were more mesenchymal. As expected, the more malignant cell lines were significantly more resistant to gemcitabine and cisplatin. Although, quite often multidrug resistance is associated to the overexpression of the membrane efflux pump P-Glycoprotein, other mechanism(s) may account for the observed resistance of DRenG2 and DDRenG2 cells.

Keywords: Lung cancer, resistance to gemcitabine and cisplatin, transition phenotypes epithelial and mesenchymal, structural cytogenetic alterations $7q^-$, $iso9q^+$, t(7;14) and $17q^+$, polyploidy and malignancy.

Resumo

O cancro do pulmão é a principal causa de morte por cancro a nível mundial. Apesar do crescente conhecimento sobre os mecanismos subjacentes ao processo tumorigénico não se tem observado alteração significativa na sobrevivência dos pacientes. É, por isso, urgente encontrar novas estratégias terapêuticas que visem superar a resistência, tanto intrínseca como extrínseca, observada com a quimioterapia corrente.

Os tumores são caracterizados pela sua heterogeneidade celular, devido à coexistência de diferentes subpopulações celulares, cuja organização hierárquica em certos tipos de cancro, levou à hipótese de que as células afetadas por mutações possam ser células estaminais e estar na origem do processo tumoral. No entanto, em outros tumores, células totalmente diferenciadas portadoras de mutações têm sido referenciadas como as células de origem. As células estaminais cancerígenas, são células com capacidade de autorrenovação, e diferenciação multipotente, que podem originar os diferentes tipos de células presentes no tumor.

Recentemente, a fim de estudar os mecanismos envolvidos na carcinogénese pulmonar por exposição ao crómio hexavalente [Cr(VI)] foi implementado, no nosso laboratório, o primeiro sistema *in vitro* de transformação maligna de uma linha do epitélio bronquial humano, BEAS-2B, induzida por Cr(VI). Subsequentemente, o potencial maligno da linha celular obtida (RenG2) foi aumentado por injecções sucessivas das células em ratinhos imunocomprometidos, estabelecendo duas novas linhas celulares malignas, derivadas da inicial (DRenG2 e DDRenG2). Inesperadamente, demonstrou-se que as células estaminais cancerígenas, podem ser obtidas por desdiferenciação de células malignas do epitélio bronquial, e/ou da percursora menos maligna RenG2.

Tendo em vista a caracterização biológica das linhas celulares: Cont-1, RenG2, DRenG2, DDRenG2 e BEAS-2B procedeu-se, ao estudo dos seus tempos de duplicação,

pelo método de azul tripano, e da evolução citogenética desde as BEAS-2B até às DDRenG2. Após a caracterização citogenética das linhas celulares o fenótipo epitelial, mesenquimal das referidas linhas foi avaliado por imunocitoquímica (MNF116, Vimentina e Oct-4). Por fim, a resistência aos agentes quimioterapeuticos convencionalmente usados no cancro do pulmão, gemcitabina (2'2' diflouro deoxicitidina) e da cisplatina [cis-$Pt(NH_3)_2Cl_2$], foi avaliada e correlacionada com a expressão da proteína de efluxo P-Glycoproteína. O método do MTT (brometo de 3- [4,5-dimetiltialzol-2-il] -2,5-difeniltetrazólio) foi utilizado para avaliar a resistência aos referidos fármacos.

A análise citogenética das linhas celulares mais malignas e proliferativas DRenG2 e DDRenG2 demonstrou que estas eram portadoras de uma alteração estrutural comum relativamente à progenitora não maligna BEAS-2B, denominada $7p^-$. As principais características citogenéticas da linha DRenG2 eram as alterações estruturais $7q^-$ e da $iso9q^+$, enquanto as da linha celular DDRenG2 eram as alterações t(7;14) e $17q^+$. As linhas celulares malignas RenG2, DRenG2 e DDRenG2 apresentaram uma ploidia de 75/76 cromossomas em oposição às linhas não malignas BEAS-2B e Cont-1.

A análise imunocitoquímica revelou que todas as linhas celulares eram MNF116- e Vimentina-positivas mas, Otc3/4- e P-Glycoprotein-negativas. Como esperado, as linhas mais malignas, apresentaram uma maior resistência à gemcitabina e cisplatina.

Palavras-chave: Cancro pulmonar, resistência à gemcitabina e cisplatina, fenótipos de transição epitelial e mesenquimal, alterações citogenéticas $7q^-$, $iso9q^+$, t(7;14) e $17q^+$, poliploidia.

1. Introduction

1.1 Cancer epidemiology

In the last decades, scientists have overcome many difficulties in order to fight the diseases that plague our world. The discovery of new techniques of investigation and the improvement of the available technology, allowed many battles to be won, even though some diseases remain incurable. Among these diseases is cancer, a worldwide concern that affects millions of persons every year. However, the cure of cancer is still unknown.

Cancer is the leading cause of death in economically developed countries. In 2008, it was responsible for 7.6 million deaths worldwide. In the past, cancer was known to be a disease of the developed countries; nerveless, it already spread into undeveloped countries where cases have been documented. Worldwide, the most commonly diagnosed cancer is lung cancer (LC) (1.61 million of incidences, corresponding 12.7% of the total) followed by breast cancer (1.38 million of incidences, 10.9% of the total) and colorectal cancer (1.24 million of incidences, 9.8% of total). Accordingly, LC is also the most frequent cause of cancer-related deaths (1.38 million deaths, 18.2% of the total), followed by stomach cancer (738 000 deaths, 9.7% of the total) and liver cancer (695 000 deaths, 9.2% of the total) (Siegel *et al*., 2011). According to the International Agency for Research on Cancer (IARC), the prevalent cancer varies between genders, being LC the most frequent among men (949 000 deaths, 22.5% of the total), while is breast cancer is the prevalent among women (459 000 deaths, 13.7% of total). In the figure 1.1 is represented the incidence (blue) and the mortality (red) of the 15 most frequent types of cancer worldwide in 2008.

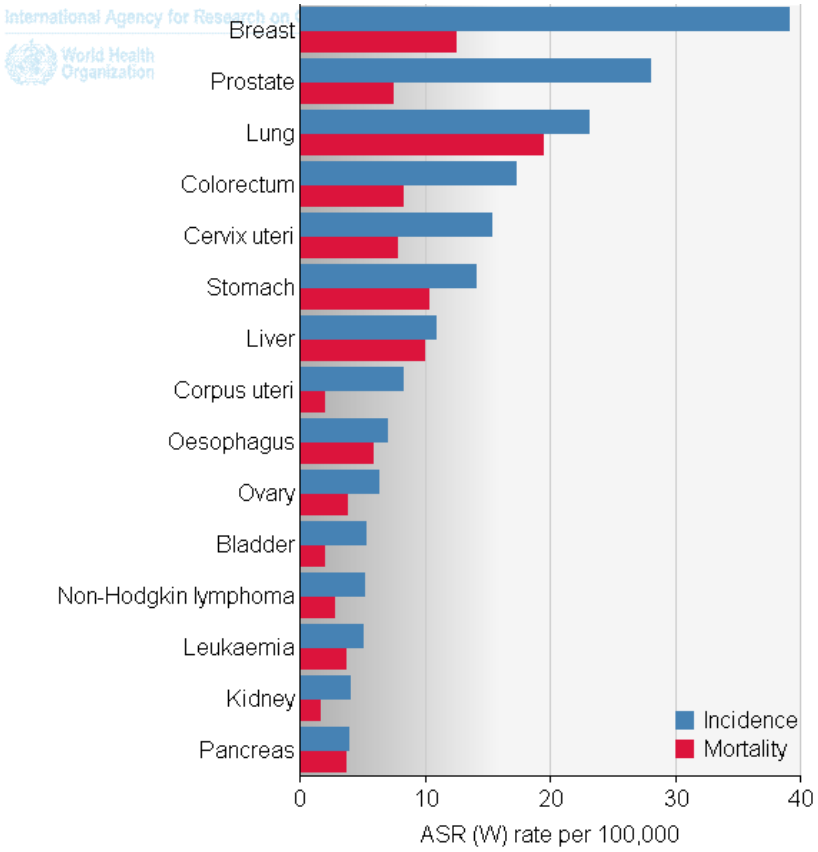

Figure 1.1 – Comparison of the most frequent cancers among genders. As depicted in this graph the main cause of cancer related deaths worldwide is the lung cancer in 2008. **ASR**: age-standardised rates. (http://globocan.iarc.fr/)

1.2 Cancer tissue-specific phenotypes etiology

The molecular and cellular mechanisms underlying the origin of tumour phenotypes remain a central question in the cancer biology field. So far two models have been proposed, to explain tissue-specific phenotype heterogeneity. The first model argues that different genetic and/or epigenetic mutations occur within the same target cell resulting in different tumour phenotypes. On the other hand, the second model states that different tumour subtypes arise from distinct cells, which will act, within the tissue, as the cell of origin (Figure 1.2) (Visvader, 2011). As these mechanisms are not mutually exclusive, they may eventually together to control tumour histopathology and behaviour.

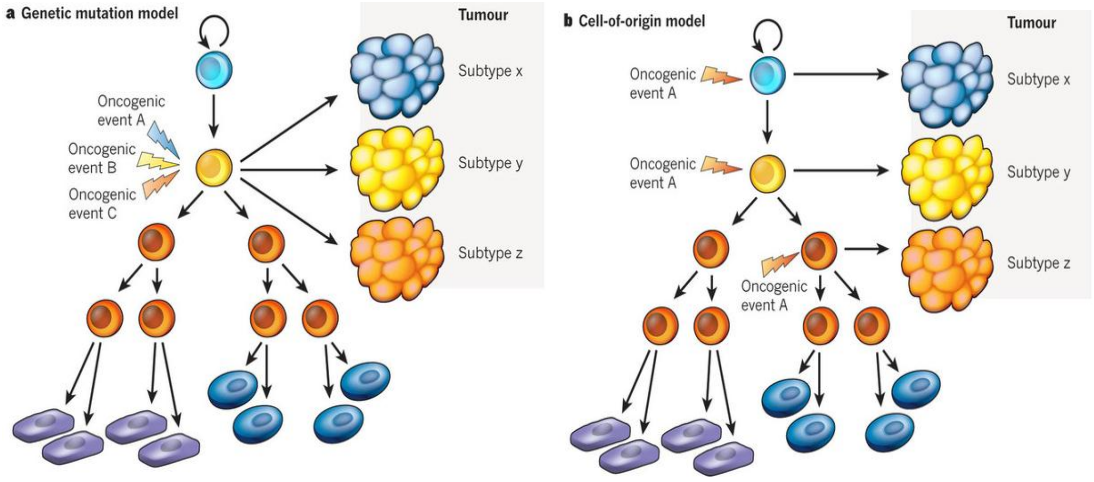

Figure 1.2 – **The two models of intertumoural heterogeneity. A**. In the genetic (and epigenetic) mutation model, mutations primarily determine the phenotype of the tumour, such that different mutations result in different tumours morphologies. **B**. In the cell-of-origin model, different cell populations along the lineage hierarchy serve as cells of origin generating different cancer populations within the organ or tissue. (Adapted from **Visvader, J.E., 2011**).

1.3 Malignant transformation etiology

1.3.1 The hallmarks of cancer

Cancer cells are characterized by a variety of genomic defects, such as inactivation of DNA repair genes, over-expression of growth promoting oncogenes, extra or missing chromosomes, abnormal number of centrosomes, aberrant mitosis and cytokinesis (Meraldi *et al*., 2004). In a recent review, Hanahan and Weinberg (**2011**) stated that all cancer cells, in spite of their remarkable diversity, have common features including: inability to respond to signals of programmed cell death and unresponsiveness to anti-proliferative signals; prevalence in a proliferative state in the absence of mitogenic signals; loss of sensors to programmed senescence; sustained angiogenesis, tissue invasion and metastasis; reprogramming of energy metabolism and evading immune destruction (Figure 1.3).

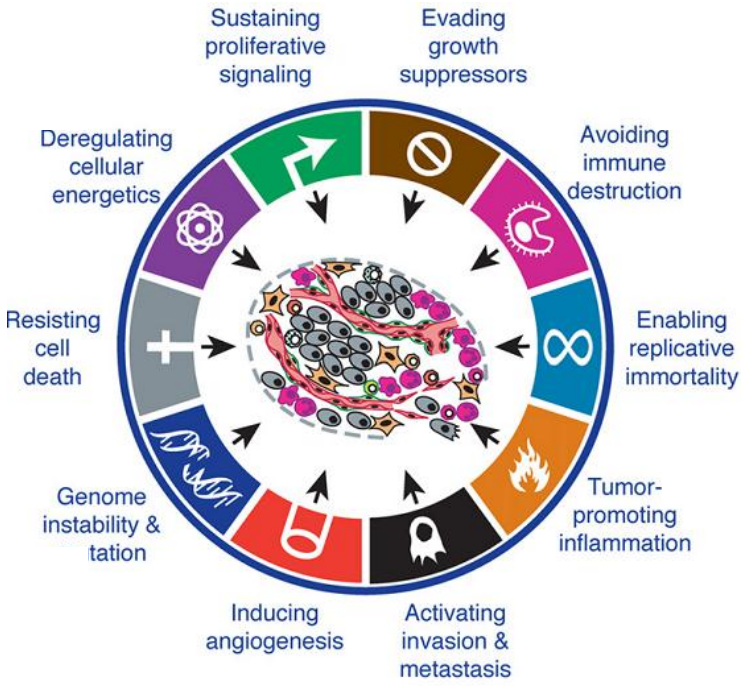

Figure 1.3 – The hallmarks of cancer (Adapted from **Hanahan and Weinberg, 2011**).

It is now accepted that the environment surrounding the cancer cells, designated as "tumour microenvironment", also contributes to tumorigenesis in cancers of epithelial origin, as known as, carcinomas (Hanahan and Weinberg, 2011**;** Hu and Polyak, 2008**;** Littlepage *et al*., 2005). In fact, contradicting the ancient belief that the recruited normal cells, which form tumour-associated stroma, were merely passive bystanders, several recent works demonstrated that the tumour microenvironment, contributes largely to tumorigenesis, acting as a regulator of the progression to invasion and metastasis (Hanahan and Weinberg, 2011**;** Hu and Polyak, 2008**;** Littlepage *et al*., 2005).

The multiple theories that tried to explain the origin of malignant transformation were, almost inevitably, in line with the more general pathological theories at the time they were postulated (Triolo, 1965). As a consequence, the establishment of cancer as a genetic disease paralleled the development of the discipline of genetics. The designation of cancer as "the disease of genes" emerged from the observation that alterations in

certain genes drive normal cells to ignore growth-controlling signals, and to the emergence of tumour masses that eventually disseminate (Shipitsin and Polyak, 2008). The failure in cellular homeostasis control, observed in cancer cells, is commonly related with mutations and/or abnormal expression of oncogenes (mutated forms of proto-oncogenes), tumour suppressor genes (*Tp53*, *Rb*, *APC* and *BCRA1*), which may be triggered by some chromosomal alterations (i.e. translocations) (Argyle and Blacking, 2007). Proto-oncogenes are important genes that are involved in the control of cell growth, proliferation and differentiation. In normal cells, proto-oncogenes can be grouped as: growth factors, growth factor receptors, protein kinases, signal transducers, nuclear proteins and transcription factors. As to tumour suppressors, they generally operate as central branches within complementary cellular regulatory circuits, responsible for cells proliferation or, alternatively, for the activation of senescence and/or apoptotic programs.

To convert a proto-oncogene into an oncogene it is only necessary a single allele mutation (punctual mutation, deletion, insertion, gene amplification and chromosomal translocation). Conversely, for the loss of function of a tumour suppressor required to initiate the malignant transformation, it is necessary that both alleles are lost. Mutations or translocations of these genes can lead to abnormal uncontrolled cell growth, and consequently to malignant transformation (Argyle and Blacking, 2007**;** Yagui-Beltrán and Jablons, 2009). However, new evidence suggests that each tumour suppressor gene operates in a larger network, and so, cells lacking a functional *Rb* or *Tp53* gene can surprisingly be free of proliferative abnormalities, despite the expectation that loss of these genes function would allow continuous abnormal cell division cycle (Hanahan and Weinberg, 2011). These cases develop abnormalities only late in life.

In addition to genetic mutations, it is now well established that epigenetic changes that occur manly in the CpG islands can be adjuvants or even surrogates of genetic

mutations. CpG islands are genomic regions that contain a high frequency of CpG sites and some CpG dinucleotides clustered in promoter regions (Yagui-Beltrán and Jablons, 2009). Whenever these regions accomodate genes that are involved in tumour suppression, their specific DNA hypermethylation leads to the silencing of these tumour suppressor genes (Herman and Baylin, 2003). If a simultaneously hypomethylation occurs at the promoter regions of oncogenes, inducing their activation and up-regulation, a generalized genomic instability takes place, favouring the malignant transformation process not only allows up-regulated transcription of oncogenes but, also, when generalized, genomic instability (Eden *et al*., 2003; Nishigaki *et al*., 2005). This additional level of complexity sheds light on the marked heterogeneity of cellular morphology, proliferative index, genetic lesions and therapeutic response of cancer cell populations present within the same tumour (Feinberg *et al*., 2006).

1.3.2 The stochastic model

Till recently the model proposed for cancer formation was the stochastic or clonal evolution model, which argues that cancer establishment, is a long term process, starting with a malignant transformation (chemical, physical or biological carcinogenic agent), which affects the genome of the cell. Then a clonal expansion takes place which might lead to tumour development and propagation, and, eventually, death. According to this model, tumour's heterogeneity is the result of genetic mutations in individual cells, and of a paracrine crosstalk between mutated cells and their microenvironment (Figure 1.4) (Argyle and Blacking, 2007).

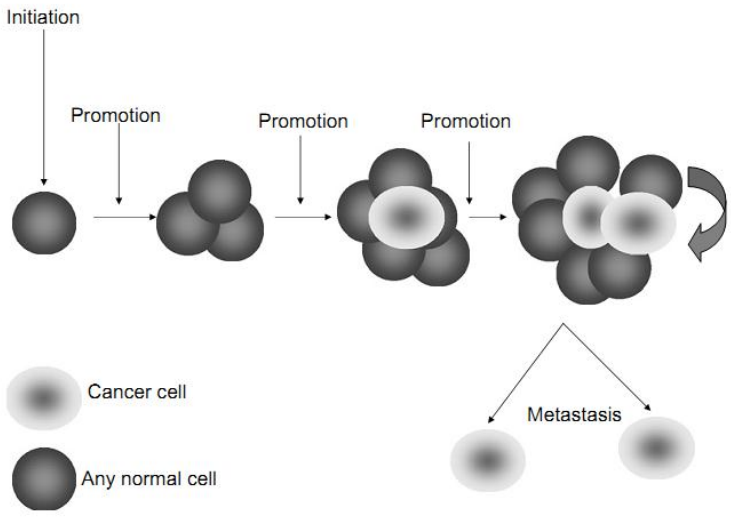

Figure 1.4 – The stochastic model of carcinogenesis. Cancer formation is the phenotypic consequence of panoply of changes that may have taken a long period of time to develop. Following an initiation step, activated by a cancer-inducing agent, a period of tumour promotion takes place. Each stage of the multi-step carcinogenic process reflects genetic and/or epigenetic changes in the cell, which will provide the selective advantage to drive the progression towards a highly malignant state. The age-dependent incidence of cancer suggests a requirement of four to seven rate limiting, stochastic events to produce the malignant phenotype (Adapted from **Argyle and Blacking**, **2007**).

1.3.3 The hierarchical or stem cell model

1.3.3.1 Stem cells

Mammals have three basic cells types in their bodies: germ cells, somatic cells and stem cells (SCs). Populations of stem cells with unique properties exist in essentially all tissues of a multicelular adult organism (Shipitsin and Polyak, 2008**;** Ashkenazi *et al*., 2008). One of the most important characteristics of SCs is their ability to generate full lineages of differentiated cells through a process called differentiation. This provides the differentiated cell, with the ability to perform a specific function, i.e., a liver cell, a blood cell, or a neuron. This process is very common during the development of a multicelular organism since its evolution from a simple zygote till a complex system of tissues and different cell types. Moreover, as the long-lived SCs subsist throughout the entire life

span of organisms, they are attractive targets of tumorigenesis, a process that often requires years to elapse (Shipitsin and Polyak, 2008; Ashkenazi *et al*., 2008).

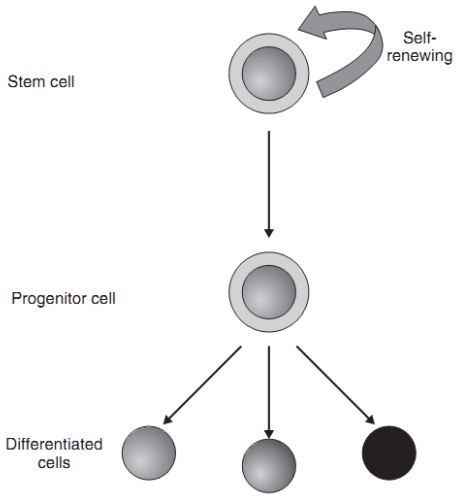

Figure 1.5 – Basic outline of stem cell functioning (Adapted from **Bapat *et al*., 2009**).

SCs are mainly characterized by their ability to go through numerous cycles of cellular division while maintaining the undifferentiated state (self-renewal), and their capacity to differentiate into specialized cell types (potency). Another important property of SCs is their homeostatic control, i.e., the existence of tight mechanisms regulating the balance between self-renewal/differentiation and quiescence/proliferation, in all tissues and organs. This mechanism is largely influenced by environmental stimuli and genetic constrains in order to maintain stem cell numbers (self-renewal capability) via the ratio of symmetric and asymmetric cell divisions (Bapat *et al*., 2009).

In asymmetric cell divisions, each stem cell divides to generate one daughter stem cell, that has the same properties as the parent cell (self-renewal), and one daughter cell intended to differentiate (Figure 1.6A). Conversely, in symmetric cell divisions, each SC can generate two phenotypically identical daughter cells that can be either stem cells (symmetric self-renewal) or differentiated cells (symmetric differentiation) (Figure 1.6B)

(Ashkenazi *et al*., 2008; Morrison and Kimble, 2006). SCs division can be either controlled by the stem cell itself (intrinsic) or by paracrine factors produced at the SC niches by stromal cells (extrinsic) (Miller *et al*., 2005).

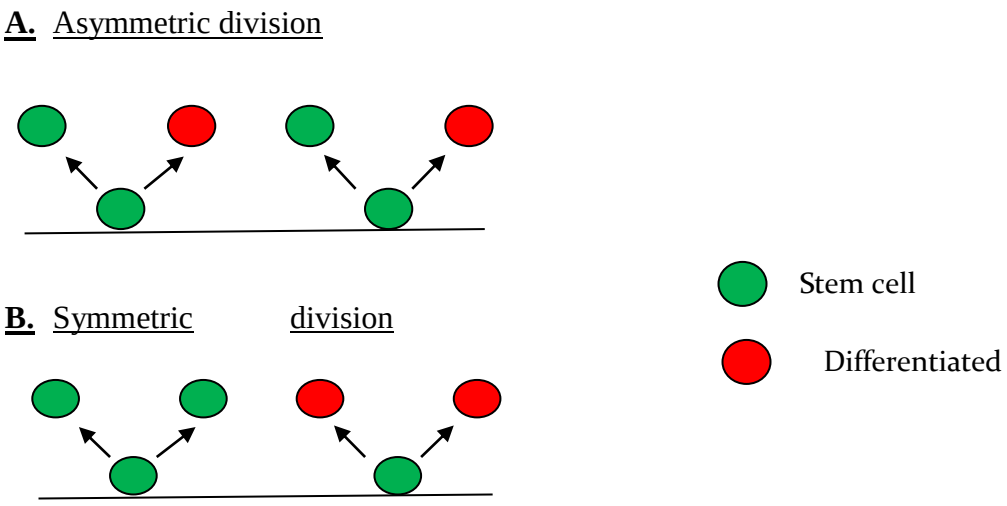

Figure 1.6 – Stem cell division models. Asymmetric self-division (A), results in one stem cell and one pluripotent cell. Symmetric division (B) can comprise symmetric self-renewal and symmetric differentiation which results in two daughter stem cells or two progenitor cells, respectively.

SC niches regulate the contribution of stem cells to tissue development, maintenance and repair (Scadden, 2006). Those niches integrate signals elicited by the surrounding environment which mediates the response of stem cells to the needs of the organisms. Therefore, dysfunctional signals from the environment play an important role on the onset of diverse pathologies, including cancer. Another important function of the SC niches is to avoid the depletion of stem cells, while protecting the host tissue from SC uncontrolled proliferation.

1.3.3.2 Cancer stem cells: What is known about?

According to the stochastic model, the malignant transformation is understood as a disruption of homeostasis during which normal cells suffer multiple genetic mutations, which will provide all cells with higher proliferative capacity (Figure 1.7a) (Reya *et al.*, 2001; Argyle, and Blacking, 2007; Miller *et al.*, 2005).

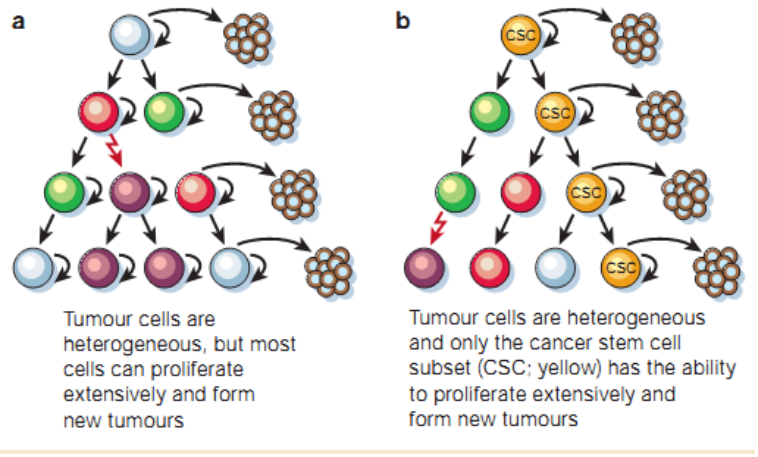

Figure 1.7 – Two general models of heterogeneity in solid cancer cells. a, Cancer cells of many different phenotypes have the potential to proliferate extensively. The model shown in **b** predicts that a distinct subset of cells is enriched for the ability to form new tumours, whereas most cells are depleted of this ability (Adapted from **Reya *et al.*, 2001**).

However, this model is unable to explain the clinical remissions and relapses of many tumours observed following chemo- and radio therapies. Additionally, the recognition that tumours are highly heterogeneous entities, composed of multiple cellular sub-populations with a wide range of malignant, tumorigenic and metastatic potentials, led to the proposal of the hierarchical or the SC model. According to this model, the stem cells or cells that acquired the SCs properties, accumulate genetic changes over long periods of time, escaping from the control of their environment, originating cellular sub-populations with dissimilar malignant, tumorigenic and metastatic potentials (Figure 1.7b and 1.8) (Reya *et al.*, 2001; Shipitsin and Polyak, 2008; Visvader, 2011). These cells,

called cancer stem cells (CSCs), due to their stem cell-like characteristics, such as, the capacity for asymmetric divisions, giving birth to a quiescent stem cell and a committed progenitor cell; expression of stem cell characteristic factors such as Oct4, Nanog, and Sox2; expression of adenosine triphosphate (ATP)-binding cassette (ABC) transporters, contributing to cellular resistance to specific cytotoxic and cytostatic drugs; extended telomeres and telomerase activity and, consequently, increased cellular life span; resistance to apoptosis as result of antiapoptotic pathways activation; and, finally, expression of surface receptors either identical to the stem cell markers or associated with homing and metastasis (Wicha *et al*., 2006; Bapat *et al*., 2009), have been proposed to start off either from malignant transformation of a normal somatic cell or a progenitor cell and, in the tumour, are credited as the only cells with tumorigenic and metastatic potential (Welte *et al*., 2010).

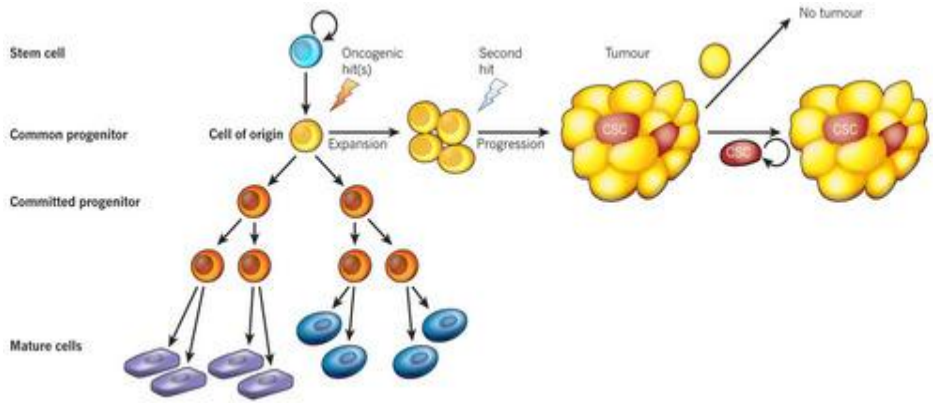

Figure 1.8– The cell of origin and the evolution of a cancer stem cell. Cellular hierarchy starting off with stem cells progressively, generating common and more restricted progenitor cells to, finally, yield all the mature cell types that constitute a specific tissue. Although the cell of origin, for a particular tumour, could be an early precursor cell such as a common progenitor, the subsequent acquisition of further genetic/epigenetic mutations by a cell, within the aberrant expanding population during neoplastic progression, may result in the emergence of a CSC. According to this model, only the CSCs (and not other tumour cells) are capable of sustaining tumorigenesis. Thus, the cell of origin, that initiates tumorigenesis, may be distinct from the CSC which propagates the tumour. (Adapted from **Visvader, 2011**).

Because normal SCs and CSCs share the ability to self-renew, it seems reasonable to propose that newly arising CSCs appropriate the machinery for self-renewing cell division that is normally expressed in normal SCs. Evidence shows that many pathways that are classically associated with cancer may also regulate normal SC development, for example, the prevention of apoptosis, by enforced expression of the oncogene Bcl-2, results in increased numbers of SCs *in vivo*, suggesting that cell death has a role in regulating their homeostasis (Domen *et al*., 1988; Domen and Weissman, 2000). Other signalling pathways associated with oncogenesis, such as the Notch, Sonic Hedgehog (Shh) and Wnt signalling pathways, also regulate stem cell self-renewal **(**Reya *et al*., 2001; Hanahan and Weinberg, 2011).Notch activation in hematopoietic stem cells (HSCs) in culture using the ligand Jagged-1 have consistently increased the amount of primitive progenitor activity that can be observed *in vitro* and *in vivo,* suggesting that Notch activation promotes HSC self-renewal, or at least the maintenance of their multipotentiality (Varnum-Finney *et al*., 2000; Karanu *et al*., 2000)**.**

CSCs, first identified in the hematopoietic system because of their unique properties have also been termed "tumour-initiating cells even though the cell of origin in which tumorigenesis is initiated may be distinct from the CSC, which propagates the tumour (Visvader, 2011). Although the origin of CSCs is rather controversial, several authors considered that CSCs are engendered by differentiation of malignant stem or progenitor stem-like cells and, as such, are sometimes represented as an intermediate state between malignant stem cells and malignant differentiated cells (Welte *et al*., 2010) (Figure 1.9). However, very recently, our group (Rodrigues *et al*., 2011) and Weinberg and collaborators (Chaffer *et al*., 2011) attested that CSCs may also arise by dedifferentatiation of malignant epithelial cells.

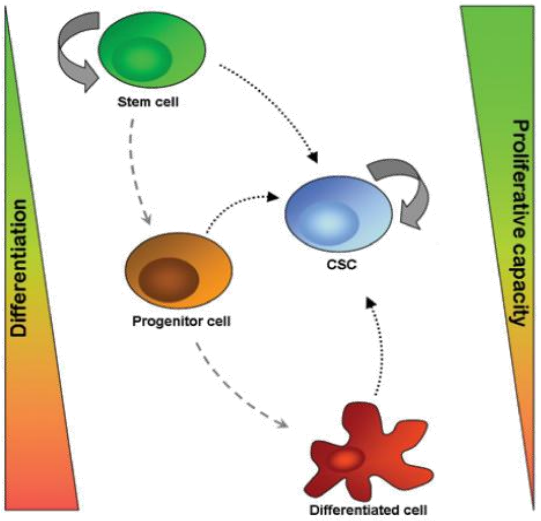

Figure 1.9 - Relationship between stem cells and their differentiation states. Stem cells are characterized by their ability to differentiate into different cellular phenotypes, originating many different types of tissues, and by their capacity to self-renew. In contrast to progenitor SCs, differentiated cell has reduced plasticity. According to some authors', cancer stem cells may arise at the interface between stem cells and progenitor cells (Adapted from **Welte *et al*., 2010)**.

More recently, the presence of sub-populations of CSCs in tumours of lung, prostate, brain, colon and in malignant cell lines from different origins have been identified by their cell surface markers, which are important biomarkers, ideal for their isolation by flow cytometry. The following Table 1 illustrates a wide range of CSCs characteristic biomarkers.

Table I – Characteristic CSCs biomarkers used to isolate CSCs from various malignancies (Adapted from **Boman and Wicha, 2008**).

Table 2. Markers to Identify and Isolate Stem Cells From Various Malignancies

	Stem-Cell Markers										
Cancer	CD44	CD24	CD133	ALDH1	ESA	B1	α6	CD138	CD34	CD166	CD20
Breast	+	−	+	+	+	+	+				
Colon	+		+	+*	+					+	
Prostate	+		+	+		+	+				
Head and neck	+			+*†							
Pancreatic	+	+	+	+	+						
Lung			+								
Brain			+								
Liver			+								
Melanoma	+		+			+					+
Multiple myeloma				+				−	+		+
Leukemia	+			+					+		

NOTE. Definitions for the human CD (cluster destination) molecules are: CD44, hyaluronate receptor (p-glycoprotein 1); CD24, heat stable antigen; CD133, prominin 1; ALDH1, aldehyde dehydrogenase 1A1; ESA, epidermal surface antigen (Flotillin/2); B1, integrin B1 chain; α6, integrin α6 chain (CD49F); CD138, heparin sulfate proteoglycan fibroblast growth factor receptor (syndecan proteoglycan 1); CD34, hematopoietic progenitor cell antigen (GP105-120); CD166, activated leukocyte cell adhesion molecule; CD20, B-lymphocyte cell-surface antigen B1, membrane-spanning 4-domains, subfamily A, member 1.
*Unpublished data: BM Boman, MA Wicha, and E Huang.
†Unpublished data: M Prince and MS Wicha.

CD133, the structural homolog of murine prominin-110, is regarded as an important marker for the identification and isolation of primitive stem and progenitor cells, present in a variety of cancers (Boman and Wicha, 2008). The CD133 positive ($CD133^{+}$) cells, ascribed as CSCs, are provided with tumour-initiating capacity in contrast with their $CD133^{-}$ counterparts (O' Brien *et al*., 2009). CD44 is another important biomarker reported as characterizing CSCs in breast, colon, prostate, head and neck, pancreatic, melanoma and leukaemia tumours. Interestingly, the presence of cells with profile $CD44^{+}/CD24^{-/low}$, which outlines the presence of CD44 and the absence of CD24 expression markers, identifies breast-tumour initiating cells (Al-Hajj and Clarke, 2004). Another important CSCs biomarkers not included in Table 1, is CD34 a sialomucin protein used to identify hematopoietic stem/ progenitor cells in clinical hematology. The profile $CD34^{+}/CD38^{-}$ identifies CSCs subpopulations present in acute myeloid leukemia (AML) (Ailles and Weissman, 2007).

Another approach used to identify CSCs is the side population (SP) assay. This assay exploits the capacity of CSCs to efflux certain drugs and dyes, such as Hoechst 33342 (Wu *et al*., 2012). Exclusion of Hoechst 33342 dye by SP cells is a dynamic process involving the multidrug resistance transporter 1 (MDR1), a member of the ABC

transporter transmembrane proteins, named P-glycoprotein (P-gp). As CSCs express high levels of cell membrane ABC transporter proteins, they are provided with the ability to efflux chemotherapeutic drugs (O' Brien *et al*., 2009; Tang *et al*., 2007). Therefore, it is expected that cells with tumour-initiating capacity exist exclusively within the SP^+ population. **Schatton** and collaborators revealed that $ABCB5^+$ SP melanoma cells expressing the 5^{th} ABC, from the subfamily B, are tumour-initiating cells in contrast with their $ABCB5^-$ counterparts (Schatton *et al*., 2009).

Despite the latest improvements in CSCs identification, investigation should continue in order to achieve a better understanding of the role of CSCs in tumours formation and proliferation.

1.3.3.3 CSCs and resistance to chemotherapy

Multi-drug resistance (MDR) is classically defined as a state of resilience against structurally and/or functionally unrelated drugs. This resistance can be inherent (intrinsic) to the cells or acquired after exposure to chemotherapeutic agents (Milane *et al*., 2011). Existing therapeutic approaches have been designed largely based on the stochastic model, since most chemotherapeutic agents are addressed to the tumour major sub-populations which have limited self-renewal and proliferative potential. As most therapeutic effects are usually transient and fail to ablate most cancers, future therapies based on the hierarchical model are thought to be much more successful. In fact, as referred above, CSCs have the ability to self-renew and to generate a wide range of cellular sub-populations within the tumour, and are responsible for sustaining tumorigenesis. So, a successful cancer therapy would necessarily have to abrogate the CSCs population (Figure 1.10). However, so far such therapy has not been developed, mainly because CSCs are highly radio- and chemo-resistant and, as such, the dose of

either chemo- or radio agent necessary to eliminate the CSC population would kill the patient. This way, while a targeted therapy is being designed, tumours often relapse after conventional treatments, and patients often die of metastatic disease (Reya *et al*., 2001; Schatton *et al*., 2009).

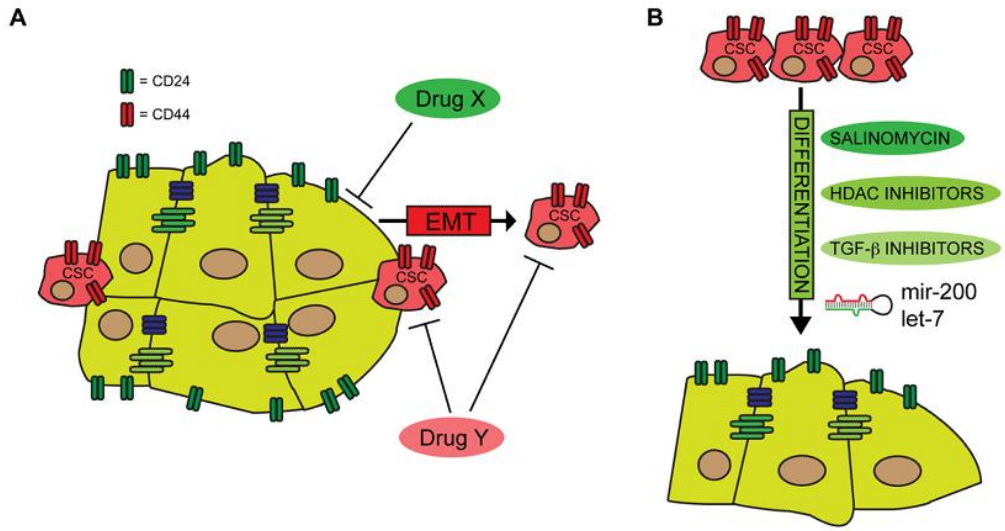

Figure 1.10 – Effective cancer therapy may require combination treatments, A. An effective therapy would be the combination of the drug X, targeting the bulk tumour cells that are well-differentiated and the drug Y, which kill cells with a CSC phenotype. Cancer cells treated with drug X may undergo EMT, in order to acquire CSC-like properties, in which case co-administration of drug Y would kill both pre-existing CSCs and those that emerge via treatment with Drug X. **B.** A novel strategy to eliminate CSCs could be to induce their differentiation, which may lead to intrinsic apoptotic cell death, or may sensitize the resulting differentiated cells to existing therapies (Adapted from **Singh and Settleman, J., 2010**).

CSCs resistance to chemotherapy relies on several mechanisms, the most important of which are the increased DNA damage recognition and repair, the alterations of cell cycle checkpoints the impairment of tumour apoptotic pathways the reduced accumulation of cytotoxic chemotherapeutic agents, through enhanced energy-dependent drug efflux and the increased Wnt/β-Catenin and Notch proliferating signalling pathways (Milane *et al*., 2011; Eyler and Rich, 2008).

It seems that a good strategy to prevent the resistance to chemotherapy and, consequently, to eliminate tumour cells relies on the inhibition of the main drug transporters (Dean et al., 2005). Alternatively, treatments that target CSCs specific markers or signalling pathways critically involved in CSCs function seem also to reduce the risk of relapse and dissemination. However, given the similarities between CSCs and normal SCs, the development of CSC-directed treatment strategies will have to be carefully implemented. Moreover, as CSCs represent a heterogeneous cell population with rather different resistance profiles, several therapeutic agents will have to be used (Singh and Settleman, 2010).

Due to what was mentioned before, a novel strategy to eliminate CSCs could be to induce their differentiation, which could lead to intrinsic apoptotic cell death or could sensitize the resulting differentiated cells to existing therapies (Singh and Settleman, 2010).

Recent advances in cancer genomes indicate a role for epigenetic regulators in driving cancer, which may result in the acquisition of additional (epi)genetic modifications (changes in gene expression that are independent of changes in DNA sequence and persist over many cell divisions, leading to drug resistance (Wilting and Dannenberg , 2012). These observations may lead to the development of new anticancer drugs, called *epigenetic drugs* that can prevent or reverse non-responsiveness to anti-cancer drugs.

Besides the epigenetic changes mentioned before, recent data showed, that drug resistance mechanisms might also be regulated by microRNAs (miRNAs) (Giovanetti *et al*., 2011**)**. miRNAs are small, non-coding and evolutionary conserved RNA molecules, and are negative regulators of gene expression through modulation of the post-transcriptional activity of multiple target mRNAs by direct cleavage or repression of

translation, so their up- and/or down-regulation influence the expression of multiple target mRNA, and consequently multiple proteins, leading to variations in chemosensitivity of cancer cells (Wang *et al*., 2010).

1.3.4 Cellular plasticity

A typical epithelium is a layer of cells, often one cell thick, lying on the top of connective tissue. The epithelium, separated by a basement membrane from the connective tissue, has cell-cell junctions and adhesions that hold tightly the neighbouring epithelial cells and inhibit the movement of individual cells away from the monolayer, thus imposing mechanical rigidity. This rigidity and the lateral migration prevent the epithelial cells to enter into the underlying extracellular matrix (ECM), unlike mesenchymal cells which can traverse individually the ECM (Yang and Weinberg, 2008; Zavadil and Bottinger, 2005). The ECM, formed by an interstitial matrix and a basement membrane where epithelial cells rest, provides structural support to the animal cells, separating tissues from one another, and regulates intercellular communication.

An important characteristic of epithelial cells is their polarity, denoting that the apical and basal surfaces are likely to be visually differently, have different functions and adhere to different molecules (Figure 1.11). In contrast, the end-to-end polarity and the absence of tight intracellular adhesions, provide the more extended and elongated mesenchymal cells with prominent migratory capacity. The migratory features of epithelial and mesenchymal cells are also somewhat different, with epithelial cells moving as a sheet “en block”, while the mesenchymal cells are rather more dynamic (Figure 1.11) (Lee *et al*., 2006). Different sets of proteins used as biomarkers, allow the distinction between these two cell types (Tiwari et al., 2012) (Figure 1.11).

The transmembrane proteins cadherins play important roles in cell adhesion, ensuring that cells within tissues are bound together. The presence of E-cadherin, in epithelial cells, is essential for the great cell-cell adhesion strength observed in epithelium when compared with to the N-cadherin-expressing mesenchymal cells (Lee et *al*., 2006). The integrins, which are heterodimeric transmembrane proteins formed by α and β subunits, are other important players in cell adhesion. These receptors mediate the attachment between a cell and the surrounding tissues, which may be other cells or the ECM. During the process of tumour cell invasion, integrins, which bind to various ECM components, critically regulate cell adhesion to the basement membrane (Maschler *et al*., 2005). Additionally, integrins play an important role in cell signalling, thereby regulating the cellular shape, motility, and cell cycle.

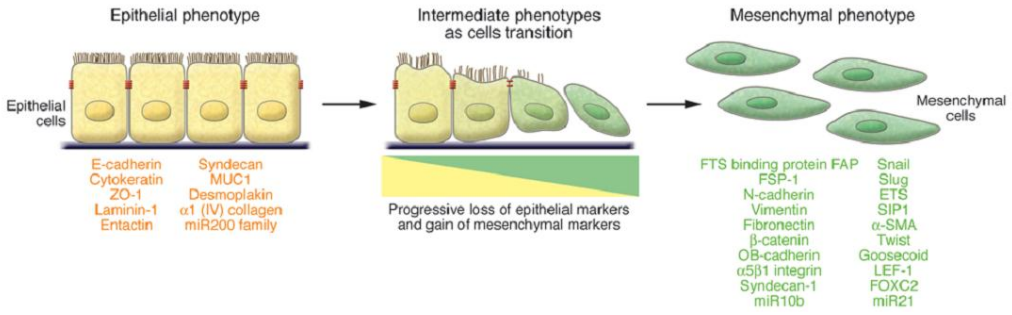

Figure 1.11 – The epithelial and mesenchymal cell biomarkers used to differentiate epithelial from mesenchymal cells. The epithelial to mesenchymal transition (EMT) encompasses the functional transition of polarized epithelial cells into the ECM component-secreting, mobile mesenchymal cells. Detection of cells expressing both sets of markers renders impossible to identify whether it is an epithelial cell or a mesenchymal cell. Fortunately, all mesenchymal cells are devised from the epithelia via EMT, as many mesenchymal cells likely lose all epithelial markers once a transition is completed (Adapted from **Kalluri and Weinberg, 2009**).

Epithelial-to-mesenchymal transition (EMT) and the reverse process known as mesenchymal-epithelial transition (MET), occur widely during embryonic development and represent clear examples of what is called cells' phenotypic plasticity (Thiery, 2002; Alison *et al.*, 2006; Yang and Weinberg, 2008). The MET process involves the conversion of mesenchymal cells to epithelial ones, and is associated with kidney formation. On the other hand the EMT process can be defined as a biologic process that allows a polarized epithelial cell, to undergo multiple biochemical changes that enable it to assume a mesenchymal phenotype (Kalluri and Weinberg, 2009). In other words, the EMT is responsible for the generation of cells with a distinct phenotype capable of allowing the invasion of the local surrounding tissues, and eventually, the systemic dissemination. (Figure 1.11) (Kalluri, 2009). The ability to migrate and invade ECM as single cells is a functional hallmark of the EMT program (Yang and Weinberg, 2008). In many carcinomas, several EMT-inducing released signals, e.g., hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming factor beta (TGF-β), appear to be essential for the induction and functional activation of a series of transcription factors, especially Snail, Twist, Goosecoid and Foxc2 (Thiery, 2002; Kalluri, 2009). In situations of tissue repair and pathological stresses, the activation of EMT programs can, also, be assisted by the disruption of cell-cell adherens junctions and cell-ECM adhesions mediated by integrins, leading to a state of cell motility (Kalluri and Weinberg, 2009).

Several distinct molecular processes are involved along the EMT process, from its beginning to its completion, namely for the activation of transcription factors the expression of specific cell-surface proteins the reorganization and expression of citoskeletal proteins (e.g., CD133 in lung epithelial cells) the production of ECM-degrading enzymes and the induction of changes on the expression of specific miRNAs

and *TGF-β* (Kalluri *et al*., 2009; Lee *et al*., 2006; Mani *et al*., 2008; Tomaskovic-Crook *et al*., 2009). Among these molecular processes, the changes on the expression of specific miRNAs and of *TGF-β* have been described to directly affect other events that occur during the EMT.

Studies mentioned that EMT involves the downregulation of microRNAs, encoding epithelial markers such as E-Cadherin, and the upregulation of microRNAs, encoding mesenchymal markers such as N-Cadherin, Vimentin, and Fibronectin (Lee *et al*., 2006; Mani *et al*., 2008). Thus, changes on cadherin-based adhesion play a key role in modulating development and organogenesis, as the activation of EMT program is high facilitated by the disruption of cell-cell adherent junctions and cell-extracellular matrix adhesions mediated by integrins (Lee *et al*., 2006; Mani *et al*., 2008). Recent discoveries revealed that malignant cells lacking E-Cadherin become more responsive to the induction of EMT, by various growth factors, and showed an increased tumorigenic and metastatic potential when these cells are injected into immunodeficient mice (Kalluri and Weinberg, 2009). Also, mutations in the E-Cadherin gene revealed to render malignant cells more susceptible to EMT and, consequently, with an increased metastatic potential (Kalluri and Weinberg, 2009**)**.

TGF-β is simultaneously an important suppressor of epithelial cell proliferation, and a positive regulator of tumour progression and metastasis, depending on the sub-pathways activated by this growth factor (Zavadil and Bottinger, 2005). There are also some evidences of a relationship between the molecular processes that control TGF-β-induced apoptosis and those that regulate EMT, as once the cell adopted a mesenchymal phenotype it no longer respond to TGF-β suppressor effects (Sánchez and Fabregat, 2010). As far as to the involvement of TGF-β family members in the EMT program, studies revealed that they play important roles in the initiation and maintenance of the

EMT in a variety of biological systems and pathophysiological situations, through the activation of major signalling pathways and transcriptional regulators integrated in extensive signalling (Sánchez and Fabregat, 2010).

Another process related with EMT is the dedifferentiation. This is a mechanism that requires a differentiated cell to revert to a more primitive stem cell phenotype, in other words, the phenotype of specialized cells can be changed, rendering them closer to their ancestors with augmented plasticity (Zhang *et al*., 2010). And, as mentioned before, this process can be responsible for the origin of CSCs in humans (Rangwala *et al*., 2010; Ben-Porath *et al*., 2008). This cellular process can also be seen in more basal life forms such as worms and amphibians, which have regenerative process (Stocum, 2004; Casimir, 1988).

1.4 Major cancer histological types

There is great diversity of histological cancer types, classified based on the function and localization of cells of origin. This way there are the carcinomas, tumours of epithelial cells, sarcomas that arise from bone, muscle, cartilage, and adipose tissue, and the leukemias, lymphomas and myelomas, tumours originated from the hematopoietic lineage. The most common histological type is the carcinoma, most likely because of the great proliferation competence of the epithelial tissue, and the easy exposure access to a wide range of chemical, physical and biological carcinogenic agents.

1.4.1 The bronchial epithelium

The pulmonary system contains a variety of epithelial cell populations residing in distinct anatomical locations. Basal, secretory, and ciliated cells are present in the trachea and the proximal conducting airways. The nonciliated, columnar Clara cells comprise the majority of the bronchiolar and terminal bronchiolar epithelium in mice, and alveolar type 1 (AT1) and type 2 (AT2) cells constitute the alveolar epithelium (Figure 1.12). The conducting airways are constituted by a specialized epithelium whose composition and function varies along the proximal to distal axis (Hong *et al*., 2004). The airway epithelium is pseudostratified in the large airways, becoming columnar and cuboidal in the small airways (Figure 1.12) (Crystal *et al*., 2008; Bannister, 1999).

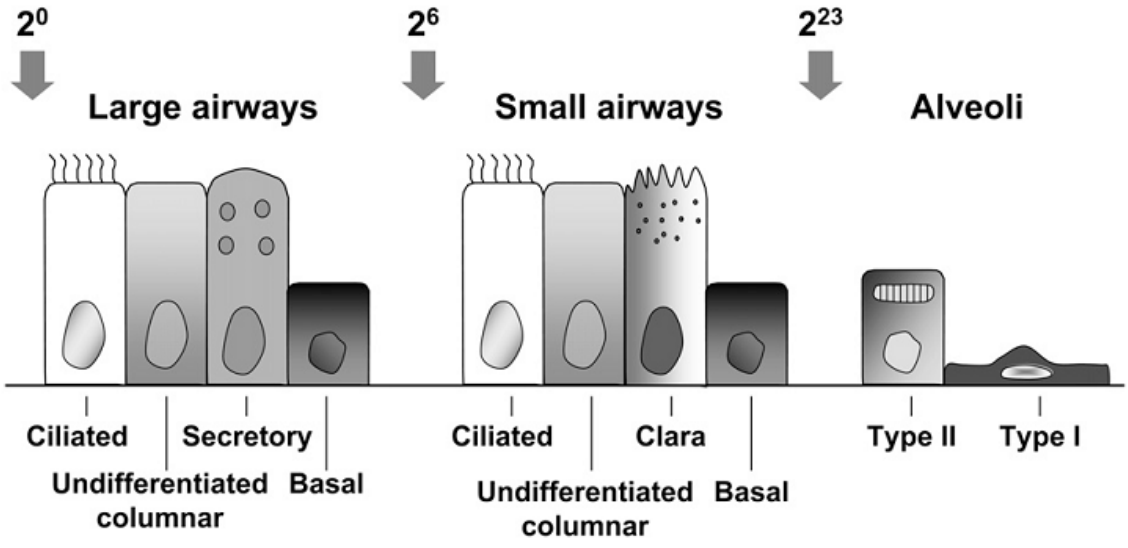

Figure 1.12 – Major cell types of the lung epithelium. In the large airways the major cell types are ciliated, undifferentiated columnar, secretory, and basal cells. In the small airways, the cell types are similar, with relatively more ciliated cells, and the secretory cells shift to the Clara cell type. In the alveoli, the airway epithelium merges with the alveolar epithelium, with type I and type II cells (Adapted from **Crystal *et al*., 2008**).

The main role of the airway epithelium is to drive the flux of air to and from the alveoli. Another important role is to protect the lung against pathogens and particulates inhaled from the atmosphere, which is achieved with the combined function of secretory and ciliated cells to sustain an efficient mucociliary clearance.

The airway epithelium is a dynamic tissue with a slow, but constant renewal, that when injured, responds quickly and effectively re-establish the normal structure and function of the epithelial layer. Strong evidence supports that stem/progenitor cells distributed throughout the airway epithelium are the source of the new epithelial cells, in other words, the mature cells of the tissue are constantly replenished by a minority population of tissue SCs (Al-Hajj and Clarke, 2004). However the process through which this replenishment occurs is still unclear. Recent murine models postulated that during normal tissue homeostasis, facultative progenitor cells (Clara cells and Type II pneumocytes), situated throughout the lung epithelium, mediate any necessary maintenance, due to their capacity to self-renew and differentiate into other lung epithelial cell types. However, progenitor cells have a restricted potential to regenerate epithelial cells, because they only can regenerate within their resident anatomical compartment. So, basal, Clara, or vCE (variant Clara cell secretory protein-expressing cells) cells only regenerate the airways while the Type II pneumocytes regenerate the alveoli (Delgado *et al*., 2011). While the maintenance of the adult murine lung is widely elucidated, many details concerning the maintenance of human lung remain uncertain because it is not possible to transpose the findings directly into humans.

1.4.2 Lung cancer

As mentioned before, LC is the most commonly diagnosed cancer worldwide and also the most frequent cause of cancer related deaths (Siegel *et al*., 2011). The high mortality rate of LC is due to the late diagnosis when the only viable options for treatment are most palliative (Wistuba and Gazdar, 2006).

Commonly, LC has an epithelial origin and, as a consequence, is classified as carcinoma (Panno, 2005**)**. Lung carcinomas normally arise in the epithelium of the major bronchi (central tumours) or in small bronchi, bronchioles, or alveoli (peripheral tumours) of the distant airway of the lung. Peripheral tumours usually metastasize widely to lymph nodes in the liver, bone and adrenal glands (Wistuba and Gazdar, 2006; Ross, 1998).

Lung carcinomas are generally divided into two major types: small cell lung cancer (SCLC), a highly aggressive and frequently lethal human tumour, which accounts for nearly 25% of all lung tumours, and non-small cell lung cancer (NSCLC), that accounts for about 75% of the total lung cancers (Risch and Plass, 2008). NSCLC can additionally be sub-divided in three major histological types: squamous cell lung carcinoma (SCC), adenocarcinoma (including the non-invasive type of bronchioloalveolar carcinoma) and large-cell lung carcinoma (LCC) (Travis, 2011).

The SCLC exhibits a more aggressive biological behaviour, disseminating widely and being seldom cured by surgical resection. SCLC recurrence after chemotherapy is highly frequent. NSCLC may be cured by surgery if diagnosed at early stages but the clinical outcome is often hard to determine (Travis, 2011). In addition, SCLC tumours have a better initial response to cytotoxic therapies than do NSCLC

The most common cause of lung cancer is long-term exposure to tobacco smoke. Other risk factors which include exposure to nickel, arsenic, chromium and polycyclic

hydrocarbons have been accepted as major causes of lung cancer (Coppé *et al*., 2008; Salnikow and Zhitkovich, 2008; Urbano *et al*., 2008).

Many substances present in tobacco and other environmental carcinogens induce mutation in important genes (e.g., in *Tp53* gene), with significant impact in lung cancer, as they play important roles in tumorigenesis of lung epithelial cells, and/or disruption of some important signalling pathways (Wnt, Hedgehog (Hh), and Notch) (Toyooka *et al*., 2003; Lemjabbar-Aloui *et al*., 2006; Muller *et al*., 2007). For example, restricted deregulation of Hh signalling, in small patches of epithelial cells, lead to the formation of SCLC and, consequently, to tumour maintenance (Magliano and Hebrock, 2003; Watkins *et al*., 2003**).** Another feature of these pathways is that they are intimately interconnected with each other (Katoh, M., *et al*., 2007).

Hh, Wnt and Notch are also accountable for triggering a small number of signalling pathways that ensure CSCs fate (Magliano and Hebrock, 2003; Reya and Clevers, 2005). Therefore, a promising and more specific anticancer therapy is addressed to Hh, Notch and Wnt pathways (Muller *et al*., 2007).

Besides the signalling pathways mentioned before other key genetic features that are commonly associated with lung adenocarcinomas, are the activation of the oncogene *KRAS*, the inactivation of the tumour suppressor genes *Tp53*, *Rb1* and *PTEN*, the loss of heterozygosity (LOH) at selected chromosomal arms, the activation of telomerase and the amplification/over-expression of *Myc*, *Cyclin D1*, epidermal growth factor receptor (*EGFR*), *C-erbB2* and *Bcl2* (Licchesi *et al*., 2008; Risch and Plass, 2008; Pleasance *et al*., 2010). The over-expression of EGFR, a receptor tyrosine kinase, of the erbB family is one of the most important events in lung cancer. This receptor dimerizes following binding to several specific ligands and phosphorylates several tyrosine residues (Arteaga,

2002). This event initiates multiple signalling pathways resulting in cells' proliferation, evasion from apoptosis and migration (Kosaka *et al*., 2004).

The first targeted therapy to be registered and later approved by the Food and Drug Administration (FDA) for use in LC was Gefitinib (Molina *et al*., 2008; Maemondo *et al*., 2010), a tyrosine cynase inhibitor (TKI), however its activity is limited to a subgroup of patients with NSCLC (Lynch *et al*., 2004; Molina *et al*., 2008).

Epigenetic alterations have also been reported to contribute to the initiation and progression of several types of cancer, especially adenocarcinomas (Brock, 2008). The most common epigenetic event which affects the chromatin condensation status is gene silencing by methylation of its promoter at the CpG islands (Risch and Plass, 2008). And it was observed that patients with several hypermethylated genes were the most likely to develop lung adenocarcinoma (Brock, 2008)**.**

1.4.2.1 CSCs in lung cancer

As mentioned in section 1.3.3.2 CSCs are present in a wide range of cancers including LC (Kim *et al*., 2005**).** Besides that, human lung tumours show a phenotypic heterogeneity, suggesting that they may originate from a multipotent cell (Berns, 2005). Other evidences include the presence, in LC, of sub-populations of SP cells (Ho *et al*., 2007), resistant to multiple chemotherapeutic agents such as cisplatin, gemcitabine, and vinorelbine, commonly used as first-line therapy in lung cancer treatment (Ho *et al*., 2007). Recently, a study revealed the presence, in LC, of a sub-population of cells with $CD133^{+}$ phenotype able to self-renew and generate an unlimited progeny of non-tumorigenic cells, as well as resistant to conventional therapy (Eramo, A., 2008). These cells were ascribed to the expression of *Oct-4* gene (Chen *et al*., 2008), an important marker for the subset population of cancer stem-like cells.

Since CSCs are responsible for the drug resistance observed in many types of cancer, including LC, it is of extreme importance to know the molecular and cellular mechanisms behind their formation and maintenance, in order to develop more efficient cancer-therapies.

1.5. Objectives

The objectives of this work is to evaluate the proliferation rate of Cont-1, RenG2, DRenG2 and DDRenG2 cell lines, and their precursor cell line BEAS-2B, the assessment of the cytogenetic evolution from BEAS-2B to DDRenG2, the immunocytochemistry characterization of these cells by assessing their epithelial/ mesenchymal status [MNF 116 and Vimentin expression], the presence of stem-like cells (Oct-4 expression) and the presence of a member of the ABC transporter transmembrane protein (P-glycoprotein expression), and finally, evaluate their resistance to gemcitabine (GEM) and cisplatin (cDDP), which are chemotherapeutic drugs usually used in LC.

2. Material and Methods

All reagents used were of analytic grade. Ultrapure water was obtained using the water purification system Simplicity™ from Millipore S.A., Molsheim, France.

2.1 Materials

Purchased to Sigma-Aldrich Química S.A., Sintra, Portugal:

- Salts for Phosphate Buffer Solution (PBS) preparation, namely KCl, NaCl, KH_2PO_4 and Na_2HPO_4;
- NaOH used for the preparation of the pH adjustment solution;
- Trypan Blue 0,4 % solution (m/v) in PBS, used in cell counting;
- Dimethyl sulphoxide (DMSO) used for cell culture cryopreservation;
- 2 % Gelatin from bovine skin used to prepare the coating solution;
- Bovine serum albumin for 1 % gelatin solution preparation;
- Immersion oil used for microscopy;
- 3-[4, 5-dimethylthiazol-2-yl]2, 5-diphenyltetrazolium bromide (MTT);
- Formamide used for slide denaturation in fluorescent *in situ* hybridization.

Purchased to Gibco® Biochrom, CA, USA, through Alfagene, Lisbon, Portugal:

- TrypLE Express 0,25 % trypsin solution to detach cells;
- Fetal bovine serum (FBS), used in fibroblasts primary cell culture;
- Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 cell culture medium, used in fibroblasts primary cell cultures;
- LHC-9 cell culture medium used for epithelial cells cultures;
- Penicillin and streptomycin used in the preparation of cell culture medium;
- Colcemide used to stop the cell cycle at pro-metaphase;

- Hank's balanced salt solution (HBSS), used in cell manipulation for cytogenetic studies.

Purchased to Panreac Química S.A., Barcelona, Spain:

- HCl used for the preparation of the pH adjustment solution;
- Absolute ethanol used for cell fixation protocol during the immunocytochemistry analysis and for fluorescent *in situ* hybridization.

Purchased to Merk, Darmstadt, Germany, through VWR International, Lisbon, Portugal:

- Methanol used into the preparations of fixation solution for cytogenetic studies;
- Acetic acid also used in the fixation solution for cytogenetic studies;
- Giemsa used to stain the chromosomes, which enhances the banding pattern;
- Gürr buffer at pH 6.8, which was used to prepare the Giemsa solution, and also, to wash the slides;
- KCl, used in the swelling of cells and their nucleus;
- KH_2PO_4 used in the solution A of the phosphate buffer pH 6.8, used in the cytogenetic analysis;
- Na_2PO_4 used in the solution B of the phosphate buffer pH 6.8, used in the cytogenetic analysis;
- NaCl for the preparation of solution 20x SSC used in fluorescent *in situ* hybridization;
- Sodium citrate for the preparation of solution 20x SSC used in fluorescent *in situ* hybridization;

- Tween 20 used in the preparation of the detergent wash solution used fluorescent *in situ* hybridization.

Purchased to European Collection of Cell Cultures (ECACC):

- BEAS-2B cell line as a cryopreserved cellular suspension.

Purchased to Orange Scientific, Braine-l'Alleud, Belgium, through Frilabo, Porto, Portugal:

- 15 and 50 mL centrifuge tubes.

Purchased to Corning Incorporated, NY, EUA, through Sigma-Aldrich Química S.A., Sintra, Portugal:

- 12-wells tissue culture plates, used for cell culture;
- T25 and T75 tissue culture flasks with vent cap, used for cell culture;
- Petri dishes with diverse diameters.

Purchased to Lab Vision Corporation, Fremont, CA, USA:

- UltraVision Large Volume Detection System Anti-Polyvalent, HRP (Ready-To-Use) Kit, for cell immunocytochemistry analysis;
- Biotin-labeled secondary antibody used to detect the primary antibody in immunocytochemistry analysis;
- Peroxidase-conjugated streptavidin for localizing the primary antibody binding in immunocytochemistry analysis;
- PBS used to wash the antibodies in immunocytochemistry analysis.

Purchased from Difco, MC, USA, through VWR International, Lisbon, Portugal:

- Trypsin, used in G-banding.

Purchased to DakoCytomation, Glostrup, Denmark,

- Monoclonal mouse anti-human Cytokeratin antibody, clone MNF116, used for cell immunocytochemistry analysis;
- Monoclonal mouse anti-human Vimentin, clone Vim 3B4, used for cell immunocytochemistry analysis.

Purchased from Novocastra Laboratories Ltd, NewCastle, UK:

- Monoclonal mouse anti-human P-glycoprotein, clone 5B12, used for cell immunocytochemistry analysis;
- 3,3-diaminobenzidine tetrahydrochloride (DAB), used to detect the primary antibody binding in immunocytochemistry analysis;
- Monoclonal mouse anti-human Oct3/4, clone N1NK, used for cell immunocytochemistry analysis.

Purchased to Hanna Instruments, Woonsocket, USA, through Hanna Instruments Portugal, Póvoa do Varzim, Portugal:

- pH meter calibration solutions.

Purchased to Cambio Lda., Cambridge, UK:

- Probes used in fluorescent *in situ* hybridization.

Purchased to Sarstedt Lda., Rio Mouro, Portugal:

- 6 and 24-well tissue culture plates, used for cell culture;
- Cryogenic vials utilized in cells' cryopreservation;
- 2 mL microcentrifuge tubes.

Purchased to Vector Laboratories, Burlingame, CA, USA

- The counterstain DAPI used in fluorescent *in situ* hybridization.

Obtained from Frilabo, Porto, Portugal:

- 2, 5, 10 and 25 mL serological pipettes.

Obtained from Menzel-Gläser, Braunschweig, Germany, through VWR International, Lisbon, Portugal:

- Coverslips and slides, used for immunocytochemistry analysis.

Obtained from Normax, Marinha Grande, Portugal:

- Coverslips and slides, used for fluorescent *in situ* hybridization;
- Coplin jars, used for fluorescent *in situ* hybridization.

Obtained from Qbiogene, Germany

- Glue utilized to seal the coverslip used in the fluorescent *in situ* hybridization.

Obtained from Schleicher & Shuell, Microscience, Dassel, Germany, through Reagente 5, Porto, Portugal:

- 0.2µm filters, model FP30 utilized in the sterilization of small volume solutions.

2.2 Equipment and software

- Bench autoclave, model Omega Media from Prestige Medical, Blackburn, UK, distributed by Ezequiel Panão Jorge, Electromédica, Coimbra, Portugal;
- Water purification system, model SimplicityTM from Millipore S.A., Molsheim, France, distributed by Interface, Equipamento e Técnica Lda, Amadora, Portugal;
- Automatic pipetting aid, model ComfoPette from Orange Scientific, Braine-l'Alleud, Belgium, distributed by Frilabo, Porto, Portugal;
- Haemocytometer from Marienfeld, Germany, distributed by Reagente 5, Porto, Portugal;
- Laboratory hotte from Ibérica Industrial Laborum, distibuited by Alfagene, Lisbon, Portugal;
- Heating plate with magnetic stirrer, model Monotherm from Variomag, Daytona Beach, Florida, USA;
- Heating plate, from Stork tronic, Germany;
- Water bath, model Hyb-bath heating from American Instrument Exchange, Inc., Haverhill, MA, USA;
- Thermal cycler, 96-well VeriFlex ™ Block from Applied Biosystems, Carlsbad, CA, USA.
- pH meter, model HI 110 from Hanna Instruments, Woonsocket, USA, distributed by Hanna Instruments Portugal, Póvoa do Varzim, Portugal;
- Precision weight balance, model Sartorius B P210 S from Sartorius AG, Goettingen, Germany, distributed by Sartorius Lda, Lisbon, Portugal;
- Centrifuge model Heraeus Instruments, Labofuge 400e from Thermo Scientific, Waltham, Massachussets, USA, distributed by SupplyLab, Cacém;

- Flux cabinet model VBH Compact Cabinet from Steril Manufacturing Division, Milan, Italy;
- Inverted microscope, model Nikon Eclipse TS100 from Nikon, USA, distributed by Nikon Portugal, Lisbon, Portugal;
- CO_2 Incubator, model CB150 from Binder-World, New York, USA;
- 96-well multiplate reader spectrophotometer, model SLT Spectra, from Alliance Analytical Inc.;
- Liquid nitrogen storage containers model Forma Cryoplus I from Thermo Scientific, Waltham, Massachussets, USA;
- Ultra-low freezer, model -86C ULT Freezer from Thermo Scientific, Waltham, Massachussets, USA;
- Graphpad QuickCalcs and Graphpad Prism 5, from Graphpad Software, La Jolla, California, USA, available free online at the website http://www.graphpad.com/quickcalcs/index.cfm;
- Nikon ACT-1 Software, from Nikon, USA, distributed by Nikon Portugal, Lisbon, Portugal;
- Microscope Photographic Camera, model Nikon Digital Camera DXM 1200F, attached to inverted microscope Nikon Eclipse 80i, both from Nikon, USA, distributed by Nikon Portugal, Lisbon, Portugal,
- Fluorescent microscope, from Nikon, USA, distributed by Nikon Portugal, Lisbon, Portugal;
- Digital photographic camera, model T-100, from Olympus, USA, distributed by Olympus Portugal, Lisbon, Portugal.

2.3 Preparation of solutions

- ✓ **Tamponized phosphate saline solution (PBS) solutions**

The 10x PBS stock solution is constituted by 2.68 mM of KCl, 4.15 mM of KH_2PO_4, 145 mM of NaCl and 8.1 mM of Na_2PO_4. Following salts solubilization in ultrapure water (miliQ water) and just before the final concentration was obtained by miliQ water addition, the solution pH was adjusted to 7.4.

The 1x PBS solution was obtained by diluting the 10x PBS stock solution with miliQ water. This solution was sterilized prior to use using an autoclave to avoid contamination during cells manipulation and in the preparation of the 1% gelatin solution.

- ✓ **2 % (w/v) Bovine serum albumin (BSA) solution**

The 2% BSA solution was obtained by powdering 2 g of BSA in 100 mL of miliQ water followed by gentle stirring in a magnetic stirrer and subsequent sterilization by filtration in a laminar flow chamber.

- ✓ **2% Gelatin solution**

To obtain this solution, 2 g of gelatin were height and dissolved in a 100 mL of miliQ water in a SCHOTT flask. The solution was gently stirred in a hot plate with magnetic stirrer to ensure complete dissolution of the gelatin. To help the solubilization process, the temperature was slightly increased. Before its use the solution was autoclaved.

- ✓ **1%Gelatin solution**

To obtain 50 mL 1 % gelatin solution, 25 mL 2 % gelatin solution was mixed with

22.5 mL of 1 % PBS and 2.5 mL 1 % BSA solution in a 100 mL SCHOTT flask or in a 50 mL Falcon tube previously sterilized.

✓ **0,4% (m/v) Trypsin solution**

In order to obtain this solution 400 mg of trypsin were dissolved in 100 mL of 1x PBS buffer pH 7.4.

✓ **Ham's F12 supplemented with Ultroser-G®**

To obtain 100 mL Ham's F12 with Ultroser-G®, 97 mL of Ham's F12 was mixed with 1 mL of Ultroser-G®, 1 mL of penicillin and streptomycin mixture containing penicillin at 100 U/mL activity and streptomycin at 100 µg/mL, 1 mL of L-Glutamine and 100µL of ampicillin B .

Note: These additives were stored in frozen aliquots.

- **Solutions for G-banding karyotyping**

In classic cytogenetic protocols for human karyotyping, G-banding is routinely used. This kind of banding gives standard light and dark bands, which are characteristic for each human chromosome.

✓ **Hypotonic solution of 5 mM KCl**

This solution was obtained by dissolution of 0.094 g of KCl in 25 mL of miliQ water.

✓ **Methanol:Acetic acid (1:3) fixation solution**

This solution was obtained by addition of 25 mL of methanol to 375 mL acetic acid, followed by homogenization in a magnetic stirrer.

✓ **Giemsa solution 10 %**

The 10 % Giemsa solution was obtained by dilution of 10 mL of Giemsa in 90 mL of the Gürr buffer pH 6.8.

✓ **Phosphate buffer pH 6,8**

This buffer was prepared from the following two stock solutions:

- **Solution A (Stock)**

  This solution was obtained by dissolving 13.5 g of H_2PO_4 in 1.5 L of miliQ water.

- **Solution B (Stock) pH 6.8**

  This solution was obtained by dissolution of 11.8 g of Na_2PO_4 in 1 L of miliQ water.

  To obtain the desirable buffer, solution B was gently stirred in a magnetic stirrer. Following the insertion of a pH electrode into the solution, solution A was added to solution B keeping the agitation to achieve the desirable pH (6.8).

✓ **pH adjustment solution of NaOH 1 M**

This solution was obtained by dilution of 20 g of NaOH in 500 mL miliQ water.

✓ **pH adjustment solution of HCl 1 M**

The preparation of this solution consists in dilute 1.74 mL of 37 % HCl in 98.26 mL miliQ water.

✓ **Solution to wash the slides – 10 % FBS in Gürr buffer**

To prepare this solution 10 mL of FBS was added to 90 mL of Gürr buffer, and the mixture homogenized carefully in a magnetic stirrer.

- **Solutions for Fluorescence *In Situ* Hybridization (FISH)**

✓ Solution 20x SSC (*Sodium chloride/sodium citrate)*

This solution was obtained by dilution of 86.7 g of NaCl and 44.1 g of sodium citrate in 500 mL of miliQ water. The solution pH was adjusted to pH 7.0 using concentrated HCl, before finalizing water volume. The solution was stored at 4ºC.

✓ Solution 1x SSC

To obtain this solution 475 mL miliQ water were added to 25 mL of a 20x SSC solution, to obtain a final volume of 500 mL. The solution pH was adjusted to pH 7.0.

✓ Solution 2x SSC

To obtain this solution 450 mL of miliQ water were added to 50 mL of a 20x SSC solution, to obtain a final volume of 500 mL. The solution pH was adjusted to pH 7.0.

✓ Solution 4x SSC

To obtain this solution 200 mL of miliQ water were added to 50 mL of a 20x SSC solution, to make up a final volume of 250 mL. The solution pH was adjusted to pH 7.0.

- ✓ Formamide solution 50%

In order to obtain this solution an equal volume (20 mL) of formamide 100 % and 2x SSC solution were added to obtain a final volume of 40 mL.

- ✓ Detergent wash solution (4x SSC/Tween 20)

To prepare this solution 25 µL of Tween 20 were added to 50 mL of 4x SSC.

- ✓ **MTT solution for viability assays**

To prepare the MTT 0.5 mg/mL solution, 75 mg of MTT were dissolved in PBS 1x in a total of 150 mL. The solution was then divided in aliquots of 10 mL and frozen. To prevent contamination of the MTT, the appropriate volume for use during the entire experiment was aseptically removed and placed in a separate tube, which was protected from light.

- ✓ **Acid isopropanol**

Acid isopropanol was obtained by addition of 37 % commercial HCl in isopropanol, to get a 0,1 M concentration.

2.4 Methods

2.4.1 Cell Culture

For the purpose of this work, the bronchial epithelial cell line BEAS-2B and BEAS-2B-derived cell lines: RenG2, DRenG2, DDRenG2 and Cont1 cells (RenG2 non-malignant control) were cultured using the appropriated cell culture medium. All cultures were implemented under aseptic conditions, preventing contamination with

microorganisms, and the cells were incubated, unless otherwise specified, at 37°C in an atmosphere with 5% CO_2

Conditions and asepsis

To ensure aseptic conditions, cell culture was performed in a cell culture room with proper ventilation, constant temperature and no direct contact with the outside. In addition, the presence of UV light allowed its sterilization between utilizations. The flux chamber used was a level 2 safety chamber, also equipped with a UV lamp that allowed the sterilization of the chamber before and after all the proceedings.

All the equipment used in the culture room was previously sterilized by means of autoclaving, bleaching or 70 % alcohol washing. All the solutions were filtered with 0.2 µM-sized porous filters before use while all proceedings were performed with sterile gloves.

2.4.1.1 Culture of BEAS-2B, Cont1 and RenG2 cell lines

Each cell line was defrosted from stock vials and plated in T75 cm^2 flasks pre-coated with 1 % gelatin (for a minimum of 2h) and containing 14 mL of previously warmed LHC-9 medium. The medium was changed 24 h after cells' plating and cells were allowed to grow in the incubator until they reach nearly 80 % confluence. Once reached the desired confluence, culture maintenance was performed as described in section 2.4.1.6.

2.4.1.2 Culture of DRenG2 and DDRenG2 cell lines

DRenG2 and DDRenG2 cells were cultured exactly as the previous cell lines, but the cell culture media utilized was the Ham's F12 supplemented Ultroser-G®, whose preparation has been described in section 2.3.

2.4.1.3 Cells sub-culturing

To guarantee the healthy preservation of the cultures, considering their growth rate and the fact that the cells' phenotype can change once confluence is attained, any cell culture must be sub-cultured when they reach about 80% confluence.

Once nearly 80 % confluence was reached, the cell culture medium was discarded and the flask was washed with 5 mL of 1x PBS to remove the remnants. 1 mL of trypsin was added to detach cells' from the matrix. When the flask surface was fully covered with trypsin, the excess was removed with a pipette, and the flask incubated at 37 ºC for 1 min. The flask was then washed with 5 mL of 1x PBS, and the PBS containing the cells was collected into a Falcon tube that was subsequently centrifuged for 5 min. at 1500 rpm. Following the discharge of the supernatant, the pellet was resuspended in an appropriate volume of medium (generally 0.5 mL to 1 mL). Whenever necessary to evaluate the cells' suspension density, a 20 µL aliquot of the cellular suspension was used for cell counting with trypan blue assay as described in section 2.4.2.

2.4.2 Cell counting

To evaluate the cellular density from each cell suspension, a 20 µL sample was collected and mixed with an equal volume of 0.4% trypan blue solution. As the dye enters into dead cells, because their membranes are damaged, and cells became easily visualized by presenting a typical blue color, it will be possible to assess their number.

Simultaneously, as living cells are surrounded by dye molecules, they will appear brighter which will also allow the evaluation of their exact number.

A Neubauer chamber, commonly named haemocytometer as it was used to count blood cells, was used to count of the cells. This device consists in a special microscope slide equipped with a lamella and two wells with a fixed volume of 10^4µL, divided into four equal quadrants. Each haemocytometer quadrant has the virtual volume of 0.1 mm^3 or 0.0001 mL and the haemocytometer contains only half of the cells' volume removed from the original cell suspension as half of the volume was the trypan blue solution. Therefore, to calculate the viable cells' concentration expressed as number of cells/volume (mL), the average number of cells were multiplied by 2 (due to dilution step) and by 10000 (due to the volume of each quadrant). For cell counting, when the cells were at the lines that limit the quadrants, the only ones counted were on the right and bottom lines (green lines) (Figure 2.1). Consequently, the cells that were on the left and up lines weren't counted (red lines) (Figure 2.1). With this in mind it was possible to determine the cells' concentration in the original suspension using a Nikon eclipse TS100 microscope.

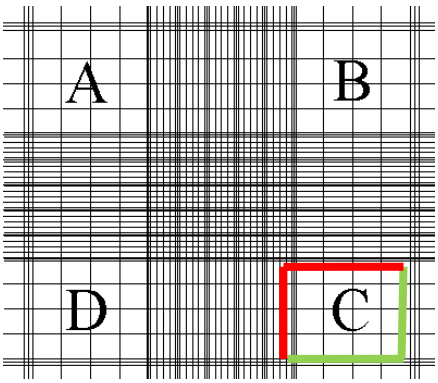

Fig. 2.1 - Schematic representation of the haemocytometer relation area/volume

2.4.3 Cells' manipulation for cytogenetic studies

The protocol encompassed several steps namely: (i) cell cycle arrest in pro-metaphase; (ii) cellular and nuclear swelling; (iii) and successive fixations in order to obtain metaphase spreads.

To obtain adequate metaphase spreads it was crucial to obtain a large number of cells in division. To this end, cells were cultured in 75 cm^2 culture flasks at an initial cells' density of 4000 cells/cm^2. When confluence reached 80%, 200 µL of colcemid were added and the cells were incubated for 30 min at 37ºC and 5 % of CO_2 to induce cell cycle arrest in pro-metaphase. During this phase, the chromosomes are condensed and, consequently, are easier to be observed in a microscope. The excess colcemid was then removed and 4 mL of 1x PBS solution was added to wash the flask content. Once discharged the washing solution, 2 mL of trypsin was added to detach cells' from the matrix. To achieve a better efficiency, the flask was incubated for 2 min. at 37 ºC and 5% of CO_2 (conditions which improves the enzymatic action of the trypsin). The flask content was washed twice with 5 mL of PBS 1x solution and the washings collected to Falcon tube. The Falcon tube was centrifuged for 5 min. at 1000 rpm to allow cell sedimentation. Once the supernatant was discarded, the pellet was resuspended in 1 mL FBS, to inhibit possible remains of trypsin to act. Subsequently, 9 mL of an hypotonic solution of KCl 5 mM were added and the tube was then placed in a stove for 17 min. at 37ºC, to allow the KCl to penetrate inside the cells and their nucleus, promoting their swelling.

After the swelling, several steps of metaphase plaques fixation with methanol:acetic acid (3:1) were performed. The first step encompassed a pre-fixation with 1 mL of fixation solution added to the hypotonic solution. This step was crucial to minimize the impact of the fixation solution in the chromosomes, which may compromise their structure. After that, the solution was centrifuged for 5 min. at 1000 rpm, the

supernatant was discarded and the pellet was resuspended in 10 mL of fixation solution and placed in the freezer during 1 h at 4 ºC. Subsequently, cells were centrifuged for 5 min. at 1000 rpm, the supernatant was again discarded and the pellet resuspended in 10 mL of fixation solution. This last step was performed twice, after which the suspension was stored overnight at 4ºC.

2.4.3.1 Spreading and banding of metaphase spreads

The process of cell spreading in slides is crucial to subsequent microscopic analysis. During this process, parameters like temperature and humidity have to be carefully controlled. The main goal in the spreading is to obtain metaphases with chromosomes well disconnected from each other in order to analyze them more easily. To this end it is necessary the use of a phase contrast microscope to evaluate the quality of mitoses in the slide, as well as to select the better ones.

The glass slides were inserted into distilled water. Two drops (7 µL each) of the cell suspension were placed at the end of the slide with the aid of a pipettor. The pipettor was leaning 30º approximately to the slide. Spreading was performed very gently and applied only when there was sufficient liquid on the slide, so that the cells were not scratched by the tip. Once spreading was accomplished, the metaphases' quality was observed using a phase contrast microscope.

Depending on the study the practical approach from here is different. Therefore, whenever the chromosomal number alterations were to be evaluated chromosomes were stained with 5% Giemsa for 3 to 4 min., followed by several washes with Gürr buffer. After drying at room temperature, the slides were ready to be visualized at microscope.

When the goal was to study the cells' karyotype, to produce a karyotype by G-banding, chromosomes were first treated before Giemsa staining. To this end

chromosome metaphases were incubated in 0.4 % trypsin to digest the euchromatin zones and leave intact the heterochromatin. Chromosomes were then stained with Giemsa, to obtain a white and pink banding pattern, corresponding respectively to euchromatin and heterochromatin. The white bands have DNA rich in G-C (guanine-cytosine) bases, while the pink bands have DNA rich in A-T (adenine-thymine) bases. Every chromosome has its unique band pattern which allows their proper identification, as well as, the structural alterations by comparison with the reference pattern.

In this work, the protocol of G-banding was always used to look for aneuploidies.

2.4.3.2. Karyotypes

After banding, the best metaphase speads were chosen. These spreads were next photographed with the aid of a camera (model LH-100C-1) incorporated in the microscope and the images obtained were analyzed with the CytoVision software to construct the cells' karyotype.

2.4.3.3 Fluorescence *In Situ* Hybridization (FISH)

The FISH protocol used in this work was the whole chromosome painting, more specifically the fluorescent *in situ* hybridization protocol for FITC labeled chromosome paints.

The treatment of metaphase chromosomes, usually prepared 3 days before, contemplated the following steps: denaturation, hybridization, washing and detection. To this end new slides were prepared with fresh metaphases spreads, which have been fixed with 3:1 methanol:acetic acid. Before slides dehydration, they were maintained at 65 ºC for 60 min. Slides dehydration was achieved by serial dipping in ethanol for 2 minutes

starting with 70 % (v/v) ethanol followed with 85 %, 90 %, and finally 5 minutes in 100 % ethanol. The slides were then air dried.

Denaturation was performed by incubating the slides in a 70 % formamide solution for 2 min. at 65 ºC (hot plate). The slides were then dipped into ice-cold 70 % ethanol (-20 ºC) for 4 min. and dehydrated by serial dipping in ethanol as mentioned before. Finally, the slides were allowed to dry.

As to the probes, following heating to 37 ºC, they were centrifuged and spin-downed for 1-3 seconds at 6000 rpm. Denaturation was performed by heating at 67 ºC for 10 min. followed by 60 min. at 37 ºC

Hybridization

To this end, each probe was added onto the slides and a coverslip applied. Carefully, the air bubbles were removed by gently pressing the coverslip before sealing with rubber cement. The slides were placed into an air tight, pre-warmed box and incubated overnight in the dark at 37 ºC.

Before washing the slides, all the solutions to be used were pre-heated in a water bath at 45 ºC, for at least 30 min. before starting. Once the slides were removed from the incubator and the rubber cement taken off, slides were immersed in 2x SSC warmed solution to remove the coverslip. The slides were washed by dipping twice for 2 min. in warmed 50 % formamide solution, followed by two washes (2 min. each) in warmed 1x SSC solution. The slides were then incubated for 4 min. in warmed detergent solution 4x SCC/Tween 20 and drained, before the counterstain solution (DAPI) was applied (10 µL). Then, a glass coverslip was applied and the air bubbles removed with the aid of forceps. Finally, the counterstain excess was removed by applying pressure on the coverslip. After that, the metaphases were observed in a fluorescent microscope (see section 2.2), and

were photographed with the aid of a camera (model LH-100C-1) incorporated in the microscope: The obtained images were treated with the CytoVision software.

2.4.4. Immunocytochemistry

Immunocytochemistry allows the identification of a specific antigen in a cytological preparation using an antigen-specific antibody. The localization of the primary antibody (and therefore the target antigen) is then visualized microscopically via a specific enzymatic or fluorescent detection system.

This method which encompasses three distinct steps, cell growing, fixation and finally, antibody staining, was used to evaluate the expression of Vimentin, Cytokeratins, Oct3/4 and P-Glycoprotein in all the five cells lines (BEAS-2B, Cont1, RenG2, DRenG2 and DDRenG2).

Two slides, previously sterilized by dipping in 100 % ethanol, were inserted in sterile Petri dishes and 5 mL of 1 % gelatin was added at least 2 h prior to the addition of the cells. Before plating the cells at an initial density of 4000 cells/cm^2, the Petri dish was washed with 5 mL of 1x PBS and 10 mL of each cell line specific cultivation medium were added. Thereafter, the cells were grown until the optimal cell confluence was achieved. The Petri dishes were observed frequently and during this time the medium was regularly changed to allow better growth conditions.

Once cultures reached nearly 80 % confluence, the medium was removed and the cells were washed 3 times with 5 mL 1x PBS. Once the slides were properly identified (initials, cell culture line, and date), they were placed into 50 mL Falcon tubes filled with 95% ethanol and allocated in the freezer. This process may cause a conformational change in the tertiary structure of proteins, therefore it may difficult the antigen-antibody

interaction, in particular when using a cross-linking fixative. So, sometimes is necessary to use mechanisms of antigen retrieval.

Afterwards, the endogenous peroxidase activity was quenched using 15 min. incubation in 3 % diluted hydrogen peroxide (H_2O_2). For blocking the nonspecific binding with primary antibodies, slides were first dipped in Ultra V Block, from the Ultra Vision Kit, for 5 min. For the staining with primary antibodies, different dilutions were used, according to the manufacturers; for Cytokeratin (MNF116) a dilution of 1:50 was used, for Vimentin the dilution was 1:200, for Oct3/4 1:100 and for P-glycoprotein 1:20. After adding the primary antibody, the slides were incubated at room temperature for 30 min. in the case of the first two antibodies and for 30 minutes and 60 minutes, in the case of Oct3/4 and P-glycoprotein, respectively. The slides were then washed with PBS from the Ultra Vision Kit, and incubated 15 min. with biotin-labeled secondary antibody, commercialized as part of the Ultra Vision Kit. Primary antibody binding was localized using peroxidase-conjugated streptavidin (Ultra Vision Kit) and 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as chromogen, according to manufacturer's instructions. Hematoxylin was used to counterstain the slides which were then dehydrated and mounted. In parallel, known positive and negative controls were used. The intensity of the staining was graded semi-quantitatively on a four point scale (0;1+,2+,3+). The percentage of immunostained cells was also registered. A final score was obtained multiplying the intensity by the percentage of cells with immunohistochemical expression and the cut off considered was 10% positive cells.

Finally, the slides were visualized with an inverted-phase microscope and photographed with the aid of a camera incorporated (Digital Camera DXM 1200F) in the microscope.

2.4.5. Cell doubling times

To evaluate the duplication times of the different cell lines, cells were cultured in T75 culture flasks at an initial cells density of 4000 cells/cm^2 and using the appropriated culture medium (LHC-9 or F12 with UG) as described in section 2.4.1. Once confluence reached nearly 80 %, the medium was discarded, and the flasks washed with 6 mL of 1x PBS to remove the remnants. 2 mL of trypsin was added to detach cells from the matrix. When the flasks' surfaces were fully covered with trypsin, the excess was removed with a pipette, and the flasks were incubated at 37 ºC for 1 min. The flasks were then washed with 15 mL of 1x PBS, and the cells suspension was collected into Falcon tubes and centrifuged for 5 min. at 1500 rpm. After discarding the supernatant, the pellets were resuspended in appropriated volumes of medium (generally 0.5 mL to 1 mL) and 20 µL aliquots were removed from each suspension to assess the number of cells by the trypan blue assay as described in section 2.4.2.

After expanding the cultures, to evaluate cells' duplication times an appropriated volume of the cell suspensions were transferred to 6 well multiwell plates (MW6) to obtain an equal initial density of $3.8x10^4$ cells/mL (4000 cells/cm^2) in each well. For each time point at least 3 wells per cell line were used.

The cells were counted in four different time points, at 24 h, 48 h, 60 h and the final count at 72 h. However, the analysis of cells' duplication times only started during the exponential phase (log phase), when the cells have reached the optimum growth conditions.

In each time point the cell culture medium was discarded and the wells washed with 1 mL of 1x PBS to remove the remnants. 0.4 mL of trypsin was added to detach cells from the matrix. When the wells' surface was covered with trypsin, the excess was removed with a pipette, and the MW plate incubated at 37 ºC for 1 min. Each well was

washed with 2 mL of 1x PBS, and each cellular suspension was collected into a Falcon tube and centrifuged for 5 min. at 1500 rpm. Following the discharge of the supernatant, the pellet was resuspended in an appropriate volume of medium (generally 0.5 mL to 1 mL). To evaluate the cells' suspension density a 20 µL aliquot of the cells' suspension was used for cell counting with trypan blue assay as described in 2.4.2.

To evaluate whether the differences of doubling times between cell lines were statistically significant, the Graphpad Quickcalcs and Graphpad Prism 5 was used.

2.4.6 Drug resistance assays

The MTT assay, a safe, sensitive and reliable method to examine cells' viability, was used to evaluate the resistance of cells to the mostly widely used anti-neoplasic drugs such as GEM and cDDP. The yellow tetrazolium compound MTT is reduced, mainly by mitochondrial dehydrogenases of metabolically active cells, to insoluble purple formazan dye crystals.

The cells were cultured in T75 culture flasks at an initial density of 4000 cells/cm^2 and using the appropriated culture medium (LHC-9 or F12 with UG) as described in section 2.4.1. Once confluence reached nearly 80 %, the cells sub-culturing protocol, described in 2.4.1, was used. After expanding the cultures, an appropriated volume of the cells' suspensions was transferred to 24 well multiwell plates (MW24) to obtain an initial density of $1.52x10^4$ cells/mL (8000 cells/cm^2), in each well. For each cell line 3 controls, plus triplicates for each drug concentration were used. Besides that, in each plate 3 wells were left only with medium (negative control - blank). 24 h after plating 10 µL of the

desired drug concentrations (0.1 µM, 10 µM and 50 µM) were added and during the following 3 days, MTT readings were made (Figure 2.2).

Before each MTT reading, the medium was discarded and the wells were washed with 200 µL of 1x PBS to remove the remnants. After that, 500 µL of MTT 0.5 mg/mL were added. Thereafter, the cells were incubated for 3 to 5 hours at 37 ºC and in 5 % of CO_2. The MTT solution was carefully removed to avoid the removal of the violet formazan crystals, and 500 µL of acid isopropanol were added to dissolve the formed crystals. The absorbance was measured at 570 nm, in a multiplate reader, using the 620 nm as the reference wavelength.

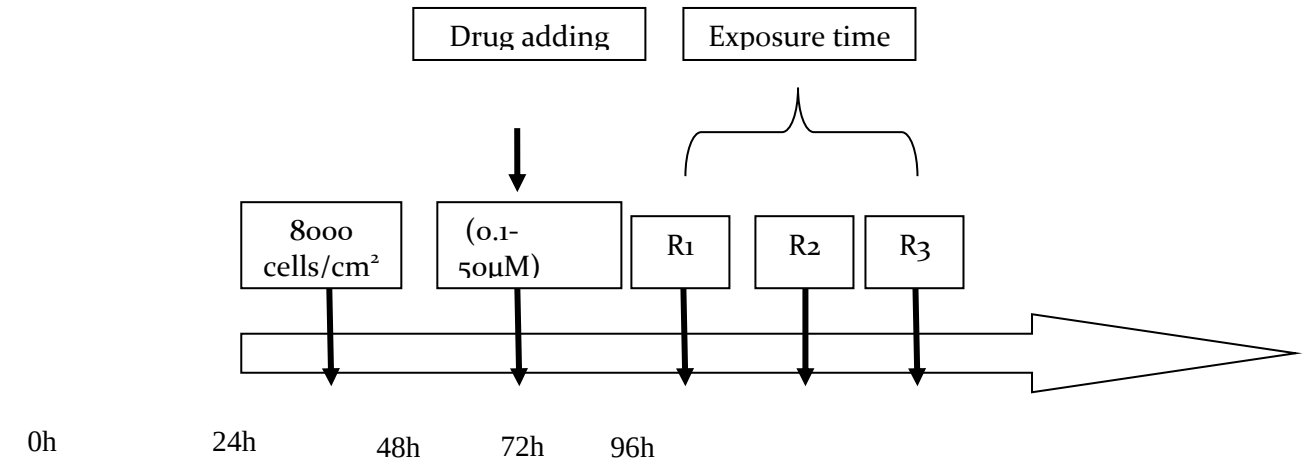

Figure 2.2 Schematic representation of the *in vitro* studies made to determine the anti-proliferative activity of the cDDP and GEM.

As the tetrazolium reduction is proportional to the cells' viability, the changes in cells' viability induced by each specific drug concentration were determined in relation to the control using the equation (1).

$$\% \text{ cell viability} = \frac{\lambda\ sample}{\lambda\ control} \times 100 \qquad \textit{Equation 1}$$

In this equation, **λ sample** represents the wavelength of the cells' sample exposed to the drug under evaluation, minus the wavelength of the blank (acid isopropanol), while **λ control** represents the wavelength of the controls (cells without drug), minus the wavelength of the blank.

2.5. Statistical analysis

The results obtained were analyzed in the *Graphpad Prism 5.00* program. The represented results are the mean ± standard deviation.

In the analysis of the obtained data, the statistic treatment was carried out by One-Way ANOVA method followed by the *Dunnet's* test (*vs* control cultures).

When aiming to see the differences between the different drugs used and their different concentrations, the method used was the One-Way ANOVA followed by Tukey test.

3. Results

Cancer cells are characterized by a variety of genomic defects, such as, inactivation of DNA repair genes, over-expression of growth promoting oncogenes, extra or missing chromosomes, abnormal number of centrosomes and aberrant mitoses and citokinesis (Meraldi *et al*., 2004), which lead some authors to classify it as the *disease of genes* (Shipitsin and Polyak, 2008). As a consequence of this definition, when studying cancer *in vitro*, through the use of different cell lines, is of extreme importance to characterize all the cellular systems under study in order to identify chromosome alterations (structural and/or numeric) that could ultimately unveil the gene alterations that drove the malignant process (*i.e.* tumor suppressor genes and oncogenes).

Recently, in our lab we observed, using the non-tumorigenic BEAS-2B cell line, immortalized with an adenovirus 12 and a *Simian virus* (SV40) viral hybrid, and their malignant derivatives RenG2, DRenG2 and DDRenG2, an unexpected degree of plasticity between stem-like and non-stem cell compartments, leading to the demonstration that differentiated cell types can convert to stem-like cells. In the light of these results it was undertaken the study of the biology of the populations of cells RenG2, obtained following prolonged treatment with Cr(VI), a known lung carcinogenic agent, of differentiated non-tumorigenic bronchial epithelial cells, (BEAS-2B), and DRenG2 and DDRenG2 cells obtained out of tumours induced in athymic nude mice by RenG2 and DRenG2 cell lines respectively.

A previously analyse of BEAS-2B cells showed an isochromosome 5 [i(5)(q10)], a terminal deletion of the short arm of chromosome X (Xp-) and additional material on the short arm of chromosomes 15 (15p+), 16 (16p+) and 22 (22p+) (Rodrigues *et al*., 2009). In addition, this cell line exhibited a trisomy of chromosome 20 in nearly 80% of the metaphases analysed, the presence of a derivative chromosome 14 with additional material on the short arm [der(14)] on 70% of the metaphases, as well as structural

alterations of the banding pattern along the long arm of chromosome 2 [der(2)] on 20% of the metaphases (Rodrigues *et al*., 2009). As to the first malignant BEAS-2B-derivative cell line, RenG2, showed a marked karyotypic change with an aneuploid phenotype (ca. 80 chromosomes in over 90% of the metaphases analyzed) (Rodrigues *et al*., 2009).

3.1 Cytogenetic characterization

In our lab the precursor cell lines, namely BEAS-2B and RenG2 cells have already been characterized, and so, the next step was the characterization of the DRenG2 and the DDRenG2 cell lines.

The first step to assess the characterization of these cell lines was taken by using conventional cytogenetic analysis (karyotype), followed by FISH.

The karyotypic characterization was made at the passage #27, #33 and #34 of DRenG2 and at the passages #30, #32, #33 and #34 of DDRenG2 cells. The FISH characterization was made at the passage #21 of DRenG2 cell line.

As to the G-Banding karyotype analysis, once obtained medium/high quality metaphase plaques, microscope observation allowed the evaluation of the numeric and structural alterations characteristic of each cell line using at least good quality 15 kariotypes for each cell line. The G-Banding results revealed that both DRenG2 and DDRenG2 cell lines predominant ploidy was 75/76 chromosomes with some chromosomes portraying a numeric pattern, in other words, these numeric alterations appear almost in all the metaphases. However, some other chromosomes have more than one numeric alteration. For both cell lines, no numeric changes were observed along passages.

3.1.1 DRenG2 cell line characterization

3.1.1.1 Karyotype

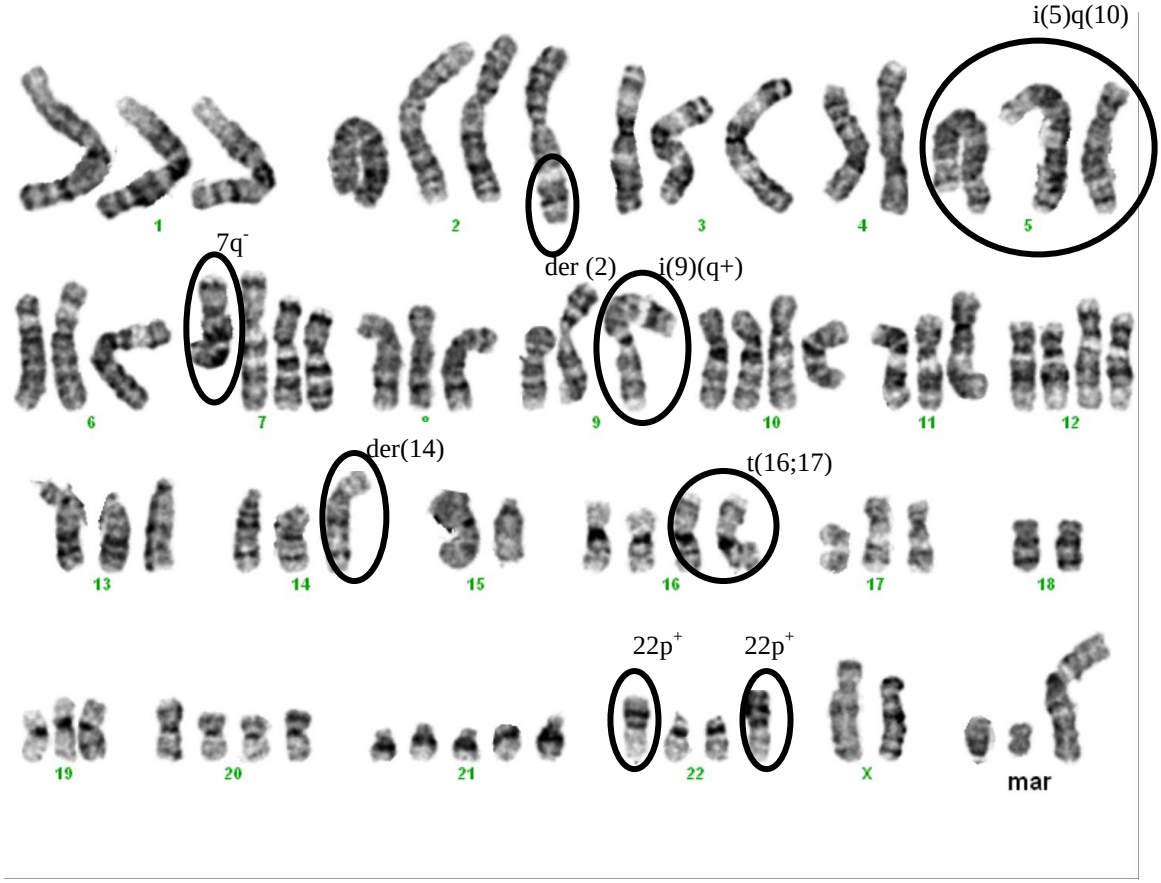

Fig. 3.1 A representative karyotype of DRenG2 cell line (#33)

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Karyotype analysis revealed that DRenG2 cell line had the characteristic isochromosome 5 [i(5)(q10)], already reported for BEAS-2B cell line **(**Rodrigues *et al*., 2009**)**, present in 87% of the metaphases analysed. Other alterations that have been already reported in BEAS-2B and RenG2 cell lines **(**Rodrigues *et al*., 2009**)** and that appear in this cell line were the additional material on the short arm of chromosome 15 ($15p^+$) and the presence of a derivative chromosome 14 with additional material on the short arm [der(14)] on 73% of the metaphases as well as structural alterations of the banding pattern along the long arm of chromosome 2 [der(2)] on 20% of the metaphases (Rodrigues *et al*., 2009). The translocation between the chromosomes 16 and 17 [t(16;17)] and between the chromosomes 21 and 22 [t(21;22)], which appear in 80% and

87% of the metaphases analysed, respectively, were also present in both BEAS-2B and RenG2 cell lines **(**Rodrigues *et al*., 2009**)**. However, new structural alterations, specific for DREnG2 cell line, were observed i.e., the terminal deletion of the long arm of the chromosome 7 ($7q^-$), and an isochromosome 9 [$i(9)(q^+)$], were identified in 20% and 60% of the metaphases analysed, respectively (Figure 1 and Table I).

Table I. The numeric and structural alterations observed in the DRenG2 cell line.

Chromosome	Numeric alterations	Structural alterations
1	4 (80%); 3 (20%)	-
2	3 (87%); 4 (13%)	der(2) (20%)
3	4 (80%); 3 (20%)	-
4	2 (60%); 3 (40%)	-
5	3 (100%)	i(5)(q10) (100%)
6	3 (87%); 4 (6.5%); 2 (6.5%)	-
7	4 (53%); 3 (40%); 5 (7%)	$7p^-$ (40%); $7q^-$ (20%)
8	3 (73%); 4 (27%)	-
9	3 (53%); 2 (47%)	$i(9)(q^+)$ (60%)
10	4 (73%); 3 (27%)	-
11	2 (33.3%); 3 (33.3%); 4 (33.3%)	-
12	4 (60%); 3 (27%); 2 (13%)	-
13	3 (53%); 2 (47%)	-
14	3 (60%); 2 (27%); 4 (13%)	der(14) (60%)
15	3 (60%); 2 (27%); 1 (13%)	$15p^+$ (60%)
16	4 (60%); 3 (40%)	t(16;17) (73%)
17	3 (87%); 2 (13%)	-
18	3 (87%); 1 (13%)	-
19	3 (67%); 2 (27%); 4(6%)	-
20	4 (60%); 3 (40%)	-
21	4 (60%); 3 (33%); 5 (7%)	t(21;22) (80%)
22	4 (40%); 3 (33%); 5 (14%); 2 (13%)	$22p^+$ (73%)
X	1 (100%)	-
Y	0	0

Regarding numeric alterations, some chromosomes display a numeric pattern; in other words, these numeric alterations appear almost in all the observed metaphases. On the other hand, some chromosomes show more than one numeric alteration.

3.1.1.2 - Fluorescence *in situ* hybridization (FISH)

Since the G-Banding karyotype analysis revealed some uncertainties concerning chromosomal structural alterations in order to, tentatively, solve these problems, another technique was used. This technique, called fluorescent *in situ* hybridization or FISH, is used mainly to detect and localize the presence or absence of specific DNA sequences on chromosomes. To accomplish this objective, fluorescent probes that bind to only those parts of the chromosome with which they have a high degree of sequence complementary are used (Langer-Safer *et al*., 1982).

Figure 3.2 consistently illustrates the presence, in DRenG2 cell line, of four chromosomes 1 (red) in accordance with G-Banding karyotype analysis. Importantly, FISH analysis revealed the presence in this cell line of two complete chromosomes 17 (green) and two halves of this same chromosome (Figure 3.2) with an additional portion of the chromosome 16. These t(16;17) has also been observed by G-Banding analysis.

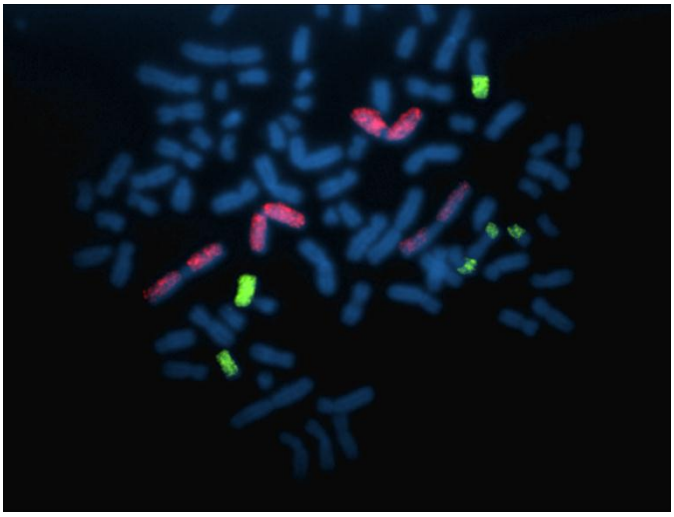

Figure 3.2 Representation of a DRenG2 cell line metaphase with an amplification of 400x. The chromosomes painted, are the chromosome 1 (red) and 17 (green).

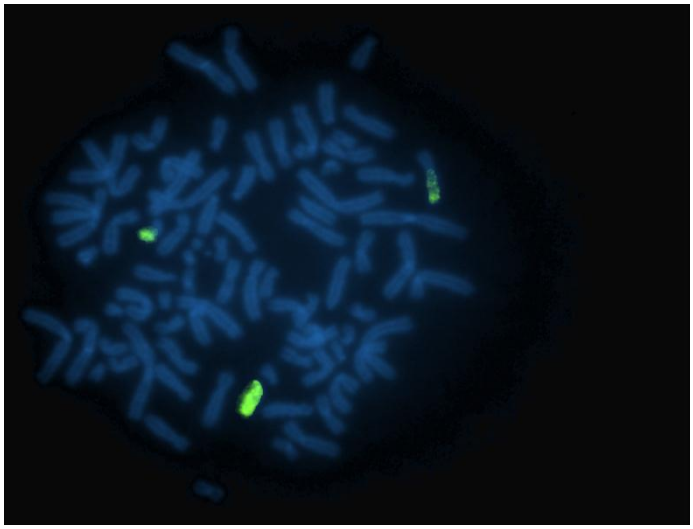

Figure 3.3 Representation of a DRenG2 cell line metaphase with an amplification of 400x. The chromosome painted in green is the chromosome 15.

Figure 3.3 illustrates FISH analysis of chromosome 15. The image illustrated revealed the presence of an entire chromosome 15 and the presence of two other chromosomes with additional material that not belongs to chromosome 15. All chromosomes appear to have different sizes. FISH analysis corroborates what was depicted in the Table I, since 60% of the metaphases appear to have a chromosomal alteration $15p^{+}$

3.1.2 DDRenG2 cell line characterization

3.1.2.1 Karyotype

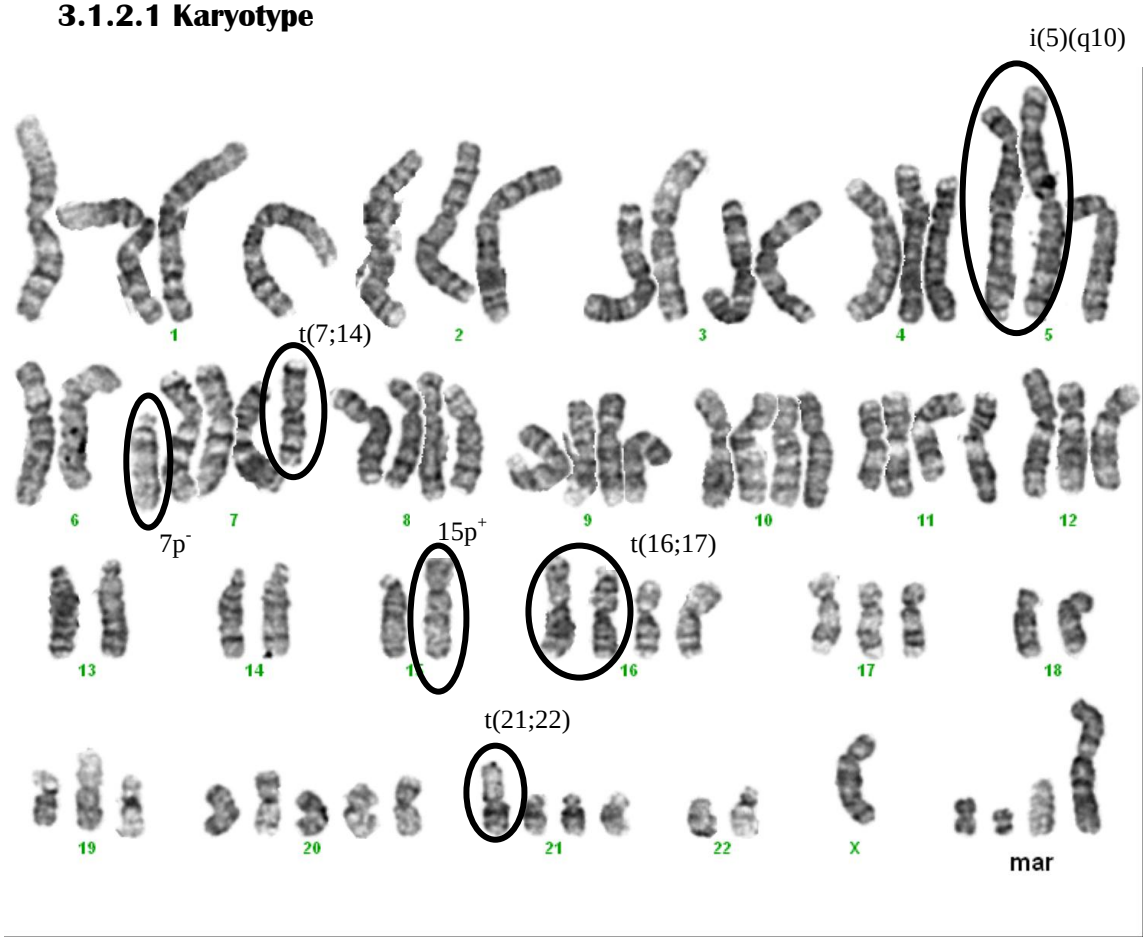

Fig. 3.4 A representative karyotype of DRenG2 cell line (#33)

Similarly to DRenG2 also some of DDRenG2 cell line structural characteristics have been also reported previously for BEAS-2B and RenG2 cell lines **(**Rodrigues *et al*., 2009**)**, namely [i(5)(q10)], additional material in chromosomes 15 and 22 i.e., $15p^+$ and $22p^+$, derivative chromosome 14 with additional material on the short arm [der(14)] (Tables I and II). The translocations [t(16;17)] and [t(21;22)] were simultaneously present in both DRenG2 and DDRenG2 cell lines, although in different percentages (Tables I and II).

Regarding *de novo* alterations, a translocation between the chromosomes 7 and 14 [t(7;14)] and a derivative of chromosome 17 with addition material on its short arm ($17q^+$) were identified in 40% and 27% of the metaphases analysed, respectively (Figure

3.4). Because they are being identified in our systems for the first time, we assume these alterations to be characteristic of the DDRenG2 cell line. Finally, another alteration observed was a terminal deletion of the short arm of the chromosome 7 ($7p^-$), which also appears in some metaphases of the DRenG2 cell line (Table II). Unlike DRenG2, the additional material on the short arm of chromosome $17q^+$ is present in DDRenG2 cell line (Tables I and II).

Characteristic of both DDRenG2 and DRenG2 cell lines was the absence of chromosome Y (Figures 3.1 and 3.4).

As for the previous cell line, also here the numeric alterations for some chromosomes are variable, while others appear to have a particular pattern.

Table II. The numeric and structural alterations observed in the DDRenG2 cell line

Chromosome	Numeric alterations	Structural alterations
1	4 (87%); 3 (13%)	-
2	3 (80%); 4 (20%)	-
3	4 (87%); 3 (13%)	-
4	3 (73%); 2 (23%)	-
5	3 (93%); 2 (7%)	i(5)(q10) (87%)
6	3 (100%)	-
7	4 (60%); 3 (40%)	$7p^-$ (27%); t(7;14) (40%)
8	4 (80%); 3 (20%)	-
9	3 (100%)	-
10	3 (87%); 2 (13%)	-
11	3 (53%); 4 (33%); 2 (14%)	-
12	3 (73%); 4 (27%)	-
13	3 (73%); 2 (27%)	-
14	3 (67%); 2 (33%)	der(14) (73%)
15	3 (80%); 2 (20%)	$15p^+$ (73%)
16	3 (87%); 4 (13%)	t(16;17) (80%)
17	3 (67%); 4 (33%)	$17q^+$ (27%)
18	2 (87%); 3 (13%)	-
19	3 (73%); 2 (14%); 1 (13%)	-
20	4 (80%); 2 (20%)	-
21	4 (80%); 5(7%); 3 (13%)	t(21;22) (80%)
22	3 (60%); 4 (40%)	$22p^+$ (73%)
X	1 (100%)	-
Y	0	0

3.2. Characterization by immunocytochemistry of the BEAS-2B, Cont-1, RenG2, DRenG2 and DDRenG2 cell lines

As described in Chapter 1, we and others observed that differentiated cell types acquired an unexpected degree of plasticity, possibly via an EMT process (Rodrigues *et al*., 2011; Chaffer *et al.*, 2011). So, following the cytogenetic characterization of the DRenG2 and DDRenG2 cell lines it was crucial to evaluate the presence or absence of biomarkers specific of the epithelial and mesenchimal phenotypes. To this end the BEAS-2B cell line and its derivative cell lines i.e., RenG2, DRenG2, DDRenG2, and Cont-1 were characterized by immunocytochemistry to assess their epithelial/mesenchymal status [MNF 116, Vimentin] and the presence of cancer stem cells biomarkers i.e. the simultaneous presence of Oct-4 (stem-like), and P-Glycoprotein (transport system-resistance to chemotherapy).

As depicted in Table III and Figures 3.5-3.8 all cell lines had fusiform and polygonal small cells. However the DDRenG2 cell line possessed also mononucleated large cells (Figure 3.6).

The BEAS-2B cell line had great number of mitotic cells and, surprisingly, like the all others cell lines it has naked nucleus i.e., cells with loss of cytoplasm (Figures 3.5-3.8).

Table III. **Table representing the immunocytochemistry analysis for the five cell lines**. (+++, >75% of cells stained; ++, 50%-75% of cells stained; +, 10%-50% of cells stained, -, there aren't cells stained)

	MNF116	Vimentin	Oct3/4	P-gp	Description
BEAS-2B	++	+	/	-	Fusiform and polygonal small cells Presence of mitotic cells
Cont-1	++	+	/	-	Fusiform and polygonal small cells
RenG2	++	++	/	-	Fusiform and polygonal small cells
DRenG2	+++	++	-	-	Fusiform and polygonal small cells
DDRenG2	+++	+++	-	-	Fusiform and polygonal small cells Mononucleated large cells are also present

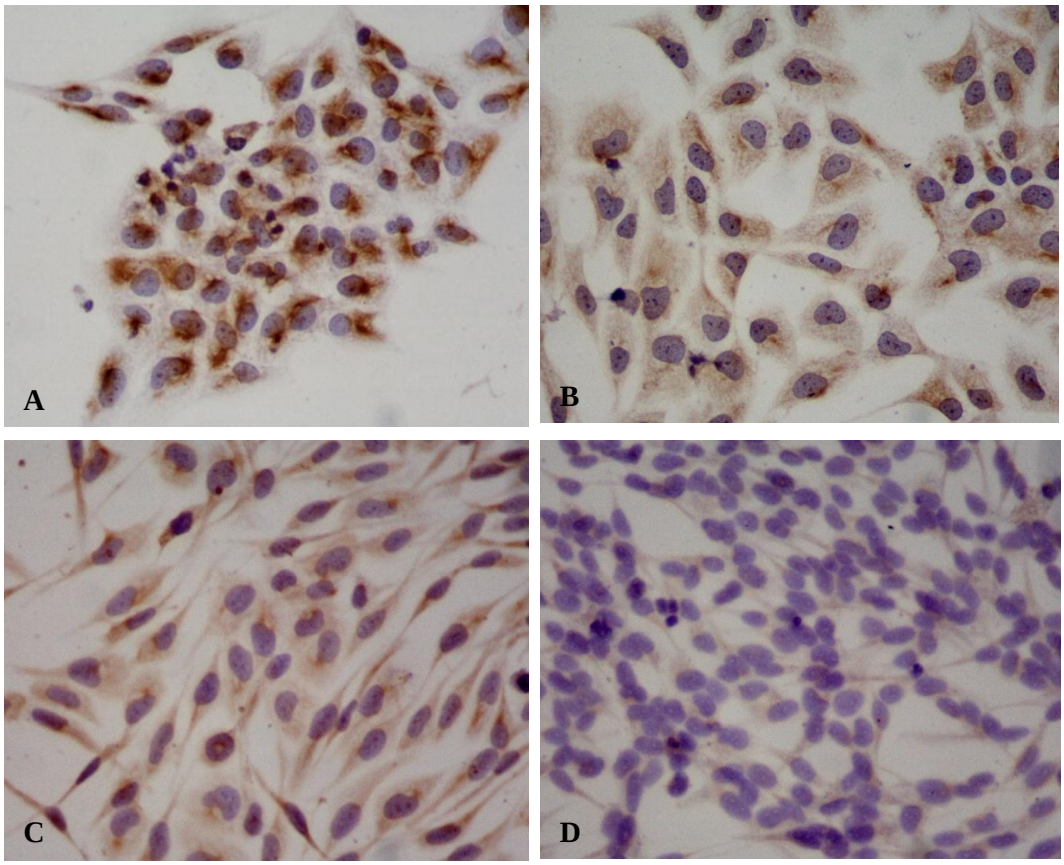

Figure 3.5 Representative images of immunocytochemical staining of BEAS-2B (A, B). and Cont-1 cell lines (C,D). Cells had been previously plated on top of microscopic slides for staining later on for Cytokeratin (MNF 116) (A and C 400x) and Vimentin (B and D 400x) as described in Materials and Methods. The blue colour represents the cell nucleus and the brown colour the antibody staining.

As illustrated in Figure 3.5 the non-malignant BEAS-2B and Cont-1 cell lines stained positive for both antibodies even though, less than 50% of the cells stained positive for Vimentin. The higher expression of Cytokeratins revealed that the vast majority of the cells in both cell lines have an epithelial phenotype.

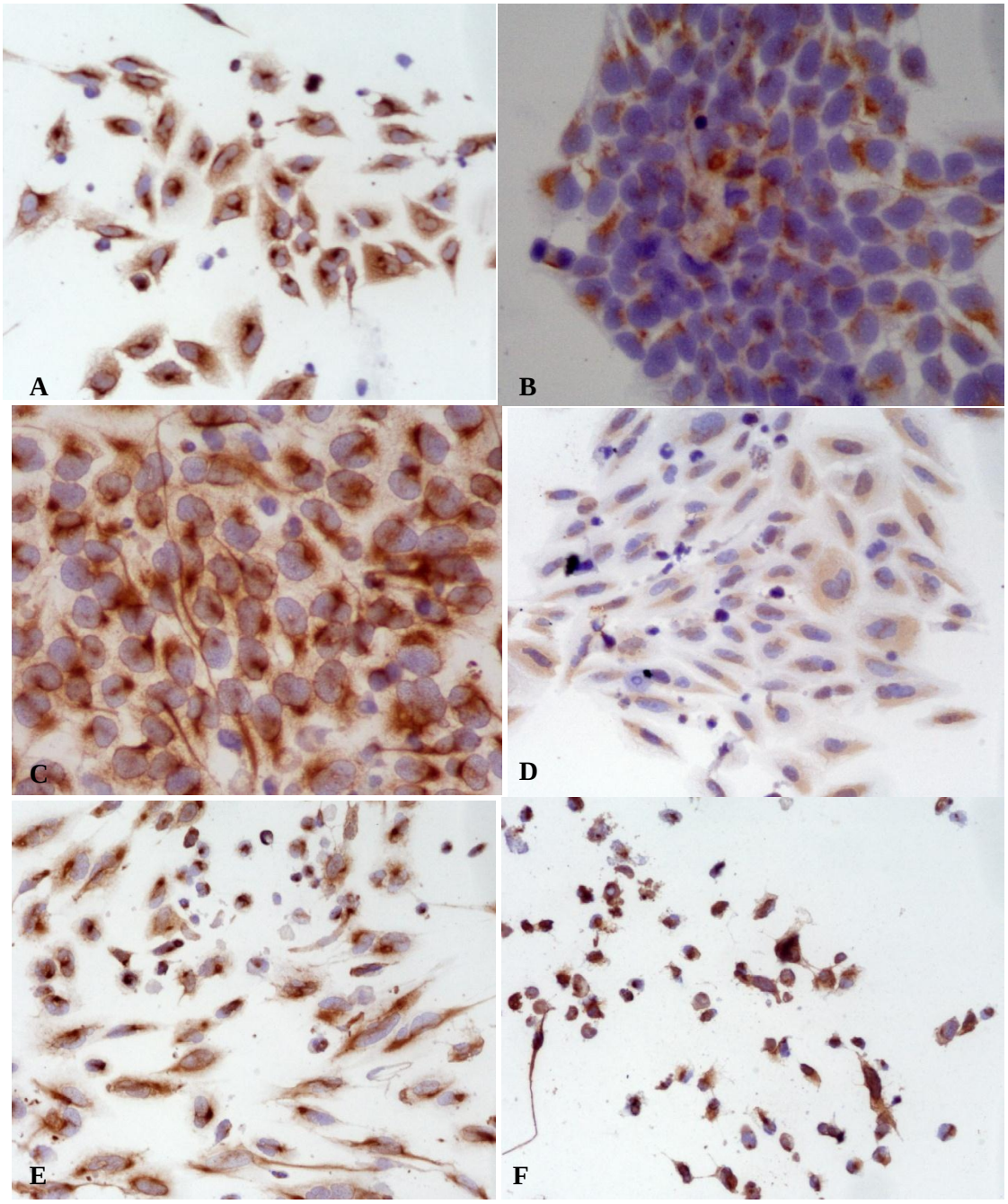

Figure 3.6 Representative images of immunocytochemical staining of RenG2 (A, B), DRenG2 (C, D) and DDRenG2 cell lines (E, F). Cells had been previously plated on top of microscopic slides for staining later on for Cytokeratin (MNF 116) (A and E 200x, C 400x) and Vimentin (B 400x, D and F 200x) as described in Materials and Methods. In blue are represented the cell nucleus and in brown the antibody staining.

As illustrated in Figure 3.6 RenG2, DRenG2 and DDRenG2 cell lines stained positive for both antibodies (MNF 116 and Vimentin). The more malignant cell lines, DRenG2 and DDRenG2, have more than 75% of the cells medium to high staining for the mesenchymal antibody (Vimentin), respectively. However, DRenG2 revealed a higher staining for MNF116 (Figure 3.6 C) than for Vimentin (Figure 3.6 D). On the other hand, the RenG2 cell line has a medium staining for both antibodies, with 50% to 75% of the cells stained.

These results just confirmed the presence, in each cell line, of different sub-population of cells with either epithelial or mesenchymal phenotypes.

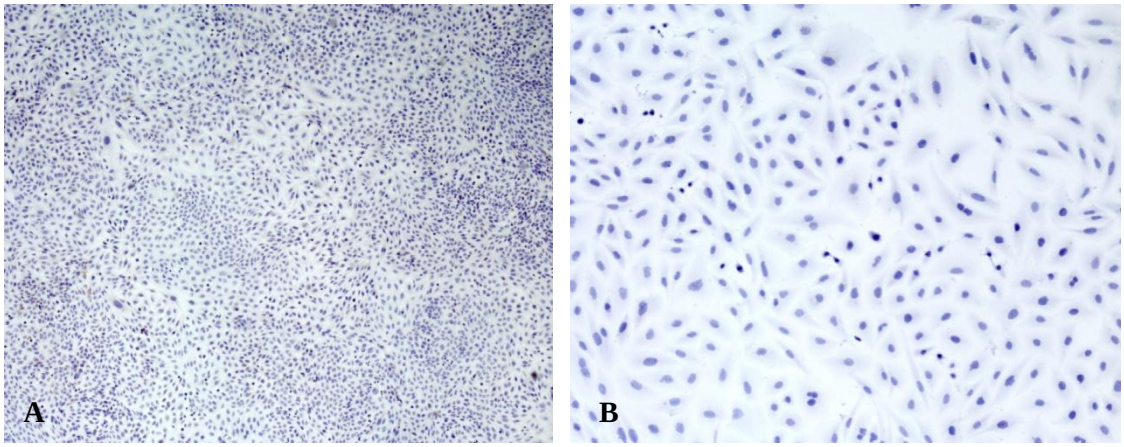

Figure 3.7 Representative images of immunocytochemical staining of DRenG2 (A), DDRenG2 (B). Cells had been previously plated on top of microscopic slides for staining later on for P-gp (A 100x and B 40x) as described in Materials and Methods.

As illustrated in Figure 3.7 both DRenG2 and DDRenG2 cell lines stained negative for the P-gp antibody. The same was observed for the BEAS-2B, Cont-1 and RenG2 cell lines (image not shown).

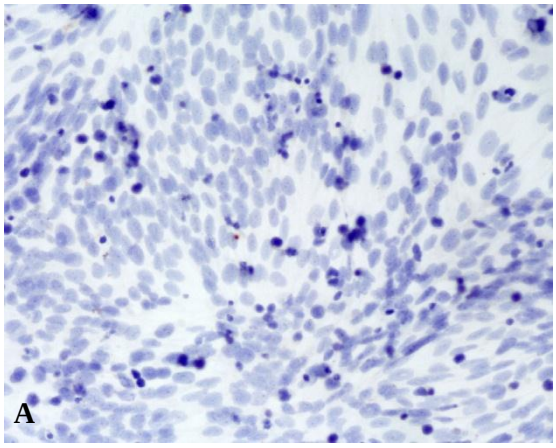

Figure 3.8 Representative image of immunocytochemical staining of DDRenG2 (A). Cells had been previously plated on top of microscopic slides for staining later on for Oct3/4 (A 200x) as described in Materials and Methods.

As illustrated in Figure 3.8 the DRenG2 cell line stained negative for the Oct3/4 antibody (image not shown). The same was observed for the DDRenG2 cell line.

3.3. Duplication time of BEAS-2B, Cont1, RenG2, DRenG2 and DDRenG2 cell lines

Cancer cells are commonly thought to have high proliferation rates even though that is not always the case. In what concerns CSCs quite often are in a quiescent non-proliferative state (Alison *et al.*, 2012). Aiming to evaluate whether the more malignant phenotypes were more proliferative than their less malignant (RenG2) counterpart, the doubling times of all cell lines including the non-malignant BEAS-2B and Cont-1 cell lines were evaluated using the trypan blue assay. Analysing the graphs illustrated in Figures 3.7 and 3.8 it is possible to observe that, even though, the lag phase lasted approximately 48h for all the cell lines the exponential growth phase slope was steeper for the more malignant cell lines i.e., DRenG2 and DDRenG2. Accordingly, as illustrated in Figure 3.12, the more malignant cell lines DRenG2 (17.04±0.38 h), and DDRenG2

(18.86±0.21 h), have statistically significant lower duplication times than their less malignant counterpart RenG2 (21.83±0.5 h) and the non-malignant BEAS-2B and Cont-1 with 23.16±0.42 h and 23.03±0.16 h, respectively.

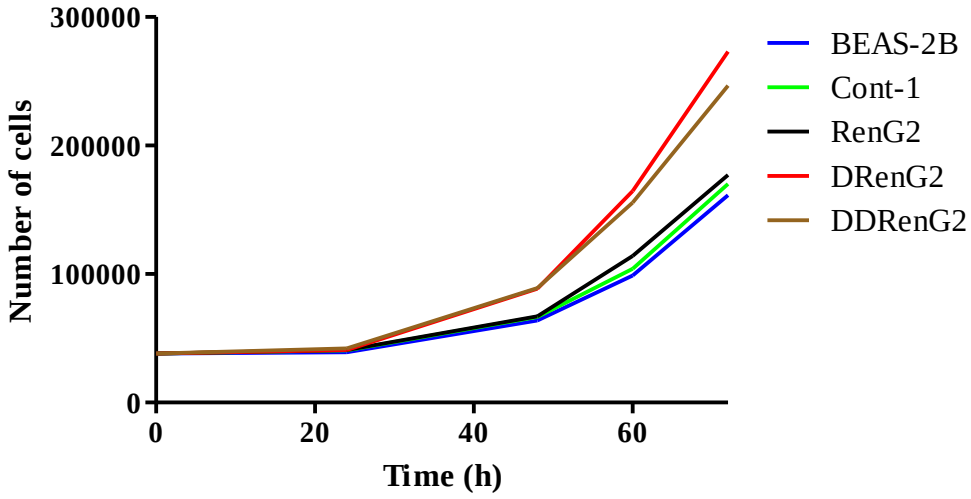

Figure 3.9 Cell doubling time of the BEAS-2B, Cont-1, RenG2, DRenG2 and DDRenG2 cell lines. This graph represents the number of viable cells versus the incubation time (72h). The number of cells was calculated by the trypan blue method as described in section 2.4.5. For each day, the number of cells obtained is the mean of the values measured in triplicates.

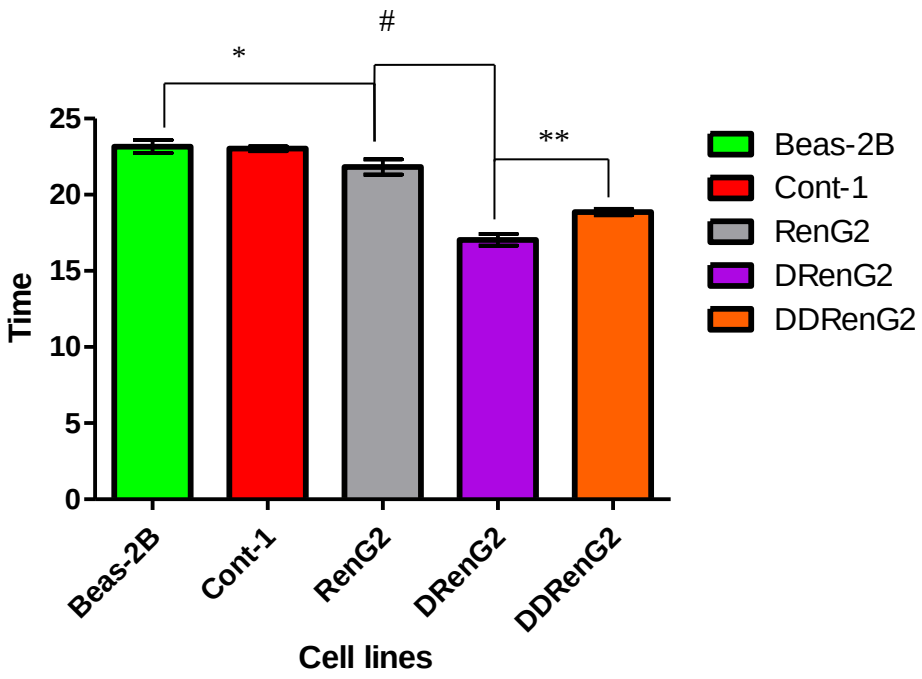

Figure 3.10 Graphic representation of the duplication time from 5 different cell lines (Beas-2B, Cont-1, RenG2, DRenG2 and DDRenG2). The results are presented as the mean of three independent experiences (± standard deviation). *p<0.05, **p<0.01, #p<0.001

3.4. Drug resistance/ cell viability

The current therapies used in cancer treatment quite often aren't effective to complete eradication of the disease, mostly because either the disease is in a very advanced stage and/or the existence of tumour subpopulations, such as the cells with stem-like properties i.e., CSCs, highly resistant to therapies. Current cell lines used in the labs (either commercial or primary cultures established from human and animal tissues) are non-homogenous representing, in a very small scale, the cellular heterogeneity observed in the tissues. As a matter of fact, the commercial BEAS-2B cell line is not homogenous and the same was observed for its derivatives, the non-malignant Cont-1 and the malignant counterparts RenG2, DRenG2 and DDRenG2 (results not shown). The most recent studies on going on our lab with these cell lines revealed the presence of

subpopulations of CSCs only in the more malignant cell lines DRenG2 and DDRenG2. Thus, the need to evaluate the resistance of all the five cell lines to the most widely used chemotherapeutic drugs against lung cancer (gemcitabine and cisplatin) to assess whether the resistance can be credited to the presence of CSCs subpopulations. To this end, several experiments were conducted adding either 0.1 µM, 10 µM and 50 µM cisplatin or gemcitabine to each cell line culture 24h after plating and evaluating the cells' viability, using the MTT assay as described in section 2.4.6, at 24h, 48h and 72 h after drugs addition.

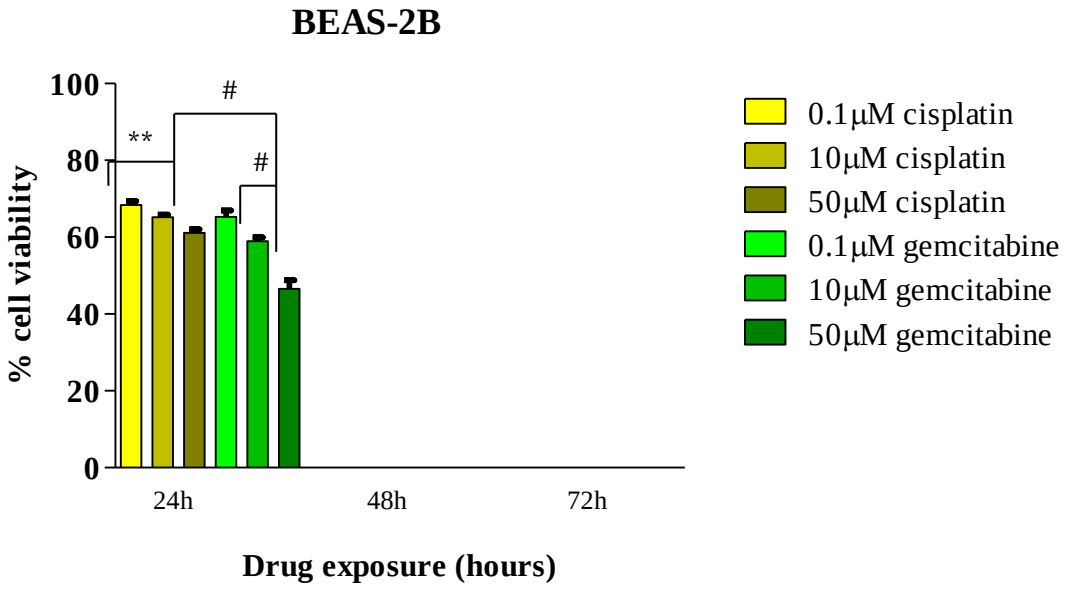

Figure 3.11 Different drug concentration effects (0.1µM, 10µM and 50µM) in the cell viability at 3 different times of drug exposure (24h, 48h, and 72h) for the BEAS-2B cell line, determined by the MTT assay. The results are represented as percentages in relation to the obtained control values. In this graph, it is also represented some statistically differences between drug concentrations and between both drugs. All the cultures were prepared with an initial cell density of $8x10^3$cells/cm^2. For each condition, the results are presented as the mean of three independent experiences (± standard deviation). *p<0.05, **p<0.01, $^{\#}$p<0.001

Figure 3.11 reveals that exposure of BEAS-2B to either cisplatin or gemcitabine decreased significantly the cells' viability 24h following the each drug addition. The drugs' effects were concentration dependent with particular emphasis for 50 µM gemcitabine. Figure also illustrates that for the highest concentration tested (50µM) gemcitabine was significantly more effective in decreasing cells' viability than cisplatin. However, independent of the drug and concentration tested BEAS-2B cells didn´t survive to exposures longer than 24h.

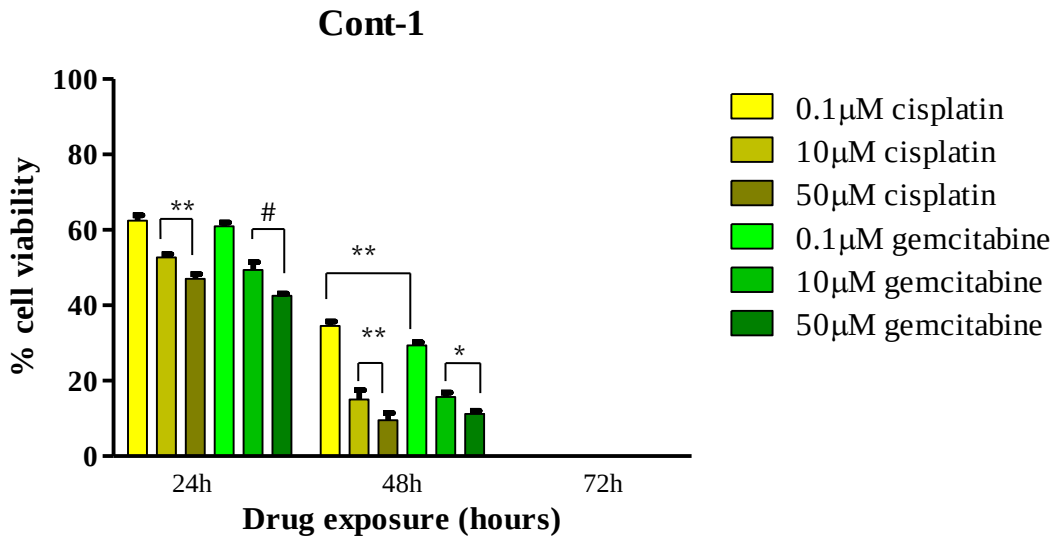

Figure 3.12 Different drug concentration effects (0.1µM, 10µM and 50µM) in the cell viability at 3 different times of drug exposure (24h, 48h, and 72h) for the BEAS-2B cell line, determined by the MTT assay. The results are represented as percentages in relation to the obtained control values. In this graph, it is also represented some statistically differences between drug concentrations and between both drugs. All the cultures were prepared with an initial cell density of $8x10^3$cells/cm^2. For each condition, the results are presented as the mean of three independent experiences (± standard deviation). *$p<0.05$, **$p<0.01$, $^{\#}p<0.001$

Concerning Cont-1 cell line the treatment with either cisplatin or gemcitabine decreased significantly the cells' viability 24h following the each drug addition. The drugs' effects were concentration dependent with particular emphasis for 50 μM gemcitabine at 24h of drug exposure. At 50μM after 24h of exposure the most effective drug (more pronounced decrease in cell viability) was the gemcitabine, however after 48h of drug exposure there wasn't significant statistically differences between the drugs.

Independent of the drug and concentration tested Cont-1 cells didn´t survive to exposures longer than 48h.

In both cell lines there were significant statistically differences (P<0.001) in cells between the control and the cells that were exposed to cisplatin and gemcitabine for 24h, 48h and 72h, independent of the drugs concentration used (graph not shown).

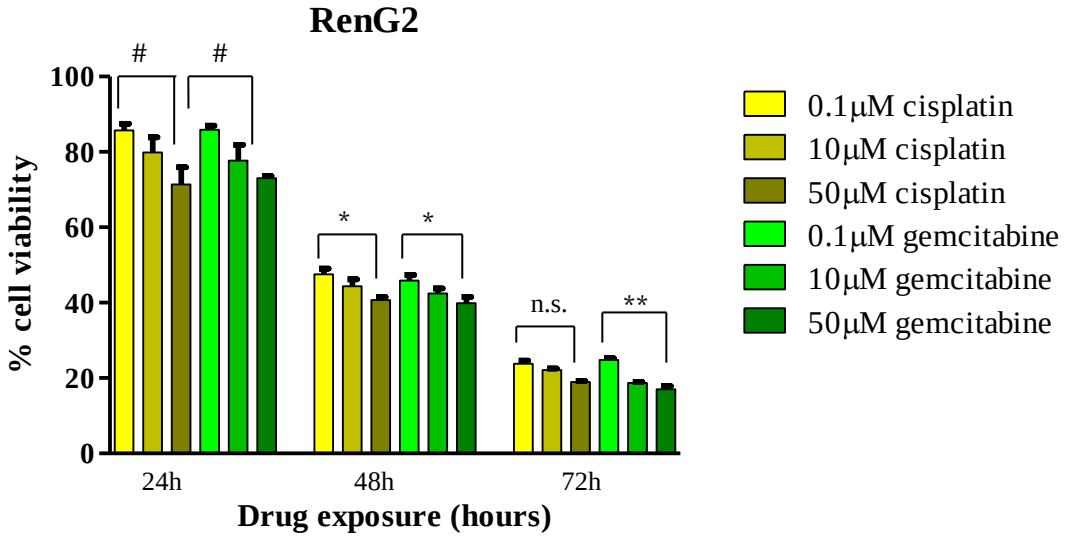

Figure 3.13 Different drug concentration effects (0.1μM, 10μM and 50μM) in the cell viability at 3 different times of drug exposure (24h, 48h, and 72h) for the RenG2 cell line, determined by the MTT assay. The results are represented as percentages in relation to the obtained control values. In this graph is also represented some statistically differences between drug concentrations and between both drugs. All the cultures were prepared with an initial cell density of $8x10^3$cells/cm^2. For each condition, the results are presented as the mean of three independent experiences (± standard deviation). *p<0.05, **p<0.01, $^{\#}$p<0.001

As to the RenG2 cell line there was also significant statistically differences (P<0.001) in cells between the control and the cells that were exposed to cisplatin and gemcitabine for 24h, 48h and 72h, independent of the drugs concentration used (graph not shown). The drugs' effects on cells' viability were also much pronounced for longer exposures i.e., 48 and 72h particularly for 72h exposures, with just 20-15% cells alive, even though, for 72h exposure to cisplatin the effects were not significantly dependent on its concentrations. Also, between the dose concentrations of 0.1 μM and 10 μM, there were only significant concentration differences for gemcitabine exposure at 24h (P<0.01). The results of Figures 3.11 and 3.12 for BEAS-2B and Cont-1 cells, respectively and the ones illustrated in Figure 3.13 for RenG2 cells clearly show that RenG2 cells are more resistant to both cisplatin and gemcitabine. Also, in contrast to BEAS-2B and Cont-1 cells, which were significantly less resistant to the highest gemcitabine concentration, RenG2 cells were similarly resistant to gemcitabine and to cisplatin.

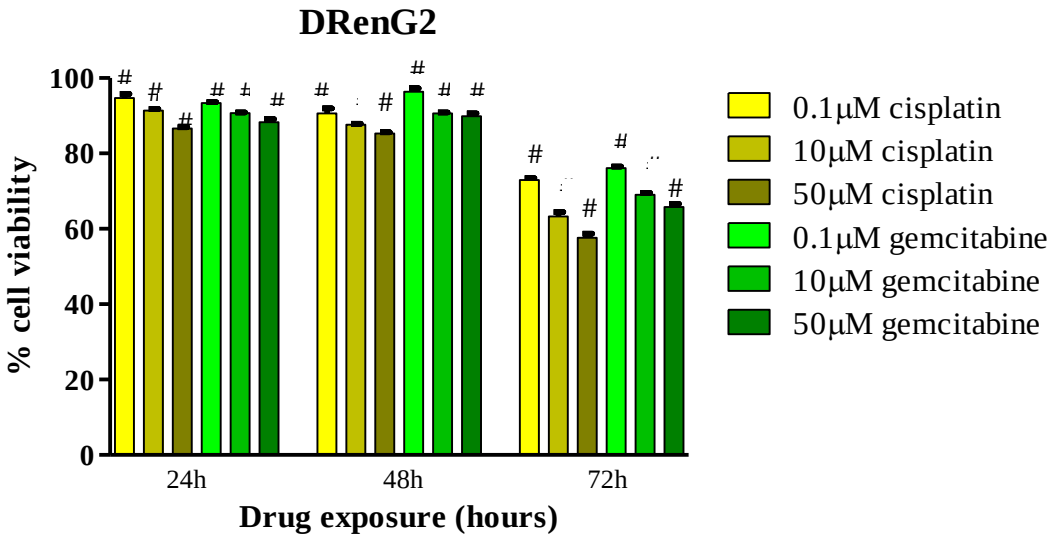

Figure 3.14 Different drug concentration effects (0.1μM, 10μM and 50μM) in the cell viability at 3 different times of drug exposure (24h, 48h, and 72h) for the DRenG2 cell line, determined by the MTT assay. The results are represented as percentages in relation to the obtained control values. All the cultures were prepared with an initial cell density of $8x10^3$cells/cm^2. For each condition, the results are presented as the mean of three independent experiences (± standard deviation). *p<0.05, **p<0.01, $^{\#}$p<0.001 vs control

Concerning DRenG2 cell line, Figure 3.14 illustrates that exposure to both 10 μM and 50 μM cisplatin or gemcitabine significantly decreased the cells' viability in relation to the control, particularly relevant for 72h exposures (P<0.001). The cells' viability at 24h and 48h is almost the same, particularly evident for 48h exposure to gemcitabine 0.1 μM. Figure 3.15 also illustrates that for the shorter exposure (24h) to 10 μM and 50 μM gemcitabine there is little statistical difference (p<0.05). Surprisingly, and in contrast to BEAS-2B, Cont-1 and RenG2, it is noticeable that DRenG2 is more resistant to gemcitabine than to cisplatin, particularly for longer exposures i.e., 72h (Figure 3.15). Clearly, when comparing the results expressed in previous Figures 3.11-3.13 is evident that DRenG2 cell line is significantly more resistant to both drugs concentrations particularly for 72h exposures.

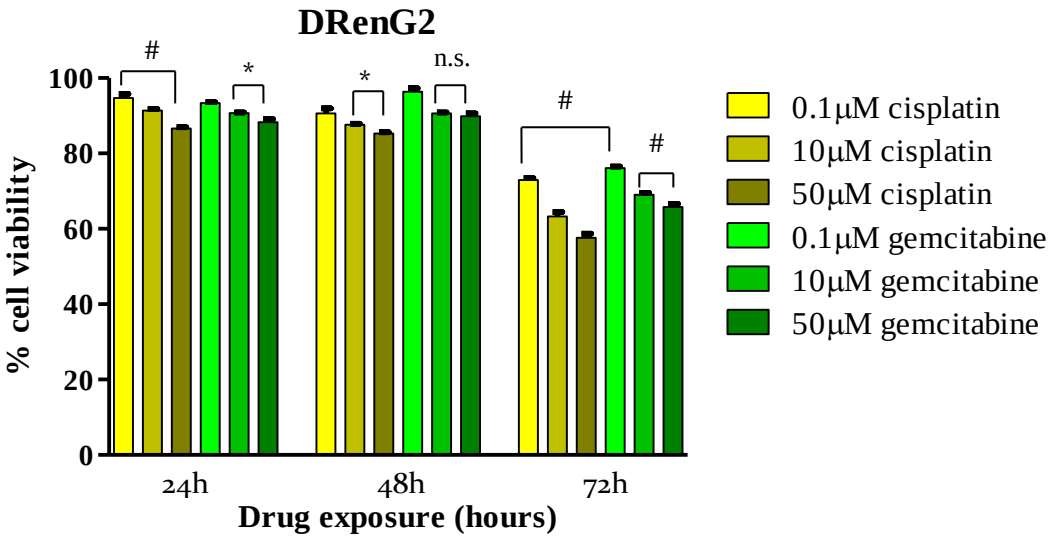

Figure 3.15 Different drug concentration effects (0.1µM, 10µM and 50µM) in the cell viability at 3 different times of drug exposure (24h, 48h, and 72h) for the RenG2 cell line, determined by the MTT assay. The results are represented as percentages in relation to the obtained control values. In this graph is also represented some statistically differences between drug concentrations and between both drugs. All the cultures were prepared with an initial cell density of $8x10^3$cells/cm^2. For each condition, the results are presented as the mean of three independent experiences (± standard deviation). *$p<0.05$, **$p<0.01$, $^{\#}p<0.001$

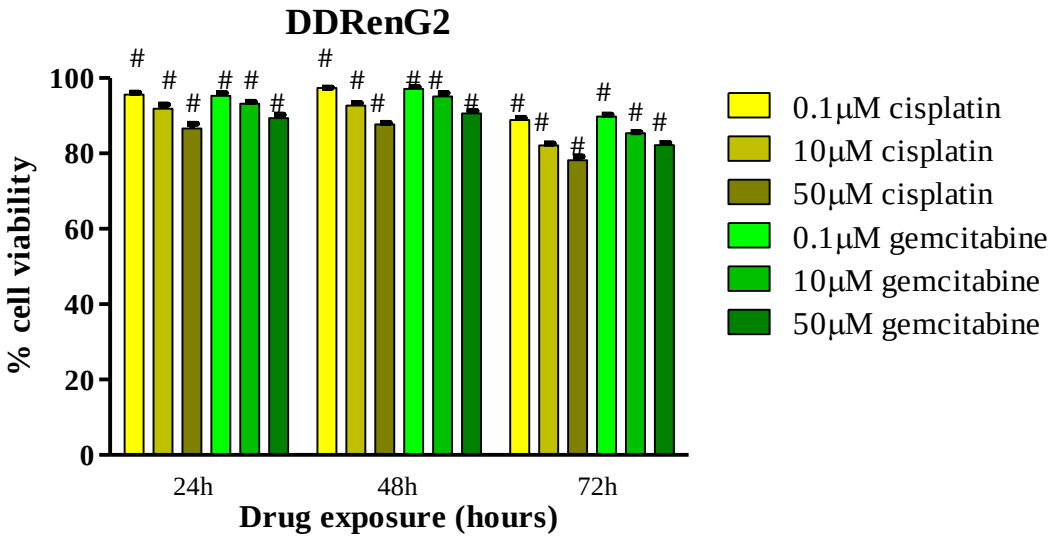

Figure 3.16 Different drug concentration effects (0.1μM, 10μM and 50μM) in the cell viability at 3 different times of drug exposure (24h, 48h, and 72h) for the DRenG2 cell line, determined by the MTT assay. The results are represented as percentages in relation to the obtained control values. All the cultures were prepared with an initial cell density of $8x10^3$cells/cm^2. For each condition, the results are presented as the mean of three independent experiences (± standard deviation). *p<0.05, **p<0.01, $^{\#}$p<0.001 vs control

Concerning DDRenG2 cell line, Figure 3.14 illustrates that exposure to both 10 μM and 50 μM cisplatin or gemcitabine significantly decreased the cells' viability in relation to the control ($p<0.001$), however despite this difference in cell viability in relation to the control, the DDRenG2 cell line, presented a higher cell viability at 72h of exposure than the DRenG2 cell line.

The cells' viability during the three days of exposure is almost the same, particularly evident for gemcitabine and cisplatin 0.1. Figure 3.15 also illustrates that for shorter exposures (24h and 48h) to 0.1 μM and 10 μM gemcitabine there is no statistical differences between them. In accordance with the DRenG2 cell line, DDRenG2 cell line is more resistant to gemcitabine than to cisplatin, particularly for longer exposures i.e., 72h (Figure 3.17). From the cell lines mentioned before, the cell which has higher cell viability is the DDRenG2 cell line.

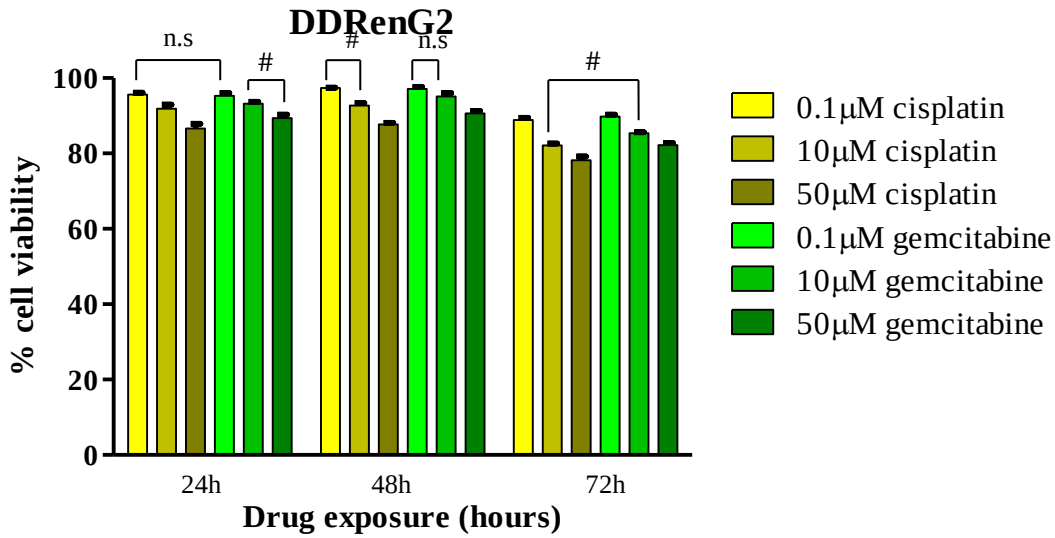

Figure 3.17 Different drug concentration effects (0.1μM, 10μM and 50μM) in the cell viability at 3 different times of drug exposure (24h, 48h, and 72h) for the RenG2 cell line, determined by the MTT assay. The results are represented as percentages in relation to the obtained control values. In this graph is also represented some statistically differences between drug concentrations and between both drugs. All the cultures were prepared with an initial cell density of $8x10^3$cells/cm^2. For each condition, the results are presented as the mean of three independent experiences (± standard deviation). *$p<0.05$, **$p<0.01$, $^{\#}p<0.001$

4. Discussion

As referred in Chapter 1, the term *cancer* refers to a broad category of diseases that arise as a result of the accumulation of mutations, chromosomal instability, and epigenetic events. Despite the fact that cancer is a common disease, the transition from a normal cell to a cancer cell is a very rare event, considering the number of cells making up an organism (Evan and Littlewood, 1998). This is mainly due to some fail-safe mechanisms within the cell that respond to DNA damage by either arresting cell cycle to allow DNA repair or to consent apoptosis (Argyle and Blacking, 2007).

The discovery of CSCs in a number of human solid tumour types has suggested a central role in tumorigenesis due to their particular properties which include: self-renewal, which drives tumorigenesis and differentiation, contributing to cancer cell heterogeneity, their ability to seed new tumours and to spawn non-CSC populations lacking tumour initiating ability. There is also increasing evidence that these CSCs mediate tumour metastasis and, by virtue of their relative resistance to chemotherapy and radiation therapy may contribute to treatment resistance and relapse following therapy (Bonet and Dick, 1997**;** Clarke *et al*., 2006**;** Reya *et al*., 2001). Importantly, CSC-rich tumours are associated with aggressive disease and a poor prognosis (Ailles and Weissman, 2007), indicating that an understanding of their biology is pertinent to the development effective therapies.

Both normal and neoplastic stem cells are thought to be self-renewing and to reside at the core of a cellular hierarchy, because through asymmetric division generate more differentiated progeny that lack self-renewal capacity (Alison and Islam, 2009**;** Tan, *et al*., 2006). Thus, intratumour heterogeneity may be explained by the ability of CSCs to generate neoplastic lineages at various differentiation stages even though, it is becoming

apparent that the differentiation states of the cells-of-origin can influence the organization of neoplastic cell populations (Ince *et al*., 2007; Lim *et al*., 2009).

Recently, subsequent to the establishment of a tumorigenic cell line (RenG2) by exposure to Cr(VI), it were successfully implemented two different cell lines with increasing degree of malignancy, DRenG2 and DDRenG2, out of tumors induced in athymic nude mice by RenG2 and DRenG2 respectively. Unexpectedly, sub-populations with stem-like properties were present only in the more malignant DRenG2 and DDRenG2 cell lines evidencing that differentiated cell types can convert to stem-like cells and an unsuspected degree of cellular plasticity.

In the present work, it was undertaken the study of the biology of the populations of RenG2 cells, obtained following prolonged treatment of differentiated non-tumorigenic bronchial epithelial cells (BEAS-2B) with Cr(VI), a known lung carcinogenic agent, and of DRenG2 and DDRenG2 cells, as well as, their precursor cell line BEAS-2B and the non-tumorigenic control of RenG2 cell line, Cont-1 cell line.

4.1. Cytogenetic characterization of the DRenG2 and DDRenG2 cell lines

Eukaryotic organisms have evolved robust mechanisms to ensure accurate segregation of the genetic material during mitosis. The cell cycle has checkpoints that delay chromosome segregation until DNA replication has been completed and the sister chromatids are properly aligned at the metaphase plate. However, these safeguards occasionally fail, resulting in unequal cell divisions that lead to developmental defects and cancer (Hardy and Zacharias, 2005).

A hallmark of human cancers is genomic instability (Hanahan and Weinberg, 2011). This instability can be represented as gains or losses of whole chromosomes or translocations/deletions/duplications of chromosomes' segments (Fang, X and Zhang,

2011). This instability may lead to a state of polyploidy, if the chromosomal alteration is a linear multiplication of the entire set of the chromosomes, or it may lead to a state of aneuploidy, which refers loss or gain of single chromosomes or fragments (usually associated to a cancer phenotype) (Ganem, *et al*., 2007; Panigrahi and Pati, 2009). In most cases, the aneuploidy can be caused by mitotic errors due to defects in the mechanisms that have evolved to ensure faithful chromosome segregation (*i.e.* spindle assembly checkpoint) (Sheltzer and Amon, 2011). Other potential mechanisms that may be involved in chromosome segregation errors are the abnormal amplification of centrosomes, with its consequent abnormal chromosome separation, and abrogated cytokinesis (Jefford and Irminger-Finger, 2006; Jung *et al*., 2007). One of the consequences of this deregulation and of the consequent changes in the karyotype is the cellular transformation, a process that enhances the proliferative capacity of a cell, which often occurs in cancer cell lines (See section 3.3) (Sheltzer and Amon, 2011). Finally, another characteristic of aneuploid cells is that unlike DNA damage, once a cell becomes aneuploid, there is almost no way to correct the lost or the gained chromosomes, which implicates that the progeny will remain aneuploid (Rasnik, 2002).

In the present work, aiming to observe the major cytogenetic events underlying the malignant transformation process and the appearance of CSCs sub-populations it was undertaken a cytogenetic study of these more malignant cell lines using conventional G-banding and FISH study addressed to the chromosomes with apparent major alterations. As mentioned in chapter 3.1, the karyotype analysis of DRenG2 and DDRenG2 cell lines showed some structural alterations that have already been described for the BEAS-2B cell line (Rodrigues *et al*., 2009). However, they also revealed some structural alterations of their own, like the $7p^-$, and the loss of the Y chromosome, for both cell lines. And the $7q^-$, $i9q^+$ for the DRenG2 cell line and t(7;14), $17q^+$ for the DDRenG2 cell line

The 7p region harbours genes for the *EGFR* (epidermal growth factor receptor) and for the *IGF* binding proteins 1 and 3 *(IGFBP1* and *IGFBP3*) at 7p12-14 (Bergamo *et al*., 1999). This region was reported to be associated with metastatic tumors, while the gain in the 7p region was suggested to be linked with a non-metastatic tumor phenotype (Bergamo *et al*., 1999).

Another recurrent chromosomal aberration is the loss of the Y chromosome, which is frequently reported in lung cancer (Luk *et al*., 2001; Berker-Karauzum *et al*., 1998).

The alterations that were characteristic of the DRenG2 were the $7q^-$ and $iso9q^+$. This is the first time that these alterations were observed in cancer cell lines, since they haven´t been described in the recent literature yet. However, rearrangements on chromosome 9 are characteristic of some cancers, including gliomas, acute lymphoblastic leukemia, and certain forms of lung cancer and finally, in the bladder tumours (Coleman *et al*., 1994). A frequent chromosome rearrangement that has been detected in these types of cancer is the loss of heterozygosity (LOH) on chromosome 9, thus suggesting that loss of one or more suppressor gene(s) on chromosome 9 may be an early or initiating event in tumorigenesis (Coleman *et al*., 1994; Simoneau *et al*., 1999). Besides that, it is known that this chromosome harbours the tumour suppressor gene *P16* (Berrieman *et al*., 2004). Finally, the gain of an isochromosome, such the ones that appear in this cell line (i5 and i9), has also been strongly associated with drug resistance (Selmecki *et al*., 2006).

The translocation (7;14) observed in the line DDRenG2 has been also observed in patients with neurocysticercosis (NCC), which is associated with a high frequency of DNA damage in human circulating lymphocytes and with the development of haematological malignancies (Herrera *et al*., 2001). A feature of chronic NCC is a constant antigen stimulation, which may increase the frequency of aberrations of

chromosomes that harbour regions constantly rearranged during T and B lymphocyte maturation *e.g*., chromosomes 7 and 14 (Herrera *et al*., 2001). A common chromosome aberration that occurs between these chromosomes is the t(7;14) due to their hypermutation (Klein, 2000).

The alterations mentioned before can be correlated with the enhanced drug resistance that was observed in the DRenG2 and DDRenG2 cell lines (See section 3.4) (Li *et al*., 2005). This is also confirmed in Duesberg paper (Duesberg *et al*., 2007), where he correlates the cancer cells karyotype with drug resistance, in other words, Duesberg and collaborators defends that when cancers cells acquire resistance against drugs, they acquire new karyorype alterations and/or they lose old ones (karyotypic theory).

Finally, it is important to give emphasis, to the fact that aneuploidy is not a mere by product of tumorigenesis, but might be its cause (Boveri, 2008). For instance, during oncogenic transformation aneuploidy provides the precancerous cells the ability to evolve into more malignant state and to adapt to the "harsh" environment (Fang and Zhang, 2011) due to the presence of extra genes.

4.2. Characterization by immunocytochemistry of the BEAS-2B, Cont1, RenG2, DRenG2 and DDRenG2 cell lines

To further understanding the biology of the cell lines, the next step was to evaluate their epithelial, mesenchymal or stem-like phenotype, and whether they express the P-glycoprotein (efflux protein) liable for their increasing resistance to chemotherapeutic drugs.

MNF116 is an anti-cytokeratin antibody that reacts with the Cytokeratins 5, 6, 8, 17 and probably also 19 (Moll *et al*, 1982). This antibody shows an especially broad

reactivity pattern towards human epithelial tissues *i.e*., from simple glandular to stratified squamous epithelium, and can be used in the detection and classification of normal and neoplastic cells of epithelial origin.

As mentioned in section 3.2, all cell lines express MNF116, which was expected, since these cell lines have an epithelial origin. However, it was expected that, in the more malignant cell lines DRenG2 and DDRenG2, the level of MNF116 staining should be weaker since these cell lines would tend to an epithelial- or mesenchymal- transition phenotype (Kalluri and Weinberg, 2009). Instead, the DRenG2 and the DDRenG2 cell lines were the ones that showed the higher MNF 116 antibody staining.

Vimentin is a type III intermediate filament protein normally expressed in cells of mesenchymal origin (Steinert and Roop, 1988). However, Vimentin expression has been described in epithelial cells involved in pathological or physiological processes which involve epithelial cell migration (Gilles *et al*., 1999).

The malignant cell lines RenG2, DRenG2 and DDRenG2, showed a higher Vimentin staining when compared to the non-malignant cell lines BEAS-2B and Cont-1. The increase on the Vimentin staining level is correlated with malignancy, as Vimentin staining of DDRenG2 is the strongest, suggesting that these cells' progress to a more malignant state involves an epithelial to mesenchymal transition (EMT), since Vimentin staining is characteristic of a mesenchymal phenotype (McInroy and Määttä, 2007).

The hallmark of cancer malignancy is the metastatic dissemination of the primary tumours, which at the beginning are inept to invade the surrounding tissue. However, following the accumulation of genetic and/or epigenetic alterations and interactions with their environment, a small number of cells may bypass fundamental rules of the normal behaviour, detaching from the neighbouring ones, allowing the migration and metastases formation (Miller *et al*., 2005; Voulgari and Pintzas, 2009; Kong, *et al*., 2011). Increasing

evidence suggests that migrating cells involved in the process mentioned acquired an EMT phenotype (Mani *et al*., 2008; Kalluri and Weinberg, 2009), which may explain the observation that cancers with strong Vimentin expression have poor prognosis (Thomas *et al*., 1999).

Oct3/4 is an essential transcriptional regulator of genes involved in maintaining the pluripotency and self-renewal of embryonic stem (ES) cells (Burdon, 2002) and may prevent the expression of genes activated during differentiation (Lee *et al*., 2010). Besides that, Oct3/4 plays a crucial role in maintaining cancer stem-like cell phenotype and the chemoresistance properties in cancers (Chen *et al*., 2008). Thus, it would be expected that the more malignant DRenG2 and DDRenG2 cell lines would stain positive for Oct3/4. Surprisingly, this wasn't observed, possibly, because CSCs are a small subset of cells population, representing a very minor sub-population of the cells, and, thus, their identification by immunocytochemistry is very difficult.

P-gp belongs to the ABC family transporters which usually are over-expressed in CSCs, leading to the MDR observed in current therapies (O' Brien *et al*., 2009) (See section 4.4). So, it would be expected to have the expression of this protein in the DRenG2 and DDRenG2 cell lines. However, this antibody stained negative for all the cell lines, which leads to the conclusion that the observed drug resistance in these cell lines (See section 3.4) is due to other mechanism than the enhanced drug extrusion promoted by this protein (See section 4.4)

4.3. Duplication cell time of 5 cell lines (Beas-2B, Cont1, RenG2, DRenG2 and DDRenG2)

Normal tissues carefully control the production and release of growth-promoting signals that allows the cells to entry into and progress through the cell cycle, thereby ensuring the cell number homeostasis and consequently the maintenance of normal tissue architecture and function (Argyle and Khanna, 2006). However, in some cases there is deregulation of these signals, leading to an uncontrolled cell proliferation, which is maintained by growth-stimulating pathways (e.g. EGF, TGF-α and bFGF) activated, independently of their normal tissue environment, culminating in cancer (Moch *et al*., 1997; Kuhn *et al*., 2003; Hanahan and Weinberg, 2011). On the other hand, CSCs, as well as normal SCs, are believed to be relatively quiescent (Signore *et al*., 2011). Although, the cell lines DRenG2 and DDRenG2 have a small subset of CSCs, surprisingly, they were the ones that showed a lower cell doubling time. Apparently, these cell lines may have gained alterations in essential genes that control cell death, cell differentiation and/or cell proliferation that increased their proliferation rate, a fundamental property of tumour cells (Sekine and Saijo, N., 2001; Panigrahi and Pati, 2009). In addition to the capability of inducing and sustaining cell proliferation through growth-stimulatory signals, cancer cells must also evade growth suppressors, which are powerful programs that negative regulate cell proliferation; many of these programs depend on the action of tumours suppressor genes, especially *Rb* and *Tp53* proteins quite often involved in apoptotic cell death (Hanahan and Weinberg, 2011). In fact, a factor that may contribute to the malignant cells lower cell doubling time is the resistance cell death, quite common in cancer cells because, usually, these cells have the apoptotic genes mutated or mis-regulated, and, consequently, the apoptotic pathways down-regulate (Panigrahi and Pati, 2009).

DNA-damage signals activates the *Tp53* gene that encodes the tumour suppressor p53, which then activates p21 expression to enforce cell cycle arrest, or activates Bax expression to enforce apoptosis, which leads to programmed cell death (PCD) (Koty *et al*., 1999). PCD is a genetically regulated pathway that is altered in many cancers, and is regulated by the ratio of PCD inducers (Bax) or inhibitors (Bcl-2). And so, an abnormally high ratio of Bcl-2 to Bax prevents PCD (Koty *et al*., 1999). Type I PCD or apoptosis is critical for cellular self-destruction for a variety of processes, namely the prevention of oncogenic transformation (Hotchkiss *et al*., 2009). A mode of apoptotic cell death is the anoikis, which is trigger in consequential to insufficient cell-matrix interactions and is a critical player in tumour angiogenesis and metastasis, since tumour cells have the ability to survive in the absence of adhesion to the ECM (Rennebeck *et al*., 2005; Sakamoto and Kyprianou, 2010).

The resisting cell death mechanisms mentioned before might largely explain the lower double cell time observed in the malignant cell lines, RenG2, DRenG2 and DDRenG2, in relation to the precursor cell line BEAS-2B, especially the possible absence of death by anoikis in the malignant cell lines, since they showed an altered morphology, to a more fibroblast cell-like (Figure 3.6), which is better suited to survive in absence of cell adhesion. So, tumour cells can escape from detachment-induced apoptosis by controlling anoikis pathways.

Other alternative forms of programmed cell death include autophagy and necrotic cell death (Portt *et al*., 2011).

4.4 Drug resistance/cell viability

Existing therapeutic approaches have been largely based on the stochastic model, since most chemotherapeutic agents are addressed to the tumour major sub-populations which have a limited self-renewal and proliferative potential. Till know, these therapeutic approaches haven't been successful, since most therapeutic effects are usually transient and fail to cure most cancers.

The chemotherapy failure is due mainly to inadequate pharmacokinetic properties of the drug, tumour cell intrinsic factors such as the expression of drug efflux pumps and tumour cell extrinsic conditions present in the tumour microenvironment, *i.e*., hostile conditions as hypoxia, acidosis and nutrient starvation that render the well adapted cancer cells particularly resistant to cytotoxic drugs (Gatti and Zunino, 2005; Gottesman, 2002).

In the present work cisplatin (cDDP) and gemcitabine (GEM), the most common anti-neoplastic agents used in lung cancer treatment were used to evaluate the chemoresistance of the cells lines. The cDDP was the first member of a class of platinum-containing anticancer drugs, followed by carboplatin and oxaliplatin. The cDDP biochemistry mechanism of action is common to many other platinum agents and their most important biological target it's the DNA (Lippard, 1982). cDDP like many DNA damaging agents, acts as a cross-linker, inhibiting both DNA replication, and RNA transcription, and leading to cell cycle arrest and apoptosis (Rosell *et al*., 2002).

GEM (2'2'-difluorodeoxycytidine), an analogue of cytosine arabinoside (ara-C), is a novel pyrimidine nucleoside antimetabolite (Huang *et al*., 1991). Unlike its analogue, GEM has demonstrated activity in a wide range of solid cancers, including the lung cancer (Lund *et al*., 1993). GEM exhibits cell-phase specificity, primarily killing cells undergoing DNA synthesis (S phase) and also blocking the progression of cells through

the G1/S-phase boundary, which leads to inhibition of DNA synthesis and cytotoxicity (Chiappori and Rocha-Lima, 2003).

As depicted in the Figures 3.11-3.17 in chapter 3.4, the more malignant DDRenG2 cell line presented the highest resistant to both drugs during the 3 days of exposure (24h, 48h,72h), followed by DRenG2 cell line. The recent findings that both DRenG2 and DDRenG2 cell lines have sub-populations of cells with stem-like properties (Rodrigues *et al*., 2011) explain the chemotherapeutic resistance to either cDDP and to GEM, and is in line with the observation that tumours that contain sub-population(s) of self-renewing and expanding stem cells known as CSCs, are highly resistance to the conventional chemotherapies.

The MDR promoted by CSCs is a serious problem in cancer chemotherapies, The CSCs resistance is mostly due to the over-expression of several energy-dependent drug efflux pumps, belonging to the ABC family of transporters, such as P-Glycoprotein and the MDR-associated proteins (MRPs), that are rightly described as *guardians of the CSCs* (Leitner *et al*., 2007; Chen *et al*., 2010). The over-expression of the ABC transporters occurs in response to drug exposure, being regulated genetically and epigenetically (Amiri-Kordestani *et al*., 2012). However, in our study none of the cell lines, including the chemoresistant DRenG2 and DDRenG2 express the P-Glycoprotein. Thus, resistance of DRenG2 and DDRenG2 to the DNA damaging agent cDDP may be due either to MRPs overexpression or to very efficient DNA damage repair systems. Unlikely DRenG2 and DDRenG2, RenG2 is much less resistant to cDDP, although the genes that code for proteins involved on DNA repair, namely RAD51, XRCC3 and OGG1 [homologous recombination (HR)], XRCC1 [base excision repair (BER)], XRCC5 (NHEJ) and MLH1 (MMR), are highly expressed (Rodrigues *et al*., 2009), as well as hypoxia-inducible factor (HIF) which, in response to hypoxia, was reported to activate the *multidrug*

resistance 1 (MDR1) gene which encodes for the membrane- resident P-Glycoprotein responsible for drug extrusion ((Rohwer and Cramer, 2011;. Comerford *et al*., 2002),

In the light of the observations, possible strategies for the successful eradication of CSCs, might be blocking EMT and targeting the self-renewal controlling pathways (Wnt, Notch and Hedgehog) (Signore *et al*., 2011; Alison *et al*., 2012; Gao *et al*., 2012).

5. Concluding remarks

Regardless the progress made in the cancer research field and the numerous theories that have emerged to explain its formation and progression, which have brought new and complementary points of view and new questions to be answered, this disease is still not fully understood.

This study demonstrated that the DRenG2 and DDRenG2 cell lines, which have a subset of CSCs populations, despite their negative staining for the Oct3/4 antibody, had marked structural and numeric alterations in relation to their precursor BEAS-2B cells, which means that through their evolution since a non-malignant state to a malignant state they acquired genetic alterations that altered their phenotype. This was demonstrated by the strong staining observed for the Vimentin antibody in the DDRenG2 cell line. These phenotypic changes are the visualized consequences of their altered intrinsic characteristics, such as resisting cell death, evading growth suppressors, and sustaining proliferative signalling. These characteristics are emphasised by the resistance to cDDP and GEM of the malignant and proliferative DRenG2 and DDRenG2 cell lines, comparatively with the other cell lines, which supports the hypothesis that CSCs are responsible for the observed resistance to the conventional therapies.

Between other things, the observed resistance in CSCs cell lines is usually due to the presence of a transport system-resistance to chemotherapy, P-gp. However, the DRenG2 and DDRenG2 cell lines stained negative for this antibody, which means that the drug resistance observed in these cell lines is correlated with other mechanism(s).

This work confirms some point of views that have been developed through the last years in the cancer field, nevertheless, further work still needs to be done to unravel the formation of the CSCs and to develop better therapies that can improve the survival of the patients.

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