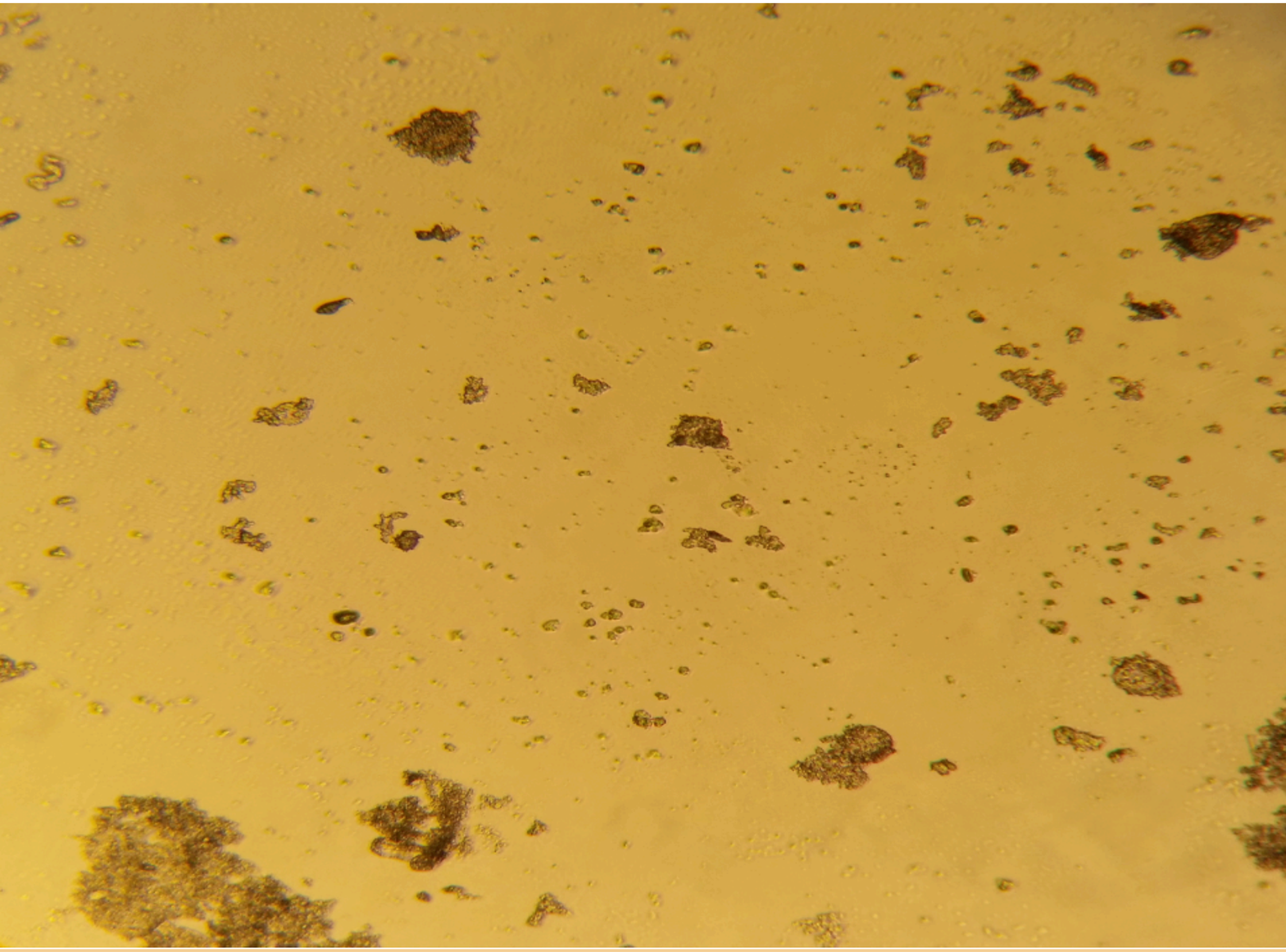

Eurico da Silva Serrano

Tracking of the intracellular cytokines' transduced signals

Tese de Mestrado em Bioquímica, orientada pelo Doutor Carlos Fernando Dias Rodrigues e pela Professora Doutora Maria Carmen Alpoim e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Julho 2016

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UNIVERSIDADE DE COIMBRA

Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Mestrado em Bioquímica

Tracking of the intracellular cytokines' transduced signals

Eurico da Silva Serrano

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre, realizada sob a orientação científica do Doutor Carlos Fernando Dias Rodrigues, Doutor associado ao Centro de Neurociências e Biologia Celular da Universidade de Coimbra, e da Professora Doutora Maria Carmen Alpoim, Professora Associada da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Julho 2016

Aos meus pais.

“Only one who devotes himself to a cause with his whole strength and soul can be a true master. For this reason mastery demands all of a person.”

— **Albert Einstein**

Abstract

Cancer stem cells (CSCs) are cancer cells that display stem-like properties and that are deeply involved in several steps of the carcinogenic progression. They are able to drive tumorigenesis and differentiation, thus explaining tumor heterogeneity, and are responsible for tumor relapse after treatment due to their high resistance to the common therapeutic approaches. Their origin is still controversial, but evidence from targeted therapy assays showed that although this population of cells may be diminished, it is able to be replenished by dedifferentiation of terminally differentiated tumor cells. The mechanisms underlying the dedifferentiation process are still unclear, but a pivotal role for the tumor microenvironment (TME) and its derived soluble factors has been proposed. In agreement, our group has previously found that interleukin 6 (IL-6), Activin-A and Granulocyte colony-stimulating factor (G-CSF) are able to drive malignant human bronchial epithelial cells' dedifferentiation.

The present study aimed to identify the intracellular signaling pathways activated during the abovementioned cytokine-driven dedifferentiation process. To this end IL-6, G-CSF and Activin-A intracellular signaling pathways were differentially blocked in Transwell® co-cultures of RenG2 cells and E2a fibroblasts, so their individual role in the RenG2 cells' dedifferentiation process was ascertained. Finally, by implementing the system in the presence of exosome-mediated communication blockers, the role of these vesicles in the intercellular communication process was assessed.

The attained results suggested that IL-6 and Activin-A were the main orchestrators of the dedifferentiation process since the presence of at least one of these cytokines led to the development of a stem cell-like population inside the RenG2 cells. Additionally, Activin-A seemed to act as a sensor of the CSCs' pool homeostasis. G-CSF, *per si*, was excluded as a driver of dedifferentiation but a role for this cytokine in the maintenance of the undifferentiated phenotype was suggested.

Resumo

Células estaminais tumorais (CETs) são células cancerígenas que exibem propriedades estaminais e que estão altamente envolvidas nos vários passos da progressão tumoral. Estas células são capazes de regular a tumorigenicidade e a diferenciação tumoral, explicando-se deste modo a diversidade celular encontrada nos tumores. Por outro lado, são ainda responsáveis pelas recidivas após tratamento devido ao seu elevado grau de resistência às terapias convencionais.

A origem das CETs é ainda alvo de controvérsia, mas estudos recentes mostraram que quando as CETs são eliminadas por terapias direcionadas, a sua população pode ser regenerada por dediferenciação de células tumorais diferenciadas. Contudo, os mecanismos que medeiam esta dediferenciação não são ainda claros.

Resultados prévios do nosso laboratório identificaram a Interleucina-6 (IL-6), a Activina-A e o Factor estimulador de colónias derivado de granulócitos (G-CSF) como mediadores do processo de diferenciação de uma linha maligna de epitélio bronquial humano. Neste sentido, o presente estudo teve por objetivo identificar o mecanismo subjacente ao processo de dediferenciação conduzido pelas citocinas supracitadas. Para isso as vias de sinalização intercelular da IL-6, do G-CSF e da Activin-A foram diferencialmente bloqueadas com vista a permitir o estabelecimento do papel individual de cada citocina no processo de dediferenciação. Finalmente, a realização das mesmas experiências na presença de inibidores da comunicação mediada por exosomas permitiu avaliar o envolvimento destas vesículas no mesmo processo.

Os resultados obtidos sugerem que a IL-6 e a Activina-A são os principais responsáveis pelo processo de dediferenciação, já que a presença de apenas uma delas leva à aquisição de fenótipo estaminal pelas células RenG2. Por outro lado, foi também possível deduzir que a Activina-A funciona como um sensor da homeostase da população de CETs. O G-CSF, por seu turno, foi excluído como mediador efetivo do processo de dediferenciação, mas parece desempenhar um importante papel na manutenção das características estaminais das CETs.

Acknowledgements

My first acknowledgment is both to **Professor Maria Carmen Alpoim** and **Doctor Carlos Fernando Dias Rodrigues** for the opportunity they gave me to work with them through this year.

To **Doctor Carlos Fernando Rodrigues,** I want to express my sincere gratitude for the opportunity, for the shared knowledge and for helping me to give the first steps in my work development. Also, for all the patience that he had to answer all my doubts and for all the work that he had especially in the final days in order to help this thesis happen.

To **Professor Maria Carmen Alpoim,** I want to express my gratitude by fueling my creativity and scientific thinking, for all the sympathy and for all the help given in the development of this work.

To **Marco Cunha** for the all the company and all the help given in the shared experience of developing a master thesis together to me this year.

To all of my friend old and new for all the support, fun, readiness to help me when I most needed and for putting up with me in the good and bad moments. I will not discriminate all of you because you are a large amount, but you know well who you are. Thanks for everything.

To **Mariana Tomás** for being one of my best friends since I came to Coimbra, thanks for all the support given through the last years, your help was always a bless. Also thanks for all the goods moments we passed together.

Para as pessoas mais importantes para mim, **Mãe e Pai**, obrigada por todo o sacríficio que fizeram para eu puder chegar a este momento e por me deixarem ter tido esta oportunidade. Também por me terem sempre ajudado sempre que possível apesar de distantes. Palavras são pouco para descrever o quanto importante são, mas sem vocês nada disto seria possível.

Obrigada ao resto da família que me suportou ao longo da vida. Apesar de não serem família, **Carmina e Paulo** um obrigada por me terem acompanhado e torcerem por mim.

Por último, mas não menos importante, ao meu **Avô** por ser aquela pessoa que esteve sempre presente desde que era pequeno, me ensinou muito e me ajudou a tornar na pessoa que sou hoje. Tenho pena que já não estejes presente para assistir ao final desta etapa, mas sei que sempre torceste por mim e quiseste o melhor para mim.

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List of Abbreviations

1. **BEAS-2B** - Bronchial epithelial airway system 2B
2. **BSA** - Bovine serum albumin
3. **CAFs** - Cancer-associated fibroblasts
4. **CETs** - célula estaminal tumoral
5. **CSCs** - Cancer stem cells
6. **DMSO** - Dimethyl sulfoxide
7. **E2A** - Human bronchial fibroblasts primary cell line
8. **ECM** - Extracellular matrix
9. **EMT** - Epithelial to mesenchymal transition
10. **ERK** - Extracellular-signal-regulated kinase
11. **FBS** - Fetal bovine serum
12. **G-CSF** - Granulocyte colony-stimulating factor
13. **IL-6** - Interleukin 6
14. **JAG1** - Jagged1
15. **MAPK** - Mitogen activated protein kinase
16. **NF-kB** - Nuclear factor-κB
17. **N-SMase** - Neutral sphingomyelinase
18. **PBS** - Phosphate buffered solution
19. **PI3-K** - Phosphatidylinositol-tri-phosphate kinase
20. **poly-HEMA** - Poly-(2-hydroxyethyl methacrylate)
21. **STAT** - Signal transducers and activators of transcription
22. **TAMs** - Tumor-associated macrophages
23. **TME** - Tumor microenvironment
24. **TGF-β** - Transforming growth factor-β

PART I

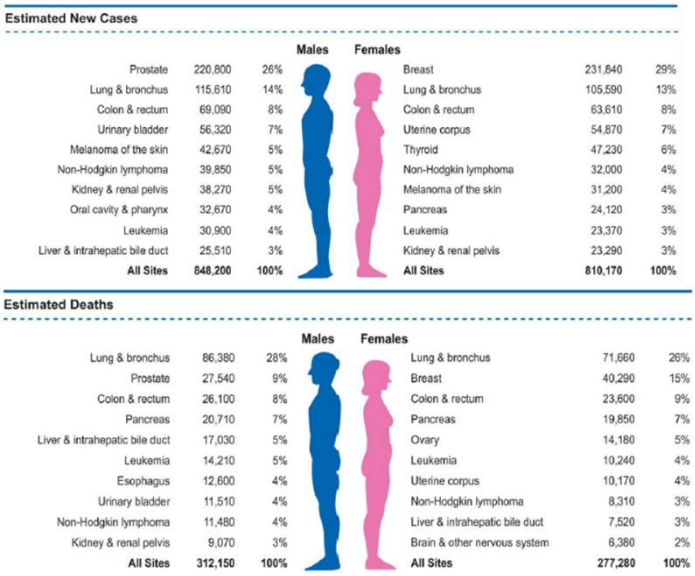
Introduction

Chapter 1

Cancer: the disease of the 21st century

1.1. Statistics and Epidemiology

Cancer is currently one of the main causes of mortality worldwide. Considering that this disease can affect people from all over the world without targeting a specific gender or age, and that it affects many organs and tissues of the human body, its current impact is not yet fully understood and a proper statistical analysis is awaited. The American Cancer Society (ACS), a major cancer consortium established to address this problem, publishes annual reports on cancer statistics in the United States. According to its latest report (Figure 1), when gender is considered, the most prevalent tumor among men is the prostate cancer, while among women the breast cancer prevails. However, in both genders, lung cancer tops the ranking of mortality.[1–3]



Estimated New Cases

Males			Females		
Prostate	220,800	26%	Breast	231,840	29%
Lung & bronchus	115,610	14%	Lung & bronchus	105,590	13%
Colon & rectum	69,090	8%	Colon & rectum	63,610	8%
Urinary bladder	56,320	7%	Uterine corpus	54,870	7%
Melanoma of the skin	42,670	5%	Thyroid	47,230	6%
Non-Hodgkin lymphoma	39,850	5%	Non-Hodgkin lymphoma	32,000	4%
Kidney & renal pelvis	38,270	5%	Melanoma of the skin	31,200	4%
Oral cavity & pharynx	32,670	4%	Pancreas	24,120	3%
Leukemia	30,900	4%	Leukemia	23,370	3%
Liver & intrahepatic bile duct	25,510	3%	Kidney & renal pelvis	23,290	3%
All Sites	**848,200**	**100%**	**All Sites**	**810,170**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	86,380	28%	Lung & bronchus	71,660	26%
Prostate	27,540	9%	Breast	40,290	15%
Colon & rectum	26,100	8%	Colon & rectum	23,600	9%
Pancreas	20,710	7%	Pancreas	19,850	7%
Liver & intrahepatic bile duct	17,030	5%	Ovary	14,180	5%
Leukemia	14,210	5%	Leukemia	10,240	4%
Esophagus	12,600	4%	Uterine corpus	10,170	4%
Urinary bladder	11,510	4%	Non-Hodgkin lymphoma	8,310	3%
Non-Hodgkin lymphoma	11,480	4%	Liver & intrahepatic bile duct	7,520	3%
Kidney & renal pelvis	9,070	3%	Brain & other nervous system	6,380	2%
All Sites	**312,150**	**100%**	**All Sites**	**277,280**	**100%**

Figure 1 – Cancer incidence and mortality. Cancer is a disease with various aetiologies, incidences and outcomes. Lung cancer is the tumour with the higher mortality rate. Adapted from Siegel 2015.[2]

1.2. Cancer hallmarks and the transformation process

Cancer is commonly understood as an abnormal growth of cells caused by multiple changes in gene expression, thus leading to a dysregulated balance between cell proliferation and cell death. Eventually, this condition yields a population of cells that can locally invade the tissues and then metastasize to distant sites, most frequently causing patients' death.

In an attempt to organize and integrate the cellular and molecular mechanisms behind cancer progression, Hanahan and Weinberg defined the hallmarks of cancer (Figure 2), meaning, the properties that cancer cells must acquire to develop and sustain their malignant phenotype. According to the authors, normal cells change gradually into a neoplastic state, successively acquiring these hallmark capabilities during the process.[4–6]

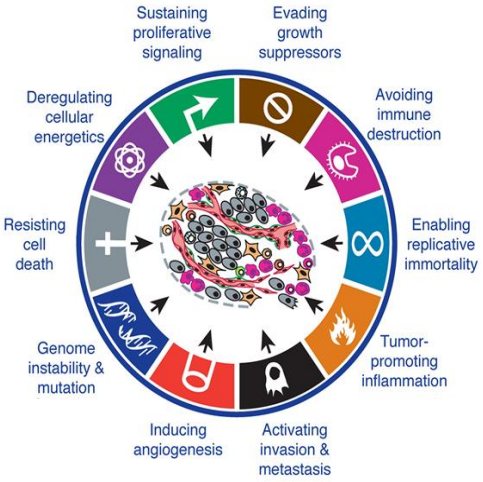


Figure 2 – The hallmarks of cancer and their enabling characteristics. The eight hallmark capabilities and the two enabling characteristics that accompany the carcinogenic process and allow the comprehension of cancer biology. Adapted from Hanahan and Weinberg 2011.[4]

Two of the first hallmarks cancer cells must acquire are the sustained proliferative signaling and the avoidance of growth suppressors' activity. Although aiming the same goal, these hallmarks are very distinct as the first involves the stimulation of growth factors' production, either by the malignant or the non-malignant cells, whilst the second is based on the evasion of the strong

programs that negatively regulate cell proliferation, normally through the loss of function of proteins encoded by tumor suppressor genes[6]. Another important hallmark is the resistance to the cellular death programs that function as a barrier to cancer progression. In this case, the ability to resist apoptosis is provided either by the overexpression of antiapoptotic genes and/or the blocking of proapoptotic factors. Combined with the high proliferative status, these characteristics contribute to the replicative immortality found in tumor cells, which *per si* is another hallmark of cancer.[4,6]

In order to ensure that the cellular energetic needs are assured and that metabolic waste products are removed, tumors activate angiogenesis. To attain this hallmark, pre-existing blood vessels are induced to sprout and a tumor-dedicated vascular web is established. However, in contrary to what was initially thought, angiogenesis' activation occurs early during the tumorigenic process, and is triggered by a process called *angiogenic switch* that involves an all-body paracrine communication with the bone marrow.[6,7] As a collateral consequence of angiogenesis, cancer cells enter the circulatory system long before a tumor is diagnosed[8]. Metastases' formation, another hallmark, is a highly dynamic and complex process in which a cell leaves the primary environment and establishes a proliferative focus in distant organs. Besides being the main cause of death in cancer patients, its precise underlying cellular and molecular mechanisms are still unknown.[6,9–11]

As a consequence of the accumulating knowledge on cancer biology, one decade later the cancer hallmarks were revisited and two additional hallmarks were added to the list. The first was the avoidance of immune destruction, which involves the dysregulated expression of the immune-checkpoint proteins, thus increasing therapeutic resistance. The second new hallmark was the deregulated cellular energetics. This hallmark comprises the reprogramming of cellular energy including the glycolytic dedifferentiation, which involves the expression of isoforms of key glycolytic enzymes, a key advantage in terms of metabolic plasticity, and the redirection of mitochondria towards biosynthetic routes. Moreover, two enabling characteristics were also identified as facilitators

of the tumorigenic process, namely the genomic instability and the inflammation. These characteristics, although not mandatory for tumor development, whenever present facilitate the acquisition of the malignant properties by the initiated cells thus fuelling the malignant transformation process.[4]

Summarizing, the acquisition of the hallmarks capabilities depends on successive rounds of genetic alterations that must happen to premalignant cells. Furthermore, depending on the tumor type, the genome changes necessary happen by distinct means and at different times, and eventually lead to the selection of clones presenting higher survival potential, which deeply contributes to the high tumor heterogeneity.[4]

1.3. Tumor heterogeneity: an overview of the explaining models

It is well established that tumors are a mass of phenotypically and functionally heterogeneous cancer cells, being this heterogeneity a subject of intense research. As a consequence, different models have been proposed, but despite the efforts to consolidate the information in one single model, they turned out to be not mutually exclusive.[12–15]

The clonal evolution model (Figure 3), one of the most well-established models, assigns the acquisition of stochastic genetic alterations as the driver of cellular heterogeneity and malignant progression, by conferring a growth advantage to the cancer cells and allowing their selection and clonal expansion. Nevertheless, although the genetic alterations are the main reason underpinning this model, epigenetic differences and microenvironmental changes have also been reported to have important roles on the events that lead to the gain of growth advantage by cancer cells and can explain the tumors' heterogeneity.[12,15,16]

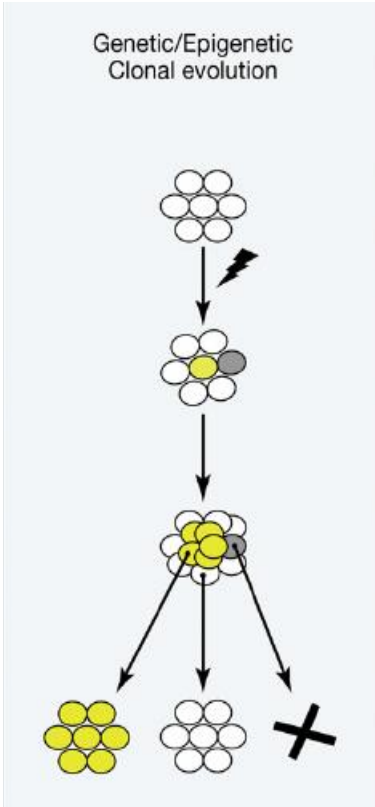


Figure 3 – The stochastic model of tumors' heterogeneity. According to this model, cancer cells acquire genetic changes that confer them growth advantages and allow their selection and expansion within the tumor. Adapted from Magee 2012.[12]

Evidence that a limited population of cells within the tumor could be responsible for tumor initiation and maintenance led to the formulation of a new theory – the hierarchical model of cancer. Contrarily to the clonal model, the hierarchical model explains that the occurrence of different degrees of cellular heterogeneity within a tumor is due to the presence of CSCs (Figure 4). More precisely, this model states that a tumor is a hierarchically organized heterogeneous entity that is coordinated by a scarse cellular population with self-renewal and multilineage differentiation abilities, the CSCs. Thus, tumors contain different subpopulations of tumorigenic and non-tumorigenic cells organized in a hierarchy, and the small population of CSCs, standing at the apex of the hierarchy, give origin to all of the phenotypically diverse cellular populations within the tumor, through their differentiation.[12,15,17]

As previously mentioned, it is important to note that these two models are not mutually exclusive (Figure 5), as CSCs may also undergo clonal evolution, and eventually either genetic and/or epigenetic alterations may provide them with a more aggressive self-renewing or growth ability, thus altering the dominant CSCs population within the tumor, as described by the stochastic model.[12,15,18]

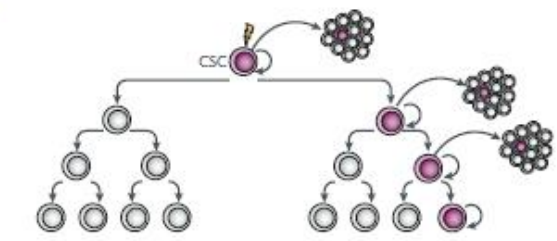


Figure 4 – The hierarchic model of tumor heterogeneity. According to this model tumors are a hierarchic entity that have at their apex CSCs. Adapted from Visvander 2008.[15]

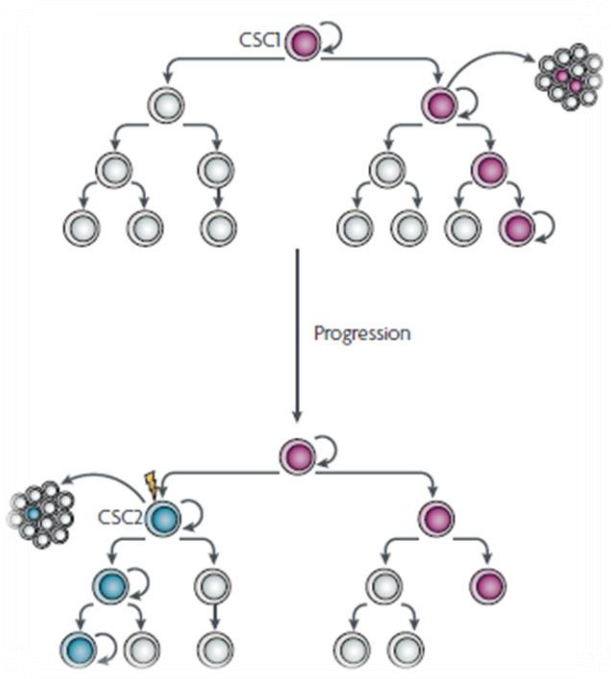


Figure 5 – The combination of the stochastic and hierarchic models of tumor heterogeneity. CSC may themselves undergo clonal evolution if a genetic alteration provides them with growth advantage. This would alter the dominant CSC pool, thus leading to a higher heterogeneity within the tumor. Adapted from Visvader 2008.[15]

More recently, with the accumulating knowledge on the role of the tumor microenvironment (TME) in the carcinogenic process, more specifically, its role on the modulation of the phenotypic and functional properties of CSCs, the dynamic hierarchic model was postulated (Figure 6). This new view of the hierarchic model of tumor heterogeneity introduces the TME as a key player in the cellular heterogeneity within the tumor. According to the dynamic cancer stem cell model, CSCs' phenotype is dependent upon signals released by the microenvironment stromal cells, which strongly modulate and regulate both CSCs' symmetric or asymmetric division, thus deciding on enriching the CSCs pool or promoting tumor heterogeneity by CSCs' differentiation. One particular aspect of this model is that it also can accommodate the more recent findings about the role of the TME on the origin and maintenance of CSCs.[19]

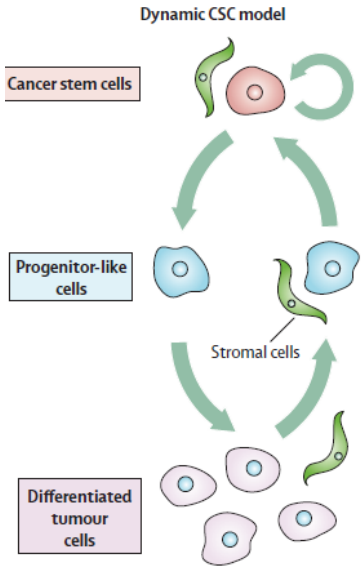


Figure 6 – The dynamic hierarchic model of tumor heterogeneity. CSCs are responsible for the tumor maintenance, but their physiology is coordinated by signals derived from the tumor stroma. Adapted from Vermeulen 2012. [19]

Chapter 2
Cancer microenvironment

2.1. The concept and the different components

Recent findings recognised that the malignant cancer cells phenotype cannot be only ascribed to changes in the genome and epigenome but that the microenvironment surrounding the cellular populations is also an important player. As a consequence, the role of the TME in the progression of the neoplastic disease has been established and several relations between its cellular populations and the different cancer hallmarks were uncovered.[5,20]

The high complexity associated to cancer mainly results from the immense crosstalk information that involves the tumor cellular populations and their microenvironment including the extracellular matrix (ECM) and tissue architecture. Academically, TME assembles the soluble factors, signaling molecules, ECM, immune and stromal cells, vascular system, as well as all the mechanical cues and dynamic constrains, that can promote neoplastic transformation, support tumor growth and invasion, protect the tumor from the host immunity, improve the therapeutic resistance and provide niches for dormant metastases to develop. It is constantly manipulated by the cancer cells so that their progression to metastasis is favored.[20–23]

The generic constituents of the TME are grouped in three general classes: the angiogenic vascular cells, the infiltrating immune cells and the cancer-associated fibroblasts (CAFs). Figure 7 correlates each of these classes with the acquisition of the different hallmarks.[5]

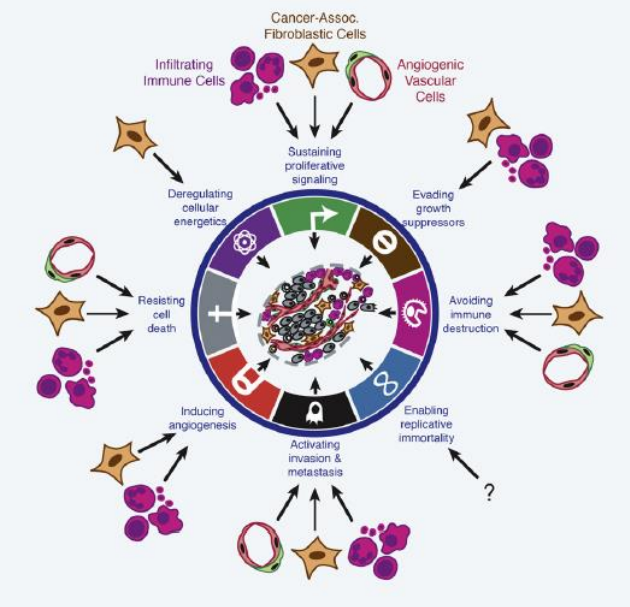


Figure 7 – Contribution of the different cellular populations of the microenvironment to the acquisition of cancer hallmarks. Each cell component from the stroma has a particular role in the gain of the different hallmark capabilities by the cancer cells. Adapted from Hanahan 2011.[4]

When tissues are wounded, fibroblasts, which are in a quiescent state, undergo activation turning into myofibroblasts and thus gaining tissue-remodeling capacity. Contrariwise, following the wound healing, myofibroblasts undergo programmed cell death. Through not yet fully clarified mechanisms, cancer is also able to activate the fibroblasts-mediated healing program fueling myofibroblasts in the TME. These cells, in the cancer-context called CAFs, are the tumor stroma major components and share with myofibroblasts similarities, such as the expression of α-smooth muscle actin. However, opposing to myofibroblasts, CAFs are not removed from the TME by apoptosis, thus accumulating there. The role of CAFs in tumor progression is a well-accepted concept and many links were established between their activity and the gain of the hallmark capabilities by the cancer cells. Evidence has shown that cancer cells can induce and maintain the activated phenotype in CAFs which, in turn, produce growth factors and cytokines that sustain tumor progression by promoting cell proliferation, ECM remodeling, angiogenesis and epithelial-to-mesenchymal transition (EMT).[5,24–29]

It was initially thought that tumor infiltration by immune cells was associated with the destruction of tumor cells, reduction of the tumor burden, and consequently, improved clinical prognosis. However, a significant number of studies have shown that the increased number of infiltrated immune cells in tumors actually helps to promote tumors' progression and invasion. In fact, it has been observed that cancer cells are able to recruit and manipulate immune cells, so they release factors that sustain tumor growth instead of the activating tumors' suppressive pathways.[5,26]

The immune cell types that infiltrate tumors are diverse and each one seems to play a distinct role in the tumorigenic process. Some of the most important immune players helping the tumorigenic process are the tumor-associated macrophages (TAMs), which are the most abundant immune cells in the TME. TAMs, similarly to CAFs, interact with a wide range of growth factors, cytokines and chemokines present in the tumor microenvironment, which will educate TAMs to become tumor-associated. Regarding their function, it is well established that TAMs are major contributors of tumors' angiogenesis, as they are highly attracted to hypoxic and necrotic areas of tumors and powerful sources of pro-angiogenic factors.[5,26,30–32]

Other important immune players are the dendritic cells. Normally, these cells work as antigen-presenters, thus stimulating the immune response in infection and cancer. However, in some cancer contexts, tumor-coopted dendritic cells were found to impair the T-cell response against tumors, and consequently compromise the all-body immune response ability.[5,26,33,34]

Regarding B cells, they are normally found in the invasive margin of tumors where they seem to inhibit immune system's response. Nonetheless, contradictory reports in the literature indicate that these cells may also be associated with a good prognosis in other tumors. In fact, the regulatory B cells were shown by different groups to be able to inhibit other immune cells through cytokines' secretion and antigen presentation, depending their action upon the tumor type and context.[34,35]

Finally, the angiogenic vascular cells are the stroma cellular components that mediate tumor angiogenesis, a crucial step in the carcinogenic process as it directly affects tumor growth. Many soluble factors present in the TME such as vascular endothelial growth factor, fibroblast growth factor and some chemokines, for instance released by CAFs, immune cells and/or cancer cells, stimulate the endothelial cells and their associated pericytes to proliferate. Mechanically, the normally quiescent blood vessel cells sense the angiogenic signals released by the malignant or inflammatory cells in response to hypoxia and activate a series of intracellular communication pathways that culminate in the sprouting of the pre-existing vessels. The new blood vessels formed, however, are generally very heterogeneous and tend to establish chaotic network structures, frequently leaky and with uneven lumens.[4,5,22,26,36–38]

Shortly, over the past years the contribution of the different cell populations of TME to the acquisition of the various hallmark capabilities by the cancer cells was established. These contributions were shown to depend on an intricate intercellular communication involving the cancer cells and the TME, which is driven by a complex and dynamic network established from cellular secreted factors such as cytokines, chemokines and growth factors, and also from the inflammatory response and action of matrix remodeling enzymes. Additionally, all of the major perturbations to the physical and chemical properties of the tissue also helps to complexity of this intercellular communication network. Thus, the comprehension of this network urges as it is believed to lead to the understanding of tumors' initiation and progression, and to open a novel range of therapeutic strategies that will increase the cancer patients' outcome.[5,22]

Chapter 3
Paracrine Communication as a source of CSCs

3.1. Cancer stem cells biology and origins

The idea that a small population of cells maintains tumors is an old hint proposed back in the XIX century. Nevertheless, it was only in 1994 that it became a recognized theory in the cancer field with the discovery of specific markers that allowed the isolation of CSCs from tumor samples of acute myeloid leukemia. From then on, several other markers were identified in different tumors, allowing the the isolation and characterization of this small cellular population from virtually all human malignancies.[15]

As previously stated, CSCs are a subpopulation of cells that share some similarities with normal stem cells. Shortly, both cells have self-renew ability conferred by their symmetric division capacity, unlimited proliferation potential and the aptitude to generate a diverse progeny of differentiated cells through asymmetric division. So, they intervene in all tumor evolution steps by generating the majority of the cellular populations within the tumor.[15,19,39,40]

The origin of these cells is controversial, and many hypotheses have been proposed (Figure 8). Initially it was proposed that the CSCs result from the transformation of endogenous normal tissue stem cells due to mutations that overactivate the self-renewal mechanisms. However, evidence suggested that CSCs may also result from restricted progenitors cells due to the acquisition of similar genetic changes.[32,41–43]

The work of Takahashi and Yamanaka showing that it is possible to induce pluripotent characteristics in differentiated cells by genetic manipulation, challenged researchers' perspective on CSCs' and a new theory was proposed. Accordingly, CSCs may result from dedifferentiation of differentiated tumor cells perhaps by the signaling of TME cues.[19,41,44–46] This dedifferentiation theory was further supported by evidence suggesting the EMT, a process by which an epithelial cell gains a mesenchymal phenotype, as a possible step in the route

to CSCs' formation. Furthermore, confirmation that stromal cells could actually induce dedifferentiation of the tumor cells by the action of several secreted factors associated with the EMT program further cemented this hypothesis.[43,45,47]

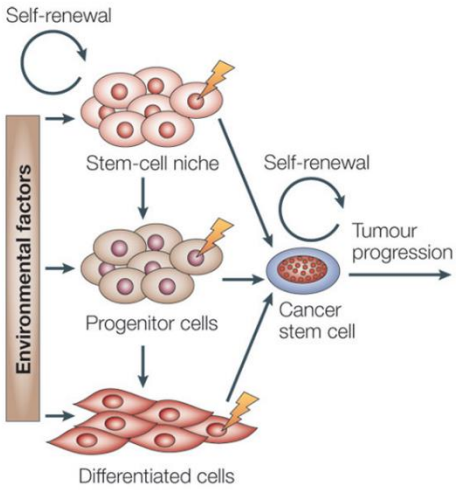


Figure 8 – Cancer stem cells' origin theories. CSCs can result either from stem cells or already compromised progenitor cells. There is also evidence that they may be obtained through dedifferentiation of differentiated cells, in a process potentially mediated by the microenvironment. Adapted from Bjerkvig 2015.[98]

3.2. Cancer stem cells' therapeutic implications

The CSCs' subpopulation has a strong impact in tumors' architecture and behaviour, as it is believed to be the responsible for tumors' therapeutic resistance and relapse (Figure 9). In fact, CSCs are tumorigenic and invasive cells that resist to the current therapies, surviving them, and subsequently, regenerating a new tumor eventually more aggressive than the initial one.[15,16,19]

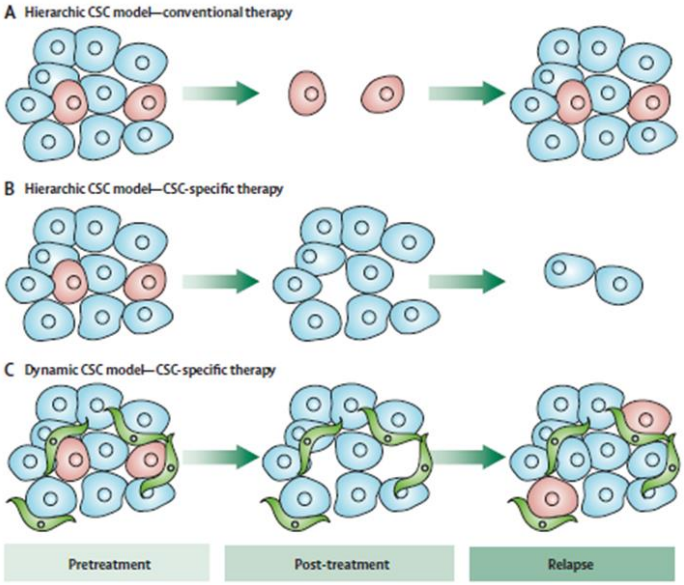


Figure 9 – Therapy resistance in the cancer stem cells' model. The CSCs' subpopulation is thought to be the responsible for tumors' relapse. In fact, CSCs survive the current therapies, and subsequently regenerate a new tumor similar to the primary tumor. Targeted therapy aimed against CSCs is also not effective as the microenvironment can modulate the emergence of a new CSCs pool. Adapted from Vermeulen 2012.[19]

The current cancer treatment protocols aim to block the proliferation potential of cancer cells and/or to prevent them of acquiring metastatic ability. As a consequence, only the bulk of the fast dividing tumor cells is eliminated and the quiescent CSCs are left untouched. Albeit these protocols succeed in shrinking tumors' size, in the majority of the cases they do not attain disease' eradication and, consequently, do not improve patients' survival outcome, as tumor CSCs-mediated relapse is a frequent result.[48,49] More dangerously, recent

studies have shown that the leftovers of these therapeutic protocols may form new tumors considerably more refractory than their progenitors to those and other therapeutic schemes, which invariably results in patients' death.[14,19,49–52]

Conversely, targeting the CSCs alone rather than the rapidly dividing cells in the tumor was hypothesized to be an effective approach to cure cancer, as hypothetically the remaining cells would not have the capacity to maintain the tumor. However, targeting CSCs is difficult, as these cells are very resistant and no specific drugs and/or therapeutic strategies have yet been developed or found. Moreover, these cells do not have the same proliferative activity as the differentiated tumor cells and are protected in their distinctive niches deep inside the tumors, which makes them refractory to the conventional antiproliferative drugs.[19,50] Nonetheless, pivotal studies with salinomycin have shown that by targeting specifically the population of CSCs tumor relapse still occurs as a new CSCs population is replenished by dedifferentiation of the differentiated malignant cells.[53]

Current knowledge argues that an effective therapy against solid tumors needs to target concomitantly the CSCs subpopulation, their niches/the TME, and the bulk of fast-dividing tumor cells.[15,16,19]

3.3. Dedifferentiation: a role of cytokines

In an attempt to create a system that allowed the study of hexavalent chromium [Cr(VI)] carcinogenesis, our laboratory cultured the bronchial epithelial airway system 2B (BEAS-2B) cells at low density in the presence of 1.0 μM of Cr(VI), thus mimicking an occupational exposure to this carcinogenic agent. Following the malignant transformation of BEAS-2B cells into the RenG2 system, proved by the acquisition of tumorigenic potential[54], the RenG2 cells were derived using serial rounds of injections in immunocompromised mice. The two attained cellular systems were progressively more malignant then their progenitor and were named DRenG2 and DDRenG2. Upon several studies it became clear that a population of CSCs that formed somehow during the

derivation of the RenG2 cells was sustaining the malignant potential of the derivative systems. Hypothesizing that the mice subcutaneous microenvironment played a role in RenG2 cells' dedifferentiation, Transwell® co-cultures were established using RenG2 cells and primary cultures of syngeneic mouse stromal fibroblasts. The cultures were kept for 2 months, thus mimicking the conditions observed during the *in vivo* studies. After that sphere isolation assay proved the acquisition of CSCs-properties by yielding spheres from the isolated RenG2 cells. As the Transwell® co-culture system only allows paracrine communication between the bottom and upper compartments, the established hypothesis was that the driver of dedifferentiation was some soluble factor released by the fibroblasts in the bottom compartment. From the analysis of all the conditioned media extracted from the co-cultures, IL-6, G-CSF and Activin-A were established as the molecular mediators of the dedifferentiation process featuring RenG2 cells.

3.4. IL-6, G-CSF and Activin-A: an overview

IL-6 is a member of the interleukin family, which mediates various physiological mechanisms, being highly associated with the inflammatory response. For instance, it is involved in the differentiation of lymphocytes, can support cell proliferation and affects the apoptotic signaling.[55,56] The binding of IL-6 to its receptor leads to the activation of the Janus kinase, which then can stimulate several protein kinase cascades such as phosphatidylinositol-tri-phosphate kinase (PI3K) and mitogen activated protein kinase (MAPK), depending on the cell type.[55,56] Also, it can directly activate the signal transducers and activators of transcription (STAT) factors STAT1 and STAT3, which are responsible for the expression of different genes involved in cell growth and apoptosis' regulation.

Considering that IL-6 and its downstream targets are involved in the regulation of cellular proliferation, survival, and metabolism, it is not unexpected that this cytokine has also been associated in tumorigenesis.[57] However, the

involvement of this cytokine in cancer has been quite controversial, as opposite roles for it in both tumor-suppressive and tumor-promoting activities have been identified.[57,58] One example is the work of Chiu and collaborators showed that IL-6 induced apoptosis in estrogen receptor-positive breast cancer cell lines after continuous treatment, thus demonstrating that IL-6 have a tumor-suppressive role in these cell lines.[59] On other hand, the work of Korkaya and colleagues demonstrating that IL-6 acts as a direct regulator of breast CSCs' self-renewal, in a process mediated by STAT3 and Nuclear factor-κB (NF-κB) pathways' activation indicates IL-6 as a tumor-promoting effector.[60] NF-κB pathway activation in the TME is known to induce the production of various cytokines that, in turn, activate other carcinogenic pathways in cancer cells to stimulate their survival and proliferation and to enhance the production of other cytokines and chemokines that recruit supporting cells to the tumor bed. Nevertheless, whether any paracrine signals activated by NF-κB contribute to self-renewal of CSCs and/or their dedifferentiation is still uncovered.[61,62]

Also, Sethi and collaborators showed that IL-6-mediated Jagged1(JAG1)-Notch1 pathway activation promotes breast cancer metastasis to bone by interfering the tumors' CSCs.[60,63] Notch signaling pathway plays an important role in cell-cell communication and is also a very important regulation pathway of the early embryonic development, cellular proliferation, differentiation, and apoptosis. In CSCs' biology, however, its role has yet to be fully demonstrated but it is plausible that stromal-mediated paracrine Notch activation may sustain CSCs' pool and renewal, thereby reinforcing the notion that TME supports tumor progression. In support of this theory, Zhang and collaborators demonstrated that this signaling pathway drives the formation and proliferation of stem cell-like populations in human gliomas.[64]

Evidence suggests that the involvement of Notch in CSCs physiology may reside on its ability to induce EMT, and thus, to form CSCs. Although Notch signaling has the same activation requirements independently of the cell type, is still unknown if the expression of Notch ligands in CSCs and non-CSCs, in different cancer etiologies, is regulated by distinct pathways. Specifically, there

is evidence that links the NF-κB pathway activity with the Notch pathway, as IL-6/STAT3 pathway has been shown to induce JAG1 expression and promote malignant stem cell growth.[18,47,61,65–67]

Activin-A is a dimeric protein belonging to the transforming growth factor-β (TGF-β) family.[68] This protein family has a wide range of biological effects at a cellular level controlling multiple aspects of cellular growth and developmental differentiation. The growth regulatory effects of ligands within this superfamily involve tumor suppression, as for instance they are capable of inhibiting epithelial cell proliferation, However, cell growth can become deregulated either by acquired resistance to the ligands regulatory effects or by dual action of the same ligands which in turn can drive oncogenic progression and metastasis.[68]

Activins exert their biological effects through activation of transmembrane receptors, which leads to the phosphorylation of the receptor-Smad proteins SMAD2 and SMAD3.[69,70] In addition to this canonical Smad pathway, other signaling pathways can be activated by Activin, namely the extracellular-signal-regulated kinase (ERK), p38 (MAPK14) and MAPK pathway.[68,71,72] Intriguingly, depending on the tissue type, Activin seems to exert either pro- or anti-tumorigenic effects. For instance, Antsiferova and Risbridger showed that Activins have a growth-inhibitory effect on breast, liver, prostate and pancreatic carcinoma cells, while Do and colleagues demonstrated that exerts the opposite effect in ovary tumors. In addition to a direct effect on tumor cells, several studies also indicate that Activin can also affect tumorigenesis by altering the TME.[71,73–75]

It has been shown that Activin-A is crucial for normal stem cells' maintenance as it drives the activation of SMAD-dependent transcription of pluripotency markers like Oct-4, Nanog, Nodal and several Nodal-signaling pathway regulators. Moreover, the Activin/Nodal pathway was also already implicated in the regulation, self-renewal and differentiation of CSCs, as well as in promoting the plasticity and metastatic potential of tumor cells.[76–79]

G-CSF is a glycoprotein produced by monocytes, mesothelial cells, fibroblasts, and endothelial cells, and its receptors are present on precursors and mature neutrophilic granulocytes, monocytes, platelets, and endothelial cells. At the myeloid progenitor cell level, G-CSF stimulates the growth of neutrophil granulocyte precursors. It also crucially regulates the survival of mature neutrophils by inhibition of apoptosis, in a process mediated by the activation of the JAK2/STAT3 and PI3K/Akt pathways and of the surface IL-6-like receptor. This activation, in turn, leads to JAK2 and STAT3 proteins' activation, dimerization and migration to the nucleus, where they affect the expression of important genes.[80–83]

3.5. Goals

Considering the findings that IL-6, Activin-A and G-CSF were major players in the dedifferentiation process behind the acquisition of stem-cell like phenotype our laboratory hypothesized that by blocking the intracellular signaling pathways activated by these molecules, the dedifferentiation process could be abrogated. If so, new therapeutic strategies may be developed to block CSCs' formation, hampering tumors' relapse and improving patients' outcome. So, the present study aimed to block IL-6, G-CSF and Activin-A intracellular signaling pathways in order to confirm their role in the dedifferentiation process driving to CSCs' formation.

PART II

Laboratory Procedures

Chapter 4

Materials and Methods

4.1. Reagents, Solutions and Mediums

All reagents used for the development of this work were cell culture recommended and of analytical grade and were purchased from Sigma-Aldrich (Sintra, Portugal) unless specifically stated. Conversely, all cell culture media and supplements were acquired from Gibco (Life Technologies, Grand Island, NY, USA) unless otherwise stated. The antibodies used were attained from R&D Systems (Minneapolis, MN, USA). Ultrapure water (milli-Q) was used any time water was needed, more precisely for the preparation of aqueous solutions. The plastics used for cell culture were attained from either Corning (Lowell, MA, USA) or SPL Life Sciences (Eumhyeon-ri, Korea).

4.1.1. 1x Phosphate buffered solution (PBS) solution

The 1x PBS solution (P3813) was obtained by reconstituting each powder pouch in 1 L of water, attaining a final 0.01 M phosphate buffered saline solution (0.138 M NaCl and 0.0027 M KCl) at pH 7.4. This solution was sterilized prior to use by autoclaving.

4.1.2. 2 % (w/v) Bovine serum albumin (BSA) solution

The 2 % BSA solution was attained by dissolving 2 g of BSA in 100 mL of water, under gentle stirring in a magnetic stirrer. Subsequent sterilization was attained by syringe filtration (0.2 µm pore size).

4.1.3. 2 % (w/v) Gelatin solution

This solution was obtained by diluting 2 g of bovine skin gelatin in 100 mL of water. The solution was gently stirred in a magnetic stirrer to ensure the complete dissolution of the gelatin. In order to help the dissolving process the temperature was increased to 37 ºC. Before its use the solution was autoclaved in order to ensure sterilization.

4.1.4. Gelatin coating solution

The gelatin coating solution was prepared by diluting the 2 % gelatin solution in 45 % 1x PBS and 5 % of 2 % BSA solution.

4.1.5. DMEM cell culture medium supplemented with 10 % FBS

500 mL of this medium were prepared by mixing 445 mL of DMEM cell culture medium (Biochrom, Berlin, Germany) with 5 mL of penicillin (10000 U/mL)-streptomycin (10 mg/mL)-amphotericin B (25 µg/mL) and 50 mL of fetal bovine serum (FBS) (Biochrom, Berlin, Germany).

4.1.6. 1 mM Progesterone solution

This solution was prepared by dissolving 0.003 g of progesterone in 1 mL of water, thus attaining a stock solution of 10 mM. The final 1 mM solution was obtained by mixing 100 µL of the initial stock solution with 900 µL of water.

4.1.7. 2 % Methylcellulose solution

300 mL of this solution were prepared by dissolving 6 g of methylcellulose in 100 mL of 80 ºC pre-warmed water, under continuous mild stirring. After the cellulose particles were dispersed, the remaining 200 mL of 4 ºC chilled water were added. The solution temperature was then brought to around 4 ºC, by

placing the solution's flask on ice. When the solution reached the desired temperature, agitation was kept for more 30 min in order to guarantee the complete dissolution of the methylcellulose particles. Autoclaving was used as a mean of sterilization.

4.1.8. CSCs' Isolation medium

The liquid phase was prepared by mixing 125 mL of DMEM medium (Biochrom, Berlin, Germany) with 125 mL of Ham's F12 medium (Biochrom, Berlin, Germany), 5 mL of penicillin (10000 U/mL)-streptomycin (10 mg/mL)-amphotericin B (25 µg/mL) solution, 10 µL of 1 mM progesterone solution and 5 mL of the commercialized insulin, transferrin, selenium (ITS) sodium pyruvate solution. Furthermore, 0.6 g of sodium bicarbonate ($NaHCO_3$) and 0.008 g of putrescine were dissolved in a small volume of either DMEM or Ham's F12 medium total volume and subsequently syringe sterilized (0.2 µm pore size) prior addition to the final liquid phase. Finally, the 2 % methylcellulose solution was used to perform the final volume of 500 mL.

4.1.9. Preparation of low adherence 6-well plates for CSCs' Isolation

As low adherence conditions were needed for the isolation of CSCs, low-adherence supports were attained by coating 6-well plates with a 2 % poly-(2-hydroxyethyl methacrylate) (poly-HEMA) solution.

In order to prepare the coating solution, 2 g of poly-HEMA were added to 100 mL of 95 % ethanol. The polymer was allowed to dissolve under mild agitation during 8 h at room temperature. After complete dissolution, 6 well-plates were coated with 0.4 mL per well of the polymer solution and were allowed to dry at room temperature over a stable bench. The plates were then sterilized by exposure to UV light in a flow chamber for 20 min. Whenever the plates were not immediately used, each one was sealed with Parafilm® and stored at 4 ºC.

4.1.10. CSCs' propagation medium

500 mL of the CSCs' propagation medium were prepared by mixing 245 mL of DMEM medium with 245 mL of Ham's F12 medium. To the mixture it was added 5 mL of penicillin (10000 U/mL)-streptomycin (10 mg/mL)-amphotericin B (25 µg/mL), 10 µL of 1 mM progesterone solution and 5 mL of the commercialized insulin, transferrin, selenium (ITS) sodium pyruvate solution. Furthermore, 0.6 g of sodium bicarbonate ($NaHCO_3$) and 0.008 g of putrescine were dissolved in a small volume of either DMEM or Ham's F12 medium and syringe sterilized (0.2 µm pore size) prior addition to the final volume.

4.1.11. Freezing solution

The solution used to freeze cells was attained by mixing cell culture medium:FBS:dimethyl sulfoxide (DMSO) at the proportion of 7:2:1.

4.1.12. 0.2 mg/mL GW4869 solution

For this solution's preparation 0.002 mg of GW4869 were dissolved in 10 mL of DMSO.

4.1.13. 50 mg/mL Xyloside solution

In order to attain this solution 0.1 g of xyloside were dissolved in 2 mL of methanol.

4.1.15. Antibodies' stocks

The blocking antibodies against IL-6 (MAB206), G-CSF (MAB214) and Activin A (MAB3381) were reconstituted following manufacturer's instructions in a 1x PBS solution to attain initial stock solutions of 0.5 mg/mL. For cell culture

use, 0.01 mg/mL solutions were prepared by mixing 20 µL of each stock solution with 980 µL of 1x PBS.

4.2. Cells and Cell Culture Procedures

The cell lines used in this project were attained using original protocols optimized in previous group works.

4.2.1. RenG2 cell line

The RenG2 cell line was developed under the scope of the group's previous projects. It resulted from the low-density culture of the BEAS-2B cell line (ECCAC, Salisbury, UK; ECCAC no. 95102433) in the presence of 1.0 µM of hexavalent chromium [Cr(VI)], thus mimicking an occupational exposure to this carcinogenic agent. The RenG2 cell line is a malignant cellular system, as proved by its ability to induce tumors in immunosuppressed mice.[54]

RenG2 cells were unfrozen from stock vials and plated in T_{75} cm^2 cell culture flasks pre-coated with 1 % gelatin and containing 15 mL of 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 the desired confluence was reached, the cell culture medium was discarded and the flask was washed with 5 mL of 1x PBS to remove any remnants. 1 mL of trypsin (Biochrom, Berlin, Germany) was then added to the flask in order to promote cellular detachment from the matrix. When the flask surface was fully covered with trypsin, the excess was removed 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 and subsequently centrifuged for 5 min at 1500 rpm (ROTOFIX 32 A, Hettich Lab Technology, Germany). Following the discharge of the supernatant, the pellet was ressuspended in an appropriate volume of medium, generally 1 mL, to be counted and used in the preparation of another flask.

4.2.2. Human bronchial fibroblasts primary cell line (E2A)

Bronchial tissue biopsies, obtained with patients' written consent, were washed several times with 1x PBS and small pieces obtained from the internal bronchial region were distributed throughout the basis of a T25 cell culture flask. A small drop of FBS was added to each one of the small fragments in order to help them attach to the plastic surface of the flask and also to provide nutrients. The flask was then turned upside-down and 5 mL of DMEM cell culture medium supplemented with 10 % FBS were added to the top surface of the flask. The fragments were allowed to attach upside-down for 24 h in the incubator and after that period the flask was gently turned to the normal position, allowing cells to contact with the medium. In the following days, cells started to slowly detach from the tissue fragments, forming a monolayer of cells in the bottom of the culture flask. When the cells covered almost all the flask's surface, they were detached with trypsin and subcultured in DMEM medium supplemented with 10 % FBS, as mentioned above.

4.3. Co-culture of RenG2 epithelial cells with the E2A fibroblasts

E2A cells were cultured in a 6-well plate to until nearly 80 % confluence was reached. Then each well was equipped with a 4.52 cm^2 Transwell® insert containing a 0.4 µm pore size membrane, above which RenG2 cells were seeded at an initial density of 4000 cells/cm^2, as represented in Figure 10. The co-cultures were kept in the incubator for 8 weeks and the media were changed every 2 weeks.

In order to test the effect of blocking either exosome release/uptake or the free cytokines on the conditioned media, different reagents and blocking antibodies were added to the co-cultures' media. The final concentrations used for both the exosomes' release blocker, GW4869, and for the exosomes' uptake blocker, xyloside, were 0.02 and 2.5 mM, respectively, and these concentrations were established according to the literature research. The blocking antibodies and follistatin final concentrations in the co-culture media were establish based

on the neutralization range given by the manufacturer and considering the amount of cytokines present in the medium, which was determined in group's previous works. Concretely, the final concentration of anti-IL-6, anti-Activin-A, anti-G-CSF added to the co-culture media were 0.6, 0.144 and 0.272 µg/mL, respectively. In the case of the blocking antibodies, co-cultures were established using the antibodies alone and combinations of two or three antibodies. Control co-cultures were also established using each of the carrier vehicles of the added compounds, namely DMSO, methanol, 1x PBS and 1x-PBS-0.1%BSA.

4.4. Sphere-forming assay

In order to ascertain the effect of the different blocking agents and neutralizing antibodies in the CSCs' formation ability, the sphere-formation assay was used. The advantage of this assay is that it takes advantage of the ability of CSCs to form tridimensional spheres when cultured under low-adherence conditions with the appropriate media and supplements, allowing the isolation of this specific cell population.

This assay made use of the low-adherence plates prepared according to the protocol described in section 4.1.9, using the CSCs' isolation medium (section 4.1.8). The tested cells were the RenG2 cells recovered from the upper chamber of the co-culture system. To this end cellular suspensions containing $3x10^4$ cells/mL were prepared in CSCs' isolation medium and 2 mL of this suspension were added to each well of the low-adherence plates. The isolation medium was supplemented with 10 ng/mL of both basic fibroblast growth factor (bFGF) (PeproTech, London) and human epidermal growth factor (EGF) and the cells were allowed to grow under normal conditions. Every two days, supplements' concentration was replaced.

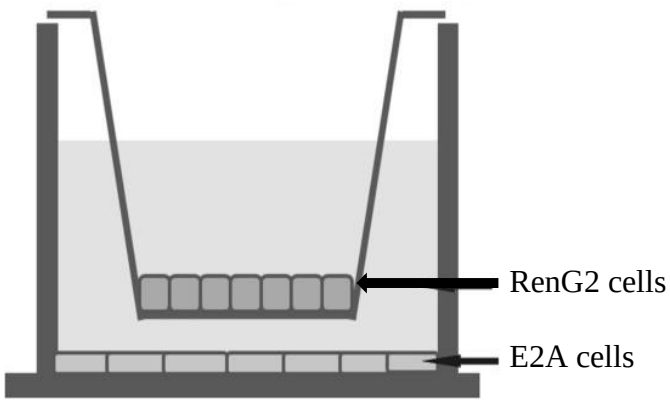


Figure 10 – Co-culture design. RenG2 and E2A cells were co-cultured for 8 weeks using 6-well plates with Transwells®. E2a cells were cultured in DMEM supplemented with 10 % FBS in the lower compartment, while RenG2 cells were plated in the upper compartment in LHC-9 medium. The different blocking agents and antibodies were added to the co-culture medium in their appropriate final concentrations. Adapted from Snyder-Talkington, 2013.[99]

PART III

Experimental Results, Discussion and Future Perspectives

Chapter 5

Dedifferentiation: finding the culprits

5.1. Introduction

CSCs display some stem-like properties and are the responsible for tumor relapse after treatment. Their origin is still controversial, but evidence suggests that they can arise from terminally differentiated cells due to a dedifferentiation process. According to the dynamic CSCs model, this process can be mediated by soluble factors released by the TME.[17,19]

There are many ways through which cells can communicate. However, the paracrine communication is one of the most important and used strategies, and is based on the interaction of cells-released soluble factors with other cells membrane-anchored receptors. Some of the crosstalk mediators are cytokines, a large group of molecules that are able to activate different cellular receptors and consequently different signaling pathways. Moreover, they are also able to convey a wide range of cellular responses by modulating changes in cells' protein expression patterns. Cytokines can be released by the cells either as free soluble factors or incorporated in microvesicles such as exosomes.[84,85]

The aim of this study was to uncover the involvement of each the three previously identified cytokines in the dedifferentiation of the malignant RenG2 cells. To this end, the Transwell® co-culture system, which only allows paracrine communication between the two cellular compartments, was again used; however, exosome release and/or uptake agents or specific neutralizing antibodies were added to the cell culture media so cytokines'-mediated paracrine communication was controlled.

5.2 Results

5.2.1. Blockage of the exosomes' release and/or uptake

Considering the hypothesis proposing that RenG2 cells' dedifferentiation was driven by exosome-mediated cytokines' release, the first experiment of the present worked aimed to block exosomes'-mediated communication. To this end the commercially available exosome release-blocking agent GW4869 was used. GW4869 is a non-competitive selective inhibitor of neutral sphingomyelinase (N-SMase), which is a key enzyme in sphingomyelin metabolism. N-SMase activity produces ceramide and phosphocholine, which have important roles in cell signalling and regulation. Moreover, ceramide is highly involved in the biogenesis of exosomes, being present in their membranes. Trajkovic and collaborators showed that by blocking N-SMases' activity, the release of exosomes was reduced.[86]

According to the literature, GW4869 is most frequently used in 10 or 20 µM concentrations. In the present work it was decided to use it in the co-culture system at a 20 µM final concentration, as it was the one showing a higher blocking effect. Moreover, the inhibitor was prepared in DMSO, following manufacturer's recommendations and a control co-culture containing only the vehicle was kept in parallel by adding 150 µL of DMSO to the total co-culture media[87–89]. Of special note is the fact that soon after GW4869 or DMSO administration, extensive cell death was observed in both cellular compartments and the colour of media of these co-cultures was markedly different of the others. Confirming these observations were the results attained in the post-co-culture sphere-forming assay performed in the very few RenG2 cells isolated from the upper compartment, that showed no ability form spheres (Figure 11B and C) in comparison to the control co-culture-resulting cells (Figure 11A).

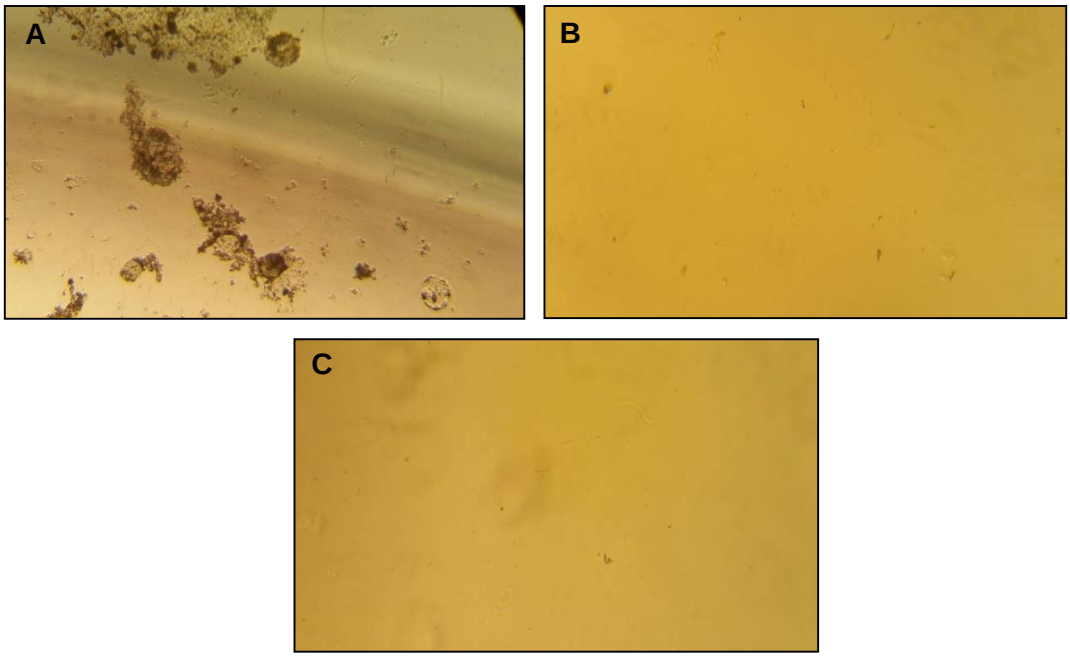


Figure 11 – Representative images of the spheres formed by RenG2 cells after co-culture in the presence of GW4869. A, control co-culture. B, co-culture to which only DMSO, the GW4869 carrier vehicle, was added to media. C, co-culture performed in the presence of GW4869. A magnification of 100x was used in all the panels (PrimoVert, Carl Zeiss Microscopy GmbH, Germany).

Another strategy implemented to block exosomes'-mediated responses was the use of an exosome-uptake blocker, in this case xyloside. Xyloside is a small hydrophobic compound that inhibits proteoglycans' biosynthesis; however, its use as an exosome-uptake blocker agent is quite recent, so only a few studies in the literature document it. The concentration of xyloside used in the present study was the same as Christianson and colleagues used to show that the blockage of proteoglycans' synthesis with agents as xyloside reduced exosomes' cellular uptake, meaning 2.5 μM, as their results showed strong consistency and no additional information was found in the literature.[27]

The co-cultures to which xyloside was added showed a reduced cell proliferation in the upper compartment, when compared to the normal control co-cultures. Moreover, the fibroblast population was also affected since a high rate of cell death was also observed in this compartment. On contrary, the co-culture to which only the vehicle of xyloside, methanol, was added in the same

volume as in the xyloside containing co-cultures, showed a cellular proliferation similar to that of the control co-cultures. After isolation, the co-cultured RenG2 cells showed ability to form spheres when subjected to the sphere-forming assay, however the attained spheres in co-cultures to which xyloside was added were small and few in relation to both controls (Figure 12B and C).

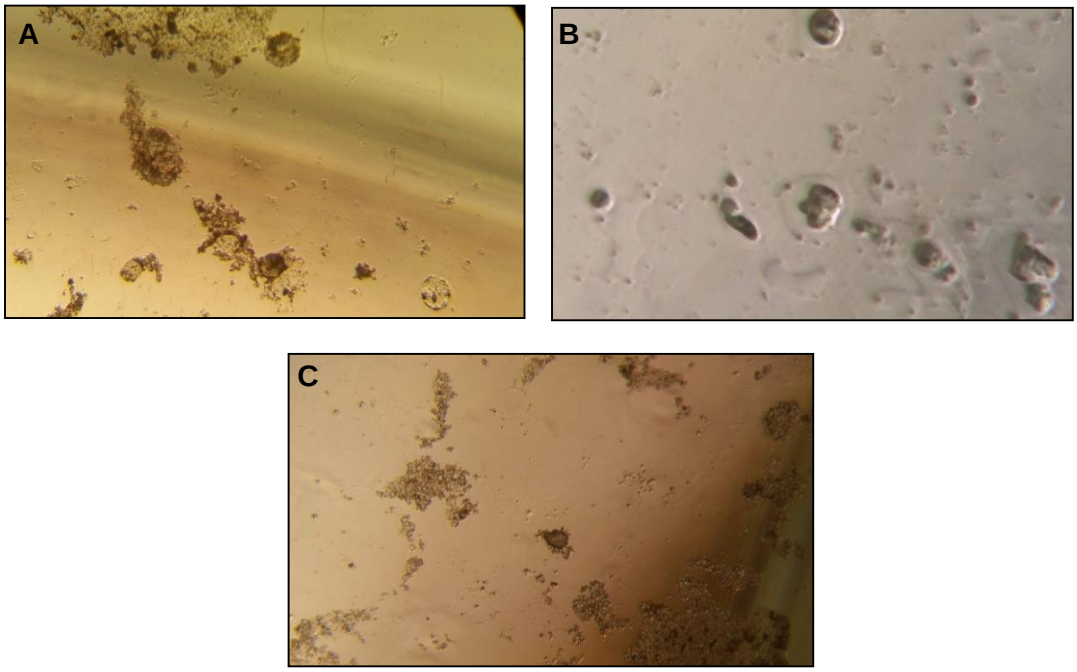


Figure 12 – Representative images of the spheres formed by RenG2 cells after co-culture in the presence of xyloside. A, control co-culture. B, co-culture to which only methanol, the xyloside carrier vehicle, was added to media. C, co-culture performed in the presence of xyloside. A magnification of 100x was used in all the panels (PrimoVert, Carl Zeiss Microscopy GmbH, Germany).

5.2.2. Neutralizing antibodies against cytokines

To guard for the hypothesis that the cytokines' release was not exosome-mediated, direct target of the principal intracellular signalling pathways activated by these cytokines was performed. To that end, neutralizing antibodies against IL-6, G-CSF and Activin-A were used to scavenge cytokines from the co-cultures' media, either alone or in combinations of two or three antibodies. The antibodies' concentrations used were established considering the cytokines' concentrations in the co-cultures conditioned media previously determined in the laboratory through Bioplex® multiplex cytokine array (BioRad, Hercules, California, United States) and ELISA (5000 pg/mL for IL-6, 300 pg/mL for G-CSF and 17000 pg/mL for Activin-A as depicted in Appendix A), and the antibodies' neutralization range provided by the manufacturer. This way, the final concentrations used for IL-6, Activin-A and G-CSF were 0.6, 0.144 and 0.272 µg/mL, respectively. As the antibodies were reconstituted in PBS, co-cultures to which only the PBS was added were also performed in parallel as controls.

The results of the sphere-forming assay applied to the RenG2 cells isolated from the co-cultures to which only PBS was added showed that these cells acquired stem potential, thus yielding spheres similar in size, number and shape to that of the control co-cultures (Figure 13A and B). Moreover, the co-cultures to which only one of the neutralizing antibodies was added, independently of the antibody, also yielded spheres (Figure 15C, D and E), as did the co-cultures in which both Activin-A and G-CSF were neutralized (Figure 14C). In fact, when only the IL-6 was present the attained spheres were bigger more abundant than those formed in both the control co-cultures and the co-cultures in which only one of the cytokines was neutralized. On contrary, when both IL-6 and G-CSF were neutralized, the attained spheres were small and few (Figure 14D); and when both Activin-A and IL-6 (Figure 14E) or all three cytokines (Figure 13C) were neutralized, no spheres were attained.

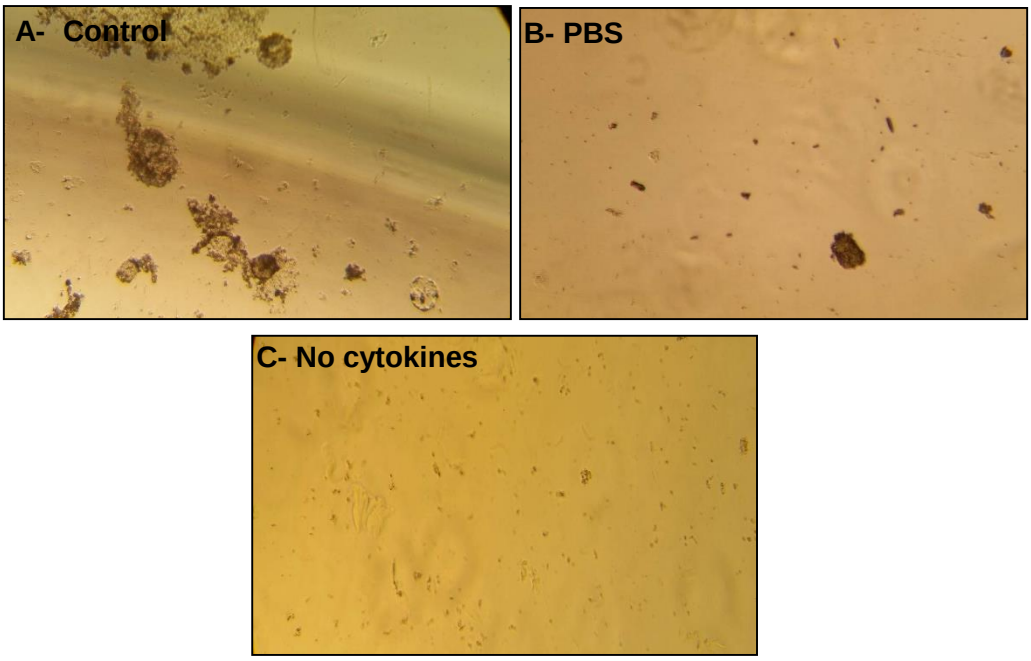


Figure 13 – Representative images of the spheres formed by RenG2 cells after co-culture with all of the cytokines in study scavenged. A, control co-culture. B, co-culture to which only PBS, the neutralizing antibodies' carrier vehicle, was added to media. C, co-cultured where the three cytokines were neutralized together. A magnification of 100x was used in all the panels (PrimoVert, Carl Zeiss Microscopy GmbH, Germany).

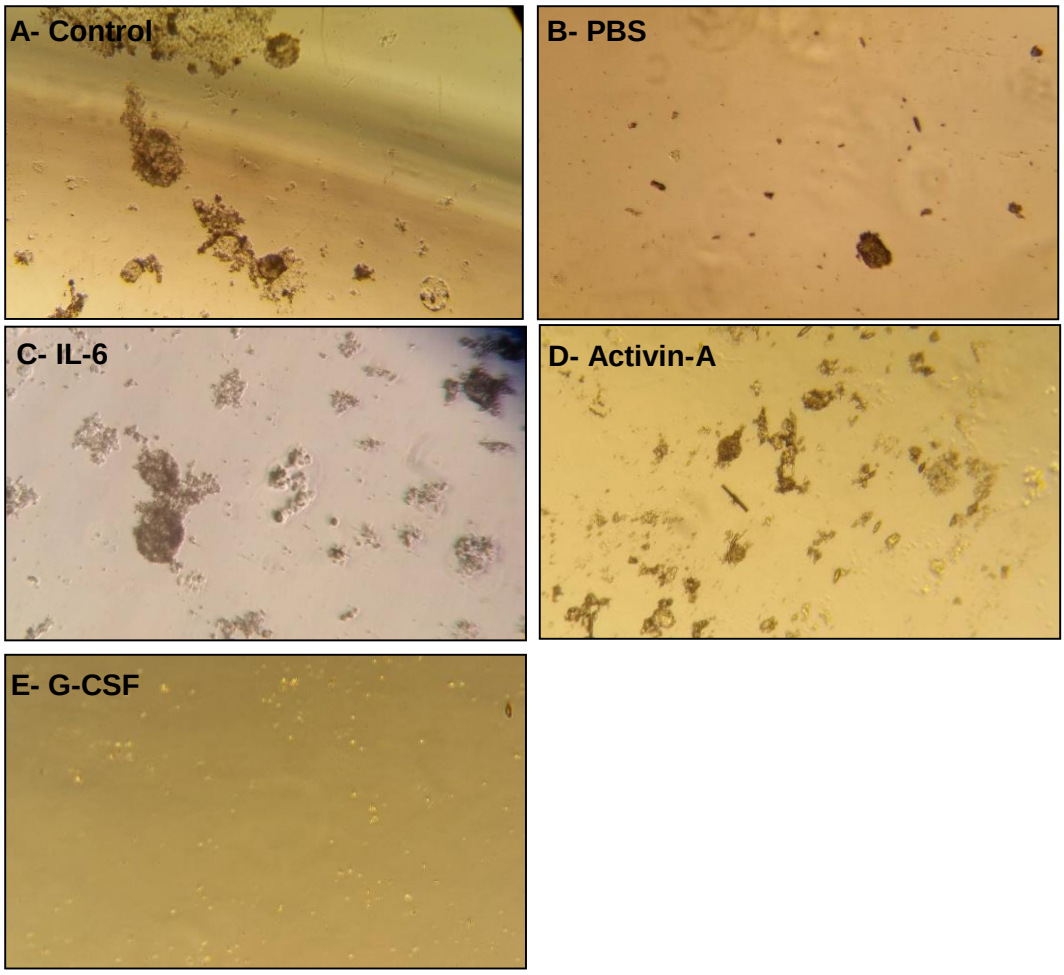


Figure 14 – Representative images of the spheres formed by RenG2 cells after co-culture with two of the three cytokines in study scavenged. A, control co-culture. B, co-culture to which only PBS, the neutralizing antibodies' carrier vehicle, was added to media. C, co-culture where both Activin-A and G-CSF were specifically neutralized together. D, co-culture where IL-6 and G-CSF were specifically neutralized together. E, co-culture where Activin-A and IL-6 were specifically neutralized together. For a better interpretation of the results, in the C, D and E panels the cytokines' name present in the media was depicted in the image. A magnification of 100x was used in all the panels (PrimoVert, Carl Zeiss Microscopy GmbH, Germany).

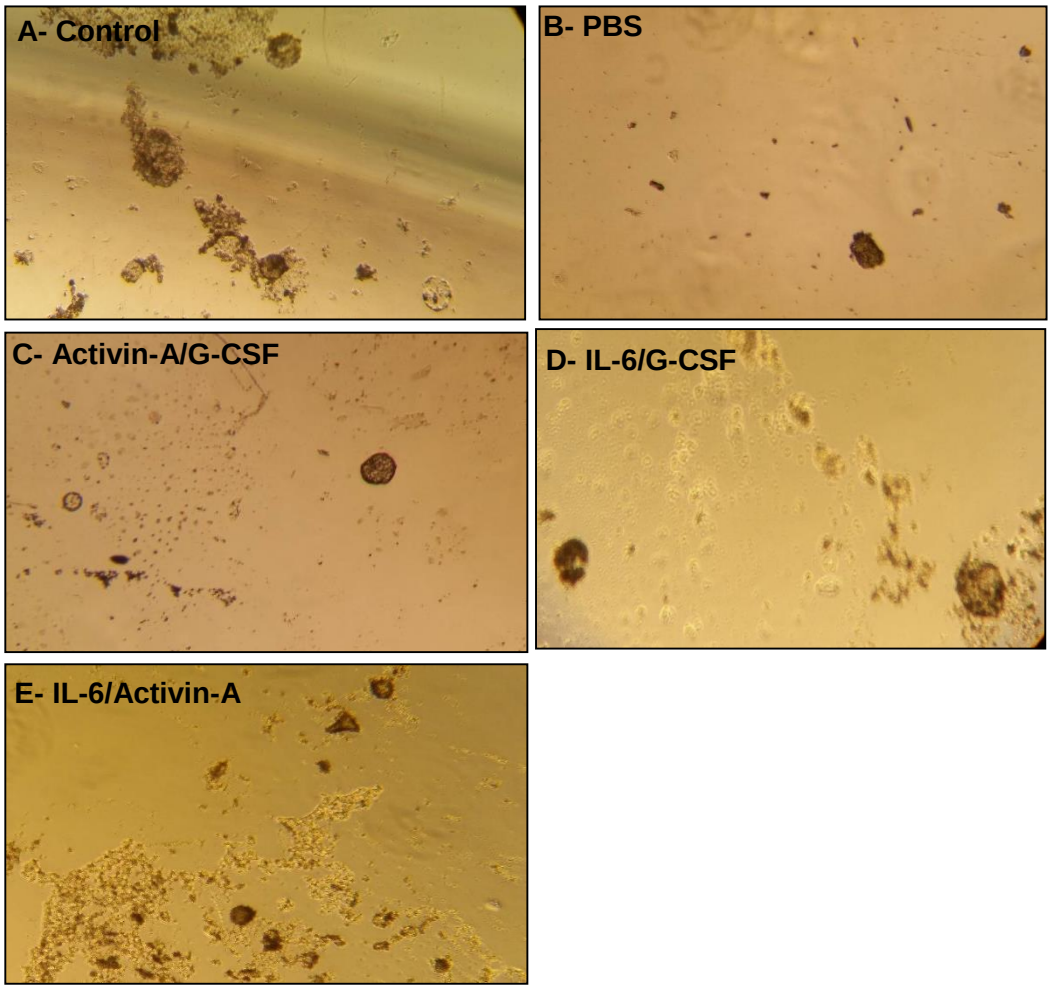


Figure 15 – Representative images of the spheres formed by RenG2 cells after co-culture with only one of the cytokines in study scavenged. A, control co-culture. B, co-culture to which only PBS, the neutralizing antibodies' carrier vehicle, was added to media. C, D and E, co-cultures where only IL-6, Activin-A or G-CSF were specifically neutralized. For a better interpretation of the results, in the C, D and E panels the cytokines' name present in the media was depicted in the image. A magnification of 100x was used in all the panels (PrimoVert, Carl Zeiss Microscopy GmbH, Germany).

5.3. Discussion

The intercellular crosstalk is an important process that is responsible for the maintenance of tissues' function and architecture, and thus for body's homeostasis. It is established throughout the entire body and uses wide panoply of biological and chemical mediators and receptors. Not surprisingly, it is deeply affected in carcinogenesis, and has recently been implicated in the formation of CSCs by dedifferentiation. In fact, CSCs have long been identified in almost all tumor types, and their attainment from already differentiated cells is nowadays unquestionable. However, the mechanisms that drive this cellular reprogramming remain unclear.

In this line of thought, our group previously found that when the RenG2 cell line, a malignant cellular system without CSCs and thus, with no sphere-forming ability, acquired stem potential when exposed to human bronchial fibroblasts in co-culture for 8 weeks. This observation lead to the hypothesis that soluble factors released by the fibroblasts were the responsible for the development of the undifferentiated phenotype, and further investigation identified IL-6, G-CSF and Activin-A as the orchestrators of the dedifferentiation process. However, the individual potential of each cytokine and its sole contribution for the overall process still needed to be ascertained, as need the mechanism behind cytokines' release, and they constituted the main goals of the present work.

The first main conclusion to be taken from the attained results is that at least one of the previously identified cytokines is responsible for CSCs' emergence in the RenG2 system following co-culture with the human fibroblasts, since when all the three cytokines were scavenged no spheres were formed (Figure 13C). Further supporting this observation in the fact that the control co-cultures using only the carrier vehicle of the neutralizing antibodies depicted sphere-formation ability (Figure 13B).

Furthermore, the experiments with cytokines' neutralizing antibodies showed that only IL-6 and Activin-A seem to promote CSCs' formation, as spheres were attained from the RenG2 cells isolated from all of the co-cultures in which at least one of these two cytokines were present, as depicted in Figures

15C-E and 14C and D. The involvement of IL-6 released in CSCs' formation was not a major surprise as several reports in the literature implicate its involvement in the acquisition of stem cell-like proprieties by cancer cells in different cancer cell types. For instance, Zhu and collaborators demonstrated that the IL-6 released from bone marrow-derived myofibroblasts was able to induce stemness in gastric cancer cells due to the activation of STAT3 pathway.[90] Also, Sansone and colleagues found that breast cancer cells whose self-renewal capabilities were abrogated by hormonal therapy, regain their stemness after exposure to IL-6 and consequent activation of the Notch signaling pathway through STAT3.[91] Moreover, Thiagalingam's group found that by inhibiting periostin in basal-like breast cancer, an often CSCs-enriched tumor, these cells lose their capacity to induce the formation of mammospheres. More specifically, these authors observed the loss of the stem potential of basal cells is dependent upon the reduction of the IL-6 levels secreted by these cells after periostin inhibition, and consequently, upon reduced STAT3 activation, thus naming this cytokine responsible for the establishment and maintenance of a CSCs'-supportive niche.[92]

The implication of Activin-A as a potential inducer of dedifferentiation, on the other hand, was rather surprising as only a few reports in the literature present this cytokine as a possible direct mediator of CSCs' formation. Nevertheless, as Activin-A is a member of the TGF-β family of proteins, which in turn are strongly associated with the EMT process, its association with CSCs' biology is extremely plausible. In agreement, Miettinen and colleagues linked the activation of the TGF-β pathway in mouse mammary epithelial cells to the gain of mesenchymal characteristics, identified by a decrease in the expression of E-cadherin and an increase in the expression of fibronectin.[93] In fact, as discussed in Chapter 3, EMT is a cellular process that can be linked with the CSCs formation, and it comprises one of the hypotheses that support the dynamic CSCs model. Furthermore, supporting that EMT's activation can drive the dedifferentiation of tumor cells is the work of Mani and collaborators in which they showed that by inducing the EMT program in human mammary epithelial cells, these cells acquired mesenchymal traits and stem cell-like proprieties, as

illustrated by the acquisition of mammospheres-forming ability. Also, Knutson's group found that EMT induced by CD8 T cells can induce breast CSCs formation upon dedifferentiation of epithelial breast cancer cell supporting the idea that CSCs can arise from fully differentiated cells due to phenotype change induced by EMT.[45,94]

Regarding G-CSF, it seems that this cytokine is not able to drive the dedifferentiation process by itself, as its presence alone in the co-culture system was not enough to provide RenG2 cells' with the ability to form spheres (Figure 14E). Yet, by its presence in the co-culture media a role of this cytokine in regulation of CSCs was not discarded. In agreement, the co-cultures where G-CSF was present along with one of the other two dedifferentiation cytokines, IL-6 or Activin-A, showed more and bigger spheres then the same co-cultures promoted in the absence of G-CSF (Figure 15C and D), and this was particularly evident in the co-culture where only Activin A and G-CSF were present. Altogether these results prompted the hypothesis that G-CSF, somehow, helps to sustain CSCs' proprieties of previously developed CSCs' pools, which is in line with the results of Agarwal and colleagues in which they show that G-CSF sustain neuroblastoma CSCs' pool through a STAT3 mechanism.[95]

When IL-6 and Activin-A were the only active players present in the co-culture media, their capacity to drive dedifferentiation was evident. Furthermore, it seems that IL-6 is more potent in inducing CSCs' formation, as this cytokine yielded more and bigger spheres than Activin-A, when both cytokines were acting alone. This way, once both cytokines were able to drive dedifferentiation, it was somehow expected that when they were both present in the co-culture media, they might work cooperatively. Against expectations, the attained results for this experiment (Figure 15E) revealed less and smaller spheres than those in which each cytokine was solely present (Figure 14C and D). Moreover, the number of spheres in the IL-6/Activin-A both present condition was smaller than that from the IL-6 only condition, (Figure 14C), further suggesting that IL-6 is even more potent alone than acting together with Activin-A.

Combined, the abovementioned results suggest that despite the fact that Activin A is able to induce CSCs' formation, it seems that this cytokine may also

act as a differentiation inducer of the pre-formed CSCs. In agreement, whenever Activin A is present the number of spheres is always minor than when it is not present, exception made to the situation where this cytokine is present. Corroborating this hypothesis are several reports in the literature indicating Activin A as a neural and pancreatic differentiation inducer.[96,97]

It is known from basic biology that the several intercellular communication mediators may be released and transported to other cells in their soluble form our inside cellular microvesicles as exosomes. To assess an eventual exosome-mediated cytokines' transport the abovementioned co-culture experiments were performed in the presence of the exosome-uptake blocker xyloside. Surprisingly, co-cultured RenG2 cells' acquisition of CSCs' traits was significantly abrogated, as the isolated cells were able to form spheres few and smaller spheres when subjected to the sphere-forming assay (Figures12A, B and C). Therefore, despite the evident fibroblasts' methanol-mediated cell death, the remaining cells were still able to produce and release cytokine-containing exosomes that drove RenG2 cells' dedifferentiation. These results corroborate others in the literature showing that the xyloside concentration used in the present work was only able to reduce approximately 50 % of the total exosome uptake.[27]

Finally, the same co-cultures were also performed in the presence of the exosome-release blocker GW4869. However, it seemed that the carrier vehicle deems this agent not usable in the selected co-culture system, as the vast majority of the cells under culture died in the presence of DMSO alone, as they did in the presence of the DMSO-dissolved GW4869 (Figure 11B and C). These unexpected results may be explained by the considerably bigger culture period implemented in the present work, which would allow DMSO to induce its known cellular toxicity. This way, the negative results attained in the sphere-forming assay in these two conditions, although promising, were biased by the cytotoxicity of DMSO and should be subjected to further confirmation.

Taken together the results attained in this project point IL-6 as the main driver of CSCs' formation in the RenG2 cellular system. Additionally, Activin A seems to be acting as a CSCs'-pool homeostasis sensor, in a way that whenever the CSCs' pool overcomes a certain threshold, Activin-A induces their

differentiation. Moreover, G-CSF is acting downstream of these two cytokines by providing the CSCs'-niche with the appropriate conditions to sustain the undifferentiated phenotype of its cells. And finally, the cytokines are released by the fibroblasts in an exosome-mediated intercellular communication process (Figure 16).

5.4. Conclusion

The use of the neutralizing antibodies for IL-6, G-CSF and Activin-A in different combinations in the co-cultures media allowed the dissection of the role of each individual cytokine in the dedifferentiation process that occurs whenever RenG2 cells are co-cultured with bronchial fibroblasts. The principal culprit in the process was IL-6 as its action alone was sufficient to induce CSCs formation. Activin-A can also induce CSCs formation but it seems to exert an additional role as a sensor of CSCs'-pool homeostasis. Also, G-CSF does not have a role as a dedifferentiation-inducer but rather acts to keep CSCs' stemness potential. Finally, the cytokines-mediated intercellular communication is mediated by exosomes.

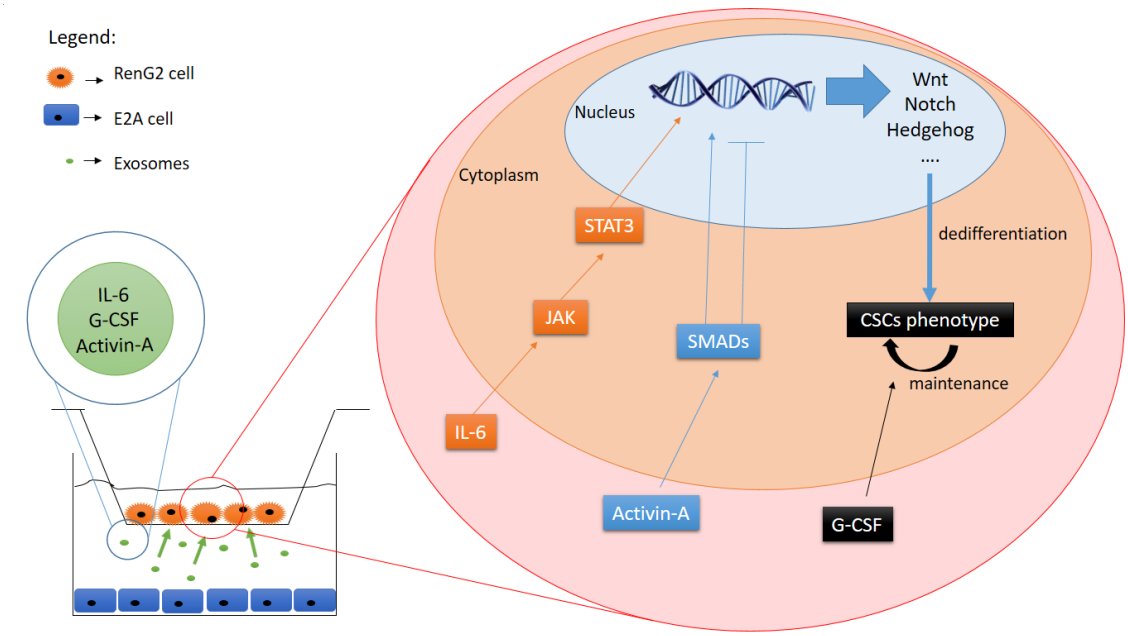


Figure 16 – Proposed model for IL-6, Activin-A and G-CSF involvement in the dedifferentiation of RenG2 cells. Upon E2A fibroblasts Transwell® co-culture with RenG2 cells, the first cells released exosomes containing IL-6, Activin-A and G-CSF, either combined or separated. These vesicles then traveled to the upper compartment where they interacted with the RenG2 cells. Then IL-6 and Activin- A most probably modulated DNA expression through STAT3 and Smad activation, respectively, and consequently activated stemness-linked pathways such as Wnt, Notch and Hedgehog. As a consequence, RenG2 cells' dedifferentiation was induced and then it was subsequently maintained by the activity of G-CSF. Finally, Activin-A seemed to act as a sensor of the CSCs' pool homeostasis, inducing their differentiation whenever a certain threshold was reached.

Chapter 6

Further Perspectives

The present study allowed the confirmation that IL-6 and Activin-A are responsible for the dedifferentiation process that occurs in the RenG2 system whenever co-cultures with bronchial fibroblasts, as well as the establishment of a mechanistic model. Nevertheless, although very appealing, the proposed model still requires further validation.

The present study only assessed which cytokine was involved in the process and provided some clues to the potential signaling pathways that are responsible for it. It would be interesting to further dissect the Nodal/Activin and IL-6/JAK1/STAT3 pathways in order to assess their downstream effectors, and eventually identify potential targets for therapeutic intervention. Furthermore, it is mandatory to deepen the study of the exosome-mediated cytokines' release, and the effect of its blockage in the dedifferentiation process. Hopefully, potential therapeutic targets may also be found in this communication pathways, thus empowering and improving the fight against tumors, and consequently, patients' outcome and welfare.

Part IV

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Appendix A

Cytokines' Levels

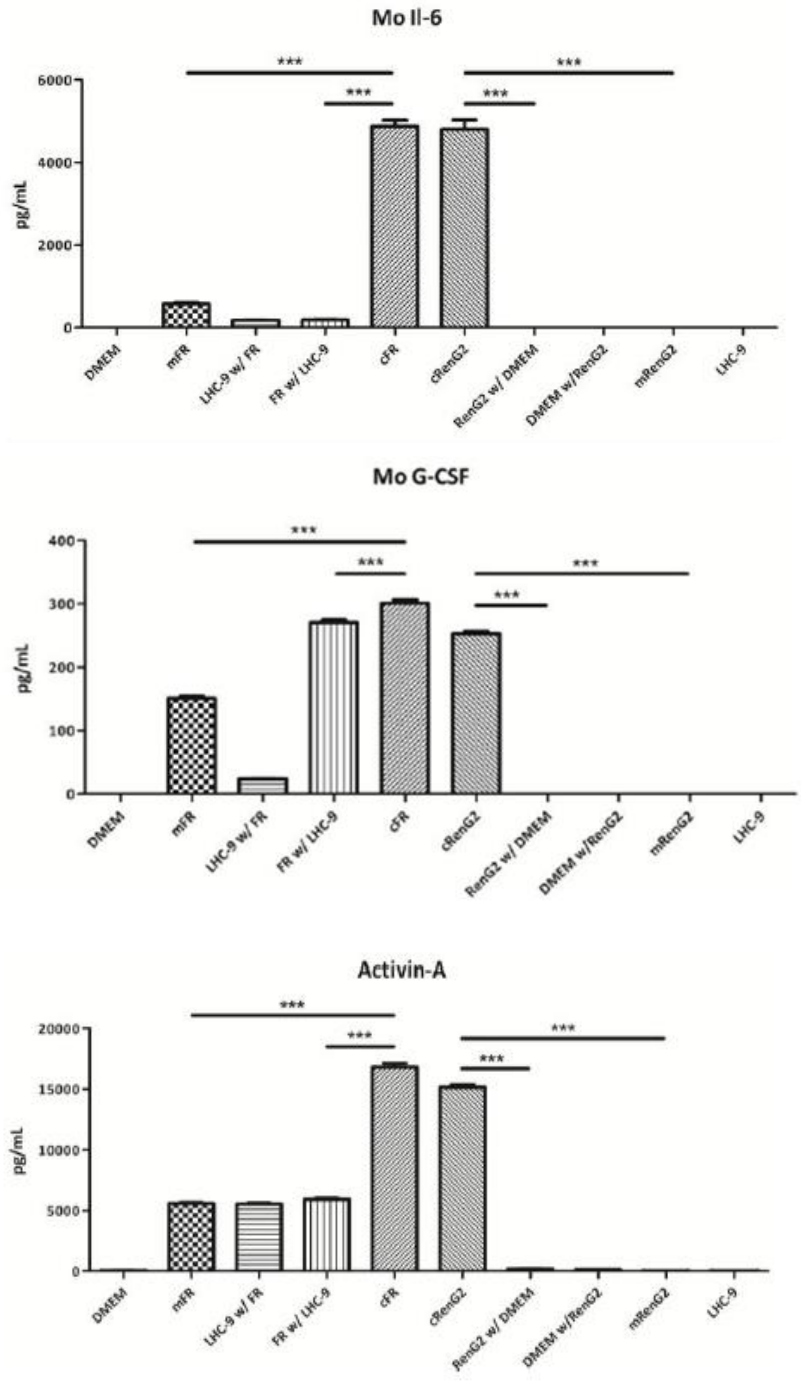

Figure 17 – Levels of IL-6, G-CSF and Activin-A present in the conditioned media of RenG2-mouse fibroblasts co-culture. Quantification of the study cytokine levels present in the co-culture media attained with ELISA (Activin-A) and Bioplex® multiplex cytokine array (IL-6 and G-CSF), 5000 pg/mL for IL-6, 300 pg/mL for G-CSF and 17000 pg/mL for Activin-A. The present results are part of a PhD thesis already submitted and are here reproduced with author's authorization.[100]