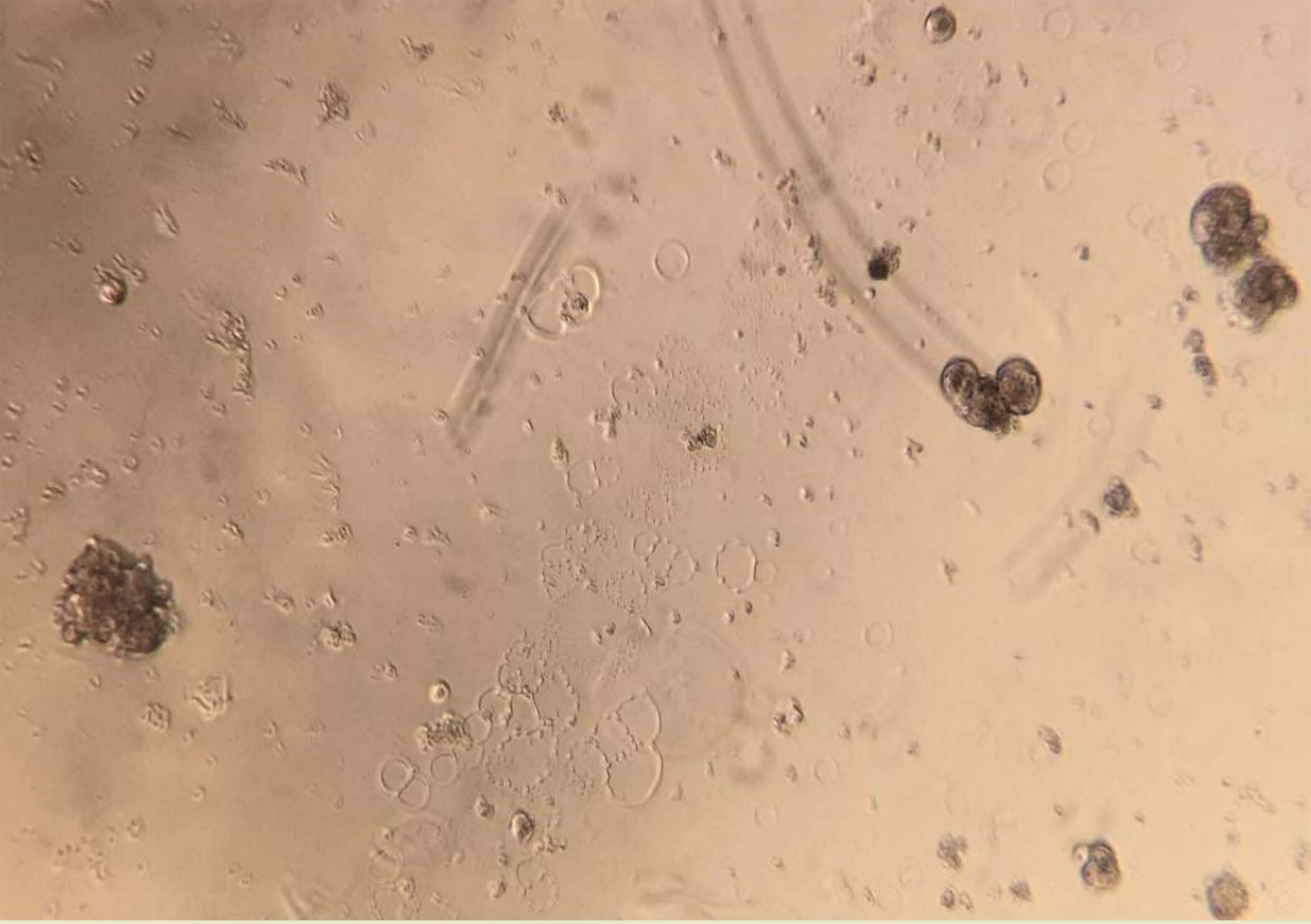

Marco Rafael Lopes da Cunha

The importance of cytokines-mediated communication in CSCs' formation

Master Dissertation presented to the Department of Life Sciences from the Faculty of Sciences and Technology of University of Coimbra.

MSc Cellular and Molecular Biology

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· U C ·

Universidade de Coimbra

Marco Rafael Lopes da Cunha

The importance of cytokines-mediated communication in CSCs' formation

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob 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 Martins de Carvalho Alpoim, Professora Associada da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Julho de 2016

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UNIVERSIDADE DE COIMBRA

"I've missed more than 9000 shots in my career. I've lost almost 300 games. 26 times I've been trusted to take the game winning shot and missed. I've failed over and over and over again in my life. And that is why I succeed."

Michael Jordan

Abstract

Evidence on the dedifferentiation process and on its implication in cancer stem cells' (CSCs) formation represents one of the most recent breakthroughs in the understanding of cancer. Our laboratory recently implicated this particular cellular population in the malignant transformation of a human bronchial epithelial cell line (BEAS-2B into RenG2), following a carcinogenic insult and serial rounds of injection in immunocompromised mice. Furthermore, it became evident that dedifferentiation was driven by a paracrine communication loop between malignant tumor cells and mice stromal cells due to the action of Interleukin-6 (IL-6), Granulocyte colony-stimulating factor (G-CSF) and Activin-A.

The main goal of the present project was to unravel the abovementioned cytokines' release dynamics, focusing on the involvement of exosomes. For this purpose, exosomes were isolated from the conditioned media of the co-culture of RenG2 cells with human bronchial fibroblasts (E2A), and their content was analyzed for the cytokines in study. Furthermore, fibroblast's extracellular matrix (ECM) was isolated and co-cultured with RenG2 cells to assess the probable reservoir function previously attributed to the ECM. Additionally, the presence of exosomes in the ECM was also evaluated in order to determine if these structures are being stored in the ECM. Finally, in order to exclude a possible non-exosome mediated release of the cytokines, the co-culture conditioned media were evaluated for the presence of free cytokines.

The results obtained allowed us to demonstrate that the cytokines of interest are being released by the fibroblasts in co-culture as exosomal cargo, which then lead to RenG2 cells' dedifferentiation upon reaching these cells. Still, the ECM seems to function as a reservoir of exosomes containing the pro-inflammatory cytokines IL-6 and G-CSF; however, Activin-A is not being stored in exosomes present in the matrix, being probably produced and released in just the right extent of the cell's needs.

Keywords: Cancer stem cells; Tumor microenvironment; Cytokines; Exosomes; Dedifferentiation.

Resumo

Um dos mais recentes avanços na área da Oncobiologia é a crescente evidência da implicação do processo de dediferenciação na formação de células estaminais tumorais (CETs). Trabalho recente do nosso laboratório mostrou que após um insulto carcinogénico e cultura baixa densidade foi possível malignizar a linha celular de epitélio bronquial humano BEAS-2B, dando origem ao sistema celular RenG2. Posteriormente, sucessivas injeções em ratinhos imunocomprometidos permitiram a derivação do sistema e a obtenção de sistemas progressivamente mais malignos e agressivos. O estudo detalhado e comparativo destes sistemas levou à identificação de uma população de CETs no seio das células RenG2, a qual foi posteriormente mostrado ter derivado de uma comunicação parácrina estabelecida entre as células RenG2 injetadas no ratinho e os fibroblastos do compartimento subcutâneo do animal, mediada pela Interleucina-6 (IL-6), o Fator estimulador de colónias granulocitárias (G-CSF) e a Activina-A.

O principal objectivo deste projeto foi determinar os mecanismos de libertação das citocinas supracitadas, com particular ênfase no envolvimento de exosomas. Com esta finalidade, o conteúdo de exosomas isolados do meio condicionado de co-culturas de células RenG2 com fibroblastos bronquiais humanos (E2A) foi analisado, e foram pesquisadas as citocinas de interesse. Para além disso, a matriz extracelular dos mesmos fibroblastos foi também isolada e co-cultivada com as células RenG2 para avaliar a provável função de reservatório previamente atribuída à matriz. Finalmente, para excluir uma libertação de citocinas não mediada por exosomas, foi também pesquisada a presença das citocinas livres nos meios condicionado das co-culturas.

Os resultados obtidos permitiram mostrar que as citocinas de interesse são libertadas no interior de exosomas pelos fibroblastos em co-cultura, fundindo-se posteriormente com as células RenG2 orquestrando a sua dediferenciação. Por outro lado, a matriz extracelular parece estar a funcionar como um reservatório de exosomas contendo as citocinas pro-inflamatórias IL-6 e G-CSF; contudo, a Activina A não é acumulada em exosomas na matriz, sendo provavelmente produzida e libertada na medida exata das necessidades celulares.

Palavras-chave: Células estaminais tumorais; Microambiente tumoral; Citocinas; Exosomas; Dediferenciação.

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I want to thank **Professor Carmen Alpoim** for being the creative mind of the group and for sharing her wisdom with me. For every new idea that she presented to the group every week and every solution for every problem that might surge. I'll be always thankful to you for this opportunity.

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To my friends, my brothers and sisters from other parents, **Cris, André**, **Torres**, **Afonseca**, **Dé**, **Ruca**, **Kika** and **Ló**, you belong to the restrict group of essential people in my life. This is over now, let's celebrate! The next round of shots is on me!!

I have, of course, to include all the friends that I have made during this journey that was this masters. I never thought I would meet such nice people when I came here to Coimbra but now I'm sure I made some friends that will be with me, hopefully, for a long time. Despite all the hard work this two years demanded form us, we always had time to party and have a good time **#aculpaédomestrado**.

Leo my little guy, you're not so little anymore now! And seeing the man you are

becoming makes me proud in a way I can't describe. Thank you for always listening to me and to deal with all my crazy ideas, but most of all thank you for letting me be you're role model, I tried my best to do a good job. Love you.

Mãe e Pai, esta tese é para vocês, mais do que isso, esta tese é vossa! Espero que vos deixe orgulhosos tanto de mim como do vosso trabalho do qual sou fruto. Agora começa uma etapa nova na minha vida na qual sei que irei ter o apoio incondicional que sempre tive da vossa parte. Obrigado soa a pouco, amo-vos!

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List of Abbreviations

ABC - ATP-binding cassette.

ACVR1 - Activin receptor type-1.

ALK4 - Activin receptor type-1B.

APC - Adenomatous polyposis coli.

AVCs - Angiogenic vascular cells.

AXL - tyrosine kinase receptor.

bFGF - Basic fibroblast growth factor.

BSA - Bovine serum albumin.

CAFs - Cancer-associated fibroblasts.

CK1 - Casein kinase 1.

Cr (VI) - Hexavalent chromium.

CSCs - Cancer stem cells.

DHH - Desert hedgehog.

DKK2- Dickkopf-related protein 2.

DLL4 - Delta-like 4 ligand.

DMSO - Dimethyl sulfoxide.

DNA - Deoxyribonucleic acid.

ECM - Extracellular matrix.

EDTA - Ethylenediamine tetraacetic acid.

EGF - Epidermal growth factor.

ELISA - Enzyme-linked immunosorbent assay.

EMT - Epithelial to mesenchymal transition.

FBS - Fetal bovine serum.

G-CSF - Granulocyte colony-stimulating factor.

GP130 - Glycoprotein 130.

GSIs - γ-secretase inhibitors.

GSK3-β - Glycogen synthase kinase 3 beta.

HH - Hedgehog.

HIF - Hypoxia-inducible factor.

IGF - Insulin-like growth factor

IHH - Indian hedgehog.

IICs - Infiltrating immune cells.

IL-n - Interleukin n.

ITS - Insulin, transferrin and selenium pyruvate solution.

JAK - Janus kinase.

LEF - Lymphoid enhancer factor

LPS - Lipopolysaccharides

MAPK - Mitogen-activated protein kinase 1.

MSCs - Mesenchymal stem cells.

MYC - Myelocytomatosis viral oncogene homolog.

NF$_K$B - Nuclear factor of kappa light polypeptide gene enhancer.

OCT-4 - Octamer-binding protein 4.

PBS - Phosphate-buffered saline.

PI3K - Phosphoinositide-3-kinase.

Poly-HEMA - Poli-(2-hydroxyethyl methacrylate).

RT - Room temperature.

SCF - Stem cell factor.

SHH - Sonic hedgehog.

Smad n - SMAD family member n.

STAT - Signal transducer and activator of transcription.

TAMs - Tumor-associated macrophages.

TAZ- Tafazzin.

TCF - T-cell factor.

TGF-β - Tumor growth factor β.

TNF-α - Tumor necrosis factor α.

VEGF - Vascular endothelial growth factor.

WNT - Wingless-type mouse mammary tumor virus integration site family.

Chapter 1

Cancer

Despite all the advances made in the understanding of tumors' biology during the last decades, cancer remains one of the biggest mysteries of modern biology and medicine. The accumulating knowledge in the field has yet not yielded an effective therapeutic approach, and cancer is still one of the leading causes of both morbidity and mortality worldwide (Prasad & Goldstein, 2014).

1.1 Cancer statistics and epidemiology

Information on new cancer cases and expected cancer related deaths at a planet scale do not exist, thus limiting the data on global cancer statistics. In order to fulfill this caveat, an online database under the control of GLOBOCAN was created. Currently, it already combines the information of 184 countries and has proved to be a very powerful tool available online for researchers from all around the world.

Data form GLOBOCAN estimates 14.1 million new cancer cases and 8.2 million cancer-related deaths to have occurred worldwide in 2012. This information led to the construction of a map that shows the regions with higher cancer prevalence in that year (Figure 1.1). Map's analysis shows that the number of new cancer cases is smaller in more developed countries (Torre *et al*., 2015).

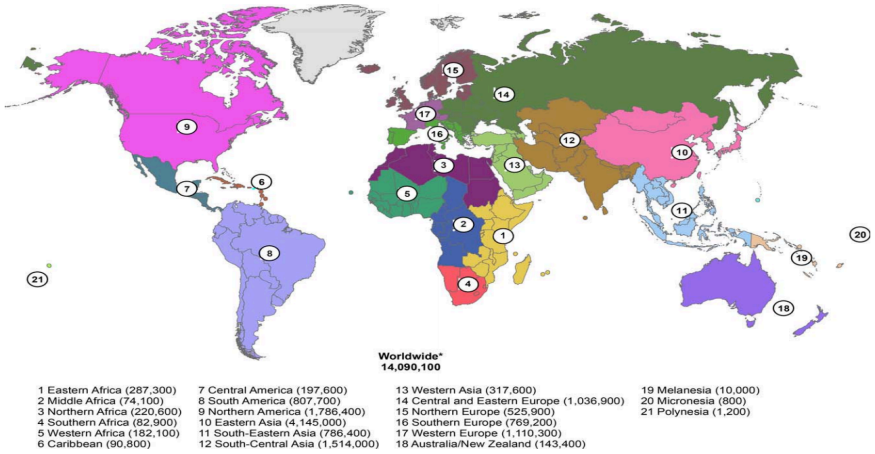

Figure 1.1 - Worldwide estimated new cancer cases in 2012. The number of new cancer cases is higher in Africa and lower in Europe. Source: GLOBOCAN 2012.

This growing incidence of cancer in less developed countries is believed to be due to the western lifestyle adopted in some of these countries. This particular lifestyle includes harmful habits like smoking, alcohol consumption and poor diet intake. The most frequently tumors diagnosed worldwide in men and women are lung and breast tumors, respectively. Moreover, although both men and women are affected by cancer, men seem to be more susceptible to cancer as the number of deaths, and also the number of new cases in almost all the different cancer types is higher in this gender (Torre *et al.*, 2015) (Figure 1.2).

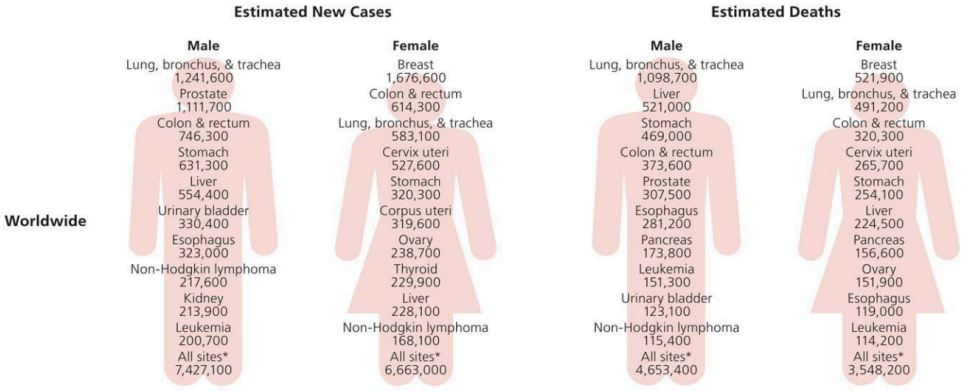

Figure 1.2 - Estimated new cancer cases and deaths worldwide. Cancer seems to be more common in men as there is a higher number of new cases and deaths in men related to cancer. Source: GLOBOCAN 2012.

The industrial revolution represents an important milestone in terms of cancer epidemiology. With its advent, the skies of the main urban centers turned darker as the black smoke emitted from coal combustion accumulated in the atmosphere. As a consequence,

respiratory diseases started to be documented and cancer incidence increased dramatically. Of particular relevance were those tumors affecting the skin and the respiratory tract, being the last ones' prevalence further fueled by the introduction of smoking as a social habit (Hajdu, 2012).

Nowadays, despite the tremendous technological advances, which were accompanied by an increase in carcinogenic agents production, the main leading causes of cancer remain the same. As a consequence, cancer is sometimes referred to as an epidemic disease that spread all over the world, affecting all kinds of animals more than any other disease.

Chapter 2

CSCs and The Hierarchical Theory of Cancer

Along the years many theories were proposed to explain how cancer arises. However, it was not until the nineties, with the discovery of tumor suppressor genes and oncogenes, that the genetic nature of cancer was revealed. More recently, the discovery of tumor cells with stem-like properties put in question the foundations of oncobiology and opened a whole new field of research.

2.1 Stochastic Model vs. Hierarchical Theory of Cancer

Until recently, the stochastic model of cancer was accepted as the best to explain cancers' etiology. According to this model, mutations, perhaps induced by environmental carcinogens, eventually induce the formation of pre-malignant clones (initiation), which, with the accumulation of further mutations (promotion), may result in cells' transformation. Transformed cells, characterized by their increased survival and growth potentials, were all biologically equivalent, and therefore have the same potential to act as cancer initiating cells (Nguyen *et al*., 2012). However, this model fails to explain intratumoral heterogeneity, which led to the emergence of the hierarchical theory of cancer as an alternative to the stochastic model (So & Suh, 2015).

According to the hierarchical theory of cancer, tumor initiation ability is restricted to a subset of cells within the tumor called CSCs (Figure 1.3). Similarly to normal stem cells, CSCs have self-renewal and multilineage differentiation capacities and inhabit discrete niches within the tumor where they live in a quiescent status maintained by a complex communication with the tumor microenvironment cells (Medema, 2013; Vermeulen *et al*., 2012). Moreover, these

cells have increased resistance to therapeutic drugs, most probably due to an over-expression of numerous ATP-binding cassette (ABC) transporters, which renders them more resistant to chemotherapy (Shigdar *et al*., 2014).

Despite all these evidences, the stochastic model and the hierarchical theory of cancer are not mutually exclusive as CSCs exhibit genomic instability and present a mutator phenotype. Consequently, as they divide they can give rise to tumorigenic and non-tumorigenic progeny, developing subclones of CSCs (Hanahan & Weinberg, 2011).

Taken together all these facts, the hierarchical model was redesigned introducing the microenvironment as a key player. According to the Emerging Dynamic CSCs model, CSCs acquire most of their characteristics due to molecules secreted by stromal cells of the microenvironment (Vermeulen *et al*., 2012) due to an intimate and complex intercommunication network. The heterogeneity of the tumoral mass results from the asymmetric division of CSCs, giving rise to progenitor cells that differentiate to a wide range of tumor cells. Otherwise, stromal cells may induce symmetric division in CSCs enriching, in this way, the pool of these cells (Vermeulen *et al*., 2012).

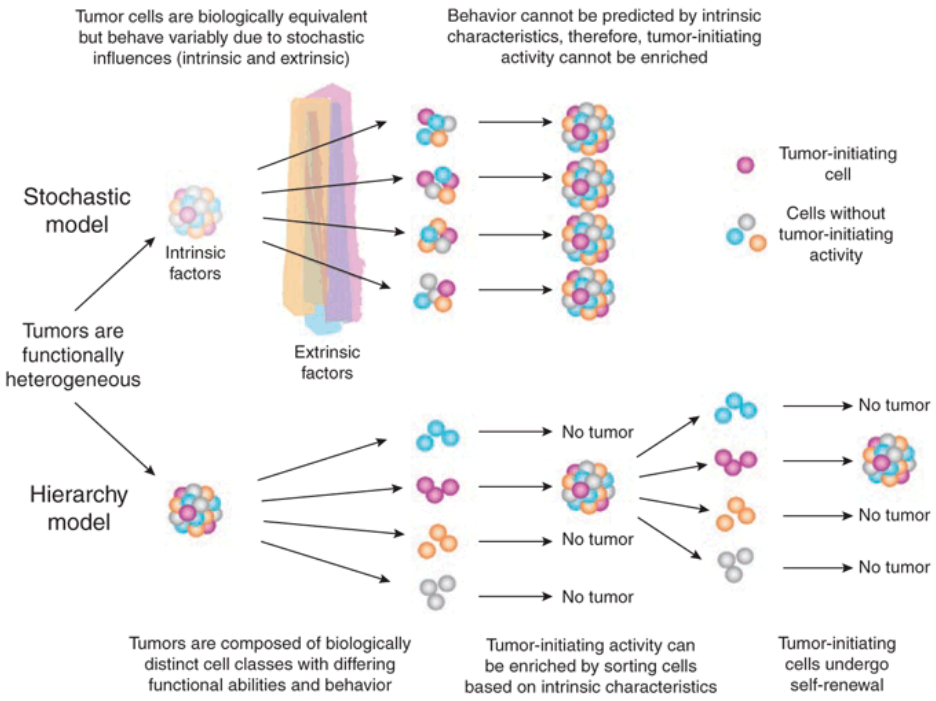

Figure 2.1- Stochastic and Hierarchic model of cancer. Tumors are composed of functionally heterogeneous cells. While the stochastic model claims that tumoral cells are biologically equivalent, the hierarchical model argues that only a small subset of cells within the tumor are responsible for tumor initiation. Adapted from Dick, 2009.

2.2 CSCs biology and underlying signaling pathways

The last decades were marked by a tremendous effort made to understand CSCs' biology. The similarities between CSCs and normal stem cells go far beyond the undifferentiated status that they present. In fact, both cells are able to undergo symmetric and asymmetric divisions (Ajani *et al*., 2015), and according to Weinberg and Medema, they also share a gene expression signature. Moreover, Wnt, Notch and Hedgehog signaling pathways, which regulate self-renewal and differentiation of stem cells, are also upregulated in CSCs, suggesting similar regulatory principles (Colak & Medema, 2014).

The Wnt signaling pathway is one of the pathways that seems to be involved in inducing and keeping CSCs. Wnt proteins bind to Frizzled to block the degradation of β-catenin through the assembly of APC/GSK3-β/CK1 complex, being this pathway therefore also known

as the Wnt/β-catenin pathway. This signaling pathway coordinates, with several other signaling systems, embryonic development and homeostasis in the adult organism. At present, there are numerous studies that demonstrate the role played by Wnt in the biology of stem cells (Wend *et al*., 2010). Furthermore, an abnormal activation of the Wnt/β-catenin pathway has been correlated with tumorigenesis and tumoral progression due to the maintenance of CSCs (Nguyen *et al*., 2012).

As several other signaling pathways, the Wnt pathway can be divided into a canonical and a non-canonical signaling pathway, being the first one the most important in the biology of CSCs (de Sousa e Melo & Medema, 2012). The canonical pathway is triggered by β-catenin, whose excess, in normal cells, is targeted for proteassomal degradation, leaving the Wnt/β-catenin pathway turned off. Alternatively, if β-catenin accumulates in the cells, it translocates into the nucleus where it is going to interact with T-cell factor (TCF)/ lymphoid enhancer factor (LEF) complex in order to modulate the transcription of genes involved in cellular proliferation and differentiation, namely *MYC* and *cyclin D1* (Figure 2.1) (de Sousa e Melo & Medema, 2012).

Hoffmeyer and collaborators established a molecular link between Wnt/β-catenin cascade and cancer associated telomerase activity by showing that β-catenin interacts with Klf4 and c-Myc on the pluripotency transcriptional network (Hoffmeyer *et al*., 2012). Moreover, recent evidence confirmed the importance of the Wnt/β-catenin cascade in brain (Gong & Huang, 2012), lung (Xu *et al*., 2013) and breast (Cai *et al*., 2013) CSCs' homeostasis, and several new Wnt/β-catenin signaling components have meanwhile been linked to stem cell functions. An example is R-spondins, proteins that activate Lgr5 stem cell receptors (de Lau *et al*., 2011), which have been implicated in the maintenance of skin (Liu *et al*., 2014), colon (Chen *et al*., 2014) and colorectal (Hirsch *et al*., 2013) CSCs. More recently, Mao and colleagues have shown that the Wnt/β-catenin pathway improves the proliferation of gastric CSCs both *in vitro* and *in vivo* (Mao *et al*. 2014), and the group of Toledo suggested an alternative way of Wnt pathway activation through *DKK2*, which they proved that can act both as agonist and antagonist of the cascade, depending on the cellular context (Cordeiro *et* al., 2014).

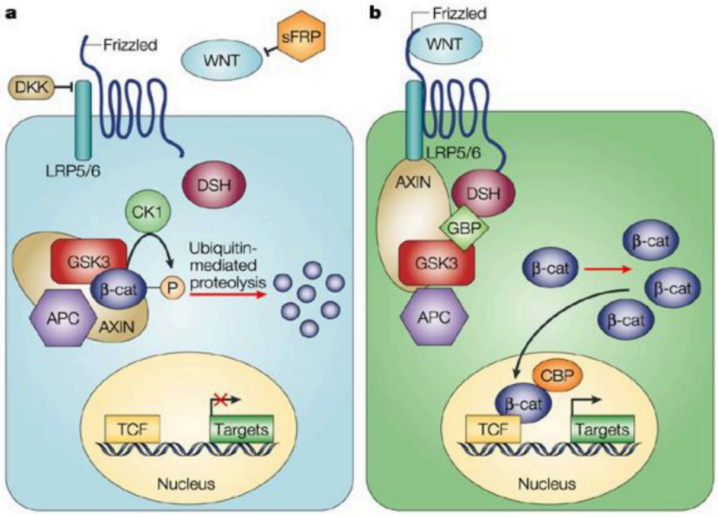

Figure 2.2 - Wnt/β-catenin canonical pathway. a) In the absence of the Wnt ligands the excess of β-catenin is phosphorylated by the APC/GSK3-β/CK1 complex and degraded by the proteasome; **b)** When Wnt ligands are present the phosphorylation of β-catenin is prevented allowing it to translocate to the nucleus where it regulates transcription. Adapted from: Moon *et al*., 2004.

The Notch pathway is a conserved cell-fate determination pathway of extreme relevance in cancer biology, namely in angiogenesis and tumor immunosurveillance (Espinoza & Miele, 2013). Regarding CSCs, this signaling pathway has been reported to play a key role in their maintenance, as the use of antibodies against delta-like 4 (DLL4) Notch ligands led to an accentuated decrease in CSCs' populations in human tumors. Additionally, using γ-secretase inhibitors (GSIs) in combination with a monoclonal antibody, Pandya and collaborators achieved to block tumor recurrence, which suggests that CSCs have been eliminated (Pandya *et al*., 2011). More recently, GSIs have been shown to have an anti-CSCs activity *ex vivo* in brain (Abel *et al*., 2014), lung (Hassan *et al*., 2013) and pancreas (Saito *et al*., 2014).

Another important pathway that is involved in the self-renewal and differentiation of stem cells is the Hedgehog (HH) pathway. Similar to the abovementioned pathways, HH pathway is also of extreme importance during embryonic development, namely in the determination of the dorsoventral body axis. There are three main HH ligands: the Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH). These ligands bind to transmembrane receptors Patched or Smoothened that will lead to the initiation of a signaling cascade that will activate numerous transcription factors that regulate genes involved in angiogenesis, proliferation and survival. In turn, the dysregulation of this pathway in adults has

been shown to be a potent inducer of tumorigenesis (Amakye *et al*., 2013). Moreover, the hedgehog cascade has also been reported to play an important role in the epithelial-to-mesenchymal transition (EMT) process (Amakye *et al*., 2013). Furthermore, there have been several evidences of the correlation of HH pathway with CSCs, namely in lung cancer and gliomas, where the downregulation of the cascade leads to a decrease in CSCs' number (Zhang *et al*., 2012; Bar *et al*., 2007). In addition, pancreatic CSCs' capacity of self-renewal has also been reported to depend on the HH pathway (Rodova *et al*., 2012). Heiden and collaborators demonstrated that the HH pathway promotes CSCs' self-renewal in thyroid cancer (Heiden *et al*., 2014). Similarly, very recently, two different groups reported that the modulation of the HH pathway sustains the self-renewal capacity of breast CSCs (Lu *et al*., 2015; Memmi *et al*., 2015).

In the recent years, Activin/Nodal pathway has also been associated with CSCs. Although this pathway is not *per si* associated with development and/or stem cells' biology, growing evidence suggests its association with CSCs. Lonardo and colleagues demonstrated that CSCs produce Activin/Nodal ligands that have an autocrine action over themselves (Lonardo *et al*., 2011). Later on, Quail and collaborators verified that this signaling loop can be induced through the co-option of the surrounding stromal cells by CSCs (Quail *et al*., 2013).

Finally, the last pathway to be connected to CSCs' biology was the insulin-like growth factor (IGF)-mediated signaling pathway. Malaguarnera & Belfiore reported that IGF helps maintaining CSCs' stemness through a cross talk with the abovementioned pathways (Malaguarnera & Belfiore, 2014).

2.3 CSCs' role in the tumorigenic process

Just like normal stem cells, CSCs have multiple mechanisms to avoid cell death. For example, they have increased apoptosis resistance, drug efflux pumps, enhanced DNA repair systems and quiescence, which together are referred to as pro-survival mechanisms (Figure 2.2) (Colak & Medema, 2014). The discovery of CSCs that express quiescence properties is the most recent and challenging problem in CSCs' understanding. Quiescence has been related by several recent studies to chemoresistance (Touil *et al*., 2014; Ehninger *et al*., 2014). However,

this less proliferative phenotype seems not to be the only characteristic of CSCs that makes them so resistant to therapy.

The association of CSCs with tumoral relapse has been supported by several studies, and further corroborated by the constant identification of these cells in the recurring tumors, namely breast, pancreas and colon (Sampieri & Fodde, 2012). Similarly, the EMT has also been linked to CSCs by numerous studies. As EMT marks the beginning of primary tumor's dissemination, and because CSCs tend to present a more mesenchymal aspect, several studies were made in order to link CSCs with metastasis. Successfully, Yan and colleagues showed that by inhibiting EMT through miR-148a, not only hepatocellular carcinoma metastasis are blocked, but also CSCs' properties (Yan *et al*., 2014a). Last year, Asiedu and collaborators further showed that the activation of the tyrosine kinase receptor AXL, which is constitutively active in breast CSCs, induces EMT through the regulation of the expression of several EMT markers (Asiedu *et al*., 2014). Currently, *in vivo* studies are needed to assess if CSCs are as sufficient to initiate the metastatic cascade *in vivo* as they have shown to be *in vitro*. According to Chinn and collaborators, CSCs are not only more tumorigenic *in vivo*, but also have a greater rate of spontaneous metastatic ability, as compared to non-CSCs (Chinn *et al*., 2015).

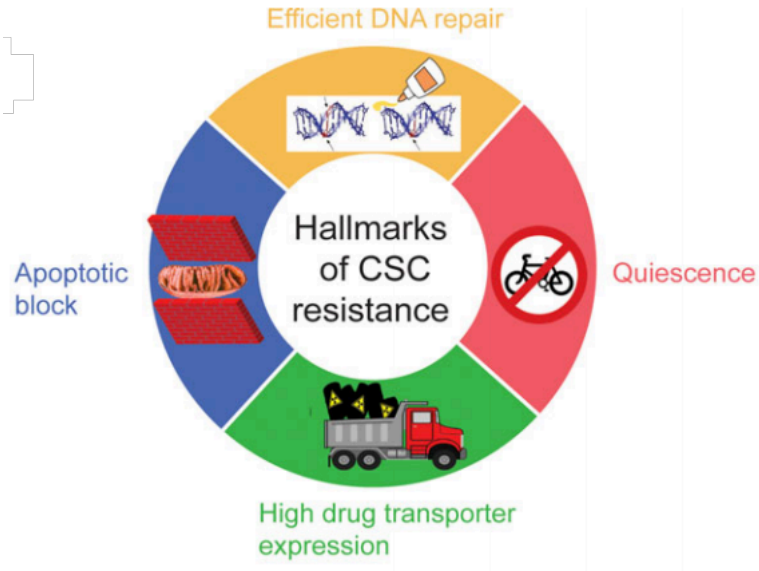

Figure 2.3 - The four hallmarks of CSCs' resistance. Four mechanisms that allow CSCs to evade cell death and resist therapy. Adapted from Colak & Medema, 2014.

In addition to those already mentioned, there are other steps of the tumorigenic process to which CSCs have been associated, namely angiogenesis. The angiogenic switch that triggers tumor's vascularization is of vital importance in tumors' development and occurs early during the tumorigenic process. Some vasculogenic factors were recently discovered and were shown to be expressed by CSCs under hypoxic conditions, thus suggesting a role for CSCs in indirectly promoting angiogenesis during the tumorigenic process. However, Shen and colleagues reported that CSCs might also participate directly in the angiogenic process, as they observed that CSCs developed vasculogenic mimicry both *in vitro* and *in vivo* (Shen *et al*., 2008). Bussolati *et al* further corroborated these observations by isolating breast CSCs with endothelial characteristics that, when cultured in the presence of VEGF, were able to grow in capillary-like structures (Bussolati *et al*., 2009). Finally, Cabarcas and colleagues were able to observe that under hypoxic conditions CSCs also express Hypoxia inducible factors (HIF), main effectors of the transforming growth factor beta (TGF-β) signaling pathway and potent drivers of angiogenesis (Cabarcas *et al*., 2011).

CSCs' resistance to chemotherapy has been the subject of several studies during the last decade. Populations of these cells isolated from liver, lung and pancreatic tumor samples have been reported to survive chemotherapeutic insults in *in vitro* assays (Ma *et al*., 2008; Bertolini *et al*., 2009; Hermann *et al*., 2007). Moreover, Samanta and colleagues observed that IL-6 and Inteleukin-8 (IL-8) signaling promote the enrichment of breast CSCs' population due to the inhibition of apoptosis induced by chemotherapy and promoting breast CSCs' phenotype (Samanta *et al*., 2014). And the group of Medema showed that after *in vitro* chemotherapeutic treatment of clonal tumor samples, only CSCs resist, and the resistant cells were able to re-establish the culture, supporting the hypothesis of CSCs as the mediators of tumors' relapse (Colak *et al*., 2014). This hypothesis was further supported by the works of Chen and collaborators showing, using labeling CSCs with a Nestin reporter construct, that these cells managed to *in vivo* repopulate the tumor after chemotherapeutic treatment. However, relapse was prevented if they selectively deleted these $Nestin^{+}$ cells (Chen *et al*., 2012). More recently, the transducer of the Hippo pathway TAZ was also reported to play an important role in chemoresistant properties of CSCs in breast cancer. Although TAZ does not confer

chemoresistance *per si*, its depletion has been shown to sensitize breast CSCs to chemotherapy (Bartucci *et al*., 2014).

There is also growing evidence of CSCs' increased radioresistance (Kurth *et al*., 2014; Bratman & Diehn, 2014). In agreement, a very recent work presented evidences that strongly support the interaction of human papilloma virus oncoproteins with stem cell signaling pathways, namely Wnt, Notch and Hedgehog, which may be responsible for radioresistance in cervical cancer (Vishnoi *et al*., 2015).

The accumulated knowledge of the complex interconnections between CSCs and the tumorigenic process will hopefully help to understand and to circumvent the high rates of malignancy imposed by these cells. Ongoing work now aims to develop a combined therapeutic strategy that may be able to concomitantly target both CSCs and the bulk of differentiated tumor cells, thus abrogating tumor growth and relapse.

2.4 The origin of CSCs

The way people think about cancer has changed since the discovery of CSCs, and nowadays, a central role is attributed to these cells in the process of tumor development. However, the origin of CSCs is still an active matter of debate inside the scientific community. There are currently three main hypotheses trying to explain the origins of CSCs, the first points normal stem cells as the most probable source of CSCs, while the second believes that they derive from committed progenitor cells. More eccentrically, a third theory defends that CSCs may derive from differentiated tumor cells by a process of dedifferentiation (Figure 2.3) (Bu & Cao, 2012).

The three main hypotheses that try to explain the origin of CSCs have acquired supporting evidences along the years. The formation of CSCs was originally proposed to be due to mutational transformation of endogenous stem cells that would induce dysregulated self-renewal properties and tumor growth (Visvader, 2011). However, there is evidence that CSCs may also arise from progenitor cells that have acquired oncogenic mutations (Pardal *et al*.,

2003). Moreover, a more recent hypothesis combines the two aforementioned models and claims that CSCs may arise from the fusion of a normal stem cell and a differentiated cell that has accumulated mutations in tumor suppressor genes or oncogenes. The resulting cell would combine characteristics of both of its progenitors (Bu & Cao, 2012).

The work of Takahashi and Yamanaka showing that through the introduction of four specific genes in fibroblasts a pluripotent cell can be attained, raised the hypothesis that a similar process may feature the formation of CSCs (Takahashi & Yamanka, 2007). In this line of thought, many works have been performed and a pivotal role has been attributed to the microenvironment in CSCs' formation. Several laboratories have shown that paracrine signaling released by microenvironmental cells is able to drive a dedifferentiation process in the tumors cells, rendering them more resistant and undifferentiated, meaning, rendering them CSCs (Rodrigues *et al*., 2013; Hanahan & Weinberg, 2011; Medema, 2013).

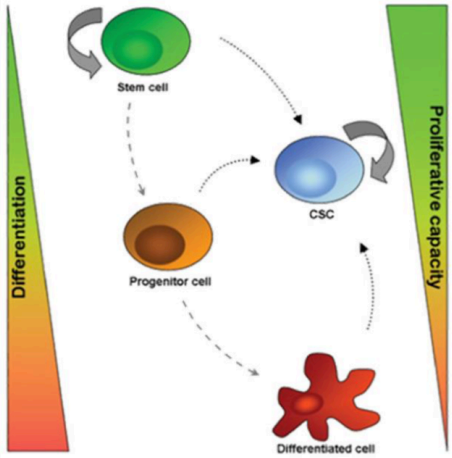

Figure 2.4 - Possible different origins of CSCs. CSCs can arise from normal stem cells through the accumulation of oncogenic mutations and in a similar way from progenitor cells. CSCs can also result from the stromal orchestrated dedifferentiation of differentiated cells. Adapted from Welte *et al*., 2010.

The dedifferentiation process has also been associated with the inflammatory response, which occurs upon the activation of NF-κB signaling pathway, which in turn will enhance Wnt signaling and consequently lead to a reprogramming process. The authors

showed that the activation of NF-κB, which induces the stabilization of β-catenin, drives the conversion of non-stem intestinal epithelial cells to intestinal epithelial CSCs (Schwitalla *et al.*, 2013).

2.4.1 Tumor microenvironment and CSCs' dedifferentiation

Currently tumors are understood as living organs composed not only of malignant cells, but also of the tumor microenvironment's non-malignant cells, which *per si* encompass a wide range of very different cells. In fact, malignant cancer cells tend to recruit different cell types in different stages of differentiation to the tumor bed, and then take advantage of each of their properties. Hanahan and Coussens grouped these cells into three categories: 1) angiogenic vascular cells (AVCs), (2) infiltrating immune cells (IICs) and cancer-associated fibroblasts (CAFs), (Hanahan & Coussens, 2012). In addition to all these different cells, the tumor microenvironment also encompasses the ECM and huge amounts of cytokines and growth factors (Figure 2.4) (Korkaya *et al.*, 2011).

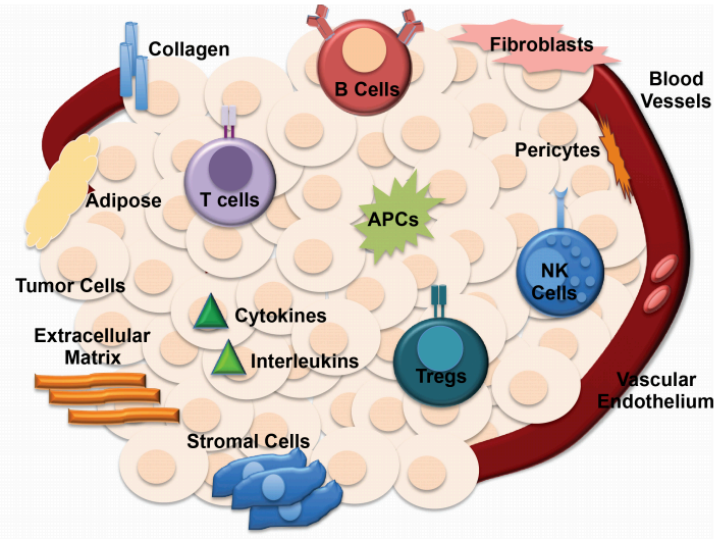

Figure 2.5 - The tumor microenvironment. The tumor microenvironment is composed of several different types of cells including immune cells, fibroblasts, mesenchymal stem cells and endothelial cells. Cytokines and the ECM are also present. Adapted from Casey *et al.*, 2015.

The tumor microenvironment is maintained by an intricate network of communication established between its different components. For instance, IICs and CAFs, which have been reported to release soluble mediators that act over endothelial cells, are key factor in regulating angiogenesis, thus maintaining nutrients income and wastes depuration. Alternatively, endothelial cells *per si* secrete a panoply of soluble factors that stimulate the self-renewal capacity of CSCs (Krishnamurthy *et al*., 2010; Lu *et al*., 2013). In the same line, Hasegawa and colleagues demonstrated that CAFs significantly increase the expression levels of CSC markers in a scirrhous gastric cancer cell line, through the activation of the TGF-β signaling pathway (Hasegawa *et al*., 2014). Finally, myofibroblasts-secreted hepatocyte growth factor is important in activating the Wnt pathway that mediates CSCs' formation through dedifferentiation in colon cancers (Vermeulen *et al*., 2010).

The principal role of the immune system is to protect the human body from its aggressors. However, the growing tumor rapidly modifies this system, thus co-opting immune cells to release factors that sustain tumor's growth (Hanahan & Weinberg, 2011). For instance, TAMs have been reported to be involved in angiogenesis and metastasis and recent results from Mitchem and colleagues revealed that the reduction in the number of TAMs proportionally reduces the number of CSCs in pancreatic cancer (Mitchem *et al*., 2013). Furthermore, Jinushi and collaborators showed that CSCs are the major activators of TAMs, inducing them to produce tumorigenic factors like IL-6 (Jinushi *et al*., 2012). Likewise, tumor necrosis factor alpha (TNF-α) secreted by TAMs in the tumor microenvironment altered the dynamic equilibrium between differentiated and dedifferentiated cells in melanomas (Landsberg *et al*., 2012).

The ECM, another member of the tumor microenvironment, assures the three-dimensional structure of the tumor and the dynamics of the tumor microenvironment. The reciprocal interaction of the cancer cells with the ECM and its components orchestrate all the tumorigenic process (Policastro *et al*., 2013). In agreement, the ECM has been reported to function as a blocking barrier for intratumoral diffusion, protecting the CSCs pool from chemotherapeutic agents (Wong & Rustgi, 2013). Moreover, Schrader and collaborators showed that the mechanical composition of the ECM influences hepatocellular carcinoma's progression, as a highly dense environment promotes cell differentiation and chemoresistance, while a softer ECM induces dormancy and promotes a more stem-like

phenotype and an enhanced clonogenic capacity (Schrader *et al*., 2011).

Mesenchymal stem cells are a very critical component of the microenvironment. These cells are responsible for the secretion of numerous cytokines, both with paracrine and autocrine functions. In addition, MSCs have been reported to produce a CSCs supportive cytokine called CXCL7 (Liu *et al*., 2011) and are implicated in several steps of the tumorigenic process (Cuiffo & Karnoub, 2012; Waterman *et al*., 2012).

Inside the tumor, CSCs inhabit an even more specific microenvironment, the so-called CSCs' niche. These niches are usually placed in the vicinity of vessel bifurcations and are essential for the regulation of CSCs' stemness and proliferation (Ye *et al*., 2014; Ishimoto *et al*., 2014). The first evidences of these niches came from Gilbertson's group that demonstrated that primary endothelial cells interacted selectively with CSCs through the secretion of soluble factors (Calabrese *et al*., 2007), and was later confirmed by Ritchie and Nör showing that CSCs localize preferentially close to the tumor vasculature (Ritchie & Nör, 2013).

2.5 Cytokine networks in the tumor microenvironment

The link between inflammation and cancer is an old concept that has been confirmed all over the last decades. Chronic inflammation is a known general cancer risk factor mainly because cytokines, the molecular mediators of inflammation, play a key role in sustaining malignant cells' viability and the tumor microenvironment (Pierce *et al*., 2009). Moreover, cells from tumor microenvironment *per si* are responsible for the production of a wide range of cytokines and growth factors, including IL-6, IL-8, G-CSF, TGF-β, VEGF and several ligands, that transform the tumor microenvironment into a pro-inflammatory ground extremely fertile for tumor growth (Ma *et al*., 2012).

The dedifferentiation process that may lie in the base of CSCs' formation seems to be a side-product of this cytokine-rich microenvironment. In agreement, Schwitalla and collaborators showed that microenvironmental NF-κB functions as regulator of CSCs' dedifferentiation (Schwitalla *et al*., 2013), while Liu and colleagues confirmed that the suppression of NF-κB in breast cancer tumors is associated with a reduction in CSCs'

population (Liu *et al*., 2010). More recently, our laboratory has shown that a paracrine loop mediated by IL-6, G-CSF and Activin-A and established between CAFs and malignant bronchial cells is enough to drive CSCs formation.

The most extensively studied inflammatory cytokine is IL-6. It is a pleiotropic cytokine that is produced by several different cell types like macrophages, B and T cells, fibroblasts and endothelial cells, and that is involved not only in immune responses and inflammatory reactions, but also in the pathophysiology of cancer (Rahim *et al*., 2015). In the tumor microenvironment IL-6 activates the Signal Transducers and Activators of Transcription (STAT) pathway, which is activated when IL-6 binds to GP130. GP130's activation, in turn, activates JAK1 and JAK2, which phosphorylate STAT3 (Guo *et al*., 2012). Interestingly, IL-6 has been reported to also signal via different signaling pathways like Notch/Jagged-1 and Wnt (Guo *et al*., 2012). As mentioned above, these signaling pathways are also associated with the CSCs' dedifferentiation process.

IL-6 has been established as a pro-angiogenic factor in several tumor types (Middleton *et al*., 2014). An example is the recent study of Nagasaki and colleagues showing that CAFs-secreted IL-6 promotes angiogenesis. Following IL-6 signaling blockage with an anti-IL-6 receptor antibody, these authors observed angiogenesis abrogation and tumor growth inhibition, with consequent metastatic cascade blockage (Nagasaki *et al*., 2014). Also relating IL-6 to the metastatic cascade are the works of Korkaya and collaborators pointing IL-6 as a major orchestrator of the EMT process in breast tumors, which in turn is the main source of CSCs', the drivers of metastasis (Korkaya *et al*., 2011).

High levels of serum IL-6 have been associated with poor patient outcome in different tumor types (Krishnamurthy *et al*., 2014; Kroon *et al*., 2013; Sanguinetti *et al*., 2015; Holmer *et al*., 2014), which is believed to depend upon IL-6-mediated NF-κB pathway activation (Yan *et al*., 2014b). Supporting this hypothesis are the studies of Yamada and collaborators showing that in biliary tract tumors IL-6 participates in a cross-talk with TGF-β that results in increased chemotherapy resistance mediated by a shift in *Smad4* functions (Yamada *et al*., 2013). Similar results were very recently attained by Nakamura's group using oral squamous cell carcinoma samples (Jinno *et al*., 2015).

Besides all the mechanisms in which IL-6 plays a key role, it is also implicated in CSCs'

biology. Krishnamurthy and collaborators showed that the secretion of IL-6 by the endothelial cells in the tumor microenvironment promotes the survival and self-renewal of head and neck CSCs (Krishnamurthy *et al*., 2014). In a similar manner, this cytokine was established as a direct regulator of CSCs' self-renewal through the activation of STAT3, as well as a key player in a positive feedback loop between MSCs and CSCs (Liu *et al*., 2011). Moreover, the amount of IL-6 in the tumor microenvironment seems to regulate the balance between CSCs and non-stem cancer cells in breast tumors (Dethlefsen *et al*., 2013), as Kim and colleagues have shown that non-stem cancer cells in breast tumors secrete IL-6 that activates the JAK1/STAT signal transduction pathway and increases CSCs' OCT-4 expression (Kim *et al*., 2013). Furthermore, evidence has been provided that IL-6 forms an autocrine loop in breast cancer cells, that requires the activation of the Notch signaling pathway, and promotes CSCs' self-renewal (Sanguinetti *et al*., 2015). A study from Kroon and collaborators reported that the JAK/STAT signaling pathway is constitutively active in prostate CSCs, which present elevated levels of IL-6 receptors, and that the blockage of this pathway leads to a decrease in the colony-forming ability of the cells (Kroon *et al*., 2013). In the same line, gliomas' CSCs were shown to produce higher levels of IL-6 than non-stem cancer cells and in addition, the invasion capacity of CSCs was attenuated using anti-IL-6 antibodies and increased upon addition of exogenous IL-6 (Qiu *et al*., 2013). Finally, Interferon regulatory factor 7, which regulates IL-6 secretion, induces glioma's stem cells formation and angiogenesis (Jin *et al*., 2012).

Another very important cytokine in the cross talk between CSCs and the tumor microenvironment is the granulocyte colony-stimulating factor (G-CSF). This cytokine is secreted by both endothelial cells and macrophages and is a major regulator of the survival, differentiation, proliferation and function of neutrophil precursor cells and mature neutrophils (Yan *et al*., 2013). After binding to the receptor protein, G-CSF activates several signaling pathways including the JAK/STAT, MAPK and PI3K/Akt pathways, some of them the same as IL-6 (Yan *et al*., 2013). More recently, G-CSF has been reported to also be produced by numerous types of tumor cells like in those isolated from liver, bladder and uterine cervix tumors (Yamamoto *et al*., 2013).

Several reports have been able to show that G-CSF may play an important role in cancer biology. Recently, G-CSF has been shown to be involved in the angiogenic process by the regulation of VEGF expression (Zhao *et al*., 2014). That has been observed in both breast

(Samineni *et al*., 2013) and hepatic tumors, where the high G-CSF was correlated with higher angiogenic and metastatic abilities (Im *et al*., 2013). Furthermore, neutrophils have been reported to have a pro-tumor function, being G-CSF, along with IL-6, one of their principal regulators. A recent report in fact showed that these two cytokines cooperate with each other to promote tumor growth and angiogenesis (Yan *et al*., 2013). Moreover, high levels of G-CSF and IL-6 have been reported to be secreted by bladder tumor cells (Matsuzaki *et al*., 2013), whereas in pancreatic adenocarcinoma MEK activation was identified as the major inducer of G-CSF secretion by cancer and stromal cells in the tumor microenvironment (Phan *et al*., 2013). Corroborating these findings, Byne and colleagues showed that the inflammatory response, which is associated with several types of cancer, is regulated by the G-SCF secreted by transformed epithelial cells in pancreatic ductal adenocarcinoma (Bayne *et al*., 2012). Earlier this year, a study performed by Beswick's group demonstrated that the inhibition of G-CSF in colon cancer might represent a potential therapeutic approach for those neoplasias (Morris *et al*., 2015).

The involvement of G-CSF in CSCs' biology has also been supported by numerous studies. Earlier this year, G-CSF has been shown to act as a CSCs' activating factor in glioblastoma through the induction of the STAT3 signaling pathway (Agarwal *et al*., 2015). Moreover, G-CSF in cooperation with stem cell factor (SCF) was demonstrated to upregulate the expression of several CSC markers in prostate cancer cell lines and to increase the sphere formation capacity of these cells (Ma *et al*., 2012).

Finally, despite all the others, there is another cytokine in the tumor microenvironment with relevant actions both in terms of tumor dynamics and CSCs' biology, called Activin-A. It is a TGF-β superfamily member that plays a major role during embryogenesis, being involved in mesoderm cell fate differentiation and reproduction (Loomans & Andl, 2014). After birth the expression of Activin-A is reduced to very low levels. Like the majority of the members of its superfamily, Activin-A binds initially to Activin-A receptors type 2, then recruiting Activin-A receptors type 1 (ALK4) (Blank & Karlsson, 2015). The signaling cascade triggered by Activin-A has a canonical and a non-canonical pathway. In the canonical pathway after Activin-A binding to ALK4, the protein complex then recruits Smad2/3, which are phosphorylated in the cytoplasm with the aid of Smad4 and then translocated into the nucleus, where they will impact gene transcription. On the other hand,

the non-canonical pathway has been associated with Wnt/β-catenin, PI3K/Akt and MAPK pathways' activation (Loomans & Andl, 2014).

TGF-β has been reported to be a potent inducer of EMT, and consequently, of cancer progression through the Smad signaling pathway, which is also activated by Activin-A (Cufí *et al*., 2010). Recent evidence shows that Activin-A activities may depend upon this cytokine's levels, and may be more complex than initially thought, as a wide range of reports in the literature associate this cytokine with different steps of the tumorigenic process. In agreement, Activin-A has been identified as an intervenient in the development of an invasive phenotype (Loomans & Andl, 2014), and Kelner and colleagues demonstrated that immunodetection of Activin-A correlates with lymph node metastasis in oral squamous cell carcinoma (Kelner *et al*., 2015). Similarly, in esophageal tumors, N-cadherin expression on the tumor cell surface is mediated by Activin-A and is associated with higher aggressiveness (Yoshinaga *et al*., 2004), and the same cytokine seems to be responsible for the upregulation of MMP-7, which degrades the ECM thus facilitating cell invasion and metastasis (Yoshinaga *et al*., 2008). Conversely, the inhibition of Activin-A in malignant mesothelioma was shown to prevent invasive growth (Tamminen *et al*., 2015).

Earlier this year Activin-A was shown to enhance pancreatic tumors' growth through a non-Smad signaling pathway (Togashi *et al*., 2015). In addition, the maintenance of the balance of Activin-A levels in the tumor microenvironment has been suggested to be involved in the conservation of epithelial homeostasis in esophageal tumors (Le Bras *et al*., 2014). Finally, Activin-A was also implicated in angiogenesis. Loomans and Andl observed that the overexpression of Activin-A in fibroblasts leads to the downregulation of VEGF, and consequently angiogenesis, in a pre-neoplastic microenvironment. However, in the presence of malignant cells, this same cytokine further supports tumorigenesis (Loomans & Andl, 2014). However, the action of Activin-A is not linear, and a wide range of controversial reports have been published in the recent years claiming for a dual action of Activin-A, most probably cell type and context-specific dependent. Supporting this hypothesis, Krneta and collaborators showed that *in vivo* breast cancer cells expressing Activin-A develop larger but less vascularized tumors in comparison to the situation in which Activin-A is inhibited (Krneta *et al*., 2006). Moreover, Islam and collaborators showed that Activin-A reduces cell proliferation in myometrium cells, but the same was not observed in leiomyoma cells (Islam *et* al., 2014).

More studies are needed to clarify the exact signaling pathways evoked by Activin-A, as well as the consequences derived from their activation.

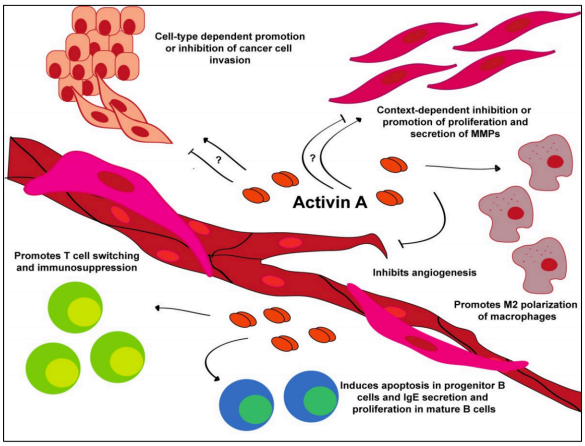

Figure 2.6 - Interactions of Actvin-A in the tumor microenvironment. Depending on the cell type and the context, Activin-A can play several different roles. Adapted from Loomans & Andl, 2014.

Considering Activin-A's involvement during embryonic development, it seems plausible to hypothesize that this cytokine may play a role in CSCs' biology. According to Lonardo and colleagues Activin-A in association with Nodal, another member of the TGF-β superfamily, were overexpressed in pancreatic CSCs, and the inhibition of the Activin/Nodal receptor Alk4/7 leads to a decrease in the self-renewal capacity of these cells (Lonardo *et al.*, 2011). More recently, a stem-like subtype of cells in hepatocellular carcinomas was identified, and their characterization resulted in the identification of a signaling circuit that includes the Activin-A receptor ACVR1, thus suggesting the involvement of Activin-A in the maintenance and formation of CSCs (Li *et al.*, 2015). In the same line, Activin-A and Nodal were reported to be secreted by stellate cells present in the tumor microenvironment promoting self-renewal capacity and invasiveness of pancreatic CSCs. This study further suggests that *in vivo* CSCs are stimulated both in a paracrine and autocrine manner by Activin-A and Nodal (Lonardo *et al.*, 2012). Finally, early this year, the miR-17-92 cluster was shown to regulate pancreatic CSCs through the targeting of different components of the Activin/Nodal pathway (Cioffi *et al.*, 2015), while in non-small cell lung cancer, the self-renewal capacity of CSCs has been demonstrated to be regulated by the upregulation of Activin-A due to NF-κB signaling during EMT (Wamsley *et al.*, 2015). Considering the accumulated knowledge on Nodal-Activin-A

interaction, Pauklin and Vallier recently proposed that the Activin/Nodal/TGF-β pathways may function as a link between cycle, self-renewal and differentiation in CSCs (Pauklin & Vallier, 2015).

At the end it is possible to conclude that there is a tightly regulated cytokine network between cancer cells and the tumor microenvironment with tremendous impact in tumor biology. Further research needs to be done to clarify the roles of each of these cytokines in the different steps of the tumorigenic process, which eventually may allow the design of new therapeutic strategies to target tumors.

Hypothesis and Goals

Recently, our laboratory has shown that a paracrine loop mediated by IL-6, G-CSF and Activin-A established between CAFs and malignant bronchial epithelial cells is enough to drive CSCs formation (Rodrigues *et al*., 2013). This project now aims to assess the molecular mechanisms underlying cytokine-mediated CSCs formation through dedifferentiation. It will focus on understanding the dynamics of cytokines' release, providing special attention to the roles of both the ECM and exosomes in the overall process. The ultimate goal is to understand the dynamics of IL-6, G-CSF and Activin-A's release and the implication of the ECM and exosomes in the overall process;

The expected results are believed to improve our understanding of cancer biology, in particular of the role played by CSCs in tumor progression. Moreover, they are expected to allow the envision of new targeted and more effective therapeutic approaches against cancer, with potential impact on clinical practice patients' care.

Chapter 3

Material and Methods

3.1 Reagents, Solutions and Mediums

The reagents used during the whole project in the various experiments were acquired from Sigma-Aldrich (Sintra, Portugal) unless stated otherwise. All cellular culture media used throughout this work were from Gibco® (Life Technologies, Carlsbad, CA, USA). Whenever water was required for the preparation of aqueous solutions, ultrapure water (mili-Q) was used. All the plastics that were used were obtained from either Corning (Lowell, MA, USA) or SPL Life Sciences (Eumhyeon-ri, Korea).

3.1.1 Gelatin Coating Solution

According to the protocol established in the laboratory, the E2A fibroblasts used in this work should be cultured over a gelatin solution coat. For its preparation commercialized 2 % type-B bovine skin gelatin solution was diluted in 45 % of 1x-phosphate-buffered saline (PBS) and 5 % of 2 % bovine serum albumin (BSA) solution.

3.1.2 DMEM Cell Culture Medium Supplemented with 10 % FBS

For every 500 mL of this medium, 444.9 mL of DMEM cell culture medium were mixed with 50 mL of fetal bovine serum (FBS), 5 mL of penicillin (5000 U/mL)-streptomycin (5000 µg/mL) and 100 µL of amphotericin B.

3.1.3 CSCs' Isolation Medium

In order to prepare the matrix of this medium 250 mL of DMEM:F12 (1:1) cell culture medium were mixed with 5 mL of penicillin (5000 U/mL)-streptomycin (5000 μg/mL), 10 μL of 1 mM progesterone solution and 5 mL of the commercialized insulin, transferrin, selenium (ITS) sodium pyruvate solution (#41400045, Gibco). Additionally, 0.08 g of putrescine together with 0.6 g of sodium bicarbonate ($NaHCO_3$) was dissolved in the liquid phase. To complete the final volume of 500 mL a 2 % methylcellulose solution was used.

3.1.4 Preparation of Low Adherence 6-well Plates for CSCs' Isolation

For the isolation of CSCs the culture conditions have to be of low adherence. This can be achieved either by buying commercially available low adherence supports or by preparing them in the laboratory. For the sake of this project the low-adherence 6-well plates were prepared by coating regular 6-well plates with a 2 % poli-(2-hydroxyethyl methacrylate) (poli-HEMA) solution.

To prepare the coating solution, 2 g of poli-HEMA were added to 100 mL of 95 % ethanol and allowed to dissolve with mild agitation for 8 hours at room temperature (RT). After completing the dissolution, 400 μL of this coating solution were added to each well of a 6-well plate and allowed to dry at room temperature over a stable bench in a sterile environment. After complete drying, plates were further sterilized by exposure to UV light for 20 minutes. Whenever the plates were not immediately utilized, they were sealed with Parafilm® and stored at 4^{o} C.

3.1.5 N2 Medium for CSCs' Growth

To prepare 500 mL of N2 medium, 5 mL of penicillin (5000 U/mL)-streptomycin (5000 μg/mL), 5 mL of the ITS solution (#41400045, Gibco®), 100 μL of amphotericin B and 10 μL of the 1 mM progesterone solution were mixed. Afterwards, 0.6 g of $NaHCO_3$ and 0.08 g of putrescine were dissolved in the medium. Sterility was guaranteed through filtration.

3.1.6 Freezing Solution

Cells were frozen in a mixture of cell culture medium, FBS and dimethyl sulfoxide (DMSO) at a proportion of 7:2:1.

3.2 Cells and Cell Culture Procedures

All the cell lines used in this project were already available in our lab. Some of them were produced as a part of a previous project using original protocols.

3.2.1 RenG2 Cell Line

The RenG2 cell line was established as an integrating part of a previous project of our group. For its attainment, the commercially available Bronchial Epithelial Airway System-2B (BEAS-2B), obtained from the European Collection of Cell Cultures (ECCAC, Salisbury, UK; ECCAC no. 95102433), was cultured at very low density in the presence of 1.0 μM of hexavalent chromium [Cr (VI)]. The resulting morphologically altered cells, showed an abnormal growth pattern and increased tumorigenic potential (Rodrigues *et al*., 2013).

3.2.2 Human Bronchial Fibroblast Cell Line (E2A)

In order to reproduce previous studies using human cells, this primary human cell line of bronchial fibroblasts was previously established in the laboratory out of a biopsy sample of lung tissue collected from Centro Hospitalar Universitário de Coimbra, with patients' written consent. For its establishment tissue fragments removed from the biopsy were washed with PBS several times and fractioned into smaller pieces that were then distributed amongst several T_{25} cell culture flasks. In order to provide the small fragments with nutrients and to help them adhere to the flask, a small drop of FBS was added next to each fragment. After turning the flask upside-down, 5 mL of DMEM supplemented with 10 % FBS was added to the top surface of the flask. The fragments were allowed to attach in this upside-down position for 24 hours at 37^{o} C being subsequently turned gently to the up-right position, thus allowing the cells to contact with the cell culture medium. During the subsequent days, cells slowly started to detach from the fragments and formed a monolayer at the bottom surface of the cell culture flask. Following trypsinization, cells were sub-cultured, amplified and several aliquots were cryopreserved for subsequent use. A 0.25 % trypsin-1 mM EDTA solution (Biochrom, Cambridge, UK) was used whenever cultures reached 80 % confluence.

3.3 Extracellular Matrix Isolation

To investigate if the cytokines under study, IL-6, G-CSF and Activin-A, are being stored in the ECM, this structure was isolated from living cells. To this end, confluent E2A fibroblasts were mitotically inactivated with a 12 μg/mL mitomycin C solution for 2.5 hours at 37^{o} C and then rinsed 5 times with PBS. Subsequently, fresh culture medium was added to the cells and they were maintained in culture for 2 more weeks aiming ECM depositing (Soteriou *et al.*, 2013). The medium was changed once a week.

After the 2 weeks ECM-producing period, ECM was isolated using the protocol

established by Meng and collaborators (Meng *et al*., 2010). Briefly, cells were stored overnight at -80 °C in cell culture medium. Subsequently, after complete thawing, the medium was removed and the ECM was carefully rinsed with PBS and air-dried in the laminar flow hood. The ECM was then treated with DNAse (6 μg/mL) for 1 hour at 37° C, carefully rinsed with PBS and stored for future use.

3.4 Co-culture of RenG2 cells with E2A fibroblasts

To assess the hypothesis that the cytokines of interest were being released by the fibroblasts through exosomes, E2A cells were co-cultured with RenG2 cells using 4.5 cm^2 Transwell® inserts (CLS3450, Corning and SPL Life Sciences) as illustrated in Figure 3.

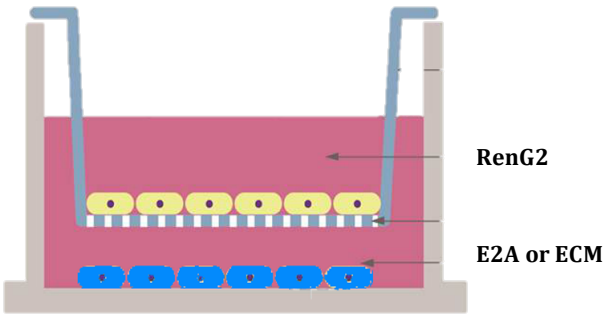

Figure 3 - Co-culture system design. RenG2 were co-cultured with E2A or isolated ECM for 2 months, using a 6-well plate adapted transwell system. While E2A cell line or the isolated ECM were cultured in the lower compartment, RenG2 cells were cultured in the upper one.

The same culture strategy was employed to dissect the possibility of ECM being used as a cytokine's reservoir. In that case, however, the previously isolated ECMs were plated on microscope coverslips placed in the bottom compartment of the co-cultures, instead of the E2A fibroblasts.

In any case, the co-cultures were maintained for 8 weeks in the incubator, changing and collecting the conditioned media from both compartments every 2 weeks. Collected media were preserved and stored at -80 °C until further use. After the co-culture period the

RenG2 cells were examined for their ability to form spheres through the sphere-forming assay as discussed in section 3.5.

Several abbreviations were used to facilitate the readings of the conditioned media of the several different conditions used in the employed co-culture system. Abbreviations are resumed in Table I.

Table I- Abbreviations of the several different conditions used in ELISA

E2A	Normal E2A medium before co-cultures' start
cE2A n weeks	Conditioned E2A medium n weeks after co-culture initiation
cRenG2 n weeks	Conditioned RenG2 medium n weeks after co-culture initiation

3.5 Sphere-Forming Assay

In order to identify the presence of CSCs amongst the RenG2 cells after co-culture with the E2A fibroblasts, the sphere-forming assay was used as previously established in the laboratory. This assay allows the isolation of CSCs by taking advantage of their intrinsic ability to form tridimensional spheres when cultured under low-adherence conditions with appropriate mediums and supplements, and it has been used to isolate CSCs from several different tumor types, including lung tumors.

CSCs' isolation medium and the low adherence 6-well plates were prepared as previously described in sections 2.1.4 and 2.1.5. Subsequently, a cellular suspension of $3x10^4$ cells/mL was prepared in 2 mL of the isolation medium and added to each well of the plate. Every two days, 10 ng/mL of both human EGF and bFGF (100-18B, PeproTech, London) were added to each well and the plates were kept in the incubator.

According to the literature, a pure clonal-derived CSCs population is only attained at the third generation of spheres. After approximately 15 days of culture the spheres reached a satisfactory size, thus being ready to be collected, washed with PBS, disaggregated and plated in a T_{25} cell culture flask with 5 mL of N2 medium. When cells reached 80 % confluence the

protocol of isolation was repeated in order to obtain second-generation spheres. This protocol was repeated three times to obtain the third generation of spheres. As a control in this experiment, the cell line MCF-7 was used, as its ability to form spheres is well documented in the literature (Rodrigues *et al*., 2013; Morrison *et al*., 2012).

3.6 Exosomes Isolation

To further test the hypothesis that the target cytokines may be being released by the E2A fibroblasts through exosomes, the conditioned media of the co-cultures were collected and their exosome content was isolated and purified. The implemented protocol was the original and most commonly used protocol for exosome purification established in 1996 by Raposo and collaborators (Raposo *et al*., 1996). Briefly, the first step consists in using successive centrifugations at increasing speed in order to eliminate large cellular debris. Subsequently, the final supernatant is ultra centrifuged at 100'000 G for 70 minutes to pellet the exosomes. The pellet was then washed abundantly with PBS in order to eliminate contaminating proteins, and centrifuged one last time at the same high speed (Théry *et al*., 2006). Isolated exosomes were stored at -20^{o} C until further use.

3.7 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA procedure was used to determine the presence of IL-6, G-CSF and Activin-A either in the co-cultures' conditioned media, or in the contents of exosomes isolated from the same co-cultures. To this end, the Human/Mouse/Rat Activin-A Quantikine ELISA Kit (#DAC00B, R&D Systems, Minneapolis, MN, USA), the Human IL-6 Quantikine ELISA Kit (#D6050, R&D Systems, Minneapolis, MN, USA) and the Human G-CSF Quantikine ELISA Kit (#DCS50, R&D Systems, Minneapolis, MN, USA) were acquired and used to process the collected media samples according to manufacturer's instructions.

To assess the cytokines in the collected co-culture media, samples were diluted in assay diluent provided by R&D in the ELISA kits, processing it according to manufacturer's

instructions.

The isolated exosomes' protein content was assessed through Bradford assay (#5000006, Bio-Rad, California, USA). To assess the cytokine levels in the exosomes isolated from the collected culture media, the same protocol performed by Subra and colleagues was performed (Subra *et al*., 2010). Briefly, 50μg of exosomal protein were incubated with 5μL protease inhibitor cocktail in 1 mL of PBS for 10 minutes at RT. After this the samples were sonicated (2 x 10s, VWR Ultrasonic Cleaner) in order to permeabilize them. This being done, ELISA was processed according to manufacturer's instructions.

3.8 Immunocytochemistry

Since antibody-based assays are very powerful techniques to identify cellular constituents, in order to assess an eventual non-exosome-mediated release of the cytokines under study, direct immunofluorescence was also performed in the decellularized E2A fibroblasts after co-culture. Additionally, exosomes' presence in the decellularized E2A fibroblasts was also assessed through direct immunofluorescence using CD63 and Rab27 markers. To this end, the microscope coverslips placed under the fibroblasts/ECM in the bottom compartment of the co-cultures were collected after the culture period and washed with PBS. Following fixation with ice-cold methanol, they were washed with a PBS-0.1 % BSA solution and then blocked and permeabilized using a PBS-1 % BSA-0.1 % Triton X solution for 45 minutes at RT. Subsequently, the coverslips were again washed with the PBS-0.1 % BSA solution and incubated with the primary antibody (1:500) for 1.5 hours at RT. After incubation, a new washing step was performed and the samples were then incubated with the secondary antibody for 1 hour at RT.

The human/mouse/rat Activin A beta A subunit antibody (#Mab3381, R&D Systems, Minneapolis, MN, USA), human G-CSF antibody (#Mab214, R&D Systems, Minneapolis, MN, USA), human/primate IL-6 antibody (#Mab206, R&D Systems, Minneapolis, MN, USA), human CD63 antibody (#Mab5048, R&D Systems, Minneapolis, MN, USA) and human Rab27 antibody (#AB7223-200, SICGEN, Cantanhede, Portugal) were all used in this project. Incubation with all these antibodies was performed for 1.5 hours at RT. The secondary antibodies (1:1000) used in

this project were Donkey anti-Mouse IgG Alexa Fluor® 488 conjugate (#A-21202, Life Technologies, Carlsbad, CA, USA) and Donkey anti-Goat IgG Alexa Fluor® 488 conjugate (#A-11055, Life Technologies, Carlsbad, CA, USA). Incubation was performed for 1 hour at RT.

Finally, and after washing the coverslips one last time, they were mounted onto microscope slides with DPX mounting medium. Cells were observed in a Nikon Eclipse 80i microscope and photographs were taken using a Nikon Digital DXM1200F coupled camera.

3.9 Data Processing and Statistics

All the data collected in this work was attained from experiments carried out in triplicate, and the statistical analysis of the results was carried out using the Graph Pad Prism software version 7 (GraphPad Inc., San Diego, CA, USA). Whenever possible, data normality was tested using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillefor correction. To assess statistical significance between means of the cell lines under study a one-way ANOVA was used followed by a Bonferroni post test. The threshold of significance for the differences was defined at a 5 % level and the *P* value was categorized according to their interval of confidence.

Chapter 4

Results

Exosomes, ECM and cytokines' release

4.1 Introduction

As described in Chapter 2, the inability of the classic stochastic model to explain tumors' heterogeneity drove the development of alternative theories, namely the hierarchical theory of cancer (So & Suh, 2015). As a consequence, tumors are actually understood as living organs, composed not only of malignant cells but also the tumor microenvironment, which, *per si*, has an important role in CSCs' formation and maintenance (Hasegawa *et al*., 2014). In order to assess tumor-stroma intercellular communication, co-culture systems have been developed and widely used in recent years. The choice of the co-culture type that best suits each experiment depends on the goal to be attained with the experiment. For instance, Transwell® systems are the best to analyze paracrine signaling as they allow two different cellular populations to communicate without physical contact. In fact, with this co-culture system, the soluble factors-enriched conditioned media may be collected after the experiment and subsequently used for further studies.

The present study used a Transwell® co-culture system of RenG2 human bronchial epithelial cells with E2A human bronchial fibroblasts. It aimed to reproduce the *in vivo* conditions where the tumoral cells are allowed to communicate with the

tumor stroma, thus permitting to dissect the mechanism behind the previously observed dedifferentiation process possibly involving IL 6, Activin A and G-CSF.

4.2 RESULTS

4.2.1 Co-culture of RenG2 with either E2A or E2A's ECM

To assess the hypothesis that the dedifferentiation of RenG2 cells was driven by exosomes-mediated cytokines' released by the E2A cells, both cells were co-cultured in a Transwell® device. Moreover, to observe if the ECM acted as a reservoir of the previously identified cytokines, thus allowing their release only upon stimulus from the RenG2 cells, the same co-culture strategy was performed, but with E2A's isolated ECM in the lower compartment.

Co-cultures were maintained for 8 weeks, after which the RenG2 cells were isolated and tested for their ability to form spheres as described in section 3.5. Approximately 6 days after cells were seeded into the low-adherence plates with the N2 medium, several small spheres appeared in both co-cultures (Figure 4). Interestingly, the RenG2 cells isolated from the co-culture with E2A's ECM yielded more and slightly bigger spheres than the RenG2 cells extracted from their culture with the E2A fibroblasts (Figure 4).

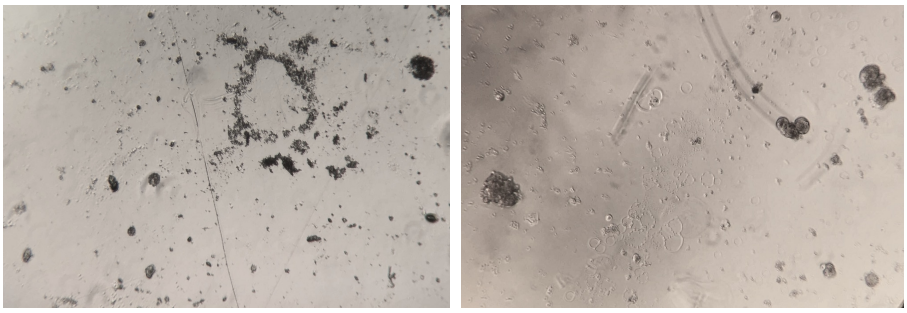

Figure 4- Representative image of the spheres formed by RenG2 cells after co-culture. RenG2 cells started to form spheres approximately 6 days after their isolation from the co-culture system, both in the co-cultures with E2A fibroblasts (left image) and with the isolated ECM (right image). A magnification of 100x was used in both panels.

RenG2 cells do have malignant potential. However, as previously demonstrated by our group, the tumorigenicity of these cells is mild and they did not show the capacity to form spheres (Rodrigues *et al*., 2013). Nonetheless, the results attained in the presented work show that the RenG2 cells isolated after 8 weeks of co-culture, either with E2A of isolated ECM, re-write their internal circuitry and change their characteristics thus adopting a more aggressive phenotype, as suggested by their ability to form spheres. In other words, the interaction of the RenG2 cells with soluble factors released from the feeder layer compartment altered their stem potential.

The fact that the co-culture of RenG2 cells with the isolated E2A's ECM led to the acquisition of sphere-forming capacity in RenG2 cells supported the underlying hypothesis that the ECM alone may act as a reservoir for cytokines.

4.3 Discussion

As discussed in Chapter 2, one of the major recent breakthroughs in cancer biology was the discovery of cells within the tumor that possess stem-like properties. Analogously to normal stem cells, these CSCs have self-renewal and multilineage differentiation capacities being maintained by a complex communication with cells of the tumor microenvironment (Medema, 2013, Vermeulen *et al*., 2012). The origin of CSCs however is still debatable being the dedifferentiation of tumor cells rendering them more resistant and undifferentiated due to microenvironmental cues the most appealing theory. In agreement, several research groups, including ours, have recently shown that paracrine signaling released by microenvironmental cells is able to drive the dedifferentiation process (Rodrigues *et al*., 2013; Hanahan & Weinberg, 2011; Medema, 2013).

Chaffer and colleagues firstly observed the dedifferentiation hypothesis when they demonstrated that differentiated mammary epithelial cells can, through a stochastic process, reconvert to a stem-like state (Chaffer *et al*., 2011). Since then, the dedifferentiation of terminally differentiated cells into a stem-like state due to factors secreted by the tumor

microenvironment has been shown by several groups in different types of tumors. In accordance, Peng and collaborators recently demonstrated that breast cancer associated myeloid-derived suppressor cells are able to enrich the CSCs population through IL-6 secretion (Peng *et al*., 2016).

Growing evidence has proved the important role played by the ECM in the maintenance and proliferation of neural and mesenchymal stem cells (Fietz *et al*., 2012; Zhou *et al*., 2016). Furthermore, in recent years, the ECM has been shown to play a major role in the tumorigenic process, particularly in its orchestration. It has been demonstrated that the composition of the ECM alone influences hepatocellular carcinoma's progression, inducing dormancy and promoting a more stem-like phenotype and an enhanced clonogenic capacity (Schrader *et al*., 2011). In the same line of thought, the acquired ability to form spheres observed in the RenG2 cells isolated from a co-culture system with E2A's isolated ECM strongly suggests that the ECM acts a principal orchestrator in dedifferentiation of RenG2 cells. This is in line with the studies of Schraeder and colleagues, as well as with those from Kumar's group, showing that ECM proteolysis is a key factor in regulating the number of CSCs within a tumor (Kumar *et al*., 2016; Schraeder *et al*., 2011).

The concept of ECMs working as a storage space for signaling molecules is not new and has been the target of many research projects in the past recent years (Schönherr & Hausser, 2000; Wiranowska & Plaas, 2008; Takawale *et al*., 2015). Corroborating those studies, the present results point for E2A cells' ECM as the reservoir for IL-6, G-CSF and Activin-A, as it alone is able to drive RenG2 cells' dedifferentiation. Furthermore, the fact that one of the cytokines involved in RenG2 cells' dedifferentiation, IL-6, is an inflammatory cytokine associated with almost every step of the tumorigenic process, further supports our hypothesis, as its storage in the ECM possibly represents a biological protection strategy, as envisioned by De Jong and colleagues (De Jong *et al*., 2014).

As important as proving that the ECM is able to store cytokines is to understand how are these cytokines stored and subsequently released. Several groups have already shown that the ECM extracted from a wide range of tumors may not only store loose cytokines, but also extracellular vesicles, namely exosomes (Clayton *et al*., 2004). In agreement, fibroblasts have

been reported to secrete exosomes to mediate their communication with tumor cells in different tumor types, as reviewed by Zhao and colleagues (Zhao *et al*., 2016). Moreover, Hu's laboratory showed that the exosomes released by fibroblasts in colorectal cancer are able to increase the sphere-forming ability and chemoresistance of the tumor cells, thus supporting the hypothesis pointing their involvement in the dedifferentiation process (Hu *et al*., 2015). Once again, in this line of thought, the results attained in the present work suggest that IL-6, G-CSF and Activin-A are stored inside exosomes in E2A cells' ECM and are released to the media upon stimulation by RenG2 cells, driving their dedifferentiation.

4.4 Conclusion

Analogously to what happens *in vivo* where the tumor microenvironment plays a vital role in tumorigenesis, namely in the dedifferentiation of CSCs, the strategy employed here showed the vital role of the tumoral stroma in CSCs' biology. The co-culture system used clearly showed the acquisition of stem potential by RenG2 cells, due to cues released by the human fibroblasts. Additionally, it seems that the ECM indeed functions as a reservoir for cytokines and exosomes that induce the dedifferentiation of RenG2 cells, thus being released upon stimulus released by RenG2 cells.

Chapter 5

Results

ECM: The Hidden Reservoir

5.1 Introduction

Intercellular communication can be achieved by several different ways, namely by the release of soluble factors such as cytokines and chemokines, or through the secretion o microvesicular bodies like exosomes (Mittelbrunn & Sánchez-Madrid, 2012). Paracrine communication is probably the most important intercellular communication process, so it came as no surprise that the tumor utilizes it in its favor.

It wasn't until recently that extracellular vesicles secreted by plant and mammals' cells have been identified to transport genetic material, exchanging it with other cells. Depending on the origin of these extracellular vesicles, they can be identified as exosomes, apoptotic bodies or shedding vesicles (Théry *et al*., 2009). Exosomes, which surge from the intraluminal budding of multivesicular bodies, are 30-100 nm in size and may contain DNA, miRNA, mRNA, lipids and/or proteins (Languino *et al*., 2016). Moreover, these vesicles can now be identified by the presence of specific proteins in their membrane, for example, CD63 (Théry *et al*., 2006).

The association of exosomes' transfer between cells and the communication between tumor cells and the tumor microenvironment came naturally. In this line, Languino and colleagues demonstrated that the exosome-mediated communication between tumor cells and the tumoral stroma leads to an increase in the TGF-β

signaling in oral cavity squamous cell carcinoma (Languino *et al*., 2016). Furthermore, cancer cells'-derived exosomes have been demonstrated to contain different biomolecules when compared to exosomes secreted by normal cells (Hannafon & Ding, 2013), more specifically, miRNAs have been shown to be enriched in cancer-derived exosomes (Melo *et al*., 2014). Despite being a relatively recent field of study, the association between exosomes and CSCs has already been made. Ramteke and collaborators observed that CAFs-secreted exosomes under hypoxic conditions induced changes in the tumoral microenvironment, increasing the aggressiveness of the prostate tumor by promoting the growth of the CSCs' population (Ramteke *et al*., 2015). Moreover, exosomes secreted by pre-adipocytes present in the tumor microenvironment potentiate the mammosphere formation capacity, thus promoting CSCs' renewal in breast cancer (Gernapudi *et al*., 2015).

One of the most powerful techniques to dissect the cellular constituents is the antibody-based immunocytochemistry. This sensitive technique allows observing the presence of any element in the cell and/or in the ECM, namely cytokines and extracellular vesicles/exosomes, using specific antibodies targeted against specific proteins.

The ELISA assay presents innumerous advantages in comparison to other immunoassay methods. The high sensitivity and specificity of ELISA is its strongest weapon as no other immunoassay method compares to it on this matter. These characteristics allow us to identify cytokines, for instance, at very low concentrations.

To further investigate the involvement of exosomes and ECM in cytokines' storage and release, a combination of immunocytochemistry and ELISA were performed.

5.2 Results

5.2.1 Immunocytochemistry Analysis of Exosome-related Proteins

Growing evidence in past decades has demonstrated that the ECM is a vital player in the tumor microenvironment and that it is responsible, through complex paracrine intercommunication loops with the tumor cells, for the appearance and maintenance of CSCs (Ye *et al*., 2014). In this intercommunication between tumor cells and its microenvironment, several molecules can be secreted either by cells in the tumor or by the tumor microenvironment itself, namely cytokines (Ma *et al*., 2012).

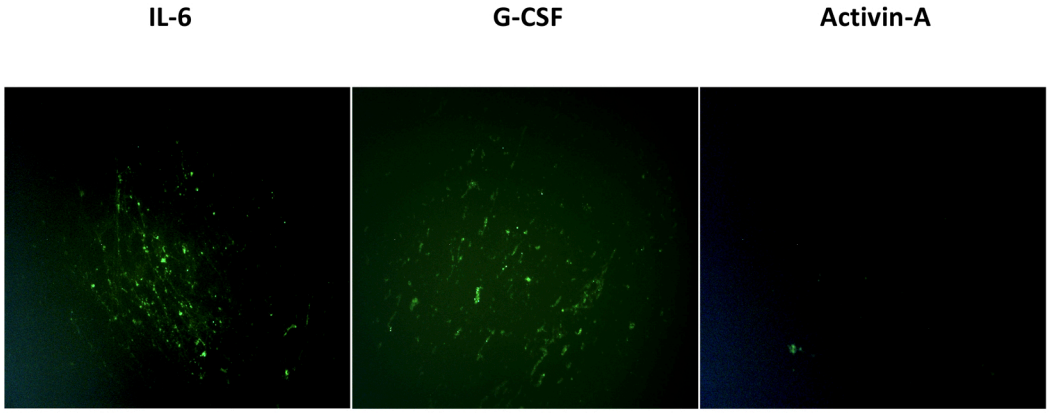

Figure 5.1- Representative images of the immunocytochemistry study of IL-6, G-CSF and Activin-A in E2A's co-cultured ECM. IL-6 and G-CSF were present in a pattern compatible with the ECM mesh. On the other hand, Activin-A did not show any specific fluorescence. A magnification of 630x was used in all panels.

Considering the hypothesis proposing that exosomes may be involved in the acquired stem potential of RenG2 cells by E2A-released IL-6, G-CSF and Activin-A, immunocytochemistry against the three cytokines was performed on the coverslips placed in the bottom chamber of the co-culture system. As depicted in Figure 5.1, IL-6 and G-CSF were undoubtedly present in the ECM extracted from the E2A fibroblasts; Activin-A, however, was not detected.

Immunofluorescence against the cytokines under study demonstrated the presence of IL-6 and G-CSF in the E2A fibroblast's ECM co-cultured with RenG2 cells. Activin-A, however, did not show any fluorescent signal similar to what was observed for the other cytokines. Taken into account that the same secondary antibody was used for the three cytokines' detection, the results observed for Activin-A support the conclusion against this cytokine's presence in E2A cells' ECM.

To indirectly assess the presence of exosomes stored in the ECM, the same procedure was implemented using primary antibodies against Rab27 and CD63. The first is a vastly described GTPase involved in the process of exosome release; CD63, on turn, is a well-known marker of intracellular vesicles' membranes in general and of exosomes in particular. As demonstrated by figure 5.2, both CD63 and Rab27 were present in the ECMs analyzed, as both markers revealed a strong staining. DAPI was used as an ECM marker, as previous experience from the laboratory as shown its retention in the ECM mesh.

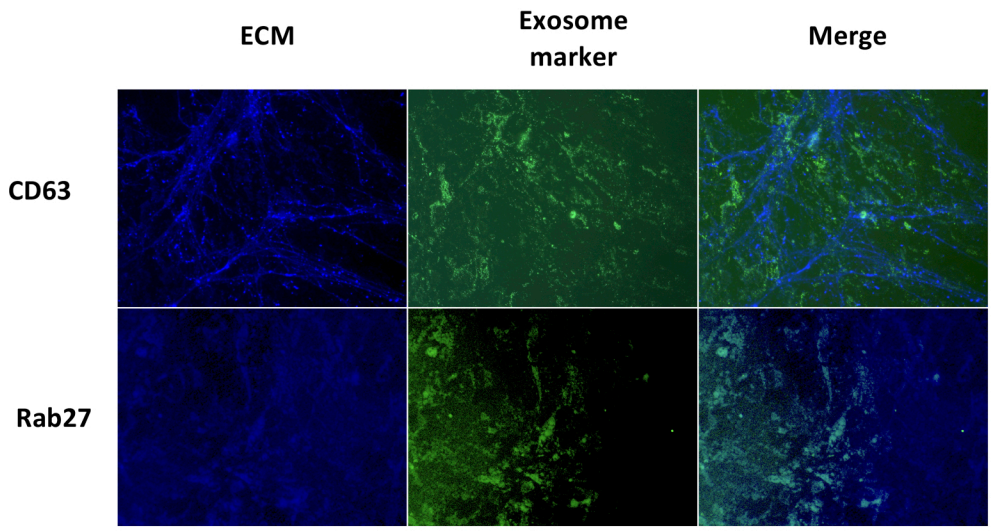

Figure 5.2- Representative images of the immunocytochemistry study of the exosome markers CD63 and Rab27. Merge images of the exosome markers (green) and the ECM structure (blue). A magnification of 630x was used in all panels.

The results observed in figure 5.2 suggested the presence of exosomes in the ECM as the staining of both markers, specially CD63, was very strong allowing to

observe the exosomes' distribution in the ECM's structure. Furthermore, Rab27 staining was also observable, however, less than CD63, suggesting that exosomes may not only be present in the ECM, but also released to the surrounding cell culture media, thus influencing tumor cells.

5.2.2 ELISA Analysis of Exosomal Contents and Conditioned Media

To definitely prove the presence of IL-6, G-CSF and Activin-A in the exosomes released by the E2A fibroblasts and/or their ECM, an ELISA assay directed towards each cytokine was used to assess the contents in the exosomes isolated from the co-culture's conditioned media. Figure 5.3 shows the concentration of the cytokines in study in the content of the exosomes isolated from the co-culture's conditioned media.

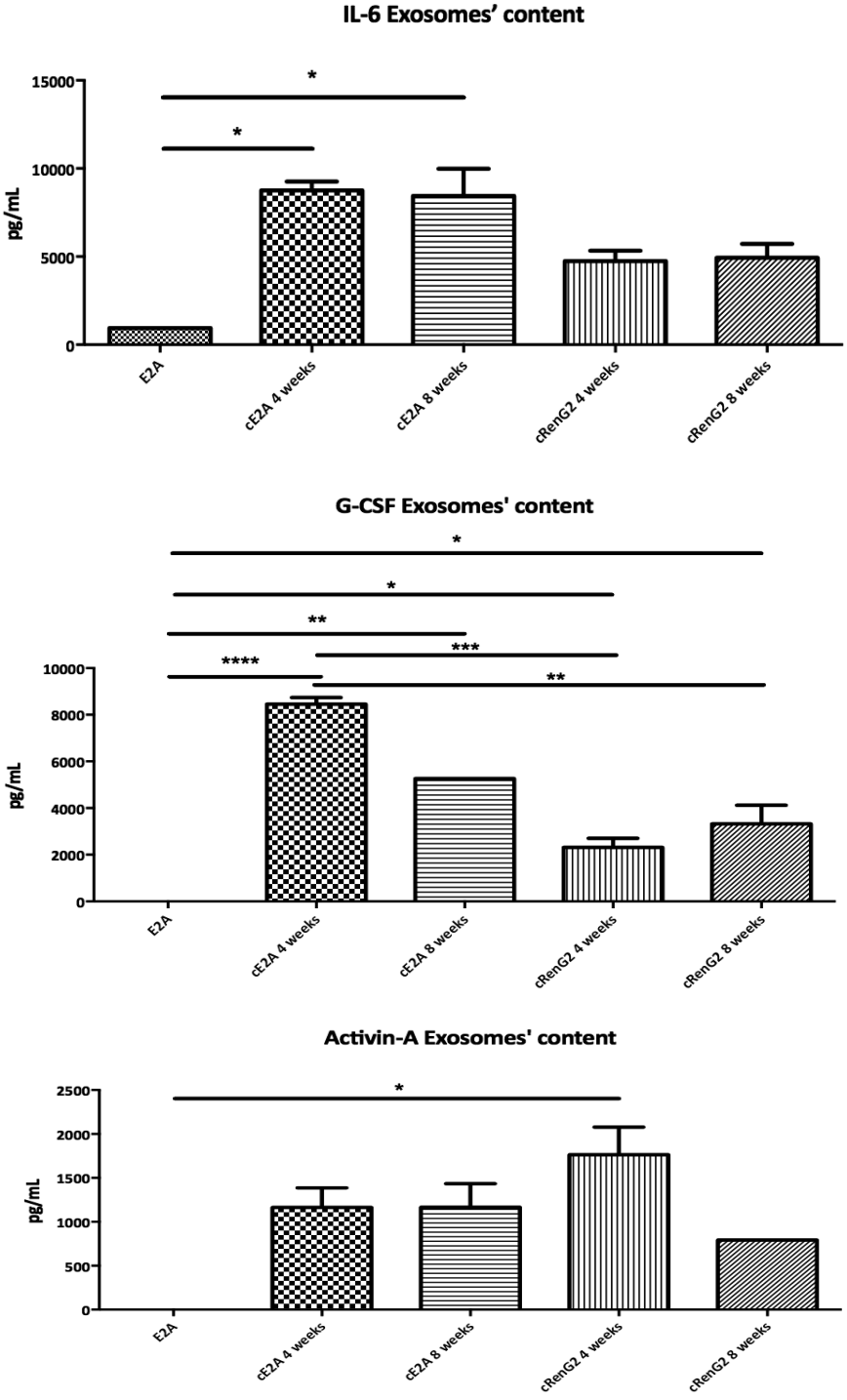

Figure 5.3 - IL-6, G-CSF and Activin-A levels in exosomes' content isolated from the conditioned co-culture media. IL-6 and G-CSF levels were significantly increased in exosomes in the upper compartment in comparison to control while Activin-A levels were significantly increased in the upper compartment after 4 weeks of co-culture. Data represent means ± SEM, Differences between the means were evaluated by one-way ANOVA followed by a Bonferroni post test.

IL-6 levels were significantly increased in the exosomes isolated from the conditioned media of E2A fibroblasts in comparison to control, being available to trespass the Transwell® and affect RenG2 cells' physiology. However, the levels of IL-6 present in exosomes did not vary significantly when comparing exosomes isolated after 4 weeks with the ones isolated after 8 weeks. Similarly to what was observed with IL-6, the levels of G-CSF present in the exosomes were significantly increased in E2A's conditioned media comparing to the control. Additionally, the presence of G-CSF in exosomes in the upper compartment of the co-culture system was also significantly increased. Despite the fact that there was a significant difference when comparing the exosomes' G-CSF concentration isolated from the upper and the bottom compartment after 4 weeks of co-culture, this difference was not observable at the final point of the co-cultures. Finally, the levels of Activin-A present in the isolated exosomes were significantly increased in relation to control only in the conditioned RenG2 media and only after 4 weeks of co-culture.

Finally, to assess an eventually non-exosome mediated cytokine release, ELISA was also applied to the conditioned co-culture media. Figure 5.4 shows the levels of IL-6 and Activin-A present in conditioned co-culture media.

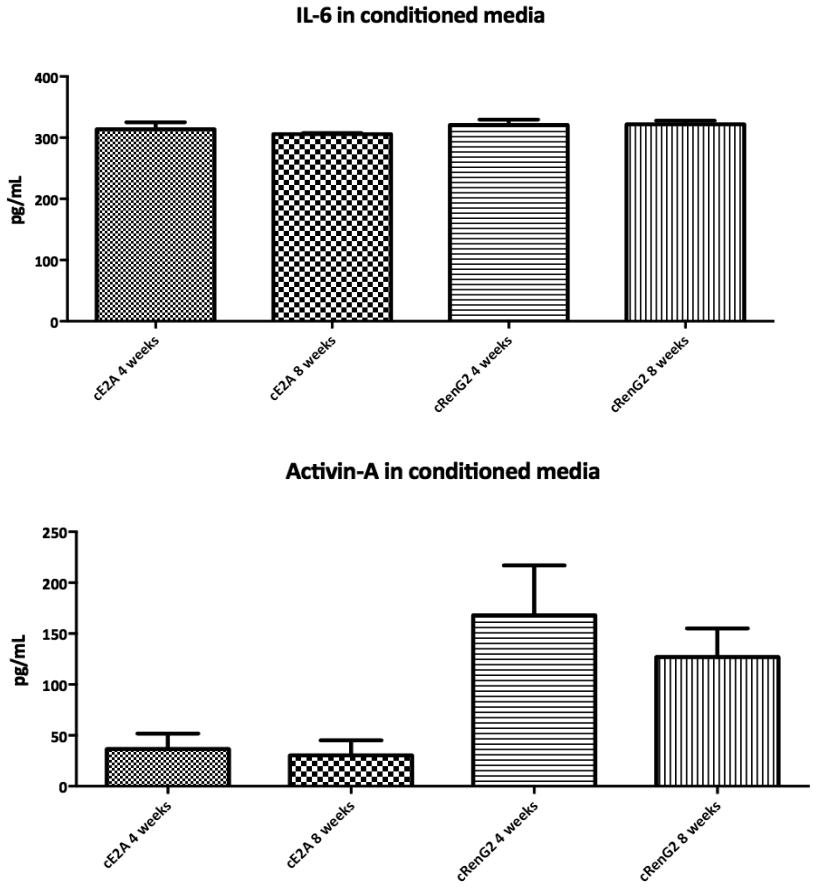

Figure 5.4 - IL-6 and Activin-A levels in co-cultures' conditioned media. There was no significant alteration in the concentrations neither of IL-6 nor of Activin-A when comparing conditioned media after 4 weeks and 8 weeks of co-culture. Data represent means ± SEM, Differences between the means were evaluated by one-way ANOVA followed by a Bonferroni post test.

The evaluation of the co-culture's conditioned media demonstrated the presence of IL-6 and Activin-A both in the upper and lower compartment of the co-culture system. No significant difference was observed in the IL-6 and Activin-A levels, when comparing media collected after 4 and 8 weeks of co-culture. G-CSF levels however, were too low above the lower calibration curve's value, thus suggesting that the main release pathway of this cytokine is exosome-mediated.

5.3 Discussion

Tumor cells have been shown to communicate paracrinally with several different cells present in the tumor stroma. Furthermore, this communication has been associated with the development of CSCs subpopulations inside the tumor, as demonstrated by Chen and collaborators that observed that cues secreted by CAFs present in the tumor microenvironment lead to the dedifferentiation of CSCs in lung cancer (Chen *et al*., 2014). In the same line of thought, Lu and colleagues observed that the ECM of the tumor stroma plays a huge part in the maintenance of the CSCs' pools as well as in angiogenesis (Lu *et al*., 2012).

Considering this information, the observed acquisition of sphere-formation ability by RenG2 cells after co-culture with the isolated E2A fibroblasts' ECM most probably results from the release of the previously identified cytokines, by the exosomes retained in the fibroblasts' ECM. In agreement, the immunocytochemistry results depicted IL-6 and G-CSF randomly dispersed across the ECM's structure. The attained results are in line with a vast panel of reports in the literature implicating IL-6 and G-CSF in the tumorigenic process, namely with CSCs' biology (Sun *et al*., 2014; Ma *et al*., 2012).

Cancer is intimately associated with the inflammatory process and both IL-6 and G-CSF are pro-inflammatory cytokines involved in diverse inflammatory responses. This fact seems to reinforce the hypothesis that these molecules remain stored in the E2A fibroblasts' ECM, and can be released in case of inflammation and/or ECM degradation (De Jong *et al*., 2014).

In the recent years, IL-6 was shown to be involved in almost every step of the tumorigenic process. Sansone's group observed that chronic suppression of the estrogen receptor in breast cancer led to a selective upregulation of autocrine IL-6 in CSCs, thus driving the self-renewal capacity of these cells in hormonal therapy resistant metastases (Sansone *et al*., 2016). Conversely, Kim and collaborators showed that the depletion of IL-6 from the cell culture media led to a decrease in the number of CSCs, resulting in a reduced ability of mammospheres formation (Kim *et al*., 2015). Moreover, CAFs-secreted IL-6 was shown to be responsible for tamoxifen resistance in breast cancer (Sun *et al*., 2014). The results attained in the present project corroborate the abovementioned observations by showing that IL-6 secreted by the fibroblasts' ECM induce RenG2 cells' dedifferentiation, thus driving the formation of a pool of CSCs inside the malignant cellular system.

Concerning the method of IL-6 release, the ELISA results demonstrated that this cytokine is indeed being transported between cells inside exosome vesicles, as its concentration inside these vesicles is more than ten-fold higher than that present in the conditioned media. In fact, the IL-6 levels inside exosomes were dramatically increased in the conditioned media of fibroblasts after 4 weeks in co-culture, in comparison to control. Additionally, IL-6 was also detected in the exosomes isolated from the conditioned media of the upper compartment, demonstrating that these structures were able to cross the Transwell® membrane upon release from the fibroblasts, and in this way effectively alter RenG2 cells' physiology. These results are in agreement with the work of Leanderson's group demonstrating the presence of IL-6 inside exosomes secreted by melanoma cells upon rWNT5A stimulation (Ekström *et al*., 2014). Another piece of evidence supporting the exosome-mediated IL-6 release hypothesis is the observation that IL-6 was not being significantly secreted as a free soluble signaling factor. Indeed, the ELISA study of the co-cultures' conditioned media did not show any significant differences along time, or between co-culture compartments, but rather identified a chemical homeostasis.

Similarly to IL-6, G-CSF levels in the isolated exosomes were also significantly increased in the E2A's conditioned media in comparison to the control. Furthermore, a significant increase in the G-CSF levels present in the exosomes from the upper compartment was also observed, further demonstrating that G-CSF-containing exosomes were being secreted by the fibroblasts, and migrating to the RenG2 cells' compartment. Moreover, a significant difference was observable between the G-CSF levels in the exosomes isolated from the upper and the bottom compartment following 4 weeks of co-culture. Interestingly, however, this significant difference was abolished after the 8 weeks of co-culture, suggesting that perhaps a boost of G-CSF is needed for the dedifferentiation process of RenG2 cells, but the subsequent maintenance of the CSCs' pool do not require such high levels of this cytokine. Also similar to IL-6 is the fact that the levels of G-CSF in the co-cultures' conditioned media were very low when compared to those inside the exosomes. In the case of G-CSF, in particular, the attained concentrations even remained below the calibration curve, further corroborating the hypothesis proposing that this cytokine is exosome-transported. The results obtained by McDonald and collaborators also support this hypothesis by demonstrating that LPS-stimulated macrophages secreted exosomes contained G-CSF (McDonald *et al*., 2014).

The third cytokine analyzed under the scope of the present work was Activin-A. Despite being the less studied of the three cytokines, Activin-A has also been associated with CSCs' biology, turning its role increasingly more important over the last years. After observing that Activin-A was the only of the three cytokines that was not being stored in the ECM, it was hypothesized that it must be released inside exosome vesicles. The ELISA results immediately confirmed this hypothesis by showing that the levels of Activin-A in exosomes isolated from the conditioned media in the upper compartment after 4 weeks of co-culture were significantly upregulated relatively to the fibroblast's medium before the start of the co-culture. Again similarly to what was observed with IL-6, no significant differences were observed when comparing free Activin-A's levels in the conditioned media from the lower and the upper compartments. Once again the most reasonable explanation is that some Activin A is in fact released to the media, either directly by exosomes' rupture, and the levels of this cytokine then establish a chemical balance between the two compartments. The experiments performed by Jones and co-workers in 2007 (Jones *et al*., 2007) somehow corroborate these findings by showing that Activin-A modulates pro-inflammatory responses upon LPS treatment in mice. Nonetheless, although some other members of the TGF-β family to which Activin-A also belongs have already been reported to be transported in exosomes (Webber *et al*., 2010; Borges *et al*., 2012), Activin-A is not one of those.

The immunocytochemistry results provided evidence that the ECM acted as a reservoir for fibroblasts'-derived exosomes and ELISA experiments further proved that those exosomes contained the three cytokines under study. This way, the overall panel of presented results confirmed the exosomal-mediated release of IL-6, G-CSF and Activin-A in the observed dedifferentiation process of the RenG2 cells (Rodrigues *et al*., 2013).

5.4 Conclusion

After the observed acquisition of sphere-formation ability by RenG2 cells, it became evident that this acquired stem potential must derive from cues released by the co-cultured ECM/fibroblasts. The present results confirmed this hypothesis by showing that both IL-6, G-CSF and Activin-A were being released by the E2A fibroblasts and accumulated in the E2A fibroblast's ECM and released to RenG2 cells' compartment, thus driving the malignant cells'

dedifferentiation. Furthermore, it was possible to conclude that two out of the three cytokines in study, IL-6 and G-CSF, were being stored in the exosomes retained in the ECM.

Chapter 6

Final Conclusion and Future Perspectives

Despite all the advances observed in the last decades in the understanding of tumorigenesis, the surface of the understanding of paracrine communication between stromal and tumoral cells has only been lightly scratched. The emergence of the CSCs' concept took this scientific field by storm and has been of vital importance in the understanding of several processes undecipherable until then. Unraveling the mechanism behind CSCs' formation may help developing new therapeutic approaches in a field where the current treatments have been maintained unchanged for decades.

Even though the CSCs' formation process is still debatable, several reports have demonstrated that an intimate and very complex communication between tumor cells and the cells of the tumor microenvironment may underline dedifferentiation (Krishnamurthy *et al*., 2010; Lu *et al*., 2013). In agreement, a previous work developed in our laboratory identified a paracrine loop mediated by IL-6, G-CSF and Activin-A, established between CAFs and malignant bronchial epithelial cells, and responsible for the formation of a CSCs' pool in the epithelial layer (Rodrigues *et al*., 2013 inside). This project focused on dissecting the mechanism behind the secretion of these cytokines by CAFs, hypothesizing exosomal and non-exosomal mediated pathways. To evaluate these hypotheses, RenG2 cells were co-cultured with either CAFs or isolated CAFs' ECM utilizing Transwell® devices. RenG2 cells' dedifferentiation became evident in both cases through their acquisition of stem potential demonstrated by the gaining of sphere-formation ability, as reported in previous studies (Kim *et al*., 2015; Ma *et al*., 2012).

The fact that the ECM alone was sufficient to drive the stem potential acquisition proved that it worked as a reservoir for the dedifferentiation-implicated cytokines. Immunocytochemistry further confirmed this hypothesis by depicting not only IL-6 and G-CSF, but also the exosome markers CD63 and Rab27. Taken together with the ELISA results, these data suggested that RenG2 cells' dedifferentiation was mediated by fibroblasts-released cytokine-containing exosomes. However, only IL-6 and G-CSF containing-exosomes were retained in the ECM, as no Activin A signal was identified in the ECM. This cytokine may perhaps only be produced in direct response to cells' needs and thereafter not accumulated.

Although the attained results mostly explained the mechanism behind RenG2's dedifferentiation, some experiments still need to be performed in order to further prove and complete it. This way, it would be very interesting to:

- Perform a proof-of-concept assay in which the three cytokines are added to monocultures of RenG2 cells and the stem potential of the system is confirmed after 8 weeks of culture;

- Assess the *in vivo* application of the attained results by establishing primary cultures of human lung tumor samples-derived ECM, co-culturing them with RenG2 cells using the same Transwell® system for 8 weeks an subsequently assess their stem potential with the sphere-forming assay;

In conclusion, the results presented in this work clearly demonstrated that IL-6, G-CSF and Activin-A induced RenG2 cells' dedifferentiation and established the tumor stroma as the main orchestrator of this process, by secreting the responsible cytokines inside of exosomes. Subsequent studies may focus on the identification of potential therapeutic targets to be used in clinics to abrogate dedifferentiation and thus improve patients' outcome and welfare.

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Appendix

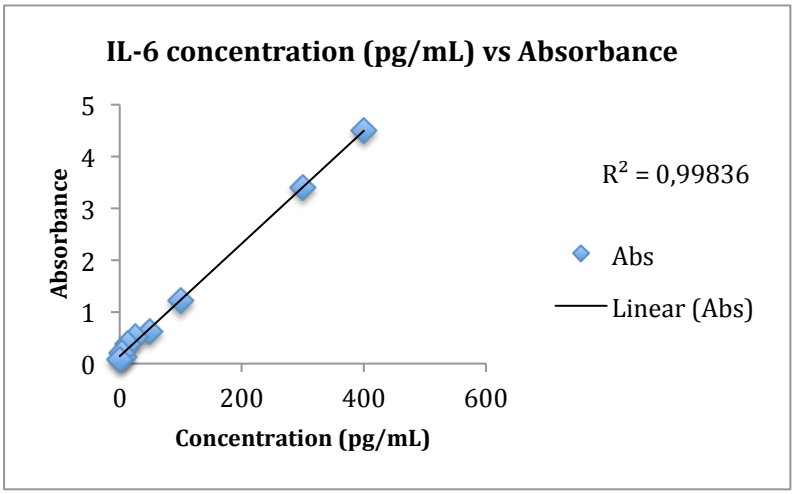

Figure S.1 – IL-6 ELISA calibration curve.

Table II – ELISA IL-6 standard values for calibration curve

Concentration (pg/mL)	Abs
400	4,5027
300	3,4011
100	1,21925
50	0,6214
25	0,51215
12,5	0,3722
6,25	0,1279
3,13	0,1986
0	0,0812

Table III - IL-6 concentrations in isolated exosomes

Samples	Absorbance	Concentration samples (pg/mL)	Dilution Factor	Final Concentration (pg/mL)
E2A	0,32685	17,15785606	18,53	317,9350728
cE2A 4 weeks n=1	3,635	320,5842898	29,2014	9361,510079
cE2A 4 weeks n=2	3,6034	317,6889965	28,806	9151,349232
cE2A 4 weeks n=3	3,3904	298,1526456	26,018	7757,335533
cE2A 8 weeks n=1	3,7463	330,7896637	28,0354	9273,820537
cE2A 8 weeks n=2	3,1009	271,5936033	20,1468	5471,742007
cE2A 8 weeks n=3	3,541	311,9595565	33,9878	10602,81901
cRenG2 4 weeks n=1	3,6197	319,1809744	17,9788	5738,490903
cRenG2 4 weeks n=2	2,861	249,5899856	19,2624	4807,702138
cRenG2 4 weeks n=3	2,4256	209,6612309	17,5404	3677,541854
cRenG2 8 weeks n=1	3,40225	299,2334124	19,9816	5979,162353
cRenG2 8 weeks n=2	3,3756	296,7890755	18,2614	5419,784024
cRenG2 8 weeks n=3	2,8538	248,9296019	13,6108	3388,131025

Table IV – IL-6 concentrations in conditioned media

Samples	Absorbance	Concentration samples (pg/mL)	Final Concentration (pg/mL)
cRenG2 4 weeks n=1	**3,452633333**	**303,8545696**	**303,85456962**
cRenG2 4 weeks n=2	3,76465	332,4727249	332,4727249
cRenG2 4 weeks n=3	3,694166667	326,0079966	326,0079966
cRenG2 8 weeks n=1	3,593866667	316,8084849	316,8084849
cRenG2 8 weeks n=2	3,570166667	314,6347219	314,6347219
cRenG2 8 weeks n=3	3,78185	334,0503082	334,0503082
cE2A 4 weeks n=1	3,7495	331,0831675	331,0831675
cE2A 4 weeks n=2	3,608033333	318,1078509	318,1078509
cE2A 4 weeks n=3	3,321566667	291,8331405	291,8331405
cE2A 8 weeks n=1	3,448633333	303,4876898	303,4876898
cE2A 8 weeks n=2	3,507466667	308,8838806	308,8838806
cE2A 8 weeks n=3	3,471933333	305,6247648	305,6247648

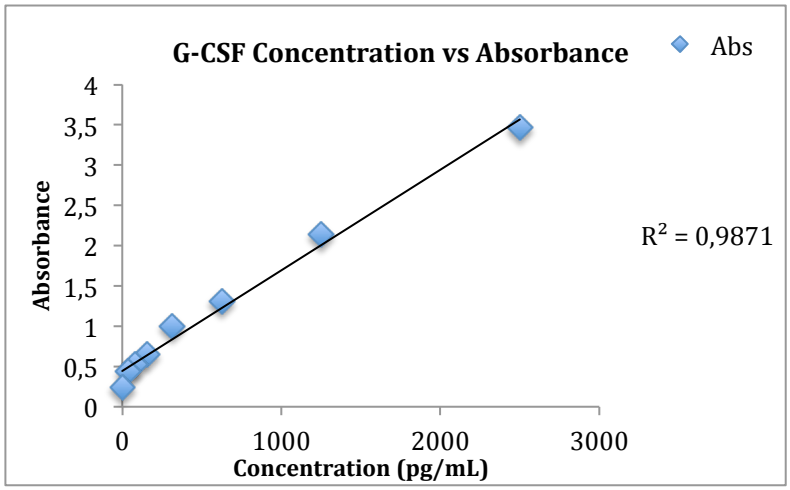

Figure S.2 – G-CSF ELISA calibration curve

Table V - ELISA G-CSF standard values for calibration curve

Concentration (pg/mL)	Abs
2500	3,46285
1250	2,1338
625	1,306
313	0,99805
156	0,65075
78	0,5253
39	0,43625
0	0,2364

Table VI – G-CSF concentrations in isolated exosomes

Samples	Absorbance	Concentration samples (pg/mL)	Dilution Factor	Final Concentration (pg/mL)
E2A	0,3017	-104,4426943	18,53	6241,901574
cE2A 4 weeks n=1	0,788266667	280,0285157	29,2014	8177,224699
cE2A 4 weeks n=2	0,81775	303,3254113	28,806	8737,591799
cE2A 4 weeks n=3	0,540566667	84,30298572	26,018	2193,395082
cE2A 8 weeks n=1	0,67125	187,5652573	28,0354	5258,467015
cE2A 8 weeks n=2	0,3278	-80,0874015	20,1468	-1561,852859
cE2A 8 weeks n=3	0,4386	3,731811412	33,9878	126,8360599
cRenG2 4 weeks n=1	0,65095	171,5247718	17,9788	3083,809568
cRenG2 4 weeks n=2	0,56625	104,5972289	19,2624	2014,793661
cRenG2 4 weeks n=3	0,5659	104,3206688	17,5404	1829,826259
cRenG2 8 weeks n=1	0,59315	125,8528476	19,9816	2514,741259
cRenG2 8 weeks n=2	0,420033333	291,833248	18,2614	-186,1514425
cRenG2 8 weeks n=3	0,81705	302,7722911	13,6108	4120,9731

Table VII – G-CSF concentrations in conditioned media

Samples	Absorbance	Concentration samples (pg/mL)	Final Concentration (pg/mL)
cRenG2 4 weeks n=1	**0,635833333**	**159,5800096**	**159,5800096**
cRenG2 4 weeks n=2	0,1974	-186,8576025	-186,8576025
cRenG2 4 weeks n=3	0,17915	-201,2782361	-201,2782361
cRenG2 8 weeks n=1	0,255566667	-140,8959487	-140,8959487
cRenG2 8 weeks n=2	0,3486	-0,787262873	-0,787262873
cRenG2 8 weeks n=3	0,3494	-66,7515042	-66,7515042
cE2A 4 weeks n=1	0,361833333	-56,92703606	-56,92703606
cE2A 4 weeks n=2	0,18275	-198,4336179	-198,4336179
cE2A 4 weeks n=3	0,3515	-65,09214363	-65,09214363
cE2A 8 weeks n=1	0,4604	20,95755446	20,95755446
cE2A 8 weeks n=2	0,39165	-33,36675	-33,36675
cE2A 8 weeks n=3	0,3425	-72,20368892	-72,20368892

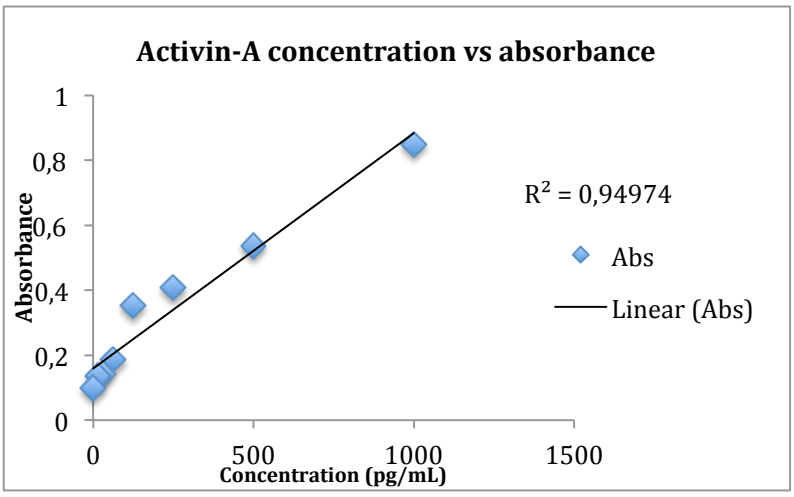

Figure S.3 – Activin-A ELISA calibration curve

Table VIII - ELISA Activin-A standard values for calibration curve

Concentration (pg/mL)	Abs
1000	0,8492
500	0,535666667
250	0,40755
125	0,352966667
62,5	0,186033333
31,3	0,1419
15,6	0,136833333
0	0,098033333

Table IX – Activin-A concentrations in isolated exosomes

Samples	Absorbance	Concentration samples (pg/mL)	Dilution Factor	Final Concentration (pg/mL)
E2A	0,114233333	-45,89204292	18,53	-850,3795553
cE2A 4 weeks n=1	0,155	7,534590209	29,2014	220,0205825
cE2A 4 weeks n=2	0,186	48,16154753	28,806	1387,341538
cE2A 4 weeks n=3	0,176866667	36,19188484	26,018	941,6404597
cE2A 8 weeks n=1	0,168866667	25,70750875	28,0354	720,7202909
cE2A 8 weeks n=2	0,191833333	55,8064051	20,1468	1124,320482
cE2A 8 weeks n=3	0,186333333	48,59839654	33,9878	1651,752582
cRenG2 4 weeks n=1	0,1988	64,93654927	17,9788	1167,481232
cRenG2 4 weeks n=2	0,223666667	97,52548493	19,2624	1878,574901
cRenG2 4 weeks n=3	0,246833333	127,8864907	17,5404	2243,180201
cRenG2 8 weeks n=1	0,179533333	39,68667687	19,9816	793,0033025
cRenG2 8 weeks n=2	0,117333333	-41,82934719	18,2614	-763,8624407
cRenG2 8 weeks n=3	0,135833333	-41,82934719	13,6108	-239,3354035

Table X – Activin-A concentrations in conditioned media

Samples	Absorbance	Concentration samples (pg/mL)	Final Concentration (pg/mL)
cRenG2 4 weeks n=1	**0,3324**	**240,0256299**	**240,02562992**
cRenG2 4 weeks n=2	0,293733333	189,3511455	189,3511455
cRenG2 4 weeks n=3	0,206466667	74,98407635	74,98407635
cRenG2 8 weeks n=1	0,204833333	72,84351623	72,84351623
cRenG2 8 weeks n=2	0,2576	141,9967135	141,9967135
cRenG2 8 weeks n=3	0,27615	166,3073605	166,3073605
cE2A 4 weeks n=1	0,1888	51,83107916	51,83107916
cE2A 4 weeks n=2	0,1656	21,42638852	21,42638852
cE2A 4 weeks n=3	0,145733333	-4,609812088	-4,609812088
cE2A 8 weeks n=1	0,1836	45,01623471	45,01623471
cE2A 8 weeks n=2	0,161233333	15,70366657	15,70366657
cE2A 8 weeks n=3	0,1417	-9,89568503	-9,89568503