UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Modelos de co-culturas de células *in vitro* para desenvolvimento de novos sistemas de entrega de fármacos

Elisabete Cristina da Rocha Costa

Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

Orientador: Professor Doutor Ilídio Joaquim Sobreira Correia
Coorientador: Mestre Vítor Manuel Abreu Gaspar

Covilhã, junho 2013

List of publications

Articles in peer reviewed international journals:

Costa, C. E., Gaspar. V. M., Marques, J.G., Coutinho, P., Correia, I. J. (2013). "Evaluation of Nanoparticle Uptake in Co-culture Cancer Models."PloS one. Article *in press*.

Poster communications:

Costa, C. E., Gaspar. V. M., Marques, J.G., Coutinho, P., Correia, I. J., Mimicking Breast Cancer Microenvironment with *In Vitro* Co-culture Models, Instituto Politécnico da Guarda (IPG), 3rd of May 2013, Guarda, Portugal.

Best poster award:

Costa, C. E., Gaspar. V. M., Marques, J.G., Coutinho, P., Correia, I. J., Mimicking Breast Cancer Microenvironment with *In Vitro* Co-culture Models, Instituto Politécnico da Guarda (IPG), 3rd of May 2013, Guarda, Portugal.

"There's a photo on my wall of a woman I've never met, its left corner torn and patched together with tape. She looks straight into the camera and smiles, hands on hips, dress suit neatly pressed, lips painted deep red. It's the late 1940s and she hasn't yet reached the age of thirty. Her light brown skin is smooth, her eyes still young and playful, oblivious to the tumor growing inside her - a tumor that would leave her five children motherless and change the future of medicine".

The Immortal Life of Henrietta Lacks - ***Rebecca Skloot***

Dedication

The completion of this work has not possible without the familiar support. Thus, I would like to dedicate this last year of my academic path to parents and to my little sister which always understood my distance.

Moreover, I would like to dedicate this work to all persons with cancer around the world. I wish that the investigation in tumor biology continues to open new windows for new anti-cancer therapies for better quality of life of cancer patients.

Acknowledgments

Firstly, I would like to thank to my supervisor Professor Ilídio Correia for the opportunity of work in his group, and for all the time and orientation provided during the aquision of my master degree.

Specially, I would like to thanks my co-supervisor, Vítor Gaspar (Ph.D. student), for supporting me in my instabilities and doubts. Furthermore, for spend great part of his time in helping me, putting in second place his own work. In fact, without his orientation I would not be the person academically and personally that I am today.

To João Marques, my co-worker and friend, I would like to thanks him for all accomplices and honesty. To him, I would like to wish a wonderful future like he deserves.

To another great influence and orientation to me, Ph.D. students and M.Sc. of the group, I thank for good working environment, entrepreneurship, good humour and in last for the friendship. We are a family.

I acknowledge to my friends and housemates which always listen my confidences.

In last, I thank to me for surpass myself.

Abstract

The demanding applications of nanocarriers in cancer biology require the existence of testing platforms that mimic the *in vivo* tumor microenvironment and its unique biological features. For this, highly informative methodologies such as animal experimentation are the current gold-standard. However, very recent reports issued by regulatory agencies appeal for the reduction of the used animal research models due to economical and ethical issues, thus evidencing the urgent necessity for novel alternatives. Co-culture cell models have the potential to bridge the gap between the required reduction of animal use and the existence of suitable models that closely reproduce *in vivo* tumors. This is a novel type of *in* vitro cell culture that is mainly characterized by the culture of cancer cells in contact with stromal cells, mimicking the tumor microenvironment *in vitro* trough the establishment of cancer-stroma synergic interactions. However, this evaluation was until now limited to co-culture systems established with precise cell ratios, not addressing the natural heterogeneity commonly found in tumors of different patients. The research work presented in this thesis describes the development and optimization of novel 2D co-culture models of breast and cervical cancers with various cell-to-cell ratios, in order to unravel the influence of heterogeneous conditions on the evaluation of nanocarrier biological performance and ultimately in the therapeutic outcome. As a proof of concept these novel platforms were used to evaluate a multifunctional gene delivery system designed for cancer therapy and revealed that in fact different co-culture ratios may influence the overall assessment of nanocarrier targeting specificity. In addition, since recent reports demonstrate the high influence of the 3D architecture of tumor masses in the response to anti-cancer drugs or delivery systems, the engineering and optimization of suitable substrates for generation of organotypic 3D co-culture models with various cancer-fibroblast cell ratios was also investigated. The 3D multicelular spheroid models of breast and cervix cancer produced at various time points, possess all the major characteristics of *in vivo* tumors including the structural rearrangement, the diffusional limit of oxygen or nutrients and most importantly, the distinctive necrotic core of solid tumors. Overall, these newly developed co-culture and 3D models assume crucial importance for the future design and optimization of new drug delivery providing a new level of *in vitro* reproducibility of *in vivo* tumors.

Keywords

2D co-cultures; 3D multicellular spheroids; Nanosized delivery systems; Tumor microenvironment.

Resumo alargado

Os recentes avanços na área da Nanotecnologia abriram novas oportunidades para o desenvolvimento de novos nano-sistemas como as nanopartículas para entrega de fármacos ou de informação genética com potencial para serem usadas futuramente na terapia do cancro. Todavia, para que as suas aplicações terapêuticas sejam significativas num contexto clínico, estes sistemas devem ser testados em modelos que representem o mais aproximadamente possível as propriedades únicas que o microambiente tumoral tem *in vivo*. Por forma a atingir este objetivo, vários protocolos experimentais usam modelos animais para avaliar a atividade biológica de nano transportadores. No entanto, recentemente, as diferentes agências regulatórias têm apelado pela aplicação da regra dos 3Rs (Reduzir, Reutilizar, Reciclar) em relação ao uso de animais como modelos para estudos experimentais em fases pré-clinicas, não só devido aos problemas económicos e legais associados ao seu uso, mas também devido a inconvenientes éticos, e à variabilidade dos resultados obtidos quando comparados com aqueles adquiridos em estudos clínicos com humanos. Neste contexto, tem-se procurado desenvolver novas alternativas que permitam reproduzir o que ocorre *in vivo*.

A cultura *in vitro* de células tumorais em co-cultura com outras células presentes no microambiente tumoral surge como uma abordagem muito promissora no que diz respeito a mimetizar as caraterísticas dos variados tipos tumores. Esta metodologia permite estudar de uma forma abrangente a biologia dos tumores, sob variadas condições e até mesmo testar, de uma forma rápida, novos fármacos ou sistemas de entrega direcionada. O contacto direto entre as células cancerígenas e as células do estroma, reproduzem as interações sinergéticas que ocorrem no microambiente tumoral. Contudo, estes sistemas de culturas celulares são usualmente desenvolvidos tendo por base um número fixo de células tumorais em relação às células do estroma. De facto, até à data apenas foram descritos testes de novos agentes anti-tumorais ou de novos sistemas de entrega em co-culturas com apenas um rácio de células, sendo que esta abordagem não permite assim analisar a heterogeneidade natural dos tumores.

Desta forma, o trabalho de investigação desenvolvido nesta tese descreve desenvolvimento e otimização de novos modelos 2D de co-culturas do cancro da mama e do colo do útero. Para tal, foram usados vários rácios de células cancerígenas células normais do estroma, com o intuito de representar a distribuição celular em diferentes tumores. Adicionalmente, pretende-se também verificar de que forma estas diferentes condições influenciam a atividade dos novos sistemas de entrega de drogas e consequentemente a sua eficácia terapêutica.

Os resultados obtidos demonstraram uma influência evidente dos fibroblastos, sobre o comportamento das células cancerígenas. Inicialmente, foi possível verificar uma alteração na organização estrutural das co-culturas, quando comparadas com as monoculturas de

controlo. Além disto, foi também observado um aumento na viabilidade celular na presença de fibroblastos, tendo sido obtida uma correlação entre o tempo de co-cultura e um aumento de proliferação celular. Após a demonstração do sucesso da otimização destas plataformas, foram testadas nanopartículas funcionalizadas nas várias co-culturas desenvolvidas. Os resultados obtidos na microscopia confocal e citometria de fluxo demonstraram que o uso de diferentes rácios celulares pode de facto influenciar a avaliação da especificidade de nano transportadores. Estes resultados evidenciam assim que, os mesmos sistemas de entrega podem atuar de forma diferente, de paciente para paciente.

Para além do desenvolvimento destes sistemas de co-culturas, foi também otimizada a produção de novos modelos de culturas tridimensionais. Estes modelos 3D, mais comumente chamados de esferoides, conseguem mimetizar os tumores sólidos, pois são constituídos por vários tipos de células e com o decorrer do tempo adquirirem propriedades únicas, nomeadamente uma superfície constituída por células com elevada proliferação, que mimetizam as zonas do tumor irrigadas por vasos sanguíneos e um núcleo necrótico, que corresponde às zinas do tumor com baixa densidade de vasos. Para promover a formação destes modelos foi utilizada a técnica de cultura celular com sobreposição líquida em conjugação com agitação horizontal. Esta nova abordagem permitiu evitar a adesão das células e promoveu a formação de esferoides 3D com morfologias bem definidas e reprodutíveis.

Para a formação destes modelos fibroblastos revelaram um papel fundamental visto que as interações que se estabelecem entre os dois tipos celulares são essenciais para formar esferoides coesos e com um gradiente de densidades celulares da periferia para o núcleo.

Em geral, os novos modelos de co-culturas desenvolvidos assumem um papel crucial no futuro desenvolvimento e investigação de novos nano transportadores, já que estes serão na prática direcionados para os tumores. Por outro lado, os modelos 3D permitem reproduzir com exatidão o que acontece *in vivo*, criando um conjunto de ferramentas que poderão contribuir para aperfeiçoar as terapias anti-tumorais.

Palavras-chave

Co-culturas 2D; Esferoides multicelulares; Microambiente tumoral; Nano sistemas de entrega direcionada.

Table of Contents

List of Figures

Chapter I - Introduction

Chapter III – Results and Discussion

List of Tables

Chapter I – Introduction

Chapter II – Methods

List of Acronyms

α-SMA	α-Smooth muscle actin
2D	Two-dimensional
3D	Three-dimensional
ADH	Atypical ductal hyperplasia
ALH	Atypical lobular hyperplasia
bFGF	Basic fibroblast growth factor
CAF	Cancer associated fibroblasts
CAM	Cell adhesion molecule
CH-H-R/pDNA	Chitosan-Histidine-Arginine/plasmid deoxyribonucleic acid
CLSM	Confocal laser scanning microscopy
CO_2	Carbon dioxide
CXCL12 (SDF-1)	Chemokine stromal derived factor-1
DC	Dendritic cell
DCIS	Ductal carcinoma *in situ*
DEMEM-HG	Dulbecco's Modified Eagle's Medium High Glucose
DMEM-F12	Dulbecco's Modified Eagle's Medium F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	European Commission
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Endothelial growth factor
EMEA	European medicines agency
EMT	Epithelial-to-mesenchymal transition
EtOH	Ethanol
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FL	Fluorescence
GFP	Green fluorescent protein
H_2O	Water

HeLa	Human cervix adenocarcinoma
hFIB	Primary normal human dermal fibroblasts
HGF	Hepatocyte growth factor
HIF-1	Tumor hypoxia-inducible factor-1
IBC	Invasive breast cancer
IGF	Insulin-like growth factor
IL	Interleukin
ISO	Organization for Standardization
KGF	Keratinocyte growth factor
LOX	Lysyl oxidase
MCF-7	Oestrogen-dependent human breast adenocarcinoma
MCP	Monocyte chemotatic protein
MCTS	Multicellular tumor spheroids
MDR	Acquired multidrug resistance
MDSC	Myeloid-derived suppressive cell
MMP	Metalloproteinase
NK	Natural killer cells
NO	Nitrogen species
O_2	Oxygen
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
pDNA	Plasmid deoxyribonucleic acid
PFA	Paraformaldehyde
PPFS	Patient progression free survival
RB	Retinoblastoma protein
RITC	Rhodamine B isothiocianate
ROI	Region of interest
ROS	Reactive oxygen species
RT	Room temperature
SEM	Scanning electron microscopy
T reg	Regulatory T cells
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α

TP53	Tumor protein p53
VEGF	Vascular endothelial growth factor
WGA-Alexa 594	Germ agglutinin conjugated Alexa 594

Chapter I

Introduction

1. Introduction

1.1. Tumor microenvironment: The driving force for cancer evolution

Cancer is nowadays a major public health problem, being the second main cause of death in developing countries (Siegel *et al.*, 2012). It is estimated that in 2020 the world population will reach a total of 7.5 billion people and a total of 15 million new cancer cases will arise (Bray and Møller, 2005). These impairing statistics contribute for the tremendous efforts put forward from behalf of the medical and scientific community to develop more effective therapeutic approaches and also to understand the mechanisms responsible for cancer development. The complex transformation of a normal cell into a malignant phenotype is generally dependent on the accumulation of multiple changes in gene expression patterns in healthy cells (Ruddon, 2007). This cascade of events termed carcinogenesis (Figure 1) encompasses all the complex intracellular signalling pathways responsible for the initiation, stimuli, and progression of cancer cells in an extended time scale (Farber, 1984; Hanahan and Weinberg, 2000).

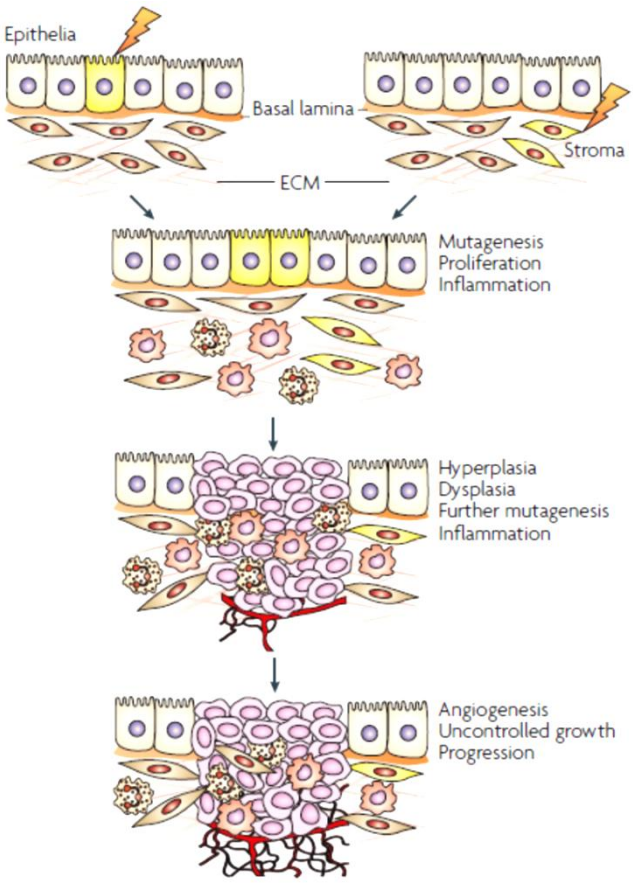

Figure 1 – Representation of carcinogenesis (Adapted from Albini and Sporn, 2007).

Cancer development, starts with changes in structure and function of deoxyribonucleic acid (DNA), usually known as mutations (Mbeunkui and Johann, 2009). These mutations usually lead to the loss of tumor-suppressor genes (tumor protein p53 (TP53), retinoblastoma protein (RB)), or activation of oncogenes (myc, RAS, AKT) (Gaspar *et al.*, 2011; Janssen and Medema, 2012), which propels malignant cells to escape from senescence signals and accept the proliferative stimuli, respectively (Levine and Puzio-Kuter, 2010). Thus, cancer cells acquire unlimited replicative potential, losing their characteristic non-dividing state (Hanahan and Weinberg, 2011). In addition, the deregulations of growth-promoting signals lead to an uncontrolled proliferation of cancer cells (Witsch *et al.*, 2010; Zhang *et al.*, 2010). In the last stages of cancer development, in order to sustain this dynamic growth and high metabolic activity, extensive neovascularisation is promoted by malignant cells in order to assure the necessary uptake of nutrients and oxygen (Annibaldi and Widmann, 2010). The angiogenesis in association with mechanisms that underlie the extravasion of cancer cells through the extracellular matrix (ECM) facilitating tumor spread into healthy tissues (Poste and Fidler, 1980; Zetter, 1998; Mbeunkui and Johann, 2009).

Recent reports demonstrate that these abilities acquired by cancer cells, i.e., sustained proliferation, cell death resistance, immortality, angiogenesis, invasion and metastization are a consequence of the unique conditions of the tumor niche. In fact, the tumor microenvironment is of critical importance for the success of cancer progression (Straussman *et al.*, 2012). Already in 1984, Dolberg and Bissel described an impaired tumor development in chicken embryos infected with an oncogene expressing Rous sarcoma virus, and hypothesized that these results were probably correlated with the absence of some additional cellular components (Dolberg and Bissell, 1984). Recent studies demonstrated that phenotypic and genotypic abnormalities of cancer cells are insufficient to induce the malignant phenotype (Ma *et al.*, 2003; Weigelt *et al.*, 2003). The influence of the tumor microenvironment in cancer development was then postulated. So far different therapies specifically directed against the tumor microenvironment have been produced (McMillin *et al.*, 2013; Sounni and Noel, 2013).

The tumor niche is characterized by the establishment of interactions between tumor cells and stromal cells. The stroma is composed of various cell types such as: i) fibroblasts; ii) endothelial cells; iii) pericytes; iv) adipocytes and v) immune cells (Figure 2), enclosed in a complex ECM (Swartz *et al.*, 2012). The closely regulated interactions between cancer cells and the surrounding healthy stromal cells originate an exclusive environment that propels tumor progression (Hogan, 2012). Moreover, tumor cells are capable of persistently shape their microenvironment according to an extracellular stimuli such as chemotherapy, thereby always maintaining an abnormal ecosystem that assures cancer cell survival (Merlo *et al.*, 2006).

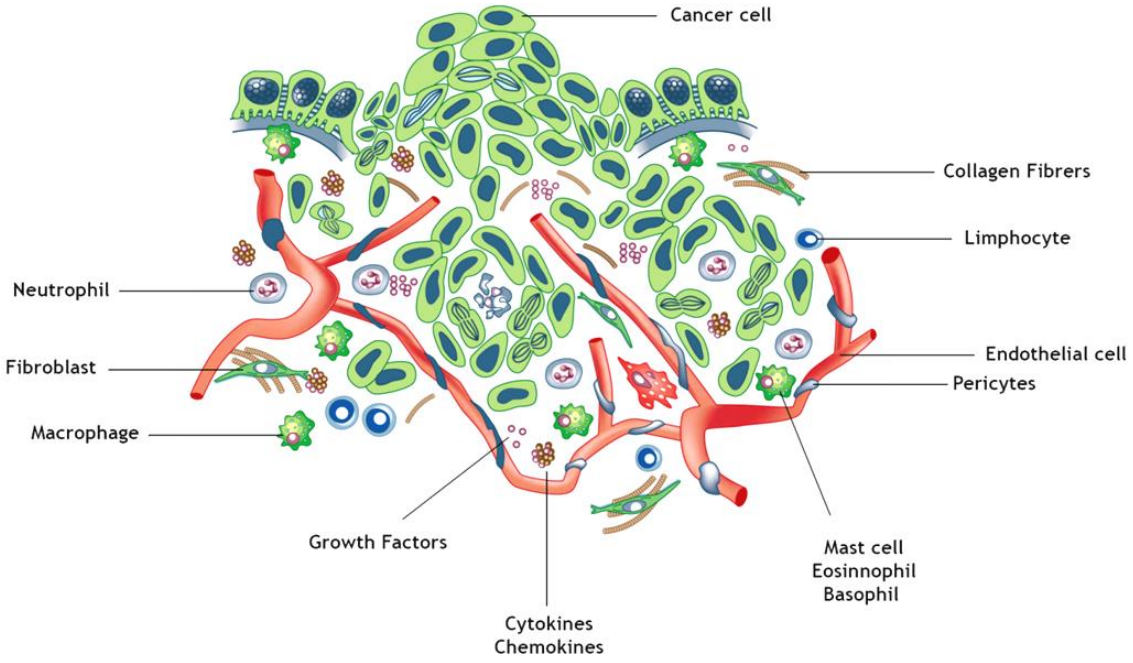

Figure 2 - Representation of tumor microenvironment (Adapted from Coussens and Werb, 2002).

The mechanisms that modulate the tumor microenvironment are highly dependent on cancer-host cells communications, and are generally formed by dynamic autocrine and paracrine signalling events (Alderton, 2012). The main mediators of these signalling cascades are soluble biomolecules, adhesion molecules, cytokines, chemokines, proteases and other degradative enzymes that are secreted by cancer cells, as well as, by stromal cells (Figure 3).

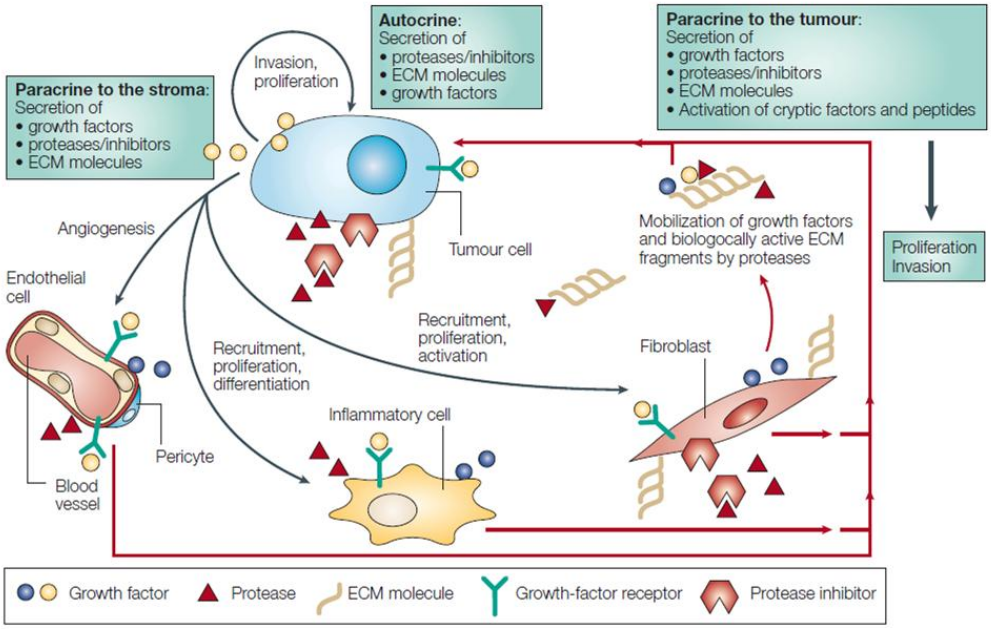

Figure 3 - General interactions established between tumour cells and stromal cells in human tumor tissue (Adapted from Mueller and Fusenig, 2004).

1.1.1. ECM in the tumor microenvironment

ECM is a complex three-dimensional (3D) network formed by macromolecular fibers (collagen, elastin, etc) as well as nonfibrous proteins (like proteoglycans and glicoproteins). This 3D matrix is present in the stroma of all tissues, either healthy or malignant (Noguera *et al.*, 2012). The ECM regulates almost all cellular behaviour (Brizzi *et al.*, 2012), and provides structural support and strength, thus allowing cellular communication, adhesion and migration (Noguera *et al.*, 2012). In tumor, the deregulation of ECM is essential for the angiogenesis and invasion of cancer cells into normal tissues (Shuman Moss *et al.*, 2012). Abnormal ECM can promote an increase in collagen deposition or stiffness. As a consequence, the integrin signalization is increased and stimulates cytoskeletal remodeling to regulate cell behaviour, thus inducing cell survival and proliferation (Lu *et al.*, 2012). The ECM also regulates the activity of immune system cells. ECM components function as chemoattractans playing an essential role in infiltration, differentiation, and functional activation of immune systems cells. In turn, these immune cells produce degradative enzymes such as metalloproteinases (MMPs), which are responsible for the degradation of ECM anti-apoptotic activities (Wang *et al.*, 2012).

1.1.2. Stromal cells

1.1.2.1. Vascular and lymphatic endothelial cells

Tumor proliferation and metastasis are dependent on the growth of blood and lymphatic vessels into the tumor mass (Weis and Cheresh, 2011; Balkwill *et al.*, 2012). The major constituent of these vessels are the endothelial cells. Particularly, in the case of blood vessels, endothelial cells are disorganized, loosely connected, branched and form a defective cellular lining on the vessel wall (Hashizume *et al.*, 2000). These cells are able to recruit immune cells due to the expression of cell adhesion molecules (CAMs) on their surface (Kobayashi *et al.*, 2007; Hanahan and Coussens, 2012). In turn, these immune cells in association with other stromal cells and cancer cells, will promote the formation of new vessels due to the activation of endothelial cells by the secretion of the main growth factor involved in angiogenesis, the vascular endothelial growth factor (VEGF) (Weis and Cheresh, 2011). Once endothelial cells become activated, they secrete their own VEGF and hepatocyte growth factor (HGF) (Li *et al.*, 2007).

The lymphatic endothelial cells are also responsible for secretion of angiogenic factors (VEGF) and for modulating the host immune cells in the tumor microenvironment (Skobe *et al.*, 2001; Balkwill *et al.*, 2012).

1.1.2.2. Perycites

Another important component of vascular vessels is the pericytes cells, which are specialized smooth-muscle cells that are present outside the vessel wall (Pietras and Östman, 2010). These cells have a potential influence on endothelial cells due to the direct contact and paracrine signalling with them, through platelet-derived growth factor subunit-B (PDGF-B),

which is a potential mitogenic factor of fibroblasts (Minami *et al.*, 2013). In addition, pericytes stabilize endothelial cells, mediating their survival, i.e., indirectly, pericytes control the formation, maturation, remodeling, stabilization and function of vascular vessels (Minami *et al.*, 2013).

1.1.2.3. Adipocytes

The influence of adipocytes in the tumor microenvironment is characterized by their capacity to secret of adipokines that promote the growth of malignant cells by providing them fatty acids (Nieman et al., 2011).

1.1.2.4. Immune System cells

Normally, inflammation serves to protect a defined region of infected or damaged tissue by recruiting cells necessary to resolve the insult, while also isolating the area to prevent the spread of infection and subsequently re-establish the normal tissue function and homeostasis (Janeway *et al.*, 2001; Sherwood, 2012). However, a non-resolved inflammation can promote genomic instability, growth, angiogenesis, survival and escape of tumor cells from immune surveillance (Figure 4) (Zamarron and Chen, 2011).

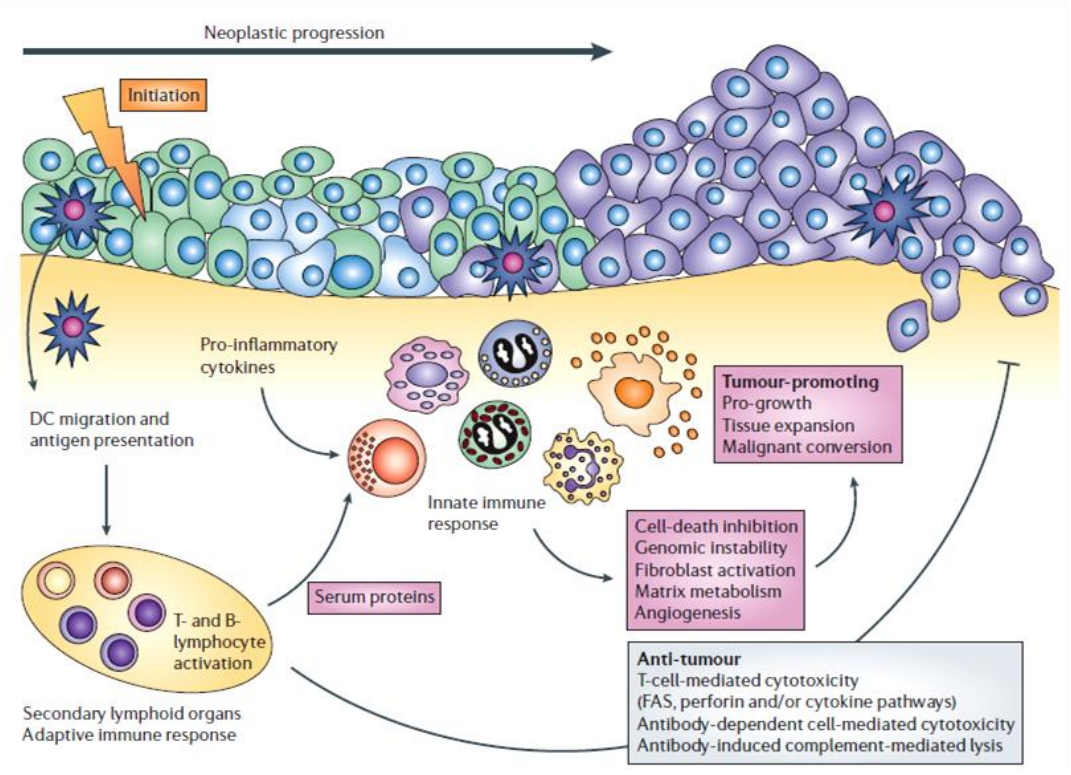

Figure 4 - Immune system behaviour in tumor microenvironment (Adapted from de Visser *et al.*, 2006).

In cancer, myeloid-derived suppressive cells (MDSCs), macrophages, mast cells, dendritic cells (DCs), eosinophils, neutrophils, lymphocytes and natural killer cells (NK) are generally recruited by malignant tumors to support their development trough signalling events

mediated by chemokines, cytokines, cytotoxic mediators and soluble mediators of cell death and proliferation (Tlsty and Coussens, 2006; Kerkar and Restifo, 2012).

The cells from the immune system induce DNA damage by production of reactive oxygen species (ROS) and nitrogen species (NO) (Alexander and Friedl, 2012). In addition, immune cells promote angiogenesis and tissue remodelling by producing growth factors such as HGF, transforming growth factor-β (TGF-β), VEGF, cytokines (tumour necrosis factor-α (TNF-α), interleukins (IL-6, IL-10) and MMPs (MMP-7, MMP-9) (Stockmann *et al.*, 2008; See *et al.*, 2012), Moreover, tumor growth can be promoted by regulatory T cells (T reg) that suppress cytotoxic T cell responses (Kerkar and Restifo, 2012), and also by humoral immune responses that increase chronic inflammation in the tumor microenvironment (Grivennikov *et al.*, 2010). All these processes are summarized in Figure 4.

1.1.2.5. Fibroblasts

It is now becoming clear that among host cells present in the tumor microenvironment, fibroblasts play crucial roles during various steps of cancer development (Strell *et al.*, 2012). These stromal constituents are elongated cells with extended and fusiform or spindle-like shapes. They are the non-vascular, non-epithelial and non-inflammatory cells of the connective tissue (Kalluri and Zeisberg, 2006). Normal stroma in most organs contains a reduced number of fibroblasts (Kalluri and Zeisberg, 2006), but in the tumor microenvironment, these cells are highly prevalent (Micke and Östman, 2005). Interestingly, the fibroblasts within the tumour stroma acquire a dynamic phenotype, similar to that of fibroblasts associated with wound healing (Cirri and Chiarugi, 2012), with high expression of α-smooth muscle actin (α-SMA) and vimentin (mesenchymal marker)(Horimoto *et al.*, 2012). These activated fibroblasts also termed cancer associated fibroblasts (CAF) (Figure 5) have been observed repeatedly in the stroma of the majority of aggressive and invasive human breast cancers (Sappino *et al.*, 2006).

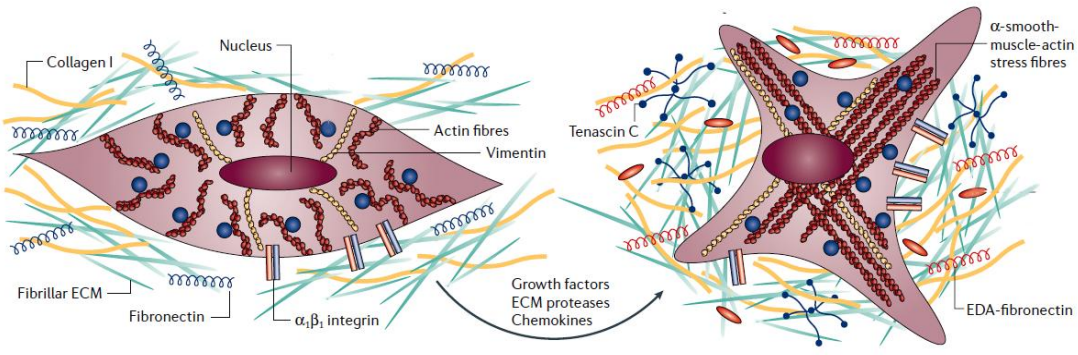

Figure 5 – Comparison of normal and cancer associated fibroblasts (CAF) (Adapted from Kalluri and Zeisberg, 2006).

The great influence of fibroblasts in cancer evolution is proved by their capability to induce the growth and activation of immortalized prostate epithelial cells *in vitro* and *in vivo* (Olumi *et al.*, 1999). Parrott and co-workers, showed in 2001, that ovarian cancer cells recruit adjacent fibroblasts from normal tissue in order to from the stroma of the tumor, demonstrating the need of other cell types to form tissue primary cancer. Furthermore, it has been also shown that, therapies against stromal fibroblasts obtained high anti-tumoral effect (Parrott *et al.*, 2001; Strell *et al.*, 2012; Mertens *et al.*, 2013).

In the tumor mass, fibroblasts interact with all other elements of the tumor microenvironment (Figure 6), affecting their action.

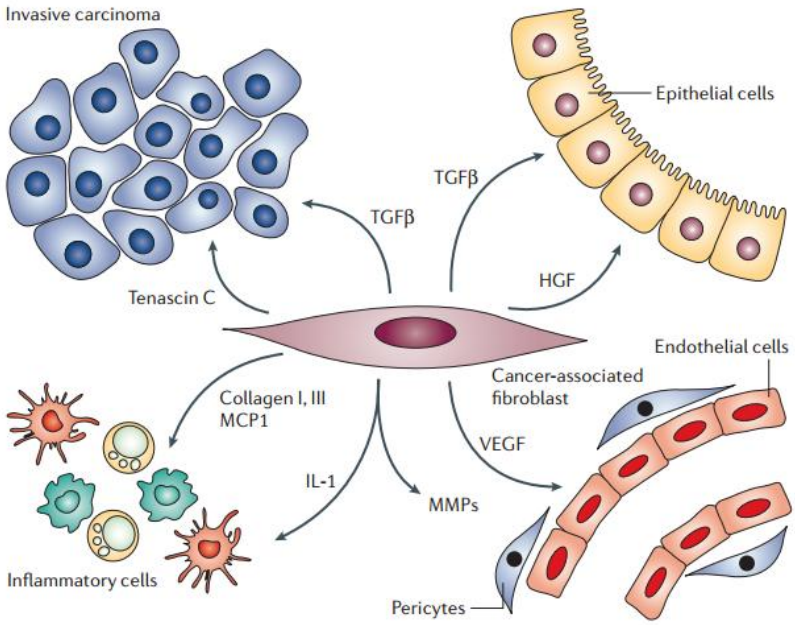

Figure 6 - Influence of fibroblasts in tumor microenvironment (Adapted from Kalluri and Zeisberg, 2006).

The interactions between fibroblasts and cancer cells are essentially mediated by growth factors (Table 1), that are secreted by these two cells to stimulate both of them and induce tumor progression. In brief, cancer cells produce growth factors such as PDGF, TGF-ß, endothelial growth factor (EGF) and basic fibroblast growth factor (bFGF), in order to activate fibroblasts. Subsequently, these fibroblasts secrete growth factors, such HGF, keratinocyte growth factor (KGF), insulin-like growth factor 1 and 2 (IGF-1 and -2), chemokines, like stromal cell-derived factor-1 (CXCL12) and glycoproteins (tenascin C) to stimulate cancer cells (Bhowmick *et al.*, 2004; Cirri and Chiarugi, 2012).

Table 1 – Interactions between cancer cells and tumor stromal fibroblasts.

Interaction	Factor	Role	Ref.
Cancer cells towards Fibroblasts	PDGF	▪ Fibroblasts activation to CAF.	(Kalluri and Zeisberg, 2006; Cirri and Chiarugi, 2012)
	TGF-β	▪ Fibroblasts activation to CAF.	(Bierie and Moses, 2006; Yin *et al.*, 2012)
	EGF	▪ Stimulates fibroblasts to produce VEGF and HGF.	(Yu *et al.*, 2012)
	bFGF	▪ Fibroblasts proliferation.	(Yu *et al.*, 2012)
Fibroblasts towards Cancer cells	HGF	▪ Proliferation; ▪ Drug resistance.	(Bhowmick *et al.*, 2004; Straussman *et al.*, 2012)
	KGF	▪ Migration; ▪ Metastasis.	(Faria *et al.*; Leyva-Illades *et al.*, 2012)
	IGF-1, -2	▪ Survival of tumor cells.	(LeBedis *et al.*, 2002; Hanahan and Coussens, 2012)
	CXCL12	▪ Growth and survival of malignant cells; ▪ Stimulate the migration of stromal cell types into the tumor microenvironment (T cells, B cells and monocyts).	(Balkwill *et al.*, 2012)
	Tenascin C	▪ Influence the capacity of tumor cells to adhere and spread through the ECM.	(Brellier and Chiquet-Ehrismann, 2012)

Besides the fact that fibroblasts induce tumor proliferation, these stromal cells also generate ROS in environments with low pH and oxygen concentration (Xing *et al.*, 2010). ROS are responsible for multiple mutations in malignance surrounding cells.

In terms of cancer cell invasion through the ECM, fibroblasts have a potential influence. CAFs retain a major role in ECM remodelling since they are mainly responsible for the production of ECM proteins (collagens and fibronectins) as well as proteases and other enzymes involved in the post-transcriptional modification of ECM proteins (Vanharanta and Massagué, 2012). In tumor, fibroblasts are responsible for the increase of matrix deposition and ECM stiffening (Cirri and Chiarugi, 2012). This stiffening is responsible for an increase in cross-linking between collagen molecules induced by lysyl oxidase (LOX) expressed by fibroblasts. These enzymes are responsible for ECM remodelling and as consequence the tumor cell migration and invasion (Cirri and Chiarugi, 2012). In addition, cancer cells induce CAF to produce MMP

(Talmadge and Fidler, 2010; Xing *et al.*, 2010), which binds to the cancer cells and it is used for degradation and invasion of malignance cells through the ECM (Pavlaki and Zucker 2003). Two other ECM components produced by fibroblasts are fibronectin and hyaluronan. Fibronectin is associated with integrin receptors and MMP secretion, thus affecting cell adhesion, migration. Within tumors, fibroblasts, secrete high concentrations of hyaluronan that are responsible for macrophage recruitment, which have also an essential role in tumor progression, as previously mentioned (Lu *et al.*, 2012; Raz and Erez, 2013). In addition to attract macrophages, fibroblasts are also responsible for mediating the inflammatory response, by secreting chemokines (monocyte chemotactic protein-1 (MCP-1)), interleukins (IL-1) and inducing immune suppression by expression of TGF-β (McClellan *et al.*, 2012; Raz and Erez, 2013). This transforming growth factor is also responsible for the orchestration of the epithelial-to-mesenchymal transition (EMT) (Figure 7) (Chaffer and Weinberg, 2011). Different studies showed the crucial role of EMT in tumor progression, since it promotes the invasion and metastasis of cancer cells (Alexander and Friedl, 2012).

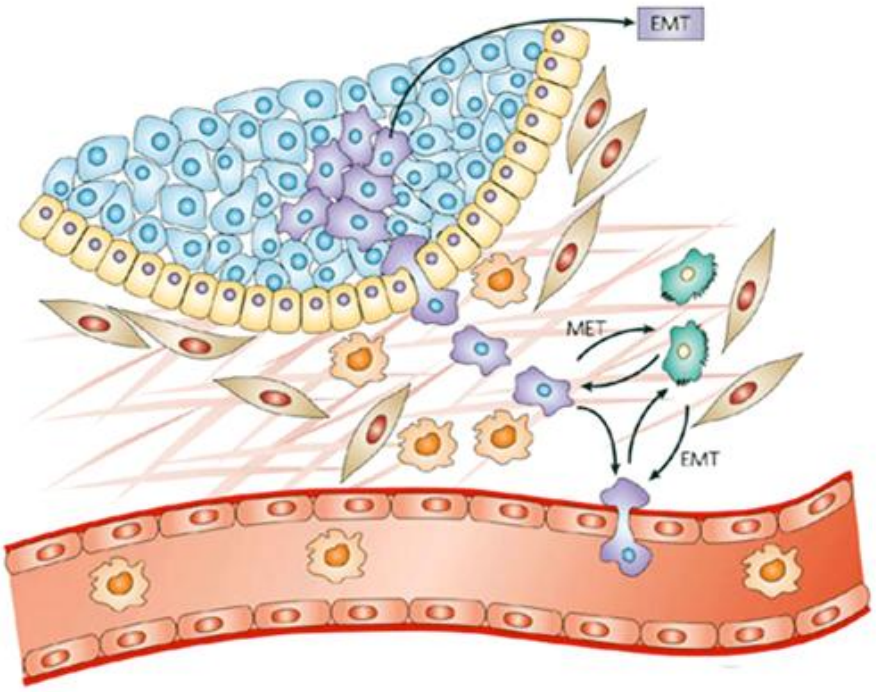

Figure 7 - Representation of the mechanism of epithelial-to-mesenchymal transition (EMT) (Adapted from (Peinado *et al.*, 2007).

EMT is the mechanism responsible for the acquisition of mesenchymal like properties of epithelial cells, in result of disruption of intercellular adhesion (adherens junctions) and the enhancement of cell motility (Alexander and Friedl, 2012).

In last, fibroblasts stromal cells also interfere in tumor vascularization. These stromal cells express VEGF, which have a potential angiogenic effect in tumor mass (Strell *et al.*, 2012).

1.2. Nanosized delivery systems as novel therapeutic approaches for cancer therapy

Advanced oncologic diseases have been for long associated with limited patient progression free survival (PPFS) (Chabner and Roberts, 2005). Over the last two decades, physicians have been focused in increasing PPFS rate through the application of multimodal treatment approaches that involve surgery, chemo- and radiotherapy, in an attempt to tackle the above mentioned adaptive and multi-resistant profile of cancer cells (Riehemann *et al.*, 2009).

The anti-cancer chemical drugs are commonly administered intravenously, being partitioned in both the tumor and major organs (e.g. liver, lungs, kidneys) due to their inability to differentiate healthy from malignant cells (Sinha *et al.*, 2006).

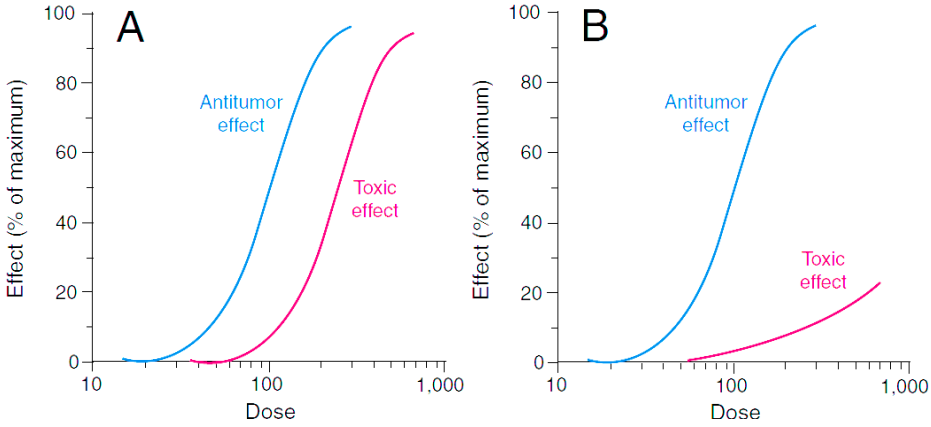

Figure 8 – Relation between drug anti-tumoral effect and toxic effect. **A)** Hypothetical dose-response curves for conventional chemotherapeutic drugs. In conventional therapies the curves of antitumor effect and toxic effect are parallel, revealing that higher doses induce higher adverse effects. **B)** Hypothetical dose-response curves for new drug delivery systems. The drug delivery systems increase the specificity for cancer cells, reducing the toxic adverse effects. (Adapted from Fox *et al.*, 2002).

This pharmacokinetic profile accounts for the adverse cytotoxicity effect attained with the majority of chemotherapeutic agents (Fox *et al.*, 2002). In addition, due to an inherent limited aqueous solubility, anti-cancer drugs present a short circulation time and sometimes their concentration in blood remains below the therapeutic window concentrations, rendering this therapy rather ineffective (Danhier *et al.*, 2012). To overcome these issues, physicians administer chemotherapy through longer time periods (usually every 21 days, with a total of 5 to 8 sessions) (Hamilton and Hortobagyi, 2005). Although, this approach has a limited success, since tumor drug accumulation remains low and cancer cells develop resistance against the effect of chemotherapeutics (Krishna and Mayer, 2000; Raguz and Yagüe, 2008). This acquired multidrug resistance (MDR) is nowadays one of the most serious problems associated with chemotherapy, having a negative impact in PPFS.

Similarly, radiotherapy-based treatments are also impaired by the lack of cell specificity (Sorensen *et al.*, 2012; Allen *et al.*, 2013). In fact, ionizing radiation penetrates within tissues indiscriminately and although it triggers cancer cell death, it also damages the surrounding

tissues and organs in such a way that new cancer cells may arise from radiation-induced mutations (Allen *et al.*, 2013; Zimmermann *et al.*, 2013).

Radiation as an anti-cancer therapy in young woman can affect reproductive organs, and as a consequence induce fertility problems (Metzger *et al.*, 2013). Another major disadvantage of these conventional therapies is their inability to eradicate circulating cancer cells that may lay dormant for a refractory period and form new metastatic cancers in other organs (Chabner and Roberts, 2005; Alexander and Friedl, 2012). Metastatic cancer cells are markedly more aggressive than their parent cells and these conventional therapies are still ineffective (Alexander and Friedl, 2012).

Thus, despite having increased expectations for cancer treatment, these traditional therapies still lack the necessary specificity and effectives to eliminate cancer cells. Therefore, recently the advent of Nanotechnology was unlocked a whole new range of opportunities to develop safe and highly effective anti-cancer treatments. In reality, gathering the unique capacity of nanoscale materials and directed it to the manufacture of novel medical devices brings forth the potential to change the effectiveness of cancer therapies (Zhang *et al.*, 2013). Moreover, nano-based platforms also make an important contribution in cancer prevention, detection, diagnosis and imaging (Yang *et al.*, 2013).

In contrast to conventional therapies, nanomedicine attempts to use sophisticated approaches to either kill specific cells or repair them, one cell at a time (Zhang *et al.*, 2013), like nanoparticles (Figure 9).

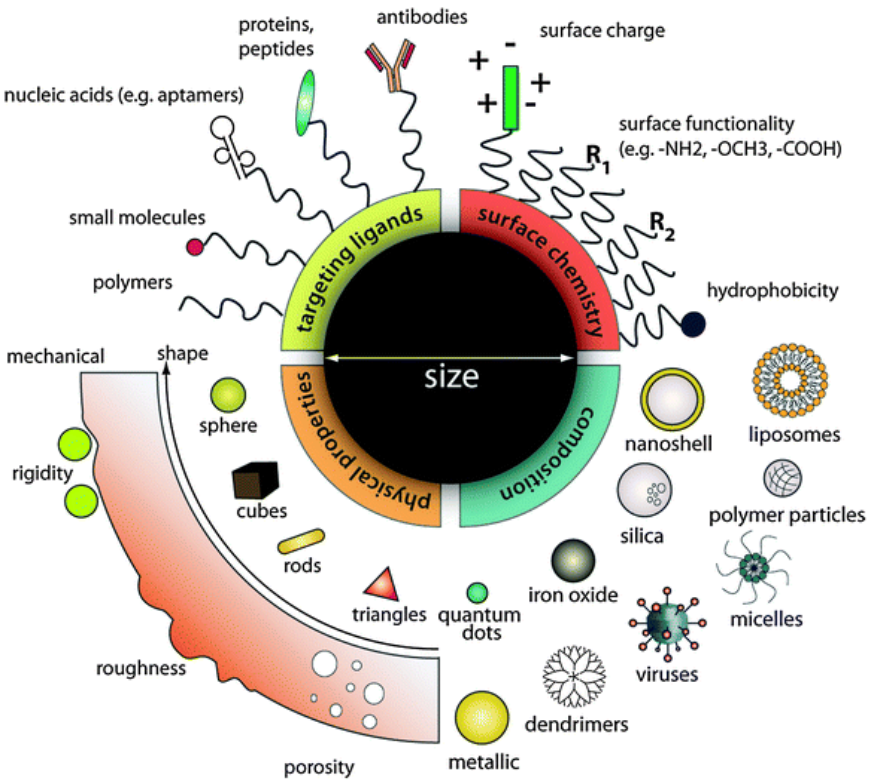

Figure 9 – Representation of the different types of nanoparticles available (Adapted from Chou *et al.*, 2011).

These nanosized particles were developed for the first time around 1970 (Couvreur, 2012) and include numerous architectural designs in terms of: i) size (from a few to several hundred nanometers), ii) shape (porosity, roughness, rigidity), iii) structure (sphere, cubes, tubes), iv) materials (polymers, metal, ceramic) and v) functionalization (hydrophobic character, surface charge, ligands) (Chou *et al.*, 2011). The different proprieties presented by each particle are responsible for different drug loading capacity, particle and drug stability, drug release profile and capacity of targeted delivery. For anti-cancer therapy, different nanocarriers have been already developed (Wang and Thanou, 2010), such as, polymeric nanoparticles, micelles, dendrimers, lipossomes, carbon nanotubes, quantum dots, magnetic nanoparticles, among others, which are able to entrap, encapsulate or attach various anti-cancer agents such as: i) Drugs, that include Doxorubicin, Paclitaxel and Cysplatin; or ii) tumor suppressor genes, that include P53, TNF-α which could induce cell death (Gaspar *et al.*, 2011).

These nanocarriers offer numerous advantages compared to conventional therapies. Particle size and surface characteristics of nanoparticles can be easily tailored (Yun *et al.*, 2012), and the sub-cellular size of these nanosystems increases the overall surface area, thereby increasing the rate of drug dissolution (Riehemann *et al.*, 2009). Nanocarriers also encapsulate several hydrophilic and hydrophobic compounds simultaneously (Kamaly *et al.*, 2012). This is a highly advantageous characteristic since a nano delivery system could co-delivery a therapeutic agent with an imaging agent, allowing physicians to track cancer cells and at the same time treat them, these systems are also known as theragnostic (Farokhzad and Langer, 2009). On the other hand, the use of multiple drugs acting in a synergistic mode could be highly useful to surpass the various defence mechanisms of cancer cells (Han *et al.*, 2013).

In addition, these nanocarriers improve the absorption of insoluble compounds and macromolecules, also improving the bioavailability and release rates, potentially reducing the amount of dose required and increasing safety by decreasing the toxic side effects (Figure 8 B) (Riehemann *et al.*, 2009). Also, the drug release could be sustained during a period of few hours to several days or weeks (Panyam and Labhasetwar, 2012), controlling in this way the therapeutic effect of the drug as intended.

In last, the best upgrade of these nano drug delivery system is their combination with cell- or tissue-specific ligands, for targeted drug delivery (Figure 9) (Farokhzad and Langer, 2009). The association of nanoparticles with these specific ligands (antibodies, molecules or nucleic acids) allows the specific recognition of cancer cells, avoiding the negative effect of the drug in normal cells (Brannon-Peppas and Blanchette, 2004).

In conclusion, with the appearance of nanomedicine new drug delivery systems have been developed, namely nanoscale particles/molecules, that improve the bioavailability and pharmacokinetics of therapeutics. Thus, these systems could improve the treatment by increasing the therapeutic efficacy and decreasing the side effects, improving patient compliance.

1.3. Experimental models to evaluate nanoparticulated delivery systems for application in cancer therapy

Nowadays, tremendous resources about the application of nanoparticles in prevention, diagnosis, and treatment of cancer are being investigated (Zhang *et al.*, 2007). The discovery and development of anti-cancer agents are the main objectives of important pharmaceutical companies (Narang and Desai, 2009). However, the introduction of new nanotechnology based pharmaceuticals creates new challenges for regulatory institutions (Lövestam *et al.*, 2010). In Europe, the European Commission (EC) and European Medicines Agency (EMEA) already define the need that all products based on nanomaterials used in humans must comply with the high level of public health and safety regulations (Hellsten, 2005). In addition, internationally, the new nanodevices need to be in agreement with the basic rules from regulatory agencies such as Food and Drug Administration (FDA) and Organization for Standardization (ISO) (Grieger *et al.*, 2009).

Clinical trials, which are prospective tests to evaluate the effect of medical devices and pharmaceutics in humans under specified conditions, have become a standard and integral part for the development and approval of new cancer therapies (Meinert, 2012). Clinical trials are divided into two main stages (pre-clinical and clinical stage). At each step, from the point of discovery and design, through the demonstration of safety and efficacy in humans, drug and nanoparticle candidates are closely scrutinized.

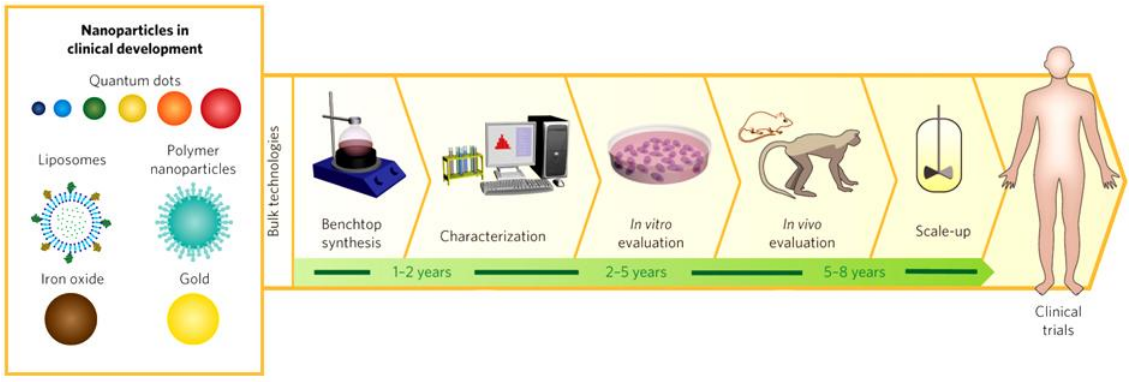

Figure 10 - Steps needed for nanoparticle development (Adapted from Valencia *et al.*, 2012).

The pre-clinical stage includes basic physicochemical studies that allow the identification and characterization of the synthesis of compounds. In this stage, *in vitro* testing, using cell lines and also *in vivo* assays, in animal models, are performed in order to characterize the pharmacokinetic/pharmacodynamic and toxic profile of the drug delivery system in biologic systems (Lee, 2007; Eifler and Thaxton, 2011).

1.3.1. *In vivo* models

International efforts have been made in other to decrease or avoid the use of animals in laboratory, as regulated by the European Directive 86/609/EEC (Stacey, 2012). Futhermore,

there are different countries where animal experimentation is not allowed (Milstein *et al.*, 1996; Stacey, 2012). Thus, nowadays the scientists are using the 3 Rs rule (Replacement, Reduction, and Refinement) in what concerns research animal experimentations (Ranganatha and Kuppast, 2012; Wolfensohn *et al.*, 2013). As a consequence, the experiments are performed only with the essential animals needed to validate the results, with fewer repetitions and during limited periods of a time (Madden *et al.*, 2012). In addition, in order to reduce maintenance costs, the studies tend to use young animals, whereas, the most difficult patients to treat are the elderly ones (Hartung, 2008).

Another major disadvantage of animal use is the difference between species (Astashkina *et al.*, 2012). At the laboratory rodent species are usually used. As demonstrated in Figure 11, it is clear that there is no apparent relationship between animal bioavailability and human bioavailability. In fact, there are several cases with high drug bioavailability in animals and low bioavailability in humans (false positives). Moreover, also low drug bioavailability in animals and high bioavailability in humans (false negatives) is also reported.

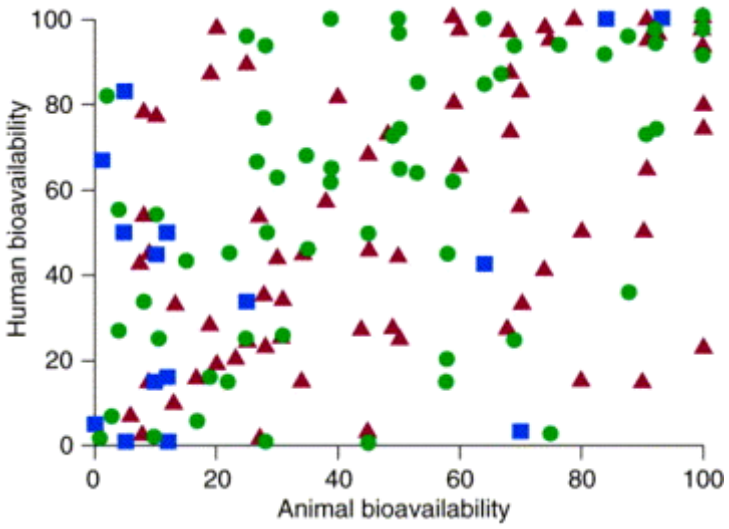

Figure 11 - Absolute bioavailability of various drugs in dogs (red triangles), primates (blue squares) and rodents (green circles) versus the absolute bioavailability reported for humans (Adapted from Sietsema, 1989).

Like these examples, there are others where the predictions offered by *in vivo* models fail categorically in humans (Johnson *et al.*, 2001; Kelland, 2004). In order to avoid this issue, *in* vivo studies could be performed with different species, but that would involve the use of more animals (Hartung, 2008).

Furthermore, some of the studies described in the literature are restricted just to one sex, and both sexes have different sensitivities to drugs, as previously reported in the literature (Hartung, 2008). So, studies should be performed in both sexes in order to obtain more realistic results. The stress caused by the experimental procedures in animals is also responsible for the variability and should be carefully addressed in these studies (Hartung, 2008).

1.3.2. *In vitro* models

Cell cultures emerged in 19th century as a valuable solution for challenging issues of *in vivo* models. The first culture was performed by Wilhem Roux in 1885, by maintaining *in vitro* chicken embryo tissue during several days (Langdon, 2004). However, only in 1922 the culture of epithelial cells was performed and in 1951 the first continuous human cancer cell line (HeLa) was obtained and cultured by George Gey (Langdon, 2004).

These *in vitro* techniques allow the growth of cells outside of their natural environment, under controlled conditions (Lindl and Steubing, 2013). These types of assays become the main force of biopharmaceutical investigation due to their few limitations, namely, bacterial and fungal contaminations (Stacey, 2011). Cell culture offers a unique testing platform to investigate the effects of different drug formulations and nanoparticle designs during pre-clinical development, under highly controlled and reproducible conditions (HogenEsch and Nikitin, 2012), such as temperature, pH, osmotic pressure, oxygen (O_2) and carbon dioxide (CO_2) tension. Moreover, cell cultures provide an easy way to manipulate numerous experimental variables in order to mimic some *in vivo* conditions, whilst avoiding ethical and legal issues associated with animal handling and experimentation (Duell *et al.*, 2011). However, up till now, the cell-cell interactions were commonly disregarded in the majority of the studies reported so far (Duell *et al.*, 2011).

1.3.2.1. Co-cultures

One of the main goals of the *in vitro* systems has been the reproduction of original tissue characteristics and its cell–cell interactions, to simulate, as close as possible, the *in vivo* environment. To overcome such limitations a new category of cell cultures, termed co-cultures, is currently being developed (Miki *et al.*, 2012). This cell culture system arises as a quite interesting alternative that allows a better method to mimic the tumor microenvironment (Duell *et al.*, 2011).

A co-culture consists of at least two different types of cells (i.e., cancer/fibroblast, epithelial cell/lymphocyte, etc.) (Miki *et al.*, 2012) and they provide a more sophisticated and sensitive system that allows to reproduce *in vitro* the *in vivo* tumor niche. By using co-cultures it is possible to recreate some of the cell inter-relationship (Tumarkin *et al.*, 2011), since heterotypic cell-cell interactions are established in close contact. These direct physical connections between cells play a pivotal role in the mechanisms of cancer invasion through actions of adhesion molecules, such as Cadherins (De Wever and Mareel, 2003; Miki *et al.*, 2012). In addition, different cells in co-culture could release cytokines, interleukins, chemokines and growth factors that are essential for the establishment of cell morphology, phenotype, metabolism and proliferation, features that are always present *in vivo* (Streuli *et al.*, 1991; Krause *et al.*, 2010; Purpura *et al.*, 2011; Miki *et al.*, 2012). In addition, co-cultures can also influence the drug resistance presented by cancer cells. In fact, Martinez-Outschoorn and his team, 2011, (Martinez-Outschoorn *et al.*, 2011) analyzed de apoptotic effect of Tamoxifen in monocultures of breast cancer cells and co-cultures of breast cancer cells and

fibroblasts. As a conclusion, they verified a markedly reduction in apoptosis of breast cancer cells when they were in co-culture, highlighting the importance of these models.

1.3.2.2. 3D cell cultures: Spheroids

In addition to the presence of various cell types encountered in the tumor microenvironment, the inclusion of ECM, which is 3D, complex and dynamic network is crucial for mimic the *in vitro* tumor (Cukierman *et al.*, 2002). So, in 1972, researchers began to explore the differences between cells grown on a flat surface versus 3D supports (Elsdale and Bard, 1972). They concluded that, ECM is not just a random mix of secreted components, but it has a specific composition of biomolecules entrapped in a very well defined geometrical structure, that stimulates specific cell responses, such as differentiation. Thus, in addition to co-localization of different cell types with cell–cell interactions and the exchange of bioactive molecules, a 3D architecture that mimics cell organization in tissues and organs is demanded to mimic tissue native proprieties using *in vitro* conditions (Kim *et al.*, 2004). Such, is the main disadvantage of two-dimensional (2D) *in vitro* models. Such models rely on the migration of cells on flat substrates, like glass or polystyrene surfaces (Freshney *et al.*, 2006), in which cells grow in two dimensions (Burdett *et al.*, 2010), which leads to the loss of spatial organization of cells and a deregulation of cell metabolism and functionality as consequence (Lee *et al.*, 2008).

To overcome such disadvantage, the 3D cell cultures have emerged as a viable option. One of the most widely used 3D *in vitro* models are the tumor spheroids (Burdett *et al.*, 2010). This unique culture is based in a small, tightly bound cellular aggregate that tends to form when cancer cells are cultured in non adherent surfaces (Trédan *et al.*, 2007; Burdett *et al.*, 2010; Fennema *et al.*, 2013). Spheroids have been used in cancer research, because the 3D architecture and extensive cell–cell interactions provided by spheroid growth closely mimic the *in vivo* cellular environment. Recently, 3D culture gene expression profiles, have been shown to reflect the clinical expression profiles, in comparison with those observed for 2D cultures (Hirschhaeuser *et al.*, 2010). In fact, the 3D cell organization can influence cell shape, gene expression, growth, morphogenesis, motility and differentiation (Table 2) (Yamada and Cukierman, 2007). In addition, cells cultured in 2D monolayers typically exhibit a lower resistance to therapy than those of *in vivo* tumors, a critical factor if novel therapeutic approaches are under evaluation (Phung *et al.*, 2011). Spheroids have also been considered an essential cell culture technique to study solid tumors. Some cancer types (sarcomas, carcinomas and lymphomas) tend to form solid tumors (Gavhane *et al.*, 2011). These tumors have a unique 3D architecture characterized by hypoxia, low pH and low levels of glucose (Box *et al.*, 2010; Yeom *et al.*, 2012).

Table 2 - The influence of 3D cell organization in cell behaviour and signalling.

Biologic Function	Observed in 3D	References
Cell Shape	▪ Loss of epithelial cell polarity; ▪ Altered epithelial and fibroblast shape.	(Yamada and Cukierman, 2007; Dhimolea *et al.*, 2010)
Gene Expression	▪ Variation in gene expression when cells are culture in 2D or 3D.	(Birgersdotter *et al.*, 2005)
Growth	▪ Cell growth is influenced by 3D structure.	(Lu *et al.*, 2012)
Morphogenesis	▪ 3D cultures are associated with formation of new vessels.	(Shaw *et al.*, 2004)
Motility	▪ Cell motility in 3D matrices is restricted.	(Khatau *et al.*, 2012)
Differentiation	▪ 3D matrix-induced cell differentiation.	(Liu and Roy, 2005; Willerth *et al.*, 2007)

Due to these characteristics, instead of normal arterioles, capillaries, and venules (Figure 12 A), the tumor vessels in solid tumors often have irregular diameters, abnormal branching patterns, and a defective wall structure (Figure 12 B) (Trédan *et al.*, 2007; De Bock *et al.*, 2011).

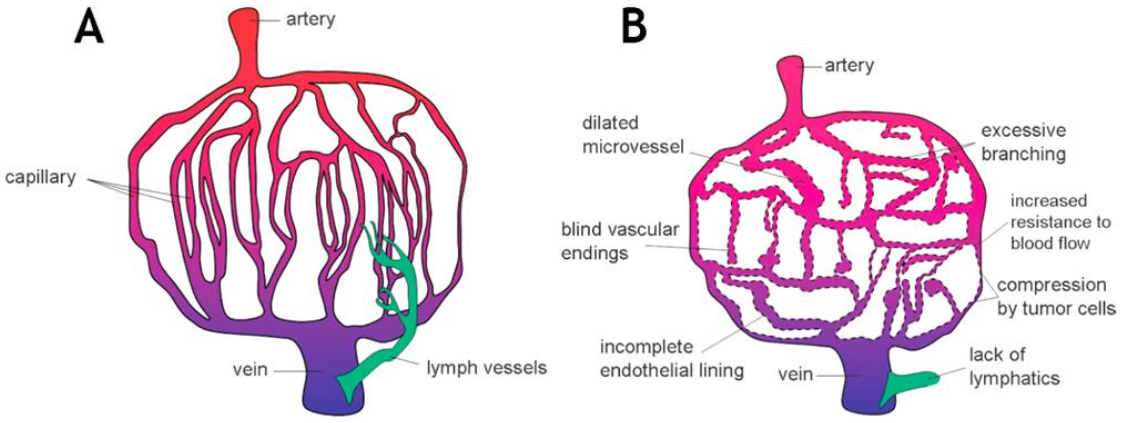

Figure 12 - Representation of the vascularization in normal **(A)** and in cancer **(B)** tissue (Adapted from Trédan *et al.*, 2007).

These irregular vessels could be responsible for the formation of hypoxic regions that are characterized by a defective supply of oxygen and nutrients (glucose and essential amino acids) (Jones and Thompson, 2009). In response, cancer cells are characterized by using lactate to obtain their energy instead of glucose - Warburg effect - (Warburg, 1956;

Hockenbery *et al.*, 2013). Such process involves CO_2 production and results in a microenvironment with a low pH (Harris, 2002).

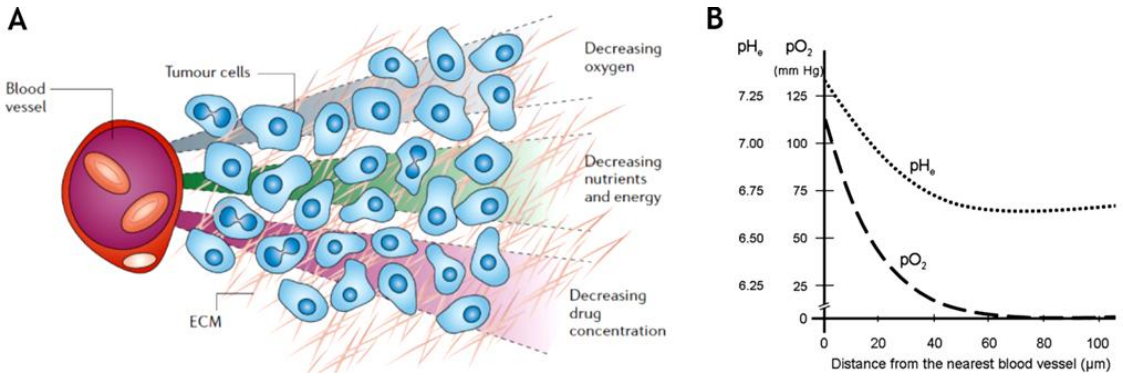

Figure 13 – Representation of the process of vascularisation in solid tumors. **A)** Distribution of tumor cells and extracellular matrix (ECM) in solid tumors. **B)** Graphical representation of pH (dotted line) and gradient of oxygen concentration (dashed line) in relation to the nearest tumor blood vessel (Adapted from Minchinton and Tannock, 2006 and Trédan *et al.*, 2007, respectively).

The majority of anticancer agents reach the cancer cells trough the vasculature (Figure 13). But, in solid tumors, the abnormal vessels difficult drug penetration. Moreover, the composition and organization of the extracellular matrix, cell–cell interactions, and the tumor cell architecture also affects drug penetration (Figure 13)(Minchinton and Tannock, 2006).

Hypoxia in tumors is known to lead to the activation of genes that are associated with angiogenesis and cell survival, and this effect is mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1)(Brahimi-Horn *et al.*, 2011). Furthermore, in the presence of oxygen, many anticancer drugs generate free radicals that damage DNA. Thus, at low oxygen concentrations, the cytotoxicity of drugs whose activity is mediated by free radicals is decreased (Kennedy, 1987; Gutteridge and Halliwell, 2010; Vera-Ramirez *et al.*, 2011).

The low pH characteristic of tumor microenvironment, can also affect the efficiency of anti-cancer drugs. Some drugs have a higher intracellular uptake and cytotoxicity in alkaline environments (Cukierman and Khan, 2010). But, in acidic tumor microenvironment, low pH (6.5-7.2) the change in the surface charge of the drug molecules can reduce their intracellular uptake.

So, the reproduction of the properties of solid tumors *in vitro* is of crucial importance to study the action of new drugs. Different methods could be used to form spheroids for example: i) Microarrays, molds constituted by hydrophobic or non-adherent materials with micro spherical wells; ii) Microfluidic chips, devices that have microchannels which due to the forces caused by fluid flow promote cell aggregation; iii) Gyratory rotation, in which cells undergo a constant circular rotation, in specific cell culture containers; iv) Hanging drop, in which cell aggregates assemble in drops hanging from a hydrophobic surface; and v) Liquid overlay, in which cells are cultured in a well covered with a non-adhesive surface that prevents cell adhesion to the bottom of the plate (Justice *et al.*, 2009; Page *et al.*, 2012;

Fennema *et al.*, 2013). This last technique is the simplest, reproducible and less expensive method for spheroid production (Perche and Torchilin, 2012). In addition, this model forms single spherical spheroids without the limitation of size or shape associated for instance with the micro fluidic and microarray-based approaches.

With 3D cell cultures it is possible to mimic this tumor microenvironment formed in *in vivo* conditions. In 1970, Sutherland and their team treated 3D multicellular tumor spheroids with radiation and observed that the therapeutic results obtained were very similar to those reported for solid tumors *in vivo* (Sutherland *et al.*, 1970).

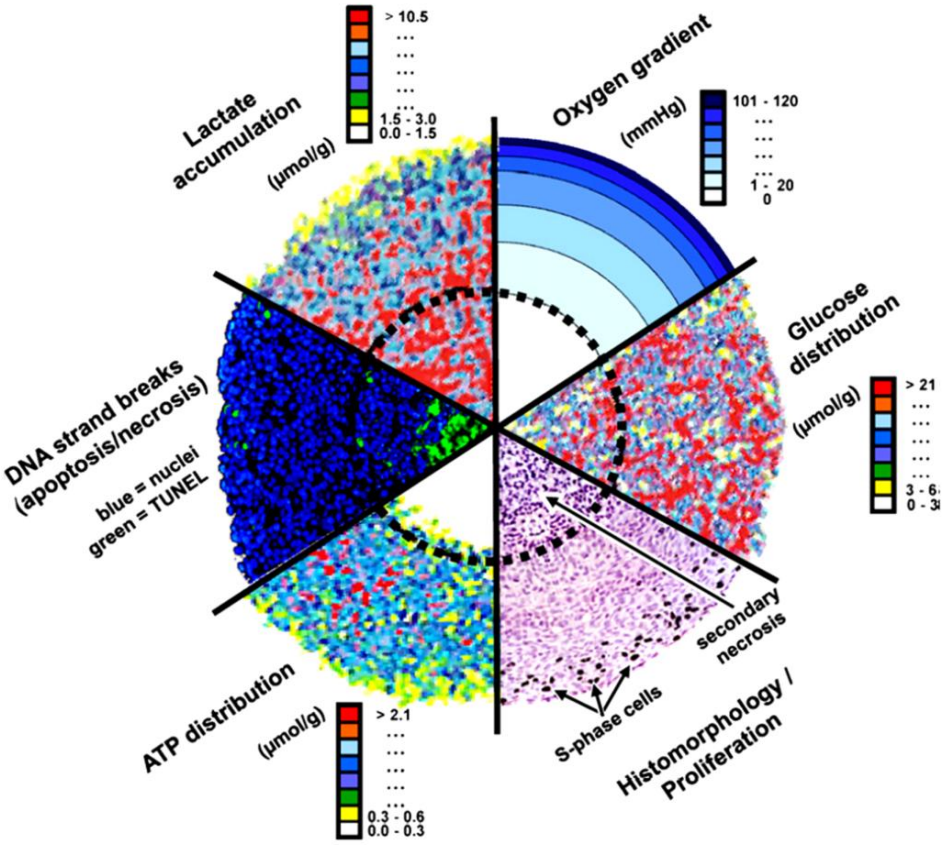

Figure 14 – Characterization of the similarities between the original tumor and the respective spheroids (Adapeted from Hirschhaeuser *et al.*, 2010).

In fact, the similarities between the original tumor and the respective spheroids include volume growth rate, cellular heterogeneity, the induction of proliferation gradients and quiescence state of cells, as well as the differentiation characteristics (Figure 14), such as the development of specific histological structures (Mueller-Klieser, 1987; Mehta *et al.*, 2012). These unique characteristics made spheroids essential models to study tumors *in vitro*.

1.4. Objectives

During the elaboration of this master dissertation, different objectives are taken into account in order to achieve the main goals proposed. The aims are summarized in the following points:

- The main aim of this thesis workplan was is the optimization of heterogenic breast and cervical 2D and 3D cell co-culture models for future development and investigation of new drugs and delivery systems for drug and gene delivery:
    - □ Develop co-culture models that mimic *in vitro* the complex tumor microenvironment found *in vivo,* by using malignant cells and stromal fibroblasts;
    - □ Evaluate the influence of co-culture conditions in cell proliferation and spatial organization;
    - □ Analyse the establishment of direct interactions between cancer and normal cells throughout extended time periods when co-culture *in vitro*;
    - □ Develop *in vitro* co-culture models of breast and cervical cancers;
    - □ Investigate the tumor targeting specificity of a multifunctional gene delivery system towards –cancer cells in 2D mono- and co-culture;
    - □ Optimize the production of 3D multicellar tumor spheroids *in vitro* by using different methodologies;
    - □ Develop 3D co-culture models of breast and cervical cancers during various time points;
    - □ Analyse the 3D tumor spheroids for the acquisition of *in vivo* mimicking characteristics of solid tumors.

Chapter II

Methods

2. Methods

2.1. Materials

Human cervix adenocarcinoma (HeLa) and oestrogen-dependent human breast adenocarcinoma (MCF-7) cells were obtained from ATCC (Middlesex, UK) and primary normal human dermal fibroblasts (hFIB) from Promocell (Heidelberg, Germany). The cell culture plates and T-flasks were obtained from Orange Scientific (Braine-l'Alleud, Belgium). Ibidi cell imaging chambers plates were acquired from Ibidi GmbH (Munich, Germany). Cacodylate, collagen Type I, Dulbecco's Modified Eagle's Medium F-12 (DMEM-F12), Dulbecco's Modified Eagle's Medium High Glucose (DEMEM-HG), ethanol (EtOH), glutharaldehyde, paraformaldehyde (PFA), phosphate-buffered saline (PBS), resazurin, rhodamine B isothiocianate (RITC) and trypsin were purchased from Sigma–Aldrich (Sintra, Portugal). Dimethyl sulfoxide (DMSO) was obtained from VWR BDH Prolabo (Madrid, Spain) and agarose was purchase from Grisp (Porto, Portugal). CellLight® Actin-Green fluorescent protein (GFP) BacMam 2.0, Germ agglutinin conjugated Alexa 594 (WGA-Alexa 594) and Hoechst 33342® were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany). All reagents were of analytical grade and used as received.

2.2. Breast cancer and cervical cancer 2D and 3D *in vitro* co-culture models optimization

2.2.1. Cell lines maintenance

All cell lines, MCF-7, HeLa and hFIB, were grown in 75 cm^2 T-flasks with a humidified atmosphere of 5% CO_2, at 37 °C. MCF-7 and hFIB were maintained in DMEM-F12 medium supplemented with 10% FBS, and 1% streptomycin and gentamycin. HeLa cell line was growth in DMEM-HG, with 10% (v/v) FBS and 1% streptomycin and gentamycin. When cells attained confluence, they were harvested using 0.18% trypsin (1:250) and 5 mM EDTA (Ethylenediamine tetraacetic acid).

2.2.2. Optimization of 2D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB) and cervical cancer (HeLa:hFIB)

Upon attaining confluence, cancer cells and normal fibroblasts were harvested using 0.18% trypsin (1:250) and 5 mM EDTA. Cells were counted by using a haemocytometer and trypan blue 4 % (w/v), in PBS.

Subsequently, co-cultures were performed. For breast cancer co-cultures, MCF-7 and hFIB were seeded onto 6-well plates, with a total number of $2x10^4$ of cells per well and with MCF-7 to hFIB ratio as summarized in Table 3. As controls, homotypic cultures of MCF-7 and hFIB

were seeded using the same total number of cells per well. All these cultures were maintained in DMEM-F12 medium supplemented with 10% (v/v) FBS, and 1% streptomycin and gentamycin.

HeLa and hFIB were seeded onto 6-well plates, with a total number of 2x 10^4 of cells per well and with HeLa to hFIB ratio as summarized in Table 4. As controls, homotypic cultures of HeLa and hFIB were seeded using the same total number of cells per well. All these cultures were maintained in DMEM-HG medium supplemented with 10% (v/v) FBS and 1% streptomycin and gentamycin.

Table 3 - MCF-7 to hFIB cell ratios used *in vitro* to mimic the breast cancer microenvironment.

Ratio	MCF-7	hFIB	References
i) 1:1	50.00%	50.00%	(Heneweer *et al.*, 2005)
ii) 1:3	25.00%	75.00%	(Ko *et al.*, 2012)
iii) 1:5	16.67%	83.33%	(Whitaker-Menezes *et al.*, 2011)
iv) 3:1	75.00%	25.00%	(Martinez-Outschoorn *et al.*, 2010)

Table 4 - HeLa to hFIB cell ratios used *in vitro* to mimic the cervical cancer microenvironment.

Ratio	HeLa	hFIB	References
i) 1:1	50.00%	50.00%	(Delinassios and Kottaridis, 1984)
ii) 1:2	33.33%	66.67%	(Delinassios and Kottaridis, 1984)
iii) 1:10	9.09%	90.91%	(Delinassios, 1987)
iv) 2:1	66.67%	33.33%	(Delinassios and Kottaridis, 1984)

2.2.2.1. Optical microscopy analysis of the distribution and morphology of 2D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB) and cervical cancer (HeLa:hFIB)

The evolution of 2D MCF-7:hFIB and HeLa:hFIB co-cultures and their controls in terms of cell distribution and morphology was analysed by using an Olympus CX41 inverted optical microscope equipped with an Olympus SP-500 UZ digital camera, at various magnifications.

2.2.2.2. Resazurin assay for analysis of cell viability of 2D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB)

The evolution of 2D MCF-7:hFIB co-cultures and their controls in terms of cell distribution and morphology were analysed using an Olympus CX41 inverted optical microscope equipped with

an Olympus SP-500 UZ digital camera. Co-cultures were seeded onto 96 well plates with MCF-7 to fibroblasts ratios in accordance to what is described in Table 3. For each MCF-7:hFIB ratio, 5 wells were seeded with $10x10^3$ cells per well (*n*=5). Cells were maintained in DMEM-F12 supplemented with 10% FBS, without antibiotics. After 24h of co-culture, the medium was replaced and 10µL of resazurin 0.1% (w/v) was incubated in each well. After an overnight incubation, the fluorescence of metabolized Resazurin was measured with a spectrofluorimeter (Molecular Devices, Spectramax Gemini XS) at an excitation/emission wavelength of λ=560/590nm, respectively. As controls, the cell viability of MCF-7 and hFIB monocultures was also determined. Cell viability was determined at 48, 72 and 96 hours (h) of MCF-7:hFIB co-culture.

2.2.3. Optimization of 3D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB) and cervical cancer (HeLa:hFIB)

Prior to all the co-culture experiments, the wells of the culture plates were coated with 300µL of 1% (w/v) agarose dissolved in double deonized and filtered water (Milli-Q water) by heating until 80ºC. Upon attaining confluence, cancer cells and normal fibroblasts were harvested using 0.18% trypsin (1:250) and 5 mM EDTA. Cells were stained with trypan blue 4% (w/v), and counted using a haemocytometer. Subsequently, co-cultures were seeded onto 24-well plates, with a total number of $5x10^3$, $10x10^3$ and $15x10^3$ cells per well, with cancer cells to fibroblasts ratios in accordance to what is described in Table 3 and Table 4. As controls, MCF-7 spheroids of cancer cells and fibroblasts alone were seeded, using the same total number of cells per well.

The MCF-7:hFIB spheroids and respective monoculture 3D model were maintained in DMEM-F12 complete medium (10% (v/v) FBS, and 1% streptomycin and gentamycin). HeLa:hFIB spheroids and their monoculture counterparts were maintained in DMEM-HG complete medium. All 3D cell cultures were maintained under horizontal agitation in a Grant-bio PMS-1000 microplate shaker (Fisher Scientific, Leicestershire, UK), in a humidified atmosphere at 37 °C with an atmosphere of 5% CO_2, during 6 days.

2.2.3.1. Optical microscopy analysis of the distribution and morphology of the 3D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB) and cervical cancer (HeLa:hFIB)

The evolution of 3D MCF-7:hFIB and HeLa:hFIB co-cultures and their controls in terms of cell distribution and morphology was analysed using an Olympus CX41 inverted optical microscope equipped with an Olympus SP-500 UZ digital camera, at various magnifications.

2.2.3.2. Scanning electron microscopy (SEM) analysis of the 3D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB)

For scanning electron microscopy (SEM) analysis of the 3D spheroid models the medium in the wells was removed and spheroids were washed with cacodylate buffer 0.1 M, in PBS 1% (w/v), and then incubated for 1h at room temperature (RT). Subsequently, the 3D spheroids were washed with PBS 1% (w/v) solution to remove traces of cacodylate. The cells were then submitted to a second fixation stage (2.5% glutharaldehyde, in PBS 1% (w/v), at RT). After 2h of fixation, the samples were washed 3 times with PBS. Then, spheroids were dehydrated with growing ethanol (50%, 60%, 70%, 80%, 90% and absolute ethanol) during 15 min. The spheroids were then subjected to critical point drying prior to visualization in the Scanning Electron Microscope. Subsequently, samples were coated with gold and observed in a Hitachi S-2700 (Tokyo, Japan) electron microscope at different magnifications.

2.2.3.3. Confocal laser scanning microscopy analysis of 3D *in vitro* cell co-culture models of cervical cancer (HeLa:hFIB)

The cell distribution and morphology of 3D HeLa:hFIB co-cultures and their controls was analysed through confocal laser scanning microscopy (CLSM) (Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss SMT Inc., USA). For this purpose, the culture medium in wells was removed and then spheroids were subjected to fixation with PFA 4% (w/v) in filtered water (H_2O). After 1h of fixation, spheroids were transferred carefully to µ-Slide Ibidi plates. All the spheroids were labelled with WGA-Alexa 594 during 30 min prior to image acquisition. 3D reconstruction and application of depth coding algorithms was performed in the Zeiss Zen software (2010) as previously mentioned.

2.3. Evaluation of Chitosan-Histidine-Arginine/pDNA nanoparticles cellular uptake in 2D breast cancer co-culture models (MCF-7:hFIB)

2.3.1. CLSM for CH-H-R/pDNA nanoparticles cell uptake analysis in 2D breast cancer co-culture models

In order to differentiate both cell types (hFIB and MCF-7), the cancer cell line was labelled with the BacMan Cell Light 2.0® Actin-GFP probe, in accordance with the manufacturer's protocol. For better cell attachment to well plates, previously to cell seeding, plates were coated with collagen type IV, for 30 min, at 37ºC. After the onset of GFP expression, various MCF-7(GFP) to hFIB ratios were sub-cultured on 8 well µ-Slide Ibidi plates, at a density of 2 x 10^4 cells per well. The cells were cultured in DMEM-F12 medium supplemented with 10% (v/v) FBS, in a humidified atmosphere (5% CO_2), at 37 ºC. After the 24 h of co-culture, the cells were incubated with chitosan polymeric nanoparticles produced accordingly to the procedure previously developed by our team (Gaspar *et al.*, 2013). Cell cultures were then

incubated with Chitosan-Histidine-Arginine/plasmid deoxyribonucleic acid nanoparticles (CH-H-R/pDNA) with RITC-labelled pDNA (1 μg/cm^2). As control, nanoparticles were also incubated in monocultures of hFIB and MCF-7.

After a 4 h, the cells were fixed with PFA 4% (w/v) in H_2O, for 15 min. Following the chemical fixation, cells were washed with PBS and stained with Hoechst 33342® nuclear probe, at room temperature. Cell imaging was performed using Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss SMT Inc., USA) using a Plan-Apochromat 40x/1.4 Oil DIC objective. The samples were acquired in z-stack mode with a slice thickness of 0.23 μm. Orthogonal sectioning and 3D reconstruction of the various Z-stacks was performed in the Zeiss LSM Zen software (2010).

2.3.2. Flow cytometry for CH-H-R/pDNA nanoparticles cell uptake analysis in 2D breast cancer co-culture models

The cellular uptake of polymeric nanoparticles was analyzed through flow cytometry by using a BD FACSCalibur flow cytometer (Becton Dickinson Inc., USA). In summary, MCF-7:hFIB co-cultures with different cancer cells to fibroblasts ratios and monocultures of both types of cells were used as controls were seeded in 6 well culture plates with a total of 2 x 10^5 cells per well. Cells were grown during 24 h in DMEM-F12 with 10% FBS. For better signal acquision, co-cultures and monocultures of hFIB and MCF-7 were used as controls to establish the correct gating and acquisition parameters in the fluorescence 1 (FL-1) (530/30nm (GFP)) and fluorescence 2 (FL-2) (585/42 nm (RITC)) channels. 24 h after seeding, different co-cultures and monocultures were incubated with nanoparticles during 4 h. For these, CH-H-R nanoparticles were prepared with freshly labeled RITC-pDNA (1 μg/cm^2) as previously described by (Gaspar *et al.*, 2013). Then, cells were extensively washed with ice cold PBS, harvested with 0.18 % trypsin/5 mM EDTA, and recovered by centrifugation. Subsequently, cells collection, cells were resuspendend in 500 μL of fresh PBS. Data acquisition was performed in the CellQuest™ Pro software, where 8 x 10^3 events were recorded in the gated regions of interest, assigned to hFIB and MCF-7 cells. The results obtained were analyzed with a trial version of FCS express v. 4 research edition software (De Novo Software, Ontario, Canada).

Chapter III

Results and Discussion

3. Results and Discussion

3.1. Breast cancer and cervical cancer 2D and 3D *in vitro* co-culture models

As previously mentioned, co-cultures unlock the possibility to mimic *in vitro* the *in vivo* tumor niche, using simple tools and cost-effective methodologies. These co-cultures are usually performed with two types of cells: cancer cells and stromal cells, namely fibroblasts which are those that mostly influence the behaviour of tumor cells. Among all types of cancer, breast cancer and cervical cancer are the most common malignancies amongst women, being responsible for a high rate of mortality at worldwide (Youlden *et al.*, 2012). From these standpoints, the aim of this thesis was the development and optimization of breast cancer and cervical cancer 2D and 3D co-cultures models, for future development of drugs and delivery systems to be applied in cancer therapy.

3.1.1. Development and optimization of 2D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB)

In order to mimic the tumor microenvironment of *in vivo* breast cancers in a more realistic mode by using cell cultures, MCF-7 adenocarcinoma cells were seeded in culture together with non-malignant human skin fibroblasts. The use of fibroblasts representative cells of the tumor stromal cells is attributed to their potential biological interaction with breast cancer cells *in vivo,* as recently reported by Straussman and co-workers, 2013 (Straussman *et al.*, 2012). Especially in breast tumor tissue, fibroblasts act as a key factor for the spatiotemporal evolution of malignant cells (Aboussekhra, 2011). Furthermore, skin fibroblasts may also be biologically altered and exhibit characteristics associated with a transformed phenotype characterized by the expression of growth factors, such as HGF, KGF, IGF, which have a potential effect in cancer cells, namely, in proliferation, metastization and drug resistance (Aboussekhra, 2011; Hanahan and Coussens, 2012). Therefore, these cells are usually used by different authors for the development of breast co-cultures models (Martinez-Outschoorn *et al.*, 2010; Olsen *et al.*, 2010; Chiavarina *et al.*, 2011). However, it is important to emphasize that in *in vivo* tumors the fraction of normal cell in relation to cancer cells is highly dynamic and changes from patient to patient. In fact, breast tumor is commonly described to be a heterogeneous mass of cancer and stromal cells in different concentrations (Weigelt *et al.*, 2010). Besides, in the literature, there are several conflicting reports concerning the actual ratio of MCF-7 to hFIB used to establish this particular *in* vitro model of breast cancer. In fact, several authors suggest the establishment of co-culture systems with equal number of normal and cancer cells, but others perform co-cultures with more cancer cells than fibroblasts, or vice-versa (Brouty-Boyé *et al.*, 1994; Sadlonova *et al.*, 2005; Krause *et al.*, 2010; Xu *et al.*,

2013). Thus, in general the described MCF-7 to hFIB cell ratios are described as follows: i) 1:1; ii) 1:3; iii) 1:5 and iv) 3:1 (Heneweer *et al.*, 2005; Martinez-Outschoorn *et al.*, 2010; Whitaker-Menezes *et al.*, 2011; Ko *et al.*, 2012; Le Droumaguet *et al.*, 2012).

Taking these discrepancies into account, co-cultures with different MCF-7:hFIB ratios were developed in an attempt to overcome the urgent necessity to mimic tumor heterogeneity. However, until presently the organization of cells in co-culture with time has never been explored (Duell *et al.*, 2011; Tumarkin *et al.*, 2011). Actually, the latter assumes further significance since an extended co-culture time is important to maximize the interactions between cell types and will surely influence the aggressiveness and resistance of cancer cells to anti-tumoral therapeutics (Kalluri and Zeisberg, 2006; Hanahan and Coussens, 2012; Strell *et al.*, 2012). This communication may occur directly by tight junctions or indirectly by paracrine inter-cellular signalling (Wang *et al.*, 2006; Pietras and Östman, 2010; Lu *et al.*, 2012).

Thus, in this study co-cultures were maintained during 10 days, in order to analyze the influence of signalling in cell organization and biological behaviour. During the establishment of the co-culture systems, the medium was not replaced by fresh medium in order to preserve the exchanged soluble mediators between cancer and stromal cells. This is a critical parameter that needs to be addressed during the production of co-culture models since various biomolecules such as growth factors, cytokines, chemokines and other soluble factors are secreted by these cells and can thus have a potential effect in cell behaviour (Hanahan and Coussens, 2012). By using this approach the tumor microenvironment is more precisely reproduced since extensive exchange of these mediators also occurs *in* vivo (Miki *et al.*, 2012).

The spatiotemporal evolution of these 2D co-cultures systems over time is presented in Figure 14.

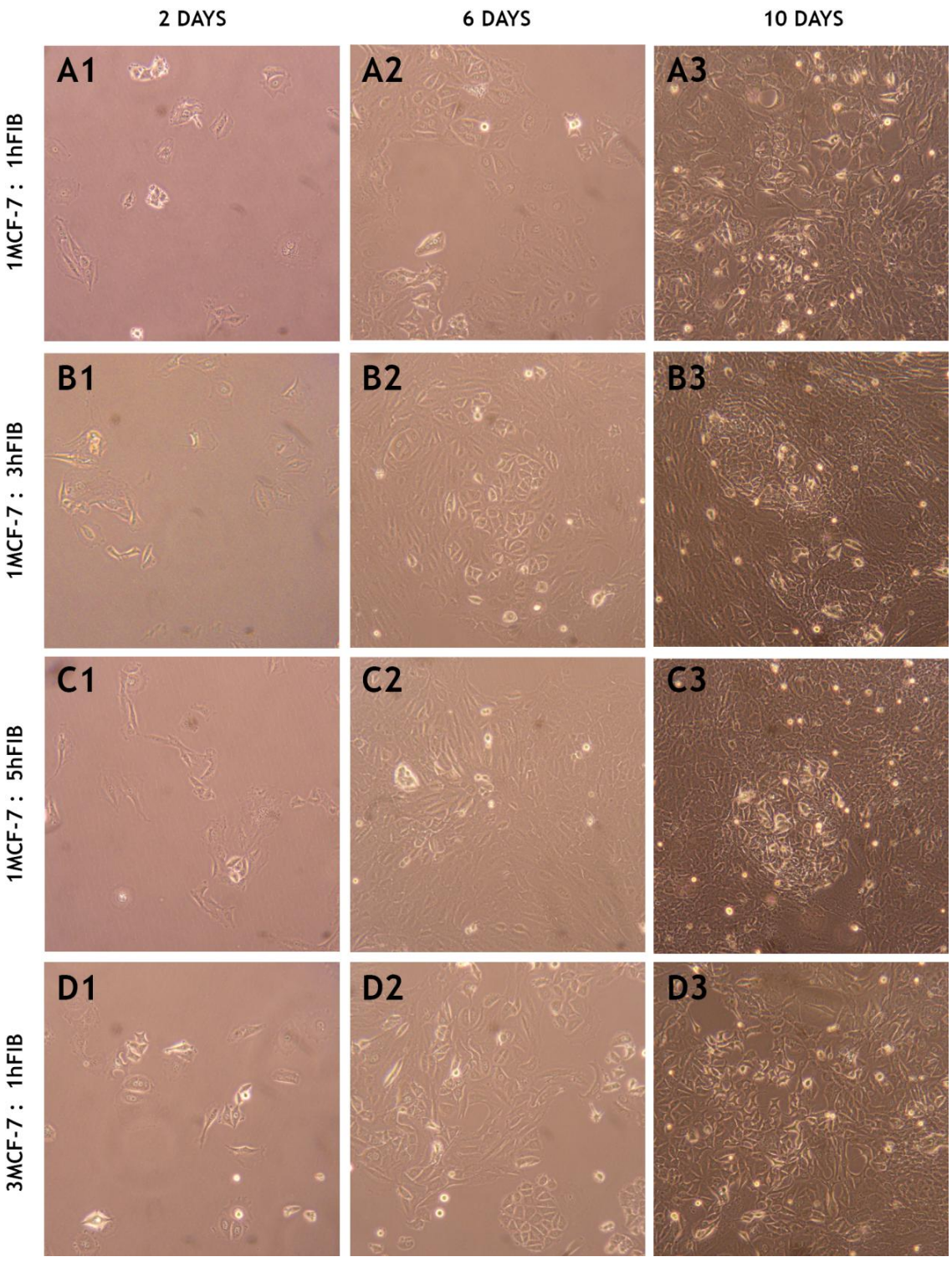

Figure 14 - Light Microscope images of 2D MCF-7 and hFIB co-cultures during 10 days of culture. Co-cultures of MCF-7 to hFIB of ratio: **A)** 1:1; **B)** 1:3; **C)** 1:5; and **D)** 3:1. Original magnification 100X.

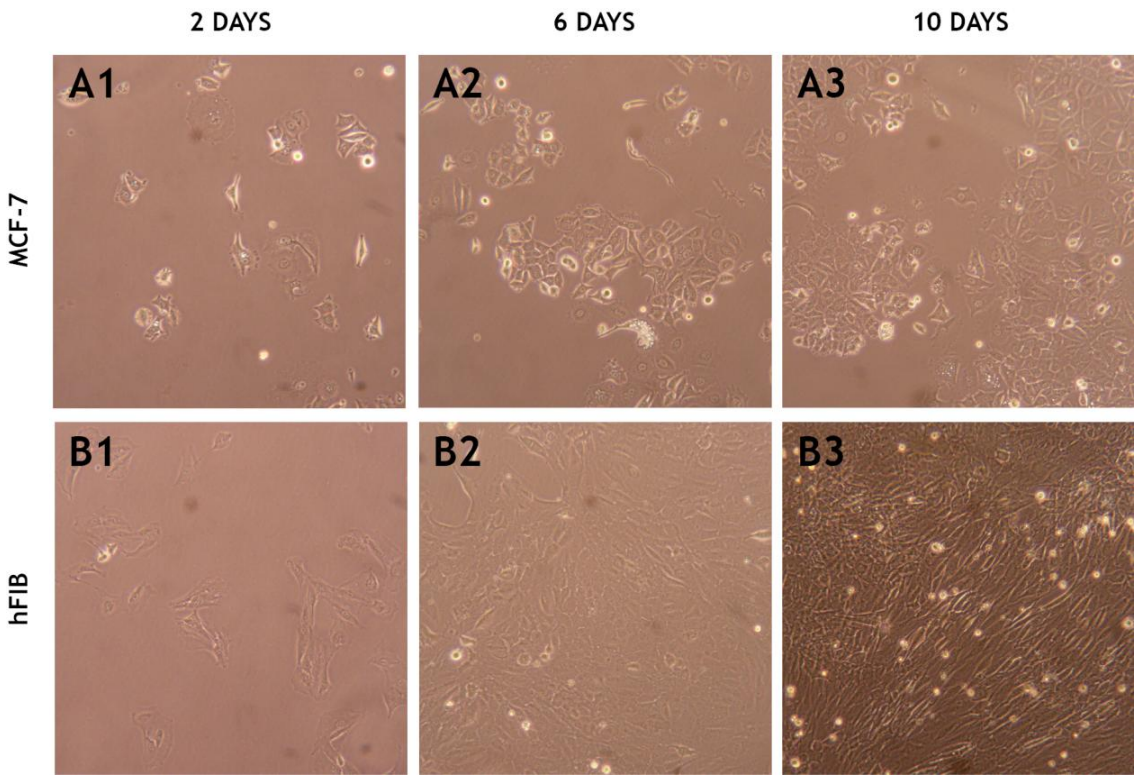

Figure 15 - Light Microscope images of 2D MCF-7 and hFIB monocultures during 10 days of culture (controls). **A)** MCF-7; **B)** hFIB. Original magnification 100X.

Through the analysis of the Figure 14 and Figure 15, it is clearly visible that all cells are adherent despite the fact that they are in monoculture or co-culture. In addition, cell morphologies of both cell types show the preservation of their phenotypic traits when co-cultured for long periods of time. Indeed, breast cancer cells remain with their characteristic polygonal epithelial morphology (Soule *et al.*, 1973; Engel and Young, 1978), whereas, fibroblasts demonstrate their distinctive spindle-shaped morphology (Kalluri and Zeisberg, 2006). These important results illustrate that it is possible to establish viable interrelationships between cancer and stromal cells in *in vitro* co-cultures.

Furthermore, the optical microscopy images demonstrate that MCF-7 and hFIB are able to remain in co-culture for long periods of time. These results are further emphasized by the viability assays.

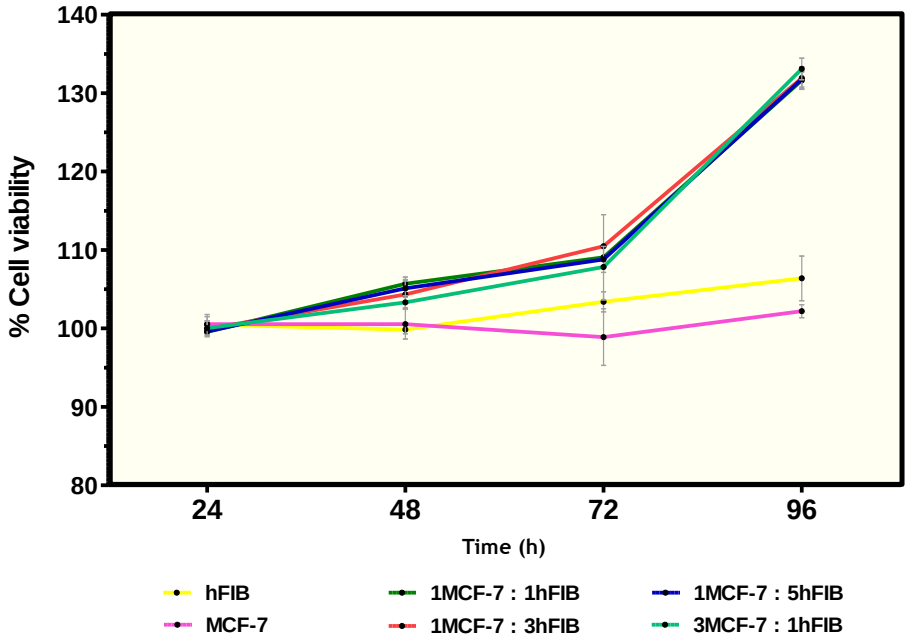

Figure 16 - Cell viability of 2D MCF-7 and hFIB co-culture models with different cell ratios at 24, 48, 72 and 96 h after their seeding. Cell viability in MCF-7 and hFIB monocultures was used as control.

Through the analysis of the viability assays shown in Figure 16, it is possible to observe that at 24h after cell seeding, both cell culture types have approximately 100% viability. Thus, when MCF-7 and hFIB were placed together in culture, no abnormal cell dead in relation to the monocultures was observed. In the course of time (48, 72 and 96 h) the proliferation rate of co-cultured cells is remarkably higher in comparison to that of monocultures. However it is important to emphasize that these results indicate that proliferation is not constant since a steep increase is obtained at 96h of co-culture. These results are in agreement with those already reported in the literature by Orimo and collaborators. They described the capacity of fibroblasts to promote an increased *in vitro* growth of breast cancer cell lines, such as MCF-7 (Orimo *et al.*, 2005). Moreover, it has also been described that cancer cell proliferation is higher when these cells are in direct contact with stromal cells, compared with monocultures and indirect co-culture systems (Fujita *et al.*, 2009).

In this case, just after 3 days of co-culture occurs probably an augment of growth factors expression, such as EGF, HGF, bFGF, between both cells. And these abnormal growth factors expression could influence tumor progression and the resistance of cancer cells against anti-cancer drugs (Straussman *et al.*, 2012). Reports have already evidenced this dynamic expression of soluble factors in co-cultures with the temporal evolution. In fact, as described by Koshida and his team, when colorectal cancer cell lines are co-culture with fibroblasts, there is a higher expression of VEGF and its expression is higher after 48h of co-culture (Koshida *et al.*, 2006).

Additionally a dynamic change of cell structural organization with the temporal evolution of co-culture was also observed. Interestingly, highly organized structures were observed after 8

days of co-culture. In *in vitro* co-cultures with more stromal cells than MCF-7 cancer cells, MCF-7 cells tend to develop agglomerates surrounded by fibroblasts, as demonstrated in Figure 17 and Figure 18.

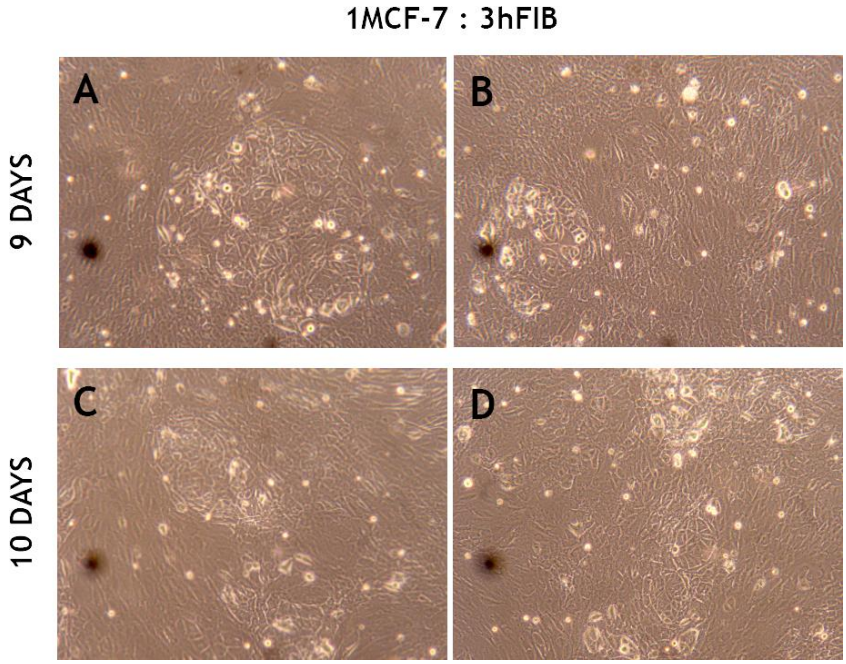

Figure 17 – Optical contrast microscopy images of 1MCF-7:3hFIB 2D co-cultures after 9 and 10 days of culture. **A, B)** 9 days in co-culture; **C, D)** 10 days in co-culture. Original magnification 100X.

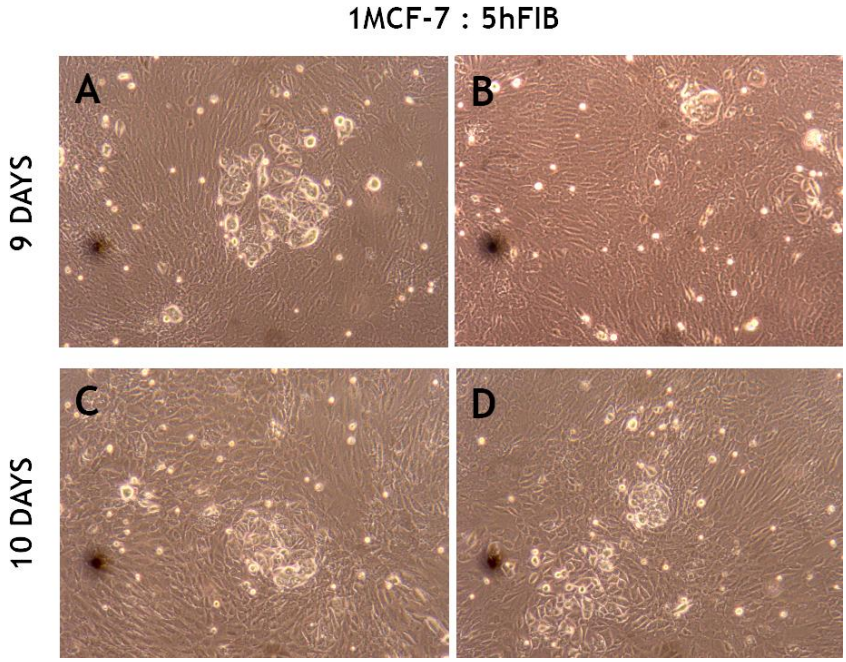

Figure 18 – Optical contrast microscopy images of 1MCF-7:5hFIB 2D co-cultures after 9 and 10 days of culture. **A, B)** 9 days in co-culture; **C, D)** 10 days in co-culture. Original magnification 100X.

This agglomeration is associated with the phenotypic characteristics of breast cancer cells that are usually organized in acinar-like structures (Figure 19) (Bissell *et al.*, 2002). As it is possible to observe in different histological images of breast cancer biopsies present in the literature, breast cancer cells are surrounded by stromal elements. The obtained results also demonstrate that, different co-culture ratios such as MCF-7:hFIB (1:3) (Figure 14 B3 and Figure 17) and MCF-7:hFIB (1:5) (Figure 14 C3 and Figure 18) have shown to be extremely similar to histological sections of invasive breast cancer (IBC) (Figure 19 E). This cancer correspond to stage I of cancer development, which occurs when cancer is constituted by small groups of cancer cells and has not yet spread outside of breast tissue, but cancer cells have are acquire metastatic potential and a more aggressive and resistant phenotype (Debnath and Brugge, 2005; Weigelt *et al.*, 2010).

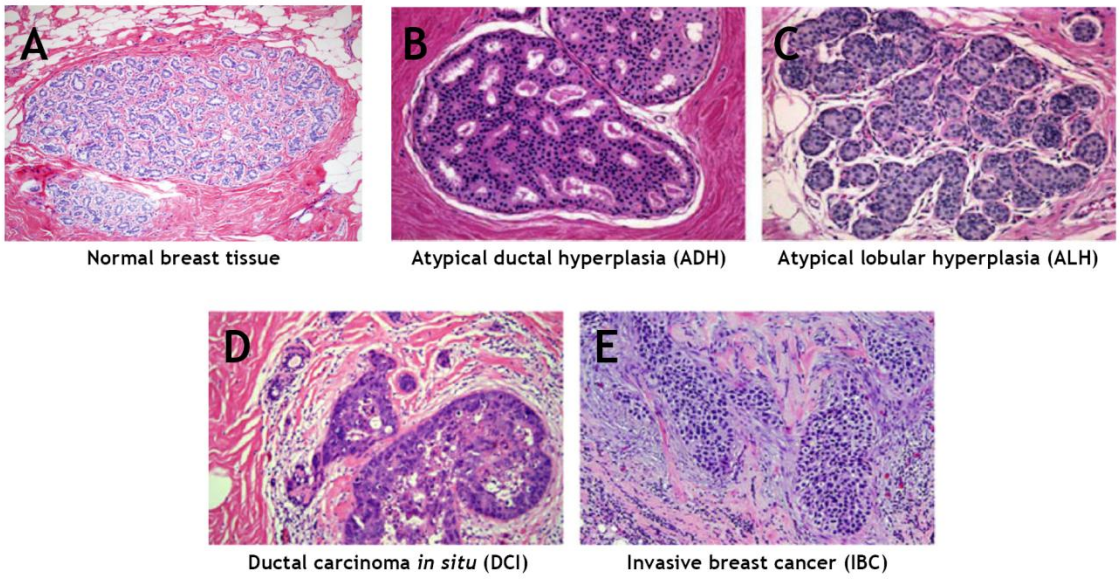

Figure 19 - Microscope images of H&E histological sections of human breast healthy and carcinoma tissue in different stages of tumor evolution. **A)** Normal breast; **B)** Atypical ductal hyperplasia (ADH); **C)** Atypical lobular hyperplasia (ALH); **D)** Ductal carcinoma *in situ* (DCIS); **E)** Invasive breast cancer (IBC). Adapted from Cichon *et al.*, 2010.

However, despite the co-cultures with less amount of fibroblasts did not form the characteristic clusters of cancer cells, they still represent viable *in vitro* models for testing novel therapeutic approaches. Actually, several recent studies have reported the production of viable co-cultures with fewer fibroblasts in comparison to breast cancer cells (Brouty-Boyé *et al.*, 1994; Sadlonova *et al.*, 2005; Krause *et al.*, 2010; Xu *et al.*, 2013). The absence of an organized structure in these co-cultures (Figure 14 A3 and D2), is actually very useful to represent breast cancers in stages after tumor be developed, which is characterized by extensive EMT and the loss of the cancer cells glomerular organization. Then, cells migrate trough ECM and stromal cell constituents, emerging a disorganized structure as presented in co-cultures performed with less initial number of fibroblasts (Figure 14 A3 and D2).

Therefore, all the co-cultures systems developed represent viable systems that mimic the heterogeneity found in breast cancers, a parameter that could not be reproduced in MCF-7 or hFIB monocultures (Figure 15). Moreover, the obtained results also demonstrate that stromal fibroblasts cells have an effect in the spatiotemporal organization of the cultures, and also, influence malignant cell proliferation throughout time. Such was not observed mono-cultured cells, mostly due to the absence of inter-cellular signalling events (Miki *et al.*, 2012). Therefore, these interesting observations render the co-culture models produced during relatively long periods of time a very attractive testing platform for screening novel drug delivery systems or novel synthetic or natural compounds with possible anti-tumoral activity in breast cancer cells.

3.1.2. Development and optimization of 2D *in vitro* cell co-culture models of cervical cancer (HeLa:hFIB)

In addition, to the establishment of MCF-7 breast cancer *in* vitro 2D co-cultures a model of co-cultures of cervix cancer was also developed. For this purpose HeLa cells were used as model cancer cells and fibroblasts were used as stromal cells. The establishment of these HeLa and hFIB co-cultures is very rare in the literature, with the studies concerning HeLa:hFIB co-cultures dating back to the late 80s. Thus, there is a need to optimize the development of HeLa co-cultures systems in order to evaluate new drugs and delivery systems that are designed for targeted therapy of cervix cancer. Thus, similarly to breast cancer co-cultures at various ratios of HeLa to hFIB that were previously reported in the literature were used: i) 1:1; ii) 1:2; iii) 1:10 and iv) 2:1 (Delinassios and Kottaridis, 1984; Delinassios, 1987). However, it should emphasized that although fibroblasts are generally grown in DMEM-F12 complete medium, for the production of these particular co-cultures cells were maintained in the same medium used for HeLa *in vitro* cultures (DMEM HG). This is an important parameter in the experimental design of different co-cultures and should be carefully addressed since it could ultimately affect cell viability. However, as the results demonstrate, despite this fact, hFIB cells self-adapted to the new environmental conditions imposed by high glucose, remaning viable along the time of the experiments, as shown in Figure 15 and 16. These results are corroborated by those also reported by Wang and co-workers, 2013, that also grew hFIB in high glucose culture mediums *in vitro* without deleterious reduction of cell viability (Wang *et al.*, 2013a).

Similarly to MCF-7 co-cultures, HeLa:hFIB co-cultures were also performed in an extended time scale. The resultant light microscopy images of these co-cultures models are shown in Figure 20.

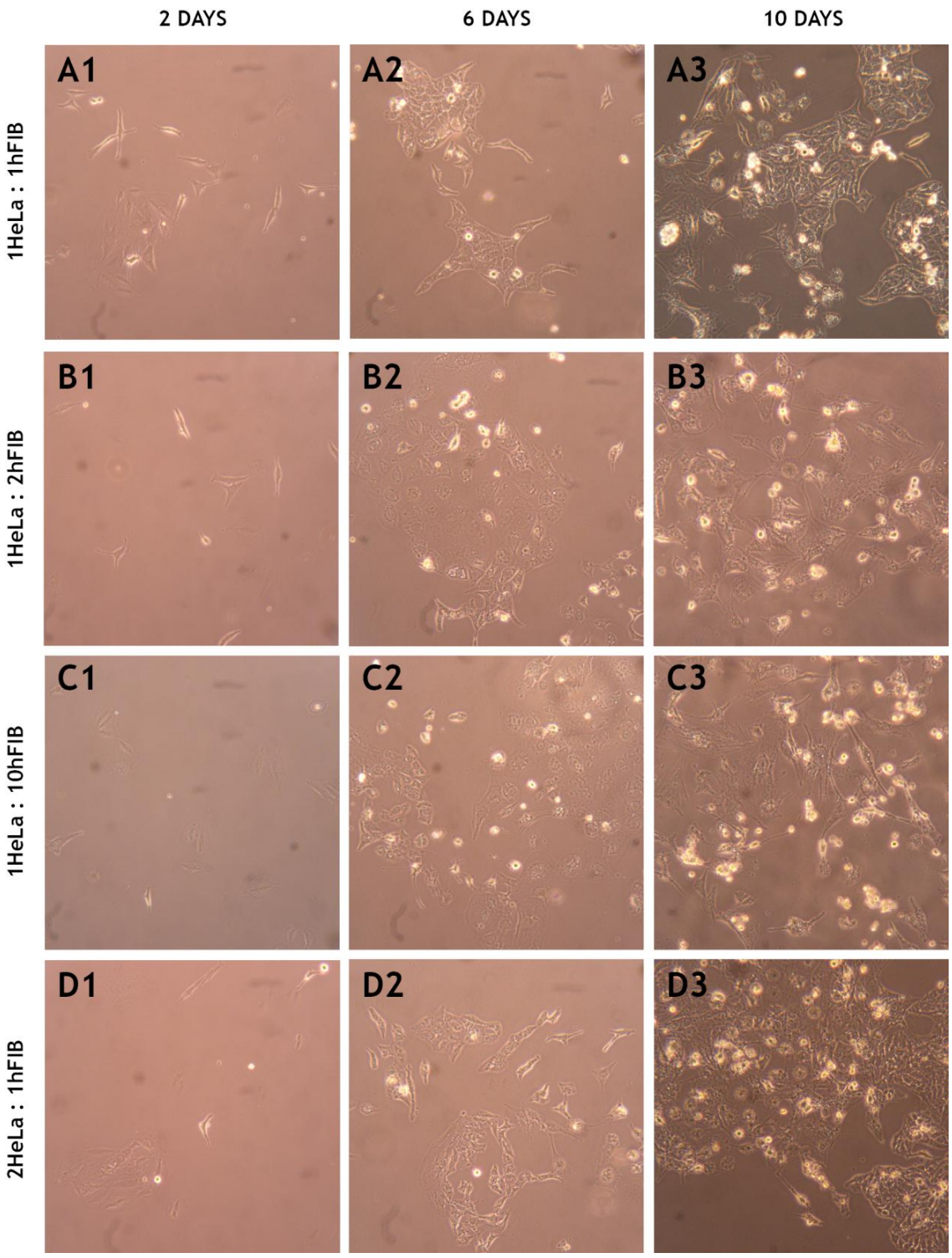

Figure 20 - Light Microscope images of 2D HeLa and hFIB co-cultures during 10 days of culture. Co-cultures with HeLa to hFIB ratio of **A)** 1:1; **B)** 1:2; **C)** 1:10; and **D)** 2:1. Original magnification 100X.

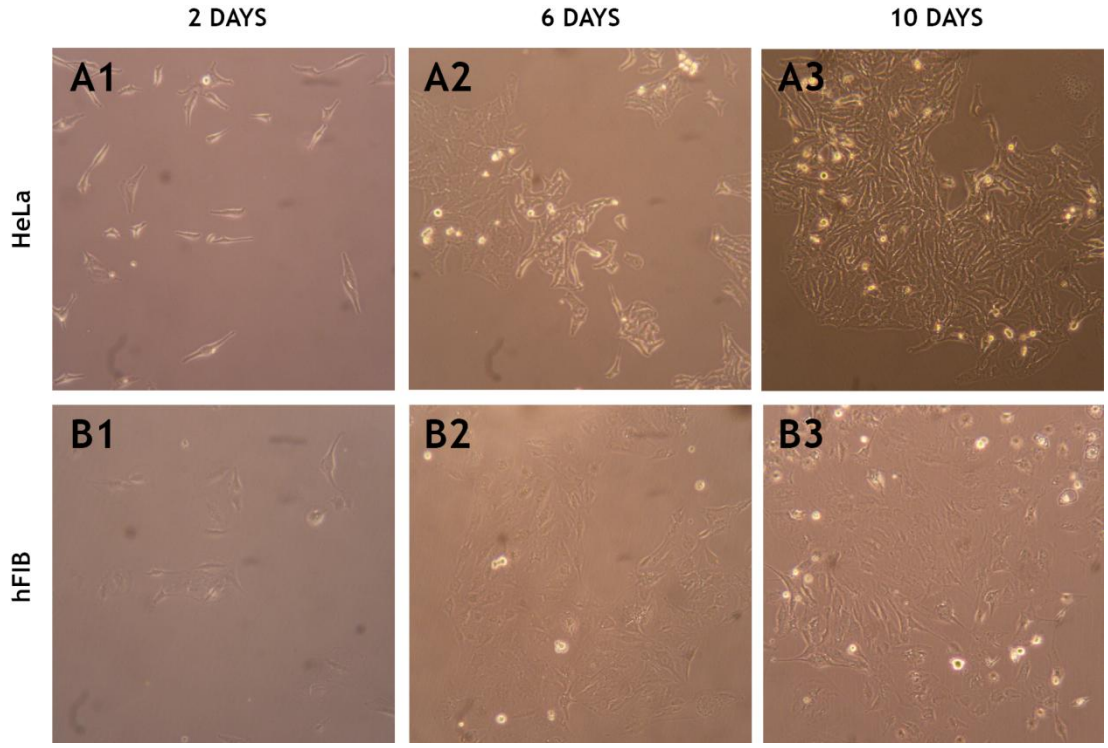

Figure 21 - Light Microscope images of 2D HeLa and hFIB monocultures during 10 days of culture (controls). **A)** HeLa; **B)** hFIB. Original magnification 100X.

Trough the analysis of Figure 21 it is possible to observe that the co-cultivation of human fibroblasts and HeLa cells *in vitro* leads to the development of a unique cellular organization, in comparison to their monocultured counterparts. In addition, in all HeLa:hFIB co-culture models cytoplasmatic projections, such as fillopodium structures between cells are evidenced in co-cultures with more fibroblasts than HeLa cervical cancer cells (Figure 22).

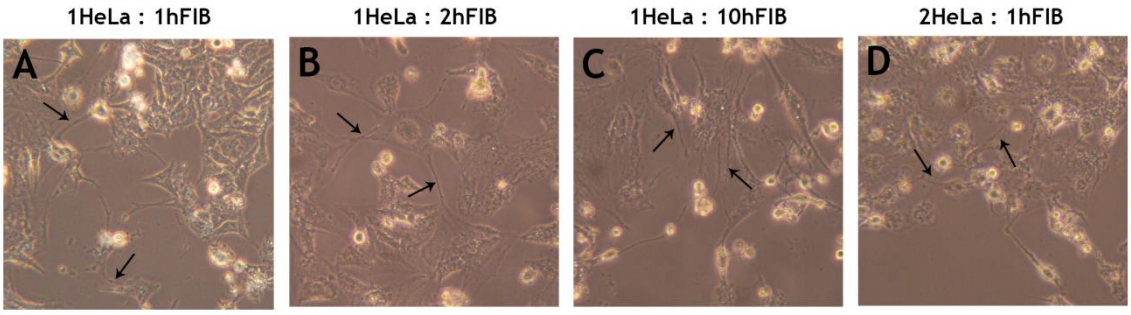

Figure 22 - Inverted Light Microscope images of fillopodium structures (arrows) of HeLa and Fibroblasts cells after 10 days of co-culture. Co-cultures with HeLa to hFIB ratio of **A)** 1:1; **B)** 1:2; C**)** 1:10; and **D)** 2:1. Original magnification 100X.

Interestingly, these HeLa:hFIB co-cultures with more fibroblasts than cancer cells demonstrate a slightly cell dead when compared with homotypic cell cultures, has demonstrated by the spherical shape cells present in Figure 20 B and C. In contrast, in co-cultures with higher concentrations of HeLa cells (Hela:hFIB co-cultures with 1:1 (Figure 20 A)

and 2:1 (Figure 20 D)), cells present a more agglomerated distribution, similarity to that observed for monocultures (Figure 21).

3.1.3. Evaluation of CH-H-R/pDNA nanoparticles cellular uptake in 2D breast cancer co-culture models (MCF-7:hFIB)

In order to take advantage of the co-culture models that were previously established, they were used to test the biological performance and cancer cell targeting capacity of a gene delivery system comprised of CH-H-R/pDNA that has been previously synthesized in our research group (Gaspar *et al.*, 2013). For this purpose breast cancer co-cultures were chosen as *in vitro* cell models since this particular gene delivery system is envisioned to be applied on breast cancer therapy in a near future.

Initially, the *in vitro* cellular uptake of the nanoparticles was analyzed by CLSM. These polymeric nanoparticles are formed by chitosan, a biocompatible polymer with positive charge that has the ability to encapsulate therapeutic nucleic acids (Borchard, 2001). Moreover, to increase the biological activity and selectivity of the system to cancer cells the polymeric backbone of chitosan was also conjugated with two functional and bioinspired ligands, namely, arginine and histidine (Gaspar *et al.*, 2013). As described in the literature, Arginine allows the establishment of electrostatic interactions with the characteristic negatively charged cancer cell membranes (Nakase *et al.*, 2012), thus, the use of Ariginine ligands in nanoparticles leads to an increased penetration of nanoparticles trough cellular membrane. Apart from grafting arginine into the chitosan polymeric backbone, since histidine pKa is in the range of the endosomal/lysosomal pH endows chitosan with improved buffering capacity and subsequently, with an improved endosomal release capacity mediated by the proton sponge effect.

This escape of nanoparticles is essential for the improvement of their therapeutic efficiency, since a large number of nanoparticles is destroyed in these compartments (Wu *et al.*, 2012). These important features could be responsible for the tendency of these nanodevices to target cancer cells, improving in this way, the cellular uptake of this delivery system in different co-culture models.

This specific targeting capacity of nanoparticles could be tested in co-cultures, in fact these platforms are considered the perfect tool to analyse the targeting specificity of drug and delivery systems designed for tumor therapy (Le Droumaguet *et al.*, 2012).

Therefore, these nanoparticles were tested the different breast cancer co-culture ratios to verify if these nanoparticles are specific to cancer cells and if this possible targeting specificity is maintained or is influenced by the heterogeneous models developed.

3.1.3.1. CLSM analysis of CH-H-A/pDNA nanoparticles cellular uptake analysis in 2D cell co-culture models of breast cancer with different MCF-7 to hFIB ratios

To perform the assays to evaluate the targeting capacity of the delivery systems, MCF-7 cells were initially labelled with an actin-GFP fluorescent probe that relies on the use of viral vectors to transfect cells, an important fact since gene expression is maintained up to 5 days in culture with this system. Moreover, since hFIB cells were not labelled, such straightforward approach distinguishes both types of cells during the envisioned analysis.

The CLSM images obtained of nanoparticle tracking in different co-cultures are shown in Figure 23.

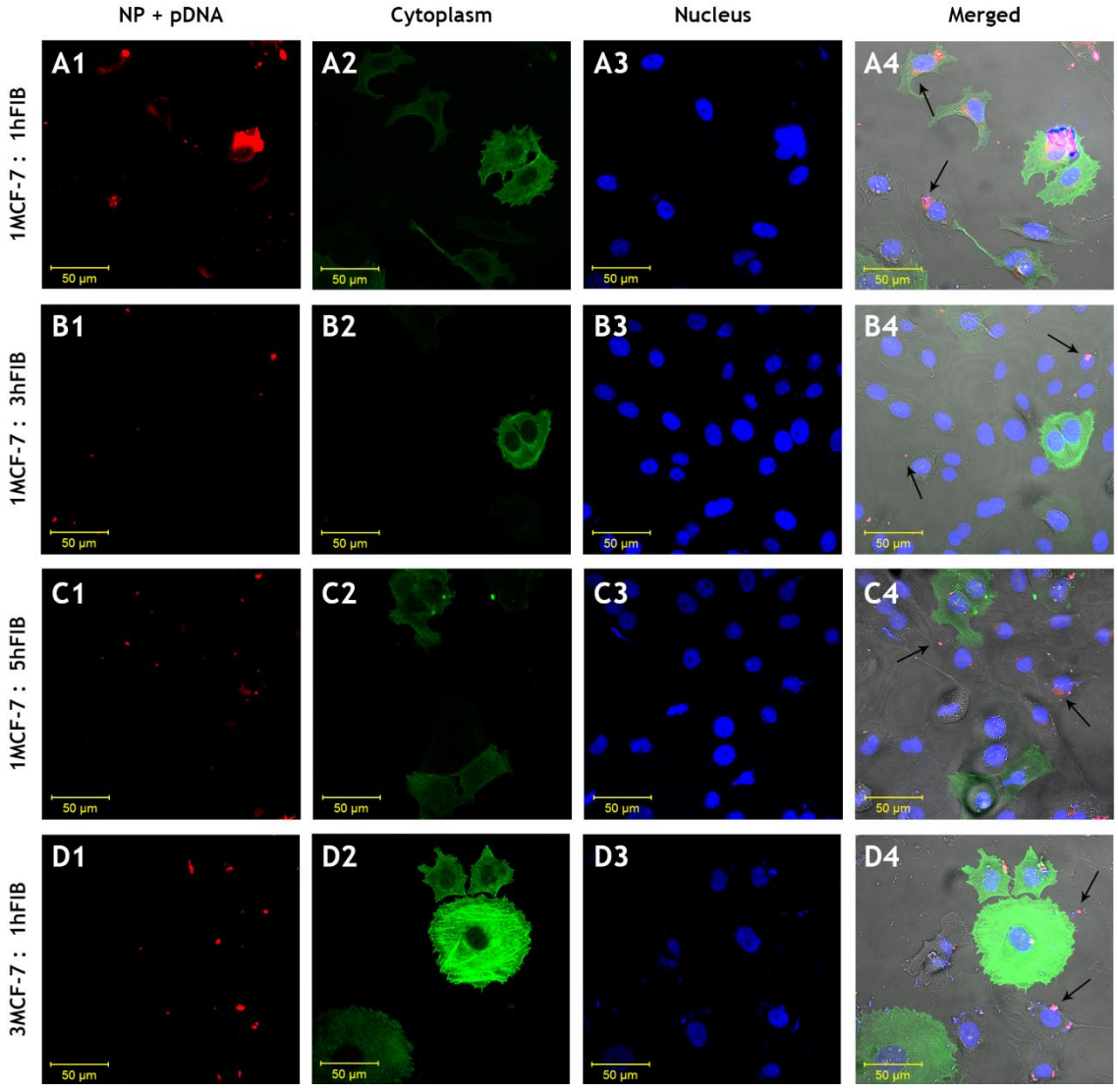

Figure 23 - Confocal Laser Scanning Microscopy images of nanoparticles cellular uptake, after 4 h of incubation in MCF-7:hFIB 2D co-cultures models, with MCF-7 to hFIB ratio of **A)** 1:1; **B)** 1:3; **C)** 1:5; **D)** 3:1. Red channel – RITC-labelled pDNA/CH-H-R nanoparticles; Green channel – Actin-GFP staining of MCF-7; Blue Channel – Hoescht 33342® nuclear staining; Merged channel – DIC + Superimposition of all channels.

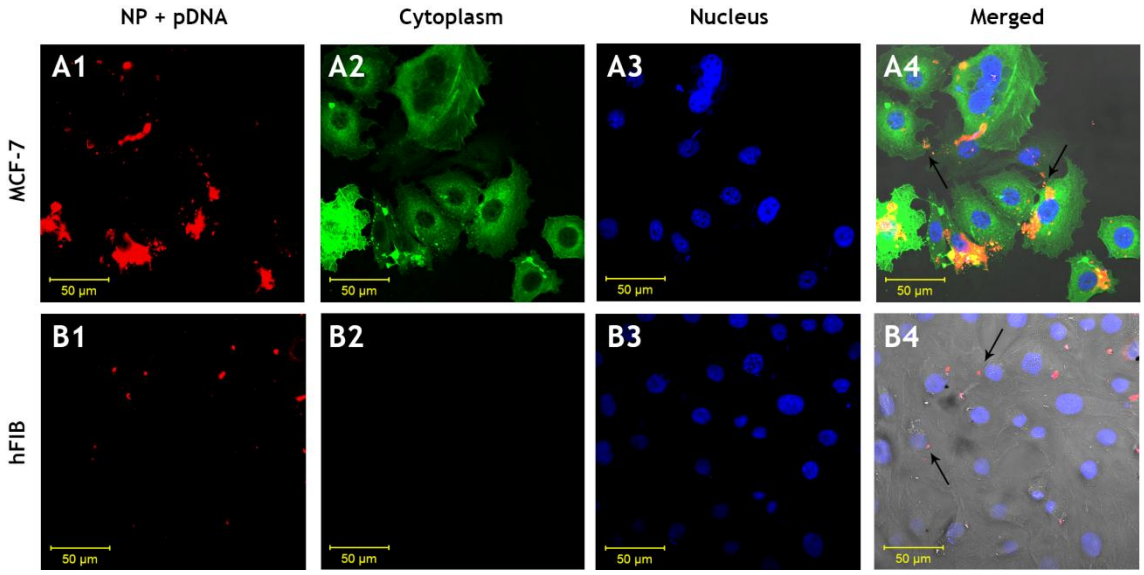

Figure 24 – CLSM images of nanoparticles cellular uptake, after 4h of incubation in MCF-7 and hFIB 2D monocultures (controls). **A)** MCF-7; **B)** hFIB. Red channel – RITC-labelled pDNA/CH-H-R nanoparticles; Green channel – Actin-GFP staining of MCF-7; Blue Channel – Hoechst 33342® nuclear staining; Merged channel – DIC + Superimposition of all channels.

Through the analysis of the various CLSM images, it is clearly visible that nanoparticle internalization is markedly higher in MCF-7 cells in comparison with hFIB. This remarkable result is also observed in all different co-culture models, evidencing the valuable biological performance of these particular delivery systems (Figure 23). Furthermore, it should be underscored that the nanoparticles are also located in the cell nucleus, an important finding since it should be taken into account that the therapeutic genes encoded in the pDNA must be located at the cell nucleus to be expressed (Figure 25). In addition, Figure 24 demonstrates that the affinity of the nanoparticles for hFIB is very low in comparison to that observed for MCF-7.

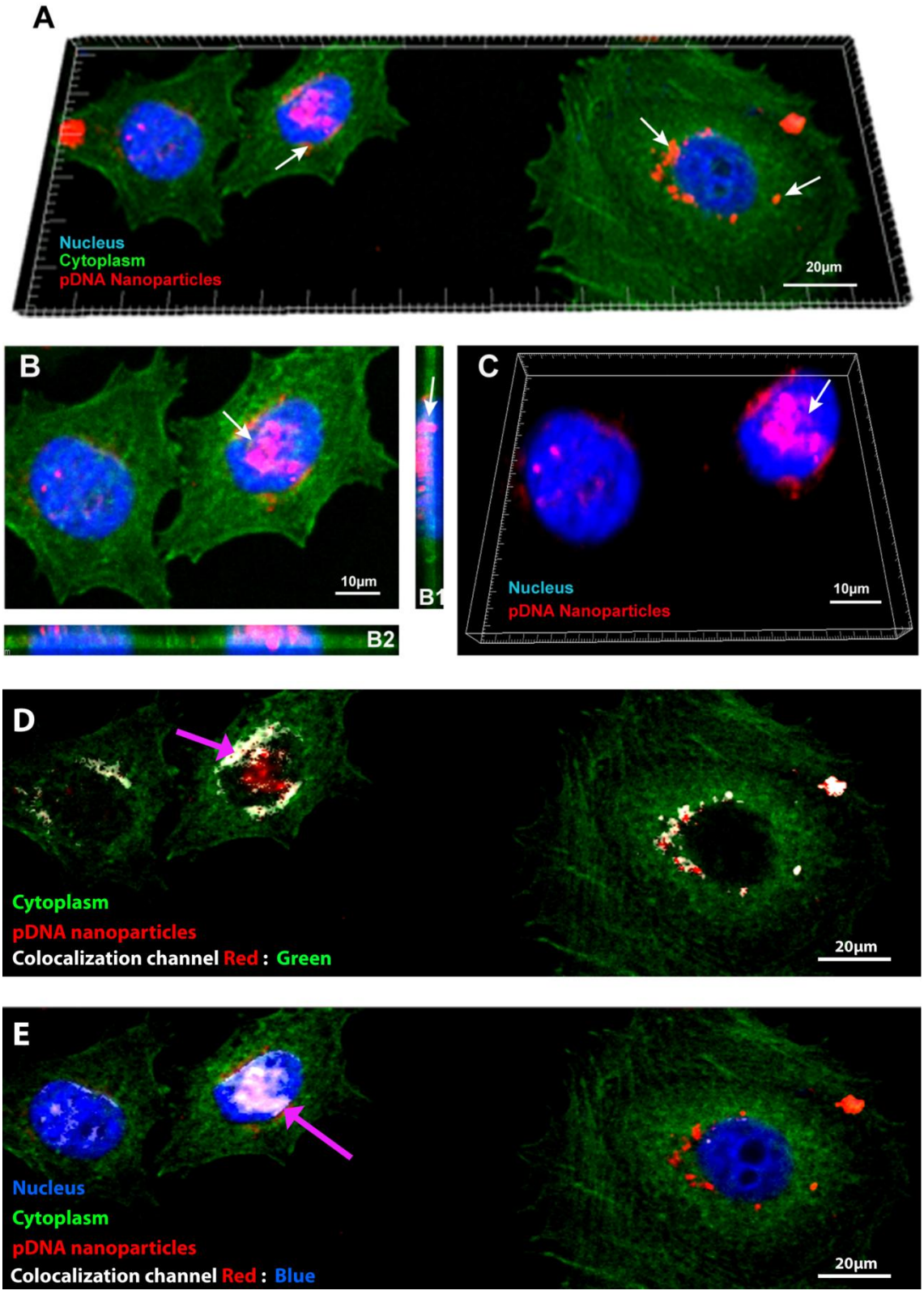

Figure 25 - CLSM images co-cultures at 1MCF-7:1hFIB ratio for nanoparticles cellular localization analysis. CLSM images of MCF-7 breast cancer cells after 4 h of incubation with nanocarriers **(A, B),** orthogonal sectioning in xy axis **(B),** 3D reconstruction of the cell nucleus **(C).** Colocalization of the red and green channels **(D).** Colocalization of the red and blue channels **(E).** Red channel – RITC labelled pDNA/CH-H-R; Green channel – Actin-GFP staining of MCF-7 and hFIB; Blue Channel – Hoechst 33342® nuclear staining. Grey channels: colocalization analysis.

In general, these results suggest that this particular nanodevice possesses tumor selectivity, a valuable characteristic that is most likely correlated with chitosan surface amino acid functionalization as previously mentioned. Nevertheless, in order to further confirm this targeting specificity a population based approach was also performed through the use of flow cytometry. This analysis is crucial since the established co-culture models are highly diverse and CLSM imaging only provide information about of the cellular events that occur in the field of view, rather than in the all population of co-cultured cells, in the various ratios.

3.1.3.2. Flow cytometry analysis of CH-H-A/pDNA nanoparticles cellular uptake analysis in 2D cell co-culture models of breast cancer with different MCF-7 to hFIB ratios

Flow cytometry is a very sensible technique that measures cell parameters under dynamic flow conditions mainly through the analysis of fluorescence (Ormerod and Imrie, 1990). This fact allows a careful and precise analysis of nanoparticles uptake in the various co-cultures with different MCF-7:hFIB ratios. Actually, flow cytometry has been widely used for the evaluation of nanoparticle uptake in *in vitro* cell models, however its use in co-culture analysis has only recently been described (Kievit *et al.*, 2009; Wang *et al.*, 2013b),

The initial transfection of cancer cells with GFP is an important parameter since it allows the separation of MCF-7 and hFIB populations, by gating the region of interest (ROI) in the GFP channel as shown in the R2 quadrant of (Figure 26). Thus, since the nanocarriers are labelled with red colour, the cells that are simultaneously GFP positive (i.e. green) and Rhodamine positive (i.e. red) are identified as MCF-7cells, with internalized nanoparticles. On the contrary green fluorescent cells represent cancer cells without internalized nanoparticles. In respect to hFIB, only those cells that present red fluorescence have the nanoparticulated systems in the intracellular space.

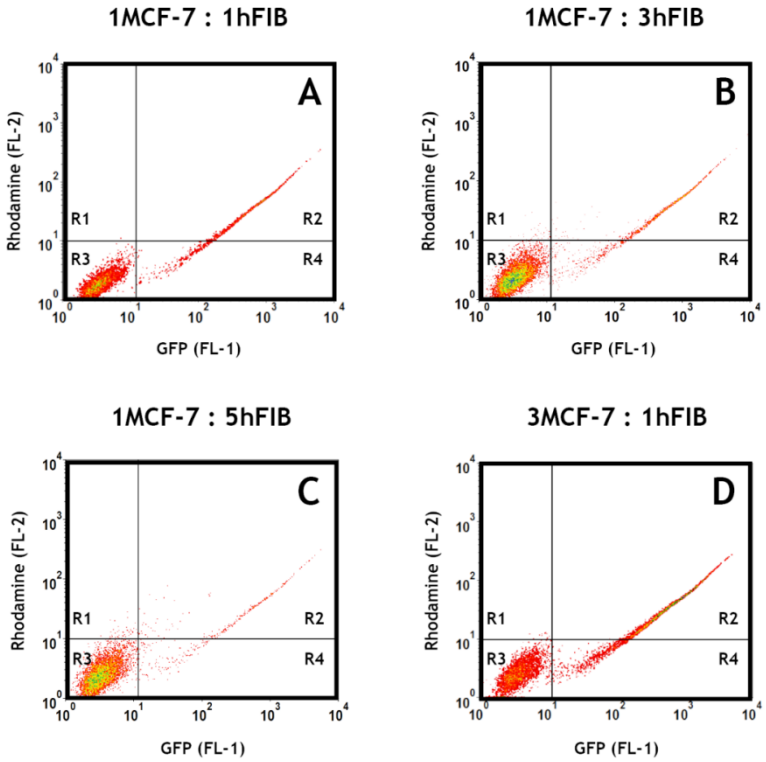

Figure 26 - Representative dot plots of nanoparticles cellular uptake analysis by flow cytometry after 4 h of incubation with RITC-labelled pDNA/CH-H-R nanoparticles in MCF-7:hFIB 2D co-cultures models, with MCF-7 to hFIB ratio of: **A)** 1:1; **B)** 1:3; **C)** 1:5; **D)** 3:1.

As controls, MCF-7 and hFIB monocultures were incubated with nanoparticles in the same conditions (Figure 27). Throughout the experiments the auto-fluorescence of cells in co-culture and monoculture has also determined since this parameter can produce analysis artefacts due to the intrinsic auto-fluorescence of eukaryotic cells in the green and red channels, a fact that is not commonly visualized in CLSM (Figure 28).

As demonstrated by the histograms of the control samples, neither hFIB, nor MCF-7 cells present very high levels of auto-fluorescence, with fluorescent signals being confined only to the first decade of the histograms (Figure 28). This fact does not affect acquisition since the gated ROI's all range from the 10^1 to the 10^4 decade, both in FL-1 and FL-2 channels. Moreover, it is possible to observe that despite the different co-culture ratios used for these experiments the auto-fluorescence signals remained similar to those observed for the mono-culture controls (Figure 28 A and B).

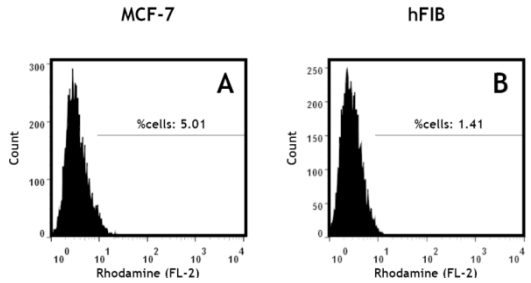

Figure 27 – Representative histograms of nanoparticle uptake in MCF-7 **(A)** and hFIB **(B)** monocultures after 4h of incubation with RITC-labelled pDNA/CH-H-R nanoparticles. Marker line represents the gated region used for data analysis.

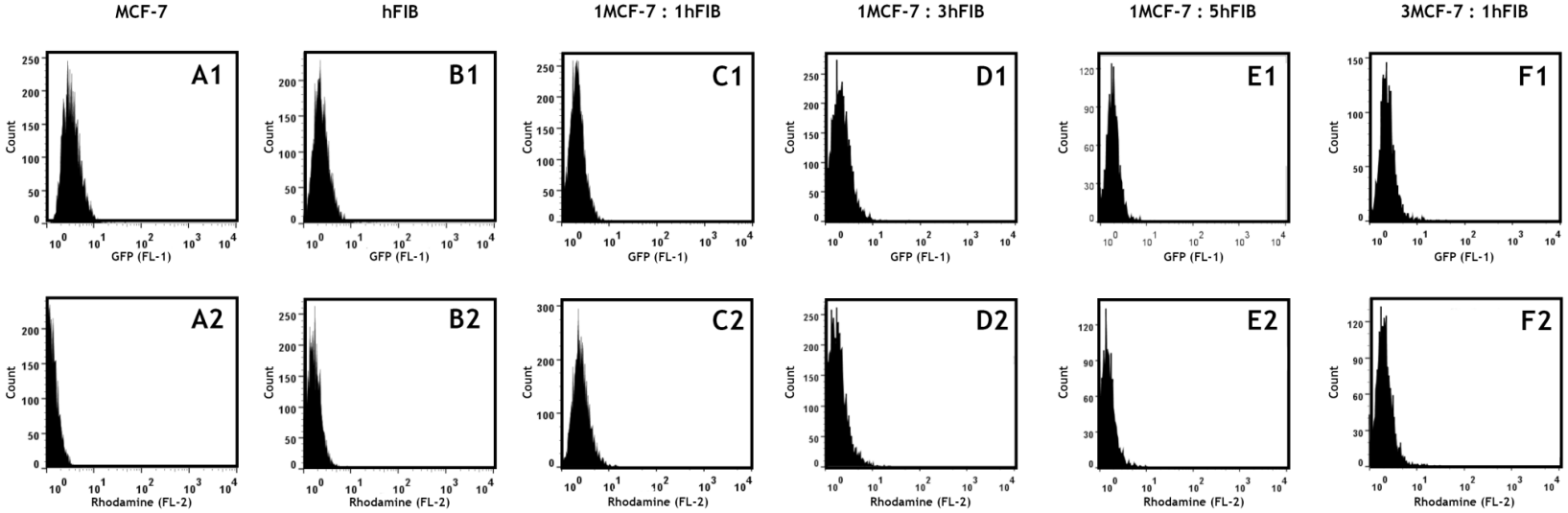

Figure 28 - Flow cytometry analysis of mono and co-cultures non- stained with GFP and non- incubated with nanoparticles. Representative histograms of non-stained and non-incubated MCF-7 and hFIB monocultures: FL-1 GFP channel **(A1, B1)**, and FL2-Rhodamine channel **(A2, B2)**, respectively; Representative histograms of non-stained and non-incubated MCF-7:hFIB co-cultures at various ratios: FL-1 GFP **(C1, D1, E1, F1)** and FL-2 Rhodamine **(C2, D2, E2, F2)**.

The analysis of the percentage of monocultured MCF-7 cells and hFIB gated in the ROI's demonstrate that the nanoparticulated carriers are localized within malignant cells with higher efficacy (MCF-7: 5.01 %; hFIB: 1.41 %). This important result suggests that the delivery systems produced can deliver their therapeutic cargo in a more effective way to cancer cells. However, in order to investigate the possible targeting specificity, the evaluation of nanoparticle uptake in the co-culture models is mandatory. Moreover, since the various ratios developed represent the heterogeneous structure of the *in vivo* tumors, the evaluation of the biological performance of the nanodevices in all these models offers a unique testing platform for a rationale design of targeted drug delivery systems.

The flow cytometry analysis of the various co-culture ratios incubated with CH-H-A/pDNA nanoparticles reveals that these systems are more internalized in malignant cells for all ratios studied (Figure 29). Such evidence demonstrates the suitability of this system for cancer therapy.

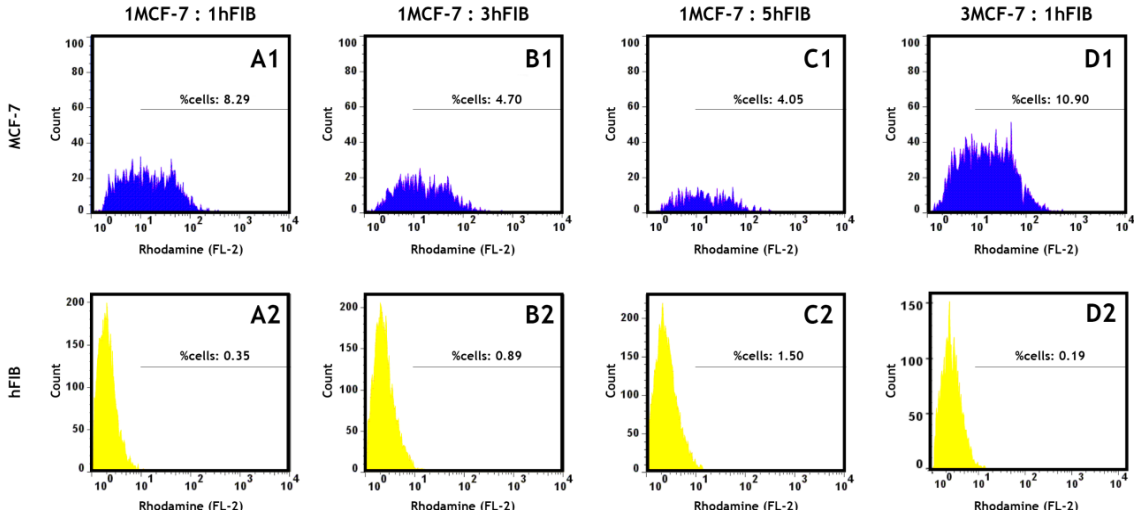

Figure 29 - Representative histograms of flow cytometry analysis of nanoparticles cellular uptake in MCF-7 **(A1-D1)** and hFIB **(A2-D2)** cell populations after 4h of incubation with RITC-labelled pDNA/CH-H-R nanoparticles in different MCF-7:hFIB 2D co-cultures models, using MCF-7 to hFIB ratio of: **A,E)** 1:1; **B,F)** 1:3; **C,G)** 1:5; **D,H)** 3:1. The R2 quadrant depicted in Figure 26 was used as a ROI for histogram analysis.

In fact, even in co-cultures with more hFIB than MCF-7 (Figure 29 B and C), nanoparticles are less attracted to fibroblasts than breast cancer cells. So, these results are in agreement with those obtained in confocal analysis (Figure 23). Interestingly, by correlating the percentages of MCF-7 (Figure 29 A1 – D1) and hFIB (Figure 29 A2 – D2) with nanoparticles in their cellular compartments, it is possible to observe a slight tendency of these percentages to be influenced by the different co-culture ratios, a result that further demonstrates the necessity of testing novel drug delivery systems in heterogeneous conditions.

Indeed, these important differences in normal and cancer cells nanoparticle uptake draw the attention to the influence of fibroblasts in co-culture and their random presence in *in vivo*

tumors. Further analysis also shows that the same delivery system may behave in a different mode according to the tumor heterogeneity that is found, i.e., depends on the MCF-7 to hFIB ratios.

Therefore, addressing different cancer to stromal cell ratios is a critical requirement to properly mimic heterogeneity and investigate the biological efficiency and specificity of a nanocarrier system under variable tumor environments. It should be underscored that the recent reports that employ co-culture models to evaluate the biological efficiency of nanodevices, disregard the possible uncertainty of the cell populations present in a tumor microenvironment (Le Droumaguet *et al.*, 2012), and thus do not utterly explore the full potential of co-cultures, as demonstrated in this thesis.

Nevertheless, despite the major improvements obtained with the various co-culture testing platforms they are still rather limited by their 2D architecture which does not entirely mimic the complexity of *in vivo* solid tumors. Therefore, in addition to the 2D systems also 3D models of multi-cellular tumors were developed.

3.2. 3D *in vitro* cell co-culture models of breast cancer and cervical cancer

Solid tumors are an abnormal mass of tissue that usually does not contain cysts or liquid areas (Gavhane *et al.*, 2011). Particularly, breast and cervix solid cancers originate from epithelial cells (Elenbaas *et al.*, 2001), more specifically, from the epithelial cells of glandular tissue, forming the so termed adenocarcinomas (Weigelt *et al.*, 2010; Ibeanu, 2011). Since the cell lines used in this research were obtained from tumor sections of human breast and cervix cancers they could have the capacity to form 3D multicellular tumor spheroids (MCTS) *in vitro*, as they do *in vivo* (Harris, 2002; Jögi *et al.*, 2012).

Similarly to co-cultures, *in vitro* 3D cell models which mimic solid tumors are essential in the pre-clinical development of new drugs and novel delivery systems for cancer therapy. However, unlike 2D cultures, 3D MCTS provide a whole new level of complexity since they represent a suitable testing platform that reproduces the complex multicellular architecture, the barriers to mass transport and the extracellular matrix deposition found in malignant tissues (Minchinton and Tannock, 2006).

Hence, after the development of 2D co-cultures, MCF-7 and hFIB cells and fibroblasts were also used in the optimization and establishment of 3D MCTS.

For the development of 3D models, it is required that adherent cells are seeded in various substrates that maintain the cells in a non adherent state, in order to form spheroids with a 3D architecture. Examples of such substrates are: i) Hidrogels, ii) Scaffolds, iii) Microspheres, or iv) Fibers that mimic the physical structure and chemical proprieties of the ECM, in a 3D volume (Yamada and Cukierman, 2007; Justice *et al.*, 2009; Rimann and Graf-Hausner, 2012). An essential mechanism for spheroid assembly is the induction of cell-cell attachment and

subsequent cell aggregation. This fact is only accomplished if cell adhesion to the common synthetic surfaces used for cell culture is avoided.

Therefore, in order to develop *in vitro* 3D MCTS which represent, as accurately as possible, the *in vivo* structure and cellular interactions in the tumor microenvironment, spheroids comprised by cancer cells and stromal cells were initially produced by the hanging drop method (Figure 30).

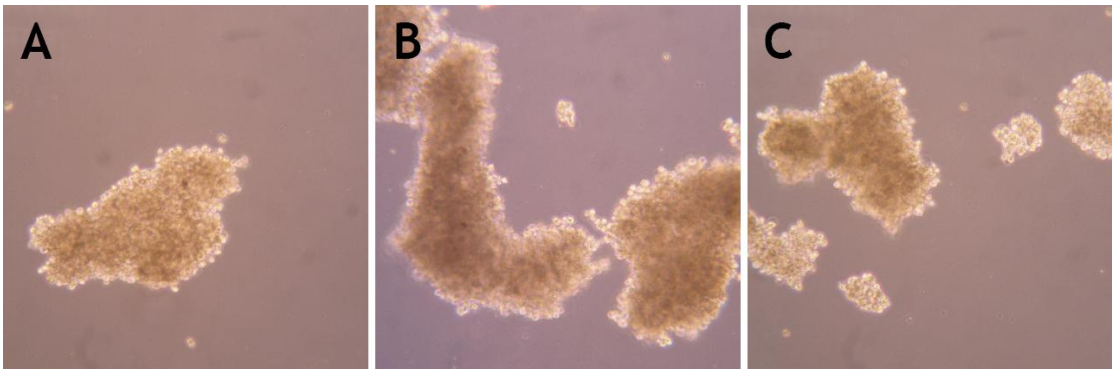

Figure 30 – Optical images of HeLa agglomerates formed using a flat hydrophobic bottom culture plate.

The results obtained with the used of the flat hydrophobic dishes reveal the formation of disorganized and random cell agglomerates with very irregular shapes (Figure 10).

The establishment of 3D MCTS with defined 3D architectures is of critical importance for the envisioned testing applications of these models. In fact, several reports underscore the importance of reproducible shapes as a factor that affects the evaluation of drug penetration in solid tumor models (Hirschhaeuser *et al.*, 2010; Mehta *et al.*, 2012; Marx, 2013). Moreover, this evaluation in well defined 3D structures also yields important information concerning the effect of tumor density, hypoxia and low pH on the therapeutic approach that is being developed.

Since the hanging drop method did not promote the production of suitable 3D models, the Liquid overlay methodology was then used to produce 3D MCTS. For this purpose 24 well plates were initially coated with low melting agarose as mentioned previously in the methods section 2.2.3 (Page 25) and schematized in Figure 31.

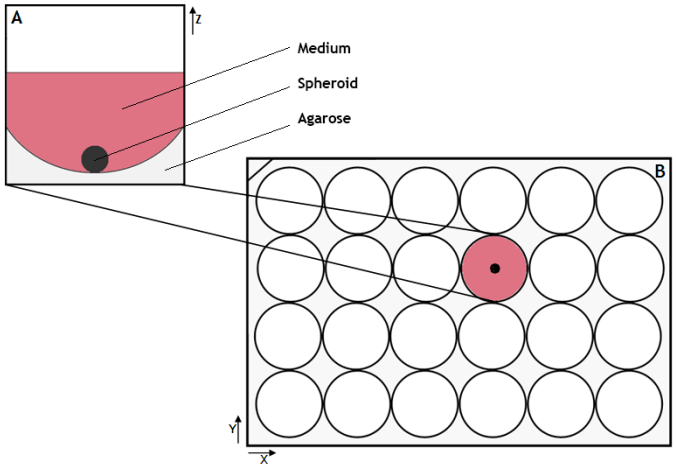

Figure 31 – Scheme of spheroids formation protocol. **A)** Well plate; **B)** 24 well plate.

Agar could also be used for this methodology, but this substrate influences the growth of fibroblasts due to the presence of sulphate groups that lead to the inhibition of cell growth as reported previously in the literature (Jou *et al.*, 2008). Moreover, in addition to the fact that agarose avoids cell adhesion, coating the surface of culture plates with this gel promotes the formation of a concave bottom. This semi-solid concave surface (Figure 31 A) is crucial for the formation of organized morphologies such as the characteristic quasi-spherical conformations of 3D spheroids. Taking the former into account, this method was optimized for the production of MCTS. Initially, agarose was used at a concentration of 1% (w/v) dissolved in water to produce a rigid gel that is capable of resisting shear stress. The optimal formation of a concave gel deposited at the bottom of the plate wells was optimized by manipulating the amount of agarose solution that was inserted in the well. However, to further promote the formation of more reproducible spheroids a final step of horizontal stirring (Giratory rotation) for 6 days was included after cell seeding on the agarose gel.

This upgrade to the liquid overlay method improved cell aggregation and facilitated the formation of quasi-spherical spheroids. This nearly spherical shape also avoided the dispersion of cells along the well, originating MCTS with more defined cell numbers. The results obtained with this strategy reveal that the speed of the horizontal stirring should be high enough to promote cell agglomeration in the centre of the wells, but also adequately to inhibit mechanical cell lysis. In addition to these experimental parameters, also the number of cells per spheroid was optimized with the aim to investigate how the overall spheroid size is influenced by this factor.

3.2.1. 3D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB)

Solid breast tumors were mimicked trough by the development of 3D MCTS comprised by MCF-7:hFIB at various ratios similarly to the co-culture models formerly described (Figure 32). As controls, monocellular spheroids of MCF-7 (Figure 32 E1,2,3; K1,2,3; Q1,2,3) and hFIB (Figure 32 F1,2,3; L1,2,3; R1,2,3) were performed in the same conditions of co-culture spheroids.

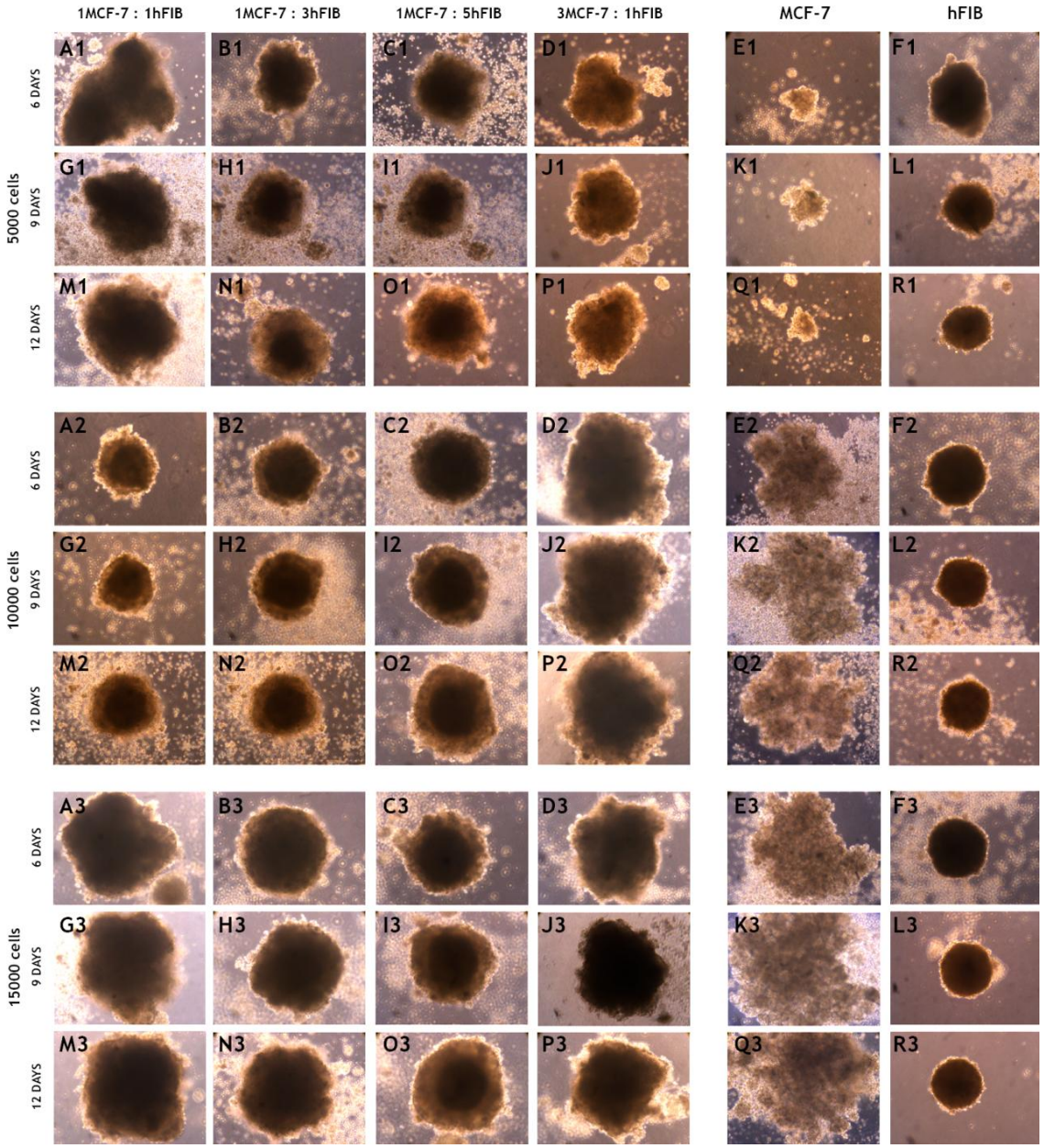

Figure 32 - Light Microscope images of 3D MCTS of MCF-7 and hFIB in mono and co-cultures grown during 12 days, using various initial cell numbers and ratios, as represented in the rows and columns. Original magnification 100X.

The results of the combinatorial approach of using agarose gels and horizontal stirring yielded 3D co-cultured MCTS that are more organized than those initially obtained by the hanging drop method (Figure 30). As shown in Figure 32, after 6 days of culture all the spheroids were formed for all conditions tested. Moreover, the results demonstrate that the 3D cultures of MCF-7 alone are not able to form cohesive and spherical 3D MCTS, as already verified by Nagelkerke and her team, MCF-7 have just the capacity to form spheroids with an irregular shape (Nagelkerke *et al.*, 2013). This is a striking finding since the monocultures of hFIB behaved in a total different way, being very stable and presenting consistent density and

quasi-spherical organization in all cases (Figure 32 F1,2,3; L1,2,3; R1,2,3). In addition, the optimization experiments emphasize that spheroid formation is highly dependent of the presence of fibroblasts. Actually, the 3D MCTS with higher fibroblast ratios (Figure 32 B1, 2, 3; H1,2,3; N1,2,3; C1,2,3; I1,2,3; P1,2,3) present an improved organization in contrast to monocultures (Figure 32 C1, 2, 3; I1, 2, 3; O1, 2, 3; D1,2,3; J1, 2, 3; P1, 2, 3). It is also important to underline that the 3D co-culture MCTS present all the unique characteristics of these models of solid tumors, i.e., there is a dense mass of apoptotic cells inside the MCTS and cancer cells with an elevated proliferation rate in the periphery (brighter halo), as shown in Figure 33.

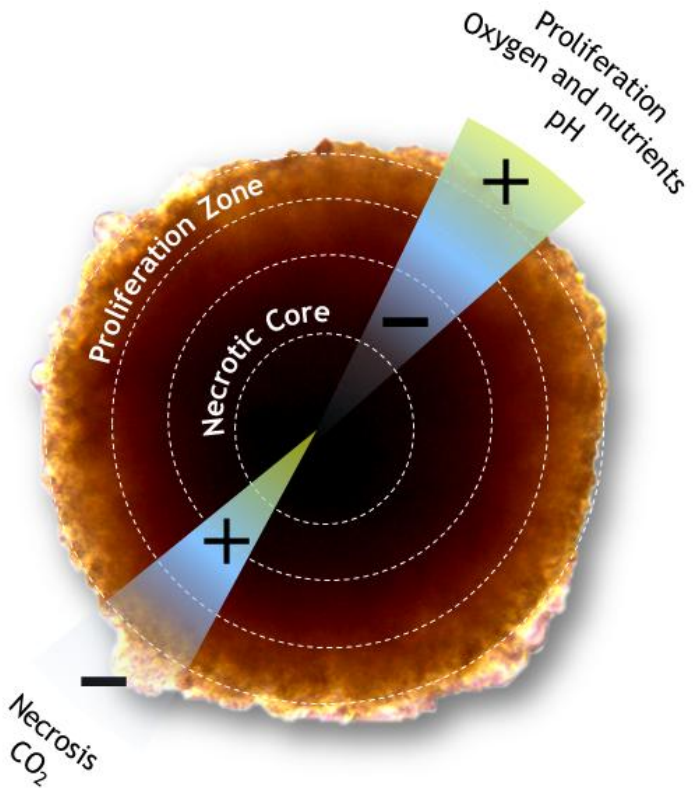

Figure 33 - Representative high resolution micrograph of a 3D MCTS produced with the modified liquid overlaid method. The spheroid structures obtained are highly representative of *in vivo* solid tumors, since they possess the characteristic necrotic core and the proliferative outer layer.

Regarding the influence of the initial cell number in the formation of 3D spheroids the results demonstrate that the MCTS size is proportional to the number of cells seeded in the agarose coated wells (Figure 32). Notably, the 3D MCTS models produced with these optimized parameters were highly reproducible and so versatile that their size could be easily adjusted by manipulation of the initial cell numbers seeded in the wells, a fact that illustrates their suitability as testing platforms for high throughput assays, that require large number of spheroids.

3.2.1.1. SEM analysis of the 3D *in vitro* cell co-culture models of breast cancer (MCF-7:hFIB)

SEM images were also acquired in order to investigate with a higher resolution the 3D MCTS produced. As shown in Figure 34, the presence of cell-cell interactions between both cell types is clearly visible. In fact, it is possible to observe the establishment of direct tight connections between both cell types due to the long filopodium-like structures formed, which play an essential role in tumor cells behaviour in both under *in vivo* or *in vitro* conditions (Figure 34 B) (Fletcher and Mullins, 2010; Mehta *et al.*, 2012). In addition, these physical interactions are essential for spheroids assembly, organization and stability, whereby, the absence of these connections in monocultured spheroids, originates a more disorganized and irregular cell agglomerates.

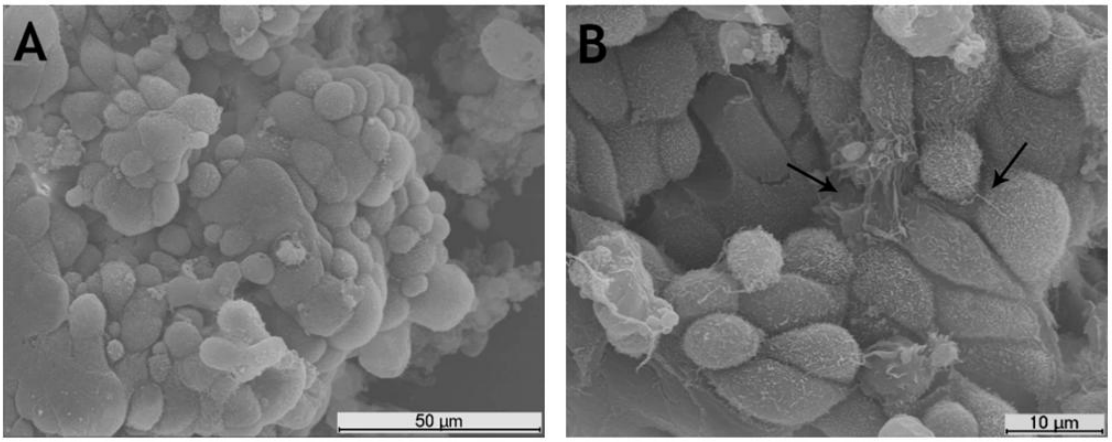

Figure 34 – Scanning Electron Microscope (SEM) images representation of 3D MCF-7:hFIB MCTS.

This is a very relevant finding that further emphasises the exchange of signals and soluble mediators between MCF-7 cells and hFIB, a fact that entirely mimics the *in vivo* tumor microenvironment.

3.2.2. 3D *in vitro* cell co-culture models of cervical cancer (HeLa :hFIB)

Apart from the 3D MCTS comprised by breast cancer cells and normal cells, HeLa:hFIB spheroids were also developed in order to obtain *in* vitro models of solid cervical adenocarcinomas (Figure 35). Similarly to breast cancer spheroids, these HeLa:hFIB 3D cell cultures were performed in the same conditions. However, it should be underscored that akin to the 2D co-culture models hFIB were also maintained in DMEM-HG medium. This parameter did not affect the cell viability of the spheroid models.

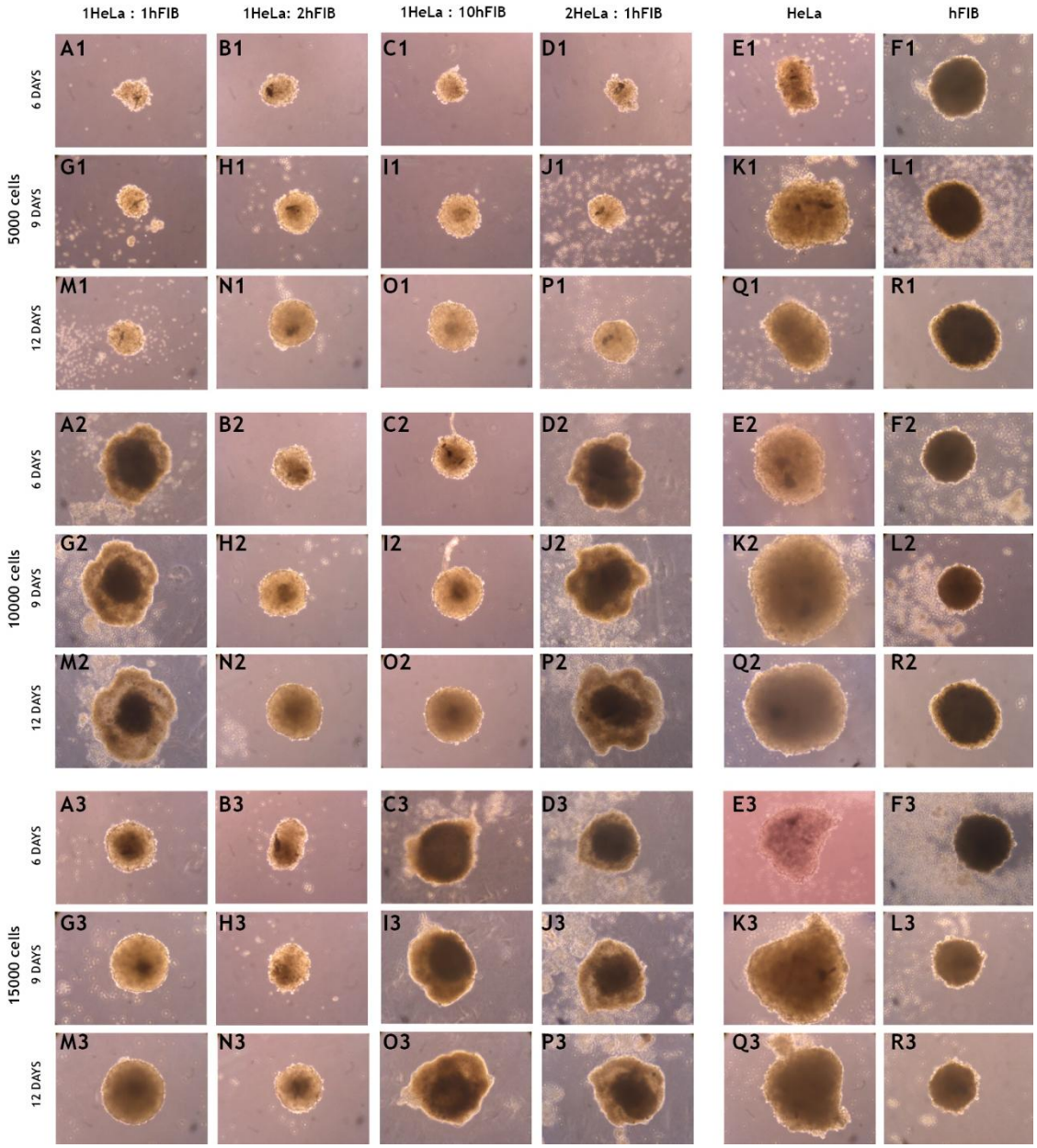

Figure 35 - Light Microscope images of 3D MCTS of HeLa and hFIB mono and co-cultures grown during 12 days using various initial cell numbers and ratios, as represented in the rows and columns. Original magnification 100X.

The results obtained in HeLa:hFIB 3D MCTS demonstrate the successful formation of spheroids in all 3D co-culture models. Along time, these spheroids tend to form more defined spherical shapes with a visible high concentration of necrotic cells in the centre of spheroid (darker areas), and highly proliferating cells around the apoptotic core. Strikingly, when the percentage of HeLa in spheroid is higher than hFIB (Figure 35 B1, 2, 3; H1,2,3; N1,2,3; C1,2,3; I1,2,3; P1,2,3), 3D structures of spheroid present a slightly less organized shape, which is particularly visible for the spheroids formulated with a initial number of cells of 5000 and 10000. Similar results are obtained in the control models (Figure 35 C1, 2, 3; I1, 2, 3; O1, 2, 3; D1,2,3; J1, 2, 3; P1, 2, 3). However, the 3D HeLa MCTS controls also present less

organized shapes even for a lower initial cell density. These results are in agreement with those obtained for MCF-7:hFIB spheroids and also emphasize the important role of fibroblasts in the formation of these unique structures. Regarding, the hFIB control spheroids, they present a highly consistent quasi-spherical morphology. HeLa 3D spheroids are particularly interesting to analyze since the characteristic dense necrotic core is entirely absent on these monoculture spheroids. This is a remarkable finding since it suggests that without the presence of fibroblasts the 3D MCTS do not entirely mimic the *in vivo* solid tumors that are characterized by their extensive necrotic cores with low oxygen and nutrient intake.
It is also important to emphasize that mono-culture spheroids are formed by the same initial number of cells than the HeLa:hFIB MCTS, thus, the bigger size of HeLa spheroids could be a result of a remarkably high proliferation rate.

3.2.2.1. CLSM analysis of 3D *in vitro* cell co-culture models of cervical cancer (HeLa:hFIB)

In order to support the previous morphological analysis of the 3D MCTS developed and provide further insights into the spheroids spatial architecture, 3D CLSM images with various slices along the MCTS structures were acquired (Figure 36 and 37). For fluorescence imaging the spheroids were labelled with WGA. This fluorescent probe is a carbohydrate-binding protein that selectively recognizes sialic acid and N-acetylglucosaminyl sugar residues that are predominantly found on the plasma membrane of cells (Favi *et al.*, 2013).

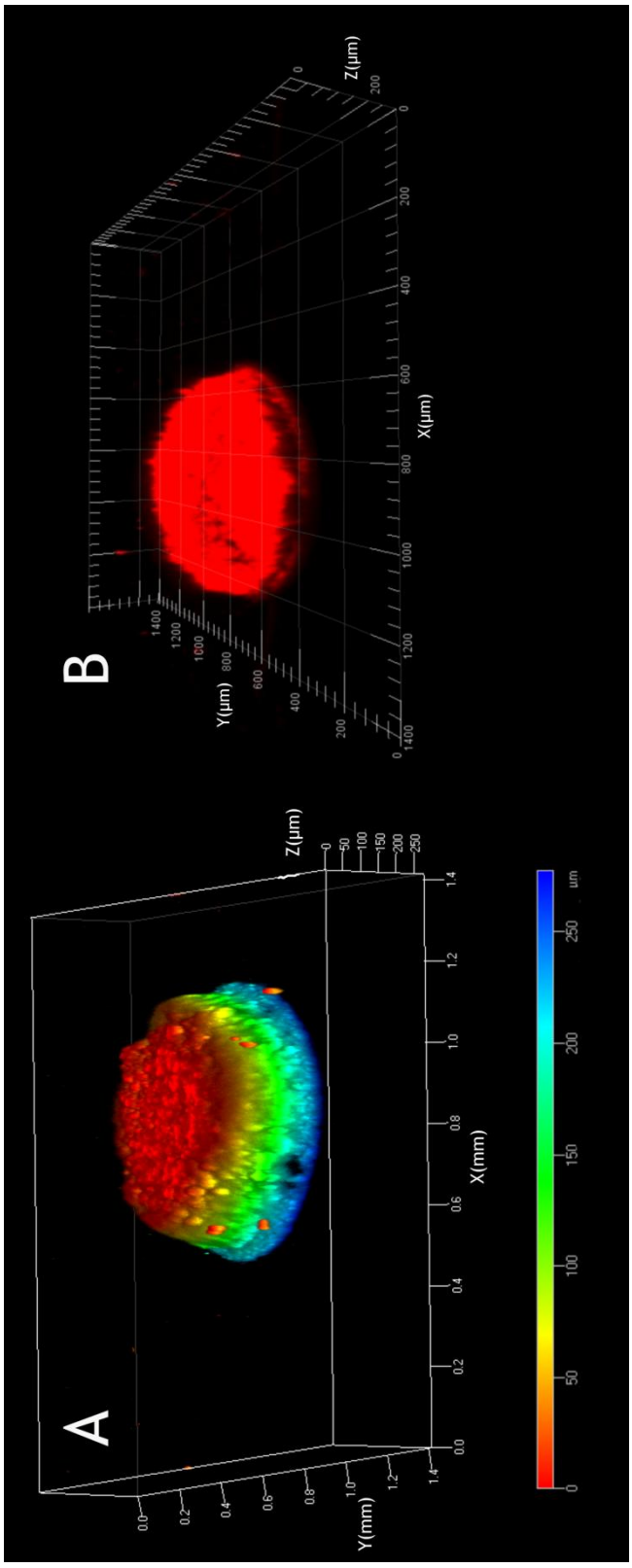

Figure 36 - CLSM images of a 3D reconstruction (**A**) and dept coding (**B**) of 2HeLa:1hFIB MTCS. Red channel –WGA staining

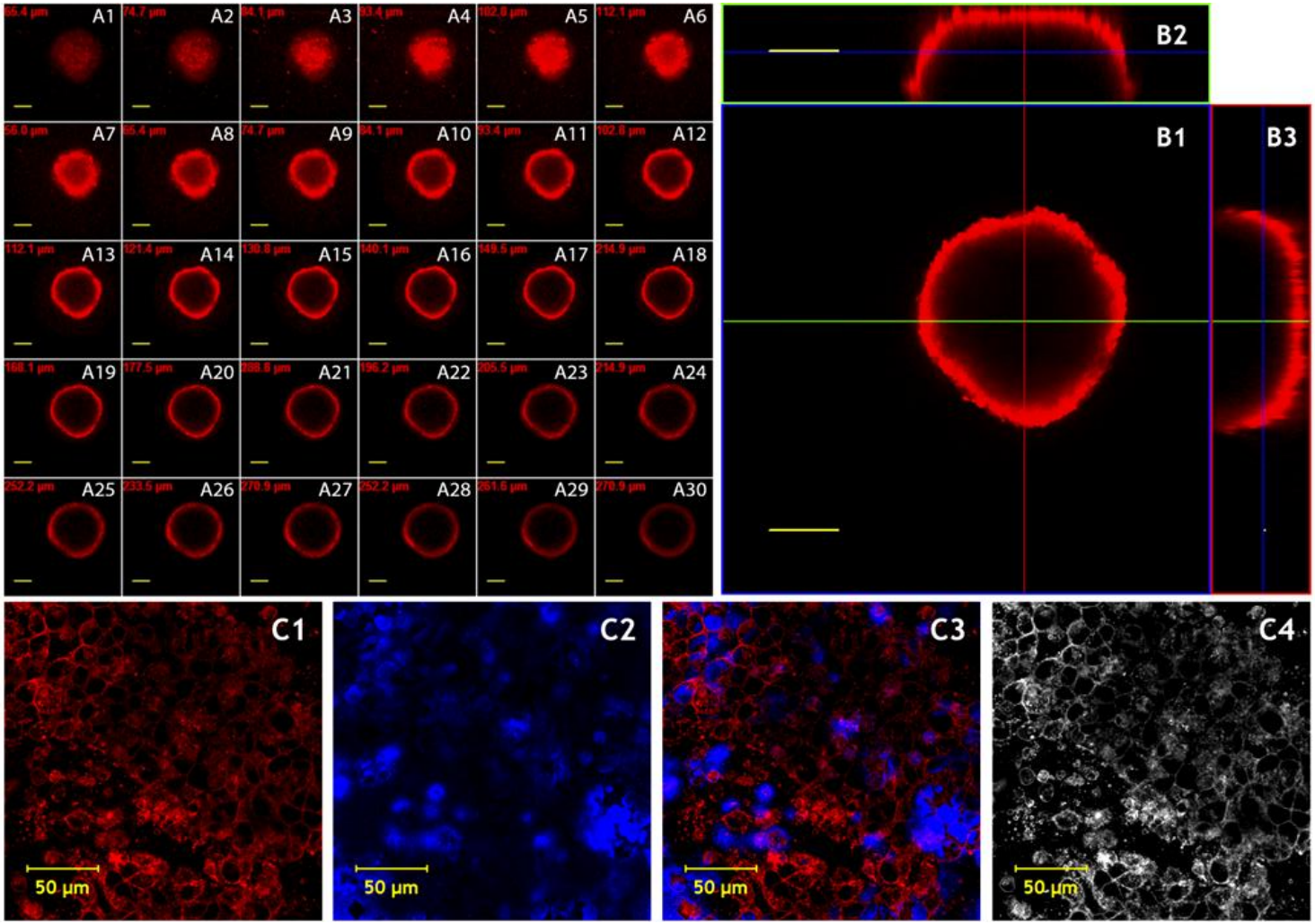

Figure 37 - CLSM of 2HeLa:1hFIB MCTS Z-stack slice representation **(A),** orthogonal sectioning in xy axis **(B)** and high resolution images **(C)** of the spheroid surface**.** Red channel – cell membranes stained with WGA; Blue Channel – Hoechst 33342® nuclear staining; Merged channel – Superimposition of all channels. In **A** and **B** scale bar correspond to 200µm.

As shown by CLSM images the produced spheroids have diameters higher than 500 µm. Regarding this parameter it has been previously described that spheroids with sizes bigger than 500 µm display all the major characteristics of *in vivo* solid tumors, becoming more compact and compartmentalized, and possessing pH, nutrient, and waste removal gradients throughout the extension of their solid structure (LaBarbera *et al.*, 2012). Moreover, as demonstrated in Figure 33, 36 and 37, spheroids have the former observed spherical shape, with a dense nucleus inside, illustrated by the fact that the fluorochrome could not penetrate deep enough into the tumor spheroid to provide a fluorescent signal. This is a notable finding since it clearly demonstrates that the penetration of anti-tumoral compounds and also drug delivery systems into the core of solid tumors is highly limited due to the diffusion barriers imposed by the solid tumor.

In brief, these spheroids possess all the unique features of solid tumors: i) uniform size and shape; ii) different zones with variable oxygen and nutrients intake, with different proliferation and necrotic rates and iii) multicellular constitution. Thus, these 3D cell cultures represent cell culture platform with promising potential for standard biological assays and screening of novel therapeutic approaches wither based on new pharmaceutics or delivery systems avoiding the need of animal models for this propose.

Chapter IV

Conclusion and Future Perspectives

4. Conclusions and Future Perspectives

New anti-tumoral drugs and delivery systems have been described for cancer therapies. They can be more effective without the risky side effects of traditional anti-cancer therapies. However, before attaining a viable clinical application, these therapeutics need to be tested both *in vitro* and *in vivo*, under tight regulations imposed by different regulatory agencies. For this, *in vitro* models have been proposed for replacing the use of laboratorial animals, due to the absence of legal authorizations, ethical constrictions and expensive costs. However, classical cell monocultures are associated with the outdated idea that the tumor tissue is comprised solely by malignant cells with a high proliferative rate, failing in this way, in the representation of the complex tumor microenvironment which plays an essential role in disease progression.

After the development of different MCF-7:hFIB and HeLa:hFIB co-culture models it was discovered that cancer cells are extremely affected by normal stromal cells, either by the physical contact or by the establishment of essential paracrine signalizing between cancer cells and fibroblasts in co-culture. In addition, the production of models with different cancer to normal cell ratios has unlocked the opportunity to evaluate the effects of tumor heterogeneity overtime. As a consequence, cells demonstrate a higher proliferate rate when in co-culture and also a different cellular organization, in accordance to fibroblasts concentration. Namely in breast 2D co-cultures systems with more fibroblasts than cancer cells, acinar structures emerged, a highly distinctive feature of breast cancer cells that was reproduced *in vitro*. After the optimization of these co-culture models, the real applicability of the developed systems was tested for the evaluation of a novel gene delivery system. The results demonstrate that in general, chitosan-H-R/pDNA nanoparticles have a tendency to target cancer cells, but the cellular uptake varies from co-culture to co-culture, evidencing the necessity of accounting for tumor heterogeneity during pre-clinical testing of therapeutic candidates.

Moreover, extending the concept of 2D *in vitro* cell cultures to 3D allowed to further mimic the physiological characteristics of a tumor *in vitro*. In fact, the results obtained demonstrate that in 3D cell cultures, cell behaviour is extremely similar to that of tumor cells formed in living organisms. Actually, beyond the cellular connections between cancer cells and tumor cells, 3D structure of tumor play a fundamental role in the establishment of cancer hallmarks, mainly in the ability of cancer cells to resist to bioactive molecules, with anti-tumoral capacity. The multicellular spheroids of MCF-7:hFIB and HeLa:hFIB demonstrated a spherical morphology constituted by a dense necrotic core and cells with high proliferation rate in the periphery, similarly to *in vivo* solid cervical tumors. In addition, it should be underscored that structural conformation of 3D MCTS is highly dependent of the number of fibroblasts.

In conclusion, it is possible to mimic breast and cervical cancer microenvironments with these established different cell co-culture models. Moreover, the results proved the essential role of tumor stroma, namely fibroblasts, in both culture systems developed here. This point

emphasize the high influence of stromal cells in cancer cells behaviour and their important role in co-cultures, suggesting that mimicking the tumor microenvironment with stroma cells actually offers a more precise evidence of what happens *in vivo*. Moreover, the temporal evolution of the co-cultures and the ratio of cancer cell to stromal cells could potentially affect the action of nanodevices, hence, these variables should be considered in future screening of delivery systems.

With the incessant creation of new anti-cancer therapies, these culture models represent descriptive, simple, and inexpensive systems to evaluate the biological performance of nanoparticles in microenvironments that closely mimic the tumor niche, being in the future a potential tool for the research and development of new pharmacologic drugs and delivery systems in large scale, with more realistic results, that can offer a perfect correlation with the clinical trials.

Chapter V

Bibliography

4. Bibliography

Aboussekhra, A. (2011). "Role of cancer-associated fibroblasts in breast cancer development and prognosis." International Journal of Developmental Biology **55**: 841-849.

Albini, A. and M. B. Sporn (2007). "The tumour microenvironment as a target for chemoprevention." Nature Reviews Cancer **7**: 139-147.

Alderton, G. K. (2012). "Tumour microenvironment: The haves and the have nots." Nature Reviews Cancer **12**: 505-505.

Alexander, S. and P. Friedl (2012). "Cancer invasion and resistance: interconnected processes of disease progression and therapy failure." Trends in molecular medicine **18**: 13-26.

Allen, C. P., A. Fujimori, R. Okayasu and J. A. Nickoloff (2013). Radiation-Induced Delayed Genome Instability and Hypermutation in Mammalian Cells, Springer.

Annibaldi, A. and C. Widmann (2010). "Glucose metabolism in cancer cells." Current Opinion in Clinical Nutrition & Metabolic Care **13**: 466-470.

Astashkina, A., B. Mann and D. W. Grainger (2012). "A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity." Pharmacology & Therapeutics **134**: 82-106.

Balkwill, F. R., M. Capasso and T. Hagemann (2012). "The tumor microenvironment at a glance." Journal of Cell Science **125**: 5591-5596.

Bhowmick, N. A., E. G. Neilson and H. L. Moses (2004). "Stromal fibroblasts in cancer initiation and progression." Nature **432**: 332-337.

Bierie, B. and H. L. Moses (2006). "TGF-β and cancer." Cytokine & growth factor reviews **17**: 29-40.

Birgersdotter, A., R. Sandberg and I. Ernberg (2005). "Gene expression perturbation in vitro—a growing case for three-dimensional (3D) culture systems." Seminars in cancer biology **15**: 405-412.

Bissell, M. J., D. C. Radisky, A. Rizki, V. M. Weaver and O. W. Petersen (2002). "The organizing principle: microenvironmental influences in the normal and malignant breast." Differentiation **70**: 537-546.

Borchard, G. (2001). "Chitosans for gene delivery." Advanced drug delivery reviews **52**: 145-150.

Box, C., S. J. Rogers, M. Mendiola and S. A. Eccles (2010). "Tumour-microenvironmental interactions: paths to progression and targets for treatment." Seminars in cancer biology **20**: 128-138.

Brahimi-Horn, M. C., G. Bellot and J. Pouysségur (2011). "Hypoxia and energetic tumour metabolism." Current opinion in genetics & development **21**: 67-72.

Brannon-Peppas, L. and J. O. Blanchette (2004). "Nanoparticle and targeted systems for cancer therapy." Advanced drug delivery reviews **56**: 1649-1659.

Bray, F. and B. Møller (2005). "Predicting the future burden of cancer." Nature Reviews Cancer **6**: 63-74.

Brellier, F. and R. Chiquet-Ehrismann (2012). "How do tenascins influence the birth and life of a malignant cell?" Journal of cellular and molecular medicine **16**: 32-40.

Brizzi, M. F., G. Tarone and P. Defilippi (2012). "Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche." Current opinion in cell biology **24**: 645-651.

Brouty-Boyé, D., C. Mainguené, V. Magnien, L. Israel and R. Beaupain (1994). "Fibroblast-mediated differentiation in human breast carcinoma cells (MCF-7) grown as nodules IN VITRO." International journal of cancer **56**: 731-735.

Burdett, E., F. K. Kasper, A. G. Mikos and J. A. Ludwig (2010). "Engineering tumors: a tissue engineering perspective in cancer biology." Tissue Engineering Part B: Reviews **16**: 351-359.

Chabner, B. A. and T. G. Roberts (2005). "Chemotherapy and the war on cancer." Nature Reviews Cancer **5**: 65-72.

Chiavarina, B., D. Whitaker-Menezes, U. E. Martinez-Outschoorn, A. K. Witkiewicz, R. Birbe, A. Howell, R. G. Pestell, J. Smith, R. Daniel and F. Sotgia (2011). "Pyruvate kinase expression (PKM1 and PKM2) in cancer-associated fibroblasts drives stromal nutrient production and tumor growth." Cancer biology & therapy **12**: 1101-1113.

Chou, L. Y. T., K. Ming and W. C. W. Chan (2011). "Strategies for the intracellular delivery of nanoparticles." Chemical Society Reviews **40**: 233-245.

Cichon, M. A., A. C. Degnim, D. W. Visscher and D. C. Radisky (2010). "Microenvironmental influences that drive progression from benign breast disease to invasive breast cancer." Journal of mammary gland biology and neoplasia **15**: 389-397.

Cirri, P. and P. Chiarugi (2012). "Cancer-associated-fibroblasts and tumour cells: a diabolic liaison driving cancer progression." Cancer and Metastasis Reviews **31**: 195-208.

Coussens, L. M. and Z. Werb (2002). "Inflammation and cancer." Nature **420**: 860-867.

Couvreur, P. (2012). "Nanoparticles in drug delivery: Past, present and future." Advanced drug delivery reviews **65**: 21-23.

Cukierman, E. and D. R. Khan (2010). "The benefits and challenges associated with the use of drug delivery systems in cancer therapy." Biochemical pharmacology **80**: 762-770.

Cukierman, E., R. Pankov and K. M. Yamada (2002). "Cell interactions with three-dimensional matrices." Current opinion in cell biology **14**: 633-640.

Danhier, F., E. Ansorena, J. M. Silva, R. Coco, A. Le Breton and V. Préat (2012). "PLGA-based nanoparticles: an overview of biomedical applications." Journal of Controlled Release **161**: 505-522.

De Bock, K., S. Cauwenberghs and P. Carmeliet (2011). "Vessel abnormalization: another hallmark of cancer?: molecular mechanisms and therapeutic implications." Current opinion in genetics & development **21**: 73-79.

de Visser, K. E., A. Eichten and L. M. Coussens (2006). "Paradoxical roles of the immune system during cancer development." Nature Reviews Cancer **6**: 24-37.

De Wever, O. and M. Mareel (2003). "Role of tissue stroma in cancer cell invasion." The Journal of pathology **200**: 429-447.

Debnath, J. and J. S. Brugge (2005). "Modelling glandular epithelial cancers in three-dimensional cultures." Nature Reviews Cancer **5**: 675-688.

Delinassios, J. and S. Kottaridis (1984). "Interactions between human fibroblasts and HeLa cells in vitro." Biology of the Cell **50**: 9-16.

Delinassios, J. G. (1987). "Fibroblasts against cancer cells in vitro." Anticancer Research **7**: 1005-1010.

Dhimolea, E., M. V. Maffini, A. M. Soto and C. Sonnenschein (2010). "The role of collagen reorganization on mammary epithelial morphogenesis in a 3D culture model." Biomaterials **31**: 3622-3630.

Dolberg, D. S. and M. J. Bissell (1984). "Inability of Rous sarcoma virus to cause sarcomas in the avian embryo." Nature **309**: 552-556.

Duell, B. L., A. W. Cripps, M. A. Schembri and G. C. Ulett (2011). "Epithelial cell coculture models for studying infectious diseases: benefits and limitations." Journal of Biomedicine and Biotechnology **2011**: 1-9.

Eifler, A. C. and C. S. Thaxton (2011). "Nanoparticle therapeutics: FDA approval, clinical trials, regulatory pathways, and case study." Methods Molecular Biology **726**: 325-338.

Elenbaas, B., L. Spirio, F. Koerner, M. D. Fleming, D. B. Zimonjic, J. L. Donaher, N. C. Popescu, W. C. Hahn and R. A. Weinberg (2001). "Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells." Genes & development **15**: 50-65.

Elsdale, T. and J. Bard (1972). "Collagen substrata for studies on cell behavior." The Journal of Cell Biology **54**: 626-637.

Engel, L. W. and N. A. Young (1978). "Human breast carcinoma cells in continuous culture: a review." Cancer research **38**: 4327-4339.

Farber, E. (1984). "The multistep nature of cancer development." Cancer research **44**: 4217-4223.

Faria, C., C. Smith and J. Rutka "The Role of HGF/c-Met Pathway Signaling in Human Medulloblastoma."

Farokhzad, O. C. and R. Langer (2009). "Impact of nanotechnology on drug delivery." ACS nano **3**: 16-20.

Favi, P. M., R. S. Benson, N. R. Neilsen, R. L. Hammonds, C. C. Bates, C. P. Stephens and M. S. Dhar (2013). "Cell proliferation, viability, and in vitro differentiation of equine mesenchymal stem cells seeded on bacterial cellulose hydrogel scaffolds." Materials Science and Engineering C: Materials for Biological Applications **33**: 1935-1944.

Fennema, E., N. Rivron, J. Rouwkema, C. van Blitterswijk and J. de Boer (2013). "Spheroid culture as a tool for creating 3D complex tissues." Trends in biotechnology **31**: 108-115.

Fletcher, D. A. and R. D. Mullins (2010). "Cell mechanics and the cytoskeleton." Nature **463**: 485-492.

Fox, E., G. A. Curt and F. M. Balis (2002). "Clinical trial design for target-based therapy." The oncologist **7**: 401-409.

Freshney, R. I., G. Vunjak-Novakovic and R. Freshney (2006). Basic principles of cell culture, John Wiley & Sons.

Fujita, H., K. Ohuchida, K. Mizumoto, T. Egami, K. Miyoshi, T. Moriyama, L. Cui, J. Yu, M. Zhao and T. Manabe (2009). "Tumor–stromal interactions with direct cell contacts enhance proliferation of human pancreatic carcinoma cells." Cancer science **100**: 2309-2317.

Gaspar, V. M., I. J. Correia, Â. Sousa, F. Silva, C. M. Paquete, J. A. Queiroz and F. Sousa (2011). "Nanoparticle mediated delivery of pure P53 supercoiled plasmid DNA for gene therapy." Journal of Controlled Release **156**: 212-222.

Gaspar, V. M., J. G. Marques, F. Sousa, R. O. Louro, J. A. Queiroz and I. J. Correia (2013). "Biofunctionalized nanoparticles with pH-responsive and cell penetrating blocks for gene delivery." Nanotechnology **24**: doi: 10.1088/0957-4484/1024/1027/275101.

Gavhane, Y., A. Shete, A. Bhagat, V. Shinde, K. Bhong, G. Khairnar and A. Yadav (2011). "Solid Tumors: Facts, Challenges and Solutions." International Journal **2**.

Grieger, K. D., S. F. Hansen and A. Baun (2009). "The known unknowns of nanomaterials: Describing and characterizing uncertainty within environmental, health and safety risks." Nanotoxicology **3**: 222-233.

Grivennikov, S. I., F. R. Greten and M. Karin (2010). "Immunity, inflammation, and cancer." Cell **140**: 883-899.

Gutteridge, J. and B. Halliwell (2010). "Antioxidants: molecules, medicines, and myths." Biochemical and biophysical research communications **393**: 561-564.

Hamilton, A. and G. Hortobagyi (2005). "Chemotherapy: what progress in the last 5 years?" Journal of clinical oncology **23**: 1760-1775.

Han, K., S. Chen, W.-H. Chen, Q. Lei, Y. Liu, R.-X. Zhuo and X.-Z. Zhang (2013). "Synergistic gene and drug tumor therapy using a chimeric peptide." Biomaterials **34**: 4680-4689.

Hanahan, D. and L. M. Coussens (2012). "Accessories to the crime: functions of cells recruited to the tumor microenvironment." Cancer Cell **21**: 309-322.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**: 57-70.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**: 646-674.

Harris, A. L. (2002). "Hypoxia—a key regulatory factor in tumour growth." Nature Reviews Cancer **2**: 38-47.

Hartung, T. (2008). "Thoughts on limitations of animal models." Parkinsonism & Related Disorders **14**: S81-S83.

Hashizume, H., P. Baluk, S. Morikawa, J. W. McLean, G. Thurston, S. Roberge, R. K. Jain and D. M. McDonald (2000). "Openings between defective endothelial cells explain tumor vessel leakiness." The American journal of pathology **156**: 1363-1380.

Hellsten, E. (2005). "Nanosciences and nanotechnologies: An Action Plan for Europe 2005-2009." Dialog zur Bewertung von synthetischen Nanopartikeln in Arbeits-und Umweltbereichen. Bonn.

Heneweer, M., M. Muusse, M. Dingemans, P. C. De Jong, M. Van Den Berg and J. T. Sanderson (2005). "Co-culture of primary human mammary fibroblasts and MCF-7 cells as an in vitro breast cancer model." Toxicological Sciences **83**: 257-263.

Hirschhaeuser, F., H. Menne, C. Dittfeld, J. West, W. Mueller-Klieser and L. A. Kunz-Schughart (2010). "Multicellular tumor spheroids: an underestimated tool is catching up again." Journal of biotechnology **148**: 3-15.

Hockenbery, D. M., M. Tom, C. Abikoff and D. Margineantu (2013). The Warburg Effect and Beyond: Metabolic Dependencies for Cancer Cells, Springer.

Hogan, C. (2012). "Impact of interactions between normal and transformed epithelial cells and the relevance to cancer." Cellular and Molecular Life Sciences **69**: 203-213.

Hogenesch, H. and A. Y. Nikitin (2012). "Challenges in pre-clinical testing of anti-cancer drugs in cell culture and in animal models." Journal of Controlled Release **164**: 183-186.

Horimoto, Y., Y. Takahashi, U. Polanska and A. Orimo (2012). "Emerging roles of the tumor-associated stroma in promoting tumor metastasis." Cell Adhesion & Migration **6**: 203-206.

Ibeanu, O. A. (2011). "Molecular pathogenesis of cervical cancer." Cancer biology & therapy **11**: 295-306.

Janeway, C. A., P. Travers, M. Walport and M. J. Shlomchik (2001). Immunobiology, Garland Science.

Janssen, A. and R. Medema (2012). "Genetic instability: tipping the balance." Oncogene: doi:10.1038/onc.2012.1576.

Jögi, A., M. Vaapil, M. Johansson and S. Påhlman (2012). "Cancer cell differentiation heterogeneity and aggressive behavior in solid tumors." Upsala journal of medical sciences **117**: 217-224.

Johnson, J., S. Decker, D. Zaharevitz, L. Rubinstein, J. Venditti, S. Schepartz, S. Kalyandrug, M. Christian, S. Arbuck and M. Hollingshead (2001). "Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials." British journal of cancer **84**: 1424.

Jones, R. G. and C. B. Thompson (2009). "Tumor suppressors and cell metabolism: a recipe for cancer growth." Genes & development **23**: 537-548.

Jou, C.-H., W.-C. Chen, M.-C. Yang, M.-C. Hwang, W.-L. Chou, S.-M. Lin and C. Y. Hsu (2008). "In vitro biocompatibility of three-dimensional chitosan scaffolds immobilized with chondroitin-6-sulfate." Polymers for Advanced Technologies **19**: 377-384.

Justice, B. A., N. A. Badr and R. A. Felder (2009). "3D cell culture opens new dimensions in cell-based assays." Drug Discovery Today **14**: 102-107.

Kalluri, R. and M. Zeisberg (2006). "Fibroblasts in cancer." Nature Reviews Cancer **6**: 392-401.

Kamaly, N., Z. Xiao, P. M. Valencia, A. F. Radovic-Moreno and O. C. Farokhzad (2012). "Targeted polymeric therapeutic nanoparticles: design, development and clinical translation." Chemical Society Reviews **41**: 2971-3010.

Kelland, L. (2004). "Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development." European journal of cancer **40**: 827-836.

Kennedy, K. (1987). "Hypoxic cells as specific drug targets for chemotherapy." Anti-cancer drug design **2**: 181-194.

Kerkar, S. P. and N. P. Restifo (2012). "Cellular constituents of immune escape within the tumor microenvironment." Cancer research **72**: 3125-3130.

Khatau, S. B., R. J. Bloom, S. Bajpai, D. Razafsky, S. Zang, A. Giri, P.-H. Wu, J. Marchand, A. Celedon and C. M. Hale (2012). "The distinct roles of the nucleus and nucleus-cytoskeleton connections in three-dimensional cell migration." Scientific reports **2**.

Kievit, F. M., O. Veiseh, N. Bhattarai, C. Fang, J. W. Gunn, D. Lee, R. G. Ellenbogen, J. M. Olson and M. Zhang (2009). "PEI–PEG–Chitosan-Copolymer-Coated Iron Oxide

Nanoparticles for Safe Gene Delivery: Synthesis, Complexation, and Transfection." Advanced functional materials **19**: 2244-2251.

Kim, J. B., R. Stein and M. J. O'Hare (2004). "Three-dimensional in vitro tissue culture models of breast cancer—a review." Breast cancer research and treatment **85**: 281-291.

Ko, Y. H., Z. Lin, N. Flomenberg, R. G. Pestell, A. Howell, F. Sotgia, M. P. Lisanti and U. E. Martinez-Outschoorn (2012). "Glutamine fuels a vicious cycle of autophagy in the tumor stroma and oxidative mitochondrial metabolism in epithelial cancer cells." Cancer Biology & Therapy **12**: 1085-1097.

Kobayashi, H., K. C. Boelte and P. C. Lin (2007). "Endothelial cell adhesion molecules and cancer progression." Current medicinal chemistry **14**: 377-386.

Koshida, Y., M. Kuranami and M. Watanabe (2006). "Interaction between stromal fibroblasts and colorectal cancer cells in the expression of vascular endothelial growth factor." Journal of Surgical Research **134**: 270-277.

Krause, S., M. V. Maffini, A. M. Soto and C. Sonnenschein (2010). "The microenvironment determines the breast cancer cells' phenotype: organization of MCF7 cells in 3D cultures." BMC cancer **10**: 263.

Krishna, R. and L. D. Mayer (2000). "Multidrug resistance (MDR) in cancer: mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs." European journal of pharmaceutical sciences **11**: 265-283.

LaBarbera, D. V., B. G. Reid and B. H. Yoo (2012). "The multicellular tumor spheroid model for high-throughput cancer drug discovery." Expert Opinion on Drug Discovery **7**: 819-830.

Langdon, S. P. (2004). Cancer cell culture: methods and protocols, Springer.

Le Droumaguet, B., J. Nicolas, D. Brambilla, S. Mura, A. Maksimenko, L. De Kimpe, E. Salvati, C. Zona, C. Airoldi and M. Canovi (2012). "Versatile and Efficient Targeting Using a Single Nanoparticulate Platform: Application to Cancer and Alzheimer's Disease." ACS nano **6**: 5866.

LeBedis, C., K. Chen, L. Fallavollita, T. Boutros and P. Brodt (2002). "Peripheral lymph node stromal cells can promote growth and tumorigenicity of breast carcinoma cells through the release of IGF-I and EGF." International journal of cancer **100**: 2-8.

Lee, J., M. J. Cuddihy and N. A. Kotov (2008). "Three-dimensional cell culture matrices: state of the art." Tissue Engineering Part B: Reviews **14**: 61-86.

Lee, J. J. (2007). "Clinical trial design for anticancer therapies." The Cancer Handbook.

Levine, A. J. and A. M. Puzio-Kuter (2010). "The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes." science **330**: 1340-1344.

Leyva-Illades, D., M. McMillin, M. Quinn and S. DeMorrow (2012). "Cholangiocarcinoma pathogenesis: Role of the tumor microenvironment." Translational Gastrointestinal Cancer **1**: 71-80.

Li, H., X. Fan and J. Houghton (2007). "Tumor microenvironment: the role of the tumor stroma in cancer." Journal of cellular biochemistry **101**: 805-815.

Lindl, T. and R. Steubing (2013). "Basic Cell Culture Techniques." Atlas of Living Cell Cultures: 5-9.

Liu, H. and K. Roy (2005). "Biomimetic three-dimensional cultures significantly increase hematopoietic differentiation efficacy of embryonic stem cells." Tissue engineering **11**: 319-330.

Lövestam, G., H. Rauscher, G. Roebben, B. S. Klüttgen, N. Gibson, J. P. Putaud and H. Stamm (2010). Considerations on a definition of nanomaterial for regulatory purposes. Joint Research Centre Reference Reports, European Commission**:** 80004-80001.

Lu, P., V. M. Weaver and Z. Werb (2012). "The extracellular matrix: a dynamic niche in cancer progression." The Journal of Cell Biology **196**: 395-406.

Ma, X. J., R. Salunga, J. T. Tuggle, J. Gaudet, E. Enright, P. McQuary, T. Payette, M. Pistone, K. Stecker and B. M. Zhang (2003). "Gene expression profiles of human breast cancer progression." Proceedings of the National Academy of Sciences **100**: 5974-5979.

Madden, J. C., M. Hewitt, K. Przybylak, R. J. Vandebriel, A. H. Piersma and M. T. Cronin (2012). "Strategies for the Optimisation of In Vivo Experiments in Accordance with the 3Rs Philosophy." Regulatory Toxicology and Pharmacology **63**: 140-154.

Martinez-Outschoorn, U. E., Z. Lin, Y. H. Ko, A. Goldberg, N. Flomenberg, C. Wang, S. Pavlides, R. G. Pestell, A. Howell and F. Sotgia (2011). "Understanding the metabolic basis of drug resistance: Therapeutic induction of the Warburg effect kills cancer cells." Cell Cycle **10**: 2521-2528.

Martinez-Outschoorn, U. E., S. Pavlides, D. Whitaker-Menezes, K. M. Daumer, J. N. Milliman, B. Chiavarina, G. Migneco, A. K. Witkiewicz, M. P. Martinez-Cantarin and N. Flomenberg (2010). "Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: Implications for breast cancer and DCIS therapy with autophagy inhibitors." Cell Cycle **9**: 2423-2433.

Marx, V. (2013). "Cell culture: A better brew." Nature **496**: 253-258.

Mbeunkui, F. and D. J. Johann (2009). "Cancer and the tumor microenvironment: a review of an essential relationship." Cancer chemotherapy and pharmacology **63**: 571-582.

McClellan, J. L., J. M. Davis, J. L. Steiner, R. T. Enos, S. H. Jung, J. A. Carson, M. M. Pena, K. A. Carnevale, F. G. Berger and E. A. Murphy (2012). "Linking tumor-associated macrophages, inflammation, and intestinal tumorigenesis: role of MCP-1." American Journal of Physiology-Gastrointestinal and Liver Physiology **303**: 1087-1095.

McMillin, D. W., J. M. Negri and C. S. Mitsiades (2013). "The role of tumour–stromal interactions in modifying drug response: challenges and opportunities." Nature Reviews Drug Discovery **12**: 217-228.

Mehta, G., A. Y. Hsiao, M. Ingram, G. D. Luker and S. Takayama (2012). "Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy." Journal of Controlled Release **164**: 192-204.

Meinert, C. L. (2012). ClinicalTrials: Design, Conduct and Analysis, OUP USA.

Merlo, L. M. F., J. W. Pepper, B. J. Reid and C. C. Maley (2006). "Cancer as an evolutionary and ecological process." Nature Reviews Cancer **6**: 924-935.

Mertens, J. C., C. D. Fingas, J. D. Christensen, R. L. Smoot, S. F. Bronk, N. W. Werneburg, M. P. Gustafson, A. B. Dietz, L. R. Roberts and A. E. Sirica (2013). "Therapeutic effects of deleting cancer-associated fibroblasts in cholangiocarcinoma." Cancer research **73**: 897-907.

Metzger, M. L., L. R. Meacham, B. Patterson, J. S. Casillas, L. S. Constine, N. Hijiya, L. B. Kenney, M. Leonard, B. A. Lockart and W. Likes (2013). "Female Reproductive Health After Childhood, Adolescent, and Young Adult Cancers: Guidelines for the Assessment and Management of Female Reproductive Complications." Journal of clinical oncology **31**: 1239-1247.

Micke, P. and A. Östman (2005). "Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy." **9**: 1217-1233.

Miki, Y., K. Ono, S. Hata, T. Suzuki, H. Kumamoto and H. Sasano (2012). "The advantages of co-culture over mono cell culture in simulating in vivo environment." The Journal of Steroid Biochemistry and Molecular Biology **131**: 68-75.

Milstein, J., V. Grachev, A. Padilla and E. Griffiths (1996). "WHO activities towards the three Rs in the development and control of biological medicines." Developmental Biology stands **86**: 31-39.

Minami, Y., T. Sasaki, J.-i. Kawabe and Y. Ohsaki (2013). Accessory Cells in Tumor Angiogenesis—Tumor-Associated Pericytes, InTech.

Minchinton, A. I. and I. F. Tannock (2006). "Drug penetration in solid tumours." Nature Reviews Cancer **6**: 583-592.

Mueller-Klieser, W. (1987). "Multicellular spheroids." Journal of cancer research and clinical oncology **113**: 101-122.

Mueller, M. M. and N. E. Fusenig (2004). "Friends or foes—bipolar effects of the tumour stroma in cancer." Nature Reviews Cancer **4**: 839-849.

Nagelkerke, A., J. Bussink, F. C. Sweep and P. N. Span (2013). "Generation of multicellular tumor spheroids of breast cancer cells: how to go three-dimensional." Analytical Biochemistry **437**: 17-19.

Nakase, I., Y. Konishi, M. Ueda, H. Saji and S. Futaki (2012). "Accumulation of arginine-rich cell-penetrating peptides in tumors and the potential for anticancer drug delivery in vivo." J Control Release **159**: 181-188.

Narang, A. S. and D. S. Desai (2009). "Anticancer Drug Development." Pharmaceutical Perspectives of Cancer Therapeutics, Springer.

Noguera, R., O. A. Nieto, I. Tadeo, F. Fariñas and T. Álvaro (2012). "Extracellular matrix, biotensegrity and tumor microenvironment. An update and overview." Histology and histopathology **27**: 693-705.

Olsen, C. J., J. Moreira, E. M. Lukanidin and N. S. Ambartsumian (2010). "Human mammary fibroblasts stimulate invasion of breast cancer cells in a three-dimensional culture and increase stroma development in mouse xenografts." BMC cancer **10**: 1-16.

Olumi, A. F., G. D. Grossfeld, S. W. Hayward, P. R. Carroll, T. D. Tlsty and G. R. Cunha (1999). "Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium." Cancer research **59**: 5002-5011.

Orimo, A., P. B. Gupta, D. C. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V. J. Carey, A. L. Richardson and R. A. Weinberg (2005). "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion." Cell **121**: 335-348.

Ormerod, M. G. and P. R. Imrie (1990). Flow cytometry, Springer.

Page, H., P. Flood and E. G. Reynaud (2012). "Three-dimensional tissue cultures: current trends and beyond." Cell and Tissue Research: 1-9.

Panyam, J. and V. Labhasetwar (2012). "Biodegradable nanoparticles for drug and gene delivery to cells and tissue." Advanced drug delivery reviews **55**: 329-347.

Parrott, J. A., E. Nilsson, R. Mosher, G. Magrane, D. Albertson, D. Pinkel, J. W. Gray and M. K. Skinner (2001). "Stromal-epithelial interactions in the progression of ovarian cancer: influence and source of tumor stromal cells." Molecular and cellular endocrinology **175**: 29-39.

Peinado, H., D. Olmeda and A. Cano (2007). "Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?" Nature Reviews Cancer **7**: 415-428.

Perche, F. and V. P. Torchilin (2012). "Accumulation and toxicity of antibody-targeted doxorubicin-loaded PEG-PE micelles in ovarian cancer cell spheroid model." Journal of Controlled Release **164**: 95-102.

Phung, Y. T., D. Barbone, V. C. Broaddus and M. Ho (2011). "Rapid generation of in vitro multicellular spheroids for the study of monoclonal antibody therapy." Journal of Cancer **2**: 507.

Pietras, K. and A. Östman (2010). "Hallmarks of cancer: interactions with the tumor stroma." Experimental cell research **316**: 1324-1331.

Poste, G. and I. J. Fidler (1980). "The pathogenesis of cancer metastasis." Nature **283**: 139-146.

Purpura, K. A., A. M. Bratt-Leal, K. A. Hammersmith, T. C. McDevitt and P. W. Zandstra (2011). "Systematic engineering of 3D pluripotent stem cell niches to guide blood development." Biomaterials **33**: 1271-1280.

Raguz, S. and E. Yagüe (2008). "Resistance to chemotherapy: new treatments and novel insights into an old problem." British journal of cancer **99**: 387-391.

Ranganatha, N. and I. Kuppast (2012). "A review on alternatives to animal testing methods in drug development." International Journal of Pharmacy and Pharmaceutical Sciences **4**: 28-32.

Raz, Y. and N. Erez (2013). "An inflammatory vicious cycle: Fibroblasts and immune cell recruitment in cancer." Experimental cell research: doi: 10.1016/j.yexcr.2013.1003.1022.

Riehemann, K., S. W. Schneider, T. A. Luger, B. Godin, M. Ferrari and H. Fuchs (2009). "Nanomedicine—challenge and perspectives." Angewandte Chemie International Edition **48**: 872-897.

Rimann, M. and U. Graf-Hausner (2012). "Synthetic 3D multicellular systems for drug development." Current Opinion in Biotechnology **23**: 803-809.

Ruddon, R. W. (2007). Cancer biology, Oxford University Press, USA.

Sadlonova, A., Z. Novak, M. R. Johnson, D. B. Bowe, S. R. Gault, G. P. Page, J. V. Thottassery, D. R. Welch and A. R. Frost (2005). "Breast fibroblasts modulate epithelial cell proliferation in three-dimensional in vitro co-culture." Breast Cancer Res **7**: 46-49.

Sappino, A. P., O. Skalli, B. Jackson, W. Schürch and G. Gabbiani (2006). "Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues." International journal of cancer **41**: 707-712.

See, A. P., J. E. Han, J. Phallen, Z. Binder, G. Gallia, F. Pan, D. Jinasena, C. Jackson, Z. Belcaid and S. J. Jeong (2012). "The role of STAT3 activation in modulating the immune microenvironment of GBM." Journal of neuro-oncology **110**: 359-368.

Shaw, K. R. M., C. N. Wrobel and J. S. Brugge (2004). "Use of three-dimensional basement membrane cultures to model oncogene-induced changes in mammary epithelial morphogenesis." Journal of mammary gland biology and neoplasia **9**: 297-310.

Sherwood, L. (2012). Human physiology: from cells to systems, Thomson Brooks/Cole.

Shuman Moss, L. A., S. Jensen-Taubman and W. G. Stetler-Stevenson (2012). "Matrix metalloproteinases: changing roles in tumor progression and metastasis." The American journal of pathology **181**: 1895-1899.

Siegel, R., D. Naishadham and A. Jemal (2012). "Cancer statistics, 2012." CA: a cancer journal for clinicians **62**: 10-29.

Sietsema, W. (1989). "The absolute oral bioavailability of selected drugs." International journal of clinical pharmacology, therapy, and toxicology **27**: 179-211.

Sinha, R., G. J. Kim, S. Nie and D. M. Shin (2006). "Nanotechnology in cancer therapeutics: bioconjugated nanoparticles for drug delivery." Molecular cancer therapeutics **5**: 1909-1917.

Skobe, M., T. Hawighorst, D. G. Jackson, R. Prevo, L. Janes, P. Velasco, L. Riccardi, K. Alitalo, K. Claffey and M. Detmar (2001). "Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis." Nature Medicine **7**: 192-198.

Sorensen, A., R. J. Mairs, L. Braidwood, C. Joyce, J. Conner, S. Pimlott, M. Brown and M. Boyd (2012). "In vivo evaluation of a cancer therapy strategy combining HSV1716-mediated oncolysis with gene transfer and targeted radiotherapy." Journal of Nuclear Medicine **53**: 647-654.

Soule, H., J. Vazquez, A. Long, S. Albert and M. Brennan (1973). "A human cell line from a pleural effusion derived from a breast carcinoma." Journal of the National Cancer Institute **51**: 1409-1416.

Sounni, N. E. and A. Noel (2013). "Targeting the Tumor Microenvironment for Cancer Therapy." Clinical chemistry **59**: 85-93.

Stacey, G. (2012). Current developments in cell culture technology, Springer.

Stacey, G. (2011). Cell culture contamination, Springer.

Stockmann, C., A. Doedens, A. Weidemann, N. Zhang, N. Takeda, J. I. Greenberg, D. A. Cheresh and R. S. Johnson (2008). "Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis." Nature **456**: 814-818.

Straussman, R., T. Morikawa, K. Shee, M. Barzily-Rokni, Z. R. Qian, J. Du, A. Davis, M. M. Mongare, J. Gould and D. T. Frederick (2012). "Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion." Nature **487**: 500-504.

Strell, C., H. Rundqvist and A. Östman (2012). "Fibroblasts-a key host cell type in tumor initiation, progression, and metastasis." Upsala journal of medical sciences **117**: 187-195.

Streuli, C. H., N. Bailey and M. J. Bissell (1991). "Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity." The Journal of Cell Biology **115**: 1383-1395.

Sutherland, R. M., W. R. Inch, J. A. McCredie and J. Kruuv (1970). "A multi-component radiation survival curve using an in vitro tumour model." International Journal of Radiation Biology **18**: 491-495.

Swartz, M. A., N. Iida, E. W. Roberts, S. Sangaletti, M. H. Wong, F. E. Yull, L. M. Coussens and Y. A. DeClerck (2012). "Tumor microenvironment complexity: emerging roles in cancer therapy." Cancer research **72**: 2473-2480.

Talmadge, J. E. and I. J. Fidler (2010). "AACR centennial series: the biology of cancer metastasis: historical perspective." Cancer research **70**: 5649-5669.

Tlsty, T. D. and L. M. Coussens (2006). "Tumor stroma and regulation of cancer development." Annual Review of Pathology-mechanisms of Disease **1**: 119-150.

Trédan, O., C. M. Galmarini, K. Patel and I. F. Tannock (2007). "Drug resistance and the solid tumor microenvironment." Journal of the National Cancer Institute **99**: 1441-1454.

Tumarkin, E., L. Tzadu, E. Csaszar, M. Seo, H. Zhang, A. Lee, R. Peerani, K. Purpura, P. W. Zandstra and E. Kumacheva (2011). "High-throughput combinatorial cell co-culture using microfluidics." Integrative Biology **3**: 653-662.

Valencia, P. M., O. C. Farokhzad, R. Karnik and R. Langer (2012). "Microfluidic technologies for accelerating the clinical translation of nanoparticles." Nature Nanotechnology **7**: 623-629.

Vanharanta, S. and J. Massagué (2012). "Field Cancerization: Something New Under the Sun." Cell **149**: 1179-1181.

Vera-Ramirez, L., P. Sanchez-Rovira, M. C. Ramirez-Tortosa, C. L. Ramirez-Tortosa, S. Granados-Principal, J. A. Lorente and J. L. Quiles (2011). "Free radicals in breast carcinogenesis, breast cancer progression and cancer stem cells. Biological bases to develop oxidative-based therapies." Critical reviews in oncology/hematology **80**: 347-368.

Wang, J., A. S. Levenson and R. L. Satcher Jr (2006). "Identification of a unique set of genes altered during cell-cell contact in an in vitro model of prostate cancer bone metastasis." International journal of molecular medicine **17**: 849-856.

Wang, M. and M. Thanou (2010). "Targeting nanoparticles to cancer." Pharmacological Research **62**: 90-99.

Wang, S., X. Hao, Y. Su, C. Yi, B. Li, X. Fan, J. Pei, Y. Song, W. Xia and B. Liu (2013a). "The Utilization of Perforated Bioinert Chambers to Generate an In Vivo Isolated Space for Tissue Engineering Involving Chondrocytes, Mesenchymal Stem Cells, and Fibroblasts." Tissue Engineering Part C: Methods **19**: doi:10.1089/ten.tec.2012.0269.

Wang, T., F. M. Kievit, O. Veiseh, H. Arami, Z. R. Stephen, C. Fang, Y. Liu, R. G. Ellenbogen and M. Zhang (2013b). "Targeted cell uptake of a non-internalizing antibody through conjugation to iron oxide nanoparticles in primary central nervous system lymphoma." World neurosurgery: doi:10.1016/j.wneu.2013.1001.1011.

Wang, Z., R. K. Kundu, M. T. Longaker, T. Quertermous and G. P. Yang (2012). "The angiogenic factor Del1 prevents apoptosis of endothelial cells through integrin binding." Surgery **151**: 296-305.

Warburg, O. (1956). "On the origin of cancer cells." Science **123**: 309-314.

Weigelt, B., F. C. Geyer and J. S. Reis-Filho (2010). "Histological types of breast cancer: how special are they?" Molecular oncology **4**: 192.

Weigelt, B., A. M. Glas, L. F. A. Wessels, A. T. Witteveen, J. L. Peterse and L. J. Van't Veer (2003). "Gene expression profiles of primary breast tumors maintained in distant metastases." Proceedings of the National Academy of Sciences **100**: 15901-15905.

Weis, S. M. and D. A. Cheresh (2011). "Tumor angiogenesis: molecular pathways and therapeutic targets." Nature Medicine **17**: 1359-1370.

Whitaker-Menezes, D., U. E. Martinez-Outschoorn, Z. Lin, A. Ertel, N. Flomenberg, A. K. Witkiewicz, R. C. Birbe, A. Howell, S. Pavlides and R. Gandara (2011). "Evidence for a stromal-epithelial" lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts." Cell cycle **10**: 1772-1783.

Willerth, S. M., T. E. Faxel, D. I. Gottlieb and S. E. Sakiyama-Elbert (2007). "The effects of soluble growth factors on embryonic stem cell differentiation inside of fibrin scaffolds." Stem Cells **25**: 2235-2244.

Witsch, E., M. Sela and Y. Yarden (2010). "Roles for growth factors in cancer progression." Physiology **25**: 85-101.

Wolfensohn, S., P. Hawkins, E. Lilley, D. Anthony, C. Chambers, S. Lane, M. Lawton, H.-M. Voipio and G. Woodhall (2013). "Reducing suffering in experimental autoimmune encephalomyelitis (EAE)." Journal of pharmacological and toxicological methods **67**: 169-176.

Wu, H., L. Zhu and V. P. Torchilin (2012). "pH-sensitive poly (histidine)-PEG/DSPE-PEG co-polymer micelles for cytosolic drug delivery." Biomaterials.

Xing, F., J. Saidou and K. Watabe (2010). "Cancer associated fibroblasts (CAFs) in tumor microenvironment." Frontiers in bioscience: a journal and virtual library **15**: 166-179.

Xu, J., Y. Lu, S. Qiu, Z.-N. Chen and Z. Fan (2013). "A novel role of EMMPRIN/CD147 in transformation of quiescent fibroblasts to cancer-associated fibroblasts by breast cancer cells." Cancer Letters **335**: 380-386.

Yamada, K. M. and E. Cukierman (2007). "Modeling tissue morphogenesis and cancer in 3D." Cell **130**: 601-610.

Yang, M., X. Jiang, L. Liang and J.-L. Yang (2013). "Application of Nanobiotechnology in Cancer: Creation of Nanooncology and Revolution in Cancer Research and Practice." World Journal of Cancer Research **1**: 24-36.

Yeom, C. J., Y. Goto, Y. Zhu, M. Hiraoka and H. Harada (2012). "Microenvironments and Cellular Characteristics in the Micro Tumor Cords of Malignant Solid Tumors." International journal of molecular sciences **13**: 13949-13965.

Yin, M., J. Soikkeli, T. Jahkola, S. Virolainen, O. Saksela and E. Hölttä (2012). "TGF-β Signaling, Activated Stromal Fibroblasts, and Cysteine Cathepsins B and L Drive the Invasive Growth of Human Melanoma Cells." The American journal of pathology **181**: 2202-2216.

Youlden, D. R., S. M. Cramb, N. A. Dunn, J. M. Muller, C. M. Pyke and P. D. Baade (2012). "The descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality." Cancer Epidemiology **36**: 237-248.

Yu, A., Y. Matsuda, A. Takeda, E. Uchinuma and Y. Kuroyanagi (2012). "Effect of EGF and bFGF on fibroblast proliferation and angiogenic cytokine production from cultured dermal substitutes." Journal of Biomaterials Science, Polymer Edition **23**: 1315-1324.

Yun, Y., Y. W. Cho and K. Park (2012). "Nanoparticles for oral delivery: Targeted nanoparticles with peptidic ligands for oral protein delivery." Advanced drug delivery reviews **65**: 822-832.

Zamarron, B. F. and W. Chen (2011). "Dual roles of immune cells and their factors in cancer development and progression." International journal of biological sciences **7**: 651-658.

Zetter, P., B.R. (1998). "Angiogenesis and tumor metastasis." Annual review of medicine **49**: 407-424.

Zhang, G., X. Zeng and P. Li (2013). "Nanomaterials in Cancer-Therapy Drug Delivery System." Journal of Biomedical Nanotechnology **9**: 741-750.

Zhang, L., F. Gu, J. Chan, A. Wang, R. Langer and O. Farokhzad (2007). "Nanoparticles in medicine: therapeutic applications and developments." Clinical Pharmacology & Therapeutics **83**: 761-769.

Zhang, X., D. Nie and S. Chakrabarty (2010). "Growth factors in tumor microenvironment." Frontiers in bioscience: a journal and virtual library **15**: 151-165.

Zimmermann, M., C. Box and S. A. Eccles (2013). Two-Dimensional vs. Three-Dimensional In Vitro Tumor Migration and Invasion Assays, Springer.