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SPARC AND SPARC DERIVED PEPTIDES INFLUENCE ON PROSTATE MALIGNANT CELLS AND ENDOTHELIAL CELLS

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To…

My Parents, my Sister and Susana.

"The art of love is largely the art of persistence."
Albert Ellis

Abstract

Prostate cancer is the second type of cancer causing more deaths among men worldwide. The primary site of metastasis for this cancer is bone, suggesting that bone microenvironment is adequate to its growth. In prostate cancer, the interactions between cancer and endothelial cells, and their microenvironment are also fundamental to understand the carcinogenesis and to discover new therapies for cancer treatment. Cancer cells grow up 1–2 mm^3 without blood vessels. To grow beyond this value angiogenesis must occur. This process begins with the release of angiogenic growth factors by cancer cells or cancer stromal cells, activating endothelial cells (ECs) that follow several steps leading to the formation of new vessels. This new blood vessels will connect to the tumor providing nutrients and oxygen and removing waste products. Therefore, angiogenesis is an important factor in the progression of cancer.

In addition, SPARC a matricellular glycoprotein is increasingly investigated related to cancer, however its role is still not fully understood. Different regions of SPARC (e.g. peptides) have distinct activities and may explain the divergent and inconsistent biological activities observed with native full-size protein in different malignancies.

The main aim of this work was to study the influence of SPARC and SPARC derived peptides FS-E and 2.3 (inhibitor and stimulator of angiogenesis, respectively) on endothelial cells and SPARC influence on prostate cancer cells in a bone-like simulated microenvironment. For that purpose, scaffolds of collagen/nanohydroxyapatite (collagen/nanoHA) were produced to mimic bone matrix. The biomaterial's network architecture allows cells to access both collagen nanofibers and HA crystals, similarly to what occurs naturally in bone environment.

Exogenous SPARC (10 and 50 μg/mL) increased proliferation of LNCaP cells in bone-like microenvironment. In contrast, concerning ECs, SPARC (10 and 30 μg/mL) decreased cell proliferation, although cell self-organized in a typical capillary-like structures. In addition, when LNCaP cells were seeded on endothelial cells, this co-culture showed increased proliferation with pre-adsorbed SPARC. Therefore, exogenous SPARC may be favorable to the survival and growth of non-bone metastatic prostate cancer cells in bone-like microenvironment.

Regarding exogenous peptide FS-E, a decrease in ECs cell proliferation for all the used concentrations (0.01, 10, 100 and 1000 μg/mL) was observed, when compared with control (at days 5 and 7). This peptide was also responsible for angiogenesis inhibition after 7 days as shown by the calcein assay. The co-culture of ECs and LNCaP cells showed that pre-adsorption of peptide FS-E at 1000 μg/mL induced a proliferation decrease.

For concentrations of 0.01 and 10 μg/mL the peptide 2.3 showed to decrease ECs proliferation, but a capillary network was observed.

Therefore, this study novelty lies on the development of a biomaterial (collagen/nanoHA) mimicking a bone-like microenvironment allowing the study of carcinogenesis in it.

In conclusion, the interactions between cancer cells and endothelial cells, and their microenvironment (stimulated through a biomaterial) are fundamental to understand the carcinogenesis and to discover new therapies for cancer treatment.

Keywords: Angiogenesis, collagen/nanohydroxyapatite, endothelial cells, peptide 2.3, peptide FS-E, prostate cancer, SPARC.

Resumo

O cancro da próstata é o segundo tipo de cancro que causa mais mortes entre os homens em todo o mundo. O osso é o principal local de metastização do referido cancro, sugerindo que esse microambiente é vantajoso para o seu crescimento. No cancro da próstata, as interações entre as células tumorais e células endoteliais, são fundamentais para estudar a carcinogénese e descobrir novas terapias para o tratamento do cancro. As células do cancro crescem 1-2 mm^3 sem vasos sanguíneos. Para crescer além deste valor é necessário que ocorra angiogénese. Este processo inicia-se com a libertação de fatores de crescimento angiogénicos pelas células do cancro ou células do estroma, da ativação de células endoteliais (ECs) conduzindo à formação de novos vasos. Estes novos vasos sanguíneos serão responsáveis por fornecer nutrientes e oxigénio e remoção de resíduos de reações celulares. Deste modo, a angiogénese é um fator importante na progressão de cancro.

A SPARC é uma glicoproteína matricelular que tem sido muito utilizada em investigação de variados cancros, no entanto o seu papel ainda não é totalmente compreendido. Diferentes regiões da SPARC (por exemplo, péptidos) têm atividades distintas e podem explicar as atividades biológicas diferentes e inconsistentes observadas quando se usa a proteína em diferentes neoplasias.

O principal objetivo deste trabalho foi estudar a influência da SPARC e dos seus péptidos FS-E e 2.3 (inibidor e promotor da angiogénese, respetivamente) em células endoteliais e influência da SPARC em células do cancro da próstata num microambiente semelhante ao osso. Para esse efeito, produziram-se *scaffolds* de colagénio/nanohidroxiapatite para mimetizar a matriz óssea. Este biomaterial permite que as células acedam quer às nanofibras de colagénio quer aos cristais de HA, como ocorre naturalmente no ambiente ósseo.

A pre-adsorção da SPARC (10 e 50 µg/mL) nos *scaffolds* aumentou a proliferação das células LNCaP. Relativamente ao comportamento da SPARC (10 e 30 µg/mL) nas células endoteliais, parece ter promovido uma redução da proliferação celular. No entanto, as células organizaram-se em estruturas de tipo capilar típicas da formação de vasos sanguíneos. Além disso, quando as células endoteliais e as células LNCaP foram usadas em co-cultura, houve um aumento da proliferação em *scaffolds* com SPARC pré-adsorvida. Portanto, a SPARC parece favorecer a sobrevivência e crescimento de células do cancro da próstata provenientes de metástases de origem não-óssea nos biocompósitos que simulam o microambiente do osso.

Relativamente ao péptido FS-E, foi observada uma diminuição na proliferação celular das ECs para todas as concentrações utilizadas (0.01, 10, 100 e 1000 µg/mL) quando comparada com o controlo (5 e 7 dias de cultura). Este péptido foi também responsável pela inibição da angiogénese após 7 dias, como mostrado pelo ensaio da calceína. A co-cultura de células ECs e

LNCaP revelou que a pré-adsorção de 1000 µg/mL de FS-E induziu uma diminuição da proliferação, relativamente aos substratos sem péptido.

Para concentrações de 0.01 e 10 µg/mL, o péptido 2.3 mostrou diminuir a proliferação de células endoteliais, observando-se, no entanto uma organização celular sob a forma de rede capilar.

A novidade deste estudo encontra-se no desenvolvimento de um biomaterial (colagénio/NanoHA) que mimetiza um microambiente ósseo permitindo o estudo de carcinogénese.

Em conclusão, as interações entre as células de cancro da próstata e células endoteliais, e o microambiente (recriado através de um biomaterial) são fundamentais para entender a carcinogénese e descobrir novas terapias para o tratamento do cancro.

Palavras-chave: Angiogénese, cancro da próstata, células endoteliais, colagénio/nanohidroxiapatite, péptido 2.3, péptido FS-E, SPARC.

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List of abbreviations

aa - Amino acids

Bcl-2 - B-cell lymphoma 2

bFGF - Basic fibroblast growth factor

BSA - Bovine serum albumin

Collagen/nanoHA – Collagen/nanohydroxyapatite biocomposite

ECs – Endothelial cells

ECM - Extracellular matrix

EDC - *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide

EGF - Epidermal growth factor

EMT - Epithelial-mesenchymal transition

FS – Follistatin

HPMEC – Human pulmonary microvascular endothelial cell

LNCaP - Lymph node carcinoma of the prostate

NHS - *N*-hydroxysuccinimide

PAI - Plasminogen activator inhibitor

PCa - Prostate cancer

PDGF - Platelet-derived growth factor

PlGF - Placenta growth factor

Rpm - Revolutions per minute

SMOC - Secreted modular calcium binding protein

SPARC - Secreted protein acidic and rich in cysteine

TGF - Transforming growth factor

TSP – Thrombospondin

VEGF – Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptors

vWF – von Willebrand factor

Chapter 1

Introduction

1.1. Cancer

Cancer occurs when cells or a group of the cells undergo changes in genes (mutations) and grow out of control and become invasive (Figure 1). Cells produce signals to control cells division, but if any of these signals are faulty or missing, cells may start to grow and proliferate excessively and form a lump (tumor) [1].

The changes that occur in cells leading a lump are alterations in genes, for example, oncogenes and tumor suppressors. They regulate cell proliferation, survival, and other homeostatic functions [2, 3].

Tumors can be malignant (cancerous) or benign. Malignant tumor grow faster and they may spread to other parts of the body though of bloodstream or lymph system. In contrast, benign tumor grow slowly and do not spread to other parts of the body [1].

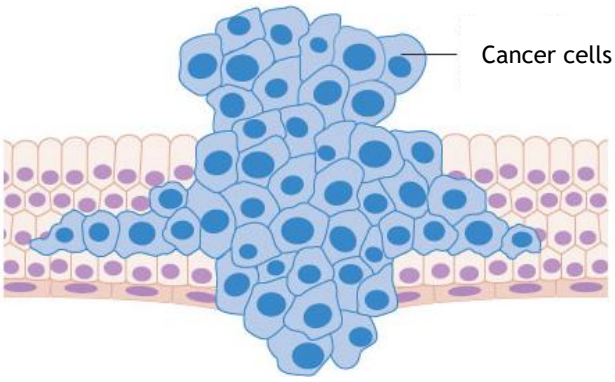


Figure 1 - Cells grow out of control. Cancer cells are characterized, for example, by acceleration of the cell cycle, genomic alterations, invasive growth, chemotaxis, and changes in the cellular surface [1, 4].

Cancer can be classified according with type of cells (Table 1) [5]. For example, carcinomas are formed by epithelial cells and they are the most common type of cancer. Carcinomas have specific names according to the type of epithelial cell: adenocarcinoma (forms in epithelial cells that produce fluids or mucus), squamous cell carcinoma or epidermoid carcinomas (forms in squamous cells), and transitional cell carcinoma (forms in transitional epithelium, or urothelium). When cancer is formed in bone and soft tissues (muscle, fat, blood vessels, lymph vessels, and fibrous tissue) is designed of sarcomas [5]. Others cancers are named and briefly described in table 1. In the case of prostate cancer, commonly, cancer starts in gland cells so it is designated adenocarcinoma [5, 6]. There are other cancer types that may be initiated in the prostate gland even as sarcomas transitional cell carcinomas or neuroendocrine tumors. However, these types of prostate cancer are rare [6].

Biology of cancer is understood through the six hallmarks (Figure 2) [7]. They include capabilities that allow tumor growth and metastatic dissemination. They consist of sustained proliferative signaling, avoiding growth suppressors, resisting cell death, allowing replicative immortality, inducing angiogenesis, and activating invasion and metastasis [7].This work will be focused on angiogenesis, one of the cancer hallmarks.

Table 1 – Cancer classification and type of cells where cancer begins [5].

Cancer classification	Type of cells
Carcinomas	Epithelial cells
Sarcomas	Bone and soft tissues
Leukemia	Blood-forming tissue of the bone marrow
Lymphoma	Lymphocytes
Multiple myeloma	Plasma cells
Melanoma	Melanocytes
Brain and spinal cord tumors	Brain and spinal cord cells
Germ cell tumors	Sperm and eggs
Neuroendocrine tumors	Cells that release hormones into the blood

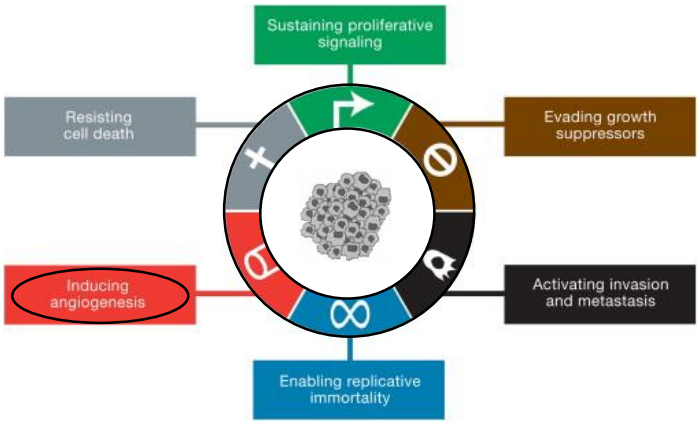


Figure 2 – The hallmarks of cancer were proposed in 2000 by Douglas Hanahan and Robert Weinberg and nowadays these six hallmarkers are still studied [7, 8].

1.2. Prostate Cancer

Prostate is a gland of male reproductive system that looks walnut [9] found below the bladder and in front of the rectum [10]. The prostate function is to make some of the fluid that nourishes and protects sperm cells in semen [6].

Prostate cancer occurs when cells grow out of control in the prostate [11]. This disease is the second type of cancer causing more deaths among men worldwide [12-14], and it is more frequent in certain regions of the world as North America, Oceania, and western and northern Europe [15-18]. The mortality is mainly a result of prostate cancer metastases [19, 20]. Metastatic prostate cancer is an incurable disease and has several consequences which consist of bone pain, spinal cord compression, outflow obstruction, renal failure and anemia [19].

This disease has some risk factors associated, for example, to age, family history of the disease, and black ethnicity [16, 21]. It shows some hallmarks such as initially slow rates of growth and in the advanced phase metastasis to bone [22].

Prostate cancer is classified in four stages according to the aggressiveness of the cancer (Figure 3) [11].

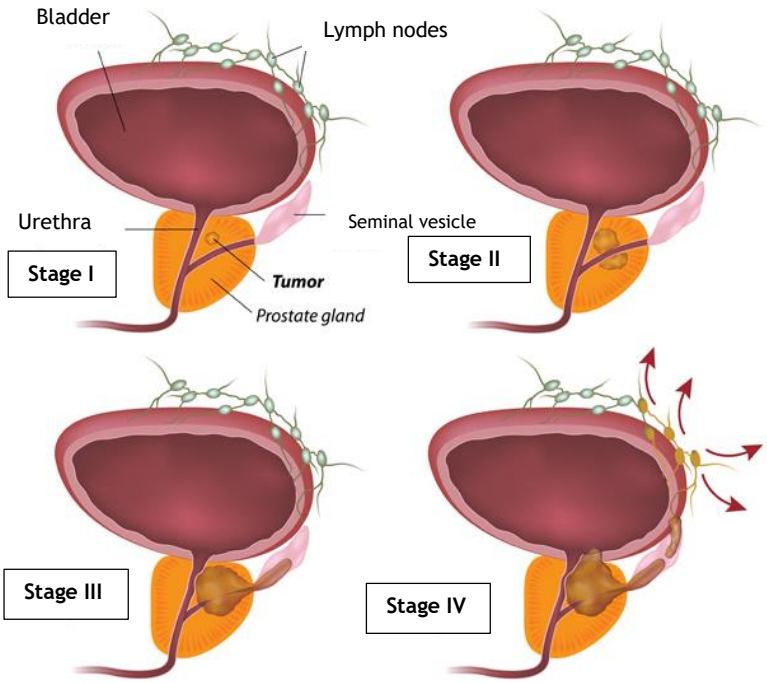


Figure 3 - Stages of cancer prostate. Stage I - Tumor is small and cancer cells aren't aggressive. Stage II - Tumor is still small but it is aggressive. Cancer cells begin to involve the gland. Stage III - Cancer cells spread to seminal vesicles or adjacent tissues. Stage IV - Cancer cells spread to near organs or to bones, lymph nodes, or lungs [11].

1.3. Angiogenesis in cancer

The tumor growth associate with angiogenesis has been investigated largely since 1970 [23].

Angiogenesis take place in normal developmental processes as well as in numerous pathologies (such as tumor growth, and metastasis) [24, 25]. It consists on the formation of new blood vessels [24, 25] by sprouting from pre-existing vessels through the migration and proliferation of endothelial cells [23]. They are aligned in columns and adjacent endothelial cell columns contact each other to form three-dimensional cords and loops that subsequently develop ducts with lumens [25].

Tumors grow up to 1–2 mm^3 in hypoxia (without blood vessels) [26-29]. Tumor requires oxygen and nutrients to maintain cell function, growth, and survival [30], and thus grows up beyond 1–2 mm^3. For it to happen, it is necessary to occur angiogenesis, otherwise, cells may become necrotic or even apoptotic. New blood vessels will penetrate into tumor providing nutrients and oxygen and removing waste products [28, 31].

Tumor angiogenesis consist of interactions between cancer cells, endothelial cells, growth factors, and ECM components [31-33]. It is characterized by a disproportion of angiogenic inhibitors and promoters resulting in ECM remodelling, endothelial cell migration, proliferation, and differentiation, initiating vessel sprouting [30].

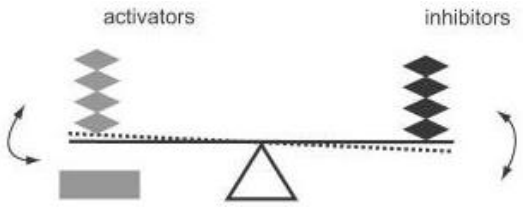
Tumor angiogenesis starts when tumor is in a state of hypoxic and need nutrients and oxygen to grow up [23, 29, 34]. Cancer cells or cancer stromal cells secrete angiogenic growth factors [30, 31, 34] activing endothelial cells [7]. This process, denominated “angiogenic switch" (Figure 4), result of balance between angiogenic inhibitors and promoters factors allowing the transition from the dormant phase to the vascularized phase [29, 35]. The most potent angiogenic growth factor is VEGF [30, 31, 36], and only its expression in high levels may start angiogenesis [37]. After growth factors release, quiescent endothelial phenotype becomes an activated mesenchymal phenotype [34]. This transition designed of EMT (epithelial cells become mesenchymal cells) allows cell migration an invasion because occurs loss of cell-cell cohesiveness [38]. Endothelial cells also secrete MMPs that degrade the basement membrane surrounding the vessel and the adjacent ECM [34]. Degradation of the ECM helps migration of endothelial cells toward the tumor, in the direction of the angiogenic stimulus [28, 32, 34]. However, MPPs not only degrade basement membranes but also they help the activation and liberation of VEGF in ECM [39]. Figure 5 shows angiogenesis steps.

Endothelial cells join to each other organized into three-dimensional structures forming the lumen in their center and these new capillaries grow towards the tumor [28, 34]. These new capillaries are envelop by smooth muscle cells and pericytes giving stability to structures [34]. Pericytes are recruited due PDGF-B produced by endothelial cells [39, 40]. PDGF is a main mitogen for connective tissue cells and other cell types [41].

Tumor blood vessels are architecturally different compared to normal blood vessels. Tumor blood vessels exhibited “irregularly shaped, dilated, and tortuous and can have dead ends” [37].

Strategies based on the fact that tumors are dependent on blood supply have been proposed for cancer treatment [37]. For example, development of therapies with endogenous inhibitors of angiogenesis and development of inhibitors of angiogenic growth factors [42].

A) Switch off



B) Switch on

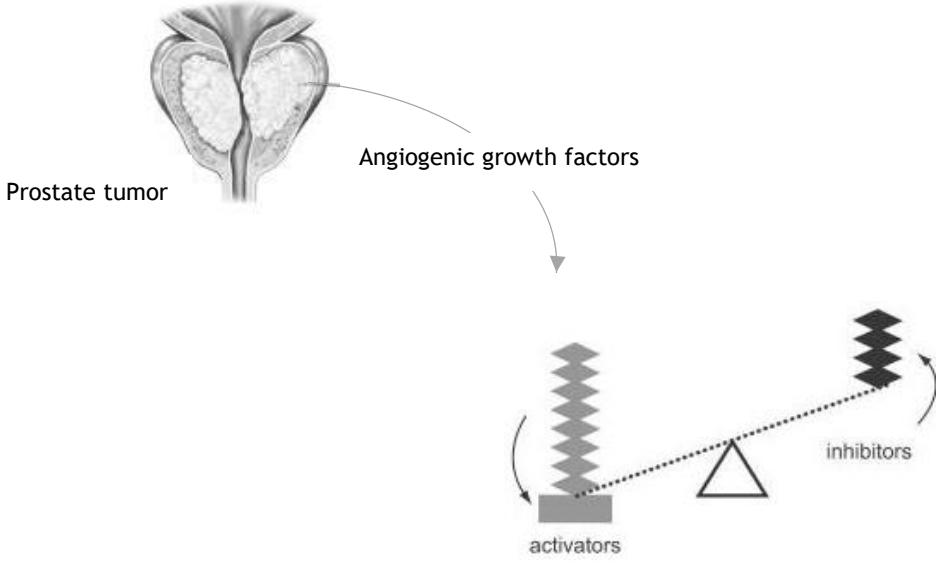


Figure 4 – Angiogenic control switch. A) Switch off - angiogenic activators and inhibitors are in balance. B) Switch on – angiogenic growth factors (activators) stimulate of angiogenesis [26, 29].

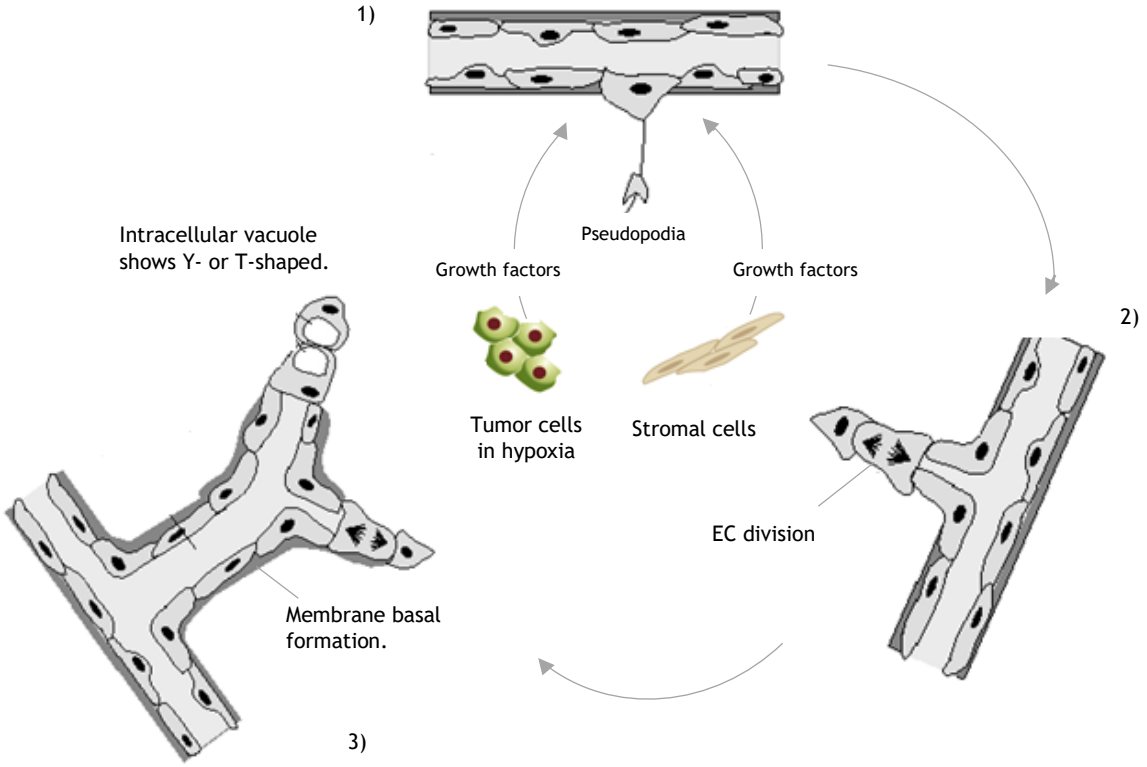


Figure 5 – Angiogenesis steps. 1) Growth factors released by tumor cells and stromal cells activate endothelial cells. These cells lose contact with adjacent cells (EMT) and spread their edges forming pseudopodia. Then, ECs secrete MMPs that degrade ECM allowing migration of endothelial cells in the direction of tumor. 2) Endothelial cells proliferate in the direction of the angiogenic stimulus. Cells contact each other to form three-dimensional cords and loops, develop ducts with lumens leading to the formation of tube-like structures. 3) Blood vessels become mature. Neighboring sprouts fuse together forming closed loops, this process is termed of anastomosis, and it "marks the onset of blood circulation in the new network" [25, 28, 32, 34, 40].

1.3.1. Angiogenic growth factors in prostate cancer

The most important angiogenic growth factors in prostate cancer are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor (TGF-β) [43].

VEGF is a growth factor responsible for the beginning of the angiogenesis leading to tumor growth [43, 44] (Figure 6). The VEGF family is the most important angiogenic factor in cancerous tissue and the adjacent stroma [26, 30, 31, 36] constituted by five glycoproteins, VEGFA or VEGF, VEGFB, VEGFC, VEGFD, and placenta growth factor (PlGF) [45]. Cancer cells secrete VEGF into the surrounding tissue and when VEGF is in contact with endothelial cells binds to their receptors [26, 39] (VEGFR-1, VEGFR-2, and VEGFR-3 [21, 45]). Binding between VEGF and their receptor stimulates endothelial cell growth, enhances vascular permeability [21] and leads to endothelial cells to produce matrix metalloproteinases (MMPs) [26]. MMPs break the ECM allowing the migration of endothelial cells [28, 32, 34] allowing vascularization. However, MMPs can inhibit endothelial cell growth by releasing angiostatin, endostatin, and tumstatin. For example, MMP-9 can activate (by release of VEGF) and inhibit ("by liberating

endogenous angiogenesis inhibitors from their parent matrix molecules") angiogenic switch [39].

Other important growth factor is basic fibroblast growth factors. bFGF is secreted by tumor cells and binds to FGFR1 and FGFR2 on endothelial cells. Binding between bFGF and their receptor activates endothelial cell proliferation [39]. In the case of prostate cancer, quite a lot of FGFs are overexpressed [35].

TGF- ß is responsible for the activation of epithelial mesenchymal transition, that can be found in three isoforms: TGF-ß1, TGF-ß2, and TGF-ß3 [45]. TGF- ß is released a more advanced tumor stage [35] by stromal cells [43] and it increases ECM production, induces angiogenesis [35], "inhibiting prostatic epithelial cell growth and inducing apoptosis" [43]. Interestingly, this growth factor favors osteoblastic bone metastases [35].

In a more advanced stage of cancer, other growth factors are released by the tumor cells leading to tumor development (Figure 6) [44].

Growth factors have been studied so they are an "attractive concept for the inhibition of tumor angiogenesis" [39, 43], for example "VEGF expression is quite high in most prostate cancers and thus serves as a target for therapeutic treatments" [43].

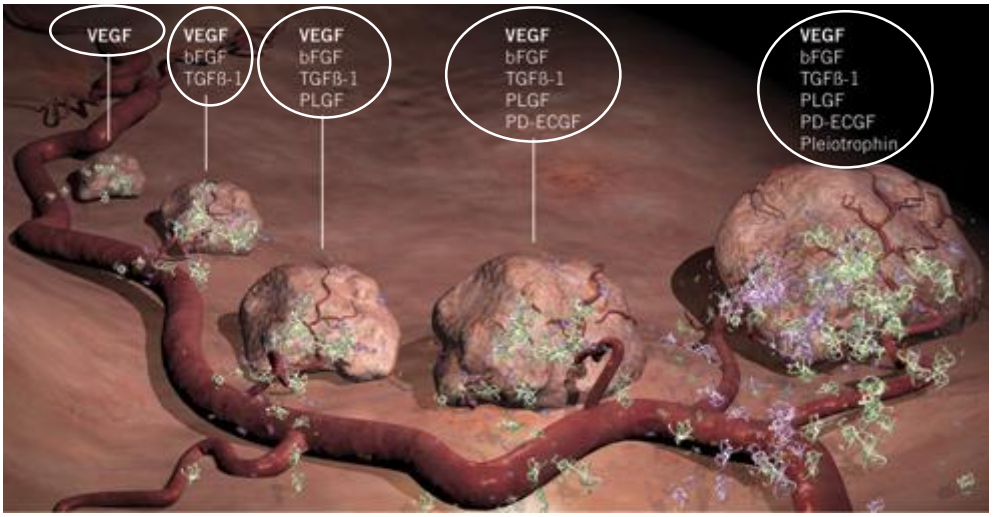


Figure 6 – Growth factors in cancer stages. In higher stages of cancer are released more growth factors. VEGF is the most potent growth factor initiating angiogenesis [44].

1.4. Bone metastases

Metastasis is defined as the process by which a malignant cell detaches the primary tumor and establishes a secondary tumor site [19, 46-48]. This ability of metastasis is considered one feature of malignancy [47, 49]. In an initial stage, prostate cancer cells are restricted to the prostate gland, but in an advanced stage they metastasize to bone [50], lymphatic system and lung and liver. Bone metastases are detected in 80 % of cases; lymph node metastases are detected in only 20 % of patients while lung and liver metastasis are relatively rare [18, 51].

Bone is a primary site of metastasis for several cancers including prostate, suggesting that the bone microenvironment is advantageous to its growth

Multiple veins (Batson's plexus) around the prostate that connect "to the venous drainage of the spine" is the anatomical characteristic that best explains the predilection of prostate

cancer cells to spread to bone [50]. Cells enter in the retrograde venous system (that connect pelvis with the vertebral spine) and they spread in bone [51, 52].

Bone microenvironment has components able to alter prostate cancer progression, [53] and are also responsible for favouring metastasis and the growth of prostate cancer cells in this microenvironment [20].

The metastasis include complex molecular events (Figure 7) such as angiogenesis on the site of the original tumor, local migration within the primary site, intravasation into the blood flow, survival within the circulation, extravasation of the tumor cells to the target organ and colonization of those cells within the new site [12, 47, 54].

After cell proliferation in the primary tumor, cancer cells detach, migrate and entry into the nearby blood or lymphatic vessels (intravasation) [12]. The cancer cells can migrate because epithelial cells lose restricted migratory capability during the malignant progression wherein epithelial cells change to mesenchymal transition (EMT) [12, 19]. Various cells of the prostate cancer microenvironment have factors [28] (for example E-cadherin [55]) that induce this change [28]. After intravasation, cells migrate in the direction of the bone through upregulation of chemokines and bone-derived factors. Then, cells extravasation into the bone target and adhere to bone matrix through integrins and cadherin, and the matrix is broken through MMPs and urokinase-type plasminogen activator. Finally, mesenchymal becomes epithelial transition and epithelial cells induce cancer cells [28] to proliferate and differentiate by molecular interactions with bone microenvironment [12]. Thus, bone is designed as secondary tumor site so cancer cells detach from prostate gland and establish in bones. For the tumor to grow in bone, a complex process such as interaction between cancer cells and bone microenvironment and angiogenesis is required [50].

The bone metastases cause lesions (osteoblastic and osteolytic lesions) the bone becomes brittle and loses density [12, 28, 51, 56]. Patients feel pain, have high blood calcium levels (so cancer cells damage the bone and calcium is released into the bloodstream) and other problems that limit their lifestyle [48].

1.4.1. Bone microenvironment

Bone is an advantageous microenvironment for cancer cell migration [57]. It is a specialized type of connective tissue, which provides structural support, protection, and plays a major role in the regulation of calcium levels in the body [57, 58].

Bone matrix includes two fractions: inorganic and organic fraction [50, 59]. Organic bone matrix consists of type I collagen (95 %), proteoglycans and others non-collagenous proteins. This matrix has no mechanical strength and so it is necessary to occur the mineralization process. Hydroxyapatite crystals are deposited in the matrix leading to bone resistance to compression [57]. Thus, the inorganic portion is constituted by calcium phosphate (in the form of non-stoichiometric, partially substituted and partially crystalline hydroxyapatite) [50].

Bone microenvironment is constituted by several cells that could contribute to the “bone metastatic niche”. They can divide into two categories: namely stromal cells (adipocytes, fibroblasts, chrondrocytes, or osteoblasts) and transient cells (erythrocytes, T cells, and platelets). Stromal cells are the ones that are derived from mesenchymal stem cells, while transient cells are the ones derived from hematopoietic stem cells [57].

Bone matrix is produced by osteoblasts [50], and integrates several growth factors, calcium ions, cell adhesion molecules, cytokines, and chemokines [57], and non-collageneous

proteins (such as SPARC, osteocalcin, fibronectin, thrombospondin-2, small integrin-binding ligand N-linked glycoproteins) [50]. The tumor growth is limited to the bone matrix [14] suggesting that components of bone matrix attract prostate cancer cells and allow them to survive and proliferate in this microenvironment. For example, SPARC has high affinity for collagen and bone mineral phase [50].

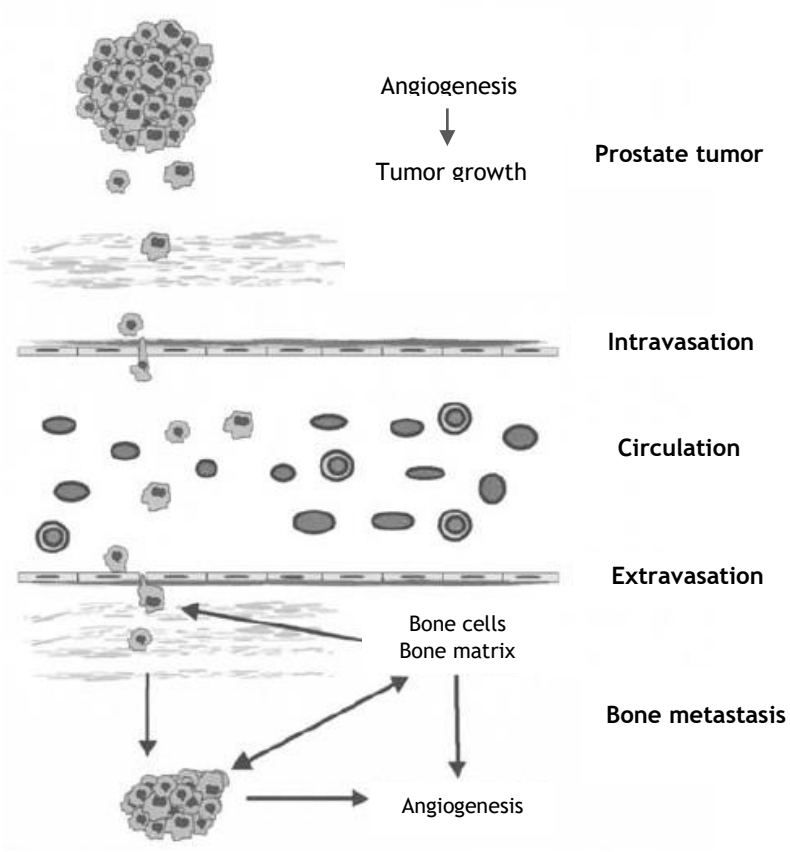


Figure 7 - Steps of prostate cancer bone metastasis. Cancer cells proliferate at primary site, intravasation, extravasation, and survive, proliferate and differentiate in bone microenvironment [48].

1.5. SPARC and cancer

The role of SPARC in cancer has been increasingly investigated, but in different cancers it is still controversial [60].

SPARC has the capacity of promoting or inhibiting tumor progression and it is dependent on the cell-type, tumor stage, and tumor microenvironment (for example, if it is produced by cancer cells or stromal cells) [60-62].

High levels of SPARC (designated as tumor promotion or progression or protumorigenic protein) are often associated with the most aggressive and highly metastatic tumors namely

breast cancer, prostate cancer [60-63], ovarian cancer, and colorectal cancer [64]. In contrast, others studies suggest that lower levels of SPARC expression (tumor suppressor or antitumorigenic protein) are also found in these types of cancers [62, 65-67]. Thus, as previously mentioned the role of SPARC in cancer is still controversial [60].

The mechanism that promotes tumor growth and involves the protein SPARC is the regulation of epithelial-mesenchymal transition (EMT) by TGF-β. In addition, SPARC can inhibit immune surveillance (Figure 8) [63]. Table 2 summarizes influence of SPARC in cancer.

In the case of prostate cancer, SPARC is mainly a protumorigenic protein [53], however, some studies described SPARC as down-regulating the proliferation [56, 67] (number of cells resulting of cell growth and division [68]) and cell invasion [56, 67].

SPARC has been shown as a chemotactic factor to promote prostate cancer migration. Several studies indicate that prostate cancer cells migrate in the direction of bone extracts containing SPARC. They show that SPARC expression has increased in bone metastasis compared with the primary tumor [53, 56, 69]. Furthermore, SPARC is expressed by prostate cancer cells obtained from metastatic cases, but it is not expressed by cells obtained from non-metastatic cases [70]. In addition, other study suggest that some factor from prostate cancer cells may influence stromal cells to reduce a secretion of SPARC [67], thus limiting cancer growth [56].

In conclusion, the roles of SPARC in prostate cancer keep on unclear [67], as a result, more research is required to understand the function of SPARC in cancer [64].

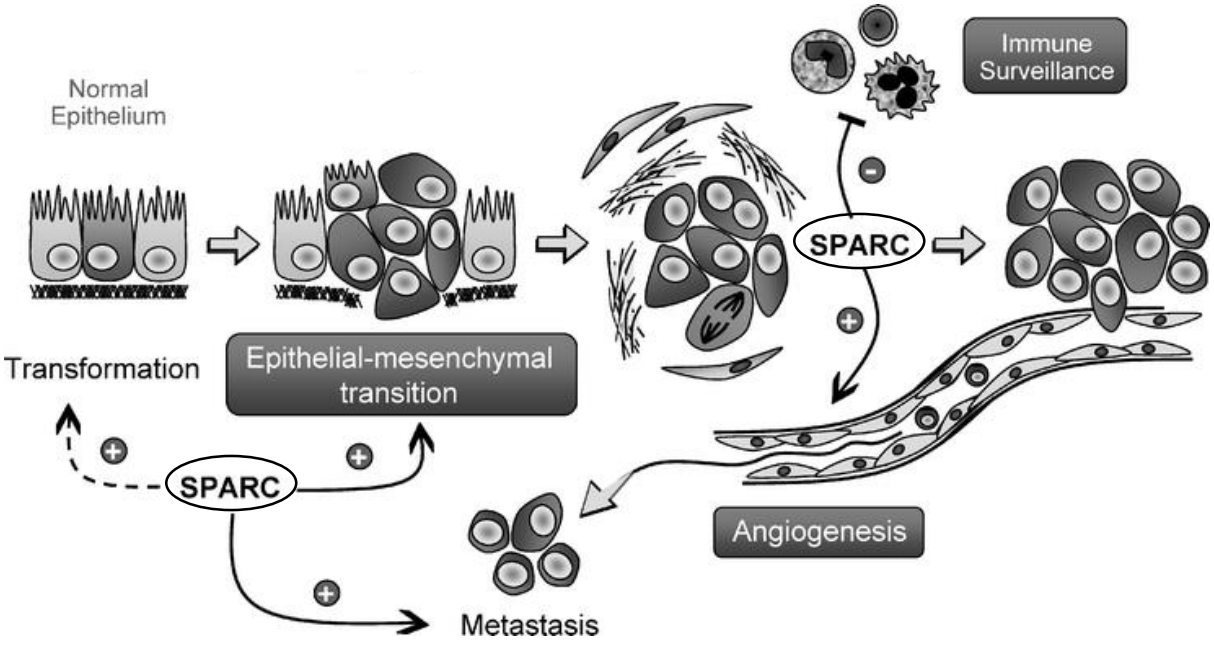


Figure 8 - Tumor progression involving SPARC. SPARC produced by tumor cells and stromal cells participates in EMT, inhibits immune surveillance and promoting angiogenesis [63].

Table 2 - Influence of SPARC in cancer.

SPARC influence in cancer	Reference
Promotes EMT.	[71].
Promotes tumor angiogenesis.	[63].
Inhibits immune surveillance.	[63].
Prostate cancer cells migration (chemotactic factor) in the direction of bone extracts containing SPARC.	[53, 56, 69, 71].

1.5.1. SPARC

SPARC (also known as osteonectin, BM-40 [49, 72-77] or 43K protein [73, 76]) is a matricellular glycoprotein that is secreted into the ECM (Figure 9) [60, 72, 74-85]. SPARC is called osteonectin because is the main non-collagenous protein of bone matrix, or known as BM-40 because it is a component of the matrix of a basement membrane tumor [74]. SPARC belongs to the SPARC family of proteins consisting of SPARC, hevin, SMOC 1 and 2, testicans 1, 2, and 3, and follistatin-like protein 1 [81].

Initially, SPARC was discovered as a non-collagenous main component of bone [86, 87], as a bone-specific protein that binds selectively to hydroxyapatite (by calcium) and collagen [88, 89]. In addition, it was one of the first known matricellular protein that modulates interactions between cells and ECM [87].

This protein is expressed during many stages of development in a variety of organisms [75, 84, 90], but in adult tissues its expression is restricted [74, 75]. During development SPARC is expressed at high levels in bone tissue, in several other tissues and cells associated with remodeling tissues [74, 79]. The expression of SPARC in adult tissues has been identified in tumors and in tissues involved in repair and high rates of turnover or to sites of disease and injury, for example, wound healing, mineralization, and angiogenesis [73-75, 78, 79, 87, 90, 91].

It was also originally considered as a protein secreted by endothelial cells *in vitro* [63], however, later studies revealed that this protein is secreted by other cells such as fibroblasts [63, 77, 92], vascular smooth muscle cells, tumor cells [92], osteoblasts, platelets [77], and macrophages [86].

SPARC interacts between the cell and ECM (Figure 9) [49, 62, 73, 81, 90], it participates in ECM assembly and turnover [61] and it is considered a calcium binding matricellular glycoprotein [72, 74]. Calcium binding proteins are present in basement membranes and contribute to their architecture [93].

This protein has several important roles, namely it inhibits cell spreading (particularly that of endothelial cells and fibroblasts), disrupts cell adhesion (antiadhesive protein), promotes changes in cell shape, inhibits the cell cycle (G1 to S-phase), regulates cell differentiation, inactivates cellular responses to certain growth factors, regulates ECM and MMP production, disrupting cell–ECM interactions [80, 87, 90, 91, 94]. It has effects on biological functions as proliferation [49, 79, 80, 87], morphogenesis [49, 62, 79, 87], tissue remodeling [49, 62, 79] (such as bone formation/mineralization [62, 92]), migration [49, 74, 79, 87, 92], wound healing [74, 92], and fibrosis [92].

SPARC is involved in angiogenesis [63, 74], adipogenesis [77, 87, 95], cancer [74, 79, 87, 92], cataractogenesis [74] and osteoblastogenesis [77, 95].

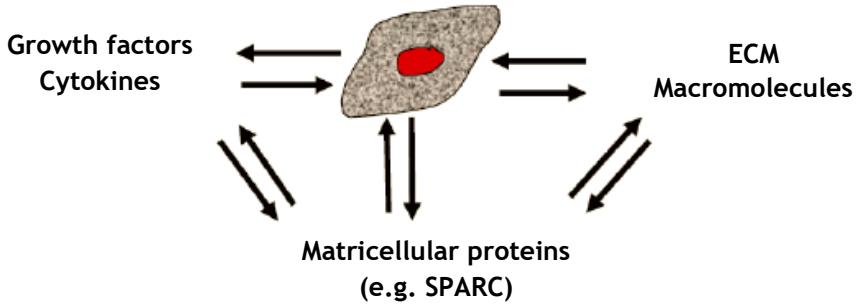


Figure 9 - ECM and matricellular proteins. Matricellular proteins (such as SPARC) are responsible for the modulation of the cell function through interaction cell-surface and they do not have structural roles in the ECM [60, 81].

1.5.1.1 SPARC structure

SPARC size is between 32 and 43 kDa [62, 72, 89, 90]. Normally, the gene encoding SPARC generates a 33 kDa protein, but this protein undergoes a process of post-translation glycosylation, consequently secreted SPARC protein has 43 kDa size in most tissues [90].

SPARC is a product of a single-copy gene mapped to mouse chromosome 11 and to the long arm of human chromosome 9 [78, 87] at locus q31-q33 [96]. SPARC is a protein with high degree of conservation among different species [76, 84, 86, 87]. There is a large selective pressure to conserve specific structural and functional features in this protein [84] indicating that this protein has an important physiological role [49, 62, 86].

The vertebrate SPARC gene encodes proteins of 298–304 amino acids (aa) and the signal sequence (constituted by the first 17 aa) is removed during processing (before the secretion of the protein) [76, 87]. The SPARC mature peptide has three different modules (module I, module II, and module III) (Figure 10, Table 4) [76, 87] which are divided according to their secondary structure and biological activities [87].

Module I (also known as N-terminal highly acidic calcium-binding domain [87]) is encoded by exons 3 and 4 [86] and it is highly acid (rich in aspartic and glutamic). Module I has a structured α-helical, binds several calcium ions with low affinity [74, 76, 86] and also binds hydroxyapatite [62, 76]. This module inhibits cell spreading, prevents chemotaxis, enhances PAI-1 and decreases fibronectin and TSP-1 [87]. Thereby, it is responsible for cartilage and bone mineralization [74]. Recent studies demonstrate that this module in interaction with Bcl-2 and caspase 8 results in the apoptosis of tumor cells [97].

Module II (also known as cysteine-rich FS-like domain [87, 96]) is encoded by exons 5 and 6, and it is constituted by 10 cysteine and a N-linked complex carbohydrate at N99 [76]. This module is responsible for inhibiting focal adhesions, promoting angiogenesis and proliferation [87]. The N-terminus region this module has a twisted ß-hairpin structure that is linked by disulfide bonds at cysteines. The C-terminus region this module has structural similarity to

Kazal family of serine proteases. It has antiparallel α-helices connected to small three-stranded antiparallel ß-sheets with disulfide bonds linking cysteines [62]. The sequence KGHK, stimulates cell proliferation and angiogenesis [74, 80]. Module II contains peptides that exert different effects on endothelial cells [76].

Module III (also known EC-binding domain [74, 87]) is encoded by exons 7, 8 and 9. Structure this module is globular, contain two EF-hand motifs and comprise of α-helices [76]. Module III has high affinity bind to calcium [74, 76, 87]. This module induces MMPs, interactions between cells and matrix, inhibits cell spreading, proliferation and adhesion [76, 86]. Thus, this module was shown to mediate antiproliferative and counteradhesive properties [90].

The summary of the modules properties is presented in Table 3.

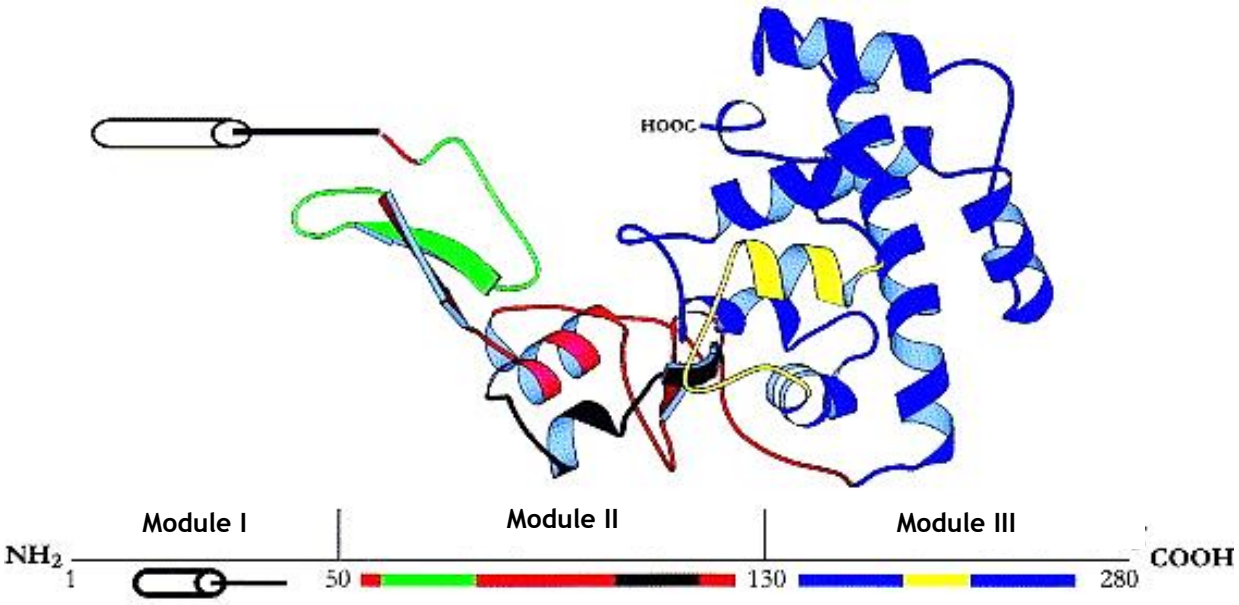


Figure 10 - Modular structure of human SPARC. SPARC is constituted by three modules (module I, module II and module III) with different propriety. The module II is shown in red except for peptide 2.1 and the KGHK angiogenic peptide which are shown in green and black, respectively. The module III is shown in blue except for peptide 4.2, which is displayed in yellow [76].

1.5.1.2. SPARC peptides

SPARC is constituted by several peptides (Figure 11) with different properties (Table 5), thus it is a multi-functional protein [62].

Peptide 1.1 (contains calcium-binding sites) inhibits cell spreading in endothelial cells and fibroblasts [62], potentiates MMP-2 activation [62, 86], does not bind to collagen III and has no effect on cell proliferation [62].

Peptide 1.3 has no effect on cell spreading [62, 98].

N-terminus peptide binds pro-caspase to activate the extrinsic and enhance the intrinsic pathway of apoptosis and inhibits binding of Bcl-2 to pro-caspase 8 to enhance apoptosis [62, 99].

Peptide 2.1 (does not contain calcium-binding sites but is disulfide-rich) inhibits endothelial cell proliferation [62, 76] with delayed entry into the S phase of the cell cycle [62, 86], prevents the mitogenic effect of VEGF-induced stimulation of cell proliferation [62], stimulates proliferation of fibroblasts, and mediates disassembly of focal adhesions on endothelial cells [86]. This peptide is placed in the N-terminal end of the cysteine-rich of module II [100].

Table 3 - Summary of the modules properties [62, 76].

Module I/N-Terminus	Module II/FS-like	Module III / EC-binding
• Has low affinity ca^{2+}-binding, • Binds hydroxyapatite, • Inhibits cell spreading, • Inhibits fibronectin, • Inhibits TSP-1, • Prevents chemotaxis, • Enhances PAI-1.	• Contains 10 cysteine residues, • Has N-linked complex carbohydrate at N99, • N-terminus region has a twisted ß-hairpin structure, • C-terminus region has structural similarity to Kazal family of serine proteases, • Abrogates adhesions, • Stimulates proliferation and angiogenesis.	• Binds calcium, • Contains two EF-hand motifs, • Comprises of α-helices, • Inhibits cell spreading, • Inhibits proliferation and adhesions, • Induces MMPs, • Induces interactions between cells and matrix.

Peptide 2.3 stimulates cell endothelial proliferation and angiogenesis due to the KGHK sequence and this peptide has high affinity with calcium [62, 76]. This peptide is located in the C-terminal end of module II [100].

Peptide 3.2 (contains a portion of the collagen-binding α-helix) induces MMP production [86] and has no effect on focal adhesion dissolution [80].

Peptide 3.4 does not bind to collagen [62], has no effect on cell rounding and has a small effect on cell proliferation [62, 91].

Peptide 4.0 (does not contain calcium-binding sites but is disulfide-rich) inhibits endothelial cell proliferation with the delayed entry into the S phase of the cell cycle [62].

Peptide 4.2 (contains calcium-binding sites) binds endothelial cells and inhibits not only endothelial cell proliferation [62, 76, 86], but also cell spreading in endothelial cells and fibroblasts [62, 98]. Furthermore, peptide 4.2 blocks the effects of VEGF, bFGF, and PDGF in multiple cell types, participates in the disassembly of focal adhesions [62, 86], exhibited differential affinity for ECM molecules [98], for example, binds to collagen but does not bind to albumin, ovalbumin, SPARC and fibronectin. This peptide also binds calcium [62]. It is found in module III [100].

Peptide Z-1 (contains a Cu^{2+} binding sequence KHGK) has a biphasic effect on endothelial cell proliferation and vessel growth (increases vessel density at 0.1 mM and inhibits vessel density at 1.0 mM) and does not stimulate endothelial cell migration [62]. Peptide Z-2 (contains the EF hand-1 calcium-binding motif) and peptide Z-3 (does not contain the Cu^{2+} binding nor the EF hand-1 motifs) inhibits endothelial cell proliferation and stimulates migration [62, 80].

Peptides FS-K and EC-N make part of the Kazal module of the module II [101]. Peptide FS-K inhibits endothelial cell proliferation [62] and peptide EC-N inhibits angiogenesis and has no effect on cell migration nor on endothelial cell apoptosis [62, 101].

Peptide FS-E has a strong inhibited endothelial cell migration *in vitro* and angiogenesis *in vivo* [62, 102], and *in vitro* [103] due to strongly inhibition of bFGF [101]. It is found in N-terminal region of module II [103].

FSEN and FSEC peptides ("correspond to the N-terminal and C-terminal regions of peptide FS-E", respectively [104]) are smaller, less structurally complex and they block angiogenesis. However FSEC is more potent [102].

Finally, peptides J1 (Cu^{2+} binds the sequence KHGK), J2 (calcium-binding EF hand-1) and J3 influence endothelial cell behavior and angiogenesis [80].

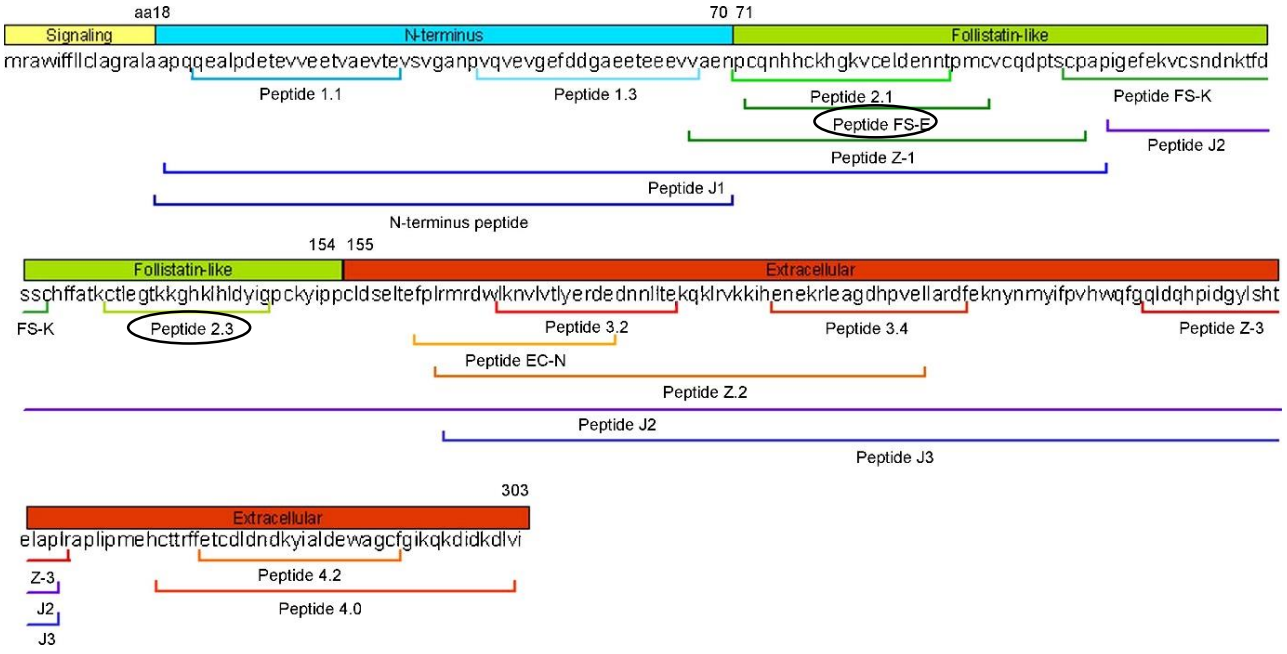


Figure 11 - SPARC peptides identified in several studies. Module I includes peptides 1.1, 1.3 and N-terminus peptide. Module II exhibit peptides 2.1, 2.3, FS-E and FS-K. Finally, module III includes peptides 3.2, 3.4, 4.0, 4.2, EC-N, Z.2, Z.3 and J.3 [62].

Table 4 – SPARC peptides and their functions [62].

Peptide	Functions
1.1	○ Inhibits cell spreading, ○ Does not bind to collagen III, ○ Induced MMP-2 activation , ○ No effect on cell proliferation.
1.3	○ No effect on cell spreading.
2.3	○ Release of (K)GHK to stimulate endothelial cell angiogenesis and proliferation, ○ Has high affinity for calcium.
FS-E	○ Inhibits endothelial cell migration in a biphasic manner, ○ No effect on endothelial cell apoptosis, ○ Potently inhibits angiogenesis, ○ Inhibits angiogenesis induced by neuroblastoma cells.
FS-K	○ Weakly inhibits endothelial cell migration, ○ No effect on endothelial cell apoptosis, ○ Weakly inhibits angiogenesis.
Z-1	○ Increases vessel density at 0.1 mM, ○ Inhibits vessel density at 1.0 mM, ○ Biphasic effect on thymidine incorporation, ○ No effect on PAE cell migration, ○ Biphasic effect on endothelial cell proliferation, ○ Did not stimulate cell migration.
3.4	○ Does not bind to collagen, ○ No effect on cell rounding, ○ Slight effect on cell proliferation.
4.0	○ Inhibits endothelial cell proliferation.
4.2	○ Loss of focal adhesions, ○ Inhibits cell spreading, ○ Inhibits endothelial cell proliferation, ○ Binds to collagen, ○ Inhibits DNA synthesis with peptide 2.1, ○ Does not bind to albumin, ovalbumin, SPARC, fibronectin, ○ Binds to calcium.
EC-N	○ No effect on cell migration, ○ No effect on endothelial cell apoptosis, ○ Inhibits angiogenesis.
Z-2	○ Minimal effects on vascular density, ○ Inhibits endothelial cell proliferation, ○ Stimulates endothelial cell migration.
Z-3	○ Minimal effects on vascular density, ○ Inhibits endothelial cell proliferation, ○ Stimulates endothelial cell migration.

1.5.2. SPARC influence on endothelial cells

The growth of endothelial cells is influenced by SPARC and some of their peptides in a concentration-dependent manner [105-108].

As previously mentioned, SPARC has several peptides that can influence endothelial cells. For example, peptide 1.1 and peptide 4.2 inhibit cell spreading [62, 98]; peptide 2.1, peptide 4.0, peptide 4.2, and peptide FS-E inhibit cell proliferation [62, 76, 86]; peptide 2.3 stimulates cell proliferation and angiogenesis [62, 76].

SPARC has several functions that play an important role in angiogenesis, including the fact that SPARC inhibits endothelial cell spreading, disrupts focal adhesions, regulates cell proliferation [73, 80]; enhances the permeability of endothelial monolayers [80]; has interactions with growth factors [62, 79, 80]; and regulates the production of integrin and MMPs [80]. SPARC binds to some growth factors reducing its interaction with its receptors [62].

During angiogenesis, SPARC inhibits TGF-β1 promoting pericytes migration from preexisting vessels to the new vessels [92, 109]. Pericytes wrap the activated endothelium (new vessel), inducing vessel quiescence (vessel maturation) and stable (Figure 12) [92, 109].

Angiogenic factors and SPARC influences on angiogenesis are shown in Table 5.

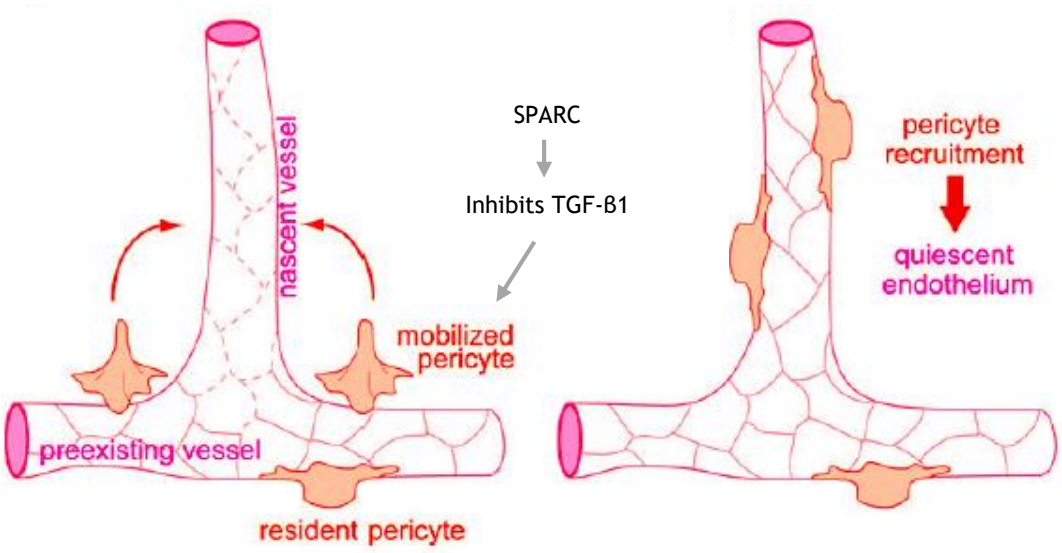


Figure 12 – Influence of SPARC in angiogenesis. SPARC influences growth factors, integrins, and TGF-β1. SPARC inhibits TGF-β1 and this inhibition allows that perycites migrate to the new (nascent) vessel [92].

1.5.2.1. Endothelial cells

The vascular system is one of the first systems to be developed during embryonic development of vertebrates. The endothelium forms the thin layer of cells that lines the interior surface of blood vessels and lymphatic duct. The cells forming the endothelium are called endothelial cells [110].

Endothelial cells provide an anticoagulant barrier between the vessel wall and blood and act as a selective filter to regulate the passage of gases, fluids, immune cells and several

molecules [111]. Endothelial cells also have an important role in angiogenesis [111-114] (including tumor angiogenesis [115]) and vasculogenesis [111-114].

Vasculogenesis occurs in the embryo when angioblasts differentiate into blood islands and then fuse to form a primitive capillary network [111]. After birth, vasculogenesis occurs following the recruitment of endothelial progenitor cells (derived from bone marrow) that are integrated into nascent vessels or which stimulate new vessel growth by releasing pro-angiogenic stimuli. Angiogenesis (as mentioned in section 1.3) is the formation of new capillaries and blood vessels derived from pre-existing blood vessels [112, 116].

These cells can exhibit a diversity of shapes during vascular development, adult homeostasis, and pathology [117].

The basement membrane matrix contains several components allowing structural and functional support and modulation of endothelial cell behavior. They are collagen IV, VIII, XV, and XVIII laminins 8 and 10, nidogens/entactins 1 and 2, heparan sulfate proteoglycans, growth factors (VEGF, TGF, FGF, and PDGF), MMPs, SPARC, fibulins 1 and 2, thrombospondins 1 and 2, and fibronectin [39, 118].

Table 5 – Angiogenic factors their role in angiogenesis and the effect of SPARC [92].

	Role in angiogenesis	**Effect of SPARC**
VEGF-A	Stimulates endothelial cell activation, Initiates angiogenesis.	SPARC inhibits the binding between VEGF-A and VEGFR1.
FGF-2	Stimulates endothelial cell activation, Initiates angiogenesis.	SPARC inhibits the binding between FGF-2 and their receptor.
PDGF	Stimulates pericyte proliferation and migration.	SPARC directly interacts with PDGF.
TGF-β1	Can stimulate endothelial cell activation, Inhibits pericyte migration and induces their differentiation.	SPARC can induce or inhibit the activation of TGF- β1.
αVβ3, and αVβ5 integrins	Induce endothelial cell survival in response to ligand interaction, Induce cell death in the absence of ligand.	SPARC can increase αVβ3, and αVβ5 - mediated migration of prostate tumor cells.

1.6. Prostate cancer cell lines and endothelial cells

Endothelial cells are used as *in vitro* model systems for several physiological and pathological processes, mainly in angiogenesis study [119]. Human microvascular endothelial cells are relevant experimental models *in vitro* culture systems for angiogenesis, inflammation, wound healing, tumor growth, and metastasis. Primary endothelial cells show unique markers such as high production of vWF, PECAM-1 or CD31, VEGF, Flt-1 and KDR. In contrast, most human endothelial cell lines have few endothelial cells features of primary endothelial cells in culture. However, the cell line HPMEC-ST1.6R displays most of the major constitutively expressed and inducible endothelial phenotypic markers. Therefore, HPMEC-ST1.6R cells are used to study

pathological mechanisms and angiogenesis of mature microvascular endothelium *in vitro* model systems [120].

The most commonly used cell lines for prostate cancer research are LNCaP, PC-3 and DU-145 cells [22]. LNCaP cells are isolated from a human prostate cancer lymph node metastasis [121, 122] and are cells androgen-sensitive [121]. The cells adhere weakly to the substrate and are easily detached through pipetting, for example. When density cellular is high, cells detach as "sheets", and after trypsinization, it is difficult to make cell counts because cells form clumps [22]. PC3 cells are isolated from a human prostate cancer bone metastasis with high malignancy [56, 121], and are androgen-insensitive [121]. PC3 cells are a good model to study how bone matrix affect the behavior of bone-metastatic prostate cancer cells [56]. And finally, DU-145 cells are derived from a human prostate cancer brain metastasis and cells are androgen-insensitive [22, 121].

1.7. State of the art

Several studies indicate the influence of endothelial cells (namely in angiogenesis process) in prostate cancer. They indicate that angiogenesis favoring tumor growth [7, 23, 26-29, 31-34].

This protein plays an important role in angiogenesis, including the fact that SPARC inhibits endothelial cell spreading [73, 80] and interacts with growth factors [79, 80]. Their peptides also influence endothelial cells. For example, peptide FS-E inhibit cell proliferation [62, 76, 86] and peptide 2.3 stimulates cell proliferation and angiogenesis [62, 76].

In prostate cancer, SPARC promotes EMT [71], tumor angiogenesis, inhibits immune surveillance [63] and it is a chemoattractant of prostate cancer cells [53, 56, 69, 71].

A recent study of endothelial cells and prostate cancer cells coculture shows that it improves the invasion ability of the prostate cancer cells. This coculture leads to an increase of interleukin-6 secretion by endothelial cells, which may result in downregulation of androgen receptor signaling in prostate cancer cells and consequently to active TGF-ß and MMP-9 signaling. This signaling increases invasion of prostate cancer cells [123].

In sum, there are studies that show the influence of SPARC in endothelial cells and in prostate cancer cells, but there is a lack of studies involving the influence of SPARC or its peptides in bone matrix under endothelial cells and prostate cancer cells.

1.8. Summary

Prostate cancer is the second more deadly type of cancer among men worldwide [12-14]. Prostate cancer occurs when cancer cells grow out of control and become invasive in the prostate [1, 11]. The primary site of metastasis for this cancer is bone, suggesting that bone microenvironment is advantageous to its growth [12, 28, 53]. This microenvironment is rich in structural and matricellular proteins capable of altering prostate cancer progression [53]. Cancer cells grow up 1–2 mm^3 without blood vessels. To grow beyond this value it is necessary to occur angiogenesis (Figure 13) [26-29]. This process begins with the release of angiogenic growth factors by cancer cells or cancer stromal cells [30, 31, 34] activing endothelial cells [7],

which follow several steps leading to the formation of new vessels. This new blood vessels will penetrate into the tumor providing nutrients and oxygen and removing waste products [28, 31].

SPARC and their peptides have been studied because of their influence in endothelial cells [62, 73, 79, 80, 86, 92, 98, 99, 105-108, 124] and also in cancer cells [53, 56, 60-64, 69]. SPARC has several functions that play an important role in angiogenesis, including the fact that SPARC inhibits endothelial cell spreading, disrupts focal adhesions, regulates cell proliferation [73, 80]; enhances the permeability of endothelial monolayers [80]; has interactions with growth factors [79, 80]; regulates the production of integrin and MMPs [80]. Their peptides also influence endothelial cells. For example, peptide 1.1 and peptide 4.2 inhibit cell spreading [62, 98]; peptide 2.1, peptide 4.0, peptide 4.2, and peptide FS-K inhibit cell proliferation [62, 76, 86]; peptide 2.3 stimulates cell proliferation and angiogenesis [62, 76].

The role of SPARC in different cancers is still controversial [60]. In the case of prostate cancer, SPARC is mainly protumorigenic protein [53], however, some studies described SPARC as down-regulater of its proliferation and invasion [56, 67]. SPARC has been shown as a chemotactic factor promoting prostate cancer migration in the direction of bone extracts containing SPARC [53, 56, 69]. Another study also indicates that regulation of EMT by TGF-ß promoting tumor growth involving the protein SPARC [63].

Biomaterials have been used in the study of co-cultures, so biomaterials have physical and biological of natural tissues allowing the study of interactions between different cell types in a particular microenvironment [125]. Through the electrospinning and electrospraying processes [126] are produced nanocomposites [127] due to the application of high potential electric [126]. Thus, it is possible to produce nanohydroxyapatite/collagen biocomposites by simultaneous electrospinning (collagen type I) and electrospraying (nanohydroxyapatite) [128]. This biocomposites mimic bone, allowing to study the influence of SPARC and its peptides in bone matrix. Thus, the main goal of this work is to study the influence of SPARC and its peptides associated to collagen/nanohydroxyapatite biocomposite mimetizing bone matrix, on endothelial and prostate cancer cells.

This dissertation is organized in five chapters, namely chapter 1 – introduction, chapter 2 - materials and methods, chapter 3 - results, chapter 4 – discussion and chapter 5 – conclusions and future work. Finally, we can find a glossary and the several references used in this work.

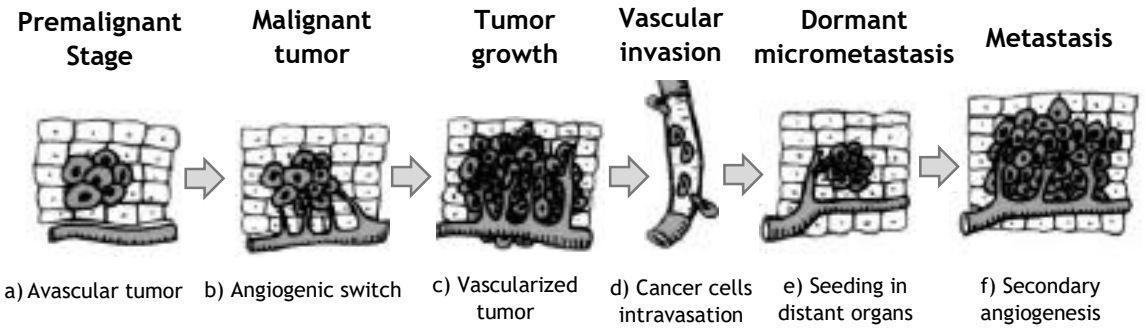


Figure 13 - Cancer angiogenesis in tumor progression. Initially, tumors can grow up to 1-2 mm^3 without blood vessels. To grow above this value angiogenesis must occur. Cancer and stromal cells release factors that activate angiogenic switch allowing the formation of new vessels. This new vessels allow the tumors to receive nutrients and oxygen, thus tumors reach more than 1-2 mm^3. In a further step, cells can enter the blood vessels and spread to distant organs. Finally, angiogenesis takes place and cells grow within the new secondary site [36].

Chapter 2

Materials and Methods

2.1. Electrospinning and electrospraying of collagen/nanoHA biocomposites

Electrospinning and electrospraying are processes that may be used to produce nanocomposites. and more specifically, to the production of polymer nanofibers and aggregates of nanoparticles, respectively [127]. Electrospinning and electrospraying can be used simultaneously using a (rotating) collector and two syringes (each with its respective solution). Those solutions are "collected in a single membrane" [129]. These techniques use a high voltage source, a collector, a syringe pump each, and a capillary or stainless steel needle each [126].

In electrospinning, the polymer solution in the capillary is induced by free charges due to high voltage potential. At the needle tip, the droplet is distorted into a conical shape (known as the Taylor cone) due to following electrostatic forces: electrostatic repulsion of like charges and the Coulombic force of the external electric field. The polymer is expelled from the needle tip towards the collector so electrostatic force counteracts the surface tension. The jet is elongated and the solvent is quickly evaporated, forming randomly oriented fibers on the grounded collector [126]. Electrospraying produces aggregates of nanoparticles due to the electrical forces, the liquid forms a fine elongated capillary, and then it is atomized into droples [127, 129].

Thus, through the techniques of electrospinning and electrospraying it is possible to simultaneously produce a biocomposite of nanohydroxyapatite/collagen (Figure 14). This biocomposite shows a high level of biomimicry with bone matrix. This biocomposite shows a high level of biomimicry with bone matrix. This novel biocomposite "allows cells access to both collagen nanofibers and HA crystals as it happens in natural bone micro-and nano-environments" [128].

The experimental conditions for optimal production of the biomaterial were optimized by Ribeiro *et al*. [128]. There are several conditions that must be taken into account in particular air humidity level (30-45 %), temperature (22 ºC), flow rate (0.1 mL/h for collagen and 2 mL/h for nanohydroxyapatite), electrospinning voltage (20 kV), needle type and size(21

G), needle-to-collector distance (120 mm), collagen solution concentration (12 % (w/v)) and solvent type (acetic acid, ethyl acetate, and water) [128]. Natural polymers are difficult to solubilize in water, so normally an acidic solvents is used to dissolve them for electrospinning. However, a recent study shows that ethyl acetate used with acetic acid in water improves the nanofibers spinnability while decreasing solvent acidity [130].

The nanohydroxyapatite/collagen biocomposites were produced according to reference [128]. Briefly a 12 % (w/v) collagen suspension and a 3.5 % (v/v) nanohydroxyapatite suspension were prepared. For that purpose , type I collagen was suspended in acetic acid, ethyl acetate, and water with a ratio of 40/30/30 % (v/v) respectively, and stirred overnight at 4 ºC. Nanohydroxyapatite was suspended in methanol and stirred during 20 minutes. Then, the solution was centrifuged (at 2500 rpm during 5 minutes) and the supernatant was subjected to a set of ultrasonic cycles (20 × 15 pulses) with an amplitude of 60 A. This set of ultrasonic cycles allows the decrease of nanoparticle agglomeration.

A syringe of 5 mL with a 21 G needle was loaded with collagen and electrospun at 0.1 mL/h, while nanohydroxyapatite was placed into a syringe of 10 mL with a 21 G needle and electrosprayed at 2 mL/h. The needles were placed at a distance of 120 mm to the rotating cylinder collector. The rotating cylinder collector was involved with aluminum foil containing coverglasses (10 mm in diameter) attached to it. The high voltage power supply was set at 20 kV and the cylinder collector was rotting at 400 rpm.

Finally, electrospinning and electrospraying process were performed simultaneously during 1 hour with a relative humidity between 30–45 % [128].

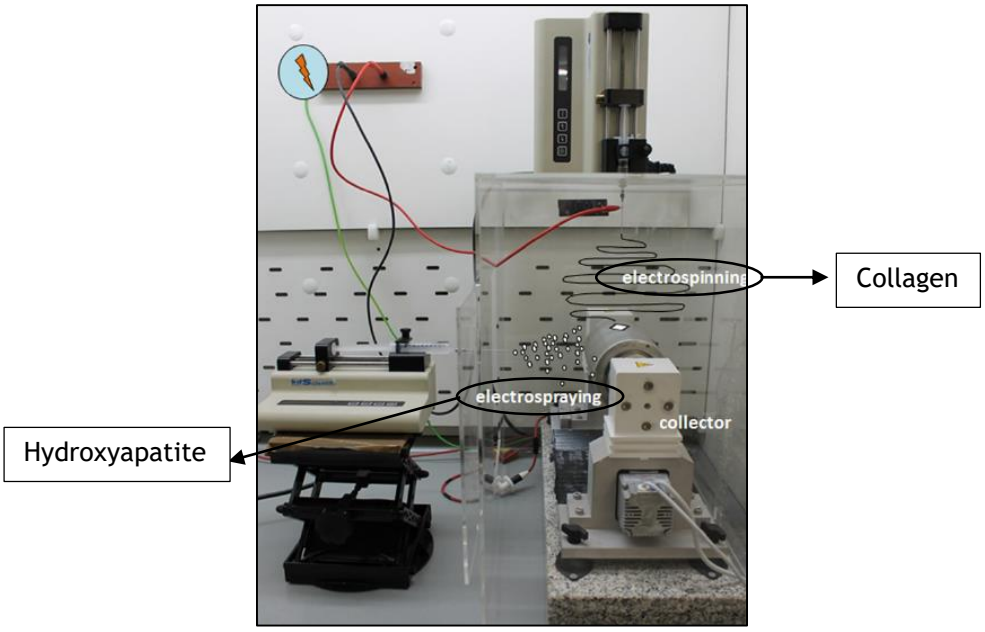


Figure 14 - Simultaneous electrospinning and electrospraying set-up [128].

2.1.1. Biomaterial cross-linking and sterilization

In order to provide mechanical strength to collagen by binding of carboxylic groups between collagen fibrils, samples are cross-linked with a solution of ethanol 90 % (v/v)

containing 20 mM EDC and 10 mM NHS (both from Fluka, BioChemika). This solution was in contact with the samples during 24 hours at 4 °C [128].

After this time period, samples were washed three times with ethanol 90 % (v/v) and twice with Millipore water.

In flow chamber, samples were sterilized with decreasing ethanol concentrations (v/v) (90, 70 and 50 %) for 10 minutes, and then washed with Millipore water and dried overnight.

2.2. *In vitro* cell culture studies

2.2.1. LNCaP cells

LNCaP cells (PCa cell line) were maintained in RPMI medium (Gibco) with 10 % (v/v) fetal bovine serum (Sigma) and 1 % (v/v) of penicillin-streptomycin (Gibco). Cells were cultured in 75 cm^2 plastic culture flasks and they incubated in a humidified incubator at 37° C and 5 % CO_2. Cells were analyzed once a month to detect mycoplasma contamination through PCR.

The cellular seeding density of 4×10^4 cells/mL corresponds to an optimized value for the used experimental conditions. This density was chosen after trying several densities namely 2×10^4,4×10^4, 6×10^4 and 12×10^4 cells/mL.

Biomaterial samples were placed into 24 well plate and biomaterial adsorbed SPARC (Sigma) with a concentration of 10 and 50 µg/mL during 1 hour. After this time, samples were washed three times with PBS solution. For control purposes, biomaterial without SPARC as well as coverglasses coated with poly-D-lysine (PDL) (Sigma) were used. Coverglasses were coated with PDL 0.1 % (v/v) for 30 minutes and afterwards they were washed twice with Millipore water.

To carry out cell seeding, confluents LNCaP were rinsed with PBS solution, then they were detached with 0.5 % (w/v) trypsin (Sigma) for 5 minutes at 37 °C, and later re-suspended in supplemented medium. Cells were counted using a Neubauer chamber (Marienfeld). Then, LNCaP (4×10^4 cells/mL) were seeded onto biomaterial with SPARC and onto control samples. Assays (resazurin and SEM) were performed at the following time points: 1, 7, and 14 days. To resazurin assay biomaterial samples and controls were incubated only with supplemented medium (without cells) and used as background.

As LNCaP cells were influenced by SPARC also it was performed an internalization assay with Quantum Dots (described in section 2.7).

Note that all coverglasses used in these assays were sterilized with ethanol 70 % (v/v) for at least 30 minutes and all biomaterial samples were sterilized as previously mentioned (section 2.1.1).

All assays were done in triplicate.

2.2.2. HPMEC-ST1.6R cells

HPMEC-ST1.6R cells (endothelial cell line) were maintained in medium 199 (Sigma) with 20 % (v/v) fetal bovine serum, 1 % (v/v) of penicillin/streptomycin (Gibco), 2 nM Glutamax-I

(Gibco), 1:1000 Geneticin (Gibco), and 1:1000 ECGS/sodium heparin (Enzifarma). Cells were cultured in 25 cm^2 plastic culture flasks coated with 0.2 % (w/v) gelatin from porcine skin (Sigma) and were incubated in a humidified incubator at 37° C and 5 % CO_2. Cells were analyzed once every month to detect mycoplasma contamination through PCR.

Firstly, it was optimized endothelial cells density in order to choose the density that is best adapted to the biomaterial. Metabolic activity (resazurin assay) was measured to several densities namely 5×10^3, 1×10^4, 2×10^4 and 5×10^4 cells/mL. Assays were performed until the 14th day. However, after 7 days, cells teared biomaterial, and so assays with endothelial cells were performed until 7 days of culture.

To perform the assays, biomaterial samples adsorbed SPARC (Sigma) and SPARC peptides (FS-E, and 2.3) (Alta Bioscience, Abingdon Health) during 1 hour. The SPARC concentrations used were 0.01, 10 and 30 µg/mL, and peptides concentrations were 0.01, 10, 100 and 1000 µg/mL. For control purposes, biomaterial without SPARC or peptides was used. To choose cell density, coverglasses coated with PDL and others with gelatin were used for controls. According to literature [106] proliferation of endothelial cells is inhibited in the presence of SPARC, particularly, Funk *et al*. it is show a high inhibition of proliferation between 10 and 30 µg/mL [106]. Thus, it was studied a lower concentration (0.01 µg/mL) and two higher concentrations (10 and 30 µg/mL) of SPARC pre-adsorbed on biomaterial of collagen/nanoHA for 1 hour. To study the influence of FS-E peptide on endothelial cells different pre-adsorption concentrations on scaffolds were used, 0.01, 10, 100 and 1000 µg/mL. It had as reference point the percentage that each peptide corresponded in SPARC protein. Each peptide corresponds to a concentration of 10 µg/mL compared to the SPARC.

To study the influence of SPARC and SPARC peptides, HPMEC-ST1.6R (1×10^4 cells/mL) were seeded (as previously mentioned) onto biomaterial with SPARC and peptides and onto control samples. 1×10^4 cells/mL density was chosen after trying several densities, as previously mentioned.

Resazurin assay, SEM analysis and calcein AM assay were performed. Resazurin assay and SEM analysis were carried out for periods of 1 day, 3 days, 5 days, and 7 days. Calcein assay was performed at 7th day of co-culture. As previously mentioned, to resazurin assay biomaterial samples and control samples were incubated only with supplemented medium (without cells) and used as background.

These assays were done in triplicate.

2.2.3. HPMEC-ST1.6R and LNCaP cells co-culture

HPMEC-ST1.6R and LNCaP cells were maintained similarly to what has indicated for the monocultures.

Endothelial cells and prostate cancer cells had different media, so it was necessary to optimize the percentages respective media that would be adequate to maintain the cells in coculture. For this optimization was perform resazurin assay until 7 day of co-culture. A mixture of 50 % of HPMEC-ST1.6R supplemented medium with 50 % of LNCaP supplemented medium was chosen, because cells were able to adhere and proliferate on the 3D scaffolds.

To perform the study of SPARC and SPARC peptides influence on endothelial cells and prostate cancer cells co-culture were chosen the following concentrations: 30 µg/mL (SPARC), 1000 µg/mL (peptide FS-E), and 100 µg/mL (peptide 2.3). These concentrations were chosen

according the results obtained with endothelial cells. Concentration of 30 µg/mL to SPARC was chosen because this concentration increase angiogenesis. For peptide FS-E it was chosen the concentration of 1000 µg/mL to know if this concentration also decrease proliferation and angiogenesis of co-culture (and thus may be applied as therapeutic target for cancer). Finally, 100 µg/mL concentration was chosen for peptide 2.3 because this peptide seemed increase both angiogenesis and proliferation of endothelial cells.

Biomaterial samples adsorbed SPARC (30 µg/mL), FS-E peptide (1000 µg/mL) and 2.3 peptide (100 µg/mL) for 1 hour. For control purposes, biomaterial without SPARC or peptides was used.

Firstly, HPMEC-ST1.6R (1×10^4 cells/mL) were seeded as previously mentioned onto biomaterial with SPARC and peptides and onto control samples. After 4 days of culture, LNCaP (4×10^4 cells/mL) were seeded onto the biomaterials previously seeded with HPMEC-ST1.6R.

Resazurin assay and immunostaining were performed for periods of 1, 3 and 5 days after LNCaP seeding. Calcein AM assay was performed at day 5. As previously mentioned, for resazurin assay, biomaterial samples and control samples were incubated only with supplemented medium (without cells) and used as background.

2.3. Resazurin assay

Resazurin assay evaluates cell metabolic activity [131-134]. Viable cells have active metabolism and reduce resazurin (blue and non-fluorescent) into the resorufin (pink and highly fluorescent) (Figure 15) [131-134]. If cells are dead, they have no ability to convert the substrate to product, and thus do not generate a fluorescent signal [131, 134]. The main advantages of this assay are that it is inexpensive; the reactant can be added directly to cells in the homogeneous solution, and it is more sensitive than other metabolic activity assays [132].

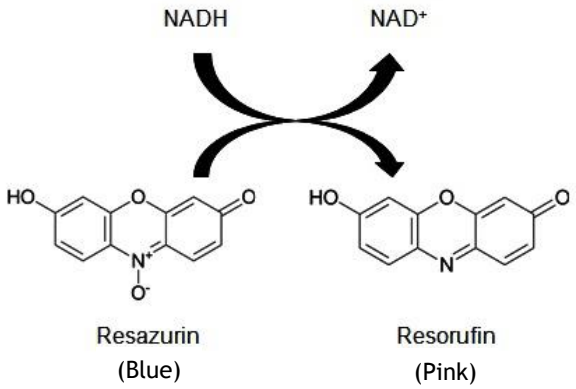


Figure 15 – Resazurin assay. Resazurin is a redox indicator. Viable cells convert resazurin (blue and nonfluorescent) into resorufin (pink and fluorescent) [132].

This assay was performed in the laminar flow hood with the lights off, and 10 % (v/v) resazurin solution was diluted in supplemented medium and added in each well.

The cell culture plates were incubated in a humidified incubator for 3 hours at 37 ºC and 5 % CO_2. Afterwards, 100 µL of supernatant were transferred into a black 96-well plate to analyze the fluorescence, measured with a fluorometer (Biotek, Synergy MX) using wavelengths of 530 nm excitation and 590 nm emission.

The units of the obtained values are RFU and allow to assess the number of viable cells. Figures for samples used as background were subtracted. As previously mentioned, six samples were used for each group, and the average of six samples measurements was considered.

The statistical analysis was assessed using nonparametric one-way ANOVA. Bonferroni´s correction was used in all statistical tests. Groups were considered statistically different when $p < 0.05$. Data were analyzed using GraphPad Prism software.

2.4. Calcein AM assay

After the time points mentioned in sections 2.2.2 and 2.2.3, HPMEC-ST1.6R and co-culture of HPMEC-ST1.6R and LNCaP were washed twice with PBS solution, and then they were stained with Calcein AM with 10 µg/mL. They were incubated for 45 minutes in the dark. After this incubation period samples were analyzed with a fluorescence microscope (Carl Zeiss Microscopy). Calcein AM (acetoxymethyl) assay allows to observe cell viability. Calcein AM (non-fluorescent) is a cell-permeant dye that when in contact with living cells is converted to a green-fluorescent after acetoxymethyl ester hydrolysis by intracellular esterases (Figure 16) [135].

Finally, images were processed using AxioVision LE software.

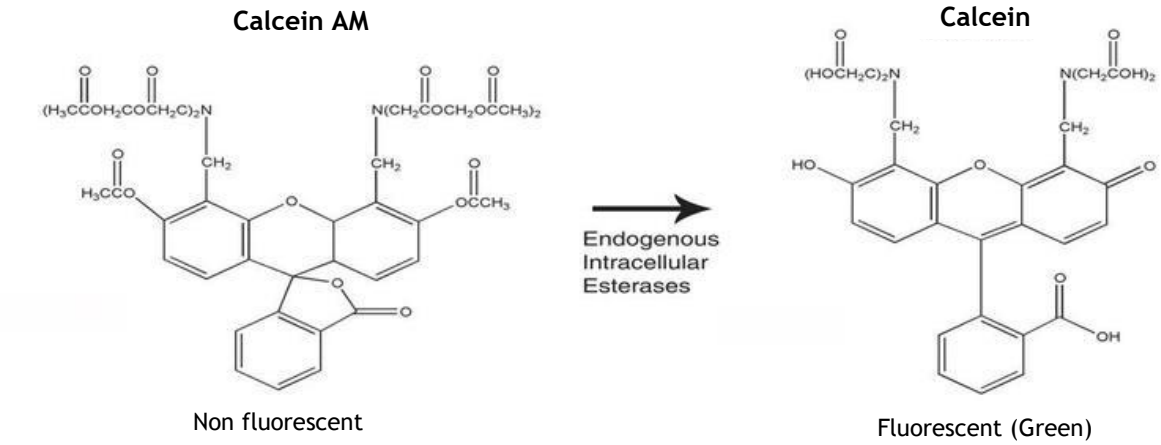


Figure 16 - Calcein AM passes through the membrane of living cells and it is hydrolyzed by endogenous intracellular esterases forming a fluorescent compound (calcein) [136].

2.5. Immunostaining

Fluorescence confocal microscopy was used to analyze morphology and distribution of cells in co-culture. It allows to obtain detailed and sharp images. Fluorochromes are added to samples in order to target and identify subcellular structures (such as the cytoplasm, nuclei, sarcoplasmic reticulum, and mitochondria). Confocal microscope has advantages when compared to other types of microscopes such as obtaining 3D images, the possibility of obtaining sections inside living cells or tissues, and tracking dynamic events (for example, cell migration and blood flow). However, it has some disadvantages such as high costs and limited depth of the sample [137].

In each time-point, cells were rinsed twice with PBS solution and fixed in 4 % (v/v) paraformaldehyde (Sigma) for 20 min. Then, they were permeabilized with 0.1 % (v/v) Triton for 5 min, and then incubated in 1 % BSA (w/v) for 30 minutes at 37 ºC.

Monocultures of LNCaP F-actin were stained with Alexa Fluor® 594 Phalloidin diluted 1:200 in 1 % (v/v) BSA for 20 minutes in the dark. Subsequently cells were rinsed with PBS solution and nuclei were counterstained with DAPI dye (Sigma) during 10 minutes in the dark. F-actin of HPMEC-ST1.6R monocultures and co-cultures were incubated in primary CD31 antibody (Santa Cruz Biotechnology) diluted 1:100 in 1 % (v/v) BSA for 45 minutes. Subsequently cells were incubated with secondary antibody, Alexa Fluor® 488 goat anti-mouse IgG1 (Gibco) diluted 1:400 in 1 % (w/v) BSA during 45 minutes in the dark. Cell nuclei were counterstained in DAPI (Sigma) for 10 minutes. Lastly, samples were rinsed in PBS solution and covered with Vectashield (Vector Laboratories) [138].

Finally, samples were analyzed on a Leica SP5 confocal microscope with 20x oil objective (Leica Microsystems), and images were processed and quantified using Leica Application Suite X software.

2.6. Scanning electron microscopy (SEM)

SEM gives information about surface morphology and chemical composition [139, 140].

At each time-point previously mentioned, cells were fixed in 1.5 % (v/v) glutaraldehyde for 30 minutes and washed twice with PBS solution. Then, they were dehydrated in increasing ethanol concentrations (50, 60, 70, 80, 90, and 99 % (v/v) for 10 minutes in each solution. After this dehydration hexamethyldisilazane was added and samples were dried overnight.

Finally, samples were attached to carbon tape in an aluminium sample holder and coated with palladium–gold film, and were observed by SEM (FEI Quanta 400FEG/EDAX Genesis X4M (Hillsboro)).

2.7. Quantum dots (QD) internalization

Quantum dots (QDs) were kindly provided and prepared by the “Center of Nanoscience, Nanotechnology, and Innovation-CeNano2I, Department of Metallurgical and Materials Engineering, Federal University of Minas Gerais, Brazil”. Briefly, SPARC was bioconjugated with chitosan using EDC as a “zerolength” crosslinking agent in the presence of N-

hydroxysulfosuccinimide sodium salt (sulfo-NHS). Then, CdS nanoparticles were stabilized with chitosan and chitosan-SPARC through an aqueous route in a reaction flask at room temperature, forming QD_Chi and QD_Chi-SPARC (10,92 µg/mL). QDs were dialyzed for 24 hours [141].

LNCaP cells (4x10^4 cell/mL of cell density) were seeded directly in the Tissue Culture Polystyrene (TCPS). They were incubated for 24 hours at 37 °C and 5 % CO_2. Different concentrations of QD_Chi and QD_Ch_SPARC were added to cells, namely 0.05, 0.5, 5, 20 and 50 %, and they were incubated for 1 hour. After this incubation period, resazurin assay and immunostaining were performed as mentioned above. To stain cellular cytoskeletons was used Alexa Fluor® 488 Phalloidin (1:40 diluted in BSA 1 % (w/v)). To detect the fluorescence of the cells QDs were excited with wavelength of 405 nm (laser irradiation).

For the flow cytometry analysis, LNCaP cells were seeded at 50×10^4 cell/mL directly on TCPS. They were incubated for 24 hours at 37 °C and 5 % CO_2. After, LNCaP cells were incubated with different QD_Ch and QD_Ch_SPARC concentrations (0.5, 5, 20 and 50 %) for 1 hour. Then, cells were detached with trypsin, fixed with 4 % para-formaldehyde, and finally suspended in PBS solution for analysis. To perform flow cytometry analysis, a flow cytometer (BD FACSCanto™ II, BD Bioscience) was used equipped with a 405 nm violet laser. “Fluorescence-activated cell sorting (FACS) is a specialized method of flow cytometry that results in separation of different cell types. It is applied to automatic sorting of heterogeneous cell mixtures depending on fluorescence characteristics of individual cells [138]”.

For control purposes, TCPS wells were seeded with cells without QDs.

Chapter 3

Results

3.1. Collagen/nanoHA biocomposites

Collagen/nanoHA biocomposites were analyzed by SEM and the morphology of collagen nanofibers and nanohydroxyapatite aggregates may be observed in Figure 17. Scaffolds were characterized by Ribeiro *et al*. [128] and nanofibers have diameters between 10 and 100 nm while the nanohydroxyapatite aggregates have dimensions of 126 ± 2 nm.

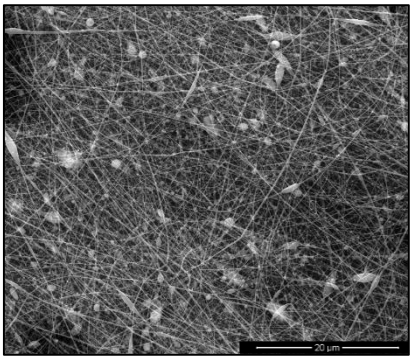


Figure 17 - SEM image of collagen/nanoHA biocomposites produced by simultaneous electrospinning and electrospraying showing collagen nanofibers and nanohydroxyapatite aggregates. Scale bar = 20 µm.

3.2. *In vitro* cell culture studies

3.2.1. LNCaP cells

These results were obtained in collaboration with Nilza Ribeiro, and they are included in a submitted article [142].

Resazurin assay suggested that SPARC pre-adsorption at 10 and 50 µg/mL, increased the metabolic activity of LNCaP cells when compared with control (collagen/nanoHA biocomposites without SPARC), especially for the later culture time points mainly 7 and 14 days (Figure 18 (1)). In addition, SPARC pre-adsorption onto collagen/nanoHA biomaterial with the highest concentration (50 µg/mL) showed the highest metabolic activity for all time points.
Cell morphology was observed by SEM (Figure 18 (2)) and after 1 day, cells with and without SPARC were elongated and spread over the scaffolds (Figure 18 A and C). Still, after 7 days, all cells were elongated and well spread on both substrates covering most of the surfaces (Figure 18 B and D). In addition, cells formed aggregates, mainly for the later culture time points (7 and 14 days).

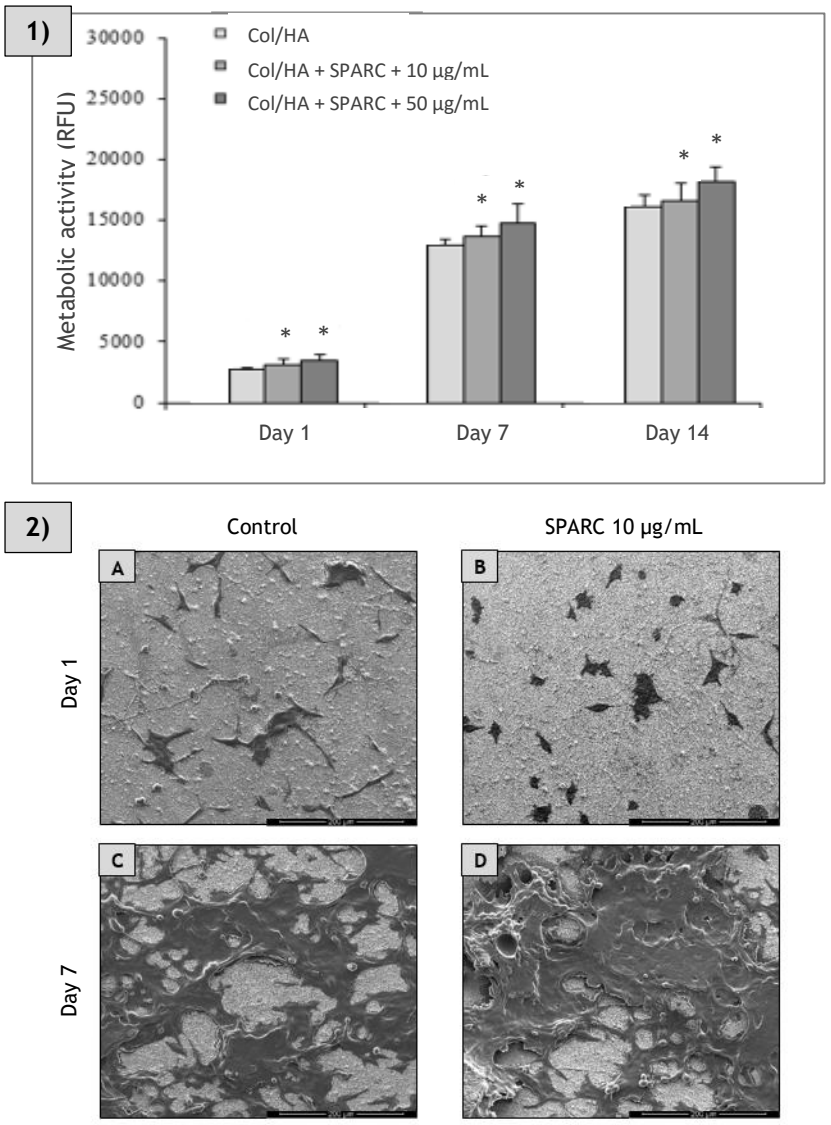


Figure 18 – 1) Metabolic activity of LNCaP cells cultured on the collagen/nanoHA composites with and without protein adsorption SPARC (10 and 50 µg/mL) pre-adsorption versus time. Assays were performed at 1, 7 and 14 days of culture. The results are expressed as relative fluorescence units (RFU). HPMEC-ST1.6R LNCaP cells cultured on collagen/nanoHA biocomposites were used as control. Values are the average ± SD of six cultures. *Indicates a statistically significant difference with respect to the control cultures ($p<0.05$). 2) SEM images of LNCaP cells morphology on collagen/nanoHA biocomposites without (control) and with and SPARC (10 µg/mL) after 1 and 7 days of culture. Scale bar = 200 µm.

Quantum dots directly functionalized with chitosan-SPARC bioconjugates were used to assess the SPARC internalization ability of LNCaP cells using 0.5, 5, 20, and 50 % of QD's. The Metabolic activity results have shown that cell viability significantly decreased for the highest concentration of QD_CHI_SPARC (50 %).

Flow cytometry (Figure 19) indicated an uptake of QD_CHI_SPARC by LNCaP cells for concentrations between 5 and 50 %. The maximum uptake occurred for 50 % of QD_CHI_SPARC (5.5 µg/mL of SPARC). In other words, flow cytometry showed that 50 % of QD_CHI_SPARC had a fluorescent positive population approximately 40 times more fluorescent on average than control. For 0.5 %, QD_CHI_SPARC were not internalized by LNCaP cells (data not shown). Confocal laser scanning microscopy (CLSM) images proved that SPARC was internalized by LNCaP cells (Figure 20).

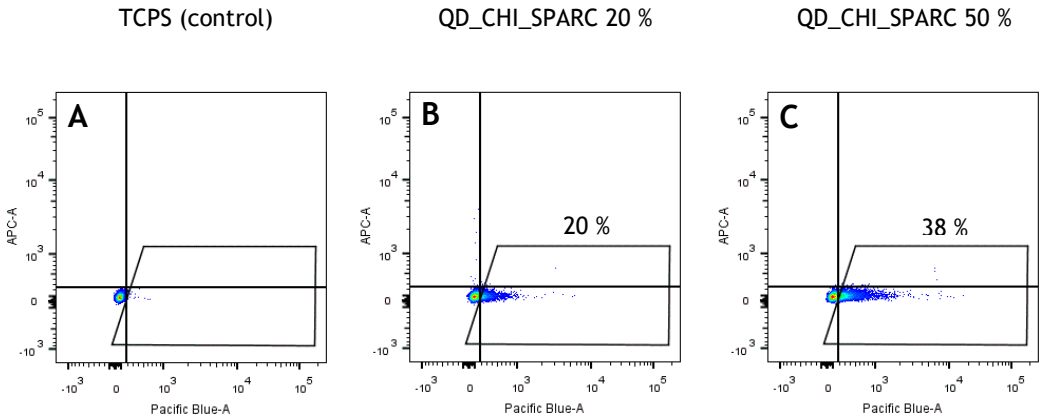


Figure 19 - Internalization of the bioconjugate QD_Ch_SPARC. LNCaP cells were incubated with QD_Ch_SPARC (0, 20 and 50 %, A, B and C) respectively) for 1 hour and internalization was assessed by flow cytometry The representative graphs of the quantitative flow cytometric analyses (QD positive - Pacific Blue and PCA-A channels) are shown.

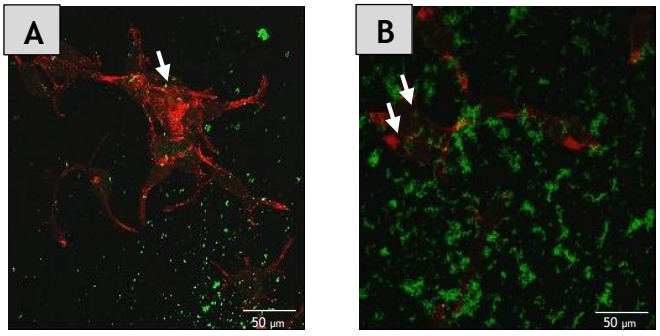


Figure 20 - Internalization of the bioconjugate QD_Ch_SPARC (20 and 50 %) by LNCaP cells after 1 hour incubation assessed by laser scanning confocal microscopy. (A) Representative image of LNCaP cells incubated with QD_Ch_SPARC 20 % and (B) with QD_Ch_SPARC 50 %, shown as the orthogonal projection of a Z-stack (of a total of 6 images). White arrows show internalized QD_Ch_SPARC. Cell cytoskeleton is shown in red (λexc: 488 nm) and QD_Ch_SPARC is shown in green (λexc: 405 nm). Scale bar: 50 µm.

3.2.2. HPMEC-ST1.6R cells

Figure 21 shows the metabolic activity of HPMEC-ST1.6R cells cultured on collagen/nanoHA scaffolds with different cell densities (5×10^3, 1×10^4, 2×10^4 and 5×10^4 cells/mL). HPMEC-ST1.6R cells were able to adhere and proliferate on 3D scaffolds, however, cells had tendency to tear the biomaterial surface, mainly at day 7 of culture. Figure 22 shows a SEM image of endothelial cells on day 7 of culture tearing the biocomposite of collagen/nanoHA.

Comparing with controls (PDL and gelatin), endothelial cells with density of 5×10^4 cells/mL cultured on collagen/nanoHA scaffolds, had a poorly proliferation. Density chosen was 1×10^4 cells/mL because cells adhered and proliferated well on collagen/nanoHA scaffolds over time. In addition, the next step is perform a crosstalk between endothelial cells and prostate cancer cells, and so a higher density (2×10^4 or 5×10^4 cells/mL) was not chosen, because a greater number of cells would tear more quickly collagen/nanoHA scaffolds.

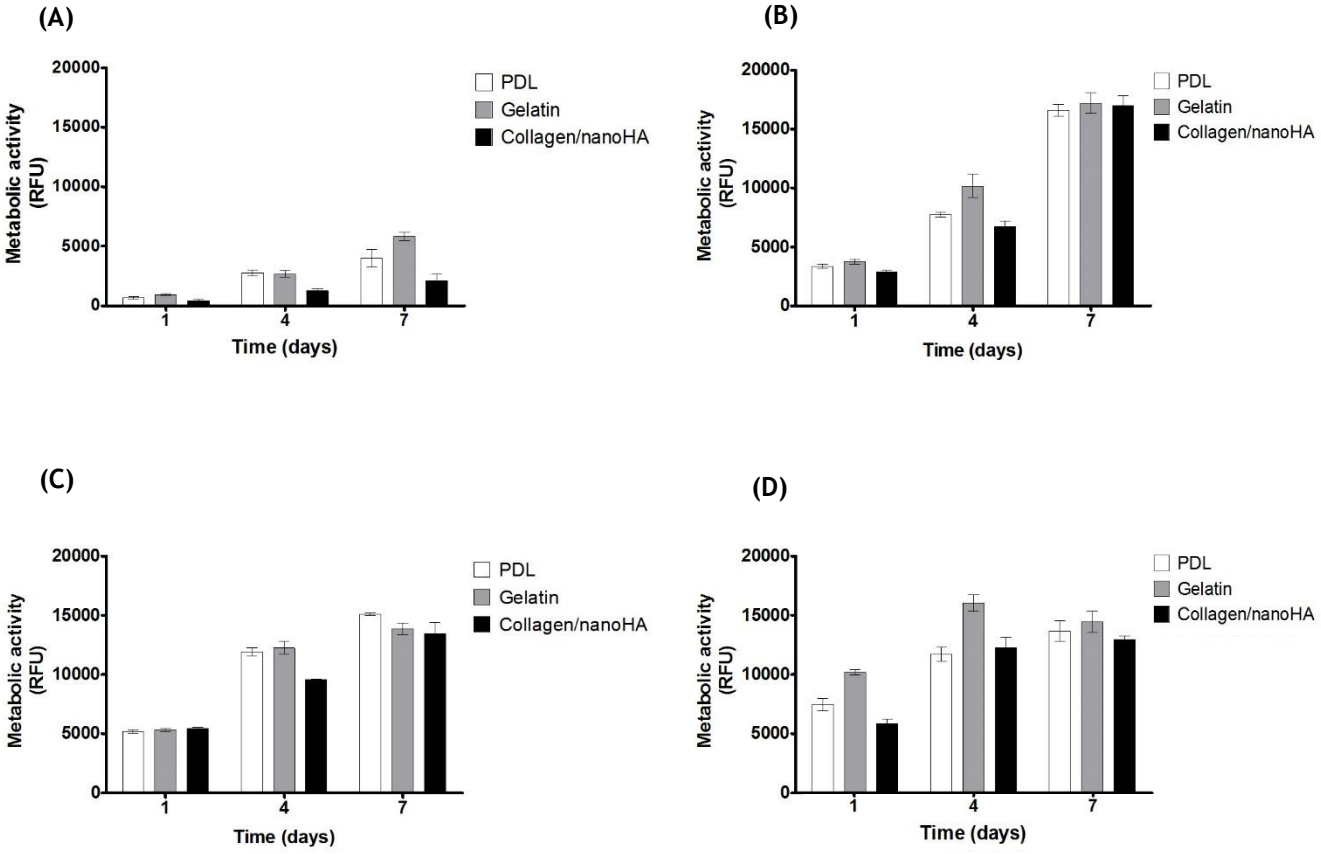


Figure 21 – Metabolic activity of HPMEC-ST1.6R cells with different cell densities. The cellular densities analyzed were 5×10^3, 1×10^4, 2×10^4 and 5×10^4 cells/mL (A, B, C and D respectively). Assays were performed after 1, 4, and 7 days of culture. Values reported are the mean of six samples and they are expressed in terms of relative fluorescence units (RFU). PDL and gelatin were used as controls.

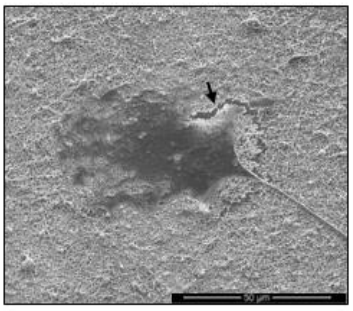


Figure 22 – SEM image of endothelial cells on day 7 of culture tearing the biocomposite of collagen/nanoHA. The black arrow shows the biomaterial tear. Scale bar = 50 μM.

3.2.2.1. Influence of pre-adsorbed SPARC on HPMEC-ST1.6R cells

The ability of SPARC pre-adsorption to mediate endothelial cells adhesion to collagen/nanoHA biocomposite was investigated using several SPARC concentrations namely, 0.01, 10 and 30 μg/mL (Figure 23). SPARC concentration had no effect on the metabolic activity up to 5 days of culture. HPMEC-ST1.6R cell metabolic activity decreased for the higher SPARC concentrations at 7 day of culture, when compared to control ($P<0.001$).

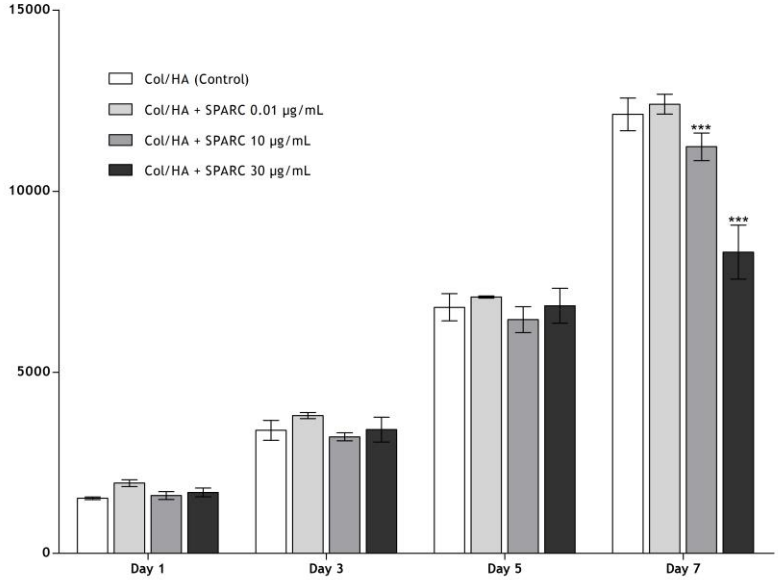


Figure 23 – Metabolic activity of HPMEC-ST1.6R cells cultured on the collagen/nanoHA composites with and without protein adsorption SPARC (0.01, 10 and 30 μg/mL) pre-adsorption versus time. Assays were performed at 1, 3, 5 and 7 days of culture. The results are expressed as relative fluorescence units (RFU). HPMEC-ST1.6R cells cultured on collagen/nanoHA biocomposites were used as control. Values are the average ± SD of six cultures. ***Indicates a statistically significant difference with respect to the control cultures ($p<0.001$).

Cell viability of HPMEC-ST1.6R cells growing on biomaterials surfaces was assessed by calcein AM assay (Figure 24). As the concentration of adsorbed SPARC increased, the ability to form the typical capillary-like structures was promoted. Cells growing on control surfaces showed no statistical significant difference when compared to the ones pre-adsorbed with 0.01 μg/mL of SPARC. However, SPARC at 10 and 30 μg/mL increased the ability to establish the

typical capillary-like structures (Figure 24 C and D). On the control (Figure 24 A) cells spread over the biomaterial, covering almost all of the surface, but only one tube formation was observed. Regarding scaffolds pre-adsorbed with SPARC, 0.01 µg/mL of SPARC concentration (Figure 24 B) a similar behavior was observed.

No morphological differences were observed between control and endothelial cells in the presence of SPARC (Figure 25). Cells with and without SPARC were elongated and spread on biomaterials at day 3 and day 7 of culture, with typical capillary-like structures (Figure 25 D, D' and D'').

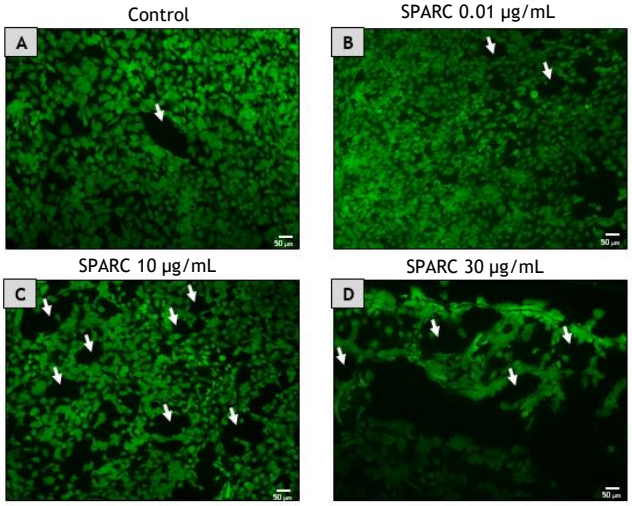


Figure 24 – Cell viability of HPMEC-ST1.6R cells cultured on the (A) collagen/nanoHA composites without protein adsorption and (B-D) with SPARC pre-adsorbed (0.01, 10 and 30 µg/mL, respectively), after 7 days of culture. Samples were analyzed by fluorescence microscopy using Calcein AM assay and cell cytoskeleton is indicated in green (calcein). HPMEC-ST1.6R cells cultured on collagen/nanoHA biocomposites were used as control. White arrows show tube formation (angiogenesis). Scale bar: 50 µm.

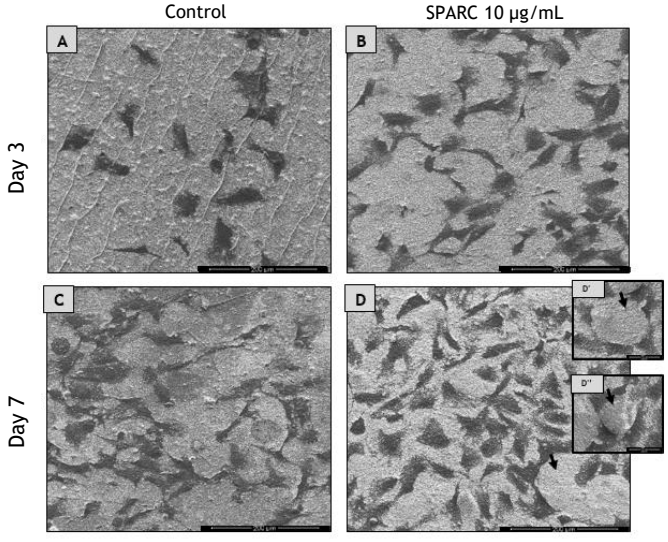


Figure 25 – SEM images of HPMEC-ST1.6R cells morphology on collagen/nanoHA composite without (A and C) and with pre-adsorbed SPARC (10 µg/mL, (B and D)) at day 3 and day 7 of culture. No morphological difference were observed. Black arrows show tube formation at 7th day of culture with 10 µg/mL SPARC concentration (D, D'and D''). Scale bar = 200 µm (A, B, C and D). Scale bar = 50 µm (D' and D'').

3.2.2.2. Influence of peptide FS-E on HPMEC-ST1.6R

The metabolic activity of cells cultured on collagen/nanoHA with pre-adsorbed peptide FS-E decreased for all the used concentrations (0.01, 10, 100 and 1000 µg/mL) at day 5 and day 7 of culture when compared to control (collagen/nanoHA biocomposite without peptide). For the 1000 µg/mL peptide FS-E concentration a marked decrease of endothelial cells metabolic activity was observed, at days 5 and 7 of culture (Figure 26).

HPMEC-ST1.6R cell viability was assessed by calcein AM assay (Figure 27) at culture day 7. Cells spread on the scaffolds 3D (without peptide FS-E) covering almost the entire surface (Figure 27 A). On the scaffolds 3D pre-adsorbed with peptide FS-E (0.01, 10 and 100 µg/mL) cells spread a little less (Figure 27 B, C and D, respectively) comparing with scaffolds 3D without peptide FS-E. 1000 µg/mL peptide FS-E concentration pre-absorbed on the scaffolds 3D were very few cells present (Figure 27 E). In addition, with this peptide no typical capillary-like structures were observed (Figure 27 B, C, D and E) contrasting with scaffolds without peptide F-SE (Figure 27 A) where cells spread on the biomaterial covering almost the entire surface.

Cell morphology on scaffolds after 3 and 7 days of culture was assessed by SEM. There were no differences in endothelial cells between control and collagen/nanoHA biocomposites pre-adsorbed with peptide FS- E (Figure 28). Cells with and without peptide FS-E were similarly elongated and spread over the biomaterial after 3 and 7 days of culture.

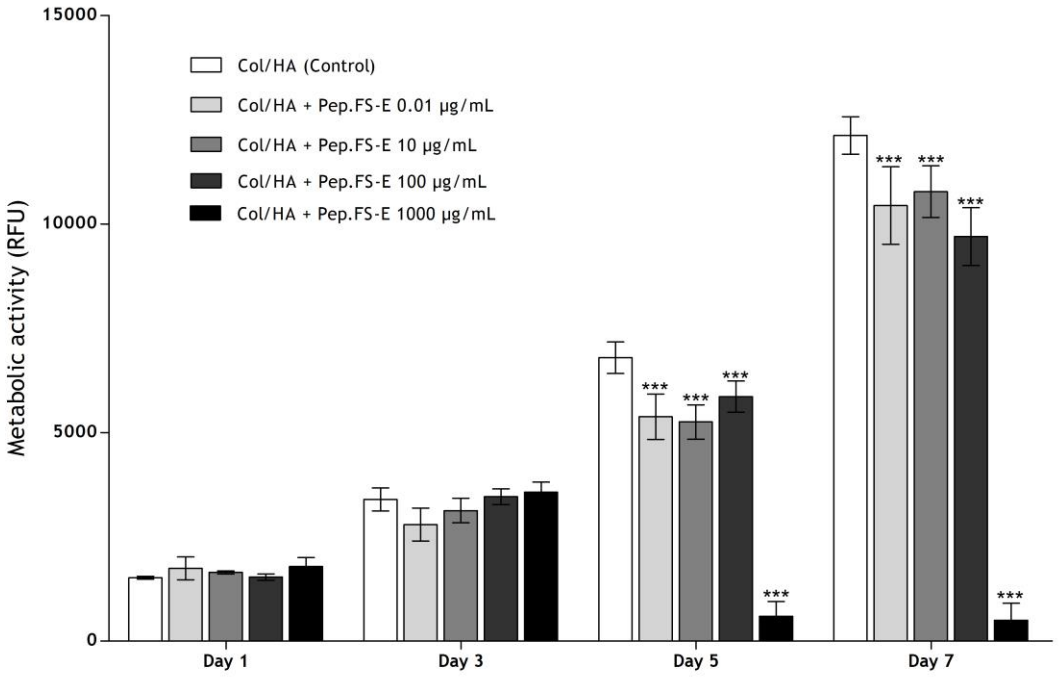


Figure 26 - Metabolic activity of HPMEC-ST1.6R cells cultured on the collagen/nanoHA composites with and without adsorption peptide FS-E (0.01, 10, 100 and 1000 µg/mL) pre-adsorption versus time. Assays were performed after 1, 3, 5 and 7 days of culture. The results are expressed in terms of relative fluorescence units (RFU). HPMEC-ST1.6R cells cultured on collagen/nanoHA biocomposites were used as control. Values are the average ± SD of six cultures. ***Indicates a statistically significant difference from the control cultures ($p<0.001$).

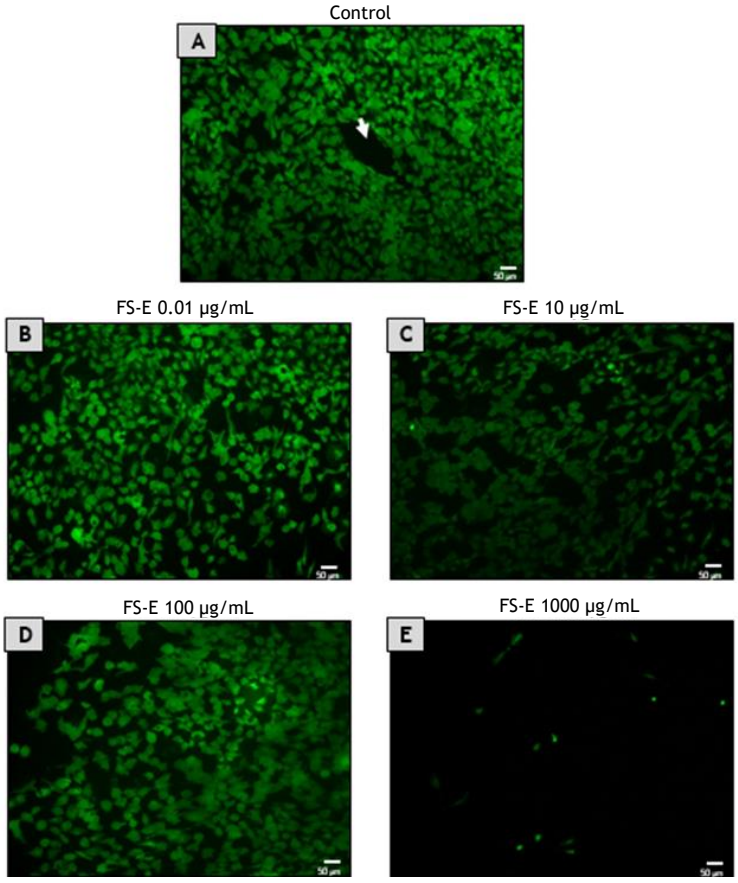


Figure 27 – Cellular viability of HPMEC-ST1.6R cells cultured on the (A) collagen/nanoHA composites without peptide FS-E and (B-E) with peptide pre-adsorbed (0.01, 10, 100 and 1000 µg/mL, respectively) after 7 days of culture. Samples were analyzed on a fluorescence microscope using calcein AM assay. Cell cytoskeleton is colored in green (calcein). HPMEC-ST1.6R cells cultured on collagen/nanoHA biocomposites were used as control. White arrow shows tube formation. Scale bar: 50 µm.

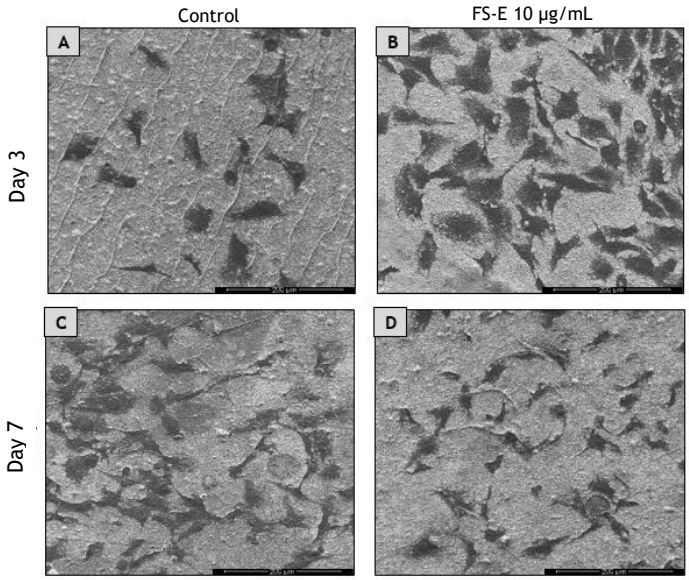


Figure 28 - SEM images of HPMEC-ST1.6R cells morphology on collagen/nanoHA composite (A, C) without (B, D) and with pre-adsorbed peptide FS-E (10 µg/mL) at day 3 and day 7 of culture. No morphological difference were observed. Scale bar = 200 µm.

3.2.2.3. Influence of pre-adsorbed peptide 2.3 on HPMEC-ST1.6R cells

To study influence of peptide 2.3 pre-adsorbed on scaffolds 3D on endothelial cells, the concentrations used were 0.01, 10, 100, and 1000 µg/mL, as previously mentioned in section (2.2.2).

For peptide concentration of 10 µg/mL a decrease in metabolic activity on day 5 and day 7 was observed with respect to control (Figure 29). Lower cell viability was also observed for the concentration of 0.01 and 10 µg/mL (Figure 30 B and C) at day 7, however typical capillary-like structures were still observed. For the peptide concentration of 100 µg/mL the highest cell viability was observed showing also vessel formation (Figure 30 D). Control scaffolds (Figure 30 A) showed cells spreading on the biomaterial, covering almost the entire surface. Concerning the scaffolds pre-adsorbed with 0.01 and 10 µg/mL of peptide 2.3 (Figure 30 B and C) cells were organized in order to generate capillary networks. More cells could be observed on scaffolds pre-adsorbed with 100 µg/mL of peptide FS-E (Figure 30) and for that reason almost the entire surface was covered with cells.

Cell morphology on scaffolds at days 3 and 7 was assessed by SEM. There were no differences in endothelial cells morphology between control (Figure 31 A and C) and collagen/nanoHA biocomposites pre-adsorbed with peptide 2.3 (Figure 31 B and D). It was also possible to observe endothelial cell organization forming vessels on collagen/nanoHA biocomposites with pre-adsorption of peptide 2.3 (Figure 31 C and D) by SEM.

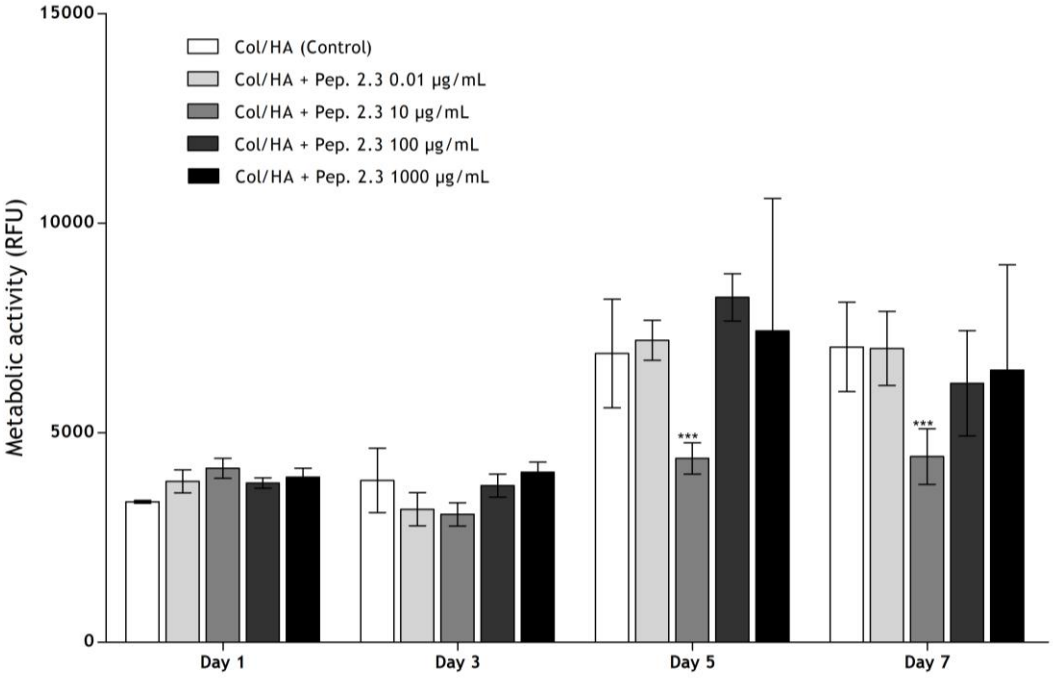


Figure 29 - Metabolic activity of HPMEC-ST1.6R cells cultured on the collagen/nanoHA composites with and without adsorption peptide 2.3 (0.01, 10, 100 and 1000 µg/mL) pre-adsorption versus time. Assays were performed at 1, 3, 5 and 7 days of culture. The results are expressed in terms of relative fluorescence units (RFU). HPMEC-ST1.6R cells cultured on collagen/nanoHA biocomposites were used as control. Values are the average ± SD of six cultures. *** Indicates a statistically significant difference from the control cultures ($p<0.001$).

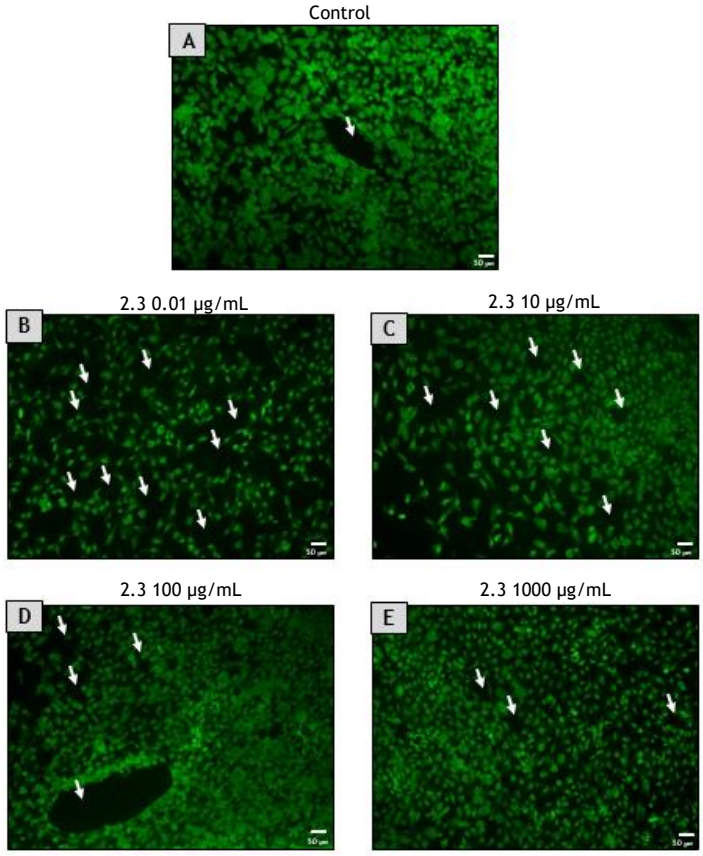


Figure 30 - Cell viability of HPMEC-ST1.6R cultured on (A) collagen/nanoHA composites without peptide 2.3 pre-adsorption and (B-E) with peptide 2.3 pre-adsorbed (0.01, 10, 100 and 1000 µg/mL, respectively), after 7 days of culture. Samples were analyzed by fluorescence microscopy using Calcein AM assay, where cell cytoskeleton was presented in green (calcein). HPMEC-ST1.6R cells cultured on collagen/nanoHA biocomposites were used as control. White arrows show tube formation (angiogenesis). Scale bar: 50 µm.

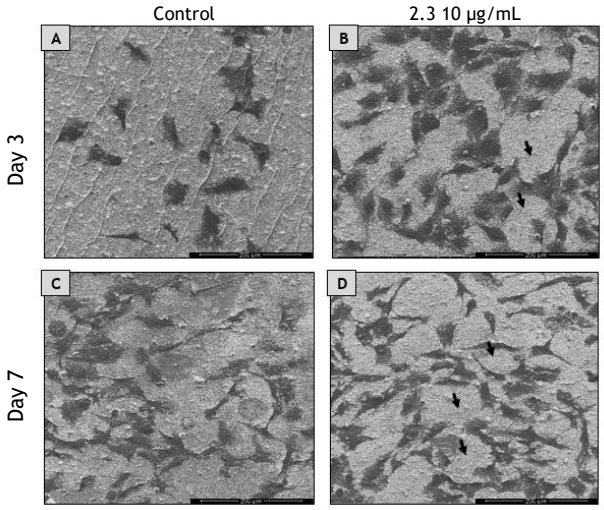


Figure 31 - SEM images of HPMEC-ST1.6R cells morphology on collagen/nanoHA composite (A, C) without (B, D) and with pre-adsorbed peptide 2.3 (10 µg/mL) at day 3 and day 7 of culture. No morphological difference were observed. Black arrows show tube formation at 3rd and 7th day of culture with peptide 2.3 concentration (B, D). Scale bar = 200 µm.

3.2.3. HPMEC-ST1.6R and LNCaP cells co-culture

During this work only preliminary results of HPMEC-ST1.6R and LNCaP co-culture were obtained. The metabolic activity of cells (HPMEC-ST1.6R and LNCaP) cultured on collagen/nanoHA with pre-adsorbed SPARC (30 µg/mL) increased at day 3. The metabolic activity of crosstalk between HPMEC-ST1.6R and LNCaP cells with pre-adsorbed peptide FS-E (1000 µg/mL) decreasing at day 1 and 3 of co-culture. In relation to peptide 2.3 (100 µg/mL), metabolic activity decreasing at day 1 (Figure 32). Metabolic activity was compared with control (cells cultured on collagen/nanoHA without SPARC or SPARC peptides).

Cell distribution was analyzed by CLSM assay after 1 and 3 days of co-culture. Compared to control, both on day 1 and day 3, SPARC presence increased cellular proliferation, while peptide FS-E decreased cellular proliferation. Concerning the scaffolds pre-adsorbed with peptide 2.3, this peptide did not show significant differences comparing with control. In addition, collagen/nanoHA biocomposite with pre-adsorbed SPARC presented a clump of cells, mainly at day 7.

Cell viability of co-cultured HPMEC-ST1.6R and LNCaP cells growing on the biomaterials surfaces was assessed by calcein AM assay on day 5 of co-culture (Figure 34). The images show the same results obtained on day 1 and 3 of the CLSM assay.

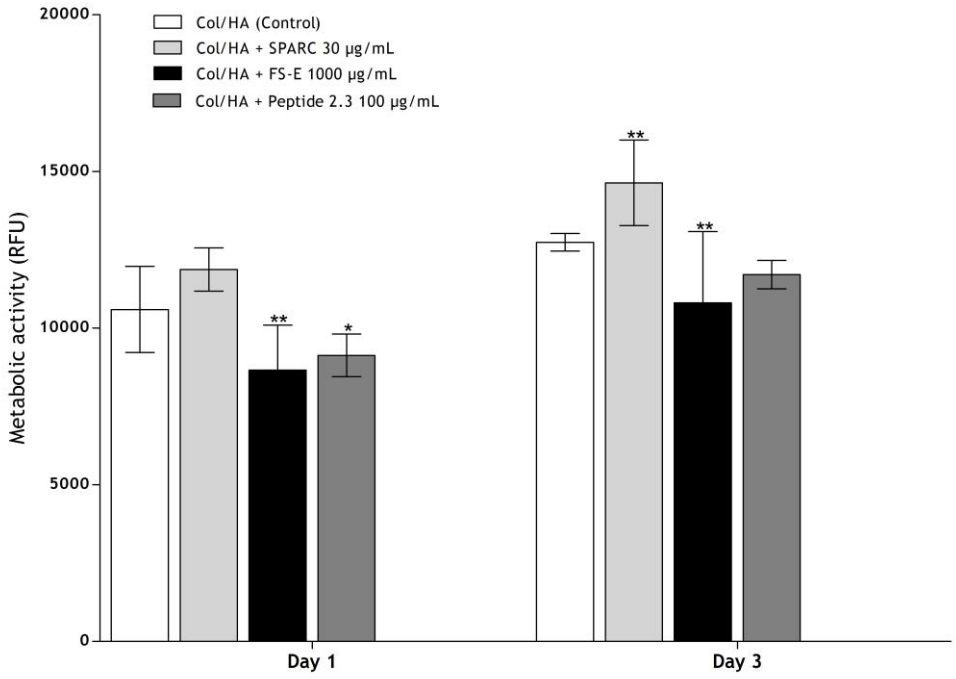


Figure 32 – Metabolic activity of co-culture of HPMEC-ST1.6R cells and LNCaP cells cultured on the collagen/nanoHA composites without SPARC or SPARC peptides pre-adsorption and with SPARC (30 µg/mL), peptide FS-E (1000 µg/mL) and peptide 2.3 (100 µg/mL) pre-adsorption versus time. Assays were performed at 1, 3, and 5 days of culture. The results are expressed in terms of relative fluorescence units (RFU). HPMEC-ST1.6R cells and LNCaP cells cultured on collagen/nanoHA biocomposites were used as control. Values are the average ± SD of six cultures. *, ** Indicates a statistically significant difference from the control cultures ($p<0.05$, $p<0.01$, respectively).

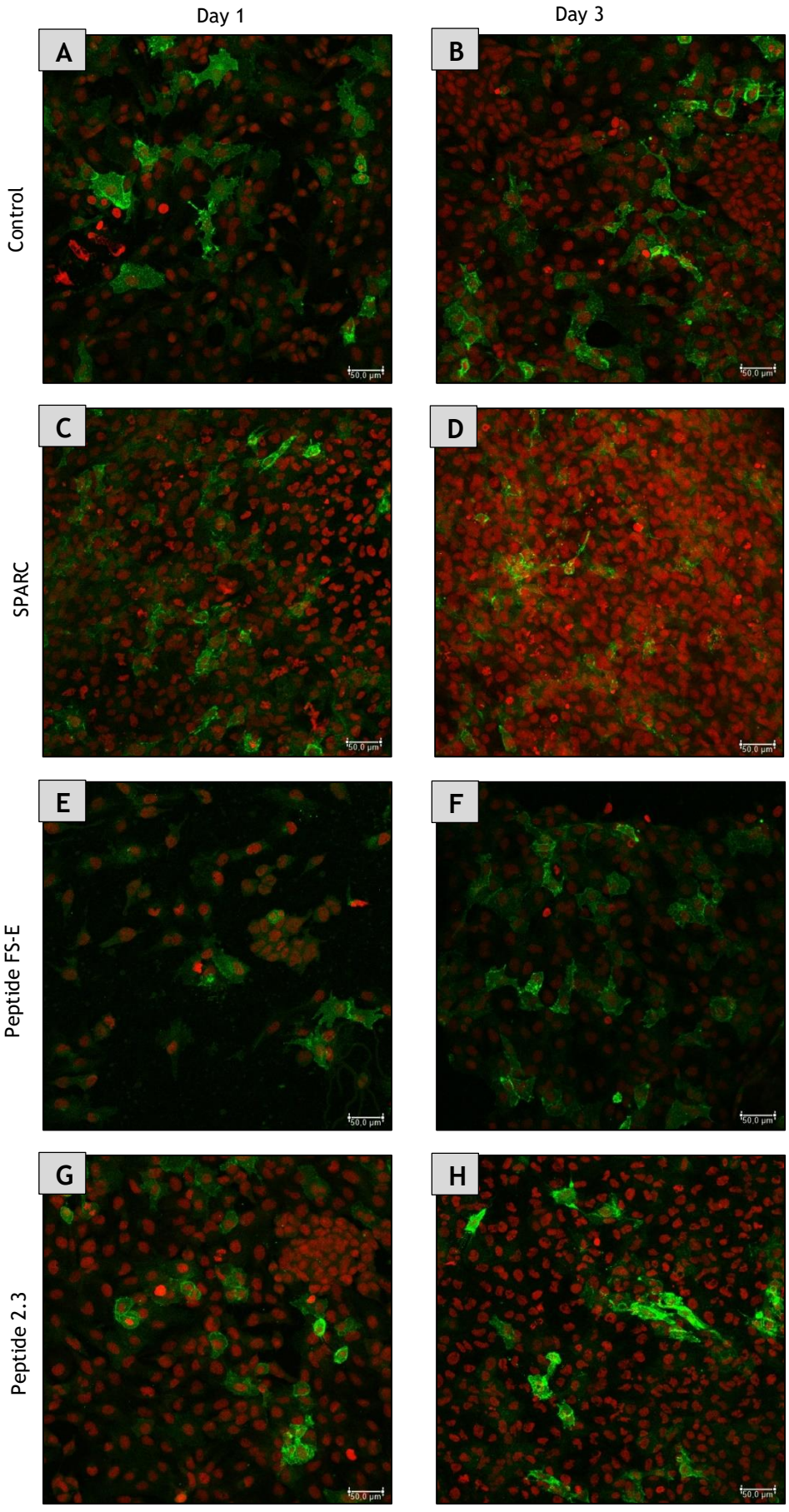


Figure 33 – Confocal laser scanning microscopy (CLSM) images of co-cultured HPMEC-ST1.6R and LNCaP cells on the (A, B) collagen/nanoHA composites without SPARC or SPARC peptides pre-adsorption and (C, D) with SPARC (30 µg/mL), (E, F) peptide FS-E (1000 µg/mL), and (G, H) peptide 2.3 (100 µg/mL), after 1 and 3 days of co-culture. Cell nuclei were stained with propidium iodide (red) while F-actin were stained were stained for CD31 (green). Scale bar = 50 µm.

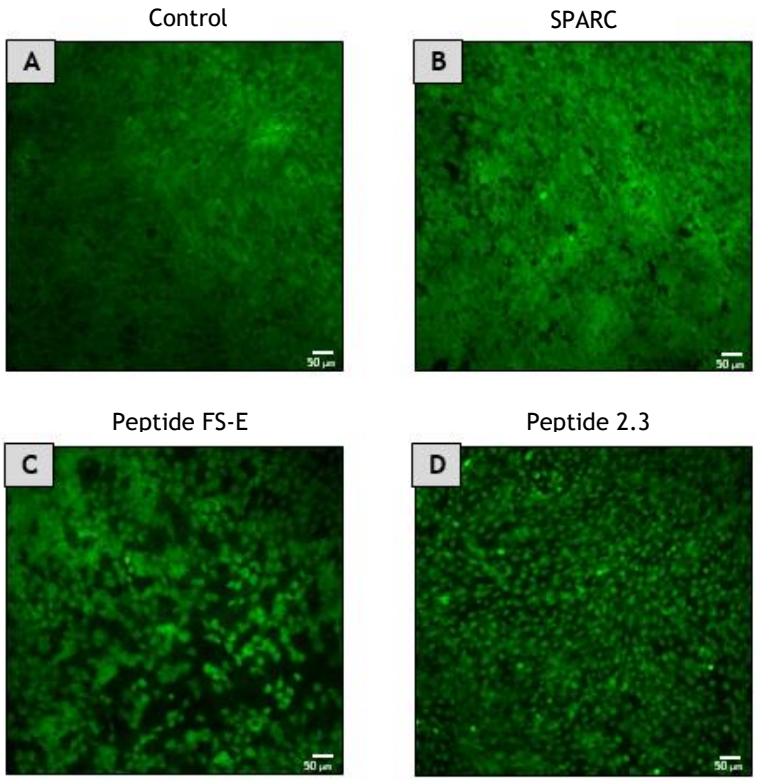


Figure 34 - Cellular viability of co-cultured HPMEC-ST1.6R and LNCaP cells on the (A) collagen/nanoHA composites without SPARC or SPARC peptides pre-adsorption and (B) with SPARC (30 µg/mL), (C) peptide FS-E (1000 µg/mL), and (D) peptide 2.3 pre-adsorbed (100 µg/mL), after 5 days of co-culture. Samples were analyzed on fluorescence microscope using Calcein AM assay and cell cytoskeleton is indicated in green (calcein). Co-culture of HPMEC-ST1.6R and LNCaP cells cultured on collagen/nanoHA biocomposites were used as control. Scale bar = 50 µm.

3.3. Influence of SPARC and SPARC peptides on LNCaP and HPMEC-ST1.6R cells

The summary of the results concerning the influence of SPARC and SPARC peptides (peptide FS-E and 2.3) pre-adsorbed onto collagen/nanoHA composites on LNCaP cells and HPMEC-ST1.6R cells is presented in Table 6 .

Table 6 – Influence of SPARC and SPARC peptides (peptide FS-E and 2.3) pre-adsorbed on collagen/nanoHA composites on LNCaP cells and HPMEC-ST1.6R cells.

	SPARC	Peptide FS-E	Peptide 2.3
LNCaP cells	Increase metabolic activity (10 and 50 µg/mL) in bone-like environment.	-	-
HPMEC-ST1.6R cells	Decrease metabolic activity (10 and 30 µg/mL) in bone-like environment. Stimulation of angiogenesis (10 and 30 µg/mL) in bone-like environment.	Decrease metabolic activity (0.01, 10, 100 and 1000 µg/mL) in bone-like environment. Does not stimulate angiogenesis (0.01, 10, 100 and 1000 µg/mL) in bone-like environment.	Decrease proliferation (0.01 and 10 µg/mL) in bone-like environment. Stimulation of angiogenesis (0.01 and 10 µg/mL) in bone-like environment. Appears to increase metabolic activity and angiogenesis (100 µg/mL) in bone-like environment.
HPMEC-ST1.6R and LNCaP cells co-culture	Increase metabolic activity (30 µg/mL) in bone-like environment.	Decrease metabolic activity (1000 µg/mL) in bone-like environment. Does not stimulate angiogenesis in bone-like environment.	Appears not influence (100 µg/mL) in bone-like environment.

Chapter 4

Discussion

Prostate cancer is the second type of cancer causing more deaths among men worldwide [12-14]. It occurs when cancer cells grow out of control in the prostate and become invasive [1, 11]. Cancer cells grow up 1–2 mm^3 without blood vessels. To grow up beyond this value angiogenesis must occurs [26-29]. When a tumor need nutrients and oxygen, angiogenic factors activate the endothelial cells, and proliferate, stabilize and develop new blood vessels. If these are non-existent, tumors will become necrotic or become apoptotic. Therefore, angiogenesis is an important factor in the progression of cancer [26]. In addition, the influence of SPARC protein has been studied on endothelial cells [62, 73, 79, 80, 86, 92, 98, 99, 105-108, 124], and it is believed that this protein plays an important role in angiogenesis [62, 73, 76, 79, 80, 86], and on cancer cells [53, 56, 60-64, 69]. Moreover, bone is the first choice as a site for metastasis of this cancer, suggesting that bone microenvironment is favorable for its growth [12, 28, 53]. The main aim of this work was to study the influence of SPARC and SPARC derived peptides FS-E and 2.3 (inhibitor and stimulator of angiogenesis, respectively) on endothelial cells and on prostate cancer cells in a simulated microenvironment. For that purpose, scaffolds of collagen/nanohydroxyapatite were produced to mimic bone matrix following a previous work by Ribeiro *et al*. [128]. Biomaterial samples were produced by simultaneously electrospinning collagen and electrospraying nanohydroxyapatite aggregate and the morphology of collagen fibers and nanohydroxyapatite agglomerates were characterized by SEM. Collagen nanofibers had mean diameters between 10 and 100 nm and agglomerates width of nanoHA was 126 ± 2 nm. These diameter values are significantly lower than those reported in the literature [143, 144], which exceed 200 nm. According study of Hartman *et al*. [143] average diameter of collagen fibers had of 319 nm [143], other study of Choi *et al*. [144] collagen fibers had diameters ranging from 275 ± 102 and 334 ± 125 nm depending rotation rate [144]. In addition, natural bone ECM exhibits collagen fibrils with diameters ranging from 20 nm to 40 μm [128], thus mean diameters of collagen fibers obtained (37.2 ± 23.2 nm) are according values of natural bone ECM .

Initially, the influence of SPARC pre-adsorbed on scaffolds on LNCaP cells was studied. LNCaP cells are a prostate cancer cell line which are isolated from a human prostate cancer lymph node metastasis (is a non-bone metastatic prostate cancer cells) [121, 122] and cells are androgen-sensitive [121]. Afterwards, the influence of SPARC and SPARC peptides pre-adsorbed on scaffolds on LNCaP cells was studied. HPMEC-ST1.6R is a cell line of human pulmonary

microvascular endothelial cells used to study pathological mechanisms and angiogenesis and it was chosen because this cell type displays most of the major constitutively expressed and inducible endothelial phenotypic markers [120, 145]. Finally, a preliminary co-culture of HPMEC-ST1.6R and LNCaP cells was performed to assess angiogenesis process so tumor angiogenesis involves interactions between endothelial cells and cancer cells [33]. This process is an important factor in cancer progression [26].

Exogenous SPARC affects the metabolic activity of LNCaP cells cultured on scaffolds of collagen/nanoHA. The proliferation of LNCaP cells increased by the pre-adsorption of SPARC. Higher levels of SPARC were found in prostate cancer cells from metastatic cases [49] and, in this study, exogenous SPARC may promote growth and survival of LNCaP cells in bone-like microenvironment.

There are some studies on SPARC internalization by some cells such as osteoclasts and their precursors, activated macrophages, sinusoidal endothelial cells, embryonic chicken cells, mouse fibroblasts [146], rat skeletal muscle progenitor cells [147], and germ cells [148]. The internalization of SPARC by LNCaP cells was studied using a bioconjugate of QD_Ch_SPARC. Assays showed internalization of SPARC by LNCaP cells.

In relation to HPMEC-ST1.6R cells, we have studied the influence of SPARC, and SPARC derived peptide FS-E, and peptide 2.3 on scaffolds of collagen/nanoHA. Peptide inhibiting angiogenesis (peptide FS-E) and stimulating angiogenesis (peptide 2.3) were chosen.

According to the literature, the proliferation of endothelial cells is inhibited in the presence of SPARC in concentrations between 10 and 30 μg/mL [106]. In this study also HPMEC-ST1.6R cells, exhibited lower proliferation with concentrations of 10 and 30 μg/mL SPARC pre-absorbed on collagen/nanoHA scaffolds. This protein inhibits cell spreading, disrupts cell adhesion, and inhibits the cell cycle (Figure 35) [80, 87, 90, 91, 94]. The ability of adherent cell to spread can regulate cell proliferation, and the cells may become quiescent or die [149]. SPARC inhibits endothelial cells in the G1 phase of the cell cycle, leading to cell proliferation inhibition [76]. In addition, SPARC modulates growth factors such as VEGF [69, 74, 124], PDGF and bFGF [69, 74, 150], and these growth factors bind to SPARC reducing the association of growth factors with endothelial cells receptors, thus leading to the inhibition of endothelial cells proliferation [69, 74, 124, 150]. Fetal bovine serum similarly to these growth factors also inhibits endothelial cell proliferation [74]. These properties may be responsible for the decreased metabolic activity.

Moreover, calcein AM assay showed that SPARC at 10 and 30 μg/mL increased typical capillary-like structures (angiogenesis). According to T. Lane *et al.*[151], SPARC stimulates the formation of endothelial cords (angiogenesis *in vitro*), corroborating this study.

Regarding peptide FS-E, resazurin assay and calcein AM assay proved that this peptide pre-absorbed on collagen/nanoHA scaffolds inhibited endothelial cells (proliferation and angiogenesis) for all studied concentrations, but showing strong inhibition for 1000 μg/mL concentration(at days 5 and 7 of culture). Peptide FS-E is found in N-terminal region of module II of SPARC [103] and it inhibits angiogenesis and proliferation of endothelial cells due to strong inhibition of bFGF [101]. This growth factor (bFGF) binds to their receptor activating endothelial cell proliferation [39]. In the presence of SPARC, bFGF binds to SPARC and reduces the association of bFGF with endothelial cells receptors, thus occurs inhibition of the proliferation of endothelial cells [69, 74, 124, 150].

Peptide 2.3 stimulates cell endothelial proliferation and angiogenesis, has high affinity for calcium [107], and it is located in the C-terminal end of module II [100]. In this study an

increase of metabolic activity was not observed, however calcein AM assay showed stimulation of angiogenesis mainly with concentration of 0.01 and 10 µg/mL at 7th day of culture. Peptide 2.3 with concentration of 100 µg/mL appeared to increase angiogenesis and cell proliferation. Thus, this peptide in contact with cancer cells may promote the growth and survival the cancer cells. Tumor angiogenesis occurs due to interactions between endothelial cells and cancer cells favoring tumor growth Other interesting suggestion is to use this peptide for bone regeneration and repair, because the formation of blood vessels through endothelial cell proliferation from extant vasculature (angiogenesis) is a pre-requisite for tissue remodeling, regeneration and repair [152].

Cell morphology on our biomaterial with SPARC and SPARC peptides was assessed by SEM. There were no significant differences in morphology of endothelial cells contacting with SPARC and SPARC peptides when compared to control (biomaterial without SPARC or SPARC peptides). Therefore, exogenous SPARC and SPARC peptides appeared not to affect cell morphology in bone microenvironment.

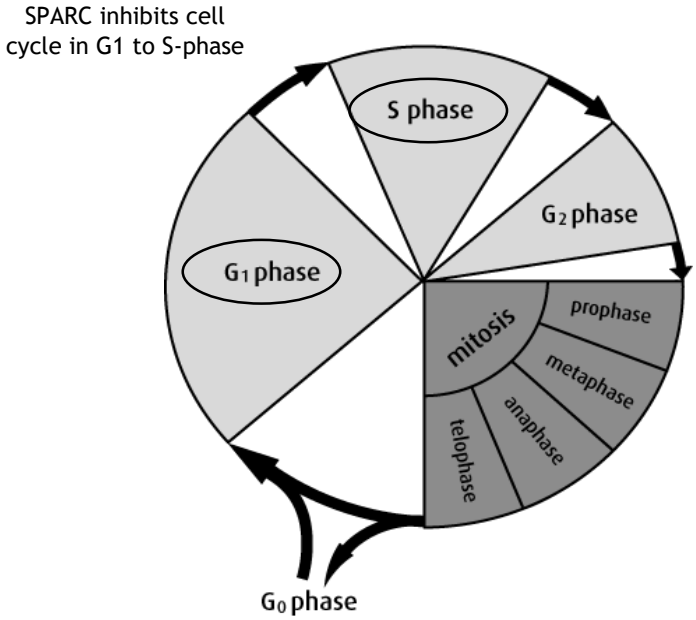


Figure 35 - Cell cycle schema. G0 - resting phase; G1 - first growth phase, S - synthesis phase, G2- second growth phase. SPARC inhibits cell cycle in G1 to S-phase decreasing cellular proliferation [62, 153].

After performing the tests with monocultures, a preliminary co-culture assay was conducted using HPMEC-ST1.6R and LNCaP cells on scaffolds of collagen/nanoHA. Co-culture systems (Figure 36) allow the study of interactions between at least two different cell populations [154]. The cells are cultured with some degree of contact between them, and if cell populations are different, growth medium must be optimized in order to choose the

medium that best sustains all the cell populations [154]. In our case, the medium was optimized as previously mentioned.

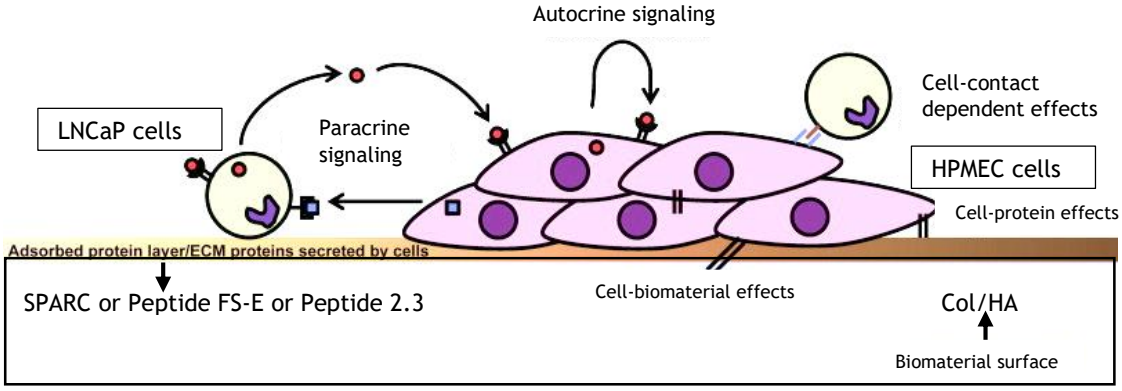


Figure 36 – Co-culture seeding on biomaterial. Cells communicate in different ways, such as cell-to-cell communication, cell-biomaterial interactions, cell-protein interactions, or autocrine effects. Biomaterials are the best model to mimic the physical and biological properties of the natural tissues. Increasingly, co-cultures have been used in biomaterials in vitro characterization allowing to study interactions between different cell types in a particular microenvironment [125].

Preliminary co-culture results showed that exogenous SPARC increased cellular proliferation at 30 µg/mL concentration, exogenous peptide FS-E decreased cellular proliferation for the higher concentration studied (1000 µg/mL), and exogenous peptide 2.3 appeared not to affect cell proliferation in co-cultures. Although SPARC decreased the metabolic activity of endothelial cells in bone matrix, when they were in contact with LNCaP cells, SPARC increased the metabolic activity of the co-culture. According to S. De *et al.* [69], SPARC can activate integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ on tumor cells and these integrins will stimulate production of VEGF supporting angiogenesis, and thus favoring growth and proliferation of prostate cancer cells. Also, SPARC (produced by tumor cell or stromal cells) can promote tumor angiogenesis favoring tumor growth [63]. Effectively, in this study, although SPARC has decreased the metabolic activity of endothelial cells, an increase in angiogenesis (shown by organization of endothelial cells in typical capillary-like structures) was observed. In addition, exogenous SPARC increased proliferation of LNCaP cells. The increase of angiogenesis when endothelial cells were in contact with cancer cells favored the co-culture metabolic activity. Moreover, the interactions between endothelial cells and cancer cells are an important area of cancer research. Endothelial cells within the tumor microenvironment control tumor formation due to the close proximity of endothelial cells and tumor cells, allowing growth factors exchange [155]. Thus, exogenous SPARC (may be modulating angiogenesis) facilitates growth of non-bone metastatic prostate cancer cells in bone-like microenvironment

Peptide FS-E (1000 µg/mL) decreased the proliferation of co-culture in bone-like microenvironment. There are no studies describing the influence of peptide FS-E in this type of co-culture. However, others studies indicate inhibition of angiogenesis in neuroblastomas [101, 102], and decrease of endothelial cells due to strong inhibition of bFGF [101] corroborating this

study. Several studies indicate the potential of antiangiogenic therapies for cancer treatment [26, 37, 42, 156, 157]. As previously mentioned, cancer cells require access to blood vessels (angiogenesis) for growth and metastasis. The discovery of angiogenesis-suppressors inhibiting tumor angiogenesis may provide means to inhibit tumor growth. Thus, this peptide FS-E could be useful in cancer therapy.

Peptide 2.3 (100 µg/mL) did not show significant differences compared to control (HPMEC-ST1.6R and LNCaP cells on scaffolds of collagen/nanoHA). In addition, others concentrations of peptide 2.3 could be studied, because this peptide is “a dose-dependent” [107], and others concentrations could have influence on the co-culture.

Chapter 5

Conclusions and future work

In prostate cancer, the interactions between cancer and endothelial cells, and their microenvironment are fundamental to understand the carcinogenesis and to discover new therapies for cancer treatment. Cancer cells grow up 1–2 mm^3 without blood vessels and to grow up beyond this value angiogenesis must take place (involving interactions between endothelial cells and cancer). In addition, SPARC is a protein that has been studied in terms of its influence on endothelial and on cancer cells. Still, SPARC and SPARC peptides have different effects depending of the cell-type and microenvironment, and they are concentration-dependent.

Initially in this work we have produced samples of nanohydroxyapatite/collagen by simultaneous electrospinning (collagen type I) and electrospraying (nanohydroxyapatite) to mimic bone-like microenvironment. This biomaterial have physical and biological properties that mimic those of bone matrix, therefore allowing the study of interactions between cells and this simulated microenvironment. Thus we have studied the influence of SPARC and peptides FS-E and 2.3 (SPARC peptides, inhibitor and stimulator of angiogenesis, respectively) on prostate cancer cells and endothelial cells in this microenvironment.

Exogenous SPARC at concentrations of 10 and 50 μg/mL increased proliferation of LNCaP cells. In contrast, concerning HPMEC-ST1.6R cells, SPARC (10 and 30 μg/mL) it decreased their proliferation, but stimulated angiogenesis. In addition, when LNCaP cells were seeded on endothelial cells, this co-culture showed increased proliferation. Therefore, exogenous SPARC may be favorable to the survival and growth of non-bone metastatic prostate cancer cells in bone-like microenvironment. In addition, this co-culture mimics the angiogenesis process and it is the first step towards tumor growth, thus, also SPARC can be associated to tumor angiogenesis process favoring growth of prostate cancer metastases in bone.

Exogenous peptide FS-E decreased the proliferation and angiogenesis of endothelial cells and co-culture, so this peptide could be used for in the therapy for the treatment of prostate cancers metastizing in bone. As this peptide decreased the proliferation and angiogenesis of endothelial cells, it could be used as a therapeutic agent in the treatment of others cancers. For example, nanoparticles (such as nanostructured lipid carriers, described in a recent study by Ferreira *et al*. [158]) is possible to bioconjugate peptide FS-E and drive them towards endothelial cells, thus inhibiting their proliferation and angiogenesis. Moreover,

peptide FS-E when in contact with endothelial cells decrease proliferation and angiogenesis, consequently may be promote tumor regression (Figure 37).

Exogenous peptide 2.3 at concentrations of 0.01 and 10 µg/mL although decreasing endothelial cells proliferation, stimulated angiogenesis and at 100 µg/mL it seemed to increase both proliferation and angiogenesis. Therefore, this peptide in contact with cancer cells may promote cancer cells growth and survival. However, in this study, 100 µg/mL did not seem to influence HPMEC-ST1.6R and LNCaP cells co-culture. In addition, others peptide 2.3 concentrations could be studied, because this peptide is a concentration-dependent and thus others concentrations could be influence of co-culture. So, this peptide stimulated angiogenesis, but more work with other concentrations is required to know if it would also promote survival and growth of non-bone metastatic prostate cancer cells in bone-like microenvironment. In addition, the formation of blood vessels through endothelial cell proliferation from extant vasculature (angiogenesis) is a prerequisite for tissue remodeling, regeneration and repair. Thus, this peptide may be used for bone regeneration and repair due to stimulating of angiogenesis.

Therefore, the interactions between the cancer and endothelial cells, and their microenvironment (stimulated through a biomaterial) are fundamental to understand the carcinogenesis and to discover new therapies for cancer treatment. In this case, it was possible to study the influence of SPARC and peptides FS-E and 2.3 in endothelial cells and in prostate cancer cells in bone-like microenvironment. SPARC and its peptides could be used in cancer therapy and for bone regeneration and repair. This study novelty lies on the development of a biomaterial (collagen/nanoHA) mimicking a bone-like microenvironment allowing the study of carcinogenesis in it.

To expand this study others assays may be performed, such as matrigel assay (to corroborate calcein AM assay to show tube formation) and *in vivo* assays (to know if SPARC and the peptides have similar influences as in the *in vitro* assays). Moreover, as the co-culture was preliminary so more assays can be performed, in order to use another marker for endothelial cells and LNCaP. Finally, it would be interesting also to assess the influence of SPARC peptides in LNCaP cells.

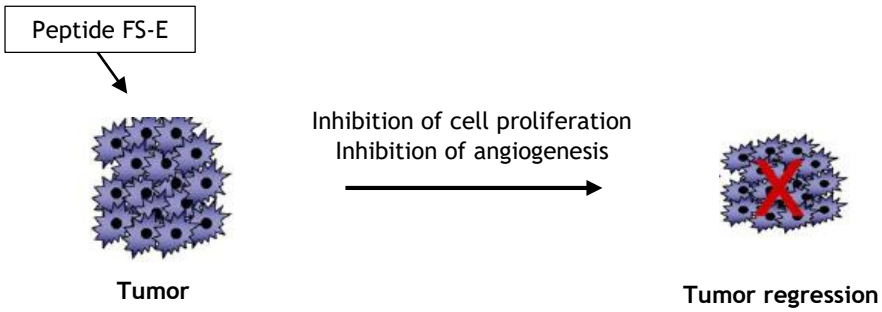


Figure 37 – Peptide FS-E in cancer therapy. Peptide FS-E inhibits proliferation and angiogenesis of endothelial cells and may be promoting tumor regression [62].

Glossary

Cancer - Outgrowth of a clonal population of cells from tissue [159].

Carcinogenesis - Development of cancer [159].

Chemokines - Chemokines represent the largest family of cytokines and are classified according location of cysteine residues. Chemokines forming a complex network for the chemotactic activation of all leukocytes [160].

Counter-adhesive properties - Characterized by modifying cell shape through the dissociation of focal adhesions, and modulates cell-matrix interactions by binding to ECM components [79].

EF-hand motifs – The EF-hand motif is the most common calcium-binding motif found in proteins. The classical EF-hand is a helix-loop-helix motif [161].

Epithelial cells – "Cells that cover the inside and outside surfaces of the body [27]."

Epithelium - Set of sheets of continuous and polarized cells along the apical–basal axis [55].

Extracellular Matrix - Three-dimensional structure of heterogeneous macromolecules. It provides "structural support to cells and tissues, supports adhesion of cells, transmits signals through adhesion receptors, and binds, stores and presents growth factors and other biologically active molecules" [39].

Flow cytometry - Allow to measure several features of single cells or particles such as nuclei, microorganisms, chromosome [162, 163].

Immune surveillance – One of the steps in the prevention of tumors that occurs by immune system. The immune system identifies and eliminates cancerous and precancerous cells [164].

Integrins - Transmembrane receptors for extracellular matrix proteins, play a key role in cell survival, proliferation, migration, gene expression, and activation of growth factor receptors. "Their functions and expression are deregulated in several types of cancer, including prostate cancer"[165].

Matricellular proteins – The matricellular proteins are secreted into the extracellular environment or matrix, and these proteins are responsible for modulation the cell function (by influencing cell adhesion, migration, proliferation, differentiation, and apoptosis) through cell-surface interaction [60, 80, 82, 83]. In contrast with collagens and laminins, matricellular proteins do not play a structural role in the ECM [60, 72, 81].

Matrigel assays -Matrigel is a solubilized basement membrane derived from Engelbreth-Holm-Swarm mouse sarcoma, that is a tumor rich in laminin, collagen IV, entactin [166, 167], heparin sulfate proteoglycans, and growth factors [166]. In the case of endothelial cells, the solubilized basement membrane has the capacity to form three-dimensional structures, in other words, tube formation [168].Thus, matrigel can be applied to in vitro and in vivo studies of angiogenesis [166].

Matrix Metalloproteinases - Metalloproteinases are proteolytic enzymes and are the primary mediators of ECM proteolysis and turnover [81, 169]

Post-translational glycosylation – Some proteins require molecular features to be changed so that they can perform their molecular function. This modification involves an enzymatic process occurring with addition of glycosyl to certain amino acid residues [170].

Primary tumor – Local where the cancer starts [1].

Quantum dots - Nanoparticles or nanocrystals of a semiconducting material with diameters in the range of 2-10 nanometers [171].

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