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Cancer-Associated Fibroblasts

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Assinatura do Tutor

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Coimbra, de Setembro de 2014

"It is, however, increasingly apparent that the growth deregulation within a tumor can only be explained once we understand the contributions of the ancillary cells present in a tumor- the apparently normal bystanders such as fibroblasts and endothelial cells- which must play key roles in driving tumor cell proliferation."

The Hallmarks of Cancer, Hanahan and Weinberg, Cell, Vol 100 (2000) 57-70.

INDEX

ABBREVIATIONS

α-SMA - α-smooth muscle actin

ATP - adenosine triphosphate

bFGF - basic fibroblast growth factor

BL - basal lamina

CAFs - cancer associated fibroblasts

COX-2 - cyclooxygenase isoform 2

ECM - extracellular matrix

EGF - epidermal growth factors

EMT - epithelial-mesenchymal transition

End-MT - endothelial-mesenchymal transition

FAK - focal adhesion kinase

FAP - fibroblast activation protein α

FSP1 - fibroblast specific protein 1

GAG - glycosaminoglycan

GF - growth factors

HGF - hepatocyte growth factors

HIF - hypoxia inducible factor

IGF - insulin-like growth factors

LOX - lysyl oxidase

MET - mesenchymal-epithelial transition

MMT - mesenchymal-mesenchymal transition

MSCs - mesenchymal stem cells

MTTs - matrix metalloproteases

NGF - nerve growth factors

PDGF - platelet-derived growth factors

PDGFR - receptor of PDGFR

RER - rough endoplasmic reticulum

TGF-β - transforming growth factor β

TIMPs - tissue inhibitors of metalloproteases

VEGFs - vascular endothelial growth factors

ABSTRACT

Cancer is one of the most challenging diseases nowadays. Millions are spent every year trying to find novel more efficient pharmaceuticals for its treatment. Despite the great effort expended, the majority of drugs available are unsatisfactory due to serious side effects and low success rate. Drugs mainly target cancerous cells or their by-products, in an attempt to avoid spreading and evolution to a metastatic state, a major cause of cancer mortality. This has been, so far, an inefficient approach. However, new insights on cancer development drew attention to fibroblasts. Cells that once were considered bystanders in the extracellular matrix are now regarded as key elements for tumor progression and decisive components regarding drug therapy success. Cancer-associated fibroblasts present themselves as potential drug targets especially in an attempt to limit tumor growth and to avoid malignancy.

Key-words: cancer; cancer-associated fibroblasts; epithelial-mesenchymal transition; extracellular matrix; myofibroblasts.

RESUMO

O cancro é uma das doenças mais desafiantes da actualidade. Milhões são gastos todos os anos numa tentativa de encontrar fármacos mais eficientes para o seu tratamento. Apesar dos grandes esforços dispensados, a maioria dos medicamentos disponíveis deixam muito a desejar visto que apresentam graves efeitos secundários e baixas taxas de sucesso. Grande parte dos fármacos tem como alvo as células cancerígenas ou produtos do seu metabolismo, tentando evitar a disseminação destes e a evolução para um estado metastático, a principal causa de mortalidade no cancro. Até agora, esta tem sido uma abordagem ineficiente. Contudo, novos conhecimentos acerca do desenvolvimento do cancro chamaram a atenção para os fibroblastos. Células que até então eram consideradas componentes inofensivos da matriz extracelular são agora vistos como elementos chave na progressão de tumores e componentes determinantes no sucesso da terapia farmacológica. Os fibroblastos associados a cancro demonstram ser potenciais alvos terapêuticos, especialmente numa tentativa de limitar o crescimento tumoral e evitar malignidade.

Palavras-chave: cancro; fibroblastos associados a cancro; matriz extracelular; miofibroblastos; transição epitelial-mesenquimal.

1. INTRODUCTION

All the systems and organs of the human body are made up of four basic types of tissue – connective, epithelial, muscular and nervous-, all sharing the presence of both cells and extracellular matrix (ECM).

The connective tissue differs from the other three types because here the main component is the ECM and not the cells themselves[1], since its main function is to provide not only a physical support to organs and other structures but also to allow the diffusion of nutrients, gases, growth factors and other components that contribute to cellular homeostasis.

Fibroblasts (Figure 1.) are the most abundant type of cell in the connective tissue[2] and their main function is the production, maintenance and remodeling of the ECM. When active, fibroblasts, now called myofibroblasts, exhibit an elongated shape with cytoplasmic projections, abundant rough endoplasmic reticulum (RER) and a well-developed Golgi apparatus, consistent with intense protein synthesis as well as a large and ovoid nucleus[3]. In contrast, when fibroblast activity is not required, they remain in a quiescent state characterized by poorly developed organelles, a smaller shape and fewer cytoplasmic projections.

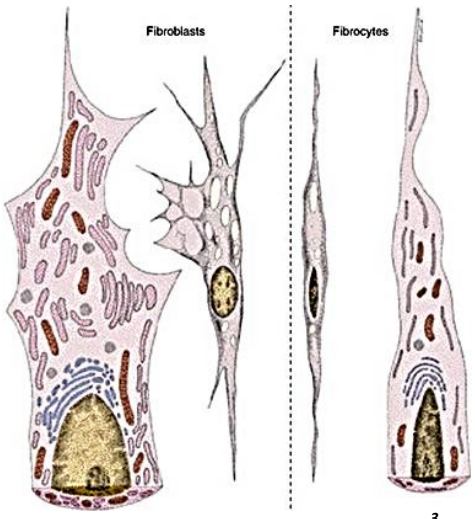

Figure 1.- Adapted from Mescher[3]

There are several stimuli responsible for the conversion of quiescent fibroblasts into myofibroblasts. When there is tissue damage and, therefore, mechanical stress, quiescent fibroblasts are recruited and activated, initiating the production of tissue remodeling substances. After the pathological condition is resolved, myofibroblasts are removed from the granulation tissue[a] by apoptosis, only remaining quiescent fibroblasts on site to be activated if necessary [2,4].

In 1999, Olumi *et al.*[5] published the first paper providing scientific evidence, using *in vitro* and *in vivo* models, that fibroblasts were necessary to tumorigenesis regarding prostate

[a] The granulation tissue is present in sites of wounds and inflammation and is composed of myofibroblasts, inflammatory cells and small vessels[2].

carcinoma[b]. In this study it is shown how cancer-associated fibroblasts (CAFs), in spite of not being able to initiate a tumor in normal epithelial cells, are capable of stimulating tumor progression and, therefore, promoting cancer development[5].

Since then, fibroblasts have been the focus of several research groups as a possible target for cancer therapy. The idea that only cancer cells contribute to the development of disease became outdated and a new hope towards cancer understanding and treatment is rising.

2. CANCER

Homeostasis of the human body is partially based on cell proliferation, differentiation and renewal[6]. Each cell has its own lifespan - from enterocytes, that have a turn-over time of 4 to 10 days[7], to memory T cells, that are supposed to live throughout one's entire life - and the ratio between the number of new cells and the ones undergoing apoptosis[c] determines a balanced and healthy status.

The maintenance of this state is possible because, in contrast to what happens in nature, cellular genetic modifications are not viewed as an evolutionary opportunity but rather as a threat to surrounding cells[2,9], jeopardizing the normal function of the system. In these situations, death of the mutated cells is induced and a physiological state is achieved once again.

Although cell growth is a tightly regulated process, sometimes cells manage to evade the normal development routes by perpetuating changes in their genomes that allow them to grow independently of other cells in the "community". When cells exhibit growing advantages respecting others, disturbing the normal cellular environment, the process of tumorigenesis is in place[1]. Despite outside the scope of this review, relevant information on cell death and cancer can be found elsewhere[6,10,11].

In 2000, Hanahan and Weinberg proposed the *Six Hallmarks of Cancer*[12], where they described the six most important modifications that a cell has to undergo in order to initiate its transformation from a normal to a malignant cell. These alterations are 1) self- sufficiency

[b] The majority of human cancers are carcinomas. This is a type of cancer that arises from the epithelial cells that line glands, ducts, and surfaces of organs[5].

[c] Genetically programmed mechanism that allows cells to commit suicide through a controlled process involving fragmentation of the DNA, shrinkage of the cytoplasm and membrane changes without damaging neighboring cells[6,8].

in growth factors (GF) - which allows cells to be independent from other cells' signaling either by synthesizing their own GFs or by overexpressing/upregulating the corresponding receptors; 2) insensitivity to anti-growth signals - so that cell growth and proliferation is not inhibited; 3) evasion of programmed cell death - which enables defective cells to proliferate; 4) limitless replicative potential- the so called immortalization; 5) sustained angiogenesis - a tumor can only go as far as its supply of oxygen and nutrients go. The ability to grow new blood vessels facilitates tumor growth and eventual spreading; 6) tissue invasion and metastasis - when primary tumors invade other tissues and form secondary masses. These are said to be malignant tumors or cancers[12].

It is worth mentioning that Hanahan and Weinbergs's classification excludes benign tumors which are the ones that exhibit a self-limited growth and, therefore, are non-invasive[8].

Since benign tumors are localized they are fairly operable and, in a great majority of cases, do not pose a threat to the individual's life[13]. On the other hand, metastases are the main cause of death in cancer patients[14], making it more appealing to devote time and funding to these types of tumors. In fact, in the United Kingdom alone, over the last five years, *Cancer Research UK* has invested over 1.6 billion pounds in research[15] towards cancer prevention and treatment which complies with the great impact tumors have in society.

Keeping in mind the benign/malignant tumor duality, it is rather interesting to analyze which differences are there between both and think about what makes it possible for some cells to just multiply *in situ* while others leave their primary settlement and migrate to other sites in the body. Clearly this is not a self-acquired ability of the cell alone given that in order to shift from one place to another, cells must move freely through places where they are not supposed to be, namely, the complex network of proteins, fibers and proteoglycans of the ECM.

So the key question is "what changes the microenvironment making it possible for cells to migrate?"

3. THE EXTRACELLULAR MATRIX

As it was previously said, the ECM has a fundamental role in nourishing and holding cells, tissues and organs together. Although it is easy to imagine the ECM as a simple glue that prevents organic systems from falling apart, it is in fact a highly complex and dynamic structure[16,17]. A myriad of biochemical and buffering reactions continuously take place[16,17] and different participants such as adhesion molecules and growth factors act together to maintain an optimal and functional environment in the body.

Since each organ carries out distinct functions, it is only logical to look at the ECM composition as variable as well, both with respect to the type of molecules found and to their spacial organization[9]. However there are some ubiquitous elements that allow us to roughly describe the general composition of the ECM.

One can distinguish two great groups of macromolecules in the ECM: proteoglycans and fibrous proteins[3,8,16]. The latter is represented mainly by collagen, followed by elastin, fibronectin and laminin fibers. Although several ECM cells contribute to the production of its components, fibroblasts are the main site of synthesis of these molecules.

Proteoglycans are macromolecules that combine a proteic core with at least one covalently attached glycosaminoglycan[d] (GAG) chain[18]. Since GAGs are highly hydrophilic molecules, they manage to form a hydrogel matrix that fills the extracellular space thus making the ECM the perfect scaffold for cells, organs and tissues due to the protection it offers against mechanical (specially compressive) forces[8,16,18]. On the other hand, the main role of fibrous proteins is to protect the ECM against tensile forces and to confer elastic properties. Not only do they protect the tissue from rupture, they also allow it to recover its original structure after a deforming stimulus has been applied.

Collagen is the most abundant protein in the body accounting for 30% of its dry weight[16]. Distinct types of collagen can be found in different tissues (so far, at least 28 types have been identified) usually as a heterogeneous mixture with one type of collagen being predominant. In the case of the ECM, collagen type I and type III are the most abundant ones [16,19]. Although collagen's molecular composition varies, a common structural feature can be found: a triple helical structure composed of three α-chains[3,19]. α-Chains are an abundant source of proline (Figure 2.) and glycine (Figure 3.), which is directly related to the physical

[d] Hyaluronic acid is an atypical GAG given that it appears in nature in an isolated form, without binding to any proteins[8].

properties collagens exhibit: proline, due to its ring structure, confers rigidity to the molecule, while glycine keeps the chains tightly packed as a result of being the smallest aminoacid in nature[8]. These structural features contribute to make collagen a highly resistant molecule that protects ECM components from separating ("fracturing") during movement.

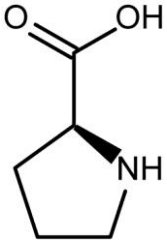

Figure 2.- Proline

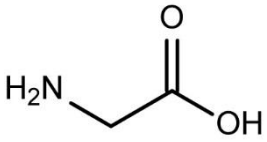

Figure 3.- Glycine

Elastic fibers from the ECM are mainly composed of the protein elastin. Although its chemical composition is somewhat similar to that of collagens, the presence of α-helical segments[8] cross linking between residues of lysine (forming the aminoacid desmosine), partially results in the elastic behavior characteristic of these fibers[3]. In a relaxed state, elastin fibers are all coiled together. When a force is applied, fibers stretch and adopt a parallel disposition. By doing this, elastin allows tissues to recoil to their normal shape after undergoing extension and deforming forces[19].

Another of the fibrous proteins found in the ECM is fibronectin, a glycoprotein formed by two polypeptide chains with almost identical composition and joined together by disulfied bonds[20]. This macromolecule exhibits domains specifically designed to bind other elements of the ECM such as collagen, integrins[e], heparin and fibrinogen. Therefore, fibronectin is associated with the organization of the interstitial space, cell adhesion and migration.

Many of fibronectin's binding domains are only exposed in a force-dependent manner and only then does it polymerize in the form of fibrils. When this happens, one end of the fibrils attaches to the cells while the other binds to ECM components like collagen, keeping the ECM structure intact[8,16,20]. This type of regulation emphasizes the importance of the quantity of fibronectin fibers: too many of these fibers would lead to an ECM that is too rigid and does not allow normal movement of its components; too few would originate cells mobile enough to migrate to other body tissues which is especially relevant in initiating cancer metastasis[22].

[e] Transmembrane adhesion proteins that work as receptors, forming structural and functional linkages between the ECM and intracellular cytoskeletal linker proteins (usually actin) [3,8,16,21].

Laminin is another abundant glycoprotein found in the ECM that, like fibronectin, shows several binding sites for other elements[3,23]. As a major component of the basal lamina (BL), laminin's main role is to keep epithelial cells together, aiding in the maintenance of its sheet-like morphology[8,24]. Although laminin is secreted in a soluble form, contact with the extracellular side of the cellular membrane serves as a polymerization nucleus. Here, laminin molecules are anchored with the aid of transmembrane proteins and receptors, such as integrins, to start the assembly of the BL[8,24].

Even though "laminin" was referred to as a single molecule, this is not the case. Instead, laminins are a family of large and flexible glycoproteins that are assembled in α, β and γ heterotrimers, held together by disulfide bonds. The genetic variety of subunits (five α, three β and three γ) originates distinct laminin isoforms that bear different properties and, therefore, are found at different developmental stages and tissues[23,24].

The ECM is in constant remodeling either by the natural lifespan of its cells or due to external influences, such as the development of a tumor mass. In fact, when a tumor is growing, not only does it change the ECM but the ECM itself influences tumor progression. Transforming growth factor β1 (TGF-β1), for example, which is a cytokine produced by several cell types, shows a double role in tumor progression. In the beginning, it starts by having a tumor suppression action, inhibiting proliferation and inducing apoptosis but, as tumor cells multiply, it switches its function enhancing tumor progression by promoting epithelial-mesenchymal transition (EMT)[f] and cell motility.

Since fibroblasts are the most abundant cell type in the ECM, a great majority of its activities are regulated by them. Furthermore, if the ECM provides biomechanical factors that allow carcinogenesis, one can safely state that fibroblasts are major contributors in this process as well.

4. FIBROBLASTS

During embryogenesis, three germ layers of cells arise: endoderm, mesoderm and ectoderm. From the mesoderm originates an immature form of connective tissue, the mesenchyme, which consists in loosely packed unspecialized cells set in a gelatinous ground

[f] Biological process that allows epithelial cells to assume a mesenchymal phenotype. This is not only a cancerous mechanism (type 3). I also happens during embryogenesis, for the creation of new tissue (type 1) or during wound healing and fibrosis (type 2) [25,26].

substance[8,27]. These mesenchymal cells then migrate during organogenesis, originating connective tissue cells (like fibroblasts) that start the production of ECM and other components, contributing to connective tissue development[3].

But fibroblasts do more than just synthesize ECM during physiological conditions. In fact, as aforementioned, there are other sources of stimuli for the transformation of fibrocytes into active fibroblasts such as tissue repair, which will be explained next, and fibrosis[g].

Wounds are always accompanied by inflammation. During this process, several cells are recruited in order to reestablish tissue homeostasis. After macrophages and neutrophils clean the inflammation site from dead cells, cellular debris, bacteria or other noxious elements, tissue repair can now start. At this point, endothelial cells and platelets begin the production of platelet-derived growth factor (PDGF), a cytokine that binds to tyrosine kinase receptors on fibrocytes, inducing their multiplication, activation into myofibroblasts and consequent production of compounds that help closing the wound and replacing the destroyed ECM[1,28]. TGF-β is also involved in fibroblastic differentiation into myofibroblasts[29].

One of the substances these myofibroblasts synthesize is α-smooth muscle actin (α-SMA), which generates the necessary contractile force to close the wound, as well as an ED-A[h] splice variant of fibronectin, that helps reestablishing gap junctions between adjacent cells[2,4,32].

When the wound is healed, myofibroblasts are no longer needed. Therefore, the balance between matrix metalloproteases (MTTs) and tissue inhibitors of metalloproteases (TIMPs), that are themselves produced by myofibroblasts[16], shifts towards ECM degradation, inducing cellular apoptosis. This is a regulation mechanism that allows myofibroblast removal after the pathological condition is corrected[4,33].

Interestingly, in a situation where cancer is present, dissipation of the granulation tissue does not happen. In fact, myofibroblasts that abnormally remain on site are shown to influence cancer progression and facilitate metastasis development. The environment persists with components that are usually only present in inflammatory or wounded states even though they are not present. Hence the concept that **cancer is a wound that does not heal**[2,4,29,32–34].

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[g] Tissue repairing accompanied by loss of function. Usually collagen fibers ("scar") replace the lacking tissue on the wounded site, holding it together but without restoring its initial characteristics or functions[1,8].

[h] Standing for Extra Domain- A, it is one of the isoform types of fibronectin, originated by alternative splicing of type III exons[30,31].

5. CANCER-ASSOCIATED FIBROBLASTS

We ended the last section introducing the relationship between altered myofibroblasts and cancer. Indeed, these tumor promoting cells, although also expressing high amounts of α-SMA[35], do not exhibit activation reversal[9]. Consequently, they form a new category of cells which do not contribute to system homeostasis as normal fibroblasts do but, instead, help cancer cells to develop and thrive.

Cancer (or carcinoma) - associated fibroblasts (CAFs) are a heterogeneous subpopulation of cells that reside within the tumor premises and have the ability to promote tumorigenesis and aid tumor growth[2,4].

It is now a consensus that CAFs have different origins[9,29,32,36–38] which in turn implies that different populations can also be found in distinct tumors' tissues and even within the stroma of a single tumor alone[29,35,39,40]. Nevertheless, due to space and content limitations, this review will summarize some of CAFs characteristics disregarding different cell subpopulations.

5.1. Origin

The first CAFs to appear in a tumor site derive from resident fibroblasts, myofibroblasts or inactive fibrocytes that are activated by cancer-derived growth factors such as TGF-β, PDGF and basic fibroblast growth factor (bFGF)[32,36][28]. This process is often called mesenchymal-mesenchymal transition (MMT)[9,32] and is responsible for the majority of CAFs in a tumor mass[40].

A second source of CAFs is bone marrow-derived mesenchymal stem cells (MSCs). As the name suggests, these cells remain highly undifferentiated and because they show an extreme susceptibility to cancer derived growth factors they can originate not only CAFs but also other stromal cell types that help its propagation. The recruitment of MSCs increases as tumors grow since the permeability of blood vessels is higher, facilitating MSCs influx to the site[4,9,29,32].

EMT (Figure 4.) was a concept already defined in this review. This is yet another interesting source of CAFs and an example of the outstanding plasticity these cells have, under environmental influence[9,32].

Type 3 EMT is associated with cancer progression and metastasis. In this process, epithelial cells, which are polarized cells in permanent interaction with the BL, acquire a

mesenchymal phenotype and lose their epithelial markers, enabling them to detach from their original place in the epithelium and migrate. These cells do not have the need for tight cell to cell contact or anchorage to survive[4]. Some of the EMT-inducing signals produced by cancer cells and fibroblast are MMPs, TGF-β, hepatocyte growth factors (HGF) and epidermal growth factors (EGF) [3,34,25,26].

Once free, these cells with mesenchymal phenotype can move to different sites where the tumor can proliferate and grow. In order to establish secondary colonies, cells must undergo MET (mesenchymal-epithelial transition) to regain their cancerous phenotype, which allows them to start the production of carcinogenic mediators that provide an optimal environment for the secondary tumor mass to grow[25].

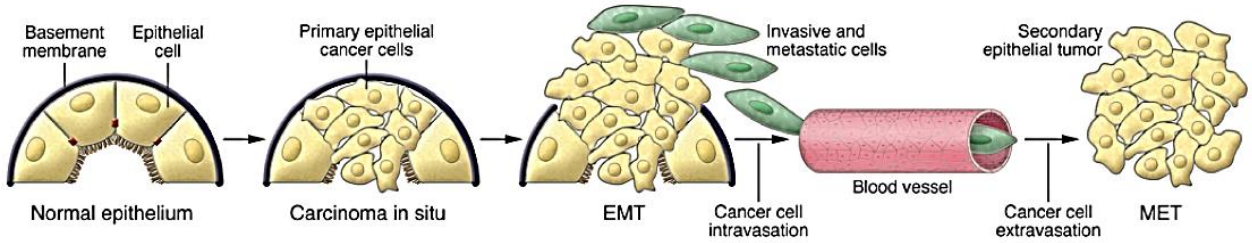

Figure 4.- Adapted from Kalluri[25]

Transdifferentiation of other cell types has been shown to be a source of CAFs such as vascular tumor cells like perycites, smooth muscle cells or even endothelial cells[4,32,33]. The latter can undergo endothelial-mesenchymal transition (End-MT), a process in which, similarly to EMT, invasion is facilitated by the loss of endothelial markers as VE-cadherin and CD31 and gain of mesenchymal markers like fibroblast specific protein 1 (FSP-1) or α-SMA[41].

Despite their different origins (Figure 5.) , CAFS synthesize mediators that promote carcinogenesis as MMPs, growth factors like HGF, insulin-like growth factors (IGF), nerve growth factors (NGF) and EGF and modulate the immune response due to the secretion of pro-inflammatory cytokines as interleukin-1,6 and 8, TNF-α and stromal cell-derived factor 1 (SDF1 also known as CXCL12) [9,22,40,42]. These are considered to be pro-invasive growth factors[32].

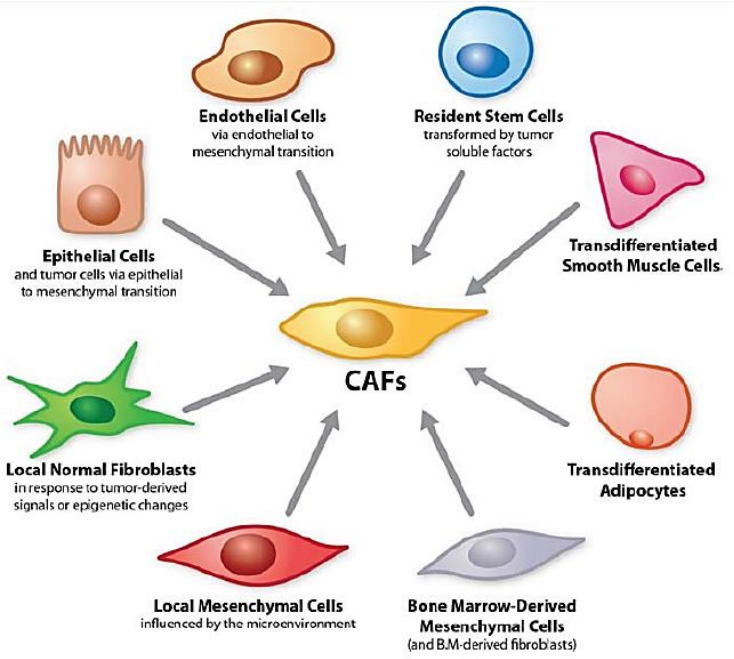

Figure 5.- Adapted from Takebe[38]

Along this section of the review, mention of markers and metastasis were a constant. In fact, CAFs markers, although not completely defined so far, are the main targets for drug therapy against cancer progression. On the other hand, this progression, as described in little detail so far, is greatly due to the influence of CAFs.

These two topics will be discussed in the following sections.

5.2. Markers

A question that has been occupying the scientific community devoted to CAFs' study is "how can one distinguish CAFs from other cells if they originate from such diverse backgrounds?". As it was said before, α-SMA is a commonly used marker to distinguish CAFs from other cells[35], although others, such as myofibroblasts, also express this protein. A more complex approach to protein expression is necessary to more accurately identify the population of cells and, therefore, other markers are often used.

Again, different subpopulations of CAFs express different markers, though there seems to be an agreement that the ones to be discussed next are the most prevalent ones.

- <u>α-SMA</u>

This marker was previously addressed. Although it is often used to identify CAFs, it is not entirely exclusive to these cells so its application is limited (unfortunately, this happens

with all markers found so far). Its main function is to provide contractile properties to, in this case, CAFs, allowing them to move around the ECM[43].

- Fibroblast activation protein α (FAP)

Also known as seprase, FAP is an inducible gelatinase expressed in activated fibroblasts and pericytes during wound healing and tumorigenisis[2].

Its location in the cell, as an integral membrane peptidase supports its role in the degradation of the ECM[44], creating tracks for cancer cells to move more easily through the tissue[34], reason because it is usually considered a poor diagnosis marker[2,39].

- FSP1

FSP1 can also be called S100A4 and it is an intracellular protein expressed by several cells besides CAFs, as macrophages and cancerous cells[39]. Although some researchers argue its utility as a drug target[39], since it is widespread in other cells, because it regulates cell progression and cytosqueleton integrity[2], aberrant forms of this protein are reported to be implicated in tumor growth and metastasis[2,39,45].

- PDGF receptor (PDGFR)

PDGF is a protein secreted mainly by platelets that acts as a mitogen, stimulating fibroblasts, pericytes and smooth muscle cells to multiply. Its role was first studied as an essential component for blood vessel repair and blood clot formation[1,8].

PDGFRs can be divided into two categories, α and β, each having its specific ligands being PDGFR-α more ubiquitous than PDGFR-β. The first one is of major importance during embryogenesis to signal the formation of epithelial tissue from mesenchymal cells[39], though its ligand PDGF-C is almost exclusive of cancer cells[46]. The second one is more restricted to pericytes and CAFs and is involved in vascular regulation[39].

In cancer, ligands from both PDGFRs are overexpressed[39], contributing for a well-vascularized and prominent stroma that enhances tumor growth[47]. Abundance of ligands for the PDGFR-β is particularly important since these compounds ultimately generate high interstitial fluid pressure next to the tumor, diminuishing drug delivery to the site[39].

5.3. Metastasis

As it can be read in the previous section, CAFs are important cells in the tumor milieu not only because they provide an ideal niche for cancer cells to thrive but also due to their ability to promote dislocation from the primary place of tumor growth to different locations in the organism.

But why do tumors have the necessity to change sites during growth? And how do CAFs facilitate this process?

When abnormal cells start to grow, and the immune system fails to eliminate them, the path is open to the propagation of these new cells. Curiously, later on, some components of the immune system turn out to help these same cells[48], as it happens with TGF-β1, already described. But, as it is known, the existence of altered cells is not enough for cancer development[9,33]. Although there is initiation, propagation requires other factors, some of which will be provided by CAFs.

In order for tumor cells to succeed in cancer formation they have to adapt their response to the surrounding environment and: 1) resist apoptosis; 2) survive in hostile environment, where hypoxia, acidity and inflammatory cytokines are abundant; 3) be plastic and mobile[9].

Resistance to apoptosis is beyond the scope of this review. Nevertheless, at least for the first cancer cells to appear, this seems to be a process that is relatively independent of CAFs since they start to form after some tumor cells have been established and initiated the production of cancer growth factors.

As a solid tumor grows in size, it eventually outgrows local blood supply and the hypoxic and non-nutritious environment starts to slow down tumor progression. As a response to this, tumor cells and CAFs undergo metabolic changes that allow them to use the glycolytic pathway as a source of ATP instead of completely catabolizing glucose by oxidative phosphorylation (a process called the "Warburg effect")[9,32]. Limited oxygen, forces cells to use more glucose than usual, in order to provide the necessary energy for cancer growth. Here, CAFs are essential in the way that the lactate resulting from their adaptation to the Warburg effect is uptaken by cancer cells and used to anabolize proteins and as an additional source of energy[9,49].

Cancer cells also initiate *de novo* angiogenesis by activation of signaling cascades mediated by the hypoxia inducible factor (HIF) family of transcription factors[9] and CAFs

potentiate this process by recruiting endothelial progenitor cells through SDF1[38]. In addition, both cell types produce vascular endothelial growth factors (VEGFs), a specific mitogen for endothelial cells[38,50].

An increase in tumor mass also means that cancer growth factors are in higher numbers, which leads to CAFs proliferation. SDF1 proved to be particularly relevant again, inducing EMT, essential for the metastatic process. Through EMT, transcription of cadherins is repressed, which reduces cell to cell adhesion and mesenchymal qualities are acquired[35]. Not only is there a repression of cadherins' synthesis, they are also cleaved by an increasing quantity of MMPs produced by CAFs[9]. Pro-proteins such as precursors of growth factors and of receptors are activated as well[32].

MMPs secretion also allows CAFs to modulate ECM integrity and content. In reality, these proteases enable CAFs to design tracks that are going to be used not only by cancer cells to escape the unfavorable conditions building up in the initial tumor site, but also to escort other types of cells, such as endothelial precursor cells (essential for angiogenesis), that will help the preparation of the secondary (metastatic) tumor site[9,32].

CAFs have also been shown to influence ECM architecture by increasing its stiffness which, in turn, facilitates tumor invasion[51–53]. As the number of CAFs and cancer cells increases, there is a higher deposition of collagen fibers (desmoplasia)[51] as well as an accumulation of lysyl oxidase (LOX) in the extracellular space. This enzyme has the ability to, not only crosslink collagen but also elastin monomers[32], reorganizing ECM fibers from a loose reticular structure to a more rigid and oriented one[34,51,53]. Increasing stiffness of the ECM leads to integrin clustering and activation of signaling pathways involving focal adhesion kinase (FAK), which is associated with ECM-mediated cell adhesion and migration in some cells[52,54]. FAK phosphorylation signals the Rho pathway[52] triggering multiple outcomes such as cell proliferation, increasing cytoskeleton contractility and promotion of microtubule stability[9,55]. Ultimately, these cascades culminate in tumor mobility.

The role of ECM stiffening and MMPs seems to be contradicting[53] though that is not the case. One can think of these two complementary processes as a tunnel that is being constructed for cancer cells to migrate: stiffening causes uniformity of the polymer that is the ECM and MMPs make the way tumor cells have to follow to initiate metastasis.

Additionally, stiffening leads to two other important outcomes: the rising in interstitial pressure close to the tumor that, as it was mentioned before, reduces drug delivery to the site, diminishing therapy's success rate; an increasing in blood marrow-

derived MSCs influx which, in turn, leads to a higher number of tumor promoting cells on site[34] available to contribute to cancer growth.

Finally, as CAFs induce EMT, some studies show that they are also involved in cancer cell stemness[51]. When this phenotype is expressed, cancer cells are capable of self-renewal, which is crucial not only in the metastatic process but also resistance to therapy, late metastases and relapses[9,35,51].

Metastasis is the ultimate adaptation cancer cells perform against the unfavorable milieu building in their surroundings[9]. An environment that should be selective and restricted from abnormal cells ends up drafting only the stronger and more adaptable cancer cells. If none of the adaptive processes described above are enough to allow cancer development, the only chance of cell survival is to change to a new body site, fresh with oxygen and nutrients.

Thanks to CAFs, the pathway is clear for cancer cells to move and, although tumor cells rely on them to spread, as the tumor grows, the need for CAFs diminishes. Ultimately, tumor cells switch their regulation manner from paracrine to autocrine, managing to sustain essential signaling themselves[2].

5.4. Drug Therapy

Given that CAFs have such an important role in tumor progression and invasion, they are logical targets for cancer therapy. In addition, their contribution to cancer resistance to drugs is another reason why these cells should not be overlooked. Besides the already mentioned increases in interstitial pressure that interfere with drug delivery, overproduction of ECM components causes blood and lymphatic vessels to collapse[51] and work as a physical barrier to drug access.

In a broad analysis, CAFs are relatively good drug targets due to their genetic stability[38], when compared to cancer cells, to their actions as regulators of the matrix around the tumor and to their role in tumor invasivness[32].

There are several drugs that target CAFs, both in clinical trials and some of them already approved, for specific types of cancer. Anti-CAFs drugs are organized in three different classes, based on their targets: class 1 compounds inhibit signal transduction pathways (TGFβ and PDGFR inhibitors, for example); class 2 target CAFs and their metabolic products more specifically (such as MMP inhibitors); and class 3 comprises other compounds that do not fit into the two previous classes (as COX-2 inhibitors)[38].

A selection of some examples of these drugs was made based on the ones thought to be more promising and based on the most mentioned targets in the analyzed papers. More detailed information on drugs against CAFs on clinical trials can be found in the literature[38].

- PDGFR inhibitors

Since PDGF stimulates cell growth, division and angiogenesis and also acts on fibroblasts and CAFs to induce the release of growth factors in a positive-feedback manner[38], targeting it has major effects on tumor growth and drug delivery[34].

There are already some marketed drugs as PDGFR inhibitors, such as the recently approved axinitib (Inlyta®), to be used on kidney cell carcinoma, but several others in Phases I and II of clinical trials[38]. Crenolanib, for example, has shown encouraging results for relapsed/refractory acute myeloid leukemia patients during Phases Ia and Ib and is expected to move into Phase II shortly[56], since it is recruiting patients at the present.

- TGF-β inhibitors

TGF-β is of main importance for recruitment and differentiation of fibroblasts into myofibroblasts and, in a sustained stimulus as it happens in cancer, into CAFs[38]. Perfenidone (Esbriet®) is approved in Europe and in the United States but for treatment of idiopathic pulmonary fibrosis, due to its anti-fibroblastic activity (not CAF specific)[33,57,58]. Other molecules are being tested for their anti-tumoral properties such as LY2157299, that finished Phase I on healthy patients and is now recruiting patients with metastatic pancreatic cancer[38,59].

- MMP inhibitors

MMPs were the most obvious targets regarding CAFs' role in cancer progression[32].

Although exciting results were gathered during pre-clinical trials, clinical trials were unsatisfactory either by lack of relevant results or by the development of serious side effects. One of the reasons for these results is the variety of MMPs as well as they ubiquitous distribution and physiological roles[4,38].

PCK3145 is a MMP-9 inhibitor with good results on Phase I trials but further information is lacking[38,60].

- Cyclooxygenase isoform 2 (COX-2) inhibitors

COX-2 is normally expressed during inflammation but is also overexpressed during cancer, firstly demonstrated by the co-culture of cancer cells with CAFs[32].

One of their tumor promoting roles has to do with the production of prostaglandin E1 that promotes HGF releasing[38]. Although new COX-2 inhibitors continue to be studied[38], celecoxib (Celebrex®) was approved by both the FDA and EMA for the treatment of familial adenomatous polyposis[61], although EMA eventually pulled back on this off-label aplication[62].

6. CONCLUSION

Cancer-associated fibroblasts and other cell types such as cancer-associated macrophages or endothelial progenitor cells[9] alert us for the fact that a therapeutic approach on cancer will not (and is not) successful when only one type of cell is targeted. In fact, this review highlights the fact that without other mediators in the tumor microenvironment, cancer would not present itself with such high mortality rates.

Nevertheless, to achieve a positive therapeutic outcome it is essential to detect cancer as soon as possible, before epigenetic changes occur, so that cells are localized and not phenotypically prepared for metastasis. This is only possible if modern medicine embraces novel molecular screenings as regular diagnostic tools and not only as research techniques. Even though some of these methodologies still need improvement for clinical use, counting on macromolecular or observational diagnosis is not a good option either since, by the time tissue alterations are visible, cellular adaptation to abnormal cells is already established.

A change in mentality towards cellular markers and protein expression has to be made, despite professional dogmas or economic status, in order to achieve better characterization of tumors and therapy rationalization and consequent success.

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