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Azurin-expressing Bone Marrow-Mesenchymal Stem / Stromal Cells in anticancer therapies

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Abstract

Current anti-cancer therapies include surgery to remove the tumors, radiotherapy and chemotherapy. However, such therapies present several inconvenients including the lack of specificity and generalized toxicity which may prevent complete regression and/or tumor recurrence. For these reason, novel approaches specifically targeting the tumor microenvironment are needed. Recently, cell-based therapies have attracted attention and, particularly, Mesenchymal Stem/ Stromal cells (MSCs) have been explored as delivery vehicles for anticancer agents due to their unique features, including immunomodulation, secretory activity, self-renewability and high migration potential towards tumors. Azurin, a bacterial protein produced by *Pseudomonas aeruginosa*, has been explored regarding its antitumoral capacity. Azurin preferentially enters cancer cells inducing little side effects either *in vitro* or *in vivo*, leading to tumor cell apoptosis and inhibiting several signaling pathways crucial for tumor progression. Here, we take advantage of the tumor tropism capacity revealed by MSCs and azurin antitumoral effect, coupling these two rationales together in an innovative cellular therapy by genetically engineering MSC towards the production and secretion of azurin. In microporated Bone Marrow-MSCs (BM-MSCs), azurin was detected in their extracellular media. Additionally, the tumor tropism of unmodified MSC towards the breast (MCF-7) and colon (HT-29) tumor cell lines was observed through indirect co-cultures. The next step will be to study the effects of azurin-producing MSCs in tumor progression through co-culture assays. By the end of this project, we aim to achieve a biological system that directly targets the tumor cells and potentiates the specificity, density and azurin's life-time in the tumor microenvironment.

Keywords: Mesenchymal Stem/Stromal Cells, Azurin, Cancer, Cell-based Therapy, Targeted Delivery

Resumo

As atuais terapias anticancerígenas incluem cirurgia para remover o tumor, radioterapia e quimioterapia. No entanto, essas terapias apresentam vários inconvenientes, incluindo a falta de especificidade e toxicidade generalizada que pode impedir a completa regressão e/ou a recorrência do tumor. Por estas razões, são necessárias novas abordagens que visam especificamente o microambiente do tumor. Recentemente, terapias celulares, particularmente as Células Estaminais do Mesênquima (CEMs), têm atraído a atenção da comunidade científica e têm sido explorados como veículos de entrega para agentes anticancerígenos, devido às suas características únicas como a atividade imunomodulatória, atividade secretora, auto-renovabilidade e alto potencial de migração para tumores. A azurina, uma proteína bacteriana produzida por *Pseudomonas aeruginosa*, tem sido explorada quanto à sua capacidade anti-tumoral. A azurina entra preferencialmente nas células cancerígenas induzindo poucos efeitos secundários, quer *in vitro* ou *in vivo*, levando à apoptose destas células e inibindo várias vias de sinalização importantes à progressão do tumor. Neste estudo, nós tiramos proveito da capacidade de tropismo tumoral revelado pelas CEMs e do efeito antitumoral da azurina numa terapia celular inovadora através da engenharia genética de CEMs para a produção e secreção de azurina. Nas (Medula Óssea-CEMs) MO-CEMs microporadas, a azurina foi detetada nos seus meios extracelulares. Além disso, o tropismo tumoral das CEMs não modificadas no sentido das linhas tumorais de mama (MCF-7) e do cólon (HT-29) foi observado por meio de co-culturas indirectas. O próximo passo será estudar os efeitos de CEMs produtoras de azurina na progressão tumoral através de ensaios de co-cultura. Até o final deste projeto, pretendemos alcançar um sistema biológico que atinge diretamente as células tumorais e potencializa a especificidade, densidade e tempo de vida da azurina no microambiente tumoral.

Palavras-chave: Células Mesenquimais Estaminais, Azurina, Cancro, Terapias celulares, Administração localizada

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List of Abbreviations

AT-MSCs – Adipose Tissue- derived Mesenchymal Stem/Stromal Cells

BBB – Blood Brain Barrier

BM- MSCs – Bone Marrow- derived Mesenchymal Stem/Stromal Cells

BSA - Bovine Serum Albumin

CAFs – Cancer Associated Fibroblasts

CSC – Cancer Stem Cells

DKK - Dickkopf protein

ECM – Extracellular Matrix

EGF – Pro-epidermal factor

EMT – Epithelial Mesenchymal Transition

FGF – Fibroblast Growth Factor

GCSF - Granulocyte colony-stimulating factor

GDEPT - gene-directed enzyme-producing therapy

HGF - Hepatocyte growth factor receptor

HSCs – Hematopoietic Stem Cells

IFNβ – Interferon β

IL – Interleukin

Lcn2 – Lipocalin 2

MSCs - Mesenchymal Stem/Stromal Cells

NGAL - neutrophil gelatinase-associated lipocalin

OPG- Osteoprotegerin

PAI – Plasminogen Activator Inhibitor

PCNA - proliferating cellular nuclear antigen

PDGF - Platelet-derived growth factor

PEDF - Pigment epithelium-derived factor

SDF – Stromal cell Derived Factor

STC - Stanniocalcin

TAMs - Tumor-Associated Macrophages

TGF – Transfroming Growth Factor

TNF – Tumor Necrosis Factor

TRAIL - Tumor Necrosis Factor-related Apoptosis-Inducing Ligand

UCB-MSCs – Umbilical Cord Blood- derived Mesenchymal Stem/Stromal Cells

VEGF - Vascular Endothelial Growth Factor

1. Introduction

1.1. Mesenchymal Stem Cells as a potential cancer therapy tool

Cancer is a malignant tumor evolving from an abnormal cell growth with the potential to invade or spread to other parts of the body. This disease represents a significant and growing public health threat worldwide with increasing incidence. Conventional therapies include surgery, radiotherapy and chemotherapy. Surgical attempts at complete excision rarely are successful and local recurrence is common, radiotherapy damages normal tissues and metastasis can recur due to radioresistance, and once the disease becomes metastatic, standard chemotherapy has little effect (Ahn *et al*, 2013; Jeong *et al*, 2015). For these reasons, several alternative therapeutic strategies have been investigated in order to develop a novel approach to treat cancer with more specificity and robustness. Tumor specificity is one of the major drawbacks of conventional cancer therapies. Due to this unspecificity various side effects on normal tissues are normally observed. To overcome these limitations, novel approaches have recently focused on selectively target tumors by applying various drug delivery systems such as stealth liposome, magnetic nanoparticles and peptides (Harati *et al*, 2015). Most recently, cell-based therapies have extensively attracted the scientific community attention. Human mesenchymal stem/ stromal cells (MSCs) hold a promising future for cell-based therapies due to their immunomodulatory properties, secretory activity, simplicity of isolation, *in vitro* expansion, selfrenewability and high migration potential towards injured sites, including tumors (Kidd *et al*, 2009; Harati *et al*, 2015). These unique features rendered MSCs to function as a new and promising therapeutic vehicle to deliver anticancer agents directly to the tumor site and their metastasis. Other advantages of using MSCs as delivery systems for cancer treatment include the ease of genetically modify them to produce a specific anti-cancer agent due to the wide variety of vectors available to transduce genes into MSCs, including retroviral, lentiviral and adenoviral and also non-viral methods such as electroporation (Kanehira *et al*, 2007), the efficiency of transduction is high, they provide a stable expression and longtime secretion of the transduced factors directly in the tumor site (Mao *et al*, 2012).

1.1.1. Defining Mesenchymal Stem Cells

Mesenchymal Stem/Stromal Cells (MSCs) are the stromal component of bone marrow and represent one of the most promising prospects for tissue regeneration and repair. They are multipotent cells that give rise to a variety of cell types such as osteoblasts, chondrocytes, myocytes and adipocytes. MSCs have a great capacity for self-renewal while maintaining their multipotency and an undifferentiated state which is an important property for their *in vitro* culture expansion in the laboratory (Caplan, 1991).

MSCs present a set of remarkable features, listed below, that assigns them a promising statute for clinical applications.

Homing Capacity

One of the most unique features of MSCs is their homing capacity, also known as tropism. Stem cell homing is a phenomenon that was initially related to hematopoietic stem cells (HSCs) since they are able to migrate through the bloodstream to different organs and return to their niches in the Bone Marrow (BM) under the guidance of chemical signaling. Similarly to HSCs, MSCs exhibit similar homing properties. This phenomenon facilitates the BM stem cells to migrate and engraft into injured tissues. When BM-MSCs are systemically administered after a stroke, the cells migrate and home towards the brain and are shown to acquire neuronal phenotype with expression of nerve cell specific markers, improving the functional outcome (Hansen *et al*, 2016). Several animal studies indicate that direct delivery of MSCs to injured tissues can significantly promote their structural and functional recovery. These cells migrate and engraft to injured tissues regardless of the causes of the injuries and the tissue type (Zhao, 2013).

Regarding the tumor microenvironment, the underlying mechanisms of MSC homing and differentiation within tumors are still not well understood. Tumor microenvironments are considered “wounds that do not heal” including a large proportion of inflammatory cells and MSC homing is thought to occur in a chemokine directed manner within the context of ongoing inflammation. The diverse factors known to be induced within tumors include: EGF, VEGF-A, PDGF, FGF, GCSF, G-MCSF, HGF, TGF-β1, CXCL7, CXCL6, CXCL5, CXCL8, CXCL12, CCL2, IL-6, and urokinase-type plasminogen activator (Studeny *et al*, 2004; Hung *et al*, 2005; Ponte *et al*, 2007; Droujinine *et al*, 2013; Studeny *et al*, 2002; Ren *et al*, 2008; Loebinger *et al*, 2009; Segers *et al*, 2006; Wang *et al*, 2009; Klopp *et al*, 2007). It is thought that combinations of these factors may help promote directed MSC recruitment. To help facilitate their directed migration, MSCs are known to be able to express virtually all chemokine receptors (CCR, CXCR, CXC3R and XCR), but the level of expression may change as a result of cell culture conditions, or with the tissue source of the MSC (von Luettichau *et al*, 2005; Fox *et al*, 2007). The CXCR4-SDF1/CXCL12 chemokine axis has been strongly linked to MSC recruitment (Shi *et al*, 2007; Kucia *et al*, 2005).

Immunomodulatory Capacity

A feature that allows MSCs to be promising targets for clinical use is their immunomodulatory capacity. Since MSCs lack immunogenicity due to the low expression of the major histocompatibility complex-I (MHC-I) and the absence of expression of MHC-II, they escape the inflammatory system from the host. This hypo-immunogenic property allows for the transplantation of MSCs allogenically, meaning that the transplant receiver may be different from the donor. Also, MSCs can modulate the

functions of the immune system by interacting with the immune cells. They have the ability to regulate the proliferation, activation and maturation of B lymphocytes and to suppress T lymphocytes proliferation, and this suppression occurs independently from the donor source. They also inhibit natural killer cells and modulate the activity of dendritic cells. Interleukin (IL)-2 is a type of cytokine signaling molecule in the immune system that regulates the activities of white blood cells. MSCs have the ability to take effect before the secretion of these molecules by the immune system avoiding the triggering of an immune response towards them (Zhao, 2013; Abdi, *et al* 2008).

Anti-apoptotic Capacity

During the process of apoptosis, Caspases, a family of cysteine proteases, are activated. MSCs are able to inhibit the activation of caspase-3, the ultimate apoptotic enzyme, thus preventing the apoptotic pathway. In a study performed by Aina He and coworkers in 2009, the role of MSCs in the prevention of cardiomyocytes apoptosis after myocardial infarction was analyzed. The cardiac function and the infarct size of myocardial infarcted rats were assessed by echocardiography after MSCs transplantation. The results showed that cardiomyocyte's apoptosis was significantly reduced by the treatment with MSCs (He A, *et al* 2009).

Anti-fibrotic Capacity

The term Fibrosis refers to the deposition of a fibrous connective tissue in an organ or tissue as a response to injury or damage. Fibrosis naturally occurs as a part of the healing process in response to injury in a process named scarring, or occurs due to the excess tissue deposition which is a pathological condition. Activated fibroblasts play an important role in the pathogenesis of pulmonary fibrosis by remodeling the tissue physiology. And the transforming growth factor-beta 1 (TGF-β1) has a predominant role in the transformation of fibroblasts into myofibroblasts that are responsible by the formation of ECM and fibers, leading to the excessive deposition of connective tissue. In a study performed by Li-Hua Dong and colleagues, the potential role of MSCs in the regression of fibrosis was assessed. After the infusion of MSCs in rats that received semi-thoracic irradiation, which induces pulmonary fibrosis, the levels of TGF- β1 decreased. The physiology of lungs was preserved without the activation of fibroblasts or collagen deposition within the injured sites. Therefore, the injection of MSC has an important role in the protection of the lung tissue from radio-induced fibrosis, thus representing a powerful tool in the prevention of this malignant condition (Dong *et al*, 2015).

1.1.2. Double role of Mesenchymal Stem Cells in cancer treatment

Considering the features of Mesenchymal Stem Cells, their application in cell-based therapies has gradually become a promising medical tool for several diseases, including cancer. As previously mentioned, mesenchymal stem cells feature tropism to specific tissues. Evidences show that MSCs have tropism towards tumor cells (Kidd *et al*, 2009; Bak *et al*, 2011; Kim *et al*, 2011; Jiao *et al*, 2011; Choi *et al*, 2012; Doucette *et al*, 2011; Yin *et al*, 2011; Park *et al*, 2011) and this raised wide interest regarding their potentialities as a delivery vehicle for anti-cancer agents. However, the role of these cells in cancer development is still very controversial. While a set of studies states that MSC may promote tumor progression, others claim their tumor suppressive effect. The discrepancy between these results may arise from issues related to the isolation of tumor cells, MSCs and tumor linages from different tissue sources, individual donor variability, the injection/ treatment timing of MSCs and study models (Yagi *et al*, 2013).

Mesenchymal Stem Cells enhance tumorigenesis

Tumor development and progression has been recognized as the product of an evolving crosstalk between different cell types within the tumor and its surrounding supportive stromal tissue. The mutual interaction between tumor cells and stromal cells via direct contact or through the production of growth factors, cytokines and chemokines in a paracrine manner are thought to modulate tumor expansion, invasion, metastasis and angiogenesis (Zhang *et al*, 2013 a.).

Nutrient deprivation and oxygen deficiency are representative characteristics of solid tumor microenvironment during cancer development. When tumors grow their carcinoma and stromal cells can undergo starvation due to the insufficient amount of nutrients in the niche. The study performed by Zhang and colleagues in 2013, was the first to address the role of hMSC in lung carcinoma cells under serum deprivation. The viability of carcinoma cells grown with MSC after serum starvation is higher than that of cells grown without the stromal cells. Also, in the cells cultured with MSC, a marked decrease in apoptosis was observed. This brought the hypothesis that MSC were protecting cancer cells through a mechanism that leads to a decrease in apoptosis. The results showed that the presence of MSCs during serum deprivation increased the levels of Beclin-1, a protein that plays a central role in autophagy. Also, the authors examined the accumulation of autophagosomes and verified an increased number in the cultures where MSC were present. The tumor cells incubated with the autophagy inhibitor 3-methyladenine (3-MA) lacked autophagic activity and showed an increased number of apoptotic cells comparing with the non-3-MA-treated groups. In conclusion, the autophagy mechanism activated by MSCs is involved in the tolerance of lung carcinoma in serum deprivation by the decrease of the apoptotic pathway (Zhang *et al*, 2013 b.). The same results were obtained by Morikawa in 2015 regarding the role of Beclin-1 expression in the microenvironment of breast cancer (Morikawa *et al*, 2015).

MSCs are known to produce a vast array of cytokines and growth factors that are deeply connected with cancer progression. The *in vitro* interaction between breast cancer cells (MCF-7 cell

line) and MSCs or its products induces major functional and structural changes in the tumor progression. The co-culture of MCF-7 and MSC induces changes in the structure and the augmentation in size of mitochondria and nuclei. This increases the potential for proliferation of these cancer cells in 30 to 40% in comparison with cancer cells cultured without MSC. Other functional feature changed by the presence of stromal cells is the low level of E-cadherin expression leading to the growth of cancer cells as single layers, whereas control cancer cells grow as cohesive monolayer clusters. This phenotype strongly potentiates the epithelial–mesenchymal transition (EMT), a necessary requirement from metastasis. When culturing MCF-7 cells with the culture media where MSC have been grown (MSC-conditioned media) the increment in proliferation and the down-regulation of E-cadherin have also been observed. This may indicate that the factors produced by mesenchymal stem cells are involved in their tumor promoting capacity. The vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) were found to enhance MCF-7 proliferation and to suppress the expression of E-cadherin, respectively when incubated with each factor separately (Fierro *et al*, 2004). VEGF plays an important role in the setup of the tumor microenvironment by inducing angiogenesis, vasculogenesis, endothelial cell growth, endothelial cell proliferation, cell migration, permeabilization of blood vessels and by inhibiting apoptosis. Also, VEGF is produced by tumor cells and is reported to enhance and direct stem cell motility to the tumor site since these cells present membrane receptors for these molecules and the depletion of this growth factor using antibodies reduced MSCs migration capacity (Yagi *et al*, 2013). Given this information we can verify that a cumulative effect of VEGF production might occur, leading to a sharp increase in the tumoral mass and development of the ideal proliferative microenvironment.

Cancer associated fibroblasts (CAFs) are involved in cancer progression and are a component of the tumor. They stimulate cancer cell growth, inflammation, angiogenesis and invasion, through specific communications with cancer cells. CAFs also secrete a variety of cytokines and growth factors such as CXCL1, CXCL2, IL-1β and IL-6, which are deeply involved in the inflammatory response, one of the hallmarks of cancer progression, and in cell proliferation. IL-6 is also involved in cancer cell migration and modulates the frequency of VEGF production, hence indirectly monitors angiogenesis. MSCs were found to home to tumors and transit into CAFs leading to the formation of a much more aggressive type of tumor (Huang *et al*, 2012).

As already observed, the conditioned medium from MSCs induces changes in the tumor expansion, angiogenesis and cell migration through the influence of VEGF and IL-6. The opposite experiment was also performed by Zhang and colleagues in 2013. The pro-angiogenic factors gene expression changes in MSCs when exposed to tumor-conditioned medium were analyzed. Interestingly, the pro-angiogenic factors TGF-β, VEGF and IL-6 had a substantial increased gene expression in MSC cells subjected to tumor-conditioned media, comparing with MSC cultured without this media (Zhang *et al*, 2013 a.). This may state that although MSCs already express these factors, the contact with tumor cells and their products is the ultimate condition for MSC to over-express these pro-angiogenic factors.

Several human cancers, including breast cancer, may arise from a population of cells that display stem cell properties such as self-renewal, which is responsible for tumorigenesis and cell

expansion, and differentiation which contributes to cancer cell heterogeneity. This population of cells is known as cancer stem cells (CSC). There are evidences that these cells are very resistant to chemotherapy and radiotherapy, thus contributing to tumor relapse and poor prognosis. As previously mentioned, MSC produce IL-6, a chemokine implicated in the regulation of CSC. Liu and colleagues investigated the interactions between MSC and breast CSC and the potential role of MSC in the CSC niche. In order to assess the ability of MSCs to affect breast CSC functionality, the authors performed co-cultures between those two types of cells and observed that these interaction lead to an increase in CSC self-renewal and that this outcome was independent on their direct contact. Although, expansion of CSC population was not reproduced when adding conditioned media obtained from a culture of MSC alone. This suggests that CSC expansion is regulated by soluble factors that are generated as a result of the interaction between MSCs and cancer cells. When analyzing the gene expression pattern after co-culture, the CXCL7 mRNA revealed a 6-fold increase in MSCs and also IL-6 revealed an increased expression. This demonstrates the important role for these two cytokines in the crosstalk between MSC and cancer cells. Interestingly, CXCL7 induces by itself a 3-fold increase in cancer cells proportion. When using a CXCL7-blocking antibody the interaction of cancer cells and MSCs is inhibited and using an IL-6-blocking the interaction in partially inhibited. The main role of CXCL7 is to mediate the production of other cytokines like CXCL1, CXCL5, CXCL6, IL6 and IL8 by the cancer cells which are directly involved in the crosstalk. The role of IL-6 in this pathway is to regulate the production of CXCL7. The addition of IL-6 produces a 10-fold increase in CXCL7 production by MSC. All these findings suggest the existence of a cytokine network that mediate the interaction between MSCs and CSC in which IL-6 produced by cancer cells interacts with a receptor expressed by MSC, which triggers the production of CXCL7 that will induce the secretion of various cytokines by both CSC and MSC, including IL6. All these secreted cytokines are able to expand CSC population. This network represents a positive feedback loop, explaining the increment in cancer cell population after the contact with MSCs *in vivo* (Liu *et al*, 2011).

Plasminogen activator inhibitor type 1 (PAI-1) levels have been shown to be increased in colorectal cancer tissue and is of particular clinical relevance in this type of cancer since high serum and tissue levels have been shown to correlate with poor prognosis. These cytokines are serine proteases inhibitors and strong inhibitors of fibrinolysis, the physiological process that degrades blood clots. MSCs are known to be present in colon tumors and to produce pro-inflammatory cytokines, including PAI-1. In order to determine the functional effect of MSC-secreted PAI-1, Hogan and colleagues assessed its outcome in colon cancer proliferation and migration. Both cell lines examined (HT29 and HCT-116) demonstrated an increasing cell migration potential in response to increasing PAI-1 concentrations (Hogan *et al*, 2013). PAI-1 can play a role in cell migration owing to its dual role in regulating the cell adhesion. It can be either pro-migratory or anti-migratory, depending on whether PAI-1 locates at the leading or trailing edge of the cell and on the PAI-1 concentration. This cytokine induces an attachment-detachment-reattachment model of integrins that is necessary for the tumor cell successive detachment and forward reattachment in the extracellular matrix, promoting migration. Regarding cell proliferation, the role of PAI-1 is very controversial since different results arise from different test cell lines. In the case of the colon cancer cell line HT-29 the presence of PAI-1 inhibits

the cell proliferation, whereas in HCT-116 there is an increment in cell proliferation (Hogan *et al*, 2013).

Stromal cell-derived factor-1α (SDF-1α) also known as CXC chemokine ligand 12 (CXCL12) is a chemoattractant produced by bone marrow mesenchymal stem (BM-MSC) cells that activates the receptor CXCR4 inducing a rapid and transient rise in the level of intracellular calcium ions leading to chemotaxis. This chemokine is strongly involved in stem cell mobilization and homing. CXCR4 allows tumor cells to access other cellular niches being pivotal for metastatic spread to organs where SDF-1α is expressed. Furthermore, SDF-1α promotes angiogenesis by recruiting endothelial cells to the tumor microenvironment and can stimulate survival and growth of neoplastic cells. Studies reveal that CXCR4 overexpression is present in several types of cancer, including ovarian, prostate, esophageal, melanoma and neuroblastoma. Bone Marrow-MSCs can attract circulating prostate cancer cells through the release of SDF-1α. The high concentration of this chemokine is recognized by the prostate cancer membrane receptor CXCR4. In the experiment performed by Mognetti and colleagues, beyond the detection of SDF-1α increase after co-culturing prostate cancer cells with BM-MSC-conditioned media, also the kinase Erk increased in 38.6% comparing with the control and kinase Akt increased in 71.2%. Akt is a kinase enrolled in biological processes like angiogenesis, cell proliferation, cellular survival and growth and Erk in several cell biological activities. When CXCR4 recognizes SDF-1α, Akt is activated by phosphorylation, triggering a kinase cascade culminating in the alteration of gene expression pattern in tumoral cells. This new expression pattern normally leads to the actin polymerization, cell skeleton rearrangement and cell migration (Mognetti *et al*, 2013). These results are also observed in human breast carcinoma (Rhodes *et al*, 2010) and human melanoma (Kucerova *et al*, 2010).

Stanniocalcin-1 (STC1) is a glycoprotein produced by MSCs that is involved in biological processes like bone development, ossification, cardiac muscle cell contraction and also has an important role in alteration of mitochondrial function by upregulating mitochondrial uncoupling protein 2 (UCP2). UCPs are mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane, leading to energy dissipation in the form of heat and they are found in some types of cancer, such as lung cancer. The finding of the upregulation of STC1 by MSCs when subjected to hydrogen peroxide (H_2O_2) lead to the hypothesis that this glycoprotein would be involved in a response pathway to oxidative stress (Block *et al*, 2009). To test this, Ohkouchi and colleagues tested the percentage of reactive oxygen species (ROS) decrease in lung cancer cells cultured with MSCs comparing with cancer cells grown alone. As expected, the co-culture with MSC displayed a 30% reduction compared with cancer cells cultured alone and observed an upregulation of UCP2 by cancer cells. Also, when knocking down STC1 expression in MSCs, the cytoprotection of cancer cells when subjected to H_2O_2 is inhibited, the increase of ROS production is observed in the cancer cells and the levels of messenger RNA for UCP2 decrease. UCP2 have a critical role in this process, since when it is not present, STC1 in unable to reduce the ROS production and maintain cancer cells viability. Therefore, STC1 produced by MSCs in tumor stroma plays a critical role in enhancing the tumor resistance to ROS by upregulating UCP2 that increases the efficiency of mitochondria in

generating a redox system found is several tumors, contributing to the proliferation of cancer cells in the presence of environments that generate ROS (Ohkouchi *et al*, 2012).

Table 1 summarizes the factors described and demonstrates the cancer type studied and the source of MSCs isolation in each study.

Table 1 Summarizing table with some of the known factors produced by MSCs involved in cancer progression. It contains the cancer type studied, the MSCs source of isolation and the reference of the study

Factor	Cancer type studied	MSC source	Reference
Beclin-1	Breast	Bone marrow	Morikawa *et al,* 2015
	Lung	Bone marrow	Zhang *et al*, 2013 **b.**
CXCL7	Breast	Bone marrow	Liu *et al*, 2015
IL-6	Breast	Bone marrow	Fierro *et al*, 2004
	Breast	Bone marrow	Liu *et al*, 2015
	Colon	Bone marrow	Huang *et al*, 2012
	Colon	Adipose tissue	Rhyu *et al*, 2015
PAI-1	Colon	Bone marrow	Hogan *et al*, 2013
SDF-1α	Breast	Bone marrow	Rhodes *et al*, 2010
	Prostate	Bone marrow	Mognetti *et al,* 2013
	Melanoma	Adipose tissue	Kucerova *et al,* 2010
STC1	Lung	Bone marrow	Ohkouchi *et al*, 2012
VEFG	Breast	Bone marrow	Fierro *et al*, 2004

Mesenchymal Stem Cells suppress tumorigenesis

Despite the several studies pointing on the pro-tumorigenic features of mesenchymal stem cells, there are also studies exploring their anti-tumoral capacity. In the present section, a set of studies found in the literature about the tumor suppressive influence of MSCs presence in the tumor microenvironment is shown.

Khakoo demonstrated in 2006 that MSCs injected in an *in vivo* model of Kaposi's sarcoma (KS), migrate towards the tumor site and arrests its growth. Protein kinase Akt is a critical mediator of KS tumor growth and survival, wherein its activity inhibition strongly suppresses KS proliferation. In this study the authors proved that bone marrow-MSCs (BM-MSCs) specifically inhibited Akt activation within KS cells and that this interaction requires direct cell contact. Thus E-cadherin has a critical role in the tumor suppressive feature of MSCs since it mediated the direct interplay MSC/KS. Although, in contrast with this effect, incubation of MSCs with a prostate tumor cell line and a breast cancer cell line had no effect on the inhibition of Akt. The blockage of Akt impairs tumor cells survival and

proliferation due to an increased measure of tumor necrosis (Khakoo *et al*, 2006). This result goes against the work developed by Mognetti, Rhodes and Kucerova, presented on the previous section, on which the presence of MSC seemed to improve the function of Akt.

Stem cells and tumor cells share many similar features. Such as the active signaling pathways that regulate self-renewal and differentiation, including Wnt, Notch, Shh and BMP pathways. Genes regulated by Wnt signaling are involved in metabolism, proliferation, apoptosis and tumorigenesis. For example, Wnt3a increases expression of the antiapoptotic protein Bcl-2 and proliferating cellular nuclear antigen (PCNA) which is an auxiliary protein of DNA polymerase increasing its processibility during the elongation phase. An aberrant activation of the Wnt signaling pathway can lead to cancer development. Qiao and colleagues observed in their study that when co-culturing a line of breast cancer cells with fetal isolated MSC conditioned media, a clear inhibition is observed. This suggests that the soluble factors produced by MSCs are capable of inhibiting tumor cell growth. Also, they observed that the expression of β-catenin (an intracellular signal transducer in the Wnt signaling pathway), c-Myc (an oncogene), Bcl-2, PCNA, and survivin proteins were down-regulated in tumor cells mediated by MSC conditioned media. Dickkopf (Dkk) is a protein that competes with the Wnt pathway, binding to LRP5/6, thus inhibits the activation of Wnt signaling pathway. This protein was observed to be up regulated in MSCs leading to the hypothesis that MSCs inhibit the Wnt signaling in tumor cells. In fact, when Dkk-1 is suppressed, a tumor progression is observed and the down-regulation of β-catenin and c-Myc is no longer observed. These findings led to the conclusion that Dkk-1 secreted by MSCs is an upstream inhibitor of Wnt signaling in breast cancer cells (Qiao *et al*, 2008 a.). Other experiment also conducted by Qiao and colleagues in 2008 showed the same outcome when subjecting hepatoma cell lines to the same MSCs used in the previous study (Qiao *et al*, 2008 b). In 2009, Zhu and colleagues also proved the inhibitory effect of AT-MSCs on breast carcinoma progression by negatively regulating the Wnt pathway through the secretion of Dkk-1 (Zhu, *et al*, 2009).

The role of the immune response in tumor progression is still a complicated subject. The inflammatory microenvironment was considered to be the seventh wallmark of cancer progression (Colotta *et al*., 2009) depending on the kind of immune system operating, since it is described that the innate immune system is pro-tumorigenic (Colotta *et al*., 2009) and the adaptive immune system is involved in tumor suppression (Mantovani *et al*., 2008). In the case of colitis-associated colorectal cancer (CAC) the role of the immune system is also very controversial. Chronic colitis accompanied by a large accumulation of T helper cells promotes neoplastic risk, whereas excessive immunosuppression regulated by Treg cells (Regulatory T cells) enhances the survival of tumor cells. MSCs modulate the behavior of immune system cells, including Treg cells, having an anti-inflammation effect on colitis and suppresses CAC. A study conducted by Tang in 2015 evaluated the influence of UCB-MSCs on a CAC mouse model. In fact, after the injection of MSCs, they migrate to the colon and decrease the incidence of colitis-related neoplasm by inhibiting the inflammatory cytokines including IL-1, IL-5, IL-6 and IL-12. Also, Treg cells are up-regulated by the presence of MSCs, leading to the supression of colitis and preventing the neoplasm development (Tang *et al*, 2015).

Human gliomas are the most commonly diagnosed malignant adult primary brain tumors since they feature resistance to induction of cell death by apoptosis in response to radiotherapy or chemotherapy. Such inauspicious prognosis encouraged the search for new treatments. Since MSCs have been used for treatment of neurological diseases such as cord injury and cerebral palsy (Gutiérrez-Fernández *et al*, 2013), Yang and colleagues investigated the influence of MSCs in glioma development, given the controversial role of MSCs towards cancer. In order to understand whether different sources of MSCs could have different outcomes regarding the development of human glioma cells, the authors analyzed two easily accessible MSCs sources, human adipose tissue-derived mesenchymal stem cells (AT-MSCs) and umbilical cord-derived mesenchymal stem cells (UC-MSCs). The study revealed that the conditioned media from both types of MSCs can significantly inhibit the growth of human glioma cell line. The results were even more positive for umbilical cord derived MSCs which inhibited the tumor growth in more than 50%. When analyzing the factors present in the MSC-conditioned media, the authors found that these cells secrete several pro-apoptotic related proteins such as bad, bax, bak and bim and pro-apoptotic proteins like caspase 3 and 9. And anti-apoptotic proteins like Survivin and XIAP were significantly lower in MSCs-conditioned media culture, comparing to tumor cells grown alone. Gathering these results, we are able to conclude that MSCs inhibit the tumor growth by promoting apoptosis of these malignant cells. Another interesting finding in this study was the increased tumor cells arrested in G0/G1 phase after the MSC-conditioned medium subjection, preventing them to proceed to the cellular expansion. Finally, the authors uncovered, unexpectedly, that after the experiments, the MSC-conditioned medium induced a rapid and complete differentiation in glioma cells in a more mature astrocyte state. This is important to partially recover the glial cell morphology and function that was impaired in the tumor cells, leading to a less malignant state. Together, these results indicate that mesenchymal stem cells efficiently induce tumor cells apoptosis, arrest tumor expansion and induce the differentiation in glioma cells. And, although apoptosis is triggered more efficiently by umbilical cord blood-MSCs (UCB-MSCs), the differentiation induction is not distinguishable between UCB-MSC and AT-MSCs (Yang *et al*, 2014).

The previous experiment was encouraged by the work of Akimoto in 2013, where a similar experiment setup was employed in glioblastoma multiforme (GBM), the most aggressive and common type of glioma. In this study the same sources for MSCs were tested, UCB-MSC and AT-MSCs. Contrarily to the previous results obtained by Yang, the co-culture of AT-MSCs with tumor cells induced tumorigenesis. Although, a decrease in tumor mass was also obtained after co-culturing with UCB-MSC. These results demonstrated that the effectiveness of cytocoxicity against GBM differs among MSCs derived from different tissues. Behind these divergent outcomes, are the different expression pattern featured in MSCs from different sources. The chemokine SDF-1α (CXCL12), and the proangiogenic factors VEGF and angiopoietin 1 (Ang-1) are significantly expressed in a higher level in AT-MSCs than in UCB-MSCs. As previously mentioned in the section Mesenchymal Stem Cells enhance tumorigenesis, SDF-1α and proangiogenic factors are critically involved in the potential pro-tumorigenic effect of mesenchymal stem cells. SDF-1α act effectively as an antiapoptotic factor in GBM development by triggering a response in these tumor cells through the CXCR7 receptor. On the other hand, the proapototic factor tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and

tumor necrosis factor (TNF) are more expressed in UCB-MSCs than in AT-MSCs. For this reason, AT-MSCs can impair tumor cell apoptosis through the activation of the SDF1-α/CXCR7 pathway, whereas, UCB-MSCs have a proapoptotic effect against tumor cells. Moreover, the presence of SDF-1α plays a role in the inhibition of the TRAIL-induced pathway in GBM leading to a growing anti-apoptotic effect of the presence of AT-MSCs in the tumor environment. The results obtained *in vitro* in this experiment were consistent with those obtained *in vivo*. When co-transplanting UCB-MSCs with tumor cells into mice, the tumor weight substantially decreased, comparing with tumor cells injected alone. Whereas, when co-transplanting AT-MSCs with tumor cells a notorious increase in tumor size was observed (Akimoto *et al*, 2013). Consistent with these findings, the study performed by Kucerova in 2010 also displays the unconformity in the suppressive/ inducing role of AT-MSCs depending on the tumor cell line in study. Whereas in melanoma cells AT-MSCs have a protective effect inducing tumor progression, in glioblastoma cells this tumor favoring effect is no longer observed (Kucerova *et al*, 2010).

The experiments performed by Akimoto, Yang and Kucerova perfectly highlighted the importance of analyzing the different outcomes that mesenchymal stem cells may protrude depending on their source of isolation and also which type of tumor cells we are aiming to annihilate, since mesenchymal stem cells from the same source manifested different results in different tumor cell types, at the same time that different mesenchymal stem cell sources induced the same result in the same tumor cell type.

Table 2 summarizes the factors described and demonstrates the cancer type studied and the source of MSCs isolation in each study.

Table 2 Summarizing table with some of the known factors produced by MSCs involved in cancer suppression. It contains the cancer type studied, the MSCs source of isolation and the reference of the study

Factor/ Effect	Cancer type studied	MSC source	Reference
Dickkopf protein	Breast	Fetal tissue	Qiao *et al*, 2008 **a**
	Hepatoma	Fetal tissue	Qiao *et al*, 2008 **b**
	Breast	Adipose tissue	Zhu *et al*, 2009
Deregulation of Akt pathway through direct contact	Kaposi's sarcoma	Bone marrow	Khakoo *et al*, 2006
Inhibition of IL-1, IL-5, IL-6 and IL-12 and up regulation of Treg cells	Colon	Umbilical cord blood	Tang *et al*, 2015

(Table 2 continuation)

Factor/ Effect	Cancer type studied	MSC source	Reference
Promotion of apoptosis	Glioma	Adipose tissue	Yang *et al*, 2014
		Umbilical cord blood	Yang *et al*, 2014; Akimoto *et al*, 2013
TRAIL and TNF	Glioblastoma multiforme	Umbilical cord blood	Akimoto *et al*, 2013

1.1.3. Mesenchymal Stem Cells and their therapeutic applications

Present cancer therapies focus on combinations of surgery, chemotherapy and radiotherapy. Although there is an improvement in the development of the conventional therapies there are several obstacles to overcome regarding the poor response of tumors and their metastasis. So the rise of new therapies becomes an urgent need.

Although there is a clear controversy in the literature regarding the influence of mesenchymal stem cells in the progression of tumorigenesis, a wide set of clinical applications have been explored. The tumor homing capacity exerted by these cells combined with their immunomodulatory capacity and lack of immunogenicity are promising features in the matter of using these cells as vehicles for anti-cancer drug therapies allowing for a more precise, more direct and less invasive administration.

One of the most common approaches regarding the use of MSC in cell-based therapies seems to be the genetic manipulation of these cells towards the production of a specific anti-cancer agent. One popular factor known to induce apoptosis of tumor cells is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Although TRAIL presents a promising feature, its clinical application is very limited due to the possibility of causing damage in normal tissues. The intravenous administration of recombinant TRAIL contains several problems like short pharmacokinetic half-life and lack of targeted delivery, leading to its frequent and high dosage administration. The hypothesis of a delivery vector of this factor holds out to solve this constrains. In 2009, the group of Loebinger suggested MSC as a delivery vehicle for this factor given their prosperous features in the field. They developed MSCs engineered to produce and deliver TRAIL to a lung metastatic cancer model using lentivirus. These cells are able to kill cancer cells *in vitro* via the extrinsic death pathway to a higher degree than the recombinant protein control and *in vivo* they reduce the growth of early subcutaneous tumors and metastasis in mice. This was the first study in the literature demonstrating a substantial reduction in metastatic tumor burden with frequent eradication of metastasis using MSCs expressing TRAIL and shows the promising potential of genetically engineered MSC therapeutical applications for the treatment of primary tumors and their metastasis (Loebinger *et al*, 2009). The same prosperous results were observed in other cancer models such as colorectal (Grisendi *et al*, 2010), pancreatic (Grisendi *et al*, 2010), mesothelioma (Lathrop *et al*, 2015) and hepatocellular cancer (Deng *et al*,

2014). Although there are some findings regarding the production of TRAIL in UCB-MSCs, the increase yield production of this factor allows for a more effective administration (Akimoto *et al*, 2013). In 2013, Ahn and colleagues developed a protocol for producing AT-MSCs expressing Interferon-β (INF-β). INF-β is a cytokine with a potent pro-apoptotic effect capable of inhibiting both tumor growth and angiogenesis. The use of MSCs as vehicles overcomes limitations in the use of this factor regarding the systemic administration such as the short half-life and toxicity for other cells than tumor cells. Compared with the other MSC sources, adipose tissue-derived MSCs are easier and simpler to isolate. In this study the authors showed the possibility of using a combination of stem cell-based therapy and chemotherapy in a canine malignant melanoma. AT-MSCs genetically engineered to produce INF-β migrate to the tumor site, reducing significantly tumor burden in an animal model. The authors constructed these cells using a lentiviral vector system. As mentioned in the previous sections, AT-MSCs seem to have the bigger outcome regarding the tumor progressive role of MSCs. Therefore, this study shows that even AT-MSC can successfully be used as a drug delivery vehicle to inhibit the tumor progression when genetically engineered. This was the first study to demonstrate the efficacy of combining a systemic chemotherapy with a stem-cell based targeted delivery of a cytokine to a malignant canine melanoma in mouse (Ahn *et al*, 2013). The same experimental set was applied by Dembinski and colleagues to successfully eradicate ovarian cancer (Dembinski *et al*, 2013).

Lipocalin 2 (Lcn2) or neutrophil gelatinase-associated lipocalin (NGAL) is a protein involved in processes such as infection, injury, asthma, arthritis, and cancer. It was shown to inhibit the pro-neoplastic factor HIF-1α and the pro-tumorigenic vascular endothelial growth factor (VEGF) synthesis. Harati and colleagues developed genetically modified BM-MSCs to produce Lcn2 and specifically track the sites of liver metastasis from colon cancer and target them to selectively inhibit its growth and development. Since the precise role of Lcn2 is not fully understood due to some controversial results in the literature, the authors decided to apply a plasmid expression system in order to transiently overexpress Lcn2, avoiding a continuous expression of Lcn2, potentially harmful. The authors observed that these engineered MSCs could home to the sites of metastasis in the liver and secrete Lcn2 triggering a significant inhibition of liver metastasis of colon cancer in injected mice, possibly due to the reduced expression of VEGF in cancer cells (Harati *et al*, 2015).

Interleukins are a group of cytokines that mediate communication between cells. Although Interleukin 2 (Zhao, 2013) (Abdi, *et al* 2008) and Interleukin 6 (Fierro *et al*, 2004) might promote tumor progression, there are multiple works showing that MSCs genetically engineered to produce other Interleukins can successfully inhibit tumorigenesis. Although Interleukins are potent anti-cancer agents, when systemically administered in at high dosages they became cytotoxic. Therefore a new therapy is urgently needed providing a specific and targeted administration of these drug and MSCs promise to accomplish this task (Hu, 2011). In 2007, the group of Kanehira developed BM-MSCs producing Interleukin 32 (NK4), an antagonist of hepatocyte growth factor (HGF) which is a multifunctional growth factor that stimulates mitogenesis, motogenesis and morphogenesis in a variety of epithelial and endothelial cells. This factor is also a strong inducer of tumor growth, angiogenesis and lymphangiogenesis. NK4 also has inhibitory effects in angiogenesis triggered by other factors such as bFGF and VEGF. The results showed that these engineered MSC could successfully migrate

to the tumor tissues and inhibit tumor progression in the lung prolonging the survival of the animals tested (Kanehira *et al*, 2007). The same positive results were also obtained by other groups when genetically modified to produce Interleukin-21, a molecule that has been applied to significantly augment antitumor immunity in multiple murine tumor models and also in clinical trials, against ovarian cancer (Hu *et al*, 2011); Interleukin-15, a cytokine with potent antitumor properties, against pancreatic tumor (Jing *et al*, 2014); Interleukin-24, an effective cancer agent, against lung cancer (Zhang *et al*, 2013 b); Interleukin-12, also very potent as an anti-tumor molecule, against metastatic hepatoma (Jeong *et al*, 2015), Ewing's Sarcoma tumors (Xiaoping Duan *et al*, 2009), glioma (Hong *et al*, 2009). Renal carcinoma (Gao *et al*, 2010) and melanoma (Elzaouk *et al*, 2006). All these studies resulted in slower tumor growth, smaller tumor volume, prolonged survival of the animal tumor model, suppressed metastasis, NK and T-cell accumulation, tumor necrosis and tumor apoptosis.

In 2012, the group of Zhu used UBC-MSCs as carriers for LIGHT, a cytokine included in the tumor necrosis factor (TNF) superfamily, leads to the proliferation of T and B-cells, natural killer cells, monocytes, and granulocytes, induces apoptosis and inhibits tumor growth. The authors engineered MSCs to produce LIGHT in order to overcome the toxicity associated with its systemic administration and to allow for a constant source to treat gastric cancer. The results demonstrated that these engineered cells could successfully home to the gastric tumor site due to the interaction between SDF-1α produced by MSCs and CXCR4 a receptor expressed by the tumor cells (Zhu *et al*, 2012).

Tumor necrosis factor (TNF) is a cytokine secreted by macrophages that induces death of certain tumor cell lines, such as gastric cancer, by causing vasculotoxicity to the tumor cells. This property provides TNF-α a promising statute for anti-cancer therapies. Although as in many other anti-cancer agents, its systemic administration would cause numerous toxic reactions to the patients. In the study performed by Mao in 2012, the manipulation of UCB-MSCs towards the production of TNF-α to target gastric tumor cells, when subcutaneously injected in tumor models, was performed. The authors observed the tumor homing capacity of these cells and a significant tumor volume decrease, a strong suppressive effect on the tumor growth and an increased tumor necrosis (Mao *et al*, 2012).
MSC engineered to express interferon (IFN)-alpha, were also shown to be effective for the treatment of lung metastasis in an experimental model of metastatic melanoma. Treated animals showed enhanced apoptosis with a reduction in proliferation and tumor vasculature (Ren *et al*, 2008).

The chemokine CX3CL1 is an immunostimulatory molecule expressed as a surface bound protein which can also be shed from the cell surface. Soluble CX3CL1 attracts T cells and monocytes, while the cell-bound chemokine promotes the adhesion of leukocytes to activated endothelial cells. Murine MSCs transduced with CX3CL1 using an adenoviral vector were intravenously injected into mice bearing lung metastases. Treatment strongly inhibited the development of lung metastases and prolonged survival of the mice (Xin *et al*, 2007). In a second study, intra-tracheal administration of CX3CL1 engineered MSCs into mice with lung metastases also strongly inhibited tumor growth and prolonged survival (Xin *et al* 2009).

Hepatocyte growth factor (HGF) promotes tumor growth in part by driving angiogenic activity. NK4 acts as a HGF antagonist and has been shown to inhibit tumor cell proliferation, induce apoptosis and reduce angiogenesis. Treatment of experimental gastric cancer xenografts with MSCs engineered

to express NK4 led to an inhibition of tumor growth that was associated with decreased micro vessel density of the treated tumors, and enhanced apoptosis of the tumor cells (Zhu *et al*, 2014).
Osteoprotegerin (OPG) can inhibit the progression of osteosarcoma. MSCs transfected with adenoviruses carrying the OPG gene were injected into the tail vein of mice bearing osteosarcoma xenograft tumors. Treatment was shown to reduce both tumor growth and to help limit general bone destruction (Qiao *et al*, 2015).
Pigment epithelium-derived factor (PEDF) has been shown to repress tumor angiogenesis, tumor growth and metastasis in several cancer types. Bone marrow-derived mesenchymal stem cells engineered to express PEDF (MSCPEDF) were tested in a model of Lewis lung carcinoma. Systemic application of BM-MSC-PEDF led to reduced tumor growth and prolonged survival of the experimental animals. Immunohistochemistry analysis showed enhanced apoptosis and decreased micro vessel density in the treated tumors (Chen *et al*, 2012).

Table 3 summarizes the factors transduced into MSCs described above including the MSCs source of isolation, the cancer type studied in each work.

Table 3 Summarizing table with some of the factors transduced into MSCs with tumor suppressive results. It contains the cancer type studied, the MSCs source of isolation and the reference of the study

MSC delivered factor	MSC source	Cancer type studied	Reference
Interferon-β	Adipose tissue	Melanoma	Ahn *et al*, 2013
	Bone marrow	Ovarian	Dembinski *et al*, 2013
Interleukin-12	Bone marrow	Hepatoma	Jeong *et al*, 2015
		Ewing's Sarcoma	Xiaoping Duan *et al*, 2009
		Melanoma	Elzaouk *et al*, 2006
		Renal carcinoma	Gao *et al*, 2010
	Umbilical cord blood	Glioma	Hong *et al*, 2009
Interleukin-15	Umbilical cord blood	Pancreas	Jing *et al*, 2014
Interleukin-21	Umbilical cord blood	Pancreas	Hu *et al*, 2011
Interleukin-24	Umbilical cord blood	Lung	Zhang *et al*, 2013 **c.**
Interleukin-32	Bone marrow	Lung	Kanehira *et al*, 2007
LIGHT	Umbilical cord blood	Gastric	Zhu *et al*, 2012
Lipocalin 2	Bone marrow	Colon	Harati *et al*, 2015

(Table 3 continuation)

MSC delivered factor	MSC source	Cancer type studied	Reference
TNF	Umbilical cord blood	Gastric	Mao *et al*, 2012
TRAIL	Adipose Tissue	Colorectal	Grisendi *et al*, 2010
		Pancreatic	Grisendi *et al*, 2010
	Bone Marrow	Mesothelioma	Lathrop *et al*, 2015
		Hepatocellular	Deng *et al*, 2014
		Lung	Loebinger *et al*, 2009
INF-α	Bone Marrow	Melanoma	Ren *et al*, 2008
CX3CL1	Bone Marrow	Lung	Xin *et al*, 2007; 2009
NK4	Bone Marrow	Gastric	Zhu *et al*, 2014
OPG	Umbilical cord (Wharton's jelly)	Osteosarcoma	Qiao *et al*, 2015
PEDF	Bone Marrow	Lewis lung carcinoma	Chen *et al*, 2012

Other approach recently explored regarding MSC anti-cancer potential is the use of gene-directed enzyme-producing therapy (GDEPT) and prodrugs. The GDEPT enzymes act on prodrugs that are converted into active therapeutic metabolites that produce a localized anti-tumor effect. Prodrugs can have advantages over conventional chemotherapy agents including increased permeability and bioavailability, reduced adverse effects and increased half-lives (Karjoo *et al*, 2015). The non-toxic prodrugs are converted in their potent anticancer derivates when subjected to the appropriate enzyme. Mesenchymal stem cells engineered to produce the enzymes, migrate and engraft in the tumor sites. Thus, only the tumor cells will be exposed to the drugs' toxic effects while normal tissues have a limited exposure level. Some examples include the CD enzyme and prodrug 5-FC (Nguyen *et al*, 2015; Abrate *et al*, 2014) and HSV-TK enzyme and GCV prodrug (Matuskova *et al*, 2010; Melo *et al*, 2015) The outcoming effects of this therapies include induced apoptosis, interference with DNA and RNA synthesis, prolonged survival and inhibition of tumor growth without major side effects.

Given the positive results achieved by MSCs in drug delivery therapies, some groups have attempted to improve the efficacy of these therapies by combining already studied approaches. One example is MSC co-expressing TRAIL and HSV/TK in a model of metastatic renal cell carcinoma (RCC). The antitumor effects of MSCs expressing TRAIL and HSV/TK alone or in combination were compared in experimental lung metastasis. The authors observed that the combined treatment exerted a stronger apoptotic response in RCC cells than MSC-TK/GCV or MSC-D-TRAIL treatment alone. The effect of MSC-D-TRAIL-TK was found to be enhanced by repeated injections, but not by increased dose, and resulted in 100% survival of tumor-bearing mice after three rounds of injection (Kim *et al*, 2013).

Together these findings allow for the conclusion that although there is a strong controversy in the literature about the role of MSCs in tumor progression, there is also growing evidences for the promising features of MSCs as delivery systems against cancer.

1.1.4. Clinical Trials

When developing a new medical treatment, its safety and efficacy has to be studied in order to be, eventually, commercialized and used in medical therapies. A limited but growing number of follow-up studies involving MSCs have been reported recently, most aimed at taking advantage of the plasticity of MSCs to be employed in disease treatment. In this section, a selection of clinical trials conducted with MSCs towards cancer therapies is shown (https://clinicaltrials.gov/).

The Phase 1 study to determine the effects of MSCs secreting Interferon Beta in patients with advanced ovarian cancer is now being conducted in the United States. The main objective of this study is to determine the highest tolerable dose of MSCs genetically modified to produce interferon beta (MSC-INFβ) that can be administered to patients with ovarian cancer. As previously mentioned in the prior section, the intratumoral production of INFβ follows the complete eradication of tumors in 70% of treated mice by inducing caspase-dependent tumor cell apoptosis. The study will enroll 21 females, between 18 and 90 years old, with histologically documented diagnosis of epithelial ovarian cancer including serous papillary, endometrioid, mucinous, clear cell, poorly differentiated or mixed adenocarcinomas. The MSC-INFβ are intraperitoneally infused through a catheter placed in the abdomen of the patient. After a period of time the patient will have a tumor biopsy to check the status of the disease (NCT02530047).

A Phase 1 study of allogeneic human bone marrow derived mesenchymal stem cells in localized prostate cancer is being carried on in the United States. The study aims to quantify the amount of systemically infused allogenic MSC DNA relative to the recipient DNA at the sites of prostate cancer in patients with localized prostate adenocarcinoma that are scheduled to undergo a prostatectomy. The objective is to determine if the MSC home to sites of prostate cancer. The investigators systemically infuse MSCs 4, 6 or 8 days before the enrolled subjects' prostectomies. Then, the relative amount of donor MSC DNA is quantified in the explanted prostate by BEAMing digital PCR. This trial will provide the foundation for future studies aimed at engineering MSCs to deliver a toxin to sites of metastatic prostate cancer. The study will enroll 30 males, between 18 and 30 years old (NCT01983709).

Phase 1 and Phase 2 studies for MV-NIS infected mesenchymal stem cells in the treatment of patients with recurrent ovarian cancer is now being conducted in the United States. The Phase I trial aims to determine the maximally tolerated dose of intraperitoneally administrated MSCs infected with a virus expressing a sodium iodine symporter (NIS) in patients with recurrent ovarian cancer. The study's Phase II is to assess the patient's progression during 4 months. The study will enroll 54 volunteers with recurrent or progressive ovarian cancer or primary peritoneal cancer after prior treatment with platinum and taxanes. The study involves patients from both genders over 18 years old (NCT02068794)

A Phase 1 trial of stem cell injection in cancer survivors is being conducted in the United States. The main purpose of this study is to examine the safety and feasibility of delivering allogenic human mesenchymal stem cells by transendocardial injection to cancer survivors with left ventricular dysfunction due to anthracycline-induced cardiomyopathy (AIC) aiming for the reparative effects of MSCs. Anthracycline is a class of drugs used in cancer chemotherapy known as one of the most effective anticancer treatments developed but their adverse effect is cardiotoxicity. This study will enroll 36 cancer survivors diagnosed with AIC between 18 and 80 years old (NCT02509156).

The Phase 1 trial of mesenchymal stem cells to repair the kidney and improve function in cisplatin-induced acute renal failure in patients with solid organ cancers. Cisplatin is a drug used in the treatment of solid tumors specially those of the ovaries, testes, head and neck. Unfortunately the cumulative nephrotoxicity is the major toxicity of this compound. MSCs were demonstrated to successfully extend to repair ischemically and cisplatin injured renal tubes in mice. The observation raises the possibility that adult-derived bone marrow cells could be administered to enhance the recovery from renal injury. The aim of this study is to test the feasibility and safety of systemic infusion of donor ex-vivo expanded MSC to repair the kidney and improve function in patients with solid organ cancers who develop acute renal failure after chemotherapy with cisplatin. The study will enroll 9 volunteers from both genders with ages between 18 and 80 years old (NCT01275612).

A Phase 1 trial of genetically modified mesenchymal stem cells therapy against head and neck cancer is being performed in Korea. In this study the safety and efficacy of the intratumoral injection of MSC genetically modified to produce the anti-tumoral molecule Interleukine-12 (IL-12) in head and neck cancer patients. The primary purpose is to determine the maximum tolerated dose of the vaccine administered in the tumor. The study will enroll 12 participants from both genders older than 19 years old (NCT02079324).

The Phase 1 and Phase 2 clinical trial about the safety and efficacy of repeated infusion of the virus Celyvir in children and adults with metastatic and refractory tumors was conducted in Spain. This virus is specially designed to eliminate selectively cancer cells. They selectively replicate inside the tumor cells. The mesenchymal stem cells are infected with the virus and expanded. After the infusion of Celyvir-MSC, they home to the tumor site, eliminating the tumor cells. This study aims to evaluate the safety of weekly infusions in children and adults with solid tumors along with the clinical response. 20 participants from both genders were engaged in this trial from 6 months to 75 years old. This study has been completed in February of this year, however no study results were already published (NCT01844661).

A pre-clinical study is being conducted in Sweden regarding haploidentical stem cells transplantation in children with therapy resistant neuroblastoma. This study focus on a novel strategy combining tumor targeted radioisotope treatment with Iodine I-131 metaiodobenzylguanidine (MIBG), a radiopharmaceutical used to identify the location of tumors such as neuroblastomas that when associated with I-131 can be used to eradicate tumor cells, and the immunotherapeutic effect of haploidentical mesenchymal stem cells followed by low-dose donor lymphocyte infusions. The aim of this clinical trial is to determine the safety and efficacy of the study. It will be enrolled 15 participants

with refractory neuroblastoma without a rapid disease progression, from both genders with ages between 6 months to 21 years (NCT00790413).

For safety reasons, the U.S. Food and Drug Administration (FDA) require a long-term follow-up for patients who have received infusions of stem cells treated with a gene transfer procedure. These patients must have long-term follow-up for at least 15 years after receiving the gene transfer. Table 4 summarizes the clinical trials using MSC as a therapeutic agent towards cancer worldwide.

Table 4 Clinical trials performed around the world regarding cell-based therapies towards cancer treatment using mesenchymal stem cells.

Study Title	Phase	Status	Location Country	Reference	Last Uptade date
Mesenchymal Stem Cells (MSC) for Ovarian Cancer	1	Recruiting	United States	NCT02530047	July 26, 2016
Allogeneic Human Bone Marrow Derived Mesenchymal Stem Cells in Localized Prostate Cancer (MSC)	1	Recruiting	United States	NCT01983709	April 13, 2016
MV-NIS Infected Mesenchymal Stem Cells in Treating Patients With Recurrent Ovarian Cancer	1, 2	Recruiting	United States	NCT02068794	July 8, 2016
Stem Cell Injection in Cancer Survivors (SENECA)	1	Recruiting	United States	NCT02509156	August 22, 2016
Mesenchymal Stem Cells In Cisplatin-Induced Acute Renal Failure In Patients With Solid Organ Cancers	1	Recruiting	Italy	NCT01275612	September 2, 2016
Genetically Modified Mesenchymal Stem Cell Theraopeutic Against Head and Neck Cancer (GX-051)	1	Active, not recruiting	Korea	NCT02079324	April 29, 2015
Safety and Efficacy of Repeated Infusion of CELYVIR in Children and Adults With Metastatic and Refractory Tumors	1,2	Completed	Spain	NCT01844661	February 18, 2016
Haploidentical Stem Cell Transplantation in Neuroblastoma	0	Active, not recruiting	Sweden	NCT00790413	March 3, 2016

1.2. Azurin, a promising anti-cancer protein

Azurin, a 128-residue bacterial protein produced by *Pseudomonas aeruginosa* has demonstrated antitumor properties. This protein has been studied in different *in vitro* and *in vivo* models, demonstrating its ability to interfere in different steps of tumor development (Punj *et al* 2004; Bernardes *et al* 2011).

Azurin enters preferentially in human cancer cells but not in the corresponding normal cells (Taylor *et al*. 2009). The preferential entry of azurin in cancer cells is mediated by the amino acids 50-77 (p28), more specifically amino acids 50-67 (Bizzarri *et al*, 2011). Azurin enters cancer cells co-localized with caveolin-1, the main component of the caveolae which are a special type of lipid rafts. These plasma membrane microdomains are rich in proteins as well as lipids such as cholesterol and sphingolipids and have several functions in signal transduction (Mehta *et al*, 2011). A wide number of signal transduction processes that play a major role in the progression of many types of tumors are dependent on lipid rafts, including cell adhesion, migration, cell survival and proliferation (Mollinedo and Gajate, 2015). Membrane rafts reorganization leads to an abnormal signaling route, which might be effective target for anti-cancer therapy. Azurin exerts an anti-cancer effect by entering the cell through endocytosis, a process that disrupt caveolae and removes from the cell membrane selective receptors that may be over-activated and are crucial for cancer progression. This impact on cancer cells is an important mechanism by which tumorigenesis is abrogated (Bernardes *et al*, 2014).

Azurin is a member of a family of copper-containing redox proteins named cupredoxins. These proteins exhibit remarkable typological similarity to a family of ligands called ephrins. Ephrin ligands bind to a family of extracellular receptor proteins known as Eph receptor tyrosine kinases. Eph and ephrin proteins have been shown to be up-regulated in many types of tumors. The Eph-ephrin complex formation leads to the trans-autophosphorylation of the tyrosine kinase domains of the receptor molecules, allowing cellular signaling that translates into a variety of pathological processes including tumor progression, angiogenesis, migration and invasion related to many types of human cancers. Azurin selectively bind to EphB2 receptor leading to the blockage of the autophosphorylation step, interfering in vascular remodeling of the tumor tissue and consequently tumor growth and migration (Chaudhari *et al*, 2007).

The tumor suppressor protein p53 is a major player in an intricate cellular network that is involved in multiple central cellular processes including transcription, DNA repair, genomic stability, cell cycle control and cell death through apoptosis. Azurin, upon entry into cancer cells, forms a complex with p53, stabilizes it to raise its intracellular level, generates enhanced levels of reactive oxygen species and induces apoptosis (Yamada *et al*, 2004). Many viral and mammalian proteins can modulate p53 function by physical interaction, although, azurin is the first bacterial protein reported to form a complex with p53. It has been demonstrated that four azurins bind per p53 monomer, which may sterically shield p53 from degrading enzymes like Mdm2 oncoprotein that inhibits its transcriptional activity, favors its nuclear export, and acts as an E3 ubiquitin ligase, targeting p53 for proteasomal degradation. This might explain the increased intracellular level this protein in the

presence of azurin (Apiyo *et al*, 2005). Although azurin preferentially enters cancer relative to normal cells, a 128-amino acid protein could display some immunogenicity, a potentially significant side effects, that compromises its pharmaceutical efficacy. Since peptide fragments of azurin could provide therapeutic molecules with the same cytotoxicity, delivery, and target specificity of the whole protein, but with potentially fewer side effects, truncated versions of azurin have been investigated for anticancer activity. Amino acids 50-77 of azurin form a peptide fragment (p28) that retains the preferential penetration of the whole protein, given by amino acids 50-67, and also its antitumor activity, given by amino acids 61-69. A spectroscopy experiment performed by Bizzarri *et al*, suggested that the bio-recognition process between azurin and p53 is given through the p28 domain. P28 already completed the phase I clinical trial in the United States. It has not only demonstrated no toxicity, but also showed tumor regression in 15 stage IV cancer patients with resistant solid tumors, substantially prolonging the lives of some patients without significant adverse effects. (Bizzarri *et al*, 2011; Warso *et al*, 2013) Also, p28 has completed a second phase I trial against pediatric brain tumor patients in 11 Children's Hospitals in the United States. Brain tumors are often highly invasive and difficult to treat because very few drugs can cross the blood-brain barrier to reach the brain tumors. P28 was given intravenously to such patients, age 3 to 21. Since the p28 was given at the adult dose as was administered during the first phase I trial, the sponsors stipulated that if p28 was found to be toxic to the pediatric brain tumor patients at this dose, or if p28 had no efficacy in reducing the growth of the tumors, they would stop the trial. The results of this second phase I trial suggest that p28 has shown acceptable toxicity and perhaps some tumor regressing effect in some of these pediatric brain tumor patients. Indeed, it is important to note that the USFDA (US Food and Drug Administration) has approved on December 02, 2015 the designation of azurin-p28 as an orphan drug for the treatment of brain tumor glioma (Fialho *et al*, 2016).

Other important target of anti-cancer therapies is angiogenic activity, and only a limited number of agents directly inhibit both angiogenesis and tumor cell growth. Vascular endothelial growth factor (VEGF) and Fibroblast growth factor (FGF) are overexpressed in solid tumors. P28 enter solid tumors' endothelial cells, co-localized with cavelolin-1 and inhibits angiogenesis by reducing VEGFR-2 tyrosine kinase activity. Inhibition of kinase activity reduces the phosphorylation of the VEGFR-2 downstream targets FAK and Akt, altering the intracellular architecture of endothelial cytoskeletal, focal adhesion, and cell contact proteins that limit endothelial cell motility and migration (Mehta *et al*, 2011).

A feature of malignant tumors it's their ability to invade surrounding tissues in a process known as metastasis. To accomplish this, cells establish effective connections with the surrounding extracellular matrix (ECM). The physical connection between cancer cells and their surrounding tissues is achieved through cell-surface receptors including integrins. Increased expression of integrin might be associated with poor prognosis, thus this protein is a suitable target for anticancer therapy. Breast cancer cells and non-small cell lung cancer carcinoma, the most common form of lung cancer, when treated with azurin demonstrate a decrease in the protein levels of integrin with the subsequent decreased ability to invade and adhere to different ECM components and to grow in anchorage-independent conditions (Bernardes *et al*, 2016) The same results were also observed in breast

cancer. Azurin decreased adhesion of cells to proteins from the ECM and altered the expression profile of integrins (Bernardes *et al*, 2014).

Certain types of cancer are very resistant to anticancer drugs, leading to the need of high dosage administration which is followed with marked side effects. Thus, finding a way to enhance the sensitivity of these cells to anticancer drugs is highly significant. Azurin has a strong synergistic anticancer effect on certain types of cancer including oral squamous cell cancinoma, when combined with 5-fluorouracil or etoposide (Choi *et al*, 2011). The synergistic effects of azurin were also observed in malignant mammary epithelial cells when co-administrated with tamoxifen (Mehta *et al*, 2010) and in non-small cell lung carcinoma when co-administrated with gefitinib and erlotinib, tyrosin kinase inhibitors which targets specifically the Epidermal Growth Factor Receptor (EGFR), one of the main targets for clinical management of this disease (Bernardes *et al*, 2016).

As outlined in this section, azurin has the ability to mediate specific high-affinity interactions with various unrelated mammalian proteins relevant in cancer. This property provides it the property of natural scaffold protein (Fialho *et al*, 2007). This is one of the most promising features of azurin allowing its broader action in several types of cancer cells and preventing the acquirement of tumor resistance. Moreover, azurin is water soluble molecule with a hydrophobic patch, which might help in its tissue penetration and clearance from the blood stream. Also is a protein with low immunogenicity since is a non-antibody recognized protein, preventing the host immune attack (Fialho *et al*, 2008). Figure 1, summarizes the mechanisms of azurin's action behind its anti-tumoral role.

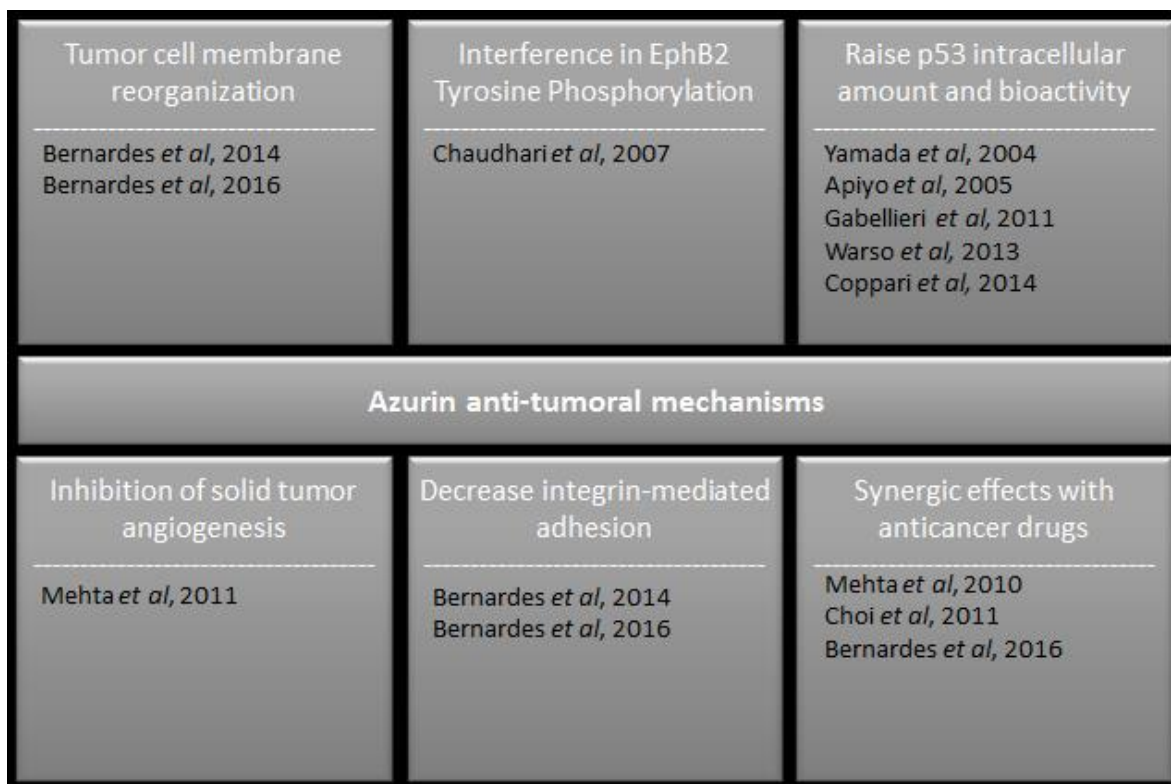


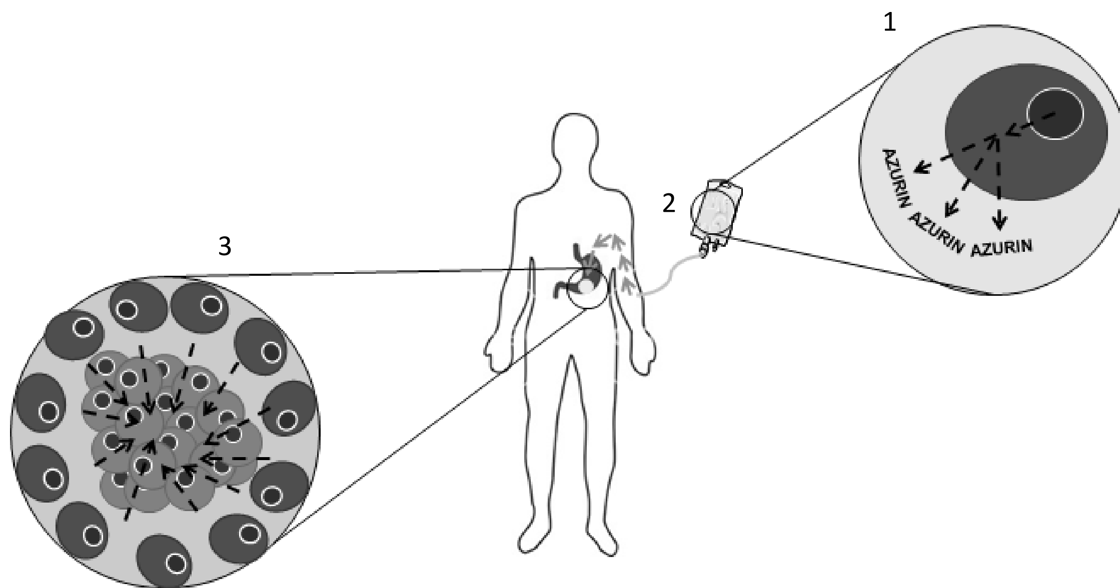
Figure 1 Azurin anti-tumoral effects mentioned in the present work and respective studies' references

2. Study's objective and strategy

The present study is part of the following research project: iBB /2015/12: "Azurin-expressing Mesenchymal Stem / Stromal Cells in anticancer therapies" and its ultimate objective is to engineer Mesenchymal Stem Cells (MSCs) towards the production and secretion of the *Pseudomonas aeruginosa* protein azurin to specifically target and annihilate tumor cells. This rationale is proposed basing on the several studies demonstrating the antitumoral capacity of azurin and the tumor tropism features observed in MSCs. An important regard explored in this work is the requirement for the azurin secretion into the tumor microenvironment in order to allow and amplify the mechanisms of azurin action. Bearing this in mind, the genetically engineering of MSCs will be accomplished with a recombinant plasmid containing the azurin gene and a secretory sequence. There's bioinformatics information supporting the recognition capacity of the natural azurin's peptide signal found in *Pseudomonas aeruginosa* by the human cells' machinery. Relying on this information, in this study azurin's natural peptide signal sequence will be coupled to azurin gene in a recombinant plasmid prior to MSCs transfection by microporation. Considering the failure possibility of this peptide signal to direct the recombinant protein to the secretory pathway of mesenchymal stem cells, an engineered secretory sequence will be additionally studied. This sequence was engineered by the group of Qiu and colleagues in 2000, and in their study there is the evidence that this sequence provides a signal for translocation of the recombinant protein into the lumen of the ER, for transport through the ER and Golgi apparatus to the extracellular environment (Qiu *et al*, 2000). Finally, Azurin-producing MSCs will be studied regarding their anti-tumoral potential in tumor progression.

Taking into consideration the fact that MSCs by secreting azurin, inevitably are going to be subjected to it in their microenvironment, a parallel study to be employed in this work is to monitor the proliferation of MSCs and azurin's entry capacity when these cells are subjected to increasing azurin concentrations. These observations have much significance since is very important that MSCs aren't affected by the presence of azurin in order to our strategy to be successful. A third parallel study to be employed in this work is to analyze the tumor tropism capacity of MSCs isolated from the Bone Marrow of healthy donors towards breast, lung and colon tumor cell lines.

In summary, this study is structured in three key points examined in parallel: the genetically engineering of MSCs and study of their anti-tumoral potential; the azurin's cytotoxic potential evaluation towards MSCs; and the tumor tropism of unmodified MSCs.



STUDY'S HYPOTHESIS

Figure 2 Schematic representation of the study's hypothesis. Azurin-Mesenchymal Stem Cells (1), when injected, migrate directly to the tumor site (2), specifically delivering azurin and restraining tumor development (3).

3. Materials and Methods

3.1 Cell lines and cell cultures

Cell lines A549, HT-29, HeLa, HEK293T and MCF-7 were obtained from ECACC (European Collection of Authenticated Cell Cultures) and cultured in DMEM media supplemented 10% of heat-inactivated FBS (Lonza, Basel, Switzerland), 100 IU/mL penicillin, 100 mg/mL streptomycin (PenStrep, Invitrogen). Cells were maintained in culture and passaged between 2 to 3 times per week, by chemical detaching with Trypsin 0.05%.

Human Bone Marrow Mesenchymal Stem Cells aspirates were obtained from adult volunteer donors, after informed consent at Instituto Português de Oncologia de Lisboa Francisco Gentil and provided to us by the personal stock of the Stem Cell Bioengineering and Regenerative Medicine Lab from iBB (courtesy of Prof Cláudia Lobato). Cells were cultured in STEM Pro XenoFree (XF) media or DMEM media supplemented with 10% FBS with a low glucose content. Three donors were tested M48A08, M83A15 and 379A15. All cell lines were grown in a humidified atmosphere at 37°C with 5% CO_2 (Binder CO_2 incubator C150).

3.2 Construction of Azurin recombinant plasmid

Two sequences were analyzed: Native Azurin construct composed by the Azurin gene, its natural peptide signal, naturally found in *Pseudomonas aeruginosa*, at the N-terminus, EcoRI recognition sequence upstream the gene and XbaI recognition sequence downstream the gene; Engineered Azurin construct composed by the azurin gene devoid of its peptide signal sequence, downstream of a secretory sequence to direct the protein to the secretory pathway (Qiu *et al*, 2000), EcoRI recognition sequence upstream the gene and XbaI recognition sequence downstream the gene. Both constructs were obtained by gene synthesis by *NZYTECH* and sent in plasmids (designated pAzurinEngineered and pAzurinNative), which followed a digestion protocol with the two enzymes selected in order to extract the gene. The vector selected for transduction was pVAX-GFP (Thermo Fisher Scientific, courtesy of Profs Miguel Prazeres and Gabriel Monteiro, iBB/IST) which was digested with EcoRI and XbaI to allow further gene cloning. The ligation protocol was accomplished using T4 enzyme (Invitrogen) in a 20μL mixture containing the azurin genes and plasmid in a 3:1 proportion, the appropriate reaction buffer (Invitrogen) and water. The mixture was them incubated for 1h at 22°C. To select the correct product from the cloning procedure, meaning pVAX containing each construct previously referred (pVAX-Azurin) classic transformation of a competent *Escherichia coli* strain DH5α was performed. The following step was the detection of the selection marker resistant *E.coli* colonies (kanamycin). Afterwards, the candidates were cultured in 20mL of liquid LB supplemented with 20μL of 50μg/mL Kanamycin and followed DNA sequencing to confirm the identity of the DNA construct obtained.

3.3 Plasmid Transformation in *E.coli*

The plasmid engineered by *NZYTECH* containing the NativeAzurin and EngineeredAzurin constructs were electroporated in an electrocompetent strain of *Escherichia coli* (XL-1 Blue) prior to DNA expansion. Regarding pVAX-GFP, pVAX and pVAX-Azurin a classic transformation of a competent *E.coli* strain DH5α was followed. After transformation with each plasmid the cultures were plated in solid LB media supplemented with 50µg/mL ampicillin for *E.coli* transformed with pAzurinEngineered and pAzurinNative; for pVAX-GFP, pVAX and pVAX-Azurin the culturing was performed in plates with solid LB media supplemented with 50µg/mL kanamycin and incubated overnight at 37°C. The colonies successfully transformed with the plasmids (those that were able to grow in the plates) were selected randomly and transferred to 100 mL of liquid LB media, supplemented with 50µg/mL ampicillin or kanamycin. These cultures were grown overnight at 37°C in an agitator at 250 rpm.

3.4 Plasmid Extraction

After the expansion of *E.coli* cultures transformed with pVAX-GFP, pVAX, pVAX-Azurin, pAzurinEngineered and pAzurinNative vectors, the plasmid DNA was extracted using the ZR Plasmid MiniprepTM-Classic kit (ZymoResearch). The procedure was performed according to the manufacturer's protocol: 2 mL of bacterial culture were collected from cultures of E.coli transformed with each plasmid and centrifuged in two clear 1.5 mL tubes at 8000 rpm for 2 minutes; the supernatant was discarded. 200µL of P1 Buffer were added to each tube to resuspend the resulting pellets. After that, 200µL of P2 Buffer were added to the tubes and the tubes were inverted 2-6 times to mix the solutions. When the solutions appeared clear, purple and viscous, 400µL of P3 Buffer were added to the tubes and the tubes were inverted again, gently but thoroughly. When the neutralization was complete, the solutions turned yellow. After 2 minutes at room temperature, the samples were centrifuged at 12000x*g* for 2 minutes. In the next step, a Zymo-SpinTM IIN column was placed in a Collection Tube, and the resulting supernatants from the previous step were transferred to a Zymo-SpinTM IIN column. Then, the Zymo-SpinTM IIN/Collection Tube was centrifuged at 12000x*g* for 30 seconds. The flow-through in the Collection Tube was discarded, and the Zymo-SpinTM IIN column was placed again in the Collection Tube. After that, 200µL of Endo-Wash Buffer were added to the column, and then the Zymo-SpinTM IIN/Collection Tube was centrifuged again at 12000x*g* for 30 seconds. Next, 400 µL of Plasmid Wash Buffer were added to the column, and after that the Zymo-SpinTM IIN/Collection Tube was centrifuged at 12000x*g* for 1 minute. Lastly, the column was transferred into a clean 1.5 mL tube and 30µL of H_2O were added to the column. The column was centrifuged 30 seconds at 13000 rpm to elute the plasmid DNA.

3.5 DNA digestion and recovery

The enzymes used to digest pVAX-GFP, pAzurinEngineered and pAzurinNative were EcoRI and XbaI. Microcentrifuge tubes containing 2µL of enzyme buffer, 0.5µL of each enzyme, ~500ng of

plasmid DNA and water making up to total volume of 20µL, were incubated overnight at 37°C which is the optimal temperature for the enzymes to function. After the digestion is complete, the samples were separated on a 1% agarose gel at 100V. After staining the DNA with Gel Red for ~20 minutes the bands with the size corresponding to the desired fragments were extracted. The last step was the recovery of the digested DNA using the Kit Nucleo Spin Extract II according to the manufacturer's protocol. After digestion, the construction of the recombinant plasmid pVAX-insert (AzurinEngineered or AzurinNative) is followed through the ligation protocol.

3.6 Bacteria growth, over-expression, extraction and purification of azurin

To produce the protein, it was made a pre-inoculum in an Erlenmeyer flask of 250 mL with 100 mL of LB media, 100µL of 150µg/mL ampicillin and an inoculum of *Escherichia coli* SURE, cloned with the plasmid pWH844, containing the gene azu (codifying for Azurin, from *Pseudomonas aeruginosa* PAO 1) (Bernardes *et al.*, 2013). This culture was grown overnight at 37°C in an agitator at 250 rpm. After 24h incubation, the inoculum was made with the pre-inoculum at an optical density at 640nm (OD640) of 0.1 in 3 L flasks with 1 L of SB media (20 g/L of yeast extract, 32 g/L of triptone and 5 g/L of NaCl) supplemented with 150µg/mL of ampicillin at 30-37°C in an agitator at 250 rpm. When the culture reached an OD640 of 0.6-0.8, 0.2 mM of IPTG (azurin's promoter inducer) was added. The culture was then grown during 4-5 hours at 30-37°C in an agitator at 250 rpm. After that, cells were collected by centrifugation (8000 rpm, 10 minutes, 4°C; Beckman J2-MC Centrifuge), and the resulting pellet was ressuspended with 15 mL of Start buffer (10 mM imidazole, 0.2 mM sodium phosphate, 0.5M NaCl, pH 7.4) and stored at -80°C until azurin´s purification. To purify the protein, cells were sonicated (Branson Sonifier Sound Enclosure) and centrifuged (17600g, 5 minutes, 4°C; Braun Sigma-Aldrich 2K15). After that, the pellet was discarded and the supernatant was again centrifuged in the same conditions during 1 hour, to remove debris. Azurin was purified in a histidine affinity column (HisTrapTM FF, GE Healthcare) and eluted with increasing concentrations of imidazole (20-500 mM). Azurin is eluted in concentrations of 100-200 mM of imidazole. In the next step, the buffer was exchanged to from Imidazole to PBS in ÄKTA system (ÄKTA Prime, Amersham Biosciences) with a desalting column (HiPrepTM 26/10 Desalting, GE Healthcare). The protein was then collected and concentrated by centrifugation (5000 rpm, 4°C, Eppendorf Centrifuge 5804R) in a 3 kDa cut-off column (Amicon Ultra Centrifugal Filter, Ultracel 3K, Milipore). To remove endotoxins from *E.coli* host strain, the sample was then passed through a detoxing column (Detoxi-GelTM Endotoxin Removing Column, Thermo Scientific) and it was concentrated again by centrifugation. The final volume of purified protein was centrifuged in a 100 kDa cut off filter, to remove eventual contaminants. The concentration was calculated reading the absorvance at 280 nm and using BeerLambert equation, where ε (280) = 9.1x103 M-1.cm-1 (van Amsterdam *et al.*, 2002). To verify if azurin had any contamination, a spot test was performed over-night at 37°C (two spots with 10 µL of azurin in a LB agar plate). Azurin was stored at 4°C until further use.

3.7 Azurin Entry Assay

Mesenchymal Stem Cells were plated (1x10^5 cells) in 6-well plastic plates and left to adhere and grow overnight at 37°C and 5% CO_2. After the 24h incubation, cells were treated with 0μM or 50μM of azurin, during 30 minutes at 37°C and 5% CO_2.

Cells, treated or not with azurin, were washed twice with 1 mL of PBS and then lysed with 100μL of catenin lysis buffer (1% Triton X-100, 1% Nonidet-P40 in deionized PBS) supplemented with 1:7 proteases inhibitor (Roche Diagnostics GmbH, Germany) and 1:100 phosphatases inhibitor (Cocktail 3, Sigma Aldrich) for 10 minutes at 4°C. After lysis is complete, lysed cells were collected and vortexed three times (10 seconds each), centrifuged (14000 rpm, 4°C, 10 min; B.Braun Sigma-Aldrich 2K15) and quantified by Bradford method (BioRad Protein Assay). ~ 20μg of total protein per sample was prepared, denatured at 95°C during 5 minutes, and then separated by SDS-PAGE. Lastly, azurin was detected by Western blotting.

3.8 Western blotting analysis

Protein lysates were separated by electrophoresis in 15% polyacrylamide gels and electrotransferred to nitrocellulose membranes (RTA Transfer Kit, BioRad), using Trans-Blot Turbo Transfer System (BioRad), following the manufacturer´s instructions. After blocking the non-specific binding sites with 5% (w/v) not-fat dry milk in PBS-tween20 or 5% BSA (Bovine Serum Albumin) in water, membranes were incubated overnight at 4°C with primary antibodies (anti-azurin [AB0048-200 SicGen] diluted 1:2000 in 5% BSA, anti-GFP diluted 1:2000 5% non-fat milk buffer and anti-GAPDH diluted 1:1000 in 5% non-fat milk buffer). Afterwards, the membranes were washed with PBS-tween-20 (0.5%) and probed with the appropriated secondary antibody, conjugated with horseradish peroxidase [anti-goat (sc-2354, Santa Cruz Biotechnology) for azurin and anti-mouse (sc-2005, Santa Cruz Biotechnology) for GAPDH and GFP, diluted 1:2000 in 0.5% PSB tween-20] at room temperature for 1 hour. After washed, the membranes were developed by adding ECL substrates (Pierce) and capture the chemiluminescence by Fusion Solo (Vilber Lourmat) equipment. The protein levels were normalized by the respective GAPDH level.

3.9 Migration/Invasion assay

The propensity of MSCs to migrate towards cells was evaluated using a 24-well-transwell migration assay. MCF-7 and HT-29 (1.5x10^5) cell lines were cultured in 24-well plates and left overnight at 37°C and 5% CO_2. Migration assay was performed using CytoSelect™ 24-Well Cell Migration and Invasion Assay with 8 μm pore size coated with Collagen, Matrigel (BD Falcon™ Cell Culture Inserts containing an 8 micron pore size PET membrane that has been treated with a Matrigel Matrix) or uncoated. Mesenchymal Stem Cells (4x10^4) were incubated in the upper compartment of the chambers, placed in the wells and left for 24 hours at 37°C and 5% CO_2. After the incubation period, non-migrated cells were removed from the upper side of the chamber's filter with a cotton swab dipped in PBS and chambers were washed with PBS. Migrated cells were fixed in cold methanol (4°C) during 10 minutes. The membrane was removed with a scalpel, placed in a microscope glass,

cells were stained with DAPI and counted under the microscope (Zeiss). In each condition, cells of 10 independent fields were counted and the average of these fields considered as the mean number of migrated cells per condition. Results are presented as the fold change in migration of cells in comparison with the tumor cells untreated with MSCs.

3.10 MTT cell viability assay

MTT [3-(4,5 dimethylthiazol-2-yl-2,5 tetrazolium bromide)] assays were used to determine viability and proliferation rate of mesenchymal stem cells upon azurin exposure and also the viability of cancer cells upon microporated MSCs. Cells were seeded in 96-well plates (Orange Scientific) at a density of $3.5x10^3$ cells/ well for MSCs, $5x10^4$ cells/ well for A549, $2x10^5$ cells/ well for MCF-7 and $1x10^5$ cells/ well for HT-29. After 24 hours, media was exchanged with 100μL of fresh azurin or MSCs' conditioned media (baseline level of 50% cancer cells' culture media; 0%, 10%, 25% and 50%).

For MSCs, after 48 hour incubation, 20μL of MMT (5mg/mL) were added to each well and incubated at 37° for 3.5h.

For cancer cells, the media was exchanged by the same concentrations of conditioned media 3 times with 24 hours interval. Afterwards, 20μL of MMT (5mg/mL) were added to each well and incubated at 37° for 3.5h.

Reaction was stopped with the addiction of 40 mM HCL in isopropanol. MTT formazan formed was spectrophotometrically read at 590 nm in a 96-well plate reader. Untreated cells were used as control, in order to determine the relative cell viability of treated cells.

3.11 HEK, MCF-7 and HeLa transfection

$5x10^5$ cells were seeded on a 6-well plate and left adhering for 24hours at 37°C and 5% CO_2. Afterwards cells were transfected with pVAX-AzurinEngineered, pVAX-AzurinNative and pVAX-GFP using LipofectamineTM 2000 reagent (Invitrogen), according to the manufacturer's instructions. Supernatants and cells were harvested at 48hours and 72hours post-transfection. Harvested cells were lysed according to the lysis protocol described in section 3.7.

GFP and Azurin expression and secretion was detected on lysed cells and supernatants through Western Blotting.

3.12 Mesenchymal Stem Cells microporation

The microporation protocol was followed according to Madeira *et al*, 2011. $7.3x10^5$cell/mL were transferred to 1.5mL microcentrifuge tube and centrifuged at 1250 rpm during 7 minutes. The supernatant was discarded, the pellet was resuspended in Resuspension Buffer R (provided by the microporator manufacturer) and 10μg of plasmid DNA (pVAX-Azurin for azurin production and pVAX-GFP as control) was added to the microcentrifuge tube. Afterwards, the cell-DNA mixture was collected with the NeonTM Pipette, carefully avoiding the entrance of air bubbles. NeonTM Transfection System (Invitrogen) was assembled according to the manufacturer instructions and the

appropriate microporation protocol was selected (Pulse voltage =1000mV; Pulse width=40 ms; Pulse number =1) in order to start cell microporation. After the delivery of the electric pulse, the NeonTM Pipette was removed and cells were immediately transferred to restoring media to increase cell viability after transfection.

After approximately 5 minutes of incubation at room temperature, the microporated cells were transferred to 75cm^2 T-Flasks previously coated with CELLstartTM and Stem Pro MSC SFM media (XF media). Cells were left incubating at 37°C and 5% CO_2 during 48 and 72hours. After that time period, the cell's conditioned media was collected, cells were harvested by centrifugation (1500 rpm, 10minutes) and stored at -80°C.

In order to analyze the microporation effectiveness, cells were lysed with catenin lysis buffer (protocol described above), prior to Western Blotting against GFP and Azurin. Conditioned media was analyzed in parallel to examine protein secretion.

3.13 RT-PCR

Total RNA was extracted from MSCs with RNeasy Micro Kit (Qiagen) and cDNA was synthesized by PCR using a Multi-Reverse transcriptase. cDNA synthesis conditions included a single cycle of 10 minutes at 25°C, 60 minutes at 37°C and 5 minutes at 95°C.

Real-time PCR amplification was done using Applied Biosystems® 7500 Real-Time PCR System (ThermoFisher) using SYBR Green Quantitative PCR kit (Invitrogen) with primers for Azurin (forward primer: 5'-*AGAACGTCATGGGACACAACTG*-3', reverse primer: 5'-*GCCATGCCGTCTGTAACGA*-3') and the control 18S (forward primer: 5'-*CGCCGCTAGAGGTGAAATTC*-3', reverse primer: 5'-*CATTCTTGGCAAATGCTTTCG*-3').

Each PCR run was followed by a dissociation curve to confirm the specificity of the amplification and the absence of primer dimers. The cycle threshold (Ct) was calculated automatically, and normalization was carried out with the 18S Ct value.

4. Results

4.1 Migratory capacity of BM-MSCs *in vitro*

Mesenchymal stem cells are intrinsically tropic for tumor cells (Kidd *et al*, 2009), which is a central feature to their utility as a reliable delivery vehicle for cancer gene therapy. The *in vitro* tumor tropism properties of 3 healthy bone marrow MSC donors (BM-MSCs) towards tumor cell lines MCF-7 and HT-29 was evaluated by a transwell migration assay using CytoSelect chambers with 8μm pores. Since the composition of expansion media is critical for the functionality, morphology and motility of MSCs (Laitinen *et al*, 2016), in this study we compared the migratory potential towards cancer cells of MSCs expanded in XenoFree (Stem Pro MSC SFM media) culture media and DMEM with FBS culture media. We also considered the presence of physiological coatings like Collagen type I (1mg/mL) and a Matrigel matrix in order to come closer to the *in vivo* situation. The Matrigel matrix is a solubilized basement membrane preparation extracted from Elgelbreta-Holm-Swarm (EHS) mouse sarcoma. It contains laminin, collagen type IV Heparin sulfate proteoglycan, entactin and growth factors, including TGFβ, basic FGF and others which occur naturally in the tumor microenvironment.

The tumor cell lines were seeded in 24-well plates and after 24 hours of incubation, chambers containing seeded MSC were added in each well with a ratio of MSCs / tumor cells = ¼ (Loebinger *et al*, 2009). These chambers have a porous membrane with an 8μm diameter pore allowing for the MSCs to cross to the tumor cells in the well. In the control condition no tumors cells were added (the corresponding media volume was added instead). After the incubation period, the non-migrated cells were removed from the upper portion of the filter and the membrane was isolated, stained with DAPI and the migrated cells were observed and counted at the microscope.

The presence of tumor cells stimulated the invasion potentialities of both DMEM and XF-MSCs as compared with the negative control where no cancer cells were added to the plates. Although, the migration specificity towards tumor cells, seems to be more pronounced in the XF-MSCs. This results were consistent in the 3 donors tested (Figure 3, 4 and 5). Also, the presence of physiological coatings such as Collagen and Matrigel increased the migration specificity. Physiological coatings provide a physical barrier to cell migration, meaning that MSCs must degrade these layers in order to reach cancer cells. Thus, the several chemoattractant factors produced by tumor cells seem to be necessary to trigger the invasive potential of MSCs.

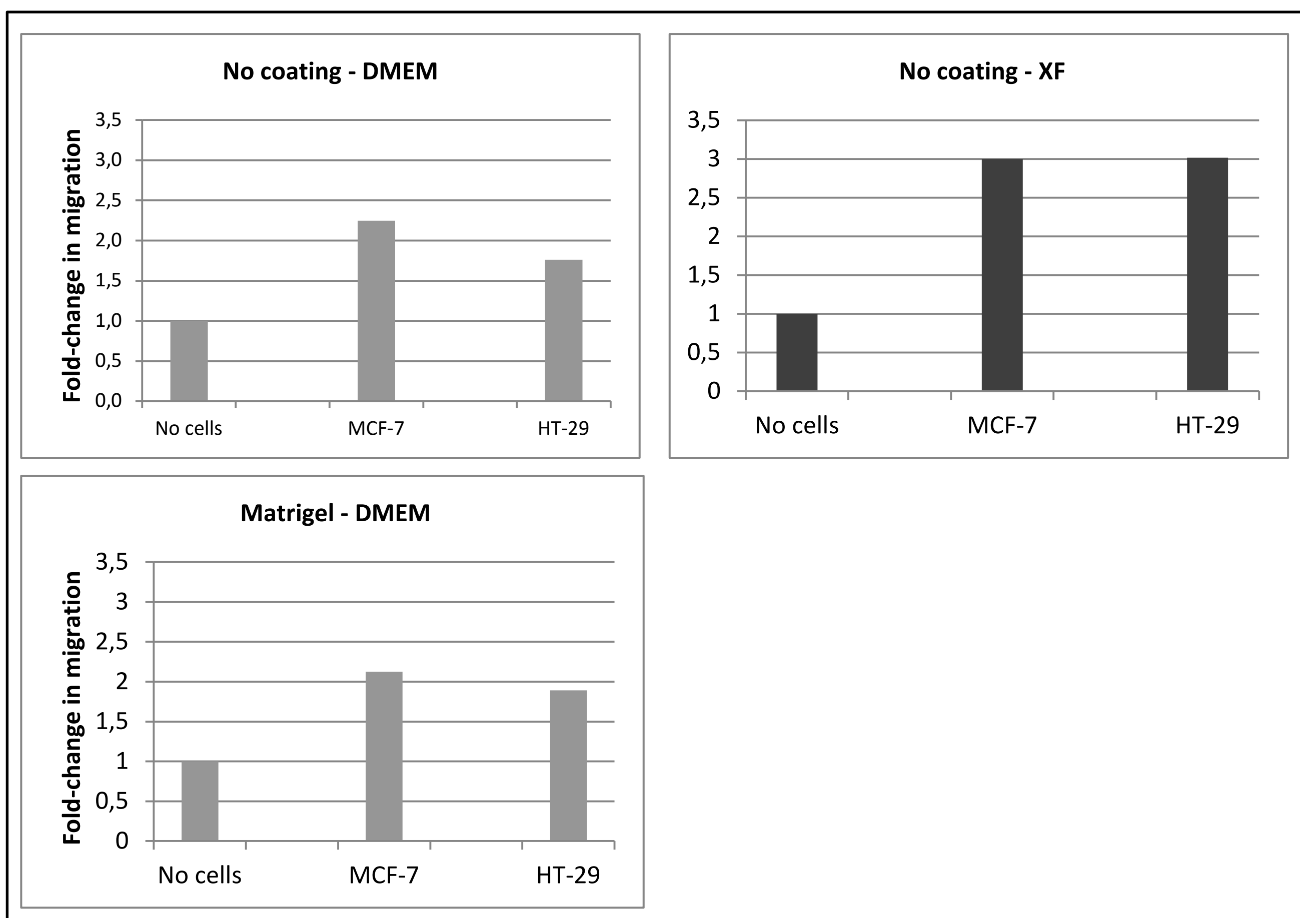


Figure 3 Invasive potential of MSCs (donor **M79A15**) towards breast cancer (MFC-7), and colon cancer (HT-29). The condition where no cancer cells were present is the negative control. The results are presented in the fold change between tumor cells and the negative control (no tumor cells in the bottom).

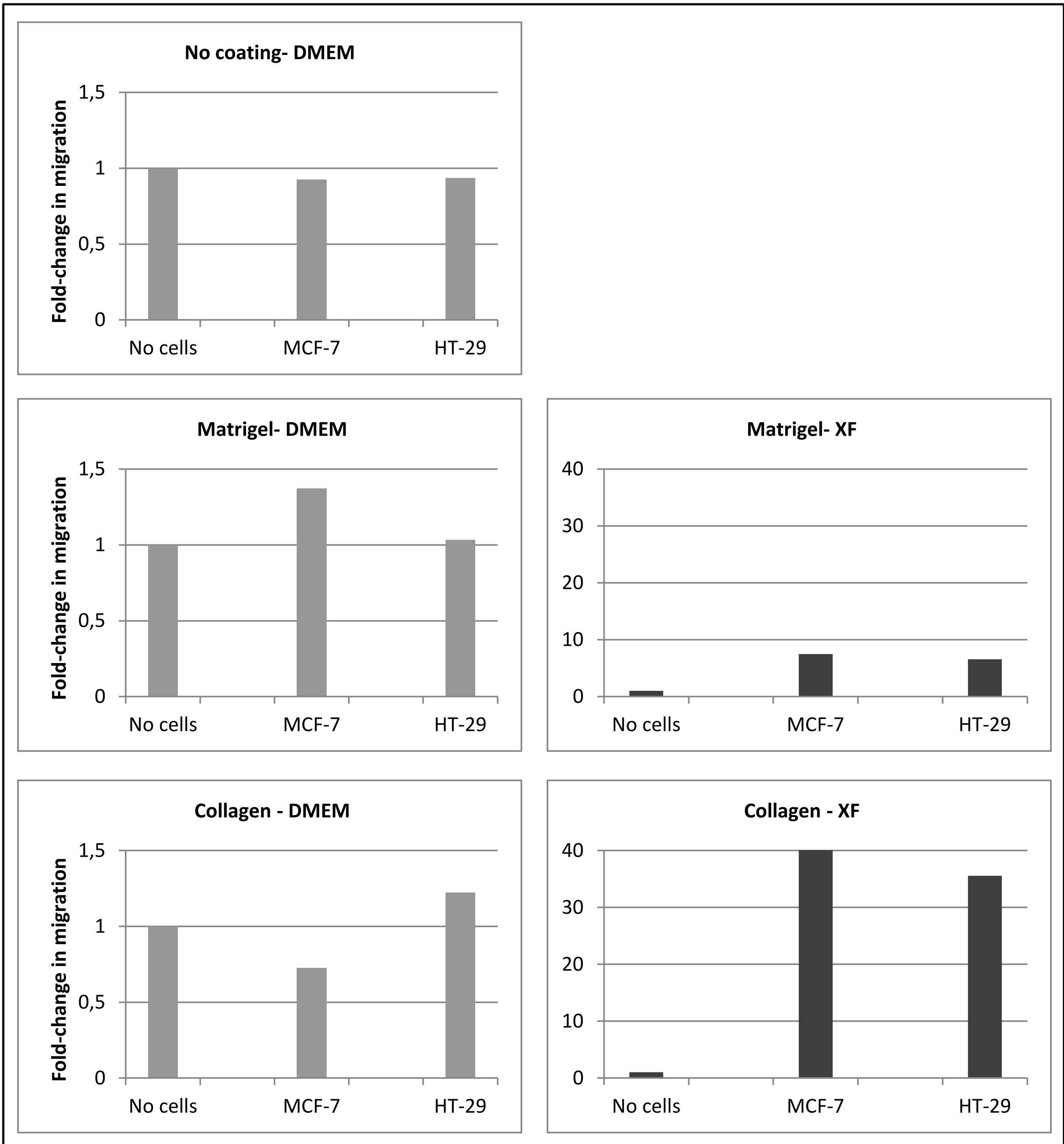


Figure 4 Invasive potential of MSCs (donor **M48A08**) towards breast cancer (MFC-7), and colon cancer (HT-29). The condition where no cancer cells were present is the negative control. The results are presented in the fold change between tumor cells and the negative control (no tumor cells in the bottom).

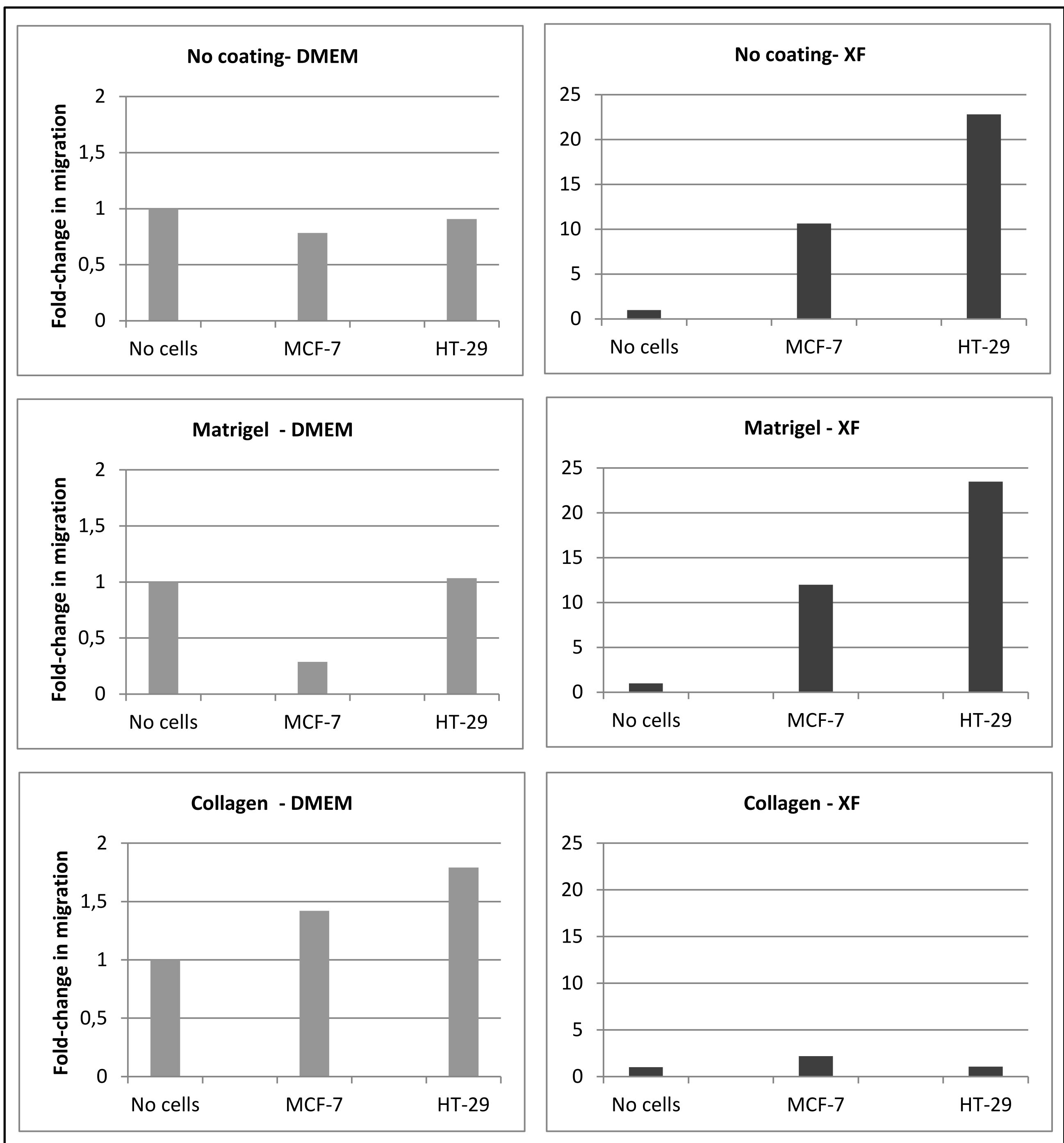


Figure 5 Invasive potential of MSCs (donor **M83A15**) towards breast cancer (MFC-7), and colon cancer (HT-29). The condition where no cancer cells were present is the negative control. The results are presented in the fold change between tumor cells and the negative control (no tumor cells in the bottom).

The migration rate is variable between donors, especially in XF-grown MSCs. Although MSCs from different donors and sources share many characteristic features, they differ in many aspects of gene expression profile and physiology. Variations such as different gene expression patterns, mutations and SNPs may originate important differences between donors that may trigger different outcomes in their role (Akimoto *et al*, 2013).

In future experiments, all the conditions tested for donor M83A15 will be assessed for the other donors (M79A15 and M48A08). In this work, it was not possible to complete all experiments. However,

with the results presented it is possible to get important observations on the tumor tropism ability of these cells and also the importance of expansion media and donors in the potential of MSCs in cancer therapies.

4.2 Azurin influence in Mesenchymal Stem Cells proliferation

As described in the objectives section, the main goal of this work is to engineer Mesenchymal Stem Cells towards the production of azurin to directly target tumor cells and specifically annihilate them. As previously described, azurin can induce apoptosis, cell cycle arrest, interfere with cell signaling pathways, among other activities that may lead cells to a lower proliferation potential (Yamada *et al*. 2004). According to our hypothesis, MSC by secreting azurin, inevitably are going to be subjected to it in their microenvironment. Thus, the first step was to understand if the proliferation rate of MSCs is influenced by the presence of increasing concentrations of azurin. This observation has much significance since is very important that MSCs aren't affected by the presence of azurin in order for our strategy to be successful.

To investigate this, a MTT cell viability assay was performed to determine the proliferation rate of Bone Marrow-MSC subjected to 0 μM, 5 μM, 10 μM, 25 μM and 50μM of azurin. A 96-well plate was cultured with BM-MSCs and incubated with the referred concentrations of azurin. To the control condition, media with no azurin was added. After incubation, the proliferation rate was relatively determined by comparison with the untreated cells.

Table 5 presents the proliferation percentage variation relatively to the control, where no azurin was added (corresponding to 100% proliferation rate).

Table 5 Proliferation rate of Bone Marrow Mesenchymal Stem Cells subjected to 0 μM, 5 μM, 10 μM, 25 μM and 50μM of azurin. The control test is the condition where no azurin was added and this value was considered to be the 100% proliferation. MSC grown in XF media and DMEM media where analyzed separately.

		Proliferation (%)	
Azurin concentration (μM)	0	100	100
	5	187.8	112
	10	174.1	113.5
	25	163.6	106.6
	50	119.2	105.9
MSC's growth media		DMEM	XF

In this study, two types of MSC-growing media were employed, STEM Pro XenoFree (XF) media and DMEM media supplemented with 10% FBS. The objective is to detect if different outcomes are protruded from different expansion media. Essentially, the difference from these two types of media is the fact that XF is completely free from animal-derived components which allow for a more

accurate result since it prevents contagious health risks from viral agents, mycoplasma and prions conferring them a more suitable statute for clinical applications. The information in Table 5 was used to build the graph represented in Figure 6.

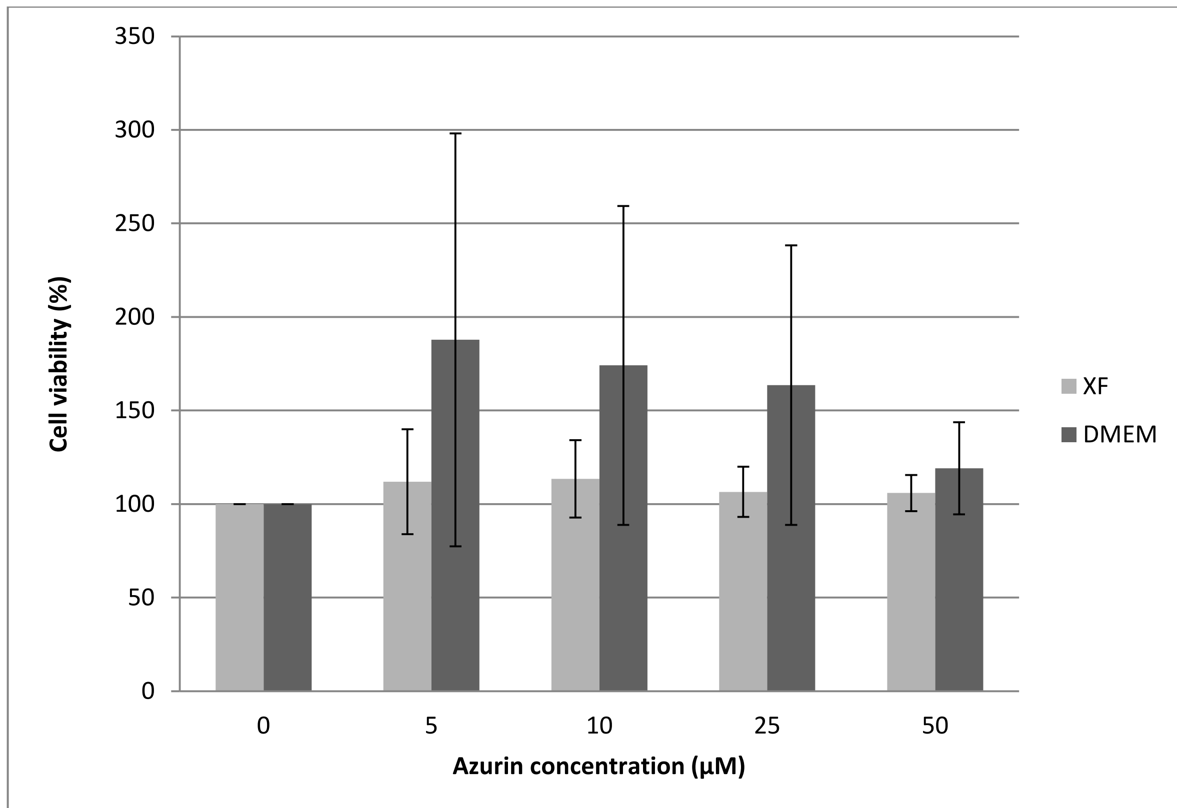


Figure 6 Cell viability assessed with MTT assay of Mesenchymal Stem Cells in the presence of azurin. Mesenchymal Stem Cells were plated in 96-well plates and exposed to diverse concentrations of azurin during 48h. Untreated cells received media without azurin and their proliferation rate was admitted as 100%.

Analyzing the results, azurin doesn't induce an inhibition in MSC viability, in fact it seems that its presence induce cell proliferation. These results allow us to precede our study maintaining the hypothesis of employing MSCs as a delivery vehicle of azurin without the risk of affecting MSCs viability due to the cytotoxic properties of azurin.

4.3 Azurin entry capacity into Mesenchymal Stem Cells

Complementing the previous result, we tested if azurin is able to enter Mesenchymal Stem Cells. An Azurin Entry Assay was performed, by subjecting XF-grown MSCs and DMEM-grown MSCs seeded in 6-well plastic plates to a 50µM azurin concentration during 30 minutes. Afterwards, the cells were washed, lysed, the total protein was quantified and a Western blot was performed, with the purpose of verifying the amount of azurin that entered the cells. GAPDH is a constitutive protein with a

very stable expression pattern in most animal cells therefore it was used as an intern control. The negative control was the condition where MSCs were treated with culture media without azurin.

Regarding both MSCs, the result was positive for the presence of azurin which means that in fact azurin is internalized by these cells. Also, it seems that there is a slight more incorporation of azurin in MSCs grown in XF media which may be due to factors present in this type of media that could function as enhancers of this mechanism. In Figure 7 the result from the Western blot is present.

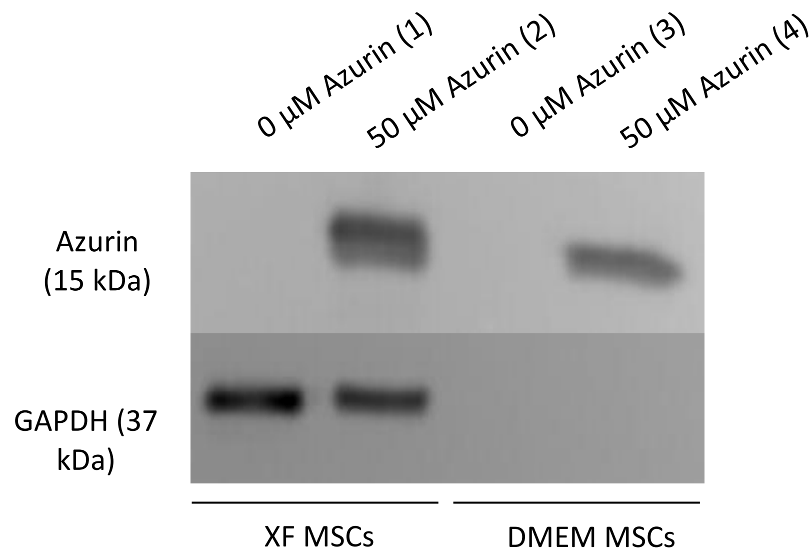


Figure 7 Azurin entry assay in XF-grown MSCs (1 and 2) and DMEM-grown MSCs (3 and 4). MSCs were treated with 50 µM azurin (2 and 4) during 30 minutes in 6-well plastic plates plastic. The negative control (1 and 3) represents the cells untreated with azurin. The protein levels were normalized by the respective GAPDH level.

Conditions 1 and 2 correspond to MSCs grown on XF media and 3 and 4 correspond to MSCs grown on DMEM media. Moreover, conditions 1 and 3 correspond to the mesenchymal stem cells treated with media without azurin (negative control) and conditions 2 and 4 correspond to MSCs treated with 50µM azurin.

The GAPDH signal was not detected in the MSCs grown in DMEM media maybe due to the absence of an essential factor for GAPDH expression or due to an error. Yet, for the purpose of this experiment, the detection of this control protein is not mandatory.

4.4 Construction of Azurin recombinant plasmid

The main objective of this work is to genetically engineer Mesenchymal Stem Cells towards the production of azurin. In order to obtain MCSs producing a gene that is not naturally expressed by them, the cloning of the azurin gene and a secretory sequence in a plasmid was performed prior to MSC's transfection.

To accomplish this, a set of genetic engineering procedures must be carried on. In Table 6 the plasmids employed in this work are shown, as well as their relevant features. And Table 7 highlights the genetic engineering steps to achieve the recombinant plasmid.

Table 6 Plasmids used in this work to accomplish the genetic engineering of MSC towards the production and secretion of azurin, their relevant features and the providers.

Plasmid	Relevant characteristics	Source
pVAX-GFP	Selected vector for MSC transduction Kanamycin selection marker.	Prof. Gabriel Monteiro and Prof Miguel Prazeres
pAzurinNative	Vector containing the Azurin gene, its natural peptide signal, EcoRI recognition sequence upstream the construct and XbaI recognition sequence downstream the construct. Ampicillin selection marker.	*NZYTECH*
pAzurinEngineered	Vector containing the Azurin gene, an engineered secretory sequence, EcoRI recognition sequence upstream the construct and XbaI recognition sequence downstream the construct. Ampicillin selection marker.	*NZYTECH*
pVAX-AzurinNative	pVAX carrying azurin gene and its natural peptide signal. Kanamycin selection marker.	This study
pVAX-AzurinEngineered	pVAX carrying azurin gene and the engineered secretory sequence. Kanamycin selection marker.	This study

Table 7 Genetic engineering phases employed to produce the recombinant vector containing the desired inserts (AzurinNative and AzurinEngineered).

Phase	Description
1	pAzurinNative, pAzurinEngineered and pVAX-GFP transformation in *Escherichia coli (E. coli)* for plasmid expansion
2	pAzurinNative, pAzurinEngineered and pVAX-GFP extraction from *E.coli*
3	pAzurinNative and pAzurinEngineered digestion with EcoRI and XbaI for construct extraction (AzurinNative and AzurinEngineered)
4	pVAX-GFP digestion with EcoRI and XbaI for GFP gene extraction and formation of cohesive ends
5	Ligation between linearized pVAX and AzurinNative or AzurinEngineered
6	Ligation product selection
7	DNA sequencing

The first step to engineer the recombinant plasmid was to select the vector. One important feature to consider is their compatibility with the expression machinery of eukaryotic cells. The plasmid selected was pVAX-GFP, carrying the GFP gene, the human cytomegalovirus (CMV) immediate early promoter for expression in a wide range of mammalian cells and the selection marker of kanamycin resistance gene. Since GFP in the construct is unnecessary for the experiment and its presence only increases the size of the vector inducing potential problems in the further steps, we decided to extract its gene. To accomplish this, the plasmid restriction map was observed. In Figure 8, pVAX-GFP restriction map is present.

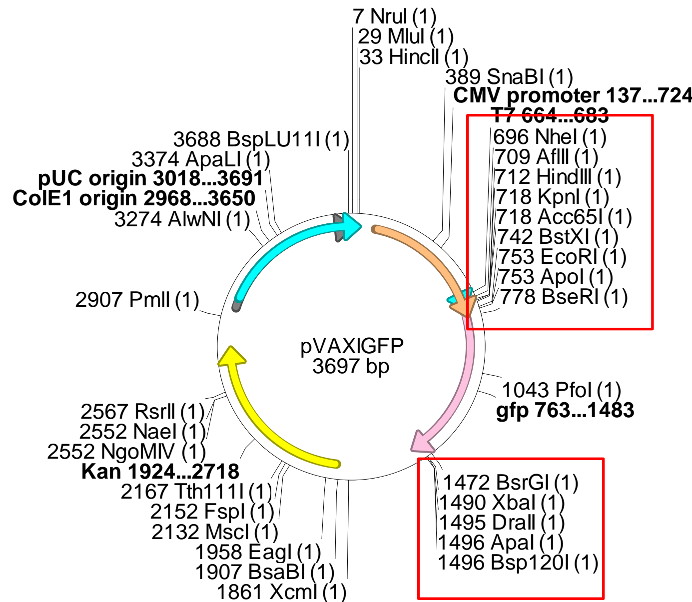


Figure 8 pVAX-GFP restriction map. The red boxes represent the enzymes that allow the GFP extraction.

The GFP gene is located between the CMV promoter and the kanamycin resistance gene. Considering the enzymes highlighted by the red box, the enzymes EcoRI and XbaI were selected to excise the GFP gene without causing any disturbance in the remaining plasmid structure. In order to allow the gene cloning, the same enzymes were selected to digest the AzurinNative and AzurinEngineered inserts.

Gene synthesis was performed in order to obtain AzurinNative and AzurinEngineered, which stands for the azurin gene and its natural peptide signal and an engineered secretory sequence (Qiu *et al*, 2000), respectively. Both constructs were provided by *NZYTECH* in plasmids. A codon optimization technology was applied in this process taking into consideration that the azurin gene is from a bacterial source and its efficiency of translation in animal cells such as MSCs can be reduced. Thus, the codon optimization algorithm towards the human codon usage is extremely important. The constructs also include the EcoRI recognition sequence upstream the construct and the XbaI recognition sequence downstream the construct to allow for the further ligation protocol in the pVAX vector. Also, the selection marker used in these plasmids is the ampicillin resistance gene.

The next step was to expand the plasmids pAzurinNative and pAzurinEngineered provided by *NZYTECH*. For this, a strain of electrocompetent *E.coli* (XL-1 Blue) was electroporated with these plasmids. The clones were selected when grown in a solid culture media containing ampicillin (150µg/mL). The colonies able to grow in these conditions are the ones that were successfully transformed with the plasmids and were selected to expand in liquid media with ampicillin (150µg/mL). The plasmids were extracted from the colonies and digested with the restriction enzymes, EcoRI and XbaI, overnight in order to isolate the construct composed by the azurin gene and their secretory

signal sequence. Afterwards the digestion products were separated in an agarose gel (1%), prior to DNA extraction.

The same procedure was applied to pVAX-GFP. An *E.coli* competent strain DH5α was transformed through classic transformation with this plasmid. The clones were selected when grown in a solid culture media containing kanamycin (50μg/mL). The colonies able to grow in such conditions were selected to expand in liquid LB media with kanamycin (50μg/mL). Then, the plasmids were extracted from the culture and the double digestion was performed in order to extract the GFP gene and create cohesive ends prior to the following cloning steps. Lastly, the digestion products were separated in an agarose gel (1%).

In Figure 9A, the pAzurinNative and pAzurinEngineered digestion products separated in the agarose gel are demonstrated. Runs number 2 and 3 are replicates from pAzurinEngineered digestion products; and run number 4 and 5 are replicates from pAzurinNative digestion products. The arrow points at the AzurinNative and AzurinEngineered genes. In Figure 9B, the pVAX-GFP digestion product is demonstrated. Runs number 2 and 3 are replicates from pVAX-GFP digestion products. The arrow points to pVAX linearized with a size corresponding to 3000 bp and the bottom band is the excised GFP gene

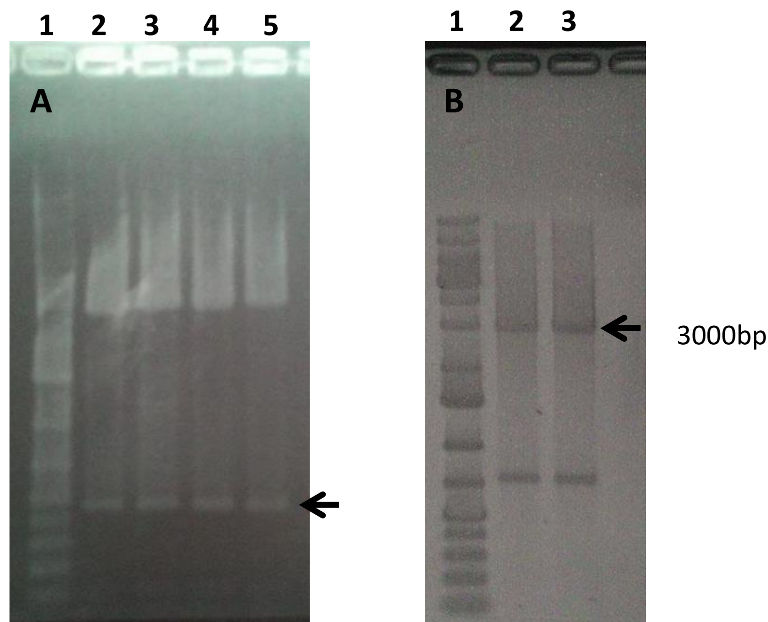


Figure 9 Digestion products from pAzurinEngineered (A-2;3), pAzurinNative (A-4;5) and pVAX-GFP (B-2;3). Arrows represent the excised genes (A) and the linearized vector (B).

After the digestion products separation in agarose gel, the bands corresponding to the desired fragments (arrows in Figure 9) were excised and the DNA was extracted from each band using a commercially available kit (Nucleo Spin Extract II).

Since both the vector and the inserts have complementary cohesive ends due to the digestion with the same proteins, the ligation protocol was accomplished using the T4 ligation enzyme in a mass proportion of 3:1 (gene:plasmid) and the appropriate reaction buffer.

The ligation products were then transformed through classic transformation in a competent *E.coli* strain DH5α. After the selection of the transformed colonies through a kanamycin selection growth, the colonies were expanded in liquid media with kanamycin (50μg/mL) and the pDNA was extracted from each culture.

The final step was the confirmation of the DNA identity by DNA sequencing to ascertain if the results obtained in fact correspond to pVAX-AzurinEngineered.

4.5 Confirmation of Azurin expression and secretion from pVAX-Azurin

With the purpose of confirming if the recombinant plasmid obtained in the present work is successfully recognized by cellular machinery, leading to azurin expression and secretion, as a first approach we used HeLa, MCF-7 and HEK cells as transfection hosts. These cell lines were selected due to the fact that they are readily transfectable with lipofectamine and the transfection protocol is optimized (Huang *et al*, 2015; Jain *et al*, 2013).

Cells were transfected alternately with the recombinant plasmids pVAX-AzurinNative, pVAX-AzurinEngineered and pVAX-GFP (transfection control) with the Lipofectamine^{TM} 2000 reagent, according to the manufacturer's instructions.

After transfection, supernatants were collected and cells were lysed prior to azurin and GFP detection through Western Blotting analysis.

In Figure 10, the result from the Western Blotting is presented regarding HeLa cells. pVAX-GFP was employed as a transfection control due to its resemblance in size to pVAX-Azurin and ease of detection. The transfection protocol was successful since GFP was detected the cell lysates. Azurin was also detected in cell lysates and supernatants, confirming that the plasmid is successfully recognized by the cellular machinery and the secretory sequence is targeting the recombinant protein to the secretory pathway properly. Although, azurin was never detected in pVAX-AzurinNative transfected cells and therefore this plasmid was exempted from further experiments.

These results where validated in the other cell lines (MCF-7 and HEK; data not shown), therefore pVAX-AzurinEngineered and pVAX-GFP where selected for MSC's microporation.

Figure 10 Confirmation of Azurin production and secretion in HeLa cells through lipofectamine transfection of pVAX-GFP (transfection control) and pVAX-Azurin. GFP production was detected in cell lysates (grey arrow), meaning that the transfection protocol was successful. Azurin was detected in both cell lysates and supernatants (red arrows), meaning that the plasmid is recognized by cellular machinery and the recombinant azurin is successfully expressed and secreted to the extracellular environment.

4.6 BM-MSCs microporation and identification of azurin expression

Having confirmed the effectiveness of the pVAX-Azurin plasmid, the next step was MSC's transfection. As in the previous section, we tested plasmids pVAX-GFP and pVAX-Azurin and in order to deliver these plasmids to BM-MSCs with a higher efficiency, we chose microporation (Madeira *et al*, 2011). After microporation, supernatants were collected and cells were lysed 48 hours and 72 hours post-transfection.

To determine if the microporation protocol was successful, GFP was detected by fluorescent microscopy 48 hours after transfection.

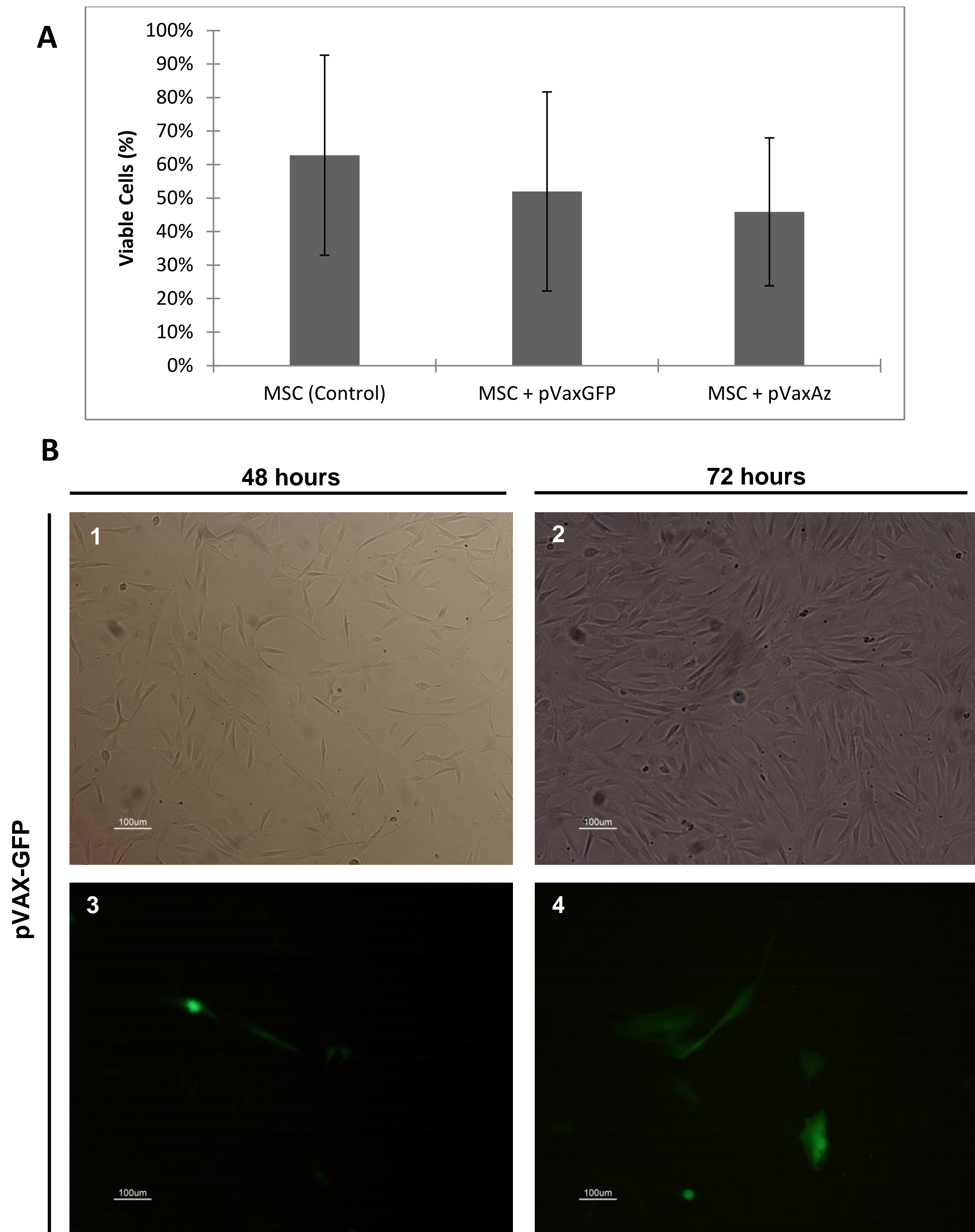


Figure 11 MSCs microporation with pVAX-GFP. Viable cells (A) were analyzed 48hours post-transfection. In B, 1 and 3 correspond to MSC's morphology 48 hours post-transfection observed under microscopy and fluorescent microscopy in the same microscopic field, respectively. 2 and 4 correspond to MSC's morphology 72 hours post-transfection observed under microscopy and fluorescent microscopy in the same microscopic field, respectively.

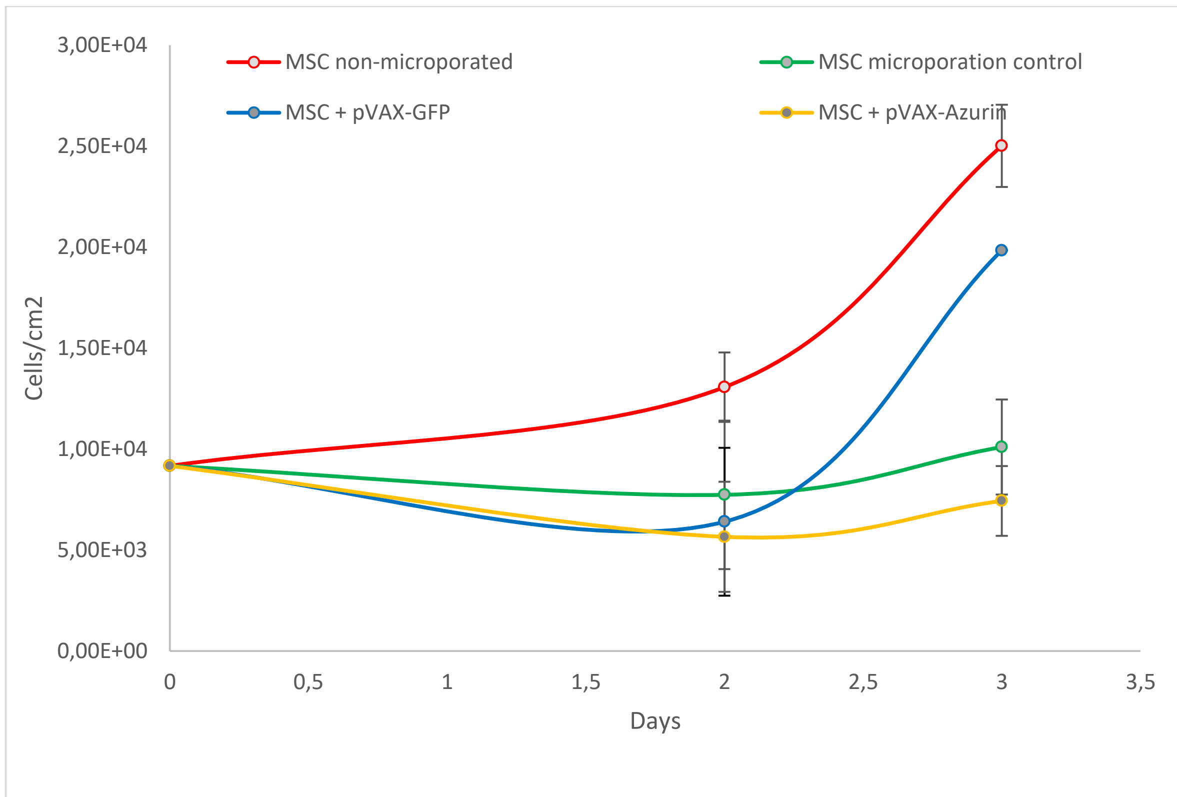


Figure 12 Mesenchymal stem cells number per cm^2 after microporation. MSC non-microporated; MSC microporation control were transfected without the plasmid DNA; MSCs + pVAX-GFP were microporated with pVAX-GFP and MSCs + pVAX-Azurin were microporated with pVAX-Azurin. Cells were initially at $9.17x10^3$cell/cm^2. Cells and supernatants were collected at day 2 and 3**. Values are Mean ± SD.**

In Figure 11A, the effect of microporation in cell viability is compared between MSCs microporated with the plasmids and without the plasmids (control). Microporation itself exerts adverse effects in cell recovery and viability and these adverse effects are enhanced by the presence of DNA. Although the seeming low percentage of viable cells, given that MSCs are hard-to-transfect cells, our results were prosperous (Madeira *et al*, 2011). Moreover, fluorescence microscopy images (Figure 11B) revealed GFP^+ cells. These results indicate that the employed microporation protocol is an effective transfection method for MSCs with little cell death.

Figure 12 compares cell growth between MSCs non-microporated and MSCs microporated without the pDNA (control) and with pVAX-GFP or pVAX-Azurin over the 72h post-transfection. The effect of microporation is considerable regarding cell regeneration. Only after 48 hours of incubation the cells were able to achieve a higher population density.

Given that the microporation protocol was well succeeded, the next step was azurin detection in cell lysates and concentrated supernatants through western blotting (Figure13).

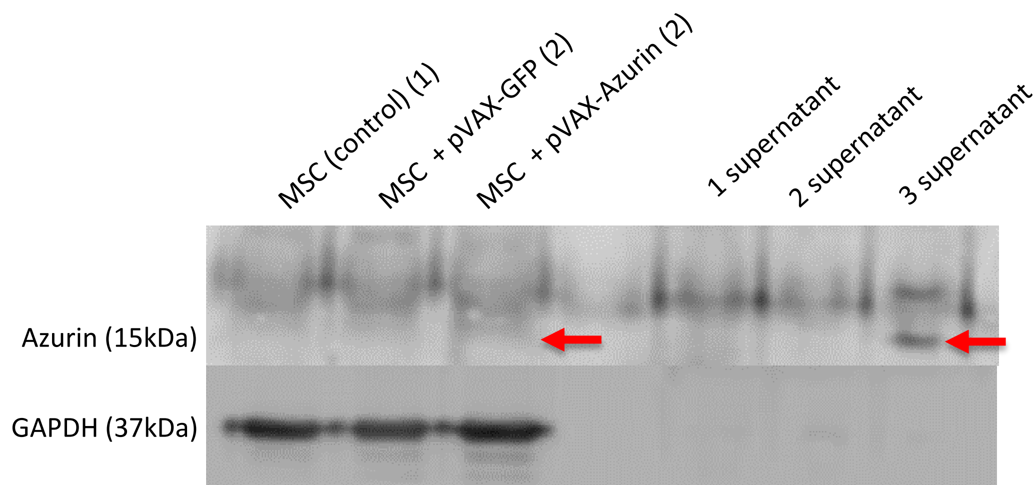


Figure 13 Confirmation of Azurin production and secretion by bone marrow mesenchymal stem cells 72hours after microporation. The first 3 conditions correspond to cell lysates: MSCs microporated without the plasmid DNA (control), MSCs microporated with pVAX-GFP and MSCs microporated with pVAX-Azurin. The last 3 conditions are the respective concentrated supernatants harvested before cell lysis. Azurin was detected in cell lysates and cell supernatants (red arrows).

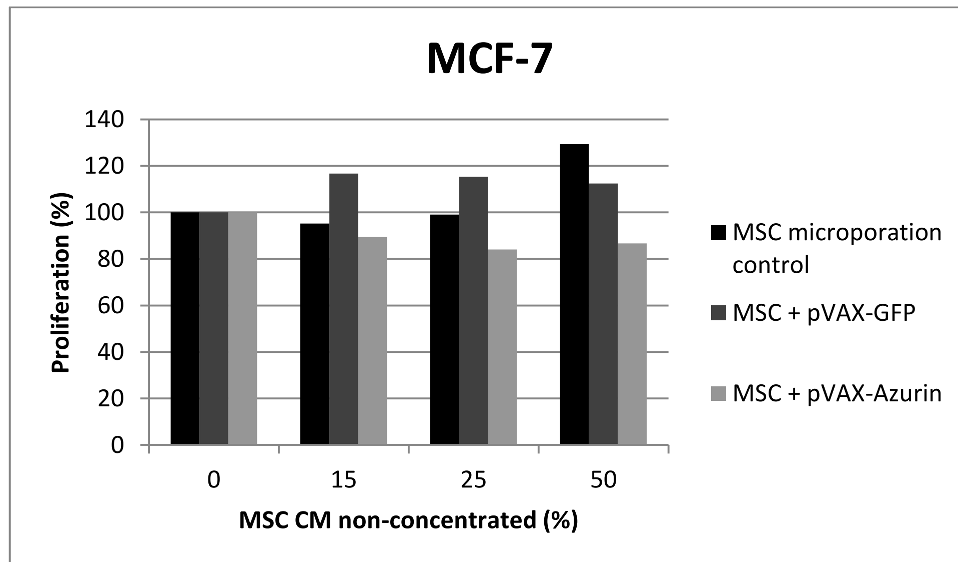
Azurin was detected in pVAX-Azurin-MSCs in both cell lysates and concentrated supernatants at 48h and 72h post-transfection, meaning that these cells successfully recognized the exogenous DNA leading to azurin synthesis and secretion to the extracellular environment. The signal seems to be stronger in supernatants which may be due to a high rate of protein secretion into the extracellular medium guided by the secretory sequence. This was further confirmed by RT-PCR to evaluate the expression of Azurin at the messenger RNA (mRNA) level.

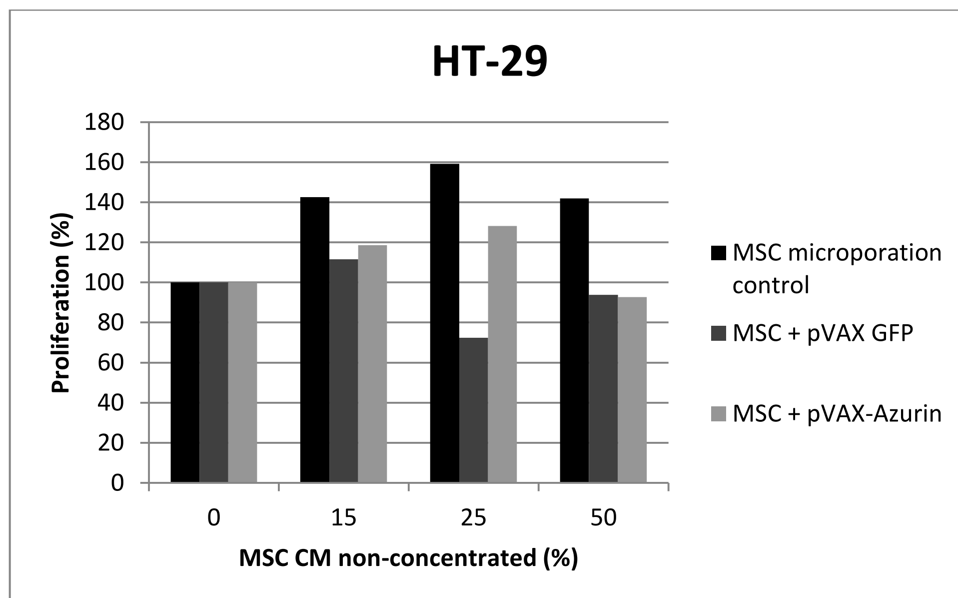
4.7 Tumor growth upon treatment with Azurin-MSC conditioned medium

To investigate whether Azurin-producing MSCs have an inhibitory effect on cancer cells' growth and viability, as a first approach we subjected tumor cell lines A549, HT-29 and MCF-7 to increasing concentrations of conditioned media from Azurin-MSC culture, harvested 72hours post-microporation.

The microporation protocol is optimized for XF grown-MSCs and given that tumor cells are cultured in DMEM with FBS, the different components present in the XF-media may exert particular effects on tumor growth that we cannot interpret. Therefore, for this experiment, we variated the Azurin-MSCs conditioned media concentration, while maintaining a baseline level of cancer cells' culture media at 50%. Thus, the potential inhibitory effects of azurin produced by MSCs are not associated with the medium change or the lack of FBS components.

After culturing cancer cells overnight, the culture media was changed by the Azurin-MSC conditioned media and cells were incubated overnight at 37°C and 5% CO_2. This step was repeated 3 times to increase the significance of MSC-azurin. Tumor development was assessed by MTT assays and the results are shown in Figure 14 with the proliferation percentage variation relatively to the control, where no Azurin-MSC conditioned media was added (corresponding to 100% proliferation rate). Conditioned media from MSCs microporated with pVAX-GFP and MSCs microporated without DNA (microporation control) were included in this experiment as a control to assess the actual effect of azurin presence relatively to tumor development.





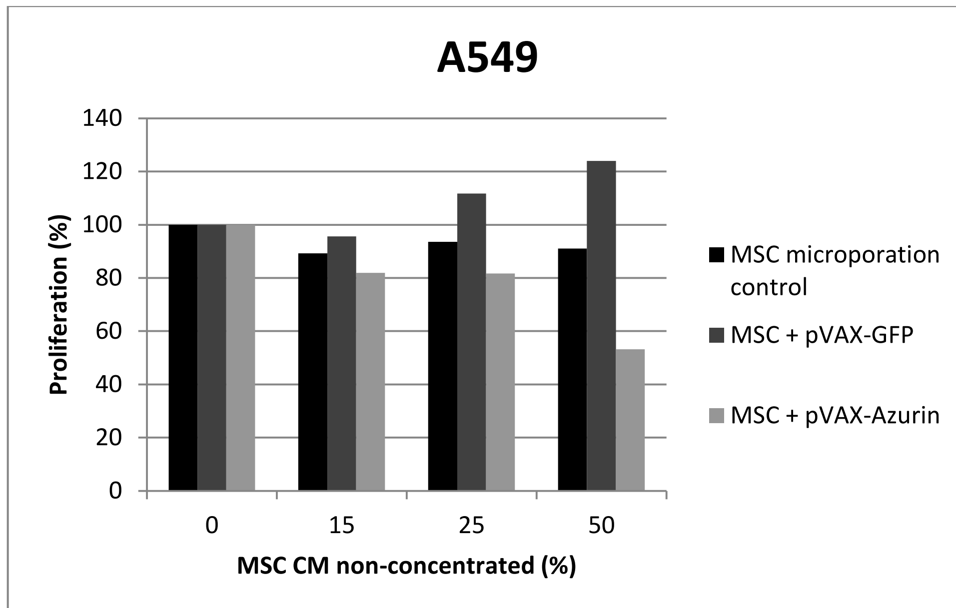


Figure 14 Cell viability assessed with MTT assay of breast cancer (MCF-7), colon cancer (HT-29) and lung cancer (A549) upon conditioned medias from MSCs microporated with pVAX-Azurin, pVAX-GFP or without DNA (microporation control). The conditioned medias where collected 72 hours post-microporation. Untreated cells received media without the conditioned media and their proliferation rate was admitted as 100%.

The effect of Azurin-MSCs' conditioned media seems to be impairing tumor proliferation and this effect is more pronounced by increasing percentage of the microporated MSCs-conditioned media. Conditioned media from MSC microporated with GFP and microporation control seem to induce, to a certain extent, tumor proliferation. As mentioned in this work, MSCs produce several factors that might be directly or indirectly related to tumor induction or repression. The balance between these two groups of factors is critical for the role of unmodified MSCs in tumor progression. This might explain a certain tendency for tumor proliferation engaged by the control conditioned media, contraposed by the seeming tumor inhibition in the cells treated with Azurin-MSCs' conditioned media.

The results were not as consistent in HT-29 cell lines, which might be explained since this cell line is a p53 mutant, meaning that this protein is not functional in this cell line, losing one of the main targets for azurin action (Rodrigues *et al*, 1990).

These results are preliminary evidences of the potential anti-tumoral effects of Azurin-MSCs that need further investigation, although they reveal important insights for the validation of our hypothesis.

5. Discussion

One of the many challenges of cancer treatment concerns the specific delivery of anticancer drugs to the tumor site. Mesenchymal stem cells (MSCs) have the ability to migrate specifically, and incorporate within tumors, they lack immunogenicity, avoiding immune rejection after transplant and they are able to self-renew. Gathered, these properties, makes them a potentially exciting drug delivery tool towards cancer, and recently MSCs have been extensively studied and used as cell/ drug delivery vehicles in cell-based therapies (Hu *et al*, 2011). MSCs do not only hold out to solve the drug delivery specificity problem, but also allow for the heightening of the drug compounds half-life in the organism; allow a lower dosage and less repeated injections to achieve meaningful responses (Jing *et al*, 2014). Despite the controversy regarding the role of unmodified MSCs in the progression of the tumor density and metastasis there is an increasing number of studies exploring MSCs and taking advantage of their features to specifically target tumor cells and deliver specific anticancer agents. One of the antitumoral proteins recently studied is azurin, a protein produced by *Pseudomonas aeruginosa*. Some of the main action mechanisms of azurin when entering in the tumor cells is to modify the cell signaling pathways by binding to the pro-apoptotic protein p53 increasing its function, by arresting cell growth in G1 phase and by interfering with the receptor tyrosine kinase EphB2-mediated cell signaling and inhibiting angiogenesis which are crucial steps for tumor development. While other anticancer proteins such as TRAIL require specific receptors in the tumor cells to recognize and penetrate into them, and those receptors are tumor cell specific, azurin enters cancer cells co-localized with caveolin-1, the main component of the caveolae, which is generally present and up-regulated in cancer cells (Mehta *et al*, 2011; Li *et al*, 2001). Also, azurin has the ability to mediate specific high-affinity interactions with various unrelated mammalian proteins relevant in cancer (Fialho *et al*, 2007). These features allow a much broader action of azurin regarding the types of tumor cells that it can affect and also supports the prevention of tumor resistance.

In this study we hypothesized the genetically engineering of MSCs towards the production and secretion of azurin. To achieve this, we engineered a recombinant plasmid containing the azurin gene and a secretory signal to direct the produced protein to the extracellular environment. Two secretory sequences were tested, azurin's natural peptide signal sequence which is naturally coupled to azurin gene in *Pseudomonas aeruginosa* and an engineered secretory sequence that provides a signal for translocation of recombinant proteins into the lumen of the ER, for transport through the ER and Golgi apparatus to the extracellular environment (Qiu *et al*, 2000). This study is the first to combine a stem cell-based approach to deliver a bacterial protein in anticancer therapy. Taking into consideration the potential dissemblance between bacterial and human cell machinery, a codon optimization technology towards the human codon usage was applied in the azurin gene by gene synthesis. After testing the effectiveness of the two azurin-plasmids in primary hosts (MCF-7, HeLa and HEK) we concluded that only the engineered sequence was capable of leading to azurin expression and secretion. Therefore, MSCs were microporated with the selected recombinant plasmid. Mesenchymal stem cells are hard-to-transfect cells, and gene delivery has been frequently accomplished by viral-based vectors, but issues regarding these vectors' safety and manufacturing have encouraged the optimization and

application of non-viral based techniques such as microporation (Madeira *et al*, 2011). The method used in this study is based on the protocol optimized at the Stem Cell Bioengineering and Regenerative Medicine Lab from iBB (Madeira *et al*, 2010), aiming BM-MSCs transfection with high efficiency without compromising cell viability and recovery. Several factors influence gene delivery efficiency, including electroporation buffer, electric pulse, pulse width, number of pulses cell density and DNA amount. All this parameters where carefully examined in order to provide the best outcome (Madeira *et al*, 2010). Regarding our experiment, microporation had a negative impact in cell growth, as was expected. However, after 48h of incubation, the cells achieved higher cell numbers. Our results on gene delivery efficiency are in agreement with those obtained in Madeira *et al*, 2010. Regarding the percentage of GFP^+ cells we obtained 58%, a cellular recovery of 46% and yield of transfection of 28% (70%, 40%, 30%, respectively in Madeira *et al*, 2010). Azurin-MSCs' supernatants and cells were collected at 48h and 72h and azurin was detected by Western Blotting and RT-PCR. In this work, we were able to successfully engineer a plasmid (pVAX-Azurin) containing an amino acid sequence that upon expression of the gene, delivers the protein to the extracellular media by a non-native hard-to-transfect host.

Following azurin confirmation in the cell supernatants, we tested the indirect influence of Azurin-MSCs in tumor progression. The three tumor cell lines MCF-7, HT-29 and A549 were subjected to increasing concentrations of Azurin-MSCs conditioned media. During 72 hours post-microporation, cells produced their native pro-tumorigenic and anti-tumorigenic factors, and also azurin. The balance between the plenitude of these factors seemed to induce tumor suppression by comparing with MSCs microporated with pVAX-GFP and MSCs microporated with no DNA, where a more pro-tumorigenic outcome was observed. Although these results need a solid confirmation with further experiments, they reveal important insights for the validation of our study and hypothesis.

Taking into consideration the fact that MSCs by secreting azurin, inevitably are going to be subjected to it in their microenvironment, a parallel study employed in this work was the proliferation monitoring of MSCs and the azurin entry capacity in MSCs when subjected to increasing azurin concentrations. By performing MTT cell viability assays we surprisingly observed an increasing proliferation rate of MSCs when subjected to azurin and its presence was detected intracellularly by Western blotting analysis. These observations have much significance since it is very important that MSCs aren't affected by the presence of azurin in order for our strategy to be successful.

MSCs' migratory capacity towards cancer cells was also tested in this work through indirect co-cultures and the results demonstrated that the presence of tumor cells stimulates the invasion potentialities of both DMEM and XF-MSCs as compared with the negative control where no cancer cells were added to the plates. The several chemoattractant factors produced by tumor cells such as EGF, VEGF-A, PDGF, FGF, HGF, TGF-β1, CXCL7, CXCL6, CXCL5, CXCL8, CXCL12, CCL2 and IL-6 (Studeny *et al*, 2004; Hung *et al*, 2005; Ponte *et al*, 2007; Droujinine *et al*, 2013; Studeny *et al*, 2002; Ren *et al*, 2008; Loebinger *et al*, 2009; Segers *et al*, 2006; Wang *et al*, 2009; Klopp *et al*, 2007) are necessary to trigger the invasive potential of MSCs. Although, the migration specificity towards tumor cells, seems to be more pronounced in the XF-MSCs. These results were consistent in the 3 donors tested. Also, the presence of physiological coatings such as Collagen type I and Matrigel

increased the migration specificity. Finally, we observed that the migration rate is variable between donors, especially in XF-grown MSCs. Although MSCs from different donors and sources share many characteristic features, they differ in many aspects of gene expression profile and physiology. Variations such as different gene expression patterns, mutations and SNPs may originate important differences between donors that may trigger different outcomes in their role (Akimoto *et al*, 2013). The experiments were all performed with 3 bone marrow MSCs healthy donors, so we must interpret the results in the perspective that a different outcome may arise from the same experiments with other MSC healthy donors from the bone marrow or other tissue sources such as adipose tissue or umbilical cord blood. Thus, the results obtained in this study are experimental evidences that need further confirmation.

Despite the obvious advantages of using MSCs as a delivery system, the role of unmodified MSCs in the progression of the tumor density and metastasis is still unclear. MSCs have the potential to differentiate into vascular endothelial cells, which may innocently participate in the neoplastic process, they present immunosuppressive properties, which may indirectly protect tumor cells from attack by the immune system and they may also produce some pro-tumorigenesis factors. But on the other hand, MSCs may also produce anti-tumoral factors, leading to the eradication of tumors. These observations create a trouble in classifying MSC's influence in the tumor progression, thus further research is necessary. So far, the best explanation for the contradictory results might be the different MSC sources being used, different tumor types, different culture methods, different experimental condition and designs, different passaging number of MSCs and tumor cells, the animal models, the individual in study and so forth (Yang *et al*, 2014). With the ease of harvest, culture and transfection of MSCs, the use of autologous cells may be realistic. Although, in practice is very difficult to evaluate in a clinical setting, as the number and quality of the MSCs differ from patient to patient making quantification of the therapeutic effect difficult to interpret . The use of allogenic MSC from healthy donors would allow greater cell numbers of better characterized cells (Loebinger, 2009).

For clinical applications of MSCs, further investigation must be performed to establish effective treatment strategies, with particular focus to the expression level of anti-cancer and pro-cancer molecules secreted by these cells, appropriate time intervals of administration and the source of MSC and tumor cell type (Akimoto *et al*, 2013). Also, the fate and long term effects of the modified MSCs should be investigated.

6. Conclusion and future perspectives

In the future is relevant to perform the same experiments explored in this work, in a wider panel of MSC donors and sources of isolation, in order to achieve a more accurate conclusion about the role of MSCs in the tumor progression and also the effects of azurin in MSCs proliferation. The source of the MSCs used is an important question. At present, most studies have made use of adipose tissue or bone marrow derived cells as delivery systems of several antitumoral factors. It is also not yet clear which tissue-MSC subtypes are the most effective in their ability to home to specific tumors without leading to tumorigenesis induction, although the results point to a less tumorigenic nature of umbilical cord blood derived-MSCs. An additional variation may be the cell culture media used for MSCs expansion. There in an increase effort to establish protocols based on xenogeneic-free culture media as a mean to produce safer therapies that are devoid of any animal-derived cell culture component, The effects that such alteration can cause to the profile of expressed cytokines and antigens may be important to establish effective therapies in the near future and should be taken in consideration when designing future experiments.

Since MSCs' transfection provides a transient expression of the recombinant protein, the monitoring of azurin expression level throughout time might be useful to determine its higher expression time-point. Azurin's transient expression may lead to the need for repeated injections and the protein level that sets in the tumor microenvironment could be relatively low. To circumvent this drawback, a possibility would be to make a permanent genome modification on MSCs by the recently explored technique, CRISPR/Cas9. This way, the azurin gene could be integrated into the cell's genome and simultaneously replace a selected gene associated with the potential pro-tumorigenic features of MSCs, such as the *VEGF* or *IL-6* genes, which indeed translate into proteins of comparable MWs with azurin. Descendants of these modified cells, therefore, will also express the new gene, resulting in a stable azurin-secreting cell line.

Azurin has a strong synergistic anticancer effect when combined with certain compounds such as 5-fluorouracil or etoposide (Choi *et al*, 2011), tamoxifen (Mehta *et al*, 2010), gefitinib, erlotinib (Bernardes *et al*, 2016), paclitaxel and doxorubicin (our group, unpublished). The peptide fragment p28, when combined with lower concentrations of drugs such as doxorubicin, dacarbazine, temozolamid, paclitaxel and docetaxel, maximizes their efficacy (Yamada *et al*, 2016). A future work could be the study of the combinatory effects of Azurin-MSCs loaded with an already studied anticancer agent. This would allow the reduction of dose-related toxicity generated by the administration of high drug dosages.

The delivery of nanomedicines to the precise tumor localization without being destroyed by the immune system and their retention within the core solid tumors is a major challenge. Different nano-formulations varying the materials used, the shape, size and surface properties have been developed to increase the efficacy. Therefore, another possible application may be the use of MSCs as vehicles for nanoparticles loaded with chemotherapeutic agents (nano-engineered MSCs). This strategy is likely to lead to an active accumulation of MSCs within tumor sites and a slow release of nanomedicines. In particular, taking in consideration that azurin demonstrates synergy with other

therapies, the combination of both strategies can also be looked as new strategy to overcome drug resistance and tumor recurrence.

Keeping all these information in mind we can state that MSCs have favorable antitumor potentialities and should be further explored in cancer therapies. However, to ensure the safety of anticancer therapies using MSCs, all the comprising factors must be thoroughly investigated before. When designing future experiments a detailed setup must be always performed regarding the MSCs source, the type of target tumor cells enrolled and all the variable factors that may lead to ambiguous outcomes.

By the end of this project, we aim to achieve a biological system that directly targets the tumor cells and potentiates the specificity, density and azurin's life-time in the tumor microenvironment, in opposition to a systemic administration in which the azurin dose that actually set in the tumor cells is lower and less effective.

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