Aus der Klinik und Poliklinik für Mund-, Kiefer- und Gesichtschirurgie - Plastische Operationen der Universitätsmedizin der Johannes Gutenberg-Universität Mainz

The effect of endothelial progenitor cells on the migration ability of different cell lines of head and neck squamous cell carcinoma

Die Auswirkung endothelialer Progenitorzellen auf die Migrationsfähigkeit verschiedener Zelllinien des Plattenepithelkarzinoms des Kopf-Hals-Bereichs

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List of Abbreviations

ºC	Celsius degree
®	Registered Trademark
µl	Microliter
AG	Aktiengesellschaft
Ang-1	Angiopoietin-1
ATCC	American Type Culture Collection
BM	Bone marrow
BM-EPC	Bone marrow-derived endothelial progenitor cell
CAC	Circulating angiogenic cell
CFU-EC	Colony-forming unit endothelial cell
$cm^{(2)}$	(Square) centimeter
CMFDA	5-chloromethylfluorescein diacetate
CO_2	Carbon dioxide
CSC	Cancer stem cell
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECFC	Endothelial colony-forming cell
EPC	Endothelial progenitor cell
FCS	Fetal calf serum
g	Gram
G	Gravity
GmbH	Gesellschaft mit beschränkter Haftung
GM-CSF	Granulocyte macrophage colony stimulating factor
GSC	Glioblastoma stem cell
h	Hour
HIF	Hypoxia-inducible factor
HNSCC	Head and neck squamous cell carcinoma
HSC	Hematopoietic stem cell
IB	Immunoblot
IHC	Immunohistochemistry
IMBEI	Institut für Medizinische Biometrie, Epidemiologie und Informatik
Inc.	Incorporated

Ltd.	Limited
min	Minute
ml	Milliliter
$mm^{(3)}$	(Cubic) millimeter
MMP-9	Matrix metalloproteinase-9
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PlGF	Placental growth factor
RKI	Robert Koch Institute
SCC	Squamous cell carcinoma
SDF-1α	Stromal cell-derived factor-1α
SPARC	Secreted protein, acidic and rich in cysteine
™	Trademark
TNM	Tumor, node, metastasis
UEA-1	Ulex Europaeus Agglutinin 1
USA	United States of America
VDA	Vascular disrupting agent
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	von Willebrand factor

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1. Introduction

Endothelial progenitor cells (EPCs) are mononuclear blood cells that display endothelial and progenitor properties. They were first isolated in 1997, by Asahara et al. (1997). In that report, the authors highlight the ability of these cells to induce, in adult life, the formation of new blood vessels through a de novo production of endothelial cells, something up to then thought to happen only during embryogenesis. This process, called vasculogenesis, can occur under physiological and pathological circumstances. Low oxygenation is usually the trigger that leads ischemic tissues (such as infarcted myocardium) or tumor cells to secret EPC-recruiting factors.

EPCs play an important role in tumor growth and metastasis. At the early stage of tumor growth, they are critical for promoting the “angiogenic switch”, a process in which tumor cells acquire an angiogenic phenotype (Carmeliet and Jain, 2000). This phenotype is the result of changes in the local equilibrium between positive and negative regulators of angiogenesis, and it stimulates the formation of new vessels (Nolan et al., 2007). Without this switch, tumors can only grow till approximately 1–2 mm^3, before their metabolic demands are restricted by the diffusion limit of oxygen and nutrients. During metastasis, EPCs promote the transition from micro- to macro-metastases. Moreover, EPCs may contribute to the development of resistance to anti-angiogenic drugs (Moschetta et al., 2014).

Given their importance to tumor development and resistance to treatment, EPCs are a promising therapeutic target. However, for treatments to be most effective, a deeper understanding of how blood vessels form, how EPCs collaborate to tumor progression and metastasis and how to best target these cells is required (Moschetta et al., 2014).

In light of the above, we aimed to investigate whether EPCs or their supernatant have an effect on the migration ability of different cells lines of head and neck squamous cell carcinoma (HNSCC), namely PCI-1, PCI-13, SCC-4 and SCC-68. For this purpose, these HNSCC cell lines were co-cultured with different concentrations of EPCs or cultivated with different concentrations of EPC supernatant and submitted to a scratch wound assay. The primary outcome of the investigation was the proportional width of the scratch at 24 and 48 hours, relative to the initial size. There were no secondary outcomes.

2. Review of the Literature

2.1. Search Strategies

For the identification of relevant studies, the databases MEDLINE and EMBASE were searched. Google was also used to search the internet for existing and potentially relevant studies. Detailed search strategies were developed for each database. These were based on the search strategy developed for MEDLINE, but revised appropriately for each database. The subject search strategy used a combination of controlled vocabulary and free text terms, based on the search strategy for MEDLINE (OVID). Databases were searched to include all languages. The reference lists of the included papers were examined, in an attempt to identify any additional studies. No further journals appropriate for this research question were identified and therefore we did not hand search any additional journals.

2.2. Cancer

Cancer, also known as malignant tumor or neoplasm, is the name given to a group of more than 100 diseases characterized by the disorganized growth of cells and the potential to invade or spread to other parts of the body. Cancerous cells tend to divide fast, being very aggressive and uncontrollable (American Cancer Society, 2016). If their spread is not halted, it can lead to death. On the other hand, (benign) tumors are localized masses of cells that slowly multiply and which resemble the original tissue, rarely being life threatening.

Presumably, most cancers originate from a single precursor mutant cell. The process through which a healthy cell becomes a cancerous one is called neoplastic transformation or carcinogenesis. This is thought to be a step-wise process, which requires epigenetic changes and alteration of genome integrity, in addition to a series of mutations in key regulatory genes, such as oncogenes, tumor suppressor genes, cell cycle genes, angiogenic factors, epithelial to mesenchymal transition genes, and DNA damage response genes (Napier and Speight, 2008). These genes play vital roles in cell division, apoptosis, and DNA repair, causing a cell to lose control of its proliferation capacity.

The chance of a gene to mutate is very low, thus the probability of several different mutations to occur in the same cell is very unlikely. This explains why the

prevalence of cancer is higher among older people, since they have had more time to accumulate the changes required to form cancer cells (American Cancer Society, 2016). After a cell becomes cancerous, as it multiplies, it may mutate further and adopt different behaviors over time. Cells that acquire an advantage in division or resistance to apoptosis, by natural selection, tend to take over the population.

External factors, such as tobacco, alcohol, infections or an unhealthy diet, as well as internal factors, like inherited genetic mutations, hormones, and immune conditions may act together or in sequence to cause cancer. Treatments include surgery, radiation, chemotherapy, hormone therapy, immune therapy, and targeted therapy (drugs that interfere specifically with cancer cell growth) (American Cancer Society, 2016).

Each year, tens of millions of people are diagnosed with cancer and more than half of them end up dying from it. In many countries, it ranks the second most common cause of death, only behind cardiovascular diseases (Global Burden of Disease Cancer et al., 2015). With significant improvements being made in the treatment and prevention of cardiovascular diseases, cancer has or will soon become the number one cause of death in many parts of the world (American Cancer Society, 2016). As the elderly are most susceptible to malignancies and population aging continues in many countries, cancer will remain a major issue around the globe.

According to a 2004 survey of the Robert Koch Institute (RKI), there was a total of 436.500 new cases of malignant tumors in Germany in 2004. With over 58.000 new cases per year, prostate cancer was the most common in men, followed by colorectal, and lung cancer. In women, breast cancer was the most prevalent malignancy, with an estimated 57.000 new cases every year, also followed by colorectal cancer. Moreover, for the first time, cancer prevalence was estimated, and current morbidity and mortality risks calculated at the federal level. According to RKI, the 5-year partial prevalence exceeds 600.000 in men; in women, the numbers are similar. Once more, the most common malignancies were prostate cancer, in men, and breast cancer, in women. The lifetime risk of developing cancer is more related to the individual and it is estimated to be higher among men (48.5%) than among women (40.3%). That means approximately every second person in Germany develops cancer during their lives. One in four men and one in five women end up dying of it (Haberland et al., 2010).

2.3. Head and Neck Cancer

Cancers of the head and neck are among the most common malignancies worldwide, with an estimated 442.760 cases and 241.458 deaths in 2012 (Ferlay et al., 2015). Head and neck squamous cell carcinoma (HNSCC) accounts for approximately 90% of all of them. Despite advances in diagnosis and treatment, the overall 5-year survival rate has not improved in the past 50 years and remains around 50% (Wu et al., 2010). Tobacco and alcohol are considered the primary risk factors and are known to be synergistic. The habit of chewing areca nut/betel quid has been strongly linked with the high incidence of oral cancer in South Asia. Other risk factors include poor diet, sun exposure and genetic predisposition (Sankaranarayanan et al., 2015).

HNSCC can be categorized according to the area of origin. These are: oral cavity, pharynx, larynx, paranasal sinuses and nasal cavity, and salivary glands. Cancers of the oral cavity and oropharynx are the most frequent types of HNSCC, while salivary gland cancers are relatively uncommon (Bose et al., 2013, Kruger et al., 2014). Moreover, salivary glands contain many different types of cells, so there are many different types of salivary gland cancer.

The natural history of HNSCC is not yet fully understood. It usually begins as an epithelial dysplasia, such as leukoplakia or erythroplakia, in the cells that line the moist, mucosal surfaces inside the head and neck (for example, inside the mouth, the nose, and the throat). The reported rates of progression to squamous cell carcinoma (SCC) vary from 1% to 36% (Napier and Speight, 2008).

2.4. Tumor Growth and Endothelial progenitor cells (EPCs)

Initial tumor growth is limited to 1–2 mm^3 by the diffusion of oxygen and nutrients (Hillen and Griffioen, 2007). To grow beyond that, tumors need to create a vascular network to attend their metabolic demands. Until recently, this was thought possible only through host endothelial cells and a direct capillary in-growth (Risau, 1997). Nonetheless, many studies now point out to the involvement of stem cells, especially EPCs (Kaur and Bajwa, 2014).

Tissue ischemia provokes an imbalance between positive and negative regulators of angiogenesis, which in turn leads to EPC recruitment and activation. Once at the tumor site, EPCs promote a change in tumor cells' phenotype to an

angiogenic state, thus stimulating the formation of new blood vessels (Carmeliet and Jain, 2000). In addition, EPCs further tumor neovascularization through vasculogenesis. On a later stage of tumor development, EPCs also promote the transition from micro- to macro-metastases. Given their importance to tumor development, EPCs may prove an important therapeutic target (Moschetta et al., 2014).

2.5. The Discovery of EPCs and their Characterization

EPCs were firstly isolated in 1997 (Asahara et al., 1997). Initially, they were characterized as “a population of postnatal mononuclear cells that adopted an adherent, endothelial morphology, when cultured for seven days in endothelial growth medium”.

Nowadays, EPCs are known to express several hematopoietic and endothelial lineage markers and are believed to derive from a common hemangioblast precursor in bone marrow (BM). EPCs isolated from BM (BM-EPCs) express the early hematopoietic marker CD133 (also referred to as AC133), representing more immature cells (Peichev et al., 2000). During culture, they start differentiating and lose this marker. After several weeks of late outgrowth, they finally turn into mature endothelial cells (Stroncek et al., 2009).

Peripheral blood EPCs is an umbrella term that encompasses several mononuclear cell types, such as CD34+ CD133+VEGFR2+ cells, subsets of monocytic cells, and cell populations with broad developmental plasticity.

Numerous in vitro methods have been described for isolating and expanding putative EPCs. By their means, at least three distinct vasculogenesis-related populations of peripheral blood mononuclear cells (PBMCs) can be isolated: EC colony-forming units (CFU-ECs), circulating angiogenic cells (CACs), and endothelial colony-forming cells (ECFCs) (Fadini et al., 2012, Prater et al., 2007).

CFU-ECs are non-adherent mononuclear cells that, when cultured on fibronectin-coated dishes for 4-9 days, form colonies with a central core of “round” cells and a periphery of more elongated “sprouting” cells. In in vivo collagen gel-based assays, they are not able to form vessels. CFU-EC-derived cells express markers specific to endothelial cells, together with the hematopoietic marker CD45 and the monocyte markers CD14 and CD115 (Moschetta et al., 2014).

CACs are whole, unfractioned mononuclear cells that are obtained after the removal of non-adherent PBMCs that have been plated in fibronectin-coated dishes and cultured in supplemented endothelial growth medium for 4 days. CACs do not form colonies, but are functionally and phenotypically indistinguishable from EC-CFUs (Moschetta et al., 2014).

Both EC-CFUs and CACs are hematopoietic-derived monocytes and macrophages that display a mixed endothelial-monocytic/hematopoietic phenotype. Genotypically, they are similar to cultured monocytes exposed to pro-angiogenic factors (Moschetta et al., 2014).

ECFCs are adherent mononuclear cells that form colonies with a cobblestone morphology, when plated (without pre-plating steps) for 14–25 days on collagen I coated dishes in medium containing endothelial growth factors. ECFCs are PBMC-derived cells with high proliferative activity and the ability to form vessels de novo (in in vivo collagen gel-based assay). Phenotypically, they are indistinguishable from cultured endothelial cells (ECs), expressing CD31, CD105, CD144, CD146, von Willebrand factor (vWF), VEGFR2, and Ulex Europaeus Agglutinin 1 (UEA-1). They do not express, however, CD45 and the monocyte markers CD14 and CD115 (Moschetta et al., 2014).

CACs and CFU-ECs present a mixed endothelial-monocytic/hematopoietic phenotype, while ECFCs' is indistinguishable from the one of cultured ECs. Genotypically, CACs and CFU-ECs are similar to cultured monocytes exposed to pro-angiogenic factors, differing from cultured ECs (Ahrens et al., 2011, Assmus et al., 2006, Bertolini et al., 2006, Ingram et al., 2004, Medina et al., 2010, Rafii and Lyden, 2003, Yoder et al., 2007). In turn, ECFCs have the ability to form vessels de novo, a feat not yet attributed to CFU-ECs (Yoder et al., 2007). These findings indicate that ECFCs are consistent with the EPC-related phenotype, while CACs and EC-CFUs represent mixed subsets of peripheral blood hematopoietic-derived cells, such as monocytes/macrophages, myeloid, and lymphoid progenitor cells, being able to indirectly promote vessel growth/repair (Basile and Yoder, 2014).

A disadvantage of studying EPCs cultured in vitro is that, due to culturing, these cells may gain or lose properties, differentiating them from their non-cultured counterpart, from which they originate (Moschetta et al., 2014).

2.6. Role of EPCs in Tumorigenesis

The recruitment of EPCs is vital to the development of the cancer-associated neovasculature, which in turn is paramount for tumor growth. EPC-mediated vasculogenesis is a multi-step process, which includes: a) mobilization of EPCs from bone marrow; b) active arrest and trans-endothelial extravasation of EPCs into the interstitial space of the growing tumor; c) incorporation of EPCs into neovessels or paracrine support of the nascent microvasculature (Moschetta et al., 2014).

The recruitment and migration of circulating EPCs to tumor sites is stimulated by angiogenic factors produced by the growing tumor, such as vascular endothelial growth factor (VEGF), stromal cell-derived factor-1α (SDF-1α), placental growth factor (PlGF), granulocyte macrophage colony stimulating factor (GM-CSF), and estrogens. These lead to an alteration in the microenvironment of the bone marrow, changing it from a quiescent state to a proangiogenic state.

The two main known pathways of BM-EPCs mobilization during cancer development are VEGF/VEGFRs and SDF-1α/CXCR4. VEGF has been shown to be the main growth signal that up-regulates other chemotactic factors, such as SDF-1α and CXCR-4 molecules. Tumor-secreted VEGF interacts with vascular endothelial growth factor receptor 1 and 2 (VEGFR-1 and VEGFR-2) expressed on EPCs. It also induces tumor cells to express matrix metalloproteinase-9 (MMP-9), resulting in the release of stem cell's membrane-bound c-kit (CD117) ligand, which in turn forces the translocation of quiescent VEGFR2+ c-kit + EPCs into the circulation for recruitment to sites of ischemia, inflammation or tumor growth (Moschetta et al., 2014).

Neoplastic growth causes tissue hypoxia. This leads to the stabilization of hypoxia-inducible factor 1α (HIF-1α) in tumor cells, which in turn induces the secretion of SDF-1α. The result is a paracrine mobilization of EPCs, given these cells widely express CXCR4, the receptor for SDF-1α (Petit et al., 2007).

A possible synergism between these two pathways has been demonstrated, by showing that, without a concurrent VEGF signal, SDF-1α is insufficient in recruiting EPCs to tumor sites (Kollet et al., 2001). Moreover, the incorporation of EPCs into the neovessels of implanted tumor has been related to HIF-1α-induced SDF-1α expression (Arbab et al., 2008).

Besides VEGF and SDF-1α, several other factors produced by tumor and surrounding cells have been suggested to play a role in EPC recruitment to tumor sites. Chemokines, such as CCL2 and CCL5, have been associated with the mobilization of

EPCs from the circulation (Spring et al., 2005). Furthermore, hormones, like 17-β-estradiol, have been reported to stimulate EPC-dependent neovascularization (Suriano et al., 2008). Finally, adiponectin, a peptide hormone secreted by adipocytes, has been shown to promote EPC numbers, migration, and mammary tumor growth in animal models (Landskroner-Eiger et al., 2009, Nakamura et al., 2009, Shibata et al., 2008).

Once at the tumor site, EPCs stimulate tumor neovascularization, either by direct incorporation into tumor vessels or through the production of proangiogenic factors and activation of tumor endothelial cells. According to Lyden et al. (2001), transplantation of β-galactosidase-positive EPCs into lethally irradiated angiogenesis defective $Id1^{+/-}$ $Id3^{-/-}$ host mice leads to significant restoration of tumor angiogenesis and growth. Moreover, donor-derived EPCs were detected throughout the tumor neovessels, with 90% of contribution. In contrast, Purhonen et al. (2008) did not find endothelial cells in the tumor endothelium in an in vivo study in mice and concluded that cancer growth does not require BM-derived endothelial progenitors. Nonetheless, other reports have shown that, although recruited in low numbers, BM-EPCs are crucial for the progression of tumor growth and angiogenesis (Gao et al., 2008). A study by Nolan et al. (2007) in a mouse glioma model has revealed that, at an early stage of tumor growth (0,5–1cm), EPCs are mostly present at the periphery of the tumor. However, once the tumor reaches the size of 1cm, vascular incorporation of administered cells is obvious. At even later stages of tumor growth, these cells are no longer detected, indicating the possibility that the administered EPC-derived vessels are adulterated with non-EPC-derived vessels.

Thus, it is safe to assume that, at very early stages, EPCs migrate from the bone marrow and reside along the tumor margins. At slightly more advanced stages, when there is an increase of chemotactic signals from the growing tumor, the rate of EPC migration from the bone marrow further increases and they are assimilated into the tumor vasculature, though at a lower incorporation rate. At this stage, EPCs may have an important and substantial paracrine function that is critical for tumor neovascularization (Rafii and Lyden, 2008, Ribatti, 2004). At further advanced stages, high numbers of EPCs are still present in the circulation, due to increased migration; however, at the tumor site, they lose their self-renewal properties and differentiate into mature endothelial cells to support tumor neovascularization, thereby not being detected there. This is further supported by the fact that high circulating levels of

VEGFR2^{+}-bone marrow-derived progenitor cells are present in patients with advanced cancer (Massard et al., 2012).

Nonetheless, Nolan et al. (2007) question the role of EPCs in tumor neovascularization and their real contribution remains controversial. Discrepancies among studies may be explained by different stages of tumor growth studied. Incorporation of EPCs into the tumor vasculature may be tumor-stage specific and their relative contribution to tumor neovascularization may be greater at earlier rather than later stages of tumor growth. Moreover, EPC-induced tumor neovascularization depends on the tumor type (Gao et al., 2009). Thus, the selection of specific mouse cancer models may be important for analyzing EPC-mediated neovascularization. Finally, differences among studies may be explained by several technical and experimental variables, like diverse strategies used to characterize EPCs (i.e. different markers used to define EPCs) or different techniques used to identify and measure EPC incorporation in the tumor blood vessels (i.e. immunofluorescence versus high resolution confocal microscopy, confocal techniques, such as 3D computer rendering, immunohistochemistry, and light microscopy).

2.7. The Role of EPCs in the Development of Resistance to Anti-Angiogenic Drugs

There is a growing interest in a possible role of EPCs in mediating resistance to chemotherapy, especially to drugs that target tumor vasculature. It has been shown that treatment with vascular disrupting agents (VDA) may lead to tumor recurrence through a regrowth of vessels, induced by EPCs mobilized by the VDAs themselves (Shaked et al., 2006). Interestingly, inhibition of EPCs by a VEGFR2-targeting anti-angiogenic drug enhances anti-tumor activity (Taylor et al., 2012). A similar effect is observed in Id1/Id3 knockout transgenic mice, which are incapable of mobilizing EPCs, suggesting that EPCs may mediate resistance to VDA therapy (Shaked et al., 2006).

According to Taylor et al. (2012), a single dose of CA-4-P, a VDA, induces two EPC peaks in the peripheral blood of mice; the first within a few hours of administration, and the second one after 3 to 4 days. Similar EPC kinetics have been observed in patients treated with another VDA, ombrabulin. Using a VEGFR2-targeting drug to inhibit the second, but not the first, EPC spike significantly enhances the efficacy of VDAs. In line with these studies, EPCs may be important biomarkers of drug activity and/or resistance, thus targeting them may increase the activity of VDAs. Besides,

VEGFR2-targeting drugs not only suppress the late EPC spike and the subsequent incorporation of EPCs into the tumor vessels, they also considerably reduce tumor infiltration by other BM-derived hematopoietic cells that may phenotypically overlap with EPCs. Furthermore, it has recently been shown that BM mobilization and tumor recruitment of Tie-expressing macrophages mediate VDA resistance, pointing out that EPCs, as well as other BM-derived cells are important in this process (Welford et al., 2011).

Some conventional chemotherapeutics, like taxanes, when administered at maximum tolerated doses, induce a quick mobilization of EPCs, both in animals and patients (Roodhart et al., 2010, Shaked et al., 2008). Besides, inhibition of BM-EPCs mobilization induced by the administration of paclitaxel with a VEGFR2-targeting drug resulted in increased anti-tumor activity in preclinical models, similar to what has been described with VDAs (Shaked et al., 2008). However, this phenomenon was only observed with drugs with vessel targeting properties, like paclitaxel, suggesting that the fast mobilization of EPCs from the bone marrow could be a result of a disruption of the tumor vasculature by some chemotherapeutics, which in turn would create the need for a prompt response by the host to repair the damage (Moschetta et al., 2012).

When administered in frequent, repetitive doses (metronomic fashion), certain chemotherapeutics, like cyclophosphamide, block the rapid EPC mobilization, exerting an anti-angiogenic effect, which supports the idea that EPC mobilization is a mechanism of resistance to some chemotherapeutics (Kerbel and Kamen, 2004). In fact, this strategy can be used to target EPCs, which partially explains why low-dose metronomic chemotherapy exert strong anti-angiogenic effects (Bertolini et al., 2003).

EPCs and other BM-derived hematopoietic cells play a role also in the development of resistance to pure anti-angiogenics drugs, like VEGFR2-targeting medications. These agents, like VDAs, can cause vessel remission, increase in intratumoral hypoxia and, in some cases, activation of HIF-1α (Bergers and Hanahan, 2008). Hypoxia and HIF-1α, in turn, lead to the secretion of SDF-1α and VEGF, which then mobilize and recruit EPCs and other BM-derived hematopoietic cells (Ceradini et al., 2004). An increase in the number of EPCs is also observed in cancer patients in drug-free intervals of treatment with VEGF inhibitors. This may promote tumor vessels regrowth and resistance to these agents (Batchelor et al., 2007).

2.8. Interactions between EPCs and Cancer Cells

The migration of EPCs to a tumor site is considerably dependent on the chemokine signaling of the tumor. In a study on glioblastomas, Folkins et al. (2009) showed that glioblastoma stem cells (GSCs) contribute to tumor angiogenesis by promoting local endothelial cell activity and systemic angiogenic processes, involving BM-EPCs in a VEGF-dependent and SDF-1α-dependent manner. Given cancer stem cells (CSC) meaningfully contribute to the release of proangiogenic factors, the number of CSCs in a tumor may importantly affect the migration of EPCs towards it. Hence, tumors with more CSCs may recruit more EPCs, raising the possibility that EPCs play a bigger role in the vascular development of tumors with a larger CSC fraction.

The role of another bone marrow-derived cell population, known as VEGFR1+, is also noteworthy in tumor metastasis. These cells are believed to arrive at the tumor site and form a premetastatic niche, even prior to CSCs activation. The migration of EPCs and CSCs that follows promotes angiogenesis and the formation of vascularized metastases (Psaila et al., 2006).

EPCs and CSCs have also been shown to be closely related. In a study by Wei et al. (2012) the authors demonstrate that CSCs secrete certain proangiogenic factors, such as VEGF, SDF-1α, angiopoietin-1 (Ang-1), which in turn promote the proliferation, migration, and tube formation of circulating EPCs. Then, the migrated EPCs further increase the self-renewal and tumorigenic capacity of CSCs by releasing more proangiogenic factors. According to Beck et al. (2011), VEGF plays an important role in stimulating cancer stemness and renewal. The authors came to this conclusion after observing that blocking VEGFR2 in CSCs leads to tumor regression, not only through reduction of microvascular density, but also through reduction of the size of the CSC pool and impairment of CSC renewal properties.

VEGF produced by tumors or CSCs stimulates the migration of EPCs and tumor vascularization, while VEGF produced by EPCs enhance the proliferative abilities of CSCs. Thus, it seems that EPCs promote tumor growth and metastasis not only through vasculogenesis, but also through stimulation of the proliferative potential of CSCs. Given the hypoxia caused by tumor growth can also mobilize EPCs from the bone marrow, EPCs may also promote the formation of vasculogenic mimicry channels from CSCs (Ribatti, 2004).

Contrary to the widely accepted role of EPCs in tumor vascularization, the results of the study by Defresne et al. (2011) must be mentioned. The authors report

that, due to their macrophage-like phagocytic capacity, EPCs may also prevent tumor metastasis in breast carcinoma. The study shows that a secreted protein, acidic and rich in cysteine (SPARC), or osteonectin, regulates the ability of EPCs to interact and phagocytose tumor cells, according to the tumor environment. In highly metastatic environments, a decrease in the SPARC-mediated macrophage potential of EPCs and an associated increase in metastatic burden is observed, while in a less angiogenic tumor niche, EPCs may behave as macrophages and assist in the clearance of tumor cells. Thus, strategies aiming only at the inherent properties of EPCs and CSCs would not be enough; it would be necessary to have well designed strategies to target the signals between EPCs and CSCs in the tumor niche as a whole.

A possible interaction between EPCs and HNSCC cells has been recently suggested by Ziebart et al. (2016). In a study comparing blood levels of EPCs between HNSCC patients and healthy controls to test this variable as a biomarker for tumor progression and stage, the authors found no difference between groups. However, a higher biological activity in the EPCs of tumor patients was detected, demonstrated by higher migration rates and quantity of formed colonies.

With evidence suggesting that EPCs could play a bigger role on tumor development than previously anticipated, by not only promoting vasculogenesis, but also interacting directly with tumor cells, we set ourselves to investigate whether EPCs affected HNSCC cells activity, more specifically, we delved into whether EPCs or their supernatant had an effect on the migration ability of PCI-1, PCI-13, SCC-4 and SCC-68 cells.

3. Materials and Methods

3.1. Materials

3.1.1. Cell Lines

- PCI-1 (kindly donated by Dr. Theresa L. Whiteside, professor of Pathology, Immunology, and Otolaryngology at the University of Pittsburgh Medical Center);
- PCI-13 (kindly donated by Dr. Theresa L. Whiteside, professor of Pathology, Immunology, and Otolaryngology at the University of Pittsburgh Medical Center);
- SCC-4 (ATCC, Manassas, USA);
- SCC-68 (ATCC, Manassas, USA).

3.1.2. Chemicals

- Accutase® (Sigma-Aldrich, St. Louis, USA);
- CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Thermo Fisher Scientific Inc., Waltham, USA);
- CellTracker™ Red CMFDX (Thermo Fisher Scientific Inc., Waltham, USA);
- EPC medium (endothelial basal medium + endothelial growth medium Single Quots (0,1% human epidermal growth factor + 0,1% hydrocortisone + 0,1% gentamicin + amphotericin-b + 0,4% bovine brain extract) + 20% fetal calf serum (FCS)) (Lonza Group AG, Basel, Switzerland);
- Fibronectin (fibronectin from human plasma 0,1%) (Sigma-Aldrich, St. Louis, USA);
- Histopaque®-1077 sterile-filtered, density: 1.077 g/ml (Sigma-Aldrich, St. Louis, USA);
- Phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, USA);
- Tumor growth medium (Dulbecco's Modified Eagle Medium + 10% FCS + 1% Penicilin/Streptomycin/Neomycin + 1% L-Glutamin) (Gibco™, Thermo Fisher Scientific Inc., Waltham, USA).

3.1.3. Consumables

- Buffy coats (Transfusion Center, University Medical Center Mainz);
- Cellometer Counting Chambers SD100 Slides (Nexcelom Bioscience LLC, Lawrence, USA);
- CELLSTAR® Filter Cap Cell Culture Flasks – 75cm^2, 175cm^2 (Greiner Bio-One, Kremsmünster, Austria);
- CELLSTAR® Multiwell Culture Plates – 6 wells (Greiner Bio-One, Kremsmünster, Austria);
- CELLSTAR® Polypropylene Tube – 50ml (Greiner Bio-One, Kremsmünster, Austria);
- CELLSTAR® Serological Pipettes – 2, 5, 10, 25ml (Greiner Bio-One, Kremsmünster, Austria);
- Eppendorf Safe-Lock Tubes – 1,5; 2ml (Eppendorf AG, Hamburg, Germany);
- Eppendorf Tube – 5ml (Eppendorf AG, Hamburg, Germany);
- PIPETMAN® tips Diamond – D10, D200, D1000 (Gilson, Villiers-le-bel, France);
- VWR® Disposable Transfer Pipet – 3ml (VWR, Darmstadt, Germany).

3.1.4. Devices

- Accu-jet® pro Pipette Controller (Brand GmbH, Neuhof, Germany);
- BZ-9000 Fluorescence Microscope (Keyence Corporation, Osaka, Japan);
- Cellometer® Auto T4 (Nexcelom Bioscience LLC, Lawrence, USA);
- Heraeus BB16 Function Line CO_2 Incubator (Heraeus Instruments GmbH, Hanau, Germany);
- Heraeus® HERAsafe HS12 Safety Cabinet (Kendro Laboratory Products GmbH, Hanau, Germany);
- Heraeus™ Megafuge™ 16 Centrifuge (Thermo Fisher Scientific LED GmbH, Osterode am Harz, Germany);
- IKA® MS 3 Basic Vortex Mixer (IKA, Staufen, Germany);
- Leica DM IL Inverted Microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany);
- PIPETMAN® Classic – P2, P10, P20, P100, P200, P1000 (Gilson, Villiers-le-bel, France).

3.1.5. Software

- AbleBits.com Random Generator (Add-in Express Ltd., Homel, Belarus);
- BZ-II Analyzer (Keyence Corporation, Osaka, Japan);
- IBM® SPSS® Statistics for Windows, Version 23.0 (IBM Corporation, Armonk, USA);
- Microsoft® Excel® 2013 (Microsoft Corporation, Redmond, USA).

3.2. Methods

3.2.1. Study Design

This was an in vitro, experimental, exploratory, non-randomized, controlled, single-blind study. Given all the cell lines used in this study were either purchased from specific companies or donated by other universities, there was no need to submit this project to ethics approval.

3.2.2. Culture of Tumor Cells

For this experiment, four HNSCC cells lines were used, namely PCI-1, PCI-13, SCC-4 and SCC-68. They were chosen out of more than 300 established HNSCC cell lines, based on convenience (Lin et al., 2007) (please refer to Table 1 for a characterization of the cells lines).

Table 1 – Characterization of the cells lines used in the study (Lin et al., 2007).

Cell Line	Patient Demographics		Site of Origin	Doubling Time (h)	TP53 Gene Mutations	
	Sex	TNM Stage			Mutation	Protein Detection IB/IHC
PCI-1	M		larynx	66		
PCI-13	M	T4N1M0	oral cavity	86	E286K	
SCC-4	M	T3N0M0	oral cavity		T150L	+
SCC-68	M		oral cavity			

Male (M); tumor, node, metastasis (TNM); immunoblot (IB); immunohistochemistry (IHC).

Using the appropriate safety equipment, the flasks containing the cells were taken from liquid nitrogen storage and submitted to a running hot water bath. The vials were thawed until there was a small amount of ice inside them, which would usually take 2-3 minutes. Then, they were disinfected with a 70% alcohol-moistened tissue and transferred into a class II safety cabinet. Next, the cells were slowly pipetted into 10ml of pre-warmed tumor growth medium and transferred to 75cm^2 bottles, which were incubated for 48h in a humidified incubator with 5% CO_2 at 37°C. When cells reached 70 – 80% confluence, subculture was conducted.

For that, the growth medium was discarded and the flasks rinsed twice with PBS. PBS was allowed to sit on the cells for at least 30 seconds to remove extracellular proteins. Next, 10ml of Accutase® were added and the bottles were taken to the incubator for 5 minutes at 37°C. When approximately 90% of the cells were rounded up, the flasks were rapped against the palm of the hand. After most of the cells had been detached, they were transferred into a sterile 50ml tube. Cells were then centrifuged at 220xG for 5 minutes. Afterwards, the supernatant was discarded and, by carefully pipetting up and down, the cell pellet was resuspended in 5 – 10ml of growth medium. A small sample was taken for cell counting, using Cellometer® Auto T4. Cells were then divided into new flasks containing tumor growth medium. One day after seeding, the growth medium was changed. The medium was regularly changed every 2 - 3 days.

3.2.3. Isolation of EPCs

EPCs were isolated by Ficoll density gradient separation from buffy coats. The content of the buffy coats was split among 50ml tubes, adding 25ml to each one. Then, 25ml of PBS were added to each tube and the two liquids mere mixed. In different 50ml tubes, 15ml of Histopaque®-1077 sterile-filtered, density: 1.077g/ml were added. Next, 25ml of the blood/PBS mixture were added to the Histopaque® tubes very slowly, so the two liquids would not mix. The tubes were then spun for 20 minutes at 740xG, with the centrifuge brakes off. While the tubes were in the centrifuge, a fibronectin coating was performed with 5ml of a PBS/fibronectin mix (in a ratio of 100:1) in ten 75cm^2 bottles, which were then taken to the incubator for 30 minutes.

After centrifugation, the interphase was pipetted from serum out of the 50ml tube and into a new 50ml tube **(Fig.1)**. Next, the latter was filled with 40ml of PBS and taken to the centrifuge. This time, the centrifuge was set to 740xG, 10 min, brakes on. After centrifugation, the supernatant was discarded and the cell pellet resuspended, by adding 10ml of PBS and pipetting it up and down. Then, the tube was once again taken to the centrifuge. This process was repeated until the liquid phase looked clear after centrifugation.

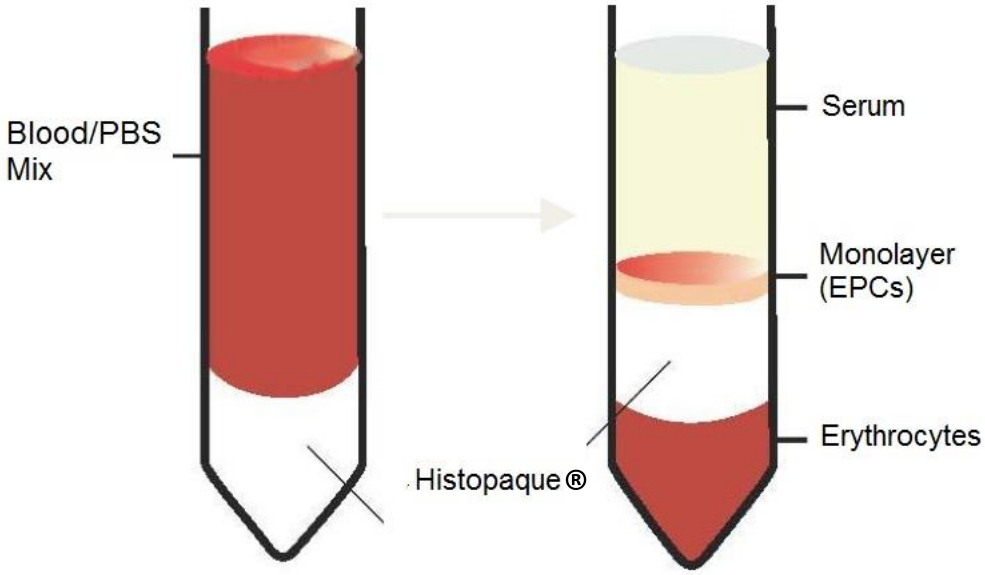


Fig.1 – EPCs isolation by Ficoll density gradient separation.

Afterwards, a small sample was taken for cell counting. The bottles were then taken out of the incubator, the PBS/fibronectin mixture was discarded, and the bottles

washed with EPC medium. Eighty million cells were then added to each bottle, together with 10ml of EPC medium. Lastly, the bottles were taken to the incubator for 72h.

3.2.4. Staining of Tumor Cells

Cells were detached with Accutase®, as previously described. They were then transferred to a 50ml tube, which was subsequently centrifuged (472xG, 5 min, brakes on). The supernatant was discarded and the cells resuspended in 10ml of cell medium. A small sample was taken for cell counting and then 6 million cells were transferred to a new tube, which was taken to the centrifuge (472xG, 5 min, brakes on).

While the tube was in the centrifuge, 2ml of medium without FCS and 5µM of CellTracker™ Green CMFDA were added to a 5ml Eppendorf tube. While working with the staining, the light of the cabinet was turned off. After centrifugation, the supernatant was discarded, and the cells resuspended in the medium/staining mixture. Next, the tube was taken to the incubator for 10 minutes. Afterwards, it was centrifuged (472xG, 5 min, brakes on) once again. The supernatant was then discarded, and the cells resuspended in 12ml of tumor medium. Subsequently, 2ml of the cells/medium mixture were added to each well in a 6-well plate, which was then wrapped in an aluminum sheet and taken to the incubator. After 24h, the 6-well plate was analyzed under an inverted-light microscope. Tumor cells should be 80% confluent, otherwise, we would wait for another 24h before proceeding with the co-culture with EPCs or the culture with EPC supernatant.

3.2.5. Staining of EPCs

EPCs were detached from the bottles with Accutase®, as previously described. Then, a small sample was taken for cell counting. The total amount of cells needed was transferred to a 50ml tube, which was taken to the centrifuge (740xG, 5 min, brakes on).

While the tube was in the centrifuge, 1ml of medium without FCS and 1µM of CellTracker™ Red CMFDX were added to a 2ml Eppendorf tube. While working with the staining, the light of the cabinet was turned off. After centrifugation, the supernatant was discarded, and the cells resuspended in the medium/staining mixture. Next, the

tube was taken to the incubator for 10 minutes. After that, the tube was centrifuged (740xG, 5 min, brakes on). Later, the supernatant was discarded, and the cells resuspended in 50ml of EPC medium.

From this tube, 10^2, 10^3, 10^4 cells were transferred to 5ml Eppendorf tubes and 10^5, 10^6 cells were transferred to new 50ml tubes. The content in the Eppendorf tubes was diluted into EPC medium, in a total of 2ml per tube. The content in the 50ml tubes was diluted into EPC medium and taken to the centrifuge. Afterwards, the supernatant was discarded, and the cell pellet resuspended in 2ml of EPC medium.

3.2.6. Scratch Wound Assay

While the tubes were in the centrifuge, a scratch was created in the monolayer of adherent tumor cells in each one of the 6 wells by gently scratching the bottom of the well with a D10 pipette tip. A different tip was used for each well. Then, the tumor medium was discarded, and the wells washed twice with PBS. Next, the EPCs were added to the plate, as below:

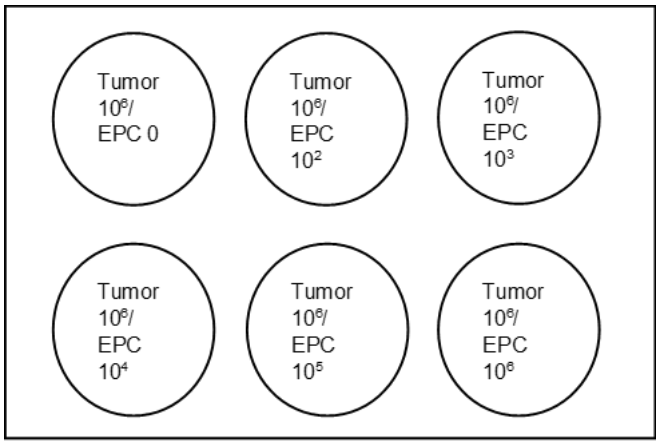


Fig.2 – EPC-treatment assignment.

Treatment randomization was not performed. Pictures were taken of each well using BZ-9000 Fluorescence Microscope, with a x2 magnification. Finally, the plate was wrapped in an aluminum sheet and taken to the incubator. After 24 and 48h, new pictures were taken from each well. Experiments were performed in triplicate for each cell line.

3.2.7. Culture of Tumor Cells with EPC Supernatant

Three days after incubation, the supernatant was transferred from the EPC bottles to a 50ml tube. This was centrifuged (740xG, 5 min, brakes on), in order to remove non-adherent cells. After centrifugation, the supernatant was transferred to a new 50ml tube and the sedimented cells were discarded. The process of centrifuging the tube and then transferring the supernatant to a new tube and disposing the sedimented cells was repeated once. Then, the supernatant was dissolved in fresh EPC medium in increasing concentrations in 5ml Eppendorf tubes. In total, each tube had 2ml of the mix of supernatant/fresh EPC medium. Concentrations were as following: 0% supernatant - 100% fresh EPC medium; 0,01% supernatant - 99,99% fresh EPC medium; 0,1% supernatant - 99,9% fresh EPC medium; 1% supernatant - 99% fresh EPC medium; 10% supernatant - 90% fresh EPC medium; 100% supernatant - 0% fresh EPC medium.

A scratch was created in the monolayer of adherent tumor cells in each one of the 6 wells by gently scratching the bottom of the well with a D10 pipette tip. A different tip was used for each tube. Then, the tumor medium was discarded, and the wells washed twice with PBS. Next, the EPC supernatant was added to the plate, as below:

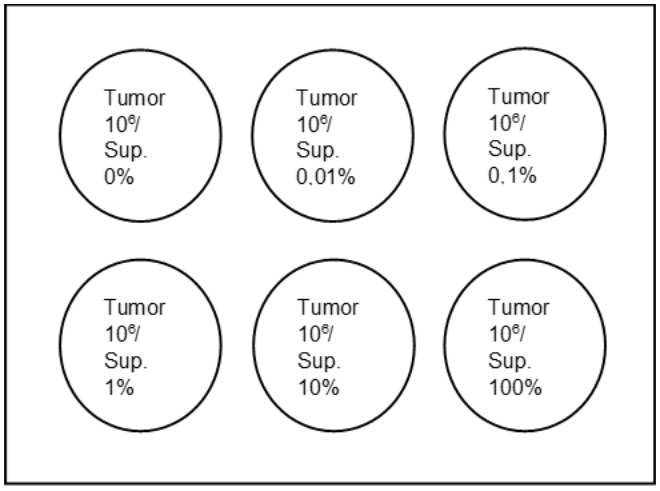


Fig.3 – EPC supernatant-treatment assignment.

Treatment randomization was not performed. Pictures were taken of each well, with a x2 magnification. Finally, the plate was wrapped in an aluminum sheet and taken to the incubator. After 24 and 48h, new pictures were taken from each well. Experiments were performed in triplicate for each cell line.

3.2.8. Calibration of the Evaluator

Seventeen pictures were randomly chosen. The distance between the two borders of the scratch was measured at three different points and a mean width of the defect was calculated for each picture. One, two and three weeks after the first measurement, new measurements were made, in a total of four per picture. Finally, the variance between the measurements for each picture was computed and the square root of this averaged variance was taken as an estimate of the standard deviation of the measurement error.

3.2.9. Scratch Wound Measurement

Pictures were anonymized using the extension AbleBits.com Random Generator, for Microsoft® Excel® 2013, so the evaluator could measure them blindly. Moreover, they were analyzed without their red layer, so the evaluator would not know how many (if any) EPCs there were in it. The pictures were evaluated using the software BZ-II Analyzer. The distance between the two borders of the scratch was measured three times for each picture, once for each third of the picture (upper, middle and lower thirds) **(Fig.4)**. Then, a mean width of the scratch was calculated. After all pictures were measured, they were deanonymized and the mean width of the scratch wound at 0h was set to 100%, while the means at 24 and 48h were adjusted as percentages of the initial value.

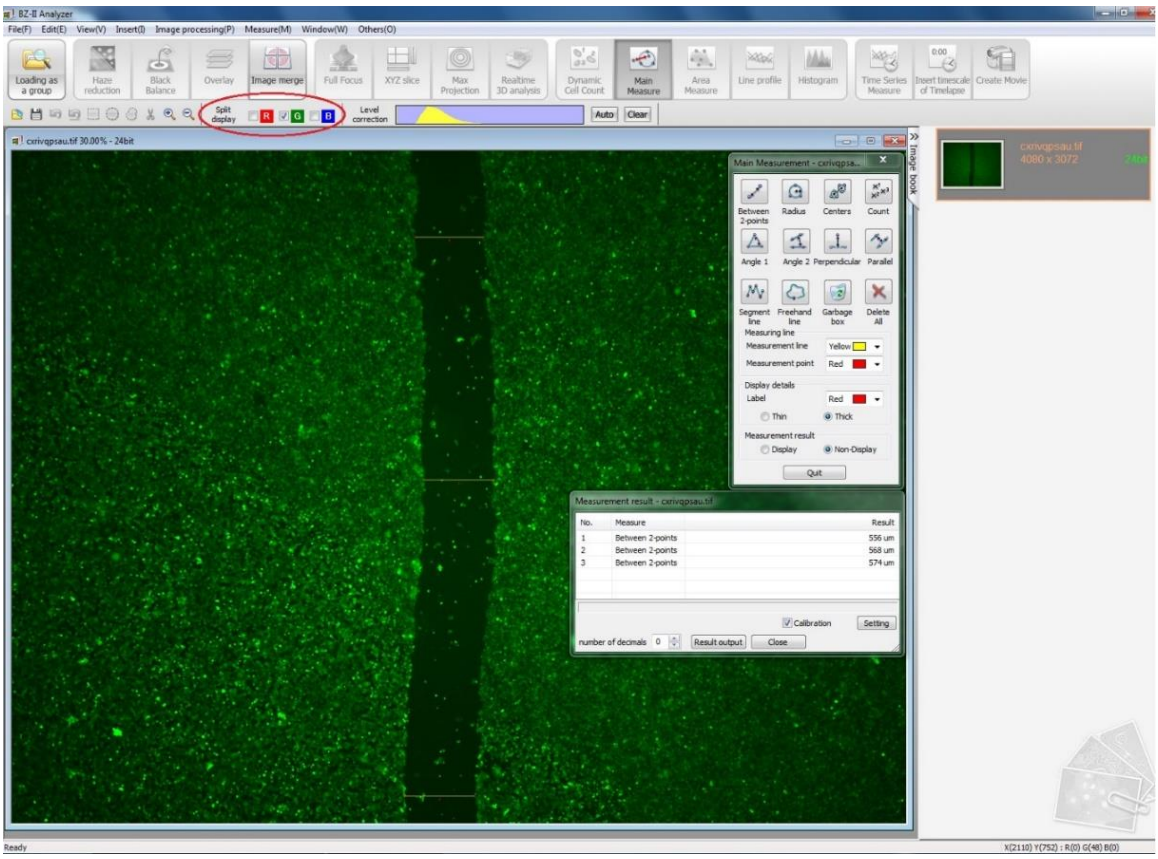
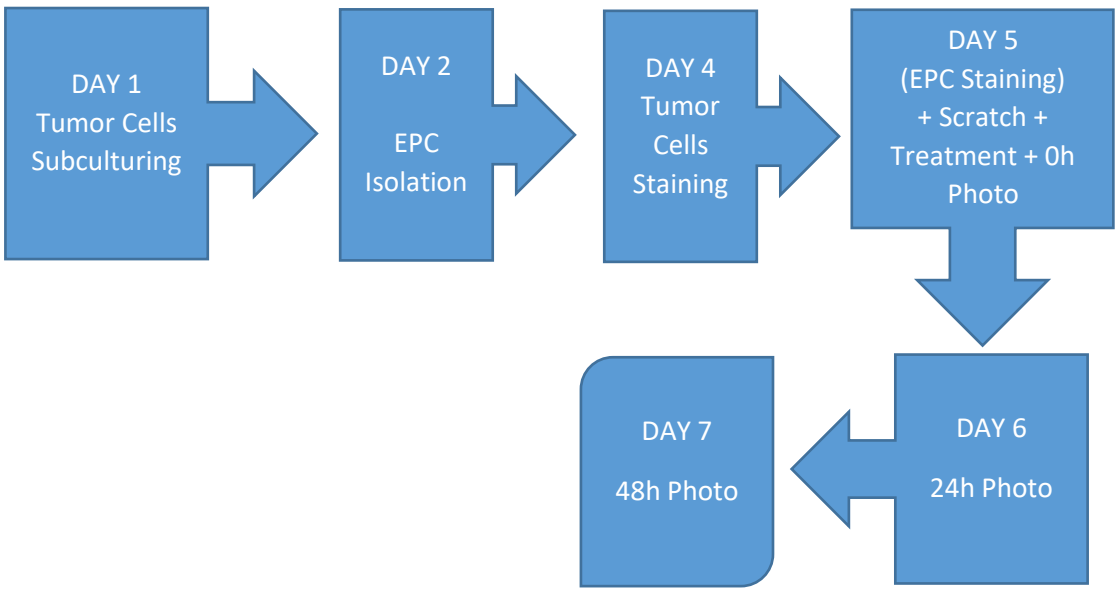


Fig.4 – Scratch wound measurement. Note in the red circle how only the green layer of the picture was used for analysis.

3.2.10. PERT Diagram



3.2.11. Statistical Analysis

Data were inserted into a Microsoft® Excel® 2013 spreadsheet and then analyzed using IBM® SPSS® Statistics for Windows, Version 23.0. The well was set as the statistical unit. The normality of data distribution was tested using the Kolmogorov-Smirnov test. The effect of the different concentrations of EPCs or their supernatant on the migration ability of the different cell lines was evaluated using a General Linear Model of Repeated Measures, having EPC concentration or EPC supernatant concentration as the independent variable and time as the within-subjects dependent variable, with 3 levels (0h, 24h, 48h). Tukey's test was used for post hoc evaluation. The statistical analysis was planned and conducted under the guidance of Prof. Dr. Manfred Berres of the Institut für Medizinische Biometrie, Epidemiologie und Informatik (IMBEI).

4. Results

4.1. Co-culture of Tumor Cells and EPCs

4.1.1. PCI-1

Table 2 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in PCI-1 monolayers treated with different concentrations of EPCs.

EPC Number	Time (h)	Mean Scratch Size (%)	Standard Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	24	75,237	12,814	47,317	103,156
	48	35,160	12,980	6,879	63,441
100	24	70,840	12,814	42,921	98,759
	48	20,727	12,980	-7,554	49,008
1000	24	75,410	12,814	47,491	103,329
	48	30,243	12,980	1,962	58,524
10000	24	79,010	12,814	51,091	106,929
	48	13,167	12,980	-15,114	41,448
100000	24	75,247	12,814	47,327	103,166
	48	14,013	12,980	-14,268	42,294
1000000	24	81,783	12,814	53,864	109,703
	48	28,520	12,980	0,239	56,801

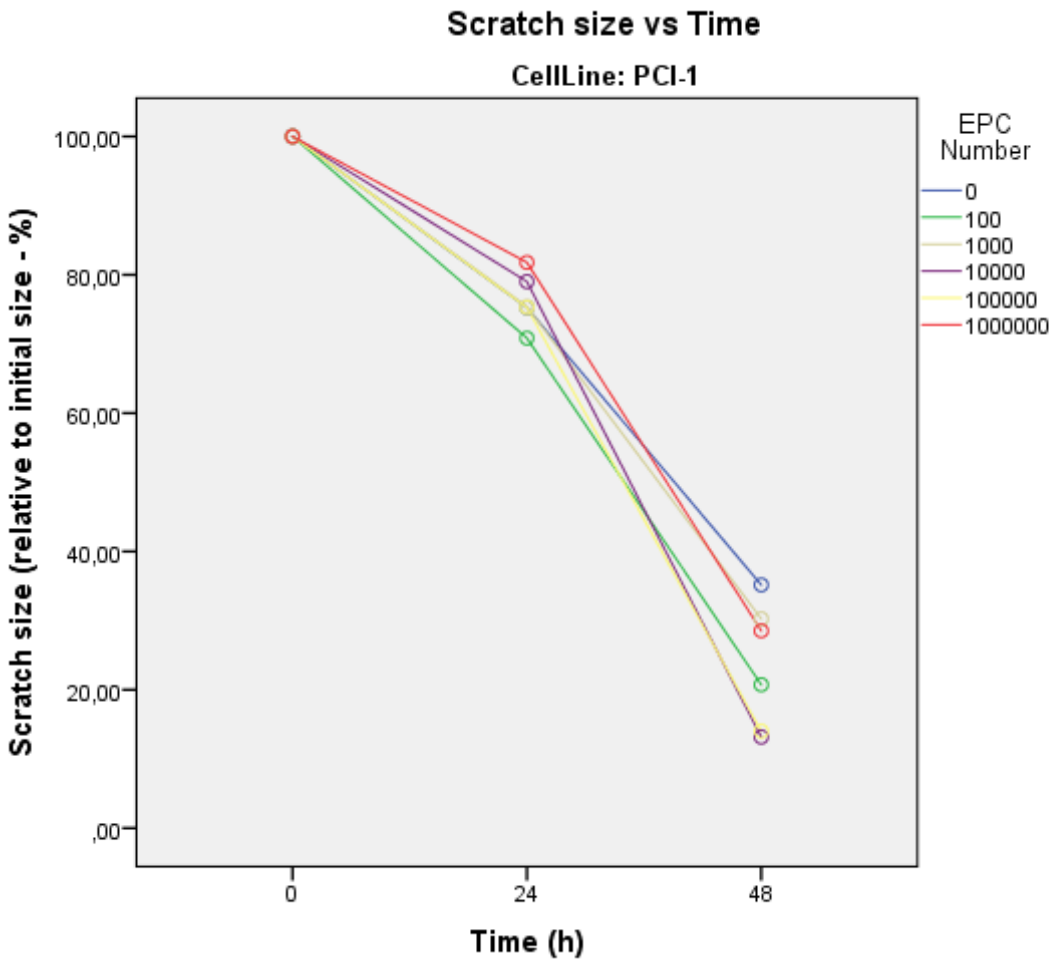


Fig.5 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in PCI-1 monolayers treated with different concentrations of EPCs.

There were no statistically significant results on the migration ability of PCI-1 cells, regardless the concentration of EPCs.

4.1.2. PCI-13

Table 3 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in PCI-13 monolayers treated with different concentrations of EPCs.

EPC Number	Time (h)	Mean Scratch Size (%)	Standard Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	24	89,280	7,560	72,808	105,752
	48	0,000	1,618	-3,525	3,525
100	24	62,967	7,560	46,494	79,439
	48	0,000	1,618	-3,525	3,525
1000	24	70,177	7,560	53,704	86,649
	48	0,000	1,618	-3,525	3,525
10000	24	70,307	7,560	53,834	86,779
	48	3,963	1,618	0,438	7,489
100000	24	67,047	7,560	50,574	83,519
	48	0,000	1,618	-3,525	3,525
1000000	24	69,913	7,560	53,441	86,386
	48	0,000	1,618	-3,525	3,525

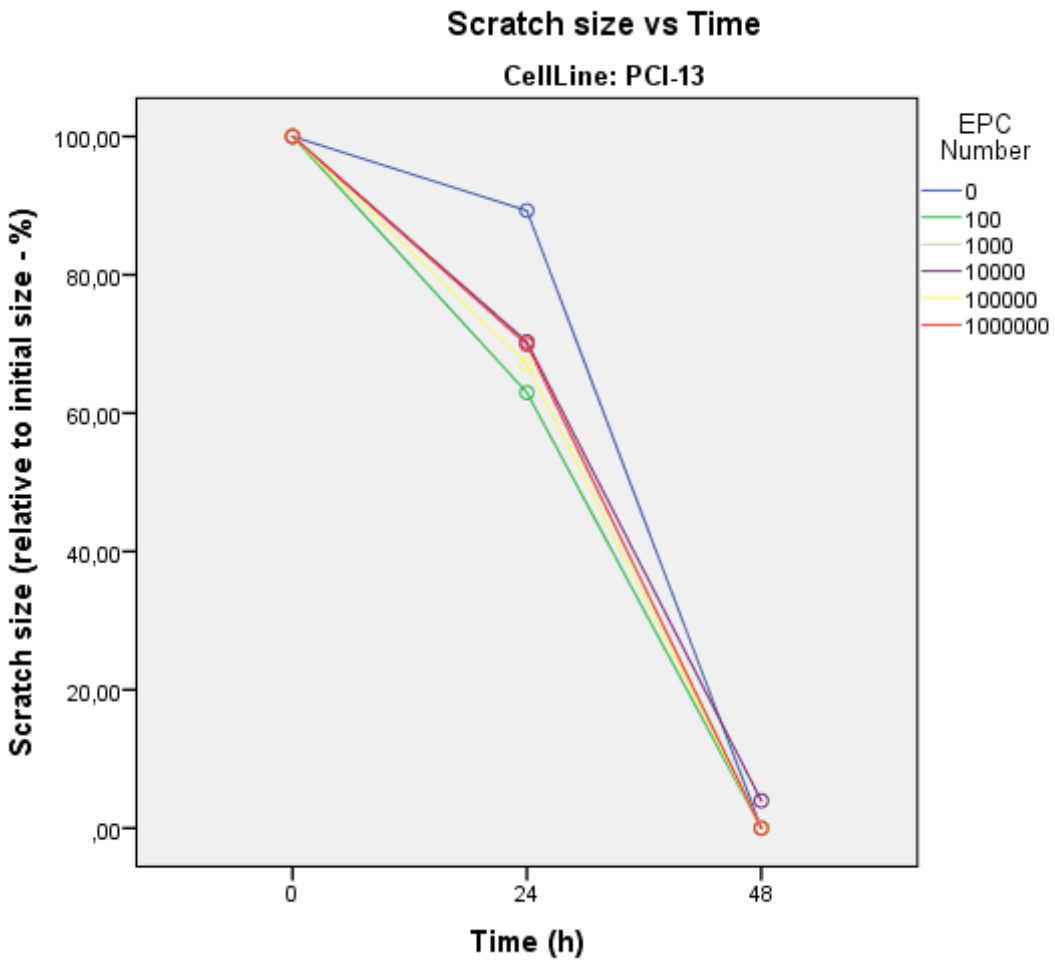


Fig.6 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in PCI-13 monolayers treated with different concentrations of EPCs.

There were no statistically significant results on the migration ability of PCI-13 cells, regardless the concentration of EPCs.

4.1.3. SCC-4

Table 4 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in SCC-4 monolayers treated with different concentrations of EPCs.

EPC Number	Time (h)	Mean Scratch Size (%)	Standard Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	24	42,333	22,626	-7,467	92,134
	48	0,000	20,333	-44,754	44,754
100	24	24,643	22,626	-25,157	74,444
	48	0,000	20,333	-44,754	44,754
1000	24	50,450	22,626	0,649	100,251
	48	0,000	20,333	-44,754	44,754
10000	24	56,303	22,626	6,503	106,104
	48	55,607	20,333	10,853	100,360
100000	24	53,197	22,626	3,396	102,997
	48	64,267	20,333	19,513	109,020
1000000	24	24,845	27,712	-36,148	85,838
	48	33,975	24,903	-20,837	88,787

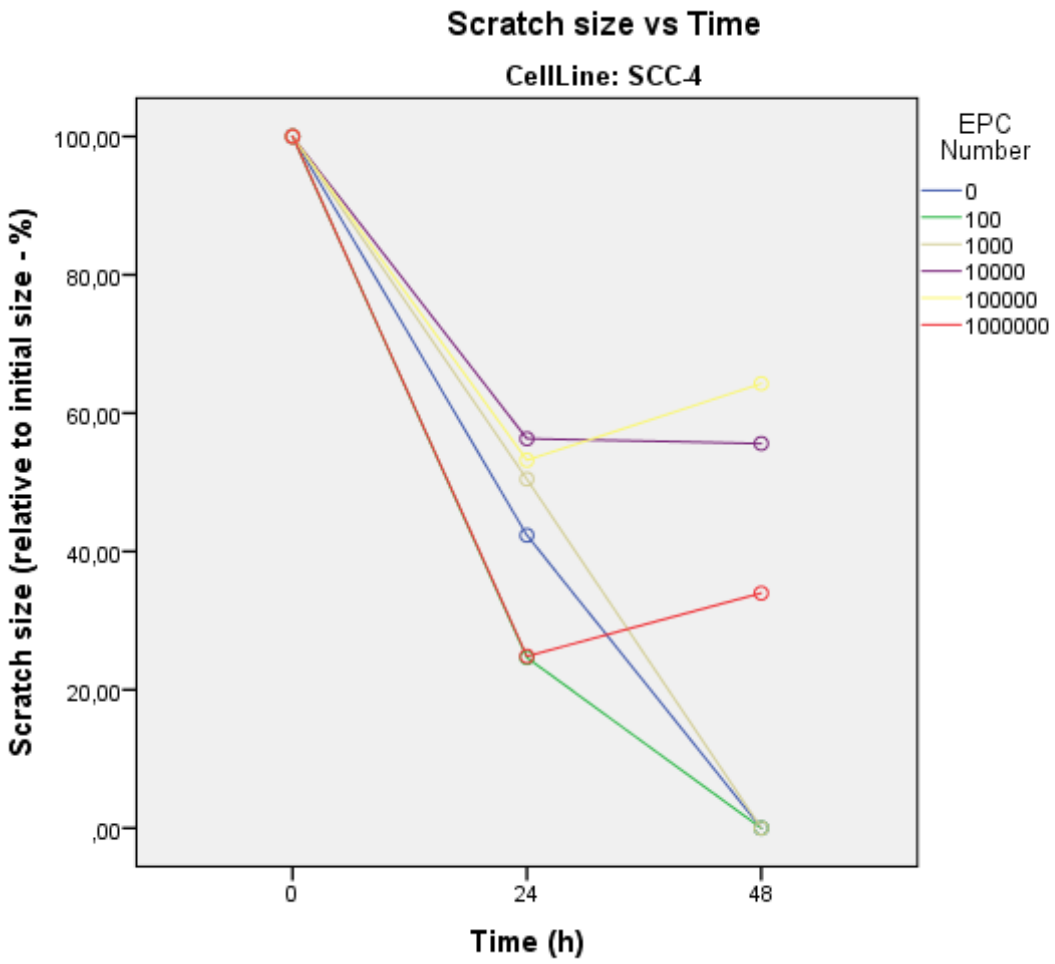


Fig.7 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in SCC-4 monolayers treated with different concentrations of EPCs.

Statistically significant results were found when comparing 100 to 10.000 EPCs ($p = 0,04$) and 100 to 100.000 EPCs ($p = 0,028$).

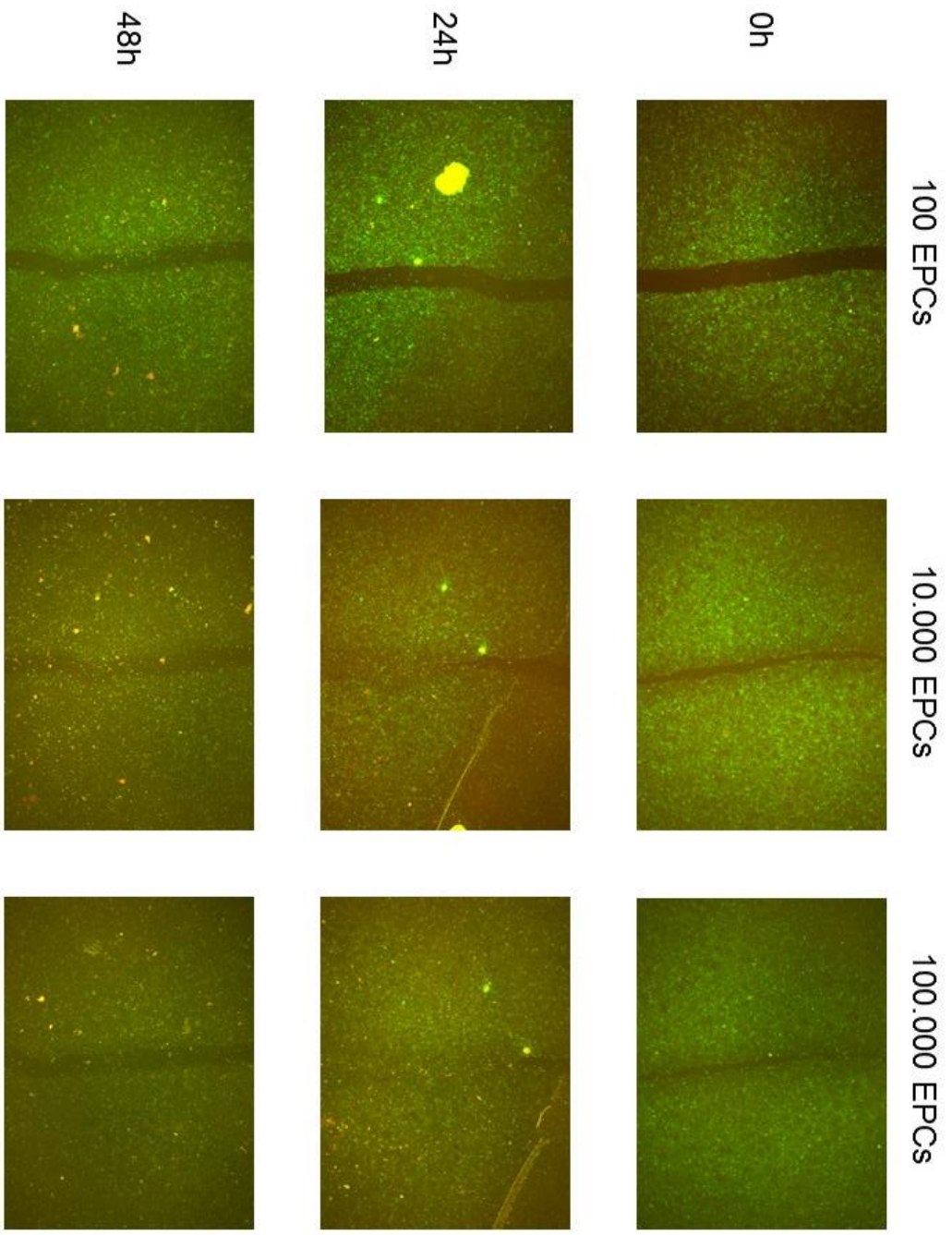


Fig.8 – Co-culture of EPCs (red) and SCC-4 cells (green). Rows show wells at different time points, while columns show different concentrations of EPCs. These pictures were chosen from one of the three plates (n=3).

4.1.4. SCC-68

Table 5 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in SCC-68 monolayers treated with different concentrations of EPCs.

EPC Number	Time (h)	Mean Scratch Size (%)	Standard Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	24	40,870	26,634	-17,751	99,491
	48	17,830	14,892	-14,948	50,608
100	24	40,100	26,634	-18,521	98,721
	48	5,930	14,892	-26,848	38,708
1000	24	54,365	32,620	-17,430	126,160
	48	0,000	18,239	-40,144	40,144
10000	24	73,143	26,634	14,523	131,764
	48	86,810	14,892	54,032	119,588
100000	24	100,377	26,634	41,756	158,997
	48	57,420	14,892	24,642	90,198
1000000	24	34,933	26,634	-23,687	93,554
	48	100,147	14,892	67,369	132,924

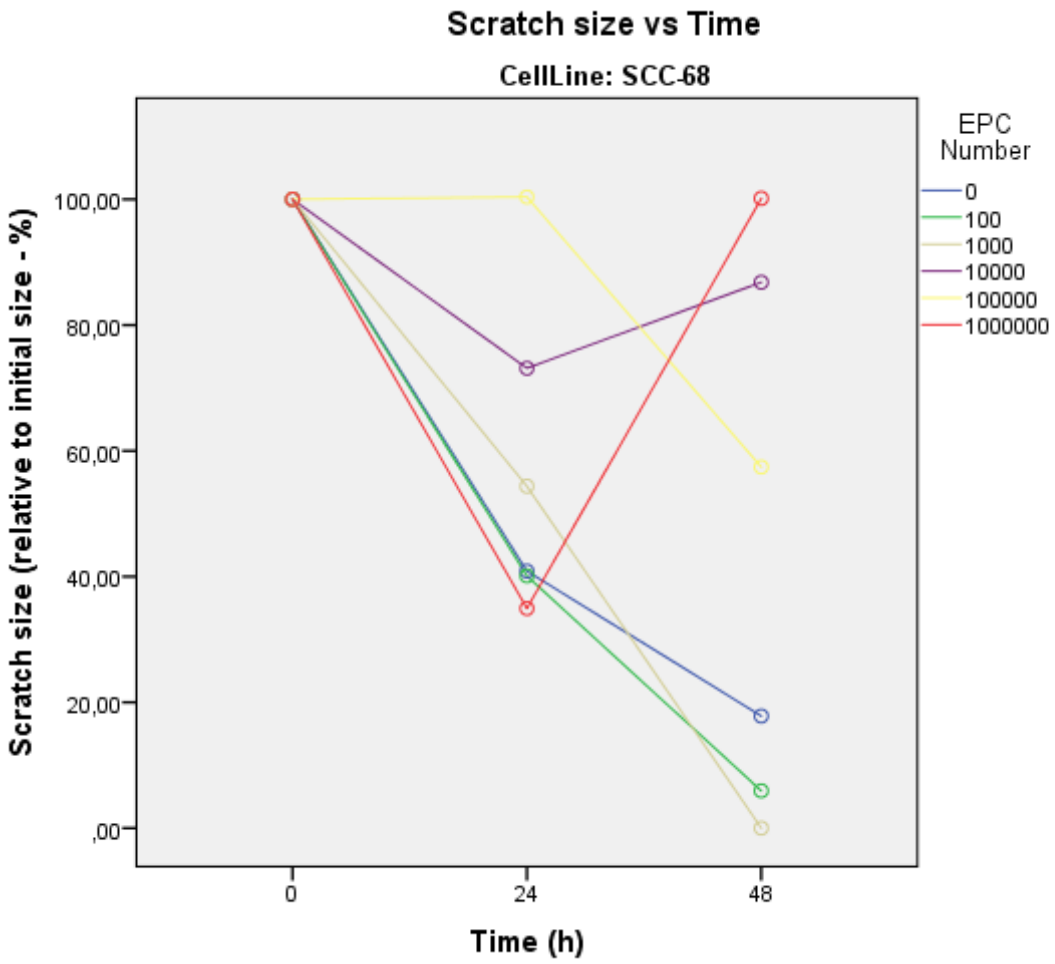


Fig.9 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in SCC-68 monolayers treated with different concentrations of EPCs.

There were no statistically significant results on the migration ability of SCC-68 cells, regardless the concentration of EPCs.

4.2. Cultivation of Tumor Cells with EPC Supernatant

4.2.1. PCI-1

Table 6 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in PCI-1 monolayers treated with different concentrations of EPC supernatant.

EPC Supernatant Concentration (%)	Time (h)	Mean Scratch Size (%)	Standard Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	24	63,203	14,607	31,052	95,354
	48	35,160	13,224	6,054	64,266
0,01	24	58,500	14,607	26,349	90,651
	48	20,727	13,224	-8,379	49,832
0,1	24	45,890	14,607	13,739	78,041
	48	30,243	13,224	1,138	59,349
1	24	51,873	14,607	19,722	84,024
	48	13,167	13,224	-15,939	42,272
10	24	57,465	17,890	18,088	96,842
	48	21,020	16,196	-14,627	56,667
100	24	64,793	14,607	32,642	96,944
	48	28,520	13,224	-0,586	57,626

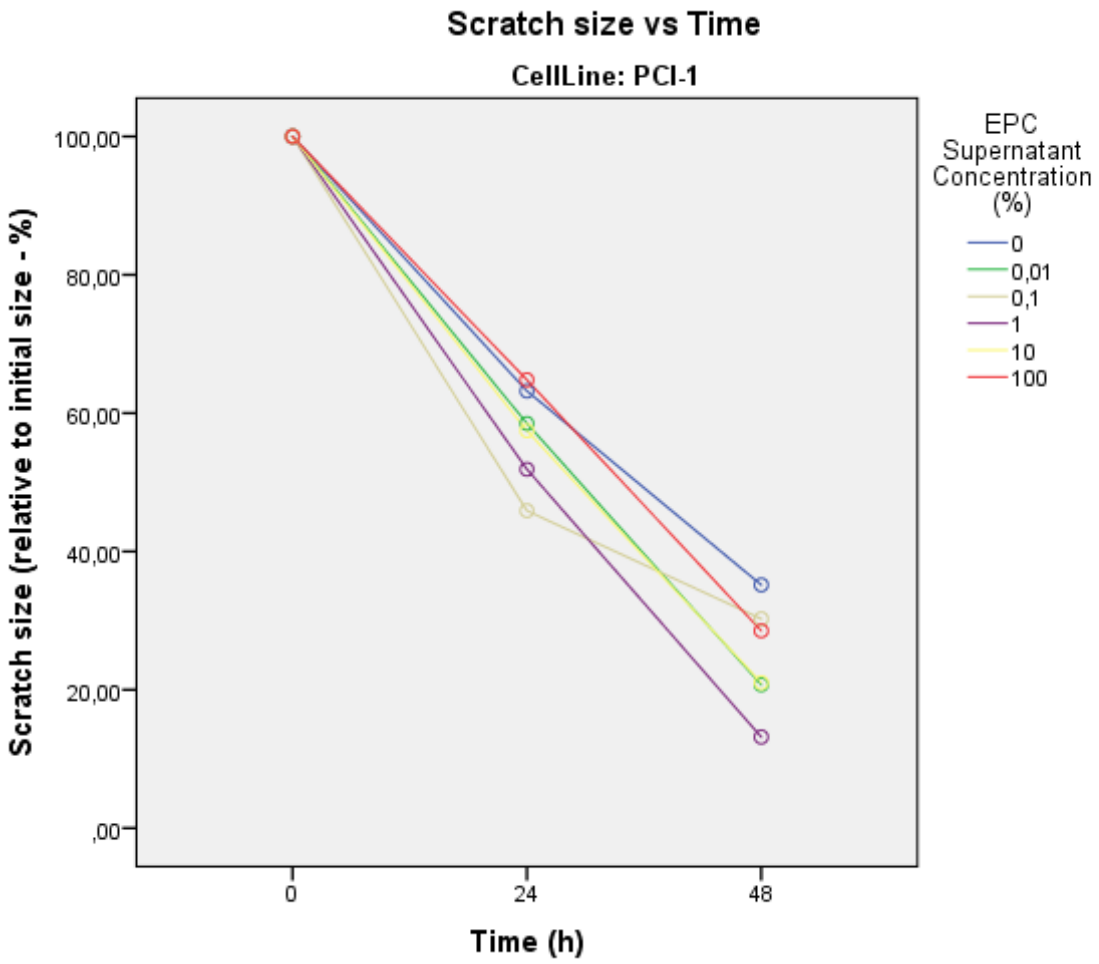


Fig.10 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in PCI-1 monolayers treated with different concentrations of EPC supernatant.

There were no statistically significant results on the migration ability of PCI-1 cells, regardless the concentration of EPC supernatant.

4.2.2. PCI-13

Table 7 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in PCI-13 monolayers treated with different concentrations of EPC supernatant.

EPC Supernatant Concentration (%)	Time (h)	Mean Scratch Size (%)	Standard Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	24	44,733	4,856	34,154	55,313
	48	0,000	1,618	-3,525	3,525
0,01	24	37,450	4,856	26,871	48,029
	48	0,000	1,618	-3,525	3,525
0,1	24	33,887	4,856	23,307	44,466
	48	0,000	1,618	-3,525	3,525
1	24	39,807	4,856	29,227	50,386
	48	3,963	1,618	0,438	7,489
10	24	34,700	4,856	24,121	45,279
	48	0,000	1,618	-3,525	3,525
100	24	62,800	4,856	52,221	73,379
	48	0,000	1,618	-3,525	3,525

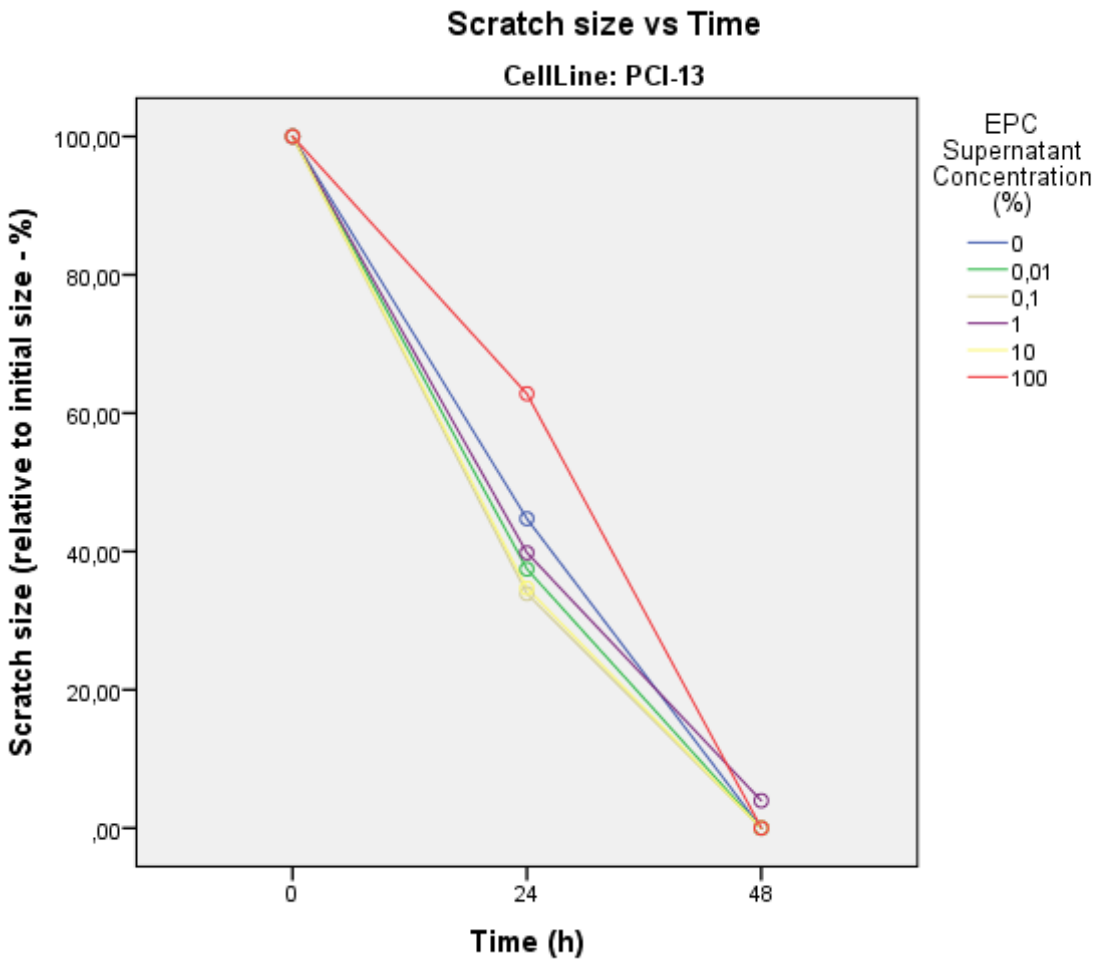


Fig.11 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in PCI-13 monolayers treated with different concentrations of EPC supernatant.

Statistically significant results were found when comparing 0,1% to 100% (p = 0,037) and 10% to 100% (p = 0,043) of EPC supernatant concentration.

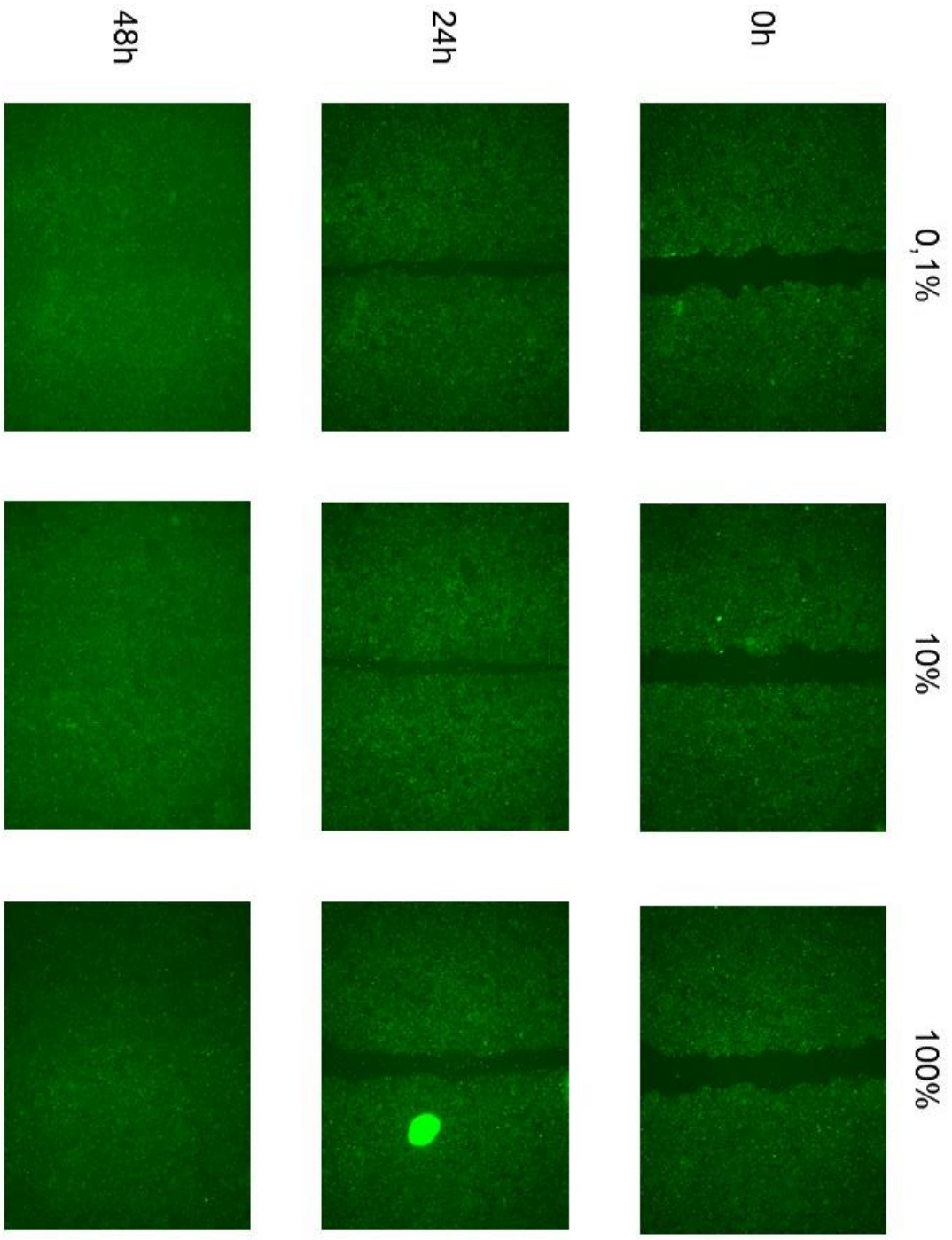


Fig.12 – Culture of PCI-13 cells with EPC supernatant. Rows show wells at different time points, while columns show different concentrations of EPC supernatant. These pictures were chosen from one of the three plates (n=3).

4.2.3. SCC-4

Table 8 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in SCC-4 monolayers treated with different concentrations of EPC supernatant.

EPC Supernatant Concentration (%)	Time (h)	Mean Scratch Size (%)	Standard Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	24	0,000	20,537	-44,746	44,746
	48	0,000	20,051	-43,686	43,686
0,01	24	0,000	20,537	-44,746	44,746
	48	0,000	20,051	-43,686	43,686
0,1	24	10,190	20,537	-34,556	54,936
	48	0,000	20,051	-43,686	43,686
1	24	55,440	20,537	10,694	100,186
	48	55,607	20,051	11,920	99,293
10	24	63,430	20,537	18,684	108,176
	48	64,267	20,051	20,580	107,953
100	24	78,867	20,537	34,120	123,613
	48	45,730	20,051	2,044	89,416

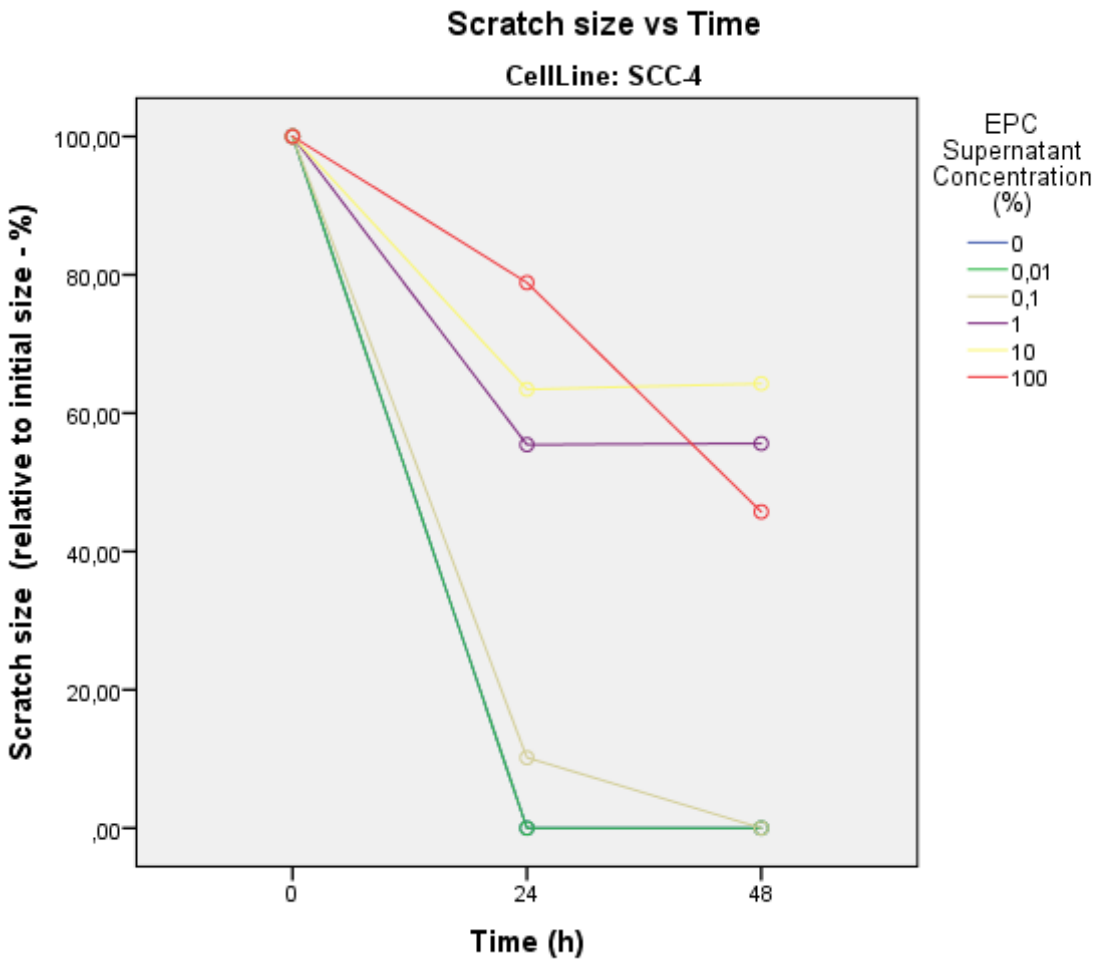


Fig.13 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in SCC-4 monolayers treated with different concentrations of EPC supernatant.

There were no statistically significant results on the migration ability of SCC-4 cells, regardless the concentration of EPC supernatant.

4.2.4. SCC-68

Table 9 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in SCC-68 monolayers treated with different concentrations of EPC supernatant.

EPC Supernatant Concentration (%)	Time (h)	Mean Scratch Size (%)	Standard Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	24	29,387	13,731	-0,836	59,609
	48	17,830	14,892	-14,948	50,608
0,01	24	17,570	13,731	-12,653	47,793
	48	5,930	14,892	-26,848	38,708
0,1	24	9,255	16,817	-27,760	46,270
	48	0,000	18,239	-40,144	40,144
1	24	99,803	13,731	69,581	130,026
	48	86,810	14,892	54,032	119,588
10	24	55,567	13,731	25,344	85,789
	48	57,420	14,892	24,642	90,198
100	24	97,347	13,731	67,124	127,569
	48	100,147	14,892	67,369	132,924

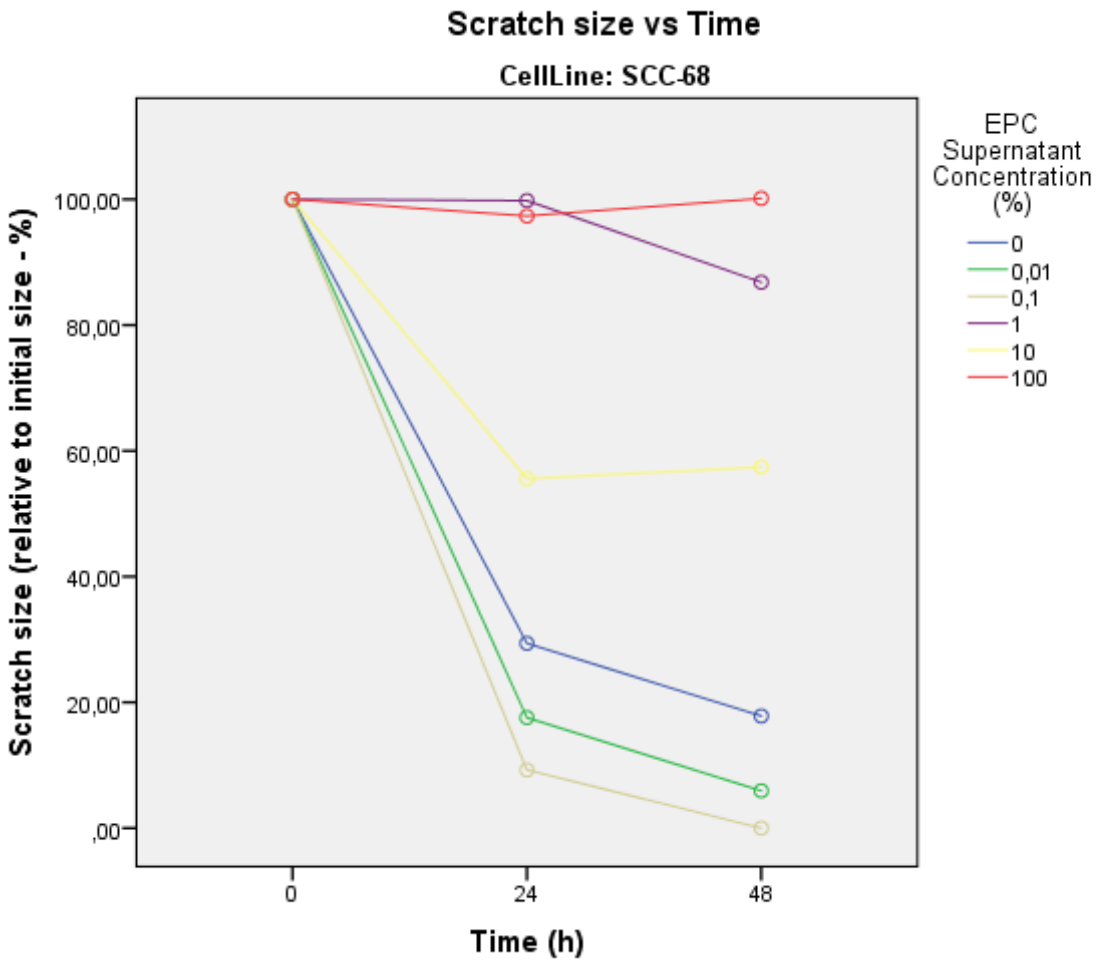
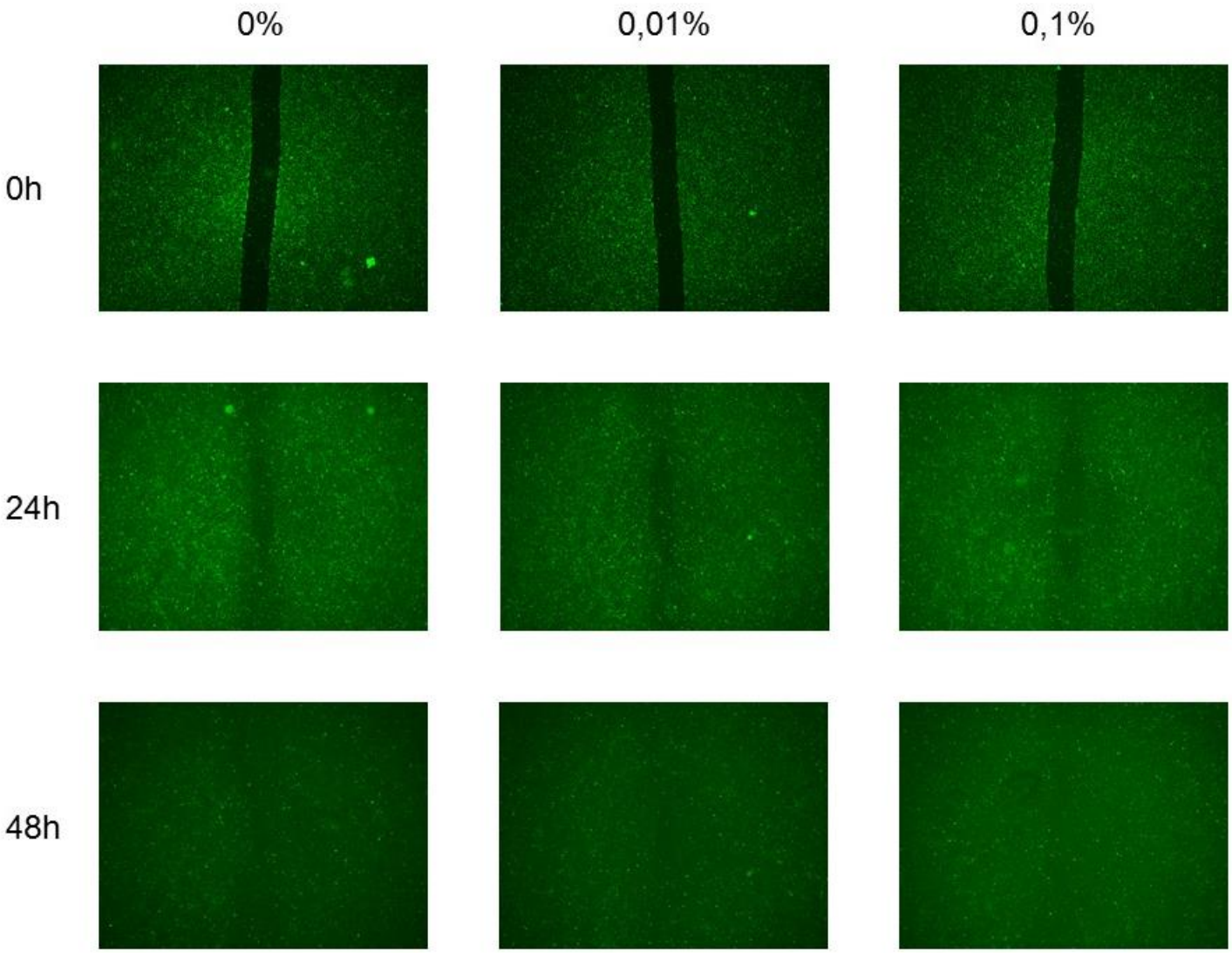


Fig.14 – Size of the scratches, relative to the initial size (100%), after 24 and 48 hours, in SCC-68 monolayers treated with different concentrations of EPC supernatant.

Statistically significant results were found when comparing 0% to 1% (p =0,041), 0% to 100% (p = 0,027), 0,01% to 1% (p = 0,016), 0,01% to 100 (p = 0,01), 0,1% to 1% (p = 0,019) and 0,1% to 100% (p = 0,013) of EPC supernatant concentration.


0%
0,01%
0,1%
0h
24h
48h

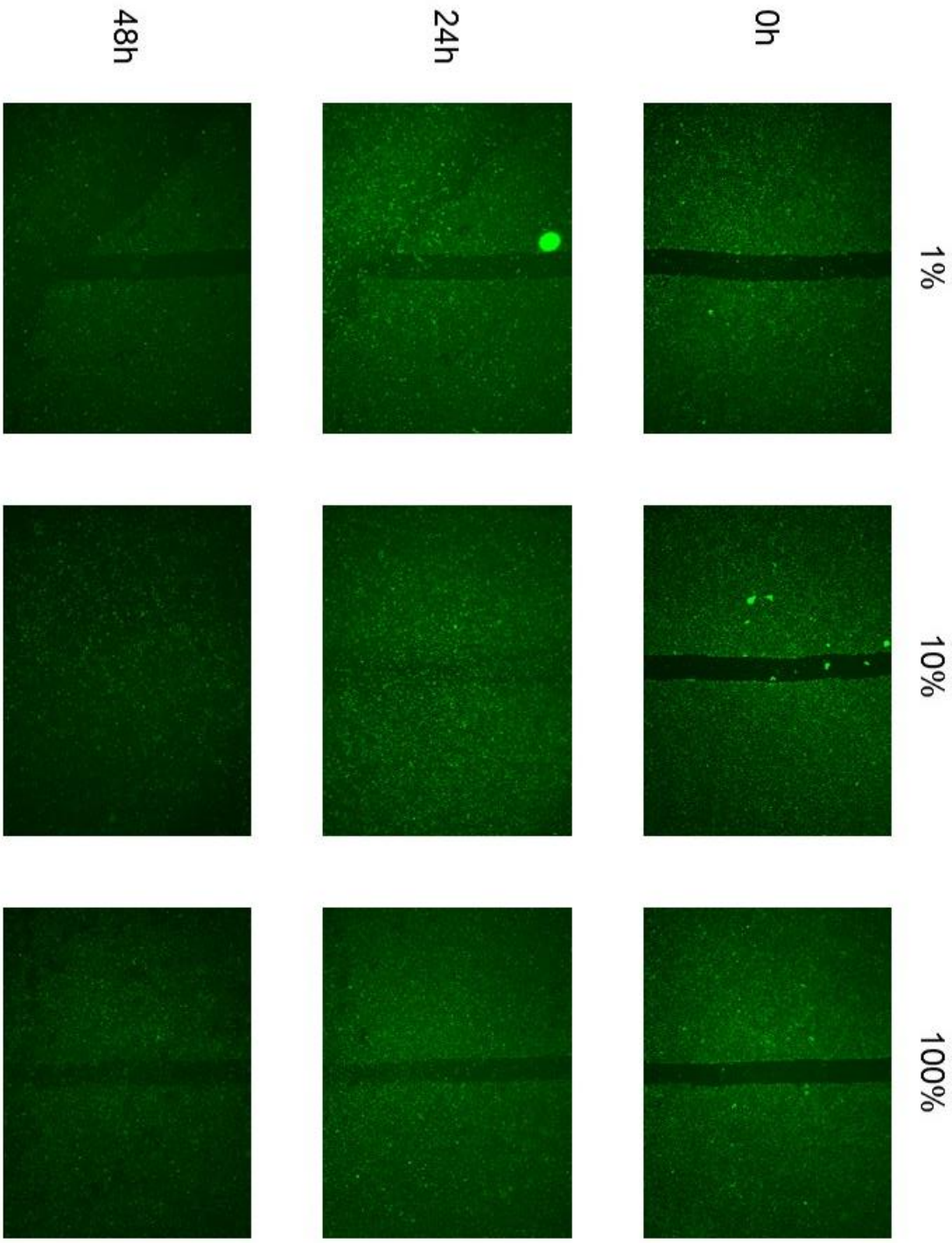


Fig.15 – Culture of SCC-68 cells with EPC supernatant. Rows show wells at different time points, while columns show different concentrations of EPC supernatant. These pictures were chosen from one of the three plates (n=3).

5. Discussion

5.1. Rationale for the Study

EPC-induced vasculogenesis is an important part of tumor growth and metastasis and, therefore, a valuable therapeutic target (Carmeliet and Jain, 2000). The problem with targeting these cells is that the efficacy of this strategy tends to be transitory: over time, most patients develop resistance to these anti-angiogenic drugs, which inevitably leads to tumor progression. To develop more effective treatments, it is paramount to better understand the mechanisms involved in tumor vessel formation, how EPCs participate in tumor progression and metastasis, and the best way to target them (Moschetta et al., 2014).

The number of circulating EPCs in healthy individuals is extremely low (between 0,01% and 0,001% of PBMCs, by flow cytometry) (Fadini et al., 2012). However, in vivo studies show much higher levels of these cells in the peripheral blood of patients with different types of solid tumors (Kaur and Bajwa, 2014, Ribatti, 2004). Brunner et al. (2008) found evidence that that might also be the case in HNSCC patients. In a pilot study to test blood levels of CECs or EPCs as surrogate markers of tumor size, lymph node metastasis, response to radiation therapy, or survival, the authors found EPC blood levels to be higher in HNSCC patients than in healthy controls, both before and after treatment. Nevertheless, there was no correlation between this finding and the clinical outcomes. Ziebart et al. (2016), on the other hand, when analyzing a possible role of EPCs as a biomarker for tumor progression and stage, did not find a difference in the blood levels of EPCs between tumor and healthy patients. The authors did find, however, a higher biological activity in the EPCs of tumor patients, demonstrated by higher migration rates and quantity of formed colonies.

The influence of cancer cells on EPCs has been demonstrated in many studies (Arbab et al., 2008, Folkins et al., 2009, Kollet et al., 2001, Moschetta et al., 2014, Petit et al., 2007). The importance of the papers by Brunner et al. (2008) and Ziebart et al. (2016) lies in the fact that they show, respectively, a similar increase in EPCs number in peripheral blood and a higher biologic activity of these cells in HNSCC patients that had been previously observed in patients with other types of solid tumors.

Notwithstanding, for a long time the interaction between cancer cells and EPCs was thought to be unidirectional. More recent studies show, though, that this interaction might be reciprocal. Wei et al. (2012) showed that EPCs that migrate to the tumor niche, in response to CSC signaling, release proangiogenic factors, like VEGF, that in

turn increase CSCs cancer stemness and renewal. The role of VEGF on cancer cells proliferative abilities had previously been demonstrated by Beck et al. (2011).

Based on the above considerations, we hypothesized that EPCs could have an effect of HNSCC cells, but when searching the literature, we found no studies on the topic. Thus, we aimed to investigate the effect of different concentrations of EPCs or their supernatant on the migration ability of four different HNSCC cell lines: PCI-1, PCI-13, SCC-4 and SCC-68.

5.2. Interactions between EPCs and HNSCC Cells

Significant differences were found when comparing SCC-4 cells co-cultured with 100 or 10.000 EPCs ($p = 0,04$), and 100 or 100.000 EPCs ($p = 0,028$). In these cases, the smaller the number of EPCs, the faster the scratch would close. The fact that the effect size was bigger when comparing SCC-4 cells co-cultured with 100 to 100.000 EPCs ($p = 0,028$) than to 10.000 EPCs ($p = 0,04$) further supports this hypothesis. This could suggest an antagonist effect, contradicting our expectations.

Given no concentration of EPC supernatant had a significant effect on SCC-4 cells, the abovementioned effects could be due to cell-to-cell interactions, rather than paracrine signaling. This is in line with what was observed in breast carcinomas by Defresne et al. (2011), according to whom EPCs present a macrophage-like, SPARC-regulated ability to interact and phagocytose tumor cells, especially in less angiogenic niches. Unfortunately, this can only be speculated, since we did not quantify the expression of SPARC.

Alternatively, it could simply be the case that, with less EPCs in the well to compete for space, tumor cells can grow more freely, given that we did not inhibit nor control for cell proliferation. Nevertheless, should that be the case, this phenomenon should have been observed more consistently in the multiple comparisons between treatments.

Another possible explanation would be assessment bias. Even though the measurements were performed by a calibrated, blinded evaluator, measurement errors can still occur. In **Fig.7**, we see that, in wells treated with 100.000 or 1.000.000 EPCs, the scratch actually grew between 24 and 48 hours, which was unexpected. On the other hand, this could also be interpreted as further evidence of phagocytosis of SCC-68 cells by EPCs.

5.3. Interactions between EPC supernatant and HNSCC Cells

Statistically significant results were also found when comparing PCI-13 cells cultured with 0,1% or 100% EPC supernatant, ($p = 0,037$) and 10% or 100% ($p = 0,043$). Here we found inconsistent results. At 24h, the scratch was narrower with a 10% concentration of EPC supernatant than with 0,1% or 100%. In fact, the scratch in the well cultured with 100% EPC supernatant was the largest one at 24h. After 48h, all scratches were closed, with exception to the one cultured with 1% EPC supernatant. This also contradicts our assumptions. Even though the content of the EPC supernatant was not analyzed, we believe it was rich in chemoattractants, specially VEGF, and thus we expected that the higher the concentration, the faster the gap would close.

When analyzing the effect of EPC supernatant on SCC-68 cells, we found the most interesting results. Statistically significant results were found when comparing SCC-68 cells cultured with 0% or 1% ($p =0,041$), 0% or 100% ($p = 0,027$), 0,01% or 1% ($p = 0,016$), 0,01% or 100 ($p = 0,01$), 0,1% or 1% ($p = 0,019$) and 0,1% or 100% ($p = 0,013$) of EPC supernatant concentration.

Here we saw that, at lower concentrations, there was an increasing effect of the supernatant's concentration on SCC-68 cells mobility. In wells treated with 0,1%, the scratch closed faster than in those with 0,01%, which in turn closed faster than in those with no supernatant. At higher concentrations, this effect seems to disappear, though. That is partially in line with our expectations. As previously mentioned, although the expression of VEGF was not measured, it is reasonable to assume that the higher the concentration of EPC supernatant, the higher the expression of VEGF, and, thus, we had anticipated a dose-dependent response.

A possible explanation for this threshold would be the existence of a perfect equilibrium between VEGF and nutrients. The lower the amount of EPC supernatant in the mixture applied to the wells, the higher the amount of EPC medium. Given the composition of EPC and tumor medium is relatively similar, in wells with a smaller concentration of EPC supernatant, tumor cells would have more access to nutrients. On the other hand, wells treated with 0,01% and 0,1% performed better than the control, so it might be that in these concentrations the cells had adequate access to nutrients and sufficient exposition to VEGF to have their migration stimulated.

5.4. Strengths and Weaknesses of the Study

5.4.1. Methods

5.4.1.1. Why Cell Migration as the Primary Outcome?

In any cell-based experiment, it is crucial to measure the health and viability of cells. To do this, several assays are available, being cell viability, apoptosis, and cytotoxicity the three majors ones. However, choosing the right one can be a daunting task, specially when investigating multiple targets.

To facilitate this decision, a few factors should be taken into consideration, such as the question the study is trying to answer, the nature of its sample, the number of samples being tested, the required sensitivity, the plates and plate readers, and the reagent costs.

Cell migration is essential to the development and maintenance of multicellular organisms. Many physiological processes, such as embryonic development, immune response, and wound healing, as well as pathological ones, like inflammation and tumor metastasis depend on it (Justus et al., 2014). The migration of cells usually occurs in response to external signals, like chemical and mechanical signals. Errors in this process may have dire consequences, like tumor formation and metastasis (Mak et al., 2016).

Given metastatic progression is the main cause of death in cancer patients, cell migration is an outcome of great importance in cancer research. For metastasis to occur, cancer cells must migrate and invade through extracellular matrix, intravasate into blood circulation, attach to a distant site, and finally extravasate to form distant foci (Bravo-Cordero et al., 2012).

Understanding the migratory and invasive capacity of tumor and stromal cells and elucidating the underlying mechanisms is paramount for the elaboration of novel strategies in cancer diagnosis, prognosis, drug development and treatment.

5.4.1.2. Scratch Wound Assay in Comparison to Other Cell Migration Assays

The scratch wound essay is an inexpensive, easy-to-implement method to study cell migration in vitro. It is specially suited for assessing the effect of cell-to-matrix and

cell-to-cell interactions on that outcome (Liang et al., 2007). One of its major advantages is it mimics to some extent cell migration in vivo. Certain cell lines even exhibit in this assay the same migration pattern they do in vivo (i.e. as a loosely connected population or a sheet of cells) (Haudenschild and Schwartz, 1979).

Another advantage of the scratch wound assay over other popular methods to assess cell migration, like the Boyden Chamber Assay, is that, unlike these, it does not disrupt cell-to-cell and cell-to-matrix interactions by previous preparation of cells in a suspension, making it particular suitable to study the role of such interactions on cell migration (Liang et al., 2007).

Lastly, one more upside to this assay is its compatibility with microscopy, including live cell imaging, which allows the analysis of intracellular signaling events during migration (Liang et al., 2007).

In contrast, there are a few disadvantages and limitations of this method in comparison to other cell migration assays. Resuming the comparison with the Boyden Chamber Assay, in the scratch wound assay, no chemical gradient is established and, thus, the latter is not suited for testing for chemotaxis (Liang et al., 2007).

Also, since one to two days are needed for the formation of the monolayer, and then another 8-18 hours for cell migration and scratch closure (in our case, 24-48 hours), the scratch wound assay is relatively more time-consuming than the alternatives (Liang et al., 2007).

Lastly, it requires a relatively large number of cells and chemicals, not being the method of choice when, for example, specialized primary cells that are difficult to obtain in sufficient numbers are being studied, or when expensive reagents are needed.

Considering its advantages and disadvantages, we concluded that the scratch wound assay was the most appropriate method to test our hypothesis. Nonetheless, it must be taken into consideration that it is just one method of testing one outcome, that is, other assays on cell migration might show different results, and no inferences can be made on the influence of EPCs and their supernatant on other characteristics of the tested HNSCC cell lines, like their viability or cytotoxicity.

5.4.1.3. Issues with EPCs Identification and Characterization

When analyzing the results of our study, it must be observed that the identification and characterization of EPCs can be problematic and its quantification exaggerated. To begin with, EPCs and hemapoietic stem cells (HSCs) have a common

precursor, the hemangioblasts and, at early stages of differentiation, these two cell lines share phenotypic and functional characteristics, which makes it difficult to differentiate between them. Moreover, peripheral blood EPCs is a loose definition, encompassing several mononuclear cell types, such as CD34+ CD133+VEGFR2+ cells, subsets of monocytic cells, and cell populations with broad developmental plasticity. Further, EPCs cultured in vitro, due to the culture process, may gain or lose properties, which would differentiate them from their non-cultured counterpart, from which they originate, namely circulating EPCs. Finally, the use of non-standardized techniques for quantifying circulating EPCs, like flow cytometry or in vitro methods, add to the uncertainty (Moschetta et al., 2014).

Many of these issues could be resolved, should there be specific markers for the putative EPC population (including circulating and resident EPCs). Of the markers currently used, none is restricted to EPCs. All antigens (or combinations of them) used for identifying EPCs are also expressed by circulating HSCs and progenitor cells, circulating mature ECs, platelets/EC-derived vesicles and some subsets of circulating hematopoietic-derived monocyte/myeloid cells (Basile and Yoder, 2014, De Palma and Naldini, 2009). Thus, cells previously identified as circulating EPCs may actually be circulating hematopoietic-derived cells.

5.5. Implications of the Study

Our results expand the understanding of the role EPCs play in tumor development, supporting the theory that they not only contribute to the creation of the vascular network that sustains the tumor, but also interact directly with the tumor cells. On one hand, we found an antagonist effect of EPCs on SCC-4, which either represents the discovery of a direct influence of EPCs on SCC-4 mobility or further supports Defresne et al. (2011) hypothesis of a SPARC-mediated phagocytosis ability of EPCs. On the other hand, we found an increasing, dose-dependent effect of EPC supernatant on SCC-68 cells mobility. These findings can help to formulate new, more efficient strategies to target EPCs in HNSCC patients.

5.6. Conclusion

EPCs apparently have cell-to-cell interactions with SCC-4 cells, with a dose-dependent, antagonist effect, with the former impairing the mobility or phagocytosing the latter. Moreover, up to a threshold of 0,1%, EPC supernatant seems to have an increasing, dose-dependent effect on the migration ability of SCC-68 cells.

Even though these are exciting results, given the limitations of our study, they should be appraised critically. Moreover, provided that our study was exploratory and not confirmatory in nature, rather than a causal relation, our discoveries can at best provide associations between EPCs and/or their supernatant and the migration of those four HNSCC cells lines. These can, in turn, be used to postulate new hypotheses for future studies. Moreover, given the novelty of our study, in principle our experiment should be reproduced by other research groups and our findings be further supported or disputed. Such studies should ideally evaluate the expression of VEGF and SPARC in the wells, control and/or inhibit cell proliferation, use additional assays for analyzing cell migration, like the Boyden Chamber Assay, and assess supplementary outcomes, like cell viability and/or toxicity. Finally, they should also have a larger sample size. In this sense, our data could be used to perform a sample size calculation.

6. Abstract

EPCs can induce, in adult life, the formation of new blood vessels through a de novo production of endothelial cells. This process can occur in physiological and pathological states, and it is usually triggered by ischemia. EPC-induced vasculogenesis is essential for tumor growth and metastasis; hence, it is a valuable therapeutic target. However, due to the development of resistance, the effect of current anti-angiogenic medications tends to be transitory. In order to create new treatments that more effectively target EPCs, it is imperative to better understand the mechanisms involved in tumor vessel formation, and how EPCs contribute to tumor progression and metastasis. With this in mind, we aimed to investigate the effect of different concentrations of EPCs or their supernatant on the migration ability of four different HNSCC cell lines: PCI-1, PCI-13, SCC-4 and SCC-68. We found that, at low concentrations (0,01% and 0,01%), the higher the supernatant's concentration, the greater the influence on SCC-68 cells mobility. However, when analyzing our results, it must be taken into consideration the difficulty to identify and characterize EPCs, which can lead to an overestimation of their numbers and effect. Moreover, our study is limited by its small sample size. Therefore, further studies with larger sample sizes are needed to confirm our findings.

Keywords: Head and Neck Neoplasms; Endothelial Progenitor Cells; Neovascularization, Pathologic; In Vitro Techniques; Cell Migration Assays

7. Zusammenfassung

EPCs können im Erwachsenenalter die Bildung neuer Blutgefäße durch eine de novo Produktion von Endothelzellen induzieren. Dieser Prozess kann unter physiologischen und pathologischen Zuständen auftreten und wird in der Regel durch Ischämie ausgelöst. Die EPC-induzierte Vaskulogenese ist essenziell für das Tumorwachstum und die Metastasierung und stellt daher ein wertvolles therapeutisches Ziel dar. Aufgrund der Resistenzentwicklung ist die Wirkung gegenwärtiger anti-angiogener Medikamente jedoch vorübergehend. Um neue Therapien zu entwickeln, die wirksamer auf die EPCs abzielen, ist es unerlässlich, die Mechanismen besser zu verstehen, die an der Bildung von Tumorgefässen beteiligt sind und wie EPCs zur Tumorprogression und Metastasierung beitragen. Vor diesem Hintergrund wollten wir die Wirkung unterschiedlicher Konzentrationen von EPCs oder ihres Überstandes auf die Migrationsfähigkeit von vier verschiedenen HNSCC-Zelllinien untersuchen: PCI-1, PCI-13, SCC-4 und SCC-68. Wir fanden heraus, dass bei niedrigen Konzentrationen (0,01% und 0,01%) der Einfluss auf die Mobilität der SCC-68-Zellen umso größer ist, je höher die Konzentration des Überstandes ist. Bei der Analyse unserer Ergebnisse muss jedoch berücksichtigt werden, dass es schwierig ist, EPCs zu identifizieren und zu charakterisieren, was zu einer Überschätzung ihrer Anzahl und Wirkung führen kann. Darüber hinaus ist unsere Studie durch ihre geringe Stichprobengröße begrenzt. Daher sind weitere Studien mit größeren Stichprobengrößen erforderlich, um unsere Ergebnisse zu bestätigen.

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