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Migration of mesenchymal stem cells towards glioblastoma cells depends on hepatocyte-growth factor and is enhanced by aminolaevulinic acid-mediated photodynamic treatment

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ABSTRACT

Hepatocyte-growth factor (HGF) is expressed by glioblastomas and contributes to their growth, migration and invasion. HGF also mediates migration of mesenchymal stem cells (MSC) to sites of apoptotic cell death. Moreover, MSC show tropism for glioblastomas, which is exploited in gene therapy to deliver the therapeutics to the tumor cells. Here, we have studied whether HGF contributes to the recruitment of MSC by glioblastoma cells and whether aminolaevulinic acid-mediated photodynamic therapy (ALA/PDT), a novel therapeutic approach that induces apoptosis in glioblastoma cells, affects HGF release and this migratory response. MSC expressed the HGF receptor MET and migrated towards U87 and U251 glioblastoma spheroids. Migration increased significantly when spheroids were subjected to ALA/PDT, which was associated with induction of apoptosis and up-regulation of HGF. Neutralizing HGF resulted in significant inhibition of MSC migration towards untreated as well as ALA/PDT-treated spheroids. Thus, glioblastoma cells express HGF, which contributes to the attraction of MSC. ALA/PDT induces apoptosis and augments HGF release causing enhanced MSC migration towards the tumor cells. ALA/PDT may therefore be exploited to improve targeting of MSC delivered gene therapy, but it may also constitute a risk in terms of beneficial effects for the tumor.

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1. Introduction

Glioblastoma is the most frequent and aggressive malignant primary brain tumor [1]. Despite multimodal therapy combining surgery, radiotherapy and alkylating chemotherapy, prognosis of patients is dismal: median survival is 14.6 months, the 2-year survival rate 27.2% [2]. Aminolaevulinic acid-mediated photodynamic therapy (ALA/PDT) is a novel therapeutic approach for glioblastoma, and early clinical results are promising [3,4]. ALA is an intermediate of the heme biosynthesis pathway. Oral uptake of ALA results in preferential accumulation of protoporphyrin IX (PPIX) in glioblastoma cells, mainly due to low ferrochelatase activity [5,6]. This preferential accumulation can be exploited for intraoperative identification of the tumor during fluorescence-guided surgery using light of 400 nm wavelength for illumination, allowing to increase the extent of resection, associated with higher progression-free survival [7]. However, when exposed to light of 635 nm wavelength, PPIX acts as a potent photosensitizer [6]. Its excitation initiates a photochemical reaction, which kills the tumor cells via the generation of singlet oxygen [5]. In addition to this direct phototoxic effect, there appears to be an immunological component to ALA/PDT, and it has been shown to influence the tumor vasculature as well as the migratory and invasive behavior of tumor cells [5,8–11].

We and others have previously shown that ALA/PDT induces apoptosis of glioblastoma cells [11,12]. The type of cell death has consequences besides killing of the tumor cells, e.g. it may affect the development of immunity [13]. Furthermore, apoptosis but not necrosis of neurons has been shown to result in up-regulation of hepatocyte-growth factor (HGF) [14,15]. HGF is a pleiotropic cytokine with anti-apoptotic activity [16]. It promotes glioblastoma growth, invasiveness and angiogenesis [17–19], and its tyrosine kinase receptor MET may represent a marker for mesenchymal and proneural glioblastoma stem cell subtypes [20]. Therefore, the HGF–MET pathway is currently evaluated as

Abbreviations: ALA/PDT, aminolaevulinic acid-mediated photodynamic therapy/treatment; HGF, hepatocyte-growth factor; MSC, mesenchymal stem cell(s).

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a potential therapeutic target in glioblastoma [21]. On the other hand, HGF is a chemoattractant for mesenchymal stem cells (MSC) [14,22,23]. MSC are multipotent stem cells found in bone marrow and other tissues. They are capable of differentiating into various types of mesenchymal cells, including osteoblasts, adipocytes and chondrocytes [24]. It is well established that MSC home to glioblastomas and this tropism is utilized to target therapeutics to the tumors, including cytokines, enzymes/pro-drugs, oncolytic viruses, toxins and others [25].

Expression of HGF by glioblastoma cells [17,19] and its activity as a chemotactic stimulus for MSC [14,22,23] as well as the observation that apoptosis, thus, the type of cell death, which is induced by ALA/PDT [11,12], is associated with induction of HGF in neurons [14,15], raises the questions whether HGF is involved in the migration of MSC towards glioblastoma cells and whether ALA/PDT-induced apoptosis results in increased HGF release by glioblastoma cells and an enhanced migratory response of MSC.

2. Materials and methods

2.1. Mesenchymal stem cells

Human bone marrow was obtained from volunteer donors after informed consent with the ethical approval of the local ethical committee. MSC were isolated from bone marrow as described previously [14]. Briefly, bone marrow cells were plated in 75-cm^2 culture flasks (Greiner, Nürtingen, Germany) and cultured at 37 °C and 5% CO_2 in a humidified atmosphere in DMEM (Lonza, Verviers, Belgium) supplemented with 30% fetal calf serum (FCS; GIBCO/Invitrogen, Karlsruhe, Germany), 50 μg/ml gentamycin and 2 mM L-glutamine (all from Lonza). After 48 h, non-adherent cells were removed and cultures continued. When reaching 80% confluence, cells were harvested with trypsin (Lonza) and re-plated at 1:3. All MSC preparations showed the immunophenotype, and osteogenic and adipogenic differentiation typical of MSC [24].

2.2. Glioblastoma spheroids and ALA/PDT

Culture and generation of spheroids as well as ALA/PDT of U87 and U251 glioblastoma cell lines were performed as described previously [10,11]. Briefly, tumor cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. To generate spheroids, cells were plated in agar-coated culture flask. After 3 days of culture, tumor spheroids with a diameter of approximately 250 μm had formed. For ALA/PDT, spheroid cultures were supplemented with 12.5 μg/ml ALA (Merck, Darmstadt, Germany) and incubated for 4 h. Spheroids were collected under microscopic control and transferred (25 spheroids/well) into agar-coated flat-bottom 96-well plates (Greiner) containing 100 μl/well of DMEM without phenol red. Exposure to laser light was performed for 625 s with an energy of 30 mW/cm^2 (equivalent to 25 J/s or 1 W on 33 cm^2) using a Ceralas 633-nm PDT diode laser (Biolitec, Jena, Germany). After laser light exposure, spheroids were used in the experiments. These ALA/PDT conditions result in induction of apoptosis in about 60% of cells [11]. Untreated spheroids and spheroids treated with ALA only or exposed to laser-light only served as controls.

2.3. Under-agarose chemotaxis assay

Chemoattraction of MSC by glioblastoma spheroids was studied using an under-agarose chemotaxis assay as described [14,15]. Briefly, 0.8% agarose (Eurogentec, Cologne, Germany) in PBS was boiled, mixed after cooling with 0.5% bovine serum albumin (BSA; Roth, Karlsruhe, Germany) in DMEM and poured into 6-well plates (Costar/Corning, Wiesbaden, Germany). Three 2 mm wide and 5 mm long slots 5 mm apart from each other were cut in the agarose of each well. 8×10^4 MSC were then added to the central slots of each well, 70 μl of chemoattractant (25 treated or untreated spheroids) in the left target slots and 70 μl of 0.5% BSA in DMEM in the right control slots. The number of cells, which migrated towards the target slot subtracted by the number of cells, which migrated towards the control slot, was determined for each well after a 12 h migration period.

To study the contribution of HGF to MSC migration towards the spheroids, neutralization studies were performed by adding neutralizing anti-HGF polyclonal antibody (2 μg/ml; goat IgG; R&D Systems, Wiesbaden) or normal goat IgG (2 μg/ml; Santa Cruz, Heidelberg, Germany) to the targets.

2.4. Detection of MET and HGF expression

MET expression on MSC was determined by flow cytometry using an anti-MET monoclonal antibody (5 μg/ml; clone 95106, IgG1; R&D Systems) and Fluorescein isothiocyanate (FITC)-conjugated F(ab)2-goat-anti-mouse IgG + M (Beckman-Coulter, Krefeld, Germany) as secondary antibody. Cells were analyzed on a FACS Canto flow cytometer (BD Biosciences, Heidelberg, Germany).

Intracellular HGF and apoptosis were detected by double labeling with FITC-conjugated Annexin V (Beckman-Coulter) and anti-HGF rabbit polyclonal antibody (15 μg/ml; Abgent, San Diego, CA) 12 h after ALA/PDT or control treatments. Phycoerythrin (PE)-conjugated goat-anti-rabbit IgG (Jackson Immuno Research, Newmarket, UK) served as secondary antibody for anti-HGF. Cells were stained first with Annexin V according to the manufacturer's protocol. Subsequently, they were fixed and permeabilized using the IntraPrep Fix/Perm kit (Beckman-Coulter), stained with anti-HGF polyclonal antibody and finally labeled with the secondary antibody prior to flow cytometric analysis.

HGF protein in conditioned media of glioblastoma spheroids obtained 12 h after ALA/PDT or control treatments was quantified by enzyme-linked immunosorbent assay (ELISA; Shino Test Corporation, Kanagawa, Japan) according to the manufacturer's protocol.

Detection of HGF transcripts by reverse transcription-polymerase chain reaction (RT-PCR) was performed as described recently [14,15]. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) served as positive control.

2.5. Statistical analysis

All data are presented as mean ± SEM for $n \geqslant 3$ unless stated otherwise. Statistical significance was determined with the Student's *t*-test using Prism Software (GraphPad, San Diego, CA).

3. Results

3.1. Attraction of MSC by glioblastoma spheroids is enhanced by ALA/PDT

To assess migration of MSC towards U87 and U251 glioblastoma spheroids, an under-agarose migration assay was performed. In three independent experiments using different MSC preparations, MSC migrated towards the spheroids (Fig. 1). Migration was significantly enhanced after ALA/PDT compared to the untreated spheroids (U87, 3.6 ± 0.6-fold, $p = 0.0096$; U251, 3.8 ± 0.4-fold, $p = 0.0018$). In contrast, control spheroids treated with exposure to laser light or incubation with ALA only showed no such effect.

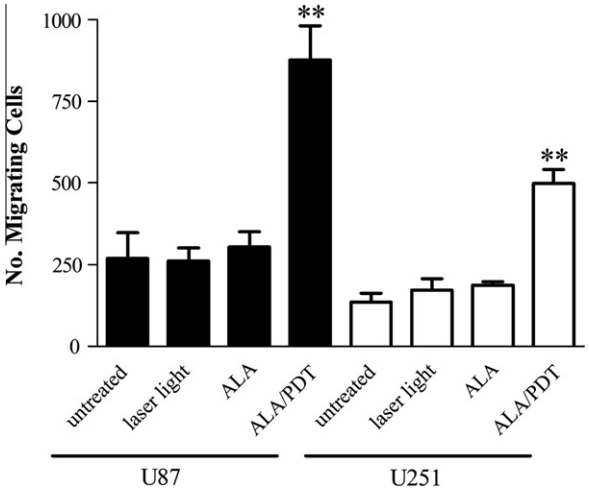

Fig. 1. ALA/PDT enhances migration of MSC towards glioblastoma spheroids. Migration of MSC towards untreated, laser light-exposed, ALA-treated or ALA/PDT-treated spheroids of the glioblastoma cell lines U87 and U251 was assessed in an under-agarose migration assay. The total number of specifically migrating MSC was determined after a 12-h migration period. Data are presented as mean ± SEM of three independent experiments. Statistical significance for the comparison with untreated spheroids is indicated, $^{**}p \leqslant 0.001$.

3.2. ALA/PDT up-regulates HGF production in glioblastoma spheroids

MSC expressed MET, the receptor for HGF, as determined by flow cytometry (Fig. 2A). RT-PCR analysis of the glioblastoma cell lines revealed expression of HGF (Fig. 2B) consistent with the detection of significant HGF protein levels in medium conditioned by untreated spheroids (Fig. 2C; U87, 5.6 ± 0.03 ng/ml; U251, 2.9 ± 0.13 ng/ml). ALA/PDT resulted in a significant increase in HGF release by the spheroids (U87, 2.6 ± 0.1-fold, $p = 0.0028$; U251, 3.1 ± 0.1-fold, $p = 0.0016$) compared to the untreated spheroids, whereas treatment with exposure to laser light or incubation with ALA only had no or only minimal effects. The presence of HGF in untreated living cells was also confirmed by intracellular staining (Fig. 2D and F). After ALA/PDT of spheroids, Annexin V staining indicated induction of apoptosis (Fig. 2E and G). HGF was present in living as well as apoptotic cells with a tendency of higher levels overall (Fig. 2E) or in apoptotic compared to living cells (Fig. 2G) after ALA/PDT.

3.3. Attraction of MSC by glioblastoma cells is mediated by HGF

To determine whether the HGF/MET axis plays a role in the tropism of MSC for glioblastoma cells and its enhancement by ALA/PDT, migration assays were performed in the presence of a neutralizing polyclonal anti-HGF antibody. Neutralizing HGF significantly inhibited migration of MSC towards U87 and U251 spheroids, irrespective of whether they were untreated or treated with laser light, ALA or ALA/PDT (Fig. 3). Inhibition was nearly complete, reaching 80.2 ± 3.1% and 74.2 ± 3.1% inhibition for ALA/PDT-treated U87 and U251 spheroids, respectively. In contrast, treatment with a control antibody had no inhibitory effects.

4. Discussion

Tropism of MSC for glioblastomas is well established. Irrespective of whether injected into the ipsilateral or contralateral carotid artery [26] or possibly even following systemic application [27], MSC have been shown to home to the tumor, to localize between the tumor and the normal brain parenchyma, and to infiltrate the tumor bed. Here, we have identified HGF as a major factor contributing to the migration of MSC towards glioblastoma cells. Moreover, we have shown that ALA/PDT of glioblastoma spheroids

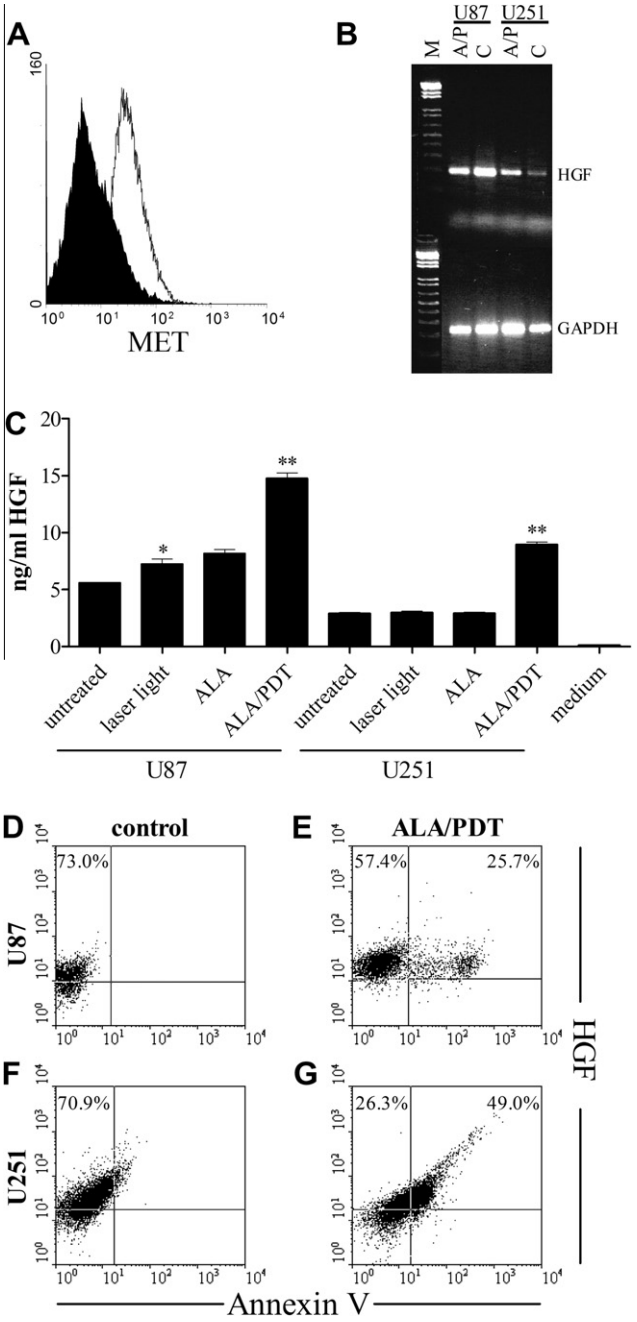

Fig. 2. ALA/PDT up-regulates HGF production in glioblastoma spheroids. Expression of the HGF receptor, MET, on MSC was determined by flow cytometry (A). Data of one of three independent experiments are shown. Cells were labeled with a MET-specific monoclonal antibody (open histogram) or an isotype control antibody (black histogram). The presence of HGF transcripts in untreated and ALA/PDT-treated U87 and U251 spheroids was determined by RT-PCR (B). GAPDH served as positive control, reactions without the addition of reverse transcriptase as negative controls (all negative, data not shown). M indicates the 100-bp ladder size marker. HGF protein levels in conditioned media derived from untreated, laser light-exposed, ALA-treated or ALA/PDT-treated spheroids of the glioblastoma cell lines U87 and U251 were determined by ELISA (C). Data represent mean ± SEM of two independent experiments. Statistical significance for the comparison with untreated spheroids is indicated, $^{**}p \leqslant 0.001$; $^{*}p \leqslant 0.05$. Apoptosis and the intracellular presence of HGF in living as well as apoptotic cells was detected by double labeling of untreated (D, F) and ALA/PDT-treated (E, G) U87 (D, E) and U251 spheroids (F, G) with Annexin V and anti-HGF antibody and flow cytometric analysis. Quadrants were set according to unlabeled controls.

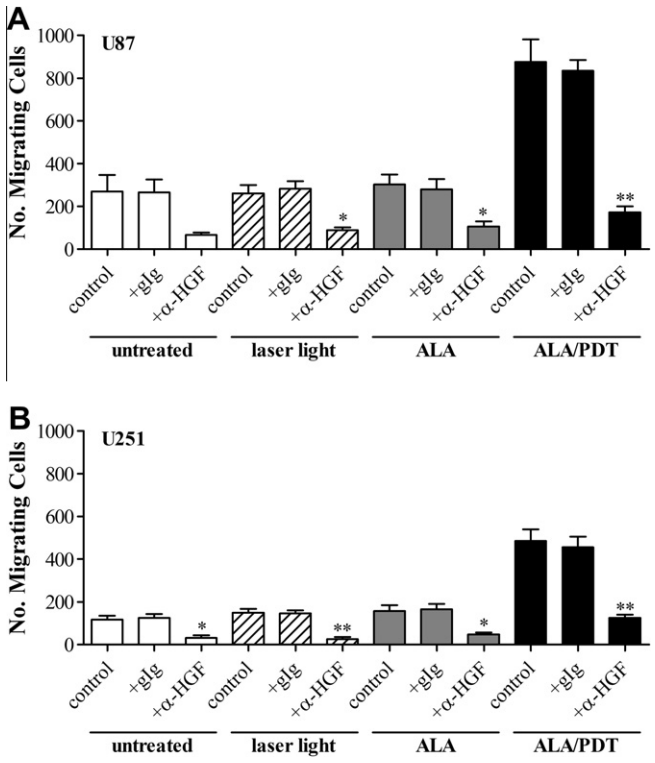

Fig. 3. MSC migrate towards glioblastoma spheroids in an HGF-dependent fashion. Migration of MSC towards untreated (open bars), laser light-exposed (hatched bars), ALA-treated (grey bars) or ALA/PDT-treated spheroids (black bars) of the glioblastoma cell lines U87 (A) and U251 (B) in the presence of a neutralizing polyclonal anti-HGF antibody or control IgG was determined. Data are presented as mean ± SEM of three independent experiments. Statistical significance for comparison with untreated and treated spheroids in the absence of antibodies is indicated, $^{**}p \leqslant 0.001$; $^{*}p \leqslant 0.05$.

induces apoptosis, augments HGF release and thereby causes an increase in MSC attraction.

Expression of HGF in glioblastoma cells, including U87 and U251 cell lines, has been described before [17,19,28,29]. Moreover, Chu et al. reported increased HGF production following gamma irradiation or hypoxia of U251 cells [29,30], and Kim and colleagues showed that irradiation of glioblastoma cells enhances attraction of MSC [31]. Thus, the ALA/PDT induced up-regulation of HGF in glioblastoma spheroids described here may represent a stress response, similar to that in response to gamma irradiation and hypoxia [29–31] and to the induction of apoptosis in neurons, which results in attraction of MSC in an HGF-dependent fashion [14]. Indeed, we could show recently [11] and confirm here that ALA/PDT of glioblastoma spheroids causes apoptosis, thus the type of cell death, which in neurons is associated with production of HGF. Tropism of other stem cell populations for glioblastomas appears to be regulated similarly: hematopoietic stem and progenitor cells [32] and neural stem cells [33] show enhanced migration towards glioblastoma cells *in vitro* as well as *in vivo* when these had been previously irradiated or subjected to hypoxic conditions before. Such stress responses, however, may also result in attraction of immune cells: we could recently show migration of immature dendritic cells towards ALA/PDT-treated glioma spheroids [10]. Thus, stress responses appear to initiate a concerted action by recruiting various cell types with regenerative or immunological activities. Interestingly, ALA/PDT appears to affect HGF release not only by the apoptotic, but also by the surviving cells. Whether this effect on surviving cells is direct or mediated indirectly by neighboring apoptotic cells requires further investigations.

Tropism of hematopoietic stem and progenitor cells for glioblastomas appears to be mediated by the CXCL12/CXCR4 axis [32], whereas for neural stem cells the CXCL12/CXCR4, vascular endothelial growth factor/vascular endothelial growth factor receptor 2 (VEGF/VEGFR2), urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor and the HGF/MET axes appear to contribute [33]. HGF has been described as chemoattractant for MSC [14,22] as well as other cell types including microglia cells [34], neural stem cells [33,35] and glioblastoma cells [17,36]. However, its role in driving migration of MSC towards glioblastomas has not been addressed before, and tropism has been attributed to other factors produced by glioblastoma cells, particularly VEGF [37], platelet-derived growth factor [38,39], CXCL12 [40] and CXCL-8 [31,41]. MSC express a multitude of chemokine and growth factor receptors involved in chemoattraction [42], some of which are expressed on subsets of cells only like CXCR4, CCR1 and CX3CR1 [43]. Moreover, there may be an interplay between the individual pathways, e.g. Tu et al. reported an up-regulation of CXCL12 in U251 glioblastoma cells after stimulation with HGF [44]. Thus, several pathways may contribute to the tropism of MSC for glioblastomas. So far, our observations are limited to *in vitro* studies and the analysis of cell lines. Although a three-dimensional spheroid model has been used, which mimics micro tumors more closely than monolayer cultures [45], differences between cell lines and primary tumor cells as well as differences in the micro milieu of the tumor *in vivo* may influence the results. Therefore, the effect of ALA/PDT on the HGF-driven tropism of MSC for glioblastomas has to be confirmed *in vivo*.

The increasing local concentration of HGF following ALA/PDT in glioblastomas and attraction of MSC to the tumors may have beneficial as well as detrimental effects. HGF contributes to glioblastoma growth, migration and invasion, and has cytoprotective, anti-apoptotic, immunosuppressive and angiogenic activity [16–19,46]. However, we could document that irrespective of the HGF expression described here, ALA/PDT causes a long-lasting inhibition of glioblastoma cell migration and invasiveness [11] and promotes anti-tumor immunity by initiating the initial steps of an adaptive immune response [10]. Moreover, Xie et al. have recently shown that expression of HGF by glioblastoma cells correlates with high levels of MET phosphorylation and predicts their sensitivity to therapeutic inhibition of the MET pathway [47]. Thus, there may be a rational for combining ALA/PDT with MET–HGF targeted therapy. Similarly, the recruitment of MSC by HGF may also have positive and negative consequences. They may contribute to angiogenesis, immunosuppression and tumor cell survival [48], but may also induce glioblastoma cell death directly [39]. Moreover, MSC are used to deliver therapeutics such as cytokines, enzymes/pro-drugs, oncolytic viruses and toxins to glioblastomas [25], and this strategy may be improved when combined with ALA/PDT, due to an enhanced tropism of the MSC. Although the net effect of ALA/PDT is currently unknown and requires further analyses, the beneficial effects of ALA/PDT may prevail as suggested by early clinical studies [3,4].

In summary, we have shown that HGF contributes to the attraction of MSC towards glioblastoma cells and that ALA/PDT enhances HGF production resulting in increased migration of MSC, which may have direct therapeutic consequences or may be exploited to improve targeted gene therapy with MSC serving as therapeutic vectors.

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