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ABSTRACT

We previously reported that OPG is involved in ischemic tissue neovascularization through the secretion of SDF-1 by pretreated-OPG endothelial colony-forming cells (ECFCs). As the vascularization is important for tumour growth and dissemination, we examined whether OPG was able to modulate the invasion of human HOS osteosarcoma and DU145 prostate cancer cell lines *in vitro* and investigated its effect *in vivo*. Cell motility was examined in Boyden chambers. Human GFP-labelled HOS cells were injected in nude mice and the tumour nodules formed were injected with OPG and/or FGF-2, or PBS. Angiogenesis was assessed by immunohistochemistry. *In vitro*, SDF-1 released by OPG-pretreated ECFCs markedly attracted both HOS and DU145 cells and also induced spontaneous migration of cancer cells. *In vivo*, tumour volumes were significantly increased in OPG-treated group compared to the control group and OPG potentiated the effect of FGF-2. Concomitantly, OPG alone or combined with FGF-2 increased the number of new vasculature compared to the control group. This study provided experimental evidence that OPG promotes tumour development in an orthotopic murine model of osteosarcoma and supports recent findings that it is actively involved in tumour vascularization.

Highlights

- OPG promotes tumour development
- OPG is actively involved in tumour revascularization
- SDF-1 released by OPG-pretreated endothelial colony-forming cells attract cancer cells
- SDF-1 induces spontaneous migration of HOS osteosarcoma cell line

Key Words: Osteoprotegerin, SDF-1, tumour angiogenesis, osteosarcoma.

1. INTRODUCTION

Osteoprotegerin (OPG) is a member of the tumour necrosis factor (TNF) receptor family which is involved in the regulation of bone remodelling, where it acts as a decoy receptor for nuclear factor-kB ligand (RANKL). OPG is expressed in a variety of tissues such as bone, heart, lung, liver, placenta, vessels and can therefore interact with a large number of cell types belonging to normal or cancerous tissues [1]. Because of its anti-resorptive properties, several studies have investigated the therapeutic potential of recombinant OPG in osteolysis associated with cancer. Preclinical studies showed that treatment with exogenous recombinant OPG inhibits osteolysis *in vivo*, associated with breast cancer metastasis or multiple myeloma, and reduces cancer cell migration *in vivo* [2]. However, therapeutic use of OPG in bone tumours remains controversial due to its ability to bind and inhibit the TNF related apoptosis inducing ligand (TRAIL) resulting in the inhibition of tumour cells apoptosis [3]. In these circumstances, OPG becomes a survival factor for tumour cells (reviewed in [4]). Hence, OPG has been described to be a survival factor of several types of cell tumour including osteosarcoma and prostate cancer cells [5].

Osteosarcoma, the most common primary malignant bone tumour, is defined as a malignant tumour of mesenchymal cells, characterized by the direct formation of malignant osteoid. Some are composed of largely fibroblastic cells, some show chondroid differentiation, and still others are highly vascular [6]. OPG production by human osteosarcoma has been previously reported [7-9]. Prostate cancer cells have been also shown to secrete OPG [4] and its is higher in metastatic tumours than in primary tumours [10]. Similarly, serum OPG levels have been positively correlated with progression and bone metastasis [11] which indicates the significant involvement of OPG in prostate cancer progression.

Angiogenesis is an important additional process contributing to tumour growth and progression. Various angiogenic cytokines, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), are potent mitogens with angiogenic activity [12]. Hence, tumour angiogenesis has been extensively investigated in solid and haematological tumours, as well as in premalignant conditions, and there are a lot of data regarding the link between tumour angiogenesis, metastasis, and overall survival (reviewed in [13]). OPG can now be added to the growing list of factors affecting tumour angiogenesis, as it has been found to be expressed in neovessels associated with malignant tumours and in angiogenic microvessels associated with inflammatory osteolytic diseases [14]. Furthermore we previously shown that OPG is involved in vasculogenesis induced by endothelial progenitor cells (EPCs) *in vivo*, that suggests a modulatory role in tumour revascularization [15].

EPCs are bone marrow-derived circulating cells involved in postnatal vasculogenesis. These cells are recruited from bone marrow to sites of active revascularization, attracted by proangiogenic factors produced by the local inflammatory response [16]. A growing body of evidence indicates that neovascularization processes associated with tumour growth are in part supported by the recruitment of endogenous EPCs, their functional incorporation into the new vasculatures and their paracrine effects (reviewed in [17]). We have already reported that OPG markedly enhances functional properties of EPCs and that these effects are correlated with overexpression and secretion of the chemokine stromal cell-derived factor-1 (SDF-1), a key player in the attraction of tumour cells [15]. Indeed, SDF-1 possesses angiogenic properties and is involved in the outgrowth and metastasis of CXCR4-expressing tumours. Consequently, CXCR4 inhibitors have been proposed as therapeutic agents to inhibit tumour growth and metastasis (reviewed in [18])[19].

Altogether, these data suggest that OPG could also promote tumour growth by promoting angiogenesis and inducing chemokines release by EPCs. These chemokines are, thereby, able to promote tumour cells spread and consequently, metastasis. So, the purpose of this study was to determine whether SDF-1 released by endothelial colony-forming cells (ECFCs; a sub-population of EPCs) after OPG treatment can modulate induce human osteosarcoma MNNG/HOS and human prostate cancer DU145 cell lines, migration and chemotaxis *in vitro*. Using a nude mouse model of human osteosarcoma xenografts, we also investigated the effect of OPG on tumour growth and vascularization *in vivo*.

2. MATERIALS AND METHODS

2.1. Reagents

Recombinant human and mouse OPG were from R&D Systems (Lille, France). Mouse basic fibroblast growth factor (FGF-2) and stromal cell-derived factor-1 (SDF-1α) were from Abcys (Paris, France). AMD3100 and porcine skin gelatine (GEL) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). DMEM and phosphate buffered saline (PBS) were provided by Invitrogen (Saint Aubin, France). Other biochemical reagents were from Sigma-Aldrich (Saint-Quentin Fallavier, France).

2.2. Cancer cell lines and treatment

The human DU145 prostate carcinoma and MNNG/HOS osteosarcoma cell lines purchased from American Tissue Cell Collection were maintained in DMEM supplemented with 10% foetal calf serum (FCS, Gibco, France). When required, AMD3100 (10µg/mL) was added 30min before the treatment with conditioned media from OPG-pre-treated ECFCs.

2.3. ECFCs isolation, culture and pretreatment

Umbilical cord bloods were collected from consenting mothers. The study was approved by local ethics committee of "Hôpital des Instructions et des Armées de Begin (France) (201008043234797) and protocol conformed to ethical guidelines of Declaration of Helsinki. ECFCs were isolated from human umbilical cord blood, expanded and characterized as previously described [20]. The endothelial cell phenotype was shown by double positivity for DiI-AcLDL uptake and BS-1 lectin binding. Further endothelial characterization was obtained by FACS analysis (FACSCalibur, Becton Dickinson) of combined expression of cell-surface antigens of the endothelial lineage, namely CD31, KDR,

Tie-2, CD144, CD34 and Flt-1. One day before experiments, ECFCs were growth-arrested for 18 hours in EBM2, 3% FCS and released from growth arrest by adding DMEM 5% FCS with or without 25ng/ml of OPG at 37°C for 48h. ECFCs conditioned media were then collected and centrifuged to be tested for cell migration assay, or kept at -80 °C to be further analysed for SDF-1 levels. All assays were performed in triplicate with cells cultured for less than 30 days.

2.4. Chemotaxis assay

Chemotaxis was examined in 24-well Boyden microchemotaxis chambers (Costar, France) with 8µm pore-size polyvinylpyrrolidone free polycarbonate Nucleopore filters. HOS and DU145 were placed in the upper chambers in their respective culture media ($1.5X10^4$ cells/chamber) and let to migrate toward the control media (DMEM, 5% FCS), control media with 100ng/ml of SDF-1 (positive control), OPG (25ng/ml) or supernatants of OPG pretreated ECFCs. When required, before seeding, HOS and DU145 were pretreated with AMD3100 (10µg/mL) for 30min. Chemoattraction was allowed to proceed for 4h at 37°C, 5% CO2. Cells remaining on the upper surface of the filters were mechanically removed, and the filters were then fixed with 1.1% formaldehyde and stained with Giemsa. The number of migrated cells was determined by counting under a high-power microscope.

2.5. Migration assay

As for chemotaxis assay, migration was examined in 24-well Boyden microchemotaxis chambers. HOS and DU145 were pretreated with the control media with or without 100ng/ml of SDF-1 (positive control), 25ng/ml of OPG or with supernatants of OPG pretreated ECFCs. When required, AMD3100 (10µg/mL) was added 30min before HOS and DU145 treatment.

24h later, $1.5X10^4$ control and pretreated-cells in suspension in the control media were placed in the upper chambers and let to migrate toward the same media. Migration was allowed to proceed for 4h at 37°C, 5% CO_2. Number of migrated cells is determined as for the chemotaxis assay.

2.6. SDF-1 ELISA

SDF-1 levels in supernatants of ECFCs were measured with enzyme-linked immunosorbent kits from R&D Systems® (France) according to the manufacturer's instructions.

2.7. Animal experiments

Animal care conformed to French guidelines (Services Vétérinaires de la Santé et de la Production Animale, Paris, France), and experiments were performed in keeping with the guidelines of Université Paris Descartes and the Institutional Committee on Animal Care and Use (C75.06.02). Human osteosarcoma MNNG/HOS cells have been previously shown to rapidly divide *in vivo*, forming subcutaneous tumours after implantation into athymic nude mice. Eight-week-old athymic nude mice were supplied by Janvier (France). $4.5X10^6$ GFP-MNNG/HOS cells were injected subcutaneously and three to five days following injection, when tumours had reached approximately 3-4mm^3 in size, 100µl of either PBS (negative control), mouse OPG (2µg/kg), mouse FGF-2 (1.4µg/kg) alone or supplemented with mouse OPG (2µg/kg) were administered by direct intra-tumour injection (7 mice per group). This procedure was repeated twice a week. Tumour volume was measured thrice per week with calipers and each volume (V) was calculated according to the following formula: V=a×b2/2, where a and b are the largest and smallest perpendicular tumour diameters. Relative tumour volumes (RTV) were calculated from the following formula: RTV=(Vx/V1), where Vx is the

tumour volume on day x and V1 is the tumour volume at initiation of treatment (day 1). After 3 weeks post initiation of treatment, host mice were euthanized, and tumour were excised and frozen by dipping for 30s in liquid nitrogen-chilled isopentane, and stored at -80C until sectioning and staining.

2.8. Tissue processing and immunofluorescence

For the analysis of tumour vascularization, frozen tumours were cut at 10-µm thickness. Sections were stained at room temperature for 1h with a rat anti-mouse CD31 monoclonal antibody (clone MEC 13.3, BD Biosciences), then with a goat anti-rat secondary antibody coupled with Alexa555 (Invitrogen, France). The DNA marker, TOPRO-3 (Invitrogen) was then applied for 10 min at room temperature. Sections were mounted in glycerol/PBS (90/10: v/v) and images were recorded on a Leica TCS SP2 confocal microscope. Eight fields were examined per section. The vessel surface area and the number of vessels were quantified with Histolab software (Microvision Instruments, Evry France). Results are expressed as the vessel surface area (%) and the number of vessels per mm^2.

2.9. Statistical analysis

Data are expressed as means ± SEM of at least three independent experiments. Differences between groups were assessed by one-way ANOVA test fallowed by Mann-Whitney test, using the statistical software package GraphPad Prism, version 5. Results were considered statistically significant at the p-values ≤ 0.05.

3. RESULTS

3.1. Endogenous SDF-1 released by OPG-pretreated ECFCs strongly attracts tumour cells.

Several studies have described the OPG and CXCR4 expression by HOS cells line [7]. We investigated the direct effect of OPG and effect of conditioned media of OPG pretreated ECFCs on HOS cells chemotaxis and migration, to determine whether OPG might be involved in tumour cell dissemination to sites of metastasis. We first analysed the SDF-1 levels in cell supernatants of OPG pretreated ECFCs (Figure 1A). As shown on figure 1B, HOS cells were maximally attracted by the conditioned media of OPG pretreated ECFCs ($P < 0.001$), more strongly than by the SDF-1 ($P < 0.05$). Furthermore, 25ng/mL of OPG stimulated HOS cells chemotaxis ($P < 0.01$). By using the AMD3100 (a specific antagonist of CXCR), we found that the effect exerted by conditioned media of OPG pretreated ECFCs is partly due to the SDF-1 released by these cells under OPG treatment ($P < 0.01$). We show in Figure 1C that SDF-1 alone as well as the SDF-1 released by ECFCs under OPG treatment are not only able to attract HOS, but can also act directly on these cells and induce their spontaneous migration, with a statistically significant effect ($P < 0.05$, $P < 0.01$, respectively).

To determine whether the observed effects were specific to HOS cells line, the same culture conditions were tested on another tumour cell line, DU145 (Figure 2). Expression of the CXCR4 by prostate cancer DU145 cell line has been reported by several studies. Hence, the SDF-1/CXCR4 axis has been described as playing a key role metastasis of prostate cancer to bone [21]. OPG is also expressed by DU145 cells, its role in the lifecycle of these cells as well as in the communication between prostate cancer cells and bone cells is well established [22]. As shown on Figure 2, conditioned media of ECFCs pretreated or not with OPG attract tumour cells. However, effect of supernatants of OPG pretreated ECFCs was more important, significant and similar to that of SDF-1 alone ($p<0.001$). This is most likely due to SDF-1

released by ECFCs under OPG treatment, since it was reduced by 30% ($p<0.05$) when the SDF-1/CXCR4 interaction was blocked by AMD3100. Pretreatment of DU145 cells with OPG or conditioned media of OPG pretreated ECFCs does not affect their spontaneous migration.

3.2. OPG enhances tumour growth in a murine xenograft model of osteosarcoma.

As described above, OPG showed greater effect on the HOS cell line motility *in vitro*. We further evaluate its effect *in vivo* in human osteosarcoma tumours developed in nude mice. The HOS xenograft showed an appreciable growth starting from the fifth day after cell inoculation and doubled its volume in about 3 days (Figure 3). To evaluate the effect of OPG in such a preclinical experimental model of osteosarcoma, a treatment protocol was applied via the intra-tumour administration, twice a week, of PBS (control), 2µg/kg mouse recombinant OPG, or 1.4µg/kg mouse FGF-2 (positive control) when the tumours reached a volume of approximately 3 to 4mm^3 (Figure 3A). As shown in Figure 3B, no appreciable variation in tumour size was observed during the first 6 days of treatment, after what, a significant increase in OPG-treated-xenograft growth was observed ($p<0.01$, at the endpoint Figure 3B).

To investigate the possible synergy of OPG/FGF-2 as previously observed in neovascularization assays *in vivo* (14), we used the same model, injecting each mouse with 1.4µg/kg of FGF-2 alone or combined with 2µg/kg of OPG. Analysis of the xenograft growth showed enhanced tumour volume in OPG/FGF-2 treated mice ($p<0.05$ Figure 3C) as compared with OPG alone ($p<0.01$ Figure 3C). It should be noted that, no appreciable sign of distress or loss of weight in mice was evidenced. This effect is correlated with tumour vascularisation. This is not surprising since blood vessels are necessary to tumour growth by providing nutrients and oxygen (Figure 4). To evaluate the implication of OPG alone or

associated with FGF-2 in tumour neovascularization, mice were sacrificed 24 days after the beginning of the treatment, and tumours were collected for immunofluorescent assays. As shown on Figure 4B-C, anti-CD31 staining clearly revealed that the vessel density as well as the number of tumour microvessels were also increased in the FGF-2/OPG-treated group as compared with FGF-2 group ($p<0.01$) and OPG group ($p<0.001$). Furthermore, mice treated with OPG alone showed enhanced vascularization compared to control mice (PBS, $p<0.05$, 52±9 vs 32±3 vessels/ml^2) (Figure 4C).

4. DISCUSSION

OPG acts as a key regulator of bone metabolism by blocking osteoclast differentiation. Several *in vitro* and *in vivo* studies attributed OPG an important role in vascular biology [15, 24]. Thereby, there is growing evidence that it underlies a possible link between the osseous and vascular systems. One of the major discoveries about OPG was its ability to bind to and inhibit the activity of TRAIL; a cytotoxic protein inducing apoptosis mostly in tumour cells, suggesting that OPG production may provide cells with a survival advantage. *In vitro* studies using a number of different tumour types have supported this hypothesis (reviewed in [25]). OPG expression is frequently altered in cancers. Investigations by several groups have shown that OPG levels hold promise as markers of cancer progression or as prognostic indicators[4].

The first aim of this study was to determine whether OPG may modulate the behaviour of cancer cells and especially human osteosarcoma cells. So, we first examined a direct effect of OPG and effect of media conditioned by OPG pretreated ECFCs compared to SDF-1 on spontaneous migration and chemotaxis of HOS cells *in vitro*. Media conditioned by OPG pretreated ECFCs attracted HOS cells more strongly than SDF-1 treatment. OPG alone induced significantly HOS cells chemotaxis, raising the possibility that OPG can intervene both directly and indirectly to modulate osteosarcoma cells attraction. It should be noted that OPG pretreated ECFCs, in addition to SDF-1, may release other factors that can modulate cells chemotaxis, since HOS cells treatment with AMD3100 not totally abolished the effect of supernatants of OPG pretreated ECFCs on cells chemotaxis. The other new element of this study is that the SDF-1 released by ECFCs under OPG treatment, at similar levels as a SDF-1 treatment, can act directly on the HOS cells and induce their spontaneous migration. We also found that SDF-1 released by ECFCs under OPG treatment significantly induces DU145 cells chemotaxis. Indicating that, the observed effects on HOS cells line can be heard on other tumour cell types.

Metastasis are the leading cause of cancer-related death, around 13–27% of the osteosarcoma patients have detectable metastasis at diagnosis, whereas 40% will develop metastases at a later stage (reviewed in [26]). Molecular pathways contributing to osteosarcoma development and progression have recently been described and the role of several cytokines and chemokines was detailed (reviewed in [27]). Although the role of OPG and SDF-1 was not detailed in this revue, the significance of CXCR4 in metastasis development in osteosarcoma has been reported. In a mouse model, the tumour cells with CXCR4 receptor were chemoattracted by SDF-1, migrated through the lymphatic and vascular system, and arrested in SDF-1 rich organs like the bone and lungs [28]. A higher CXCR4 expression in metastasis compared with primary osteosarcoma was also reported [29]. In an analysis of Ewing sarcoma, another bone cancer, and in chondrosarcoma of bone, CXCR4 correlated with metastasis [30]. Taken together with the present study, these findings support the increasing evidence of the role of SDF-1/CXCR4 axis in osteosarcoma metastasis.

Furthermore, Namløs *et al* report that infiltrating stroma (macrophages) can be the major source of chemokine expression in osteosarcoma [31]. ECFCs are found to be recruited into tumour environment as they are actively involved in tumour vascularization [17]. OPG, previously described to be released in osteosarcoma [7], is therefore able to induce the SDF-1 release by ECFCs in osteosarcoma environment. Suggesting that, like macrophages, ECFCs can also represent a source of chemokines and actively participate in tumour cells migration and evidencing that OPG can clearly be, directly or indirectly, involved in osteosarcoma metastasis development. The same role of OPG has been reported in gastric cancer by the Reiko team's, which showed that strong expression of OPG in cancer tissue was closely associated with deep invasion, nodal metastasis, advanced stage and poor prognosis [32].

We and others have previously described the effect of OPG on new blood vessel formation which may occur through angiogenesis, defined as the sprouting of endothelium

from pre-existing vasculature and involves the mature endothelial cells, or vasculogenesis in which entirely new vessels develop from ECFCs which circulate and ultimately contribute to tumour development and metastasis [33]. OPG has been shown to promote both angiogenesis [34, 35] and vasculogenesis since it mediates the mobilization and differentiation of ECFC [15, 24]. Therefore, it may ultimately have implications for tumour angiogenesis, a key process in cancer development. Studies conducted on the OPG involvement in osteosarcoma are limited to found the best therapy permitting to benefit of its role on osteoclastogenesis inhibition and eliminate its effect on tumour cells survival [8, 32, 36, 37]. To our knowledge, none of these studies have considered the possible involvement of OPG in tumour angiogenesis. So, in our *in vivo* study, using a xenograft model of osteosarcoma in nude mouse, was undertaken to evaluate the biological effects of OPG on osteosarcoma growth and vascularization. We confirmed that OPG alone or associated with FGF-2; a growth factor previously described to potentiate the OPG proangiogenic effect *in vivo* [15], induces xenograft growth and angiogenesis.

Initially, tumour growth relies on diffusion of oxygen and nutrients from the surrounding tissues, and don't need new blood vessels formation. Under these conditions, a tumour can grow to a size of 2–3 mm^3. Thereafter, the growing metabolic demands associated with tumour growth are satisfied through growth and establishment of new blood vessels. So, tumour cells undergo the angiogenic switch, where they acquire an angiogenic phenotype that changes the local equilibrium between positive and negative regulators of angiogenesis, and stimulates the formation of new vasculatures necessary for sustainable tumour growth (reviewed in [17]). In the present study, we noticed that tumours grow slowly during the first 5 days after cell inoculation, to attain a size of 3 to 4 mm^3, approximately. Thereafter, tumours size doubled in just 3 days. This would probably correspond to a beginning of vasculature development. Surprisingly, no significant difference was observed between the

different groups during the first 6 days of treatment, after what the FGF-2/OPG treated xenografts showed exponential growth, followed by the FGF-2 and OPG treated ones. The apoptosis inhibitory effect, survival extension of endothelial cells and neoangiogenesis induced by OPG alone or associated with FGF-2 have therefore established suitable environment for tumour cells proliferation and consequently tumour growth. This hypothesis is supported by the Anti-CD31 staining which clearly revealed that xenografts treated with OPG alone or associated with FGF-2 displayed a greater number of blood vessels, than those injected with only the vehicle.

In summary, our findings suggest that OPG may participate in tumour growth and invasion, possibly through inhibition of tumour cell apoptosis but also by promoting tumour angiogenesis. The effects of OPG on tumour neovascularization include augmented chemotaxis of ECFCs [15, 24]. The maintain of ECFCs in the tumour environment can not only promote the vasculature and consequently the tumour growth, but also the release of cytokines and chemokines permitting potentially the tumour cells spread and metastasis.

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FIGURE LEGENDS

Figure 1: SDF-1 released by ECFCs after OPG exerts a strong chemoattraction on osteosarcoma cells. (A) OPG markedly increased SDF-1 release by ECFCs: SDF-1 levels were detected by ELISA in supernatants of ECFCs pretreated or not with OPG (25ng/ml) for 48h. **(B)** This SDF-1 strongly attracts HOS cells: Before the migration assay, HOS cells were pretreated or not with AMD3100 (10µg/ml) for 30min, placed in the upper Boyden chambers in RPMI, 5% FCS than let to migrate toward the control media (RPMI, 5% FCS), control media with 100ng/ml of SDF-1 (positive control), OPG (25ng/ml) or supernatants of OPG pretreated (or untreated) ECFCs during 4h. HOS cells were maximally attracted by the conditioned media of OPG pretreated ECFCs ($P<0.001$), more strongly than by the SDF-1 ($P<0.01$). This effect was strongly reduced, by 50%, after HOS treatment with AMD3100 ($P<0.01$). 25ng/mL of OPG stimulated HOS cells chemotaxis ($P<0.01$). **(C)** SDF-1 released in the supernatants of OPG pretreated ECFCs induces HOS cell migration: HOS cells were pretreated with RPMI, 5% FCS with or without 100ng/ml of SDF-1 (positive control), 25ng/ml of OPG or with supernatants of OPG pretreated ECFCs. When required, AMD3100 (10µg/mL) was added 30min before HOS treatment. 24h later, control and pretreated- cells in suspension in RPMI, 5% FCS were placed in the upper chambers and let to migrate toward the same media. Migration was allowed to proceed for 4h. SDF-1 alone ($P<0.05$) and SDF-1 released by ECFCs under OPG treatment ($P<0.01$) induce HOS cells spontaneous migration. Effect of supernatants of OPG pretreated ECFCs was reduced by 30% ($p<0.05$) when the SDF-1/CXCR4 interaction was blocked by AMD3100.

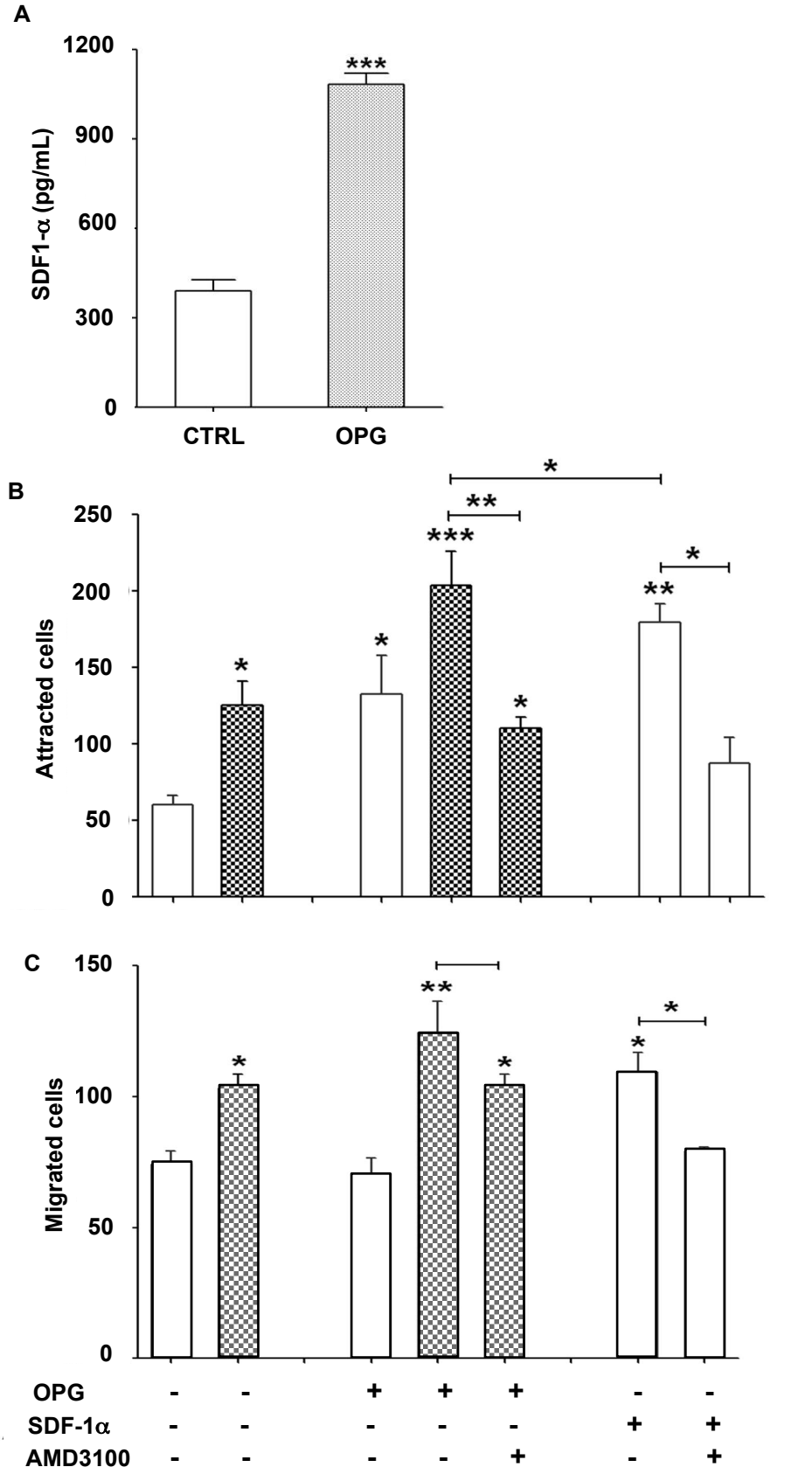
Figure 2: SDF-1 released in the supernatants of OPG pretreated ECFCs modulates the motility of prostate cancer cells. Before the migration assay, DU145 cells were pretreated or not with AMD3100 (10µg/ml) for 30min, placed in the upper Boyden chambers in DMEM, 5% FCS than let to migrate toward the control media (DMEM, 5% FCS), control media with 100ng/ml of SDF-1, OPG (25ng/ml) or supernatants of OPG pretreated (or untreated) ECFCs during 4h. Conditioned media of ECFCs pretreated with OPG strongly attract tumour cells ($p<0.001$). This effect was reduced by 30% ($p<0.05$) when the SDF-1/CXCR4 interaction was blocked by AMD3100. OPG alone has no effect on the DU145 cells chemotaxis. Mean ± SEM, n=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$

Figure 3: OPG alone or associated with FGF-2 induces tumour growth in a xenograft osteosarcoma model. HOS cells were injected subcutaneously into mice. When tumours had reached approximately 3-4mm^3 in size, 100µl of either PBS (negative control), mouse OPG (2µg/Kg), mouse FGF-2 (1.4µg/Kg) alone or supplemented with mouse OPG (2µg/Kg) were administered by direct intra-tumour injection. This procedure was repeated twice a week. Tumour volume was measured thrice per week and each volume (V) was calculated according to the following formula: $V=a\times b2/2$ (a and b are the largest and smallest perpendicular tumour diameters). Relative tumour volumes (RTV) were calculated from the following formula: $RTV=(Vx/V1)$; Vx is the tumour volume on day x and V1 is the tumour volume at initiation of treatment (day 1). **(A)** Example photographs of tumours excised from mice of each group at the endpoint. **(B)** Tumour growth curves as a function of time in peri-tumour treated with OPG (2µg/Kg) or control vehicle: a significant increase in OPG-treated-xenograft growth was observed from day 7 after injection (about 90% with respect to control at the endpoint, $P<0.01$). **(C)** Tumour growth curves as a function of time, peri-tumour treated with OPG (2µg/Kg), FGF-2 (1.4µg/Kg) or OPG+FGF-2: administration of OPG associated with

FGF-2 showed a more important effect on xenograft growth than the injection of FGF-2 alone ($P < 0.05$) and the OPG alone ($P < 0.01$). Mean ± SEM, n = 7, *p<0.05, **p<0.01, ***p<0.001.

Figure 4: OPG increases tumour vascularization. A 10µm thickness sections from frozen tumours were stained with an anti-mouse CD31 monoclonal antibody and TOPRO-3. Images were recorded on a Leica TCS SP2 confocal microscope. Eight fields were examined per section. The vessel surface area and the number of vessels were quantified. **(A)** Representative photomicrographs of cryosections of xenograft tumours from CTRL, OPG, FGF-2 and OPG/FGF-2 mice. Vessels were stained in red with an anti-CD31 antibody and with a secondary antibody coupled to alexa-555. Nuclei were stained with TOPRO-3. **(B)** Quantitative analysis of the vessel surface area (%, vs CTRL), showed an increase in tumour angiogenesis in OPG/FGF-2 group ($p<0.001$) fallowed by the FGF-2 ($p<0.05$) and the OPG ($p=0.059$) ones. **(C)** Analysing the number of vessels per mm^2 confirmed the results of the vessel surface analysis. Mean ± SEM, n=4, *p<0.05, **p<0.01, ***p<0.001.

Figure 5: SDF-1 plays a key role in the biological activity of OPG in tumour development. OPG secreted by cancer cells (1) increases SDF-1 release by endothelial colony-forming cells located in the tumour microenvironment (2) which binds to CRCX4 expressed by cancer cells and in turn exerts a chemoattractant activity on cancer cells (3). Concomitantly, OPG in close collaboration with endothelial colony-forming cells contributes to the formation of new blood vessels (4) and to an increase of intra-tumour vasculature and then to an increase of tumour development (5).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1200
900
600
300
0
SDF1-α (pg/mL)
***
CTRL
OPG
B
250
200
150
100
50
0
Attracted cells
C
150
100
50
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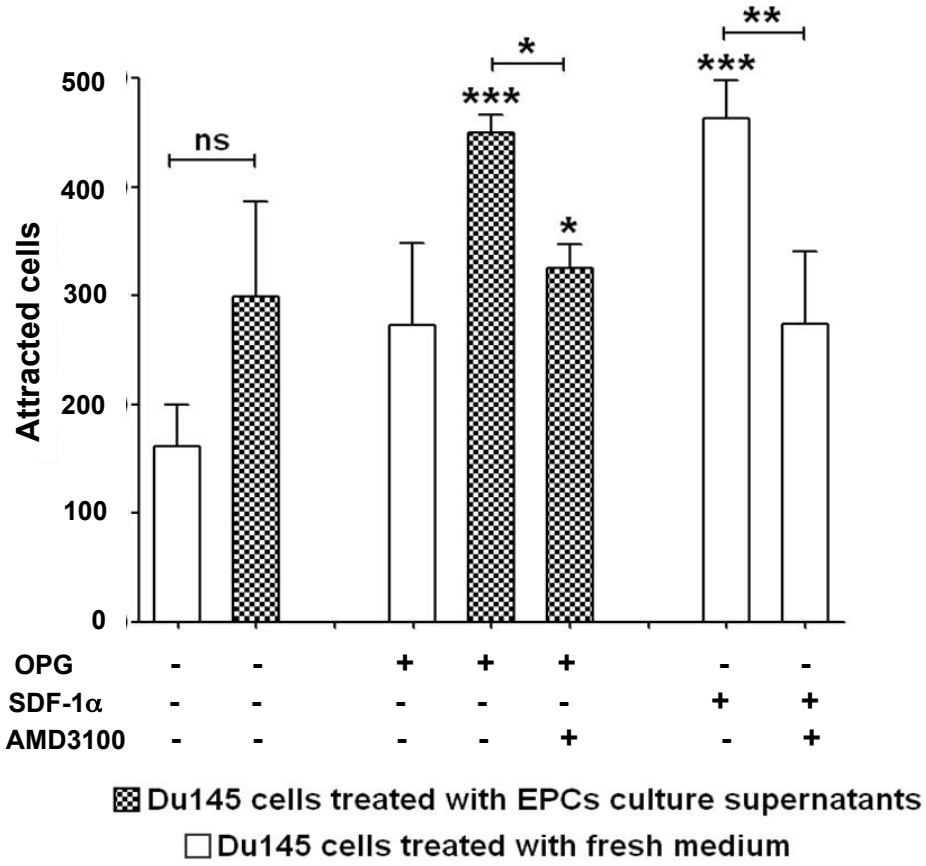
Migrated cells
OPG
SDF-1α
AMD3100
HOS cells treated with EPCs culture supernatants
HOS cells treated with fresh medium

Figure 2


Attracted cells
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400
300
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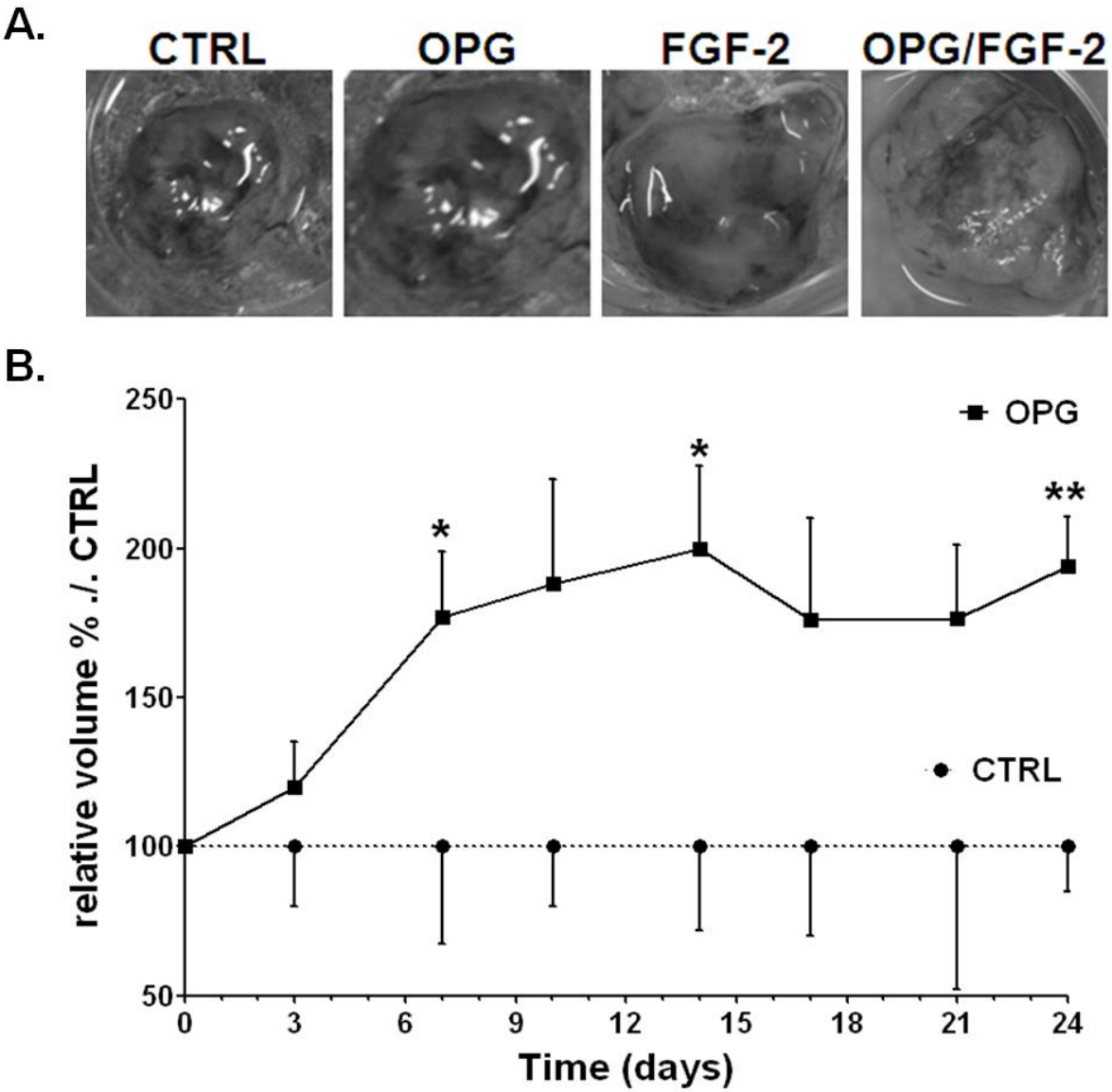
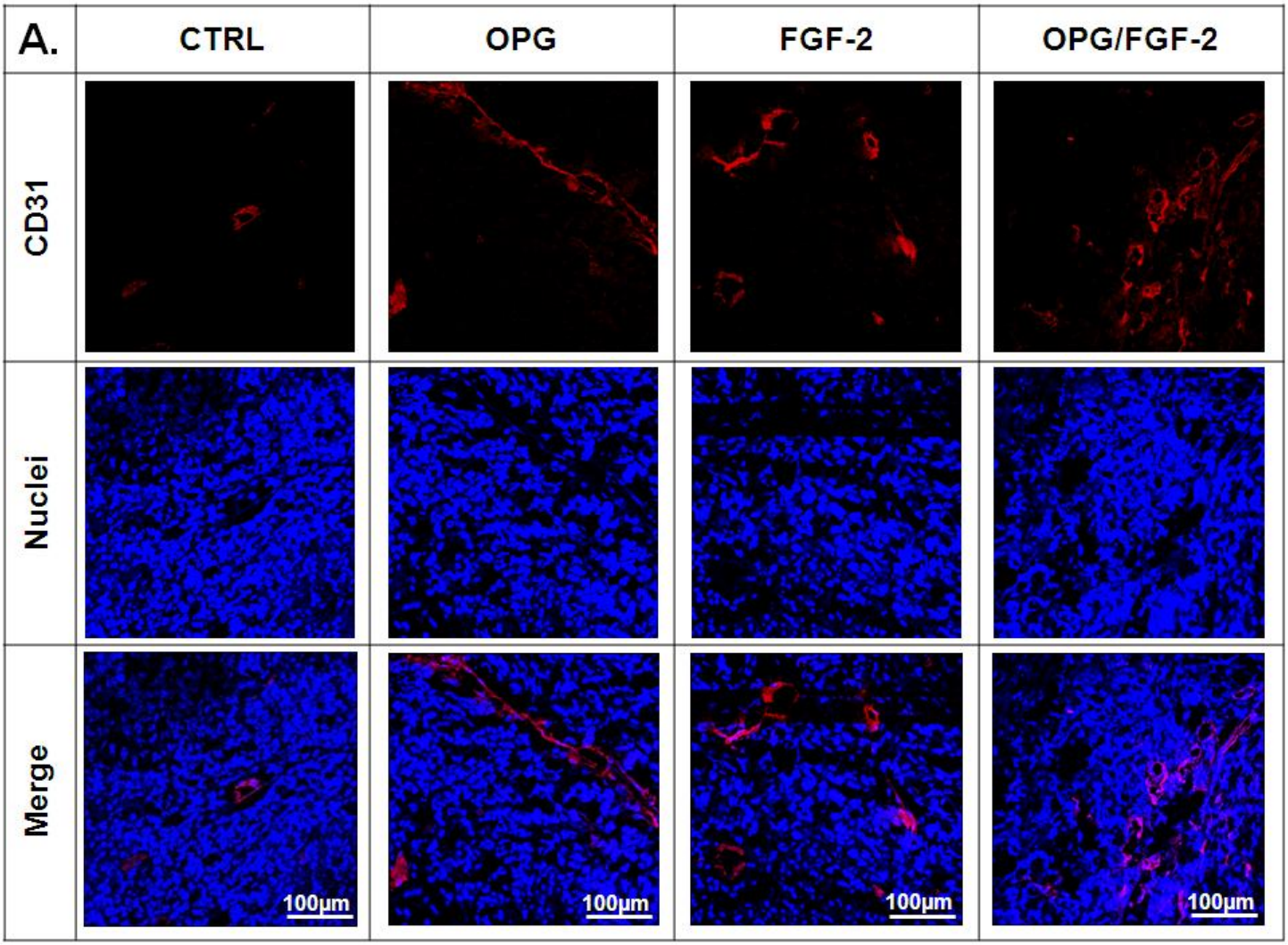
AMD3100 - - - - + - +
Du145 cells treated with EPCs culture supernatants
Du145 cells treated with fresh medium


A.
CTRL
OPG
FGF-2
OPG/FGF-2
B.
OPG
CTRL
relative volume % ./. CTRL
Time (days)
C.
FGF-2
OPG
OPG/FGF
relative volume (Vx/V1)
Time (days)
* OPG/FGF-2 VS OPG, # OPG/FGF-2 VS FGF-2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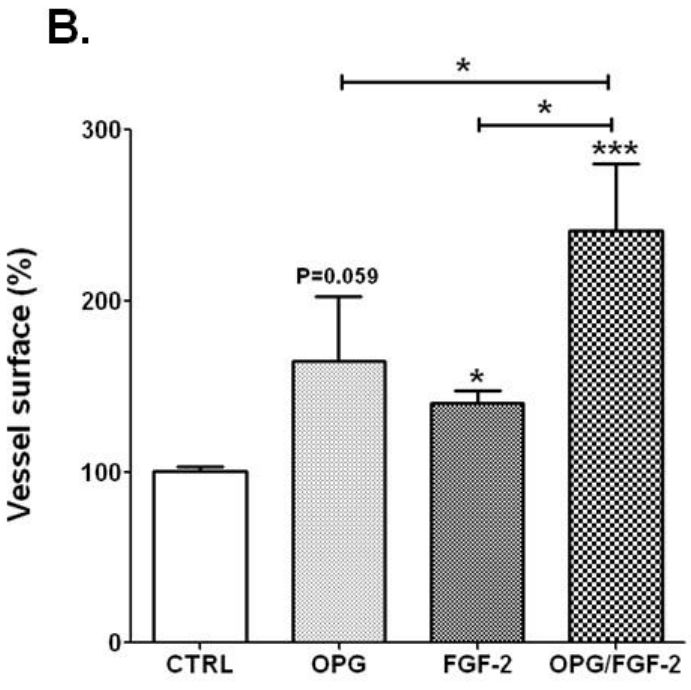
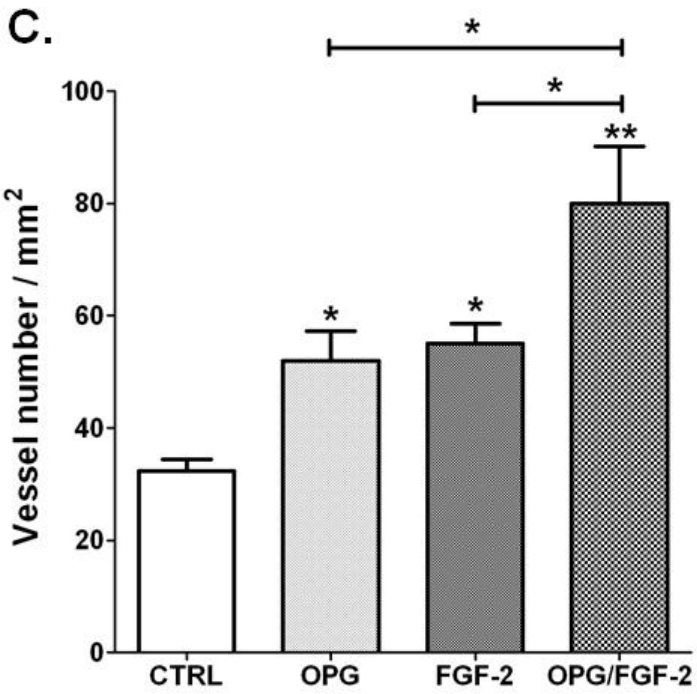
CTRL
OPG
FGF-2
OPG/FGF-2
CD31
Nuclei
Merge
100µm
100µm
100µm
100µm


B.
Vessel surface (%)
300
200
100
0
P=0.059
*
*
*
***
CTRL
OPG
FGF-2
OPG/FGF-2


C.
Vessel number / mm2
100
80
60
40
20
0
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*
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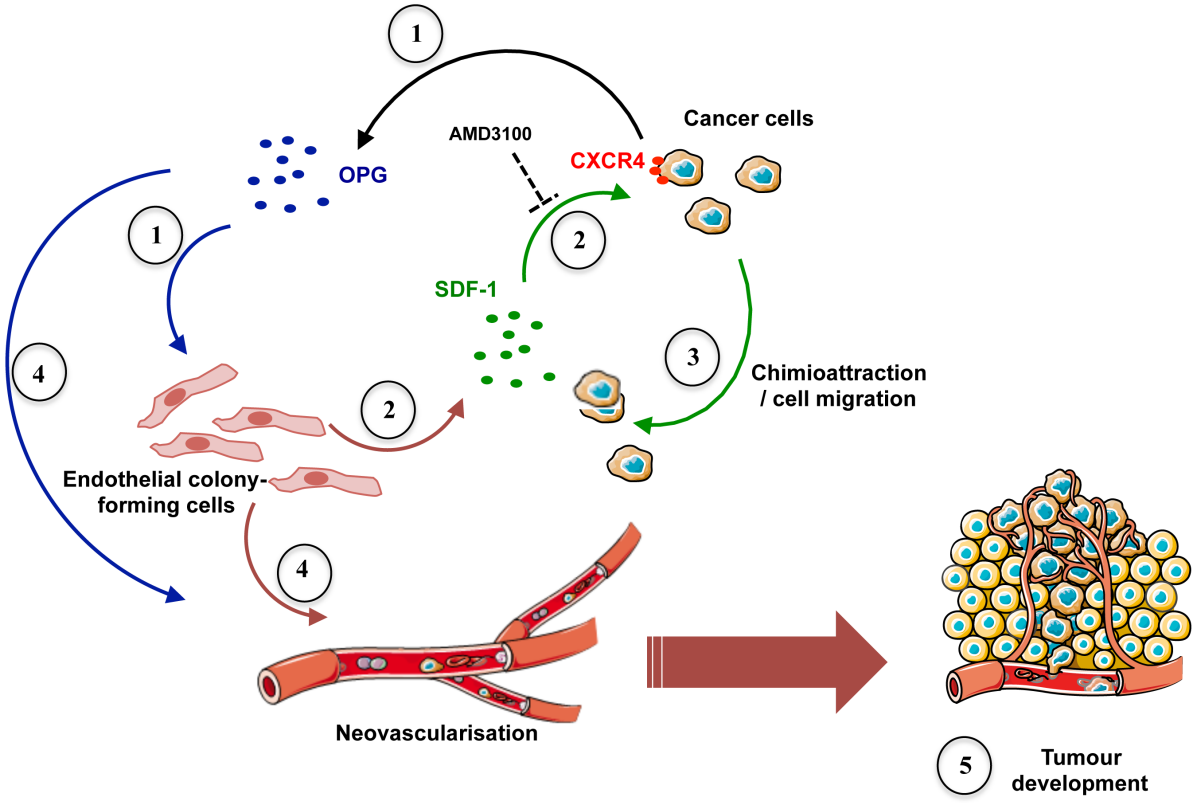
CTRL
OPG
FGF-2
OPG/FGF-2


1
Cancer cells
AMD3100
CXCR4
OPG
1
2
SDF-1
4
3
Chimioattraction
/ cell migration
2
Endothelial colony-
forming cells
4
Neovascularisation
5
Tumour
development