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Osteoprotegerin regulates cancer cell migration through SDF-1/CXCR4 axis and promotes tumour development by increasing neovascularization

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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 one of the key factor influencing the tumour growth and cancer cell dissemination, we investigated whether OPG was able to modulate the invasion of human MNNG-HOS osteosarcoma and DU145 prostate cancer cell lines *in vitro* and *in vivo*. Cell motility was analysed *in vitro* by using Boyden chambers. Human GFP-labelled MMNG-HOS cells were inoculated in immunodeficient mice and the tumour nodules formed were then injected with OPG and/or FGF-2, AMD3100 or 0.9% NaCl (control group). Tumour growth was manually followed and angiogenesis was assessed by immunohistochemistry. *In vitro*, SDF-1 released by OPG-pretreated ECFCs markedly attracted both MNNG-HOS and DU145 cells and 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. Interestingly AMD3100, an inhibitor of SDF-1, prevented the *in vivo* effects of OPG induced by SDF-1 This study provides experimental evidence that OPG promotes tumour development trough SDF-1/CXCR4 axis.

Highlights

- OPG promotes tumour development

- OPG is actively involved in tumour revascularization

- SDF-1 released by OPG-pretreated endothelial colony-forming cells chemoattract cancer cells

- SDF-1 induces spontaneous migration of osteosarcoma and carcinoma cell lines

- OPG promotes tumour development trough SDF-1/CXCR4 axis

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

1. INTRODUCTION

Osteoprotegerin (OPG) is a member of the tumour necrosis factor (TNF) receptor superfamily which is involved in the regulation of bone remodelling, where it acts as a decoy receptor for nuclear factor-κB ligand (RANKL) [1]. OPG is considered as an ubiquitous factor which is then expressed in a variety of tissues and organs such as bone, heart, lung, liver, placenta, vessels and interacts with numerous cell types belonging to normal or cancerous tissues [2]. By its blocking activity of RANKL induced osteoclastogenesis, pre-clinical and clinical studies have investigated the therapeutic potential of recombinant OPG in osteolysis associated with cancer [1]. Preclinical studies demonstrated that the administration of recombinant OPG inhibited osteolysis associated with breast cancer metastasis or multiple myeloma and reduced cancer cell migration *in vivo* [3]. 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 [4]. OPG could be considered as a survival factor for tumour cells (reviewed in [5]). Hence, OPG has been described to be a survival factor of several types of cell tumour including osteosarcoma and prostate cancer cells [6].

Osteosarcoma, the most common primary malignant bone tumour, is defined as a malignant tumour with mesenchymal origin producing a malignant osteoid matrix [7]. Some are composed of largely fibroblastic cells, some show chondroid differentiation, and still others are highly vascular [8]. Whether the production of OPG by human osteosarcoma has been previously reported [9-11], carcinoma cells (e.g. prostate cancer) secrete also OPG [5] with a higher level in metastatic foci than in primary tumours [12]. Accordingly, serum OPG levels in prostate carcinoma patients have been positively correlated with the progression of the disease and the establishment of bone metastases [13]. These results strengthen strongly the potential involvement of OPG in cancer progression.

Angiogenesis is a key 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 [14]. Hence, tumour angiogenesis has been extensively investigated in solid and haematological tumours, as well as in premalignant conditions, and numerous publications describing the link between tumour angiogenesis, metastasis, and overall survival are already available [reviewed in 15]. OPG should 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 [16]. We previously demonstrated that OPG expressed by endothelial progenitor cells (EPCs) stimulated the migration of endothelial cells, then exerted a chemoattraction activity on these cells and stimulated the vascular cord formation *in vitro and in vitro* as shown in Matrigel plug assay [17]. OPG-mediated angiogenic activities were mediated by a MAPK, Akt and mTOR signaling cascade. OPG-induced proangiogenic activities were significantly blocked by preincubation with the CXCR4 antagonist AMD-3100 and by prior heparan sulphate proteoglycan disruption, demonstrating that OPG activities on EPCs were partly mediated by syndecan-1 and SDF-1/CXCR4 pathway [18]. Furthermore, OPG markedly enhanced functional properties of EPCs and these effects were correlated with overexpression and secretion of the chemokine stromal cell-derived factor-1 (SDF-1), a key player in the attraction of tumour cells [17]. Overall, these observations suggest a modulatory role of OPG in tumour revascularization. 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 [19,20].

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 [21]. A growing body of evidences indicates that neovascularization processes associated with tumour growth are partly supported by the recruitment of endogenous EPCs as well as their functional incorporation into new vasculatures and their paracrine effects [reviewed in 22].

The aim of the present study was to determine whether SDF-1 released by endothelial colony-forming cells (ECFCs; a sub-population of EPCs) after OPG treatment can modulate human osteosarcoma MNNG/HOS and human prostate cancer DU145 cell lines, migration and chemotaxis *in vitro*. In addition, using a nude mouse model of human osteosarcoma xenografts, the effect of OPG on tumour growth and vascularization *in vivo* was investigated.

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, NaCl 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 30 min 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 [23]. 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 25 ng/mL of OPG at 37°C for 48 hours. 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 analysed in 24-multiwell Boyden microchemotaxis chambers with 8 µm pore-size polyvinylpyrrolidone free polycarbonate Nucleopore filters (Costar, France). MNNG-HOS and DU145 were seeded in the upper chambers in their respective culture media (1.5×10^4 cells/chamber) to allow the cell migration towards the control media (DMEM, 5% FCS), control media with 100 ng/mL of SDF-1 (positive control), OPG (25 ng/mL) or supernatants of OPG pretreated ECFCs placed in the lower chambers. When required, before seeding, MNNG-HOS and DU145 were pretreated with AMD3100 (10 µg/mL) for 30 min. Chemoattraction was analysed after 4 hours 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 manual counting under a high-power microscope.

2.5. Migration assay

As for chemotaxis assay, migration was examined in 24-multiwell Boyden microchemotaxis chambers. MNNG-HOS and DU145 were pretreated with the control media with or without 100 ng/mL of SDF-1 (positive control), 25 ng/mL of OPG or with supernatants of OPG

pretreated ECFCs. When required, AMD3100 (10 µg/mL) was added 30 min before MNNG-HOS and DU145 treatment. After 24 hours, 1.5 x 10^4 control and pretreated-cells in suspension in the control media were placed in the upper chambers to follow the cell migration towards the same media placed in the lower chambers. Migration was allowed to proceed for 4 hours at 37°C, 5% CO_2. The number of migrated cells was determined as previously described 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. 4.5 x 10^6 MNNG-HOS cells were injected subcutaneously in eight-week-old athymic nude mice (Elevage Janvier, France). Three to five days following injection, when tumours had reached approximately 3-4 mm^3 in size, 100 µl of either 0.9% NaCl (negative control), mouse OPG (2 µg/kg), mouse FGF-2 (1.4 µg/kg) in the presence of absence of mouse OPG (2 µg/kg) were administered by direct intra-tumour injection (7 mice per group). This procedure was repeated twice a week for 3 weeks. To block the effects of OPG mediated by secreted SDF-1, the selective CXCR4 antagonist AMD3100 (0.5 mg/kg) or saline vehicle

(0.9% NaCl) was injected 30 minutes before OPG injection, followed by one intraperitoneal administration of AMD3100 (5 mg/kg) 8 hours later. Tumour volume was measured three times 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 primary antibody. When required, a secondary antibody coupled with Alexa Fluor® 555 or Alexa Fluor® 488 fluorochrome was used (Abcam, France). Rat Anti-CD31 monoclonal antibody (clone MEC 13.3), FITC-rabbit monoclonal anti-SMA antibody (clone 1A4) and polyclonal rabbit anti-von Willebrand factor antibody were purchased from BD Biosciences, Sigma (France) and Dako (France) respectively. 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

We first measured the SDF-1 secretion by EFCFs cells in the presence or absence of OPG (Figure 1A). SDF-1 was basic produced spontaneously by ECFCs and OPG significantly upregulated the secretion of this soluble factor (Figure 1A). We then investigated the potential effect of OPG and conditioned media of OPG-pretreated ECFCs on MNNG-HOS cells chemotaxis and migration, to determine whether OPG might be involved in tumour cell dissemination. As shown in Figure 1B, osteosarcoma cells were attracted by 100 ng/mL of SDF-1 or 25 ng/mL of OPG ($p<0.05$ and $p<0.01$ respectively). Interestingly, MNNG-HOS cells were also markedly attracted by the conditioned media of OPG-pretreated ECFCs ($p<0.001$). In addition, OPG stimulated significantly MNNG-HOS cells chemotaxis in the same manner as SDF-1α ($p<0.01$). By using AMD3100, a specific antagonist of CXCR, we found that the effect exerted by conditioned media of OPG-pretreated ECFCs was partly due to the SDF-1 released by these cells under OPG treatment ($p<0.01$) As shown in Figure 1C, SDF-1 alone as well as 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 ($p<0.05$ and $p<0.01$ respectively).

Expression of the CXCR4 by prostate cancer DU145 cell line has been reported by several studies (expression confirmed by RT-qPCR in the present study, data not shown). Hence, the SDF-1/CXCR4 axis has been described as playing a key role metastasis of prostate cancer to bone [24]. 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 [25]. To determine whether the observed effects were specific to osteosarcoma cells, similar investigations were carried out on DU45 prostate carcinoma cells (Figure 2). As shown in

Figure 2, conditioned media of ECFCs pretreated or not with OPG attracted DU45 carcinoma cells. The supernatant of OPG-pretreated ECFCs induced a more potent attraction of DU45 than SDF-1 alone ($p<0.001$). This activity was 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 did not affect their spontaneous migration (Data not shown).

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

As described above, OPG showed greater effect on the MNNG-HOS cell motility *in vitro*. We further evaluate its effect *in vivo* in human osteosarcoma tumours developed in nude mice. The MNNG-HOS xenograft showed an appreciable growth starting from the fifth day after cell injection and doubled its volume after 3 days in all mice inoculated (Figure 3). To determine the effect of OPG on tumour growth *in vivo*, intra-tumour administration of 0.9% NaCl (control), 2 µg/kg of mouse OPG, or 1.4 µg/kg of mouse FGF-2 (positive control) was performed twice a week for 3 weeks, when the tumours reached a volume of approximately 3 to 4 mm^3 (Figure 3A). As shown in Figure 3B, intra-tumour administration induced a significant increase of subcutaneous tumour growth from 6 days post cell inoculation ($p<0.01$, 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 1.4 µg/kg of murine FGF-2 alone or combined with 2 µg/kg of OPG. Analysis of the tumour growth showed enhanced tumour volume in OPG/FGF-2 treated mice ($p<0.05$ Figure 3C) compared to OPG alone ($p<0.01$ Figure 3C). No sign of distress or loss of weight in mice was evidenced.

Blood vessels are necessary to tumour growth by providing nutrients and oxygen. Double-labelled fluorescent immunohistochemistry was undertaken to first determine whether CD31 staining correlated with endothelial cell positive labelling. As shown in Figure 4A-C, von Willebrand Factor and CD31 colocalization was detected in endothelial cells in tumour blood vessels. Similarly, a-SMA (smooth muscle) and CD31 (endothelial cells) were colocalized in large tumoural blood vessels of tumour plugs (Figure 4D-F).

To evaluate the implication of OPG 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. The effects observed in Figure 3 were correlated with an increase of tumour vascularisation. As shown in Figure 5A,B, 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 ($p<0.05$) and OPG group ($p<0.05$). Furthermore, mice treated with OPG showed enhanced vascularization compared to control mice (Figure 5B, $p<0.05$).

3.3. OPG pro-angiogenic activity associated to the tumour growth is dependent of SDF-1/CXCR4 pathway

To determine the role of SDF-1/CXCR4 axis in the OPG-induced tumour growth, 100 µl of either 0.9% NaCl (negative control), mouse OPG (2 µg/kg) with or without pre-injection of the AMD3100 antagonist for CXCR4 (0.5 mg/kg), were administered into the tumour tissues. Similarly to 0.9% NaCl, AMD3100 did not modulate the tumour growth when administered alone (Figure 6). As expected OPG increased significantly the tumour volume and interestingly AMD3100 abolished the OPG-increase tumour growth demonstrating that OPG-dependent tumour growth was mediated by SDF-1/CXCR4 axis.

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 [17, 18, 27]. Thereby, there is growing evidence that it underlies a potential link between the osseous and vascular systems [27, 28]. 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 [29, 30]). 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[5].

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 MNNG-HOS cells *in vitro*. Media conditioned by OPG pretreated ECFCs attracted MNNG-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 MNNG-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 MNNG-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 [31]). Molecular pathways contributing to osteosarcoma development and progression have recently been described and the role of several cytokines and chemokines was detailed (reviewed in [32]). 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 [33]. A higher CXCR4 expression in metastasis compared with primary osteosarcoma was also reported [34]. In an analysis of Ewing sarcoma, another bone cancer, and in chondrosarcoma of bone, CXCR4 correlated with metastasis [35]. 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) could be the major source of chemokine expression in osteosarcoma [36]. ECFCs are found to be recruited into tumour environment as they are actively involved in tumour vascularization [17, 18]. OPG, previously described to be released in osteosarcoma [9], 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 Reiko

et al who showed that strong expression of OPG in cancer tissue was closely associated with deep invasion, nodal metastasis, advanced stage and poor prognosis [37].

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 [38]. OPG has been shown to promote both angiogenesis [39, 40] and vasculogenesis since it mediates the mobilization and differentiation of ECFC [17, 18]. 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 and are mainly focused its role on osteoclastogenesis inhibition and related impact on tumour cell survival [11, 37, 41, 42]. To our knowledge, none of these studies have considered the possible involvement of OPG in tumour angiogenesis. In the present *in vivo* study, using a xenograft model of osteosarcoma in nude mice, 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* [17, 18], induces xenograft growth and angiogenesis through a SDF-1/CXCR4 axis.

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 needs associated with tumour growth are satisfied through establishment and expansion of new blood vessels. 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 reach a size of 3 to 4 mm^3, approximately. Thereafter, tumours size doubled in then next 3 days. This observation could be related 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 groups. 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. Figure 7 summarizes the role of SDF-1 in the pro-tumoural activity of OPG. SDF-1 release by EPC located in the surrounded tissue is increased by the OPG secreted by cancer cells (1). It binds to CXCR4 expressed by cancer cells and allows these cells to attract cancer cells (3). Locally released OPG enhances the formation of blood vessels (4) which increases the intra-tumour vasculature and then the tumour development (5).

In summary, our findings demonstrate that OPG contributes to the tumour growth and invasion by promoting tumour angiogenesis though SDF-1/CXCR4 axis in addition to its inhibitory effect of tumour cell apoptosis [43]. The effects of OPG on tumour neovascularization include increased chemotaxis of ECFCs [17, 18]. 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 (25 ng/mL) for 48h. **(B)** SDF-1 strongly attracts MNNG-HOS cells: before the migration assay, MNNG-HOS cells were pretreated or not with AMD3100 (10 μg/mL) for 30 min, placed in the upper Boyden chambers in RPMI, 5% FCS to allow migration towards the control media (RPMI, 5% FCS), control media with 100 ng/mL of SDF-1 (positive control), OPG (25 ng/mL) or supernatants of OPG-pretreated (or untreated) ECFCs placed in the lower chambers for 4h. MNNG-HOS cells were attracted by the conditioned media of OPG-pretreated ECFCs ($P<0.001$) with a more efficiency SDF-1 ($P<0.01$). This effect was strongly reduced, by 50%, after MNNG-HOS treatment with AMD3100 ($P <0.01$). 25 ng/mL of OPG stimulated MNNG-HOS cell chemotaxis ($P <0.01$). **(C)** SDF-1 released in the supernatants of OPG-pretreated ECFCs induces MNNG-HOS cell migration: MNNG-HOS cells were pretreated with RPMI, 5% FCS with or without 100 ng/mL of SDF-1 (positive control), 25 ng/mL of OPG or with supernatants of OPG-pretreated ECFCs. When required, AMD3100 (10 μg/mL) was added 30 min before MNNG-HOS treatment. 24h later, control and pretreated- cells in suspension in RPMI, 5% FCS were placed in the upper Boyden chambers to allow migration towards the same media placed in the lower chambers for 4h. SDF-1 alone ($P <0.05$) and SDF-1 released by ECFCs under OPG treatment ($P <0.01$) induces MNNG-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. Mean ± SEM, n=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 2: SDF-1 released in the supernatants of OPG pretreated ECFCs modulates the motility of prostate cancer cells. Before the migration assay, DU145 prostate carcinoma cells were pretreated or not with AMD3100 (10 µg/mL) for 30 min, placed in the upper Boyden chambers in DMEM, 5% FCS to allow cell migration towards 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 placed in the lower chambers 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. MNNG-HOS cells were injected subcutaneously into mice. When tumours had reached approximately 3-4 mm^3 in size, 100 µl of either 0.9% NaCl (negative control), mouse OPG (2 µg/Kg), mouse FGF-2 (1.4 µg/Kg) alone or supplemented with mouse OPG (2 µg/Kg) were administered by direct intra-tumour injection. This procedure was repeated twice a week for 3 weeks. Tumour volume was measured thrice per week and each volume (V) was calculated according to the following formula: $V=a\times b^2/2$ (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 (2 µg/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 (2 μg/kg), FGF-2 (1.4 μg/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 : **Co-localisation of von Willebrand factor (vWF) with CD31 and α-SMA in the endothelial cells of neoformed tumour blood vessels.** Immunohistochemistry of VWF, CD31 and α-SMA coupled to confocal microscopy analysis were performed on 10 μm cutting temperature coumpound frozen sections of tumours from control and treated-mice. (A) anti-CD31, (**B**) vWF, (**C**) merged picture. (D-F) Immunohistological staining of α-SMA (FITC) and CD31 expression in the endothelial cells of tumoural blood vessels. (D) anti-CD31, (E) α-SMA, (F) merged picture. Note co-localisation of CD31 and a-SMA in endothelium of tumoural blood vessels, indicating high specificity of CD31 antibody for blood vessels. Note the co-localisation of CD31 with vWF and α-SMA in endothelium of tumour blood vessels, indicating high specificity of CD31 antibody for blood vessels.

Figure 5: OPG increases tumour vascularization. 10 μm 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 MNNG-HOS tumours from Control, 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 of tumour angiogenesis in OPG/FGF-2 group ($p<0.001$) followed by the FGF-2

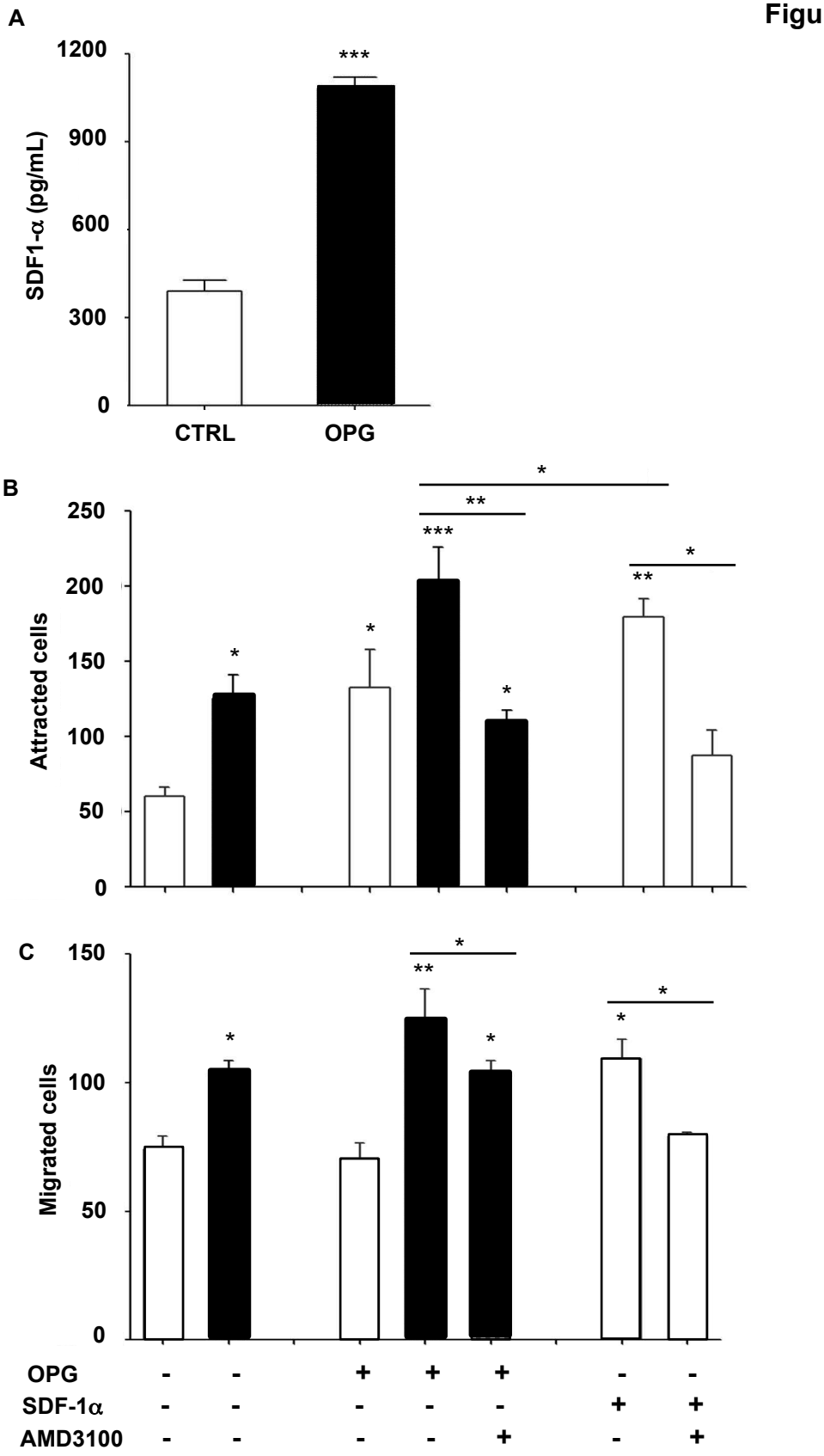
($p<0.05$) and the OPG ($p=0.059$) ones. **(C)** The Number of vessels per mm^2 confirmed the results of the vessel surface analysis. Mean ± SEM, n=4, *p<0.05, **p<0.01, ***p<0.001.

Figure 6: OPG promotes tumour development trough SDF-1/CXCR4 axis. MNNG-HOS cells were injected subcutaneously into mice. When tumours had reached approximately 3-4 mm^3 in size, 100 µl of either 0.9% NaCl (negative control), mouse OPG (2 µg/kg) with or without pre-injection of AMD3100 (0.5 mg/kg) the antagonist for CXCR4 (30 min before OPG administration) followed by one intraperitoneal administration of AMD (5 mg/kg) 8 hours later, were administered directly into intra-tumour masses. This procedure was repeated twice a week during 3 weeks. Tumour volume was measured three times per week and each volume (V) was calculated according to the following formula: V=a×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). Treatment of animals with AMD3100 abolished the effect of OPG on tumor growth, demonstrating that SDF-1/CXCR4 signaling is necessary for OPG-promoted tumor growth. Mean ± SEM, n = 7, *p<0.05, **p<0.01, ***p<0.001.

Figure 7: 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**). Released SDF-1 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**) which increases the intra-tumour vasculature and then the tumour development (**5**).

Figure 1


A
1200
900
600
300
0
SDF1-α (pg/mL)
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CTRL
OPG
B
250
200
150
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50
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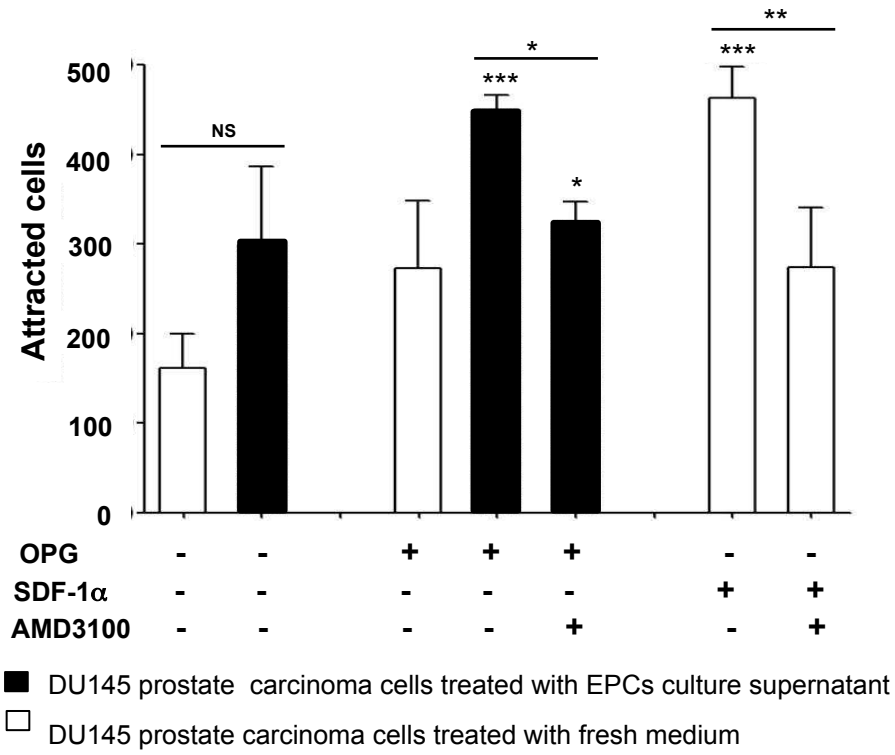
Attracted cells
C
150
100
50
0
Migrated cells
OPG - - + + + - -
SDF-1α - - - - - + +
AMD3100 - - - - + - +
MNNG-HOS osteosarcoma cells treated with EPCs culture supernatant
MNNG-HOS osteosarcoma cells treated with fresh medium

Figure 2


Attracted cells
500
400
300
200
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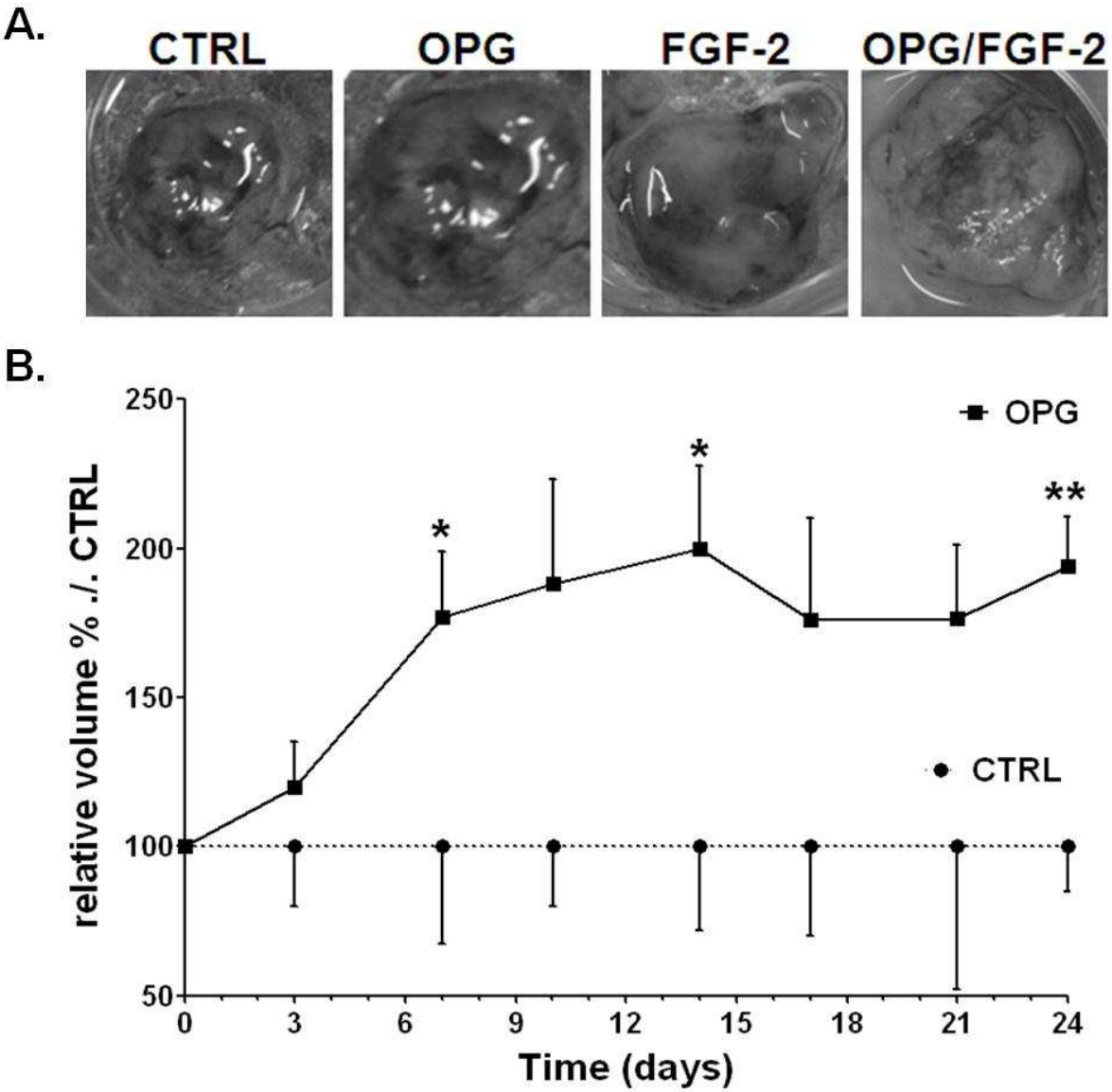
OPG - - + + + - -
SDF-1α - - - - - + +
AMD3100 - - - - + - +
DU145 prostate carcinoma cells treated with EPCs culture supernatant
DU145 prostate carcinoma cells treated with fresh medium


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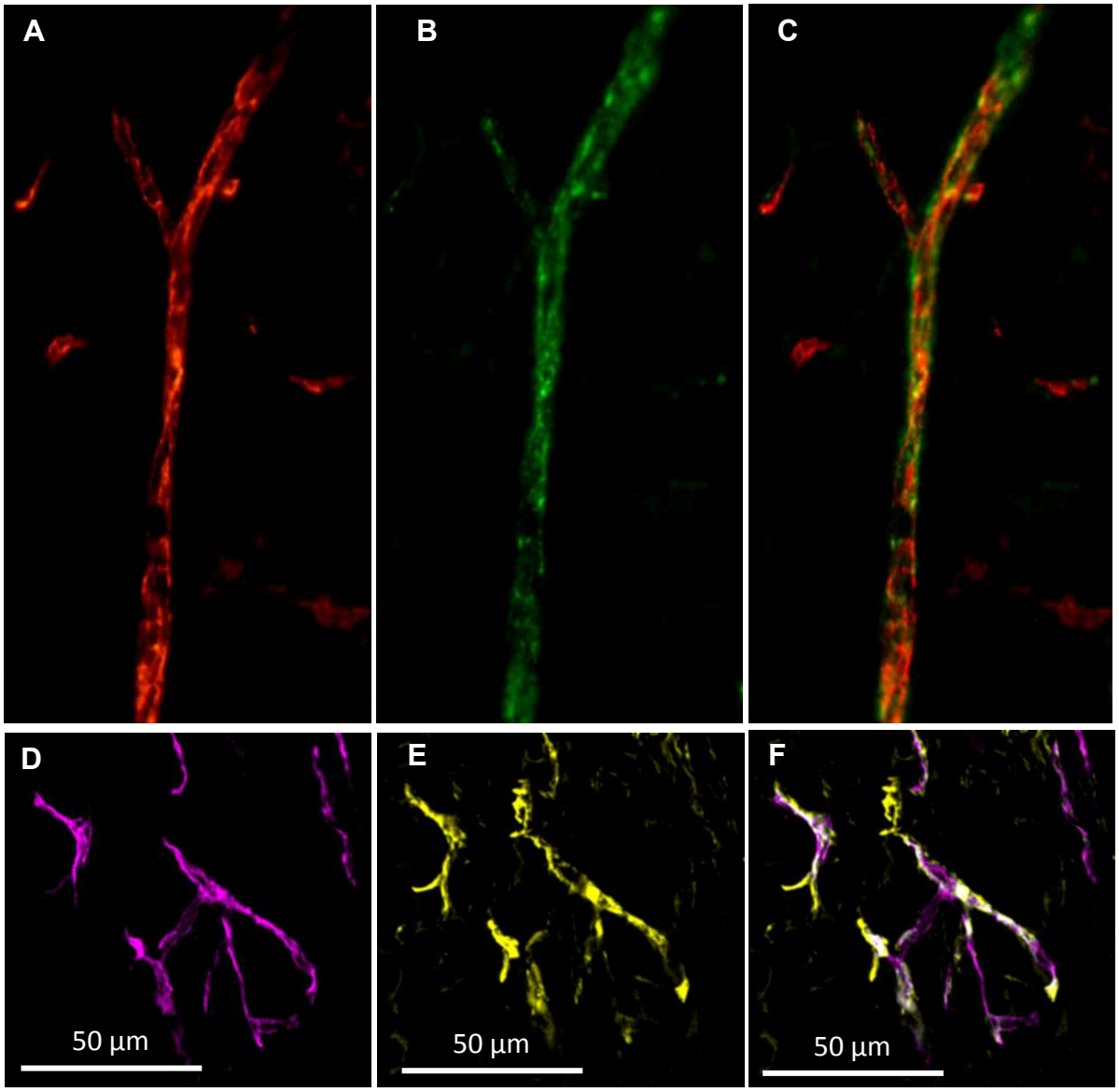
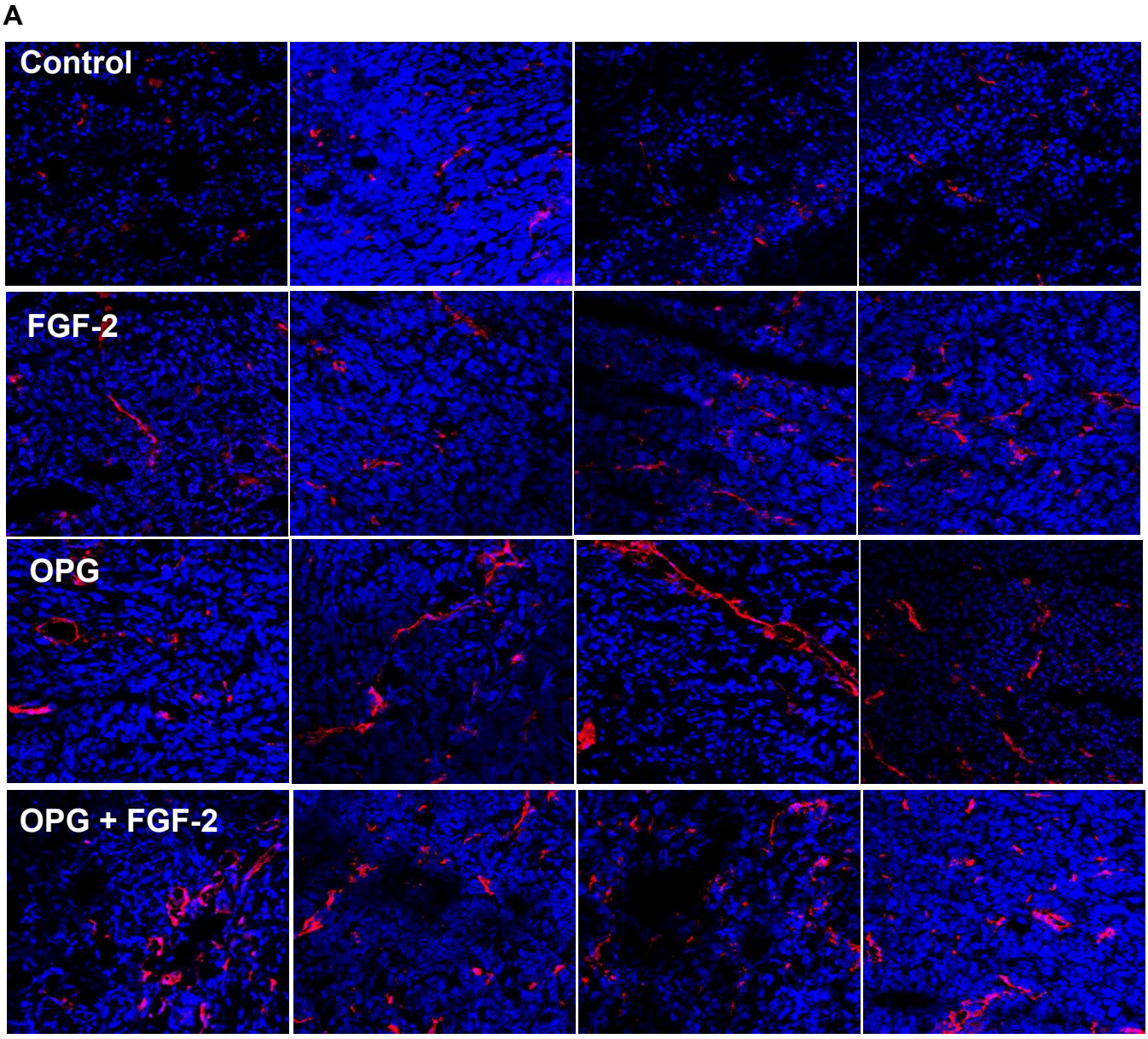
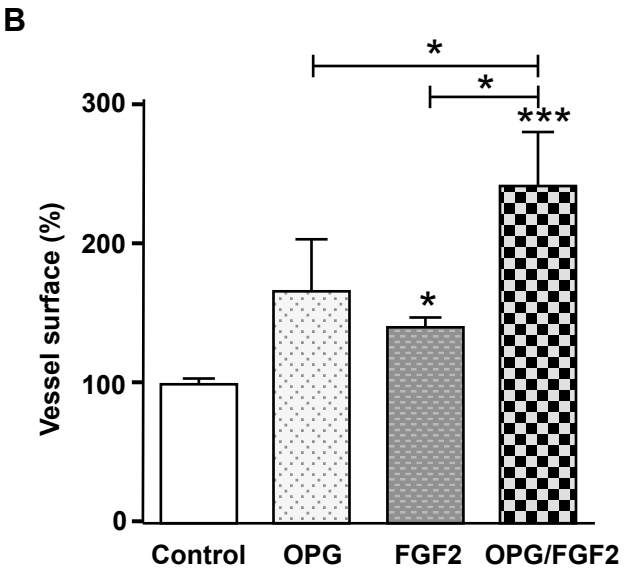
relative volume % ./. CTRL
Time (days)
C.
FGF-2
OPG
OPG/FGF
relative volume (Vx/V1)
Time (days)
* OPG/FGF-2 VS OPG, # OPG/FGF-2 VS FGF-2

Figure 4




A
Control
FGF-2
OPG
OPG + FGF-2


B
Vessel surface (%)
300
200
100
0
*
*
***
*
Control
OPG
FGF2
OPG/FGF2

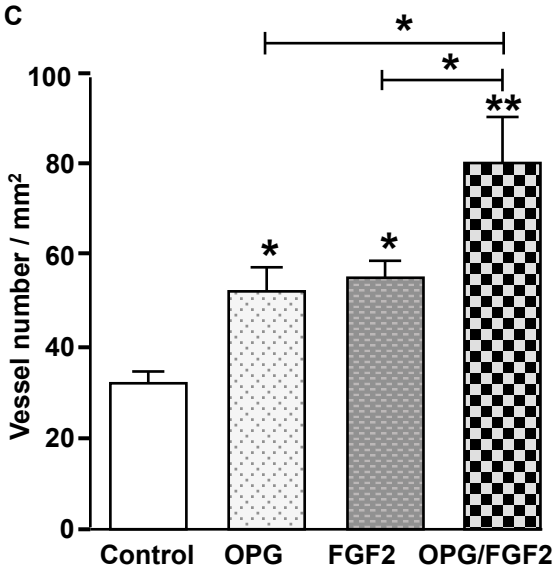
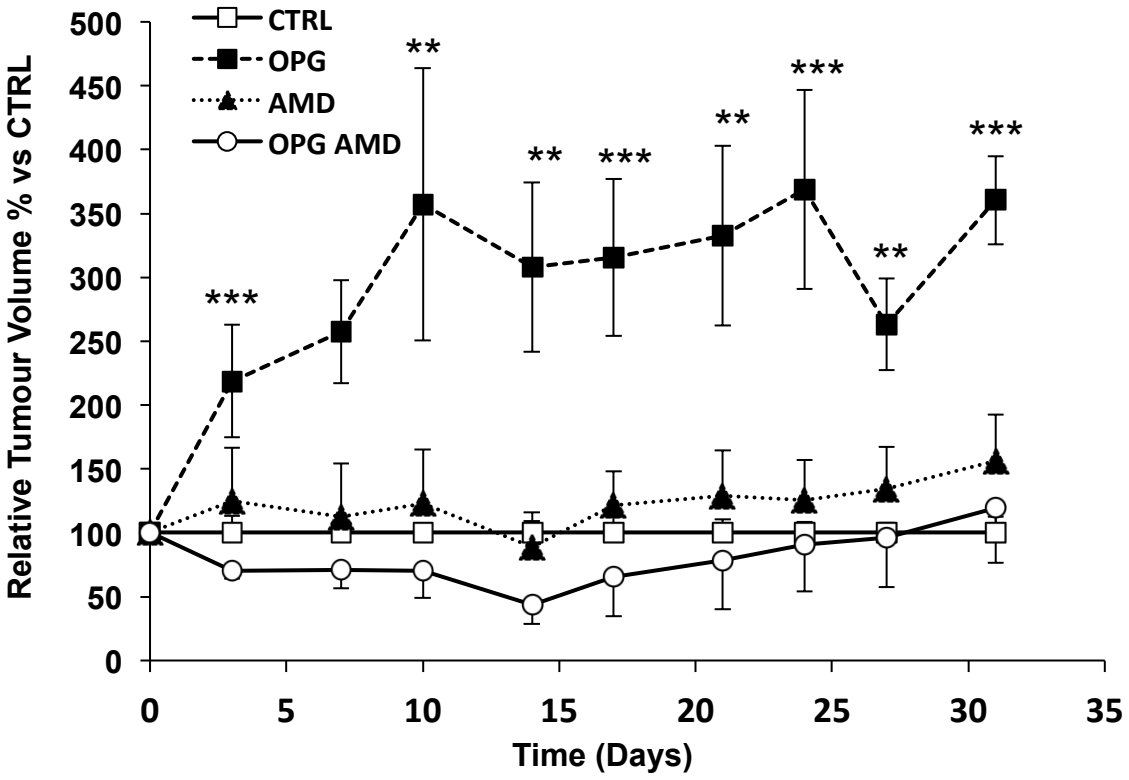

C
Vessel number / mm²
100
80
60
40
20
0
*
*
**
*
*
Control
OPG
FGF2
OPG/FGF2

Figure 6


CTRL
OPG
AMD
OPG AMD
Relative Tumour Volume % vs CTRL
Time (Days)
0
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100
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200
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400
450
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5
10
15
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35
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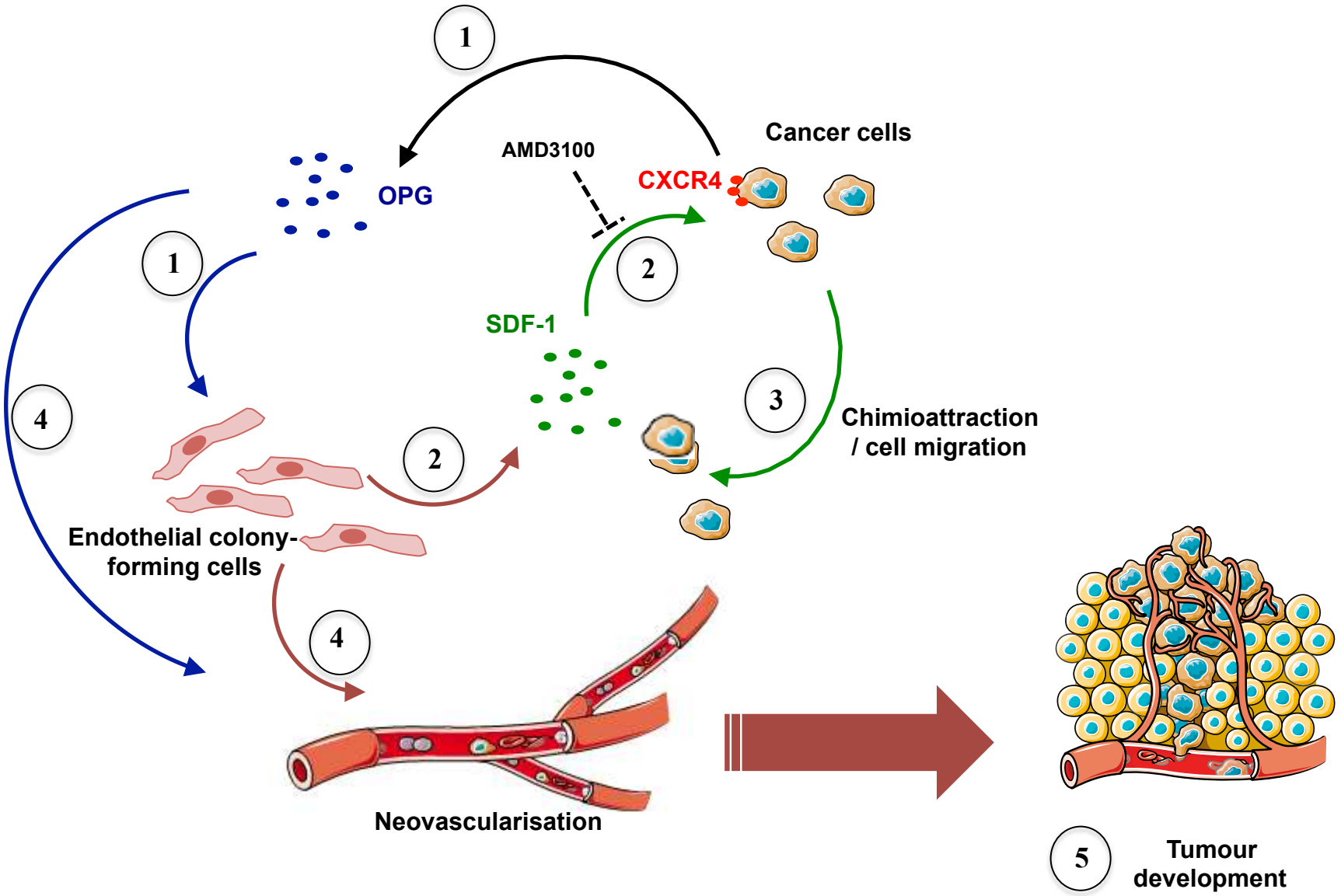
Figure 7


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