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# Osteopontin promotes gastric cancer metastasis by augmenting cell survival and invasion through Akt-mediated HIF-1α up-regulation and MMP9 activation

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## Abstract

Osteopontin (OPN) is a secreted, integrin-binding matrix phosphorylated glycoprotein. OPN has been shown to facilitate the progression and metastasis of malignancies and has prognostic value in several types of cancer, including gastric cancer. However, the functional mechanism of OPN mediated metastatic growth in gastric cancer remains unclear. Here, using multiple *in vitro* and *in vivo* models, we report that OPN strongly promoted the progression and metastasis of gastric cancer. Immunohistochemical staining revealed that OPN, matrix metalloproteinase (MMP)9 and hypoxia-inducible factor (HIF)-1α have statistically significant different expression patterns between well- and poorly differentiated tissue samples ($P < 0.05$). Correlations existed between OPN and MMP9, and between OPN and HIF-1α ($r_1 = 0.872$, $p_1 < 0.01$ and $r_2 = 0.878$, $p2 < 0.01$). Furthermore, OPN dramatically increased colony formation and invasion of gastric cancer cells *in vitro* and promoted tumour growth and metastasis *in vivo*. In addition, OPN potently protected gastric cancer cells from serum depletion-induced apoptosis. Further study shows that OPN activated phosphoinositide 3-kinase/Akt survival pathway and up-regulated HIF-1α *via* binding to $\alpha_v\beta_3$ integrins in gastric cancer cells. Moreover, we found that OPN could activate MMP9 and up-regulate MMP2. Taken together, our results suggest that the survival-promoting function is crucial for OPN to promote the development of gastric cancer, and HIF-1α and MMP9 may play key roles during this process. Thus, targeting OPN and its related signalling network may develop an effective therapeutic approach for the management of gastric cancer.

**Keywords:** Akt • gastric cancer • HIF-1α • MMP9 • osteopontin (OPN) • survival

## Introduction

Osteopontin (OPN), also known as early T-cell activation-1, was originally discovered as an inducible marker of transformation of epithelial cells. It is a secreted, integrin-binding matrix phosphorylated glycoprotein involved in a number of cell functions including cell adhesion and migration, anti-apoptosis, inflammation, angiogenesis, tissue remodelling and tumour development (see reviews [1–5]). Recent findings showed that OPN expression correlates with tumour progression in breast [6], lung [7], prostate [8], liver [9], stomach [10], colon [11], cervices [12], ovary [13], and that OPN concentration in the plasma of patients with metastatic disease is significantly higher than that in normal serum [14]. Studies have indicated that OPN has multifunctional properties in promoting cell survival, cell adhesion, and cell migration through its interaction with the integrins and CD44 receptors [4, 5]. The C-terminal fragment of OPN binds directly to variant isofroms of CD44 [15], which could interact with matrix metalloproteinase (MMPs). MMPs are extracellular matrix degrading enzymes that play a crucial role in embryogenesis, tissue remodelling, inflammation, tumour growth, angiogenesis and metastasis.

OPN binds to integrins and induces various cellular signalling events. Multiple signalling molecules are activated by OPN which may contribute to tumour progression and metastatic behaviour. For example, the phosphorylation and activation of various kinases, such as phosphoinositide 3-kinase (PI3-K), nuclear factor inducing kinase, phospholipase C, protein kinase C, mitogen-activated

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protein kinase (MAPK) [3], induce the DNA binding and transactivation potential of various transcription factors, including nuclear factor-κB and activator protein-1 [16]. Thus, OPN contributes to malignancies through both inhibition of apoptosis and activation of various matrix-degrading proteases such as urokinase plasminogen activator and MMPs [17], leading to tumour cell motility, and metastatic growth. PI3-K is a central regulator in OPN-induce survival, cell motility and invasion. It has been documented that OPN protects cells from apoptosis by activating PI3-K/Akt pathway in BA/F3 murine pro-B-cell lines [18]. OPN induces PI3-K activity and PI3-K-dependent Akt phosphorylation through the $\alpha_v\beta_3$ integrin-mediated pathway in breast cancer cells [19].

During the process of tumour development, cancer cells have to overcome a number of stresses [20]. Hypoxia is an inevitable stress that tumour cells have to face during the development of solid tumour. HIF-1α is induced by exposure of cells to hypoxia or growth factors [21]. Because HIF-1α undergoes rapid ubiquitination and degradation by proteasomes, it is maintained at a low level in normoxic cells. On the contrary, under hypoxia or treatment with iron chelators, the praline hydroxylases keep inactivated, so the HIF-1α activity is stable [22]. Studies have shown that activation of some oncogenes increases HIF-1α activity through activating PI3-K/Akt, MAPK or other pathways [23, 24]. Although PI3-K/Akt and HIF pathways share many common features, such as promoting cell survival, angiogenesis and tumour malignance and metastasis, how PI3-K/Akt regulates HIF activity remains controversial [25, 26].

Gastric cancer is one of the most common malignancies around the world [27]. In China, it remains the most frequently diagnosed cancer and the second-most common cancer-related cause of death with a high case fatality [27]. As to gastric cancer treatment, the combination of chemotherapeutic agents is the major strategy. However, these agents at clinically effective doses are often accompanied by severe toxicity. This lack of specificity has stimulated the development of a new strategy that targets the molecular signalling pathways in cancer cells, and thus, tends to be less toxic to normal cells than conventional chemotherapies. Recent findings showed that OPN overexpression contributed to the development of gastric cancer. Two studies using DNA microarrays identified OPN as an overexpressed gene in gastric cancer tissues and in highly metastatic gastric cancer cell lines [28, 29]. Other studies have reported that OPN expression at mRNA and protein level and its plasma concentration were all closely related to invasion and metastasis, indicating that OPN may have the potential to be developed into an effective prognostic factor for gastric cancer [10, 30, 31]. However, the functional significance and molecular mechanisms of OPN contributed to gastric carcinogenesis remain unknown.

Here, using multiple *in vitro* and *in vivo* models, we examined the roles of OPN expression in the progression of gastric cancer development, and revealed the cellular and molecular mechanisms by which OPN promotes gastric cancer development. Our study demonstrated that OPN increased the survival of gastric cancer cells through PI3-K/Akt pathway activation and HIF-1α up-regulation *via* binding to $\alpha_v\beta_3$ integrins. Furthermore, we found OPN could activate MMP9 and up-regulate MMP2 to promote metastatic growth in gastric cancer.

# Materials and methods

## Materials

Human recombinant OPN protein and rabbit anti-phospho-Akt (Ser 473) antibody were purchased from R&D System (Abingdon, UK). Akt specific inhibitor (1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate), and the PI3-K inhibitor LY2940002 were purchased from Calbiochem (San Diego, CA, USA). Soft agar was purchased from Merck (Darmstadt, Germany), Mouse anti-Flag, anti-Akt, anti-β-actin and Goat anti-OPN were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse anti-HIF-1α and anti-rabbit IgG, antimouse IgG and anti-goat IgG antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Surgical specimens of gastric cancer and normal tissues were selected randomly from Division of Tumor Pathology, Zhongshan Hospital (Xiamen, China). Tissue procurement was approved by the institutional review board of Xiamen University. *In vivo* animal experiments with female Balb/c nude mice were carried out in the Cancer Research Center, Xiamen University Medical College, China.

## Immunohistochemical staining

The tissue sections of the primary gastric cancers, including the well- and poorly differentiated gastric cancer tissues, and the matched non-cancer gastric tissues were fixed in 10% formaldehyde and embedded in paraffin. Sections were then cut and immunohistochemical staining was performed as described previously [31]. Briefly, Unmasking of antigens was performed by microwave heating of sections in citrate buffer (pH 6.0). Sections were incubated overnight at 4°C with the primary antibodies and were then incubated with the secondary antibodies for 30 min. at room temperature. After rinsing, the sections were incubated with DAB, counterstained with haematoxylin, dehydrated, and then mounted. As a negative control, the primary antibody was replaced with normal mouse IgG.

## Cell culture and generation of OPN-producing gastric cancer cells

The human gastric cancer cell line SGC7901 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences in Shanghai, and was cultured as previously described [32]. The full open reading frame of human OPN cDNA (a kind gift from Dr. Xiao-Fan Wang, Duke University Medical Center) was cloned into pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, CA, USA) with a Flag tag at C-terminus of OPN protein. Cell transfections were performed with FuGene 6 reagent (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's instruction. The OPN/pcDNA3.1 plasmid or the vector control was introduced into SGC7901 gastric cancer cells, and the stable cell clones were obtained by 200 μg/ml hygromycin B (Amresco, Solon, OH, USA) selection in the culture medium. Three OPN-expressing clones and control clone were chosen for the subsequent experiments.

## Cell survival assay

The cell viability was measured by 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) method as previously described [33]. The other method for cell survival assay was done as follows, the cell numbers were determined after trypan blue staining of viable cells in parallel plates. The experiment was repeated at least three times and triplicate assays were performed for each group and data were presented as mean ± S.D.

## Soft-agar growth

Cells ($1 \times 10^3$ cells/dish) were plated in 35-mm-diameter dishes with a top layer of 0.3% agar and a bottom layer of 0.6% agar in medium. A total of 0.3 ml of medium was supplemented every 3 days and the dishes were examined under microscope. After 2 weeks, the number of cell clusters (⩾50 cells per cluster) per dish was counted, and cell clusters were photographed. The results were quantified from three independent experiments. Colony formation efficiency was calculated as follows: number of clusters/number of plated cells × 100%. Assays were performed in triplicate for each group of cells and data were presented as mean ± S.D.

## Cell invasion assay

Cell invasion assay was performed with Matrigel™-coated invasion chamber according to the standard procedure [34]. Briefly, the cell suspension was added to the upper chamber of the Matrigel™ coated pre-hydrate polycarbonate membrane filter. The lower chamber was filled with fronectin-contained condition medium, which acted as chemoattractant. Cells were added to the upper chamber, and then incubated for 24 hrs. The non-invaded cells from the upper side of the filter were scraped and removed using moist cotton swab. The invaded cells in the reverse side of the filter were fixed with methanol and stained with haematoxylin and eosin. The invaded cells on the filter were counted under an inverted microscope. Data were presented as mean ± S.D. of three separate experiments to obtain the statistical results.

## Zymography analysis

The gelatinolytic activity was measured as described previously [35]. Briefly, to check the effect of OPN on MMP2 and MMP9 expression and activation, the equal OPN-expressing cells were incubated in serum-free medium or the equal parental cells were treated with 400 ng/ml OPN in serum-free medium, and all the cells were incubated for 24 hrs. The equal condition medium was colleted by centrifugation, and loaded onto zymographic SDS-PAGE containing gelatin (0.1% (w/v)). The gels were washed and incubated in incubation buffer for 48 hrs. The gels were stained with Coomassie Blue and destained. The zones of gelatinolytic activity were shown by negative staining.

## Western blot analysis

Western blot was performed as described before [33, 36]. After harvesting, the experimental cells were washed and lysed in lysis buffer. The total lysate was mixed with 4× loading buffer, and pre-heated at 95°C for 10 min. The samples were then loaded onto SDS-PAGE. The proteins were transferred onto a PVDF membrane by semi-dry transfer system (Bio-Rad, Hercules, CA, USA). The membrane was blocked in 5% milk, and then incubated 1 hr or overnight at 4°C with primary antibody. After hybridization with primary antibody, the membrane was washed and incubated with HRP-labelled secondary antibody. Final detection was performed with ECL™ (enhanced chemiluminescence) Western blotting reagents (Amersham, Pharmacia Biottech, San Francisco, CA, USA). The blot was then stripped at 50°C for 30 min. The membranes were re-blotted for detection of other proteins after extensive wash.

## Xenograft assays in nude mice

*In vivo* tumourigenesis experiments were conducted in nude mice. Two OPN-overexpressing clones (nos. O3 and O16), control vector and Parental cells were used. Eighteen female Balb/c nude mice, aged 8 weeks were randomly divided into three groups. Two groups were injected *via* subcutaneous injection with the equal cells of OPN-expressing SGC7901 and the control clone Vec/SGC7901 ($2 \times 10^6$/mice) in the left and the right flanks respectively. In separate experiments, the SGC7901 cells were pre-treated with 400 ng/ml OPN for 24 hrs, and injected into the left flanks. Meanwhile, the same number of untreated cells was injected into the right flanks as control. 400 ng/ml OPN was again injected into the tumour sites twice a week for up to 5 weeks.

In other experiments, OPN-expressing SGC7901 (nos. O3 and O16) ($6\times10^6$/mice) and the same number of the control Vec/SGC7901 were injected into nude mice by intraportal vein injection. Four nude mice were used in each set of experiments. All nude mice were killed 5 weeks after injection and examined for the growth of subcutaneous tumours and micrometastases formation.

# Results

## Correlations of OPN, MMP9 and HIF-1α expression in human gastric cancer tissues

To assess the correlation of OPN, MMP9 and HIF-1α expression in human primary gastric cancer, we examined a series of matched tissue sections, including well-differentiated gastric cancer, poorly differentiated gastric cancer, and the adjacent normal gastric tissues from the same patients by immunohistochemical analysis. The anti-OPN, anti-MMP9 and anti-HIF-1α antibodies were shown to specifically recognize the corresponding proteins (Fig. 1A–C, middle and right panels). The immunohistochemical staining was performed on 32 cases of primary gastric cancer tissues, including 20 cases of well differentiated, and 12 cases of poorly differentiated tumour tissues, as well as 32 cases of matched non-cancer gastric tissues. As shown in Fig. 1, the immunostaining analyses indicated that high levels of OPN, MMP9 and HIF-1α were present in areas containing cancer cells of the primary gastric tumours, and more intense protein staining was seen in the poorly differentiated tumour tissues (Fig. 1A–C, right panels). In contrast, OPN, MMP9 and HIF-1α were hardly detectable in all matched normal gastric tissues. Overall, in the total 32 cases of primary gastric cancer tissues include both well- and poorly differentiated samples, 21 (65.6%) cases showed

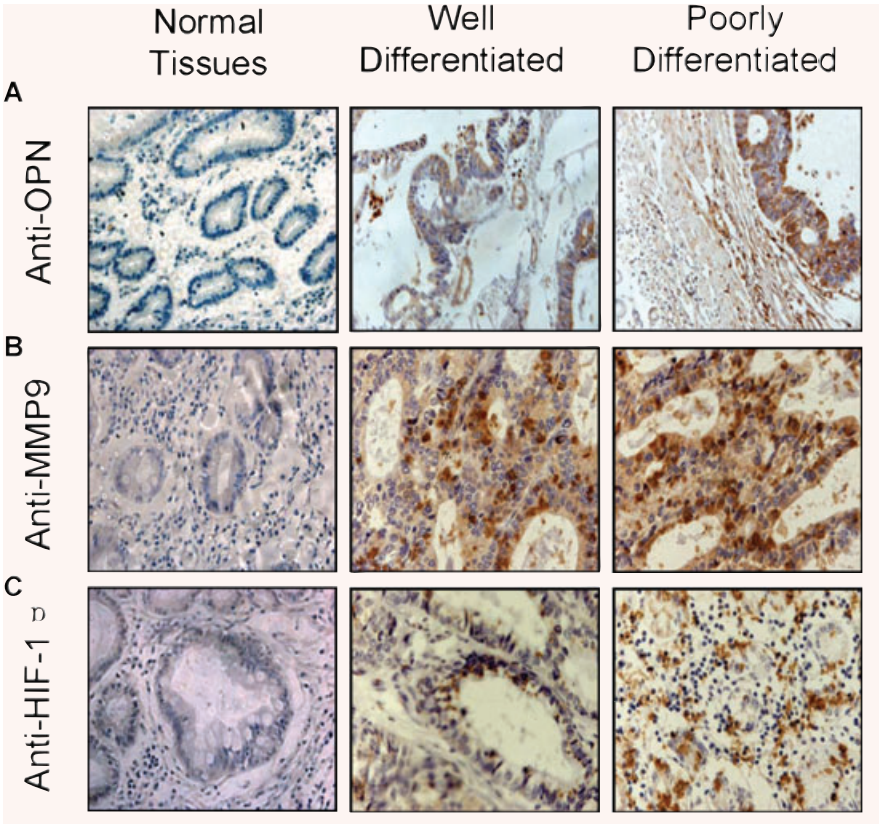


**Fig. 1** Immunohistochemical staining of OPN, MMP9 and HIF-1α in human gastric cancer tissues. The tissue sections of the primary gastric cancers, including the well- and poorly differentiated gastric cancer tissues, and the matched non-cancer gastric tissues, were immunostained with anti-OPN (**A**), anti-MMP9 (**B**) and anti-HIF-1α (**C**) antibodies respectively. The positive staining for OPN, MMP9 or HIF-1α proteins were shown in brown colour. All sections were counterstained with haematoxylin showing in blue colour (×100).

OPN$^1$ expression, 26 (81.3%) cases showed MMP9$^+$ expression, and 21 (65.6%) cases showed HIF-1α$^+$ expression (Table 1). Moreover, all cases of poorly differentiated tumour tissues showed OPN-, MMP9- and HIF-1α$^+$ expression, which presented statistically significant differences compared to the well-differentiated gastric cancer ($P < 0.01$, Table 1).

Furthermore, the statistical analysis was shown in Table 1, there was a correlation between OPN and MMP9, since the majority of cases (19 cases) with MMP9$^+$ expression (26 cases) also expressed high OPN levels ($r = 0.872$, $P < 0.01$). Another correlation was also found between OPN and HIF-1α, since high HIF-1α expression occurred predominantly (65.6%) in association with high levels of OPN ($r = 0.878$, $P < 0.01$). Taken together, these observations indicate that OPN expression was associated with MMP9 and HIF-1α expression.

## Overexpression of OPN promoted gastric cancer cellular survival

To further assess the functional significance of elevated OPN expression during the development of gastric cancer, we investigated whether stable overexpression of OPN in a human gastric cancer cell line could alter cellular survival in either nomoxia or hypoxia condition *in vitro*. We selected a human gastric cancer cell line SGC7901, which expresses a very low level of endogenous OPN (Fig. 2A, middle band). A C-terminal Flag-tagged OPN expression construct was transfected into the SGC7901 cells. Three stable clones (nos. O3, O16 and O19) and vector-transfected controls (V) were verified with specific antibodies against Flag-tag and OPN (Fig. 2A).

We next examined whether OPN overexpression and $CoCl_2$-induced hypoxia could affect gastric cancer cell survival in serum-depleted condition. As shown in Fig. 2B and C, results from three independent experiments revealed that hypoxia promoted SGC7901 cells are indeed more resistant to serum starvation in comparison with control ($P < 0.05$). Similarly, OPN-producing SGC7901 cells were also shown to prevent serum depletion-induced apoptosis (Fig. 2B and C). These observations suggest that OPN could act to promote cellular survival for gastric cancer cells by mimicking the effect of hypoxia.

## OPN activated PI3-K/Akt pathway and up-regulated HIF-1α *via* the αvβ3 integrins

To further explore the molecular mechanisms by which OPN promotes cellular survival, we examined the potential effect of OPN

**Table 1** Correlations analysis of OPN, MMP9 and HIF-1α expression in human gastric cancer tissues

| Proteins | Cases | | | *r*-value | *P*-value |
|---|---|---|---|---|---|
| | Total | (+) | (−) | | |
| OPN | 32 | 21 | 11 | − | − |
| MMP9 | 32 | 26 (19*) | 6 | 0.872 | $P < 0.01$ |
| OPN | 32 | 21 | 11 | − | − |
| HIF-1α | 32 | 21 (18**) | 11 | 0.878 | $P < 0.01$ |

*Indicates the co-expression cases of OPN and MMP9; **indicates the co-expression cases of OPN and HIF-1α.

on the activity of Akt and HIF-1α. We found that OPN activated PI3-K/Akt pathway and increased the cellular level of HIF-1α protein. As shown in Fig. 3A and B, Ser 473 phosphorylation, which indicates the activation of Akt pathway, was readily detected and HIF-1α was up-regulated in SGC7901 cells overexpressing OPN under either normoxic conditions or $CoCl_2$-induced hypoxia conditions. To define whether the impact of OPN on Akt pathway activation and HIF-1α up-regulation is a primary effect or secondary to other cellular effects induced by OPN expression, we examined the effect of exogenous human recombinant OPN on Akt activation and HIF-1α expression in the parental SGC7901 cells, and the similar results were observed (Fig. 3C).

To elucidate the possible role of the PI3-K/Akt pathway on OPN-mediated HIF-1α up-regulation under normoxic conditions, we used PI3-K inhibitor LY2940002 to block the PI3-K activity and examined its effect on the OPN-mediated HIF-1α up-regulation. As shown in Fig. 3D, LY2940002 inhibits OPN-induced Akt phosphorylation and HIF-1α up-regulation dramatically, suggesting that PI3-K and Akt activity are required for OPN to enhance HIF-1α expression. To further determine whether the $\alpha_v\beta_3$ integrins are involved in the regulation of OPN in gastric cancer, we pre-incubated the SGC7901 cells with antibody against the $\alpha_v\beta_3$ integrins and measured the OPN-induced Ser473 phosphorylation of Akt and expression of HIF-1α. As shown in Fig. 3E, antibody blockade of OPN and $\alpha_v\beta_3$ integrins interaction reduces the activation of Akt and HIF-1α expression, indicating that OPN up-regulates HIF-1α *via* Akt pathway through interaction with the $\alpha_v\beta_3$ integrins.

## OPN potently promoted gastric cancer cells growth *in vitro* and *in vivo*

To further assess the biological significance of elevated OPN expression during the development of gastric cancer, we investigated whether stable overexpression of OPN in human gastric cancer cells or introduction of exogenous human recombinant OPN could alter the tumour growth *in vitro* and *in vivo*. Firstly, we examined whether OPN overexpression could affect the colony formation efficiency in soft agar. The colony formation efficiency in soft agar indicates the ability of cells to grow and is correlative with the tumour malignant degree [37]. As shown in Fig. 4A, colony formation analysis indicated that the clone size of SGC7901 cells transfected with vector construct is smaller than that of OPN overexpressing SGC7901 cells. The colony formation efficiency was notably different between vector control and OPN/ SGC7901 clones ($P < 0.01$). To further investigate the impact of OPN on tumour growth *in vivo*, we injected the gastric cancer cells into the nude mice to assess the potential differences in tumour growth between the OPN-overexpression cells and the vector control cells. As shown in Fig. 4B, OPN overexpression appeared to significantly enhance the subcutaneous tumour growth over the controls and the average ratio of tumour weight between OPN-expressing clones and vector clone was more than twofold ($P < 0.05$). In separate experiments, the SGC7901 cells were pre-treated with exogenous human recombinant OPN, and were then injected into the nude mice. Exogenous OPN was repeatedly injected into the tumour sites twice a week for up to 5 weeks, and the similar results were observed (Fig. 4C). Taken together, OPN overexpression or introduction of exogenous human recombinant OPN could enhance gastric tumour growth *in vivo*.

## Overexpression of OPN in gastric cancer cells activated pro-MMP9 to promote tumour metastasis

To examine how OPN regulates MMPs and promotes gastric cancer metastasis, we evaluated whether stable overexpression of OPN in human gastric cancer cells or introduction of exogenous human recombinant OPN could alter the *in vitro* tumour cells invasion and *in vivo* tumour metastasis. *In vitro* cell invasion assay was performed with Matrigel™-coated invasion chamber as described in 'Materials and methods'. As shown in Fig. 5A, the significant difference in invasiveness was observed between OPN/SGC7901 clones and vector control ($P < 0.01$). We next examined whether OPN overexpression could affect the metastatic potential and growth of the cancer cells *in vivo*. Equal numbers of cells ($6 \times 10^6$/mice) of two OPN-producing gastric cancer clones or the vector controls were introduced into the nude mice *via* intrasplenic injection. Five weeks after the inoculation of tumour cells, the animals were killed for the examination of metastatic growth. Strikingly, tumour metastases were detected in the intestine and lung of the survived mice with injection of the OPN-producing SGC7901 cells (Fig. 5B and C). In contrast, no visually observable metastatic tumours were found in the intestine and the lung of the control mice injected with the vector control cells (Fig. 5B and C). Furthermore, we examined OPN and MMP9 expression on the sections of those metastatic tumours by using immunohistochemical staining. As shown in Fig. 5D and E with the representative staining pattern of OPN and MMP9 expression, the metastatic tumours of the intestine and lung tissues derived from the OPN-producing gastric cancer cells have much more intensely

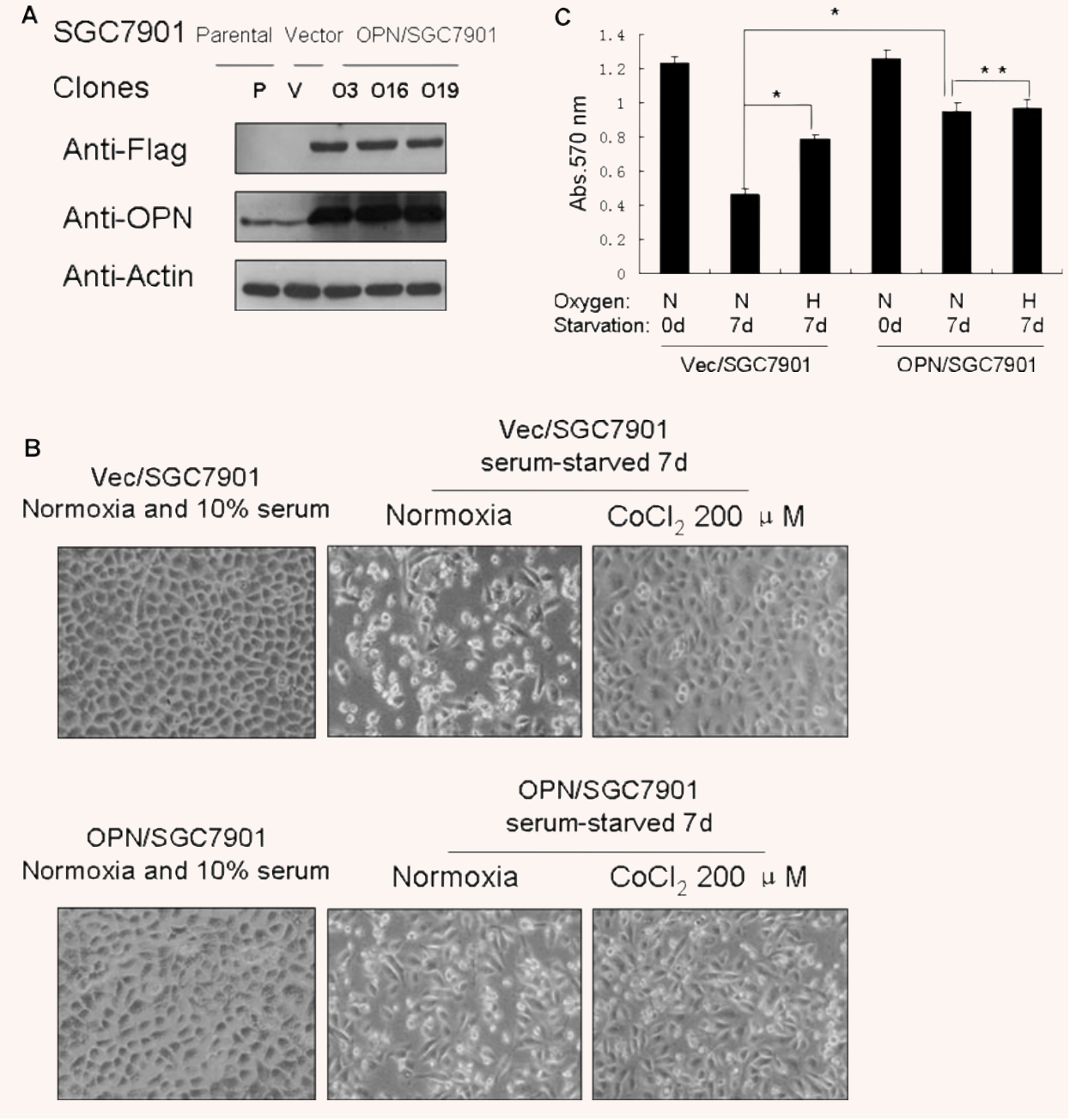


**Fig. 2** Overexpression of OPN promoted gastric cancer SGC7901 cellular survival in serum depleted condition. (**A**): Characterization of OPN-overexpressing SGC7901 clones and vector control clone. Equivalent number of parental SGC7901 cells (P), one vector control SGC7901 clone (V) and three OPN-producing SGC7901 clones (nos. O3, O19 and O16) were seeded in culture medium in 100 mm culture dishes. Twenty-four hours later, cells were washed with PBS and undergone starvation in 4 ml serum-free medium for another 48 hrs. Then the conditional media from the culture were subjected to Western blot assay using anti-OPN and anti-Flag antibodies. Levels of β-actin were monitored as loading control. (**B**) and (**C**): Effects of serum starvation and hypoxia on OPN/SGC7901 cell viability. The Vec/SGC7901 and OPN/SGC7901 cells were serum-starved for one week in the presence or absence of 200 μM $CoCl_2$. Cells were observed and the cell micrographs were taken in random microscopic fields (×100), and the cell numbers were counted by MTT assay. $^{*}P < 0.05$ and $^{**}P > 0.05$ (SPSS 11.0).

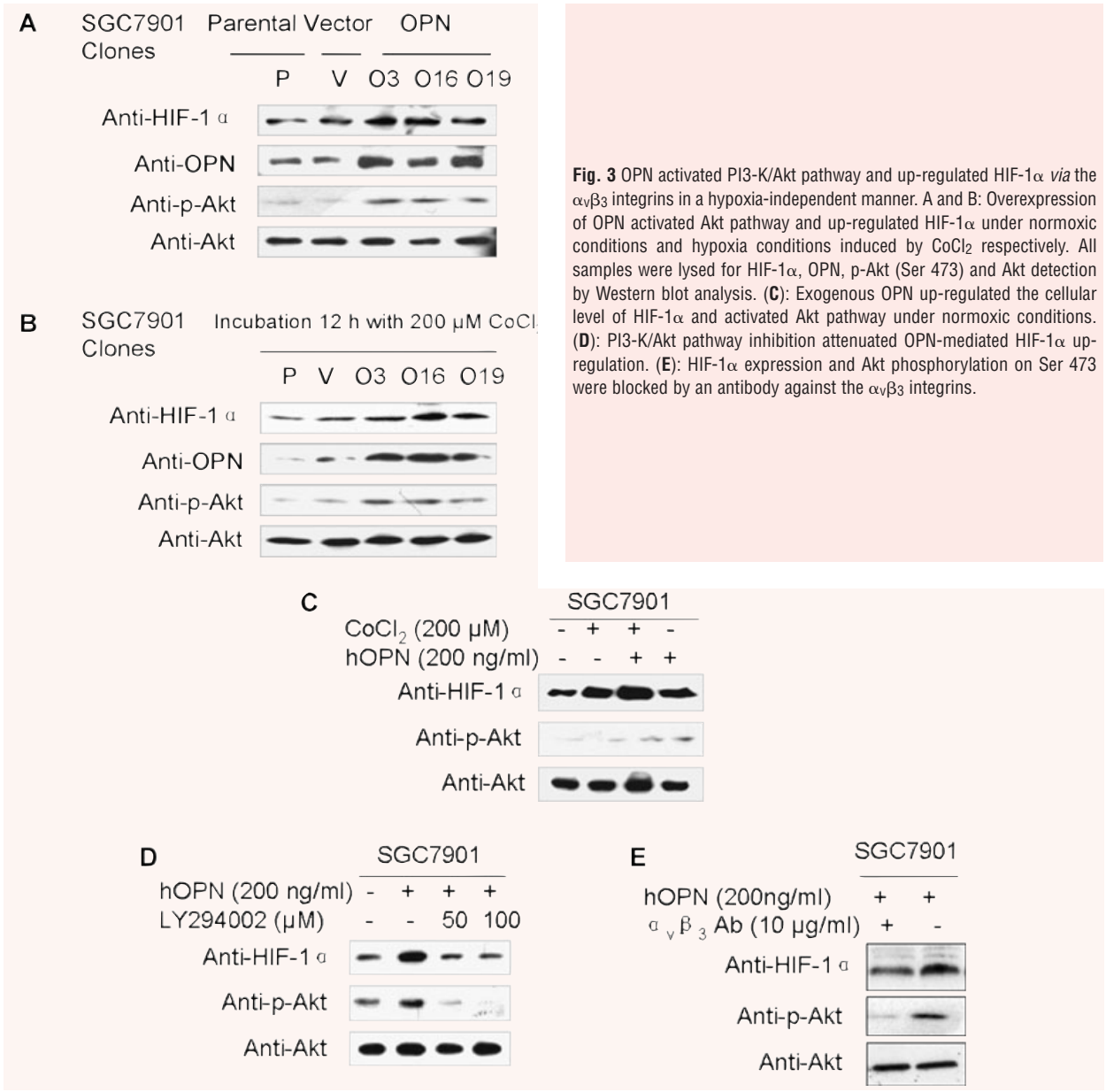


**Fig. 3** OPN activated PI3-K/Akt pathway and up-regulated HIF-1α *via* the $\alpha_v\beta_3$ integrins in a hypoxia-independent manner. A and B: Overexpression of OPN activated Akt pathway and up-regulated HIF-1α under normoxic conditions and hypoxia conditions induced by $CoCl_2$ respectively. All samples were lysed for HIF-1α, OPN, p-Akt (Ser 473) and Akt detection by Western blot analysis. (**C**): Exogenous OPN up-regulated the cellular level of HIF-1α and activated Akt pathway under normoxic conditions. (**D**): PI3-K/Akt pathway inhibition attenuated OPN-mediated HIF-1α up-regulation. (**E**): HIF-1α expression and Akt phosphorylation on Ser 473 were blocked by an antibody against the $\alpha_v\beta_3$ integrins.

expression pattern of MMP9 than that of normal intestine and lung tissues. These results suggest that overexpression of OPN in gastric cancer cells strongly promoted tumour metastatic growth *in vivo*, supporting a critical role for OPN in the late stage of tumour progression.

Previous reports indicated that MMPs, including MMP9 and MMP2, which are activated by OPN *in vitro*, play a key role in cancer progression [17, 36]. To determine whether MMP9 and MMP2 are involved in the mediation of OPN action in gastric cancer progression, we next examined the potential effect of OPN on the activity of MMP9 and MMP2 using zymography and Western blot analysis. The equal numbers of OPN-expressing cells were incubated in serum-free medium or the equal parental cells were treated with exogenous human recombinant OPN in serum-free medium. The conditioned media were collected, and the gelatinolytic activity of MMP9 and MMP2 were measured by zymography. Increased levels of MMP2 expression and MMP9 activation (92 kD pro- and 86 kD active forms) were observed in OPN-expressing cells or parental cells treated with exogenous human recombinant OPN, respectively (Fig. 6A and B). In contrast, almost no active MMP9-specific bands or pro-MMP2 bands were detected in the control cells. OPN-expressing cells and control cells were incubated in serum-free

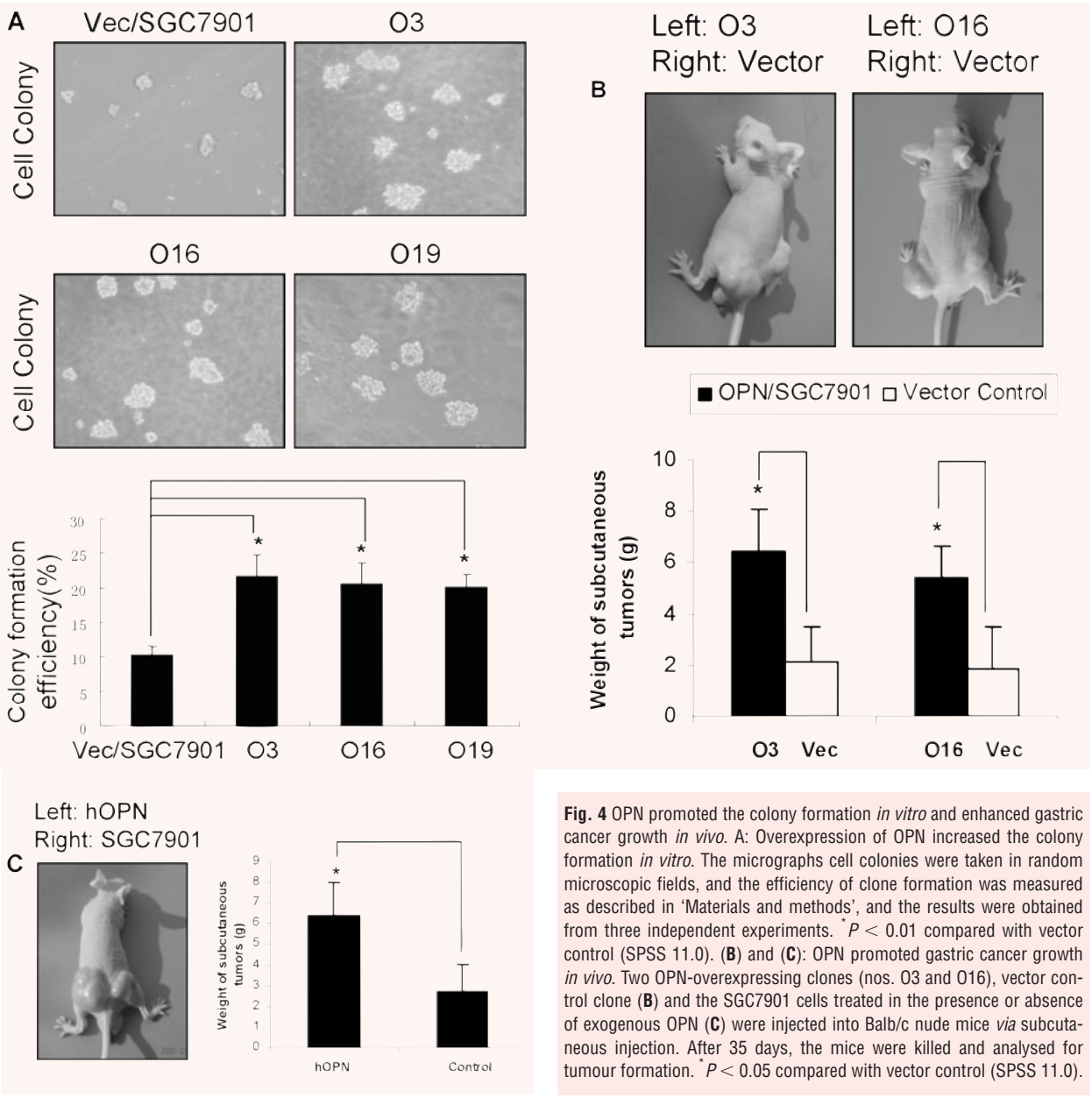


**Fig. 4** OPN promoted the colony formation *in vitro* and enhanced gastric cancer growth *in vivo*. A: Overexpression of OPN increased the colony formation *in vitro*. The micrographs cell colonies were taken in random microscopic fields, and the efficiency of clone formation was measured as described in 'Materials and methods', and the results were obtained from three independent experiments. $^{*}P < 0.01$ compared with vector control (SPSS 11.0). (**B**) and (**C**): OPN promoted gastric cancer growth *in vivo*. Two OPN-overexpressing clones (nos. O3 and O16), vector control clone (**B**) and the SGC7901 cells treated in the presence or absence of exogenous OPN (**C**) were injected into Balb/c nude mice *via* subcutaneous injection. After 35 days, the mice were killed and analysed for tumour formation. $^{*}P < 0.05$ compared with vector control (SPSS 11.0).

medium and lysed for Western blot analysis, and similar results were observed and shown in Fig. 6C and D.

## Discussion

Cancer cells commonly resist cell death either through disruption of apoptotic processes or activation of survival signals. Within a growing tumour mass, the sequential acquisition of a number of genetic and epigenetic alterations during tumour progression also enable cancer cells to gain the ability to escape from apoptosis, induce angiogenesis and metastasize to distant organs [20, 38]. We have previously demonstrated that a secreted protein called periostin which is overexpressed in colon cancers can activate the Akt signalling pathway through the $a_vb_3$ integrins to augment the survivals of both cancer cells and endothelial cells, and dramatically enhance metastatic growth of colon cancers [20]. OPN is

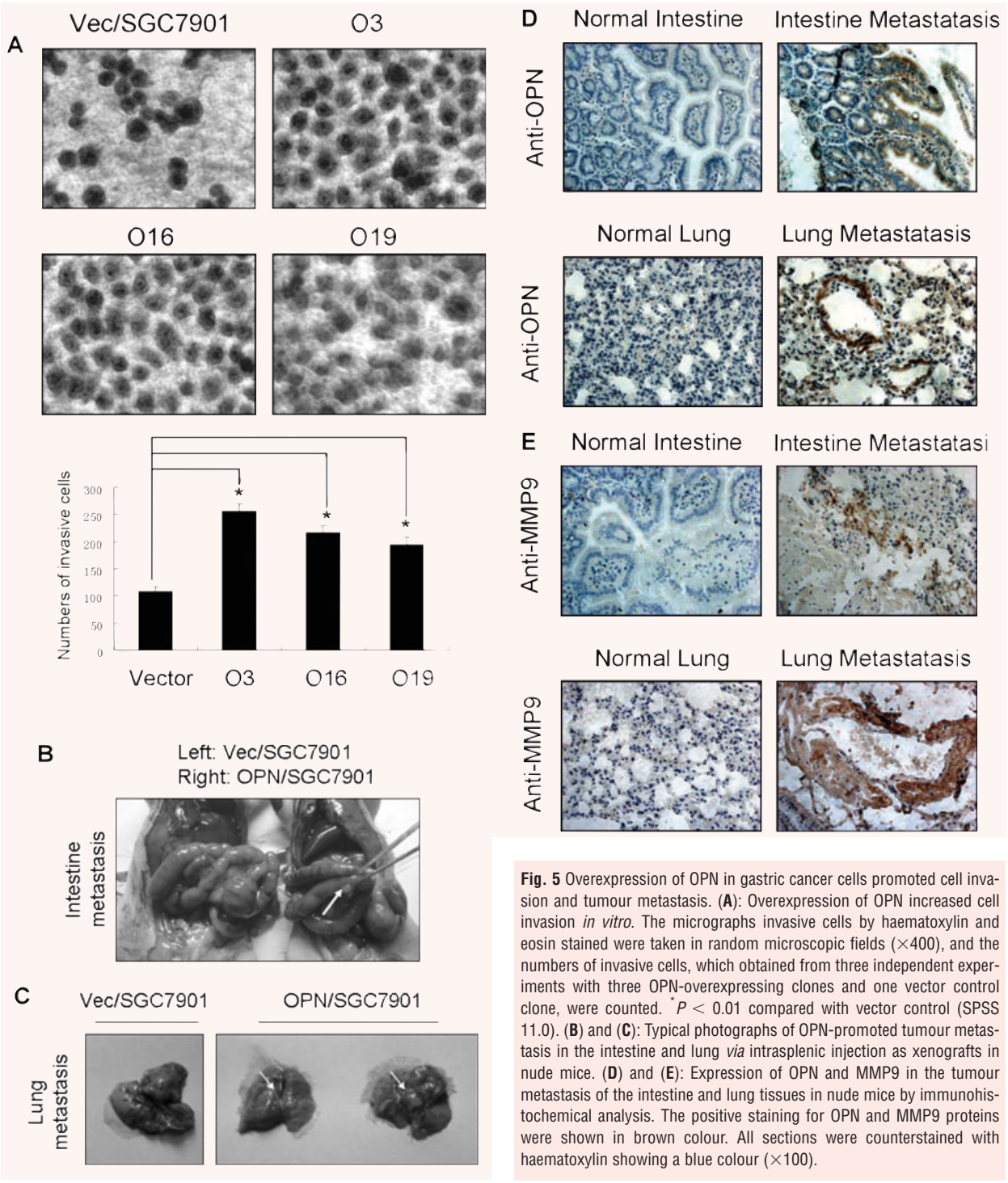


**Fig. 5** Overexpression of OPN in gastric cancer cells promoted cell invasion and tumour metastasis. (**A**): Overexpression of OPN increased cell invasion *in vitro*. The micrographs invasive cells by haematoxylin and eosin stained were taken in random microscopic fields (×400), and the numbers of invasive cells, which obtained from three independent experiments with three OPN-overexpressing clones and one vector control clone, were counted. $^{*}P < 0.01$ compared with vector control (SPSS 11.0). (**B**) and (**C**): Typical photographs of OPN-promoted tumour metastasis in the intestine and lung *via* intrasplenic injection as xenografts in nude mice. (**D**) and (**E**): Expression of OPN and MMP9 in the tumour metastasis of the intestine and lung tissues in nude mice by immunohistochemical analysis. The positive staining for OPN and MMP9 proteins were shown in brown colour. All sections were counterstained with haematoxylin showing a blue colour (×100).

another secreted protein that plays an important role in the progression of tumour development [1, 3, 5]. Our results suggest that the underlying mechanism of OPN-mediated promotion of tumour development is largely associated with the ability of Akt activation to enhance cell survival under stresses. In this regard, acquired expression of OPN and similar types of proteins may enable tumour cells to thrive in the hypoxic microenvironment. The discovery of a link between OPN, HIF-1$\alpha$ and the PI3-K/Akt signalling

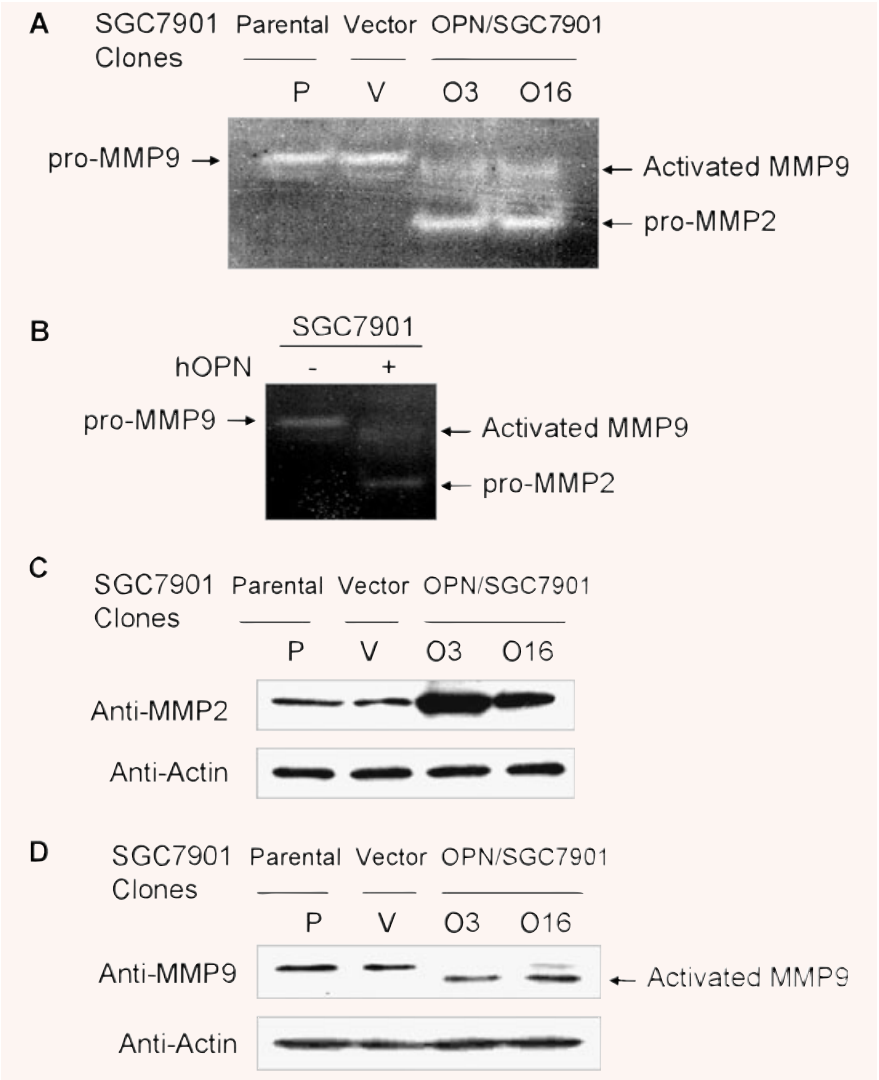


**Fig. 6** OPN activated pro-MMP9 and up-regulated MMP2 in gastric cancer cells. (**A**) and (**B**): Overexpression of OPN and exogenous human recombinant OPN protein activated pro-MMP9 and up-regulated MMP2 in gastric cancer cells by zymography analysis as described in Materials and Methods. (**C**) and (**D**): Overexpression of OPN up-regulated MMP2 and activated MMP9 in gastric cancer cells shown by Western blot analysis.

pathway may provide a molecular explanation for the pro-survival activity of OPN in the context of tumour progression.

The inner microenvironment of solid tumour is commonly hypoxic. How cancer cells adapt to the hypoxic condition in a solid tumour is critical for tumour progression. Currently, intratumoural hypoxia has been increasingly considered as a critical microenvironmental factor which promotes tumour aggressiveness and metastasis. Stabilization of the HIF-1$\alpha$ transcription complex can promote tumour progression and metastasis, leading to treatment failure and mortality in various human cancers [22, 39, 40]. Here we found that OPN could also increase the expression of HIF-1$\alpha$, which may be another mechanism by which OPN stimulates the metastatic growth of gastric cancer *in vivo*. Therefore, the discovery of OPN-mediated HIF-1$\alpha$ up-regulation may provide a molecular explanation for the pro-survival activity of OPN.

In addition to playing a critical role in the tumourigenicity, OPN appears to be involved in metastasis, as a recent report showed that a significant increase of OPN expression in patients with metastatic tumours [5]. The ability for cancer cells to survive and grow in the distant organ is critical for the successful development of metastases. Here, we observed that tumour metastases were detected in the intestine and lung of the survived mice with injection of the OPN-producing SGC7901 cells *via* intrasplenic injection. In contrast, no visually observable metastatic tumours were found in the survived mice with injection of the OPN-producing SGC7901 cells *via* subcutaneous injection. Our results indicate that the presence of OPN did not significantly affect the initial formation of micrometastases in the animal models, but that it did stimulate metastatic growth and development at later stage by promoting cancer cell survival. Obviously, OPN is intimately

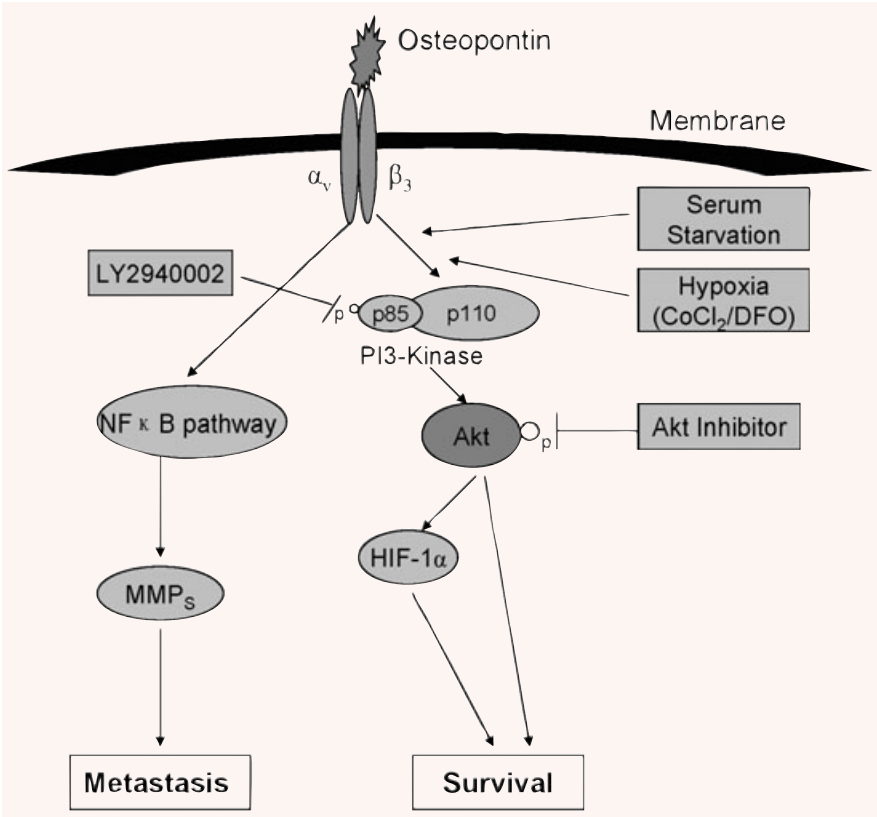


**Fig. 7** Molecular mechanisms of OPN promoted gastric cancer metastatic growth through MMPs activation and Akt-mediated HIF-1α up-regulation. Binding of OPN to $\alpha_v\beta_3$ integrin receptor induces phosphorylation and activation of PI3-K, which subsequently activates Akt through phosphorylation. HIF-1α is then up-regulated, thus promotes cell survival. In addition, recent studies showed that OPN promoted cell motility and invasion through nuclear factor-κB pathways [21, 42]. Here, OPN regulated pro-MMP9 activation and up-regulated MMP2 in gastric cancer cells, thus regulated cell motility, invasion and tumour growth.

involved in the critical steps of tumour metastatic process. OPN could be a potential target for the development of novel treatment for metastatic gastric cancers.

Previous studies indicated that OPN binds to a variety of cell surface receptors including integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and CD44 [15, 41, 42]. OPN functions are primarily executed through a division of labour between integrin $\alpha_v\beta_3$ and CD44 [43–45]. Recently, correlative studies suggest that $\alpha_v\beta_3$ may be particularly associated with increased tumour aggressiveness [46, 47]. However, the relative contribution of these integrins to gastric cancer cell malignancy remains uncertain. To gain further insight into the role of OPN in gastric cancer cell survival, we pre-incubated the SGC7901 cells with antibody against the $\alpha_v\beta_3$ integrins and measured the phosphorylation of Akt on Ser 473 and the expression of HIF-1α induced by the presence of OPN. The results demonstrated that, without binding to the $\alpha_v\beta_3$ receptor, OPN can neither activate the PI3-K/Akt kinase cascade nor increase the expression of HIF-1α. The *in vitro* results indicate that HIF-1α up-regulation *via* PI3-K/Akt pathway by OPN is mediated primarily through the $\alpha_v\beta_3$ integrins (Fig. 7). Moreover, we observed that OPN could activate MMP9 and up-regulate MMP2 in our gastric cancer model. MMPs, including MMP9 and MMP2, which are activated by OPN *in vitro* [17, 36], play a key role in cancer progression. Indeed, the link we found in gastric cancer model between MMP9 and OPN may provide the biological basis in the biological diagnosis and prognosis of gastric cancer.

In conclusion, the present studies demonstrate that acquired expression of OPN may enable gastric cancer cells to thrive under stress conditions by activating PI3-K/Akt survival pathway and up-regulating cellular level of HIF-1α *via* binding to $\alpha_v\beta_3$ integrins. Moreover, OPN could activate MMP9 and up-regulate MMP2 to promote metastatic growth in gastric cancer. These findings indicate that OPN is closely associated with the progression of gastric cancer and may be considered as a useful molecular target for the therapeutics of gastric cancer.

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