International Journal of Pharmaceutics 431 (2012) 197–203

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Suppression of tumor growth in xenograft model mice by small interfering RNA targeting osteopontin delivery using biocompatible poly(amino ester)

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ARTICLE INFO

Article history:
Received 16 December 2011
Received in revised form 30 March 2012
Accepted 9 April 2012
Available online 15 April 2012

Keywords:
Cancer gene therapy
Osteopontin
siRNA
Poly(amino ester)

ABSTRACT

Gene therapy using small interfering RNA (siRNA) is a novel strategy for effective anticancer therapies. However, low gene transfection efficiency and technical difficulties linked to siRNA delivery limit their practical application for gene delivery. Therefore, development of effective siRNA carriers is required. Overexpression of osteopontin (OPN) and its association with tumorigenesis has been reported in a majority of breast cancers. In this study, we used siRNA against OPN (siOPN) and investigated the possible OPN-dependent signaling pathway and the potential use of poly(amino ester) (PAE) based on glycerol propoxylate triacrylate (GPT) and spermine (SPE) for siRNA delivery. The GPT–SPE could effectively condense siRNA and protect the siRNA from RNaseA enzyme degradation. GPT–SPE/siRNA complexes showed good intracellular uptake and high gene silencing efficiency *in vitro*. Furthermore, in the breast cancer xenograft model, intratumoral injection of GPT–SPE/siOPN significantly inhibited tumor growth. These results demonstrated that silencing of OPN effectively suppressed the growth of breast cancer cells and further suggested that delivery of siRNA using GPT–SPE may act as an effective gene carrier for cancer therapy.

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1. Introduction

Osteopontin (OPN) is a non-collagenous glycophosphoprotein which plays a crucial role in determining the oncogenic potential of different cancers and, is known as a potential prognostic marker during cancer development (Chakraborty et al., 2008; Rangaswami et al., 2006). Substantial data have linked enhanced expression of OPN with both progression and severity of many cancers, including breast cancer (Cook et al., 2005; Weber et al., 2010). Highly metastatic human breast cancer cell lines have been reported to express higher level of OPN, compared with low metastatic ones (He et al., 2006). In breast cancer, increased OPN expression is believed to be correlated with metastasis, tumorigenesis and angiogenesis (Cook et al., 2005). In addition, it has been demonstrated that down regulation of OPN expression by small interfering RNA (siRNA) reduced the metastasis in CT26 colon cancer by decreasing tumor cell invasion (Wai et al., 2005); however, the inhibitory effect of down-regulated OPN on tumor growth and angiogenesis is not well defined in breast cancer cells. To address this issue, we used siRNA to silence the expression of OPN and investigated the effect of decreased OPN on growth of MDA-MB-231-derived tumors in a xenograft mouse model.

Several studies have showed that siRNA targeting oncogene molecules resulted in significant inhibition of tumor growth *in vivo*, which further supported the therapeutic potential of RNAi-based methods for cancer therapeutics (Gong et al., 2008; Liu et al., 2010a; Pirollo and Chang, 2008; Yu et al., 2010). The efficiency of RNAi mostly depends on the effective delivery of intact siRNA into mammalian cells (Duan et al., 2008). However, because of the low

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http://dx.doi.org/10.1016/j.ijpharm.2012.04.028

permeability through the cellular membranes and low stability of siRNA against enzymatic degradation, the efficacy of naked siRNA remains insufficient (Oishi et al., 2005). Therefore, it is important to develop efficient and safe approaches for delivery of siRNA. Recently, non-viral vectors have been shown to be promising vectors for introducing nucleotides into different cell types or tissues (Merdan et al., 2002). Among them, poly(amino ester)s, as delivery agents, have been shown to generally possess low cytotoxicity and high transfection efficiency. They can be easily synthesized by the conjugate addition of a primary or bis (secondary) amine to either diacrylate or triacrylate (Anderson et al., 2003). In a previous study, biocompatible PAE-based on glycerol propoxylate triacrylate (GPT) and spermine (SPE) demonstrated good capabilities for transfection of plasmid DNA (Jiang et al., 2011), suggesting the possibility of this vector for siRNA transfection, both *in vitro* and *in vivo*. Therefore, in this study, GPT–SPE was assessed as a siRNA delivery carrier. The physicochemical properties of GPT–SPE/siRNA complexes, their cellular uptake and transfection efficiency were analyzed. Furthermore, *in vivo* therapeutic effect was investigated using siRNA against OPN (siOPN) and after intra-tumor injection of GPT–SPE/siOPN complexes in xenograft model mice.

2. Materials and methods

2.1. Preparation of GPT–SPE and characterization of copolymer and GPT–SPE/siRNA complex

The GPT–SPE copolymer was synthesized using the Michael addition method as described previously (Jiang et al., 2011). Complexes were prepared by adding siRNA solution to equal volume of polymer solution, vortexing gently. After 30 min incubation at room temperature, complexes were loaded on 3% agarose gel and subjected to electrophoresis. siRNA protection and release assay was performed using a previously reported modified method (Jiang et al., 2007). Briefly, one unit of either RNaseA or DEPC H_2O was added to naked siRNA or polymer/siRNA complex solution, and the protection ability of polymer was analyzed by 3% agarose gel electrophoresis (1 h at 50 V). The morphology of GPT–SPE/DNA complexes was observed by energy-filtering transmission electron microscope (EF-TEM, LIBRA 120, Carl Zeiss, Germany).

2.2. Cellular uptake and silencing studies in vitro

For confirmation of intracellular uptake of GPT–SPE/siRNA complex, FITC-labeled GPT–SPE/siRNA complex was delivered and visualized using confocal microscopy. Briefly, cells were seeded in 24-well plates at an initial density of 10×10^4 cells/well. After incubation for 18 h, the media were replaced with serum-free media with polymer/siRNA (100 pmol) complex and additionally incubated for 4 h. Then, the media were exchanged by fresh media, and allowed to incubate for 24 h for confocal microscopy measurement. *In vitro* silencing studies were performed by checking silencing efficacy in GFP-positive A549 cell line. For silencing study, GPT–SPE/siGFP, PEI25K/siGFP and lipofectamine/siGFP complexes were prepared at 100 pmol siGFP concentration, by incubating for 30 min. The complexes were transferred to each well of 24-well plates and incubated for 4 h. Then, the media were changed with fresh media. After 24 h incubation, the efficiency of silencing was measured by FACS.

2.3. In vivo tumor xenograft studies

Six-week-old nude mice were obtained from (Joongang Laboratory Animal Inc. Seoul, South Korea). The mice were maintained in the pathogen-free animal facility at least one week before use. For xenograft study, mice were inoculated s.c. with (5×10^6) MDA-MB-231 cells in 200 μl culture medium. When solid tumors were established, mice were randomized into three groups. Two groups were intratumorally injected with either GPT–SPE/siOPN or GPT–SPE/siSCR (15 μg of siRNA/mouse) every 3 days, whereas control group received PBS. Tumor volume of each mouse was measured with calipers at regular intervals and calculated, as described previously (Kim et al., 2010). Upon termination of the experiment, mice were sacrificed and individual tumors were weighted and collected for further analysis. All methods used in this study were approved by the Animal Care and Use Committee at Seoul National University (SNU-110610-2). Synthetic siRNAs were purchased from ST Pharm (Genolusion Pharmaceuticals, Seoul, KOREA). The siRNA sequence targeting OPN mRNA was 5′-CGAGGTGATAGCTTGGCTTAT-3′ and the scrambled sequence was 5′-GATAGCAATGACGAATGCGTA-3′.

2.4. Western blot and ELISA

Tumors were homogenized then digested in three volumes of lysis buffer (Promega, Madison, USA), and total protein concentration was determined with Bio-Rad protein assay reagent (Bio-Rad, Hercules, USA). Equal amounts of protein lysates (50 μg) were loaded onto SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Amersham Pharmacia, Cambridge, UK). The membranes were blocked with 5% non-fat dry milk in Tris buffered saline + Tween 20, for 1 h at room temperature. The membranes were then incubated with specific antibodies for 3 h at room temperature or overnight at 4 °C. After washing, the membranes were incubated for 1 h with horseradish peroxidase-labeled secondary antibody and visualized using the Westzol enhanced chemiluminescence detection kit (Intron, Sungnam, Korea). The bands were detected with LAS-3000 (Fujifilm, Tokyo, Japan). Quantification of Western blot bands analysis was performed by Multi Gauge version 2.02 software (Fujifilm). Antibodies against proliferation cell nuclear antigen (PCNA), OPN, VEGF, COX-2, MMP-9, and Actin were obtained from

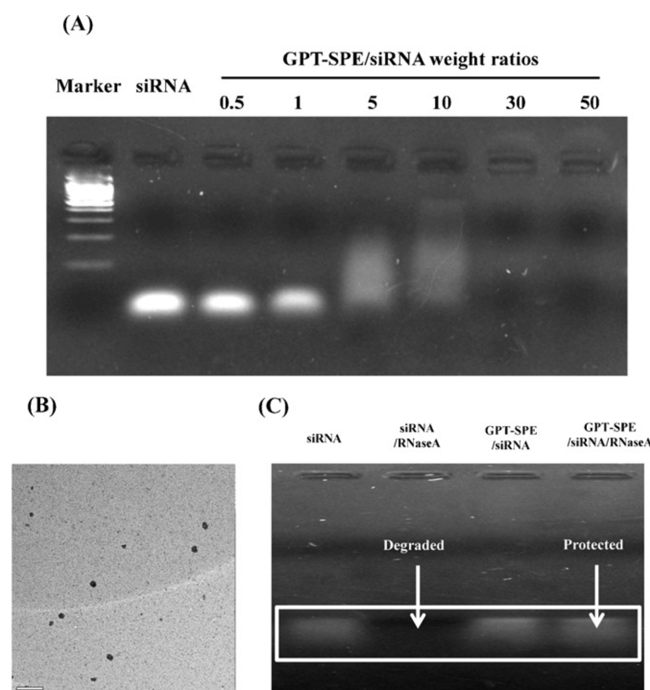

Fig. 1. Characterization of GPT–SPE/siRNA complexes (A) Agarose gel electrophoresis of GPT–SPE/siRNA complex at various weight ratios. (B) EF-TEM images of GPT–SPE/siRNA complexes at weight ratio 30 (scale bar = 0.5 μm). (C) Protection and release assay of GPT–SPE/siRNA complex.

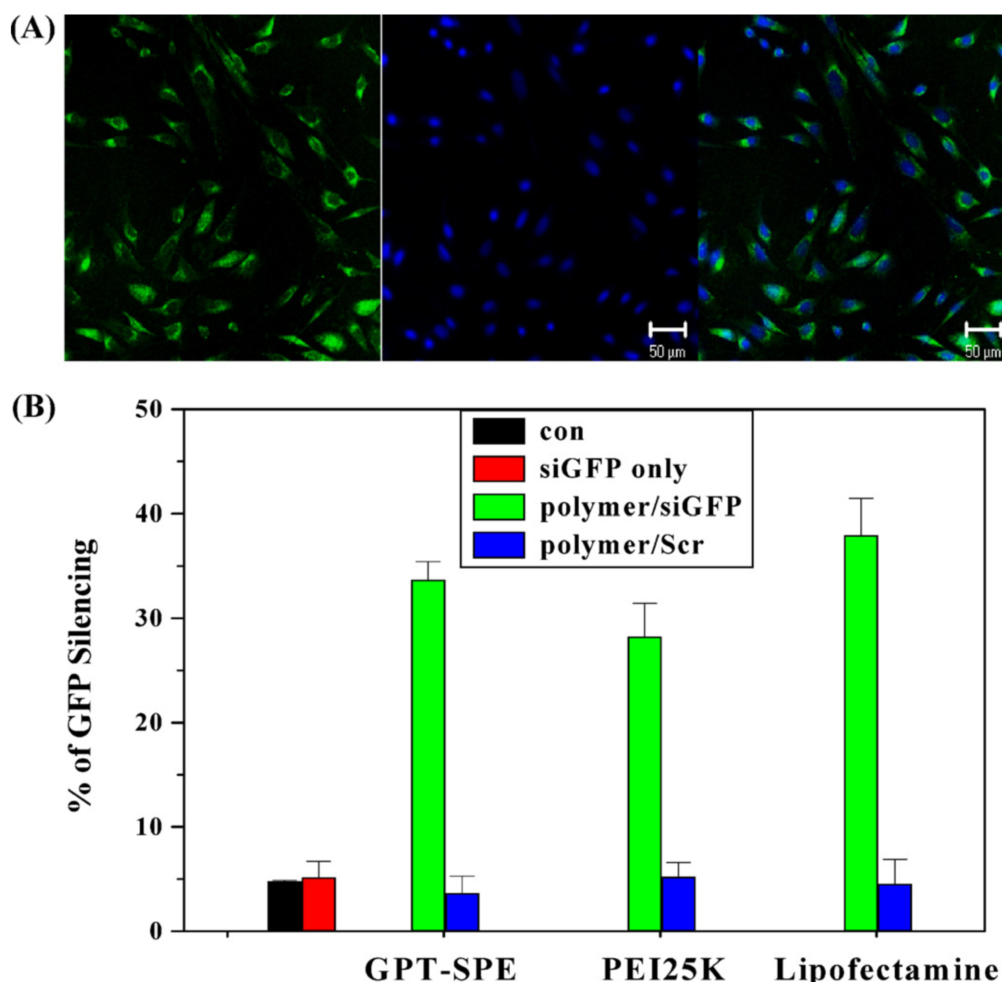

Fig. 2. Cellular uptake and gene silencing studies (A) Cellular uptake study of GPT–SPE/siRNA complexes. GPT–SPE was labeled with FITC, and nucleus was stained with DAPI (scale bar = 50 μm). (B) Gene silencing study of GFP stable A549 cells by GPT–SPE/siGFP complex (mean ± SD, $n = 3$).

Santa Cruz Biotechnology (Santa Cruz, CA, USA). CD-31 antibody was purchased from Abcam (Cambridge, MA, USA).

OPN levels were measured using a commercially available ELISA kit (Uscn Life Science Inc. Wuhan, China) according to the manufacturer's protocol.

2.5. Immunohistochemistry

Tumors were removed and fixed in 10% neutral phosphate-buffered formalin overnight and embedded in paraffin. Paraffin-embedded tissue sections were cut and transferred to plus slides. The slides were dewaxed by immersion in xylene, rehydrated through a graded series of ethanol and washed with water, then were immersed in 3% H_2O_2 for 10 min. After washing, non-specific binding was blocked by incubating the sections in 3% bovine serum albumin (BSA) for 1 h at room temperature. Then, appropriate primary antibodies (1:200 dilution in BSA) were applied on tissue sections for 3 h, followed by secondary HRP-conjugated antibodies (1:50; Invitrogen) for 1 h at room temperature. After careful washes, the sections were incubated for 5–10 min at room temperature with 3,3′-diaminobenzidinetetrahydrochloride substrate (DAB) (Biosesang, Sungnam, Korea), followed by 1% Mayer's hematoxylin (DAKO, Carpinteria, CA, USA) counterstain. Cover slips were then mounted using Permount (Fisher Scientific, USA), and the slides were reviewed using light microscope (Carl Zeiss, Thornwood, NY, USA).

2.6. Statistical analysis

Statistical analyses were performed with Student's *t*-test for experiments consisting of two groups (Graphpad Software, San Diego, CA). $^{*}P < 0.05$ was considered statistically significant, compared with corresponding control values. Quantification of Western blot analysis was performed using Multi Gauge software version 2.02 (Fujifilm).

3. Results

3.1. Evaluation of GPT–SPE for siRNA delivery

The condensation capability of GPT–SPE with siRNA was evaluated using 3% agarose gel electrophoresis. Migration of siRNA was completely retarded when weight ratio of the GPT–SPE/siRNA complexes was about thirty (Fig. 1A). GPT–SPE/siRNA complexes showed well-formed spherical shapes and compact structure, as detected by TEM image (Fig. 1B). Our results clearly showed that siRNA was protected from nucleases in the complexes, whereas control naked siRNA was completely degraded (Fig. 1C).

3.2. In vitro cellular uptake and silencing studies

To confirm intracellular uptake of GPT–SPE/siRNA complex, FITC-labeled GPT–SPE/siRNA complex was delivered and visualized using confocal microscopy. As shown in Fig. 2A, significant

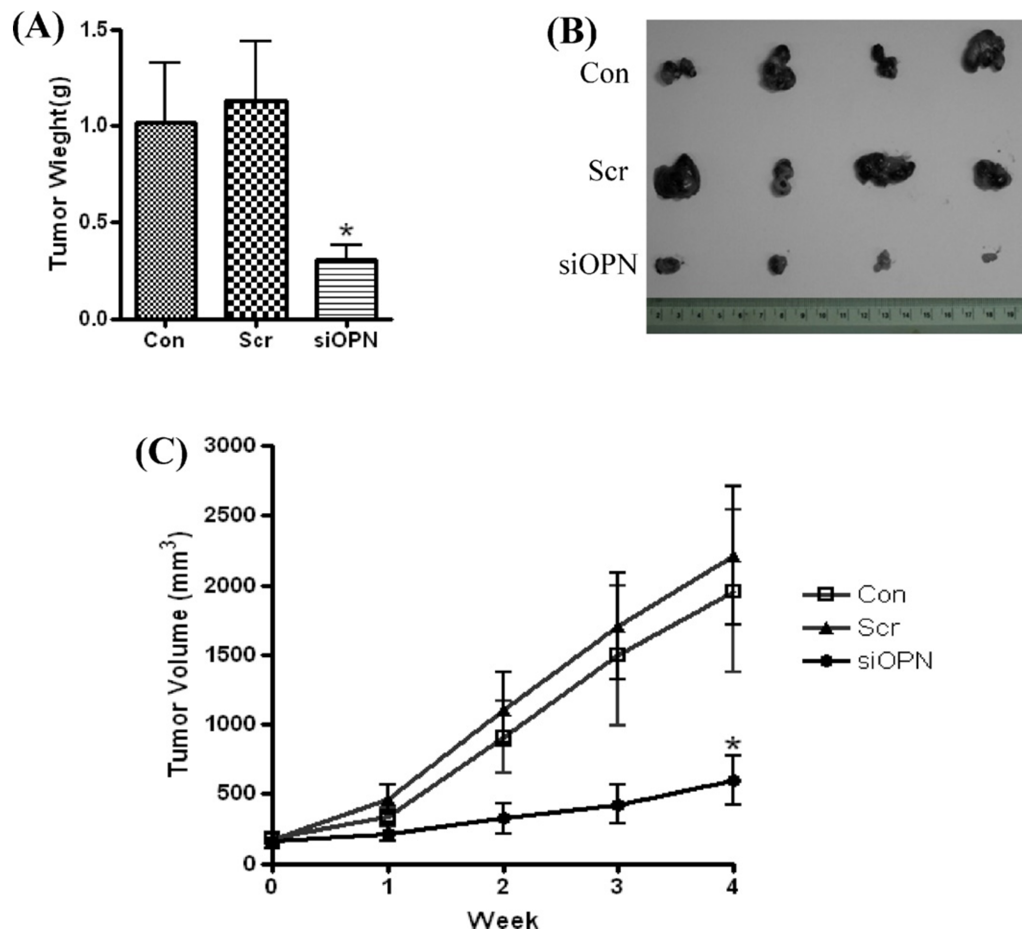

Fig. 3. Effects of siRNA against OPN on tumor growth (A) MDA-MB-231 cells were inoculated subcutaneously into nude mice and injected (intratumorally) with GPT–SPE/siOPN, GPT–SPE/siSCR and PBS. Tumor growth was monitored and volumes were calculated, as described in Section 2 (B) Tumor weights were measured and represented on the bar graph. Each bar represents the mean ± SEM ($n = 6$). *$P < 0.05$ was considered statistically significant, compared with corresponding control values. (C) Representative photograph of tumors in nude mice. Con, Control; Scr, GPT–SPE/siSCR-injected mice; siOPN, GPT–SPE/siOPN-injected mice.

fluorescence of labeled complex demonstrated that high intracellular uptake occurred. Fig. 2B indicated the silencing efficiency of GPT–SPE/siGFP complex in GFP stable cells. GFP siRNA complexed with GPT–SPE showed higher silencing efficiency compared to either naked siGFP or PEI/siGFP, while, scrambled siRNA complexes did not show any silencing effect on GFP expression.

3.3. OPN siRNA suppressed tumor growth in vivo

The effect of OPN siRNAs on tumor growth was evaluated using breast cancer xenograft model. GPT–SPE/siOPN gene therapy significantly inhibited tumor growth, as shown in Fig. 3C. Tumors from mice treated with GPT–SPE/siOPN were smaller in size and weight, compared with mice injected with GPT–SPE/siSCR or control (Fig. 3A and B).

3.4. OPN siRNA decreased proliferation and angiogenesis in vivo

We showed that expression level of OPN was significantly decreased in the GPT–SPE/siOPN treated mice, compared with GPT–SPE/siSCR and control groups, as determined by Western blot analysis and ELISA (Fig. 4A, B and Supplementary data 1). We next examined the change of PCNA expression in tumor homogenates. Our results indicated that the expression of PCNA was significantly decreased in mice treated with GPT–SPE/siOPN. Densitometric analysis clearly confirmed our Western blot result (Fig. 4A and B). It has been demonstrated that several OPN-dependent proteins, such as MMP-9, VEGF, FGF-2, COX-2 and CD-31 play important roles in angiogenesis, invasion and growth of tumor cells. Therefore, we investigated whether OPN siRNA altered the expression of these proteins in the breast tumor xenograft model. Our results clearly showed that siOPN significantly decreased the expression levels of MMP-9, VEGF, and FGF-2, as determined by both Western blot and densitometric analysis (Fig. 4A and B). Furthermore, immunohistochemistry analysis showed decreased expression levels of CD31 and COX-2 in GPT–SPE/siOPN-injected tumors (Fig. 4C).

4. Discussion

Previous studies have indicated that introduction of siRNAs into mammalian and human cells resulted in effective silencing of the corresponding mRNA molecule and proteins (Liu et al., 2004; Yin et al., 2003). OPN was overexpressed in the blood and primary tumors of breast cancer patients and has been shown to correlate with metastasis and poor prognosis (Beausoleil et al., 2011; Tuck et al., 2007). Therefore, targeting OPN and inhibiting its downstream signaling pathway by introducing siRNA directed against OPN could be a good strategy for cancer therapy. Delivery of siRNA into the cell can be achieved through exogenous application of synthetic siRNA (Sioud, 2005); recently, intratumoral delivery of siRNA indicated good prospect in cancer therapy, particularly *in vivo* (Cabon et al., 2003; Gillespie et al., 2008). In this study, we used GPT–SPE for delivery of siRNA of OPN and demonstrated that intratumoral injection of siOPN suppressed breast tumor growth

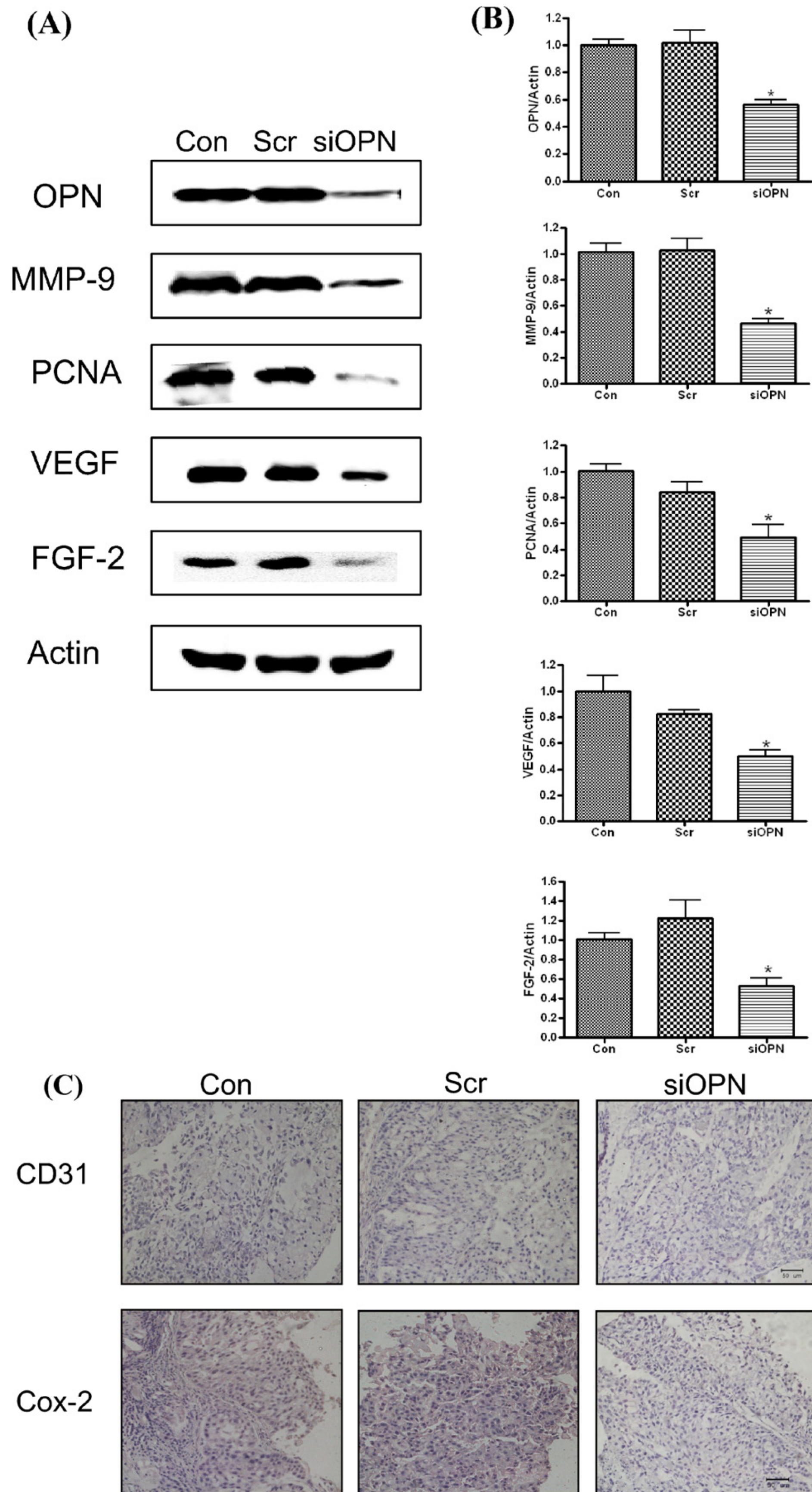

Fig. 4. Effects of siRNA against OPN on angiogenesis-invasion related proteins. Tumors homogenates were subjected to Western blot analysis. Blots were probed with antibodies, as indicated. (A) Expression levels of OPN, MMP-9, PCNA, VEGF and FGF-2. (B) The bands of interest were further analyzed by densitometry. Each bar represents the mean ± SEM ($n = 3$). *$P < 0.05$ was considered statistically significant, compared with corresponding control values. (C) Immunohistochemistry analysis of COX-2 and CD31 (magnification 200×; *bar* = 50 μm). Con, Control; Scr, GPT–SPE/siSCR-injected mice; siOPN, GPT–SPE/siOPN-injected mice.

and angiogenesis in the breast cancer xenograft mouse model. It may be crucial to employ an efficient gene delivery system for introducing siRNA into target cells. The optimal *in vivo* delivery system should be biocompatible and low immunogenic, the delivery system should improve stability, efficient cellular uptake, and the pharmacokinetics of siRNA, finally inside the cells the system should enhance endosomal release of siRNA to the cytosol, allowing the binding of siRNA with its intended mRNA targets (Akhtar and Benter, 2007; Juliano et al., 2008; Oh and Park, 2009). The use of non-viral vectors is considered a promising tool in cancer gene therapy, because they have been reported to express genes efficiently into variety of cancer cells (Kim et al., 2010; Liu et al., 2010b; Poulain et al., 2000). In this study, we assessed the transfection efficiency of siRNA using GPT–SPE. This copolymer was previously demonstrated to have high DNA plasmid transfection properties (Jiang et al., 2011). Although there are several structural similarities of RNA and plasmid DNA, the two nucleic acid species may show different behavior regarding complex formation and condensation (Spagnou et al., 2004; Utku et al., 2006). Therefore, it is important to determine the transfection properties and formulation requirements for siRNA oligomers.

One prerequisite of polymeric gene carrier is gene condensation. Our results clearly showed spherical shape and strong binding ability of GPT–SPE/siRNA complexes at weight ratio 30 (Fig. 1A and B). The protection ability is also an important factor for non-viral vectors. For effective gene expression, the gene in the gene vehicle should be protected from enzyme degradation (Jiang et al., 2007, 2009). As shown in Fig. 1C, siRNA was protected from RNaseA attack in the complex, whereas naked siRNA was completely degraded. This result suggests that GPT–SPE copolymer could deliver intact siRNA into cells, without degradation. For confirmation of post-transfectional siRNA uptake by cells, FITC-labeled GPT–SPE/siRNA complex was delivered and visualized by confocal microscopy. As expected, GPT–SPE/siRNA complex induced high intracellular uptake (Fig. 2A). Moreover, GPT–SPE-mediated siGFP delivery reduced GFP fluorescence intensity stronger than that of PEI25K, substantiating high siRNA transfection efficiency of GPT–SPE carrier. Taken together, these results in RNAi studies suggested good potential for siRNA delivery of GPT–SPE carrier.

Tumor growth and metastasis are known to be angiogenesis-related processes. VEGF is well known as an important molecule in both tumor endothelial cell survival and angiogenesis (Ferrara, 1999; Rugo, 2004). In fact, VEGF is an endothelial cell mitogen that increases vascular permeability and acts as a potent prosurvival factor for endothelial cells in newly formed vessels (Alon et al., 1995; Bamberger and Perrett, 2002; Kupprion et al., 1998). Co-expression of OPN and VEGF has been demonstrated to be correlated with angiogenesis and tumoregeneis in patients with breast cancer (Hoeben et al., 2004; Shijubo et al., 2000; Yu et al., 2010). COX-2 can also be modulated during angiogenesis through increased production of proangiogenic factors, such as VEGF (Basu et al., 2006; Cianchi et al., 2001; Tsujii et al., 1998). Furthermore, several studies have documented that FGF-2 is largely distributed in tumoral tissues and is involved in growth stimulation and differentiation of several cell types (Liu et al., 2002; Okada-Ban et al., 2000). *In vivo*, FGF-2 can be detected in pericellular basal lamina of capillaries and in capillary endothelium of different tumors indicating that FGF-2 can regulate angiogenesis with an autocrine mode of action (DiMario et al., 1989; Seghezzi et al., 1998). MDA-MB-231 cells were shown to secrete high amount of MMP-9, which can be associated with both angiogenesis and invasion potential of several cancers, including breast cancer. MMP-9 contributes to the metastasis and malignant behavior of breast cancer by regulating neovessel formation and boosting tumor growth (Adams et al., 2010; Belotti et al., 2003; Folgueras et al., 2004). MMP-9 has been demonstrated to enhance the bioavability of growth factors and different cytokines, and to alter bioactive molecules and protein function by proteolytic process (Huang et al., 2002). CD31 is also a potent stimulator of angiogenesis and plays an important role in tumor growth and progression (DeLisser et al., 1997; Marchetti et al., 2006). Our results clearly showed decreased expression of MMP-9, FGF-2, CD31, COX-2 and VEGF (Fig. 4), suggesting that suppression of OPN by siRNA using GPT–SPE can effectively inhibit angiogenesis and invasion in GPT–SPE/siOPN-injected tumors. PCNA has been widely used as a marker for tumor proliferation and survival in a variety of cancers (Gong et al., 2008; Kelman, 1997), PCNA functions as a molecular platform, interacting with proteins involved in cell cycle control and DNA repair (Paunesku et al., 2001; Zhao et al., 2011). The rate of tumor growth mainly depends on proliferative activity and the death rate of cells (Guzinska-Ustymowicz et al., 2008; Kelman, 1997). Our results clearly indicated that GPT–SPE/siOPN significantly decreased expression of PCNA; therefore, combined with effective suppression of several angiogenesis and invasion related proteins, silencing of OPN by siRNA using GPT–SPE could inhibit tumor growth in vivo. Taken together, these results suggest that delivery of siRNA using GPT–SPE may have potential as an effective gene carrier for cancer gene therapy.

Acknowledgements

This work was partly supported by the National Research Foundation (NRF-2012-0000102), Ministry of Education, Science and Technology (MEST) as well as by the Technology Development Program for Agriculture (Agriculture Center Program), the Ministry for Food, Agriculture, Forestry and Fisheries, Korea. M.H. Cho was also partially supported by the Research Institute for Veterinary Science, Seoul National University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.04.028.

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