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# Osteopontin Stimulates Tumor Growth and Activation of Promatrix Metalloproteinase-2 through Nuclear Factor-κB-mediated Induction of Membrane Type 1 Matrix Metalloproteinase in Murine Melanoma Cells*

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**Matrix metalloproteinases (MMPs) degrade the extracellular matrix (ECM) and play critical roles in tissue repair, tumor invasion, and metastasis. MMPs are regulated by different cytokines, ECM proteins, and other factors. However, the molecular mechanisms by which osteopontin (OPN), an ECM protein, regulates ECM invasion and tumor growth and modulates MMP activation in B16F10 cells are not well defined. We have purified OPN from human milk and shown that OPN induces pro-MMP-2 production and activation in these cells. Moreover, our data revealed that OPN-induced membrane type 1 (MT1) MMP expression correlates with translocation of p65 (nuclear factor-κB (NF-κB)) into the nucleus. However, when the super-repressor form of IκBα (inhibitor of NF-κB) was transfected into cells followed by treatment with OPN, no induction of MT1-MMP expression was observed, indicating that OPN activates pro-MMP-2 via an NF-κB-mediated pathway. OPN also enhanced cell migration and ECM invasion by interacting with $\alpha_v\beta_3$ integrin, but these effects were reduced drastically when the MMP-2-specific antisense *S*-oligonucleotide was used to suppress MMP-2 expression. Interestingly, when the OPN-treated cells were injected into nude mice, the mice developed larger tumors, and the MMP-2 levels in the tumors were significantly higher than in controls. The proliferation data indicate that OPN increases the growth rate in these cells. Both tumor size and MMP-2 expression were reduced dramatically when anti-MMP-2 antibody or antisense *S*-oligonucleotide-transfected cells were injected into the nude mice. To our knowledge, this is the first report that MMP-2 plays a direct role in OPN-induced cell migration, invasion, and tumor growth and that demonstrates that OPN-stimulated MMP-2 activation occurs through NF-κB-mediated induction of MT1-MMP.**

Cell migration and extracellular matrix invasion are some of the major steps in embryonic development (1, 2) and wound healing and cancer cell metastasis (3, 4). However, the exact molecular mechanisms that regulate these processes are not well understood. In the past, several investigators have shown that matrix metalloproteinases (MMPs)[1] and the tissue inhibitor of matrix metalloproteinase (TIMP) play a major role in the regulation of cancer cell migration, extracellular matrix (ECM) invasion, and metastasis by degrading the ECM proteins (3–5). Current investigations have focused on the understanding of molecular mechanism(s) by which osteopontin (OPN), an ECM protein, regulates MMP expression both *in vitro* and *in vivo* and controls invasiveness and tumor growth in B16F10 cells.

OPN is a noncollagenous, sialic acid-rich, and glycosylated phosphoprotein (6, 7). It has an N-terminal signal sequence, a highly acidic region consisting of nine consecutive aspartic acid residues, and a GRGDS cell adhesion sequence predicted to be flanked by the β-sheet structure (8). This protein has a functional thrombin cleavage site and is a substrate for tissue transglutaminase (7). OPN binds with type I collagen (9), fibronectin (10), and osteocalcin (11). Several highly metastatic transformed cells synthesize higher levels of OPN compared with non-tumorigenic cells (12). It has been shown that OPN also interacts with CD44 receptor globulin (13). OPN causes cell adhesion and migration, ECM invasion, and cell proliferation by interacting with its receptor $\alpha_v\beta_3$ integrin in various cell types (14). Integrins are noncovalently associated, heterodimeric, cell-surface glycoproteins with α- and β-subunits. Integrins are a superfamily of transmembrane glycoproteins found predominantly on the surface of leukocytes that mediate cell-cell and cell-substratum interactions. Until today, ~12 α-subunits, 8 β-subunits, and 20 αβ-heterodimers were documented in the literature (15).

MMPs are a family of enzymes that are classified under different subgroups (16). MMP-2 (also called type IV collagenase or gelatinase A) degrades several ECM proteins such as fibronectin, laminin, type I collagen, and proteoglycans. It plays critical roles in embryogenesis, tissue remodeling, inflammation, periodontitis, and metastasis (16). Several reports have indicated that the increased levels of MMP-2 correlate with the invasive properties of certain tumor cell types (16, 17). TIMP-2 is the specific inhibitor of MMP-2. TIMP-2 is a non-glycosylated protein (21 kDa) that forms a complex with both the inactive and active forms of MMP-2 (18).

Previous studies have demonstrated that the ligation of $\alpha_v\beta_3$ integrin in melanoma cells by anti-$\alpha_v\beta_3$ integrin antibodies enhances the expression of MMP-2, resulting in increased lev-

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[1] The abbreviations used are: MMPs, matrix metalloproteinases; TIMP, tissue inhibitor of matrix metalloproteinase; ECM, extracellular matrix; OPN, osteopontin; MT1, membrane type 1; NF-κB, nuclear factor-κB; ASMMP-2, MMP-2-specific antisense *S*-oligonucleotide; SMMP-2, MMP-2-specific sense *S*-oligonucleotide; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Pen, penicillamine.

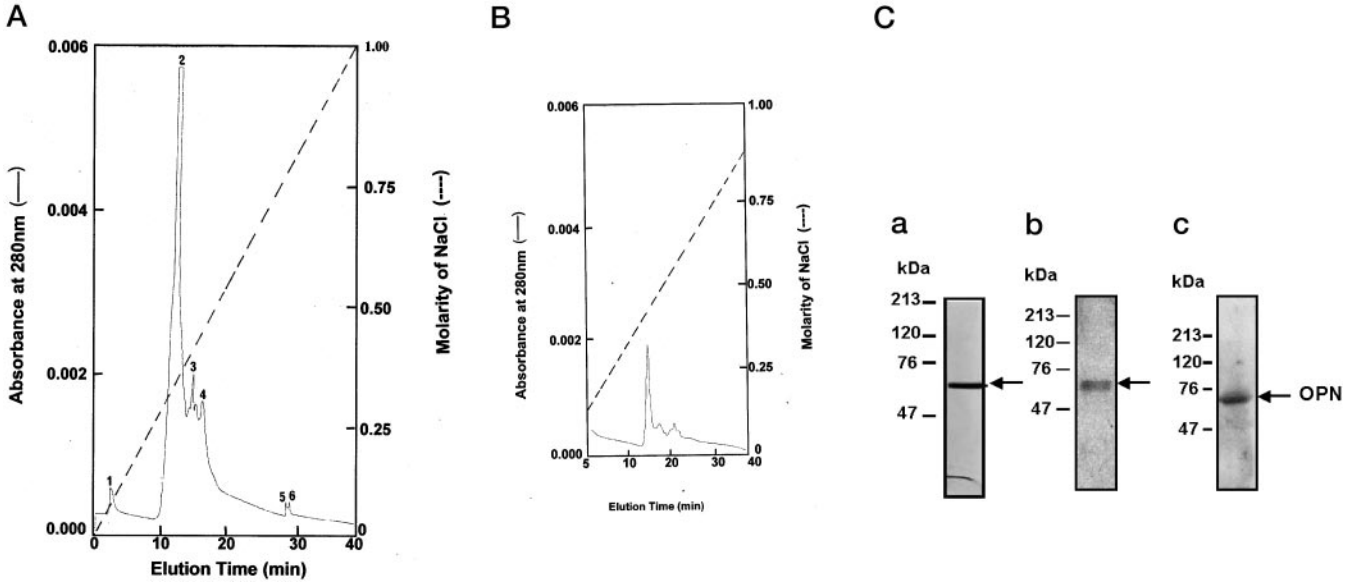


FIG. 1. **Purification and characterization of OPN from human milk.** *A* and *B*, shown are typical separation patterns of OPN in FPLC Resource-Q chromatograms. OPN was partially purified from cleared human milk by DEAE-Sephadex chromatography. The partially purified samples were purified further on an FPLC Resource-Q column with a linear gradient of 0–1 M NaCl in 20 mM Tris-HCl (pH 7.6) over a period of 40 min (*A*). The fraction containing OPN was rechromatographed on an FPLC Resource-Q column (*B*). *C*, the purity of OPN was checked by SDS-PAGE, followed by Coomassie Blue staining (*panel a*). The purified protein was further characterized by Western blot analysis using in-house rabbit anti-OPN antibody (*panel b*) and established commercially available goat IgG-purified anti-OPN antibody (*panel c*).

els of cellular invasiveness (17). Furthermore, treatment of melanoma cells with vitronectin induces the expression of MMP-2 and TIMP-2 as well as enhances cellular invasiveness in a dose-dependent manner (19). Takahashi *et al.* (20) showed that treatment of human melanoma cells with monoclonal anti-CD44 antibody induced the expression of MMP-2 and enhances cell migration and ECM invasion. However, treatment of the same melanoma cells with recombinant OPN-glutathione *S*-transferase fusion protein failed to induce the expression of MMP-2. It was speculated that MMP-2 expression did not occur probably because of the glutathione *S*-transferase fusion with osteopontin (20). Recently, Maquoi *et al.* (21) have shown that type IV collagen induces the activation of MMP-2 in human fibrosarcoma cells.

Here we report that treatment of B16F10 cells with purified human OPN (but not with other ECM proteins such as fibronectin, type I collagen, and laminin) induced the expression of pro-MMP-2 and active MMP-2. We provide evidence that OPN induced membrane type 1 (MT1) MMP expression by stimulating the nuclear factor-κB (NF-κB) pathway. OPN also enhanced cell migration and ECM invasion by interacting with the $\alpha_v\beta_3$ integrin receptor in these cells. Transient transfection of B16F10 cells with the MMP-2-specific antisense *S*-oligonucleotide (ASMMP-2), but not with the sense *S*-oligonucleotide (SMMP-2), caused pronounced inhibition of MMP-2 protein expression and drastic suppression of OPN-induced cell migration and ECM invasion. Interestingly, OPN also induced tumor growth, and expression of MMP-2 in tumors of OPN-injected nude mice was significantly higher than in controls. Moreover, when anti-MMP-2 antibody was injected into OPN-induced tumors, the size of the tumors and the levels of active MMP-2 were reduced dramatically compared with controls. Similarly, when ASMMP-2-transfected cells were injected into the mice, the size of the tumors and the levels of MMP-2 were also suppressed compared with SMMP-2- or LipofectAMINE Plus-injected cells. Taken together, these data indicate that OPN-induces pro-MMP-2 expression and activation and enhances cell migration, ECM invasion, and tumor growth. These data further demonstrate that MMP-2 is mechanistically involved in the regulation of these processes.

## EXPERIMENTAL PROCEDURES

*Materials*—Fibronectin, type I collagen, laminin, GRGDSP, GPen-GRGDSPCA, GRGESP, and LipofectAMINE Plus reagent were purchased from Life Technologies, Inc. Mouse monoclonal anti-MMP-2 (Ab-3) and anti-MT1-MMP antibodies were obtained from Oncogene Research. Rabbit anti-human $\alpha_v\beta_3$ integrin antibody was from Chemicon International. Goat anti-human $\alpha_v$ integrin, mouse monoclonal anti-$\beta_3$ integrin, and rabbit polyclonal anti-NF-κB p65 antibodies were obtained from Santa Cruz Biotechnology. The IgG-purified anti-OPN antibody was purchased from R&D Systems. The anti-OPN antibody was also raised against purified intact human OPN in rabbits and characterized in our laboratory. $Na^{125}I$ (carrier-free; 3.7 GBq/ml) was purchased from Board of Radiation and Isotope Technology (Mumbai, India). Boyden-type cell migration chambers were obtained from Corning Corp. BioCoat Matrigel™ invasion chambers were from Collaborative Biomedical. The nude mice (NMRI, nu/nu) were obtained from the National Institute of Virology (Pune, India). All other chemicals were analytical grade.

*Cell Culture*—The murine melanoma cells (B16F10) were obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C.

*Purification and Detection of OPN in Human Milk*—Human milk was collected from a local hospital. OPN was purified by DEAE-Sephadex chromatography, followed by FPLC. Briefly, the milk sample was centrifuged, and the cleared sample was loaded onto the DEAE-Sephadex column. The column was washed with 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl and eluted with the same buffer containing 500 mM NaCl. The fractions containing OPN were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (22), followed by Western blot analysis. Briefly, the OPN sample was resolved by SDS-PAGE and electrotransferred from the gel to a nitrocellulose membrane. The membrane was incubated with established IgG-purified anti-OPN antibody (1:5000 dilution). It was washed, incubated further with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000 dilution), and detected using an ECL detection system (Amersham Pharmacia Biotech). Partially purified OPN was purified further on an FPLC Resource-Q column with a linear gradient of 0–1 M NaCl in 20 mM Tris-HCl (pH 7.6) over a period of 40 min and rechromatographed under the same conditions on the Resource-Q column. The final purity of OPN was checked by SDS gel and Western blot analyses using both established and in-house anti-OPN antibodies as described above. The concentration of OPN was measured by the Brad-

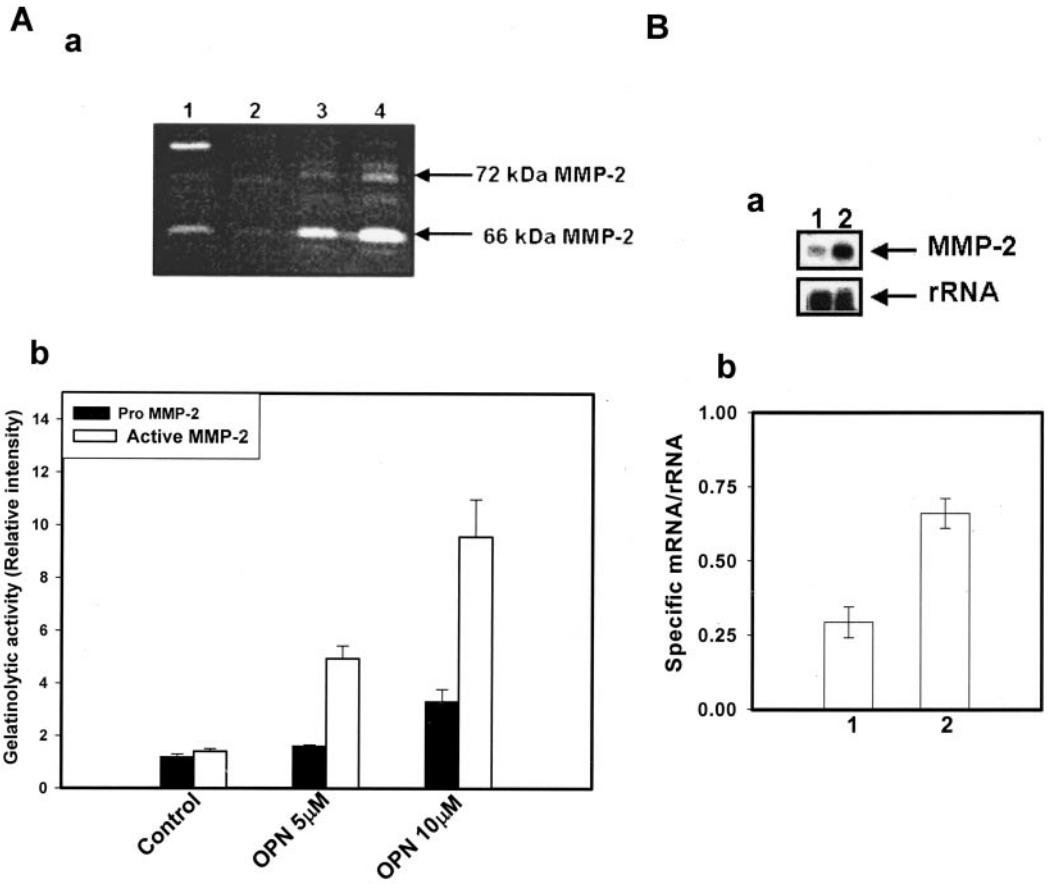


FIG. 2. *A*, detection of MMP-2 expression in B16F10 cells by zymography. *Panel a*, the cells were treated in the absence or presence of OPN (0–10.0 $\mu$M); the conditioned media were collected; and MMP-2 activity was analyzed by gelatin zymography. *Lane 1*, purified MMP-2 as a standard; *lane 2*, untreated cells; *lanes 3*, cells treated with 5 $\mu$M OPN; *lane 4*, cells treated with 10 $\mu$M OPN. The *arrows* indicate both 72- and 66-kDa MMP-2-specific bands. *Panel b*, the levels of MMP-2 (pro and active) were quantified by densitometric analysis. *B*, expression of MMP-2 mRNA by Northern blot analysis. *Upper panel a*, shown are the faint and intense MMP-2-specific mRNA bands in the untreated (*lane 1*) and OPN-treated (*lane 2*) cells. *Lower panel a*, the rRNA bands are shown as a control. *Panel b*, RNA bands were quantified by densitometric analysis, and the ratio of specific mRNA to rRNA is represented in the form of a *bar graph*. In both *panels b*, the mean values of three experiments are calculated.

ford method (Bio-Rad protein assay) according to the manufacturer's instructions.

*Zymography Experiments*—To check the effect of OPN on MMP-2 expression and activation, the B16F10 cells were treated with varying concentrations of purified OPN (0–10.0 $\mu$M) in serum-free medium and incubated at 37 °C for 24 h. The conditioned media were collected by centrifugation, concentrated, and dialyzed. The dialyzed samples containing an equal amount of total proteins were mixed with sample buffer in the absence of reducing agent, incubated at room temperature for 30 min, and loaded onto zymographic SDS gel containing gelatin (0.5 mg/ml) as described (23). The gels were washed and incubated in incubation buffer (50 mM Tris-HCl (pH 7.5) containing 100 mM $CaCl_2$, 1 $\mu$M $ZnCl_2$, 1% (v/v) Triton X-100, and 0.02% (w/v) $NaN_3$) for 16 h. The gels were stained with Coomassie Blue and destained. The zones of gelatinolytic activity were shown by negative staining. Purified MMP-2 was used as a control. In separate experiments, the cells were treated with other ECM proteins (5.0 $\mu$M each fibronectin, type I collagen, or laminin) or peptides (10.0 $\mu$M each GRGDSP, GPenGRGDSPCA, or GRGESP) and incubated under the same conditions described above. The conditioned media were collected and used for zymography.

*Northern Blot Analysis*—To analyze the levels of MMP-2 and TIMP-2 mRNAs in untreated and OPN-treated cells, the cells were first treated with OPN (10 $\mu$M), and total RNA was extracted using the RNAzol method (Tel-Test) according to the manufacturer's instructions. RNA concentration was measured spectrophotometrically at absorbances of 260 and 280 nm. An equal amount of total RNA (20 $\mu$g) was denatured in formaldehyde and resolved by electrophoresis on formaldehyde-agarose gels. The RNA samples were transferred from the gels to nylon membranes and cross-linked by UV irradiation. Hybridization was performed using an $\alpha$-$^{32}$P-labeled mouse MMP-2 or TIMP-2 probe. The 708-base pair MMP-2 cDNA probe was obtained by polymerase chain reaction amplification, followed by hybridization with an MMP-2-specific probe. The 508-base pair TIMP-2 probe was obtained by digesting plasmid pBluescript SK containing partial-length TIMP-2 cDNA (nucleotides 1173–1680) (a generous gift from Prof. M. Seiki, University of Tokyo, Tokyo, Japan) with *Hin*dIII. The DNA was purified by agarose gel electrophoresis. The probe was labeled with [$\alpha$-$^{32}$P]dCTP using a random primer labeling kit (Bangalore Genei, Bangalore, India) according to the manufacturer's instructions. The blots were exposed to Eastman Kodak X-Omat AR x-ray film and autoradiographed.

*Nuclear and Cytoplasmic Extracts, Transfection, and Western Blotting*—The nuclear extracts were prepared using a modification (24) of the method of Dignam (40). Briefly, cells were incubated in the absence or presence of OPN (5 $\mu$M) in serum-free medium at 37 °C for 24 h. Cells were scraped, washed with PBS, resuspended in hypotonic buffer (10 mM Hepes (pH 7.9), 10 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol), and allowed to swell on ice for 10 min. Cells were homogenized in a Dounce homogenizer. The nuclei were separated by spinning at 3300 $\times$ *g* for 5 min at 4 °C and resuspended in low salt buffer (20 mM Hepes (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 1.2 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol), followed by the addition of an equal volume of high salt buffer. The lysed nuclei were centrifuged at 12,000 $\times$ *g* for 5 min at 4 °C, and the cleared supernatant was collected. The post-nuclear supernatant was used for cytoplasmic extraction. To this supernatant was added 0.1 volume of cytoplasm extraction buffer (0.3 mM Hepes (pH 7.9) containing 30 mM $MgCl_2$ and 1.4 M KCl). The sample was centrifuged, and the cleared supernatant was collected. The protein concentrations in the supernatants of both nuclear and cytoplasmic extracts were measured by the Bio-Rad protein assay. The level of NF-$\kappa$B p65 in both nuclear and cytoplasmic extracts was detected by Western blot analysis using rabbit polyclonal anti-NF-$\kappa$B p65 antibody as described above. In other experiments, the super-repressor form of I$\kappa$B$\alpha$ fused downstream to a FLAG epitope in an expression vector

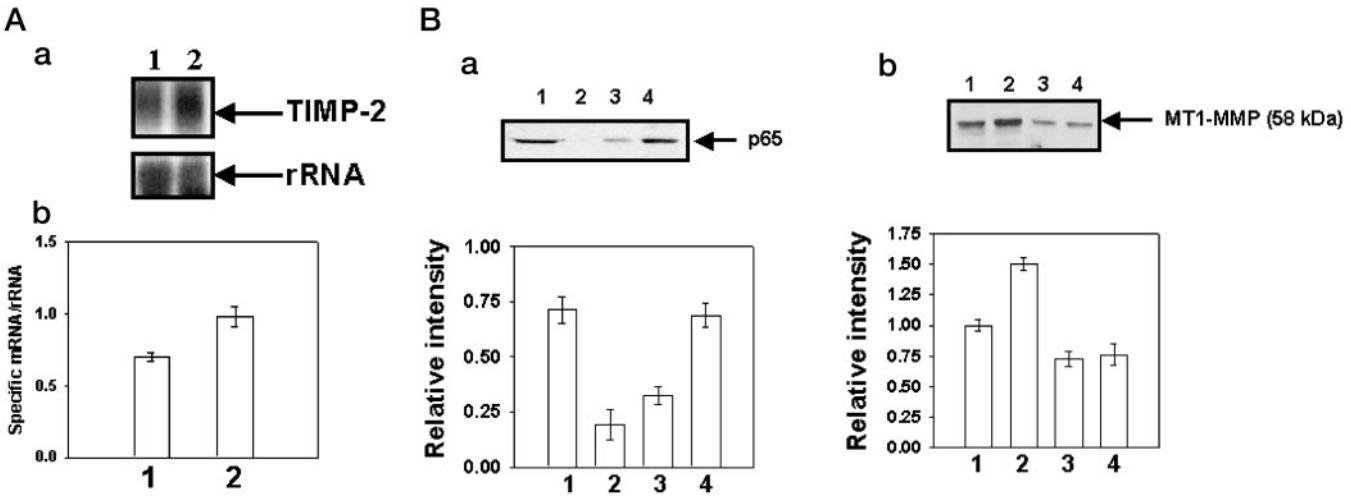


FIG. 3. *A*: expression of TIMP-2 mRNA by Northern blot analysis. *Upper panel a*, TIMP-2-specific mRNA bands are shown in *lane 1* (untreated cells) and *lane 2* (OPN-treated cells). *Lower panel a*, rRNA bands are shown as a control. Note that there was an increase in TIMP-2 mRNA expression in the OPN-treated cells. RNA bands were quantified by densitometric analysis, and the ratio of specific mRNA to rRNA is represented in the form of a bar graph (*panel b*). These experiments were performed in triplicate, and the mean value was analyzed statistically. *B*: *panel a*, effect of OPN on cellular localization of NF-κB p65. Nuclear and cytoplasmic extracts from untreated and OPN-treated cells were immunoblotted with rabbit polyclonal anti-NF-κB p65 antibody (*upper panel a*). In the untreated cells, the majority of the p65 was detected in the cytoplasm, but not in the nucleus (*lanes 1* and *2*, respectively). In contrast, upon OPN treatment, p65 was translocated from the cytoplasm to the nucleus (*lanes 3* and *4*, respectively). The relative intensity of p65 in the cytoplasm and nucleus was analyzed and is represented in the form of a *bar graph* (*lower panel a*). *Panel b*, inhibition of OPN-induced MT1-MMP expression by the NF-κB super-repressor IκBα. The cells were transfected with the super-repressor form of IκBα in pCMV4 and then treated in the absence or presence of OPN. The cell lysates were used for the detection of MT1-MMP by Western blot analysis (*upper panel b*). *Lane 1*, untreated non-transfected cells; *lane 2*, OPN-treated non-transfected cells; *lane 3*, untreated transfected cells; *lane 4*, OPN-treated transfected cells. Note that there was an increase in MT1-MMP expression in OPN-treated cells (*lane 2*) compared with control cells (*lane 1*), whereas in transfected cells, no differences in expression of MT1-MMP were observed in both untreated (*lane 3*) and OPN-treated (*lane 4*) cells. The bands were analyzed by densitometry and are represented in the form of a *bar graph* (*lower panel b*). The mean values of triplicate experiments are indicated in both *lower panels a* and *b*.

(pCMV4) was transiently transfected into cells using LipofectAMINE Plus reagent according to the manufacturer's instructions. Briefly, the vector containing IκBα was mixed with plus reagent in Opti-MEM I and incubated at room temperature for 15 min. LipofectAMINE was mixed with DNA plus reagent and incubated further at room temperature for 15 min. The LipofectAMINE Plus-DNA complex was added to cells and mixed by gentle agitation. After 48 h, the cells were treated in the absence or presence of OPN (5 μM) and incubated further at 37 °C for 24 h. Cells were extracted with radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride) at 4 °C for 2 h. The supernatant was collected by centrifugation and used for detection of MT1-MMP by Western blotting using mouse monoclonal anti-MT1-MMP antibody as described above.

*Cell Migration Assay*—The migration assay was conducted using a Transwell cell culture chamber according to the standard procedure described (25). Briefly, the confluent monolayer of B16F10 cells was harvested with trypsin/EDTA and centrifuged at 800 × *g* for 10 min. The cell suspension ($5 \times 10^5$ cells/well) was added to the upper chamber of a pre-hydrated polycarbonate membrane filter. Purified intact OPN (0–10 μM) was added to the upper chamber. The lower chamber was filled with fibroblast-conditioned medium, which acted as a chemoattractant. To ascertain whether OPN-stimulated migration occurred via $\alpha_v\beta_3$ integrin, the cells were pretreated with anti-human $\alpha_v\beta_3$ integrin antibody (40 μg/ml) for 20 min, and then OPN was added (5.0 μM). Similarly, the cells were also pretreated with monoclonal anti-MMP-2 antibody (50.0 μg/ml), and then OPN was added. In other experiments, the cells were individually treated with GRGDSP, GRGESP, or GPenGRGDSPCA (5.0 μM each) for 20 min and then incubated with OPN (5.0 μM). After treatment, the cells were incubated in a humidified incubator in 5% $CO_2$ and 95% air at 37 °C for 24 h. The non-migrating cells on the upper side of the filter were scraped and washed. The migrating cells on the reverse side of the filter were stained with Giemsa. The migrating cells on the filter were counted and a photomicrograph was taken under an Olympus inverted microscope. In separate experiments, SMMP-2- or ASMMP-2-transfected cells ($5 \times 10^5$ cells/well) were added to the upper chamber, and a migration assay was performed under the same conditions as described above. The experiments were repeated in triplicate. Preimmune IgG served as a nonspecific control.

*ECM Invasion Assay*—The ECM invasion assay was performed using commercially available 24-well plates that consist of upper and lower chambers as described (26, 27). The two chambers were divided by a porous filter, the upper surface of which was precoated with a layer of artificial basement membrane, Matrigel™ (Collaborative Biomedical). The cell suspension ($5 \times 10^5$ cells/well) was added to the upper chamber. The lower chamber was filled with fibroblast-conditioned medium. which acted as a chemoattractant. The cells were treated with varying concentrations of purified intact OPN (0–10.0 μM) and then added to the upper chamber. In some experiments, the cells were pretreated with anti-human $\alpha_v\beta_3$ integrin antibody (40.0 μg/ml), monoclonal anti-MMP-2 antibody (50.0 μg/ml), GRGDSP (5.0 μM), GRGESP (5.0 μM), or GPenGRGDSPCA (5.0 μM) for 20 min and then incubated with OPN (5.0 μM) at 37 °C for 24 h as described above. The non-migrating cells and Matrigel from the upper side of the filter were scraped and removed using a moist cotton swab. The invaded cells in the lower side of the filter were stained with Giemsa and washed with PBS (pH 7.6). The invaded cells were then counted, and photomicrographs were taken under the Olympus inverted microscope. In other experiments, SMMP-2- or ASMMP-2-transfected cells ($5 \times 10^5$ cells/well) were added to the upper chamber, and the invasion assay was done under the same conditions as described above. The experiments were repeated in triplicate. Preimmune IgG was used as a nonspecific control.

*Proliferation Assay*—The proliferation assay was performed as described previously (14). Briefly, serum-starved B16F10 cells were incubated in the absence or presence of increasing concentrations of OPN (0–10 μM) at 37 °C for 24 h. After 4 h, [$^3$H]thymidine (1 μCi/ml) was added, and the cells were maintained in culture for another 20 h. After removing the supernatants, the cells were washed with basal medium and lysed in 50% trichloroacetic acid. The acid-precipitable cell-bound radioactivity was measured with a scintillation counter (Packard Instrument Co.).

*Cell-surface Labeling*—To detect the level of $\alpha_v\beta_3$ integrin in B16F10 cells, the cells were surface-labeled with $Na^{125}I$ and IODO-BEADS as described (28). The cells were lysed in lysis buffer composed of 1% Triton X-100 solution containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM EDTA and immunoprecipitated individually with anti-human $\alpha_v$ or monoclonal anti-$\beta_3$ integrin antibody (Roche Molecular Biochemicals) according to the manufacturer's instructions. The samples were boiled in SDS sample buffer, electrophoresed, and autoradiographed.

*Radioreceptor Assay*—Purified human OPN (20 μg) was radioiodi-

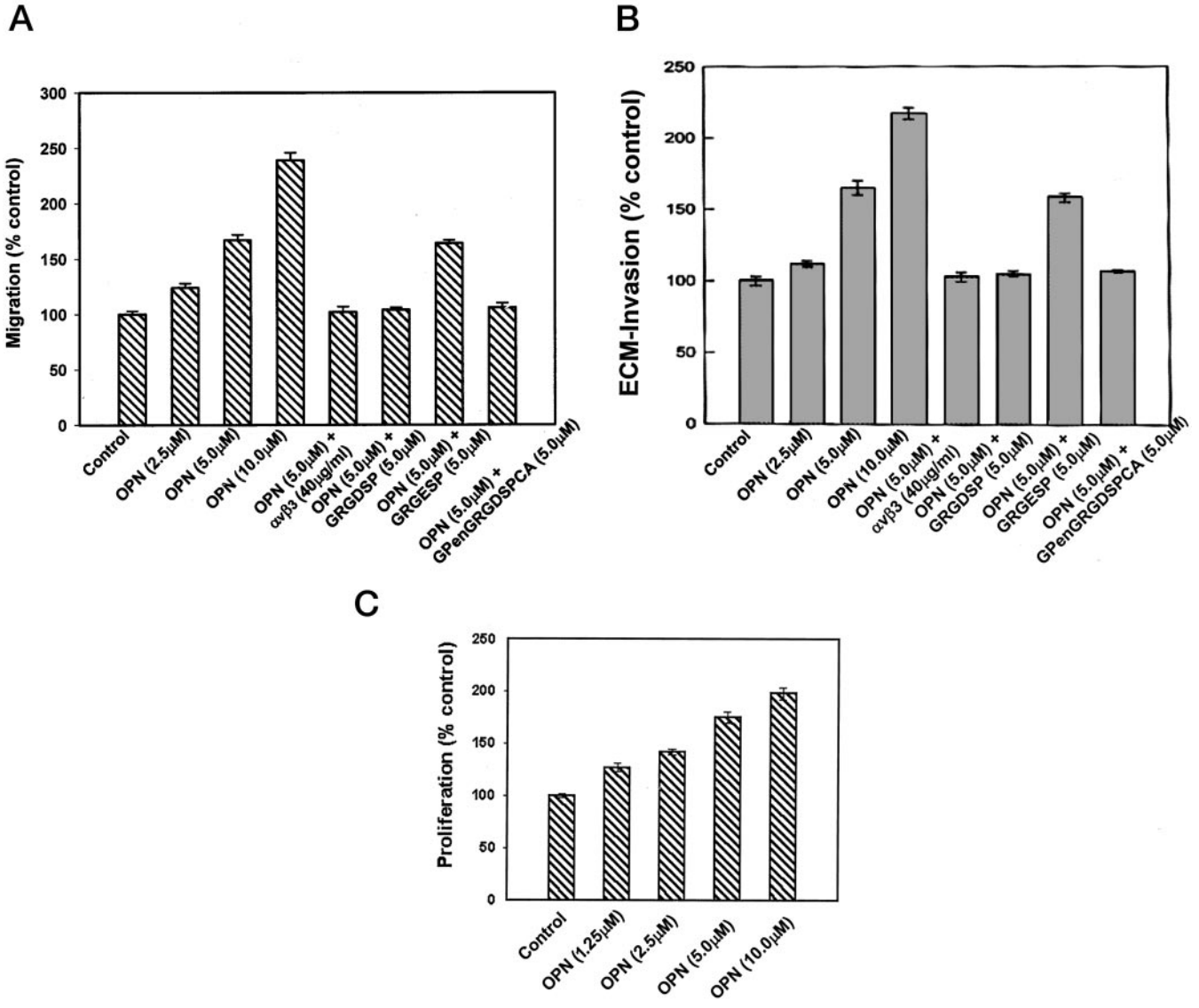


FIG. 4. **Effect of OPN on cell migration (*A*), ECM invasion (*B*), and proliferation (*C*).** *A*, the migration assay was performed using either untreated cells ($5 \times 10^5$ cells/well) or cells treated with three different concentrations of purified human OPN (2.5–10.0 $\mu$M) in the upper chamber. In separate experiments, the cells were pretreated individually with anti-$\alpha_v\beta_3$ antibody (40 $\mu$g/ml), GRGDSP (5.0 $\mu$M), GRGESP (5.0 $\mu$M), or GPenGRGDSPCA (5.0 $\mu$M) and then treated with OPN (5.0 $\mu$M) in the upper chamber. The results are expressed as the means ± S.E. of three determinations. *B*, the ECM invasion assay was done using either untreated cells ($5 \times 10^5$ cells/well) or cells treated with three different concentrations of purified human OPN (2.5–10.0 $\mu$M) in the upper chamber. In separate experiments, the cells were also pretreated with anti-$\alpha_v\beta_3$ integrin antibody (40 $\mu$g/ml), GRGDSP (5.0 $\mu$M), GRGESP (5.0 $\mu$M), or GPenGRGDSPCA (5.0 $\mu$M) and then treated with OPN (5.0 $\mu$M). The results are expressed as the means ± S.E. of three determinations. *C*, the proliferation assay was carried out by treating the cells with different doses of OPN (1.25–10.0 $\mu$M) in serum-free medium. The results are expressed as the means ± S.E. of three determinations. In all three experiments, preimmune IgG served as a control.

nated using $Na^{125}I$ (carrier-free; 2 mCi) and chloramine T as described (29). $^{125}$I-OPN was purified by Sephadex G-50 column chromatography, and radioactivity was measured in a $\gamma$-counter (Beckman Instruments) with a counting efficiency of ~85%. The specific activity of carrier-free purified monoiodinated OPN was 78 $\mu$Ci/$\mu$g. For binding assays, subconfluent cultures of B16F10 cells were incubated with $^{125}$I-OPN ($3.0 \times 10^5$ cpm/well) in the absence or presence of increasing concentrations of unlabeled purified OPN in 1.0 ml of Hanks' balanced salt solution (pH 7.6) containing 0.1% bovine serum albumin. After incubation at 37 °C for 2 h, the reactions were stopped by rapid removal of medium containing unbound radiolabeled OPN. The cells were solubilized with 1 N NaOH and neutralized with 1 N HCl, and radioactivity was measured in the $\gamma$-counter. Specific binding was calculated by subtracting nonspecific binding from total binding. The $K_d$ value was determined by Scatchard analysis using the LIGAND computer program (30).

*Oligonucleotide Treatments*—Murine ASMMP-2 (5′-CCA CTC GTG CCT CCA TCG TT-3′) and SMMP-2 (5′-AAC GAT GGA GGC ACG AGT-3′) oligonucleotides with phosphorothioate linkages were synthesized (Gemini Biotech). These oligonucleotides were purified by column chromatography, and purity was checked by polyacrylamide gel electrophoresis. The B16F10 cells were pretreated with OPN (10 $\mu$M) at 37 °C for 6 h and then transfected transiently with the sense or antisense *S*-oligonucleotide using LipofectAMINE Plus according to the manufacturer's instructions. Briefly, the sense or antisense *S*-oligonucleotide was mixed with plus reagent, and then oligonucleotide reagent plus was incubated with LipofectAMINE. The LipofectAMINE Plus-oligonucleotide complex was added to the cells and incubated further at 37 °C for 8 h. The control cells received LipofectAMINE Plus alone. The cell viability was detected by a trypan blue dye exclusion test. After incubation, the oligonucleotide-containing medium was removed, and the cells were refed with fresh medium and cultured for an additional 12 h. The serum-free conditioned medium was used for the detection of MMP-2 by zymography, and the cells were used for migration, ECM invasion, and tumorigenicity experiments as described above. To check the dose-dependent response, a separate transfection experiment was performed with different doses (0–10 $\mu$g) of ASMMP-2.

*In Vivo Tumorigenicity*—The tumorigenicity experiments were performed as described (31). The cells were grown in monolayer and

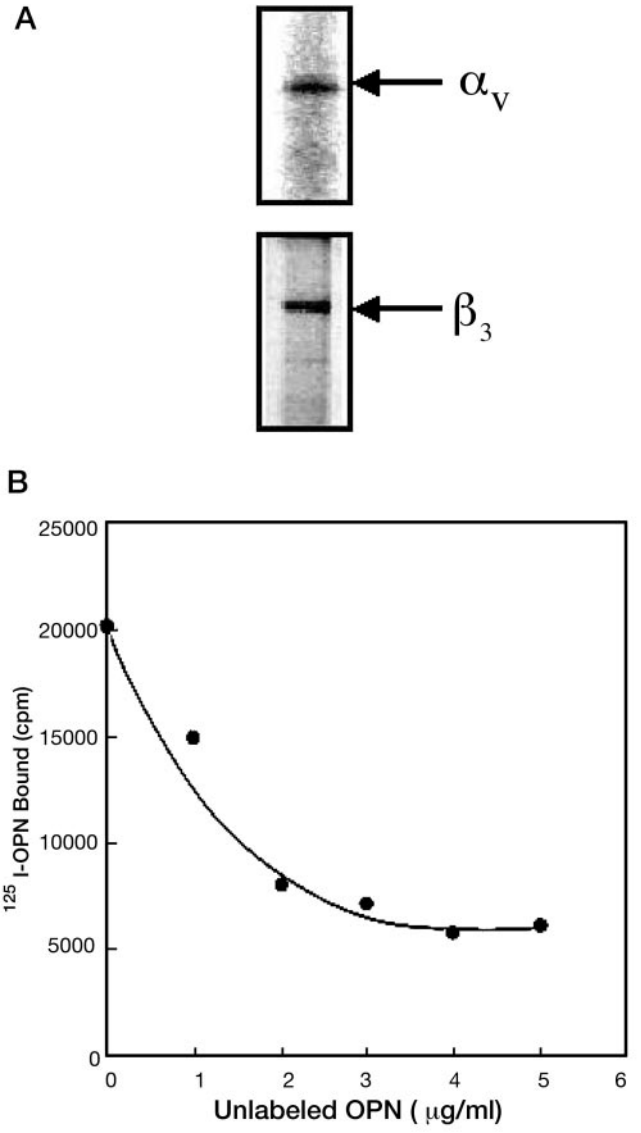


FIG. 5. *A*, cell-surface labeling of $\alpha_v\beta_3$ integrin in B16F10 cells. The cells were labeled with $Na^{125}I$, individually immunoprecipitated with anti-$\alpha_v$ (*upper panel*) or anti-$\beta_3$ (*lower panel*) integrin antibody, resolved by SDS-PAGE, and autoradiographed. *B*, binding of OPN to its receptor in B16F10 cells. $^{125}$I-OPN was incubated with B16F10 cells in the absence or presence of increasing concentration of unlabeled OPN (0–5.0 μg/ml) at 37 °C for 2 h. The data are from three experiments, and each *point* represents the mean of triplicate determinations.

treated in the absence or presence of purified human OPN (10 μM) in serum-free medium. The cells were incubated at 37 °C for 24 h. After that, the cells ($5 \times 10^6$ cells/0.2 ml) were detached, centrifuged, washed, and injected subcutaneously into the flanks of male athymic NMRI (nu/nu) mice (6–8 weeks old). Four mice were used in each set of experiments. The mice were kept under specific pathogen-free conditions. OPN (10 μM) was again injected into the tumor sites twice a week for up to 4 weeks. In other experiments, the cells were treated with OPN (10 μM) and injected into mice. A mixture (0.2 ml) of anti-MMP-2 antibody (50 μg/ml) and OPN (10.0 μM) was injected into the same tumor sites twice a week for up to 4 weeks. In separate experiments, the LipofectAMINE Plus-oligonucleotide (ASMMP-2 or SMMP-2) complex was injected into the tumors of the nude mice. After 4 weeks, the mice were killed, and the tumor weights were measured. The tumor tissues were homogenized; lysed in lysis buffer composed of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 15 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride; and centrifuged at 12,000 × *g* for 10 min. The clear supernatants were collected, and MMP-2 expression was detected by Western blot analysis (32). Briefly, the sample containing an equal amount of total proteins was resolved by SDS-PAGE. The protein was electrotransferred from the gel to a nitrocellulose membrane. The membrane was incubated with mouse monoclonal anti-MMP-2 antibody (1:250 dilution). It was washed, incubated further with horseradish peroxidase-conjugated anti-mouse IgG (1:2000 dilution), and detected using the ECL detection system as described above. The levels of MMP-2 in tumor samples were also analyzed by zymography and Northern blot analysis as described above.

## RESULTS

*OPN Purification and Western Blot Analysis*—Native OPN was purified from human milk by DEAE-Sephadex chromatography, followed by final purification on an FPLC Resource-Q column. The cleared milk sample was passed through a DEAE-Sephadex column. The protein sample was eluted with 10 mM sodium phosphate buffer (pH 7.0) containing 500 mM NaCl. The presence of OPN was checked by SDS-PAGE, followed by Western blot analysis using both in-house and established anti-OPN antibodies. The major fraction containing OPN was rechromatographed on an FPLC Resource-Q column, and the chromatogram is shown in Fig. 1*A*. Of six fractions in Fig. 1*A*, fraction 3 showed the OPN-specific band as judged by SDS-PAGE and Western blot analysis (data not shown). Fraction 3 was rechromatographed using a Resource-Q column and showed a major single symmetrical peak (Fig. 1*B*). The concentration of OPN was measured by the Bio-Rad protein assay. The final purity of OPN was checked by SDS-PAGE, followed by Coomassie Blue staining (Fig. 1*C*, *panel a*), and further characterized by Western blot analysis using rabbit anti-OPN (*panel b*) and established goat IgG-purified anti-OPN (*panel c*) antibodies.

*OPN Induces MMP-2 Expression at the Protein and mRNA Levels*—To check the effect of OPN on pro-MMP-2 production and activation, the B16F10 cells were treated with increasing concentrations of OPN (0–10.0 μM). The conditioned media were collected, and the gelatinolytic activity of MMP-2 was detected by zymography. Increased levels of MMP-2 expression (72-kDa pro and 66-kDa active forms) were observed when the cells were treated with two different concentrations of OPN (Fig. 2*A*, *panel a*, *lanes 3* and *4*). Minor bands of pro-MMP-2 and active MMP-2 were detected in the untreated cells (*lane 2*). Purified MMP-2 was used as a positive control (*lane 1*). The levels of active MMP-2 and pro-MMP-2 expression (gelatinolytic activity) were quantified densitometrically (Kodak Digital Science) and analyzed statistically. As compared with controls, there were at least 5- and 9.8-fold increases in MMP-2 activation when the cells were treated with 5.0 and 10.0 μM OPN, respectively (*panel b*). To ascertain the specificity of induction of MMP-2 expression, the cells were individually treated with fibronectin, type I collagen, laminin, GRGDSP, GPenGRGDSPCA, or GRGESP prior to zymography. No significant levels of MMP-2 expression were observed by zymography (data not shown).

The OPN-induced MMP-2 expression in these cells was reconfirmed by Northern blot analysis. The intensity of the MMP-2-specific band was increased when the cells were treated with 10 μM OPN (Fig. 2*B*, *upper panel a*, *lane 2*). However, a minor MMP-2-specific band was observed in the untreated cells (*lane 1*). The low level of MMP-2 mRNA expression in the untreated cells was not due to degradation of RNA because strong bands of rRNA were obtained, a result that was virtually identical in each of the untreated and OPN-treated samples (*lower panel a*). The level of MMP-2 mRNA was also quantified by densitometry and analyzed statistically. There was at least a 2.8-fold increase in MMP-2 mRNA expression in OPN-treated cells compared with control cells (*panel b*). These data were corroborated by the zymography data (Fig. 2*A*).

*OPN Induces MT1-MMP Expression by Activating NF-κB*—Since the mechanism for activation of pro-MMP-2 involves complex formation with MT1-MMP and TIMP-2, we sought to

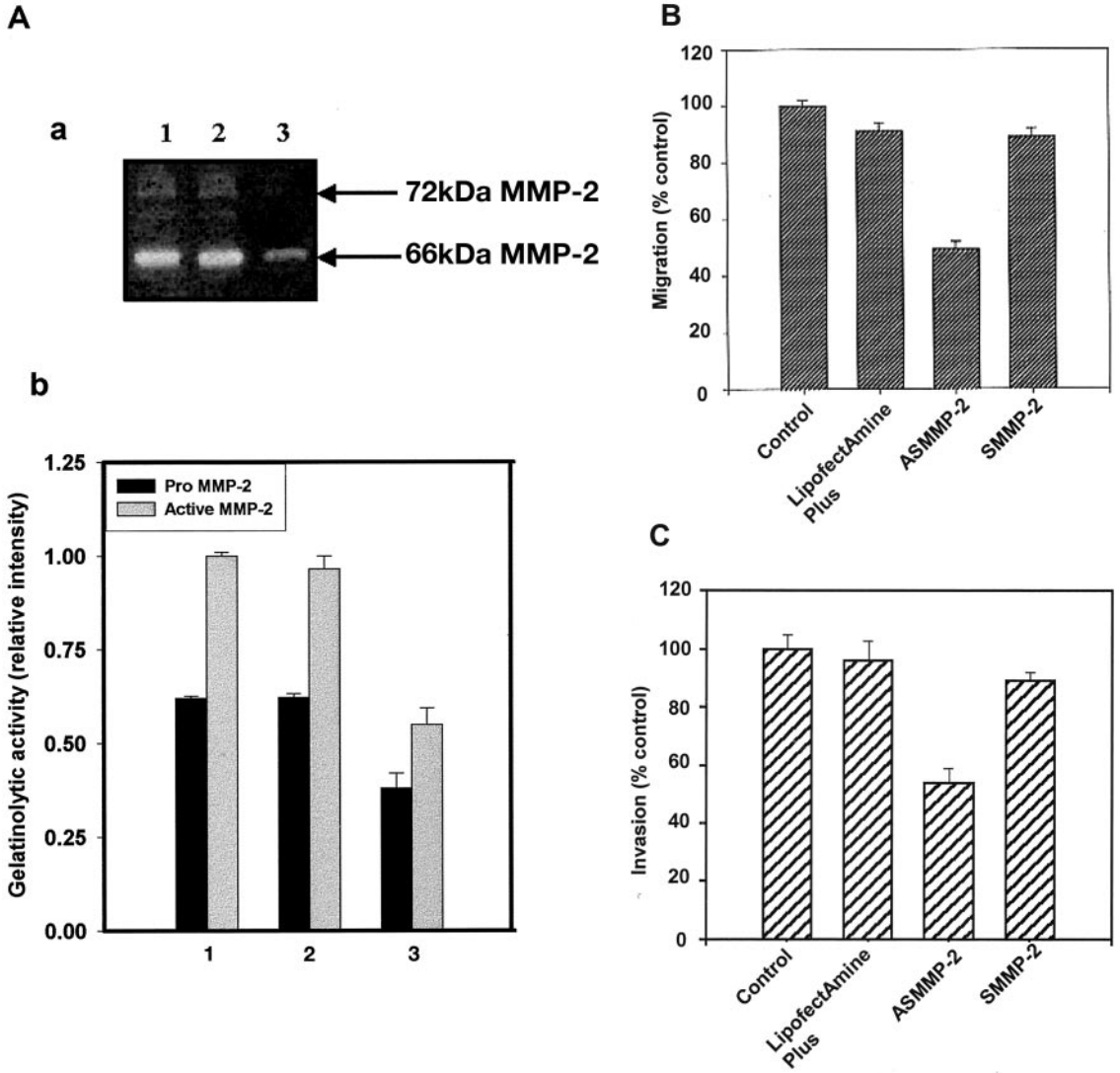


FIG. 6. *A*, detection of MMP-2 expression in MMP-2-specific *S*-oligonucleotide-transfected cells by zymography. *Panel a*, cells were pretreated with OPN and then transfected with LipofectAMINE Plus alone (*lane 1*) or with SMMP-2 (*lane 2*) or ASMMP-2 (*lane 3*). The conditioned media were collected and used for zymography. The *arrows* indicate the 72-kDa (pro) and 66-kDa (active) MMP-2 bands. *Panel b*, the levels of MMP-2 were quantified by densitometric analysis and are represented in the form of a *bar graph*. The mean value of triplicate experiments is indicated. *B*, effect of transfection of OPN-treated B16F10 cells with MMP-2-specific *S*-oligonucleotide on cell migration. Cells were pretreated with OPN and then transfected with LipofectAMINE Plus alone or with SMMP-2 or ASMMP-2 as described above. Both non-transfected and transfected cells were used for the cell migration assay as described under "Experimental Procedures." The cells transfected with ASMMP-2 showed dramatic reduction of cell migration compared with non-transfected cells or cells transfected with LipofectAMINE Plus alone or with SMMP-2. The results are expressed as the means ± S.E. of three determinations. *C*, effect of transfection of OPN-treated cells with MMP-2 specific *S*-oligonucleotide on ECM invasion. Cells were pretreated with OPN, transfected, and then used for the ECM invasion assay as described above. The cells transfected with ASMMP-2 showed dramatic reduction of invasion compared with non-transfected cells or cells transfected with LipofectAMINE Plus alone or with SMMP-2. The results are expressed as the means ± S.E. of three determinations.

determine whether OPN has any role in MT1-MMP expression. The Western blot analysis data indicated that the level of MT1-MMP expression (58 kDa) was 1.5 times higher in OPN-treated cells than in untreated cells (Fig. 3*B*, *upper panel b*, *lanes 1* and *2*, respectively). Since it has been previously shown that OPN stimulates NF-κB signaling by interacting with $\alpha_v\beta_3$ integrin in endothelial cells (33), we therefore speculated that NF-κB might be involved in OPN-induced MMP-2 activation in B16F10 cells. Our Western blot data revealed that p65 was localized mostly in the cytoplasm (*upper panel a*, *lane 1*) compared with the nucleus (*lane 2*) in the OPN-untreated cells. However, it was translocated from the cytoplasm (*lane 3*) to the nucleus (*lane 4*) in the OPN-treated cells. The Western blot data were further quantified by densitometric statistical analyses (*lower panel a*).

To check whether OPN-induced MT1-MMP expression occurs through the NF-κB/IκBα pathways, we transiently transfected the cells with the super-repressor form of IκBα and then treated them with OPN. The expression of MT1-MMP in untreated and OPN-treated cells was almost identical (Fig. 3*B*, *upper panel b*, *lanes 3* and *4*). These Western blot data were also analyzed (*lower panel b*). These results suggest that OPN activates the NF-κB signaling pathway, leading to induction of MT1-MMP and subsequently activating pro-MMP-2.

*OPN Stimulates TIMP-2 Expression in B16F10 Cells*—To determine whether OPN has any role in induction of TIMP-2 expression in B16F10 cells, the RNA was isolated from the untreated and OPN-treated cells, and expression of TIMP-2 was analyzed by Northern blot analysis. The level of TIMP-2 expression was increased in the OPN-treated cells (Fig. 3*A*, *upper panel a*, *lane 2*), but the level of TIMP-2 expression was lower in the untreated cells (*lane 1*). The rRNA bands are

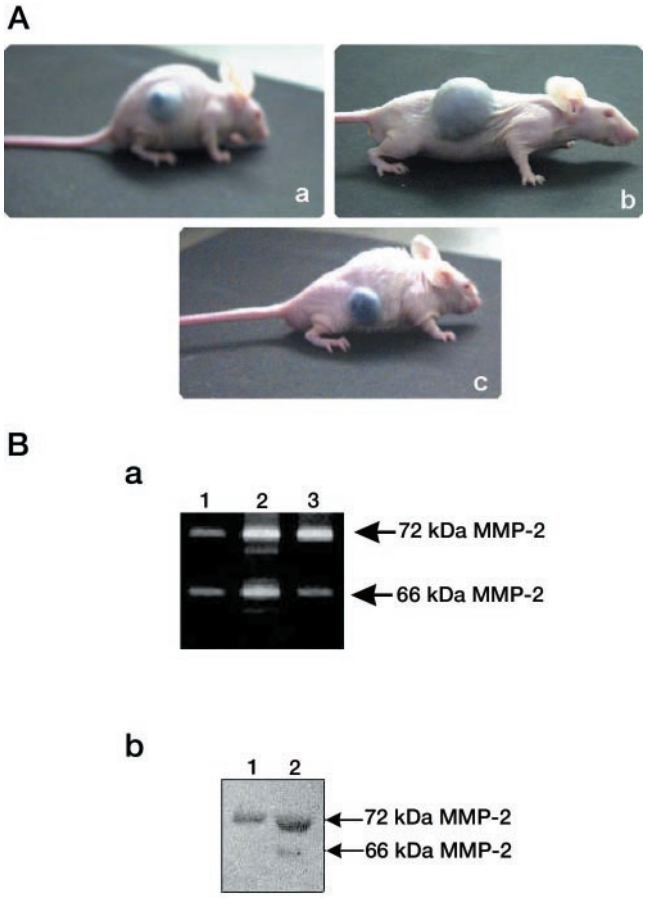


FIG. 7. *A*, typical photographs of OPN-induced tumors in nude mice. The B16F10 cells were treated in the absence or presence of OPN (10.0 $\mu$M) and injected subcutaneously into the flanks of nude mice. In separate experiments, the OPN-treated cells were injected into the nude mice, and then anti-MMP-2 antibody (10.0 $\mu$g/0.2 ml) was injected into the tumor sites. Four mice were used in each set of experiments. *Panel a*, cells with PBS; *panel b*, cells with OPN; *panel c*, cells with OPN and anti-MMP-2 antibody. *B*, detection of MMP-2 expression in the tumors of nude mice by gelatin zymography (*panel a*) and Western blotting (*panel b*). *Panel a*, the tumor samples from *A* were lysed in lysis buffer and analyzed by gelatin zymography. Equal amount of total proteins was used in each lane. *Lane 1*, PBS alone; *lane 2*, with OPN; *lane 3*, with OPN and anti-MMP-2 antibody. The *arrows* indicate the 72- and 66 kDa-MMP-2-specific bands. *Panel b*, the tumor samples from *A* were lysed. Equal amounts of total proteins were electrophoresed and transferred to the nitrocellulose membranes. The membranes were immunostained with mouse monoclonal anti-MMP-2 antibody and detected by ECL detection system as described under "Experimental Procedures." *Lane 1*, PBS alone; *lane 2*, with OPN.

shown as a control (*lower panel a*, *lanes 1* and *2*). The densitometric data indicated that there was a slight increase in TIMP-2 expression in the OPN-treated cells, whereas the untreated cells expressed low levels of TIMP-2 (*panel b*).

*OPN Enhances in Vitro Cell Migration, ECM Invasion, and Cell Proliferation*—To examine the effect of OPN on B16F10 cells, we used established *in vitro* assay systems to evaluate the effects of this protein on cell migration, ECM invasion, and proliferation. Fig. 4*A* shows the dose-dependent increases in cell migration (123–239%) when increasing concentrations of OPN (2.5–10.0 $\mu$M) were used in the upper chamber. The OPN-untreated cells are used as controls (100% migration). Interestingly, the OPN-induced migration was drastically reduced when the cells were pretreated individually with anti-human $\alpha_v\beta_3$ integrin antibody, GRGDSP, or GPenGRGDSPCA (but not with GRGESP) prior to the addition of OPN (Fig. 4*A*). Similarly, there was a dramatic increase in ECM invasion (114–219%) when the cells were pretreated with increasing concentrations of OPN (2.5–10 $\mu$M), as shown in Fig. 4*B*. The OPN-untreated cells were used as controls (100% invasion). The OPN-induced ECM invasion was also suppressed when the cells were individually pretreated with anti-human $\alpha_v\beta_3$ integrin antibody, GRGDSP, or GPenGRGDSPCA (but not with GRGESP) prior to the addition of OPN (Fig. 4*B*). These data indicate that OPN-induced migration and ECM invasion occur through interaction with the OPN receptor ($\alpha_v\beta_3$ integrin) and that the RGD sequence of OPN is involved in both of these processes. When preimmune IgG used in the upper chamber, no migration and ECM invasion were observed, suggesting that the migration and ECM invasion of melanoma cells are OPN-specific (data not shown). To examine whether OPN has any role in the growth rate of these cells, we performed the proliferation assay by treating the cells with different doses of OPN (0–10 $\mu$M). The results indicate that OPN induces cell growth (121–198%) in a dose-dependent manner in these cells (Fig. 4*C*).

*Characterization of the $\alpha_v\beta_3$ Integrin Receptor*—Because OPN induces cell migration and ECM invasion by interacting with $\alpha_v\beta_3$ integrin, we sought to determine whether these cells express any functional $\alpha_v\beta_3$ integrin receptor. Accordingly, the cells were surface-labeled with $Na^{125}I$ and immunoprecipitated individually with anti-$\alpha_v$ or anti-$\beta_3$ integrin antibody. The immunoprecipitated complex was resolved by SDS-PAGE and autoradiographed. Fig. 5*A* represent the $\alpha_v$ (*upper panel*) and $\beta_3$ (*lower panel*) integrin expression in these cells. In addition, we performed the displacement binding experiments using radiolabeled OPN as a ligand. The binding data were analyzed by the LIGAND computer program, and the results indicate that OPN binds to its receptor with high affinity and specificity, with a $K_d$ of 1.1 nM (Fig. 5*B*).

*MMP-2 Plays a Crucial Role in in Vitro Cell Migration and ECM Invasion*—To assess the direct role of MMP-2 in cell migration and ECM invasion, the OPN-treated cells were transiently transfected with LipofectAMINE Plus containing SMMP-2 or ASMMP-2 with phosphorothioate linkage. The conditioned media were collected after transfection, and the level of MMP-2 was analyzed by zymography. Both latent and active MMP-2 expression was reduced when the cells were transfected with ASMMP-2 (Fig. 6*A*, *panel a*, *lane 3*), but MMP-2 expression remained the same in cells transfected with LipofectAMINE Plus (*lane 1*) or SMMP-2 (*lane 2*). The data were also quantified by densitometric analysis (*panel b*). The transfected cells were also used for cell migration (Fig. 6*B*) and ECM invasion (Fig. 6*C*) assays. The migration of non-transfected cells treated with 10.0 $\mu$M OPN was considered to be 100% (control). There was a dramatic suppression of OPN-induced migration (49%) when ASMMP-2-transfected cells were used, but no significant differences were observed in LipofectAMINE Plus- or SMMP-2-transfected cells (Fig. 6*B*). Similarly, a dramatic reduction of OPN-induced invasion (44%) was observed in ASMMP-2-transfected cells, but no significant changes were noted in LipofectAMINE Plus- or SMMP-2-transfected cells (Fig. 6*C*). These results demonstrate that MMP-2 plays a critical role in OPN-induced cell migration and ECM invasion.

*Tumorigenicity Experiments*—The *in vitro* results prompted us to examine whether OPN has any role in tumor growth and regulating the expression of MMP-2 in an *in vivo* system. Accordingly, B16F10 cells were treated with OPN (10.0 $\mu$M) and then injected subcutaneously into the flanks of nude mice. Fig. 7*A* (*panels a–c*) shows typical photographs of tumors grown in 4-week-old nude mice. After 4 weeks, the mice were killed, and tumor weights was measured. The weights of the OPN-induced tumors were increased at least 3.1-fold compared with the tumors of the non-OPN-injected mice (Table I). How-

TABLE I
*Effect of OPN and anti-MMP-2 antibody on tumor growth*

B16F10 cells were treated with OPN and injected into nude mice (NMRI). In separate experiments anti-MMP-2 antibody (Ab) was injected into the OPN-induced tumor sites. The injections were performed twice a week for 4 weeks. The mice were killed, and the tumor weights were measured and analyzed statistically by Student's *t* test ($p < 0.005$). Mice injected with cells in PBS were used as controls.

| No. nude mice | Treatment | Tumor wt (-fold changes) |
|---|---|---|
| Four | Control (PBS) | 1.0 ± 0.25 |
| Four | OPN | 3.1 ± 0.15 |
| Four | OPN and anti-MMP-2 Ab | 0.93 ± 0.1 |

ever, the weights of the OPN-induced tumors were reduced dramatically when anti-MMP-2 antibody was injected into the sites of the tumors (Table I). Four mice were used in each set of experiments. To detect the levels of pro-MMP-2 and active MMP-2 expression in the tumors, the samples were lysed, and MMP-2 expression was analyzed by zymography (Fig. 7*B*, *panel a*). The levels of both the pro and active forms of MMP-2 in the tumors produced by OPN (10.0 $\mu$M) were significantly higher (*panel a*, *lane 2*) compared with the levels of MMP-2 in the tumors in non-OPN-injected mice (*lane 1*). However, the levels of MMP-2 (especially the active form) were reduced significantly in the anti-MMP-2 antibody-injected mice (*lane 3*). MMP-2 expression in tumors was further confirmed by Western blot analysis (*panel b*). Both latent and active MMP-2 expression was much higher in the tumors of the OPN-injected mice (*lane 2*) than in the tumors of the control mice (*lane 1*), and these data were corroborated by the zymography data (*panel a*). Moreover, MMP-2 mRNA expression in tumors was analyzed by Northern blot analysis, and it appeared that the level of mRNA expression was also significantly higher in the tumors of the OPN-injected mice compared with those of the control mice (data not shown). Taken together, these data strongly suggest that OPN-induced MMP-2 (both latent and active) expression in tumors occurs at both the transcriptional and post-transcriptional levels, and these values correlate with tumor growth (melanoma formation) in nude mice. Moreover, the data also demonstrate that when ASMMP-2-transfected cells were injected into mice, the size of the tumors was reduced drastically (Fig. 8*c*) compared with LipofectAMINE Plus-injected (Fig. 8*a*) or SMMP-2-injected (Fig. 8*b*) mice. The -fold changes in tumor weight caused by injection with LipofectAMINE Plus or different *S*-oligonucleotides are shown in Table II. The tumor weights of ASMMP-2-injected mice were reduced to one-third of the tumor weights of LipofectAMINE- or SMMP-2-injected mice (Table II). Four mice were used in each set of experiments. The expression levels of pro-MMP-2 and active MMP-2 in ASMMP-2-injected mice were much lower than in LipofectAMINE Plus- or SMMP-2-injected mice as detected by zymography and Western blot analysis (data not shown).

## DISCUSSION

Signals transduced by cell adhesion molecules play an important role in tumor cell attachment, motility, and invasion, all of which are key steps in the process of tumor metastasis. Cell-matrix interactions play a major role in tissue remodeling, cell survival, and tumorigenesis. OPN, an extracellular matrix protein, plays a significant role in cell adhesion, migration, and metastasis. It has been shown that overexpression of OPN is associated with various cancers and their metastatic potentials (12). MMPs are a family of $Zn^{2+}$-dependent endopeptidases that are responsible for remodeling of the extracellular matrix and degradation of ECM proteins. Several studies have shown the correlation between MMP-2 expression and metastatic potential and suggested that MMP-2 may act as a useful marker for the prognosis or diagnosis of cancers (34, 35). Therefore, we sought to determine whether OPN has any role(s) in the regulation of MMP-2 activation and control of cell motility, invasiveness, and tumor growth using B16F10 cells.

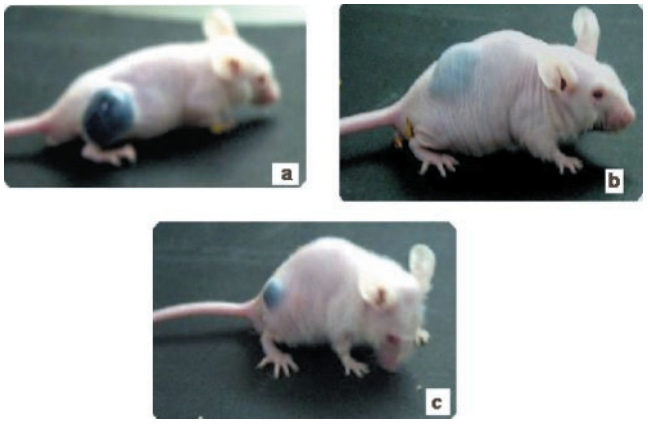


FIG. 8. **Typical photographs of MMP-2-specific *S*-oligonucleotide-injected tumors in nude mice.** The cells were treated with OPN and transfected with LipofectAMINE Plus containing SMMP-2 or ASMMP-2. The transfected cells were injected into the nude mice. Four mice were used in each set of experiments. *Panel a*, LipofectAMINE Plus; *panel b*, LipofectAMINE Plus and SMMP-2; *panel c*, LipofectAMINE Plus and ASMMP-2.

TABLE II
*Effect of MMP-2-specific S-oligonucleotides on tumor growth*

The cells were treated with OPN, transfected with LipofectAMINE Plus containing SMMP-2 or ASMMP-2, and then injected into nude mice. The LipofectAMINE Plus-oligonucleotide complex was injected into the tumor sites in the nude mice twice a week for 4 weeks. The mice were killed, and the tumor weights were measured and analyzed statistically by Student's *t* test ($p < 0.007$). The LipofectAMINE Plus-injected mice were used as controls.

| No. nude mice | Transfected cells | Tumor wt (-fold changes) |
|---|---|---|
| Four | LipofectAMINE Plus | 1.0 ± 0.05 |
| Four | SMMP-2 | 0.95 ± 0.1 |
| Four | ASMMP-2 | 0.37 ± 0.02 |

We have demonstrated that purified intact human OPN induces pro-MMP-2 production and activation in a dose-dependent manner in these cells. However, other ECM proteins such as fibronectin, type I collagen, laminin, and RGD-containing non-cyclic or cyclic peptides (GRGDSP and GPenGRGDSPCA) are unable to induce MMP-2 production and activation. Since OPN induces MMP-2 activation, we investigated whether OPN enhances cell migration and ECM invasion in B16F10 cells. The results indicate that OPN enhances cell migration and ECM invasion in a dose-dependent manner. Previous reports have shown that vitronectin induces MMP-2 and TIMP-2 secretion and enhances invasiveness in B16F1 and B16F10 cells (19). Maquoi *et al.* (21) demonstrated that type IV collagen induces MMP-2 activation in human fibrosarcoma cells. These data are supported by our present findings demonstrating direct dose-dependent increases in MMP-2 activation, cell migration, and ECM invasion by OPN in B16F10 cells. We also found that pretreatment of cells with anti-$\alpha_v\beta_3$ integrin antibody blocked OPN-induced cell migration and ECM invasion, suggesting that OPN-enhanced cellular responses occur through interaction with the $\alpha_v\beta_3$ integrin receptor. Therefore, we analyzed whether these cells express any functional $\alpha_v\beta_3$ integrin receptor. Cell-surface labeling and immunoprecipitation with anti-$\alpha_v$ and anti-$\beta_3$ integrin antibodies showed that these cells express $\alpha_v\beta_3$ integrin. Binding data revealed that radiolabeled OPN interacts with its receptor with high affinity ($K_d$ = 1.1 nM) and specificity. The OPN-derived RGD-containing peptides

(GRGDSP and GPenGRGDSPCA) suppressed OPN-enhanced cell migration and ECM invasion.

Transfection data showed that OPN-induced MMP-2 production can be regulated at the transcriptional level, resulting in lower OPN-enhanced cell migration and ECM invasion These data clearly demonstrate that MMP-2 is required in these cellular processes. Similarly, pretreatment of cells with anti-MMP-2 antibody blocked MMP-2 activation and thereby reduced OPN-induced migration and invasion (data not shown).

To determine the function of OPN in tumor growth and to check the status of MMP-2 in OPN-induced tumors, several experimental approaches were used. These include the tumor growth assay and specific inhibition of tumor growth by anti-MMP-2 antibody and ASMMP-2. OPN-induced tumor growth was suppressed by anti-MMP-2 antibody or ASMMP-2, suggesting that MMP-2 contributes to tumor growth. Proliferation data indicated that OPN stimulates the growth rate in B16F10 cells, and these data may partly explain the OPN-induced tumor growth in nude mice. Gardner *et al.* (36) have shown that antisense OPN expression in transformed malignant rat fibroblasts correlates with decreases in tumor growth, and these data support our experimental results. The levels of pro-MMP-2 and active MMP-2 in OPN-induced tumors were significantly higher than in controls. Moreover, the level of active MMP-2 in tumors was reduced dramatically in anti-MMP-2 antibody-injected mice. This reduction is probably due to anti-MMP-2 antibody blockage of the activation of MMP-2 by inhibiting the activation pathways. It has been shown previously that the localization of active MMP-2 to the cell surface by binding to $\alpha_v\beta_3$ integrin is essential for tumor growth (37, 38). It is possible that anti-MMP-2 antibody may block the active MMP-2 that is available for binding to the cell surface, thereby reducing tumor growth.

We have delineated for the first time the biochemical mechanism by which OPN induces cell migration, ECM invasion, and tumorigenicity by inducing MMP-2 expression and activation. Activation is necessary for the functional consequence of MMP-2 expression. Activation of MMP-2 requires its cell-surface localization and cleavage by cell membrane-bound MT1-MMP. TIMP-2 is involved in this process by forming a receptor for pro-MMP-2 (39). We have detected an increased level of TIMP-2 expression in OPN-treated B16F10 cells. The balance between TIMP-2 and MMP-2 levels is critical in determining the activation status of MMP-2 because overexpression of TIMP-2 would lead to MMP-2 inhibition. We have also detected increased levels of MT1-MMP expression by Western blot analysis. Increased pro-MMP-2 activation reflects up-regulation of MT1-MMP expression in OPN-treated cells, implying that OPN facilitates a shift in balance toward increasing proteolytic activity of MMP-2. The mechanism by which OPN activates MMP-2 by inducing ternary complex formation is not well understood. We observed that increased expression of MT1-MMP by OPN correlates with translocation of NF-$\kappa$B into the nucleus. Moreover, the NF-$\kappa$B super-repressor blocked OPN-induced MT1-MMP expression, suggesting that OPN-stimulated pro-MMP-2 activation is mediated by activation of NF-$\kappa$B.

In summary, we have demonstrated for the first time that native purified human OPN induces *in vivo* tumor growth and stimulates *in vitro* cell migration and ECM invasion using B16F10 cells. Moreover, increased invasiveness and tumor growth correlate with enhanced expression of pro-MMP-2 and active MMP-2 both *in vivo* and *in vitro*. We provide evidence showing that OPN increases the gelatinolytic activity by inducing MT1-MMP expression through the NF-$\kappa$B pathways. Furthermore, the suppression of MMP-2 gene expression by ASMMP-2 reduces the OPN-induced cell migration and ECM invasion in these cells. Blocking the functional activity of MMP-2 by its specific antibody also abrogates cell migration and ECM invasion. Both the tumor size and MMP-2 production and activation in OPN-induced tumors were abruptly reduced when anti-MMP-2 antibody- or ASMMP-2-treated cells were injected into the nude mice, and these data further support our *in vitro* data. Finally, these data indicate that MMP-2 plays an important role in OPN-enhanced cell migration, ECM invasion, and tumor growth and further demonstrate that OPN-induced activation of MMP-2 occurs via NF-$\kappa$B-mediated pathways.

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