p38 Kinase is Crucial for Osteopontin-induced Furin Expression that Supports Cervical Cancer Progression

Vinit Kumar¹, Reeti Behera¹, Kirti Lohite², Swapnil Karnik² and Gopal C. Kundu¹*

¹National Center for Cell Science, Pune, India and ²Grant Medical Foundation, Ruby Hall Clinic, Pune, India

Running title-OPN promotes p38 MAPK dependent cervical cancer progression

Keywords- Osteopontin, p38MAPK, furin and cervical cancer growth

*Corresponding Author: Gopal C. Kundu, National Center for Cell Science, NCCS Complex, Pune 411 007, India; Phone: 91-20-25708103; Fax: 91-20-25692259; E-mail: kundu@nccs.res.in
Abstract

p38 kinases activated by growth factors, hormones, and environmental stresses exert diverse functions in regulating normal and malignant cell pathophysiology. Enhanced levels of activated p38 isoforms have been linked with poor prognosis in breast cancer, although the mechanistic basis for this association is poorly understood. In this study, we report that p38 activation in cervical cancer cells is driven by osteopontin (OPN), an extracellular matrix-associated cytokine that drives invasive progression. OPN regulates CD44-mediated p38 phosphorylation that induces NF-κB activation and NF-κB dependent expression of furin, an extracellular protease implicated in HPV processing that enhances cervical cancer cell motility. OPN induces CD44-mediated MKK3/6 phosphorylation which in turn phosphorylates p38 in these cells. OPN-induced furin expression and cell motility was impeded by blockades to MKK3/6, p38α/β or NF-κB signalling. In a mouse xenograft model of human cervical cancer, tumor growth was enhanced by OPN overexpression and blocked by shRNA-mediated OPN silencing. Furin overexpression similarly augmented tumor growth in the model, whereas blocking MKK3/6, p38, or furin reduced OPN-induced cervical tumor growth. Analysis of clinical specimens revealed that enhanced expression of OPN, phosphorylated NF-κB, p65 and furin correlated with cervical cancer progression, further strengthening the in vitro and in vivo results. In summary, our findings offer a proof-of-concept for targeting OPN and its downstream p38 signaling as a novel therapeutic strategy to manage cervical cancer.
Introduction

Cervical cancer is one of the leading causes of death among women in the developing countries and around the world (1). Therefore, there is an urgent need to identify signaling mechanism regulating cervical cancer progression which may lead to the advances in therapeutics. p38 kinase is the member of mitogen activated protein kinase (MAPK) family of serine/threonine kinase (2). Four mammalian isoforms of p38 MAPK have been reported such as p38α, p38β, p38γ and p38δ (2-7). p38 MAPK is induced by various stimuli including inflammatory cytokines, growth factors as well as physical and chemical stresses resulting in cellular proliferation, differentiation, migration and apoptosis (2, 5, 8). The activation of p38 is regulated by MAP kinase kinase 3 (MKK3) and MKK6 in a selective manner within Thr-Gly-Tyr motif present in kinase domain (4, 7). The increased activation of p38 has been reported in breast and bladder cancers (9-11). p38 kinase is also involved in inducing cell invasion through activation of MAPK activated protein kinase (MAPKAPK)-2 and HSP-27 in prostate cancer (12). However, the role of p38 in regulating cervical cancer progression is not well defined.

Osteopontin (OPN) is a member of SIBLING family of cytokine like matrix-associated phosphoglycoprotein (13-15). The N-terminal region of OPN binds to integrins while C-terminal region interacts with CD44 (13, 16). Previous reports have shown that enhanced expression of OPN correlates with cervical cancer progression (17-19). Upon interacting with integrins, OPN triggers series of signaling events leading to expression of matrix metalloproteinase (MMP)-2 (20), MMP-9 (21), cyclooxygenase-2 (22), vascular endothelial growth factor (23), ICAM-1 (24) and
cyclin D1 as well as Bcl2 (25) ultimately inducing cell migration, angiogenesis and tumor progression. However, the mechanism by which OPN regulates cervical cancer progression is not studied well.

CD44 is a single chain transmembrane protein involved in cell-cell as well as cell-matrix interactions and lymphocyte homing. It interacts with various ECM components like hyaluronic acid, collagen and OPN (26, 27). CD44 has been found to be implicated in the progression of various cancers including glioblastoma (27), prostate (28), oral (29), lung (30) and cervical (31). Moreover, increased level of CD44 phosphorylation has been correlated with the aggressiveness of melanoma (32). Hyaluronan-mediated CD44 signaling leading to tumor progression is reported earlier (28, 33). However, the signaling mechanism by which OPN regulates CD44 dependent cervical cancer progression is not well documented.

NF-κB is well known to modulate various genes influencing cell invasion, angiogenesis and metastasis (34, 35). Furin is a member of proprotein convertases (PC) family and plays crucial role in regulation of tumor progression, metastasis and angiogenesis (36, 37). Enhanced expression of furin is correlated with aggressiveness of various cancers (37). The mechanism by which OPN regulates NF-κB dependent furin expression leading to cervical cancer progression is not well understood.

In the present study, we provide evidence that OPN regulates CD44-mediated MKK3/6 and p38 dependent NF-κB activation leading to furin expression which ultimately augments cervical cancer cell migration. Our in vivo data revealed that p38 and furin played crucial role in OPN-induced cervical tumor growth in mice xenograft model. The clinical specimen analysis established the correlation between enhanced expression of OPN, phosphorylated NF-κB, p65 and furin with cervical cancer
progression. Taken together, this study provides a novel mechanism of p38-mediated furin expression driven by OPN leading to cervical cancer progression.

Materials and Methods

Cell lines and transfections

Human cervical cancer (HeLa and SiHa) cells were obtained from American Type Culture Collection. The dominant negative (DN) mutants of MKK3 in pRSV and MKK6 in pcDNA3 (Dr. Roger J. Davis, University of Massachusetts Medical School), super repressor (SR) form of IkBα in pCMV4 (Dr. Dean W. Ballard, Vanderbilt University School of Medicine) and furin promoter P1 luciferase construct in pGL2-P1 (Dr. Claire M. Dubois, University of Sherbrooke) were transiently transfected in HeLa and SiHa cells using Lipofectamine-2000. The transiently transfected cells were used for in vitro experiments. Stable clones of wild-type OPN in pCR3.1 (Dr. Georg F. Weber, University of Cincinnati), OPN shRNA in pSUPER (Dr. Lalita A. Shevde, University of South Alabama), MKK3 DN, MKK6 DN and wild-type furin were used for in vivo tumorigenicity experiments.

Purification of human OPN

The human OPN was purified from breast milk with minor modification and used throughout the study (25).

Small interfering RNA

Both HeLa and SiHa cells were transfected with small interfering RNA (siRNA) specifically targeting OPN (siGENOME SMARTpool human SPP1, Dharmacon International), CD44 (CD44 siRNA), furin (furin siRNA) and p65 (p65 siRNA) (Santa Cruz Biotechnology) according to the manufacturer’s instructions.

Immunoprecipitation, western blot and EMSA

The immunoprecipitation, western blot and EMSA were performed as described (23).
Immunofluorescence

The immunofluorescence was carried out as previously described (25).

Luciferase reporter assay

The furin luciferase reporter assay was performed using HeLa cells as described earlier (22).

Cell migration and wound migration assays

The migration assay using Transwell cell culture chamber and wound migration assay were performed as previously described (23, 25).

In vivo tumorigenicity and immunohistochemistry

The tumorigenicity and immunohistochemistry experiments were performed as described (22, 25). Briefly, HeLa (2x10⁵) cells were stably transfected with wild-type OPN, OPN shRNA, MKK3 DN, MKK6 DN or wild-type furin and then injected subcutaneously into the flanks of female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. In some experiments, OPN (1 μmol/L) was injected at the site of tumor twice a week up to seven weeks. The mice were housed in pathogen-free condition in the experimental animal facilities of National Center for Cell Science as per institutional guidelines. In separate experiments, HeLa-Luc (1x10⁶) (Xenogen Corp., Alameda, CA) cells were injected into the flanks of NOD/SCID mice. OPN was injected either alone or along with p38 inhibitor, SB203580 (4 μmol/kg body wt) or furin inhibitor I (4 μmol/kg body wt) to the tumor site twice a week. Tumor volume was calculated using the formula, V= l x b x h.

After 7 weeks, mice were sacrificed by cervical dislocation, tumors were excised and weighed. The expression of OPN, phospho-p38, phospho-p65 and furin from tumors was analyzed by immunohistochemistry and western blot.

In vivo bioluminescence imaging
*In vivo* bioluminescence imaging was performed on a cryogenically cooled IVIS system (Xenogen Corp.) using Living Image acquisition and analysis software. D-Luciferin (15 mg/mL) was administered via intraperitoneal injection into the NOD/SCID mice at a dose of 150 mg/kg body weight. Images were acquired and bioluminescence was measured, analyzed and represented in the form of flux (photons/sec).

**Human cervical cancer specimen analyses**

Human cervical cancer specimens were collected with the help of oncopathologist from the local hospital with informed consent. The samples were analyzed by immunohistochemistry and western blot (23, 25).

**Statistical analysis**

Statistical analysis was performed by Student’s *t*-test using Sigma Plot software. The data was considered statistically significant if *p* <0.05. All bands were analyzed densitometrically (Kodak Digital Science) and fold changes were calculated.

**Results**

**OPN induces CD44 mediated MKK3/6 dependent p38 phosphorylation**

Previous reports have shown that phosphorylation of CD44 at Ser-325 is crucial for hyaluronan-mediated melanoma cell migration (38). The data also revealed that OPN regulates cell survival through CD44 dependent phosphatidylinositol 3-kinase/PKB-mediated pathway (39). Therefore we sought to investigate whether OPN plays any role in CD44 phosphorylation and CD44 dependent MAPK activation in cervical cancer cells. The results indicated that OPN stimulates CD44 phosphorylation (Fig. 1A). MAPKs are known to be involved in regulation of OPN driven signaling through CD44 and integrins in various cancer cells (14, 40). In this study, we investigate the possible role of OPN in CD44-mediated MKK3/6 activation in cervical cancer cells.
The data showed that OPN induces MKK3/6 phosphorylation in HeLa (Fig. 1C) and SiHa (Supplementary Fig S1A) cells. To examine the specificity of OPN-induced CD44-mediated MKK3/6 phosphorylation, siRNAs targeting OPN and CD44 were used (Fig. 1C; Supplementary Fig. S1C). The expression of OPN and CD44 in siRNA transfected cells was analyzed as controls (Fig. 1B; Supplementary Fig. S1B).

MKK3/6 has been reported to selectively phosphorylate p38 (4). To delineate whether OPN regulates p38 phosphorylation and CD44 and MKK3/6 are involved in this process, HeLa cells were individually transfected with CD44 siRNA, DN forms of MKK3 and MKK6 and then treated with OPN. Cell lysates were analyzed by western blot using anti-phospho-p38 antibody. The results revealed that OPN induces CD44-mediated MKK3/6 dependent p38 phosphorylation in these cells (Fig. 1D). The time dependent OPN-induced p38 phosphorylation was also performed in SiHa cells (Supplementary Fig. S2A).

**OPN induces CD44-mediated MKK3/6 and p38 dependent NF-κB activation**

Previous reports suggested that various external stimuli activate NF-κB and NF-κB regulated target gene expression (34, 35). To examine whether OPN regulates NF-κB phosphorylation and CD44, MKK3/6 and p38 are involved in this process, HeLa cells were treated with OPN alone or individually transfected with CD44 siRNA, DN forms of MKK3, MKK6, p38α and p38β and then treated with OPN and analyzed by western blot. The results revealed that OPN induces p65 phosphorylation and OPN-induced phosphorylation was inhibited by CD44 siRNA, DN forms of MKK3/6 and p38α/β (Fig 2A)

To decipher the role of OPN in nuclear translocation of phosphorylated p65, cells were treated with OPN for 0-240 min and immunofluorescence was performed.
The results suggested the involvement of OPN in phospho-p65 nuclear translocation (Fig 2B). The OPN-induced p65 nuclear translocation in SiHa cells was shown by western blot (Supplementary Fig. S2B). To further study the role of OPN in NF-κB-DNA binding, HeLa cells were either treated with OPN alone or transfected with CD44 siRNA or pretreated with p38 inhibitor, SB203580 and then treated with OPN and analyzed by EMSA. The results indicated that OPN indeed induces NF-κB-DNA binding through CD44 and p38 dependent pathway (Fig. 2D). The OPN-induced time dependent NF-κB-DNA-binding was also shown in HeLa cells (Fig. 2C).

**p38 and NF-κB play important role in OPN-induced CD44-mediated furin expression**

Furin is a serine protease known to activate many proprotein substrates including MMPs (36). Its elevated level has been correlated with metastatic potential of various cancers (37). OPN is also reported to induce expression and activation of various proteases such as MMP-2 and -9 (20, 21). Therefore, we sought to determine the possible role of OPN in furin expression in cervical cancer cells. Accordingly, both HeLa and SiHa cells were transfected with CD44 siRNA or IκBα super repressor (SR) or pretreated with SB203580 and treated with OPN and expression of furin was analyzed by western blot. The data indicated that p38 inhibitor, IκBα SR or CD44 siRNA attenuates OPN-induced furin expression suggesting that CD44, p38 and NF-κB play crucial role in this process in these cells (Fig. 3B and C). This was further confirmed by using OPN siRNA. In separate experiments, OPN-induced furin expression was shown in a time dependent manner in HeLa cells (Fig. 3A).

**OPN induces MKK3/6 and p38 dependent NF-κB-mediated furin promoter activity**
To determine the role of MKK3/6, p38 and NF-κB on OPN-induced furin promoter activity, HeLa cells were transfected with furin luciferase construct either alone or along with CD44 siRNA, IκBα SR and DN forms of MKK3/6 and p38α/β and luciferase activity was measured. The result revealed that OPN augments furin promoter activity through CD44-mediated MKK3/6, p38 and NF-κB dependent pathway (Fig. 3D).

**Furin plays crucial role in OPN-induced MKK3/6, p38 and NF-κB dependent cervical cancer cell migration**

Furin has been found to be involved in cell migration as inhibiting furin activity led to reduce cell motility (37). Previous data revealed that OPN enhances cancer cell migration through αvβ3 integrin (13). To examine the role of furin in OPN-induced cell migration and whether MKK3/6, p38 and NF-κB are involved in this process, HeLa cells were transiently transfected with CD44 siRNA, MKK3/6 DN, p38α/β DN or IκBα SR or pretreated with furin inhibitor I and used for cell migration. OPN was added in the upper chamber. Migrated cells were photographed, quantified, represented in the form of bar graph and analyzed statistically. The data indicated that inhibiting CD44 or its downstream molecules such as p38, MKK3/6, NF-κB and furin resulted in suppression of OPN-induced cell migration (Fig. 4A and B). In separate experiments, wound migration assays were performed using HeLa cells transfected under same conditions as described above. In another experiments, SiHa cells were transfected with p65 siRNA or furin siRNA and treated with OPN and wound assay was performed. The migrated cells towards the wound were photographed, quantified and represented in the form of bar graph and analyzed statistically. The data indicated that MKK3/6 DN, p38 DN, p65 siRNA or furin siRNA reduced the OPN-induced cell
migration further suggesting that these molecules are crucial in regulating wound migration (Fig. 4C, 4D, panel I and Supplementary Fig. S3 and S4). The expression of p65 and furin in siRNA transfected cells was analyzed by western blot (Fig. 4D, panels II and III).

**OPN stimulates MKK3/6, p38 and furin dependent cervical tumor growth in mice xenograft model**

Our *in vitro* results prompted us to investigate the roles of MKK3/6, p38 and furin in OPN-induced cervical tumor growth in mice xenograft model. First, to examine the specificity of OPN in cervical tumor growth, HeLa cells were stably transfected with wild-type OPN or OPN shRNA and injected into NOD/SCID mice. The expression of OPN and furin in stably transfected clones is shown in Supplementary Fig. S5A. In separate experiments, HeLa cells stably transfected with wild-type furin or DN forms of MKK3 and MKK6 were injected into mice and then OPN was injected to the tumor site twice a week upto 7 weeks. Tumor volumes were calculated and growth kinetics showing the fold change in tumor volume versus time in weeks is represented in the form of graph (Fig. 5A and 6A). The error bars represent the SEM. Similarly, changes in tumor weights were calculated and represented in the graphical form with statistical analysis (Fig. 5B and Supplementary Fig. S5D). The results clearly indicated that cells overexpressing OPN enhanced whereas knocking down by OPN shRNA suppressed tumor growth suggesting that both exogenous and intrinsic OPN play crucial role in cervical tumor progression (Fig. 5A). Similarly, DN forms of MKK3 and MKK6 reduced whereas wild-type furin enhanced tumor growth in response to OPN (Fig. 6A).

In separate experiments, HeLa-Luc cells were injected subcutaneously into mice. OPN either alone or along with SB203580 or furin inhibitor I was injected at
the site of tumor twice a week upto 7 weeks. The tumor volumes and weights were measured, analyzed statistically and represented in the form of graphs (Supplementary Fig. S5B and C). In vivo bioluminescence imaging was performed as described under “Materials and Methods”. The typical bioluminescence images of representative mice were shown in Fig. 6C, panel I. The bioluminescence was calculated, analyzed statistically and represented in form of flux (photons/sec) versus treatment (Fig. 6C, panel II). OPN significantly enhanced whereas SB203580 or furin inhibitor I reduced flux in response to OPN. These data confirmed our earlier in vivo data and further emphasized that both p38 and furin are involved in OPN-induced cervical tumor progression.

Tumor specimens were analyzed by immunohistochemistry and the results showed that OPN enhanced whereas MKK3/6 DN suppressed the expression of phospho-p38, phospho-p65 and furin in response to OPN (Fig. 5D and 6B, panel I). Similarly, SB203580 suppressed OPN-induced phospho-p65 and furin expression (Fig. 6D). The immunohistochemistry results were further confirmed by western blot (Fig. 5C and 6B, panel II). The data clearly suggested the role of MKK3/6 and p38 in OPN-induced furin expression leading to cervical tumor progression in mice models. The level of furin in stably transfected cells is shown in Fig. 6B, panel III.

Expression profiles of OPN, phospho-p65 and furin in clinical specimens and their correlation with cervical cancer progression

Our in vitro and in vivo results further prompted us to analyze the human cervical cancer specimens. The data revealed that enhanced expression of OPN (4 out of 6), phospho-p65 (5 out of 6) and furin (4 out of 6) was observed in the tumor tissues as compared to peripheral normal tissues (Fig. 7A). The western blot data were further confirmed by immunohistochemistry (n=11). The data showed enhanced expression...
of OPN, furin and phospho-p65 in tumor specimens as compared to peripheral tissues (8 out of 11) (Fig. 7B), suggesting their roles in cervical cancer progression. The expression of intracellular OPN in some of the clinical specimens is also observed (Fig. 7A). Interestingly, the nuclear localization of phosphorylated p65, NF-κB was detected in tumor specimens (Fig. 7B). The details of analyses of OPN and furin expression and their correlation with phospho-p65 activation and cervical cancer progression were summarized in Supplementary Table S1.

**Discussion**

Cervical cancer is one of the fatal diseases in women especially in the developing countries. Human papilloma virus (HPV) is considered as the major risk factor for carcinogenesis of cervix and thus, to date, Pap smear is one of the most effective tests for early diagnosis of this cancer. Unfortunately, in developing countries due to lack of HPV awareness, the lethality of this disease is more severe (1). Therefore, the in-depth insight into the molecular mechanism is inevitable for the development of effective therapy against cervical cancer.

OPN has widely been reported to be involved in promoting various cancers mediated through its integrin receptors (13). Interestingly, increased expression of OPN has also been correlated with the invasiveness and metastasis of cervical cancer (17-19). Previous reports have shown that plasma OPN levels are generally increased in patients with head and neck and cervical cancers (41). Furthermore, Wong et al have performed the genome-wide gene expression profiling of cervical cancer in Hong Kong population and showed the differential expression of OPN (42). In this study, we have delineated the in depth molecular mechanism by which OPN regulates cervical cancer cell migration and tumor growth.
Previous reports indicated that HeLa cells lack the expression of functionally active αvβ3 integrin heterodimer (43). Ibrahim et al have shown that IL-1, -4, -13 and TGF-β1 upregulate the expression of CD44 in HeLa cells (44). Earlier results also revealed that TGF-β1 induces the expression of OPN in rat osteosarcoma cells (45). Furthermore, serine phosphorylation of CD44 is crucial for cell migration and mutation of CD44 at Ser-325 resulted in blockade to hyaluronan-dependent melanoma cell migration (38). Therefore we sought to determine whether OPN regulates CD44 phosphorylation and CD44 dependent cell migration using HeLa cells. Our results suggested that OPN enhances CD44-mediated cervical cancer cell migration through downstream signaling events.

p38 MAPK has been involved in many cellular processes including cancer progression (2). It regulates cell proliferation in estrogen receptor (ER) negative breast cancer cells and cell invasion in bladder and prostate cancers through MAPKAPK-2 activation (3, 10, 12). Moreover, p38 kinase activation is also required for hyaluronan-induced CD44-mediated MMP-13 expression in articular chondrocytes (46). In this paper, we have reported that OPN induces CD44-mediated MKK3/6 dependent p38 phosphorylation in cervical cancer.

Earlier reports suggested that OPN regulates αvβ3 integrin mediated NF-κB activation in various cancer and endothelial cells (20-24, 47). The integration of signaling by JNK and p38 MAPK plays crucial role in the development of many cancers (48). Our results revealed that p38 is the key molecule in inducing NF-κB activation, its nuclear localization and DNA binding triggered by OPN in HeLa cells. Interestingly, silencing CD44 expression resulted in reduced OPN-induced p38 dependent NF-κB activation in cervical cancer cells.
Furin, a ubiquitously expressed type I transmembrane protein, is a member of proprotein convertases (PC) family of calcium dependent serine endoproteases (36). It processes inactive precursor proteins to their mature or functional forms at a paired basic amino acid site within the Golgi/trans-Golgi network secretory pathway (37). The expression of furin has been found to be upregulated in various cancers such as ovarian, lung, head and neck, glioblastoma and breast (36). Earlier reports indicated the role of furin in HPV infection (49). In this study, we have demonstrated that OPN regulates furin expression through CD44-mediated p38 and NF-κB dependent signalling pathway in cervical cancer cells.

OPN is reported to be involved in breast cancer cell migration through αvβ3 integrin (23, 25). Moreover, it has been documented that autocrine activation of OPN-CD44-Rac pathway enhances invasion and transformation of NIH 3T3 cells (40). Here, we provide evidence that OPN induces CD44-mediated migration of cervical cancer cells and further demonstrate the critical roles of MKK3/6, p38, NF-κB and furin in this process. The migration of HeLa cells towards the wound is slower as compared to SiHa that might be because of lack of functional expression of αvβ3 integrin heterodimer in HeLa cells (43). Moreover, HeLa cells have less invasive potential than SiHa cells (43).

The molecular mechanisms by which OPN regulates breast, melanoma and prostate tumor progression have been thoroughly studied earlier (20-23, 25), however, the signaling pathway driven by OPN leading to cervical tumor growth is still the subject of intense investigation. Our in vivo mice xenograft experiments and bioluminescence imaging described the collective roles of MKK3/6, p38 and furin in OPN-induced cervical tumor progression. This was further confirmed by the fact that cells overexpressing OPN enhanced whereas shRNA-mediated silencing of OPN
suppressed cervical tumor growth. Clinical specimen analysis further strengthened our findings confirming that enhanced levels of OPN, phospho-NF-kB, p65 and furin correlate with the malignancy of cervical cancer. Earlier reports indicated that alternative translation of a single OPN mRNA generates a secreted and intracellular isoform of OPN (50). Our results also indicated the presence of both secretory as well as intracellular OPN in cervical cancer specimens. However, the study of the mechanism by which secretory and intracellular OPN regulate cervical tumor progression is in further progress.

In conclusion, we demonstrate that OPN regulates CD44-mediated p38 MAPK activation followed by NF-κB dependent furin expression leading to cervical cancer progression (Fig. 7C). The in vitro and in vivo findings are further corroborated with clinical studies indicating that the higher expression of OPN, phospho-NF-κB, p65 and furin correlate with enhanced cervical cancer progression. Thus, understanding this molecular mechanism of OPN regulated MKK3/6 dependent p38 MAPK activation followed by furin expression could be a potential therapeutic strategy for the treatment of cervical cancer.

**Disclosure of Potential Conflicts of Interest:** No potential conflicts of interest were disclosed.

**Acknowledgements:**

We thank Dr. B. Ramanamurthy, In-charge, Experimental Animal Facility, NCCS for breeding and maintaining the NOD/SCID mice. We also thank Ms. Aswani Atre, Senior Technician, NCCS for assistance in confocal microscopy.

**Grant Support**
Department of Biotechnology, Government of India (G. C. Kundu), Council of Scientific and Industrial Research, Government of India (V. Kumar) and University Grant Commission, Government of India (R. Behera).

References


27. Xu Y, Stamenkovic I and Yu Q. CD44 attenuates activation of the hippo signaling pathway and is a prime therapeutic target for glioblastoma. Cancer Res 2010;70:2455-64.


Figure legends

Figure 1. OPN stimulates CD44-mediated MKK3/6 and p38 phosphorylation in HeLa cells. A, cells were treated with 0.5 μmol/L of OPN for 0-60 min, cell lysates containing equal amount of total proteins were immunoprecipitated with anti-CD44 antibody followed by western blot with anti-phospho-Ser antibody. B, panels I and II, levels of OPN and CD44 in cells transfected with their specific siRNA were analyzed by western blot. Actin was used as control. C, cells were either treated with OPN alone or transfected with OPN siRNA (OPNi) or CD44 siRNA (CD44i) followed by OPN treatment and level of phospho-MKK3/6 was analyzed by western blot. The blots were reprobed with anti-MKK3/6 antibody. D, cells were transfected with CD44 siRNA, MKK3 DN or MKK6 DN and then treated with OPN and phospho-p38 level was detected by western blot. Control siRNA (Coni) was used as negative control. All data are representation of three experiments. Fold changes were calculated.

Figure 2. OPN induces CD44-mediated MKK3/6 and p38 dependent NF-κB, p65 phosphorylation, nuclear translocation and DNA binding in HeLa cells. A, cells were individually transfected with CD44 siRNA, MKK3/6 DN and p38α/β DN and then treated with OPN. The phospho-p65 in cell lysates was detected by western blot. B, cells were treated with OPN and nuclear localization of phospho-p65 was studied by immunofluorescence. phospho-p65 was stained with Cy3 (red) while nuclei were stained with DAPI (blue). C, cells were treated with OPN for 0-240 min, nuclear extracts were prepared and analyzed by EMSA. D, cells were either treated with OPN alone or transfected with CD44 siRNA or pretreated with SB203580 (p38 inhibitor)
and treated with OPN. Nuclear extracts were analyzed by EMSA. The data represent three experiments exhibiting similar results.

**Figure 3.** p38 and NF-κB play important role in OPN-induced CD44-mediated furin expression and promoter activity. A, HeLa cells were treated with OPN for 0-48 h and furin expression was analyzed by western blot. B and C, both HeLa and SiHa cells were independently treated with OPN alone or transfected with OPN siRNA, CD44 siRNA or IκBα super repressor or pretreated with SB203580 and then treated with OPN and furin expression was analyzed by western blot. D, HeLa cells were cotransfected with CD44 siRNA, MKK3/6 DN, p38α/β DN or IκBα super repressor along with furin-luciferase construct and then treated with OPN. The luciferase activity was measured. The values were normalized to Renilla luciferase activity and the fold changes were calculated, and represented in the form of bar graph. Columns, means of three independent experiments; bars, SEM (*, p<0.05 versus control, **, p<0.05 versus OPN).

**Figure 4.** Furin plays crucial role in OPN-induced CD44-mediated MKK3/6, p38 and NF-κB dependent cell migration. A and B, HeLa cells were individually transfected with CD44 siRNA, MKK3/6 DN, p38α/β DN and IκBα super repressor or pretreated with furin inhibitor I and used for cell migration assay. OPN was added to the upper chamber. After 24h, migrated cells were stained, counted, photographed and quantified statistically. Columns, mean of three independent experiments; bars, SEM (*, p<0.05 versus control; **, p<0.05 versus OPN). C, HeLa cells were treated or transfected under the same conditions as described in panels A and B and used for wound assay. Wound photographs were taken and area of migrated cells was quantified. The error bar represents SEM. * and **, p<0.05. D, SiHa cells were
transfected with p65 siRNA (p65i) or furin siRNA (furini) and then treated with OPN and wound assay was performed (panel I). *, p < 0.05, **, p < 0.01. D, panels II and III, expression of p65 and furin in cells transfected with their specific siRNA was analyzed by western blot.

**Figure 5.** Wild type OPN enhances whereas OPN shRNA suppresses cervical tumor growth in mice xenograft model. A, tumor volumes were calculated, analyzed statistically and represented as tumor volume (fold change) versus time in weeks. Error bars, SEM, *, fold changes in 6th week (p < 0.05), **, fold changes in 7th week (p < 0.05). B, tumor weight in fold change versus transfection was plotted. The error bars represent SD. *, p < 0.001. C, expression of OPN, phospho-p38 and furin in tumor lysates was analyzed by western blot. D, immunohistochemical analyses of OPN, phospho-p38 and furin in tumor samples stained with Cy3-conjugated IgG (red).

**Figure 6.** MKK3/6, p38 and furin are crucial for OPN-induced cervical tumor growth in mice model. A, tumor volumes were calculated and analyzed statistically. Error bars, SEM, *, fold changes in 6th week (p < 0.05), **, fold changes in 7th week (p < 0.007). B, panel I, immunohistochemical analyses of phospho-p38, phospho-p65 and furin in tumor samples stained with Cy3-conjugated IgG (red). Panel II, expression of phospho-p38, phospho-p65 and furin in tumor lysates was analyzed by western blot. Panel III, cells stably transfected with wild type furin were analyzed by western blot. C, panels I and II, photographs of bioluminescence imaging of representative mice. The bar graph depicts fold changes in bioluminescence expressed in flux (photons/sec) versus treatments. Error bars, SEM, *, p < 0.05 versus control, **, p < 0.05 versus OPN. Six mice were used in each set of experiments. D, immunohistochemical analyses of phospho-p65 and furin in tumor samples stained with Cy3-conjugated IgG (red).
**Figure 7.** Expression profiles of OPN, phospho-p65 and furin in human clinical specimen and their correlation with cervical cancer progression. **A,** expression of OPN, phospho-p65 and furin in tumor lysates of human cervical cancer specimens (T) and peripheral normal tissues (N) was performed by western blot. **B,** immunohistochemical analyses of OPN, phospho-p65 and furin in human cervical tumor specimens using their specific antibodies followed by staining with diaminobenzidene. Arrow heads (red) indicate nuclear localization of phospho-p65. **C,** schematic representation of OPN-induced CD44-mediated MKK3/6, p38 and NF-κB-dependent furin expression leading to cell migration and cervical tumor progression.
Fig. 2. Kumara et al.
p38 Kinase is Crucial for Osteopontin-induced Furin Expression that Supports Cervical Cancer Progression

Vinit Kumar, Reeti Behera, Kirti Lohite, et al.

_Cancer Res_ Published OnlineFirst October 27, 2010.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-10-1470

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/10/27/0008-5472.CAN-10-1470.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.