Advances are made by answering questions. Discoveries are made by questioning answers

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8. SUMMARY AND CONCLUSION

The most important phenomenon of malignant progression of cancer cells are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of apoptosis, immortality, sustained angiogenesis, tumor invasion and metastasis. Thus, the regulation of the tumor-endothelial and tumor-host interaction in the tumor microenvironment plays a crucial role in tumor progression. Angiogenesis or formation of new blood vessel from existing one has been considered as one of the key step during cancer progression. There are several substantial preclinical and clinical evidences that support the central role of angiogenesis in tumor formation and metastasis. Experimental and clinical data demonstrated that breast cancer is an angiogenesis-dependent disease and that the vascular endothelial growth factor (VEGF) and its receptors play a key role in this process. Thus, understanding the molecular mechanism underlying the regulation of VEGF expression in cancer cells may help to develop novel anticancer therapeutic approach.

Osteopontin (OPN) a member of SIBLING family of protein has been considered as one of the major oncogenic molecule during breast cancer progression. Elevated expression of OPN has been observed in several cancers and the expression status of this protein is not only associated with metastatic potential of tumor cells but also regulate the expression profile of several metastatic associated proteins including matrix metalloproteinase (MMP-2 and -9), urokinase plasminogen activator (uPA), cyclooxygenase 2 (COX-2) and prostaglandins (PGE2). Therefore, the expression profile of OPN in blood or in the tumors of breast cancer patients can be used for the prognosis as well as diagnostic factor of cancer. Thus, the integration of information about the contribution of OPN to breast tumor progression and metastasis, with improved knowledge of individual steps involved in OPN-regulated cell signaling pathways and tumourigenic processes, may lead to the identification of novel molecular therapeutic targets and can clarify the potential utility of targeting OPN in different phases of cancer progression. This study provides valuable insights into the potential role of OPN in regulating VEGF expression and its receptors neuropilin-1 (NRP-1)/KDR mediated breast cancer progression and angiogenesis via autocrine, paracrine and juxtacrine mechanism. Moreover, in this study we have showed that down-regulation of tumor as well as host-derived OPN significantly inhibits breast cancer progression in vitro as well as in vivo mice models.
The following aspects has been addressed in this study

- To delineate whether OPN augments expression of VEGF in human breast cancer cells.
- To understand the role of NIK-IKKα/β-NF-κB signaling pathway in OPN-induced VEGF promoter activity and expression.
- To examine whether breast tumor specific kinase like Brk and transcription factor like ATF-4 play any function in VEGF expression in response to OPN.
- To investigate whether there would be any crosstalk between NF-κB and ATF-4 in response to OPN.
- To delineate the role of OPN-induced tumor-derived VEGF on tumor and endothelial cell motility and in vivo angiogenesis through VEGF receptors (NRP-1 and KDR)-mediated autocrine, paracrine and juxtacrine mechanisms.
- To study the role of tumor-derived and exogenous OPN on VEGF dependent in vivo orthotopic breast tumor growth and angiogenesis in nude mice model.
- To delineate the function of host-derived OPN on breast tumor growth and angiogenesis by generating orthotopic breast tumors in wild type and OPN-knockout mice.
- To examine whether naturally occurring carcinogen like pristane can promotes breast tumor growth and whether OPN plays any role in this process.
- To understand the expression profile of OPN in various grades of breast tumor specimens and its correlation with the activation and expression profile of NF-κB, ATF-4, Brk, NRP-1, VEGF and various other oncogenic molecules and tumor angiogenesis.

The major findings of the study are as follows

- The study showed that purified human OPN augments VEGF expression both at transcriptional and translational levels in human breast cancer cells.
- Both soluble and matrix associated OPN were capable of inducing VEGF expression.
- OPN also induced VEGF expression in various other human cancer cells like MCF-7, A375, LNCaP and HT1080.
- These data suggested that both soluble and matrix associated OPN-induced VEGF expression in human breast cancer and various other cancer cells.

- OPN interacts with cells surface integrin receptors and induce c-Src mediated activation of PI 3-kinase in breast cancer cells which ultimately regulates VEGF expression.
- OPN regulates αvβ3 integrin-c-Src-PI 3-kinase mediated NIK activation.
MDA-MB-231 cells transfected with wild type (wt) NIK or IKKα/β showed enhanced whereas those transfected with mutant (mut) NIK or dominant-negative (dn) IKKα/β or IκBα super repressor (sup.rep.) showed significant reduction of OPN-induced VEGF promoter activity. 

**Taken together, these data showed that OPN stimulates αvβ3 integrin-c-Src-PI 3-kinase dependent NIK activation which in turn regulates IKKα/β and NF-κB mediated VEGF expression both at transcriptional and translational levels.**

- OPN stimulates αvβ3-integrin dependent but c-Src and PI 3-kinase independent Brk phosphorylation and kinase activation.
- OPN-induced phosphorylated-Brk interacts with NIK and controls NIK phosphorylation.
- Brk plays crucial role in OPN-induced NIK dependent NF-κB activation.
- OPN promotes ATF-4 nuclear localization and DNA binding.
- Cells transfected with wt Brk enhances but kinase mutant (KM) Brk suppresses OPN-induced ATF-4 DNA binding.
- OPN regulates crosstalk between ATF-4 and NF-κB, which is directed towards ATF-4.
- Brk and ATF-4 play crucial role in OPN-induced VEGF promoter activity and expression.

**These data demonstrated a novel signaling pathway by which OPN regulates Brk mediated NF-κB dependent/independent ATF-4 activation, which in turn augments VEGF expression.**

- OPN-induced tumor-derived VEGF via autocrine pathway binds with tumor cell surface receptor (NRP-1) and augments tumor cell motility.
- Through a paracrine mechanism, tumor-derived VEGF, which is produced in response to OPN, interacts with endothelial cell surface receptor KDR and promotes its phosphorylation and induces endothelial cell motility.
- OPN-induced VEGF controls tumor endothelial cell interaction via NRP-1-KDR mediated juxtacrine mechanism.
- OPN-induced VEGF in tumor cells promotes in vivo angiogenesis in Matrigel plug assay whereas VEGF blocking antibody suppresses this effect.

**From this point of view, it can be postulated that OPN-induced tumor-derived VEGF via autocrine, paracrine and juxtacrine mechanism promotes tumor-endothelial cell interaction and regulates in vivo angiogenesis. Moreover,**
through a juxtacrine mechanism OPN-induced tumor-derived VEGF act as a bridge between tumor cell surface receptor NRP-1 and endothelial cell surface receptor KDR and promotes endothelial cell migration towards tumor cells which ultimately control tumor angiogenesis.

- OPN-induced orthotopic breast tumors are characterized by enhanced VEGF expression and elevated neovascularization (vWF expression).
- Intratumoral injection of anti-VEGF blocking antibody or NRP-1 siRNA significantly suppressed OPN-induced orthotopic breast tumor growth in nude mice, which further indicated that VEGF and its receptor NRP-1 plays crucial role in OPN-induced \textit{in vivo} breast tumor progression.
- OPN siRNA injected tumors showed significant depletion of NF-κB, ATF-4 and AP-1 activation, VEGF, MMP-2, MMP-9, MT1-MMP and uPA expression and reduced angiogenesis.

These data suggested that both exogenous as well as tumor-derived OPN promotes VEGF dependent breast tumor progression and angiogenesis and targeting tumor-derived OPN by siRNA could be a potential approach for breast cancer therapeutics.

- There was a significant reduction of breast tumor growth observed in OPN knockout mice as compared to wild type mice.
- Tumor generated in OPN knockout mice showed significant reduction in VEGF expression and angiogenesis.
- Activation of NF-κB, ATF-4 and AP-1 were considerably suppressed in OPN knockout mice as compared to wild type one.
- Enhanced expression of MMP-2, MMP-9, MT1-MMP and uPA were observed in wild type but not in the tumors of OPN knockout mice.

These results showed that the potential role of host OPN in breast tumor growth and angiogenesis.

- Carcinogenic compound pristane induced breast tumor growth in nude mice, which is significantly suppressed by intratumoral injection of OPN siRNA.
Enhanced expression of MMP-2, MMP-9, uPA, MT1-MMP, elevated activation of ERK, Akt, NF-κB and AP-1 were observed in pristane induced but not in pristane-induced OPN siRNA injected tumors.

There was a significant reduction of pristane induced breast tumorigenicity in OPN knockout but not in wild type mice.

**This observation demonstrated that OPN is important for the external carcinogen induced mammary tumorigenesis in nude mice and both tumor-derived and host OPN play crucial role in this process.**

- Higher Scraff-Bloom-Richards (SBR) grades of human breast cancer clinical specimens are characterized by enhanced OPN expression, which is further correlated with tumor angiogenesis.
- The data also showed that enhanced activation of NF-κB, ATF-4 and AP-1 observed in higher grades of human breast cancer clinical specimens and further correlated with elevated OPN expression.
- Expression of OPN in human breast cancer clinical specimens is further correlated with expression profiles of VEGF, NRP-1, Brk, MMP-2, MMP-9, uPA and MT1-MMP.

**This study showed that OPN could be an important prognostic marker for human breast cancer and further suggested the therapeutic potential of OPN for breast cancer treatment.**

Our experimental strategy was initiated with the purpose of better understanding of the molecular mechanism underlying OPN induced breast tumor progression and angiogenesis. Our study demonstrates the signaling cascades and molecular mechanism, at least in part, by which OPN regulates VEGF expression and VEGF dependent breast tumor progression. Moreover, using multiple in vivo approaches we have demonstrated that exogenous, tumor-derived and host OPN act as a crucial factor for breast tumor progression and angiogenesis. Thus understanding the molecular mechanism underlying OPN-induced breast tumor progression and angiogenesis may assist in development of novel therapeutic approaches, which might help us to spawn a new strategy for generation of next generation of cancer management.