"The man of science has learned to believe in justification, not by faith, but by verification.”

Thomas H. Huxley
7. SUMMARY AND CONCLUSION

Melanoma is one of the most aggressive cancers with high mortality rate since it is highly resistance to chemotherapy and it also shows poor response to immune therapy. Melanoma has potential immune-modulating effects and during melanoma progression, interaction between tumor and immune cells produce a unique microenvironment that support tumor growth, metastasis and angiogenesis by secreting a wide array of growth factors, chemokines, and proteases.

Clinical and experimental evidences revealed that TAMs have multifaceted roles in tumor development, particularly associated with tumor angiogenesis and invasion but the molecular mechanism underlying this association remains unclear. While multiple studies have focused on elucidating the role of infiltrating macrophages in angiogenesis and tumor growth, there has been least characterization of soluble mediators released by macrophages and signaling events driven by these soluble mediators. Identification of a number of accompanying molecular changes occurred in macrophages may help us to discover the novel therapeutic targets for melanoma management.

OPN is widely expressed in various cell types including activated immune cells such as T cells and macrophages. Role of tumor derived OPN in various cancer progressions is well reported but the function of macrophage derived OPN is paradoxical as it participates in both tumorigenesis and tumoricidal activities. COX-2 is constitutively expressed in various cancers, predominantly by stromal cells thereby promoting tumor growth and metastasis. It has been reported that macrophages are one of the major source of COX-2 in various cancers. Recent data demonstrates that COX-2 inhibition altered TAMs phenotypes by redirecting TAMs toward M1 phenotype in the polyps of \( Apc^{Min/+} \) mice. Recent study indicated that tumor derived PGE\(_2\) is responsible for differentiation of monocytes to M2 macrophages.

In this study, we delineated the role of OPN signaling in regulating macrophages phenotype which further controls melanoma growth and angiogenesis. In this study, we report that genetic ablation of stromal OPN suppresses melanoma growth in mice and macrophages are the crucial component in stroma responsible for melanoma growth. This study has provided insights into the crucial role of OPN activated macrophages in melanoma progression and angiogenesis by enhancing COX-2 dependent PGE\(_2\) production. Furthermore, we identify \( \alpha 9\beta 1 \)
integrin as a functional receptor for OPN that activates intracellular ERK and p38 signaling that ultimately leads to COX-2 expression in macrophages. The major role played by OPN and PGE₂ in angiogenesis was further amplified by upregulation of MMP-9 expression. OPN activated macrophages promote the migration and collagen adherence of cancer cells via PGE₂.

In this study the following aspects have been addressed.

1) The role of stromal OPN in melanoma growth.
2) The effect of genetic ablation of stromal OPN on macrophage infiltration, microvessel density and its correlation with melanoma growth.
3) Effect of COX-2 inhibition on melanoma growth and its correlation with macrophage infiltration and angiogenesis.
4) The effect of melanoma conditioned media on OPN and COX-2 expression in macrophages.
5) Role of OPN and COX-2 in macrophage migration in response to soluble factors secreted by melanoma cells.
6) The role of OPN in regulation of COX-2 expression and PGE₂ production.
7) Effect of OPN induced ERK1/2 and p38 phosphorylation on AP-1 activation and AP-1 mediated COX-2 expression and COX-2 dependent MMP-9 expression.
8) The effect of OPN activated macrophages induced PGE₂ on endothelial cell motility and angiogenesis through paracrine mechanism.
9) Role of PGE₂ in OPN activated macrophages induced melanoma cell migration and collagen adhesion through paracrine mechanism.
10) The correlation between increased infiltration of OPN+ TAMs with melanoma progression and angiogenesis.

The major findings of the present study are as follows-

- The study demonstrates that genetic ablation of stromal OPN suppresses melanoma growth.
- Microvessel density was significantly higher in melanoma tissue from Wt mice compared to OPN −/− mice.
Infiltration of COX-2 positive macrophages was significantly suppressed in melanoma tumor tissue from OPN\(^{-/-}\) mice compared to wild type mice.

Serum level of PGE\(_2\) was strikingly reduced in melanoma tumor bearing OPN\(^{-/-}\) mice compared to Wt mice.

This data provides the genetic evidence that ablation of stromal OPN suppresses tumor growth and reduces microvessel density by attenuating the infiltration of COX-2 positive macrophages.

COX-2 inhibitor significantly curbs melanoma growth.

Inhibition of COX-2 significantly suppresses microvessel density and macrophage infiltration in melanoma.

Serum level of PGE\(_2\) was reduced in mice supplemented with etoricoxib compared to control mice.

These results revealed that COX-2 and its main downstream mediator PGE\(_2\) play significant role in melanoma growth and angiogenesis.

OPN and COX-2 are highly elevated in macrophages upon co-culture with melanoma cells.

Soluble factors secreted by melanoma cells induce migration of macrophages.

Macrophages transfected with OPN or COX-2 cDNA show increased migration whereas cells transfected with OPN and COX-2 siRNA significantly loses their ability to migrate in response to soluble factors secreted by melanoma cells.

These data suggest that OPN and COX-2 play crucial role in macrophage migration in response to soluble factors secreted by melanoma cells.

CM of melanoma enhances COX-2 expression in macrophage and our data revealed that the enhanced COX-2 expression may not be solely regulated by OPN present in melanoma but by other soluble factor.
Silencing macrophage derived OPN curbs melanoma CM induced COX-2 expression in macrophages.

Expression of COX-2 in macrophages derived from OPN KO mice was significantly less compared to wild type mice upon co-culture with melanoma cells.

Collectively, these data suggested that co-culture of macrophages with melanoma, elevates OPN expression in macrophages which in turn upregulate COX-2 expression in an autocrine manner.

Exogenous OPN induces COX-2 expression in a dose dependent manner in RAW264.7, peritoneal macrophages and IC-21 cells, in a comparable way as did the co-culture.

Consistent with the induction of COX-2, OPN enhanced PGE₂ production in macrophages.

OPN has no effect on COX-2 expression in melanoma cells.

OPN or CM of melanoma does not influence COX-2 expression in fibroblast cells.

This data revealed that OPN specifically induced COX-2 expression in macrophages but has no effect on COX-2 expression in melanoma as well as in fibroblast cells.

OPN induced COX-2 expression is not through αvβ3 integrin and CD44.

OPN induced COX-2 expression is mediated through α9β1 integrin in RGD independent manner.

Co-culture of macrophages with melanoma cells results in increased expression of α9 integrin in macrophages.

These results suggest that α9β1 is the functional receptor for OPN that mediates COX-2 expression in macrophages and soluble mediator in melanoma CM not only stimulates OPN expression but also upregulate its receptor expression which in turn effectively enhanced the autocrine effect.
- OPN stimulates phosphorylation of ERK and p38 in macrophages.
- OPN induced COX-2 expression is mediated through ERK and p38 pathways but not through JNK and Akt.
- OPN enhance expression of c-Fos but not c-Jun in macrophages.
- OPN-induced c-Fos expression and nuclear translocation is mediated through ERK and p38 pathways.
- OPN induced COX-2 expression was significantly suppressed in cells transfected with A-Fos and dn c-Jun, however there was an increased expression of COX-2 in cells transfected with wt c-Jun.
- OPN-induced ERK, p38 mediated COX-2 expression and COX-2 dependent MMP-9 expression in macrophages.

Taken together, these data showed that OPN stimulates α9β1 integrin dependent ERK and p38 kinase activation which in turn regulates AP-1 mediated COX-2 expression and COX-2 dependent MMP-9 expression.

- OPN activated macrophages induced ICAM expression and ICAM dependent endothelial cell migration via PGE2.
- OPN activated macrophages induced PGE2, regulates angiogenesis through paracrine mechanism.
- OPN activated macrophage induced melanoma cell migration and collagen adhesion via COX-2/PGE2.

Osteopontin activated macrophages in the tumor stroma could promote angiogenesis and melanoma cell migration and collagen adhesion via PGE2.
Melanoma clinical specimens analysis revealed the increased infiltration of OPN positive macrophage in malignant tumor compared to peripheral normal specimens that correlates with increased angiogenesis, corroborating our *in vitro* and *in vivo* results.

Taken together these results indicates that macrophage derived OPN might be one of the important players in tumor microenvironment which educates macrophages towards cancer promoting phenotype through COX-2, a potent inflammatory molecule apparently involved in cancer progression. Our results emphasize the potential role of macrophage in the modulation of the tumor microenvironment via secretion of OPN, PGE$_2$, and MMP-9 which suggest that OPN signaling blockade may provide a mean of targeting tumor growth and angiogenesis.