7 Study on the role of stromal OPN in melanoma progression and angiogenesis

7.1 Introduction

The progression and spreading of the cancer cells from the site of origin to distant organ not only depends on the intrinsic factors produced by cancer cells but the stromal factors derived from host environment also fuels cancer progression (234-238). It has been hypothesized that tumor development is dependent upon the mutual interaction between the genetically altered malignant cells and the dynamic microenvironment in which they grow (234, 237). Although the “seed and soil” hypothesis of cancer progression had been proposed by Paget more than hundred years ago, but to date, the role of soil (stromal microenvironment) in cancer progression is not understood clearly as compared to the function of seed (tumor cell) in this process (239). Therefore determining the role of host/stromal environment as well as stromal factors in the development of tumor malignancies not only helps in understanding the molecular mechanism of cancer progression but may also spawn a new era of prognostic and therapeutics targets in next generation of cancer management (240). Hence, prognostic factors are required to identify those patients who are at high risk from metastatic spread so that they can be treated more aggressively than those with indolent, non-metastatic carcinomas. One of the challenges in the development of new cancer therapies is to identify specific stromal factors that regulate key processes leading to tumor aggression and reoccurrence. Very recently, Gao et al. showed that bone marrow derived endothelial progenitor cells act as critical regulators of angiogenic switch and that ultimately regulate pulmonary metastasis of cancer cells (241) and further indicated that tumor-stromal interaction played crucial role in tumor metastasis and angiogenesis. Moreover, using an activity based protein profiling approach Jessani et al. showed the elevated enzymatic activity of human breast cancer cells in host environment of mouse mammary fat pad that ultimately regulates enhanced breast cancer progression in vivo (242). However, the role of stroma/host-derived OPN in regulation of melanoma progression is not clearly understood. Therefore, role of stromal factors in regulation of tumor progression is under intense investigation.
One such secretory factor is OPN which modulates other stromal factors that play a key role in regulation of malignancy. OPN a member of small integrin binding ligand N-linked glycoprotein (SIBLING) family of ECM associated protein has been known to play crucial role in various physiological as well as pathological functions (41-43, 69, 175). Recent studies have indicated the multifaceted role of OPN in regulation of various cancers and manifested the importance of OPN in these processes (41-44). OPN activates multiple signaling cascades that regulates the expression of various oncogenic and angiogenic molecules ultimately leading to tumor progression (42, 175). Moreover, highly malignant tumors express enhanced OPN expression as compared to benign ones (47-50). Furthermore, recent studies have shown that targeted disruption or inhibition of tumor derived OPN significantly curbs tumor progression, metastasis and angiogenesis in vitro as well as in vivo (77-85). Recently, Grassinger et al. have demonstrated that thrombin cleaved OPN acts as a chemoattractant for stem and progenitor cells (243). Furthermore using OPN knockout mice, the role of OPN as a negative regulator of hematopoietic stem cells proliferation has been demonstrated (105, 106). However, Sumitomo et al. have observed that transcriptional mediator subunit MED1/TRAP220 in stromal cells promotes hematopoietic stem/progenitor cell growth through OPN expression (107). Likewise Saika et al. have demonstrated that loss of OPN in an injured mouse lens epithelium perturbs the epithelial-mesenchymal transition suggesting the importance of OPN in EMT (244). Recently, it has been shown that OPN derived from senescent fibroblast stimulates preneoplastic cell growth through CD44 receptor and activation of MAPK pathway, highlighting the importance of stromal OPN on tumorigenesis (101, 102). Moreover, adding exogenous OPN to cancer cells promotes cell survival and proliferation in contrast to stem cells (77-79). However, the effect of stromal OPN on cancer stem cells mediated cancer progression has not been studied so far.

We have tried to understand the role of stroma/host derived OPN in regulation of tumor progression, metastasis and angiogenesis using murine melanoma as a model system. We have found the crucial role of stroma-derived OPN in these processes, thereby regulating the tumor initiating cells and that may help to determine the prognostic and therapeutic significance of stromal/host-derived OPN in the treatment of cancer.
7.2 Results

7.2.1 Abrogation of melanoma growth and angiogenesis in OPN knockout mice

The role of tumor derived OPN in regulation of tumor progression is well established, however, role of host derived OPN in tumorigenesis is not clearly understood. Recently Chakraborty et al. have shown that abrogation of host OPN significantly suppressed in vivo breast tumor growth in mice model (78). Earlier Hayashi et al. reported that serum collected from OPN+/+ mice induced in vitro migration of B16F10 cells whereas serum from OPN−/− mice suppressed this event (100) which further suggested the potential role of host OPN in tumor progression. Serum was collected from OPN+/+ and OPN−/− mice and analysed for OPN expression by Western Blotting. The data showed significant OPN expression in the serum of OPN+/+ whereas negligible expression was detected in the serum of OPN−/− mice (Fig. 24B). Therefore, to explore the role of host derived OPN in subcutaneous melanoma growth, B16F10 cells were injected to the OPN+/+ and OPN−/− mice. Tumors growth was measured weekly and plotted graphically (Fig. 24 B). The data showed that deficiency of host OPN significantly slowed down melanoma growth in vivo. Tumor weights were measured and represented graphically and the data reflects at least 4 fold reduction of melanoma load that occurs due to deficiency of host OPN (Fig. 24 C). The tumor sections were analyzed histopathologically by H&E staining (Fig. 24 A, middle panel) and the results showed that host OPN induced higher infiltration, and poorly differentiated structure in OPN+/+ mice as compared to OPN−/− one. To determine whether host OPN promoted tumor angiogenesis in melanoma model, tissue sections were stained with anti-CD31 antibody (an endothelial cell marker) and the results showed that host OPN induced significant tumor angiogenesis (higher CD31 positive areas) whereas reduction of angiogenesis (less CD31 positive areas) were observed in OPN−/− mice (Fig. 24 A, lower panel). Taken together the data suggested that host OPN is crucial for melanoma growth and angiogenesis in murine melanoma model.

7.2.2 Stromal OPN promotes lung and liver metastasis in experimental mice model

Several reports suggested that tumor derived OPN augments metastasis in various cancer models by inducing the expression of varieties of oncogenic molecules, through
multiple signaling cascades (75-85, 175). However, it is not clear whether host OPN can promote tumor metastasis. Therefore to determine the role of host OPN in experimental melanoma metastasis, B16F10 cells were injected at intracardiac position in OPN+/+ and OPN−/− mice.

**Figure 24** Suppression of melanoma growth and angiogenesis in OPN−/− mice. (A) B16F10 cells were injected subcutaneously into wild type and OPN knockout mice (n=6 mice per genotype). Tumors were allowed to grow for 5 weeks and at the end, mice were sacrificed, tumors dissected out, photographed, weighed and analyzed histopathologically. Upper panel: representative image of tumor from the cohorts of OPN+/+ and OPN−/− mice. Middle panel: tumors dissected were sectioned and stained with hematoxylin and eosin, and analyzed histopathologically. Images were captured at 10x and 60x magnification. Lower panel: Tumor angiogenesis were studied from the cryo-sectioned tumor tissue using anti-CD31 antibody. CD31 are stained with DAB (brown) whereas nuclei are stained with hematoxylin (blue) Photographs were captured at 10x magnification. (B) Tumor volumes in these mice were measured weekly and represented graphically. Inset: Western blot analysis of OPN in the serum of OPN+/+ and OPN−/− mice. *P<0.001 (C) Isolated tumors were weighed, analyzed statistically and represented in the form of bar graph. *P<0.001
After termination of experiments, mice were sacrificed; organs such as lungs and liver were dissected and photographed. Significantly enhanced melanoma metastasis were observed in the lung of OPN+/+ as compared to OPN−/− mice (Fig. 25A). The lung and liver section were analyzed histopathologically by H&E staining and significant number of metastatic foci were visible in the lung of OPN+/+ mice as compared to knockout mice. The data further showed that metastasis foci of melanoma (indicated by arrow) were observed in liver of OPN+/+ but not in OPN−/− mice (Fig. 25B).

**Figure 25 Stromal OPN promotes melanoma metastasis.** (A) B16F10 cells were injected intracardiacly to OPN+/+ and OPN−/− mice. Mice were dissected; vital organs were collected and photographed after experimental metastasis assay. *Upper panel:* Lung photographs of representative experimental mouse group. Uninjected mice were used as a control. *Middle and Lower panel:* histochemical analysis of lung sections to study metastasis. Images were captured at 10x and 60x magnification. (B) Analysis of liver metastasis was performed histopathologically, and images were captured at 10x and 60x magnification. Metastasis foci in liver sections were indicated by arrow. (C) Photographs of primary culture from the tumor tissue derived from subcutaneous injection of B16F10 cells into OPN+/+ and OPN−/− mice.

### 7.2.3 Functional characterization of primary melanoma cells isolated from OPN+/+ and OPN−/− mice tumors

Jessani et al. have shown that breast tumor cells derived from orthotopic xenograft tumors exhibit profound differences in their enzymatic profile and showed enhanced
aggressiveness as compared to the parental cell line (242). Moreover, it has been postulated
that the stromal factors derived from the host microenvironment of tumor is responsible for
aggressive behavior (242). Therefore, to explore the role of host derived OPN in induction of
higher metastatic and aggressive ability of melanoma, cells were isolated from subcutaneous
melanoma tumors of OPN+/+ and OPN−/− mice and cultured at in vitro environment. Cells
were isolated and cultured from OPN+/+ and OPN−/− mice termed as B16-WT and B16-KO
respectively throughout the study. Morphologically B16-WT and B16-KO cells do not show
any significant differences from parental B16F10 cells (Fig. 25C). These observations
prompted us to determine other biological characterization of B16-WT and B16-KO cells.
Our data showed that B16-WT cells exhibited higher in vitro migratory activity in wound
assay than parental B16F10 or B16-KO cells (Fig. 26A & B). Transwell migration assay
using Boyden chamber demonstrated that B16-WT cells showed profound migration as
compared to parental B16F10 or B16-KO cells (Fig. 26C & D). Taken together, the data
demonstrated that melanoma cells derived from tumor generated in OPN+/+ mice showed
enhanced aggressive behavior than parental cell line. In comparison, the cells derived from
tumors grown in OPN−/− mice did not exhibit any significant functional differences as
compared to the parental cells which further indicates the crucial role of host derived OPN in
regulation of melanoma progression.

7.2.4 B16-WT cells exhibit vasculogenic mimicry and tumor-endothelial
cell interaction

Previous studies have suggested that highly aggressive melanoma cells formed ECM-
rich tubular network that can ensure blood and nutrient supply to tumors and this phenomena
is termed as vasculogenic mimicry (245). Vasculogenic mimicry is correlated with poor
prognosis and enables melanoma growth, at least in part, independent of angiogenesis (246).
Recently Rothhammer et al. have showed that highly aggressive melanoma cells efficiently
formed capillary like network on matrigel coated plate and indicated that these cells have the
potential to exhibit vasculogenic mimicry (247). Therefore to determine whether the
enhanced aggressiveness of B16-WT cells reflects the phenomenon of vasculogenic mimicry;
cells (B16F10, B16-WT and B16-KO) were plated on matrigel coated tissue culture dishes
and incubated in humidified environment. Data showed that B16-WT cells produce tube like
network within 8 h (Fig. 26E & F). No significant tube-like network formation was found in
parental B16F10 or B16-KO cells even after 24 hrs. This suggested that B16-WT cells exhibit vasculogenic mimicry and the phenomenon further reflects the enhanced aggressive behavior of these cells.

Figure 26 Characterization of tumor cells from the B16F10 generated tumors of OPN+/+ and OPN−/− mice. (A) Artificial wounds were made in confluent monolayer of the B16F10, B16-WT and B16-KO cells. Migration of the melanoma cells towards the wound were photographed after 12 h of wounding. (B) Wound closure was quantified using ImagePro plus software, analyzed statistically and represented graphically. *P<0.001 vs. control. (C) Melanoma cells (B16F10 or B16-WT or B16-KO) were plated on the upper chamber of transwell whereas lower chamber were filled with medium containing 2% FBS. Migrated cells in the opposite side of upper chamber were fixed, stained with Giemsa and photographed. (D) Migrated cells were counted, analyzed statistically and represented in the form of bar graph. *P<0.001 vs. control. (E) Parental as well as primary cultures were plated on the matrigel coated plate for 8 h. Cord formed were photographed. (F) The tubes formed were counted using ImagePro plus software, analyzed statistically and represented graphically. *P<0.002 vs. control
Figure 27 B16-WT cells exert enhanced interaction with endothelial cells. (A) Western blot analysis for OPN and VEGF expression from B16F10, B17-WT and B16-KO cell lysates. Actin was used as a loading control. (B & C) Melanoma cells were seeded in the lower chamber whereas HUVEC (1 X 10⁵) were plated in the upper chamber of modified Boyden chamber or matrigel coated invasion chamber and incubated for 12 h. Migrated or invaded HUVEC on the opposite side of upper chamber were stained with Giemsa and photographed. (D) Migrated and invaded endothelial cells were counted, analyzed statistically and represented in the form of bar graph. *P<0.001 vs. control.

Interaction between tumor and endothelial cells plays a crucial role in determining tumor growth and angiogenesis (78, 79). During metastasis, tumor cells interact with the nearby endothelial cells of blood vessels, invade and metastasize through the blood stream to distant organs (239). Therefore to determine the direct interaction between melanoma and endothelial cells, co-migration and co-invasion assays were performed. Melanoma cells were plated in the lower chamber whereas HUVEC were plated on upper chamber of modified Boyden chamber and matrigel coated invasion chamber. The migrated and invaded HUVEC on the reverse side of upper chamber were stained with Giemsa, photographed (Fig. 27B & C) in three hpf, counted, analyzed and represented in the form of bar graph (Fig. 27D). The data showed that B16-WT cells promote endothelial cells migration and invasion towards it
and further suggested that these cells exhibit enhanced angiogenic potential as compared to B16F10 and B16-KO cells. Taken together the data suggested that host OPN induced angiogenic property in melanoma cells.

### 7.2.5 Expression and activation of protein kinases in parental and primary melanoma cells

Our previous results showed that B16-WT cells exhibit superior oncogenic as well as angiogenic behaviors as compared to parental B16F10 or B16-KO cells but no significant morphological differences have been observed among these cells. Therefore we hypothesized that host derived OPN might induces some constitutive alteration of the expression profile of various oncogenic molecules which in-turn regulates the aggressive behavior of B16-WT cells. Accordingly lysates of these cells were analyzed by Western blot and the data showed that B16-WT cells exhibit significant higher levels of OPN and VEGF expression as compared to B16F10 and B16-KO cells (Fig. 27A). Then we checked the signaling pathways that altered in these cells. Accordingly, Western blot analysis for ABCG2, p-Akt and p-ERK were performed from the lysates of these cells. The results showed that ABCG2 and ERK signaling are upregulated in B16-WT cells as compared to B16F10 and B16-KO cells, suggesting the importance of stromal OPN in regulation of these molecules. Interestingly, no significant difference was observed at the level of p-Akt among these cells (Fig. 31 E-G).

### 7.2.6 Reintroduction of B16-WT cells in OPN−/− mice exhibit enhanced tumor growth, angiogenesis and metastasis

To study the in vitro tumorigenicity of these cells, matrigel based colony formation assay was performed. Interestingly, B16-WT cells shows considerable higher in vitro colony formation with respect to B16-KO or parental B16F10 cells (Fig. 28A). Our in vitro observation prompted us to determine the tumor growth and metastatic potential of B16-WT/B16-KO cells under in vivo condition. For this, we have chosen OPN−/− mice so that host OPN has no effect in the growth kinetics of tumor. Accordingly, cells (B16F10, B16-WT and B16-KO) were implanted subcutaneously to the OPN−/− mice. Tumor growths were measured weekly up to five weeks. The results showed that B16-WT cells showed comparatively enhanced tumor growth as compared to other cells (Fig. 28B-D). Mice were sacrificed and the tumors were photographed (Fig. 28B), weighed and represented in the form of bar graph...
The data showed that B16-WT cells exhibit significantly higher tumor load as compared to B16-KO or parental B16F10 cells which further supports our \textit{in vitro} observation. Tumor sections were analyzed by histopathological and immunohistochemical studies using anti-CD31 antibody and the data indicated that B16-WT cell derived tumor exhibit enhanced angiogenesis (CD31 positivity, Fig. 29A) as compared to B16F10 or B16-WT cells.

To examine the comparative metastatic potential of B16-WT and B16-KO cells, \textit{in vivo} metastasis assay was performed. Cells were injected intracardially in OPN\textsuperscript{-/-} mice and were kept for 21 days. After termination of experiments, mice were sacrificed and
photographed (Fig. 29B). The result showed comparatively higher lung metastasis in B16-WT cells injected mice. To further confirm this observation, histopathological analysis were performed and the data showed enhanced lung metastasis in B16-WT cells injected mice as compared to B16-KO cells (indicated by arrows). However we were unable to detect any significant metastasis in the injected mice (data not shown). Taken together the data demonstrated that B16-WT cells exhibit higher level of tumor growth and metastasis as compared to other two cells, suggesting the importance of stromal OPN in melanoma growth.

Figure 29 B16-WT cells showed enhanced angiogenic and metastatic property in experimental mice model. (A) Tumor isolated were subjected to cryo-sectioning and stained with anti-CD31 antibody. Blood vessels were visualized with DAB staining and nuclei with Hematoxylin. Photographs were captured at 10x magnification. (B) B16F10, B16-WT and B16-KO cells were injected at intracardiac position of OPN/− mice to study lung metastasis. After 21 day, mice were sacrificed and lung was dissected out. Upper panel: Photographs of representative Lung isolated from experimental mice. Lower panel: Lungs were analyzed histopathologically and photographs were taken at 60x magnification.
7.2.7 Stromal OPN selectively enriches SP phenotype in murine melanoma cells

Our Western blot result demonstrated that B16-WT cells have enhanced expression of ABCG2, suggesting the possibility of having higher efflux capacity in this cells (Fig. 31E). Recent advancement in cancer stem cell biology suggests the correlation between Side Population (SP) and tumor initiating cells. SP is a set of cells with differential exclusion of Hoechst dye through ABC transporter and is expected to exhibit the property of cancer stem cells. Recently, Dou et al. have reported the presence of SP phenotype in B16F10 melanoma cells (248). Emerging evidences suggest the crucial role of stromal-OPN in regulation of stem or progenitor cells (105-107, 243). However, the impact of stromal-OPN on the regulation of cancer stem cell or tumor initiating cells has not been studied so far. Therefore, to study this effect, B16F10, B16-WT and B16-KO cells were stained with Hoechst in presence or absence of reserpine and analyzed by flowcytometer. To confirm the existence of SP, reserpine is used in each experimental setup. A549 cells were used as positive control for
SP phenotype. The percentage of SP phenotype observed was 24.9%, 8.3%, 21.4% and 9.3% in A549, B16F10, B16-WT and B16-KO cells respectively (Fig. 31A-D). The data clearly demonstrated that stromal OPN selectively enriches SP cells whereas lack of OPN has no effect on it. Several reports have suggested that host cells particularly endothelial and fibroblast cells exhibit this SP phenomenon.

Figure 31: Host OPN selectively enriches stem-like cancer cells. Cells were stained with the Hoechst 33342 dyes in the presence or absence of 50 µg/ml reserpine. (A) A549 used as a positive control for SP phenotype (-reserpine). Inset, reserpine treated A549 cells were stained with Hoechst. (B) B16F10 cells were stained with Hoechst in absence or presence (Inset) of reserpine. (C) B16-WT cells were stained with Hoechst in absence or presence (Inset) of reserpine. (D) B16-KO cells were stained with Hoechst in absence or presence (Inset) of reserpine. The average size of the SP was around 9% in B16F10 and B16-KO cells whereas it was 19% in B16-WT cells. The size of SP is directly correlated with the expression of ABC transporters which exclude out the Hoechst dyes. (E), (F) and (G) Western blot analysis for ABCG2, p-ERK and p-Akt expressions from the lysates of B16F10, B16-WT and B16-KO cells. Actin, ERK2 or Akt2 were used as internal controls.
Accordingly, to overrule that increase in SP percentage is due to enrichment rather than accompanying host cells, B16F10 cells stably transfected with GFP were injected into wild type mice. Primary culture from this tumor (B16-WT-GFP) was used for SP analysis. SP analysis was performed for GFP positive and non-GFP cells were excluded. The result demonstrated almost similar percentage of SP phenotype as in B16-WT cells (data not shown); suggesting the increase in percentage is due to enrichment of SP cells in presence of stromal-OPN.

7.2.8 Functional characterization of sorted SP murine melanoma cells

7.2.8.1 Efflux of mitoxantrone from melanoma SP cells

The enhanced expression of ABC transporter proteins indicates that the SP cells should have high efflux capacity for antineoplastic drugs. To investigate this possibility, SP cells were sorted and treated with mitoxantrone with or without reserpine for 24h, and non-SP cells were treated with mitoxantrone and cell viability experiment were performed. The result showed increase in inhibition of cell growth in non-SP cells with increased doses of mitoxantrone, whereas no significant difference was observed in mitoxantrone treated SP cells. However, reserpine and mitoxantrone treated cell showed increase in growth inhibition of SP cells (Fig. 32A). This suggested that resistance to mitoxantrone is due to its efflux by the ABC-transporter, which when blocked with reserpine, the cells became susceptible to drug.

7.2.8.2 SP regenerates into SP and non-SP cells

To compare the repopulation ability of SP cells, we cultured the sorted SP cells under the same culture condition for a week before it was restained with Hoechst 33342 dye and reanalyzed. SP cells were viable in culture, and regenerated both SP and a non-SP cells with a higher fraction size than original population (Fig. 32B), whereas cultured non-SP cells produced mainly non-SP cells (data not shown). This suggested that SP cells have the property of differentiation which is a typical feature of stem or progenitor cells.

7.2.8.3 SP cells are more tumorigenic in nature

To further test whether SP cells are enriched for tumorigenic cells, in vitro colony formation assay was performed. The data showed that SP cells have high colony formation capacity (Fig. 32C & D) indicating enhanced in vitro tumorigenicity as compared to non-SP
cells. To extend our study further, sorted SP and non-SP (1x10^3) cells were injected subcutaneously into OPN^{+/+}, OPN^{-/-} and NOD/SCID mice and allowed to grow for 5 weeks. Tumor incidence obtained from different mice models are summarized in Table 2. Tumor generated in OPN^{+/+} and OPN^{-/-} mice were sacrificed, tumors were dissected, weighed and tumor volume was calculated. The result depicted enhanced tumor growth in OPN^{+/+} as compared to OPN^{-/-} mice (Fig 33A-C), suggesting that SP cells are highly enriched with tumorigenic properties.

**Figure 32: In vitro characterization of SP cells.** Both SP and non-SP cells were sorted and characterized. (A) Sorted non-SP cells were seeded onto 96 well plates and treated with mitoxantrone. SP cells were treated with mitoxantrone (0-50 nM) with or without Reserpine (5 µM) and cell viability was checked by MTT assay. (B) Repopulation assay was performed with sorted SP cells to study the differentiating property of stem cells. (C) In vitro tumorigenicity of SP cells was checked by Matrigel base colony formation assay with sorted cells. Colonies formed were photographed at 10x a magnification. (D) Colonies were counted, analyzed statistically and represented in the form of bar graph. *P<0.012
7.2.8.4 SP cells display increased lung metastasis in mice model

To investigate possible differences in metastasis between SP and non-SP, intravenous injection was done in NOD/SCID mice with sorted SP and non-SP cells and monitored for 21 days. IVIS analysis was performed, mice were sacrificed, lung and liver were dissected, and again imaging was done with these organs. Fig. 33D shows that SP cells have the ability to metastasize to lung and liver but not the non-SP cells.

Figure 33: SP cells enhanced tumorigenicity and metastatic potential than non-SP cells. (A) Sorted SP cells were injected into the OPN\(^{+/+}\) and OPN\(^{+/-}\) and kept for 5 weeks. Mice were sacrificed and tumors were collected and photographed. (B) Mean tumor weight was calculated, analyzed statistically and represented graphically. \(*P<0.007\) (C) Mean tumor volume was calculated, analyzed statistically and represented in the form of bar graph. \(*P<0.007\) (D) Sorted SP and non-SP cells were injected intravenously into NOD/SCID mice and metastasis to lungs and liver was analyzed using IVIS system. Lower panel: Mice were sacrificed and lung/liver dissected out and further confirms lung and liver metastasis using IVIS.
7.2.9 Stromal OPN regulates SP phenotype by upregulating ABCG2 expression through ERK signaling

To examine the signaling pathway involved in enrichment of SP phenotype, primary cells (B16-WT) were treated either with PI3 kinase inhibitor (wortmannin) or MEK/ERK inhibitor (U0126) for 24 h, stained with Hoechst and analyzed by flowcytometer for SP phenotype. The result indicated that blocking PI3-kinase have no effect on SP pool size but when treated with U0126, SP phenotype significantly decreased (Fig. 34A-C), suggesting that ERK pathway is involved in regulation of enhanced SP phenotype. We then examined for ABCG2 expression in the wortmannin or U0126 treated B16-WT cells lysates, and the result suggests that inhibiting PI3-kinase signaling has no effect but blocking ERK signaling significantly reduced ABCG2 expression (Fig. 34D). This further confirms that stromal OPN enhanced SP phenotype is associated with ABCG2 expression through ERK signaling.

To further examine whether B16-WT cells has the ability to enrich SP phenotype in parental B16F10 cells, conditioned media (CM) collected from B16-WT cells were used to treat B16F10 cells, stained with Hoechst dyes and analyzed by flowcytometer. There were almost two fold (8% to 19.4%) increase in SP phenotype in CM treated B16F10 cells as compared to untreated parental B16F10 cells (Fig. 35A & B). Next, we treated B16F10 cells with either wortmannin or U0126 along with CM of B16-WT for 24 h. Treated cells were stained with Hoechst dyes and SP analysis was performed. The results showed that blocking PI3K signaling has no effect on SP size of CM treated B16F10 cells (Fig. 35C), whereas inhibiting MAPK pathway by U0126 significantly reduces the SP phenotype to 2.5% in CM.

<table>
<thead>
<tr>
<th>Cells injected (1x10^3 cells/mice)</th>
<th>No of Tumor in OPN^+^ mice</th>
<th>No of Tumor in OPN^−^ mice</th>
<th>No of Tumor in NOD/SCID mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>10/12</td>
<td>8/12</td>
<td>8/8</td>
</tr>
<tr>
<td>NSP</td>
<td>2/12</td>
<td>0/12</td>
<td>1/6</td>
</tr>
</tbody>
</table>

Table 2: Summary of tumor incidence in different mice model injected with SP and non-SP cells.
treated B16F10 cells (Fig. 35D). This observation further strengthens the finding and suggests that stromal-OPN regulates SP phenotype through ERK pathway.

Figure 34: stromal OPN regulates SP phenotype in murine melanoma through ERK pathway. (A) B16-WT cells were stained with Hoechst and SP analysis was performed. (B) B16-WT cells were treated with wortmannin for 24 h, stained with Hoechst and analyzed for SP using flowcytometer. (C) B16-WT cells were treated with MEK-ERK inhibitor (U0126) and SP analysis was performed. Inset: SP analyses were performed with reserpine treated respective cells. (D) Western blot analysis of ABCG2 expression in the lysates of B16F10, B16-KO and B16-WT cells treated with either wortmannin or U0126.

7.2.10 ERK2 but not ERK1 regulates SP phenotype in response to stromal-OPN in B16F10 cells

To further dissect ERK signaling in response to stromal-OPN in B16F10 cells, loss of function studies were performed. For this B16F10 cells were stably transfected with kinase
negative ERK1 (ERK1-dn) and kinase negative ERK2 (ERK2-dn), treated with CM of B16-WT cells, stained with Hoechst dyes and analyzed for SP phenotype with flowcytometer.

Unexpectedly, no significant difference in SP size (15.6%) in ERK1-dn transfected melanoma cells were observed but drastic reduction in SP phenotype (5.3%) was observed in ERK2-dn transfected melanoma cells (Fig. 36A & B). This suggests that ERK2 is playing an important role in regulation of SP phenomena.

To further confirm our observations, gain of function studies were performed with B16F10 cells stably transfected with ERK1-wt and ERK2-wt. These clones generated were treated with CM of B16-WT cells, stained with Hoechst dyes and analyzed for SP. Surprisingly, only 22.7% SP was observed in ERK1-wt transfected melanoma cell which is almost similar to that observed in B16-WT cells (Fig. 36C). In contrast, drastic increase in SP

Figure 35: B16-WT cell enhances SP phenotype in parental B16F10 cells through ERK pathway. (A) Flowcytometric analysis of SP phenotype in B16F10 cells. (B) B16F10 cells were treated with conditioned media of B16-WT cells for 24 h and SP analysis was performed. (C) B16F10 cells were treated with conditioned media of B16-WT cells and wortmannin for 24 h and SP analysis was performed. (D) SP analysis was performed with B16F10 cells treated with U0126 and conditioned media of B16-WT cells. Inset: control setup for SP treated with reserpine
phenotype (35.6%) was observed in ERK2-wt transfected melanoma cells (Fig. 36D). Since we have shown that SP phenotype is associated with ABCG2 expression, we sought to check the expression of ABCG2 in these clones. Accordingly, these clones were treated with conditioned media collected from B16-WT and Western blot analysis was performed. As expected, loss or gain of ERK1 function has no impact on ABCG2 expression.

Figure 36: ERK2 but not ERK1 regulates SP phenotype in B16F10 cells. (A) B16F10 cells stably transfected with ERK1-dn were treated with conditioned media of B16-WT cells, stained with Hoechst and SP phenotype was analyzed using flowcytometer. (B) B16F10 cells stably transfected with ERK2-dn were treated with CM of B16-WT cells, stained with Hoechst and SP phenotype was analyzed using flowcytometer. (C) B16F10 cells stably transfected with ERK1-wt were treated with CM collected from B16-WT cells, stained with Hoechst and SP phenotype was analyzed using flowcytometer. (D) B16F10 cells stably transfected with ERK2-wt were treated with CM of B16-WT cells, stained with Hoechst and SP phenotype was analyzed using flowcytometer. *Inset*: control setup for SP treated with reserpine (E) Western blot analysis of ABCG2 expression from lysates of B16F10 cells either treated with CM of B16-WT cells or stably transfected with ERK1-dn, ERK2-dn, ERK1-wt and ERK2-wt. Actin was used as a loading control.
But gain of ERK2 function leads to upregulation whereas loss of ERK2 function results in downregulation of ABCG2 expression (Fig. 36D). This result coincides with the flowcytometry results on SP phenotype, and further suggested that stromal-OPN enriches SP phenotype through ERK2 dependent signaling.

7.3 Discussion

The role of OPN in regulation of tumor growth and angiogenesis has been considered as the field of intense investigation for last few years (42, 43, 69, 175). However, most of the studies have demonstrated the crucial role of tumor derived or exogenous OPN in this process (43, 175). The function of host derived OPN in regulation of tumor progression is not clearly understood. Earlier, Nemoto et al. showed the reduced tumor metastasis in bone and lung of OPN deficient mice (249, 250). Moreover, Chakraborty et al. have observed significant reduction of breast tumor growth and angiogenesis in OPN−/− mice (99). In contrast, Natasha et al. have not able to detect any significant alteration of tumor metastasis in bone of OPN−/− mice as compared to OPN+/− (251). However, variation of the previous observations might be due to the nature of tumors or origin of OPN knock out mice but the molecular mechanism by which host OPN regulates tumor growth is not clearly understood.

In the current study, we have observed significant reduction of melanoma growth in OPN−/− mice as compared to wild type mice. Earlier Jessani et al. have demonstrated that human breast cancer cells isolated from orthotopic xenograft tumor in SCID mice, exhibit profound differences in their enzyme activity profiles and showed enhanced tumor growth and metastasis as compared to parental cells (242). Our in vitro data revealed that melanoma cells isolated from the tumors generated in OPN+/− mice (B16-WT cells) exhibit profound aggressive behavior compared to the parental B16F10 cells or B16-KO (cells isolated from tumor generated in OPN−/− mice) cells. Consequently, we have proposed three hypotheses regarding such belligerent behavior of B16-WT cells: (i) stromal OPN might induce constitutive changes in melanoma cells resulting in enhanced activation of kinases and transcription factors and elevated expression of oncogenic molecules which in turn regulates the belligerent behavior of B16-WT cells, (ii) Stromal-OPN might select the aggressive subpopulation from the heterogeneous parental B16F10 cells and produce a highly tumorigenic population of melanoma cells which exhibit enhanced belligerent behavior of
tumor, (iii) stromal OPN might selectively promote the growth of cancer stem cell population of B16F10 cells and exhibit the aggressive behavior.

Although previous researchers have demonstrated that stromal OPN can regulate hematopoietic stem cells in mice model, its role on cancer stem cell has not been addressed completely. We extended our study and checked the SP phenotype, and found that the B16F10 cells exhibit SP phenotype and stromal-OPN selectively enriches it in the mice model system. We further found that B16-WT cells have the capacity to regulate SP phenotype in B16F10 cells. Further we demonstrated that ERK2 but not ERK1 is capable of regulating the SP phenotype in B16F10 melanoma cells in response to stromal-OPN.

In conclusion, using in vitro and in vivo approaches we have demonstrated, at least in part, the crucial role of stroma derived OPN in regulation of melanoma growth, angiogenesis and metastasis. Moreover we have shown that stromal OPN regulates constitutive changes which resulted in acquisition of an aggressive or tumor initiating phenotype leading to rapid tumor growth, angiogenesis and metastasis. These results indicated that the expression profile of stromal-OPN can be used as an early prognostic marker and suggested the intriguing possibility of stromal-OPN targeted therapy in cancer management.