“Research is what I’m doing when I don’t know what I’m doing.”

Werner Von Braun
8. Summary and Conclusion

Semaphorins play multifaceted roles in tumor progression and angiogenesis apart from regulating the development of central nervous system. Sema 3A originally identified as chemorepellent during axonal guidance is also known to be a candidate tumor suppressor which attenuates breast tumor progression. Sema 3A binds with Neuropilin-1 (NRP-1) which couples with plexin A1 to serve as the signal transducing unit for Sema 3A/NRP-1 complex. However, the underlying mechanism by which it suppresses breast cancer progression is not clearly understood. Our data revealed that Sema 3A regulates PTEN and FOXO 3a phosphorylation and induces their nuclear translocation. The data also suggested that Sema 3A-induced FOXO 3a activation is dependent on NRP-1 and is mediated by PTEN. The cell migration data revealed that PTEN and FOXO 3a play crucial role in Sema 3A/NRP-1-mediated breast cancer cell migration. Chip assay data depicted that FOXO 3a binding site is present on MelCAM promoter. The results also indicated that Sema 3A-induced MelCAM expression is dependent on NRP-1 and mediated by PTEN and FOXO 3a. Using various in vivo mice models, we have either overexpressed or silenced Sema 3A and observed the similar expression pattern of Sema 3A regulated molecules like pPTEN, FOXO 3a and MelCAM. Clinical specimen analysis revealed that reduced expression of Sema 3A and p-PTEN correlate with breast cancer progression, further strengthening our in vitro and in vivo findings. We have for the first time linked a series of tumor suppressors like PTEN, FOXO 3a and MelCAM in Sema 3A/NRP-1-mediated breast tumor suppression. In second part of our study, we have elucidated an apoptotic signaling mechanism where Sema 3A triggers anti-cancer activity of sulforaphane (SFN) when used in combination in breast cancer cells. Our in vitro findings revealed that Sema 3A in combination with SFN inhibits breast cancer cell motility and induces apoptosis by regulating the expression of various pro- and anti-apoptotic molecules. The results indicated that Akt and ERK pathways are crucial for induction of apoptosis-induced by Sema 3A in response to SFN in breast cancer model. Our in vivo results revealed that Sema 3A in combination with SFN attenuated breast tumor growth in NOD-SCID mice model by modulating Akt and MAPK signaling.
The following aspects have been addressed in the first part of the study

- To examine whether Sema 3A inhibits breast tumor (MDA-MB-231 and MCF-7) cell migration.
- To study whether Sema 3A regulates Akt activation and Akt dependent PTEN activation in breast cancer cells.
- To investigate the role of Sema 3A in Akt dependent FOXO 3a and MelCAM expression in these cells.
- To examine the role of PTEN, FOXO 3a and MelCAM in Sema 3A regulated wound migration in breast cancer cells.
- To study whether Sema 3A regulated signaling controls breast tumor growth and angiogenesis in NOD-SCID mice model.
- To study the expression profile of Sema 3A and other Sema 3A regulated molecules in various grades of breast cancer clinical specimens and how it correlates with breast cancer progression.
- To further understand the molecular mechanism by which Sema 3A controls tumor growth and angiogenesis and how Sema 3A regulated signaling may act as a therapeutic target for management of breast cancer.

The major findings of the first part of the study are as follows:

- Sema 3A phosphorylates PTEN and induces its nuclear translocation in MDA-MB-231 cells.
- Sema 3A-induced PTEN phosphorylation is inhibited by blocking NRP-1 expression.
- Sema 3A inhibits Akt phosphorylation.

Taken together, these results suggested that Sema 3A regulates NRP-1 dependent PTEN phosphorylation and induces its nuclear translocation in breast cancer cells.

- Sema 3A inhibits FOXO 3a phosphorylation.
- Sema 3A induces FOXO 3a nuclear translocation and DNA binding.
- NRP-1 regulates Sema 3A-induced FOXO 3a nuclear translocation and DNA binding in MDA-MB-231 cells.
Sema 3A dependent FOXO 3a nuclear translocation is mediated by PTEN.

These data indicated that Sema 3A-induced FOXO 3a nuclear translocation is dependent on NRP-1 and mediated by PTEN in breast cancer cells.

- Sema 3A induces MelCAM expression both at protein and mRNA levels.
- NRP-1 plays a key role and PTEN/FOXO 3a serve as downstream molecules in Sema 3A-induced MelCAM expression.
- Identification of FOXO 3a binding site on MelCAM promoter.

The data showed that FOXO 3a binding site is present on MelCAM promoter and suggested that Sema 3A-induced MelCAM expression is dependent on NRP-1 and mediated via PTEN and FOXO 3a.

- NRP-1 mediates Sema 3A attenuation of MDA-MB-231 cell migration.
- Overexpression of Sema 3A inhibits MDA-MB-231 cell motility.
- Blocking endogenous Sema 3A expression enhances MCF-7 cell migration.

Overall, these results highlighted the tumor suppressive role of Sema 3A and indicated that Sema 3A attenuation of breast cancer cell migration is dependent on NRP-1.

- FOXO 3a overexpression enhances Sema 3A inhibition of MDA-MB-231 cell migration and vice versa.
- Sema 3A regulates tumor-endothelial cell interaction through NRP-1-mediated paracrine mechanism.

Thus, these data showed that PTEN and FOXO 3a play crucial role in modulating Sema 3A-mediated breast cancer cell migration and Sema 3A
regulates tumour-endothelial cell interaction through NRP-1 dependent paracrine mechanism.

- Sema 3A overexpression attenuates breast tumor growth and angiogenesis.
- Blocking endogenous Sema 3A downregulated p-PTEN and MelCAM expression and FOXO 3a expression in MCF-7 cells.
- Silencing of endogenous Sema 3A augments breast tumor growth in NOD-SCID mice model.
- Sema 3A suppresses VEGF-induced angiogenesis and capillarogenesis.

Taken together, these results suggested that overexpression of Sema 3A attenuates whereas silencing endogenous Sema 3A augments breast tumor growth and angiogenesis, modulates the expression of various Sema 3A regulated molecules in breast cancer cells.

- Clinical specimen analysis revealed that Sema 3A and p-PTEN expression are correlated with breast cancer progression suppresses VEGF-induced angiogenesis and capillarogenesis.

In conclusion, our study highlighted the molecular mechanism by which Sema 3A and NRP-1 regulates breast cancer progression and angiogenesis. Our study has established a functional correlation between chains of tumor suppressor genes with attenuation of breast cancer progression.

The following aspects have been addressed in the second part of the study:

- To analyze the effect of Sema 3A on MDA-MB-231 cell viability and migration in presence of SFN.
- To decipher the signaling mechanism involved in the apoptosis-induced by Sema 3A and SFN treatment in MDA-MB-231 cells.
- To investigate the expression profile of various pro-apoptotic and anti-apoptotic molecules in response to Sema 3A and SFN-induced cell death.
- To analyze the effect of both Sema 3A and SFN on breast tumor growth using in vivo mice model.
The major findings of the second part of the study are as follows:

- Sema 3A in combination with SFN inhibits MDA-MB-231 cell viability.
- Sema 3A in combination with SFN inhibits colony formation on matrigel.
- Sema 3A in presence of SFN significantly increased DNA fragmentation and induced apoptosis in MDA-MB-231 cells.
- Sema 3A in combination with SFN enhanced the expression of various pro-apoptotic molecules and downregulated the expression of many anti-apoptotic molecules.
- SFN in combination with Sema 3A suppressed MDA-MB-231 cell migration.

Overall the results indicated that Sema 3A in combination with SFN induces apoptosis and inhibits breast cancer cell migration.

- Akt and ERK signaling pathways are crucial for induction of cell death in response to both Sema 3A and SFN treatment in MDA-MB-231 cells.
- Sema 3A in combination with SFN suppresses breast tumor growth in orthotopic mice model by modulating Akt/ERK signaling.

Taken together, the data suggested that Sema 3A in combination with SFN inhibits breast tumor growth by regulation of Akt/ERK pathways.

Thus, in summary our study provided a novel mechanism of regulation of tumor growth by anti-cancer compound, SFN and a tumor suppressor gene, Sema 3A. The data indicated that Sema 3A triggers anti-carcinogenic effect of SFN in breast cancer cells. Sema 3A in combination with SFN induces cell death and suppresses breast cancer growth. Thus, Sema 3A in combination with SFN could unravel many questions that can help in designing new drugs for the treatment of breast cancer.