Chapter 5

Summary and Conclusions
Summary

This study examined molecular mechanisms underlying metastasis of primary breast tumors to distant organs. The study had two parts: first, identification of a key regulator of metastasis; and second, investigation of molecular pathways contributing to tumor spread. Analysis of patient samples, animal models, cell lines, and signaling pathways operating within breast cancer cells identified NM23H2 as a crucial regulator of breast cancer metastasis. At a molecular level, NM23H2 negatively controlled TGF beta signaling through TGFBR2, opposed epithelial to mesenchymal transition (EMT), and decreased metastatic proclivity of breast cancer cells.

Part 1: Identification of key regulator of breast cancer metastasis

A. Gene expression datasets from breast tumors of 927 patients was analyzed to assess which of >30 metastasis suppressor genes (MSGs) showed reduced expression during tumor progression. The tumors were first classified into two categories: early (stage I and II) and advanced (stage III and IV). The analysis indicated significantly depleted level of NM23H2 in advanced compared to early stages of tumors across multiple datasets. Notably, NM23H2 was the only MSG which showed consistently reduced expression among >30 MSGs.

B. Quantitative RT PCR of breast tumors confirmed reduced expression of NM23H2 in advanced tumors compared to early stage tumors.

C. Immunohistochemical analyses of lymph node metastasis and the primary tumors from patients showed significantly diminished level of NM23H2 in metastasis.

D. Breast cancer cells with reduced expression of NM23H2 showed higher metastatic potential in vivo compared to cells with enhanced expression of NM23H2. In particular, studies in nude mice model indicated efficient formation of metastatic
foci in lung by breast cancer cell with reduced expression of NM23H2 in comparison to cells with enhanced expression of NM23H2.

E. Expression of NM23H2 in primary breast tumor related with over all patient survival; higher level of NM23H2 associated with better overall patient survival (n=728).

**Part 2: Identification of molecular mechanisms involved in metastasis**

Following identification of NM23H2 as a key regulator of metastasis potential of breast cancer cells, investigations revealed that molecular basis of metastasis control by NM23H2 involved modulation of TGF beta signaling.

A. Gene expression profiling after enhanced expression of NM23H2 showed wide spread changes in transcriptome of breast cancer cells (MDA-MB-231); 1608 genes were differentially expressed; 996 genes were up regulated and 612 genes were down regulated ($P<0.01$).

B. Analysis of gene expression showed conspicuous enrichment of genes implicated in EMT among NM23H2 target genes. This suggested NM23H2 could regulate EMT and modulate aggressiveness of breast cancer cells.

C. Gene Ontology analysis showed evidence of genes related to TGF beta signaling as one of the most enriched among NM23H2 targets. This suggested NM23H2 mediated control of TGF beta signaling as a candidate for regulation of EMT pathway.

D. Enhanced level of NM23H2 led to decreased expression of TGF beta receptor II (TGFBR2). The decreased expression was both at mRNA and protein level suggesting transcriptional regulation of TGF beta receptor II (TGFBR2) by NM23H2.
E. A bioinformatic approach identified a sequence motif for NM23H2 binding in the promoter of TGF beta receptor II (TGFBR2) 1490 bases upstream of TSS. ChIP for NM23H2 followed by PCR with primers that flanked the motif confirmed NM23H2 occupancy of the TGF beta receptor II (TGFBR2) promoter suggesting NM23H2-mediated transcriptional repression.

F. The phosphorylation of SMAD3 was diminished in presence of NM23H2 showing impaired relay of signals downstream of TGF receptor II.

G. ChIP assays revealed reduced SMAD4 localization on the E-cadherin promoter in cells with enhanced expression of NM23H2.

H. Altered regulation of TGF beta signaling was independently confirmed using a SMAD reporter construct; ~60% reduced activity of the SMAD reporter was noted in NM23H2-induced cells.

I. Quantitative Real time PCR and western blot of MDA-MB-231 cells expressing NM23H2 showed increase in several markers of the epithelial state; concomitantly, several mesenchymal markers decreased in expression. This suggested a shift in state of MDA-MB-231 cells which are known to be mesenchymal breast cancer cells with aggressive metastatic potential.

J. Cells with enhanced expression of NM23H2 showed a pronounced epithelial morphology compared to cells with reduced level of NM23H2.

K. Invasiveness across extra cellular basement membrane matrix and endothelial cells was found to be diminished on NM23H2 induction. These observations suggested that enhanced expression of NM23H2 compromised efficiency of breast cancer cells to successfully complete initial steps of metastatic cascade.
L. Independent observations in MDA-MB-468 cells also showed decreased invasiveness under NM23H2-induced conditions. This suggested opposition of EMT by NM23H2 as common theme of metastasis suppression in breast cancer cells.

M. The relationship between NM23H2 and TGFBR2 expression extended to patient samples. Analysis of 728 patient tumor gene expression datasets confirmed inverse relation between the two.

N. NM23H2 interacts with LSD1 and enhanced recruitment of LSD1 on TGFBR2 promoter brings about loss of histone marks (H3K4Me3) which signals for gene activation.

O. The promoter of NM23H2 is hyper methylated in highly aggressive MDA-MB-231 cells compared to MCF-7 cells.

**Conclusions**

The results described here yield novel insights into breast cancer metastasis. NM23H2 emerged as a MSG that regulates EMT and dictates the metastatic potency of cancer cells. These results are relevant in the context of previous observations as well which although implicating NM23H2 in metastasis suppression did not provide a molecular explanation for the same. The results here showed NM23H2 as a decisive regulator of EMT in breast cancer cells. Once activated, EMT program confers an enhanced ability to migrate, invade through basement membrane, oppose apoptosis and mediate resistance to cancer drugs in cancer cells. Herein, it was noted that increased expression of NM23H2 in metastatic breast cancer cells led to molecular events that favored an epithelial organization and ensured a decrease in aggressiveness.
The significance of current project stems from observations that metastasis from primary tumors in epithelial cells kill 90% of cancer patients. It is thus conceivable that reduced/loss of expression of NM23H2 during tumor progression would lead to increased TGF beta signaling and thus favor metastatic dissemination. On the other hand, strategies that increase the expression of NM23H2 may impede TGF beta signaling and hence oppose aggressive growth.