Metavivor FINAL progress report. Heide L. Ford

The original aims of the Metavivor proposal were as follows (all progress since 6 month report is shown in blue text):

**Aim 1. To determine whether and which members of the miR106b-25 cluster contribute to Six1-induced TGFβ signaling and metastasis *in vivo*, and to assess whether the miRs, when targeted, can reverse Six1 induced metastasis, as well as which miRs within the cluster most efficiently reduce metastasis when targeted.**

As noted in the 6 month progress report, we initially began these experiments by collaborating with MiRagen, a microRNA based company that was able to generate locked nucleic acids (LNAs) for us to test in vivo. LNA technology has demonstrated superior stability *in vivo,* and increased specificity in target recognition, therefore making these molecules of great potential clinical use for the purpose of miRNA inhibition (127). Thus, miRagen designed and manufactured specific LNA oligonucleotides that would inhibit the miR-106b-25 cluster miRNAs for our use *in vivo*. Importantly, when designing these LNAs for inhibition, we decided to first target the miRNA families that are represented in the miR-106b-25 cluster (trying to start with the most broad stroke, after which we intended to inhibit each miR separately to see which miR plays the most important role in Six1-induced metastasis). The families we inhibited were the miR-17 family (comprised of miR-17, miR-20a, miR-20b, miR-106a, miR-106b, and miR-93), and the miR-25 family (comprised of miR-25, miR-92a, and miR-363). Reasons for this particular design include the possibility of redundancy for miRNAs that contain the same seed sequences, and therefore may target the same genes and facilitate similar phenotypes. Therefore, for inhibition of the miR-106b-25 cluster miRNAs and their family members, we used two LNA oligos, miR-106b Fam LNA (targeting the miR-17 family members, including miR-93 and miR-106b) and miR-25 fam LNA (targeting the miR-25 family members). In this way, we were able to break up effects of miR-25 (which we had previously shown did not have a significant effect on TGFβ signaling, and miR-106b/miR-93, which we had shown increased the tumor promotional effects of TGFβ. Before using the LNAs *in vivo*, we tested their efficacy and specificity *in vitro* using luciferase reporters, and all LNAs were able to target the appropriate seed sequences in this context (and not non-specific sequences).

To determine if the miR-106b-25 miRNAs are necessary for Six1-mediated breast cancer metastasis, we utilized our established intracardiac metastasis model with luciferase tagged MCF7-Ctrl and MCF7-Six1 cells in nude mice. All mice were implanted with estrogen pellets prior to cell injection, and cells were then injected into the left ventricle of each mouse. To inhibit the miRNAs, we delivered the LNA miRNA inhibitors through subcutaneous injection starting on day 2 after cell injection. For control groups, the scrambled LNA control (trunc_ctrl) was injected into both MCF7-Ctrl and MCF7-Six1 injected mice. For the experimental group, the miR-106b fam and the miR-25 fam LNAs were injected subcutaneously into MCF7-Six1 injected mice. We also included an additional saline only control in MCF7-Six1 injected mice. Unfortunately, within the first week of the experiment, we observed dark lesions on the hind flanks of mice specifically only on the sides injected with the miR-106b-25 LNA, suggesting that this LNA caused an unexpected side effect. As a result, the experiment was not properly completed (we were asked by our animal facility to halt injections for a period of time), and thus we were unable to assess whether the miRs are critical to mediate Six1 induced metastasis using this method. Instead, we are developing lines in which we stably inhibit the specific miRs, in order to ask this question in a genetic manner first (rather than attempting first to use the more therapeutic approach), while we attempt to determine the cause for the side effects in the mice treated with LNAs that target miR106b/93 family members.

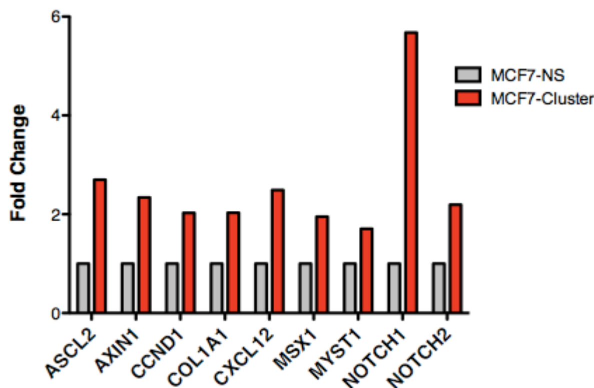


**Figure 1. Tumor initiating cell related genes are enriched in miR-106b-25 overexpressing MCF7 cells**. Genes important for stem cell maintenance, growth and differentiation are increased in MCF7-Cluster cells as compared with MCF7-NS cells, as determined by a stem cell qPCR array. Data are represented as fold change in gene expression in MCF7-Cluster cells as compared with control MCF7-NS cells from three replicate plates of each condition. The Genes shown exhibit at least a twofold induction in MCF7-Cluster cells as compared with the MCF7-NS cells.

**Aim 2. To determine whether miRs within the miR106b-25 cluster can predict response to TGFβ inhibitors, and to determine which of the miRs have the most predictive value.**

This aim has also taken an unexpected turn, although a very exciting one. While we do know that TGFβ signaling mediates the later stages of metastasis (Micalizzi et al., JCI 2009) downstream of Six1, and that the miR106b-25 cluster is important to mediate TGFβ signaling downstream of Six1, we also know that Six1 mediates tumor initiation (an important component of metastasis as tumors need to initiate at secondary sites to grow out), and thus we were most concerned with first with proving that TGFβ signaling was required downstream of the miR106b-25 cluster of microRNAs to mediate tumor initiation properties. Part of reason to examine that question was that we had also determined that the same cluster of microRNAs leads to upregulation of Notch signaling, which is also an important mediator of tumor initiation/stemness (see Fig. 1 above). Because we would like to target the tumor initiating cells (TICs), often referred to as cancer stem cells (CSCs), as these are believed to be critical for metastasis, we first asked whether TGFβ or Notch signaling played a more important role in mediating the tumor initiating phenotypes downstream of miR106b-25. To this end, we performed a mammosphere assay (an in vitro assay) to measure functional TIC capability. As can be seen in Fig. 2 below, when we inhibit Notch signaling using DAPT (a gamma secretase inhibitor that inhibits Notch signaling), we inhibit the ability of the miR106b-25 cluster to mediate mammosphere formation of MCF7 cells, a functional readout of TICs. However, when we inhibited TGFβ signaling, using SB431542 (an inhibitor of the TGFβ Type I receptor), we did not inhibit the ability of the cluster of microRNAs to induce mammosphere formation. Similarly, when we added both inhibitors together, we saw effects that were in line with the effects we observed with DAPT alone. Together, these data suggest that the miR106b-25 cluster influences TIC properties primarily through its ability to induce Notch signaling, rather than TGFβ signaling. We have also examined whether Notch signaling is important for TIC phenotypes in additional cell lines, so that we know the effect is broader than in just the context of MCF7 cells. Fig. 3 below shows that the cluster of miRs induces TIC phenotypes (as measured by mammosphere formation) in SUM159PT breast cancer cells also (primary assay shown- secondary is currently underway), and that Notch signaling is critical for this phenotype in this setting as well as in the MCF7 cells.

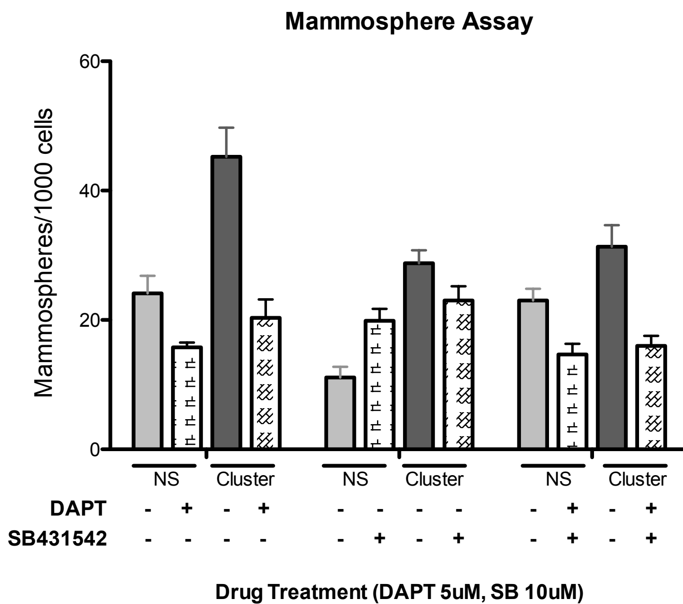


**Fig. 2. Mammosphere assays** demonstrate that the miR106b-25 cluster enhances mammosphere formation via its ability to effect Notch signaling. DAPT- gamma secretase inhibitor that targets Notch signaling. SB431542- TβRI kinase inhibitor.

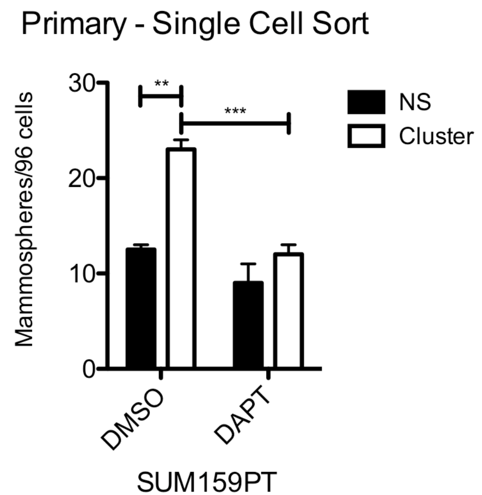


Fig. 3. The miR106b-25 cluster enhances mammosphere formation via Notch signaling in SUM159PT cells.

Interestingly, we can observe the effect of miR106b-25 on Notch signaling in numerous breast cancer cell lines, beyond the two shown above (Fig. 4), suggesting that this cluster of miRs affects Notch globally in breast cancer. Thus, we have begun to carefully examine the effects of the individual miRs within the miR106b-25 cluster on the ability to influence

Notch signaling. We have found that similar to what is observed with TGFβ signaling, miR-106b and miR-93 mediate the effects on Notch signaling, whereas miR-25 does not significantly affect the pathway (not shown). At the time of our

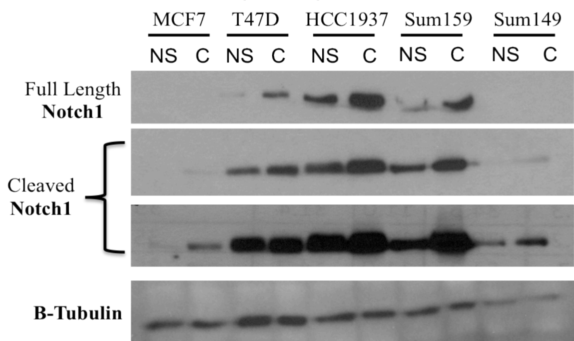


**Fig. 4.** Both full length and intracellular cleaved Notch are increased with stable overexpression of the miR106b-25 cluster in numerous breast cancer cell lines, demonstrating the broad generality of the ability of the miRs to increase Notch signaling. Shorter and longer exposures of cleaved Notch are shown.

last progress report, we had begun to dissect the mechanism by which miR-106b and miR-93 influence Notch signaling, focusing on Itch, which we have found is targeted by miR-106b and miR-93 (which share the same seed sequence), and which is a negative regulator of Notch by ubiquitinating it and targeting it for degradation. Interestingly, however, while we could prove that Itch is targeted by miR106b/miR93, our data suggested that it is not the major mechanism through which the miRs are activating Notch signaling. We thus examined whether miR106b-25 influences Notch signaling via additional mechanisms, and found that the cluster downregulates p63, a negative regulator of Notch signaling (Fig. 5).

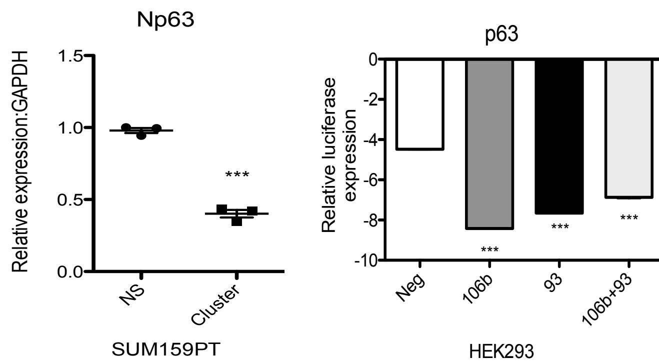


**Fig. 5.** P63 is inhibited by miR106b and miR93. Left panel shows real-time PCR of levels of p63 mRNA in SUM159PT cells either expressing a non-silencing control vector or the miR106b-25 cluster of microRNAs. The right panel shows a luciferase reporter containing the 3'UTR of p63, demonstrating that it can be inhibited by miR106b and miR93.

Thus, downregulation of deltaN-63a (a specific isoform of p63) would enable activation of Notch signaling. We have further found that the cluster of miRs leads to upregulation of Jagged, thus apparently affecting both the receptor and ligand in this pathway. We continue to work on the mechanism by which Notch is regulated by the cluster of microRNAs, focusing on miR106b and miR93, as the major regulators of the Notch phenotype. As we move forward, we will continue to elucidate the mechanism by which miR-93 and miR-106b influence Notch signaling and cancer stem cell properties, and will likely now focus on the miRs that are predictive of response to Notch inhibitors, as a more targeted way of inhibiting tumor stem cells (and thus breast cancer metastasis), as opposed to focusing primarily on TGF-beta inhibitors. In addition, we will determine whether the miRs correlate with increased Notch signaling in human breast cancers, particularly metastatic breast cancers. We have begun to look in human breast cancers, and have seen weak correlations with Notch1 mRNA levels. However, we feel the better approach in human breast cancers is to see whether the microRNAs correlate with Notch response signatures (as a read-out of Notch activity in human breast cancers). Examining datasets in this manner is ongoing.

To demonstrate clearly that Notch signaling, downstream of miR106b-25, is responsible for both tumor initiation and breast cancer metastasis, we have generated stable cell lines in which we knocked down Notch1 downstream of the cluster of microRNAs is both MCF7 and SUM159PT cells. Stable lines are currently being propagated in order to put into animals. Before generating these lines, we tested whether a transient knockdown of Notch1 in the lines containing miR106b-25 would lead to reversal of TIC phenotypes (thus suggesting that in vivo they will reverse both tumor initiation and metastasis. Fig. 6 demonstrates that a transient knockdown (KD) of Notch1 in setting plus or minus miR106b/93 (introduced using mimics) reverses the ability of miR106b/93 to induce cancer stem cell phenotypes, such as increased numbers of cells expressing the stem cell marker signature CD44+/CD24-. These data strongly suggest that stable KD of Notch1 will reverse the TIC phenotypes induced by miR106b-25, thus likely inhibiting tumor initiation in vivo as well as in vivo metastasis. In the next few months, we plan to perform the in vivo experiments, continue mining human data, as well as to definitely identify the mechanism by which miR106b and miR93 induce the TIC phenotype. Once this work is

completed, we plan to submit our first manuscript on this project, on which the Metavivor foundation will be credited. We will then move on to the inhibitor work outlined in our original proposal, either in patient derived xenograft models or in explants.

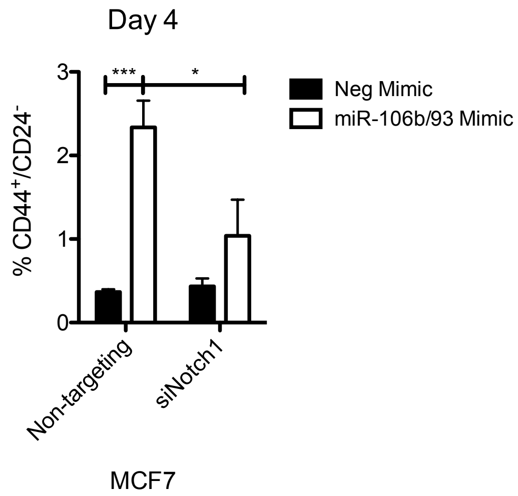


**Fig. 6.** miR106b/93 dramatically increases the CD44+/CD24- TIC phenotype in MCF7 cells, which is reversed by KD of Notch 1 using siRNAs.