Tracking Uptake and Regulating Release of Acute Myeloid Leukemia-derived Extracellular Vesicles

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Introduction and Background

The bone marrow is a complex microenvironment in which many different forms of cell-cell communication can be observed. The release of extracellular vesicles (EVs) from both leukemic and hematopoietic stem and progenitor cells (HSPCS) in the bone marrow is an extremely important area of research due to the role of EVs as a major form of cell-cell communication. This cargo, delivered by either leukemic cells or stem cells, is suspected to cause changes in growth signaling pathways in leukemic cells and cause different stress responses. [2]

In this project, three different drugs were hypothesized to have an effect on leukemic cell EV release. GW4869 (GW) and MHY1485 (MHY) are two drugs we hypothesize will decrease vesicle release. GW operates through inhibiting the production of ceramide, an important player in the budding of exosomes from multivesicular bodies. MHY functions as an mTOR activator. A continuous activation of MTOR has been shown to cause an inhibition of vesicle release,[1] and so we hypothesize that MTOR activation via pharmacological methods will have a similar effect. Rapamycin (RAPA) is the third drug that was investigated in this experiment. Rapamycin functions as an mTOR inhibitor and is hypothesized to have the opposite effect as MHY, and therefore a decrease in vesicle release in MOLM-14 cells is hypothesized. We used MOLM-14 cells as they are a human line of acute myeloid leukemia cells.

While it is important to examine how EV release effects cells, it is also important to analyze where the secreted EVs go. Therefore, another aspect of this experiment involved visualizing EV uptake. We utilized a Cre-lox reporter system to trace EV uptake into recipient cells. Donor cells were produced by introduction of a Cre recombinase gene by transfection into cells. Cre mRNA was confirmed to be present in EVs secreted from these donor cells. Concurrently, recipient cells were produced by transduction of of a lentiviral plasmid encoding a DsRed gene flanked by loxp sites and followed by an eGFP gene, which in the presence of Cre recombination switches from DsRed to eGFP expression. DsRed-expressing recipient cell uptake of EVs containing Cre mRNA will result in translation of Cre recombinase protein and subsequent eGFP fluorescence. Proof of principle and initial validation experiments of this model was conducted using HEK293T cells, an easily genetically modifiable immortalized epithelial kidney cell line.

Altogether these experiments improve upon our understanding of the regulation of EV release in acute myeloid leukemia cells, and validate a Cre-lox model system to further explore the routes of uptake in recipient cells.

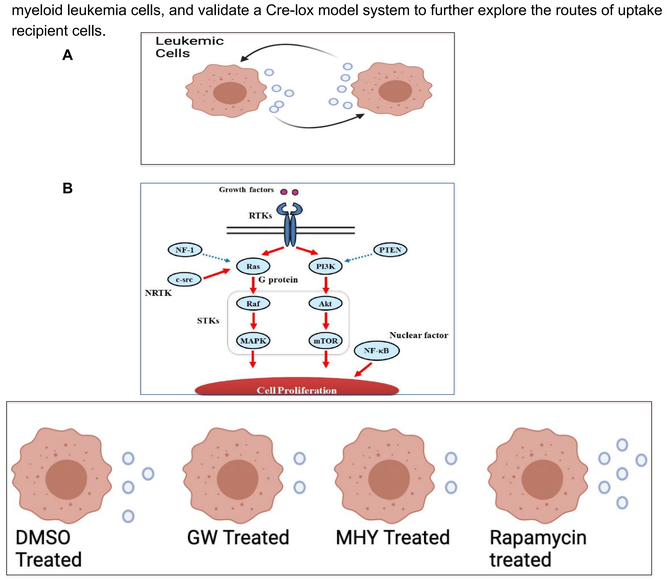

Figure 1. EV release and their impact on signaling pathways. A) In acute myeloid leukemia, leukemic cells release EVs that carry cargo that we hypothesize will impact the growth signaling pathways of other surrounding leukemic cells and themselves (autocrine signaling). In this project, we used MOLM-14 cells specifically as they are human acute myeloid leukemic cells. B) A description of MAPK/ mTOR pathway is presented. MTOR plays a crucial role in the growth and proliferation of a cell as it is activated in the final step of cell proliferation. Based on this, mTOR's phosphorylated form "pmTOR" is one of the proteins we probed for in order to visualize the effect of EVs from leukemic cells. C) An illustration of our hypothesis: Rapamycin we predict will cause an increase in vesicle release, whereas treatment with GW and MHY we hypothesize will cause a decrease in vesicle release.

References

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Butler, John T et al. "Extracellular vesicles in the hematopoietic microenvironment." *Haematologica* vol. 103,3 (2018): 382-394. doi:10.3324/haematol.2017.183335 [2]

Methods

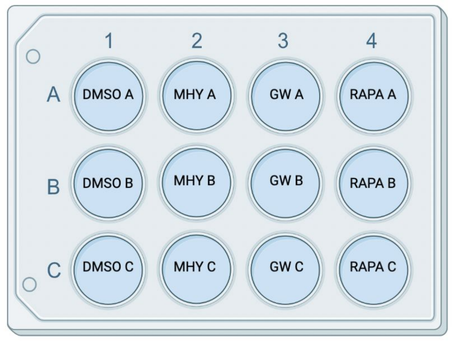


Figure 2. Growth curve plate set up. On day 1, 50,000 MOLM-14 cells were seeded in 1mL of media in 12 different wells in a 12 well plate. Three wells were treated with Dimethyl Sulfoxide (DMSO) as a control as the rest of the drugs used in treatment were reconstituted in DMSO. MHY, GW, and Rapamycin were then administered in triplicate to the remaining 9 wells. The cells were recounted and recorded every 24 hours for seven days and underwent treatment with the drug compound every three days.

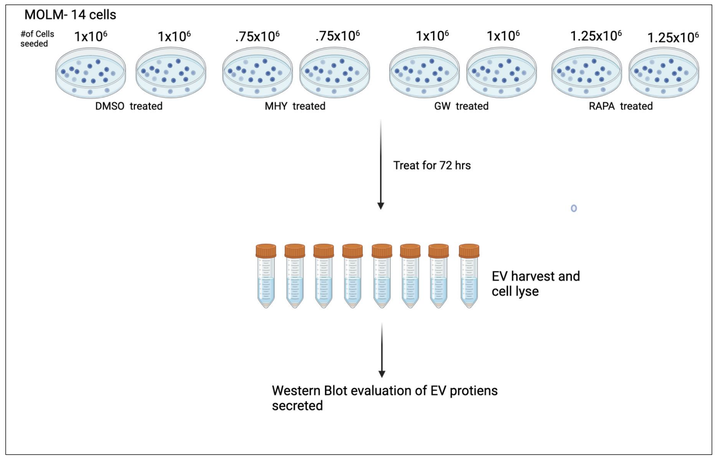


Figure 3. Drug treatment experiment set up. On day 1, according to the the growth curve results, adjusted MOLM-14 cell numbers were seeded in 10 cm plates. Each drug, MHY, Rapamycin, DMSO control, and GW, was administered to cells in duplicate (A and B replicates). Each well was treated for 72 hours then was EV harvested following this protocol: a 500g spin for 5 minutes keeping the supernatant, a 2000g spin for 10 minutes keeping the supernatant, a 10,000g spin for 30 minutes keeping the supernatant, followed by an overnight incubation at 4℃ with polyethylene glycol, a 3000g spin for 1 hour at 4℃ keeping the pellet, and one last clean up ultacentriugation at 100,000g for 90 minutes keeping the pellet. These EVs were then lysed in non-reducing sample buffer and ran on a western blot to be evaluated for EV proteins secreted. The two proteins that were probed for were CD9 and CD63, glycoproteins expressed on EV membranes. Differences in the expression of these proteins would indicate a difference in EV secretion.

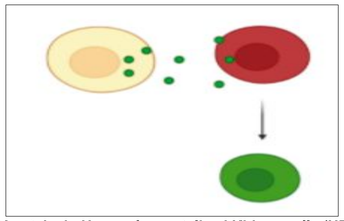


Figure 4. Visualizing EV uptake in Human Immortalized Kidney cells (HEK 293T Cells) In order to introduce Cre recombinase (Cre-IRES-PuroR) into the 293T cells, (an easily genetically modifiable cell line) and their secreted EVs, a transfection method was used. To test for the presence of Cre recombinase in the cells and secreted vesicles, a polymerase chain reaction (PCR) testing of DNA extracted from transfected cells was run. In order to test for where the EVs are taken up, target cells were transduced with the loxP-dsred-stop-loxP-eGFP plasmid. If the EV-associated Cre recombinase is taken up by the target cells, we measured a switch in recipient cells from dsred fluorescence expression to GFP expression. This change in fluorescence expression was measured through flow cytometry analysis.

Results

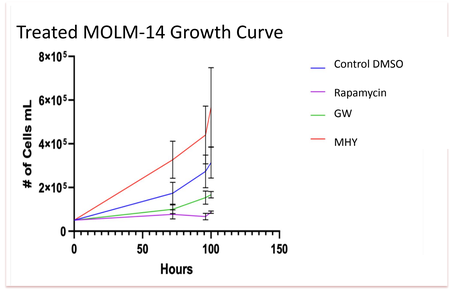


Figure 5. MOLM-14 Cell Growth Curve. Each treatment is represented by a different line. We were able to show that MHY does cause a statistically significant increase in cell growth, as well as the fact that rapamycin treatment causes a statistically significant decrease in cell growth. GW did not cause a significant change from DMSO growth.

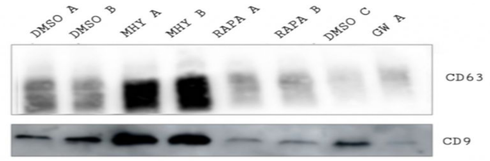


Figure 6. MOLM-14 EV Western blot results. The western blot analysis showed a decrease in EV marker proteins (CD9 and CD63) as a result of treatment with GW. It also appeared to indicate an increase in EV marker proteins in MHY treated cells and no significant difference in Rapamycin treated cells vs. its control. This raised questions as to whether the differences in cell growth of Rapamycin treated cells and MHY treated cells in comparison to their DMSO control was the real driving factor of the apparent changes in EV release due to treatment with these drugs.

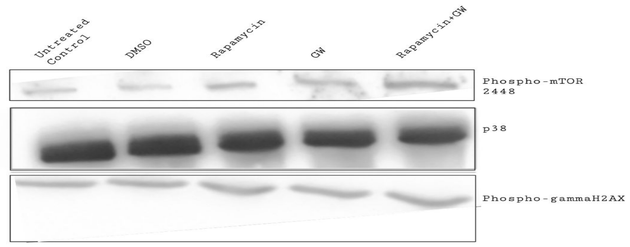
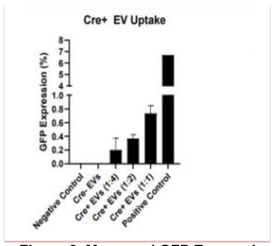


Figure 7. MOLM-14 drug treated cell lysates. Rapamycin and GW treatment resulted in increased levels of activated phosphorylated mTOR protein, that was synergistically elevated with combined treatment. Minimal effects of drug treatment were seen on p38 (a MAP kinase) and phospho-gamma H2AX (a DNA damage response protein) levels.

A



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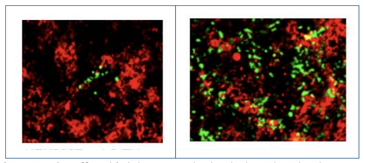

Figure 8. Measured GFP Expression in Cre EV treated cells. A) A bar graph depicting the the increase in GFP expression when treated with increasing doses of Cre+ EVs is presented. It showed a correlational increase in GFP expression with a corresponding increase in vesicle number during treatment. The positive control was a transfection of a dsred transduced cell with Cre plasmid. B) Fluorescent imaging of control Dsred untreated cells (left) vs dsred cells treated with Cre + Evs (right). This shows a clear increase in GFP expression in Dsred cells that were exposed to Cre+ EVs.

Conclusion

Together these findings begin to address the experimental questions of the mechanisms of EV secretion and uptake. We demonstrate that mTOR activation and inhibition significantly impacts myeloid leukemic cell growth, and simultaneously has an effect on EV secretion. Inhibition of ceramide synthesis using GW also appears to decrease vesicle secretion from the AML cell line used in this study. Finally, we have validated a Cre-lox model for beginning to study EV uptake in relevant biological systems.