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Stabilin-mediated cellular internalization of phosphorothioate-modified Antisense Oligonucleotides (ASOs)

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The authors don't have anything to disclose with the presented information.

Abstract

Introduction: Antisense oligonucleotides (ASOs) are short chemically modified oligonucleotides (5-7.4 kDa) that can produce a pharmacological effect by binding to RNA and affecting intermediary metabolism. Over 35 phosphorothioate (PS) ASOs are at various stages of clinical development for use as therapeutic agents and pharmacological tools. Antisense therapy is a progressing area of research, as these small strands of nucleotide oligomers can be produced to silence genes that aggravate chronic disorders or infections. An important distinction for ASOs compared to DNA is the substitution of the phosphodiester (PO) backbone with the PS modification. This sulfur substitution allows for these polar polyanionic molecules to have high stability in biological fluids and selective binding to cell surfaces. Although there is a premium for clinical development of these short chain molecules, the current understanding of the pathways for their ability to traverse plasma membranes remains unresolved. Injected gymnotic ASOs have been shown to accumulate in the liver via the organ's functional scavenging mechanism in highly endocytically active sinusoidal endothelial cells (SECs) and Kupffer cells (KC) compared to hepatocytes. Our work outlines how a non-DNA binding class of scavenger receptors known as Stabilins binds to, and internalizes these small PS ASOs. **Methods:** Primary cells from rat and mouse were isolated and cultured with ^{125}I-ASOs. Stable cell lines expressing Stabilin-1 and two Stabilin-2 isoforms (315-HARE and 190-HARE), were used to analyze binding affinity, endocytosis and degradation of ^{125}I-ASOs in the cell. Co-localization was also done to analyze trafficking of ASOs once internalized within the cell by use of fluorescent ASO and lysotracker. **Results:** It was determined that PS ASOs bind with high affinity to the Stabilin class receptors with the majority of internalization performed by clathrin-mediated endocytosis. Binding was determined to be dependent on proper folding of the receptor, along with relying on salt-bridge formation. Once inside the cell via the Stabilin receptors, co-localization analysis showed ASOs being trafficked for degradation in the lysosome. Increased internalization rates of an ASO targeting the non-coding RNA of *malat-1*, in the Stabilin-expressing cell lines reduced *malat-1* expression more efficiently, indicating not all ASOs are trafficked to the lysosome after internalization. **Conclusion:** Our work shows that ASOs are internalized into the cells of the liver through clathrin-mediated endocytosis. The understanding of the pathway(s) for chemically modified ASO internalization and trafficking with cell surface receptors will aid in future clinical design of ASOs as therapeutic agents.

Introduction: Stabilin receptors

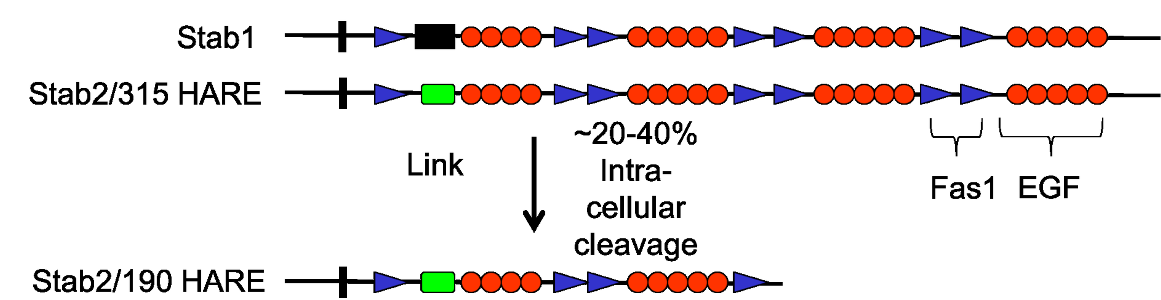

Fig. 1: The Stabilin receptors. Stab1 and Stab2 are expressed in endothelium of liver, lymph node, spleen and bone marrow. Both receptors are comprised of the same domain organization, although Stab1 does not have a functional LINK domain and cannot be categorized as a hyalectin. Both receptors bind to heparin, GDF-15, phosphatidylserine, advanced glycation end-products (AGE), and modified LDL. Individually, Stab1 binds to lactogen and SPARC and Stab2 binds with HA, CS, and collagen pro-peptides. Neither receptor binds natural DNA or RNA with phosphodiester backbones. Both receptors undergo proteolytic cleavage/maturation with Stab1 expressed as isomers of nearly the same size in a 1:1 ratio and Stab2 expressed as 315- and 190-kDa receptors in a 5:1 to 2:1 ratio depending on the tissue. ● = EGF domain, ► = Fas-1 domain, | = transmembrane domain, ▬ = Link domain

Introduction: Antisense Oligonucleotides (ASO)

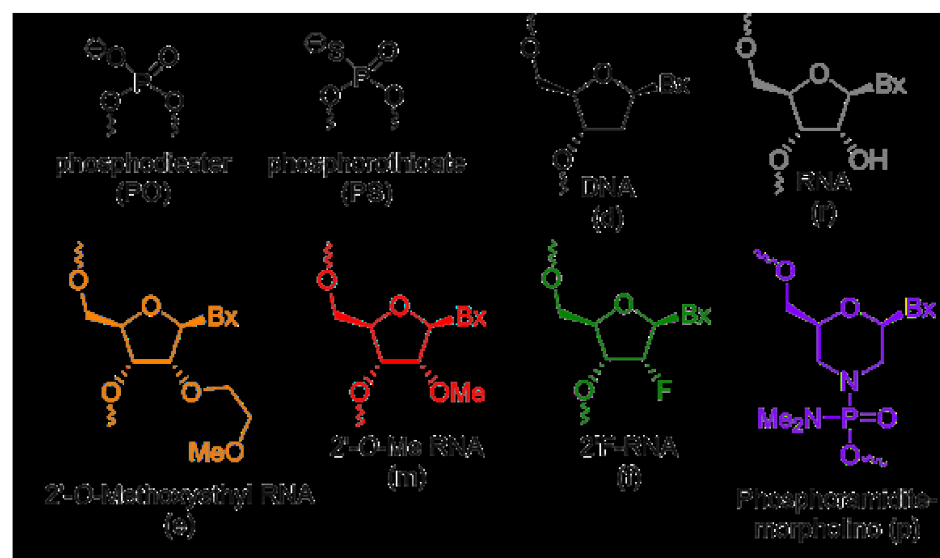

Fig. 2: Components of ASOs

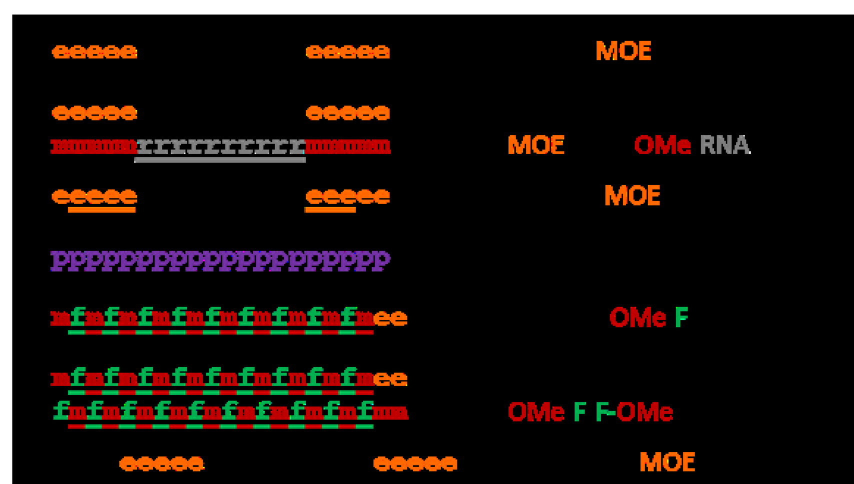

Table. 1: ASO polymers used in this study.

Results

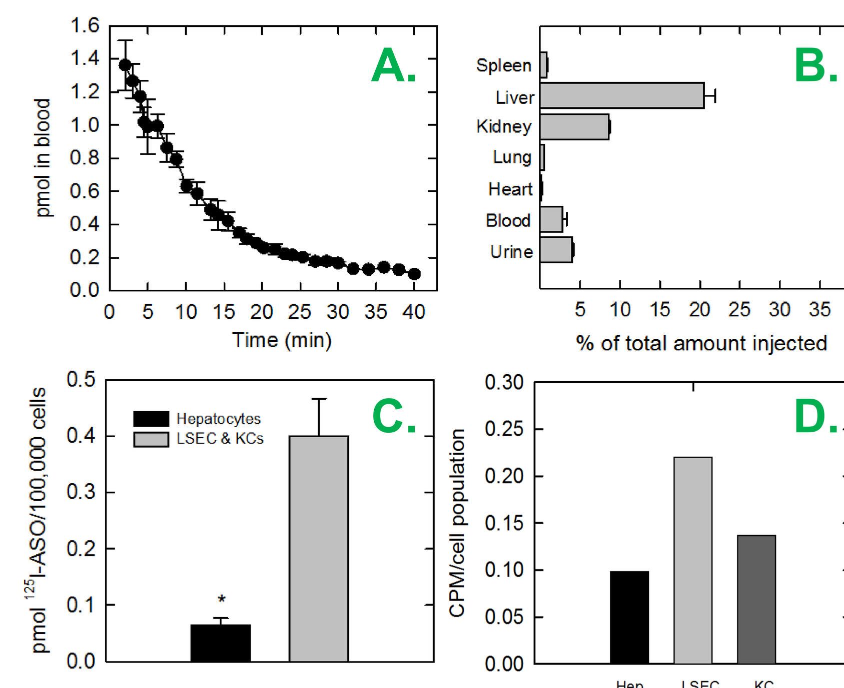

Fig. 3: Half-life and tissue distribution in mice. A) Intravascular injections in mice with 1.0 mg/kg ^{125}I-ASO resulted in a half-life of 9 min in the blood. B) Approximately 20% of all ASO was contained in the liver. C) Within the liver, over 80% of the ASO was in the non-parenchymal fraction and D) the highest distribution of ASO was in the LSECs.

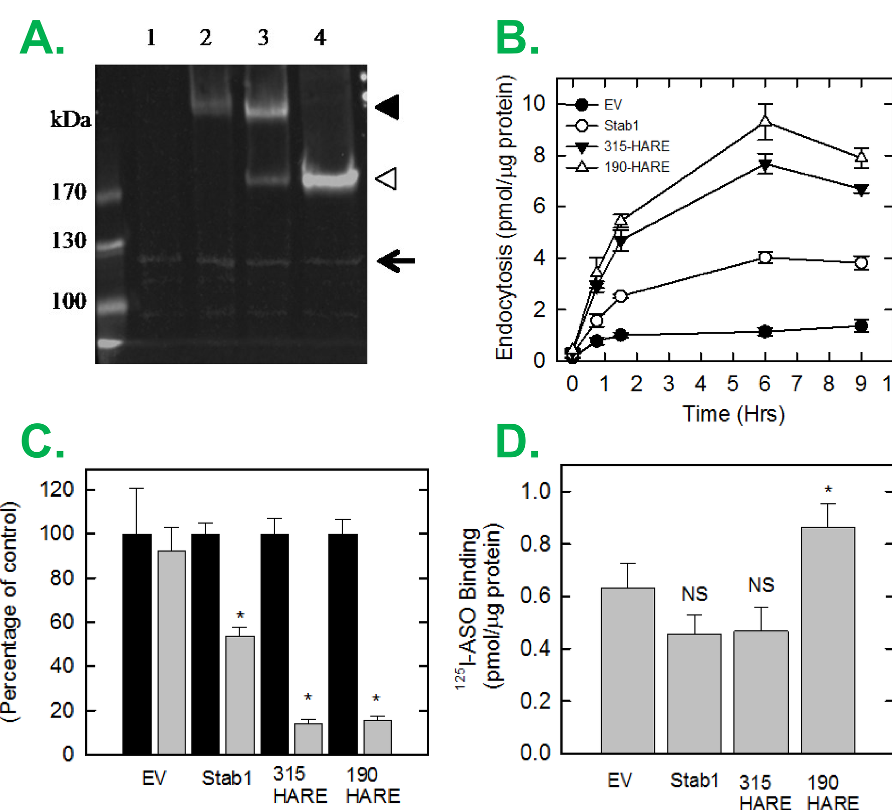

Fig. 4: Stabilin receptor internalize ASOs. A) Cell lysates on 5% SDS-PAGE from HEK Flp-In 293 cells expressing parent pcDNA5 (empty vector (EV)) (lane 1), Stabilin-1 (lane 2), Stabilin-2, 315-HARE (lane 3, black arrowhead), or Stabilin-2, 190-HARE (lane 4, open arrowhead) were probed with anti-V5 to visualize recombinant protein expression and anti-vinculin as load control (black arrow). B) All four cell lines were subject to 0.1 µM ^{125}I-ASO over 9 hrs. C) At least half of the internalization is regulated by clathrin-mediated endocytosis (gray bars) in contrast to untreated controls (black bars). D) A small increase in binding occurs with the 190-HARE cells and not with the other cell lines when compared with EV indicating that Stabilin receptors are primarily involved with endocytosis of ASO from the cell surface. NS = not significantly different from control. * is p=0.038.

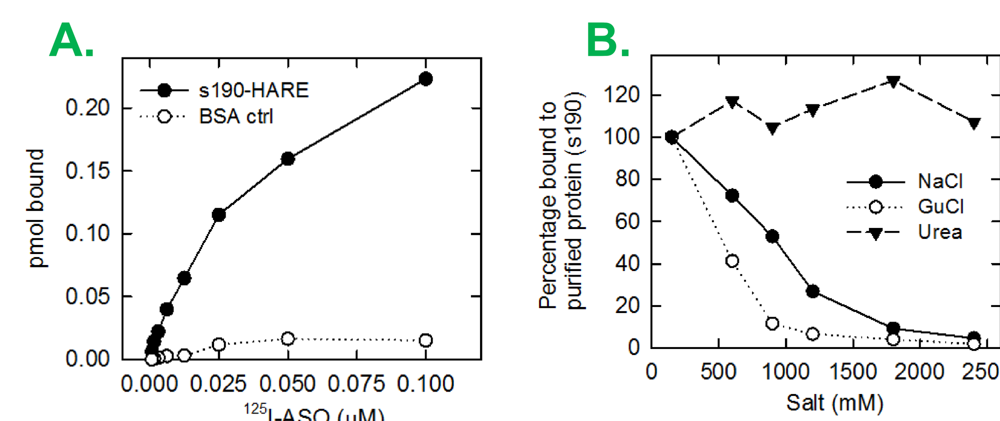

Fig. 5: Binding characteristics of ASO and 190-HARE. A) Purified 190-HARE or BSA was plated in polysorp wells and incubated with increasing concentrations of ^{125}I-ASO. B) 0.05 µM ^{125}I-ASO was incubated with increasing concentrations of NaCl to disrupt ionic bonding, urea to disrupt hydrogen bonding, or guanidinium chloride to disrupt protein folding.

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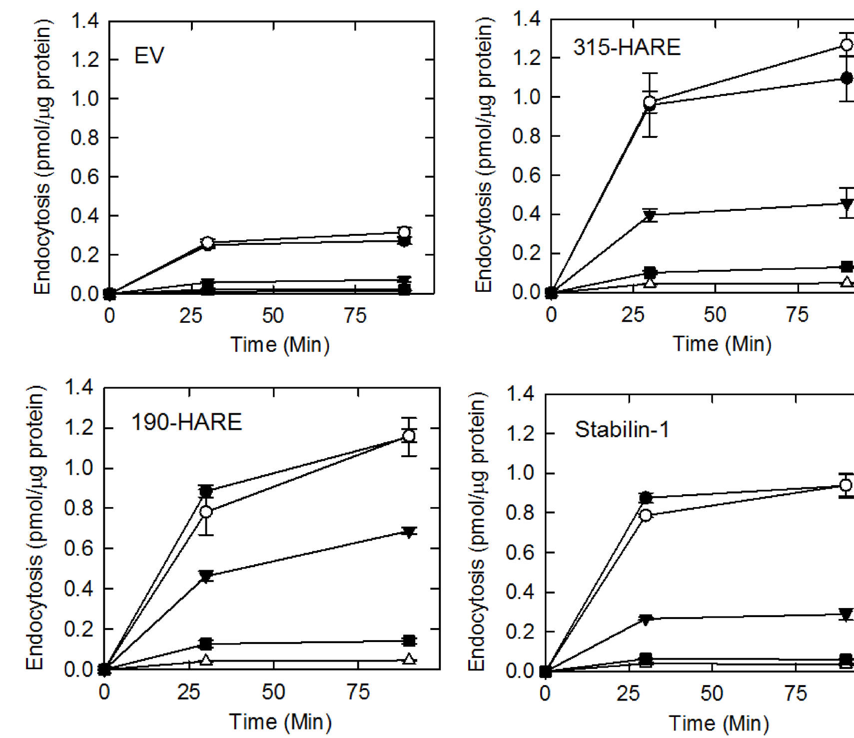


Fig. 6: Endocytosis with competition. All four cell lines were incubated with 0.1 µM ^{125}I-ASO in the presence of 20 µM competitor as indicated for up to 90 min.

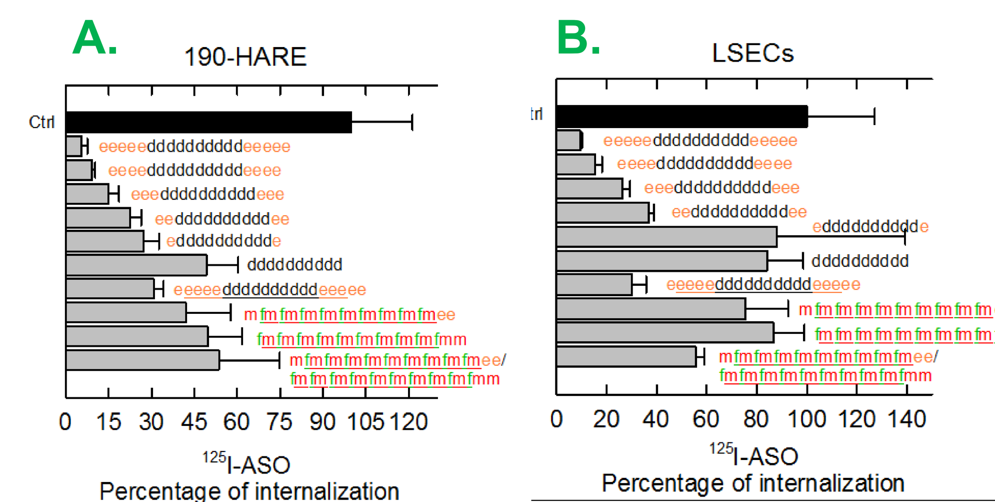
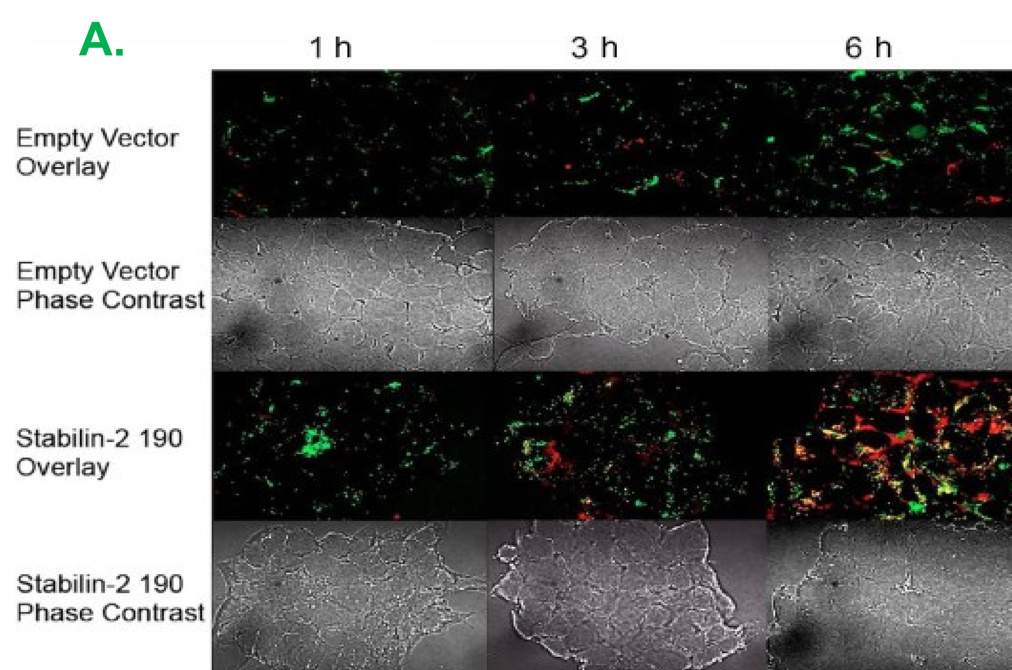


Fig. 7: Competition in both 190-HARE and rat primary LSECs. A) 190-HARE and B) rat LSECs were incubated with 0.1 µM ^{125}I-ASO with 20 µM the indicated competitors for 90 min showing similar uptake values.



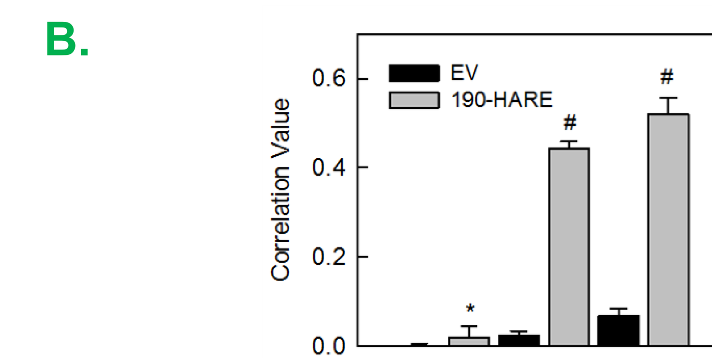


Fig. 8: Stabilin receptors traffick ASO to lysosomes. A) EV and 190-HARE cells were incubated with 0.05 µM Cy3-ASO (red) for 1, 3, or 6 hrs and then incubated with 1.0 µM Lysotracker (green) for 25 min. and imaged by confocal microscopy. Images were processed with Fiji Coloc-2 software and the colocalization data was quantified in B.

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There are no financial disclosers to report associated with this work

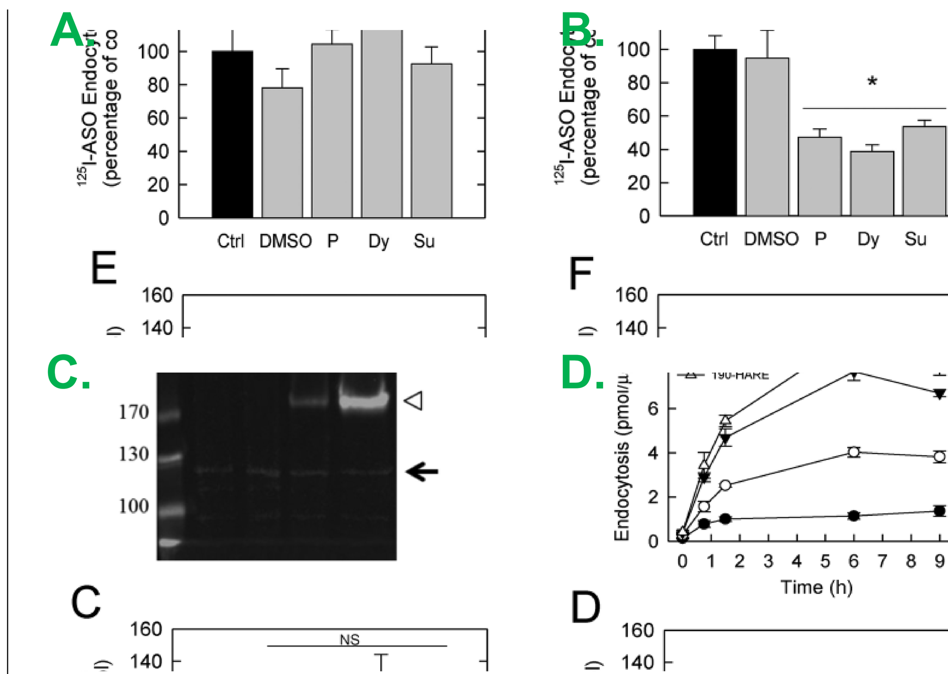

Fig. 9: Endocytosis with Drug Inhibitors. Endocytosis of 0.1 µM 125I-ASO in EV (A), Stabilin-1 (B), 315-HARE (C), and 190-HARE (D) cell lines at one time point (90 mins) as either untreated control (black bars) or with drug/conditions (grey bars) that inhibit clathrin-mediated endocytosis. Dimethyl sulfoxide solvent control, P- 30.0 µM PitStop2, Dy-300 µM-Dynasore, Su-0.4 M sucrose.

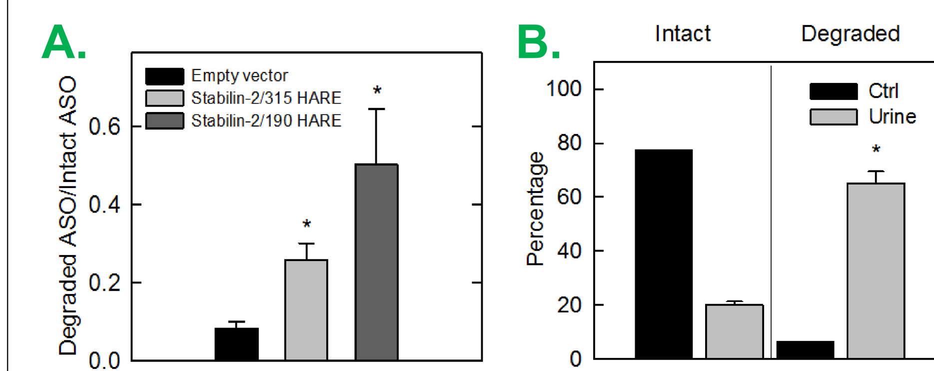

Fig. 10: Degradation of ASO in cells. EV, 190-HARE and 315-HARE cells were incubated with 0.1 µM 125I-ASO for 2 hrs (pulse), washed with PBS, and incubated in fresh medium for 6 additional hrs (chase). The chase medium was subject to DEAE column chromatography to capture the ASO. Degraded and partially degraded ASO was eluted with 0.4 M NaCl and intact ASO was eluted with 2.0 M NaCl. A) The ratio of degraded to intact ASO was highest in the Stab2 expressing cell lines in contrast to the EV. B) Left: ^{125}I-ASO was assessed for intrinsic degradation in the preparation indicated that at least 80% was intact (black bars). Mice were injected with 1.0 mg/kg ^{125}I-ASO and urine was collected over a 2 hr period resulting in the majority of ASO in urine is degraded.

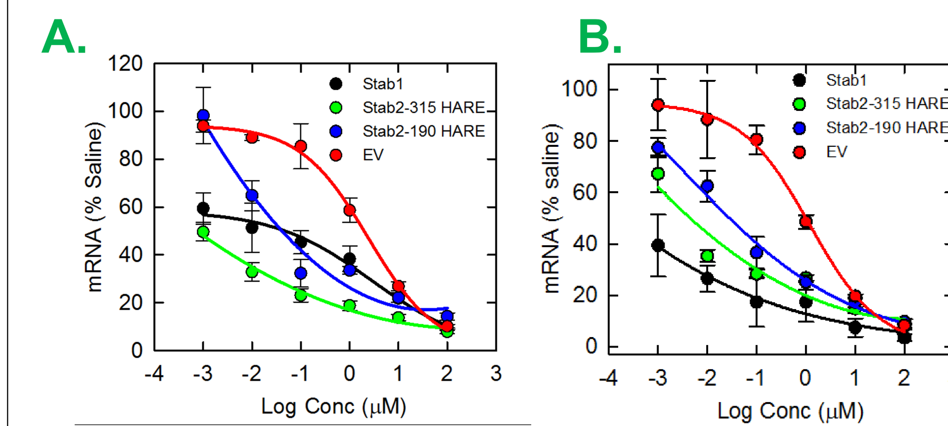

Fig. 11: Efficient gene expression knockdown in Stabilin-expressing cells. All four cell lines were incubated with 0.001-100 µM ASO against *malat-1*, a long non-coding RNA, for A) 24 or B) 48 hrs and assessed for *malat-1* expression levels by qPCR.

Conclusions

- **Liver is a primary site for ASO sequestration from blood.**
- **Stabilin-1 and both Stabilin-2 receptors increased internalization of ASOs from the cell surface.**
- **ASOs bind Stabilins via ionic bonding**
- **ASOs composed of phophorothioate linkages and methoxyethyl modifications have higher affinities for the Stabilins than the morpholinos or phosphodiester ASOs.**
- **Internalization by the Stabilin receptors increases lysosome delivery which is a dead-end for ASOs, but receptor internalization also enhances gene knock-down.**