INHIBITION OF PRODUCTIVE/COMPETITIVE ENDOCYTIC PATHWAYS ENHANCES SIRNA DELIVERY AND CELL SPECIFIC TARGETING

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While the use of short interfering RNAs (siRNAs) for laboratory studies is now common practice, development of siRNAs for therapeutic applications has slowed, due in part to a still limited understanding of the endocytosis and intracellular trafficking of siRNA-containing complexes. As a result, it is difficult to design delivery vehicles for specific cell types, resulting in inefficient delivery, cytotoxicity, or immunogenicity when used *in vivo*. Our aim is to identify which endocytosis and intracellular trafficking pathways lead to active silencing by siRNA-containing complexes. Our work explores the preferential mechanism of endocytosis (whether by clathrin, caveolin, Arf6, Graf1, flotillin, or macropinocytosis) across multiple cell types (HeLa (cervical), H1299 (lung), HEK293 (kidney), and HepG2 (liver)).

Using Lipofectamine 2000 (LF2K), fluorescently-labeled siRNAs were delivered to cells stably expressing green fluorescent protein (GFP). Chemical inhibitors (Filipin, Dynasore, Cytochalasin D, Chlorpromazine, Amiloride, and Methyl-β-cyclodextrin) were used to identify the specific endocytic pathway internalizing the complexes. By measuring the effect of inhibitors on both intracellular levels of siRNA and GFP silencing, we were able to categorize pathways as being productive/competitive according to their functional role in facilitating gene silencing. In productive pathways, siRNAs are actively delivered to a cell and silence a target protein, whereas in competitive pathways, siRNAs are endocytosed but do not lead to silencing.

To further validate the findings from our inhibitor assay, we overexpressed the following endocytic proteins and quantified their effect on siRNA uptake: Dynamin, Actin, AP2, Clathrin, Caveolin, Flotillin 1, Flotillin 2, Arf6, and Graf1. Together our findings suggest that LF2K-siRNA complexes are internalized through multiple pathways in all cells but that only one of the pathways is productive, leading to GFP silencing (Table 1). Interestingly, our data suggests that the relative expression of endocytic proteins within a cell line may indicate which endocytotic pathway is productive.

Table 1: Productive/competitive pathways utilized for LF2K-mediated RNAi.

Cell Line	Productive Pathway	Competitive Pathway
HeLa	Arf6	Clathrin
HepG2	Arf6	Clathrin
H1299	Flotillin	Clathrin
HEK293	Graf1	Arf6

Additionally, we explored the use of inhibitors in co-cultured cell populations. Based on our results, we were able to selectively inhibit GFP silencing for a specific cell line by targeting its productive pathway (Figure 1). Furthermore, inhibiting siRNA uptake in one targeted cell type enhanced the overall bioavailability of siRNA complexes and GFP silencing in the other cell population.

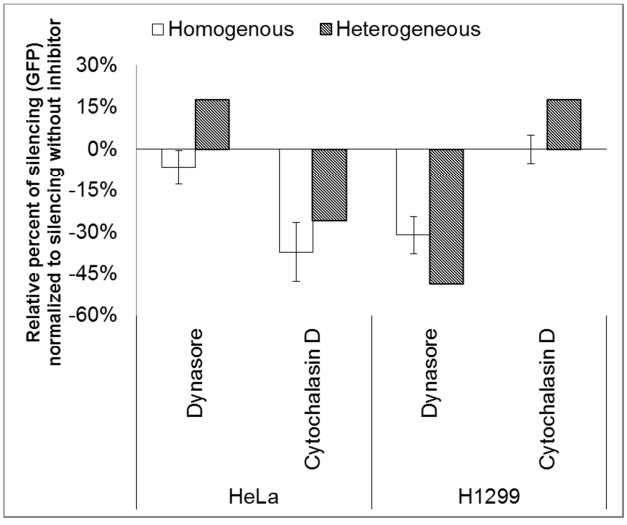

Figure 1: Effect of inhibitors on GFP silencing between single and co-culture (HeLa and H1299) cell populations.