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14. ABSTRACT The most significant finding of the research period reported here is that delivery conjugates can be assembled that can direct siRNA molecules to target cells, including HER2+ human breast cancer cells, in culture in the presence of serum, while non-target cells are avoided. The successful siRNA delivery in serum resulted in substantial reduction of target gene expression in the specific cells being aimed at by these missile-like molecules. These findings suggest that the approach of using our lab's novel targeting and cell penetration proteins may be effective at targeting specific tumor cells in vivo, which will be tested in our upcoming funding periods.					
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INTRODUCTION:

The purpose of this research is to test the hypothesis that recombinant Ad5 capsid proteins targeted to HER2+ breast cancer induce tumor cell-specific death through: receptor-targeted binding and cell entry; siRNA-mediated “knock-down” of specific gene transcripts; and cytokine-mediated cytotoxicity. The main objective of this study is to demonstrate that heregulin-directed proteins target siRNA delivery to HER2+ cells *in vitro* and in a xenograft model of breast cancer in nude mice.

BODY:

Research Accomplishments Associated with Task 1

Task 1. To test whether recombinant heregulin-modified capsid proteins assemble with siRNA to form “siRNA-missiles”, and undergo targeted cell binding and entry *in vitro*. (Months 1-12)

The targeted carrier protein, HerPBK10, undergoes high cell binding and entry of HER2+ but not HER2- human breast cancer cells.

We designed the recombinant protein, HerPBK10, to penetrate HER2+ cells by using the ‘Her’ domain for binding heregulin receptors, which have an increased ligand affinity on HER2+ tumor cells, and the membrane penetration and intracellular trafficking features of the ‘PB’ domain, which is derived from the cell-penetrating adenovirus capsid. To verify the specificity and internalization activity of the protein on human breast cancer cells, we treated HER2+ (MDA-MB-435) and HER2- (MDA-MB-231) cells in culture with HerPBK10 and observed the cell binding, internalization, and intracellular trafficking activities using immunofluorescence and confocal microscopy. MDA-MB-435 cells displayed substantially greater HerPBK10 bound to the cell surface than MDA-MB-231 (**Fig. 1A**). While MDA-MB-231 cells are not deficient in HER2, the preponderance of HER2 subunit is found intracellularly rather than on the cell surface (**Fig. 1B**). Moreover, these cells display relatively high levels of HER3 subunit (**Fig. 1C**), which is capable of binding heregulin albeit at a dramatically lower affinity when HER2 is low to absent [2] [3,4]. Accordingly, HerPBK10 fluorescence was minimal to negligible on MDA-MB-231 cells. HerPBK10 internalization into each cell line likewise reflected the level of cell binding, with substantial protein uptake observed in MDA-MB-435 cells while minimal to negligible levels of uptake were detected in MDA-MB-231 cells (**Fig. 1A**).

HerPBK10 transports labeled oligonucleotide into MDA-MB-435 cells.

To examine the capacity of HerPBK10 to facilitate direct transport of small nucleic acids into cells, a Cy3-labeled oligonucleotide (Cy3-oligo) was incubated with HerPBK10 to mediate assembly via electrophilic interaction, then the resulting complex was added to MDA-MB-435 cells in culture. Confocal fluorescence microscopy of treated cells shows that HerPBK10 and the Cy3-oligo colocalize after uptake (**Fig. 2**), suggesting that the oligonucleotide is bound to HerPBK10 after cell entry. As it is possible that the oligo may enter cells by fluid-phase uptake with HerPBK10, we examined more closely the binding interaction between small nucleic acids and HerPBK10.

Recombinant targeted proteins form a stable assembly with siRNA.

As a goal of this project is to assemble a noncovalent siRNA conjugate, we examined the interaction of a commercial siRNA duplex with HerPBK10 by gel mobility shift assay. The duplex, which was obtained from Invitrogen (Carlsbad, CA, USA), has been established elsewhere to specifically eliminate HER2 transcripts and thus induce apoptosis [5,6]. Constant concentrations of the HER2 siRNA were incubated with increasing concentrations of HerPBK10 and each mixture was then analyzed on either an agarose or acrylamide gel. A dose-dependent decrease in siRNA electromobility was evident as HerPBK10 concentration increased (**Fig. 3A and B**). Likewise, the protein, PBK10, which lacks the Her domain, also dramatically decreased siRNA mobility, while PB did not (**Fig. 3A**), indicating that the decalysine, or 'K10', domain is responsible for binding the siRNA. As we have demonstrated in our previous studies using plasmid DNA and labeled oligonucleotides, both HerPBK10 and PBK10 can bind nucleic acids likely via electrophilic interactions between the positively charged polylysine and the negatively charged nucleic acid phosphate backbone [7,8].

To demonstrate that the protein-siRNA complex formed a stable assembly that could be separated from free siRNA, the mixture was subject to high speed centrifugation onto filter membranes with specified molecular weight exclusion ranges of either a 30K or 100K molecular weight cut-off (mwco). Both HerPBK10-siRNA and free siRNA are retained on 30K mwco filters (**Fig. 4A and B**) whereas the 100K filter retains the conjugate but allows the free siRNA to flow through (**Fig. 4A**). The ability of the conjugate to withstand high speed centrifugation, as determined by low to no detection of free siRNA in 100K filtrates (**Fig. 4A**), indicates that this conjugate can withstand high shear forces without releasing the siRNA molecule.

siRNA missiles mediate knock-down in HER2+ but not HER2- cells in culture.

To determine whether the HerPBK10-siRNA conjugate is capable of mediating specific gene "knock-down" in target cells, the conjugate delivering HER2 siRNA was compared to a standard transfection agent (Lipofectamine 2000) on MDA-MB-435 cells in culture. Knock-down of HER2 was analyzed by immunoblotting of cell lysates. The transfection agent mediated very modest reductions of HER2 whereas the targeted conjugate reduced HER2 to undetectable levels (**Fig. 5**). A scrambled siRNA had no effect on HER2 levels when delivered by either the transfection agent or HerPBK10 (**Fig. 5**). Interestingly, HER3 levels were also nearly completely reduced by the targeted conjugate whereas the transfection agent had no detectable effect on this subunit (**Fig. 5**), raising the possibility that protein levels of HER2 may regulate those of HER3. HerPBK10 alone had no effect on HER2 and HER3 levels (**Fig. 5**), thus ruling out the possibility that HerPBK10 may somehow induce down-regulation of the receptor subunits by virtue of its binding and internalization through HER.

HER2 immunofluorescence was compared between MDA-MB-435 cells and MDA-MB-231 cells when each was treated with either the targeted conjugate or transfection reagent. As mentioned earlier, MDA-MB-231 are not HER2 deficient but rather express HER2 intracellularly but not on the cell surface. Whereas the transfection reagent delivering HER2 siRNA mediated 50-60% reduction of HER2 in both cell lines, the targeted conjugate reduced

HER2 nearly 60% in MDA-MB-435 cells but had little to no effect on HER2 in MDA-MB-231 cells (**Fig. 6**). HerPBK10 alone and targeted conjugate delivering scrambled siRNA had little to no effect on either cell line. Importantly, all siRNA delivery experiments in culture were performed in complete (i.e. serum-containing) media, thus suggesting that serum may not have an inhibitory effect on the conjugate.

HerPBK10 augments lipoplex-mediated siRNA delivery in high HER2-expressing cells.

We wanted to examine whether conjugate activity could be expanded to other HER2+ cell lines. Using immunofluorescence, we determined that SKBR3 and SKOV3 cell lines expressed nearly 3-times higher HER2 compared to MDA-MB-435 cells (**Fig. 7A**). Whereas a transfection reagent delivering HER2 siRNA had no detectable effect on HER2 levels in SKBR3 cells, the targeted conjugate reduced levels nearly 50% (**Fig. 7B**). The transfection reagent used here is specified for siRNA delivery (Silentfect; Bio-Rad Laboratories, Hercules, CA, USA). To see if HerPBK10 could improve delivery by the transfection reagent, increasing concentrations of HerPBK10 were incubated with the lipoplex, resulting in a dose-dependent decrease (up to 80-85% reduction) in HER2 when added to SKBR3 cells (**Fig. 7B**). The transfection reagent fared better in SKOV3 cells, resulting in nearly 50-55% reduction of HER2 whereas the targeted conjugate mediated nearly 50% reduction (**Fig. 7C**). Combining HerPBK10 with the lipoplex augmented HER2 reduction, resulting in nearly a 70% HER2 knock-down (**Fig. 7C**).

KEY RESEARCH ACCOMPLISHMENTS:

- Assessment of HER subunit levels on a panel of HER2+ and HER2- cell lines
- Assessment of HerPBK10 (carrier protein) entry into HER2+ cells
- Determination that HerPBK10 can transport small nucleic acids into HER2+ cells
- Assembly of an siRNA conjugate targeted to heregulin receptors
- Determination that HerPBK10 facilitates delivery of siRNA into HER2+ cells and mediates specific knock-down of target (HER2) protein
- Demonstration that the targeted siRNA conjugate mediates specific delivery and knock-down in target (HER2+) cells but not non-target (HER2-) cells
- Demonstration that HerPBK10 can augment lipoplex delivery of siRNA and mediate enhanced knock-down of target (HER2) protein

REPORTABLE OUTCOMES:

- Manuscript in preparation based on data generated from this work
- Informal presentation at Women's Cancer Research Institute meeting

CONCLUSION:

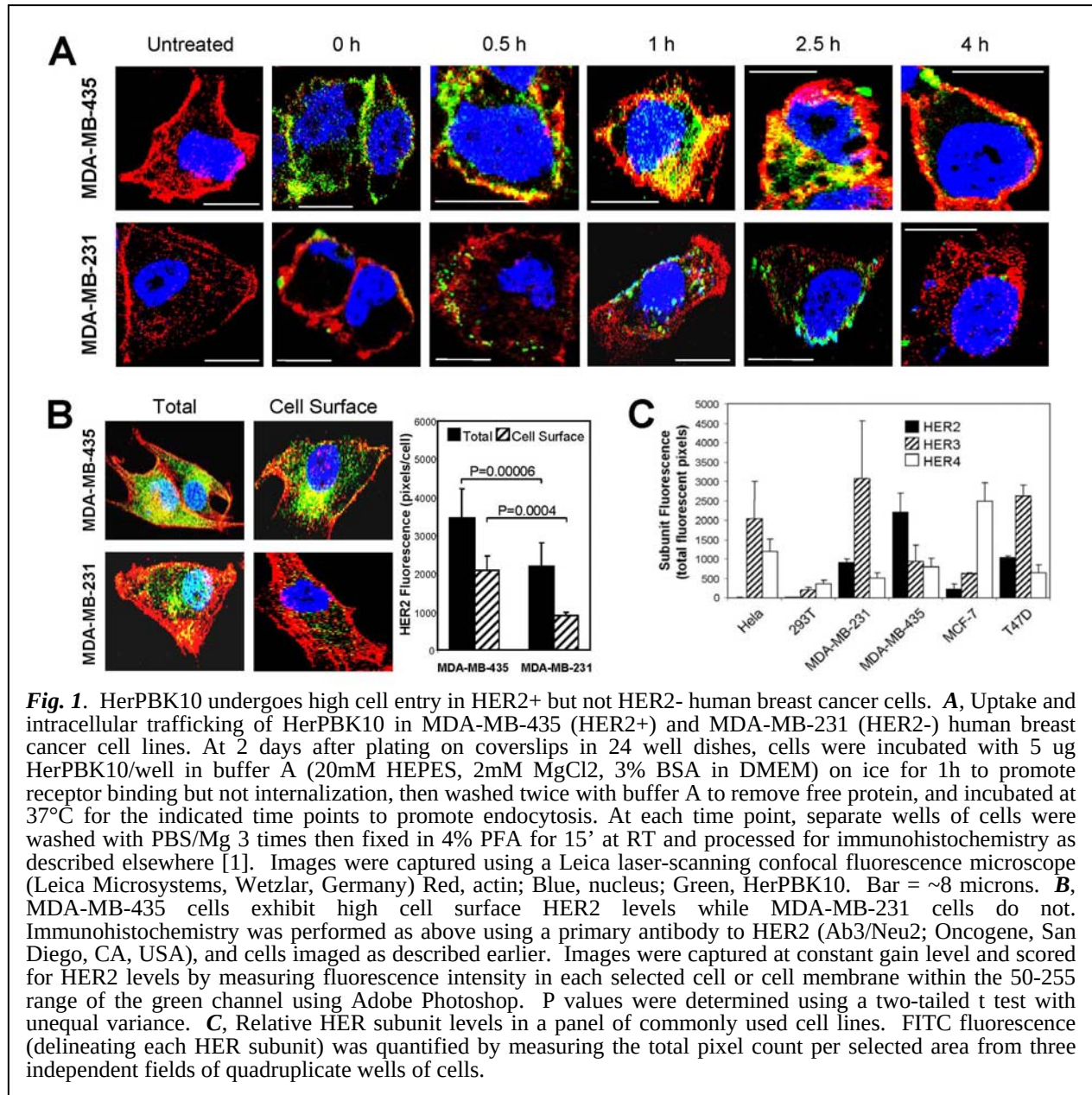
Thus far we have been able to demonstrate that we could assemble conjugates targeted to HER2+ breast cancer cells that can deliver an siRNA that mediates specific knock-down in the target cells. The importance of this work is that we may be able to develop this conjugate into a targeted therapeutic that can deliver siRNA molecules specifically to HER2+ tumor cells in the body while avoiding delivery to non-target tissue. This could be an improvement over current

therapies because standard treatments affect normal tissue as well as tumor cells, whereas this treatment may concentrate the therapy in the tumor only. The anticipated therapeutic effect of the siRNA molecule will be the suppression of specific genes that would result in tumor cell death, while preserving normal, healthy cells.

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FIGURES



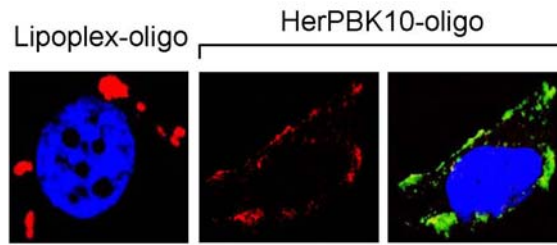
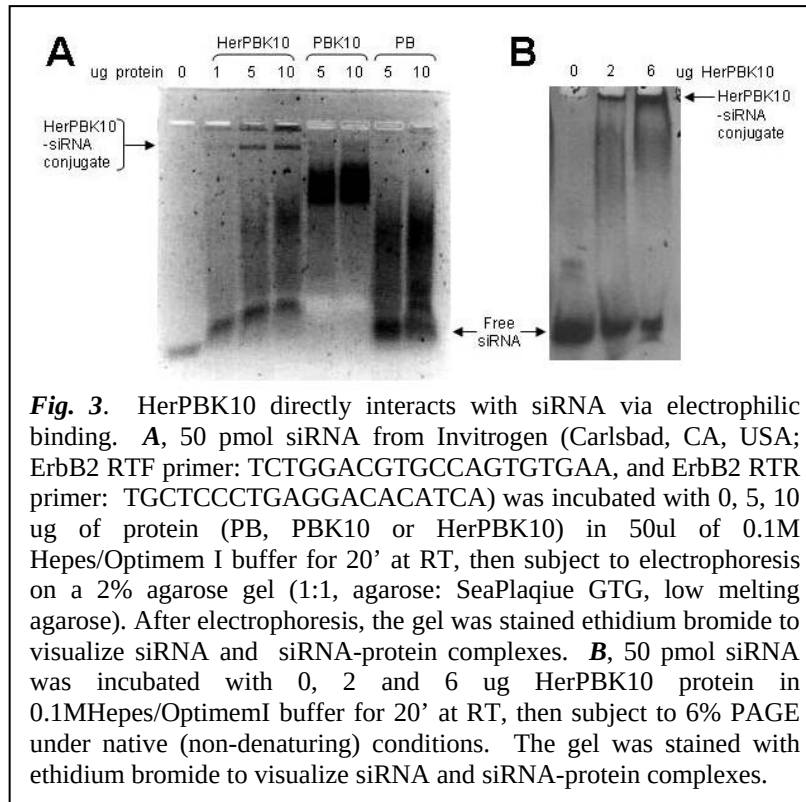
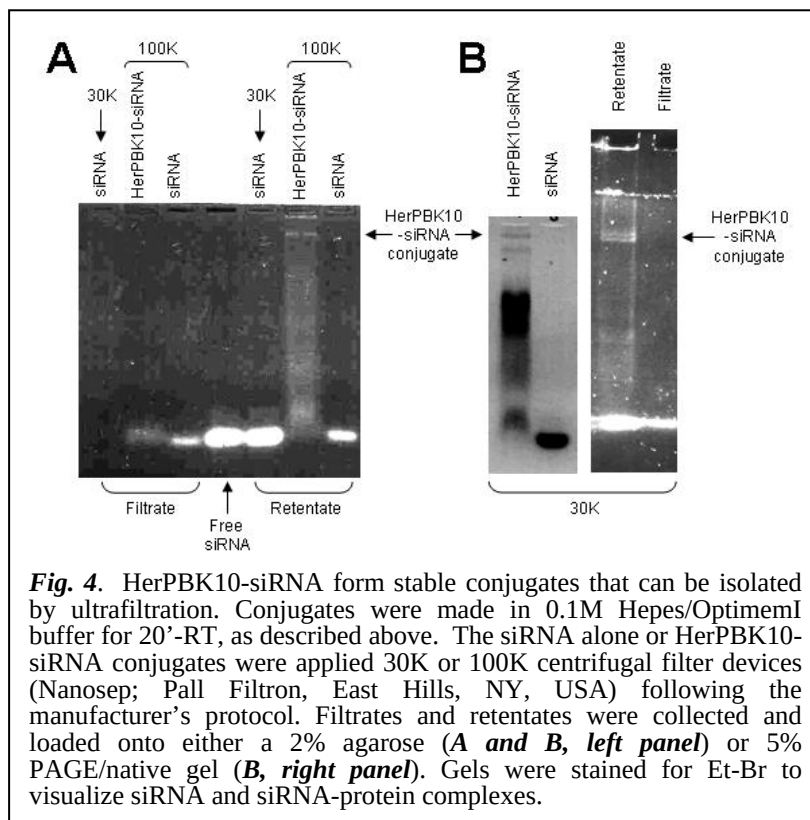


Fig. 2. HerPBK10 transports a labeled oligonucleotide in MDA-MB-435 cells. A Cy3-labeled oligonucleotide (50 pmol) was incubated with either HerPBK10 (5 ug) or a commercial transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA, USA) in 0.1 M HEPES/Optimem I (Invitrogen; Carlsbad, CA, USA) for 20 minutes at RT. The resulting mixture was added to MDA-MB-435 cells and incubated for 1h at 37°C. Cells were fixed in 4%PFA for 15' at RT and processed for immunohistochemistry and confocal microscopy as described in Fig. 1. Red, oligonucleotide; Green, HerPBK10; Blue, nucleus.





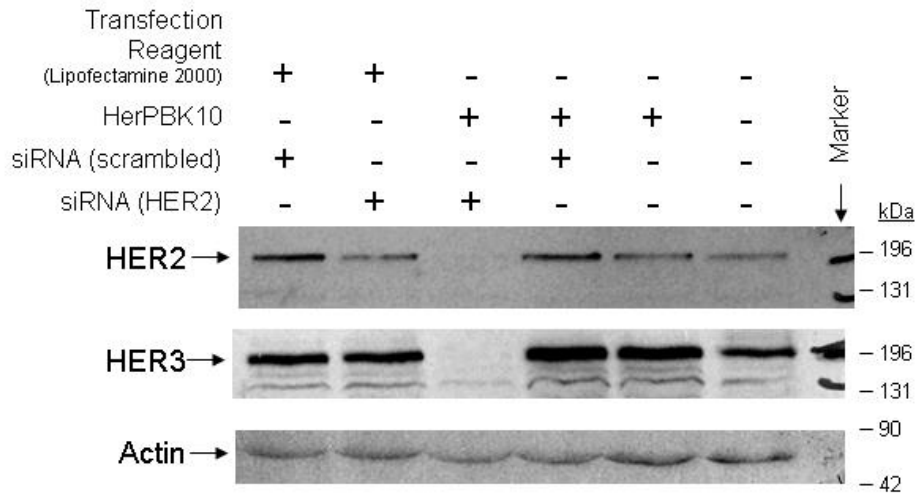
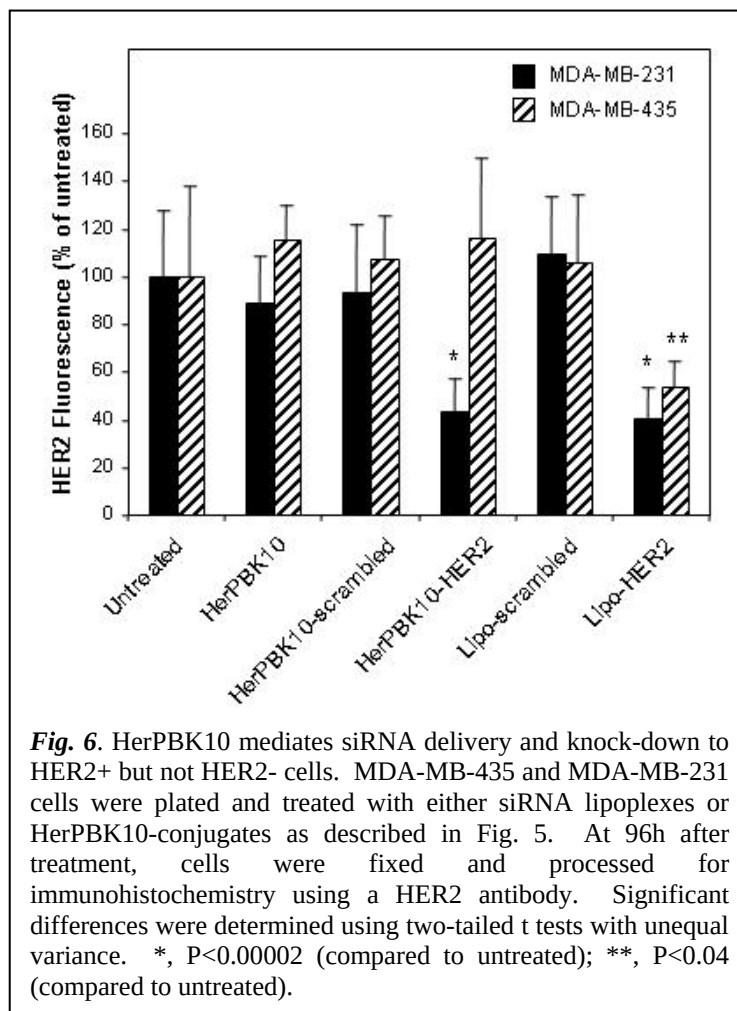


Fig. 5. HerPBK10 facilitates siRNA-mediated knock-down in targeted HER2+ cells. At 2 days after plating, MDA-MB-435 cells were treated with either siRNA lipoplexes or HerPBK10-siRNA conjugates in complete media and incubated for 48h at 37°C, after which the media was exchanged for fresh media. At 96h after transfection, cells were analyzed for HER2 and/or HER3 subunit levels by Western blotting. Lipoplexes were formed by incubating 100 pmol siRNA with Lipofectamine 2000 in Optimem I, following the manufacturer's protocol. HerPBK10-siRNA conjugates were formed by incubating 100 pmol siRNA with 20 ug HerPBK10 at 1:2 or 1:4 (siRNA:protein) molar ratios in 100mM Hepes in Optimem I buffer, at RT for 20' before adding to cells in complete media. For Western blotting, cells were lysed with RIPA buffer (150mM NaCl, 50mM Tris base pH 8.0, 1mM EDTA, 0.5% sodium deoxycholate, 1% NP40, 0.1% sodium dodecyl sulfate, 1mM DTT, 1mM PMSF, and 1mM Na3VO4) supplemented with complete Protease Inhibitor cocktail. Protein concentration was determined using a Bio-Rad protein assay reagent. The cell lysate proteins were separated by 10% PAGE (25 ug of total protein loaded per well), followed by electrotransfer (140 mA for 2 hours) to a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked in PBS containing 3% dry milk for 1 h at room temperature with constant agitation. The blotted nitrocellulose was incubated with 1 ug/ml Anti-ErbB2/Her2 or Anti-erbB3/Her3 (Upstate/Millipore, Billerica, MA, USA) diluted in PBS-milk agitating at 4°C overnight. The membranes were washed twice with water, and incubated with secondary antibody for 1.5hours at room temperature. The membranes were washed twice with water, then with PBS-0.05% Tween for 3-5 minutes. The nitrocellulose was rinsed with water 4-5 times, and processed for chemiluminescence.



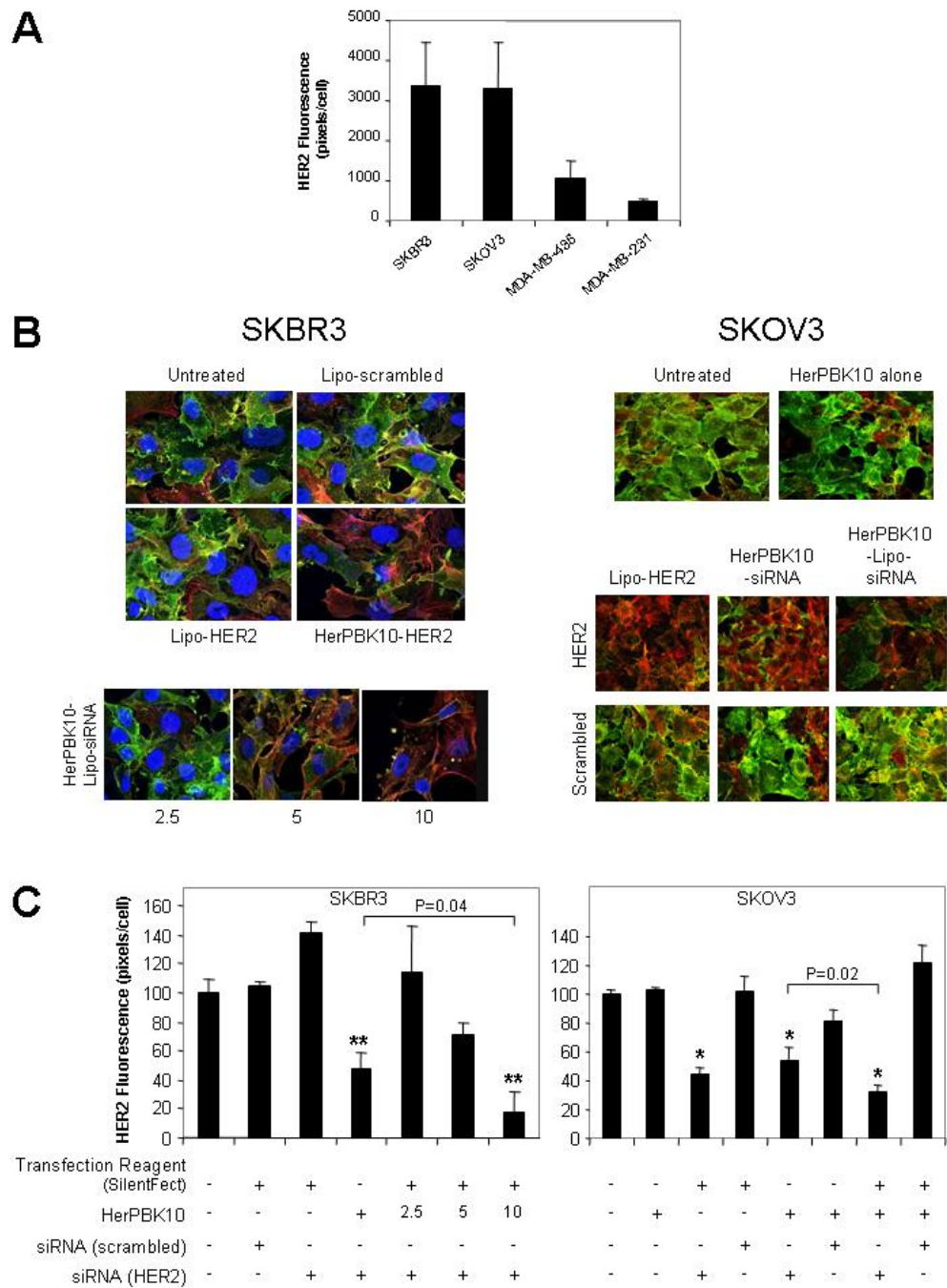


Fig. 7. HerPBK10 mediates siRNA delivery to high HER2-expressing cell lines, and augments lipoplex-mediated knock-down. **A**, HER2 levels of SKBR3 and SKOV3 cell lines in comparison to MDA-MB-435 and MDA-MB-231 cells. Cells were processed for immunohistochemistry using a HER2 antibody and scored for HER2 levels as described in Fig. 1. **B**, Immunofluorescence of HER2 protein after delivery of lipoplexes or targeted conjugates. SKBR3 and SKOV3 cells were plated and treated with either siRNA lipoplexes or HerPBK10-conjugates as described in Fig. 5. At 96h after treatment, cells were fixed and processed for immunohistochemistry using a HER2 antibody. Conjugates containing HerPBK10 with lipoplexes were formed by incubating either 1.5, 5, or 10 micrograms of HerPBK10 with siRNA lipoplexes, equating a final molar ratio of either 1:1:1, 1:2:1, or 1:4:1 (siRNA:HerPBK10:Lipofectamine), respectively. These complexes were formed by incubating HerPBK10 with lipoplexes at RT for an additional 20 min after lipoplex formation, then complexes were added to the cells. Control cells were incubated in 100mM HEPES/Optimem I buffer alone, or with HerPBK10 only or siRNA only. Cells received either HER2/neu-specific or scrambled siRNA. Red, actin; Green, HER2; Blue, nucleus. **C**, Quantification of HER2 knock-down. Cells were scored for HER2 immunofluorescence as described in Fig. 1. Statistical significance was determined using a two-tailed t test at unequal variance. *, $P < 0.00006$ (compared to untreated); **, $P < 0.04$ (compared to untreated).