Enhanced Tumor Targeting Using Clustered Integrin Binding in Non-Viral Vectors

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Introduction: The ability to target non-viral vectors towards tumors that highly express $\alpha_v\beta_3$ integrin receptors would increase the applicability of non-viral vectors in cancer treatment by targeting metastatic tumors and the tumor vasculature. We have designed a strategy to increase targeting of non-viral gene delivery vectors to $\alpha_v\beta_3$ expressing cells and tumors, which uses clustered integrin-binding peptides. In our approach, gold nanoparticles are modified with the tri-peptide sequence Arg-Gly-Asp (RGD) to form RGD nanoclusters that are subsequently used to modify the surface of DNA/polyethylenimine (PEI) polyplexes (**Figure 1A**). Thus, the resulting RGD nanocluster modified polyplexes target cells through RGD peptide clusters rather than individual RGD peptides.

Methods:

RGD nanocluster modified polyplexes: The peptides CCVVVT-COOH (Cap) and Ac-CCVVVTGRGDSP-SSK-COOH (Cap-RGD) were used to modify the surface of citrate stabilized gold nanoparticles. The resulting RGD nanoclusters were immobilized to the surface of the DNA/PEI polyplex via a UV-activated heterobifunctional crosslinker (NHS-ASA) bound to the Cap-RGD peptide (Cap-RGD-ASA). DNA/PEI polyplexes were formed by mixing equal volumes of plasmid DNA with 25kDa branched PEI to get an N/P of 10 in milliQ water. PEI was added to the DNA solution, vortexed for 10 seconds, and incubated at room temperature for 15 minutes. Specified concentrations of Au-Cap, Au-Cap-RGD or Au-Cap-RGD-ASA nanoparticles were added to the DNA/PEI polyplexes and exposed to ambient light for 15 minutes.

In vitro transfection: In vitro gene transfer was assessed using a plasmid encoding for luciferase and a luciferase assay. DNA/PEI-Au-Cap-RGD polyplexes (1.3 µg/well, N/P = 10) were added to plated HeLa cells with varying concentrations of Au-Cap-RGD bound to the surface of the polyplex. As controls, transfections with untargeted DNA/PEI and DNA/PEI modified with electrostatically bound Au-Cap and Au-Cap-RGD particles (no ASA group) were used.

In vivo biodistribution: Positron emission tomography (PET) was used to quantify the *in vivo* biodistribution of the modified polyplexes. DOTA-NHS was conjugated to branched PEI and mixed with ^{64}Cu Chloride prior to polyplex formation and RGD nanoparticle conjugation. Micro-PET/CT imaging was performed with a micro-PET FOCUS 220 PET scanner (Siemens, Malvern, PA) and a MicroCAT II CT scanner (Siemens). Subcutaneous tumors were formed in SCID mice above the left and right shoulders by injection of HeLa (medium $\alpha_v\beta_3$) and U87 (high $\alpha_v\beta_3$) cells in Matrigel. ^{64}Cu labeled DNA/PEI-Au polyplexes (50 µg DNA) in 5% glucose was injected into the tail vein into anesthetized SCID mice. Mice were scanned after 40 min and at 22 hrs.

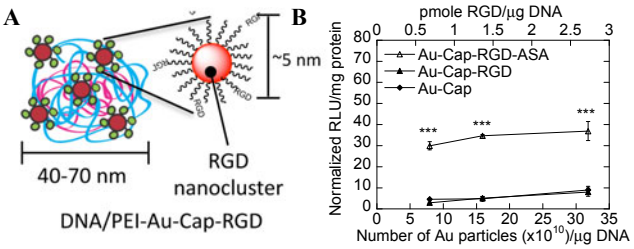

Figure 1 Schematic of the proposed targeting strategy (**A**) and gene transfer efficiency (**B**) are shown. RGD nanoclusters that are covalently attached to the DNA/PEI polyplex (Au-Cap-RGD-ASA) show significantly higher gene expression than electrostatically attached (Au-Cap-RGD) or Au without RGD (Au-Cap). The symbol *** represents statistical significance to a level of $p<0.001$.

Results: DNA/PEI polyplexes with attached RGD nanoclusters (**Figure 1A**) resulted in up to 40-fold increase in gene transfer compared to untargeted polyplexes (**Figure 1B**). Further, compared to polyplexes modified with Au-Cap or Au-Cap-RGD through electrostatic interactions, the covalently modified polyplexes, (Au-Cap-RGD-ASA) resulted in a statistically significant increase in transgene expression demonstrating that the covalent bond is necessary for efficient targeting.

PET scans of ^{64}Cu labeled DNA/PEI-Au polyplexes show an increase in targeting towards tumors with high $\alpha_v\beta_3$ integrin receptor density (U87), compared to untargeted polyplexes (**Figure 2 A,C** vs **B,D**). Further, the level of RGD nanoclustered targeted polyplexes in the U87 tumor was similar to that observed in the liver, which further points to effective targeting.

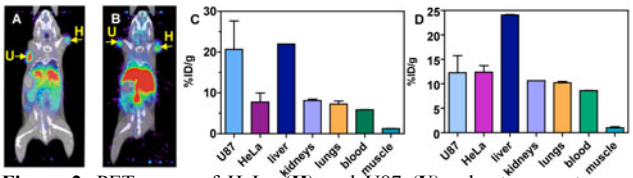

Figure 2. PET scans of HeLa (**H**) and U87 (**U**) subcutaneous tumor mice after injection of ^{64}Cu labeled DNA/PEI-Au polyplexes. Scans of DNA/PEI-Au polyplexes (**A**,**C**) after 22-hrs show enhanced accumulation in U87 tumors compared to untargeted DNA/PEI polyplexes (**B, D**), which showed the same accumulation in U87 and HeLa tumors. **C** and **D** show the biodistribution of the labeled particles after 22-hrs of injection.

Conclusions: The use of clustered integrin binding in non-viral vectors has shown to increase transfection efficiency *in vitro* for medium level $\alpha_v\beta_3$ integrin expressing HeLa cells and displayed improved targeting towards high level $\alpha_v\beta_3$ integrin expression (U87) tumors *in vivo*. Our approach for clustering is versatile and can be used to introduce alternative ligands to target other receptors by simply modifying the Cap peptide with the ligand of interest, which we believe will be an ideal approach to enhance targeting and gene transfer efficiency of non-viral vectors.