Introduction to Non-Viral Gene Therapy Or Plasmid Based Gene Delivery

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Plasmid-Based Gene Therapy

- What are plasmid-based gene delivery vehicles?
- What are the barriers to plasmid gene delivery and how are they overcome
- How are these gene delivery vehicles administered and how do they perform?
- How can these systems be used to treat disease?

Plasmid-Based Gene Delivery Vehicles

- Naked DNA
- Naked DNA + Electroporation or Ultrasound
- Hydrodynamic
- Polymer/Plasmid Complex
- Cationic Liposome/Plasmid Complex
- Drug/Plasmid Complex

- Dosage Form
  - Liquid
  - Lyophilized Cake

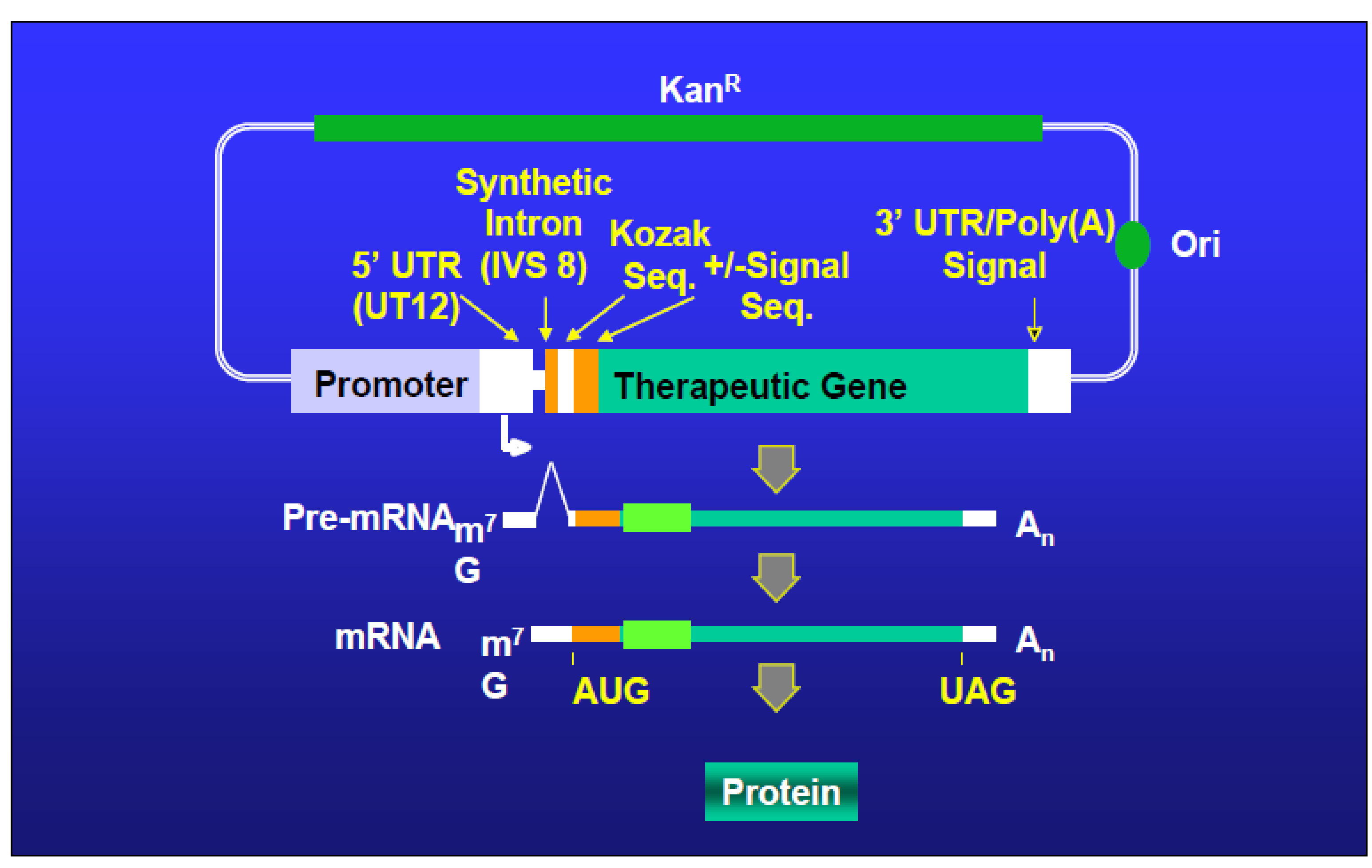
Analytical Methods for Detection of Genes and Gene Products

- Quantitative PCR-quantitation of plasmid biodistribution-sensitivity 1 copy per 1000 copies.
- RT-PCR-qualitative and quantitative measurement of transgene RNA transcripts.
- ELISA-quantitation of protein gene product with sensitivity on the order of 0.01 to 0.1 ng/gm of tissue. Sensitivity is protein dependent.
- Biological Assay

Clinical Status of Plasmid-Based Gene Therapy

Vehicle	Disease Application	Stage of Development	Company
Plasmid DNA Alone	Vaccine-Infectious Disease Cardiovascular Disease	Phase I Phase I	Merck Wyeth Ayerst Vascular Genetics
Cationic Lipid/ Plasmid DNA Complexes	Cancer Cystic Fibrosis	Phase II Phase II Phase I Phase I	Vical Valentis Valentis/Glaxo Genzyme
Polymer/Plasmid DNA	Cancer	Phase I	Valentis
Gold Particle/ Plasmid DNA Biolistics	Vaccines-HIV,HBV,HSV, influenza	Phase I	Powderject

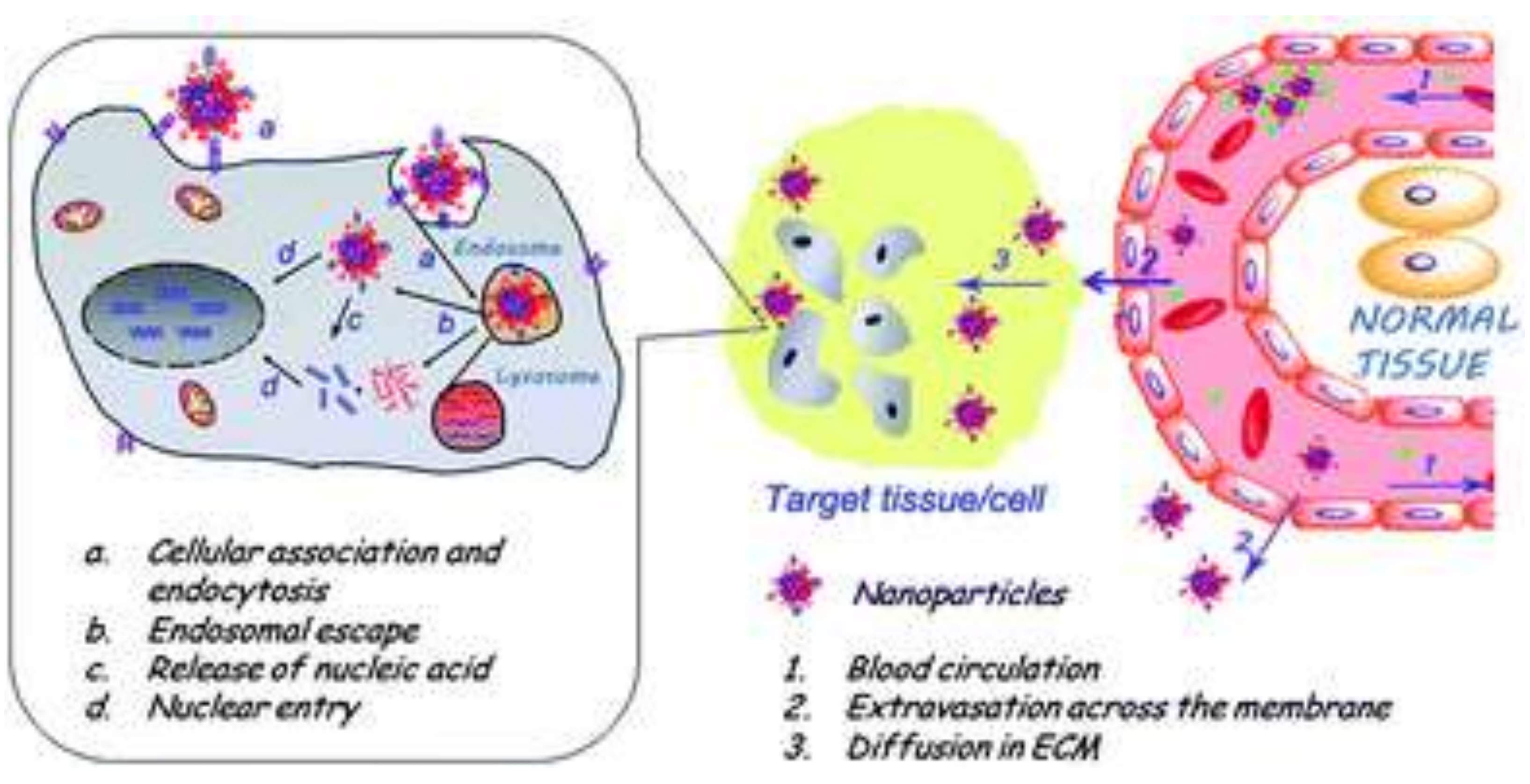
The Features of Plasmid DNA



siRNA

From Nature Publishing

Barriers to Delivering Nucleic Acids



Du et al., Soft Matter, 2010, **6**, 835-848

Barriers to Delivering Nucleic Acids

- The most important goal of gene therapy is to develop site-specific gene delivery systems for the controlled expression of transgenes in targeted cells or tissues
- Nonviral vectors rely on the basics of supramolecular chemistry in which anionic DNA molecules are condensed into compact, ordered nanoparticles that are 50−200 nm in diameter by complexing DNA with an appropriately designed cationic molecule.
- Since very large DNA molecules can be condensed into compact particles, nonviral vectors permit the incorporation of the gene regulatory regions that may afford better control of gene expression.
- Once DNA condensation has been established with synthetic vectors, it is possible to incorporate functional groups into the carrier molecules so that cell-specific targeting and nuclear localization can be facilitated
- Nonviral systems should be sufficiently stable to serum inactivation and provide protection of DNA from degradation

“Naked DNA”or Plasmid Alone

I. Plasmid Alone

Local Administration: Intramuscular

Application: Genetic Vaccine

II.Hydrodynamic Gene Transfer

Systemic Administration **Hepatocyte Transfection**

Transient Gene Expression

Testing Therapeutic Gene

Local Administration **Muscle**

Long Term Gene Expression

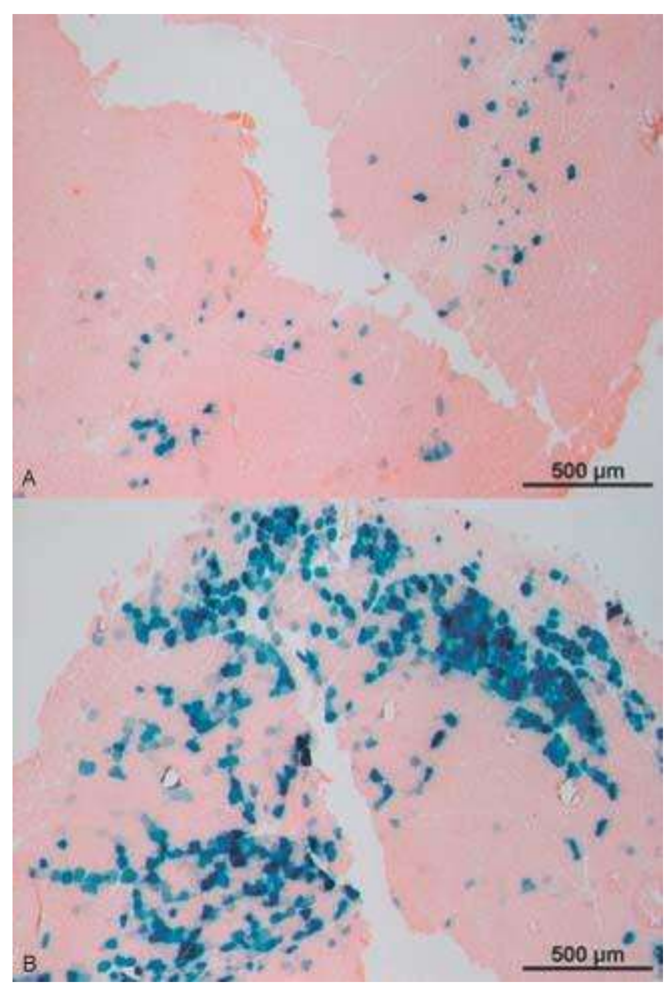
Expression of Dystrophin

III. Plasmid + Device (Electroporation or Ultrasound)

Local Administration **Intramuscular Systemically active secreted protein Long Term Gene Expression in mice 1 year.**

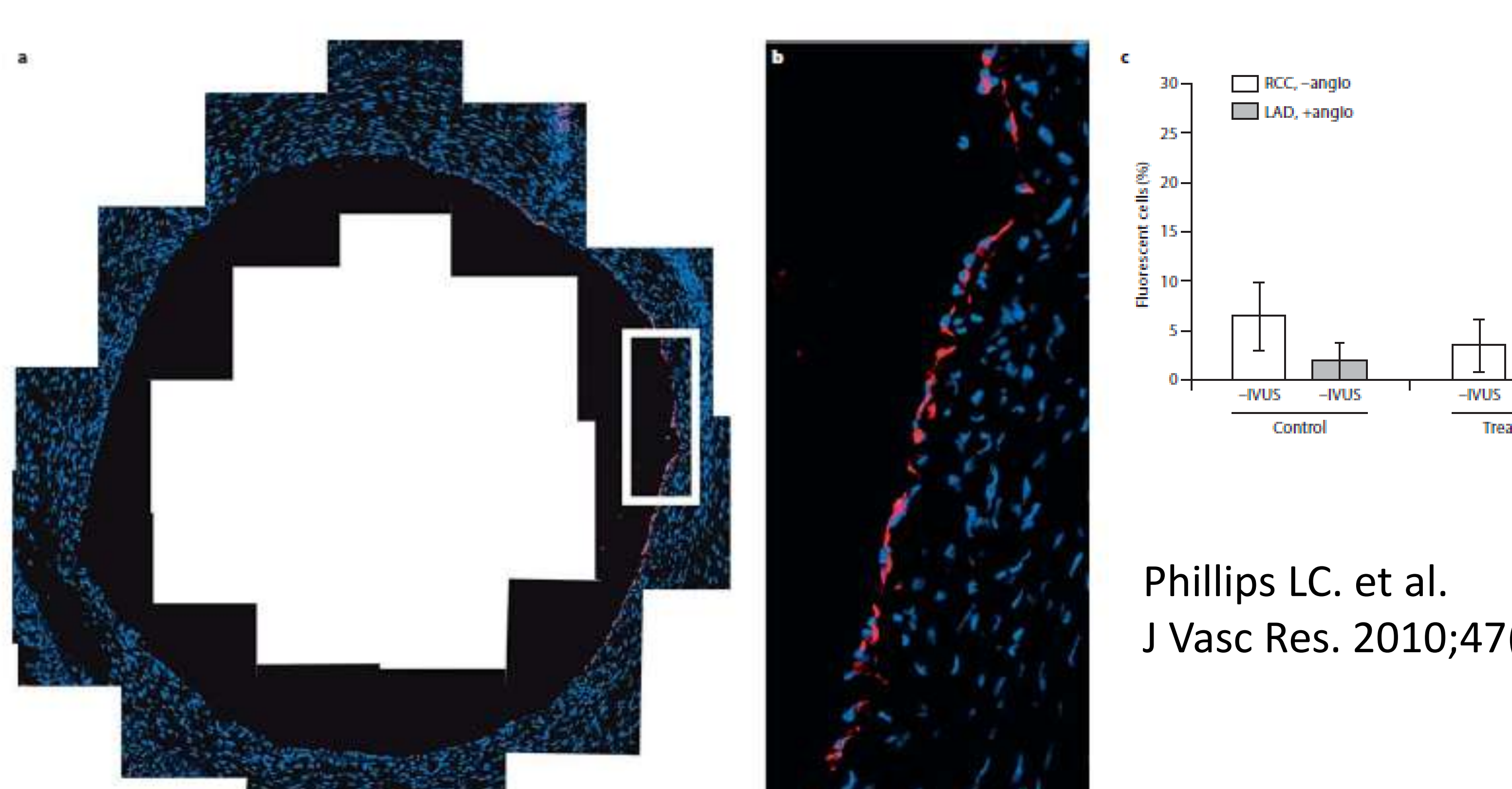
Physical methods

- Electroporation
  - Applied electrical field
  - Causes increased electrical conductivity and permeability

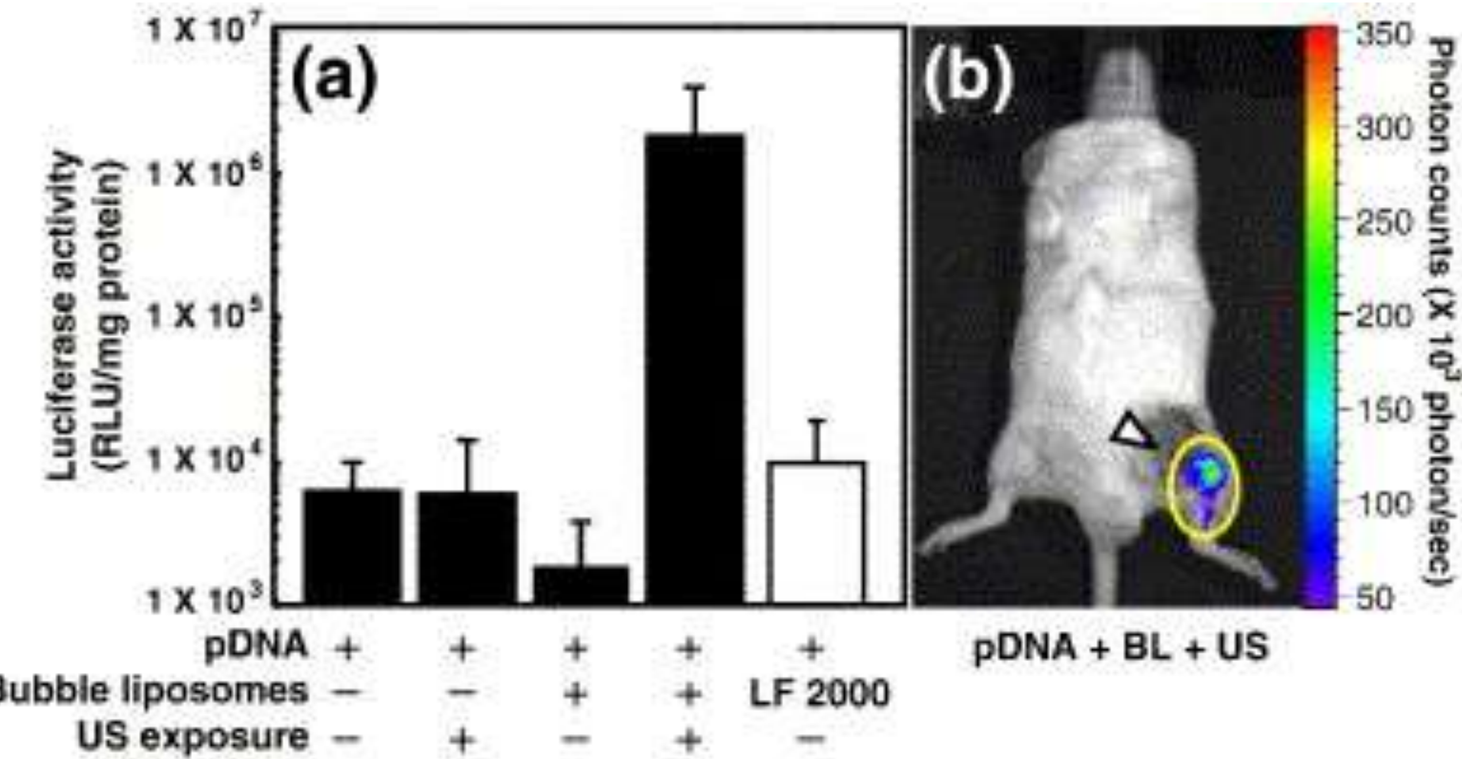


Ratanamart J, Huggins CG, Shaw JA.
J Gene Med. 2010 Apr;12(4):377-84.

- Ultrasound
  - The use of sound to modify the permeability of the plasma membrain
  - employs the acoustic cavitation of microbubble



Phillips LC. et al.
J Vasc Res. 2010;47(3):270-4.

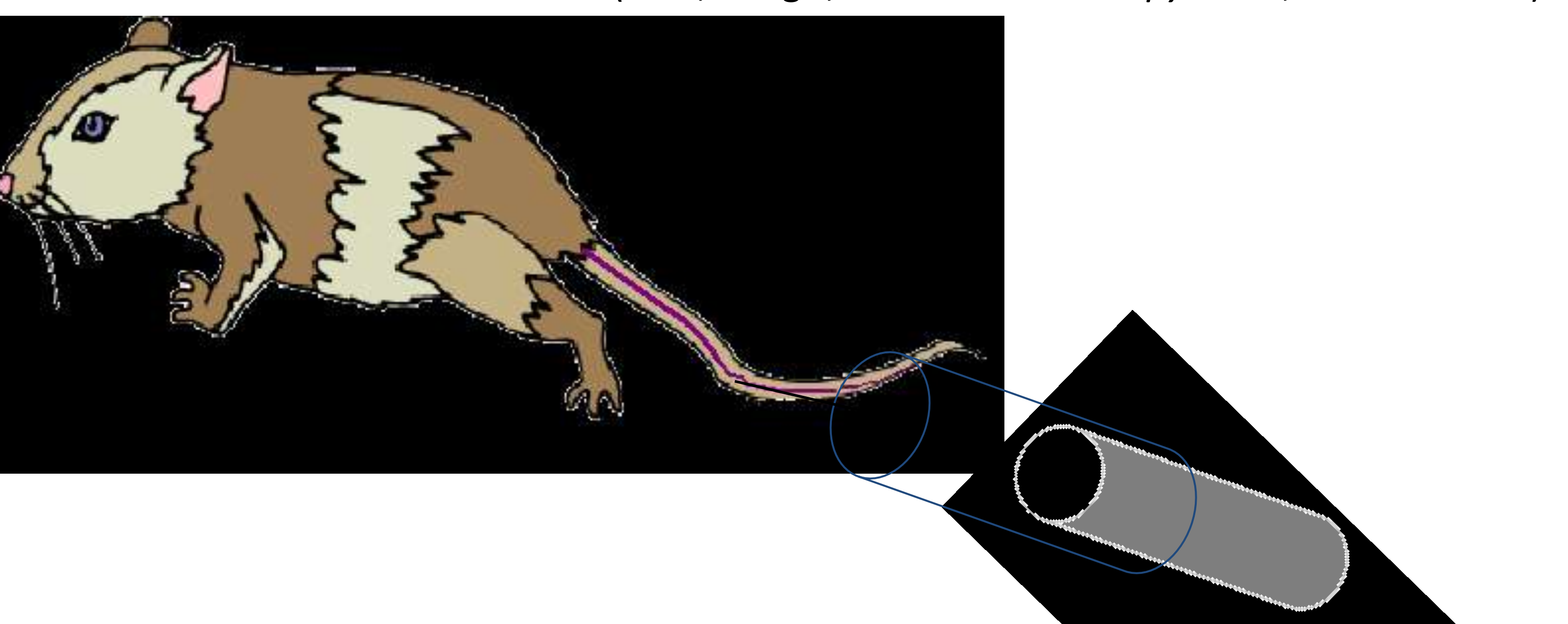


Gene delivery to femoral artery with perfluoropropane Bubble liposomes. Each sample containing plasmid DNA 10 µg was injected into femoral artery. In the same time, ultrasound (frequency, 1 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1 W/cm^2; time 2 min) was exposed to the downstream area of injection site. (a) Luciferase expression in femoral artery of the ultrasound exposure area at 2 days after transfection.Data are shown as means ± S.D. (*n* = 5). (LF2000: Lipofectamine 2000) (b) In vivo luciferase imaging at 2 days after transfection in the mouse treated with plasmid DNA, Bubble liposomes and ultrasound exposure. The photon counts are indicated by the pseudo-color scales. Arrow head shows injection site and circle shows ultrasound exposure area BL, Bubble liposomes; US, Ultrasound.

Hydrodynamic Gene Delivery

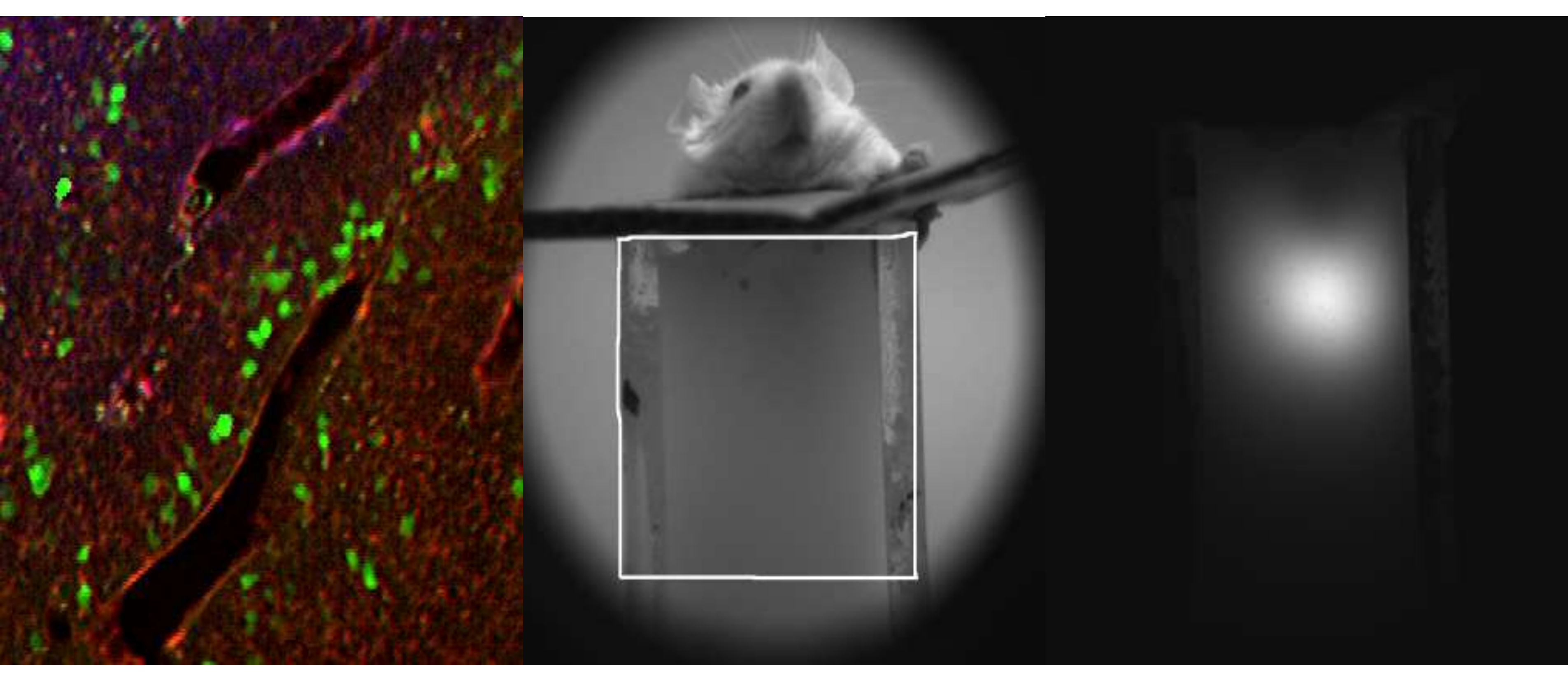
- Injecting a large volume (10% body weight)
- Small amount of plasmid DNA (10μg)
- Short time period (5-10 seconds)

(Liu F, Song Y, Liu D. *Gene Therapy* 1999; 6: 1258-1266)

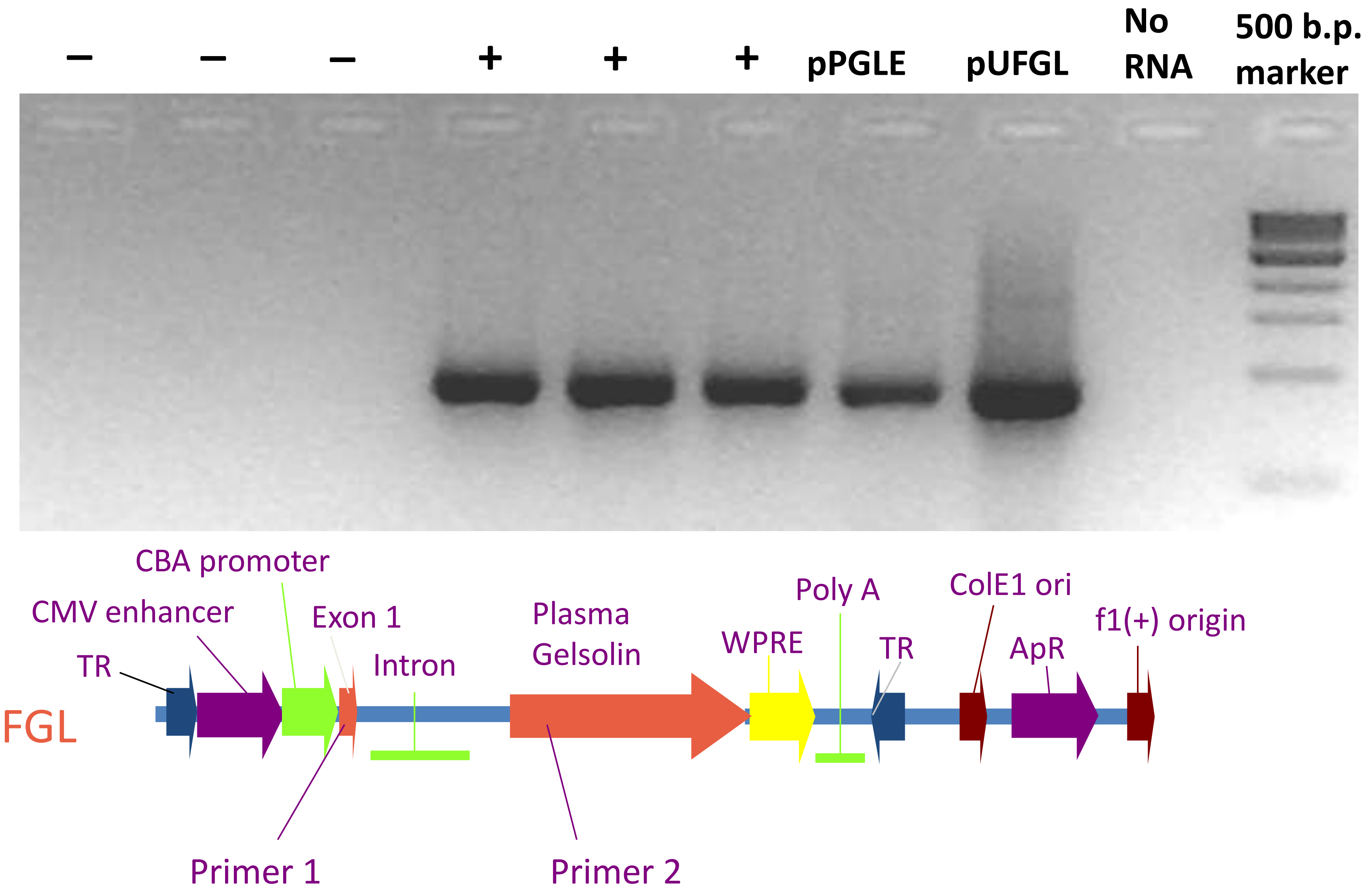


Mouse Light Bulb

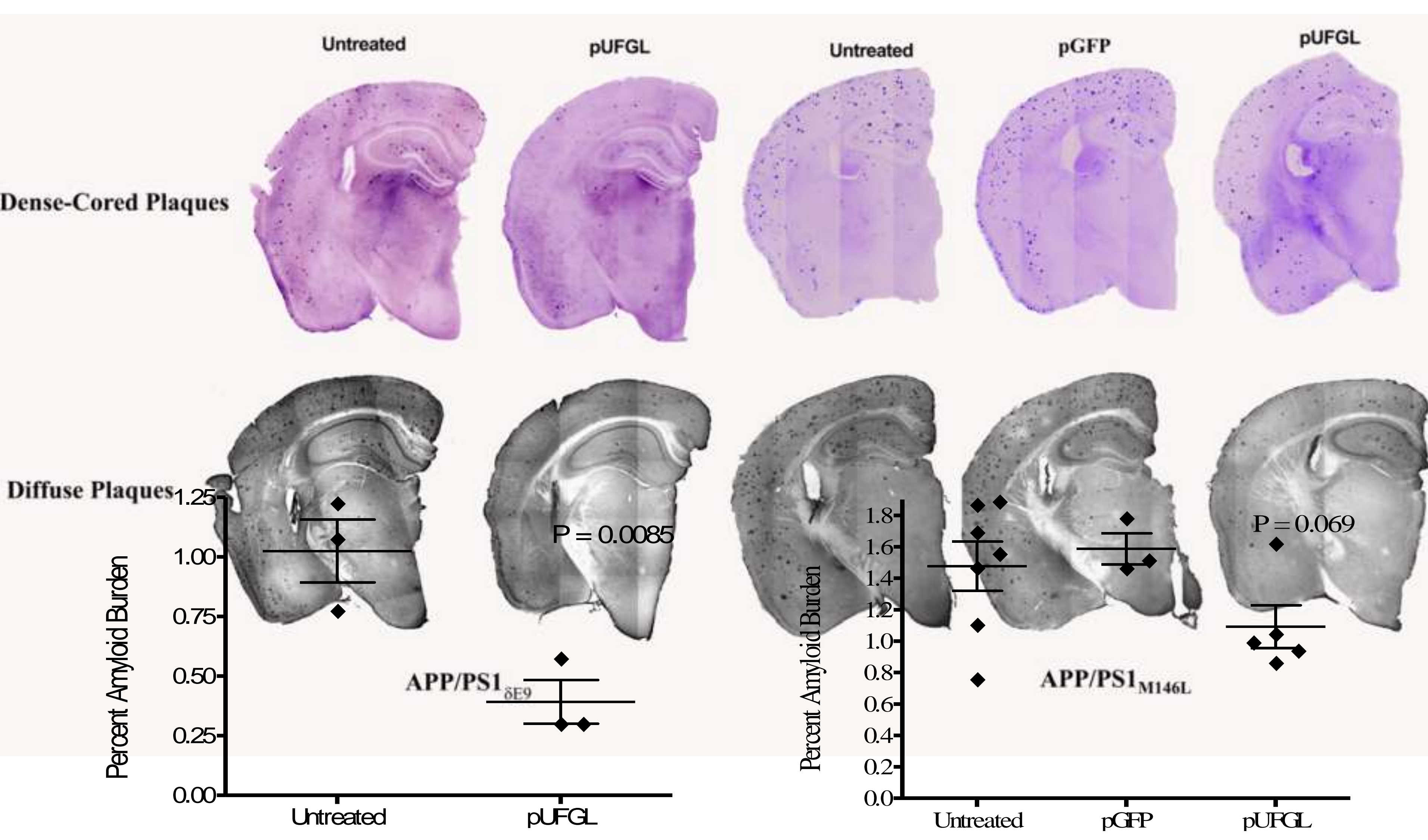
- High levels of transgene expression in the liver



Expression in $_{m/h}APP_{K594N,M595L}/PS1_{\delta E9}$ Transgenic Mice



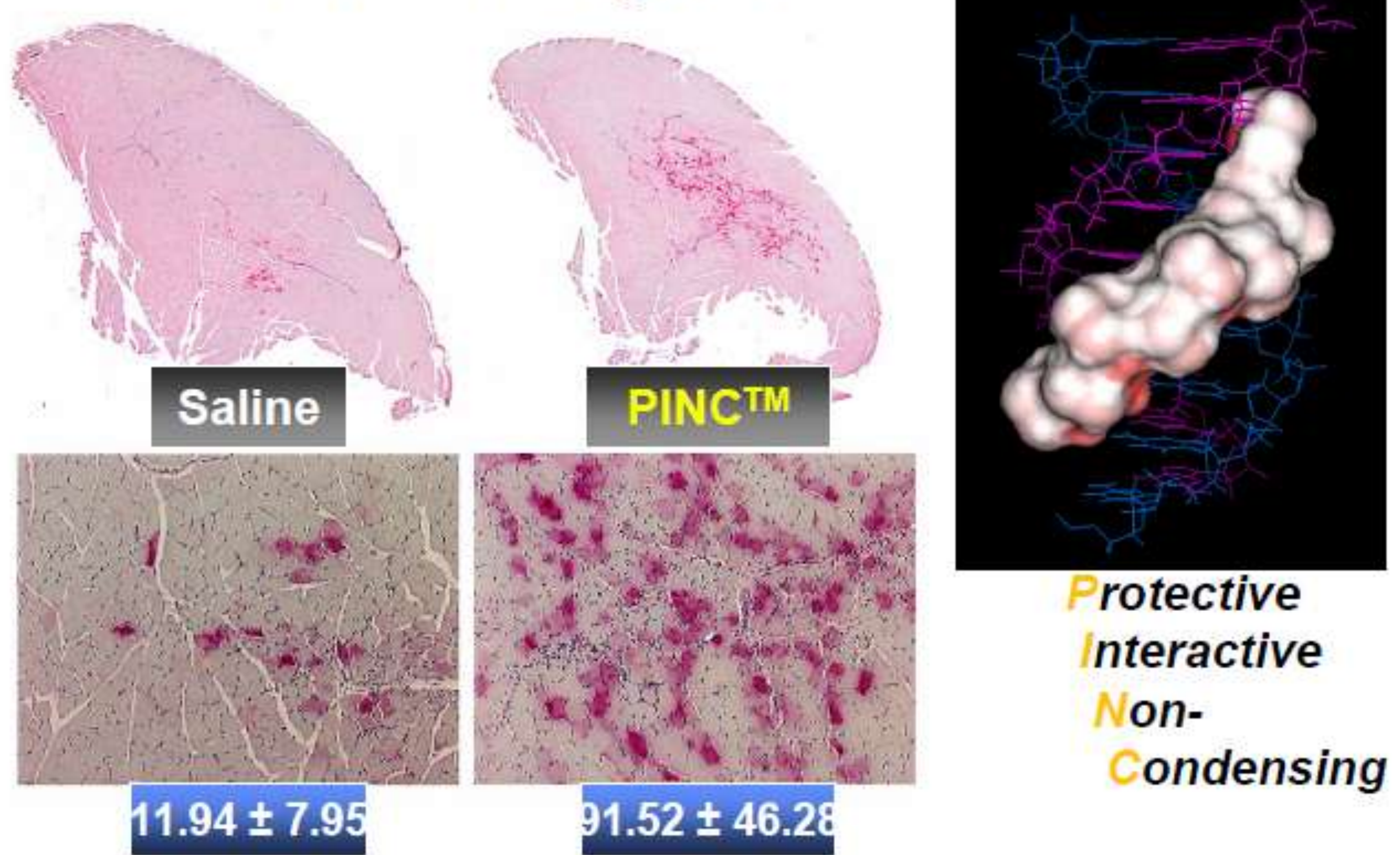
Quantification of Amyloid Pathology



Chemical Delivery Methods

- Condensing and noncondensing
- Interactive and noninteractive
- Lipids and polymers

Enhanced Distribution of DNA with PINC Polymers



β–Galactosidase Positive Muscle Cells (n=6)

(7 days after single IM administration in rats)

Mumper et al., Journal of Controlled Release, 52 (1998) 191-203

Poloxamer or Pluronic

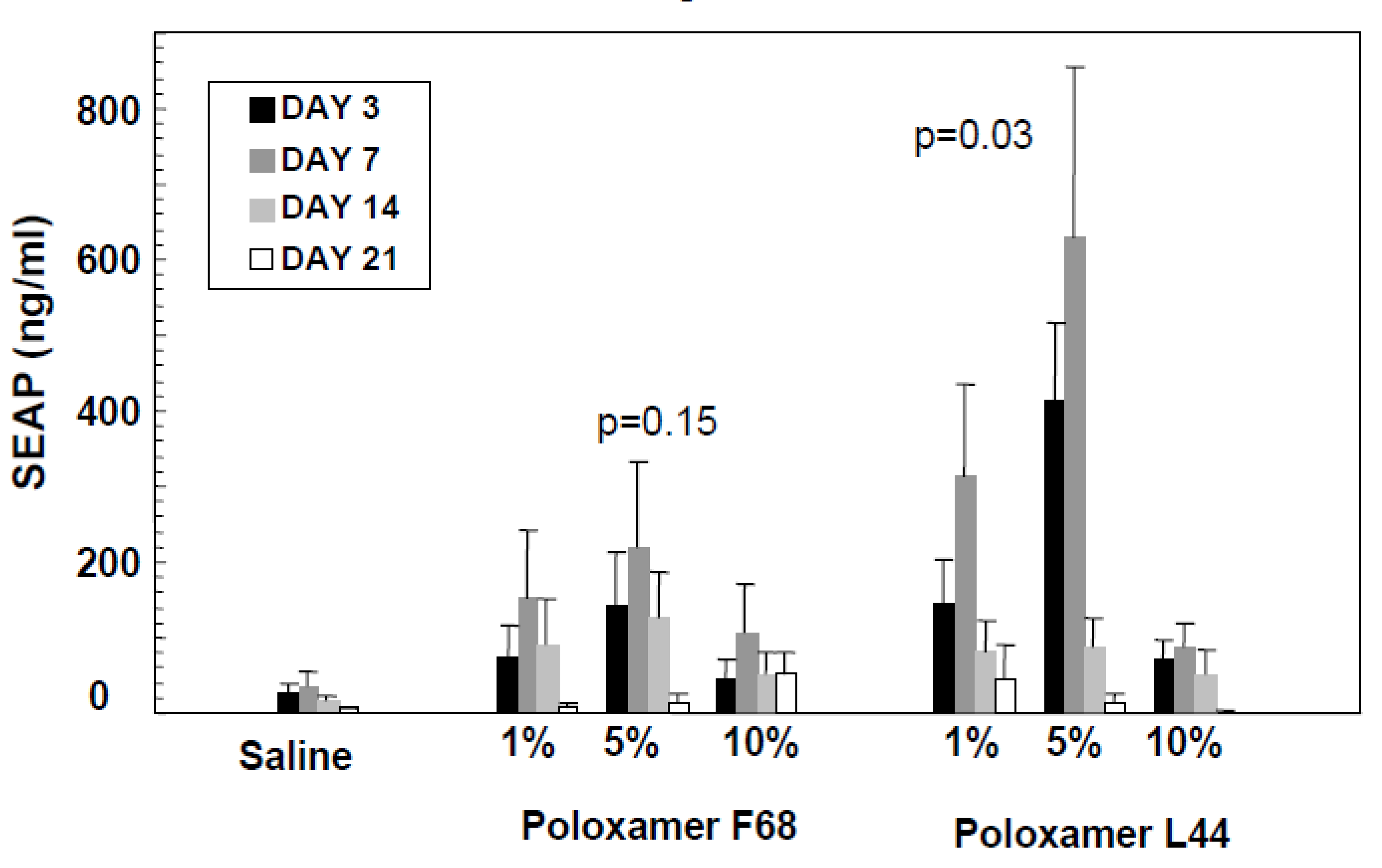
- The Pluronic. block copolymers are synthetic copolymers of ethylene oxide and propylene oxide represented by the following chemical structure:

HO(C2H4O)a(C3H6O)b(C2H4O)aH

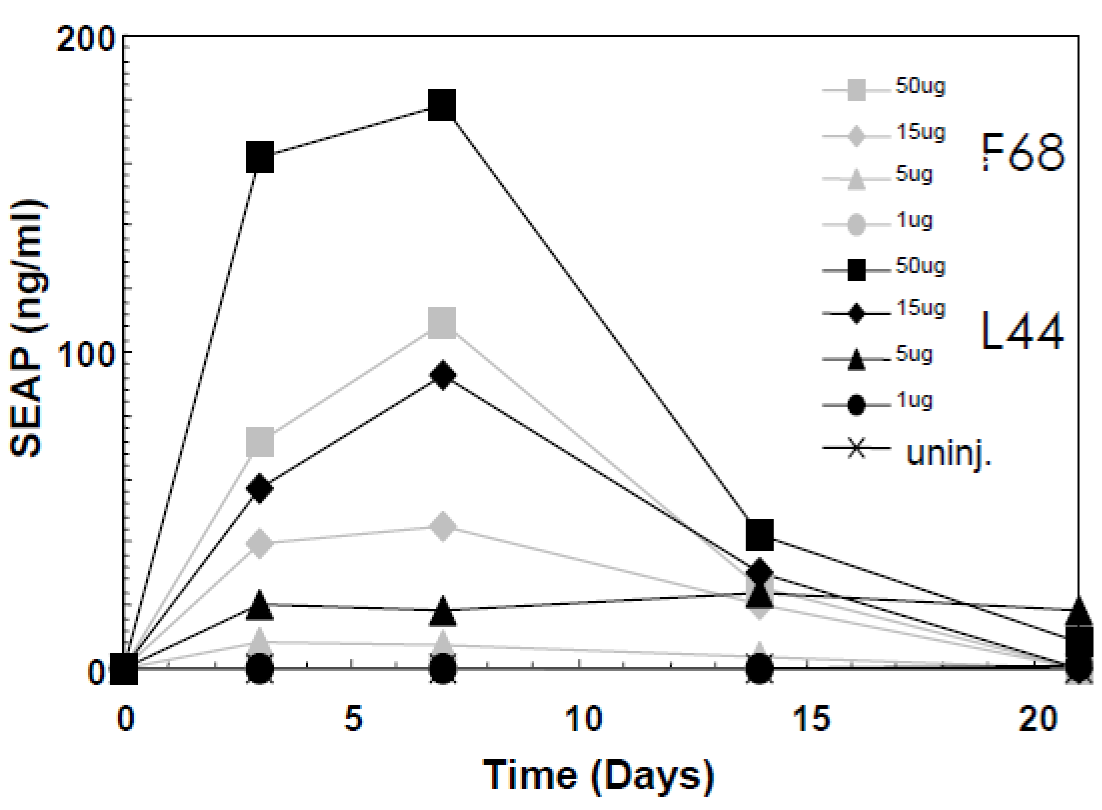
Typical properties					
Pluronic® Surfactant	L44NF	F68NF	F87NF	F108NF	F127NF
Form	Liquid	Prill	Prill	Prill	Prill
Average molecular weight	2200	8400	7700	14600	12600
Specific gravity*	1.05	1.06	1.04	1.06	1.05
Viscosity cps*	440	1000	700	2800	3100
Pour/melt point	16°C	52°C	49°C	57°C	56°C
Cloud point (1% aqueous)	65°C	>100°C	>100°C	>100°C	>100°C
Surface tension (0.1% aqueous at 25°C) dynes/cm	45	50	44	41	41
HLB	12–18	>24	>24	>24	18–23
Solubility in water at 25°C	>10%	>10%	>10%	>10%	>10%

*liquid at 25°C, prill at 70°C

Effect of Poloxamer Concentration on SEAP Expression



Plasmid Dose Response for SEAP Expression



DNA Condensing polymers

- Poly-L-Lysine
- Peptides
- Polyethyleneimine

PolyEthyleneImine

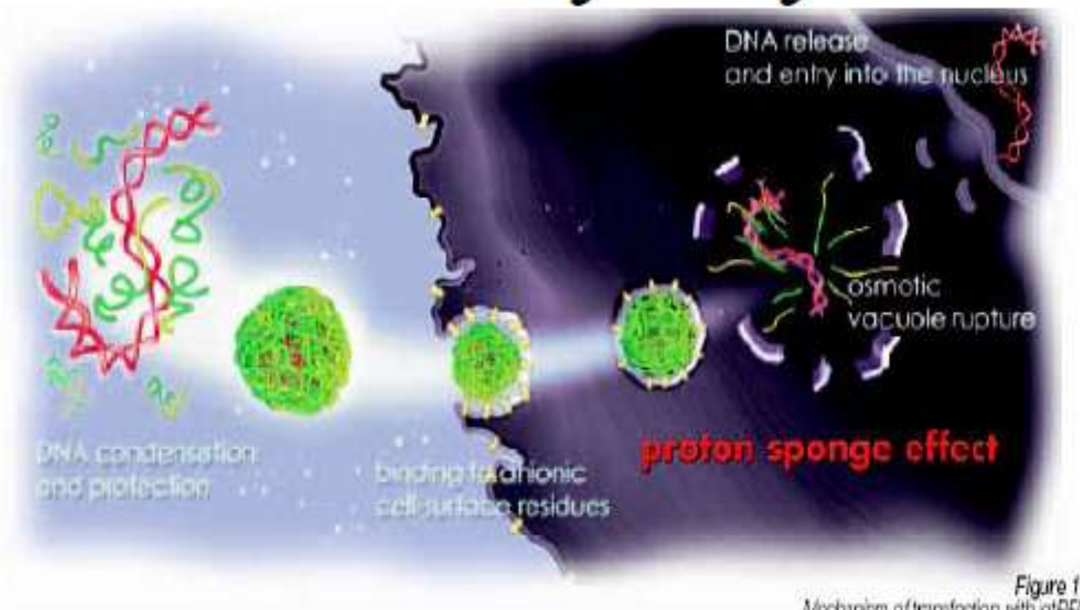


Figure 1
Mechanism of transfection with jetPEI

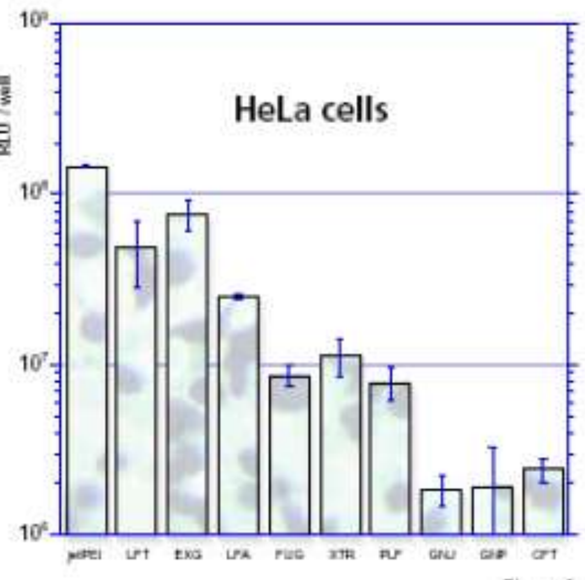
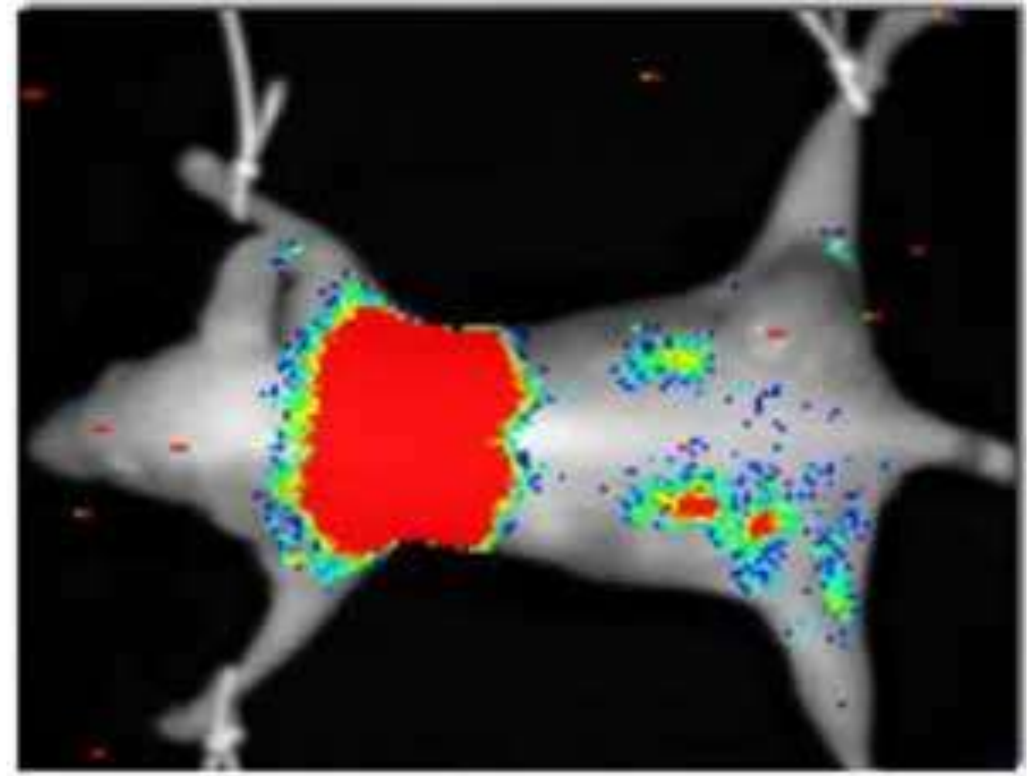


Figure 3
HeLa cells were transfected in 24-well plates in the presence of 10% serum, using 1µg pCMV-luciferase and following the manufacturer's protocol. Luciferase expression was measured after 24h incubation. The data are plotted as the mean relative light units per well (+- s.d.) of triplicate experiments.

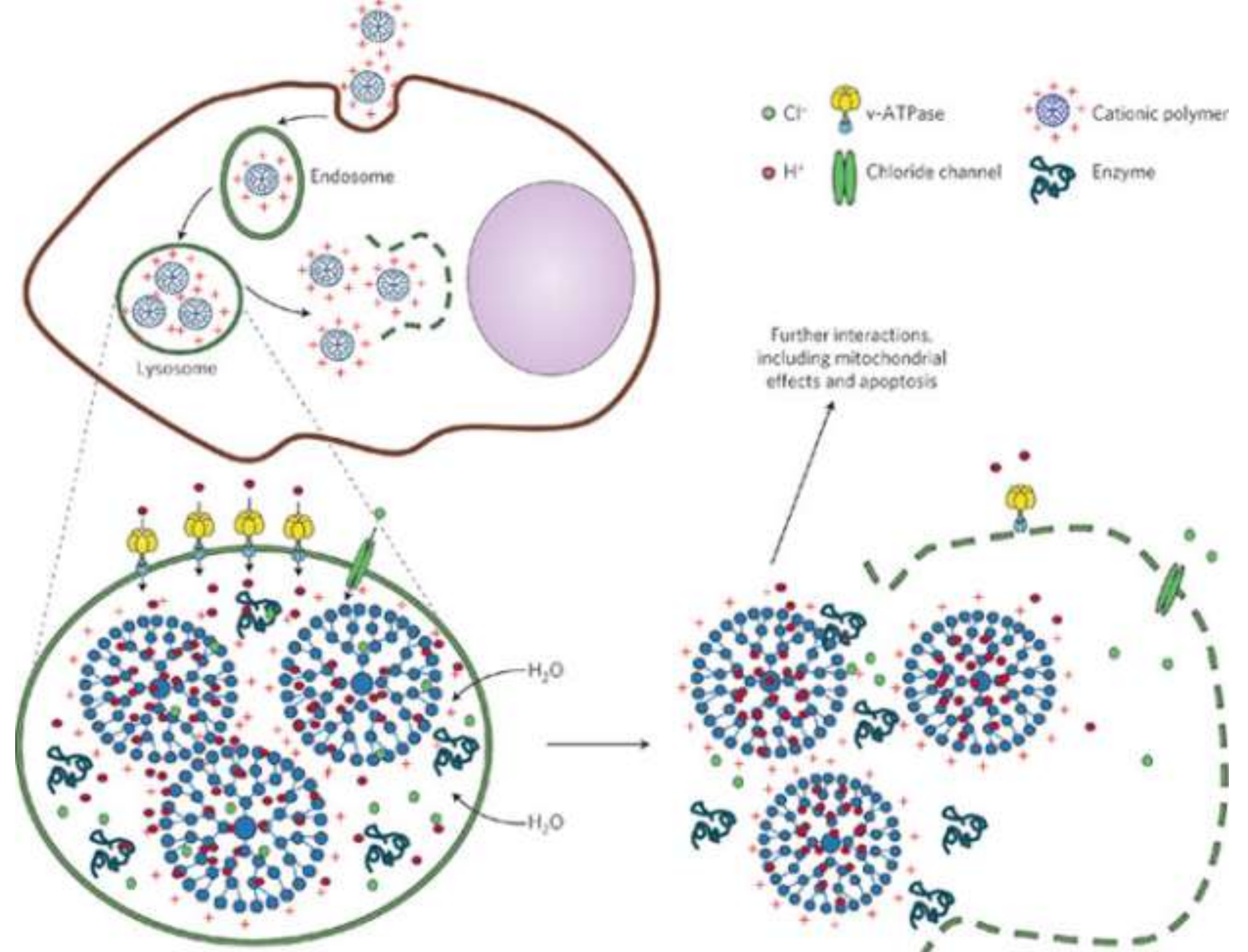
H_2N–(CH₂)₂–(NH–(CH₂)₂)$_n$–NH–(CH₂)₂–NH_2

Figure 2
L-PEI Chemical structure

http://www.qbiogene.com/literature/profiles/pdf/profile-jetpei.pdf



Systemic delivery using *in vivo*-jetPEI™. Bioluminescent imaging of luciferase expression in living Balb/C mouse using a cooled camera 24 h after gene delivery. pCMVLuc (50 µg) was complexed with *in vivo*-jetPEI™ in 400 µl of 5% glucose solution and injected into the tail vein. Courtesy J.L. Coll.



Cationic (for example PEI-coated) particles bind with high affinity to lipid groups on the surface membrane and are endocytosed in the tight-fitting vesicles. Once these cationic nanoparticles enter into an acidifying lysosomal compartment, the unsaturated amino groups are capable of sequestering protons that are supplied by the v-ATPase (proton pump). This process keeps the pump functioning and leads to the retention of one Cl^- ion and one water molecule per proton. Subsequent lysosomal swelling and rupture leads to particle deposition in the cytoplasm and the spillage of the lysosomal content. Nel et al., Nature Materials 8, 543 - 557 (2009)

Table 1. Peptides Used for Guided Gene Delivery*

Function	Peptide	Sequence	Reference
DNA Condensation	Polylysine	$K_{90\ to\ 450}$	5,7,12-16
	Polylysine-containing peptides	$YKAK_8WK$	17
		$(KKK)_2KGGC$	18
		CWK_{18}	19,20
		$CWK_{17}C$	7,21
Endosomolytic	Histidine-rich peptides	CHK_6HC	21
		CHK_6HC	22
		H5WYG	23-25
Fusogenic	Influenza HA-2	GLFGAIAGFIENGWEGMIDGWYG	26-28
	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	29-32
	Tat (48-60)	GRKKRRQRRRPPQ	33-38
	Penetratin	RQIKIWFQNRRMKWKK	9,39,40
	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	41,42
	GALA	WEAALAEALAEALAEHLAEALAEALEALAA	43-45
	KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	46
	JST-1	GLFEALLELLESLWELLLEA	17
	ppTG1	GLFKALLKLLKSLWKLLLKA	47
	ppTG20	GLFRALLRLLRSLWRLLLRA	47
Proteasome	Gly-Ala repeat	$CWK_{18}(GA)_4$	48
Monopartite NLS	SV40 T antigen	PKKKRKV	49-52
	SV40 Vp3	KKKRK	53
	Adenovirus E1a	KRPRP	54
	Human c-*myc*	PAAKRVKLD, RQRRNELKRSP	55
Bipartite NLS	Nucleoplasmin	KRPAATKKAGQAKKKK	56
	Xenopus N1	VRKKRKTEEESPLKDKDAKKSKQE	57
	Mouse FGF3	RLRRDAGGRGGVYEHLGGAPRRRK	58
	PARP	KRKGDEVDGVDECAKKSKK	59
Nonclassical NLS	M9	NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY	60
Cellular targeting	RGD	ICRRARGDNPDDRCT	61,62
	Integrin binding	PLAEIDGIELTY	63
	Secretin	HSDGTFTSELSRLRDSARLQRLLQGLV	64
	GE7 (from EGF)	NPVVGYIGERPQYRDL	65
	NL4	CTTTHTFVKALTMDGKQAAWRFIRIDTAC	66
	Neurotensin	ELYENKPRRPYIL	67
	LOX-1 binding	LSIPPKA, FQTPPQL, LTPATAI	68

Cationic Lipid/Plasmid DNA Transfection Complex Manufacture

Critical Parameters for Cationic Lipid Mediated Systemic Gene Transfer

- Helper Lipid
  - -Phospholipid
  - -Cholesterol
- Surface Charge
  - -Positively charged surface
- Particles Size
  - -Less than 150 nm diameter

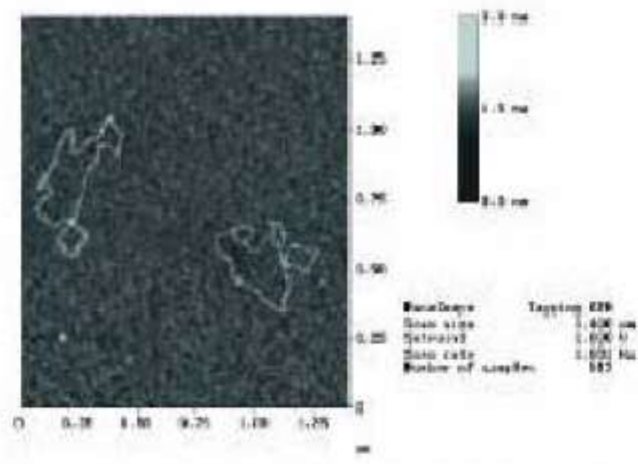


Figure 1 *Images of plasmid DNA pCAT obtained by Tapping - Mode AFM.Scan size:1.4 µm.*

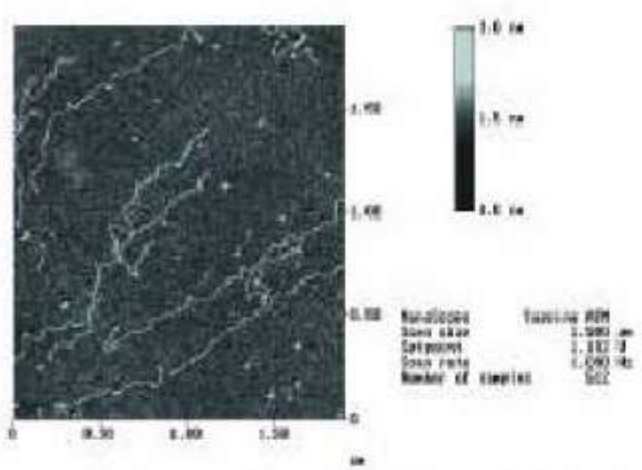


Figure 2 *TappingMode image of plasmid DNA pBR322 cut by EcoRI.Scan size:1.9 µm.*

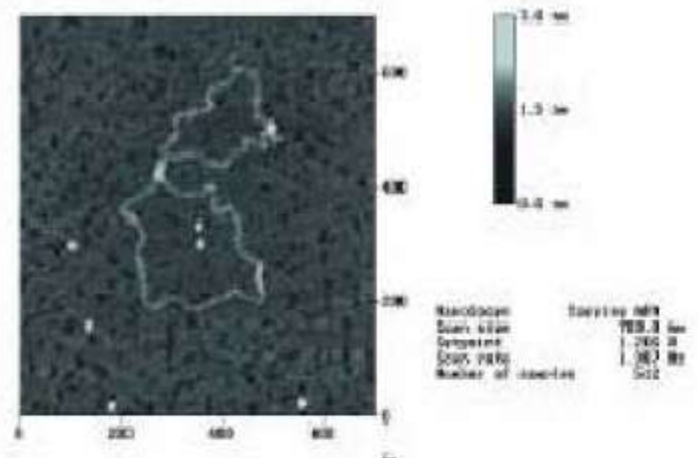


Figure 3 *TappingMode image of plasmid DNA pBR322 com - plexed with EcoRI.Scan size:1.9 µm.*

Dimensions of Linear and Supercoiled Plasmid DNA

Conformations of Phospholipids: Micelles, Liposomes, Hexagonal Phase,

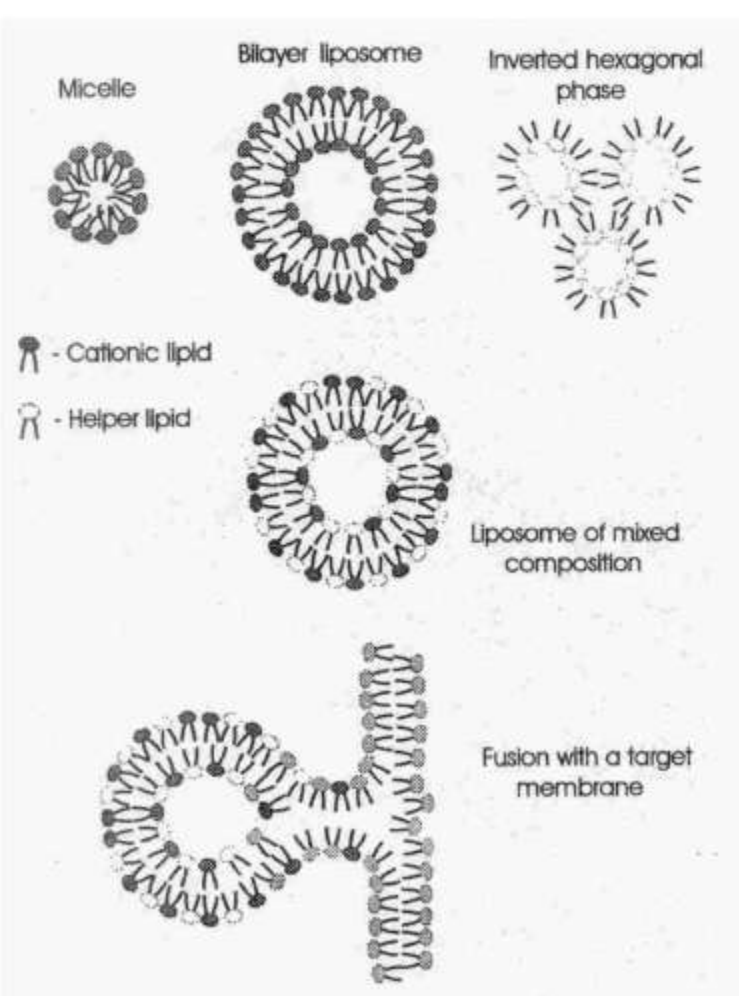


Figure 2 Cartoons of cationic lipids and helper lipids shown as a micelle, single composition and mixed composition liposomes, and in an inverted hexagonal phase. Proposed lipid mixing model of fusion of cationic liposome with a target, i.e. endosomal, membrane.

Xu Y et.al., Biochem 1996;35:7143-7151

Basic structure of cationic lipids

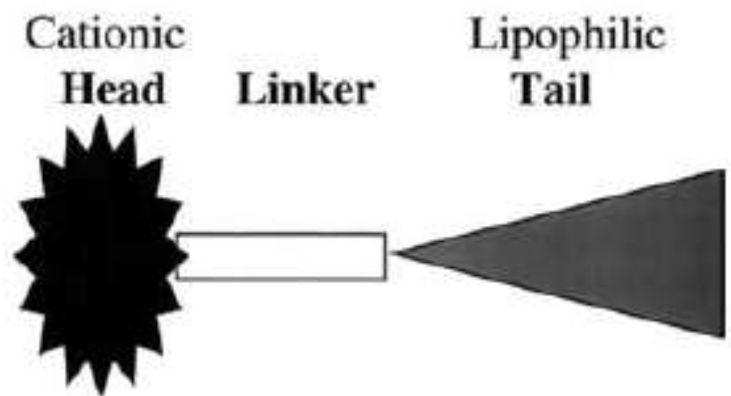
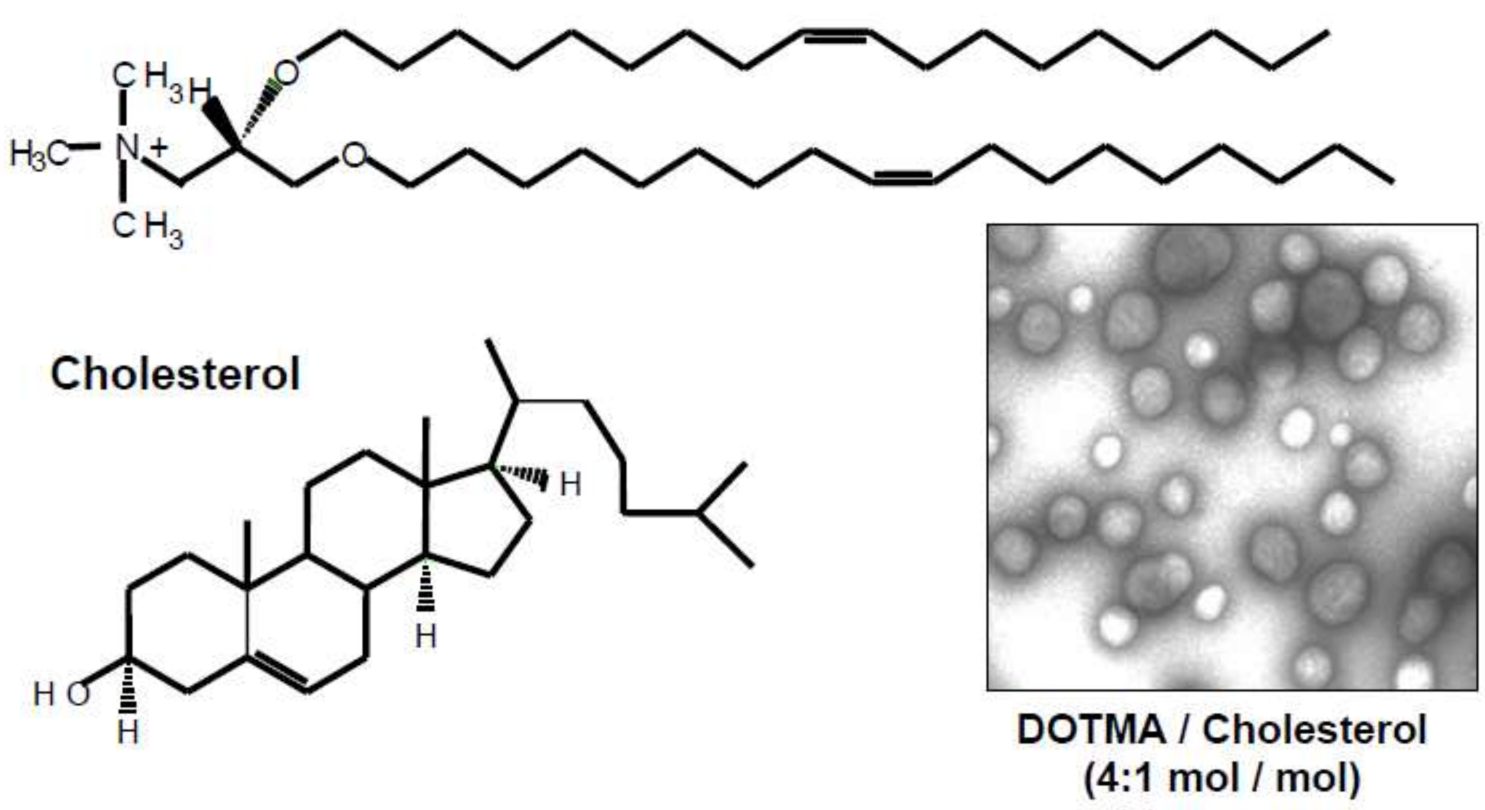


Table 1 Cationic Lipids

Lipid	Lipid/DNA Ratio	
DOTMA	2.67 mol:1 mol (in COS-7 cells) 1.21 mol:1 mol (in CV-1 cells) 0.6 mol:1 mol 0.5 to 2.67 mol:1 mol	Felgner, PL, et.al. PNAS 1987;84:7413
DC-Chol		Gao X et.al. BBRC 1991;179:280
DOGS	1.65 mol:1 mol 1–3 mol/1 mol	Behr J-P, et.al. PNAS 1989;86:6082
GL-67	2–3 mol/1 mol	Behr J-P, et.al. DNA Cell Biol. 1993;12:553
DMRIE	0.67 mmol/4.8 mmol 0.09–0.7 mol/1 mol	Lee ER, et.al. Hum.Gene Ther. 1996;7:1701 Felgner, PL, et.al. J.Biol.Chem.1994;269:2550

Examples of Cationic Lipids and Optimal Lipid/DNA Ratios For In Vitro Gene Transfer

Cationic Liposomes

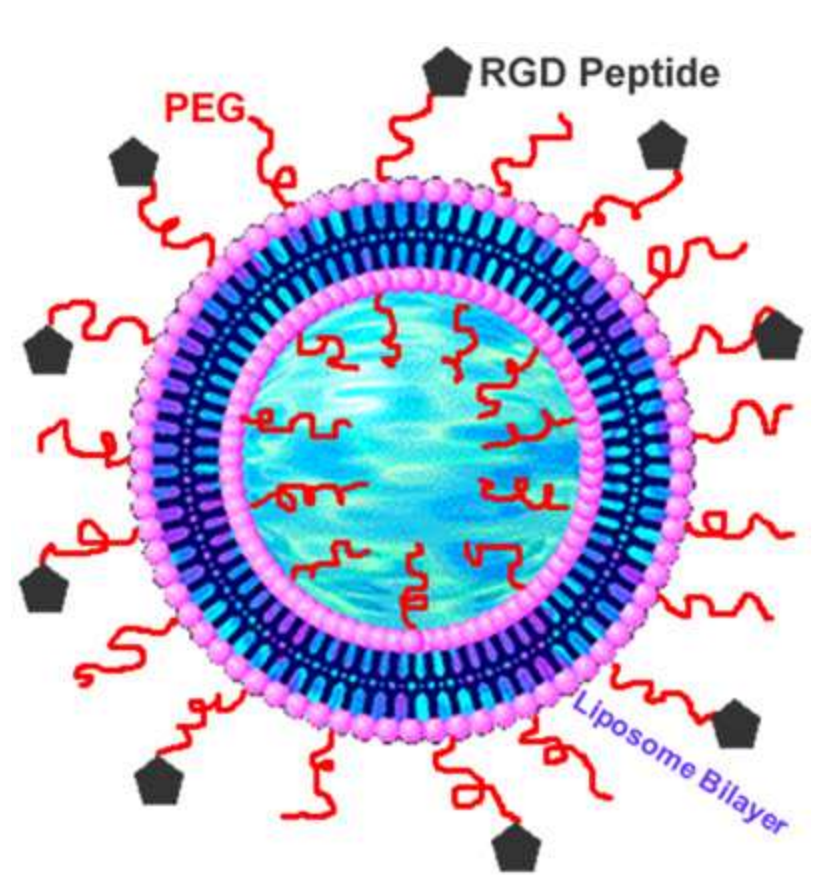


DOTMA / Cholesterol (4:1 mol / mol) Liposomes

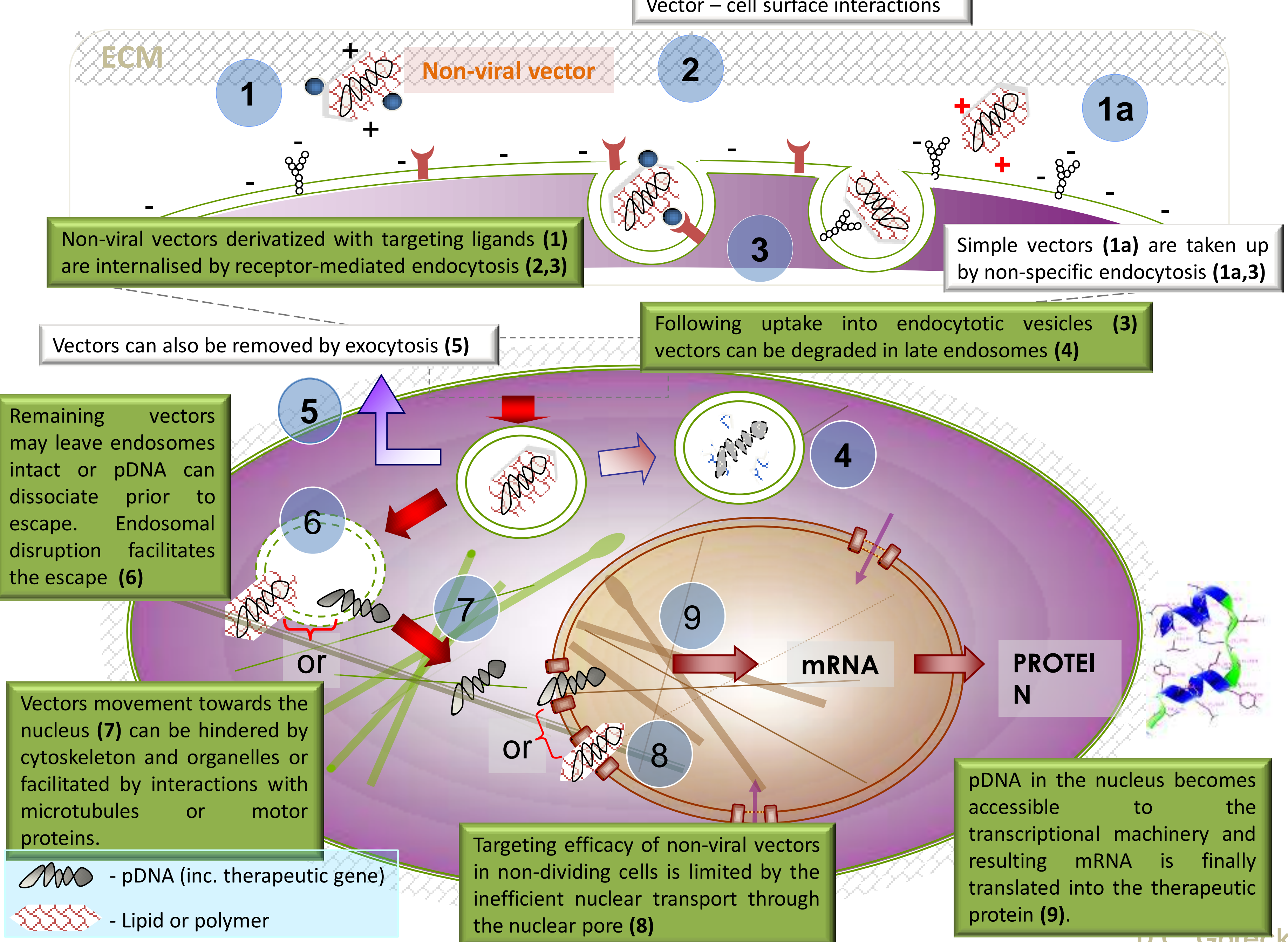
Cationic Liposomes

- In Vivo cationic liposomes have a very short half-life
- positive surface charges cause lipoplexes to aggregate once exposed to proteins in the plasma
- Trigger opsonization
- Rapidly cleared by the reticular endothelial system

Stealth lipids



- PEG offers steric stabilization by decreasing self andnon-self interactions
- PEG also offers a handle to attach targeting ligands



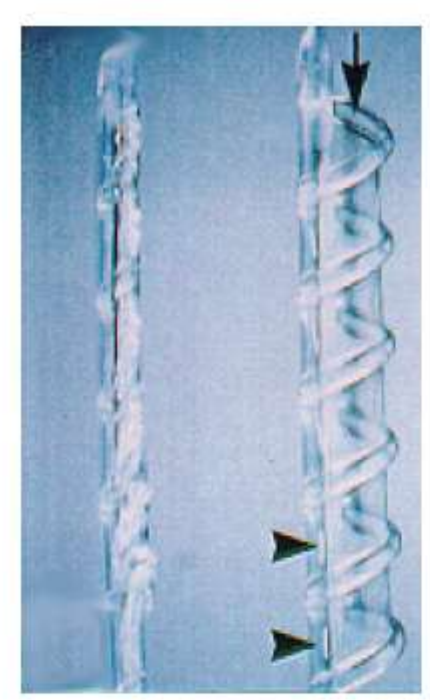
Vector – cell surface interactions
ECM
1
Non-viral vector
2
1a
3
Non-viral vectors derivatized with targeting ligands (1) are internalised by receptor-mediated endocytosis (2,3)
Simple vectors (1a) are taken up by non-specific endocytosis (1a,3)
Following uptake into endocytotic vesicles (3) vectors can be degraded in late endosomes (4)
Vectors can also be removed by exocytosis (5)
5
4
6
Remaining vectors may leave endosomes intact or pDNA can dissociate prior to escape. Endosomal disruption facilitates the escape (6)
7
or
9
mRNA
PROTEI N
or
8
Vectors movement towards the nucleus (7) can be hindered by cytoskeleton and organelles or facilitated by interactions with microtubules or motor proteins.
- pDNA (inc. therapeutic gene)
- Lipid or polymer
Targeting efficacy of non-viral vectors in non-dividing cells is limited by the inefficient nuclear transport through the nuclear pore (8)
pDNA in the nucleus becomes accessible to the transcriptional machinery and resulting mRNA is finally translated into the therapeutic protein (9).
D.C. Gorecki

Gene Therapy for Restenosis

- Localized intravascular (catheter) or extravascular (collar) delivery
- Localized up-regulation of NO via $VEGF_{165}$ or eNOS
- Safe and well-tolerated in ongoing clinical trials

Catheter Delivery of $VEGF_{165}$ Gene Medicine for Prevention of Restenosis

- Ongoing Phase IIb trials in CAD (N=90) and PVD (N=75)
- Delivery of gene therapy via Infusion-perfusion coil balloon catheter
- Placebo, 2 x 10^{10} pfu Ad-VEGF or 2 mg PI-VEGF/DOTMA:Dope formulation
- $VEGF_{165}$ chosen based on anti-proliferative effects
- Endpoints include IVUS, angiography, and exercise tolerance

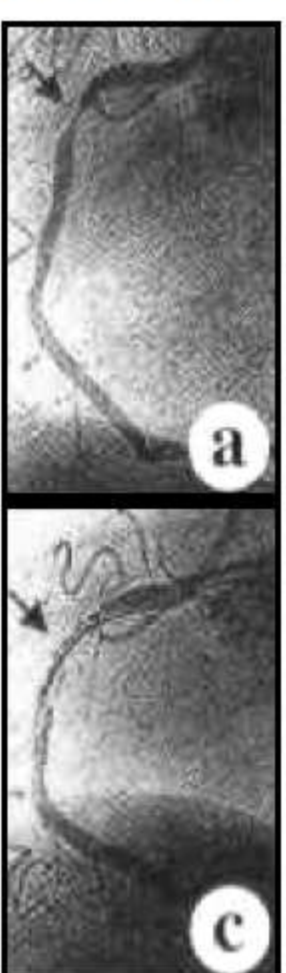


Coil balloon catheter

Catheter-Mediated Intra-Coronary Gene Delivery in Humans

Patient with 90% stenosis in the right coronary artery

Dispatch catheter inflated for a 10 min gene transfer



Dilated angioplasty catheter at the site of stenosis

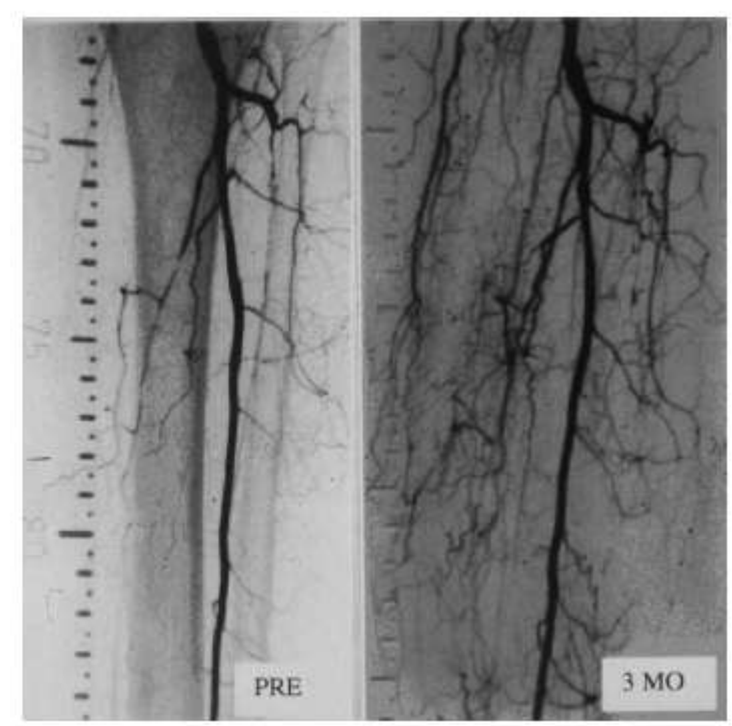
Final angiography after procedure

Lentinen et al., Hum. Gene Ther., 11 (2000)263-270

VEGF$_{165}$ Gene Medicine Stimulates Collateral Vessels in Phase IIb Trial

	+ Angiogenesis
Placebo	0/5
Plasmid	3/5*
AdV	4/5*

**p=0.014 placebo vs. VEGF groups*



Makinen et al., AHA 1999

Patient treated with VEGF plasmid post-angioplasty

Non-Viral/Viral Chimeric Gene Transfer System

HVJ/
Plasmid DNA/
Liposome
Chimeric Gene Transfer System

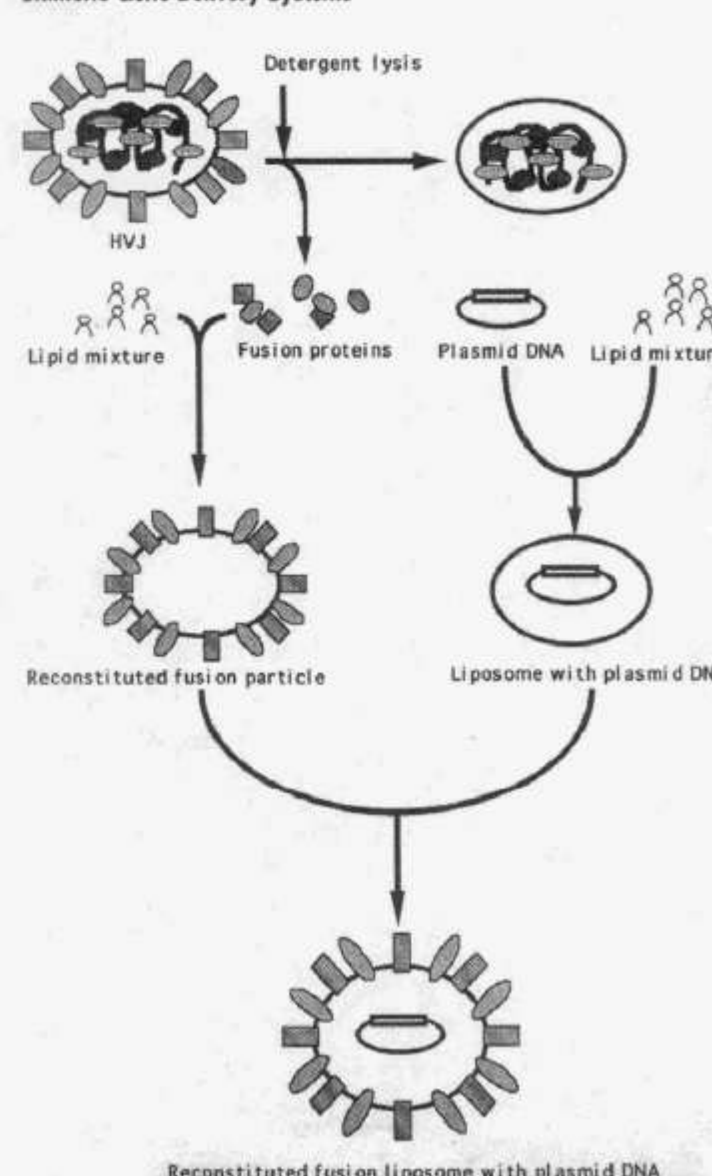


Figure 2 Preparation of reconstituted fusion liposomes. Fusion proteins (HN and F) of HVJ are isolated by detergent lysis of HVJ particles. The proteins are inserted into liposomes by dialysis to form the reconstituted fusion particles. Liposomes containing plasmid DNA or FITC–ODN are constructed and fused with the reconstituted fusion particles. The reconstituted fusion liposomes with plasmid DNA or FITC-ODN are then used as gene transfer vehicles in vitro and in vivo.

Proposed Mechanism For Positively Charged vs. Negatively Charged Chimeric Gene Delivery System

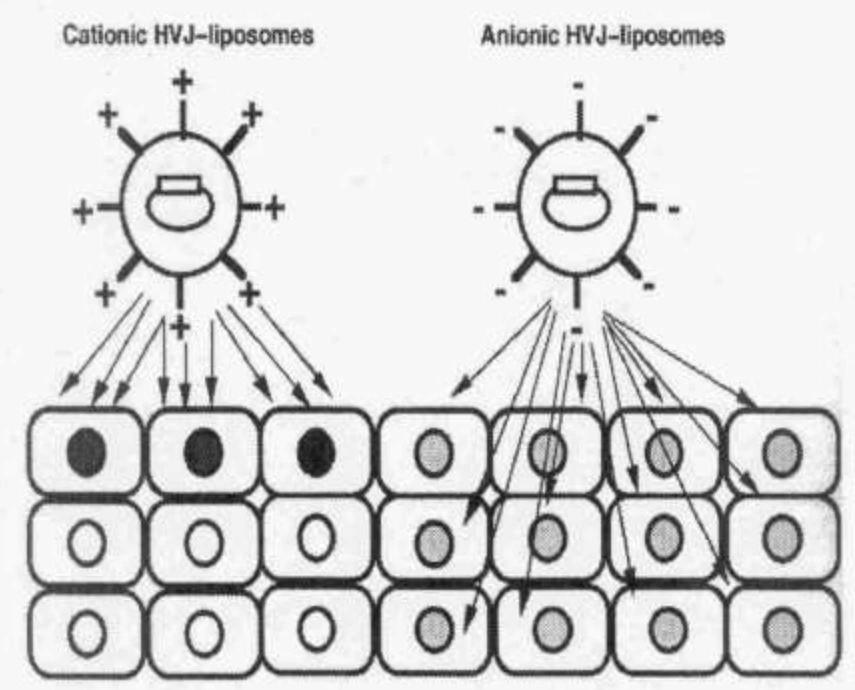


Figure 3 Schema of gene transfer to organs by HVJ–anionic liposomes (right) or HVJ–cationic liposomes (left). It is thought that HVJ-anionic liposomes can better penetrate tissues and distribute to broader areas than cationic liposomes. These HVJ–anionic liposomes gradually fuse with many cells to transfer DNA. However, the level of gene expression per cell is much lower than that observed with HVJ–cationic liposomes. HVJ–cationic liposomes cannot penetrate tissues and remain localized at the injection sites. Therefore, gene expression is detected only at the surface or restricted areas of tissues. Gene expression per cell is much higher with HVJ-cationic liposomes than that with HVJ–anionic liposomes.

In Vivo Gene Transfer Using Chimeric Gene Delivery System

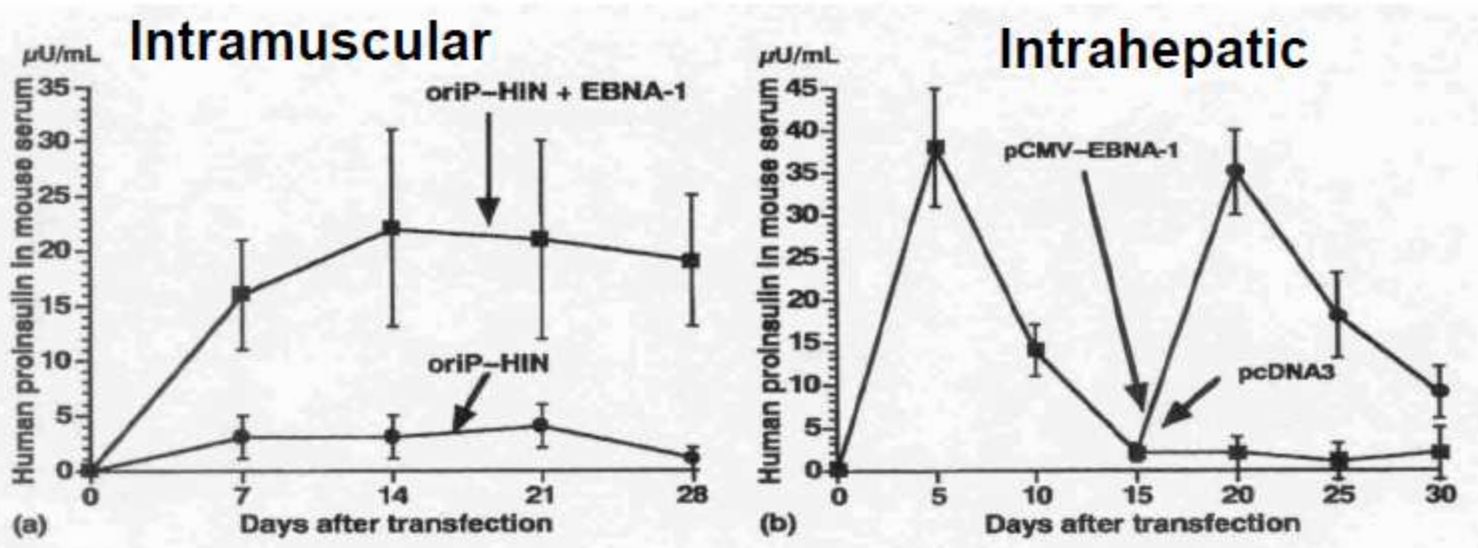


Figure 4 Human proinsulin levels in mouse sera. (a) Approximately 3.8 μg of poriP–CMV–HIN (oriP-HIN) was cotransfected with 12 μg of pCMV–EBNA-1 (EBNA-1) (■) (n = 4) or 8.6 μg of pcDNA3 (●) (n = 4) by HVJ–anionic liposomes into the quadriceps. At 1, 2, 3, and 4 weeks after transfer, the human proinsulin level (μU/ml) was measured by ELISA. (b) Approximately 7.6 μg of poriP–CMV–HIN was transferred directly with 24 μg of pCMV–EBNA-1 (●) (n = 6) to one lobe of the liver. On day 15 after transfer, 24 μg of pCMV–EBNA-1 alone (●) (n = 3) or 17.2 μg of pcDNA3 alone (■) (n = 3) was transferred to one lobe of the liver by HVJ-anionic liposomes. The mean value and standard deviation of samples from four mice are indicated to each time point. A concentration of 1 μU/mL corresponds to 40.67 pg of human proinsulin/mL of mouse serum.

Summary

- Naked DNA can be used by itself or in combination with electroporation or ultrasound.
- Plasmid DNA can be formulated with cationic lipids for systemic administration although clinical trials have tested intratumoral administration.
- Polymer mediated gene transfer can use interactive and non-interactive polymers for local and systemic administration
- Chimeric systems combine viral components with plasmid based gene delivery system to facilitate gene transfer intramuscularly and systemically.