

Hypothesis: Inserting bacterial natural transformation protein complexes into human cells for efficient gene therapy using naked DNA

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Abstract

Naked DNA is a non-toxic vector for therapeutic gene delivery. However, current methods of transfection with naked DNA reach a limited range of susceptible tissues and have a low efficiency. The transfection of clinically important post-mitotic cells is particularly challenging because in these cells DNA need to pass the nuclear barrier. Thus, new principles for the transfer of naked DNA into human cells are required and can be found among the genetic exchange mechanisms in bacteria, where gene entry into cells via pick-up and transfer of naked DNA is known as "transformation". In a number of bacteria, dedicated molecular machinery facilitates cell entry of free DNA by the process of "natural transformation". In transformation-competent bacterial cells, specialised protein complexes mediate the binding of free double-stranded DNA, its fragmentation, cell entry and conversion to single-stranded DNA. I propose to exploit bacterial natural transformation machinery for a two-step transfection of human cells with therapeutic naked DNA. Firstly, the bacterial transformation protein complexes are inserted into the plasma membranes or nuclear envelopes of the target human cells and, secondly, the double-stranded vector DNA is supplied for the processing by the installed DNA transfer apparatus. I hypothesize that non-toxic bacterial transformation complexes residing in their new human *milieu* can promote the ultra-efficient transfer of exogenous therapeutic naked DNA. As the introduction of DNA into mammalian cells by non-viral means is called "transfection", I propose to name the bacterial transformation complexes functioning in their new eukaryotic surroundings as "transfectosomes". The initial step of the gene delivery should exploit the modern methods of extraneous protein insertion into mammalian cells, such as cell painting, engineering of cell permeable proteins with targeted intracellular localization, physical techniques of protein transfer like electroinsertion and electroporation. Sequence-selective natural transformation systems are known and can be taken advantage of to exclude

undesired (e.g. gene silencing) portions of vector DNA from entering human nucleoplasm. Improved transfectosomes can possibly be engineered for better establishment and performance in human membranes. The hypothesis can be tested by comparing the naked DNA transfer efficiency into the transfectosome-bearing and the naive human cells in *ex-vivo* and *in-vivo* gene therapy settings. Immunogenicity of the transfectosomes can be modulated by protein engineering. As the delivered fragments of single-stranded DNA are highly recombinogenic, the confirmation of the hypothesis can lead to a breakthrough in gene repair therapy of dominantly inherited familial hypercholesterolemia, polycystic kidney disease and trinucleotide repeat disorders.

Background

Gene therapy is achieved by the transfer of genetic material to human cells and subsequent correction of the disease-causing faulty genes or expression of therapeutic transgenes. Genetic material can be delivered after packaging into virions, complexing with transfer-promoting substances or as naked DNA. Transfection with naked DNA is an attractive gene therapy approach because of pure DNA's lack of toxicity. It was discovered that some tissues (e.g. skeletal muscle, heart muscle, thyroid, solid tumours) are capable of pure DNA uptake after a simple injection, although with a low efficiency [1]. Physical methods of pure DNA delivery like high-volume high-pressure "hydrodynamic" injection, electroporation, microbubble-assisted sonoporation and jet-injection can broaden the spectrum of the susceptible tissues and increase the efficiency of gene delivery [2, 3]. However, further improvements in naked DNA transfer technology are needed to foster therapeutic applications.

Common therapeutic targets are non-dividing or rarely-dividing human cells where, in addition to the

external plasma membrane, the inward bound vector DNA need to pass through the nuclear envelope barrier in order to reach its destination in the nucleoplasm. There is no evidence that the currently available procedures can provide efficient transfer of naked DNA to nuclei. Normally, the delivery of genes into postmitotic cells requires transduction with some viral vectors or transfection involving the complexing of DNA with nuclear transfer promoting substances [4, 5]. These nuclear transfer methods suffer from high toxicity. Therefore, new principles for the transfer of naked DNA into human cells are required.

In search of such principles one can look at the genetic exchange mechanisms in bacteria, where gene entry into cells via pick-up and transfer of naked DNA is known as "transformation". Some bacterial species developed in their evolution a process of "natural transformation" with specialised transformation protein complexes, which reside in the bacterial membranes, mediating the binding of extraneous double-stranded DNA, its fragmentation, cell entry and conversion to a highly recombinogenic single-stranded form [6, 7]. Natural bacterial transformation systems are known in Gram-positive genera *Bacillus* and *Streptococcus*, Gram-negative genera *Acinetobacter*, *Haemophilus*, *Helicobacter*, *Neisseria*, *Pseudomonas* and many others. In all well-studied cases transformation complexes are discrete self-assembled membrane-associated protein ensembles. Transformation complexes can be composed of integral membrane proteins (e.g. secretins), traffic NTPases and structural proteins like pilins. The particular details of the natural transformation machinery vary between various bacterial species, especially between Gram-positive and Gram-negative bacteria, which have disparate cell wall structures. Portions of such bacterial transformation protein complexes are sometimes observable microscopically and are called "transfosomes" in *Haemophilus influenza*.

The hypothesis

I propose to exploit bacterial natural transformation machinery for a two-step transfection of human cells with therapeutic naked DNA. Firstly, the bacterial transformation protein complexes are inserted into the plasma membranes or nuclear envelopes of the target human cells and, secondly, the double-stranded vector DNA is supplied for the processing by the installed DNA transfer apparatus. I hypothesize that non-toxic

bacterial transformation complexes residing in their new human *milieu* can promote the ultra-efficient transfer of exogenous therapeutic naked DNA. As the introduction of DNA into mammalian cells by non-viral means is called transfection, I propose to name functional bacterial transformation complexes in their new eukaryotic setting as "transfosomes". Improved transfosomes can possibly be engineered offering better establishment and performance in human membranes providing for both efficient gene repair and therapeutic transgene expression. Protein engineering can also be used to reduce immunogenicity of transfosomes, if required.

Evaluation of the hypothesis

Transformation protein complexes can be harvested from their original host bacteria. If the competent state of the bacteria is not constitutive, it should be induced prior to the harvesting of the transformation complexes. Alternatively, individual proteins for the transformation complexes can be expressed in heterologous bacteria, yeast, insect or plant cells. In this case self-assembly of the complexes can be accomplished either immediately in the producer cells, or with purified proteins *in vitro*, or after insertion into the human cells.

Transformation complexes from various bacterial species should be compared to find the most suitable foundation for the generation of transfosomes with the best performance in the human membrane setting. The selection criteria might also include non-pathogenicity of the bacterial host, which could simplify the complexes' production. Mutant versions of the transformation ensembles with an increased efficiency of DNA transfer can be selected in their native bacterial background or in cells of heterologous organisms.

The absence of a rigid cell wall in mammalian cells allows unobstructed insertion of proteins and protein complexes into the plasma membrane of the cells. Integral membrane proteins and their complexes can be delivered to the human plasma membrane in a lipid or detergent carrier by the procedure known as "cell painting" [8-10]. Physical methods of protein transfer like electroinsertion [11] and electroporation [12] can also be employed for the protein delivery. Artificial modification of natural transformation complexes might be required to prepare effective transfosomes adapted to the correct establishment and DNA transfer within mammalian plasma membrane *milieu*. For example, protein modification with

glycosyl-phosphatidylinositol (GPI) membrane anchors and/or other lipidations can be used. Glycosylated derivatives of the transfectosome proteins can be generated to provide a signal for the correct orientation of the protein complex in the membrane. The elements of the transformation complex and its auxiliary proteins can be delivered into human cytoplasm if supplied with a "protein transduction" (PTD) domain, as successfully used for Cre recombinase [13] and FLP recombinase [14]. A combination of different protein delivery approaches might be the optimal option.

Theoretically, it is possible to introduce bacterial transgenes to direct the biosynthesis of transfectosomes in human cells. However, the need for transfectosomes is transient, and so the insertion of "pre-made" proteins into human cells looks more attractive than transfectosome biosynthesis *in situ*. The protein delivery option benefits from the possibility to modulate the self-assembly of the transformation complexes *in vitro*, while the choice of the *in situ* biosynthesis option might result in insertional mutagenesis or potentially deleterious long-term expression of bacterial transgenes in human cells. Nevertheless, stable mammalian cell lines permanently or inducibly expressing transfectosomes can be generated for ultra-efficient transfection needs, e.g. for transgene expression studies, viral gene vector production or gene repair research.

For efficient transfection of postmitotic cells, it would be advantageous to insert bacterial transformation complexes into the nuclear envelope to drive the efficient transport of the DNA into the nucleoplasm. In this scenario, the transformation complexes need to be artificially modified to reach the nuclear envelope and to acquire the functional state there. In particular, bacterial transformation complexes should be supplied with targeting signals for outer and inner nuclear membranes [15, 16]. Under standard circumstances, incoming DNA should be double-stranded prior to the entry into bacterial transformation complexes and it is expected to be single-stranded after being processed by the complexes. Therefore, the concomitant employment of the transfectosomes in plasma membrane and nuclear envelope is complicated by the requirement of either unusual cytoplasmic synthesis of the second DNA strand or an essential modification of the plasma membrane transfectosomes with a block of conversion of the double-stranded DNA into the single-stranded form.

Some systems of natural transformation are known for their DNA sequence selectivity, e.g. competent cells of *Neisseria gonorrhoeae* require 10-bp sequence 5'-GCCGTCTGAA-3' to be present in the extraneous DNA for efficient cell incorporation. Such sequence-specific transformation machinery can be used for DNA transfer into human cells or nuclei, provided the required sequence tags are inserted into vector DNA. As the incoming DNA is fragmented by the transformation machinery, the tagged portions of exogenous DNA would be able to enter the cells while the non-tagged DNA portions would be denied access. This sequence-selectivity can be exploited for the advantage of gene therapy. For example, as an origin of replication and a marker gene of a bacterial plasmid, used for gene delivery, were reported to induce undesired transgene silencing [17], the tag sequences could be arranged to be excluded from the bacterial plasmid backbone DNA sequences.

The hypothesis should be tested by experiments *in vitro* and *in vivo* with view of *ex-vivo* and *in-vivo* gene therapy. Human cells should be furnished with transformation complexes with the excess extraneous protein removed by washing or by epitope-targeted immunoprecipitation to minimise the premature binding of the transformation complexes and DNA. Transfectosome-bearing cells should be incubated with pure DNA and the efficiency of gene transfer should be evaluated by standard methods relying on the chromosome recombination events or transgene expression in the treated cells. As an additional model system, transformation complexes can be assembled into proteoliposomes and the conditions for the DNA transfer into their interior compartments can be studied.

The proposed bipartite transfection strategy is expected to combine ultra-high efficiency of gene delivery with the advantages of naked DNA vectors because pure DNA would be used without complexing by cytotoxic polycationic transfectants or packaging into cytotoxic virions. The insertion of bacterial transformation complexes into human cells is not likely to be toxic. In many *in-vivo* gene therapy scenarios, immune response associated with gene delivery is detrimental. Thus, pre-existing immunity to the transformation protein complexes should be avoided by the thoughtful choice of the bacterial source for the transformation complexes and their purposeful re-engineering. The transfectosomes are required only for a short period of time during DNA delivery and are

likely to be degraded by the time a substantial immune response is mounted against them. Conversely, in gene therapy of cancer, immune response after gene delivery is often beneficial, so in this situation, the immunostimulatory potential of the transfectosome-insertion step should be taken advantage of.

Once in the nucleoplasm, the single-stranded DNA generated by the transfectosomes can be immediately available for gene repair by strand displacement. Alternatively, single-stranded DNA can be built up to the double-stranded form *in vivo* and mediate transgene expression. It is well-documented that genes delivered as single-stranded DNA by adeno-associated virus (AAV) vectors [18] and filamentous bacteriophages [19] can be expressed in mammalian cells. Nevertheless, the anticipated fragmentation of DNA and its conversion to the single-stranded form by transfectosomes might have a negative effect on the expression of large therapeutic transgenes. Therefore, gene correction treatment, which can rely on smaller DNA fragments and which can benefit from the highly recombinogenic status of the single-stranded DNA, is a particularly attractive application of the transfectosome-mediated gene transfer. The confirmation of the hypothesis can lead to a breakthrough in gene repair therapy of dominantly inherited familial hypercholesterolemia, polycystic kidney disease and trinucleotide repeat disorders, such as Huntington's disease.

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