



Shielding of non-target cells using RNA vectors conferring gene transfer resistance: A strategy to enhance targeting accuracy and reduce side-effects in therapeutic gene delivery

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Introduction

Accumulating knowledge about molecular mechanisms of human diseases and their genetic underpinnings creates enticing promises to cure these diseases using genetic modification of human cells. Genetic medicines, delivered to cells by gene vectors, consist of a variety of therapeutic genes, such as genes for complementation of recessive genetic defects, genes blocking expression of pathological dominant mutations, genes for cell growth factors, drug-sensitivity genes to destroy cancer cells and genes for human gene-editing. Although there are some successes in genetic treatment [1,2], many gene therapy attempts are thwarted by a long list of problems, including: low yield and instability of the chosen gene vector during its production stage [3]; vector inactivation by serum complement and other elements of the intra-body environment [4]; low efficiency of gene transfer [5]; vector and/or transgene cytotoxicity [6]; adverse immune reactions due to several forms of pre-existing and acquired immunity to gene vectors [7,8]; failure of transgenes or transgenes' products to evade intracellular molecular surveillance machinery, e.g. degradation in acidified endosomes [9]; failure of gene vectors to enter nuclei in non-dividing cells [10]; silencing of transgene expression [11]; tumorigenicity [12]; and accumulation of potentially hazardous unwanted 'genetic litter' [13]. Some of these problems, most prominently cytotoxicity, tumorigenicity and adverse effects of immunogenicity, fall under an umbrella term of 'undesirable side-effects of gene delivery'. Side-effects present a

particularly notable hurdle when repeated gene vector administrations are required to top-up shutting down expression of therapeutic transgenes. Balancing the scale of the curative action and the undesirable side-effects of this curative action is the important part in the creation of the optimal treatment protocol. Much can be achieved by the strategic choice of the suitable target cell population for therapeutic gene delivery. The selected target cell population should be: 1) critical for the effectiveness of a curative action; 2) associated with minimal unwanted side-effects; 3) amenable for efficient gene delivery, that is, accessible and receptive for gene transfer.

Typically, both for viral vectors, which deliver genes to recipient cells through 'transduction', and non-viral vectors, which deliver genes to recipient cells through 'transfection', side-effects positively correlate with the therapeutic gene vector dose. Often the target cell population requiring genetic treatment is small, giving the chance to reduce many side-effects through the coupling of the reduction of the total administered gene vector dose and the accurate targeting of gene delivery on the target cells. So, due to the intense focusing of the employed gene vector particles on the target cells, gene delivery to these cells can be performed with high efficiency even with a reduced total amount of used vector. If a still higher efficiency of gene delivery to the curatively important cells is desired, then accurate targeting can create a higher vector load at the target site without an increase in the total amount of used vector. As in targeted gene delivery vector particles are not wasted on non-target cells, targeting brings the additional benefit of associated

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reduction in vector expenditure to achieve the desired efficiency of gene transfer, therefore, providing economy of gene vector preparation, which could be available only in a small amount and/or expensive.

In addition to the amelioration of side-effects, targeting of gene vectors contributes to tackling another problem in gene delivery, the silencing of transgene expression. Indeed, therapeutic transgenes are typically equipped with expression control elements capable of providing lasting expression only in specific tissues and, therefore, are likely to shut down in mis-targeted cell populations [11]. Therefore, delivery of transgenes to the correct cellular address of the chosen target cells increases their chances of long-term expression. Furthermore, the targeting of a vector on the appropriate cell population ensures that a genetic cargo is correctly expressed in the fitting epigenetic environment (e.g. transgene products are being correctly processed) and does not generate undesired off-target side-effects through incorrect expression in mis-targeted cells [11].

A radical solution to accomplishing gene vector targeting is gene transfer *ex vivo* [14]. In this scenario, the targeting of gene delivery is implemented through isolation of the target cell population from the body, subsequent gene transfer *ex vivo* and, finally, implantation of the genetically modified population back into the body. Unfortunately, for many diseases, particularly affecting terminally differentiated cells, *ex vivo* gene therapy is either extremely technically challenging or impossible to carry out. Thus, in numerous disease settings, gene therapy should be performed *in vivo*, with direct therapeutic gene delivery into the curatively important cells within the human body.

Methods of targeted delivery with gene vectors *in vivo* can be broadly categorised into two groups. Firstly, targeting can be at the cellular level, with targeted entries of gene vectors into recipient cells relying on specific binding events between targeted vectors and the cells from the target population [15]. This type of targeting depends either on tissue-specific tropism of naturally occurring viruses or on the decoration of gene vectors with cell-specific ligands, such as: 1) small molecules binding to their cognate cell-surface receptors; 2) single-chain antibodies; 3) cell-binding domains of toxins and other proteins; and 4) artificial cell-binding peptides selected in molecular display procedures. Secondly, targeting can be at the body-site level, where gene vectors are targeted to a particular spatial zone within the body. This type of targeting relies on 'physical' methods such as purposefully chosen administration route [16], real-time image-guided administration [17], localised gene delivery through vector-releasing depots [18], electroporation (electrotransfer) *in vivo* [19], microbubble-assisted sonoporation [20] and *in vivo* gene focusing using magnetic force [21].

Some benefits of targeted gene delivery can also be garnered through engineering and delivery of transgene expression cassettes which, relying on regulatory elements such as tissue-specific transcription enhancers or tissue-specific miRNAs, ensure selective transgene expression only in the desired therapeutically important cell populations [22]. In this scenario, even with untargeted, 'carpet', gene delivery, it could be possible to re-create an exact replica of the outcome of the genuinely targeted gene delivery through the pre-programming of mis-targeted transgene modules to self-destruct in an unsuitable epigenetic environment of non-target cells [13].

Various targeting strategies can be used together in order to benefit from the maximal possible targeting efficiency. For example, cell-specific targeting relying on the natural kidney-specific tropism of a viral vector based on Adeno-Associated Virus (AAV) 9 and physical targeting through renal vein injection were effectively combined for therapeutic gene delivery to the cortical and medullary tissues in the kidney [23]. Similarly, it was suggested that the accuracy of targeted gene delivery could be enhanced through the prior transfer of helper-transgenes which were capable of guiding subsequent cell-specific therapeutic gene delivery through tissue-specific expression of 'ear-marking' extracellular receptors [24]. Thus, in this scenario, 'scout' vector particles could be programmed to probe the epigenomes of recipient cells and then to send signals to circulating therapeutic vector particles via the

expression of an ear-marking cell-surface receptor, thereby permitting therapeutic gene transfer to true target cells and precluding therapeutic gene transfer to non-target cells [24].

A wide-ranging 'split vector' strategy, which is applicable at both the body-site level and the cellular level, was suggested to improve the accuracy of gene delivery targeting [25]. In a 'split vector' gene delivery system, the gene transfer vector is divided into two or more components, which, taken individually, are necessary but not sufficient for gene transfer. Overall targeting enhancement is achieved through independent targeted administrations and independent spread of the gene vector components, which self-assemble into functional gene transfer machinery only where the spreads overlap and, thus, ensure therapeutic gene transfer only in a highly restricted target body-site or a highly restricted target cell population [25]. It appears that such superposition of independent subordinate targeting actions to boost overall targeting accuracy can be generalised to a universal principle of combinatorial targeting, which is, in fact, not limited just to the self-assembly of the essential 'split vector' components at the intersections of their spreads *in situ*.

Thus, in this paper I propose that enhanced targeting of therapeutic genes in delivery *in vivo* could be achieved through the extension of the combinatorial targeting principle to include independent administrations and independent spreads of a therapeutic gene vector and a shielding vector. The shielding vector, administered earlier, would make non-target cells unreceptive to gene transfer with its cognate therapeutic vector, administered later. So, hypothetically, optimal shielding from therapeutic gene delivery vectors, providing a boost of accuracy for therapeutic gene delivery, could be achieved through pre-administration of specialised shielding gene vectors capable of blocking gene transfer with therapeutic gene vectors into non-target cells. Indeed, the resultant targeting enhancement would be a novel form of combinatorial targeting, as the improvement in targeting accuracy would be achieved through a combination of two independently targeted vectors: one prohibiting therapeutic gene delivery in the undesired cell populations and another one accomplishing it in the desired cell population.

Hypothesis

I propose that overall targeting accuracy of therapeutic gene delivery could be boosted through the targeted pre-administration of shielding RNA gene vectors, conferring transient gene transfer resistance to non-target cells. With non-target cells being shielded, subsequent independently targeted administration of therapeutic gene vectors is hypothesized to provide more accurate curative gene transfer into target cells. Shielding could be implemented in two modes: 1) 'strict non-admittance' of therapeutic vector particles into non-target cells, with the return of entry-banned therapeutic vector particles back into the circulating transducing/transfecting pool; 2) obstruction of cell entry, maintenance and/or expression of the genetic elements of the therapeutic gene vector, without the return of the incoming therapeutic gene vector particles back into the circulating transducing/transfecting pool. The shielding RNA vectors are expected to provide three-dimensional (3D) protection of compactly-located or widely-distributed non-target cells from the therapeutic gene delivery vectors, thereby ameliorating the side-effects of therapeutic gene delivery, including cytotoxicity, tumorigenicity and adverse immunogenicity.

Evaluation of the hypothesis

Theoretical exploration of the possibilities of implementing shielding to improve targeting of curatively important cell populations with therapeutic gene vectors within the 3D space of the human body

Targeted gene delivery is typically achieved through the focusing of gene transfer on a specific 3D target zone or a specific cell population.

While the significance of such focusing for effective gene therapy is widely appreciated, the substantial potential for enhanced targeting through support of the focusing of gene transfer by shielding of non-target body zones or non-target cell populations is currently under the radar in gene therapy research. This is rather surprising, as targeting through shielding is used extensively in modern technology, e.g. through the use of masking materials for finely-patterned etching in the fabrication of integrated circuits from wafers. Perhaps, the employment of shielding as a targeting strategy in drug delivery *in vivo* is hindered by the scarcity of therapeutically relevant two-dimensional (2D) targeting settings and the typical need to achieve targeting in the 3D space of the human body.

However, if interpreted broadly, shielding of non-target zones is not an entirely new approach in therapeutic gene transfer. Indeed, shielding of non-target cells from gene transfer can be implicitly accomplished through the spatial confinement of gene transfer, that is, the confinement of the administered vector particles in a specific body compartment harbouring exposed target cells exploiting body's natural barriers for the shielding of the outlying non-target cells. Thus, targeting of gene transfer through the purposeful choice of administration routes [16] tacitly relies on the shielding of non-target zones by intra-body features with low penetrability for the gene vectors. In a dramatic example, gene vector administration into a horse joint cavity by intra-articular injection resulted in the localised intra-cavity spread of the vector and targeted gene transfer into cartilage cells with 99.7% of the vector remaining within the injected joint [26].

The 2D version of shielding using a solid screen would be straightforward to implement in gene delivery to skin or other temporarily exposed tissue surface, e.g. when using a 'gene gun' (biolistic) technique [27]. A membrane with a pinhole positioned under the barrel ring of the gene gun would be sufficient to limit the area of bombardment with gene vectors to the pinhole's outline. However, vector-impenetrable artificial shields would be difficult to implement in 3D intra-body gene delivery *in vivo* because of the inevitable blocks to the essential blood flows delivering oxygen and nutrients to cells. And, importantly, if the target cells are not compactly localised at a specific body-site and instead are widely distributed throughout the body, mechanical shielding would not be altogether possible.

Nevertheless, the shielding of non-target cells holds a significant promise to increase the sharpness of the 3D spatial profile of therapeutic gene delivery. This view is supported by the analogy of targeting and imaging. Indeed, targeting can be viewed as the reverse of imaging: while in imaging, information about the space distribution of one or more parameters is being collected from an object, in targeting, information about the space distribution of parameters is being impacted onto an object. 'Target resolution' of gene delivery can be defined similarly to resolution of 3D-printing either through the diameter of the smallest targetable volume feature [28] or through volume pixels, known as voxels [29], with a single voxel corresponding to an individual cell or a group of cells. Thus, strategies to increase target resolution can be analogous to the strategies that are used to increase image resolution. The mainstay of modern diagnostic radiography, such as magnetic resonance imaging or computer tomography, is the improvement of image resolution through a 'contrast procedure', which consists of pre-administration of 'contrast materials' ('contrast dyes') with extra-high or extra-low imaging density. Considering imaging density as analogous to intensity of gene transfer, it is reasonable to surmise, that, similarly to the enhancement of image resolution with 'contrast dyes' of extra-low imaging density, the required 'contrast'-based enhancement of target resolution could be achieved through the pre-administration of gene transfer blocking agents, such as proposed shielding RNA vectors, to prevent therapeutic gene transfer to the chosen non-target cells.

In contrast to mechanical contraptions or any other 'physical' method of shielding, which at best could protect non-target cells from therapeutic gene transfer exclusively at the body-site level, the

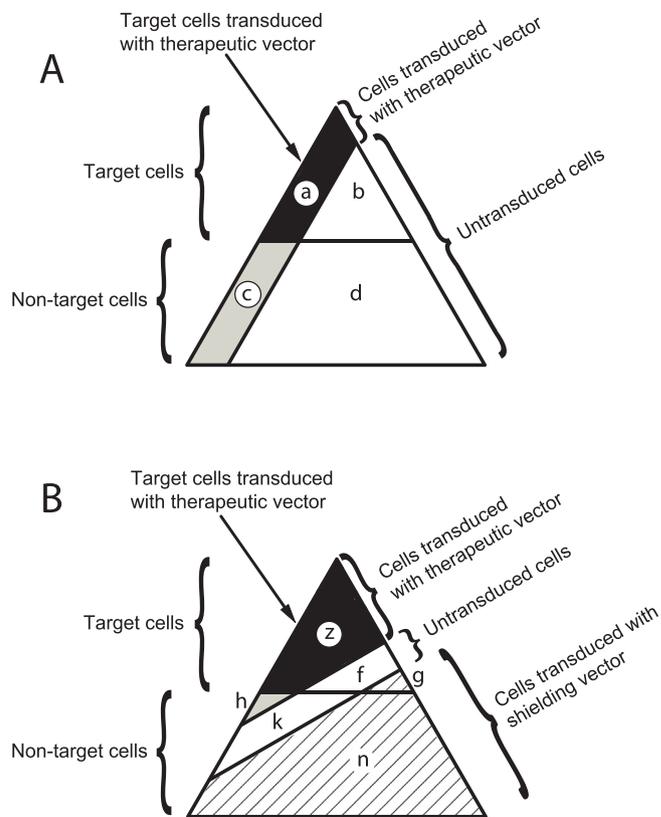


Fig. 1. Euler diagrams for various sets of cells in a targeted therapeutic gene delivery. Triangular table format is chosen for the Euler diagrams to reflect the typical numerical preponderance of non-target cells versus target cells. Vectors are presumed to be viral; for non-viral vectors the word 'transduced' in the annotations of the diagrams should be replaced with 'transfected'. **A.** Euler diagram for the sets of cells in a simple, therapeutic-vector-only, gene delivery. Horizontal rows show the sets of target and non-target cells. Oblique columns show the sets of non-transduced cells and cells transduced by a therapeutic vector. These four sets of cells intersect to generate four subsets. There is no intersection between the two row sets, because cells cannot simultaneously have 'target' and 'non-target' statuses. Similarly, there is no intersection between the two column sets, because cells are either transduced or untransduced. The curatively important subset, which is marked in black, is the set of target cells transduced by the therapeutic vector. The set of non-target cells transduced by a therapeutic vector is the product of mis-targeting of the therapeutic vector. Each bottom-rank cell set is assigned an individual letter as an algebraic representation of the number of cells in the particular set. Efficiency of gene delivery with therapeutic vector, R , is the ratio of all transduced cells to all treated cells; using chosen parameters: $R = \frac{a+c}{a+b+c+d}$. Efficiency of targeting with therapeutic vector, T , is the ratio of transduced target cells to all transduced cells; using chosen parameters: $T = \frac{a}{a+c}$. Hence, efficiency of targeted gene delivery with therapeutic vector, D , the ratio of transduced target cells to all treated cells, is given by $D = \frac{a}{a+b+c+d} = R * T$. This equation shows that the efficiency of targeted gene delivery with therapeutic vector, D , is directly proportional to the efficiency of gene delivery with therapeutic vector, R , and efficiency of targeting with therapeutic vector, T . **B.** Euler diagram for the sets of cells in a shielding-vector-supported targeted therapeutic gene delivery. Horizontal rows show the sets of target and non-target cells. Oblique columns show the sets of non-transduced cells, cells transduced by a therapeutic vector and cells transduced by a shielding vector. These five sets intersect to generate six subsets. There is no intersection between the two row sets, because 'target' and 'non-target' statuses of cells are mutually exclusive. There are no intersections between the three column sets, as, firstly, cells cannot be transduced and untransduced at the same time and, secondly, once they are transduced by a pre-administered shielding vector, they cannot be transduced by a therapeutic vector. The curatively important subset, which is marked in black, contains target cells transduced by the therapeutic vector. The small sets of non-target cells transduced by the therapeutic vector and target cells transduced by the shielding vector are the products of mis-targeting of the respective vectors. Each bottom-rank cell set is assigned an individual letter as an algebraic

representation of the number of cells in the particular set. Efficiency of gene delivery with shielding vector, H, is the ratio of all shielded cells to all treated cells; using chosen parameters: $H = \frac{g+n}{z+h+k+f+g+n}$. Efficiency of targeting with shielding vector, A, is the ratio of shielded non-target cells to all shielded cells; using chosen parameters: $A = \frac{n}{g+n}$. Hence, efficiency of targeted gene delivery with shielding vector, S, the ratio of shielded non-target cells to all treated cells, is given by $S = \frac{n}{z+h+k+f+g+n} = H * A$. This equation shows that efficiency of targeted gene delivery with shielding vector, S, is directly proportional to the efficiency of gene delivery with shielding vector, H, and the efficiency of targeting with shielding vector, A. Efficiency of targeting with therapeutic vector, T, is the ratio of target cells transduced with the therapeutic vector to all transduced cells; using chosen parameters: $T = \frac{z}{z+h}$. Considering a scenario where cells are shielded without strict non-admittance of therapeutic vector, efficiency of gene delivery with therapeutic vector, R, is the ratio of all cells transduced with therapeutic vector to all treated cells; using chosen parameters: $R = \frac{z+h}{z+h+k+f+g+n}$. Hence, in the scenario without strict non-admittance of therapeutic vector, efficiency of targeted gene delivery with therapeutic vector, D, the ratio of target cells transduced with therapeutic vector to all treated cells, is given by: $D = \frac{z}{z+h+k+f+g+n} = R * T$. In an alternative scenario where cells are shielded with strict non-admittance of therapeutic vector, the pool of cells treated with therapeutic vector can be assumed to be reduced from $z+h+k+f+g+n$ to $z+h+k+f$. Hence, in the strict non-admittance shielding scenario, efficiency of gene delivery with therapeutic vector, R', is the ratio of all cells transduced with therapeutic vector to the reduced pool of treated cells; using chosen parameters: $R' = \frac{z+h}{z+h+k+f}$. Therefore, in the scenario with strict non-admittance, efficiency of targeted gene delivery with therapeutic vector, D', the ratio of target cells transduced with therapeutic vector to the reduced pool of treated cells, is given by: $D' = \frac{z}{z+h+k+f}$ or $D' = R' * T$. It is straightforward to show algebraically, that, $D' = \frac{D}{1-H}$ showing how efficiency of targeted gene delivery with therapeutic vector in the strict non-admittance shielding scenario (D'), compared to the shielding scenario without strict non-admittance (D), increases hyperbolically with increasing H through the coefficient $\frac{1}{1-H} = \frac{z+h+k+f+g+n}{z+h+k+f}$, where H is efficiency of gene delivery with shielding vector.

proposed shielding RNA vectors could be targeted in a cell-specific manner and, therefore, could provide protection both at the body-site level and the cellular level, accordingly enhancing the accuracy of therapeutic targeting at both of these levels. A unified view of gene delivery targeting at both the body-site level and the cellular level can be provided by set theory [30]. The sets of target cells, non-target cells, therapeutically transduced (transfected) cells, therapeutically untransduced (untransfected) cells and all their existing intersections are presented as an Euler diagram in Fig. 1A. All existing intersections of the above four sets and the set of cells, which were transduced (transfected) by a shielding vector, are presented in Fig. 1B. Each set can be a compact group of cells within a body-site, e.g. when adjacent cells form a tissue, or can exist as a composite, widely-distributed population of individual cells intermingling with cells from other sets. The spatial view of shielding compact non-target zones around a compact body-site target is presented in Fig. 2A. The spatial view of shielding a distributed population of non-target cells, intermixed with a distributed population of target cells, is presented in Fig. 2B.

Targeting enhanced through shielding can be regarded as a version of combinatorial targeting [25], where the increased information content of the more accurate overall targeting is derived from the accumulating information content of two or more subordinate independent targeting actions. This information theory perspective underlines that, for the overall shielding-enhanced targeting to be effective, shielding gene delivery must be: 1) targeted to non-target cells to evade target cells; 2) targeted independently from the targeted therapeutic gene delivery. A comparison of the 'split vector' combinatorial targeting system, suggested previously [25], and the 'shielding' combinatorial targeting system, proposed in this paper, is presented in Fig. 3AB. For simplicity of representation, Fig. 3AB shows only the targeting of

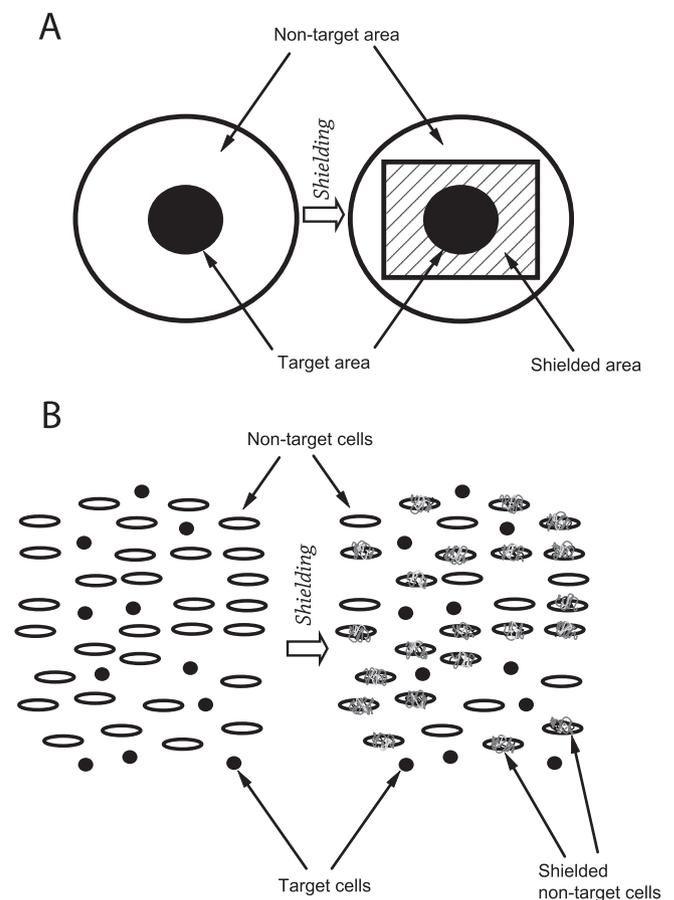


Fig. 2. Shielding of compact and distributed targets. 3D targets are presented as 2D projections. The diagrams demonstrate that, while compact-zone shielding is suitable for a compact target, a distributed target of the widely dispersed cell population requires cell-specific shielding. For both compact and distributed targets, shielding is presumed to be not completely effective and with absent erroneous shielding of target cells. **A.** Shielding of a single compact zone target, composed exclusively of target cells, which is surrounded by the compact non-target zone, composed exclusively of non-target cells. **B.** Shielding of a distributed target, composed of dispersed individual target cells, which are intermixed with dispersed individual non-target cells.

compact body-sites. However, both 'split vector' and 'shielding' targeting strategies are expected to be applicable for the targeting of widely distributed cell populations using techniques for cell-specific vector-cell attachment. Notably, in a 'split vector' system, self-assembly of the vector components within the intersection of the vector components' spreads occurs irrespective of the order in which the component administrations are performed. So, superposition of independent targeting administrations of the essential components of a 'split vector' system can be called 'commutative'. In contrast, for the overall targeting accuracy to be improved in a shielding-based system, the administration of a shielding vector must be performed prior to the administration of a therapeutic gene vector, making superposition of the subordinate targeting actions 'non-commutative'.

Why RNA vectors could be the optimal choice for shielding of non-target cells in vivo?

A key feature of the optimal shielding vectors, providing the desired protection of non-target cells from the therapeutic gene transfer at the level of distributed cell populations or at the body-site level, is the temporal nature of the required shielding. Thus, pre-administration of shielding vectors should result only in transient resistance to therapeutic gene transfer, enabled only within the timeframe of

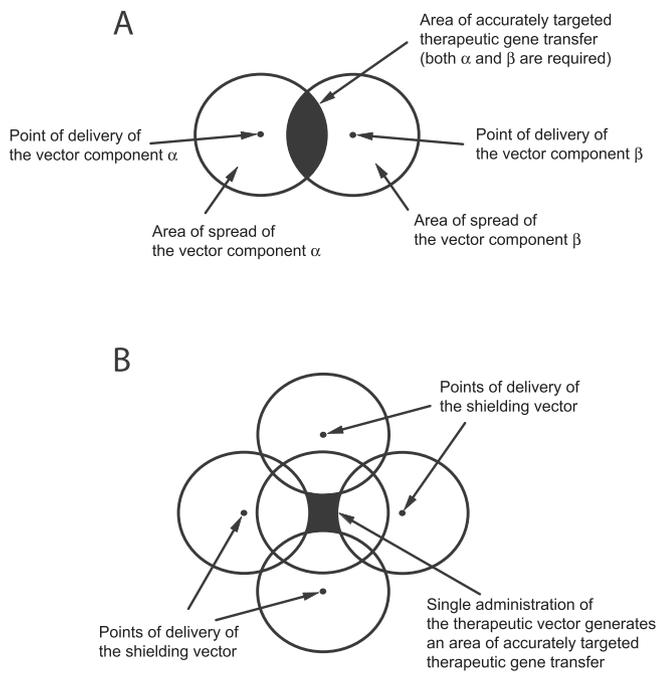


Fig. 3. Comparison of two implementations of the combinatorial principle for the enhanced accuracy of body-site targeting of gene delivery *in vivo*: a ‘split vector’ gene delivery system versus a shielding/therapeutic vector pair. **A.** Accurate spatial targeting of therapeutic gene transfer using a binary ‘split vector’ system. Components α and β of the binary vector system are co-administered in two separate points next to the target zone. The ‘split vector’ system components α and β are assumed to be spreading as expanding spheres; 2D projections of the 3D spread zones are presented. Therapeutic load is contained within one or both components α and β . For the gene transfer to occur, components α and β need to interact with each other via recipient cells; this requirement is expected to result in a tightly-defined zone of gene transfer (marked in black) at the intersection of the spread zones of the individual vector components. Thus, in the presented example of gene delivery with a binary ‘split vector’, the enhanced accuracy of targeting is due to re-creation of the intersection within a bipartite 3D Venn diagram. **B.** Accurate spatial targeting of therapeutic gene transfer using pre-administration of a shielding vector blocking gene transfer into non-target zones. Treatment with a shielding vector is followed by a single administration of a therapeutic vector to the target zone. The shielding vector is applied in four separate vector injection points along the perimeter area of the target zone. Vector particles are assumed to be spreading as expanding spheres; 2D projections of the 3D spread zones are presented. Therapeutic gene transfer occurs exclusively in the tightly-defined zone (marked in black) that is left unprotected after the prior administrations of the shielding vector.

administration of the therapeutic vector. Indeed, after shielding has played its part in the enhanced accuracy of therapeutic gene delivery, there would be no reason to make non-target cells permanently resistant to therapeutic gene delivery, as the continuously expressed shielding transgenes could interfere with the cells’ tissue function or normal epigenetic trajectory. In addition, permanently shielding non-target cells could complicate their possible future genetic treatment, when they could become the next therapeutic target. Finally, the employment of stable genetic material for transfer by shielding vectors would result in the accumulation of unwanted and potentially hazardous ‘genetic litter’ [13]. Therefore, vectors of the ‘hit-and-run’ type, making non-target cells transiently non-receptive to therapeutic gene transfer and then vanishing without a trace of their genetic material, would be the adequate shielding vectors.

Gene vectors containing RNA as their genetic material [31,32] offer precisely such a ‘hit-and-run’ feature of brief transgene expression and eventual complete disappearance of the transgenes. This special feature is sufficient to short-list RNA-based vectors as a choice for the

implementation of the shielding vectors. It holds true for both viral and non-viral RNA vectors and is due to the two core functional differences between RNA and DNA: 1) intrinsic instability of RNA in comparison with DNA [31]; 2) a lack of RNA replication in normal cells. There is one more core functional difference between RNA and DNA: 3) in contrast to DNA, RNA-mediated transgene expression does not normally involve the passage of newly-arrived genetic messages in and out of the cell’s nucleus [31]. The implications of this third core difference for potential shielding employment of RNA vectors are substantial. Indeed, the immediate extra-nuclear expression of the delivered RNA, whether it is protein-coding mRNA or RNA-destroying siRNA, means that RNA-based vectors can offer the following five advantages in their role as the shielding vectors: 1) there are no potentially mutagenic genomic insertions; 2) RNA-coded transgenes are expressed equally well in dividing cells without nuclear envelope and in non-dividing cells with an intact nuclear envelope; 3) RNA-coded transgene expression is insensitive to the major gene silencing mechanisms, which operate in the nucleus, resulting in more uniform distribution of the level of transgene expression among the transduced/transfected recipient cells; 4) RNA expression after delivery into the cell’s cytoplasm has a quick start; 5) since there is no need to disturb nuclear envelope, procedures for RNA transfection could be simpler and/or less stressful to cells than comparative procedures for DNA transfer. These advantages, which make RNA vectors promising shielding vectors, should be considered together with advantages and drawbacks of other two classes of potential cell-level shielding agents: shielding DNA vectors and non-genetic shielding agents, such as cell membrane penetrating proteins equipped with a protein transduction domain or antibodies directed against cellular receptors of therapeutic gene vectors (Table 1). Advantages 1, 2 and 3 of RNA vectors seem to be pivotal, particularly in conjunction with proclivity of all gene vectors for cross-talk with intracellular biomarkers, which could be exploited to discriminate between non-target and target cells among the recipient cells. Let us examine these three advantages in greater detail.

Firstly, it is critical for the genetic material of the shielding vectors not to integrate randomly into the cellular genome. This is because chromosomal integrations can potentially result in insertional mutagenesis or undesired position effects like gene activation in the neighbourhood of a chromosomally integrated transgene, thereby creating a risk of malignant transformation. In general, it would be absurd to administer shielding vectors with a declared aim of minimisation of undesired side-effects of the therapeutic vectors through improved targeting, at the same time making a substantial contribution to the said side-effects. While RNA vectors do not leave undesired long-lasting genomic traces, a number of them might still have some cytotoxicity and/or undesired immunogenicity, including through induction of pro-inflammatory stimuli. A possible complicating circumstance is that large shielding vector doses might be required to provide protection for all chosen non-target cells, e.g. along the perimeter area of a target body-site. Therefore, it is vital that the shielding vectors are constructed using RNA vector backbones that are known to have only negligible cytotoxicity and immunogenicity.

Secondly, a substantial number of cells in human body are non-dividing or dividing only rarely. It is likely that among potentially many types of the non-target cells requiring protection by shielding vectors, at least some non-target cells would be non-dividing. The closed nuclear envelope of postmitotic cells, such as neurons or cardiomyocytes, presents a serious barrier to the genetic cargo of DNA vectors, which need to reach the nucleoplasm to be transcribed and expressed. In contrast, as no nuclear import and export is normally required for the expression of incoming RNA, the genetic cargo of RNA vectors can be expressed both in dividing and non-dividing cells, enabling their equally effective shielding.

Thirdly, the major mechanisms of transgene silencing, e.g. DNA methylation or chromatin remodelling, are entirely intra-nuclear and, thus, are irrelevant for the expression of RNA-coded transgenes in the

Table 1

Enhancing targeting of gene delivery through shielding of non-target cells: Benefit comparison of non-genetic gene transfer blocking agents, RNA-based and DNA-based shielding vectors.

Benefit/harm criterion	Gene transfer blocking agents		
	Non-genetic gene transfer blocking agents	RNA-based shielding gene vectors	DNA-based shielding gene vectors
Can these agents leave permanent genetic trace in shielded cells, thereby creating a risk of harmful insertional mutagenesis?	No	No	Yes
Can these agents block gene transfer to terminally differentiated (non-dividing) cells, thereby offering the shielding benefit to these cells?	Yes	Yes	No Exception: DNA vectors equipped with nuclear entry apparatus can shield non-dividing cells
Are these agents functional irrespective of the major intranuclear silencing mechanisms?	Yes	Yes	No
Can these agents offer the 'quick start' benefit of their gene transfer blocking action?	Yes	Yes	No
Can these agents be applied in comparatively mild administration conditions, without stress to the treated cells?	Yes	Yes	No Exception: Naked DNA delivery to muscle cells <i>in vivo</i>
Can these agents be of strict non-admittance type, thereby offering a smaller chance of undesired side-effects of their own?	Yes Example: Receptor-specific antibodies, which block receptors for therapeutic gene vectors	Yes	Yes
Can these agents discriminate between non-target cells and target cells through direct access to intra-cellular biomarkers of their recipient cells?	Yes, but with fewer possibilities than gene vectors Example: Protein agents equipped with a protein transduction domain can enter recipient cells and have a chance of being activated or repressed through interaction with intra-cellular biomarkers	Yes	Yes

cytoplasm. Typically, differential silencing of DNA-coded transgenes in transfected or transduced cells results in wide heterogeneity in the level of transgene expression [11]. To achieve effective enhancement of the targeting accuracy with shielding vectors, the implemented shielding should not be patchy due to non-uniform distribution of the expression of shielding transgenes within the non-target cell populations requiring protection. Thus, RNA gene vectors could bring greater uniformity of shielding transgene expression through their ability to evade silencing, providing for effective sheltering of non-target cells.

What could be the molecular mechanisms for the shielding RNA vectors to achieve their *raison d'être*, that is, to shield non-target cells from gene transfer with therapeutic vectors? Clearly, the effective genetic cargo for the shielding vectors should be the genetic machinery capable of blocking cell entry of therapeutic gene vectors and/or obstructing therapeutic transgene expression. Ideally, cell entry of therapeutic gene vectors into non-target cells should be prevented altogether, so that after unsuccessful attempts to enter non-target cells, therapeutic gene vector particles would nonetheless retain their 'infectivity', that is, their ability to transduce or transfect the desired target cells. In this way, 'strict non-admittance' gene transfer resistance conferred by the shielding vectors would allow the reduction of the effective therapeutic vector dose, which would expectedly be accompanied by reduced side-effects. However, even if therapeutic gene vectors would be 'admitted' to non-target cells, a block of their further entry, maintenance or transgene expression might still be a valuable shielding tool. Indeed, such a block could eliminate the particular side-effects, which could be due to the expression of the genetic elements of the therapeutic vector backbone or, in fact, the therapeutic transgenes in non-target cells.

The molecular machinery for the 'strict non-admittance' gene transfer resistance can be borrowed from 'strict non-admittance' viral superinfection interference systems. For example, a number of naturally occurring viruses use sialic acid residues on extracellular glycoproteins as cellular receptors [33]. It is common for such viruses to block viral

gene transfer by superinfecting viral particles through knockdown of extracellular sialoglycoproteins by viral enzymes neuraminidase or sialate-*O*-acetyl-esterase. These enzymes arrive into cells as elements of primarily-infecting virions and disrupt modification of glycoproteins by sialic acid residues within the cellular vesicular network, thereby preventing secondary infections by homologous viruses. Thus, shielding vectors, delivering mRNA coding for neuraminidase or sialate-*O*-acetyl-esterase, would be able to knockdown sialic acid receptors from the cell surface of the desired non-target cells, thereby disabling the receptivity of these cells for transduction by a number of prospective therapeutic viral vectors. In another hypothetical model, mimicking the superinfection interference mechanism of ecotropic murine leukemia virus (MLV), mRNA coding for the envelope protein Env of ecotropic MLV could be delivered by the shielding vectors to achieve intracellular sequestration of the extracellular virus receptor CAT-1 [34]. The ensuing knockdown of the receptor-active forms of CAT-1 protein from the cell's surface would result in the impossibility of gene transfer by an appropriate therapeutic gene vector, which otherwise uses CAT-1 as a cellular receptor. Many viruses employ multi-component cell surface receptors, with each component being necessary but not sufficient for efficient viral entry [35]. Thus, the development of an effective pair of a shielding vector and a therapeutic vector might involve the incorporation of sialic acids residues or CAT-1 as necessary components into the receptor component hierarchies of the engineered therapeutic gene vectors, which are not normally dependent on sialic acids or CAT-1 for their cell entry. In this way, therapeutic vectors which are frequently used in clinical trials, such as viral AAV vectors, could be modified to become effective partners in the shielding-assisted therapeutic gene delivery.

In order to block the expression of therapeutic vector functions, shielding RNA vectors could be used to deliver siRNA [36–39] directed against RNA transcribed from the backbones and the transgene modules of DNA-containing therapeutic vectors or against genomic RNA of RNA-containing therapeutic vectors. Thus, maintenance of therapeutic

retroviral and lentiviral vectors could be undercut by a siRNA-led attack on genome vector RNA to prevent its reverse transcription [40] and in that way to block genomic integration. Virosomal vectors based on Sendai virus [41] seem to be an appropriate shielding vector choice to deliver siRNA. Another option to deliver siRNA is the employment of suitably targeted bacteriophage Q β virus-like particles [42].

In general, both viral and non-viral vectors can be used to deliver mRNA. In contrast, the cargo of synthetic siRNA is typically delivered by non-viral vectors. However, the distinction between viral and non-viral vectors is somewhat blurred. So, several types of *bona fide* non-viral vectors, like the above-mentioned virosomes and virus-like particles, might, in fact, contain some significant viral elements. Viral vectors are known to have rigid packaging size constraints for nucleic acids during their encapsidation. These constraints do not present a serious obstacle to the production of viral vectors for delivery of shielding mRNAs, as the mRNAs are expected to be shorter than the genomes of naturally occurring viruses used to build shielding gene vectors. Viral RNA vectors typically benefit from the simplicity of their administration and the efficiency of gene transfer [43]. Modern methods of delivery of non-viral RNA vectors include electrotransfer *in vivo* [19], microbubble-assisted sonoporation [20] and magnetofection *in vivo* [21]. Importantly for their role in targeted shielding of non-target cells, these non-viral gene delivery methods are at the same time methods of 'physical' targeting [44–46].

High efficiency of gene delivery for the shielding vectors is highly desirable, so that only minimal gaps are left in the protected spatial zones and cell populations requiring shielding. To achieve the high shielding efficiency, substantial doses of the shielding vectors can be needed. In turn, this places a high emphasis on the requirement for the shielding vectors to confer only minimal side-effects.

In what gene therapy scenarios would shielding-enhanced targeting of therapeutic gene delivery be particularly useful?

In practical terms, it is simpler to use a single therapeutic gene vector than a combination of a therapeutic gene vector and a shielding vector. Therefore, the situations, in which extra-accurate targeting using pre-administration of a shielding vector is unequivocally worthwhile, should be clearly identified.

One such clear setting is where the side-effects of therapeutic gene delivery are expected to be particularly strong and/or undesirable. For example, the particularly adverse side-effects could be due to either the known high immunogenicity of the employed therapeutic vectors or to the known individual allergies of the patient. Employment of 'strict non-admittance' shielding vectors for better targeting in these circumstances could facilitate the reduction of the therapeutically effective vector dose and, hence, the reduction of the associated side-effects. In addition, homing of the shielding action on particular non-target cells could be enhanced through cell-specific expression of the shielding genes. For example, triggering of the expression of the delivered shielding mRNA by a specific intracellular molecular signature could be achieved through the inclusion of an appropriate tissue-specific 'cap-independent translational enhancer' [32] within the shielding mRNA.

Another set of circumstances, which encourage the use of the shielding vectors, is where targeting opportunities are otherwise limited. This could happen when therapeutic gene vectors of a particular variety, which are being used, e.g., primarily because of their high efficiency of gene delivery, have broad cell tropism. For example, VSV-G pseudotyped lentiviral vectors are capable of infecting virtually all types of cells [47]. Many serotypes of adenoviruses also have a broad cell range due to the ubiquity of their main cell surface receptors and their ability to utilise alternative receptors [48].

Finally, a prospective field for the employment of shielding vectors is their use for genetic treatments, which are deemed to have completely absent mis-targeting of therapeutic gene vectors to non-target cells. Shielding vectors in these circumstances could be thought of as

analogous to decorators' 'masking tape'. However, the 'genetic masking' action to achieve ultra-accurate targeting would have to be performed in 3D and the target/non-target cell sets could be located not only in immediately adjacent body-sites but could also be widely spatially distributed.

Clearly, accurate targeting using shielding RNA vectors should be just one tool in the targeting toolbox of the gene therapist. That tool should not be used where there are no specific indications. There is hardly any need to employ shielding vectors while delivering therapeutic genes using an effectively targeted vector, which generates minimal or no side-effects.

In what settings would it be straightforward to test the hypothesis?

The key verifiable implications of the hypothesis are: 1) reduction of transfer and/or expression of genetic elements of a therapeutic gene vector in non-target cells after pre-administration of a shielding vector, without concomitant reduction in the efficiency of therapeutic gene transfer to target cells; 2) amelioration of the side-effects of gene delivery by the therapeutic vector due to the improved accuracy of targeting created by the shielding vector. The first implication should be tested in an animal model, where it is straightforward to measure efficiencies of gene transfer to target and non-target cells by immunocytochemistry or by cell sorting. For simplicity, it is possible to replace therapeutic genes with a gene for an easily detectable marker, such as bacterial lactate dehydrogenase or a fluorescent protein. Transduction or transfection by the shielding vector could also be confirmed through the employment of an alternative easily detectable marker. The second implication should be tested in animal models and subsequently in clinical trials. An easily detectable side-effect, such as tumorigenicity due to lentiviral vectors [12], should be selected for a proof of principle study in animals. It is important to take into account that, for a pair of a shielding and a therapeutic vector to become successful instruments in targeted therapeutic gene delivery, mutually-dependent adjustments and re-adjustments of both vectors might be required for a better fit.

Future perspectives

In future, the purpose of targeted genetic modification of human somatic cells is likely to expand from the classic gene therapy to therapeutic epigenetic re-programming *in vivo* [32] and somatic human genetic enhancement, the latter being motivated by life-style choices and/or adaptation needs. An example of such enhancement, which seems to be within the reach of modern genetic technology, is frostbite-resistant skin for life in the Arctic, Antarctic and high-altitude environments [49]. The new applications of gene delivery *in vivo* might invoke additional reasons for accurate targeting, such as: 1) extreme minimisation of the genetically or epigenetically modified body zone (e.g. 'genetic retuning' of cochlea to expand the hearing frequency range); 2) fine spatial or cell-specific separation of incompatible genetic or epigenetic modifications (e.g. segregated transdifferentiation of retinal pigment epithelium cells into rod and cone photoreceptors); 3) ultra-fine patterns of genetic modification (e.g. genetic and epigenetic re-arrangement of the human brain).

Within the framework of combinatorial targeting of gene delivery, shielding RNA vectors are to be used in conjunction with vectors loaded with genes for genetic or epigenetic modification. However, in general, shielding RNA vectors can also be used in a stand-alone single vector format as anti-viral drugs for protection against viral infections. Dangerous viruses causing Ebola, Influenza and Dengue fever would be a priority in the development of genetic medicines based on the stand-alone shielding RNA vectors, which could be engineered to block cell penetration, inactivate RNA genomes or disrupt gene expression of these pathogenic viruses.

Declaration of Competing Interest

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