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Design and Production of mRNA-based Gene Vectors for Therapeutic Reprogramming of Cell Fate

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Abstract

Recent advances in therapeutically important cell fate reprogramming fuelled a renaissance in the use of mRNA-based gene vectors. Thus, mRNA vectors were successfully employed to induce lasting epigenetic changes in various target cells making them short-listed vector candidates for the manufacture of therapeutic engraftment materials for autologous transplantation, artificial human tissues for drug discovery via high-throughput screening projects and also for therapeutic cell trans-differentiation directly in the human body. De-differentiation of cells into 'induced pluripotent stem cells', transgene-directed differentiation and trans-differentiation require the simultaneous delivery of a number of regulatory factors, and, favourably, potent reprogramming vector cocktails can be straightforwardly assembled from a selection of mRNA species. In addition, several proteins can be conveniently expressed from a single mRNA using internal ribosome entry sites (IRESes) or, alternatively, fusion proteins supplemented with 'polypeptide-cleaving' ribosome skipping sequences. This review is focused on the design and production of cell-fate changing mRNAs.

Keywords: Epigenetic reprogramming; Forward programming; mRNA gene vectors; Induced pluripotent stem cells; Transdifferentiation; Regenerative medicine; Drug discovery

Background

One of the pivotal discoveries in molecular medicine was made by Takahashi and Yamanaka in 2006, who showed that stable epigenetic reprogramming of mammalian cells could be achieved using the delivery of just a few transgenes coding for regulatory factors [1]. Initially, the new approach was used only for de-differentiation to generate artificial stem cells, so-called 'induced pluripotent stem cells' (iPS cells), from differentiated cells. However, later it became apparent that gene transfer can also be used to direct, to 'coax', stem cells, including iPS cells, into differentiation in vitro [2,3]. Such 'gene-transfer-forced' differentiation was called 'forward programming'. The employment of gene transfer to achieve cell differentiation considerably expanded the possibilities offered by traditional in vitro differentiation protocols relying on protein and small molecule factors in the cell culture medium. Finally, it was discovered that on some occasions gene transfer can be used for the direct reprogramming of differentiated cells in order for them to become differentiated cells of another type [4]. Epigenetic changes of this type are classified as 'transdifferentiation'. The above modes of epigenetic transformation, which can be achieved through gene transfer, are therapeutically important because of the possibilities they open for regenerative medicine and high-throughput drug screening projects with artificial human tissues. For example, a common scenario in regenerative medicine involves the imbedding of differentiated iPS cells, forward-programmed stem cells or directly trans-differentiated cells, which were originally obtained from individual patients, into a suitable scaffold for subsequent autologous therapeutic transplantation back into individual cell donors.

A variety of gene vectors can be used to deliver transgenes to cause epigenetic shifts in recipient cells. Recently, gene vectors based on synthetic messenger RNA (mRNA) have gained prominence as an effective and safe tool for epigenetic reprogramming of cells. Vectors based on mRNA were well-tested, as over the last 20 years substantial experience has been acquired in the use of these vectors for the delivery of foreign genes into a variety of mammalian cells [5,6]. Crucially, in contrast to most other types of non-viral gene vehicles, mRNA vectors do not need to pass through the barrier of the bi-membrane nuclear envelope to enter nucleoplasm for successful transgene expression. This property has multiple beneficial implications for induced epigenetic switches. Indeed, standard mRNA vectors: 1) achieve rapid transgene expression; 2) are effective for gene transfer into slowly dividing and non-dividing cells; 3) evade silencing at a transcriptional level, which is an impediment typically associated with DNA vectors; 4) are incapable of either integrating into chromosomes or long-term episomal maintenance and, thus, do not leave a permanent genetic trace after epigenetic modification of target cells; 5) offer improved transfection efficiency in comparison to DNA-mediated non-viral gene delivery as the latter relies either on the short window of the nucleoplasm's accessibility during mitosis or on as yet inefficient artificial molecular prop-ups for the passage through the nuclear envelope. However, the efficient and reliable manipulation of cell fate still faces many practical challenges, many of which can be addressed by bespoke and optimized gene vectors. Thus, the employment of mRNA-based gene vectors in the future medical applications of epigenetic engineering requires refinement of their design and production, which we review below.

Design and production of vector mRNA

mRNA directs the biosynthesis of specific proteins in cells by translation. Thus, clearly, mRNA is the core component of any mRNA-based gene vectors. For eukaryotic translation to start, the

formation of a translation complex is required, which, in addition to mRNA, consists of some protein initiation factors, the initiator tRNA and the 40S ribosomal subunit. A molecular structure called a 'cap' at the upstream mRNA end is important for the translation of many functional eukaryotic mRNAs with some exceptions, which we discuss below. In general, a standard cap has the structure m7G(5')ppp(5')N, which includes an unusual 5'-5' tri-phosphate bridge between the terminal m7-guanosine and the rest of the mRNA. The well-established functions of the cap are the protection of mRNA from RNAses, export of mRNA from the nucleus and translation initiation. 'Natural' formation of the cap in the nucleus involves the activities of RNA terminal phosphatase, RNA guanylyl transferase and RNA guanine methylase, with the latter enzyme being responsible for the methylation of the nitrogen atom in position 7 of the guanine base [7]. Another common structural feature of mRNAs is a homopolymeric sequence of A nucleotides known as 3'-poly(A) tail. Similarly to the cap, the functions of the poly(A) tail include stimulation of translation, protection of mRNA from degradation and export of mRNA from the nucleus. 'Natural' poly(A) tails are formed through a concerted action of a number of enzymatic activities, with nucleotides being added through polyadenylate polymerase (PAP) activity [8].

While both the cap and the poly(A) tail play a role in the export of mRNA out of the nucleus, natural eukaryotic mRNA might contain some additional signals for its export out of the nucleus (e.g., 'post-transcriptional response elements', PRE). In contrast to the cap and the poly(A) tail, which are normally essential for mRNA stability and initiation of translation, the presence of such additional nuclear export signals is not required within artificial mRNA vectors, as they are designed to be exclusively cytoplasmic. Similarly, the delivery of artificial mRNA directly into the cytoplasm necessitates its synthesis in a spliced form with possible omission of redundant splicing signals in artificial mRNA vectors.

Segments of mRNA encoding an amino acid sequence are called 'cistrons'. In eukaryotic translation of nuclear-sourced mRNAs, AUG is the start codon and UAG (amber), UGA (opal) and UAA (ochre) are the stop codons. In active eukaryotic mRNAs, the start codon AUG is imbedded within the 'Kozak consensus sequence'. The Kozak sequence is one of the key translation initiation signals in eukaryots and, as such, is an important element of artificial mRNA vectors. One of the efficient versions of the Kozak sequence is 5'-GCCGCCAUGG-3' where AUG is the start codon.

Synthesis of vector mRNA

The synthesis of mRNA using methods of traditional polymer chemistry is time-consuming and inefficient. Therefore, DNA-template directed polymerase-led transcription processes are normally used for the generation of artificial mRNA gene vectors.

Eukaryotic cells harbour all the elements of transcription machinery and, as an example, yeast cells can certainly be used as mRNA vector factories. However, the drawbacks of such an intact-cell-based approach for the generation of mRNA are a relatively low mRNA yield and a need to purify specific mRNAs from a multitude of contaminants including biochemically-similar undesired mRNAs. In contrast, cell-free in vitro transcription systems can provide an ultra-high yield mRNA production and also drastically reduce the number of contaminating molecules. Although mRNA that is active in human cells is required, the standard choice for in vitro synthesis of pre-designed mRNA is a prokaryote-based system, as transcription machinery in eukaryotes is more complex and, therefore, more difficult to adapt to in vitro use. Particularly attractive are bacteriophage-derived transcription systems, which offer superior productivity, the simplicity of DNA-template elements required for the bacteriophage-encoded transcription machinery and the ready availability of RNA polymerases. Thus, a typical strategy is employing prokaryotic enzymes like RNA polymerases of bacteriophages T7, T3 or SP6 for in vitro synthesis of the major portion of mRNA, followed by the introduction of appropriate add-ons and modifications, which make the resultant mRNA suitable for efficient expression in eukaryotic cells. Common commercially available systems include mMESSAGE mMACHINE® kit and a more sophisticated mMESSAGE mMACHINE® T7 Ultra kit (Invitrogen Life Technologies-Ambion), both of which exploit bacteriophage T7 RNA polymerase. Linear DNA templates, such as plasmid fragments or PCR amplicons are preferred because the high processivity of bacteriophage RNA polymerases could result in undesired extra-long multimeric transcripts produced by repeated cycling of RNA polymerases along circular templates [9].

Normally, prokaryotic mRNAs are not capped or polyadenylated. Therefore, the addition of both the cap and poly(A) tail to synthetic mRNA is required for its successful translation in eukaryotic cells. To cap RNA in vitro, Invitrogen Life Technologies-Ambion recommends supplementation of the transcription with an analogue of 7-methyl guanosine (5') triphospho-(5') guanosine. The name of the latter compound is abbreviated as m7G(5')ppp(5')G and its analogue is known as 'anti-reverse cap analogue' (ARCA). Indeed, m7G(5')ppp(5')G can be used by RNA polymerase as a building block for synthesized mRNA. As desired, due to space constraints, m7G(5')ppp(5')G can only be incorporated into the first position of the nascent transcript. However, bacteriophage-T7-based in vitro transcription system installs m7G(5')ppp(5')G in two alternative orientations (incorporating either G or m7G into the nucleotide chain), with only one of the orientations (with the chain-incorporated G) corresponding to the natural translation-competent cap. Thus, chemical modification of m7G(5')ppp(5')G is used to obtain ARCA that is suitable for directional insertion. The desired translation-compatible modification can be achieved, for example, by replacement of one of the hydroxyl groups in the m7G moiety of m7G(5')ppp(5')G with a methoxyl group to block the RNA chain growth via this group. So, the employment of ARCA ensures a single and correct orientation of the cap in the synthesized RNA molecules, thus increasing the activity of the obtained mRNA in translation.

One of the structural features of the eukaryotic mRNA is a 3'-poly(A) tail of at least 30 nucleotides. Supplementation of synthetic mRNA with poly(A) is often important for the stability of mRNA and for the efficiency of mRNA translation in target cells.

One possible strategy for the addition of the poly(A) tail is to mimic the natural process of poly(A) synthesis in mRNA. In eukaryotes, the formation of the natural poly(A) tail in vivo requires a polyadenylation start signal sequence within template DNA. The mechanics of polyadenylation initiation is currently not entirely understood. The sequences 5'-AAUAAA-3' or 5'-AUUAAA-3' are often found in polyadenylation signals [10]. They are located 10-30 nucleotides upstream of the poly(A) attachment site, are highly conserved but not strictly required for the polyadenylation. A role in polyadenylation initiation might also belong to the secondary structures within RNA [11]. In general, eukaryotic polyadenylation is somewhat complicated for reconstruction in vitro, with polyadenylate polymerase providing just one of the required enzymatic activities in the polyadenylation complex. Therefore, prokaryotic E.coli-derived template-independent

poly(A) Polymerase (EcPAP) is often a preferred tool to furnish artificial mRNA with a poly(A) in a simple in vitro procedure [12]. The alternative practical strategy for attachment of the 3'-poly(A) tail to the synthetic mRNA is to generate the tail together with the rest of the RNA during transcription via inclusion of the poly(A) sequence within the DNA template [13].

In many practical situations, a weak point of synthetic mRNA gene vectors is their instability. Encouragingly, it was shown that the instability can be substantially reduced through the incorporation of specific sequences from the 3' untranslated terminal region (3'-UTR) of Venezuelan Equine Encephalitis Virus (VEEV) into artificial mRNAs [14].

Purification of mRNA after its synthesis

After in vitro transcription, attachment of the synthetic cap and polyadenylation, synthetic mRNA need to be purified. The standard steps are digestion of the DNA template with DNAse I, RNA precipitation with LiCl and washing with 70% ethanol. After digestion it is important to inactivate DNAseI, for example, by addition of a chelating stop solution and heating. The step of RNA precipitation with LiCl is particularly useful because the precipitation is selective; that is, NTPs, DNA and substantial fraction of proteins are not precipitated [15]. However, epigenetic engineer should be aware that mRNAs with a highly compact 3D structure might be poorly precipitated by LiCl. In this case, the employment of a chaotropic agent such as formaldehyde might be required to destroy secondary structures of the heavily folded mRNA and, thus, to improve its precipitation.

Alternative methods, which might be useful for high-throughput or large-scale purification and concentration of synthetic mRNA, include gel filtration, ultrafiltration, binding to silica-gel membranes, treatment with StrataCleanTM resin and dialysis. StrataCleanTM resin (Agilent Technology) is a silica-based resin with negatively charged hydroxyl groups capable of binding macromolecules such as positively charged proteins. Production of ultra-pure mRNA might also rely on poly(A)-targeted oligo(dT) affinity columns and cap-targeted affinity columns with immobilised cap-binding eukaryotic initiation factor 4E (eIF4E). Column affinity-based purification systems can be substituted for affinity-based paramagnetic beads, a robot-friendly format. Dialysis can be particularly important for the removal of small ionic impurities from the mRNA preparations prior to gene transfer by electroporation because ionic impurities can be responsible for undesired electric current, which increases the temperature of the electroporated cells and, hence, decreases their viability. Small volume dialysis can be conveniently performed in Slide-A-LyzerR MINI Dialysis units (Thermo Scientific).

The RNA concentration can be established and the size homogeneity of mRNA can be rapidly confirmed with small sample consumption using microfluidic chips on BioanalyzerTM system (Agilent Technology). An alternative way to quantify mRNA is to use RNA-specific fluorescent ligands, e.g., in conjunction with purpose-designed QubitTM fluorometer (Invitrogen Life Technologies).

For the isolation of small amounts of mRNA, e.g., from cells and tissues, 'carrier RNA' can be employed to improve the binding of mRNA to column matrices, efficient precipitation and/or for protection from RNAses. A reasonable choice for the carrier RNA is poly(I), a polymer of inosine. This nuclear acid is genetically inert because, although III triplets can be interpreted by the translation machinery as a glycine codon GGG, poly(I) lacks the Kozak translation start signal. Alternative translationally-inert carriers are glycogen, the cocktail of yeast tRNAs and the cocktail of E.coli ribosomal RNAs [16]. Commercial suppliers, which produce mRNA vectors and cocktails of mRNA, include Stemgent, System Biosciences (SBI) and Stemedica Cell Technologies.

In contrast to DNA vectors, many of which contain plasmid DNA generated in bacteria, mRNA vectors, which are produced using in vitro transcription systems or eukaryotic cells, have a reduced chance of contamination with immunogenic and toxic bacterial lipopolysaccharides (LPS). Clearly, this is particularly advantageous in those therapeutic applications of mRNA-based vectors, which involve intra-body gene delivery.

Use of non-canonical mRNAs as gene vectors

Some eukaryotic mRNAs can get involved in translation not only through the cap structure but also through internal ribosome entry sites (IRESes) in their 5'-untranslated terminal regions (5'-UTRs). Some researchers consider such 5'-UTR IRESes as sufficiently different from the bona fide inter-cistron IRESes to warrant them a special name, cap-independent translational enhancers (CITEs) [17]. Some CITEs appear to be reasonably efficient, attracting up to three translation initiation complexes on a single mRNA [18]. It is difficult to define a specific sequence motif for CITEs and they are presumed to act through secondary structures. Research on 5'-UTR sequences also shows that on some occasions secondary structures associated with 5'-UTR IRESes can impede 'ribosome scanning' through RNA and so can interfere with cap-dependent translation [19]. Clearly, the interplay between cap-dependent and cap-independent initiation of translation can be a control point to regulate gene expression, e.g., for tissue-specific expression [20] or a response to environmental stimuli [13].

As vector mRNA is often produced in vitro, it is straightforward to engineer a CITE element in its 5'-UTR to avoid the in vitro capping step. Alternatively, both the cap and a 5'-UTR IRES can be employed, e.g., emulating genomic transcripts of the wild type HIV-1 virus [21]. Tissue-specificity of transgene expression is often an important therapeutic factor in genetic medicine, so CITEs can potentially be exploited in the design of tissue-specific mRNA vectors.

Histone mRNAs are notorious for lacking poly(A) tails and instead containing secondary structures at their 3'-ends. Clearly, these or similar structures can be used to substitute for a poly(A) tail in mRNA vectors. It is possible that histone-style 3'-ends of mRNA can provide superior protection from RNases and, thus, enhance mRNA stability and ensure more efficient protein synthesis.

Multigene delivery using mRNA vectors

Typically, epigenetic reprogramming requires the simultaneous expression of several transgenes. Another common scenario for the simultaneous delivery and expression of several messages is the combined transfer of reprogramming genes and a marker gene. In general, the strategies to unite the delivery of several messages include assembly of mRNA cocktails, engineering of polycistronic mRNA vectors and construction of mRNAs coding for fusion proteins.

Fortuitously, mRNA vectors offer the benefits of straightforward combination of several messages in a single multi-gene cocktail. For example, spiking of mRNA for ecotropic retroviral receptor mCAT-1 with mRNA for a marker protein GFP was successfully used to

monitor the efficiency of mCAT-1 gene transfer [22]. The same co-delivery strategy can be applied to the transfer of cocktails of reprogramming mRNAs, which can be supplemented with an 'internal control' marker gene mRNA. Bouquets of reprogramming mRNAs can contain multiple synthetic mRNAs with a broad range of concentrations and also can be supplemented with mRNA-extracts from specific mammalian cell populations.

Engineering of polycistronic mRNA is often a convenient strategy to combine several reasonably short messages. Translation of a downstream cistron in eukaryots can occur only after re-initiation on IRES sequences positioned between the cistrons. In artificial constructs, an IRES element is typically embedded within a sequence of about 500 bases. The translation initiation efficiency of the IRES elements can be controlled through modification of the size of the inter-cistronic sequence [23]. Multiple cistrons were previously successfully assembled within a single efficient IRES-joined transcription module [24]. As in the mRNA-cocktail-based gene co-delivery strategy, one of the common scenarios in polycistronic gene co-delivery is a combination of a 'payload gene' and an easily detected marker as an 'internal control' for convenient gauging of the level of the 'payload gene' expression. However, the epigenetic engineer should be aware that in IRES-based gene co-delivery, the expression level of the upstream transgene is not necessarily equal to the expression level of the downstream transgene.

Another feasible approach to combine several messages for concurrent expression is to design and to generate a single mRNA for a fusion protein, e.g., the 'payload protein' fused with a protein possessing an easily detectable activity. Fusion proteins can be split into individual polypeptide chains using viral 'ribosome skipping elements' like 2A peptide sequence [25].

Gene co-delivery with synthetic mRNA vectors compares favourably to co-delivery using competing 'hit-and-run' transfer systems which leave no ultimate genetic residue in recipient cells, namely, cell transduction with virally packaged RNAs and direct transfer of proteins to cells relying on 'protein transduction domains' (PTDs). Indeed, all viral vectors have nucleic acid size packaging constraints preventing encapsidation of extra-long polycistronic RNAs. Furthermore, the direct transfer of several proteins into cells necessitates equipping each of the proteins with an individual PTD, a laborious and time-consuming procedure.

The choice of the marker gene

Marker genes are required to estimate the efficiency of gene delivery and expression. The expression of marker genes can also be a useful tracer of the delivery area after in vivo gene transfer. The transiency of mRNA-mediated transgene expression means that the detection time frame is fairly short. Therefore, markers with an extended period of evaluation, such as cellular drug-resistance markers, are often not suitable for measuring the efficiency of mRNA-based gene delivery. In contrast, genes for enzymes and fluorescent proteins are particularly convenient.

Luciferases are enzymes, which catalyse the conversion of a substrate (luciferin) into a product (oxyluciferin) with concomitant emission of light. The commonly used luciferases originate from the North American firefly Photinus pyralis and sea pansy Renilla reniformis. Luciferase activity can be detected using specialized luminometers either in a solution or within a tissue. Other enzymes, for example, chloramphenicol acetyl transferase (CAT) and E.coli β-galactosidase, can also be employed to read-out the efficiency of mRNA delivery. Secreted enzymes, such as secreted luciferase, allow time-course measurements of the transgene expression in the same population of live cells.

Currently used fluorescent proteins are mostly derived from the green fluorescent protein (GFP) of the jellyfish Aequoria victoria and dsRed protein of coral Discosoma sp. Fluorescence can be read-out by both microscopy and fluorescence activated cell sorting (FACS). Intracellular targeting of fluorescent proteins is often used to increase the specificity of microscopic detection and quantitative examination by automated high-throughput microscopy and image analysis (e.g., using the software of Cellomics robot or Definiens software package). The available targeting motifs include nuclear, peroxisomal, mitochondrial and plasma membrane localisation sequences. Genes for extra-cellular domains of cell surface proteins can be used for detection by fluorescently labelled antibodies. Binding of macromolecules in vivo can be detected through microscopy, relying on the fluorophore-proximity-dependent fluorescence wavelength shift called 'fluorescence (also Förster) resonance energy transfer' (FRET).

Most of the marker proteins are from non-mammalian organisms where the translation machinery is adapted to the respective non-mammalian profiles of codon frequencies. In human cells, unusual codons, which are also called 'hungry codons', can slow down translation and thereby reduce the efficiency of gene expression [26]. Thus, the optimization of codon frequencies for the genes, which are born on mRNA vectors, is thought to be advantageous. If the frequencies in a coding sequence are adjusted to match the human codon usage profile, the sequence is said to be 'humanized'. For the reliable expression of DNA-delivered transgenes, optimization of the coding sequence also involves the abrogation of consensus-predicted sites for undesired binding of transcription factors exploiting the degeneracy of the genetic code. However, in contrast to DNA-delivered transgenes, this modification of the coding nucleotide sequence is not required for mRNA produced in vitro using a DNA template and purified RNA-polymerase, as no transcription factor binding sites are known within mRNA. Consequently, as the removal of transcription binding sites can compromise the optimal codon choice, optimization of transgene expression is more straightforward with mRNA vectors than with DNA vectors.

In tissue culture and animal experiments, fluorescent substances, such as fluorescein, can be included into the vector complexes instead of marker genes in order to visualise the area of distribution of vector particles and also to visualize the intracellular trajectories of the vector particles. In addition, fluorescent dyes can be targeted to transfected cells through the fusion of the transgene products to the HaloTag® peptide, which can bind fluorescent labels attached to the HaloLinkTM adapter (Promega). Similarly, colloidal carbon (e.g., the same as used as ink in tattoo parlours), can be added to the administered vector preparations to mark the site of the vector administration in vivo.

Particular features of the design of cell-fate reprogramming mRNA vectors

Cell-fate reprogramming is used in regenerative medicine and production of testing human tissue for high-throughput drug screening. The requirements of these applications should be kept in

mind while designing mRNA vector molecules and mRNA-based vector complexes.

Cell-fate reprogramming through gene transfer for the needs of regenerative medicine

It is widely appreciated that clinical regenerative medicine is plagued by the scarcity of human donor tissue. Indeed, research in stem cell technologies is driven, to a large extent, by the need for tissue and cell material for transplantation.

Germ line cells are totipotent, that is, they have an epigenetic potential to differentiate into all existing types of cells. To achieve the goals of regenerative medicine, it is often sufficient to be in possession of cells, which are pluripotent, that is, have an epigenetic potential to differentiate into a number of specific types of cells. For example, cells extracted from early stage embryos and propagated in vitro, embryonic stem (ES) cells, are pluripotent. Various other strategies to procure pluripotent cells are currently available. The choice of a particular type of pluripotent cells is dictated by many parameters, including ethical considerations, the number of initially available cells, the number of cells required for the transplantation material, their level of pluripotency, non-carcinogenicity and the availability of an appropriate differentiation protocol. Regrettably, the use of highly pluripotent ES cells, which are capable of highly reproducible differentiation into a range of specific cell types, is mired with ethical concerns related to human embryo destruction. In addition, ES cells have a dangerous proclivity to produce teratomas after grafting in vivo. Thus, the development of stringent methods for removal of potentially tumorigenic undifferentiated cells in the human transplant material (e.g., via engineering of inducible suicidal cell clean-up systems) can be an appropriate strategy to generate safe transplant material [27]. Favourably, pluripotent cells that are resident in adult individuals, so-called 'adult progenitor cells', are not prone to tumorigenesis. However, generation of transplantation material from progenitor cells could be complicated by the scarcity of the patient-derived starting cell material and by the absence of a reliable differentiation protocol.

An important breakthrough in regenerative medicine has occurred when it was shown that pluripotent cells can be produced artificially by de-differentiation of adult differentiated cells in vitro [1]. De-differentiation was achieved through the introduction of several developmental genes into the cells and the resultant pluripotent cells were named 'induced pluripotent stem cells' (iPS cells). At first, the iPS cells were generated from fibroblasts [28,30]. Later, the techniques were perfected to produce iPS cells from other cell types, e.g., T-lymphocytes [31,32]. Issues affecting the successful generation of iPS cells include mutations and DNA rearrangements in terminally differentiated cells, both in the source cells, particularly in old adult cell donors, and the cells growing in vitro. It was shown that the generation of de novo mutations and chromosomal abnormalities can be alleviated through incubation of the cells in hypoxic conditions, where the burden of the mutagenic reactive oxygen species is reduced.

Reprogramming factors to produce iPS cells in the original combination of K. Takahashi and S. Yamanaka were Oct3/4 (also called Oct4), Sox2, Klf4, c-Myc, which were introduced with retroviral vectors [1]. Later, iPS cells were successfully generated with the same combination of transgenes using mRNA vectors [33]. Other formulas of reprogramming factors were used to produce iPS cells, e.g., a combination of Oct4, Sox2, LIN28 and Nanog [34,35]. The motivation for the search of alternative de-differentiating combinations of factors is driven by: 1) the desirability for increased efficiency of iPS cell generation; 2) the unsafe potential of c-Myc to induce malignant transformation [36]. Encouragingly, c-Myc-free combinations of factors did not have a reduced reprogramming efficiency [37]. One more practical option is the employment of a transformation-deficient reprogramming-competent mutant version of c-Myc [38]. Various source cells for the generation of iPS cells are expected to require different optimal combinations of reprogramming developmental factors [39,40].

After pluripotent cells are obtained in vitro or pinpointed in vivo, the next issue on the therapeutic agenda is their differentiation into specialized cells to confer the desired function to the human body. Traditional differentiation protocols often depend on a combination of various factors in the cell growth medium and a defined schedule of their administration. A more recent approach for 'coaxing' of pluripotent cells, such as iPS cells, into specific differentiation pathways is their 'forward programming' using gene delivery [41], e.g., with mRNA vectors.

Clearly, the generation of transplantation material through de-differentiation of specialized cells into iPS cells with their subsequent differentiation into desired specialized cells is an elaborate and costly strategy. Direct trans-differentiation of one type of specialized cells into another type could be an attractive shortcut in the production of cell grafts. In an important breakthrough, gene transfer-based direct trans-differentiation, also called 'direct reprogramming', was used to change postnatal cardiac or dermal fibroblasts into cardiomyocytes without recourse to stem cells, using reprogramming factors GATA4, Mef2c and Tbx5 [4]. So far, it was accomplished in several cell systems in vitro and only recently in vivo [42]. Another group of researchers achieved effectively similar trans-differentiation of embryonic fibroblasts into cardiomyocytes using a protocol for epigenetic reprogramming relying on the extreme shortening of the iPS-cell-stage, the latter being attained through expression of the standard combination of Oct4, Sox2, Klf4 and c-Myc transgenes [43]. Developments in protocols for direct reprogramming of patients' cells into cardiomyocytes have potentially high clinical significance [44,45]. Direct reprogramming was successfully accomplished in other types of cells, with neurons and neuron-related cells obtained through trans-differentiation of Sertoli cells [46], melanocytes [47] and fibroblasts [48,49].

Once transgene combination required for trans-differentiation is established ex vivo, the same gene cocktail can be delivered in vivo for trans-differentiation in situ. Direct reprogramming through targeted gene delivery in vivo is extremely attractive as therapeutic benefits of regeneration can be achieved without the potential complications associated with transplantation procedures. So, instead of using an external graft, this healing strategy relies on the generation of the replacement cells by in situ trans-differentiation of the patient's own cells. For example, the attained direct reprogramming of cardiofibroblasts into cardiomyocytes in the heart [42] could be an alternative to cardiac cell therapy, where the cell retention rate is often low due to poor cell homing [50]. Thus, therapeutic epigenetic reprogramming in vivo could become a safer and cheaper therapeutic option than traditional transplantation technology. Indeed, in eye conditions due to the degeneration of the neuroretina, it would be very attractive to trans-differentiate retinal pigment epithelium (RPE) cells into neuroretina cells after intraocular administration of a reprogramming gene cocktail (of course, with the condition that RPE

cells have not degenerated themselves); this procedure was successfully accomplished in chicks [51]. Direct reprogramming through gene delivery in vivo is also incomparably faster than the route with de-differentiation and subsequent differentiation, as this latter route unavoidably involves time-consuming cell expansion in vitro. In the clinical setting, time is of the essence; for example, failure of pancreas often requires urgent therapeutic intervention. Encouragingly, it was possible to generate pancreatic cells through gene-transfer-mediated trans-differentiation in vivo [52]. In conclusion, it appears that gene-transfer in vivo, such as mRNA-mediated gene transfer in vivo, can direct therapeutic trans-differentiation directly in the human body, combining the advantages of autologous transplantation and in vivo gene therapy.

Cell-fate reprogramming through gene transfer for the needs of drug screening

Regenerative medicine is not the only medical field where donor tissue is required. Another very important application for human stem-cell derived material is high-throughput robotic screening of chemical compound libraries to discover new drugs [53]. For example, iPS cells can be used to generate fragments of human heart and these fragments can be employed to screen extensive libraries of various chemicals to find the best substances with the desired pharmacological action [54]. Clearly, millions of heart disease patients could benefit from the results of such screening.

Although cells of non-human origin, human-animal hybrid cells and human cancer cells can be used for drug screening, undoubtedly, the results obtained with non-cancerous human tissue offer more reliable guidance on the potential value of a specific drug in human medicine. In general, high quality human tissue material for drug screening can be obtained through gene-transfer-led differentiation of hES cells, donated human adult stem cells and in-vitro-generated iPS cells. Advancing technologies might enable bespoke high-throughput screening projects and the generation of personalised drugs using iPS-cells-derived differentiated material or trans-differentiated material from individual patients.

Where do mRNA-based vectors stay among other gene vectors for cell-fate reprogramming?

Various types of gene vectors were used to deliver reprogramming gene cocktails for iPS cell production (de-differentiation), differentiation coaxing (forward programming) and trans-differentiation. The majority of gene vector types leave a permanent genetic trace in target cells because of the irreversible chromosomal integration of their genetic material. Integration can be mediated by specialized enzymatic machinery, e.g., in retroviral [55], lentiviral vectors [56] and eukaryotic transposons [57]. After gene delivery with some other vector types, such as plasmid vectors [58] or minicircle vectors [59,60], chromosomal integration occurs through spontaneous target-cell-mediated recombination. As remodelling of cell fate requires only transient expression of the reprogramming factors and, in fact, the continuously expressed transgenes are likely to interfere with the desired epigenetic trajectory of the cells, permanent presence of the transgenes within chromosomal DNA necessitates assembly and employment of complicated inducible systems of transgene expression. Another, even more serious, problem of chromosomal integration events is their propensity to generate adverse mutations, e.g., mutations leading to the malignant transformation.

Gene vectors providing the exclusive extra-chromosomal existence of transgenes, so-called episomal vectors [61], might offer a solution to the insertional mutagenesis problem. However, as expression of developmental factors would, ideally, terminate in an eventual shutdown, these vectors would still require genetic machinery for the controlled end of transgene expression. In general, the ideal vectors for genes to induce epigenetic switches should not just be episomal but should possess a 'hit-and-run' property, leaving no genetic residue after successful reprogramming. A permanent genetic trace can be avoided with direct delivery of developmental factors in the form of cell-permeable proteins to the cytoplasm of the target cells [62-64]. However, engineering cell-permeable versions of all the desired developmental proteins could be cumbersome. Delivering the genetic cargo directly to the cytoplasm can also be achieved with viral RNA-carrying vectors, such as negative-strand-RNA-containing Sendai virus vectors with ablated or temperature sensitive replication [65]. Notably, synthetic cytoplasmic gene delivery vectors, such as mRNA vectors, appear to be the flexible tools required for 'hit-and-run' epigenetic reprogramming [66]. The perceived safety of synthetic mRNA vectors in comparison to viral vectors is undoubtedly an important feature, which can make mRNA vectors a favoured option in the choice of the vector type for the clinical applications of therapeutic epigenetic reprogramming. In addition, numerous genes can be efficiently delivered simultaneously to target cells for co-transfection with cocktails of synthetic mRNA vectors, while it might be challenging to perform co-transduction with multiple viral vectors in one go [67]. There are two general explanations, which can be offered for the low efficiency of co-transduction with viral vectors. Firstly, since individual viral vector particles penetrate the membrane barrier of the target cells in an individual manner, their co-transduction might follow the higher order reaction kinetics. Secondly, co-transduction with some individual viral vectors could be distinctly uncooperative because of the presence of infection interference mechanisms in their cognate viruses and the incomplete inactivation of these mechanisms in the descendant viral vectors. Synthetic mRNA vectors have been used to establish a solid track record of efficient gene delivery and, importantly, have been successfully employed for cell fate reprogramming [66,68]. The upside of the mRNA vectors is also the fact that their nucleic acid sequences are easily amenable for chemical modification. Thus, mRNA vectors with some modified nucleobases, which are known to reduce innate immune responses, were shown to be effective tools of epigenetic reprogramming [69].

No doubt, delivery of developmental factors with mRNA vectors is only one element in successful epigenetic switches. Cocktails of small molecular weight chemicals and the addition of extracellular protein factors can certainly be used to augment the transformation of the epigenetic state through the action of mRNA vectors. In particular, DNA de-methylation agents 5-azacytidine and zebularine were reported to be capable of trans-differentiation enhancement [70], while histone deacetylase inhibitor valproic acid was used to potentiate de-differentiation [71]. In addition, cell fate reprogramming was shown to be augmented by miRNAs [72]. Clearly, miRNAs can be delivered in conjunction with mRNA. It is expected that robotic systems for combinatorial screening can be used to search for suitable reprogramming mixtures of mRNA vectors, protein factors, miRNAs and small molecular weight molecules [72].

Future perspectives

Undoubtedly, mRNA vectors should be a short-listed vector choice for the generation of iPS cells and other epigenetically modified cells in regenerative medicine and high-throughput drug screening with artificial human tissues. Control of mRNA abundance during a short time-frame immediately after the delivery of mRNA vectors to human cells is a key feature, which makes them attractive to use for cell fate reprogramming. In addition, clinical applications of mRNA-based gene transfer, in particular generation of engraftment materials, draw vital safety benefits from the non-mutagenic, 'hit-and-run' and cytosol-targeted, gene delivery that is characteristic of mRNA vectors.

Further improvements in the design and production of mRNA vectors for therapeutically important epigenetic switches are expected and are likely to include:

- Development of more stable, highly deliverable and gene-expression-proficient forms of mRNA vectors, incorporating ligands for cell-specific targeting, efficient cell penetration and positioning in the intracellular milieu and
- Automated screening for new cocktails of mRNA vectors and other molecules for bespoke tasks of therapeutic epigenetic reprogramming.

A very attractive goal in regenerative medicine is therapeutic trans-differentiation in vivo with targeted delivery of reprogramming gene vectors directly to the body site requiring a regenerative intervention. So far in vivo trans-differentiation was achieved only in animal models. The inherent safety of mRNA-based vectors might make them a highly suitable tool for the transfer of the technology to human patients.

Conflict of interest

The authors declare that there are no conflicts of interest.

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