# Concise Review: Application of Chemically Modified mRNA in Cell Fate Conversion and Tissue Engineering

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**Key Words.** Nucleic acid therapy • Cell programming and reprogramming • Differentiation • iPSCs • Nanoparticles • Regenerative medicine

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## ABSTRACT

Chemically modified RNA (cmRNA) has potential as a safe and efficient tool for nucleic acid-based therapies and regenerative medicine. Modifications in the chemistry of mRNA can enhance stability, reduce immunogenicity, and thus facilitate mRNA-based nucleic acid therapy, which eliminates risk of insertional mutagenesis. In addition to these valuable advantages, the mRNA-based method showed significantly higher efficacy for reprogramming somatic cells to pluripotency compared with DNA- or protein-based methods. These findings suggest cmRNA can provide a powerful and safe tool for cell programming and reprogramming. Delivery methods, particularly using lipid nanoparticles, provide strategies for cell and organ-specific targeting. The present study comprehensively compares studies that have used cmRNAs for cell fate conversion and tissue engineering. The information should be useful for investigators looking to choose the most efficient and straightforward cmRNA-based strategy and protocol for tissue engineering and regenerative medicine research.
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## SIGNIFICANCE STATEMENT

Chemically modified mRNA (cmRNA), as a less immunogenic and more stable form of mRNA with no risk of insertional mutagenesis, offers new strategies for the field of nucleic acid therapy. With respect to cell engineering, the safety is accompanied by high efficiency for reprogramming to pluripotency. The present study comprehensively compares a variety of studies that used cmRNAs for cell fate conversion and tissue engineering. The information should be useful for investigators looking to choose a cmRNA-based protocol for tissue engineering, and could enhance future investigations using cmRNAs for regenerative medicine in a timely and efficient manner.

## INTRODUCTION

Recent studies have pursued mRNA therapeutics as an alternative strategy for DNA-based gene therapy, due to outstanding advantages of mRNA. Foremost, introducing mRNA does not harbor the risk of genomic integration, which remains a major concern for DNA-based gene therapy. Second, in contrast to DNA, delivery to the nucleus is not required for mRNA function, as it is translated in the cytoplasm [1, 2]. However, high levels of mRNA instability and immunogenicity hindered its broad use for therapeutic investigation and clinical applications [3]. These problems were largely mitigated by introduction of a number of structural modifications in mRNA molecules, including a m7G 5'-Cap structure, untranslated regions (UTRs), poly(A) tail, and perhaps most importantly, modified nucleotides. The structural modifications significantly improve mRNA stability,

reduce its immunogenicity, and enhance translational efficiency [4–6]. The improvement in translational efficiency of modified mRNA depends on the encoded protein and the type of modifications. For example, Warren et al. demonstrated a fourfold increase in fluorescence intensity when using either 5-methylcytidine or pseudouridine in the structure of *GFP* mRNAs, whereas the intensity was 10-fold higher when using both modified nucleotides [7]. The modified mRNA, hereafter referred to as chemically modified mRNA (cmRNA), is synthesized via in vitro transcription. Figure 1 illustrates a generic structure of cmRNA. However, the rules for modification remain a work in progress and likely depend on the application. The full range of possible modifications in the structures of mRNAs, especially the type of modified nucleotides, are not yet completely explored and different research groups have used various sets of cmRNA modifications

**Figure 1.** General structure of chemically modified mRNA (cmRNA). Modifications increase the stability, decrease the immunogenicity, and in some cases increase the translational efficiency. Typical components of a cmRNA include, with color-coding: 7-methylguanosine (m7G) cap structure (5′-Cap). Untranslated regions (5′-UTR and 3′-UTR), usually derived from β-globin mRNAs. Open reading frame, coding sequence for the gene of interest, containing optimized codons and/or chemically modified nucleotides. Polyadenylated tail (Poly [A] tail), stretch of 100–200 adenine nucleotides.

[6]. Overall, there remains much chemistry to explore to find the optimal set of modifications useful for a specific cmRNA indication. Direct cmRNA modifications include the following.

## 5′-Cap Structure

During natural transcription, the 5′ end of eukaryotic mRNA links to a 7-methylguanosine (m7G 5′) cap. The cap structure can dramatically affect translational efficiency by two mechanisms. First, binding of cap to translation initiation factor 4E (eIF4E) is essential for mRNA translation. Second, binding of cap to mRNA decapping enzymes initiates mRNA decay. Therefore, choosing the appropriate cap structures that have high affinity for binding to eIF4E and are resistant against decapping enzymes can significantly increase the translational efficiency as well as cmRNA stability [6, 8].

## UTRs

Due to the presence of regulatory sequence elements, impacting tertiary structures such as hairpin loops, and interaction with RNA-binding proteins, 5′- and 3′-UTRs can affect both stability and translational efficiency of cmRNAs. To this end, many studies have used the highly stabilizing UTRs derived from α/β-globin genes to increase stability and efficiency of translation of desired cmRNAs [9] .

## Poly(A) Tail

Eukaryotic mRNAs naturally bear a poly(A) tail structure at the 3′ end, which plays an important role in mRNA stability and translation. The poly(A) tail, with an optimum length of 120–150 nucleotides, can be added to synthetic cmRNA in either of two ways, encoding the poly(A) structure in the template vector of the target cmRNA, or enzymatically adding adenine nucleotides post-transcription of cmRNA [6].

## Modified Nucleotides

Unmodified mRNAs can be detected by a subclass of Toll-like receptors (TLRs), such as TLR7, and thus are highly immunogenic. Incorporation of modified nucleotides such as 5-methylcytidine (5mC), pseudouridine (Ψ), 5-methyluridine (5mU), or N6-methyladenosine in the structure of cmRNAs has been shown to significantly decrease the immunogenicity by avoiding activation of TLRs [6, 9].

The advent of modified mRNA has substantially accelerated cmRNA-based therapeutic investigation both in academia and industry. cmRNA is already being investigated for cancer immunotherapy, mRNA vaccines, protein replacement, gene editing, cell fate conversion, and regenerative medicine [6]. Application of cmRNA for cancer immunotherapy and development of mRNA-based vaccines are already in clinical trials, a topic that has been previously reviewed [10, 11].

Here, we focus on application of cmRNAs for cell fate conversion (cell differentiation or reprogramming) and regenerative medicine (Fig. 2). First, we discuss application of cmRNAs for cell reprogramming (a form of dedifferentiation) toward stem cell fate, which has been validated as a safe and efficient method for production of induced pluripotent stem cells (iPSCs). Various studies are compared according to the type of mRNA modifications, delivery vehicles, number of transfections, and the efficiency and duration of iPSC colony formation. Second, we summarize studies that used cmRNAs for directed differentiation or trans-differentiation of stem/progenitor cells to defined cell lineages, including for tissue regeneration. Again, various criteria are considered, such as types of mRNA modification, delivery vehicles, and type of biomaterials used for tissue engineering, to compare different cmRNA-applied methods for cell differentiation. Finally, we discuss strategies for in situ applications of cmRNAs encoding anabolic factors to suppress degenerative diseases, as well as using cmRNA to improve homing of stem cells in the injury area. By providing key information about how cmRNA can be applied for cell and tissue engineering at a glance, we hope to inspire future investigations using cmRNAs for regenerative medicine in a timely and efficient manner.

### CELL REPROGRAMMING USING MRNA

Cell reprogramming, also known as cell dedifferentiation, has long been a topic of interest to developmental biologists. In the 1960s, John Gurdon showed for the first time that differentiation of a cell can be reversed by transferring a cell nucleus of an adult frog into an enucleated egg to derive cloned frog embryos [12]. In 1996, the research group of Ian Wilmut cloned the first mammal using adult somatic cell nuclei to generate the sheep named Dolly [13]. In a similar strategy, using fused (heterochromatic) somatic cells, Blau et al. showed that gene expression in differentiated cells is plastic and can be altered by modulations in the cytoplasm [14].

More recently, this phenomenon has been rediscovered as an important and feasible strategy for biotechnology, whereby a specialized differentiated cell is reverted to a more primitive state of stem or progenitor cell. The self-renewal properties along with the capability to differentiate to multiple different cell types are features that make stem cells unique tools for regenerative medicine [15]. In 2006, Takahashi and Yamanaka demonstrated that forced expression of the four transcription factors *OCT3/4, SOX2, KLF4,* and *c-MYC* (now often referred to as Yamanaka factors) can reprogram mature fibroblasts to pluripotency [16], generating what are referred to as iPSCs.

The discovery of iPSCs was a remarkable achievement in biotechnology with major repercussions for regenerative medicine. iPSCs can be generated from a patient's own cells, and thus should mitigate much of the risk of genetic incompatibility or
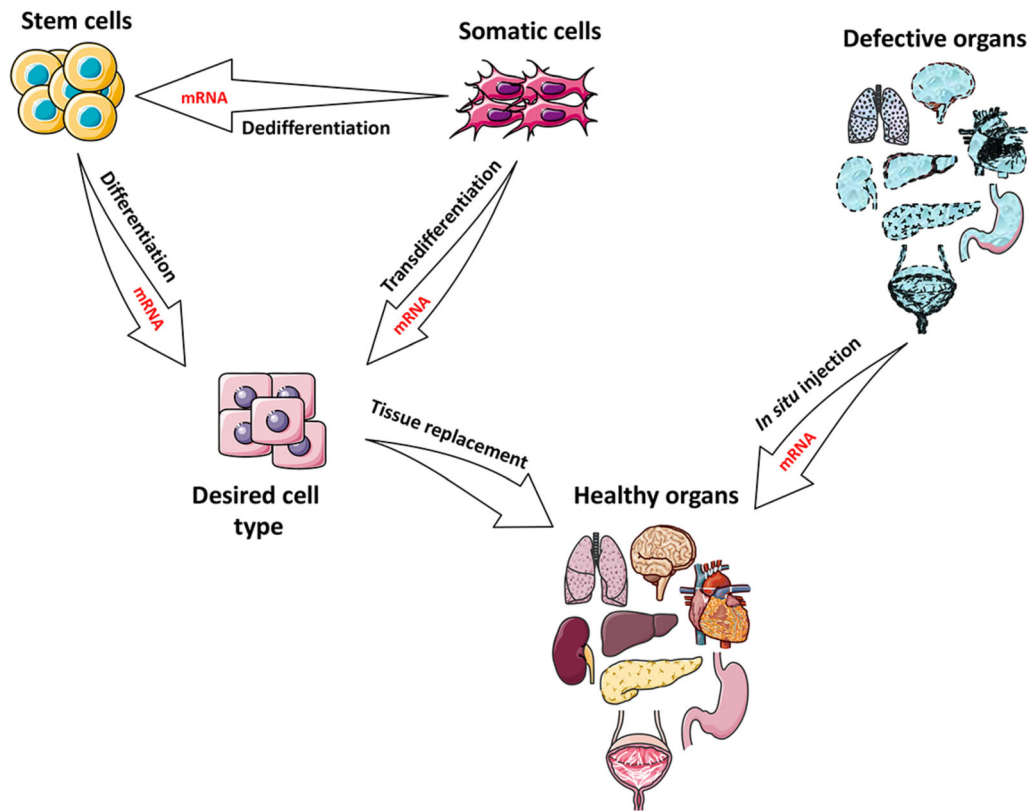
**Figure 2.** Application of mRNAs in cell and tissue engineering. mRNA can be used for reprogramming (dedifferentiation) of somatic cells to stem cells or directed differentiation of stem cells to the desired cell type. In addition, somatic cells can be directly reprogrammed to a distinct somatic cell type (trans-differentiation) using mRNAs. Direct injection of therapeutic mRNAs to defective organs (in situ mRNA delivery) may also trigger tissue regeneration. This figure was made in part by using the Servier medical art free image collection.

**Table 1.** Comparison of different protocols for reprogramming human fibroblasts to iPSCs, based on delivery of proteins, DNAs, and cmRNAs of Yamanaka factors

| Reprogramming protocols | Protein [17] | DNA virus (retrovirus/lentivirus) [18–20] | RNA virus (Sendai virus) [21–23] | cmRNA [7, 24] |
|---|---|---|---|---|
| Time course for colony isolation | 8 weeks | 2–4 weeks | 4 weeks | 2 weeks |
| Reprogramming efficiency | 0.001% | 0.01%–0.1% | 0.01%–1% | Up to 4.4% |
| Risk of genome integration | No | Yes | No | No |

Abbreviations: iPSCs, induced pluripotent stem cells; cmRNA, chemically modified RNA.

immune rejection for cellular derivatives that are subsequently transplanted. The patient's own genome is retained in the cells, which can be exploited for patient-specific disease modeling. Unlike embryonic stem cells (ECSs), production of iPSCs does not require use of embryos, relieving potential ethical concerns [15]. However, iPSCs reprogrammed by viral vector delivery of cDNA (for example retroviral, lentiviral, or adenoviral vectors) are prone to insertional mutagenesis and residual viral vector contamination, which in turn limit the clinical application of such iPSCs. Therefore, a number of studies have been performed to establish an integration-free method for reprogramming to pluripotency. In 2009, for instance, human iPSCs were first generated by direct protein delivery of Yamanaka factors. Although the method presented no risk of genome insertion, low efficiency and lengthy procedure limit the protein-based reprogramming protocols from most applications (Table 1) [17]. Subsequently, the efficiency of this protocol was to some extent improved using partially purified recombinant protein produced in mammalian cells

[25]. Also in 2009, Sendai virus, a type of RNA virus, was first used for delivery of Yamanaka factors and reprogramming to iPSCs. Although Sendai virus reprogramming is integration-free and the efficiency was quite improved, the method still suffers from an overall relative low efficiency (maximum 1%) and lengthy procedure (Table 1) [21].

The first successful reprogramming of somatic cells to iPSCs with cmRNAs encoding Yamanaka factors was performed in 2010 [7]. With this protocol, fibroblasts underwent a "daily transfection regime" for 14 consecutive days using cationic lipid nonviral vectors for cmRNA delivery, and iPSC colonies were isolated at day 18 [24]. The resulting transgene-free iPSCs with no obvious risk of mutagenicity and no viral residual, along with high reprogramming efficiency (up to 4.4%), make this method a candidate preferential procedure for iPSC reprogramming in future clinical applications (Table 1). Accordingly, a number of commercially available kits have been designed, and a human iPSC line has been established, based on the above-mentioned mRNA reprogramming protocol [26].

Although the cmRNA-based reprogramming protocol has advantages over protein- or DNA-based strategies with higher safety and efficiency, the protocol is relatively complicated and laborious as it requires a daily transfection regimen over 2 weeks. In addition, the use of feeder cells and in some cases antibiotic-free culture increases the risk of bacterial and biological contamination [7, 24]. As a result, continued efforts have sought to simplify the process and optimize the cmRNA reprogramming protocols. Parameters that have been modified include fewer numbers of transfections, fewer reprogramming factors, shorter time frame for reprogramming, using feeder free culture, various vectors, and various modifications of mRNAs (Table 2). Note that not all of these studies were pursued until colony characterization or testing for capacity of teratoma formation. Indeed, some studies only detected elevated expression levels of endogenous reprogramming genes as markers for initiation of reprogramming.

In addition to reprogramming to iPSCs, cmRNAs can be used for reprogramming to other multipotent stem cells. To this end, Kim et al. reprogrammed human mesenchymal stem cells (MSCs) to induced neural stem cells (iNSCs) using SOX2 cmRNA [40]. This study used the same modifications as the Mandal and Rossi protocol [24]. The resulting integration-free iNSCs can be used for studying neural pathogenesis or potentially for treatment of neurodegenerative disease [40].

Recently, mRNAs have been used not only for making iPSCs, but also for gene editing of the resulting iPSCs using CRISPR/Cas9 technology. In contrast to plasmid DNA, transient delivery of mRNA encoding Cas9 provides an integration-free platform for further editing cmRNA-reprogrammed iPSCs, to either introduce a specific gene mutation for disease modeling, or to correct a gene mutation via homology directed repair [41]. Both approaches are highly relevant in regenerative medicine.

As mentioned before, other integration-free reprogramming methods such as using nonintegrative viral vectors and excisable vectors have also been evaluated to find the safest and the most efficient strategy for iPSC production. However, the risk of random genome insertion of segments of viral vectors, the additional efforts to remove the excisable vectors, as well as the low efficiency of these methods limit their applications [42]. Using self-replicating mRNA is another approach to simplify the procedure by elimination of daily transfections. In this method, a single long mRNA encoding all four reprogramming factors was used based on the noninfectious and self-replicating Venezuelan Equine Encephalitis (VEE) virus RNA replicon. Nonetheless, this method requires conditioned media containing B18R and Puromycin for blocking the innate immune response and retention of VEE RNA, respectively [43]. In addition, the time interval for reprogramming to pluripotency using self-replicating mRNA was almost two times longer compared with the mRNA protocol (iPSC colony isolation on day 25–30 or day 15–18 using self-replicating mRNA method or cmRNA protocol, respectively [24, 43]). Thus, reprogramming to iPSC fate using cmRNAs remains the most efficient and safest method for cell dedifferentiation.

## CELL PROGRAMMING AND TISSUE ENGINEERING USING MRNA

In parallel with cell reprogramming, cmRNAs have been investigated for their use in cell programming, also known as cell differentiation. In these studies, cmRNAs were used to differentiate stem cells to the desired cell lineages for application in tissue engineering and regenerative medicine (summarized in Table 3).

In most of the studies listed in Table 3, mRNAs contained modified nucleotides, either 100% pseudouridine (Ψ) and 5-methylcytidine (5mC), or 25% 2-thiouridine (2TU) and 5mC. Very recently, 7.5% 5-iodo-cytidine and 35% 5-iodo-uridine modification of BMP2 mRNA has been used for bone regeneration [49]. Nonetheless, differentiation to endothelial progenitor cells using ETV2 cmRNA harboring no modified nucleotides has also been reported [53].

Various methods have been used for delivery of cmRNAs to cells in vitro, ex vivo, and in vivo. Mechanical methods such as electroporation and gene gun have been widely used for cmRNA delivery. However, these are expensive methods, to some extent invasive, and clearly not translatable for in vivo clinical applications. Chemical methods for cmRNA delivery, on the other hand, are cheaper, easier, and impose lower toxicity and immunogenicity. Most importantly, chemical methods are capable of noninvasive cmRNA delivery in vivo. In these methods, cationic lipids or polymers bind electrostatically with negatively charged cmRNA molecules and form nanoparticles [9]. The use of lipid or polymer nanoparticles can arguably be considered the current gold standard for delivery of nucleic acids including cmRNAs [57]. Such complexes are biocompatible, biodegradable, with a natural propensity to interact with cellular membranes to facilitate cargo uptake through an endocytic pathway.

For translation, nanoparticles provide advantages for targeting cmRNA to specific cells or organs, which is a very important consideration for clinical applications, and represents a very active area of current research (Fig. 3). Hydrophilic or electrostatic binding of the cmRNA nanoparticles to the cell membrane can initiate endocytosis and lead to internalization of nanoparticle to the cell cytoplasm [9]. The nanoparticle can be delivered systematically via blood injection/administration, in which case the predominant cells of uptake are liver hepatocytes [58]. If other cell types are to be targeted in vivo, other strategies can be used, for example direct local tissue injection for muscle [59] or inhalation delivery to target the lung epithelium [60]. Modifying the surface charges of the cmRNA nanoparticles is an emerging strategy for targeting cmRNA to defined cell types. Chemical traits that can impact targeting include the specific lipid-amine compound, the amount of Polyethylene glycol (PEG) included in the formulation, the defined PEG structure, and the molar amount of cholesterol. In an elegant and promising recent study, Sago et al. used a high throughput method to screen over 250 lipid nanoparticle formulations that incorporated DNA barcodes. Following delivery, they could screen organs and cells for biased targeting based on the coincorporation of the bar-code, and were successful at identifying two nanoparticles that efficiently delivered mRNA to endothelial cells [61]. Finally, lipid nanoparticles loaded with mRNA can be decorated with targeting moieties, such as monoclonal antibodies, as was accomplished recently to target expression of Interleukin-10 in inflammatory leukocytes as a potential therapy for inflammatory bowel disorder [61].

Beyond lipid nanoparticles, many in vivo studies have benefited from exploring biomaterials to facilitate the delivery of cmRNAs to a desired tissue. Using biomaterials can reduce the off-target toxicity and enhance delivery of cmRNA to the specific site. Biomaterials can also work as a "reservoir" for sustained delivery of cmRNA, where the delivery efficiency

**Table 2.** Summary of studies on reprogramming to iPCS using mRNA

| Transcription factors | Transfection reagent | Duration between first TF and colony formation/pick up | Number of transfections | Using feeder cells | Cell source | Differentiation to three germ layers/teratoma formation | mRNA modifications | References |
|---|---|---|---|---|---|---|---|---|
| KLF4, c-MYC, OCT4, SOX2, LIN28 | Cationic lipid | Colony formation day 17/colony pick up day 20 | 17 | Yes | BJ1, dH1f, Detroit 551 (D551), and MRC-5 fibroblast, and skin cells of a cystic fibrosis patient (CF) | Yes | 100% 5mC and Ψ, 5'-UTR containing Kozak sequence, α-Globin 3'-UTR, Poly(A) tail, Cap | [7] |
| KLF4, c-MYC, OCT4, SOX2, LIN28 | Cationic lipid | Colony formation day 6–9/colony pick up day 15–18 | 14 | Yes | Human primary fibroblasts | No | 100% 5mC and Ψ, 3'-UTR, 5'-UTR, Poly(A) tail, Cap | [24]a |
| OCT-4, NANOG, KLF-4, c-MYC, SOX-2, hTERT | Electroporation and lipofection | Colony formation day 30 | 4 | Yes | Human foreskin cells, adult Huntington fibroblasts, adult skin fibroblasts of healthy donors | Yes | Poly(A) tail, Cap | [27] |
| OCT4, SOX2, KLF4, c-MYC | Cationic lipids | Not mentioned/not continued | 3 | Yes | Mouse embryonic fibroblasts (MEFs) | No | Poly(A) tail, Cap | [28] |
| OCT4, LIN28, SOX2, NANOG | Cationic lipids | Colony formation day 12–19/colony pick up day 21 | 5 | Yes | Human foreskin Fibroblasts (hFF) | No | Poly(A) tail, Cap, IRES sequence | [29] |
| KLF4, c-MYC, OCT4, SOX2, LIN28 | Cationic lipids | Colony formation around day 30 | 18 | No | MSCs derived from adipose tissue of a 50-year-old patient | Yes | 100% 5mC and Ψ, Cap | [30] |
| OCT4, SOX2, c-MYC, KLF4, SV40 large T (LT) | Electroporation | Colony formation day 30 | 1 | No | Human fetal skin fibroblasts (HuF1), Human embryonic lung fibroblasts (MRC5), Human foreskin fibroblasts (HFF) | No | Poly(A) tail, Cap, 5'- and 3'-UTRs of Xenopus β-Globin | [31] |
| OCT4, SOX2, KLF4, c-MYC | Lipofectamine 2000 | Colony pick up day 22 | 5 | No | Goat embryonic fibroblasts (GEFs) | Yes | Poly(A) tail, Cap | [32] |
| M3Ob, SOX2, KLF4, c-MYC-T58A, LIN28, NANOG | Cationic lipids | Colony formation day 9–13 | 9 | No | BJ neonatal Fibroblasts, HDF-f fetal fibroblasts, HDF-n neonatal fibroblasts, HDF-a Adult fibroblasts, XFF xeno-free neonatal fibroblasts | Yes | 100% 5mC and Ψ, 5'-UTR containing Kozak sequence, α-Globin 3'-UTR, Poly(A) tail, Cap | [33]c |

*(Continues)*

**Table 2** (Continued)

| Transcription factors | Transfection reagent | Duration between first TF and colony formation/pick up | Number of transfections | Using feeder cells | Cell source | Differentiation to three germ layers/teratoma formation | mRNA modifications | References |
|---|---|---|---|---|---|---|---|---|
| OCT4, SOX2, c-MYC, KLF4 Or Total mRNA or RNA extracted from cells expressing Yamanaka factors | Graphene oxide-polyethylenimine (GO-PEI) using a dynamic suspension culture | Colony formation day 18, Colony pick up day 24 (for hADFs cells) | 3 | Yes | Human adipose tissue-derived fibroblasts (hADFs), rat ADFs (rADFs), and mouse embryonic fibroblasts (MEFs) | Yes | 3′-UTR, 5′-UTR, Poly(A) tail, Cap | [34] |
| KLF4, c-MYC, OCT4, SOX2, LIN28 | RNAiMAX | Colony formation day 8, Colony pick up day 12–16 | 8–12 | No | BJ human fibroblasts, GM13325 fibroblasts, human adult fibroblasts (HUF1 and HUF58) | Yes | 100% 5mC and Ψ, Cap | [35] |
| OCT4, KLF4, SOX2, c-MYC, LIN28 | RNAiMAX | Colony formation day 15, colony pick up day 18–21 | 18 | Yes | Bone marrow derived-MSCs from a patient with β-thalassemia | Yes | 100% 5mC and Ψ, Poly(A) tail, Cap | [36] |
| KLF4, c-MYC, OCT4, SOX2, LIN28, MBD3 SiRNA | RNAiMAX | Colony pick up day +7 | 2 | Yes and no (same efficiency) | Adult Human patient-specific fibroblasts | Yes | Not mentioned | [37] |
| KLF4, c-MYC, OCT4, SOX2, LIN28 | RNAiMAX | Colony formation day 18 | 18 | Yes | Human amniotic fluid-derived stem cells (AFSC) | Yes | 100% 5mC and Ψ, 5′-UTR containing Kozak sequence, α-Globin 3′-UTR, Poly(A) tail, Cap | [38] |
| M3O, SOX2, KLF4, cMYC, LIN28A, NANOG, mWasabi, miRNA-367/302 seconds | RNAiMAX | Colony formation day 18 | 7 | No | Primary fibroblasts form healthy donors and patients[d] | Yes | 100% 5mC and Ψ, 5′-UTR containing Kozak sequence, α-Globin 3′-UTR, Poly(A) tail, Cap | [39] |

5mC and Ψ stand for 5-methylcytidine and pseudouridine, respectively.

[a]This publication is a protocol based on reference 7.

[b]Oct4 incorporating an N-terminal MyoD transactivation domain.

[c]Different reprogramming regimens with and without using feeder layer were tested to find the method with highest efficiency and easiest handling.

[d]Primary neonatal fibroblast lines: FN1, FN2, and FN5. Healthy primary adult fibroblast lines: F40, F62, and F50. Fibroblasts derived from patients with inherited skin blistering disorders and Down's syndrome.

**Table 3.** mRNA applications in cell differentiation and tissue regeneration

| mRNA | Type of cell differentiation or tissue regeneration | mRNA modification | Transfection reagent/method | Biomaterial | Number of transfections | References |
|---|---|---|---|---|---|---|
| BMP2 | Osteogenic diff. Bone reg. | 100% Ψ and 5mC, Poly(A) tail, Cap | Branched PEI | Collagen | 1 | [44] |
| BMP2 | Osteogenic diff. Bone reg. | 25% 5mC and 2TU, Poly(A) tail, Cap | In vitro: DreamFect gold (DF-gold) ± magnetic nanoparticles In vivo: C12-EPE | Fibrin gel | 1 | [45] |
| BMP2 or BMP9 | Osteogenic diff. Bone reg. | 100% Ψ and 5mC, Poly(A) tail, Cap | PEI | Collagen | 1 | [46] |
| BMP2 | Osteogenic diff. Bone reg. | 25% 5mC and 2TU, Poly(A) tail, Cap | A proprietary lipid | Collagen | 1 | [47] |
| BMP2 | Osteogenic diff. | 25% 5mC and 2TU, Poly(A) tail, Cap | DF-gold | Fibrin gel and micro–macro biphasic calcium Phosphate (MBCP) granules | 1 | [48] |
| BMP2 | Osteogenic diff. Bone reg. | 7.5% 5IC and 35% 5 IU Poly(A) tail, Cap[a] | A proprietary lipid | Collagen | 1 | [49] |
| hVEGF-A | Vascular reg. Cardiac reg. Cardiovascularendothelial and endovascular diff. | 100% Ψ and 5mC, Poly(A) tail, Cap | RNAiMAX | None | 1 | [50] |
| VEGF-A | Endothelial diff. Vascular reg. | 100% 5mC and 2TU Cap Poly(A) tail | RNAiMAX | Matrigel | 1 or 2 | [51] |
| NEUROG1 NEUROG2 NEUROG3 NEUROD1 NEUROD2 | Neuronal cell diff. | 100% Ψ and 5mC, Poly(A) tail, Cap | Lipofectamine messenger max | None | 2 | [52] |
| ETV2 | Trans-diff. of fibroblasts to endothelial progenitor cells | Poly(A) tail, Cap | Nucleofection, FuGENE HD | None | 1 | [53] |
| MYOD | Myogenic diff. | 100% Ψ and 5mC, Poly(A) tail, Cap | RNAiMAX | None (plate coated with gelatin) | 3 | [7] |
| Gata4, Mef2c, Tbx5 | Trans-diff. of cardiac fibroblasts to cardiomyocyte-like cells | 100% Ψ and 5mC, Poly(A) tail, Cap | c-lipo (=lipofectamine 2000 + CRPPR-R9[b]) | None (plate coated with gelatin) | 14 | [54] |
| Pdx1, Neurogenin3, MafA | Trans-diff. of pancreatic nonendocrine cells into insulin-producing β-cells | 100% Ψ and 5mC, Poly(A) tail, Cap | Lipofectamine messenger-MAX mRNA transfection reagent | None | 10 | [55] |
| MAFA[c] | Trans-diff. of human pancreatic duct-derived cells into insulin-secreting β-cells | 100% Ψ and 5mC, Poly(A) tail, Cap | jetPEI | None | 7 | [56] |

[a]5IC and 5 IU stand for 5-iodo-cytidine and 5-iodo-uridine, respectively. Further modifications in this study are included: omitting an upstream ORF in the 5′-UTR and a polyadenylation element along with an AU-rich tract in the 3′-UTR. Furthermore, a translation initiator of short UTRs (TISU) was incorporated in mRNA structure.
[b]CRPPR-R9, a polyarginine-fused heart-targeting peptide.
[c]V-Maf musculoaponeurotic fibrosarcoma oncogene homolog A.
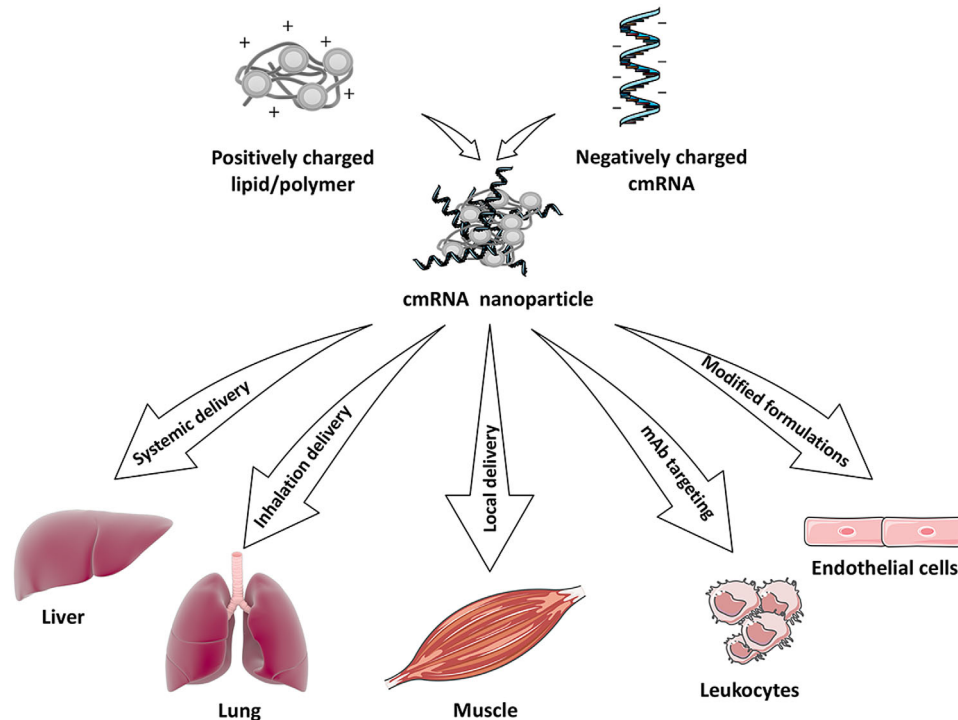Abbreviations: Diff., differentiation; Reg., regeneration.

**Figure 3.** Noninvasive and targeted delivery of chemically modified mRNAs (cmRNAs) in vivo. Negatively charged cmRNA molecules bind electrostatically with positively charged polymers or lipids. Various strategies have been used to target the resulting nanoparticles to certain tissues. Systemic (blood), inhaled, or local injections have been used to preferentially target liver hepatocytes, lung epithelium, or skeletal muscle, respectively. Decorating the nanoparticles with monoclonal antibodies has been used to target leukocytes expressing defined antigens, whereas defined lipid/polymer formulations can bias delivery to distinct cells and tissues, including endothelium. See text for details and relevant references. This figure was made in part by using the Servier medical art free image collection and the library of Science & Medical Illustrations.

can be controlled by porosity and/or biodegradability of the biomaterials. To this end, Badieyan et al. applied transcript activated matrices preloaded with cmRNA complexes. They established a sustained cmRNA delivery collagen matrix with a plateau of almost 1 week of protein production for application in tissue engineering [47]. Different criteria need to be considered to choose the right biomaterial for each application. These criteria include: biocompatibility, biodegradability, in situ gelation upon injection, and physical and mechanical properties such as permeability and mechanical response. Considering these factors, natural biopolymers such as collagen and fibrin have often been used to deliver cmRNAs to tissues [9].

cmRNAs have been used for differentiation and transdifferentiation into a variety of cell types including neurons [52], β-cells [55], endothelial progenitors [53], cardiovascular cells [50, 54], and myogenic cells [7, 54]. However, the majority of studies using cmRNA for cell differentiation and tissue engineering have been performed on bone, and to a lesser extent, on heart regeneration. The effect of *BMP2* on bone regeneration was previously shown using gene [62] or protein [63] therapy. Recent publications demonstrated successful bone regeneration following application of cmRNAs encoding BMP2 in femur [45, 47] and calvarial [44, 46] bone defect models. Regarding cardiovascular regeneration, Hadas et al. reported successful differentiation of cardiomyocytes and heart regeneration following application of *VEGF* cmRNA [8]. They suggested that cmRNA is an efficient tool for boosting endogenous regeneration after myocardial infarction (MI). This is due to high protein production efficiency of cmRNA as

well as fast kinetics of expression that closely meet the time frame of MI pathology. In addition, cmRNA has a transient effect and thus eliminates the risk of malignancy which could occur following induced cell proliferation due to over-expression of the delivered gene over long periods [8]. In addition to cardiovascular regeneration, Lui et al. showed enhancement of engraftment with reduction of apoptosis in human heart progenitor cells after delivery of *VEGF-A* mRNA in vivo [51]. A recent study characterized the optimal type of modifications as well as doses of cmRNA for cardiovascular delivery. According to this, applying cmRNAs containing N1-methylpseudouridine-5′-triphosphate nucleotide modification with 100 μg per mouse heart (1.6 μg cmRNA per microliter in 60 μl total) in sucrose-citrate buffer led to the best result for in vivo delivery of cmRNA into mouse heart [64].

In situ delivery of mRNAs encoding anabolic markers has also been used for controlling degenerative diseases. To this end, direct injection of mRNA encoding Runt-related transcription factor 1 (RUNX1) has been applied to modify osteoarthritis (OA). *RUNX1* mRNA formulated with polyplex nanomicelles was injected into knee joints of an OA mouse model. The expression of *RUNX1* as a cartilage-anabolic factor significantly reduced the progression of OA in mice, and suggested in situ mRNA delivery as a potential approach for regenerative medicine [65]. Another example of in situ application of mRNA was direct injection of vasopressin mRNA into the hypothalamus to temporarily invert diabetes insipidus in Brattleboro rats. This animal model presents with diabetes insipidus due to a mutation in the propressophysin gene that makes them unable to secret vasopressin in hypothalamic neurons [66].

Another study used in situ delivery of cmRNA encoding IGF1 to damaged myocardial cells after MI. Among other roles, IGF1 can elicit pathways of cell survival and eventually cardiomyocyte repair. The protein production following delivery of nanoparticles of jetPEI and *IGF1* cmRNA significantly reduced cell apoptosis and thus promoted the survival of cardiac cells after MI in an in vivo murine mode [67]. Kariko et al. also showed mRNA encoding erythropoietin significantly increases production of red blood cells and decreases the risk of anemia [68].

In a number of studies mRNAs were used for migration and homing of MSCs to the site of inflammation and brain tumors. The improvement of homing of MSCs can in turn enhance the capability for regeneration of damaged tissue. To this end, Ryser et al. showed that MSCs transfected with mRNA encoding chemokine (C-X-C motif) receptor 4 (CXCR4) can improve migration of MSCs to inflammation sites, and thus can potentially improve cell therapy in the inflammatory area [69]. CXCR4 is a chemokine receptor with high affinity to bind to stromal derived factor-1, which is highly expressed in inflammatory sites, such as an ischemic area. In a similar study, MSCs transfected with mRNA encoding P-selectin glycoprotein ligand-1 (PSGL-1) and α-(1,3)-fucosyltransferase (FUT7) improved the tethering and rolling and consequently the homing of MSCs in the inflammatory region [70]. FUT7 enzyme increases the secretion of Sialyl-Lewisx (SLeX). PSGL-1, further modified with SLeX, facilitates the interaction with P-selectin that is often upregulated on endothelial cells during inflammation. Likewise, Nowakowski et al. improved MSC migration to the brain ischemic area by using integrin a4 (*ITGA4*) mRNA-transfected MSCs [71]. Expression of *ITGA4* facilitates adhesion of MSCs to the VCAM-1 ligand of endothelial cells and thus improves migration of MSCs to injured tissue such as ischemic brain.

## CONCLUSION

Although application of mRNA in cell fate conversion and regenerative medicine is a fast progressing field, existing hurdles remain that will need to be managed for eventual translation. The high cost of the modified mRNAs and high sensitivity to heat and ubiquitous nucleases may limit the enthusiasm of industrial and academic groups to invest time and effort on cmRNA-based applications, as a relatively new field of research. Moreover, the optimal carrier for delivery of mRNA may need to be determined for each single application, particularly for in vivo studies. Even with enhanced engineered delivery vehicles, challenges remain, for example to control release of cmRNA into the cytoplasm following endocytosis, and to control levels of expressed proteins. When reprogramming of cells or regeneration of tissue needs a relatively long time-course, the ideal biomaterials for prolonged delivery of mRNA should be determined to compensate for the transient effect of mRNA and mitigate the need for multiple transfections. Overall, the accumulating knowledge of enhanced mRNA synthesis, modification, delivery and targeting, should overcome these obstacles and fulfill promise of a bright future for translation of cmRNA toward clinical applications.

## AUTHOR CONTRIBUTIONS

Z.S.B.: conception and design, collection and/or assembly of data, manuscript writing; T.E.: conception and design, financial support, manuscript editing.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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