Review

Cell Reprogramming and Differentiation Utilizing Messenger RNA for Regenerative Medicine

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Review

Cell Reprogramming and Differentiation Utilizing Messenger RNA for Regenerative Medicine

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Abstract: The COVID-19 pandemic stimulated attention to the medicinal applications of messenger RNA (mRNA). mRNA is expected to be applied not only to vaccines but also to regenerative medicines. The purity of mRNA is important for its medicinal application. However, the current mRNA synthesis techniques have problems, *e.g.*, contamination of undesired 5′-uncapped mRNA and double-stranded RNAs. Recently, our group developed a completely capped mRNA synthesis technology that contributed to the progress of mRNA research. The introduction of chemically modified nucleosides, *e.g.*, *N*1-methylpseudouridine and 5-methylcytidine, have been demonstrated by Dr. Karikó and Dr. Weissman which opened the practical application of mRNA for vaccines and regenerative medicines. Dr. Yamanaka reported the production of Induced Pluripotent Stem Cells (iPSCs) by the introduction of 4 types of genes using a retrovirus vector. iPSCs are widely used for research about regenerative medicines and the preparation of disease models to screen new drug candidates. Of the Yamanaka factors, Klf4 and c-Myc are oncogenes, and there is a tumor development risk if these are integrated into genomic DNA. Therefore, regenerative medicines using mRNA, which has no risk of genome insertion, have attracted attention. In this review, we summarized the synthesis of mRNA and its application for regenerative medicine.

Keywords: messenger RNA; cell regeneration; pluripotent cells; cellular differentiation; direct reprogramming; regenerative medicine

1. Introduction

Messenger RNA (mRNA) is produced in living organisms by transcription from genomic DNA and proteins are produced based on the sequence information of mRNA. With the recent spread of the Coronavirus Disease 2019 (COVID-19) pandemic, attempts to apply mRNA as a medicine have attracted attention [1]. In addition to being used as a vaccine against cancer, and viral and bacterial infections [2], mRNA is also expected to be used as a drug for protein replacement therapy, which enables the replacement of missing proteins in genetic diseases [3]. Research and development of mRNA therapeutics have been interesting for a long time, but it has dramatically progressed under the COVID-19 pandemic situation [4].

mRNA is composed of several regions, such as a 5' cap structure, 5'-untranslated region (UTR), protein-coding region, 3'-UTR, and poly-A tail (Figure 1)[5]. The 5' cap structure is a characteristic structure present at the 5' end of mRNA, in which 7-methyl guanosine is linked via a 5'-5' triphosphate bond[6]. The 5' cap structure was discovered by Furuichi et al in Japan, and it has been reported that it has a variety, such as Cap0, Cap1, and Cap2, depending on the presence or absence of 2'-*O*-methyl modification[7, 8]. This structure is essential for practical use as an mRNA therapeutics because it is involved in the intracellular stability of mRNA, translation initiation, splicing, and innate immune responses. Although the 5'/3'-UTR does not directly encode proteins, it has the function of controlling the translation activity of mRNA[9-11]. For example, internal ribosome entry sites (IRES) [12] are being actively studied and play an important role in recruiting ribosomes and translational initiation. The poly-A tail is involved in mRNA stability and translation initiation[13].

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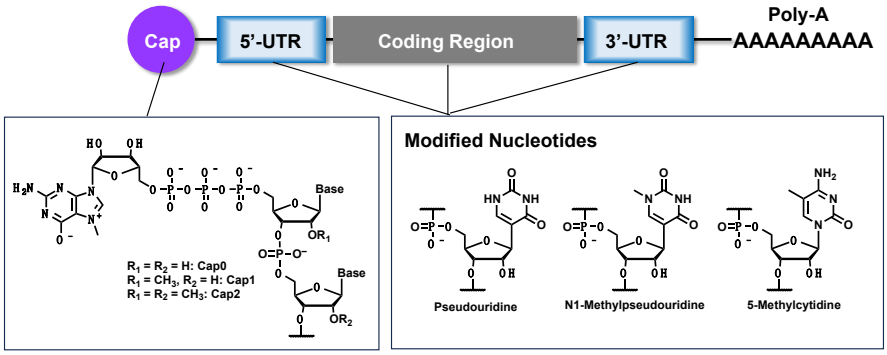

Figure 1. Structure and Established Chemical Modifications of mRNA.

On October 2023, Karikó and Weissman received the Nobel Prize in Physiology or Medicine for their discoveries important to the development of mRNA vaccines[14]. They discovered that mRNA therapeutics containing the natural uridine base showed a very high inflammatory response, whereas mRNA therapeutics in which the uridine base converted to pseudouridine or *N*1-methylpseudouridine suppressed the inflammatory response (Figure 1). This made it possible for the first time to apply mRNA to practical medicine, making a great contribution to humanity [15]. It has been suggested that mRNA therapeutics can apply not only to vaccines but also to the fields of protein replacement therapy and regenerative medicine. In this paper, we will discuss the current progress of mRNA therapeutics development and research toward regenerative medicine using mRNA.

2. Synthesis of mRNA

General mRNA synthesis methods are *in vitro* co-transcription, and post-capping using Vaccinia Capping Enzyme (VCE) [6]. In the *in vitro* co-transcription capping method, a cap analog called ARCA[16] or CleanCap[17] is added to a transcription reaction solution with nucleoside 5′-triphosphates (NTPs), and the transcription reaction is carried out by RNA polymerases in the presence of template DNA. The transcription reaction is initiated from the cap analogs yielding 5'-capped mRNA. However, the cap analogs and guanosine 5′-triphosphate (GTP) are competitively incorporated resulting in a mixture of capped mRNA and undesired uncapped mRNA. To overcome this problem, the authors developed a new mRNA synthesis method, named PureCap method, that can obtain completely capped mRNA [18]. In the PureCap method, an *in vitro* transcription reaction is performed using a novel cap analog with an *o*-nitrobenzyl group which functions as a hydrophobic purification tag, at the 7-methylguanosine moiety. *o*-Nitrobenzyl group is introduced only into the 5′-terminus of capped mRNA, making it significantly more hydrophobic than uncapped RNA. As a result, differences in retention time can be observed on reverse-phase high-performance liquid chromatography (RP-HPLC), making clear separation of capped and uncapped mRNA possible (Figure 2). It has been revealed that the PureCap method can produce highly purified mRNA with a Cap2-type structure, which was difficult to synthesize using conventional synthetic methods. Additionally, completely capped mRNA can be applied to the structure-activity relationship (SAR) study of between mRNA 5′-cap structure diversities and protein translation activity. Furthermore, the purified fully capped mRNA showed a translation activity more than 10 times higher than that of mRNA produced using conventional cap analogs. The authors aim to contribute to mRNA therapeutics research by making it possible to produce highly pure mRNA by applying the PureCap method.

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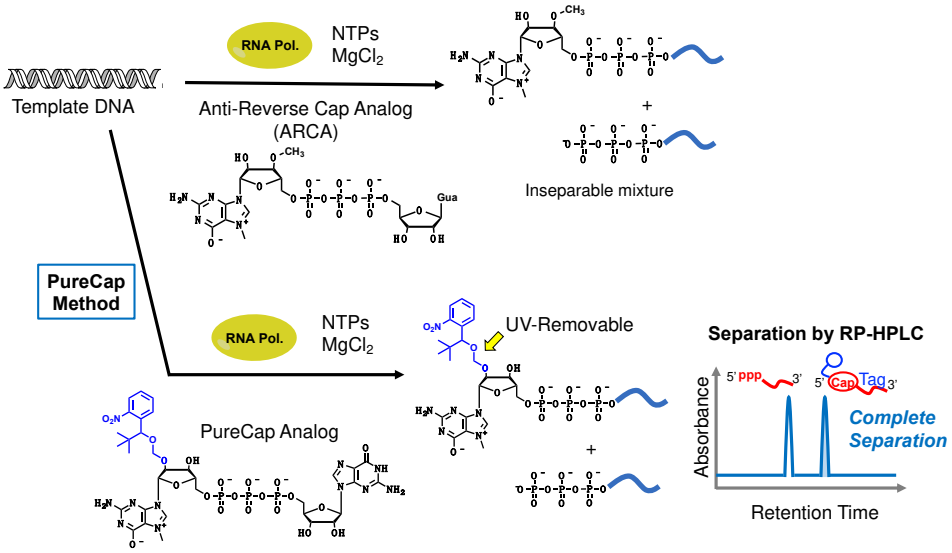

Figure 2. Synthetic Methods of mRNA by Using Co-Transcriptional Cap Analogs.

Capping method using VCE is also widely used for mRNA preparation. VCE is a capping enzyme derived from the vaccinia virus, which uses 7-methylguanosine 5'-triphosphate to add a cap structure to the 5' end of transcriptionally synthesized RNA[19]. VCE has 3 enzymatic activities, RNA triphosphatase, guanylyltransferase, and guanine methyltransferase activities, and can assemble the Cap0 structure. More recently, Ohno et al have focused on post-capping methods using VCE, and have investigated the introduction of various chemically modified cap structures[20]. They investigated the acceptability of chemical modifications when introducing a cap structure using VCE, and the effects of chemical modifications of the cap structures on translational activities and decapping resistance. The research progress related to mRNA synthesis is important for accelerating mRNA therapeutics development, and further studies are expected with the participation of many chemists in the future.

3. Key Technologies for mRNA Therapeutics

An early discovery regarding mRNA vaccine development was the demonstration in 1984 of mRNA synthesis by Krieg and Melton et al using a virus-derived RNA synthase [21]. Furthermore, in 1989, Malone et al showed that mRNA mixed with lipid droplets was taken into frog embryos, suggesting the possibility of externally adding mRNA as a drug [22]. However, it has been recognized that synthetic mRNA is generally unstable in serum and difficult to apply as a medicine or vaccine. Such mRNA instability has been overcome with the discovery of mRNA delivery techniques using lipid nanoparticles composed of phospholipids, cholesterol, ionized lipids, and PEG lipids. In the present day, lipid nanoparticles are an indispensable technology for the development of mRNA therapeutics. LNP formulates mRNA and protects it against nuclease digestion [23]. mRNA formulating LNP is incorporated into cells by the endocytic mechanism. The technology was developed primarily by Cullis et al. Since the 1990s, they have been working on the development of LNP as a technology to deliver short oligonucleotides, antisense oligonucleotides, that control gene expression in cells [24]. Nowadays, several oligonucleotides have been approved as LNP therapeutic agents for genetic diseases [25]. In 2012, Cullis et al started the experiment with applying the LNP technology to mRNA delivery. In 2012, Geall et al successfully prepared the first LNPs encapsulating an mRNA vaccine [26]. LNPs developed in this way are used in current COVID-19 vaccines.

The development of LNP has made it possible to relatively stably administer mRNA into cells. However, LNPs taken into cells by the endocytic mechanism reside within endosomes. Therefore, it is necessary to release mRNA from within the endosome, and the problem is that the endosomal escape efficiency is generally only about 2% at most [27]. Furthermore, although delivery to the liver and spleen has been achieved, delivery to other tissues is difficult, and it is necessary to develop LNPs

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with tissue specificity. To solve these problems, further research and development on LNP is important for the medicinal application of mRNA, and many researchers are getting involved[28].

Although mRNA therapeutics research has progressed with the development of LNP, the expression of inflammatory responses when mRNA is administered to cells has also been pointed out as a serious problem. Karikó and Weissman received the 2023 Nobel Prize in Physiology or Medicine, and their co-workers succeeded in solving this problem. Starting in the 1990s, they refined Malone's protocol and were able to demonstrate expression of therapeutic proteins in cells [29] [30]. However, in 1997, when trying to develop an mRNA vaccine against HIV and administering synthetic mRNA to mice, a severe inflammatory response occurred [31]. The reason for this phenomenon was considered to be that immune sensor receptors, including toll-like receptors, which are nucleic acid receptors present in the cytoplasm, recognized the administered synthetic mRNA as a non-self RNA[32]. Then, in 2005, they discovered that this inflammatory response could be suppressed by converting uridine in mRNA to a base-modified nucleoside called pseudouridine [33]. By replacing uridine 5'-triphosphate with pseudouridine 5'-triphosphate during *in vitro* transcriptional RNA synthesis, pseudouridine modification can be introduced into the entire mRNA[15]. In fact, in the COVID-19 vaccine, all of Moderna's mRNA has modified bases introduced. On the other hand, the development of mRNA therapeutics that do not use modified mRNA is also attracting attention. Hertlein and co-workers hypothesized that by adding the appropriate 5' cap structure to unmodified mRNA and removing all impurities, they should be able to create mRNA therapeutics that would be as effective as modified mRNA. In 2016, Heartlein, Anderson, and their co-workers showed that what matters is the quality of the RNA and that unmodified mRNA has greater activity than pseudouridine-modified mRNA if the mRNA is highly purified [34]. Our group has reported that mRNA synthesized using the PureCap method showed lower inflammatory responses and higher translational activity than conventional mRNA[18].

In this way, the development of mRNA vaccines involves a lot of developments in chemical technologies. Delivery methods, modified base introduction methods, and high-purity mRNA production methods are important not only for mRNA vaccines but also for regenerative medicine, and these technologies are being applied.

4. mRNA-Based Protein Supplementation for Regenerative Medicine

Regenerative medicine is a treatment method that restores function by regenerating tissues and organs lost due to accidents or diseases, and cell transplantation treatment using stem cells is known[35]. However, regenerative medicine requires a special cell culture facility, leading to rising medical costs, the risk of mutations occurring during cell culture, and slow supply speeds that depend on the time required for culture[36-38]. Stem cell is a general term for cells that have both self-renewal and multipotency and includes somatic stem cells (adult stem cells, tissue stem cells), Embryonic Stem Cells (ES cells), induced Pluripotent Stem Cells (iPSCs), etc. Somatic hepatocytes are cells that can modify and regenerate tissues by differentiating into new cells, and treatments using mesenchymal cells are being performed. ES cells are cells created by collecting cells from embryos that are in the process of becoming fertilized eggs and are used from fertilized eggs that were not used for infertility treatment[39]. iPSCs developed by Professor Yamanaka and co-workers in 2006[40, 41], is a kind of stem cell that has been artificially returned to an undifferentiated state by incorporating multiple genes into mature somatic cells and were the first in the world to undergo clinical research in 2014. In these current treatments, the patient's stem cells are differentiated into cells with the desired function outside the body, cultured, and then transplanted into the patient's tissue to restore that function[42]. By using the patient's cells, rejection after transplantation can be suppressed, but not all cells can be used. Additionally, it has been pointed out that cells may be difficult to proliferate and maintain *in vitro* due to limitations in their ability to proliferate. Therefore, regenerative medicine using mRNA is attracting attention[43]. Therapeutic effects are expected by injecting mRNA into cells and expressing proteins that lead to the regeneration of lost tissue.

Examples of regenerative medicine using mRNA drugs include heart failure treatment and fracture treatment. Moderna is actively working on developing mRNA treatments for heart

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failure[44]. Heart failure is a disease that occurs when blood vessels in the heart become clogged or hardened, and one treatment option is surgery to replace them with artificial blood vessels. In contrast, when an mRNA drug (AZD8601) encoding vascular endothelial growth factor (VEGF-A) is directly administered to a patient's myocardium, VEGF-A is produced from the administered mRNA, which can promote cardiac repair and regeneration [45]. Researchers at the Mayo Clinic are working on applications for fracture treatment. In a study using rats, researchers confirmed that administering mRNA encoding bone morphogenetic protein 2 (BMP-2), which promotes bone formation, to the fracture site promoted bone regeneration [46].

Most recently, Itaka et al succeeded in rapid bone regeneration in animal bone defect models by administering mRNA-encoding proteins that promote bone induction and angiogenesis [47]. They synthesized mRNA expressing 2 types: osteoinductive transcription factor (Runx2), a therapeutic protein that exhibits bone regeneration effects, and vascular endothelial growth factor (VEGF), a secreted protein that plays a role in angiogenesis. These mRNAs were administered to undifferentiated osteoblasts and their ability to induce osteogenic differentiation was evaluated. Even when Runx2-mRNA and VEGF-mRNA were administered alone, the expression of bone differentiation markers (osteopontin, osteocalcin, etc.) was increased, suggesting that not only Runx2 but also VEGF can induce bone differentiation. When both were administered simultaneously, even higher expression of osteogenic differentiation markers was observed, suggesting that both acts synergistically to induce osteogenic differentiation of cells. Next, the bone regeneration effects of these mRNAs were verified using rats in which a bone hole with a diameter of 4 mm was formed in the jawbone. As a result, even when Runx2-mRNA or VEGF-mRNA was administered alone, better bone regeneration was achieved in the bone defect compared to the no-treatment group or the control mRNA administration group. Furthermore, consistent with the results with cells, the most vigorous bone regeneration was observed in the group that received both in combination. Furthermore, histological evaluation suggested that VEGF-mRNA played a role in both angiogenesis in bone defects and osteoinduction, and when combined with Runx2-mRNA, the two acted synergistically.

Although some results have been reported in applied research on regenerative medicine using mRNA, there are still some challenges [48-58]. Only short peptide fragments and proteins can be expressed by mRNA, and tissue regeneration does not involve only peptides and proteins, but multiple factors. To link expressed peptides and proteins to tissue regeneration, it is required a deeper understanding of the tissue regeneration mechanism itself (Figure 3).

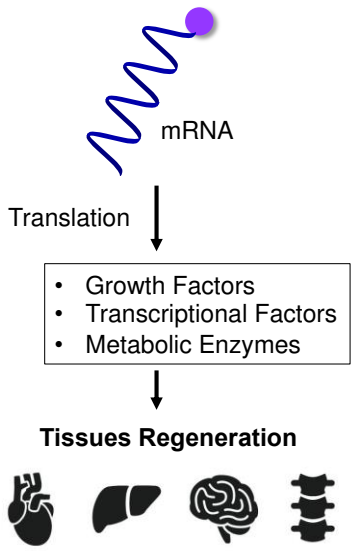

Figure 3. mRNA-Induced Regeneration.

5. mRNA for Cell Reprogramming

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ES cells are created by collecting cells from an embryo that is in the process of becoming a fertilized egg, and there are ethical issues involved [59]. On the other hand, iPSCs are cells with ES cell-like pluripotency and proliferation ability that are established by introducing several types of factors into somatic cells and culturing them [60]. Unlike ES cells, which require early embryos, they can be created from all somatic cells, so there are no ethical issues or fears of immune rejection. iPSCs can be differentiated into cells of almost any tissue or organ, so they are expected to have a wide range of applications [61]. For example, regenerative medicine that creates damaged tissues and organs from iPSCs, technology that easily examines activity and safety by creating disease model cells and administering drug candidates, and differentiation of patient-derived cells into disease-related cells. To generate iPSCs, reprogramming technology is required to initialize epigenetic modifications of differentiated cells. iPSCs were first developed by Yamanaka and co-workers [40]. They discovered four genes called Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) and injected them into fibroblasts using retroviral vectors to induce cell reprogramming. However, with retrovirus-based techniques, there is a possibility that the transgene will integrate into nuclear genomic DNA. Among the Yamanaka factors, there is concern that if Klf4 and c-Myc, which are involved in carcinogenesis, are accidentally inserted into genomic DNA, there is a risk of disease onset and tumor formation [62] [63].

Since then, various techniques for creating iPSCs have been developed (Table 1). For example, methods for introducing plasmid vectors and proteins are also known, but although these are safer than viral vectors, their cell introduction efficiency and reprogramming efficiency are insufficient [64]. Other reprogramming techniques using small molecules have been reported, such as ACTH 1-24 peptide (fragment of adrenocorticotropic hormone) [65, 66], A83-01 (selective inhibitor of Activin receptor-like kinase) [67], CHIR99021 (inhibitor of Glycogen synthase kinase 3β) [68], SU5402 (FGF receptor inhibitor) [69], DAPT (inhibitor of γ-secretase) [70], LDN193189 (inhibitor of bone morphogenetic protein) [71], PD0325901 (selective inhibitor of MEK/MAPKK) [72, 73], and SB431542 (activin receptor-like kinase inhibitor) [74, 75], SU5402 (tyrosine kinase inhibitor specific to fibroblast growth factor receptor) [76], and Thiazovivin (improves the survival rate of human ES cells against trypsin treatment) [77]. Although reprogramming methods using such small molecules are extremely simple and innovative methods, it is necessary to confirm the required dosage and the presence or absence of cytotoxicity [78-80]. Furthermore, it is difficult to say that existing small-molecule reprogramming can cover all applications. It is necessary to expand the types of proteins and receptors that can be targeted and to search for further compounds.

MicroRNA-induced reprogramming from somatic cells by injecting the mir-302 family (mir-302a, 302b, 302c, 302d, pre-microRNA cluster) is also reported[81]. The mir-302 family is highly expressed in slowly proliferating human ES cells, and rapidly decreases as the cells differentiate and proliferate. Reprogramming using microRNAs is an effective method, but the discovered microRNAs, that are involved in reprogramming, are only mir-302[81], mir-372[82], miR-17-92 cluster[83], mir-19[84], mir-524[85], mir-371[86], mir-31[87], therefore it is necessary to explore the applicability of various microRNAs in the future [88].

Table 1. Advantages and disadvantages of cell reprogramming strategies.

Reprogramming Methods	Advantages	Disadvantages
Retroviral Vectors	• Well-investigated and established • High cellular introduction efficiency	• Undesired transgene into nuclear genomic DNA • Carcinogenesis and a risk of tumor formation
Plasmids Vectors	• Low risk of genome insertion	• Insufficient cellular introduction and reprogramming efficiency
Small Molecules	• Simple handling	• Required relatively high-dosage

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	• Low cost • High cellular introduction efficiency	• Necessary to care for dose-dependent cytotoxicity • Difficult to cover all applications
microRNA	• Fast reprogramming • No risk of genome insertion	• Low physiological stability • Fewer examples relative to other methods
mRNA	• Fast reprogramming • No risk of genome insertion and tumor development • High reprogramming efficiency	• Required more effective intracellular delivery methods • Required multiple injections (every day)

Therefore, a reprogramming method using mRNA has been developed in recent years (Figure 4a)[89-108]. Similar to mRNA vaccines, the mRNA reprogramming method creates iPSCs by introducing mRNA containing genetic information for creating cell reprogramming factors into cells and expressing the target proteins. Compared to the retrovirus delivery method of DNA encoding reprogramming factors, mRNA is unstable within cells and degrades gradually, so it does not remain in iPSCs[109]. As a result, mRNA does not cause mutations in genomic DNA, there is no risk of tumor development. It is also known that the reprogramming efficiency is higher than existing methods using viral vectors[93]. In reprogramming using mRNA, the introduction of chemical modifications is recommended, just as when using mRNA as a vaccine[110-112]. Coupled with this year's Nobel Prize in Physiology or Medicine, the use of chemically modified mRNA in COVID-19 vaccines has been attracting attention[15, 34], but the application of chemically modified mRNA to cell reprogramming has also been considered since around 2010[93]. LNP formulations similar to mRNA vaccines, electroporation, and liposomes, have been reported as cellular introduction methods. The mRNA used for cell reprogramming is loaded with 5-methylcytosine, pseudouridine, and 5'-Cap structure[110]. In addition to OCT4, SOX2, KLF4, and c-MYC, LIN28 is commonly added as a reprogramming factor encoded by mRNA. Examples of mRNA-mediated reprogramming that have been reported to date include somatic cells such as fibroblasts [113-118], adipose-derived stem cells (ADSCs) [119], bone marrow-derived mesenchymal stem cells (BMSCs) [120], and amniotic fluid stem cells [121]. On the other hand, there are challenges for creating iPSCs using mRNA from blood cells which are generally used to create iPSCs because it is easy to culture. mRNA needs to be injected every day due to its biological instability. Blood cells are resistant to cationic lipids [122], so lipofection cannot be used, and electroporation is the method of choice, but multiple electroporation increases the risk of cell death[123]. Therefore, it is necessary to improve the intracellular stability of mRNA to ensure sustainable protein expression and reduce the number of administrations. Although there have been some successful cases of reprogramming using mRNA, there are limitations in its applicability, and further research and development are necessary.

A recent one of the succeeded examples of mRNA-induced reprogramming is establishment of iPSCs derived from Alzheimer's disease patients. In 2022, Supakul et al established iPSCs for patients with mild Alzheimer's disease using an iPSCs establishment kit sold by Reprocell biotech company[124]. They succeeded in establishing iPSCs from cells collected from a patient's urine by administering an mRNA cocktail by lipofection. Until now, most iPSCs have been produced from fibroblasts found in the skin or blood. Since urine is easier to collect than skin or blood, it is expected that it will become easier to generate iPSCs from patients with diseases and children, which were previously difficult to collect[125]. Research using the generated iPSCs is thought to provide clues to solving social problems associated with aging, such as the increasing number of patients with dementia. In the future, by accumulating more examples of reprogramming using mRNA, it is expected that this will lead to the elucidation of the mechanisms of development of various diseases and their application to therapeutic research.

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6. mRNA-Induced Cell Differentiation from iPSCs

To apply iPSCs to regenerative medicine, technology to induce differentiation of reprogrammed iPSCs is also required. There are broadly 3 methods for inducing differentiation of iPSCs into target tissue cells. The first is to prepare a cell culture medium containing a combination of various cell growth factors, cell differentiation factors, and small molecule drugs, and to culture pluripotent stem cells in this medium[126]. In many cases, cells are sequentially differentiated by exposing them to different culture solutions one after another. The second method is to create clusters or aggregates of pluripotent stem cells, which allow the cells to change and interact with each other within the cell cluster (self-organization) to differentiate into various cells[127, 128]. These methods require multiple steps, so it takes time to differentiate into the desired cells, and it is necessary to confirm whether the cells are the same as the original cells that exist in the body. The third method takes advantage of the fact that genes called transcriptional regulators determine the differentiation state of cells, and induces cell differentiation by activating these genes in pluripotent stem cells[129-131]. This method directly manipulates transcriptional regulatory factors that determine the differentiation state of cells, resulting in rapid differentiation. However, since it requires genome editing technology such as the clustered regularly interspaced short palindromic repeats /CRISPR associated proteins（CRISPR/Cas9） system, there is a risk of cancer or malfunction due to the introduction of off-target mutations that cleave and edit sequences other than the target sequence[132, 133]. On the other hand, these problems may be overcome by introducing mRNA encoding transcriptional regulatory factors. Due to the reason, research on differentiation induction using mRNA is attracting attention (Figure 4b).

An example of the usefulness of differentiation induction using mRNA is a report in 2017 that showed that neurons could be rapidly generated from iPSCs derived from Gaucher disease patients[134] [135]. Gaucher disease is caused by mutations in the glucocerebrosidase (GBA) gene, which is an enzyme that decomposes the glycolipid glycosylceramide [136]. Glycolipids cannot be broken down, and the main symptoms include enlargement of the liver and spleen, anemia, and thrombocytopenia, but neurological symptoms may also appear, and are classified into 3 types depending on their presence and severity (I ~Type III) [137]. Although type I Gaucher disease is a relatively mild type and does not cause neurological symptoms, it is known that the risk of developing Parkinson's disease is extremely high at 9% to 12% as people get older [138]. It has been suggested that excessive accumulation of glycolipids in the brain influences the onset of Parkinson's disease, but the mechanism is unknown. They investigated the relationship between glycolipid accumulation and α-synuclein using nerve cells generated from iPSCs derived from type I Gaucher disease patients[134]. When they synthesized and administered mRNA encoding a transcription factor that promotes neural differentiation, they were able to confirm the accumulation of glycolipids just 10 days after the start of differentiation. Although α-synuclein aggregation had not been detected at this point, it was found that the phosphorylation modification of α-synuclein involved in it was enhanced, making it susceptible to neurodegeneration. In addition, by forcing the normal GBA gene to promote glycolipid degradation, α-synuclein phosphorylation could be suppressed, suggesting that glycolipid accumulation is directly involved in the onset of Parkinson's disease. On the other hand, it has been revealed that with conventional neural differentiation techniques, glycolipids accumulate after 60 days after the start of differentiation. With this method, it takes more than a month for neurons to form, so it takes even longer to detect the phenotype. Thus, it was shown that the synthetic mRNA differentiation method not only enables short-term differentiation but also is effective in rapidly reproducing disease-related phenotypes.

A recent research result is the successful creation of sperm stem cell precursors from iPSCs of the marmoset, an experimental primate animal[139]. They induced marmoset iPSCs to become primordial germ-like cells (PGCLCs) by transfecting them with mRNA encoding the SOX17 gene, a master regulator of primordial germ cells. The created marmoset PGCLCs were transplanted under the kidney capsule of an immunodeficient mouse, and they succeeded in developing pre-spermatogonia (sperm stem cell precursors). Gene expression and DNA methylation analysis revealed that it almost faithfully reproduces the *in vivo* germ cell development process (up to the newborn stage), and the newly developed method is useful for research on early germ cell

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development in primates. In primates including humans, sperm production from iPSCs has not yet been achieved, and the process has only progressed to the production of pre-spermatogonia. We hope to advance development toward sperm production, which will lead to the investigation of the causes of infertility and applications in reproductive medicine in the future.

7. mRNA for Both Cell Reprogramming and Differentiation Induction

We introduced examples in which mRNA is used for reprogramming and differentiation induction. It is also possible to generate functional tissues by administering mRNA as a differentiation-inducing factor to iPSCs that have been reprogrammed and established with mRNA. That is, mRNA can act as both a reprogramming and differentiation-inducing factor. In 2010, Warren et al reported the transformation of fibroblasts into embryonic stem cells, which then differentiated into contractile muscle tissue, using modified mRNAs[118]. They synthesized mRNAs encoding the Yamanaka factors Oct4, Sox2, Klf4, and c-Myc. In this mRNA, cytidine was completely replaced with 5-methylcytidine and uridine was completely replaced with pseudouridine. When these mRNAs were administered to cells, immunostaining showed that the Yamanaka factor was expressed and localized in the nucleus. Furthermore, protein expression by this mRNA peaks 12 to 18 hours after introduction, and then rapidly decreases, indicating that the mRNA is degraded within 10 hours after administration and does not remain in the cells. They have also successfully reprogrammed somatic cells. A 5-factor cocktail (KMOSL) containing 4 Yamanaka factors plus mRNA encoding LIN28 was used in Detroit 551 (D551), MRC-5 fetal fibroblasts, BJ neonatal fibroblasts, and primary cells from adult patients with cystic fibrosis. When the KMOSL-mRNA cocktail was introduced daily into four cultured skin-derived fibroblast-like cells (CF cells), many human ES cell-like colonies appeared, and more than 10 iPSCs from each somatic cell line appeared. Furthermore, the established iPSCs express OCT4, SOX2, NANOG, hTERT, the Oct4 gene is demethylated, and have pluripotency-related genes including SOX2, REX1, NANOG, OCT4, LIN28, and DNMT3B. Transcripts were elevated to levels comparable to those of human ES cells and showed that mRNA-reprogrammed iPSCs are more similar to human ES cells than to virus-generated iPSCs. In addition, BJ fibroblasts introduced with the 5-factor mRNA cocktail had a conversion efficiency of 2% or more to iPSCs regardless of the presence or absence of the Rho-associated kinase (ROCK) inhibitor Y-27632. It was found to be 2 orders of magnitude more efficient than conventional virus-based methods. Next, we introduced KMOS-mRNA or KMOS retrovirus into dH1f fibroblasts in parallel, and found that ES cell-like colonies began to appear after 2 weeks in those into which mRNA had been introduced, and on day 16, transfection occurred. By the last day of transfection, there was an outgrowth of ES cell-like colonies, whereas when using the KMOS retrovirus, no ES cell-like colonies appeared by this time point and only from day 24 after gene transfer. The iPSCs establishment efficiency by counting the beginning of colony appearance and TRA-1-60 positive colonies was 1.4% and 0.04% for KMOS-RNA and KMOS retrovirus, respectively, with KMOS-mRNA being 36 times more efficient. Fibroblast growth factor FGF was removed from the medium of the iPSC line established using mRNA, serum was added, the medium was spread on a gelatin coat, and mRNA encoding the muscle differentiation-inducing MyOD gene was introduced. An additional 3 days of culture under low serum conditions showed that myogenin and MyHC double-positive myotubes appeared with high efficiency. These results indicate that mRNA directly differentiates pluripotent stem cells into terminally differentiated cells.

8. mRNA-Induced Direct Reprogramming without Passage through Pluripotent Stem Cells

Induction into tissue cells via ES cells and iPSCs is expected to be applied to regenerative medicine. However, there are concerns about the risk of tumor formation due to undifferentiated cells and the low engraftment efficiency of treatments using these pluripotent stem cells[107]. Direct reprogramming is attracting attention as a reuse method to solve the problems of stem cell-derived cell transplantation[140]. This is a method for directly producing desired cells from fibroblasts, etc., without using iPSCs, and it is possible to produce tissues *in vivo* by introducing genes into the target sites. The concept of direct reprogramming was proposed in 1987. The first report was that MyoD

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was identified as a master factor for skeletal muscle and that by forcing the MyoD gene to be expressed in fibroblasts, they succeeded in producing fibroblasts, which are the precursors of skeletal muscle[141].

In 2010, Ikeda et al were able to coax fibroblasts into beating heart muscle. Using retroviral vectors, they revealed that Gata4, Mef2c, and Tbx5 genes (GMT) are essential for myocardial direct reprogramming. By introducing these 3 factors into fibroblasts, a cardiac muscle-specific gene expression pattern was observed, as well as the expression of cardiac muscle-specific structural proteins such as α-actinin and cardiac troponin (cTnT), and sarcomere structure[142]. Since then, efforts have been made to find factors that promote direct reprogramming. In 2014, Muraoka et al reported the use of microRNA as a factor that promotes direct reprogramming of the heart muscle[143]. They revealed that adding miR-133 to GMT efficiently induced myocardium in a short period. Enhancement of direct reprogramming using lower-cost small molecules is also being investigated.

In 2015, Zhao et al hypothesized that fibroblast plasma maintenance mechanisms inhibit reprogramming into the myocardium. By using the small molecules that suppress TGF-β and ROCK pathways, which promote fibrosis, they succeeded in improving the efficiency of guiding mouse fetal fibroblasts to the myocardium[144]. Furthermore, in 2019, Muraoka et al showed that the nonsteroidal anti-inflammatory drug diclofenac suppresses age-related inflammation, thereby improving the efficiency of direct reprogramming from adult mouse fibroblasts to myocardium, which has been difficult to induce[145]. Cardiomyocyte induction using only small molecules without using any genes has also been reported. In 2016, Cao et al reported that they could induce human fibroblasts to become functional heart muscles by introducing 9 small molecules[146]. This method has the advantage of being safer and relatively easy to control cell culture conditions because it does not use genes or viral vectors. On the other hand, direct reprogramming using mRNA, which has less risk of gene insertion, is also attracting attention (Figure 4c).

In 2014, Simeonov et al reported direct reprogramming of human fibroblasts to Hepatocyte-like cells using synthetic mRNA[147]. They confirmed the generation of Hepatocyte-like cells by lipofection of 3 types of mRNAs consisting of HNF1A and 2 genes among FOXA1, FOXA3, and HNF4A, into human fibroblast cells in an optimized haptic growth medium. In addition, in 2017, Pham et al achieved direct reprogramming of endothelial progenitor cells from skin fibroblasts using mRNA encoding the ETV2 gene[148]. Endothelial progenitor cells are important for angiogenesis, but their abundance in the human body is limited. With the development of this technology, it is expected that it will be applied to autologous transplantation by administering mRNA to skin fibroblasts. Only recently has research been conducted on direct reprogramming using mRNA. Several applied studies for regenerative medicine using model animals have also been reported. In 2021, Kaur et al demonstrated that direct reprogramming from non-cardiomyocytes to cardiomyocytes by using mRNAs encoding 4 cardiac reprogramming genes (Gated, Mef2c, Tbx5, Hand2) and 3 reprogramming-helper genes (dominant-negative-TGFb, dominant-negative-Wnt8a, acid ceramidase). Using a lineage-tracking model, they administered an mRNA cocktail at the time of myocardial infarction and found that 25% of cardiomyocyte-like cells in the scar area were reprogrammed. As a result, it was observed a significant improvement in cardiac function, scar size, long-term survival rate, and capillary density. Through this research, we can expect the development of safe and highly efficient regenerative medicine for ischemic diseases using mRNA[149]. In August 2023, Qabrat et al demonstrated that direct reprogramming of mouse fibroblasts to myogenic progenitor cells (iMPC) by administering MyoD-expressing mRNA and small molecules that promote myoD expression (cyclic AMP agonist Forskolin, TGF-β receptor inhibitor RepSox, and GSK3 inhibitor CHIR99210). The generated iMPCs were shown to express a series of myogenic stem cell markers and differentiate into contractile myotubes. Furthermore, iMPCs strongly engrafted into skeletal muscle in a mouse model of Duchenne muscular dystrophy and restored dystrophin expression in hundreds of myofibers[150].

To improve the efficiency of direct reprogramming using mRNA, it is important to innovate the technology for introducing mRNA into cells. In 2015, Lee et al were able to induce cardiomyocyte

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cells from cardiac fibroblast cells in mice by adding polyarginine-fused heart-targeting peptide (CRPPR-R9) to lipofectamine, a common lipofection reagent. They administered mRNA encoding the Gata4, Mef2c, and Tbx5 genes (GMT) for 2 weeks. They showed that by adding CRPPR-R9, the efficiency of intracellular introduction was approximately 2 times higher than that of conventional lipofection, and as a result, translational efficiency was confirmed to be approximately 3 times higher. In this way, the development of highly efficient delivery technology is expected to lead to direct reprogramming within the human body[151].

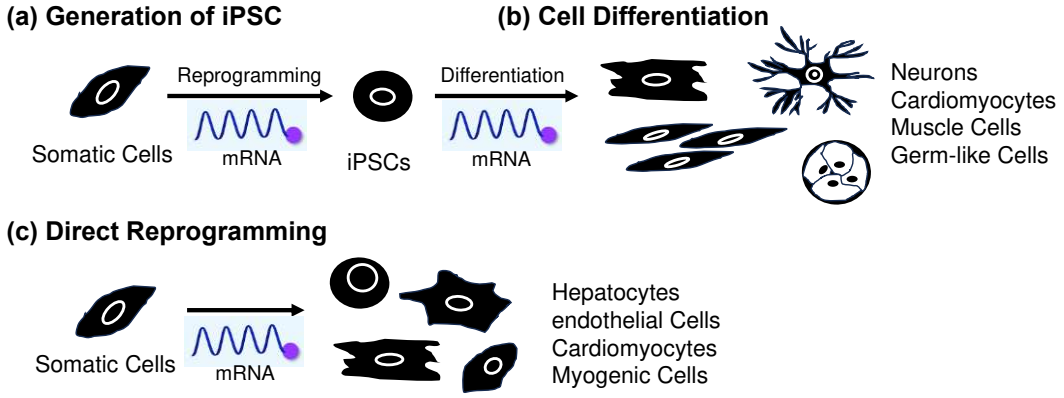

Figure 4. Application of mRNA for (a) cell reprogramming, (b) differentiation and (c) direct reprogramming.

9. mRNA-Based Purification Method of iPSCs and iPSC-Derived Cells

iPSCs can differentiate into various cells, but after induction of differentiation, they also contain cells other than the target cells. Therefore, cells are sorted by identifying antigens on the cell surface using a flow cytometer[152, 153]. However, when using a flow cytometer, there is a possibility that unintended cells or impurities may be mixed in during the operation of sorting each cell, which is expensive, and it is difficult to prepare the necessary amounts of cells for transplantation. Also, it may take several hours to several days to get the required amounts of cells. Furthermore, it is often difficult to identify antigens specific to target cells. iPSCs express microRNAs specific to cell tumors, and Saito et al have developed an mRNA switch technology that can identify these microRNAs and control gene expression, it was shown that purification of iPSCs is possible (Figure 5)[154].

They synthesized mRNAs in which the genes for expressing Barnase (Bn), a lethal ribonuclease that causes cell death, and Barstar (Bs), a protein that inhibits Bn, were incorporated into the switch[155]. When purifying HeLa cells, we introduced the Bn gene into the microRNA response OFF switch that responds to miR-21, and the Bs gene into the microRNA response ON switch. HeLa cells that have miR-21[156] produce Bs protein that inhibits Bn due to the miRNA response ON switch, and cells that do not have miR-21 produce Bn due to the microRNA response OFF switch, resulting in cell death (Figure 5a). When purifying 293FT cells, they introduced the Bs gene into the microRNA response OFF switch that responds to miR-21, and the Bn gene into the microRNA response ON switch. In 293FT cells that do not have miR-21, the Bs protein that inhibits Bn is produced by the miRNA response OFF switch, and in HeLa cells that have miR-21, Bn is produced by the microRNA response ON switch, resulting in cell death (Figure 5b). In addition, as a method to purify iPSCs established by reprogramming HeLa cells, they introduced the Bn gene into the microRNA response OFF switch that responds to miR-302a, and the Bs gene into the miRNA response ON switch. iPSCs that have miR-302a produce Bs protein that inhibits Bn due to the miRNA response ON switch, and HeLa cells that do not have miR-302a produce Bn due to the miRNA response OFF switch, resulting in cell death.

In this way, they achieved high-purity cell sorting without using a flow cytometer. This method can be applied to the purification of various cells. For example, they have also succeeded in purifying cardiomyocytes differentiated from iPSCs. This technology takes advantage of the characteristics of mRNA, which has a low risk of insertion into the genome is easily degraded within cells, and does

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not remain, making it a highly safe and practical method for purifying various cell types for transplantation.

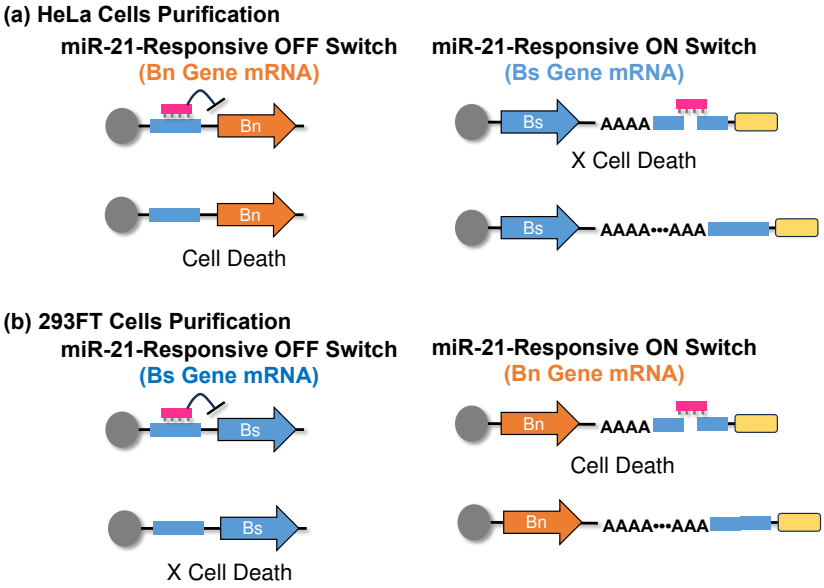

Figure 5. Purification of specific cells by microRNA-responsive mRNA switch system: (a) Purification of HeLa cells, Hela cells have miR-21 which inhibits the expression of Bn, and express Bs to inhibit Bn; (b) Purification of 293FT cells, 293FT cells doesn't have miR-21, they express Bn and inhibit Bs.

5. Conclusions

In this paper, we outlined the progress of mRNA therapeutics design and synthesis methods and introduced the application of mRNA to vaccines, cell reprogramming, differentiation, and regenerative medicine. The medical application of mRNA has been developed over many years of scientific and technological progress. The intracellular delivery technology of mRNA and the introduction of chemically modified bases that exhibit anti-inflammatory effects, which are currently in practical use as vaccines, are important technologies not only for mRNA vaccines but also for cell reprogramming and differentiation. Regenerative medicine using mRNA has significant advantages over conventional methods in terms of manufacturing cost, manufacturing speed, cell reprogramming efficiency, differentiation efficiency, safety, etc. Additionally, once the mRNA production method, delivery technology, and chemical modifications, are established, a variety of applications can be expected by simply changing the introduced gene to match the target. For the future development of this field, it will be important to develop new science and technology related to mRNA therapeutics and practical mRNA production technology, and the authors are also working on the development of these technologies [18, 157]. Application of mRNA to regenerative medicine requires research and development of methods for separating differentiated and undifferentiated cells using the mRNA switch technology recently developed by Saito et al, and direct reprogramming that does not involve pluripotent stem cells[154]. In particular, it is expected to be applied to therapeutic techniques that can regenerate and restore dysfunction in intractable diseases such as neurodegenerative diseases and various fibrotic diseases that are difficult to treat with existing technologies. To this end, it is important to conduct further basic research on regenerative medicine using mRNA and collect applicable examples.

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