Molecular Therapy
Review

mRNA-Based Genetic Reprogramming

Luigi Warren[1] and Cory Lin[1]

[1]Cellular Reprogramming, Inc., Pasadena, CA, USA

The discovery that ordinary skin cells can be turned into pluripotent stem cells by the forced expression of defined factors has raised hopes that personalized regenerative treatments based on immunologically compatible material derived from a patient's own cells might be realized in the not-too-distant future. A major barrier to the clinical use of induced pluripotent stem cells (iPSCs) was initially presented by the need to employ integrating viral vectors to express the factors that induce an embryonic gene expression profile, which entails potentially oncogenic alteration of the normal genome. Several "non-integrating" reprogramming systems have been developed over the last decade to address this problem. Among these techniques, mRNA reprogramming is the most unambiguously "footprint-free," most productive, and perhaps the best suited to clinical production of stem cells. Herein, we discuss the origins of the mRNA-based reprogramming system, its benefits and drawbacks, recent technical improvements that simplify its application, and the status of current efforts to industrialize this approach to mass-produce human stem cells for the clinic.

Just over a decade has elapsed since the publication of Shinya Yamanaka's groundbreaking work[1] showing that ordinary skin cells can be "reprogrammed" by the expression of a cocktail of transcription factors into induced pluripotent stem cells (iPSCs) capable of giving rise to any cell or tissue of the body. Already, the first regenerative therapies based on these cells, focused on age-related macular degeneration, ischemic heart disease, and Parkinson's disease, have progressed to the stage of clinical trials.[2] The advent of cellular reprogramming holds out the tantalizing prospect it might be possible to turn a patient's own cells into a limitless supply of physiologically rejuvenated, immunologically compatible stem cells that can be coaxed to become specialized cells, tissues, and organs for transplant back into the donor, enabling new treatments for a wide range of diseases and for the maladies of old age.

Clearly, there is a long road ahead before this futuristic vision can be fully realized, with many technical, financial and regulatory obstacles to be overcome. A major hurdle to any clinical application of iPSCs made with Yamanaka's original method was its dependence on integrating viral gene expression vectors to effect reprogramming, as the resulting heritable changes to cellular DNA would entail an unacceptable risk of tumorigenicity were the iPSCs or their differentiated progeny to be transferred into a human host. The goal of achieving "footprint-free" reprogramming obsessed the field for several years in the wake of Yamanaka's breakthrough. This once-daunting challenge can now be considered a solved problem. One of the first compelling solutions presented relies on the sustained delivery of synthetic mRNA encoding Yamanaka's reprogramming factors. Today, mRNA reprogramming vies with other well-established "non-integrating" methods, but it remains one of the most promising ways of making pluripotent stem cells for clinical use, based on the unambiguously transient character of the vector, the superior speed and efficiency of iPSC generation it provides, and the supple control it affords over reprogramming factor dosing, stoichiometry, and time course. This review surveys the emergence of mRNA reprogramming, looking at the roadblocks that had to be circumvented to bring it to fruition, improvements that have been made to the method since it was first described, areas where work remains to be done to address outstanding limitations, and progress toward the industrialization of this technique and its application in a clinical setting.

It is appropriate here to recap the general character of cellular reprogramming, highlighting why techniques that alter the targeted cells' DNA were employed in its early embodiments, and why this is a problem from a clinical perspective. Yamanaka asked if there is a way to take a differentiated cell such as a skin fibroblast and revert its gene expression profile back to an early embryonic state, so it becomes a blank slate with the potential to produce any of the cell lineages that make up the body. That this might be possible in principle is implicit in the fact that changes in gene expression rather than changes in DNA sequence underpin almost all known changes in cellular lineage and phenotype during development. More concretely, Sir John Gurdon's work in the 1960s on somatic cell nuclear transfer revealed that the global expression profile of a nuclear genome isolated from a differentiated cell can be reset when it is transferred into an enucleated egg, presumably by factors such as proteins in the cytoplasm of the egg, allowing the formerly specialized genome to support development of the entire organism.[3] Subsequently, decades of research into the character of cellular differentiation have identified a class of nuclear proteins designated "transcription factors" that regulate the overall gene expression profile of every cell. In many cases, it has proved possible using classical genetic techniques to pinpoint specific transcription factors or small networks of cross-regulating factors that act as master regulators of cell fate. There were also a few published experiments, going back to the 1980s, in which transgene constructs were used to ectopically express master regulator factors within cells *in vitro*, resulting in broad phenotypic changes or putative

https://doi.org/10.1016/j.ymthe.2018.12.009.

Correspondence: Luigi Warren, Cellular Reprogramming, Inc., 2265 E. Foothill Boulevard, Pasadena, CA 91107, USA.
E-mail: luigi.warren@cellular-reprograming.com

cell-lineage conversions.[4] Notably, transient expression of the muscle-specific transcription factor MyoD was shown to induce cultured fibroblasts to undergo a remarkable and enduring remodeling and conversion into multinucleate myotube-like cells.[5]

The emergence of retroviral gene expression vectors as a powerful method for producing strong, heritable ectopic gene expression in cultured cells gave Yamanaka the means to conduct a large-scale screen for transcription factors that might trigger differentiated cells to activate genes associated with embryogenesis and pluripotency. Focusing on factors known to be associated with the embryonic state, Yamanaka transduced random combinations of transcription factor expression cassettes into cultured fibroblasts. He went on to identify the factors that had integrated into the genome of emergent colonies expressing selection markers indicative of embryonic reversion, then winnowed down these factors by a process of elimination to find especially potent factor combinations. He discovered that a cocktail of four factors (the so-called Yamanaka factors: Oct4, Sox2, Klf4, and c-Myc) can reliably reprogram a small fraction of murine and human fibroblasts into cells that are almost indistinguishable from the embryonic stem cells (ESCs) cultured from early embryos.[1,6,7] These rapidly growing, immortalized cells form compact colonies that can be picked, replated, and expanded, then differentiated under appropriate conditions to produce cells representative of all three embryonic germ layers (mesoderm, ectoderm, and endoderm), satisfying the basic criteria for pluripotency.

The ability of integrating retroviral vectors to sustain gene expression at a high level over multiple rounds of cell division was crucial to the success of Yamanaka's strategy. Even the modest levels of reprogramming efficiency seen in this early work, in which on the order of 0.01% of fibroblasts gave rise to iPSC clones, required weeks of continuous co-expression of the most potent factor combination. Expression of reprogramming factors from the transgenes does not have to be sustained indefinitely because they gradually activate a self-reinforcing "pluripotency network" of endogenous transcription factors that maintains the global embryonic gene expression pattern. In the retroviral system, native genome-defense pathways eventually methylate and silence the transgenes, which is crucial because their continued forced expression would inhibit differentiation of the iPSCs. Nonetheless, the presence of these transgenes in iPSC genomes entails serious risks from the standpoint of clinical application. In the first place, the silencing of the retroviral cassettes is not always an irreversible process. Sporadic reactivation of the Yamanaka transgenes, some of which are known to have oncogenic and immortalizing effects, could easily lead to the formation of tumors. Even the disruption of native DNA sequence brought about by the integration of retroviruses at random genomic sites bears some risk of dysregulating normal gene expression and, again, raises the specter of tumorigenicity.

The scientific community swiftly embraced the potential for applying iPSCs to make almost any type of cell with any desired genetic background for use in developmental studies, disease modeling, and drug screening. For these types of research applications, the problem of the carryover of integrated transgenes from the reprogramming process is a marginal concern. Still, it was also recognized that novel reprogramming methods would have to be developed to overcome the problem of genome modification before iPSCs could become the basis for a new era of personalized regenerative medicine.

The first steps in this direction involved the use of two well-established, non-integrating DNA-based gene expression vectors, plasmids and adenovirus.[8,9] Owing to the relatively inconstant gene expression afforded when these vectors are delivered into rapidly dividing cells, these substitute vectors gave even lower iPSC yields than integrating retrovirus and lentivirus. This drawback was subsequently addressed by the development of plasmid-like vectors called "episomes," which incorporate a eukaryotic origin of replication to promote replication of the vector with the host cell.[10] Nonetheless, all of these DNA-based reprogramming vectors suffer from the basic weakness that recombination between vector and genome can occur, albeit at very low frequency, implying a need for rigorous screening, possibly involving whole-genome sequencing, before iPSCs made using these systems can be exploited in a clinical setting.[11–13]

An end run around the problem of genome modification was heralded in 2009 by reports of reprogramming using specially modified Yamanaka factor proteins supplied to target cells via culture media rather than expressed from vectors introduced into the cells.[14,15] The chimeric factors used in this system incorporated "cell-penetrating peptide" moieties to promote their attachment to the cell membrane, leading to subsequent endocytic internalization of the protein by the cells. Unfortunately, this system suffered from extremely low iPSC induction efficiency as originally presented, a hurdle that has not been fully overcome in the years since and that may reflect inherent technical limitations of the protein transduction system.[16] There has since been only minimal adoption of this technique.

Early hopes that a small-molecule-based reprogramming system might by developed to overcome the need for ectopic gene expression altogether have yet to be convincingly realized, at least in the human system.[17] This is probably because of the complexity of the genetic network remodeling required to achieve the induction of pluripotency, as suggested by the fact that a four-transcription factor cocktail is typically required to produce iPSC colonies with anything but a vanishingly low level of efficiency.

As of now, the prime candidates to have emerged for a reasonably efficient, clinically relevant reprogramming system that sidesteps the issue of genomic modification are all RNA based. These include, principally, systems based on synthetic mRNA, Sendai virus (a cytosolic virus with a completely RNA-based life cycle), and synthetic RNA "replicons" adapted from alphaviruses.[18–20] Because this review is focused on mRNA reprogramming, we will only touch on the characteristics of the Sendai and replicon systems here. Both employ self-replicating vectors that give a prolonged burst of transgene expression after being introduced into target cells in a single delivery step.

Therefore, they offer the convenience of "single-shot" reprogramming. The long-lasting transgene expression resulting from the burst of vector replication also allows them to reprogram cell types that can only be transfected with low efficiency, including cells of the blood lineages. The problem of assuring complete vector clearance after reprogramming is probably the main drawback to these systems from a clinical standpoint.[13]

mRNA Reprogramming

The emergence of the mRNA reprogramming system in 2010 took the stem cell field by surprise. Synthetic mRNA did not feature in the standard toolkit of cell biologists at the time, in contrast with retroviruses, lentiviruses, plasmids, small interfering RNAs (siRNAs), soluble proteins, and small molecules. In fact, synthetic mRNA has been produced and applied in niche research applications going as far back as the 1980s.[21] By the early 2000s, a small coterie of researchers in Europe and the United States had come to envisage it as a versatile therapeutic tool with near-term applications in the field of cancer immunotherapy. These researchers established ground rules for making long-lived, efficiently expressed mRNA, providing insight and empirical data on the important roles played in these artificial transcripts by features copied from natural mRNA, such as the 5′ cap, polyA tail, and UTRs, and describing optimized techniques for mass-producing synthetic mRNA and delivering it to cultured cells.[22,23] The rush to apply mRNA transfection to the problem of making footprint-free iPSCs would likely have been a stampede had there been greater awareness of this body of work. As it turned out, a handful of groups did see the potential. They quickly ran into the key hurdle to realizing mRNA reprogramming, which is that synthetic mRNAs delivered to cultured mammalian cells powerfully activate broadly expressed antiviral defense pathways, which in turn suppress protein translation from the exogenous transcripts and trigger a cascade of cytotoxic and cytostatic responses that are inimical to reprogramming.

Upon optimization, efficient delivery of synthetic mRNA can usually be achieved in adherent cells such as fibroblasts with cationic transfection vehicles like those routinely used for transfecting other nucleic acids such as plasmids and siRNAs. Employing mRNAs manufactured according to established best practices regarding capping, tailing, and UTR content, researchers found it relatively straightforward to produce robust levels of protein expression, comparable with those given by plasmid and viral vectors, upon initial delivery of mRNA into cultured cells. Because even longer-lived mRNAs degrade in the cytoplasm on a timescale of 12–24 hr, the perdurance of protein expression after mRNA transfection is short compared with that from plasmid delivery, let alone compared with the expression attained with integrating viral vectors. Looking at the time course of protein expression from a single mRNA transfection, researchers could infer that many repeat rounds of mRNA delivery might be needed to derive iPSCs with useful levels of efficiency. Further, given that the protein half-life of transcription factors is typically quite short, daily dosing is required to avoid massive seesawing in the levels of the reprogramming factors using the mRNA system.

Publications from the 2010–2012 period describe abortive attempts to make iPSCs by mRNA transfection and report that cytotoxic responses limited the transfection time course to just a few days.[24,25] These studies detected robust expression of reprogramming factors from transfected mRNA and document some activation of endogenous pluripotency genes. There are also claims dating from this period of abbreviated mRNA protocols giving colonies that stained positive for stem cell markers, but overall the data in these studies do not convincingly show that true iPSCs were ever obtained.[26]

On closer investigation, it emerged that the difficulties experienced in sustaining effective protein expression by repeat mRNA transfection arose from the mRNA itself and not from (for example) vehicle toxicity. Angel and Yanik[27] showed that a battery of innate immune response genes becomes activated when mRNA is delivered to fibroblast cultures, setting in play a positive feedback loop mediated by autocrine and paracrine signaling via type I interferons that further sensitizes the cells to follow-up challenges. This was an unexpected finding because the prevalent view regarding innate immunity at the time was that, to the extent this system is stimulated by RNA, the major agonist is usually double-stranded RNA (dsRNA), not single-stranded RNA (ssRNA).[28] This picture is easily rationalized given that dsRNA is a distinctive feature in the life cycle of many viruses, whereas ssRNA (including endogenous mRNA) is ubiquitous within the cytoplasm of uninfected cells. It is now apparent that this notion of the roles of dsRNA versus ssRNA in triggering antiviral defenses is oversimplified. Innate immunity has been honed by evolution to register exogenous ssRNA, as well as dsRNA, based on cues such as compartmental localization, the presence or absence of non-canonical, modified nucleosides, and various other moieties or structural motifs. A further complication, generally unappreciated at the time, is that the *in vitro* transcription reactions used to make synthetic mRNA also give rise to double-stranded side products that carry over into mRNA preparations unless specialized purification techniques are employed.[29]

Angel and Yanik[27] went on to show that the antiviral response to transfected mRNA can be abrogated by siRNA knockdown of key genes within the innate immune defense network, and demonstrated that such countermeasures could be used to prolong the expression of reprogramming factors attainable by mRNA transfection, although the goal of iPSC derivation itself remained elusive.

Warren et al.[18] published the first rigorous demonstration of iPSC induction by mRNA transfection in late 2010. As with the Angel study, this research grappled with the problem of the immunogenicity of exogenous mRNA, which likewise motivated the pursuit of countermeasures to enable prolonged transfection regimens. Viruses have evolved diverse genes to subvert innate immunity. B18R,[30] a decoy receptor for type I interferons identified in the Vaccinia genome, has been extensively characterized in the literature and is available commercially in recombinant form. Employed as a media additive, B18R turned out to be a useful tool for slowing the buildup of the inflammatory response to transfected mRNA. A second and more fundamental strategy was also brought to bear in this study, based on the use of "modified

mRNA." The modified-RNA gambit was inspired by the findings of Karikó and Weissman,[31–33] revealing that the RNA-specific sensors of innate immunity are more sensitive to "plain vanilla" RNA featuring canonical nucleobases than they are to RNA that contains various non-standard nucleobases, many of which occur frequently in eukaryotic transcripts. In the setting of human fibroblast reprogramming trials, a striking reduction in mRNA immunogenicity was seen when the non-standard nucleosides pseudouridine and 5-methylcytidine were substituted for uridine and cytidine in synthetic transcripts. When this "stealthy" modified mRNA was deployed in conjunction with the application of B18R interferon inhibitor, it proved possible to keep daily mRNA transfections going at functionally relevant doses for more than 2 weeks without provoking a marked immune response. Given this foundation, a remarkably efficient and speedy mRNA reprogramming system was quickly attained and presented in the literature.[18,34] By highlighting the untapped potential of a hitherto relatively obscure technology, this breakthrough had an impact well beyond the iPSC research community, stimulating a groundswell of interest and investment moved by the vision of synthetic mRNA as the basis for an entirely new class of pharmaceutical drugs.[35]

The published "modified mRNA" reprogramming protocol incorporates several optimizations informed by earlier iPSC research, including an mRNA cocktail stoichiometrically weighted in favor of the most potent reprogramming factor, Oct4, and the use of 5% oxygen culture.[36,37] Applied to several low-passage fibroblast lines, this regimen gave impressive results, with full-fledged iPSC colonies emerging in under 20 days, compared with the ~30 days more typical of traditional viral protocols, and with colony productivity in some cases as much as two orders of magnitude higher than routinely obtained with integrating viral vectors.

The productivity and rapidity of iPSC induction achieved with mRNA reprogramming was unexpected because other early approaches based on transient vectors performed poorly by these metrics. The striking performance of the mRNA system (at least as applied to low-passage human fibroblasts) has never been fully explained. One possibility is suggested by reports that activation of antiviral or pro-inflammatory pathways, which still occurs at a low level even when countermeasures like modified mRNA and B18R are employed, might actually facilitate reprogramming.[38–41] If this idea is valid, the window in which such benign effects outweigh the cytotoxic and cytostatic effects of innate immune stimulation must be quite narrow. It also seems possible that collective effects involving cell-cell communication are at work, linked to the homogeneity of factor expression achieved in the mRNA system—a speculative hypothesis prompted by the lockstep, population-wide phenotypic shifts characteristic of mRNA reprogramming cultures.

Despite the availability of commercial reagents such as pre-made synthetic mRNA that greatly simplified adoption of the mRNA reprogramming system, its uptake by the research community has been limited by comparison with the episomal and Sendai systems. This can be attributed mainly to the demanding nature of the early protocols, which mandated around 2 weeks of consecutive daily transfections. For the research-oriented iPSC user, the greater hands-on time and the discipline needed to stick to transfection regimens 7 days a week often outweigh the benefit of getting more iPSC colonies in a shorter time. Further, although the productivity of mRNA reprogramming is usually excellent with high-quality, low-passage fibroblast lines, the minor fraction of "real-world" lines that prove refractory to reprogramming tends to be higher with this system—or, at least, that was the experience using early versions of mRNA reprogramming.[42] Finally, as blood has gained in popularity as an alternative starting point for iPSC derivations, the limited progress so far achieved in adapting the mRNA system to the reprogramming of blood-derived cell types has emerged as a significant limitation of the system.

Considerable work has now gone into developing enhanced mRNA protocols that address the weak points of the protocol originally described by Warren et al. in 2010.[18] A major focus has been to further accelerate the rapid induction seen with the original system by potentiating the RNA cocktail through incorporation of additional reprogramming factors, use of "engineered" chimeric transcription factors with extra transactivating domains, and co-transfection of microRNAs (miRNAs) that synergize with the protein factors to promote mesenchymal-epithelial transition and pluripotency.[43–45] In some instances, these approaches support robust iPSC induction from human fibroblasts with as few as four transfections. These accelerated protocols much reduce hands-on time and lower reagent costs. Compressing the reprogramming timeline has also enabled the development of streamlined protocols in which iPSC derivation is performed in a single culture vessel coated with a defined substrate without any need for feeder cells. Feeder-free derivation is now the standard for mRNA reprogramming, as it is for most competing systems. The newer protocols have already been used to derive iPSCs from hundreds of patient-specific fibroblast lines with a very high success rate, testifying to their robustness in practice.

Lowering the number of transfections needed to induce pluripotency is the most promising route to extending the applicability of the mRNA system to hard-to-transfect cell types, which, as noted, remains a weak point of the mRNA system. To date, mRNA reprogramming has been performed mostly on human fibroblasts, although success has also been reported in several other cell types including murine fibroblasts[46] and human chondrocytes, mesenchymal stromal cells, vascular endothelial cells, urine-derived cells, and dental pulp cells.[45,47–49] Blood cells tend to be somewhat refractory to transfection with cationic reagents, and this presents a major hurdle to their reprogramming with mRNA. Electroporation can achieve efficient mRNA delivery into blood cells,[50,51] but the harshness of this procedure raises similar difficulties when repeat dosing is necessary. Novel RNA delivery methods may yet render this problem more tractable. For example, over the last decade several promising membrane disruption-based delivery approaches, in some cases realized within lab-on-a-chip platforms, have been described in the literature.[52] Meanwhile, mRNA reprogramming of the relatively transfectable endothelial progenitor cells that can be isolated from peripheral blood

has recently been reported,[45] but there is still an unmet demand for an mRNA protocol that works with true blood lineages.

Much progress has been made in the last few years toward adapting mRNA protocols to industrial-scale use for mass-producing clinical-grade iPSCs. Highly automated mRNA reprogramming-based iPSC production lines have been implemented and characterized in the scientific literature,[53] cGMP-compliant mRNA reprogramming and iPSC expansion protocols have been described,[54,55] and the dedication of specialized facilities for iPSC production using such methods has been announced.

One important technical choice facing the architects of these facilities is whether the iPSC colonies generated within their production lines should be individually picked, graded, and selectively expanded (representing a "classical" approach), or whether it is better to pool the iPSCs from each derivation and expand them together without establishing clonal iPSC lines. The New York Stem Cell Foundation, which has done extensive work on industrializing the mRNA system, has argued for a polyclonal approach in describing a prototype automated iPSC derivation pipeline and has presented data suggesting that the averaging effect of pooling alleviates problems associated with clonal dominance that attend traditional colony-picking strategies.[53] The mRNA method certainly lends itself to this approach, not least because the reprogramming system is itself inherently non-clonal. Pooling takes maximum advantage of the high efficiency of mRNA reprogramming, which amounts to a bulk conversion of the input cells in some instances, potentially slashing the number of population doublings required to produce any given number of iPSCs for clinical use. Fewer doublings implies less *in vitro* selection and potentially fewer mutations before differentiated iPSC progeny are administered to a patient. Although the data from different groups are mixed, there is evidence that mRNA reprogramming is associated with a lower burden of spontaneous mutations than other methods.[42,56,57] This might reflect the faster timelines involved in the process, and it may be that the shorter expansion timelines achievable by polyclonal culture can extend this advantage. Doubtless it will be some time before the relative merits of the alternative iPSC expansion strategies can be accurately assessed.

Conclusions

Of the techniques so far demonstrated that can make iPSCs with useful efficiency, mRNA transfection affords the cleanest solution to the problems associated with gene expression vector persistence, obviating any need to screen for residual traces of vector and minimizing any concerns that the reprogramming system will leave an imprint on the iPSCs. From this standpoint alone, it appears to be a strong contender for application to iPSC production in the clinical arena. It also offers advantages with respect to speed and efficiency that may translate to benefits at the level of genomic integrity in a mass-production setting, assuming the polyclonal iPSC expansion strategy described above gains favor. The labor-intensive character of the original protocol, perhaps the biggest drawback to the technique, has been greatly alleviated in more recent versions of the system. The difficulty of reprogramming blood lineages with mRNA remains a significant challenge, but it is by no means clear that blood will be the starting material of choice for future clinical-grade iPSC production. In conclusion, the mRNA reprogramming system offers an attractive path around one of the main stumbling blocks to future iPSC-based therapeutics and, accordingly, continues to deserve and receive the attention of scientists working to bring that dream to reality.

CONFLICTS OF INTEREST

L.W. is founder and CEO of Cellular Reprogramming, Inc., of Pasadena, CA, an mRNA reprogramming service provider.

REFERENCES

1. Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.
2. Normile, D. (2018). First-of-its-kind clinical trial will use reprogrammed adult stem cells to treat Parkinson's. Science. , Published online July 30, 2018. https://doi.org/10.1126/science.aau9466.
3. Gurdon, J.B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J. Embryol. Exp. Morphol. *10*, 622–640.
4. Graf, T., and Enver, T. (2009). Forcing cells to change lineages. Nature *462*, 587–594.
5. Davis, R.L., Weintraub, H., and Lassar, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell *51*, 987–1000.
6. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.
7. Yamanaka, S. (2012). Induced pluripotent stem cells: past, present, and future. Cell Stem Cell *10*, 678–684.
8. Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., and Yamanaka, S. (2008). Generation of mouse induced pluripotent stem cells without viral vectors. Science *322*, 949–953.
9. Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., and Hochedlinger, K. (2014). Induced pluripotent stem cells generated without viral integration. Science *322*, 945–949.
10. Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, I.I., and Thomson, J.A. (2009). Human induced pluripotent stem cells free of vector and transgene sequences. Science *324*, 797–801.
11. Mitani, K., and Kubo, S. (2002). Adenovirus as an integrating vector. Curr. Gene Ther. *2*, 135–144.
12. Würtele, H., Little, K.C.E., and Chartrand, P. (2003). Illegitimate DNA integration in mammalian cells. Gene Ther. *10*, 1791–1799.
13. Kehler, J., Greco, M., Martino, V., Pachiappan, M., Yokoe, H., Chen, A., Yang, M., Auerbach, J., Jessee, J., Gotte, M., et al. (2017). RNA-generated and gene-edited induced pluripotent stem cells for disease modeling and therapy. J. Cell. Physiol. *232*, 1262–1269.
14. Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., et al. (2009). Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell *4*, 381–384.
15. Kim, D., Kim, C.H., Moon, J.I., Chung, Y.G., Chang, M.Y., Han, B.S., Ko, S., Yang, E., Cha, K.Y., Lanza, R., and Kim, K.S. (2009). Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell *4*, 472–476.
16. Leontovyc, I., Habart, D., Loukotova, S., Kosinova, L., Kriz, J., Saudek, F., and Koblas, T. (2017). Synthetic mRNA is a more reliable tool for the delivery of DNA-targeting proteins into the cell nucleus than fusion with a protein transduction domain. PLoS ONE *12*, e0182497.
17. Ma, X., Kong, L., and Zhu, S. (2017). Reprogramming cell fates by small molecules. Protein Cell *8*, 328–348.
18. Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., et al. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell *7*, 618–630.

19. Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., and Hasegawa, M. (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc. Jpn. Acad., Ser. B, Phys. Biol. Sci. *85*, 348–362.

20. Yoshioka, N., Gros, E., Li, H.R., Kumar, S., Deacon, D.C., Maron, C., Muotri, A.R., Chi, N.C., Fu, X.D., Yu, B.D., and Dowdy, S.F. (2013). Efficient generation of human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell *13*, 246–254.

21. Yisraeli, J.K., and Melton, D.A. (1989). Synthesis of long, capped transcripts in vitro by SP6 and T7 RNA polymerases. Methods Enzymol. *180*, 42–50.

22. Holtkamp, S., Kreiter, S., Selmi, A., Simon, P., Koslowski, M., Huber, C., Türeci, O., and Sahin, U. (2006). Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood *108*, 4009–4017.

23. Elango, N., Elango, S., Shivshankar, P., and Katz, M.S. (2005). Optimized transfection of mRNA transcribed from a d(A/T)100 tail-containing vector. Biochem. Biophys. Res. Commun. *330*, 958–966.

24. Plews, J.R., Li, J., Jones, M., Moore, H.D., Mason, C., Andrews, P.W., and Na, J. (2010). Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach. PLoS ONE *5*, e14397.

25. Tavernier, G., Wolfrum, K., Demeester, J., De Smedt, S.C., Adjaye, J., and Rejman, J. (2012). Activation of pluripotency-associated genes in mouse embryonic fibroblasts by non-viral transfection with in vitro-derived mRNAs encoding Oct4, Sox2, Klf4 and cMyc. Biomaterials *33*, 412–417.

26. Yakubov, E., Rechavi, G., Rozenblatt, S., and Givol, D. (2010). Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. Biochem. Biophys. Res. Commun. *394*, 189–193.

27. Angel, M., and Yanik, M.F. (2010). Innate immune suppression enables frequent transfection with RNA encoding reprogramming proteins. PLoS ONE *5*, e11756.

28. Kawai, T., and Akira, S. (2007). Antiviral signaling through pattern recognition receptors. J. Biochem. *141*, 137–145.

29. Karikó, K., Muramatsu, H., Ludwig, J., and Weissman, D. (2011). Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res. *39*, e142.

30. Symons, J.A., Alcamí, A., and Smith, G.L. (1995). Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. Cell *81*, 551–560.

31. Karikó, K., Buckstein, M., Ni, H., and Weissman, D. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity *23*, 165–175.

32. Karikó, K., Muramatsu, H., Welsh, F.A., Ludwig, J., Kato, H., Akira, S., and Weissman, D. (2008). Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol. Ther. *16*, 1833–1840.

33. Karikó, K., and Weissman, D. (2007). Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development. Curr. Opin. Drug Discov. Devel. *10*, 523–532.

34. Mandal, P.K., and Rossi, D.J. (2013). Reprogramming human fibroblasts to pluripotency using modified mRNA. Nat. Protoc. *8*, 568–582.

35. Dolgin, E. (2015). Business: The billion-dollar biotech. Nature *522*, 26–28.

36. Papapetrou, E.P., Tomishima, M.J., Chambers, S.M., Mica, Y., Reed, E., Menon, J., Tabar, V., Mo, Q., Studer, L., and Sadelain, M. (2009). Stoichiometric and temporal requirements of Oct4, Sox2, Klf4, and c-Myc expression for efficient human iPSC induction and differentiation. Proc. Natl. Acad. Sci. USA *106*, 12759–12764.

37. Yoshida, Y., Takahashi, K., Okita, K., Ichisaka, T., and Yamanaka, S. (2009). Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell *5*, 237–241.

38. Lee, J., Sayed, N., Hunter, A., Au, K.F., Wong, W.H., Mocarski, E.S., Pera, R.R., Yakubov, E., and Cooke, J.P. (2012). Activation of innate immunity is required for efficient nuclear reprogramming. Cell *151*, 547–558.

39. Lee, J., Xu, L., Gibson, T.M., Gersbach, C.A., and Sullenger, B.A. (2016). Differential effects of toll-like receptor stimulation on mRNA-driven myogenic conversion of human and mouse fibroblasts. Biochem. Biophys. Res. Commun. *478*, 1484–1490.

40. Zhou, G., Meng, S., Li, Y., Ghebre, Y.T., and Cooke, J.P. (2016). Optimal ROS signaling is critical for nuclear reprogramming. Cell Rep. *15*, 919–925.

41. Brady, J.J., Li, M., Suthram, S., Jiang, H., Wong, W.H., and Blau, H.M. (2013). Early role for IL-6 signalling during generation of induced pluripotent stem cells revealed by heterokaryon RNA-Seq. Nat. Cell Biol. *15*, 1244–1252.

42. Schlaeger, T.M., Daheron, L., Brickler, T.R., Entwisle, S., Chan, K., Cianci, A., DeVine, A., Ettenger, A., Fitzgerald, K., Godfrey, M., et al. (2015). A comparison of non-integrating reprogramming methods. Nat. Biotechnol. *33*, 58–63.

43. Warren, L., Ni, Y., Wang, J., and Guo, X. (2012). Feeder-free derivation of human induced pluripotent stem cells with messenger RNA. Sci. Rep. *2*, 657.

44. Warren, L., and Wang, J. (2013). Feeder-free reprogramming of human fibroblasts with messenger RNA. Curr. Protoc. Stem Cell Biol. *27*. Unit 4A.6.

45. Poleganov, M.A., Eminli, S., Beissert, T., Herz, S., Moon, J.I., Goldmann, J., Beyer, A., Heck, R., Burkhart, I., Barea Roldan, D., et al. (2015). Efficient reprogramming of human fibroblasts and blood-derived endothelial progenitor cells using nonmodified RNA for reprogramming and immune evasion. Hum. Gene Ther. *26*, 751–766.

46. El-Sayed, A.K., Zhang, Z., Zhang, L., Liu, Z., Abbott, L.C., Zhang, Y., and Li, B. (2014). Pluripotent state induction in mouse embryonic fibroblast using mRNAs of reprogramming factors. Int. J. Mol. Sci. *15*, 21840–21864.

47. Boreström, C., Simonsson, S., Enochson, L., Bigdeli, N., Brantsing, C., Ellerström, C., Hyllner, J., and Lindahl, A. (2014). Footprint-free human induced pluripotent stem cells from articular cartilage with redifferentiation capacity: a first step toward a clinical-grade cell source. Stem Cells Transl. Med. *3*, 433–447.

48. Heng, B.C., Heinimann, K., Miny, P., Iezzi, G., Glatz, K., Scherberich, A., Zulewski, H., and Fussenegger, M. (2013). mRNA transfection-based, feeder-free, induced pluripotent stem cells derived from adipose tissue of a 50-year-old patient. Metab. Eng. *18*, 9–24.

49. Gaignerie, A., Lefort, N., Rousselle, M., Forest-Choquet, V., Flippe, L., Francois-Campion, V., Girardeau, A., Caillaud, A., Chariau, C., Francheteau, Q., et al. (2018). Urine-derived cells provide a readily accessible cell type for feeder-free mRNA reprogramming. Sci. Rep. *8*, 14363.

50. Van Tendeloo, V.F.I., Ponsaerts, P., Lardon, F., Nijs, G., Lenjou, M., Van Broeckhoven, C., Van Bockstaele, D.R., and Berneman, Z.N. (2001). Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood *98*, 49–56.

51. Rabinovich, P.M., Komarovskaya, M.E., Wrzesinski, S.H., Alderman, J.L., Budak-Alpdogan, T., Karpikov, A., Guo, H., Flavell, R.A., Cheung, N.K., Weissman, S.M., and Bahceci, E. (2009). Chimeric receptor mRNA transfection as a tool to generate antineoplastic lymphocytes. Hum. Gene Ther. *20*, 51–61.

52. Stewart, M.P., Sharei, A., Ding, X., Sahay, G., Langer, R., and Jensen, K.F. (2016). In vitro and ex vivo strategies for intracellular delivery. Nature *538*, 183–192.

53. Paull, D., Sevilla, A., Zhou, H., Hahn, A.K., Kim, H., Napolitano, C., Tsankov, A., Shang, L., Krumholz, K., Jagadeesan, P., et al. (2015). Automated, high-throughput derivation, characterization and differentiation of induced pluripotent stem cells. Nat. Methods *12*, 885–892.

54. Durruthy-Durruthy, J., Briggs, S.F., Awe, J., Ramathal, C.Y., Karumbayaram, S., Lee, P.C., Heidmann, J.D., Clark, A., Karakikes, I., Loh, K.M., et al. (2014). Rapid and efficient conversion of integration-free human induced pluripotent stem cells to GMP-grade culture conditions. PLoS ONE *9*, e94231.

55. Ni, Y., Zhao, Y., Warren, L., Higginbotham, J., and Wang, J. (2016). cGMP generation of human induced pluripotent stem cells with messenger RNA. Curr. Protoc. Stem Cell Biol. *39*, 4A.6.1–4A.6.25.

56. Bhutani, K., Nazor, K.L., Williams, R., Tran, H., Dai, H., Džakula, Ž., Cho, E.H., Pang, A.W., Rao, M., Cao, H., et al. (2016). Whole-genome mutational burden analysis of three pluripotency induction methods. Nat. Commun. *7*, 10536.

57. Steichen, C., Luce, E., Maluenda, J., Tosca, L., Moreno-Gimeno, I., Desterke, C., Dianat, N., Goulinet-Mainot, S., Awan-Toor, S., Burks, D., et al. (2014). Messenger RNA- versus retrovirus-based induced pluripotent stem cell reprogramming strategies: analysis of genomic integrity. Stem Cells Transl. Med. *3*, 686–691.