Feeder-Free Derivation of Human Induced Pluripotent Stem Cells with Messenger RNA

Luigi Warren1, Yuhui Ni1,2, Jiwu Wang1,3 & Xirong Guo2

1Allele Biotechnology & Pharmaceuticals, Inc., San Diego, CA, 2Nanjing Maternity and Child Health Medical Institute, Nanjing Medical University Affiliated Nanjing Maternity and Child Health Care Hospital, 3The Scintillon Institute, San Diego, CA.

The therapeutic promise of induced pluripotent stem cells (iPSCs) has spurred efforts to circumvent genome alteration when reprogramming somatic cells to pluripotency. Approaches based on episomal DNA, Sendai virus, and messenger RNA (mRNA) can generate "footprint-free" iPSCs with efficiencies equaling or surpassing those attained with integrating viral vectors. The mRNA method uniquely affords unprecedented control over reprogramming factor (RF) expression while obviating a cleanup phase to purge residual traces of vector. Currently, mRNA-based reprogramming is relatively laborious due to the need to transfet daily for ~2 weeks to induce pluripotency, and requires the use of feeder cells that add complexity and variability to the procedure while introducing a route for contamination with non-human-derived biological material. We accelerated the mRNA reprogramming process through stepwise optimization of the RF cocktail and leveraged these kinetic gains to establish a feeder-free, xeno-free protocol which slashes the time, cost and effort involved in iPSC derivation.

Shinya Yamanaka’s discovery that differentiated cells can be reverted to a pluripotent state by the expression of a select group of transcription factors opened up the prospect that patient-specific cells might be turned into cells of any desired type for use in the study of genetic disease and ultimately for cell-replacement therapy. In Yamanaka’s original work the expression of reprogramming factors was achieved using viral vectors which integrate into the genome1, and iPSC derivations are still typically performed with integrating retrovirus or lentivirus. Modification of the genome represents a hurdle to therapeutic application of iPSCs, while the possibility of reactivated expression from integrated viral expression cassettes is a concern even for in vitro studies. Considerable progress has been made in the development of novel RF expression vectors to alleviate this problem. Lentiviral vectors are now available which encode the multiple factors required for iPSC induction within a single polycistronic cassette flanked with recombination sites, allowing almost-seamless excision of the transgene after reprogramming by transient expression of a recombinase2–3. Transgene insertion with subsequent excision can also be effected by using a transposon vector followed by brief expression of transposase4–8. Non-integrating vectors based on adenovirus, plasmids and episomal DNA have been developed to transiently express RFs for a time sufficient to induce pluripotency5–10, and it has proved possible to generate iPSCs by repeated transduction of cells with recombinant RF proteins incorporating cell-penetrating peptides, albeit with low efficiency11,12. Relatively efficient iPSC conversion can now be achieved using Sendai virus13–15, which has a completely RNA-based reproductive cycle, and by sustained transfection of synthetic mRNA transcripts encoding the Yamanaka factors16.

The use of synthetic mRNA for cell fate manipulation is appealing because this system allows cellular expression of transcription factors to be modulated on a daily basis simply by adjusting the payload of transcripts added to the culture medium. Once transfection of a particular factor is terminated ectopic expression in the target cells ceases in short order due to the rapid degradation of mRNA in the cytoplasm. In contrast to non-integrating DNA vectors or RNA viruses, no cleanup phase is entailed when reprogramming with mRNA, nor is there any risk of random genomic integration or persistent viral infection. These advantages assume greater significance when we envisage that multiple rounds of ectopic RF expression may ultimately be employed to derive specialized cells of interest from a patient sample via an iPSC intermediate. Nonetheless, there are drawbacks to mRNA-based reprogramming as currently practiced. Expression of RFs is typically robust for about 24 hours after mRNA is transfected, yet it takes about two weeks of factor expression to induce pluripotency in human cells, and the hands-on time involved in performing the repeated transfections needed to generate iPSCs is therefore relatively
high. Not all cell types and culture media are equally conducive to transfection, and this is an obstacle to mRNA-based reprogramming of certain cell types, including blood cells. It has also so far been necessary to employ a feeder layer of mitotically-arrested fibroblasts when reprogramming cells using mRNA. The feeder cells buffer the population density of the culture as the target cells grow out from a low starting density over the extended time course required for iPSC induction, evening out the per-cell dosage of RNA and transfection reagent (both of which have associated toxicities) and supporting the viability of the target cells in the face of pro-apoptotic and cytostatic forces engendered by the reprogramming process. This requirement adds complexity and setup time to the procedure and introduces an important source of technical variability, especially given that the feeders are themselves subject to transfection. The presence of a feeder layer also impedes monitoring and analysis of the reprogramming process. Finally, although human feeder cells are currently the standard for mRNA reprogramming, even these cells are a potential source of xeno-biological contamination when non-human animal products are used in their derivation and expansion. We reasoned that the weaknesses inherent in current mRNA-based protocols could be mitigated if reprogramming were accelerated through potentiation of the RF cocktail delivered to the cells. Several recent publications have described novel reprogramming factors or factor combinations which can enhance the kinetics and efficiency of iPSC derivation. Optimization of the established mRNA RF cocktail based on these findings and our own experiments has enabled us to define a revised protocol that compresses and streamlines the mRNA reprogramming process, and which supports the rapid production of footprint-free iPSCs from human fibroblasts without the use of feeder cells or other potentially xeno-contaminated reagents. This work will extend the appeal of the mRNA method and help clear remaining roadblocks to the therapeutic application of iPSC technology.

Results

Our experiments initially focused on the question of whether mRNA reprogramming could be enhanced using engineered variants of Oct4 incorporating an N-terminal MyoD transactivation domain or a C-terminal triple repeat of the VP16 transactivation domain, or by augmenting the cocktail with transcripts for two additional factors, Rarg and Lrh-1. To this end, we carried out a test to compare the performance of six different mRNA cocktails comprising transcripts for the five RFs currently used in mRNA reprogramming (Oct4, Sox2, Klf4, c-Myc-T58A and Lin28), or a 7-factor cocktail including Rarg and Lrh-1, each combination being tried in three variations based on wild-type Oct4 and the MyoD- and VP16-Oct4 fusion constructs (designated M1O and VPX3, respectively). BJ fibroblasts plated at 10K cells per well were transfected in feeder-based cultures for 11 days, by which time advanced morphologies were apparent in several of the wells. Over the next few days colonies with characteristic human embryonic stem cell (hESC) morphology emerged in wells transfected with the cocktails based on wild-type Oct4 and M1O, while the target cells in the VPX3-transfected cultures retained a fibroblastic morphology—albeit while showing accelerated growth and some tendency to aggregate into foci—and no colonies emerged (data not shown). The 5-factor and 7-factor versions of the wild-type Oct4 and M1O-based cocktails demonstrated similar colony productivity, hence no advantage accrued from the inclusion of Rarg and Lrh-1. However, the M1O cocktail gave several times the number of colonies produced with wild-type Oct4. Colonies were picked from the M1O 5-factor well for expansion and further analysis (Fig. 1). Pluriptocity of M1O-derived colonies was confirmed by staining for nuclear and cell-surface markers (Fig. 1b), and by in vitro differentiation into the three primary germ layers (Fig. 1c-e and Supplementary Movie 1). Six expanded clones were subjected to karyotype analysis and DNA fingerprinting, karyotypic normality and BJ lineage being confirmed in all cases (data not shown).

In a follow-up experiment we investigated whether the potency of the mRNA cocktail could be further enhanced by the inclusion of Nanog transcripts. Four wells containing 50K BJ fibroblasts on feeders were transfected with wild-type Oct4 or M1O-based 5-factor or 6-factor cocktails for six days, and each culture was then passaged 1:6 onto fresh feeders to populate a 6-well plate (Fig. 2a). Transfection was continued for up to 5 more days within each plate. Cultures were fixed and stained with TRA-1-60 antibodies on day 18 (where day 0 corresponds to the first transfection) to assess the impact of the different cocktails and transfection time courses on iPSC productivity (Fig. 2b-c). The results showed that adding Nanog to the cocktail was highly beneficial regardless of the Oct4 variant employed, while the greatest conversion efficiency was attained when M1O and Nanog were used together.

We confirmed the efficacy of the M1O-based 5-factor or 6-factor cocktails in additional feeder-based experiments using three additional human fibroblast lines (data not shown). Reprogramming kinetics and efficiency were improved with these low-passage lines (HDF-f, HDF-n and XFF), which displayed faster population doubling times than the relatively late-passage BJ cells in expansion culture. In some cases, hESC-like colonies were obtained following as little as six days of transfection, although the yields were higher when transfection was continued for a few more days. These experiments did reveal a drawback to the M1O-based cocktail in that attrition of the mitotically-arrested feeder cells was markedly accelerated when using the engineered Oct4 (data not shown). The rapid loss of the feeder layer appeared to have an adverse impact on the survival and proliferation of the target cells, suggesting that the performance advantage from using M1O over wild-type Oct4 might be heightened if we could either find a way to sustain the feeder layer or switch to a feeder-independent system. Experiments involving periodic reinforcement of the feeder layer by the addition of fresh cells suggested that while such a strategy might be of some benefit, this would be offset by the added complexity of the protocol (data not shown). We therefore decided to focus on applying the more potent cocktail to establish a streamlined, feeder-free protocol.

Feeder-independent iPSC derivation has generally proved somewhat challenging regardless of the reprogramming technology employed, but raises special difficulties in the context of a sustained transfection regimen. There is a lower limit to the density at which fibroblasts can be plated without compromising cell viability and proliferative activity. The propensity of cells to undergo mitotic arrest or apoptosis in sparse cultures is exacerbated when they are stressed by transfection and by ectopic expression of reprogramming factors. Moreover, RNA doses which are well tolerated at the high cell densities characteristic of feeder-based reprogramming produce more severe cytotoxic effects when distributed among fewer cells. At the same time, the penetrance of expression achieved with mRNA transfection declines after fibroblasts reach confluence, perhaps due to down-regulation of endocytosis associated with contact inhibition and cell-cycle arrest. Reduced permissiveness to transfection in crowded cultures tends to be alleviated during mRNA reprogramming once cells undergo mesenchymal-to-epithelial transition (MET). However, the ~7 days required for human fibroblasts to reach MET using current mRNA cocktails renders it difficult to avoid fibroblastic overgrowth even when cells are plated at the lowest survivable initial densities. Cultures can be thinned by passaging to postpone this fate, but the plating efficiency of highly stressed reprogramming intermediates is unpredictable, and a split-based protocol would in any case sacrifice convenience and scale poorly to high-throughput applications.

Phenotypic indications of MET (involution of fibroblastic processes and the emergence of foci and cobblestone morphologies) were accelerated in experiments with enhanced cocktails, so we
attempted feeder-free derivations using the 6-factor M3O cocktail without passaging steps starting with fibroblast cultures seeded at from 50K to 150K cells per well. The fate of the reprogramming cultures proved highly sensitive to the initial seeding density, presumably because the deleterious effects of excessive cytotoxicity and fibroblastic overgrowth compound over the course of days of transfection. In an early experiment employing a uniform RNA dosing regimen, dozens of hESC-like colonies were obtained from neonatal HDF-n and XFF fibroblasts plated at 100K per well, while the population density in 50K wells crashed and 150K wells succumbed to fibroblastic overgrowth, these sub-optimal wells produced only a few colonies each (data not shown). When the same protocol was applied to two other fibroblast lines, BJ and HDF-a (an adult-derived line), even the most promising (150K) cultures became largely quiescent shortly after reaching confluence, subsequently yielding only sporadic colonies with delayed kinetics. In our follow-on experiments we switched from ambient to 5% oxygen culture and ramped to full RNA dosing from a quarter dose over the first four days of transfection, in efforts to minimize the impact of stress-induced cellular senescence. In the first trial using these new conditions we tested if substitution of wild-type L-Myc for c-Myc-T58A could further improve reprogramming efficiency, and in parallel evaluated a simplified transfection scheme by which RNA was added to cells with daily media changes rather than being delivered in a separate step four hours earlier. RNA dosing was scaled down in 24-hour transfection wells to compensate for an expected increase in cytotoxicity, and two different transfection reagents were tested with this regimen (RNAiMAX and Stemfect). XFF cells were plated at 50K per well and transfected for 9 days. Productivity was assessed by counting TRA-1-60 colonies at the experiment end-point (Fig. 3a–b). The more conventional 4-hour transfection regime yielded about 250 TRA-1-60 colonies per well by day 15 using the c-Myc-based cocktail, equivalent to a reprogramming efficiency of 0.5% (CV = 21%, n = 3), while the corresponding L-Myc cultures displayed an average reprogramming efficiency of only ~0.02%. Reprogramming kinetics and efficiency were improved across the board with the 24-hour transfection regime, and these cultures were fixed for analysis on day 11 to avoid overgrowth in the most productive wells. Using the c-Myc cocktail, the RNAiMAX condition gave on the order of a thousand colonies per well at all three RNA dosages tested, corresponding to an iPSC conversion efficiency of ~2%. The yield of TRA-1-60 colonies was still higher using the Stemfect reagent, making it impossible to count colonies in the most productive wells. In the best-performing c-Myc 400 ng/ml condition, the culture was almost completely overrun with hESC-like cells as early as day 9, one day after the last transfection (Fig. 3c). The mechanistic basis for the...
Figure 2 | Comparison of Reprogramming Efficiency Using 4 Different mRNA Cocktails. (a) Flowchart summarizing the feeder-based 4-cocktail comparison experiment described in the text. The 5-factor reprogramming cocktails comprised wild-type Oct4 or M3O plus Sox2, Klf4, c-Myc-T58A, and Lin28 mRNA transcripts, while 6-factor cocktails included these RFs plus Nanog. (b) Immunofluorescence imaging showing the yield of TRA-1-60 colonies produced by the four different cocktails at day 18 of the experiment. The total number of days of transfection is indicated for each culture. (c) Chart showing the yield of TRA-1-60 colonies at day 18 versus the number of days of transfection using the four cocktails.
superior performance of the 24-hour regimen is unknown, but it may be relevant that dose ramping was achieved within these wells by delivering a reduced volume of medium containing a fixed concentration of RNA, and this might have increased the effective density of thinly-plated cultures. When the preferred conditions were applied in a follow-up derivation on HDF-a adult-derived fibroblasts, 50K and 100K cultures gave a handful of colonies each, but wells seeded with 75K cells produced abundant hESC-like colonies with similar kinetics to the highly proliferative XFF cells (Fig 3d). The rapid kinetics of colony formation using these conditions is apparent in time-lapse imaging showing a subsequent XFF-based derivation presented in Supplementary Movie 2.

Discussion

We have doubtless only scratched the surface in terms of the potential for technical improvements to RNA-based reprogramming. Size-based purification of synthetic mRNA may further reduce activation of cellular antiviral responses and any associated priming of senescence pathways, while possibly obviating the need for an interferon inhibitor. Numerous candidate RFs have been identified which might yield additional performance gains if incorporated into mRNA cocktails, while the advantage realized by substituting M3O for wild-type Oct4 offers fresh support for the application of engineered chimeric factors to cell-fate direction. Acceleration of the reprogramming process could likewise come about through optimization of factor stoichiometries, and we note that the mRNA method opens up the possibility of fine-tuning cocktails to address early and late phases of iPSC induction independently. Finally, the reagents and approach applied here for sustained delivery of mRNA can also be employed to co-transfect other types of RNA including miRNA, which has proven utility for iPSC generation. The methodology we describe already represents a major advance over current protocols. It cuts mRNA reprogramming time by as much as a half with even greater reductions in hands-on time and material costs, while removing troublesome steps from the procedure and allowing mRNA to be handled as a media supplement with almost the same facility as growth factors and cytokines. At the same time, the feeder-free protocol meets a key requirement for moving iPSCs from laboratory to clinic by eliminating potential sources of non-human contamination from the derivation process. If this fast, economical footprint-free iPSC origination technique can be combined with advances in high-throughput propagation and quality assurance of pluripotent clones, the remaining barriers to large-scale, cost-effective production of therapeutically-qualified patient-specific iPSC lines should be few indeed.

Methods

Generation of IVT templates. Plasmid constructs to serve as the basis for generating linear in vitro transcription (IVT) templates were constructed using Ligation Independent Cloning (LIC). We first constructed a parental plasmid (pIVT) incorporating 5' and 3' untranslated regions (UTRs) flanking an insertion site designed to accept an open reading frame (ORF) encoding a protein of interest. The ORF-flanking sequences are as described previously, comprising a low secondary structure 5' leader and strong Kozak site and the murine 9-globin 3' UTR. A linearized version of the pIVT vector bearing 5' overhangs was produced by melting...
Characterization of iPSC colonies. To assess iPSC colony productivity in reprogramming plates, cells were fixed with 4% paraformaldehyde (PFA) in DPBS with calcium and magnesium and immunostained with StainAlve TRA-1-60 Alexa 488 antibody (Stemgent) diluted 1:100. Colony picking, expansion, and subsequent immunostaining and trilineage differentiation assays for molecular and functional validation of pluripotency were carried out by the Sanford Burnham Medical Research Institute. For pluripotency immunostaining panels, clones were cultured on Matrigel (BD Biosciences) in mouse embryonic fibroblast (MEF)-conditioned medium, fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 (for nuclear markers), then stained with primary antibodies against SSEA3 (Millipore), TRA-1-81 (Cell Signaling), Oct3/4 (Santa Cruz) and Sox2 (Cell Signaling). Ectodermal differentiation was performed by breaking up Dispase-detached colonies into chunks and culturing them in neural progenitor cell (NPC) media without low-attachment plates for 4 days, after which the resulting NPCs were seeded to a tissue culture plate coated with Fibronectin (Sigma). The plated cells were stained after 2–3 days using primary antibodies against Nestin and Pax6 (Abcam) diluted 1:200. Endodermal differentiation was performed by breaking up Dispase-detached colonies into chunks and then re-anneling two PCR products amplified from the plasmid using tailed template PCRs to generate linear IVT template amplicons incorporating a T7 promoter, a UTR-flanked ORF, and a T120 polynucleotide stretch to drive addition of a poly(A) tail, as described previously. The T120 sequence was introduced by use of a tailed reverse primer. PCR product stocks were maintained at ~100 ng/μL.

Production of mRNA cocktails. The mRNA synthesis procedure is summarized in Supplementary Fig. S1. Synthetic mRNA was generated in IVT reactions using a 4:1 ratio of ARCA cap analog to GTP to give a high percentage of capped transcripts. Complete substitution of 5m-CTP for CTP and Pseudo-UTP for UTP in the nucleotide triphosphate (NTP) mix was employed to reduce the immunogenicity of the RNA products. Cap analog and modified NTPs were purchased from Trilink Biotechnologies. A T7-IVT-NTP mix was prepared (ARCA/ATP:5m-CTP:GTP:Pseudo-UTP at 15:53:75:3.75:75:3.75 mM) to replace the standard NTPs provided with the MEGAscript T7 Kit (Ambion) used to perform IVT reactions. Each 40 μL IVT reaction comprised 16 μL NTP mix, 4 μL 10X T7 Buffer, 16 μL DNA template and 4 μL T7 polymerase. Reactions were incubated 4–6 hours at 37 °C and treated with 2 μL TURBO DNase for a further 15 minutes at 37 °C before being purified on MEGAclear (Ambion) spin columns, the RNA products being eluted in a volume of 100 μL. To remove immunogenic 5′-triphosphate moieties from uncapped transcripts, 10 μL of Antarctic Phosphatase reaction buffer and 3 μL of Antarctic Phosphatase (NEB) was added to each prep. Phosphatase reactions were incubated for 30 minutes at 37 °C and the IVT products were purified. RNA yield was quantitated by Nanodrop (Thermo Scientific), and the prep were subsequently adjusted to a standardized working concentration of 100 ng/μL by addition of TE pH 7.0 (Ambion). IVT product quality was checked by running 500 ng of RNA per lane on 2% agarose SYBR Safe Gel-E gels (Invitrogen). Cocktails were assembled by pooling prep replicates with matching ratios of the RFs in the desired concentrations. The concentration of each RF used took into account the predicted molecular weight of the respective transcript, all RFs being equimolar except for Oct4 and its derivatives, which were included at 3X molar concentration. A 10% spike of mRNA encoding a nuclearized monomeric LanFYP fluororescent protein was added to cocktails to facilitate monitoring of transfection efficiency during reprogramming trials.

Cells and growth media. Cells targeted for reprogramming included BJ neonatal fibroblasts (ATCC), HDF-f fetal fibroblasts, HDF-n neonatal fibroblasts and HDF-a adult fibroblasts (ScienCell), and XFF xenogeneic neonatal fibroblasts (Millipore). Expansion medium was 1:1 split in M:SW7 (~50% MEM + 10% FBS). Cells were plated at 250K per well in the first BJ reprogramming experiment using FBS-containing 20% FBS) for 7–10 days in low-attachment plates, then plating the EBs to tissue culture plates coated with 0.1% gelatin in EM media. Cardiomyocytes were stained with 1:200 diluted mouse anti-human β-Actinin primary antibody (Sigma).

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Acknowledgements
We thank Drs. Brandon Nelson and Yang Liu of the Sanford Burnham Medical Research Institute and Dr. Kim Thien Ly for their assistance with the isolation, propagation and analysis of iPSC clones, and WiCell for DNA fingerprinting and karyotype analyses. This work was supported in part by grants from the National Natural Science Foundation of China (81100618, 30973231) and the Jiangsu medical research program (BJ201108) to XG.

Author contributions
LW and JW planned the project. YN and XG participated in discussion. XG provided funding and support. LW conducted the majority of the experiments. JW and YN performed certain plasmid construction work. LW wrote the manuscript; YN helped with the videos; JW finalized the paper.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: LW is a consulting scientist and YN and JW are employees of Allele Biotechnology & Pharmaceuticals, Inc., San Diego, CA. A patent has been filed on the reported technologies.

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How to cite this article: Warren, L., Ni, Y., Wang, J. & Guo, X. Feeder-Free Derivation of Human Induced Pluripotent Stem Cells with Messenger RNA. Sci. Rep. 2, 657; DOI:10.1038/srep00657 (2012).