Conversion of adult human fibroblasts into neural precursor cells using chemically modified mRNA

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Abstract

Direct reprogramming offers a unique approach by which to generate neural lineages for the study and treatment of neurological disorders. Our objective is to develop a clinically viable reprogramming strategy to generate neural precursor cells for the treatment of neurological disorders through cell replacement therapy. We initially developed a method for directly generating neural precursor cells (iNPs) from adult human fibroblasts by transient expression of the neural transcription factors, SOX2 and PAX6 using plasmid DNA. This study advances these findings by examining the use of chemically modified mRNA (cmRNA) for direct-to-iNP reprogramming. Chemically modified mRNA has the benefit of being extremely stable and non-immunogenic, offering a clinically suitable gene delivery system. The use of SOX2 and PAX6 cmRNA resulted in high co-transfection efficiency and cell viability compared with plasmid transfection. Neural positioning and fate determinant genes were observed throughout reprogramming with ion channel and synaptic marker genes detected during differentiation. Differentiation of cmRNA-derived iNPs generated immature GABAergic or glutamatergic neuronal
phenotypes in conjunction with astrocytes. This represents the first time a cmRNA approach has been used to directly reprogram adult human fibroblasts to iNPs, potentially providing an efficient system by which to generate human neurons for both research and clinical application.

Keywords: Biotechnology, Neuroscience, Stem cell research

1. Introduction

Direct reprogramming of somatic cells provides the potential for the generation of patient-specific disease models for the study of disease pathogenesis, the identification of novel therapeutic targets, and for drug development and screening [1, 2, 3, 4, 5]. Furthermore, direct reprogramming provides the exciting possibility of an autologous cell source for cell replacement therapy bypassing the need to use human fetal tissue, and removing concerns such as tumorigenesis associated with the use of human pluripotent stem cells obtained either from embryos or through induced pluripotent stem cell reprogramming.

For the study and treatment of neurological and neurodevelopmental disorders, researchers have focused on the direct lineage conversion of non-ectodermal tissue to functional neural cells. Neurons were the first cells demonstrated to be directly converted from mouse fibroblasts by forced expression of the neural lineage-specific transcription factors ASCL1, BRN2, and MYT1L [6], illustrating that direct lineage conversion is possible between cell types representing different germ layers. This was extended to direct conversion of human fibroblasts to neurons with the addition of NEUROD1 [7]. However, while direct conversion of human neurons is a viable tool for investigating neurological disorders, induced neurons are post-mitotic and cannot be expanded on mass for drug screening assays or used for cell replacement therapies.

To address this, we and other groups have identified direct reprogramming strategies by which to generate populations of induced neural precursor cells (iNPs) from fibroblasts. The objective is to provide a direct source of non-pluripotent, expandable iNPs with the capability of generating multiple neural lineages. Two main approaches have been used to generate iNPs; either transient expression of the four pluripotent factors OCT4, KLF4, SOX2, and cMYC [8, 9, 10, 11, 12, 13, 14, 15], or forced expression of either neural-specific [16, 17, 18, 19, 20, 21, 22, 23] or non-neural transcription factors [24]. Regardless of the strategy or transcription factor combination employed, each protocol results in the production of either bi- or tri-potent iNPs.

Our protocol utilises the neural promoting transcription factors SOX2 and PAX6 to directly reprogram adult human fibroblasts to a neural precursor cell-like state [18, 23]. SOX2 and PAX6 were identified based on their prominent roles in human neural
development [25, 26, 27, 28, 29, 30, 31, 32], and forced expression in adult human fibroblasts results in the generation of bipotent iNPs with the capability to differentiate into GFAP-positive astrocytes and mature region-specific neurons [18, 23]. A key feature of this protocol for clinical translation is the utilization of non-viral plasmid DNA transfection to transiently over-express SOX2 and PAX6 in adult human dermal fibroblasts, without requirement of oncogenic-promoting transcription factors [18, 23]. Although DNA transfection-based methodologies significantly reduce the risk of genomic recombination or insertional mutagenesis, considerable limitations accompany non-integrative cell reprogramming strategies [33, 34, 35, 36, 37, 38]. In particular, while the use of plasmid DNA provides a robust and transient mechanism by which to generate human iNPs [18, 23], the major limitation is a low level of transfection efficiency. We propose the use of chemically modified mRNA as an alternative strategy for the generation of clinically acceptable cells. While the use of non-viral mRNA gene delivery has had several technical challenges to overcome including efficient RNA synthesis, stability, and translation [39, 40], and lack of immunogenicity [41, 42, 43, 44, 45], chemically modified mRNA eliminates the risk of genomic integration and insertional mutation inherent to all DNA-based methodologies. In addition, chemically modified mRNA technology allows cell reprogramming without residual tracers of transgenes, making it an attractive option for cell-based therapies in translational research. This study represents the first time a chemically modified mRNA approach has been used to directly reprogram adult human fibroblasts to iNPs, providing a rapid, safe and efficient system by which to generate human neurons, particularly for clinical application to treat neurological disorders.

2. Materials and methods

2.1. Chemically modified mRNA transfection of adult human dermal fibroblasts

Human dermal fibroblasts cells (HDFs) were isolated from the abdomen of five adult donors (age 33–56; n = 3 female, n = 2 male) obtained from Cell Applications Inc. GFP, SOX2, and PAX6 cmRNA were designed and manufactured by Ethris GmbH (Supp. Fig. 1). Prior to transfection, Neural Reprogramming Media comprising of Neurobasal-A (NBA; Thermofisher) or BrainPhys™ (Stem Cell Technologies) with 1 mM valproic acid, 1% penicillin/streptomycin/glutamine, 2% B27 supplement, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL fibroblast growth factor 2 (FGF2), and 2 μg/mL heparin was applied to cells. Chemically modified mRNA (0.01–2 μg cmRNA) transfections were performed using Lipofectamine RNAiMAX (Life Technologies) in OptiMem media (Invitrogen). Transfections were performed in 1–4 cycles with either a 5 hr or 24 hr incubation time. Three days following the final transfection, cells were passaged and subsequently every seven days until full iNP colony formation was achieved. Reprogramming efficiency
was determined by quantification of cells at full colony formation compared to original plating density described as a percentage.

PAX6 cDNA (Life Technologies) and SOX2 cDNA (Addgene) were cloned into pEGFP-N1 (Clontech) after removal of eGFP expression cassette driven by a CMV promoter. Cells were transfected with plasmid DNA for 5 hrs using Lipofectamine LTX reagent (Life Technologies).

2.2. FACS and Alamar Blue assays

To assess transfection efficiency, FACS analysis was performed in triplicate 24 hrs following cmRNA transfection using a BD LSRII Flow Cytometer (BD Bioscience). Cells were stained using either an eGFP antibody (AbCam), or antibodies against SOX2 (AbCam) and PAX6 (BioLegend). The number of fluorescently expressing cells (eGFP+, SOX2+, PAX6+) are described as a percentage of total cell number in the sample.

Cell viability was assessed using the Alamar Blue® assay (ThermoFisher Scientific). Alamar Blue® dye was performed in triplicate 48 hrs following transfection, as per manufacturer instructions. Fluorescence (excitation wavelength 544 nm; emission 590 nm) was measured on a FluoStar (BMG Labtech) plate reader. Percentage of cell viability was obtained by comparing transfected to non-transfected control wells.

2.3. Quantitative RT-PCR

Total RNA was isolated from 2 to 3 independent iNP cell lines during reprogramming and differentiation, and HDF control lines using the Nucleospin RNA kit (Ma-cherey Nagel). Total RNA was also isolated from human embryonic- and induced pluripotent- stem cell derived neurospheres (provided by Assoc Profs Mirella Dottori and Alice Pebay, University of Melbourne) during striatal differentiation. cDNA was synthesized from total RNA using Superscript III reverse transcriptase (Life Technologies). Duplex qPCR reactions were performed for each independent sample using the TaqMan® system (Applied Biosystems) with ribosomal 18S rRNA as the internal standard and an equivalent of 4–10 ng mRNA per reaction, in triplicate. Fold change in gene expression is presented as ΔΔCT relative to HDF or Day 0 of differentiation.

2.4. Differentiation of iNP cells

Following reprogramming, iNPs were mechanically dissociated and plated out for differentiation on GelTrex-coated coverslips (50,000–80,000 cells/cover-slip) to either a cortical or a striatal phenotype. Differentiation was performed on n = 2 independent cell lines in triplicate.
2.4.1. Cortical differentiation

Induced NPs were differentiated either in NBA or Astrocyte Conditioned Media (ACM) supplemented with 1% penicillin/streptomycin/glutamine (Gibco), 2% B27 supplement (Life Technologies), 0.01 mM retinoic acid (Sigma-Aldrich), 1% N2 supplement (Life Technologies), 20 ng/mL BDNF (PeproTech), 20 ng/mL GDNF (PeproTech), 10 μM Forskolin (Sigma-Aldrich) and 200 nM ascorbic acid (Sigma-Aldrich).

2.4.2. Striatal differentiation

From days 0–2 of differentiation, iNPs were differentiated in NBA supplemented with 1% Penicillin/Streptomycin/Glutamine, 2% B27 supplement, 1% N2 supplement, 200 ng/mL sonic hedgehog (SHH) (PeproTech), 100 ng/mL Dkk1 (PeproTech), 30 ng/mL BDNF (PeproTech) and 10 μM Y27632 (Sigma-Aldrich). From days 2–10, the cells continued to be incubated in the same media, but with the removal of Y27632. From day 10 onwards, the cells were differentiated in NBA supplemented with 1% Penicillin/Streptomycin/Glutamine, 2% B27 supplement, 1% N2 supplement with 10 μM Forskolin, and 50 ng/mL BDNF. This is based on the protocol by Lin and colleagues [46].

2.5. Immunocytochemistry and quantification

Cells were fixed in 4% paraformaldehyde for 10 minutes and processed for immunocytochemistry. The cells were first permeabilised in phosphate buffered saline with 0.5% Triton X-100 for 5 min. The following human specific primary antibodies were used: SOX2 (AbCam), PAX6 (BioLegend), FOXG1 (AbCam), SOX1 (AbCam), SOX3 (Merck Millipore), Ki67 (DAKO), Nestin (AbCam), NGN2 (R&D Systems), ASCL1 (EMD Millipore), TUJ1 (BioLegend), S100β (AbCam), GFAP (DAKO), MAP2AB (Chemicon), NSE (DAKO), vGLUT1 (MBL), DARPP32 (Novus). The species appropriate Alexa Fluor™ secondary antibody were used for visualisation of the primary antibody. At least 8 random images were acquired with an inverted Nikon TE2000 fluorescence microscope and at least 100 DAPI+ cells counted per image to obtain a total cell count. Lengths of neurites from TUJ1+ cells were measured in ImageJ and averaged to obtain an average neurite length in microns per condition.

2.6. Electrophysiology

Whole-cell patch-clamp recordings were performed at 28–62 days after differentiation of iNPs. Data was acquired using pClamp 9.3 software with multiclamp 700B amplifier and Digidata 1330 digitizer (Molecular Devices). Glass electrodes with resistance between 5 and 8 MΩ were pulled from the standard wall Borosilicate
Fig. 1. Chemically modified mRNA demonstrates high transfection efficiency and cell viability. (A) Graph demonstrating the percentage of transfected cells 24 hrs following a single or multiple transfection (TF) of eGFP cmRNA (0.01–2 μg). (B) Graph demonstrating the percentage of viable cells 48 hrs following a single or multiple transfection of eGFP cmRNA (0.01–2 μg). (C) Graph comparing transfection efficiency 24 hrs following either eGFP plasmid or eGFP cmRNA transfection, or following
tubing filamented glass (Sutter Instrument). Cells were continuously perfused at room temperature with an artificial cerebrospinal fluid (aCSF) containing the following (in mM): 119 NaCl, 2.5 KCl, 1 Na₂HPO₄, 1.3 MgSO₄, 2.5 CaCl₂, 26.2 NaHCO₃, 11 D-(-)glucose. The patch pipette contained (in mM): 120 K gluconate, 40 HEPES, 2 Na₂ATP and 0.3 NaGTP at pH 7.2 with KOH. Membrane potentials were typically kept at −60 mV to −70 mV. In current-clamp whole-cell configuration, action potentials were induced by injection of step currents that shifted membrane potentials from hyperpolarising −100 mV to depolarising 0 mV. Data was collected in Clampex and analysed in Clampfit (Molecular Devices).

2.7. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics v23 (IBM Corporation). Levene’s test for equality of variances was performed on all data. A one-way or two-way analysis of variance was used for comparison of treatment and/or time. Post-hoc analyses were performed with the Bonferroni test. All data are presented as mean ± s.e.m. Results were considered significant if p < 0.05.

3. Results

3.1. Chemically modified mRNA results in high transfection efficiency and cell viability of adult human dermal fibroblasts

The optimal concentration of chemically modified mRNA (cmRNA) to effectively transfect adult HDFs was determined by either single or multiple transfections of HDFs for 5 hrs using a concentration range of eGFP cmRNA (0.01–2 µg). FACS analysis demonstrated that eGFP cmRNA transfection was most effective at a concentration of 0.5–2 µg, resulting in ~90% of HDFs expressing eGFP following a single transfection (n = 3 lines; Fig. 1A). There was no difference in efficiency between 0.5 and 2 µg of cmRNA. Multiple transfections of 0.5–2 µg either SOX2/PAX6 plasmid or SOX2/PAX6 cmRNA co-transfection. (D) Graph demonstrating the percentage of transfected cells 24 hrs following a single transfection of either eGFP cmRNA, SOX2 cmRNA, PAX6 cmRNA or SOX2/PAX6 cmRNA. (E) Graph comparing the percentage of viable cells 48 hrs following either eGFP plasmid or eGFP cmRNA transfection, or following either SOX2/PAX6 plasmid or SOX2/PAX6 cmRNA co-transfection. (F) Graph comparing the percentage of viable cells following either 1 or 4 repeat transfections of eGFP cmRNA with either a 5 hr or 24 hr transfection incubation. (G) Graph comparing the percentage of viable cells following either 1 or 4 repeat transfections of PAX6 cmRNA, SOX2 cmRNA or SOX2/PAX6 cmRNA with either a 5 hr or 24 hr transfection incubation. (H) Image of eGFP protein expression 24 or 48 hrs after a single transfection with 0.5 µg eGFP cmRNA. (I) Image of SOX2 (green) and PAX6 (red) co-expression 24 hrs after SOX2/PAX6 cmRNA co-transfection. Scale bar = 50 µm. N = 3 independent lines; 3 repeats each line. A one-way analysis of variance was used for comparison of conditions. *p < 0.05; **p < 0.01; ***p < 0.001.

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cmRNA RNA did not increase transfection efficiency, with 78—92% of HDFs expressing eGFP (Fig. 1A). The effect of cmRNA on cell viability was also examined across a range of concentrations (0.5—2 μg) and compared between single or multiple transfections using the Alamar Blue® assay (n = 3 lines; Fig. 1B). We observed no difference in cell viability between 0.5 and 2 μg eGFP cmRNA, or between single and multiple transfections with viability ranging from 71 to 110% (Fig. 1B). Transfection of HDFs with either eGFP cmRNA or the combined transfection of SOX2/PAX6 cmRNA (1 μg cmRNA) resulted in a significant increase in both transfection efficiency and cell survival when compared to transfection with plasmid DNA (1 μg cDNA) (Fig. 1C and E). We compared the transfection efficiency of SOX2 cmRNA only, PAX6 cmRNA only and SOX2/PAX6 cmRNA combined with eGFP cmRNA. We observed no difference in transfection efficiency between SOX2, PAX6 or eGFP cmRNA single transfections, or when SOX2/PAX6 cmRNA was co-transfected (Fig. 1D). To assess the stability of cmRNA following transfection, eGFP protein expression was examined following a single 5hr transfection of eGFP cmRNA (0.5 μg). Protein expression was detected 18 hrs following transfection, peaked at 24 hrs and steadily declined up to 48 hr post-transfection (Fig. 1H). When HDFs were transfected up to four times at 24 hr intervals, eGFP expression persisted for no more than 48 hrs after each transfection. This is in agreement with the duration of mRNA expression reported by Yakubov and colleagues [47]. The presence of SOX2 and PAX6 protein was confirmed by immunocytochemistry 24 hr after transfection (Fig. 1I).

To ensure maximum cell transfection, we examined whether HDFs could be successfully transfected 4 times with cmRNA (0.5 μg/transfection), and whether cmRNA incubation could be extended to 24 hrs without affecting cell viability. A single transfection of eGFP cmRNA for 24 hrs significantly reduced cell viability compared to control HDFs. However, there was no difference in cell viability when we compared 5 hr versus 24 hr incubations for either single or multiple eGFP cmRNA transfections (n = 3 lines; Fig. 1F). We also compared single versus multiple (4x) transfection of either SOX2 cmRNA, PAX6 cmRNA or the combination of SOX2/PAX6 cmRNA (0.5 μg/transfection) following incubation for either 5 or 24 hrs. A reduction in cell viability was observed for cultures following either a single 24 hr transfection of SOX2 cmRNA, 4 × 24 hr transfections with PAX6 cmRNA or a single 24 hr transfection of SOX2/PAX6 cmRNA when compared to non-transfected HDFs (n = 3 lines; Fig. 1G). We also observed a significant reduction in cell viability between 1 × and 4 × 24 hr transfection of PAX6 cmRNA and 4 × 5 hr compared to 4 × 24 hr PAX6 cmRNA (Fig. 1G). All other conditions displayed consistent cell viability of >70%, suggesting that the number of transfections and the duration of cmRNA incubation has little effect on cell viability. Based on these results we chose to use a concentration of 0.5 μg of SOX2 and PAX6 cmRNA with transfections performed on 4 consecutive days for all further experiments.
3.2. Transfection of adult human dermal fibroblasts with SOX2/PAX6 cmRNA generates neural precursors

Adult HDFs (n = 3 lines) were transfected with 0.5 μg of SOX2/PAX6 cmRNA, cultured for up to 28 days in reprogramming media, and passaged every 7 days. Over the first 7 days following transfection, the cells retained a fibroblast-like morphology until the commencement of weekly passaging at day 7 (passage 1). After this point, cells transitioned to compact adherent epithelial-like morphologies and formed semi-adherent or floating aggregates that resembled neurospheres (Fig. 2A). Cultures were passaged weekly with reformation of neurosphere-like colonies within 2 days. Cultures could be passaged up to 10 passages before senescence occurred. This is in contrast to plasmid cDNA transfection which took up to 6 weeks post-transfection (PT) to generate colonies, with full colony formation not seen until weeks 8–9 PT (Fig. 2B) [18]. Adult HDFs transfected with eGFP cmRNA did not form colonies at any stage post-transfection (Fig. 2C). Hence these cells were not further examined. Based on the number of fibroblasts initially transfected with SOX2/PAX6 cmRNA, we estimate reprogramming efficiency to be 0.3%. In comparison, the reprogramming efficiency obtained using SOX2/PAX6 plasmid cDNA was 0.05% [18].

To confirm whether SOX2/PAX6 cmRNA is able to induce the expression of pro-neural genes as seen following transfection with SOX2/PAX6 plasmid DNA [18], real-time quantitative PCR (qPCR) was used to examine gene expression during reprogramming following either 5hr or 24 hr incubations with 0.5 μg SOX2/PAX6 cmRNA (n = 3 lines). We first confirmed the expression of the transgenes SOX2

![Fig. 2. (A) Images demonstrating the progression of iNP formation following SOX2/PAX6 cmRNA transfection. Cells passaged every 7 days. (B) Images demonstrating the progression of iNP formation following SOX2/PAX6 cDNA transfection. (C) Lack of iNP colony formation by passage 4 (28 days post transfection; PT) in human dermal fibroblasts transfected with eGFP cmRNA. Scale bars = 100 μm.](https://doi.org/10.1016/j.heliyon.2018.e00918)
and PAX6. Both transgenes were highly expressed across all time points examined (Fig. 3A and C) with the expression of SOX2 significantly greater following a 24 hr incubation than a 5 hr incubation (p = 0.012).

When we examined the expression of a range of pro-neural genes (Fig. 3C and D), we observed that FOXG1 and SIX3 were induced following either 5 hr or 24 hr incubations of SOX2/PAX6 cmRNA when compared to HDFs. OTX2, however, was expressed at low levels following either 5 hr or 24 hr incubations with SOX2/PAX6 cmRNA. We also observed expression of LHX2, NGN2 and the dorsoventral positioning gene GSH2 following both 5 hr and 24 hr incubations. In contrast, ASCL1 and the lateral ganglionic eminence (LGE) lineage gene DLX2 were reduced compared to HDFs following both 5 hr or 24 hr incubations. The hindbrain positioning gene GBX2 was not expressed at any time point during reprogramming. Furthermore, the pluripotent factor OCT3/4 was not expressed at any time following transfection with SOX2/PAX6 cmRNA (not shown).

To further assess the expression of pro-neural genes, immunocytochemical analysis was conducted after transfection with 0.5 μg SOX2/PAX6 cmRNA. SOX2 and PAX6 positive cells could be seen 24 hr after the last transfection (Fig. 4A), as could positive staining of cells for FOXG1 (Fig. 4B), SOX1, and OTX2 (Fig. 4C), and

![Fig. 3. SOX2/PAX6 iNPs express a range of neural genes. (A & C) Temporal profile of SOX2 and PAX6 transgene expression following either a 4 × 5 hr (A) or a 4 × 24 hr (C) transfection with SOX2/PAX6 cmRNA. (B and D) Temporal profile of neural gene expression following either a 4 × 5 hr (B) or a 4 × 24 hr (D) transfection with SOX2/PAX6 cmRNA. N = 3 independent lines; 3 repeats each line. *p < 0.05.](image-url)
Nestin (Fig. 4D). Positive staining for GSH2 was seen at both passages 1 and 4 (Fig. 4E). In agreement with mRNA levels, a proportion of cells expressed NGN2 by passage 4 (Fig. 4E). Positive staining for the LGE marker MEIS2 was also seen at both passage 1 and passage 4 (Fig. 4E). These findings demonstrate that within 28 days of SOX2/PAX6 cmRNA transfection, adult HDFs are reprogrammed to a heterogenous population of neural precursor cells expressing neuroectoderm and neural positioning markers.

3.3. Differentiating iNPs express neural lineage-specific genes as well as genes for ion channels and synaptic markers

Based on the expression of forebrain genes observed following SOX2/PAX6 cmRNA transfection, we examined whether SOX2/PAX6 derived iNPs were able to be differentiated either to a cortical or a striatal neuronal fate. We also wanted to determine whether there was a difference in the extent of neuronal differentiation for iNPs that had been reprogrammed following repeated (4x) transfection with SOX2/PAX6 cmRNA for either 5 hr or 24 hr incubations.

We first examined whether transfection of HDFs with SOX2/PAX6 cmRNA for either 5 hrs or 24 hrs affected differentiation when iNPs were cultured in either
cortical or striatal neuronal differentiation media (n = 3 lines cortical differentiation; n = 1 line striatal differentiation). Surprisingly we found that iNPs generated from the 4 x 24 hr transfection protocol did not undergo differentiation and died within a week of plating. In contrast, iNPs generated from the 4 x 5 hr transfection protocol underwent differentiation when plated out at either passage three or passage four. The inability for iNPs generated from the 4 x 24 hr protocol to differentiate is not in accordance with the comparable level of pro-neural gene expression detected in these cells relative to the 4 x 5 hr protocol. However, we did observe significantly higher levels of SOX2 in cells transfected for 24 hrs. This may inhibit the induction of lineage-specific neural genes required for iNPs to proceed to differentiation. While we are unable to distinguish between ectopic and endogenous SOX2 and PAX6 expression, our observation that eGFP protein expression is diminished by 48 hr post-transfection suggests that the lack of differentiation is due to high levels of endogenous transcription factor expression induced by the 4 x 24 hr protocol (Fig. 3A). Based on these observations we chose to use the 4 x 5 hr transfection protocol for the remainder of the study.

We next examined the gene expression profile of iNPs undergoing either cortical or striatal differentiation. HDFs (n = 3 lines cortical differentiation; n = 1 line striatal differentiation) were transfected with 0.5 μg SOX2/PAX6 cmRNA over 4 consecutive days with a 5 hr incubation period each day. Following 4 weeks of reprogramming, iNPs were cultured in either cortical or striatal neuronal differentiation media for 21 days. The gene expression profile of the differentiating cells was assessed weekly using qPCR.

In cells undergoing cortical differentiation we observed an up-regulation at various time points of differentiation of the lineage markers ASCL1, DLX2, MEIS2, TBR2 and vGLUT1 while NGN2 and BRN2 remained at low or undetected levels of expression (Fig. 5A–D, E1, F1 and H1). We also observed a reduction in expression of NCAM1 and TUJ1 at day 14 of differentiation (Fig. 5J1 and K1). During striatal differentiation, we observed the expression of ASCL1, DLX2, MEIS2 and GAD67 (Supp. Fig. 2A, B, D and E). The lack of neuronal genes observed during 21 days of differentiation is concerning and suggests that extended differentiation is required for maturation. However, what is interesting is that the profile of neuronal lineage markers expressed during the differentiation of hES- or iPSC- derived neural precursor cells (Fig. 5J1&2, K1&2, L1&2) is similar to that seen during the differentiation of directly reprogrammed iNPs suggesting that more time may be required for these markers to be expressed.

We also examined the expression of a range of ion channels and synaptic markers during differentiation. During cortical differentiation, we saw an increase in the expression of the calcium voltage-gated channel subunit 1αC (CACNA1C) and the synaptic genes SAP97, PSD95 and SHANK2 (Fig. 6A1, F1, G1 and H1). The
Fig. 5. Graphs demonstrating the level of neural gene expression in iNPs during cortical differentiation (A–D, E1–L1; n = 3 independent lines). Graphs demonstrating the level of neural gene expression during cortical differentiation of either iPSC- or hES- derived neurospheres (E2–L2; n = 1 line) at the equivalent stage of differentiation as iNPs. Fold change in gene expression is presented as ΔΔCT relative to HDF or Day 0 of differentiation. *p < 0.05; **p < 0.01; ***p < 0.001.
voltage-gated sodium channel gene SNC8A and synaptophysin (SYP) were reduced over time (Fig. 6B1 and D1). During striatal differentiation, we observed the expression of CACNA1C, SCN8A, and SAP97 (Supp. Fig. 2I, J and N).

### 3.4. Differentiation of SOX2/PAX6 cmRNA induced neural precursor cells results in the generation of immature vGLUT1+ glutamatergic and DARPP32+ striatal neurons

To investigate whether the duration of reprogramming affected the yield of neurons generated during differentiation, HDFs (n = 2 lines, 3 repeats) were transfected with 0.5 μg of SOX2/PAX6 cmRNA using the 4 × 5 hr transfection protocol. Reprogrammed cells were plated out for differentiation either at passage 2, 3 or 4 and cultured for 21 days in cortical differentiation media. At the end of differentiation, immunocytochemistry was performed for TUJ1 and the proportion of TUJ1+ cells determined relative to the total cell count (DAPI). Differentiation of iNPs after 14 days of reprogramming resulted in 11% of cells expressing TUJ1. In contrast, iNPs reprogrammed for either 21 or 28 days significantly increased the generation of TUJ1+ neurons to 38% and 31% respectively. This indicates that increased reprogramming time is required to provide optimal induction of neural genes for efficient differentiation. Further supporting this, we observed that reprogramming for over 35 days reduced the survival of iNPs providing a sub-optimal population of cells for differentiation (data not shown).

Following the culture of iNPs in cortical differentiation media for 21 days we observed a population of both TUJ1+ neurons and GFAP+ or S100β+ astrocytes (Fig. 7A, D and E). During differentiation we observed co-expression of S100β in a proportion of TUJ1+ neurons (Fig. 7L and M) suggesting the presence of a mixed population of radio-glial progenitors and immature neurons. By 21 days of differentiation we also observed the expression of the mature neuronal markers MAP2AB and NSE (Fig. 7B and C) indicating the presence of a more mature neuronal phenotype. Interestingly, the use of astrocyte conditioned media (ACM) as the base for the cortical differentiation media promoted the proportion of TUJ1+ neurons from 19% in NBA media to 84% in ACM, and reduced the population of S100β+ astrocytes (80% in NBA media; 16% in ACM). We also observed enhanced maturation of TUJ1+ neurons when cultured in ACM differentiation media, as defined by the presence of long multi-process neurites extending from the cell body, that were not seen in TUJ1+ neurons differentiated in NBA media (Fig. 7E and F; NBA media average

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**Fig. 6.** Graphs demonstrating the level of synaptic and ion channel gene expression in iNPs during cortical differentiation (A1–H1; n = 3 independent lines). Graphs demonstrating the level of synaptic and ion channel gene expression during cortical differentiation of either iPSC- or hES-derived neurospheres (A2–H2; n = 1 line) at the equivalent stage of differentiation as iNPs. Fold change in gene expression is presented as ΔΔCT relative to HDF or Day 0 of differentiation. *p < 0.05; **p < 0.01; ***p < 0.001.
Fig. 7. SOX2/PAX6 iNPs can generate either cortical or striatal immature neurons. (A–C) Images of (A) TUJ1 positive, (B) MAP2AB positive, and (C) NSE positive neurons after 21 days of cortical differentiation. (D) Image of GFAP positive astrocyte after 21 days of cortical differentiation. (E & F) Images demonstrating the presence of TUJ1 positive neurons and S100B positive astrocytes cultured in cortical differentiation media with either NBA (E) or astrocyte conditioned base media (ACM; F). Extended neurites can be seen from TUJ1 positive neurons cultured in ACM (arrows). (G) Image of TUJ1 positive neurons co-expressing the striatal marker DARPP32 35 days following striatal differentiation. (H–K) Images demonstrating TUJ1 positive neurons expressing vGLUT1 after 21 days of cortical differentiation.
neurite length = 39.46 μm, ACM media average neurite length = 109.95 μm). Following differentiation in ACM media, a population of TUJ1+ neurons co-expressed the glutamatergic marker vGLUT1 (85%; Fig. 7H–K).

We previously observed the level of gene expression was lower in neurons following 21 days of striatal differentiation than following cortical differentiation. Based on this, we extended our striatal differentiation protocol to a three-step 60+ day protocol and assessed the generation of DARRP32+ striatal neurons. After 30 days of differentiation we obtained a yield of 34% TUJ1+ cells from DAPI+ cells which was reduced to 5% by 60 days of differentiation, suggesting a possible loss of cell viability with the extended culture duration. Cells were co-stained for the GABAergic medium spiny neuron marker DARPP32. From days 30–62 of differentiation 70–90% of TUJ1+ cells co-expressed DARPP32 and exhibited multi-branching processes (Fig. 7G).

To assess whether any iNP cells remained in the differentiated cultures, cells were stained for SOX1, SOX2, SOX3, PAX6 and NESTIN at both 30 and 45 days of differentiation. We did not detect the expression of any of these precursor cell markers (data not shown). We also stained for the proliferative marker Ki67 which is readily observed in iNP cells. This was also absent at both 30 and 45 days of differentiation (data not shown).

To determine whether iNP-derived striatal neurons exhibited excitable membrane properties equivalent to mature human neurons, whole cell patch clamping was performed. Recordings were made at 36 and 50 days of differentiation (Fig. 7N and O). A few cells elicited single action potential-like responses upon the injection of depolarizing currents, where peak amplitudes increased with longer period of differentiation in culture (−7.93 mV at Day 36; 9.34 mV at Day 50). However, none of cells displayed multiple action potentials and many aspects with regards to a functionally mature action potential such as threshold, amplitude, and kinetics were still lacking. While this indicates the presence of an immature neuronal fate, extended culture time is required for full maturation.

4. Discussion

This study represents the first time a cmRNA approach has been used to directly reprogram adult human fibroblasts to iNPs, with the potential to generate region-specific neurons and glia. Cell reprogramming has predominantly been performed using integrative retroviral or lentiviral gene delivery systems [48]. Although very efficient and
stable, the use of integrative delivery systems can generate reprogrammed cells that carry randomly distributed viral transgene insertions with potential for tumorigenesis. Even if properly silenced, viral transgenes can be reactivated during differentiation leading to tumour formation. The use of Cre-deletable or inducible lentiviruses has resolved some of these issues, but in the context of therapeutic application, integrative viral delivery systems are not considered suitable [48, 49]. Recently, researchers have started using integration-defective Sendai viral vectors to address the issues associated with retroviral and lentiviral vectors [35, 50, 51, 52]. While efficient at transducing a wide range of host cells, the Sendai virus is not easily eliminated, making it a challenge to properly isolate transgene-free clones [35].

The issue of viral transgene integration and reactivation is more of a concern for the generation of iNPs through delivery of the potential oncogenic factors KLF4 and MYC, than through the use of the transcription factors SOX2 and PAX6. Regardless, the aim of the current study was to identify and confirm an efficient, non-integrative gene delivery approach for direct-to-iNP reprogramming to prevent the risk of genomic recombination or insertional mutagenesis, and provide a neural cell source suitable for neurological disease modelling and therapeutic application. We have previously demonstrated the use of plasmid DNA and recombinant protein delivery methods by which to generate human iNPs [18, 23]. While these strategies allowed for transgene-free and vector-free reprogramming, the major limitation associated with the use of plasmid DNA was a low level of transfection efficiency. We also observed that while the use of recombinant protein delivery resulted in iNP reprogramming [18], this was a very slow approach.

In regards to both safety and efficiency, RNA provides an ideal non-viral, non-integrating delivery system for cell reprogramming [53]. An initial study by Warren and colleagues [40] demonstrated that synthetic mRNA could provide the efficient conversion of different human somatic donor cells into induced pluripotent stem cells (iPSCs) with higher efficiency than that achieved by other non-integrative systems. To prevent the endogenous antiviral cell defence response to ssRNA, Warren and colleagues [40] synthesized mRNAs incorporating modified ribonucleoside bases. In addition, media supplemented with a recombinant version of B18R protein, a Vaccinia virus decoy receptor for type 1 interferons, further increased cell viability of RNA transfection.

In contrast to the system used by Warren and colleagues [40], we have used differently modified mRNA which has allowed for mRNA transfection without immune response inhibition through the replacement of uridine and cytidine residues with 2-thiouridine and 5-methylcytidine analogues, respectively, reducing the activation of an innate immune response and increasing mRNA stability [41]. Similar to Warren and colleagues [40], we obtained high transfection efficiency and cell viability after repeated cmRNA transfection. We performed 4 repeat transfections with adult HDFs
before culturing them in reprogramming media for an additional 21–28 days. In contrast, Warren and colleagues [40] continued to transfect cells with modified mRNA until the generation of iPSC colonies at 17 days. This was done to achieve a constant transgene expression to induce reprogramming. However, based on the high expression levels of SOX2 and PAX6 obtained after 4 repeat transfections we were concerned that extended repeat transfections would prevent later stage neural positioning genes to be expressed during reprogramming due to continual SOX2 and PAX6 expression inhibiting this progression. Indeed, the lack of differentiation observed with iNPs induced following 4 repeated transfections over 24 hr incubations supports this concern. We observed that the level of SOX2 expression was \( \sim 5 \times \) greater after the 4 \( \times \) 24 hr incubation compared to the 4 \( \times \) 5 hr incubation, which was then reflected by the expression of SIX3 also being a \( \sim 5 \) fold greater. There is a direct regulatory link between SOX2 and SIX3 [54] and up-regulation of SIX3 maintains neural progenitor cells in an undifferentiated state [55]. It is therefore necessary for ectopic expression of SOX2 and PAX6 to be transient and allow for the induction of endogenous neural patterning and region-specific genes to ensure full iNP reprogramming and subsequent differentiation.

As the gene expression profile of SOX2/PAX6 iNPs indicated the ability to generate a telencephalic fate, we investigated the potential for iNPs to differentiate into both cortical and LGE lineages. In accordance with the high expression of the cortical progenitor marker TBR2 and the glutamatergic gene VGLUT1, we observed that 21 days of cortical differentiation supplemented with ACM resulted in the induction of immature glutamatergic neurons (85%). In contrast, 21 days of differentiation did not induce sufficient upregulation of LGE lineage genes and so we proposed that differentiation was extended up to 60 days using a three-step protocol. This resulted in \( \sim 70–90\% \) of TUJ1+ neurons expressing DARPP32, a significantly enhanced yield compared to previous iPSC studies in which a yield of 0.5–27% of DARPP32+ neurons have been reported [56, 57, 58]. However, electrophysiological analysis indicated that further maturation is required to generate a mature striatal phenotype.

### 5. Conclusions

In conclusion, the current study provides proof-of-principle that cmRNA can be effectively used to directly reprogram adult HDFs to a neural precursor fate. The use of cmRNA for direct-to-iNP reprogramming has several key advantages over the use of integrative delivery systems for both neurological disease modelling and for cell replacement therapy to treat neurodegenerative disorders such as Parkinson’s disease and Huntington’s disease. For disease modelling, the initial donor cell population may be limited, so efficient methods are required to generate the disease cell line; in the current study, we have shown that cmRNA provides both high
transfection efficiency and cell survival to address this requirement. For cell replacement therapy, safety is the major issue, and it is imperative that genetic integrity is maintained in donor cells; this can be achieved through the use of cmRNA.

While further optimisation is required, and the generation of functional mature neurons needs to be confirmed, the use of cmRNA for cell reprogramming is extremely appealing for its simplicity and efficiency, as well as its high level of safety for clinical use in cell replacement therapy. We therefore believe the use of cmRNA has the potential to become a major technology for cell reprogramming and regenerative medicine. In the future we will extend this work to generate a range of neuronal phenotypes, with particular emphasis on striatal and dopaminergic fates, as well as the generation of oligodendrocytes, for cell replacement therapy to treat Huntington’s disease, Parkinson’s disease and spinal cord injury respectively.

Declarations

Author contribution statement

Bronwen Connor: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Erin Firmin, Amy McCaughey-Chapman, Ruth Monk, Kevin Lee, Sophie Liot: Performed the experiments.

Johannes Geiger, Carsten Rudolph: Contributed reagents, materials, analysis tools or data.

Kathryn Jones: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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