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Non-viral generation of neural precursor-like cells from adult human fibroblasts

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Abstract

Recent studies have reported direct reprogramming of human fibroblasts to mature neurons by the introduction of defined neural genes. This technology has potential use in the areas of neurological disease modeling and drug development. However, use of induced neurons for large-scale drug screening and cell-based replacement strategies is limited due to their inability to expand once reprogrammed. We propose it would be more desirable to induce expandable neural precursor cells directly from human fibroblasts. To date several pluripotent and neural transcription factors have been shown to be capable of converting mouse fibroblasts to neural stem/precursor-like cells when delivered by viral vectors. Here we extend these findings and demonstrate that transient ectopic insertion of the transcription factors SOX2 and PAX6 to adult human fibroblasts through use of non-viral plasmid transfection or protein transduction allows the generation of induced neural precursor (iNP) colonies expressing a range of neural stem and pro-neural genes. Upon differentiation, iNP cells give rise to neurons exhibiting typical neuronal morphologies and expressing multiple neuronal markers including tyrosine hydroxylase and $GAD_{65/67}$. Importantly, iNP-derived neurons demonstrate electrophysiological properties of functionally mature neurons with the capacity to generate action potentials. In addition, iNP cells are capable of differentiating into glial fibrillary acidic protein (GFAP)-expressing astrocytes. This study represents a novel virus-free approach for direct reprogramming of human fibroblasts to a neural precursor fate.

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Non-Viral Generation of Neural Precursor-like Cells from Adult Human Fibroblasts

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Recent studies have reported direct reprogramming of human fibroblasts to mature neurons by the introduction of defined neural genes. This technology has potential use in the areas of neurological disease modeling and drug development. However, use of induced neurons for large-scale drug screening and cell-based replacement strategies is limited due to their inability to expand once reprogrammed. We propose it would be more desirable to induce expandable neural precursor cells directly from human fibroblasts. To date several pluripotent and neural transcription factors have been shown to be capable of converting mouse fibroblasts to neural stem/precursor-like cells when delivered by viral vectors. Here we extend these findings and demonstrate that transient ectopic insertion of the transcription factors *SOX2* and *PAX6* to adult human fibroblasts through use of non-viral plasmid transfection or protein transduction allows the generation of induced neural precursor (iNP) colonies expressing a range of neural stem and pro-neural genes. Upon differentiation, iNP cells give rise to neurons exhibiting typical neuronal morphologies and expressing multiple neuronal markers including tyrosine hydroxylase and GAD$_{65/67}$. Importantly, iNP-derived neurons demonstrate electrophysiological properties of functionally mature neurons with the capacity to generate action potentials. In addition, iNP cells are capable of differentiating into glial fibrillary acidic protein (GFAP)-expressing astrocytes. This study represents a novel virus-free approach for direct reprogramming of human fibroblasts to a neural precursor fate.

Introduction

Cellular differentiation and lineage commitment have traditionally been considered as robust and irreversible processes. Recent work however has demonstrated that mature somatic cells can be reprogrammed to a pluripotent embryonic stem cell-like state by forced expression of the stem cell associated genes *OCT3/4*, *SOX2*, *KLF4* and/or *C-MYC*.[1-3] Using this technology, a range of cell types have been reprogrammed including human fibroblast cells, [2,4,5] keratinocytes, [6] mesenchymal cells, [7] peripheral blood cells ,[8] melanocytes [9] and adipose stem cells. [10] In addition, it has been demonstrated that forced expression of lineage-specific transcription factors can convert fibroblasts directly to blood progenitor cells, [11] mature cardiomyocytes, [12] hepatocyte-like cells [13], neural stem/precursor-like cells [14-18] and mature neurons [19-24] without first reverting to an intermediate pluripotent state. As such, somatic cell reprogramming provides a unique platform by which to dissect the molecular mechanisms that underlie epigenetic reprogramming and lineage specification as well as the exciting opportunity to personalize human cells for both research and clinical application.

Several studies have reported direct reprogramming of human fibroblast cells to mature neurons by the introduction

of defined neural genes.[19-24] Further, neuronal cell subtypes such as functional spinal motor neurons [24] or dopaminergic neurons [20,22] have been generated by over-expression of specific transcription factor combinations. Cell culture systems utilizing induced mature neurons have potential in the areas of neurological disease modeling and drug development. However, a clear limitation of postmitotic induced neurons is their inability to expand once reprogrammed. To date expandable neural stem/precursor-like cells have only been generated from mouse fibroblasts by viral transduction of pluripotent and neural transcription factors.[14-18] We therefore investigated whether adult human fibroblasts could be directly reprogrammed to become neural precursor cells through ectopic expression of specific neural transcription factors using non-viral technologies.

Different combinations of up to seven pluripotent and neural transcription factors have been identified to be capable of converting mouse fibroblasts into neural stem/precursor-like cells.[14-18] However, current knowledge regarding the key factors required to initiate neural stem/precursor fate determination in mouse and particularly in human cells is very limited. We hypothesized that genes which are involved in directing the differentiation of embryonic stem cells to the neuroectoderm lineage may also serve to reprogram human fibroblasts towards a neural stem/precursor-like state. Based on

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previous work, [25,26] we identified the neural genes *SOX2* and *PAX6* as playing a key role in the early stages of neurogenesis. *SOX2* is a key factor in reprogramming cells to a pluripotent state [2,5] and is also associated with multipotent and unipotent stem cells.[27-29] *PAX6* is essential for neural stem cell proliferation, multipotency and neurogenesis.[30] *SOX2* and *PAX6* are also expressed when embryonic stem cells undergo differentiation to a neural lineage.[25,27-32] Further, we have previously observed that neural induction of human embryonic stem cells results in mutually exclusive expression of *PAX6* and the pluripotency factor *OCT3/4,* [25] indicating the involvement of *PAX6* in neural lineage determination.

The current study provides proof-of-concept that transient expression of *SOX2* and *PAX6* in adult human fibroblasts generates induced neural precursor-like (iNP) colonies expressing a range of neural stem and precursor genes, and immature neural markers. Upon differentiation, iNP colonies give rise either to mature neurons capable of generating action potentials upon current injection or to astrocytes as indicated by GFAP expression. This was achieved using either non-viral plasmid transfection or recombinant protein transduction technology alleviating concerns of insertional mutation associated with the use of genome-integrating viral vectors. Furthermore, we have optimized cell culture conditions allowing us to generate iNP colonies in the absence of an animal or human feeder cell layer, an essential requirement for the transfer of reprogrammed cell lines to clinical use. This technology provides a novel and ethically viable option for obtaining neural precursor cells for neurological research and clinical application.

Materials & Methods

Cell culture

Human dermal fibroblast cells (HDF) isolated from the abdomen of two adult donors were obtained from Cell Applications Inc. and maintained in human HDF Growth Media (Cell Applications, Inc.). Plasmid DNA transfections and protein transduction were performed at passage 8 or earlier. Media was changed to proliferation medium comprising Neurobasal-A (Gibco), 1% D-glucose (Sigma-Aldrich), 1% Penicillin/Streptomycin/Glutamine (Gibco), 2% B27 supplement (Gibco), 0.2% EGF (PeproTech), 0.08% FGF-2 (PeproTech), 0.2% Heparin (Sigma-Aldrich) and VPA (Sigma-Aldrich) to a concentration of 1µM three days post-transfection or post-transduction. Media was changed three-times weekly, and cells were replated regularly during iNP colony formation.

Transcription factor delivery

HDF cells at 1×10^5 cells per well were seeded in polystyrene 6-well plates (Nunc - Thermo Fisher Scientific) 24 hrs prior to transfection or transduction. Media was changed to Optimem (Invitrogen) 2 hrs prior to the transfection procedure. Nonviral plasmid transfections were carried out using Lipofectamin LTX, Plus reagent (Invitrogen) and plasmid constructs encoding *SOX2* , *PAX6* or *eGFP* driven by a CMV promoter at a ratio 3:1:1 (v/v/w) and a total

plasmid DNA amount of 2µg. pCMV-SPORT6 comprising *PAX6* cDNA was purchased from Invitrogen. *SOX2* cDNA was purchased from Addgene and cloned into pEGFP-N1 (Clontech) after removal of *eGFP* expression cassette from pEGFP-N1. Protein co-transductions were carried out in four transduction cycles using a mix of SOX2-TAT protein (PeproTech) and PAX6 protein (Abnova) or PE control protein (OzBioscience) with ProDeliverIn (OzBioscience) in a ratio 1:1 and a total protein amount of 5µg per transduction cycle.

Quantitative RT- PCR

Total RNA was isolated from $2.5 – 9.0 \times 10^3$ colonies of four independent iNP cell lines and two independent control cell lines by RNAeasy Mini Kit (Invitrogen), respectively. cDNA was synthesised from total RNA using Superscript reverse transcriptase (Invitrogen). Three independent duplex qPCR reactions were performed in triplicates for each independent cell line using the TaqMan system (Applied Biosystems) with ribosomal 18S rRNA as the internal standard and an equivalent of 4ng mRNA per reaction.

Immunocytochemistry

Immunocytochemical staining was carried out by standard procedures using the following human-specific primary antibodies: CD133 (Abcam), SOX2 (R&D Systems), PAX6 (Covance), NGN2 (R&D Systems), MASH1 (Chemicon), TUJ-1 (Covance), NSE (DAKO), MAP2 (Chemicon), $GAD_{65/67}$ (Chemicon), TH (Chemicon), and GFAP (DAKO).

Differentiation of iNP cells.

Induced neural precursor colonies were generated by *SOX2*/*PAX6* plasmid transfection and cultured for 6 weeks in neural precursor proliferation media medium comprising of NBA media, 2% B27, 1% D-glucose, 1% Penicillin/Streptomycin/Glutamine, 0.2% EGF, 0.08% FGF-2, 0.2% Heparin, 1µM VPA and 25ng/ml Midkine (Peprotech). For neuronal differentiation, the cells were mechanically dissociated and transferred onto poly-ornithine/laminin-coated (Sigma-Aldrich/ Invitrogen) surface following full colony formation and differentiated using a two-step protocol as previously described.[33] Briefly, cells were cultured in NBA media supplemented with 250ng/ml SHH (R&D systems), 100ng/ml DKK1 (PeproTech), 20ng/ml BDNF (PeproTech) and 10µM Y27632 (Calbiochem). After 10 days, culture conditions were changed to NBA media containing 0.5mM dibutryl-cyclic AMP (Sigma-Aldrich), 0.5µM VPA, 20ng/ml BDNF and 10µM Y27632.

For glial differentiation iNP colonies were mechanically dissociated following full colony formation and plated onto on fibronectin-coated surface. Astroglial differentiation was carried out in Glasgow minimum media (Sigma-Aldrich), supplemented with 10% FBS and 1% Penicillin/Streptomycin/Glutamine for 14 days. To promote oligodendroglial differentiation, cells were cultured in 3 stages: NBA media supplemented with 2% B27, 1% D-glucose, 1% Penicillin / Streptomycin / Glutamine, 0.08%

FGF-2 and 0.1% retinoic acid (Sigma-Aldrich) for 2 days, followed by DMEM/F12 media containing 0.5% N2 supplement (Invitrogen), 10ng/mL FGF-2, 10ng/mL PDGF, 0.5mM dibutryl-cyclic AMP for 4 days and DMEM/F12, 30ng/mL 3,3',5-Triiodothyronine (Sigma-Aldrich), and 200µM Ascorbic acid (Sigma-Aldrich) for an additional 4 days.

Electrophysiology

Whole cell patch clamping was performed as previously described [34] on iNP–derived neurons after 13, 21 and 30 days of differentiation. Briefly, cells were bathed in artificial cerebrospinal fluid (in mM: 120 NaCl, 3 KCl, 2 CaCl2, 1.25 NaH2PO4, 2 MgSO4, 20 glucose, 26 NaHCO3). Internal solution consisted of (in mM: 120 K gluconate, 40 HEPES, 5 MgCl2, 2 NaATP, 0.3 NaGTP). Cells were visualised on an Olympus BX51W1. Electrode resistance was 5 MΩ. Action potentials were induced in current clamp mode by a 10 ms current injection using a Multiclamp 700B Commander (Molecular Devices). Series resistance was constantly monitored and results were not included if significant variation (>20%) occurred during an experiment. Data acquisition and analysis was performed using pClamp9 software.

Results

SOX2 and PAX6 convert human fibroblasts to neural precursor-like cells.

SOX2 and PAX6 are key transcription factors in the development and maintenance of neural precursor cell identity. We therefore investigated the potential of ectopic SOX2 and PAX6 expression to directly convert adult human

fibroblasts to neural precursor-like cells. A homogenous population of adult human dermal fibroblasts previously used to induce pluripotency by Yamanaka and colleagues, and shown to be free of cell cross-contamination was used in this study.[2,35] Protein transduction and non-viral plasmid transfection systems were utilized to provide transient ectopic expression of SOX2 and PAX6 in adult human fibroblasts (Figure 1A). Optimization of transfection / transduction efficiencies and control experiments were performed using either an eGFP plasmid or red-fluorescent phycoerythrin (PE) protein, respectively (Figures 1B, 1C).

Neural differentiation protocols for human embryonic stem (hES) cells suggested the use of defined neural culture conditions in the absence of a mouse embryonic fibroblast (MEF) feeder layer to drive differentiation towards a neuroectodermal fate.[36,37] Thus, fibroblasts were allowed to recover in human fibroblast growth media for 3 days after SOX2/PAX6 delivery before being transferred to specific neural precursor cell culture conditions consisting of Neurobasal A media (NBA) with B27 supplement, FGF2, EGF and valproic acid (VPA). For both SOX2/PAX6 plasmid transfection and protein transduction, we observed significant changes in fibroblast density and morphology between days 5 to 10 following transfer to neural precursor cell culture conditions. The changes occurred in a temporal order with decreasing cell density, altered morphology to a more compact shape and grouping of cells (Figure 2A). Strikingly, we observed the formation of domed and compact neurosphere-like cell colonies 4 weeks after protein delivery and 8 to 11 weeks after ectopic gene expression of SOX2
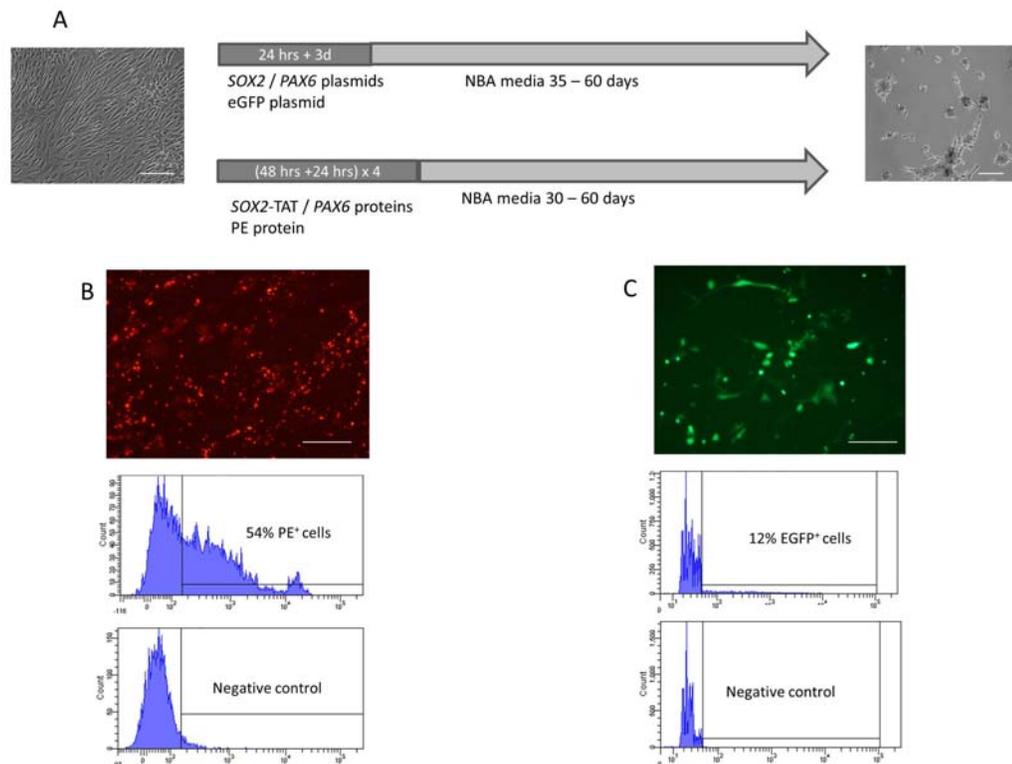

Figure 1: Timeline of protein / plasmid DNA delivery and generation of iNP cell colonies. (A) Transcription factors were introduced into fibroblast cells by either plasmid DNA transfection or protein transduction. Plasmid DNA transfections were carried out in human dermal fibroblast growth media using Lipofectamin LTX with a lipoplex incubation time of 24 hrs. Cells were subsequently cultured in human dermal fibroblast growth media for another 3 days before culture conditions were changed to NBA media to allow colony formation within 35-60 days. Protein transduction was performed in four cycles over a period of 12 days using ProDeliverin. Transductions were performed for 48 hrs in human dermal fibroblast growth media. Cells were cultured in human dermal fibroblast growth media for additional 24 hrs after transduction procedure. Transductions were carried out in four cycles of 72hrs each. (B) Phycoerythrin protein was utilised to optimize and assessed ProDeliverIn mediated protein transduction into human dermal fibroblast cells. FACS analysis revealed a protein transduction efficiency of 54%. (C) Nonviral plasmid transfection was optimized to an efficiency of 12% using pCMV-EGFP-N1 plasmid and Lipofectamine LTX. Scale bars: 100µm

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and *PAX6*. Colonies induced by plasmid transfection and protein transduction both appeared with a regular, round morphology with a diameter between 50 - 500μm (Figure 2B). Interestingly, fibroblasts transfected with *eGFP* control plasmid or transduced with PE control protein and cultured in neural culture conditions for up to 11 weeks occasionally developed cell colony structures (Figure 2C). However, colony formation in control cultures occurred significantly later, with lower efficiency and a less uniform morphology than *SOX2*/*PAX6*-induced colonies. In contrast, fibroblasts cultured in human fibroblast growth media only grew in a monolayer and showed no colony formation at any time point (Figure 2D).

To investigate the proliferative capability of induced cell colonies, primary colonies were mechanically dissociated and replated as single cells. Within 2-3 days, individual cells generated secondary colonies with increased cell diameter (Figure 2E). Repeated dissociation and replating gave rise to tertiary and quaternary colonies. Formation and growth of uniform colonies could be increased and accelerated by

and *PAX6* expression exhibited features of neural precursor cells, we investigated the transcriptional signature and phenotypic characteristics of *SOX2*/*PAX6* induced colonies compared to *eGFP* or PE controls. Total RNA from three fully developed independent colony lines was isolated at 65, 75 or 85 days respectively following plasmid transfection, and an additional colony line was isolated 27 days following protein transduction. A range of neural stem cell and precursor genes and positional markers within the neuroectodermal tube were analyzed and compared by quantitative PCR (Figure 3A). We observed that colonies transduced with SOX2 and PAX6 protein exhibited *SOX2* and *PAX6* mRNA expression in contrast to the PE control (Figure 3A). Due to the transient nature of protein transduction in contrast to delivery of genetic material, this observation strongly indicates the activation of endogenous *SOX2* and *PAX6* mRNA expression by ectopic protein transduction. Similar to protein transduction, *SOX2* and *PAX6* mRNA expression levels were found in independent *SOX2/PAX6* induced colony lines formed at different time points after transient
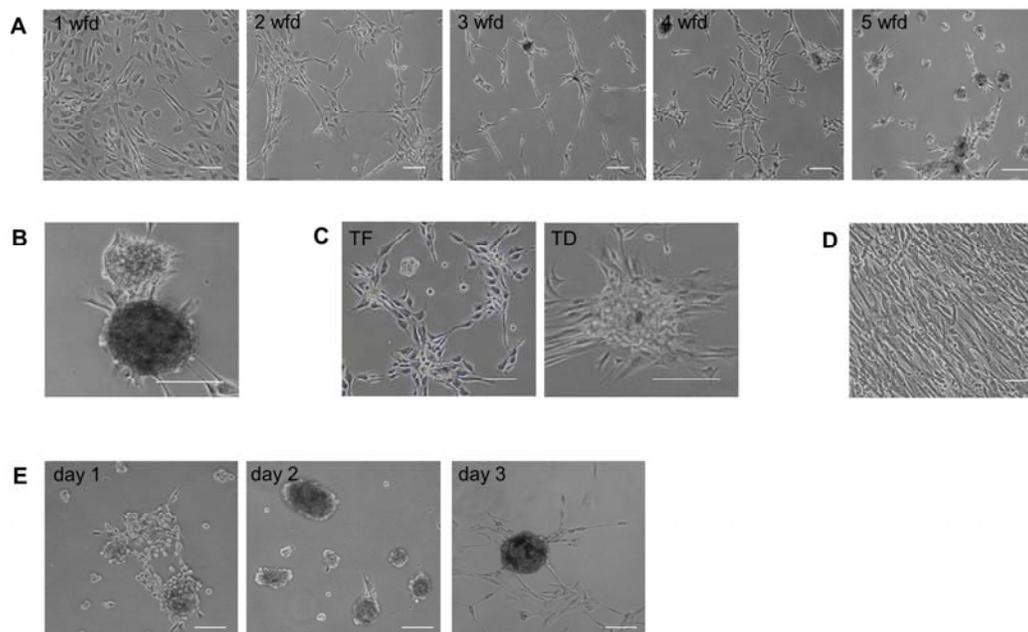

Figure 2: Generation of induced neural precursor colonies from human fibroblasts. (A) Temporal change of fibroblast morphology from 1 - 5 weeks following *SOX2*/*PAX6* delivery (wfd). (B) Fully developed iNP colonies 6 weeks after *SOX2*/*PAX6* delivery. (C) Fibroblast morphology 11 weeks after either *eGFP* plasmid transfection (TF) or PE protein transduction (TD). (D) Untreated fibroblasts maintained their morphology when cultured in human dermal fibroblast growth media. (E) Secondary iNP colonies reformed within 3 days following mechanical dissociation. Scale bars: 100 μm.

regular replating of cells to an efficiency of 0.05% of the number of fibroblasts initially transfected/transduced with *SOX2*/*PAX6*. Regular cell replating also decreased the time required for full colony formation to 5 - 8 weeks following plasmid transfection.

These results demonstrate that transient ectopic delivery of *SOX2* and *PAX6* can direct the generation of uniform induced colonies resembling neural precursor cell neurosphere colony formation.[38,39] Furthermore, colony formation was achieved in the absence of a MEF feeder cell layer.

Induced colonies show neural precursor-like characteristics.

To assess if cell colonies induced by transient ectopic *SOX2*

plasmid DNA transfection with the highest relative expression observed in colony lines isolated 65 days after transfection and gradually decreasing in colony lines isolated 75 and 85 days post transfection (Figure 3A). Quantitative PCR analysis further demonstrated elevated mRNA expression levels of the neural markers neurogenin2 (*NGN2*), *SOX1*, *HOXB9*, *NKX6.1* and *SIX3*. *eGFP*- and PE-treated controls showed either no mRNA expression or relatively lower levels of these markers (Figure 3A). In contrast, *HES1* and *IRX3* mRNA levels were reduced in iNP colonies generated by SOX2/PAX6 protein transduction compared to PE control cells (Figure 3A). Transcriptional profile determination of three independent colony lines generated by plasmid transfection revealed
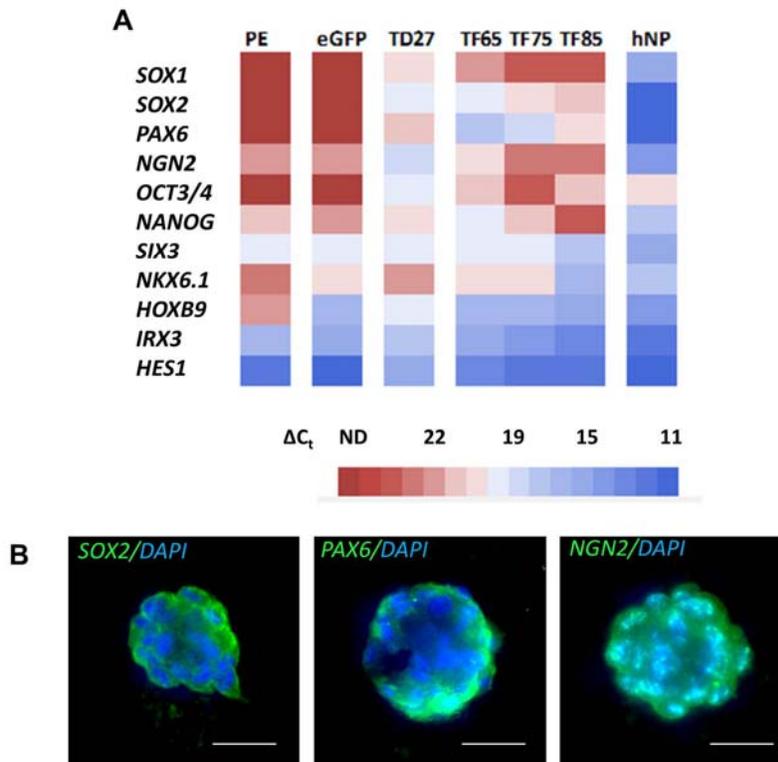
Figure 3: Characterization of induced neural precursor cells. (A) Gene expression profiles of independent iNP colony lines generated by protein transduction (TD) or plasmid transfection (TF) at different time points post transcription factor delivery compared to *eGFP* transfection (eGFP), PE transduction (PE) and hESC-derived neural precursor cell (hNP) controls. Heatmap represents the average cycle threshold values normalized to internal ribosomal 18S rRNA control (ΔCt) obtained from three independent qPCR reactions, respectively. (B) Expression of SOX2, PAX6 and NGN2 in iNP colonies. Scale bars: 25 µm.

a distinctive pattern of neural gene expression dependent on the duration following delivery of *SOX2/PAX6* (Figure 3A). Colony lines analyzed 65 days following *SOX2/PAX6* delivery exhibited elevated mRNA expression levels of *SOX2*, *PAX6*, *SOX1*, *HOXB9*, *NKX6.1* and reduced *HES1* and *IRX3* expression relative to *eGFP* controls. Conversely, colony lines analyzed at 85 days showed a shift towards higher *HES1*, *IRX3*, *SIX3* and lower *PAX6*, *SOX2*, *NGN2* and *SOX1* expression levels compared to the colony lines analysed 65 days post *SOX2/PAX6* treatment. In accordance, colony lines examined 75 days following *SOX2/PAX6* treatment displayed intermediate transcription factor expression levels. This suggests a progressive transition of neural gene expression towards a more mature phenotype over time following the initial induction of *SOX2* and *PAX6* expression. The gene expression pattern of the iNP colony lines was also compared to noggin-primed hES-derived neural precursor (hNP) cells (Figure 3A). Quantitative PCR analysis demonstrated higher mRNA expression levels of *SOX2*, *PAX6*, *SOX1* and *NGN2* in hNP cells compared to iNP cells. This may reflect either the presence of partially reprogrammed iNP cells or a population of non-reprogrammed fibroblasts present in the culture.

It has been shown that the putative pluripotent marker *OCT3/4* is expressed in neural stem cells and that expression of *NANOG* is partially regulated by a *SOX2-OCT3/4* complex.[40-42] Thus, we assessed if expression of *SOX2* in iNP colonies is associated with the expression of *OCT3/4* and/or *NANOG*. *OCT3/4* mRNA levels were found to be up-regulated in iNP colony lines isolated 65 days after

plasmid transfection and in colony lines generated by protein transduction when compared to controls. However, analysis of iNP colony lines isolated 75 days following *SOX2/PAX6* gene delivery revealed no increase in *OCT3/4* expression (Figure 3A). Similar results were found for *NANOG*, with an increase in *NANOG* mRNA expression only observed in iNP colony lines isolated at 65 or 75 days post transfection and in protein-treated colony lines when compared to controls (Figure 3A).

To further assess the expression of neural precursor markers, immunocytochemical analysis was conducted. Positive staining of iNP colonies for SOX2, PAX6 and NGN2 confirmed qPCR results at the protein level (Figure 3B). However, irregular expression patterns were observed in a small number of colonies which may also suggest partial reprogramming. Combined, these results demonstrate that transient expression of *SOX2* and *PAX6* in human fibroblasts induce cell colonies that display the transcriptional and phenotypic signature of neural precursor cells.

Induced neural precursor colonies have the capacity to differentiate into mature neurons and astrocytes.

To investigate the capacity of iNP colonies to differentiate into mature neurons and glial cells, we tested several differentiation conditions. We have previously demonstrated efficient differentiation of neural precursor cells derived from the subventricular zone of the adult rat brain using standard neural differentiation media.[43,44] As such, induced neural
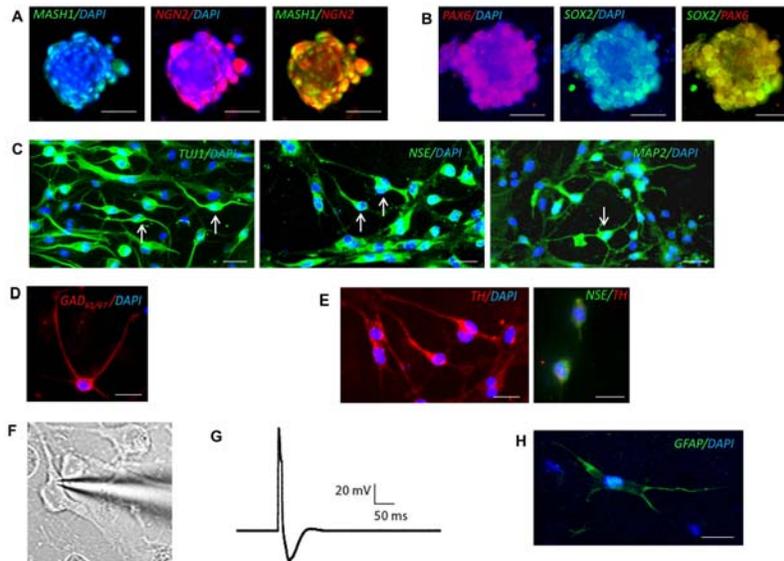
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Figure 4: Neuronal and astroglial differentiation of induced neural precursor cells. Co-expression of (A) MASH1 and NGN2 and (B) SOX2 and PAX6 in differentiating iNP colonies. (C) iNP-derived neurons express the neuronal markers TUJ1, NSE, and MAP2. Arrows indicate cells exhibiting a typical neuronal morphology. (D) iNP-derived neurons express $GAD_{65/67}$ or (E) tyrosine hydroxylase (TH) with NSE co-expression. (F) Image of iNP-derived neuron undergoing patch clamp recording after 30 days of differentiation. (G) Representative trace of action potential generated in response to current injections from iNP-derived neuron after 30 days of differentiation. (H) Astrocytes derived from iNP cells express the astroglial marker GFAP. Scale bars: 25 μm.

precursor colonies generated by plasmid transfection were cultured in standard neural differentiation media for two weeks. Immunocytochemical analysis demonstrated co-expression of the pro-neural markers *MASH1* and *NGN2* (Figure 4A) and enduring expression of *SOX2* and *PAX6* (Figure 4B) following two weeks of differentiation. However, iNP colonies did not differentiate into either neurons or glia as indicated by a lack of staining for the markers MAP2, GFAP and RIP, and remained in their colony assemblage.

Several studies suggest the use of sonic hedgehog (*SHH*) to efficiently regulate neuronal differentiation of hES-derived neurospheres.[33,45] Thus, we examined whether a modified two-staged differentiation protocol which has previously been demonstrated to generate mature neurons from iPSCs[33] could drive efficient neuronal differentiation of iNP cells. Induced neural precursor colonies were mechanically dissociated and cultured in neural differentiation media supplemented with SHH, DKK1, BDNF and Y27632 for 10 days. The cells were subsequently exposed to dibutryl-cyclic AMP, VPA, BDNF and Y27632. After 13 days of differentiation a proportion of iNP cells developed a neuron-like morphology with tangential and radial processes. Continued differentiation up to 30 days resulted in an increase in the proportion of iNP cells exhibiting a typical neuronal morphology and the generation of a more mature phenotype with the appearance of multiple arborizing dendrites and a single axon from the cell soma. Further confirmation of neuronal differentiation was demonstrated by robust expression of the neuronal markers TUJ-1, NSE and MAP2 (Figure 4C). A population of iNP-derived neurons also expressed the GABAergic marker $GAD_{65/67}$ (Figure 4D) or the catecholaminergic marker tyrosine hydroxylase (TH) (Figure 4E). To determine whether iNP-derived neurons exhibit excitable membrane properties similar to mature neurons, whole cell patch clamping was performed. Initial recordings

performed after 13 or 21 days of differentiation revealed the presence of functionally immature cells. The average resting membrane potentials were depolarized (-39.8 ± 4.3 mV, n = 6 cells and -38.5 ± 3.3 mV, n = 11 cells respectively) and no action potentials could be evoked by current injection. However, after 30 days differentiation functionally mature neurons were able to fire single action potentials upon current injection (Figures 4F, 4G). Moreover, the average resting membrane potential had shifted to significantly more hyperpolarised potentials (-50.8 ± 2.9 mV, n = 15 cells) compared to recordings performed at days 13 and 21 (p < 0.05). This was accompanied by a significant increase in membrane capacitance (from 164.5 ± 48.0 pF to 869.3 ± 334.2 pF; p < 0.05) consistent with significant increases in the amount of neuronal membrane as the cells develop a more mature neuronal morphology.

To determine the glial differentiation potential of iNP colonies, we mechanically dissociated iNP cells and cultured them in astroglial or oligodendroglial differentiation media, respectively. We observed a small proportion (< 1%) of cells expressing the astrocytic marker GFAP and exhibiting an astrocyte-like morphology after 14 days of differentiation (Figure 4H). However, no cells were found expressing the oligodendrocyte markers O4 or RIP when cultured under oligodendroglial conditions (data not shown).

Discussion

The current study demonstrates a method to directly convert adult human fibroblasts into neural precursor cells. This was achieved by exogenous application of the transcription factors *SOX2* and *PAX6*. In order to avoid genome interference, non-viral plasmid transfection or protein transduction techniques were chosen for ectopic expression

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of *SOX2* and *PAX6*. The time period required for fibroblasts to form iNP colonies was comparable with previous studies using protein transduction and plasmid transfection to generate iPSCs, indicating a significant impact of methodology on the reprogramming process.[46,47] In contrast, the efficiency of iNP colony generation of 0.05% of the initial transfected/transduced fibroblast cells was considerably higher than the induction of iPSCs using non-viral methods.[46,47]

Others have recently demonstrated the direct generation of neural stem and precursor cells from either embryonic [14-17] or adult [18] mouse fibroblast cells. This was achieved by viral transduction of either the four pluripotent transcription factors *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* in a transient manner [15], or viral transduction of up to eleven neural stem cell-associated factors.[16-18] In two other studies the proto-oncogene *BMI1* was identified as a key factor in reprogramming mouse fibroblasts into either induced neural stem or precursor cells.[17,18] Interestingly, Han and colleagues [14] observed morphological transformation of mouse embryonic fibroblasts 2-3 weeks after viral delivery of *SOX2* and *PAX6*. However, the cells were not analyzed or cultured to a stage later than 3 weeks post transduction. Further, a recent study has demonstrated the reprogramming of mouse embryonic fibroblasts and human fetal foreskin fibroblasts into multipotent neural stem-like cells following retroviral transduction of *SOX2* when cultured on a mitomycin C-treated MEF feeder layer.[48] This is the first study to demonstrate the generation of neural stem-like cells from human fibroblasts and supports the findings presented in the current study. Using both *SOX2* and *PAX6*, we now extend these findings to the generation of iNP cells from adult human fibroblasts by non-viral delivery without the requirement of a MEF feeder layer.

In the current study, iNP colonies exhibited elevated expression of the neural stem and pro-neural markers *SOX1*, *SOX2*, *PAX6*, *NGN2*, *HOXB9* and *NKX6.1*, with down-regulation of *HES1* and *IRX3* mRNA after SOX2/PAX6 protein delivery or 65 days following plasmid transfection. Due to the transient nature of both the plasmid transfection and protein transduction procedures utilized in this study, we were able to determine the effect of endogenous *SOX2* and *PAX6* mRNA expression in the resultant iNP cells. Interestingly, temporal analysis of mRNA expression revealed an inverse shift of expression with reduced relative mRNA levels of *PAX6*, *SOX2*, *NGN2* and *SOX1* and concurrent elevation of *HES1*, *IRX3*, *SIX3* expression in independent colonies analyzed at 75 and 85 days compared to 65 days following *SOX2/PAX6* delivery. *PAX6* has previously been demonstrated to regulate *NGN2* expression in a concentration-dependent manner [49], reflected by the temporal correlation of *PAX6* and *NGN2* expression observed in iNP colonies. Further, down-regulation of the transcriptional repressor *HES1*, with opposing functions to *PAX6*, is in accordance with *PAX6* up-regulation and *PAX6/HES1* regulation of neurogenesis. [30] Elevated mRNA expression of the putative pluripotent markers *OCT3/4* and *NANOG* was also detected with gradually decreasing levels in cells from 65 to 75 days following *SOX2/PAX6* delivery. *OCT3/4* and *NANOG* expression in iNP cells was in agreement with studies demonstrating that *SOX2* and *OCT-3/4* work cooperatively to regulate their own transcription by a positive feedback loop, and that *OCT3/4* and *SOX2* in turn

activate *NANOG* expression.[40,50] Interestingly, 85 days post *SOX2/PAX6* transfection *OCT3/4* levels were found to be increased whereas *NANOG* and *SOX2* expression levels were decreased compared to 75 days post transfection.

To prove the capacity for iNP cells generated by *SOX2/PAX6* delivery to form mature neurons, we tested several differentiation conditions and assessed the expression of mature neuronal markers as well as the electrophysiological properties of differentiated iNP cells. Mature neurons were able to be derived from iNP colonies by a differentiation protocol which included SHH and BDNF. Differentiated cells showed a distinctive neuronal morphology including multiple arborizing dendrites and expression of the neuronal markers TUJ1, NSE and MAP2 with a population of iNP-derived neurons expressing TH or GAD$_{65/67}$. Most importantly, iNP-derived neurons demonstrated the ability to fire action potentials in response to current injection. Measurement of the electrical properties of iNP-derived neurons at different time points during differentiation confirmed a gradual maturation process over a period of 30 days. In addition, iNP cells were able to give rise to a small population of astrocytes as demonstrated by staining for the astrocytic marker GFAP. However, we were not able to detect oligodendroglial differentiation. Lujan and colleagues [16] also observed a lack of oligodendrocyte differentiation from neural precursor cells which were generated from mouse embryonic fibroblasts by viral delivery of the two transcription factors *SOX2* and *FOXG*. Oligodendroglial differentiation was only achieved by addition of *BRN2*. It would be interesting to further assess whether a combination of *BRN2*, *PAX6* and Sox2 can also successfully convert human fibroblasts to iNP cells capable of oligodendrocyte differentiation.

Conclusion

The current study provides proof-of-concept that transient expression of the neural transcription factors *SOX2* and *PAX6* directly converts adult human fibroblasts into neural precursor-like cells. The use of either non-viral plasmid transfection or recombinant protein transduction alleviates concerns of insertional mutation associated with the use of genome-integrating viral vectors. Further studies will be necessary to thoroughly optimize the efficiency for human iNP cell generation and neural differentiation. Such studies will facilitate the application of this method for future use for *in vitro* disease modeling, drug screening and as a ready source of human autologous neural precursor cells for cell replacement therapy.

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