Attenuation of PRRX2 and HEY2 enables efficient conversion of adult human skin fibroblasts to neurons

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The direct conversion of accessible cells such as human fibroblasts to inaccessible cells, particularly neurons, opens up many opportunities for using the human model system to study diseases and discover therapies. Previous studies have indicated that the neuronal conversion of adult human skin fibroblasts is much harder than that for human lung fibroblasts, which are used in many experiments. Here we formally report this differential plasticity of human skin versus lung fibroblasts in their transdifferentiation to induced neurons. Using RNAseq of isogenic and non-isogenic pairs of human skin and lung fibroblasts at different days in their conversion to neurons, we found that several master regulators (TWIST1, TWIST2, PRRX1 and PRRX2) in the fibroblast Gene Regulatory Network were significantly downregulated in lung fibroblasts, but not in skin fibroblasts. By knocking down each of these genes and other genes that suppress the neural fate, such as REST, HES1 and HEY2, we found that the combined attenuation of HEY2 and PRRX2 significantly enhanced the transdifferentiation of human skin fibroblasts by ASCL1 and p53 shRNA. The new method, which overexpressed ASCL1 and knocked down p53, HEY2 and PRRX2 (ApH2P2), enabled the efficient transdifferentiation of adult human skin fibroblasts to MAP2+ neurons in 14 days. It would be useful for a variety of applications that require the efficient and speedy derivation of patient-specific neurons from skin fibroblasts.

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1. Introduction

Ectopic expression of Brn2, Ascl1 and Myt1l (BAM) directly converts mouse fibroblasts to induced neurons (iN) [1]. Since this seminal discovery, human fibroblasts have been successfully reprogrammed to induced neurons [2] and various subtypes of neurons including dopaminergic neurons (iDA) [3–5], serotonergic neurons (i5HT) [6,7], motor neurons (iMN) [8] and excitatory cortical neurons (iCtx) [9], by different combinations of transcription factors, which generally include the pioneer factor ASCL1 and various lineage-specific transcription factors [10,11]. Several ASCL1-independent methods, such as miR-124 overexpression [12] or PTB/ nPTB knock-down [13], also transdifferentiate human fibroblasts to neurons, including cortical neurons, striatal medium spiny neurons [14], and spinal cord motor neurons [15]. Our previous studies have shown that overcoming reprogramming barriers, such as p53 and cell cycle [16], significantly improves the transdifferentiation of human fibroblasts to induced dopaminergic neurons [17] and induced serotonergic neurons [6]. The majority of human fibroblasts used in past reprogramming experiments are lung fibroblasts, particularly fetal lung fibroblasts such as MRC5 and IMR90. Despite the huge success in reprogramming human fetal lung fibroblasts to various types of neurons [2,16], the efficiency to convert adult human skin fibroblasts, the most easily accessible fibroblasts from live human subjects, remains very low for unknown reasons.

In this study, we performed RNA sequencing (RNAseq) on both isogenic and non-isogenic pairs of human lung and skin fibroblasts during ASCL1-induced neuronal conversion to investigate their
differential plasticity in transdifferentiation. Analyses of RNAseq data collected at various stages of reprogramming revealed several master transcription factors in the fibroblast Gene Regulatory Network (GRN) and several genes that suppress the neural fate as barriers for the conversion of human skin fibroblasts to induced neurons. Through two rounds of siRNA screens targeting these genes, we found that the double knockdown of HEY2 and PRRX2 significantly enhanced the transdifferentiation of adult human skin fibroblasts to induced neurons by ASCL1 and p53 shRNA. The optimized condition — overexpression of ASCL1 plus knocking down p53, HEY2 and PRRX2 (Aph2P2) — provided an efficient method to generate patient-specific MAP2+ mature neurons in 14 days. It would be very useful for a variety of applications, especially in biomarker discovery studies where fast and efficient derivation of neurons from a large number of human subjects is desired. The method would facilitate the adoption of patient-specific neurons for the study of many brain disorders.

2. Methods

2.1. Materials

DIBUTYRIL CYCLIC-AMP (dc-AMP) was purchased from sigma. Dorsomorphin dihydrochloride, purmorphamine, CHIR99021, SB431542, PD 0332991, 3 mM doxycycline. For generation of induced dopaminergic neurons, medium was changed every other day for the duration of the culture period. The medium was changed to neural induction medium (DMEM/F12, 1x N2, 1x B27, 1xNEAA, 0.5 mM dorsomorphin, 2.5 mM PD 0332951, 3 mM CHIR99021, 0.2 mM vitamin C, 10 mM Y27632, 0.5 mM dCAMP, 20 ng/ml NGF, 20 ng/ml GDNF, 20 ng/ml BDNF, 1 µg/ml doxycycline). For generation of induced dopaminergic neurons, 2 µM purmorphamine and 1 ng/ml TGFβ3 were used in addition. The medium was changed every other day for the duration of the culture period.

2.3. Immunofluorescence

Cells were fixed at day 10 after doxycycline induction with 4% paraformaldehyde for 10 min at room temperature (RT), permeabilized and blocked in blocking solution (3% BSA in PBS) containing 0.1% Triton X-100 for 1 h at RT, and then incubated in primary antibodies (anti-TUJ1, AB78078 from Abcam; anti-TUJ1, BioLegend 801201; anti-MAP2, Santa Cruz sc-74421; anti-TH, Thermo Fisher OPA1-04050) diluted 1:1000 in blocking solution overnight at 4°C, then secondary antibodies (Alexa 488 donkey anti-rabbit, Thermo Fisher A21206; Alexa 546 donkey anti-mouse, Thermo Fisher A10036) diluted 1:2000 in blocking solution for 2 h at RT and DAPI for 5 min at RT. Fluorescence images were acquired on a Leica AF6000 Inverted Microscope. Stained cells were counted from five randomly selected fields at 10× magnification for each condition.

2.4. Validation of the knockdown efficiency of shRNAs

Neonatal dermal fibroblasts, AG22056, were plated at 5 × 10^3/cm^2 in 6 well plates for 24 h and infected for 16 h with lentivirus for each plKO/shRNA or plKO/scramble at MOI of 20. Cells were harvested 4 days after infection. Total RNA was extracted using Trizol and reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Biorad). Expression levels of targeted genes were measured by q-PCR in triplicates using IQ™ Supermix (Biorad) with gene specific primers (REST: GTGACCGCTGCCGCTAATCTAA and GGACAAGTACGGCTTAATGAG; HEY2: GTGCCGGCTGTGCTCATTCA and GCCGAATGACCATGGCTGA; PRRX2: CCTCGGCCTGGTCTTTTG; TWIST1: AGAGTGATGTCAGCGTGAGG and AACTAGCCATTGGCCTGTA; TWIST2: CTCGTCGTCGTGCGTTTG; NURR1: TRCN0000020869; PRRX1, TRCN0000436440; PRRX2, TRCN00000014872; HEY2, TRCN0000002049; HES1, TRCN0000018989 and REST, TRCN0000014783) were selected from TRC (RNAi Consortium) shRNA library [18] and purchased from Sigma.

2.5. RNAseq and bioinformatics analysis

Total RNA were extracted at indicated time points during conversion using RNAasy Mini kit (Qiagen). Library preparation, sequencing, raw reads alignment and RPKM calculation were performed by University at Buffalo Genomics and Bioinformatics Core facility following its standard pipeline [20]. RPKMs were log2 transformed with base line set at 0.3. Accumulative Expression (AE) of a given gene was defined as the sum of the log transformed RPKMs at all four time points. Differentially expressed genes between lung and dermal fibroblasts were defined using the following criteria, AE(lung) - AE(dermal) >= 3.5. Genes with annotation of GO:0003700, DNA-binding transcription factor activity, were considered as transcription factors. All bioinformatics analyses were performed using R. RNAseq data on AG04452 and AG04451 are deposited to GEO under accession GSE125692. RNAseq data on C002 and MRC5 are deposited to GEO under accession GSE129358.

3. Results

3.1. Neuronal conversion is much less efficient for human skin fibroblasts than for human lung fibroblasts

With our previously established protocol for the derivation of induced dopaminergic neurons using ASCL1, NURR1, LMX1A, miR124 and p53 shRNA (ANLmp) [17], two lines of primary human
Establishing adult human skin as an alternative source for transdifferentiation is important because of the ethical concerns with using embryonic tissues. To compare the efficiency of transdifferentiation between human lung fibroblasts and human skin fibroblasts, we performed transdifferentiation experiments on five different lines of primary human skin fibroblasts (MRC5 and IMR90) and five different lines of human lung fibroblasts (AG22056, AG16146, and GM00731), under the same condition, only 32.5 ± 3.3% of cells generated from human skin fibroblasts exhibited neuron-like morphology and 19.4 ± 1.5% of cells expressed TH for the three lines of skin fibroblasts on average (Fig. 1A). Furthermore, almost all of TUJ1+ cells generated from human lung fibroblasts (e.g. MRC5) exhibited typical neuronal morphology, characterized by long and thin processes (Fig. 1B and D). In contrast, only 32.5 ± 3.3% of cells generated from human skin fibroblasts (e.g. AG22056) had neuron-like morphology and 19.4 ± 6.8% had fibroblast-like morphology, even though both kinds of cells were TUJ1+ (Fig. 1C and D). The results showed that ASCL1-driven transdifferentiation of human fibroblasts had significantly different efficiencies, depending on the tissue origins of the fibroblasts, with human skin fibroblasts much harder to reprogram than human lung fibroblasts.

3.2. Fibroblast gene regulatory network is more stably maintained in human skin fibroblasts during transdifferentiation to neurons

To explore the cause for the different efficiencies in the direct conversion of human skin vs. lung fibroblasts to neurons, we performed RNAseq to compare transcriptomic changes in human lung and dermal fibroblasts at days 0, 1, 2 and 4 of transdifferentiation to iDA neurons by ANLmp (Fig. 2A). We profiled transcriptomes from two pairs of human lung/dermal fibroblasts, one isogenic pair and one non-isogenic pair (Fig. 2B). This experimental design allowed us to identify changes that are dependent on cell type, but not on genetic background. We first analyzed the expression levels of the three transcription factors (ASCL1, NURR1 and LMX1A) that drive transdifferentiation. They were induced by doxycycline to similar levels in human skin and lung fibroblasts (Fig. 2C). This excludes the possibility of insufficient expression of transgenes in human skin fibroblasts. Based on cumulative expression level (details in methods), we found that 488 genes were differentially expressed between skin and lung fibroblasts in a consistent manner among the two pairs of skin and lung fibroblasts during conversion.

Among them, 258 genes showed higher expression in lung fibroblasts than in skin fibroblasts, and 230 genes exhibited higher expression in skin fibroblasts than in lung fibroblasts (Table S1, Common Differentially Expressed Genes, CoDE-genes). Gene Ontology (GO) analysis revealed lung fibroblasts had stronger expression of neural development-related genes, which is consistent with efficient transdifferentiation of lung fibroblasts to neurons. GO analysis also revealed that many genes related to immune response and interferon pathway were strongly expressed in skin fibroblasts. However, suppression of immune response by dexamethasone or inhibition of interferon pathway by STAT1 or IRF9 knockdown had no significant effect on the neural conversion of human skin fibroblasts (data not shown).

We then focused on the 86 transcription factors among the 488 differentially expressed genes and found that 42 transcription factors were expressed at significantly higher levels in lung fibroblasts, while 44 transcription factors were expressed at significantly higher levels in skin fibroblasts (Table S2, Common Differentially Expressed Transcription Factors, CoDE-TFs). Among these transcription factors, four previously identified fibroblast master transcription factors [21,22], TWIST1, TWIST2, PRRX1 and PRRX2, remained highly expressed in human skin fibroblasts during transdifferentiation, but were suppressed rapidly in human lung fibroblasts after doxycycline induction of reprogramming factors (Fig. 2D). It indicates that fibroblast Gene Regulatory Network (GRN) was more stable in human skin fibroblasts than in lung fibroblasts during neural conversion.
3.3. Double knockdown of PRRX2 and HEY2 facilitates neuron conversion of human neonatal dermal fibroblasts

Based on the data above, we hypothesized that destabilizing fibroblast GRN in human skin fibroblasts may improve their transdifferentiation to neurons. To test this, we knocked down the four transcription factors identified in bioinformatics analysis to be critical for fibroblast GRN [21,22], TWIST1, TWIST2, PRRX1 and PRRX2, using a lentivirus expressing shRNA targeting each of them (Fig. 3A). In addition, we knocked down transcription factors that suppress neural fate, such as REST [23,24], HES1 [25] and HEY2 [26], by a lentivirus expressing shRNA against each of the genes (Fig. 3A). Significant knockdown of each candidate gene was observed in AG22056 human skin fibroblasts (Fig. 3A). We tested whether each of these shRNAs changed the reprogramming efficiency (Fig. 3B) and yield (Fig. 3C) over the results produced by ASC1 and p53 shRNA (Ap) [6,17] in AG22056, a line of primary neonatal foreskin fibroblasts. Reprogramming efficiency is defined by the percentage of cells that were MAP2⁺ at 14 day of transdifferentiation. Reprogramming yield is defined by the average number of MAP2⁺ neurons in a frame of view. Both criteria are needed, because some conditions resulted in significant cell death, but the remaining cells had higher percentages of neurons, thus increasing reprogramming efficiency without increasing reprogramming yield. We found that on top of Ap, the addition of shRNA against HEY2 (H2), PRRX2 (P2) or REST (R) significantly improved both the efficiency and yield of MAP2⁺ cells, among which HEY2 knockdown had the most potent effect (Fig. 3B and C). The addition of shRNA against TWIST2 (T2) or HES1 (H1) resulted in severe cell death and dramatic reduction of MAP2⁺ cells (Fig. 3B and C). They were thus excluded from further experiments. We then tested whether the advantageous effect of Ap and H2 (ApH2) could be enhanced further by the addition of another shRNA. The addition of PRRX2 shRNA (P2) significantly improved reprogramming efficiency (Fig. 3D) and yield (Fig. 3E). Knockdown of PRRX1 (P1) did not significantly change the effect of ApH2. The addition of shRNA for TWIST1 (T1) or REST (R) worsened reprogramming by causing marked cell death (Fig. 3E). Thus, the biggest enhancement over the basal condition of ASC1 and p53 shRNA (Ap) (Fig. 3F) was the addition of HEY2 and PRRX2 shRNAs (ApH2P2) (Fig. 3G), as evidenced by quantification of percentage of Tuj1⁺ cells among all cells at day 14 (Fig. 3H). The addition of HEY2 and PRRX2 shRNAs significantly increased the share of MAP2⁺ neurons, while decreasing the fibroblast-like or neuron-like Tuj1⁻/MAP2⁻ populations of partially reprogrammed cells.

3.4. Double knockdown of HEY2 and PRRX2 improves the transdifferentiation of human adult skin fibroblasts to neurons

We next tested whether the double knockdown of HEY2 (H2) and PRRX2 (P2) enhances the ability of ASC1 and p53 shRNA (Ap) [6,17] to convert adult human skin cells to neurons. Two different lines of adult human skin fibroblasts, GM00731 (Fig. 4A–C) and AG16146 (Fig. 4D–F), were transduced with lentiviruses expressing Ap or ApH2P2. Compared to the reprogramming efficiency of Ap, the addition of HEY2 and PRRX2 shRNAs significantly increased the percentages of Tuj1⁺ cells at Day 14 from 32.2±5.8% to 82.1±9.2% in GM00731 (Fig. 4C) and from 23.9±3.4% to 70.0±8.4% in GM00371 (Fig. 4C).
performed RNAseq on different days at the early stage in the (Fig. 4C) and AG16146 (Fig. 4F), respectively. Thus, the optimal conversion of lung skin knockdown of HEY2 and PRRX2 signiﬁcantly enhances the direct conversion of human skin broblasts to MAP2+ mature neurons in 14 days.

4. Discussion

Direct conversion of cell types across lineages has shown that it is possible to generate many inaccessible types of cells, such as neurons, from readily available cells, such as fibroblasts, without going through stem cells. Adult skin fibroblasts, cultured from skin biopsy, are the most accessible type of cells for reprogramming studies, particularly for the generation of patient-speciﬁc cells for disease modeling or development of cell replacement therapy. Robust conversion protocols have been developed to convert primary human ﬁbroblasts to various types of neurons. Most of the human ﬁbroblasts being tested in previous studies are lung ﬁbroblasts. Despite similarities in gene expression proﬁle [27], morphology and cell culture behavior, adult human skin ﬁbroblasts were much harder to be reprogrammed to induced neurons [6,17], as shown in Fig. 1. To understand the underlying differences, we performed RNaseq on different days at the early stage in the transdifferentiation of lung or skin ﬁbroblasts to induced neurons. Both isoegenic pairs and non-isoegenic pairs were used to facilitate the identiﬁcation of key differences between lung and skin ﬁbroblasts, irrespective of genetic backgrounds. We found that 488 genes were differentially expressed in a consistent way between lung and skin ﬁbroblasts; 258 genes had higher expression in lung ﬁbroblasts than in skin ﬁbroblasts, while 230 genes showed the opposite pattern. About 20% of the differentially expressed genes are transcription factors (42 showed higher expression in lung ﬁbroblasts and 44 had lower expression in lung ﬁbroblasts). Among these transcription factors, four master regulators (TWIST1, TWIST2, PRRX1 and PRRX2) in the ﬁbroblast Gene Regulatory Network (GRN) [21,22] were rapidly downregulated during the conversion of lung ﬁbroblasts to neurons, but remained high in skin ﬁbroblasts (Fig. 2D). We reasoned that lowering the expression of these genes may render skin ﬁbroblasts similar to lung ﬁbroblasts. Although not differentially expressed, genes that suppress the neural fate, such as REST, HEY2 and HES1, were speculated to be inhibiting the conversion. Through two rounds of screens with shRNA targeting these seven genes, we found that the combined knockdown of HEY2 and PRRX2 signiﬁcantly improved the baseline condition of ASCL1 plus p53 shRNA in the conversion of neonatal skin ﬁbroblasts (Fig. 3F–H) and adult skin ﬁbroblasts (Fig. 4). Our previous studies have shown that the attenuation of p53 signiﬁcantly enhances the direct conversion of human ﬁbroblasts to induced dopaminergic neurons [17] and induced serotonergic neurons [6]. Thus, the new method identiﬁed in this study (over-expression of ASCL1 plus the knockdown of p53, HEY2 and PRRX2) represents an efﬁcient way to generate induced neurons from skin ﬁbroblasts in 14 days. The method can be readily adapted to screen large numbers of human subjects for biomarker discovery research and many other applications.

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Appendix A. Supplementary data

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Transparency document

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