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## ATTENUATION OF PRRX2 AND HEY2 ENABLES EFFICIENT CONVERSION OF ADULT HUMAN SKIN FIBROBLASTS TO NEURONS

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### Abstract

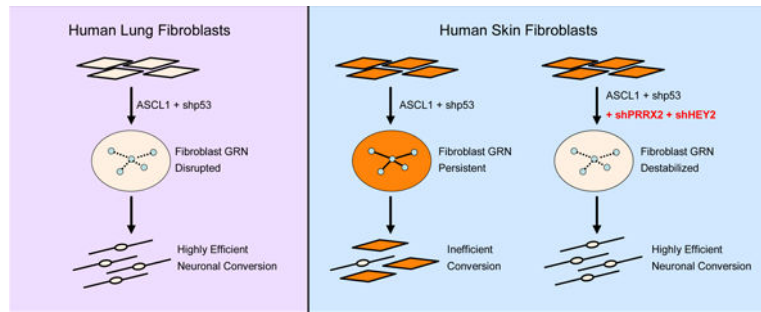
The direct conversion of accessible cells such as human fibroblasts to inaccessible cells, particularly neurons, opens up many opportunities for using the human model system to study diseases and discovery therapies. Previous studies have indicated that the neuronal conversion of adult human skin fibroblasts 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 induced 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<sup>+</sup> 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.

### Graphical Abstract

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## Keywords

Direct conversion; transdifferentiation; induced neurons; skin fibroblasts; lung fibroblasts; HEY2; PRRX2

## 1. Introduction

Ectopic expression of Brn2, Ascl1 and Myt11 (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, particular 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 stage 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<sup>+</sup> 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

Dibutyl cyclic-AMP (dc-AMP) was purchased from sigma. Dorsomorphin dihydrochloride, pumorphamine, CHIR 99021, SB431542, PD 0332991 and Y27632 were purchased from Tocris. Recombinant human bFGF, NGF, GDNF, BDNF and TGFβ3 were purchased from PeproTech., pLKO.1/p53shRNA, pLKO.1/scrambled shRNA, pMD2.G and psPAX2 were purchased from Addgene. FUW-tetO-LoxP-cDNA (hAscl1, hNurr1, hLmx1a and hmiR-124) and FUW-LoxP-M2rtTA were generated previously [17]. pLKO/shRNA (TWIST1, TRCN0000378362; TWIST2, TRCN0000020869; PRRX1, TRCN0000436440; PRRX2, TRCN0000014782; HEY2, TRCN0000020249; HES1, TRCN0000018989 and REST, TRCN0000014783) were selected from TRC (RNAi Consortium) shRNA library [18] and purchased from Sigma.

### 2.2. Generation of induced neurons from human fibroblasts.

Primary human fibroblasts MRC5 (from American Type Culture Collection), IMR90, AG22056 (both from Coriell) were cultured in DMEM containing 10% FBS and 2mM L-glutamine (both from Thermo Fisher). AG16146 and GM00731 (both from Coriell) were cultured in DMEM containing 20% FBS, 2mM L-glutamine and 10ng/ml bFGF. Lentivirus production was performed as described previously [19]. Briefly, 293FT cells (Thermo Fisher) in one 10-cm dish were cotransfected with 10 μg transfer plasmids (pLKO or FUW) with 2.5μg pMD2.G and 7.5μg psPAX2 using Lipofectamine 2000. Viruses were collected every 12 hours between 12h to 48h after transfection and titred using p24 ELISA kit (ZeptoMetrix Corporation, Buffalo, NY). Induced neurons were generated with previously established protocol [17]. Briefly, fibroblasts were plated at  $5 \times 10^3/\text{cm}^2$  and infected 1 day later for 16h with the indicated combinations of lentiviruses (MOI 10 for pLKO/p53shRNA, MOI 20 for all others) in the presence of 8 μg/ml polybrene. Cells were then washed three times with DMEM/F12 to remove remaining serum and cultured in DMEM/F12 for 24h to arrest cell cycle. Then medium was changed to neural induction medium (DMEM/F12, 1x N2, 1x B27, 1xNEAA, 0.5 μM dorsomorphin, 2.5 μM SB431542, 1 μM PD 0332991, 3 μM CHIR99021, 0.2mM vitamin C, 10 μM Y27632, 0.5mM 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 pumorphamine 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 1h 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 546 donkey anti-rabbit, Thermo Fisher A21206; Alexa 488 donkey anti-mouse, Thermo Fisher A10036) diluted 1:2000 in blocking solution for 2h 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  $5 \times 10^3/\text{cm}^2$  in 6 well plates for 24 hrs and infected for 16h 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: GTGACCGCTGCGGCTACAATACTAA and GGACAAGTAGGATGCTTAGATTTGA; HES1: ACGTGCGAGGGCGTTAATAC and GGGGTAGGTCAATGGCATTGA; HEY2: GTGCGGCTTGTGTCTCATCTC and CTGCTGCTGCTGCGTTTG; PRRX1: GAGACGTGACTGCTGTGGAG and AAGTAGCCATGGCGCTGTA; PRRX2: CCTCGCTGCTCAAGTCCTAC-and-AGGGTGGCACTGTGCTGT;-TWIST1: AAGGCATCACTATGGACTTTCTCT-and-GCCAGTTTGATCCCAGTATTTT;-TWIST2: TCTGAAACCTGAACAACCTCAG-and-CTGCTGTCCCTTCTCTCGAC;-GAPDH: AGCCACATCGCTCAGACAC and GCCCAATACGACCAAATCC). Raw  $C_t$  values of each gene were normalized to the  $C_t$  value of GAPDH in the same sample to control for loading variation.

#### 2.5. RNAseq and bioinformatics analysis

Total RNA were extracted at indicated time points during conversion using RNeasy 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,  $|AE(\text{lung}) - AE(\text{dermal})| \geq 3.5$ . 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 lung fibroblasts, MRC5 and IMR90, were directly converted to neurons. On

average for the two lines of lung fibroblasts,  $87.0 \pm 6.1\%$  of the cells at day 10 were TUJ1<sup>+</sup> neurons, while  $56.5 \pm 3.9\%$  of the cells were TH<sup>+</sup> dopaminergic neurons (Fig. 1A). When we reprogrammed three different lines of primary human skin fibroblasts, AG22056, AG16146 and GM00731, under the same condition, only  $48.0 \pm 3.3\%$  of cells were TUJ1<sup>+</sup> and  $11.7 \pm 1.5\%$  of cells expressed TH for the three lines of skin fibroblasts on average (Fig. 1A). Furthermore, almost all of TUJ1<sup>+</sup> 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  $32.5 \pm 3.3\%$  of cells generated from human skin fibroblasts (e.g. AG22056) had neuron-like morphology and  $19.4 \pm 6.8\%$  had fibroblast-like morphology, even though both kinds of cells were TUJ1<sup>+</sup> (Fig. 1 C 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 the 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 accumulative 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 focus 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 ASCL1 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<sup>+</sup> at 14 day of transdifferentiation. Reprogramming yield is defined by the average number of MAP2<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cell (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 ASCL1 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<sup>+</sup> cells among all cells at day 14 (Fig. 3H). The addition of HEY2 and PRRX2 shRNAs significantly increased the share of MAP2<sup>+</sup> neurons, while decreasing the fibroblast-like or neuron-like Tuj1<sup>+</sup>/MAP2<sup>-</sup> 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 ASCL1 and p53 shRNA (Ap) [6, 17] to convert adult human skin cells to neurons. Two different lines of adult human skin fibroblasts, GM00371 (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<sup>+</sup> cells at Day 14 from 32.2 ± 5.8% to 82.1 ± 9.2% in GM00371 (Fig. 4C) and from 23.9 ± 3.4% to 70.0 ± 8.4% in AG16146 (Fig. 4F). Ap generated no MAP2<sup>+</sup> neurons, while ApH2P2 generated 17.2 ± 5.6% and 5.8 ± 3.0% MAP2<sup>+</sup> neurons in GM00371 (Fig. 4C) and AG16146 (Fig. 4F), respectively. Thus, the optimal combination of reprogramming factors (ApH2P2) identified in neonatal skin fibroblasts was able to reprogram adult skin fibroblasts to MAP2<sup>+</sup> 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 isogenic pairs and non-isogenic 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 fibroblast (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 (overexpression 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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## Abbreviations

<b>RNaseq</b>	RNA sequencing
<b>iDA neurons</b>	induced dopaminergic neurons
<b>iN</b>	induced neurons
<b>GRN</b>	Gene Regulatory Network
<b>MOI</b>	multiplicity of infection
<b>RT</b>	room temperature

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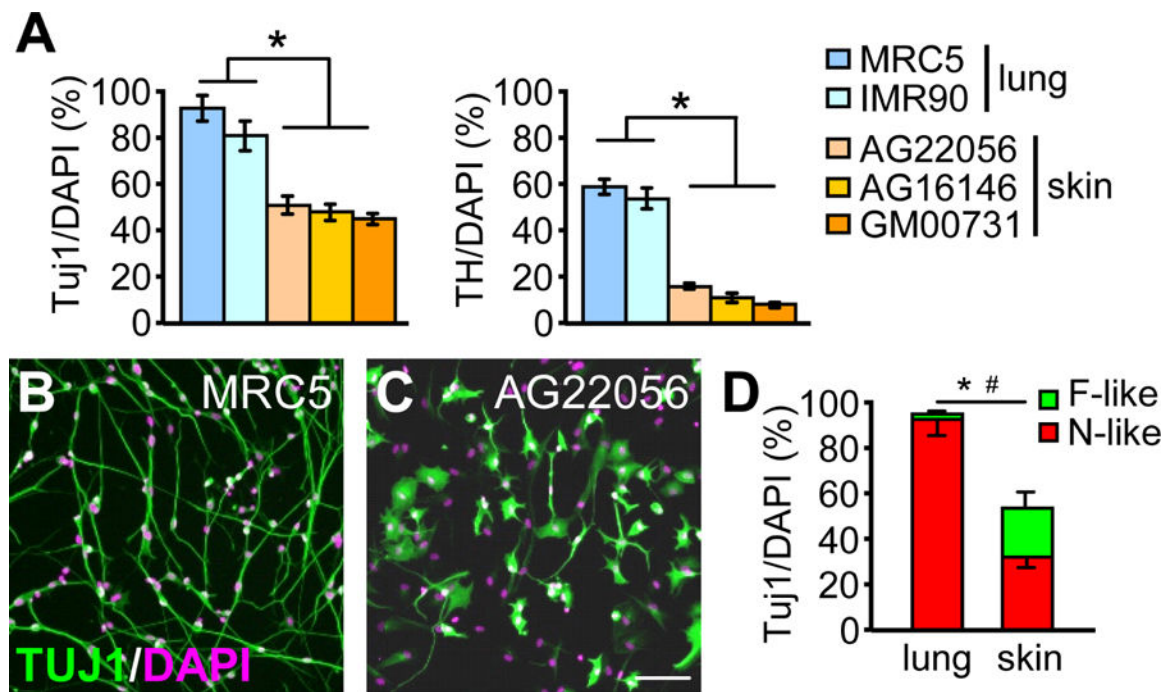
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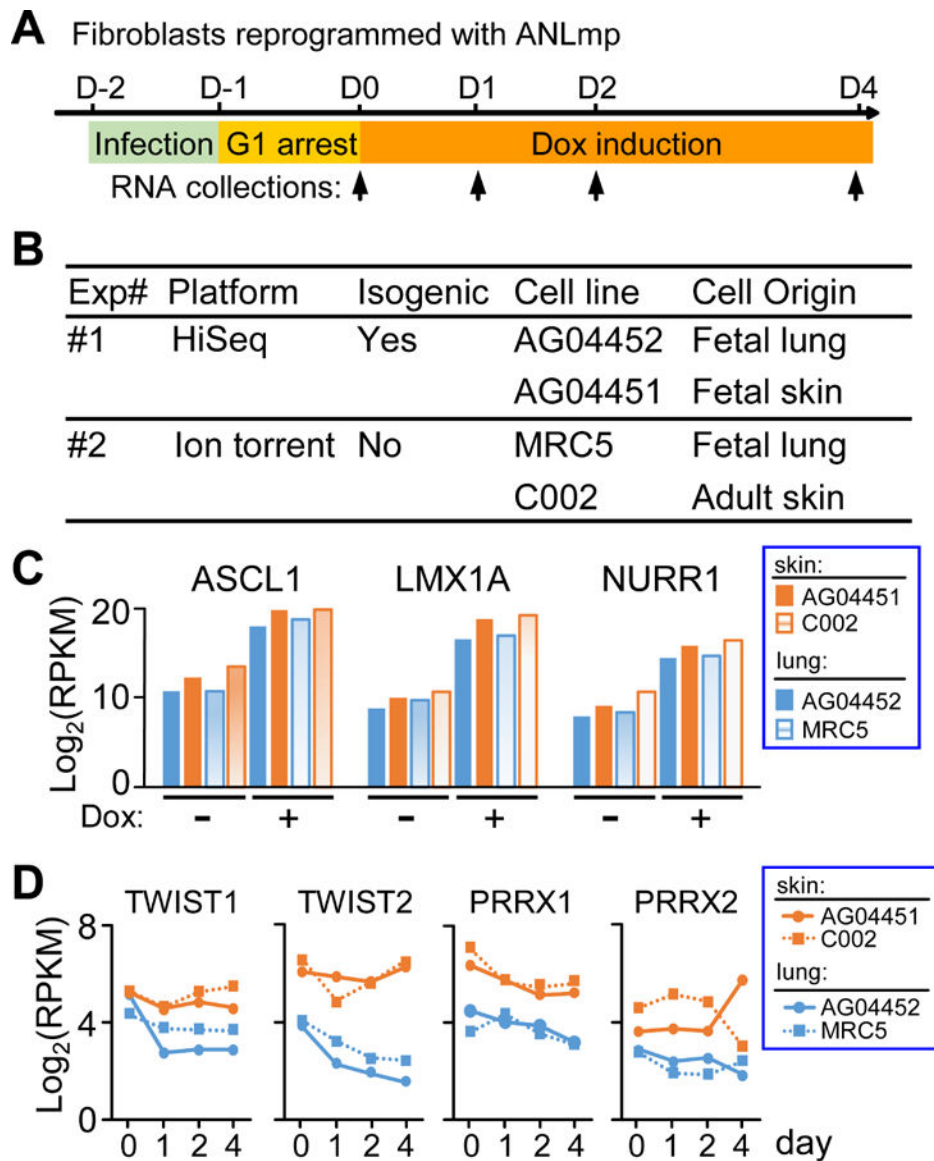
### Highlights

- Neuronal conversion of human skin fibroblasts is much harder than lung fibroblasts.
- Fibroblast Gene Regulatory Network is more stable in skin than lung fibroblasts.
- PRRX2 and HEY2 shRNAs enhance conversion of human skin fibroblasts to neurons.
- The optimal condition is ASCL1 overexpression and knockdown of p53, PRRX2 and HEY2.



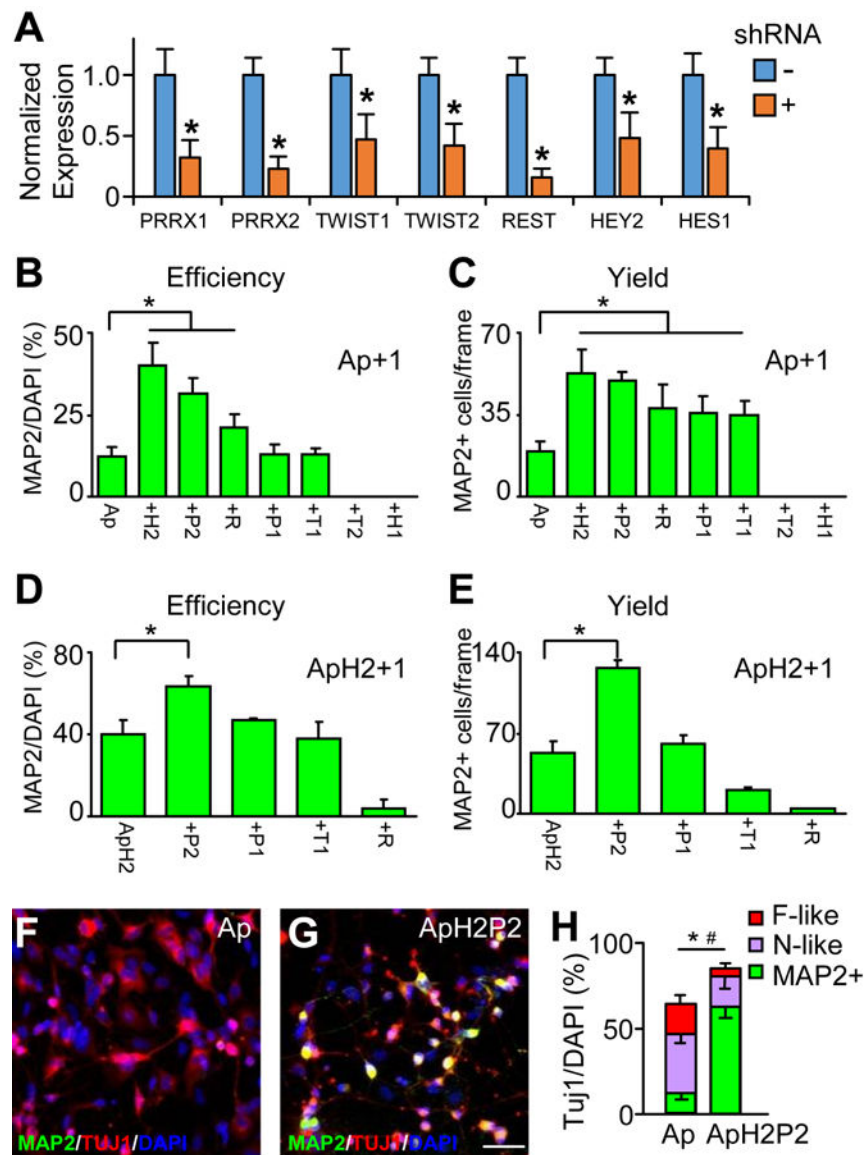
**Fig. 1. Human lung fibroblasts and skin fibroblasts have significant difference in their ability to be reprogrammed to induced neurons.**

Five different lines of primary human lung fibroblasts or skin fibroblasts were reprogrammed with ASCL1, NURR1, LMX1A, miR124 and p53shRNA (ANLmp) to induced dopaminergic neurons [17]. (A) Conversion efficiency for the indicated fibroblasts was quantified by the percentage of TUJ1<sup>+</sup> or TH<sup>+</sup> cells among all cells (DAPI<sup>+</sup>) at day 10. \*,  $p < 0.001$ , one-way ANOVA with Tukey's test,  $n = 5$  experiments for each line. (B-D) Induced neurons derived from the human lung fibroblast MRC5 (B) and the human skin fibroblast AG22056 (C) at day 10 were stained as indicated. Bar, 20  $\mu$ m. (D) TUJ1<sup>+</sup> cells were quantified by their morphology into two categories, fibroblast-like (F-like) or neuron-like (N-like). \*, total TUJ1<sup>+</sup> cells,  $p < 0.05$ , Student's  $t$ -test; #, F-like TUJ1<sup>+</sup> cells,  $p < 0.05$ , Student's  $t$ -test.



**Fig. 2. Differential expression of key transcription factors in fibroblast gene regulatory network during the conversion of human skin and lung fibroblasts to induced neurons.**

(A) Schematics for the transdifferentiation of human fibroblasts to induced dopaminergic neurons using lentiviruses for ASCL1, NURR1, LMX1A, miR124 and p53shRNA (ANLmp). RNA was collected at indicated time points. (B) Summary of samples used in the RNAseq experiments. (C) Expression levels of the three reprogramming factors in the indicated fibroblasts before and after doxycycline induction for 24 hours. (D) Time course for expression levels of the indicated transcription factors that are critically involved in the fibroblast gene regulatory network. Please note the  $\log_2$  scale of Y-axis.

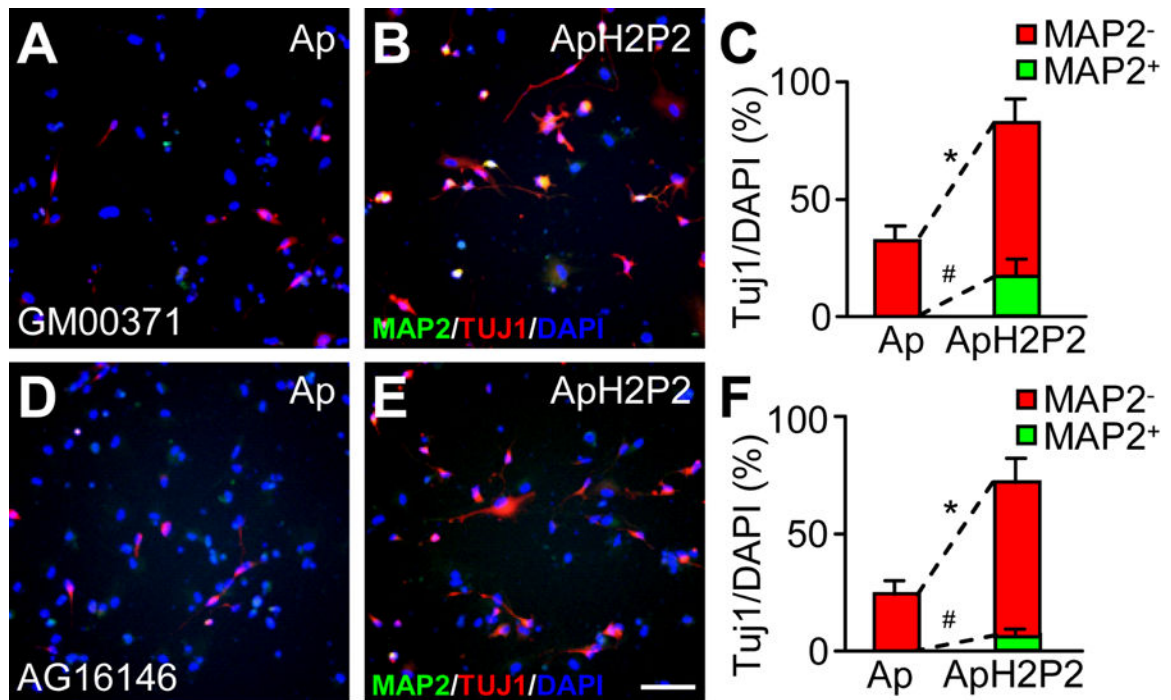


**Fig. 3. Double knockdown of PRRX2 and HEY2 improve the transdifferentiation of neonatal human skin fibroblasts to induced neurons.**

(A) Knockdown efficiency of shRNAs for the indicated genes. \*,  $p < 0.05$ , student's  $t$ -test. (B-C) Reprogramming efficiency (percentage of MAP2<sup>+</sup> neurons in DAPI<sup>+</sup> cells) (B) and reprogramming yield (number of MAP<sup>+</sup> neurons per frame) (C) in the transdifferentiation of AG22056 neonatal human skin fibroblasts to induced neurons by ASCL1 and p53 shRNA (Ap) plus another shRNA were quantified at day 14. H2, HEY2 shRNA; P2, PRRX2 shRNA; R, REST shRNA; P1, PRRX1 shRNA; T1, TWIST1 shRNA; T2, TWIST2 shRNA; H1, HES1 shRNA. \*,  $p < 0.05$ , one-way ANOVA with Tukey's test. (D-E) Reprogramming efficiency (D) and yield (E) in the conversion of AG22056 cells to induced neurons by ApH2 plus another shRNA were quantified at day 14. \*,  $p < 0.05$ , one-way ANOVA with Tukey's test. (F-H) AG22056 neonatal skin fibroblasts were reprogrammed with Ap (F) or ApH2P2 (G) and costained as indicated. Bar, 20  $\mu$ m. (H) At day 14 TUJ1<sup>+</sup> cells were

quantified according to their morphology, fibroblast-like (F-like) or neuron-like (N-like). \*, total TUJ1<sup>+</sup> cells,  $p < 0.05$ , Student's  $t$ -test; #, F-like TUJ1<sup>+</sup> cells,  $p < 0.05$ , Student's  $t$ -test.





**Fig. 4. Double knockdown of PRRX2 and HEY2 improves the direct conversion of adult human skin fibroblasts to induced neurons.**

(A-C) GM00371 adult human skin fibroblasts were reprogrammed with ASCL1 and p53 shRNA (Ap) (A) or ASCL1 and shRNAs against p53, HEY2 and PRRX2 (ApH2P2) (B) and costained as indicated. The percentages of TUJ1<sup>+</sup> cells among all DAPI<sup>+</sup> cells, as well as the percentages of MAP<sup>+</sup> neurons among all DAPI<sup>+</sup> cells were quantified at day 14 (C). \*,  $p < 0.05$  for TUJ1<sup>+</sup> cells; #,  $p < 0.05$  for MAP<sup>+</sup> cells, both Student's *t*-test. (D-F) AG16146 adult human skin fibroblasts were reprogrammed with ASCL1 and p53 shRNA (Ap) (D) or ASCL1 and shRNAs against p53, HEY2 and PRRX2 (ApH2P2) (E) and costained as indicated. The percentages of TUJ1<sup>+</sup> cells among all DAPI<sup>+</sup> cells, and the percentages of MAP<sup>+</sup> neurons among all DAPI<sup>+</sup> cells were quantified at day 14 (F). \*,  $p < 0.05$  for TUJ1<sup>+</sup> cells; #,  $p < 0.05$  for MAP<sup>+</sup> cells, both Student's *t*-test.