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Direct Reprogramming of Mouse Embryonic Fibroblasts Into GABAergic Neurons

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Introduction

The process of pluripotent stem cell differentiation was long thought to be unidirectional and irreversible. It was believed that progenitor cells became locked into a single lineage as they divided, eventually losing their capacity to differentiate into new cell types. This model was overturned with the discovery of induced pluripotent stem cells (iPSCs). The expression of four transcription factors, *Oct4*, *Sox2*, *Klf4* and *c-Myc*, in mouse embryonic fibroblasts was shown to produce a small population (0.02% efficiency) of pluripotent stem cells [1]. Subsequent studies have demonstrated potential uses for this technique, including the generation of iSPCs from human fibroblasts [2-4], creating disease models by generating disease-specific iSPCs from individual patients [5-7], and bypassing many of the ethical dilemmas surrounding the use of embryonic stem cells. Advances in cellular reprogramming also led to the discovery that mouse embryonic fibroblasts (MEFs) could be directly converted into neurons by using the neural-associated factors *Ascl1*, *Brn2* and *Myt1L* (BAM pool) [8]. Though similar to iPSC generation, directly reprogramming MEFs into induced neurons (iNs) was faster, more efficient (7-19%), and required fewer steps to form mature, functional neurons. These findings led to further experiments using human fibroblasts, which successfully generated iNs, though the addition of the factor *NeuroD1* to the BAM pool was required [9-11].

With the BAM pool established as a foundation for direct neural reprogramming, researchers are now attempting to derive specific neuronal types. The BAM pool alone produces predominantly glutamatergic neurons [8], but additional factors have been found to induce the production of additional cell types, including dopaminergic neurons [12], tyrosine-hydroxylase expressing cells [13], motor neurons [14], and neural progenitor cells [15-16] (Fig. 1). It has not yet been determined what factors are necessary to enrich production of GABAergic neurons.

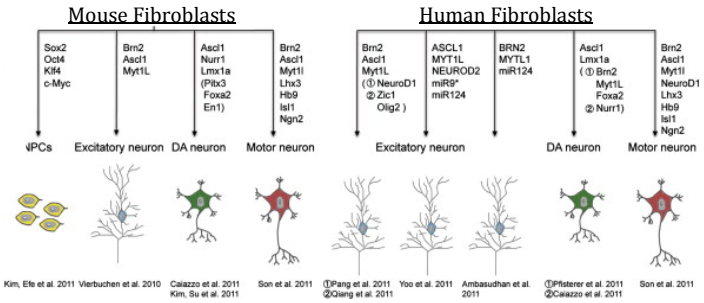

Figure 1: Established neural cell-reprogramming pathways. Each is listed with the necessary transcription factors; parentheses indicate alternative factors that yield the same cell type. Figure adapted from [17].

The neurotransmitter γ-aminobutyric acid (GABA) is used as a primary inhibitory signal in a number of essential brain functions, including structural and functional development of the cortex [18], modulation of cortical output [19], and plasticity [20]. Abnormal GABAergic signaling has been linked to epilepsy [21-23], schizophrenia [24-26], autism spectrum disorder [27,28], and anxiety disorders [29]. Though the development and differentiation of GABAergic neurons is not fully understood (due in part to the diversity of GABAergic interneuron subtypes), several factors have been implicated in 5-HT-specific differentiation (Table 1).

Table 1: GABAergic Candidate Factors

Transcription Factor	References
Lbx1	30, 31
Pax2	19, 31
Lhx1	32
Lhx2	32
Dlx1	19, 32-35
Dlx2	19, 32-34, 36
PitX2	37, 38
Pft1a	31, 39-41

We examined these possible GABAergic factors to identify which, if any, are capable of directly reprogramming MEFs into GABAergic iNs. Simultaneously transfecting all nine factors plus the BAM pool resulted in cell death (data not shown). Thus, groups of four potential factors for determining GABAergic cell fate (Table 2) were combined with the BAM pool

Table 2: Transfection Groups

Group #	Factors
1	BAM + *Lbx1*, *Pax2*, *PitX2*
2	BAM + *Lbx1*, *Pax2*, *Pft1a*
3	BAM + *Lbx1*, *Dlx1*, *Lhx1*
4	BAM + *Lbx1*, *Dlx2*, *Lhx2*

and transfected into Tau-EGFP MEFs. Resulting iNs were tested for interneuron-specific markers to determine if these factors were sufficient to induce GABAergic neuronal cell fate. Individual factors were then removed in order to determine the minimum combination of factors necessary to generate GABAergic iNs.

Methods

Mouse embryonic fibroblast isolation & culture: Homozygous TauEGFP (Jackson Laboratories) MEFs [42] were isolated from E14 embryos under a dissection microscope (Olympus). The head, vertebral column (containing the spinal cord), dorsal root ganglia, and all internal organs were excised to ensure the removal of all potentially neurogenic cells from culture. The remaining tissue was dissociated in 0.5% trypsin at 37°C for 30 minutes. The resulting cell suspension was plated on 1x Gelatin-coated 6-well plates in Fibroblast Media containing DMEM (Invitrogen), 10% FBS (Atlanta Biologicals), β-mercaptoethanol (Sigma), MEM NEAA, sodium pyruvate and penicillin/streptomycin (all Invitrogen). Cells were grown at 37˚C until confluent, and then frozen. Upon thawing, cells were grown in antibiotic-free Fibroblast Media until confluent, at which point the cells were transfected. The MEFs were transfected before their third passage. Two days after transfection, cells were passaged 1:4 and put onto laminin-coated coverslips. Five days after transfection cells were switched to Neural Differentiation Media, containing DMEM/F12 and supplemented with 1% each N2, B27, and MEM NEAA, 0.1% Ascorbic Acid, and 0.01% each BDNF, GDNF and cAMP (all Invitrogen).

Gene cloning & transfection: Eight transcription factors associated with GABAergic cell fate (Table 1) were chosen for transfection along with the BAM pool. Each factor was inserted into a plasmid, under the ubiquitous CAG promoter. The inserts were flanked with terminal repeats; upon the addition of PiggyBac transposase (PBase), the cDNA between these flanking repeats was excised and inserted into the fibroblast genome. Without PBase, the plasmids remain episomal, with no genomic incorporation. Plasmid cDNA transfection was carried out using FuGene6 (Roche). The reagent was first diluted in Opti-MEM basal media (Invitrogen) at a 3:100 ratio, and then incubated at room temperature for 5 minutes. Plasmid DNA was then added to the solution at a ratio of 1 µg of DNA for every 3 µL FuGene6. This was incubated at room temperature for 15 minutes. 100 µL of complex was added to the Fibroblast Media in each well of a 6 well plate. Media was changed 24 hours after transfection.

Immunocytochemistry & cell counts: Cells were fixed in 4% paraformaldehyde for 12 minutes at room temperature. After washing, cells were treated with blocking solution containing 0.2% TritonX-100 (Sigma) and 5% goat serum (Sigma) for 30 minutes at room temperature. Primary and secondary antibodies were diluted in blocking solution. Typically, primary and secondary antibodies were applied for one hour and 30 minutes, respectively, both at room temperature. Between primary and secondary staining cells were washed three times with PBS. The following antibodies were used: rabbit anti-GABA (Sigman, 1:4,000), mouse anti-MAP2 (Sigma, 1:500), mouse anti-NeuN (Millipore, 1:100), rabbit anti-Tuj1 (Covance, 1:1,000), mouse anti-Tuj1 (Covance, 1:1,000), guinea-pig anti-vGLUT1 (Millipore, 1:2,000). Alexa-488- and Alexa-568-conjugated secondary antibodies were obtained from Inivitrogen. Cells were kept in neural media for 1-4 weeks prior to paraformaldehyde fixation and ICC analysis.

Efficiency calculation: Efficiency of neuronal induction was calculated between 7 and 20 days after transfection. The total number of $Tuj1^+$ cells with neuronal morphology (circular, three-dimensional appearance with thin processes at least three times longer than width of the cell body) found on 10 different 6-well coverslips were quantified. This number was divided by the number of cells plated before transfection to get the percentage of the starting cell population that adopted neuron-like properties. Efficiency of GABAergic reprogramming was calculated by dividing the number of $GABA^+$ iNs by the total number of $Tuj1^+$ cells.

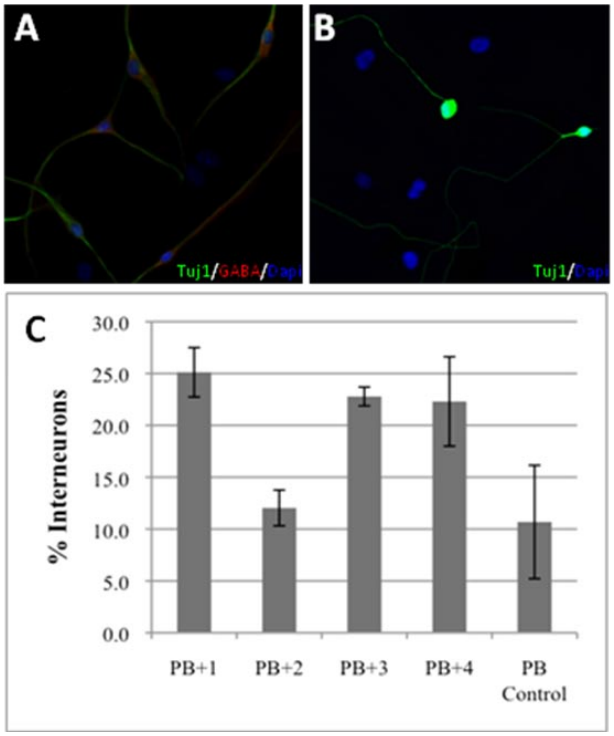

Figure 2: Some induced neurons displayed the interneuron marker GABA (A), while others remained Glutamatergic in appearance (B). (C) Efficiency of $GABA^+$ expression following transfection with groups 1-4. Error bars represent standard error across all trials (n=3). PB+2 coverslips included non-neuronal material, confounding cell counts.

Condition	Factors
A	BAM + *Lbx1*, *Pax2*
B	BAM + *Lbx1*, *Pft1a*
C	BAM + *Pax2*, *Pft1a*
D	BAM+ *Lbx1*
E	BAM + *Pax2*
F	BAM + *Pft1a*

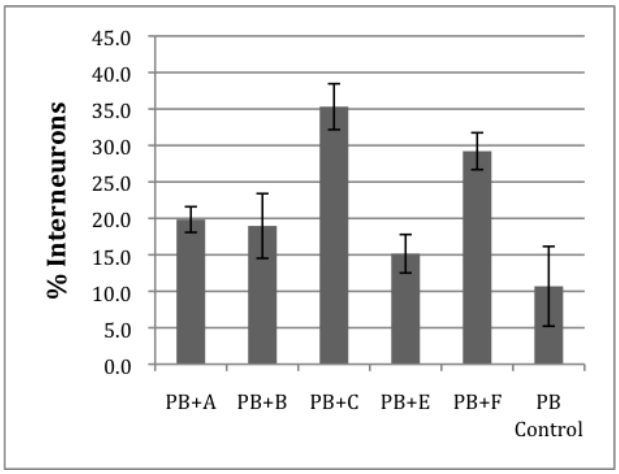

Figure 3: Factors in Group 2 were transfected individually and in pairs (table). Efficiency of $GABA^+$ expression following transfection with groups A-F (graph). Error bars represent standard error across all trials (n=3; n=4 for PB+F). PB+D cells did not survive to transfection date.

Results

Twenty days after transfection, all coverslips contained $Tuj1^+$ iN cells with subpopulations of $GABA^+$ cells. A marked difference in $GABA^+$ cells was found between groups 1, 3, and 4, compared to controls (Fig. 2). No notable difference was seen between group 2 and controls, though these results differed considerably from preliminary experiments (data not shown).

Several groups of factors enriching for interneuron production led to further transfections, in which each group of factors was broken first into pairs, then individual factors. This would elucidate the role of the individual factors in GABAergic iN production. In order to assess the validity of the initial group 2 findings, the factors that formed group 2 were the first to be re-grouped and added to the BAM pool. Component factors were transfected individually and in pairs, along with the BAM pool (Fig. 3). Individual and paired group 2 factors resulted in noticeably higher yields of $GABA^+$ cells, with the combination of *Pax2* and *Pft1a* producing the greatest percentage of $GABA^+$ iNs. *Pax2* alone failed to produce comparably high derivation of $GABA^+$ cells, but *Pft1a* alone did result in an increased percentage of $GABA^+$ iNs compared to controls.

Discussion

Groups containing all eight transcription factors of interest have demonstrated some capacity for enriching production of $GABA^+$ iNs, though the most efficient factor combination must still be determined. Of every group of factors tested here, group C yielded the highest percentage of $GABA^+$ neurons. The fact that this combination (BAM + *Pax2*, *Pft1a*) was derived from the least efficient of the initial groups indicates that more experiments will be necessary to elucidate which factors are required to generate $GABA^+$ iNs.

The enhanced enrichment of $GABA^+$ cells in groups C and F indicate that *Pft1a* may

play a crucial role in determining interneuron cell fate. The addition of *Pax2* alone to the BAM pool did not greatly increase the percent yield of $GABA^+$ cells. When *Pax2* and *Pft1a* were added together, however, a greater percentage of $GABA^+$ iNs were generated than when *Pft1a* was added alone. As a result *Pax2* cannot be discounted as a potential factor for enrichment of $GABA^+$ iNs, and must be further studied along with *Pft1a*.

Additional experiments will be required to fully characterize the effects of these target factor pools. Factors from the other three groups must be transfected individually and in pairs in order to discover which factors yield the greatest GABAergic iN enrichment, most likely including *Pft1a*. Once all maximizing factors are identified, removal of individual BAM factors from the transfection pool will determine if these novel factors are sufficient to replace any of the BAM factors and still induce neuronal reprogramming. Electrophysiological analysis will elucidate the functionality of these $GABA^+$ iNs, and interneuron-specific immunostaining will identify any interneuron subtypes generated by these factors.

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