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The effect of substrate topography on direct reprogramming of fibroblasts to induced neurons

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ABSTRACT

Cellular reprogramming holds tremendous potential for cell therapy and regenerative medicine. Recently, fibroblasts have been directly converted into induced neurons (iNs) by overexpression of the neuronal transcription factors *Ascl1*, *Brn2* and *Myt1L*. Hypothesizing that cell–topography interactions could influence the fibroblast-to-neuron reprogramming process, we investigated the effects of various topographies on iNs produced by direct reprogramming. Final iN purity and conversion efficiency were increased on micrograting substrates. Neurite branching was increased on microposts and decreased on micrograting, with a simplified dendritic arbor characterized by the reduction of $MAP2^{+}$ neurites. Neurite outgrowth increased significantly on various topographies. DNA microarray analysis detected 20 differentially expressed genes in iNs reprogrammed on smooth versus micrograting, and quantitative PCR (qPCR) confirmed the upregulation of *Vip* and downregulation of *Thy1* and *Bmp5* on micrograting. Electrophysiology and calcium imaging verified the functionality of these iNs. This study demonstrates the potential of applying topographical cues to optimize cellular reprogramming.

1. Introduction

Neurodegenerative disorders caused by the loss of specific cells in the nervous system are on the rise in the aging population – presenting an increasingly urgent challenge for the society. Wernig et al. in 2010 directly reprogrammed mouse fibroblasts into functional neurons by lentiviral delivery of the transcription factors (TFs) *Brn2, Ascl1,* and *Myt1l* - referred to as induced neuronal (iN) cells [1]. Further studies confirmed that iNs could be obtained from adult human cells [2–4]. Continuing progress saw the generation of induced functional dopaminergic neurons (iDAs) [5–8], cholinergic motor neurons (iMNs) [9] and induced oligodendroglial cells (iOPCs) [10] from various cocktails of transcription factors. Neuronal reprogramming of fibroblasts from patients with familial Alzheimer's disease (fAD) could generate a model that recapitulated hallmarks of the disease [2]. These advances of generating a new source of functional neurons render cell replacement therapy a promising approach to combat neurodegenerative diseases.

We [11] and Maucksch et al. [12] have respectively demonstrated that iNs and neural precursor-like cells can be produced without viral vectors, paving the way to a clinically viable cell source for translation. Another issue is the continuing need to optimize the reprogramming process to yield a therapeutic quantity of iNs. To date, all neuronal reprogramming has been conducted on conventional tissue-culture dishes. During reprogramming a drastic change in cell morphology and gene expression occurs. Considering the recent findings that cell–topography interactions modulated many cell phenotypes [13,14] and improved induced pluripotent stem cell (iPSC) generation [15], we hypothesized that topographical cues can influence the neuronal conversion process and the subsequent phenotype of the induced neurons.

In vivo, all adherent cells encounter topographical features from neighboring cells and the extracellular matrix. The function of cells in general – and of neurons in particular – depends on their shape and polarity. Neurons explore their immediate extracellular environment to make the appropriate connections to build functional

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neural circuitry. For instance, neurons and PC12 cells polarized along gratings [16] and this polarity is controlled by the confinement of focal adhesions on the gratings [17,18]. Focal adhesions have been shown to play an important role in topography-sensing [19]. In this study we investigated if topographical features imprinted on polystyrene would affect the direct reprogramming of fibroblasts to neurons by means of lentiviral vectors. We chose posts and grating topographies because these have been shown to have an effect on neuronal cells [20]. Here we report the resultant changes compared to conventional planar tissue-culture polystyrene (TCPS), in terms of efficiency, morphology, and gene expression, suggesting a role of cell–topography interactions in shaping iNs.

2. Materials and methods

2.1. Substrate preparation

2×2 cm^2 metal masters of pits of 1 μm diameter spaced by 4 μm (F (1.4)) and pits of 4 μm diameter spaced by 1 μm (F (4.1)) were produced by a lithographic process reference in Suppl. Text. A master for the 5 μm grating features was produced by UV photolithography. PDMS substrates were fabricated from the masters and used for imprinting onto polystyrene (PS) for cell culture.

2.2. Cell culture

Primary mouse embryonic fibroblasts (PMEFs) were isolated as previously described [1] or purchased (ATCC, Manassas, VA). PMEF (Passage 2–4) at 15,000 cells cm^{-2} were seeded on PS substrates at 37 °C and 5% CO_2 in complete PMEF medium as previously described [1]. For neurite tracing experiments a seeding density of 7500 cells cm^{-2} was used to enable the attribution of the neurites to their cell body. 24 h after seeding, PMEFs were infected in PMEF medium containing 8 mg mL^{-1} sequabrene (Sigma, St. Louis, MO) with TFs in lentiviral vectors *Ascl1* (Addgene, Cambridge, MA, plasmid # 27150), *Brn2* (Addgene plasmid # 27151), *Myt1L* (Addgene plasmid # 27152) [1], and *M2rttA* (Addgene plasmid # 20342) [21]. 24 h after infection, PMEF medium was replaced with PMEF medium containing 2 mg mL^{-1} doxycycline (Sigma). 72 h after infection, PMEF medium containing doxycycline was replaced with neurogenic N3 medium containing: DMEM/F-12 (Life Technologies/Invitrogen), 25 μg mL^{-1} bovine insulin (Gemini Bio-Products, West Sacramento, CA), 50 μg mL^{-1} human apo-transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine (Sigma), 10 ng mL^{-1} human bFGF2 (Stemgent, Cambridge, MA), 25 μg mL^{-1} gentamicin (Life Technologies/Invitrogen) and 2 mg mL^{-1} doxycycline (Sigma). After 48 h in N3 medium with doxycycline, the medium was exchanged every 48 h with N3 medium without doxycycline. To identify iNs from non-converted fibroblasts, the cells were infected with a synapsin promoter-driven RFP expression plasmid (Addgene, plasmid # 22909) [22] at day 10 in electrophysiology, microarray and flow cytometry experiments. In some experiments, 25 μM blebbistatin (Millipore, Billerica, MA) – an inhibitor of non-muscle myosin II ATPase – was added to the culture medium throughout the entire cell culture. Three independent experiments with triplicate substrates were performed for each analysis.

2.3. Immunocytochemistry and antibodies

Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized, and stained in a blocking solution containing the antibodies, 0.03 g/ml bovine serum albumin (BSA, Sigma), 10% goat serum (Sigma), and 0.3% Triton X-100 (Sigma) in PBS for 2 h. The samples were washed before incubation with secondary antibodies for 1 h at RT. The samples were then mounted in Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA, USA) for fluorescent imaging.

Antibodies against the following proteins were applied for immunofluorescence (IF). Monoclonal antibodies: Neuronal class III β-tubulin (Tuj1) 1:500 (Covance, Princeton, NJ), MAP2 1:500 (BD Biosciences, Franklin Lakes, New Jersey). Rabbit polyclonal antibodies: Neuronal class III β-tubulin (Tuj1) 1:500 (Covance). Secondary antibodies used for IF were Alexa Fluor 594 goat anti-rabbit secondary antibody 1:200 (Life Technologies/Invitrogen), or Alexa Fluor 488 goat anti-mouse secondary antibody 1:200 (Life Technologies/Invitrogen) and cell nuclei were stained with 4,6-Diamidino-2-phenylindole (DAPI) 1:5000 (Life Technologies/Invitrogen).

2.4. Image analysis

Widefield images were collected for measurement of Tuj1$^+$ cells using a Nikon Eclipse TE2000-U fluorescence inverted microscope and a 4× objective. Large areas corresponding to 6 single images were scanned with an automated stage and the images stitched together from each of three replicate substrates: planar PS, 5 μm gratings PS, F (1.4) PS and F (4.1) PS. At least 705 cells from three independent experiments with at least 235 cells per substrate were analyzed for each condition using a *FIJI* macro [23], implemented by a tester blinded to the identity of each condition. Briefly, Bernsen's thresholding method was used to define regions of high local contrast within each image, corresponding to the cell bodies. Using *FIJI's* "Analyze Particles" command, ellipses were fitted to these outlines, and an angle of deviation from the horizontal axis was measured for each, with 0° (90°) denoting a cell parallel (perpendicular) to the grating direction or line of posts. If an angle was greater than 90° then the angle was subtracted from 180°, so all angles fell between 0° and 90°. Very small and large (non-cell) debris particles were excluded with a size threshold. For Fig. 1M all measured angles were averaged. An average angle of deviation of 45° represents a random orientation with respect to the horizontal axis. For Fig. 1N the distribution of the measured angles from 0° to 90° is displayed. To quantify cell attachment, cells were fixed and DAPI-stained 24 h after seeding. Images of DAPI-stained cell nuclei were acquired by scanning each of the three replicate substrates. Bernsen's thresholding method was used to define regions of high local contrast within each image corresponding to the cell's nucleus, and the "Analyze Particles" command was used to count the number of nuclei for each substrate. For neurite tracing, an initial fibroblast seeding density of 7500 cells cm^{-2} was used and the iNs produced were stained for Tuj1 expression. Higher magnification images of iNs were collected by wide field microscopy by an experimenter blinded to the experimental conditions. A blinded experimenter traced neurites using the "Simple Neurite Tracer" plugin in *FIJI*. The cell soma was chosen as starting point and the longest neurite was designated as the primary neurite. The number of neurites per soma was determined by the sum of primary and secondary neurites extending from the cell body. The primary neurite length was determined by comparison of the average length of the primary neurite per iN for each substrate topography. The length of the neurite arbor was determined by comparison of the length of all neurites per iN for each substrate topography.

2.5. Electrophysiology

iNs for patch clamp analysis were identified by synapsin promoter-driven RFP expression after 12 days of culture in N3 medium. Micropipettes with resistances between 3 and 7 MΩ were filled with internal solution containing 130 mM $KMeSO_3$, 10 mM HEPES, 10 mM sodium phosphocreatine, 4 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_2GTP, 3 mM sodium L-ascorbic acid, with pH 7.24 and an osmolarity of 290 mM. Giga-ohm membrane seals were formed under voltage-clamp conditions. Action potentials were then recorded using an Axon Multiclamp 700B Microelectrode Amplifier (Molecular Devices, Sunnyvale, CA) by stepwise whole-cell current clamp injections, and analyzed with MATLAB programs developed in-house. The cells were perfused with artificial cerebral spinal fluid (ACSF) saturated with 5% O_2 and 95% CO_2 and containing: 130 mM NaCl, 2.5 mM KCl, 2 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM glucose, 2 mM $CaCl_2$, and 2 mM $MgCl_2$.

2.6. Calcium imaging

Cells were infected with a GCaMP5 construct under control of a MAP2 promoter at day 9 after infection with the *Ascl1, Myt1L,* and *Brn2*. Imaging experiments were performed 4–5 days thereafter in imaging solution (NaCl 140 mM, KCl 5 mM, 0.8 mM $MgCl_2$, Hepes 10 mM, $CaCl_2$ mM, glucose 10 mM). Calcium imaging was performed using a Nikon Eclipse TE2000-U fluorescence inverted microscope and a 20× objective. The time-series images were acquired at a rate of 5 fps. 50 mM KCl, 50 mM NaCl and 1 mM glutamate in imaging solution was directly pipetted onto the iN during perfusion. Calcium imaging was performed at room temperature. To produce ΔF/F traces, ROIs were drawn with *FIJI*, containing either the iN cell body or the background, and the average fluorescent intensity was measured for each time point.

2.7. Flow cytometry

Cells were washed briefly with phosphate-buffered saline (PBS) (Mediatech, Washington, DC), and released from the substrates with 0.25% Trypsin-EDTA (Invitrogen/Life Technologies). Trypsin was inactivated with serum-containing media and the cells were centrifuged at 4 °C, and resuspended in ice-cold N3 medium if they were to be sorted for RNA extraction, or fixed in ice-cold PBS containing 1% paraformaldehyde (PFA) for 10 min for flow cytometric analysis. Cells were analyzed for tau promoter-driven expression of EGFP, or synapsin promoter-driven expression of RFP with FACS Canto II (Becton Dickinson, Franklin Lakes, NJ), or sorted and analyzed with FACSVantage SE (Becton Dickinson, Franklin Lakes, NJ), and collected for RNA extraction. For each analysis we conducted three independent experiments with triplicate substrates and at least 930 cells were analyzed.

2.8. Microarray

Total RNA samples were assessed for quality with an Agilent 2100 Bioanalyzer G2939A (Agilent Technologies, Santa Clara, CA) and Nanodrop 8000 spectrophotometer (Thermo Scientific/Nanodrop, Wilmington, DE). Hybridization targets were prepared with MessageAmp™ Premier RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX) from total RNA, hybridized to GeneChip® Mouse 430A 2.0 arrays in an Affymetrix GeneChip® hybridization oven 645, washed in an Affymetrix GeneChip® Fluidics Station 450, and scanned with an Affymetrix GeneChip® Scanner 7G according to standard Affymetrix GeneChip® Hybridization, Wash, and Stain protocols (Affymetrix, Santa Clara, CA). Data processing and statistical analysis: Partek Genomics Suite 6.5 (Partek Inc., St. Louis, MO) was used to perform data