

**Research Article** 

## No Detection of Potential Cancer Risk for Free-Viral Reprogrammed Stem Cell-Derived Dopaminergic Neurons from Adult Mice Fibroblasts

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### Abstract

**Objective:** Stem cell replacement therapy through the reprogramming of somatic cells is a significant prospective therapy for neurodegenerative diseases and personalized medicine. As virus-carrying reprogramming genes impart a considerable risk for tumor formation, current approaches tend to replace the viral pattern with a non-viral system. However, there is still great concern regarding the oncogenic properties of these reprogramming genes. At present no direct evidence can verify that non-viral systems do not influence genomic DNA integration and mutagenesis events in the final products after reprogramming.

**Methods:** We evaluated the potential cancer risk of reprogrammed stem cell-derived dopaminergic neurons using a unique non-viral vector containing four reprogramming genes. Reprogrammed stem cell-derived dopaminergic neurons were the final products after 50 days of cell culture from adult mice fibroblasts. After 6 months of culture, these cells were assessed for cancer risk.

**Results:** Overall cancer risk assessments were first examined using multiple stem cell biomarkers. We did not detect any overexpression of these biomarkers. Moreover, we assessed whether or not the most important neurotransmission factors were expressed after 6 months in mass cell culture. Our results determined that only robust gene expression of tyrosine hydroxylase (TH), as the rate-limiting enzyme in dopamine synthesis, was detected. Furthermore, the final product of TH-positive dopaminergic neurons was confirmed by sequencing TH genomic DNA. Our data suggested that none of the four reprogramming genes integrated into the host cell's genomic DNA and that no other mutagenesis events occurred after gene sequencing TH genomic DNA related complete 13 coding exons with two-ended partial untranslated regions.

**Conclusion:** Re-programmed adult mouse fibroblasts-derived dopaminergic neurons were proven to be a safe technology and approach as a potential therapy for neurodegenerative diseases.

**Keywords:** Free-viral; Re-programming genes; Fibroblasts; Tyrosine hydroxylase-positive-dopaminergic neurons; Cancer risk

**Abbreviations:** AD: Alzheimer's Disease; BDNF: Brain Derived Neurotrophic Factor; Chat: Choline Acetyltransferase; c-Myc: cellular Myelocytomatosis Oncogene; DA: Dopamine; Dcx: Doublecortin; iPSC: Induced Pluripotent Stem Cell; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; Klf4: Kruppel-like Factor 4; Oct4: Octamer-binding Transcription Factor 4; PD: Parkinson Disease; RFU: Relative Fluorescence Units (real time qPCR); SLC6A2: Norepinephrine; SLC6A4: Serotonin; Sox2: SRY (sex determining region Y)-box 2; TH: Tyrosine Hydroxylase; Vector 20866: Vector pCAG2LMKOSimO; UTRs: Untranslated Regions

### Introduction

Stem cell replacement therapy has potential clinical applications by inducing neuroplasticity in neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD) and spinal cord injury, and possibly neuropsychiatric illnesses [1,2]. Re-programming stem cells from somatic cells of individual patients with specific diseases reveals innovative profits for the personalized medicine as there are less immune response rejections to such cell replacement.

One critical limitation of the use of induced pluripotent stem cells (iPSCs) derived from re-programmed somatic cells for longterm cell culturing is the fact that the viral-based (usually retroviral) transfection methods applied in the host cells pose a considerable risk of tumor formation. This is attributable to the random and permanent integration of viral genomic DNA into the host cells' genome. Although Wernig et al. [3] successfully prevented toxin-induced Parkinsonism by transplanting iPSCs into rat brains that were generated through retroviral re-programming; this may increase the potential risk of tumor formation due to genomic insertion to the host cells.

Recent years have indicated a clear inclination that the viral vector system would benefit from substitution of a non-viral system in a variety of approaches [4-12]. Although posing far less risk of causing cancer than the more conventional viral-based iPSCs-generating systems, it is quite unclear whether or not virus-free reprogramming methods have the potential to cause genomic insertion due to the lack of direct evidence [4,9,13]. Indeed, this kind of risk still exists since re-programming genes cellular Myelocytomatosis oncogene (c-Myc) and Kruppel-like factor 4 (Klf4) are oncogenic genes [4,13-15]. Further, there is no straight proof that a non-viral reprogramming system does not have a proficient to inspire genomic DNA integration and mutagenesis events for the final product such as re-programmed dopaminergic neurons, in particular, in the long-term *in vitro* cell culture surroundings.

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Received January 27, 2015; Accepted June 03, 2015; Published June 05, 2015

**Citation:** Liu G (2015) No Detection of Potential Cancer Risk for Free-Viral Reprogrammed Stem Cell-Derived Dopaminergic Neurons from Adult Mice Fibroblasts. J Stem Cell Res Ther 5: 286. doi:10.4172/2157-7633.1000286

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After careful review of current publications, we considered that the non-viral system that was created by Kaji et al. [4] had the most advantages: high re-programming efficiency; one vector (for most easy opening) packed with four transcription factor genes - cMyc, Klf4, Oct4 (Octamer-binding transcription factor 4), and Sox2 (SRY sex determining region Y-box 2), the most important of re-programming genes; and the subsequent excision of these re-programming factors; as well as one fusion of vector marker mOrange with the re-programming genes in the same reading-frame for common gene expressions. Hence, we concluded this system showed promise to be a good model for us to evaluate potential cancer risks in free-viral reprogrammed stem cellderived dopaminergic neurons from adult mice fibroblasts in longterm cell culture circumstances.

### Materials and Methods

### **Plasmid structure**

One free-viral vector pCAG2LMKOSimO (vector 20866, Addgene) was used for re-programming somatic cells into iPSCs for our experiments [4]. This vector was constructed with free-viral within one single vector frame c-Myc-Klf4-Oct4-Sox2-(MKOS)-IRES-mOrange, flanked by loxP sites with a 2A-peptide-linked reprogramming cassette. Their expressions were transcribed by driving universally synthetic CAG enhancer/promoter [16,17]. This vector holds more exclusive advantages as described above.

### Primary cell cultures

The fibroblast tail-tips from two-month adult (CD1) mice were segregated. The protocols were permitted by the Carleton University Animal Care Committee, in accordance with The Animals for Research Act of Ontario and the Guidelines of the Canadian Council on Animal Care (CCAC). The matrix of  $100 \times 60$  mm plates or 6-well plates was prepared by coating culture vessels with laminin (final coating concentrations from 1  $\mu$ g/cm<sup>2</sup> of surface area, Invitrogen 23017-015) and Poly-D-Lysine (final coating concentrations from 1  $\mu$ g/cm<sup>2</sup> of surface area, Millipore A-003-E). Fibroblasts were composed with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/ F12, Invitrogen, 10565-018). Tissues were chipped into small pieces on a plate with 4 mL DMEM/F12, and then were enzymatically digested using 2 mg/mL of filter-sterilized papain (Worthington, LS003119) at 37°C for 45 minutes. Consequently, tissues were removed and spun down, and the supernatant re-suspended in 5 mL of knockout DMEM (Invitrogen, 10829-018). Tubes containing cells were additionally centrifuged for 4 minutes at 200×g, after the supernatants were removed and cell pellets re-suspended in 41.5 mL DMEM/F-12 (Invitrogen, 10565-018). A total of 50 mL medium of DMEM/F-12 was prepared by adding 7.5 mL knockout<sup>™</sup> serum replacement (Invitrogen, 10828-028), 0.5 mL MEM Non-Essential Amino Acids Solution (NEAA) (Invitrogen, 11140-050), 0.5 mL L-glutamine (Invitrogen, 25030-081), 91 µl (final 0.1 mM)  $\beta$ -mercaptoethanol (Invitrogen, 21985-023), and 50  $\mu l$  of 10  $\mu g/mL$  (final 10 ng/mL) bFGF (Fibroblast growth factors, Invitrogen 13256-029). Cells were raised at 37°C and 5% CO<sub>2</sub> in an ambient humidified atmosphere; and were freshly nourished every third day with the proper medium.

### Initiation of pluripotency by non-viral reprogramming genes

Cells at 90% confluence were transfected with/without reprogramming genes containing vector 20866 by applying lipofectamine<sup>®</sup> 2000 Transfection Reagent (Invitrogen, 11668-027). Cells were continually fed with fresh media to maintain re-programming and the induction of pluripotency. The cluster-appearances of stem-cell-similar Page 2 of 8

colonies expressing the mOrange-positive marker were observed starting from Days 5-6 after transfection. The iPSCs were confirmed by expressions of SSEA-1 (stem cell stage-specific antigens, Millipore SCR002) and GF141 (Stem Cell Factor Protein, Millipore GF141). The mOrange-positive iPSCs at Day 20 were further distinguished by live staining with GF141, and then were transferred to cultures containing a Neural Induction Medium on freshly gelatin-coated plates (Attachment Factor is sterile 1X solution containing 0.1% gelatin, Invitrogen, S-006-100).

Cells from Days 1 to 14 were freshly nurtured with complete N2B27 Medium (Invitrogen 11330-057, plus N-2 Supplement 17502-048, B-27<sup>®</sup> Supplement 17504-044, knockout<sup>™</sup> serum replacement, L-glutamine, NEAA, β-Mercaptoethanol, in the amounts described above), enhanced with a CHALP cocktail, which significantly enriched re-programming efficiency [18]. The CHALP cocktail comprised of: A-83-01 (TGF-β/Activin/Nodal receptor inhibitor, 0.5 μM, Stemgent, 04-0014); PD0325901 (MEK inhibitor, 0.5 µM, Stemgent 04-0006); CHIR99021 (GSK3β inhibitor, 3 μM, Stemgent 04-0004); HA-100 (ROCk inhibitor, 10 µM, Santa Cruz, sc-203072); hLIF (human leukemia inhibitory factor, 10 ng/mL, Millipore LIF1005); and bFGF (100 ng/mL). From Days 15 to 20, the medium was altered to the Essential 8<sup>™</sup> Medium (Prototype), comprising of DMEM/F-12 (HAM) 1:1, Essential 8<sup>™</sup> Supplement (50X) (Invitrogen, A14666SA), N-2 Supplement, B-27<sup>®</sup> Supplement, knockout<sup>™</sup> serum, L-glutamine, NEAA, and β-Mercaptoethanol, in the amounts described above. All mediums above were made fresh and replaced every other day.

### Neural induction

Pluripotency iPSCs were induced into the neural progenitor cells under the Neural Induction Medium from Days 21 to 28. This medium consisted of 50 mL Neurobasal<sup>®</sup> medium (Invitrogen, 21103049) with bFGF, heparin solution (Sigma H3149, 50  $\mu$ l of 2 mg/mL), N-2 supplement, glutaMAX<sup>™</sup>-I supplement, NEAA, knockout<sup>™</sup> serum replacement, and  $\beta$ -Mercaptoethanol. The medium was made fresh and replaced every 3 days.

### Differentiation of neuronal cells

To transform into dopamine neural progenitors, the neural progenitor cells from Days 29–35 were cultured with the Dopamine Neuronal Progenitor Medium, comprising of a total of 50 mL of neurobasal\* medium, with 200 ng/mL sonic hedgehog (SHH, R&D systems, 1314-SH-025), 100 ng/mL FGF-8b (Invitrogen, PHG0271), 50  $\mu$ l heparin solution, 0.5 mL N-2 supplement without vitamin A (Invitrogen, 12587-010), and 0.5 mL NEAA.

Furthermore, the dopamine neural progenitor cells were converted into mature dopamine neurons by adding the Dopamine Neuronal Differentiation Medium from Days 36 to 50. This medium contained a total of 50 mL of Neurobasal<sup>\*</sup> medium, with 0.5 mL L-glutamine, 1 mL N-2 supplement without vitamin A (Invitrogen 12587-010), 1 mL NEAA, recombinant human BDNF (Invitrogen, PHC7074, 50  $\mu$ l of 25- $\mu$ g/mL), recombinant human GDNF (Invitrogen, PHC7045, 50  $\mu$ l of 20- $\mu$ g/mL), ascorbic acid (Sigma A4403, 50  $\mu$ l of 200 mM), and dcAMP (Dibutyryl cyclic-AMP) (Sigma, D0627, 50  $\mu$ l of 1-mM).

### Immunocytochemistry

In order to assess stem cell markers, Embryonic Stem (ES) Marker Sample Kits (Millipore, SCR002 and GF141) were employed. The kits are respectively comprised of monoclonal antibodies for the detection of cell-surface Stage-Specific Embryonic Antigens-1 (SSEA-1), and the expression of Stem Cell Factor (SCF) protein antigens (GF141). Mouse Nestin Antibody (R & D system, MAB2736) was applied as a neural progenitor mark for the verification of neuronal progenitor cells. For the tyrosine hydroxylase (TH)-positive dopaminergic neurons marker, TH antibody (ImmunoStar, 22941) was utilized. Briefly, the primary antibodies were diluted at a 1:25 ratio in a blocking solution (1X PBS/4% normal goat serum/0.3% Triton<sup>™</sup> X-100), and then incubated for 1 hour at room temperature following 3 washes with 1X Rinse Buffer. Furthermore, the samples were incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibodies for 1 hour at room temperature. Fluorescence images were shot using an Olympus 1x2-UCB series microscope with Media Cybernetics image analysis software. Living cell culture samples were evaluated using a Zeiss Axiovert 40 CFL microscope with Lumenera Infinity software.

### Quantitative real-time PCR

Total RNA from culture cells was segregated using a GeneJET RNA Purification kit (Thermo scientific, K0732), and then quantitative Real-time PCR (qRT-PCR) was measured by applying a LightCycler' 480 RNA Master Hydrolysis Probes kit (Roche, 04991885001). All TaqMan-gene-specific primers (Supplementary Table 1) were designed by using Integrated DNA Technologies' web application PrimeTime qPCR Assays. The values of gene expressions were normalized to total levels of a control housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All tests were also normalized for PCR sample loading and reaction. Relative fluorescence unit (RFU) is a unit of measurement used in analysis of fluorescence detection in real time PCR. This data is represented in one of three independent experiments for ct (threshold cycle; at 35 cycles) plots in the Figures.

### Genomic DNA analysis of tyrosine hydroxylase

Under the forced direction described above, the final product was dopaminergic neurons throughout the entire progressive staging cell culturing. Since it is difficult to measure dopamine using current technology due to its instability, tyrosine hydroxylase (TH, ratelimiting enzyme for dopamine synthesis) represents TH-positive dopaminergic neurons. Consequently, TH-positive dopaminergic neurons were investigated in order to evaluate the potential risk of four re-programming genes for the host cell's genomic integration or mutagenic incidences. Thus, cells were continually cultured long-term for 6 months following their conversion into mature dopamine neurons.

Specifically, re-programmed stem cells-derived dopamine neuronal cells and parallel original tail-tip cells, which were cultured in the absence of the re-programming factors as one control for cell culture conditions, were sequenced for investigation, along with additional two tissue sample controls that were comprised of untreated *in vivo* brain tissue (midbrain) and tail tissue. A total of four groups were individually analyzed at the same time. We examined the TH genomic DNA sequences with full 13-coding regions of cDNA (ID numbers Exons 1-13) and their two-ended partial UTR regions. The design of specific PCR primers (Supplementary Table 1) were determined according to the alignment of Genbank ID BC156668, 7191 bp of Chromosome 7, position 142,892,776 - 142,899,966, which were identified by the tool (UCSC Genome Browser, http://genome.ucsc.edu), as well as Multiple Sequence Alignment, according to http://www.ebi.ac.uk/Tools/msa/ clustalo/.

PCR reactions for producing genomic DNA TH were applied with Phusion<sup>®</sup> High-Fidelity PCR Master Mix with High-Fidelity Buffer (New England Biolab M0531), according to the manufacturer's protocols. Briefly, all reaction components were set up on ice and quickly transferred to a thermocycler, which was preheated to the denaturation temperature (98°C). All components were mixed and centrifuged prior to use. The Phusion Master Mix was added on the last step in order to prevent any primer degradation caused by the 3' $\rightarrow$  5' exonuclease activity. The thermocycling conditions for a routine PCR were: initial denaturation 98°C, 30 seconds; 35 cycles for 98°C 10 seconds, 65°C 25 seconds, and 72°C 25 seconds; final extension 72°C 2 minutes; hold 4°C. DNA templates for a 50 µl reaction were 200 ng. Sanger Sequencing was conducted using Applied Biosystem's 3730×l DNA Analyzer technology at The McGill University Innovation Centre.

### Statistical analyses

All calculations were produced using StatView version 6.0, a statistical software package from the SAS Institute Inc. Significant findings were followed up on using the Bonferonni analyses.

## Results

# Virus-free reprogramming genes converted mice mature fibroblasts into iPSCs

In order to optimize current re-programming methods established by Kaji et al. [4], the tail-tip fibroblast cells from 2-month-old adult mice (Figure 1a) were transfected with the free-viral vector 20866 containing the following four re-programming transcription factors: Oct4, Sox2, Klf4, and c-Myc. The iPSC identity during Days 17-20 was identified by the expression of the fluorescent vector mOrange marker (Figure 1b and 1c) and further verified by stem cell markers – GF141 (Figure 1d), and SSEA-1 (Figure 1e). The colonies were further selected and flourished on freshly gelatin-coated plates, which offered effective reprogramming. The non-programmed cells were cultured in parallel in the same conditions (Figure 1f).

## Neural progenitor cells derived from iPSCs

Upon successfully generating pluripotency cells by using freeviral re-programming system, our auxiliary interests were to produce neuronal phenotype cells. By applying the specific Neural Induction Medium, iPSCs were transformed into neural progenitor cells from Days 20 to 35 (Figure 2a, with nestin staining as a neural progenitor mark; and Figure 2b), suggesting significant differences with the control group in the morphology (Figure 2c).

## Generation of mature dopaminergic neurons from neural progenitor cells

Following the transformation of the neural progenitor cells, cells were further steered to differentiate into midbrain-like mature dopaminergic neurons (Figure 3a) by using the Dopamine Neuronal Differentiation Medium from Day 36 to 50. The cells developed to form mature neural networks (Figure 3b). The presence of the neuronal marker tyrosine hydroxylase (TH) was detected for verification (Figure 3c). However, no significant modification was observed in the original non-reprogrammed adult fibroblasts in the parallel cultures (Figure 3d).

## Long-term cell culture for preparation of cancer risk assessments

Long-term observation of these cell lines until 6 months after the initial fibroblast cell re-programming revealed that the cultured dopaminergic neurons still had a pronounced ability to form mature neuronal networks. In contrast, at the 6-month mark, tail-tip fibroblast cells grown in control cultures (i.e., without cell re-programming and





**Figure 2:** Differentiation of neural progenitor cells from iPSCs. Producing neural progenitor cells from iPSCs were manufactured using the Neural Induction Medium during Days 20-35 (Figure 2a and 2b; 2a with nestin antibody staining), and there was no significant alteration in the morphology of the the parallel cultured control group (Figure 2c).



Figure 3: Transformation of dopaminergic neurons from neural progenitor cells. From Day 36 to 50, dopaminergic neurons were formed from neural progenitor cells under the forced conditions (Figure 3a), and cells were outgrowing to form mature neural networks (Figure 3b). The cells were verified through staining of the neuronal marker tyrosine hydroxylase (Figure 3c). These neural cells presented significant different morphology when compared with the parallel cultured non-reprogrammed adult fibroblasts (Figure 3d).

differentiating media) maintained their original shape and failed to form any visible cellular networks (Figure 4a). The TH genomic DNA of total Exons 1-13 were further abstracted to produce PCR products on gel from these long-term culturing cells (Figures 4a and 4b) for cancer risk assessments (Figure 4c).

### Overall assessment for potential cancer risk

We assessed overall potential cancer risk by the expression of four re-programming genes cMyc, Oct4, Sox2, and Klf4 (all 4 genes for both exogenous and endogenous gene expressions), plus two stem cell bio-markers SSEA1 and Nanog (all two genes for endogenous gene expressions) for 6-month cultured mass iPSC-derived neurons. These were compared with 3 control groups: original non-reprogrammed mice tail-tip cells from parallel cell cultures, original mice midbrain tissues, and original tail tissues. The results suggested that six gene expressions of the 6-month cultured dopaminergic neurons did not differ from that of the three control groups in mRNA level by real time qPCR (Figure 5).

### Screening and identifying final product at end of cell culture

During the first 50 days under the forcibly directed conditions of the cell cultures, the re-programmed stem cells developed in five different stages: tail-tip fibroblasts, iPSCs, neural progenitor cells, dopamine neural progenitors, to dopaminergic neurons. The dopaminergic neurons were the final product. After 6 months, we further screened out and distinguished gene expressions for the most important neurotransmission factors: TH, doublecortin (Dcx), choline acetyltransferase (Chat), serotonin (SLC6A4), and norepinephrine (SLC6A2) from the samples of the cultured iPSC-derived neurons. We measured mRNA expressions of these genes by RT-qPCR-TaqMan with specific primers. Our results detected that there was only a boost in TH gene expression; the other genes did not present any expressions (Figure 6).

### Tyrosine hydroxylase genomic DNA analysis

Based on the overall assessment for potential cancer risk and elevated TH as the final product, we further scrutinized the TH



genomic DNA through gene sequencing. We examined whether or not the re-programming genes occurred with incidents of genomic DNA integration into host cells and/or mutagenesis of host cells. Our results (Figure 7 and Supplementary data) demonstrated that all cells and tissues had identical genomic DNA sequences, with no any evidence of integration or mutagenesis for TH genomic DNA after reprogramming.

### Discussion

At present, the re-programming of genes for transformation of

### Page 6 of 8



Exon3-Ref tgggaggcagagtatcctgctgggcgggggggacgaccctgaagtgtccacagagttg Cell-D-3-F tgggaggcagagtatcctgctgggcggggggggacgaccctgaagtgtc------Cell-T-3-F tgggaggcagagtatcctgctgggcggggggggacgacctgaagtgt-------Tissue-D-3-F tgggaggcagagtatcctgctgggcgggggggggacgaccctgaagtgtccacagagttgg Tissue-T-3-F tgggaggcagagtatcctgctgggcggggggggacgaccctgaagtgtccacagagttgg

**Figure 7:** Sequencing genomic DNA of tyrosine hydroxylase. The genomic DNA encoding of the mouse TH gene was extracted from 6-month cultured iPSCderived dopaminergic neurons (from Figure 4b). Three additional groups acted as controls: original non-reprogrammed mouse tail-tip cells from parallel 6-month control culturing (from Figure 4a), and non-reprogrammed in vivo tissues – from midbrain and tail. The primers were designed for examining the sequences of all 13 of the coding Exons plus partial regions of the respective Intronic sequences flanking in both sides 5' and 3' untranslated regions (UTRs) (Figure 4c). These events were investigated 3 times for each genomic DNA by gene sequencing. The meanings of abbreviations were similarly applied in Figure 7 (as an example) and in the Supplementary data (complete genomic DNA sequences).

#### Page 7 of 8

somatic cells into stem cells are predominantly connected with viral vectors, which are a safety concern for potential cancer risks due to viral genomic DNA integration and mutagenesis for host cells. This has seriously limited potential clinical applications. Recently, researchers have made great advances to create non-viral vector models to replace viral models. However, the risks are not completely eliminated, which has attracted our attention for compulsory evaluation. In addition, the mechanism of non-viral re-programming is still unclear. In order to reach these conclusions, we chose and applied an existing excellent non-virus-vector system with four re-programming genes (vector 20866) for our studies.

This system efficiently re-programmed adult mouse fibroblasts (tailtips) to produce iPSC, progressively differentiated neuron progenitor cells, and TH-positive dopaminergic neuron cells, which was the final product under the forcibly directed conditions of the cell cultures. Our experiments showed that only TH gene expression was detected, while the other important neurotransmitters (Dcx, Chat, serotonin, and norepinephrine) did not present any expressions. The results did not surprise us, since the conversion of tail-tip fibroblasts into neuronal cells was being cultured under forced conditions for producing only unique neuron cells and further dopamine cells [19-34].

Significantly, there have been no published reports demonstrating the exact mechanisms of "gene re-programming" for non-viral systems. The possibilities are thought to include: 1) the insertion of foreign genes; 2) the modification of existing genomic DNA; and 3) the activation or silencing of existing genes. We observed a tissueand- cell-specific pattern of TH expression, which could be viewed as lending support to an epigenetic mechanism of reprogramming. Tail-tips and re-programmed stem cell-derived dopaminergic neurons showed the exact same genomic sequences of TH, however, only the re-programmed stem cell-derived dopaminergic neurons presented gene expression. These findings suggest that the mechanism of reprogramming may make silence TH expression in the tails to present boosted gene expression in the re-programmed dopaminergic neurons. Although we did not know the detailed pathways for gene silencing, the re-programmed stem cell-derived dopaminergic neurons may not involve changes in the actual genetic code at the genomic DNA level. However, this could potentially involve epigenetic factors, wherein methylation or acetylation of DNA sequences could be influencing TH expression following re-programming. Explicitly, the mechanism of re-programming might involve epigenetic changes that occur via a pattern of synthesized mRNA and protein, which acts to promote gene silencing in adult mice fibroblast tail-tips, which may then be converted into the activated expression of TH-positive dopaminergic neurons.

Of note, two directions may become apparent for the complete procedure: 1) the re-programming genes such as cMyc, Klf4, Oct4 and Sox2 may be present from gene expression to silence, and their functional role is only for "starting" cell life as embryotic cells, but not for developing into "mature" and "specific" types of cells such as neuron, heart, and lung, etc. 2) Gene TH-positive dopaminergic neurons may advance into phenotype from silence to activity, which are fully developed into "mature" and "specific" cell types under the forcedly direction conditions such as specific mediums. The contradictory directions cause development for re-programmed somatic cells to develop into specific types of cells, while re-programming genes become into silence. The entire developing stages handle progression from genotype to phenotype.

Given the association of cancer with mutagenic events, our findings support the argument that our free-viral pluripotency-inducing system may provide a lower risk of genetic pathology than the commonly used alternate viral procedures. Thus, the ultimate significance is that our results may provide numerous potential downstream clinical applications. However, this is speculative and it is still unclear as to the nature of the gene-transcription factor interactions occurring during (and after) re-programming. Free-viral methods lessen the potential risk of detrimental mutagenic events and the re-programming mechanism could involve the activation of previous silent genes from tail-tip cells to dopaminergic neurons. In order to confirm that this method carries less risk, the indication of tumorigenesis with varied approaches such as immunocompromised mice and soft agar assays should be further to provide further supporting evidence.

In conclusion, we determined that the non-viral system was an efficient and safe system by which to re-programmed somatic cells into iPSC and subsequent developing neuron cells. This system may become an innovative method for future therapeutic applications, such as neuroplasticity and neuroprotection.

### Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research.

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Page 8 of 8

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