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Original Article

Full small molecule conversion of human fibroblasts to neuroectodermal cells via a cocktail of Dorsomorphin and Trichostatin A

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ABSTRACT

A revolutionary new approach to produce efficient cells is to induce transdifferentiation to make it conventional in therapeutic strategies. In this paper, we describe a brief cocktail of small molecules including Dorsomorphin (DSM) and Trichostatin A (TSA) to produce safe neuroectodermal cells as a resource to produce various types of nervous system cells for a safe cytotherapy. Furthermore, in order to optimize this strategy, we implemented a cocktail of neurotrophic factors to enhance the viability of the cell. This modification was accompanied by pretreatment of the culture dishes with a combination of poly-L-ornithine and laminin and fibronectin. In order to decrease the length of protocol and trans-differentiation variation concomitantly, TSA was utilized as an epigenetic modulator. Finally, this improved protocol mediated neuroectodermal conversion of human fibroblasts within 12 days with an average efficiency of 24%, promising a fast strategy to produce neuroectodermal cells applicable for therapeutic purposes in neural damages. Here we induce neural cells by a cocktail consists of two small molecules of DSM and TSA. Our protocol is a 12 day protocol with the efficiency of 24% which is a more efficient one in comparison to previous protocols inducing neural cells. Consequently, our protocol shortens the path of in vitro and preclinical studies in the field of neural conversion and neuroregeneration.

1. Introduction

Regenerative neuroscience is a flourishing area in interdisciplinary medicine. It is more likely a panacea for neurodegenerative diseases with no definitive cures such as Amyotrophic Lateral Sclerosis (ALS), Parkinson's disease (PD) or spinal cord physical injuries causing paralysis since it provides cell sources which have potentials to conceal the scars and regenerate the whole organ or tissue. There were many attempts by regenerative neuroscientists aimed to target autologous conversion/trans-differentiation (TD) of human fibroblasts to neural cells in order to supply safe cell pools for cell transplantations, with few yields. The former studies mainly applied transgene systems to produce neural cells [1–4], but recently there is a tendency to apply small molecule cocktails to produce specific safe cell sources approaching experimental procedure for clinical purposes [5,6]. The most important feature of a full small molecule TD system which burst these systems into

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Abbreviations

ALS	Amyotrophic Lateral Sclerosis
BDNF	brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BMPR1	BMP receptor 1
BMPR2	BMP receptor 2
CNTF	ciliary neurotrophic factor
DMEM/F12	Dulbecco's modified Eagle medium and Ham's F-12
DSM	Dorsomorphin
ECM	Extra Cellular Matrix
FBS	fetal bovine serum
FN	fibronectin
GDNF	glial cell line-derived neurotrophic factor
HFFs	Human foreskin fibroblasts
LN	laminin
L-Glu	L-glutamine
MAPK	mitogen activated protein kinase
NTFs	neurotrophic factors
NEAA	non-essential amino acids
PD	Parkinson's disease
PLL	poly-l-Lysine
PLO	poly-l-ornithine
TD	trans-differentiation
TSA	Trichostatin A

prominence is an acceptable package of safety, accessible cell source and being more economical.

One of the small molecules which has been applied to induce neuroectodermal cells from human and monkey embryonic stem cells is Dorsomorphin (DSM) [7]. DSM is a strong inhibitor for Bone morphogenetic protein (BMP) signaling pathway through interacting with BMP receptor 1 (BMPR1) [8], thereby bans the activatory effects of BMP pathway on Smad1/5/8. To our knowledge, BMP signaling pathway is responsible for mesoderm formation during development and its downstream molecules are expressed, active and responsive to the BMP receptor manipulation in human fibroblasts and keratinocytes [9]. Notably, DSM is not capable to inhibit BMP receptor 2 (BMPR2) which is an activator of Mitogen Activated Kinases (MAPKs eg. ERK1/2, JNK, P38). MAPKs are the signaling pathways with definitive and determinative role in differentiation especially in neural differentiation [10,11]. Hence, fibroblast treatment with DSM could be effective to decrease mesodermal features of human fibroblasts by inhibiting any possible endodermal inductions and ameliorates ectodermal induction. Therefore, this study aims to examine the ability of DSM in conversion of human fibroblasts to neuroectodermal cells. Here, we show that DSM solely, is able to switch human fibroblasts to neuroectodermal cells. To elaborate this procedure, we decided to utilize an efficient epigenetic modulator. Previous studies have introduced Trichostatin A (TSA) [12]. Therefore, in the present study, we introduce an efficient protocol to convert human fibroblasts to single human neuroectodermal cells by means of a simple cocktail of DSM and TSA through a 12 day protocol which is more efficient and less time consuming.

2. Methods

2.1. Ethical approval statement

Ethical approval for this study to use the human foreskins was obtained from the institutional review board of Royan institute and the samples were obtained from the donors after signing consent by their parents.

2.2. Cell culture and treatment

Human foreskin fibroblasts (HFFs) were isolated from foreskin samples which were collected from 9 male infants' circumcision within 3–12 months age, under aseptic condition as primary cultures. For each experiments, different samples from 3 different persons (triplicate) were used to consider personal variation in our protocol so final results will be generalized for different people considering personal variations. HFFs were passaged and were used in passage 2–4 for all experiments.

HFF cells were maintained in fibroblast medium containing 15% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 1% L-glutamine (L-Glu), 1% penicillin/streptomycin (Pen/Str), Dulbecco's modified Eagle medium and Ham's F-12 (DMEM/F12). A flow cytometry test was performed for Vimentin and PAX6 markers for every sample on day 0 to demonstrate that sources of human fibroblast cells are at least higher than ninety percent pure and there is no cross contamination from other types of cells as sources for neuro-ectodermal cells (Supplementary Fig. 1). To assess the optimal concentration of DSM, and to avoid the presumable toxic effects of DSM, MTS assay was carried out according to the kit protocol (Promega). Prior to perform MTS assay, appropriate cell number for fibroblasts was quantified based on the growth curve achieved by MTS assay (Supplementary Fig. 2). Different concentrations of DSM (Sigma) were dissolved in DMSO. Meanwhile, to adjust suitable concentration of Trichostatin A (TSA) (Sigma) treatment, neural conversion of fibroblasts were carried out in presence of several amount of TSA (50, 100 and 200 nM) and relative expression of *PAX6* was calculated. For Neuroectodermal conversion of fibroblast cells induction and Dorsomorphin (Sigma) treatment, medium replaced by neural induction medium containing 1% N2, 1% B27, 1% Pen/Str, 1% L-Glu, 1% NEAA, Neurobasal medium (Gibco) (1:1) DMEM/F12 (Gibco). Both of DSM and TSA were diluted in DMSO. To improve the efficiency of cell viability during the conversion of HFFs, a cocktail of neurotrophic factors (NTFs) including brain-derived neurotrophic factor (BDNF, Sigma, 10 ng/mL), glial cell line-derived neurotrophic factor (GDNF, Sigma, 10 ng/mL) and ciliary neurotrophic factor (CNTF, Sigma, 10 ng/mL) were added to the media. Additionally, a number of extracellular matrixes (ECMs) were implemented for coating of the culture dishes. Briefly on day 6, cells were seeded on treated culture dishes with three components in two combination of poly-L-lysine (PLL) (Sigma, 0.01%) or poly-L- ornithine (PLO) (Sigma, 0.01%) with a cocktail of laminin (LN) and fibronectin (FN) [each 10 or 20 μg/mL].

2.3. Morphological tracking of the cell

Cells were treated with various concentrations of DSM under a 21 day protocol as depicted in Fig. 1A. Cells were passaged based on their confluency on day 6. At the first step, morphological changes of human fibroblast cells to neural like cells were tracked with light microscopy during the procedure.

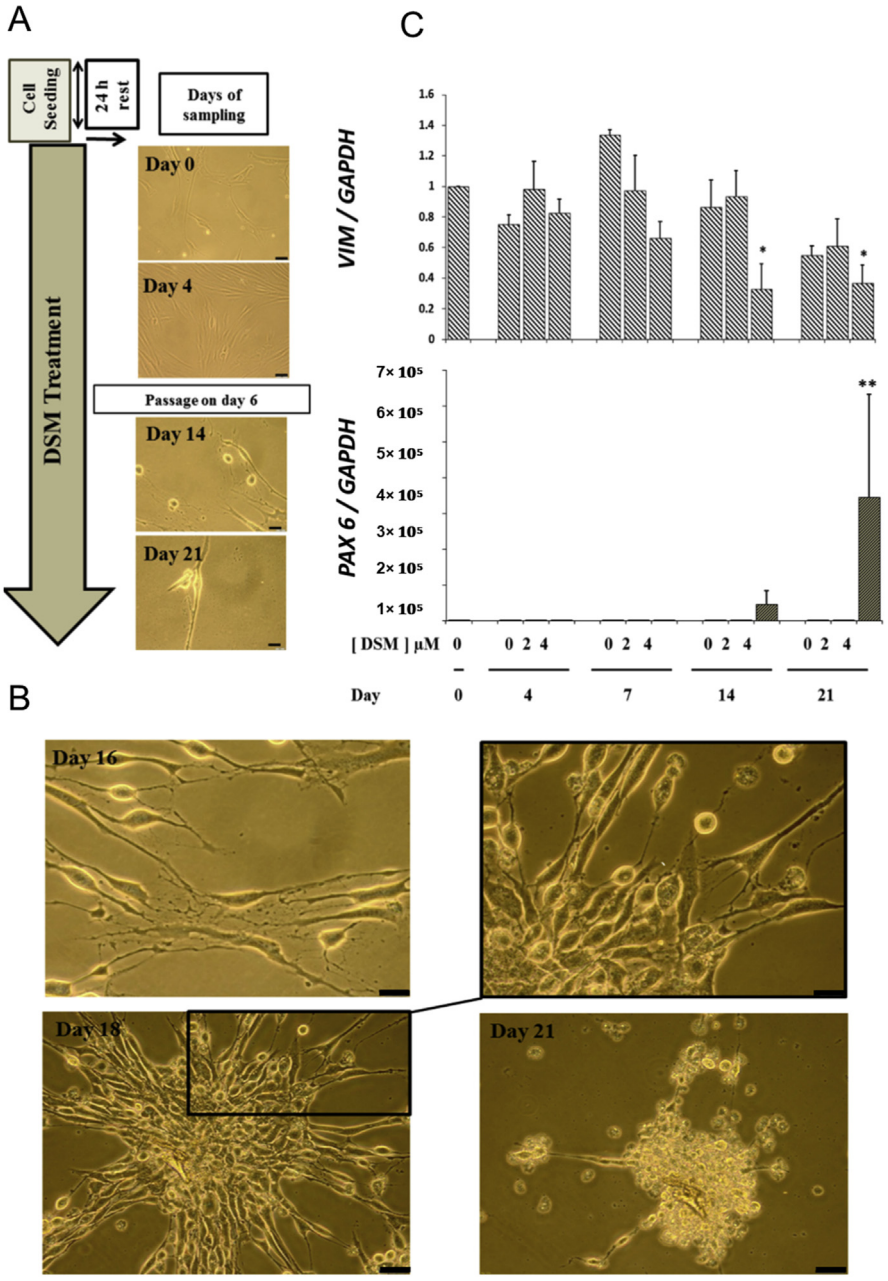

Fig. 1. Primary protocol of DSM application to induce conversion of fibroblasts. A- Schematic representation of the protocol used for conversion of hFFs. To start the experiment, 10^4 cells/cm^2 were seeded as described in Supplementary Fig. 2. Next day, cells were treated with different concentration of DSM (2 and 4 µM). Half of Medium change was carried out every two days and cells were passaged on day 6 to dissociate the cell. Similar treatment with DSM was repeated by day 21. To examine the characterization of the cells, harvesting was performed on day 0, 4, 14 and 21. B-Manifestation of neural-like cells during DSM treatment. As observed, bipolar morphology of the cells is evident mostly on day 18. Also, there was an increasing rate of the cell death on day 21. Bar = 50 µm. C- The pattern of Vimentin and *PAX6* expression as specific fibroblastic/mesodermal and definite neuroectodermal markers respectively. * and ** indicate significant difference between samples and control (day 0) at $p < 0.05$ and 0.01, respectively. All measurements and experiments were performed in triplicate, from three separate cultures.

2.4. Gene expression profiling

In order to track cell characteristics, sample pick up was carried out on days 4, 7, 14 and 21. The expression of specific lineage and cell type markers including Vimentin, as a fibroblastic-mesodermal marker [13], and PAX6 as definitive neuroectodermal marker [14], were assessed by real time quantitative PCR as described [10]. The list and sequence of the primers are presented in Table 1.

2.5. Cell staining and flowcytometry

Cells were washed with Mg^{2+}/Ca^{2+} free PBS containing EGTA and then treated with Accutase to be dissociated from the culture plate and collected. Cells were fixed and stained accordingly [10]. The primary antibodies were against PAX6 (Abcam, 1:1000)/its isotype (Abcam, 1:200) and Vimentin (Sigma, 1: 60)/its isotype (Millipore, 1: 300). To avoid the false positive result, cells were

Table 1
Primers used in this study.

Gene	Primer Sequence	Annealing temperature
PAX 6	F: 5′-TTGCTGGAGGATGATGAC-3′ R: 5′-CTATGCTGATTGGTGATGG-3′	60 °C
Vimentin	F: 5′-GGCTCGTCACCTTCGTGAAT-3′ R: 5′-GAGAAATCCTGCTCTCCTCGC-3′	60 °C
GAPDH	F: 5′-CCACTCCTCCACCTTTGACG-3′ R: 5′-CCACCACCCTGTTGCTGTAG-3′	60 °C
Nestin	F: 5′-TTCCCTCCGCATCCCGTCAG-3′ R: 5′-GCCGTCACCTCCATTAGC-3′	60 °C
Rex1	F: 5′-GCGGAGGAGAACAAACAG-3′ R: 5′-TGTGAGGCGGTAGTAGGA-3′	55 °C

F and R represent forward and reverse primers respectively. Also, annealing temperature for each PCR is indicated.

washed with PBS containing tween 0.05% prior to incubating with second antibody conjugated FITC (Millipore, 1:50) and PE (Sigma, 1:20). Cell observation was performed under a fluorescent microscope (Olympus, Tokyo, Japan). The percentage of neuroectodermal cells was calculated according to the data obtained by flowcytometry by subtracting of the average percentage of Vimentin negative cells from the average percentage of PAX6 positive cells in the same population of cells. Flow cytometry was carried out with the same cell population and analyzed on Becton Dickinson FACSCalibur flow cytometer (USA).

2.6. Statistical analysis

Statistical analysis was performed by SPSS (Version 16.0, USA) and data were shown as mean ± SEM for each group in three independent replicates for each experiment. Comparisons between groups were analyzed by independent-sample *t*-test and a One-Way ANOVA, which were considered to be significant at $p < 0.05$.

3. Results

3.1. DSM induced neural-like morphology in HFFs

Six day treatment with DSM (2 and 4 μM), manifested neural-like morphology in human fibroblast populations. This morphological change was apparent after two weeks. These neural-like cells were mostly bipolar (Fig. 1A and B). However, a robust death was observed after 18 days until day 21 of incubation with DSM.

3.2. Gene expression analysis showed a robust expression of the neuroectodermal marker, PAX6, and decrement of fibroblast marker, Vimentin

Transcript level of *PAX6*, a definite neuroectodermal marker, upregulated two weeks post treatment of DSM. This increasing wave was sharp by day 21 in association with decrease in *Vimentin* expression (Fig. 1C and D). Data clarified that DSM increased neuroectodermal marker expression and reduced fibroblast marker in an anti-parallel correlation. However, an obvious variation in the value of these changes in different populations of DSM treated fibroblasts demonstrated that this protocol needs to be improved.

3.3. A variety of culture dish coating components with several neurotrophic factors were implemented to shorten duration of neuroectodermal cells manifestation and increase the viability of neuroectodermal cells

Previous studies well established that NTFs can be used as supportive component to increase the efficiency of neural cell formation [2,15,16]. To improve cell survival during this protocol, we examined whether a cocktail of three NTFs including BDNF, GDNF

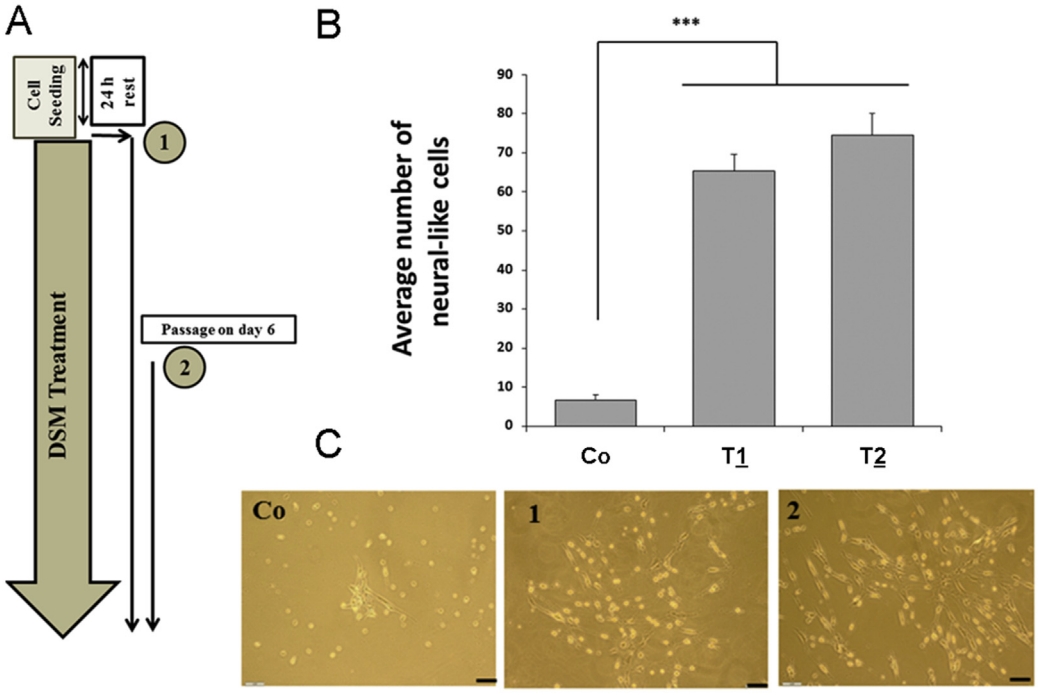

Fig. 2. Modification of the proposed protocol with combination of NTFs. A- Similar protocol as Fig. 1 A was implemented for DSM treatment in presence of 10 ng/mL of each NTF including GDNF, BDNF and CNTF. Please note that two different time table was set for application of NTFs (treatment 1 (T1): 15 days and treatment 2 (T2): 21 days supplementation with NTFs). B- Average number of neural-like cells increased upon NTFs supplementation. Cell number average was calculated with cell counting of at least ten different fields on day 21 in three independent replicates of experiment. *** indicates significant difference between samples and control (day 0) at $p < 001$. As observed there was no significant difference between two treatments, thus we preferably used treatment 1 for the rest of experiments C- Morphological observation of treated cells. As obvious the survival rate of the cells were increased by implementation of the NTFs. Bars are 50 μm.

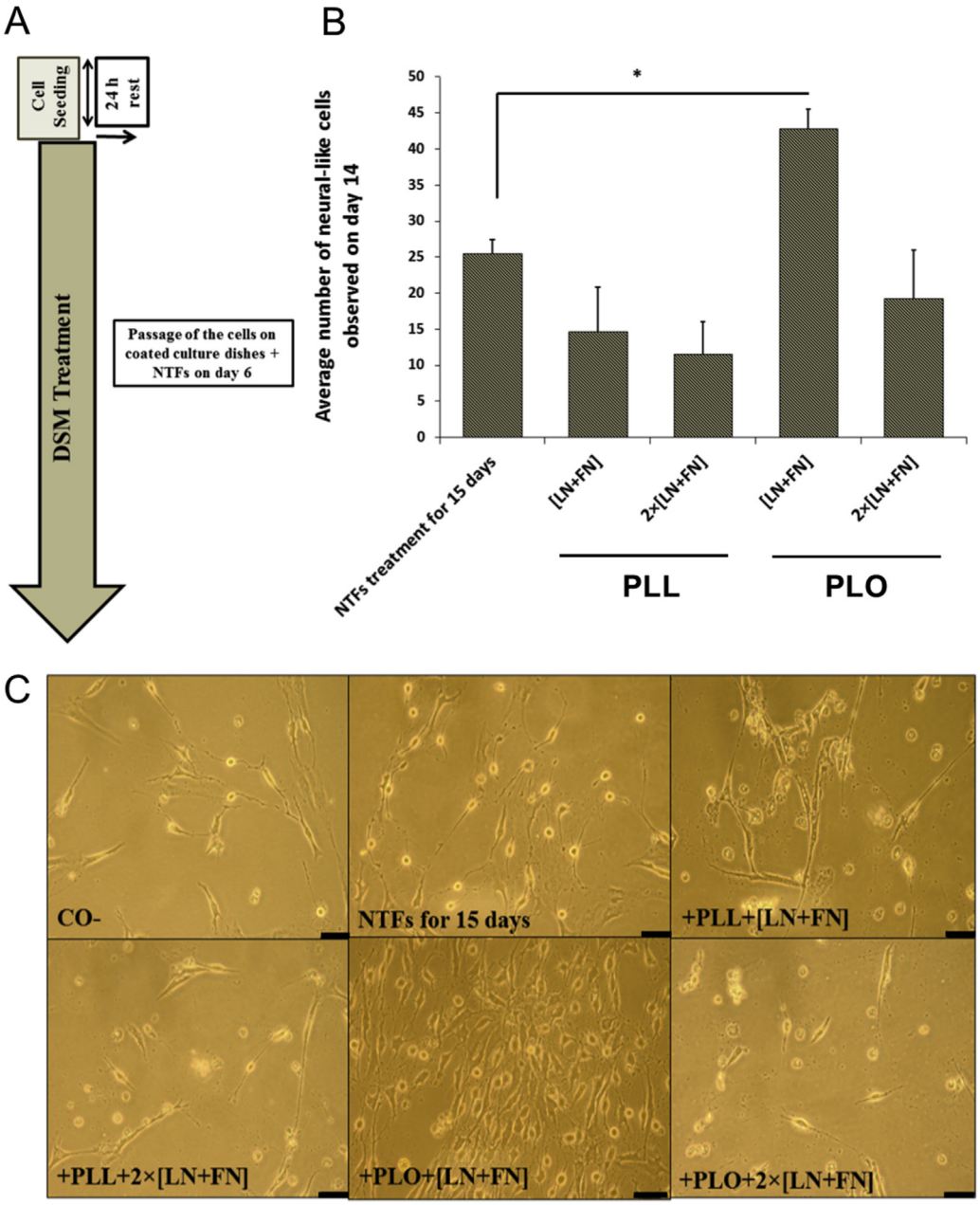

Fig. 3. Improvement of the proposed protocol with implementing a cocktail of ECM components. A- The proposed Protocol in Fig.2A was improved by pre-treatment of the culture dishes with various extracellular matrixes. B- Average number of neural-like cells during DSM and NTs treatment in presence of various cocktails of ECM components on day 14. Please note that as the number of cells increased efficiently, cell counting was performed on day 14. Group 1 = Control (−), group 2 = PLL+[LN + FN], group 3 = PLL+2 × [LN + FN], group 4 = PLO + [LN + FN], group 5 = PLO + 2 × [LN + FN]. All measurements and experiments were performed in triplicate, from three separate cultures C- Morphological observation of treated cells as elucidated above. Bars are equal to 50 μm.

and CNTF is able to enhance the efficacy of TD (Fig. 2A). Interestingly, this combination increased neural-like cells survival significantly (Fig. 2B and C). During three weeks treatment of HFFs with DSM, there was a massive reduction in neural-like cell survival and adherence. While the ratio of the neural-like cells augmented following two weeks significantly, the adherence and subsequently the survival of these cells decreased after day 18. In order to compensate this apparent reduction, a number of matrix components [16] were evaluated for coating of the culture dishes on day 6 (Fig. 3A), where improvement in cell adhesion was yielded with PLO + [Laminin (LN) + Fibronectin (FN)] 10 μg/mL (Fig. 3B and C).

3.4. TSA efficiently improved conversion of HFFs to neuroectodermal cells

According to data obtained in Fig. 1C, significant variation in transcript level of *PAX6* was evident. Furthermore, duration (21 days) of the proposed protocol was time-consuming. Therefore, TSA was applied as an epigenetic modulator to accelerate this process as beneficial effects of TSA was already approved [12]. Interestingly, cellular response to treatment was more orchestrated, since TSA along with DSM was used to induce neuroectodermal conversion of HFFs in a shorter 12 day procedure

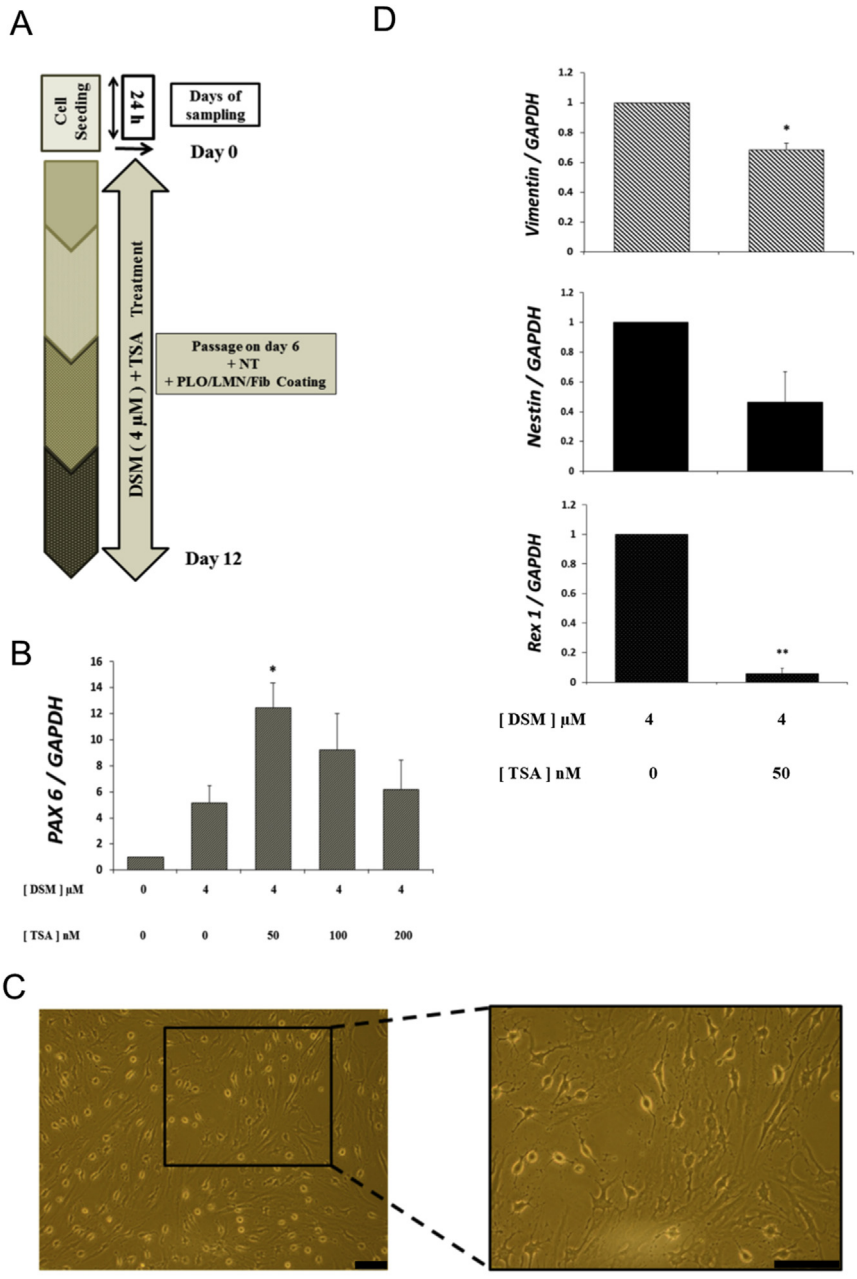

Fig. 4. Neuroectodermal conversion of HFFs by co-treatment of DSM (4 µM) with TSA (50 nM). A- Modified protocol used for transdifferentiation of HFFs to neuroectodermal cells. Please note that duration of modified protocol was shortened accordingly. B- The optimization of TSA amount for neuroectodermal cells production was carried out according to the relative expression of *PAX6*. Data indicated that maximal expression of *PAX6* was yielded when 50 nM of TSA was used. Hence this concentration was used for further experiments. C- Cellular morphological change observation by application of 50 nM of TSA. Neural like cells were emerged upon this procedure on day 12. Bars are 50 µm. D-F- Relative expression pattern of *Vimentin*, fibroblast marker (D), *Rex1*, pluripotency marker (E) and *Nestin*, neural progenitor marker (F). * indicates significant difference between samples and control (day 0) at $p < 0.05$. All measurements and experiments were performed in triplicate, from three separate cultures.

(Fig. 4A and B). Hence, TSA reduced the variation of behavior of different lines of HFFs in response to DSM treatment (Fig. 4C and D). The optimal concentration of TSA for this procedure was 50 nM (Fig. 4C). This concentration was used for further analyses. In order to validate the conversion process, *Rex1* expression, determinative marker for pluripotency, was assessed and showed a significant reduction (Fig. 4E). Notably cells were not transited to neural progenitor state as RNA level of Nestin [17] was not changed significantly (Fig. 4F). To verify data obtained by real time PCR, flow cytometry was exerted on cells during day 0 (HFFs) and day 12 of the protocol. Data showed approximately 99% of cells on day 12 were positive for *PAX6* marker (Fig. 5A). Also, approximately 25% of the cells in same population (day 12) were negative for Vimentin (Fig. 5B). Therefore, 24% of the cells on day 12 were PAX6 positive and Vimentin negative which were considered as human neuroectodermal cells (Fig. 5C). Indirect immunofluorescent staining was

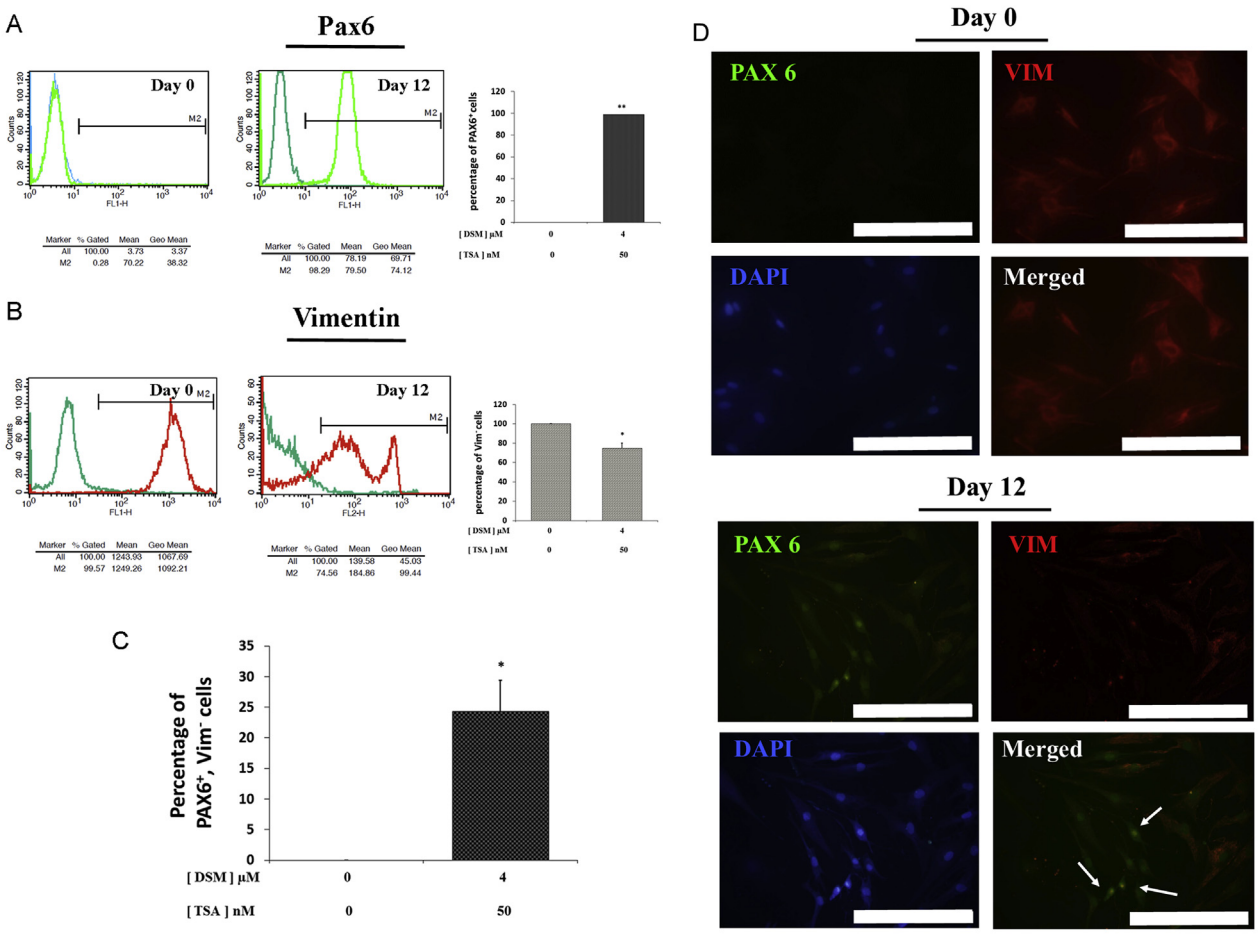

Fig. 5. Pax6 +/Vimentin − cells were emerged on day 12 as a subpopulation of converted HFFs to neuroectodermal cells. A- Flow cytometric plot of PAX6 -expressing cells on day 0 (left plot) and day 12 (right plot). B- Flow cytometric plot of Vimentin -expressing cells on day 0 (left plot) and day 12 (right plot). C-Average percentage of Vimentin- cells subtracted from the average percentage of PAX6+ cells as the neuroectodermal cells by this protocol was calculated to be around 24%. Data are the average of three independent experiments. * and ** indicate significant difference between samples and control (day 0) at $p < 0.05$ and 0.01, respectively. Fig. 5D- Immunofluorescent staining of the cells on day 0 and day 12 for PAX6 and Vimentin (VIM) markers and DAPI as described in materials and methods. Bars are 1 µm. Arrows indicate neuroectodermal cells which are positive for PAX 6 and negative for Vimentin.

carried out on cells of day 12 compare with day 0. As indicated, a number of the cells were definitely PAX6 positive and Vimentin negative on day 12 (See the arrows, Fig. 5D).

There are some guidelines to avoid potential pitfalls of this protocol available in Table 2.

4. Discussion

A full small molecule TD system could be applicable for therapeutic purposes in regenerative medicine. Such approach will help to overcome invasive surgeries for cell therapy of tissues that have reduced regeneration capacity. For elaborate neuroectodermal conversion of human fibroblast cells, herein we report a strategy by implementing of two small molecules including DSM and TSA.

DSM is a very exclusive inhibitor for BMP signaling pathway, which has already recognized to have a determinative role in mesoderm and endoderm induction [18,19]. To our knowledge, BMP receptor has two types, the BMPR1 and BMPR2. BMPR1 activation results in triggering of SMAD1/5/8 activation, which plays a key role in mesodermal and endodermal induction. On the other hand, BMPR2 activation results in MAPKs activation, a group of signaling molecules involved in cell differentiation especially neural and cardiac differentiation. In contrast to Noggin and Chordin, which are natural inhibitors of BMP receptors, DSM only inhibits BMPR1 receptor. Consequently, DSM blocks induction and maintenance of mesoderm and endoderm, while allows BMPR2 to play its role as an activator for differentiation. Therefore, DSM is supposed to induce neural TD of human fibroblast cells. To verify this hypothesis, we treated fibroblast with 4 µM of DSM and observed neural-like cells which became positive for *PAX6*, a neuroectodermal marker, on day 14 in a contradictory pattern with VIM expression which reduced in the same population of the cell.

To improve the viability of neural-like cells during the performance of protocol, we applied NTFs (GDNF, BDNF and CNTF). Also, cells were replated on PLO+ 10 µg/mL (LN + FN) coated dishes in order to increase cellular viability which significantly improved neural-like cell survival by around 2 fold.

In order to reduce inter-assay variations and shorten the duration of TD, cells were treated with TSA as a potent epigenetic modulator [12]. TSA application proceeded TD of HFFs efficiently, shortening duration of protocol. Finally, characterization of the cells at the end of protocol was performed again by expression analysis of *PAX6*, *VIM*, *Nestin*, and *Rex1*. Eventually, we produced neuroectodermal cells via conversion of HFFs by means of a simple

Table 2
Potential pitfalls of protocol and troubleshooting.

Pitfalls	Troubleshooting
Clamp formation	Shake cell culture dishes after seeding the cells because they adhere to the surface very fast by ECM production especially during the 24 h seeding step.
Delay in neuroectodermal induction	• Prepare medium freshly. • Vortex DSM and TSA vials during dilution with DMSO. • Avoid temperature decrement during changing media. • Avoid fluorescent light while handling DSM and TSA.
Cell death	• Handle and pipet cells during passage on day 6 gently. • Avoid temperature decrement during handling cells on day 6.
Low RNA concentration	Solve the cells completely on day 12 in RLT by pipetting and dissociating them from ECM.

cocktail of two small molecules within a brief 12 day protocol. There are published studies on full small molecule conversion so far [5,6,20]. One of them has used mouse fibroblasts as cell sources for conversion [20] while the next one has benefited from human fibroblasts where mature human neurons was produced [5]. There are three important privileges in our protocol. First, we produced neuroectodermal cells which are appropriate source for generation of all types of the nervous system cells. Second, our proposed cocktail consists of two small molecules of DSM and TSA, compared to Hu and coworkers who implemented eight different small molecules to achieve induced neurons. Third, our protocol has been shortened reasonably (12 days) with the efficiency of 24% in contrast to the protocol with 28 day and 5% efficiency as reported earlier [5]. However to obtain a more comprehensive protocol using DSM and TSA, RNA-seq analysis and *in vivo* experiments are needed to be performed for further characterization of yielded cells.

5. Conclusion

Taken together, the efficacy and being less time consuming aspects of this protocol made it a reasonable strategy to be applied in regenerative medicine for drug delivery and cytotherapy purposes. However, more characterization and *in vivo* experiments are needed to be carried out to assess the feasibility of this protocol in treatment of neural damages. Our next step is to get a grant to overcome the shortcomings and to facilitate the RNA-seq analysis, co-culture and *in vivo* experiments in order to assess the best applications of our neuroectodermal cells in human cytotherapy.

Ethical approval and consent to participate

Approval for this study was obtained from the Institutional Review Board of Royan Institute (Tehran, Iran). Informed consent for foreskin biopsies was obtained from parents of all individual donors included in the study.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

None of the authors has any conflict of interest to disclose, and all authors support submission to this journal.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2020.05.003.

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