Directed neural differentiation of induced pluripotent stem cells from non-human primates

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Abstract

Induced pluripotent stem cells (iPS cells) are important for the future development of regenerative medicine involving autologous cell therapy. Before autologous cell therapy can be applied to human patients, suitable animal models must be developed, and in this context non-human primate models are critical. We previously characterized several lines of marmoset iPS cells derived from newborn skin fibroblasts. In the present studies, we explored methods for the directed differentiation of marmoset iPS cells in the neuroectodermal lineage. In this process we used an iterative process in which combinations of small molecules and protein factors were tested for their effects on mRNA levels of genes that are markers for the neuroectodermal lineage. This iterative process identified combinations of chemicals/factors that substantially improved the degree of marker gene expression over the initially tested combinations. This approach should be generally valuable in the directed differentiation of pluripotent cells for experimental cell therapy.

Keywords: induced pluripotent stem cells, non-human primates, differentiation, cell therapy

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Introduction

Non-human primate iPS cells in regenerative medicine

Induced pluripotent stem cells (iPS cells) are believed to represent a major approach to the future development of regenerative medicine.[1] It is widely thought that a form of autologous cell therapy will be possible, in which iPS cells would be derived from the patient's cells, in order to provide a source for cells that could be transplanted back to the patient to restore function to the heart, central nervous system, hematopoietic system or other organs that are affected by disease or aging. Following the discovery of iPS cells, it was almost immediately realized that this discovery opened the way to autologous cell therapy. A review in 2007 stated: 'If this method can be translated to humans, patient-specific stem cells could be made without the use of donated eggs or embryos'.[2] It is assumed that if the cells are accepted as 'self' then they would represent the best possible functional outcome of a transplant: cells that function in their natural environment, without eliciting chronic immune or inflammatory reactions, and without the problems that would result from the use of immunosuppressive drugs.

Before it would be possible to consider applying autologous cell therapy to human patients, the properties of iPS cells must be thoroughly explored in suitable animal models, in order to make sure that autologous cell therapy is both safe and effective. It has been generally recognized that clinically relevant experiments should be performed in a non-human primate (NHP) rather than a rodent. NHPs are thought be ideal for such preclinical trials because of their relatedness to humans and their similar physiology, particularly with respect to the central nervous system. Long-term studies of transplanted cell function (3 years) will be possible in NHPs, but are impossible in rodents. Thus there is a clear path from basic to translational studies in iPS cell-based regenerative medicine in NHPs. Of the various NHPs that could be used, the common marmoset (*Callithrix jacchus*) has several advantages, as previously described.[3] For this reason, we previously characterized several lines of marmoset iPS cells derived from newborn skin fibroblasts.[4]

Directed differentiation of pluripotent stem cells

A universal need in regenerative medicine is the large-scale production of differentiated cells from a pluripotent cell source, either embryonic stem cells or iPS cells. The requirements for such cells are that they should be sufficiently

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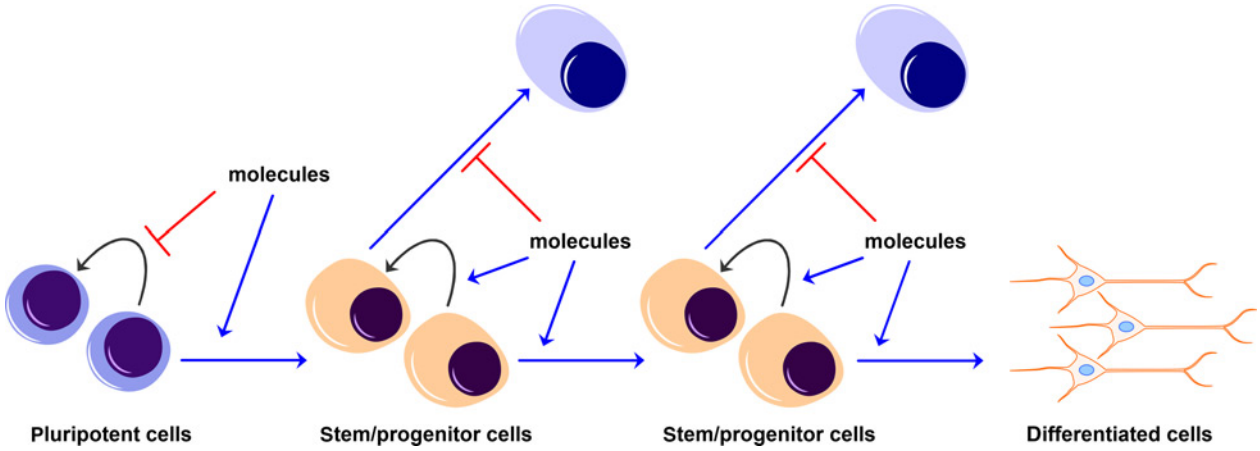

Figure 1 The basic principle underlying protocols that aim for efficient and rational differentiation of pluripotent cells. A typical differentiation protocol goes through multiple stages, attempting to mimic embryonic development by using molecules to stimulate the pathways that are required, while using the same or other molecules to block unwanted differentiation to other pathways at each stage. In this diagram, intermediate cell populations are termed 'stem/progenitor cells' although they may not directly correspond to any population of cells found in actual tissues *in vivo*, either in the embryo or the adult. At the end of the process illustrated here the aim is to have a population of cells that are suitable for cell therapy or for other purposes. Diagram adapted from Zhu *et al*.[6] (A color version of this figure is available in the online journal)

differentiated to accomplish the goals of cell therapy, while not being too differentiated as to impair successful transplantation. In particular, teratoma-forming undifferentiated pluripotent cells must be eliminated prior to cell therapy being initiated. Protocols for differentiation in a variety of desired lineages have been evolving rapidly. Early protocols used mostly undefined materials and mixtures, while more recent emphasis has been on a rational approach involving interventions in cellular pathways known to be or hypothesized to be involved in differentiation, using chemicals/drugs or defined protein factors[5,6] (Figure 1). These protocols have lowered the time taken to form a differentiated cell population from several weeks to much shorter periods of 10–20 days.

A significant advance in the field of neural differentiation from pluripotent cells was the introduction of the concept of dual SMAD inhibition.[7] Initially this was accomplished by the combination of noggin and SB431542, while later versions substituted chemical inhibitors such as dorsomorphin for the more expensive noggin.[8,9] This forms one example of how, over the last few years, an armamentarium of small-molecule agents has been developed that are available for differentiation protocols.[5,6] The need now is to be able to rationally combine selected compounds in protocols that take advantage of results of prior studies and of increasing knowledge of the pathways that have been identified as being involved. In many ways the experimenter attempts to mimic the multiple stages of actual embryonic development in the dish (Figure 1). This also requires the identification of suitable molecular markers needed for each developmental stage. Furthermore, the adaptation of protocols across species (for example, from humans to NHPs) requires adjustments in concentrations of inducing molecules and in the timing of their addition.

Optimizing differentiation protocols

As protocols for directed differentiation of pluripotent cells have proliferated, the number of factors available to the experimenter for testing has greatly increased.[5] Each factor should be used at an optimal concentration. Therefore, the potential number of combinations of factors and concentrations that might be tested in differentiation protocols can become very large.[10] As the list of molecules useful to control stem cell fate decision gets longer, so does the list of factor interactions that need to be understood.[11] Moreover, protocols can be very sensitive to small changes in concentrations of inducing factors. For example, with reference to a protocol for oligodendrocyte differentiation:[12] 'The most surprising aspect is how sensitive the cells are to the concentration of the reagents . . . Very small changes give you precise results'.[13] Systematic approaches to handling large numbers of factors and concentrations are needed, with the aim of significantly reducing the number of experiments required to arrive at meaningful results.[10,14] Instead of conducting experiments in a traditional way, where factors are varied one by one and independently, an experimental approach in which multiple factors are changed simultaneously in a controlled manner becomes very attractive. Combinations should be tested simultaneously, not sequentially, because of possible interactions among the factors, which may be both predicted and unexpected. Several examples of ways factors can interact are described by Audet.[11] For example, one factor might induce the receptor for a second factor. In that case, testing the second factor in the absence of the first could result in a lack of a response. On the other hand, iterative testing of the two factors together can yield an optimal combination that maximizes the induction of the receptor by the first factor and the resultant response to the second factor. Methodologies involving simultaneous testing and iterative optimization have been widely adopted in bioprocess optimization, and to some extent in drug screening, but far less frequently in stem cell research.[14]

In an example of the use of iterative approaches to searching for an optimal combination of multiple factors and multiple concentrations, Tsutsui *et al.*[15] studied five factors implicated in maintaining pluripotency and avoiding

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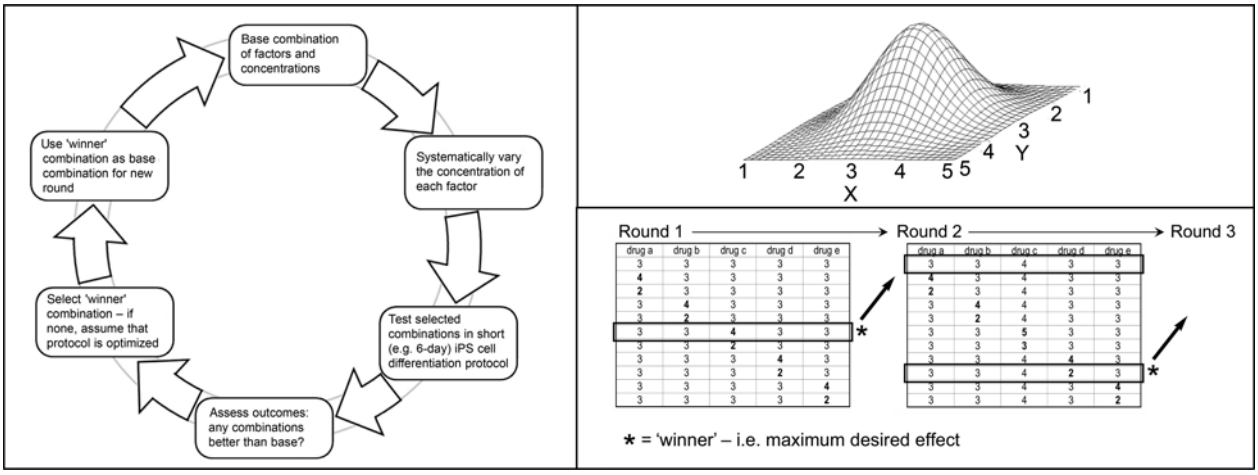

Figure 2 A process for optimizing differentiation protocols that use combinations of multiple small molecule drugs or protein factors. The panel on the left outlines the process by which combinations of chemicals/factors are iteratively tested with respect to a desired outcome, i.e. the level of differentiation as assessed by suitable markers. At each round the outcome is assessed, and a new base combination is selected to begin the new round. The 'winner' combination in each round is chosen according to a hill climbing algorithm. This depends on simultaneous, rather than sequential, optimization of the drug combinations. The rationale for simultaneous optimization is illustrated in the image in the upper right panel. Two drugs (X and Y) are depicted that strongly synergize at concentration '3' but less so or not at all at other concentrations. If each drug were tested first in the absence of the other, only weak responses (shown as the elevation at different places on the hill) would be recorded, while testing of the two together can result in a rapid determination of the optimum combination ('hill climbing', as described in detail in the text). The method is shown in the lower panel on the right. In this case the optimization of five drugs used in combination is shown. The first round begins with each drug used at concentration '3' representing a presumably effective concentration previously determined from the literature (see Table 1 for actual examples). Apart from the 33333 combination, 10 other variations are tested in which each drug is varied by increasing the concentration by one step (to level '4') and by decreasing one step (to level '2'). After the 'winner' combination is identified, this combination is used as the base combination in round 2. Rounds may be repeated as necessary until no further improvement in the differentiation is obtained

unwanted differentiation of human embryonic stem cells. Starting with concentrations of the five factors thought to be useful based on prior literature, in each round of experiments they systematically varied the concentrations of each of the five factors and assessed the resultant level of pluripotency by OCT4 expression and alkaline phosphatase staining. Identification of an effective small molecule combination for maintenance of pluripotency required testing 192 combinations in six rounds; if they had used a 'brute-force' approach it would have been necessary to test 7776 possible combinations.[15]

In adapting the approach of Tsutsui *et al.*, we opted to employ a simpler 'hill-climbing' algorithm for optimizing chemical/factor combinations in differentiation protocols. We assume that the degree of differentiation of the cells following combination treatments is evaluable as a single value – a theoretical concept, but useful for the purpose of illustration. The metaphor of hill climbing refers to the concept that the values representing the outcomes of all possible chemical/factor combinations can be plotted as a three-dimensional surface, i.e. a fitness landscape. The highest point in the landscape represents the optimal combination for the measures of differentiation being used (Figure 2).

Hill climbing is a technique that is applicable to a wide variety of situations where optimization is desired. The 'hill climb' begins with any (suboptimal) solution to the problem and then iteratively improves the solution until some condition is maximized (the top of the hill is reached). Hill climbing is an example of an informed search method because it uses information about the search space to search in a reasonably efficient manner.[16] Using the metaphor of climbing a real hill in fog, the algorithm can be stated as 'check the height 1 foot away from your current location in each direction; move to the point that is highest; then repeat the algorithm. If all directions lead to a point lower than the current position it is assumed that the summit has been reached'. Hill climbing algorithms can run afoul of foothills, plateaus and ridges in the fitness landscape.[16] For this reason more complex algorithms may be preferred, which use strategies to avoid landscape features that represent local maxima as opposed to a global maximum.[10] Where prior knowledge of the nature of the landscape exists, more complex approaches might be necessary; for example, to determine drug combinations that inhibit vesicular stomatitis virus infection and combinations of cytokines that regulate NF-κB.[17] In a second example where hill climbing was not thought to be suitable as an algorithm, an iterative approach was used to select an optimal combination from among 82,950 possible combinations by exploring 0.5% of the space in experiments to optimize the formulation of amphotericin B in lipids.[18] On the other hand, hill climbing was shown to be applicable to a search for effective combinations of 19 anti-cancer drugs.[19] As these authors point out, to be useful, the algorithm needs only to find combinations that have fitness levels that are 'good enough' with respect to the aim of the experiments, and to be more efficient than a random screen. In the absence of examples to the contrary, it is simpler to begin with the assumption in differentiation protocols that there is a single global optimum for drug/factor combinations, rather than any more complex situation. In such a case, hill climbing is the fastest method to determine the optimal combination (Figure 2).

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Table 1 Concentrations of small molecules/protein factors used in two neuroectodermal differentiation protocols for marmoset induced pluripotent stem cells

Protocol #1	RA (μmol/L)	SB (μmol/L)	DM (μmol/L)	FGF (ng/mL)	SAG (μmol/L)
1	0.001	0.1	0.1	2	0.1
2	0.01	0.32	0.32	6.3	0.32
3	0.1	1	1	20	1
4	1	3.2	3.2	63	3.2
5	10	10	10	100	10

Protocol #2	DMH1 (μmol/L)	SB (μmol/L)	BIO (μmol/L)	PD (μmol/L)
1	0.05	1	0.2	0.1
2	0.16	3.2	0.63	0.32
3	0.5	10	2	1
4	1.6	32	6.3	3.2
5	5	100	20	10

RA, all-*trans* retinoic acid; SB, SB431542; DM, dorsomorphin HCl; FGF, FGF2; SAG, Sonic hedgehog agonist; DMH1, dorsomorphin analogue; BIO, (2′Z,3′E)-6-bromoindirubin-3′-oxime; PD, PD0325901. The first column (1 through 5) shows the code used for the specific concentrations of the chemicals. For example, '33333' in protocol #1 refers to the combination of 0.1 μmol/L RA, 1 μmol/L SB, 1 μmol/L DM, 20 ng/mL FGF, and 1 μmol/L SAG

Methods

Marmoset-induced pluripotent stem cells

A line of marmoset iPS cells was grown as previously described.[4] At the beginning of the differentiation protocols, cells were removed from the dish with Accutase.[20] Cells were then transferred into different differentiation media, as described below.

Differentiation Protocol #1

In this protocol, cells were permitted to aggregate into embryoid bodies using 384-well hanging-drop plates (3D Biomatrix, Ann Arbor, MI, USA). Cells were placed in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 20% Knockout Serum Replacement (KSR; Invitrogen, Carlsbad, CA, USA), 0.32 μmol/L dorsomorphin HCl (Tocris, Bristol, UK), 0.32 μmol/L SB431542 (Selleck, Houston, TX, USA), 1 nmol/L all-*trans* retinoic acid (Sigma, St Louis, MO, USA), 10 μmol/L Y-27632 (Abcam Biochemicals, Cambridge, MA, USA), 20 ng/mL FGF2 (Stemgent, Cambridge, MA, USA), 25 μg/mL insulin (bovine, Sigma). Each well of the hanging-drop plate received 3000 cells in 30 μL of this medium. Plates were placed in a humidified incubator at 37.5°C for 72 h. Following this incubation period, the resultant early embryoid bodies were collected from the plate. They were then dissociated to single cells by incubation in 1 mL 0.25% trypsin EDTA for 30 min at room temperature. Cells were transferred into DMEM/F12 medium containing 20% KSR, 2% B27 supplement (Invitrogen), 15 μg/mL transferrin (human, Sigma), and various concentrations of retinoic acid, SB431542, dorsomorphin, FGF2 and SAG (Sonic hedgehog agonist; EMD Chemicals, Billerica, MA, US). Cells in these various drug/factor combinations were plated on Matrigel-coated 35-mm plates at 10,000 cells/cm^2 and were maintained in the same media for 72 h. Following this period, cells were harvested to make RNA (RNA-Bee, Tel-Test Inc., Friendswood, TX, USA).

Differentiation Protocol #2

In this protocol, cells were permitted to aggregate in U-bottom 96-well plates (Greiner BioOne #650161), previously treated for 24 h with 10% (w/v) Pluronic F68 (Sigma) which prevents attachment of the cells to the wells.[21] Marmoset iPS cells were dissociated with Accutase. Each well received 3000 cells in 150 μL differentiation medium. Differentiation medium comprised DMEM/F12 with 20% KSR, 10 μmol/L Y-27632 and 1 nmol/L all-*trans* retinoic acid, together with variable concentrations of DMH1 (dorsomorphin analog, Millipore, Billerica, MA, USA), SB431542 (Selleck), BIO ([2′Z,3′E]-6-bromoindirubin-3′-oxime; Enzo, Farmingdale, NY, USA) and PD0325901 (Selleck). On days 2 and 4, each well received an additional 75 μL of medium containing the same combination of factors as originally added on day 0. After six days, cells were harvested to make RNA. Levels of mRNAs were determined using standard quantitative polymerase chain reaction techniques and are reported as cycles versus β-actin, using marmoset gene-specific primers.

Results

Small molecule/protein factor combinations for neuroectodermal differentiation of marmoset iPS cells

We based our search for optimal differentiation cocktails on the work of Tsutsui *et al.*[15] The approach is illustrated in Figure 2. As in Tsutsui *et al.*, we started with level '3' of each factor (Table 1) and then varied the concentrations systematically. The starting concentration '3' is a concentration based on values shown to be effective in the literature, although those experiments typically did not systematically investigate different concentrations. The other levels (1, 2, 4, 5, etc.) are based on the concentration '3' by decreasing or increasing the concentration by a factor of square root of 10, except for retinoic acid, which was increased or decreased by a factor of 10. Therefore, the combination 33333 for protocol #1 comprises 0.1 μmol/L retinoic acid, 1 μmol/L SB431542, 1 μmol/L dorsomorphin, 20 ng/mL FGF2, and 1 μmol/L SAG. We systematically varied each factor by one step up and down in each iteration; therefore with combinations of five molecules we used 11 combinations in each round.

In protocol #1 we tested the combinations of five factors (Table 1) beginning with concentrations ('3') that were previously determined to be useful in neuroectodermal

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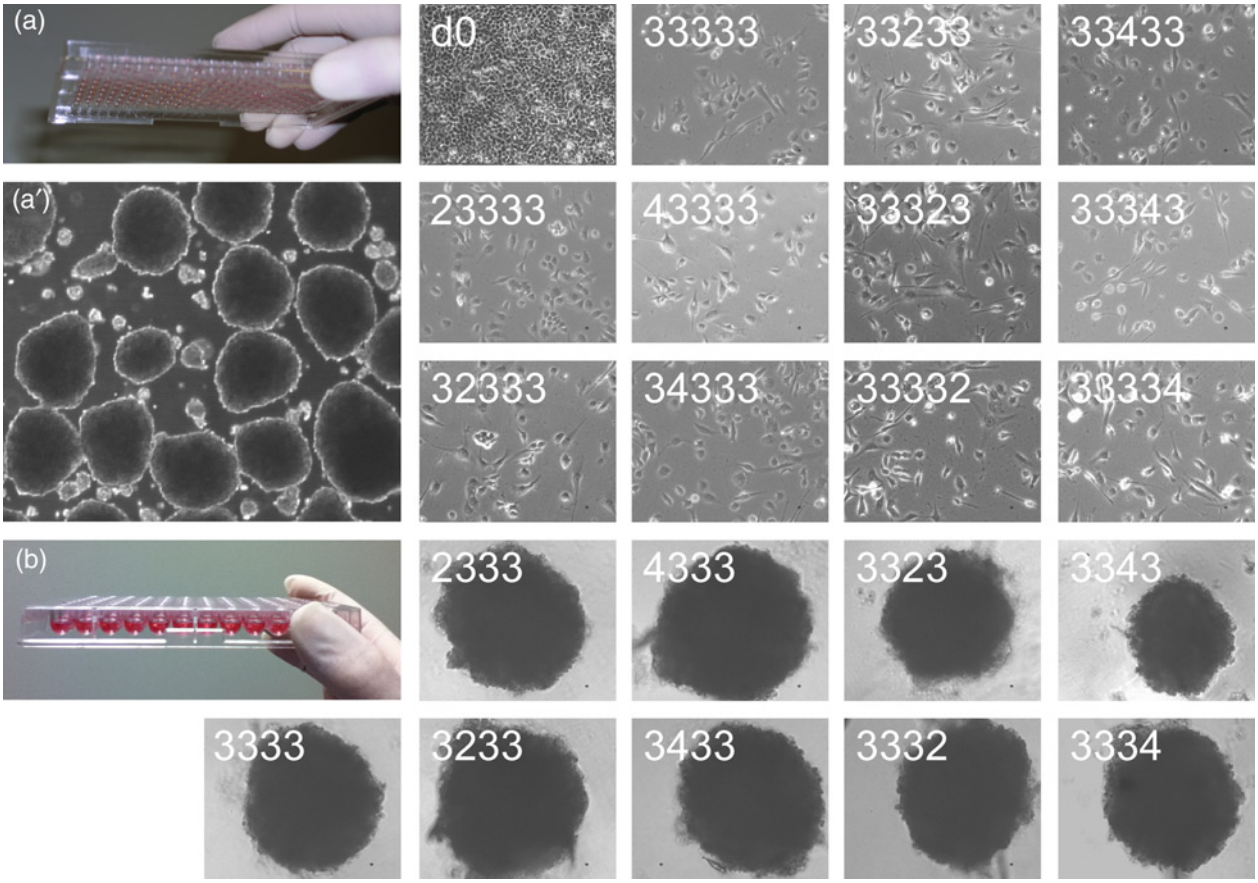

Figure 3 Representative images of marmoset iPS cells subjected to two different differentiation protocols using chemical/factor combinations. In (a), a hanging-drop plate in which EBs are formed from dissociated iPS cells is shown (protocol #1). As described in Methods, EBs were collected after three days and dissociated for subsequent plating. (a)' Shows the appearance of the collected EBs before dissociation. During the subsequent monolayer stage, the cells were treated with small molecule combinations. The cell morphology at the end of the protocol (6 days) is shown above for 11 different combinations (see Table 1). 'd0' refers to day 0, the appearance of the marmoset iPS cells in feeder-free conditions before dissociation. In (b), a 96-well plate is shown in which EBs were formed and simultaneously treated with small molecule combinations for six days (protocol #2). The appearance of representative EBs for each combination used is shown at the end of the protocol (6 days). iPS cells, induced pluripotent stem cells; EBs, embryoid bodies. (A color version of this figure is available in the online journal)

differentiation in the literature. At each round, each of the five factors was varied by one increased step and one decreased step (Figure 2). At each round, cells were harvested for RNA preparation followed by qPCR of a set of genes that were selected based on prior knowledge that they may be informative in differentiation in this lineage. Based on an assessment of the levels of mRNA for these genes a 'winner' combination of the round was chosen. In these experiments we assessed 'winners' by an overall assessment of the set of genes. In a future expansion of this work, this could potentially be done mathematically; however, at the present stage of development of this approach we do not have sufficient information to formulate a purely mathematical method for 'winner' selection.

We tested two different protocols for assessing combinations of drugs and protein factors in differentiation. Both protocols are based on schemes for the differentiation of pluripotent cells in the neuroectodermal lineage.[22] Both are also based on the concept that the initiation of differentiation in this lineage is enhanced by formation of a three-dimensional structure. These aggregates, if allowed to remain as three-dimensional structures, will eventually form mature embryoid bodies (EBs). The two protocols differ in the following respect. In the first, a mass production of early EBs under standardized conditions for three days was followed by dissociation of the cells and individual treatments of the plated cells (Figure 3). Thus, no variations in treatment occurred over the first three days, but variations occurred in the three days the cells spent as monolayer-plated cells. The mass production of the EBs, using commercially available 384-well hanging-drop plates, is efficient, but is not convenient for assessing the effects of varying treatments during the three days of hanging-drop EB formation, as opposed to the subsequent plated cell step (total 6 days protocol). In protocol #2, variations in the treatments during EB formation are possible because the cells were allowed to form EBs in 96-well plates. In this case the format is adaptable to variations in the treatment of the cells during EB formation. Medium containing fresh additives was re-added to the wells at intervals. In this case the same additions were made over the period of the experiment, but this could be varied. Re-addition is not possible using hanging-drop plates, as the medium for the drops cannot be changed during EB formation and the time is limited to three days because of concerns about excessive evaporation over longer time periods.

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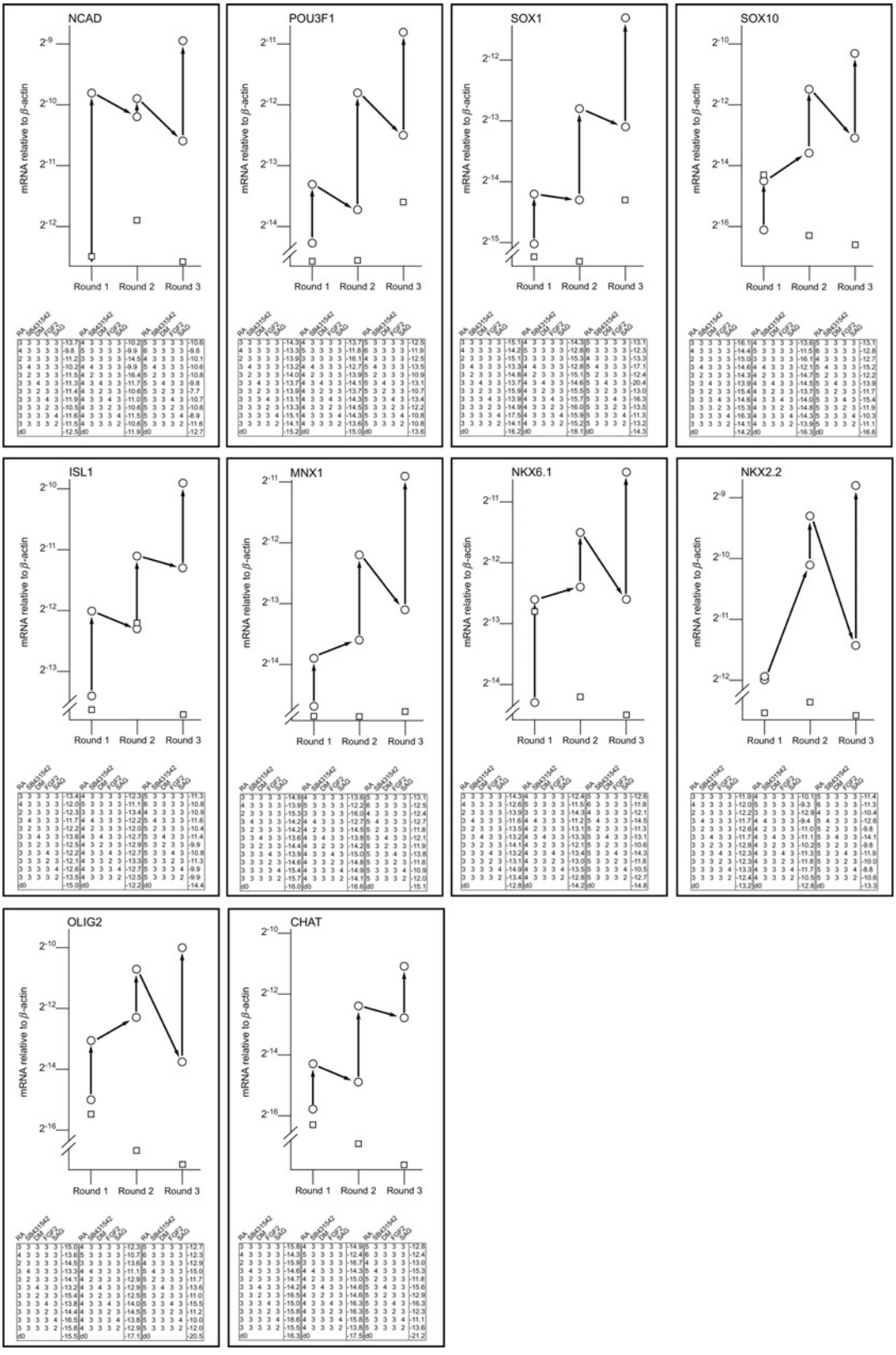

Figure 4 Levels of neuroectodermal gene mRNAs resulting from differentiation of marmoset induced pluripotent stem cells via protocol #1. For each gene, the tables list the mRNA levels as measured by qualitative polymerase chain reaction for various chemical/factor combination in each of three rounds. The values in the tables are given as $C_{t(\beta\text{-actin})} - C_{t(\text{gene})}$. The numeric code is explained in the text and is shown in Table 1. The day 0 (d0) mRNA levels, in the undifferentiated cells, are plotted as open squares. The mRNA levels in the cells at six days are plotted as open circles, both for the starting combination in each round and for the 'winner' combination for that round. For round 1, the open circles show the mRNA levels for the 33333 combination and the 'winner' combination, 43333; for round 2, levels for 43333 and the 'winner' combination, 53333; for round 3, levels for 53333 and the 'winner' combination, 53334

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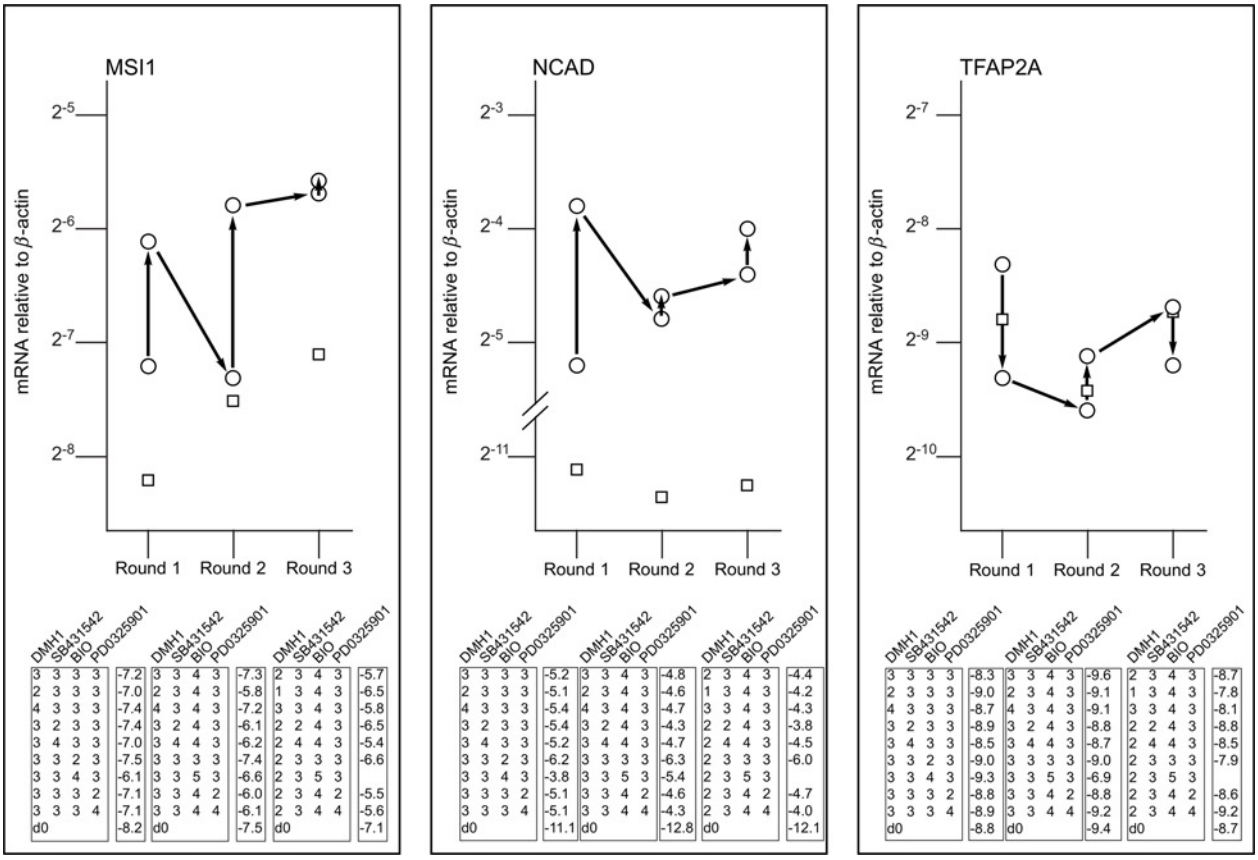

Figure 5 Levels of neuroectodermal gene mRNAs resulting from differentiation of marmoset induced pluripotent stem cells via protocol #2. For each gene, the tables list the mRNA levels as measured by qualitative polymerase chain reaction for various chemical/factor combination in each of three rounds. The values in the tables are given as $C_{t(\beta\text{-actin})} - C_{t(gene)}$. The numeric code is explained in the text and is shown in Table 1. The day 0 (d0) mRNA levels, in the undifferentiated cells, are plotted as open squares. The mRNA levels in the cells at six days are plotted as open circles, both for the starting combination in each round and for the 'winner' combination for that round. For round 1, the open circles show the mRNA levels for the 3333 combination and the 'winner' combination, 3343; for round 2, levels for 3343 and the 'winner' combination, 2343; for round 3, levels for 2343 and the 'winner' combination, 2344

Protocol #1: Hanging-drop plate protocol

In the hanging-drop protocol, we screened five small molecules/factors for their effects on expression of 10 genes (Figure 4). These genes are involved in neuroectodermal differentiation generally, and more specifically in motor neuron differentiation.[23,24] For most of the genes studied, there was a rapid increase in the extent of increase in mRNA levels over two rounds. Most of the improvement was in the range of 2^3–2^4, i.e. eight-fold to 16-fold increases over the level of induction observed with the starting combination ('33333'); however, some exceeded this improvement (e.g. *SOX10*, which showed an overall improvement in induction of 2^6, i.e. 64-fold, and *OLIG2*, which showed an overall improvement in induction of 2^5, i.e. 32-fold).

Protocol #2: 96-well plate protocol

The results of the approach in the 96-well protocol (Figure 5) showed generally small improvements over the starting combination ('3333'; Table 1). 'Winner' combinations were much less obvious than in protocol #1. *MSI1* (musashi) mRNA increased by about three-fold, comparing round 3 versus the starting combination. *NCAD* (N-cadherin) showed a two-fold improvement. *TFAP2A* was generally unchanged, which appeared to be because this mRNA is not responsive to the combinations of small molecules used in these tests.

Discussion

These studies address the problem of optimizing the differentiation of pluripotent cells for cell therapy, including models of autologous cell therapy in NHPs. While protocols for pluripotent cell differentiation have been evolving rapidly, systematic approaches to optimizing differentiation have so far been uncommon. We propose that systematic approaches, similar to that described here, will be necessary in order to avoid the twin problems of low rates of differentiation into desired lineages and protocols that take very long time periods. In this study we used combinations of molecules (drugs and protein factors) in an iterative scheme, with the aim of improving neuroectodermal differentiation of NHP iPS cells. Our aim was to maximize expression of markers of the neuroectodermal lineage using short (6-day) treatment protocols. For future cell therapy, it is desirable to achieve as much differentiation as possible in the desired lineage using drug/factor treatments, thereby reducing dependence on subsequent

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processing to isolate specific desired subpopulations of cells. While cell sorting will almost certainly be required for the future use of cells in experimental cell therapy and in human regenerative medicine, the initial approach should be to optimize differentiation using protocols that are rapid, simple, and scalable.

The results of these studies show that the approach is feasible and simple to implement. We used a straightforward algorithm for iterative testing of small molecule/protein factor combinations. The results showed rapid improvements in neuroectodermal gene induction in a two-stage protocol involving three days of EB formation in hanging-drop culture followed by three days of drug/factor treatment of the dissociated cells in monolayer culture. The improvement was more modest in a six-day protocol in which the cells were maintained throughout as EBs in 96-well plates. The rapid improvement in the case of the hanging-drop protocol was mostly the result of the increase in retinoic acid concentrations over the three rounds of the experiment. Based on the literature, the initial concentration was set at 100 nmol/L, while 10 μmol/L eventually proved to be much more effective. The initial concentration has been shown to be optimal in some systems (e.g.[25,26]) while the higher concentration has been used in others.[27,28] Presumably, because 10 μmol/L is greatly in excess of the physiological concentration of retinoic acid, cross-reaction with another pathway may be involved; further studies are needed to clarify this point. In the case of the six-day EB protocol, the combination initially used may already have been close to optimal, thus reducing the chance of finding a more effective combination. Again, further experience with the system is needed to establish whether this is a common occurrence, and additional factors must be tested in both protocols.

Because the process *in vitro* is intended to recapitulate a series of developmental stages in the embryo, the *in vitro* process may require multiple stages with changing strategies; both different structures (e.g. monolayers or EBs) and differing combinations of chemicals/factors may be needed at different stages of the process. Iterative approaches to optimization could be used at each stage. Because of the complexity of many differentiation protocols, and because the molecular targets of the drugs/factors used are not always known, it is not yet clear whether a simple 'hill climbing' iterative approach to optimizing differentiation will always be appropriate. More complex algorithms might be necessary. This will require enough testing to ensure that the theoretical possibility that the search becomes 'stuck' on a local maximum rather than a global maximum is unlikely. However, if practical experience shows that this happens with some frequency, algorithms should be adjusted to avoid this.

Extensions of this approach can be envisioned in which synthetic mRNAs are used in combinations, essentially employing synthetic mRNAs like other drugs and small molecules.[29] It was already suggested, in the context of transdifferentiation (reprogramming) protocols using transcription factors, that 'a subset of factors may be selected for retesting in the reprogramming assay', i.e. an iterative approach as employed here.[30] By combining optimal small molecules and mRNA treatment, very efficient and rapid differentiation protocols may be feasible for future regenerative medicine studies.

Author contributions: All authors participated in the design and interpretation of the studies, analysis of the data and wording of the manuscript; SLF and ZQ conducted the experiments, AM provided excellent assistance with all aspects of the experiments and PJH was responsible for the overall planning of the experiments and the manuscript. All individuals who made contributions to this study are included as authors. SLF and ZQ contributed equally to this work.

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