

Influence of Initial Seeding Density on Gene Expression during Neuronal Priming

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Abstract

Derivation of neurons from pluripotent stem cells has moved from a niche expertise to a widely used technology, prompting a surge to create novel and timely treatments for neurologic disorders such as Parkinson's disease. However, current methods for derivation of neurons, though widely available, remain inefficient, resulting in sub-optimal yields and purities of potentially therapeutic cells. Optimising cell processing methods to improve yield and purity is necessary and in this work, we assessed the impact of Initial Cell Seeding Density (ICSD) on differentiation of pluripotent cells (human embryonic and human induced pluripotent stem cells) towards a neuronal specification using a recognised laboratory protocol. We found small but significant differences in expression of specific genes related with pluripotency and neuronal lineage commitment associated with ICSD. By reducing ICSD, it may be possible to improve yield from less start material.

Keywords: Neuronal differentiation; Initial cell seeding density; Optimization; Gene expression; Translation; Pluripotent stem cells

Introduction

Regenerative medicine holds promise to treat many currently unmet clinical indications and has seen major change in the last decade, with expectations of the science only increasing with each new discovery and clinical trial undertaken. With these advancements stem cell research has grown from being driven by biological discovery to developing and optimizing processes and protocols to enable their application in the therapeutic arena. As cell therapy process technology develops and researchers look to transplant specific cell types generated using *in-vitro* differentiation protocols, it will become more and more critical to ensure process efficiencies are optimized in order to achieve the scale of production needed to meet the clinical demand.

In order to successfully address the challenges of cell therapy manufacture at the industry scale, it is important to ensure all stages of the whole bioprocess are optimised first at the bench scale. The very first stage of many expansion or differentiation processes involves plating the cells onto an appropriate substrate for attachment and growth. The substrate is critical, so for example human pluripotent cells are often plated onto Mouse Embryonic Fibroblast (MEF) feeders [1,2] or are specially adapted for survival and growth on acellular material such as matrigel [3]. In addition to substrate, Initial Cell Seeding Density (ICSD) can have a significant impact on cell behaviour and is reported to alter proliferation and differentiation responses of various adult cells during 2D culture [4] and in 3D culture for tissue engineering purposes [5]. In the case of pluripotent cells, passaging hESC colonies at lower density of 1:8 resulted in a high degree of spontaneous differentiation versus those passaged at high density of 1:3 [6]. Using mouse 46C ESCs, Veraitch et al. demonstrated that lower seeding density specifically enhanced neuroectoderm differentiation, identified by enhanced Sox1 expression [7].

ICSD is important for a second reason too. Scalable expansion of therapeutic cell lines to achieve necessary clinical quantities is still fraught with difficulty [6] with adherent and suspension culture methods currently being investigated [8,9]. This is not just the case for primary somatic cell populations, where expansion is hampered by limited replicative potential and biological changes accompanying cell

aging [10], but also for pluripotent cell lines that are prone to karyotypic abnormalities with extended periods in culture [11,12].

Attempts to reduce the start material needed to produce a cellular therapeutic will be of substantial benefit to the industry. Therefore, the aim of this study was to determine the impact of ICSD on expression of key genes during neuronal induction of human pluripotent cell lines. We based our work on an established protocols, which use SMAD signalling inhibition along with dopaminergic factors to induce neuronal differentiation [13,14] and mapped the gene expression profile associated with the shift away from a pluripotent state, during neuronal priming and induction of a specialised phenotype over the first 11 days of differentiation.

Methods

Human pluripotent cell lines

Two cell lines were used to assess the effect of ICSD on neuronal priming, the expression of pluripotent markers, early neuronal and mature markers as well as specialized dopaminergic markers: the human induced pluripotent stem cell (hiPSC) line, MSUH001 (a kind gift from Dr Cibelli, Cellular Reprogramming Laboratory, Michigan State University); and the human embryonic stem cell (hESC) line, Shef6 obtained from UK Stem Cell Bank. Both hESC and hiPSC pluripotent cell lines (Figure 1A and 1B) were cultured on Mitomycin-C (1 mg/mL, Sigma-Aldrich, Poole, UK) inactivated MEFs and cultured in Knockout serum replacement medium (KSRM, Knockout-DMEM,

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Received September 22, 2014; **Accepted** December 23, 2014; **Published** December 26, 2014

Citation: Thwaites JW, Ruban L, Mason C, Dalby P, Habib N, et al. (2014) Influence of Initial Seeding Density on Gene Expression during Neuronal Priming. J Bioprocess Biotech 5: 195 doi: [10.4172/2155-9821.1000195](https://doi.org/10.4172/2155-9821.1000195)

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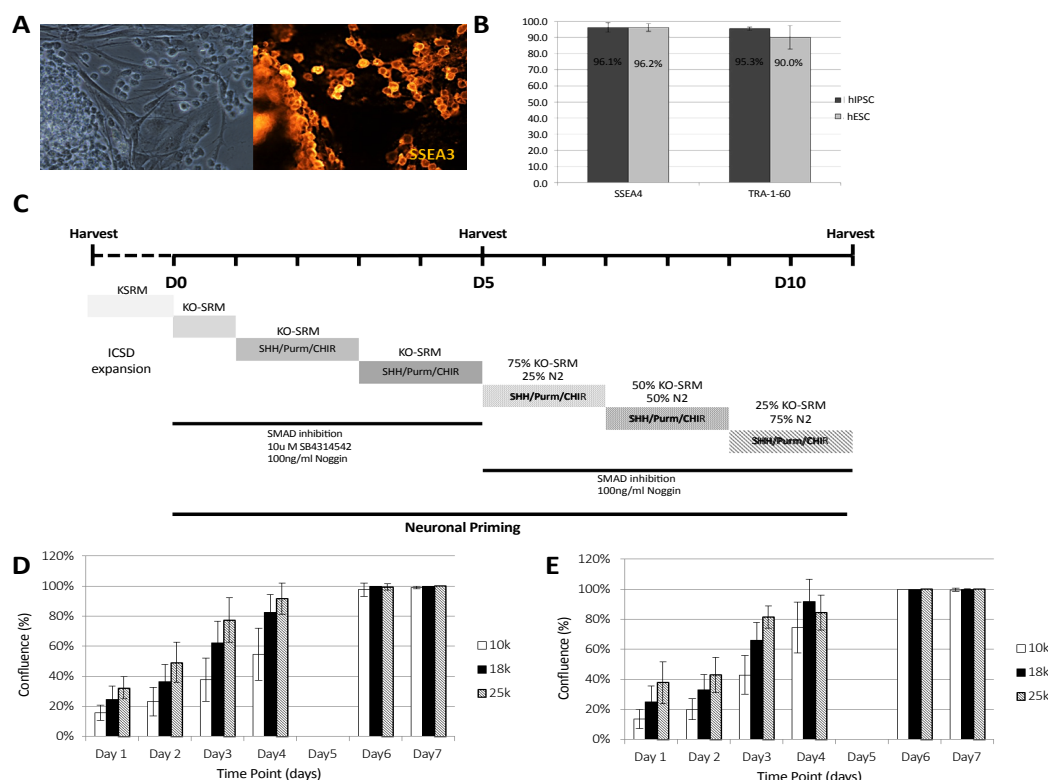


Figure 1: A Phase contrast image of iPS colony (magnification $\times 10$, scale bar = 400 μm) and ICC for Oct4 pluripotency marker. B Graph representing flow cytometry data for hESC and hiPSC culture expression of pluripotent surface markers SSEA4 (n=3) and TRA-1-60 (n=2). Error bars represent SD ± 1 . C Diagram demonstrating the stages of the priming protocol. D Growth profile of the hES cell line over 7 days of culture expansion. E Growth profile of the iPS cell line over 7 days of expansion (Bars indicated Mean $\pm$ SEM).

10% KO-Serum replacement, 1% Glutamax, 1% NEAA and 100mM β -mercaptoethanol (All Invitrogen, Paisley, UK) supplemented with basic Fibroblast Growth Factor (bFGF, 4 ng/mL, Prospec-Tany technogene Ltd. East Brunswick, USA). Cells were mechanically passaged twice weekly and maintained at 37°C, 5% (v/v) CO_2 prior to initiation of the protocol.

Neuronal priming

Priming briefly consisted of the cells being expanded in T25 flasks (Nunc., VWR International Ltd, Leicestershire, UK) in KSRM supplemented with 4 ng/mL bFGF and cultured until colonies compacted. Upon commencement of priming, cells were disassociated into single cell suspension using Tryple Express (Invitrogen) and passed through a cell strainer (40 μm , BD Falcon, Oxford, UK) to ensure a single cell suspension. Cells were resuspended in filter sterilised MEF-conditioned media (CM media, KSRM supplemented with 4 ng/mL bFGF incubated with inactivated MEFs for 3 days, removed and filter sterilised) supplemented with bFGF (10 ng/mL) and ROCK-inhibitor (10 μM , Y27632, Stemolecule, Miltenyi Biotec, Surrey, UK,) (complete CM media), then plated onto gelatin-coated 6 well plates (Nunc) and incubated at 37°C for 1 hour. Non-adhered cells were removed and the plates rinsed gently with fresh media to remove any remaining non-attached pluripotent cells. Feeders remained adhered to the gelatin-coated surface. The pluripotent cell suspensions were centrifuged at 1,200 rpm for 3 minutes and resuspended in complete CM media, then counted using a haemocytometer. Human pluripotent cell populations were plated in duplicate into wells pre-coated with Matrigel (BD, Oxford, UK) 24 well plates (Nunc) at ICSD of 10 k/cm², 18 k/cm² and

25 k/cm² in complete CM media. Media was replaced after 24 hours, removing Y-27632 supplement.

On day 3 of the population expansion when three clear confluence densities were observed across the investigated ICSD, the media was changed to initial differentiation media, KSRM supplemented with noggin (500 ng/mL, R&D Systems, Abington, UK) and TGF- β inhibitor (10 μM , SB431542, Miltenyi Biotec). Day 1 media was supplemented with Sonic Hedgehog (100 ng/mL SHH) (R&D Systems) and Purmorphamine (2 μM , Miltenyi Biotec). Media was maintained until day 3, when CHIR99021 (3 μM , Miltenyi Biotec) was added. After an additional 2 days, the media was modified to contain 25% N2, neuronal supplement (PAA, Somerset, UK) in addition to factors: Noggin, SHH, Purmo and CHIR, but excluding SB431542. N2 concentration was incrementally increased over the following 6 days (to day 11) through 25%, 50% and 75% of the media constituent.

Confluence analysis

Analysis of confluence was performed using a phase contrast microscope (Nikon, Eclipse TI-E) with an inbuilt automated platform. 20 random images (10 $\times$ magnifications) were taken of each duplicate well. Images were processed using the PHANTAST software [15]. Briefly, cell regions on phase contrast microscopy images are characterised by high variations in intensity as opposed to image background regions that are mostly homogeneous. These regions of high variability were detected using a local contrast filter. Image confluence was computed as the ratio of the number of cell pixels to the total number of pixels. Images of biological duplicates were processed and averages were taken

of all readings for a given ICSD. Readings were taken over a time course up to 7 days, to ascertain the growth profile of the two cell lines and to determine the appropriate time point to commence priming the cells.

Quantitative Polymerase Chain Reaction

Cells were mechanically harvested from the culture well and processed for total RNA extraction using RNAqueous micro kit (Applied Biosystems, Warrington, UK), according to manufacturer's instructions. Total RNA content and purity was then quantified using a Nanodrop 1000 spectrophotometer (Thermoscientific, Wilmington, USA). Reverse transcription was achieved using QuantiTect Reverse transcription Kit (Qiagen, Manchester, UK) and gene expression analysis was performed using QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. The QuantiTect primers (all Qiagen) used were: housekeeper genes ubiquitin C (UBC-1-SG), *gapdh* & β -actin; pluripotency markers, *nanog*, *sox2* & *oct4*; epiblast marker, *fgf5*; neuronal markers, *otx2*, *pax6* & *sox1*; and dopaminergic markers, *TH* and *lmx1a*. Expression data was normalised against three housekeeping genes (β -Actin, GAPDH and UBC-1-SG) and calculated relative to day 0. All samples were processed in triplicate.

qPCR data was collected using the BioRad CFX Connect Real-Time System (Bio-Rad, Hertfordshire, UK) and was categorised and processed using CFX Manager Data analysis software (Bio-Rad). Samples were categorised using a number of criteria: biological set (biological replicate number); cell type; technical replicate; target gene and sample name. Relative expression ($\Delta\Delta Cq$) was calculated using the BioRad Gene study programme, normalising target gene expression against housekeeping gene levels. Significance was found when $p \leq 0.05$. Data is shown against a logarithmic scale ($\log_{10}$).

Results

To investigate the effect of ICSD on the expression of target genes through a neuronal priming protocol on pluripotent stem cell populations (Figure 1A-1C) we initially investigated the growth profiles of two human pluripotent cell lines at 3 different ICSDs over a period of 7 days. It was found that both cell lines reached confluence by 6 days at all ICSDs tested (Figure 1D and 1E). In line with expectation, at earlier time points lower ICSD resulted in lower confluence compared with cells seeded at higher ICSD. Therefore, subsequent neuronal differentiation experiments were commenced from day 3 when differences in confluence could be identified.

Expression levels of target genes were assessed at day 0, 5 and 11 following induction of neuronal differentiation. In the case of pluripotency genes, *nanog* were significantly down-regulated ($p < 0.01$) at day 5 and 11 following induction for all ICSDs in hESCs and hiPSCs (Figure 2A and 2D respectively). Similarly, *oct4* was significantly down-regulated ($p < 0.05$) and a further time-dependent effect was observed with the degree of silencing increasing from day 5 to day 11 ($p < 0.01$) in both hESC and hiPSC (Figure 2B and 2E). In both cell lines *sox2* expression increased, though only marginally (Figure 2C and 2F). In terms of differences between ICSDs, only a very marginal increase in *nanog* down-regulation was seen for 10 k/cm^2 and 18 k/cm^2 versus 25 k/cm^2 ICSD ($p < 0.05$) in hESCs (Figure 2A) and conversely small increases in *sox2* up-regulation were seen at 25 k/cm^2 versus 10 k/cm^2 for hESCs ($p < 0.05$, Figure 2C).

Epiblast marker *fgf5* underwent moderate time-dependent up-regulation over the 11 day neuronal induction protocol for only 25 k/cm^2 for both hESCs and hiPSCs (Figure 3A and 3B). Notably, differences in gene expression between different ICSDs were observed,

with a consistent trend of increased *fgf5* expression at 25 k/cm^2 versus 10 k/cm^2 (at day 5 for hiPSCs ($p < 0.05$) and at day 11 for both hESCs ($p < 0.01$) and hiPSCs ($p < 0.05$)).

Next, expression of neuronal genes was assessed to determine whether changes in population-wide gene expression were evident at different ICSDs. There was a positive increase in expression of neuronal markers *sox1*, *pax6* and *otx2* at days 5 and 11 versus day 0 (Figure 4A-4F). Very small, but significant increases in expression of *sox1* (Figure 4A) and *otx2* (Figure 4C) were seen in hESCs at day 11 at the lower seeding density of 10 k/cm^2 versus 25 k/cm^2 ICSD ($p < 0.05$). Similar differences were not seen for hiPSCs. Furthermore, a general increase in gene expression was seen from day 5 to day 11 for hESCs (*sox1* and *pax6* $p < 0.01$, *otx2* $p < 0.05$) but this was not the case for hiPSCs. In fact, a significant decrease in expression of *otx2* ($p < 0.01$) was observed from

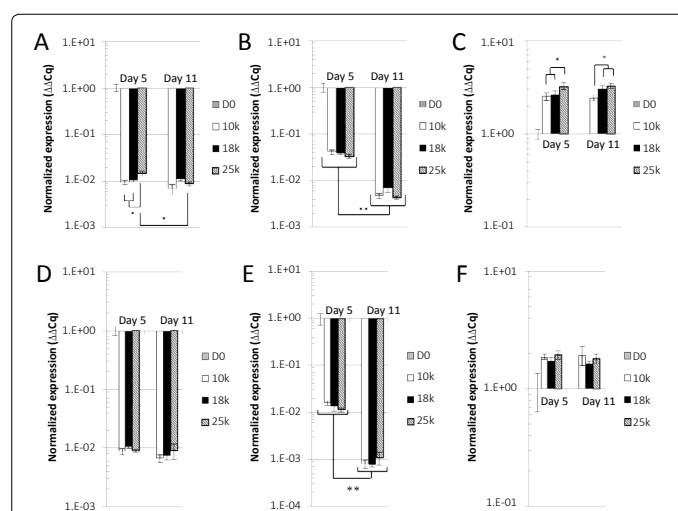


Figure 2: qPCR analysis of the effect of ICSD on pluripotent gene expression over 11 days of neuronal priming. Day 5 and day 11 columns represent *nanog* (A/D), *oct4* (B/E) and *sox2* (C/F) expression relative to day 0 expressions at the different ICSD in hESC and iPSC lines respectively. Expression of both *nanog* and *oct4* was shown to decrease significantly in both hESC and hiPSC. *Oct4* was shown to continually decrease over time whereas *nanog* remained stable. *Sox2* expression was shown to increase slightly in both hESC and hiPSC. ($p < 0.05$ & $**p < 0.01$, Bars represent Mean $\pm$ SEM, $n=3$)

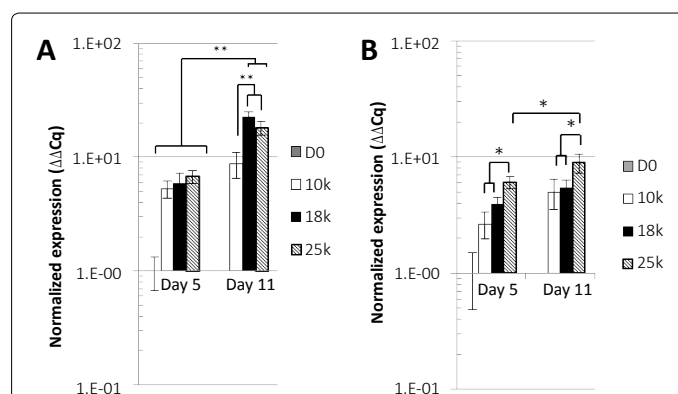
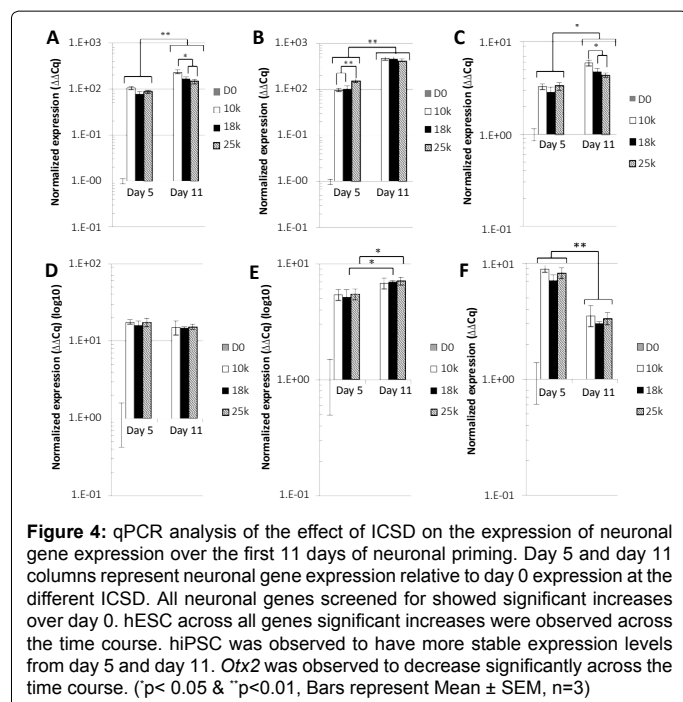


Figure 3: qPCR analysis of the effect of ICSD on hESC and hiPSC *fgf5* gene expression over the first 11 days of neuronal priming. Day 5 and day 11 columns represent *fgf5* expression relative to day 0 at the different seeding densities. hESC *fgf5* expression was shown to increase significantly over time for both 18 k/cm^2 & 25 k/cm^2 . At day 11 10 k/cm^2 was shown to have decreased expression relative to the other two ICSD. hiPSC did not show an increase over time for all ICSD. It was shown that 25 k/cm^2 had significantly greater expression at both time points. ($*p < 0.05$ & $**p < 0.01$, Bars represent Mean $\pm$ SEM, $n=3$)



day 5 to day 11 across all three ICSDs (Figure 4F).

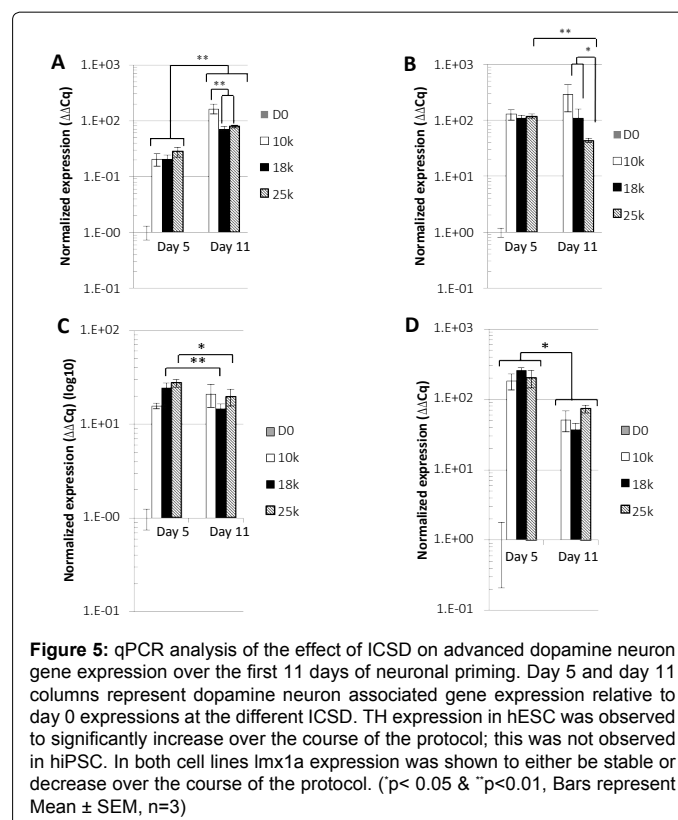
Finally, expression of specialized dopaminergic neuronal genes was assessed to determine whether induction of these late markers was at all affected by ICSD over the first 11 days. The markers commonly used for dopaminergic neurons, TH and *lmx1a*, were significantly up-regulated at both days 5 and 11 relative to day 0 (Figure 5A-5D). TH gene expression in hESC consistently increased over time irrespective of ICSD ($p < 0.01$, Figure 5A). However, for hiPSCs there was a drop in TH gene expression for 18 k/cm^2 and 25 k/cm^2 ($p < 0.01$ and $p < 0.05$ respectively) from day 5 to day 11 (Figure 5C). Interestingly, for hESCs both TH and *lmx1a* were expressed at significantly greater levels at ICSD of 10 k/cm^2 versus the higher ICSD of 25 k/cm^2 (TH $p < 0.01$ and *lmx1a* $p < 0.05$) at day 11 (Figure 5A,5B). Notably hiPSC showed a significant time dependent drop from day 5 to day 11 (Figure 5D) across all ICSD.

Discussion

Multiple bioprocess parameters can impact on the responses of human cells during processing [7]. ICSD and degree of confluence at the point of initiation of differentiation protocols should also be considered. In this study, three different ICSDs were studied and the time at which neuronal priming was initiated was determined according to the point at which three different levels of in-culture density were seen. At the start of the neuronal priming protocol, SMAD inhibitors were supplemented to the differentiation media [13,14] and were observed to have no effect of population viability.

Over the course of the priming protocol pluripotency markers and markers of the neuronal lineage were assessed. It was found that consistent, significant drops in pluripotency markers *oct4* and *nanog* occurred for both hESC and hiPSC cultures (Figure 2). This would indicate that the cultures shifted from expressing genes associated with maintained pluripotency towards a differentiated state. Moreover, it was accompanied by a concurrent increase in expression of *fgf5* (Figure 3), indicating differentiation to the late epiblast stage and early ectoderm phase [16]. Increased expression of *sox2* (Figure 2) is

also associated with acquisition of a neuronal progenitor phenotype [17] and further supports a shift away from pluripotency towards the ectodermal/neuronal lineage. The higher seeding density of 25 k/cm^2 led to higher population-wide expression of *fgf5*, which may seemingly suggest that a higher ICSD results in a more efficient differentiation for both hiESCs and hiPSCs. However, combined with the increased expression of later neuronal markers at lower ICSD, it is possible that in the higher ICSD investigated, a higher proportion of the population stay in this late epiblast/early ectoderm stage, whereas at 10 k/cm^2 , stagnated expression of *fgf5* possibly indicates differentiation past the epiblast stage. The suggestion that lower *fgf5* expression in hESCs at 10 k/cm^2 indicates a move down the ectoderm lineage is enforced by the significant difference in *sox1* expression observed at day 11 (Figure 4A). This trend was repeated for *otx2* at 10 k/cm^2 where higher expression was noted compared to the other ICSDs (Figure 4C), possibly indicating more rapid differentiation towards mature neuronal states at lower ICSD. This supports previous observations in mouse ESCs, where increased neuroectoderm differentiation was noted at low ICSD [7]. Expression of neuronal genes by hiPSCs was not as prominent as those observed for hESCs, with relative expression of the majority of genes screened never showing greater than $1 \times 10^2 \Delta\Delta Cq$ increase. This may indicate a reluctance of the cell type to move down the ectoderm lineage. In the case of hiPSCs, the reduction in *otx2* expression over time (Figure 4F) would suggest that differentiation is not toward a mid-neuronal tube phenotype. This assumption in hiPSCs is backed up by the expression profile of TH and *lmx1a* (Figure 5C,5D) with TH showing significantly reduced expression at both 18 and 25 k/cm^2 and *lmx1a* expression showing a significant drop across all ICSDs within the hiPSC populations. In the case of hESCs, the trend for TH and *lmx1a* (Figure 5A,5B) was similar to that found for the other neuronal genes; the lowest ICSD of 10 k/cm^2 led to significantly greater expression of TH and *lmx1a* than the higher ICSDs. The highest ICSDs showed



the only significant drop in *lmx1a* (Figure 5B) expression observed in hESCs, potentially reiterating that lower ICSD and level of confluence at initiation of differentiation can improve the efficiency of lineage specification needed to drive towards a dopaminergic neuronal cell phenotype.

Conclusion

In the current study, low ICSD resulted in overall improved neuronal differentiation in hESCs, with greater expression of TH and *lmx1a* indicating a potential move towards a dopaminergic lineage. hESCs had a greater propensity to differentiate towards an ectoderm cell type than MSUH001 hiPSCs, when assessing population-wide gene expression. Even though substantial differences are evident between different pluripotent cell lines, this data still provides evidence that ICSD can affect target gene expression and hence differentiation during neuronal lineage commitment.

Acknowledgements

J.T. acknowledges support from the Engineering and Physical Science Research Council (EPSRC) Industrial Doctoral Training Centre in Bioprocess Engineering Leadership (EP/G034656/1) and the UK Stem Cell Foundation. I.W. is supported by a Priority Research Centers Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (grant No.2009-0093829).

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