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Rational bioprocess design for human pluripotent stem cell expansion and endoderm differentiation based on cellular dynamics

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Abstract

We present a predictive bioprocess design strategy employing cell- and molecular-level analysis of rate-limiting steps in human pluripotent stem cell (hPSC) expansion and differentiation, and apply it to produce definitive endoderm (DE) progenitors using a scalable directed-differentiation technology. We define a bioprocess optimization parameter (L; targeted cell Loss) and, with quantitative cell division tracking and fate monitoring, identify and overcome key suspension bioprocess bottlenecks. Adapting process operating conditions to pivotal parameters (single cell survival and growth rate) in a cell-line specific manner enabled adherent-equivalent expansion of hPSCs in feeder- and matrix-free defined-medium suspension culture. Predominantly instructive differentiation mechanisms were found to underlie a subsequent 18-fold expansion, during directed differentiation, to high-purity DE competent for further commitment along pancreatic and hepatic lineages. This study demonstrates that iPSC expansion and differentiation conditions can be prospectively specified to guide the enhanced production of target cells in a scale-free directed differentiation system.

Keywords

bioprocess; pluripotent stem cells; differentiation; endoderm; expansion

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Introduction

The regulatory functions of endocrine and exocrine tissues of the endoderm-derived pancreas and liver are essential for human health. In the United States type I diabetes, resulting from loss of the insulin-producing pancreatic beta cells, affects an estimated 1 million people, with an annual economic cost of \$14.9 billion (Dall et al., 2009), while liver disease leads to approximately 45,000 deaths per year (Kim et al., 2002). These tissues are consequently of great interest as targets of regenerative therapies and, in the case of the pancreas, a model system for human islet transplantation already exists in the Edmonton protocol (Shapiro et al., 2000). While success in basic research often consists of the generation of a few thousands to millions of cells of a given type, therapeutic interventions such as transplantation of *in vitro* derived islets are expected to require on the order of 10^8 to 10^9 or more cells per treatment, per patient (Street et al., 2004). Herein we develop a cell-generation strategy, based on systematic investigations of the cellular dynamics and mechanisms of suspension-based differentiation processes, and apply it to the scalable production of PSC-derived definitive endoderm (DE) progenitors.

Substantial effort has been expended to engineer approaches to increase the numbers of PSC available for differentiation (reviewed in (Azarin and Palecek, 2010; Kirouac and Zandstra, 2008)). Ultimately these technologies rely on either providing increased surface for adherent cell growth (microcarrier approaches)(Storm et al., 2010) or empirically specifying the formation of suspension aggregates, aiming for sufficiently rapid aggregation to support PSC survival, while avoiding the formation of excessively large aggregates that inhibit cell growth (Kehoe et al., 2010; Zweigerdt et al., 2011).

Similarly intense research has provided proof-of-concept for the production of hepatic and pancreatic cell types from PSC (Cai et al., 2007; Chen et al., 2009; D'Amour et al., 2006; Kroon et al., 2008; Liu et al., 2011; Si-Tayeb et al., 2010). A microcarrier-based approach to integrated hPSC expansion and endoderm differentiation recently reported conditioned medium- and FBS-dependent yields of 0.4 endoderm cells per input hESC (Lock and Tzanakakis, 2009). While these efforts are promising, generation of functionally appropriate cells in the quantities that will be required for therapeutic interventions has remained elusive.

We hypothesized that the systematic design of expansion / differentiation processes would result in significant improvements in yield and scalability over typically employed empirical approaches. We enabled this concept via quantitative measurements of PSC survival, proliferation and differentiation, using differentiation efficiency and target cell yields to score process improvements. We applied scale-independent microwell-suspension technologies (Ungrin et al., 2008) to reduce experimental variability and enable population-level measurements as proxies for single cell analyses. In contrast to conventional stirred-suspension approaches, in this system the aggregates are held in individual microwells (without adhering to the adjacent surfaces). For simplicity we will use the term “suspension culture” to refer to this substrate-independent static suspension culture system. To undertake quantitative studies of cellular dynamics, we defined a novel cell therapy bioprocess optimization parameter (L ; targeted yield Loss) and employed it with quantitative cell division tracking and fate monitoring to hPSC expansion and differentiation. We demonstrated that primarily instructive (as opposed to selective) mechanisms underlie cytokine-driven hPSC endoderm commitment. Output cells were C-KIT+/CXCR4+/CD31- by flow cytometry, and significantly upregulated SOX17, FOXA2, Cerberus and GATA3 but not PDGFRA, KDR, SOX7, FOXA3 or SOX1. Importantly, bioprocess-generated cells remained competent for subsequent pancreatic and hepatic differentiation. Cell losses during differentiation generally resulted from non-selective cell survival-compromising

manipulations, and could be predictively overcome using -size control and stage-specific medium optimization. Our integrated expansion (>12-fold every 4 days; sustainable over more than 5 weeks) and differentiation (>18 output cells per input hPSC) system is capable of generating the quantity of endoderm progenitors required to support pre-clinical studies.

Materials and Methods

Aggregate formation

Microwells (AggreWell™, StemCell Technologies Inc, Vancouver, BC, or custom variants) were loaded with 0.4 (24-well format) or 2 mL (6-well format) of 5% w/v Pluronic F127 (Sigma, cat# P2443), centrifuged for 2 minutes at 1,740 x g to remove air bubbles, and incubated for at least 30 minutes at room temperature. After Pluronic coating, microwells were washed once in PBS, loaded with either 0.4 or 2 mL of the selected aggregate formation medium, and again centrifuged for 2 minutes at 1,740 x g. Cells were dissociated with TrypLE (Invitrogen, cat# 12563-029), and passed through a 40 micron filter (BD Falcon, cat# 352340) to remove any remaining clumps. Required cell numbers, determined by multiplying the desired cluster size by the number of microwells per well (1,200 for 24-well format, 6,000 for 6-well format), were suspended in 0.4, 2 mL or 3mL medium and added to the wells, taking care to achieve a homogeneous suspension across the well. The plate was then centrifuged for 5 minutes at 200 x g, and incubated overnight to form aggregates.

Cell culture (aggregate suspension expansion)

Uniform aggregates were formed in growth medium (HES2 cells: 78% DMEM-F12 (Gibco, cat# 11330) containing 1 x penicillin/streptomycin (Invitrogen, cat# 15140-148) and 2mM Glutamax (Invitrogen, cat# 35050-061, 20% Knockout serum replacement (KOSR, Invitrogen, cat# 10828-028), 100µM β-mercaptoethanol (Sigma-Aldrich, cat# M7522), 100 µM non-essential amino acids (NEAA, Invitrogen, cat# 11140-050), supplemented with 20ng/mL FGF2 (Peprotech, cat# 100-18B); H9 cells: as for HES2 cells except no antibiotics were used and FGF2 was added at a final concentration of 4ng/mL). Medium (3mL) was supplemented with 10µM Y27632 (EMD Chemicals, cat# 688000) during the first day of aggregate formation, and replaced with growth medium daily.

Cell culture (monolayer)

Cells were cultured on an irradiated mouse embryonic fibroblast (MEF) feeder layer as described elsewhere (Thomson et al., 1998), in KO-DMEM (Invitrogen, cat# 10829-018), 20% KOSR, 100 µM NEAA, 2 mM Glutamax, 100 µM β-mercaptoethanol with 4 ng/mL FGF2. In all cases, efforts were made to ensure even seeding of hPSC across the well, relatively uniform colony size, and an absence of large clumps of cells.

Carboxyfluorescein succinimidyl ester (CFSE) staining

Cells were dissociated to single cells, and incubated in the presence of 10µM CFSE (Invitrogen, cat# 293-VE-050) for 10 minutes in DMEM-F12, and then washed once with growth medium.

Differentiation to Anterior Primitive Streak (APS) fate

Differentiation employed a base differentiation medium (BDM) consisting of X-Vivo10™ (BioWhittaker cat# 04-380Q) containing 1x Glutamax (Invitrogen, cat# 35050061), 50 µg/mL Ascorbic acid (Sigma, cat# A4544), and 39 ppm Monothioglycerol (Sigma, cat# M6145), and was carried out at 37 °C in a humidified incubator under 5% O₂ and 5% CO₂. In the initial protocol, cellular aggregates were formed in 200 micron microwells in BDM

with 10ng/ml BMP4 (R&D Systems, cat# 314-BP) for 24h, and then extracted and transferred to BDM with 0.5 or 0.25 ng/mL BMP4, 2.5 ng/mL FGF2 and 100 ng/mL Activin A (R&D Systems, cat# 338-AC/CF) for a further 72h. Finally, they were re-fed with BDM with 0.5 or 0.25 ng/mL BMP4, 5 ng/mL FGF2, 100 ng/mL Activin A and 10 ng/mL VEGF (R&D Systems, cat# 293-VE-050), incubated a further 24-48h, and harvested for analysis. Under the optimized protocol, the above sequence was maintained, however hPSC were first clustered in maintenance medium for 24h, and rather than being extracted, were cultured in the 400 micron microwells in which they were formed for the entire duration of the process. Plating 10^5 cells / cm^2 on gelatin for 48 hours in BDM with 0.25 ng/mL BMP4, 5 ng/mL FGF2, 50 ng/mL Activin A and 10 ng/mL VEGF resulted in maturation to definitive endoderm expressing maximal SOX17 and FOXA2.

Differentiation to pancreatic fate

Cells were passed through successive stages of differentiation according to the protocol of Nostro and co-workers, as published elsewhere (Nostro et al., 2011).

Differentiation to hepatic fate

Cells were passed through successive stages of differentiation according to the protocol of Cai and co-workers, as published elsewhere (Cai et al., 2007).

Flow Cytometry

Antibodies, suppliers and working dilutions used in flow cytometric analysis are listed in Supplementary Table S1.

Quantitative RT-PCR

Total RNA was extracted from the cells by homogenization in Trizol Reagent (Invitrogen cat# 15596-026) and extraction with chloroform followed by precipitation with iso-propyl alcohol as per the manufacturer's instructions. Purified RNA was used to generate cDNA using Superscript-III reverse transcriptase (Invitrogen, cat# 18080-093) as per the manufacturer's instructions, which was used in PCR reactions with iQ-SYBR-green master mix (BioRad, cat# 170-8882) or Power SYBR Green PCR Master Mix (Applied Biosystems, cat# 4368702). Relative expression of various marker genes was determined by delta-delta Ct method with the expression of beta-actin as internal housekeeping reference in comparison to undifferentiated hESC controls. INS levels were at or below the limit of detection in input cells, thus lower bounds for INS upregulation are reported, and the reported error does not incorporate error in the measurement of INS levels in input cells as this value is not available. The primer sequences are listed in Supplementary Table S2.

Statistical analysis

Statistical significance of differences between conditions was assessed using the non-parametric Mann-Whitney U test. Analyses were performed in Minitab 15, MATLAB 2009, and R version 2.10.1 (2009-12-14). Standard error propagation methods were employed as appropriate.

Results

Cellular-dynamics analysis yields insight into mechanisms of hPSC differentiation

We utilized our scalable microwell-based technology to generate uniform size-controlled cellular aggregates (Ungrin et al., 2008) (also referred to as spheroids or microtissues) (Markway et al., 2010) (Fig. 1A). Aggregate suspension culture approaches have the advantages of high density, scalability and lack of a requirement for expensive and / or

undefined surface or matrix components. We reasoned that losses in target cell yield would bracket our culture parameter space; the minimal aggregate size being a function of hPSC survival when dissociated into single cells (Amit et al., 2000), and the maximal aggregate size being due to either transport limitations (Sherar et al., 1987), or off-target endogenous signaling events (ten Berge et al., 2008; Itskovitz-Eldor et al., 2000). We therefore imposed control over aggregate size and homogeneity at both input and output.

Definitive endoderm is the antecedent to the gut tube and its derivatives, including the liver and pancreas, and is an intermediate step in the staged differentiation of PSC to these clinically important tissues. It is characterized by expression of FOXA2, Cerberus, GATA3 and SOX17 in the absence of the primitive endoderm marker SOX7 (D'Amour et al., 2006; Kroon et al., 2008; Sherwood et al., 2007). We started with an Activin A-based protocol for differentiation of hPSC to the immediate precursor of DE, anterior primitive streak (APS) (Nostro et al., 2011), which is assessed by co-expression of C-KIT (CD117) and CXCR4 (CD184) (D'Amour et al., 2005), in order to lay a foundation for clinical scale production for cells of this lineage. The process resulted in a yield of 0.5 ± 0.3 C-KIT⁺CXCR4⁺ cells per input hESC after a six day differentiation, via unknown cellular mechanisms (Fig. 1B).

During differentiation, the yield of a desired cell type results from a convolution of cell survival, proliferation, and differentiation. As causes and timing of cell loss during PSC expansion and differentiation are poorly understood, it is unclear whether differentiation proceeds according to an instructive model, where equipotent cells take direction from exogenous signals; or via a selective process, where only a subset of the input population is competent for a given fate (Enver et al., 1998; Suda et al., 1983). Under the former model, losses indicate targets where yield may be improved, while in the latter, selective elimination provides an upper limit on theoretical production from a given input population. The occurrence and timing of cell loss is therefore also informative, permitting discrimination between different potential cellular population dynamics models that give similar overall yield (Fig. 1B, i-iii). To determine where efforts to improve yield could most effectively be focused, we defined the targeted yield loss parameter L:

$$L_i(C, \Delta PD) = \Delta PD - \log_2 \left(\frac{C_i}{C_{i-1}} \right) \quad \text{Eqn. (1)}$$

where for a given observation *i*, *C* is the number of live cells counted, and *PD* is the cumulative number of population doublings that have occurred. Measurements of *PD* were obtained using division tracking cytometry, where live cells stained with a pulse of Carboxyfluorescein succinimidyl ester (CFSE) (Ko et al., 2007) lose fluorescence intensity as they divide and the dye is partitioned equally between daughter cells (Figs. 1C; S3). The value of *L* at a given time point is thus the number of additional cell population doublings that would be obtained if the cells lost in the preceding interval were recovered (for example, *L*=2 signifies a 2²-fold loss in cell yield due to death or off-target differentiation – Fig. 1D).

To identify bottlenecks in PSC differentiation to definitive endoderm we monitored cell proliferation (Fig. 1E), cell output, and differentiation status over a six-day aggregate suspension HES2 differentiation culture, and calculated the *L*-parameter at each day. The resulting *L* profile (Fig. 1F) suggested that differentiation followed a sub-population outgrowth model, with a wave of initial cell death (Fig. 1B-iii). Elevated *L* values at the end of the process also suggested a second, smaller wave of cell yield loss. This profile indicated that substantial increases in yield could be expected if the initial wave of cell death could be prevented (for an instructive differentiation model), or if a specific competent sub-population could be isolated (for a selective differentiation model).

Widespread endoderm competence in hPSC is supportive of an instructive mechanism

Hypothesizing that differentiation followed an instructive model, we predicted that non-specifically enhancing cell survival would enhance overall yield of C-KIT⁺CXCR4⁺ differentiated cells. We further reasoned that physico-chemical limitations on access to diffusible factors including oxygen, metabolites and growth factors define an upper limit to aggregate size, and that losses in theoretical yield at later stages of the differentiation process would result if this limit were exceeded. Accordingly, we focused on enhancing viability over the first 24h, while minimizing long-term accumulation of inter-aggregate variability. By maintaining consistent aggregate size, average terminal aggregate size may approach an upper limit as closely as possible. In order to prevent inter-aggregate fusions we had observed in suspension culture (data not shown), we performed the entire process - including full medium exchanges - within the microwells in which the aggregates were formed. To allow space for aggregate growth, we also increased microwell size from 200 to 400 microns (Fig. 2A). The combined effects of cluster-size optimization, formation of aggregates in the same medium in which the cells were cultivated and inclusion of the ROCK inhibitor Y27632 (10 μ M) during the first day of aggregate formation, which was previously shown to enhance survival of dissociated hESC (Watanabe et al., 2007), resulted in 36-fold increase in yield (Fig. 2B). In absolute cell numbers, a 23-fold expansion occurred during differentiation of input PSC population to >80% C-KIT-CXCR4 double positive cells. This represented a yield of 18 target cells (C-KIT-CXCR4 double positive cells) per input PSC. Both expansion and yield improved with decreasing aggregate size, however below a certain size (twenty cells per aggregate) there was a sharp drop in both parameters (Fig. 2C). The significant synergy between aggregate formation in supportive medium, ROCK inhibition, size optimization and use of 400 micron microwells is shown by the dramatic effects of detuning the optimized protocol along any of these axes (Fig. 2D). These results indicate that a substantial portion of the observed cell loss is non-specific, supporting an instructive model of differentiation. We also investigated the competing selective differentiation hypothesis, by flow-sorting for populations expressing high, medium or low levels of SSEA3 or TG30, markers potentially correlated with hPSC expansion and differentiation potential (Enver et al., 2005; Laslett et al., 2007). Neither of these markers was capable of prospectively isolating a population to which endoderm competence was restricted (Fig. S4).

Scalable aggregate culture of hPSC

In order to integrate expansion and differentiation into a single scalable process, we next explored the potential to maintain HES2 hESCs in this system. We examined the effect of aggregate size (60-100- and 200-cell aggregates) on the retention of marker expression over ten serial passages (35 days) in suspension culture in defined maintenance medium (Fig. 3A). Cell growth was dependent on aggregate size and consistent (Fig. S5A) over the 35-day cultures. For all aggregate sizes the percentage of OCT4/SSEA3 double positive cells remained above 85% (Fig. S5B). Net yield of pluripotent cells over this period, defined as the number of OCT4/SSEA3 double positive cells produced per single input cell, was 9.9×10^6 -fold for cells cultured as 60-cell aggregates (Fig. 3A). In subsequent experiments, we observed a unimodal distribution in the yield of OCT4/SSEA3 double positive cells after each four day passage, peaking at 12.4 ± 1.1 output cells for each input cell from clusters initially seeded with twenty cells (Fig. 3B), corresponding to a predicted output of 3.6×10^9 double positive cells over a 35 day culture protocol. Expansion of control adherent cultures on mouse embryonic fibroblast (MEF) feeder cells was independent of presence of the ROCK inhibitor Y27632, and gave lower yields (10.5 ± 3.5 OCT4/SSEA3 double positive cells per input cell every four days; $P = 0.048$, Mann-Whitney U-test) than those observed in the twenty-cell aggregate suspension microwell system (Fig. 3B, **inset**). Importantly, this aggregate based serial expansion could be robustly applied to other pluripotent cells lines,

albeit in a cell line specific aggregate size-dependent manner (Fig. S6). We have furthermore validated that PSC that had been serially passaged through six rounds of microwell-based growth (fold expansions of >230,000 times, equivalent to approximately 7-11 standard expansions (Zweigerdt et al., 2011)) maintained normal karyotypes (consistent with those of MEF-expanded control cells)(Fig. S7). Expanded cells remained responsive to in vitro differentiation signals, and remained capable of producing all three embryonic germ layers in teratoma assays (Fig. S8). Thus, our aggregate-based suspension expansion protocol is capable of producing very large quantities of pluripotent cells in a suspension-based matrix-free manner for use in clinically-relevant differentiation procedures. As a final step in the validation of our integrated expansion differentiation system we confirmed that the OCT4⁺SSEA3⁺ hPSC produced in microwell suspension culture could be directly integrated into the microwell based definitive endoderm differentiation process. Four rounds of serial expansion as 40-cell aggregates followed by differentiation as aggregates under our optimized protocol, starting with ten-cell clusters demonstrated a net production of approximately 65,000 target cells per initial PSC (Fig. 4).

Suspension-produced definitive endoderm is capable of hepatic and pancreatic differentiation

We next confirmed the lineage specification and developmental potential of our integrated cell production system. Microwell suspension differentiated cells expressed C-KIT and CXCR4 in the absence of CD31 by flow cytometry analysis (Fig. 5A), and after plating on gelatin (see Methods) stained positive for the definitive endoderm markers FOXA2 (Ang et al., 1993) and SOX17 (Kanai-Azuma et al., 2002) (Fig. 5B). Quantitative RT-PCR results were consistent with a highly enriched definitive endoderm population (CXCR4⁺, SOX17⁺, Cerberus⁺, FOXA2⁺, GATA3⁺); lacking markers of pluripotency (OCT4, E-cadherin), neuroectoderm (SOX1), mesoderm (PDGFRA, KDR) or primitive endoderm (SOX7, FOXA3) (Fig. 5C).

Given the significant potential applications for PSC-derived pancreatic and hepatic cell types, we confirmed the competence of our microwell-differentiated cells for these fates. Directed differentiation along the pancreatic lineage according to the method of Nostro and co-workers (Nostro et al., 2011) resulted in sequential upregulation of markers of gut tube, pancreatic endoderm, and mature pancreas, including PDX1, insulin (INS), glucagon (GCG) and somatostatin (SST), with fully differentiated cells exhibiting insulin transcript levels six orders of magnitude higher than the input population (Fig. 5D) and staining for the presence of c-peptide (Fig. 5E). Directed differentiation along the hepatic lineage according to the method of Cai and co-workers (Cai et al., 2007) was also effective, resulting in upregulation of hepatocyte markers including AFP, albumin and the cytochrome p450 CYP3A4 (Fig. 5F). This data indicated that the microwell suspension-produced cells could yield functional derivatives that may be useful for pre-clinical evaluation.

Kinetics of optimized differentiation

We next compared our optimized suspension microwell protocol with a previously published endoderm differentiation protocol (Kroon et al., 2008), which differs in multiple aspects including use or absence of serum, monolayer vs. suspension culture, and duration of differentiation. We observed a significant improvement in expansion during differentiation, with our optimized method giving close to a 10-fold improvement over the previously published method (23.5 ± 4.5 vs 2.5 ± 0.3 fold respectively; $P = 0.00013$, Mann-Whitney U-test). In order to identify the cellular mechanisms (e.g. alterations in fate decisions or proliferation rate) underlying these differences, we performed L-profile analysis on both the suspension (Fig. 6A) and monolayer (Fig. 6B) processes. Parallel differentiation analysis indicated that in suspension differentiation during the first three days, OCT4 levels

remained high and markers of differentiation remained low, followed by a rapid decline in OCT4 with a concomitant increase in C-KIT and CXCR4 levels (Fig. 6C). In contrast, under monolayer conditions marker transition began immediately (Fig. 6D). Data from the L-profile analysis collected during the three-day marker transition phase (suspension culture days S4-S6, monolayer culture days M1-M3) showed similar cell division rates in the suspension and monolayer systems (Fig. 6E), however the suspension system exhibited a substantially greater increase in cell numbers (Fig. 6F, 8.3 ± 1.7 fold vs 2.7 ± 0.9 fold in total). The higher cell specific productivity in the suspension system can thus be attributed to lower non-specific cell losses over this period. Interestingly in both conditions, cell division measurements and observed cell proliferation exhibited an unexpected increase during the first day of the differentiation phase, with cells undergoing over two rounds of division in this 24h period ($T_d = 11.20 \pm 0.24$ hours)(Fig. 6E, S4 and M1). Based on this observation, we considered the hypothesis that a cumulative cell division threshold was involved in the differentiation process; however no correlation was observed between CXCR4 status and CFSE-assessed proliferative history in cells in the process of differentiation (Fig. S9). Overall, use of the L parameter as a cell production guide allowed us to deconstruct divergent differentiation protocols and identify key enhancements required to increase cell yield.

Quantitative prediction of differentiation outcome in an additional cell line

Cell production and differentiation protocols should be robustly applicable to different cell lines. The typical need to re-optimize protocols for each cell line significantly limits broad translation of cell production systems. We next hypothesized that output cell numbers available for DE production should be predictable based on two input parameters - cell yield after 24h of aggregate formation, and subsequent aggregate cell proliferation rate. We formalize this model by expressing the expected DE expansion (E) as a function of survival (S) over the initial 24h and P_5 , the projected expansion over the subsequent 5 days, derived from the hPSC growth rate under suspension maintenance (undifferentiated) conditions:

$$E(S, P_5) = k \times S \times P_5 \quad \text{Eqn. (2)}$$

As these variables do not account for the transient acceleration in proliferation at the onset of C-KIT/CXCR4 upregulation (Fig. 6E), we incorporate a constant (k) to represent this effect. Initial survival for the twenty-cell clusters used in our optimized protocol was 0.60 ± 0.16 (Fig. 7A). Calculating P_5 based on expansion numbers from 20-cell HES2 aggregates (Fig. 3B) gave a value of 23.3 ± 2.6 . For $k = 1$, the predicted expansion is 14.0 ± 4.0 fold, which falls as expected slightly below the observed result of 23.5 ± 4.5 (Fig. 2B). We can then calculate a value for k of 1.7 ± 0.6 , which is empirically reasonable from our cell tracking analysis (Fig. 6E,F; day S4).

We then proceeded to validate our quantitative approach to set differentiation parameters in the widely-used H9 (Thomson et al., 1998) cell line. Assessing 24-hour survival, we observed very low survival in 20-cell clusters, rising to approximately 40% in 60-cell clusters (Fig. 7A). Employing Equation (2) with the value of k determined above, and a 24h survival of 0.45 ± 0.14 , coupled to daily expansion numbers of approximately 1.49-fold (Fig. S6) for the remaining five days results in a prediction of 5.5 ± 3.3 -fold expansion. Performing the experiment, we observed expansion and yield values of 5.7 ± 1.1 -fold and 3.8 ± 0.9 -fold respectively (Fig. 7B). We also confirmed differentiation with the H1 (Thomson et al., 1998) line and the 38-2 iPS line (Nostro et al., 2011) under the same conditions (Fig. 7B). These results demonstrate the importance of simple parameters in assessing the capacity of a given cell line to differentiate towards a specific fate.

Discussion

The findings reported herein clearly demonstrate the effectiveness of our quantitative, mechanistic approach to bioprocess improvement, as well as the underlying culture technology we have developed to support it.

As a foundation for our rational differentiation process optimization strategy, we performed in-depth investigation of the cellular dynamics underlying an integrated process of hPSC expansion and differentiation towards DE. It is not common practice within the field to communicate differentiation efficiencies, instead only the purity of the output populations is generally reported. Indeed it is not possible to determine efficiency without first obtaining data on cell proliferation and loss over the course of a given process, which to our knowledge has not previously been assessed for PSC differentiation. This information forms the essential foundation for the consideration of the underlying cellular mechanisms of differentiation and their application to bioprocess improvement. In cases where differentiation is instructive and cell loss is due to non-specific factors, efforts must be focused on preventing this cell loss. By contrast, should differentiation occur following a selective mechanism, process improvement would hinge on the ability to prospectively identify the competent sub-population, and engineer preceding steps to enhance its prevalence in the input population. We thus developed a loss monitoring parameter (L), with which we identified and quantified the timing and magnitude of process inefficiencies, and determined that cell division continues throughout differentiation, counterbalanced by a wave of initial cell loss. In investigating the source of this loss, we assessed the contributions of instructive and selective models of differentiation (Enver et al., 1998; Suda et al., 1983) and observed a substantial instructive component, with differentiation competence widespread (should it have been due to specific loss under a selective mechanism, this approach would have resulted in an increase in total cell numbers without increasing yield of DE cells, and output purity would have been in the range of only two to three percent). Differentiation also does not appear to be triggered by a division-counting mechanism, with heterogeneities in cell-division history and differentiation status showing no apparent correlation.

Based on these results, we have been able to increase yield by thirty-six fold (Fig. 2B), to 18.5 ± 3.5 output cells per input hPSC. Using prototype large-scale (65 cm^2) microwell bioreactors output numbers on the order of 10^8 cells are easily achievable using standard laboratory equipment, and we are in the process of extending this by additional orders of magnitude. Preliminary results indicate a simple linear relationship between yield and microwell surface area (data not shown), thus we are focusing our efforts on the development of stackable modular designs compressing maximal surface area into a given volume. We have further shown that maintenance culture as uniform aggregates of controlled, optimal size can significantly enhance yield of hPSC in suspension (Fig. 3B), and have obtained an improvement of several orders of magnitude in yield over the current state-of-the-art, without employing serum, feeders or matrix. Recent studies by Steiner *et al* (Steiner et al., 2010) report expansion of approximately 100-fold over five weeks, while the values reported by Zweigerdt *et al* (Zweigerdt et al., 2011) give between 1.5×10^4 and 6.4×10^6 fold expansion at low densities, or 30 to 240-fold at densities comparable to ours, over this same time period. In comparison, our optimized expansion conditions are capable of 3×10^9 -fold expansion during this interval. We have further demonstrated integration of our microwell-suspension culture system with this microwell-suspension differentiation process, with four successive rounds of expansion as aggregates followed by our optimal differentiation protocol yielding approximately 65,000 DE cells per input hPSC in a total of 22 days (Fig. 4). Contrasting our results with previously published monolayer differentiation methods (Kroon et al., 2008), we determined that a substantial portion of the increase in

yield per PSC we observe in our system arises during the marker transition phase of differentiation. While cell cycling rates are similar in both processes, actual increase in cell numbers is greater in our microwell-suspension process, likely a consequence of non-specific increases in cell survival due to the increased physical space available for expansion in a three-dimensional system, and purity of the resulting populations are similar at the end of both processes (Fig. 6C/D).

In our integrated process model (Fig. 7C) cell clusters formed and cultured in individual microwells follow a trajectory through an “optimal envelope” bounded by aggregate size. The upper bound in aggregate size is likely due to a combination of competition with endogenous signals (Coucovanis and Martin, 1995), the tendency of larger aggregates to self-organize (Bauwens et al., 2011; Conley et al., 2004; Li et al., 2001; Ungrin et al., 2008), and heterogeneities arising from mass-transport restrictions (Carmeliet and Jain, 2000; Sachlos and Auguste, 2008). As aggregate sizes exceed the optimum, uncontrolled cell-cell contact and signaling increases in the endoderm differentiation process, the ability to control differentiation with exogenous factors is reduced, and purity decreases (yield of target cells declines faster than overall expansion - Fig. 2C). Aggregate size consistency is retained over the course of the process (Feret’s diameter 74 ± 14 microns, Circularity 0.79 ± 0.08), permitting calculation of approximate inter-aggregate distances (Fig. S10). Interestingly, the minimum free path between two adjacent aggregates at the end of the process is on the order of 200 microns for aggregates in 200 micron microwells, as opposed to 500 microns in the 400 micron microwells (or 250 vs 600 for center-to-center distances). We have previously determined that in patterned two-dimensional cultures of murine and human ESC, inter-colony communication effects are lost when center spacing exceeds 300 to 400 microns (Peerani et al., 2009; Peerani et al., 2007), consistent with a role for accumulation of inter-aggregate signals in the decreased yield when differentiation is performed in two hundred micron microwells. We have also observed an increased incidence of aggregates escaping from two hundred micron microwells and fusing with one or more adjacent aggregates (data not shown). Use of the ROCK inhibitor Y27632 is also a significant factor (Fig. 2D). Interestingly in our early experiments, using the unoptimized protocol with aggregates formed from clusters of 60 – 200 cells each, we observed that Rho-kinase inhibition resulted in increased total cell numbers but a decrease in purity, which we initially considered to support a selective cell loss event. We now recognize this loss of purity as a secondary effect of excessive aggregate size, as it does not occur when starting with smaller initial cell numbers. The dramatic effect of removing ROCK inhibition from the optimized protocol may be related to the significant decrease in cell output for clusters starting with less than twenty cells (Fig. 2C), and is consistent with the effect of ROCK inhibition on survival of dissociated hESC (Watanabe et al., 2007).

In the expansion process PSC yield declines in parallel with overall cell numbers in excessively large aggregates. The shape of the trajectory is a cell-line specific parameter, as we have shown, determined by interactions between upper and lower boundaries, the amount of cell loss during aggregation, and rate of cell division. The optimum number of input cells is therefore the smallest number able to retain viability through the process of aggregate formation, permitting maximum scope for expansion. Interestingly, these values are similar to the 20-45 cells of the inner cell mass of day 5-7 blastocysts (Hardy et al., 1989), inviting future investigation into potential quorum-sensing mechanisms and their connection to development *in vivo*. The quantitative predictions of this model are borne out by our assessment of H9 cell behavior, with observed results (Fig. 7B) consistent with predicted outcomes. These results also raise the intriguing possibility that apparent inconsistencies in the differentiation capacity of different hPSC lines may be due at least in part to relatively simple input parameters. For example, variations in survival under stress, cell cycle duration, or inter-cellular affinity will affect aggregate size distributions,

particularly in the context of conventional non size-controlled aggregate formation conditions. These variations could derive from intrinsic differences between the original cells from which the hESC were derived, the protocols used in the derivation process, and / or changes during post-derivation culture (Allegrucci et al., 2007). In addition, we have previously demonstrated in a two-dimensional model system that accumulation of autocrine factors that affect differentiation is lost in sub-threshold size cell groupings (Peerani et al., 2009), likely due to reduced autocrine signaling impact. A line that appears unable to efficiently generate progeny of a given type may simply reflect sub-optimal values for these important parameters (Fig. 2C) under the specific assay conditions used.

This observation highlights the importance of consistency and size control in aggregate formation, both between and within experiments. The ability to manipulate these parameters also bears significant potential advantage in subsequent steps in the progression towards implantable hPSC-derived liver and pancreas substitutes, as aggregation has been reported to enhance the formation of physiologically-relevant structures from hepatocytes (Lazar et al., 1995), while the engraftment potential of cadaveric islets has been reported to show significant size effects (Korsgren et al., 2008; Lehmann et al., 2007). Such “organoids” may be formed from a single cell type, or from mixtures of cell types or cells and bioactive microparticles (Bratt-Leal et al., 2011).

We report here a generally applicable strategy for quantitative investigations into the cellular mechanisms of hPSC differentiation, and have validated this strategy in a DE differentiation model. We have determined that competence for this fate is widespread and follows a substantially instructive model, that cell division continues during the differentiation process, and that substantial counterbalancing cell loss arises when aggregate size deviates from the cell-line-specific optimum. Our coupled expansion and differentiation systems employ only defined culture components, and in large-scale bioreactor systems we are developing, will permit the generation of over 10^9 C-KIT⁺CXCR4⁺ double positive cells from 1.5×10^4 input cells in 22 days, providing a solid foundation for future pre-clinical and clinical studies. The aggregates themselves are confined to unit cells with dimensions $400 \times 400 \times 283$ microns, giving final densities exceeding 8×10^6 cells per mL. To facilitate the rapid incorporation of additional future process and culture improvements, we focused on scale-independent solutions and “open-access” approaches that are easily replicated, improved, and extended upon without requiring unconventional technical capabilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Cellular mechanisms of endoderm differentiation. The initial protocol, where cells were formed into 60-cell clusters in 200-micron microwells, extracted and cultured in suspension (A) produced less than one C-KIT⁺CXCR4⁺ cell per input hPSC, over the course of a six day differentiation via unknown cellular mechanisms (B). Illustrative models yielding this result include *proliferation-independence* (B-i, (L profile remains at zero throughout the process); *compensatory elimination* (B-ii) (constant, non-zero L profile), and *sub-population outgrowth* (B-iii) (dynamic L profile with initial peak). Live cells stained with CFSE (Ko et al., 2007) lose fluorescence intensity as they divide and the fluorophore is partitioned between daughter cells (C). At the single-cell level, population doubling (ΔPD) over time is thus calculated as a function of fluorescence (F), with correction for signal loss over time. This value can then be combined with cell count data to calculate an L-profile (D). CFSE-derived cell division histories (E; Supplementary Figure S3) depict a population progressing en masse through a consistent trajectory, and exhibiting a period of accelerated growth on the fourth day of differentiation ($T_d = 11.20 \pm 0.24$ hours). An L profile was obtained over the course of the 6-day differentiation process (F), and showed significantly elevated cell

loss on days one and six (Mann-Whitney U, $P=0.024$, 0.047 and 0.024 d1 vs d2-4 and $P=0.024$, 0.17 and 0.024 d6 vs d2-4 respectively, $N \geq 3$ for each time-point).

Figure 2.

Differentiation towards definitive endoderm exhibits a substantial instructive component. A 36.3 ± 21.1 fold increase in yield was obtained in HES2 cells with 20-cell clusters formed in growth medium (GM), in the presence of ROCK inhibitor, in 400 micron microwells (**A,B**, $N=9$ and 32 original and optimized respectively, Mann-Whitney U, $P=6.2 \times 10^{-6}$). Initial cluster size effects exhibited a unimodal distribution with maximum yield obtained from 20 cell clusters (**C**, $N=3$ for each size). The contributions of individual components of the optimization were assessed (**D**) by contrasting the optimized protocol (Opt, $N=32$) with the same protocol with aggregates formed in differentiation medium (no GM, $N=6$), without ROCK inhibitor (no RI, $N=6$), with 60-cell clusters (60-cell, $N=6$), and in 200-micron microwells (200 micron, $N=6$). All performed significantly less well than the optimized protocol (yield, Mann-Whitney U, $P=0.00013$).

Figure 3.

Expansion of undifferentiated hPSC in the microwell system. Serial rounds of microwell-based expansion over 5 weeks yield nearly 10⁷-fold expansion of undifferentiated hPSC (A) – comparable to that observed utilizing traditional feeder-based protocols (inset). These proof-of-principle experiments show that the serum- and feeder-free production of clinically-relevant numbers of undifferentiated hPSC is within reach using our methodology. Under supportive (serum- and feeder-free) conditions, yields of OCT4⁺SSEA3⁺ cells increased with time, and were unimodally distributed according to initial aggregate size (B). Maximal yields of undifferentiated hPSC were produced from initial clusters of 20-40 cells after four days in culture. Yields obtained under control conditions (cells cultured on mouse embryonic fibroblast feeder layer in the presence or absence of the ROCK inhibitor Y27632, under comparable seeding densities) were significantly lower than those observed during the microwell-based expansion experiments (inset)(Mann-Whitney U test, $P=0.048$, $N\geq 4$ for each condition).

Figure 4.

Cells from four rounds of serial microwell-based expansion were successfully coupled to our differentiation process, generating a combined expansion and differentiation output of approximately 65,000 APS cells per input PSC (N=4).

Figure 5.

Validation of output cells. The optimized bioprocess generates cells that are positive for C-KIT and CXCR4 by flow cytometry (A), and proceed to express the definitive endoderm markers FOXA2 and SOX17 (B – scale bar represents 100 microns). Comprehensive qRT-PCR analysis normalized to hESC and β -actin shows downregulation of pluripotency markers (OCT4, E-CAD) and upregulation of definitive endoderm markers (CXCR4, SOX17, CER, FOXA2, GATA3), and lack of markers of neural (SOX1), mesodermal (PDGFRA, FLK1/KDR) or primitive endoderm (SOX7, FOXA3) fates (C). Differentiation to APS and thence definitive endoderm fate was confirmed by capacity to undergo subsequent differentiation along the pancreatic and hepatic trajectories. Cells were able to express markers such as INS, PDX-1 and SOX9 in the expected chronological order during pancreatic differentiation (D; samples 1: endoderm precursors, 2: definitive endoderm, 3: fore/midgut endoderm, 4: pancreatic endoderm, 5: endocrine progenitors, 6: endocrine cells, 7: as 6 with dorsomorphin – INS PCR results represent a minimum value for upregulation relative to the input cells, in which INS expression was at or below the limit of detection; and E: immunofluorescence staining for the proinsulin c-peptide fragment demonstrates the presence of INS-expressing cells at the end of the process, scale bar represents 100 microns). Cells were also able to express markers of the hepatic lineage such as AFP, Albumin, and the cytochrome C Cyp3A4 (F; samples 1: differentiated aggregates, 2: following five days HGF, 3: following two days OSM+Dex, 4: following five days OSM +Dex).

Figure 6.

Expansion during differentiation is substantially higher in suspension culture than in monolayers. We compared our optimized process with the monolayer endoderm differentiation approach of Kroon and co-workers (Kroon et al., 2008) (process steps shown above for reference). While this protocol differs from ours in several respects including the use of serum, we sought to determine if there were any obvious cellular mechanisms behind the increased expansion of our process (23.5 ± 4.5 C-KIT⁺CXCR4⁺ cells vs 2.5 ± 0.3). Applying L-profile analysis we see both processes exhibit some ongoing losses, implying further improvement may be possible in both cases (A,B). Marker expression indicates that in our feeder-free serum-free suspension process, the population remains almost entirely OCT4⁺ and C-KIT⁺CXCR4⁺ for the initial three days (C), whereas differentiation begins immediately under adherent / serum / feeder conditions (D). Aligning expansion data on the basis of marker expression, we assessed theoretical (CFSE-based) and observed population doublings in our suspension process and in monolayer differentiation culture. While theoretical expansion was broadly similar (E), real expansion in suspension culture was low during the initial OCT4⁺ period, and substantially greater than in the monolayer system during the period in which marker expression was changing, with a significant increase in observed doubling at S4 (Mann-Whitney U, $P=0.0049$, $N=6$ for S4 vs S3, or $P=0.00036$, $N=6$ and 24 for S4 vs S2/3/5/6 respectively) (F). This difference accounted for the majority of the difference in overall expansion and may reflect increased spatial freedom to expand in the suspension system.

Figure 7.

Bioprocess parameters allow predictive adaptation to other cell lines. Survival was assessed after twenty-four hours in growth medium in 20-cell HES2 clusters (**A**; 0.60 $\pm$ 0.16, N=6). For H9, viable cell numbers were too low to count after clustering of 20 cells, while approximately 40% survival was attained with clusters of 40, 60 and 100 cells, with highest coefficient of variance at the 40-cell size (**A**; N=4, CV = 0.21, 0.14, 0.18 respectively). Differentiation of H9 cells from 60-cell clusters was successful, with expansion and yield of approximately 5.65-fold and 3.75-fold respectively, and similar results from IPS and H1 lines (**B**; N=11, 22 and 6 respectively). In our model (**C**), optimal aggregate size is a function of trajectory through the “optimal envelope”. Efficiency of cell production is maximal when aggregates approach the upper size limit, beyond which viability and / or purity are adversely impacted, immediately prior to harvest. The trajectory followed from a given starting point then depends on the amount of cell loss experienced after aggregate formation, and the rate at which real expansion of cell numbers occurs. Aggregate size may be reset by dissociation and re-aggregation to allow for repeated cycles of expansion (left side) or subsequent differentiation steps (right side).

Supplementary Table S1. Antibodies and supplier information.

Supplementary Table S2. Primer sequences employed for quantitative real-time polymerase chain reaction quantification of transcript levels.

Supplementary Figure S3. In the course of this differentiation process, CFSE peak spreading precluded assessment of population doublings via peak counting. We therefore obtained expansion data from the population fluorescence profile by treating the measured profile on a given day as a linear combination of contributions from a reference population shifted by different numbers of cell divisions, after compensating for a CFSE half-life of 72 hours. Population doubling history was obtained by minimizing the squares of the residuals (**A-F**: hatched – measured profile; white – reference profile; red line – best fit line; bar plot – fitted relative contributions).

Supplementary Figure S4. Differentiation competence cannot be isolated prospectively on the basis of SSEA3 or TG30 expression levels. In order to test the selective differentiation hypothesis, equal numbers of cells were differentiated after flow sorting the input population (**A,B**) into low (**C,D** - bottom 15th percentile), medium (**E,F** - middle 70%) and high (**G,H** - top 15th percentile) on the basis of expression levels of SSEA3 or TG30. Neither marker permitted prospective isolation of the competent population of cells (**I,J**), although the population with the lowest SSEA3 expression did exhibit a statistically significant reduction in yield (**I** - Low vs Medium / High, Mann-Whitney U, $P=0.026$ and 0.0022 respectively, $N=6$), consistent with the expected presence of contaminating differentiated cells in this fraction. These results are consistent with non-specific cell loss in an instructive-differentiation model.

Supplementary Figure S5. Mann-Whitney U tests were used to compare all combinations of cell yields from 60-, 100-, and 200-cell aggregates expanded during 10 serial passages (**A**). Our analysis demonstrates that there is no consistent pattern of significant differences such as would be observed should changes in proliferation or self-renewal rates accumulate over successive passages (for example, an increase in proliferation rates with time would lead to a consistent pattern of black squares [significant differences] when comparing later to earlier passage results – see inset for hypothetical dataset showing this property). This data supports the notation that fundamental cell properties (single cell survival, cell proliferation rate) do not change during the study. Representative FACS plots of the expression of the pluripotency markers OCT4 and SSEA3 do not alter appreciably during serial passaging (**B**), suggesting that cells do not lose their pluripotent state during the 35 day culture period.

Supplementary Figure S6. Expansion of H9 hPSC through 5 serial passages results in a unimodal distribution with aggregate size, consistent with observations made using HES2 hPSC. Mean expansion was maximal with an initial aggregates size of 60 cells, and not significantly different from expansion of H9 cells cultured on MEF ($P > 0.2$). H9 cells were expanded under the same conditions as HES2, other than minor differences in medium composition noted in the Materials and Methods section under Cell culture (aggregate suspension expansion).

Supplementary Figure S7. Karyotype analysis demonstrates the genetic stability of hPSCs cultured serially in the microwell system. HES2 (top row) and H9 cells were cultured over 6 serial passages either on MEF or in microwells, and processed for karyotype analysis (0.05 mg / mL Colcemid for one hour; hypotonic KCl solution for 30 minutes, desiccated overnight at room temperature, then at 90°C for 90 minutes, and treated with 0.4x Pancreatin for 3 minutes 15 seconds). Representative karyotypes from each condition are as indicated.

Supplementary Figure S8. Teratoma analysis of HES2 cells serially passaged in the microwell system. Tumours (**A**) resulting from the injection of HES2 cells serially passaged using conventional passaging on MEF (**B**), as 40-cell aggregates (**C**), as 100-cell aggregates (**D**), or as 200-cell aggregates (**E**) contain cells from all germ layers, contrasting with a growth arising from the injection of proliferating MEF cells (**F**). Tumours 1-5 in (**A**) correspond to panels (**B-F**) respectively.

Supplementary Figure S9. Differentiation is not controlled by a simple cell-division counting mechanism. Cells on the 5th day of differentiation (**S5**) were assessed flow cytometrically for CFSE intensity (FITC channel) and CXCR4 staining (APC channel). A control (CFSE alone) sample indicated that CFSE signal did not have a substantial effect on APC fluorescence intensity (not shown), and no substantial correlation between cell division history and differentiation status that might have indicated a division-counting mechanism active in fate decisions was observed.

Supplementary Figure S10. Aggregate dimensions remain consistent after 6 days of differentiation. Particle analysis was performed using ImageJ (1.44i) on images captured at 10x magnification, which were calibrated, thresholded automatically, subjected to the binary Close function (iterations = 10, count = 4) to close internal holes, and segmented using the

Watershed algorithm. An example image (**A**) shows close correspondence between input image and best-fit ellipses (red). Aggregates remain highly uniform (Feret's diameter $73.8 \pm 13.7 \mu\text{m}$) and symmetrical (Circularity 0.79 ± 0.08) ($N=351$ for both measurements) (**B**). Approximating aggregates as uniform spheres of radius r , we can calculate the minimum free path (MFP) between aggregates, defined as the shortest path connecting the surfaces of two adjacent aggregates that does not pass through the microwell substrate (**C**). We first calculate the height (H) of the aggregate centre above the base of the microwell, and then determine MFP for a given well depth (WD) and pitch (P) trigonometrically. Using the calculated Feret's diameter (**C, upper panel, middle sphere**) gives $MFP_{200} = 179 \mu\text{m}$ in a $200 \mu\text{m}$ microwell, or $MFP_{400} = 519 \mu\text{m}$ in a $400 \mu\text{m}$ microwell (**C, lower panel**). Repeating this calculation with aggregates one standard deviation below (**C, upper panel, left sphere**) or above (**C, upper panel, right sphere**) the average gives $MFP_{200} = 152 \mu\text{m}$ and $208 \mu\text{m}$ and $MFP_{400} = 488 \mu\text{m}$ and $551 \mu\text{m}$ respectively.