Stem Cell Research (2010) 4, 165–179

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REGULAR ARTICLE

Up-scaling single cell-inoculated suspension culture of human embryonic stem cells

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Received 21 December 2009; received in revised form 3 March 2010; accepted 4 March 2010

Abstract We have systematically developed single cell-inoculated suspension cultures of human embryonic stem cells (hESC) in defined media. Cell survival was dependent on hESC re-aggregation. In the presence of the Rho kinase inhibitor Y-27632 (Ri) only ~44% of the seeded cells were rescued, but an optimized heat shock treatment combined with Ri significantly increased cell survival to ~60%. Mechanistically, our data suggest that E-cadherin plays a role in hESC aggregation and that dissociation and re-aggregation upon passaging functions as a purification step towards a pluripotency markers-enriched population. Mass expansion of hESC was readily achieved by up-scaling 2 ml cultures to serial passaging in 50 ml spinner flasks. A media comparison revealed that mTeSR was superior to KnockOut-SR in supporting cell proliferation and pluripotency. Persistent expression of pluripotency markers was achieved for two lines (hES2, hES3) that were used at higher passages (>86). In contrast, rapid down regulation of Oct4, Tra-1-60, and SSEA4 was observed for ESI049, a clinically compliant line, used at passages 20-36. The up-scaling strategy has significant potential to provide pluripotent cells on a clinical scale. Nevertheless, our data also highlights a significant line-to-line variability and the need for a critical assessment of novel methods with numerous relevant cell lines.

Introduction

The clinical applications of hESC and their derivatives require their mass production in scalable suspension cultures. Processing of anchorage-dependent cells such as ES cells is facilitated by dissociation into single cells for passaging. This has implications for a controlled scale-up process and automation, where it is important to seed bioreactors with reproducible numbers of evenly distributed cells.

Experience with murine embryonic stem cell (mESC) cultures over the past three decades has recently resulted in the development of relatively large-scale processes of up to

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doi:10.1016/j.scr.2010.03.001

2 liters, in stirred bioreactors (Schroeder et al., 2005; zur Nieden et al., 2007; Niebruegge et al., 2008). mESC are routinely passaged as single cells generated by enzymatic treatment and remain vital when seeded in suspension.

Three-dimensional (3D) suspension culture of ES cells results in the formation of aggregates, a process which can be controlled by several factors including cell concentration at inoculation, bioreactor and impeller design, and stirring speed (Schroeder et al., 2005). Another parameter of key importance is that of the culture medium. The addition of Leukemia Inhibitory Factor (LIF) that activates the Jak/Stat pathway largely prevents mESC differentiation in both conventional plastic adherent 2-dimensional (2D) culture, as well as in aggregates in 3D suspension culture (Taiani et al., 2009). Unfortunately, LIF fails to maintain self-renewal of human ESC (Daheron et al., 2004).

Research to define the multi-factorial network of growth factors and downstream signaling in hESC determined that the addition of fibroblast growth factor (FGF) family members, particularly basic FGF (bFGF), and the blocking of BMP-signaling by noggin or activin, are required to sustain proliferation of undifferentiated hESC (Xu et al., 2008a). However, physical interactions with the extracellular matrix have also been shown to play a role in hES cell fate (Chin et al., 2009). Subsequently, laboratory scale hESC culture is predominantly performed in 2D on a layer of feeder fibroblasts or on matrices such as matrigel, fibronectin, laminin, or heparan sulfate, in media supplemented with bFGF.

In recent studies, investigators have either focused on automation of 2D hESC cultures (Terstegge et al., 2007; Thomas et al., 2009) or adaptation to 3D suspension by microcarriers (MCs). MCs are particles which allow the translation of anchorage-dependent cells into suspension culture by providing an enlarged attachment surface. In a first study using coated polystyrene MCs (Phillips et al., 2008), cell-to-carrier attachment and initial cell expansion was achieved, but cell growth ceased over successive passages. Continuous long term propagation of hESC on cellulose MCs was established by Oh and coworkers (Oh et al., 2009). However, cell attachment and propagation relied on MC-coating with matrigel, a poorly defined basement membrane. Historically, MC-technology has been applied to up-scale suspension cultures of conventional cell lines for the production of recombinant proteins, vaccines, or antibodies (Zweigerdt, 2010), whereby the final product is ultimately separated from the cells. In contrast, MCs might impose technical and regulatory hurdles with respect to (stem)cell generation for clinical purposes, as cells in such situations, comprise the final, therapeutic treatment.

Thus, the primary objective of this study was to establish hESC propagation and expansion in scalable, stirred suspension cultures, independent of additional substrates (Mahlstedt et al., 2010) or MCs. By using an inhibitor of Rho kinase (Y-27632; Ri) in combination with heat shock treatment, we have systematically developed a method of forming controlled aggregates in suspension from single hESC inoculations in defined, non-conditioned culture media, i.e. KnockOut-SR (KO) and mTeSR.

Using three independent hESC lines of early to late passages, and performing a high number of experimental repeats, conditions were systematically optimized in 2 ml static cultures and progressively up-scaled to 10 ml, and finally 50 ml stirred suspension cultures (schematically presented in Fig. 1). Expression of pluripotent markers and the ability to form teratomas also persisted over at least 5 serial passages in 50 ml bioreactors. Interestingly, these features were found to be strongly cell-line dependent.

Results

Optimized heat shock treatment combined with Ri significantly improves cell survival in suspension

It has been reported that Ri supports survival of hESC dissociated into single cells (Watanabe et al., 2007) and the formation of aggregates in suspension culture (Li et al., 2009). However, the efficacy of this process has been poorly studied. To address this, we added Y-27632 at a 10 μM concentration in hESC culture medium (KO) post single-cell dissociation. Formation of aggregates and cell survival in suspension culture were analyzed 24 hrs later. There was no aggregate formation in control cultures, and only about 2% (+/−1.5%) vital cell recovery was observed (Fig. 2A). Ri addition resulted in robust aggregate formation and a 41-44% (+/−4.6%) recovery of vital hES2 or hES3 cells, respectively (Fig. 2B). Pre-treatment with Y-27632 before hESC dissociation was not more advantageous as compared to direct addition to suspended cells (data not shown).

A significant increase in the number of surviving cells was further induced by combining Ri with a heat shock treatment applied 2 hrs after cell dissociation; trypan blue staining revealed 52-60% (+/−4.0%) vital cells at 24 hrs, respectively (Fig. 2A). A modified heat shock treatment (at 0, 4, or 8 hrs after cell dissociation) in combination with Ri did not outperform the Ri with heat shock at 2 hrs (S) protocol (termed Ri+S=RiS). RiS was thereafter applied throughout the study. Notably, heat shock treatment alone, which was also most efficient when applied 2 hrs after cell dissociation, induced a modest but significant increase in cell recovery over controls (7.3+/−1.1% vs 2.0+/−1.5%) for hES3 cells (Fig. 2A).

To challenge the general significance of RiS versus Ri, an additional cell line, ESI049, and another defined medium, mTeSR, were included in the 24 hrs vitality assay (Fig. 2B). The trends shown in Figure 2B became highly significant when the cell recovery data were combined and statistically analyzed (Fig. 2C). Over all cell lines, RiS treatment resulted in a significantly higher cell count of about 13.0% (+/−0.2%) above Ri (n=18 for each treatment; Fig. 2C). A culture medium-centered analysis resulted in a 14.0% (+/−0.2%) increase in cell recovery in mTeSR over KO (n=36 for each medium). More drastically, a comparison of Ri / KO versus RiS / mTeSR conditions (n=18 for each set) showed 28% (+/−1.6%) better cell recovery in the latter setup in static suspension culture (Fig. 2C).

High cell vitality and elevated E-cadherin levels in aggregates

Aggregates from static cultures in 6 well plates resulting from Ri or RiS treatment at 24 hrs were separated from the remaining single cells, and both populations were analyzed. While the vitality of single cells was rather low (<10%),

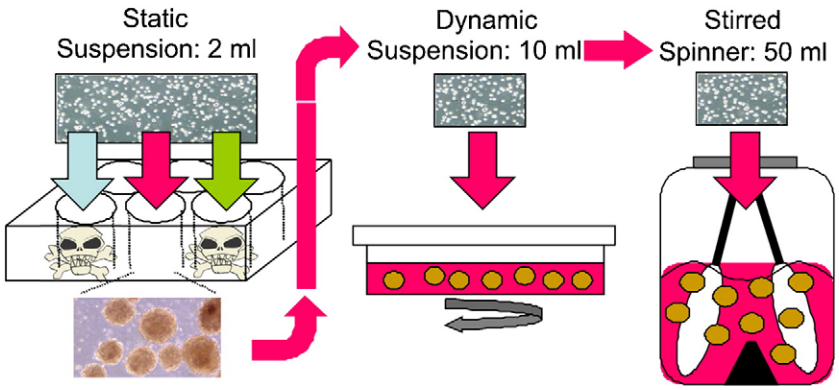


Figure 1 Schematic presentation of the experimental strategy used to optimize and up-scale single cell-inoculated suspension culture of hESC. Single-cell dissociated hESC die off under most conditions (symbolized by a skull in the 6-well platform) when seeded in suspension culture. Optimized treatments resulting in highest cell vitality and cell aggregation in 6-well static suspension were tested for up-scaling to dynamic suspension in a 10 ml culture volume and finally to 50 ml spinner flasks.

essentially all cells that had formed clusters appeared vital (data not shown). This observation might suggest that 1) Ri/RiS treatment induces cell aggregation, and consequently results in increased cell survival; or 2) survival of single cell-dissociated hESC is generally supported (and/or prolonged) by the treatment, but only cells that form clusters by cell-cell attachment (within a relative short time window post inoculation) can ultimately survive in suspension.

To address these possibilities, protein levels of E- and N-cadherin (E-cad, N-cad) were analyzed by flow cytometry. Both trans-membrane proteins are known to mediate Ca^{2+}-dependent cell-cell adhesion and are expressed in hESC (Eastham et al., 2007; Avery et al., 2008). hESC harvested from conventional 2D culture on feeders - the inoculum - were compared to aggregates (generated by Ri addition) which were formed from this inoculum after 24 hrs in suspension culture. For all 3 cell lines tested, an apparent enrichment for an E-cad-high cell population was found in aggregates (Fig. 2D). This suggests that cells with a higher E-cad level have a higher likelihood to agglomerate and subsequently survive in suspension. Notably, aggregate-derived cells also showed higher levels of Tra-1-60 (Fig. 2D) and Oct4 (not shown) compared to the inoculum, whereas N-cad and SSEA4 levels were high (>95%) and essentially unchanged in both populations (data not shown).

Static suspension culture resulted in up to 2.5-fold hESC expansion in 7 days

To investigate the effect of suspension culture on cell expansion, we next followed aggregate formation and cell proliferation in static suspension for 7 days, corresponding to our passaging cycle of hESC on feeder cells. The following parameters were analyzed: 3 hESC lines, 4 treatment conditions at inoculation (control, S, Ri, RiS), and 2 types of culture media (KO, mTeSR). Cell counts were performed on day 1-7, for 6 replicates each.

In contrast to non-treated controls (Fig. 3A) or heat shock only (data not shown), robust aggregate formation and growth was observed for all 3 hESC lines in both media after Ri or RiS treatment (Fig. 3A). The average number of cells per aggregate increased relatively homogeneously for all cell lines and conditions (Fig. 3B). On day 1, a range of 200-400 cells per aggregate was observed. This number increased by about 5-10 fold until day 7, resulting in 1500-2500 cells per aggregate, respectively (Fig. 3B).

Growth kinetics revealed an expected cell loss on day 1. Thereafter, a continuous increase in cell numbers was observed, resulting in a 2–2.5-fold expansion of the inoculated cells over 7 days, respectively (Fig. 3C). While minor line-to-line variability was discovered (highest expansion for ESI049 and lowest for hES2), cell counts generally increased with RiS treatment relative to Ri alone. A higher cell yield was also detected in mTeSR cultures compared to KO medium. Subsequently, a statistical analysis over all cell lines revealed a significant, 34% (+/−1.6%) higher cell yield at RiS / mTeSR conditions compared to Ri/KO (Fig. 3D).

In summary, a noteworthy net expansion of hESC was observed in static suspension, suggesting that up-scaling using a 3D culture platform was feasible.

Formation of homogeneous aggregates, but reduced cell expansion in dynamic suspension culture

As static suspension culture generally resulted in non-homogenous aggregates, we investigated the impact of dynamic culture conditions. 2 ml static cultures were scaled-up to a stirred, 10 ml platform in Petri dishes. Consistent with our previous data, aggregate formation and cell expansion was only observed after Ri or RiS treatments (Figs. 4A and B). The inoculation density of 5×10^5 cells/ml, successfully applied in static culture was found to be unfavorable for aggregate formation in stirred dishes (data not shown) and was consequently increased to 1×10^6 cells/ml (1×10^7 cells per Petri dish) for these studies.

Light microscopy showed that the dynamic culture platform supported the formation of more homogeneous aggregates as compared to static controls (see Figs. 4A and 3A at day 7). At the end of a passage, on day 7, aggregates consisted of about 3000 cells per cluster regardless of cell line and culture medium (Fig. 4B). These results were comparable to data obtained from the static suspension

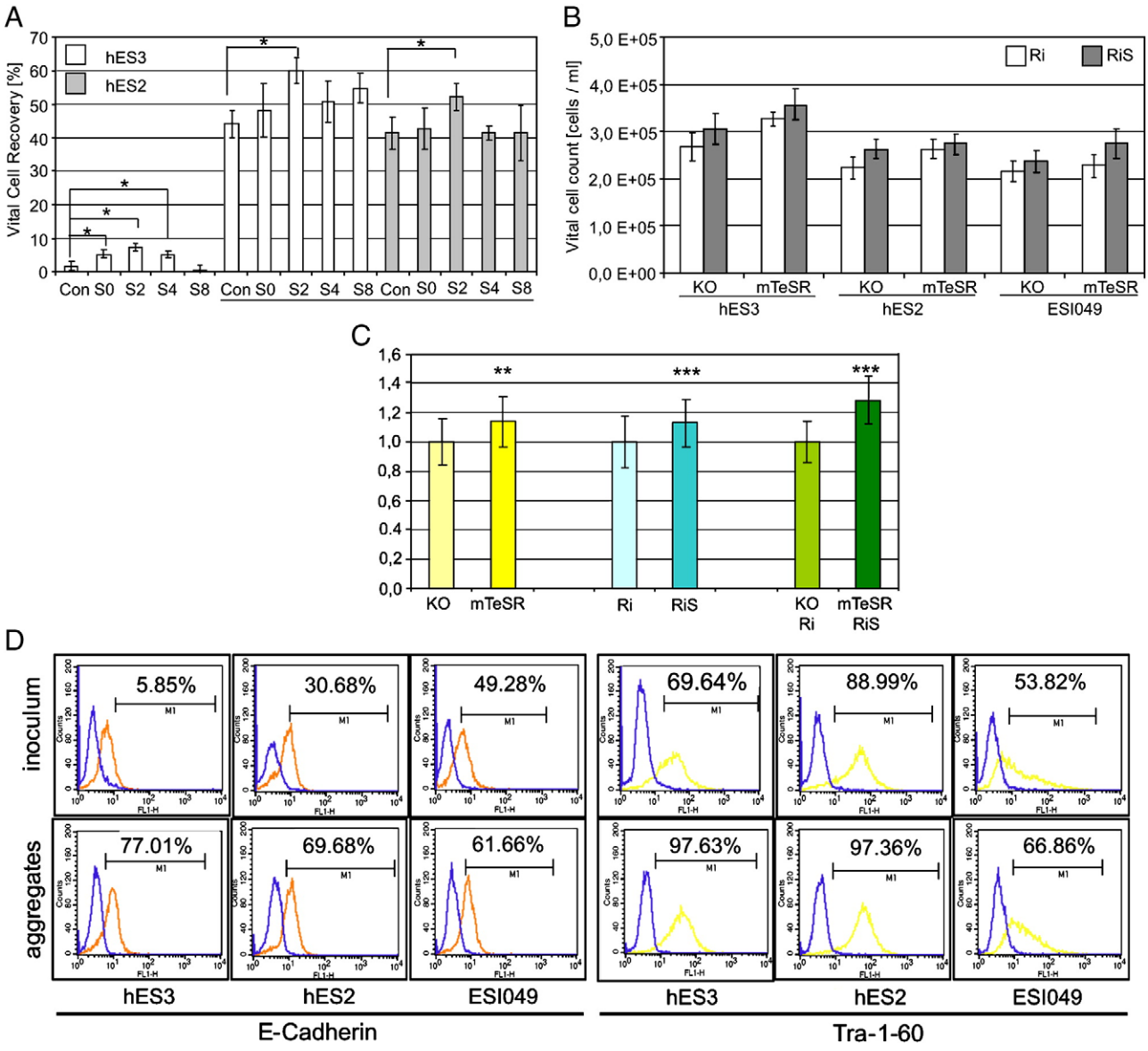


Figure 2 Vital cell recovery at 24 hrs post single cell inoculation in static suspension culture. A. Percentage of surviving cells compared to the inoculated cell number (100%) in KO medium. Single heat shock pulses (S) were applied at 0, 2, 4 or 8 hrs (S0, S2 etc) after dissociation into single cells. Pulses applied at 2 hrs yielded the highest vital cell counts over non-treated controls (Con) alone or in combination with Ri-addition; Ri-addition is as underlined. n = 6 for each bar. B. Mean vital cell counts for hES3, hES2, and ESI049 cell cultured in KO or mTeSR medium either with Ri addition only or Ri plus heat shock pulse applied at 2 hrs (RiS). RiS treatment was generally superior to Ri only, and mTeSR resulted in a higher vital cell count over KO medium. n = 6 for each bar. C. The trends shown in 2b became highly significant when the cell recovery data were combined and statistically analyzed. The mean for the vital cell count in KO, Ri, or KO/Ri conditions was calculated over all 3 cell lines (n = 36 for each bar), normalized to 1 and compared to mTeSR, RiS, or mTeSR/RiS conditions, respectively. Unpaired two-tailed t-test indicated a level of significance of ** $p \leq 0.01$ or *** $p \leq 0.001$, respectively. D. Flow cytometry analysis. hESC from conventional cultures on feeders (inoculum) were compared to aggregates (generated by Ri addition) formed from the inoculum after 24 hrs in suspension culture. For all three cell lines tested, an apparent enrichment for an E-cad-high cell population (red line) was found in aggregates (compare upper and lower row). Aggregate-derived cells also showed higher Tra-1-60 levels (yellow line) and an elevation in Oct4 levels (not shown). The blue line represents the isotype controls.

cultures. However, larger clusters consisting of 4000-5000 cells were observed for hES2 in mTeSR (Fig. 4B).

Surprisingly, only about $1\text{-}1.3 \times 10^7$ vital cells per Petri dish were recovered for most conditions after a 7-day passage interval (Fig. 4C). This represented a modest 30% net expansion, as compared to the 2.5-fold increase in static suspension culture. Higher cell counts were only found to occur for the hES3 line in mTeSR medium, yielding about $1.6\text{-}2.0 \times 10^7$ cells/dish (about 2-fold expansion per passage) after RI or RiS treatment, respectively (Fig. 4C).

In summary, a combination of RiS and mTeSR outperformed Ri / KO for all cell lines, confirming static suspension culture results. In addition, dynamic suspension culture also resulted in a statistically significant, albeit modest, cell expansion within one passage. Based on our prior trials (data not shown), increasing the rotational speed beyond an optimum speed produced detrimental effects. Thus, our results suggests that cell vitality and proliferation may have been limited by shear stresses induced by the rotating Petri dish platform that were absent in static suspension.

mTeSR supports long-term expression of pluripotency markers in hES2 and hES3, but not in ESI049 cells

To assess the expression of pluripotency markers, aggregates derived from rotating suspension cultures in 10 cm plates were dissociated on day 7 (passage 1) and analyzed for the presence of Oct4, SSEA4, and Tra-1-60 antigens by flow cytometry. As outlined in Figure 5A, 80-100% of hES2 and hESC3 cells expressed these markers regardless of culture or treatment conditions. In contrast, a drastic decrease in Oct4 and Tra-1-60 positive cells to less than 65% and 30% respectively, was observed for ESI049 in KO medium (irrespective of Ri or RiS; n=3) after only one passage (Fig. 5A). No apparent reduction in these markers was observed in parallel ESI049 cultures in mTeSR, suggesting that this medium supports pluripotency marker expression under the conditions tested.

In consequence, serial cultures were propagated for up to 7 passages in mTeSR medium combined with RiS treatment. At every passage, aggregates were harvested, dissociated into single cells, and re-seeded with 10 million cells per Petri dish on the agitated platform (n=3 replicates each). Expression of Oct4, SSEA4, and Tra-1-60 was usually above 90% for the lines hES2 and hES3 at passage 1-5 (Fig. 5B). At passage 7, Oct4 and Tra-1-60 expression was somewhat reduced to about 62-70% in both lines and SSEA4, the least sensitive marker in our studies, was also slightly down regulated. For ESI049 cultures, however, the down regulation of pluripotency markers was more pronounced. Oct4 expression had reduced to <80% at passage 3 and subsequently dropped to 45% and 35% at passages 5 and 7, respectively. This drastic reduction was even more pronounced for Tra-1-60, which varied from 12–40% between passages 3–7. These findings were contrasted by SSEA4 levels, which remained stably expressed by 87-97% of cells in the population (Fig. 5B).

Despite this loss of pluripotency for one cell line, our suspension culture strategy showed potential for further up-scaling and was adapted to the spinner flask platform.

Up-scaling to 50 ml stirred flasks resulted in a 2-fold net expansion per passage

For translation to bioreactors, RiS treated cells were seeded at 1×10^6 cells/ml in 50 ml mTeSR medium into stirred spinners (5×10^7 cells/flask). Optimization of the stirring speed resulted in extensive cell aggregation at 30 rpm (data not shown). Increasing the stirring spread to 40 rpm reproducibly resulted in the formation of relatively uniform aggregates for all 3 cell lines (Fig. 6A). As with the Petri dish platform, increasing the stirring speed was detrimental to the cells (data not shown). Under these conditions, an average cluster size of approximately 15,000 – 25,000 cells per aggregate was observed on day 7 (Fig. 6A), being approximately 10-fold larger as compared to the previous platforms.

Serial passaging by aggregate dissociation every 7 days consistently resulted in the re-formation of aggregates. Cell expansion per passage was approximately 2-fold, yielding 1×10^8 - 1.2×10^8 cells per spinner flask, irrespective of the hESC line. This was a substantial improvement compared to the agitated Petri dish platform. Importantly, the cell yield remained extremely stable over 5 passages (Fig. 6C), highlighting the robustness and high reproducibility of the culture system. Karyotype analysis was performed to assess the genomic integrity of cells after 5 passages in spinner flasks and no abnormalities were detected in any of the 3 lines (Fig. 7C).

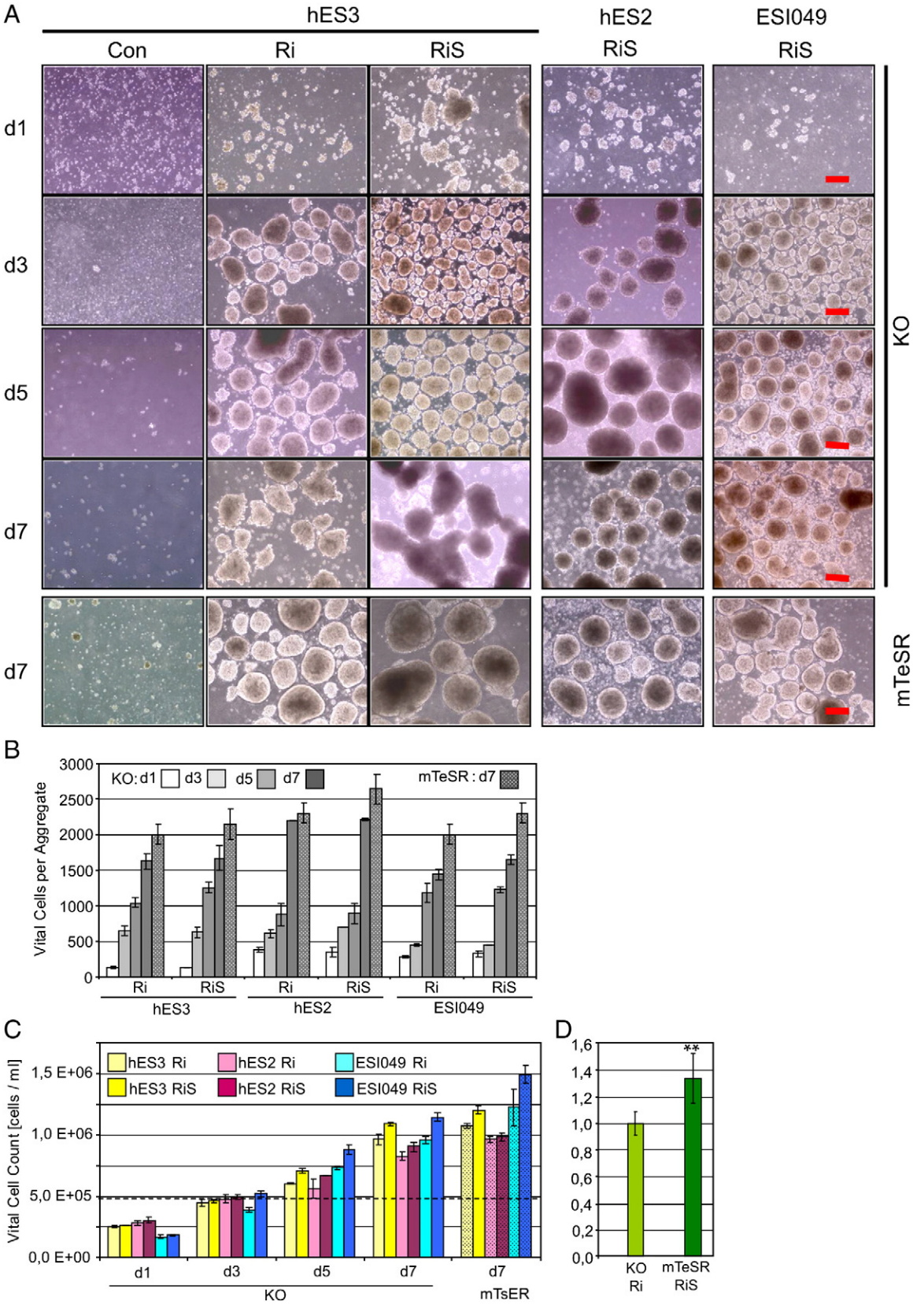
However, consistent with our Petri-dish culture results, flow cytometry revealed a significant reduction of pluripotency marker expression for ESI049 cells cultured in spinner flasks. While no apparent reduction compared to controls was observed after passage 1, Tra-1-60 expression dramatically decreased to 3% at passage 3. Although Oct4 expression persisted at passage 3, the percentage of positive cells dropped below 20% by passage 5 (Fig. 6D). Furthermore, the least sensitive marker SSEA4 decreased to below 60% at this stage. Immunofluorescence staining specific to these markers at passage 5 depicted the presence of relatively large, cell-free cysts in ESI049-derived aggregate-sections, as compared to respective hES2/3-derived controls (Fig. 6B). More importantly, only small clusters of cells from ESI049-derived sections stained positive for these pluripotency markers (Fig. 6B), supporting the flow cytometry results.

In contrast, flow cytometry definitively showed that the expression of pluripotency markers remained high (>90%) over 5 spinner flask passages for hES2 and hES3 cells (Fig. 6D). This observation was further confirmed by immunofluorescence staining specific to these markers on aggregate-sections collected from passage 5/day 7 cultures (Fig. 6B). While a "rim" consisting of 1-2 cell layers was negative, relatively uniform staining was observed throughout the entire sections. In contrast to medium-receiving controls, the injection of passage 5/day 7 cells into SCID mice resulted in teratoma formation in 5 out of 5 mice. Successful *in vivo* differentiation into derivatives of all 3 germ layers strongly underscored the pluripotency of our suspension-derived cells (Fig. 7A).

Given our interest in cardiomyogenic differentiation, we also performed *in vitro* differentiation of hES3 cells, a line known to have a higher efficiency of cardiomyocyte formation. Aggregates from passage 5 spinner flask cultures were differentiated in a clinically-compliant medium (Xu et al., 2008b,c) and contracting aggregates were observed after 10-12 days. Cardiomyocyte formation in differentiated EB-like aggregates derived from spinner flasks was unequivocally proven by immunocytology specific to cardiac markers (Fig. 7B)

Proceeding further with the extensive spinner flask approach with 3 cell lines and the respective number of experimental repeats beyond passage 5 was beyond the logistical capabilities

of this study. To test the feasibility of our method for additional passages we have thus employed the 6-well static culture platform initially used for process optimization. After 12 passages following the established protocol (7d per passage in mTsER with intermittent by single-cell dissociation of aggregates and RiS treatment) we have observed constant expansion rates or ~2-3fold per passage, normal karyotype patterns, and sustained pluripotency marker expression of hES2 and hES3



cells. ESI049 cultures were abandoned at passage 7 due to the loss of pluripotency marker expression (data not shown).

Discussion

The major focus of this work was to test the feasibility of single-cell based inoculation and serial propagation of hESC as aggregates in defined suspension culture, and to gain insights into mechanisms that may govern this process. Subsequent adaptation to dynamic conditions and scalability to bioreactors was investigated.

Cell survival post single-cell inoculation was the first key requirement for the method to work. Analysis at 24 hrs revealed that in the presence of Ri only about 44% of the seeded hESC were recovered. We determined that an optimized heat shock treatment significantly increased the number of surviving hESC in an autonomous, Ri-independent manner; heat shock application was also found to enhance cell survivability when used in conjunction with the Ri.

It has been established that stress response of cells involves molecular chaperones and co-chaperones, especially heat shock proteins (Hsp). Hsp are ubiquitous molecules involved in the modulation of protein conformational and complexation states (Morimoto et al., 1997). The functions of Hsp are well characterized in differentiated cells, while their role in stem cells remains unclear. But it appears that ESC exhibit increased stress tolerance and concomitant high levels of chaperone expression (Prinsloo et al., 2009). In an effort to identify novel hESC-specific cell-surface markers, it was found that hESC express abundant levels of heat shock 70-kDa protein-8 isoform-1 (HSP70), which is down regulated upon differentiation (Son et al., 2005). Interestingly, HSP70 can neutralize the apoptosis-inducing factor (AIF), an intermembrane flavoprotein that induces caspase-independent apoptosis (Cande et al., 2002). Experimental inactivation of AIF renders ESC resistant to cell death following growth factor withdrawal. Moreover, AIF is essential for programmed cell death during cavitation of embryoid bodies and the very first wave of (caspase-independent) cell death indispensable for mouse morphogenesis (Cande et al., 2002).

A definite role (or roles) of Rho kinase (ROCK) in apoptosis and cell survival has been described (Krawetz et al., 2009a). Although the function of ROCK in these signaling cascades is not fully elucidated, a caspase-induced activation of ROCK (by cleaving ROCK and constitutively activating the kinase domain) has been shown (Chang et al., 2006). In a hypothetical model, ROCK inhibition might thus diminish caspase-mediated apoptosis signals, whereas heat-shock treatment, through Hsp, blocks caspase-independent apoptogenetic activity in hESC, consistent with the additive effect of the combined ROCKi+ heat shock treatments described here.

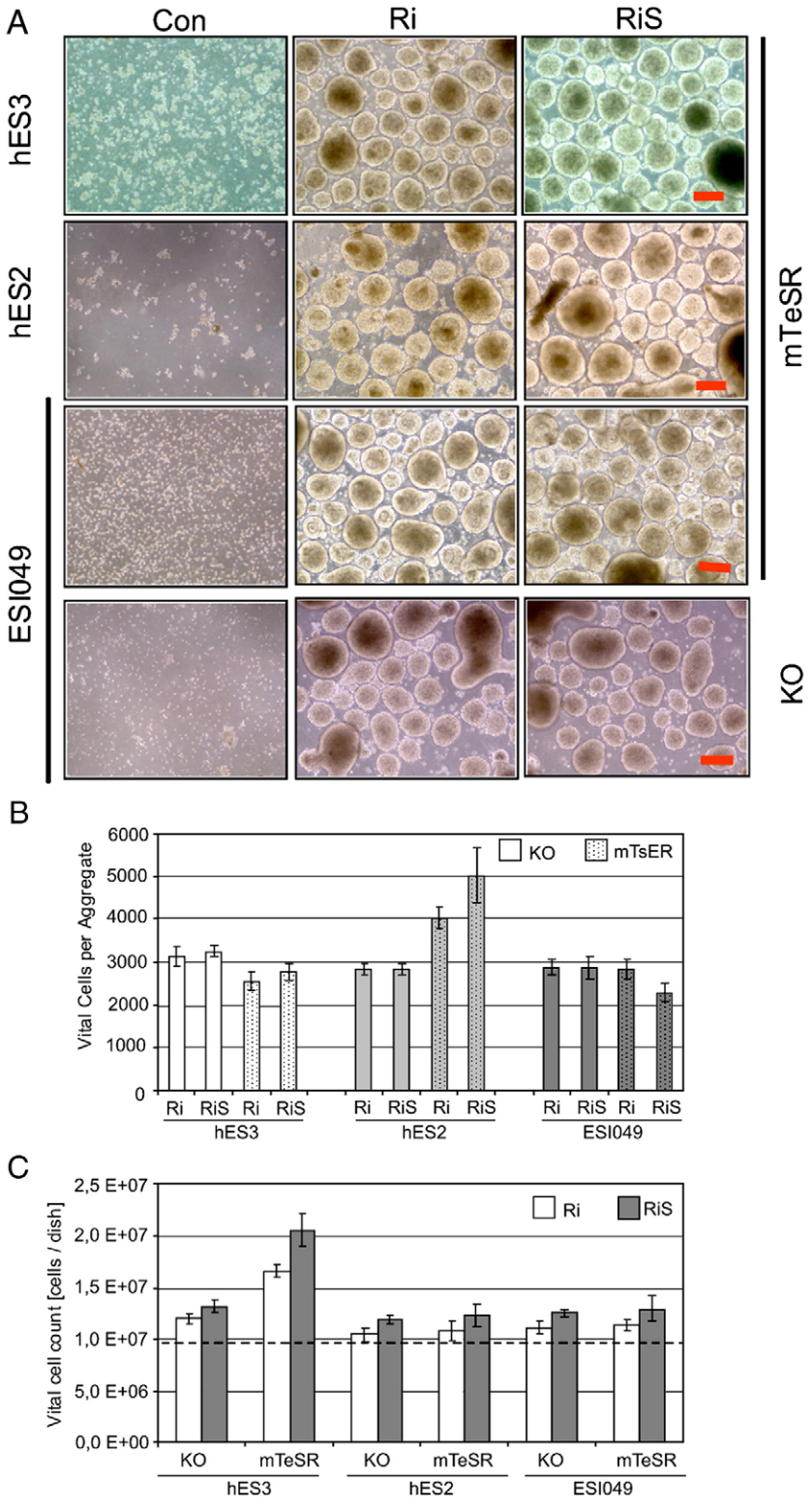
Recently, it was hypothesized that Ri does not protect cells against apoptosis but rather increases the adhesive properties of hESC, thereby enhancing aggregate formation and consequently reducing the extent of anoikis (Krawetz et al., 2009a). Although we can not rule out these possibilities, our data are most compatible with a modified scenario. Analysis of both E- and N-cad levels on conventional 2D hESC cultures versus aggregates derived thereof, revealed an apparent enrichment for an E-cad-high cell population in aggregates (Fig. 2D). We thus propose that: 1) Ri or RiS treatment generally supports/prolongs transient survival of single-cell dissociated hESC in suspension. 2) Cells with a relatively high E-cad level in the suspended population have a higher likelihood to adhere and agglomerate, and are thus enriched in aggregates; non-aggregated cells die quickly as shown by our 24 hrs vitality analysis. 3) Aggregated, E-cad-enriched cells are also enriched for pluripotency marker levels including Tra-1-60 and Oct4. This finding is in accordance with the known expression of E-cad in undifferentiated hESC and its corresponding down regulation upon differentiation (Costa et al., 2005). In summary, these findings suggest that passaging via single cell dissociation and re-aggregation comprises a "purification step" towards more pluripotent cells. Aggregate formation of mouse and human embryonic stem cells is traditionally applied to induce cell differentiation. In differentiation cultures, aggregates are referred to as embryoid bodies (EBs) due to the embryo-like multi-lineage differentiation observed therein. The proposed purification step based on our data provides a feasible mechanism, which, at least partially, explains why our culture method is applicable for the serial passaging and maintenance of hESC, while not promoting differentiation. Interestingly, in mouse ESC, it was also demonstrated that the undifferentiated state of ESC aggregates in suspension can be controlled by constant dissociation and reaggregation upon passaging. It was suggested that the mechanism is likely E-cadherin dependent (Fok and Zandstra, 2005).

Another key factor balancing the culture towards pluripotent cell expansion, as opposed to differentiation, is that of the culture medium. We proved that mTeSR outperforms KO

Figure 3 Aggregate formation and growth kinetics in static suspension culture. A. Light microscopy analysis of aggregate formation in static 6 well-plates over 7 days (one passage). Non-treated controls (Con) and Ri-only treatment is represented for hESC3. Respective results were observed for all three cell lines. Representative results for mTeSR are only depicted on day 7 (d7) due to space limitations. Red scale bars represent 250 μm. B. Increase in the average number of cells per aggregate over time. Analysis on days 1, 3, 5, and 7, revealed that relatively consistent aggregates were formed throughout all 3 cell-lines in KO medium. Data for mTeSR are presented on day 7 to highlight the effect of the medium; see complete mTeSR data set in supplementary Figure S1a. The mean cell number of a sample containing 100-300 aggregates was determined; each bar represents 3 independent samples. C. Growth kinetics representing vital cell count over time (7 day passage cycle) for all three cell lines in KO medium is depicted; results for mTeSR are highlighted on day 7, to highlight the effect of the medium (see full data set in Fig. S1b). The dashed line represents the cell concentration at inoculation. After the expected loss of about 50% of the vital cell count on day 1, an approximate 2-fold net expansion was achieved for all cell lines on day 7. Notably, ESI049 cells treated with RiS and grown in mTeSR resulted in a 3-fold expansion. n = 6 repeats for each bar. D. Statistical analysis of the data shown in 3c on day 7. Mean Ri in KO medium (normalized to 1) was compared to Mean RiS in mTeSR by unpaired two-tailed t-test; $p = 0.02$. On average, cell yields were about 34% higher in mTeSR/Ris conditions.

with respect to short-term cell recovery and long-term expansion over several passages in all culture platforms. Furthermore, only mTeSR medium – optimized for feeder-free, long-term expansion of hESC on defined matrices in 2D (Ludwig et al., 2006) – supported, at least transiently, pluripotency marker expression for the cell line ESI049, being the most differentiation-prone line in our study. This result is very promising, as it indicates the feasibility of maintaining pluripotency even in differentiation-prone hESC lines by altering culture media components.

The observed line-to-line variability, reported here as well as in other studies (Allegrucci et al., 2007), emphasizes the importance of a critical and thorough assessment of new culture methods prior to claiming a general applicability.



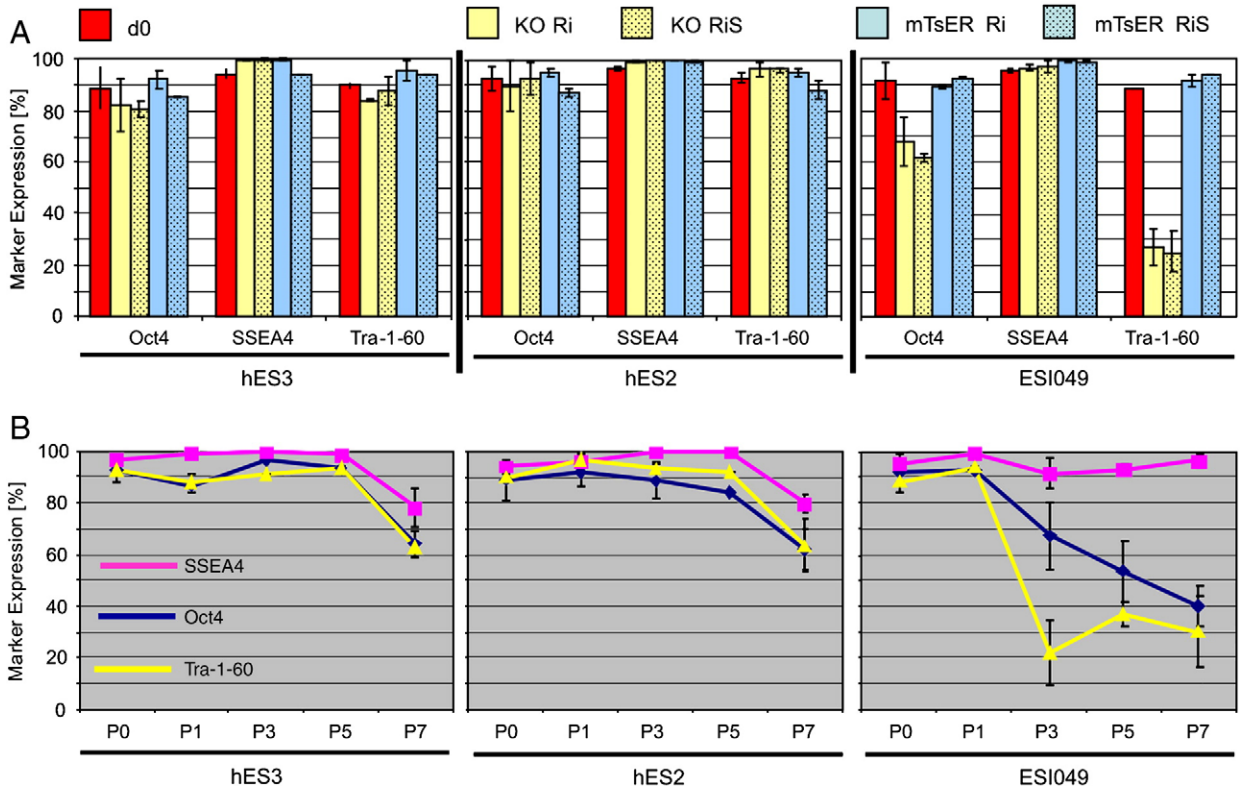


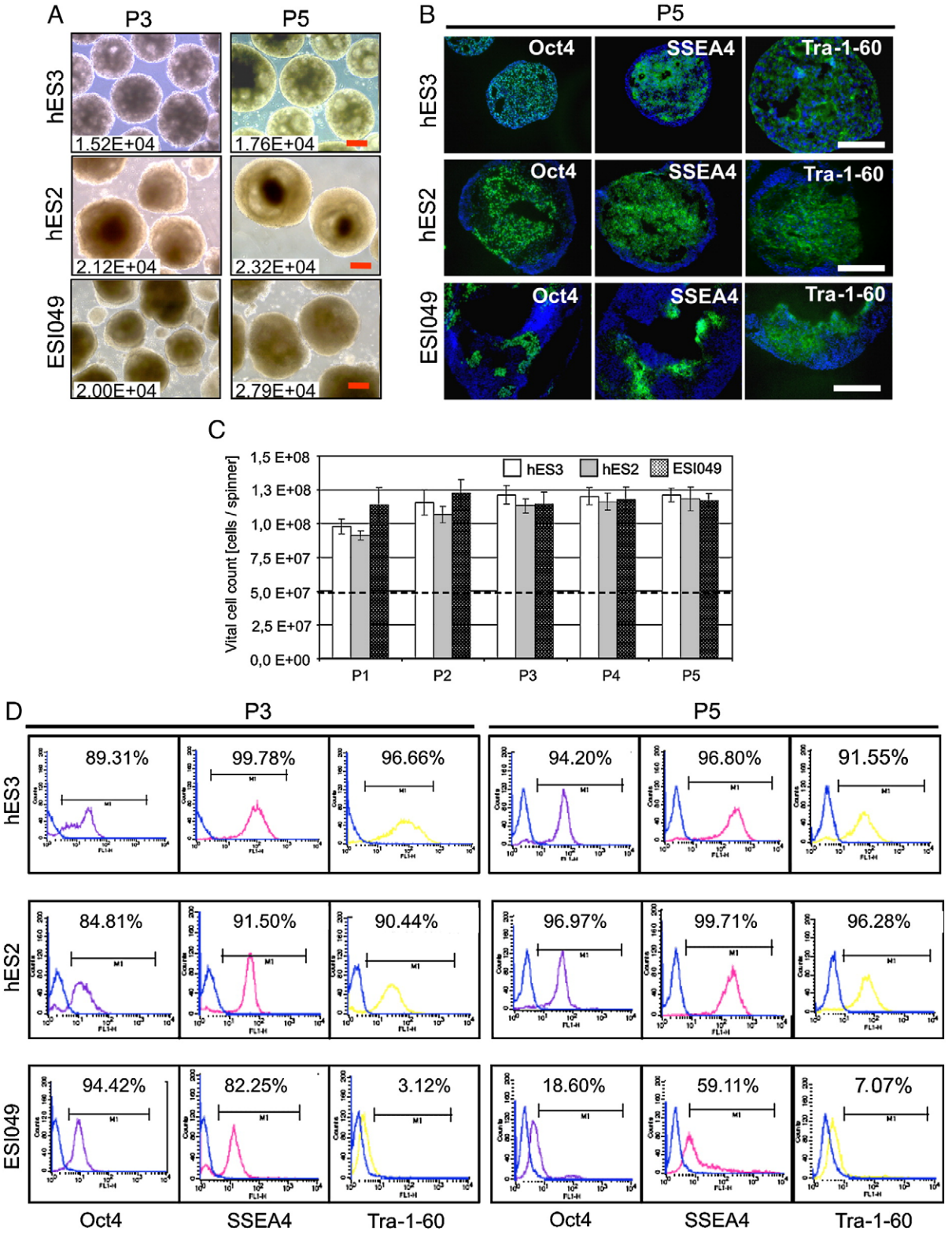
Figure 5 Drastic reduction in pluripotency markers expression in ESI049 cells at passage 3 in dynamic 10 ml suspension cultures. A. Flow cytometry analysis revealed a drastic reduction in pluripotency markers Oct4 and Tra-1-60 expression in ESI049 cells after only one passage in KO medium (yellow bars). In contrast, marker expression was retained in this cell line when cultured in mTeSR (blue bars). Also, no reduction in pluripotency markers expression was observed in hES2 and hES3 cells cultured in both media under identical culture conditions. Red columns represent the expression of the respective markers at inoculation (cells from 2D feeder culture). n = 3 for each bar. B. Subsequently, cells were cultured for 7 passages in mTeSR medium only. hES3 and hES2 cells showed stability in maintaining pluripotency markers expression for up to 5 passages, after which the expression of all three markers was reduced by about 20% or more. In ESI049 cells a drastic drop in Oct4 and Tra-1-60 expression was readily noticed at passage 3, whereas SSEA4 expression remained relatively stable.

Here, we have established a direct, pre-adaptation free translation from 2D-feeder culture into feeder-free suspension for all three hESC lines tested. Cell vitality post inoculation, and cell yields over several passage was highly reproducible and suggested platform-inherent, rather than cell line variability.

In 2D feeder-based cultures utilizing KO medium, the split ratio for all hESC lines was 1:4–1:5 (7 day passage cycle), which is in line with published data for hES2/3 under equivalent conditions (Chin et al., 2009). Since hESC colonies were only partially dissociated for passaging in the 2D platform, the absolute cell yield was not routinely defined, but the established, stable split ratio indicated a 4-5 fold expansion per passage. In single cell inoculated suspension culture, expansion rates dropped to ~2 fold per passage, although the passage duration (7 days) and the culture medium (KO) remained unchanged (Fig. 3C; in mTeSR medium expansion rates were slightly higher). This might suggest that translation to suspension, which includes deprivation of feeder cells, growth in aggregates and other factors, resulted in reduced hESC proliferation rates. Notably, however, a ~50% drop in vital cell count was observed in single-cell inoculated suspension despite the optimized RiS treatment (Fig. 3C), whereas in 2D feeder-

Figure 4 Aggregate formation and growth kinetic in Dynamic 10 ml suspension culture in Petri dishes. A. Light microscopy analysis of aggregates formed on day 7 in mTeSR medium; representative results of ESI049 cells in KO medium are also presented. Note the formation of more homogeneous aggregates in dynamic suspension compared to static 6 well-plates shown in Figure 2A. Red scale bars represents 250 μm. B. The average number of cells per aggregate on day 7 is presented. A relative homogeneous size of about 3,000 cells per aggregate was observed for all three cell lines, with the exception of hES2 in mTeSR medium resulting in 4,000-5,000 cells per aggregate after Ri or RiS treatment, respectively. For data generation, the average cell number of a sample containing 100-300 aggregates was determined; each bar represents 3 independently derived samples. C. Cell yield on day 7. With the exception of hES3 cells in mTeSR, only a marginal cell expansion over the inoculated cell count per dish (indicated by the dashed line) was achieved. This might suggest a detrimental effect of the culture platform on cell vitality. RiS treatment was generally superior to Ri-only. Results for non-treated controls (Con) and heat shock only samples are not presented. n = 6 repeats for each bar.

based controls no such cell loss occurred (data not shown). These findings must be accounted for in growth kinetics assessment. Seeding 5×10^5 single hESC/ml in static suspension (6-well platform) resulted in $\sim2.5\times10^5$ vital cells/ml recovery at 24 hrs ($\sim$50% of inoculated cells, Fig. 3C). Applying the vital cell count at 24 hrs as the relevant starting population revealed that a 4-5 fold expansion was indeed achieved in suspension, yielding 1-1.25 $\times10^6$ vital cells/ml on day 7 in KO medium (Fig. 3C; even higher yields were observed in mTeSR). Equivalent results were also achieved in spinner flask (Fig. 6C). This calculation suggests that hESC expansion rates in 2D-feeder cultures were essentially



conserved in 3D suspension, but the cell loss induced by single-cell inoculation entailed the ostensibly limited expansion rates.

Both parameters, the reduction in vital cell count 24 hrs post single-cell inoculation and the expansion rates per passage, remained extremely stable for all cell lines over 7 passages in rotated Petri dishes and 5 passages in spinner flasks. Importantly, this strongly suggests no selective enrichment for a highly proliferative hES cell population via the single cell inoculation-based passaging strategy, which was also supported by a normal karyotype analysis (Fig. 7C). Prolonged single cell-inoculated suspension cultures for up to 12 passages (applying optimized RiS treatment and mTeSR) in the resource-friendly 6-well suspension culture platform also showed no indication of culture adaptation and karyotype changes (data not shown).

Although mTeSR significantly increased vital cell counts post inoculation and per passage compared to KO (Figs. 2B and c, 3c and d), the effect was moderate, resulting in a 20-30% improvement. This is in some contrast to recent findings by Chin and co-workers (Chin et al., 2009). They found that adaptation to feeder-free cultures in mTeSR resulted in an 8-10 fold split ratio of hES2/3 per passage (7 days), as compared to a 4-5 fold split ratio on feeder cell-based controls in KO medium. This (reversible) elevation of hESC proliferation was notably observed in 2D cultures either on matrigel or the more defined extracellular matrix component fibronectin. The presence of matrigel, coupled with the effect of the relatively high surface areas of microcarriers, might also explain the relative high splitting rates of 1:8-1:10 (7d passage) observed by the same group for hES3 cells passaged as clumps on microcarrier in suspension (Oh et al., 2009). Vital cell loss post single-cell inoculation and the formation of aggregates without any external addition of matrigel and microcarriers might consequently explain the lower splitting/expansion rates observed in our study. On the other hand we have achieved a remarkable cell density of >2 million cells/ml in spinner flasks. This density is in the range of 1.5-3.5 million cells/ml observed on matrigel-coated microcarriers (Oh et al., 2009) and at least 4 fold higher compared to the maximal density of ~0.5 cells/ml which was achieved in a single cell-inoculated spinner flask platform for the H9 cell line (Krawetz et al., 2009b).

Our focus on up-scaling demonstrated that despite a ~50% initial loss of vital cells at every inoculation, a greater than 2-fold net expansion per passage was still achieved. The duplication of cell numbers at every passage and the remarkable cell density achieved also accommodates an envisioned up-scaling to larger bioreactors. Scaling from an initial 50 ml culture to 125, 250, 500, and 1000 ml volumes over 5 passages (in respective vessels), can therefore be considered to be highly feasible. Thus, the initial inoculum of 5×10^7 cells per spinner flask would hypothetically result in a substantial cell yield of at least 1.6×10^9 cells over 5 passages, per vessel. Continuation of our comprehensive analysis beyond 5 passages, for 3 parallel spinner cultures per cell line, was beyond the scope of this study due to logistical and economical limitations. However, the calculated yield of $\sim1.6\times10^9$ cells after 5 passages in bioreactors readily comprises a useful quantity of "raw material" for the generation of therapeutic progenies for a single patient (Zweigerdt, 2009).

Long-term maintenance of pluripotency marker expression was only achieved for hES2 and hES3 cells; both lines were only available to us at high passage numbers. Although we did not detect any karyotypic abnormalities in any of the suspension culture-expanded lines, progressive accumulation of epigenetic heterogeneity, as well as genomic alterations at a sub-chromosomal level in hESC cultures have been reported (Allegrucci et al., 2007; Maitra et al., 2005; Tanasijevic et al., 2009). Such changes, relative to early-passage lines, are also known to exhibit altered responses to growth factors, and the subsequent differentiation of hESCs. This may explain why only lines at relatively high passage numbers were ultimately compatible with undifferentiated expansion in suspension aggregates. In contrast, ESI049 cells utilized at passage numbers 20-36 were found to differentiate relatively quickly, despite the use of mTeSR and the observed enrichment for pluripotent cells following re-aggregation after passaging. This observation is of importance, since ESI049 is a recently derived, clinical-grade hESC line (Crook et al., 2007), which is considered to be ideal for regenerative medicine. Thus, our study highlights the need for extensive testing of novel culture platforms, particularly by using cell lines at low passage numbers that would be relevant to therapeutic applications.

During the progress of this study, the cultivation of hESC in suspension cultures that also utilized Y-27632 was reported (Krawetz et al., 2009b). In line with our findings, the addition of Ri at a 10 μM concentration resulted in aggregate formation from dissociated cells. Surprisingly, and

Figure 6 Growth kinetic, flow cytometry, and immunohistochmical analysis of cells generated in stirred 50 ml spinner flasks in mTeSR medium. A. Light microscopy analysis of aggregates at day 7 passages 3 and 5, respectively. Relative round and homogeneous aggregates were formed in spinner flasks. The numbers in the lower left corner indicated the average number of cells per aggregate. Notably, these numbers were about 10-times higher compared to aggregates generated in the other platforms. Aggregates also appeared more structured including light or dark areas suggest the formation of cavities. Red scale bars represent 250 μm. B. Cavities were indeed observed on aggregate sections derived from passage 5, day 7 cultures. However, as expected from the flow cytometry analysis (Fig. 5B), relative homogeneous anti-pluripotency marker-specific immunofluorescence staining (depicted in green) was observed throughout sections, as obtained from hES2 and hES3 aggregates. However, an outer rim was notably negative for the markers tested. Aggregates from ESI049 cells had much larger cavities (cysts) and only a relatively small cluster of positive stained cells were observed. White bars represent 500 μm; nuclear DAPI stain is depicted in blue. C. Depicted are cell yields obtained at the end of passages 1-5. An approximate 2-fold expansion of the inoculated cell number (indicated by a dashed line) was observed for all cell lines, suggesting a high reproducibility of the culture system. n = 3 independent spinner flasks runs were performed in parallel for each cell line for all passages. d. Although the cell yield remained stable over 5 passages, a drop in Tra-1-60 expression was observed for ESI049 cells at passage 3. Analysis at passage 5 further revealed a drastic reduction in Oct4 and SSEA4 expression in this cell line. In contrast, all markers remained highly expressed in hES2 and hES3 cells.

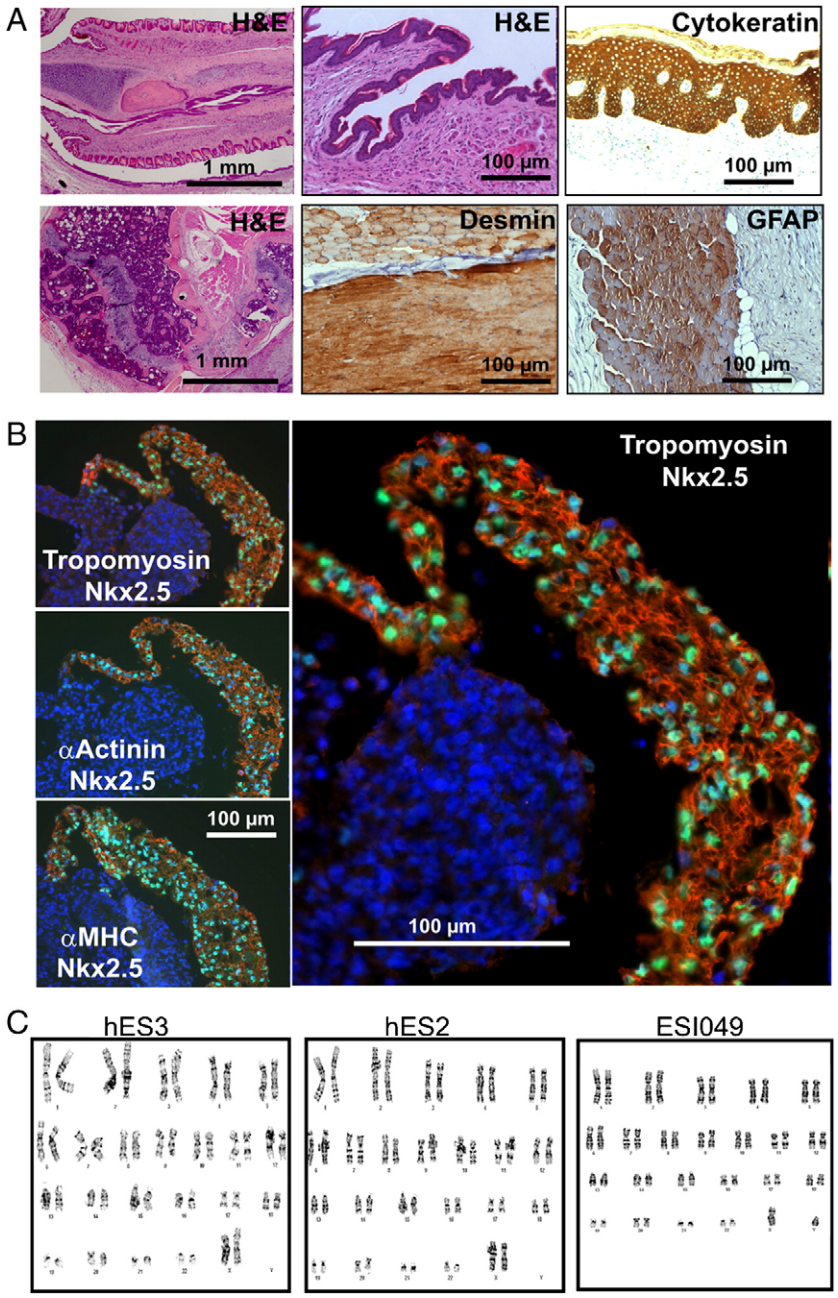


Figure 7 In vivo and in vitro differentiation and karyotype analysis of cells generate in spinner flasks at passage 5, day 7. A. Teratoma formation was observed at 5 weeks after intramuscular cell injection. Upper and lower pictures on the left depict overview graphs of teratoma sections showing the formation of typical epithelial and mesenchymal structures, suggesting the formation of tissues representing all 3 germ layers; higher magnification of an H&E staining (top middle) showing epithelial structures in more detail. Specific anti-Cytokeratin (endoderm, upper right), anti-Desmin (mesoderm, lower middle), and anti-glial fibrillary acidic protein (GFAP, ectoderm, lower right) immunostaining was also detected (Diaminobenzidine stain shown in brown). B. Spinner flask-derived aggregates were exposed to directed differentiation applying a protocol supporting cardiomyogenesis. Contracting embyoid bodies at day 12 of differentiation were sectioned and immunofluorescence staining specific to cardiomyocyte markers Tropomyosin, α-MHC (α-myosin heavy chain), and α-Actinin was performed (red). Nuclear anti-Nkx2.5-specific staining is shown in green. A higher magnification of the Tropomyosin / Nkx2.5-specific staining is depicted to display specificity of the stain and cytological structure of the preparation. Cell nuclei were stained with Hoechst (blue) dye. C. Karyotype analysis revealed chromosomal integrity of all three cell lines after spinner flask expansion. At least 20 Metaphase spreads for every cell line were prepared and no macroscopic aberrations were identified.

in apparent contrast to our study, the addition of Rapamycin was mandatory to maintain persistent aggregate integrity. It remains to be tested whether this observation is specific to the hESC line H9, as used in the aforementioned study (unfortunately without providing information on passage numbers), or if it was inherent to other experimental

parameters. This, once again, illustrates the importance of robust testing using different lines and passage numbers during platform development.

Another aspect of the present study is the observed relative sensitivity of suspension cultured hESC to shear stresses. Inoculation of cells under the same conditions with respect to the inoculation density, medium quality, and medium change frequency resulted in a modest net expansion on agitated Petri dishes, whereas stirring with a bulb-shaped pendulum in spinner flasks reproducibly resulted in a higher, minimum 2-fold net cell expansion. In previous studies, mouse ESC appeared to be robust and showed equivalent growth characteristics on several platforms, including rotating Petri dishes, stirred spinners, and bioreactors of up to 2-litre capacity, stirred by pitch-blade impellers (Schroeder et al., 2005; zur Nieden et al., 2007; Niebruegge et al., 2008; Fok and Zandstra, 2005; Zandstra et al., 2003; Zweigerdt et al., 2003). This highlights that, in apparent contrast to mouse ESC, hESC cultures might be more sensitive to the respective culture platform. Thus, impeller design, the type of culture aeration (i.e. sparger-based bubble generation vs. bubble free), and other bioreactor characteristics will play a pivotal role in the future translation to larger reactor vessels. To date, only very few studies on hESC in controlled bioreactors exist, which focus mostly on differentiation (Gerecht-Nir et al., 2004; Niebruegge et al., 2009).

Conclusions

By focusing on the translation to stirred systems, we have shown that our methodology supports the mass expansion of pluripotent cells in defined suspension culture. Moreover, the novel application of heat shock in conjunction with a small molecule ROCK inhibitor was found to enhance cell survival and increase overall cell yield. We further demonstrated that both *in vivo* and *in vitro* differentiation of bioreactor expanded cells could be performed, highlighting that our up-scaling methodology is highly robust, and yet flexible, thereby potentially enabling numerous other differentiation applications. Importantly, our study exemplifies the need for extensive testing of novel culture platforms. While hES3 and hES2 cells were successfully up-scaled and yet maintained high levels of pluripotency, ESI049 cells (used at low passage numbers) exhibited loss of pluripotency markers upon serial passaging. In summary, our results clearly suggest that our up-scaling strategy is highly robust, practical and has significant potential for the mass expansion of pluripotent cells. Nevertheless, our data underscore the established variability of hESC cell lines with respect to culture conditions. Moving forward, cell lines at low passage numbers (potentially of high clinical relevance) should be subjected to similarly stringent levels of testing.

Material and Methods

hESC culture

2D hESC cultures were dissociated into single cells with TrypLE SelectTM (Invitrogen), strained through a 40 µm straiThree hESC lines were used: hES2, hES3, and ESI049 (ES Cell International, Singapore), with passage numbers ranging from 138-166, 83-123 and 20-36, respectively. For conventional 2D culture, cells were grown on γ-irradiated human foreskin fibroblasts in KO medium (KnockOut DMEM, 20% KnockOut Serum Replacement, 50 ng/ml bFGF, 2 mM L-glutamine, and 1% MEM Non Essential Amino Acids; Invitrogen) and passaged every 7 days, with a split ratio of 1:3 or 1:4.

Experimental Platforms

2D hESC cultures were dissociated into single cells with TrypLE SelectTM (Invitrogen), strained through a 40 µm strainer (BD Falcon), and resuspended in either KO or mTeSR (Stemcell Technologies). The ROCK inhibitor Y-27632 (Merck) was added to the single-cell suspension at 10 µM. Heat shock treatment was performed for 30 min at 43 °C in a cell culture incubator at 0-8 hrs after cell dissociation. While media changes were performed daily, the addition of the Ri or heat shock application were only performed once at culture inoculation. For passaging, aggregates were treated with Collagenase IV (1 mg/ml) for 45 min and dissociated with TrypLE SelectTM for 10-30 min. Cell counts were performed with a hemocytometer (Improved Neubauer, Brand, Germany) after trypan blue staining, applying a standard protocol.

Static suspension cultures: ultra-low attachment plates (Corning) were seeded with 2 ml of cell suspension at a concentration of 2.5×10^5 cells/ml per well.

Stirred Petri dish cultures: dishes (BD Falcon) were inoculated with 10 ml cell suspension at 1×10^6 cells/ml and rotated at 35 revolutions per minute (rpm). Trials demonstrated the formation of irregular aggregates at 20-30 rpm, while 40-50 rpm produced detrimental effects.

Spinner Flask cultures: 100 ml spinner flasks were inoculated with 5×10^7 cells in 50 ml culture volume (1×10^6 cells/ml) in mTeSR and stirred at 40 rpm using a single stirring pendulum (CELLSPIN, IBS Integra Biosciences). Initial trials revealed that a stirring speed below 40 rpm resulted in extensive cell clumping while 50 rpm and above yielded lower cell numbers compared to 40 rpm.

For static suspension and stirred Petri dish cultures 6 independent experiments – that is duplicate experiments of 3 biological repeats - were performed for every cell line at each time point and experimental setting; 3 single biological repeats were performed for the spinner flask platform.

Cardiomyogenic differentiation: Aggregates from spinner flasks were cultured overnight in serum-free medium and further supplemented with SB203580 (Sigma) as previously described (Xu et al., 2008b,c).

Flow Cytometry

Cells were treated with the Cytofix/Cytoperm™ Permeabilization Kit (BD Biosciences) and incubated with primary antibodies specific to: Oct4 (1:10, Santa Cruz), SSEA4 (1:50, Millipore), Tra-1-60 (1:40, Millipore), E-Cadherin (1:50, Santa Cruz), and N-Cadherin (1:50, Sigma). The secondary antibody used was goat anti-mouse FITC conjugated (1:100

Invitrogen). Analysis was performed on a FACSCalibur machine (BD Biosciences).

Teratoma studies

5×10^6 cells suspended in 100 µl KO were injected into the right hind leg quadriceps of male SCID mice as previously described (Hentze et al., 2009); Teratomas were harvested and fixed in Bouin's Solution for analysis. Studies were approved by the Biological Resource Center, Biopolis Singapore, Institutional Animal Care and Use Committee (IACUC No. 080314), and the National University of Singapore Institutional Review Board (NUS IRB No. 08-188).

Immunohistochemistry

Aggregates were fixed in 4% PFA, preserved in 25% sucrose, snap-frozen in OCT (Leica), and sectioned at 6 µm. After blocking, sections were incubated with primary antibodies against Oct4, SSEA4, Tra-1-60 (see above), αMHC (1:200, Developmental Studies Hybridoma Bank), α-Actinin (1:800, Sigma Aldrich), Tropomyosin (1:500, Sigma Aldrich), and Nkx2.5 (1:500, Santa Cruz). Secondary antibody: goat anti-mouse FITC conjugated (1:100 Invitrogen).

For teratoma analyses paraffin sections were generated. Primary antibodies: Polyclonal Rabbit anti-Cytokeratin (1:250), Desmin Clone D33 (1:100), and Rabbit anti-GFAP (1:250) all from Dako. Secondary antibodies: EnVision+HRP anti-Rabbit, and EnVision+HRP anti-Mouse (all undiluted from Dako). Samples were incubated with Chromogen DAB (Dako) and counter-stained with Haematoxylin.

Karyotyping

Cytogenetics analysis was performed at the Cytogenetics Labs at the KK Women's and Children's hospital, Singapore. Actively growing cells derived from the respective suspension culture platform(s) were arrested in metaphase following incubation with colcemid. 20 metaphase spreads were examined for each condition.

Statistics

MINITAB® software release 14.13 was used. Two-tailed paired and unpaired t-tests were applied respectively. Asterisks indicate the level of significance as follows: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. The respective number of experimental replicates is indicated in the results.

Acknowledgments

We gratefully thank Prof. Birgit Lane (Institute of Medical Biology) and Prof. Alan Colman (Singapore Stem Cell Consortium) for their exceptional support. We thank Drs. Keith Rogers and team and Declan Lunny for the teratoma analysis, and Dr. Suzanne Dorfman for proof-reading this manuscript. The entire project was funded by.the Agency for Science, Technology and Research (A*STAR).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2010.03.001.

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