Efficient Propagation of Single Cells Accutase-Dissociated Human Embryonic Stem Cells

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ABSTRACT Human embryonic stem cells (hESCs) hold great promise for cell-based therapies and drug screening applications. However, growing and processing large quantities of undifferentiated hESCs is a challenging task. Conventionally, hESCs are passaged as clusters, which can limit their growth efficiency and use in downstream applications. This study demonstrates that hESCs can be passaged as single cells using Accutase, a formulated mixture of digestive enzymes. In contrast to trypsin treatment, Accutase treatment does not significantly affect the viability and proliferation rate of hESC dissociation into single cells. Accutase-dissociated single cells can be separated by FACS and proliferate as fully pluripotent hESCs. An Oct4–eGFP reporter construct engineered into hESCs was used to monitor the pluripotency of hESCs passaged with Accutase. Compared to collagenase-passaged hESCs, Accutase-treated cultures contained a larger proportion of undifferentiated (Oct4-positive) cells. Additionally, Accutase-passaged undifferentiated hESCs could be grown as monolayers without the need for monitoring and/or selection for quality hESC colonies. *Mol. Reprod. Dev.*

Key Words: human embryonic stem cells; single cells dissociation; FACS separation

INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass of human embryos at the preimplantation stage that are capable of in vitro differentiation into virtually every cell type found in the adult body (Thomson et al., 1998). Initially, hESCs were isolated and passaged by mechanical dissociation (Thomson et al., 1998). Since the development of this technique, several variations for the derivation and maintenance of hESC were reported (Xu et al., 2001, 2005; Amit et al., 2004; Klimanskaya et al., 2005; Stojkovic et al., 2005). However, most approaches require close human supervision of the quality of hESC colonies. To maintain the high quality of hESC cultures, colonies having a healthy, undifferentiated morphology are visually identified, manually selected, and passaged as clusters (alternatively, colonies having poor differentiated morphologies are removed from culture). Recently, an automated method for mechanical hESC passaging was proposed (Joannides et al., 2006). However, this approach requires specialized, custom-produced equipment and the ability of this system to maintain the pluripotency of hESC cultures over many passages has not been documented.

Passaging of hESCs as clusters imposes major limitations for downstream applications requiring single cell preparations, such as cell separation by flow cytometry (FACS) or the dispensing of live hESCs into multiwell plates for use in high throughput screening. However, in contrast to murine ESCs, which can be efficiently passaged by dissociation into single cells, the dissociation of hESCs into single cells limits their ability to survive and expand as pluripotent cultures likely resulting from physical damage (Amit et al., 2000). Recently, the derivation of 17 lines that can be propagated using trypsin dissociation has been described (Cowan et al., 2004), but these hESCs are also propagated as small clusters rather than single cells. Dissociation of hESCs to single cells with trypsin results in a dramatic decrease in plating efficiency (<3%) during the first 5–10 passages and the emerging sublines that arise invariably acquire detectable karyotypic abnormalities after 30 passages (Hasegawa et al., 2006).

Here we apply Accutase (a commercially available cell detachment solution) for single cell propagation of pluripotent hESCs. Contrary to trypsin dissociation, Accutase treatment does not significantly affect the plating efficiency of hESC dissociation into single cells. Using an hESC line engineered with an Oct4–eGFP reporter construct demonstrates that cultures dissociated with Accutase to single cells at each passage maintain a higher proportion of Oct4/eGFP-positive cells as compared to collagenase-passaged hESCs. Accutase-treated hESCs can be grown to a high density as monolayers, and yet retain their pluripotency. The single cell dissociation with Accutase does not require human supervision for selection of colonies with

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undifferentiated morphologies and thus could be easily adopted by any laboratory currently using regular cell culture techniques.

RESULTS

Single Cell Accutase Dissociation of hESCs Does Not Decrease Plating Efficiency

Treatment of hESC for H9 line colonies with Accutase resulted in the complete dissociation of each colony to a single cell (Fig. 1A) suspension over 5–10 min. In contrast to collagenase, which digests the extracellular matrix, Accutase digests cell surface proteins that mediate contacts between cells as well as cell-matrix contacts. Thus, Accutase treatment allows the efficient dissociation of cells both in the middle of the hES cell colony and those cells on the periphery (Fig. 1A). Cells with differentiated morphologies (e.g., feeder mouse embryonic fibroblasts (MEFs)) require less time to detach from the plastic, allowing efficient cleaning of loose cells with intermediate PBS washes (Fig. 1A). After gentle trituration, hESCs can be easily dispersed into small clusters and additional trituration resulting in single cell suspensions (Fig. 1A).

To determine if dissociation of hESCs to single cells with Accutase affects cell viability, the proliferation kinetics of collagenase-, Accutase- and trypsin-passaged hESCs were compared. In contrast to trypsin dissociation, Accutase-dissociated single hESCs maintained a high proliferation capacity from the first day in culture (Fig. 1B). In fact, no significant differences were observed between collagenase and Accutase propagated hESCs (Fig. 1C). Similar kinetics was observed for the second consecutive passage (Fig. 1D,E). In contrast, trypsin-dissociated single hESCs failed to proliferate and form colonies, in agreement with the very low plating efficiency and proliferation rate reported earlier (Hasegawa et al., 2006). In subsequent passages collagenase- and Accutase-treated cells also had similar proliferation kinetics (data not shown). These results suggest that, in contrast to trypsin dissociation, the Accutase treatment does not result in massive cell death. The majority of hESCs present in the culture continue to proliferate and form numerous colonies. Several different hESC lines were propagated with Accutase for various numbers of passages; all retained their proliferation rate and undifferentiated morphology of hESC colonies, as well as the ability to terminally differentiate (Supplementary Table 1). No significant differences occurred between various hESC lines in any of the above outcomes following Accutase treatment. The focus of this study is on the H9 clone engineered with an Oct4–eGFP expression cassette and propagated with Accutase by dissociation to single cells for over 20 passages.

Generation and Validation of hESCs Engineered With an Oct4–eGFP Cassette

H9 cells engineered to express eGFP under the control of 4 Kb Oct-4 promoter, which is known to drive specific reporter expression in blastocyst and pluripotent ES cells (Scholer et al., 1990; Yeom et al., 1996; Ovitt and Scholer, 1998; Fuhrmann et al., 1999; Gerrard et al., 2005), were used to monitor the propagation of pluripotent hESCs. To verify the fidelity of the Oct4–eGFP reporter, immunostaining with Oct4-specific antibodies was used to detect endogenous Oct4 protein levels as compared to eGFP reporter activity (Supplementary Fig. 1).

eGFP was expressed in all cells that were positive for endogenous Oct4 protein. A few cells weakly positive for eGFP and negative for endogenous Oct4 protein, were found scattered throughout the colony. Individual cell counts showed that weakly eGFP-positive cells represented less than 0.1% of all eGFP-positive cells (i.e., 99.9% of eGFP-positive cells are also positive for the endogenous Oct4). The slow degradation of eGFP is likely to be responsible for these weakly eGFP-positive, Oct4-negative cells. Differentiated cells (e.g., day 6 embryoid body) entirely lack Oct4–eGFP fluorescence (data not shown), demonstrating that the lentiviral Oct4–eGFP cassette faithfully labels pluripotent hESCs as positive for the endogenous Oct4 protein.

Accutase-Passaged hESCs Form Monolayers

To gain insight into the formation and growth of colonies after Accutase-induced dissociation of hESCs, Oct4–eGFP hES cell colonies were monitored using bright field and fluorescent microscopy. As expected, Accutase-treated hESCs retained Oct4–eGFP fluorescence after dissociation to single cells (Fig. 2A). No substantial decrease in plating efficiency of Accutase-dissociated single hESCs was observed. Collagenase-passaged cells, propagated as small clusters, were monitored in parallel. Accutase-treated single hESCs attached to the plates after 1 day and started to grow as small colonies (Fig. 2B, also see Fig. 7). Twice daily observations of individual growing colonies provided evidence that the majority of colonies grew from individual single cells and did not form via aggregation. Because the Accutase-treated cultures began as single (possibly few) cells, the hESC colonies were smaller in these cultures as compared to collagenase-treated cultures at similar time points. No excessive floating debris or dead cells were observed in these cultures. When compared to collagenase-passaged cultures, the single cell-initiated Accutase colonies grew in denser arrays of smaller colonies. Nine to 10 days after Accutase treatment, the colonies began to fuse, eventually forming a monolayer culture (Fig. 3A). The edges of the fused colonies remained well-formed and maintained the morphology of undifferentiated hESCs. To confirm that the hESCs remained pluripotent in monolayer culture, we examined the eGFP fluorescence of Oct4–eGFP–H9 cells grown in monolayer. Fluorescence microscopy showed that all cells in the monolayer cultures remained as pluripotent, Oct4-positive hESCs (Fig. 3B). Cells within the monolayer had characteristic hESC morphology with clearly distinct nuclei and cytoplasm, and exhibited different phases of cell cycle

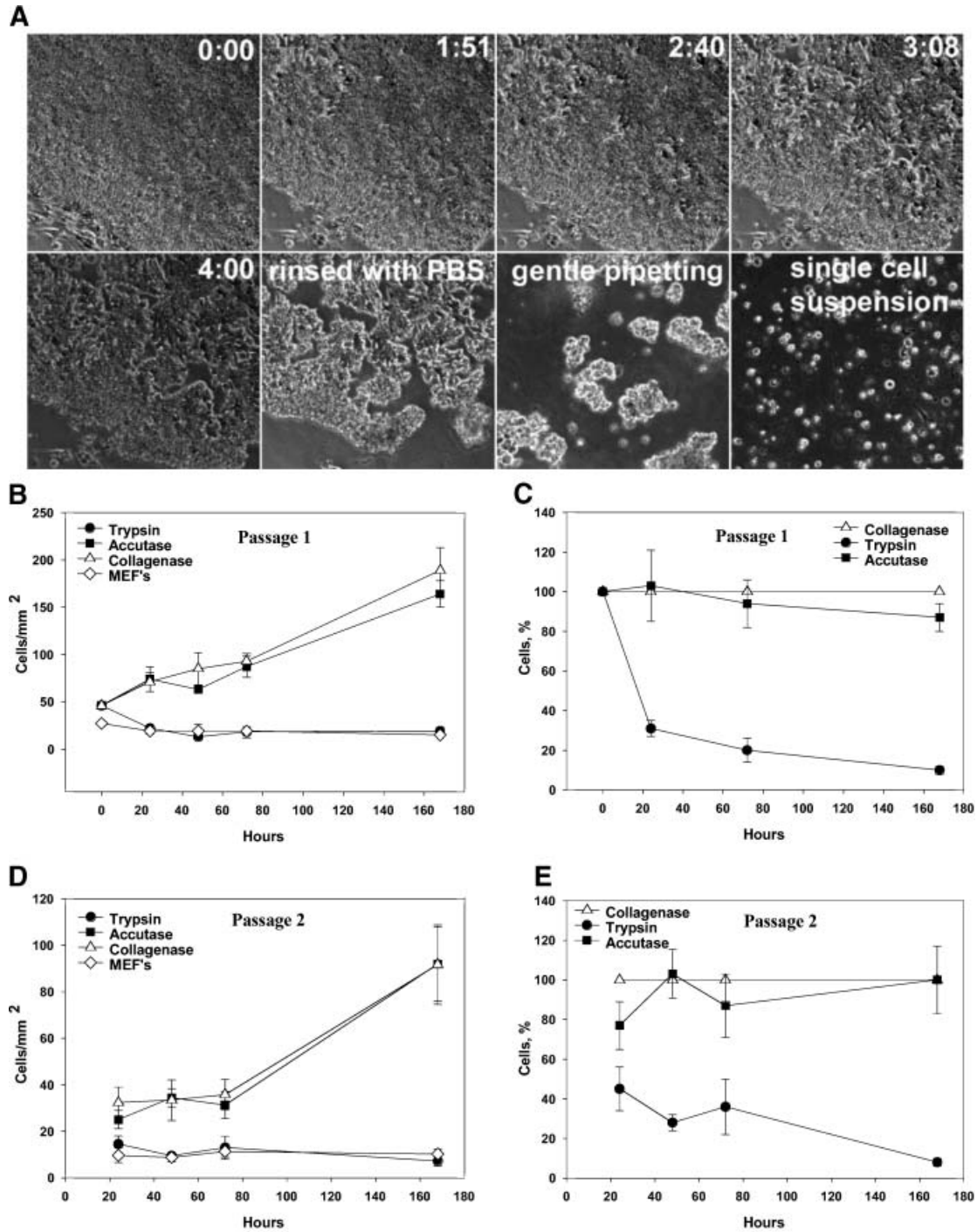

Fig. 1. Accutase dissociation to single cells maintains the proliferation rate of hESCs. **A**: Dissociation of hESC colony with Accutase. Bright field time lapse images (time in minutes:seconds as indicated) of cells in an hESC colony (H9 cells) during Accutase treatment. Note the clear borders of undifferentiated hESC colonies and the absence of feeder and differentiated cells after PBS rinse. At this stage gentle pipetting resulted in small hESC clusters. Additional trituration dissociated hESCs clusters into single cells. **B**–**E**: Standard cultures of hESCs (passaged with collagenase) were split into three equal parts and propagated by dissociation to single cells with Accutase or trypsin (gentle dissociation with 0.05% trypsin). The collagenase-treated cultures were maintained in parallel. B: Total number of cells in each culture over the first 7 days. C: Percentage of hES cells growing in Accutase and trypsin cultures compared to collagenase cultures (always 100%) over the first 7 days. D,E: Similar plots to A and B for the second consecutive passage of hESCs. hESCs were grown on irradiated (mitotically inactive) MEF feeder cells equally present in each culture.

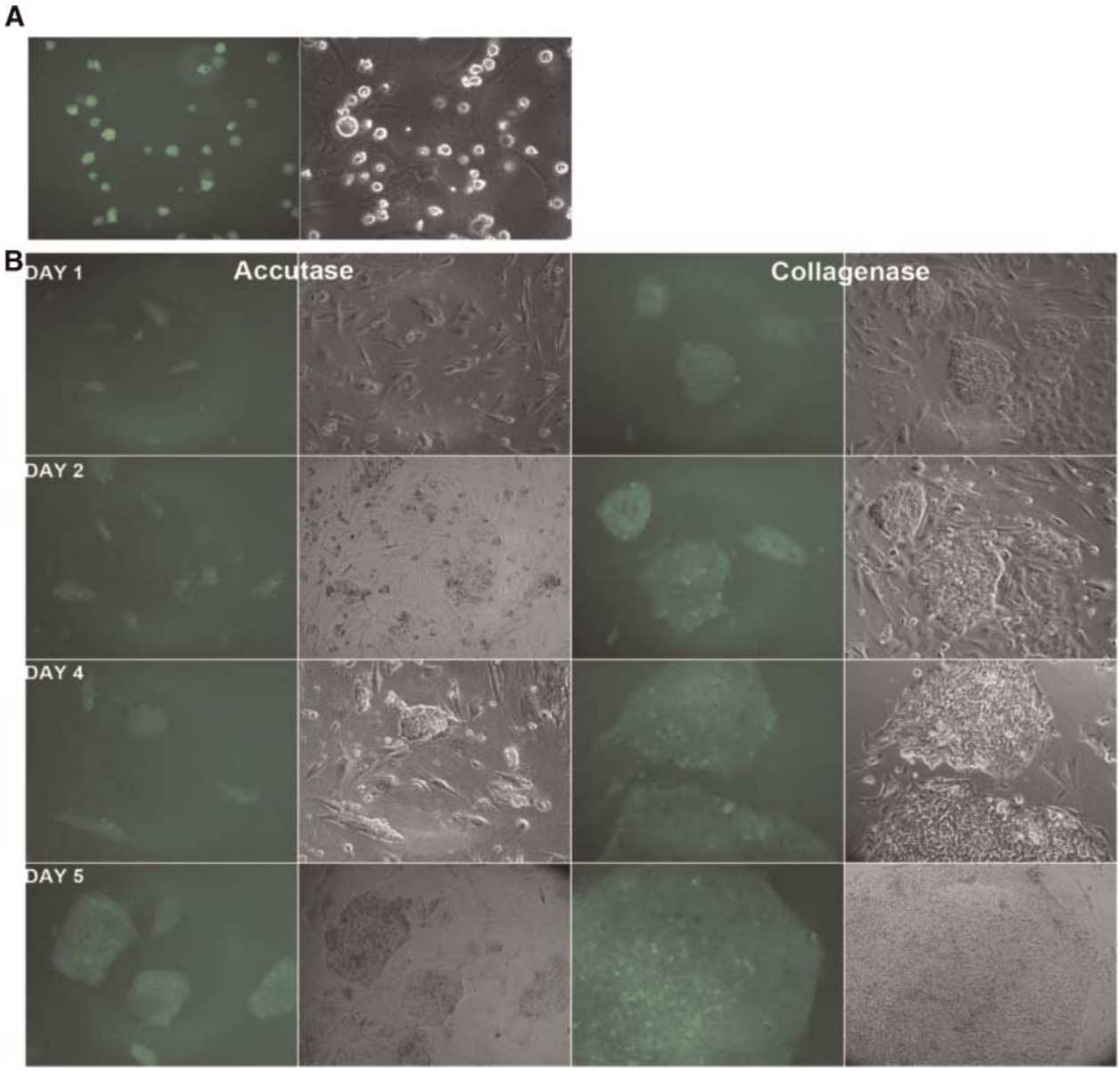

Fig. 2. Visualization of hESC colony growth after passaging. Human ESCs engineered with Oct4–eGFP were treated in parallel with Accutase or collagenase and followed over the first 5 days of culturing with bright field and fluorescence microscopy. **A**: Single cells after dissociation with Accutase (day 0). **B**: Sequential images of hESC colonies after passaging with Accutase (left columns) or collagenase (right columns). Accutase-treated hESC colonies started as single cells and are significantly smaller than the collagenase-treated hESCs colonies, which began as small clusters. The Oct4–eGFP reporter shows the pluripotent state of hESC colonies at all times. At days 4 and 5 after plating Accutase-treated hESCs formed colonies having amorphology characteristic of undifferentiated hESC. [See color version online at www.interscience.wiley.com.]

(Fig. 3C). The formation of hESC monolayers depended on the plating density. When split at a 1:3 ratio, Accutase-dissociated hESCs routinely formed a monolayer around 11–12 days and could be propagated in this way for as many passages as necessary. No adverse effect of monolayer cultures on hESC viability or pluripotency was detected.

Passaging With Accutase Maintains a High Proportion of Pluripotent hESCs

Colonies of Human ESCs passaged with Accutase maintained the characteristic undifferentiated morphology after 15 passages (Fig. 4A). Flow cytometry was used to quantify the amounts of undifferentiated cells within Oct4–eGFP H9 cultures propagated with collagenase and Accutase from passages 9 to 11. A higher percentage of eGFP-positive cells were observed in cultures passaged with Accutase (>90%) as compared to collagenase (~60%; Fig. 4B). In addition, Accutase-passaged Oct4–eGFP hESCs appeared more homogenous and showed a smaller percentage of cells displaying intermediate fluorescence levels (Fig. 4C). To obtain an independent confirmation that hESCs propagated with Accutase remain undifferentiated, we used a H9

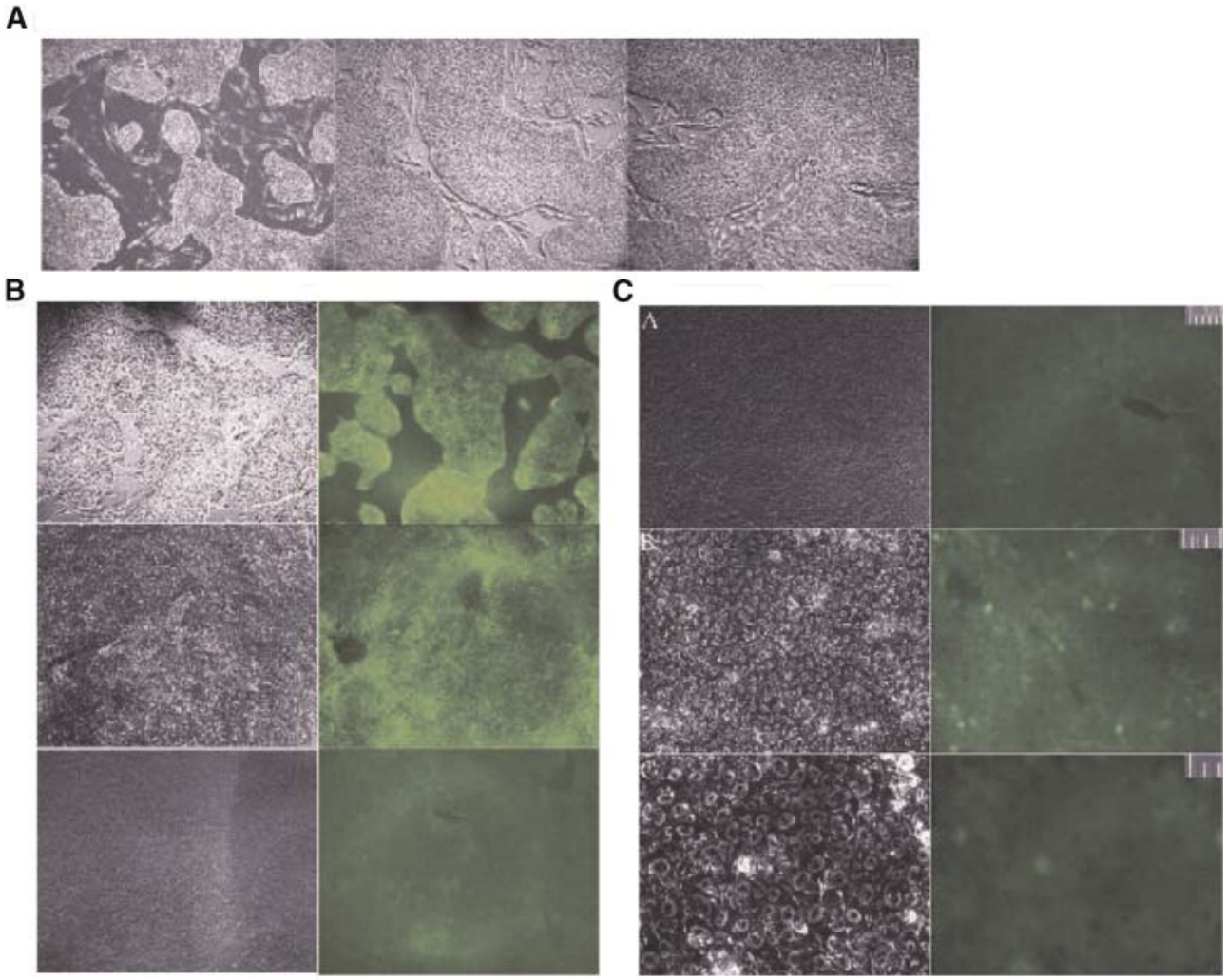

Fig. 3. Accutase-propagated hESCs form monolayers. **A:** Bright field images of day 9 colonies of hESCs (H9) at different passage ratios, 1:9, 1:6, and 1:3, from left to right. Increasing cell density accelerates monolayer formation. **B:** Sequential images of hESC monolayer formation with eGFP fluorescence. The images were captured at day 7, day 9, and day 11; left column, bright field images, right column, eGFP fluorescence. **C**: Images of hESC monolayers at different magnifications (the distance between the small bars in the upper right corner is 10 μm). The nuclei and cytoplasm of the hESCs are clearly visible at higher magnification. [See color version online at www.interscience.wiley.com.]

hESC line that was treated with Accutase for 10 passages. Immunostaining for the key markers of pluripotent hESCs: Oct4, SSEA4, Nanog, and Sox2 (Fig. 5) revealed that a high proportion of cells within hESCs colonies were positive for these markers. These data strongly suggest that hESC cultures passaged with Accutase maintained a higher proportion of undifferentiated cells. In addition, our comparative analysis indicates that Accutase-passaged hESC cultures maintained a higher proportion of undifferentiated (Oct4-positive) cells than collagenase-passaged cultures.

FACS Isolation and Propagation of Viable Single hESCs

Prospective isolation of cells by flow cytometry is critical for numerous applications. However, by the definition of this technique, FACS procedure requires single cell preparations of viable cells. This single cell requirement is a major obstacle for the efficient application of flow cytometry techniques for separating hESCs. We investigated whether Accutase-dissociated single hESCs can be efficiently separated by flow cytometry. The Oct4–eGFP–hESC colonies were dissociated by Accutase and sorted into eGFP-positive and eGFP-negative cells using a Vantage Diva FACS sorter (7-aminoactinomcin D (7-AAD) was used to monitor cell viability). The single cells were deposited directly onto feeder layers of MEFs (Fig. 6, 1 day). FACS-isolated single hESCs quickly attached to the plate and grew as colonies with growth kinetics similar to that seen in Accutase-passaged hESCs that did not undergo FACS (Fig. 6, days 2–12). For example, the small colonies started to fuse and form continuous monolayers around day 10–11. The eGFP-negative cells failed to form well-growing colonies (data not shown). The monolayer cultures formed by FACS-isolated Oct4–eGFP

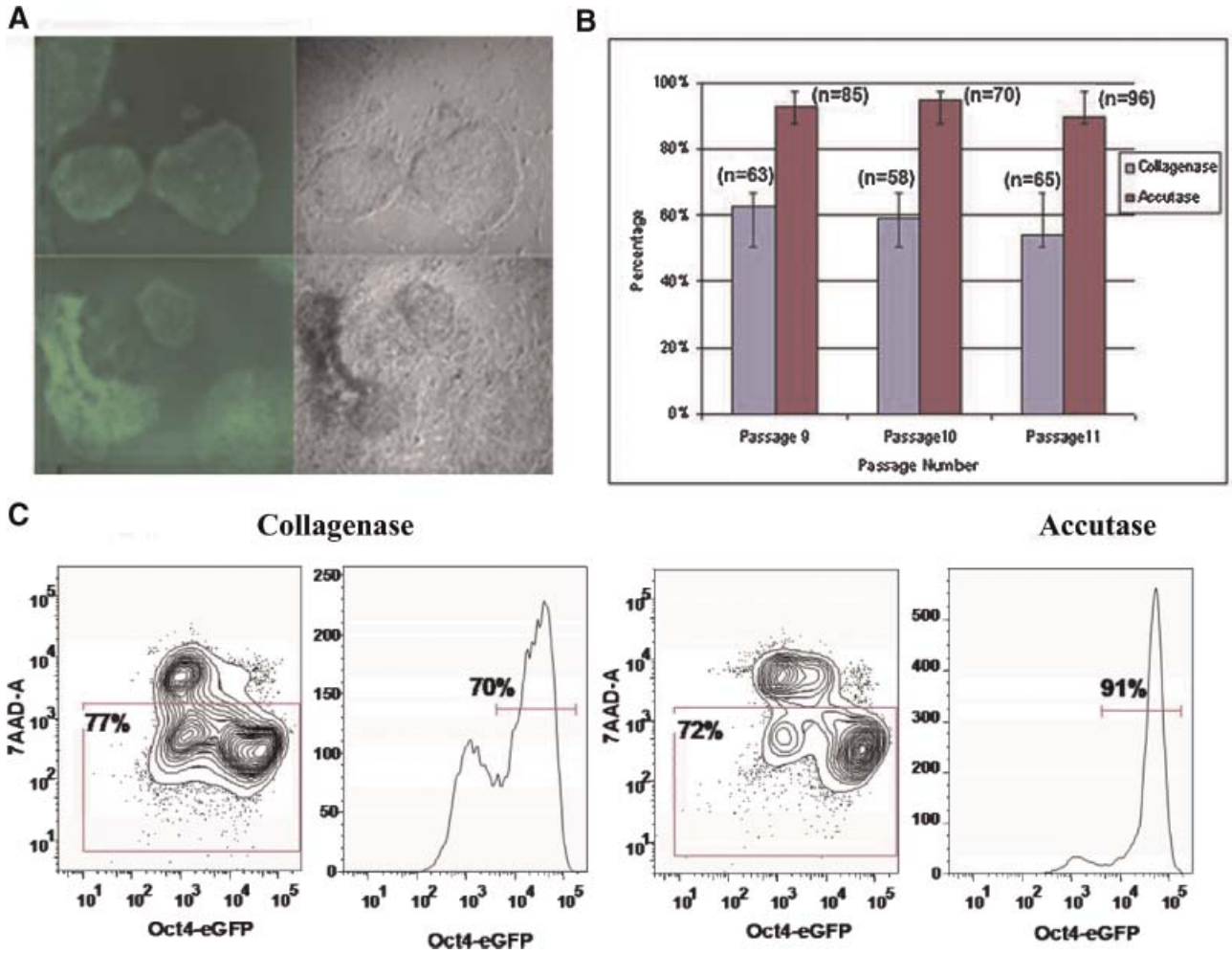

Fig. 4. Accutase-passaged hESCs maintain high levels of undifferentiated cells. **A**: Comparable colonies from Accutase and collagenase treated cultures; most Accutase-passaged cultures (upper part) show uniformly undifferentiated round colony morphology while collagenase-passaged cultures (lower part) often show some differentiation. **B**: The numbers of undifferentiated colonies were counted in both Accutase- and collagenase-passaged hESCs using combined morphological and Oct4–eGFP criteria. The ratio of undifferentiated colonies to the total number of colonies in seven microscopic fields was calculated for each culture at 9–11 passages. Error bars represent standard deviation. **C**: Representative FACS analysis (contour plots at 5% probability and histograms) of eGFP fluorescence of Accutase-passaged hESCs (day 10) and collagenase-passaged hESCs (day 7). The histogram profiles of 7-AAD negative live cells (pink gates in contour plots) are shown along with the percentage of eGFP-positive cells (70% and 91% for collagenase and Accutase, respectively). [See color version online at www.interscience.wiley.com.]

hESCs were analyzed and found to have a similar fluorescence pattern with ~90% of cells falling within the narrow peak of eGFP-positive, undifferentiated hESCs (Fig. 6, inset). The FACS-sorted hESCs were maintained for an additional five passages and behaved identically to the nonsorted hESCs passaged with Accutase. These results demonstrate that hESCs dissociated with Accutase to single cells can be isolated by flow cytometry as viable single cells and propagated as undifferentiated hESC cultures that are indistinguishable from the original cultures.

Differentiation of Accutase-Treated hESCs Into Three Major Germ Layers

Finally, we determined if human ES cells propagated with Accutase were capable of differentiation into the three major germ layers: ectoderm, mesoderm, and endoderm. The human ES cell line H9 and Oct4–eGFP–H9 cells were maintained for 15–20 passages before assaying the differentiation into three germ layers (Fig. 7). The differentiation into neuronal precursors was performed using a protocol recently described by Conti et al. (2005). Under these conditions, Accutase-propagated cells efficiently differentiated into neuroectoderm, as monitored using a Nestin-specific antibody. The differentiation into the mesoderm and endoderm was performed using a classical embryoid body formation protocol (Doetschman et al., 1985). Mesodermal differentiation was monitored using markers for squamous epithelial and myelomonocytic lineages (CD15), muscle (MF20), and endothelium (FLK-1). The differentiation into the primitive endoderm/paraxial mesoderm was assessed using PDGF Rα, labeling during development first the primitive

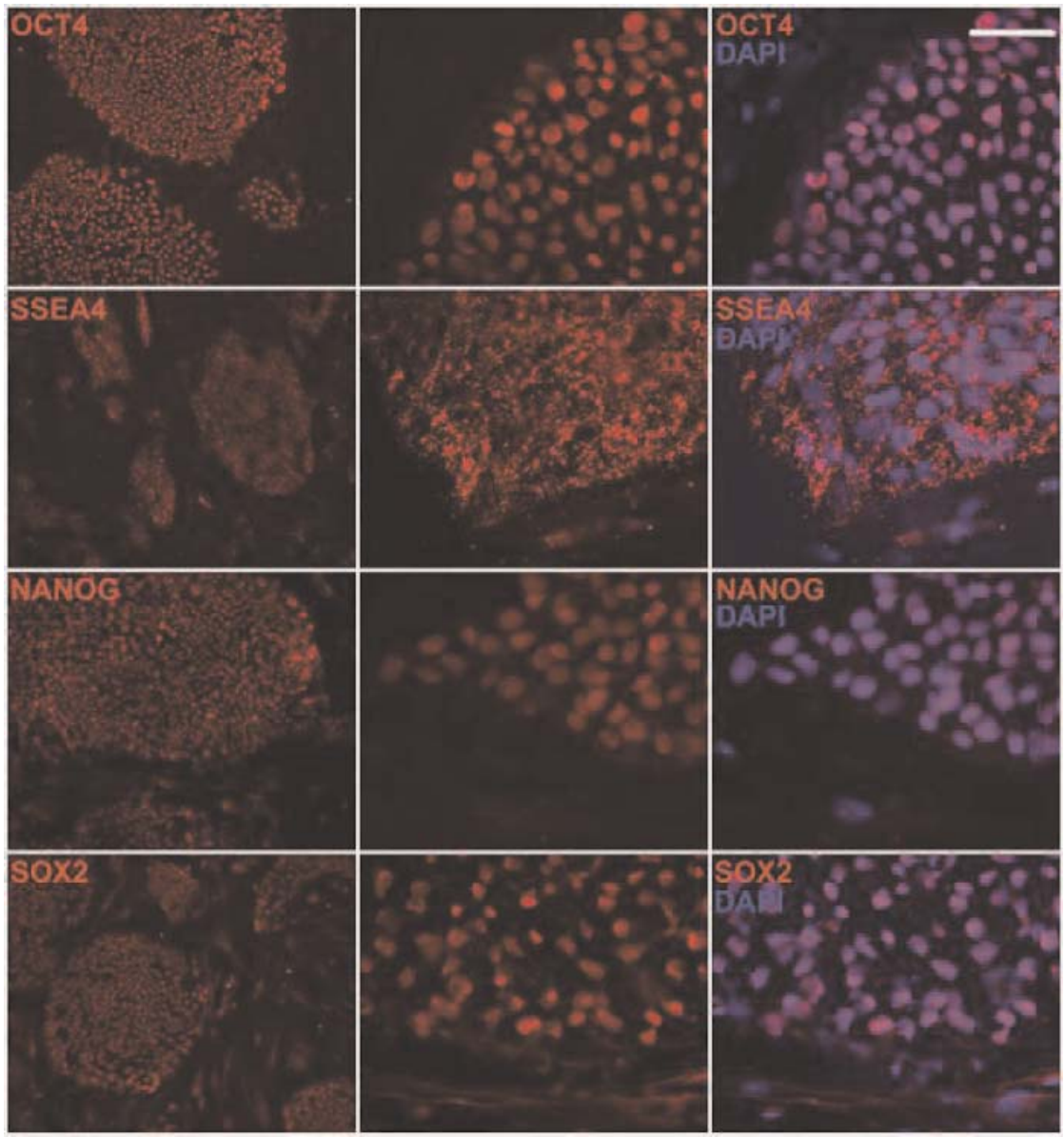

Fig. 5. Accutase-passaged hESCs express markers of pluripotentcy. The H9 hESCs propagated with Accutase over 10 passages were stained with antibodies to Oct4, SSEA4, Nanog, and Sox2. All cells in the colonies were positive for nuclear Oct4, Nanog, and Sox3, and membrane SSEA4. Filamentous structures positive for Sox2 (second column) are the background staining of MEF feeders. Left column, 10× magnification view of a representative colony. Two right columns, 40× magnification, bar is 30 μm. [See color version online at www.interscience.wiley.com.]

endoderm, then paraxial mesoderm, and finally many cell types in the embryonal mesenchyme (Takakura et al., 1997). Differentiation into the endoderm (likely the primitive endoderm) was also monitored using immunostaining against AFP. In summary, the staining revealed that the cells were positive for markers of all germ layers in differentiated hESC cultures propagated with Accutase (Fig. 7). The efficiency of directed differentiation into neural precursors was similar to that of collagenase-treated hESCs (data not shown).

DISCUSSION

Here we describe a technique for hESC propagation with Accutase that allows the simple and routine dissociation of hESC colonies into single cells at each passage. Accutase is a mixture of enzymes with proteolytic, collagenolytic, and DNAse activities and is commercially available from several manufacturers (e.g., Innovative Cell Technologies, San Diego, CA, Chemicon/Millipore, Temecula, CA). Accutase was used previously for passaging different cell types, for example

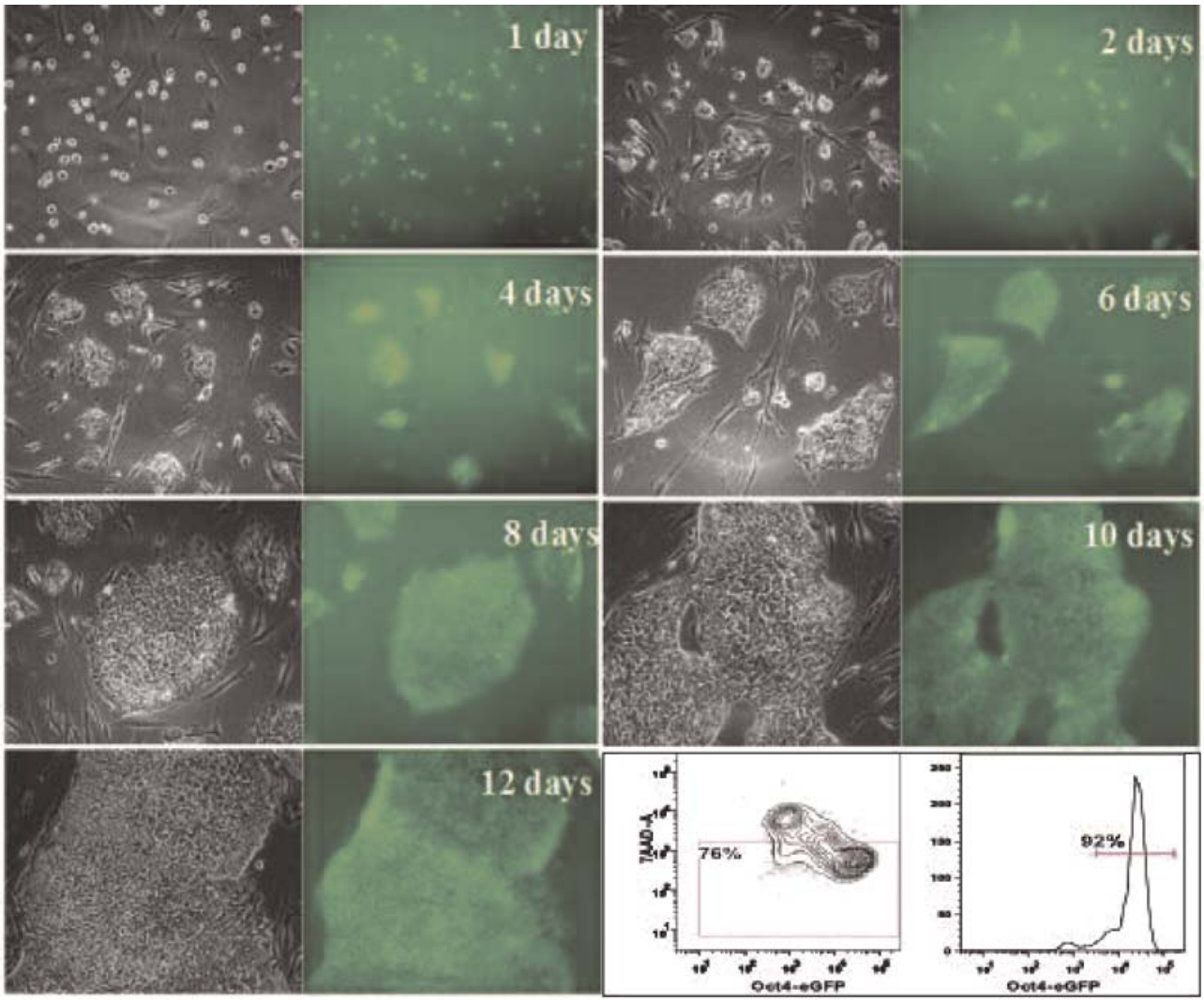

Fig. 6. Isolation of Accutase-passaged hESCs by flow cytometry. Colonies of hESCs engineered with Oct4–eGFP were dissociated into single cells and sorted into eGFP-positive and eGFP-negative cells using FACS instrument. Sorted cells were plated in standard hESC conditions and sequential images of the colonies were captured using bright field and fluorescence microscopy. Inset in lower right corner: representative FACS analysis of eGFP-positive sorted cells re-grown as colonies of pluripotent hESCs. The histogram profiles of 7-AAD negative live cells (pink gates in contour plots at 5% probability) are shown along with the percentage of eGFP-positive cells (92%). The eGFP-negative cells failed to form viable colonies (data not shown). [See color version online at www.interscience.wiley.com.]

neural precursor cells (Wachs et al., 2003), and offered reduced cell death and improved culturing conditions.

Single cell dissociation of hESC colonies with Accutase resulted in continuous cultures of hESCs that were viable and highly pluripotent for at least 20 passages (the longest propagation time assayed in this work). Critically, no decrease in the proliferation rate after switching from collagenase to Accutase propagation of hESCs (i.e., from small clusters to single cells) was observed. This is in sharp contrast to conventional trypsin dissociation, which resulted in $<3\%$ plating efficiency and massive cell death during the initial 5–10 passages (Hasegawa et al., 2006). The emerging hESC sublines with higher plating efficiency (up to 60%) that arose after trypsin treatment inevitably acquired karyotypic changes after 30 passages (Hasegawa et al., 2006). The dramatic cellular "crisis" period of 5–10 passages during the trypsin dissociation likely results from the selection of rare cells having karyotypic abnormalities that could confer a growth advantage. In contrast, Accutase-dissociated hESCs do not undergo this cellular crisis. In fact, we did not observe significant differences in the cell viability and cell proliferation kinetics between collagenase- and Accutase-passaged hESCs. These results alleviate the previous concerns regarding the selection and propagation of a subpopulation of genetically abnormal hESCs under the conditions of bulk culturing (Buzzard et al., 2004; Mitalipova et al., 2005). However, it is not clear whether karyotypic abnormalities or subtle genetic alterations (Maitra et al., 2005) can be induced by the single cell culturing with Accutase, which would result in the slow

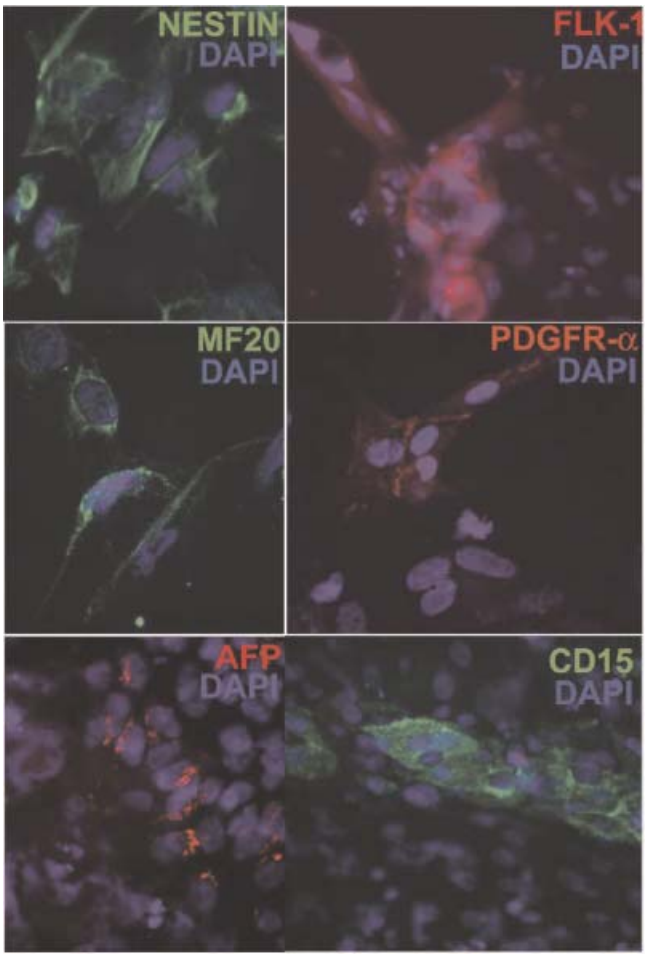

Fig. 7. Differentiation of Accutase-treated cells into three germ layers. hESCs were propagated with Accutase for 15–20 passages and then differentiated into ectodermal (Nestin), mesodermal (CD15, MF20, FLK-1) and endodermal, likely primitive endoderm (PDGFRα, AFP) derivatives. [See color version online at www.interscience.wiley.com.]

accumulation of abnormal cells. Long-term cultures of Accutase-treated hESCs are currently being characterized to address this concern.

Accutase-passaged hESCs exhibit a higher proportion of undifferentiated hESCs as compared to collagenase-passaged cells. This is likely due to a faster detachment of differentiated cells from plastic that was observed in hESC cultures treated with Accutase. Ongoing experiments will determine the enzyme specificity in the Accutase mixture and their contribution to the dissociation of hESC colonies and differentiated cells.

Single cell dissociation of hESCs with Accutase should help developing clonal hESCs lines. Indeed, our preliminary experiments using co-infection with two lentiviral preparations harboring PGK-mCherry and PGK-GFP expression cassettes suggests that green-only and red-only colonies can be obtained and isolated into a clonal hESC sublines (data not shown).

Accutase-passaged hESCs can be grown at high density as monolayers, with viable single ES cells isolated by FACS to be further cultured as pluripotent hESCs. The ability to grow hESCs as monolayers allows a much higher cell density as compared to hESC growth in conventional clusters, which greatly increases the numbers of cells that can be recovered under the same culturing conditions. Passaging of hESCs with collagenase requires the periodic inspection of cultures to remove colonies with poor morphology indicative of the presence of differentiated cells. In contrast, all single hESCs after Accutase dissociation can be used for plating, thus allowing "unsupervised" hESC propagation. Indeed, the Accutase protocol does not require previous experience growing hESCs (e.g., selecting the hESCs colonies having healthy morphologies) and as such can be quickly adopted by any personnel who are well-versed in tissue culture techniques. Passaging with Accutase should allow a true automation of hESC growth, which will be indispensable for obtaining the large amounts of cells for clinical applications and high throughput drug screening. The latter application will especially benefit from deposition of precisely counted numbers of single cells into 96 or 384 well plates. Finally, dissociation to single cells will provide better control over the cellular environment for the purpose of directed differentiation and will improve gene delivery into hESCs (e.g., infection and transfection procedures).

METHODS

hESC Culturing

hESCs were cultured on growth factor-reduced Matrigel coated plates (Becton Dickinson, Franklin Lakes, NJ) and inactivated (irradiated) MEFs (Specialty Media, Phillipsburg, NJ) in Knockout DMEM (GIBCO, Carlsbad, CA), 20% Knockout serum replacement (GIBCO), 10 mM nonessential amino acids(GIBCO), 200 mM L-glutamine (GIBCO), 0.1 mM 2-mercaptoethanol (GIBCO), 100 units/ml of each penicillin/streptomycin (GIBCO), and 25 μg/L recombinant human basic Fibroblast Growth Factor (Sigma, St. Louis, MO). The medium was changed daily.

Passaging of hESCs With Collagenase

Human ESCs were treated using 1 mg/ml collagenase IV solution (GIBCO, dissolved in Knockout DMEM) and incubated for approximately 5 min until the edges of colonies began to curl. The collagenase IV solution was removed and the colonies washed with dPBS twice. The differentiated cells were removed mechanically with micro pipette tips and then undifferentiated colonies were cut into small pieces with the 2 ml plastic pipette and detached from the plastic by trituration. The hESC clusters were transferred to a new plate containing standard hESC medium (see above).

Single Cell Passaging of hESCs With Accutase

Human ESCs were washed twice with Ca^{2+}- and Mg^{2+}-free PBS and treated with Accutase™ (Chemicon) in a 37°C incubator for ~5–10 min to dissociate the colonies into single cells. After gentle tritutation the

cells were collected by centrifugation (800 rpm for 5 min) and replated at $\sim 1 \times 10^5$ cells/ml (5×10^5 cells per well of a six well plate) using standard hESC culturing conditions. Cells were passaged between day 5 and 10, depending on the initial plating density and the experimental setup. The cultures were washed with PBS every time before changing medium as soon they reached 70% confluency.

FACS Analysis and Single Cell Sorting

Both the Accutase- and the collagenase-treated hESCs engineered with the Oct4–eGFP reporter were dissociated to single cells using Accutase. Single cells were collected by centrifugation and washed twice with PBS. 7-AAD (Molecular Probe, Carlsbad, CA) was added to each sample according to manufacturer instructions. The cells were sorted into eGFP-positive and eGFP-negative fractions using FACS Vantage-Diva (BD Bioscience, San Jose, CA) and plated in standard hESC culturing conditions. Single hESCs were analyzed using a FACSCanto instrument (BD Bioscience).

Immunofluoresence Assay/Antibody Staining

hESC colonies were fixed with 4% paraformaldehyde for 5 min at room temperature and washed with Ca^{2+}- and Mg^{2+}-free PBS. The fixed cells were stained overnight at 4°C using primary antibodies specific for Oct4, SSEA4, Nanog, Sox2, Nestin, MF20, FLK-1, PDGFRα, AFP, and CD15 followed by incubation with an appropriate secondary fluorescent-conjugated antibodies. All primary antibodies are from Chemicon, except Oct4 and SSEA4 specific antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary, FITC/Rho conjugated antibodies were from Jackson Laboratories and 488/Cy5 conjugated antibodies were obtained from Molecular Probes.

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