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The Nature of Floating Cells in Human Embryonic Stem Cell Culture

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Abstract

A consistent presence of floating cells is a common phenomenon in cultures of human embryonic stem cells (hESCs). However, little attention has been paid to their existence. It is currently believed that unavoidable imperfections in culture conditions lead the cells to undergo senescence and apoptosis resulting in unattached cells floating in the culture medium. Inspired by recent studies on mitotic activities in human embryonic stem cell colonies, we believe the existence of floating cells is not simply the result of unfavorable growth conditions but an intrinsic phenomenon resulted from maintaining the pluripotency of hESCs under the culture conditions. We tested this hypothesis with a set of systematic experiments and discovered: 1) the ratio of floating cells to attached cells was significantly increased with culture time; 2) the number of floating cells could be manipulated. For example, we were able to reduce the number of floating cells by providing the colonies with more horizontal or vertical cultural spaces and maintaining the cells' pluripotency. The results open a new avenue to increase the stem cell culture efficiencies by rescuing the floating cells. On the other hand, by placing a physical barrier on the top of colonies, the number of floating cells was decreased, at the same time, hESCs also showed signs of differentiation. In addition, when inducing cells to differentiate with retinoic acid, the number of floating cells no longer increased with prolonged culture time. Taken together, these results suggested that continuous cell division across the colonies is responsible for the emergence of floating cells during hESC culture. This is quite different from the bacterial colony growth where the cells in the center of colonies are quiescent. Our results indicated that continuous cell division, even at the cost of floating cells formation, is essential for human embryonic stem cell proliferation.

Keywords: Floating cells; Human embryonic stem cells; Colony growth; Colony repair

Introduction

The ability of culturing human Embryonic Stem Cells (hESCs) under controlled laboratory conditions opened a new era of cell-based therapy. Researchers in optimizing culture conditions for efficient proliferation, developing methods for directed differentiation to specific lineages, and exploring in vivo function, tissue integration and cell therapy [1-4] have boosted the potential clinical applications of hESCs. At the same time, the success of generating induced Pluripotent Stem Cells (iPSCs) from adult tissues has attracted great attention due to immune incompatibility and ethical concerns [5-12]. Both hESCs and iPSCs share the same characteristic growth as distinctive colonies rather than monocultures. Currently, these characteristic growth patterns are not very well understood including the interplay between growth, morphology and cellular responses to factors in the culture medium. Furthermore, questions related to colony expansion pattern, structure, niche and micro-environment have yet to be addressed [13,14]. This paper is aimed to address some of the above-mentioned questions.

We are particularly interested in a persistent phenomenon associated with hESC culture, i.e. existence of floating cells in hESC culture. It is assumed by most that these floating cells are dead cells resulting from apoptosis or cellular differentiation due to imperfections in culture conditions [15] and thus very little attention has been paid to their existence. Recently, we performed a systematic and quantitative study as part of continuing efforts to elucidate embryonic stem cell colony growth mechanism by examining whether different levels of mitotic activities, assessed by the percentage of S-phase cells at any given time point, existed in different regions of hESCs colonies. Our data showed that S-phase cells were distributed randomly across the colonies and the mitotic activities of hESC cultures were time independent under current growth conditions [16]. For cells in the center of a colony with continuous mitotic divisions and limited lateral space, the fate of newly divided daughter cells in the center of a colony remains unclear. To reveal how these cells contribute to floating cells, we evaluated the percentage of floating cells over a seven day culture period. To demonstrate whether the limited space caused cells to float, we experimentally provided additional space either by surgically removing the central part of a colony or by overlaying a membrane inserts in the Z direction, respectively. We revealed that floating cells were competent to form new colonies. Furthermore, we studied the relationship between the percentage of floating cells and the undifferentiated state of hESCs.

Materials and Methods

Carotid flow phantom

To estimate the average diameter and depth of the common carotid artery (CCA), we scanned 10 healthy subjects, 6 males (mean age 33 ± 5 years, body mass index (BMI) 23.4 ± 3) and 4 Females (mean age 30 ± 4 years, BMI 24.3 ± 2.3) (Table 1), using B-mode ultrasound (Philips ATL5000 HDI) with L7-4 linear array probe. The mean CCA diameter

was 5.9 ± 0.5 mm and depth was 17 ± 3 mm. Figure 1 shows an example of B-mode image.

Based on these measurements, a flow-phantom was constructed using an agar-based tissue-mimicking material (TMM) which has a backscatter similar to soft tissue , attenuation was 0.5 ± 0.03 dB/cm (0.5 db/cm/MHz, soft tissue average), and speed of sound 1541 ± 3 cm/s (1540 cm/s in soft tissue) (Ramnarine, Anderson et al. 2001).

	Day 3	Day 4	Day 5	Day 6	Day 7
Floating rate Floating cells/ × 100% Total attached cells	23.78 ± 1.1	30.4 ± 2.1	36.26 ± 3.1	61 ± 3	66.74 ± 2.5
Attachment rate Newly attached cells/ × 100% Total attached cells	10.7 ± 2.8	9.6 ± 3.5	52.23 ± 1.55	24.3 ± 4.2	17.67 ± 3.6

Table 1: Percentage of floating and newly attached cells from Days 3-7 after passaging

Cell lines and reagents

The hESCs used in this study were H_9 cells from Wicell Research Institute (Madison, WI). Primary Mouse Embryonic Fibroblast (MEF) cells were obtained from Promega (Madison, WI). Cell culture plates and membrane inserts were bought from BD Biosciences (San Jose, CA). Antibodies for OCT-4 (sc-9081) and SSEA-4 (sc-21704) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All alexa fluor 594 and alexa fluor 488 labeled secondary antibodies were obtained from Invitrogen (Carlsbad, CA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). hESC culture and maintenance was carried as described elsewhere [13,16]. To ensure the pluripotency of stem cells, they were periodically examined for the presence of lineage-restricted stem cell markers such as octomer-binding transcription factor 4 (OCT-4) and stage-specific embryonic antigen-4 (SSEA-4). Cells were passaged every seven days by incubation with 1 mM Ethylenediamine Tetra Acetic Acid (EDTA) in Dulbecco's Phosphate-Buffered Saline (D-PBS) for 3 minutes followed by scrapping of colonies with a glass pipette. Cells from a single well of a 6-well plate were passaged to three 6-well plates that had been seeded with mitotically arrested MEF cells 24 h prior to passaging. For experiments with membrane cell culture inserts, passaging was done after removing the peripheral MEF cells by scrapping with glass pipette on the culture plate in order to prevent new hESC colony formation along the edge of the culture well. hESC differentiation was induced by the presence of 10 μM Retinoic Acid (RA) in the culture medium. After passaging, cells were kept in the induction medium for five days (Days 2-6 of the experiment).

Immunocytochemistry and imaging

On Day 7, cells were fixed with 4% paraformaldehyde in D-PBS for 20 minutes at room temperature. The cells were washed twice with DPBS followed by fixing them in 4% paraformaldehyde for 20 min at room temperature. These cells were blocked and permeabilized overnight at 4°C with 10% goat serum and 0.3% triton in D-PBS, followed by incubation with primary antibodies raised against OCT-4 (1:500 of rabbit IgG,) and SSEA-4 (1:400 of mouse monoclonal IgG1) for 2 h, and secondary antibodies at 1:2000 (Alexa Fluor 594 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-mouse) for 3 h. After which the cells were washed thrice with DPBS, stained for nucleus using Hoechst 33342 (1:10000). The cells were imaged with an Olympus IX70 confocal microscope with appropriate filter and excitation wavelength settings using 20x and 60x objectives. The scope was equipped with a SPOT camera (Diagnostic Instruments, Inc.).

Counting viable cells, floating cells and attached cells

The cell viability was assessed with the vital stain Trypan Blue. Live cells with intact membranes did not take up the dye and any non-colored cells were therefore counted as viable using hemocytometer; any blue cells were presumed dead. The percentage of viable cells was calculated as the number of non-blue cells divided by the sum of blue and non-blue cells. The mean value was calculated from eight rounds of hESC passages. To quantify the number of floating cells, culture medium (2.5 ml) was collected from each well, centrifuged, re-suspended in an appropriate volume of fresh medium, and cells were counted with a hemocytometer with Neubauer ruling. The same corresponding sets of culture plates were then used to quantify attached cells. Attached cells were first trypsinized with 1 ml of trypsin-EDTA for 4 minutes, and the resultant cell soup was treated as mentioned above for cell number quantification. The number of newly attached cells (the net gain of attached cells) was calculated by subtracting the number of attached cells of the previous day from the number of attached cells of the given day. Cell numbers per well were obtained from two 6-well plates each for days 3 and 4 and one plate each for days 5-7 after passage. The data was collected over 8 passages and cell numbers represented is the mean of these experiments.

Cutting the colonies

On Day 4 after passage, cells at the center (one-half the diameter of the colony) were completely detached from the culture well by using needles and syringes under aseptic conditions. The detached cells were collected and counted as described earlier, and the remaining cells were allowed to grow under the same condition. The percentage of floating cells in cut wells was compared to un-cut wells from Days 1-3 after cutting. Cell numbers were calculated from one 6-well plate for day 5 to day 7 and this was repeated 7 times more and represented as mean percentage.

Plating MEF on cell culture inserts

Cell culture membrane inserts used for 6-well plate had a pore size of 0.1μm. After washing with D-PBS, the inserts with or without the attached glass cover were turned upside down in a sterile container and seeded with MEF feeder cells and kept overnight in an incubator at 37°C and 5% CO_2. On the next day, the inserts were washed twice in the original container with D-PBS, and then flipped and moved to a 6-well plate with hESCs seeded and grown for two days. Due to the design of the inserts, there was a gap of about 800 μm between the lower side of the membrane insert and the bottom of the 6-well plate.

Results

The percentage of floating cells increased with culture time

Only cultures with colonies having sharply defined edges and being positive for OCT-4 and SSEA-4 were used in this study. The presence of floating cells was continuous phenomenon and numerous floating cells kept emerging during the seven-day growth period. In order to evaluate the number of floating cells quantitatively at different time points, floating cells were collected and counted from Days 3-7 after passaging. As shown in Figure 1, the absolute number of floating cells increased from 2.4×10^4 to 2.9×10^5 over the 5-day period. The floating rate, calculated as the percentage of floating cells divided by the number of total attached cells for any given day, increased from 23.78% (first row in Table 1). The cell attachment rate, calculated as the percentage of newly attached cells divided by the number of total attached cells (the second row in Table 1), increased from 10.7% at day 3 to 17.67% ($P<0.05$). Therefore, to our surprise, the floating rate during the 24 h period was 2-4 times greater than the cell attachment rate for the same given day, with the only exception at Day 5. It was also interesting to notice that cell attachment rate matched well with the rate of colony expansion. For example, most colonies, which were about 246-308 mm2 big on Day 6, grew to about 314-393 mm^2 on Day 7. The net area expansion was 68-85mm2, approximately about 20% bigger than on Day 6. This was very close to the calculated cell attachment rate for the same time period (17.67%). The results indicated that more newly formed cells would rather join floating cells or replenish the previously attached cells than contribute to colony expansion.

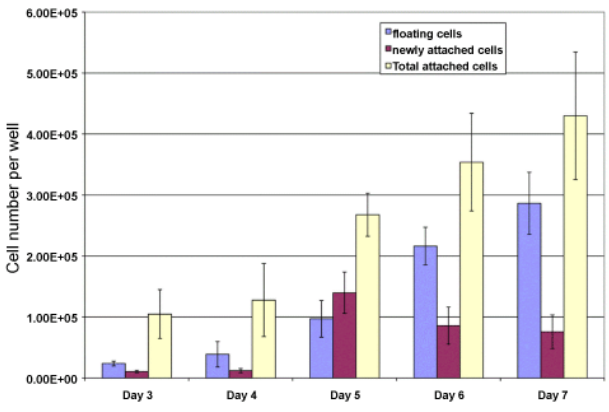

Figure 1: The number of floating cells, attached cells and newly attached cells from Days 3-7 after passaging. From day 3 after passage, floating cells, attached cells were collected and counted and represented as mean cell number for each population. Bars represents mean cell number ± SEM. The cell attachment rate, increased from 10.7% at day 3 to 17.67 % ($P< 0.05$). The floating rate during the 24 h period was 2-4 times greater than the cell attachment rate on any given day, with the exception of Day 5.

Space limitation was a key factor for the emergence of floating cells

To identify the source of floating cells, we created space by cutting and removing the center part of colonies. The "damaged" colonies were cultured under normal conditions for three days. Figure 2 illustrates the colony morphologies at Days 4 and 7. It was repeatedly observed that the hESCs can fully "repair" the excised areas in the culture and fill the space within the next three days or sooner. Both floating cells and attached cells from uncut and cut cultures were collected and counted on Days 5-7. The numbers of floating cells and attached cells are listed in Table 2. The floating rate at Day 7 was about 65% for uncut culture but only about 22% for the center cut culture. The lower percentage of floating cells was possibly due to the availability of extra space, so that those otherwise floating cells could now deposit and fill the space. In addition, the total numbers of attached cell in both damaged and uncut control cultures were very similar (within the experimental errors). Furthermore, the expression of stem cell marker OCT-4 in the repaired colonies was comparable to the uncut colonies (Figure 2). This result supports the hypothesis that space availability is affecting the emergence of floating cells. If there is ample space available for each newly divided cell for attachment, fewer cells end up floating in the culture medium. The rapid catch-up of total attached cell numbers in damaged colonies echoed our conclusion.

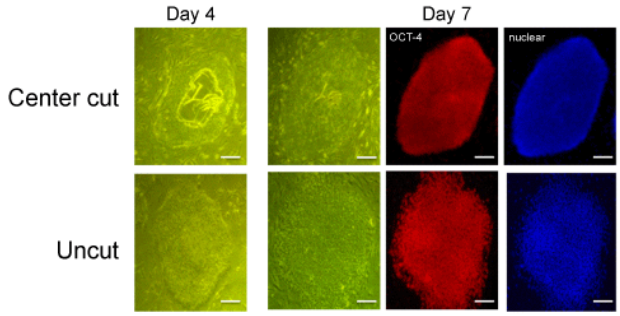

Figure 2: Morphology and stem cell marker staining of center cut and non-cut colonies. Left: Phase contrast images showing the result of surgically removing the central part of the colony on Day 4 and control colony was undisturbed. Right: Confocal images of colonies stained with the nuclear dye Hoechst 33342 and fluorescently labeled antibodies against OCT-4 at Days 3 and 7 after cutting. Scale bar represents 100 μm.

	Uncut culture			center cut culture	
	Floating cells	Attached cells	Floating rate	Floating cells	Floating rate

			Floating cells Attached cells/ × 100% Attached cells		Floating cells Attached cells/ × 100% Attached cells	
Day 5	9.7×10^4 $\pm 2.7 \times 10^3$	2.5×10^5	38.8	9×104 $\pm 2.7 \times 10^3$	2.58×10^5	35
Day 6	2.1×10^5 $\pm 3.4 \times 10^3$	3.5×10^5	60	8.4×104 $\pm 2.2 \times 10^3$	3.6×10^5	23
Day 7	2.8×10^5 $\pm 4.8 \times 10^3$	4.3×10^5	65	9.2×104 $\pm 3.1 \times 10^3$	4.1×10^5	22

Table 2: Percentage of floating cells from uncut and center cut hESC cultures from Days 5-7 after passaging. The central part of the colony was removed at Day 4 after passaging.

Many floating cells are viable and can form new colonies

To test the hypothesis that floating cells are dead, we stained floating cells with the vital dye Trypan Blue. Contrary to common belief, we found that about 40% of the cells were impermeable to the vital dye, indicating that they were still alive. We postulate that the percentage of live cells could be even higher by avoiding cell death during the experimental procedure involving collection (centrifugation) of cells and trypan blue staining. If a large percentage of floating cells was indeed viable, what was their fate? Could they form new colonies? To examine this question, we provided additional vertical space by placing the membrane insert with mitotically arrested MEF feeder cells face down onto the existing colonies. As shown in Figure 3, we observed a significant lower percentage of floating cells when the MEF-coated membrane was placed over existing colonies (P=0.0049). Correspondingly, we found numerous new colonies on the MEF-coated membrane (Figure 4). Furthermore, most of these colonies were exact replicas of the colonies in the culture well. They exhibited normal morphology and expression of OCT-4 and SSEA-4. These results suggest that most floating cells were entirely competent to form new colonies if suitable space and extracellular matrix was immediately available after cell floating. When a rigid cover slip was placed on the underside of culture inserts, due to the weight of the glass cover slip, the gap between coverslip and culture plate was too close that cells were no longer freely floating and dividing. Although we observed lower floating cell number, the hard surface of the coverslip did not favor stem cell colony formation on them. As a result, only few cells without the distinctive morphology of undifferentiated hESCs were found attached to the coverslip. Cells from these coverslips had weaker expression of OCT-4 and SSEA-4, presumably due to the suppressed cell division and limited flotation of cells at the Z direction (Figure 4).

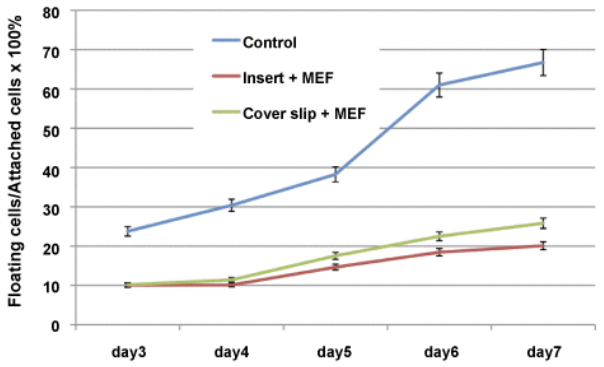

Figure 3: The percentage of floating cells from different hESC cultures- control culture without membrane insert, with membrane insert pre-coated with MEF (pink) and with membrane insert but partially covered by MEF-coated cover slip (orange). The floating cell rate was high in cultures without cell culture inserts. The cultures with coverslips coated with MEF had lower floating cell rate when compared to control cell culture but the morphology of colonies from these cultures did not show intact border of stem cell colony. The cultures with MEF inserts not only had lower floating cell rate but also had colony morphology similar to the control cultre.

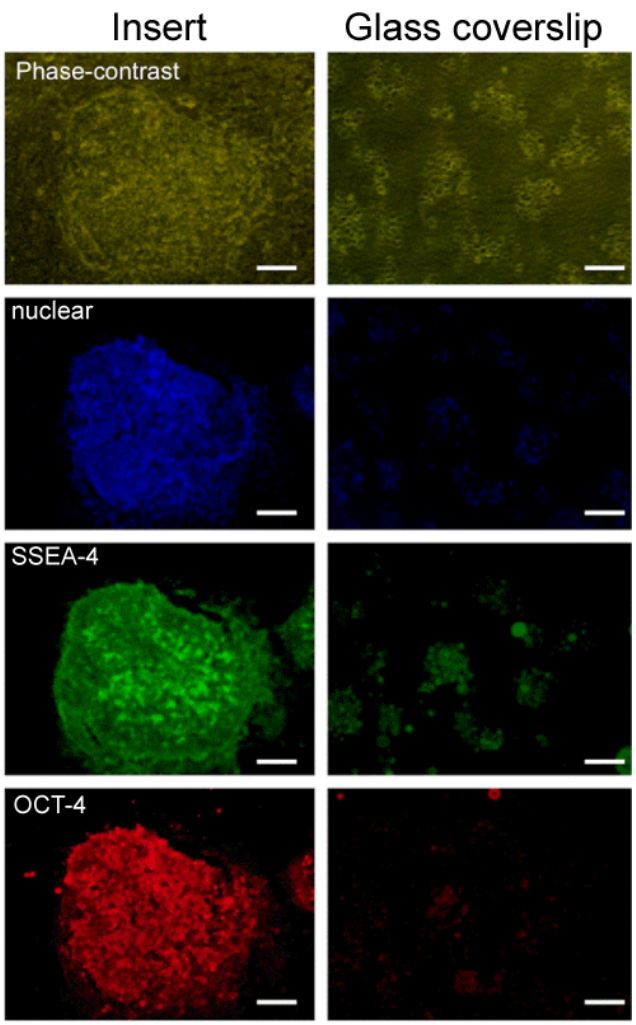

Figure 4: hESC attachment and colony formation on inserts/glass cover-slip pre-coated with MEF. Floating cells in cultures using inserts with MEF formed colonies but did not form in cultures with glass cover slip. Although there were few cells attached to glass surface, they were not pluripotent based on weak staining for OCT-4 and SSEA-4. Scale bar represents 100 µm.

The percentage of floating cells was affected by the developmental status of hESCs

Since hESCs are characterized among others, by continuous cell division and floating cells and floating cells are inevitable under current culture conditions, we examined the possibility that the developmental status of hESCs might affect the emergence of floating cells. Differentiation of hESCs was induced by the presence of 10µM Retinoic Acid (RA) in the culture medium, a treatment that has previously been demonstrated by our group as well as others to result in differentiated hESCs [16]. Cells were grown with RA in the culture medium for five days and the number of floating cells was quantified each day. At the same time, colonies from corresponding plates were fixed on Day 2 (start of RA treatment) and on Day 7 (end of experiment) and inspected under the confocal microscope for the expression of hESC markers OCT-4 and SSEA-4. After five days of growth in the induction medium, undifferentiated hESCs had differentiated as indicated by the morphological and organizational changes of the colonies (Figure 5a). Weaker staining of OCT-4 and SSEA-4 and therefore lower expression levels of two markers for undifferentiated hESCs confirmed this conclusion (Figure 5b). As already shown in previous experiments (Figure 1), the percentage of floating cells increased continuously from 15-67% for the undifferentiated control culture, whereas there was no increase at all in the differentiated culture. The percentage of floating cells remained around 15% throughout the entire growth period (Figure 5b). These data show that the percentage of floating cells under RA treatment was no longer increasing with prolonged culture time as under normal culture condition. The lower percentage of floating cells was likely associated with fewer cell divisions and more RA-induced cell differentiation.

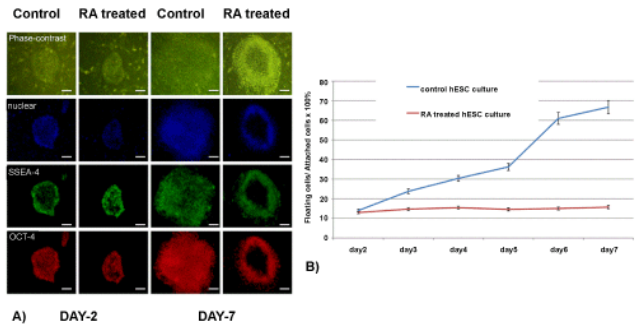

Figure 5. (5a) Human embryonic stem cell colony on day 2 and day 7 after passage in control and retinoic (RA) treated cultures. On the day 2 the stem cell colony of comparable size and morphology (left). RA treated results in loss of pluripotent genes as seen in loss of OCT-4 (red) and SSEA-4 (green). Scale bar represents 100 µm. (5b) the percentage of floating cells from control and retinoic acid-treated hESC cultures. Floating cells from control and RA treated hESC culture from day 2 to day 7 after passage were collected, counted and represented as floating cells/ attached cells x 100.

Our interest in the phenomenon of floating cells in hESC culture arose from literature reports as well as our own observations that mitotic activities, assessed by the percentage of S-phase cells at any given time point, existed evenly across the hESC colonies [16-21]. This is quite different from other types colony growth such as bacterium colony growth [22,23], where it is well accepted that the cells located at the colony center are in the cessation of their division and the colony expansion is sustained by growth limited to the peripheral zone of the colony. We were therefore interested to know how and where newly divided cells are deposited in the center of a colony. It is reasonable to assume that some newly divided cells can only deposit in the direction perpendicular to the substrate as the cell density on the substrate is typically much higher at the center of the colony and leaves no room for the newly divided cells. Without appropriate local environment, such as extracellular matrix or paracrine signals from surrounding/ supporting cells, these cells become floating cells. Such a floating cell model matches well with the experimental observations. The percentage of floating cells increased with prolonged culture time because the increased cell density at later stage of cell growth impeded more cell attachment and thus stimulated more cells to float. The

floating cell model assumes that unattached cells in the culture medium are dead but when cells were given additional space to attach (horizontal or vertical), the percentage of floating cells was significantly reduced (P=0.004). This implies that some if not many floating cells are viable, a conclusion that was supported by the results from staining with the vital dye Trypan Blue and further corroborated by the fact that some floating cells were able to form new colonies.

When the hESCs were induced to differentiate, fewer floating cells were observed compared to undifferentiated cultures. Likewise, when cells were prohibited from floating by placing a physical barrier over the colony, cells showed clear signs of differentiation. These results suggest that the degree of floating cells was directly associated with the developmental status of hESCs under the current culture conditions. They also indicate that continuous cell division was required for the maintenance of the undifferentiated state of hESCs. It was interesting to observe that damaged hESC colonies could self-repair. When one fourth of biomass in the central part of the colony was removed, the colony could heal the "wound" while maintaining its peripheral expansion. By Day 7, the cut colonies were of similar size compared to control colonies without any damage. Apparently, space played the key role here as the cells in center-cut colonies could deposit their newly divided cells in both inner and outer colony spaces. This resulted in filling up the damage area instead of forming floating cells, as well as colony expansion at the periphery.

Contrary to common belief [15], we demonstrated that many floating cells were alive and could be harvested to form a "normal" stem cell colony by providing an additional MEF substrate. When membrane with MEF was placed over the hESCs, floating cell numbers decreased in culture medium. Instead, distinctive colonies on the MEF coated membrane inserts were visible. The colonies stripped from the insert were further able to establish new colonies (data not shown). These results suggest that the hESCs might be destined to grow in three dimensions rather than in two [24-27]. We hypothesis that due to the lack of suitable support/substrate in two dimensional culture conditions, floating cells arise as they are unable to retain their cell to cell contact which finally leads to cell death.

We demonstrated that the existence of floating cells is an intrinsic property of embryonic stem cells cultured under currently established protocols. Our results directly support the hypothesis that in order for hESCs to maintain their pluripotency, cells have to be dividing continuously. We further demonstrated the number of the floating cells can be manipulated. Floating cells could initiate colony growth provided appropriate extracellular matrix and space was available. Similar studies that aim at iPSCs are currently under investigation.

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