Random Mitotic Activities Across Human Embryonic Stem Cell Colonies

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A systemic and quantitative study was performed to examine whether different levels of mitotic activities, assessed by the percentage of S-phase cells at any given time point, existed at different physical regions of human embryonic stem (hES) cell colonies at 2, 4, 6 days after cell passaging. Mitotically active cells were identified by the positive incorporation of 5-bromo-2-deoxyuridine (BrdU) within their newly synthesized DNA. Our data indicated that mitotically active cells were often distributed as clusters randomly across the colonies within the examined growth period, presumably resulting from local deposition of newly divided cells. This latter notion was further demonstrated by the confined growth of enhanced green fluorescence protein (EGFP) expressing cells amongst non-GFP expressing cells. Furthermore, the overall percentage of mitotically active cells remained constantly at about 50% throughout the 6-day culture period, indicating mitotic activities of hES cell cultures were time-independent under current growth conditions.

Introduction

INDEFINITE GROWTH OF HUMAN pluripotent cells in culture is a pure in vitro process, in contrast to the transient existence of human embryonic stem (hES) cells in the inner cell mass of blastocyst in a developing embryo [1]. It is expected that genetic changes that promote self-renewal and limit differentiation or apoptosis in a colony may happen during the transition from in vivo embryo development to laboratory cultures. In this regard, we view a hES cell colony as a quasi developmental organ, which is a complex cell community consisting of multiple cell populations with coordinated functionalities. This type of complexity is viewed as a necessary tactic for hES cells to maintain their self-renewal and pluripotency through forming unique microenvironments [2–4]. Thus, a comprehensive study of the nature of embryonic stem cell colony holds the promise for understanding the mechanism of stem cell self-renewal and differentiation. Such a study may also provide clues to disclosing any potential differences between hES and induced pluripotent stem (iPS) cells [5–7]. Accordingly, a very fundamental study of revealing how a hES colony expands is essential.

As hES cell colony is viewed as a complex community, various cell cycle-related features, such as the entire and individual cell cycle phase duration, the proportion of

S-phase cells or mitotically active cells, the successful deposition rate of newly divided cells, as well as their deposition manner are believed to be different for cells located at different physical regions within a colony [2,3,8]. All these difference will affect how a human stem cell colony is finally expanded and looked. In this study, we focus on revealing which physical regions, the edge or the center, have a higher fraction of mitotically active cells at different growth period, thus contributing more to the expansion of the colony. This question has been partially addressed by various research groups [9,10]. Heng et al. [9] reported that there were more mitotically active cells at the center than at the periphery of the colonies, whereas Ozolek et al. [10] concluded that more cells at the periphery were in S-phase. Such discrepancy on one hand indicated the expansion of hES cell colonies might be colony-specific and affected by multiple factors, such as the culture age and the quality of the examined colonies. On the other hand, it might suggest more detailed studies be needed. Hence, we conducted systemic and quantitative studies to reveal how many cells at different physical regions within a colony are in S-phase during 6-day growth period, how these mitotically active cells are distributed across the colonies, whether the fraction of S-phase cells are affected by culture age and spatial locations, and more interestingly

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whether the newly divided cells undergo massive migration before they were deposited within colonies.

Materials and Methods

hES cell culture and induced differentiation by retinoic acid treatment

The hES cells used in this study were of the H9 line from Wicell Research Institute (Madison, WI). Cells were routinely cultured in 5% CO₂ at 37°C with high humidity in hES cell medium consisting of 80% Dulbecco's modified Eagle's medium (DMEM/F12), 20% knockout serum replacement, 2 mM L-glutamine, 1% nonessential amino acids, 0.1 mM β -mercaptoethanol (Sigma, St. Louis, MO), 4 ng/mL basic fibroblast growth factor (all from Invitrogen unless otherwise indicated) on a layer of mitomycin C-inactivated primary mouse embryonic fibroblasts (MEF) feeder cells, as indicated in Wicell protocols. To ensure their undifferentiated state, cells were periodically examined for the presence of stem cell-specific markers, such as Oct-4, SSEA-4, Tra-1-81, and the absence of SSEA-1 [11]. Cells were passaged on average 5–7 days by incubation with 1 mM EDTA in Dulbecco's phosphate-buffered saline (D-PBS) for 3 min followed by dissociation with 5-mL glass pipettes. Cells between passages 30 and 35 were used for all experiments described below. To initiate differentiation, hES cells were fed with hES cell medium containing 10 μ M retinoic acid from day 2 to day 6 after passaging.

RT-PCR

Total RNA was extracted from hES cells using the RNeasy mini kit (Qiagen, Valencia, CA), and digested with DNase I to remove contaminating genomic DNA. The first strand cDNA synthesis was carried out using the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA). One-tenth of the cDNA mix was subjected to PCR amplification with DNA primers specific for genes *Oct-4* (forward: 5'-CGACCATCTGCCGCTTGAG, reverse: 5'-CCCCCTGTCCCCATTCCTA), *Rex1* (forward: 5'-GGAGTGCAATGGTGTGATCTC, reverse: 5'-GCTGGA CTGTGAGCACTACTAG), *Sox2* (forward: 5'-CCCCCG GCGGCAATAGCA, reverse: 5'-CTCGGCGCCGGG GAGATA), *Egf4* (forward: 5'-CAGCAAGGGCAAGCTCT ATGG, reverse: 5'-GCACCAGAAAAGTCAGAGTTG), *Nanog* (forward: 5'-GACAGCCCTGATTCTTCCAC, reverse: CAGTTGTCATGTTTCATGGAG), *Afp* (forward: 5'-CAGAACCTGTCAAGCTGTG, reverse: 5'-GACAGCA AGCTGAG GATGTC), and internal control β -actin (forward: 5'-CCACACCTTCTACAATGAGC, reverse: 5'-CGTCATA CTCCTGCTTGCTG). These primers were designed to be intron-spanning to preclude the amplification from genomic DNA. PCR cycles consisted of an initial denaturation step at 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, 58°C for 30 s, and 68°C for 1 min.

Immunocytochemical staining

Cells in 6-well culture plate were fixed in 4% paraformaldehyde/D-PBS for 20 min at room temperature, and blocked with 10% goat serum in D-PBS for the detection of surface markers or in PBS/10% goat serum/0.3% Triton

X-100 for the detection of intracellular markers for overnight at 4°C. Cells were then incubated with primary antibody diluted in PBS/2% goat serum at room temperature for 3 h followed by 2 h incubation with Alexa Fluor 488 or 594 conjugated secondary antibodies diluted in PBS/2% goat serum. Most primary antibodies, including mouse anti-SSEA-1, mouse anti-SSEA-4, mouse anti-Tra-1-81, and rabbit anti-Oct-4 were purchased from Santa Cruz biotechnology (<http://www.scbt.com>). All Alexa Fluor conjugated secondary antibodies were purchased from Invitrogen. Immunofluorescence images were observed on Olympus IX70 microscope equipped with appropriate filters and excitation using SPOT software (Diagnostic Instruments, Inc., Sterling Heights, MI).

BrdU incorporation assay

hES cells in culture plate were pulsed with 10 μ M BrdU in hES cell medium for 30 min at 37°C at different growth period, namely 2, 4, and 6 days after passaging. Retinoic acid-treated hES cells were pulsed with 10 μ M BrdU at day 6 only. Cells were then fixed with 4% paraformaldehyde, denatured in 4 N HCl at room temperature for 20 min, washed extensively with D-PBS, and then subjected for double detection of Oct-4 and BrdU, following the immunocytochemical staining procedure described above.

Flow cytometry (FCM) analysis

Cells previously exposed to 10 μ M BrdU at different growth period were detached from culture plates by incubation with trypsin/EDTA for 5 min, fixed in 70% ethanol for 20 min on ice, and denatured in 2 N HCl at room temperature for 20 min. After neutralization by extensive wash with PBS, cells were permeabilized in D-PBS containing 4% goat serum and 0.1% Triton X-100 for 30 min at room temperature, and subjected for double labeling following the procedure described above except the incubation time with antibodies reduced to 30 min. BrdU was detected by using Alexa Fluor 488 conjugated anti-BrdU monoclonal antibody (Invitrogen), while Oct-4 was detected by using rabbit anti-Oct-4 and Alexa Fluor 594 goat anti-rabbit IgG. Two hours before FCM analysis, stained cells were spun at 1,500 rpm for 5 min, and subsequently resuspended in 500 μ L propidium iodide (PI, 50 μ g/mL) containing 0.1 mg/mL RNase A (Invitrogen) and 0.05% Triton X-100 for 40 min at 37°C. Then cells were collected, resuspended in 500 μ L PBS, and analyzed at University of Chicago flow cytometry facility using a Becton Dickinson LSRII analyzer equipped with a 488 nm laser for excitation of Alexa Fluor 488 and PI, and a 561 nm laser for excitation of Alexa Fluor 594. Standard emission filters for these fluors were used. Results were analyzed using FlowJo software. A single cell population was identified using PI-width versus PI-area measurements and was subsequently gated for Oct-4-positive staining. Single Oct-4-positive cells were further subjected for PI-based cell cycle analysis and the calculation of S-phase percentage identified by BrdU incorporation. This experiment was repeated 3 times.

Statistical analysis

To determine which physical regions had more mitotic activities within the colonies, the dual staining images of BrdU and Oct-4 were merged in Photoshop. All Oct-4-

positive or Oct-4 BrdU double-positive cells were counted for the first outmost cell layer, the 3 outmost cell layers, the center circular region with the diameter of 200 μm , and the entire colonies. The fraction of S-phase cells was calculated as the number of BrdU Oct-4 double-positive cells divided by the total number of Oct-4-positive cells. At least 10 colonies from each time point were examined and the experiment was repeated with 3 independent batches of cells. The percentage of S-phase cells was then averaged and plotted against different time points and physical regions within colonies.

Derivation of EGFP-expressing cells

hES cells previously grown on MEF feeders were treated 2 min with PBS/EDTA at 37°C, then scraped off and seeded onto Matrigel-coated plate. The next day, cells were transfected with YPL2-EGFP plasmid (a generous gift from Yiping Liu, University of Wisconsin, Madison; [12]) in which the expression of EGFP was under the control of the constitutive promoter EF-1 α , by using Fugene 6 following the same procedure as described by Liu et al. [12]. Neomycin-resistant colonies were obtained through 2 weeks selection in neomycin containing medium. Five colonies with strongest expression of EGFP were picked and individually passaged for 5 more passages on Matrigel as described [12,13]. Then they were adapted to grow and maintain on MEF feeder cells as described above.

Mixed growth of EGFP-expressing and non-EGFP-expressing hES cells

Non-EGFP-expressing hES cells were allowed to establish colonies in 6-well plate at low density (about 50 colonies per well in 6-well plate) for 3 days after passaging. The center part of colonies was subsequently removed by scratching cells off with 21-gauge needles under microscope, and then received EGFP-expressing cells that were dissociated from a separate well and had been grown for 6 days. Any colonies, which had green cells landed in the hollow center, were

marked on next day and traced for 3 days to monitor the distribution of green cells.

Results and Discussion

General characterization of hES cell colonies

hES cells routinely grown in our lab were passaged by PBS/1 mM EDTA treatment. We found this passaging method was extremely gentle, which resulted in a very high recovery rate. We usually passaged cells at the split ratio of 1 to 22 every 5 to 7 days, in contrast to 1 to 6 used by most enzymatic or mechanical dissociation methods [11,14–16]. The cell growth was closely monitored by cell morphology and colony shape. Majority cells at most colonies were positively stained with stem cell-specific markers Oct-4, TRA-1-81, and SSEA-4, but negatively stained with SSEA-1, indicating cells were maintained under undifferentiated state (Fig. 1A). Our RT-PCR analysis further indicated that these cells expressed all stem cell-specific genes examined [17,18], including *Oct-4*, *Sox2*, *Nanog*, *Rex1*, and *Fgf4* (Fig. 1B).

Mitotically active cells were distributed as clusters randomly across the colony

To address one of the fundamental questions about how many cells are mitotically active at any given time point and where they are spatially located within hES cell colonies, we performed a detailed systemic study to delineate the colony growth pattern. Cells grown for different period of time, that is 2, 4, and 6 days, respectively, were exposed to BrdU, and then fixed and double labeled with BrdU and Oct-4 antibodies. As only mitotically active cells incorporated BrdU within their newly synthesized DNA, the percentage of S-phase cells was calculated as labeled BrdU cells divided by total number of undifferentiated cells (Oct-4-positive cells) at any designated physical regions. As shown in Figure 2, statistically speaking, the percentage of S-phase cells at the first or 3 outmost layers was about 45%–50%, approximately 10%–15% lower than that in the designated

FIG. 1. Characterization of human embryonic stem (hES) cells by immunocytochemistry staining with stem cell markers (A) and RT-PCR analysis (B). hES cells grown for 6 days were fixed and incubated with Oct-4, SSEA-4, Tra-1-81, and SSEA-1 antibodies followed by incubation with Fluor 594 or 488 conjugated secondary antibodies. The phase and corresponding fluorescence images were taken by IX70 Olympus microscope equipped with appropriate filters. To perform RT-PCR analysis, total RNA was extracted and transcribed using Superscript First strand cDNA Synthesis kit (Invitrogen). PCR amplification was performed by using primers corresponding to *Oct-4* (lane 1), *Sox2* (lane 2), *Rex1* (lane 3), *Nanog* (lane 4), *Fgf4* (lane 5), β -Actin (lane 6), and *Afp* (lane 7). *Afp* was used as a negatively expressed control and β -Actin as an internal PCR control.

FIG. 2. Statistical analysis of the percentage of S-phase cells at different spatial location and growth period. hES cells from the same passage batch were exposed to BrdU at day 2, day 4, and day 6, respectively, followed by immunocytochemistry staining with Oct-4 and 5-bromo-2-deoxyuridine (BrdU) antibodies on 6-well plates, or flow cytometry (FCM) analysis after dissociated into single cells. Ten colonies were then randomly picked for each time point and imaged under different channels. Corresponding images were then merged in Photoshop and the percentages of S-phase cells were calculated for the first outmost layer, 3 outmost layers, the center circular region with the diameter of 200 μ m and the entire colonies. The chart shown here was the average percentage of 30 colonies from 3 independent batches. For FCM analysis, only single cells stained with Oct-4-positive were gated for S-phase percentage analysis based on BrdU incorporation and DNA content. The chart shown here was the average percentage from 3 independent batches of cells. Color images available online at www.liebertonline.com/scd.

center region. However, this tendency only applied to cells examined at day 2 and day 6, but not at day 4. The lower percentage of S-phase cells at the periphery at day 2 might be in part due to environmental changes caused by passaging. Periphery cells were more disturbed than center cells during passaging, thus required more time to adapt to a new environmental setting. By day 4, with cells grown under optimum conditions, the difference between the periphery and central cells became marginal, resulting in very similar percentage of mitotically active cells. As cells grew to late stage such as day 6, more spontaneous differentiation might have occurred at the periphery of colonies, leading to a lower percentage of S-phase cells at the periphery than at the center, as differentiated cells generally possess slower DNA synthesizing rate [19]. However, at this point, we do not exclude the possibility that the difference of S-phase percentage we observed might not be intrinsic to physical regions for the following reasons. First, when each colony was examined carefully, we realized that S-phase cells were usually distributed as clusters, which was more apparent at day 2 (Fig. 3). If there happened to have clusters of BrdU-positive cells (green cells) included in the designated center regions, the percentage of S-phase cells would be higher than that of the edge. Likewise, the edge could have higher percentage of S-phase cells. Therefore, we can easily find areas both in the center and in the edge with S-phase percentage as high as 80% or as low as 30%. Generally speaking, these cluster cells were often arranged as circular shape. Due to the way we defined the spatial location, there was certainly more chance for clustered green cells to be included and counted for the central region due to its circular shape, and less chance for outmost layers due to its noncircular shape. Second, the difference between the edge and the center might be just an indication of the stem cell qualities. In one of our separated experiments, cells were purposely induced to be differentiated by treating with

retinoic acid [20]. As shown in Figure 4, the center cells were heavily differentiated as revealed by very weak Oct-4 staining and apparent SSEA-1 staining. Correspondingly, the percentage of S-phase cells in heavily differentiated entire center region (not just a circular region with the diameter of 200 μ m as defined for undifferentiated center regions) was drastically reduced to about 20% or lower. When cells were

FIG. 3. Mitotically active cells were distributed as clusters randomly across the colonies. Cells at day 2, day 4, and day 6 after passaging were exposed to BrdU, and then fixed and stained with Oct-4 and BrdU antibodies. Images were taken by Olympus IX70 and merged in Photoshop. Scale bars: 100 μ m.

grown under undifferentiated conditions, spontaneous differentiation, as reported elsewhere, was almost inevitable [19,21]. Although we have applied Oct-4 labeling to exclude those heavily differentiated cells within colonies, we could not ensure that every Oct-4-positive cells were undifferentiated. It has been well-known that this transcription factor takes some time to shut down RNA transcription in differentiating hES cells [3,22–26]. Therefore, some low S-phase percentage area might be either due to the lack of clustered S-phase cells or certain extent of spontaneous differentiation. The latter notion was consistent with the observation that areas with a lower percentage of S-phase cells were often associated with weaker staining of Oct-4, and also consistent with the notion that spontaneous differentiation was more common in the periphery than in the other parts of colonies [7]. In addition, the discrepancy reported in literature might be also due to the different quality standard used by different groups. Therefore, some of them reported that center part was more mitotically active, while others reported vice versa [9,10]. Third, the percentage of S-phase cells we obtained was not only largely dependent on how we defined the center area, but it also inevitably contained certain extent of manual counting error. Taking the colony from day 2 shown in Figure 3 as an example, the S-phase percentage in the center part was 65% for the left top corner, 58% for the right top corner, 55% for the left lower corner, and 41% for the right lower corner. Difference within the same colony was as large as 24%. This drastic standard deviation demonstrated from the other hand that S-phase cells were randomly distributed as clusters throughout the colonies.

FIG. 4. Lower percentage of S-phase cells was likely caused by differentiation. hES cells were purposely induced to be differentiated by retinoic acid treatment, as indicated by weak staining with Oct-4 and apparent staining with otherwise nondetectable SSEA-1 at the center part of colonies (left panel). When retinoic acid-treated colonies were exposed to BrdU, the BrdU incorporation rate was significantly lower in the center part compared to the edge part of colony (right panel) or nontreated colony (Fig. 3). Scale bars: 100 μ m.

Mitotic activities during 6-day growth period were time independent

When we manually counted the percentage of S-phase cells for the entire colony at different time points after passaging, we surprisingly discovered that they were fairly close, with 53% at day 2 and day 4, and 52% at day 6 (Fig. 2). To exclude possible counting error as well as ensure the BrdU-positive cells were indeed S-phase cells, we applied FCM analysis to examine the percentage of S-phase cells by both double labeling of Oct-4 and BrdU as well as PI-based cell cycle analysis. Only Oct-4-positive cells were gated for analysis. As shown in Figure 5, the percentage of S-phase cells for this particular batch was about 51% at day 2, about 52% at day 4, and about 51% at day 6, if calculated by the percentage of BrdU and Oct-4 double-positive cells (second column), or by the percentage of BrdU-positive cells with S-phase DNA content in the single Oct-4-positive cell population (third column). If calculated by PI cell cycle analysis (fourth column), the percentage of S-phase cells at day 6 is about 51%, lower than day 2 (56%) and day 4 (59%) for this particular batch. But the average percentage from 3 independent experiments was very close at different growth period, indicating mitotic activities during 6-day growth period were time-independent (Fig. 2).

Newly divided cells deposited locally

There are possibly 2 ways for newly divided progeny cells involving the expansion of hES cell colonies. First, the newly divided cells deposit locally, namely where the cell division occurs, especially at the early stage of colony expansion when unoccupied lateral space is available. The distribution of S-phase cells as clusters suggested in a way that newly divided progenies likely deposited locally and hence relatively synchronized at cell cycle phases. Thus at any given time points, the majority of cells in a confined area were either in S-phase or not in S-phase. Second, the newly divided cells do not deposit locally, but float to the medium, migrate for a short or long distance, and deposit wherever space is available, such as the periphery of the colony or empty space within and between colonies. To aid our understanding on the deposition of newly divided cells, we constructed an EGFP-expressing cells of H9 line and examined the growth pattern when EGFP-expressing cells were confined by non-EGFP-expressing parental cells. It is anticipated that if newly divided cells are to be floated before deposited, green and nongreen cells should be mingled together forming mosaic pattern. Otherwise, green cells would be still confined by nongreen cells. As shown in Figure 6, we observed EGFP-expressing cells were still predominantly localized in the center part of the colony. There were no additional green colonies, clusters, or cells emerging in other parts of colonies, supporting newly divided cells likely deposited locally. Of course, since the EGFP cell line we constructed, as most current cell lines, has a certain percentages of cells that lose the expression of EGFP with passaging [27], we cannot completely rule out of the possibility that minority of cells might be deposited away from the dividing place after released to the medium. Nevertheless, the percentage is low if at all.

The fact that a healthy hES cell colony grows as a monolayer under most circumstances suggests that the newly

FIG. 5. Mitotic activities during 6-day growth period were time-independent. hES cells grown for 2, 4, and 6 days after passaging were exposed to BrdU, and then dissociated into single cells and stained with BrdU and Oct-4 antibodies or the corresponding isotype. Two hours before flow cytometry (FCM) analysis, labeled cells were further stained with PI. Results were analyzed by FlowJo software. Only single Oct-4-positive cells were gated for further analysis. The percentage of BrdU and Oct-4 double-positive cells, as well as BrdU-positive cells with typical S-phase DNA content, was marked on each corresponding images.

divided cells from the middle part of colony do not promote 3D growth of a dome colony, and rather they deposited locally, thus increase the cell density in the middle of the colony, and eventually push the biomass of the colony for colony growth. Such a model of hES cell colony expansion had been postulated by Heng et al. [9]. In our lab, we indeed observed the cell density increase from about 130 cells at day 2 to about 180 cells at day 6 within the same radius of 200 μm . However, given the doubling time of hES cells is about 15–16 h [22] and the percentage of S-phase cells is about 50% at any given time, local deposition is unlikely the only destiny for the newly divided cells derived from the center cells within a colony. In addition, it will take a significant amount of energy for inner cells to push the colony biomass for colony expansion. Therefore, our model for hES colony expansion is

mainly dominated by the way of peripheral growth, yet cells across the entire colony are equally mitotically active. For cells in the center zone of the colony, where the lateral space is not available, their newly divided cells end up in vertical direction and float to the medium. Due to the unavailability of extracellular matrix or paracrine signals from surrounding cells [3], these floating cells might eventually lose the opportunity to join the expansion of colonies and become part of floating cells, which might explain the existence of abundant floating cells in standard stem cell culture. Our preliminary data indeed indicated some of these floating cells were able to reform into colonies provided appropriate extracellular matrix and space were immediately available for cells to attach after cell division. In addition, we believe the existence of floating cells in hES cell culture is possibly

FIG. 6. Newly divided cells likely deposited locally. The center part of non-EGFP hES cell colonies were scratched off and received the enhanced green fluorescence protein (EGFP)-expressing cells at day 0. The growth of EGFP-expressing cells were monitored for 3 consecutive days and found out to be predominantly localized where they were seeded, indicating newly divided cells unlikely undergo long-range traverse before they were deposited. Scale bars: 200 μ m. Color images available online at www.liebertonline.com/scd.

an intrinsic nature of the embryonic stem cells. In order to maintain their pluripotency, hES cells have to be constantly mitotically active, which might contribute to the “stemness” of the cells in the colony. Indeed, when hES cell culture was induced to be differentiated by retinoic acid treatment, the number of floating cells versus the total attached cells was about 50% less than cells grown under undifferentiated condition (a separated manuscript in preparation).

In conclusion, our data demonstrated that the percentage of S-phase cells remained constantly about 50% throughout 6-day growth period. Mitotically active cells were distributed randomly as clusters, presumably resulting from the local deposition of newly divided cells, at different physical regions across the entire stem cell colony. In addition to local deposition, some newly divided cells might float into the medium and be committed to quiescence, terminal differentiation, senescence, or cell death. Future focus will be in-depth analysis of cell cycle phases (G1, S, G2, M) at different growth period, in situ monitor of colony expansion, and the mechanisms why hES cells grow as colonies.

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Author Disclosure Statement

No competing financial interests exist.

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