Pluripotency of a polyploid H1 (ES) cell system without leukaemia inhibitory factor

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Abstract

Objectives: Tetraploid cells are strictly biologically inhibited from composition of embryos; by the same token, only diploid cells compose embryos. However, the distinction between diploid and tetraploid cells in development has not been well explained. To examine pluripotency of polyploid ES cells, a polyploid embryonic stem (ES)-cell system was prepared.

Materials and methods: Diploid, tetraploid, pentaploid, hexaploid, octaploid and decaploid H1 (ES) cells (2H1, 4H1, 5H1, 6H1, 8H1 and 10H1 cells, respectively) were cultured for about 460 days in L15F10 medium without leukaemia inhibitory factor (LIF). The cells cultured under LIF-free conditions were denoted as 2H1(–), 4H1(–), 5H1(–), 6H1(–), 8H1(–) and 10H1(–) cells, respectively. Pluripotency and gene expression were examined.

Results: Ploidy alteration of H1(–) cells was similar to that of H1 cells. The polyploid H1(–) cells showed positive activity of alkaline phosphatase, suggesting that they maintained pluripotency *in vitro* without LIF. The polyploid H1(–) cells formed teratocarcinomas in mouse abdomen, suggesting they could differentiate in mouse abdomen *in vivo*. 2H1, 4H1 and polyploid H1(–) cells expressed *nanog*, *oct3/4* and *sox2* genes, suggesting that they fulfilled the criteria of ES cells. Nanog gene was significantly over-expressed in 4H1 and polyploid H1(–) cells, suggesting that overexpression of nanog gene was a characteristic of polyploid H1 cells.

Conclusion: Polyploid H1 (ES) cells retained pluripotency *in vitro*, without LIF with nanog over-expression.

Introduction

H-1 (ES) cells, mouse germline-transmissible embryonic stem (ES) cells, were established from blastocysts of C3H/He mice, and it has been confirmed that the cells have the ability to differentiate into neural cells, epithelial cells, muscle cells, hair follicles and chondrocytes (1). H-1 (ES) cells cultured in Leibovitz's L15:Ham's F10 mixture (7:3) medium were named H1 cells. Tetraploid H1 cells (4H1) were established from diploid H1s (2H1 cells) through polyploidization, using demecolcine (DC) (2). Octaploid H1 cells (8H1s) were established from 4H1 cells (3). Pentaploid H1 cells (5H1 cells) (4) were accidentally established from an 8H1 cell as well as hexaploid H1 cells (6H1 cells) (5). Decaploid H1 cells (10H1 cells) were established from 5H1 cells (6). Pluripotency of 2H1, 4H1, 5H1, 6H1, 8H1 and 10H1 cells was ascertained by the positive activity of alkaline phosphatase (AP) and the ability to form teratocarcinomas in the mouse abdomen (2).

Leukaemia inhibitory factor (LIF) has been used to maintain mouse ES cells *in vitro* (7,8). LIF causes heterodimer formation on the LIF receptor, activates signal transducer and transcription factors, and maintains the equivalent renewal of ES cells (9,10). Removal of LIF causes irreversible commitment and resulted in the differentiation of diploid ES cells (7). 4H1 cells cultured in medium without LIF showed AP activity and formed teratocarcinoma in mouse abdomen (11), suggesting the cells maintained pluripotency in LIF-free medium. We were interested in whether higher-polyploid H1 cells cultured without LIF retain pluripotency.

Embryo construction of tetraploid cells was found to be forbidden, and only diploid cells constructed embryo body (12). However, the distinction between diploid and tetraploid cells in development has not been well

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explained. ES cells in culture expressed *nanog* gene as well as *oct3/4* and *sox2* genes (13). Nanog is a homeodomain protein found in mammalian pluripotent cells, and acts in inner cell mass construction (14,15). Deletion of *nanog* caused early embryonic lethality; in contrast, overexpression of *nanog* gene could maintain the pluripotency of ES cells in medium without LIF (16). Gene expression in polyploid H1 cells has not been well examined. Thus, we were interested in *nanog* expression of polyploid H1 cells in a medium without LIF.

In this study, 2H1, 4H1, 5H1, 6H1, 8H1 and 10H1 cells were cultured in medium without LIF for about 460 days, and the cell systems were examined for pluripotency and gene expression.

Materials and methods

Cells

H-1 (ES) cells established from blastocyst of a C3H/He mouse (1) were purchased from RIKEN Bio Resource Center (Institute of Physical and Chemical Research, Tsukuba, Japan) and maintained in a humidified atmosphere of 5% CO₂ at 37 °C in Leibovitz's L15:Ham's F10 mixture (7:3) medium supplemented with 10% foetal bovine serum (FBS; CELLECT GOLD, ICN Biomedicals, Aurora, OH, USA), 2-mercaptoethanol (0.1 mM), streptomycin (50 µg/ml), penicillin (50 U/ml) and LIF (500 U/ml; ESGRO, Chemicon International Inc., Funakoshi, Japan). This medium is denoted as L15F10 medium with LIF.

4H1, 5H1, 6H1, 8H1 and 10H1 cells that had been stored at -135 °C were thawed and cultured under the same culture conditions as 2H1 cells. The population doubling level (PDL) of these polyploid cells stored at 135 °C about 50. Adaptation of cells to L15F10 medium without LIF was performed through a gradual decrease of LIF concentration to 500, 100, 25 and then 0 U/ml. 2H1, 4H1, 5H1, 6H1, 8H1 and 10H1 cells cultured in L15F10 medium without LIF are named 2H1(-), 4H1(-), 5H1(-), 6H1(-), 8H1(-) and 10H1(-) cells, respectively. 2H1 and 4H1 cells were used as controls.

Cell culture for 460 days

Cells were washed twice in divalent cation-free phosphate-buffered saline (PBS⁽⁻⁾), treated with 0.3 ml trypsin-EDTA (0.17% and 30 mM, respectively) for 1 min at room temperature and suspended in L15F10 medium without LIF. Cells were subcultured almost every day by 1:2 or 1:4 dilution in culture flasks (25 cm²; Corning Costar Co., Acton, MA, USA). Cell number was counted using a haemocytometer at time of subculturing. The cell density was maintained at around 5 × 10⁶ cells/flask.

DNA histograms of cell populations were obtained using flow cytometry (FCM), in which G₁ peak of 2H1 cells was set to 2C (C, complement).

Flow cytometry

Cells were fixed in 20% ethanol, incubated in 0.25% RNase (Type II-A; Sigma, St. Louis, MO, USA) for 3 h at 4 °C, then counted using a haemocytometer. Immediately before measurements, cells were stained with propidium iodide (7.5 × 10⁻⁵ M) and examined for red fluorescence by FCM. Under these staining conditions, signal due to residual double-stranded RNA is negligible and relative intensity of red fluorescence corresponds to DNA content (17).

Fluorescence from individual cells was measured using FACSORT (Becton Dickinson Immunocytometry Systems, Franklin Lake, ND, USA). The red fluorescence of individual cells irradiated with focused laser light at a wavelength of 488 nm, was detected using a photomultiplier tube. The relative intensity of red fluorescence (FL2H) was measured and DNA histograms were obtained.

Cell cycle analysis

FCM data of FL2H, through a logarithmic amplifier for 10,000 cells were inputted into CASL software written by Mathematica (Wolfram Research Inc., Champaign, IL, USA) for cell cycle analysis of DNA histograms on a log scale using the transfer software FACS to ASCII (free-ware), and the DNA histograms were decomposed into phase fractions based on DNA content (18). The algorithm of CASL is similar to Fried's method (19) except that normal distribution functions with the same half-width instead of the same coefficient of variation value are used as components. To calculate durations of G₁, S and G₂/M phases of exponentially growing cells, doubling time T_d was used instead of cell cycle time (20).

Cell morphology and histochemistry

The morphology of logarithmically growing cells, in culture dishes was photographed using a phase contrast microscope (CK2; Olympus, Tokyo, Japan) equipped with a digital camera system (C4040; Olympus). The cells were trypsinized, fixed in 20% ethanol and resuspended in PBS⁽⁻⁾ for cell volume measurements. The distribution of cell volumes was measured using a Coulter Counter (ZM/256; Coulter Electronics, Fullerton, CA, USA).

Exponentially growing cells in Lab-Tek Chamber Slides (Nalge Nunc International, Naperville, IL, USA) were washed twice in PBS⁽⁻⁾ and fixed in fixative

(methanol:formalin:acetic acid = 9000:1000:1). Cells in chamber slides were stained for alkaline phosphatase activity using the standard method (Histofine; Nichirei Bio Science Co., Tokyo, Japan). Cells stained without substrate were used as a negative controls. Cells were photographed under a microscope (BX 60; Olympus) equipped with a digital camera system (C4040; Olympus). Images were transferred to a personal computer and were printed out.

Teratocarcinoma formation

The DNA histograms of exponentially growing cells that were to be injected were previously obtained. Solid tumours formed by H1 (ES) cells after intraperitoneal injection into normal C3H/He male (5w) mice (Japan SLC Inc., Shizuoka, Japan) were sliced as several histological sections and stained with haematoxylin-eosin (HE) using a standard method. The morphology of tumours was photographed using a microscope (BX 60, Olympus) equipped with a digital camera system and mouse abdomens were also photographed using a digital camera (μ 720; Olympus). Images were entered into a personal computer and printed out. Portions of solid tumours and other organs, kidney, heart and liver, were cut, minced using scissors, filtered through a 40- μ m nylon mesh and prepared for FCM.

Western blotting for Nanog, Oct3/4 and Sox2 proteins

Logarithmically growing cells were lysed using Laemmli buffer (4% sodium dodecyl sulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.0125 M Tris-HCl pH 6.8), and the lysate was loaded at equal protein content into wells of polyacrylamide gel. The SDS-conjugated cellular proteins were electrophoresed on SDS-polyacrylamide gel, and proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The proteins were stained with anti-Nanog (#5232 rabbit; Cell Signaling Technology Japan (CSTJ), Tokyo, Japan), anti-oct4A (#5677 rabbit; CSTJ), or anti-sox2 (#S1451 mouse; Sigma-Aldrich Japan, Tokyo, Japan) monoclonal antibodies, followed by peroxidase-conjugated secondary antibody. Immunoreacted proteins were visualized using ECL Plus reagents (GE Healthcare Japan, Tokyo, Japan). Beta-actin band stained with Coomassie Brilliant Blue on the membrane was used to check loaded protein content.

Quantitative RT-PCR analysis for nanog, oct3/4 and sox2 mRNA

Total RNA of exponentially growing cells was obtained using RNeasy Mini Kit (Qiagen, Valencia, CA, USA).

Relative mRNA densities for *nanog*, *oct3/4* and *sox2* genes were measured using 7900HT Real-Time PCR system (Applied Biosystems, Foster, CA, USA) with One Step SYBR PrimeScript RT-PCR Kit II (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. The mRNA of *oct3/4* and *sox2* genes was quantified by normalization to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase, housekeeping gene). Primers for quantitative RT-PCR were purchased from Takara Bio Inc., and their sequences were as follows: *nanog*-F (5'-AATTCTGGAACGCCTCATC-3') and *nanog*-R (5'-CCTTGTCAGCCTCAGGACTTG-3'); *oct3/4*-F (5'-CAATGCCGTGAAGTTGGAGA-3') and *oct3/4*-R (5'-CTCGAAGCGACAGATGGTG-3'); *sox2*-F (5'-CATGAGAGCAAGTACTGGCAAGA-3') and *sox2*-R (5'-AACGATATCAACCTGCATGGAC-3'); GAPDH-F (5'-TGTGTCCGTCGTGGATCTGA-3') and GAPDH-R (5'-TTGCTGTTGAAGTCGCAGGAG-3').

Results

To adapt 2H1, 4H1, 5H1, 6H1, 8H1 and 10H1 cells (2-10 H1 cells) to L15F10 medium without LIF, cells were cultured in gradually decreasing LIF concentrations of 500, 100, 25 and then 0 U/ml. Representative examples of DNA histograms of 10H1 cells in the adaptation process are shown in Fig. 1a. No remarkable alteration was observed in the DNA histogram pattern, suggesting that the fractions of G₁, S and G₂M phases were maintained. 2-8H1 cells showed similar responses to the adaptation process (data not shown). 2H1, 4H1, 5H1, 6H1, 8H1 and 10H1 cells that survived in the LIF-free medium were denoted as 2H1(-), 4H1(-), 5H1(-), 6H1(-), 8H1(-) and 10H1(-) cells (2-10H1(-) cells), respectively. It should be noted that removal of LIF immediately led to cell death, suggesting that gradual adaptation of the cells was required (data not shown).

To examine ploidy alteration, DNA histograms were measured for more than 460 days. 2H1(-) cells exhibited an altered DNA histogram pattern in which the S phase fraction decreased, suggesting that 2H1(-) cells changed their cell cycle parameters (Fig. 1b, Table 1). 5H1(-) and 6H1(-) cells showed constant DNA content during 461 days (Fig. 1e,f, respectively). 4H1 cells were exposed to LIF-free medium after culturing for more than 29 days, during which the cells showed gradual decline in DNA ploidy level (gradual ploidy decline). The 4H1 cells were in abrupt ploidy elevation stage at $t = 0$. The cells retained hyperploidy until 165 days, then exhibited gradual ploidy decline until 461 days (Fig. 1d). 8H1 cells were exposed to LIF-free medium after culturing for 107 days, during which the cells showed abrupt decline in DNA ploidy level (abrupt ploidy decline); 8H1(-) cells maintained

Figure 1. DNA histograms. DNA histograms of 10H1 (a), 2H1(-) (b), 2H1 (c), 4H1(-) (d), 5H1(-) (e), 6H1(-) (f), 8H1(-) (g) and 10H1(-) (h) cells in long-term culture. The polyploid cells stored in the early stages of establishment were re-cultured after thawing. 2H1 cells were also thawed and cultured. Column a represents changes in DNA histograms of 10H1 cells in the process of weaning from L15F10 medium with leukaemia inhibitory factor, LIF. Numerals on the upper-left hand side represent LIF concentration (U/ml). Numerals on the upper-right hand side represent the days from start of long-term culture without LIF. Minus days represent the day before LIF removal. Abscissa represents relative the DNA content (C, complement, logarithmic scale). Longitudinal dotted lines were drawn to facilitate comparison of ploidy levels of respective cells.

Table 1. Cell cycle parameters of 2H1(-), 4H1(-), 5H1(-), 6H1(-), 8H1(-), 10H1(-) and 2H1 cells in long-term culturing ($t = 461$ days)

Cells Fraction (Duration, h)	G ₁	S	G ₂ /M
2H1(-) cells (Td = 17 h)	0.456 (6.3)	0.315 (5.6)	0.229 (5.1)
4H1(-) cells (Td = 17 h)	0.295 (3.9)	0.463 (7.8)	0.242 (5.3)
5H1(-) cells (Td = 17 h)	0.267 (3.5)	0.455 (7.5)	0.277 (6.0)
6H1(-) cells (Td = 17 h)	0.305 (4.1)	0.435 (7.3)	0.260 (5.7)
8H1(-) cells (Td = 17 h)	0.264 (3.5)	0.514 (8.6)	0.223 (4.9)
10H1(-) cells (Td = 17 h)	0.292 (3.9)	0.462 (7.7)	0.246 (5.4)
2H1 cells (Td = 17 h)	0.193 (2.5)	0.512 (8.2)	0.295 (6.3)

DNA histograms shown in Fig. 1 were used. Phase fractions were determined omitting those for cells with other ploidy levels in the cell population. Phase duration was calculated through conventional equations using doubling time instead of cycle time (20). Td, doubling time, was calculated from daily subculturing (data not shown).

hypo-DNA content until 461 days (Fig. 1g). 10H1(-) cells showed gradual ploidy decline until 216 days, abrupt ploidy decline at 377 days, then retained hypoploidy until 461 days (Fig. 1h). These H1(-) cells in various ploidy stages were used in the following experiments.

To examine cell cycle, DNA histograms at $t = 461$ days were analysed (Table 1). The cell cycle parameters were essentially the same among polyploid H1(-) cells except 2H1(-) cells, suggesting that the cell cycle of polyploid H1(-) cells was maintained independently of DNA content.

To examine changes in cellular characteristics, cell volume, morphology and alkaline phosphatase (AP) activity were measured (Fig. 2). The cell volume of 2-10H1(-) cells was roughly correlated with DNA content of cells at that time (Fig. 2a). The growth morphology of 4-10H1(-) cells was altered in the long-term culturing (Fig. 2b,c). 2H1(-) cell populations had a mixture of flagstone-like, spindle-shaped and spherical cells, suggesting that 2H1(-) cells had differentiated to several kinds of cells. Alkaline phosphatase activity was measured for cells at $t = 182$ days (Fig. 2d). All of the polyploid cells showed positive alkaline phosphatase activity, suggesting that the pluripotent potential was preserved in these polyploid H1(-) cells *in vitro*. In contrast, 2H1(-) cells lost their activity, suggesting that they lost pluripotency.

Figure 2. Cellular characteristics. Volume distribution (a) phase contrast micrographs (b, c) and alkaline phosphatase activity (d) of logarithmically growing 2H1(-), 4H1(-), 5H1(-), 6H1(-), 8H1(-) and 10H1(-) cells at $t = 14$ days (a, b), 359 days (c) and 182 days (d). Cell volumes were measured using a Coulter counter. Vertical lines and scale of column (a) were drawn to facilitate understanding. Inserts at upper-right of columns (b) and (c) are lower-magnification photographs of respective main fields. In panel (d), cells with alkaline phosphatase activity are stained red. Upper right inserts of (d) are light micrographs of respective main fields. Lower right inserts of column (d) represent negative control in which cells were stained without substrate.

To examine teratocarcinoma formation *in vivo*, 2–10H1(-) cells were inoculated into mouse abdomens at $t = 133$ days for 2H1, 2H1(-) and 4H1(-) cells, and $t = 375$ days for 5–10H1(-) cells (Fig. 3). Solid tumours were confirmed in the mouse abdomen for 4–10H1(-) cells. The tumours contained many types of differentiated cell as well as undifferentiated cells, suggesting that the tumours were teratocarcinomas. DNA content of tumour cells was almost the same as that of inoculated cells, suggesting that the exact ploidy of these polyploid cells was not altered in the teratocarcinoma formation processes here (upper-right DNA histogram in each panel). 2H1 cells were used as a control of the experiment (Fig. 3a); note that 2H1(-) cells did not show any tumours (data not shown).

To examine expression of *nanog*, *oct3/4* and *sox2* genes, western blotting and quantitative RT-PCR

techniques were applied to 2–10H1(-) cells at $t = 347$ –357 days (Fig. 4). 2H1 and 4H1 cells were used as controls. The Oct4A and Sox2 proteins were equivalently expressed in all of cells except 2H1(-) cells, suggesting that protein density of Oct4A and Sox2 was constant in pluripotent H1 cells. The Nanog expression was decreased in the order of 4H1(-), 5H1(-), 6H1(-), 8H1(-) and 10H1(-) cells, suggesting that Nanog density was not constant in the H1(-) cell system. 2H1(-) cells did not show positive expression of Nanog, Oct4A and Sox2 proteins, suggesting that they had differentiated.

Relative mRNA contents of *nanog*, *Oct3/4* and *Sox2* genes of 2–10H1(-) cells were compared to those of 2H1 cells (Fig. 4E). The mRNA levels of *nanog* gene in polyploid H1(-) cells were significantly higher than those of 2H1 cells, suggesting that *nanog* gene was overexpressed

Figure 3. Teratocarcinoma formation. Exponentially growing 2H1 (a), 4H1(–) (b), 5H1(–) (c), 6H1(–) (d), 8H1(–) (e) and 10H1(–) cells (f) were injected intraperitoneally into a C3H/He mouse at $t = 133, 133, 375, 375, 375$ and 375 days, and solid tumours were confirmed in mouse abdomen at $t = 159, 166, 408, 411, 410$ and 408 days, respectively. Upper left and upper right DNA histograms are of inoculated cells and of tumour shown in the photograph (upper middle), respectively. Insert DNA histograms in upper right are of kidney (b), heart (c) and liver (d–f). In DNA histograms, abscissa represents DNA content (C, complement). Main frames represent micrographs of HE-stained histological sections of the tumour. The tumours consisted of heterogeneous conformation of cells, suggesting that they were teratocarcinomas. Undifferentiated cells increased with the ploidy.

in polyploid H1(–) cells. The Oct3/4 and sox2 mRNA levels in 4–10H1(–) cells were approximately constant and comparable to those of 2H1 cells. 2H1(–) cells showed cessation of gene activation of *nanog*, *oct3/4* and *sox2*. Overexpression of *nanog* mRNA was observed in both 4H1 and 4H1(–) cells, suggesting that *nanog* overexpression was a characteristic of tetraploid H1 (ES) cells.

Discussion

DNA content of mammalian diploid cells is well preserved during subculturing; however, polyploid cells sometimes exhibit gradual and occasionally abrupt decrease in ploidy. Moor *et al.* (21) concluded that near-triploid is the terminal ploidy of near-tetraploid Ehrlich's ascites tumour cells. How is cellular DNA stability deter-

mined? DNA content of 4H1, 8H1 and 10H1 cells declined gradually and abruptly (15, 3, 6, respectively), but that of 5H1 and 6H1 cells was DNA-stable (4, 5, respectively). 4–10H1(–) cells in this study showed similar DNA responses, suggesting that LIF had no influence on ploidy alteration in polyploid H1 cells. We propose that DNA stability depends on the chromosome configuration on the basis of the DNA fractal hypothesis (22) constructed mainly from reports by Myhra & Brogger (23), Bak *et al.* (24), Wollenberg *et al.* (25) and Nagele *et al.* (26). The DNA alteration in polyploid H1(–) cells in this study was in harmony with this hypothesis.

In long-term culturing without LIF, 2H1(–) cells lost pluripotency; however, the polyploid H1(–) cells maintained it. How could these polyploid H1(–) cells retain pluripotency *in vitro*? These polyploid cells showed

Figure 4. Gene expression. Western blotting (A–D) and real-time RT-PCR (E) measurements for *nanog*, *oct3/4* and *sox2* genes of 2H1 (a), 4H1 (b), 2H1(–) (c), 4H1(–) (d), 5H1(–) (e), 6H1(–) (f), 8H1(–) (g) and 10H1(–) cells (h). Exponentially growing cells at $t = 347$ days (A–D) and $t = 357$ days (E) in long-term culture were used. 2H1 and 4H1 cells in the early stage of culturing were used as controls. Panels A–D represent western blotting for Nanog (A), Oct4A (B) and Sox2 (C), and Immobilon-P membranes stained with Coomassie Brilliant Blue (D). Equivalent protein contents were loaded for western blotting experiments (A–D). Open arrow in panel D indicates the position of beta actin. Numerals under each blot represents relative optical density of blot. Numbers on the right side of panels B and D represent molecular weight (kD). Panel E represents relative intensities of mRNA expression for *nanog* (closed squares), *oct3/4* (gray squares) and *sox2* (open squares). Total RNA content was equivalently loaded. Column a₀ represents relative mRNA content in 2H1 cells. Columns a–h represent relative mRNA contents of *nanog*, *oct3/4* and *sox2* normalized against those of 2H1 cells. The relative mRNA content for 2H1(–) cells was multiplied by 1000. Longitudinal bars on columns represent error bars.

overexpression of *nanog* gene that could maintain pluripotency of ES cells in medium without LIF (16). It is probable that overexpression of *nanog* gene provides pluripotency in polyploid cells in LIF-free medium over the long-term. The polyploid H1(–) cells that retained pluripotency *in vitro* were differentiated in mouse abdomen, suggesting that mouse abdomen was more suitable for differentiation than LIF-free L15F10 medium. At this stage, we cannot explain how differentiation-resistant polyploid H1(–) cells could differentiate *in vivo*.

Nanog protein density decreased in order for 4H1(–), 5H1(–), 6H1(–), 8H1(–) and 10H1(–) irrespective of overexpression of *nanog* mRNA, suggesting that Nanog was degraded in cells. Gradual decrease in key materials

in differentiation might be useful to control the direction of differentiation. Overexpression of *nanog* gene was observed in both 4H1 and 4H1(–) cells, suggesting that it was an intrinsic characteristic of tetraploid H1 cells. The different characteristics between diploid and tetraploid H1 cells might match the principle of tetraploid complementation in which only diploid cells enable construction of an embryo (12,27).

Gene regulation in polyploid cells that have multiple homologous chromosomes are not yet well known. Abductive speculation may be useful for understanding the phenomena observed in this study. Embryo composition of tetraploid cells was strictly inhibited, and only diploid cells only form embryo body (12), suggesting that

full differentiation was restricted in tetraploid cells. Oct3/4 and Sox2 correlating to Nanog protein may cause the differentiation inhibition. Nanog protein is usually consumed and exhausted in diploid cells without LIF presence, whereas tetraploid cells that have duplicated genes may produce twice as much nanog mRNA (overexpression). Excess production of nanog mRNA in tetraploid cells leaves a Nanog residue, resulting in development inhibition.

It should be emphasized that these results regarding cellular responses of polyploid H1 cells can be applied only to particular types of proliferating cell under specific culture conditions with L15F10 medium.

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