Establishment of a mammalian cell line suitable for industrial production of recombinant protein using mutations induced by high-energy beam radiation

Yasuhito Chida · Keiichi Takagi · Satoshi Terada

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Abstract Mammalian cells are extensively used for production of biopharmaceuticals. Most cells used in industry have infinite proliferative capacity, which provides a high number of cells and corresponding productivity. However, infinite cells will continue to multiply even after cell density reaches sufficient levels. This excess proliferation aggravates the culture environment and induces low productivity. Therefore, after cell density reaches sufficient levels, downregulation of proliferation would prevent such aggravation and extend the culture period and improve productivity. To realize such suitable proliferation, we aimed to establish a novel cell line whose proliferation was spontaneously downregulated after reaching a sufficient population level. Mutagenesis using high-energy beam irradiation was used. CHO-DP12 cells were irradiated with 2.5 Gy X-rays and screened with hydroxyurea and 5-fluorouracil to eliminate any cells multiplying after confluence and to concentrate desired mutants. One clone was established and named CHO-M1. Cell cycle analysis

indicated that CHO-M1 cells had a similar cell cycle profile in the exponential growth phase, but cells rapidly accumulated in G1 phase just before confluence and did not progress through the cell cycle. This suggested that until confluence, proliferation of CHO-M1 was similar to parental CHO, but after confluence, it was inhibited and under G1 arrest. The specific antibody production rate of CHO-M1 was kept high, even after confluence, while that of parental CHO was drastically decreased in stationary phase. These results suggest that the desired cell line was successfully established and that high-energy beam irradiation could be an efficient mutagenic technique for breeding industrial cells.

Keywords X-ray · Mutation · Proliferation · CHO cells · High-energy beam irradiation

Introduction

Biopharmaceuticals including antibody therapeutics are mainly produced by mammalian cell culture. Because of the increase in demand for therapeutic proteins, techniques to achieve higher productivity are required. Towards this goal, different approaches including host cell engineering have been developed (Fujita et al. 1996; Kuystermans et al. 2007; Chusainow et al. 2009), as well as proliferation control using cytokines (Terada et al. 1996). Currently, CHO cells are extensively used in industry to produce therapeutic

Y. Chida · S. Terada (✉)
Department of Applied Chemistry and Biotechnology,
Graduate School of Engineering, University of Fukui,
3-9-1 Bunkyo, Fukui 910-8507, Japan
e-mail: terada@u-fukui.ac.jp

K. Takagi
Biology Group, The Wakasa Wan Energy Research
Center, 64-52-1 Nagatani, Tsuruga, Fukui 914-0192,
Japan

proteins, partly because they can provide stable and accurate glycosylation of proteins. As well as other mammalian cells and microorganisms, CHO cells have infinite proliferative capacity, which leads to high cell numbers and consequent high productivity. However, in general, infinite cells continue to multiply even after the cell density reaches sufficient levels. This excess proliferation aggravates the culture environment, leading to ineffectual consumption of nutrients, accumulation of byproducts such as ammonia and lactic acid, and worthless synthesis of descendant cell bodies, resulting in shorter culture periods and lower productivity. Therefore, after the cell density reaches sufficient levels, downregulation of the proliferation prevents the culture from such aggravation, extends the culture period, and improves productivity (Takahashi et al. 1994).

Since the discovery that X-rays induce mutagenesis, various kinds of mutagenic sources have been developed and successfully used for breeding various kinds of organisms. For this purpose, gamma rays and X-rays have been widely used as mutagens. In addition, ion beams including protons and carbon ions are also used because they have several advantages, such as higher linear energy transfer (LET) and relative biological effectiveness in comparison to gamma rays (Fokas et al. 2009; Lacombe and Le Sech 2009).

The purpose of the present work was to establish a new cell line suitable for producing recombinant proteins. We used high-energy beam irradiation to induce mutation. As described above, downregulation of proliferation improves productivity. To establish a cell line with such capacity, CHO cells were irradiated to induce mutations.

A screening method was designed using hydroxyurea (HU) and 5-fluorouracil (5-FU) to kill the proliferating cells after confluence. Both HU and 5-FU are metabolic antagonists and are used as anticancer drugs. HU also has the effect of synchronizing the cell cycle at the boundary between the G1 and S phases. 5-FU has greater cytotoxic activity than HU.

Both agents were used in turn to kill the undesired cells that continued to proliferate after confluence. Irradiated cells were expanded to confluence and then treated with HU to synchronize their cell cycle. After an interval of several hours to allow for re-entry into S phase, the cells were again treated with HU to kill the

multiplying cells. The surviving cells were then treated with 5-FU. If the cells had the desired ability, they would not enter S phase after becoming confluence and could survive treatment with HU and 5-FU.

Materials and methods

Cell culture and media

The CHO cell line DP12 (CRL-12445), producer of recombinant humanized interleukin-8 antibody, was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in 5 % fetal bovine serum containing Dulbecco's Modified Eagle's Medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 200 nM methotrexate (MTX, Wako Pure Chemical Industries, Osaka, Japan).

Irradiation for mutagenesis

Accelerated protons (200 MeV) and carbon ions (660 MeV) were generated and irradiated using the Multipurpose Accelerator System with Synchrotron and Tandem (W-MAST), and X-rays were generated using the X-ray source, Hitachi Medico MBR-1520R-3 (Tokyo, Japan), at the Wakasa Wan Energy Research Center (WERC) in Fukui, Japan. Several dose points were selected for clonogenic survival analysis. Excess radiation dose could cause too much DNA damage and mutations and reduce the yield of mutated cells; therefore, cells should be irradiated with the proper level of proton beam to produce 50–90 % cell death (Bartkowiak et al. 2001). Similarly, the irradiation intensity of the carbon beams and X-rays was determined.

Clonogenic survival assay

The cells were inoculated into 25- or 12.5-ml culture bottles (Sumitomo Bakelite Co., Tokyo, Japan) and irradiated using three types of radiation. Following irradiation, the cells were cultured until colony formation. The colonies were fixed using 4 % para-formaldehyde (Wako Pure Chemical Industries) and then stained using 1 % methylene blue (Kanto Chemical Co., Tokyo, Japan). The colonies were observed under a phase contrast microscope, and colonies containing >20 cells were scored as survivors.

Screening

The cells were inoculated onto 9-cm culture dishes (Sumitomo Bakelite Co.) in a semi-confluent state and then irradiated to mutate the genes involved in proliferation control. After irradiation, the medium was replaced, and the cells were incubated at 37 °C for 24 h.

The cells were treated with 4 mM hydroxyurea (HU) (Wako Pure Chemical Industries) for 24 h to synchronize the cell cycle at the boundary between the G1 and S phases. The medium was changed to HU-free and cells were cultured for 6 h to allow entry into S phase, followed by additional treatment with 4 mM HU for 24 h. After the surviving cells expanded and reached confluence, they were treated with 1 mM 5-fluorouracil (5-FU) (Wako Pure Chemical Industries) for 48 h. The clones were isolated with a cloning cylinder (AGC Techno Glass Co., Shizuoka, Japan).

Cell number, viability and cumulative viable cell density

The viable and dead cell densities were determined by counting in a hemocytometer under a phase contrast microscope using the trypan blue exclusion method. The cumulative viable cell density (CVC) was calculated using the trapezoidal rule for the integral given by the following equation:

$$CVC = \int_0^{t_f} X dt \quad (1)$$

where t_f was the final time of cultivation and X the viable cell density.

Doubling time was calculated using following equation:

$$\text{Doubling time} = \frac{t}{\log_2 \left(\frac{N(t)}{C} \right)} \quad (2)$$

where $N(t)$ was the cell number at time t , C the initial cell number, and t the time of culture period.

Measurement of antibody productivity

Culture supernatant was collected to analyze antibody productivity of the cells. Secreted humanized IgG (h-IgG) concentration in the culture supernatant was measured by ELISA. The antibody was sandwiched by rabbit anti-human IgG antibody (Bethyl Laboratories,

Montgomery, TX, USA) and horseradish-peroxidase-conjugated goat anti-human IgG antibody (American Qualex Antibodies, La Mirada, CA, USA).

Specific production rate of h-IgG (q_{h-IgG}) was calculated by the following equation:

$$q_{h-IgG} = \frac{1}{\bar{X}} \frac{dP}{dt} = \frac{P_f}{CVC} \quad (3)$$

where $\bar{X}$ was the mean cell density, P the increase in h-IgG concentration during culture period for dt and P_f the total h-IgG concentration during culture.

Flow cytometric analysis

The trypsinized cells were collected by centrifuging at an average of 2,000 rpm for 10 min and then washed once with PBS. The cell pellets were gently resuspended in 200 μ l PBS, which was slowly dripped into 2 ml ice-cold methanol with agitation, and were stored in an ice bath for 30 min for fixation. Fixed cells were centrifuged at 2,000 rpm for 10 min and rinsed in PBS. The final cell pellets were resuspended in sodium acid citrate solution and stained by 50 μ g/ml propidium iodide (Wako Pure Chemical Industries), and incubated in an ice bath for $\sim$ 30 min. Cell cycle analysis of each cell was performed using FACSCalibur (Beckton Dickinson Japan, Tokyo, Japan).

Analysis of cell metabolism

The culture supernatants were collected to measure the substrates in the culture supernatants and analyze cell metabolism. Concentration of the substrates in the culture supernatants, glucose, lactate, glutamine, glutamate, pyruvate and ammonia, were measured by a Biosensor BF-6 M (Oji Scientific Instruments, Amagasaki, Japan).

Results and discussion

Determination of irradiation intensity of X-ray and ion beams

The survival curves of CHO-DP12 cells irradiated with X-ray and ion beams were obtained from the colony-forming ability. Results for survival versus absorbed dose are shown in Fig. 1. Survival of cells irradiated with X-ray and ion beams decreased

exponentially with dose. The survival curves for proton-beam and X-ray irradiation were similar and showed a characteristic shoulder at low doses. In contrast, the survival curve for carbon ion beams had no shoulder region, which was the characteristic feature of high LET irradiation. Excess irradiation dose might cause too much DNA damage and mutation and reduce yield of mutated cells, therefore, irradiation intensity for mutation was determined at the level that induced 50–90 % cell death. From the survival curves, irradiation intensity of proton beams, carbon beams and X-rays was determined between 1 and 3 Gy, 0.5 and 2 Gy, and 2.5 Gy, respectively.

Determination of screening conditions

Screening was performed using HU and 5-FU. Both agents are known to kill the cells in S phase as well as to inhibit S-phase progression, but in a different manner. It has also been reported that HU synchronizes the cell cycle at the boundary between G1 and S phases (Adams and Lindsay 1967). The screening was done in the following manner.

First, irradiated cells were treated with HU to eliminate multiplying cells and to concentrate the desired mutants. Irradiated cells were expanded to confluence and then treated with HU for 24 h to kill multiplying cells and to synchronize the cell cycle at the boundary between G1 and S phases. After an interval of several hours to allow for re-entry into S phase, the cells were again treated with HU to kill multiplying cells. After the second treatment with HU,

more cells were killed than during the first treatment, because most of the cells had entered S phase. The surviving cells were cultured in the absence of HU until confluence and then treated with 5-FU so that multiplying cells were selectively killed.

Cells with the desired ability of spontaneous proliferation control remained in G1 phase and did not enter S phase after confluence, and therefore could survive both HU and 5-FU treatment. Thus, we expected that this strategy would kill only the proliferating cells and effectively concentrate the desired mutants.

To determine these screening conditions, the effect of HU and 5-FU on CHO-DP12 cells was examined and the results are shown in Fig. 2. First, the concentration of HU and the interval required for synchronizing the cell cycle for entry of the multiplying cells into S phase. CHO-DP12 cells were treated with HU at various concentrations for 24 h and after the medium was changed to HU-free, the cell cycle of the treated cells was serially analyzed.

The effect of various concentration of the first HU treatment on cell proliferation and death was analyzed (data not shown). Cells treated with 1 or 2 mM HU survived and within 24 h, started to proliferate, while most of the cells treated with 8 or 16 mM HU were killed, indicating that 1 and 2 mM would be too low and 8 and 16 mM would be too high. Treatment with 4 mM HU successfully resulted in the growth arrest. Among various concentration of HU examined in this report, only 4 mM had the desired effect to stop the proliferation and to synchronize the cell cycle without killing the cells.

As shown in Fig. 2a, CHO-DP12 cells treated with 4 mM HU for 24 h were arrested at G1 phase, and the population of cells in G1 phase was more than that of the controls (Fig. 2a-1, 2). At 6 h culture after HU depletion, the treated cells re-entered S phase. At 8 h culture, some cells re-entered G2/M phase, and many of them at 12 h (Fig. 2a-3, 4, 5). Therefore, an interval of 6 h after 4 mM HU treatment was decided.

After the 6-h interval from the first HU treatment, the concentration dependency of the second HU treatment for cell death was analyzed (Fig. 2b). Cells treated with 1 mM HU survived and then proliferated, whereas treatment with 2 mM HU resulted in more effective cell killing, and the greatest effect was observed when the cells were treated with 4 and 8 mM HU. Although both the 4 and 8 mM HU treatments killed the cells completely (viable cells were not

Fig. 1 Surviving fraction versus doses for CHO-DP12 cells irradiated with protons *square*, carbon beams *circle* and X-rays *triangle*

Fig. 2 Effects of HU and 5-FU on CHO-DP12 cells. **A** Representative results of serial DNA histograms of CHO-DP12 cell line obtained after treatment with 4 mM HU. Cells were treated with HU for 24 h and incubated in the absence of HU for 0, 6, 8 and 12 h (A2–A5). **B** Induction of cell death by HU for cells synchronized at S phase. Treatment with 1 *black diamond*, 2 *black square*, 4 *black triangle*, and 8 *black circle* mM HU and no treatment *white square*. *Error bars* indicate SD. No symbol indicates that viable cells were not detected. **C** Induction of cell death by 5-FU. The symbols *black diamond*, *black square*, *black triangle*, *black circle*, *white square* and *white triangle* represent no treatment, 0.5, 1, 2, 4, and 8 mM 5-FU treatment, respectively. *Error bars* indicate SD. No symbol indicates that viable cells were not detected

detected after 312 h culture), we decided upon a concentration of 4 mM for the second HU treatment because a lower concentration would have resulted in less cytotoxicity.

Similarly, concentration dependency of 5-FU treatment for cell death was investigated. The purpose of this step was to kill all of the proliferating cells. We decided upon a period of 48 h for 5-FU treatment, which was 3–4 times longer than the doubling time of CHO cells, because doubling time is an average value and some cells multiply slower than the doubling time. In this study, all of the multiplying cells had to be treated with 5-FU during S phase to be killed.

As shown in Fig. 2c, 5-FU treatment killed the cells and after 10 days culture, no viable cells were detected

Fig. 4 Proliferation profiles of obtained cell lines: CHO-DP12 diamond, CHO-M1 square, CHO-M2 triangle and CHO-M6 circle

Fig. 3 Clones established by screening. **A** A representative colony from the cells irradiated with 2.5 Gy X-rays and treated with HU and 5-FU. Microscopic image on day 0 (upper left), day

4 (upper right), day 9 (lower left), and day 19 (lower right) after 5-FU treatment. **B** Morphology of the clones obtained from this study, CHO-M1 (left), CHO-M2 (middle) and CHO-M6 (right)

after treatment with >1 mM 5-FU. Similar to the second HU treatment, we decided upon a concentration of 1 mM 5-FU for the second treatment to avoid damage to the desired cells.

Clones established by screening

It has been reported that mutation occurs more significantly in relaxed than in condensed chromatin (Spotheim-Maurizota and Davidková 2011). DNA regions concerned with proliferation are relaxed in sub-confluent cells compared with confluent cells; therefore, we irradiated the cells during sub-confluence. Irradiated cells were cultured for a further day prior to screening, because it is reported that inappropriate repair of the DNA damage suffered by radiation is fixed as a mutation on the day after radiation (UNSCEAR 2000).

Irradiation with X-ray, proton and carbon beams, followed by screening, was attempted several times. Among these irradiations and screenings, only X-rays yielded surviving cells. After irradiation with 2.5 Gy X-rays and screening with 4 mM HU, six colonies survived.

The surviving colonies were additionally treated with 1 mM 5-FU and Fig. 3a shows a representative colony during this screening. During screening, the medium was periodically changed to remove any 5-FU that had leaked out from dead cells. Just after treatment with 5-FU, the colony was small (i.e. >50 cells), but at day 4, several of the cells in the colony were dead and the colony became smaller. At day 9, the colony started to become larger again and at day 19, the colony became even larger and the cells were layered.

Three of the six colonies survived and were named CHO-M1, M2 and M6. The resultant cell lines are shown in Fig. 3b. Density of CHO-M2 cells was higher than that of CHO-M1 and CHO-M6 cells.

Proliferation profile and antibody production of the three cell lines obtained

The proliferation and antibody production of the three cell lines obtained were analyzed (Fig. 4). Proliferation profiles showed that the maximum cell density of CHO-M1 and CHO-M6 cells was lower than that of CHO-DP12 cells, whereas it was equivalent to that of CHO-M2 cells. CHO-M1 and CHO-M6 cells

proliferated in the monolayer state, whereas CHO-M2 cells were stacked (Fig. 3b). These results suggested that CHO-M1 and CHO-M6 cells did not proliferate after they reached confluence.

ELISA was used to determine antibody concentrations in the culture supernatant, and the specific antibody production rates were calculated (Table 1). Specific antibody production rates for CHO-DP12 and CHO-M2 cells were high during growth phase (days 0–6), but decreased significantly after they reached confluence (days 6–9); those of CHO-DP12 cells were 0.34 pg/cell/day during growth phase versus 0.14 pg/cell/day during stationary phase; and those of CHO-M2 cells were 0.21 pg/cell/day during growth phase versus 0.18 pg/cell/day during stationary phase. In contrast, antibody production rate of CHO-M1 cells did not decrease after confluence (0.28 pg/cell/day during growth phase versus 0.31 pg/cells/day during stationary phase). Antibody production of CHO-M6 cells was not detectable, suggesting that they had lost antibody production potency, probably because the necessary genes were disrupted by the irradiation.

Flow cytometric analyses of cell lines obtained

To investigate the ability to control cell proliferation, cell cycle analysis using flow cytometry was performed (Fig. 5). Cell cycle analysis indicated that CHO-M1 cells were quickly accumulated in G1 phase just before confluence and then did not progress through the cell cycle, suggesting that these cells proliferated normally until confluence, but did not proliferate further.

In comparison with CHO-M1, cell cycle profiles of CHO-M2 were relatively similar between the growth and decline phases, and similar between the decline and stationary phases. This indicated that CHO-M2

Table 1 Comparison of specific antibody production rates of CHO-DP12 and obtained cell lines

Cell line	Specific antibody production rate (ng/10 ⁴ cells/day)	
	Day 0-day 6	Day 6-day 9
CHO-DP12	3.43	1.37
CHO-M1	2.83	3.09
CHO-M2	2.11	1.85
CHO-M6	N.D	N.D

Fig. 5 Flow cytometric analyses of CHO-DP12 and obtained cell lines. Representative results from three independent experiments are shown. Histograms show the results of cell

cycle analysis of each cell line in growth phase (*left*), sub-confluence (*middle*), and confluence (*right*)

cells continued to proliferate after confluence. CHO-M2 cells might have acquired some resistance against screening reagents, including HU and 5-FU, during irradiation. Clearly, CHO-M2 was not our desired cell line.

Cell cycle analysis of CHO-M6 resembled that of CHO-M1, suggesting that CHO-M6 cells proliferated normally until confluence, and did not proliferate thereafter. However, as mentioned above, CHO-M6 cells lost the ability to produce antibodies; therefore, they cannot be used.

Long-term culture of CHO-M1 cells by repeated batch culture as a model of industrial manufacture

CHO-M1 cells had the capacity to control their proliferation and high antibody productivity even after confluence. These results led us to study CHO-M1 further. Long-term culture of CHO-M1 cells was performed using repeated batch culture as a model of industrial manufacture (Fig. 6). The cells were cultured until day 38 and the medium was changed on days 8, 14, 20, 26 and 32.

Viable cell density of both cell lines remained at the same level after confluence; that of CHO-DP12 was $\sim 1,000,000$ cells/ml and that of CHO-M1 was lower, $\sim 400,000$ cells/ml. However, specific antibody production rates for the two cell lines differed significantly. Although the specific antibody production rate of CHO-DP12 cells was drastically decreased soon after confluence, that of CHO-M1 cells remained at a high level. Finally, the total amount of antibody production in CHO-M1 cells over the culture period exceeded that of CHO-DP12 cells. These data imply that the rapid arrest of proliferation of CHO-M1 cells enabled them to prevent aggravation of the culture and thereby to maintain productivity for longer. These data also indicate the possibility that longer culture contributes toward more antibody production.

Metabolism of glucose and glutamine in the repeated batch cultures of CHO-M1 cells

To analyze why CHO-M1 cells maintain high antibody production after confluence, major metabolites in the culture supernatants—glucose, lactate, glutamine and glutamate—were examined at the times of culture medium change (Fig. 7). Transitions in the concentration of glucose did not differ between the

Fig. 6 Long-term culture of CHO-DP12 white diamond and CHO-M1 black diamond cells by repeated batch cultures as a model of industrial manufacture. **A** Proliferation profiles of CHO-DP12 and CHO-M1 cells during long-term culture. Medium was changed on days 8, 14, 20, 26 and 32. **B** Specific antibody production rate during culture. **C** Total antibody production of CHO-DP12 and CHO-M1 cells during culture

two cell lines, except for CHO-M1, which was consumed earlier during the first period (0–100 h). CVC of CHO-M1 cells was lower than that of CHO-

Fig. 7 Metabolism of glucose and glutamine in repeated batch cultures of CHO-DP12 *white square* and CHO-M1 *black square* cells. **A**, **B**, **C**, and **D** represent transitions of concentration of glucose, L-lactic acid, glutamate and glutamine, respectively

DP12 cells, and glucose consumption rates of the former were faster, indicating that CHO-M1 cells could use more glucose per cell.

Akt, a serine/threonine-specific protein kinase, plays a key role in multiple cellular processes including cell survival and transcription. Bathmell et al. reported that Akt can promote cell survival induced via glucose metabolism (Rathmell et al. 2003). High glucose consumption in CHO-M1 might be caused by activation of Akt, and it might contribute prolonged cell survival and higher antibody production.

Accumulation and consumption levels of lactic acid, a byproduct of glucose, were similar between the two cell lines. In other words, the rate per cell of accumulation and consumption of L-lactic acid of CHO-M1 cells was faster than that of CHO-DP12 cells. It has been reported that lactate consumption in cell culture correlates with enhanced product yields in fed batch culture (Mulukutla et al. 2012). In a survey of data from >200 manufacturing runs, a strong

positive correlation between superior productivity and the switch from lactate production to consumption in late culture was observed. Additionally, they found that the shift to lactate consumption occurred upon the rapid cessation of proliferation and under conditions of low glycolysis flux and high extracellular lactate concentrations. Assuming that the same phenomenon happened in the present study, this result might imply that rapid cessation induced by self control of proliferation might enable the cells to switch the metabolism of L-lactic acid without damage, which results in high L-lactic acid consumption and high specific antibody production.

Transition of the concentration of glutamine did not differ between the two cell lines. Similar to glucose, CHO-M1 cells could consume glucose efficiently. In glutamine metabolism, glutamine is deamidated to glutamate, and ammonia is released. Subsequently, glutamate is converted into α -ketoglutarate, one of the TCA cycle intermediates, followed by further release

of ammonia. Glutamate is known to accumulate during culture, and is used as an energy source after glutamine is consumed. For measurement of glutamate, lower accumulation, even considering the low CVC, was observed after 193 h. This result indicates that glutamine was not greatly converted to glutamate, and therefore production of ammonia was suppressed, maintaining specific antibody production rate.

Conclusion

We tried to establish a novel cell line whose proliferation was spontaneously downregulated after reaching sufficient numbers. We mutated the cells with high-energy beam irradiation and screened with HU and 5-FU and established the novel cell line, CHO-M1, which proliferated normally until confluence and then was downregulated, as we expected. Additionally, CHO-M1 cells maintained their high antibody production rate over 5 weeks. It can be concluded that we successfully established the CHO-M1 cell line, and it is suitable for industrial production of recombinant protein, indicating that energy-beam irradiation and screening using HU and 5-FU are effective for establishing new CHO cell lines.

In this study, we got only one ideal cell line within three surviving colonies. To increase the possibility of establishing new cell lines, several strategies would be effective. In radiation step, it would be important to induce mutations in the genes up-regulating the proliferation of the cells in sub-confluent state, indicating that the cells should be irradiated during sub-confluence. In screening step, damage to the desired cells should be avoided. Especially, 5-FU itself and the proteases released from the dead cells adversely affect the cellular survival. So toward improving the probability of establishment, early pick-up of the cell colony would effectively contribute, as well as proper exchanges of spent medium for fresh one.

Recently, a draft genomic sequence of the CHO cell line was uncovered (Xu et al. 2011). Using this information, the identification of the specific mutations in CHO-M1 cells could contribute to cellular engineering.

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