Improvement of a method to reproducibly immortalize human T cells by oncogene transfection

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(Received 7 July 1999; accepted 1 March 2000)

Key words: human T cells, immortalization, oncogenes

Abstract

The method to immortalize human T cells efficiently and reproducibly by oncogene transfection was improved. T cells were first grown selectively from peripheral blood lymphocytes population of healthy donors and atopic asthma patients, and from lymph node lymphocytes population of lung cancer patients by activating with mitogens (phytohemagglutinin and concanavalin A) and recombinant human interleukin-2 (rhIL-2) for five days. Plasmids expressing oncogenes, such as c-Ha-ras, c-myc, c-fos, v-myb and v-jun under the control of human cytomegalovirus promoter, were then introduced into these stimulated lymphocytes either separately or in various combinations by electroporation. After culturing these transfected lymphocytes for recovery for 1 day, they were fed every 3–4 days. Although all the control cells died within one month, oncogene-transfected lymphocytes continued to proliferate actively even for more than several months, indicating that oncogene-transfected lymphocytes were successfully immortalized. Flowcytometric analyses revealed that most of the immortalized lymphocytes were T cells expressing CD3⁺ surface antigen. The ratios of CD4⁺ and CD8⁺ subpopulations in immortalized T cells derived from healthy donors varied, depending on the kinds of oncogenes used. However, CD8⁺ subpopulation in immortalized T cells derived from cancer patients and atopic asthma patients were dominant, independent of the kinds of oncogenes. These immortalized T cells showed different proliferative responses in the presence or absence of exogenous human rhIL-2, depending on their origin of donors. Furthermore, immortalized T cells derived from healthy donors showed stronger cytotoxicity against K562 cells, suggesting that MHC-nonrestricted killer T cells in T cell population were also immortalized. Immortalized T cell lines, which proliferate continuously without stimulation of a mitogen or antigen in medium containing a low concentration of rhIL-2, have been maintained for more than 2 years without any growth rate decrease.

Abbreviations: BME, bone marrow extract; Con A, concanavalin A; LSM, lymphocyte separation medium; PHA, phytohemagglutinin; YLP, yolk lipoprotein; rhIL-2, recombinant human interleukin-2.

Introduction

Establishment of an efficient and reproducible method to immortalize functional T cells will contribute to the study of various functions of T cells in the immune system. Although the most general method to immortalize T cells is stimulation of T cells by specific

antigen in the presence of antigen presenting cells, only biased T cell lines depending upon the antigens could be obtained. Until now, several other methods to immortalize human T cells have been reported as follows:

- (1) treatment with carcinogens (Russo et al., 1988),
- (2) irradiation of UV light, X-rays or γ -rays (Borek et

- al., 1980),
 (3) transfection with SV40 large T-antigen (Shay et al., 1989), and
 (4) infection with specific viruses such as Epstein-Barr virus (Stevenson et al., 1986) or Herpes virus saimiri (Troidl et al., 1994).

However, these conventional methods have several problems such as the loss and/or alteration of functionality of T cells after immortalization, poor reproducibility, and low immortalizing efficiency.

We have reported that cotransfection of oncogenes resulted in immortalization of human lymph node derived T lymphocytes (Okano et al., 1993b). However, this method also had several disadvantages such as: (i) only cotransfection of *myc* and *ras* oncogenes were effective to immortalize T lymphocytes, but transfection of single oncogene or any other combination of oncogenes resulted in obtaining no immortalized T cell lines; (ii) conditions of cell culture and transfection methods used were not fully optimized, and finally (iii) this method had poor reproducibility. Here, we succeeded to improve the oncogene-transfection method to immortalize T cells, derived from different human individual origins by optimizing the cell culture conditions and the medium components, and realizing high efficiency and reproducibility. Furthermore, we characterized these immortalized human lymphocytes and evaluated the variances caused by immortalization.

Materials and methods

Oncogene expression plasmids

The genomic oncogene clones such as avian *v-myb*, human *c-Ha-ras*, human *c-fos*, human *c-myc* and avian *v-jun* were obtained from the Japanese Cancer Research Resources Bank (JCRB; Tokyo, Japan). The construction of each oncogene expression plasmid (pCMV-*v-myb*, pCMV-*c-Ha-ras*, pCMV-*c-fos*, pCMV-*c-myc* and pCMV-*v-jun*) was described previously (Yano et al., 1994).

Preparation of bone marrow extract and yolk lipoprotein

A fresh cow bone marrow was obtained from a cattle slaughter-house. The external membrane was removed and then the separated bone marrow was cut into small pieces. An equal volume of phosphate-buffered saline

(PBS) was added to the chopped bone marrow and homogenized. This homogenate was kept overnight at 4 °C and then centrifuged at $250 \times g$ for 30 min. The supernatant (bone marrow extract, BME) was sterilized with a $0.45 \mu\text{m}$ pore filter and stored at -20°C .

Yolk lipoprotein (YLP) was prepared by the method described by Murakami et al. (1988). Briefly, after separating the yolk membrane from an unfertilized egg using a sterile forceps, 2 ml of PBS was then added to 10 ml of the egg yolk. The egg yolk was mixed and centrifuged at $102,000 \times g$ for 24 hours at 4 °C. The upper and middle layers of yolk were mixed with 2 ml of PBS, and then used as YLP after membrane filtration ($0.22 \mu\text{m}$ pore).

Preparation of mononuclear cells

Human peripheral blood mononuclear cells were obtained by directly subjecting peripheral blood from healthy donors and atopic asthma patients to density gradient centrifugation using lymphocyte separation medium (LSM: Organon Technica Co., Durham, NC), according to the manufacturer's protocol. Lymph nodes from lung cancer patients were separated from connective tissues, sliced into small pieces, squeezed between two slide glasses, and mononuclear cells were then isolated by density gradient centrifugation as described above.

These separated mononuclear cells were cultured in 60 mm petri dishes (Becton Dickinson, Lincoln Park, NJ) at a density of 1.0×10^7 cells ml^{-1} in ERDF medium (Kyokuto Pharmaceuticals, Tokyo, Japan), containing 10% fetal bovine serum (FBS, Irvine Scientific, Santa Ana, CA), $10 \mu\text{g ml}^{-1}$ of concanavalin A (Con A), $10 \mu\text{g ml}^{-1}$ of phytohemagglutinin (PHA) and 10 U ml^{-1} of recombinant human IL-2 (rhIL-2) (Genzyme, Cambridge, MA) for 5 days.

Transfection of oncogenes into lymphocytes

Lymphocytes stimulated with Con A, PHA and rhIL-2 were washed twice with electroporation buffer (0.25 M mannitol, 0.1 mM CaCl_2 , 0.1 mM MgCl_2 , and 0.2 mM Tris-Cl, pH 7.4) and resuspended at a density of 1.0×10^7 cells ml^{-1} . After adding $15 \mu\text{g}$ of oncogene expression plasmid and $10 \mu\text{l}$ of TRANSFECTAM (Sepracor Inc. Marlborough, MA) to 0.4 ml of the cell suspension, the cells were subjected to a pulse of 190 V/cm using an electrocellmanipulator 600 (BTX electroporation system, CA) followed by chilling on ice for 3 min. The control cells (without

plasmids containing oncogenes) were also subjected to electroporation under the same conditions as described above. This pulse strength yielded as much as 70% viability for transfected lymphocytes. Transfected lymphocytes were cultured for recovery in ERDF medium containing 10% FBS, $1.5 \mu\text{l ml}^{-1}$ of BME, $1.0 \mu\text{l ml}^{-1}$ of YLP, $5 \mu\text{g ml}^{-1}$ of insulin (Novo Nordisk, Tokyo, Japan), $20 \mu\text{g ml}^{-1}$ of transferrin, 20 mM ethanolamine, 25 nM sodium selenite and 10–20 U ml^{-1} of recombinant human interleukin-2 (rhIL-2) for 24 hours at 37 °C under humidified 5% CO_2 /95% air atmosphere. The transfected cells were then plated into 96-well flat bottom tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) at the density of 1.0×10^4 cells/well. Control cells were also plated into the top row column of every plate. These cells were then fed every 3–4 days.

Immortalizing efficiency of transfected lymphocytes

Immortalizing efficiency of transfected lymphocytes were evaluated by counting the number of wells, containing large and round shaped viable colonies in each 96-well plate every 2 weeks for a period of 3 months. This rough but convenient evaluation was continued, until no remarkable variation was observed in the number of wells, containing viable colonies on each plate. The final immortalizing efficiency (%) was calculated by dividing the number of the wells, containing viable colonies by the total number of wells inoculated with oncogene transfected lymphocytes.

Flowcytometric analyses

Surface marker antigens expressed on the immortalized lymphocytes were analyzed by a flowcytometer (EPICS 752; Coulter Electronics Inc., Hialeah, FL, and FACS Calibur; Becton Dickinson, San Jose, CA). Simultest (CD3/CD19) (Becton Dickinson, San Jose, CA) which contains FITC-labeled anti-CD3 antibody (T cells surface antigen marker) and PE-labeled anti-CD19 antibody (B cells surface antigen marker), simultest (CD4/CD8) (Becton Dickinson) and FITC-labeled anti-IL-2 receptor (Becton Dickinson) antibodies were used for determining the type of lymphocytes immortalized by this procedure.

Proliferative responses of immortalized T cells

Proliferative responses of immortalized T cells in the presence of Con A and rhIL-2 were assessed in triplicate for a period of 2 weeks by WST-1 assay (Dojindo,

Kumamoto, Japan), according to the manufacturer's protocol. Briefly, the immortalized T cells (shown in Figure 2B) at a density of 1.0×10^4 cells ml^{-1} , were stimulated with $10 \mu\text{g ml}^{-1}$ of Con A in the presence or absence of 10 U ml^{-1} of rhIL-2 in 96-well microplates, and kept for 2 weeks at 37 °C without changing medium in a humidified 5% CO_2 incubator. Twenty μl of WST-1 dye solution was directly added to the cell culture. After 4 h incubation, the absorbance at 450 nm was measured.

Cytotoxicity assay of immortalized T cells

Cytotoxicity assays were performed in triplicate by using a non-radioactive assay kit (Promega Corp., Madison, WI), according to the manufacturer's manual. Briefly, after optimizing the cell density of the immortalized T cell lines (effector cells) against tumor cell lines (target cells): an erythroleukemia cell line, K562 (Japanese Cancer Research Resources Bank; JCRB, Tokyo, Japan) and a Burkitt's lymphoma cell line, Daudi (JCRB), the effector cells and the tumor target cells were mixed into ERDF medium without phenol red supplemented with 5% FBS and 20 U ml^{-1} rhIL-2. Cytotoxicity assays were carried out in triplicate in round-bottomed 96-well microplates with the effector-to-target cell (E/T) ratio being 10:1. This assay is able to quantitatively measure the level of lactate dehydrogenase (LDH) that is released upon cell lysis in the same way as ^{51}Cr released in radioactive assays. The percentage of specific cytotoxicity was calculated, using the formula: % cytotoxicity = $100 \times (\text{Experimental} - \text{Effector spontaneous LDH release} - \text{Target spontaneous LDH release}) / (\text{Target maximum LDH release} - \text{Target spontaneous LDH release})$.

Results

Immortalizing efficiency of human lymphocytes

The immortalizing efficiency of human lymphocytes by oncogene transfection was assessed by counting the percentages of the number of the wells, which contained vigorously proliferating colonies every 2 weeks for a period of 3 months (Figure 1). Transfection of single c-Ha-ras, c-myc, c-fos, v-jun and v-myb oncogene, as well as transfections with the various combinations of oncogenes resulted in efficient and reproducible immortalization of human lymphocytes. No significant variance was observed in the immortalizing efficiencies depending upon the transfected

Figure 1. Immortalization efficiencies of oncogene-transfected cells. Immortalization efficiencies of lymphocytes isolated from the healthy donor (A), the cancer patient (B) and the atopic patient (C) were evaluated at the denoted time period by counting the % of the wells containing viable colonies.

oncogenes, perhaps because of the rough evaluation method employed. On the other hand, the immortalizing efficiencies were remarkably varied depending upon the individual origin of the lymphocytes. In order to examine the reproducibility of this immortalizing method, lymphocytes derived from 3 different individuals in each group of healthy donors, atopic

patients and lung cancer patients, were immortalized and high reproducibility and similar immortalizing efficiencies were demonstrated. Although all the lymphocytes, derived from different human individual origins, could be immortalized by this oncogene transfection method, lymph node lymphocytes from cancer patients were most effectively immortalized.

Figure 2. Phase-contrast microscopic analyses of immortalized lymphocytes derived from the peripheral bloods of the healthy donor. (A) vigorously propagating cells ($\times 200$); (B) colony forming cells ($\times 200$).

Phase contrast microscopic analysis of immortalized lymphocytes

Phase-contrast microscopic analysis shows vigorously propagating (Figure 2A) and colony forming (Figure 2B) immortalized lymphocytes 3 months after transfection. These results demonstrated that the immortalized lymphocytes exhibited uniform morphology and formed a number of large clumping colonies. These vigorously propagating and colony forming abilities of the immortalized cells may explain the IL-2 independent proliferation as discussed below.

Surface marker antigens on immortalized human lymphocytes

Flowcytometric analysis revealed that immortalized human lymphocytes derived from healthy donors, atopic patients and cancer patients were all $CD3^+$, indicating that T cells could be immortalized by any of the oncogene combinations used in this study (Figure 3). Grid lines on vertical and horizontal axes in

Figure 3 were set on the position, including 90% of $CD19^+$ and $CD3^+$ primary lymphocytes derived from healthy donors, respectively. Figure 3 shows that some of cells from healthy donors and cancer patients were $CD3-CD19^-$, while those from the atopic patients did not contain those double negative cells. The types of those double negative cells are unknown. The ratios of $CD4^+$ and $CD8^+$ subsets in the population of immortalized T cells, derived from healthy donors, did not reflect the profile of the primary lymphocytes, rather they were variable depending on the kinds of oncogenes used (Figure 4). The immortalized T cells, derived from atopic asthma patients and cancer patients, were shown to be dominantly $CD8^+$, regardless of which combination of oncogenes were used for transfection. Furthermore, analysis of surface expression of the IL-2 receptor (IL-2R) demonstrated that $IL-2R^+$ T cells subpopulation could be detected in the immortalized T cells derived from cancer patients (Table 1).

Table 1. Surface antigens of immortalized T cells

Transfected oncogenes	Healthy donors			Cancer patients			Atopic patients		
	CD3	CD19	IL-2R	CD3	CD19	IL-2R	CD3	CD19	IL-2R
<i>ras</i>	+++	–	–	+++	–	++	+++	–	ND
<i>myc</i>	ND	ND	ND	+++	–	ND	ND	ND	ND
<i>fos</i>	ND	ND	ND	+++	–	ND	ND	ND	ND
<i>jun</i>	ND	ND	ND	+++	–	ND	ND	ND	ND
<i>myb</i>	ND	ND	ND	+++	–	ND	ND	ND	ND
<i>ras+myc</i>	+++	–	–	+++	–	+	+++	–	ND
<i>ras+fos</i>	+++	–	–	+++	–	++	+++	–	ND
<i>ras+jun</i>	+++	–	–	+++	–	+	+++	–	ND
<i>ras+myb</i>	+++	–	–	+++	–	–	+++	–	ND
<i>myc+fos</i>	+++	–	–	+++	–	+	+++	–	ND
<i>myc+jun</i>	+++	–	–	+++	–	–	+++	–	ND
<i>myc+myb</i>	+++	–	–	+++	–	++	+++	–	ND
<i>fos+jun</i>	+++	–	–	+++	–	+	+++	–	ND
<i>fos+myb</i>	+++	–	–	+++	–	+	+++	–	ND
<i>jun+myb</i>	+++	–	–	+++	–	+	+++	–	ND
<i>control</i>	+++	–	ND	+++	–	++	+++	–	ND

ND: Not determined.

Relative amounts of human lymphocytes with specific surface marker antigens were evaluated by flowcytometric analysis and the results are shown as follows: +++: > 70%; ++: 20–70%; +: < 20%; and –: not detected.

Figure 3. Flowcytometric analyses of immortalized lymphocytes. Immortalized lymphocytes were simultaneously stained with FITC-labeled anti-CD3 antibody (T cell marker) and PE-labeled anti-CD19 antibody (B cell marker). Immortalized T cells derived from healthy donors (A), from cancer patients (B) and from atopic patients (C), respectively. Analyses (A) and (B) were obtained using EPICS 752 and analysis (C) was obtained using FACS Calibur.

Proliferative responses of immortalized T cells

Proliferative responses of immortalized T cells derived from healthy donors (shown in Figure 2B) in the presence and absence of rhIL-2 were assessed by WST-1 assay (Figure 5). Immortalized T cells derived from healthy donors showed vigorous propagation,

both in the presence and absence of exogenous rhIL-2. However, immortalized T cells derived from cancer patients showed high proliferative responses only in the presence of exogenous rhIL-2, indicating that these cells are consistent with their surface expression of IL-2R, and might be related to the high expression of CD8 antigen (data not shown). On the other hand, the im-

CD4, ■; CD8, □. ND, not determined.

Figure 4. Flowcytometric analyses of immortalized human T cells. Immortalized T cells derived from healthy donors (A), from cancer patients (B) and from atopic patients (C) were simultaneously stained with FITC-labeled anti-CD4 antibody and PE-labeled anti-CD8 antibody. Analyses (A) and (B) were obtained using EPICS 752 and analysis (C) was obtained using FACS Calibur.

immortalized T cells derived from atopic patients showed low proliferative responses, both in the presence and absence of exogenous rhIL-2 (data not shown). Decrease of cell number around 8 days will be related to depleted nutrition because no medium change was performed for a period of 2 weeks.

Cytotoxicity assay

The cytotoxic activities of oncogene-transfected immortalized T cells derived from healthy donors, atopic asthma patients and cancer patients against tumor cell lines such as K562 and Daudi, were shown in Figure 6. Immortalized T cells from cancer patients showed mild cytotoxicity against K562 and Daudi cells. Low cytotoxicity against K562 and Daudi cells

were exerted by immortalized T cells derived from atopic asthma patients. Whereas, immortalized T cells derived from healthy donors showed stronger cytotoxicity against K562 cells but only slightly to Daudi cells, suggesting that these immortalized T cell population contained T cells exhibiting natural killer (NK)-like activity.

Discussions

Immortalizing ability is termed as the power to convert a cell line of limited replicative potential *in vitro* into one, which can be passaged without limit (Weinberg et al., 1985). Rodent-derived cells can be easily immortalized (Rohme et al., 1981), however, it is difficult

Figure 5. Proliferative responses of immortalized T cells derived from healthy donors against Con A in the presence (○) or absence (●) of rhIL-2. The x axis denotes the culture periods of immortalized T cells in the presence of mitogen, and y axis demonstrates the proliferative responses of these T cells.

to immortalize human cells (Wright et al., 1989). Although some of the reported methods could be adopted to immortalize human cells, establishment of immortal human cell lines is quite hard due to the strict finite life span of human somatic cells. Cellular senescence is by far dominant over immortality (Pereira-Smith et al., 1988). Recently telomere shortening has been revealed to function as biological clock for aging and to be involved in cellular senescence. An enzyme telomerase,

which adds essential telomeric sequences, is believed to be involved in maintaining telomere length stability in germ line, immortal cells in vitro and most cancer cells, but not in normal cells (Smith and Pereira-Smith, 1996). The length of telomere in blood cells is also reported to be shortened with aging (Hastie and Allshire, 1989). Recently it has been reported that telomerase activity is detectable at low levels in normal human T and B cells, increases by in vitro mitogenic stimula-

Figure 6. Cytotoxic activities of immortalized human T cells derived from healthy donors, cancer patients and atopic patients against K562 cells (A) and against Daudi cells (B). Results are presented as mean values $\pm$ standard errors.

tion, increases in hematopoietic progenitor cells upon their proliferation and differentiation, and decreases with aging (Hiyama et al., 1995). Nuclear oncogenes such as *myc*, *fos*, *jun*, and *myb* code for nuclear transcription factors and *ras* oncogene code for cytoplasmic p21 protein which act a signal transduction molecule upstream of nuclear oncogenes (Joneson and Bar-Sagi, 1997). These oncogenes have potential to immortalize cells by stimulating cell cycles (Jones and Kane, 1996).

Here, we reported a simple, reproducible and highly efficient method to immortalize human T cells by oncogene transfection. Two manipulations were needed for successful immortalization of human T cells as follows: (1) stimulation of primary lymphocytes with specific mitogens and IL-2 before oncogene transfection for the generation of immortalized lymphocytes with the desired phenotype; (2) culture for recovery after electroporation for 24 h in the complete medium; and (3) development of optimum medium for maintaining the immortalized T cell lines.

We stimulated the selective growth of T cells with the mitogen and rhIL-2, which improved the immortalization efficiency of T cells. Although it was possible to obtain immortalized T cell lines, the immortalizing efficiency was low. When lipopolysaccharide (LPS) ($10 \mu\text{g ml}^{-1}$) and pokeweed mitogen ($2 \mu\text{g ml}^{-1}$) were used as mitogens, B cells could be immor-

talized by oncogene transfection (unpublished data). We found that addition of transfectam on electroporation resulted in improvement of the transfection efficiency. Recovery culture after electroporation was also important to improve the efficiency and reproducibility of this method, because culture at high cell density for 24 h before screening of immortalized cells, will be useful for the quick recovery of the partially damaged or weakened cells after the electroporation.

At the beginning of this study, we performed several preliminary experiments, seeking for the optimization of the culture conditions and the medium components suitable for the growth of oncogene-transfected human T cells, although human lymph node-derived T cells cotransfected with oncogenes, have been maintained in the ERDF medium containing 10% FBS (Okano et al., 1993b). However, practically this medium was not suitable for the growth of oncogene-transfected human T cells, derived from the peripheral blood of healthy donors and atopic asthma patients and from lymph node lymphocytes of lung cancer patients. After reviewing preliminary experiments, several other media components such as BME, YLP, insulin, transferrin, ethanolamine, selenite and rhIL-2 were also supplemented to this basal medium. We termed this medium as 'complete medium' for stably maintaining immortalized human T cells. BME is thought to have a growth promoting activity

of human T cells. Insulin, transferrin, ethanalamine and selenite were generally used as growth factors for serum-free medium. YLP along with these components was shown to be very active for human cell growth (Murakami et al., 1982, 1988). IL-2 was generally considered to be a T cell growth factor (Smith et al., 1980). Although these immortalized T cells do not require rhIL-2 for their growth as shown in Figure 5, addition of low concentration of rhIL-2 (5-10 U/ml) to the complete medium stimulated the growth of these cells. These results suggested that addition of low concentration of rhIL-2 might be needed for the maintenance of these immortalized T cells, retaining differentiated functions such as cytokine-mediated growth in a long-term culture.

Based on these improvements, reproducibility and efficiency for immortalizing human T cells were greatly increased, resulting in establishing 317, 154 and 932 lines of immortalized human T cells derived from healthy donors, atopic asthma patients and cancer patients, respectively. A single oncogene transfection was previously shown to be not effective for immortalizing human T cells (Okano et al., 1993b). In this study, we found that transfection of single oncogene cause immortalization of human T cells, perhaps because of improved immortalization efficiencies.

The immortalized T cells, derived from healthy donors, exhibited stronger cytotoxicity against K562 cells, suggesting that these immortalized cell population might contain T cells having NK-like activity (Alam et al., 1999). Cloning of these functional immortalized T cell lines have been successfully performed using standard limiting dilution method (Alam et al., 1997). The oncogene-transfected T cells could be maintained in long-term culture for 28 months (healthy donors-derived T cells), 22 months (atopic asthma patients-derived T cells) and 20 months (cancer patients-derived T cells), although the control primary cells without oncogene survived for only one month. Other researchers judged that the cells were immortalized when the cells were passaged for more than 150 population doublings (Shay et al., 1991; Wright et al., 1992). The immortalized T cells have already been passaged 200–360 doubling times, depending on the transfected oncogenes and their donor's origins. Even after long-term culture, the immortalized T cells exhibited characteristic functions of T cells such as IL-2 dependent proliferation, cytokine secretion stimulated by mitogens, proliferation concomitant with recognition of antigen of target cells, cytotoxic activity for target cells etc. (Alam et al.,

1997; 1999). Our method to establish immortalized human T cells will be valuable to investigate the functions of T cells. The T cell receptor (TCR) cDNA was cloned from an immortalized T cell line which exhibit specific cytotoxicity to a human breast cancer cell line, MCF-7 to demonstrate that the TCR protein recognize a target antigen of 32 kDa on MCF-7 cells (Okano et al., 1993a).

Finally, we were able to improve the method to efficiently and reproducibly immortalize human T cells by oncogene transfection, however, the mechanisms underlying this successful immortalization have not clearly been elucidated yet. Further works will be needed to clarify these mechanisms.

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