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*Original Research*

# Chicken egg-white extracts promote OCT4 and NANOG expression and telomeres growth in 293T cells

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**Abstract:** It will have broad applications in cell biology if one of egg cell extracts has the roles to promote cell proliferation and reprogramming. It will provide a new method for easier reprogramming somatic cells and promote cell proliferation. We found chicken egg-white extracts have roles to promote cell proliferation and reprogramming. The different ingredients were then assessed for cell proliferation activity and somatic cell reprogramming. Chicken egg-white extract ingredients that were less than 3 kDa (LT3K) promoted cell proliferation. Those ingredients that were greater than 3 kDa (GT3K) promoted the increased expression of pluripotency factors in somatic cells and promote telomeres growth in 293T cells. Chicken egg-whites can be separated into ingredients of LT3K, which act to promote cell proliferation, and GT3K, which can be used to promote somatic cell reprogramming.

***Key words:*** Chicken egg-white extract; Ingredients; Cell proliferation; Cell reprogramming; Telomeres growth; Stem cell.

## Introduction

To date, many studies have reported on the ingredients and roles of chicken egg-white extracts (1-7), whereas few studies have examined whether chicken egg-white extracts have roles in promoting somatic cell reprogramming (8-13). Previous research demonstrated that human somatic cells can be directly reprogrammed back into an induced pluripotent stem cell (iPSC) state through exogenous expression of a small number of transgenic factors(14, 15). The ability of these cells to differentiate into any human cell type highlights their promise for future autologous cellular therapies. Nevertheless, the continued presence of potentially oncogenic transgenic elements following reprogramming represents a safety concern that must be addressed prior to clinical applications(16).

There is currently ongoing an extensive search for new methods for reprogramming such as a reduced number of defined factors(17), adenoviral or plasmid-based transient gene delivery (18-20), or oocyte-free, nonviral inducers like small molecules(21-23) and proteins(24) that could be safely used. There were also reports that pig egg extracts can reprogram somatic cells (25). But scholars are still looking for simple new reprogramming approach.

We found that after the egg-white extract co-cultured with cell, the pluripotent factors expression can be increased, and the telomere length is increased, so that the somatic cells converted into young stem cells. It provides a new approach for reprogramming somatic cells. Through our research (26) on chicken egg-white, egg-yolk and whole-egg extracts, we found that chicken egg-white and whole-egg extracts could promote cell proliferation(27, 28) and the expression of pluripotency factors. Subsequent studies found that chicken egg-white extract played the key role in this process, though the specific ingredients that are responsible remain unknown. We conducted intensive studies in which chicken egg-white extracts were ultrafiltrated and divided into fractions containing different ingredients with molecular weights that were either greater than 3 kDa (GT3K) or less than 3 kDa (LT3K) in order to identify those that are important for cell reprogramming. We found that chicken egg-white extract ingredients of LT3K function to promote cell proliferation and survival, whereas ingredients GT3K function to promote the increased expression of pluripotency factors. Our findings could facilitate the development of cell culture additives to promote cell growth and proliferation. We can also determine an egg cell extract to promote the elevated expression of pluripotency factors NANOG and OCT4.

No previous studies used mouse spleen cells permeabilized by chicken egg-white extracts. We previously found that when mouse spleen cells were permeabilized by chicken egg extracts, the mouse spleen cells increased their expression of the pluripotency factor OCT4 (26). Electrophoresis of the chicken egg extract showed different molecular weight protein bands; however, which protein was responsible for OCT4 upregulation could not be determined. Hence, we used ultrafiltration to separate GT3K proteins and LT3K small molecules and cultured these fractions with 293T cells. Subsequent

quantitative PCR experiments were performed to detected changes in the expression levels of the pluripotency genes OCT4 and NANOG in the 293T cells. Cell proliferation assays were also performed by culturing cells with different concentrations of the extracts, and the results are reported below.

## Materials and Methods

### Chicken egg-white extract preparation

The egg-white and egg-yolk of a chicken egg were isolated under sterile conditions. An equal volume of lysis buffer (50 mM NaCl, 5 mM MgCl2, 100 mM HEPES pH 8.2, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail) was added to the egg-white. The solutions were fully mixed and subjected to three rounds of freezing and thawing. Then, the solutions were centrifuged, and the supernatants were stored at 4 °C to produce the chicken egg-white extract.

### Separation of chicken egg-white extracts ingredients

Chicken egg-white extract was separated using ultrafiltration tubes (3-kDa cut off) with a processing capacity of 15 ml. Briefly, 15 ml of chicken egg-white extract was applied to the ultrafiltration tube, followed by centrifugation at 4000 rpm for 30 min. The fraction at the bottom of the ultrafiltration tube contained ingredients LT3K, and the fraction remaining at the top of the ultrafiltration tube contained ingredients GT3K.

### SDS-PAGE of chicken egg-white extract

The protein concentrations of the ingredients of the LT3K and GT3K of chicken egg-white extract fractions were measured using the Bradford method. Based on the measured concentration, the chicken egg-white extract and the GT3K fraction were diluted 20-fold. The LT3K fraction was not diluted because the LT3K fractions almost do not contain protein. Next, 50 µL of extract was added to 50 µL of 2 × loading buffer and incubated in a boiling water bath for 5 min. Then, 5 µL of sample was loaded into the wells. The upper gel was run at 80 V, and the lower gel was run at 120 V. The gel was stained for three hours, destained overnight, and then photographed.

### Detection of cell proliferation activity

The 293T cells were adjusted to a concentration of $1\times10^5$/mL, and 50 µL of cells was added to each well (i.e., $5\times10^3$ cells per well). Furthermore, 100 µL of PBS was added to the edges of the 96-well plates to prevent evaporation. A total of 50 µL of extract was then added to reach a final concentration of 50 %, 25 %, and 5 %. Each extract was added to 10 wells, and the plates were incubated at 37 °C for 3 days. Finally, 20 µL of the cell viability detection reagent from the Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was added per well, followed by incubation at 37 °C for three hours and colorimetric detection at 490 nm. The control media is DMEM-F12 media supplemented with 10% FBS.

### Co-culturing of 293T cells with different ingredients of chicken egg-white extract

A total of $1\times10^5$ 293T cells were added to each well of a 24-well plate in a total volume of 500 µL per well, to which 500 µL of the different extracts was added. The GT3K and LT3K ingredients were present at a final concentration of 50 %. Co-culturing was performed for 2, 3, 4 and 5 days to collect cells for RNA extraction using Baitaike kits. Reverse transcription to cDNA and quantitative PCR were carried out using Promega reagents.

### Relative expression of the pluripotent genes NANOG and OCT4 detected by quantitative PCR

The primer sequences are based on the literature, and the sequences are as Table 1.

### Telomere relative length detection in 293T cells

293T cells co-cultured with different extracts were collected. Cells are extracted RNA, reverse transcription into cDNA. Quantitative PCR was performed using telomere primers and internal control 36B4 primer.

**Table 1.** The primer sequences and product length.

| gene | sequences | Product length |
|---|---|---|
| OCT4 | F: AAGCGATCAAGCAGCGACTAT<br>R: GGAAAGGGACCGAGGAGTACA | 163bp |
| NANOG | F: CAAAGGCAAACAACCCACTT<br>R: TCTGCTGGAGGCTGAGGTAT | 158bp |
| GAPDH | F: TCGGAGTCAACGGATTTGGT<br>R: TTGCCATGGGTGGAATCATA | 148bp |

**Table 2.** Primer sequences of Telomere and internal control.

| Genes | Primer sequences |
|---|---|
| Telomere | Forward<br>5’-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3’<br>Reverse<br>5’-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3’ |
| 36B4 | Forward 5’-CAGCAAGTGGGAAGGTGTAAATCC-3’<br>Reverse 5’-CCCATTCTATCATCAACGGGTACAA-3’ |

**Table 3.** The protein concentrations of the extracts

| Extract | Protein concentration (mg/mL) |
|---|---|
| chicken egg-white extract | 24 |
| GT3K ingredients | 31 |
| LT3K ingredients | 0.14 |

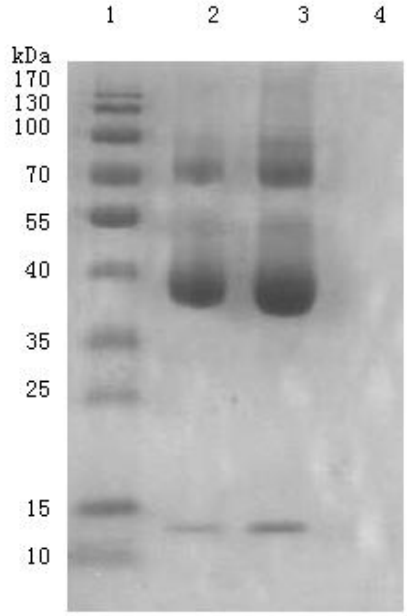


**Figure 1.** Electrophoresis of the different ingredients of chicken egg-white extract. Lane 1, protein molecular weight standards; lane 2, chicken egg-white extract; lane 3, chicken egg-white ingredients GT3K; lane 4, chicken egg-white ingredients LT3K.

Annealing temperature of telomere is of 54 degrees; of internal control 36B4 is of 58 degrees. By quantitative PCR, the relative length of telomere was calculated.

The primer sequences are based on the literature, and the sequences are as Table 2.

### Flow cytometry detection of pluripotent factor expression induced by egg-white extract

293T cells cultured for 3 days with media and 50% final concentration of egg-white extract respectively. The cells were collected, centrifuged, the supernatant was discarded. The cells were labeled with SSEA-4-PE (from BD Company), OCT4-PE (from eBioscience Company) and NANOG-PE (from BD Company). The cells were incubated at room temperature for 1 hour, washed by PBS, and detected by flow cytometry.

### Western Blot detection of pluripotent factor expression induced by egg-white extract

293T cells cultured for 3 days with media and 50% final concentration of egg-white extract respectively. The cells were collected, centrifuged, the supernatant was discarded. The cells were extracted proteins. 20 μg cellular proteins each well performed SDS-PAGE electrophoresis. After electrophoresis, the proteins were transfered to PVDF membrane. PVDF membranes were blocked for 1 hour with 5% nonfat dry milk in TBS. The antibodies 1: 200 was added 2% nonfat dry milk in TBS. The antibodies are rabbit monoclonal antibody against human OCT4 and NANOG (purchased from Abcam Company) respectively. PVDF membranes were shaken for one hour at 37 degrees. TTBS washed three times, each time 5min. 2 of the membranes were added to the second antibody of goat anti-rabbit IgG-HRP antibodies. The secondary antibodies were 1: 200 in 2% nonfat dry milk in TBS, shaken for 1 hour at 37 degrees. TTBS washed three times, each time 5min, added chemiluminescent substrate ECL, placed in chemiluminescence imager and photographed.

### Statistical Analysis

The data are shown as the mean ± SD. The groups were compared using one-way ANOVA with the SPSS 17.0 statistical software. A $P < 0.05$ was considered statistically significant.

## Results

### SDS-PAGE of chicken egg-white extract

Based on the Bradford method, the protein concentrations of the extracts were as Table 3.

The chicken egg-white extract was separated by ultrafiltration into GT3K and LT3K ingredients. Electrophoresis revealed that the chicken egg-white extract comprises three main bands: 90 kDa, 40 kDa, and 12 kDa (Figure 1).

### Relative NANOG and OCT4 gene expression in 293T cells co-cultured for 2, 3, 4, 5 d with the different extracts

Statistical analyses indicated that the ingredients GT3K can increase the expression of NANOG and OCT4 compared with the LT3K ingredients (* P <0.01) (Figure 2).

As seen from Figure 2A, the ingredients GT3K promote the expression of pluripotency genes NANOG. This effect is stronger than that for the total chicken egg-

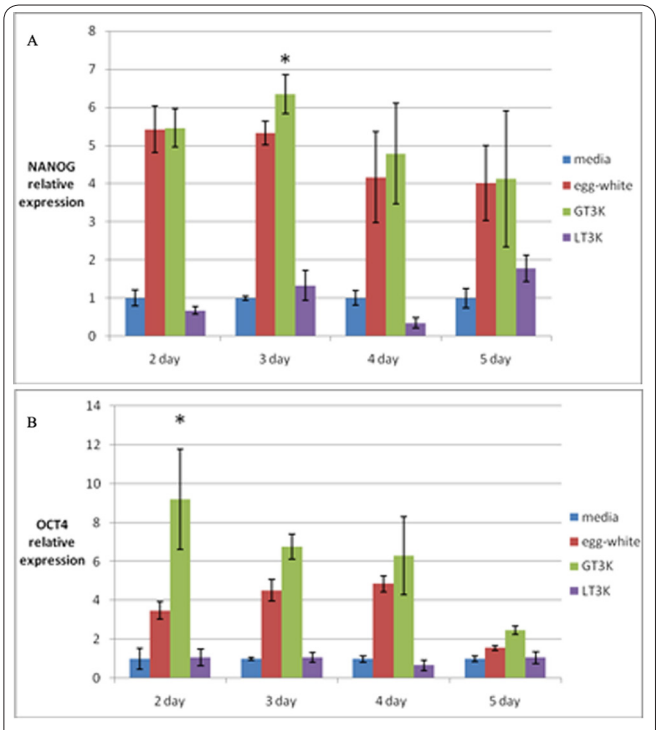


**Figure 2.** The relative expression of the NANOG and OCT4 genes in 293T cells co-cultured for 2, 3, 4, 5 d with the extracts (n=5). A. Relative expression of the NANOG gene. NANOG gene expression is strongest at 3 d, and at 5 d, the expression was reduced. B. Relative expression of the OCT4 gene. OCT4 expression was strongest at 2 d and was decreased at 5 d.

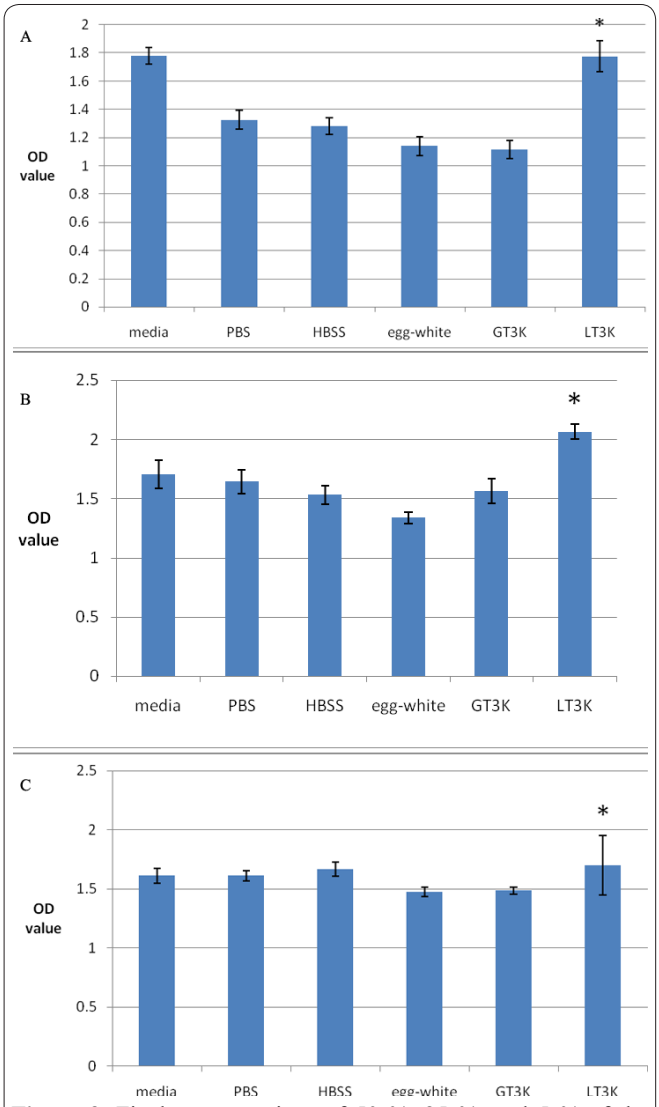


**Figure 3.** Final concentrations of 50 %, 25 %, and 5 % of the chicken egg-white extract ingredients differentially promote proliferation. A Chicken egg-white extract each ingredients at a final concentration of 50 % co-cultured with 293T cells for 3 d (n=10) (* P < 0.01 compared with the other groups, P = 0.973 compared with the media). B Chicken egg-white extract each ingredients at a final concentration of 25 % co-cultured with 293T cells for 3 d (n = 10) (* P < 0.01 compared with the other groups, P < 0.05 compared with the media). C Chicken egg-white extract each ingredient at a final concentration of 5 % co-cultured with 293T cells for 3 days (n = 10) (* P < 0.01 compared with GT3K and chicken egg-white, P > 0.05 with the rest of the groups).

white extract. NANOG gene expression is strongest at 3 d, and at 5 d, the expression was reduced. As seen from Figure 2A, the GT3K ingredients are the main ingredients involved in promoting the increased expression of NANOG pluripotency genes.

As seen from Figure 2B, the GT3K ingredients promote the increased expression of the pluripotency gene OCT4. This effect is stronger than that for the total chicken egg-white extract. OCT4 expression was strongest at 2 d and was decreased at 5 d. As seen from figure 2B, the GT3K ingredients promote the increased expression of OCT4.

As seen from Figure 2, the GT3K ingredients increased the expression of the pluripotency genes NANOG and OCT4, whereas the LT3K ingredients had no effect.

## Final concentrations of 50 %, 25 %, and 5 % of each chicken egg-white extract ingredient exhibit different effects on proliferation

As seen from figure 3A, at a final concentration of 50 %, the LT3K chicken egg-white extract ingredients have the same effect as the media (P = 0.973) and a stronger effect than PBS, HBSS, the GT3K ingredient and chicken egg-white extract (P <0.01). The control media is DMEM-F12 media supplemented with 10% FBS. At a final concentration of 50 %, the LT3K chicken egg-white extract ingredients with 5 % FBS have the same effect as the media (P = 0.973) with 10 % FBS, so we speculate the LT3K promote 293T cells proliferation.

As seen from figure 3B, a 25% final concentration of LT3K chicken egg-white extract ingredients promoted cell proliferation better than the media (P < 0.05) and better than PBS, HBSS, chicken egg-white extract and the GT3K ingredients (P < 0.01). This suggests that the chicken egg-white extract LT3K ingredients can be used as additives to cell culture media, given the enhanced cell proliferation at a final concentration of 25 %. At the final concentration of 25 %, the LT3K components at 7.5 % of FBS promoted stronger cell proliferation role than the medium at 10 % of FBS (P < 0.05). These results suggest that the LT3K components of chicken egg white extract are a potential additive for cell culture media, better promoting cell proliferation than media supplemented with 10 % FBS.

As seen from figure 3C, the chicken egg-white LT3K ingredients extract at a final concentration of 5 % has the same effect as the culture media, PBS, and HBSS (P > 0.05), but a stronger effect than the chicken egg-white and GT3K ingredient fractions (P < 0.01). At a final concentration of 5 %, the LT3K chicken egg-white extract ingredients with 9.5 % FBS have the same effect as the media with 10% FBS, so we speculate the LT3K promote 293T cells proliferation.

## The test results of the telomere relative length

293T cells were cultured for 3 days with four extracts, the test results of relative telomere length showed that 50% of the final concentration of egg-white extract, GT3K and LT3K ingredients co-cultured with 293T

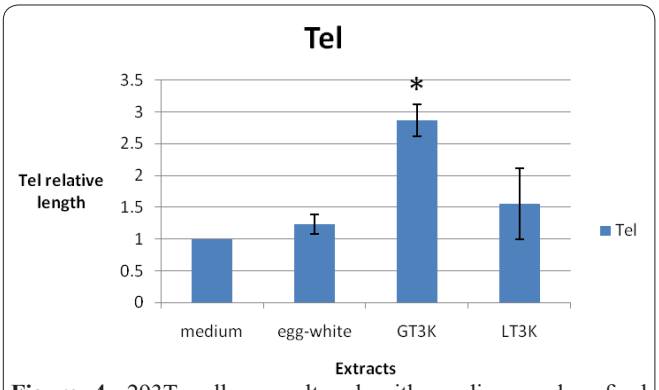


**Figure 4.** 293T cells co-cultured with medium and a final concentration of 50% three extracts for 3 days, the test results of relative telomere length. (n = 5, * P <0.01 compared to other groups). 50% of the final concentration of egg-white extract, GT3K and LT3K ingredients co-cultured with 293T cells for 3 days, the relative telomere length in cells increased. While GT3K ingredients were co-cultured with 293T cells for 3 days, the relative length of the telomeres is the longest.

cells for 3 days, the relative telomere length in cells increased, while GT3K ingredients were co-cultured with 293T cells for 3 days, the relative length of the telomeres is the longest, indicating that GT3K ingredients can promote telomere growth in 293T cells, make cells transformed into a younger stem cells (Figure 4).

## Flow cytometry results

Cultured in media for three days, SSEA-4-PE positive rate was of 0.305%. 50% final concentration of egg-white extract was incubated for 3 days, SSEA-4-PE positive rate was of 5.79%, and the positive rate was significantly increased. Cultured in media for three days, OCT4-PE positive rate was of 1.93%. 50% final concentration of egg-white extract was incubated for 3 days, OCT4-PE positive rate was of 7.54%, the positive rate was significantly increased. Cultured in media for three days, NANOG-PE positive rate was of 0.539%. 50% final concentration of egg-white extract was incubated for 3 days, NANOG-PE positive rate was of 4.10%, the positive rate was significantly increased (Figure 5). It showed pluripotency factor expression in some of cells was significantly increased, occurred the phenomenon of reprogramming.

## Western Blot test results

Media for three days almost did not express OCT4 protein, 50% final concentration of egg-white extract cultured for 3 days, OCT4 protein expression was positive (Figure 6A). Media for three days did not express NANOG protein, 50% final concentration of egg-white extract cultured for 3 days, NANOG protein expression was positive (Figure 6B). It showed the cells occurred reprogramming, pluripotent protein expression openned.

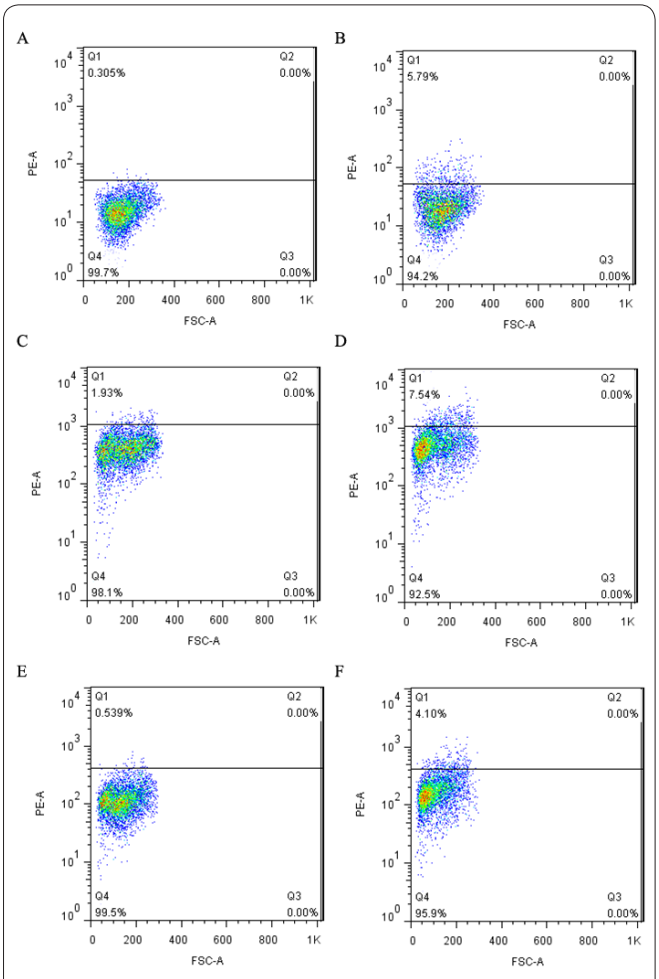


**Figure 5.** Flow cytometry results. A and B labeled with SSEA-4-PE; C and D labeled with OCT4-PE; E and F labeled with NANOG-PE. A, C and E cultured in media; B, D and F cultured in 50% egg-white extract, the positive rate was significantly increased.

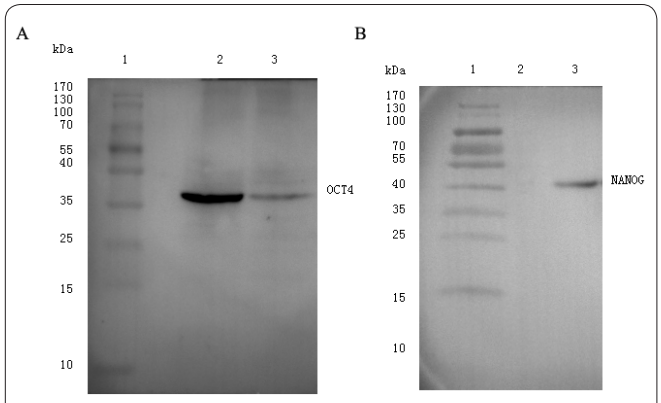


**Figure 6.** Western Blot test results. A the primary antibody was rabbit anti-human OCT4 monoclonal antibody. 1 marker. 2 induced for three days (50% egg-white extract co-culture). 3 control (media co-culture); B the primary antibody was rabbit anti-human NANOG monoclonal antibody. 1 marker. 2 control (media co-culture). 3 induced for three days (50% egg-white extract co-culture). It showed OCT4 and NANOG protein expression was significantly increased after induction.

## Discussion

The chicken egg yolk is the largest component of the egg, and the yolk membrane has similar functions to those of the cell membrane. The egg-white and egg-shell, which have roles in nutrition and protection, are formed from oviduct secretions. We prepared chicken egg-white, egg-yolk, and whole-egg extracts, used them to treat 293T cells, and observed the effects on cell survival and differentiation. The results showed that chicken egg-white extract contained substances that promoted 293T cell survival and proliferation. The effects were significantly different than those observed with the control. We speculated that these substances are proteins or small molecules in the chicken egg. In our follow-up studies, we found that chicken egg-white extract plays an important role in promoting cell survival.

In the present study, we found that chicken egg-white extract could promote cell proliferation, though the specific ingredient is not known. In this experiment, we separated the chicken egg-white into GT3K and LT3K ingredients, which were then co-cultured with cells at different final concentrations. The results showed that LT3K ingredients promoted cell proliferation, with LT3K ingredients at a final concentration of 25 % exhibiting a stronger effect on proliferation than that of the media, showing that the LT3K ingredients of chicken egg-white extract can be used as cell culture additives at a final concentration of 25 % to better promote cell proliferation. At the final concentration of 25 %, the LT3K components at 7.5 % of FBS promoted stronger cell proliferation role than the medium at 10 % of FBS ($P < 0.05$). We compared the media to the effects of balanced salt PBS and HBSS, as well as different ingredients of chicken egg-white extract, and GT3K and LT3K on proliferation. The results of the balanced salt solution and

extract solutions at final concentrations of 50 %, 25%, and 5 % were remarkably stable. The LT3K ingredients displayed good activity in promoting cell proliferation at different final concentrations. Proliferation increased as the final concentration of the ingredients gradually decreased, with a final concentration of 25 % showing a stronger proliferation effect than the media. When the final concentration was decreased further, the effect was the same as that of the media. The effect is stronger than that of chicken egg-white extract, and GT3K ingredients ($P < 0.05$). At a final concentration of 5 % of each ingredient, the effect of LT3K was the same as that of the culture media, PBS, and HBSS ($P > 0.05$) but stronger than those for the chicken egg-white extract and the GT3K ingredients ($P < 0.01$). The 25 % final concentration of the LT3K ingredient had a better effect, indicating its use as an additive to promote cell proliferation in culture media.

In the cell proliferation assay, the extract was added to a final concentration of 50 %, 25 %, or 5 %, and the cells were cultured for 3 d. Because the traditional MTT assay is cumbersome, with many factors affecting the test results, we chose to use the Promega cell viability detection kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay). This kit simplifies the procedure and does not require siphoning off the supernatant and dissolving the precipitate. Instead, this assay generates a colorimetric readout directly upon co-culture, which makes the results more accurate and more reproducible. Due to the presence of the 96-edge effect, we excluded the edge of the well by using 100 µL of PBS to prevent evaporation and obtain accurate results. Each extract concentration was added to 10 wells, and the mean ± standard deviation was determined to make the results more comparable.

Our results indicate that the LT3K ingredients in chicken egg-white extract at a final concentration of 25 % have a stronger function than the media in promoting cell growth. We can therefore consider separating chicken egg-white extract into LT3K ingredients as a cell medium additive to promote cell growth.

In earlier studies, we found that chicken egg extracts (egg-white, whole-egg) increased OCT4 expression in permeabilized mouse spleen cells. Later studies also showed that chicken egg extracts (egg-white, egg-yolk, whole-egg) promote increased expression of the pluripotency genes NANOG and OCT4 in 293T cells(13), and in-depth studies found that chicken egg-white extract has the strongest effect. Chicken egg-white is easily accessible, and the obtained extract is stable for more than 1 month at 4 °C. Furthermore, chicken egg-white can be used for cell culture without the need for filter sterilization. However, the active ingredient in chicken egg-white extract is unclear. Hence, we used 3-kDa MWCO ultrafiltration to separate the chicken egg-white extract into GT3K ingredient and LT3K ingredients for cell culture. The results showed that the GT3K ingredients function to promote increased NANOG and OCT4 gene expression, whereas the LT3K ingredients have no effect. Thus, it may be possible to isolate the GT3K ingredients for cell co-culture to induce cell reprogramming. The detection of increased NANOG and OCT4 gene expression levels via quantitative PCR further demonstrates that the chicken egg-white extracts have a role in reprogramming somatic cells.

Some studies have reported that animal egg cell extracts can reprogram somatic cells. The chicken egg yolk is the largest egg cell. The egg yolk membrane is a cell membrane. The egg-white and eggshell, which have roles in nutrition and protection, are formed from oviduct secretions. In our previous study, chicken egg extracts (egg-white, egg-yolk, whole-egg) were prepared and used in cell permeabilized culture (26). However, the yolk contains more lipid, the extraction process is complex and influenced by many factors, and the ability of yolk extracts to promote cell activity is not strong; therefore, we prepared chicken egg-white extracts for cell co-culture, as chicken egg-white is easy to extract in large quantities, and after the extraction process can be used in cell culture without need additional sterilization.

Studies have shown that telomere growth is cell rejuvenation performance (29, 30). 293T cells co-cultured with medium and a final concentration of 50% three extracts for 3 days, 50% of the final concentration of all three extracts promote telomere growth in 293T cells. Among them, GT3K ingredients promote telomeres the most significant growth rate in 293T cells, indicating egg-white extract has some ingredients to promote telomeres growth in 293T cells, has the role of reprogramming somatic cells. The cells after co-culture with the extracts occurred phenomenon to transform into more youthful stem cells. The effect of GT3K ingredients was the most obvious among three extracts. In the future we can focus on study the mechanisms of GT3K ingredients promote 293T cells transformation into younger stem cell.

Our study highlights a new method and tools to reprogram somatic cells using egg cell extracts. We expect to identify new active ingredients to reprogram somatic cells or promote cell survival and proliferation.

Our study indicated that in co-culture, the GT3K egg-white extract ingredients function in promoting somatic cells reprogramming. The LT3K ingredients stably promote cell proliferation, and the results showing that the different molecular ingredients of chicken egg-white extracts have different roles will help us choose different ingredients for use in cell co-culture.

**Authors' contributions**

Guang-ping Ruan drafted the manuscript. Ju-fen Liu carried out the SDS-PAGE. Jian-yong Yang and Rongqing Pang participated in the PCR. Xiang Yao and Jun Shu participated in the design of the study and performed the statistical analysis. Xing-hua Pan conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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**Interest Conflict**

The author(s) declare that they have no competing interests.

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