Treatment with chicken-egg-white or whole-egg extracts maintains and enhances the survival and differentiation of spleen cells

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Abstract The identification of egg extracts with the ability to maintain and enhance the survival and differentiation of cells would be widely useful in cellular biology research. In this study, we compared the different abilities of spleen cells to survive and differentiate in vivo after permeabilization by five different types of egg extracts. Five types of egg extracts were prepared. The spleen cells from male GFP-transgenic mice were permeabilized by the extracts for 30 min, cultured for 12 days, and then transfused into irradiated female mice. At varying days after transplantation, the percentage of GFP-expressing surviving spleen cells was detected in the peripheral blood by flow cytometry. At 120 days after transplantation, bone marrow cells from the female mice were analyzed for the presence of cells containing the Y chromosome. Surviving GFP-positive spleen cells that had been permeabilized with either chicken-egg-white or whole-egg extracts could be detected in the female mice after transplantation. A lower percentage of GFP-positive cells was also detected after permeabilization by the other extracts tested, and no GFP-positive cells were found in the female mouse transfused with spleen cells permeabilized with

Hank's Buffered Salt Solution (HBSS) as a control. At 120 days after transplantation, the percentage of cells containing a Y chromosome in the bone marrow positively correlated with the percentage of GFP-positive cells in the peripheral blood. After permeabilization by chicken-egg-white or whole-egg extracts, spleen cells demonstrated significantly enhanced survival and differentiation functions compared with the spleen cells treated with the other egg extracts tested. These results show that chicken-egg-white and whole-egg extracts have roles in maintaining and enhancing the survival and differentiation of spleen cells. Therefore, these two types of extracts may be of future use in maintaining the function of stem cells.

Keywords GFP-transgenic mice · Spleen cells · Extracts · Cell survival · Cell differentiation

Introduction

Stem cells are a potential source of biological material for regenerative medicine. However, stem cells are easier to differentiate into mature cells than to revert to a pluripotent state in culture. Studies have shown that the extracts of mammalian oocytes (Miyamoto et al. 2009) and *Xenopus* oocytes (Alberio et al. 2005) have the potential to reprogram cells. Therefore, we speculated that the extracts of chicken and/or fish eggs may also have roles in maintaining the original function of the spleen cells. Mouse spleen cells contain a number

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of hematopoietic stem cells (Huang et al. 2007). We have previously shown these cells can reconstruct a hematopoietic stem cell population but are easier to differentiate into mature cells than to revert to a hematopoietic state in culture. Therefore, we prepared egg extracts from different species and observed the roles of these extracts in the protection of hematopoietic stem cells from the spleen.

All cells within GFP-transgenic mice are fluorescent (Balic and Mina 2010; Smeti et al. 2010; Takaishi et al. 2010; Wilkosz et al. 2011). Fluorescent cells from GFP-transgenic male mice can be transfused into irradiated female mice, and the infused cells can be tracked by two methods. The first detects the GFP-positive cells in the peripheral blood, and the second detects the presence of cells containing a Y chromosome in the bone marrow. Our results show that the rate of GFP-positive cells in the peripheral blood positively correlates with the percentage cells containing a Y chromosome in the bone marrow. Therefore, simply detecting GFP-positive cells in the peripheral blood can reflect the extent of hematopoietic reconstitution. In this study, we compared the different abilities of spleen cells to survive and differentiate *in vivo* after permeabilization by five types of egg extracts.

Many studies have shown that egg extracts, including cell-free extracts from mammals (Miyamoto et al. 2009), *Xenopus laevis* (Danilchick et al. 1991; Lohka and Masui 1983), *Drosophila melanogaster* (Leno 1998; Ulitzur and Gruenbaum 1989), and other non-mammalian oocytes (Cameron and Poccia 1994; Iwao and Katagiri 1984) can partially induce nuclear reprogramming in somatic cells. Therefore, we wanted to determine if chicken and/or fish-egg extracts have the same function because chicken, carp and rainbow trout eggs produce larger extract volumes and are easier to obtain. We observed the effect of treatment with these extracts on mouse spleen cells. By infusing the spleen cells from fluorescent male mice into female mice, we can easily quantify the survival and differentiation ability of the spleen cells *in vivo*. We used five types of extracts to permeabilize the spleen cells, cultured the permeabilized cells for 12 days and then transfused the cells into irradiated female mice. The results indicated that treatment with the chicken-egg-white and whole-egg extracts maintained the functional activity of the treated spleen cells and protected the hematopoietic progenitor cells within the splenocytes. Therefore, the use of chicken-

egg-white and whole-egg extracts may have potential uses for cellular reprogramming in the future.

Materials and methods

Extract preparation

The egg white and egg yolk of a chicken egg were sterilely isolated. 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 phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail) was added to the egg white, and lysis buffer was added to the egg yolk at a 1:3 yolk to buffer ratio. The solutions were fully mixed and stored at 4 °C. After 3 days, the solutions were centrifuged and the supernatants were stored at 4 °C to produce the chicken-egg-white and egg-yolk extracts. For the whole-egg extract, another chicken egg was sterilely isolated, lysed with a 1:3 egg to lysis buffer ratio, fully mixed and stored at 4 °C. After 3 days, the solution was centrifuged, and the supernatant was stored at 4 °C. For the carp-egg and rainbow-trout-egg extracts, the respective eggs were washed with saline, and equal volumes of saline were mixed with the eggs using a homogenizer to make the fish oocyte solutions. The solutions were frozen at −80 °C, thawed, and lysis buffer was added to the supernatants at a 3:1 or 1:2 egg-to-buffer ratio for the carp and rainbow trout oocytes, respectively. The solutions were then stored at 4 °C for 10 min, centrifuged and the supernatants were collected and filtered using a 0.22 µm filter. The protein concentrations of the extracts were measured using the Bradford method. The extracts were then marked with their protein concentrations and preparation times, aliquoted and stored at −20 °C until use. Immediately prior to use, the protein concentrations of the extracts were adjusted to 10 mg/ml using HBSS, and the extracts were diluted with H₂O to adjust the osmolality to approximately 300 mOsm.

SDS-PAGE analysis of the five extracts

For the SDS-PAGE gels, the polyacrylamide concentrations of the upper and lower gels were 5 and 15%, respectively. The samples were added to the wells, subjected to electrophoresis, stained with Coomassie Brilliant Blue Staining for 3 h and destained overnight.

Preparation of the spleen-cell suspensions from GFP-transgenic male mice

The welfare of the mice in this study was monitored by our institution's regulatory framework. All experimental protocols were approved by the Experimental Animal Ethics Committee of Kunming General Hospital. Using sterile technique, the spleen was removed using double antibiotics-soaked instruments, placed on a 100-line screen, homogenized with a syringe needle, and washed with basal medium (incomplete DMEM/F12 medium). The cells were then put on a filtration screen for collection and washed again with basal medium. The red blood cells were lysed by adding lysis buffer (NH_4Cl 8 g, NaHCO_3 0.84 g, Na_2EDTA 0.37 g, distilled water added to 1,000 ml).

The permeabilization of fluorescent spleen cells from GFP-transgenic male mice by five types of egg extracts

The cells were permeabilized according to a previously published protocol (Freberg et al. 2007; Taranger et al. 2005) with minor modifications. Briefly, 500,000 spleen cells were washed in 500 μl PBS, subsequently washed in 500 μl of ice-cold Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (HBSS) and resuspended in 475 μl of ice-cold HBSS. The samples were placed in a 37 °C H_2O bath for 2 min, and 25 μl of Streptolysin O (SLO; 200 $\mu\text{g}/\text{ml}$ stock diluted 1:20 in cold HBSS; Sigma-Aldrich) was added to a final concentration of 500 ng/ml. The samples were then incubated horizontally in a 37 °C H_2O bath for 30 min with occasional agitation and placed on ice. The optimal SLO concentration and incubation time were adjusted for each SLO batch. The samples were subsequently diluted with 1 ml of cold HBSS, and the cells were sedimented at $120 \times g$ for 5 min at 4 °C. The permeabilized cells (500,000) were resuspended in 500 μl of the different egg extracts containing an ATP-regenerating system and 1 mM of each nucleotide triphosphate. The samples were incubated horizontally in a 37 °C H_2O bath for 30 min with occasional agitation. To reseal the cellular membranes, the extracts were diluted with complete DMEM/F12 medium containing 2 mM CaCl_2 , and 250,000 cells/well were seeded in a 12-well plate. After 4 h, the supernatants were removed, and the plated cells were cultured in complete DMEM/F12 medium.

The detection of oct-3/4 and c-myc double-positive spleen cells by flow cytometry after 7, 10 or 12 days in culture

After incubation for 7, 10 or 12 days, the spleen cells were centrifuged, the supernatants were discarded and the precipitates were resuspended in 200 μl of 4% paraformaldehyde in PBS at room temperature for 10 min. The cells were then washed once with SAP buffer (a sterile solution containing 0.1% (w/v) saponin and 0.05% (w/v) NaN_3 in Hank's Balanced Salt Solution (HBSS)), and the samples were stained with 10 μl of an oct-3/4-PE conjugated antibody (R&D Systems, Inc.) and 20 μl of a c-myc-perCP conjugated antibody (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions. Then, the cells were stored at room temperature for 30 min, and positively stained cells were detected by flow cytometry. The experiments were repeated after 7, 10 or 12 days in culture, and the results are shown as the mean $\pm$ SD ($n = 3$).

Female mice irradiation and transfusion of cultured cells

Twenty-four female 8- to 12-week-old C57BL mice weighing from 18 to 22 g were provided by the Experimental Animal Center at Kunming General Hospital of PLA. The 24 female mice were divided into six groups of 4 mice. The irradiation was performed with a linear accelerator with a dose of 600 cGy at a rate of 50 cGy/min. The distance to the top of the box and the center of the mice was 98.5 and 100 cm, respectively. The individual mice groups were infused with either spleen cells permeabilized with HBSS or spleen cells permeabilized with chicken-egg-white, chicken-egg-yolk, chicken-whole-egg, carp-egg, or rainbow-trout-egg extracts. Each mouse was transfused with 2×10^6 spleen cells.

The detection of GFP-positive cells in the peripheral blood after transplantation

At either 21, 34 or 55 days after transplantation, blood samples were collected from the tails of the 24 female mice and hemolyzed by ammonium chloride. The percentage of GFP-positive cells was detected by flow cytometry.

The detection of CD4 and CD8 positive cells within the transplanted GFP-positive cells

At either 21, 34 or 55 days after transplantation, blood samples were collected from the tails of the 24 female mice. The blood samples were divided into two tubes, and 1.5 µl of a CD4-PE conjugated antibody (Biolegend) was added to one sample while 1.5 µl of a CD8-PE conjugated antibody (Biolegend) was added to the other sample for staining according to the manufacturer's instructions. After staining, the tubes were incubated at room temperature for 30 min. The samples were then hemolyzed with ammonium chloride. GFP and CD4 double-positive cells or GFP and CD8 double-positive cells were detected by flow cytometry.

The detection of cells containing a Y-chromosome in the bone marrow

One out of the 4 mice transplanted with HBSS permeabilized cells died by day 60. Therefore, 120 days after transplantation, the Y-chromosome-positive rates in the bone marrow were detected in 3 mice within each of the six treatment groups. A biotin-labeled mouse Y chromosome specific probe (50–100 ng) was diluted in 12 ml of hybridization buffer (50% deionized formamide, 10% dextran sulphate, $2 \times$ SSC and 0.5 mol/L phosphate buffer, pH 7.3). The probe was then denatured at 65 °C for 10 min and preannealed by incubation at 37 °C for 30–60 min. The slides were then denatured by incubation in a 70% formamide/ $2 \times$ SSC solution at 65 °C for 1–2 min, quenched in ice-cold 70% ethanol and dehydrated with a 70, 90 and 100% ethanol series. The preannealed probe was applied to the slides and allowed to hybridize overnight at 37 °C. After hybridization, the slides were washed with two 5-min incubations in 50% formamide/ $2 \times$ SSC at 45 °C and two 5-min incubations in $2 \times$ SSC at 45 °C. The biotin-labeled probe was visualized using Cy3-avidin (1:1,000 Amersham). After avidin binding, the slides were mounted in Vectashield mounting medium with DAPI (4',6-diamidino-2-phenylindole). The images were captured using a CytoVision system (Applied Imaging) with a CCD camera mounted on a Zeiss Axioplan 2 microscope. The results are shown as the mean $\pm$ SD ($n = 4$).

Statistical methods

A two-way analysis of variance (ANOVA) was carried out using SPSS software for Windows Version 17.0 to determine statistical significance.

Results

The SDS-PAGE results showed that the five extracts contain different proteins with different molecular weights (Fig. 1). The significance of the specific protein bands with roles in cellular reprogramming will require further investigation.

The expression of c-myc and oct-3/4 were examined in the permeabilized spleen cells after 7, 10 or 12 days in culture. The spleen cells that had been permeabilized with the chicken-egg-white or whole-egg extracts showed significantly higher expression of c-myc and oct-3/4 compared with the spleen cells permeabilized with HBSS ($p = 0.005$) (Fig. 2). The experiments were repeated after 7, 10 and 12 days in culture, and the results are shown as the mean $\pm$ SD ($n = 3$).

GFP-transgenic spleen cells from male mice were permeabilized with the five different types of egg extracts, cultured for 12 days and then transplanted into irradiated female mice. At 21, 34 or 55 days after transplantation, the percentage of GFP-positive cells

Fig. 1 The SDS-PAGE results of the five egg extracts. Lane 1: protein marker. Lane 2: chicken-egg-white extract. Lane 3: chicken-egg-yolk extract. Lane 4: whole-egg extract. Lane 5: carp-egg extract. Lane 6: rainbow-trout-egg extract

Fig. 2 c-myc and oct-3/4 expression in the permeabilized spleen cells cultured for 7, 10 or 12 days. The chicken-egg-white and whole-egg extract permeabilized spleen cells showed statistically significant higher expression of c-myc and oct-3/4

compared with the spleen cells permeabilized with HBSS ($p = 0.005$). The experiments were repeated after 7, 10 and 12 days in culture, and the results are shown as the mean $\pm$ SD ($n = 3$)

in the peripheral blood of the female mice was determined by flow cytometry. After transfusion, the spleen cells that had been permeabilized by the egg-white or whole-egg extracts were detected at a significantly higher rate in vivo than the spleen cells that had been treated with either HBSS as a negative control or the other tested extracts. An analysis of variance showed that the GFP-positive percentages among each treatment group were significantly different ($p = 0.01$) (Figs. 3, 4, 5). The results are shown as the mean $\pm$ SD ($n = 4$).

At 21, 34 or 55 days after transplantation, the spleen cells that had been permeabilized with the various types of extracts were analyzed by flow cytometry for the presence of GFP and CD4 positive staining. The results demonstrated that the spleen cells that had been treated with the chicken-egg-white or whole-egg extracts had significantly higher percentages of cells

with double-positive staining for GFP and CD4 than the spleen cells that had been treated with the other extracts ($p = 0.015$) (Fig. 6). The results are shown as the mean $\pm$ SD ($n = 4$).

At varying days after transplantation, the spleen cells that had been permeabilized with the various types of extracts were analyzed by flow cytometry for the presence of GFP and CD8 positive staining. The results showed that the spleen cells that had been treated with the chicken-egg-white or whole-egg extracts had significantly higher percentages of cells with double-positive staining for GFP and CD8 than the spleen cells that had been treated with the other extracts ($p = 0.019$) (Fig. 7). The results are shown as the mean $\pm$ SD ($n = 4$).

At 120 days after transplantation, the bone marrow from the transplanted female mice was analyzed for the presence of cells containing a Y-chromosome, and

Fig. 3 The percentage of GFP-positive transplanted cells in the peripheral blood of the female mice at varying days after transplantation. The results are shown as the mean $\pm$ SD ($n = 4$)

◀ **Fig. 4** The percentage of GFP-positive cells in the peripheral blood of the female mice at 34 days after transplantation. **a** HBSS. **b** Chicken-egg-white extract. **c** Chicken-egg-yolk extract. **d** Whole-chicken-egg extract. **e** Carp-egg extract. **f** Rainbow-trout-egg extract. The spleen cells were permeabilized by the different extracts before transplantation, and the percentage of GFP-positive cells was detected by flow cytometry

the peripheral blood was also analyzed for the presence of GFP-positive cells. The percentage of GFP-positive cells in the peripheral blood positively correlated with percentage of cells containing a Y chromosome in the bone marrow (Figs. 8 and 9). The results are shown as the mean $\pm$ SD ($n = 3$). These results demonstrated that the transplanted stem cells that had been treated with the chicken-egg-white or whole-egg extracts were capable of long-term survival in the female mice.

Discussion

In the present study, we report the effects of different types of egg extracts on the survival of spleen cells in vivo after transplantation. Different extract-treated spleen cells showed different survival rates in vivo, which indirectly demonstrated the effects of the different extracts on maintaining the function of the spleen cells. The survival rates were defined as how many cells among the 2×10^6 spleen cells survived in vivo after transplantation. The identification of egg extracts with the ability to maintain and enhance the survival and differentiation of cells would be widely useful in cellular biology research. Our results demonstrated that the chicken-egg-white and whole-egg extracts had roles in maintaining the differentiation capability of the spleen cells. The spleen cells were permeabilized for 30 min with the five different types of egg extracts or HBSS as a control and cultured for 7, 10 or 12 days. Using flow cytometry, we found that the spleen cells treated with HBSS did not have oct-3/4 or c-myc expression, unlike the spleen cells treated with the chicken-egg-white or whole-egg extracts that did show oct-3/4 and c-myc positive expression. This difference was statistically significant, and these results demonstrate that after permeabilization, spleen cells are able to be reprogrammed.

Many studies have reported that the animal egg extracts are able to induce the reprogramming of

somatic cells (Hansis et al. 2004; Miyamoto et al. 2009). The chicken egg yolk is the largest egg cell, where the yolk membrane comprises the cell membrane, and the egg white and eggshell, which have nutrition and protection roles, are formed by oviduct secretions. Therefore, we prepared chicken-egg-white, egg-yolk, whole-egg, carp-egg and rainbow-trout-egg extracts to permeabilize mouse spleen cells and observe the effects on cellular survival and differentiation. We found that spleen cells after permeabilization by different types of egg extracts had different survival and differentiation abilities in vivo. Our study showed that spleen cells treated with chicken-egg-white or whole-egg extracts maintained their functions better than spleen cells treated with the other tested extracts. These results indicate that the chicken egg white and whole egg contain some substances required to maintain the original function of the spleen cells. We hypothesize these substances may be proteins or small molecules in the chicken egg. The percentage of GFP-positive spleen cells that survived in vivo after treatment with the chicken-egg-white or whole-egg extracts was not significantly different. Therefore, we inferred that the chicken egg white plays a major role in facilitating cellular survival. The chicken egg white contains necessary nutrients for embryo development and may also contain key factors for the maintenance of embryo pluripotency. We hypothesize that the key factors for the maintenance of embryo pluripotency are proteins or small molecules in the chicken egg. Currently, little is known about the molecular mechanisms underlying the reprogramming process facilitated by egg extracts. Our SDS-PAGE analysis of the five egg extracts demonstrated large differences between the protein contents and molecular weights among the five extracts. In the process of permeabilization, the chicken-egg-white and whole-egg extracts maintained the pluripotency of the spleen cells and thus, contributed to the survival of the spleen cells in the female mice after transfusion. Determining whether the results were from the protection of the spleen hematopoietic progenitor cells or the induction of cell-reprogramming is not possible at present. If the results were from the protection of the spleen hematopoietic progenitor cells, the chicken-egg-white and whole-egg extracts could be applied for the protection of stem cells. However, if the results were from the induction of cell reprogramming, in the future, we can consider

◀ **Fig. 5** The percentage of GFP-positive cells in the peripheral blood of the female mice at 55 days after transplantation. **a** HBSS. **b** Chicken-egg-white extract. **c** Chicken-egg-yolk extract. **d** Whole-chicken-egg extract. **e** Carp-egg extract. **f** Rainbow-trout-egg extract. The spleen cells were permeabilized by the different extracts before transplantation, and the percentage of GFP-positive cells was detected by flow cytometry

using chicken-egg-white and whole-egg extracts to permeabilize a variety of cells to induce cell reprogramming.

Among the surviving GFP-positive cells in the female mice, some spleen cells that had been permeabilized with the egg-white or whole-egg extracts had converted into CD4+ and CD8+ T cells. This conversion rate was significantly higher than the other extract-treated groups ($p = 0.019$). The spleen cells permeabilized with the chicken-egg-white or whole-egg extracts were converted into hematopoietic cell lines in vivo, which were indicated by the presence of the CD4+ and CD8+ T cells (Ning et al. 2010).

Until now, the use of chicken-egg-white, egg-yolk and whole-egg extracts to permeabilize spleen cells,

along with the subsequent study of these cells in vivo, has not been investigated (Cho et al. 2010; Freberg et al. 2007; Miyamoto et al. 2009; Taranger et al. 2005). However, we found that the chicken-egg-white and whole-egg extracts were capable of protecting the CD4+ and CD8+ T cells within the splenocytes. Additionally, 120 days after transplantation, the percentage of cells containing a Y-chromosome in the bone marrow of the different extract-treated groups was statistically significant. Therefore, we inferred that the egg-white and whole-egg extracts can maintain the activity of hematopoietic stem cells within the spleen cells; however, the specific mechanisms behind these processes will require further investigation.

In this study, the use of carp-egg and rainbow-trout-egg extracts had no significant effects on maintaining the functional activity of the spleen cells, unlike the results found with the use of the chicken-egg-white or whole-egg extracts. Therefore, in the future, we can consider using chicken-egg-white or whole-egg extracts to permeabilize a variety of cells to maintain stemness. Cumulatively, the chicken-egg-white and whole-egg extracts have shown clear advantages in

Fig. 6 The percentage of CD4 and GFP double-positive cells at varying days after transplantation. The results are shown as the mean $\pm$ SD ($n = 4$)

Fig. 7 The percentage of CD8 and GFP double-positive cells at varying days after transplantation. The results are shown as the mean $\pm$ SD ($n = 4$)

Fig. 8 The percentage of cells in the bone marrow of the transplanted female mice containing a Y chromosome at 120 days after transplantation. The percentage of cells with a

Y-chromosome in the bone marrow positively correlated with the percentage of GFP-positive cells in the peripheral blood. The results are shown as the mean $\pm$ SD ($n = 3$)

Fig. 9 The Y chromosome was detected in metaphase spreads from cells collected from the bone marrow of female mice. The red chromosome indicates the Y chromosome. **a** A representative

metaphase spread with a Y chromosome. **b** A representative metaphase spread without a Y chromosome

maintaining the function and pluripotency of hematopoietic stem cells.

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