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Full Paper

Bioscience Microflora Vol. 30 (2), 37–44, 2011

Establishment and Evaluation of an *in vitro* M Cell Model using C2BBe1 Cells and Raji Cells

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Received October 18, 2010; Accepted December 8, 2010

***In vitro* M cell models, consisting of co-cultures of Caco-2 cells and lymphoid cells, were developed and examined to observe bacterial transport. However, under our experimental conditions, the differentiation of Caco-2 cells into M cell-like cells could not be induced efficiently. To obtain a functionally stable M cell model based on human cells, C2BBe1 cells were screened and co-cultured with human Raji cells. In our co-cultures, increased sialyl Lewis A antigen expression and decreased *Ulex europeaus* agglutinin 1 binding were observed. Regarding the functional properties of the model, microsphere and lactic acid bacteria transport across the C2BBe1 co-cultures were increased compared with the levels seen in monocultures. The C2BBe1 monolayers that were co-cultured with Raji cells exhibited some M cell features; therefore, we consider our M cell model to be useful for investigating the interactions of bacteria with M cells.**

Key words: M cell; C2BBe1; Raji; co-culture

INTRODUCTION

Immunization via the oral route offers several important advantages. In particular, unlike parenteral routes, specific immune responses to vaccine antigen are induced in the mucosa *(1)*. Therefore, a variety of oral vaccines that were generated from genetically modified bacteria have been reported *(2)*. We generated recombinant lactic acid bacteria (LAB) for use in an oral vaccine. These recombinants induced protective immunity and exhibited adjuvant properties *(3, 4)*. However, no practical oral vaccines that have used LAB as an antigen delivery vehicle have been established. The first step in the induction of protective intestinal immune responses is the uptake and transport of antigens to gut-associated lymphoid tissue (GALT). Hence, it is thought that efficient recombinant LAB transport to immuno-competent cells is necessary for effective vaccination.

It is generally thought that M cells, which are located in the follicle-associated epithelium (FAE) of Peyer's patch, play a major role in the uptake of luminal antigens *(5)*. M cells have a characteristic morphology and different functions compared with other intestinal enterocytes. M cells lack a well-organized brush border, have a thick glycocalyx, and display low levels of digestive enzymes, such as alkaline phosphatase and sucrase-isomaltase *(6–9)*. In addition, M cells have intraepithelial pockets containing lymphocytes, macrophages, and dendritic cells. The antigens internalized by M cells are transferred to these underlying immune cells, and antigen-specific immune responses are initiated *(10)*. Therefore, it is considered that these processes are key triggers of the induction of intestinal mucosal immunity. In addition, M cells are targeted by invasive pathogens, which exploit their uptake mechanisms to gain access to the body *(11)*. However, the uptake mechanisms of M cells are little known except for those of a few pathogens such as *Yersinia* and type-I-piliated bacteria *(12, 13)*. Due to the low number of M cells in the human intestine and the difficulty in culturing M cells, the characterization of M cells including their antigen uptake mechanisms has not advanced very far in *in vivo* or *in vitro* studies *(14, 15)*.

The human colon carcinoma cell line Caco-2 is widely used as a model of intestinal epithelial cells in studies of bacterial adhesion, invasion, and drug absorption *(16–18)*. In 1997, Kernéis et al. co-cultured Caco-2 cells with isolated murine Peyer's patch lymphocytes and proposed an *in vitro* human FAE model *(19)*. In this model, Caco-2 cells showed similar features to M cells, such as apical microvilli disorganization, the disappearance digestive enzymes, and the ability to transport microspheres and *Vibrio*. Based on this model, a human intestinal M cell model was established using co-cultures of Caco-2 cells and human Raji B cells instead of murine cells *(20)*. Subsequently, further M cell models with improved culture conditions have been developed, for example

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using Caco-2 subclones, and used to evaluate the transport of several pathogens and proteins *(21–23)*.

C2BBe1 cells were cloned from Caco-2 cells, and this cell line shows a more homogeneous brush border expression than the parental Caco-2 cells *(24)*. C2BBe1 cells have also been co-cultured with murine Peyer's patch lymphocytes in order to establish an M cell model *(25)*. In this study, to obtain a functionally stable M cell model based on human cells, we attempted to establish an M cell model by co-culturing C2BBe1 cells with Raji B cells.

MATERIALS AND METHODS

Bacterial strain and culture

Recombinant *Lactobacillus casei* IGM393 harboring pLPEmpty was grown in MRS broth (Difco) containing 5 μg/ml of erythromycin at 37 °C *(3)*.

Cell culture conditions

C2BBe1 cells were obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10 % fetal bovine serum (FBS; JRD), 1 × Glutamax I (Gibco BRL), 1 × nonessential amino acids (Gibco BRL), penicillin (100 U /ml), and streptomycin (100 μg/ml) (Gibco BRL). The human Burkitt's lymphoma cell line Raji (RCB1647) was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. The Raji cells were cultivated in RPMI1640 (Sigma) supplemented with 10 % FBS, 1 × nonessential amino acids, 1 × Glutamax I, penicillin (100 U /ml), and streptomycin (100 μg/ml) (Gibco BRL). All cells were grown in a humidified 5 % CO_2 atmosphere at 37 °C.

Induction of M cell features in C2BBe1 cells co-cultured with Raji cells

The induction of M cells from C2BBe1 cells was performed according to the methods of Corr et al. *(25)*. C2BBe1 cells were seeded (1×10^5 cells) onto transwell membranes (12-mm membrane diameter, 3.0-μm pore size, Corning) and cultured until they had fully differentiated. The medium was changed every 2 days. The transepithelial electrical resistance (TEER) of the C2BBe1 cells was measured with a Millicell-ERS (MILLIPORE) to confirm their differentiation and the integrity of the monolayer. After the TEER value of the C2BBe1 monolayer had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartment (Fig. 1). The co-cultures were maintained for 3–6 days. The upper medium was changed every day.

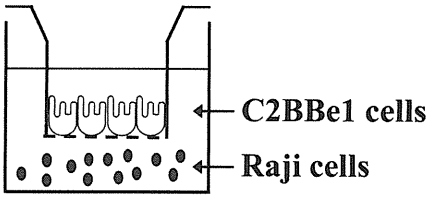

Fig. 1. Schematic of C2BBe1 cell and Raji cell co-culture model. Raji cells were added to basolateral side of C2BBe1 cell monolayers and co-cultured for 3–5 days.

Immunofluorescence

For immunofluorescence microscopy, samples were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS. After fixation, the samples were quenched with 50 mM NH_4Cl for 10 min and washed with PBS. The samples were then blocked with 2% bovine serum albumin in PBS for 60 min, before being incubated with a sialyl Lewis A (SLAA) antibody, β1 integrin antibody, or FITC-conjugated *Ulex europeaus* (UEA-1) for 60 min at room temperature. Then, the samples were washed and incubated for 60 min with an Alexa Fluor 488 goat anti-mouse IgG1 antibody (dilution: 1:2000). Transwell membranes were removed with a scalpel and mounted on glass slides. The slides were observed by fluorescence microscopy (Biozero; KEYENCE), and the stained area was measured by imageJ software *(26)*.

Fluorescent microsphere transport

Microsphere transport was observed in Hank's Balanced Salt Solution (HBSS) buffered to pH 7.4. After equilibration, the HBSS on the donor side was replaced with 500 μl of prewarmed microsphere suspension. The number of particles transported across the cell monolayer was then quantified in a Fluorescent Activated Cell Scan (FACScan, Becton-Dickinson).

Measurement of Lactobacillus casei IGM393 transport

L. casei IGM393 that had been cultured overnight were collected by centrifugation, washed three times with PBS, and resuspended in DMEM, before the bacterial cell concentrations were adjusted to 2×10^8 CFU/ml. A 500-μl volume of the bacterial suspension was added to the apical side of the C2BBe1 monolayers and incubated for 3 h. The basolateral media were then sampled and spread onto MRS-agar plates to estimate the number of colony-forming units.

Statistical analysis

Data were evaluated with Student's t-test and p values of less than 0.01 were considered statistically significant.

RESULTS

Monitoring the transepithelial electrical resistance of C2BBe1 monolayers during growth on a transwell membrane

The differentiation of C2BBe1 cells and the integrity of the monolayers were confirmed by measuring their transepithelial electrical resistance. The TEER values of the C2BBe1 cells had reached 300 $\Omega \times cm^2$ at 21 days (Fig. 2). After the C2BBe1 cells had been co-cultured with Raji cells, the TEER values of the co-cultures were similar to those of the monocultures (Fig. 3).

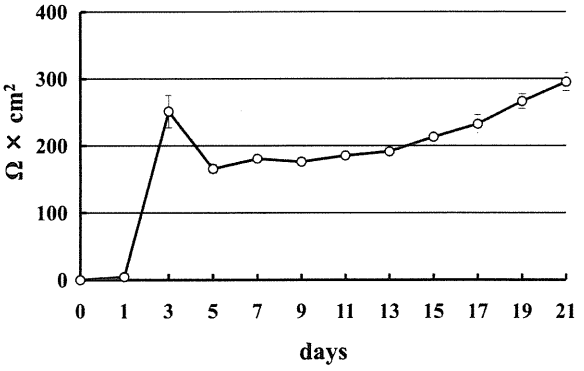

Fig. 2. TEER values of C2BBe1 monolayers grown on transwell membranes.

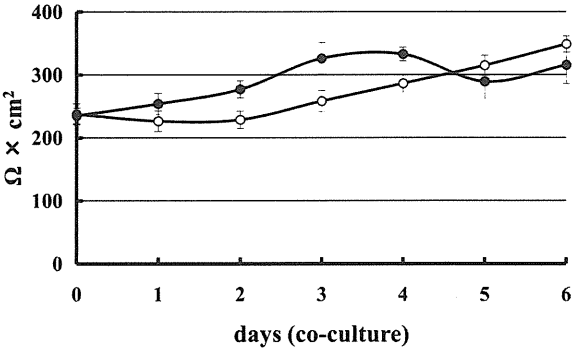

Fig. 3. Comparison of the TEER values of C2BBe1 monocultures and co-cultures. After the TEER values of the monolayer had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartment. The TEER values of co-cultures were measured everyday (closed circles). Monocultures of C2BBe1 monolayers were used as controls (open circles).

Expression of M cell markers

To investigate the effects on the C2BBe1 monolayer of co-culture with Raji cells, the expression levels of characteristic phenotypic markers of human M cells were examined. The expression of SLAA was increased by approximately 3-fold in the co-cultures compared with the monocultures (Fig. 4), and the binding of UEA-1 was decreased in apical membrane of the co-cultures (Fig. 5). There was no clear difference in the apical localization of β1 integrin in the C2BBe1 monolayers between the monoculture and co-culture conditions.

Transport of fluorescent microspheres

In order to confirm that the C2BBe1 cells had acquired M cell functional features, the number of transported fluorescent microspheres was measured. The transport of particles was increased 100-fold in the co-cultures compared to the C2BBe1 monocultures (Fig. 6).

Quantification of L. casei IGM393 transport across C2BBe1 monolayers

The ability of the *in vitro* M cell model to translocate *L. casei* IGM393 was examined. *L. casei* IGM393 were added to the apical side of the C2BBe1 monolayers. The C2BBe1 monolayers cultured with Raji cells had transported 10^3 CFU *L. casei* IGM393 after 3 h incubation at 37°C (Fig. 7). On the other hand, little bacterial transport was observed in the C2BBe1 monolayers cultured alone.

DISCUSSION

Observations of the internalization of the bacteria into non-phagocytic cells have mainly been performed using epithelial cell monolayers. However, in the intestine, a number of bacteria invade the host through M cells, and the morphology and function of M cells are markedly different from those of epithelial cells. Hence, a simple epithelial cell monolayer is insufficient as an M cell model, and a model system resembling M cells is necessary to observe bacterial internalization *in vitro*.

In vitro M cell models have been generated by co-culturing a variety of Caco-2 subclones with mouse Peyer's patch or human B cells. We attempted to establish an M cell model using Caco-2 cells in a preliminary study. However, as the Caco-2 monolayer was unstable during co-culture, we found it difficult to establish an M cell model using this technique. Therefore, Caco-2 clones were screened to see if they could be used to produce a stable model.

C2BBe1 cells form a polarized monolayer with an

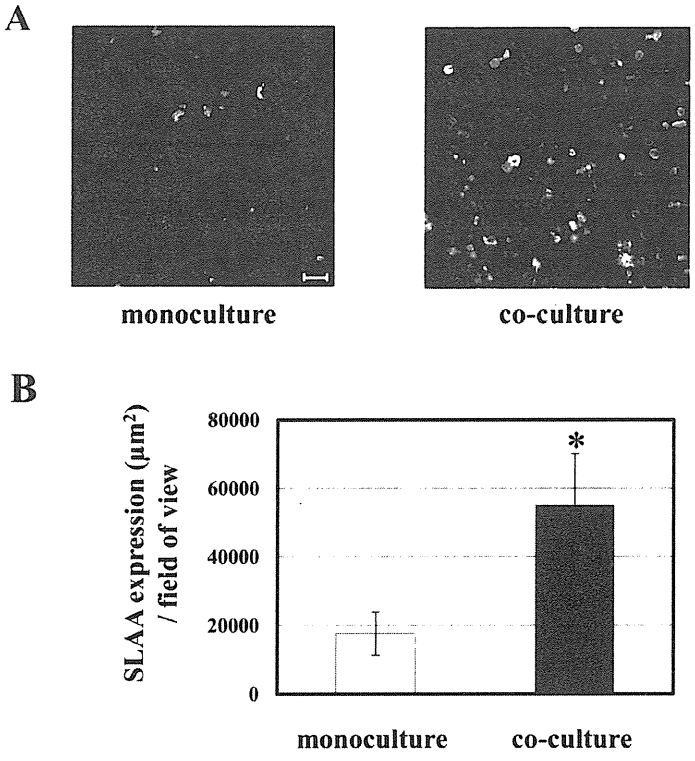

Fig. 4. Observation and quantification of sialyl Lewis A antigen expression. A) Immunohistochemistry of SLAA expression in C2BBe1 monocultures and co-cultures was observed by fluorescence microscopy. Scale bar: 50 μm. B) SLAA expression in the apical membranes of co-cultures compared with that observed in monocultures.

apical brush border that is morphologically comparable with that of the human colon and have been used to investigate bacterial adhesion and invasion *(27, 28)*. In the present study, we investigated whether human Raji B cells can induce C2BBe1 cells to differentiate into M cell-like cells.

First, the TEER values of C2BBe1 cells cultured on transwell membranes were measured as an indicator of cell monolayer integrity because we consider careful monitoring to be important for the establishment of a stable and reproducible model *(29)*. The TEER value increased rapidly within 3 days of the cells being seeded on the transwell membranes and gradually increased thereafter (Fig. 2). The C2BBe1 cells grew slowly and more stably over the long-term than other Caco-2 clones (data not shown). After the TEER value had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartments of the C2BBe1 monolayers. Monolayers of other Caco-2 clones could not be used because the TEER values of their co-cultures were extremely low, and the integrity of the differentiated monolayers was lost (data not shown). On the other hand, the TEER values of C2BBe1 co-cultures were between 250 and 300 $\Omega \times cm^2$, which was similar to that of the C2BBe1 monocultures (Fig. 3). The reduction in the Caco-2 cell co-culture TEER has been suggested to be due to the conversion of Caco-2 cells into M cells, whereas the C2BBe1 co-cultures seemed to maintain their integrity *(21)*.

In order to investigate the effects of Raji cells on C2BBe1 monolayers, the expression of M cell markers was examined. Several M cell markers have been reported, and in our experiment we observed that the apical expression of SLAA was significantly increased in co-cultures compared to monocultures (Fig. 4). The binding of UEA-1, which is a mouse and rabbit M cell marker, was decreased in the apical membranes of the co-cultures (Fig. 5). These results were also observed in a number of human M cell models *(20, 30)*. On the other hand, we were not able to find clear differences in the localization of β1 integrin between the co-cultures and

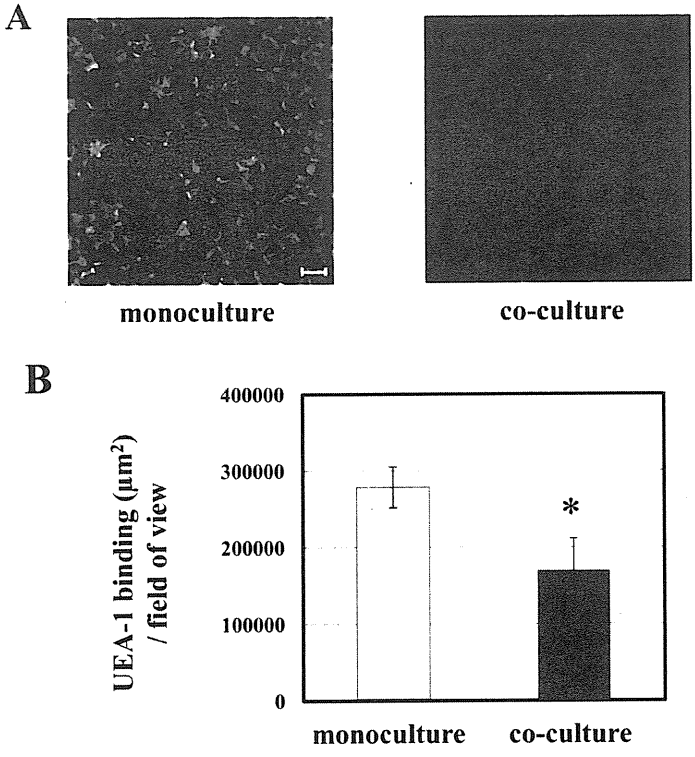

Fig. 5. Binding of UEA-1 lectin to C2BBe1 cells in monocultures and co-cultures. A) The binding of UEA-1 conjugated with FITC to C2BBe1 cell monolayers was observed by fluorescence microscopy. Scale bar: 50 μm. B) UEA-1 binding in C2BBe1 co-cultures compared with that observed in monocultures

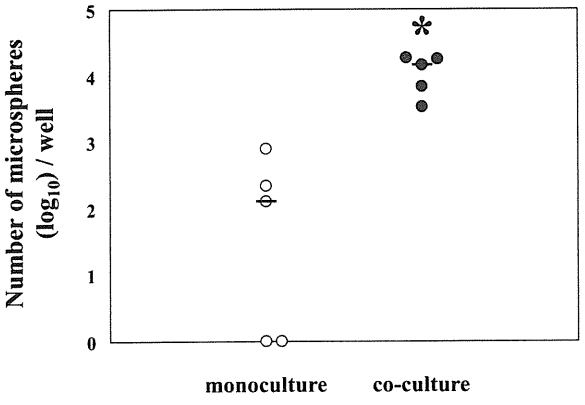

Fig. 6. Transport of microspheres across C2BBe1 monocultures and co-cultures. Mono- and co-cultures were incubated with microspheres for 3 hr at 37°C. The number of transported microspheres was evaluated by FACS.

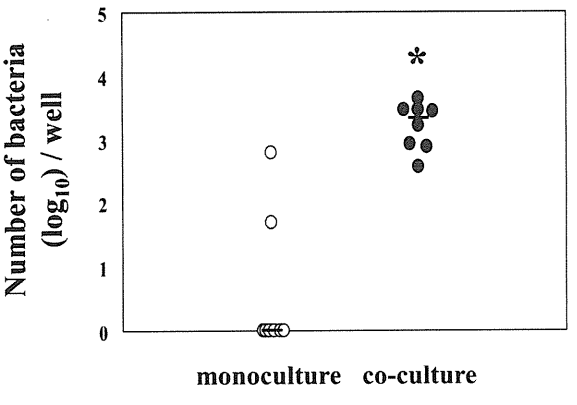

Fig. 7. Transport of *Lactobacillus casei* IGM393 across C2BBe1 monocultures and co-cultures. Mono- and co-cultures were incubated with *L. casei* IGM393 for 3 hr at 37°C. The number of transported bacteria was measured by CFU.

mono cultures.

Furthermore, to investigate the transport function of our model, microsphere transport was examined in both the co-cultures and monocultures. The number of transported particles was significantly increased in the co-cultures (Fig. 6). Before and after the particle transport

assay, the TEER values of each monolayer were not change, indicating that the integrity of the monolayers was maintained during the transport assay. Increased particle transport is a typical feature of M cell models *(20, 31)*. These results suggest that Raji cells induce C2BBe1 cell differentiation.

Finally, to investigate whether the co-cultures are able to transport non-invasive bacteria, we observed *L. casei* IGM393 transport across the monolayers. The number of transported *L. casei* was significantly increased in the basolateral compartments of the co-cultures compared to those of the monocultures (Fig. 7). Consequently, it was shown that the co-cultures were capable of incorporating even non-pathogenic and non-invasive lactic acid bacteria. However, in a few monocultures, a similar level of *L. casei* transport was found. It was reported that the differentiation of Caco-2 cells into M cell like-cells occurred without lymphocyte treatment, and a similar phenomenon was also seen in our experiment *(22)*.

Recently, it has been suggested that the induction of M cell features in Caco-2 monolayers is mediated by direct contact between Caco-2 and Raji cells, soluble factors such as those found in the Raji cell culture supernatant, and/or macrophage migration inhibitory factor (MIF) *(21, 30, 32)*. However, neither the Raji cell culture supernatant nor MIF efficiently induced the differentiation of C2BBe1 monolayers in our experiment. The differences in the results between the above studies and ours might have been due to the different Caco-2 subclones and culture conditions used including differences in the FBS used. At the very least, the presence of Raji cells is important in our C2BBe1 model.

C2BBe1 cells co-cultured with murine Peyer's patch lymphocytes showed M cell-like features such as disordered apical membrane brush borders and bacterial transport *(25)*. That model was constructed with an established human cell culture and primary mouse cells which were isolated from mouse Peyer's patch. On the other hand, our model was based entirely on established human cell lines. Hence, it is thought that our model is a homologous co-culture like the *in vitro* human M cell model compared with previous murine Peyer's patch model reported by Corr et al. in 2006 (*25*).

However, the LAB transport efficiency of our model is lower than that of the murine Peyer's patch model. This difference might be due to the induction efficiency of C2BBe1 differentiation during co-culture because Peyer's patches contain a variety of immunocompetent cells. To obtain an efficient differentiation model, improvements in the culture conditions such as ensuring the close contact of C2BBe1 cells and Raji cells will be necessary *(21)*. Alternatively, there might be differences between the abilities of *L. salivarius* and *L. casei* to adhere to intestinal epithelial cells and Peyer's patch cells *(33, 34)*. However, it remains to be determined whether the uptake of LAB by M cells is a specific or non-specific response.

In this study, to establish a more homologous co-culture model using C2BBe1 cells, C2BBe1 cells were co-cultured with Raji B cells. We demonstrated that Raji cells induced C2BBe1 cells to differentiate in a manner similar to Caco-2 cells that had been co-cultured with murine Peyer's patch cells and the cells used in a number of other *in vitro* M cell models. Therefore, we consider that our C2BBe1 co-cultured model is a useful M cell model. As the interactions between M cells and LAB are poorly understood, investigations of these interactions would help to elucidate the mechanisms of immunostimulation by lactic acid bacteria. Furthermore, our M cell model might contribute not only to examinations of the factors that affect the adhesion and uptake of lactic acid bacteria by M cells but also to studies selecting M cell targeted bacterial strains as vehicles for mucosal vaccine delivery.

ACKNOWLEDGMENT

This study was supported by a grant from the Food Safety Commission of Japan and partly by a grant from the Ministry of Health, Labour, and Welfare of Japan (Research on Food Safety).

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http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.010102

Characteristics of Novel Chicken Embryonic Stem Cells Established Using Chicken Leukemia Inhibitory Factor

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Chickens are an ideal animal model study tool for developmental biology, and a farm animal with excellent productivity. Researchers have therefore long sought to establish chicken embryonic stem cells (cESCs) to enable the creation of genetically modified chickens. Here, we derived novel cESCs from chicken blastodermal cells (CBCs) cultured with chicken leukemia inhibitory factor (chLIF). These cESCs have the capacity for long-term (100 days or more) successive subculture and express both chicken Nanog (chNanog) and chicken vasa homolog (Cvh) mRNAs and proteins. The cESCs showed a capacity for chimeric formation during a transplant experiment that used a fertilized egg. Transfer of the enhanced green fluorescent protein (EGFP) gene to cESCs enabled green fluorescence to be observed among primordial germ cells (PGCs). These results indicate that novel cESCs should have the capacity to differentiate into germ cells.

Key words: chicken embryonic stem cells, chicken Nanog, chicken vasa homolog, leukemia inhibitory factor, primordial germ cells

J. Poult. Sci., 48: 64-72, 2011

Introduction

In 1981, mouse embryonic stem (ES) cell lines were established by culturing the mouse inner cell mass (Evans and Kaufman, 1981; Martin, 1981). These mouse ES cells have an infinite proliferative capacity to differentiate into various cells derived from the ectoderm, mesoderm, and endoderm, both *in vitro* and *in vivo*. Genetically modified mice that are created from ES cells allow us to analyze the functions of specific genes *in vivo*, thereby contributing to the field of life science research. While attempts have been made to establish ES cells using animals other than mice, successful differentiation of ES cells into germ cells has only been achieved for mice (Evans and Kaufman, 1981; Martin 1981; Nichols *et al.*, 1990), rats (Buehr *et al.*, 2008; Li *et al.*, 2008), and monkeys (Thomson and Marshall 1998; Suemori *et al.*, 2001; Adachi *et al.*, 2006; Teramura *et al.*, 2007; Yamauchi *et al.*, 2009).

During the early stages of chicken development, no cells correspond to the ICM of mammals. However, chicken blastodermal cells (CBCs), which derive from stage X embryo (Eyal-Giladi and Kochav, 1976) have the capacity to differentiate into all somatic cells and germ cells when transplanted into a recipient embryo of the same developmental stage (Petitte *et al.*, 1990; Kagami *et al.*, 1995). Thus, attempts have been made to establish chicken ES cells (cESCs) by incubating CBCs (Pain *et al.*, 1996; van de Lavoir *et al.*, 2006a). Unfortunately, after long-term culture, cESCs lose the expression of chicken vasa homolog (Cvh) protein as the most specific marker for germ cells (Tsunekawa *et al.*, 2000), as well as their capacity for differentiation into germ cells (van de Lavoir *et al.*, 2006a, b).

Researchers have previously used leukemia inhibitory factor (LIF), isolated from mice or rats, to preserve the pluripotency of cESCs. However, our cloning of chicken LIF (chLIF) found it to share only 38% identity with mouse LIF (mLIF) (Horiuchi *et al.*, 2004). During the culture of CBCs, phosphorylation of signal transducer and activator of transcription 3 (STAT3) occurred in the presence of chLIF but not mLIF, while chLIF was shown to be more important than mLIF in preserving the pluripotency of cESCs (Horiuchi *et al.*, 2004; Yamashita *et al.*, 2006).

Received: October 12, 2010, Accepted: October 26, 2010
Released Online Advance Publication: December 25, 2010
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In the present study, we report on the characteristics of novel cESCs that we successfully established using chLIF, as well as on the potential of creating genetically-modified chickens using ES cells.

Materials and Methods

Experimental Animals, Tissues, and Cells

White Leghorn (WL) and Barred Plymouth Rocks (BPR) chickens were maintained in an isolated facility at the University Animal Farm, Hiroshima University, Japan. Female New Zealand white rabbits (approximately 2.5 kg) and 8-week old female BALB/c mice were purchased from Shimizu Laboratory Supplies Co. (Kyoto, Japan), and used to raise antibodies. Animals were maintained, bred, and used in experiments in accordance with Hiroshima University guidelines, and the study protocol was approved by its experimental animal committee (Authorization No. D07-19).

Freshly laid, fertilized, unincubated WL and BPR eggs were purchased from Akita Co. (Fukuyama, Japan) and incubated at 38℃. Their embryonic stage was defined according to Eyal-Giladi and Kochav (1976) (shown as roman numerals) and Hamburger and Hamilton (1951) (shown as arabic numerals). CBCs were collected from stage X embryos as described previously (Horiuchi *et al.*, 2006). Primordial germ cells (PGCs) were collected from the blood of stages 13-15 embryos (after about 50 h incubation) and concentrated by Ficoll density centrifugation as described previously (Tajima *et al.*, 1993).

Production of Recombinant chLIF

The chick embryo cell line CHCC-OU2 (Ogura and Fujiwara, 1987), originally supplied by Dr. Fujiwara (Okayama University, Okayama, Japan), was routinely grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Hyclone; Thermo Fisher Scientific Inc, Walthan, MA), 100 μg/ml penicillin and 70 μg/ml streptomycin at 37℃ in 5% CO_2. The coding region of chLIF was amplified using the following primers: sense primer for chLIF, GCGCTAGCCATGAGGCTCATCCCC; reverse primer for chLIF, GTCGACCGCGGGGCTGAGGTGAGGTA. PCR products were digested with *Nhe* I and *Sal* I and sub-cloned into the pSecTag2A plasmid (Invitrogen) containing the histidine tag. The myc-epitope was excised from the pSecTag2A plasmid using restriction enzymes. The recombinant plasmid was transfected into CHCC-OU2 cells using Polyfect Transfection Reagent (Qiagen, Hilden, Germany) and recombinant cells were selected in medium containing 0.25 μg/ml zeocin (Invitrogen). Several stable CHCC-OU2 cell lines secreting biologically active chLIF were selected and recombinant chLIF (rchLIF) was purified from culture supernatants on ProBond resin (Invitrogen).

Incubation of Chicken ES Cells

Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) cells (American Type Cell Collection, Manassas, VA) were cultured to 80% confluence in low-glucose DMEM containing 10% FBS. Feeder cells were prepared by treating STO cells with mitomycin C (Sigma-Aldrich, St. Louis, MO) and culturing them at a cell density of 1.5×10^4 cells/cm^2. The reason to select STO cells as feeder cells is to exclude contamination of the genes in chick-derived feeder cells when the expression of the genes in CBCs and cESCs is analyzed.

Using a filter paper ring, stage X CBCs were collected from one egg of BPR chickens as described previously (Horiuchi *et al.*, 2006). CBCs were gently suspended using a micropipette, disseminated on a 12-well plate, and cultured in a CO_2 incubator. The chicken ES cell medium (CECM) contained high-glucose DMEM supplemented with 20% KnockOut Serum Replacement (Invitrogen), 2% heat-inactived chicken serum (Invitrogen), 0.1 mM sodium pyruvate, nucleocide (8 μg/ml adenosine, 8.5 μg/ml guanosine, 7.3 μg/ml cytidine, 7.3 μg/ml uridine, 2.4 μg/ml thymidine), 0.05 mM non-essential amino acid, streptomycin (70 μg/ml), penicillin (100 μg/ml), 0.1 mM β-mercaptoethanol and rchLIF (20 ng/ml). Cultured cells were serially subcultivated every second or third day to ensure optimal culture and to inhibit differentiation. The cells were separated gently using the tip of a micropipette and disseminated onto a new plate. Half or all of the incubation medium was replaced daily with fresh medium.

RT-PCR Analysis

Total RNA was extracted from cESCs using ISOGEN-LS (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. RNA was used for reverse transcription PCR (RT-PCR) with the SuperScript™III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. PCR was carried out using the ABI PRISM 7700 sequence-detection system (Applied Biosystems) and SYBR Green core reagents (Applied Biosystems). The cDNA amounts were normalized to chicken GAPDH SYBR Green PCR, according to the manufacturer's instructions, and equal quantities were then used as templates in PCR reactions. PCR amplification was carried out with 0.4 μM primers, 0.2 mM dNTPs, 1 mM $MgCl_2$, 10 x *Ex Taq* buffer (Mg^{2-} free) and 1 U *Ex Taq* (Takara) in a final volume of 25 μl using a Gene Amp PCR System 9700 (Applied Biosystems). The PCR reaction consisted of 22, 24 or 28 cycles (specified below) of 30 s at 94℃, 30 s at 58 or 60℃, and 1 min at 72℃. The PCR products were analyzed on 1.5% agarose gels and visualized with EtBr. The upstream and downstream primer pairs, annealing temperature and cycle numbers used for each gene were as follows: chNanog (TGACCACAGAGCAGAAAACG, CAGCCATGAACGGATACAGG, 58℃, 24 cycles); Cvh (CAGCCATGAACGGATACAGG, ATGAATGTGCTGTTGAGATGTC, 60℃, 28 cycles); GAPDH (GCACGCCATCACTATCTTCCAG, CGGCAGGTCAGGTCAACAACAG, 58℃, 22 cycles). We confirmed that 22, 24 or 28 PCR cycles represented the exponential phase of the amplification. Chicken GAPDH gene amplification products were used as internal controls.

Production of Specific Anti-chNanog and Anti-Cvh Antibodies

Recombinant chNanog (rchNanog) and Cvh (rCvh), as antigens for antibody production, were expressed in the pMAL-c2X plasmid (New England Biolabs, Ipswich, MA) containing the maltose binding protein (MBP) gene and in the pGEX-6P-1 plasmid (GE Healthcare, Buckinghanshire, UK) containing the glutathione S-transferase (GST) gene, according to the manufacturer's instructions. The entire translated region of rchNanog was expressed and purified as MBP-rchNanog or GST-rchNanog fusion proteins. The partial translated region (amino acids 116–464) of rCvh was expressed and purified as MBP-rCvh or GST-rCvh fusion proteins.

Anti-Cvh mouse monoclonal antibody (mAb) was developed by immunizing 8-week-old female BALB/c mice intraperitoneally with 50μg GST-rCvh mixed with an equal volume of complete Freund's adjuvant. After three bi-weekly boosts with the same dose of the immunogens in 0.1 m*l* PBS, mice with high serum titers of antibodies against MBP-rCvh were identified in enzyme-linked immunosorbent assays (ELISA). These mice were boosted intravenously with 50μg GST-rCvh in PBS and, three days later, their splenocytes were fused with SP2/0 Ag14 myeloma cells (Shulman *et al.,* 1978) using established methods (Galfre *et al.,* 1977). Hybridomas secreting specific antibodies were identified by ELISA (Yamashita *et al.,* 2006), and cloned by limiting dilution. The immunoglobulin isotype of the mAb was determined using a mouse monoclonal antibody isotyping kit (GE Healthcare).

Anti-chNanog polyclonal antibody (pAb) was raised by immunizing female NZW rabbits subcutaneously with 300 μg MBP-rchNanog mixed with an equal volume of complete Freund's adjuvant. After four bi-weekly immunizations with the same dose of the immunogens, antisera from the rabbits were purified by affinity chromatography using GST-rchNanog-conjugated agarose beads and designated anti-chNanog pAb.

Western Blot Analysis

Nuclear and cytoplasmic extracts were prepared from freshly isolated CBCs, STO cells and chicken embryo fibroblast (CEF) using a Nuclear Extract Kit (Active Motif, Carlsbad, CA), according to the manufacturer's instructions. Testis and ovary lysates were prepared from newly hatched chicks using extraction buffer (0.15 M NaCl, 50 mM Tris-HCl (pH7.4), 1 mM EDTA, 0.1% sodium deoxycholate and 1% Nonidet P-40). One tablet of a complete protease-inhibitor cocktail (Roche, Mannheim, Germany) was added per 25 m*l* extraction buffer. Cell extracts or tissue extracts were separated on 10% SDS-PAGE gels under reducing conditions and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 25 mM EDTA, 0.2% Tween 20 and 4% nonfat milk overnight at 4℃, washed three times in Tween-PBS, and incubated with Anti-Cvh mAb (hybridoma culture supernatants) diluted 1:5 or anti-chNanog pAb diluted 1:1,000 in blocking buffer containing 1% nonfat milk (dilution buffer) for 1 h at room temperature (RT). After 3 washes as before, blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit immunoglobulin (Ig) (KPL, Gaithersburg, MD), diluted 1:4,000 in dilution buffer, for 1 h at RT, washed 3 times, and incubated with ECL Plus Western blotting detection reagent (GE Healthcare). Results were recorded using a LAS-3000 image reader (Fujifilm, Tokyo, Japan).

Immunofluorescence Analysis

cESCs were incubated for two days on Cellware 8-well CultureSlides (BD Biosciences, San Jose, CA). Cells were fixed using PBS containing 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) and permeabilized with PBS containing 0.1% Triton X-100 (Sigma-Aldrich). PBS containing 3% bovine serum albumin (BSA) (Nacalai Tesque) was added to block for 30 min at room temperature. An anti-chNanog pAb (1:100), an anti-Cvh mAb (1:5) and/or an anti-GFP rabbit pAb (1:100, Clontech, Mountain View, CA) diluted with 1% BSA-PBS were added to the cells and cultured at 37℃ for 1 h. The secondary antibody was also diluted with 1% BSA-PBS and incubated with the cells at 37℃ for 1 h. To test the reactions to the secondary antibody, we used Alexa Fluor488-conjugated anti-mouse IgG antibody (1:200, Invitrogen), tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG antibody (1:100, Southern Biotech, Birmingham, AL), Alexa Fluor594-conjugated anti-mouse IgG antibody (1:200, Invitrogen) and/or Alexa Fluor488-conjugated anti-rabbit IgG antibody (1:200, Invitrogen).

Blood containing PGCs was collected from ten chimeric embryos at stages 13 to 15 (Hamburger and Hamilton, 1951). Red blood cells were lysed and examined under fluorescent light. Stages 31 to 34 (Hamburger and Hamilton, 1951) chimera embryos were fixed using PBS containing 4% paraformaldehyde and embedded with paraffin. The sample was sliced to 5μm, dewaxed and dewatered using a xylene-ethanol series. After blocking for 1 h using 3% BSA-PBS, the anti-Cvh mAb (1:5) and the anti-GFP pAb (1:100) was incubated overnight at 4℃. The reaction was induced using an Alexa Fluor594-conjugated anti-mouse IgG antibody (1:200) and Alexa Fluor488-conjugated anti-rabbit IgG antibody (1:200) as a secondary antibody, and the sample was examined using a fluorescent microscope BX51 (Olympus, Tokyo, Japan,) or BZ-9700 (Keyence, Osaka, Japan).

Gene Transfer

cESCs were suspended in 700μ*l* OPTI-MEM (Invitrogen). A Gene Pulser XCell electroporator (Bio-Rad, Hercules, CA) was used for transfection with the following conditions: square wave, 200 V, 15 ms, 20μg linearized pCAG-EGFP-IP vector (a kind gift from Dr Niwa, CDB, RIKEN, Kobe, Japan). Selection was applied approximately 24 h after transfection using 0.1–0.6μg/m*l* puromycin. Six to 14 days after selection, colonies with high

resistance were transferred to a new plate to proliferate.

Cloning of Chicken ES Cells

cESCs were treated for 2 h with 10μM p160-Rho-associated coiled-coil kinase (ROCK) inhibitor (Y-27632; Wako, Osaka, Japan,) before being dissociated. PBS containing 1 mM EGTA (Nacalai Tesque) with 0.025% trypsin (Invitrogen) was added to the cells and they were separated into single cells. They were then suspended in new CESM with Y-27632, and disseminated onto a 96 -well plate at 1 cell/well. Six to seven days after dissemination, cells were serially subcultivated into a 24-well plate. This procedure was repeated at least twice. The cloning efficiency was evaluated by counting the number of survival cESCs colonies for triplicate samples±the standard deviation (SD). The statistical significance (P values) in mean values of two-sample comparison was determined with Student's t-test (Microsoft Excel).

Creation of Chimera Chicken Using Chicken ES Cells

cESCs derived from BPR chickens were prepared at 5,000 cells/μl using DMEM containing 2% inactivated chicken serum. Two microliters of suspended cells was injected into the subgerminal cavity of a WL chicken embryo recipient. Stage X recipient embryos were moved into a substitute shell, which was filled with egg white and sealed with clear wrap. For the culturing procedure, we modified systems II and III devised by Rowlett and Simkiss (1987), Perry (1988), and Borwornpinyo *et al.* (2005). The transplanted embryo was cultured at 38.5℃ for three days at 60% relative humidity. It was then gently moved into a new substitute shell (30–35 g and above), and sealed with clear wrap. The embryo was cultured at 38.5℃ and 60% relative humidity until incubation.

Results

mRNA Expression of chNanog and Cvh During Primary Culture of CBCs

CBCs were cultured for eight days with or without rchLIF. mRNA expression of chNanog and Cvh was analyzed by RT-PCR. As shown in Figure 1, chNanog and Cvh mRNA were expressed in the presence or absence of rchLIF until the second day of culture. Expression then decreased radically after four days of culture without rchLIF, but decreased gradually and was preserved until the eighth day with rchLIF.

Characterization of Anti-chNanog and Anti-Cvh Specific Antibodies

In western blots, the anti-chNanog pAb reacted a band in nuclear extracts of CBC, and an apparent molecular weight was approximately 40 kDa (Fig. 2A). No chNanog band was detected in STO cells or CEF (Fig. 2a). The anti-Cvh mAb reacted as a band with an apparent molecular weight of approximately 75 kDa (Fig. 2B), and did not react with somatic tissue (lung, heart, kidney and liver, data not shown). The mAb was an IgG1-k immunoglobulin and could be used in various immunological techniques including ELISA, western blotting, immunoprecipitation and immunohistochemistry.

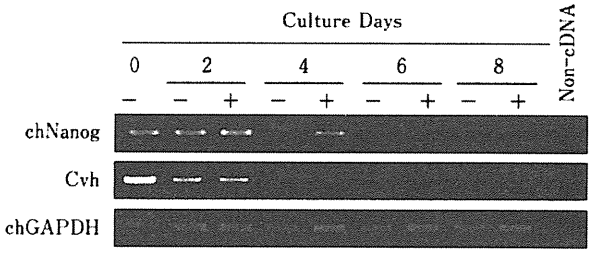

Fig. 1. **Changes in chNanog and Cvh mRNA expression during CBCs culture.** CBCs were cultured with (+) or without (−) rchLIF for eight days. mRNA was isolated for RT-PCR using chNanog, Cvh or chGAPDH gene specific primers. chGAPDH gene amplification products were used as an internal control.

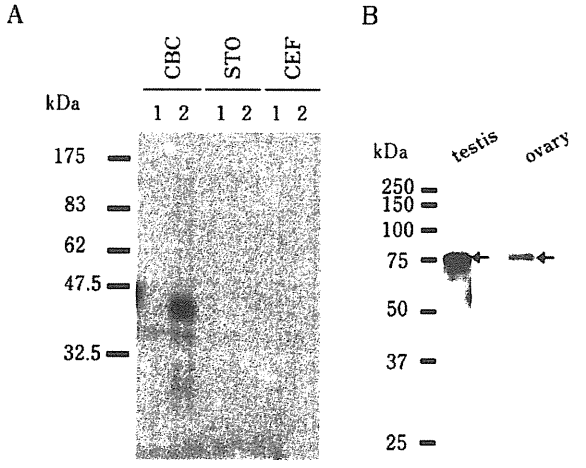

Fig. 2. **Characterization of anti-chNanog and anti-Cvh specific antibodies.** (A) The cytoplasmic fractions (1) and nuclear fractions (2) from CBCs, STO cells and CEF were separated on a 10% SDS-PAGE gel and analyzed by western blotting using the anti-chNanog pAb. (B) Western blot analysis with newly hatched chick testis and ovary lysates using the anti-Cvh mAb. The relative mobility of protein markers is indicated on the left.

Long-term Culture of CBCs and Their Characteristics

CBCs obtained from one egg were cultured on STO cells feeder cells using CECM with rchLIF. The growth morphology of CBCs was similar to primate ES cells and consisted of a circular colony with a clear contour, and each individual cell visible (Fig. 3-2a). Cells had to be subcultured every second or third day to maintain their proliferative potential. After cryopreservation, cells maintained the same morphology when recultured (data not shown).

Using CBCs that were successively subcultured for 100 days or more, we analyzed the expression of chNanog and Cvh mRNA. As shown in Figure 3-1, we confirmed ex-

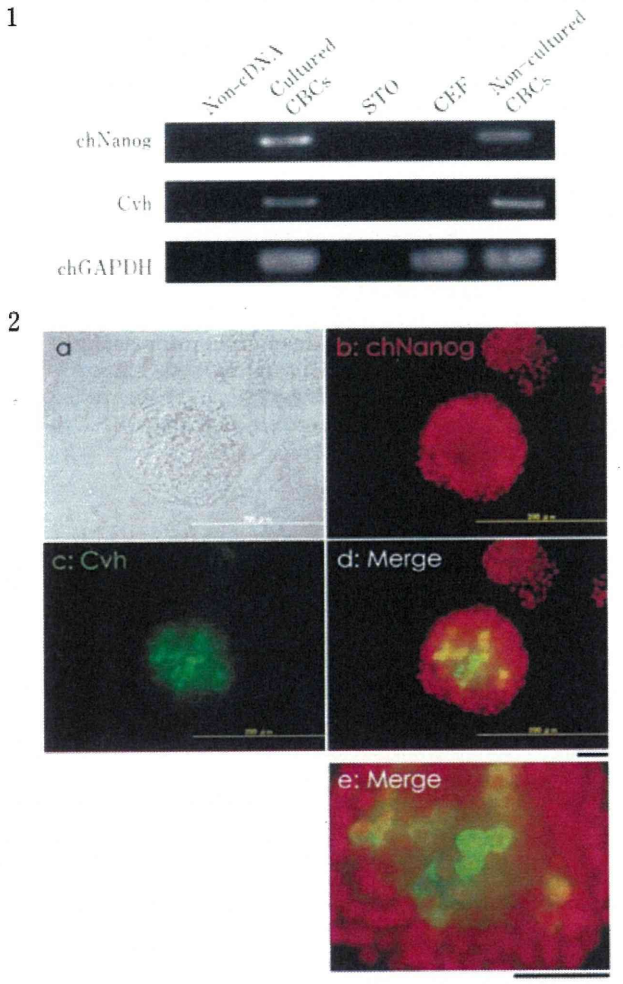

Fig. 3. **Characterization of long-term cultured CBCs.** (1) Expression analysis of chNanog and Cvh mRNA. RNA was isolated from CBCs successively subcultured for 212 days and used for RT-PCR with chNanog, Cvh and chGAPDH gene specific primers. RNA isolated from STO cells, CEF and non-cultured CBCs was also examined with the same primers. (2) (a) A circular colony of cultured CBCs. Immunofluorescence analysis of chNanog (b) and Cvh (c) protein. CBCs successively subcultured for 105 days were tested with double fluorescent dye staining using anti-chNanog pAb and anti-Cvh mAb. (d) Double immunofluorescence imaging. (e) High magnification. Scale bar = 50μm.

pression of chNanog and Cvh mRNA immediately after collection of the CBCs. As shown in Figure 3-2, chNanog was detected in the nucleus of most cells that formed a colony (Fig. 3-2b), while Cvh expression was confirmed in the cytoplasm of cells that existed around the central area of the colony (Fig. 3-2c). The cultured CBCs showed a capacity for chimeric formation during a transplant experiment that used a fertilized egg derived from WL

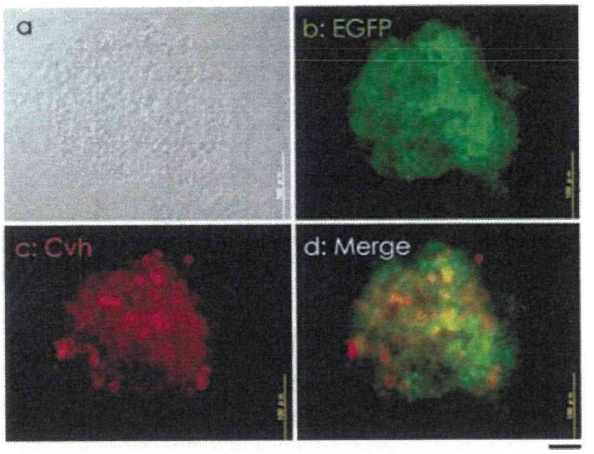

Fig. 4. **Characterization of EGFP-cESCs.** Anti-Cvh mAb fluorescence was examined using EGFP-cESCs. (a) A circular colony of EGFP-cESCs. (b) Expression of EGFP. (c) Immunofluorescence analysis of Cvh protein. (d) Double fluorescence imaging. Scale bar = 50μm.

chickens as a recipient (data not shown). We therefore named the cultured CBCs cESCs and used them for the following study. While CBCs used in this study were all collected from fertilized eggs derived from BPR chickens, it is also possible to establish cESCs with the same characteristics from CBCs derived from WL or Bovans Nera chickens using the same culture methods.

Characteristics of Chicken ES Cells Harboring the EGFP Gene

In order to create genetically-modified chickens using established cESCs, it was deemed necessary to preserve the expression of chNanog and Cvh even after gene transfer. Moreover, the transfer of reporter genes such as enhanced green fluorescent protein (EGFP) gene into cESCs enables their differentiation into germ cells to be traced. We electroporated linearized pCAG-EGFP-IP carrying the EGFP and puromycin resistance genes into cESCs and performed selection with puromycin. After 6-14 days, we obtained puromycin-resistant cESCs that proliferated by forming a circular colony like pre-transfer cells (Fig. 4a). Expression of EGFP was detected in almost all cells that formed a colony (Fig. 4b). Immunofluorescent staining with anti-Cvh mAb confirmed expression of Cvh (Fig. 4c, d) after gene transfer. In the same way, chNanog was expressed in the gene-transfer cESCs (data not shown).

Cell Cloning of Chicken ES Cells and Characteristics of Cloned EGFP-cESCs

Cell cloning is essential in homogenizing the areas of gene insertion of gene-transfer cESCs. Moreover, it may also be possible to induce only Cvh-positive cESCs to proliferate. We therefore tried to clone cESCs transfected with EGFP (EGFP-cESCs) by limiting dilution. Three kinds of EGFP-cESCs (#1, 2 and 3) derived from differ-

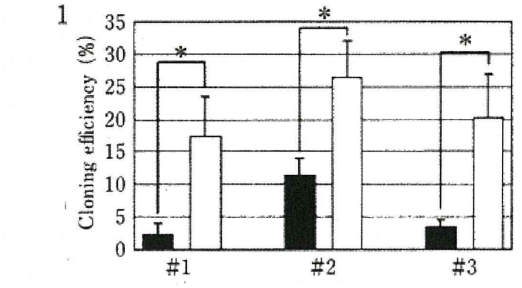

Fig. 5. **Cloning efficiency of EGFP-cESCs and Cvh-expression of cloned EGFP-cESCs.** (1) Cloning efficiency of EGFP-cESCs (#1, 2 and 3), with (white bar) and without Y-27632 (black bar) (*, $p<0.05$ versus control, n=3). (2) Expression of Cvh protein analyzed by immunofluorescence staining using anti-Cvh mAb. (a) A circular colony of EGFP-cESCs. (b) Expression of EGFP. (c) Immunofluorescence analysis of Cvh protein. Scale bar=50μm.

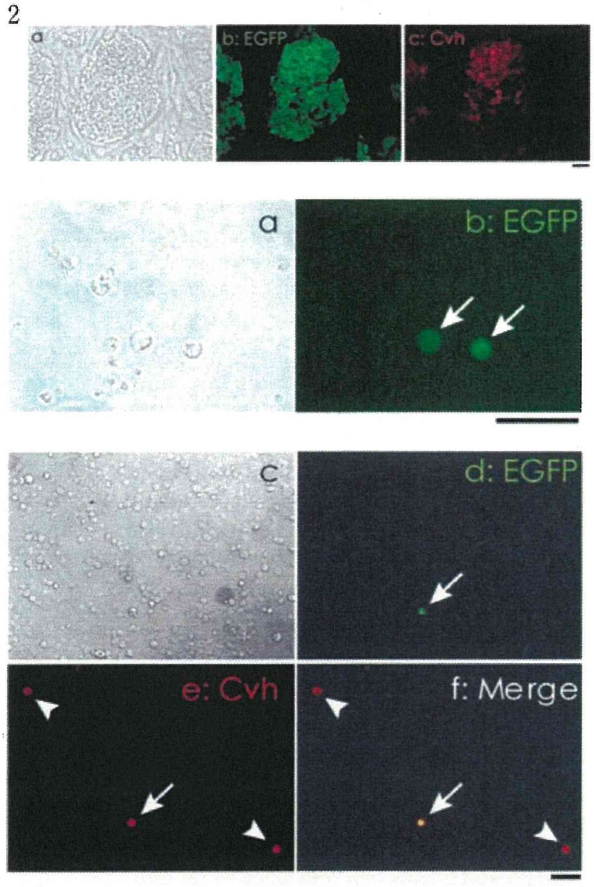

Fig. 6. **EGFP expression in migrating PGCs.** (a) Whole blood was collected from stages 13–15 chimeric embryos. (b) Living cells were observed directly under a fluorescent microscope. Arrows indicate EGFP-positive cells. Scale bar=50μm. (c) Smear sample of embryo blood collected from 2.5-day incubated embryos. (d) Expression of EGFP. (e) Immunofluorescence analysis of Cvh protein. Arrowheads indicate recipient PGCs (Cvh-positive cells). (f) Double fluorescence imaging. Arrow indicates PGC derived from cloned EGFP-cESCs among Cvh-positive cells. Scale bar=50μm.

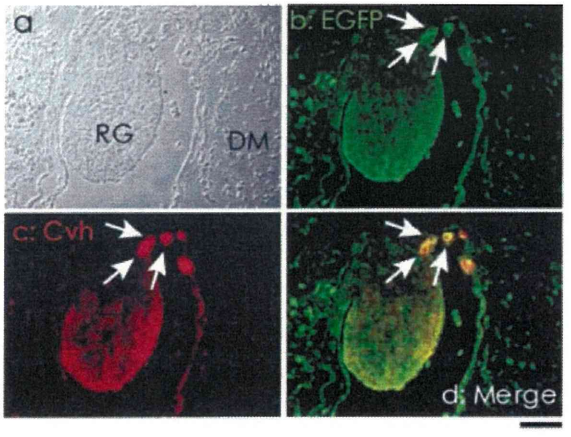

Fig. 7. **EGFP expression into transverse section of a sixth day incubated embryo.** Anti-Cvh mAb fluorescence was examined using a tissue slice (a) near the gonadal ridge of a sixth day old incubated chimera embryo. (b) Expression of EGFP. (c) Immunofluorescence analysis of Cvh protein. (d) Double immunofluorescence imaging. Arrows indicate PGC derived from cloned EGFP-cESCs among PGC (Cvh-positive cells). DM; dorsal mesentery, RG; right gonad. Scale bar=50μm.

ent eggs were disseminated into 96-well plates at 1 cell/well. Only cells that formed a colony were counted. In cases where limiting dilution was performed only with CESM, the cloning efficiency was 15% or lower. In many cases, cells were destroyed during successive subcultures. When Y-27632 was added to CESM, the cloning efficiency increased significantly to 15% or higher (Fig. 5-1, $p < 0.05$). Subsequent successive subculture was also successful. In immunofluorescence staining, cloned EGFP-cESCs was all EGFP- and chNanog-positive cells that formed colony (Fig. 5-2a, b). Some of these EGFP-expressing cells were also Cvh-positive (Fig. 5-2c). By repeating this method more than twice, we acquired cloned EGFP-cESCs that were used for the following study.

EGFP Expression in PGCs and Capacity of Cloned EGFP-cESCs to Form a Chimera

Based on the above findings, we transplanted cloned EGFP-cESCs to a recipient embryo of a fertilized egg derived from WL chickens. In collecting stages 13 to 15 embryo blood, living EGFP-positive cells were shown to have the morphological characteristics of PGCs (Fig. 6a, b). In immunofluorescence staining using smear of embryo blood cells, we confirmed expression of EGFP deriving from cloned EGFP-cESCs among Cvh-positive cells (Fig. 6c-f). Tissues near the gonadal ridge of an incubated egg in its sixth day revealed expression of EGFP among Cvh-positive cells, migrating through the dorsal intestinal membrane to the gonadal ridge (Fig. 7a-d). In its eighth day, some EGFP-positive cells were observed among many Cvh-positive cells in the gonadal ridge (data not shown). This indicated that Cvh-positive cells among cloned EGFP-cESCs migrate into the gonadal ridge.

Expression of EGFP was observed all over the transplanted embryo on its fifth day of incubation, indicating that cloned EGFP-cESCs possess the capacity for pluripotency (Fig. 8a, b). Continuation of the incubation showed the transplanted embryo to develop normally, and we observed the hatching of a chimera chicken with black feathers, which is an expression of BPR chickens (Fig. 8c, d).

Discussion

In 2006, van de Lavoir and others reported the establishment of cESCs that differentiated into somatic cells at a high frequency (2006a). However, these ES cells could not differentiate into germ cells and were unable to maintain their potential for germline transmisson. Subsequent research has found that these established ES cells contained very few Cvh-positive cells (2006b). Unlike mammals, stage X CBCs contain approximately 30 Cvh-positive cells (0.1%) that are thought to be precursor PGCs (Stepinska and Olszanska, 1983; Tsunekawa *et al.*, 2000). Many researchers have shown that it is possible to create germline chimera chickens by transplanting cells from these CBCs to a recipient embryo (Petitte *et al.*, 1990; Kagami *et al.*, 1995).

To establish cESCs from CBCs that will contribute to germ cells, it is considered essential to develop a culture method that can preserve the characteristics of stage X CBCs, in other words to preserve Cvh-positive cells (Lavial *et al.*, 2009). This study confirmed that it is possible to maintain expression of chNanog and Cvh mRNA by using rchLIF in the primary culture of CBCs (Fig. 1). Although this expression gradually decreases after six days of culture, we found that successive subcultures performed within one to three days enabled long-term culture and expression of Cvh. In the case of CBC culture using CESM without rchLIF, the expression of chNanog and Cvh mRNA decreased extremely (Fig. 1). Yamashita and others reported that chLIF mRNA expression decreased extremely after 3 days of CBC culture, and suggested that CBCs themselves produced chLIF in culture, which acted as an autocrine signal (2006). These results suggest that the expression of chNanog and Cvh mRNA is maintained for a short culture time by autocrine signal of chLIF. The effect of LIF on the cESCs established in this study produced characteristics that were closer to those of mouse ES cells than primate ES cells (Niwa *et al.*, 1998; Smith *et al.*, 1988; Thomson and Marshall, 1998; Matsuda *et al.*, 1999; Suemori *et al.*, 2001; Horiuchi *et al.*, 2004; Yamashita *et al.*, 2006). However, the tendency of cESCs to be destroyed when the colony or its morphology is separated into single cells shows a close resemblance to primate ES cells.

The biggest advantage of creating genetically-modified animals from ES cells is that gene targeting by recombining homologous genes is made possible (Thomas and Capecchi, 1987; Mansour *et al.*, 1988). However, while cell cloning is an essential technology to achieve this, the ready destruction of cESCs due to colony dissociation makes the incorporation of this difficult. In 2007, Watanabe and others reported that the addition of ROCK inhibitor was effective in culturing human ES cells from single cells (2007). Therefore, we used ROCK inhibitor for the cloning of cESCs in this study and effectively cultured and cloned cESCs from single cells (Fig. 5).

We next hypothesized that it might be possible to clone only Cvh-positive cESCs. However, not all Cvh-positive cells turned into cloned cESCs but were scattered inside the colony, rather like pre-cloned cells. This may have resulted from the fact that only Cvh-positive cells showed clonal proliferation during the cloning process while others lost expression of Cvh. Alternatively, the culture method established in this study might have caused some of the cESCs to become Cvh-positive. Whether it is true or not, it is certain that Cvh-positive cells among our established cESCs keep proliferating *in vitro* (Fig. 3, 4, 5-2). We aim to use our culture system to determine the development of PGCs among birds.

By transferring the EGFP gene into cESCs, we were able to trace expression of EGFP to PGCs in the migration stage (Fig. 6, 7). In immunostaining of the gonadal ridge, the whole gonad was seen green (Fig. 7). The rea-

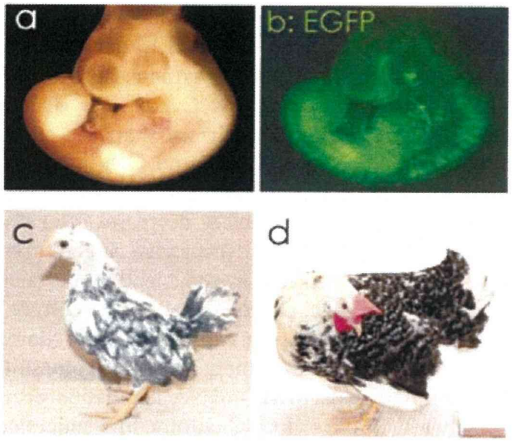

Fig. 8. **Capacity of cloned EGFP-cESCs to form a chimera.** EGFP-cESCs transplanted to a stage X recipient embryo of WL chickens were examined for their capacity to form chimeras. (a, b) Expression of EGFP was observed by using the transplanted embryo on its fifth day of incubation. A chimeric chicken with black feathers while is an expression of BPR chickens. (c) Forty days old. (d) Six-month old.

son for this result should be that the cESCs frequently contributed the somatic cells of the gonad. We should transplant only Cvh-positive cells among EGFP-positive cells to a recipient embryo for the future. More recently, in preliminary results, we have detected EGFP gene in sperm genomes and EGFP-expression in oocytes of chimeric chickens (our unpublished data, 2010), and are confident in creating genetically-modified chickens using cESCs established in the present study.

In conclusion, we show the derivation of cESCs from CBCs cultured with chLIF. The cESCs express chNanog and Cvh and are capable of long-term successive subculture, after gene transfer and cell cloning. They can also migrate into gonadal ridge and can be transplanted into recipient embryos for the development of a chicken chimera. These results indicate that novel cESCs have the potential of creating genetically-modified chickens.

Acknowledgments

The authors thank Dr Hitoshi Niwa for kindly donating the pCAG-EGFP-IP vector and Dr Hirofumi Suemori for valuable discussion. This work was supported by a Grant-in-Aid for Scientific Research (B) (No. 16580279) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Biotechnology Cluster in Central Hiroshima, Hiroshima Prefecture, Japan, and Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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