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2

Maintenance of Chicken Embryonic Stem Cells In Vitro

Hiroyuki Horiuchi, Shuichi Furusawa, and Haruo Matsuda

Summary

In this chapter, we describe the methods we have used to show that chicken leukemia inhibitory factor (LIF) maintains chicken embryonic stem (ES) cells in an undifferentiated state in culture. Recombinant chicken LIF (rchLIF) was expressed as a fusion protein linked to glutathione *S*-transferase (GST) and purified to greater than 90% purity in two chromatography stages, the first an affinity step using the GST tail, which was cleaved before further purification by gel chromatography. Chicken ES cells were obtained by culturing chicken blastodermal cells isolated from stage X embryos of freshly laid chicken eggs. These cells can be maintained in media containing rchLIF for at least 9 d without any other cytokines or feeder cells. Chicken ES cells were characterized by the expression of alkaline phosphatase activity, stage-specific embryonic antigen (SSEA)-1 and embryonal carcinoma cell monoclonal antibody-1. In addition, the phosphorylation of signal transducers and activators of transcription-3 by LIF, which is sufficient to maintain the undifferentiated state of ES cells, was detected by Western blotting analysis.

Key Words: Alkaline phosphatase (AP); chicken; chicken blastodermal cells (CBC); embryonic stem (ES) cells; leukemia inhibitory factor (LIF); stage-specific embryonic antigen (SSEA)-1; signal transducers and activators of transcription-3 (STAT3).

1. Introduction

The avian embryo represents an important model system in developmental and cell biology because of its ease of manipulation and its similarity to mammalian development. In addition, the production of transgenic birds using early embryos is an important technology in both fundamental and applied avian biology. Transgenic chickens are particularly in the spotlight at present because of their potential as bioreactors. For this research to progress, the development of transgene technology for use in the early embryo is necessary. Different methods have been employed to introduce transgenes into avian embryos, including microinjection, retroviruses, and transfection of avian blastodermal or primordial germ cells. However, which technique will be most generally applicable is not yet clear.

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Mouse embryonic stem (ES) cells can be maintained in culture without feeder cells in the presence of fetal bovine serum and mouse leukemia inhibitory factor (LIF) *(1)*. The injection of ES cells into blastocysts can give rise to chimeric mice, and the ES cells can contribute to all tissues, including the germ cells. Mutant mouse ES cells have been used to produce mouse strains with disrupted genes. The relative ease with which one can manipulate ES cells in vitro has made them a powerful tool for the targeted disruption of endogenous genes, and this led to a dramatic increase in the number of transgenic ("knockout") mice during the 1990s.

In chickens, the existence of pluripotent ES-like cells in the stage X *(2)* chicken blastoderm has been demonstrated directly by immunostaining *(3)* and indirectly by the ability of chicken blastodermal cells to contribute to the germline as well as somatic ectodermal, mesodermal, and endodermal lineages *(4–7)*. Pain et al. *(8)* first reported that pluripotent avian stem cells could be produced and maintained by long-term culture of stage X blastodermal cells with several cytokines: chicken stem cell factor (SCF), bovine basic fibroblast growth factor (bFGF), mouse interleukin (IL)-6, human IL-11, and mouse LIF. However, there are no reports of chicken ES cell lines contributing to the germline after extended culture, although there have been some promising developments.

More recently, the chicken homologue of mammalian LIF was cloned, and the effects of recombinant chicken LIF (rchLIF) and mouse LIF on chicken ES-like cells, chicken blastodermal cells (CBCs), were compared *(9)*. In these experiments, rchLIF was found to maintain chicken ES cells in an undifferentiated state, whereas mouse LIF did not. This finding should contribute to the establishment of chicken ES cell lines and the development of transgenic chicken technology. In this chapter, we describe protocols for the expression and purification of rchLIF in *Escherichia coli*, the culture of CBCs, and the detection in these cells of markers characteristic of an undifferentiated state.

2. Materials

2.1. Cloning, Expression, and Purification of rchLIF

2.1.1. Reverse Transcription Polymerase Chain Reaction

1. Thermal cycler (Applied Biosystems, Foster City, CA; model 9700).
2. Agarose gel apparatus and reagents.
3. Isogen-LS (100 mL; Wako, Osaka, Japan; cat. no. 311-02621) (*see* **Note 1**).
4. Chloroform (500 mL; Sigma, St. Louis, MO; cat. no. 05-3400-5).
5. 2-Propanol (500 mL; Sigma, cat. no. 15-2320-5).
6. Diethyl pyrocarbonate (DEPC)-H_2O.
7. Oligotex-dT30 <Super> Kit (Roche, Mannheim, Germany; cat. no. 489991).
8. 70 and 100% ethanol.
9. SuperScript First-Strand Synthesis System for reverse transcriptase polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA; cat. no. 11904-018). Package contents: 0.5 µg/µL Oligo $(dT)_{12\text{-}18}$, 10 m*M* deoxyribonucleoside triphosphate (dNTP) mix, 10X first-strand buffer, 0.1 *M* dithiothreitol (DTT), 40 U/µL RNase OUT (inhibitor), 50 U/µL SuperScript II reverse transcriptase.

10. PCR primers diluted to 10 pmol/μL with sterile distilled water (dH_2O).
11. Takara Ex Taq (5 U/μL; Takara, Shiga, Japan; cat. no. RR001A). Supplied reagents: 10X Ex Taq buffer, 2.5 m*M* dNTP mix.

2.1.2. Ligating Plasmid and Target DNA and Transformation

1. pGEX-6P-1 plasmid (Amersham Pharmacia Biotech, Piscataway, NJ; cat. no. 27-4597-01).
2. Competent cells, *E. coli* BL21 strain (Novagen, Madison, WI; cat. no. 70232-3).
3. *Bam* HI (30–60 U/μL; Takara, cat. no. 1010AH). Supplied reagents: 10X K buffer.
4. *Xho* I (30–60 U/μL; Takara, cat. no. 1094AH). Supplied reagents: 10X K buffer.
5. T4 DNA ligase (1 U/μL; Invitrogen, cat. no. 15224-017). Supplied reagents: 5X ligase reaction buffer.
6. 100 × 15-mm plastic Petri dishes (Becton Dickinson, Bedford, MA; cat. no. 351999).
7. Super optimal catabolite (SOC) medium (Invitrogen, cat. no. 15544-034).
8. LB agar (500 g; Invitrogen, cat. no. 22700-025).
9. Ampicillin solution, 100 mg/mL stock: dissolve 200 mg Ampicillin sodium salt in 2 mL sterile H_2O.
10. Ampicillin-LB plate. For 100 mL: mix 3.2 g LB agar in 100 mL dH_2O and autoclave. Remove the medium from the autoclave and swirl it gently (*see* **Note 2**). Allow the medium to cool to 50–60°C and then add 100 μL ampicillin solution (100 mg/mL stock) and swirl. Pour 10–15 mL of medium per 100 × 15-mm plastic Petri dish. When the medium has completely set, invert the dishes and store them at 4°C until needed.

2.1.3. Expression and Purification

1. LB broth base, powder (500 g; Invitrogen, cat. no. 12780-052). For 1 L: mix 20 g LB broth base in 1 L dH_2O and autoclave. Remove the medium from the autoclave and swirl it gently (*see* **Note 2**). Store the medium at room temperature until needed.
2. Isopropyl β-D-thiogalactoside (IPTG) (100 m*M* IPTG stock): dissolve 0.5 mg IPTG in 20 mL dH_2O and sterilize by passing it through a 0.22-μ*M* disposable filter. Dispense the solution into 1-mL aliquots and store at −20°C.
3. Ampicillin solution (100 mg/mL stock) (*see* **Subheading 2.1.2., item 9**).
4. Protease inhibitor (20 tablets; Roche, cat. no. 1-697-498).
5. PreScission protease (2 U/μL; Amersham Pharmacia Biotech, cat. no. 27-0843-01).
6. PreScission cleavage buffer: 50 m*M* Tris-HCl, pH 7.0, 150 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA)·2Na, 1 m*M* DTT. For 100 mL: combine 33 mL 5 *M* NaCl, 0.2 mL 500 m*M* EDTA·2Na, 1 mL 100 m*M* DTT, and 5 mL 1 *M* Tris-HCl, pH 7.0.
7. Glutathione-Sepharose 4B (100 mL; Amersham Pharmacia Biotech, cat. no. 27-4574-01).
8. PD-10 empty column (Amersham Pharmacia Biotech, cat. no. 17-0435-01).
9. HiLoad 26/60 Superdex 75 pg (Amersham Pharmacia Biotech, cat. no. 17-1070-01).
10. Dulbecco's phosphate-buffered saline (PBS), pH 7.4 (Nissui, Tokyo, Japan; cat. no. 08190). For 1 L: dissolve 9.6 g Dulbecco's PBS powder in 1 L dH_2O and autoclave.

2.2. Cell Culture

1. Iscove's modified Dulbecco's medium (IMDM) (1 L; Gibco BRL, Carlsbad, CA; cat. no.12440-046).
2. Dulbecco's modified Eagle medium (DMEM) (500 mL; Gibco BRL, cat. no.11965-092) (*see* **Note 3**).
3. Fetal bovine serum (FBS) (500 mL; Hyclone, Logan, UT; cat. no. SH30070.03) (*see* **Note 4**).

4. Chicken serum (500 mL; Gibco BRL, cat. no.16110-082) (*see* **Note 4**).
5. 24-well culture plates (Becton Dickinson, cat. no. 353047).
6. 10 m*M* minimum essential medium (MEM) nonessential amino acid (NEAA) solution (100 mL; Gibco BRL, cat. no. 11140-050).
7. 100 m*M* MEM sodium pyruvate solution (100 mL; Gibco BRL, cat. no. 11360-070).
8. 2-Mercaptoethanol (100 mL; Sigma, cat. no. M7522).
9. Adenosine (5 g; Sigma, cat. no. A4036).
10. Cytidine (1 g; Sigma, cat. no. A4036).
11. Guanosine (5 g; Sigma, cat. no. G6264).
12. Thymidine (1 g; Sigma, cat. no. T1895).
13. Uridine (5 g; Sigma, cat. no. U3003).
14. 100 μ*M* nucleotide stock solution. For 100 mL: mix 80 mg adenosine, 73 mg cytidine, 85 mg guanosine, 24 mg thymidine, and 73 mg uridine in 100 mL dH_2O, and dissolve the nucleotide mixture at 37°C. Sterilize by passing it through a 0.22-μm disposable filter, dispense the solution into 1-mL aliquots, and store at −20°C. Thaw nucleotide stock at 37°C before use.
15. Fertilized, freshly laid chicken eggs.
16. Lipopolysaccharide (LPS) from *E. coli* serotype O127:B8 (100 mg; Sigma, cat. no. L3880).
17. Penicillin-streptomycin 100X (100 mL; Gibco BRL, cat. no. 15140-122).
18. 100 × 20-mm tissue culture dish (Becton Dickinson, cat. no. 353003).
19. 15-mL centrifuge tube (Sumilon, Tokyo, Japan; cat. no. MS-56150).
20. Filter paper rings (*see* **Note 5**).
21. Glass or plastic tube (*see* **Note 6**).

2.2.1. Media

1. Tissue culture medium for IN24 cell line: IN24 cells (chicken monocytic leukemia cells) (*10*) are maintained in IMDM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin (10% FBS-IMDM). To prepare 100 mL 10% FBS-IMDM, combine 10 mL heat-inactivated FBS, 1 mL penicillin-streptomycin, and 89 mL IMDM.
2. Tissue culture medium for CBCs: CBCs are maintained in DMEM supplemented with 10% heat-inactivated FBS, 2% heat-inactivated chicken serum, 0.1 m*M* MEM sodium pyruvate solution, 0.05 m*M* MEM NEAA, 1 μ*M* nucleotide stock solution, and 1% penicillin-streptomycin. This medium is called cytokine-free chicken embryonic stem cell medium (CESM). To prepare 100 mL CESM, combine 10 mL heat-inactivated FBS, 2 mL heat-inactivated chicken serum, 0.1 mL MEM sodium pyruvate solution, 0.5 mL MEM NEAA, 1 mL nucleotide stock solution, 1 mL penicillin-streptomycin, and 85.4 mL DMEM. When CBC are cultured, 100 mL CESM is added with 0.7 μL 2-mercaptoethanol and 2 mL purified rchLIF (1 μg/mL).

2.3. Detection of Markers of Undifferentiated CBC

2.3.1. Alkaline Phosphatase Reaction

1. Naphthol AS-BI alkaline solution (10 mL; Sigma, cat. no. 86-1).
2. Fast Red violet-alkaline solution (10 mL; Sigma, cat. no. 86-2).
3. Sodium nitrite solution (10 mL; Sigma, cat. no. 91-4).
4. Hematoxylin solution, Gill no. 3 (50 mL; Sigma, cat. no. GHS-3).
5. Citrate solution (50 mL; Sigma, cat. no. 91-5).
6. Acetone (500 mL; Wako, cat. no. 016-00346).
7. 37% formaldehyde solution (500 mL; Nacalai tesque, Kyoto, Japan; cat. no. 16223-55).

8. Citrate-acetone-formaldehyde fixative solution: to 25 mL citrate solution, add 65 mL acetone, 8 mL 37% formaldehyde solution. Place in a glass bottle and cap tightly. Store in a refrigerator (2–8°C). Warm to 18–26°C prior to use.

2.3.2. Immunofluorescence

1. PBS at pH 7.4 (*see* **Subheading 2.1.3., item 10**).
2. 10% formaldehyde neutral buffer, pH 7.0 (1 L; Nacalai tesque, cat. no. 37152-51).
3. Block Ace (250-mL; Dainippon Pharmaceutical Co., Osaka, Japan; cat. no. UK-B25).
4. Anti-stage-specific embryonic antigen (anti-SSEA)-1 mouse monoclonal antibody (ascites) (Developmental Studies Hybridoma Bank, Iowa City, IA; cat. no. MC-480).
5. Embryonal carcinoma cell monoclonal antibody (EMA)-1 mouse monoclonal antibody (partially purified immunoglobulin [Ig]) (Developmental Studies Hybridoma Bank, cat. no. EMA-1).
6. Fluorescein isothiocyanate-conjugated sheep antimouse Ig $F(ab')_2$ fragment (1-mL vial; Silenus, Victoria, Australia; cat. no. DDAF).

2.3.3. Western Blotting Analysis

1. Cell extraction buffer: 10 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1 m*M* EDTA·2Na, 1 m*M* Na_3VO_4, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40. For 100 mL: combine 33 mL 5 *M* NaCl, 0.2 mL 500 m*M* EDTA·2Na, 1 mL 0.1 *M* Na_3VO_4, 1 mL 10% sodium deoxycholate, 1 mL 10% SDS, 1 mL Nonidet P-40, and 1 mL 1 *M* Tris-HCl, pH 7.4. Add one tablet of protease inhibitor (*see* **Subheading 2.1.3., item 4**) to 25 mL prior to use.
2. PVDF membrane (Bio-Rad, Hercules, CA; cat. no. 13236).
3. Precision plus protein standards (protein marker) (1 mL; Bio-Rad, cat. no. 161-0363).
4. Blocking buffer: 20 m*M* Tris-HCl, pH 7.4, 140 m*M* NaCl, 25 m*M* EDTA·2Na, 0.2% Tween-20, 3% bovine serum albumin. For 100 mL: combine 3 mL 5 *M* NaCl, 5 mL 0.5 *M* EDTA·2Na, 0.2 mL Tween-20, 3 g bovine serum albumin, and 2 mL 1 *M* Tris-HCl, pH 7.4.
5. Anti-STAT3 (749-769) rabbit antibody (1 mg/mL; Calbiochem, San Diego, CA; cat. no. 57609).
6. Anti-Phospho-STAT3 (Tyr705) antibody (100 µL; Cell Signaling Technology, Beverly, MA; cat. no. 9131).
7. Horseradish peroxidase-labeled antirabbit IgG (1 mg/mL; KPL, Gaithersburg, MD; cat. no. 474-1516).
8. ECL plus Western blotting detection reagents (Amersham Pharmacia Biotech, cat. no. RPN2132).

3. Methods

3.1. Cloning, Expression, and Purification of rchLIF

3.1.1. Isogen RNA Extraction

Chicken LIF cDNA was cloned using mRNA from LPS-stimulated IN24 cells, and chicken LIF mRNA was most abundant in adult chicken liver and thymus *(9)*. The following procedure of RNA extraction was derived from the manufacturer's protocol (*see* **Note 7**).

1. Aspirate the culture medium from IN24 culture dishes and rinse the dishes three times with IMDM.
2. Add 10 mL of 10 µg/mL LPS-IMDM and culture for 16–24 h at 38.5°C in a CO_2 incubator.

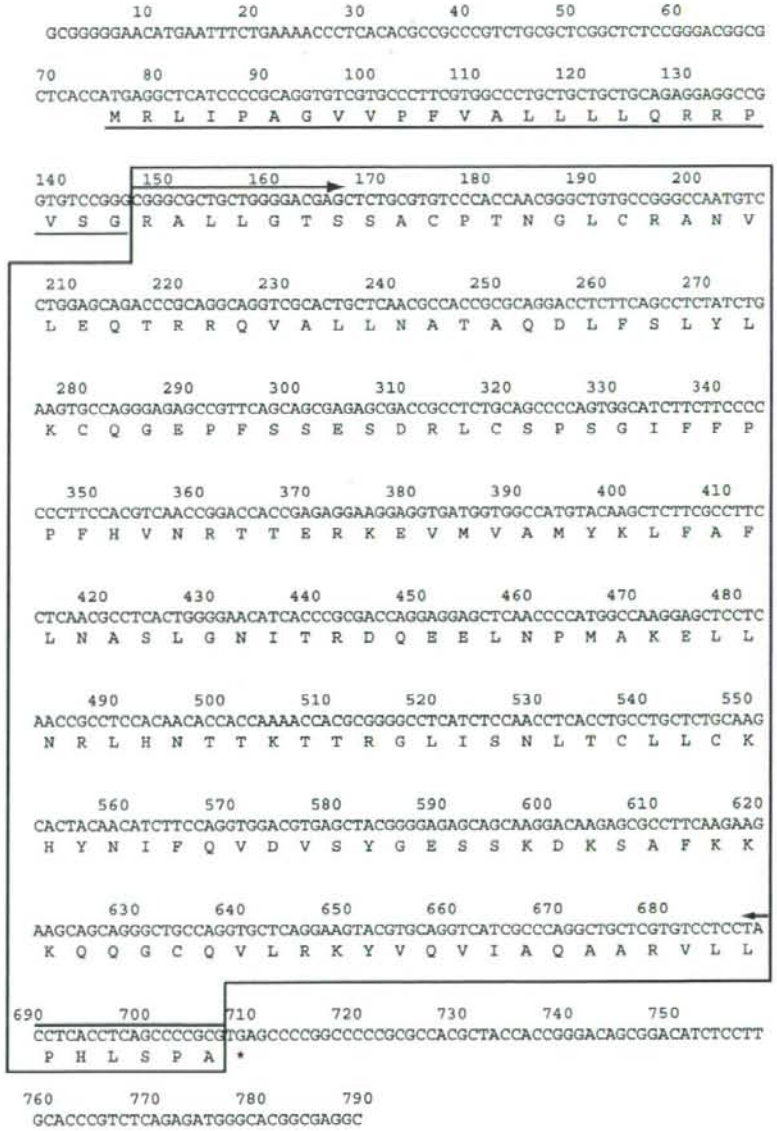
10 20 30 40 50 60
GCGGGGGAACATGAATTTCTGAAAACCCTCACACGCCGCCCGTCTGCGCTCGGCTCTCCGGGACGGCG
70 80 90 100 110 120 130
CTCACCATGAGGCTCATCCCCGCAGGTGTCGTGCCCTTCGTGGCCCTGCTGCTGCTGCAGAGGAGGCCG
M R L I P A G V V P F V A L L L L Q R R P
140 150 160 170 180 190 200
GTGTCCGGGCGGGCGCTGCTGGGGACGAGCTCTGCGTGTCCCACCAACGGGCTGTGCCGGGCCAATGTC
V S G R A L L G T S S A C P T N G L C R A N V
210 220 230 240 250 260 270
CTGGAGCAGACCCGCAGGCAGGTCGCACTGCTCAACGCCACCGCGCAGGACCTCTTCAGCCTCTATCTG
L E Q T R R Q V A L L N A T A Q D L F S L Y L
280 290 300 310 320 330 340
AAGTGCCAGGGAGAGCCGTTCAGCAGCGAGAGCGACCGCCTCTGCAGCCCCAGTGGCATCTTCTTCCCC
K C Q G E P F S S E S D R L C S P S G I F F P
350 360 370 380 390 400 410
CCCTTCCACGTCAACCGGACCACCGAGAGGAAGGAGGTGATGGTGGCCATGTACAAGCTCTTCGCCTTC
P F H V N R T T E R K E V M V A M Y K L F A F
420 430 440 450 460 470 480
CTCAACGCCTCACTGGGGAACATCACCCGCGACCAGGAGGAGCTCAACCCCATGGCCAAGGAGCTCCTC
L N A S L G N I T R D Q E E L N P M A K E L L
490 500 510 520 530 540 550
AACCGCCTCCACAACACCACCAAAACCACGCGGGGCCTCATCTCCAACCTCACCTGCCTGCTCTGCAAG
N R L H N T T K T T R G L I S N L T C L L C K
560 570 580 590 600 610 620
CACTACAACATCTTCCAGGTGGACGTGAGCTACGGGGAGAGCAGCAAGGACAAGAGCGCCTTCAAGAAG
H Y N I F Q V D V S Y G E S S K D K S A F K K
630 640 650 660 670 680
AAGCAGCAGGGCTGCCAGGTGCTCAGGAAGTACGTGCAGGTCATCGCCCAGGCTGCTCGTGTCCTCCTA
K Q Q G C Q V L R K Y V Q V I A Q A A R V L L
690 700 710 720 730 740 750
CCTCACCTCAGCCCCGCGTGAGCCCCGGCCCCCGCGCCACGCTACCACCGGGACAGCGGACATCTCCTT
P H L S P A *
760 770 780 790
GCACCCGTCTCAGAGATGGGCACGGCGAGGC

Fig. 1.

3. Lyse the cells in the culture dish by adding the appropriate amount of Isogen (1 mL per 10 cm^2).
4. Transfer 1-mL samples of the homogenate to microcentrifuge tubes and incubate them for 5 min at room temperature.
5. Add 0.2 mL chloroform per 1 mL of Isogen.
6. Cap the samples securely and shake the microcentrifuge tubes vigorously by hand for 15 s, then incubate at room temperature for 3 min.
7. Centrifuge at 12,000*g* for 15 min at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA remains exclusively in the aqueous phase.
8. Transfer the aqueous phase to a fresh tube. Precipitate the RNA by adding 0.5 mL RNase-free 2-propanol per 1 mL of Isogen used for the initial homogenization. Incubate the samples for 5–10 min at room temperature.
9. Centrifuge at 12,000*g* for 10 min at 4°C. The RNA forms a pellet on the bottom and the sides of the tube.
10. Remove the supernatant and wash the pellet with 1 mL of RNase-free 70% ethanol. Mix the sample by vortexing and centrifuge at 12,000*g* for 5 min at 4°C.
11. Remove the supernatant and air-dry or vacuum-dry the pellet and resuspend it in DEPC-H_2O.
12. Measure the optical density (OD) at A_{260} and A_{280}. The ratio of ODs at A_{260}:A_{280} should be between 1.6 and 2. Determine the concentration (*see* **Note 8**).

3.1.2. Reverse Transcription

The following procedure was derived from the manufacturer's protocol for SuperScript II reverse transcriptase:

1. Add the following components to a microcentrifuge tube: 1.0 µL Oligo $(dT)_{12\text{-}18}$ (0.5 g/mL), 1.0 µL 1–5 µg/µL total RNA, 1.0 µL 10 m*M* dNTP mix, and 11.0 µL dH_2O.
2. Heat the mixture to 65°C for 5 min, then quickly chill it on ice. Collect the contents of the tube by brief centrifugation and add 2.0 µL 10X first-strand buffer, 2.0 µL 0.1 *M* DTT, and 1.0 µL RNase inhibitor (40 U/µL).
3. Mix contents of the tube gently and incubate at 42°C for 2 min.
4. Add 1.0 µL (50 U) of SuperScript II reverse transcriptase, mix by pipetting gently up and down, and incubate for 50 min at 42°C.
5. Inactivate the reaction by heating at 70°C for 15 min and then store at 4°C.

3.1.3. Polymerase Chain Reaction

1. Design and synthesize the appropriate oligonucleotide primer for amplification of mature chicken LIF (*see* **Fig. 1**). For cloning into the expression vector, modify the 5′-end sequence of the primers: forward primer 5′-CG*GGATCC*CGGGCGCTGCTGGGGACGAG-3′ (italics, *Bam* HI restriction site); reverse primer 5′-CC*CTCGAG*TTATCACGCGGGGCTGAG-GTGAGGTA-3′ (italics, *Xho* I restriction site; underline, double stop codon).

Fig. 1. Nucleotide and deduced amino acid sequences of chicken leukemia inhibitory factor (LIF) cDNA and primer positions for reverse transcriptase polymerase chain reaction (RT-PCR). The termination codon (TGA) is marked with an asterisk. The numbers above refer to the nucleotide sequence, and the putative signal peptide is underlined. The mature chicken LIF sequence is boxed, and primer positions used for RT-PCR are marked with arrows. The nucleotide sequence has been submitted to the GeneBank/EBI Data Bank with accession number BD187371.

2. Prepare the PCR mixture (50 µL final volume) into the amplification tube: 1.0 µL template DNA (approx 100 ng), 5.0 µL 2.5 m*M* dNTPs, 5.0 µL 10X Ex Taq buffer, 1.0 µL forward primer (10 pmol/µL), 1.0 µL reverse primer (10 pmol/µL), 0.5 µL Takara Ex Taq DNA polymerase (5 U/µL), and 36.5 µL dH_2O.
3. In the thermal cycler, heat the samples to 98°C for 6 min and then run 30 amplification cycles in the linear range of 10 s at 98°C (denaturation), 30 s at 60°C (annealing), and 1 min at 72°C (polymerization). Finally, hold for 10 min at 72°C as an extension step and then store at 4°C.
4. Analyze the amplification products on a 1.0–1.5% agarose gel. The correct amplification product should be approx 600 bp.
5. Dilute the amplification product to 100 µL with distilled water.
6. Add 100 µL of phenol-chloroform (1:1 v/v) and vortex.
7. Centrifuge at 12,000*g* for 5 min at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube. Do not remove any of the interface with the aqueous layer.
8. Add an equal volume of chloroform and vortex.
9. Centrifuge at 12,000*g* for 3 min at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube.
10. Precipitate the cDNA from the aqueous layer by adding the following: 10 µL 3 *M* sodium acetate and 250 µL 100% (v/v) ethanol.
11. Vortex and leave to precipitate for 15 min at −80°C.
12. Centrifuge at 12,000*g* for 10 min at 4°C. The cDNA forms a pellet on the bottom and the sides of the tube.
13. Remove the supernatant and wash the pellet with 1 mL 70% ethanol. Mix the sample by vortexing and centrifuge at 12,000*g* for 5 min at 4°C.
14. Remove the supernatant and air-dry or vacuum-dry the pellet and resuspend it in TE buffer.
15. Measure the OD at A_{260} and A_{280}. The A_{260}:A_{280} ratio should be between 1.6 and 2. Determine the concentration of the cDNA (*see* **Note 9**).

3.1.4. Expression of rchLIF

We succeeded in producing rchLIF using a GST gene fusion system. The following procedure was derived from the manufacturer's protocol.

3.1.5. Ligating cDNA Into the Expression Vector

1. For restriction digestion of pGEX-6P-1 vector (*see* **Fig. 2**) and inserting cDNA, prepare the following reaction mixtures separately for the vector and insert: 5.0 µg pGEX-6P-1 vector or 0.3 pmol/µL insert cDNA, add 5.0 µL 10X K buffer, 1.0 µL *Bam* HI, 1.0 µL *Xho* I, and make up to 50 µL with distilled water.
2. Incubate at 37°C for 16 h.
3. Electrophorese the digested DNAs on 1.0% agarose gels and purify the DNAs using a commercially available gel extraction kit.
4. Determine the concentration of the DNAs using the spectrophotometer (*see* **Note 9**).
5. To anneal the linearized vector and insert DNA, they should be mixed at a vector:insert molar ratio of 1:5. Mix the components listed in a microcentrifuge tube: 100 ng plasmid vector, 61 ng insert DNA (chicken mature LIF cDNA), 4.0 µL 5X T4 DNA ligase buffer, 0.1 µL T4 DNA ligase (100 U/µL). Make up to 20 µL with distilled water.
6. Mix gently and incubate at 23–26°C for 1 h and store the unused portion of the cDNA at −20°C.

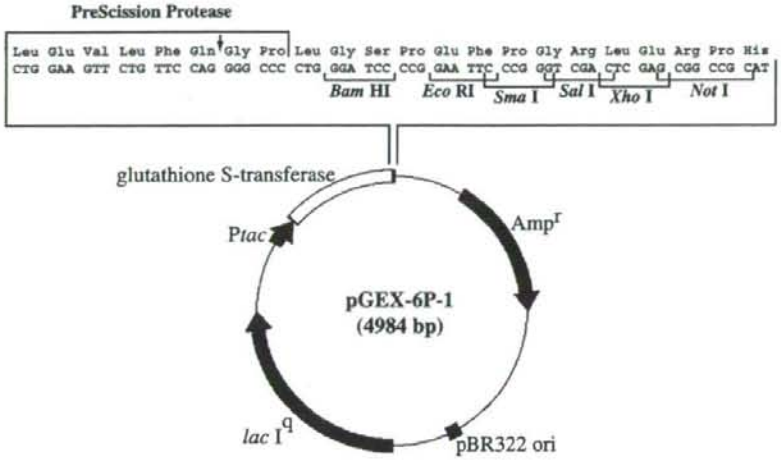

Fig. 2. Map of pGEX-6P-1 vector showing the reading frames and main features. The vector contains a glutathione-*S*-transferase (GST) coding sequence, an ampicillin resistance marker (Amp^r), a *Tac* promoter (P*tac*), and a PreScission protease recognition site for cleaving the desired product from the fusion protein. Mature chicken leukemia inhibitory factor nucleotides were cloned into the *Bam* HI-*Xho* I sites.

3.1.6. Transformation of Competent Cells

1. Place a clean microcentrifuge tube and a tube of BL21-competent cells on ice.
2. Let the competent cells thaw on ice and mix gently to ensure that the cells are evenly suspended.
3. Pipet 100-µL aliquots of cells into the prechilled microcentrifuge tube.
4. Add 5 µL of the ligated DNA solution to the cells immediately. Stir gently to mix.
5. Leave the tube on ice for 20 min.
6. Heat the tube for exactly 30 s in a 42°C water bath, do not shake.
7. Place on ice for 2 min.
8. Add 400 µL of room temperature SOC medium to the tube.
9. Culture at 37°C for 1 h with vigorous shaking.
10. Plate 100-µL aliquots of the diluted, transformed cells onto ampicillin-LB plate.
11. Incubate the plates at 37°C overnight.
12. Select colonies of BL21 cells containing a recombinant plasmid (*see* **Note 10**).

3.1.7. Preparation of Large-Scale Bacterial Sonicates

1. Use a single colony of BL21 cells containing a recombinant plasmid to inoculate 10 mL ampicillin-LB medium.
2. Incubate for 12–15 h at 37°C with vigorous shaking.
3. Dilute the culture 1:100 into 1 L of fresh ampicillin-LB medium (approx 330 mL in a 1-L flask) and grow in a 20°C shaking incubator until the A_{600} reaches 0.5.

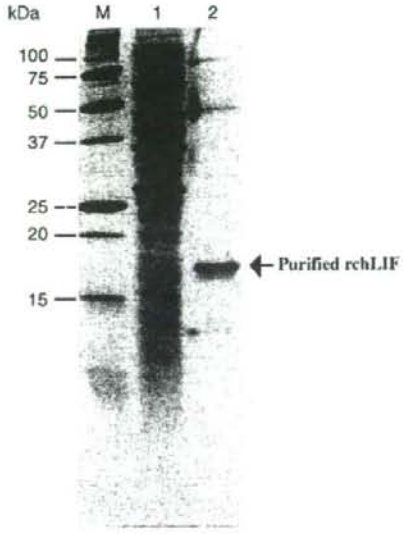

Fig. 3. Sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) analysis of bacterial lysate (**lane 1**) and purified recombinant chicken leukemia inhibitory factor (rchLIF) (**lane 2**). The samples were analyzed on a 15% SDS-PAGE stained with Coomassie blue. The molecular sizes of marker proteins (**lane M**) are shown on the left.

4. Add 100 m*M* IPTG to a final concentration of 0.1 m*M* and continue incubation for an additional 10–15 h.
5. Transfer the culture to appropriate centrifuge containers and centrifuge at 7700*g* for 10 min at 4°C.
6. Discard the supernatant and drain the pellet. Place on ice.
7. Using a pipet, completely suspend the cell pellet by adding 50 mL ice-cold 1X PBS in which one tablet of protease inhibitor has been dissolved.
8. Disrupt the resuspended cells using a sonicator, on ice, in short bursts.
9. Centrifuge at 12,000*g* for 10 min at 4°C. Transfer the supernatant to a fresh container. Save aliquots of the supernatant for analysis by SDS-PAGE (polyacrylamide gel electrophoresis) (*see* **Fig. 3, lane 1**).

3.1.8. Two-Step Purification of rchLIF

rchLIF is expressed as a fusion protein linked to GST and purified to greater than 90% purity in a two-stage (affinity and gel) chromatography method, which includes removal of the GST affinity tail. Using this method, 1–5 µg purified rchLIF can be obtained from 1 L of *E. coli* culture medium.

3.1.9. Affinity Purification Using Glutathione-Sepharose 4B

To facilitate the removal of the GST affinity tail from rchLIF, the GST fusion protein used contains the PreScission protease recognition site. This site is recognized by human rhinovirus 3C protease, which is used to cleave rchLIF from the GST affinity

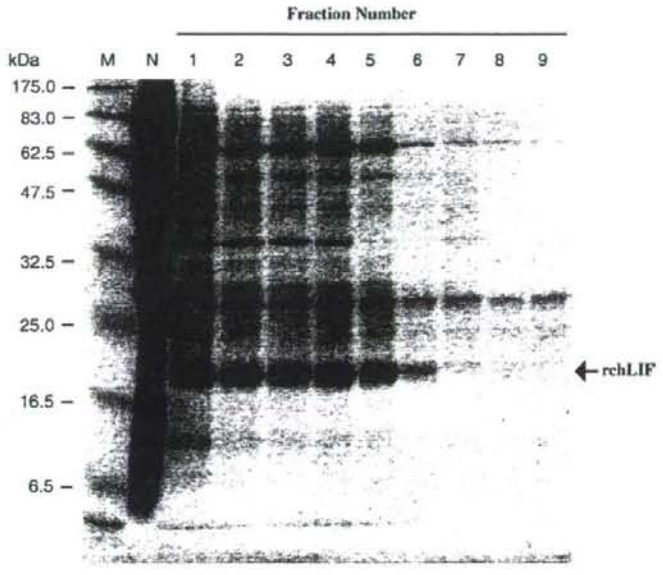

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of eluted fractions from affinity chromatography with glutathione-Sepharose 4B. The samples were analyzed on a 15% SDS-PAGE stained with Coomassie blue. The molecular sizes of marker proteins (**lane M**) are shown on the left. **Lane N** is the flow-through fraction.

tail and is eluted from the column, leaving the GST affinity tail and the protease bound to the glutathione-Sepharose 4B (*see* **Note 11**).

1. Gently shake the bottle of glutathione-Sepharose 4B to resuspend the matrix.
2. Use a pipet to remove sufficient slurry to form a 5-mL bed volume in the disposable column supplied with the purification kit.
3. Remove the bottom cap and allow the column to drain.
4. Wash the glutathione-Sepharose 4B by adding 50 mL cold (4°C) PBS and allow the column to drain.
5. Use a pipet to apply the bacterial sonicate (*see* **Subheading 3.1.7.**) to the column.
6. Remove the bottom cap and allow the sonicate to flow through the column.
7. Wash the matrix by adding 150 mL cold (4°C) 1X PBS. Allow the column to drain. The GST-rchLIF fusion protein will have bound to the glutathione-Sepharose matrix.
8. Replace the bottom cap on the washed column and add 5 mL PreScission protease reaction mixture; mix 200 µL (400 U) of PreScission protease with 4.8 mL of ice-cold PreScission cleavage buffer.
9. Replace the top cap and gently rotate (10 cycles/min) the suspension at 4°C for 14 h.
10. Remove the bottom cap and collect the eluate in 1-mL fraction in new tubes.
11. Analyze by SDS-PAGE to estimate the yield and purity of every fraction (*see* **Fig. 4**).

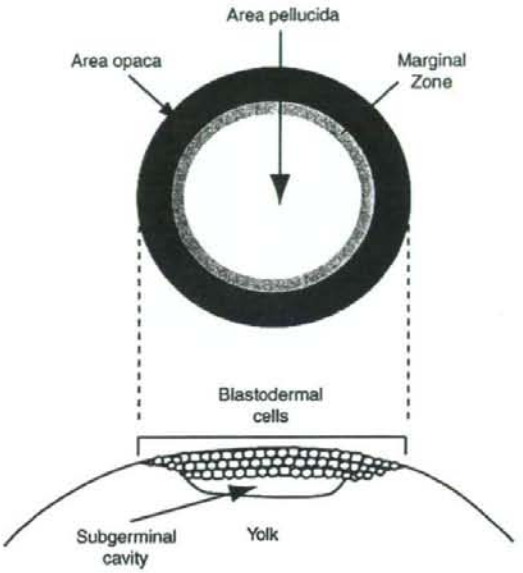

Fig. 5. Schematic illustration of stage X chicken embryo. The upper figure shows the ventral view, and the lower figure shows the cross section. Two distinct regions of blastoderm can be identified, the area pellucida and the area opaca, consisting of the darker cells at the margin of the blastoderm and yolk. Between the area pellucida and the yolk is a space called the subgerminal cavity.

3.1.10. Gel Chromatography

As shown in **Fig. 4**, several bands in addition to rchLIF are present on gels following elution from glutathione-Sepharose 4B. Highly purified rchLIF is obtained after a final gel chromatography step using the following procedure.

1. Pool the major fractions containing rchLIF in a clean tube.
2. Dialyze against cold PBS at 4°C overnight.
3. Equilibrate a Superdex 75 column with 1 L PBS.
4. Apply 5 mL of the dialyzed sample filtered through a 0.45-μm filter to the Superdex 75 column and elute with PBS at a flow rate of 1.0 mL/min.
5. Monitor by measuring the absorbance at 280 nm and collect 1.5-mL fractions.
6. Fraction eluted between 200 and 215 mL should contain rchLIF (*see* **Fig. 3, lane 2**).

3.2. Cell Culture

3.2.1. Method for Isolation of CBCs

Freshly laid fertilized chicken eggs are used to isolate CBCs from stage X embryos, as defined by Eyal-Giladi and Kochav *(2)* and illustrated in **Fig. 5**. Stage X blastodermal cells are pluripotent cells containing primordial germ cells or their immediate precursor cells. We use the stage X blastodermal cells in the area pellucida to isolate and cultivate CBCs.

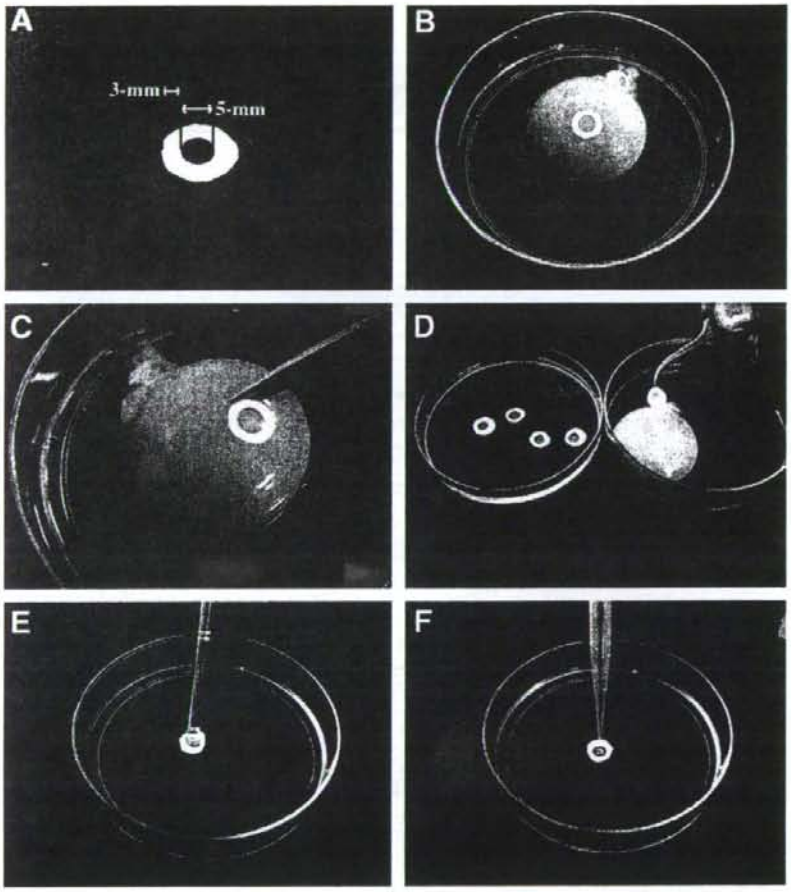

Fig. 6. Method for isolation of chicken blastoderm. (**A**) Dimensions of a filter paper ring. (**B**) Yolk positioned with embryo at the top with a filter paper ring placed centrally over the blastoderm. (**C**) Cutting the blastoderm along the periphery of the ring with scissors. (**D**) Lifting and inverting the ring and transferring it to a dish with PBS. (**E**) Punching out the area pellucida with the bottom of a glass pipet. (**F**) Collecting the area pellucida with a micropipet.

1. Prepare filter paper rings (*see* **Note 5** and **Fig. 6A**).
2. Autoclave and dry the prepared rings.
3. Break the fresh fertilized egg and put the contents into a 100-mm culture dish.
4. Rotate the yolk so the blastoderm is on the top surface.
5. Place a filter paper ring centrally over the blastoderm (*see* **Fig. 6B**).
6. Cut along the periphery of the paper ring with scissors to separate the blastoderm from the yolk (*see* **Fig. 6C**).

7. Pick up the blastoderm and filter paper together using a pair of tweezers, invert them, and transfer them to a 100-mm culture dish containing 10 mL PBS (*see* **Fig. 6D**).
8. Remove any yolk granules by washing carefully twice with PBS.
9. Cut the area pellucida from the blastoderm using a glass or plastic tube, such as the bottom of a pipet, as a punch (*see* **Note 6** and **Fig. 6E**).
10. Collect the area pellucida with a micropipet (*see* **Fig. 6F**) and transfer it to a 15-mL tube with 5 mL of serum-free CESM.
11. Dissociate the area pellucida by pipetting with a Pasteur pipet and then centrifuge at 120*g* for 10 min to pellet the cells.
12. Remove the supernatant, add 10 mL serum-free CESM, and gently resuspend.
13. Estimate the cell number with a hemocytometer. Approximately 2×10^4 cells should be obtained from each embryo.

3.2.2. Maintenance of CBCs

CBCs can be maintained in CESM containing 20 ng/mL rchLIF for at least 9 d without the addition of other cytokines or feeder cells.

1. After counting the cells, centrifuge the cell suspension (*see* **Subheading 3.2.1.**) at 120*g* for 10 min.
2. Remove the supernatant and gently resuspend the cell pellet at 4×10^4 cells/mL in 20 ng/mL rchLIF-CESM.
3. Distribute 1 mL to each well of a 24-well culture plate and incubate at 38.5°C in 5% CO_2 and 90% humidity.
4. The medium is partially replaced (by 50%) on every third day.

3.3. Detection of Markers of Undifferentiated CBCs

Alkaline phosphatase (AP) activity, the differentiation antigens recognized by EMA-1 and SSEA-1 are useful markers of chicken ES cells *(6)*. As another method for identifying undifferentiated cells, we measured the level of phosphorylation of STAT3. The advantage of this method is that it directly reflects LIF activity because LIF is responsible for this phosphorylation in ES cells. In fact, the activation of STAT3 by LIF is sufficient to maintain the undifferentiated state of both chicken and mouse ES cells *(9,11,12)*.

3.3.1. AP Reaction of CBCs

1. Measure 45 mL dH_2O and adjust temperature to 18–26°C.
2. Prepare diazonium salt solution by adding 1 mL sodium nitrite solution to 1 mL of Fast Red violet-alkaline solution and mixing gently by inversion. Allow to stand for 2 min.
3. Add diazonium salt solution (**step 2**) to the 45 mL dH_2O (**step 1**).
4. Add 1 mL naphthol AS-BI alkaline solution to diluted diazonium salt solution (**step 3**) and mix thoroughly.
5. Remove the medium from the CBC culture plate (*see* **Subheading 3.2.2.**) and carefully rinse twice with PBS.
6. Add 500 µL alkaline-dye mixture (**step 4**) per well to the washed culture plate and incubate at 18–26°C for 15 min. Protect the culture plate from direct light.
7. Discard the alkaline-dye mixture and rinse three times for 2 min with 1 mL dH_2O per well.
8. Counterstain with 500 mL hematoxylin solution for 2 min and rinse three times for 2 min with 1 mL dH_2O.
9. View and photograph the cultures in distilled water (*see* **Fig. 7**).

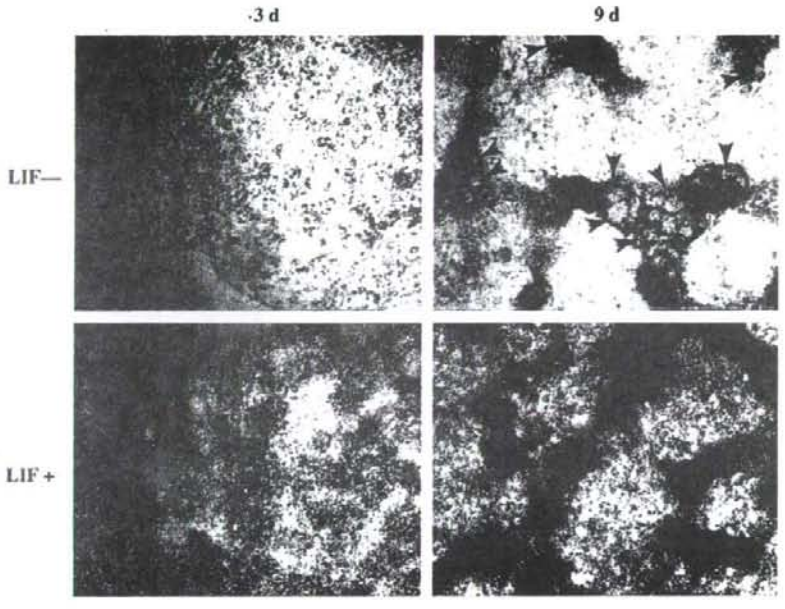

Fig. 7. AP staining of chicken embryonic stem (ES) cells after 3 and 9 d in culture. When ES cells stain positive for alkaline phosphatase (AP), this indicates that they are in an undifferentiated state. Cultures with rchLIF (LIF+) contained a high number of AP-positive cells compared to cultures without rchLIF (LIF–). After 9 d in culture, almost all of the ES cells formed cystlike embryoid bodies (arrowheads) in the absence of LIF, but very few appeared in the presence of LIF.

3.3.2. Immunofluorescence of CBCs

1. Remove the medium from the CBC culture plate (*see* **Subheading 3.2.2.**) and carefully rinse three times with cold (4°C) PBS.
2. Fix the cells with cold (4°C) 10% formaldehyde neutral buffer for 30 min at 4°C.
3. For blocking, add 500 µL/well of cold (4°C) 1% skimmed milk and 10% BlockAce-PBS and incubate for 30 min at 4°C.
4. Remove the blocking solution and add 300 µL/well of diluted antibody (anti-EMA-1 [1:50] or anti-SSEA-1 [1:30]) in 10% BlockAce-PBS. Incubate for 1 h at 4°C.
5. Rinse the wells three times with PBS to remove unbound primary antibody.
6. Add 300 µL/well of diluted fluorescein isothiocyanate-conjugated sheep antimouse Ig (1:50) in 10% BlockAce-PBS. Incubate for 30 min at 4°C.
7. Rinse the wells three times with PBS to remove unbound secondary antibody.
8. View and photograph the cultures in PBS (*see* **Fig. 8**).

3.3.3. Detection of Activated STAT3 Using Western Blotting

We have detected activated STAT3 in both CBCs immediately after isolation (*see* **Subheading 3.2.1., step 13**) and cultured CBCs (*see* **Subheading 3.2.2.**). However,

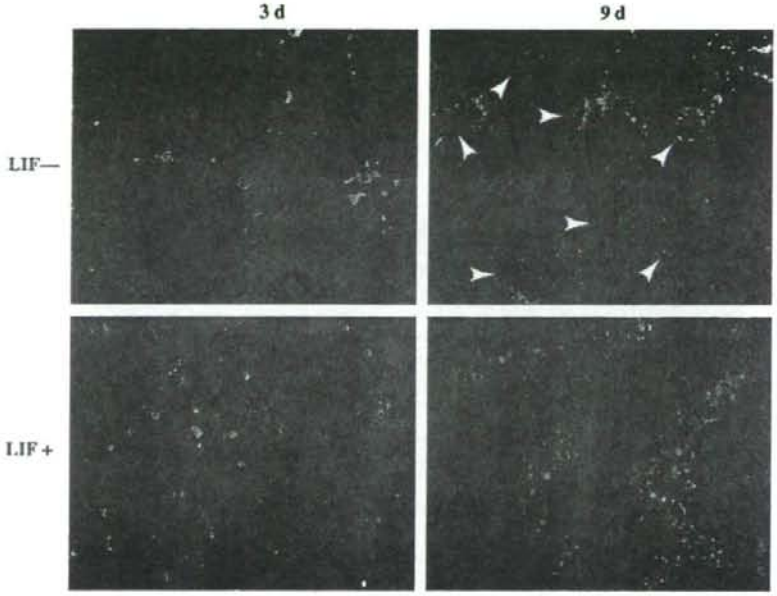

Fig. 8. Immunofluorescence, after labeling with anti-SSEA-1 antibody, of chicken embryonic stem (ES) cells after 3 and 9 d in culture. ES cells that are anti-SSEA-1-positive are in an undifferentiated state. Cultures with recombinant chicken leukemia inhibitory factor (rchLIF) (LIF+) contained a large number of anti-SSEA-1-positive cells compared to cultures without rchLIF (LIF–). After 9 d of culture, almost all of the ES cells formed cystlike embryoid bodies (arrowheads) in the absence of LIF, but very few appeared in the presence of LIF.

when CBCs are cultured without chicken LIF for 24 h or more, the levels of endogenous activated STAT3 decrease. We describe here the method used to detect activated STAT3 in fresh CBCs.

1. Incubate isolated CBC (*see* **Subheading 3.2.1., step 13**) with serum-free CESM containing rchLIF for 15 min at 38.5°C.
2. Lyse the cells ($1–2 \times 10^5$ cells) with 400 µL cold (4°C) cell extraction buffer in a microcentrifuge tube and rotate (10 cycles/min) for 16 h at 4°C.
3. Centrifuge the tube at 14,000*g* for 10 min and transfer the supernatant to a new tube on ice.
4. Add 300 µL cell lysate to 60 µL of 6X SDS loading buffer, vortex briefly, and heat for 5 min at 90–100°C.
5. Centrifuge briefly, then load 10 µL of the sample onto a 7.5% SDS-polyacrylamide gel.
6. Transfer the separated proteins from the electrophoresis gel to PVDF membrane.
7. Transfer the membrane onto which the protein has been blotted into an appropriate container, such as a Petri dish.
8. Add 50–100 mL of blocking buffer to the container and incubate for 2 h at 37°C with gentle shaking.

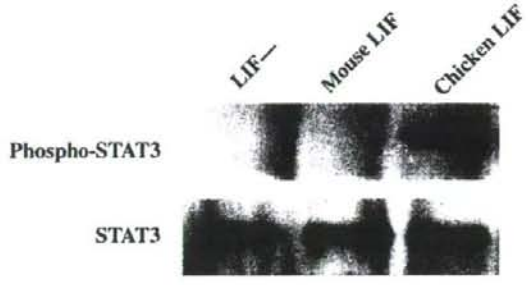

Fig. 9. Detection of activated STAT3 using Western blotting. The CBCs were incubated in serum-free CESM with or without 20 ng/mL recombinant chicken LIF or 20 ng/mL mouse LIF for 15 min at 38.5°C. The application of equivalent amounts of proteins was confirmed by determining nonphosphorylated STAT3 (STAT3). Only treatment with recombinant chicken LIF resulted in the phosphorylation of STAT3 (Phospho-STAT3).

9. Decant and discard the blocking buffer and add 50 mL of diluted anti-STAT3 antibody (1:1000) or diluted anti-Phospho-STAT3 antibody (1:1000) in fresh blocking buffer. Incubate for 10–16 h at 4°C with gentle shaking.
10. Decant and discard the first antibody solution.
11. Rinse the membrane twice by shaking for 15 min in 50 mL fresh blocking buffer.
12. Decant and discard the blocking buffer and add 50 mL diluted horseradish peroxidase-labeled antirabbit antibody (1:3000) in fresh blocking buffer. Incubate for 1 h at 37°C with gentle shaking.
13. Decant and discard the second antibody solution.
14. Rinse the membrane twice by shaking for 15 min in 50 mL fresh blocking buffer.
15. Develop the blot with ECL plus Western blotting detection reagent (*see* **Fig. 9**).

4. Notes

1. Isogen-LS reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissue. The reagent, a monophasic solution of phenol and guanidine isothiocyanate, is an improvement on the single-step RNA isolation method, similar to Invitrogen's Trizol reagent. In this method, Trizol reagent should work equally well, but we have used Isogen-LS as it is made in Japan.
2. Be careful in case it boils over.
3. This media is high in glucose (4500 mg/L D-glucose) and contains L-glutamine and pyridoxine hydrochloride.
4. Both FBS and chicken serum are heat inactivated by thawing the bottle of serum and incubating at 56°C for 30 min, with agitation every 10 min.
5. To isolate the chicken blastoderm from the yolk, we use filter paper rings (*see* **Fig. 6A**). To make these, we use a paper punch to make many holes (5-mm diameter) in the filter paper (Advantec, cat. no. 023634007), then we cut around each hole to make rings approx 3 mm wide.
6. To isolate the area pellucida from the chicken blastoderm, we use the bottom of a glass or plastic pipet with a 2- or 3-mm diameter opening (*see* **Fig. 6E**).
7. The key to successful purification of intact mRNA is speed to avoid degradation by endogenous RNase in the cells. It is crucial during this extraction procedure to use RNase-free instruments and solutions and to change gloves often to minimize the risk of RNase contamination from other sources.

8. Determine the concentration of the RNA using the following equation:

$$\text{RNA concentration } (\mu g/\mu L) = [A_{260} \times 40 \times \text{Dilution factor}] / 1000$$

9. Determine the concentration of the cDNA using the following equation:

$$\text{DNA concentration } (\mu g/\mu L) = [A_{260} \times 50 \times \text{Dilution factor}] / 1000$$

10. The 5′ pGEX sequencing primer and the 3′ pGEX sequencing primer can be used to screen transformants rapidly by colony PCR. We recommend determining the sequence of the clones obtained using these sequencing primers.
11. We have used the bulk GST purification module, but the appropriate module should be selected for the scale of your experiment.

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Development and Evaluation of Event-Specific Qualitative PCR Methods for Genetically Modified Bt10 Maize

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In 2005 it was reported that the genetically modified (GM) maize strain or 'event' called Bt10 had been distributed inadvertently in the United States over the previous 4 years. In order to ensure that grain for food and feed production did not contain trace amounts of Bt10 maize and complied with the applicable regulation, highly sensitive and specific detection of Bt10 maize was required. Accordingly, we developed a novel qualitative PCR system for specific detection of Bt10 maize. Moreover, we amply evaluated the performance characteristics of two PCR systems, our own and the one provided by the developer of Bt10, Syngenta Co. Ltd. It was confirmed that both of the qualitative PCR systems can specifically detect Bt10 maize, and the results of a single-laboratory examination suggested that the limit of detection was approximately less than 0.05% for both methods. To evaluate the reproducibility of the methods, we organized an interlaboratory study with the participation of 6 laboratories and analysis of 240 blind test samples. In this paper, we report, for the first time, the statistical analysis of the qualitative PCR data obtained from the interlaboratory study. The results of this analysis also revealed that there was no significant difference in the sensitivity between the two aforementioned methods and that the limit of detection of both the methods was less than 0.05%. Thus, we conclude that both of the methods are equally suitable for correct identification and sensitive detection of the unapproved GM maize Bt10 event in test samples.

INTRODUCTION

Recent progress in recombinant DNA and plant transformation techniques has accelerated the development of various genetically modified (GM) organisms (GMOs) and of products derived from them. GM crops, in particular, herbicide-tolerant or insect-resistant soy, maize, rapeseed, and cotton, have already been approved by many countries for agricultural use for about a decade (*1*). However, the safety, in terms of use in foods/feeds, and the environmental effects of GM crops has been a matter of public concern because of anxiety regarding new technologies and lack of understanding of technical information. Therefore, an increasing number of countries have implemented regulatory frameworks to assess the safety of these GMOs prior to declaring them safe for the environment, for human consumption, and for use in animal feed. In addition, some countries have adopted the policy of informing their consumers of the GM origin of a food product's ingredients by labeling, supported by testing, and traceability of GM and conventional crops throughout the supply chain (*2*, *3*). Sensitive and specific analytical methods to test commercialized GM crops strains or 'events' are available at commercial and governmental laboratories (*4*).

On the other hand, no specific analytical methods are, in general, required for testing the presence of particular GM events that have not undergone safety assessment for approval, since these events are not intended to be commercialized for agricultural use and do not enter the food chain. However, in the event of accidental contamination of an unapproved GM event due to unforeseen circumstances, regulatory organizations should be well prepared with a testing system to monitor the distribution of the unapproved GM event. It will be challenging or perhaps impossible for commercial and governmental laboratories to develop such testing system without the assistance of the developer. Development and evaluation of a new analytical method is likely to be required, based upon experiments with authentic material containing the GM event in question. An incident where specific testing system for an unapproved GM event became necessary was the accidental distribution of Bt10 maize reported in 2005 (*5*). Syngenta Co. Ltd., one of the world's largest agricultural biotechnology companies, reported that they inadvertently produced and

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