Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities

B. Pain1,*, M. E. Clark2, M. Shen2, H. Nakazawa3, M. Sakurai4, J. Samarut1 and R. J. Etches2

1Laboratoire de Biologie Moléculaire et Cellulaire, Ecole Normale Supérieure de Lyon, UMR 49 CNRS/ENS, LA INRA 913, Lyon, France
2Animal and Poultry Science Department, University of Guelph, Ontario, Canada
3International Agency for Research on Cancer, World Health Organisation, Lyon, France
4National Institute of Animal Health, Tsukuba, Japan

*Author for correspondence (e-mail: bpain@ens-lyon.fr)

SUMMARY

Petitte, J. N., Clarck, M. E., Verrinder Gibbins, A. M. and R. J. Etches (1990; Development 108, 185-189) demonstrated that chicken early blastoderm contains cells able to contribute to both somatic and germinal tissue when injected into a recipient embryo. However, these cells were neither identified nor maintained in vitro. Here, we show that chicken early blastoderm contains cells characterised as putative avian embryonic stem (ES) cells that can be maintained in vitro for long-term culture. These cells exhibit features similar to those of murine ES cells such as typical morphology, strong reactivity toward specific antibodies, cytokine-dependent extended proliferation and high telomerase activity. These cells also present high capacities to differentiate in vitro into various cell types including cells from ectodermic, mesodermic and endodermic lineages. Production of chimeras after injection of the cultivated cells reinforced the view that our culture system maintains in vitro some avian putative ES cells.

Key words: embryonic stem cells, ES cells, chicken, quail, in vitro culture

INTRODUCTION

Experimental modification of the genome of animal species is one of the major tools to investigate embryonic development, gene expression and tissue differentiation and to develop new approaches of animal selection. Two strategies have been used to date: (1) injecting DNA into oocytes and (2) genetically engineered embryonic stem cells. In avian species the injection of DNA into oocytes successfully generated transgenic chickens but the technique is tedious and the yield of transgenic animals is low (Love et al., 1994). The development of ES cells would provide invaluable tools to manipulate the genome of avian species.

ES cells were first isolated in mouse in cultures of inner cell mass of preimplantation embryos (Evans and Kaufman, 1981; Nichols et al., 1990). When implanted into host blastocysts, these cultured cells can participate in the development of all cell lineages including the germ line. Numerous mouse ES cell lines from several strains have been isolated and maintained in vitro using various protocols (Smith, 1991; Robertson, 1987; Kawase et al., 1994). Recently, ES-like cells, designated as EG, were derived in culture from murine PGC (Matsui et al., 1992; Donovan, 1994). These cells are distinct from mouse Embryonic Carcinoma (EC) cells derived from teratocarcinomas, although the ability to contribute to all somatic tissues and the germ line is shared by ES and EG cells (Bradley et al., 1984, Robertson, 1987). Cultures of EC cells or ES-like cells have been developed to date from mink (Sukoyan et al., 1993), hamster (Doetschman et al., 1988), rat (Iannaccone et al., 1994), pig (Wheeler, 1994) and bovine (First et al., 1994), and recently from monkey (Thomson et al., 1995) and zebra fish (Sun et al., 1995). Although these cells share several features with murine ES cells, they have not yet been shown to reconstitute the germ line.

Cells derived from the early chick blastoderm will contribute to the somatic and the germ line when injected into recipient embryos to form chimeras (Thoraval et al., 1994; Carsience et al., 1993; Watanabe et al., 1992; Petitte et al., 1990). To date, blastodermal cells have been maintained in vitro for only brief periods (Etches et al., 1996) and have not yet been cytologically identified.

In the present work, we characterised embryonic stem cells derived from culture of chicken and quail embryos. The culture system allows long-term LIF-dependent growth of cells with histochemical and antigenic markers and morphogenetic potentialities similar to those described for murine ES cells.

MATERIALS AND METHODS

Medium

ESA medium was composed of Glasgow-MEM (Gibco, UK) containing 10% foetal bovine serum (Techgen batch no F 93246, France),
2% chicken serum (Valbiotech, France), 1% bovine serum albumin (Boehringer, Germany), 20 ng/ml conalbumin (Sigma, USA), 1 mM sodium pyruvate (Gibco, UK), 1% non-essential amino acid (Gibco, UK), 1 μM of each nucleotide adenosine, guanosine, cytidine, uridine, thymidine (Sigma, USA), 10 mM Hepes pH 7.6 (Gibco, UK), 0.16 mM β-mercaptoethanol (Sigma, USA), 100 U/ml penicillin (Gibco, UK), 100 μg/ml streptomycin (Gibco, UK) and 10 mg/ml gentamycin (Boehringer, Germany). ESA medium refers to as medium without addition of growth factors. ESA complete medium refers to as medium supplemented with 10 ng/ml bFGF, 20 ng/ml h-IGF-1, 1% vol/vol avian-SCF and 1% vol/vol h-LIF, 1% vol/vol h-IL-11.

Growth factors
bFGF, IGF-1, m-SCF, IL-6, CNTF and OSM were purchased from PeproTech Inc. (NJ, USA) or from R&D Systems (UK), ESGRO from Gibco (UK) and IL-11 was kindly provided by Genetics Institute (MA, USA).

For routine maintenance, some growth factors were provided as supernatants of transfected Cos-7 cells. Expression vectors of the cDNAs of avian SCF (Zhou et al., 1993), LIF (kindly provided by A. Smith, Edinburgh, UK), IL-11 and IL-6 (kindly provided by Genetics Institute, Cambridge, MA, USA) were transiently transfected in Cos-7 cells (ATCC collection) by a calcium precipitate procedure and, 3 days later, conditioned medium was filtered and stored in aliquots at –20°C.

Anti-retinoic acid monoclonal antibody (ARMA)
ARMA hybridoma (kindly provided by Dr Ide, Tohoku University, Japan) was maintained in RPMI 1640 with 10% FBS, 1% glutamine and 100 μM penicillin (Gibco, UK) and 100 μg/ml streptomycin (Gibco, UK). The IgG fraction was purified from the conditioned medium through an affinity column of protein G Sepharose 4 Fast Flow (Pharmacia, Sweden).

Preparation of culture dishes and feeder cells
Dishes (Costar or Nunc) were coated with 0.25% sterile bovine skin gelatine (Sigma, USA) solution. STO feeder cells (ATCC collection) were maintained at 37°C in 7.5% CO2 in the presence of DMEM containing 4.5 g/l of glucose, 10% FBS, 2 mM L-glutamine, 100 μM penicillin and 100 μg/ml streptomycin, and treated with 10 μg/ml of Mitomycin C (Sigma) for 90 minutes at 37°C or irradiated by exposure to 45 Gy (Cobalt source). Cells were used at 10^5 cells/cm^2 as feeder.

Blastodermal cells
Freshly laid unincubated eggs from Barred Rock or White Leghorn chicken or Coturnix coturnix japonica quail were used. The stages of the chicken embryos were estimated according to Eyal-Giladi and Rama (1986), by Dr J. L. Duband (Paris, France) for N-CAM, by Dr C. Ziller (Nogent/Marne, France) for 13F4 and by Dr T. Graf (Heidelberg, Germany) for MEP21 (Graf et al., 1992).

Murine ES cells
The CGR-8 ES cell line (kindly provided by A. Smith, Edinburgh, UK) was maintained according to Smith (1991).

Culture
The blastodermal cells were maintained at 37°C in 7.5% CO2 and 90% humidity. Half of the medium was partially replaced after 24 hours in culture. Fresh blastodermal cells were added in half of the original volume of ESA complete medium 48 hours later. The medium was then changed partially (50%) on the third day and totally every day thereafter. To recover the cells, cultures were washed with PBS-G and incubated in a solution of pronase (0.025% w/v, Boehringer, Germany). Dissociated cells were seeded on new feeder cells or on gelatine-coated dishes for longer culture or laid on L3M gradient (Nycoprep, Norway). The density gradients were centrifuged at 2500 g and the interface was collected to isolate putative stem cells for injection into recipient embryos.

Development of embryoid bodies in vitro
Cells were enzymatically dissociated after several passages, washed in PBS-G and plated in non-tissue-culture dishes at 10^5 cells/ml of ESA medium without LIF. Fresh medium was added every 2 days. After 7 days, floating masses of cells were collected, washed carefully and plated into wells to allow them to attach and differentiate.

Alkaline phosphatase reaction
After washing, cultured cells were fixed with cold 4% paraformaldehyde for 15 minutes at 4°C. Alkaline phosphatase staining solution containing 100 mM NaCl, 100 mM Tris-HCl pH 9.5, 5 mM MgCl2, 1 mg/ml NBT, 0.1 mg/ml BCIP was added after washing and incubating at 37°C. The reaction was incubated for 5-30 minutes at 37°C, stopped by addition of 10 mM EDTA and the wells were washed with PBS. Coloured colonies were scored using an inverted microscope (Leica, Germany).

Immunofluorescence analysis
Cultured cells were fixed with cold 4% paraformaldehyde for 15 minutes at 4°C. Reactions were performed with cold PBS-containing 1mg/ml BSA at 4°C. The first diluted antibody was added overnight. After washing, the second antibody D2AF-labelled (Jackson Laboratories, USA) was added for 1 hour and the cells were re-washed and then observed using an inverted Leica fluorescence microscope.

Cytofluorometry analysis
Blastodermal cells were dissociated into a monocellular suspension with pronase and then washed and incubated in complete ESA medium for 1 hour at 37°C until the epitopes were reconstituted. Cells were then washed again in PBS-G buffer and fixed with cold 4% paraformaldehyde. Immunofluorescence was analysed using a FACSscan (Becton Dickinson), equipped with an argon laser (488 nm) and a band pass filter at 530 nm. Data were analysed using the CellQuest program (Becton Dickinson).

Antibodies
Antibodies were bought from the Developmental Studies Hybridoma Bank (Iowa University, USA) for SSEA-1, SSEA-3 (Solter and Knowles, 1978) and TROMA-1 (Brulet et al., 1980) or kindly provided by Dr R. Kemler (Freiburg, Germany) for ECMA-7 (Kemler et al., 1981), by Dr A. Eddy (NIH, NC) for EMA-1 and EMA-6 (Hahnel and Eddy, 1986), by Dr J. L. Duband (Paris, France) for N-CAM, by Dr C. Ziller (Nogen/Marne, France) for 13F4 and by Dr T. Graf (Heidelberg, Germany) for MEP21 (Graf et al., 1992).

Telomerase activity
Cells from various passages were dissociated by pronase, counted and washed extensively. Cell pellet (5x10^6 cells) was washed with 1 ml PBS, then with ice-cooled telomere-repeat-amplification-protocol (TRAP) washing buffer (Kim et al., 1994). Dried pellets could be stored at –80°C. 100 μl of ice-cooled TRAP lysis buffer was added to fresh or dried pellets. Samples were homogenised 3 times on ice with a plastic pestle for 1 minute each and kept on ice for 30 minutes. After the lysates were cleared by centrifugation at 70 000 revs/minute for 30 minutes at 4°C, the supernatants were collected and kept at –80°C until use. PCR-based TRAP assay was performed as described by Kim et al. (1994). The PCR products (15 μl out of 50 μl) were
electrophoresed on a 8% polyacrylamide gel, run for 1 hour at 200 V. The gel was then dried, exposed for 2-3 hours and analysed by a phosphorimager (with scanner Control) and ImageQuant soft wares, Molecular Dynamics.

### RT-PCR

CEFs (primary chicken embryonic fibroblasts), prepared as previously described (Gandrillon et al., 1989), embryoid bodies and 9-day-old embryonic blood cells were used for extraction of RNA by guani
dinium thiocyanate acidic phenol procedure. 2 μg of heat-denatured total RNA were reverse transcribed for 1 hour at 37°C in 20 μl 1× RT buffer (Promega) containing 1 mM each dNTP, 200 pM d(N)6, 1 U RNAsin (Promega), 10 U M-MVL RT (Promega). 5 μl of mixture were then used for PCR amplification in 1× GoldStar buffer (Euro
tec, Belgium) containing 2 mM MgCl₂, 1 mM each dNTP, 1 μg of each oligonucleotide and 0.3 U Goldstar thermostable polymerase (Euro
tec, Belgium). Oligonucleotides (5’ to 3’) were purchased from Genset (France): α-globin 9(s) GTCTGTAACAGAAGAAC,
CAAG, β-globin 307(as) CCAATGCTTCCTGGTTG (Dognog and E, 1983), β-globin 425(s) TCTCCCAGTCCGAAC, β-globin 1415(as) TAGTGTCCTGGATCTCTCATC (Dolan et al., 1983), ε-globin 332(s) GGTTCCGTGCTCATGGTAAG, ε-globin 1575(as) CTAATGAGCTGGTCGTCTG (Dognog and Engel, 1983), c-S17 61(as) TACACCCGTTCGGCAACGAC, c-S17 169(as) CCGCTG
GATGCCGCTCATAC (Trueb et al., 1988), c-vil 301(as) TCTG
GTGGCGCGTGGCAC, c-vil 840 (as) GACCTGATGTCTCTCATC (Bazari et al., 1988). Reaction was performed on a Perkin Elmer 9600 thermocycler during 25 cycles for c-S17, α-globin, β-globin and ε-globin and 30 cycles for c-villin. Parameters were 30 seconds at 94°C for denaturation, 30 seconds at 62°C (c-S17, β-globin) or 30 seconds at 60°C (α-globin and ε-globin) and 30 seconds at 58°C (c-villin) for annealing and 30 seconds at 72°C for elongation. The last step of elongation was realised for 5 minutes. PCR products were analysed on an agarose gel (2% Nusieve, 1% agarose).

### Injection into recipient embryo

Stage X White Leghorn embryos from newly laid eggs were used as recipient embryos for the Barred Rock cultures. Recipient embryos were irradiated at 6 Gy (Cobalt source), according to Carsience et al. (1993). A small window was made on the lateral part of the egg and shell membrane was removed. Between 1 and 3 μl of cell suspension at 2×10⁶ cells/ml were injected into the subgerminal cavity using a micropipet (20 μl borosilicate capillaries, Denmark). The window was then closed with two layers of shell membrane and sealed with cement for plastic model (Testors, USA). When dried, the window was then closed with two layers of shell membrane and sealed with cement for plastic model (Testors, USA). After 3 days of culture, cells were fixed and stained for alkaline phosphatase activity and the positive colonies with a compact and round morphology were scored. When added alone, none of the factors significantly increased the number of positive colonies. However, the combination of h-LIF, bFGF and either avian or murine SCF strongly enhanced the number of alkaline-phosphatase-positive colonies. This combination of growth factors was then shown to induce the growth of alkaline-phosphatase-positive cells in CEC cultures (data not shown). These culture conditions were then used to develop routinely CEC or QEC cultures.

LIF belongs to a family of cytokines (Piquet-Pellorce et al., 1994) including ciliary neurotrophic factor (CNTF), onco
cstatin (OSM), interleukin-6 (IL-6) and interleukin-11 (IL-11). Since all of the members of the family of cytokines are known to provide their stimulatory transduction signal through very similar receptors and associated molecules (Kishimoto et al., 1994; Yoshida et al., 1994), the effects of OSM, CNTF, LIF; IL-11 and IL-6 on avian embryonic cells were evaluated. The cultures were grown on gelatine-coated dishes either in the presence or absence of STO feeders. The data illustrated in Fig. 2B and C indicate that, in both conditions, OSM, LIF, IL-11 and IL-6 added individually provided the greatest enhancement of the number of alkaline-positive colonies. We further observed that LIF and IL-11 had a slight synergistic effect on the growth of alkaline-phosphatase-positive cells (data not shown) and these two cytokines were then routinely used in combination. As shown in Fig. 2C, the STO feeder strongly promoted the development of alkaline-positive colonies (Fig. 2B).

When comparing different cultures of feeder, it appeared that the mitomycin C or irradiated inactivated STO feeder provided the most regular and reproducible morphology and highest number of alkaline-phosphatase-positive colonies. Avian
feeders, like primary CEFs or QEFs, or established cells QT6 (Moscovici et al., 1977), QBr-3 (Guilhot et al., 1993) or LMH (Kawaguchi et al., 1987) also provided high numbers of alkaline phosphatase colonies. Because of their highly transformed phenotype, QT6, QBr-3 and LMH cells are inappropriate for generation of stable adherent feeders.

Effect of anti-retinoic acid antibody on CEC growth

Retinoic acid is a strong inducer of differentiation especially on the murine ES cells, which then rapidly lose their totipotency. In order to neutralise endogenous retinoic acid in the culture medium, we added a monoclonal antibody against retinoic acid (ARMA; Tamura et al., 1990). This procedure has been used successfully to maintain selfrenewing hematopoietic cells in culture (Gandrillon et al., 1994; unpublished data). As shown in Fig. 2D, the addition of a low concentration of this antibody (1 μg/ml) into the CEC cultures enhanced the number of alkaline-phosphatase-positive colonies. In contrast, the addition of an excess of retinoic acid (10^{-6} M final) into the medium for 2 days completely abolished the presence of alkaline-phosphatase-positive colonies (data not shown). Depletion of retinoic acid using activated charcoal or anionic exchange resin strongly decreased the ability of treated sera to sustain the growth of blastodermal cells. Therefore, the media were routinely supplemented with ARMA.

Characterisation of epitopes expressed by grown blastodermal cells

Cells maintained in CEC cultures were characterised using antibodies known to identify murine ES cells. Blastodermal cells were plated in ESA complete medium. After 5 days, the cultures were fixed and analysed for expression of both alkaline phosphatase and epitopes known to be specific of murine ES cells. Cultures of murine ES cells were used as controls. The epitopes recognised by the monoclonal antibodies ECMA-7 (Kemler et al., 1981) and SSEA-1 (Solter and Knowles, 1978) which are expressed by non-differentiated murine ES cells were also expressed on colonies in CEC cultures (Fig. 3A-D). Another antibody, SSEA-3, which recognises a specific epitope on murine ES cells also labelled CEC in culture (not shown). The EMA-1 and EMA-6 antibodies recognise epitopes specifically on primordial germ cells (PGC) in both mouse and chicken (Hahnel and Eddy, 1986; Urven et al., 1988). We observed that these two antibodies also label murine ES cells (Fig. 3E,F, and data not shown) and CEC cultures (Fig. 5G,H). In general, the epitopes expressed by undifferentiated cells disappeared following exposure to retinoic acid within 12-18 hours for ECMA-7, 24 hours for...

Fig. 1. Endogenous alkaline phosphatase activity in ES and CEC cultures. (A) Murine ES cells plated at low density. Spontaneously differentiated cells (D) were observed between areas of non-differentiated cells (ND). Cells were stained for alkaline phosphatase activity. (B) Alkaline-phosphatase-positive colonies in CEC culture grown for 5 days.

Fig. 2. Effect of culture conditions on the development of alkaline phosphatase colonies. (A) QEC (0.75 embryo/ml) were plated in the presence or absence of growth factors as indicated (10 ng/ml for bFGF, 10 ng/ml for mSCF, 1% V/V aSCF and 1% hLIF. After 3 days, alkaline-phosphatase-positive colonies were scored. (B,C) CEC (1 embryo/ml) were plated in complete ESA medium on gelatine-coated dishes in the presence (C) or absence (B) of inactivated STO feeder cells. Cytokines were added as indicated. After 5 days, alkaline-phosphatase-positive colonies were numbered. (D) QEC (0.75 embryo/ml) were plated in ESA complete medium in the presence or absence of ARMA (1 μg/ml) on non-coated (− gelatine) or gelatine-coated (+ gelatine) dishes. After 5 days, alkaline-phosphatase-positive colonies were numbered.
Long-term culture of avian embryonic stem cells

SSEA-1 and 36-48 hours for EMA-1 (not shown). The TROMA-1 antigen is specifically expressed on cells derived from differentiation-induced murine ES cells. This antigen was also observed on CEC cells 48 hours following treatment with RA.

We observed that the expression of ECMA-7, SSEA-1 and EMA-1 was localised in the alkaline-positive CEC colonies, as illustrated on Fig. 3A and B for ECMA-7. However, in primary culture, around 20% and 40% of the alkaline-phosphatase-positive foci were labelled by the ECMA-7 and EMA-1 antibodies, respectively. As the cultures were passaged, the frequencies of ECMA-7 and EMA-1-positive foci increased strongly making the cultures phenotypically more homogeneous (not shown).

LIF is necessary for long-term in vitro growth of CEC with ES-like markers

LIF is necessary for the growth and long-term maintenance of murine ES cells in culture (Nichols et al., 1990). We therefore tested its importance in CEC culture. CEC cultures were initiated in ESA complete medium and then passed every 4 to 7 days by subculturing. After 12 passages, one half of the culture was subcultured for several passages in identical conditions. The other half was seeded in ESA complete medium devoid of LIF and regularly subcultured in this same medium. Growth kinetics were then followed in each conditions. As shown in Fig. 4A, the cultures devoid of LIF showed a much slower growth and the number of cells was more than ten times lower than in LIF-supplemented cultures. The phenotype of the cultures was also progressively altered in the absence of LIF as the colonies became less compact, cells became flat, lost their nucleoli and increased their nucleocytoplasmic ratio. In addition, the expression of antigenic epitopes was profoundly altered by deprivation of LIF. As shown in Fig. 4B, the cultures maintained in the presence of LIF contained 10-15 times as many cells that were positive for ECMA-7, EMA-1 and SSEA-1 than cultures maintained in the absence of LIF. As a consequence, we observed that the cultures regularly maintained in the presence of LIF progressively selected a more homogeneous population of cells harbouring the ECMA-7 and EMA-1 epitopes (not shown). Growing cultures containing ECMA-7-, SSEA-1- and EMA-1-positive cells could be maintained for at least 35 passages, i.e. more than 160 days in the presence of LIF. Therefore the mammalian LIF is required for the long-term growth of avian embryonic cells and the expression of antigens characteristic of ES cells.

Fig. 3. Epitope profile of cultured CEC. Expression of various epitopes was tested on CEC after 5 days in culture. (A,B) Alkaline phosphatase staining and ECMA-7 detection; (C,D) phase contrast and SSEA-1 detection; (E,F) murine ES cells in phase contrast and stained anti-EMA-1 (scale, 20 μm).

Fig. 4. Effect of LIF on long-term growth of CEC. (A) Kinetics of growth CEC were grown in complete ESA medium. After 11 passages, cells were plated at similar density with or without LIF. The number of cells was determined during 24 days of culture. At day 12 of culture, samples of cells were tested for epitope analysis. (B) Epitope analysis. Each bar represents the ratio between the number of labelled cells in culture with LIF versus number of labelled cells in culture without LIF.
CEC contain a high telomerase activity
Telomerase activity is high in human immortalised and cancer cells and in mouse germ cells and ES cells (Kim et al., 1994; Prowse and Greider, 1995; Chadeneau et al., 1995; our unpublished data). To further characterise our CEC as putative ES cells, we tested their telomerase activity and compared it to the activity of the same cells taken 48 hours after retinoic-acid-induced differentiation. Normal CEF and carcinogen-transformed established quail fibroblasts QT6 (Moscovici et al., 1977) were used on controls. The data are presented in Fig. 5.

Telomerase activity was undetectable in CEF and very high in QT6 cells. In two different CEC cultures at several passages, telomerase activity was high. This activity totally disappeared in the same cultures treated with retinoic acid. These observations therefore provide an additional marker suggesting the occurrence of stem cells in the long-term CEC cultures maintained in conditions that prevent their differentiation.

In vitro differentiation of cultured CEC
Murine ES cells can be induced to differentiate in vitro into various lineages. One way is to remove LIF and to prevent the adhesion of ES cells to the culture dish. This procedure leads to the formation of embryonic bodies (EB) which can then be induced to attach again and to differentiate into various cell types including muscle, hematopoietic and nerve cells (Doetschman et al., 1985; Sanchez et al., 1991; Rohwedel et al., 1994; Fraichard et al., 1995).

To test whether CEC could differentiate into several lineages, we adapted this procedure to develop embryoid bodies from cultures at different passages (see Materials and Methods). When seeded in non-tissue culture dishes without LIF, CEC developed floating organised structures resembling embryoid bodies (Fig. 6). These EB-like structures could be maintained in these conditions for more than 10 days. Spontaneous differentiation occurred in these floating masses. By RT-PCR analysis (Fig. 7B), we could detect the expression of villin, an early endodermic marker (Ezzel et al., 1992; Maunoury et al., 1988). When transferred onto tissue-culture dishes, these EB-like structures attached and within a few days generated various cell phenotypes that were characterised with specific antibodies. As shown in Fig. 6C and D, some cells exhibited morphological features of nerve cells and were stained with antibodies against N-CAM. GFAP could also be detected in these conditions (not shown). Some other cells exhibited markers of muscle cells as revealed by staining with an anti-myosin antibody (Fig. 6G,H). Rhythmic contractile activity could also be observed confirming the development of functional muscle cells. Finally, immature hematopoietic cells were revealed by the expression of the antigenic marker MEP-21 (Fig. 6E,F) (Graf et al., 1992) and mature hemoglobinised erythrocytes were identified (data not shown). The presence of hemoglobin was corroborated by the detection by RT-PCR of mRNA for α, β and ε globins (Fig. 7A). All these cell types were identified from embryoid bodies derived from cultures maintained during at least 12 passages in the presence of LIF.

These results suggest that these cells were able to differentiate, at least in vitro, into endoderm, mesoderm and ectoderm lineages.

In vivo differentiation of cultured CEC
In order to test the morphogenetic potentialities of the cultured blastodermal cells, we injected these cells into recipient embryos. For these series of experiments, the cultured CEC originated from Barred Rock black strain embryos and were maintained routinely on STO feeder in ESA complete medium. Cells were collected from cultures after 1-3 passages. 100-500 cells were injected into the subgerminal cavity of irradiated stage X White Leghorn recipient embryos. Several of the grafted embryos hatched, some of which exhibited a chimeric plumage phenotype (Fig. 8). Regardless of the number of passages, more than 50% of the hatched recipient embryos were chimeras with nearly 33% of the plumage from donor phenotype. The extent of donor phenotype in the chimeric chickens did not change appreciably with the duration of culture. To date, among a high number of chimeras tested, we have obtained 2 chickens from 7-day-old culture, which gave rise to chicks of the donor-derived phenotype demonstrating a germ-line transmission. These data thus demonstrate that some of the cultured CEC are able to provide germ-line transmission. The ability of long-term cultures to give rise to chimeric animals is currently under investigation.

DISCUSSION
The purpose of this work was to set up culture conditions allowing the growth and characterisation of putative avian ES cells. Although the occurrence of totipotent cells in early avian embryos has been demonstrated, these cells have not yet been identified and maintained in culture. We were able to cultivate...
avian embryonic cells with biochemical and biological features reminiscent of those associated with ES cells in the mouse.

Murine ES (mES) cells are characterised by their round and small shape, their large nucleus, the presence of one or two prominent nucleoli and their small cytoplasm amount (Robertson, 1987). In addition, mES cells possess strong endogenous alkaline phosphatase activity similar to the EC-derived cells (Strickland et al., 1980). In our quail and chicken blastodermal long-term cultures, we could detect large colonies of small cells, tightly packed in nests with this typical “ES-like” morphological features and a high endogenous alkaline phosphatase activity. The conclusion that the endogenous alkaline phosphatase activity, observed in colonies of blastodermal cells, reflects ES-like properties was further supported by the presence of the ECMA-7 and SSEA-1 epitopes, known to be characteristic of non-differentiated murine ES cells (Kemler et al., 1981; Solter et al., 1978). The expression of ECMA-7 and SSEA-1 epitopes has not yet been reported on avian cells and strongly suggests that ES-like features are shared by cells derived from the inner cell mass of murine embryos and the blastoderm of avian embryos. This interspecies cross reactivity suggests that these epitopes fulfil highly conserved functions in embryonic progenitor cells. The presence of EMA-1 and EMA-6 epitopes (Hahnel and Eddy, 1986) on cultured mES cells and on chicken blastodermal cells suggests that these epitopes can also be used to characterise non-differentiated embryonic stem cells (Fig. 3E,F). As soon as mES cells enter differentiation, they rapidly lose their characteristic morphology, their endogenous alkaline phosphatase activity and expression of the ECMA-7, SSEA-1 and EMA-1 epitopes. In contrast, the epitope TROMA-1 is induced in these conditions (Brulet et al., 1980). The same changes in expression of all these epitopes were observed in the CEC cultures induced to differentiate either by removal of LIF or by treatment with retinoic acid. To exclude any contamination of our CEC cultures by mES cells, we checked the karyotype of our cells. All the examined metaphases showed the expected macrochromosomes, characteristic of chicken.

The long-term growth of alkaline-phosphatase-positive cells and of cells harbouring ES-cell-specific epitopes in the CEC cultures is dependent upon specific growth factors and cytokines like bFGF, SCF and (mostly) LIF. bFGF and SCF have been previously reported to stimulate proliferation and prevent differentiation of murine EG cells and PGC (Matsui et al., 1992; Donovan, 1994). The ability of this combination to promote proliferation and to prevent differentiation is reinforced by the addition of ARMA. LIF produced either under soluble or membrane-associated form is essential for the growth of murine ES (Rathgen et al., 1990). We show here that mammalian LIF is required to maintain cells with ES features in CEC cultures. The addition of IL-11, another member of the LIF family of cytokines, also facilitates the maintenance of ES-like properties of avian blastodermal cells. The effect of LIF and IL-11 on chicken blastodermal cells may be similar to that of CNTF, which is also a member of the LIF family of cytokines and which is also active on murine ES cells (Wolf et al., 1994; Conover et al., 1993). This observation further supports the assumption that our cultures maintain embryonic stem cells very similar to ES cells. It is interesting to see that a mammalian LIF can activate a signalling pathway in avian cells, which suggests that some molecular mediators of cytokine signal transduction are structurally conserved between species. This proposal is strengthened by the recent identification of a chicken membrane receptor for GPA (GPA R0), similar to the CNTF receptor (Heller et al., 1995).

The culture conditions, which included the use of mouse embryonic feeder cells and the inclusion of LIF, IL-11, SCF, bFGF, IGF-1 and ARMA in the medium, facilitated the proliferation of cells with an undifferentiated phenotype during more than 35 passages, i.e. more than 160 days. Life span of primary chicken cells in culture is usually limited to 30-40 generations and spontaneous immortalisation of these cells is extremely rare (Shay et al., 1991). These long-term cultures could be generated only with some strains of chickens at a frequency of more than 40% of the initial starting cultures.

![Fig. 6. Differentiation of CEC in culture. (A) CEC culture in the presence of LIF (scale: 10 μm); (B) embryoid body-like structures after 8 days (scale: 1.5 mm); (C,D) nerve cells in phase contrast and after staining with anti-N-CAM (scale: 1.5 mm); (E,F) hematopoietic cell colony in phase contrast and after staining with anti-MEP21 (scale, 20 μm); (G,H) muscle cells in phase contrast and after staining with anti-myosin (scale, 20 μm).](Image)
similar variation exists among mouse strains for the establishment of ES cell cultures (Kawase et al., 1994). Therefore, the culture conditions described above are likely to promote the growth of a specific kind of cells endowed with extended growth potential like ES cells. In this context, the high telomerase activity detected only in non-differentiated CEC suggests that we maintained in culture some cells with similar properties to mouse germ cells and ES cells (Kim et al., 1994; Prowse and Greider, 1995; Chadeneau et al., 1995; our unpublished data). The conclusion that the cultured CEC were undifferentiated cells was tested by providing an environment in which differentiation would be induced in vitro and in vivo. Differentiation of CEC in vitro was induced by preventing adhesion of blastodermal cells, conditions that promote formation of embryoid bodies and differentiation of cells into neural, muscular and hematopoietic lineages. Since these lineages are derived from the ectoderm and mesoderm, it can be concluded that the long-term CEC cultures contained precursors of both lineages. Moreover, detection of chicken villin mRNA in the EB-like structures suggest that cells derived from endoderm were also generated. Since the stage X embryo contains only the epiblast, it seems reasonable to conclude that cells derived in vitro from such embryo were multipotential. This conclusion was supported by the production of somatic chimeras when cultured CEC were injected into recipient embryos. Previously, similar chimeras have been produced after grafting fresh blastodermal cells and these chimeras contained donor-derived contribution to the ectoderm, mesoderm, endoderm and germ line (Kagami et al., 1995; Watanabe et al., 1992; Carsience et al., 1993; Petite et al., 1990; Thoraval et al., 1994). In the present work, the evidence for donor-derived contributions from cultured cells was observed for the ectoderm and the germ line in at least two chickens. Even though this germ-line contribution was detected at a low frequency, it demonstrates the ability of these cells to be the equivalent of the murine ES cells. The low frequency of the germ-line transmission observed so far might have several explanations. It is possible that the germ line has already differentiated within the stage X embryo and this view is supported by the presence of cells that exhibit the SSEA-1 and EMA-1 epitopes at this stage of development (Urven et al., 1988). Alternatively, we may imagine that germ-line precursors are lost preferentially from the culture. The karyotype of chicken blastodermal cells might have been impaired during the cultivation altering the ability of CEC to generate viable germ cells, as is often observed in murine ES cell clones. The polyclonal nature of grafted cells might also block the development of functional germ cells, a hypothesis strengthened by the observation that some chimeric hens were sterile. We have been unable so far to maintain clonal growth of CEC, which could suggest that some specific avian growth factors are necessary and produced by the blastodermal cells themselves. Work is in progress to improve the culture conditions allowing clonal selection of stem cells and further work is required to improve donor-derived cells so that they can enter readily and at a high frequency into the germ line.

In conclusion, we showed in this work that cells derived from stage X avian blastoderm exhibit a morphology and epitope profiles that are similar to those of murine ES and EG cells after extended periods in culture (Kagami et al., 1995; Watanabe et al., 1992; Carsience et al., 1993; Petite et al., 1990; Thoraval et al., 1994). In the present work, the evidence for donor-derived contributions from cultured cells was observed for the ectoderm and the germ line in at least two chickens. Even though this germ-line contribution was detected at a low frequency, it demonstrates the ability of these cells to be the equivalent of the murine ES cells. The low frequency of the germ-line transmission observed so far might have several explanations. It is possible that the germ line has already differentiated within the stage X embryo and this view is supported by the presence of cells that exhibit the SSEA-1 and EMA-1 epitopes at this stage of development (Urven et al., 1988). Alternatively, we may imagine that germ-line precursors are lost preferentially from the culture. The karyotype of chicken blastodermal cells might have been impaired during the cultivation altering the ability of CEC to generate viable germ cells, as is often observed in murine ES cell clones. The polyclonal nature of grafted cells might also block the development of functional germ cells, a hypothesis strengthened by the observation that some chimeric hens were sterile. We have been unable so far to maintain clonal growth of CEC, which could suggest that some specific avian growth factors are necessary and produced by the blastodermal cells themselves. Work is in progress to improve the culture conditions allowing clonal selection of stem cells and further work is required to improve donor-derived cells so that they can enter readily and at a high frequency into the germ line.

Fig. 7. RT-PCR analysis of globin and villin mRNAs. Total mRNA from CEF, EB-like structures, CEC culture and embryonic blood was tested by RT-PCR for chicken α globin, β globin and ε globin (A) and chicken villin (B). The chicken ribosomal protein S17 RNA was tested as control.

Fig. 8. Chimeric chickens generated after grafting of cultured CEC. (A) Control normal White Leghorn chick; (B-D) chimeric chicks derived from White Leghorn embryos grafted with Barred Rock CEC cultivated during 3 to 19 days.
eration and differentiation of chicken blastodermal cells have been established opening the way to isolation and utilisation of avian pluripotential embryonic stem cells.

We thank Dr A. Smith for reading the manuscript and for his comments. This work was supported by grants from ARC, INRA and the FITT program of Region Rhone Alpes to J. S., in part by the Ontario Ministry of Agriculture, Food and Rural Affairs and an Operating Grant from the Natural Sciences and Engineering Research Council of Canada for R. E. During the tenure of this work, R. E. held the visiting Chair Marcel Merieux at ENS de Lyon, supported by the Fondation Merieux.

REFERENCES


Matsudaira, P., T3R and RARs control commitment of mouse primordial germ cells defined by two monoclonal antibodies. Gamete Research 15, 25-34.


Matsudaira, P., T3R and RARs control commitment of mouse primordial germ cells defined by two monoclonal antibodies. Gamete Research 15, 25-34.


2348  B. Pain and others


(Accepted 24 May 1996)