

Isolation and Genetic Transformation of Primordial Germ Cell (PGC)-Derived Cells from Cattle, Goats, Rabbits and Rats

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ABSTRACT : At present embryonic stem (ES) cells with confirmed pluripotential properties are only available in the mouse. Recently, we were able to isolate, culture and genetically transform primordial germ cell (PGC)-derived cells from pig embryos and demonstrate their ability to contribute to chimera development in the pig. In order to determine whether the system we developed could be used to isolate embryonic germ (EG) cells from other mammalian species, we placed isolated PGCs from cattle, goats, rabbits and rats in culture. Briefly, PGCs were isolated from fetuses of cow (day 30-50), goat (day 25), rabbit (day 15-18) and rat (day 11-12), and plated on STO feeder cells in Dulbecco's modified Eagle's medium (DMEM): Ham's F10 medium (1:1) supplemented with 0.01 mM nonessential amino acids, 2 mM L-glutamine, 0.1 mM β -mercaptoethanol, soluble recombinant human stem cell factor (SCF; 40ng/ml), human basic fibroblast growth factor (bFGF; 20ng/ml) and human leukemia inhibitory factor (LIF; 20ng/ml). For maintenance of the cells, colonies were passed to fresh feeders every 7-10 days. In all species tested, we were able to obtain and maintain colonies with ES-like morphology. Their developmental potential was tested by alkaline phosphatase (AP) staining and *in vitro* differentiation assay. For genetic transformation, cells were electroporated with a construct containing the green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter. GFP-expressing colonies were detected in cattle, rabbits and rats. These results suggest that PGC-derived cells from cattle, goats, rabbits and rats can be isolated, cultured, and genetically transformed, and provide the basis for analyzing their developmental potential and their possible use for the precise genetic modification of these species. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 5 : 587-594*)

Key Words : Primordial Germ Cell, Embryonic Germ Cell, Alkaline Phosphatase Staining, Embryoid Body, Genetic Transformation, Green Fluorescent Protein

INTRODUCTION

The ability to introduce precise modification in the mouse genome by the use of homologous recombination in embryonic stem cells has greatly influenced the understanding of developmental biology and biomedical science. A critical element of these genetic modification systems is the availability of pluripotential embryonic cell lines that can be maintained *in vitro* for genetic manipulation without losing their ability to contribute to chimera formation.

In mice, the embryo-derived, embryonic stem (ES) cells are available (Evans and Kaufman, 1981; Martin, 1981). While, isolation of ES cells have been attempted in the rat (Iannaccone et al., 1994), mink (Sukoyan et al., 1993), rabbit (Giles et al., 1993), hamster (Doetschman et al., 1989; Piedrahita et al., 1990a), primate (Thomson et al., 1995), sheep (Piedrahita et al., 1990b; Handyside et al., 1987), cattle (Evans et al., 1990; Strelchenko and Stice, 1994; Stice et al., 1996), pig (Piedrahita et al., 1990b, 1990c; Notarianni et al., 1990; Gerfen and Wheeler, 1995; Talbot et al., 1993a; Moore and Piedrahita, 1997) and human (Shamblott et al., 1998), only in the mouse, have germline chimeras been produced (Bradley et al., 1984). An alternate source of pluripotent stem cells are embryonic germ (EG) cells derived from primordial germ cells (PGCs) of the developing mouse fetus. Mouse PGCs can be identified by the expression of tissue nonspecific alkaline phosphatase (TNAP) at 7.0 days post coitus (d.p.c.) (Ginsburg et al., 1990). The PGCs are first detected in the extraembryonic mesoderm and migrate towards the developing embryonic gonad over the next five days of embryonic development (Chiquoine, 1954). During migration, PGCs actively proliferate and increase in cell numbers about 300 fold (Tam and Snow, 1981). Matsui et al. (1992) and Resnick et al. (1992) reported that they could establish pluripotent cell lines from mouse PGCs and named them embryonic germ

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(EG) cells. Mouse EG cells exhibit all of the morphological characteristics of the pluripotent ES cells and were shown to express alkaline phosphatase (AP), a marker of the undifferentiated phenotype (Matsui et al., 1992; Resnick et al., 1992). Also mouse EG cells can be induced to differentiate *in vitro* (formation of embryoid body) as well as *in vivo* (teratocarcinoma development). More importantly, when introduced into host blastocysts, EG cells can also contribute to the formation of germline chimeras (Matsui et al., 1992; Stewart et al., 1994; Labosky et al., 1994).

Pig (Takagi et al., 1997), cattle (Lavoit et al., 1994), rabbit (Moens et al., 1997) and rat (Kemper and Peters, 1987) PGCs have been collected and characterized by their morphology and immunohistochemistry. We and others have previously showed successfully that porcine EG cell lines can be isolated (Piedrahita et al., 1998; Shim et al., 1997), genetically transformed, and contribute to chimera development in the pig (Piedrahita et al., 1998). Also the isolation of putative bovine EG cells has been reported (Cherny et al., 1994; Strelchenko, 1996). However, to date, genetic transformation of PGC or PGC-derived cells has not been reported in domestic animals other than the pig.

We attempted to demonstrate whether the system we developed for porcine EG cells could be used to isolate EG cells from other mammalian species. We isolated PGCs from cattle, goats, rabbits and rats, and determined their ability to develop into PGC-derived colonies with ES-like morphology, positive AP activity, and ability to differentiate *in vitro*. Additionally, experiments were carried out to demonstrate the wide applicability of the green fluorescent protein (GFP) as a marker gene for identification of transgenic PGCs from cattle, rabbits and rats.

MATERIALS AND METHODS

Isolation of PGCs

Fetuses were collected from day 30-50 slaughterhouse cows (determined by crown-rump length), day 25 goats, day 15-18 New Zealand White rabbits, day 11-12 Sprague-Dawley rats, and 9.5-12.5 days post coitus (d.p.c.) ICR mice. Urogenital ridges, which contain most of the PGCs, were collected from the fetuses and PGCs were isolated either by mechanical (cattle, goats) or enzymatic (rabbits, rats, mice) disruption of the tissue. First, isolated ridges were washed several times with PES medium prior to the collection of PGCs. The PES medium is comprised of Dulbecco's modified Eagle's medium (DMEM): Ham's F10 medium (1:1) supplemented with 0.01 mM nonessential amino acids, 2 mM L-glutamine, 0.1 mM β -mercaptoethanol (all media and supplements were

obtained from Gibco BRL, Gaithersburg, MD) and 15% fetal bovine serum (FBS; batches selected on the basis of their ability to maintain mouse ES cells, Sunnit Biotechnology, Fort Collins, CO). PGCs were then isolated either by incubation in 0.25% Trypsin/1 mM EDTA solution (Gibco BRL) followed by gentle pipetting, or by mechanical dissociation of the ridges into small fragments followed by gentle pipetting. After tissue disruption, cells were centrifuged for 3-5 min at 250 g to remove tissue debris. The supernatant containing most of the single cells was collected and centrifuged at 1,000 g for 5-10 min. The resulting pellet was resuspended with PES medium containing soluble recombinant human stem cell factor (SCF; 40 ng/ml), human basic fibroblast growth factor (bFGF; 20 ng/ml) and human leukemia inhibitory factor (LIF; 20 ng/ml) (all growth factors are from R&D Systems, Minneapolis, MN).

In vitro culture of PGCs and maintenance of PGC-derived colonies

Cell suspensions were plated on irradiated mouse STO feeders (3×10^6 cells per 35 mm well), prepared as previously described (Piedrahita et al., 1998) and cultured in a humidified environment of 5% CO₂ in air, 38°C. PGC-derived colonies with ES-like morphology after 7-10 days (cattle, goats, rabbits) or 4-6 days (rats, mice) of culture were passaged to fresh feeders for establishment of cell lines. PGC-derived colonies were dissociated with 0.25% Trypsin/1 mM EDTA (Gibco BRL) for 10 min (cattle, goats) or 0.05% Trypsin/0.5 mM EDTA for 5 min (rabbits, rats, mice). The state of differentiation was determined by morphology and by expression of alkaline phosphatase (AP). To freeze PGCs, after trypsinization, cells were resuspended with PES medium with 10% DMSO and froze at the rate of 1°C per minute to -70°C before storing them in liquid nitrogen. When thawing cells, cryovials were plunged into a 37°C water bath and shaken until the contents are completely thawed. Then, cells were washed with PES medium and placed in culture.

AP staining

AP activity was determined as described previously (Moore and Piedrahita, 1997). Briefly, culture plates were rinsed twice in PBS and fixed in 4% formaldehyde in PBS for 15 min at room temperature. Fixed cells were washed twice with PBS and stained in naphthol AS-MX phosphate (200 μ g/ml; Sigma) and Fast Red TR salt (1 mg/ml; Sigma) in 100 mM Tris buffer, pH 8.2 for 30 min at room temperature. Staining was terminated by washing cultures in PBS.

In vitro differentiation of PGC-derived colonies

PGC-derived colonies were tested for their ability

to differentiate *in vitro* using suspension culture, as well as cultured on plastic in the absence of feeders. PGC-derived colonies were detached from the plate by gentle trypsinization and cultured on nonadhesive bacterial plates with PES medium lacking growth factors and with calf serum (CS) instead of FBS. The medium was changed daily and the cultures were observed daily for signs of differentiation. The PGCs were also grown directly on plastic, in PES without growth factors, and observed daily for morphological changes.

Genetic transformation of PGCs

Freshly isolated and early passage PGC-derived colonies were transfected by electroporation as described previously (Piedrahita et al., 1998). Linearized DNA plasmid (5 nM) was mixed with PGC suspensions ($1-10 \times 10^5$) in PES medium and electroporated at 400 V, 250 μ FD (Farad; unit of capacitance) for cattle and goats, or 300 V, 250 μ FD for rabbits, rats and mice (Genepulser, BIO-RAD Laboratories, Hercules, CA). The plasmid contains the humanized green fluorescent protein (GFP) marker gene driven by the cytomegalovirus promoter (CMV) and the bovine growth hormone poly adenylation signal for stable mRNA (Piedrahita et al., 1998). Cells were plated on fresh feeder layers after electroporation and cultured as described previously. Following 7-10 days of culture, colonies were observed by fluorescence microscopy using FITC (Fluorescein isothiocyanate) filters for detection of transgenic colonies.

RESULTS

Isolation and characterization of PGC-derived cells

The morphology of freshly isolated PGC was characterized by microscopy. In all species, isolated PGCs showed morphology similar to that of mouse PGCs, such as large size (10-20 μ m) compared to surrounding somatic cells, high nuclear to cytoplasmic ratio, and a round shape. In all species except cattle, pseudopod from PGCs were detected. Also, in all species, freshly isolated PGCs stained for alkaline phosphatase activity.

After 7-10 days of culture (cattle, goats) or 4-6 days of culture (rabbits, rats), colonies with ES-like morphology developed. In all species, typical ES-like morphology was observed including a well-delineated colony composed of a multilayer of cells with large nuclei and prominent nucleoli (figures 1, 2, 3, 4). Also, in all species except the rat, colonies of irregular shape were observed (figure 3). Mouse EG cell colonies were more rounded and grape-like during primary culture but showed normal ES-like morphology in later passages (figure 4). The colonies were then trypsinized and passed onto fresh feeder

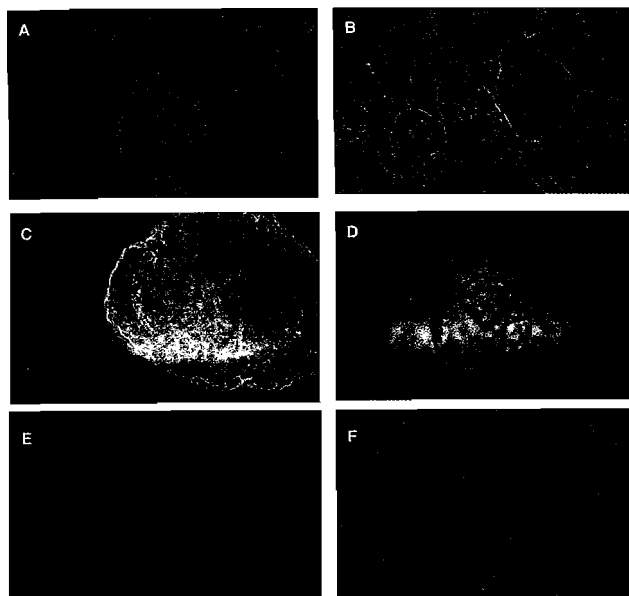


Figure 1. Cattle PGC-derived cells. PGCs isolated from the fetuses at 30-50 days of gestation were placed on STO feeder cells. Seven to ten days later, colonies with ES-like morphology were identified. Colonies were passaged at least twice before morphology and AP activity were detected. A), B) Cattle PGC-derived colonies; 200X. Notice morphology typical of ES cells with small cytoplasm, prominent nucleoli and distinct outline. C) Simple embryoid body after 5 days of suspension culture; 200 \times . Notice the clear demarcation between the differentiating endoderm (outer layer) and remaining undifferentiated colony. D) Cystic embryoid body showing the typical fluid filled cavity that develops in simple embryoid bodies after prolonged culture; 200 \times . E) Transgenic cattle PGC-derived colony under fluorescent light after electroporation with GFP expression plasmid and cultured on STO feeders for 7-10 day; 200 \times . F) Same colony as in E) under transmitted light; 200 \times . Notice the morphology is indistinguishable from that of non-transgenic undifferentiated colonies as in A) and B).

layers in 5-10 days intervals. The characteristic morphology was maintained with repeated passages but with each passage, the proportion of differentiating colonies tended to increase. Cell lines were maintained with characteristic morphology up to 8, 4, 9 and 11 passages for cattle, goats, rabbits and rats, respectively (table 1).

Morphological characteristics of the PGC-derived colonies were verified using AP activity, a marker of the undifferentiated phenotype (Talbot et al., 1993b). In a previous study, porcine PGC colonies were stained for AP and showed intense red staining in the cytoplasmic area (Piedrahita et al., 1998). Same results

Table 1. Characteristics of PGC-derived cells from cattle, goats, rabbits, rats and mice

Species	AP Staining ¹	Passage No.	Survival after freeze-thaw	<i>In vitro</i> differentiation	Genetic transformation
Cattle	+	8	Yes	SEB ³ , CEB ⁴	Yes
Goat	+	4	Yes	SEB	ND ²
Rabbit	+/-	9	Yes	SEB	Yes
Rat	+	11	Yes	None	Yes
Mouse	+	ND ²	Yes	CEB ⁵	Yes

¹ -=signal not detected, +/-=weak signal, +=signal present; ² ND=not determined; ³ SEB=simple embryoid body; ⁴ CEB= cystic embryoid body; ⁵ Matsui et al. (24)

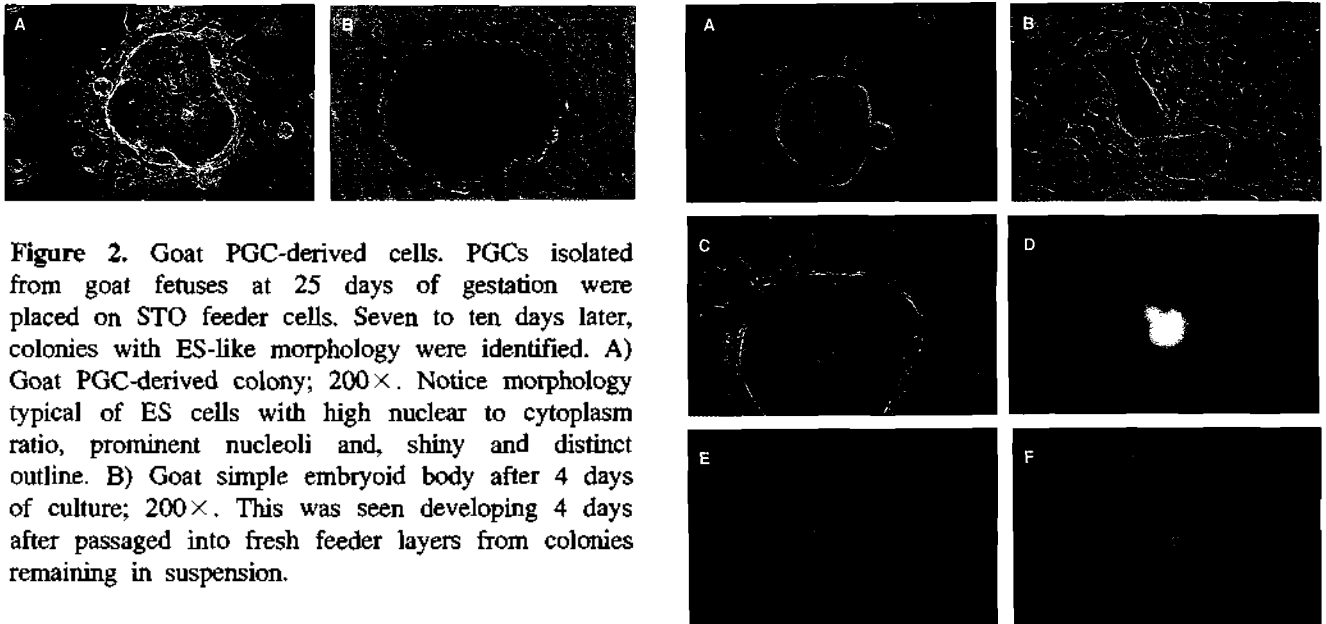


Figure 2. Goat PGC-derived cells. PGCs isolated from goat fetuses at 25 days of gestation were placed on STO feeder cells. Seven to ten days later, colonies with ES-like morphology were identified. A) Goat PGC-derived colony; 200 \times . Notice morphology typical of ES cells with high nuclear to cytoplasm ratio, prominent nucleoli and, shiny and distinct outline. B) Goat simple embryoid body after 4 days of culture; 200 \times . This was seen developing 4 days after passaged into fresh feeder layers from colonies remaining in suspension.

were obtained from PGC-derived colonies from all species (figure 5). The control mouse ES and EG cells were positive for AP staining (figure 5). They all stained with AP and exhibited the same pattern of staining as porcine PGC colonies. Although AP activity was found in all species, the intensity of the signal was drastically reduced in rabbit PGC-derived colonies. The AP activity decreased in differentiating colonies of all species.

In vitro differentiation of PGC-derived colonies

Pluripotency of PGC-derived colonies were tested using *in vitro* differentiation. When PGC-derived colonies were placed into suspension culture without growth factors and with calf serum instead of fetal bovine serum, they differentiated into simple embryoid bodies (EB) over a period of several days in all species except the rat (figures 1, 2, 3). Colonies rounded up and developed an outer layer of endoderm, the first indication of differentiation. The cystic embryoid bodies, EB with fluid-filled cavities, were not detected in any species except cattle. Also, in goats and rabbits, simple embryoid bodies were observed during culture at later passages. Differentiation

Figure 3. Rabbit PGC-derived cells. PGCs isolated from rabbit fetuses at 15-18 days of gestation were plated on STO feeder cells. After culture for 7-10 days, colonies with ES-like morphology were identified. A) and B) Rabbit PGC-derived colonies; 200 \times . Notice the typical ES morphology with small cytoplasm, large nucleus, prominent nucleoli and well-delineated outline. Also in B), irregular shaped colony was observed developing linear-like shape rather than typical tight circular shape. C) Rabbit simple embryoid body after 6 days of culture; 200 \times . D) Transgenic rabbit PGC-derived colony under fluorescent light after electroporation with GFP expression plasmid and plated on STO feeders for 7-10 days; 200 \times . Note that part of the cells in colony are expressing GFP, indicating that the colony likely originated from more than a single cell. E) Same colony as in D) under transmitted light; 200 \times . Notice the morphology is indistinguishable from that of non-transgenic undifferentiated colonies as in A) and B). F) Same colony as in D) under both transmitted and fluorescent light.

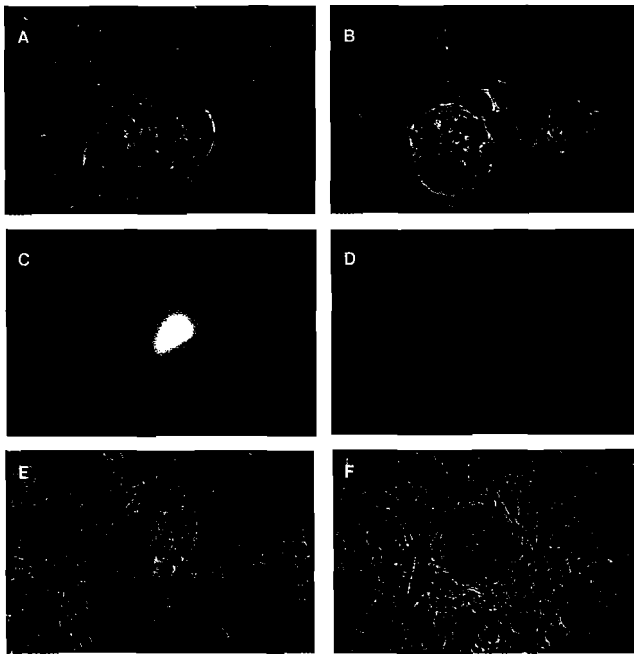


Figure 4. Rat and mouse PGC-derived cells. Rat and mouse PGCs isolated from the fetuses at 11-12 and 9.5-12.5 days of gestation, respectively, were plated on STO feeder cells. After culture for 4-6 days, colonies with ES-like morphology were identified. A), B) Rat PGC-derived colonies with typical morphology of ES cells; 200 \times . Notice the large nucleus, small cytoplasm, prominent nucleoli and well-delineated outline. C) Transgenic rat colony under fluorescent light after electroporation with GFP expression plasmid and cultured on STO feeders for 7-10 days; 200 \times . Notice that there are difference in expression of GFP level within the colony meaning that this colony is originated from at least two different transgenic cells. D) Same colony as in C) under transmitted light; 200 \times . E) Mouse PGC-derived cells after 4 days of primary culture isolated from 10.5 d.p.c. mouse; 200 \times . Notice the grape-like shape of colony. F) Typical mouse EG cells isolated from 9.5 d.p.c. mouse; 200 \times . Notice the same morphology as that of mouse ES cells.

was also tested by plating PGCs or PGC-derived colonies on plastic in the absence of STO feeder layers. PGCs attached to the plastic and differentiated into AP-negative epithelial- or fibroblast-like cells.

Genetic transformation of PGC-derived colonies

Freshly isolated PGC and early passage PGC-derived colonies were transfected with linearized construct (pGFP3; 32) containing the marker gene GFP driven by the CMV promoter. After electroporation, PGCs were cultured for 7-10 days prior to detection of transgene expression using fluorescence microscopy.

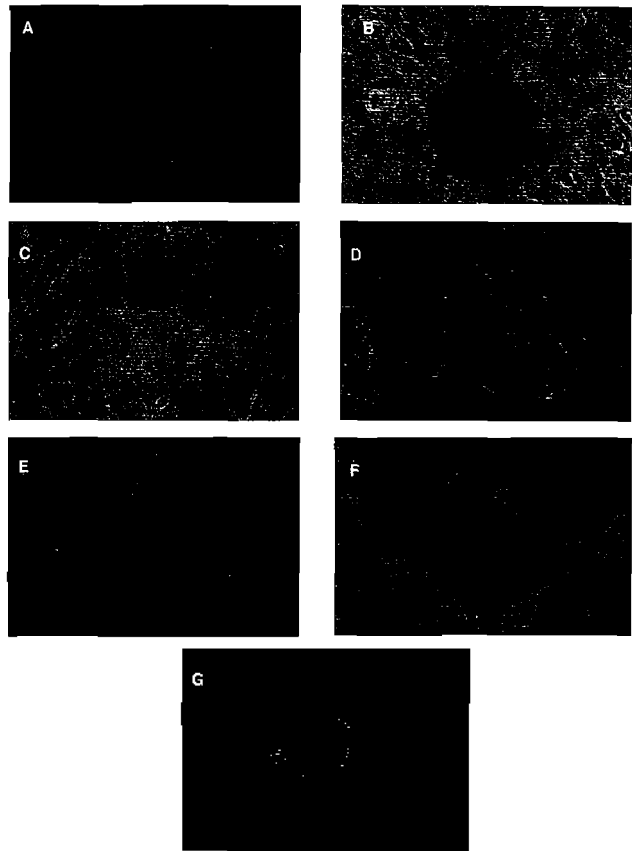


Figure 5. Alkaline phosphatase staining of PGC-derived colonies. PGC-derived colonies from cattle, goats, rabbits and rats are stained for alkaline phosphatase as described in text. A) Cattle PGC-derived colony. B) Goat PGC-derived colony. C), D) Rabbit PGC-derived colonies. Notice in D) that partial expression of AP even though the morphology of the colony is like typical undifferentiated ES cells. E) Rat PGC-derived colony. F) Mouse EG cells. G) Mouse ES cells.

Colonies with bright green fluorescence under the FITC filter were regarded as transgenic colonies and they were observed in all species tested (figures 1, 3, 4; transformation was not attempted in goats). Transgenic colonies varied in their intensity of expression as well as the pattern of expression. Most of the transgenic colonies were a mixture of GFP-positive and GFP-negative cells (figures 1, 3, 4).

DISCUSSION

Results reported here are the first demonstration of successful genetic transformation of PGCs from cattle, rabbits and rats. Also PGCs from these species can maintain their pluripotency *in vitro* as demonstrated by AP staining and *in vitro* differentiation ability.

The morphology of PGCs from all species tested showed common characteristics to those of mouse PGCs (Chiquoine, 1954). They exhibited a rounded shape, bigger size than surrounding somatic cells, a large nucleus and several prominent nucleoli. These characteristics were also detected in previous studies (pig: Takagi et al., 1997; cattle: Lavoit et al., 1994; rabbit: Moens et al., 1997; rat: Kemper and Petters, 1987). There are some species specific characteristics of PGCs, such as the cytoplasmic vesicles of cattle (Lavoit et al., 1994), dense vesicles in mouse and pig (Clark and Eddy, 1975; Pelliniemi, 1976) and lipid droplets of rabbit PGCs (Moens et al., 1997). One of the characteristics of migratory cells is the presence of pseudopods or cytoplasmic extrusion. Since mouse PGCs emerge from extraembryonic mesoderm and actively migrate through the hind gut and dorsal mesentery to the genital ridge, PGCs during migration show typical pseudopods (Chiquoine, 1954). In this study, goat, rabbit and rat PGCs showed typical pseudopods, meaning that these cells are migratory or have just colonized the genital ridge. However, pseudopods were not detected in PGCs from cattle and this may be due to the relatively old age of the fetuses, meaning that PGCs already completed their colonization of the genital ridge. In cattle, PGCs can be detected during days 35-55 (less than 5 cm crown-rump length). Bovine day 35 gonads are comparable to those of murine day 11.5. At this stage, germ cells have just arrived in the genital ridge (Lavoit et al., 1994). In rabbits, PGCs reach the genital ridge at day 14 and undergo two major waves of mitotic activities between day 16 and 18, and between day 22 and 26. Mitosis continues in rabbit germ cells throughout gestation and meiosis is delayed until birth (Moens et al., 1996). According to Kemper and Peters (1987), the characteristics of PGCs from rat are similar to those of mouse PGCs but with 1.5-2 day discrepancy. For example, rat PGCs from day 11-12 are at a similar stage as mouse PGCs from day 9.0-10.5. Alkaline phosphatase is a widely used marker of undifferentiated germ cells (Talbot et al., 1993b; Lawson and Hage, 1994). PGCs from all species tested stained with alkaline phosphatase right after isolation. Chretien (1968) reported that rabbit germ cells show no alkaline phosphatase activity. However, in this study, although the signal was reduced, rabbit PGCs isolated during day 15-18 stained for AP.

To culture PGCs *in vitro*, feeder cells, expressing membrane bound form of stem cell factor (SCF), are required (Donovan, 1994). Additionally, the growth factors SCF (soluble form) and LIF are also required for survival and maintenance of the undifferentiated state of the PGCs *in vitro* (Donovan, 1994). However, until successful isolation of embryonic germ (EG) cell lines from mouse PGCs using SCF, LIF and basic

fibroblast growth factor (bFGF) (Matsui et al., 1992; Resnick et al., 1992), PGCs could only be cultured for 4-5 days even with feeder layers, SCF and LIF. Thus, to proliferate indefinitely *in vitro*, bFGF is needed but the exact mechanism of bFGF action is not known yet. Interestingly, Shim et al. (1997) used only porcine LIF to isolate cell lines from pig PGCs and Cherny et al. (1994) used feeder cells expressing human LIF to isolate bovine EG cells. In our laboratory, pig PGCs were effectively cultured in the presence of mouse embryonic fibroblast feeder layers with PES medium supplemented with LIF, SCF and bFGF (Piedrahita et al., 1998). We used human recombinant form of growth factors as these factors are highly conserved among mammals (LIF; Willson et al., 1992, SCF; Zhang and Anthony, 1994, bFGF; Bechtner et al., 1993). This same system was effective for culturing PGCs from cattle, goats, rabbits and rats. The PGC-derived colonies showed typical ES-like morphology; tight, compact, well delineated colonies comprised of multiple layers of cells with large nuclei and prominent nucleoli. Additionally, PGC-derived colonies from all species were successfully passaged and survived after frozen-thaw, making isolation and manipulation of cell lines possible. Except for goat, all PGC-derived colonies maintained their typical morphology for more than 8 passages even though they had a tendency to increase the number of differentiating colonies. Cherny et al. (1994) maintained bovine PGC-derived cells and detected AP activity up to the 8th passage. Rabbit and rat PGCs have been cultured for only 24 and 48 hrs *in vitro*, respectively (Moens et al., 1997; Kemper and Petters, 1987). The successful application of pig PGC culture system to other mammalian species indicates that mammalian PGCs show common physiological and biochemical behavior *in vitro*, in addition to having common morphological characteristics.

One of the critical aspects of establishing embryonic cell lines is the pluripotency/totipotency of the cell. To demonstrate the developmental potential of the cell *in vitro*, the cells can be stained with markers of undifferentiated phenotype and/or induced differentiation *in vitro* using suspension culture. All PGCs tested stained positively with alkaline phosphatase, a marker of undifferentiated phenotype (Talbot et al., 1993b). However, in rabbit PGC-derived colonies, a reduced AP signal was observed, consistent with the results of AP staining of freshly isolated PGCs. Moreover, it was common to observe colonies with ES-like morphology those were AP-negative. As shown in figure 5 D), even within the same colony, and in spite of very similar morphology, some cells were AP-positive and some AP-negative. Thus, at this time we feel that AP staining in the rabbit may not be as good a marker as in other species tested.

Isolated PGC-derived colonies from cattle, goats and rabbits were differentiated into simple embryoid bodies with typical endodermal formation. However except in cattle, they showed limited formation of cystic embryoid bodies. This has also been observed in pig PGCs (Piedrahita et al., 1998). It is likely that the inability to form cystic embryoid bodies is not an indication of a lack of differentiation potential as we have been able to obtain chimeras with porcine EG cell line, unable to differentiate into cystic embryoid bodies in our differentiation system (Piedrahita et al., 1998).

Cattle, rabbit and rat PGCs were genetically transformed with GFP as a marker by electroporation. The parameters used for electroporation were chosen based on previous experiences. These parameters gave us best results in the pig (400 V, 250 μ FD) and the mouse (300 V, 250 μ FD), so we used the same parameters for cattle/goats and rabbits/rats/mice, respectively. Both freshly isolated and passaged PGCs from colonies were successfully transformed with marker gene. As reported previously (Piedrahita et al., 1998), a wide range in the level and pattern of expression of GFP was observed. GFP expression varied from non-detectable to intense. The differences in the level of expression may be due to 1) Non-clonal development of the colony. This means that colonies originate from more than one cell. This is a common characteristic of PGC grown in vitro. The reasons for this are that PGCs have a tendency to make a close contact with each other at the beginning of culture, and PGC colonies trypsinize into clumps of cells rather than single cells. 2) Transgene incorporation after one or more cell divisions - a common reason of mosaicism of pronuclear injection embryos. 3) Expression of the transgene by some cells but not others - positional effect of transgene expression. The promoter used to derive the GFP was CMV promoter. From the porcine PGC studies, it was shown that this promoter is heavily influenced by neighboring chromatin environments (Piedrahita et al., 1998). At present, trials are underway to identify a promoter which can maintain high levels of expression independent of chromosomal location. The marker gene used in this study was GFP which can be used in single cells and whose assay is less invasive. However, in order to show up over autofluorescence, it requires 10^5 to 10^6 GFP molecules per cell (Niswender et al., 1995).

The results presented here indicate that PGCs from cattle, goat, rabbit and rat can be isolated, cultured without loss of developmental potential, and genetically manipulated. Also, according to their morphology and behavior in culture, mammalian PGCs share common physiological and biochemical characteristics. Consequently, the availability of genetically manipulated PGC-derived cells will be of great benefit for the

introduction of precise genetic modification in these species.

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