BIOLOGY OF REPRODUCTION 57, 1089–1095 (1997)

Isolation of Pluripotent Stem Cells from Cultured Porcine Primordial Germ Cells[1]

Hosup Shim,[3] Alfonso Gutiérrez-Adán,[3] Lih-Ren Chen,[3] Robert H. BonDurant,[4] Esmail Behboodi,[3] and Gary B. Anderson[2,3]

Department of Animal Science[3] and Department of Population Health and Reproduction,[4] University of California, Davis, California 95616

ABSTRACT

Embryonic germ (EG) cells are undifferentiated stem cells isolated from cultured primordial germ cells (PGC). To date, EG cells have been isolated only in the mouse. Murine EG cells share several characteristics with embryonic stem (ES) cells, including morphology, pluripotency, and the capacity for germ-line transmission. We report here the isolation of porcine EG cells. PGC collected from Day 24 or 25 porcine embryos were cultured on mitotically inactivated murine fibroblasts. Four EG cell lines were isolated from repeated subculture of porcine PGC. Porcine EG cells morphologically resembled murine ES cells and consistently expressed alkaline phosphatase activity. These cell lines maintained a normal diploid karyotype and survived after cryopreservation. Porcine EG cells were capable of differentiating into a wide range of cell types in culture, including endodermal, trophoblast-like, epithelial-like, fibroblast-like, and neuron-like cells. In suspension culture, porcine EG cells formed embryoid bodies. When injected into host blastocysts, the EG cells were able to differentiate and contribute to tissues of a chimeric piglet. Both in vitro and in vivo evidence demonstrates that the isolated EG cells were pluripotent. These cells are potentially useful for genetic manipulation in pigs.

INTRODUCTION

Embryonic stem (ES) cells provide not only useful models for the study of embryonic development but also can serve as vehicles for germ-line transfer of foreign DNA. Such cells were first isolated from blastocyst-stage embryos in the mouse [1, 2]. ES cells remain undifferentiated in repeated subcultures, and under the appropriate culture conditions they can differentiate into a wide range of cell types. In vivo pluripotency, demonstrated by transmission of ES cell genotypes to chimeric offspring, has been reported in the mouse [3], rat [4], rabbit [5], and pig [6]. To date, only murine ES cells have been conclusively demonstrated to colonize the germ line of chimeras [3]. Transplantation of nuclei from cultured inner cell mass (ICM) cells into enucleated oocytes has produced embryos capable of developing to term after transfer to recipients [7]. Pluripotency of embryo-derived cells from cattle [8] and sheep [9] has been demonstrated by bovine conceptus development and birth of live lambs, respectively, after nuclear transfer.

Primordial germ cells (PGC) are embryonic cells that migrate from the root of the allantois to the genital ridge, where they ultimately give rise to gametes. PGC can be distinguished throughout PGC migration from surrounding somatic tissues by expression of alkaline phosphatase (AP) activity [10]. Pluripotent stem cells have been isolated from murine PGC [11, 12]. These cells are referred to as embryonic germ (EG) cells to distinguish them from undifferentiated stem cells of blastocyst origin. EG cells share several important characteristics with ES cells, including their morphology, pluripotency, and capacity to contribute to the germ line of chimeras when injected into blastocysts [13, 14].

Because of their potential use for targeted gene manipulation, isolation of ES cells in livestock species could have numerous agricultural and biomedical applications. Use of ES cell technology in livestock may overcome current limitations to efficient gene transfer by providing an abundance of pluripotent stem cells to be genetically manipulated by conventional recombinant DNA techniques [15]. However, progress toward establishment of ES cell lines from species other than the mouse has been slow [16]. Since EG cells are highly similar to ES cells in their characteristics, PGC may provide an alternative source of pluripotent stem cells. As compared with the situation for the conventional method for isolating ES cells from ICM cells, PGC are available in large numbers per embryo.

Bovine PGC have been collected from fetal ovaries and identified by morphology and histochemistry [17]. The isolation of putative bovine EG cells has also been reported [18]. These cells were capable of in vitro differentiation, and they displayed AP activity, a murine ES cell marker. When injected into the blastocoele of blastocysts, PGC-derived cells were incorporated into the ICM of the host blastocyst. Spontaneously aborted fetuses between 38 and 60 days in gestation were recently reported [19] after transfer of nuclei from putative bovine EG cells into enucleated oocytes. To date, only results of short-term culture of PGC are available in the pig. Under conventional culture conditions, porcine PGC were reported to survive barely more than 24 h [20].

In this study, the isolation and characterization of porcine EG cells are reported. Pluripotency of isolated EG cells was tested both in vitro and in vivo.

MATERIALS AND METHODS

Collection of PGC

Embryo donors were Hampshire × Yorkshire crossbred gilts (approximately 6 mo of age) prepared as described previously [19]. Animals were slaughtered on Day 24 or 25 of gestation, and embryos were dissected from the uteri. Genital ridges, if visible, were dissected from the embryos; otherwise, dorsal mesentery was removed [21]. Isolated tissues were washed once with PBS and incubated in 0.02% EDTA solution (Sigma Chemical Co., St. Louis, MO) for 20 min at room temperature. After incubation, PGC were dissociated by gentle disruption of the tissues using fine forceps. The suspension containing dissociated cells was collected and centrifuged at 800 × *g* for 5 min. The pellet was resuspended in PGC culture medium, Dulbecco's Modified Eagle's medium (DMEM) containing 15% fetal bovine

Accepted July 8, 1997.
Received March 3, 1997.
[1]This research was supported by USDA NRICGP Grant 94–37205–1029.
[2]Correspondence. FAX: (916) 752–0175; e-mail: gbanderson@ucdavis.edu

serum, L-glutamine (1 mM), MEM nonessential amino acids (0.1 M), 2-mercaptoethanol (10 μM), penicillin (100 U/ml), and streptomycin (0.5 mg/ml). A total of 174 embryos dissected from 17 gilts were used to collect PGC and ultimately to isolate EG cell lines.

AP Histochemistry

Prior to seeding of PGC onto feeder cells, samples of the cell suspension were assessed for presence of PGC using morphological criteria [22]. To confirm the morphological assessment, samples were stained for AP activity using an AP histochemistry kit (Sigma) according to the manufacturer's protocol. Putative EG cells were also tested for AP activity. Monolayers containing PGC-derived colonies and feeder cells were fixed with 80% ethanol [23] and stained for AP activity.

Feeder Cells

Feeder cells were prepared and maintained as described elsewhere [24]. Briefly, STO cells (subcultures of stock provided by Dr. G.R. Martin, University of California, San Francisco) were inactivated by incubation in medium containing 10 μg/ml of mitomycin C (Sigma) for 2 h. A day before PCG were seeded, inactivated STO cells were plated at a density of 5×10^4 cells per well in a 96-well plate (Falcon, Franklin Lakes, NJ) or at 2.5×10^5 cells per well in a 4-well multidish (Nunclon, Roskilde, Denmark).

Isolation of EG Cell Lines

For primary culture, cells collected from the embryos of the same gilt were pooled. The number of PGC equivalent to that collected from one embryo (approximately 15 000 PGC) was seeded per well of a 96-well plate containing feeder cells. Approximately 4–7 days after PCG were seeded, densely packed EG-like colonies obtained from primary culture were picked from the feeder layer and disaggregated in a microdrop of 0.25% trypsin-EDTA (Gibco BRL, Grand Island, NY) for 10–15 min at 39°C with the aid of a micropipette. The cells disaggregated from the colonies were seeded onto a fresh feeder layer in a 4-well multidish. Putative EG colonies were passed as described above for 1–3 passages until colonies reached more than 50% confluence. For further subculture, at 4- to 7-day intervals, the plates containing colonies and feeder cells were washed with PBS and treated with 0.25% trypsin-EDTA for 10–15 min at 39°C. Cells were removed from the plates and centrifuged at $800 \times g$ for 5 min, and resulting pellets were resuspended in PGC culture medium prior to plating onto a fresh feeder layer in a 4-well multidish. All cultures were maintained at 39°C in 5% CO_2:95% air with PGC culture medium changed every other day. EG cell lines were isolated and maintained in the medium with or without supplementation of porcine leukemia inhibitory factor (LIF; 1000 U/ml; a gift from Alexion Pharmaceuticals, New Haven, CT).

Cryopreservation

To ensure availability of EG cells for later use, cells were cryopreserved at each passage, beginning as early as passage 3. Porcine EG cells were frozen and thawed as described elsewhere [25].

Karyotypic Analysis

At passages 8–12, porcine EG cells in a 4-well multidish were cultured overnight in PGC culture medium containing 0.02 μg/ml colcemid (Gibco BRL) at 39°C in 5% CO_2:95% air. The cells were trypsinized and harvested as described above. The resulting pellet was resuspended in hypotonic solution (0.56% KCl in H_2O, w:v) and was incubated for 20 min at 20°C. The cells were pelleted by centrifugation at $800 \times g$ for 5 min at 20°C and fixed in cold Carnoy's fixative (3:1 vol of absolute methanol to glacial acetic acid) for 5 min. After another wash by centrifugation, the cells were resuspended in 0.5 ml of fixative. To prepare slides, the cell suspension was dropped onto microscope slides prewashed with fixative. After air drying, the slides were stained in Giemsa staining solution (Gibco BRL). The stained slides were rinsed with tap water, air dried, and observed at ×400 magnification with oil immersion. Approximately 10–20 metaphase spreads from each EG cell line were examined for the presence of structural and numerical abnormalities of chromosomes. Photographs were taken on Kodak (Eastman Kodak, Rochester, NY) Technical Pan B&W film, and karyotypes from each EG cell line were arranged as described elsewhere [26].

In Vitro Differentiation

To induce differentiation on a monolayer, EG cells were cultured for more than 2 wk without passage. To induce differentiation in suspension culture, EG cells were passed once as described above onto a 0.1% gelatin-coated plate to eliminate possible contamination by fibroblasts. After 4–7 days in culture, colonies were gently dislodged from the plate with the aid of a micropipette and were disaggregated by incubation in 0.25% trypsin-EDTA for 10–15 min at 39°C. Dissociated cells were cultured in a microdrop of PGC culture medium containing 0.3 μM retinoic acid (Sigma) on a 35-mm nonadhesive petridish (Falcon). Suspension cultures were monitored daily for embryoid body formation, with medium changed every other day.

Chimera Production

The ability of porcine EG cell lines to differentiate in vivo was tested by injection into host blastocysts. Duroc gilts (approximately 6 mo of age) artificially inseminated with mixed semen from 2 to 3 Duroc boars were used as host blastocyst donors. Estrus in blastocyst donors and recipient sows was induced as described previously [27]. Skin-pigmentation markers were used for preliminary identification of chimeric piglets. Porcine EG cell lines were isolated from embryos of Hampshire × Yorkshire crossbred pigs (black and white pigmentation; both are codominant alleles), and host blastocysts were Durocs (red pigmentation; recessive). Blastocysts were collected from the Duroc gilts 6 days after the first day of estrus. Porcine EG cell lines at passage 7–15 were used for injection. All cell lines used had been cryopreserved once or more prior to the blastocyst injection. Colonies of EG cells picked off the feeder layer were incubated in 0.25% trypsin-EDTA for 5–10 min and dissociated into small clumps containing approximately 10–20 cells each. One EG cell clump each was injected into the blastocoele of a host blastocyst as described previously [28]. Injected blastocysts were surgically transferred to the uteri of recipients on Day 4 of their estrous cycle (i.e., 2 days behind donor gilts). Host blastocysts were pooled across donors after injection with EG

cells to ensure a sufficient number of embryos to sustain pregnancy after transfer. At birth, piglets were examined for skin-pigmentation chimerism (i.e., black or white among red pigmentation).

DNA Marker

A piglet with overt skin-pigmentation chimerism was subjected to analysis of DNA from various tissues. Since host blastocysts collected from 6 gilts had been pooled for the embryo transfer that produced the skin-pigmentation chimera, parentage analysis was performed to identify the sire and dam of the host blastocyst that produced the skin-pigmentation chimera. After polymerase chain reaction (PCR) amplification of microsatellites (MS) S0036, S0099, SW157, and SW871 from blood samples of the skin-pigmentation chimera and its potential parents, the amplified fragments were analyzed using PAGE [29] (primers were provided by Dr. M.F. Rothschild, Iowa State University, Ames, IA). These MS were selected for analysis on the basis of their having a high degree of polymorphism and therefore a likelihood of being the most informative. Each MS was amplified individually. Five gilts and two boars could be excluded as possible parents of the Duroc host blastocyst on the basis of MS DNA incompatibilities. The remaining gilt and boar were genetically comparable with the Duroc host blastocyst and were identified as the parents. The presumptive chimera was killed 5 days after delivery, and tissue samples were excised from the brain, pituitary gland, lung, liver, heart, spleen, kidney, muscle, testis, epididymis, pancreas, intestine, thyroid gland, and skin. After washing of tissue samples with PBS, DNA was isolated from each tissue sample and analyzed by PCR amplification of MS SW871 as described above.

RESULTS

Isolation of EG Cell Lines

With or without supplementation of LIF in the culture medium, porcine EG cell lines were isolated and maintained in long-term culture (Table 1). Although PGC readily formed colonies resembling murine ES cells in primary culture, most colonies were lost prior to the fourth passage (Table 1). Four EG cell lines isolated from PGC of different gilts survived in long-term culture; one cell line survived for more than 29 passages. These cells proliferated indefinitely in repeated subculture carried out over a period of more than 6 mo.

The EG cell lines produced densely packed colonies similar to murine ES cells (Fig. 1A), but porcine EG colonies were flatter and more translucent than murine ES cells. Porcine EG cells did not contain lipid-like vacuoles, which often appear in both murine and porcine ES cells [30]. The size and shape of EG colonies varied, and individual EG cells were 5–15 μm in diameter, approximately a third the size of a STO feeder cell. As described for porcine ES cells [30], each porcine EG cell contained a large nucleus with prominent nucleoli and a relatively small amount of cytoplasm. One and three cell lines were isolated from PGC collected from the dorsal mesentery and genital ridge, respectively (Table 2). Among isolated EG cell lines, no obvious differences were observed in their morphology, proliferation, and AP activity. Porcine EG cells consistently expressed AP activity (Fig. 1B) whereas STO feeder cells did not. When the EG cells differentiated in vitro, they rapidly lost AP activity. After 8–12 passages, all four isolated EG cell lines had the normal porcine complement of 38 chromosomes (36 autosomes and 2 sex chromosomes). No obvious abnormalities were found in chromosomes from the isolated EG cells. Three cell lines possessed normal diploid male karyotypes, and one cell line had a normal diploid female karyotype (Table 2). After cryopreservation, EG cells survived and proliferated without overt changes in their characteristics.

TABLE 1. Progressive loss of porcine EG cell lines after several passages in medium with and without porcine LIF.

Growth factor supplemented	No. primary cultures*	No. cell lines surviving to passage				
		1	2	3	4	>14
None	10	9	7	6	3	3
Porcine LIF	7	5	3	2	1	1

* Each primary culture included PGC from pooled embryos of a different embryo donor.

In Vitro Differentiation

In prolonged culture without passage, porcine EG cells occasionally differentiated into several cell types. As evidenced by morphology, the EG cells gave rise to at least five differentiated phenotypes, including endodermal, trophoblast-like, epithelial-like, fibroblast-like, and neuron-like cells. The neuron-like cells had several long neurites that emerged from cell bodies (Fig. 1C) and often formed neural rosettes. The fibroblast-like cells grew rapidly and elongated in culture (Fig. 1D). They easily mixed with feeder cells and rapidly dominated undifferentiated stem cells in culture. The epithelial-like cells formed a monolayer of polygonal cells with visible borders between cells (Fig. 1E), whereas typical undifferentiated EG cells did not show distinct boundaries between cells. The trophoblast-like cells were occasionally found in loosely packed colonies in which individual cells were larger than EG cells (Fig. 1F). When EG colonies formed tent-like protrusions with multilayers, the colonies often resulted in formation of an endodermal layer at the boundaries of the colonies (Fig. 1G). AP expression was rapidly reduced with differentiation of EG cells. Differentiation could also be induced in suspension culture. After approximately 7 days in suspension culture, the EG cells formed simple embryoid bodies (Fig. 1H), each containing an outer layer of large endodermal cells separated from a core of undifferentiated stem cells.

TABLE 2. In vivo differentiation of porcine EG cell lines after injection into blastocysts.

	EG cell line			
	PEGC142*	PEGC273	PEGC367	PEGC62‡
Origin of PGC†	GR	GR	GR	DM
Karyotype (2n)	38,XY	38,XY	38,XY	38,XX
No. of embryos transferred	105	25	56	NT
No. of recipients	4	1	2	NT
No. of pregnant recipients	4	1	1	NT
No. of piglets born	20	10	11	NT
No. of chimeras born	1	0	0	NT

* Cell line was isolated with supplementation of porcine LIF in the culture medium.
† GR, genital ridge; DM, dorsal mesentery.
‡ Not tested.

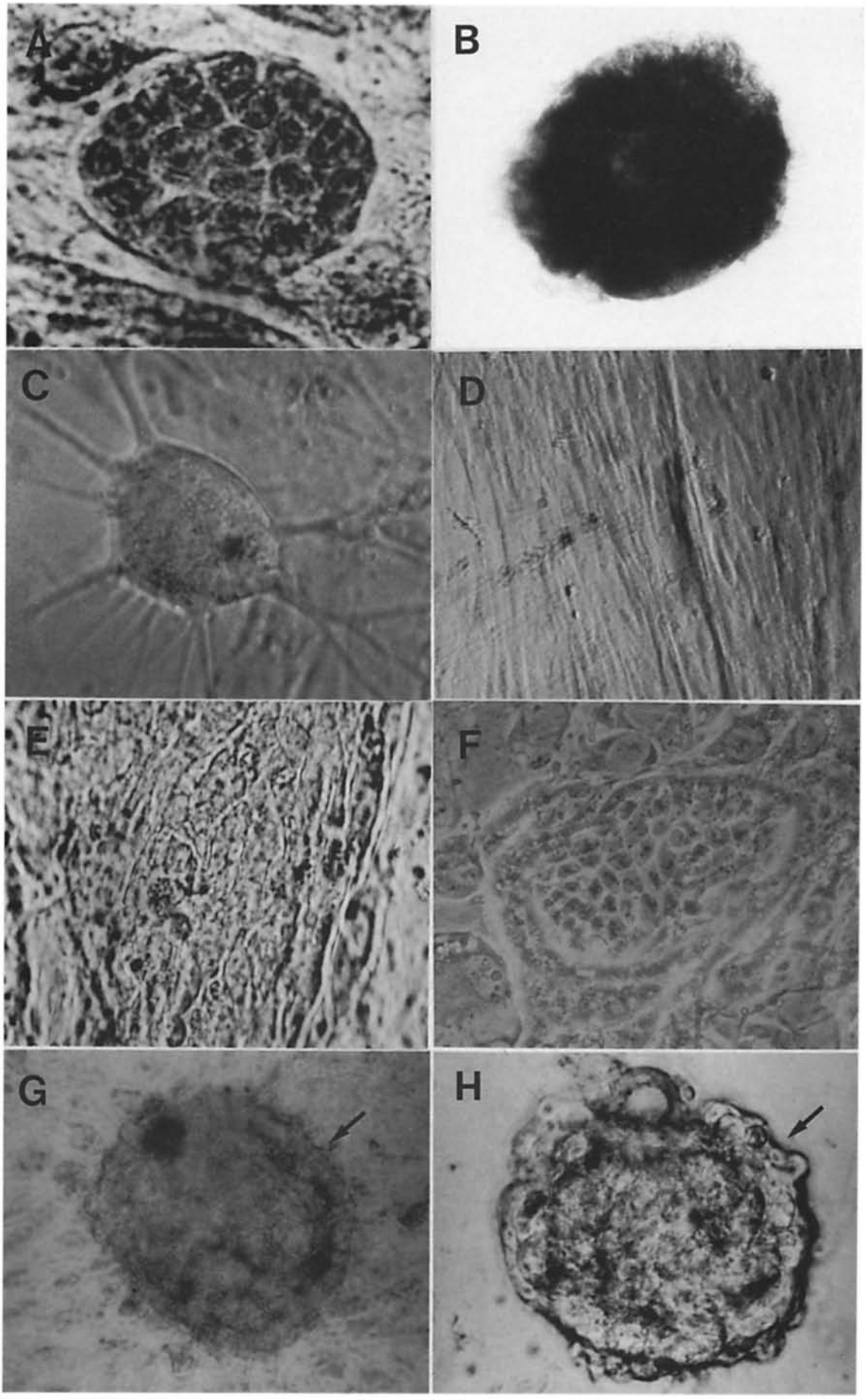

FIG. 1. Porcine EG cells. **A**) Colony of EG cells each with a large nucleus and prominent nucleoli. Stained with hematoxylin and eosin. ×400. **B**) AP histochemical staining of porcine EG cells. Dark stain represents AP activity of undifferentiated stem cells. ×200. **C–G**) In vitro differentiation of porcine EG cells. **C**) Neuron-like cells. Long neurites emerged from a cell body. ×200. **D**) Fibroblast-like cells elongated and mixed with feeder cells. ×100. **E**) Epithelial-like cells forming a monolayer of polygonal cells. ×200. **F**) Trophoblast-like cells. Colony was loosely packed, and individual cells were larger than EG cells. ×100. **G**) Endodermal cells. A layer of endodermal cuff (arrow) was differentiated at boundaries of the EG colony. ×100. **H**) Simple embryoid body formation induced by suspension culture of porcine EG cells. Note an outer layer of large endoderm cells (arrow). ×200. (Reproduced at 90%.)

Chimera Production

Porcine EG cell lines were tested for in vivo pluripotency by injection into blastocysts that were subsequently transferred to recipients for development to term. As shown in Table 2, a total of 186 host blastocysts were injected with EG cells from three cell lines and transferred to seven recipients. Six recipients were pregnant (pregnancy rate, 86%), and 41 piglets were born (embryo survival rate, 22%; 4 were dead at birth). One male (piglet 363) showed overt skin-pigmentation chimerism resulting from the injection of the crossbred EG cell line PEGC142 into a Duroc host blastocyst. White stripes derived from the EG cells were observed on the flank and back of the piglet and most prominently on the left hind leg (Fig. 2).

The chimeric piglet failed to thrive postnatally, and at 5 days of age it was killed for collection of tissue samples. At necropsy, several developmental abnormalities were observed, including a ventricular septal defect and shortened caudate vertebrae. MS profiles of parents of the host blastocyst, the EG cell line, and tissue samples from the skin-pigmentation chimera confirmed chimerism. A 120 base-pair allele of MS SW871 was present both in the injected EG cell line (PEGC142) and in various tissues from the skin-pigmentation chimera but was absent in parents of the host blastocyst (Fig. 3). Porcine EG cells contributed to tissues derived from all three germ layers, including blood, brain, pituitary gland, lung, kidney, muscle, testis, epididymis, pancreas, intestine, thyroid gland, and skin. Contributions of the EG cells to development of the heart, liver, and spleen were not detectable.

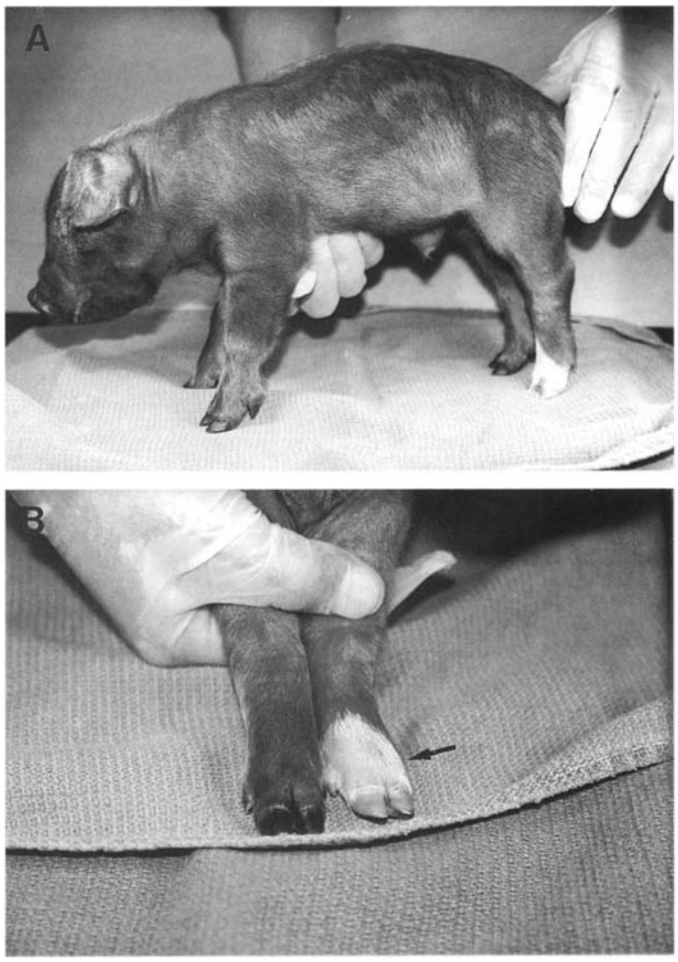

FIG. 2. Chimeric piglet (piglet 363) that developed from a Duroc blastocyst (red pigmentation) injected with Hampshire × Yorkshire (black and white pigmentation) EG cells. **A**) Chimeric male with white stripes interspersed among areas of red across flanks and back. **B**) White pigmentation derived from EG cells was most prominent on left hind leg (arrow).

DISCUSSION

LIF has been shown to be an essential growth factor for isolation and maintenance of murine ES cells [31]. STO cells produce LIF, but cell culture medium is often supple-

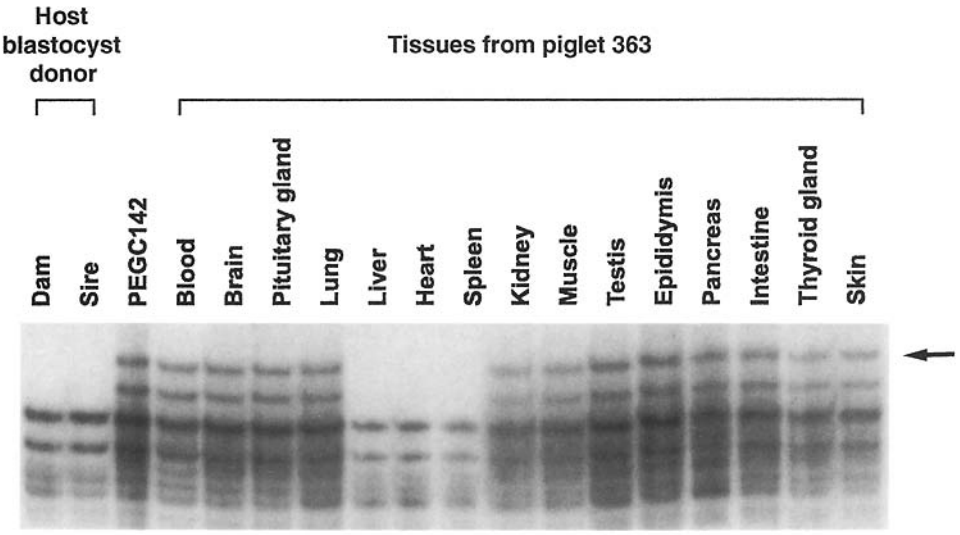

FIG. 3. PCR analysis of DNA from the parents of the Duroc host blastocyst, the EG cell line injected into the blastocyst, and tissues of piglet 363 with skin-pigmentation chimerism. A 120-bp allele (arrow) of MS SW871 was present both in the injected EG cell line PEGC142 and in various tissues of piglet 363 but absent in parents of the host blastocysts. MS profile of the piglet 363 confirms observations of overt pigmentation chimerism.

mented with additional LIF when ES cells are isolated and passed [32]. Moreover, murine EG cells have been successfully isolated only in instances in which PGC were cultured with a combination of three growth factors, including LIF, stem cell factor, and basic fibroblast growth factor [11, 12]. These three growth factors have also been used to establish bovine PGC-derived cell lines [19]. However, cell lines have also been isolated by culturing bovine PGC over a feeder layer of either murine or bovine fibroblasts without growth factor supplementation [18]. Porcine PGC can proliferate in short-term culture on STO feeder layers without supplementation of growth factors in the culture medium (unpublished results). In this long-term culture experiment, a feeder layer of STO cells provided sufficient support to allow isolation and maintenance of porcine EG cell lines. It remains to be determined whether these and other growth factors have a long-term effect on efficiency in isolation of EG cells, on contribution of EG cells to normal in vivo differentiation, and on colonization of the germ line by EG cells.

Markers for pluripotent cells are often useful to identify stem cells in culture. Expression of AP has been demonstrated in ES and ES-like cells in the mouse [33, 34], rat [35], pig [36], and cow [37]. AP activity has also been detected in murine PGC [11], murine EG cells [12, 13], and porcine PGC (unpublished results). In the present study, AP activity was consistently expressed in primary cultures and subcultures of EG cells. With in vitro differentiation of EG cells, AP activity was rapidly lost. In conjunction with morphological evaluation of EG cell colonies, AP expression was a convenient marker to identify undifferentiated stem cells in culture.

A significant proportion of newly established ES cells has been reported not to contain a normal diploid karyotype [32], a phenomenon associated with their rapid proliferation in culture. For germ-line genetic manipulation using ES cells (e.g., gene targeting), normal diploid cell lines (preferably male) are required. As evidenced by Giemsa staining and karyotypic analysis, all porcine EG cell lines in this study contained a normal diploid karyotype, and three of four cell lines were male.

Murine ES cells are capable of differentiating in vitro into multiple cell types, including skeletal muscle-, cardiac-, neuron-, and hematopoietic-like cells [2, 38, 39]. Cell lines derived from epiblast showed in vitro differentiation into a variety of cell types in the cow [37], pig [36, 40], and sheep [36]. In this study, porcine EG cells were capable of in vitro differentiation into various cell types. Formation of simple embryoid bodies with endodermal differentiation was also induced from porcine EG cells. The capacity for in vitro differentiation demonstrated by EG cells suggested that they were pluripotent.

One male piglet was confirmed by skin pigmentation and DNA analysis to be a chimera from injection of porcine EG cells into blastocysts. An association between observed developmental abnormalities and contribution of EG cells could be neither confirmed nor excluded. Congenital cardiac malformations have been observed in natural swine populations at a frequency of 4.35%; 7% of these were ventricular septal defects as seen in the chimera described here [41]. Occasional abnormalities including stunted growth, skeletal abnormalities, and sterility in chimeras derived from murine EG cells have been reported [11]. Varying degrees of chimerism observed among organs, and at times lack of chimerism in some organs, have been described in chimeric mice [42], rabbits [43], and pigs [44]. Pigmentation chimerism was not extensive throughout the body, but in vivo pluripotency of porcine EG cells was clearly demonstrated by DNA analysis. Descendants of EG cells were incorporated into several tissues derived from all three germ layers, including ectoderm (brain and skin), mesoderm (skeletal muscle, kidney, testis, and epididymis) and endoderm (lung, liver, and intestine).

The efficiency of producing chimeras appeared to be low on the basis of expression of the pigmentation markers (Table 2). Injection of fresh ICM cells into blastocysts has given rise to 10–11% chimeric pigs among offspring [27, 44], but a low efficiency (5% and 2.5%, respectively) of producing chimeras was observed after injection of rabbit ES cells [5] and PGC [43] into blastocysts. In mice, efficiency of producing chimeras and frequency of germ-line transmission vary among strains from which ES cells are derived [45]. The incidence and degree of chimerism vary among EG cell lines in the mouse [13]. It is not yet clear whether the low frequency of chimerism was due to developmental potency of porcine EG cells in general or of the particular cell lines tested, to challenges of manipulation and transfer of embryos in large animal species, or simply to the relatively smaller number of embryo transfers that is frequently feasible in large animal species as compared to mice.

In this study, porcine EG cells were successfully isolated and maintained in long-term culture. These cells demonstrated many important features of pluripotent stem cells, including AP activity, capacity of in vitro differentiation, and production of chimeric offspring with EG cells contributing to various cell lineages. This report is the first to document isolation of EG cells from a species other than the mouse. Further research is required to enhance the efficiency of chimera production and to document germ-line transmission of EG cells, but results of this study provide an important step toward targeted modification of the porcine genome.

REFERENCES

1. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981; 292:154–156.
2. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned to teratocarcinoma stem cells. Proc Natl Acad Sci USA 1981; 78:7634–7638.
3. Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germline chimeras from embryo-derived teratocarcinoma cell lines. Nature 1984; 309:255–256.
4. Iannaccone PM, Taborn GU, Garton RL, Caplice MD, Brenin DR. Pluripotent embryonic stem cells from the rat are capable of producing chimeras. Dev Biol 1994; 163:288–292.
5. Schoonjans L, Albright GM, LI J, Collen D, Moreadith RW. Pluripotential rabbit embryonic stem (ES) cells are capable of forming overt coat color chimeras following injection into blastocysts. Mol Reprod Dev 1996; 45:439–443.
6. Wheeler MB. Development and validation of swine embryonic stem cells: a review. Reprod Fertil Dev 1994; 6:563–568.
7. Sims M, First NL. Production of calves by transfer of nuclei from cultured inner cell mass cells. Proc Natl Acad Sci USA 1993; 90: 6143–6147.
8. Stice SL, Strelchenko NS, Keefer CL, Matthews L. Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. Biol Reprod 1996; 54:100–110.
9. Campbell KHS, McWhir J, Ritchle WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996; 380:64–66.
10. Chiquoine AD. The identification, origin, and migration of the primordial germ cells. Anat Rec 1954; 118:135–146.
11. Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 1992; 70:841–847.

12. Resnick JL, Bixter LS, Cheng L, Donovan PJ. Long-term proliferation of mouse primordial germ cells in culture. Nature 1992; 359:550–551.
13. Stewart CL, Gadi I, Bhatt H. Stem cells from primordial germ cells can reenter the germ line. Dev Biol 1994; 161:626–628.
14. Labosky PA, Barlow DP, Hogan BLM. Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development 1994; 120:3197–3204.
15. Seamark RF. Progress and emerging problems in livestock transgenesis: a summary perspective. Reprod Fertil Dev 1994; 6:653–657.
16. Stice SL, Strelchenko NS. Domestic animal embryonic stem cells: progress toward germ-line contribution. In: Miller RH, Pursel VG, Norman HD (eds.), Biotechnology's Role in the Genetic Improvement of Farm Animals. Beltsville Symposia in Agricultural Research, Savoy: American Society of Animal Science; 1996: 189–201.
17. Lavoir MC, Basrur PK, Betteridge KJ. Isolation and identification of germ cells from fetal bovine ovaries. Mol Reprod Dev 1994; 37:413–424.
18. Cherny RA, Stokes TM, Merei J, Lom L, Brandon MR, Williams L. Strategies for the isolation and characterization of bovine embryonic stem cells. Reprod Fertil Dev 1994; 6:569–575.
19. Strelchenko N. Bovine pluripotent stem cells. Theriogenology 1996; 45:131–140.
20. Leichthammer F, Baunack E, Brem G. Behavior of living primordial germ cells of livestock in vitro. Theriogenology 1990; 33:1221–1230.
21. Cooke JE, Godin I, Ffrench-Constant C, Heasman J, Wylie CC. Culture and manipulation of primordial germ cells. Methods Enzymol 1993; 225:37–58.
22. De Felici M, McLaren A. Isolation of mouse primordial germ cells. Exp Cell Res 1982; 142:476–482.
23. Buehr M, McLaren A. Isolation and culture of primordial germ cells. Methods Enzymol 1993; 225:58–77.
24. Hogan B, Beddington R, Costantini F, Lacy E. Manipulating the Mouse Embryo: A Laboratory Manual, 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1994: 253–290.
25. Robertson EJ. Embryo-derived stem cell lines. In: Robertson EJ (ed.), Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. Oxford: IRL Press; 1987: 71–112.
26. Eldridge FE. Cytogenetics of Livestock. Westport: AVI Publishing Company; 1985: 219–242.
27. Anderson GB, Choi SJ, BonDurant RH. Survival of porcine inner cell masses in culture and after injection into blastocysts. Theriogenology 1994; 42:204–212.
28. Butler JE, Anderson GB, BonDurant RH, Pashen RL, Penedo MCT. Production of ovine chimeras by inner cell mass transplantation. J Anim Sci 1987; 65:317–324.
29. Rohrer GA, Alexander LJ, Keele JW, Smith TP, Beattie CW. A microsatellite linkage map of the porcine genome. Genetics 1994; 136: 231–245.
30. Gerfen RW, Wheeler MB. Isolation of embryonic cell-lines from porcine blastocysts. Anim Biotech 1995; 6:1–14.
31. Williams RL, Hilton DJ, Pease S, Wilson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 1988; 336:684–687.
32. Abbondanzo SJ, Gadi I, Stewart CL. Derivation of embryonic stem cell lines. Methods Enzymol 1993; 225:803–823.
33. Wobus AM, Holzhausen H, Jakel P, Schoneich J. Characterization of a pluripotent stem cell line derived from a mouse embryo. Exp Cell Res 1984; 152:212–219.
34. Pease S, Braghetta P, Gearing D, Grail D, Williams RL. Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). Dev Biol 1990; 141:344–352.
35. Ouhibi N, Sullivan NF, English J, Colledge WH, Evans MJ, Clarke NJ. Initial culture behaviour of rat blastocysts on selected feeder cell lines. Mol Reprod Dev 1995; 40:311–324.
36. Talbot NC, Rexroad CE, Purcel VG, Powell AM. Alkaline phosphatase staining of pig and sheep epiblast cells in culture. Mol Reprod Dev 1993; 36:139–147.
37. Talbot NC, Powell AM, Rexroad CE. In vitro pluripotency of epiblasts derived from bovine blastocysts. Mol Reprod Dev 1995; 42:35–52.
38. Smith AG, Hooper ML. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol 1987; 121:1–9.
39. Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. Embryonic stem cells express neural properties in vitro. Dev Biol 1995; 168:342–357.
40. Talbot NC, Rexroad CE, Purcel VG, Powell AM, Nel ND. Culturing the epiblast cells of the pig blastocyst. In Vitro Cell Dev Biol 1993; 29A:543–554.
41. Hsu FS, Du SJ. Congenital heart diseases in swine. Vet Pathol 1982; 19:676–686.
42. McLaren A. Mammalian Chimaeras. Cambridge: Cambridge University Press; 1976: 105–117.
43. Moens A, Betteridge KJ, Brunet A, Renard JP. Low levels of chimerism in rabbit fetuses produced from preimplantation embryos microinjected with fetal gonadal cells. Mol Reprod Dev 1996; 43:38–46.
44. Onishi A, Takeda K, Komatsu M, Akita T, Kojima T. Production of chimeric pigs and the analysis of chimerism using mitochondrial deoxyribonucleic acid as a cell marker. Biol Reprod 1994; 51:1069–1075.
45. Stewart CL. Production of chimeras between embryonic stem cells and embryos. Methods Enzymol 1993; 225:823–855.