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Short Contribution

Incorporation of cultured embryonic cells into transgenic and chimeric, porcine fetuses

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ABSTRACT The derivation of murine, embryonic stem cells, and their use in the generation of transgenic mice, are well-established procedures. Application of these methodologies to non-murine species, however, remains to be fully realized. Non-murine embryonic stem cells would be of considerable value to studies in comparative development, and would raise important implications for agriculture and biotechnology. Here we report the achievement of chimerism and transgenesis in the domestic pig *(Sus scrofa)* at a fetal stage by the embryonic stem-cell methodology, using an embryonic cell line which previously we described as having the capacity to differentiate *in vitro*. This entailed: transformation, by electroporation, of a cell line, PE1, with the bacterial gene for *neomycin*-resistance; re-introduction of a polyclonal population of *neo*-PE1 cells into host blastocysts, followed by transfer of reconstructed embryos into a pseudopregnant recipient; and subsequent derivation of transgenic and chimeric porcine fetuses, as determined by two independent molecular assays of fetal genomic DNA. In the first instance, chimerism was revealed in one fetus by the presence of the transgene, as detected by Southern blotting; and in the second instance, in that and another fetus by the presence of supernumerary alleles for the class II major histocompatibility locus, *SLA-DQB*C*, by single-strand conformation polymorphic analysis. The contribution of *neo*-PE1 cells to the first chimeric and transgenic fetus was approximately 25%, and to the second chimeric fetus, below the level of detection by Southern blotting (i.e. less than 10%). The results indicate that, at the time of embryo reconstitution, a proportion of *neo*-PE1 cells were pluripotent and of the primary ectodermal lineage.

KEY WORDS: *cell culture, chimerism, domestic pig, embryonic stem cells, transgenesis*

Murine ES cells (Evans and Kaufman, 1981; Martin, 1981) offer a direct route for the introduction of mutations into the germ line owing to their capacities to be maintained *in vitro* during strategies for genetic manipulation, and to contribute specifically to the germ cells (Bradley *et al.*, 1984; Gossler *et al.*, 1986). Using this technology, great advances have been made in molecular and somatic-cell genetics of the mouse (Robertson, 1991). Although the isolation of stable, ES-like cell lines has been described for diverse species (reviewed by Notarianni, 1996), the derivation of proven, germ-line competent, non-murine ES cells remains a desirable goal. Reports have been made of the generation of chimeric animals using certain rat (Iannaccone *et al.*, 1994) and porcine (Wheeler, 1994) cell lines; however, the germ-line transmission of the genomes of any non-murine ES cells, and the subsequent derivation of transgenic chimeras or offspring, have not been demonstrated. Equally, the derivation and validation of non-murine homologs of EG cells, the pluripotent stem cells from primordial germ cells, has yet to be achieved (Cherny *et al.*, 1994).

We have previously described the establishment, from normal, pre-implantation porcine embryos, of cell lines having the capacity for morphological differentiation *in vitro* (Notarianni *et al.*, 1990). Our objective therefore was to test the pluripotency of one such cell line, PE1 (Fig. 1), *in vivo* by assaying at a fetal stage for its ability to chimerize host embryos. To this end a genetic marker, the bacterial *neomycin*-resistance gene, *neo*, was introduced into the cell line by electroporation with the plasmid, pSV2-*neo* (Fig. 2A), so that chimerism and transgenesis in fetuses would be revealed by the presence of *neo* sequences in the genomic DNA. Southern analysis of genomic DNA extracted from the pool of G418-resistant, *neo*-PE1 cells (Fig. 2B, lanes 1 and 2) revealed that at least three integration events had occurred for the *neo* gene, as three distinct Eco RI fragments were detected, 10, 7.8 and 6 kb in size (lane 2). (There are no Eco RI sites within the linearized molecule of pSV2-*neo*; Fig. 2A).

Abbreviations used in this paper: ES, embryonic stem; ICM, inner cell mass; *neo*, *neomycin*-resistance gene.

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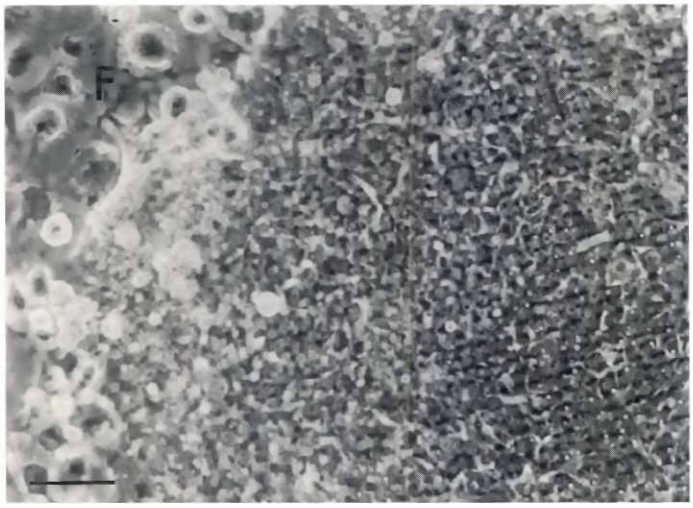

Fig. 1. Morphology of porcine embryonic cells, *neo*-PE1, grown in the presence of feeder layers of mitomycin-C-inactivated STO cells. *Feeder cells are labeled 'F'. Bar, 100 μm. The porcine embryonic cell line, PE1, was isolated and cultured from an 8-day-old blastocyst, as described previously (Notarianni et al., 1990), and utilized for electroporation and blastocyst injections at low passage generations (numbers 17 and 20, or 34 and 40 cell-doublings, respectively). The PE1 cell line has a normal, diploid complement of 38 chromosomes, and is female.*

To assess the capacity of the *neo*-PE1 cell line to differentiate *in vivo*, cells were introduced by microinjection into the blastocoel cavities of seven host blastocysts. The reconstructed embryos, together with two uninjected blastocysts to act as carriers to maintain the pregnancy (Polge *et al.*, 1996), were transferred to a non-pregnant recipient. A litter of five normal fetuses was retrieved from the recipient at 30 d of pregnancy.

For analysis of chimerism in the porcine fetuses, genomic DNA was digested with Eco RI and Pst I, and probed by Southern analysis for the presence of *neo* sequences. Specifically, a probe was used which maps within the *neo* coding sequences (Fig. 2A), and which would detect two fragments 2.2 kb and 923 bp in size in Pst-I-digested DNA containing the *neo* gene (Fig. 2B, lane 15). The DNA of the third fetus showed hybridization to a 6 kb Eco RI fragment at an estimated level of 0.25 copies per cell (Fig. 2B, lane 8). The 2.2 kb and 923 bp Pst I fragments were also detected (lane 7), confirming the presence of *neo* sequences in the DNA. This result indicates that the fetus had been colonized by one or more cells in which the *neo* gene was integrated, and therefore was transgenic and chimeric. It is also inferred that the original pool of G418-resistant cells was polyclonal with respect to the integration of *neo* sequences, consisting of at least two distinct populations, one of which carried a single integration event and was incorporated into the chimeric fetus.

Weak hybridization was detected also in the Eco RI-digested DNA of fetuses four and five (lanes 10 and 12) after prolonged exposure of the autoradiogram (not shown). No hybridization was detected in DNA from the pseudopregnant recipient (lanes 13 and 14).

An additional, independent marker system was sought to corroborate chimerism in the fetal tissues. Conventional analysis for chimerism by PCR amplification of hypervariable microsatellites (Delhaise *et al.*, 1993) using available sequences proved uninformative for genomic DNA from *neo*-PE1 cells and the litter of 5 fetuses, reflecting the high degree of inbreeding in the line of pigs used in these experiments. Therefore we developed an alternative PCR-based system for the analysis of chimerism in the domestic pig based on polymorphisms in the MHC Class II *SLA-DQB*C* allele (Shia *et al.*, 1995). Specifically, we analyzed an 181 bp sequence from within exon 2 (Hoelzel *et al.*, in preparation): nucleotide sequence polymorphisms were detected by mobility shift analysis of single-stranded DNA following non-denaturing polyacrylamide gel electrophoresis. Single-strand conformation

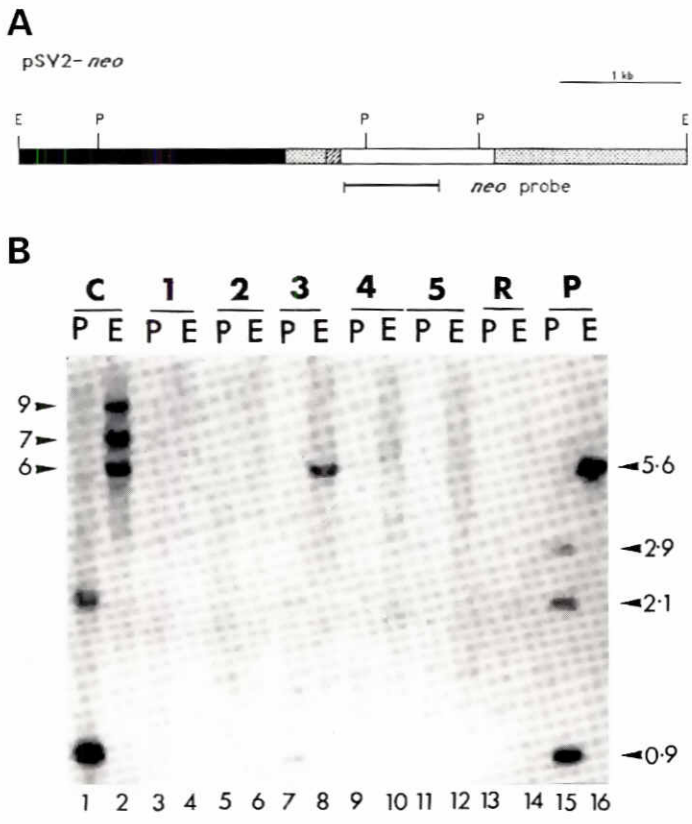

Fig. 2. Detection of chimerism by Southern blotting. (A) *Description of the Eco RI-linearized sequences of pSV2-neo (Southern and Berg, 1982). The plasmid (5.7 kb) consists of, from left to right in the diagram: a 2.3 kb, Eco RI-Pvu II fragment of pBR322 (solid bar); a 340 bp, Pvu II-Hind III fragment of SV40 (stippled bar) with the viral origin of replication and early promoter (map units 0.71-0.65); a 120 bp, Hind III-Bgl II fragment from the plasmid, pSV2-gpt (hatched bar), from which this vector was constructed; a 1.3 kb, Bgl II-Bam HI fragment containing the neo gene (open bar); and a 1.6 kb, Bgl II-Eco RI fragment of pSV2-gpt (stippled bar), with the t-antigen splice sites and polyadenylation signal of SV40. Eco RI (E) and Pst I (P) restriction sites, and the 787 bp neo sequence which was used as the probe in Southern analysis (lower bar), are indicated.* **(B)** *Autoradiogram showing Southern analysis of Eco RI and Pst I digest of genomic DNA from neo-PE1 cells, fetuses, recipient animal and plasmid vector. Sizes of restriction fragments from circular plasmid DNA are indicated on the right, those from genomic DNA on the left. DNA samples are as follows: lanes 1 and 2, neo-PE1 cells ('C'; 10 μg); 3 to 12, fetuses 1 to 5, respectively (10 μg); 13 and 14, recipient ('R'; 10 μg); 15 and 16, pSV2-neo (50 pg). Odd-numbered lanes show Pst I digests ('P'), even-numbered lanes Eco RI digests ('E').*

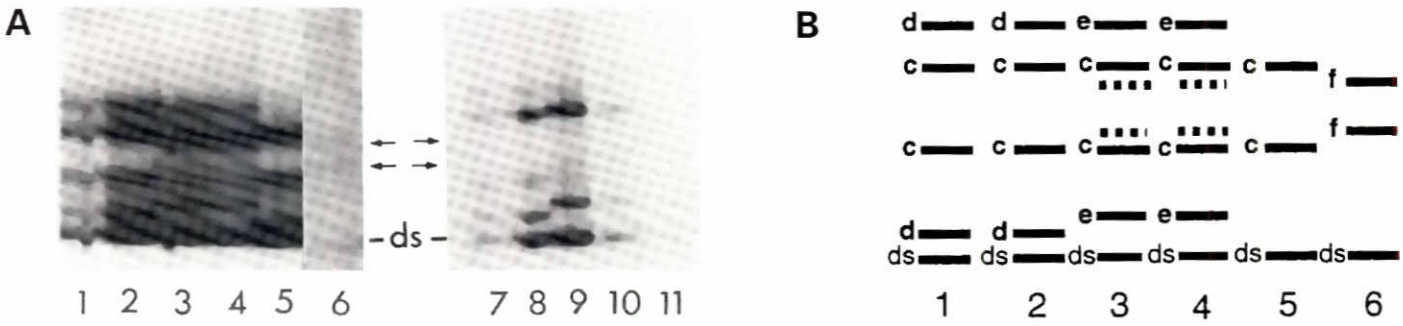

Fig. 3. Detection of chimerism by SSCP analysis of *SLA-DQß*C* sequences. (A) *Autoradiogram showing SSCP analysis of polymorphisms in the SLA-DQB*C allele genomic DNA from fetuses, feeder cells and neo-PE1 cells. DNA samples are as follows: (lanes 1 to 5) fetuses 1 to 5, respectively; (lane 6) neo-PE1 cells. (Lanes 7 to 11) Re-amplification and analysis of genomic DNA from fetuses, 1 to 5. The co-migrating, double-stranded DNA is indicated to the right of track 6, and the left of track 7. Supernumerary SSCP's in lanes 3, 4, 9 and 10 are indicated by arrows.* **(B)** *Assignation of polymorphisms in the SLA-DQB*C allele in lanes 1 to 6,* **(A)**, *according to the convention of Orita et al. (1990). Supernumerary SSCP's deriving from allele f are indicated by stippled bars in lanes 3 and 4.*

polymorphisms (SSCP's) are variants of true Mendelian traits (Orita *et al.*, 1990), and therefore serve as genetic markers. Heterozygosity is revealed by the presence of 3 or more predominant, non-co-migrating, SSCP's in the amplified products of DNA samples, and homozygosity by 2. In principle, therefore, the method was applied to distinguish additional alleles in chimeric fetuses contributed by colonizing *neo*-PE1 cells. Figure 3A,B shows SSCP analysis of polymorphisms in the *SLA-DQB*C* allele in genomic DNA from the porcine fetuses (lanes 1 to 5) and *neo*-PE1 cells (lane 6). The assignment of alleles based on these SSCP's is indicated (B): 3 alleles (c,d and e) were identified in the litter of fetuses, from which it is deduced that the genotypes of the parental animals were cd and ce; 4 of the fetuses were heterozygous (cd, cd, ce and ce; lanes 1 to 4), and 1 was homozygous (cc, lane 5). None of these SSCP's co-migrated with those present in the amplified product from the *neo*-PE1 cell line (lane 7), which was homozygous (ff). However, the f allele was detected as supernumerary SSCP's in the amplified products from two fetuses (Fig. 3A, lanes 3 and 4; stippled bars, Fig. 3B, lanes 3 and 4). The detection of supernumerary SSCP's in these two fetuses was reproducible in further amplification reactions (lanes 9 and 10). This confirms chimerism in one fetus, and further reveals chimerism in a second fetus, at a level undetectable by Southern analysis (i.e. <10%). These results represent the first report of the attainment of transgenesis in the pig at fetal stages, by means other than pronuclear injection (Hammer *et al.*, 1985). Further efforts to reproduce this result using the *neo*-PE1 cell line proved unsuccessful, and later analysis revealed that the cells were overgrown with porcine trophectoderm (Fléchon *et al.*, 1995). This is deduced to have arisen either by the presence of trophectoderm cells in the original isolate, or to spontaneous differentiation from ICM (Nicols and Gardner, 1984; Chisholm *et al.*, 1985).

Non-murine ES cells, once established, will greatly facilitate research into comparative embryology and development, and will provide enormous opportunities for advancement in the field of gene therapy, and for the provision of models of human disease syndromes, expanding the scope of mammalian molecular genetics beyond achievements that were pioneered in the mouse.

Experimental Procedures

Transformation of PE1 cells by electroporation

PE1 cells were transformed by electroporation (Joyner *et al.*, 1989) using the plasmid pSV2-*neo* (Southern and Berg, 1982) (Fig. 2A): 0.5×10^6 PE1 cells were suspended in 1 ml of phosphate-buffered saline (PBS) with 1 µg of Eco RI-digested pSV2-*neo* (Pharmacia); electroporation was performed using a Bio Rad Gene Pulser set at 500 µF and 240 V (Joyner *et al.*, 1989), resulting in approximately 50% cell lethality. Cells were propagated onto *neo*-transformed feeder cells in the presence of G418 (Gibco) at 300 µg/ml. Selection was maintained for 3 weeks. G418-resistant cells showed no morphological changes or differences in growth rates, and the calculated efficiency of electroporation was 0.6×10^6 (for the three observed integration events).

Reintroduction of neo-PE1 cells into host blastocysts

All pigs used were of the Large British White breed. Nine unhatched, donor blastocysts were surgically removed from an 8-month-old, British Large White gilt (mated naturally 24 h after the onset of estrus, on day 1), by perfusing both uterine horns with modified Dulbecco's PBS, at day 5 of pregnancy. Five to 10 *neo*-transformed PE1 cells were introduced by microinjection into the blastocoel cavities of each of 7 embryos via a micropipette, using a Narishige hydraulic manipulation system. A total of 9 embryos were surgically transferred into the tip of one uterine horn of a recipient gilt which had undergone estrus 48 h after the donor gilt. Blastocyst collection, injection and transfer were performed within 3 hours at room temperature. The successful pregnancy was terminated at 30 days of gestation.

Southern blotting

Genomic DNA was prepared from whole fetuses, from a sample of the pseudopregnant recipient's kidney, and from *neo*-PE1 cells passaged to feeder cells, using phenol/chloroform extraction and ethanol precipitation (Ausubel *et al.*, 1992). The DNA was digested with Eco RI or Pst I, electrophoresed in a 0.8% agarose gel, subjected to acid depurination, and transferred to Hybond C Extra (Amersham). The filter was hybridized (in 0.9 M NaCl at 65°C) to a ^{32}P-radiolabeled *neo* probe (Fig. 2A) which had been labeled to a specific activity of 1×10^9 cpm/µg using a Multiprime kit (Amersham), and was used at 5×10^5 cpm/ml. The filter was washed in 2XSSC and 1% SDS at 65°C, then 0.1%SSC at 65°C. Exposure was for 4 weeks.

PCR amplification of genomic DNA using SLA-DQB*C primers

Upstream primer 5'TCG-TGT-TCC-AGT-TTA-AGG-GC-3'.
Downstream primer 5'ACG-TCC-TTC-TGG-CTG-TTC-CA-3'.
Amplified fragment lengths, 181 bp (Hoelzel *et al., in preparation*).

Genomic DNA samples were amplified in a total volume of 10 µl, overlaid with 50 µl of mineral oil to prevent evaporation. Reaction mixtures contained: 150 ng genomic DNA; 1x reaction buffer (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl); 0.1 U "Perfect Match" (Stratagene); 1 pmol of *SLA-DQB*C* primers; 200 µmol of dCTP, dGTP and dTTP; 0.25 µCi of "Redivue" [α-^{33}P]dATP (1-3,000 Ci/mmol) (Amersham); and 0.25 U of Taq polymerase. After initial denaturation at 94°C for 2 min, 35 cycles were conducted, consisting of denaturation at 93°C for 45 sec, annealing at 55°C for 1 min, and primer extension at 72°C for 2 min. After amplification, 2 µl of loading buffer (95% formamide, 1XTBE, 0.01% xylene cyanol and 0.01% bromophenol blue) were added to each tube. Samples were denatured at 98° for 5 min, and snap-cooled prior to loading. Aliquots of 6-8 µl were electrophoresed in pre-heated, 0.4 mm-thick non-denaturing gels (consisting of 4.6% acrylamide (37.5:1 of acrylamide to bis-acrylamide), 1xTBE and 10% glycerol), keeping a constant current of 25 mA, sufficient to maintain a temperature between the ambient and 40°C. After electrophoresis, the gels were dried onto Whatman 3MM paper, and exposed by autoradiography using KODAK X-Omat AR film, with intensifying screens, for 4 to 16 h.

Acknowledgments

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