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Development of Culture Conditions for the Isolation of Pluripotent Porcine Embryonal Outgrowths from *In Vitro* Produced and *In Vivo* Derived Embryos

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Abstract. In the present study we examined the effect of culture media and protein source on the formation of pluripotent primary outgrowths from *in vitro* produced and *in vivo* derived porcine embryos as the first step towards the isolation of embryonic stem cells (ESCs). To do this we compared high glucose Dulbeccos Modified Eagles Medium (DMEM) with Minimal Essential Alpha Medium (α MEM) both supplemented with fetal bovine serum (FBS) or serum replacement (SR) in a 2×2 factorial design. Culture in DMEM or α MEM supplemented with 10% SR resulted in the establishment of homogenous populations of cells which expressed Oct 4 and Nanog. In contrast culture in either media with FBS resulted in the formation of embryonal outgrowths composed entirely of differentiated cells or a mixture of differentiated cells and putative ESCs which grew poorly and could not be passaged. Using α MEM medium containing 10% SR and culturing in 5% oxygen, putative ESC lines were isolated from *in vitro* and *in vivo* derived embryos at efficiencies of 2 and 10% respectively.

Key words: Embryonic stem cells, Nanog, Oct-4, Porcine

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Embryonic stem cells (ESCs) are pluripotent cells derived from preimplantation embryos. These cells can be maintained indefinitely in culture and can contribute to all the cell types in the body including the germ cells. ESCs were initially isolated in mice [1, 2] and subsequently for a variety of species including humans [3]. However germline transmission has only been demonstrated in mice [1, 2] and more recently in rats [4]. Putative ESCs have also been isolated for variety of domestic animals including the pig [5–11]. However germline transmission has not been demonstrated for any of these lines limiting their use for multiple rounds of gene targeting (reviewed in [12]). The advent of somatic cell nuclear transfer has allowed us and others to produce gene knockout pigs without using ESCs [13]. However SCNT suffers from the limitation that somatic cells have a limited lifespan in culture which makes it impossible to perform multiple rounds of gene targeting, which is required for producing pigs expressing multiple transgenes for xenotransplantation research (reviewed in [14]), highlighting the need for ESCs for this as well as other applications.

The overall objective of the current study was to isolate porcine ESC lines which could ultimately be used to perform multiple rounds of gene targeting for research purposes. As a first step, the present study aimed to develop conditions for the isolation of homogenous pluripotent outgrowths from whole embryos, as the majority of methods that have been used so far result in heteroge-

neous outgrowths which may inhibit isolation (reviewed in [15]). To do this we compared DMEM medium, which has been widely used for ESC isolation, with α MEM medium, which has shown promising results in previous studies by us [16]. Secondly, as foetal bovine serum (FBS) is believed to promote spontaneous differentiation of ESCs because of its undefined nature [17], we compared FBS with serum replacement (SR) in this experiment. Finally, we compared the efficiency with which primary cell lines could be established from *in vitro* versus *in vivo* derived whole embryos using the conditions developed in the previous experiment.

Materials and Methods

Experimental design

Two experiments were undertaken to develop an isolation method which would result in homogeneous embryonal outgrowths of ESC progenitor cells and to determine the efficiency with which lines could be established from these following cryopreservation at passage two. Experiment 1 used IVP embryos and determined the effect of media and protein source on these two parameters by comparing DMEM with α MEM containing 20% FBS or 10% SR in a 2×2 factorial design. Experiment 2 used the isolation method determined be optimal from Experiment 1 namely α MEM containing 20% FBS or 10% SR and compared embryonal outgrowth rate and the efficiency with which cell lines could be established from these for IVP and *in vivo* derived embryos.

In vitro embryo production

Porcine ovaries from slaughtered mature sows were collected

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from a local abattoir and transported to the laboratory in phosphate buffered saline (PBS) solution, containing 1% of an antibiotic-antimycotic solution (Invitrogen, New Zealand) at between 33 and 37 C. Follicles with a diameter between 3 and 6 mm were aspirated with a 21G needle, through which a constant suction (1 l/min) was applied, and follicular contents were pooled in a collection tube. Cumulus-oocyte complexes (COC) with at least three uniform layers of compact cumulus cells were recovered from the collected fluid and matured for approximately 40–42 h in groups of 50 in 500 μ l of M199 medium (Invitrogen, USA) supplemented with 5 ng/ml insulin, 0.5 mM cysteamine, 0.2 mM Na-pyruvate, 75 mg/ml Penicillin-G, 50 mg/ml Streptomycin sulphate, 10 ng/ml recombinant human epidermal growth factor (hEGF) (all from Sigma, USA), 5 mg/ml Follicle Stimulating Hormone (ICP bio, New Zealand), and 10% sow follicular fluid under mineral oil (Sigma, USA) in a humidified atmosphere of 5% CO₂, 5% O₂, the balance N₂ at 38.5 C. Sow follicular fluid was collected from 3–6 mm follicles, stored at –70 C, thawed and filter sterilized (Millipore, USA; filter pore size 0.22 μ m) immediately prior to use.

Mature COCs were treated briefly with 0.1% hyaluronidase (Sigma, USA), washed twice in HEPES buffered NCSU23 + 10% FBS (JRH Biosciences, KA, USA), twice in the TALP-PVA fertilization medium [18] supplemented with 3 mM calcium lactate and 2 mM caffeine-sodium benzoate (Sigma, USA), then placed into 90 μ l drops of fertilization medium (30 oocytes per drop) under mineral oil. Freshly collected and extended semen was obtained from a Landrace and a Hampshire boar (Sabor, Clare, SA, Australia). The semen was allowed to come to room temperature (23 C) from its storage temperature (18 C). It was then centrifuged at 1100 g for 5 min and the supernatant discarded. The pellet was resuspended in 10 ml of M199 (Invitrogen, USA) medium containing 1 mg/ml sodium pyruvate, 9 mg/ml calcium-lactate, 0.075 mg/ml penicillin G, 0.05 mg/ml streptomycin sulphate (all from Sigma) and 10% FBS then centrifuged and resuspended a second time. The sperm was diluted with TALP-PVA medium and 10 μ l of this added to the fertilization drop to give a final concentration of 5×10^5 sperm/ml. The oocytes and sperm were left to co-incubate for 6 h in a humidified atmosphere of 5% CO₂, 5% O₂, the balance N₂ at 38.5 C, after which any remaining cumulus cells were removed by manual pipetting and the prospective embryos washed and placed into culture drops. The culture medium used was as previously described [19]. A modified version of NCSU23 medium [20], contained 0.2 mM pyruvate, 5.7 mM lactic acid, 0.6 mM glucose (all from Sigma, USA) and MEM-nonessential amino acids (Invitrogen, USA) was used for day 1 to 3 of culture, and NCSU23 medium containing 5.6 mM glucose (Sigma, USA) and MEM non-essential and essential amino acids (Invitrogen, USA) was used for day 3 to 6. 30 embryos were cultured in 50 μ l droplets under mineral oil (Sigma, USA) in a humidified atmosphere of 5% CO₂, 5% O₂ and the balance N₂. A low oxygen tension was chosen because we have previously shown that this is superior compared with atmospheric oxygen for the isolation and culture of porcine fibroblasts [13] and the establishment of primary embryonal outgrowths (unpublished results).

In vivo embryo collection

Experiments were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [21]. Late morulae and early blastocysts were collected from one multiparous Landrace $\times$ Large White sow on each of three days and were then cultured for 1 day to the expanded blastocyst stage. The sows were synchronized by weaning, given 1000 IU of eCG (Folligon, Intervet Australia, NSW, Australia) on the day of weaning, and 750 IU of hCG (Chorulon, Intervet Australia) 80 h later. Both injections were given intramuscularly. The sows were mated by artificial insemination during estrus using semen from the Hampshire boar during standing estrus which usually commenced 24 h after hCG treatment. The embryos were collected surgically approximately 5 days after the onset of standing estrus. Each uterine horn was flushed with 50 ml of PBS containing 2% inactivated FBS (56 C for 30 min; JRH Biosciences, USA) and the collected fluid searched using a dissecting microscope. Recovered embryos were washed in HEPES-buffered NCSU23 containing MEM essential and non-essential amino acids and 10% FBS and then held in 3 ml of the same medium in a 5 ml tube in a portable incubator at 38.5 C until surgery was completed and all the collected embryos were transported to the laboratory. Embryos were then cultured overnight as described above.

Establishment of embryonal outgrowths

Primary embryonal outgrowths were established from day 6 *in vivo* derived expanded blastocysts or day 7 IVP expanded or hatching blastocysts which had a defined inner cell mass, large blastocoele cavity and a large number of trophectoderm cells (good grade or better; Fig. 1A). Following removal of the zona pellucidae mechanically by repeated pipetting using a hand drawn glass pasteur pipette whose diameter was approximately half that of the embryo, embryos were plated on mitotically inactivated mouse embryonic fibroblast (MEF) feeder layers by gently pressing the whole embryo into the feeder layer using a 30G needle (Fig. 1B). The isolation media used consisted of α MEM medium with ribo- and deoxyribonucleosides or DMEM medium both supplemented with either 10% SR (Invitrogen, USA) or 20% FBS, (Hyclone, USA), 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml hEGF, 1 $\times$ Insulin-Transferrin-Selenium solution, 55 μ M 2-Mercaptoethanol, 1 $\times$ MEM non-essential amino acids, 1 $\times$ Glutamax (all from Invitrogen, USA) 10 ng/ml human recombinant LIF (Millipore, USA), and 10 ng/ml Activin A (R&D Systems, USA). Cultures were conducted in a humidified atmosphere of 5% CO₂ and 5% O₂. The media were changed every 2–3 days. After 12–15 days, primary outgrowths which had reached 2–5 mm in diameter were mechanically passaged onto fresh feeder layers. This was done by cutting outgrowths into approximately 80–100 μ m square pieces using a hand drawn glass pasteur pipette, transferring these onto new feeder layers and then gently pressing the pieces into the feeder using a 30G needle. Colonies were passaged every 10–12 days. Putative ESC in primary outgrowths were identified according to morphological criteria described previously for bovine ES cells [22, 23], namely polygonal in shape, a relatively small (10–15 μ m) diameter, a small cytoplasmic/nuclear ratio, a single nucleus with multiple nucleoli, and multiple lipid inclusions in the cyto-

Fig. 1. Isolation and expansion of porcine putative ESC lines. Day 7 IVP porcine expanded and hatching blastocysts (a) which had their zona pellucida removed were plated onto mitotically inactivated mouse embryonic fibroblast (MEF) feeder layers (b). After 2–3 days most of the embryonic cells detached from the feeder layer and degenerated leaving groups of putative ESCs clustered together (c). Over the next 3–5 days these clusters divided to form a single homogeneous outgrowth which grew as monolayer and whose cells had a morphology characteristic of ESCs (d). Primary colonies (e) were passaged and plated onto new MEF feeder layers after 13–21 days. ESCs grew initially as clumps (f) and then as monolayers after 12–15 days. Scale bars: a=40 μm ; b=80 μm ; c=100 μm ; d=200 μm ; e and f=500 μm .

plasm. Homogeneity of outgrowths was assessed morphologically initially and then examined for Oct 4 and Nanog co-expression.

At passage 2, putative ESC colonies were manually dissected and pieces vitrified by solid surface vitrification using the Cryologic Vitrification System (CVM; Cryologic Pty. Ltd., Victoria, Australia) described previously [34]. Cell lines which survived vitrification and warming and continued to grow were considered viable.

Immunofluorescence analysis and confocal microscopy

Oct-4 and Nanog expression were examined on 10 outgrowths obtained from IVP embryos using immunofluorescence analysis and confocal microscopy. 20 *in vitro* produced embryos were used as positive controls for each measure. Primary outgrowths or porcine embryos were fixed in 4% paraformaldehyde (Sigma, USA) in PBS (Sigma, USA) supplemented with 0.3% saponin (Calbiochem, Switzerland) for 5–20 min at room temperature. To facilitate antibody entry into cells, saponin was included in all solutions. Cells were then washed twice in PBS for 10 min. Non-specific antibody binding was blocked using PBS supplemented with 1% bovine serum albumin (BSA; Invitrogen USA) and 3% normal donkey

serum (Jackson Immuno Research Labs, USA) or FBS for 30 min at room temperature. Cells were then incubated overnight at 4 °C with primary antibodies in the blocking solution. The primary antibodies were rabbit antibody to Nanog (Millipore, USA; 1:200 dilution), and goat antibody to Oct 4 (Santa Cruz Biotechnology, USA; 1:100 dilution). After washing, porcine primary outgrowths or porcine embryos were incubated for 30 min with secondary antibodies at room temperature. Secondary antibodies were goat rabbit specific antibody conjugated to Texas Red (Southern Biotech, USA; 1:200 dilution) and donkey goat specific antibody conjugated to FITC (Santa Cruz, USA; 1:200 dilution). After a final wash, porcine primary outgrowths were mounted in Prolong Gold mounting medium with DAPI (Invitrogen, USA) on glass cover slips. Imaging analysis was done using a confocal laser microscope SP5 (Leica, USA) using Z-stack sectioning with 0.4 μm sections.

Statistical analysis

The effect of culture media, protein source and embryo source on attachment rate, embryonal outgrowth formation and the number of primary cell lines produced were analysed using a one way chi square test for small samples using GraphPad Prism 5 software

Table 1. Effect of culture media and protein source on embryonal outgrowth formation and putative ESC line establishment

Culture system	Number of blastocysts	Number of primary outgrowths (%)	Number of homogeneous outgrowths (%)	Number of putative ESC lines isolated (%)
DMEM + 20%FBS	35	11 (31.4) ^b	0 ^a	0
DMEM + 10%SR	36	2 (5.6) ^a	2 (5.6) ^a	0
α MEM + 20% FBS	57	10 (17.5) ^b	0 ^a	0
α MEM + 10% SR	75	28 (37.3) ^c	28 (37.3) ^b	2 (2.7)

Percentages calculated from number of blastocysts used. Values with different superscripts in the same column are significantly different, $P < 0.05$.

(GraphPad Software, USA). A value of $P < 0.05$ was considered to be statistically significant. Experiments were replicated at least three times and the data pooled.

Results

Experiment 1. Effect of culture media and protein source on embryonal outgrowth formation and putative ESC line isolation

Experiment 1 examined primary outgrowth formation and putative ES cell line isolation rate using *in vitro* produced blastocysts cultured in DMEM or α MEM containing 20% FBS or 10% SR. The results for this comparison are shown in Table 1. No passagable primary outgrowths were obtained in the DMEM plus 20% FBS group. After 9–12 days, embryonal outgrowths in this group consisted entirely of differentiated cells or small clusters of putative ESCs surrounded by differentiated cells which were too small to passage. Culture in DMEM plus 10% SR group resulted in two outgrowths from 36 embryos plated (efficiency 5.6%) that were large enough to be passaged (1–1.5 mm in diameter). In contrast to the outgrowths obtained using FBS, these outgrowths consisted entirely of cells which resembled ESCs in their morphology. Culture in α MEM with 20% FBS resulted in similar outgrowths obtained with DMEM plus FBS which were also small in size and consisted of clusters of putative ESCs surrounded by differentiated cells. Culture with α MEM plus 10% SR resulted in 28 outgrowths from 75 embryos plated (efficiency 37.3%). After 2–3 days most of embryonic cells detached from the feeder layer and degenerated leaving groups of putative ESCs clustered together (Fig. 1C). Five to 7 days after plating, the cell clusters formed outgrowths. These outgrowths were homogeneous and consisted of cells which resembled bovine ESCs in their morphology namely, polygonal in shape, a relatively small (10–15 μ m) diameter, a small cytoplasmic/nuclear ratio, a single nucleus with multiple nucleoli, and multiple lipid inclusions in the cytoplasm (Fig. 1D, [22, 23]). Over the next 5–8 days these colonies grew in size up to 3–5 mm in diameter (Fig. 1E). At this stage outgrowths were passaged onto fresh feeder layer to establish porcine putative ES cell lines (Fig. 1F). In addition embryonal outgrowths from IVP embryos were examined for pluripotent marker expression at this stage. As shown in Fig. 2, these outgrowths consisted entirely of putative ESCs which expressed Oct4 and Nanog.

Only two outgrowths obtained following culture in α MEM plus 10% SR medium were able to be maintained after passage 1 and

following vitrification at passage 2 whereas embryonal outgrowths in the remaining groups failed to grow following passaging.

Experiment 2. Effect of embryo source on embryonal outgrowth formation and putative ESC line isolation

Experiment 2 examined embryonal outgrowth formation and putative ESC line isolation rate for *in vitro* produced embryos and *in vivo* derived embryos using α MEM containing 10% SR determined to be optimal in Experiment 1. The results are shown in the Table 2. Although the number of passagable homogeneous outgrowths were the same for both groups of embryos, the number of putative ESC lines that could be established from these was significantly higher for *in vivo* derived embryos compared with IVP embryos (10 vs. 2.6%, $P < 0.05$).

Discussion

The present study was undertaken to develop culture conditions which could be used to establish homogenous outgrowths of ESC progenitor cells as a first step towards isolating embryonic stem cells from pigs. To do this we compared DMEM with α MEM and FBS with SR in a factorial design. The most significant finding in this study was that culture with α MEM plus 10% SR resulted in embryonal outgrowths which formed colonies consisting entirely of putative ESCs as evident by their morphology and pluripotent gene expression. Furthermore these could be passaged resulting in the establishment of several putative ESC lines. In contrast, culture in DMEM or α MEM with FBS resulted in populations of differentiated cells or mixed populations of differentiated cells and putative ES progenitor cells which grew poorly and could not be maintained after initial passaging. This is a major finding as the majority of methods developed previously for pigs as well as other domestic species have resulted in heterogeneous outgrowths which continue to grow as mixed populations during early passages [9, 24–26]. Whether this was due to the use of α MEM, SR, low oxygen tension or as yet undefined factors remains to be determined. However based on our findings we suggest that all three are likely to have an additive effect.

The embryonic stage used in our study may have also contributed to the establishment of these homogenous outgrowths. In mice, ESCs are isolated around 4.5 days post coitum when the inner cell mass has differentiated into the epiblast and the hypoblast [27]. In contrast, formation of the porcine epiblast occurs later, when the blastocyst begins to hatch, and is maintained until around

Fig. 2. Co-expression of Oct-4 and Nanog in primary embryonal outgrowths derived from IVP embryos. Primary embryonal outgrowths were fixed in 4% paraformaldehyde and Oct4 and Nanog expression examined using immunofluorescence analysis and confocal microscopy. Figure is a representative image of all outgrowths assessed. a: Phase contrast of fixed primary embryonal outgrowth. b: Nuclei of primary embryonal outgrowth stained with DAPI. c: Oct-4 expression in nuclei of primary embryonal outgrowth. d: Nanog expression in nuclei of primary embryonal outgrowth. Scale bar=25 μ m.

Table 2. Effect of embryo source on embryonal outgrowth formation and putative ESC line isolation rate

Source of embryos	Number of blastocysts	Number of homogeneous outgrowths (%)	Number of putative ESC lines isolated (%)
<i>In vitro</i> produced	156	13 (8.3) ^a	4 (2.6) ^a
<i>In vivo</i> derived	20	4 (20.0) ^a	2 (10) ^b

Percentages calculated from number of blastocysts. Values with different superscripts in the same column are significantly different, $P<0.05$.

day 12 [28]. In the pig, extraembryonic endoderm cells first appear around day 7 *in vivo* but are not detected in day 7 *in vitro* produced embryos [29] because of their delayed development. Hence by using day 7 *in vitro* produced and day 5.5 *in vivo* derived embryos we may have bypassed any possible contamination of outgrowths with extraembryonic endoderm. While all non-extraembryonic

cells in the pig blastocysts express Oct 4 at this stage, only some of those in the inner cell mass express both Oct 4 and Nanog and are presumably ESC progenitors cells [30]. Furthermore we have found that these cells are negative for Gata 6, suggesting that the inner cell mass has not begun to differentiate into ectoderm and extraembryonic endoderm (unpublished results). However further

work is required to confirm this. Our method also resulted in the majority of trophoblast cells failing to attach to the feeder when the embryos were pressed into it with any remaining trophoblast cells degenerating over the next few days, resulting in a homogeneous population of putative ESCs. This may also be an important feature of our method because the trophectoderm is generally removed to prevent these cells from overgrowing ESCs in culture. The failure of these cells to attach to the feeder layer may have been a direct consequence of using SR which does not contain attachment factors such as fibronectin or vitronectin which are found in FBS [17].

In the present study, putative ESC lines were established from *in vitro* as well as *in vivo* derived embryos using these conditions. The efficiency with which these were obtained was lower for *in vitro* produced embryos. However both efficiencies were similar to that obtained for 129Sv mice and higher than that reported for other so called non permissive mouse strains [31]. The finding that putative ESC lines could be derived from *in vitro* produced embryos is important as these are relatively inexpensive to produce and as such may make them an ideal large animal model for human ESC research. Previous attempts to isolate ES cells from *in vitro* produced pig embryos have been unsuccessful [11, 32] possibly because these contained too few ES cell progenitor cells as a consequence of the culture system used. However recent improvements in the porcine preimplantation culture conditions used by our laboratory have resulted in increases in cell number which have resulted in acceptable pregnancy rates and litter sizes following their transfer to recipient animals [33].

In conclusion, we have developed a new method for the isolation of porcine embryonic stem cells. This method can be used for *in vitro* and *in vivo* derived embryos and differs from the methods developed previously for pigs in that it results in the establishment of homogeneous outgrowths consisting of pluripotent progenitor cells from which putative ESC lines can be established following vitrification. The *in vitro* and *in vivo* characterisation of these cell lines including the production of chimaeric pigs is described elsewhere [34].

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