A comparison of the number of inner cell mass and trophectoderm cells of preimplantation Meishan and Yorkshire pig embryos at similar developmental stages

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Day 12 blastocysts from Meishan gilts contain fewer cells than do day 12 blastocysts from Yorkshire gilts. The purpose of this study was to evaluate the effect of breed on the relative numbers of inner cell mass and trophectoderm cells in Meishan and Yorkshire embryos at similar stages. Embryos were collected on days 5.5–6.5 of gestation and were subjected to image analysis and differential cell staining. No breed differences were detected in the thickness of zona pellucida or in the areas of the perivitelline space, embryo proper, blastocoel and inner cell mass at any of the developmental stages examined (compact morula, early blastocyst or blastocyst). However, differences were observed in the pattern of growth of embryos from Meishan versus Yorkshire gilts. The total number of cells of Meishan embryos from Meishan gilts increased progressively from the compact morula through the blastocyst stage, whereas the total number of cells of embryos from Yorkshire gilts remained constant from compact morula through to early blastocyst, and then increased markedly from the early blastocyst to the blastocyst stage. At the blastocyst stage, Meishan embryos contained fewer ($P < 0.05$) cells than did Yorkshire embryos, and this lower number of cells was due entirely to fewer ($P < 0.05$) trophectoderm cells. As the number of inner cell mass cells increased during embryonic growth, Meishan embryos exhibited a slower ($P < 0.02$) increase in the number of trophectoderm cells than did Yorkshire embryos. These results demonstrate that the reduced number of cells present in Meishan embryos results from a selective reduction in the number of trophectoderm, but not inner cell mass, cells.

Introduction

Chinese Meishan pigs are more prolific than European pig breeds, averaging from three to five more piglets per litter (Cheng, 1983). Recent studies from our laboratory have demonstrated a reduced developmental rate of Meishan embryos versus those of European pig breeds both *in vivo* and *in vitro*. Youngs *et al.* (1993) reported that Meishan embryos collected on day 2 after oestrus and cultured *in vitro* to the early blastocyst stage exhibited a lower total number of cells when compared with Yorkshire embryos. In another study, Anderson *et al.* (1993) showed that on day 12, average littermate embryonic diameter and DNA content were less for Meishan embryos than for embryos from European white crossbred sows, although the range of embryonic sizes was similar between the two breeds. Those researchers also reported that Meishan embryos produced markedly less oestradiol than did embryos from European white crossbred sows, even when comparing embryos of the same diameter.

During embryonic development, differentiation begins at the time of compaction with the formation of tight cell junctions

between the outermost cells of an embryo. These outer cells are subsequently referred to as trophectoderm cells, and the remaining inner cells of the embryo are known as inner cell mass cells. The inner cell mass cells give rise to the embryo proper, yolk sac and other extraembryonic tissues, and the trophectoderm cells give rise to the chorionic ectoderm (Gardner *et al.*, 1973; Bard and Kaufman, 1994) and are the first site of embryonic oestrogen secretion (Gadsby *et al.*, 1980). Oestrogen, produced by the trophectoderm cells of larger littermate embryos, alters the uterine environment, possibly through changes in endometrial secretory products, making it unsuitable for smaller littermate embryos (Geisert *et al.*, 1982a). An initial, rapid mitotic rate of trophectoderm cells, followed by their morphological changes, results ultimately in elongation and spacing of pig embryos within the uterus (Geisert *et al.*, 1982b). It has been suggested (Ford and Youngs, 1993; Youngs *et al.*, 1994) that Meishan embryos may have the ability to elongate from fewer cells than Yorkshire embryos. Recently, Wilson *et al.* (1995) confirmed that Meishan embryos elongate from fewer cells and reach a reduced length on day 14 than do Yorkshire embryos.

The objective of the present study was, therefore, to determine whether there are differences in the relative numbers

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Table 1. Reproductive characteristics of Meishan and Yorkshire pig embryo donors^a

Measurement	Meishan (<i>n</i> = 12)	Yorkshire (<i>n</i> = 11)
Number of oestrous cycles	3.1 ± 0.4 (median = 2)	2.9 ± 0.4 (median = 2)
Number of corpora lutea	14.8 ± 0.8	16.2 ± 0.8
Percentage recovery of embryos (embryo ^b /corpora lutea)	84 ± 4	86 ± 4
Number of embryos subjected to differential staining ^c	138	113

^aValues are presented as least squares means ± SEM. No differences were observed in any of the parameters measured (*P* > 0.20).

^bThe mean does not include two unfertilized ova. All recovered embryos were of good to excellent quality.

^cAn additional 11 embryos from the last two Meishan donors and 40 embryos from the last five Yorkshire donors were randomly selected for use in an unrelated study and were not subjected to differential staining.

of inner cell mass and trophoctoderm cells among compact morula, early blastocyst and blastocyst stage Meishan and Yorkshire embryos.

Materials and Methods

Animals

Meishan (*n* = 12) and Yorkshire (*n* = 11) gilts of similar reproductive age (two to six postpubertal oestrous cycles; Table 1) were checked for oestrus twice a day (08:00 and 17:00 h) with an intact boar. The first observation of behavioural oestrus, irrespective of the time of day, was designated as the beginning of day 0. Gilts were hand-mated to a boar of the same breed at day 0 and were mated again 24 h later.

Collection of embryos

In our population of pigs, the ovulation time following the onset of oestrus is similar for both Meishan and Yorkshire gilts (Youngs *et al.*, 1994), yet Meishan embryonic development has been shown to be slower (Anderson *et al.*, 1993; Youngs *et al.*, 1993). Therefore, to correct for known breed differences in developmental rate, embryos from Meishan gilts were surgically collected on days 6.0 (*n* = 94) or 6.5 (*n* = 44) while embryos from Yorkshire gilts were collected on days 5.5 (*n* = 50) or 6.0 (*n* = 63; Table 1). Meishan and Yorkshire gilts were sedated and anaesthetized as described by Youngs *et al.* (1994). The uterus was exteriorized via midventral laparotomy, and an incision was made in the antimesometrial region of the uterine wall 2–3 cm from the utero-tubal junction. A borosilicate glass cannula (10 mm o.d. and 10 cm long) was inserted into the incision pointing away from the oviduct and was kept in place with a silk ligature. Twenty millilitres of Dulbecco's PBS (Biowhittaker, Walkersville, MD) containing 2% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic (10 000 U penicillin ml⁻¹; 10 000 mg streptomycin ml⁻¹ and 25 mg amphotericin-B ml⁻¹; AGTECH Inc., Manhattan, KS) was injected via a blunt 18-gauge needle inserted at the base of the cannulated uterine horn. The embryos were moved to the tip of the uterine horn by firmly pushing the flushing medium

along the length of the horn, where medium was collected through the cannula into a sterile glass Petri dish.

Classification of developmental stage of embryos

After collection, the embryos were immediately transferred to modified PBS (mPBS; 0.0036% (w/v) sodium pyruvate, 0.0023% (w/v) L-glutamine, 0.4% (w/v) BSA and 1% (v/v) penicillin-streptomycin). The stage of development was determined in all embryos using the International Embryo Transfer Society method (Stringfellow and Seidel, 1990) with modifications made in our laboratory. The criteria used to classify the embryos were: (i) compact morula: embryo had a large perivitelline space, blastomeres had compacted, cell definition was lost, and no blastocoel was present; (ii) early blastocyst: an evident perivitelline space, embryo had two distinct populations of cells, a small blastocoel and (iii) blastocyst: embryo had very little, or no, perivitelline space, a large blastocoel and no evidence of thinning of the zona pellucida.

Immunosurgical lysis and differential staining

The zona pellucida of each embryo was removed by incubation at 39°C for 45 s in an acidic Tyrode's solution (0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.024% (w/v) CaCl₂ · H₂O, 0.01% (w/v) MgCl₂ · 6H₂O, 0.1% (w/v) glucose, 0.4% (w/v) polyvinyl pyrrolidone; pH 2.5; Hogan *et al.*, 1986). After removal of the zonae pellucidae, embryos were washed three times in mPBS. The embryos were subjected to immunosurgery and were differentially stained by the method of Hardy *et al.* (1989) with modifications determined to be necessary for pig embryos. Briefly, the embryos were incubated on ice for 30 min in 10 mmol trinitrobenzene sulfonic acid l⁻¹ (TNBS; Sigma Chemical Co, St Louis, MO) to label cell surface proteins with covalently bound trinitrophenol (TNP) groups. This allowed the use of an antiserum against dinitrophenol (DNP) which crossreacts with TNP-labelled proteins. Embryos were then washed three times in mPBS and placed in an anti-DNP antibody solution (ICN Immunobiologicals, Costa Mesa, CA; 0.1 mg ml⁻¹ in mPBS) for 30 min at 39°C. The embryos were again washed three times in mPBS and were then placed in an mPBS solution containing guinea-pig complement serum (1:5 dilution; Sigma Chemical Co.) and propidium iodide

(0.01 mg ml⁻¹; Sigma Chemical Co.) at 39°C for 20 min. This step resulted in the lysis of the trophoctoderm cells and the red staining of trophoctoderm cell nuclei. Immediately after this step, embryos were placed in absolute ethanol containing the fluorochrome bisbenzimidazole (0.05 mmol l⁻¹, Hoechst 33258; Sigma Chemical Co.) for 1 h at 4°C. This resulted in the staining of all cell nuclei (intact inner cell mass and lysed trophoctoderm) and the fixation of the embryo, with the inner cell mass cell nuclei appearing blue while the trophoctoderm cell nuclei appeared pink (due to the combination of the blue and red). Finally, embryos were removed from this solution and were washed by placing them in absolute ethanol for 30 min at 4°C. Various controls were conducted to validate the differential staining technique. Controls consisted of omissions of TNBS, antibody or complement individually, or in combination, with the embryo solutions, resulting in the prevention of trophoctoderm cell lysis and subsequent blue staining of both trophoctoderm and inner cell mass cell nuclei.

Each embryo was individually mounted in a 5 µl drop of glycerol on a glass slide, and the two types of nuclei (trophoctoderm and inner cell mass) were visualized and counted by use of a fluorescent microscope (Nikon Diaphot-TMD) fitted with a UV2A filter combination having a 330–380 nm excitation filter and a 420 nm barrier filter. All nuclei of each embryo were counted independently by two operators, and the counts were averaged. Overall, counts of inner cell mass cell nuclei and trophoctoderm cell nuclei of the operators were not different ($P > 0.45$ and $P > 0.77$, respectively).

Image analysis

Embryos collected from the last six Meishan and last six Yorkshire donors were available for morphological analysis. A randomly selected portion of embryos from some donors were used in another experiment, but the remaining whole or partial litters were photographed for subsequent analysis (Nikon N2020 camera, loaded with Kodak TMX 100 black and white print film). Embryos in which differential staining was successfully accomplished ($n = 35$ Meishan and $n = 33$ Yorkshire) were subjected to image analysis as described by Youngs *et al.* (1987). Each photographic negative was illuminated using a ChromaPro 45 light box, and the image was conveyed to the computer using a Sony DXC-3000A video camera equipped with a Contax 60 mm Macro lens. The captured image of each embryo was then traced, and each image was evaluated with a Zeiss SEM-IPSA Image Analysis System (Zeiss-Kontron: IBAS version 2.0). The image analysis data were used to determine the thickness of the zona pellucida, and the areas of the perivitelline space, embryo proper, blastocoel and inner cell mass.

Statistical analyses

Data from the subset of embryos subjected to image analysis were analysed using zona pellucida thickness, perivitelline space area, area of embryo proper, blastocoel area and inner cell mass area as dependent variables. Data were analysed using the General Linear Model (GLM) procedure of the Statistical Analysis Systems (SAS, 1985). Breed effects were tested using

Fig. 1. A differentially stained day 6 blastocyst from a Yorkshire pig ($\times 200$). The blue objects are the nuclei of the inner cell mass cells, and the pink objects are the nuclei of the trophoctoderm cells.

gilt within breed as the error term. Furthermore, Student's *t* test was used to determine whether there was an effect of operator on the numbers of inner cell mass and trophoctoderm cells per embryo, if the number of total cells differed between and within breeds at a particular stage of development, and if the slopes of the regression lines were different. Results were considered significant at the $P < 0.05$ level. The results are presented as least squares means $\pm$ SEM.

Results

The number of ovulations and the number of embryos recovered did not differ ($P > 0.05$) between breeds (Table 1). Embryos collected from Meishan gilts on days 6.0–6.5 yielded the same range of developmental stages (compact morula, early blastocyst and blastocyst) as embryos collected from Yorkshire gilts on days 5.5–6.0. Not all attempts at differential staining were successful, as many compact morulae yielded only pink nuclei and some other embryos became fragile and the nuclei were disrupted. In the latter case, these embryos were excluded from the analysis to avoid influencing biological interpretation of the data because of methodological difficulties. Differential staining was successfully accomplished in 94 embryos from each breed (Fig. 1) and was not affected by breed of embryo ($P > 0.10$). Image analysis demonstrated no breed differences at any of the embryonic stages examined in the thickness of the zona pellucida, or in the area of the perivitelline space, blastocoel, embryo proper and inner cell mass (Table 2).

Breed differences were observed in the pattern of embryonic growth from the compact morula to the blastocyst stage (Fig. 2). The total number of cells of Meishan embryos increased ($P < 0.01$) from the compact morula to the early blastocyst and again from the early blastocyst to the blastocyst stage ($P < 0.01$). In contrast, the total number of cells of Yorkshire embryos remained relatively constant from the compact morula through the early blastocyst stage but exhibited a marked

Table 2. Morphological characteristics of embryos from Meishan and Yorkshire gilts^a

Measurement	Meishan			Yorkshire		
	Compact morula (n = 13)	Early blastocyst (n = 12)	Blastocyst (n = 10)	Compact morula (n = 3)	Early blastocyst (n = 8)	Blastocyst (n = 22)
Thickness of zona pellucida (μm)	10.58 ± 0.51	10.65 ± 0.87	9.39 ± 0.90	8.93 ± 0.93	10.92 ± 0.95	10.20 ± 0.69
Area of perivitelline space (μm ²)	4443 ± 265	3276 ± 510	1434 ± 821	5386 ± 481	3381 ± 559	3043 ± 629
Area of embryo proper (μm ²)	9883 ± 679	10 896 ± 351	15 817 ± 2506	8991 ± 1493	10 460 ± 468	12 317 ± 2155
Area of blastocoel (μm ²)	—	3474 ± 1853	5017 ± 1284	—	2627 ± 1644	2892 ± 933
Area of inner cell mass (μm ²)	—	2490 ± 836	4201 ± 1323	—	2287 ± 966	4882 ± 1154

^aData were obtained through image analysis, and values presented are least squares mean ± SEM. No differences were observed in any of the characteristics measured ($P > 0.1$).

Fig. 2. Growth patterns (number of nuclei counted per embryonic stage) of compact morulae, early blastocysts and blastocysts of (□) Meishan and (■) Yorkshire gilts on which image analysis was performed. Each bar represents the least squares mean ± SEM of the number of cells in embryos at each developmental stage. The number of embryos used to generate the mean is indicated inside each bar. Values with different letters are significantly different ($P < 0.01$) within a breed in the number of total cells from one developmental stage to the next. Asterisks denote differences ($P < 0.05$) between breeds for that developmental stage.

increase ($P < 0.01$) from the early blastocyst to the blastocyst stage. By the blastocyst stage, the total number of cells in Meishan and Yorkshire embryos differed ($P < 0.05$), and blastocysts from Meishan gilts had approximately 11 fewer cells than did Yorkshire blastocysts. The reduction in the total number of cells observed in Meishan blastocysts was due entirely to a decrease in the number of trophectoderm cells (44.1 ± 2.71 versus 52.55 ± 3.68 ; $P < 0.05$) and not to a difference in the number of inner cell mass cells (10.80 ± 1.46 versus 13.63 ± 2.01 ; $P > 0.10$) in Meishan versus Yorkshire blastocysts, respectively.

As the number of cells of the inner cell mass increased, the number of trophectoderm cells in Meishan embryos accumulated more slowly ($y = 1.40x + 19.25$; $r^2 = 0.77$) than those of Yorkshire embryos ($y = 2.82x + 16.18$; $r^2 = 0.83$) resulting in regression lines (Fig. 3) with different ($P < 0.02$) slopes. The

Fig. 3. Distribution of trophectoderm cells as the number of inner cell mass cells increase in (●) Yorkshire and (▲) Meishan embryos. Each point on the graph represents an average of 2–8 Yorkshire embryos collected from each of 2–7 Yorkshire gilts or an average of 3–10 Meishan embryos collected from each of 2–8 Meishan gilts.

inner cell mass cell to trophectoderm cell ratio increased in Meishan embryos from the compact morula to the early blastocyst stage (0.29 and 0.36, respectively) and then decreased at the blastocyst stage (0.25). Yorkshire embryos demonstrated a different pattern of growth from Meishan embryos with a continuous decrease in the inner cell mass cell to trophectoderm cell ratio from the compact morula (0.32) to the early blastocyst (0.30) and blastocyst (0.25) stages of development.

Discussion

Although it is known that embryos from Meishan pigs contain fewer cells than do contemporary Yorkshire embryos throughout the preimplantation period (day 6: Youngs *et al.*, 1993; and day 12: Anderson *et al.*, 1993; Youngs *et al.*, 1994), the present study is the first to report the specific cell type(s) involved. To accomplish this goal, we modified a differential staining technique (originally adapted for human embryos) that enabled the number of inner cell mass and trophectoderm cells comprising

Meishan and Yorkshire embryos to be quantified and compared. The embryonic developmental stages observed in this experiment (compact morula, early blastocyst and blastocyst) were comparable to those found by other investigators on the same days of early gestation in the pig (Heuser and Streeter, 1929; Papaioannou and Ebert, 1988; te Kronnie and de Boer, 1993). In addition, image analysis data provided corroboration of similarities in morphology between Meishan and Yorkshire embryos at each developmental stage, consistent with previous findings (Youngs *et al.*, 1993). Furthermore, the number of cells comprising individual embryos from the compact morula to the blastocyst stages of development were in the range of those reported previously for pig embryos by other researchers (Papaioannou and Ebert, 1988; te Kronnie and de Boer, 1993; Rath *et al.*, 1995; Tao *et al.*, 1995).

These data demonstrated that the lower number of cells observed in Meishan versus Yorkshire blastocysts was predominantly due to fewer trophoctoderm cells. In addition, breed differences were observed in the pattern of embryonic development from the compact morula to the blastocyst stage. As embryos developed from the compact morula to the early blastocyst stage, both the inner cell mass cell and trophoctoderm cell populations increased in Meishan embryos. In contrast, no increases were observed between these stages in Yorkshire embryos. From the early blastocyst to the blastocyst stage, however, patterns of embryonic cell growth were similar in both Meishan and Yorkshire embryos, as the only observed change was an increase in the number of trophoctoderm cells. Although the number of Yorkshire compact morulae was limited, data from the present study are consistent with those of Heuser and Streeter (1929), who reported that the increase in the total number of cells observed from day 5 to day 6 for pig embryos reflected increases in smaller and more flattened outer cells (that is, trophoctoderm cells).

In a number of studies performed in our laboratory over a period of years, we have consistently found a reduced total number of cells in Meishan compared with Yorkshire (or white crossbred) embryos. Meishan early blastocysts have fewer cells than do Yorkshire early blastocysts (Youngs *et al.*, 1993), and Meishan embryos possess fewer cells than Yorkshire embryos on day 12 following natural mating (Anderson *et al.*, 1993) or embryo transfer (Youngs *et al.*, 1994). However, data on the number of cells contained by day 7–9 Meishan and Yorkshire embryos have not been collected owing to limitations of currently available technologies (that is, differential staining and DNA analysis). During our refinement of the differential staining technique we found that it was very difficult to count > 200 cells (that is, embryos beyond day 7 of gestation). Furthermore, DNA analysis is not sufficiently sensitive to assess DNA in individual embryos before day 10 of gestation. Recently, however, we have analysed day 10.5 blastocysts of Meishan and Yorkshire gilts and found that, consistent with our observations on day 6 and day 12 embryos, Meishan embryos contained less DNA than did Yorkshire embryos (S. P. Ford, unpublished). In addition, Wilson *et al.* (1995) demonstrated that the mitotic rate (as measured by proliferating cell nuclear antigen) of trophoctoderm cells was significantly lower in day 11.5 Meishan versus Yorkshire blastocysts. Thus, the lower number of trophoctoderm cells observed in day 12 Meishan embryos seems to result from both a slower

mitotic rate and a smaller initial population of trophoctoderm cells at the time of blastocyst formation.

Data on the comparative growth rate of preimplantation embryos from Meishan and European pig breeds have been collected in other laboratories. Bazer *et al.* (1988) reported that Meishan embryos were smaller than Large White embryos on day 8 but were larger than Large White embryos on days 10–12. In contrast, Wilmut *et al.* (1992) found no difference in the size or developmental stage of Meishan and European pig embryos collected 18–219 h after the estimated time of ovulation. The results of the present study are difficult to compare directly with those of Bazer *et al.* (1988) and Wilmut *et al.* (1992) because of asynchrony between breeds in the estimated time of ovulation and, hence, the time of embryo collection. Had the Meishan embryos in the present study been collected several hours later than Yorkshire embryos, similar results may have been obtained. In several studies, however, no obvious breed differences in the time of ovulation have been observed between our Meishan and Yorkshire females (Anderson *et al.*, 1993; Youngs *et al.*, 1993, 1994).

Although experiments are needed to test the hypothesis, we propose that events occurring during early embryogenesis directly influence embryonic survival and litter size in the Meishan breed. Meishan embryos with a reduced number of trophoctoderm cells (present study) apparently give rise to day 12 embryos that possess fewer cells and contain less oestradiol (Anderson *et al.*, 1993) than do embryos from European pig breeds. A reduced embryonic oestradiol secretion presumably results in a more gradual change in uterine environment that is more conducive to embryonic survival (Pope, 1992). Furthermore, Meishan embryos elongate from a reduced total number of cells (Youngs *et al.*, 1994) and develop into conceptuses that contain approximately 50% as many cells and are about 70% as long on day 14 as Yorkshire embryos (Wilson *et al.*, 1995). These observations are biologically consistent with numerous other studies reporting a reduction in conceptus size for Meishan versus European pig breeds at day 30 (Ashworth *et al.*, 1990, 1992; Ford *et al.*, 1994) and day 90 of gestation (Rivera *et al.*, 1994), as well as at term (Biensen *et al.*, 1995).

The number of trophoctoderm cells present at elongation may dictate the maximum length of an embryo and, thus, the space within the uterine horn it ultimately occupies. It has been suggested that each fetus in European breeds of pig requires a certain length of uterine horn to survive (Knight *et al.*, 1977; Dziuk, 1985; Wu *et al.*, 1989). Furthermore, about 10% of prenatal mortality in domestic pigs occurs after day 30 of gestation as a result of fetal overcrowding (Bazer *et al.*, 1969; Fenton *et al.*, 1972; Dziuk, 1985). Bazer *et al.* (1988) reported no breed differences in uterine horn length and width, or in endometrial surface area, on day 30 of gestation between Meishan and Large White gilts. However, they demonstrated that embryo survival and average litter size favoured Meishan over Large White gilts, suggesting that conceptus factors rather than uterine morphology played an important role in embryonic and fetal survival. Collectively, these data suggest that a reduction in the number of trophoctoderm cells during early embryogenesis may result in a reduced placenta size and an increased litter size in prolific breeds such as the Meishan.

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