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Abstract
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Lack of effect of sex on pig embryonic development *in vivo*

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The effect of sex on pig conceptus development to day 12 of gestation was investigated. On day 2 of gestation, reciprocal embryo transfers were performed resulting in four groups (Yorkshire–Yorkshire, Yorkshire–Meishan, Meishan–Yorkshire and Meishan–Meishan). Conceptuses at day 12 were recovered from each recipient and diameter, as well as DNA, protein and oestradiol content were determined for individual conceptuses. The sex of individual conceptuses at day 12 was determined by amplification of a fragment of the pig *SRY* gene, using the polymerase chain reaction. Embryos developed more rapidly to day 12 in Yorkshire recipients, but there was no detectable effect of sex on the diameter, DNA, protein or oestradiol content of conceptuses from any transfer group. Thus, no sex effect was apparent under conditions either promoting or retarding the rate of early pig blastocyst growth. These results provide strong evidence that pig embryonic development occurs at a rate determined by uterine environment and not by sex of the conceptus.

Introduction

It is widely accepted that for many mammals males grow faster and are larger than females owing to the higher concentrations of circulating androgens synthesized by the testes. Testicular androgen synthesis is developmentally regulated and, although increases in testicular steroidogenesis accompany puberty, significant production occurs periodically at much earlier stages of development. Enzymes directly involved in androgen synthesis are present at high concentrations in pig fetal testes at the time of parturition and at the start of the second trimester, consistent with increases in fetal androgen concentrations at these times (Conley *et al.*, 1994). However, it has been suggested that there are sex-associated differences in growth rates before differentiation of the fetal gonads, and that increased growth rates are exhibited by male embryos. Mittwoch (1969) showed that the early differentiation of the male rat gonad is preceded by an enhanced growth rate of the undifferentiated testes. Faster growth rates of male over female rat and mouse preimplantation embryos have been demonstrated by Scott and Holson (1977), Valdivia *et al.* (1993) and Tsunoda *et al.* (1985), and similar data have been reported for cattle (Avery *et al.*, 1989; Xu *et al.*, 1992; Yadav *et al.*, 1993) and pigs (Cassar *et al.*, 1994).

Breed differences in birth weights argue strongly for genetic effects on embryonic and fetal development. Maternal breed is a major component of this effect which is mediated by changes in the uterine environment. Recent experiments in our laboratory, using cross-transfer of Meishan and Yorkshire embryos, illustrate this point (Ford and Youngs, 1993; Youngs *et al.*, 1994). The rate of development of Meishan embryos to day 12 was accelerated in uteri of Yorkshire gilts and embryonic development of Yorkshire pigs was depressed in uteri of Meishan gilts. It is hypothesized that the rate of embryonic development can affect embryo survival in pigs, particularly when differences in growth rate occur within a litter, leading to developmental asynchrony among littermates (Pope, 1988). This is particularly relevant at day 12, when embryonic oestrogen synthesis occurs, because oestrogen induces changes in uterine histotroph (Geisert *et al.*, 1990). Therefore, advanced embryos may initiate oestrogen synthesis first and alter uterine environment in a way that disadvantages further development of less advanced littermates. The potential for faster growth of male pig embryos, perhaps coupled to the earlier onset of oestrogen synthesis, suggests that sex may influence survival at day 12. Furthermore, this may be more apparent when embryonic growth is accelerated or retarded.

Therefore, the objective of this study was to investigate the effects of sex on embryonic development under conditions promoting or retarding growth rate, by re-examining the day 12 Meishan and Yorkshire embryos collected in our previous cross-transfer experiments. This study was made possible by the development and validation of a polymerase chain reaction (PCR)-based technique for the detection of the Y chromosome in small samples of pig tissues.

Materials and Methods

Animals

Meishan and Yorkshire gilts of similar reproductive age (first 2–5 postpubertal oestrous cycles) were checked for oestrus daily and donor gilts were hand-mated to a boar of the same breed at the onset of oestrus (day 0) and 24 h later. Recipient

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gilts were treated similarly but were not mated. All embryo collections and transfers were performed as described by Youngs *et al.* (1994).

Collection of day 12 embryos

Yorkshire recipient gilts were anaesthetized with 6.6 mg thiamylal sodium kg^{-1} body mass administered i.v., whereas Meishan gilts received an i.m. injection of ketamine hydrochloride (1.1 mg kg^{-1} body mass). Gilts were maintained on a mixture of O_2 and halothane (2–5% Fluothane) on a closed-circuit system. Embryos were flushed from the uteri with PBS, as described by Youngs *et al.* (1994). After collection, the diameter of each embryo was determined with an ocular micrometer. Each embryo was then individually centrifuged at 11 750 ***g*** to facilitate pellet formation After removing the excess fluid, embryos were lyophilized and resuspended in 1 ml high salt buffer (PBS with 2 mol NaCl l^{-1} and 2 mmol EDTA l^{-1}), sonicated for 30 s and frozen at −80°C until assayed for oestradiol, DNA and protein. The remainder of the embryo sample was frozen for determination of sex by PCR as described below.

Assays

Embryonic tissue content of oestradiol as well as the protein and DNA content of individual day 12 conceptuses were determined, and these data were published by Youngs *et al.* (1994). The remaining day 12 conceptus tissue was used for determination of embryonic sex (see below). The oestradiol content of day 12 conceptuses was performed using the fully characterized antiserum (No. 030073, Eli Lily Inc., Indianapolis, IN, oestradiol antiserum; Youngs *et al.*, 1994). Inter- and intra-assay coefficients of variation (CVs) for pooled day 30 allantoic ($n = 5$) and amniotic ($n = 4$) fluid were 8.0% and 4.7% and 3.3% and 4.9%, respectively. The sensitivity of the assay, defined as the amount of steroid yielding 95% of the radioactivity in control tubes, was 2 pg per tube.

Identification of sex of embryo by polymerase chain reaction

After completion of protein and DNA assays, sonicated day 12 conceptuses were prepared for PCR analysis. Although the sex of all embryos was determined, only those from litters containing six or more embryos were used in subsequent analyses. For optimum amplification, preparation necessitated dialysis against TE (10 mmol Tris l^{-1}, 1 mmol EDTA l^{-1}) for 24 h. The dialysate was centrifuged at 11 750 ***g*** for 1 min in a microcentrifuge; the supernatant was removed; the pellet was resuspended in 400 µl PBS and then centrifuged once again for 1 min. The pellet was resuspended in 100 µl sterile water. Twenty microlitres of the sample was then added to 200 µl of InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and incubated for 30 min in a 56°C shaking water bath. The sample was vortexed at high speed (10 s), placed in a boiling water bath (8 min), vortexed again and centrifuged for 2 min. Five microlitres (20–50 ng DNA) of the supernatant was used for each PCR amplification.

All PCR reactions were performed using a Perkin Elmer Cetus (Norwalk, CT) Gene Amp Core Reagent Kit in a 25 µl final reaction volume overlaid with 35 µl mineral oil. InstaGene purified embryo samples were coamplified with sex-determining region Y (*SRY*) and Zinc Finger Y (*ZFY*, X chromosome linked homologue) oligonucleotide primers (20 pmol), reaction buffer (10%), 100 µmol of each deoxyribonucleotide triphosphate, 1.15 mmol $MgCl_2$ and 0.2 units Taq polymerase. The pig *SRY* and *ZFY* genes were cloned and sequenced in our laboratory (Pomp *et al.*, 1995). Each amplification consisted of a 2.5 min hot start followed by 35 cycles of a 1 min 95°C denaturation, a 1°C annealing and a 1 min 72°C extension. Polymerase chain reaction products were resolved by electrophoresis on a 2.5% agarose gel and stained with ethidium bromide (10 mg ml^{-1} in buffer). The accuracy of this technique was determined in an independent experiment in which chorioallantoic membrane was collected from 41 fetuses from day 90 of gestation, whose sex was visually apparent. Samples were prepared as described above, except that they were coded so that identification of sex by PCR was performed in a blind test.

Statistical analysis

Data were analysed using the general linear models (GLM) procedure of SAS (1985). Individual day 12 conceptus data were averaged by sex within litter to examine differences in littermate diversity. The independent variables for analysis of day 12 included sex of embryo, embryo breed and recipient breed. Dependent variables for day 12 conceptuses included diameter (mm), DNA (µg per embryo), protein (µg per embryo) and oestradiol content (pg per embryo).

Results

After optimization of PCR conditions, single bands representing products of the *SRY* (157 bp) or *ZFY* (445 bp) genes were observed in all day 90 chorioallantoic membranes sampled. This enabled the successful identification of sex in all cases (25 male and 16 female samples). Male tissues exhibited a strong band at 157 bp and a fainter band at 445 bp, whereas in female tissues only a band at 445 bp was obvious. This banding pattern was identical to that obtained when male and female genomic DNA was used as template (Fig. 1).

The data collected from day 12 conceptuses by Youngs *et al.* (1994) were re-evaluated in the present study with respect to sex. Recovery and developmental parameters were re-calculated to ensure that the exclusion of the conceptuses from the smaller litters (22% of those originally collected) would not eliminate the previously observed growth differences. Recovery rates were similar across recipient breeds (Meishan 73%, $n = 69$; Yorkshire 75%, $n = 77$) for those embryos sexed, and the results of the analysis of this subset were the same as those obtained previously, with recipient breed having a major influence on embryo development. Specifically, embryos collected from Meishan recipients were much less developed with respect to embryo diameter ($P < 0.001$), DNA ($P < 0.001$) and protein ($P < 0.001$) than

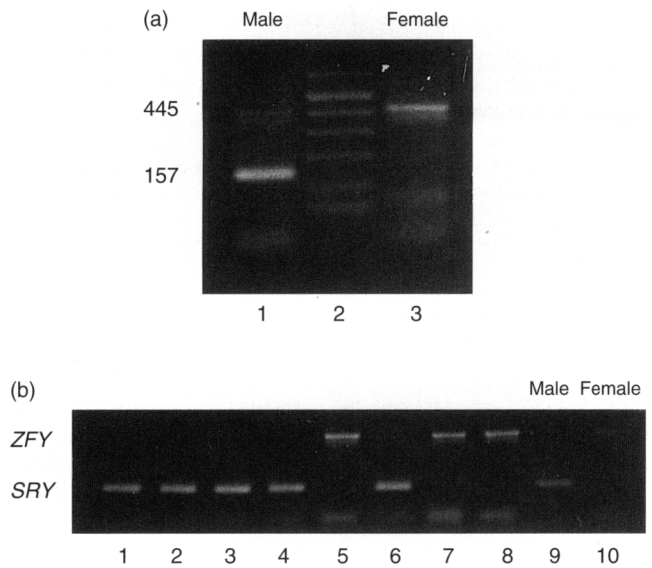

Fig. 1. Determination of pig embryonic sex by PCR. (a) Lane 1: pig male genomic DNA. Lane 2: molecular weight markers. Lane 3: pig female genomic DNA. Bands at 445 base pairs (bp) indicate the amplification product for the nonspecific sequence of the *ZFY* gene. The band at 157 bp is the amplification product for the male specific *SRY* gene. (b) PCR amplification products for (lanes 1–8) a litter of day 12 pig conceptuses, lanes 9 and 10 are the amplification products of male and female genomic DNA, respectively.

embryos recovered from Yorkshire recipients, regardless of the breed of embryos (Table 1). The oestradiol content of embryos collected from Yorkshire recipients was also greater than that of embryos collected from Meishan recipients ($P < 0.001$; Table 1). In addition, there was a significant uterine type by embryo breed interaction ($P < 0.005$), whereby Meishan embryos recovered from Yorkshire uteri had a greater oestradiol content than those of any other transfer group (Table 1). Therefore, the embryos sexed reflected the larger group in terms of the previously noted maternal effects on development and differentiation with respect to oestradiol content.

The potential effect of sex on embryonic growth was investigated at day 12 of gestation. Despite the demonstration of differences in embryo development induced by recipient breed at day 12, no effect of sex was observed on diameter, DNA or protein content of embryos from any of the transfer groups. In all, these analyses included examination of a total of 146 embryos collected from 12 litters on day 12, comprising 83 males and 63 females. Although diameter, protein and DNA content of male conceptuses were numerically higher in three of the four transfer groups, these differences were not significant ($P > 0.4$). Similarly, no differences were observed between male and female embryos with respect to oestradiol content.

Discussion

Reports in several mammals, including pigs (Cassar *et al.*, 1994), have demonstrated that the development of male embryos occurs more rapidly than for females early in gestation. However, contrary to the results of Cassar *et al.* (1994), no differences in embryo development could be detected between male and female preimplantation pig embryos in the present study. The reason for this discrepancy in results is unknown, although several methodological differences are notable. The determination of embryonic sex by PCR (Pomp *et al.*, 1995), as verified in the present study, allows for the accurate and repeatable amplification of a segment of the Y chromosome-specific *SRY* gene from a minimal quantity of DNA. Cassar *et al.* (1994) were able to sex only 58% of the embryos examined, and no indication was given of the accuracy of the technique. In the present experiment, PCR was demonstrated to be 100% accurate in determining the sex of 42 fetuses collected on day 90 of gestation. In addition, in contrast to the surface area measurement used by Cassar *et al.* (1994) as an index of blastocyst development, we used several parameters, including diameter, DNA and protein content. It is impossible to consider differences in embryo development between studies, because no actual size data were reported in the study by Cassar *et al.* (1994). Furthermore, size categories were arbitrarily established in that study, within which sex

Table 1. Influence of sex, embryo breed and uterine type on development of day 12 pig conceptuses

Sex	Embryo breed	Uterine type	*n*	Diameter* (mm)	DNA* (μg)	Protein* (μg)	Oestradiol*† (pg per embryo)
Female	Meishan	Meishan	5	3.56 ± 0.18	2.47 ± 0.22	42.87 ± 5.67	228.67 ± 25.71
Male	Meishan	Meishan	5	4.01 ± 0.16	3.13 ± 0.10	61.18 ± 3.74	327.30 ± 67.89
Female	Meishan	Yorkshire	4	6.17 ± 0.79	6.32 ± 0.45	147.14 ± 10.89	1585.74 ± 170.56
Male	Meishan	Yorkshire	4	5.47 ± 0.45	6.23 ± 0.70	153.80 ± 6.75	1764.38 ± 301.03
Female	Yorkshire	Meishan	2	4.41 ± 0.26	3.22 ± 0.49	67.59 ± 22.28	485.53 ± 167.00
Male	Yorkshire	Meishan	2	5.05 ± 0.05	4.00 ± 0.52	82.19 ± 19.99	628.40 ± 149.10
Female	Yorkshire	Yorkshire	3	5.99 ± 0.94	6.66 ± 0.68	165.91 ± 13.60	1346.69 ± 248.16
Male	Yorkshire	Yorkshire	3	5.74 ± 1.43	5.97 ± 1.19	160.23 ± 26.99	1134.23 ± 308.73

n = Number of litters averaged.
*Effect of uterine type ($P < 0.001$).
†Effect of uterine type × embryo breed ($P < 0.05$).

ratios were analysed, whereas diameter, protein and DNA content were treated as continuous variables in the present study.

The lack of effect of sex on embryonic development suggested by the results of the present study are also consistent with at least two other reports. Pomp *et al.* (1995) examined a total of 209 pig embryos collected on day 10 or 11 of gestation, but could not detect differences in diameter between males and females. Similarly, 8–16-cell pig embryos cultured *in vitro* matured to the blastocyst stage at equivalent rates irrespective of sex (Stumpf *et al.*, 1994). In addition, unpublished data from our laboratory demonstrate that sex has no effect on fetal or placental mass in day 30 embryos, although at this stage of development we have examined too few embryos to draw firm conclusions. These data appear to be consistent with the relatively small differences in masses between male and female piglets at birth, which average 2–5% and require extremely large samples to demonstrate statistical significance (Bereskin *et al.*, 1973; Rohde Parfet *et al.*, 1990). It remains possible that differences in growth rates between male and female pig embryos may be evident only under certain uterine conditions or at much later developmental stages.

The present study evaluated embryos at day 12, whose rate of development had been retarded or accelerated by reciprocal transfer between Meishan and Yorkshire breeds, as reported by Youngs *et al.* (1994). Consistent with the reports of Pomp *et al.* (1995) and Stumpf *et al.* (1994), the present study failed to detect significant differences in the development of male versus female pig conceptuses. Therefore, we suggest that pig embryonic growth is not influenced by sex during the preimplantation stage of development, and thus may differ fundamentally from that of cattle and rodents.

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