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Associations between fetal size, sex and both proliferation and apoptosis at the porcine feto-maternal interface

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ABSTRACT

Introduction: Inadequate fetal growth has severe consequences for both neonatal and adult development. It is hypothesised that the feto-maternal interface associated with the lightest and male fetuses will undergo more apoptosis and less proliferation than those supplying the closest to mean litter weight (CTMLW) and female fetuses respectively.

Methods: Placental and endometrial samples associated with the lightest and CTMLW (gestational day (GD) 18 and 30), male and female (GD45, 60 and 90) Large White X Landrace conceptuses or fetuses were obtained. The mRNA expression of candidate genes involved in apoptosis or proliferation (*BAX*, *BCL2*, *P53* and *KI67*) was quantified by qPCR. TUNEL staining was performed on placental samples supplying the lightest and CTMLW fetuses (GD45 and 60), of both sex (GD60).

Results: Placentas associated with the lightest fetuses had decreased *P53* and *KI67* expression compared to the CTMLW fetuses at GD45. At GD60, *P53* expression was increased in placentas supplying the lightest compared to CTMLW fetuses. *P53* expression was increased in endometrial samples associated with the lightest compared to the CTMLW fetuses at GD45. At GD30 and GD60 respectively, *BAX* expression was increased and *BCL2*, *P53* and *KI67* expression were decreased in endometrial samples associated with females compared to their male littermates. TUNEL staining revealed no association between fetal size or sex, and apoptotic cell number.

Discussion: This study has highlighted dynamic associations between fetal size, sex, and apoptosis and proliferation at the porcine feto-maternal interface. Further studies should be performed to improve the understanding of the mechanisms behind these findings.

1. Introduction

The establishment and maintenance of pregnancy requires significant changes in uterine structure and function, including the formation of new blood vessels in both the endometrium and placenta. Apoptosis can occur by activation of either extrinsic or intrinsic pathways [1]. The intrinsic apoptosis pathway is tightly regulated by pro- and anti-apoptotic members of the B-cell lymphoma 2 (BCL2) family of proteins [1]. In response to cell stressors, tumour suppressor protein 53 (P53) is upregulated which initiates the intrinsic pathway.

Apoptosis has been described in the human placenta where it is essential for trophoblast invasion, differentiation and survival [2–9]. Similarly, apoptosis and proliferation have been suggested to play a role in implantation in the pig [10–13]. As pigs exhibit non-invasive epitheliochorionic placentation, extensive remodelling must occur at the porcine feto-maternal interface to ensure that adequate nutrient transfer can occur to meet fetal demands [14]. This remodelling is likely to involve extensive apoptosis and proliferation. Cristofolini et al. [15], demonstrated that apoptotic cells are present in the porcine placenta throughout gestation and that the number of apoptotic cells relative to the total cell number in placental villi was associated with gestational day (GD). A high number of apoptotic cells was observed at GD28, presumably reflecting the extensive remodelling that occurs at the feto-maternal interface during placentation. They also suggested that BCL-2-associated X protein (BAX) is expressed at the feto-maternal interface in early and late gestation, which would provide further evidence for the activation of the intrinsic apoptotic pathway.

Appropriately regulated proliferation is essential for the establishment and maintenance of a successful pregnancy. Several factors known to be expressed at the feto-maternal interface during implantation have been shown to activate Phosphatidylinositol-3 kinase (PI3K) - protein kinase B (AKT) and Mitogen-activated Protein Kinase (MAPK) signaling pathways to induce proliferation and/or migration of porcine trophectoderm cells *in vitro* [reviewed by Ref. [16]]; highlighting the role

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of proliferation in pregnancy establishment in the pig.

Dysregulated apoptosis has been linked to pregnancy complications including gestational trophoblast disease [17,18], preeclampsia [19–23] and intrauterine growth restriction (IUGR) [21,24–30]. Placentas associated with term IUGR human infants have increased apoptosis compared to those supplying normally-grown infants [27,31]. Decreased placental BCL2 expression [29,30], accompanied by increased BAX [29] expression has been observed in term IUGR placentas compared to those supplying normally-grown infants. In the pig, it has been suggested on a protein level that components of the proliferation pathway are downregulated, accompanied by increased apoptotic stress in placentas associated with IUGR fetuses compared to normal-body weight fetuses at GD60, 90 and 110 [32].

Recent studies have revealed sexual dimorphism in human placentas [33,34], with fetal sex influencing the expression of placental genes and the inflammatory response [35,36]. Intriguingly, sexual dimorphism has been demonstrated in P53 knockout mice, with decreased implantation rate, pregnancy rate and litter size observed when matings were carried out using female P53 null mice, but not with male P53 null mice [37]. On occasions where human pregnancy is complicated by preeclampsia and IUGR, male offspring have increased perinatal mortality and morbidity [36,38]. Although it is proposed that male new-born piglets have a survival disadvantage compared to their female littermates [39], the possibility of sexual dimorphism in porcine placental development is poorly understood. However, recent investigations in our laboratory have revealed striking relationships between fetal sex and both placental and endometrial vascularity [40].

This study aimed to improve the understanding of the relationship between fetal size, sex and apoptosis and proliferation at the porcine feto-maternal interface. It is hypothesised that IUGR in the pig occurs due to aberrant conceptus attachment. Specifically, it is hypothesised that apoptosis and proliferation pathways will be up- and down-regulated respectively in the feto-maternal interface associated with the lightest compared to the closest to mean litter weight (CTMLW) conceptus or fetus and that the feto-maternal interface associated with male fetuses will have increased apoptosis and decreased proliferation compared to those supplying female fetuses throughout gestation.

2. Materials and methods

All procedures were performed with approval from The Roslin Institute (University of Edinburgh) Animal Welfare and Ethical Review Board and in accordance with the U.K. Animals (Scientific Procedures) Act, 1986.

2.1. Experimental animals and sample collection

Large White X Landrace gilts (age 11–14 months; n = 31) were observed daily for signs of oestrus and were housed in groups of 6–8 animals per pen. Oestrous cyclicity and ovarian function were controlled in accordance with routine normal practice at The Roslin Institute Large Animal Unit. In a subset of gilts (distribution between the GD investigated indicated in Supplementary Table 1) oestrus was synchronised by daily feeding of 20 mg Altrenogest (Regumate, Hoechst Roussel Vet Ltd., Milton Keynes, U.K.) for 18 days followed by injection of pregnant mare serum gonadotrophin (PMSG, Intervet UK Ltd, Milton Keynes, U.K.) and human chorionic gonadotrophin (hCG; Intervet UK Ltd, Milton Keynes, U.K.) [41]. All gilts were inseminated twice daily for the duration of oestrus with semen from one of four sires (Large White). The sires used were equally distributed throughout the GD to attempt to minimise the effect of sire. The first day of insemination was assigned as GD0 and samples were obtained at GD18, 30, 45, 60 and 90 (n = 5, 6, 6, 11, and 8 respectively). Gilts were euthanised at the GD of interest with sodium pentobarbitone 20% w/v (Henry Schein Animal Health, Dumfries, U.K.) at a dose of 0.4 ml/kg by intravenous injection via a cannula inserted in the ear vein. Following confirmation of death, mid-ventral incision revealed the reproductive tract. The tract was lifted from the body cavity and placed in a dissecting tray. Both uterine horns were dissected from the ovary towards the cervix.

At GD18, the uterine tract was rinsed with saline and pieces of string were used to tie the end of the right and left uterine horns at the bifurcation. The uterine horns were cut between the two pieces of string and each uterine horn was placed in a floatation device containing a solution to preserve the integrity of the RNA. This solution was prepared by dissolving 700 g ammonium sulphate (SLS, Nottingham, U.K.) in 935 ml of RNase free water. Once dissolved, 25 ml of 1 M sodium citrate (Fisher Scientific, Loughborough, U.K.) and 40 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA) were added, and the solution was adjusted to pH 5.2 using concentrated sulphuric acid. Using dissection scissors, the uterine horn was opened along the mesometrial side, and the conceptuses floated upwards in the solution. The conceptuses were removed and weighed in a cryovial (Starlab, Milton Keynes, U.K.). The uterine lumen was occluded between each conceptus to ensure that endometrial samples associated with individual conceptuses could be identified. The lightest and CTMLW conceptus was identified based on weight, and endometrial samples were taken from each conceptus of interest. Samples were snap-frozen in liquid nitrogen and stored at −80 °C for RNA extraction or fixed in Bouin's (Sigma Aldrich, St Louis, Missouri, U.S.A.) for histology.

On the remaining GD investigated, the uterine lumen was occluded between each feto-placental unit by tying with string to ensure that tissues associated with individual conceptuses or fetuses could be identified later. Fetuses were identified as 'live' or 'dead' based on their morphology at the time of dissection and were weighed. At GD45, 60 and 90, sex was determined morphologically. DNA was isolated from the GD30 fetuses using the DNeasy Blood and Tissue DNA extraction kit (Qiagen, Manchester, U.K.), and PCR was performed for the sex-determining region Y (SRY) region of the Y chromosome [42]. The lightest and CTMLW fetus (GD30), of both sex (GD45, 60 and 90) were identified based on fetal weight. From the anti-mesometrial side, placental and endometrial samples were taken from each feto-placental unit of interest and snap-frozen in liquid nitrogen or fixed in Bouin's (Sigma Aldrich).

2.2. Analysis of candidate gene expression by qPCR

2.2.1. Total RNA extraction and cDNA synthesis

RNA was extracted from 20 to 50 μg of tissue from snap-frozen samples as previously described [43], with the addition of a DNase treatment step (RNase-free DNase, Qiagen, Manchester, U.K.). The RNA was quantified spectrophotometrically using a Nanodrop ND-1000 (Labtech International Ltd., Heathfield, U.K.), and the quality assessed electrophoretically using a Tapestation 2200 (Agilent Technologies, Edinburgh, U.K.) (Supplementary Table 2). If the RINe value obtained was lower than the desired ranges (Supplementary Table 2), the sample was excluded from the analyses.

Complementary DNA (cDNA) was prepared from 0.3 μg of RNA with SuperScript III reverse transcriptase (Life Technologies, ThermoFisher Scientific, Altrincham, U.K.). Each reaction contained 250 ng random primers (Promega, Southampton, U.K.) and 40 units RNaseIn (Promega, Southampton, U.K.). Negative controls without reverse transcriptase were included to check for genomic contamination. Reverse transcription was performed in duplicate for each sample and pooled.

2.2.2. Relative expression of candidate genes

Quantitative PCR was performed on a Stratagene MX3000 instrument using Platinum SYBR Green SuperMixUTG (Life Technologies, ThermoFisher Scientific, Altrincham, U.K.) using cDNA from placental samples at GD30, 45, 60 and 90, and endometrial samples at GD18, 30, 45, 60 and 90 (n = 5, 6, 6, 6 and 8 litters respectively). The samples were associated with the lightest and CTMLW conceptuses or fetuses at GD18 and 30, and the lightest and CTMLW fetuses of both sex at GD45,