CSIRO PUBLISHING

Reproduction, Fertility and Development, 2015, **27**, 655–666
http://dx.doi.org/10.1071/RD14293

Arginine increases development of *in vitro*-produced porcine embryos and affects the protein arginine methyltransferase–dimethylarginine dimethylaminohydrolase–nitric oxide axis

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Abstract. Culture systems promote development at rates lower than the *in vivo* environment. Here, we evaluated the embryo's transcriptome to determine what the embryo needs during development. A previous mRNA sequencing endeavour found upregulation of solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (*SLC7A1*), an arginine transporter, in *in vitro*- compared with *in vivo*-cultured embryos. In the present study, we added different concentrations of arginine to our culture medium to meet the needs of the porcine embryo. Increasing arginine from 0.12 to 1.69 mM improved the number of embryos that developed to the blastocyst stage. These blastocysts also had more total nuclei compared with controls and, specifically, more trophectoderm nuclei. Embryos cultured in 1.69 mM arginine had lower *SLC7A1* levels and a higher abundance of messages involved with glycolysis (hexokinase 1, hexokinase 2 and glutamic pyruvate transaminase (alanine aminotransferase) 2) and decreased expression of genes involved with blocking the tricarboxylic acid cycle (pyruvate dehydrogenase kinase, isozyme 1) and the pentose phosphate pathway (transaldolase 1). Expression of the protein arginine methyltransferase (PRMT) genes *PRMT1*, *PRMT3* and *PRMT5* throughout development was not affected by arginine. However, the dimethylarginine dimethylaminohydrolase 1 (*DDAH1*) and *DDAH2* message was found to be differentially regulated through development, and the DDAH2 protein was localised to the nuclei of blastocysts. Arginine has a positive effect on preimplantation development and may be affecting the nitric oxide–DDAH–PRMT axis.

Additional keywords: amino acid, arginine transporter, culture, gene expression, metabolism, Warburg effect.

Received 9 August 2014, accepted 14 February 2015, published online 13 March 2015

Introduction

Culture *in vitro* is at the heart of many assisted reproductive technologies. However, current systems still do not adequately mimic an *in vivo* environment, resulting in reduced blastocyst and pregnancy rates (Kikuchi *et al.* 1999). In addition, genetic and epigenetic effects due to culture are well documented (for a review, see Fleming *et al.* 2004). Therefore, to produce an ideal culture system, there is a need to understand what the embryo needs *in vivo*. In an effort to identify ways to improve culture conditions, a next-generation sequencing analysis was completed using *in vivo*-produced embryos that were cultured to the blastocyst stage *in vitro* (IVC) or *in vivo* (IVV; Bauer *et al.* 2010). The arginine transporter solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (*SLC7A1*) was found to be upregulated by at least 63-fold in IVC compared with IVV blastocyst stage embryos. Arginine is a vital amino acid for many metabolic processes in the cell, such as protein synthesis, creatine production, polyamine synthesis and nitric oxide (NO) generation (Wheatley and Campbell 2003). Removal of arginine from the culture medium by either medium formulation or arginase treatment quickly leads to death in 80% of tumour cell lines (Scott *et al.* 2000). Because early rapidly dividing embryos appear to be metabolically similar to cancer cells (Krisher and Prather 2012; Redel *et al.* 2012), we hypothesised that *in vitro*-produced embryos also require significant amounts of arginine.

Arginine is used to produce NO by NO synthases (NOS). There are three NOS isoforms, namely neuronal (NOS1), inducible (NOS2) and endothelial (NOS3). Expression of all three NOS isoforms has been detected in murine and bovine preimplantation embryos from the zygote to blastocyst stage (Tranguch *et al.* 2003; Tesfaye *et al.* 2006). L-Arginine is depleted from porcine embryo culture medium *in vitro*, suggesting that it is metabolised

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during embryonic development (Humpherson *et al.* 2005). Manser and Houghton (2006) used the NO-sensitive probe 4-amino-5-methylamino-2′,7′-difluorofluorescien diacetate (DAF-FM-DA) and found that NO was present at all stages of murine preimplantation development. Supplementation of culture media with additional arginine also improves porcine embryo development, suggesting that this amino acid plays a critical role in preimplantation development (Bauer *et al.* 2010).

NO production is regulated, in part, by the production of endogenous NOS inhibitors through the protein arginine methyltransferase (PRMT)–dimethylarginine dimethylaminohydrolase (DDAH)–NO axis. Proteins that contain arginine methylation via PRMTs release methylated arginine residues upon proteolysis. These residues, specifically monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA), then act on NOS within the cell to reduce NO production. DDAH1 and DDAH2 degrade excess MMA and ADMA within the cell. The *DDAH1* null mutation is embryonic lethal, whereas *DDAH2*-null mice reproduce normally (Breckenridge *et al.* 2010). This reveals an important role for DDAH and proper regulation of NO in the early embryo.

In the present study we investigated these pathways in embryos that were produced *in vitro* and show that arginine can enhance the development of these embryos and that these embryos are developmentally competent. We also present evidence supporting a functional PRMT–DDAH–NO axis in early porcine embryonic development.

Materials and methods

Chemical components

Unless indicated otherwise, all chemical components were purchased from Sigma Chemical (St Louis, MO, USA).

In vitro *embryo production*

Prepubertal porcine oocytes were obtained from ovaries collected from a local slaughterhouse and were subjected to *in vitro* maturation as described previously (Zhang *et al.* 2010). Cumulus–oocyte complexes (COCs) were aspirated from follicles of ovaries collected from the local slaughterhouse. COCS were selected on the basis of multiple layers of cumulus cells and evenly distributed cytoplasm; they were a washed in Tyrode's lactate (TL) HEPES medium supplemented with 0.1% polyvinyl alcohol (PVA). Between 200 and 250 COCs were cultured in 2 mL maturation medium (TCM-199 with 0.1% PVA, 3.05 mM glucose, 0.91 mM sodium pyruvate, 10 μg mL^{-1} gentamicin, 0.57 mM cysteine, 10 ng mL^{-1} epidermal growth factor, 0.5 μg mL^{-1} LH and 0.5 μg mL^{-1} FSH) for 42–44 h in a humidified atmosphere with 5% CO_2 in air at 38.5°C. Forty four hours after culture in maturation medium, mature oocytes were identified by extrusion of a polar body and washed in modified Tris-buffered medium (mTBM) containing 2 mg mL^{-1} bovine serum albumin (BSA) and 2 mM caffeine (IVF medium). Thirty oocytes were placed into 50-μL droplets of IVF medium covered with mineral oil and incubated at 38.5°C until spermatozoa were added. The spermatozoa used for fertilisation were obtained from a single boar and were used throughout the entire experiment. For IVF, a 0.1-mL frozen semen pellet was thawed in 3 mL sperm washing medium (Dulbecco's phosphate-buffered saline (dPBS; Gibco, Grand Island, NY, USA) supplemented with 0.1% BSA). Spermatozoa were washed twice by centrifugation. The sperm pellet was resuspended with fertilisation medium to 0.5×10^6 cells mL^{-1}. Finally, 50 μL sperm suspension was added to the oocytes in IVF medium, giving a final concentration of 0.25×10^6 cells mL^{-1}. Spermatozoa and oocytes were incubated together for 5 h.

Embryo culture

After fertilisation, oocytes were removed from the droplets and washed in porcine zygote medium 3 (PZM3; Yoshioka *et al.* 2002). Fifty presumptive zygotes were then cultured in each well of a four-well dish in PZM3 in a humidified atmosphere with 5% CO_2 in air for 28–30 h at 38.5°C. After the 28–30 h culture, embryos that cleaved and were at the 2- to 4-cell stage were selected and 15 cleaved embryos were moved to 25-μL droplets in one of five treatment groups: (1) PZM3 (0 mM arginine); (2) PZM3 control (0.12 mM arginine); (3) PZM3 (0.36 mM arginine); (4) PZM3 (0.72 mM arginine); or (5) PZM3 (1.69 mM arginine), hereafter referred to as MU1. Embryos were cultured in a humidified atmosphere of 5% CO_2, 90% N_2 and 5% O_2 at 38.5°C until Day 6. The concentration of 1.69 mM arginine is the highest physiological concentration found in Day 3 oviductal fluid in gilts (Li *et al.* 2007) and thus was used as our high arginine concentration *in vitro*. On Day 6 after fertilisation, blastocysts from each treatment group were collected and the percentage of embryos that had developed to the blastocyst stage was recorded. To determine the effect of arginine on development, the percentage of blastocysts in each treatment group was analysed using PROC GENMOD in SAS (SAS Institute, Cary, NC, USA). A least significant difference (LSD) post-test comparison was performed to determine whether significant differences existed between treatment groups, with significance set at two-tailed $P < 0.05$. For subsequent experiments, embryos were cultured in one of three treatment groups: (1) PZM3 (0 mM arginine); (2) PZM3 control (0.12 mM arginine); or (3) MU1. The blastocysts were then collected and used for RNA isolation or stained for determination of nuclear number.

Differential nuclear staining

A comparison of the number of trophectoderm (TE) and inner cell mass (ICM) nuclei for embryos cultured in each of the three treatment groups was conducted after differential nuclear staining using procedures described previously (Macháty *et al.* 1998). Briefly, the zona pellucida was removed using physiological saline lowered to pH 1.79. Zona-free embryos were exposed to a 1 : 7 dilution of rabbit anti-pig whole serum for 60 min (Bauer *et al.* 2010). The embryos were then washed three times for 5 min each time in TL HEPES medium. Finally, embryos were incubated in a 1 : 10 dilution of the guinea-pig complement containing 10 μg mL^{-1} propidium iodide and 10 μg mL^{-1} bisbenzimide for 35 min. The embryos were then observed under ultraviolet (UV) light at ×40 magnification using a Nikon Eclipse E600 inverted microscope (Nikon, Tokyo, Japan). ICM nuclei stained blue, whereas TE nuclei stained pink. Mean ICM, TE, total cell number and the ratio of

TE/ICM were first analysed for normality using the UNIVARIATE procedure in SAS. The data were then $\log_2$ transformed and analysed by analysis of variance using the MIXED procedure in SAS. An LSD post-test comparison was then performed for each variable to determine whether significant differences ($P < 0.05$) existed between groups.

Extraction and amplification of RNA for real-time polymerase chain reaction of SLC7A1

Three replicates were obtained for each treatment group, namely PZM3 (0 mM arginine), PZM3 control (0.12 mM arginine) and MU1. Total RNA was extracted from pools of 10 embryos in each replicate using the AllPrep Genomic DNA/RNA Micro Isolation Kit (Qiagen, Germantown, MD, USA). Total RNA was suspended in 12 μL of RNase free water, and 5 μL was amplified using the WT-Ovation Pico RNA Amplification System (NuGEN Technologies, San Carlos, CA, USA). After amplification, samples were purified using Micro Bio-Spin P-30 Columns (Bio-Rad Laboratories, Hercules, CA, USA). Real-time polymerase chain reaction (PCR) was then performed using these amplified embryo pools to determine message abundance of *SLC7A1* and genes that regulate the Warburg effect (WE; Bauer *et al.* 2010).

Relative real-time PCR

Relative real-time PCR was conducted on each of the amplified samples for the genes involved with the WE and embryo metabolism using IQ SYBR Green Supermix (Bio-Rad Laboratories) and the amplified cDNA from each biological replicate (diluted to 5 ng μL^{-1}) as template. Primers were designed using Integrated DNA Technology (Coralville, IA, USA) software and real-time PCR was run in triplicate for every biological replicate on the MyiQ Single-Colour Real-Time PCR Detection System (Bio-Rad Laboratories) to verify the differential abundance of the chosen transcripts. Primer efficiency tests were completed for each primer set by generating a standard curve using 1 : 10 dilutions of the 5 ng μL^{-1} reference cDNA pool. Real-time PCR was run in triplicate for each concentration (5, 0.5 and 0.05 ng μL^{-1}) to validate each primer set. Abundance was calculated for each mRNA transcript as described by Bauer *et al.* (2010) relative to the reference sample and the housekeeping gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma (*YWHAG*; Whitworth *et al.* 2005; Bauer *et al.* 2010). *YWHAG* has been used as a stable reference message in many of our pig oocyte and embryo studies. Whitworth *et al.* (2005) found, by both microarray and real-time PCR, that *YWHAG* expression did not differ among samples, and Bauer *et al.* (2010) found that *YWHAG* expression did not differ between treatments in mRNA deep sequencing. For these reasons, *YWHAG* was used as our reference gene in the present study. The reference sample contained four biological replicates of *in vivo*-fertilised and then *in vivo*-cultured blastocysts and *in vivo*-fertilised and then *in vitro*-cultured blastocysts pooled together (Bauer *et al.* 2010). Expression levels between treatments were determined using the comparative threshold cycle (C_T) method for each gene. The $2^{-\Delta\Delta CT}$ values were analysed for normality before being log transformed if not normally distributed. The resulting values were then analysed using the general linear model (PROC GLM) in SAS. The significance of differences in expression was evaluated using the least-squares means (LSMeans) generated by PROC GLM; significance was set at $P < 0.05$.

Quantitative real-time PCR of PRMT *and* DDAH *transcripts*

To examine the expression of *PRMT1*, *PRMT3* and *PRMT5*, as well as *DDAH1* and *DDAH2*, quantitative real-time PCR analysis was performed on three pools each of 18–22 MII oocytes, 4-cell embryos and blastocyst stage embryos cultured in either control (0.12 mM arginine) or MU1 (1.69 mM arginine). Briefly, RNA was isolated using the Dynabeads mRNA Direct micro kit (Life Technologies, Carlsbad, CA, USA) and cDNA was synthesised using Superscript III (Life Technologies). Real-time PCR was performed using iQ SYBR Green Supermix (BioRad) and run on a BioRad platform using a two-step protocol with melting curve analysis. Reactions were run in triplicate with the following program: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. A melting curve analysis followed, with temperature increments of 0.3°C from 55 to 95°C. Quantitative real-time PCR data were analysed using the $\Delta\Delta C_t$ method with comparison against *YWHAG* expression as an endogenous control (Whitworth *et al.* 2005). Relative mean expression was compared by analysis of variance (ANOVA) after log transformation.

Immunocytochemistry

To examine DDAH2 protein localisation, Day 6 blastocysts were collected and the zona pellucida removed using low-pH (pH 1.79) PBS. Embryos were then fixed for 20 min in 4% paraformaldehyde, washed twice in TL HEPES medium and held at 4°C until processing. After permeabilisation in 0.1% Triton in PBS for 3 h at 37°C, embryos were washed through PBS with 0.01% Tween and 3% BSA and then incubated in 2 M HCl for 30 min at room temperature. This was followed by incubation in 100 mM TRIS-HCl, pH 8.5, for 10 min at room temperature. Embryos were then blocked for 1 h at room temperature (blocking buffer 0.01% Tween and 3% BSA) before being incubated overnight in primary antibody (1 : 100; goat polyclonal DDAH2; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking buffer. After three 10-min washes with PBS containing 0.01% Tween and 3% BSA (with rocking), embryos were incubated in secondary antibody (1 : 250; donkey anti-goat Alexa fluor 488; Life Technologies). After three 10-min washes with rocking, embryos were counterstained with propidium iodide and mounted using Prolong Antifade (Life Technologies). Images were taken at 12-μm cross-sections using a Zeiss (Zeiss United States, Thornwood, NY, USA) LSM 510 META NLO two-photon point scanning confocal microscope.

DAF-FM imaging and fluorescence analysis

For DAF imaging, cleaved embryos were cultured in one of four treatments: (1) PZM3 control (0.12 mM arginine); (2) MU1; (3) 50 μM Arginine N-Methyltransferase Inhibitor-1 (AMI-1) (PRMT1 inhibitor) in 0.12 mM arginine; or (4) 100 μM ADMA (NOS inhibitor) in 0.12 mM arginine. Embryos were moved 30 h after insemination to 25-μL culture drops and cultured to either Day 2 (4-cell) or Day 6 (blastocyst). On Day 2 or 6, embryos

were washed through TL HEPES medium and into 500-μL wells of PZM3 with 0.1% PVA (no BSA) plus 10 μM DAF-FM-DA (Life Technologies). Embryos were incubated in DAF-FM-DA for 40 min in 5% O_2 at 38.5°C and then washed into PZM3 without DAF-FM-DA and incubated for an additional 15 min in 5% O_2 at 38.5°C. For imaging, embryos were placed individually in 5-μL drops of TL HEPES medium under mineral oil, held in place using a microinjection holding pipette and imaged with a Nikon inverted fluorescence microscope. All treatments were present throughout the incubation and imaging steps. Images were taken using an exposure time that was the average for embryos treated with 5 mM N^G-nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor, to control for background fluorescence. The resulting images were analysed using ImageJ (National Institutes of Health, Bethesda, MD, USA), and corrected fluorescence intensity values were calculated according to Burgess *et al.* (2010) as integrated density – (area of embryo measurement × background). Values were then log transformed and compared using PROC GLM ANOVA with fixed effects of treatment and IVF group.

Embryo transfer

Day 6 after fertilisation, blastocysts cultured in MU1 were selected and placed in 3 mL manipulation medium (9.50 g TCM-199, 0.05 g NaHCO3, 0.75 g Hepes, 1.76 g NaCl, 3.00 g BSA, 1 mL gentamicin and 1000 mL Milli Q H_2O) in polystyrene tubes (BD Falcon 352054, San Jose, CA, USA). Embryos were transported at 37°C to the University of Missouri Swine Research Complex, where they were loaded with a minimal volume of medium into a tomcat catheter and surgically transferred to the ampullary–isthmic junction of the cycling gilt on Day 5 or 6 of her oestrous cycle (Spate *et al.* 2010; Lee *et al.* 2013). Pregnancy was monitored by ultrasound after Day 25 and checked weekly until the gilt returned to oestrus or farrowed. Sex and birthweights were recorded.

Results

Supplementing our current culture (PZM3 with 0.12 mM arginine) with 1.69 mM arginine increased the development of porcine embryos to the blastocyst stage above no arginine (Table 1), and increased the number of TE nuclei and total nuclei above the control PZM3 without significantly changing the ratio of ICM/TE (Table 2). The higher arginine concentration (1.69 mM) in culture produced embryos that were developmentally competent because they produced live piglets (Table 3). A comparison of term development from these two culture systems was beyond the scope of the present study.

Message for *SLC7A1* was decreased in embryos cultured in 1.69 mM arginine compared with 0 or 0.12 mM arginine (Fig. 1). The abundance of two WE-related genes, namely transaldolase 1 (*TALDO1*) and pyruvate dehydrogenase kinase, isozyme 1 (*PDK1*), were decreased by culture in the presence of 1.69 mM arginine, whereas expression of hexokinase 1 (*HK1*), hexokinase 2 (*HK2*) and glutamic pyruvate transaminase (alanine aminotransferase) 2 (*GPT2*) was increased (Table 4).

The abundance of message for *PMRT1* and *PMRT3* decreased from the MII to 4-cell stage, whereas expression of *PRMT5* increased from the MII to 4-cell stage (Fig. 2). The abundance of *PRMT1* then increased from the 4-cell to blastocyst stage. There was no effect on *PRMT1*, *PRMT3* or *PRMT5* message abundance if embryos were cultured in the presence of 0.12 or 1.69 mM arginine. There was no difference in the

Table 1. Blastocyst development depends on arginine concentration during culture

Two-cell stage embryos were cultured in various concentrations of arginine to the blastocyst stage and assessed. Data are the mean ± s.e.m. of five replicates ($n = 148$, 163, 150, 120 and 134). Within columns, values with different superscript letters differ significantly ($P \leq 0.02$). PZM3, porcine zygote medium 3

Treatment	% Blastocysts
PZM3 + 0.0 mM arginine	$57 \pm 10^{b,c}$
PZM3 + 0.12 mM arginine	50.9 ± 9.8^{c}
PZM3 + 0.36 mM arginine	$67 \pm 11^{a,b}$
PZM3 + 0.72 mM arginine	$67 \pm 12^{a,b}$
PZM3 + 1.69 mM arginine	70 ± 11^{a}

Table 3. Pregnancy results for Day 6 blastocyst transferred to recipient gilts

Piglet no.	Sex	Birth weight (kg)	Wean weight (kg)
1	Male	0.9	3.7
2	Male	1.1	6.6
3	Male	1.3	7.9
4	Male	0.5	Died (runt)
5	Female	0.8	Crushed by mother

Table 2. Effects of arginine concentration on the number of nuclei in blastocyst stage embryos

Data are the mean ± s.e.m. Within columns, values with different superscript letters differ significantly ($P \leq 0.03$). Values shown are untransformed values; however, statistical analyses were performed on log-transformed data. ICM, inner cell mass; TE, trophectoderm; PZM3, porcine zygote medium 3

Treatment	No. ICM nuclei	No. TE nuclei	Total cell number	TE : ICM ratio
PZM3 + 0 mM arginine ($n = 20$)	5.8 ± 0.5^{a}	26.0 ± 2.2^{a}	31.8 ± 2.3^{a}	4.7 ± 0.5
PZM3 + 0.12 mM arginine ($n = 27$)	7.1 ± 0.3^{b}	25.2 ± 1.5^{a}	32.3 ± 1.6^{a}	3.6 ± 0.3
PZM3 + 1.69 mM arginine ($n = 24$)	7.0 ± 0.4^{b}	31.8 ± 1.7^{b}	38.8 ± 1.8^{b}	4.6 ± 0.4

expression of *DDAH1* between the MII oocytes and 4-cell embryos, but both were higher than expression at the blastocyst stage (Fig. 3; $P < 0.01$). In contrast, *DDAH2* expression remained low in MII oocytes and 4-cell embryos, but was then fivefold higher by the blastocyst stage ($P < 0.01$). DDAH2 protein localisation was analysed using confocal imaging of Day 6 *in vivo*-derived, PZM3 (0.12 mM arginine) and MU1 cultured IVF embryos. The DDAH2 protein was localised to the nucleus in both *in vivo*- and *in vitro*-produced blastocysts, and this localisation appeared to be less abundant in Day 5 *in vitro*-produced blastocysts cultured in MU1 compared with Day 5 blastocysts cultured in 0.12 mM arginine (Fig. 4). In both treatments, DDAH2 nuclear localisation appeared to be lower in ICM cells compared with TE. A similar pattern of protein expression was seen in Day 6 *in vivo*-derived blastocysts.

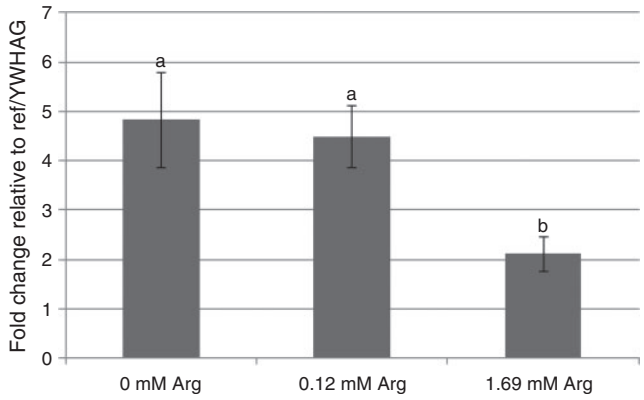

Fig. 1. Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (*SLC7A1*) transcript levels depend on arginine concentration. Data are the mean ± s.e.m. Different letters represent significant differences in *SLC7A1* message abundance ($P \leq 0.0001$). The values shown are untransformed values; however, statistical analyses were performed on log-transformed data.

To explore the role of NO production during development, 2-cell embryos were cultured in 2, 5 and 10 mM L-NAME until the blastocyst stage. At 5 and 10 mM, L-NAME inhibited development to the blastocyst stage ($P < 0.05$; Fig. 5). NO production in 4-cell *in vitro*-produced embryos was estimated using DAF-FM fluorescence compared with embryos cultured with 5 mM L-NAME (Fig. 6). Quantification of fluorescence on Day 2 of culture (4–6 cells) revealed an increase in NO production in embryos cultured in either 0.12 mM arginine (PZM3) or 1.69 mM arginine (MU1) compared with embryos cultured in 0.12 mM arginine plus 5 mM L-NAME. There was also a tendency for greater NO production in 1.69 mM arginine-treated embryos compared with those treated with 0.12 mM arginine treatment, however the difference did not reach statistical significance ($P = 0.11$). AMI-1, an inhibitor of class 1 PRMTs, and ADMA dihydrochloride did not reduce

Table 4. Primer sequences used for real-time polymerase chain reaction

PKM2, Pyruvate kinase, muscle; *M1*, Pyruvate kinase M1 variant; *M2*, Pyruvate kinase M2 variant; *SLC2A2*, solute carrier family 2 (facilitated glucose transporter), member 2; *HK1*, hexokinase 1; *HK2*, hexokinase 2; *TKT*, transketolase; *PDK1*, pyruvate dehydrogenase kinase, isozyme 1; *PDK3*, pyruvate dehydrogenase kinase, isozyme 3; *GPT2*, glutamic pyruvate transaminase (alanine aminotransferase) 2; *LDHA*, lactate dehydrogenase A; *TALDO1*, transaldolase 1; *YWHAG*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma; *PRMT1*, protein arginine methyltransferase 1; *PRMT3*, protein arginine methyltransferase 3; *PRMT5*, protein arginine methyltransferase 5; *DDAH1*, dimethylarginine dimethylaminohydrolase 1; *DDAH2*, dimethylarginine dimethylaminohydrolase 2

Transcript Annotation	Accession no.	Primers	
		Sense	Antisense
PKM2 M1 and *M2*	XM_003356683[A] and XM_001929069[B]	GAAGATGATGATTGGGCGGTGCAA	AGTCAGCTCCATCCAAGACTGCAT
PKM2 M1	XM_003356683[A]	TGCTGGAGAGCATGATCAAGAAGC	TGCCAGACTCCGTCAGAACTATCA
PKM2 M2	XM_001929069[B]	ATGCAGTCTTGGATGGAGCTGACT	ATTCAAATGGTAGATGGCGGCCT
SLC2A2	NM_001097417	TTCATGTCGGTGGGACTTGTGCTA	AATTGCGGGTCCAGTTGCTGAATG
HK1	NM_001012668	TCTTGATCGACTTCACCAAGAGGG	TCGCTCTCGATCTGCGAGAGATACTT
HK2	NM_001122987	GAATTTGATGCGGCCGTGGATGAA	CCAGGTACATGCCGCTGATCATTT
TKT	NM_001112681	AAGCGATGGTGTGGCTACAGAGAA	GGACCACCTTGGCTTGTCCAATTT
PDK1	NM_001159608	ACCAGGACAGCCAATACAAGTGGT	ACGTGGACTTGAATAGGCGGGTAA
PDK3	XM_001927439	TTTGCCCCTGACAACTTAGGCCTGA	TAAGAGACAGGAGGGGCACTGGGA
GPT2	XM_003126995	AGGAGTCCTTTGAGCAGTTCAGCA	GCAGTGAATTCCCGGAACTTGGTT
LDHA	NM_001172363	TTCAGCCCGGTTCCGTTACCTAAT	TTCTTCAGGGAGACACCAGCAACA
TALDO1	XM_003122399	TGAAGCGGCAGAGGATGGAGAGC	TCGTCGATGGCGTTGAAGTCGC
YWHAG	NM_012479	TCCATCACTGAGGAAAACTGCTAA	TTTTTCCAACTCCGTGTTTCTCTA
PRMT1	XM_003127320.1	TGATTCCTACGCTCACTTCGGCAT	GTGCCGGTTGTGAAACATGGAGTT
PRMT3	XM_003122919.1	TCAGCTCATACGGGCATTATGGGA	GCTCCAGCTTTCGCAGCAAACATA
PRMT5	NM_001160093.1	TCCTCAAGTTGGAGGTGCAGTTCA	AAGAGTTCGTAGGCATTGGGTGGA
DDAH1	XM_003125937	AGGACAAATCAACGAGGTGCTGAG	TCATCTGCTGCATGATCTTGAGGG
DDAH2	KP696756	TGGATGGCACCGATGTTCTCTTCA	ACGATCTCAGCTCCACGGTGATT

[A]Ensembl Transcript ID: ENSSSCT00000002161.
[B]Ensembl Transcript ID: Q29582_PIG (ENSSSCT00000002160).

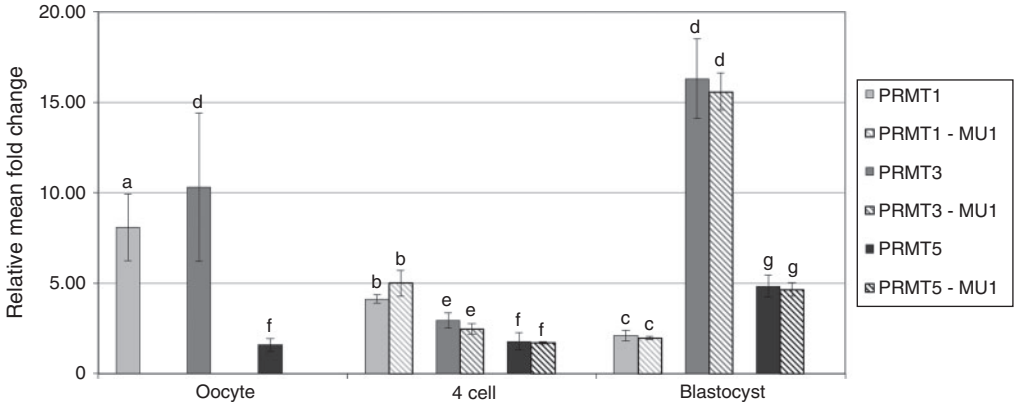

Fig. 2. Abundance of protein arginine methyltransferase 1 (*PRMT1*), protein arginine methyltransferase 3 (*PRMT3*) and protein arginine methyltransferase 5 (*PRMT5*) in oocytes, 4-cell embryos and blastocysts. Two-cell stage embryos were cultured in control porcine zygote medium 3 (PZM3; 0.12 mM arginine) or PZM3 plus 1.69 mM arginine. Data are the mean ± s.e.m. Bars with different letters within gene of interest are differ significantly ($P < 0.05$; a,b,c = *PRMT1*, d,e = *PRMT3*, f,g = *PRMT5*).

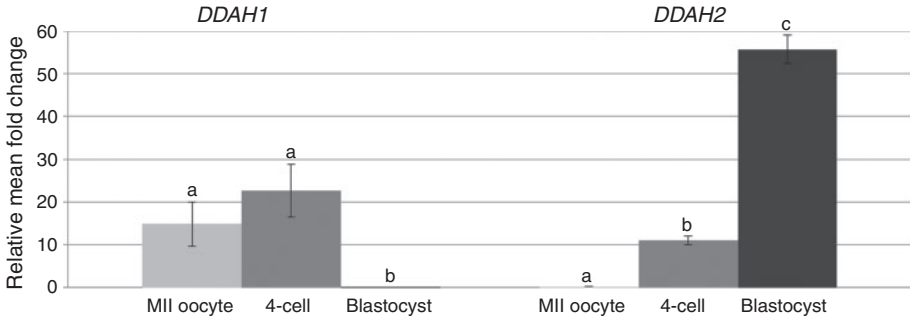

Fig. 3. Abundance of message for dimethylarginine dimethylaminohydrolase 1 (*DDAH1*) and dimethylarginine dimethylaminohydrolase 2 (*DDAH2*) in oocytes, 4-cell embryos and blastocysts. Data are the mean ± s.e.m. The values shown are untransformed relative mean fold changes; however, statistical analyses were performed on log-transformed data. Bars with different letters within gene of interest are significantly different (*DDAH1* $P < 0.01$; *DDAH2* $P < 0.05$).

DAF-FM fluorescence in embryos cultured in 0.12 mM arginine. Culture of embryos treated with DAF-FM-DA to Day 6 resulted in a 70% blastocyst rate (data not shown), not only confirming that developmentally competent embryos were chosen for imaging, but also that the DAF did not have a negative effect on development.

Discussion

Our overall goal is to better understand the metabolism of the embryo so that embryo culture conditions can be improved to increase the efficiency of pig production. One way that this can be done is by assessing the transcriptional profile of IVC and IVV embryos. A previous transcriptional profiling study found a transcript involved with arginine transport was increased in IVC compared with IVV embryos (Bauer *et al.* 2010). Using those data, we assessed the effect additional arginine would have on embryo development *in vitro*. Here, we provide evidence illustrating the positive effect that the addition of arginine to porcine embryo culture can have on development.

Arginine is a nutritionally essential amino acid for conceptus growth and development (Wu *et al.* 2010). Arginine is a precursor not only for protein synthesis, but also for NO, urea, proline, glutamate, creatine and agmatine (Wu and Morris 1998). Most of the L-arginine is transported by the Na^+-independent system y+ for cationic amino acids into cells. This cationic amino acid transporter (CAT) system contains three different members encoded by the *SLC7A1*, *SLC7A2*, and *SLC7A3* genes. Message for *SLC7A1* and *SLC7A2* has been previously identified in mouse preimplantation embryos (Van Winkle 2001). The *SLC7A1* message has also been detected in ovine conceptuses between Days 13 and 18 of pregnancy (Gao *et al.* 2009), and in porcine conceptuses on Days 12 and 15 of pregnancy (Bazer *et al.* 2013). More recently, *SLC7A1* was found to be the key transporter of arginine by ovine TE (Wang *et al.* 2014*a*). Using *in vivo* morpholino antisense oligonucleotide-mediated knockdown of *SLC7A1*, Wang *et al.* (2014*a*) found that the conceptuses were retarded with abnormal function compared with controls. They concluded that arginine is essential for conceptus survival and development (Wang *et al.* 2014*a*).

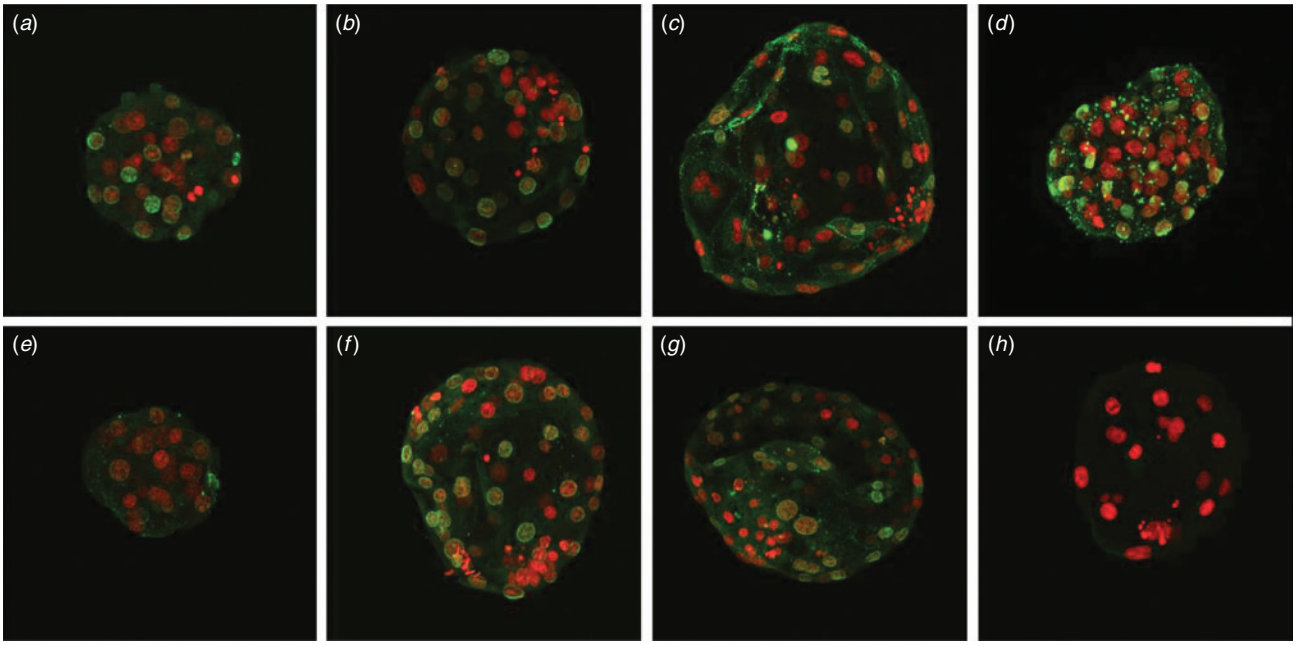

Fig. 4. Dimethylarginine dimethylaminohydrolase 2 (DDAH2) protein localisation in *in vitro*- and *in vivo*-derived blastocysts. Embryos were cultured in porcine zygote medium 3 (PZM3) supplemented with either 0.12 mM arginine (*a*–*c*) or 1.69 mM arginine (*e*–*g*), or were *in vivo* derived on Day 6 (*d*). Embryos were processed for immunocytochemistry on Day 5 (*a*, *e*), Day 6 (*b*, *f*) and Day 7 (*c*, *g*) of *in vitro* culture. Green indicates antibody localisation, red indicates propidium iodine staining of DNA. (*h*) The secondary antibody only control is also shown for comparison.

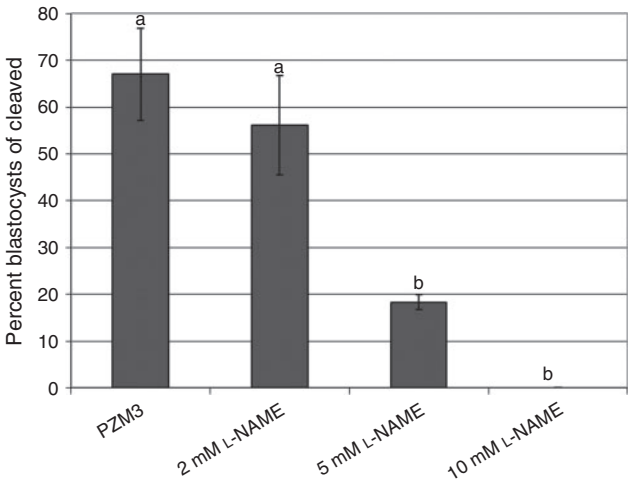

Fig. 5. Effects of nitric oxide synthase inhibition on blastocyst development. Two-cell embryos were cultured to the blastocyst stage in porcine zygote medium 3 (PZM3; 0.12 mM arginine) or PZM3 (0.12 mM) containing 2, 5 or 10 mM N^G-nitro-L-arginine methyl ester (L-NAME). Data are the mean ± s.e.m. of four replicates ($n = 20$, 26, 16, 18). Bars with different letters differ significantly ($P < 0.05$).

In a previous study, a threefold greater concentration of arginine than used in PZM3 was added to evaluate the effect on *SLC7A1* message levels (Bauer *et al.* 2010). Increasing arginine from 0.12 to 0.36 mM and culturing *in vivo*-produced 2-cell embryos to the blastocyst stage decreased the *SLC7A1* message level to that seen in *in vivo* embryos; however, *SLC7A1* expression did not differ from what was seen following *in vitro* culture with 0.12 mM arginine (Bauer *et al.* 2010). Therefore, a higher concentration of arginine was used to evaluate the effects on *in vitro*-produced embryo development. Five different treatments were used in the present study: PZM3 with 0, 0.12 (control), 0.36, 0.72 or 1.69 mM arginine. Embryos were also cultured in the presence of 2.5 and 5 mM arginine to see whether there was even more of an improvement in embryo development; however, there was no difference compared with embryos cultured in 1.69 mM arginine (data not shown), so 1.69 mM was used for the remainder of the experiments. Li *et al.* (2007) characterised the concentrations of amino acids in porcine oviductal and uterine fluid on Day 3 and Day 5 after insemination and found that the concentration of arginine ranged from 0.22 mM in Day 5 uterine fluid to 1.69 mM in Day 3 oviductal fluid. For this reason, 1.69 mM arginine, which is 14-fold higher than the control concentration, was used as the high physiological level of arginine. Embryos cultured with 1.69 mM arginine had decreased levels of *SLC7A1* message compared with embryos cultured in 0 or 0.12 mM arginine. This validates our hypothesis that by adding arginine to our current culture medium, we will decrease the expression of this arginine transporter. It appears as though the embryo is trying to overcompensate for the lack of arginine in the 0.12 mM arginine-containing medium and upregulating *SLC7A1* message compared with *in vivo* embryos.

Adding additional arginine to culture improved the percentage of embryos that developed to the blastocyst stage. All three treatments cultured with additional arginine had higher blastocyst percentages than the control embryos. Specifically, embryos cultured in 1.69 mM arginine had the highest percentage of blastocysts compared with each of the treatments, with 70% of

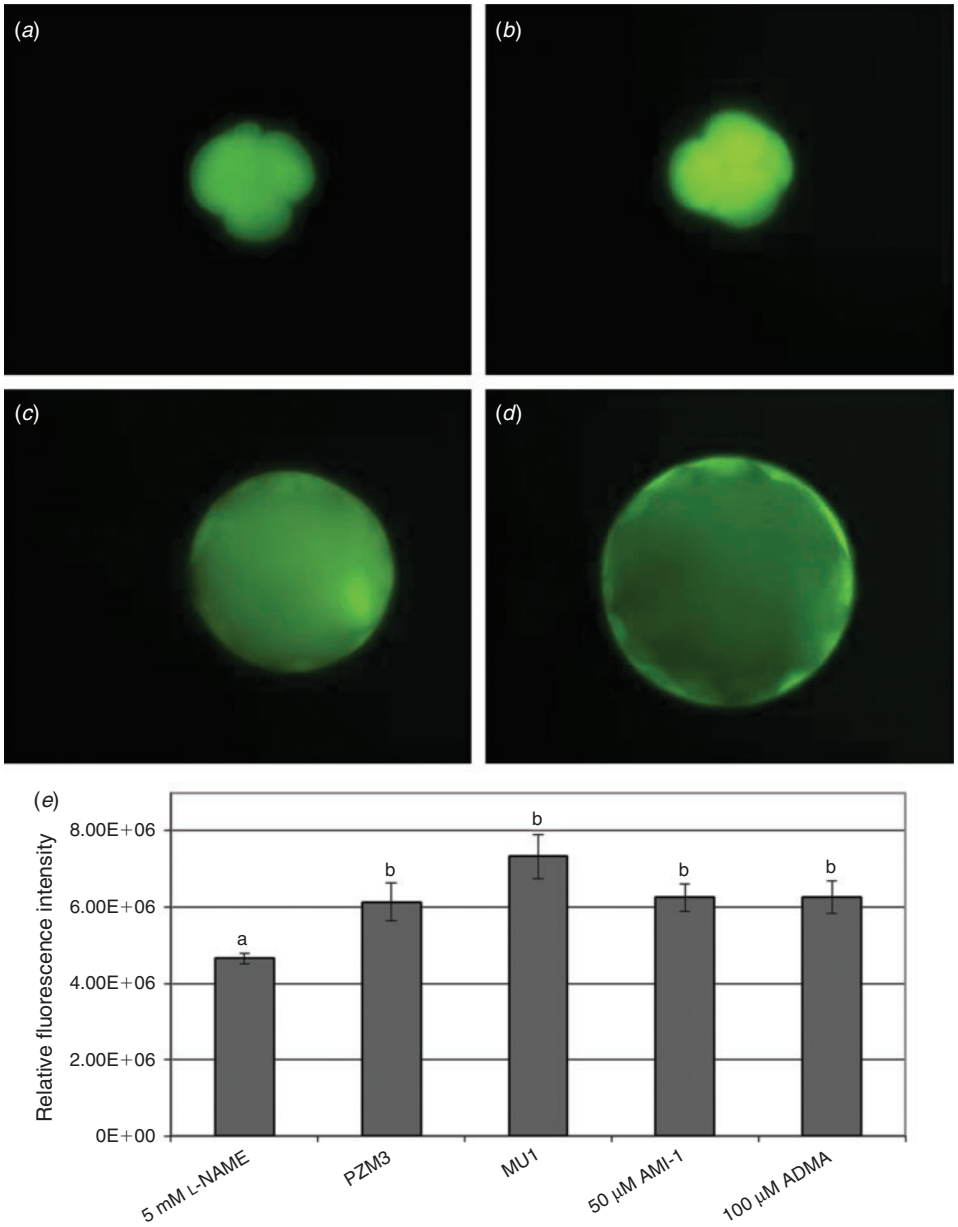

Fig. 6. Nitric oxide (NO) production viewed by using (*a–d*) 4-amino-5 methylamino-2′,7′-difluorofluorescien (DAF-DM) staining of 4-cell embryos and blastocysts treated with either 5 mM N^G-nitro-L-arginine methyl ester (L-NAME; *a*, *c*) or 1.69 mM arginine (*b*, *d*) and (*e*) relative intensity of DAF staining to measure NO production in embryos on Day 2 of culture. DAF intensity was determined in Day 2 (4–6-cell) embryos cultured in porcine zygote medium 3 (PZM3) containing 0.12 mM arginine or one of four other treatments: 5 mM L-NAME, 1.69 mM arginine (MU1), 0.12 mM arginine with 50 μM Arginine N-Methyltransferase Inhibitor-1 (AMI-1) or 0.12 mM arginine with 100 μM asymmetric dimethylarginine (ADMA). Data are the mean ± s.e.m. of five replicates ($n = 16, 19, 18, 21, 17$). Values shown are untransformed values; however, statistical analyses were performed on log-transformed data. Bars with different letters differ significantly ($P < 0.05$).

cleaved embryos developing into blastocysts (vs ~51% for controls). A few studies have completed amino acid profiling of porcine embryo culture media and found that arginine was depleted from the media (Booth *et al.* 2005, 2007; Humpherson *et al.* 2005). Booth *et al.* (2005) found that arginine was consistently depleted from the medium at each preimplantation stage of development and morulas producing ≥25% blastocysts had more arginine depleted than morulas producing ≤14% blastocysts. Depletion of arginine from the medium is consistent with what is illustrated here, in that providing the embryos additional arginine in culture can improve the blastocyst percentage.

An indicator of embryo quality is total cell number of the resulting blastocysts. Adding a higher concentration of arginine (1.69 mM) increased the average total cell number compared with control blastocysts. After differential staining, the two different cell types in the blastocyst were analysed and there was no effect on ICM number, but there was an increase in the TE number. A porcine TE-derived cell line was cultured in the presence or absence of arginine in a customised medium and when 2 mM arginine was added, there was increased cellular proliferation by approximately eightfold compared with cells cultured in the presence of 0 mM arginine (Kim *et al.* 2013). This, again, is consistent with what was demonstrated in the present study, because the TE cells appeared to be the cells with increased proliferation compared with controls. There was no difference in the number of ICM between control embryos or embryos cultured in the presence of high arginine.

Arginine has been shown to stimulate cell signalling via the AKT1/mammalian target of rapamycin complex (mTORC) 1/mTORC2 pathway to affect survival and development of the conceptus (Bazer *et al.* 2013). Arginine induces the phosphorylation of proteins in the mammalian target of rapamycin (mTOR) cell signalling pathway, including ribosomal protein S6 kinase (RPS6K) and ribosomal protein S6 (RPS6) in ovine TE cells (Kim *et al.* 2011), as well as in porcine TE cells (Kong *et al.* 2012; Kim *et al.* 2013). mTOR is a highly conserved serine/threonine protein kinase and is a regulator of mRNA translation. mRNA translation is a key event in the regulation of protein synthesis (Wu 2010). Porcine embryos undergo rapid proliferation, elongation and cellular remodelling, and we propose that the mTOR pathway stimulates TE proliferation, mRNA translation, protein synthesis and cytoskeletal remodelling (Kong *et al.* 2012; Bazer *et al.* 2013; Kim *et al.* 2013).

Like embryos, cancer cells have stimulated mTOR signalling to drive cellular proliferation (Guertin and Sabatini 2007). These cancer cells have an altered metabolism that illustrates the unique characteristics of the WE (Vander Heiden *et al.* 2009), that is, they have increased glucose uptake and decreased metabolism through the tricarboxylic acid (TCA) cycle. mTORC1 responds to the nutritional status of the cell and is often deregulated in cancer cells (Zoncu *et al.* 2011). Arginine has been shown to activate mTORC1 via the lysosome amino acid transporter solute carrier family 38, member 2 (SLC38A9) (Wang *et al.* 2015). Once mTORC1 is stimulated, AKT is activated, promoting glucose uptake (Howell and Manning 2011). Thus, arginine may be directly affecting the WE pathway. To determine whether arginine was affecting transcripts associated with the WE, real-time PCR was conducted on WE-defining genes (Krisher and Prather 2012; Redel *et al.* 2012; Table 5). There was an upregulation of *HK1* and *HK2*; these are hexokinases involved with the first step of glucose metabolism. *HK2* is the main hexokinase expressed in cancer cells and, in these Day 6 blastocysts, it is the more highly abundant compared with *HK1*. This is consistent with the WE because *HK2* is required for tumour initiation and maintenance in cancer cells (Patra *et al.* 2013). Cancer cells also have increased lactic acid and alanine production. Here, additional arginine caused an increase in the *GPT2* message in embryos, which is consistent with cancer cells metabolising pyruvate to alanine and away from the TCA cycle (Beuster *et al.* 2011). Additional arginine decreased *PDK1* expression. This enzyme is important in blocking pyruvate from entering the TCA cycle and shunting it towards alanine or lactate production. The *TALDO1* message was also decreased in embryos cultured in 1.69 mM arginine. This enzyme is part of the pentose phosphate pathway that, in rapidly dividing cells, is important for producing NADPH and ribose to assist with the increased cellular proliferation (Krisher *et al.* 2014). Although only gene expression was measured, and changes seen in transcript abundance do not always translate to changes in protein levels, the results do give a predictor of what could be occurring at the protein and/or enzyme level.

Table 5. mRNA abundance of various Warburg effect-related genes, as determined by real-time polymerase chain reaction in porcine blastocyst stage embryos

Data are the mean ± s.e.m. relative to the reference gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma (*YWHAG*). *PKM2*, Pyruvate kinase, muscle; *M1*, Pyruvate kinase M1 variant; *M2*, Pyruvate kinase M2 variant; *SLC2A2*, solute carrier family 2 (facilitated glucose transporter), member 2; *HK1*, hexokinase 1; *HK2*, hexokinase 2; *TKT*, transketolase; *TALDO1*, transaldolase 1; *PDK1*, pyruvate dehydrogenase kinase, isozyme 1; *PDK3*, pyruvate dehydrogenase kinase, isozyme 3; *GPT2*, glutamic pyruvate transaminase (alanine aminotransferase) 2; *LDHA*, lactate dehydrogenase A

Gene	0.12 mM Arginine	1.69 mM Arginine	*P*-value
PKM2 M1	0.000 ± 0.001	0.002 ± 0.001	0.15
PKM2 M2	0.46 ± 0.10	0.37 ± 0.10	0.88
PKM2 M1 and *M2*	0.41 ± 0.09	0.37 ± 0.09	0.77
SLC2A2	4.90 ± 0.63	4.90 ± 0.63	0.94
HK1	0.13 ± 0.08	0.72 ± 0.08	<0.0001
HK2	1.9 ± 0.4	3.6 ± 0.4	0.006
TKT	0.78 ± 0.15	0.95 ± 0.15	0.45
TALDO1	7.5 ± 0.9	3.6 ± 0.9	0.02
PDK1	5.6 ± 0.3	0.8 ± 0.3	<0.0001
PDK3	0.5 ± 0.2	0.8 ± 0.2	0.96
GPT2	0.5 ± 0.5	4.6 ± 0.5	0.0002
LDHA	1.4 ± 0.6	2.8 ± 0.6	0.12

Overexpression of PRMTs has been associated with many different cancers. Recently, there has been research linking the deregulation of PRMTs in cellular processes such as proliferation, transformation and anti-apoptotic processes that promote tumourigenesis (Yang and Bedford 2013). In the present study, *PRMT1* expression was highest at the MII stage and then decreased, suggesting that less arginine methylation (and therefore less ADMA) is being produced as the embryo progresses through development (Fig. 2). Conditional knockout of *PRMT1* in mice led to embryonic lethality, emphasising a need for this protein in the early stages of development (Breckenridge *et al.* 2010). Further investigation in mouse *PRMT1*-null embryonic fibroblasts revealed chromosomal aberrations and hypersensitivity to DNA damage (Yu *et al.* 2009), both of which would be detrimental to the early embryo. The expression of *PRMT3*, which adds ADMA to arginine residues, also decreased from the oocyte to 4-cell stage, but then markedly increased by the blastocyst stage. This suggests a particular role for this PRMT later in development. PRMT5 is the main symmetric arginine

methyltransferase, and *PRMT5*-knockout mice die very early during development, probably when the maternal RNA pool is depleted (Yang and Bedford 2013). Arginine concentration had no effect on the abundance of the *PRMT5* message.

DDAH1 did not appear to be present in porcine blastocysts, but was expressed in oocytes and 4-cell embryos; which could be in response to higher NO production in early embryos, because DAF staining of porcine embryos appeared to have a higher intensity at the 4-cell stage that at the blastocyst stage (Figs 3, 6). In contrast, *DDAH2* appears to be expressed throughout development, with a marked increase between the oocyte and 4-cell stages, as well as an increase from the 4-cell stage to blastocyst. This is consistent with earlier work using microarray analysis to determine differences in gene expression between oocytes, 4-cell embryos and blastocysts, where a significant upregulation of *DDAH2* was found between the oocyte and 4-cell stages (Whitworth *et al.* 2005). *DDAH2* has been found to be expressed primarily in fetal rather than adult tissues, and has also been found to be upregulated in rapidly dividing cells, such as melanoma cells (Tran *et al.* 2003). *DDAH2* overexpression also enhanced proliferation and migration of endothelial cells (Hasegawa *et al.* 2006), which suggests that this upregulation could be involved in the dividing embryo.

DDAH2 protein localisation was analysed using confocal imaging of Day 6 *in vivo*-derived IVF embryos cultured in the presence of PZM3 (0.12 mM arginine) and MU1. *In vitro*-derived embryos were also analysed on Days 5 and 7 of development. The DDAH2 protein was localised to the nucleus in both *in vivo*-derived and *in vitro*-produced blastocysts, suggesting a possible role for this protein in gene regulation. This expression pattern is in contrast with that seen in endothelial cells, where DDAH2 protein was found only in the cytosol (Chen *et al.* 2005). However, localisation of DDAH2 in the nucleus has been reported in endothelial cells of mesenteric vessels as well as vascular smooth muscle, and translocation of DDAH2 protein localisation to the mitochondria has been reported with interleukin-1β treatment in chondrocytes (Palm *et al.* 2007; Cillero-Pastor *et al.* 2012). More importantly, DDAH2 has been shown to be localised in the nucleus of oral squamous cell carcinoma cells, and this localisation relates early embryos to tumour cells (Khor *et al.* 2013). In the present study, immunocytochemical imaging of porcine blastocysts also showed localisation of DDAH2 primarily in the TE rather than ICM cells. Analysis of a differentially methylated region (DMR) in the promoter of *DDAH2* in mice revealed epigenetic regulation of this gene in trophoblast cells (Tomikawa *et al.* 2006). Further analysis will be needed to determine whether this expression pattern is due to epigenetic differences between these two embryonic cell types.

To examine the role of NO production in the early porcine embryo, the non-specific NOS inhibitor L-NAME was added during *in vitro* culture. Addition of varying concentrations of L-NAME to porcine embryo culture starting on Day 1 of culture revealed a dose-dependent decrease in blastocyst percentages. Although a low concentration (2 mM) of L-NAME did not affect development rates, the addition of 5 mM L-NAME significantly ($P < 0.05$) reduced the ability of cleaved embryos to progress to the blastocyst stage. Furthermore, increasing this concentration to 10 mM L-NAME completely abolished blastocyst development (Fig. 5). Similar levels of L-NAME inhibited mouse and bovine embryonic development when added to *in vitro* culture, supporting a role for NOS in embryonic development *in vitro* (Amiri *et al.* 2003; Manser *et al.* 2004; Schwarz *et al.* 2010). NOS3 has been shown to be a key enzyme for NO production in ovine TE. Knockdown using an *in vivo* morpholino antisense oligonucleotide for *NOS3* resulted in thin, small and underdeveloped conceptuses (Wang *et al.* 2014*b*). These conceptuses also had decreased *SLC7A1* message in the TE. In our transcriptional profiling database, we found that IVC blastocysts had 12 reads that aligned to the *NOS3* transcript compared with three reads aligned for IVV blastocysts ($P = 0.2$; Bauer *et al.* 2010). This may illustrate a role for *NOS3* in porcine embryos.

Examination of embryos on Day 2 of culture (4- to 6-cell stage) using DAF-DM-DA staining revealed a significant increase in DAF fluorescence in embryos cultured in either 0.12 or 1.69 mM arginine-supplemented medium compared with 0.12 mM arginine-supplemented medium containing 5 mM L-NAME ($P < 0.05$). DAF fluorescence is an effective means of measuring NO production in live porcine embryos, and the findings suggest dynamic production of NO during early embryonic development. DAF fluorescence also demonstrates a marked response of embryos to NOS inhibitor treatment because L-NAME was only present during DAF incubation. Embryos exposed to 1.69 mM arginine during DAF incubation tended to have a higher amount of DAF fluorescence than control 0.12 mM embryos, but this increase was not significant ($P = 0.11$). This could reflect a need for embryos to have a longer exposure to higher arginine concentrations before NO measurement. Culture in 0.12 mM arginine with inhibitors of the PRMT–DDAH–NOS axis (i.e. AMI-1 and ADMA) did not affect NO production, as assessed by DAF staining, and these embryos still exhibited significantly higher DAF fluorescence than embryos treated with 5 mM L-NAME. This may be due to differences in the effects or activity of exogenous and endogenous forms of these inhibitors in aqueous culture.

Optimising the *in vitro* environment is crucial to promote embryo development. By evaluating the embryo's transcriptome, we were able to modify our culture conditions to improve embryo development to the blastocyst stage by adding arginine to our culture medium. Future studies need to determine the exact mechanism by which arginine stimulates increased embryo development to the blastocyst stage, but here we provide the framework to show a dynamic role for the NO–PRMT–DDAH axis in preimplantation porcine embryos.

Acknowledgements

The authors thank Jiude Mao, Jennifer Hamm and Alyssa Davis for their assistance in this project. This work was funded, in part, by Food for the 21st Century at the University of Missouri and by the National Institutes of Health (U42 OD011140).

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