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Arginine Increases Development of *In Vitro* Produced Porcine Embryos and Affects the PRMT-DDAH-NO 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 endeavor found *SLC7A1*, an arginine transporter, to be up-regulated in *in vitro* compared to *in vivo* cultured embryos. We added different concentrations of arginine to our culture medium to meet the needs of the 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 to controls and specifically, more trophectoderm nuclei. Embryos cultured in 1.69 mM arginine had lower *SLC7A1* levels and had higher abundance of messages involved with glycolysis (*HK1, HK2*, and *GPT2*) and decreased expression of genes involved with blocking the TCA cycle (*PDK1*) as well as the pentose phosphate pathway (*TALDO1*). *PRMT1*, *PRMT3* and *PRMT5* expression throughout development was not affected by arginine. *DDAH1* and *DDAH2* message was found to be differentially regulated through development and DDAH2 protein was localized to the nuclei of blastocysts. Arginine has a positive effect on preimplantation development and may be affecting the NO-DDAH-PRMT axis.

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 both reduced blastocyst rates, and reduced pregnancy rates (Kikuchi *et al.* 1999). In addition, genetic and epigenetic effects due to culture are well documented (Reviewed in (Fleming *et al.* 2004). Therefore to produce an ideal culture system a need exists for understanding what the embryo needs *in vivo*. In an effort to identify ways to improve culture conditions, a next-generation sequencing analysis was completed by using *in vivo* produced embryos that were cultured to the blastocyst stage *in vitro* (IVC) or *in vivo* (IVV) (Bauer *et al.* 2010). An arginine transporter, *SLC7A1*, was identified as up-regulated by at least 63 fold in IVC compared to IVV cultured blastocyst stage embryos. Arginine is a vital amino acid for many metabolic processes in the cell; examples include protein synthesis, creatine production, polyamine synthesis and nitric oxide generation (Wheatley and Campbell 2003). Removal of

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arginine from culture medium by either medium formulation or arginase treatment quickly leads to death in 80% of tumor 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 hypothesize that *in vitro*-produced embryos also require significant amounts of arginine.

Arginine is used to produce nitric oxide (NO) by nitric oxide synthase (NOS) enzymes. Three isoforms of NOS include neuronal (NOS1), inducible (NOS2) and endothelial (NOS3). Expression of all three NOS isoforms has been detected in both murine and bovine preimplantation embryos from the zygote to the 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 metabolized during embryonic development (Humpherson et al., 2005). Manser and Houghton (2006) utilized the nitric oxide sensitve probe DAF-FM diacetate and found that NO was present at all stages of murine preimplantation development. Supplementation of culture media with additional arginine improves porcine embryo development as well, suggesting that this amino acid plays a critical role in preimplantation development (Bauer et al., 2010).

Nitric oxide production is regulated in part by the production of endogenous NOS inhibitors through the protein arginine methyltransferases (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 enzymes within the cell to reduce NO production. The enzymes DDAH1 and 2 degrade excess MMA and ADMA within the cell. The *DDAH1* null mutation is embryonic, lethal whereas *DDAH2* null mice reproduce normally. This reveals an important role for DDAH and proper regulation of NO in the early embryo.

Here 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 otherwise indicated, all the chemical components were purchased from Sigma Chemical Company (St. Louis, MO).

In Vitro Production of Embryos

Pre-pubertal porcine oocytes were obtained from ovaries that were 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 a local slaughterhouse. COCS were selected based on multiple layers of cumulus cells and evenly distributed cytoplasm and then were washed in Tyrode's Lactate (TL) Hepes medium supplemented with 0.1% polyvinyl alcohol (PVA). Two hundred-250

COCs were cultured in 2 mLs of maturation medium (TCM-199 with 0.1% PVA, 3.05 mM glucose, 0.91 mM sodium pyruvate, 10 μg/ml gentamicin, 0.57 mM cysteine, 10 ng/mL EGF, 0.5 μg/ml LH and 0.5 μg/ml FSH) for 42–44 hours in a humidified atmosphere with 5% CO_2 in air at 38.5°C. Forty four hours later, mature oocytes were identified by extrusion of a polar body and washed in modified Tris-buffered medium (mTBM) containing 2 mg/mL 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 sperm were added. The sperm used for fertilization was obtained from a single boar, and was used throughout the entire experiment. For IVF, a 0.1 mL frozen semen pellet was thawed in 3 mL of sperm washing medium (Dulbecco's phosphate-buffered saline (dPBS; Gibco) supplemented with 0.1% BSA). The sperm were washed twice by centrifugation. The spermatozoa pellet was resuspended with fertilization medium to 0.5×10^6 cells/mL. Finally, 50 μL of the sperm suspension was added to the oocytes in the IVF medium giving a final concentration of 0.25×10^6 cells/mL, and sperm and oocytes were incubated together for 5 hours.

Embryo Culture

After fertilization, the 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 hours at 38.5°C. After the 28–30 hr 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 of 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, in humidified atmosphere of 5% CO_2, 90% N_2 and 5% O_2 at 38.5°C until day 6. Arginine at 1.69 mM 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 post-fertilization, blastocysts from each treatment group were collected and the percent of embryos that developed to the blastocyst stage was recorded. To determine the effect arginine had on development the percentage blastocyst for each treatment group was analyzed by using PROC GENMOD in SAS (SAS Institute, Cary, NC). A least significant difference post-test comparison was completed to determine if significant differences existed between treatment groups ($p<0.05$). For subsequent experiments embryos were cultured in 1 of 3 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 3 treatment groups was conducted after differential nuclear staining. The differential staining technique was described earlier (Machaty *et al.* 1998). All zona pellucidae were removed by using a physiological saline lowered to a pH of 1.79. Zona-free embryos were exposed to a 1:7 dilution of rabbit anti-pig whole serum for 60 minutes (Bauer *et al.* 2010). Embryos were then washed three times for 5 minutes in Tyrode's Lactate (TL) Hepes medium. Finally, embryos were incubated in a 1:10 dilution of the guinea pig complement, propidium iodide, and bisbenzimide for 35 minutes. The

propidium iodide and bisbenzimide were added to the guinea pig complement at a concentration of 10 μg/mL. The embryos were then observed in UV light under 40x magnification by using a Nikon Eclipse E600 inverted microscope (Nikon Corp., Tokyo, Japan). Inner cell mass nuclei stained blue while TE nuclei stained pink. Mean ICM, TE, total cell number, and ratio of TE/ICM were first analyzed for normality by using the UNIVARIATE procedure in SAS. The data were then log2 transformed and analyzed by an analysis of variance by using the MIXED procedure in SAS. A least significant difference post-test comparison was then completed for each variable to determine if significant differences ($p<0.05$) existed between the groups.

RNA Extraction and Amplification for Real-time PCR of *SLC7A1*

Three replicates were obtained for each treatment group: PZM3 (0 mM arginine); PZM3 control (0.12 mM arginine); and MU1. Total RNA was extracted, from pools of 10 embryos in each replicate, by using the AllPrep™ genomic DNA/RNA micro isolation kit (Qiagen, Germantown, MD, USA). Total RNA was suspended in 12 μL, and 5 uL was amplified by using the WT-Ovation ™ Pico RNA Amplification System (NuGEN Technologies, Inc., San Carlos, CA). After amplification, the samples were purified by using Micro Bio-Spin P-30 Columns (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was then completed for the *SLC7A1* and genes that regulate the Warburg Effect (WE) to determine message abundance by using these amplified embryo pools (Bauer *et al.* 2010).

Relative Real-Time PCR—Real-time PCR was conducted on each of the amplified samples for the genes involved with the WE and embryo metabolism by using IQ SYBR Green Supermix (Bio-Rad Laboratories) and the amplified cDNA from each biological replicate (diluted to 5 ng/μL) as template. Primers were designed by using Integrated DNA Technology (Coralville, Iowa) software and real-time PCR was run in triplicate for every biological replicate on the MyiQ™ Single-Color 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 by using 10 ng dilutions of our 5 ng/μL reference cDNA pool. Real-time PCR was run in triplicate at each concentration (5 ng/μL, 0.5 ng/μL, 0.05 ng/μL) to validate each primer set. Abundance for each mRNA transcript was calculated in the same manner as Bauer et al. (Bauer *et al.* 2010) relative to the reference sample and the housekeeping gene, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (*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 was identical in all samples. Bauer *et al.* (2010) found *YWHAG* expression did not differ between treatments in mRNA deep sequencing. For these reasons, *YWHAG* was used as our reference gene for this experiment. The reference sample contained four biological replicates of *in vivo* fertilized and then *in vivo* cultured blastocysts and *in vivo* fertilized and then *in vitro* cultured blastocysts pooled together (Bauer *et al.* 2010). Expression levels between treatments were determined by using the comparative threshold cycle (C_T) method for each gene. The $2^{-\Delta\Delta CT}$ values were analyzed for normality before being log transformed if not normally distributed. The resulting values were then analyzed by using the general linear model (PROC GLM) in the

SAS. Differences in expression were found by by using the Least Squares Means (LSMeans) generated by PROC GLM ($p<0.05$).

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 3 pools each of 18–22 metaphase II oocytes, 4-cell stage embryos, and blastocyst stage embryos cultured in either control (0.12 mM arginine) or MU1 (1.69 mM arginine). Briefly, RNA was isolated by using the Dynabeads mRNA Direct micro kit (Life Technologies), and cDNA was synthesized by using Superscript III (Life Technologies). Real-time PCR was performed by using iQ SYBR green supermix (BioRad) and run on a BioRad platform by using a 2-step protocol with melt curve analysis. Reactions were run in triplicate by using a cycling program as follows: 95°C for 2 minutes, 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. A melt curve analysis followed, with temperature increments of 0.3 °C from 55 °C to 95 °C. Quantitative real-time PCR data was analyzed by using the ΔΔCt method with comparison to expression of *YWHAG* as an endogenous control (Whitworth et al., 2005), and relative mean expression levels were compared by using an analysis of variance (ANOVA) after a log transformation.

Immunocytochemistry

To examine DDAH2 protein localization, day 6 blastocysts were collected and the zona pellucidae removed by using low pH phosphate buffered saline (PBS) (pH 1.79). Embryos were then fixed for 20 minutes in 4% paraformaldehyde, washed twice in TL Hepes medium and held at 4°C until processing. After permeabilization in 0.1% Triton in PBS for 3 hours at 37°C, embryos were washed through PBS with 0.01% Tween and 3% BSA and then incubated in 2 M HCl for 30 minutes at room temperature. This was followed by incubation in 100 mM Tris-HCl pH 8.5 for 10 minutes at room temperature. Embryos were then blocked for 1 hour 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) diluted in blocking buffer. After 3 washes of 10 minutes each (with rocking), embryos were incubated in secondary antibody (1:250; donkey anti-goat Alexa fluor 488, Life Technologies). After three 10 minute washes with rocking, embryos were counterstained with propidium iodide and mounted by using Prolong Antifade (Life Technologies). Images were taken at 12 μm cross sections by using a Zeiss 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 4 treatments: 1) PZM3 control (0.12 mM arginine) 2) MU1, 3) 50 μM AMI-1 (PRMT1 inhibitor) in 0.12 mM arginine, or 4) 100 μM ADMA (NOS inhibitor) in 0.12 mM arginine. Embryos were moved at 30 hours post insemination to 25 μL culture drops and cultured to either day 2 (4-cell) or 6 (blastocyst). On day 2 or day 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 (4-Amino-5-Methylamino-2′,7′-Difluorofluorescein Diacetate, Life Technologies). Embryos were incubated in DAF-FM-DA 40 minutes in 5% O_2, then washed into PZM3 without DAF-FM-

DA and incubated an additional 15 minutes in 5% O_2. For imaging, embryos were placed singly in 5 μL drops of TL Hepes medium under mineral oil, held in place by using a microinjection holding pipette and imaged by using a Nikon inverted fluorescent microscope. All treatments were present throughout the incubation and imaging steps. Images were taken by using an exposure time that was the average for embryos treated with 5 mM N -nitro L-arginine methyl ester (L-NAME) (a nonspecific NOS inhibitor) to control for background fluorescence. Resulting images were analyzed by using ImageJ software, and corrected fluorescence intensity values were calculated as integrated density – (area of embryo measurement X background (Burgess *et al.* 2010). Values were then log transformed and compared by using PROC GLM ANOVA with fixed effects of treatment and IVF group.

Embryo Transfer

Day 6 post-fertilization blastocysts cultured in MU1 were selected and placed into 3 mL of manipulation medium in polystyrene tubes (BD Falcon 352054). The embryos were transported at 37°C to the University of Missouri Swine Research Complex where they were loaded with 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 estrous 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 estrus or farrowed. Sex and birth weights 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 trophectoderm nuclei and total nuclei above the control PZM3 without significantly changing the ratio of ICM to TE (Table 2). The higher arginine concentration (1.69 mM) in culture produced embryos that were developmentally competent as they produced live piglets (Table 3). A comparison of term development from these two culture systems was beyond the scope of this project.

Message for *SLC7A1* was decreased in embryos cultured in 1.69 mM arginine as compared to 0 or 0.12 mM arginine (Figure 1). The abundance of two WE-related genes, *TALDO1* and *PDK1*, were decreased in response to 1.69 mM arginine, while *HK1, HK2* and *GPT2* were increased (Table 4).

The abundance of message for *PMRT1* and *PMRT3* decreased from the MII oocyte to 4-cell stage, while *PRMT5* increased from the MII oocyte to 4-cell stage (Figure 2). The abundance of *PRMT1* then increased from the 4-cell to blastocyst stage. There was no effect on *PRMT1*, *3* or *5* message abundance if the embryos were cultured in 0.12 or 1.69 mM arginine. *DDAH1* expression was not different between the MII oocyte and 4-cell stage, but both were higher as compared to blastocyst stage embryos (Figure 3; $p<0.01$). In contrast, *DDAH2* remained low in MII oocytes and 4-cell stage embryos, but was then 5-fold higher by the blastocyst stage ($p<0.01$). DDAH2 protein localization was analyzed by using confocal imaging of day 6 *in vivo* derived, PZM3 (0.12 mM arginine) and MU1 cultured IVF embryos. The DDAH2 protein was localized to the nucleus in both in vivo and in vitro produced blastocysts, and this localization appeared to be less abundant in day 5 in vitro

produced blastocysts cultured in MU1 as compared to day 5 blastocysts cultured in 0.12mM arginine (Figure 4). In both treatments, DDAH2 nuclear localization appeared to be lower in ICM cells versus trophectoderm. A similar pattern of protein expression was also seen in Day 6 in vivo derived blastocysts.

To explore the role of NO production during development, 2-cell stage embryos were cultured in 2, 5 and 10 mM L-NAME until the blastocyst stage. Five and 10 mM L-NAME inhibited development to the blastocyst stage ($p<0.05$, Figure 5). Nitric oxide production in 4-cell stage *in vitro* produced embryos was estimated by using DAF-FM fluorescence compared to embryos cultured with 5 mM L-NAME (Figure 6). Quantification of fluorescence on day 2 of culture (4- to 6-cells) revealed an increase in NO production in embryos cultured in either 0.12 mM arginine (PZM3) or 1.69mM arginine (MU1) as compared to embryos cultured in 0.12 mM arginine plus 5mM L-NAME. Embryos also tended towards more NO production in 1.69 mM arginine treated embryos as compared to 0.12 mM arginine treatment; however, this difference was not significant ($p=0.11$). AMI-1, an inhibitor of class 1 PRMTs, and ADMA dihydrochloride, also did not reduce DAF-FM fluorescence in the 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 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 produced embryos. A previous transcriptional profiling study found a transcript involved with arginine transport was increased in IVC embryos compared to IVV embryos (Bauer *et al.* 2010). By using this data, we assessed the effect additional arginine would have on embryo development *in vitro*. Here we provide the evidence that illustrates 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 not only a precursor for protein synthesis but also for nitric oxide, 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: *SLC7A1*, *SLC7A2*, and *SLC7A3* genes. Message for *SLC7A1* and *SLC7A2* has been previously identified in mouse pre-implantation stage embryos (Van Winkle 2001). *SLC7A1* message has also been detected in ovine conceptuses between days 13–18 of pregnancy (Gao *et al.* 2009) and in porcine conceptuses on days 12 and 15 of pregnancy (Bazer 2013). More recently, *SLC7A1* was found to be the key transporter of arginine by ovine TE. By using *in vivo* morpholino antisense oligonucleotide mediated knockdown of *SLC7A1*, the authors found that the conceptuses were retarded with abnormal function compared to controls. They concluded that arginine is essential for conceptus survival and development (Wang *et al.* 2014a).

In our previous study, three times the concentration of the arginine in PZM3 was added to see the effect on *SLC7A1* message levels (Bauer *et al.* 2010). Increasing arginine from 0.12 mM to 0.36 mM and culturing *in vivo* produced 2-cell stage embryos to the blastocyst stage decreased the *SLC7A1* message level to that of the *in vivo* embryos, but was also not different from the *in vitro* 0.12 mM message level (Bauer *et al.* 2010). Therefore, a higher concentration of arginine was sought to see the effects on *in vitro* produced embryo development. Five different treatments were used: PZM3 with 0 mM, 0.12 mM (control), 0.36 mM, 0.72 mM, or 1.69 mM arginine. Embryos were also cultured in 2.5 mM and 5 mM arginine to see if there was even more of an improvement in embryo development however, there was no difference compared to embryos cultured in 1.69 mM arginine (data not shown), so 1.69 mM was used for the remainder of the experiments. Li and coworkers characterized the concentrations of amino acids in porcine oviductal and uterine fluid on day 3 and day 5 post-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 (Li *et al.* 2007). For this reason, 1.69 mM arginine, which is 14 times higher than the control concentration, was used as the high physiological level of arginine. Embryos cultured with 1.69 mM arginine had decreased level of *SLC7A1* message compared to 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 up-regulating *SLC7A1* message compared to *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 to each of the treatments with 70% of cleaved developing into blastocysts compared to about 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; Humpherson *et al.* 2005; Booth *et al.* 2007). One of these studies found that arginine was consistently depleted from the medium at each preimplantation stage of development and morulae producing ≥25% blastocyst percentage had more arginine depleted than morulae producing ≤14% blastocyst percentages (Booth *et al.* 2005). 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 to control blastocysts. After differential staining, the two different cell types in the blastocyst were analyzed, 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 customized medium and when 2 mM arginine was added, there was increased cellular proliferation by approximately 8 fold compared to 0 mM arginine cells (Kim *et al.* 2013). This again is consistent with what was shown in this present study as the TE cells appeared to be the cells with increased proliferation compared to controls. There was no difference in the number of ICM between control or high arginine cultured embryos.

Arginine has been shown to stimulate cell signaling via AKT1/mTORC1/mTORC2 pathway to affect survival and development of the conceptus (Bazer 2013). Arginine induces the phosphorylation of proteins in the MTOR cell signaling pathway including RPS6K and 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 remodeling, and we propose that the MTOR pathway stimulates TE proliferation, mRNA translation, protein synthesis and cytoskeletal remodeling (Kong *et al.* 2012; Bazer 2013; Kim *et al.* 2013).

Like embryos, cancer cells also have stimulated mTOR signaling 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), i.e. they have an increased glucose uptake and decreased metabolism through the TCA cycle. The mechanistic target of rapamycin complex 1 (mTORC1) responds to the nutritional status of the cell and is often de-regulated in cancer cells (Zoncu *et al.* 2011). Arginine has been shown to activate mTORC1 via a lysosome amino acid transporter 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 if 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). There was an up-regulation of hexokinase 1 (*HK1*) and hexokinase 2 (*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 to *HK1*. This is consistent with the WE as *HK2* is required for tumor initiation and maintenance in cancer cells (Patra *et al.* 2013). Cancer cells also have an increase in lactic acid and alanine production. Here, additional arginine caused an increase in alanine amino transferase (*GPT2*) message in embryos; which is consistent with cancer cells metabolizing pyruvate to alanine and away from the TCA cycle (Beuster *et al.* 2011). Additional arginine decreased pyruvate dehydrogenase kinase 1 (*PDK1*). This enzyme is important in blocking pyruvate from entering the TCA cycle and shunting it towards alanine or lactate production. Transaldolase 1 (*TALDO1*) message was decreased as well in embryos cultured in 1.69 mM arginine. This enzyme is part of the pentose phosphate pathway where 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, it does give a predictor of what could be occurring at the protein/enzyme level.

Overexpression of PRMTs have 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 tumorigenesis (Yang and Bedford 2013). Here, *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 (Figure 2). A conditional knockout of *PRMT1* in mice led to embryonic lethality, emphasizing a need for this protein in the

early stages of development. 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. *PRMT3*, which adds ADMA to arginine residues, also decreases in expression from the oocyte to the 4-cell stage, but then dramatically increases by the blastocyst stage. This suggests a particular role for this PRMT later in development. PRMT5 is the main symmetric arginine methyltransferases enzyme, 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 *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, as DAF staining of porcine embryos appeared to have a higher intensity at the 4-cell stage as compared to the blastocyst stage (Figures 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 4-cell to blastocyst. This is consistent with earlier work by using microarray analysis to determine differences in gene expression between oocytes, 4-cell embryos, and blastocysts, where a significant up-regulation of *DDAH2* was found between the oocyte and 4-cell stages (Whitworth *et al.* 2005). *DDAH2* has been found to be primarily expressed in fetal versus adult tissues, and also has been found to be up-regulated in rapidly dividing cells such as melanoma cells (Tran *et al.* 2003). *DDAH2* overexpression also enhanced proliferation and migration of endothelial cells, which suggests that this up-regulation could be involved in the dividing embryo.

DDAH2 protein localization was analyzed by using confocal imaging of day 6 *in vivo* derived, PZM3 (0.12 mM arginine) and MU1 cultured IVF embryos. In *vitro* derived embryos were also analyzed on days 5 and 7 of development. The DDAH2 protein was localized 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 to that seen in endothelial cells, where DDAH2 protein was found only in the cytosol (Chen et. al., 2005). However, localization 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 localization to the mitochondria has been reported with IL1β treatment in chondrocytes (Palm *et al.* 2007; Cillero-Pastor *et al.* 2012). More importantly, DDAH2 has been shown to be localized in the nucleus of oral squamous cell carcinoma cells, and this localization relates early embryos to tumor cells (Khor *et al.* 2013). Immunocytochemistry imaging of porcine blastocysts also showed localization of DDAH2 primarily in the TE versus 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 if this expression pattern is due to epigenetic differences between these two embryonic cell types.

To examine the role of nitric oxide production in the early porcine embryo, the nonspecific NOS inhibitor L-NAME was added during *in vitro* culture. Addition of L-NAME to porcine embryo culture starting on day 1 of culture at varying concentrations revealed a dose-dependent decrease in blastocyst percentages. Although a low concentration (2 mM) 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 completely abolished blastocyst development (Figure 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). Nitric oxide synthase 3 has been shown to be a key enzyme for NO production in ovine TE. Knockdown by using an *in vivo* morpholino antisense oligonucleotide for *NOS3* resulted in thin, small and under developed conceptuses (Wang *et al.* 2014b). These conceptuses also had decreased *SLC7A1* message in the TE as well. In our transcriptional profiling database, we found that IVC blastocysts had 12 reads that aligned to the *NOS3* transcript vs. 3 reads aligned for IVV blastocyst (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) by using DAF-DM-DA staining revealed a significant increase in DAF fluorescence in embryos cultured in either 0.12 mM or 1.69 mM arginine treated medium compared to 0.12 mM arginine medium including 5 mM L-NAME ($p<0.05$). DAF fluorescence is an effective means of measuring NO production in live porcine embryos, and suggests dynamic production of NO during early embryonic development. DAF fluorescence also demonstrates a dramatic response of embryos to NOS inhibitor treatment as 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, 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 prior to NO measurement. Culture in 0.12 mM arginine with inhibitors of the PRMT-DDAH-NOS axis (AMI-1 and ADMA) did not affect NO production as assessed by DAF staining, but were still significantly higher 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.

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

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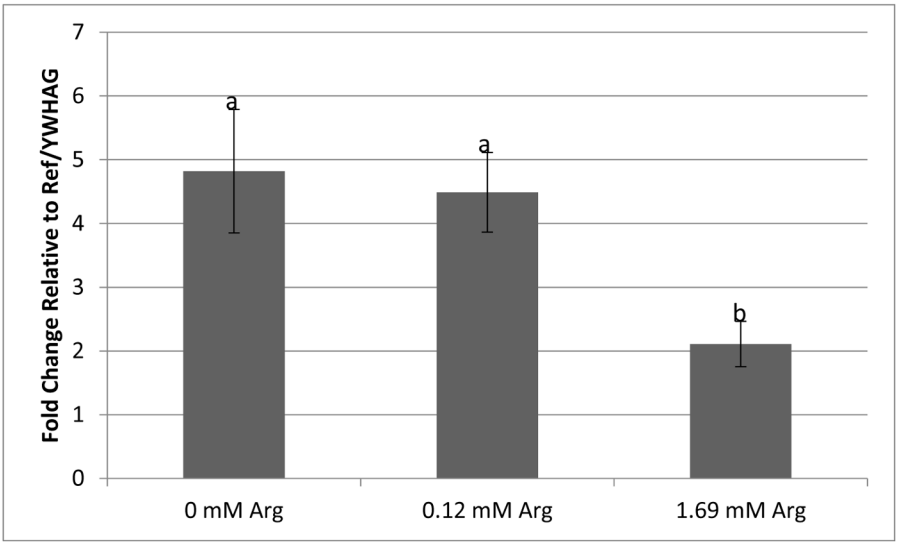

FIGURE 1. *SLC7A1* transcript levels depend on arginine concentration

Different letters represent significant differences in *SLC7A1* message abundance ($p \leq 0.0001$). The values depicted here are untransformed values however; the statistical differences were detected by using the transformed data.

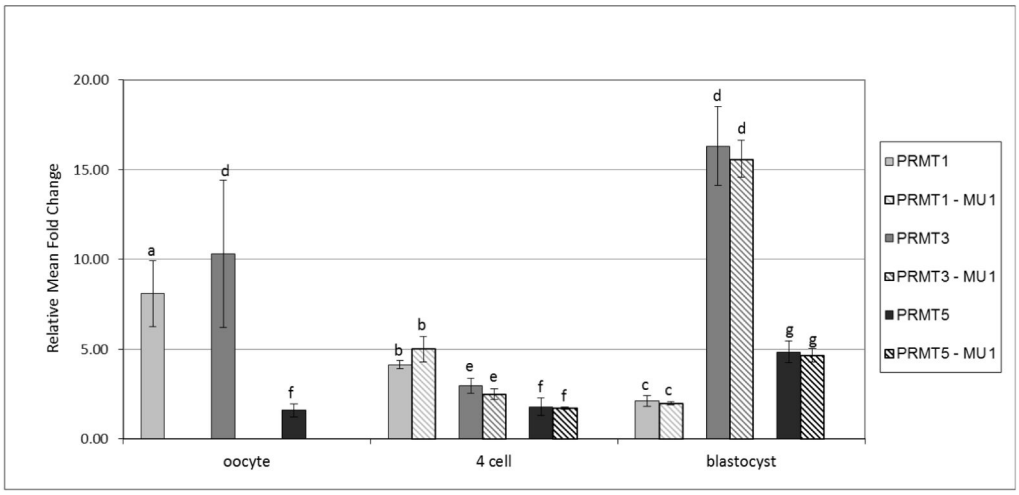

FIGURE 2.

Abundance of *PRMT 1, 3* and *5* in oocytes, 4-cell and blastocyst stage embryos. Two-cell stage embryos were cultured in control PZM3 (0.12 mM arginine) or MU1 (1.69 mM arginine). Bars with different letters within gene of interest are significantly different ($p < 0.05$; a = PRMT1, a′ = PRMT3, a″ = PRMT5).

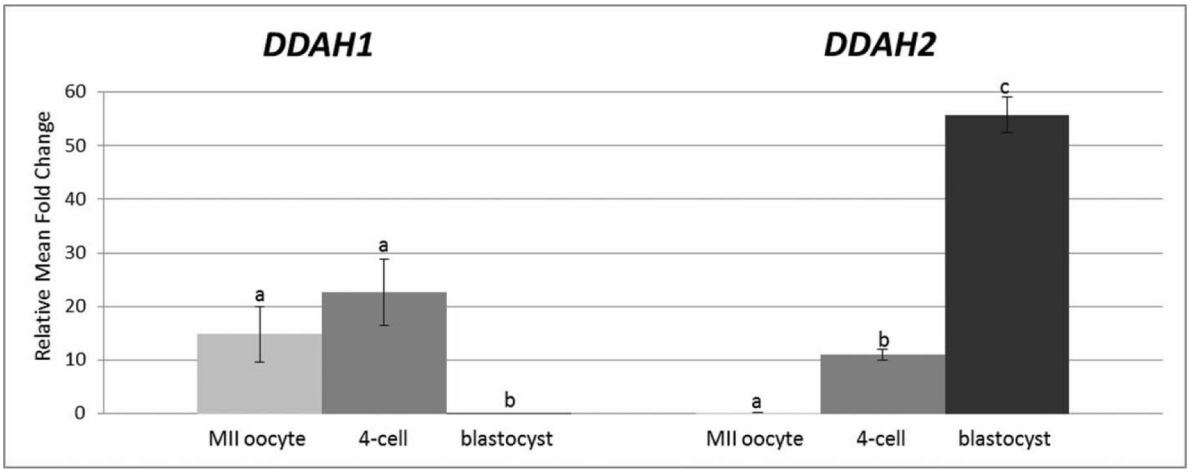

FIGURE 3.

Abundance of message for *DDAH1* and *DDAH2* in oocyte, 4-cell and blastocyst stage embryos. The values depicted here are untransformed relative mean fold change values; however, statistical differences were detected by using the transformed data. Bars with different letters within gene of interest are significantly different (*DDAH1* $p<0.01$; *DDAH2* $p<05$).

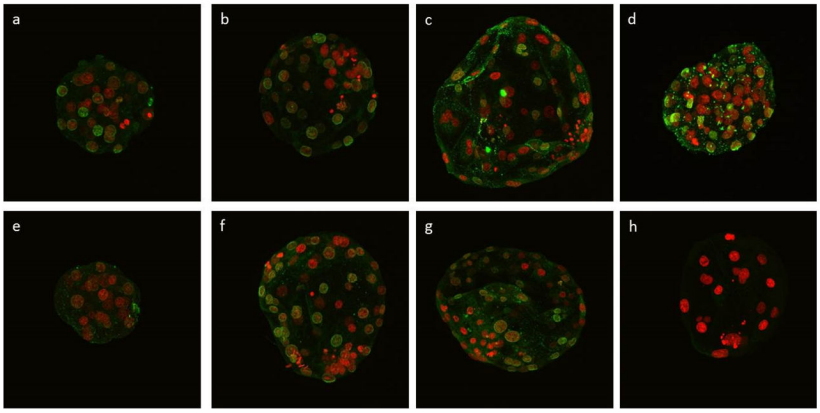

FIGURE 4.

DDAH2 protein localization in *in vitro* and *in vivo* derived blastocysts. Embryos were cultured in either PZM3 with 0.12 mM arginine (a, b, c) or 1.69 mM arginine (e, f, 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 is antibody localization, red is propidium iodine staining of the DNA. Secondary antibody only control is also shown for comparison (h).

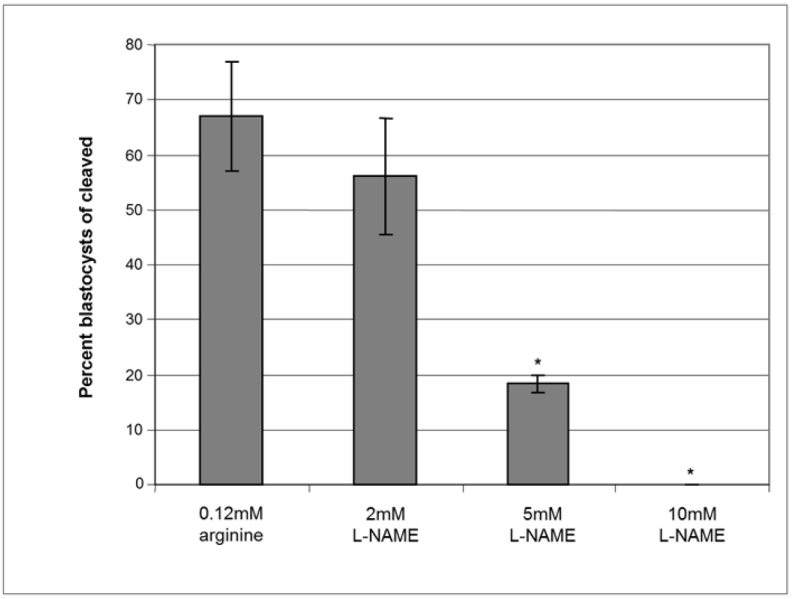

FIGURE 5. Effect of NOS inhibition on blastocyst development

Two-cell stage embryos were cultured to the blastocyst stage in PZM3 (0.12 mM arginine) or PZM3 (0.12 mM) containing 2, 5 or 10 mM L-NAME. Bars with different letters are significantly different ($p<0.05$). Four replications; n=20, 26, 16, 18, respectively.

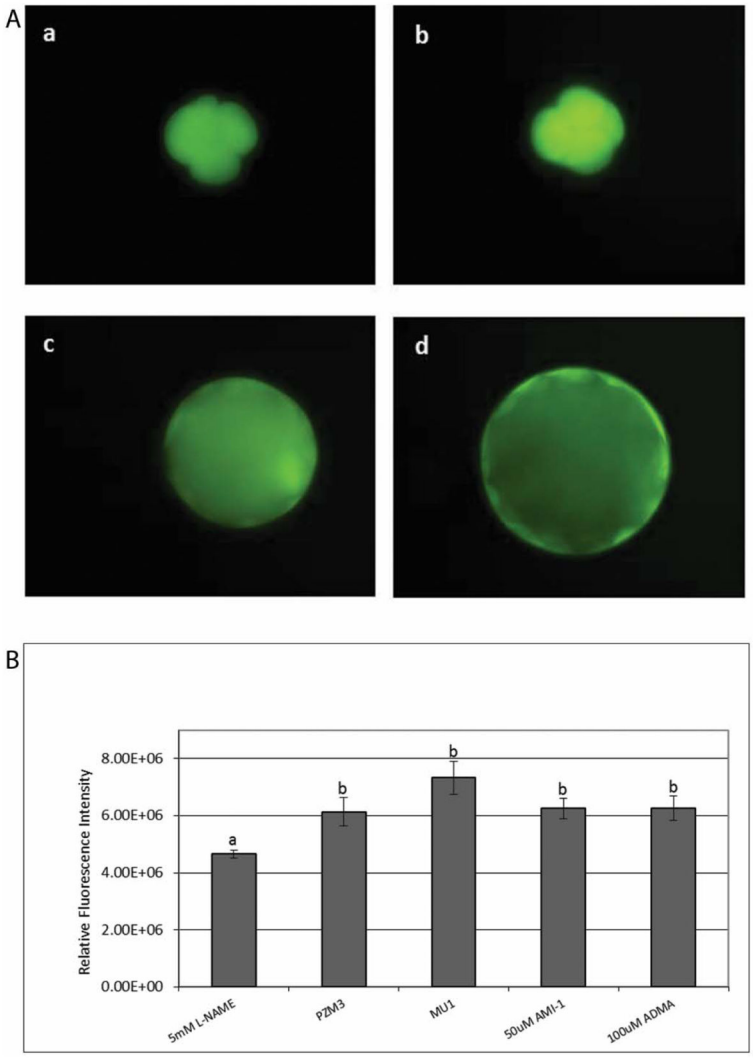

FIGURE 6.

Nitric oxide production viewed by using (A) DAF-FM staining of 4-cell and blastocyst stage embryos treated with either 5 mM L-NAME (a, c) or 1.69 mM arginine (b, d) and (B) the relative intensity of DAF staining to measure nitric oxide production in embryos on day 2 of culture. DAF intensity of day 2 (4–6 cell) embryos cultured in 0.12 mM arginine (PZM3) or one of five treatments: 5 mM L-NAME, 1.69 mM arginine (MU1), 0.12 mM arginine with 50 μM AMI-1 or 0.12 mM arginine with 100 μM ADMA. Values depicted here are untransformed values; however statistical differences were detected by using the transformed data. Bars with different letters are statistically significant (p-value <0.05). Five replicaitons; n=16, 19, 18, 21, 17 respectively.

TABLE 1

Blastocyst development depends on arginine concentration during culture.

	Mean Percent Blastocyst ± S.E.M.
PZM3 + 0.0 mM Arg	56.8 ± 10[b,c]
PZM3 + 0.12 mM Arg	50.9 ± 9.8[c]
PZM3 + 0.36 mM Arg	66.7 ± 11[a,b]
PZM3 + 0.72 mM Arg	66.7 ± 12[a,b]
PZM3 + 1.69 mM Arg	70.2 ± 11[a]

Two-cell stage embryos were cultured in various concentrations of arginine to the blastocyst stage and assessed.

[a,b,c]Values within a column having different superscripts were found to be significantly different (P≤0.02). Five replications; n= 148, 163, 150, 120 and 134, respectively.

TABLE 2

Effect of arginine concentration on the number of nuclei in blastocyst stage embryos.

Treatment	Mean ICM # (±S.E.M.)	Mean TE # (±S.E.M.)	Mean Total Cell # (±S.E.M.)	Mean Ratio TE:ICM (±S.E.M.)
PZM3 + 0 mM Arg (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 Arg (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 Arg (n=24)	7.0 ± 0.4^{b}	31.8 ± 1.7^{b}	38.8 ± 1.8^{b}	4.6 ± 0.4

^{a,b}Values within a column having different superscripts were found to be significantly different ($p \leq 0.03$). Values depicted here are untransformed values; however, the statistical values were generated from log transformation.

TABLE 3

Pregnancy result of day 6 blastocyst transfer to recipient gilt.

Piglet #	Sex	Birth Wt (Kg)	Wean Wt (Kg)
1	M	0.9	3.7
2	M	1.1	6.6
3	M	1.3	7.9
4	M	0.5	Died (runt)
5	F	0.8	Crushed by mother

TABLE 4

Primer sequences used for real-time PCR.

Transcript Annotation	Accession no.	Sense Primers	Antisense Primers
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

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

[A] Ensembl Transcript ID: ENSSSCT00000002161.

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

TABLE 5

Abundance of mRNA (Real-time PCR) of various Warburg Effect-related genes.

Gene	0.12 mM Arg	1.69 mM Arg	p-value
PKM2 M1	0±0.0009	0.002±0.0009	0.15
PKM2 M2	0.46±0.1	0.37±0.1	0.88
PKM2 M1 & 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±0.35	3.6±0.35	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	0.84±0.3	<0.0001
PDK3	0.50±0.2	0.82±0.2	0.96
GPT2	0.52±0.5	4.6±0.5	0.0002
LDHA	1.4±0.6	2.8±0.6	0.12

Data is presented as the mean ± S.E.M. relative to the reference/*YWHAG*.