Supplemental Information

Replacement of bovine serum albumin with N-methyl-D-aspartic acid and homocysteine improves development, but not live birth.

L.D. Spate, B.K. Redel, A.N. Brown, C.N. Murphy, R.S. Prather;
Division of Animal Sciences University of Missouri, Columbia, MO, USA

Introduction

The ability to efficiently and reproducibly create pig embryos in vitro that are competent to result in normal offspring is a goal that may facilitate a variety of technological advances (e.g. in vitro fertilization with gender sorted sperm, production of cloned embryos by nuclear transfer, creation of transgenic embryos, development of embryos followed by nonsurgical embryo transfer, etc…). Unfortunately in vitro produced embryos are developmentally delayed and less developmentally competent when compared to their in vivo produced counterparts (Armant 2005; Machaty et al. 1998; Wheeler et al. 2004). Most culture systems use components such as bovine serum albumin (BSA) or fetal calf serum (FCS) and the quality of these components vary between lots (Moore-Scott et al. 2003; Rorie et al. 1994). In addition, they may have numerous contaminants that could confound cell culture experiments. Indeed, serum itself can be detrimental to the development of cloned embryos (Farin et al. 2001).

Improvements in culture conditions for the creation and development of mammalian embryos have often been an empirical exercise of trial and error with little overall biological direction. Here we propose to develop a culture system that is based on results obtained from a global scan of gene transcription in early pig embryos (Whitworth et al. 2005b). In those studies, several specific receptor-mediated signaling system components were identified. The fact that certain ligands and their cognate receptors are abundant or are differentially expressed in the early pig embryo probably reflects their involvement during early embryonic development and, therefore, makes them attractive candidates for improving porcine embryo culture methodology. An underlying assumption herein is that the selective activation of these receptor systems can be used to better mimic the in vivo environment and promote embryo development in vitro. Such a strategy has helped improve in vitro conditions in the past. For example, the abundance of SPP1 (osteopontin) message was significantly higher in cDNA libraries from day 0 oviducts as compared to day 3 (Hao et al. 2006). When we added purified SPP1 back to the fertilization system we could significantly reduce polyspermy and increase the overall efficiency of fertilization (Hao et al. 2006). We also evaluated global changes in transcript abundance in the germinal vesicle (GV) oocyte, in vivo derived 4-cell and blastocyst stage pig embryos (Whitworth et al. 2005a) and identified the low density lipoprotein (LDL) receptor as increasing during development. Addition of LDL to the culture system partially replaced BSA (Spate et al. 2010). More recently we used deep sequencing to discover another gene (*SLC7A1*) that lead to improved formulations of culture

media (adding more arginine) that improved in vitro development (Bauer et al. 2010a).

Further mining of the EST and transcriptional profiling data (Whitworth et al. 2004; Whitworth et al. 2005b) from pig embryogenesis showed that the message level for GRIN3A was higher in both in vitro and in vivo produced 4-cell and blastocyst stage embryos as compared to germinal vesicle stage oocytes. GRIN3A (aka NR3A; a glutamate receptor, ionotropic, N-methyl-D-aspartate 3A (NMDA)). GRIN3A assembles *to form excitatory glycine receptors* that are unaffected by glutamate or NMDA, and inhibited by D-serine, a coactivator of conventional NMDA receptors (Kew and Kemp 2005). Agonists for the *glutamate recognition* site include L-glutamic acid, D-serine, L-alanine and NMDA among others. Antagonists for the glutamate recognition site include (R)-2-amino-5-phosphonopentanoate (D-AP5) and (±)-cis-4-phosphonoheptanoic acid (NPC 12626) (Mayer and Armstrong 2004). NMDA receptors belong to the superfamily of glutamate-regulated ion channels and are present in neurons throughout the central nervous system. Stimulation results in increases in intracellular Ca^{2+} and Na^{2+}. A glutamate binding domain is at the junction of GRIN1 and GRIN2x subunits (See Figure S1). In addition to glutamate the NMDA receptor requires a co-agonist, glycine, to bind to allow the receptor to function. The glycine binding site is on the GRIN1 subunit.

It is clear that addition of glycine to the culture medium has beneficial effects on development (Iwasaki et al. 1999; Xia et al. 1995). It had been thought that improved development may be the result of glycine acting as an organic osmolyte (Steeves and Baltz 2005). However, since message for GRIN3A increased from the oocyte to the 4-cell stage (relative abundance increased from 1.6 to 3.4 (p=0.01)) and then was at a similar level at the blastocyst stage (3.1), glycine may be enhancing embryo development via an unexpected pathway, i.e. the GRIN3A assembled with GRIN1. Interestingly Zn^{2+} contamination in culture medium has been shown to be inhibitory to embryo development (Azuma et al. 2002; Matsukawa et al. 2002). Zn^{2+} binds to the NMDA receptor and modulates its ion channel function (Paoletti et al. 1997; Paoletti et al. 1995; Paoletti et al. 2000).

The amount of DNA methylation in bovine embryos that were cultured to the blastocyst stage in homocysteine (HC) was dramatically increased (Ikeda et al. 2010). Since our preliminary data showed that removal of BSA resulted in a decrease in DNA methylation, we decided to add HC to the culture medium to restore the level of DNA methylation. Two members (MAT2B and AHCY) of all methylation pathway enzymes (methionine adenosyltransferase 1A (MAT1A), MAT2A, MAT2B, S-adenosylhomocysteine hydrolase (AHCY), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), betaine-homocysteine methyltransferase (BHMT), serine hydroxymethyltransferase 1 (SHMT1), SHMT2, and 5,10-methylenetetrahydrofolate reductase (MTHFR))

were selected for real time PCR to determine if transcript levels were altered as a result of culture in NMDA and HC.

Results

The first experiment was to determine if NMDA could promote development to the blastocyst stage. While removal of BSA from the culture medium decreased both the percent blastocyst and the total cell number; the experiment demonstrated that NMDA could partially replace BSA in the culture medium as development of embryos in PZM4+NMDA was not different from PZM3 (Table S1).

A follow up experiment again showed that removal of BSA and replacement with PVA resulted in a 33% drop in development to the blastocyst stage (PZM3 vs PZM4: Fig. 1 and Table S2). Addition of NMDA or HC significantly improved development above PZM4. In addition, HC and NMDA+HC resulted in development that was not different from PZM3. There was no difference in total cell number with an overall mean of 31.4.

Because the embryos in the PZM4+HC appeared to be larger, another set of embryos was created in the various media and the surface area of the blastocysts was measured by using Nis Elements BR3.0 software under 20X magnification. The measurements were created using arbitrary units and showed

that indeed, the PZM4+HC blastocysts had a greater surface area than PZM4+NMDA+HC (Fig. 1 and Table S3).

Since HC has been shown to affect global DNA methylation of bovine embryos (Ikeda et al. 2010) we stained additional blastocyst stage embryos for 5-methylcytidine. The mean intensities were lower for PZM3 as compared to PZM4. Interestingly the PZM4+NMDA+HC brought the level of methylation back down to a level not different from PZM3 (Fig. 1 and Table S4).

Real time PCR was conducted to examine a few genes that are thought to be involved with the decrease in global methylation that was observed in the PZM4+NMDA+HC treatment. S-Adenosylhomocysteinase (AHCY) catalyzes the hydrolysis of S-adenosylhomocysteine to adenosine and homocysteine. Methionine adenosyltransferase II, beta (MAT2B) is involved in biosynthesis of S-adenosylmethionine from methionine and ATP. Finally, neither AHCY nor MAT2B showed any change in gene expression in response to the PZM4+NMDA+HC treatment (Table S5).

Since NMDA and HC appear to be having an effect on the development of pig embryos to the blastocyst stage, we next wanted to determine if the embryos were developmentally competent, but could not produce any piglets.

Materials and Methods

Oocyte maturation, in vitro fertilization and embryo culture.

Ovaries were obtained from prepubertal gilts at slaughter (Premium Standard Farms, Milan, MO). Follicles were aspirated as per laboratory standards (Hao et al. 2003) and washed in TL-HEPES supplemented with polyvinyl alcohol (PVA) rather than BSA (TLHP) and only oocytes with homogenous cytoplasm and multiple layers of cumulus cells were then put into maturation media (Hao et al. 2006). Fifty oocytes were cultured per 500 µL medium covered with light mineral oil for 42hrs. Then the cumulus cells were striped and only those showing a polar body (MII stage) were placed in fertilization. After they were selected the oocytes were washed in the IVF media, Modified Tris Buffered Saline (MTBM) supplemented with BSA and caffeine (Hao et al. 2006), and placed into groups of 30 in 50 µL drops of MTBM. Fifty µL of frozen thawed semen were then added so the final sperm concentration was ~0.25X10 ^{6}, and cultured for 4-6hrs prior to washing in Porcine Zygote Medium 3 (108 mM NaCl, 10 mM KCl, 0.35 mM $CaCl_2$*$2H_2O$, 0.4 mM $MgSO_4$*$7H_2O$, 25.07 mM $NaHCO_3$, 0.2 mM Na-pyruvate, 2 mM Ca-$(lactate)_2$*5 H_2O, 1 mM L-glutamine, 5 mM hypotaurine, 20 mL/L BME (Sigma B6766), 10 mL/L MEM (Sigma M7145), 0.05 mg/mL gentamicin, 3 mg/mL BSA) except with 0.1% PVA supplemented in place of BSA (PZM4). The presumptive zygotes were then cultured in Nunc 4-well plates containing 500 µL PZM4 covered with light mineral oil. After 28-30hrs, cleaved embryos (2- to 4-cell stage) were selected and then allocated to the various treatment groups and cultured to day 7 (~20 embryos per 25 µL microdrop) at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2 90% N_2.

Assessment of development and cell numbers.

On day 6 embryos were visually assessed for morphological stage. In addition the number of nuclei was determined. The zona pellucida of blastocyst stage embryos was removed using physiological saline at pH 1.79, and then rinsing in PBS with BSA to restore pH. Then the embryos were placed in 500 µL TLHP (Hagen et al. 1991) for 30 minutes. The embryos were then transferred to microscope slides (Fisher 1255034) and a cover slip was placed over the embryos and compressed to flatten the embryos out as much as possible. The cover slip was then sealed to the slide with fingernail polish. The total number of cells for each embryo was visualized under a florescence microscope and data was recorded.

Effect of NMDA and HC on surface area.

On day 6 of the culture the blastocyst stage embryos from each treatment group were placed in their own individual micro drop of TLHP (100 µL) and assigned an identification number and an image was taken using Nis Elements BR3.0 software under 20X magnification. At a later date the images were then measured for total surface area using the same software.

Effect of NMDA and HC on Global methylation.

On day 6 the zona pellucida of all blastocysts was removed as described above and then fixed in 2% paraformaldehyde (Electron Microscopy Sciences #15710),

washed in TLHP and stored at 4°C. All steps from this point further were done in Sarstedt 96 well tissue culture plates in order to minimize antibody volume. Fixed blastocysts were permeablized with 0.1% Triton X-100 in TLHP for 30 minutes then washed in TLHP. After exposure to 2 M HCl for 30 minutes at room temperature and neutralized in tris HCl at a pH of 8.5 for 10 minutes at room temp, they were transferred to blocking solution (TL-HEPES with 2% non-fat dried milk and 2% goat serum) at 4°C overnight. Blastocysts were then moved to a 1:500 dilution of Anti 5 Methylcytidine (Eurogentec anti 5-MECY-0100) for 1 hr at 38.5°C, washed 3 times in PBS with 0.1% Triton X100 plus 0.1%PVA, before moving them to a 1:1000 dilution of secondary antibody (Santa Cruz Biotechnology SC-2781) for 40 minutes. After a wash in PBS with PVA and 0.1% Triton 100X they were transferred to slides and depressed with a cover slip and sealed with nail polish. Embryos were visualized by UV light with a Texas red filter and intensity was measured by using Nis Elements BR3.0 software under 20X magnification, and the data was analyzed by SAS Proc GLM.

Real time PCR

Blastocyst stage embryos were processed for real time PCR as previously described (Whitworth et al. 2005b) and YWHAG was used as a control. Primers used for amplification were AHCY Forward: CCT GCC CAA GAA GCT GGA TGA AGC, Reverse: TCA CGA GAC ATG CCC AGG TAC TGG, and for MAT2B Forward: GGC CAC AGT GTG TCG TCA GTT AGC, Reverse: TGC CAG ACC AGT GAA AGG TTC CCT.

206

207 *Embryo Transfer*

208 Blastocyst stage embryos were surgically transferred to the oviducts of

209 synchronized surrogates as previously described (Li et al. 2006; Li et al. 2009;

210 Spate et al. 2010).

Tables

Table S1. Preliminary data not reported in the Correspondence. Development of 2-cell stage embryos to the blastocyst stage in vitro with or without NMDA.

Treatment	N	Blastocysts	Total Cell Number
PZM3	260	47.5%[a]	25.8 ± 1.4 [a]
PZM4	220	29.6%[b]	19.6 ± 1.7 [b]
PZM4 + 0.5 mM NMDA	300	36.1%[c]	36.1 ± 1.7 [ab]

[a,b] P<0.05

Table S2. Development of 2-cell stage embryos to the blastocyst stage in vitro with or without NMDA and HC.

Treatment	N	Blastocysts	Total Cell Number
PZM3	120	63.3% ± 5.2[d]	31.2 ± 1.2
PZM4	135	29.7% ± 4.9[e]	30.3 ± 1.3
PZM4 + 0.5 mM NMDA	120	46.1% ± 5.3[c]	33.5 ± 1.3
PZM4 + 0.5 mM NMDA + 10 µM HC	135	55.6% ± 5.1[dc]	31.5 ± 1.3
PZM4 + 10 µM HC	120	49.2% ± 5.2[dc]	30.5 ± 1.3

[c,d,e] P<0.05

Table S3. Surface area of blastocyst stage embryos (arbitrary units)

Treatment	N	Surface area ± SE
PZM3	33	296,180 ± 15,042 [ab]
PZM4	26	295,114 ± 16,228 [ab]
PZM4 + 0.5 mM NMDA	37	303,451 ± 14,810 [ab]
PZM4 + 0.5 mM NMDA + 10 µM HC	31	271,913 ± 14,884 [b]
PZM4 + 10 µM HC	32	316,773 ± 14,632 [a]

[a,b] P<0.05

Table S4. Global DNA methylation of pig embryos cultured in various media.

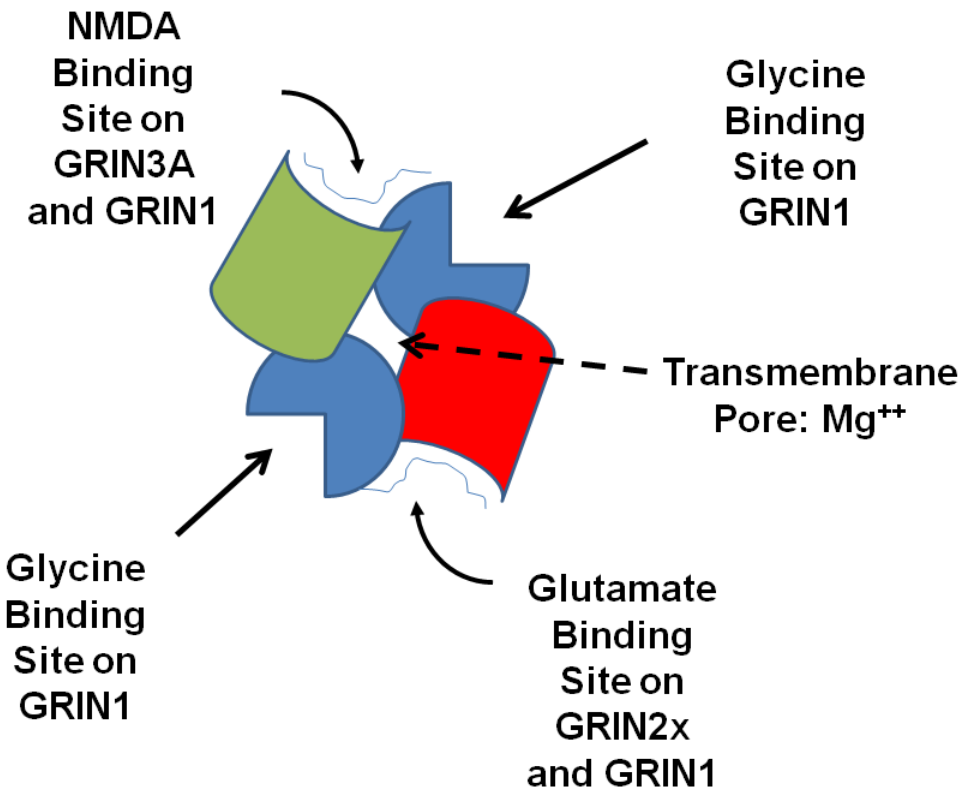
Treatment	N	Mean Intensity ± SE
PZM4	33	26.7 ± 4.1 [g]
PZM3	26	16.9 ± 3.2 [f]
PZM4 + 0.5 mM NMDA	37	19.3 ± 3.5 [fg]
PZM4 + 0.5 mM NMDA + 10 µM HC	31	13.6 ± 2.9 [f]
PZM4 + 10 µM HC	32	19.4 ± 2.9 [fg]

[f,g] P<0.05

Table S5. Real time PCR levels of AHCY and MAT2B in blastocyst stage embryos relative to YWHAG.*

Gene	GO biological process description	PZM3		PZM4 + NMDA + HC	
		Mean	Standard Error	Mean	Standard Error
AHCY	Regulates the intracellular S-adenosylhomocysteine (SAH) concentration thought to be important for transmethylation reactions	1.11	2.49	1.10	2.49
MAT2B	Biosynthesis of S-adenosylmethionine from methionine and ATP. This protein is the regulatory beta subunit of MAT	2.82	0.35	3.31	0.35
*There was no difference between treatments P>0.10.					

Figure S1. Cartoon of the NMDA receptor. The NMDA receptor is composed of 4 subunits: two GRIN1 subunits, a GRIN3A subunit and a GRIN2x where the x can be either A, B, C or D. Message for GRIN1, GRIN2A, GRIN2B and GRIN2D has been identified in blastocyst stage pig embryos (Bauer et al. 2010b).



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