

Glycine Supplementation In Vitro Enhances Porcine Preimplantation Embryo Cell Number and Decreases Apoptosis but Does Not Lead to Live Births

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SUMMARY

Most in vitro culture conditions are less-than-optimal for embryo development. Here, we used a transcriptional-profiling database to identify culture-induced differences in gene expression in porcine blastocysts compared to in vivo-produced counterparts. Genes involved in glycine transport (*SLC6A9*), glycine metabolism (*GLDC*, *GCSH*, *DLD*, and *AMT*), and serine metabolism (*PSAT1*, *PSPH*, and *PHGDH*) were differentially expressed. Addition of 10 mM glycine to the culture medium (currently containing 0.1 mM) reduced the abundance of *SLC6A9* transcript and increased total cell number, primarily in the trophectoderm lineage ($P = 0.003$); this was likely by decreasing the percentage of apoptotic nuclei. As serine and glycine can be reversibly metabolized by serine hydroxymethyltransferase 2 (*SHMT2*), we assessed the abundance of *SHMT2* transcript as well as its functional role by inhibiting it with aminomethylphosphonic acid (AMPA), a glycine analog, during in vitro culture. Both AMPA supplementation and elevated glycine decreased the mRNA abundance of *SHMT2* and tumor protein p53 (*TP53*), which is activated in response to cellular stress, compared to controls ($P \leq 0.02$). On the other hand, mitochondrial activity of blastocysts, mtDNA copy number, and abundance of mitochondria-related transcripts did not differ between control and 10 mM glycine culture conditions. Despite improvements to these metrics of blastocyst quality, transfer of embryos cultured in 10 mM glycine did not result in pregnancy whereas the transfer of in vitro-produced embryos cultured in control medium yielded live births.

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Abbreviations: AMPA, aminomethylphosphonic acid; mtDNA, mitochondrial DNA; MU1, porcine zygote medium 3 plus 1.69 mM arginine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling. **Genes:** *AMT*, aminomethyltransferase, glycine cleavage T; *ATIC*, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP BAD, B-cell CLL/lymphoma 2-associated agonist of cell death; *CCNB1*, cyclin B1; *CCND3*, cyclin D3; *DLD*, dihydrolipoamide dehydrogenase; *GCSH*, glycine cleavage system protein H; *GLDC*, glycine dehydrogenase (decarboxylating); *GPX6*, glutathione peroxidase 6; *GSS*, glutathione synthetase; *IMMT*, inner membrane protein, mitochondrial; *MFN2*, mitofusin 2; *MTHFD1[L]*, methylenetetrahydrofolate dehydrogenase (NADP⁺ dependent) 1, methylenetetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase [like]; *PAM16*, presequence translocase-associated motor 16 homolog; *PARP2*, poly-(ADP-ribose) polymerase 2; *PINK1*, PTEN induced putative kinase 1; *PCNA*, proliferating-cell nuclear antigen; *PHGDH*, phosphoglycerate dehydrogenase; *PSAT1*, phosphoserine aminotransferase; *PSPH*, phosphoserine phosphatase 1; *SHMT2*, serine hydroxymethyltransferase 2; *SLC6A9*, solute carrier family 6 member 9; *SMUG1*, single-strand-selective monofunctional uracil-DNA glycosylase 1; *SSBP1*, single stranded DNA binding protein 1, mitochondrial; *TP53*, tumor protein p53; *TXNRD1*, thioredoxin reductase 1.

INTRODUCTION

In vitro embryo culture environments remain suboptimal to those found in vivo. The process of oocyte maturation and subsequent embryo culture, in particular, is inefficient for producing developmentally competent embryos. While in vitro-produced embryos do develop under sub-standard conditions, these environments have negative consequences on development. For example, high rates of loss occur for many mammalian embryos, with up to 80% of in vitro-matured and inseminated oocytes failing to progress to the blastocyst stage (Watson et al., 2004). Recent adaptations have been made to culture systems that improve embryo development and subsequent in vivo development (Bauer et al., 2010a,b; Spate et al., 2010; Lee et al., 2013; Redel et al., 2015; Spate et al., 2015), but these improvements have not kept up with the demands associated with the increasing use of nuclear transfer and genetic editing technology to produce animal models for human clinical applications, such as for xenotransplantation and cystic fibrosis (Lai et al., 2002; Rogers et al., 2008), and to protect agricultural animal production by creating pigs resistant to the porcine reproductive and respiratory syndrome virus (Whitworth et al., 2016).

One approach to improve in vitro culture conditions is to identify the metabolic requirements of preimplantation embryos so the culture environment can be tailored to increase the number and viability of embryos that develop to the blastocyst stage, and will lead to subsequent offspring. Mammalian preimplantation embryos are proposed to utilize metabolism similar to that of many cancers—for example, the Warburg Effect, which favors anaerobic glycolysis involving lactic acid-dependent production of ATP rather than oxidative glycolysis involving pyruvate (Krisher and Prather 2012; Redel et al., 2012). This hypothesis stems from the rapid proliferation of cells that is a hallmark of both cancer and preimplantation embryos, and provided a framework to better understand the metabolism of preimplantation embryos.

Glycine, a non-essential amino acid, is an essential precursor for the synthesis of proteins and nucleic acids, and is required for the rapid proliferation of cells. Indeed, Jain et al. (2012) found that glycine consumption correlated

with proliferation rates of 60 human cancer cell lines, based on the metabolic profile of their spent media. Thus, the glycine demand likely exceeds the endogenous capacity of these highly proliferative cancer cells to de novo synthesize glycine. Zhang et al. (2012) also found that tumor-initiating cells rely on glycine metabolism, and that glycine decarboxylase (GLDC) activity can drive tumorigenesis. The hypothesized shared metabolic profiles between proliferating cancer cells and preimplantation embryos was reinforced by the number of genes predicted to be involved with glycine metabolism, as identified by RNA sequencing, that were found to be mis-regulated in in vitro-cultured embryos compared to their in vivo counterparts (Table 1) (Bauer et al., 2010a). We therefore focused on the effects of glycine on embryo development, specifically addressing how glycine supplementation to our culture medium affects development, alters gene expression, and affects mitochondrial function. We further asked how glycine contributes to development by culturing embryos with aminomethylphosphonic acid (AMPA), a glycine analog that inhibits serine hydroxymethyl transferase 2 (SHMT2), thereby reducing the catalysis of glycine to serine. Finally, embryo transfers were conducted to assess the potential of blastocysts created by glycine-supplemented culture to implant and produce offspring.

RESULTS

Effect of Glycine on Embryo Development

A study by Li et al. (2007) previously revealed that glycine concentrations ranged from 1.7 mM to 5.4 mM in porcine oviductal and uterine fluid on Days 3 and 5 post-insemination. Our current culture medium (MU1, porcine zygote medium 3 containing 1.69 mM arginine; the control condition) is formulated to have a 0.1 mM glycine, so we first tested if additional glycine affected in vitro embryo development. Glycine supplementation did not improve the percent of presumptive zygotes that developed to the blastocyst stage compared to the basal concentration of 0.1 mM glycine (Fig. 1); however, increasing glycine concentrations (≥ 5 mM) resulted in blastocyst rates that were

TABLE 1. Relative Abundance of Message for Selected Genes in Day-6 Blastocysts Cultured In Vitro or Produced In Vivo

Glycine-related genes	Number of aligned reads (mean \pm standard error)		P-value
	In vitro	In vivo	
Solute carrier family 6 member 9 (<i>SLC6A9</i>)	46.6 \pm 4.2	1.7 \pm 0.6	0.003
Glycine dehydrogenase (<i>GLDC</i>)	127.8 \pm 24.3	241.5 \pm 35.3	0.002
Aminomethyltransferase, glycine cleavage T (<i>AMT</i>)	128.1 \pm 13.2	248.9 \pm 26.3	0.02
Glycine cleavage system protein H (<i>GCSH</i>)	563.9 \pm 240.3	1047.1 \pm 50.2	0.13
Dihydroliipoamide dehydrogenase (<i>DLD</i>)	297.7 \pm 59.2	503.7 \pm 47.2	0.07
Phosphoglycerate dehydrogenase (<i>PHGDH</i>)	249.8 \pm 64.7	12.6 \pm 9.4	0.05
Phosphoserine phosphatase 1 (<i>PSPH</i>)	61.3 \pm 2.8	1.5 \pm 0.7	0.003
Phosphoserine aminotransferase (<i>PSAT1</i>)	188.5 \pm 46.3	1.1 \pm 0.6	0.06

RNA sequencing data were normalized, from Bauer et al. (2010a).

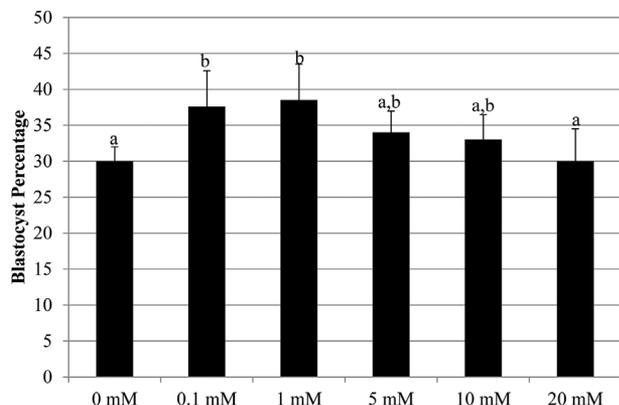


Figure 1. Percentage of embryos developing to the blastocyst stage after culture in different concentrations of glycine. Values were determined from 7 replicates (0, 0.1, 1, 5, and 10 mM treatments; $n = 298$ embryos per treatment) or 5 replicates (20 mM glycine; $n = 218$ embryos). Data show mean \pm standard error. Different letters indicate statistical differences ($P < 0.05$).

indistinguishable from suboptimal conditions without any glycine (0 mM).

A significant increase in the number of nuclei in Day-6 blastocysts was observed when embryos were cultured with 5 mM, 10 mM, or 20 mM glycine compared to controls (Fig. 2; Table 2). Specifically, embryos cultured in 10 mM glycine tended to have the highest number of nuclei, with an average of 72 per blastocyst. There was no difference in the number of nuclei associated with the inner cell mass among treatments, whereas the number of trophectoderm nuclei and the trophectoderm-to-inner-cell-mass ratio was higher for embryos cultured with 10 mM glycine (MU1 + 10 mM glycine) (Table 2). Thus, in vitro glycine supplementation

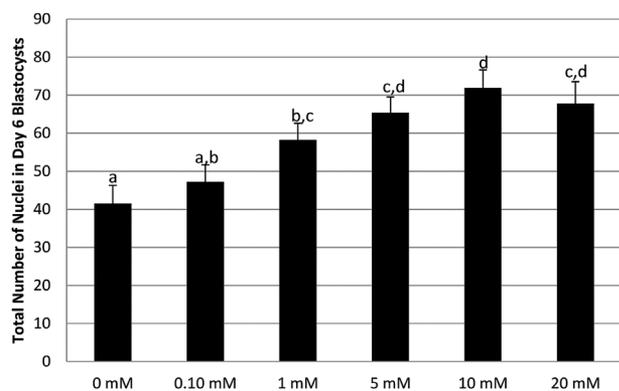


Figure 2. Total number of nuclei in blastocyst-stage embryos cultured in different concentrations of glycine. Values were determined from three replicates ($n = 20, 22, 23, 27, 21,$ and 15 for 0, 0.10, 1, 5, 10, and 20 mM glycine, respectively). Data show mean \pm standard error. Different letters indicate statistical differences ($P < 0.04$).

promotes cell proliferation primarily in the trophectoderm lineage.

Effects of Osmolality on Embryo Culture

Changes in amino acid composition can impact the osmolality of culture media, thus indirectly affecting embryo development. We therefore tested how control MU1 or MU1 + 10 mM glycine, at three different osmolalities—achieved with adjustments to sodium chloride concentration—affected blastocyst development. No change in blastocyst rates was observed among under different osmolalities (Fig. 3), although the same glycine-dependent increase in the number of nuclei was observed under all three osmolalities ($P \leq 0.08$) (Fig. 4)—which turns out to be due to glycine alone since a significant increase in the number of nuclei ($P = 0.003$) was observed in this dataset after removing variation due to osmolality as an effect in the statistical model.

Effect of Glycine on Apoptosis

The number of apoptotic nuclei in embryos cultured in MU1 and MU1 + 10 mM glycine was determined by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (Fig. 5). Embryos cultured in control MU1 had more ($P = 0.003$) ($14 \pm 1.5\%$) TUNEL-positive nuclei compared to embryos cultured with 10 mM glycine ($8 \pm 1.4\%$), but the absolute number of TUNEL-positive cells between treatments did not differ: MU1 (5.3 ± 0.5) versus MU1 + 10 mM glycine (4.7 ± 0.6) ($P = 0.46$).

Effect of Glycine on Gene Expression

We next used quantitative reverse-transcription PCR to assess how glycine supplementation affected gene expression of components needed to handle the increased glycine load. The functions addressed included glycine transport (*SLC6A9*), glycine metabolism (*GLDC*, *GCSH*, *DLD*, *AMT*), serine metabolism (*PSAT1*, *PSPH*, *SHMT2*, *PHGDH*), cell cycle (*PCNA*, *CCND3*, *CCNB1*), reactive oxygen species (*GSS*, *TXNRD1*, *GPX6*), apoptosis (*PARP2*, *BAD*, *TP53*), and one-carbon metabolism (*MTHFD1*, *MTHFD1L*, *ATIC*, *SMUG1*). Based on the relationship between glycine and serine metabolism, we expected that embryos cultured in increased glycine would show some changes in gene expression. Indeed, significant decreases in the transcript abundance of *SLC6A9*, *GLDC*, *GCSH*, *PSAT1*, and *PSPH* (all related to glycine or serine metabolic processes) were observed in the 10 mM glycine-treated group (Figs. 6 and 7). *TXNRD1* and *SMUG1* levels were also significantly decreased following culture with glycine, and a lower abundance of *BAD* was observed in the same group ($P = 0.08$) (Fig. 8). Transcript abundance of all the other genes showed no change between treatment groups.

Effect of AMPA on Embryo Development and Gene Expression

The reciprocal relationship between overabundance of glycine and reduced expression of genes involved in

TABLE 2. Differential Staining Results From Embryos Cultured in MU1 or MU1 + 10 mM Glycine

Treatment	Number of inner cell mass nuclei	Number of trophectoderm nuclei	Total cell number	Trophectoderm-to-inner cell mass ratio
MU1	13.7 ± 1.6	32.7 ± 2.2 ^a	48.6 ± 2.9	2.4 ± 0.3 ^a
MU1 + 10 mM Gly	15.8 ± 1.3	42.2 ± 2.2 ^b	55.9 ± 2.8	3.9 ± 0.4 ^b

Data are aggregated from five experimental replicates (n = 22 to 26). Means ± standard error are reported. Different letters indicate statistical differences ($P < 0.01$).

glycine and serine metabolism (Fig 6) lead us to ask if glycine supplement promoted the increased cell proliferation through SHMT2 activity. Culture with 50 mM AMPA, an inhibitor of SHMT2, completely abolished blastocyst development, but 10 mM AMPA did not affect blastocyst development (data not shown); therefore, 10 mM was used for the experiment. While embryos cultured in MU1 + 10 mM glycine had significantly more cells than control embryos produced in MU1 ($P = 0.01$), MU1 + 10 mM AMPA resulted in a phenotype that was indistinguishable from either MU1 or MU1 + 10 mM glycine groups (Fig. 9). Quantitative reverse-transcription PCR revealed that AMPA does not alter the abundance of *BAD* mRNA, but did decrease the expression of *SHMT2* and *TP53* compared to control embryos (Fig. 10). We predicted the reduction in *SHMT2* due to feedback from the inhibitor, but were surprised by the change in *TP53* levels.

Effect of Glycine on Mitochondria

Metabolism also depends on mitochondrial function, so we evaluated different metrics of the embryos' mitochondrial health following their culture in 10 mM glycine. Mitochondrial activity, as measured by MitoTracker Red CMXRos staining in live embryos (Fig. 11), was not different between blastocysts cultured in MU1 (2,212,748 ± 514,749 fluorescence units) or MU1 + 10 mM glycine

(2,853,625 ± 489,377 fluorescence units) ($P = 0.93$). Mitochondrial DNA (mtDNA) copy number, a proxy for the quantity of these organelles, showed a trending increase in embryos cultured with glycine across seven replicates of MU1– (609,700 ± 95,520 mitochondrial genomes among 41 individual blastocysts) versus MU1 + 10 mM glycine-cultured embryos (984,236 ± 200,058 mitochondrial genomes among 42 individual blastocysts) ($P = 0.12$). An F-test indicated that the variation between treatments was different ($P = 0.01$)—that is, there was more variation within the glycine-treated embryos than within the control embryos. On the other hand, no differences were observed in the transcript abundance of mitochondria-related genes—*IMMT*, *SSBP1*, *MFN2*, *PAM16*, and *PINK1*—in three 10-blastocyst pools of embryos cultured in MU1 + 10 mM glycine compared to MU1 (Fig. 12).

Embryo Transfer

Eleven embryo transfers were completed, involving 22–57 in vitro-produced blastocysts cultured in MU1 + 10 mM glycine, to test the competency of these glycine-enhanced blastocysts produce live offspring. Each of the recipient gilt's estrus cycle was extended past 21 days, but there was never a pregnancy detected and thus no live births. One additional animal was flushed on Day 15 to observe the MU1 + 10 mM glycine-cultured embryos when

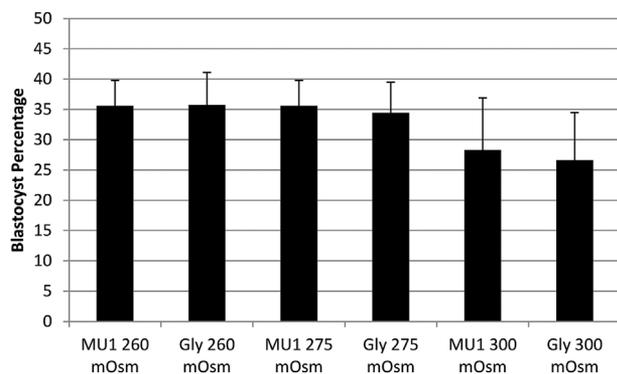


Figure 3. The effect of osmolality on blastocyst development in embryos cultured in MU1 or MU1 + 10 mM glycine (Gly). Values were determined from 5 replicates (n = 253, 254, 297, 297, 255, and 258 from left to right, respectively). Data show mean ± standard error. No differences in development were observed between treatments $P > 0.05$.

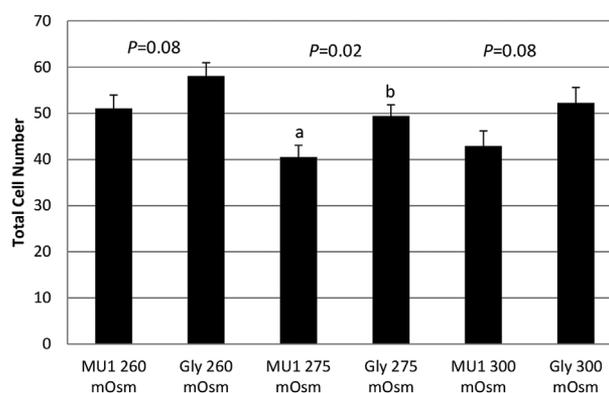


Figure 4. Effect of osmolality on total cell number in embryos cultured in MU1 or MU1 + 10 mM glycine (Gly). Values were determined from 5 replicates (n = 44, 43, 54, 58, 35, and 34 from left to right, respectively). Data show mean ± standard error. The main effect of glycine was different ($P = 0.003$).

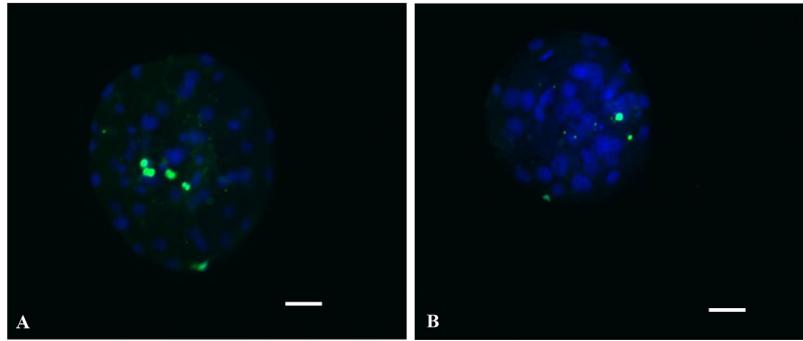


Figure 5. Representative images of TUNEL staining of Day-6 blastocysts cultured in (A) MU1 or (B) MU1 + 10 mM glycine. Scale bars, 50 μ m.

attachment normally occurs; a clump of elongated embryos was obtained. In contrast, 14 control in vitro-fertilized transfers were completed during this same period: 4 recipients contained viable fetuses (5, 6, 7, and 10 fetuses) on Day 45 of gestation, and 4 maintained pregnancy to term (delivering 4, 4, 5, and 5 live piglets).

DISCUSSION

Cancer cells and embryos utilize a metabolism that is distinct from the system used by most somatic cells. Highly proliferative cancer cells preferentially use the pentose phosphate pathway and glycolysis, regardless of the oxygen tension; this bias is characteristic of the Warburg Effect (Levine and Puzio-Kuter 2010), and is thought to be important for the production of macromolecules, for the protection against reactive oxygen species, and to support proliferation and growth. One attribute of the metabolic profile of cancer and tumor-initiating cells is their reliance

on glycine for proliferation and tumorigenesis: Jain et al. (2012) reported that isotope-labeled glycine is directly incorporated into the purine backbone of nucleotides. Zhang et al. (2012) found that glycine decarboxylase (GLDC), which converts glycine to 5, 10-methylenetetrahydrofolate for thymidine synthesis from deoxyuridine in concert with pyrimidine biosynthesis, metabolized most of the available glycine. Indeed, up-regulation of *GLDC* alone promotes cellular transformation by overcoming a deficiency in nucleotides. By analogy, exogenous glycine is likely needed to sustain the rapid cellular proliferation occurring in early embryos.

Many zygotes do not develop to blastocysts in vitro; those that do are often developmentally delayed or have an altered developmental program that may impact later development. Mining a previous transcription-profiling comparison of blastocysts that were in vivo-fertilized and in vivo-cultured or in vivo-fertilized and in vitro-cultured (Bauer et al., 2010a) revealed differentially expressed genes involved with glycine and serine metabolism (Table 1).

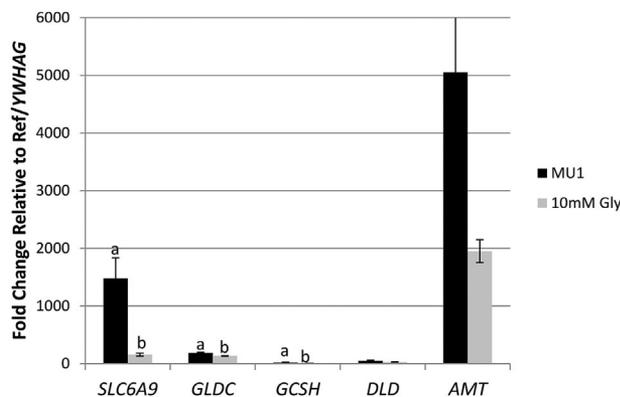


Figure 6. Relative abundance of transcripts involved with glycine transport and glycine metabolism from embryos cultured in MU1 or MU1 + 10 mM glycine. Data show mean \pm standard error. Different letters indicate statistical differences ($P < 0.05$).

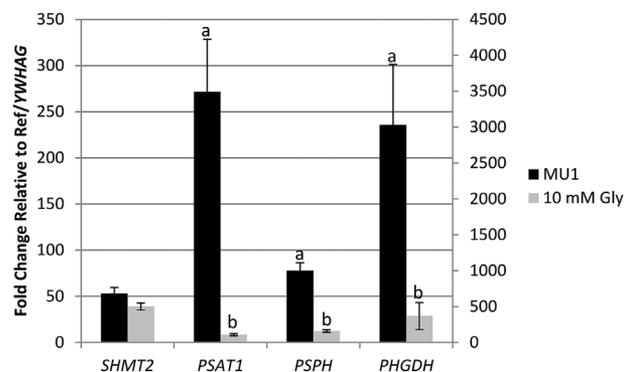


Figure 7. Relative abundance of transcripts involved with serine metabolism from embryos cultured in MU1 or MU1 + 10 mM glycine. Data show mean \pm standard error. Different letters indicate statistical differences ($P < 0.02$). Note that the Y axis on the left is for *SHMT2*, *PSAT1*, and *PSPH*; while the Y axis on the right is for *PHGDH*.

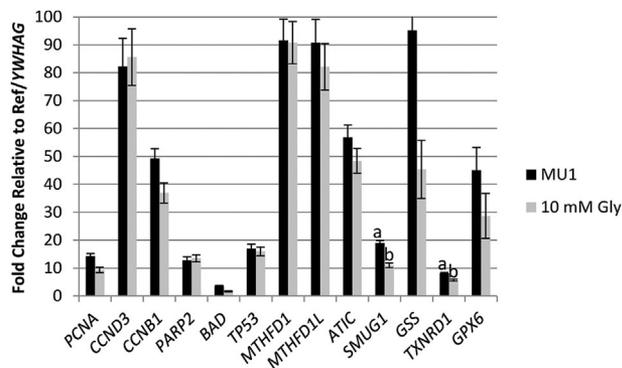


Figure 8. Relative abundance of transcripts involved with cell cycle, apoptosis, reactive oxygen species, and one-carbon pool from embryos cultured in MU1 or MU1 + 10 mM glycine. Data show mean ± standard error. Different letters indicate statistical differences ($P < 0.04$).

Culturing fertilized oocytes with different concentrations of glycine profoundly affected pre-implantation development (Fig. 2), which is consistent with the predicted impairment in glycine metabolism. Indeed, embryos cultured in 10 mM glycine possessed 1.5-fold more cells per blastocyst than our current optimized medium (72 vs. 42 cells per blastocyst). This elevated proliferation in response to glycine is consistent with reports for cancer and tumorigenic cells grown in the presence of additional glycine. Another study found that adding glycine and glucose together in a chemically defined medium lacking bovine serum albumin (PZM5) resulted in increased pig blastocyst development, increased hatching of blastocysts, increased cell number, and decreased apoptotic index compared to control embryos; unfortunately, no embryo-transfer results were reported (Mito et al., 2012). Surprisingly, these authors did not find any difference with glycine alone.

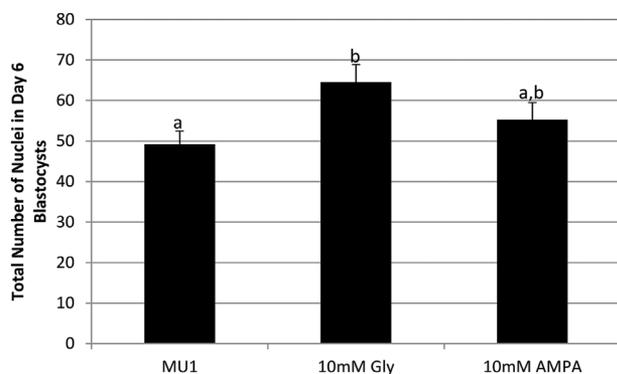


Figure 9. Effect of culture with AMPA on the number of nuclei in blastocysts. Data were from 3 replicates ($n = 28, 31,$ and 30 for MU1, MU1 + 10 mM glycine, and MU1 + 10 mM AMPA, respectively). Data show mean ± standard error. Different letters indicate statistical differences ($P = 0.02$).

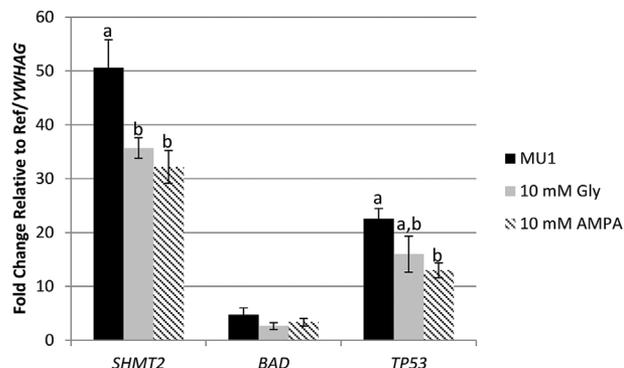


Figure 10. Effect of AMPA on the transcript abundance for genes involved with serine metabolism and apoptosis. Data show mean ± standard error. Different letters indicate statistical differences ($P < 0.02$).

Glycine was previously shown to benefit mouse embryo development by acting as an organic osmolyte (Van Winkle et al., 1990; Dawson et al., 1998; Steeves et al., 2003; Moravek et al., 2012). In porcine embryos, high and low osmolalities tended to yield blastocysts with more cells from the glycine-supplemented media compared to control media; indeed, media at 275 mOsm with additional glycine was associated with a significant increase in the total embryonic cell number (Fig. 4). After correcting for variation due to osmolality, glycine was found to significantly affect overall blastocyst cell number, and thus likely promotes proliferation through a mechanism that is independent of osmolality (Fig. 13).

Glycine can contribute to many processes during embryo development, and its availability likely influences de novo glycine and serine metabolism. The decreased expression of *SLC6A9*, which encodes glycine transporter, in embryos cultured with MU1 + 10 mM glycine is consistent with the hypothesis that control MU1-cultured embryos are up-regulating *SLC6A9* expression in response to insufficient available glycine—and mirrors the response previously reported for low availability of arginine (Bauer et al., 2010a,b). Expression of genes encoding glycine cleavage enzymes *GLDC* and *GCSH* are similarly lower because of the abundance of glycine. Genes related to de novo serine metabolism—*PHGDH*, *PSAT1*, and *PSPH*—were also down-regulated in the presence of supplemental glycine, which is consistent with the model that more available free glycine requires less conversion of serine to glycine.

Physiological levels of reactive oxygen species (ROS) are essential for regulating transcription factors and serving as second messengers in signal transduction (Peng et al., 2014). Under metabolic stress, however, ROS can induce large amounts of cellular damage to proteins, lipids, and nucleic acids. Two small-thiol systems, utilizing glutathione or thioredoxin, protect against damage associated with excess ROS. Thioredoxin reductase 1 (*TXNRD1*) was recently found to be critical for protecting mouse embryonic

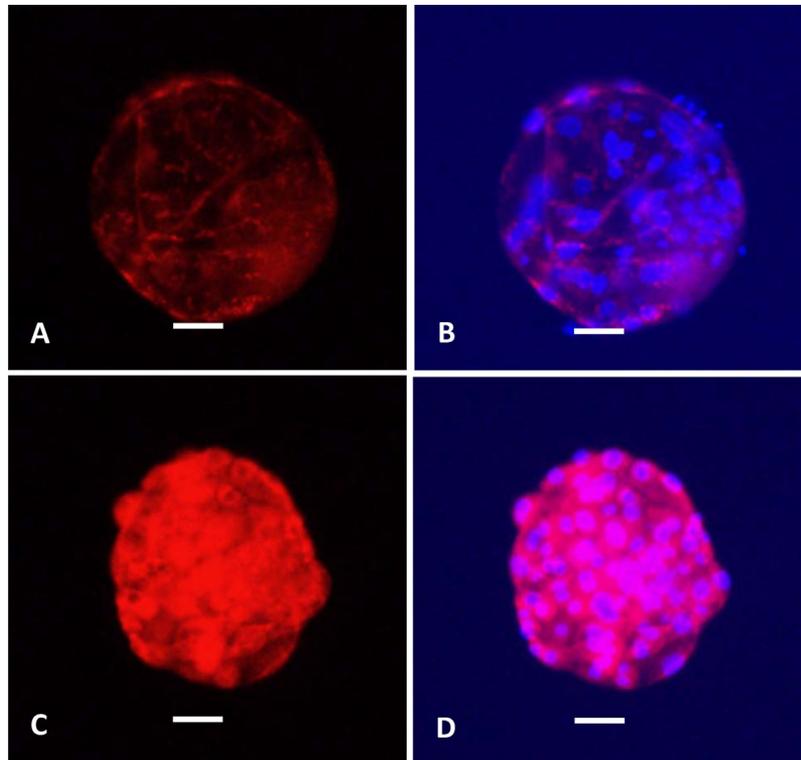


Figure 11. MitoTracker Red CMXRos staining of Day-6 blastocysts culture in either (A, B) MU1 or (C, D) MU1 + 10 mM glycine. Left images (A, C) show MitoTracker staining alone; right images (B, D) show MitoTracker staining merged with stained nuclei. Scale bars, 50 μ m.

fibroblasts from glucose-induced hydrogen peroxide production (Peng et al., 2014). We observed a decrease in *TXNRD1* transcript abundance in porcine blastocysts cultured in MU1 + 10 mM glycine, suggesting that supplemental glycine may be reducing the accumulation of ROS in these embryos, thus requiring less thioredoxin to protect against oxidative stress.

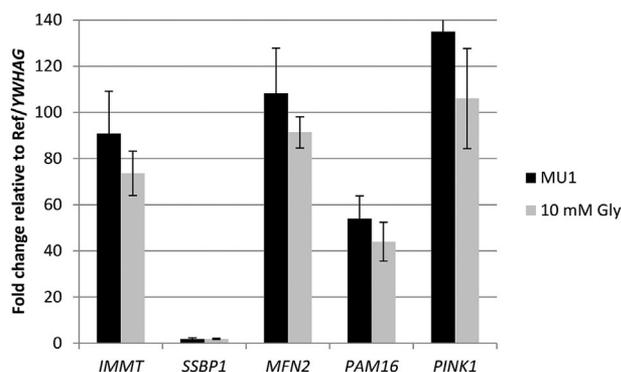


Figure 12. Effect of glycine on abundance of mitochondria-related transcript abundance.

DNA replication requires sufficient levels of one-carbon units for the de novo synthesis of purine or thymidylate, a pyrimidine precursor; a shortage of these one-carbon units could suppress mitotic rates and retard DNA repair, thereby reducing cellular proliferation and promoting genomic instability (Beaudin and Stover 2007). One-carbon units may be limiting in in vitro-cultured embryos since they possess higher expression of single-strand-selective monofunctional uracil-DNA glycosylase (*SMUG1*), a DNA base-excision-repair enzyme that removes mis-incorporated uracil from DNA (Vinson and Hales 2002), compared to their in vivo-produced counterparts (Bauer et al., 2010a). This phenotype suggests that concentrations of 5, 10-methylenetetrahydrofolate are disrupted or depleted in in vitro-cultured embryos. Increasing glycine in the culture medium lowered *SMUG1* transcript levels, suggesting that thymidylate may no longer be limiting, thus implying an abundance of purine and pyrimidine nucleotides that ultimately results in less mis-incorporation of uracil into DNA. This hypothesis is consistent with the lower percentage of apoptotic nuclei and reduction in pro-apoptotic *BAD* transcript in blastocysts cultured in MU1 + 10 mM glycine compared to controls cultured in MU1. Overall, these metrics point to healthier embryos.

SHMT2 is important for production of glycine from serine in the mitochondria, and its abundance is associated with

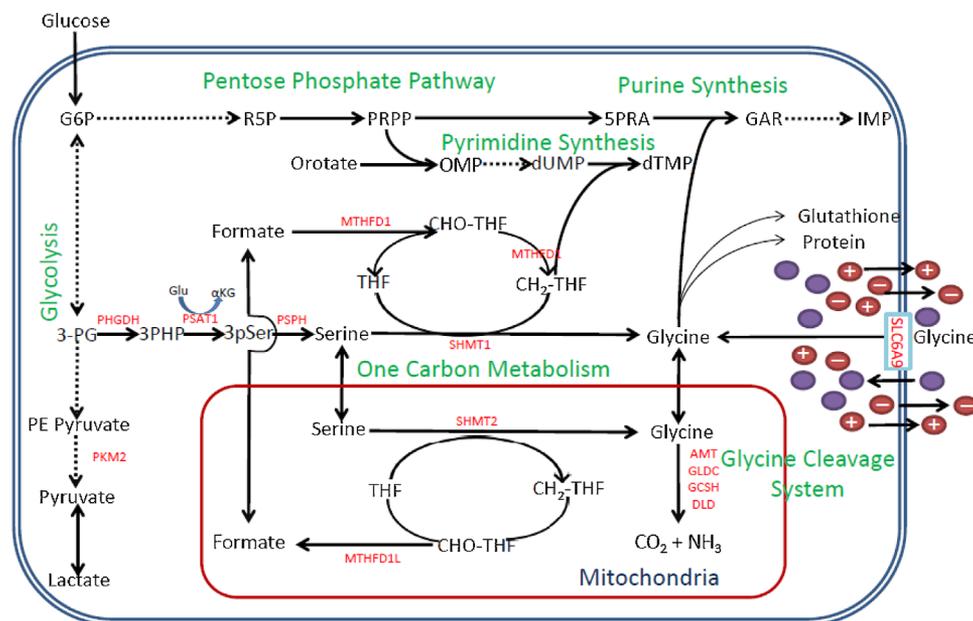


Figure 13. Potential effects of glycine on embryonic cells. Adapted from Tomita and Kami (2012).

the proliferation rate of cancer cells (Jain et al., 2012; Amelio et al., 2014). The glycine analog AMPA inhibits SHMT2, and selectively retards proliferation of cancer cells, but not normal somatic cells, at 25 mM (Li et al., 2013). When embryos were cultured with the less-toxic concentration of 10 mM AMPA, the resulting blastocysts contained a total cell number that fell between and did not differ from embryos cultured in control or glycine-supplemented conditions. Both glycine- and AMPA-treated embryos did, however, exhibit reduced *SHMT2* abundance compared to controls, which is consistent with the model that increased availability of glycine in the medium decreases the need for SHMT2 to convert serine to glycine, thereby reducing expression of the *SHMT2* gene.

Respiration in rapidly proliferating cancer cells is hypothesized to rely on endogenous glycine synthesis via SHMT2 located in the mitochondria (di Salvo et al., 2013). Glycine also condenses with succinyl-CoA in mitochondria to form 5-aminolevulinic acid, the universal precursor of heme that is required for cytochrome function. Considering the importance of glycine for mitochondrial function, we asked if supplemental glycine affected the function of this organelle in blastocysts. Although numerically higher fluorescence intensity was observed in embryos cultured with 10 mM glycine, the level did not differ from the control group due to the high variation in intensity among embryos cultured under either condition. Similarly, mtDNA copy number tended to be higher in embryos cultured with 10 mM glycine, but the variation within this experimental group confounded the detection of any significant change. Only small amounts of mitochondrial DNA replication occur during porcine preimplantation development, although a slight increase in this

metric was reported at the blastocyst stage (Spikings et al., 2007). The high variation observed in mtDNA levels following culture with glycine supplement could therefore indicate the first movement of this measure away from baseline; perhaps a difference in mitochondrial function and replication would be detected later in development. On the other hand, quantitative reverse-transcription PCR for the mitochondria-related genes *IMMT*, *SSBP1*, *MFN2*, *PAM16*, and *PINK1* revealed no difference in the abundance in embryos cultured in 10 mM glycine versus control conditions (Fig. 12), suggesting that these trends may be a consequence of the generally higher variation observed in cultured embryos compared to their in vivo counterparts (Bauer et al., 2010a). Consequently, significant differences are more difficult to detect under this expanded variance.

None of the eleven embryo transfers conducted with embryos cultured in 10 mM glycine resulted in a successful pregnancy, indicating that in vitro glycine supplementation may ultimately have had a detrimental effect on establishing and maintaining pregnancy in vivo. *GLDC* was found to be significantly down-regulated under 10 mM glycine compared to control conditions, but reduced *GLDC* activity could also cause an accumulation of excess glycine that may have ultimately proved to be lethal due to the formation of the toxic intermediates aminoacetone and methylglyoxal (Kim et al., 2015). Instead, these results reinforce the possibility that total cell number and overall appearance of an embryo may not be good indicators of developmental capabilities in vivo (Spate et al., 2012). Given the failure to achieve pregnancy or live birth, supplemental glycine could be considered as a contraceptive.

Glycine is utilized in many ways in an early embryo (Fig. 13). Our results indicate that this amino acid does not contribute solely as an osmolyte in pig embryo culture: it is a precursor to nucleotide synthesis—as suggested by decreased *SMUG1* levels, which correlate with increased uracil incorporation to DNA—and dramatically influences the expression of serine- and glycine-metabolism genes without affecting the expression of mitochondria-related genes. Glycine-cultured embryos also undergo less apoptosis than control embryos. How glycine promotes these improvements in preimplantation development and why these embryos are not able to establish pregnancy and produce live piglets still need to be determined.

MATERIALS AND METHODS

Chemical Components

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

In Vitro Production of Embryos

Ovaries were obtained from pre-pubertal gilts according to the study protocol and standard operating procedures reviewed and approved by the Institutional Animal Care and Use Committee of the University of Missouri. Cumulus-oocyte complexes were aspirated from follicles 2–8 mm in diameter, and subjected to in vitro maturation, as described previously (Zhang et al., 2010). Cumulus cells were denuded from matured oocytes by vortexing briefly in 0.03% hyaluronidase in Tyrode's Lactate (TL)-Hepes buffered saline (Lai and Prather 2003). Metaphase-II oocytes were selected based on the extrusion of the first polar body, and were subsequently used for in vitro fertilization.

Mature oocytes were washed in IVF medium (modified Tris-buffered medium containing 2 mg/mL bovine serum albumin (BSA) and 2 mM caffeine). Thirty oocytes were placed into 50- μ L droplets of IVF medium, covered with a mineral oil overlay, and incubated at 38.5°C until sperm were added. The sperm used for fertilization was obtained from a single boar, and used for all experiments. For in vitro fertilization, a 0.1-mL frozen semen pellet was thawed in 3 mL of sperm washing medium (Dulbecco's phosphate-buffered saline (Gibco) supplemented with 0.1% BSA and 10 μ g/mL gentamicin). The sperm were washed twice by centrifugation: first in a 45% Percoll solution, and then in modified Tris-buffered medium. The spermatozoa pellet was resuspended with IVF medium to 0.5×10^6 cells/mL. Finally, 50 μ L of the sperm suspension was added to the oocytes in the IVF medium, giving a final concentration of 0.25×10^6 cells/mL. Gametes were incubated together for 4–5 hr.

Effect of Glycine on Embryo Development Embryo Culture

Inseminated oocytes were removed from the droplets and washed in PZM3 (Yoshioka et al., 2002) containing 1.69 mM arginine (MU1) (Bauer et al., 2010b; Redel et al.,

2015). Fifty presumptive zygotes were then cultured in MU1 with 0, 0.1 (the concentration of glycine normally in MU1; our control), 1, 5, 10, or 20 mM glycine in a four-well dish in humidified atmosphere with 5% CO₂ in air for 28–30 hr at 38.5°C. After 28–30 hr of culture, the embryos were moved to a humidified atmosphere of 5% CO₂, 90% N₂, and 5% O₂ at 38.5°C until Day 6 post-fertilization, when blastocysts from each treatment group were collected and the percent of embryos that developed to the blastocyst stage was recorded. To determine the effect that each concentration of glycine had on development, the percentage of blastocysts in each treatment group was analyzed by using a general linear model PROC GENMOD in SAS (V9.3, SAS Institute, Cary, NC). A Least Significant Difference (LSD) post-test comparison was completed to determine if significant differences existed between treatment groups ($P < 0.05$).

Embryos for all subsequent experiments were cultured in either MU1 + 0.1 mM glycine (referred to as MU1, or the control condition) or MU1 + 10 mM glycine. The blastocysts were then collected and used for RNA isolation or stained to quantify cell numbers.

Differential Nuclear Staining

The number of trophectoderm and inner cell mass nuclei for embryos cultured in either MU1 or MU1 + 10 mM glycine were assessed by differential nuclear staining (Machaty et al., 1998; Bauer et al., 2010a). Zona pellucidae were removed using a physiological saline lowered to a pH of 1.79. Zona-free embryos were exposed for 60 min to a 1:7 dilution of rabbit anti-pig whole serum (Bauer et al., 2010a), and then washed three times for 5 min in TL-Hepes buffered saline. Finally, embryos were incubated for 35 min in a 1:10 dilution of guinea pig complement, propidium iodide (10 μ g/mL), and bisbenzimidide (10 μ g/mL). This complement-mediated lysis causes trophectoderm cells to lyse, which allows them to take up propidium iodide, whereas cells of the inner cell mass should remain alive. All cells take up bisbenzimidide. The embryos were then observed with ultraviolet light under 40 \times magnification using a Nikon Eclipse E600 inverted microscope (Nikon Corp., Tokyo, Japan). Inner-cell-mass nuclei stained only with bisbenzimidide (blue) while trophectoderm nuclei stained with both propidium iodide and bisbenzimidide (pink). The number of nuclei belonging to the inner cell mass, trophectoderm, total embryo (total cell number), and the ratio of trophectoderm-to-inner cell mass were first analyzed for normality by using the UNIVARIATE procedure in SAS. The data were then transformed and analyzed by analysis of variance using the mixed-linear model procedure in SAS. An LSD post-test comparison was completed for each variable to determine if significant differences ($P < 0.05$) existed between the groups.

Effects of Osmolality on Embryo Culture

An experiment assessing the effects of high and low osmolality of culture medium was conducted to assess if

glycine is acting as an osmolyte in our culture system, as it does for other species. Fertilized embryos were cultured in media with low (260 mOsm), normal (275 mOsm), or high (300 mOsm) osmolalities, which were obtained by adjusting the concentration of NaCl. Presumptive zygotes were cultured in one of six treatments: MU1 ± 10 mM glycine at 260 mOsm; MU1 ± 10 mM glycine at 275 mOsm; or MU1 ± 10 mM glycine at 300 mOsm. The number of embryos that developed to the blastocyst stage and the total number of nuclei were counted for each treatment group.

RNA Extraction and cDNA Synthesis for Quantitative PCR

Three biological replicates of Day-6 blastocysts were obtained for both MU1 and MU1 + 10 mM glycine conditions. Total RNA was extracted from pools of 10 embryos per replicate for the glycine and serine metabolism genes or from pools of 40 embryos per replicate for the remaining genes, using an RNeasy micro kit (Qiagen, Germantown, MD). Total RNA was suspended in 12 µL, and was then converted to cDNA using the SuperScript VILO kit (Life Technologies, Carlsbad, CA).

Relative Quantitative PCR

Relative quantitative PCR was conducted on each of the samples for the genes involved with glycine metabolism, serine metabolism, glutathione production, cell cycle,

folate-related metabolism, and apoptosis-related genes using IQ SYBR Green Supermix (Bio-Rad Laboratories) and unamplified cDNA from each biological replicate as template. Primers were designed using Integrated DNA Technology software (Itdna.com; Coralville, IA), 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 expression of the chosen transcripts (Table 3). Primer efficiency tests were completed for each primer set by generating a standard curve using 10 ng dilutions of our 5 ng/µL reference cDNA pool. Quantitative PCR was run in triplicate at each concentration (50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, 0.005 ng/µL) to validate each primer set.

The abundance of each mRNA transcript was calculated relative to the reference sample and the housekeeping gene *YWHA*G (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide) (Whitworth et al., 2005; Bauer et al., 2010a). The reference sample was pooled from four biological replicates of in vivo-fertilized/in vivo-cultured blastocysts and in vivo-fertilized/in vitro-cultured blastocysts (Bauer et al., 2010a). Transcript abundance between treatments was determined using the comparative threshold cycle (C_T) method for each gene. The $2^{-\Delta\Delta C_T}$ values were analyzed for normality, and log transformed if not normally distributed. The resulting values were then analyzed using the general linear model (PROC GLM) in SAS. Differences in expression

TABLE 3. Transcripts Selected for Real-Time Quantitative PCR

Gene	Forward primer	Reverse primer	Accession number
<i>SLC6A9</i>	5'-TGCGGCCACCTGACTAGTGTT	5'-GAGGAGGTGGCAACCACAGATAAT	XM_005656191
<i>GLDC</i>	5'-CCAGGTGCTGATCTCCATA	5'-AGAGAGGTGGCAGCATT	NM_000170.2
<i>GCSH</i>	5'-GGTGCTTTGGAAAGTGTGAAAGCTGC	5'-CATCCAGTTCTGAAGGTTACTGA	XR_130503.3
<i>DLI</i>	5'-GGACTAGAAGAGCTTGGAAATTG	5'-CATCCCTTCCACACAGATAATG	NM_214062.1
<i>AMT</i>	5'-TCCCACATGCTGCAGACCAAGATA	5'-CCCTGATTTGGCTTTAACTCGGCA	NM_001244426.1
<i>SHMT2</i>	5'-GAGAGTCTATCAGACAGTGACC	5'-CGAGTACTTGTGTTTCAGACAG	XM_005663918.1
<i>PSAT1</i>	5'-TGGCAACACCAAGGAGATGATGC	5'-TGCTCATAGCTGGTGCATCTCCAA	XM_001928079.3
<i>PSPH</i>	5'-AAGCCTGTCCTCCTGCTGATGTTT	5'-TGATGTACCATTCCGCGTTGTCCT	NM_001243221.1
<i>PHGDH</i>	5'-CTTCCATGAAGGATGGCAAAT	5'-AGACCTCTGGAGCAATGATAG	NM_001123162
<i>PCNA</i>	5'-GGCTCCCAAGATCGAAGATGAA	5'-AGTATGTGCTGGCATCACCGAA	NM_182649.1
<i>CCND3</i>	5'-GATTCCTGGCTCTGATTCTC	5'-GGTACATGGCAAAGGTGTA	NM_001078678.1
<i>CCNB1</i>	5'-ACTGGCTAGTGCAGGTTTCAGAT	5'-AACATGGCAGTGACACCAACCA	NM_031966.2
<i>PARP2</i>	5'-CGTGTACAGGAGCTGATAAAG	5'-GCCCGAAT CAATCCTCAAT	XM_005656314.1
<i>BAD</i>	5'-TTGCCAGCCGAGATTAACCCCTAAC	5'-CACGCGGGCTTTATTAGCACGTTT	XM_003122573.2
<i>TP53</i>	5'-GGAACAGCTTTGAGGTGCGTGTTT	5'-ATACTCGCCATCCAGTGGCTTCTT	NM_213824.3
<i>MTHFD1</i>	5'-AGTGGAGAGTGCAGAGCATTTCCT	5'-CAGGTTACCAATGGGCTTTGGCTT	XM_001924329.3
<i>MTHFD1L</i>	5'-AGTACCGCAGGTGGAACTTCACT	5'-ACGTACTTTGGCTTTGCTTCTGCC	XR_300085.1
<i>ATIC</i>	5'-TCCATGCTGGAATCTTGGCTCGTA	5'-ACATCTGGAGAAGCCACTGTCTC	NM_001130736.1
<i>SMUG1</i>	5'-AGGGAGAGAGAATTCAGTGTGGCA	5'-TTGGAGAGGTGCGTCAACTCATCT	XM_001927219.3
<i>GSS</i>	5'-GCCAAGATCCTCTCCAATAATC	5'-GGCCAGTAGTCTATTCTCTA	NM_001244625.1
<i>TXNRD1</i>	5'-CTCAAGTGGGAGAAGGAAAC	5'-GATCGTCCATTCCACAAAGA	NM_214154.3
<i>GPX6</i>	5'-AAACAAGAACCAGCAAAGAAC	5'-GGAGTTCTTCAGGAAGGTAAAG	NM_001137607.1
<i>IMMT</i>	5'-GGTGGAAAGGGATGAGTATTT	5'-CAGCTGTATCTCACTTCTAT	XM_003481170.2
<i>SSBP1</i>	5'-GAAAGTAGACTATGGCGAATACA	5'-TCAGAGTATCCAGCTTCTT	XM_003134602.4
<i>MFN2</i>	5'-ATGCTCTGGACAAAGTGCTGC	5'-TTGACGCTCCTCTTCTCCTCTGA	XM_005665006.1
<i>PAM16</i>	5'-AGGCCAGCAGATTCTCAATGTCT	5'-TTTGACTGCAGGTAGAAGGAGCCA	XM_003124646
<i>PINK1</i>	5'-GATGGAGTCAGAGGTCTAA	5'-GATGCTATGCAGAGACCTAATC	XM_005665091.1
<i>CYTB</i> (for standard)	5'-GGAATCTCATCAGACATAGAC	5'-GAGGTCTGCTACTAGTATTC	KJ789952
<i>CYTB</i> (for samples)	5'-ACCTACTAGGAGACCCAGACAAC	5'-TGAACGTAGGATAGCGTAGGCGAA	KJ789952

were determined by using the Least Squares Means (LSMeans) generated by PROC GLM, and $P < 0.05$ was considered significant.

TUNEL Staining

Apoptotic cells in Day-6 blastocysts were identified using the In Situ Cell Death Detection Kit Fluorescein (Roche Diagnostics). Embryos were fixed in 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. A positive-control group was achieved using 32 Kunitz units of DNase I and 25 μL of the TUNEL solution. A negative control was obtained by incubating with only the "label" solution. The permeabilized blastocysts were washed in TL-Hepes buffered saline with 0.1% polyvinyl alcohol (PVA). Embryos were then incubated in 25 μL of this solution at 38.5°C for 1 hr in a dark, humidified atmosphere. Embryos were washed with TL-Hepes buffered saline with PVA, and the nuclei were stained with 10 μg /mL bisbenzimidazole for 20–30 min. The embryos were then washed and mounted on a glass slide for viewing by epifluorescence illumination.

Culture AMPA

SHMT2 reversibly catalyzes the conversion of serine to glycine, and may be responsible for the intracellular glycine content. AMPA, a glycine analog, inhibits more than 95% of nuclear thymidylate synthesis, suggesting that this compound inhibits SHMT2 (Li et al., 2013). We, therefore, used AMPA to test if glycine was promoting cell proliferation through SHMT2. Oocytes were collected and fertilized as described above. Presumptive zygotes were then cultured for 28–30 hr in a four-well dish incubated in a humidified atmosphere with 5% CO_2 in air at 38.5°C, in either MU1, MU1 + 10 mM glycine, or MU1 + 10 mM or 50 mM AMPA. After 28–30 hr, embryos were moved to a humidified atmosphere of 5% CO_2 , 90% N_2 , and 5% O_2 at 38.5°C until Day 6 post-fertilization, when blastocysts from each treatment group were collected and the percent of embryos that developed to the blastocyst stage was recorded. The blastocyst percentage for each treatment group was analyzed by using PROC GENMOD in SAS. An LSD post-test comparison was completed to determine if significant differences existed between treatment groups ($P < 0.05$). Total number of nuclei was also counted from Day-6 embryos, as described above. Three replicates of 28, 31, and 30 blastocysts were counted for MU1, MU1 + 10 mM glycine, and MU1 + 10 mM AMPA, respectively. Day-6 blastocysts were then collected for gene expression analysis. Three replicates of pools of 10 blastocysts for each treatment were collected and processed for quantitative PCR, as described above, for *SHMT2*, *TP53*, and *BAD*.

MitoTracker Red CMXRos Staining

The effect glycine had on mitochondrial membrane activity was evaluated because glycine is the precursor to heme, a critical component for cytochrome synthesis and

oxidative phosphorylation. Live embryos were stained with 125 nM MitoTracker Red CMXRos (Life Technologies). Six replicates were performed, generating 17 blastocysts from MU1 and 16 blastocysts from MU1 + 10 mM glycine conditions. A control group of embryos was left unstained to capture background levels. Each treatment group was then fixed in 4% paraformaldehyde, and stained with bisbenzimidazole to count total nuclei in each embryo. Embryos were mounted on slides and imaged using a fluorescent microscope. The sum intensity was collected for each stained blastocyst, and background intensity was subtracted from each embryo. This adjusted-sum-intensity value was imported into SAS, and a mixed-linear procedure was used to determine if differences existed between culture groups. The data were log transformed before the analysis due to their non-normal distribution. The raw mean intensity is presented in the text, but the statistical analysis was derived from the log-transformed values.

Mitochondrial DNA Copy Number

Analysis of mtDNA copy number in Day-6 blastocysts cultured in either MU1 or MU1 + 10 mM glycine was performed in a manner similar to described previously (Mao et al., 2012). The zona pellucidae were removed from the blastocysts using a physiological saline solution at pH 1.79, and then the embryos were washed in TL-Hepes-buffered saline. An individual blastocyst in 1 μL diethylpyrocarbonate-treated phosphate-buffered saline with 1% PVA was then placed in a 0.5-mL tube and snap frozen in liquid N_2 . An external standard was generated to create a standard curve for PCR quantification. Primers were designed to amplify a 369-bp fragment of the mitochondrial cytochrome B gene (*CYTB*) (Table 3). Reactions included 7.5 μL GeneAmp Fast PCR Master Mix (ABI, Foster City, CA), 0.5 mM of each primer, and 1 μL DNA template in 15 μL . Plasmid-standard concentrations were determined on a NanoDrop 1000 Spectrophotometer (NanoDrop products, Wilmington, DE); 1 ng of the plasmid standard (3 kb vector + 0.369 kb PCR product) was assumed to contain 2.72×10^8 molecules. Serial 10-fold dilutions were used to construct a standard curve for PCR quantification (Mao et al., 2012).

mtDNA was quantified from embryos in the same manner as Mao et al. (2012). Primers were designed that anneal inside of the amplified 369-bp *CYTB* fragment (see Table 3). Single-embryo lysates were used as a template, and quantitative PCR reactions were completed as described above. A linear-regression analysis of all standard curves for samples with copy numbers between 10^2 and 10^7 showed a high correlation coefficient ($r^2 = 0.999$). A water control was included within each assay. Absolute mtDNA copy number in a single blastocyst was calculated from the standard curve derived from the quantitative PCR amplifications using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA). Statistical analysis was completed by first assessing the data for normality by using the Shapiro–Wilks test. The mtDNA copy number for each embryo was then analyzed using the mixed linear procedure in SAS.

Embryo Transfer

On Day 6 after fertilization, blastocysts cultured in MU1 + 10 mM glycine were selected and placed in 3 mL of 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 H₂O) in polystyrene tubes (BD Falcon 352054, San Jose, CA). Embryos were transported at 37°C to the University of Missouri Swine Research Complex, where they were loaded into a tomcat catheter and surgically transferred to the ampullary-isthmic junction of a cycling gilt on Day 5 or 6 of her estrous cycle (Spate et al., 2010; Lee et al., 2013). Pregnancy was determined by heat checking and monitored by ultrasound after Day 25. Pigs were checked weekly until the gilt returned to estrus or farrowed.

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