BIOLOGY OF REPRODUCTION (2015) **92**(2):51, 1–10
Published online before print 30 December 2014.
DOI 10.1095/biolreprod.114.125971

Uterine Histotroph and Conceptus Development. I. Cooperative Effects of Arginine and Secreted Phosphoprotein 1 on Proliferation of Ovine Trophectoderm Cells via Activation of the PDK1-Akt/PKB-TSC2-MTORC1 Signaling Cascade[1]

Xiaoqiu Wang,[3,4] Greg A. Johnson,[5] Robert C. Burghardt,[5] Guoyao Wu,[3,4] and Fuller W. Bazer[2,3,4]

[3]Center for Animal Biotechnology and Genomics, Texas A&M University, College Station, Texas
[4]Department of Animal Science, Texas A&M University, College Station, Texas
[5]Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, Texas

ABSTRACT

The greatest limitation to reproductive performance in most mammals, including humans, is embryonic mortality, which, in general, claims 20%–40% of the embryos during the peri-implantation period of pregnancy. Both arginine and secreted phosphoprotein 1 (SPP1) are multifunctional molecules that increase significantly in ovine uterine histotroph during early pregnancy. However, little is known about the relationship and underlying mechanisms for synergistic effects of arginine and SPP1, if any, on conceptus (embryo/fetus and associated extraembryonic membranes) development. Therefore, we conducted in vitro experiments using our established ovine trophectoderm cell line (oTr1) isolated from Day 15 ovine conceptuses to determine their proliferative response to individual and synergistic effects of arginine and recombinant SPP1 (rSPP1) that contains an RGD binding sequence. At physiological concentrations, arginine (0.2 mM) stimulated oTr1 cell proliferation 1.7-fold ($P < 0.05$) at 48 h, whereas rSPP1 (10 ng/ml) had no such effect. However, an additive effect on oTr1 cell proliferation was induced by combination of arginine and SPP1 as compared to the control (2.1-fold increase; $P < 0.01$), arginine alone (1.3-fold increase; $P < 0.05$), and rSPP1 alone (1.5-fold increase; $P < 0.01$). This additive effect was mediated through cooperative activation of the PDK1-Akt/PKB-TSC2-MTORC1 cell signaling cascade. Collectively, results suggest that arginine and SPP1 in histotroph act cooperatively to enhance survival, growth, and development of ovine conceptuses.

arginine, MTORC1, sheep, SPP1, trophectoderm

INTRODUCTION

In all mammalian species, the greatest constraint to reproductive performance is embryonic mortality, which, in most cases, claims 20%–40% of embryos [1]. Of these embryonic losses, two-thirds occur during the peri-implantation period of pregnancy due to factors that include asynchrony between conceptus (embryo/fetus and associated extraembryonic membranes) and uterine signals that regulate conceptus elongation and uterine receptivity to implantation, resulting in defects in conceptus development and implantation [1, 2]. Conceptus elongation, migration, attachment, and, in many species, invasion must be intricately orchestrated and carefully timed during the window of implantation in all mammalian species [3, 4]. Ovine conceptuses undergo rapid elongation from spherical to tubular and filamentous forms that requires proliferation, migration, and cytoskeleton reorganization of trophectoderm (Tr) cells, and these structural changes are supported by the transport or secretion of key nutrients or molecules (histotroph) from the uterus into the lumen and conceptus-uterine interactions for pregnancy recognition signaling and implantation [2, 5]. Failure of conceptuses to implant is the major limiting factor in assisted reproduction in humans that accounts for 50%–75% of failures to establish pregnancy [6]. Thus, an understanding of the intrauterine environment and molecular mechanisms responsible for appropriate conceptus development, as well as successful implantation, is necessary if clinicians and animal scientists are to enhance fertility by reducing embryonic mortality during the peri-implantation period of pregnancy [7].

Histotroph, the major source of nutrients required for conceptus development, is a complex mixture of molecules secreted or transported into the uterine lumen by uterine luminal (LE), superficial glandular (sGE), and deeper glandular (dGE) epithelia [5, 8]. Of the nutrients provided in histotroph, arginine is a conditionally essential amino acid for most mammals, including sheep, and it increases significantly during the peri-implantation period of pregnancy [9–12]. In addition to being a building block for protein synthesis, arginine has indispensably functional roles for normal development of conceptuses during the peri-implantation period of pregnancy. Arginine exerts effects on conceptuses, as it is the substrate for production of nitric oxide (NO) via NO synthases and polyamines via arginase and ornithine decarboxylase (ODC1) [13–17] in sheep [18–20], pigs [21, 22], rats [23, 24], and mice [25]. Our in vitro studies using ovine trophectoderm (oTr1) cells demonstrated that arginine 1) stimulates cell proliferation and protein synthesis via its metabolism to NO and polyamines, particularly putrescine, and 2) stimulates production of interferon tau (the pregnancy recognition signal in ruminants) [26]

During the peri-implantation period of pregnancy, attachment of Tr to uterine LE is facilitated by a mosaic of interactions between integrins and extracellular matrix (ECM) proteins that contribute to stable adhesion for implantation [27–29]. It has been established that uterine synthesis of secreted phosphorprotein 1 (SPP1; also known as osteopontin) increases significantly during the peri-implantation period of most mammals studied [30]. SPP1 is a multifunctional ECM protein that binds to cell surface integrin receptors via its Arg-Gly-Asp

[1]This research was supported by Agriculture and Food Research Initiative competitive grants 2011-6705-20028 and 2011-67015-20067 from the U.S. Department of Agriculture National Institute of Food and Agriculture.
[2]Correspondence: E-mail: fbazer@cvm.tamu.edu

Received: 19 October 2014.
First decision: 8 November 2014.
Accepted: 22 December 2014.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

TABLE 1. Antibodies and dilutions used for immunocytochemical analyses.

Antibody	Catalog number	Dilution	Source
Primary antibody			
pAkt[a]	9275	1:50	Rabbit
pMTOR[a]	2971	1:50	Rabbit
pPDK1[a]	3061	1:50	Rabbit
pRaptor[b]	sc-130214	1:100	Rabbit
pTSC2[a]	3615	1:100	Rabbit
Akt[a]	9272	1:100	Rabbit
MTOR[a]	2983	1:100	Rabbit
PDK1[a]	3062	1:100	Rabbit
α-Tubulin[c]	T5168	1:500	Mouse
Secondary antibody			
Alexa Fluor 488[d]	A11008	1:250	Goat-anti-rabbit
Alexa Fluor 594[d]	A11037	1:250	Goat-anti-rabbit
Alexa Fluor 488[d]	A11029	1:250	Goat-anti-mouse

[a] Antibody from Cell Signaling Technology.
[b] Antibody from Santa Cruz Biotechnology.
[c] Antibody from Sigma-Aldrich.
[d] Antibody from Life Technologies.

(RGD) amino acid sequence to regulate cell proliferation, migration, adhesion, differentiation, survival, and immune function in many physiological systems [30–37]. In sheep, implanting conceptuses secrete interferon tau, prolonging the life span of corpus luteum (CL). Progesterone released by CL then induces SPP1 synthesis and secretion from the endometrial GE into the uterine lumen, where SPP1 supports conceptus attachment to the uterus for implantation. Using an ovine trophectoderm cell line (oTr1), it has been demonstrated that SPP1 binds the αvβ3 integrin heterodimer and increases cell adhesion and migration but not proliferation [30, 34–37].

Although arginine and SPP1 are prominent factors within the intrauterine environment during the peri-implantation period, little is known about whether the arginine and SPP1 act cooperatively (synergistic and/or additive effects) to affect development of peri-implantational conceptuses or about the underlying mechanism, if any, responsible for those effects. Therefore, we treated our established oTr1 cells with arginine and recombinant SPP1 (rSPP1) containing an RGD binding sequence to determine their individual and cooperative effects on cell proliferation. Our results indicate that arginine and rSPP1 act cooperatively to increase cell proliferation and that this synergistic effect is mediated through activation of the PDK1-Akt/PKB-TSC2-MTORC1 cell signaling cascade.

MATERIALS AND METHODS

Cell Culture

An established mononuclear ovine trophectoderm (oTr) primary cell line from Day 15 sheep conceptuses was developed, propagated, and used in the present in vitro studies as described previously [26, 37–39]. One cell line, referred to as oTr1, that exhibits numerous properties of ovine trophectoderm cells in vivo was cultured in complete medium (CM; Dulbecco modified Eagle medium/Nutrient Mixture F-12; Gibco BRL, Grand Island, NY), with 10% fetal bovine serum (FBS; Gibco BRL), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM each for nutritionally nonessential'' amino acids, 1 mM sodium pyruvate, 2 mM glutamine, and 4 μg/ml insulin. The medium was replaced every 2 days. When the density of cells in the dishes reached about 80% confluence, subcultures of cells were prepared at a ratio of 1:3, and frozen stocks of cells were preserved at each passage.

For the experiments, monolayer cultures of oTr1 cells (between passages 8 and 15) were grown in CM to 20%–30% confluence in 96-well plates (Costar#3595; Corning, Corning, NY) or Lab-Tek II 4-well chamber slides (154534; Sigma-Aldrich, St. Louis, MO). Cells were serum and insulin starved for 24 h in customized medium, further deprived of arginine for an additional 6 h, and then treated with arginine and/or rSPP1 [30, 40, 41] at indicated concentrations in basal medium (BM; arginine-free customized medium containing 5% FBS and 1 ng/ml insulin). For each experiment, the design was replicated in three independent experiments.

Proliferation Assay

The oTr1 cells were seeded (15 000 cells/0.2 ml/well) on 96-well plate (Costar#3595; Corning) in complete medium (CM) until the monolayer reached up to 30% confluence and then switched to serum- and insulin-free customized medium. After starvation for 24 h, cells were further deprived of serum, insulin, and arginine for additional 6 h. Cells (n = 3 wells per treatment) were then cultured in 50 μl BM at 0 min as a starting point control and then treated with arginine (200 μM), rSPP1 (10 μg/ml), or arginine plus rSPP1. Cells grown in BM and CM served as negative and positive controls, respectively. The medium were changed every 2 days, and treated cells were maintained for 48 h. Cell numbers were determined as described previously [42]. Briefly, medium was removed from cells by vacuum aspiration, and cells were fixed in 50% ethanol for 30 min, followed by vacuum aspiration of the fixative. Fixed cells were stained with Janus Green B in PBS (pH 7.2) for 3 min at room temperature. The stain was immediately removed using a vacuum aspirator, and the whole plate was sequentially dipped into water and destained by gentle shaking. The remaining water was removed by shaking, after which stained cells were lysed immediately in 0.5 N HCl (0.3 ml/well), and absorbance readings were taken at 595 nm using a microplate reader. As described previously [42], cell numbers were calculated from absorbance readings using the following formula: cell number = (absorbance − 0.00462)/0.00006926. The entire experiment was repeated independently three times with different batches of oTr1 cells between passages 9 and 12.

Quantitative Immunocytochemistry

The oTr1 cells were seeded onto Lab-Tek II four-well chamber slides (154534; Sigma-Aldrich). After serum and insulin starvation for 24 h followed by additional deprivation of arginine for an extra 6 h, cells were then treated with arginine (200 μM), rSPP1 (10 μg/ml). or arginine plus rSPP1. Cells cultured in BM served as control. After 12, 24, and 48 h, cells were fixed with −20°C methanol for 10 min and rinsed with 0.02 M PBS containing 0.3% Tween (PBST) for 5 min. The cells were blocked in 5% normal goat serum for 2 h at room temperature and rinsed, and then immunofluorescence staining was performed using primary antibodies listed in Table 1 overnight at 4°C. Purified nonrelevant rabbit or mouse IgG was substituted for the primary antibody as a negative control. Cells were then incubated with secondary antibodies listed in Table 1 for 1 h at room temperature and then rinsed in PBST and overlaid with Prolong Gold Antifade with DAPI if it was for single staining procedure. For the double-staining procedure, separate antibody incubations were used. Briefly, after being blocked in 5% normal goat serum for 2 h and probed with first primary antibody overnight and then first secondary antibody for 4 h, cells were rinsed and incubated with second primary antibody overnight and second secondary antibody for 4 h and then rinsed in PBST and overlaid with Prolong Gold Antifade with DAPI. Images were captured using a Zeiss Axioplan 2 microscope with an Axiocam HR camera and Axiovision 4 software (Carl Zeiss Microscopy, Thornwood, NY). All parameters during image acquisition were the same. Signals were quantified by Image J software (Version 1.47; National Institutes of Health, Washington, DC) using standardized procedures described previously [43–45]. Briefly, the region of cells of interest (ROI; number of pixels for the selected cell) was defined by the Freehand selection tool. The next step was to split the image into the three color channels (RGB merge/split function) to gain one image per channel and then obtain the density value (DV; average signal per pixel for the selected cell) of each of the molecules, depending on fluorescent color of secondary antibodies. In parallel, average signal per pixel for a region selected just beside the cell of interest was measured as background signal for subtraction. Finally, the corrected total cell fluorescence (CTCF; arbitrary units) was calculated based on the following formula: CTCF = (DV − background signal) × ROI, indicating the level of protein expression per cell analyzed. The quantitative data were derived from analysis of 10 fields per well, and a total of three wells were analyzed.

Statistical Analyses

Normality of data and homogeneity of variance were tested using the Shapiro-Wilk test and Brown-Forsythe test, respectively, in SAS 8.1 (SAS Institute, Cary, NC). Data were analyzed by least-squares one-way ANOVA as well as the Fisher least significant difference as post hoc analysis, with each well identified as an experimental unit. The synergism (interaction between arginine and rSPP1) was analyzed by least-squares two-way ANOVA [46]. All analyses were performed using SAS. Data are expressed as means ± SEM, and values of $P < 0.05$ were considered significant.

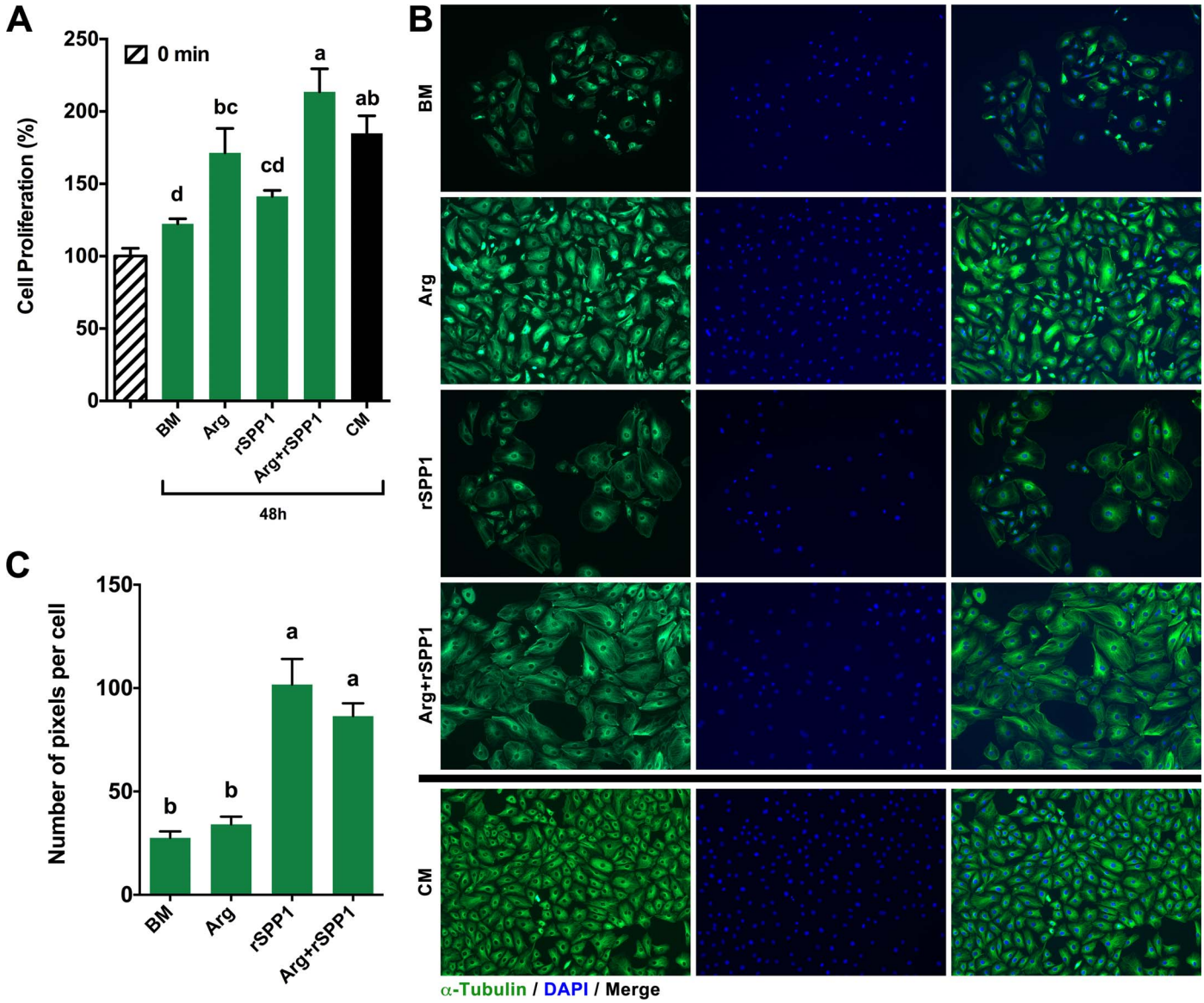

FIG. 1. Cooperative effects of arginine and rSPP1 on proliferation of oTr1 cells at 48 h of incubation. The oTr1 cells (n = 3 wells) were seeded at 30% confluence in 96-well plates. After serum and insulin starvation for 24 h followed by additional deprivation of arginine for an extra 6 h, cells were cultured in BM with or without arginine (Arg; 200 μM), rSPP1 (10 μg/ml), or arginine plus rSPP1. Cells grown in BM and CM served as negative and positive controls, respectively. After 48 h of incubation, cell numbers were determined by proliferation assay (**A**) and also visualized by immunofluorescence imaging using an antibody against α-tubulin (**B**). Data are expressed as a percentage relative to nontreated control cells at 0 min. Arginine increased cell proliferation, and rSPP1 increased cell size, while their cooperative effects were to increase both number and size of oTr1 cells (**C**). In addition, images of oTr1 cells grown in CM are presented for a reference (**C**). Different superscript letters denote significant ($P < 0.05$) differences in cell proliferation or cell size (number of pixels per cell) due to treatment. Data are presented as means ± SEM. BM, basal medium; CM, complete medium. Width of image field = 900 μm.

RESULTS

Arginine and rSPP1 Act Additively to Increase Proliferation of oTr1 Cells at 48 h of Incubation

We first investigated the effects of arginine and rSPP1, independently and in combination, on oTr1 cell proliferation after 48 h of incubation (Fig. 1). Arginine increased ($P < 0.05$) oTr1 cell proliferation by 1.7-fold at 48 h, whereas rSPP1 had no effect ($P > 0.05$; Fig. 1A). However, an additive increase ($P < 0.05$) in oTr1 cell proliferation occurred in arginine plus rSPP1 treatment as compared to BM (2.1-fold increase; $P < 0.01$), arginine alone (1.3-fold increase; $P < 0.05$), and rSPP1 alone (1.5-fold increase; $P < 0.01$). In addition, rates of cell proliferation were not different ($P > 0.05$) between arginine plus rSPP1-treated and CM-positive control cells. These results are consistent with immunofluorescence imaging results that allow visualization of the numbers and morphology of oTr1 cells after 48 h of incubation (Fig. 1B) and that showed that arginine increased oTr1 cell numbers, whereas the effect of rSPP1 was to increase cell spreading (Fig. 1C). The additive effects were achieved by a combination of arginine and rSPP1 whereby the numbers and spreading of oTr1 cells increased as compared to BM control cells (Fig. 1).

Arginine and rSPP1 Synergistically Stimulate oTr1 Cell Proliferation via the MTORC1 Signaling Pathway

Next, we evaluated the abundance of total and phosphorylated mechanistic target of rapamycin (pMTOR) as well as

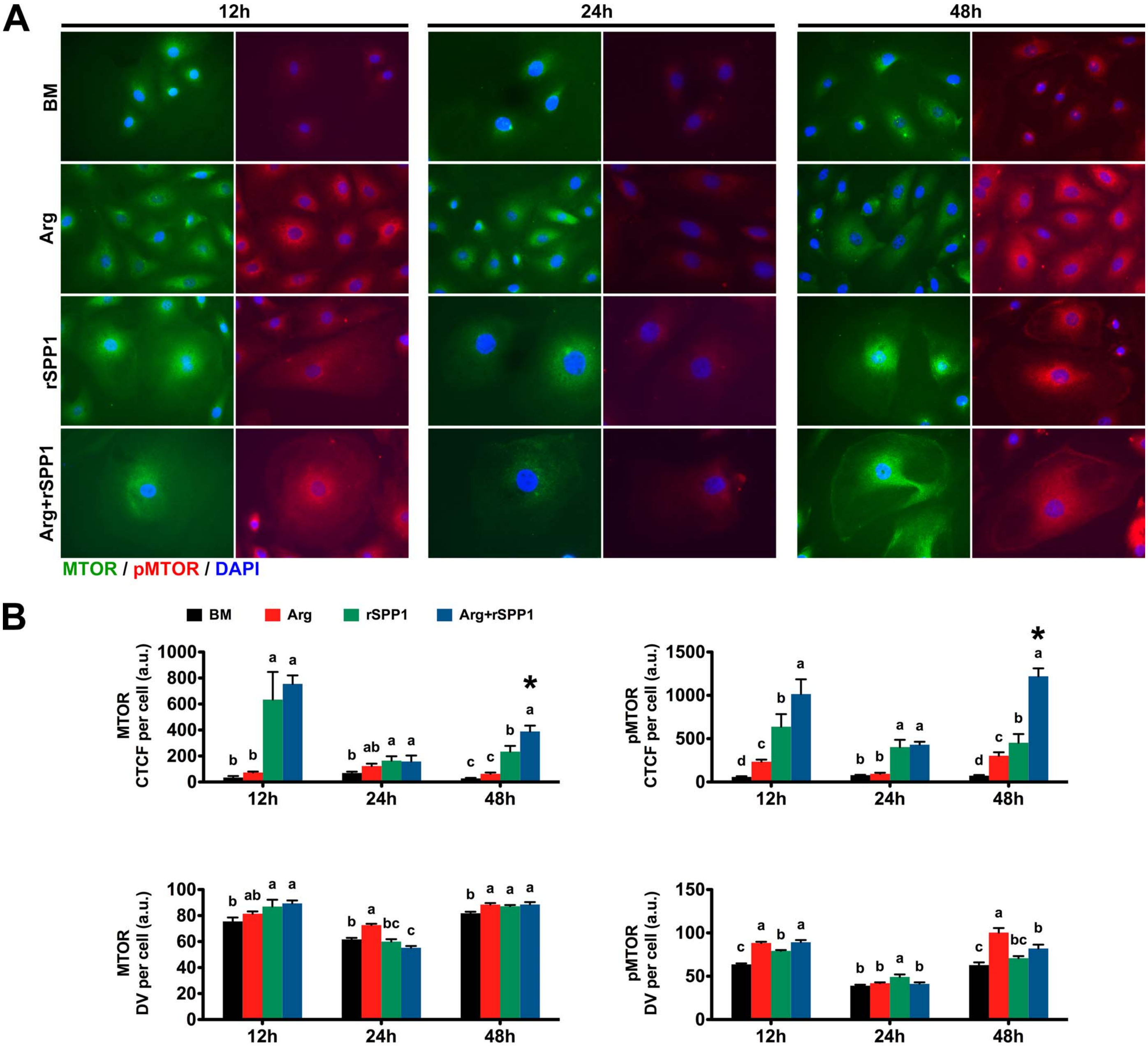

FIG. 2. Arginine and rSPP1 activate MTOR signaling pathway in oTr1 cells. The oTr1 cells (n = 3 wells) were seeded at 30% confluence onto Lab-Tek II four-well chamber slides. After serum and insulin starvation for 24 h followed by an additional deprivation of arginine for 6 h, cells were treated in BM with or without arginine (Arg; 200 μM), rSPP1 (10 μg/ml), or arginine plus rSPP1. Images (**A**) and quantification (**B**) of immunocytochemical analyses demonstrated increased abundances of pMTOR in oTr1 cells treated with either arginine at 12 and 48 h or rSPP1 at all three time points compared to BM control; however, the additive and synergistic increases of pMTOR were observed only at 12 and 48 h, respectively. Total MTOR was not different between arginine-treated and BM cells but increased in oTr1 cells treated with rSPP1 at 12, 24, and 48 h. However, the synergistic increase in MTOR occurred only at 48 h. CTCF, corrected total cell fluorescence; DV, density value; a.u., arbitrary units. Width of image field = 220 μm. Different superscript letters denote significant ($P < 0.05$) differences among treatment groups at the respective time points. An asterisk (*) denotes significant ($P < 0.05$) synergistic effects of arginine and rSPP1 at the respective time points. All data are presented as means ± SEM. For clarity, global contrast and brightness are adjusted uniformly on all images of total MTOR at all time points.

phosphorylated regulatory-associated protein of MTOR (pRaptor) using quantitative immunocytochemical analyses (Figs. 2 and 3). After 12, 24, and 48 h of incubation, the pMTOR increased 3.9- ($P < 0.01$), 1.2- ($P > 0.05$), and 4.2-fold ($P < 0.01$) in the arginine-treated cells; 10.6-. 5.1-, and 6.2-fold ($P < 0.01$) in the rSPP1-treated cells; and 16.9-, 5.5-, and 16.7-fold ($P < 0.05$) in the arginine plus rSPP1-treated cells as compared to BM control cells at each of the time points, respectively (Fig. 2). The additive and synergistic increases in pMTOR were observed at 12 and 48 h, respectively. Total MTOR in oTr1 cells was not affected significantly by arginine alone (Fig. 2) but increased 18.3-, 2.4-, and 8.5-fold ($P < 0.05$) in rSPP1-treated oTr1 cells and 21.8-, 2.3-, and 14.1-fold ($P < 0.05$) in arginine plus rSPP1-treated oTr1 cells compared to BM control cells at 12, 24, and 48 h, respectively. However, the synergistic increase in total MTOR was observed only at 48 h of incubation. Furthermore, pRaptor increased 23.2-, 1.9-, and 3.1-fold ($P < 0.05$) in arginine treatment oTr1 cells; 23.1-,

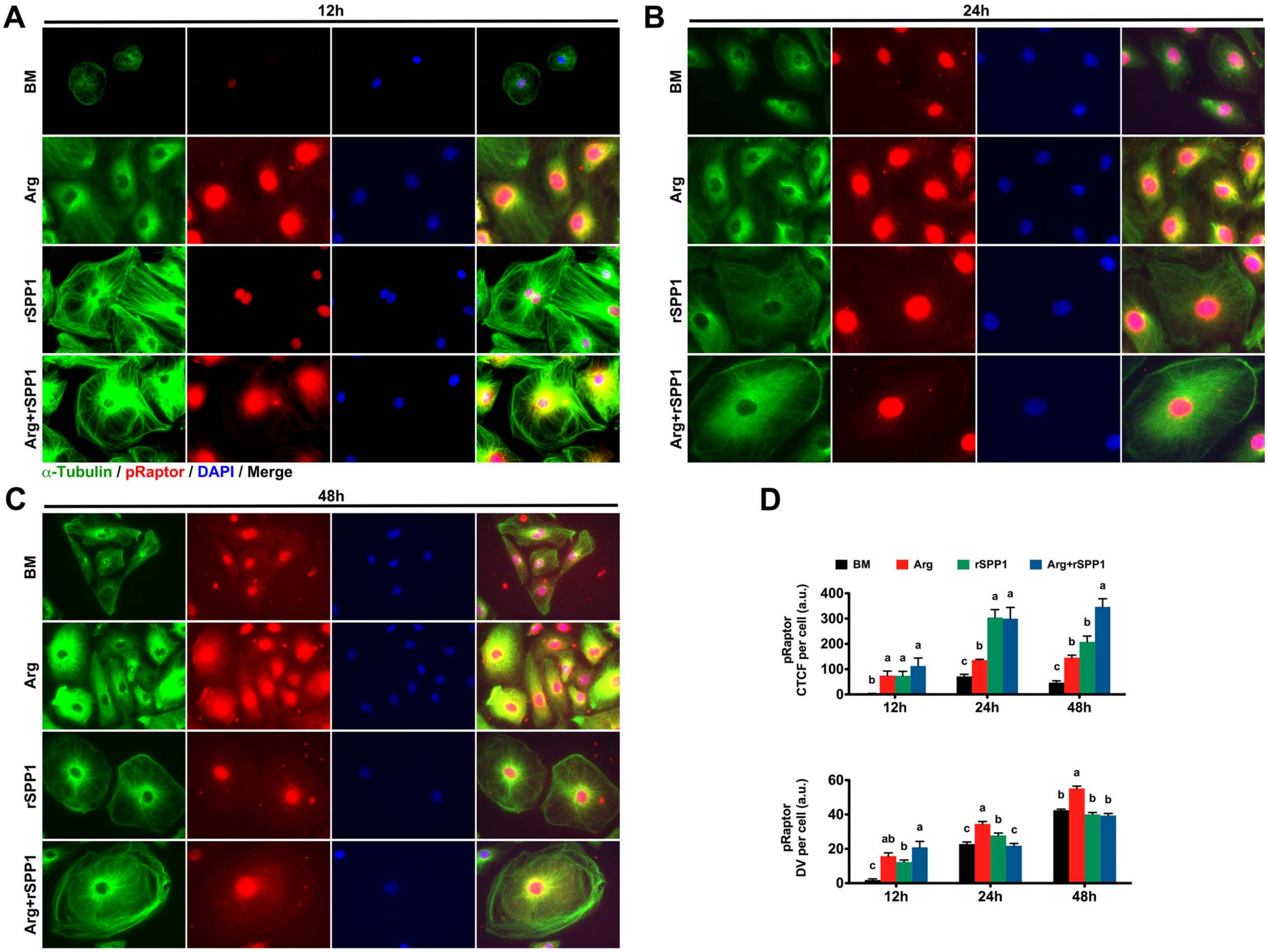

FIG. 3. Arginine and rSPP1 activate the MTORC1 signaling pathway in oTr1 cells via phosphorylation of Raptor. The oTr1 cells (n = 3 wells) were seeded at 30% confluence onto Lab-Tek II four-well chamber slides. After serum and insulin starvation for 24 h followed by an additional deprivation of arginine (Arg) for 6 h, cells were treated in BM with or without arginine (200 μM), rSPP1 (10 μg/ml), or arginine plus rSPP1. Images (**A**–**C**) and quantification (**D**) of immunocytochemical analyses revealed increased abundances of pRaptor in oTr1 cells treated with either arginine or rSPP1 at all three time points; however, the additive increases in pRaptor were observed only at 48 h. Cell shape and size were visualized after incubating oTr1 cells with antibody against α-tubulin. CTCF, corrected total cell fluorescence; DV, density value; a.u., arbitrary units. Width of image field = 220 μm. Different superscript letters denote significant ($P < 0.05$) differences among treatment groups at the respective time points. Data are presented as means ± SEM. For clarity, global contrast and brightness are adjusted uniformly on all images of pRaptor at all time points.

4.3-, and 4.4-fold ($P < 0.05$) in rSPP1-treated oTr1 cells; and 35.3-, 4.2-, and 7.4-fold ($P < 0.05$) in arginine plus rSPP1-treated oTr1 cells as compared with BM control oTr1 cells at 12, 24, and 48 h, respectively (Fig. 3). Again, the additive increase in pRaptor occurred only at 48 h of incubation. Antibody against α-tubulin was used for visualization of cell shape when the abundance of pRaptor was detected in oTr1 cells at each time point (Fig. 3, A–C).

Arginine and rSPP1 Synergistically Activate the MTORC1 Signaling Pathway via Increased Phosphorylation of TSC2 in oTr1 Cells

We then investigated the abundance of phosphorylated TSC2 (pTSC2) using quantitative immunocytochemical analyses (Fig. 4). The pTSC2 increased 5.5-, 2.1-, and 2.7-fold ($P < 0.05$) in the arginine-treated oTr1 cells compared to BM control oTr1 cells at 12, 24, and 48 h, respectively, whereas pTSC2 increased 2.5- and 5.4-fold ($P < 0.05$) in the rSPP1-treated oTr1 cells only at 24 and 48 h, respectively. However, pTSC2 increased 8.7-fold ($P < 0.01$) due to the additive effects of arginine plus rSPP1 on oTr1 cells at 12 h, whereas the effects of arginine plus rSPP1 on oTr1 cells were synergistic at 24 and 48 h when pTSC2 increased 6.9- and 9.4-fold ($P < 0.01$), respectively, as compared to BM control oTr1 cells.

Arginine and rSPP1 Act Synergistically to Increase Phosphorylation of TSC2 via Activation of Akt in oTr1 Cells

The abundances of total and phosphorylated Akt (pAkt) were evaluated by immunocytochemical analyses (Fig. 5). Compared with BM control oTr1 cells, pAkt increased 6.0-, 4.6-, and 3.8-fold ($P < 0.05$) in oTr1 cells in response to arginine treatment at 12, 24, and 48 h, respectively. It also increased 5.8- and 7.6-fold ($P < 0.05$) in rSPP1-treated oTr1 cells at 24 and 48 h, respectively. However, the greatest

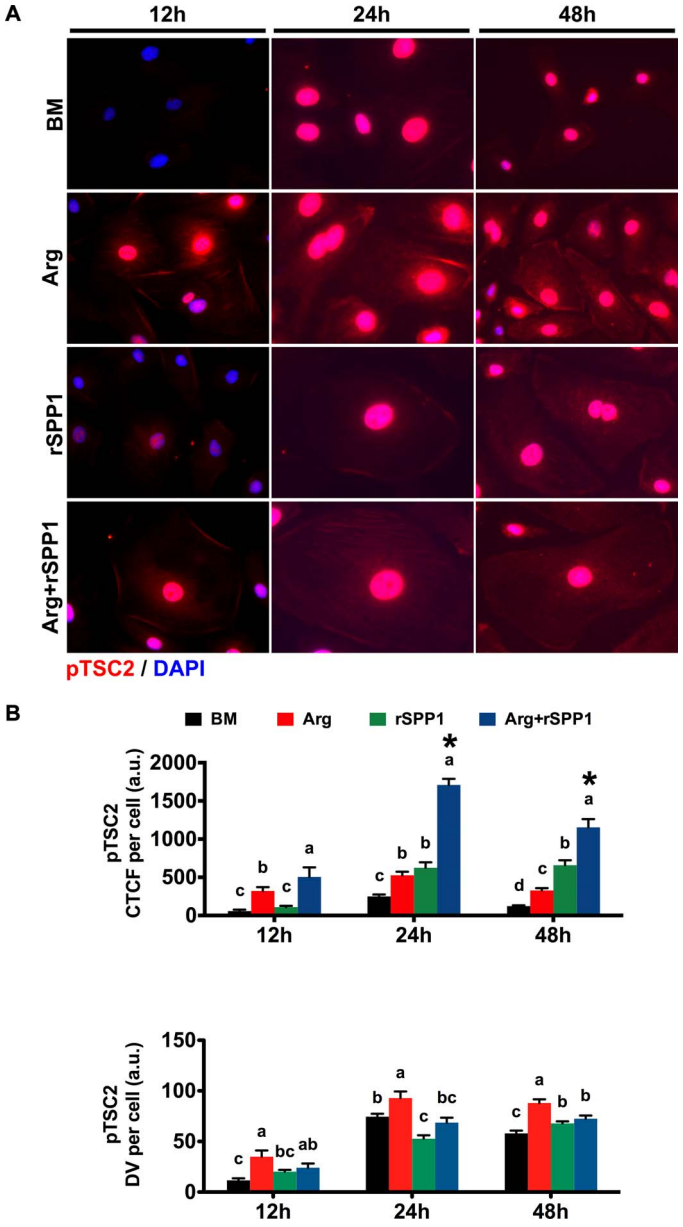

FIG. 4. Arginine and rSPP1 stimulate MTOR cell signaling pathway in oTr1 cells by increasing phosphorylation of TSC2. The oTr1 cells (n = 3 wells) were seeded at 30% confluence onto Lab-Tek II four-well chamber slides. After serum and insulin starvation for 24 h followed by an additional deprivation of arginine (Arg) for 6 h, cells were treated in BM with or without arginine (200 μM), rSPP1 (10 μg/ml), or arginine plus rSPP1. Images (**A**) and quantification (**B**) of immunocytochemical analyses revealed increased abundance of pTSC2 in oTr1 cells treated with either arginine at 12 and 24 h or rSPP1 at 24 and 48 h as compared to BM cells. The synergistic effects of arginine and rSPP1 on increased abundance of pTSC2 in oTr1 cells were observed at 24 and 48 h, whereas only additive effects of arginine and rSPP1 were detected at 12 h. CTCF, corrected total cell fluorescence; DV, density value; a.u., arbitrary units. Width of image field = 220 μm. Different superscript letters denote significant ($P < 0.05$) differences among treatment groups at the respective time points. Asterisk (*) denotes significant ($P < 0.05$) synergistic effects of arginine and rSPP1 at the respective time points. Data are presented as means ± SEM.

synergistic increases in pAkt were achieved in the arginine plus rSPP1-treated oTr1 cells at 12 and 24 h but not at 48 h, with 17.9-, 16.6-, and 9.2-fold changes ($P < 0.01$) compared to BM control oTr1 cells, respectively. Total Akt was not affected significantly by arginine treatment alone but increased 2.2-, 4.8-, and 3.1-fold ($P < 0.05$) in rSPP1-treated oTr1 cells and 3.0-, 6.7-, and 5.3-fold ($P < 0.05$) in arginine plus rSPP1-treated oTr1 cells compared to BM control oTr1 cells at 12, 24, and 48 h, respectively. The additive increases in total Akt were observed only at 24 and 48 h of incubation.

Arginine and rSPP1 Synergistically Activate the Akt Signaling Pathway via Increased Total and Phosphorylated PDK1 Abundance in oTr1 Cells

We next evaluated the abundance of total and phosphorylated 3-phosphoinositide-dependent protein kinase 1 (pPDK1) in oTr1 cells at 12, 24, and 48 h of incubation (Fig. 6). The pPDK1 increased 5.7-, 2.3-, and 4.4-fold ($P < 0.05$) in arginine-treated oTr1 cells, 6.7-, 3.3-, and 16.1-fold ($P < 0.05$) in rSPP1-treated oTr1 cells and 16.8-, 3.8-, and 25.8-fold ($P < 0.01$) in arginine plus rSPP1-treated oTr1 cells compared to BM control oTr1 cells at 12, 24, and 48 h, respectively. The synergistic increases in pPDK1 induced by arginine plus rSPP1 occurred at 12 and 48 h. Total PDK1 was not affected significantly by arginine treatment alone but increased 6.0-fold ($P < 0.05$) in response to rSPP1 at 48 h of incubation. However, the synergistic effects of arginine and rSPP1 on the abundance of total PDK1 were achieved at 12 and 24 h with 3.5-fold changes ($P < 0.01$) at each of those time points.

DISCUSSION

The results of this study provide the first in vitro evidence to support that conceptus development during the peri-implantation period benefits from the cooperative actions of arginine and SPP1. Although it is well established that arginine and SPP1 are individually pivotal to conceptus elongation and implantation in sheep, interaction between these factors to alter conceptus development is a new finding that significantly advances our understanding of the roles of histotroph to support successful pregnancy.

During the peri-implantation period of pregnancy, the conceptus undergoes rapid elongation from spherical to tubular and filamentous forms immediately prior to attachment to uterine LE and implantation [2, 5]. Failed elongation or inappropriate development of the peri-implantation conceptus results in early embryonic losses in most mammals and accounts for 50%–75% of failures to establish pregnancy [6]. This process is highly correlated with the composition of histotroph, a complex mixture of molecules that are secreted or transported into the uterine lumen by uterine LE, sGE, and dGE, including but not restricted to enzymes, growth factors, adhesion proteins, hormones, transport proteins, amino acids, and glucose. In most mammalian species, both arginine (a conditionally essential amino acid) and SPP1 (the multifunctional ECM protein) increase significantly during the peri-implantation period of pregnancy [9–12, 30, 34–37]. Results of our previous studies demonstrated that arginine is an indispensable amino acid required for normal conceptus development, whereas SPP1 plays a fundamental role in adhesion, migration, and cytoskeletal remodeling of conceptus Tr, which are essential for conceptus development and implantation. However, implantation is an intricately timed event that requires the cooperative actions of many factors to orchestrate synthesis and secretion of histotroph, conceptus signaling for pregnancy recognition, and uterine receptivity to implantation, and it is reasonable to predict the existence of cooperative (synergistic and/or additive) effects between arginine and SPP1 to support these events. Therefore, the present study was performed to gain insight into the cooperative roles of arginine and rSPP1 in stimulating ovine

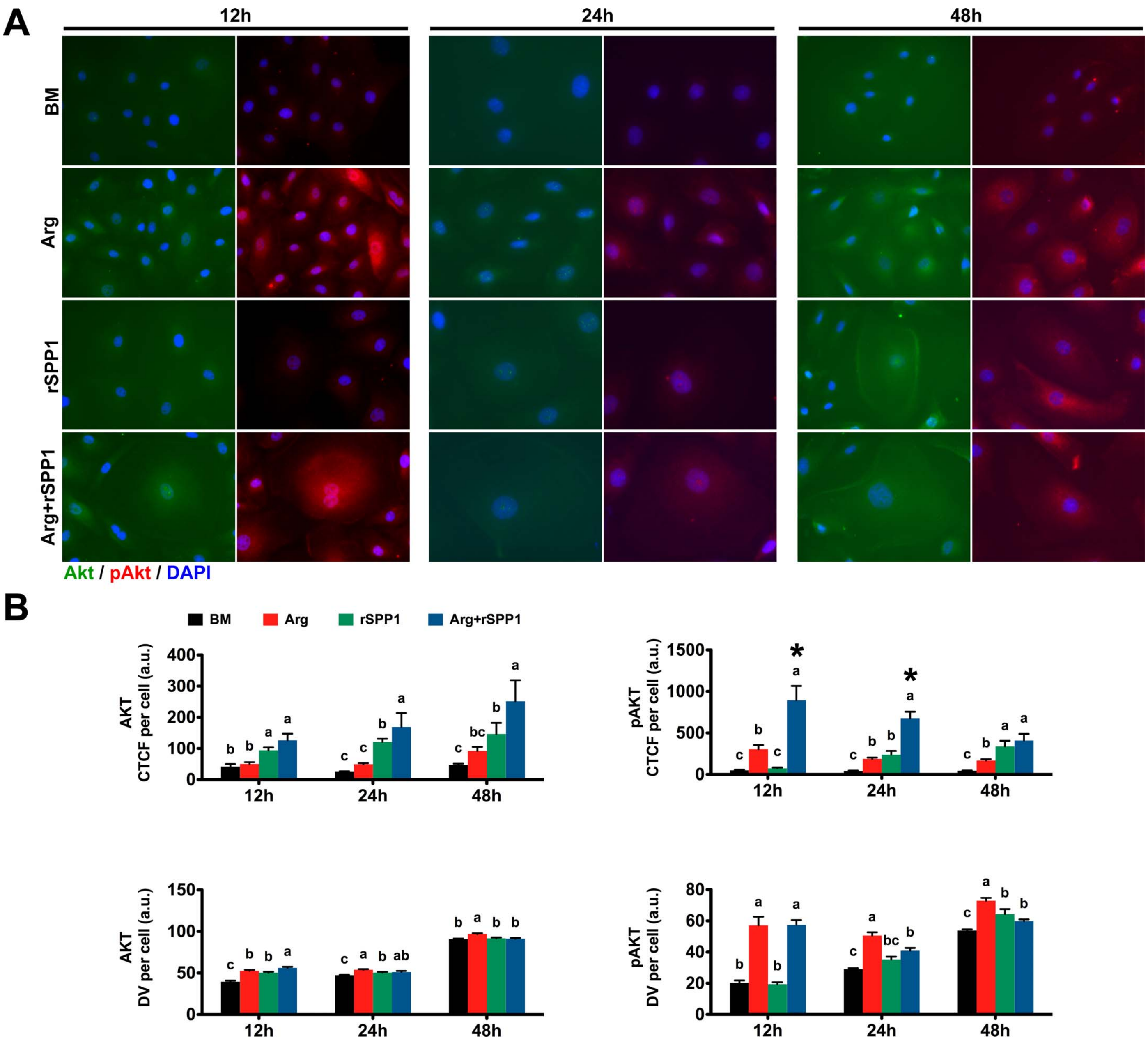

FIG. 5. Arginine and rSPP1 activate Akt cell signaling independently and synergistically at 12, 24, and 48 h of incubation. The oTr1 cells (n = 3 wells) were seeded at 30% confluence onto Lab-Tek II four-well chamber slides. After serum and insulin starvation for 24 h followed by an additional deprivation of arginine (Arg) for 6 h, cells were treated in BM with or without arginine (200 μM), rSPP1 (10 μg/ml), or arginine plus rSPP1. Images (**A**) and quantification (**B**) of immunocytochemical analyses demonstrated increased abundances of pAkt in oTr1 cells treated with arginine at all three time points, or rSPP1 at 24 and 48 h, whereas the synergistic effects of arginine and rSPP1 to increase pAkt were observed only at 12 and 24 h. The total Akt was not different between arginine and BM groups; however, it was increased by rSPP1 at all the time points, and the additive effects were observed at 24 and 48 h. CTCF, corrected total cell fluorescence; DV, density value; a.u., arbitrary units. Width of image field = 220 μm. Different superscript letters denote significant ($P < 0.05$) differences among treatment groups at the respective time points. The asterisk (*) denotes significant ($P < 0.05$) synergism between arginine and rSPP1 at the respective time points. All data are presented as means ± SEM. For clarity, global contrast adjustment is processed uniformly on all images of total Akt at 48 h.

trophectoderm cell proliferation and the underlying mechanisms using our established oTr1 cell line.

The results of this study provide the first in vitro evidence that arginine and rSPP1 act cooperatively to increase oTr1 cell proliferation via activation of the PDK1-Akt/PKB-TSC2-MTORC1 signaling cascade. First, arginine increased oTr1 cell proliferation significantly, whereas rSPP1 per se did not stimulate proliferation but increased spreading of those cells (Fig. 1). However, the combination of physiological levels of both rSPP1 and arginine increased oTr1 cell proliferation to a greater extent than either one of them alone (Fig. 1) and also increased oTr1 cell size, indicated by the increases in both cell spreading and signal abundance. This additive effect of arginine and rSPP1 on proliferation of oTr1 cells might be due to the fact that arginine stimulates protein synthesis via activation of MTOR, particularly MTORC1 cell signaling, whereas rSPP1, an adhesion protein, facilitates signal transduction (proliferation, migration, and attachment) by forming focal adhesions, thereby cooperatively amplifying the proliferative effects of arginine. To test this hypothesis, a series of

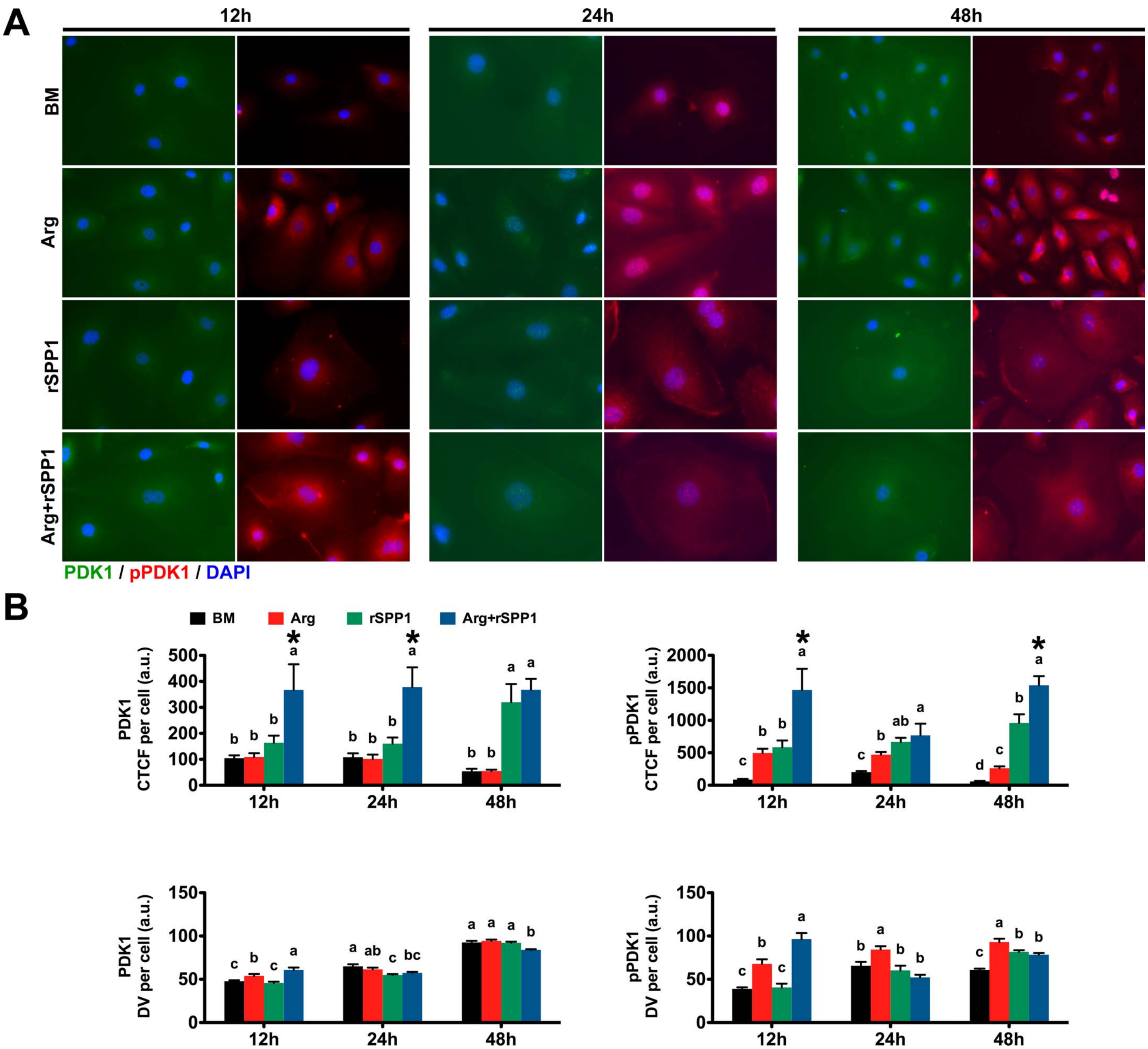

FIG. 6. Arginine and rSPP1 activate PDK1 signaling independently and synergistically at 12, 24, and 48 h of incubation. The oTr1 cells (n = 3 wells) were seeded at 30% confluence onto Lab-Tek II four-well chamber slides. After serum and insulin starvation for 24 h followed by an additional deprivation of arginine (Arg) for 6 h, cells were treated in BM with or without arginine (200 μM), rSPP1 (10 μg/ml), or arginine plus rSPP1. Images (**A**) and quantification (**B**) of immunocytochemical analyses demonstrated increased abundances of pPDK1 in oTr1 cells treated with either arginine or rSPP1 as compared to BM at all three time points, whereas synergistic increases in pPDK1 induced by arginine and rSPP1 were observed only at 12 and 48 h. Total PKD1 was not different among oTr1 cells treated with arginine, rSPP1, and BM at any of the time points except that rSPP1 induced an increase in PKD1 at 48 h; however, synergistic increases of total PDK1 were observed at 12 and 24 h. CTCF, corrected total cell fluorescence; DV, density value; a.u., arbitrary units. Width of image field = 220 μm. Different superscript letters denote significant ($P < 0.05$) differences among treatment groups at the respective time points. The asterisk (*) denotes significant ($P < 0.05$) synergism between arginine and rSPP1 at the respective time points. All data are presented as means ± SEM. For clarity, global contrast adjustment is processed uniformly on all images of total PDK1 at 48 h.

immunocytochemical analyses were performed to examine molecules related to MTORC1 signaling cascade in oTr1 cells treated with arginine, rSPP1, and arginine plus rSPP1.

MTOR functions as a serine/threonine kinase, which is one of the most essential proteins regulating mRNA translation and cell division. Intensive studies have shown that MTOR is a nutrient-sensing system stimulated by molecules, including insulin-like growth factor 2 (IGF2) and selected amino acids (i.e., arginine, leucine, and glutamine), to support blastocyst development [6, 26, 37, 39, 47–53]. Further, MTOR is the catalytic subunit of two structurally distinct complexes: MTOR complex 1 (MTORC1) and MTOR complex 2 (MTORC2). MTORC1 is composed of MTOR, regulatory-associated protein of MTOR (Raptor) and mammalian lethal with SEC13 protein 8 (MLST8) as the core components. Alternatively, MTORC2 is composed of MTOR, rapamycin-insensitive companion of MTOR (Rictor), MLST8, and mammalian stress-activated protein kinase interacting protein 1 (MSIN1). In

flies, yeasts, and mammals, such as porcine and ovine trophectoderm cells during the peri-implantation period of pregnancy, MTORC1 is key to the nutrient-sensing signaling network that controls cell metabolism [26, 52, 54–57], and of the core components in MTORC1, multisite phosphorylation of Raptor serves as a biochemical rheostat to modulate MTORC1 signaling [58, 59]. In the present study, arginine and rSPP1 both independently and cooperatively increased ($P < 0.05$) the abundances of pMTOR and pRaptor, demonstrating that their cooperative effects on proliferation of oTr1 cells may be mediated by activation of MTORC1 signaling (Figs. 2 and 3). Interestingly, rSPP1 alone increased the abundance of pMTOR and pRaptor but had no effect on cell proliferation. This suggests that its role in oTr1 cell proliferation is probably as a facilitator rather than the primary driving force. Results of a previous study demonstrated that SPP1 binds integrins on oTr1 cells to induce focal adhesion assembly and alter MTOR signaling, leading to increased oTr cell migration [37]. It is plausible to predict that assembly of focal adhesions in response to SPP1 provides a structural framework within the cell that allows the proliferative effects of arginine to be enhanced.

Tuberous sclerosis complex 2 (TSC2) acts as a GTPase activating protein that stimulates GTPase activity of the small GTPase Rheb [60–63]. Since Rheb in its GTP bound form is an activator of MTORC1 [61], TSC2, in complex with TSC1, is inhibitory to the MTORC1 signaling cascade, and phosphorylation of TSC2 removes that inhibition. In the present study, increases in pTSC2 suggest that arginine and rSPP1, both independently and synergistically, activate the MTORC1 signaling pathway by increasing phosphorylation of TSC2, thereby preserving Rheb in its GTP bound form in oTr1 cells (Fig. 4). Inhibition of TSC2 activity usually results from Akt-mediated phosphorylation and membrane partitioning [64]. Therefore, we next investigated the abundances of total and phosphorylated Akt (v-akt murine thymoma viral oncogene homolog, also known as protein kinase B [PKB]), a serine/threonine kinase that controls physiological processes, such as cell growth, survival, and motility. Akt/PKB is fully activated by phosphorylation at two vital sites: Thr308, which is phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) [65], and Ser473, which is phosphorylated by MTORC2 (Rictor-MTOR complex) and DNA-dependent protein kinase (DNA-PK) [66]. Increases in both total and phosphorylated Akt suggest synergistic effects of arginine and rSPP1 on Akt-TSC2-MTORC1 signal transduction (Fig. 5). In addition, alterations in the abundance of total and phosphorylated PDK1 may account for Akt activation since Akt phosphorylation occurred at Thr308. This prediction is consistent with results that total and phosphorylated PDK1 increase significantly in response to treatment of oTr1 cells with arginine plus rSPP1 (Fig. 6). These findings help elucidate the mechanisms for beneficial effects of dietary arginine supplementation to enhance embryonic survival and growth in mammals [67].

In summary, arginine added into culture medium at a physiological concentration significantly stimulated oTr1 cell proliferation, whereas rSPP1 alone did not have such an effect. However, the combination of arginine and rSPP1 resulted in an additive effect on proliferation in oTr1 cells. We hypothesize that arginine is the driving force for cell proliferation and that SPP1 facilitates cell proliferation. The additive effects of arginine and rSPP1 were achieved via cooperative stimulation of the PDK1-Akt/PKB-TSC2-MTORC1 cell signaling pathway, which provides important insight into understanding the orchestrated events and mechanisms responsible for conceptus-uterine signaling during implantation in support of conceptus development, pregnancy recognition signaling, and implantation. The present study shows that rSPP1 affects the shape and spreading of oTr1 cells, while arginine increases the proliferation and abundance of cytoskeleton proteins in those cells (i.e., α-tubulin; Figs. 1A and 3), and together they act cooperatively to increase the proliferation and even size of oTr1 cells. Future studies will address the question of whether there are cooperative effects of arginine and SPP1 on migration and attachment of oTr1 cells for implantation and the potential mechanisms related to MTORC2 and cytoskeletal reorganization during the critical phase of conceptus elongation during the peri-implantation period of pregnancy in sheep.

ACKNOWLEDGMENT

The contributions of graduate students and postdoctoral fellows from the Laboratory of Uterine Biology and Pregnancy (Texas A&M University) are gratefully acknowledged.

REFERENCES

1. Bazer FW, First NL. Pregnancy and parturition. J Anim Sci 1983; 57(suppl 2):425–460.
2. Spencer TE, Bazer FW. Uterine and placental factors regulating conceptus growth in domestic animals. J Anim Sci 2004; 82(suppl):E4–E13.
3. Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. Mol Hum Reprod 2010; 16:135–152.
4. Lee KY, DeMayo FJ. Animal models of implantation. Reproduction 2004; 128:679–695.
5. Bazer FW. Uterine protein secretions: Relationship to development of the conceptus. J Anim Sci 1975; 41:1376–1382.
6. Bazer FW, Wu G, Johnson GA, Kim J, Song G. Uterine histotroph and conceptus development: select nutrients and secreted phosphoprotein 1 affect mechanistic target of rapamycin cell signaling in ewes. Biol Reprod 2011; 85:1094–1107.
7. Fazleabas AT. Physiology and pathology of implantation in the human and nonhuman primate. Semin Reprod Med 2007; 25:405–409.
8. Roberts RM, Bazer FW. The functions of uterine secretions. J Reprod Fertil 1988; 82:875–892.
9. Wu G. Functional amino acids in nutrition and health. Amino Acids 2013; 45:407–411.
10. Wu G, Bazer FW, Dai Z, Li D, Wang J, Wu Z. Amino acid nutrition in animals: protein synthesis and beyond. Annu Rev Anim Biosci 2014; 2: 387–417.
11. Gao H, Wu G, Spencer TE, Johnson GA, Li X, Bazer FW. Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. Biol Reprod 2009; 80: 86–93.
12. Wu G, Bazer FW, Cudd TA, Meininger CJ, Spencer TE. Maternal nutrition and fetal development. J Nutr 2004; 134:2169–2172.
13. Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. Select nutrients in the ovine uterine lumen. V. Nitric oxide synthase, GTP cyclohydrolase, and ornithine decarboxylase in ovine uteri and peri-implantation conceptuses. Biol Reprod 2009; 81:67–76.
14. Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. Select nutrients in the ovine uterine lumen. III. Cationic amino acid transporters in the ovine uterus and peri-implantation conceptuses. Biol Reprod 2009; 80:602–609.
15. Conrad KP, Vill M, McGuire PG, Dail WG, Davis AK. Expression of nitric oxide synthase by syncytiotrophoblast in human placental villi. FASEB J 1993; 7:1269–1276.
16. Wu G, Pond WG, Flynn SP, Ott TL, Bazer FW. Maternal dietary protein deficiency decreases nitric oxide synthase and ornithine decarboxylase activities in placenta and endometrium of pigs during early gestation. J Nutr 1998; 128:2395–2402.
17. Dye JF, Vause S, Johnston T, Clark P, Firth JA, D'Souza SW, Sibley CP, Glazier JD. Characterization of cationic amino acid transporters and expression of endothelial nitric oxide synthase in human placental microvascular endothelial cells. FASEB J 2004; 18:125–127.
18. Wang X, Frank JW, Little DR, Dunlap KA, Satterfield MC, Burghardt RC, Hansen TR, Wu G, Bazer FW. Functional role of arginine during the peri-implantation period of pregnancy. I. Consequences of loss of function of arginine transporter SLC7A1 mRNA in ovine conceptus trophectoderm. FASEB J 2014; 28:2852–2863.
19. Wang X, Frank JW, Xu J, Dunlap KA, Satterfield MC, Burghardt RC, Romero JJ, Hansen TR, Wu G, Bazer FW. Functional role of arginine

during the peri-implantation period of pregnancy. II. Consequences of loss of function of nitric oxide synthase NOS3 mRNA in ovine conceptus trophectoderm. Biol Reprod 2014; 91:59.
20. Wang X, Ying W, Dunlap KA, Lin G, Satterfield MC, Burghardt RC, Wu G, Bazer FW. Arginine decarboxylase and agmatinase: an alternative pathway for de novo biosynthesis of polyamines for development of mammalian conceptuses. Biol Reprod 2014; 90:84.
21. Li X, Bazer FW, Johnson GA, Burghardt RC, Frank JW, Dai Z, Wang J, Wu Z, Shinzato I, Wu G. Dietary supplementation with L-arginine between days 14 and 25 of gestation enhances embryonic development and survival in gilts. Amino Acids 2014; 46:375–384.
22. Kong X, Wang X, Yin Y, Li X, Gao H, Bazer FW, Wu G. Putrescine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells. Biol Reprod 2014; 91:106.
23. Zeng X, Wang F, Fan X, Yang W, Zhou B, Li P, Yin Y, Wu G, Wang J. Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. J Nutr 2008; 138:1421–1425.
24. Zeng X, Mao X, Huang Z, Wang F, Wu G, Qiao S. Arginine enhances embryo implantation in rats through PI3K/PKB/mTOR/NO signaling pathway during early pregnancy. Reproduction 2013; 145:1–7.
25. Gonzalez IM, Martin PM, Burdsal C, Sloan JL, Mager S, Harris T, Sutherland AE. Leucine and arginine regulate trophoblast motility through mTOR-dependent and independent pathways in the preimplantation mouse embryo. Dev Biol 2012; 361:286–300.
26. Kim JY, Burghardt RC, Wu G, Johnson GA, Spencer TE, Bazer FW. Select nutrients in the ovine uterine lumen. VIII. Arginine stimulates proliferation of ovine trophectoderm cells through MTOR-RPS6K-RPS6 signaling cascade and synthesis of nitric oxide and polyamines. Biol Reprod 2011; 84:70–78.
27. Lessey BA, Castelbaum AJ. Integrins and implantation in the human. Rev Endocr Metab Disord 2002; 3:107–117.
28. Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE, Bazer FW. Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. Cells Tissues Organs 2002; 172:202–217.
29. Aplin JD, Kimber SJ. Trophoblast-uterine interactions at implantation. Reprod Biol Endocrinol 2004; 2:48.
30. Johnson GA, Burghardt RC, Bazer FW, Spencer TE. Osteopontin: roles in implantation and placentation. Biol Reprod 2003; 69:1458–1471.
31. Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. FASEB J 1993; 7:1475–1482.
32. Senger DR, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L. Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. Mol Biol Cell 1994; 5:565–574.
33. Sodek J, Ganss B, McKee MD. Osteopontin. Crit Rev Oral Biol Med 2000; 11:279–303.
34. Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW. Ovine osteopontin: II. Osteopontin and alpha(v)beta(3) integrin expression in the uterus and conceptus during the periimplantation period. Biol Reprod 1999; 61:892–899.
35. Johnson GA, Spencer TE, Burghardt RC, Bazer FW. Ovine osteopontin: I. Cloning and expression of messenger ribonucleic acid in the uterus during the periimplantation period. Biol Reprod 1999; 61:884–891.
36. Johnson GA, Spencer TE, Burghardt RC, Taylor KM, Gray CA, Bazer FW. Progesterone modulation of osteopontin gene expression in the ovine uterus. Biol Reprod 2000; 62:1315–1321.
37. Kim J, Erikson DW, Burghardt RC, Spencer TE, Wu G, Bayless KJ, Johnson GA, Bazer FW. Secreted phosphoprotein 1 binds integrins to initiate multiple cell signaling pathways, including FRAP1/mTOR, to support attachment and force-generated migration of trophectoderm cells. Matrix Biol 2010; 29:369–382.
38. Kim J, Song G, Gao H, Farmer JL, Satterfield MC, Burghardt RC, Wu G, Johnson GA, Spencer TE, Bazer FW. Insulin-like growth factor II activates phosphatidylinositol 3-kinase-protooncogenic protein kinase 1 and mitogen-activated protein kinase cell Signaling pathways, and stimulates migration of ovine trophectoderm cells. Endocrinology 2008; 149:3085–3094.
39. Kim JY, Burghardt RC, Wu G, Johnson GA, Spencer TE, Bazer FW. Select nutrients in the ovine uterine lumen. VII. Effects of arginine, leucine, glutamine, and glucose on trophectoderm cell signaling, proliferation, and migration. Biol Reprod 2011; 84:62–69.
40. Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE, Burghardt RC. Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. Biol Reprod 2001; 65:820–828.
41. McFarland RJ, Garza S, Butler WT, Hook M. The mutagenesis of the RGD sequence of recombinant osteopontin causes it to lose its cell adhesion ability. Ann N Y Acad Sci 1995; 760:327–331.
42. Raspotnig G, Fauler G, Jantscher A, Windischhofer W, Schachl K, Leis HJ. Colorimetric determination of cell numbers by Janus green staining. Anal Biochem 1999; 275:74–83.
43. Potapova TA, Sivakumar S, Flynn JN, Li R, Gorbsky GJ. Mitotic progression becomes irreversible in prometaphase and collapses when Wee1 and Cdc25 are inhibited. Mol Biol Cell 2011; 22:1191–1206.
44. Gavet O, Pines J. Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. Dev Cell 2010; 18:533–543.
45. Burgess A, Vigneron S, Brioudes E, Labbe JC, Lorca T, Castro A. Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. Proc Natl Acad Sci U S A 2010; 107:12564–12569.
46. Slinker BK. The statistics of synergism. J Mol Cell Cardiol 1998; 30: 723–731.
47. Nielsen FC, Ostergaard L, Nielsen J, Christiansen J. Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway. Nature 1995; 377:358–362.
48. Kimball SR, Shantz LM, Horetsky RL, Jefferson LS. Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. J Biol Chem 1999; 274:11647–11652.
49. Martin PM, Sutherland AE, Van Winkle LJ. Amino acid transport regulates blastocyst implantation. Biol Reprod 2003; 69:1101–1108.
50. Bazer FW, Song G, Kim J, Erikson DW, Johnson GA, Burghardt RC, Gao H, Satterfield MC, Spencer TE, Wu G. Mechanistic mammalian target of rapamycin (MTOR) cell signaling: effects of select nutrients and secreted phosphoprotein 1 on development of mammalian conceptuses. Mol Cell Endocrinol 2012; 354:22–33.
51. Kim J, Song G, Wu G, Bazer FW. Functional roles of fructose. Proc Natl Acad Sci U S A 2012; 109:E1619–E1628.
52. Bar-Peled L, Sabatini DM. Regulation of mTORC1 by amino acids. Trends Cell Biol 2014; 24:400–406.
53. Howell JJ, Manning BD. mTOR couples cellular nutrient sensing to organismal metabolic homeostasis. Trends Endocrinol Metab 2011; 22: 94–102.
54. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 2008; 320:1496–1501.
55. Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL. Regulation of TORC1 by Rag GTPases in nutrient response. Nat Cell Biol 2008; 10: 935–945.
56. Binda M, Peli-Gulli MP, Bonfils G, Panchaud N, Urban J, Sturgill TW, Loewith R, De Virgilio C. The Vam6 GEF controls TORC1 by activating the EGO complex. Mol Cell 2009; 35:563–573.
57. Kong X, Tan B, Yin Y, Gao H, Li X, Jaeger LA, Bazer FW, Wu G. L-arginine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells. J Nutr Biochem 2012; 23:1178–1183.
58. Frey JW, Jacobs BL, Goodman CA, Hornberger TA. A role for Raptor phosphorylation in the mechanical activation of mTOR signaling. Cell Signal 2014; 26:313–322.
59. Foster KG, Acosta-Jaquez HA, Romeo Y, Ekim B, Soliman GA, Carriere A, Roux PP, Ballif BA, Fingar DC. Regulation of mTOR complex 1 (mTORC1) by raptor Ser863 and multisite phosphorylation. J Biol Chem 2010; 285:80–94.
60. Huang J, Manning BD. A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans 2009; 37:217–222.
61. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 2003; 17: 1829–1834.
62. Huang J, Manning BD. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochem J 2008; 412:179–190.
63. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003; 115:577–590.
64. Cai SL, Tee AR, Short JD, Bergeron JM, Kim J, Shen J, Guo R, Johnson CL, Kiguchi K, Walker CL. Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J Cell Biol 2006; 173:279–289.
65. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002; 2:489–501.
66. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005; 307: 1098–1101.
67. Wu G, Bazer FW, Satterfield MC, Li X, Wang X, Johnson GA, Burghardt RC, Dai Z, Wang J, Wu Z. Impacts of arginine nutrition on embryonic and fetal development in mammals. Amino Acids 2013; 45:241–256.