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Analysis and characterization of differential gene expression during rapid trophoblastic elongation in the pig using suppression subtractive hybridization

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Abstract

During late peri-implantation development, porcine conceptuses undergo a rapid (2–3 hrs) morphological transformation from a 10 mm sphere to a thin filamentous form greater than 150 mm in length. Elongation of the conceptus is important for establishing adequate placental surface area needed for embryo and fetal survival throughout gestation. Genes involved with triggering this unique transition in conceptus development are not well defined. Objective of the present study was to utilize suppression subtractive hybridization (SSH) to characterize the change in gene expression during conceptus transformation from spherical (8–9 mm) to tubular (15–40 mm) to early filamentous (>150 mm) morphology. Spherical, tubular, and filamentous conceptuses were collected from pregnant gilts and subjected to SSH. Forward and reverse subtractions were performed to identify candidate genes differentially expressed during spherical to tubular and tubular to filamentous transition. A total of 384 transcripts were differentially screened to ensure unique expression. Of the transcripts screened, sequences were obtained for 142 that were confirmed to be differentially expressed among the various morphologies. Gene expression profiles during rapid trophoblastic elongation were generated for selected mRNAs using quantitative real-time PCR. During the transition from tubular to early filamentous conceptuses, s-adenosylhomocysteine hydrolase and heat shock cognate 70 kDa expression were significantly enhanced. A novel unknown gene was isolated and shown to be significantly up-regulated at the onset of rapid trophoblastic elongation and further enhanced in filamentous conceptuses.

Background

As in the majority of mammalian species, successful embryonic development in the pig requires temporally and spatially specific gene expression essential to placental and embryonic differentiation during early gestation. Expressing the appropriate transcripts during development of the pig conceptus is essential for expansion of the trophoblast and placental attachment to the uterine surface. Prenatal mortality in the pig ranges from 20% to 46% by term [1], the majority of which occurs during peri-implantation conceptus development [2]. The peri-implantation period is the most critical stage of conceptus development

as a rapid morphological transformation of the trophoblast occurs just prior to conceptus attachment to the uterine surface [3]. Rapid transformation of the trophoblast, termed trophoblastic elongation, occurs during days 11 to 12 of gestation. Trophoblastic elongation is initiated when a conceptus reaches a 10 mm sphere and then rapidly transforms into a long filamentous thread greater than 150 mm in length within 2–3 hrs [3]. The process of trophoblastic elongation is characterized by four distinct morphological stages (spherical, ovoid, tubular and filamentous). Elongation of the conceptus is a short-lived phenomenon that results from cellular remodeling and migration, rather than through cellular hyperplasia [3]. Secretion of the conceptus produced maternal recognition signal, estrogen, occurs simultaneously with rapid elongation of the trophoblastic membrane [4,5]. Conceptus release of estrogen induces an acute phase response by the endometrium that alters the uterine environment, which may be unfavorable for less developed littermates [1,6]. Because the pig has a diffuse epitheliochorial type of placentation, rapid trophoblastic elongation provides an essential biological function to satisfy the conceptus' necessity for maximal placental-uterine contact to ensure adequate nutrient exchange throughout gestation [2].

It has been estimated that approximately 10,000 genes must be appropriately expressed for successful pre-implantation and early fetal development [7]. A number of mRNAs hypothesized to be involved with early porcine conceptus development have been evaluated. Yelich et al. [8] characterized gene expression for retinoic acid receptors (RAR) α, β and γ as well as retinal binding proteins (RBP) during early porcine conceptus development and trophoblast elongation. Results indicate expression of RARα and RBP increase during transition to conceptuses of filamentous morphology. Estrogen receptor-β has been localized in the porcine conceptus and its expression appears to be enhanced during trophoblastic elongation [9], which follows a pattern similar to aromatase expression [10].

At present, knowledge of genes regulating rapid trophoblastic elongation in the pig is far from complete. Due to the limited insight on the transcriptional regulation of this critical developmental process, the objective of the present investigation was to utilize suppression subtractive-hybridization (SSH) to characterize and analyze differentially expressed genes during rapid trophoblastic elongation in the pig. Identification and characterization of gene expression patterns during rapid trophoblastic elongation in the pig will provide a better understanding of the events required for successful implantation and embryonic survival.

Materials and Methods

Conceptus Collection

Research was conducted in accordance with and approval by the Oklahoma State Institutional Animal Care and Use Committee. Twenty-five crossbred, cyclic gilts were checked for estrus twice daily in the presence of an intact boar and naturally mated at the onset of the second estrus and again 24 hrs later. Gilts were hysterectomized between day 11 and 12 of gestation as previously described for our laboratory [11]. After removal of the uterine horns, conceptuses from each uterine horn were flushed into a sterile petri dish with 20 mL of physiological saline. Due to the limited time frame when conceptuses are in tubular transitional development (2–3 hrs) and difficulty in determining when tubular conceptuses are in the uterus following mating, one uterine horn was removed on day 11.5 of gestation in a subset of gilts. Conceptuses were flushed into a sterile petri dish from the uterine horn and evaluated to determine an appropriate time-delayed removal of the second horn corresponding to the predicted time conceptuses would be in a tubular morphology as described by Geisert et al. [3]. Morphology of conceptuses collected following flushing from the uterine horns was recorded and conceptuses of identical morphologies were transferred to cryogenic vials, snap-frozen in liquid nitrogen, and stored at -80°C until extracted for RNA.

Table 1: Primer and probe sequences used for real-time RT-PCR

Target	Primer Sequence	Reporting Dye	Product Size(bp)
SAHH	Forward 5'-TGTTGCTTTTATGTCTCTCTGG-3' Reverse 5'-GCTTGGCATTCTCTTAAACC-3'	SYBR Green	154
HSC70	Forward 5'-GTCTTCCTTGCTCAAACG-3' Reverse 5'-AACTCACAGGCATACCTCC-3'	SYBR Green	164
OSU-T1-50	Forward 5'-TCACGGTTAGTGTCGCATGA-3' Reverse 5'-ACCCATGAACAGGTCCTGAA-3'	6-FAM-CGTGTTTCCTACGTTGGGCGTCC-TAMRA	72

Primer and probe sequences used for real-time RT-PCR, reporting dye used for detection of amplified product and product size for s-adenosylhomocysteine hydrolase (SAHH), heat shock cognate 70 kDa (HSC70) and the novel OSU-T1-50.

RNA Isolation

Total RNA was extracted from individual conceptuses and pools of conceptuses of identical morphologies as previously described in our laboratory [8]. Briefly, conceptuses were denatured for 15 min on ice using 500 μl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-β-mercaptoethanol), 500 μl phenol, 70 μl 2 M sodium acetate (pH 4.0), and 140 μl chloroform/iso-amyl-alcohol (49:1 fresh dilution). The aqueous phase was recovered following centrifugation at 14 000 rpm for 20 min at 4°C and added to a tube containing 500 μl of chloroform, vortexed briefly and centrifuged at 10 000 rpm for 10 min at 4°C. The aqueous phase was recovered, placed in a sterile tube, and 7 μl of Rnaid binding matrix (BIO 101, La Jolla, CA) was added, vortexed briefly, and rotated for 25 min at 22–25°C. Following rotation, the suspension was centrifuged at 10,000 rpm for 2 min and the aqueous phase was discarded. The remaining pellet containing the glass beads bound to total RNA was washed three times using 250 μl of 50% RNA wash (BIO 101, La Jolla, CA) and 50% ethanol solution followed by centrifugation at 10,000 rpm for 2 min at 22–25°C. The pellet was dried at 22–25°C for 10 min and re-suspended in 50 μl of nuclease-free H_2O. The re-suspended solution was heated at 56°C for 5 min and centrifuged at 10,000 rpm for 2 min. Approximately 40 μl of the aqueous phase containing the purified total RNA was transferred to a sterile tube and stored at -80°C. Isolated RNA concentrations were estimated based on absorbance at the 260 nm wavelength. Purity of RNA was determined from the 260:280 ratio.

Suppression Subtractive Hybridization

Suppression Subtractive Hybridization was performed using the Clontech PCR-Select cDNA Subtraction Kit (K-1804-1, Clontech Laboratories Inc., Palo Alto, CA) as described previously [12]. Forward and reverse subtractions were conducted for two different comparisons during trophoblast elongation. The first comparison was made between spherical (8–9 mm) and tubular (15–40 mm) morphologies allowing the isolation of gene products differentially expressed during the onset of rapid trophoblastic elongation. The second comparison was made between tubular (15–40 mm) and filamentous (>150 mm) morphologies encompassing gene expression changes during the transitional completion when the conceptuses have began to cover the surface area of the uterine horns. Driver and tester cDNA was produced from 10 μg total RNA for each morphology of each comparison following the manufacturers' guidelines. Briefly, synthesized cDNA was digested with the restriction enzyme Rsa1 and the tester cDNA populations were divided into two tubes and ligated to both adaptor 1 or adaptor 2R. Prior to ligation at 16°C overnight, 2 μl from each adaptor ligation for each tester population were combined to serve as an unsubtracted control and were diluted into 1 mL sterile water following ligation. The subtractive hybridization was performed by adding 1.5 μl driver cDNA to each tube, one containing 1.5 μl of adaptor 1 and the other containing 1.5 μl adapter 2R-ligated tester cDNA (tester cDNA was approximately 30 times less concentrated than driver cDNA) in 1 μl 4X hybridization buffer. Samples were denatured at 98°C for 1.5 min, then allowed to anneal at 68°C for 8 hours. Following the first hybridization, the two samples were combined simultaneously with the excess addition of 1 μl freshly denatured driver cDNA and hybridization was continued at 68°C overnight. Products from the second hybridization were diluted in 200 μl of dilution buffer (20 mM HEPES pH 8.3, 50 mM NaCl and 0.2 mM EDTA) and heated at 68°C for an additional 7 min and stored at -20°C.

PCR Amplification of Subtracted Products

Two PCR amplifications were performed on the subtracted and unsubtracted tester products. Primary PCR amplifications were conducted for each tester using diluted subtracted products following the second hybridization or the diluted unsubtracted cDNA. One microliter of sample was added to 24 μl PCR master mix prepared using the reagents supplied in the kit, and cycling conditions commenced as follows: 75°C for 5 min to extend the adaptors; 94°C for 25 sec; and 27 cycles at 94°C for 10 sec, 66°C for 30 sec, and 72°C for 1.5 min. Amplified products were diluted 10-fold in sterile water and 1 μl of diluted primary PCR products were added to 24 μl of secondary PCR master mix containing nested primers, 1 and 2R, to ensure specific amplification of double-stranded templates containing both adaptors. Secondary PCR was performed at 94°C for 10 sec, 68°C for 30 sec and 72°C for 1.5 min (cycle number varied between 12 and 27 cycles). Primary and secondary PCR products were analyzed on a 2% agarose gel.

Cloning of Subtracted cDNA Templates

Following the secondary PCR amplification, subtracted products from each tester cDNA population were cloned into the PCR IV vector of the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and used in the transformation of competent DH5α *Escherichia coli* cells. Colonies were grown overnight at 37°C on Luria Broth (LB) agar plates containing carbanocillin, X-gal (5-bromo 4-chloro 3-indoyl-β-D-galactopyranoside) and isopropyl-β-D-thio-galactopyranoside for blue/white colony screening. Ninety-six plasmids for each tester were randomly selected and plasmid DNA was extracted (Wizard SV96 Plasmid DNA Purification System, Promega Corporation, Madison, WI) and eluted into 100 μl sterile nuclease-free water.

Differential Screening

To confirm unique expression of the subtracted products, all transcript clones were subjected to differential screening. Plasmid DNA was denatured by adding 250 μl denaturing solution (0.5 M NaOH, 1.5 M NaCl) and incubating for 10 min at 22–25°C. Equal amounts of denatured, subtracted cDNA from each tester were spotted on four separate, positively charged nylon membranes (Roche Applied Science, Indianapolis, IN) using the Bio-Dot apparatus (BioRad, Hercules, CA) and neutralized with 200 μl neutralization solution (1 M Tris-HCl, pH 8.0, 1.5 M NaCl). Following DNA spotting and neutralization, the membranes were washed by placing on filter paper saturated in 2X SSC for 2 min, removed from filter paper, UV cross-linked and stored at 4°C. Digoxigenin (DIG) labeled probes were created from subtracted and unsubtracted secondary PCR products using the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Applied Science, Indianapolis, IN). Prior to DIG-labeling, PCR products were digested with RsaI for 6 hours at 37°C to remove adaptors and purified using PCR purification columns (Qiagen, Valencia, CA). Denatured template DNA (1 μg) was labeled with DIG at 16°C for 20 h. Labeling efficiency was determined by comparison to known concentrations of herring sperm DIG labeled DNA. The four membranes for each subtracted tester were hybridized overnight at 42°C with 125 ng DIG labeled DNA diluted in 5 mL DIG Easy Hyb (25 ng/mL) from subtracted and unsubtracted template products of morphologies for each comparison. Immunological detection of hybridization was conducted in accordance with manufacturers' protocol (Roche Applied Science, Indianapolis, IN) and membranes were exposed to X-ray film (X-Omat LS, Kodak, Rochester, NY) to visualize template-probe binding. Binding intensities were quantified using a densitometer and differential gene expression of cloned template was confirmed when binding intensity was 5X greater when probed with labeled subtracted tester cDNA compared to the intensity of subtracted driver cDNA and a greater binding intensity when hybridized with unsubtracted tester cDNA compared to unsubtracted driver cDNA. Plasmids containing confirmed differentially expressed templates were re-cultured and plasmid DNA was extracted (Wizard Plus Mini-prep DNA Purification System, Promega Corporation, Madison, WI) and subjected to dideoxy chain termination sequencing (Applied Biosystems, Model 373A Automated Sequencer, Oklahoma State University Recombinant DNA/Protein Resource Facility). Basic Local Alignment Search Tool (BLAST) [13] was used to confirm sequence homology of each differentially expressed template.

Quantitative One-Step RT-PCR

Quantitative analysis of clones of interest, specifically, s-adenosylhomocysteine hydrolase (SAHH), heat shock cognate 70 KD protein (HSC70), and one unknown transcript, OSU-T1-50, were assayed using quantitative real-time RT-PCR [14]. Individual and small pools of conceptuses were evaluated at the four morphologically distinct stages; spherical (n = 8), ovoid (n = 2), tubular (n = 5) and early filamentous (n = 6). The PCR amplification was conducted using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The unknown transcript was evaluated using a dual-labeled probe designed to have a 5' reporter dye (6-FAM) and a 3' quenching dye (TAMRA). SAHH and HSC70 were evaluated using the SYBR green reporter assay kit available from Qiagen (Valencia, CA). Sequences of the primer and probe set used for amplification of the unknown transcript and the primers for SAHH and HSC70 are presented in Table 1. Fifty nanograms of total RNA were assayed for each sample in duplicate. Thermal cycling conditions using the dual labeled probe were 48°C for 30 min and 95°C for 10 min, followed by 40 repetitive cycles of 95°C for 15 sec and a combined annealing/extension stage, 60°C for 1 min. Cycling parameters using SYBR green detection were 50°C for 30 min followed by 95°C for 15 min then 40 repetitive cycles at 95°C for 15 sec, 51°C for 30 sec, 72°C for 30 sec, and a fluorescent data acquisition step following a brief 15 sec incubation at 78.5°C. Following PCR cycling with SYBR green a melting curve analysis was conducted using the following parameters: 95°C for 15 sec followed by 51°C for 30 sec to 95°C for 15 sec with a ramp time of 19 min 59 sec. Continuous fluorescent data acquisition was collected during final ramp enabling the generation of the melting curve graph to confirm that detectable fluorescence was strictly from amplified target cDNA. 18S ribosomal RNA was assayed as a normalization control to correct for loading discrepancies.

Following RT-PCR, quantitation of gene amplification was made by determining the cycle threshold (C_T) based on the fluorescence detected within the geometric region of the semilog view of the amplification plot. Relative quantitation of target gene expression was evaluated using the comparative C_T method as previously described by Hettinger et al. [14]. The ΔC_T value was determined by subtracting the target C_T of each sample from its respective ribosomal 18S C_T value. Calculation of $\Delta\Delta C_T$ involves using the highest sample ΔC_T value as an arbitrary constant to subtract from all other ΔC_T sample values (Table 4). Fold-changes in gene expression of the target gene are equivalent to $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Quantitative RT-PCR ΔC_T values were analyzed using the Mixed Procedure of the Statistical Analysis System [15]. Since uterine flushings of a few gilts contained multiple morphological stages, which were utilized in the analysis of gene expression, gilt was initially included in the statis-

Table 2: Putative identity, size and homology of porcine conceptus cDNA clones from spherical to tubular subtraction.

Expression Pattern	Identity	Clone Number	Base Pairs submitted to BLAST	GenBank Accession Number	Homology
Down-Regulated					
	F1Fo-ATP synthase complex	OSU-S-6	313	S70448	Bovine 93% (205/220)
				NM_006476	Human 93% (204/219)
				BC031384	Mouse 92% (128/139)
	Similar to keratin 18	OSU-S-14*	324	BC009754	Human 87% (134/154)
	Thymosin Beta 4	OSU-S-7*	308	BC022857	Human 91% (158/173)
				X16053	Mouse 84% (140/167)
				NM_031136	Rat 85% (122/143)
	16S Ribosomal RNA	OSU-S-31	427	AY011178	Pig 99% (374/375)
	Mitochondrial Cytochrome B	OSU-S-54	241	AJ314556	Pig 98% (182/185)
	Complete Mitochondrial DNA	OSU-S-11*	679	AF304203	Pig 99% (678/679)
Up-Regulated					
	28S Ribosomal RNA	OSU-T1-3*	436	X00525	Mouse 100% (350/350)
				M11167	Human 100% (350/350)
	16S Ribosomal RNA	OSU-T1-7*	250	AY011178	Pig 99% (212/214)
	18S Ribosomal RNA	OSU-T1-8*	342	AF102857	Pig 99% (293/294)
				X00686	Mouse 99% (293/294)
				X03205	Human 99% (293/294)
	Unknown	OSU-T1-50	702	AC009682	Human 87% (179/206)

* Indicates transcripts with multiple clones sequenced

tical model. However, given that gilt, as a random effect, did not significantly alter the variation due to the model and not all gilts were represented across all stages of development, gilt was deleted from the model. The statistical model used in the analysis tested only the fixed effect of morphology (spherical, ovoid, tubular, and filamentous). Significance ($P < 0.05$) was determined by probability differences of least squares means between morphologies on conceptus gene expression of s-adenosylhomocysteine hydrolase, heat shock cognate 70 kDa protein and OSU-T1-50. Results are presented as arithmetic means ± SEM.

Results

Suppression Subtractive Hybridization

Following SSH, subtracted products were cloned and 96 template clones for each subtracted product of each comparison were randomly selected and differentially screened. Screening indicated a total of 42 templates confirmed to be down-regulated and 18 up-regulated during the spherical to tubular transition. A total of 69 and 43 templates were confirmed up and down-regulated, respectively, during the tubular to filamentous transition. In all, 142 templates were subjected to dideoxy chain termination sequencing. Information regarding the identity and homology of morphologically specific genes, during spherical to tubular and tubular to filamentous is presented in Tables 2 and 3.

During the spherical to tubular transitions several genes were indicated using SSH to be down-regulated, such as thymosin-beta 4, mitochondrial cytochrome B, and F1Fo-ATP synthase complex mRNA. Also, numerous mitochondrial DNA template clones were down-regulated during this transition. Several ribosomal RNA transcripts were detected to be up-regulated during the onset of rapid trophoblastic elongation as well as a novel gene, which is referred to as OSU-T1-50. An approximate 200 base pair region of OSU-T1-50 had significant homology (87%) to the human gene clone RP11-72O13 (GenBank accession #AC009682) but had no significant homology compared to over 5.8 million non-human and non-mouse expressed sequence tags.

Few genes, myeloid cell leukemia (Bcl-2 related protein) and a ribosomal gene, were detected to be down-regulated during the tubular to filamentous transition. Numerous transcripts were up-regulated in conceptuses of filamentous morphology including, pro-interleukin-1β, interleukin-1β (IL-1β), s-adenosylhomocysteine hydrolase (SAHH), heat shock cognate 70 kDa protein (HSC70), metallopanstimulin-1 (MPS-1) and elfin. The three transcripts selected to be investigated using quantitative one-step real time RT-PCR were OSU-T1-50, SAHH and HSC70.

Real Time RT-PCR Quantification

Messenger RNA expression profiles of SAHH, HSC70 and OSU-T1-50 were generated using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Following amplification, C_T, ΔC_T and $\Delta\Delta C_T$ values were calculated as described previously in *Material and Methods* (Table 4).

Table 3: Putative identity, size and homology of porcine conceptus cDNA clones from tubular to filamentous subtraction.

Expression Pattern	Identity	Clone Number	Base Pairs submitted to BLAST	GenBank Accession Number	Homology
Down-Regulated					
	16S Ribosomal RNA	OSU-T2-19*	242	AY011178	Pig 100% (182/182)
	Myeloid Cell Leukemia	OSU-T2-40*	636	AF144096	Rat 96% (227/236)
	(Bcl-2Related Protein)			AJ307006	Pig 93% (151/163)
				BC005427	Mouse 91% (465/510)
				NM_021960	Human 88% (175/199)
Up-Regulated					
	Pro-interleukin-1β	OSU-F-1*	723	X74568	Pig 97% (319/328)
	Interleukin-1β	OSU-F-2*	684	M86725	Pig 90% (623/686)
				AB028216	Dolphin 87% (427/492)
				M37211	Cow 84% (448/531)
				X54796	Sheep 83% (418/501)
				U92481	Horse 85% (298/349)
				NM_000576	Human 85% (280/329)
	S-adenosylhomocyteine hydrolase	OSU-F-3*	452	AJ422131	Pig 99% (355/356)
				BC010018	Human 88% (238/272)
				NM_016661	Mouse 83% (66/79)
	Heat Shock Cognate Protein 70kD	OSU-F-7*	650	AF352832	Human 91% (501/546)
				X53335	Cow 92% (482/522)
				NM_024351	Rat 90% (493/546)
				M19141	Mouse 89% (479/534)
	Ribosomal protein 8S	OSU-F-21	442	NM_031706	Rat 92% (260/281)
				NM_001012	Human 92 % (260/281)
				NM_009098	Mouse 91% (263/289)
	Metallopanstimulin-1	OSU-F-30	382	L19739	Human 94% (244/259)
	Elfin	OSU-F-54*	540	NM_020992	Human 85% (321/379)
	(PDZ and LIM domain 1)				

* Indicates transcripts with multiple clones sequenced

Table 4: Quantitative RT-PCR analysis of gene expression during rapid trophoblastic elongation for SAHH, HSC70, and OSU-T1-50.

Transcript	Morphology	Average Target C_T *	Average 18S rRNA C_T *	ΔC_T‡¶	$\Delta\Delta C_T$§
SAHH	Spherical	23.48 ± 0.23	17.47 ± 0.10	6.01 ± 0.21[a]	-0.20
	Ovoid	23.62 ± 0.82	17.78 ± 0.24	5.84 ± 0.58[a]	-0.37
	Tubular	23.20 ± 0.51	16.99 ± 0.14	6.21 ± 0.51[a]	0.00
	Filamentous	20.72 ± 0.55	17.24 ± 0.12	3.47 ± 0.58[b]	-2.76
HSC70kD	Spherical	20.86 ± 0.28	17.47 ± 0.10	3.38 ± 0.25[c]	0.00
	Ovoid	21.02 ± 0.80	17.78 ± 0.24	3.24 ± 0.57[c]	-0.15
	Tubular	20.17 ± 0.52	16.99 ± 0.14	3.18 ± 0.48[c]	-0.21
	Filamentous	17.35 ± 0.46	17.24 ± 0.12	0.11 ± 0.48[d]	-3.27
OSU-T1-50	Spherical	33.22 ± 0.48	17.47 ± 0.10	15.75 ± 0.64[e]	0.00
	Ovoid	32.60 ± 1.01	17.78 ± 0.24	14.82 ± 1.22[ef]	-0.93
	Tubular	29.21 ± 0.63	16.99 ± 0.14	12.22 ± 0.80[f]	-3.53
	Filamentous	26.30 ± 0.57	17.24 ± 0.12	9.05 ± 0.80[g]	-6.69

Quantitative RT-PCR analysis comparing gene expression across morphologies during rapid trophoblastic elongation for three of the transcripts identified using suppression subtractive hybridization: s-adenosylhomocysteine hydrolase, heat shock cognate 70 kDa, and an unknown transcript identified by its clone number, OSU-T1-50. * C_T = Cycle Threshhold. Indicates cycle number in which amplification crosses the threshold set in the geometric portion of amplification curve. ‡ ΔC_T = Target transcript C_T – 18S ribosomal C_T: Normalization of C_T for target gene relative to ribosomal 18S RNA C_T. ¶Statistical analysis of normalized expression levels between morphologies. Values with different superscripts for each of the target genes differ significantly: [ab]($P < 0.01$), [cd]($P < 0.001$), [ef]($P < 0.004$),[eg]($P < 0.001$), and [fg]($P < 0.01$). §$\Delta\Delta C_T$ = Mean ΔC_T – highest mean ΔC_T value: The mean value for the morphology with highest ΔC_T (lowest expression levels for target) was used as a calibrator to set the baseline for comparing mean differences in the ΔC_T values across all morphologies.

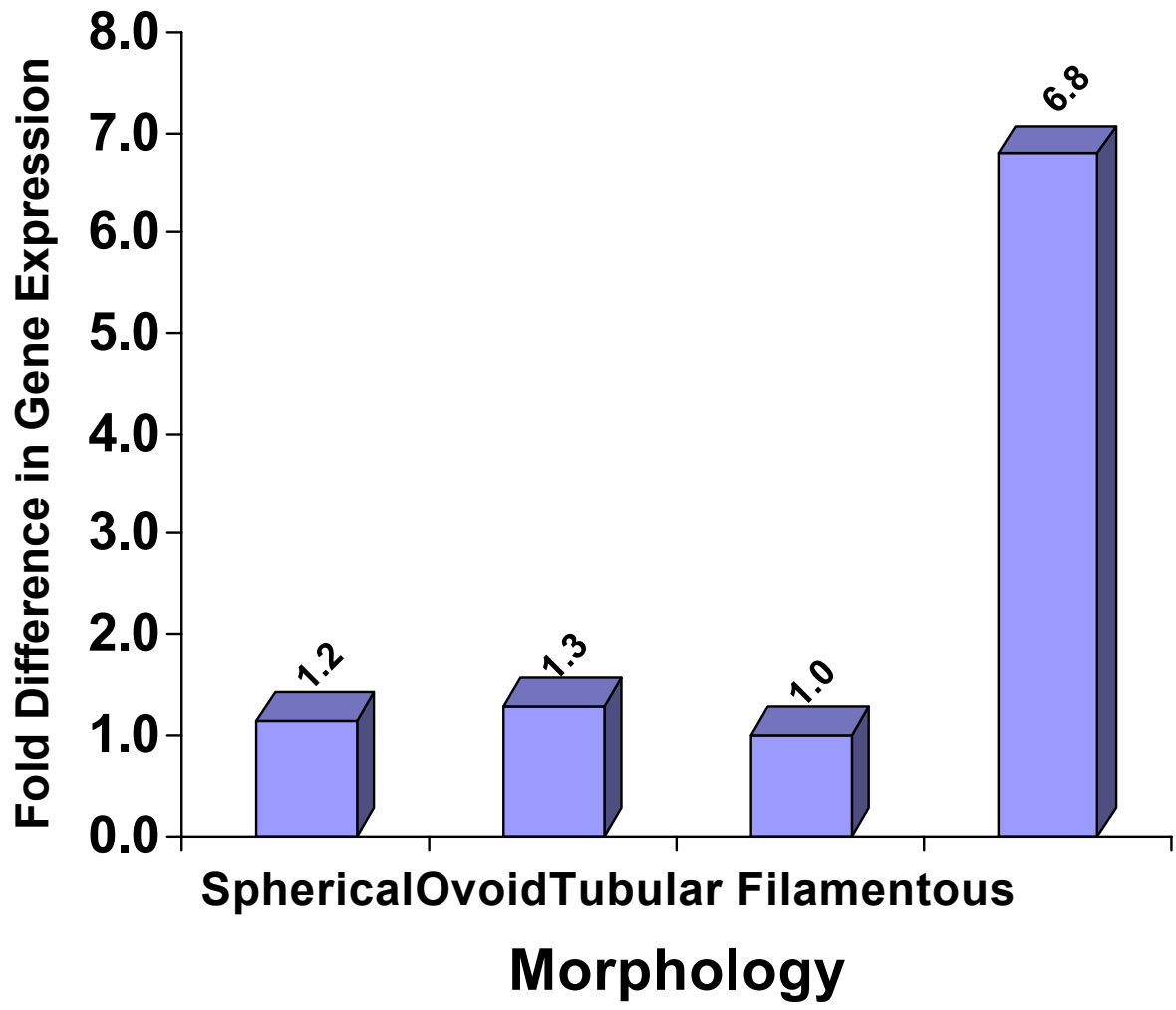

Figure 1
Fold gene expression differences for SAHH. Fold difference in conceptus produced gene expression for s-adenosylhomocysteine hydrolase detected using one-step real-time RT-PCR (2–8 pools or individual conceptuses/morphology). The fold differences in gene expression were calculated as described in *Materials and Methods*.

Based on normalization with 18S ribosomal RNA, SAHH mRNA expression was greater ($P < 0.01$) in filamentous conceptuses compared to all other morphologies evaluated (Table 4). There was an approximately 7-fold increase of SAHH gene expression in filamentous conceptuses compared to spherical conceptuses (Figure 1). HSC70 gene expression was also significantly enhanced ($P < 0.001$) in filamentous conceptuses (Table 4), which displayed an approximate 10-fold increase in gene expression compared to spherical, ovoid and tubular morphologies (Figure 2). Gene expression for OSU-T1-50 was greater ($P < 0.004$) in tubular conceptuses compared to spherical while filamentous conceptus expression was greater ($P < 0.01$) compared to all other morphologies evaluated (Table 4). When compared to spherical conceptuses, gene expression for OSU-T1-50 was approximately 11 and 104-fold greater in tubular and filamentous morphologies, respectively (Figure 3).

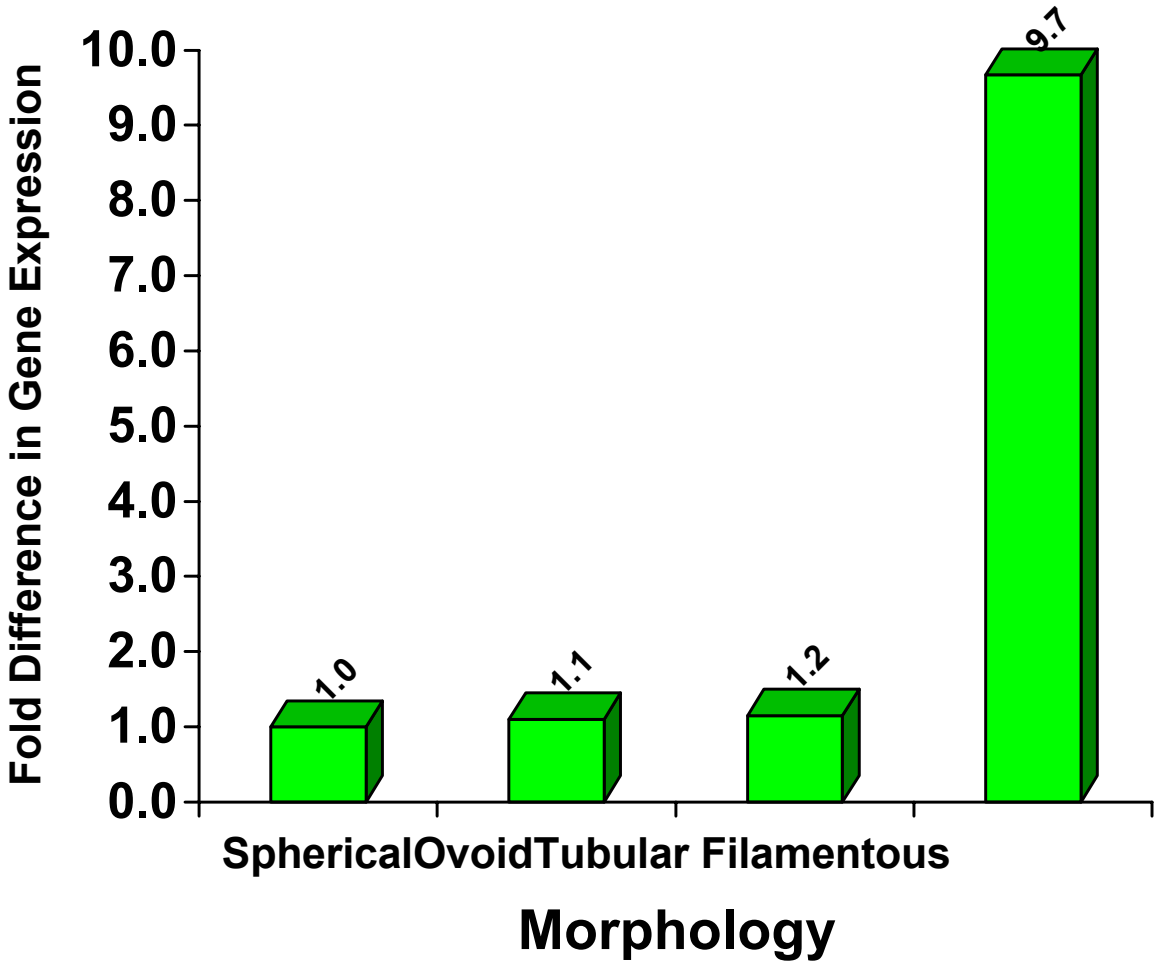

Figure 2
Fold gene expression differences for HSC70. Fold difference in conceptus produced gene expression for heat shock cognate 70 kDa detected using one-step real-time RT-PCR (2–8 pools or individual conceptuses/morphology). The fold differences in gene expression were calculated as described in *Materials and Methods*.

Discussion

During early porcine development a rapid morphological transformation of the conceptus from a spherical (9–10 mm) to filamentous (> 150 mm) morphology is required to establish adequate placenta to uterine contact necessary for survival [2]. Characterization of the specific subset of genes regulating peri-implantation conceptus development and trophoblastic elongation in the pig provides valuable information concerning key developmental events essential to embryonic survival after trophoblastic elongation [1].

Previous information regarding genes critical to development in early pig pregnancy is limited. Using semi-quantitative RT-PCR, Yelich et al. [10] evaluated peri-implantation gene expression profiles for 17α-hydroxylase, aromatase, brachyury and leukemia inhibitory factor receptor, all of which are more greatly expressed in filamentous conceptuses. Messenger RNA for retinoic acid re-

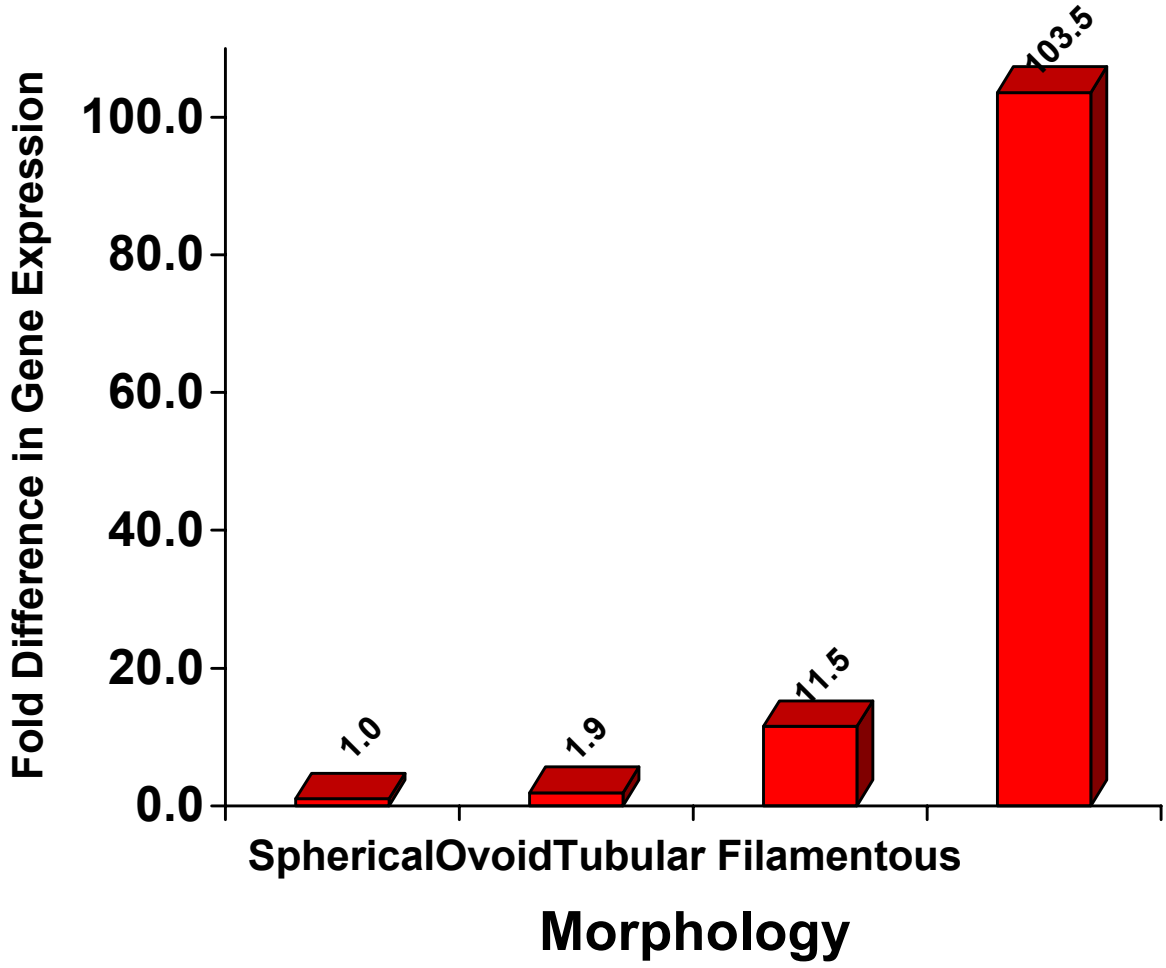

Figure 3
Fold gene expression differences for OSU-T1-50. Fold difference in conceptus produced gene expression for the novel gene, OSU-T1-50 detected using one-step real-time RT-PCR (2–8 pools or individual conceptuses/morphology). The fold differences in gene expression were calculated as described in *Materials and Methods*.

ceptor α, retinal binding protein and transforming growth factor β-3 is also increased in filamentous conceptuses [8]. Using a ribonuclease protection assay, Wilson et al. [16] indicated enhanced cyclooxygenase-2 expression was specific to filamentous conceptuses. An autocrine effect of conceptus estrogen synthesis has been suggested as filamentous conceptuses have greater gene expression for estrogen receptor β compared to its spherical and tubular counterparts [9].

Suppression subtractive hybridization allowed isolation of candidate genes expected to be differentially expressed during rapid trophoblastic elongation. Differentially expressed genes identified using SSH striking particular interest were interleukin-1β (IL-1β) and metallopanstimulin-1 (MPS-1), both of which are suspected to be up-regulated in the elongated conceptus. MPS-1 expression is notable in proliferating cells and has been identified as a nuclear protein that binds to DNA [17]. Further analysis indicates cells stimulated with transforming growth factor β1 (TGF-β1) exhibit a 8-fold in-

crease in MPS-1 gene expression [18]. TGF-β1 and its receptors are localized in the trophectoderm of porcine conceptuses between days 10–14 of gestation [19]. MPS-1 may have roles in transcriptional-mediation of the embryonic response to TGF-β1 having effects on continued intra-uterine elongation. However, MPS-1 gene expression needs to be quantitatively analyzed across the stages of conceptus development. IL-1β was the most predominant clone sequenced during SSH and was also the most abundant clone represented when mapping expressed sequence tags derived from a porcine early embryonic cDNA library in a study by Smith et al. [20]. We have more thoroughly examined and confirmed IL-1β gene and ligand expression in developing conceptuses as well as the endometrium. Changes in conceptus IL-1β gene expression and ligand release in the uterine horn dramatically increase during the process of elongation (Ross and Geisert, unpublished results). Increase in IL-1β gene expression was specific to the conceptus as endometrial expression was unchanged between cyclic and pregnant gilts. Following completion of conceptus elongation, IL-1β gene expression and secretion rapidly decline and expression levels are more than 2000-fold lower in day 15 compared to day 12 filamentous conceptuses. It is possible IL-1β plays an important role in triggering conceptus elongation and initiating uterine-conceptus "cross-talk" in the pig.

In the present study, three transcripts; OSU-T1-50, SAHH and HSC70 were confirmed to be differentially expressed analogous to the pattern predicted using SSH. Although no confirmed identity was found in GenBank, the novel gene, OSU-T1-50 displayed a very dynamic enhancement of gene expression during trophoblastic elongation. When compared to spherical conceptuses there was almost a 12-fold increase in mRNA expression for OSU-T1-50 in tubular conceptuses while expression in filamentous conceptuses increased over 100-fold. Given the substantial increase in gene expression and temporal relationship to conceptus development, OSU-T1-50 may play an important role in trophoblastic elongation. Future studies will attempt to identify the gene and its translated protein to determine the biological function during conceptus development.

Gene expression of SAHH was similar between spherical, ovoid and tubular conceptuses, but there was nearly a 7-fold increase in gene expression in filamentous conceptuses. SAHH may have significant impact on the conceptuses ability to use folates during this transitional stage of development. Folates have long been known to be an essential requirement for developing embryos, predominately during neurulation [21]. Vallet and coworkers [22] have previously shown the increase in maternal folate binding protein activity occurs in the uterine lumen of cyclic and pregnant gilts between days 10–12 post-estrus. The increase of folate in the uterine lumen is temporally associated with the increase in conceptus SAHH gene expression.

S-adenosylhomocysteine (SAH) is the resultant product following the release of a methyl group from s-adenosylmethionine (SAM), a universal methyl donor [23]. Methyl donation from SAM has crucial developmental impacts governing DNA methylation [24] as well as methylation of amino acids, proteins, carbohydrates and polysaccharides [23]. S-adenosylhomocysteine hydrolase is the only known enzyme capable of SAH hydrolysis. The breakdown of SAH, which is reversible, results in the release of free homocysteine which is converted to methionine while being used as a substrate for the reduction of 5-methylenetetrahydrofolate (5-MTHF) to tetrahydrofolate (THF). Elevated levels of methionine can then be used in the synthesis of SAM while THF has downstream effects on uracil to thymidine conversions involved with DNA repair and synthesis [24].

SAHH functions as a protective enzyme for adenosine toxicity by preventing nuclear accumulation of SAH [25] and may explain the nuclear localization of SAHH in cells that are transcriptionally active in Xenopus embryos [26]. Vanaerts et al. [27] demonstrated that high concentrations of homocysteine are associated with embryotoxicity during early gestation in rats. These authors suggest the toxicity may be associated with the reverse hydrolysis of homocysteine to SAH resulting in the dramatic reduction of the SAM/SAH ratio to a point where methylation reactions are inhibited. Miller and co-workers [28] have revealed that the lethal nonagouti (a^x) mutation in mice is characterized by deletion of the SAHH gene resulting in embryonic death prior to implantation. Addition of an inhibitor to SAHH, 3-deazaaristeromycin, inhibits inner cell mass proliferation and differentiation during in vitro development of non-mutant embryos [28].

As in other species, SAHH is likely a biological regulator of the SAM/SAH ratio controlling the occurrence of transmethylation reactions to the degree of which they are necessary for successful porcine conceptus development. Establishment of the maternal-fetal interface is extremely competitive among littermates in early swine gestation. Advanced conceptuses, those that elongate first, have a much greater advantage with regards to acquiring ample placental:uterine contact and also limit the available uterine capacity for those conceptuses lagging in development [2]. While gene expression for SAHH was present at all stages evaluated, an approximate 7-fold increase in relative expression for this transcript over a 2–3 hour time period is noteworthy. It is possible that increased expression of SAHH by filamentous conceptuses serves as a protective function at the level of the nucleus by reducing SAH to ho-

mocysteine thereby maintaining the SAM/SAH ratio at appropriate levels for SAM-mediated transmethylation reactions to occur. Increased SAHH expression by advanced conceptuses suggests there would also be increased homocysteine released into the uterine lumen that may have an embryo-toxic effect on neighboring conceptuses lagging in development.

Gene expression for HSC70 was similar in spherical, ovoid and tubular conceptuses followed by a 10-fold increase in filamentous conceptuses. Gene expression changes for HSC70 during early development have previously been associated with neurulation in Xenopus [29] and chick [30] embryos. Negative mutations of HSC70 in the nervous system of Drosophila larvae resulted in both developmental defects and lethality [31]. The increased HSC70 gene expression during trophoblastic elongation is temporally associated with neural tube development in pig conceptuses.

Traditionally, heat shock proteins are known for their function during cellular stress as molecular chaperones responsible for the folding, re-folding and transport of newly synthesized proteins. HSC70 is a constituitively expressed member of the 70 kDa heat-shock protein (HSP70) family. Members of the HSP70 family have also been proposed to be involved with HSP90 chaperones regulating signal transduction pathways [32]. Unlike HSP70, HSC70 does not exhibit increased gene expression when exposed to heat stress or other agents such as sodium arsenate in the gastrula and neurula Xenopus embryo [33]. Tsang [34] suggested because of their ability to bind and transport folded and un-folded cellular proteins, HSP70 family members may function as a cross-linker to couple cellular proteins to the cytoskeletal matrix. Association of HSP70 with the cytoskeleton suggests that conceptus produced HSC70 may be directly involved with the complex process of conceptus remodeling during trophoblastic elongation.

Through the method of SSH, we have detected several genes that may serve a vital role in differentiation, neurulation, as well as attachment and maintainence of pregnancy in the pig. Detection and confirmation of IL-1β, SAHH, HSC70 and OSU-T1-50 as being differentially expressed during the period of rapid trophoblastic elongation contributes important information towards understanding the mechanisms involved with this essential biological event in the pig.

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