

Comparative Transcriptome Analysis of In Vivo- and In Vitro-Produced Porcine Blastocysts by Small Amplified RNA-Serial Analysis of Gene Expression (SAR-SAGE)

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ABSTRACT Production of embryos in vitro has enormous potential for research and commercial applications. Unfortunately, in vitro production of porcine embryos is extremely inefficient. Despite the characterization of distinct phenotypes, little is known about the molecular mechanisms and altered physiological processes of in vitro-produced embryos. The objective of this study was to compare global gene expression patterns from in vivo- (IVO) and in vitro-produced (IVP) porcine embryos using small amplified RNA-serial analysis of gene expression (SAR-SAGE). Whole-cell RNA from pools of Day 6 IVO and IVP blastocysts was used to construct SAR-SAGE libraries. Sequence analysis of the IVO and IVP libraries yielded 98,771 and 98,408 tags, respectively. A total of 20,029 and 23,453 putative transcripts were detected in the IVO and IVP libraries, respectively. Statistical analyses of SAGE tag frequencies between the IVO and IVP libraries indicated that 938 and 193 tags were differentially expressed at a $P < 0.05$ and $P < 0.001$ level of significance, respectively, suggesting significant deviations in transcriptome profiles from IVO and IVP embryos. Categorization of differentially expressed transcripts into functional groupings indicated a significant deviation in gene expression from IVP blastocysts compared with IVO blastocysts for a number of biological processes including cellular metabolism, organization, and response to stress. Real-time PCR confirmed differential expression for several transcripts from independent IVO and IVP blastocysts. These results demonstrate compromised gene expression in IVP blastocysts compared with IVO blastocysts for a number of biological processes, particularly processes involved in mitochondrial function; thereby providing potential target pathways for improvement of IVP methods. *Mol. Reprod. Dev.* 75: 976–988, 2008. © 2007 Wiley-Liss, Inc.

Key Words: in vitro fertilization; embryo culture; gene regulation; transcript profiles

INTRODUCTION

Production of embryos in vitro has enormous potential for research and commercial applications in livestock. Developing efficient in vitro techniques for producing embryos is essential for the production of transgenic animals using gene manipulation and nuclear transfer technologies. Transgenic livestock can be utilized for a number of biotechnological applications such as enhancing growth and disease resistance in livestock, manufacturing biomedical products and xenografts for humans (Wall, 1996). Despite recent advancements, the overall efficiency of in vitro embryo production in pigs remains extremely low when compared with other livestock species (Swain et al., 2002).

Pig embryos produced in vitro are inferior to their in vivo-produced (IVO) counterparts and develop a number of abnormal phenotypes (Day, 2000; Abeydeera, 2002). Compared with IVO embryos, in vitro-produced (IVP) pig embryos have a high incidence of polyspermy (Suzuki et al., 1994; Abeydeera and Day, 1997), significant chromosomal abnormalities (McCauley et al., 2003), abnormal blastomere development (Wang et al., 1999) with decreased numbers of cells per blastocyst (Wang et al., 1999; Kikuchi, 2004), and altered cellular metabolism (Swain et al., 2002). As a result, the transfer of IVP blastocysts results in very low efficiencies with less than 25% of transferred blastocysts

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typically resulting in the birth of live piglets (Rath et al., 1995; Kikuchi et al., 2002; Yoshioka et al., 2003).

Several mechanisms have been associated with abnormal phenotypes observed in IVP pig embryos. Inadequacies in embryo culture conditions likely play a major role in the development of inferior embryos compared with IVO embryos (Machaty et al., 1998; Swain et al., 2002). Embryo culture conditions include media formulations, gas levels, pH concentration, temperature and specific macromolecule supplementation, all of which can confer sub-optimal culture conditions that lead to impaired cellular function and energy production (Lane and Gardner, 2005). Disrupted cellular metabolism, as measured by substrate utilization, has been identified following the culture of sheep (Thompson et al., 1991), cattle (Khurana and Niemann, 2000), pig (Swain et al., 2002), and mouse (Gardner and Leese, 1990) embryos. Alterations in cellular metabolism of IVP embryos has been associated with decreased developmental capacity (i.e., the ability to generate live offspring) of these embryos (Barnett and Bavister, 1996). Swain et al. (2002) demonstrated that throughout early preimplantation development (i.e., first cleavage up to the blastocyst stage), porcine IVO embryos, especially morulae and blastocysts, utilized greater amounts of energy substrates via glycolysis than IVP embryos. Regardless of treatment, Krebs cycle utilization of glucose was substantially less than that utilized by glycolysis (Swain et al., 2002). But once again, IVO embryos had an increased oxidative conversion of glucose via Krebs cycle compared with IVP embryos (Swain et al., 2002).

Because IVP embryos display significant deviations in phenotypes compared with IVO embryos, it is likely that alterations in gene expression play a role in the perturbation of abnormal phenotypes observed in IVP embryos. In cattle, several studies have identified aberrant gene expression between IVO and IVP blastocysts (Lazzari et al., 2002; Rizos et al., 2002; Lonergan et al., 2003; Wrenzycki et al., 2004). Altered genes observed in IVP bovine embryos included genes associated with a number of biological processes, such as metabolism, cytokine signaling and stress adaptation (Wrenzycki et al., 2004). Recent studies utilizing expressed sequence tags (Whitworth et al., 2004) and a cDNA microarray experiment (Whitworth et al., 2005) have demonstrated only limited variations between IVO and IVP pig blastocysts transcript profiles. Most noteworthy were alterations in the expression level of transcripts associated with cellular metabolism and transcriptional regulation between IVO and IVP pig blastocysts (Whitworth et al., 2005). For instance, Whitworth et al. demonstrated that the ATP synthase, H⁺ transporting, mitochondrial F1 (*ATP5A1*) mRNA level was greater in IVO blastocysts compared with IVP blastocysts and suggested that IVO embryos may have a higher metabolic rate compared with less developmentally competent IVP embryos (Whitworth et al., 2005). Similarly, these authors found that mRNA expression for high mobility group box 1 (*HMGB1*) as well as small

nuclear ribonucleoprotein D (*LSM2*) were greater in IVO blastocysts compared with IVP blastocysts and suggested that this may result from less nuclei or limited transcriptional activity within cells of IVP embryos compared with the cells of IVO embryos (Whitworth et al., 2005).

Unlike microarrays, which is a "closed" system that relies on previously identified genes/transcripts, serial analysis of gene expression (SAGE) is an "open" system which utilizes high throughput transcriptome analysis to enable identification and quantification of both known and novel genes (Velculescu et al., 1995; Blomberg and Zuelke, 2004). The SAGE technique is sensitive enough that it also allows for the identification of rarer, less abundant transcripts (Velculescu et al., 1995). Taken together, these SAGE attributes permit one to develop a more complete picture of biological systems. Several modifications to the traditional SAGE protocol have been developed to enable the analysis of limited starting amounts of RNA (Vilain et al., 2003). Our laboratory recently compared SAGE amplification methodologies using Day 11.5 tubular, porcine embryos and determined that small amplified RNA (SAR)-SAGE compared with PCR-based Micro-SAGE provided the most reliable correlation (75% and 43%, respectively) compared with an unamplified SAGE library (Blomberg et al., 2006).

We hypothesized that significant deviation in transcriptome profiles would be identified between IVO and IVP porcine blastocysts, suggesting that alterations in phenotypes of IVP embryos may be driven by the altered expression of specific genes involved in important developmental processes. Therefore, the primary objective of the current study was to compare transcriptome profiles from IVO and IVP porcine blastocysts using SAR-SAGE. In particular, we were interested in characterizing the global gene expression patterns to further assess the biology of the IVP embryo and potentially identify compromised physiological processes that could be targeted to improve IVP methods.

MATERIALS AND METHODS

Production of Embryos

All animal protocols were approved by the Beltsville Area Animal Care and Use Committee and met the United States Department of Agriculture and National Institutes of Health guidelines for the care and use of animals. For in vivo embryo production, normal cycling, White crossbred gilts 6 months of age or older and weighing at least 100 kg were synchronized, super-ovulated and artificially inseminated as previously described (Blomberg et al., 2005). Gilts were artificially inseminated using pooled semen collected from six distinct White crossbred boars across the experiment. On Day 6 of gestation, pregnant gilts were slaughtered and their reproductive tracts were removed. Embryos from each individual uterine horn were immediately flushed into a Petri-dish using Beltsville embryo recovery, rehydration and transfer medium (BERRTs)

for swine (Blomberg and Zuelke, 2005). Approximately 10–20 transferable (Grade 1, 2 and 3) mid- to expanded-blastocysts (Stringfellow and Seidel, 1998) from each gilt ($n = 3$) were collected together in $< 5 \mu\text{l}$ of PBS, snap frozen and stored in liquid N_2 until whole cell RNA (wcRNA) was extracted.

For in vitro embryo production, random ovaries from predominantly White crossbred sows were obtained from a commercial abattoir and transported to the laboratory in 0.9% (w/v) saline at $\sim 32^\circ\text{C}$. Cumulus-oocyte complexes (COC) were aspirated, matured, and fertilized in vitro as previously described (Brad et al., 2003; Herrick et al., 2003). Briefly, COCs were aspirated from 3 to 8 mm follicles and washed twice in synthetic oviductal fluid (SOF)-HEPES-buffered medium. Groups of 50 COCs were matured for 42 hr in 500 μl of TCM-199 (GibcoBRL, Grand Island, NY) supplemented with 10% porcine follicular fluid, 0.1 U/ml of each porcine LH and FSH (Sioux Biochemicals, Sioux Center, IA), 10 ng/ml epidermal growth factor (EGF, Sigma, St. Louis, MO), 0.57 mM cysteine (Sigma), and 1.0% (v/v) PSA (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.25 ng/ml amphotericin; GibcoBRL). For oocyte maturation, COCs were incubated in 5% CO_2 :95% air at 39°C under mineral oil (Sigma). Following the maturation period, oocytes were denuded by repeated pipet aspiration in SOF-HEPES containing 100 $\mu\text{g}/\text{ml}$ hyaluronidase (Sigma). Denuded oocytes were washed an additional three times in SOF-HEPES. Fresh semen from an individual White crossbred boar was diluted in 1.5 ml Dulbecco's PBS (GibcoBRL) without CaCl_2 , supplemented with 0.1% (w/v) BSA. Three different White crossbred boars were represented in the five IVP replicates. Motile spermatozoa were collected using a 45%:90% Percoll gradient with centrifugation at 1,000g for 20 min. Spermatozoa (5.0×10^5 sperm/ml) and oocytes (20 oocytes/well) were coincubated for 6 hr in 5% CO_2 :95% air at 39°C in 50 μl droplets of fertilization medium [modified Tris-buffered medium (mTBS) supplemented with 3.5 mM caffeine (Sigma) and 0.2% (w/v) BSA, fraction V (Sigma)]. Following incubation, presumptive zygotes were washed in NCSU23 (Petters and Wells, 1993) culture medium supplemented with 4 mg/ml Pentex BSA (Serologicals, Kanakakee, IL) to remove excess spermatozoa. Zygotes were in vitro cultured (10 per 50 μl droplet of culture medium) under mineral oil in 5% CO_2 , 10% O_2 , and 85% N_2 at 39°C for 144 hr. Approximately 10–30 heterogeneous, transferable (Grade 1, 2, and 3) mid- to expanded-blastocysts (Stringfellow and Seidel, 1998) from five IVP embryo replicates were collected together in $< 5 \mu\text{l}$ of PBS, snap frozen and stored in liquid N_2 until wcRNA was extracted.

Processing of Whole Cell RNA

wcRNA was isolated from a collective pool of IVO embryos (consisting of 77 total blastocysts from three gilts artificially inseminated using pooled semen represented by six different boars) or IVP embryos (consisting of 79 total blastocysts from five IVP replicates fertilized using fresh semen represented by three different boars)

using the RNeasy Micro kit (Qiagen, Valencia, CA) following the manufacturer's protocol. For removal of genomic DNA contamination, DNase-I treatment was performed on the spin column using DNase provided by the manufacturer (Qiagen). Quantification and analysis of the integrity of wcRNA were performed with an RNA 6000 Pico LabChip kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA; data not included).

Generation of SAR-SAGE Libraries

Two SAGE libraries from either pooled IVO or IVP blastocysts were generated using the SAR-SAGE approach previously described (Vilain et al., 2003). Briefly, ~ 40 ng of wcRNA from pools of IVO and IVP blastocysts were used as starting material to generate amplified libraries. A T7 adapter containing the T7 RNA polymerase promoter, confirmed in silico not to correspond to any known porcine genes, was ligated to the 5' cohesive end of the *Nla*III-cleaved cDNA (Vilain et al., 2003; Blomberg et al., 2006). One round of mRNA amplification was performed using the T7-Megascript kit (Ambion, Austin, TX) following the manufacturer's protocol. Amplified, sense mRNA was purified using the RNeasy Micro kit (Qiagen) and quantified using the Agilent 2100 Bioanalyzer and RNA 6000 Pico LabChip kit (Agilent Technologies; data not included).

Approximately 250 ng of amplified mRNA generated from the IVO and IVP wcRNA pools was used to construct traditional IVO and IVP SAGE libraries based on the methodology of Velculescu et al. (1995) using the I-SAGE kit (Invitrogen). All procedures for the amplification of SAGE concatemers and sequencing of SAGE tags was performed as previously described (Long et al., 2003), except purification of concatemer PCR products was carried out using the QuickStep2 384-Well Purification kit (Edge Biosystems, Gaithersburg, MD) and sequence analysis was performed using 3.2 pmol of M13-20 Forward primer (5'-CCCAGTCAGACGTTGTAACCG-3').

Processing and Analysis of SAGE Tag Sequences

The SAGE tag's nucleotide sequence quality was assessed using PHRED and vector sequences were trimmed using `cross_match`, as previously described (Long et al., 2003; Blomberg et al., 2005). Processed SAGE tag nucleotide sequences were converted to text files, extracted and quantified using SAGE 2000 software, version 4.12 (<http://www.invitrogen.com/sage>). Tag sequences and frequency data were output to Microsoft Access for comparison between IVO and IVP libraries.

Annotation of SAGE Tags

Prior to annotation, the *Nla*III-recognition sequence (CATG) was appended to the 5' end of each SAGE tag. Initially, BLAST comparison of SAGE tags was performed using the species-specific porcine gene index (SsGI, release 11: 01/18/05) available through The Institute of Genome Research (TIGR, Rockville, MD;

<http://www.tigr.org/tdb/tgi/ssgi>) assigning SAGE tags to tentative consensus (TC) contigs. Only SAGE tags that had a plus–plus orientation and perfect 14-bp match in a TC contig were considered in the final data set. For cross-species comparison, positively identified pig TC contigs were masked to human mRNA sequences using RepeatMasker Open-3.0 (1994–2004; <http://www.repeatmasker.org>) prior to BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>) comparison of pig TC contigs and human mRNA sequences to assign tentative annotation. For a nonannotated TC contig, human UniGene databases (<http://www.ncbi.nlm.nih.gov/entrez/>) were queried for the most homologous sequences with a word size of 9, a minimum alignment of 40 bp and $P < 0.05$. The resulting human mRNA sequences and UniGene IDs with high similarity to pig TC contigs were used to assign gene ontology (GO) annotations for biological processes. Since the initial BLAST comparison of the SAGE tags, the SsGI (TIGR, release 11) has been updated. Therefore, the annotation of transcripts reported in this study were verified and updated with TC contig ID available through the Dana-Farber Cancer Institute porcine gene index (DFCI SsGI, release 12: 06/20/06; <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig>).

Real-Time PCR

Validation of transcript expression levels (SAGE tag frequencies) between IVO and IVP blastocysts was carried out utilizing real-time PCR analysis of wcrRNA obtained from individual, Grade 1, expanded blastocysts (Stringfellow and Seidel, 1998) produced in vivo or in vitro. For real-time PCR analysis, embryos were produced in a similar manner described above. To verify that the transcript profiles attributed to IVO or IVP embryos were consistent, single embryos were collected

from four different gilts artificially inseminated using pooled semen from three different boars for the IVO treatment. Similarly, a different replicate of IVP embryos fertilized with fresh semen from a different boar were used for the IVP treatment. Thus, the starting mRNA for real-time PCR represents a distinct source of mRNA that was used for generating SAGE libraries. Because of the limited amount of wcrRNA present in individual embryos (<2.0 ng), the Eberwine technique (Van Gelder et al., 1990) of linear mRNA amplification (aRNA) was utilized to generate adequate amounts of antisense mRNA for the analysis of a large panel of transcripts from individual embryos. Briefly, wcrRNA was extracted using the RNeasy Micro kit (Qiagen) from blastocysts. For removal of genomic DNA contamination, DNase-I treatment was performed on the spin column using DNase provided by the manufacturer (Qiagen). Individual wcrRNA samples served as template for two rounds of RNA amplification using the MessageAmpII aRNA Amplification kit (Ambion) following manufacturer's protocol. Quantification and analysis of the integrity of aRNA was performed with an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies; data not included).

Porcine-specific primers (Table 1) were designed using Primer Express Software version 2.0 (Applied Biosystems, Foster City, CA) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), cytochrome c oxidase subunit VIIb (*COX7B*), cytochrome c, somatic (*CYCS*), alpha tubulin 1 (*TUBA1*), high-mobility group nucleosomal binding domain 2 (*HMGN2*), heat shock protein 10 kDa 1 (*HSPE1*), heat shock protein 60 kDa 1 (*HSPD1*), heat shock protein 90 kDa 1 alpha (*HSPCA*), and ribosomal protein, large P2 (*RPLP2*) based on sequences obtained through the SsGI (DFCI). A two-step, real-time RT-PCR method was used for transcript expression analysis in

TABLE 1. Porcine-Specific Primer Sequences Used for Real-Time PCR Validation of Selected Transcripts

Gene symbol ^a	DFCI SsGI TC no.	Primer sequences
<i>GAPDH</i>	TC298767	F 5'-CATTGCCCTCAACGACCACTT-3' R 5'-TGGAGGCCATGTGGACCAT-3'
<i>COX7B</i>	TC280843	F 5'-GGCAAGGCAGAACCACCAGAA-3' R 5'-ATGCCCATACAGCAACACAGAAAAG-3'
<i>CYCS</i>	TC278484	F 5'-AGAACTACCGGATGTGTGTGATTGA-3' R 5'-CTGACGGTACAGCTCCCCTACTTT-3'
<i>TUBA1</i>	TC298934	F 5'-TGGACCACAAGTTTGACCTGATG-3' R 5'-GTCCTCACGGCCTCAGAAA-3'
<i>HMGN2</i>	TC290635	F 5'-ACAAATGCCTTGTGGTGTGGAA-3' R 5'-GGTCTCTCTGGGATTAAGGGAGTCA-3'
<i>HSPE1</i>	TC298933	F 5'-TGCCCCATTCCACTGAAGT-3' R 5'-TGGATGTCATCAGTTTGGATATCATTAG-3'
<i>HSPD1</i>	TC252092	F 5'-GCGGCGTTCCTCACCAATAA-3' R 5'-TTTCATAAATGTCAGCCAGCCTTT-3'
<i>HSPCA</i>	TC297882	F 5'-AAAGCCCACCTAGGCATGTG-3' R 5'-CGGCGCTACATCTCCACTCA-3'
<i>RPLP2</i>	TC284388	F 5'-GCTGCAGCAGAGGAGAAGAAAGA-3' R 5'-TTTGCAGGGAGCAGGACTCTAGT-3'

F, forward primer; R, reverse primer.

^aAccording to human Gene ID (NCBI).

which real-time PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems). Reverse transcription was performed with 500 ng of aRNA from individual IVO and IVP blastocysts ($n = 7$ for each treatment) using 100 μM of random decamers (Ambion), preferred for antisense mRNA, and SuperScriptIII reverse transcriptase (Invitrogen) under conditions recommended by the manufacturer. Each real-time PCR was assayed in duplicate and consisted of 5 ng equivalents of cDNA, 0.25 μM of the appropriate forward and reverse primer, 12.5 μl of $1\times$ iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA) in a 25- μl reaction. All PCR conditions included denaturation (95°C for 2 min) followed by amplification (95°C for 15 sec, 60°C for 15 sec, and 70°C for 45 sec) for 30–40 cycles. Melt-curve analysis and gel electrophoresis were used to confirm amplification of a single product of the predicted size (data not included). PCR products from a representative sample (a randomly selected, Day 6 IVP blastocyst) were verified by sequence analysis to confirm amplification of the correct cDNA. Data from SAGE tag frequencies (Table 2) and real-time PCR expression levels (Table 5) indicated that *RPLP2* was constitutively expressed in IVO and IVP blastocysts; therefore, *RPLP2* was used as the reference control transcript. Expression of mRNA for each transcript was determined using the Relative Quantification software version 1.1 (Applied Biosystems) and was based on threshold cycle (C_T) values. For calculation of the relative quantity (RQ) value, based on the comparative C_T method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen, 2001), the ΔC_T [difference between the reference control transcript (*RPLP2*) C_T and target transcript C_T] and $\Delta\Delta C_T$ [difference between the ΔC_T of a calibrator (random individual IVO sample) and ΔC_T of individual samples] were determined. The formula $2^{-\Delta\Delta C_T}$ was used to determine the final RQ values for each target transcript. The formula $2^{-\Delta C_T}$ was used to determine the final RQ values for the reference control transcript from four assays with an interassay coefficient of variation of 14.8%.

Statistical Analysis

Frequency data for SAGE tags from the IVO and IVP libraries were analyzed for significant distribution differences ($P < 0.05$) using statistical tools in the SAGE 2000 software based on a Chi-square analysis combined with Monte-Carlo simulations (Audic and Claverie, 1997). The Monte-Carlo simulations provide estimates of the P values for each tag based on normalized tag counts, which minimize the number of assumptions and account for the large number of comparisons being made between the SAGE libraries (Zhang et al., 1997). Proportional data for accrual rates were analyzed using Chi-square analysis (Steel et al., 1997; SAS, 2003). Real-time PCR data were analyzed using the general linear model procedures for analysis of variance (Steel et al., 1997; SAS, 2003). Results of real-time PCR are reported as least-squares means \pm SEM and means were considered significantly different at $P \leq 0.05$.

RESULTS

The SAGE IVO and IVP porcine blastocyst libraries were sequenced to 98,771 and 98,408 tags, respectively. There were 20,029 tags identified in the IVO library and 23,453 tags identified in the IVP library, representing putative transcripts. Complete tag sequences and frequencies from these libraries are reported in Supplemental Table S1 and have been deposited for public access in the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo>; GSM Accession # GSM129476 and GSM129478, for IVO and IVP libraries, respectively). In both libraries, $\sim 30\%$ of all tags were detected at a frequency ≥ 2 . To assess the representation of unique tags (i.e., individual genes) relative to the overall size of each library, the accrual rate was plotted as a percentage of genes detected relative to the cumulative tag count for the IVO and IVP libraries. The accrual rate of unique tags decreased in a similar pattern for the IVO and IVP libraries (Fig. 1).

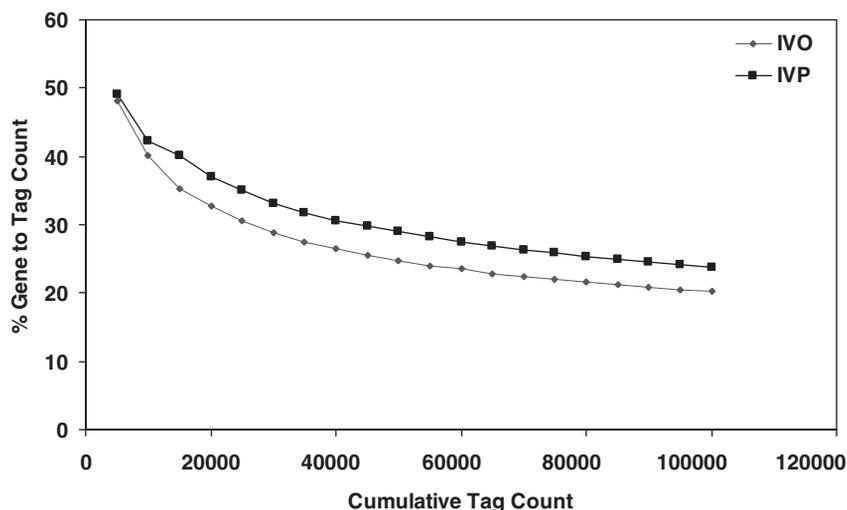


Fig. 1. Accrual rates of SAGE tags plotted as a percentage of genes detected relative to the cumulative tag count for SAGE libraries from in vivo- (IVO) and in vitro-produced (IVP) porcine blastocysts.

TABLE 2. Most Abundant, Annotated SAGE Tags From IVO and IVP Blastocysts

SAGE tag sequence	Transcript (gene symbol ^a)	DFCI SsGI TC no.	Tag frequency		Fold diff.
			IVO	IVP	
TAGGTTGTCT	Tumor protein, translationally-controlled 1 (<i>TPT1</i>)**	TC301215	962	1454	1.5
CACCCCTGAG	Creatine kinase, brain (<i>CKB</i>)*	TC270802	514	565	1.1
CCAGGCGTCC	Cathepsin D (<i>CTSD</i>)**	TC301566	566	457	1.2
GGCTTCGGCT	Ribosomal protein, large, P2 (<i>RPLP2</i>) ^b	TC284388	511	501	1.0
GTGGGAAACT	Ribosomal protein L27a (<i>RPL27A</i>) ^b	TC302651	476	490	1.0
TTTCGGGTTA	Ferritin, heavy polypeptide 1 (<i>FTH1</i>)**	TC282657	392	549	1.4
AGAGCCTTGA	ATP synthase, beta polypeptide (<i>ATP5B</i>)**	TC246092	555	359	1.6
GCCGGCCTGG	Ribosomal protein S15 (<i>RPS15</i>)**	TC279520	373	526	1.4
GATTCCTAGT	Ribosomal protein L13a (<i>RPL13A</i>) ^b	TC271171	451	477	1.0
AGTGGTGTTA	Uridine phosphorylase 1 (<i>UPP1</i>)**	TC251315	567	321	1.8

^aAccording to human Gene ID (NCBI).

^bNonsignificant.

* $P < 0.05$.

** $P < 0.001$.

The final accrual rates were not significantly different ($P = 0.49$) between the IVO and IVP libraries (20.3% and 23.8%, respectively, after sequencing ~100,000 tags per library) with an average accrual rate of 22% (43,482/197,179). This indicated that the initial mRNA amplification step did not introduce significant bias.

Combining the SAGE tags from both libraries resulted in a total of 197,179 tags corresponding to 34,480 unique tags or putative transcripts. Of the total tags sequenced, only 9,003 (26%) were in common between the two libraries, leaving 11,027 (32%) and 14,450 (42%) putative transcripts unique to the IVO and IVP libraries, respectively. The majority (~70%; 24,203/34,480) of the unique tags were detected only once within either the IVO or IVP libraries, indicating that most of the putative transcripts expressed in IVO and IVP blastocyst were expressed at very low levels. Furthermore, the significant number of singletons may explain why only 26% of the tags were in common between the two libraries. Statistical analysis of SAGE tag frequencies was performed on 10,277 putative transcripts that were expressed at threshold frequency of ≥ 2 tags within a single library. Statistical analysis of SAGE tag frequencies between the IVO and IVP libraries indicated that 9% (938/10,277) of the putative transcripts were differentially expressed at a $P < 0.05$ level of significance. Of the differentially expressed tags ($P < 0.05$), 514 tags were up-regulated and 424 tags were down-regulated in the IVO library compared with the IVP library. Using a more stringent cutoff for significance ($P < 0.001$), 2% (193/10,277) were differentially expressed with 118 tags up-regulated and 75 tags down-regulated in the IVO library compared with the IVP library.

An initial comparison of constitutively and differentially expressed SAGE tags with a frequency ≥ 2 in a single library against the TIGR SsGI resulted in 11,339 TC contigs that matched a SAGE tag. Many of the tags matched multiple TC contigs resulting in ambiguity. Tags that matched only one distinct TC annotation were examined further and the resultant was 5,025 constitu-

tively and differentially expressed tags. Tags were classified as being abundant if they constituted $\geq 0.5\%$ of the total number of tags sequenced. Of all expressed tags, 18 tags were considered abundantly expressed, of which 10 matched a unique TC (Table 2). Three tags, annotated as ribosomal protein, large, P2 (*RPLP2*), ribosomal protein L27a (*RPL27A*), and ribosomal protein L13a (*RPL13A*) were constitutively expressed between the IVO and IVP libraries. Tags for cathepsin D (*CTSD*), ATP synthase, beta polypeptide (*ATP5B*), and uridine phosphorylase (*UPP1*) were more abundant in the IVO library compared with the IVP library. In contrast, tags for tumor protein, translationally-controlled 1 (*TPT1*), creatine kinase, brain (*CKB*), ferritin, heavy polypeptide 1 (*FTH1*), and ribosomal protein S15 (*RPS15*) were more abundant in the IVP library compared with the IVO library.

BLAST sequence alignment of the 938 differentially expressed ($P < 0.05$) SAGE tags resulted in 55% (517/938) of the tags that matched a unique TC annotation. Of those, 329 were up-regulated and 188 were down-regulated in the IVO library compared with the IVP library. These TCs were compared against human mRNA sequences resulting in 89% (460/517) of the TCs that aligned with a similar human ortholog. Corresponding human mRNA sequences were used to assign GO annotation based on biological process. Of the 460 differentially expressed TCs that matched human annotation, 373 were assigned to a biological process. Categorization of the predominant functional groupings (greater than 25 TCs per grouping) based on the fourth level GO biological process annotation is presented in Figure 2. The majority of the differentially expressed TCs categorized to a biological process were associated with cellular metabolism (GO:0044237), followed by cellular transport (GO:0006810), regulation of cellular physiological process (GO:0051244), signal transduction (GO:0007165), cellular organization and biogenesis (GO:0016043), response to stress (GO:0006950), cell cycle (GO:0007049), and organogenesis (GO:0009887), respectively (Fig. 2). Of these TCs, approximately 70%

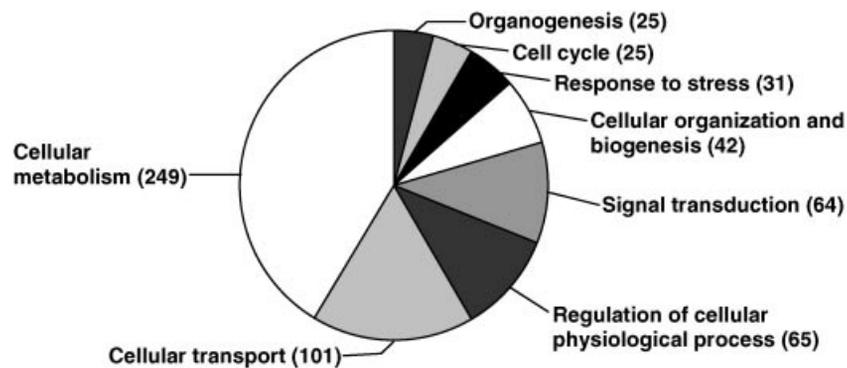


Fig. 2. Categorization of the predominant (>25 TCs) functional groups based on the fourth level GO annotation for biological process identified for the differentially expressed SAGE tags from IVO and IVP porcine blastocysts. Parentheses indicate the observed number of differentially expressed TC sequences observed between the SAGE libraries.

were up-regulated in IVO blastocysts compared with IVP blastocysts (Table 3).

A representative number of genes identified in the biological process subcategories are presented in Table 4. These genes met the following criteria: (1) a minimum frequency of 15 tags within one library, (2) twofold difference in expression between libraries, and (3) each tag matched only one unique TC annotation. A number of these transcripts were identified within multiple categories of the fourth level biological processes. For instance, tags, annotated as apolipoprotein E (*APOE*), HSP 60 kDa 1 (*HSPA1B*) and HSP 90 kDa 1, alpha (*HSPCA*) were all associated with cellular metabolism, cellular transport and response to stress indicating complexity of these biological systems. To avoid confusion, transcripts identified within multiple categories are listed in only one category.

Because the greatest deviation in SAGE tags expression between the IVO and IVP libraries was observed for cellular metabolism, further analysis of tags differentially expressed associated with catabolism of glucose (glycolysis, Krebs cycle, and oxidative phosphorylation; eighth level GO annotation) was performed. Table 5 summarizes all transcripts associated with the catabolism of glucose that were identified as differentially

expressed between the IVO and IVP libraries. Some deviations in gene expression of transcripts associated with glycolysis (3 transcripts) and Krebs cycle (1 transcript) were observed between the IVO and IVP libraries. Interestingly, the majority (78%; 14/18) of the differentially expressed genes associated with the glucose catabolism were involved in oxidative phosphorylation and all were depressed in IVP blastocysts compared with the IVO blastocysts. These transcripts included a number of ATP synthases, cytochrome c oxidases, NADH dehydrogenases, and ubiquinol-cytochrome c reductases (Table 5).

To validate the SAGE results obtained from pooled IVO and IVP blastocysts, quantitative real-time PCR of a select number of differentially expressed genes was performed on mRNA from individual IVO and IVP blastocysts collected from different gilts and IVP replicate for the IVO and IVP treatments, respectively. Transcripts selected for validation met the following criteria: (1) a tag frequency of >0.05% or greater within both libraries and (2) significant differential expression at $P < 0.001$ level between the IVO and IVP libraries. These transcripts included coverage from several of the characterized biological processes identified by the SAGE analysis. Transcripts were associated with

TABLE 3. Pattern of Differential Expression Between IVO and IVP Blastocysts for TCs Identified as a Biological Process

Biological process ^a	Total # TCs	Number of up-regulated TCs (% of total)	
		IVO	IVP
Cellular metabolism	249	177 (71)	72 (29)
Cellular transport	101	80 (79)	21 (21)
Regulation of cellular physiological process	65	44 (68)	21 (32)
Signal transduction	64	45 (70)	19 (30)
Cellular organization and biogenesis	42	29 (69)	13 (31)
Response to stress	31	26 (84)	5 (16)
Cell cycle	25	17 (68)	8 (32)
Organogenesis	25	18 (72)	7 (28)

^aCategorization of biological process based on the fourth level GO annotation.

TABLE 4. Categorization of Differentially Expressed SAGE Tags From IVO and IVP Blastocysts[†]

SAGE tag sequence	Transcript (gene symbol ^a)	DFCI SsGI TC no.	Tag frequency		
			IVO	IVP	Fold diff.
<i>Cellular metabolism</i>					
CCATTGGGGC	Mitochondrial ribosomal protein L40 (<i>MRPL40</i>)**	TC252406	77	38	2.0
CGCGTCATTC	Mitochondrial ribosomal protein S6 (<i>MRPS6</i>)**	TC256076	64	31	2.1
GAGCGTCTCTG	Cytidine deaminase (<i>CDA</i>)**	TC239819	78	24	3.2
TAATTCTTCT	Chaperonin containing TCP1 (<i>CCT</i>)**	TC290948	64	23	2.9
<i>Cellular transport</i>					
CGTAATGTAA	Translocase inner mitochondrial membrane 8A (<i>TIMM8A</i>)**	TC279187	50	15	3.3
GAAGGTGTGC	Translocase outer mitochondrial membrane 7 (<i>TOMM7</i>)*	TC281429	19	6	3.2
GCCGCCCGCT	Apolipoprotein A-1 (<i>APOA1</i>)**	TC297386	28	2	14.0
CGGCGCCAGT	Apolipoprotein E (<i>APOE</i>)**	TC238987	22	1	22.0
<i>Cellular organization</i>					
TCTCAAAAAG	Alpha tubulin (<i>TUBA1</i>)**	TC298934	224	81	2.8
GACAGGTGTG	Beta tubulin (<i>TUBB</i>)**	TC250666	57	29	2.0
ATTGTTTATG	High-mobility group nucleosomal binding domain 2 (<i>HMGN2</i>)**	TC290635	148	47	3.3
ATTTTGGTGT	Serine/threonine kinase 6 (<i>STK6</i>)*	TC246084	15	5	3.0
<i>Response to stress</i>					
TCTGTCTTTT	Hsp10 kDa 1 (<i>HSP10</i>)**	TC298933	92	22	4.2
TACCAATGTA	Hsp60 kDa 1 (<i>HSP60</i>)**	TC252092	100	37	2.7
TAAGTTAGTA	Hsp70 kDa 1B (<i>HSPA1B</i>)**	TC238433	27	7	3.9
TGGTAAAGCT	Hsp90 kDa 1A (<i>HSP90A</i>)**	TC297882	135	41	3.3

[†]Categorization of transcripts based on GO annotation within Biological Processes (fourth level).

^aAccording to human Gene ID (NCBI).

* $P < 0.05$.

** $P < 0.001$.

TABLE 5. Transcripts Associated With Catabolism of Glucose That Were Differentially Expressed Between the IVO and IVP Libraries[†]

SAGE tag sequence	Transcript (gene symbol ^a)	DFCI SsGI TC no.	Tag frequency		
			IVO	IVP	Fold diff.
<i>Glycolysis</i>					
TGTGCCACA	Enolase 1, alpha (<i>ENO1</i>)*	TC239860	105	131	1.3
TACCATCAAT	Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)**	TC298767	179	98	1.8
CTTAGTTTAA	Lactate dehydrogenase B (<i>LDHB</i>)**	TC254983	232	133	1.7
<i>Citrate cycle</i>					
TGACAGCTTT	Succinate dehydrogenase complex, subunit C (<i>SDHC</i>)*	TC239427	65	42	1.6
<i>Oxidative phosphorylation</i>					
AGAGCCTTGA	ATP synthase, beta polypeptide (<i>ATP5B</i>)**	TC246092	555	359	1.6
CCCGGTGCTG	ATP synthase, F0 complex, subunit 9, isoform 1 (<i>ATP5G1</i>)**	TC269281	519	238	2.2
AATACGTTCC	ATP synthase, F0 complex, subunit F6 (<i>ATP5J</i>)*	TC288381	28	13	2.2
AAAATCTTTA	ATP synthase, F1 complex, O subunit (<i>ATP5O</i>)**	TC261232	99	49	2.0
GAAGTTTGTC	Cytochrome c oxidase subunit VIc (<i>COX6C</i>)*	TC280926	118	79	1.5
ATTGAAGAAA	Cytochrome c oxidase subunit VIIb (<i>COX7B</i>)**	TC280843	120	38	3.2
GAAGGCTTAA	Cytochrome c, somatic (<i>CYCS</i>)**	TC278484	97	22	4.4
TTGTATTGTA	NADH dehydrogenase 1 alpha subcomplex, 4 (<i>NDUFA4</i>)*	TC294252	266	213	1.3
AACATTAACA	NADH dehydrogenase 1 alpha subcomplex, 8 (<i>NDUFA8</i>)*	TC242967	21	9	2.3
AAGGATGTGC	NADH dehydrogenase 1 alpha subcomplex, 13 (<i>NDUFA13</i>)*	TC272204	16	4	4.0
TACTGGAAAA	NADH dehydrogenase (ubiquinone) Fe-S protein 2 (<i>NDUFA2</i>)*	TC239361	56	24	2.3
ACAATAAAAT	Ubiquinol-cytochrome c reductase, complex III (<i>UQCRCQ</i>)**	TC250554	116	47	2.5
GACCACCTTT	Ubiquinol-cytochrome c reductase complex [7.2 kD] (<i>UCRC</i>)*	TC294237	48	29	1.7
TGAAGTAAAT	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur (<i>UQCRCF1</i>)**	TC282033	43	18	2.4

[†]Categorization of transcripts based on GO annotation within Biological Processes (eighth level).

^aAccording to human Gene ID (NCBI).

* $P < 0.05$.

** $P < 0.001$.

cellular metabolism (*GAPDH*, *COX7B*, and *CYCS*), cellular organization (*TUBA1* and *HMGN2*), and response to stress (*HSPE1*, *HSPD1*, and *HSPCA*). Validation of the up-regulation of *COX7B*, *CYCS*, *TUBA1*, *HSPE1*, *HSPD1*, and *HSPCA* transcripts in IVO blastocysts compared with IVP blastocysts observed with SAGE analysis was confirmed using real-time PCR (Table 6). Although SAGE analysis demonstrated a significant up-regulation of *GAPDH* and *HMGN2* in IVO blastocysts compared with IVP blastocyst, real-time PCR analysis failed to reach significance due to great variability in transcript expression level between individual blastocyst. However, *GAPDH* and *HMGN2* transcripts levels from real-time PCR (Table 6) did show a trend of up-regulation in IVO blastocysts (7.8 and 5.5-fold increase compared with IVP blastocysts, respectively), confirming a similar expression pattern with SAGE analysis (1.8 and 3.3-fold increase compared with IVP blastocysts, respectively).

DISCUSSION

Porcine embryos produced in vitro develop a number of abnormal phenotypes, such as increased incidences of polyspermy, development of abnormal blastomeres, and decreased numbers of cells per blastocysts compared with embryos produced in vivo (Day, 2000; Abeydeera, 2002). This study characterized in-depth transcriptome profiles of porcine blastocysts produced in vivo or in vitro using SAR-SAGE to identify physiological processes that may be disrupted. The final accrual rate of putative transcripts in IVO and IVP SAGE libraries were similar with an average accrual rate of 22%. Similar accrual rates indicated that these libraries generated from amplified mRNA had similar representation of uniquely expressed tags relative to the overall library size. Our laboratory has previously confirmed using aliquots of the same pool of Day 11.5 tubular embryos that mRNA amplification by T7 polymerase, utilized in SAR-SAGE,

provided the most reliable correlation of transcript levels than PCR-based MicroSAGE (76% and 43%, respectively) when compared with an unamplified library (Blomberg et al., 2006). Thus, minimal bias should have been introduced as a result of SAR-SAGE. Vilain et al., demonstrated similar correlations (75%) by comparing unamplified SAGE libraries and SAR-SAGE amplified library from mouse thyroid tissue furthering indicating minimal bias as a result of mRNA amplification from the SAR-SAGE (Vilain et al., 2003). In addition, SAGE libraries generated from the IVO and IVP embryos were represented by multiple females and males suggesting any gilt or boar effect on transcriptome expression profiles was limited. Furthermore, IVO and IVP embryos produced for real-time PCR validation were from completely independent sources of females and males, which indicated further that the gilt or boar effect on transcriptome expression profiles was minimal, if any.

Comparison of the IVO and IVP SAGE libraries demonstrated distinct patterns of gene expression with only 26% of the total putative transcripts detected in common between the two libraries. The high number of SAGE tag singletons indicated the majority of transcripts are rare, thus, it is difficult to determine if they are truly statistically unique to IVO or IVP embryos. However at a threshold of ≥ 2 tags in a single library, approximately 9% of the putative transcripts were differentially expressed between IVO and IVP blastocysts. These findings indicate significant deviations in transcriptome profiles of more prevalent transcripts from porcine embryos produced in vivo or in vitro. These findings contrast those of Whitworth et al. (2005), who observed only limited deviations in gene expression between IVO and IVP porcine blastocysts from a cDNA microarray experiment. The discrepancy in results between the current study and Whitworth et al. (2005) may be attributed to differences in methodology for

TABLE 6. Comparison of Real-Time PCR Analysis and SAGE Analysis of Transcripts Associated With Catabolic Metabolism, Cellular Organization, and Response to Stress in IVO and IVP Blastocysts

Transcript ^a	Real-time PCR analysis				SAGE analysis ^c		
	IVO RQ ^b	IVP RQ ^b	Fold diff.	P-value	IVO tag frequency	IVP tag frequency	Fold diff.
<i>Cellular metabolism</i>							
<i>GAPDH</i>	5.7 ± 2.0 (17.0)	0.7 ± 2.3 (18.5)	7.8	0.13	179	98	1.8
<i>COX7B</i>	12.8 ± 1.0 (18.4)	8.2 ± 1.1 (19.0)	1.6	0.01	120	38	3.2
<i>CYCS</i>	13.6 ± 1.7 (12.4)	3.6 ± 1.8 (14.0)	3.8	0.002	97	22	4.4
<i>Cellular organization</i>							
<i>TUBA1</i>	4.1 ± 0.7 (14.8)	1.2 ± 0.7 (15.6)	3.4	0.02	224	81	2.8
<i>HMGN2</i>	2.2 ± 0.8 (17.5)	0.4 ± 0.8 (19.2)	5.5	0.14	148	47	3.3
<i>Response to stress</i>							
<i>HSPE1</i>	9.8 ± 1.0 (13.0)	2.5 ± 1.0 (14.8)	3.9	<0.001	92	22	4.2
<i>HSPD1</i>	7.1 ± 1.3 (12.7)	2.7 ± 1.2 (14.9)	2.6	0.03	100	37	2.7
<i>HSPCA</i>	14.2 ± 2.6 (13.3)	5.3 ± 2.8 (14.4)	2.7	0.04	135	41	3.3
<i>Reference control</i>							
<i>RPLP2</i>	1.0 ± 0.2 (13.3)	1.2 ± 0.2 (13.1)	1.2	0.35	511	501	1.0

^aTranscript abbreviation according to human Gene ID (NCBI).

^bNumber in parentheses indicate the average C_T value for each transcript.

^cP < 0.001 between IVO and IVP SAGE tag frequencies.

measuring gene expression (i.e., SAGE vs. cDNA microarray) and differences in source of embryos (i.e., culture conditions and embryo population).

Of the differentially expressed SAGE tags that matched a unique TC annotation, the majority (64%) of these transcripts were up-regulated in IVO blastocysts compared with IVP blastocysts, suggesting decreased gene expression from porcine embryos produced in vitro. A similar pattern of down-regulation in gene expression has been observed following microarray analysis of mouse embryos cultured in Whitten's medium compared with in vivo controls (Rinaudo and Schultz, 2004). Categorization of differentially expressed transcripts from our SAGE libraries into functional groupings based on biological processes indicated that the majority (67%) of these transcripts were associated with cellular metabolism. Furthermore, most (71%) of the differentially expressed transcripts associated with cellular metabolism were up-regulated in the IVO blastocysts compared with IVP blastocyst, suggesting that embryos produced in vitro have decreased cellular metabolism. This finding is not surprising since an abnormal culture environment likely causes disruptions in metabolism, thereby resulting in lower developmental competence of IVP embryos (Barnett and Bavister, 1996).

Development of mammalian blastocysts requires significant amounts of energy to increase protein synthesis and increase Na^+/K^+ -ATPase activity that are important for the formation of the blastocoel cavity (Machaty et al., 2001). Energy production in animal cells is primarily derived from the production of ATP via catabolism of glucose (i.e., glycolysis, Krebs cycle, and oxidative phosphorylation; Horton et al., 1996). During glycolysis, GAPDH in the presence of inorganic phosphate and NAD, initiates the recovery of energy (ATP) stored in glyceraldehyde 3-phosphate through a series of downstream steps (Horton et al., 1996). Additionally, the NADH produced in the catabolism of glyceraldehyde 3-phosphate can serve as an electron acceptor for respiratory electron transport in the generation of ATP via oxidative phosphorylation. NADH can also be reoxidized to regenerate NAD by converting pyruvate to lactate via lactate dehydrogenase (Horton et al., 1996). Elevated levels of glucose have been shown to inhibit the development of embryos in vitro for a number of species including hamster, mouse, cattle, and sheep (for review see, Barnett and Bavister, 1996). In contrast, glucose has been shown to not inhibit embryo development in the pig but rather is necessary for pig embryos to develop to the blastocyst stage in vitro (Petters et al., 1990). However, a negative effect of phosphate on blastocyst development has been observed when in the presence of glucose in the culture media (Petters et al., 1990). Interestingly, the embryo culture media (NCSU23; Petters and Wells, 1993) used in the current study as well as a numerous other studies used for generating porcine embryos in vitro (Abeydeera et al., 1998a,b; Machaty et al., 1998; Swain et al., 2001, 2002; Sturmey and Leese, 2003) contains significant amounts of glucose (5.5 mM) as well as phosphate (1.19 mM).

Our SAGE results indicated that transcripts encoding glycolytic proteins, *GAPDH* and *LDHB*, were found to be significantly down-regulated in IVP blastocysts compared with IVO blastocysts. Furthermore, real-time PCR examination of *GAPDH* expression from an independent collection of embryos indicated a trend toward the inhibition of *GAPDH* expression in IVP blastocysts. If the protein levels mimic the mRNA expression, this might account for the lower glycolytic activity from in vitro-derived blastocysts compared with their in vivo-derived counterparts described by Swain et al. (2002). As a consequence of glucose underutilization in IVP blastocysts, oxidative phosphorylation may be compromised in IVP blastocyst due to the limited availability of glucose-derived metabolites. Still unresolved is the mechanism by which *GAPDH* and *LDHB* are down-regulated. Perhaps the combination of glucose and phosphate in the culture media is inhibitory to *GAPDH* and *LDHB* expression. On the other hand, limited availability of electron carriers (NAD) or phosphate needed for the catalytic mechanism of GAPDH and LDHA may suppress their expression.

Oxidative phosphorylation, the terminal pathway of cellular respiration, occurs within the mitochondria and provides the major source of ATP production (Horton et al., 1996). Sturmey and Leese (2003) have demonstrated that the majority of the ATP produced by pig embryos (zygote to blastocyst) results from oxidative metabolism rather than from glycolysis. Analysis of SAGE tag frequencies revealed a significant inhibition of numerous transcripts associated with oxidative phosphorylation in IVP blastocysts compared with IVO blastocysts (Table 5). These transcripts included a number of ATP synthases, cytochrome c oxidases, NADH dehydrogenases, and cytochrome c reductases. Real-time PCR from independent embryos confirmed similar pattern of down-regulation in two oxidative phosphorylation transcripts, *COX7B* and *CYCS*, in IVP blastocysts as was observed by the SAGE analysis. The decreased expression of transcripts associated with oxidative phosphorylation observed in IVP blastocysts compared with IVO blastocysts suggests ATP production would be limited in embryos produced in vitro. Interestingly, our SAGE analysis clearly indicated greater deviations in transcripts associated with oxidative phosphorylation than glycolysis suggesting that the primary mechanism for generating ATP may be inefficient. Thus, the developmental incompetence of IVP embryos may be a result of an energy deficit due to mitochondrial dysfunction or oxygen availability. It is unlikely that oxygen availability is the primary reason for decreased expression of transcripts associated with oxidative metabolism because the oxygen level (10%) for the current culture system was greater than those observed in the oviducts and uteri of mammals (~8% and ~5%, respectively; Fischer and Bavister, 1993). However, it is not known whether the elevated oxygen level (10%) of the current culture system was inhibitory to oxidative metabolism.

Alternatively, mitochondrial dysfunction has been suggested to reduce oxidative metabolism resulting in decreased developmental competence observed from embryos produced in vitro (Lane and Gardner, 2005). Compared with in vivo controls, significant deviations in the morphology of mitochondria have been observed in cattle (Crosier et al., 2001) and pig (Ott et al., 2002) blastocysts following embryo culture. Furthermore, SAGE analysis from the current study demonstrated that expression of transcripts associated with mitochondrial metabolism (*MRPL40* and *MRPS6*) and transport (*TIMM8A* and *TOMM7*) were significantly decreased from IVP blastocysts compared with IVO blastocysts, suggesting that abnormal mitochondrial function in embryos produced in vitro may play a key role in decreased expression of transcripts associated with oxidative phosphorylation.

The ability of animal cells to undergo proper cytoplasmic reorganization is essential for regulating energy production and thus is instrumental in determining cell viability (Barnett et al., 1997). Cytoplasmic organization of animal cells is regulated by a dynamic interaction between actin filaments and microtubules (Mayer and Jurgens, 2002). Although the effects of in vitro embryo culture on microtubule function are unclear in the pig, Sun et al., (2001) have demonstrated that preventing microtubule assembly by the administration of nocodazole resulted in the inhibition of in vitro porcine oocyte maturation due to inadequate translocation of mitochondria to the inner cytoplasm (Sun et al., 2001). Microtubule assembly occurs via the polymerization of the heterodimers α - and β -tubulin (Mayer and Jurgens, 2002). Newly formed tubulin polypeptides are processed through a series of folding reactions that are catalyzed by number of chaperones including chaperonin-containing t-complex polypeptide 1 (CCT; Yokota et al., 2001). Our SAGE results indicated that α -tubulin (*TUBA1*), β -tubulin (*TUBB*), and *CCT* were all significantly decreased in IVP blastocysts compared with IVO blastocysts. Furthermore, decreased expression of *TUBA1* in IVP blastocysts compared with IVO blastocysts was confirmed by real-time PCR from independent embryos. These findings suggest that porcine blastocysts produced in vitro may have abnormal microtubule assembly. Inadequate microtubule assembly could affect the ability of IVP blastomeres to undergo proper cytoplasmic reorganization, thereby, resulting in decreased energy production potential. Given that microtubules play a key role in organelle localization, particularly the mitochondria (Sun et al., 2001), it is possible that limited energy production potential from IVP blastocysts may be due to improper localization of mitochondria as a result of limited microtubule assembly.

Transcription, involving the interaction of a number of molecular components, such as chromatin structure, RNA polymerase II and various transcript factors (Kanka, 2003), increases in the embryo throughout the preimplantation period (Mohamed et al., 2001). The conformation of chromatin (heterochromatin or euchro-

matin) governs the accessibility of the basal transcription machinery, thereby, controlling transcription (Kanka, 2003). Although histones and their modification play a major role in chromatin organization, the nucleus also contains a number of nonhistone proteins identified as high mobility group nucleosomal (HMGN) proteins that bind to DNA and/or the nucleosomes and change the structure of chromatin (Bustin, 2001). Recent evidence in mouse embryos demonstrated that HMGN1 and HMGN2 are important for maintaining transcription levels throughout the preimplantation period (Mohamed et al., 2001). Antisense oligonucleotides for either *HMGN1* or *HMGN2* caused a transient reduction of both RNA and protein synthesis that resulted in delayed embryonic development rather than developmental arrest or lethality (Mohamed et al., 2001). In the present study, SAGE analysis demonstrated that *HMGN2* was significantly decreased in the IVP blastocysts compared with IVO counterparts and a similar trend in decreased expression of *HMGN2* was observed in IVP embryos by real-time PCR from independent embryos. Given that HMGNs are associated with transcriptional regulation and inhibition of HMGN results in delayed embryo development, decreased expression of *HMGN2* in IVP blastocyst suggests porcine IVP embryos may have altered transcription that potentially leads to delayed embryonic development.

Embryos produced in vitro are exposed to a variety of stresses as a result of inappropriate culture conditions that may alter cellular homeostasis and metabolism and thus developmental competence (Lane and Gardner, 2005). Normally, cells exposed to harmful insults respond by producing a number of protective proteins, heat shock proteins (HSPs), initially named due to their induction in cells exposed to high heat (Parcellier et al., 2003). Furthermore, HSPs have been shown to play essential roles as molecular chaperones regulating a number of key housekeeping functions such as the transport of proteins to cellular compartments, protein folding, and degradation of unstable proteins (Parcellier et al., 2003). SAGE analysis between IVO and IVP blastocysts demonstrated that expression of several HSP transcripts (*HSPE1*, *HSPD1*, *HSPA1B*, and *HSPCA*) were significantly decreased in IVP blastocyst compared with IVO blastocysts. Furthermore, real-time PCR from independent IVO and IVP blastocysts demonstrated similar pattern of expression for *HSPE1*, *HSPD1*, and *HSPCA* as was observed for the SAGE analysis. Therefore, porcine embryos produced in vitro may be compromised in their ability to respond to in vitro culture stresses resulting in decreased developmental competence of these embryos compared with in vivo counterparts. Or as molecular chaperones, limited HSP expression may lead to abnormal protein assembly or conformation as well as incorrect cellular localization of proteins in IVP blastocysts resulting in aberrant cellular metabolism and/or structure.

In summary, SAGE analysis has demonstrated significant deviations in transcriptome profiles between

Day 6 porcine blastocysts produced *in vivo* or *in vitro*. Of the differentially expressed transcripts identified by SAGE analysis, there was a significant depression in expression of these transcripts in blastocysts produced using our *in vitro* system compared with IVO blastocysts. Provided that protein levels for these transcripts follow similar patterns, these results present some explanation as to why IVP embryos display abnormal phenotypes compared with IVO counterparts. A number of potentially compromised biological processes were identified that may begin to provide evidence of alterations in molecular pathways, in particular oxidative phosphorylation, that may lead to decreased developmental competence of porcine embryos produced *in vitro*. We hypothesize a potential model (Fig. 3) from our gene expression profiles that may account for decreased developmental competence of porcine embryos produced using the current *in vitro* system in

which altered function of the mitochondria leads to decrease energy production, thereby, decreasing the developmental competence of these embryos. Ultimately, the hope is that the in-depth analyses of IVO and IVP embryos (i.e., morulae, 8-cell, 4-cell, 2-cell, and zygotes) and oocyte transcriptomes would identify biomarkers of competence and provide potential target pathways to improve IVP methodology in the pig.

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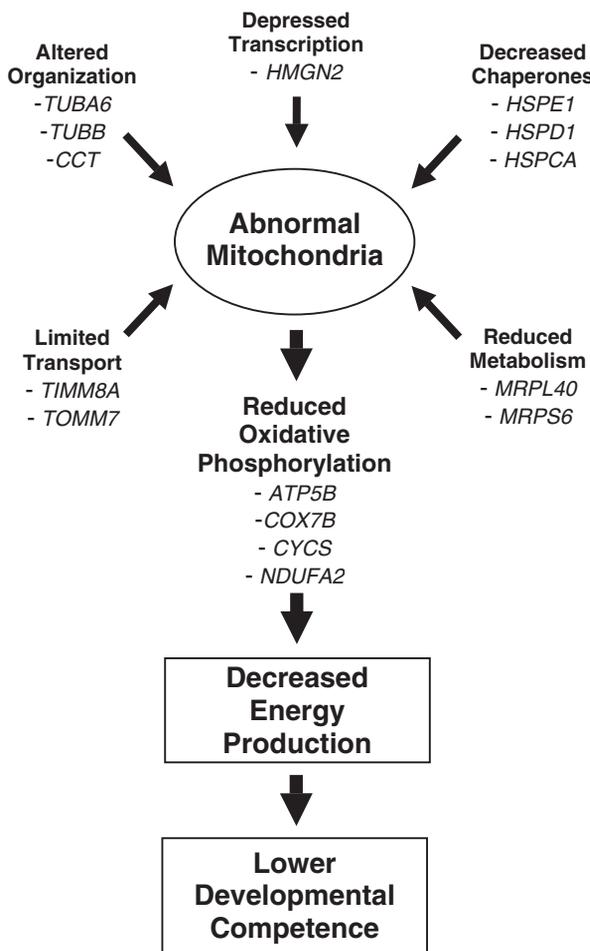


Fig. 3. Proposed hypothesis based on gene profiles from the IVO and IVP SAGE libraries that may account for decreased developmental competence observed for porcine embryos produced *in vitro* compared with IVO embryos. Compared with *in vivo* controls, significant deviations in transcript expression occur in embryos produced *in vitro* that results in abnormal function of the mitochondria, thereby leading to decreased energy production potential (ATP), which ultimately decreases the developmental competence of embryos produced using our *in vitro* system.

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